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Julius-Kühn-Archiv

Proceedings of the 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops

July 5 - 10, 2009
Neustadt, Germany

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants
Institute for Plant Protection in Fruit Crops and Viticulture
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Preface

The XXIst International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops was the first one held under this new title but was a continuation of a series of conferences that originated with a meeting in 1954 in Wädenswil, Switzerland. This meeting was entitled Symposium on Fruit Tree Virus Diseases and was held by a small group of international plant pathologists who were interested in the few virus diseases of deciduous fruit trees then known to occur in Europe and North America. The subsequent meetings became progressively wider in scope and increasingly concerned with viruses *per se* and virus diseases of fruit trees and small fruit crops worldwide. Furthermore, the scope of these meetings came to include diseases caused by phytoplasmas and viroids when these organisms were identified as new and serious pathogens of fruit crops.

In 1973 the organizing committee accepted the invitation of the International Society for Horticultural Science to become one of the working groups set up by its Plant Protection Commission. This meeting was entitled IXth International Symposium on Fruit Tree Virus Diseases.

Proceedings of the first eight meetings were published as supplements of different phytopathological journals. Extended abstracts were published for the meetings in 1954 and in 1958. With one exception, the proceedings since the 9th meeting were published by ISHS in Acta Horticulturae.

After discussions and approval by the current Scientific Committee it was decided to return to a meeting organization outside the auspices of an international organization and, thus, the 2009 conference was held not in association with the ISHS. This decision was approved during the business meeting at the 2009 Conference in Neustadt. At the same time it seemed appropriate to identify and establish a new title for the conference. The goal was to establish one name covering both the Tree Fruit and Small Fruit Crops subject areas and to include viroid and phytoplasma diseases, that previously were referred to as virus-like diseases when little or no information was available on these pathogens.

It was therefore decided to hold the 2009 and future meetings under the title International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops. A new organization, the International Council for the Study of Virus and other Graft Transmissible Diseases of Fruit Crops (ICVF) was formed. The organization is represented by a Management Board consisting of two chair persons and an elected secretary. One chair will always be the organizer of the previous meeting and the second chair is the organizer of the following meeting. The secretary is elected for three years during the Conference Business Meeting. The ICVF Management Board addresses issues relevant to the ICVF between conferences.

The Management Board is supported by a Scientific Committee. It has a maximum of 25 members, who must be active researchers in their field of expertise, and who should be selected so as to provide a balance of representation across geography, crops and specialty. Retirees will automatically leave the Scientific Committee at the next conference and, at the same time, a total of five members will be replaced by newly elected members. The Scientific Committee will propose the appointment of new members, who must be approved at the Conference Business Meeting.

The members of the Scientific Committee discuss and advise on scientific, policy and organizational issues that are relevant to the continued development of the ICVF. They are also responsible for peer review of proceedings from the ICVF meetings and all other scientific information that is posted on the ICVF website.

The current Management Board set up a permanent website at: http://icvf.jki.bund.de/.

This website offers membership to scientists interested in diseases of fruit crops caused by viruses, viroids, phytoplasmas or unknown graft transmissible agents. Any issues related to future meetings of ICVF will be posted on the website.

The papers contained in this volume of the "Julius-Kühn-Archiv" report the Proceedings of the XXIst International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops. The manuscripts submitted were reviewed by the organizers of the 2009 Conference with the support of the Scientific Committee and some professional language editing. All papers will be available for download as PDF documents with no charge through the ICVF website. The complete Programme and Abstracts of the Conference are also available for download.

The organizers of the 2009 Conference

Wilhelm Jelkmann and Gabi Krczal

Participants at Schwetzingen castle and gardens

In Memoriam Dr. Luigi Carraro

Dr. Luigi Carraro, Gigi to us, was born in Sagrado (GO) in 1960; a date that is easy to remember in the relative scale of the ages of some researchers of the DIPI of Udine. We looked like we were set up in an orderly line, separated by twenty years: Luigi, the undersigned, and Prof. Refatti. Defining the course of his life also seemed just as straightforward and foregone to us. Instead it was an unbearable, unexpected passing on 28 November 2009.

Luigi completed his secondary school studies at the scientific college of Gorizia and graduated from the Faculty of Agriculture at the University of Udine. In line with his measured ways of understanding life, he did not even seek degree honours. He wanted to earn them later, in the field, during his work as a researcher, which he conducted with exemplary commitment and seriousness, and with love and intelligence in particular. We understood that he knew how to abandon the superficiality of more common thoughts, led as he was by an exceptional sensitivity, constantly in search of more profound and intimate spheres. Only a few words were needed to perceive the level of his considerations. Besides, that's best since he was a man of few words, not even to convince the incredulous or in search of greater popularity. He was as though he was destined to be honest.

He devoted much of his time to research, which he virtually continued non-stop, going from his degree thesis to the experience enforced and conducted as a researcher at the Department of Biology and Plant Protection at the University of Udine (DIPI). His main research concerned the viruses and viroses of the graminaceae and fruit trees and the phytoplasmas and phytoplasmoses of trees and herbaceous plants. Vectors and their relationships with plants and studies into epidemiology were his great love. It isn't a coincidence that he was the first and main person to discover the phytoplasma vector of the European Stone Fruit Yellows. He published in leading scientific journals, which often asked for his contributions. And he loved scientific conferences, where he came to life.

His death is such a shame; the result of an unacceptable error by higher decision-making powers, at least for us.

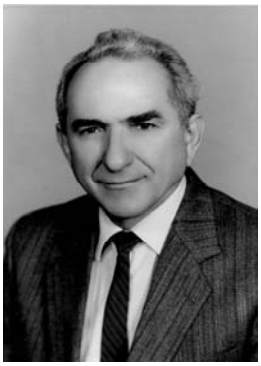
He will be missed by many, as a person and as a figure, the latter increasingly harder to find, as someone who knew how to work inside and outside the laboratory.

But he will be most sorely missed by his young daughter Carolina, whom he adored and who has suddenly been faced by this turn of fate, and his wife Nazia.

Prof. Ruggero Osler

University of Udine

In Memoriam Prof. Dragoljub D. Sutic



In the pursuit of knowledge, Professor Dragoljub Sutic completed three faculties. Firstly, he graduated from Military Academy in 1940. Having returned from war captivity where he served as a prisoner of war for four years, he graduated from Faculty of Agriculture, Belgrade in 1949. During the studies he became particularly interested in botany and phytopathology, scientific fields which marked his scientific and pedagogical life, and to which he remained devoted to the very end. His professional work began at Faculty of Agriculture where he began his career as a teaching assistant in the field of phytopathology. Always eager to expand his knowledge of biology, he, concurrently with the work on doctoral thesis, graduated from Faculty of Science in Belgrade (1954), whereas in the following year, he defended his doctoral thesis at Faculty of Agriculture. In the years that followed, he was admitted to academic titles of Assistant Professor (1959), Associate Professor (1961) and Full Professor (1969), and was later retired.

As an academic, he delivered lectures in phytopathology at undergraduate, graduate and postgraduate courses of study. He published a large number of books and textbooks, including the following: "Plant Viruses, Handbook of Plant Virus Diseases and Anatomy, and Physiology of Diseased Plants", out of which the latter two were translated into English and published in the USA.

He was proficient in a number of European languages (French, German, Italian, Russian and English) which, besides his high professional qualifications, helped him to easily make connections and maintain exceptional international cooperation with phytopathologists worldwide. He was a leader or a participant in numerous domestic and 4 international projects, and also participated in a large number of international scientific meetings. He was a member in a wide range of international professional and scientific organizations and editorial boards of renowned scientific journals.

Dragoljub Šutić was a pioneer of the plant virology of Serbia and former Yugoslavia. He was among the first to attempt to discriminate PPV isolates, and it was him who distinguished three kinds of PPV strains depending on kinds of local lesions induced on herbaceous indicator *Chenopodium foetidum*. His scientific work also encompasses the study of relationship between host plants and viruses (particularly of *Plum pox virus*), i.e. detection of new herbaceous and woody plants hosts, identification of the different pathogenicity of some PPV isolates/strains for particular species of hosts of *Prunus* genus, the investigation of resistance of stone fruits to PPV, the study of epidemiology and the virus spread by leaf aphids and the developing control measures. He made an enormous contribution to the study, detection and identification of types and strains of viruses and bacteria in vegetable and fruit crops. He was the author and co-author of 165 scientific and a number of professional papers. Dragoljub Sutic was highly merited scientist both in domestic and scientific community abroad, which resulted in his appointment for the corresponding member of Agricultural Academy of France and a huge number of awards.

His extraordinary knowledge of phytopathology and high competence in describing complex phenomena in an extraordinary simple and understandable way, his openness to discussion, respect he showed for other people's work and knowledge, readiness to help the young and open new realms of science, professionalism, his devotion to new experiences, his swift conceiving of new practical issues and possibilities of application of scientific results provided him an extraordinary merit both among colleagues and students. All of them were happy enough to have known him, especially those who were honoured to work with him, and all of them certainly feel great respect for him. He will live in their memory as a powerful person and an accomplished scientist.

Svetlana Paunovic

Fruit Research Institute, Republic of Serbia

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Association of *Tobacco ringspot virus*, *Tomato ringspot virus* and *Xiphinema americanum* with a decline of highbush blueberry in New York

Fuchs, M.
Department of Plant Pathology and Plant-Microbe Biology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456

Abstract

Plantings of highbush blueberry cultivars 'Patriot' and 'Bluecrop' showing virus-like symptoms and decline in vigor in New York were surveyed for the occurrence of viruses. *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) from the genus *Nepovirus* in the family *Comoviridae* were identified in leaf samples by DAS-ELISA. Their presence was confirmed by RT-PCR with amplification of 320-bp and 585-bp fragments of the RNA-dependent RNA polymerase genes, respectively. Comparative sequence analysis of viral amplicons of New York isolates indicated moderate (80.7-99.7 %) to high (90.8-99.7 %) nucleotide sequence identities with other ToRSV and TRSV strains, respectively. Soil samples from the root zone of blueberry bushes contained dagger nematodes and cucumber bait plants potted in soil samples with identified *X. americanum* became infected with ToRSV or TRSV. Altogether, ToRSV, TRSV, and their vector *X. americanum sensu lato* are associated with the decline of highbush blueberry in New York.

Keywords: *Vaccinium corymbosum* L., dieback, DAS-ELISA, RT-PCR, RNA-dependent RNA polymerase gene

Introduction

In the spring of 2007, decline and virus-like symptoms were observed in plantings of mature highbush blueberry (*Vaccinium corymbosum* L.) cvs. 'Patriot' and 'Bluecrop' in New York. Symptoms consisted of stunted growth, top dieback or mosaic and dark reddish lesions on apical leaves. Necrosis of leaf and flower buds and reduced fruit yield were also noticed. These observations prompted a survey of highbush blueberry cvs. 'Patriot' and 'Bluecrop' for the occurrence of viruses and their vectors.

Materials and methods

Plantings of highbush blueberry cv. 'Bluecrop' and 'Patriot' were surveyed for the occurrence of viruses by double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) with specific antibodies (Bioreba, Reinach, Switzerland).

The presence of viruses identified by DAS-ELISA was confirmed by reverse transcription (RT) polymerase chain reaction (PCR) using total RNA extracted from leaf tissue and appropriate primers. RT-PCR was carried out using the OneStep kit (Qiagen, Valencia, CA) with a 30 min heating step at 50 °C and a 15 min heating step at 95 °C followed by 35 cycles of 30 sec melting at 94 °C, 1 min annealing at 50°C, and 1 min elongation at 72 °C with a final extension of 10 min at 72 °C. The reaction products were resolved by electrophoresis in 1.5 % agarose gels.

Viral DNA amplicons obtained by RT-PCR were extracted from agarose gels and sequenced bidirectionally. Sequences were analyzed and compared using the DNASTAR Lasergene® v7.2 software package and TRSV strain bud blight (GenBank accession no. U50869) and ToRSV strain raspberry (GenBank accession no. L19655**)** as reference strains.

Soil samples were collected from the root zone of blueberry bushes and analyzed for the occurrence of dagger nematodes, which were identified based on morphological parameters (Lamberti et al., 2002) and counted under a dissecting microscope at 40X magnification. Blueberry soil samples collected from the root zone of blueberry bushes were used in cucumber baiting assays in a greenhouse.

Results

Decline and virus-like symptoms were observed in plantings of mature highbush blueberry cvs. 'Patriot' and 'Bluecrop' in New York in the spring of 2007. Symptoms in cultivar 'Patriot' consisted of a severely reduced vigor, shoot defoliation, distorted leaves and chlorosis of apical leaves, vein clearing or mosaic. Some bushes also showed a top dieback with poor blossom development or necrotic flower buds, and a few of them were dead. The decline symptoms were observed throughout the planting of blueberry cv. 'Patriot'. Symptoms in cultivar 'Bluecrop' consisted of mosaic or dark reddish lesions on apical leaves and a general decline.

Preliminary DAS-ELISA results indicated the presence of *Tomato ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) in a few leaf samples surveyed in the fall of 2007. Since these tests were run late in the season, an extensive

survey was conducted in the spring of 2008 to confirm preliminary findings and determine the incidence of these two virus species from the genus *Nepovirus*, family *Comoviridae* in every bush of the two blueberry plantings. Thirty-seven of the 528 blueberry leaf samples (7 %), including 439 from cv. 'Patriot' and 89 from cv. 'Bluecrop', reacted positively to ToRSV (3 %, 17 of 528) or TRSV (4 %, 20 of 528) in DAS-ELISA. Nine ToRSV-infected samples were from Patriot (2 %) and eight from Bluecrop (9 %); twelve Patriot (3 %) and eight Bluecrop (2 %) samples were infected with TRSV. The two target viruses were found in symptomatic and asymptomatic blueberry bushes, suggesting no strict association between virus detection by DAS-ELISA and symptom development. Seven soil samples from the Patriot planting and three from the Bluecrop planting were tested for the presence of nematodes. Species identified were primarily *Pratylenchus* spp., *Helicotylenchus* spp. and *Hoplolaimus* spp. but members of the *Xiphinema americanum* group were also found. *X. americanum* populations varied from 40 to 280 per kg of soil collected and were all from the root zone of Patriot bushes. Cucumber baiting assays with each of the four soil samples containing *X. americanum* demonstrated transmission of TRSV or ToRSV.

The presence of TRSV was confirmed by RT-PCR in blueberry leaf samples with amplification of a 320-bp DNA fragment of the RNA1-encoded RNA-dependent RNA polymerase (RdRp) using primers MF05-21-R (5' CAATACGGTAAGTGCACACCCCG 3') and MF05-22-F (5' CAGGGGCGTGAGTGGGGGCTC 3'). Similarly, the presence of ToRSV was confirmed in blueberry leaf tissue by immunocapture RT-PCR and primers ToRSV-R (5' CCACCACACTCCACCTACC 3') and ToRSV-F (5' ACTTCTGAAGGCTACCCGTT 3') to characterize a 585-bp fragment of the RNA1-encoded RdRp gene.

The viral gene amplicons obtained by PCR-based assays from four ToRSV and eleven TRSV isolates were sequenced. A multiple sequence alignment of ToRSV isolates from blueberry characterized in this study and those available in GenBank indicated 80.7-99.7 % and 90.2-99.5% sequence identity at the nucleotide (585 nts) and amino acid (194 residues) levels, respectively. Phylogenetic analyses showed a clustering of ToRSV isolates into three groups. Notwithstanding, ToRSV haplotypes from blueberry did not group with corresponding haplotypes from other crops, suggesting a genetic differentiation according to host. A multiple sequence alignment of TRSV isolates from blueberry characterized in this study and those available in GenBank indicated 90.8-99.7 % and 91.4-100 % sequence identity at the nucleotide (316 nts) and amino acid (105 residues) levels, respectively. Phylogenetic analyses inferred a clustering of TRSV isolates into a single group.

Discussion

ToRSV and TRSV are known to occur in fruit crops in New York, including grapevines (Gilmer et al., 1970; Uyemoto et al., 1977a) and *Prunus* spp. (Cummins and Gonsalves, 1986; Uyemoto et al., 1977b). ToRSV is also described in *Malus* spp. (Rosenberger et al., 1989) but, to our knowledge, this is the first report on the occurrence of TRSV and ToRSV in highbush blueberry in New York. In the USA, TRSV was reported in blueberry in Connecticut, Illinois, Michigan, Arkansas, Oregon and New Jersey (Ramsdell, 1985a). Also, ToRSV was described in blueberry in Oregon, Washington and Pennsylvania (Ramsdell, 1985b). The distribution of the two nepovirus species in the major blueberry producing areas in the United States is likely explained by the use of noncertified planting material.

The infection rate of TRSV and ToRSV was low in apical leaves in spite of a severe degeneration in cv. 'Patriot' and a slow decline in cv. 'Bluecrop'. Highbush Bluecrop is known to recover from TRSV infection (Lister et al., 1963). This recovery phenomenon likely results from the manifestation of RNA silencing. Interestingly, a complete or partial recovery of *N. tabaccum* and *N. benthamiana* from TRSV infection was described recently (Siddiqui et al., 2008) as well as a recovery of *N. benthamiana* from ToRSV infection (Jovel et al., 2007). It is conceivable that RNA silencing could be active in highbush blueberry cvs. 'Patriot' and 'Bluecrop' infected with TRSV or ToRSV, affecting systemic spread, reducing virus titers in apical leaves, in spite of a marked decline. *X. americanum*, the nematode vector of TRSV and ToRSV, was detected in the root zone of highbush blueberry cv. 'Patriot'. Populations of *X. americanum* were described in vineyards (Uyemoto et al., 1977a) and in apple, peach and cherry orchards (Molinari et al., 2004) in New York. To our knowledge, this is the first report of the dagger nematode *X. americanum senso lato* in blueberry in New York. The population density of *X. americanum* in the blueberry plantings surveyed in this study was low, but similar to findings in other crops (Evans et al., 2004; Pinkerton et al., 2008). In spite of the low population density detected, it is conceivable that *X. americanum* could contribute to the decline of highbush blueberry cvs. Patriot and Bluecrop by feeding on actively growing rootlets, thus weakening the root system and affecting plant vigor.

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A new member of the family *Reoviridae* may contribute to severe crumbly fruit in red raspberry, *Rubus idaeus* 'Meeker'

Quito, D.[1]; Jelkmann, W.[2]; Alt, S.[2]; Leible, S.[2]; Martin, R.R.[2]
[1] Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, OR USA;
[2] Julius Kuhn-Institut, Institute for Plant Protection in Fruit Crops and Grapevine, Dossenheim, Germany
[3] USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR, USA

Abstract

A virus induced crumbly fruit disease of considerable importance in 'Meeker' and other cultivars of red raspberry has been observed in northern Washington, USA, and British Columbia, Canada and to a lesser extent in the Willamette Valley of Oregon. Raspberry bushy dwarf virus (RBDV), a pollen-borne virus, has been considered the causal agent of the disease. However, dsRNA extractions from raspberry plants exhibiting severe crumbly fruit in northern Washington revealed multiple bands in addition to those corresponding to RBDV (5.5kb and 2.2kb). Sequence analyses of these dsRNAs showed the presence of two additional viruses. One has significant amino acid identity to proteins encoded by Rice ragged stunt virus (RRSV), a ten-segmented dsRNA *Oryzavirus* that belongs to the family *Reoviridae*. Thus far, all dsRNA segments, except the one that corresponds to S6 of RRSV, have been fully sequenced and found to have characteristic features of other plant reoviruses genomes. In addition, Raspberry leaf mottle virus (RLMV), a recently characterized member of the *Closteroviridae*, has also been identified from raspberries with severe crumbly fruit. These findings along with the lack of severe crumbly fruit symptoms in 'Meeker' red raspberry singly infected with RBDV in Oregon, suggest the existence of a novel virus complex associated with severe crumbly fruit in red raspberries. The complex may involve RBDV, RLMV and/or this new reovirus, provisionally named Raspberry latent virus (RpLV).

Keywords: Raspberry crumbly fruit, Raspberry bushy dwarf virus, Raspberry leaf mottle virus, Raspberry leaf spot virus, plant reoviruses.

Introduction

There are more than 40 viruses known to infect *Rubus* spp. a number of which have been described based on symptom development, mode of transmission and particle morphology (Converse, 1987). Others are less well characterized and based on symptoms caused on indicator plants. One of the viruses in this latter group, Raspberry leaf spot virus (RLSV), has been reported as a component of Raspberry mosaic disease virus complex in Europe but not in North America (Jones, 1982). Raspberry plants that tested negative for RBDV but exhibiting leaf mottling symptoms were observed in production areas in Washington, USA and British Columbia, Canada. Leaf tissue from these symptomatic plants was used for dsRNA extractions and cloning. Partial sequence information obtained from cloning revealed the presence of a new plant reovirus. Interestingly, primers made to detect the virus failed to detect RLSV in symptomatic plants in Europe, suggesting that the symptoms observed in plants in North America were caused by a different virus (Martin and Jelkmann, personal communication).

The above mentioned primers have been used as part of the routine virus testing program in raspberry plants in the USA, to detect the virus that, mistakenly, has been referred to as RLSV. Hereafter, we will refer to this new virus as Raspbery latent virus (RpLV), since it does not cause symptoms in several indicators and cultivars of Rubus in single infections. Note that the RLSV in Europe has been shown to be a variant of Raspberry leaf mottle virus (RLMV) (Tzanetakis et al., 2007) and thus, due to precedence in original description, RLMV should be the name used for these viruses (See MacFarlane – these proceedings). Interestingly, raspberry plants showing severe crumbly fruit symptoms from different fields in northern Washington have tested positive for RpLV and RLMV as well as for RBDV. Raspberry bushy dwarf virus (RBDV), a pollen-borne *Ideovirus*, has been associated with crumbly fruit disease in most red raspberry cultivars worldwide (Daubeny et al., 1982). With the finding that severely crumbly fruited 'Meeker' plants are infected with multiple viruses, the role of these additional viruses in crumbly fruit should be investigated in other cultivars and other production areas. These findings, along with the lack of severe crumbly fruit in 'Meeker' red raspberry singly infected with RBDV in Oregon, suggest a possible new virus complex associated with severe crumbly fruit in the susceptible raspberry cultivar 'Meeker' and possibly other cultivars. The complete characterization of RpLV is necessary to elucidate possible interactions with other common raspberry viruses and its implications in raspberry diseases. This communication reports the partial characterization of RpLV, including dsRNA extractions, sequence analyses, grafting onto indicators, and possible vectors.

Materials and methods

Virus source: Root cuttings from symptomatic 'Meeker' raspberry plants were obtained from production fields in northern Washington. The roots were planted, grown in one gallon pots and maintained in a greenhouse under standard conditions.

Double-stranded RNA extraction: Twenty grams of fresh leaf tissue was powdered in liquid nitrogen. DsRNA was extracted using phenol/STE buffer, and recovered by cellulose CF-11 chromatography, as previously described (Morris and Dodds, 1979). The dsRNA was treated with ribonuclease T1 from Aspergillus oryzae (Sigma) and DNAse 1 from bovine pancreas (Sigma) to remove single-stranded RNA and dsDNA, respectively. After the digestions the dsRNAs were again purified on CF-11 cellulose columns and precipitated with EtOH. The dsRNA was pelleted, dried and separated by electrophoresis through 1% agarose gels stained with ethidium bromide and visualized under UV light. All bands except the ones corresponding to RBDV (5.5kb and 2.2kb) and RLMV (17kb and 1.2kb) were gel extracted using a DNA gel extraction kit (Genescript).

cDNA synthesis, cloning and sequencing: The reverse transcription (RT) reaction was primed with the universal random primer 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3' (Froussard, 1992) and the methodology described by Tzanetakis et al., (2005) was used. Briefly, a mixture containing the purified dsRNA and primers was denatured with CH_3HgOH at room temperature for 30 min. Then, a second mix, containing reverse transcription buffer, DTT, dNTPs, and Superscript III RT (Invitrogen) was added to the denaturant mix and incubated for 50 min at 50C. The reaction was terminated by heating at 75C for 10 min. The RNA template was digested with RNAse H (Invitrogen), and then a PCR reaction containing the anchor primer 5'-GCCGGAGCTCTGCAGAATTC-3'was carried out to amplify the cDNA products. The PCR products were cloned into the TOPO TA vector (Invitrogen) and sequenced.

Genome assembly: The sequences were assembled into contigs using the assembly program CAP3, the contigs were then compared to the database at NCBI GenBank using BLAST, which returned Rice ragged stunt virus (RRSV) as the nearest relative. Contigs were aligned with RRSV and then specific primers were made to fill in the gaps. The 5' and 3' termini were obtained by poly (A) tailing of the 3' ends of dsRNAs, as described (Isogai et al., 1998), followed by RT-PCR using primers developed to known sequence near the ends and oligo dT. To confirm the last base, a 3' blocked DNA oligonucleotide was ligated to both 3' ends of the dsRNAs followed by RT-PCR using its complementary primer and specific primers for each end.

Grafting assays: Plants with single infections of RpLV or mixed infections (RLMV and RpLV) were grafted onto the *Rubus* virus indicators 'Norfolk Giant', 'Malling Landmark' and 'Munger'. Symptom development was monitored for nine weeks.

Aphid transmission assays: The large raspberry aphid Amphorophora agathonica was tested for its ability to transmit RpLV. This aphid is common in the main raspberry production areas in northern Washington where severe crumbly fruit and RpLV are very common. Also, this aphid and RpLV are much less common in the Willamette Valley of Oregon. Nonviruliferous *A. agathonica* were placed on an RpLV-infected 'Meeker' raspberry plant and allowed to feed for two weeks. Then, approximately 60 aphids were transferred to a virus-tested 'Meeker' plant and allowed a 1.5 h inoculation access period. Then the aphids were transferred serially to four additional healthy 'Meeker' plants and allowed inoculation access times of 2.5 h, 8 h, 12 h, and 24 h, respectively. After each inoculation access feeding period, six aphids were collected and tested for the presence of RpLV by RT-PCR. The aphid transmission test plants were tested for RpLV 6, 10 and 14 weeks post-inoculation by RT-PCR.

Results

Double-stranded RNA (dsRNA): In addition to the bands corresponding to RBDV (5.5kb and 2.2kb) and RLMV (17kb and 1.2kb) multiple bands were observed from crumbly-fruited raspberry plants when separated by electrophoresis on agarose gels. These additional bands migrated between ~4kb and ~1100bp of the dsDNA marker (Figure 1).

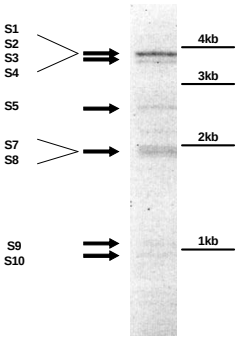

Fig. 1 DsRNA from symptomatic 'Meeker' plants separated by electrophoresis (1% agarose gel).

Sequencing: Sequencing analyses showed significant amino acid identity to different proteins encoded by Rice ragged stunt virus (RRSV). RRSV is a ten-segmented dsRNA Oryzavirus that belongs to the family Reoviridae. Terminal sequences revealed that RpLV has the conserved sequences AGUU/A and GAAUAC at the 5' and 3' termini of each RNA segment, respectively (Table 1).

Tab. 1 Conserved terminal sequences of RpLV

Genome segment	5' terminus	3' terminus
S1	**AGU**UUUA	**GCGAAUAC**
S2	**AGU**UUAU	**GGGAAUAC**
S3	**AGU**GAAA	**GCGAAUAC**
S4	**AGU**UUUA	**GCGAAUAC**
S5	**AGU**UUUU	**GUGAAUAC**
S7	**AGU**AAAA	**GCGAUUAC**
S8	**AGU**UUUA	**GCGAAUAC**
S9	**AGU**UAAA	**GUGAAUAC**
S10	**AGU**UAAA	**GCGAAUAC**

In addition, each segment has an inverted repeat that consists of 6 to 8 nucleotides adjacent to the conserved region (Figure 2). Thus far, nine dsRNA segments have been completely sequenced; the putative proteins encoded by each segment are presented in table 2. The relatedness of RpLV to RRSV and other plant reoviruses is underway and completion of the elucidation of the tenth genomic segment if it exists.

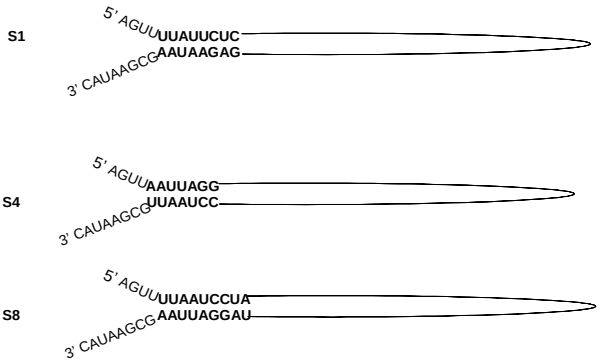

Fig. 2 Inverted repeats of genome segments S1, S4, and S8 of RpLV. The positive sense strand is illustrated. Regions adjacent to the conserved terminals are complementary.

Tab. 2 Putative proteins encoded by each genomic segment of RpLV based on amino acid identity to RRSV

RRSV Genome seg (bp)	Protein encoded by segment	Raspberry latent virus genome (bp)	Amino acid score/identity (bits/%)
1 (3849)	Spike	3948	299/35
2 (3808)	Structural	3650	251/27
3 (3699)	Structural	3566	229/29
4 (3823)	RdRp	3818	323/39
5 (2682)	Capping enzyme	2563	80/27
6 (2157)	RNA-binding	not detected	unknown
7 (1938)	Non structural	1936	79/26
8 (1814)	Autocatalytic enz	1997	30/28
9 (1132)	Spike	1141	62/24
10 (1162)	Unknown	1205	58/23

Grafting: Grafting assays showed that indicators grafted with leaves from plants singly-infected with RpLV did not exhibit symptoms; whereas plants containing mix infections with RLMV and RpLV developed symptoms when grafted onto the raspberry virus indicator 'Malling Landmark' (Figure 3), but was symptomless in 'Norfolk Giant'.

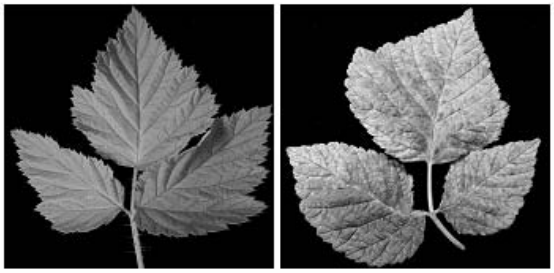

Fig. 3 Indicator raspberry 'Malling Landmark' 6 weeks post graft-inoculation. Left: Leaf grafted with RpLV only. Right: Leaf from plants grafted with RpLV and RLMV

Aphid transmission assays: RpLV was detected by RT-PCR in the fourth plant in the serial transmission tests, which had an inoculation access time of twelve hours. The virus was not detected after access feeds of 1.5, 2.5 and 8 hours and nor was there any transmission after 12 hours. Interestingly, the aphids tested positive for RpLV by RT-PCR throughout the transmission experiment. These experiments are being repeated as well as transmission tests with leafhoppers.

Discussion

The initial dsRNA extractions and limited sequence of RpLV was obtained from raspberry plants as early as 1988. However, recent observations of plants affected with severe crumbly fruit and the presence of this virus along with RLMV suggested that a virus complex may be responsible for the disease (Martin, personal communication).

Grafting assays suggest a possible synergistic effect of these two viruses when found in mix infections. It is believed, that RpLV in conjunction with RLMV and RBDV may cause severe crumbly fruit in 'Meeker'. This hypothesis will be studied by real-time PCR, fruit quality and yield evaluations of plants with single or mix infections of all combinations of the three viruses, RpLV, RLMV and RBDV.

Plant reoviruses possess terminal sequences, which are conserved at the genus level, and segment-specific inverted repeats adjacent to the conserved termini (Kudo et al., 1991; Yan et al., 1992). Sequencing results of nine dsRNA segments obtained from symptomatic raspberry plants, show those characteristics. The first three nucleotides at the 5' end (AGU) are similar to those of other reoviruses. However, the 3' terminal of RpLV seems to be unique, which indicates that the virus probably belongs to a new genus of the plant *Reoviridae*. Most viruses belonging to the family *Reoviridae* contain 10-12 dsRNA genome segments (Mertens et al., 2005). Recently, a new reovirus isolated from the mosquito *Aedes pseudoscutellaris* was found to have a genome comprised of 9 dsRNA segments (Attoui et al., 2005). Our sequence results indicate that RpLV may also have nine genome segments, although this is not definitive. One additional experiment with 'Deep Sequencing' will be performed in an effort to identify a tenth genomic segment for RpLV.

The high aphid populations in northern Washington, where severe crumbly fruit is more prevalent, prompted us to conduct transmission assays with the common raspberry aphid *Amphorophora agathonica*, despite the fact that all known plant reoviruses are transmitted by various species of leafhoppers. The result obtained from this preliminary experiment is quite interesting. If reproducible, it would indicate that the virus is transmitted by aphids in a semi-persistent manner since first several and the last serial transfers were negative. An alternative explanation could be that A. agathonica is an inefficient vector of the RpLV. Experiments with leafhoppers as vectors are underway, as well as repeating the aphid transmission tests.

At this time, the role of RpLV in severe crumbly fruit in red raspberry is unclear. Plants singly infected with RBDV, RLMV, and RpLV, are being used to create mix infections in all possible combinations. These plants will be planted in field experiments to study the impact of various virus combinations on fruit quality and yield, and also to study the interaction between viruses using real time PCR.

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The biology of *Cixius wagneri*, the planthopper vector of 'Candidatus *Phlomobacter fragariae*' in strawberry production tunnels and its consequence for the epidemiology of strawberry marginal chlorosis

Salar; P.[1]; Danet, J.-L.[1]; Pommier, J.-J.[2]; Foissac, X.[1]
[1]UMR1090, INRA and Université Bordeaux2, BP81, F33883, Villenave d'Ornon, France
[2] Hortis Aquitaine, Maison Jeannette, F-24140, Douville, France

Abstract

«Candidatus *Phlomobacter fragariae*» is the prevalent agent of strawberry marginal chlorosis (SMC) and is transmitted by the planthopper *Cixius wagneri*. Because the insect vector biology was unknown, a field experiment was set up to determine if it was able to reproduce on strawberry plants, to determine the number of insect generations per year and the ability of nymphs to transmit SMC. During spring 2004, 80 *C. wagneri* adults were delivered into 4 small insect-proof tunnels containing 30 healthy plants. Fifteen percent of the delivered insect population were carrying the pathogen. In October 2004, only 3 young L1 instar nymphs were found in the first tunnel, demonstrating there were no new insect generations during the summer. In April 2005, 330 *C. wagneri* of early L1 to late L5 nymph instars were collected at the roots of the plants,clearly indicating that a single insect generation had overwintered as larvae andemerged at the following spring. All instars were shown to carry 'Ca. *P. fragariae*' (70 to 75 % of the larvae) and were able to transmit SMC as assessed by transmission assays. An insecticide treatment was applied in March 2005 in a third tunnel and a fourth tunnel was kept as a control. More than 120 *C. wagneri* adults were collected in the control tunnel 4 in June 2005 confirming that an insect generation arose in the tunnel, whereas no insects could be found in the treated tunnel 3. All plants were kept for two years, surveyed for symptom expression and tested for 'Ca. *P. fragariae*' infection by 16S-PCR. Results indicated a reduced mortality and SMC incidence in tunnel 3, and a higher mortality and SMC incidence in tunnel 2 than in tunnel 1, attesting that *C. wagneri* larvae had spread SMC and that an early insecticide treatment could control the disease.

Keywords: Phloem-restricted bacteria, planthopper, insect vector, *Fragaria x ananassa*

Introduction

Marginal chlorosis has affected strawberry production in France since the early eighties (Nourrisseau et al., 1993). A phloem-restricted uncultured bacterium, "Candidatus *Phlomobacter fragariae*" is associated with the disease (Zreik et al., 1998). A large survey conducted from 1996 to 2001 for marginal chlorosis in French strawberry production fields and nurseries revealed that symptoms of SMC were most frequently induced by "Ca *P. fragariae*" in strawberry production tunnels but that the stolbur phytoplasma could also cause SMC in nurseries (Danet et al., 2003). Whereas the transmission of stolbur phytoplasma to strawberry plants is certainly achieved by its main planthopper vector *Hyalesthes obsoletus* (Fos et al., 1992), «Ca. *P. fragariae*» is transmitted in production tunnels by the planthopper *Cixius wagneri* (Danet et al., 2003). To investigate the ability of *C. wagneri* to grow in strawberry production tunnels and of *C. wagneri* larvae to transmit «Ca. *P. fragariae* », we intended to establish infectious *C. wagneri* populations in insect-proof mini-tunnels and recover infectious *C. wagneri* nymphs. The efficiency of an early insecticide treatment to reduce insect vector population and disease spread was also evaluated.

Material and methods

Design of tunnel experiments:Four small insect-proof tunnels were installed in April 2004 in a 6 meter wide plastic tunnel under normal production conditions. Each tunnel contained 30 healthy strawberry plants of the cultivar 'Cijosée'. In May and June 2004, *C. wagneri* were captured in organic tunnels and groups of insects were introduced, with an equal sex ratio, into the 4 insect-proof mini-tunnels. A total of 80 insects were introduced in each tunnel. Twenty insects, representative of the collected population were kept for "Ca P. fragariae" detection. After a period of 4 months (Tunnel 1, October 2004), 10 months (Tunnel 2, April 2005) and 12 months (Tunnel 3 and 4, June 2005), insects were collected using D-Vac aspiration, and plants were pulled out and their root system and surrounding soil examined for the presence of *C. wagneri* larvae. Finally, plants were planted in individual pots, sprayed with insecticide and kept in a greenhouse for 18 months. Mortality and symptoms were recorded after 12 and 18 months. Plants were submitted to "Ca. *P. fragariae*" detection after 12 months of incubation. Tunnel 3, received a single insecticide treatment with endosulfan (organochlorine) in March 2005.

"Ca. *P. fragariae*" transmission assays: *C. wagneri* larvae collected in tunnel 2 in April 2005 were caged on a healthy strawberry plant in groups of 10 (L1-L2 larvae) or 20 (L3, L4-L5 larvae) until all insects died. Plants were then kept in the greenhouse, examined for SMC symptoms and PCR tested for "Ca. *P. fragariae*" infection.

"Ca. *p. fragariae*" detection in plants and insects: Nucleic acids were extracted from strawberry plants and from individual insects as described previously (Maixner et al., 1995). Primers

Fra4 (5' CTCCTCTGTCTCTAAAGG-3') and Fra5 (5'-AGCAATTGACATTAGCGA-3')

from the 16S rDNA sequence of "Ca. *P. fragariae*" were used for the amplification of DNA extracted from strawberry plants under the following conditions: 35 cycles of 1 min at 92 °C, 1 min at 52 °C, and 1 min at 72 °C (Zreik et al., 1998). The amplified DNA was visualized under UV light after electrophoresis on 1 % agarose gels stained with ethidium bromide. Spot-PCR was carried out for "Ca. *P. fragariae*" detection in *C. wagneri* as previously described (Foissac et al., 2000).

Results

Fifteen percent of the *C. wagneri* population introduced into the small tunnels was infected with "Ca *P. fragariae*" as revealed by Spot-PCR detection performed on 20 individuals randomly selected from the collected populations. In October 2004, only 3 larvae of early development stage L1 and an old adult were found at the upper part of the root system. Therefore no C. wagneri summer generation had emerged at fall. In April 2005, 80 larvae of early L1-L2 stages, 110 larvae of L3 stages and 140 larvae of late L4-L5 stages were collected on the soil just under the plastic cover. This result indicated that a single C. wagneri generation is overwintering as eggs or young larvae and is emerging at spring under the production tunnel. Infectivity of insects was assessed by transmission assays to strawberry plants. Six of the 8 plants caged with 10 larvae of stages L1-L2 developed SMC after 6 months and 7 out of 8 tested positive for "Ca *P. fragariae*" infection. Similarly, 5 plants out of 6 that had been caged with 20 L3 larvae finally developed SMC and were infected by "Ca *P. fragariae*". All the 6 plants caged with 20 L4-L5 larvae developed SMC and were infected by "Ca *P. fragariae*". Insects dead during the transmission period were collected and submitted to "Ca *P. fragariae*" PCR detection. It revealed that 70 % of the L3-L4 larvae (developed from L1 & L2), 75 % of L4-L5 larvae (developed from L3) and 19 % of adults (developed from L4 & L5) were infected with "Ca. *P. fragariae*".

In June 2005, no *C. wagneri* could be found in the insecticide-treated tunnel 3, whereas 120 *C. wagneri* adults were captured in the untreated Tunnel 4. This confirmed that *C. wagneri* can reproduce and develop on strawberry plants under production conditions and that an early insecticide treatment can control *C. wagneri* population. In June 2006, 9 plants of the 30 plants of tunnel 3 tested positive for "Ca *P. fragariae*" infection and only 3 % of the plants died after 18 months, while 17 plants of the 30 plants of the control tunnel 4 tested positive for "Ca *P. fragariae*" infection and 50 % died after 18 months.

Discussion

In conclusion, the planthopper *C. wagneri* reproduces on strawberry plants under tunnels in the French strawberry production system. As *C. wagneri* is the vector of "Ca. *P. fragariae*", one of the bacterial agents of SMC, its ability to multiply on strawberry plants explain the epidemic spread of SMC on the crop. One insect generation occured per year and insects overwintered as larvae on plant roots under their plastic covers. Larvae of early stages were infectious and could efficiently transmit the disease. The high proportion of larvae infected by "Ca. *P. fragariae*" was certainly due to the acquisition of the proteobacterium during winter when larvae were feeding on the roots of the infected strawberry plants. However, we cannot exclude the possibility of transovarial transmission of "Ca. *P. fragariae*" by infected females to their progeny. It was also shown that a single insecticide treatment targeting the nymphal stage efficiently reduces the insect population and disease spread. Other means such as biological control of the insect vector should also be investigated in order to provide alternative measures compatible with integrated pest management.

Acknowledgements

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The seasonal detection of strawberry viruses in Victoria, Australia

Constable, F.E.[1]; Bottcher, C.[1]; Kelly, G.[1]; Nancarrow, N.[1]; Milinkovic, M.[1]; Persely, D.M.[2]; Rodoni, B.C.[1]
[1] Department of Primary Industries, Knoxfield, Private Bag 15 Ferntree Gully Delivery Centre, Victoria 3156, Australia
[2] Department of Primary Industries and Fisheries, 80 Meiers Rd, Indooroopilly 4068, Queensland, Australia

Abstract

PCR tests were adopted from international, peer-reviewed literature and developed for the detection of *Strawberry mottle sadwavirus* (SMoV), *Strawberry crinkle cytorhabdovirus* (SCV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), *Beet pseudos yellows crinivirus* (BPYV), and *Strawberry pallidosis associated crinivirus* (SPaV) in Victoria, Australia. The PCR tests were applied to 23 positive control plants infected with one or more viruses and these plants have been tested monthly from May 2005 to April 2007. Our results have indicated that the viruses were most reliably detected by PCR during May-October. In November, December and January of each year a decline in the number of positive PCR results for BPYV, SVBV and SPaV was observed. Twelve positive control plants maintained at the AQIS post entry quarantine screenhouse at Knoxfield, Victoria, and also infected with one or more viruses, were tested monthly from August 2006 to April 2007. A similar decline in the ability to detect SMoV, SVBV and SPaV in the AQIS positive control plants was observed in 2006/07 and November was the least reliable month for detection of strawberry viruses in these plants. These results indicate that spring and autumn may be the optimal times for PCR detection of strawberry viruses in south east Australia.

Keywords: *Strawberry mottle sadwavirus; Strawberry crinkle cytorhabdovirus; Strawberry mild yellow edge potexvirus; Strawberry vein banding caulimovirus; Beet pseudos yellows crinivirus; Strawberry pallidosis associated crinivirus*; detection; polymerase chain reaction; PCR; certification

Introduction

In Australia certified strawberry runners are supplied through the Victorian Strawberry Certification Authority (VSICA) and the Queensland Strawberry Runner Certification Scheme. The strawberry runners are certified on the basis of the high health status of nucleus collections, which are held by each scheme and are indexed annually for the major fungal, bacterial and virus-associated diseases of strawberries known to occur in Australia. These pathogen tested schemes have been operational in Australia for over 40 years and have contributed greatly to increased yields for strawberry growers due to the exclusion of these pathogens from industry and the ongoing supply of high health planting material. Both nucleus collections are tested annually in spring for virus-associated diseases including: Strawberry mild yellow edge, Strawberry mottle, Strawberry crinkle, Strawberry vein banding and Pallidosis. Each of these diseases is associated with one or more viruses for which they are tested via the biological indexing method of petiole grafting onto sensitive indicator species (Frazier, 1974; Converse, 1987). While this method is reliable and sensitive, it is labour intensive, expensive, time consuming (takes 6-8 weeks to return a result) and can only be reliably done in the spring and early summer months of each year. Plants are also tested for Strawberry necrotic shock virus (SNSV, formally thought to be a strain of Tobacco streak virus, TSV) using herbaceous indexing. Recent advances in molecular techniques have been published overseas for the detection of most of the viruses that infect strawberry plants. Molecular indexing via the polymerase chain reaction (PCR) offers the Australian strawberry industry a more rapid and cost effective method of indexing the strawberry nucleus collection. PCR returns a diagnosis in 1-2 days resulting in a much reduced cost to industry for the annual indexing of the nucleus collection. Detection of plant pathogens can be influenced by changes in season, associated with environmental changes such as temperature and/or with physiological changes in the plant (Dal Zotto et al., 1999; Heleguera et al., 2001; Posthuma et al., 2002; Tzanetakis et al., 2004a). These changes may have a direct effect on pathogen concentration or on the presence of plant compounds which can inhibit enzymes used in molecular detection when co-extracted with nucleic acid.

To determine if such variation exists for the detection of strawberry viruses in Australia we tested 23 positive virus infected strawberry plants each month during two years and an additional 12 plants during nine months for *Strawberry mild yellow edge virus* (SMYEV), *Strawberry crinkle virus* (SCV), *Strawberry mottle virus* (SMoV), *Strawberry vein banding virus* (SVBV), *Strawberry pallidosis associated virus* (SPaV) and *Beet pseudos yellows virus* (BPYV).

Materials and methods

Sampling: Twenty three strawberry plants containing one or a combination of SMYEV, SCV, SMoV, SVBV, SPaV, SNSV and BPYV were maintained during 24 months in a glass house at 24°C and normal day length. Every month from May 2005 until April 2007, 2-3 leaves, with petioles, were collected from each plant for virus testing. Plants were not tested in July 2006. In addition 12 strawberry plants containing one or a combination of the same viruses were maintained in a screen house in the Australian Quarantine Inspection Service (AQIS) post entry quarantine (PEQ) screenhouse located at Knoxfield, Victoria. Every month from August 2006 until April 2007, 2-3 leaves, with petioles, were collected from each AQIS plant for virus testing.

Nucleic acid extraction: RNA was extracted from 0.3g of infected strawberry plant material using the RNeasy® Plant Mini Kit (QIAGEN Pty Ltd, Doncaster, VIC Australia) as described previously (MacKenzie et al., 1997).

Pathogen primers: Table 1 lists the PCR primers used for each test, their orientation, the annealing temperature for each primer pair, the expected size of the product and the reference from which the primer sequence was sourced. The final concentration for each pathogen primer for all one step RT-PCRs was 0.4 µM.

Tab. 1 List of viruses that were tested for using PCR techniques, the primers used and the associated annealing temperature, the genomic region of the virus that was amplified, the expected PCR product size and reference cited for each test.

Pathogen	Primer name	Orien-tation	Primer sequence (5'-3')	Tm	Region amplified	Expected product-size	Refe-rence
Housekeeping gene	AtropaNad2.1a AtropaNad2.2b	F R	GGACTCCTGACGTATACGAAGGATC AGCAATGAGATTCCCCAATATCAT	55°C	NADH dehydrogenase ND2 subunit	188bp	1
Beet pseudo yellows virus	BP CPm F BP CPm R	F R	TTCATATTAAGGATGCGCAGA TGAAAGATGTCCACTAATGATA	55°C	Coat protein	334pb	2
Strawberry crinkle virus	SCVdeta SCVdetb	F R	CATTGGTGGCAGACCCATCA TTCAGGACCTATTTGATGACA	60°C	Polymerase	345bp	3
Strawberry mottle virus	SmoVdeta SMoVdetb	F R	TAAGCGACCACGACTGTGACAAAG TCTTGGGCTTGGATCGTCACCTG	50°C	Non-coding region	219bp	4
Strawberry mild yellow edge	SYEupstcp1a SYEPolyTb	F R	CCGCTGCAGTTGTAGGGTA TTTTTTTTTTTTTTTAAGAAAAAGAAA AACAAAC	50°C	Coat protein	913bp	5
Strawberry vein banding virus	SVBVdeta SVBVdetb	F R	AGTAAGACTGTTGGTAATGCCA TTTCTCCATGTAGGCTTTGA	55°C	Coat protein	422bp	6
Strawberry pallidosis virus	SP 44 F SP 44 R CP5' CPn731R	F R F R	GTGTCCAGTTATGCTAGTC TAGCTGACTCATCAATAGTG AGCTAGAACAAGGCAAGTC GCCAATTGACTGACATTGAAG	52°C 52°C	Heat shock protein 70 homolog Coat protein	517bp 752bp	7 8
Strawberry necrotic shock	SNSV CPbeg F SNSV CPend R	F R	GAGTATTTCTGTAGTGAATTCTTGGA ATTATTCTTAATGTGAGGCAACTCG	55°C	Coat protein	823bp	9

[1]Thompson et al., 2003; [2]Tzanetakis et al., 2003; [3]Thompson et al., 2003; [4]Thompson et al., 2003; [5]Thompson and Jelkmann 2004; [6]Thompson et al., 2003; [7]Tzanetakis et al., 2004a; [8]Tzanetakis et al., 2004a; [9]Tzanetakis et al., 2004b

One step RT-PCR: The SuperScript™ One-Step RT-PCR System (Invitrogen) was also used for detection of viruses and used for the detection of NAD mRNA. One step RT-PCR was done according to the manufacturer's instructions except the total reaction volume was 25 µl. Cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at denaturing at 94 °C for 1 min, annealing for 40 s at the appropriate temperature for each primer pair (see table 1), elongation at 72 °C for 40 s and a final elongation step at 72 °C for 5 min.

Gel electrophoresis: After amplification, 10 µl from each PCR reaction was subjected to electrophoresis in a 1 % agarose gel using 0.5 x TBE (0.045 M Tris-borate, 1 mM EDTA, pH 8.0) running buffer. Products were stained with ethidium bromide that was incorporated in the gel and visualized by UV transillumination. Water controls, in which no nucleic acid was added to the PCR mix, were also included. DNA markers used were DNA Molecular Weight Marker X (Roche Diagnostics).

Weather data: Average monthly maximum and minimum temperatures for each month from May 2005 until July 2007 were obtained form the Bureau of Meteorology for the Scoresby weather station, which is located at DPI, Knoxfield.

Results

Strawberry crinkle virus: Four of the 23 positive control plants maintained in the glasshouse tested positive for SCV at least once during the 23 months of testing and the maximum number of SCV positives at any one sampling time was two. At least one of each plant tested positive in each month except August 2006 and December 2006 (Figure 1a). In July, September and October in 2005 and February, May and September 2005 SCV was detected in 2/4 plants. In the remaining months SCV was only detected in one plant. Two of the four plants in which SCV was detected were positive in 11 and 12 months of the 23 months of testing. The remaining two plants each tested positive on two occasions, in July and October 2005. None of the positive control plants maintained in the AQIS PEQ screen house tested positive for SCV.

Strawberry vein banding virus: Twenty of the 23 positive control plants from the glasshouse tested positive for SVBV during the 23 months of testing. At least one plant tested positive in all months except in November and December 2005 and in February 2007. In each year the greatest number of positive plants occurred in winter and 10-11 plants were positive in June-August 2005 and 12 and 14 plants were positive in May and June 2006 respectively (Figure 1b), indicating that the PCR test had a 50-75 % efficiency for detection. In spring the number of SVBV positive plants decreased. Five of the plants tested positive once for SVBV and this did not occur in the same month. Nine of the SVBV-infected plants were positive in 13-18 of the 23 months in which they were tested. Six plants were positive in 2-6 of the 23 months in which they were tested. All of the plants maintained in AQIS tested positive for SVBV during the nine months they were tested. The largest number of positive plants (9/12) was obtained in September 2006 (Figure 2a). The number of positive plants then decreased and SVBV was not detected in November 2006.

Strawberry mottle virus: SMoV was only detected in 3/23 plants maintained in the glasshouse and was infrequently detected. SMoV was only detected in May and October 2005, May 2006 and March 2007. One plant tested positive on two separate occasions (May 2006 and March 2007). It is possible that the primers for SMoV are unreliable with our isolate due to variation at the primer binding sites. It may also be that SMoV does not replicate well under the glasshouse conditions in which it was maintained and titres were too low for reliable detection. SMoV was detected in 8/12 of the plants maintained in the AQIS PEQ screenhouse. Four plants tested positive in 5-8 months of the nine month testing period and four plants only tested positive once. The largest number of positive plants was obtained in March 2007, when 5/8 (63 %) SMoV infected plants tested positive (Figure 2b). SMoV was not detected in November 2006.

Beet pseudos yellows virus: BPYV was detected in 15/23 plants and 12/15 plants were positive in 10-16 months of the 24 month testing period. Two plants tested positive on two and five occasions each and one plant tested positive once. The largest number of positive results was obtained in May and August 2005 and May and September 2006 when 12-13 of the 15 plants (80-87 %) tested positive (Figure 1c). BPYV was not detected in any plant in November 2005 or January and February 2007. BPYV was not detected in the AQIS positive control plants.

Strawberry mild yellow edge virus: Two primer pairs were used for detection of SMYEV. Initial sequencing of the PCR product generated by the SMYEVdeta/ SMYEVdetb primer pair and screening of the positive control plants using this primer pair indicated that they were specific for SMYEV detection. However, during many of the first 12 months of testing PCR products similar to the expected size for SMYEV were generated in all plants, which was unexpected. Cloning and sequencing of this PCR products from three plants suggested that they were associated with plant nucleic acid and this primer pair was not used for the remainder of the experiment. Consequently the SYEupstcp1a/SYEPolyTb primers (Thompson and Jelkmann, 2004) were trialled for detection of SMYEV. This primer pair did not generate false positive results and was used to test all samples during the 24 month testing period. SMYEV was only detected in 3/24 plants using the SYEupstcp1a/SYEPolyTb primer pair. All three plants tested positive in August 2005 and only one plant tested positive again in April 2007. SMYEV was not detected in the AQIS positive control plants and SMYEV may not have been present in these plants. It is possible that the SYEupstcp1a/SYEPolyTb primers did not detect isolates of SMYEV in the AQIS or DPI plants due to genetic variation at the primer binding sites.

Strawberry pallidosis virus: SPaV was detected in all 23 positive control plants by both primer pairs used for detection. However, variation in the reliability of detection was observed between the two primer pairs, but neither primer pair was consistently more reliable than the other (Figure 1d). When the results of both primer pairs are combined SPaV was most reliably detected in September in 2005 (20/23 plants) and 2006 (21/23 plants). In both years detection of SPaV was unpredictable in most other months. SPaV was detected in eight of the PEQ plants using the CP5'/CPn731R primer pair and in nine plants using the SP 44 F/SP 44 R primer pair. When the results of both primers pairs are combined SPaV was most reliably detected in August (8/9 plants) and September 2006 (8/9 plants), after which a reduction in the number of positive plants occurred and detection between the two primer pairs was variable (Figure 2c).

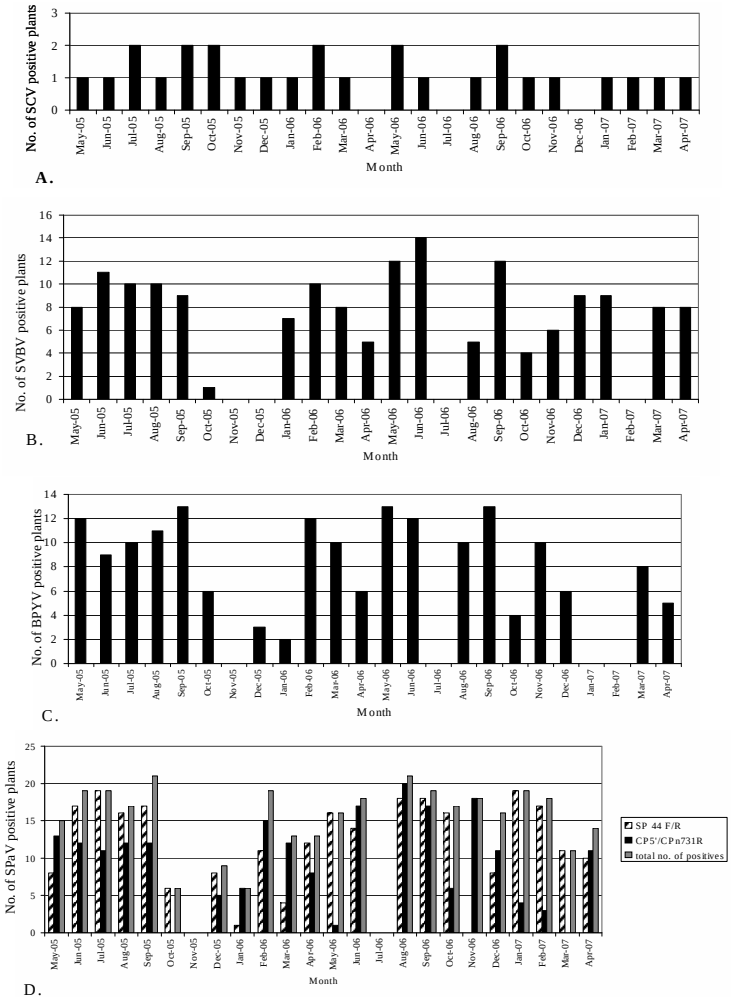

Fig. 1 The number of DPI virus positive plants in which viruses were detected during 24 months from May 2005 until April 2007. **(a)** *Strawberry crinkle virus* (SCV): SCV was detected in a total of 4 plants; **(b)** *Strawberry vein banding virus* (SVBV): SVBV was detected in a total of 20 plants; **(c)** *Beet pseudos yellows virus* (BPYV): BPYV was detected in a total of 15 plants; **(d)** *Strawberry pallidosis associated virus* (SPaV): SPaV was detected in 23 plants using the SP 44 F/R primers and the CP5'/CPn731R primers. Plants were not tested in July 2006.

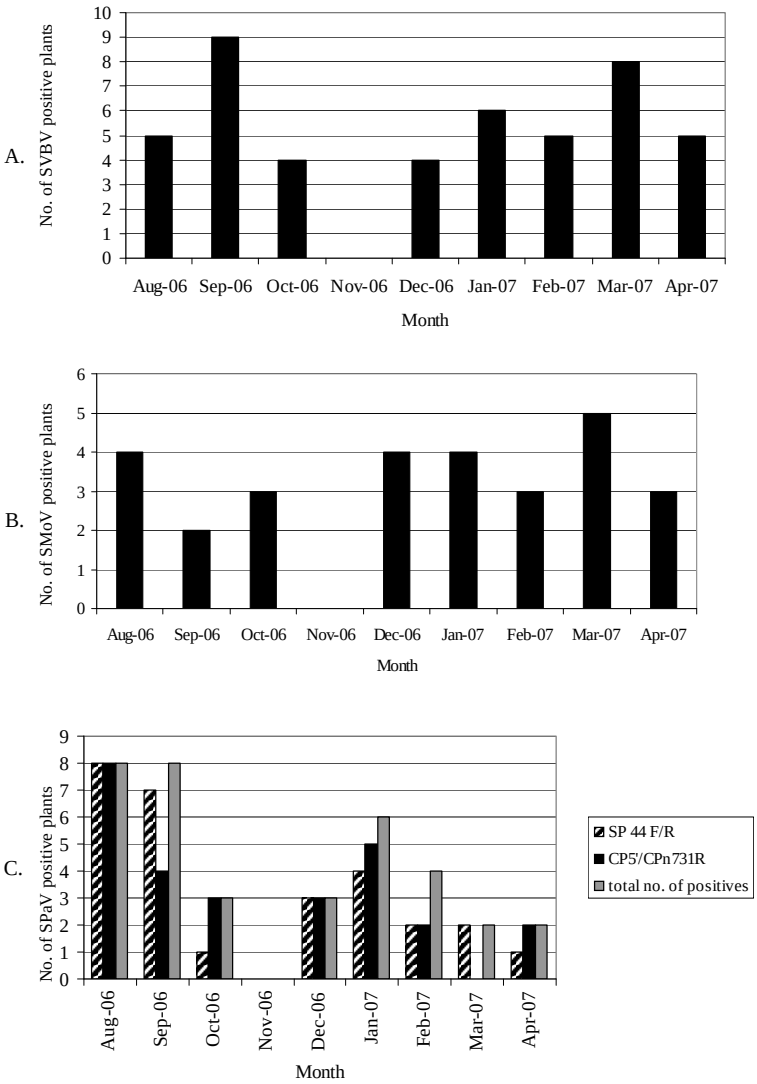

Fig. 2 The number of AQIS virus positive plants in which viruses were detected during 9 months from August 2006 until April 2007. **(a)** *Strawberry vein banding virus* (SVBV): was SVBV was detected in a total of 12 plants; **(b)** *Strawberry mottle virus* (SMoV): SMoV was detected in a total of 8 plants; **(c)** *Strawberry pallidosis associated virus* (SPaV): SPaV was detected in SPaV was detected in nine plants with the SP 44 F/R primers and in eight plants with the CP5'/CPn731R primers.

Weather data: The average minimum and maximum temperatures observed for each month from May 2005 until July 2007 were compared (Figure 3). In October, November and December 2005, when a greater decline in the detection of strawberry viruses was observed, the average monthly minimum temperatures were 3.1 °C, 2 °C and 1.2 °C warmer than in the same months in 2006 and the average monthly minimum temperatures were 0.7 °C cooler in October, 0.9 °C warmer in November and the same in December in 2006. The average maximum temperatures, for all years, in August and September were 15 °C and 18 °C respectively and 27 °C for December and January. The average minimum temperatures, for all years, in August and September were 7 °C and 8 °C respectively, and 11 °C and 14 °C for December and January respectively.

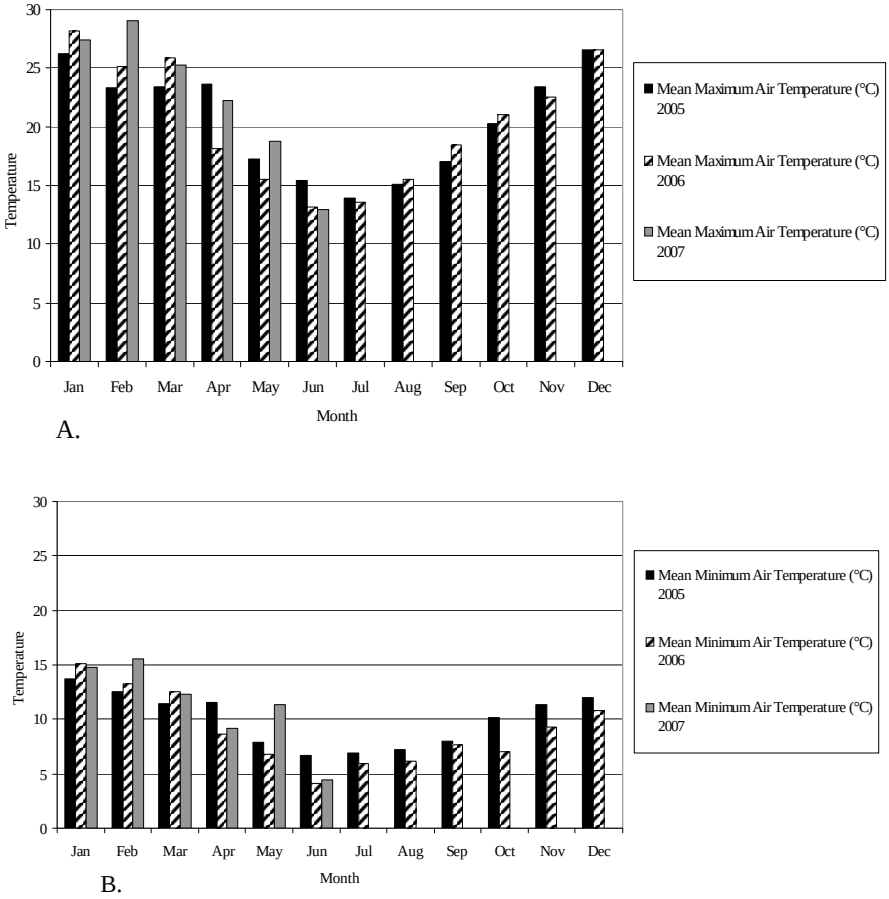

Fig. 3 The mean monthly (a) maximum and (b) minimum air temperatures at Knoxfield for 2005, 2006 and 2007.

Discussion

The PCR tests that were developed have been applied to strawberry plants infected with one or more viruses and these plants have been tested monthly since May 2005. The PCR results suggest that spring or autumn may be ideal times for virus detection by PCR in strawberry plants in south east Australia. Although positive results can be obtained during winter for each virus using the PCR tests that were developed, winter is not suitable for virus testing for certification in south east Australia because mother plants destined for the nucleus collection are placed in cold storage to induce dormancy and meet chilling requirements there is no material available.

In November, December and January of each year a decline in the number of positive PCR results for SPaV, SVBV and SPaV was observed. November was the least reliable month for detection of strawberry viruses in these plants. The number of positive results obtained from the positive control plants for SPaV, SVBV and BPYV began to increase again from to April in each year. These results indicate that there is a seasonal effect on detection of some viruses in strawberries and it is possible that the seasonal effect was related to the higher temperatures that were observed in summer compared to spring and autumn, however the mechanism of this effect is not understood.

The decline in the number of positive results for SPaV, BPYV and SVBV in the DPI plants in Spring 2006 was not as marked as in Spring 2005, indicating that there may also be changes in the seasonal trend for detection from year to year, even under glasshouse conditions. This could be due to climatic differences from year to year affecting plant physiology and/or virus titre. Although the DPI positive control plants were maintained in a glasshouse the temperature control is unreliable and fluctuates with outside air temperature. It is possible that the greater drop in virus detection in November and December in 2005, compared to the same months in 2006, was due to the slightly warmer minimum temperatures observed during this time, which were between 1-3 °C warmer in 2005 compared to 2006. It is possible that temperature could influence virus replication directly or indirectly via an influence on plant physiology and change the rate of replication and titre, however, no work has been done yet to support this hypothesis.

SCV was detected in 4/23 DPI plants and SMYEV was detected 3/23 DPI plants and neither virus was detected in the AQIS positive control plants. Based on the small number of infected plants, we cannot conclude which time of year is best for detection of these two viruses.

At other times of the year the number of positives fluctuated from month to month. This inconsistency of positive results for some viruses may have been due to uneven titre or location of the strawberry viruses within the host plant and changes in the way plants are sampled (e.g. sampling more leaves) might reduce the risk of false negative results.

A 100 % efficiency of detection by PCR was rarely obtained for any of the viruses at any time of the year. The lower efficiency may have been due to environmental factors affecting virus titre at each sampling period. It is also possible that uneven distribution of the viruses in the plant may have affected PCR efficiency and that sampling techniques need to be improved. Improved extraction techniques, to reduce the amount of co-extracted compounds that can inhibit PCR may also improve the efficiency of the PCR tests. It is possible that strain variation of viruses occur within a plant and between plants and that some strains may be less efficiently detected by the PCR tests due to sequence differences at the primer binding sites. It is also possible that these variants may respond differently to environmental factors affecting virus and strain titre at each sampling period. Further work to determine the strain variation within the virus species found in Australian strawberry plants is required. In the meantime, we recommend that plants are tested at least twice per year, in spring and in autumn when the highest number of positive results were obtained, to improve that chance of detection by PCR.

The high proportion of SPaV and BPYV in the DPI positive control plants is not unexpected as these viruses are transmitted by greenhouse whitefly (Duffus, 1965; Tzanetakis et al., 2004c), which has occurred sporadically in the DPI glasshouse. It is interesting to note that BPYV was not detected in the AQIS plants. It is possible that the presence of this virus in the DPI plants was due to their exposure to another source of BPYV from a different host plant species to which the AQIS plants were not exposed. However it is also possible that strain variation contributed to false negative results in the AQIS plants and it may be useful to trial other primers for the detection of BPYV in strawberries.

The results generated by the RT-PCR tests for SPaV, when they were compared with each other, were variable throughout the two years and in many months some plants tested positive with one test but not the other. However neither test was more reliable than the other over the entire 24 months. As a consequence both primer pairs should be used for detection of SPaV, until an improved RT-PCR test is developed. The reason for the unpredictability of detection between the two primers pairs is unknown. It is possible that the variation is a reflection of a change in the population of strains of the virus in the plant hosts that can be detected by each primer pair. More work will be done to determine the extent of variation in Australian SPaV isolates and the effect on detection by PCR.

Currently, before certification can be granted for a new variety, three years of negative biological indexing results must occur. The new variety is then incorporated into the nucleus stock, which continues to be tested annually. The increased sensitivity of PCR may allow for the reliable detection of pathogens during spring and autumn and could lead to several tests being conducted within the one season, resulting in the early detection of virus infected plants. PCR techniques will also assist the screening of plants during post-entry quarantine and during virus eradication procedures by quickly identifying infected plants, which can be discarded, and investing in the “likely” uninfected plants which would still undergo biological indexing. This will result in a reduced cost to importers of new varieties and to the Australian strawberry certification schemes and breeders. This will also give strawberry fruit growers a competitive edge in local

and overseas markets and ensure that the industry has the fastest possible access to new popular varieties without compromising plant health. Based on our results we recommend PCR should be used to identify or confirm virus infected plants and assist the interpretation of symptoms on biological indicators. However, our results indicate that virus titres and distribution may fluctuate throughout the season and that the distribution of virus within the plant maybe uneven, consequently virus infection may at times fall below levels that are detectable by PCR. The seasonal fluctuation of virus titre and distribution may also affect the reliability of the biological indexing, and we have experiments underway to examine this possibility. Our results also suggest the possibility that strain variation within a viral species may influence detection by PCR. Therefore we recommend that PCR testing for virus detection be used in combination with biological indexing during three years to certify new accessions as “high health planting material”.

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Detection of phloem restricted bacteria responsible for strawberry marginal chlorosis (SMC) by real-time PCR in a single assay

Danet, J.-L.[1*]; Fimbeau, S.[2]; Pommier, J.-J.[2]; Couture, C.[1]; Foissac, X.[1]
[1] UMR1090, INRA and Université Bordeaux[2], BP81, F-33883, Villenave d'Ornon, France
[2] Hortis Aquitaine, Maison Jeannette, F-24140, Douville, France

Abstract

Two uncultured phloem restricted plant pathogens, the *γ3* proteobacterium «Candidatus *Phlomobacter fragariae* » and the stolbur phytoplasma (group 16SrXII-A) are associated with strawberry marginal chlorosis (SMC) in France. As "Ca. *P. fragariae*" and stolbur phytoplasma induce identical symptoms, the only way to identify the pathogen infecting a given diseased plant is to perform conventional PCR assays. Because using two PCR techniques for detecting separately each of the two bacteria is time consuming and because specificity and sensitivity of the detection test needed to be improved, a new approach using triplex real time PCR was developed for the routine detection of "Ca. *P. fragariae* " and stolbur phytoplasma. The real time PCR has the advantage of being faster reduces the risks of producing false positives. Furthermore, real-time PCR techniques provide the possibility of multiplexing by using probes with different compatible fluorescent dyes. Here, we present a new sensitive Taqman® method which permits the simultaneous amplification of three DNA targets in one test: the *map* gene of stolbur phytoplasma, the *spo*T gene of "Ca. *P. fragariae*" and the *cox* gene of strawberry chloroplast taken as an internal control. The specificity and the efficiency of this method were determined.

Keywords: Strawberry Marginal Chlorosis, Triplex taqman® PCR ,Candidatus *Phlomobacter fragariae*, stolbur phytoplasma.

Introduction

During a survey of the French strawberry production carried out from 1996 to 2001, using conventional PCR detection methods, we showed that marginal chlorosis symptoms could be induced by two different pathogens: "Ca. *P. fragariae*" and the stolbur phytoplasma. "Ca. *P. fragariae*" predominated in French strawberry production fields whereas stolbur phytoplasma was prevalent in nurseries (Danet et al., 2003). However, it was later shown that the 16S-rDNA PCR used for the detection of "Ca. *P. fragariae*" also detected the proteobacterium associated to the Syndrome "Basses Richesses" of sugar beet (SBRp) (Gatineau et al., 2002). In addition, SBRp was recently detected in strawberry plants affected by SMC in northern Italy (Terlizzi et al., 2007) and a new phytoplasma "Candidatus *Phytoplasma fragariae*" that belongs to the same taxonomic group as stolbur phytoplasma had been detected in Lithuania on yellowing strawberry plants (Valiunas et al., 2006). As the 16S primers used for the detection of stolbur phytoplasma are conserved in the 16S-rDNA of 'Ca. *Phytoplasma fragariae*', the PCR assay is expected to detect both phytoplasma species. It was therefore decided to develop a new detection test aimed to be more specific for stolbur phytoplasma and "Ca. *Phlomobacter fragariae*'. Triplex real-time PCR was preferred in order to gain sensitivity and perform the simultaneous detection of the two bacteria as well as an endogenous plant analytical control.

Materials and methods

Plant material: Healthy strawberry plants (*Fragaria* x *ananassa* Dutch) were produced by meristem tip culture and *in vitro* propagation and maintained as a control in a greenhouse. Strawberry plants infected with "Ca. *P. fragariae*" or with stolbur phytoplasma were collected in strawberry production fields or nurseries.

Insect DNA extract: The origin of DNA extracts of insects, carrying proteobacteria related to "Ca. *P. fragariae*" and used to evaluate triplex Taqman specificity for "Ca. *P. fragariae*", have been described by Salar et al. (2009). These extracts consist of DNA of *Pentastiridius leporinus* infected by SBRp, DNAs of *Trialeurodes vaporarorium*, *Diaphorina citri*, *Conomelus anceps* and *Mocydia crocea*.

Plant DNA extraction: Petioles of the most symptomatic leaves were detached with a razor blade and washed with water. Total DNA of one gram of petioles was extracted using cethyl-trimethyl-ammonium bromide (CTAB) (Maixner et al., 1995). The final total DNA pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). DNA from healthy plants was also extracted as negative control.

Design of oligonucleotides and probes: Primers and TaqMan® LNA probes used for triplex real-time PCR are detailed in Table 1. They were designed using the "beacon designer" software version 5.1. The primers and probe for stolbur phytoplasma were described in a previous work (Pelletier et al., 2009), except that the probe has been modified by LNA (Locked Nucleic Acid) bases (Braasch and Corey, 2001) and extended by 4 bases at the 5' end. The probe was 5' labelled with VIC™ reporter dye. The amplified fragment was 72bp long. Primers and probe for "Ca. *P. fragariae*" were determined by comparing the *spo*T gene sequences of 5 reference bacteria, the SBRp, the *Trialeurodes vaporarorium* proteobacterium, the *Diaphorina citri* proteobacterium, the *Conomelus anceps* proteobacterium, and the *Mocydia crocea* proteobacterium (respectiveGenbank accession number FM992680, FM992677, FM992682, FM992678, FM9926679). The probe was 5' labelled with FAM™ reporter dye. The amplified fragment was 75 bp long. Primers and probe sequences for the amplification of the endogenous control (EC) were determined on the basis of the alignment of *cox* genes of 10 plant species : *Solanum tuberosum, S. lycopersicum, Pisum sativum, Brassica juncea, Oenothera berteroana, Populus tremuloides, Beta vulgaris, Oryza vulgaris* and *Zea mays* (respective Genbank accession numbers X83206, X54738, AF338446, X14409, AY300014, X05465, U77623, DQ381450, X15990 and AF542203). The probe was 5' labelled with TEXAS RED ™ reporter dye. The amplified fragment was 81 bp long. All probes were 3' labelled with a non-fluorescent quencher (BHQ).

Tab. 1 Primer and probe sequences for simultaneous detection of "Ca. *P. fragariae*", stolbur phytoplama and a plant endogenous control by real-time PCR.

Name	**Target**	**Sequence 5'→ 3'**
Coxfrag-F	Plant	CGTCGCATTCCAGATTATTCC
Coxfrag-R	mitochondrial	CCCAACTACGGATATATAAGAGC
Coxfrag-TexasRed-LNA	*cox* gene	TEXAS RED-AAAL*TGCZ*AAGGGCATTCCA-BHQ-2
MapStol-F	Stolbur phytoplasma	ATTTGATGAAACACGCTGGATTAA
MapStol-R	map gene	TCCCTGGAACAATAAAAGTYGCA
MapStol-VIC-LNA		VIC-CAE*AE*AE*CL*CE*CE*AAE*TGC-BHQ-1
SpoTphlomo-F	"Ca. *Phlomobacter*	AGGTCATGCCTGTGTTGGAG
SpoTphlomo-R	*fragariae*"	TGGTTTGCCGGTACTTAAC
SpoTphlomo-FAM-LNA	spoT gene	FAM-TGGGACAP*AGGAE*AGGGTTGACGA-BHQ-1

*LNA modified bases:L=C-LNA; Z=T-LNA;E=A-LNA and P=G-LNA.

PCR assays: The current official detection method for "Ca. *P. fragariae*" using the PCR primers pair Fra4-Fra5 was performed as previously described (Zreik et al., 1998). Primers Stol11F3 andStol11R2 were used to perform the current official PCR detection method for stolbur phytoplasma which consists of a direct PCR developed from a protocol previously published for the detection of stolbur phytoplasma in grapevine (Clair et al., 2003). The TaqMan triplex real-time PCR reaction was performed in a final volume of 25 µl comprising 12.5 µl of QuantiTect Multiplex PCR buffer (Qiagen), primers and probes at a final concentration of 0.2 µM and 5 µl of purified DNA. Amplification and detection were performed using the CFX 96 Real-Time system apparatus (Bio-Rad). The thermal cycle consisted in a pre-step of 15 min at 95 °C for Hot Start Taq DNA polymerase activation, followed by 40 cycles of 60 s denaturation at 94 °C and 90 s hybridization and elongation at 59 °C. Each reaction included at least one blank assay without template and two negative controls corresponding to healthy plants. The software Bio-Rad CFX manager was used for fluorescence acquisition and estimation of threshold cycles (Ct). For this estimation, the baseline was automatically set and the fluorescent threshold was set manually for each individual target to intersect with the linear part of all amplification curves.

Evaluation of the Taqman real-time assays efficiencies: The efficiency and the linear range of each real-time PCR reaction were evaluated by constructing dilution curves of DNA extracts from two different samples: one strawberry plant infected with stolbur phytoplasma and one "Ca. *P. fragariae*" -infected strawberry plant. 10-fold serial dilutions were performed and each dilution was tested in duplicates. The slope (k) of the linear regression line between logarithmic values of the dilution factor (x-axis) and estimated Ct values (y-axis) was used to calculate the amplification efficiency, $E = (10^{-1/k}-1) \times 100$ (Pfaffl, 2004).

Results

Specificity of the triplex real-time PCR assay: The specificity of the stolbur phytoplasma real-time PCR assay was previously tested (Pelletier et al., 2009). The specificity of "Ca. *P. fragariae*" real-time PCR was tested on DNA extracts from healthy plant and on DNA extracts from insects carrying proteobacteria phylogenetically related to "Ca. *P. fragariae*". In this case, the Ct value was 27.8 for the strawberry DNA infected by "Ca. *P. fragariae*" whereas no significant signal was observed for the other bacteria (Figure 1).

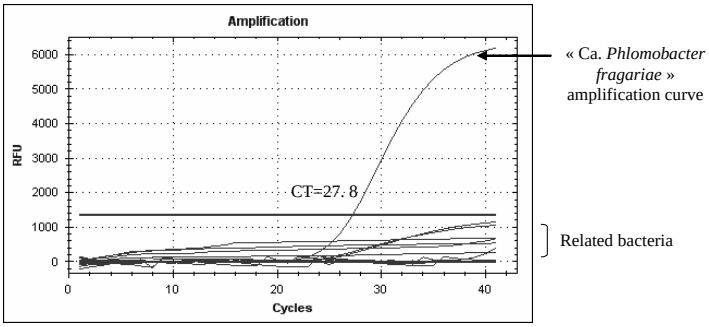

Fig. 1 Taqman® PCR amplification curves of proteobacteria phylogenetically related to "Ca. *P. fragariae*": SBR proteobacterium, *Trialeurodes vaporarorium* proteobacterium, *Diaphorina citri* proteobacterium, *Conomelus anceps* proteobacterium, *Mocydia crocea* proteobacterium

Performance of the triplex real-time PCR assay in comparison with the simplex real-time PCR assay: Calibration lines were constructed for each target by analyzing ten times serial dilutions of total DNA extracted from one strawberry plant infected by "Ca. *P. fragariae*" and one strawberry plant infected by stolbur phytoplasma. PCR efficiency values were 100.6 %, 100.2 % and 101.7 % for the "Ca. *P. fragariae*", stolbur phytoplasma and EC target respectively when they were tested in simplex real-time PCR and 101.71 %, 102.5 % and 112.9 % when they were tested in a triplex real-time PCR. Therefore, these results indicated that multiplexing did not reduce the efficiency value.

Triplex real-time PCR detection sensitivity in comparison with the conventional PCR official test: Sensitivities of the triplex real-time PCR assay and the official PCR test were compared by analysing the same serial dilutions. Figure 2 shows results obtained for "Ca. *Phlomobacter fragariae*" and stolbur phytoplasma detection on these dilutions. For "Ca. *Phlomobacter fragariae*", the triplex Taqman PCR gave positive amplification up to a dilution of 10^{-4} whereas the official PCR detected the bacterium DNA up to a dilution of 10^{-2}. For stolbur phytoplasma, the Triplex Taqman PCR gave a positive amplification up to a dilution of 10^{-6} whereas the official PCR detected the phytoplasma DNA up to a dilution of 10^{-5}. The sensitivity of the triplex real-time PCR is therefore 100 times higher than the current PCR for the "Ca. *Phlomobacter fragariae*" and 10 times higher for the detection of the stolbur phytoplasma.

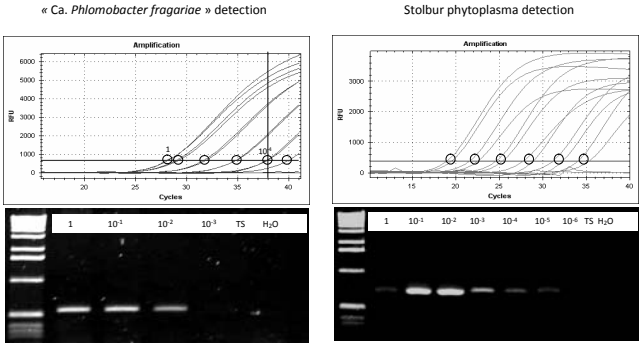

Fig. 2 Taqman® PCR amplification curves of tenfold dilutions of strawberry plant DNA infected with "Ca. *P. fragariae*" or with stolbur phytoplasma (top). Agarose gel electrophoresis of the official conventional PCR assay on the same DNA dilutions (bottom)

Discussion

A new real-time PCR method was developed for the simultaneous detection of "Ca. *P. fragariae*" and stolbur phytoplasma in strawberry plants with an endogenous control. The use of the TaqMan technology allowed the multiplexing of three different targets: *spo*T gene of "Ca. *P. fragariae*", *map* gene of stolbur phytoplasma and *cox* gene

for the endogenous control. Specificity was promoted by the use of LNA conjugates (Braasch and Corey, 2001) and by the choice of a non-ribosomal target. Indeed, variability in the *spo*T gene helped discriminate "Ca. *P. fragariae*" from among bacteria of its phylogenetic group, whereas the PCR detection based on the 16SrDNA did not (Foissac et al., 2000). The triplex real-time PCR showed lowered limits of detection in comparison to the official PCR assay: up to 10 and 100 times lower for the stolbur phytoplasma and "Ca. *P. fragariae*" respectively. This new multiplex assay, improved both in specificity and sensitivity, will be used to re-evaluate the relative incidence of the two phloem-limited bacteria associated with SMC in French strawberry production.

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Sequencing studies for the identification and characterization of new and old Rubus viruses

MacFarlane, S.A.; McGavin, W.J.
Plant Pathology Programme, SCRI, Invergowrie, Dundee DD2 5DA, Scotland

Abstract

In Europe, raspberry plants are commonly infected with a complex of aphid-transmitted viruses that together cause raspberry mosaic disease (RMD). During the previous 30 years, by grafting and vector transmission to a range of red and black raspberry cultivars, these viruses have been loosely characterized and identified as Raspberry leaf spot virus (RLSV), Raspberry leaf mottle Virus (RLMV), Black raspberry necrosis virus (BRNV) and Rubus yellow net virus. An additional, very widespread virus, Raspberry vein chlorosis virus (RVCV), is spread by a different aphid vector. Recently some sequence data have been obtained for RYNV, BRNV and Raspberry mottle virus (RMoV), a virus found in plants showing RMD symptoms. We have carried out sequencing studies using random amplification and mass analysis approaches and will present information on the relationship between RMoV, RLSV and RLMV, as well as the first data for RVCV and a novel, possibly segmented minus-strand RNA virus infecting raspberry.

Kewords: raspberry viruses, RLMV, RLSV, RVCV, RLBV

Soft fruit virology at SCRI

The purpose of soft fruit virus research at SCRI is to identify and characterize viruses causing disease in the soft fruit industry in Scotland and elsewhere, to obtain sequence information from these viruses enabling us to design diagnostic tools for testing of UK Soft Fruit Nuclear Stock material (maintained as a collection at SCRI), and to use this new knowledge ultimately to identify new or improved sources of virus resistance in raspberry and blackcurrant germplasm that can be used by fruit breeders at SCRI. Our current work looks at a range of different viruses, some that have been known for a long time and others are newly identified. For example, two of the most well understood viruses we study are Raspberry bushy dwarf virus (RBDV), a pollen transmitted virus for which an effective antibody test is available, and Blackcurrant reversion virus (BRV), currently the most important virus affecting blackcurrant which has been addressed by breeding for resistance to its vector, the gall mite *Cecidophyopsis ribis*. Viruses that are known to cause disease in raspberry but for which little or no sequence information is available include Raspberry leaf spot virus (RLSV), Raspberry leaf mottle virus (RLMV) and Raspberry vein chlorosis virus (RVCV). In addition, our work has identified two new viruses, Rubus chlorotic mottle virus (RuCMV) and Raspberry leaf blotch virus (RLBV), which are now undergoing more extensive study.

Clarification of relationship between Raspberry leaf spot virus (RLSV), Raspberry leaf mottle virus (RLMV) and Raspberry mottle virus (RMoV): Leaf spotting is a common disease symptom in raspberries, attributed in Europe to two viruses, RLMV (Cadman, 1951) and RLSV (Cadman, 1952). These viruses have the same aphid vector, the large raspberry aphid *Amphorophora idaei*, and are similarly heat-labile but are differentiated by host reaction – RLMV causes chlorotic spots on the red raspberry cultivars Malling Delight, Malling Landmark and St. Walfried, whereas, RLSV causes chlorotic spots on the cultivars Burnetholm, Glen Clova and Norfolk Giant. Recently a new closterovirus, RMoV, was isolated from an asymptomatic red raspberry plant in Washington state, USA and the complete sequence of the virus was determined (Tzanetakis et al., 2007). As part of this study, material containing RLSV from Scotland was tested by RT-PCR and found to contain the same closterovirus. To examine the possibility that these three viruses are strains of the same closterovirus we have carried out a sequencing study comparing RLSV and RLMV from the SCRI virus collection, together with plant samples showing leaf spot symptoms newly collected at various farms in the UK. Using PCR primers based on the RMoV sequence we amplified and sequenced from RLMV and RLSV a 3kb region including the genes encoding the coat protein homologue (CPh), minor coat protein (CPm) and major coat protein (CP). In addition the same region was sequenced from a new RLMV isolate (PM1) that was collected from the field.

The results of this study showed that the RLMV and RLSV RNAs are 99 % identical over the 3kb region, and both are 97 % identical to RMoV. In addition, one of the field isolates, PM1, was found to be only 75 % identical to the other isolates in this region. Isolate PM1 was found in all locations that were sampled, RLMV was found in fewer locations and samples from some locations contained both isolate types. Furthermore, the CP amino acid sequences are 98 % identical between RLMV, RLSV and RMoV but there was only 78% CP sequence identity between PM1 and the three other viruses. Our conclusions from this work are that RLMV, RLSV and RMoV are all isolates of the same virus, and we propose all should now be referred to as RLMV as this was the earliest name to be given in the literature. The

taxonomic situation of isolate PM1 is less clear. Current ICTV guidelines suggest closterovirus species are demarcated by a CP amino acid sequence difference of 10 % or more which would mean that PM1 should be considered as a different virus to RLMV.

Design of a RT-PCR diagnostic for Raspberry vein chlorosis virus (RVCV): RVCV was first described in 1952 in Scotland, is extremely common in Europe and Russia, and also is present in Canada, and New Zealand. It is transmitted by the small raspberry aphid *Aphis idaei*, causing loss of vigour, fruit yield and fruit set in some cultivars. Large bacilliform particles found in infected plants and in sections of vector aphid (Jones *et al*., 1974; Murant and Roberts, 1980), suggested RVCV might be a rhabdovirus, an enveloped, negative-strand RNA virus, able to replicate in both insect and plant hosts.

Several approaches were taken in order to obtain some sequence information for RVCV. We were unable to clone the virus using RNA extracted from material collected from virus-infected plants using polyethylene precipitation or gradient centrifugation, or by isolating dsRNA from infected plants or by RT-PCR amplification with rhabdovirus-specific degenerate primers of total RNA isolated from infected plants. Subsequently, we used rhabdovirus-specific degenerate primer RT-PCR of total RNA extracted from small raspberry aphids reared on RVCV-infected plants. This approach was successful, and RVCV-specific primers designed from the cDNA cloned obtained in these experiments are able to prime amplification of the virus both from infected plants and aphids. Phylogenetic analysis confirms that RVCV is a rhabdovirus that is most closely related to Strawberry crinkle virus (SCV).

A newly discovered virus of raspberry – Raspberry leaf blotch virus (RLBV): Leaf samples were collected from red raspberry plants growing at several farms in Scotland and showing strong symptoms of leaf malformation, leaf necrosis and blotchy discolouration that was most apparent on the underside of affected leaves.

Double-stranded RNA was extracted from these leaves and cloned by semi-random primer amplification. In addition, extracts of these leaves were inoculated to a range of herbaceous plants. Inoculation to *Nicotiana benthamiana* resulted in obvious symptoms of a systemic yellow-green mosaic or blotching. The infection could be passaged a limited number times from *N. benthamiana* to *N. benthamiana*, and did not survive freezing. DsRNA cloning identified one small (200nt) cDNA with sequence related to wheat mosaic virus (previously High Plains Virus). This is a new group of viruses with a multipartite, negative-strand RNA genome that also includes pigeonpea sterility mosaic virus (PPSMV), fig mosaic virus (FMV) and European mountain ash ringspot-associated virus (EMARAV).

We have named this new virus raspberry leaf blotch virus (RLBV), have completed the sequencing of the RLBV nucleocapsid RNA, have raised an antibody to the nucleocapsid protein, and have designed a RT-PCR diagnostic that detects the virus in infected plants. The symptoms seen on infected raspberry plants were previously associated with an eriophyid mite, raspberry leaf and bud mite (*Phyllocoptes gracilis*). WMV, FMV and PPSMV are also associated with eriophyid mites, and experiments to show whether *P. gracilis* is responsible for transmission of RLBV are underway.

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Emerging strawberry virus and virus-like diseases in the world

Tzanetakis, I.E.
Department of Plant Pathology, Division of Agriculture, University of Arkansas, AR, USA

As strawberry demand and production increases , so too do the virus diseases that affect them. In the last ten years there has been significant work towards the characterization and detection of graft-transmissible diseases and today the number of strawberry viruses has more than doubled compared to the number we knew of at the turn of the century (Martin and Tzanetakis, 2006). Another significant change in the last years is the presence of multiple virus infections that synergistically cause severe disease and losses in several strawberries-producing areas. This review aims to summarize the information on strawberry viruses and diseases that was accumulated during the first part of the 21st century.

The most important strawberry viruses are those transmitted by aphids. This virus group has always been a major problem wherever the strawberry aphid (*Chaetosiphon fragaefolii*) is present (Converse, 1987). There are now seven aphid transmitted viruses, Strawberry mild yellow edge (SMYEV), Strawberry vein banding (SVBV), Strawberry crinkle (SCV), Strawberry mottle (SMoV), Strawberry chlorotic fleck (SCFV), Strawberry pseudo-mild yellow edge (SPMYEV) and Strawberry latent C (SLCV).

SMYEV is an aphid-borne potexvirus (Jelkmann et al., 1992) and SVBV is a caulimovirus (Petrzik et al., 1998). In the last decade, a study with several SMYEV isolates revealed significant virus diversity and this information was used to develop primers that allow detection of all diverse isolates identified (Thompson and Jelkmann, 2004). This information has been invaluable in certification programs for elimination of the virus given its significance in strawberry production.

SCV, another major strawberry virus, was known to be a persistently transmitted *Cytorhabdovirus*, but now there is sequence information (Schoen et al., 2004) that has been used to develop new detection techniques that allow fast and sensitive detection (Posthuma et al., 2002; Klerks et al., 2004; Mumford et al., 2004). A major strawberry disease, mottle, now known to be caused by SMoV, a semi-persistent virus that is closely related to *Satsuma dwarf virus* and *Black raspberry necrosis virus* (Thompson et al., 2002). Several isolates have been characterized and this information has been used for the development of a robust detection method of the virus in certification programs and the field (Thompson and Jelkmann, 2003). SCFV, a closterovirus, was isolated and characterized from the only chlorotic fleck diseased plant known to exist, but the presence of several other viruses in this plant prevented the determinating whether CF is the sole cause of chlorotic fleck disease (Tzanetakis et al. 2007). Detection protocols for the virus have been employed in the North America and Europe and the virus was found in both continents in small number of plants (this meeting, Martin and Tzanetakis; Ratti, personal communication). This information indicates that the virus is probably not a major problem for strawberry production. The other two aphid-borne viruses, SPMYEV and SLCV are poorly characterized but there is information on their phylogenetic placement. SPMYEV is a carlavirus (Yoshikawa and Inouye, 1986) and SLCV a nucleorhabdovirus (Yoshikawa and Inouye, 1988). An antiserum has been developed for SPMYEV and there are efforts under way to characterize SPMYEV and SLCV at the molecular level.

A new group of viruses, members of the genus *Crinivirus* have emerged as a new threat to strawberry in areas where whiteflies, crinivirus vectors, are present. To date all criniviruses are transmitted by whiteflies in the genera *Trialeurodes* and *Bemisia*. There are four new criniviruses discovered in strawberry, *Strawberry pallidosis associated virus* (SPaV) (Tzanetakis et al., 2004), *Beet pseudo-yellows virus* (BPYV) (Tzanetakis et al., 2003) and strawberry criniviruses 3 and 4 (Tzanetakis and Martin, unpublished). SPaV and BPYV have been fully characterized, including their virus-vector relationships (Tzanetakis et al., 2006), whereas there is only limited sequence information available for strawberry criniviruses 3 and 4. SPaV and BPYV are the most common of the four and are present in both the New and Old World. SPaV has a limited host range but some of the alternative hosts are common strawberry field weeds (Tzanetakis et al., 2006). BPYV has a wide host range ranging from strawberry and blackberry to beet and spinach. Both viruses are transmitted semi-persistently by the greenhouse whitefly (*Trialeurodes vaporariorum*). Although there are both immunological and molecular (reverse transcription polymerase chain reaction/RT-PCR) tests available for SPaV and BPYV, the preferred detection method is RT-PCR against conserved polymerase sequences because of the low titer of the viruses and the genetic diversity observed between different isolates that can be lower than 80% in the nucleotide level.

The pollen-borne ilarviruses that infect strawberry include Strawberry necrotic shock (SNSV), Apple mosaic (ApMV), Tobacco streak (TSV) and Fragaria chiloensis latent (FClLV). SNSV is the predominant ilarvirus in the United States whereas FClLV has significant presence in Chile (this meeting, Martin and Tzanetakis). TSV is uncommon in

strawberry in the United States (Tzanetakis et al., unpublished) and previous reports of the virus in the crop were probably actually due to SNSV (Tzanetakis et al., 2004). ApMV was first found naturally infecting strawberry a few years ago (Tzanetakis and Martin 2005), although it was known that the virus can replicate in strawberry through grafting experiments. The virus was found in the only known plant with strawberry leafroll disease, but as was the case with chlorotic fleck the plant was also infected with SPaV and BPYV that might have contributed to the observed symptomatology. Several hundred plants have been tested for ApMV infection in the United States and the infection percentage was miniscule. The high titer ilarviruses reach in strawberry, the great diversity observed with many ilarviruses (Petrzik and Lenz, 2002) and the excellent antisera available for these viruses make immunological detection methods, such as ELISA, the preferred detection method for these viruses.

Modern strawberry cultivation has minimized the impact of nematode-borne viruses but the reduced use of methyl bromide and other soil fumigants may lead to the re-emergence of this group of viruses in the future. There are five nematode transmitted viruses found in the crop, *Tomato ringspot* (ToRSV), *Strawberry latent ringspot* (SLRSV), *Arabis mosaic* (ArMV), *Raspberry ringspot* (RpRSV) and *Tomato black ring* (TBRV). All but ToRSV are primarily found in Europe. The major discovery concerning nematode-borne viruses in strawberry in the last decade was the discovery of SLRSV in the United States and Canada (Martin et al., 2004). Like ilarviruses ELISA is the preferred detection method for strawberry nematode-borne viruses.

Three other viruses have been found in strawberry, Tobacco necrosis, Fragaria chiloensis cryptic and Fragaria latent. They have not been reported to cause significant losses as is also true for the still uncharacterized agent that causes feather-leaf disease.

Tolerance of modern cultivars to single virus infections together with much of the cultivation now done on an annual system have minimized the effect of viruses in the crop. However, there have been cases of severe outbreaks that have caused losses in the tens of millions of dollars to growers. Such is the case of the virus-caused decline that occurred in the west coast of North America in the 2002-2003 seasons and led to losses that exceeded $50 million (Martin and Tzanetakis, 2006). The new information developed on strawberry viruses over the last decade is being applied to improve certification schemes, which are the cornerstone in controlling virus disease of perennial crops such as strawberry.

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Introduction of certification program in production of plum planting material

Jevremović, D.; Paunović, S.
Fruit Research Institute, Kralja Petra I 9, 32000 Čačak, Republic of Serbia

Abstract

Certification program for the production of fruit planting material has not been fully established in the Republic of Serbia. Despite this fact, Fruit Research Institute, Čačak has initiated the introduction of certification into the production of plum planting material of cultivars developed at the Institute. The main goal is to establish plum mother plantations with basic material satisfying the EPPO recommendations and national certification standards.

Propagated material from pomologically selected trees in commercial and experimental orchards was collected and grafted onto virus-free *Myrobalan* rootstock. Candidate clones are kept in screen house which ensure absence of infection. Fifteen plum (*Prunus domestica*) cultivars are included in this study: 'Čačanska Lepotica', 'Čačanska Rodna', 'Čačanska Najbolja', 'Čačanska Rana', 'Valjevka', 'Valerija', 'Čačanski Šećer', 'Jelica', 'Timočanka', 'Boranka', 'Mildora', 'Krina', 'Pozna Plava', 'Požegača', 'Stanley', and perspective hybrid 14/21.

All tests were done according to the EPPO recommendations. Selected clones were tested on woody indicators *Prunus tomentosa*, *P. persica* and *P. serrulata* cv. Shirofugen. ELISA test was duly performed for the detection of the following viruses: *Plum pox virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Apple chlorotic leaf spot virus*, *Apple mosaic virus* and *Myrobalan latent ringspot virus*. To increase the sensitivity of *Plum pox virus* detection, IC-RT-PCR was used. The material was also tested for the presence of '*Candidatus* Phytoplasma prunorum' by nested-PCR method.

The presence of viruses was found in 8 plants. ELISA test revealed that four plants of cv. 'Jelica' were found to be positive on the presence of *Apple chlorotic leaf spot virus*. Latent infection with *Plum pox virus* was detected by IC-RT-PCR in 4 candidate clones (1 plant of each of cvs 'Valerija', 'Čačanska Rodna', 'Čačanska Lepotica' and 'Požegača'). The rest of the material was free of all other viruses. The infection with '*Candidatus* Phytoplasma prunorum' was not evidenced in any of the tested plants.

Keywords: certification, plum, viruses, phytoplasmas.

Introduction

The production of fruit and grapevine planting material has had a long history in Serbia. Over the past decades, numerous nurseries have intensively been producing planting material. The material is not only producing for domestic growers but also for export. An ever increasing demand for rootstocks and propagation material (buds, graft wood) for this production, especially for certified category, has been evidenced in Serbia over the past few years. There are two ways for obtaining this material: one is import from abroad and the other one relies on domestic production.

Early work on the production of healthy fruit planting material in Serbia began in - '70'-s at Fruit Research Institute, Čačak. Using biological tests, as the only method available at that time, virus-free material was produced for the establishment of mother plantations (Ranković, 1981). The implementation of certification program in the production of fruit planting material according to recommended EPPO certification schemes began in 2002 through a project funded by the Ministry of Science and Technological Development. Later work was implemented in activities within another research project. In part these activities relate to plum as major and traditional fruit culture in Serbia and major fruit species in breeding program in Fruit Research Institute Čačak.

Material and methods

The material for this study includes 89 plum trees that represent the collection of candidate clones. The collection was formed by grafting buds from pomologically selected trees in commercial and experimental orchards onto virus-free *Myrobalan* rootstock. Selected plants were visually inspected for any symptoms suggesting possible presence of viruses. Candidate clones were kept in screen house where plants were safe from infection. Fifteen plum varieties were included: 'Čačanska Lepotica', 'Čačanska Rodna', 'Čačanska Najbolja', 'Čačanska Rana', 'Valjevka', 'Valerija', 'Čačanski Šećer', 'Jelica', 'Timočanka', 'Boranka', 'Mildora', 'Krina', 'Pozna Plava', 'Požegača', 'Stanley', and perspective hybrid 14/21. From the very beginning all plants were tested for all pathogens listed in EPPO certification scheme for almond, apricot, peach and plum (OEPP/EPPO, 2001). Three types of tests were performed: biological, serological and molecular.

Woody indicators *Prunus tomentosa*, *P. persica* GF305 and *P. serrulata* cv. 'Shirofugen' were used for biological testing. Two buds per each tested clone were grafted on the above indicators. Grafting on *Prunus tomentosa* and *P. persica* indicators was performed in glasshouse in three repetitions per each plant-indicator combination, while testing on the *P. serrulata* cv. Shirofugen was done in open field. After grafting, all indicator plants were visually inspected for the presence of symptoms caused by pathogens.

Serological ELISA testing (Clark and Adams, 1977) was done every year. Testing was performed in the appropriate time for the detection of six viruses: *Plum pox virus* - PPV, *Prune dwarf virus* - PDV, *Prunus necrotic ringspot virus* - PNRSV, *Apple chlorotic leaf spot virus* - ACLSV, *Apple mosaic virus* – ApMV and *Myrobalan latent ringspot virus* - MLRSV. Reagents from BIOREBA AG, Switzerland were used for detection of PPV, PDV, PNRSV, ApMV and ACLSV, whereas for the detection of MLRSV, reagents from BIORAD, France were used. Testing was done according to the manufacturer's recommendations. Samples (1:20) were homogenized in PBS-Tween + 2 % PVP buffer. OD values were recorded on the Multiskan MCC340 plate reader.

To increase the sensitivity of *Plum pox virus* detection we used IC-RT-PCR with P1/P2 primer set (Wetzel et al, 1991; OEPP/EPPO, 2004).

All candidate clones were tested for the presence of 'Candidatus *Phytoplasma prunorum*' by nested-PCR method. DNA extraction was performed according to Angelini et al., 2001. Nested-PCR was done with two primer sets: P1/P7 for the first round and R16(X) F1/R16(X) R1, for the second one (Schneider et al, 1995; Lee et al, 1995). PCR products were analyzed in 5 % polyacrylamide gel electrophoresis and staining with silver-nitrate (Schumaher et al, 1986).

Results

None of the tested plants was positive in biological testing. All inoculated indicators were symptomless while positive controls showed clear symptoms corresponding to the inoculated viruses. Indicators were visually inspected after inoculations in two growing seasons.

Serological tests were performed every year after the formation of candidate clones collection. Of all analyzed plants only 4 plants of 'Jelica' were positive for presence of *Apple chlorotic leaf spot virus*. Serological testing was performed before planned test on woody indicators. The plants were removed from the collection and hereupon Sharka-like symptoms caused by ACLSV appeared on leaves. In all other plants none of the tested viruses (PPV, PDV, PNRSV, ApMV, ACLSV and MLSRV) were found in repeated tests.

IC-RT-PCR test was done using leaves as test sample. In 4 out of 89 analyzed plants latent infection with *Plum pox virus* was detected. One plant of each of cvs 'Valerija', 'Čačanska Rodna', 'Čačanska Lepotica' and 'Požegača' were found to be positive for PPV. None of these plants was positive either on woody indicators or in ELISA test. Analyzing the PCR results in polyacrylamide gel, very slight bands of expected size 243 bp appeared. Four positive plants were removed from the collection. In nested-PCR test for the presence of '*Candidatus* Phytoplasma prunorum' no positive samples were found.

Obtained virus-free plants, maintained in screen-house present nuclear stock material of 15 plum cultivars and one perspective hybrid. Basic material for the establishment of mother plantation will be produced by the multiplication of this material under controlled conditions

Discussion

Besides true-to-type, healthy planting material is a major precondition for successful fruit production. A number of field and laboratory work is needed to check health status of selected clones. Biological indicators are compulsory in certification programs. Indicators *Prunus tomentosa*, *P. persica* and *P. serrulata* cv. 'Shirofugen' are recommended by EPPO and are also listed in Serbian bylaws. *Prunus tomentosa* and *P. persica* are used for the detection of a wide range of pathogens. *Prunus persica* GF305 is recommended for the detection of numerous pathogens, such are ACLSV, ApMV, MLRSV, PPV, PDV and PNRSV. This indicator is also used for the detection of phytoplasma which causes European stone fruit yellows disease. *Prunus tomentosa* is suitable indicator for PPV and other viruses (Damsteegt, 1997; Ranković, 1980). *Prunus persica* is not a suitable indicator for PPV-Rec strain. PPV-Rec causes no or very mild symptoms on this indicator (Glasa et al., 2005). *Prunus serrulata* cv. 'Shirofugen' is recommended indicator for PDV and PNRSV. In our tests none of these indicators showed symptoms.

ELISA test is a suitable method for routine detection allowing large-scale testing for viruses for which antiserum is available. The correct time for testing and appropriate sample type ensures successful of detection. Early detection of ACLSV in 'Jelica' reduced time for detection, and time-consuming biological test was avoided.

IC-RT-PCR method was used to increase sensitivity of biological and ELISA test for PPV detection. PPV is the most detrimental pathogen of stone fruits. In Serbia it is commonly found in plum, peach and apricot (Jevremović et al., 2008). It is often present at low concentrations and it is unevenly distributed in young trees. Four of the analyzed samples were found positive in IC-RT-PCR testing, nonetheless but not in biological and serological tests did not present such result. Negative result suggests its uneven distribution and very low concentration in these plants, or just a 'false positive' in IC-RT-PCR. To be completely reliable in health status of the analyzed plants, we removed 4 plants were removed from the collection.

The presented results showed that a majority of selected plants of plum cultivars are not infected with viruses. The past work on the production of virus-free material has laid a good foundation for the present investigations and adjustments of law regulation system using all available techniques. At present, there are no specialized institutions for certification of crops in Serbia. This study on the implementation of certification program according to EPPO in the production of fruit planting material is currently the only one in Serbia. Fruit Research Institute is leading institution in the field of fruit breeding and pomology which possesses laboratory equipment, indicators, screen-houses and researchers indispensable for performing all steps in certification. According to the current Law on Plant Health, higher categories of planting material ('basic') are needed for the establishment of mother plantation (Official Gazette of RS 41/09). Major objectives of this study are the establishment of mother plantation with 'basic category' plants for production of certified plum reproductive material and commercialization and full evaluation of newly recognized plum cultivars.

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Confirmation of the elimination of *Apple stem grooving virus* from apple trees by *in vitro* chemotherapy

James, D.
Sidney Laboratory - Centre for Plant Health, Canadian Food Inspection Agency, 8801 East Saanich Road, Sidney, BC, Canada, V8L 1H3

Abstract

Apple stem grooving virus (ASGV) is widespread in its distribution in apple trees. The virus causes tree decline and graft union necrosis in certain combinations of scion and rootstock, and attempts are made usually to control the virus where apples are grown commercially. ASGV is difficult if not impossible to eliminate by heat therapy. In 1996, *in vitro* cultures of apple infected with ASGV were grown for 9-12 weeks on media containing quercetin and ribavirin (10 µg/mL of each), then cultured on media free of these chemicals. Analysis by immunocapture (IC) RT-PCR failed to detect the presence of ASGV, while all untreated controls were positive. Treated and untreated cultures were subcloned, rooted, hardened, and eventually planted in the field. The plants were observed and tested annually by IC/RT-PCR from 1998 - 2008. The treated plants were consistently negative by IC/RT-PCR, while untreated plants tested positive for ASGV. After 11 years of testing by the sensitive IC/RT-PCR assay it is safe to say that *in vitro* chemotherapy with quercetin and ribavirin is effective for the elimination of ASGV from apple.

Keywords: *Apple stem grooving virus*, *in vitro* chemotherapy, quercetin, ribavirin, immunocapture RT-PCR

Introduction

Apple stem grooving virus (ASGV) is an important target in any *Malus* (apple) virus certification program. The virus is widely distributed and is transmitted by budding or grafting, so use of healthy propagating material is essential for controlling the movement of the virus (Nemeth, 1986; Welsh and van der Meer, 1989). ASGV is symptomless in most commercial cultivars (Welsh and van der Meer, 1989), but may cause tree decline and graft union necrosis in susceptible scion/rootstock combinations (Yanase et al. 1990). Since ASGV infection is common, certain apple cultivars may not be readily available free of ASGV infection. In this case virus therapy may be considered. ASGV has been described as one of the most difficult viruses to eliminate by any procedure (Knapp et al., 1995a, b). Reports of ASGV elimination from apple and citrus by heat therapy (Campbell, 1968; Miyakawa, 1980) have not been confirmed by long term confirmatory testing using sensitive diagnostic assays. Heat therapy is one of several methods used for plant virus elimination which includes; shoot tip culture, meristem tip culture, chemotherapy, thermotherapy, or various combinations of the above (Spiegel et al., 1993).

James et al. (1997) claimed successful elimination of ASGV by using a combination of the antiviral chemicals quercetin and ribavirin. Shoot tip cultures of ASGV-infected apple and shoot tip cultures of ASGV-infected Nicotiana occidentalis (a herbaceous host) were treated by in vitro chemotherapy using ribavirin and quercetin at a concentration of 10 µg/mL of each. The plants were exposed to the chemicals for a period of 9-12 weeks, and subsequent testing by ISEM, herbaceous host indexing, RT-PCR, and immunocapture RT-PCR did not detect ASGV in any culture subjected to this treatment (James et al., 1997). Untreated control plants were all positive. Attempts at plant virus elimination may suppress the concentration of a virus to levels that are not detectable. Positive results may be obtained if the plants are grown for an extended period of time subsequent to being treated, then re-tested (Knapp et al. 1995b). To confirm virus elimination it is essential therefore that treated plants be propagated for an extended period, free of any antiviral chemicals in the case of chemotherapy, and delayed testing carried out to allow replication and detection of any virus particles that may have remained after treatment (Hansen, 1989). This is especially important in woody fruit trees where: a) virus replication and recovery may be slow, and b) virus detection is inefficient due to the presence of inhibitors. The use of sensitive diagnostic assays improves the validity of claims of virus elimination (James et al. 1997).

In this study apple cultures that gave negative results by immunocpature RT-PCR after *in vitro* chemotherapy were subcloned, rooted, hardened, and eventually planted in the field. The results of annual IC/RT-PCR testing of the treated plants which were maintained in the field for over 10 years confirm the elimination of ASGV.

Materials and methods

Field propagation: Shoot cultures that were treated for 9 – 12 weeks with quercetin and ribavirin (10 µg/mL of each), and gave negative results when tested by IC/RT-PCR, were rooted and planted in the field in 1997 (James, 2001). Untreated cultures that gave positive results were rooted and planted also, as positive controls. There were a total of 15 trees (9 derived from treated cultures, and 5 derived from untreated cultures) and these were planted randomly with no indication of plant status. They were identified by a number only (#1 - #15, Table 1).

Tab. 1 IC/RT-PCR analysis from 1998 – 2008 (11 years) of apple trees derived from *in vitro* cultures treated with quercetin and ribavirin (10 µg/mL of each), and untreated positive controls.

Tree #[A]	***Status***[B]	IC/RT-PCR Resultc[C]
1	T	-
2	T	-
3	UT	+
4	T	-
5	T	-
6	UT	+
7	UT	+
8	T	-
9	UT	+
10	UT	+
11	T	-
12	T	-
13	UT	+
14	T	-
15	T	-

[A] Trees derived from treated and untreated *in vitro* cultures were planted randomly and identified by numbers only for purposes of testing; [B] T = trees derived from *in vitro* cultures treated with quercetin and ribavirin (10 µg/mL of each), UT = Trees derived from *in vitro* cultures that were untreated positive controls; [C] Consistent results were obtained for the annual testing conducted from 1998 – 2008; - = negative, and + = positive.

The plants were included in the regular schedule of maintenance of field grown apple trees maintained at the Sidney Laboratory - Centre for Plant Health. The plants were pruned annually and the row of trees top-dressed with a granular fertilizer. Pest control treatments were applied on a regular basis, including treatments for apple scab, apple codling moth, and powdery mildew. The plants were blind tested annually (May – June) by various technicians for the period 1998 – 2008. Healthy apple plants were used as negative controls. Also apple plants known to be infected with ASGV were included as additional positive controls.

Immunocapture (IC)RT-PCR: IC/RT-PCR was carried out using essentially procedure A as described by James (1999). ASGV polyclonal antiserum, cross-absorbed with clarified healthy *Chenopodium quinoa* sap (*C. quinoa* was the propagation host for the purified virus used for PAb production), was purified and adjusted to 2 µg/mL. This was diluted 1:100 and 100 µL added to each 0.5 ml microfuge tube. See James (1999) for further details.

Results

The results obtained by IC/RT-PCR testing for ASGV for the 11 year period from 1998 – 2008 were consistent. All 9 trees derived from *in vitro* cultures treated with a combination of ribavirin and quercetin (10 µg/mL each) gave negative results when tested for the presence of ASGV by IC/RT-PCR. The results obtained in 2008 are shown in Figure 1. The 5 trees derived from untreated *in vitro* cultures were consistently positive (Fig. 1, 2008 results). Additional controls were always included in each assay. These included known infected plants (Fig. 1A and B, indicated as +), healthy apple (Fig 1B), and water controls (Fig 1B). The results expected with these controls were observed.

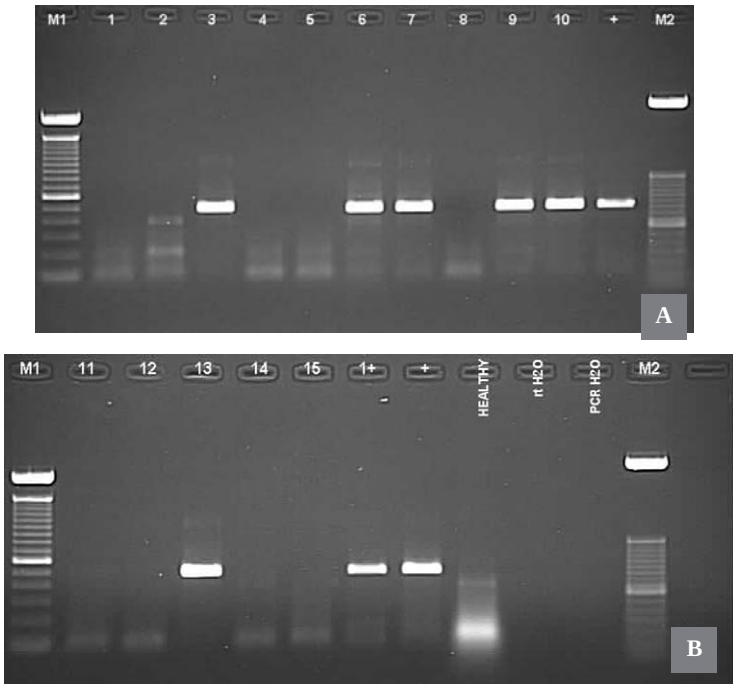

Fig. 1 Agarose gel analysis of the products of an IC/RT-PCR assay performed in 2008, of 15 randomly planted apple trees derived from *in vitro* cultures treated with quercetin and ribavirin, 10 µg/mL of each (Fig. 1A, lanes 1, 2, 4, 5, 8; Fig. 1B, lanes 11, 12, 14, 15); and apple trees derived from untreated positive controls (Fig. 1A, lanes 3, 6, 7, 9, 10; Fig. 1B lane 13). Additional positive controls (+) and water controls were included also.

Discussion

The wide distribution of ASGV (Nemeth, 1986; van der Meer, 1989) means that desirable virus-free germplasm may not always be available. Under such conditions having a reliable procedure for virus elimination may prevent the need to destroy valuable material and reduce the likely hood of planting infected material. ASGV is difficult to eliminate (Knapp et al., 1995a & b) and no well validate procedure for the elimination of ASGV has been described. ASGV elimination was claimed by James et al. (1997). Evidence of elimination was obtained for ASGV infected apple and *N. occidentalis*. In this study testing was conducted annually, over an 11 year period (1998 – 2008), by IC/RT-PCR. IC/RT-PCR is a very sensitive assay (Candresse et al., 1994; James 1999), and the use of random blind samples in this study provides increased confidence in the results. Eleven years of testing provided consistent results where the trees derived from cultures treated with quercetin and ribavirin remained negative, while trees derived from untreated cultures gave positive results consistently. The accumulated data confirms that virus elimination was achieved.

Ribavirin (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a base anolog of adenine or guanine and was developed specifically for use as an antiviral chemical (Hansen, 1989). Several modes of action have been hypothesized including the disruption of virus replication; by inhibiting nucleic acid synthesis, inhibiting RNA-dependent RNA polymerase, or inhibiting 5'-capping of viral RNA. Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a natural product flavonoid. Flavonoids have been shown to be effective antivirals against several plant viruses (French et al., 1991; French and Towers, 1992; Malhotra et al., 1996). Flavonoids enhance cAMP levels by inhibiting cAMP phosphodiesterase (Mucsi and Pragai, 1985) and this may affect virus replication. Ribavirin has been effective against a range of viruses, but in many cases the results have been disappointing (Hansen, 1989). James et al. (1997) found that some cultures treated with only ribavirin gave negative results by RT-PCR, but were positive by IC/RT-PCR. Also the concentration of the

virus increased to RT-PCR detectable levels when cultures were grown on media free of ribavirin. Quercetin was found to be more effective than ribavirin in tomato ringspot virus (TomRSV) inhibition studies (Malhotra et al., 1996).

Trees derived from *in vitro* cultures treated with ribavirin and quercetin appeared normal, with normal flowers and fruits. There was no evidence of unusual phenotypic changes or abnormalities, further confirming the observations of James (2001).

This study confirms that the antiviral chemical combination of ribavirin and quercetin (10 µg/mL of each) was effective for the elimination of ASGV from apple. ASGV was eliminated from infected *N. occidentalis* cultures by this treatment (James et al., 1997), and so it is not host specific. Treatment for 9 – 12 weeks was effective, which agrees with the findings of Malhotra et al. (1996) who found that 12 weeks was optimal for quercetin inhibition of TomRSV. It would be interesting to determine if this combination of chemicals in *in vitro* chemotherapy is effective for the elimination of other plant viruses.

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Detection of olive tree viruses in Egypt by one-step RT-PCR

Youssef, S.A.[1]; Moawed, S.M.[2]; El-Sayed, M.[3]; Shalaby, A.A.[1]
[1] Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.
[2]Central Administration of Seed Test and Certification (CASC), Giza, Egypt.
[3]Horticulture Research Institute, Agricultural Research Center, Giza, Egypt.

Abstract

Olive (*Olea Europaea* L.) is a major and economically important crop for the new reclamation land in Egypt. The estimated acreage of cultivated olive trees in Egypt is above one hundred and thirty five thousand Feddens of which total production is five hundred thousand tons per year (Ministry of Agriculture statistics, 2007). Olive trees are affected by several viruses and virus-like diseases. To date, 15 viruses in eight genera have been isolated from olive trees. In a preliminary study for the assessment of the sanitary status of olive trees in five locations in Egypt, shoots from 300 trees of 9 cultivars were collected. Using virus-specific primers, a one-step RT-PCR assay was used to detect and identify each of the eight viruses most commonly found in olives. Namely, *Cucumber mosaic virus* (CMV), *Olive latent ringspot virus* (OLRSV), *Olive latent virus*-1 (OLV-1), *Olive latent virus*-2 (OLV-2), *Olive leaf yellowing-associated virus* (OLYaV), *Strawberry latent ringspot virus* (SLRSV), *Cherry leaf roll virus* (CLRV), and *Arabis mosaic virus* (ArMV). Among the eight viruses assayed, OLRSV (6.7 %), OLV-1 (5.7 %), CLRV (4.7 %), OLV-2 (2.7 %), SLRSV (2.3 %), OLYaV (1.3 %) and ArMV (0.7%) were detected. The most common virus detected was CMV which prevailed with a high incidence of 24.7 % in olive orchards. The use of one step RT-PCR was efficient and reliable to detect the eight olive viruses found in Egypt. Surprisingly, the infection rate found is lower than expected, if we take into consideration previous surveys conducted in the Mediterranean area. This technique is useful for detection of olive viruses for production of certified plant propagative material in certification programs.

Keywords: Olive cultivars, olive virus detection, olive viruses in Egypt, one step RT-PCR.

Introduction

Olive (*Olea europaea* L.) trees are hosts to number of diseases caused by viruses, phytoplasmas, bacteria, fungi, and agents of diseases of unknown etiology which disseminate by propagating material, and are the object of certification programs in many countries, including Egypt. Sensitive and reliable detection methods of olive tree viruses are needed in these programs. Enzyme-linked immunosorbent (ELISA) is routinely used in certification as it allows sensitive, specific, and simultaneous analysis of many samples (Garnsey and Cambra, 1991). However, molecular methods based on polymerase chain reaction (PCR) amplification of the pathogen nucleic acid (Olmos et al., 1999) enable greater sensitivity especially when the target is in low concentration, or the pathogen has uneven distribution as in asymptomatic olive trees. Among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting RNA of the target in infected plants (Hadidi and Candresse, 2001). Thus, the use of PCR technology is an important step to optimize and speed up olive tree virus diagnosis. In this study, we applied a one-step RT-PCR protocol to detect the eight most common olive tree virus species that belong to five genera: *Cucumovirus*. *Cucumber mosaic virus* (CMV) (Savino and Gallitelli, 1983); *Sadwavirus*, *Strawberry latent ringspot virus* (SLRSV); *Nepovirus*, *Arabis mosaic virus* (ArMV) (Savino et al., 1979), *Olive latent ringspot virus* (OLRSV), and *Cherry leaf roll virus* (CLRV) (Savino and Gallitelli, 1981); *Necrovirus*, *Olive latent virus*-1(OLV-1) (Gallitelli and Savino, 1985); *Oleavirus*, *Olive latent virus*-2 (OLV-2) (Savino et al., 1984), and *Closteroviridae*, *Olive leaf yellowing associated virus* (OLYaV) (Savino et al., 1996).

Materials and methods

Source of plant material: Shoots from 300 symptomatic or asymptomatic olive trees were collected from five different locations in Egypt (Giza, Fauom, Nubaria, Behera and Ismailia). The trees represented 9 different cultivars, covering two native cultivars (Aggazi and Maraki) and seven imported cultivars (Dolce, Kalamata, Koronaki, Koratina, Manzanello, Picual and Sorany).

Viral RNA preparation and one-step RT-PCR amplification: Phloem tissue from young shoots were scraped and powdered in liquid nitrogen. About 100 mg of each sample was used for total RNA extraction using the Plant Total RNA Mini Kit, according to the manufacturer's protocol (Real Biotech, Corp., Taiwan). RNA was finally eluted with 50 µl of RNase- free water, and stored at -20 ºC until used. RT-PCR was carried out on RNA preparations with Reverse-iT™One-Step RT-PCR Kit (ABgene®UK). This allows RT and amplification to be performed sequentially in the same

tube. In particular, 2.5 µl of total RNA containing the target RNA was mixed with 12.5 µl 2x RT-PCR Master mix containing 1.25U/50µl Thermoprime Plus DNA Polymerase; 1.5mM $MgCl_2$, 0.2 mM each dNTPs, 10µM specific forward and reverse primers (Table 1); 0.5 µl Reverse-iT™ RTase Blend (50U/µl); RNase/DNase-free water to a volume of 25 µl. Synthesis of cDNA was performed at 47 ºCfor 30 min, followed by denaturation at 94 ºC for 2 min. Amplification was carried out for 35 cycles under the following conditions: denaturation at 94 ºC for 30 sec, annealing at 50 °C for 45sec (55 ºC in case of OLV-2), extension at 72 ºC for 60 sec, followed by a final extension for 7 min at 72 ºC. Amplified products were detected by 1-1.5 % agarose gel electrophoresis in TBE buffer, stained with ethidium bromide and visualized by gel documentation system (Bio-Rad, USA).

Tab. 1 Sequence of specific primers used for the detection of olive tree viruses.

Virus	Primer sequence	Expected size	Amplification region
OLV-1F	5'-GTGGACTGCGCTCGAATGGA-3'	230 nt	CP gene
OLV-1R	5'-CTCACCATCGTTGTGTGG-3'		
OLV-2F	5'-CCGTTCTGTGGCCTTTGAGA-3'	220nt	RdRp
OLV-2R	5'-AACACGATCCTCACCC-3'		
OLYaVF	5'-ACTACTTTCGCGCAGAGACG-3'	346nt	
OLYaVR	5'-CCCAAAGACCATTGACTGTGAC-3'		
OLRSVF	5'-AAGAATTCTGCAAAACTAGTGCCAGAGG-3'	492nt	3'terminal
OLRSVR	5'-AAAAGCTTGCATAAGGCTCACAGGAG-3'		
SLRVF	5'-AAAAGCTTCAAGGAGAATATCCCTGGCCC-3'	525nt	CP gne
SLRVR	5'-AAGGATCCTAAGTGCCAGAACTAAACC-3'		
CLRVF	5'-AAAAGCTTGGCGACCGTGTAACGGCA-3'	431nt	non coding region
CLRVR	5'-AAGAATTCGTCGGAAAGATTACGTAAAA-3'		
ArMVF	5'-TTGGCCCAGATATAGCGTAAAAAT-3'	519 nt	
ArMVR	5'-CAGCGGATTGGGAGTTCGT-3'		
CMVF	5'-GCCGTAAGCTGGATGGACAA-3'	~499nt	CP gene
CMVR	5'-TATGATAAGAAGCTTGTTTCGCG-3'		

Results and discussion

Natural infections of olive tree with viruses are mostly symptomless; those viruses that elicit symptoms in certain cultivars (e.g. SLRSV, OLYaV) are latent in others. Thus it is difficult to base diagnosis on symptoms expression. Biological indexing of olive viruses on woody differential indicators is not done as they are currently not available. The diagnostic bioassay that has been used extensively up to a recent past is mechanical transmission. This assay, however, is unreliable because of the low intrinsic sensitivity (Felix et al., 2001).

To detect viruses using PCR, it is important that nucleic acid samples extracted from the original source of plant material is free of appreciable amounts of oil, polysaccharides, phenolic compounds, and other PCR Inhibitors (Wilson, 1997). These inhibitors are present in olive tissue extracts (Amiot et al., 1989; De Niro et al., 1997). They must be removed in order to detect the viral RNA targets by PCR. The choice of an extraction technique that can be used for routine testing of a large number of samples must take into account simplicity of use and rapidity of execution. The results of this study showed that RNA extraction procedure developed is suitable for routine use in diagnostic laboratories. One step RT-PCR analysis was simple and fast; it allowed testing of hundreds of samples to be done in a relatively short time. Virus infected olive trees were found in all investigated locations and cultivars in Egypt. Each pair of the eight selected primer pairs amplified successfully its specific target RNA from total RNAs extracted from infected tissues with the Plant Total RNA Mini Extraction kit.(Figure 1). Noticeably, the majority of olive viruses were isolated from symptomless trees. The average incidence of infection for each virus tested was 24.7 % for CMV, 6.7 % for OLRSV, 5.7 % for OLV-1, 4.7 % for CLRV, 2.7 % for OLV-2, 2.3 % for SLRSV, 1.3 % for OLYaV and 0.7 % for ArMV (Table 2; Figure 2). Mixed infection was observed in large number of tested trees. The reliability of this detection method allowed increased investigations on distribution of olive viruses by conducting several surveys in different geographical areas in Egypt.

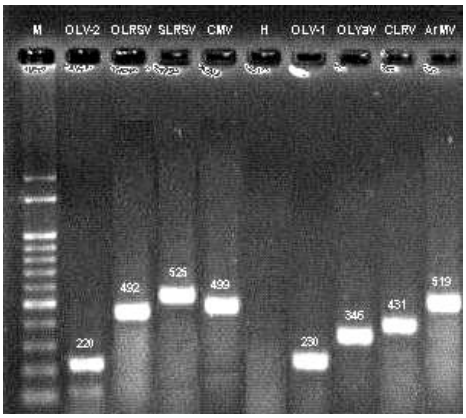

Fig. 1 Agarose gel electrophoresis analysis of amplified products obtained by one step RT-PCR using primers for the eight olive tree viruses (Table 1). Lane M, 100 bp DNA marker

Tab. 2 List of analyzed cultivars (Number of infected/number of tested) of olive trees and percentage of infection obtained by one-step RT-PCR assay for the detection of CMV, OLRSV, OLV-1, CLRV, OLV-2, SLRSV, OLYaV, and ArMV.

Cultivars	**Virus Tested**							
	CMV	**OLRSV**	**OLV-1**	**CLRV**	**OLV-2**	**SLRSV**	**OLYaV**	**ArMV**
Kalamata	8/38	6/38	0/38	2/38	2/38	0/38	1/38	0/38
Koronaki	10/48	4/48	8/48	5/48	1/48	0/48	0/48	1/48
Koratina	9/39	4/39	0/39	1/39	1/39	2/39	0/39	0/39
Picual	15/46	1/46	1/46	3/46	4/46	0/46	0/46	0/46
Manzanello	12/46	2/46	5/46	3/46	0/46	4/46	3/46	1/46
Dolce	4/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
Sorany	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Aggazi	8/31	0/31	1/31	0/31	0/31	1/31	0/31	0/31
Maraki	7/37	1/37	2/37	0/37	0/37	0/37	0/37	0/37
% of infection	24.7	6.7	5.7	4.7	2.7	2.3	1.3	0.7

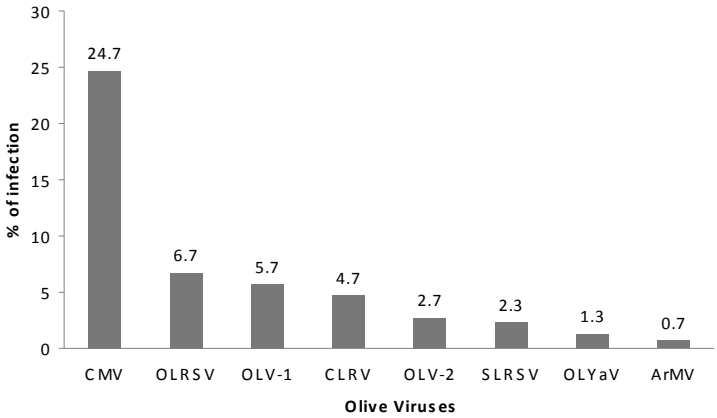

Fig. 2 Percentage of infection of the eight detected viruses (CMV, OLRSV, OLV-1, CLRV, OLV-2, SLRSV, OLYaV and ArMV) obtained in this investigation.

Theses results coupled with the sensitivity and the absence of contamination risks (since the assay is done in a single tube), made this technique very suitable for large-scale investigation (Bertolini et al., 2001; Ragozzino et al., 2004). The one-step RT-PCR protocol confirmed, for all tested olive tree viruses, its rapidity and reliability (Faggioli et al., 2005). Since the genomic sequences of the majority of viruses, including the somewhat rare OLV-1, OLV-2 and OLRSV, are known (Grieco et al., 1995, 1996a,b), the design and use of adequate PCR primers is now possible, as exemplified by the successful identification of CMV, CLRV, ArMV, SLRSV, OLYaV, OLV-1 and OLV-2 by single step RT-PCR (Sabanadzovic et al., 1999; Grieco et al.,2000; Bertolini et al., 1998, 2001).

Throughout the increasing international demand for olive plants and legislation enacted require that all olive propagative material produced in nurseries must be free of all viruses. This led to the development of sensitive diagnosis techniques to assist in selection, improvement and sanitary certification of olive planting material. Reliable virus detection is also needed in epidemiological studies and in-establishing strategies for control and certification programs.

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Validation of a microarrays protocol for detection and genotyping isolates of *Plum pox virus*

Pasquini, G.[1]; Faggioli, F.[1]; Luigi, M.[1]: Gentili, A.[1]; Hadidi, A.[2]; Canini, I.[3]; Gabriele, L.[3]; Czosnek, H.[4]; Tiberini, A.[1]; Çağlayan, K.[5]; Mazyad, H.[6]; Anfoka, G.[7]; Barba, M.[1]

[1] CRA-Centro di Ricerca per la Patologia Vegetale, Rome, Italy;

[2] Agricultural Research Service, United States Department of Agriculture, Beltsville, MD, USA

[3] Istituto Superiore di Sanità. Dipartimento di Biologia cellulare e Neuroscienze. Rome, Italy

[4] Institute of Plant Sciences and Genetics in Agriculture, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

[5] Department of Plant Protection, Faculty of Agriculture, Mustafa Kemal University, 31034 Antakya-Hatay, Turkey

[6] Plant Pathology Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza12619, Egypt

[7] Department of Technology, Faculty of Agricultural Technology, Al-Balqa' Applied University, Al-Salt 19117, Jordan

Abstract

A genomic strategy for PPV identification has been recently developed (Pasquini et al., 2008). The method is based on using a 70-mer oligonucleotide DNA microarray chip capable of simultaneously detecting and genotyping PPV strains. Universal and specific probes have been identified and used with a sensitive protocol of hybridization using an indirect fluorescent labelling of cDNA product with cyanine able to enhance the sensitivity of the virus detection avoiding the use of the PCR amplification step. In order to evaluate the protocol fitness for diagnostic use, about 30 samples belonging to a PPV isolates collection, including M, D, EA and C strains, have been used for its validation, that was determined, estimating the performance criteria that include the following parameters: diagnostic sensitivity (D-SN), diagnostic specificity (D-SP) and diagnostic accuracy (D-AC).

Keywords: oligonucleotides chip, PPV, sensitivity, specificity, accuracy, performance criteria.

Introduction

The developing of innovative viral diagnostic methods is one of the major objectives of plant virologists to increase the sensitivity and rapidity of detection of low titer viruses. In recent years, great emphasis has been made towards the development of technologies to identify simultaneously a wide range of different pathogens, including plant viruses in a single sample, thus avoiding the use of parallel tests. The availability of systems that identify a large number of plant virus targets in a single event is the prerequisite for the control of viruses in quarantine to prevent their introduction in a country through the international movement of germplasm and in national and local certification programs to reduce their spread within a country. Furthermore, a single simultaneous diagnostic protocol could be useful in the certification programs which include testing of a large number of viruses in evaluated germplasm.

Several techniques were developed for the simultaneous detection of viruses. They consist essentially of multiplex-PCR or multiplex-real time PCR, however, the number of virus targets that can be identified is limited by possible primers interactions (multiplex-PCR) or by the number of fluorochromes that can be added to the reaction (multiplex-real time PCR). Currently, DNA microarrays represent the major known technology able to identify in a single event a large number of viruses and other pathogens (Boonham et al., 2007). They were first described in 1995 (Schena et al., 1995) for simultaneous analysis of large-scale gene expression patterns. Since then, this technology was developed to extend its use to other fields including detection of human and plant viruses (Barba and Hadidi, 2007, 2010; Hadidi and Barba, 2008; Hadidi et al., 2004)

An oligonucleotide microarrays chip for simultaneously detection and genotyping strains of *Plum pox virus* (PPV) has been recently developed using PPV universal and strain-specific 70-mer oligonucleotides probes (Pasquini et al., 2008). The method has a sensitive hybridization protocol, based on the indirect fluorescent labeling of cDNA incorporating cyanines. The protocol, which avoids the use of a PCR amplification step, was sensitive and specific and may represent a model for further technology developments.

In this paper the above mentioned system was evaluated to validate its performance. The validation of a protocol is the evaluation of its fitness for diagnostic use. It is determined by estimating performance criteria that include the following parameters: diagnostic sensitivity (D-SN), diagnostic specificity (D-SP) and diagnostic accuracy (D-AC). D-SN is the proportion of known infected reference samples that test positive in the assay. D-SP is the proportion of uninfected

reference samples that test negative in the assay. D-AC is the average of D-SN and D-SP which gives a complete evaluation of the protocol.

Materials and methods

Reference samples: The number and source of reference samples used to validate a protocol are significant. The reference samples ideally should represent known infected and non-infected plants from the pathogen population. A series of reference samples, target (representing all genomic and geographical PPV variability) and non-target (representing other viruses and healthy controls), have been selected to perform all experiments (Table 1).

Tab. 1 List of PPV target and other virus and healthy non target samples used to obtain the validation parameters.

N°	Name	Specie	Variety	Origin	Pathogen	Strain
1	Ispave 2	peach	Bela c1	Ex-Yugoslavia	PPV	M
2	Ispave 8	plum	P2774	Hungary	PPV	D
3	Ispave 11	peach	Marcus	Greece	PPV	M
4	Ispave 12	apricot	Canino	Spain	PPV	M
5	Ispave 13	plum	Delickia	Austria	PPV	M
6	Ispave 17	apricot	Tolda di Castigliole	Italy	PPV	D
7	Ispave 21	plum	Centenar	Italy	PPV	D
8	Ispave 29	apricot	Canino Ancian	France	PPV	M
9	Ispave 32	plum	-	Italy	PPV	D
10	Ispave 38	apricot	Priana	Spain	PPV	D
11	Ispave 39	apricot	El Amar	Egypt	PPV	EA
12	Ispave 40	plum	Plodiuv	Bulgaria	PPV	M
13	Ispave 42	peach	Big Top	Italy	PPV	M
14	Ispave 46	plum	Goccia d'oro	Italy	PPV	D
15	Ispave 51	peach	Calipso	Italy	PPV	D
16	Ispave 53	peach	-	Greece	PPV	M
17	Ispave 148.2	N. benthamiana	-	Italy	PPV	C
18	-	apricot	-	Egypt	PPV	EA
19	-	Brassica rapa	-	Italy	TuMV	-
20	-	artichoke	C3	Italy	ArLV	-
21	-	peach	GF 305	Italy	ApMV	-
22	-	peach	GF 305	Italy	ACLSV	-
23	-	peach	GF 305	Italy	PNRSV	-
24	-	peach	GF 305	Italy	PDV	-
25	-	peach	GF 305	Italy	PLMVd	-
26	-	peach	GF 305	Italy	HSVd	-
27	-	peach	GF 305	Italy	healthy	
28	-	plum	??	Italy	healthy	
29	-	apricot	??	Italy	healthy	

Target samples were as follow:

- PPV-infected isolates originated from different Mediterranean countries and maintained as the PPV collection at the C.R.A.–Plant Pathology Research Centre of Rome, Italy (PPV-ISPaVe collection). The isolates represented four PPV strains (PPV-D, PPV-M, PPV-EA and PPV-C) and different host species (peach, plum, apricot, *Nicotiana benthamiana*).

Non-target samples were as follow:

- species of Potyviruses (*Turnip mosaic virus* - TuMV and *Artichoke latent virus* - ArLV) to verify possible cross-reaction with homologous viruses of the same genera;
- viruses commonly infect stone fruits to verify possible cross-reactions with pathogens potentially present in the assayed vegetative hosts:
- c.1. Ilarvirus species (*Prunus necrotic ringspot virus* – PNRSV, *Prune dwarf virus* – PDV, *Apple mosaic virus* – ApMV);
- c.2. Trichovirus species (*Apple chlorotic leafspot virus* – ACLSV) and
- c.3. viroid species (*Hop stunt viroid* – HSVd and *Peach latent mosaic viroid* – PLMVd);
- healthy samples from different species (peach, plum and apricot) to verify the interferences due to the assayed matrices.

DNA Microarrays protocol: Experiments were performed using microarray chips prepared with UltraGAPS Microarray Slides (Corning, NY, USA) printed at the Istituto Superiore di Sanità, Dipartimento di Biologia Cellulare e Neuroscienze, Rome, Italy with twenty-one 70-mer oligonucleotides (Pasquini et al., 2008).

The microarray design was as follows:

- The chip contained eight rows with four sub arrays (total of 32 subarrays), each sub array contained five rows of twelve spots. Each oligonucleotide was printed randomly in 12 replicated spots on sub arrays.
- Total RNA was extracted from 100 mg of each virus infected and uninfected leaf tissue using a RNeasy Plant mini kit (Qiagen Inc., Valencia, CA). A cDNA was obtained from the above plant.
- Total RNAs by using oligodT and random primers as described in Pasquini et al., 2008.
- The indirect chemical labelling of cDNA was done by adding 8 µl of cDNA to 1 µl of $Na(HCO_3)_2$ buffer 0.1M pH 9.0 and 1 µl of Cyanine 3 (Cy3) or Cyanine 5 (Cy5) dye (Amersham Bioscience, Buckinghamshire, UK) suspended in DMSO buffer and incubated for 18 h at room temperature. The labelled cDNA was purified using a QiaQuick PCR cleaning kit (Qiagen).
- Slides were pre-hybridized for 45 min at 55 ºC with a pre-heated (55 °C) blocking buffer (1 % BSA, 0.1 % SDS, 5X SSC).
- The slides were then washed and dried by centrifugation.
- The hybridization buffer, contained 1 µg of each purified labelled cDNA (typically 30 pmoles of incorporated dye), 50 % formamide, 0.1 % SDS, 5X SSC, was applied to the slide after denaturation and covered with a cover slip (HybriSlip, Schleicher and Schuell Bioscience, Keene, NH). Hybridization was performed for 20 h at 55 ºC in the dark.
- The slides were then washed with pre-heated 2X SSC, 0.1 % SDS at 55 °C (5 min, twice), followed by 0.5X SSC, 0.1 % SDS (10 min at room temperature) and finally with 0.05X SSC (5 min at room temperature, four times).
- The hybridized slides were scanned using a GenePix 4200 A array scanner (Axon Instruments Ltd., Aberdeen, Scotland, UK).
- The parameters 'mean signal–mean local background' (mean Cy3 minus B or mean Cy5 minus B) and the 'mean local background' (B) were used in further calculations. Local background was calculated using the adaptive circle method. To estimate D-SN, D-SP and D-AC the cut-off point of reaction (positive/negative threshold) was established to be at least five fold fluorescent signal above the local background.

Establishment of performance criteria: The 29 target and non-target samples were assayed in 36 experiments performed by scientists in two laboratories (CRA-PAV and Istituto Superiore di Sanità) to verify the reproducibility of the protocol. The same array scanner read the slides.

In each experiment two samples, previously labeled with the two different fluorochromes Cy3 and Cy5, were combined in the hybridization mixture. Eighteen experiments were performed by mixing target samples and eighteen experiments by mixing target and non-target samples.

The D-SE, D-SP and D-AC parameters were calculated, on the averages values of the 36 experiments, using a two-way (2x2) table (Table 2). Results of the test were classified as TP (true positive) or TN (true negative) if they were in agreement with the oligonucleotide specificity determined in the set up of the chip (Pasquini *et al.*, 2008). Alternatively, they were classified as false positive (FP) or false negative (FN) if they disagreed with the previously determined oligonucleotides strain-specificity.

Tab. 2 Two-way table for calculating performance criteria

+ obtained/+ expected (TP*)	+ obtained/- expected (FP)
- obtained/+ expected (FN)	- obtained/- expected (TN)
D-SE = TP / (TP + FN)	D-SP = TN/(FP + TN)
D-AC = TP + TN / (TP + FP + FN + TN)	

*TP = true positive; FP = false positive; FN = false negative; TN = true negative
D-SE = diagnostic sensitivity; D-SP 0 diagnostic specifity; D-AC = diagnostic accuracy

The performance criteria were estimated for each single probe and for the entire chip. The probes designed on the basis of the alignment of the RNA genome of a member of PPV-W strain were not included in the experiments because of the unavailability of isolates of this strain.

Results

In all experiments good hybridizations were always obtained, without any background problems or weak signals. No significant differences were obtained in experimental results performed in the two laboratories (data not shown). Eleven

probes showed D-AC percentages from 97 to 100 %. The lower percentages of D-AC were always determined by the lower values of D-SE, as oligonucleotide probes did not hybridize occasionally with some infected reference samples. Four probes showed very low D-AC percentages (83-87 %) due to lower percentages both of D-SE or D-SP. Two of these probes are specific for PPV-D strain (V3 and P2) and were both designed on P3 gene of the PPV genome, whereas the other two probes (F3 and D3) recognized more strains and were designed on two different genes (HCPro and in the region between CP and 3'UTR, respectively) (Table 3).

Tab. 3 Percentages of validation parameters of each probe

Probe name	PPV genes	% D-SE*	% D-SP	% D-AC
D-CP (D)	CP	96	99	97
F-2 (D)	CP	97	98	97
V-3 (D)	P3	90	85	87
P-2 (D)	P3	83	84	83
F-3 (D + M)	HCPro	82	87	85
D-3 (D + M + C)	CP-3'UTR	84	83	83
V-2 (D + M + C + EA)	3'UTR	96	100	98
M-1 (M)	NIb	99	99	99
M-CP (M)	CP	98	99	98
SoC-1 (C)	P1	100	100	100
Soc-2 (C)	HCPro	99	100	99
SwC-1 (C)	NIb	99	100	99
SwC-2 (C)	P3	100	100	100
EA-1 (EA + D)	CI	96	99	97
EA-CP (EA)	CP	98	99	98

* = D-SE: diagnostic sensitivity; D-SP: diagnostic specificity; D-AC: diagnostic accuracy

Oligonucleotide probes specific for PPV-C isolates showed the highest D-AC percentages, but only one homologous reference sample was used from *N. benthamiana.* Also the two PPV-M specific probes showed high percentages of D-AC. The V2 universal oligonucleotide probes, designed on the conserved 3'UTR region of the PPV genome, showed a D-SE of 96 %, a D-SP of 100 % and a D-AC of 98 %. When the percentages of D-AC of each probe were correlated with the percentage of sequence homologies of each oligonucleotide probe it was evident that a cut off of 80% can be established for the genotyping of the virus in this set up of DNA microarrays system (Table 4).

Tab. 4 Comparison among percentages of probe sequence homology to PPV strain sequence and percentage of D-AC

	% Homology				
Probe name	**PPV-D**	**PPV-M**	**PPV-C**	**PPV-EA**	**D-AC**
D-CP (D)	100	77	64	65	97
F-2 (D)	98	77	62	61	97
V-3 (D)	100	82	74	74	87
P-2 (D)	100	82	74	74	83
F-3 (D + M)	100	100	74	84	85
D-3 (D + M + C)	97	90	90	88	83
V-2 (D + M + C + EA)	95	91	91	90	98
M-1 (M)	80	100	72	80	99
M-CP (M)	77	100	35	67	98
SoC-1 (C)	71	65	100	65	100
Soc-2 (C)	70	42	98	31	99
SwC-1 (C)	62	71	98	65	99
SwC-2 (C)	72	72	98	77	100
EA-1 (EA + D)	88	78	77	100	97
EA-CP (EA)	65	67	24	100	98

The D-SE, D-SP and D-AC of the complete chip were established on the averages of each probe values. On the whole the protocol showed: D-SE of 94 %, a D-SP of 96 % and a D-AC of 95 %. These parameters were obtained including also the lower values of the V3, P2, F3 and D3 oligonucleotide probes.

Discussion

The 36 experiments performed in two different laboratories confirmed the versatility of the protocol as good results were always obtained, which indicated the reproducibility of the system, using different scientists, equipment and environmental conditions.

The parameters obtained by assaying the designed oligonucleotide probes against a wide range of PPV isolates substantially confirmed the probe strain-specificity reported previously (Pasquini et al., 2008). The universality of the V2 probe, designed on the conserved 3' UTR region of PPV genome, was confirmed, as it reacted with all strains and, practically, with all tested isolates. Also the specificity of the probes directed against a single PPV strain (M1 and M-CP specific for PPV-M strain; EA1 and EA-CP specific for PPV-EA strain; Soc1, Soc2, Sw1, Sw2 specific for PPV-C strain and D-CP specific for PPV-D strain) was confirmed in this investigation. The values obtained with the probes V3, P2, F3 and D3, respectively, were lower than expected which may be explained by the percentage of sequence homology of the probe. A threshold of 80% of sequence homology is need for PPV genotyping in these protocol conditions.

The validation parameters obtained for the PPV microarrays chip were very good, as the values of D-SE, D-SP and D-AC were high. These data confirmed the usefulness of this innovative technique in the diagnosis and typing of PPV and it has a great potential to be applied to other plant viruses as well as viroids and phytoplasmas. The possibility to test an infected plant sample against an high number of probes is a new research field in plant pathology without frontiers. The DNA microarrays for plant pathogen detection are a technology in a developing stage. The set up protocol, without the use of commercial kits and the amplification steps, is not expensive and very easy to hande. It means that it can be performed also in phytosanitary laboratories without the necessity of specialized personal. Moreover, the technology cost is decreasing because have been developed chips re-usable more than one time. For all these reasons DNA microarrays technology represents a diagnostic tool with great potential, as it could be used to test simultaneously in a single event all possible pathogens of a crop as previously suggested (Hadidi and Candresse, 2001, 2003).

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Real-time RT-PCR quantitative analysis of plant viruses in stone fruit tissues

Jarošová, J., Gadiou, S., Kumar, J.K.
Department of Virology, Crop Research Institute, Drnovská 507, 161 06 Prague, The Czech Republic

Abstract

Real-time PCR assays aiming at quantifying the level of plant infection by pathogens are becoming more and important. Within microbiology, the application of Real-time PCR has had the biggest impact upon the field of virology. However, Real-time PCR application in fundamental plant virology studies is still lagging behind. The use of relative and absolute quantification is discussed in this study. Also, case studies including Plum pox virus in transgenic plums C5 are presented.

Keywords**:** Plum pox virus, C5, Real-time PCR, quantitation

Introduction

Real-time PCR with melting curve analysis has already proved to be a simple, rapid, and reliable technique for the detection of plant RNA viruses (Varga and James, 2005). These highly sensitive assays were employed only for virus detection rather than quantification of virus inoculum. Real-time PCR assays aiming at quantifying the level of plant infection by pathogens have been increasing for the last few years (Gachon et al., 2004) in multiple areas of viral pathogenesis. For means of quantifying a virus in tissues, absolute quantification is contemporarily, beyond controversy, the preferred way of Real-time PCR. Here, it should be kept in mind that the obtained 'absolute numbers' are always calculated relative to the standard (RNA, cDNA, plasmid DNA, genomic DNA) and largely depend on the accuracy of the used standard (Klein, 2002). For many applications, where there is no need to know the exact number of copies in the sample, a relative quantification using a comparative quantification method (ΔΔCT-method) is sufficient (Mackay, 2004). To ensure the accuracy of the obtained data, the reaction efficiencies of the two assays either should be similar or a correction factor must be introduced into the calculation (Klein, 2002). Relative quantification in virology was neglected for a long time, even though, when conducted carefully, its data can be at least as precise as the data of absolute quantification. The aim of this study was to compare absolute and relative quantification by Real-time PCR and uniformity of results reached by both methods. For this, a case study monitoring Plum pox virus (PPV) titre in transgenic plum trees C5 was chosen. PPV is the causal agent of sharka, one of the most important diseases affecting commercial stone fruit (Németh, 1986). Six different strains of PPV have been described until now (Candresse et al., 1998; Glasa et al., 2004; James and Varga, 2005; Kerlan and Dunez, 1979; Nemchinov and Hadidi, 1996; Wetzel et al., 1991). Transgenic C5 cv. 'Honey Sweet' is a clone of *Prunus domestica* L. transformed with the *Plum pox virus* strain D coat protein gene (PPV-CP) (Hily et al., 2004; Malinowski et al., 2006; Ravelonandro, 1997; Ravelonandro et al., 2000). C5 viral resistance is based on RNA silencing (Scorza et al., 2001), i.e. a sequence-specific RNA degradation mechanism widely observed in animals, fungi and plants.

Material and methods

Nine C5 transgenic plum trees grown in an experimental orchard were bud-grafted with plum cv. 'St Julien' infected with PPV-Rec strain (Polak et al., 2008). The leave samples from the trees were collected during years 2006, 2007 and 2008. Always, three samples were collected per one tree - leaves of the 'St Julien' infectious bud (the non-transgenic part), leaves of bottom part of the C5 (leaves close to the infectious bud, transgenic part) and leaves of top part of the C5 (far from the infectious bud, transgenic part). The material was grinded in liquid nitrogen and several aliquots of 0.1g were stored at -80 °C. Thereafter, total RNA extraction was performed by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in Mekuria et al. (2003). Concentration of the RNA samples was determined and diluted to 50 ng/µl. All the samples were treated with DNase I (DNAfree, Ambion) and stored at -80 °C until further manipulation.

18S ribosomal RNA was used as the endogenous control for the relative quantification.
The primer pair for 18S ribosomal RNA gene Pru18SF1 5'-CGTCACACGCCGTTGCCCCC-3' and Pru18SR1 5-GAGCCGAGCATTTTTTCGAGCCC-3' amplifying a PCR fragment of 199 bp (NCBI Acc. Number EF211087) and primer pair specific for PPV-Rec targeting (Cter) NIb-(Nter) CP 8532-8669 (NCBI Acc. Number AY028309) RecJF: 5'-AATGATATTGATGATAGCCTTGAC-3' and
RecJR 5'-AGCTGGTTGAGTTGTTGCCAC-3' amplifying a 138bp product. Specificity of the PPV-Rec primers was checked by Real-time RT-PCR on PPV-D, PPV-M and PPV-Rec isolates (data not shown).

For the purpose of absolute quantification, a specific PPV-Rec fragment described above was inserted into the vector pGem-T (Promega Inc.) and cloned into E. coli JM-109. The plasmid was linearized at the Rsa I site and used as target in an in vitro transcription reaction performed with Megascript T7 kit (Ambion Inc., TX) followed by DNase I treatment (DNAfree, Ambion). The amount of RNA was quantified by UV densitometry. Conversion of microgram of single stranded RNA to picomole was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (Nb). The following mathematical formula was applied: pmol of ssRNA= µg (of ssRNA)×(106 pg/1µg)×(1 pmol/340 pg)×(1/Nb). Avogadro constant was used to estimate the number of transcripts (6.023×1023 molecules/mol) (Olmos et al., 2005). Subsequently, ten-fold serial dilutions of the transcripts were prepared and used. Real-time RT-PCR was performed with SYBR Green I, using a 7300 Real-time PCR System (Applied Biosystems, CA, USA) and Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystem, USA) according to the recommendation of the manufacturer.

The relative ratios were calculated by a mathematical model, which includes an efficiency correction for Real-time PCR efficiency of the individual transcripts (Pfaffl, 2001). Amplification efficiency was established for each of the targets from serial dilutions of C5 plum leaves and ranged between 0.80 and 1.0. The relative viral gene's expression was then transformed into absolute values by simple proportion. All the results were then analyzed by paired t-test as well as by two way ANOVA test. Thereafter Bonferroni post-test was applied on the data to compare the value of each column (factor *"position of the collected leaves"*) and each row factor *"year"*. P>0.05 were considered as non significant (ns), P<0.01 as significant and P<0.001 as extremely significant.

Results

The expression of the 18S ribosomal gene was quite stable among all the tested samples, with the C_t ranging from 15 to 18 cycles. In the previous in-house study, no significant differences in gene expression between virus-free and virus-infected plants had been recorded (data not shown). The C_t values for the PPV-Rec, on the other hand, varied greatly, ranging from 13 to 36 cycles. After calculating the relative viral gene abundance in the samples, the difference between the two most differing samples was 10 million fold. There were significant differences among the three parts of the tree. The virus was the most abundant in the non-transgenic parts of the trees, followed by the bottom parts of the transgenic trees and the scarcest in the top parts of the C5s. If the mean relative quantity of the virus in the top parts of the trees is assumed to be 1, then the mean relative quantity in the bottom parts of the trees is 1.3 and in the non-transgenic 'St. Julien' infectious bud it is 44. Furthermore, statistical analysis by two way ANOVA confirmed that the non-transgenic 'St. Julien' part of the C5 trees and the actual transgenic C5 parts varied in their PPV-Rec quantity levels. The differences between the virus level in 'St. Julien' infectious bud and the transgenic tree were found to be extremely significant in all years, independently on whether the samples were collected close to of far from the bud. On the other hand, there were no significant differences found between the virus titre in the bottom and top parts of the transgenic trees (see Table 1).

Tab. 1 Results of the Bonferroni post-test performed after two-way ANOVA statistical analysis.

		Bonferroni Post-test	
		Absolute quantification	**Relative quantification**
St Julien	2006	ns	***
vs	2007	**	***
C5 (Top)	2008	***	***
St Julien	2006	ns	***
vs	2007	**	***
C5 (Bottom)	2008	***	***
C5 (Top)	2006	ns	ns
vs	2007	ns	ns
C5 (Bottom)	2008	ns	ns

ns: Not significant; ** (P<0.01); *** (P<0.001)

For the absolute quantification, standard dilutions were obtained within a range from 1.78×10^6 to 1.78×10^1 copies of PPV-Rec partial RNA. Then correlation between the logarithm of copy number and number of cycle was found with R^2=0.99 and PCR efficiency = 104 %, and was used to evaluate the copies number of unknown samples. The determined number of PPV-Rec RNA copies varied from only 1 copy up to almost million copies in the sample. The mean determined number of virus copies in the top part of the C5 was 8500, in the bottom part of the C5 it was 23 000, and in the non-transgenic part it was more than ten times more, 240 000 PPV-Rec RNA copies (see Figure 1). Statistical

analysis confirmed that the differences between the virus level in 'St. Julien' infectious bud and the transgenic tree were found to be extremely significant in 2008, significant in 2007 and not significant in 2006, independently on whether the samples were collected close to of far from the bud. On the other hand, there were no significant differences found between the virus titre in the bottom and top parts of the transgenic trees and no significant differences were recorded for all the parts in 2006 (see Table 1).

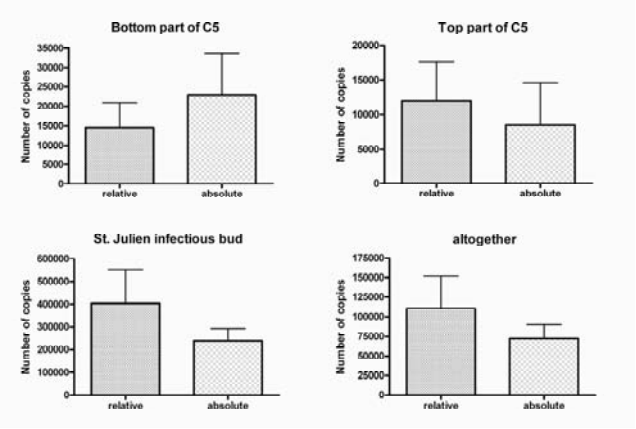

Fig. 1 Comparison of mean PPV-Rec viral RNA copies as determined by relative and absolute Real-time RT-PCR in different parts of the C5 transgenic trees sampled from 2006 to 2008.

Both methods were compared by a paired T-test, in which the numbers of viral RNA copies determined by absolute and by relative quantification were compared. In all analyses carried out (infectious buds, bottom part of C5, top part of C5, all samples together), no statistically significant differences were recorded, and in all the cases, the pairing was significantly effective. The mean values are shown in Figure 1.

Discussion

To avoid bias, relative quantification by Real-time PCR is referred to one or several internal control genes, which should not fluctuate during treatments. Ideally, the conditions of the experiment should not influence the expression of this internal control gene. However, many studies showed that internal standards could vary with the experimental conditions (Faccioli et al., 2007; Nicot et al., 2005; Sturzenbaum and Kille, 2001; Thellin et al., 1999). On the other hand, with absolute quantification, there is usually no normalizing factor and we only assume that our samples were prepared uniformly and the results are thus accurate. The drawbacks of both methods are therefore lying in normalisation. The differences between the results of the absolute and relative quantification carried out in this study were not tremendous; in almost all cases even the statistics confirmed the uniformity. However, sometimes, the statistical results reached were not in agreement, as in the case of 2006 samples. Further studies are needed in order to confirm accuracy of each method. Nevertheless, careful choice of endogenous control including stability of gene expression testing, as well as uniform and precise sample preparation for quantification by both absolute and relative quantification are highly recommended.

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Improvement of the reverse transcription loop mediated isothermal amplification (RT-LAMP) method for the detection of *Peach latent mosaic viroid* (PLMVd)

Boubourakas, I.N.[1]; Fucuta, S.[2]; Luigi, M.[3]; Faggioli, F.[3]; Barba, M.[3]; Kyriakopoulou, P.E.[1]
[1] Agricultural University of Athens, Department of Plant Production Science, Plant Pathology Laboratory, 11855 Athens, Greece
[2] Biotechnology Group, Aichi Agricultural Research Center, 1-1 Sagamine, Yazako, Nagakute, 480-1193 Aichi, Japan
[3] CRA – Centro di Ricerca per la Patologia Vegetale, Via C.G. Bertero, 22 – 00156 Rome, Italy

Abstract

Peach latent mosaic viroid (PLMVd) is the most known peach viroid. Among the diagnostic techniques used for its detection, the most recent described being the reverse transcription loop-mediated isothermal amplification method (RT-LAMP). Several modifications were done on the basic protocol proposed by Boubourakas et al. (2009), additional experiments were preformed in order to further evaluate the method. Namely, the reaction time was further reduced and traces of leaf tissue, taken by a sterile toothpick, instead of tRNA were used as the intial material. Moreover, the AMV reverse transcriptase proved to be more effective than Thermoscript, while restriction enzyme analysis was performed on the RT-LAMP products in order to confirm that products had the respective sequences of the selected target. Finally, the extremely high efficiency and sensitivity of RT-LAMP proved to be sufficient for the detection of PLMVd in hosts other than peach.

Keywords: PLMVd, RT-LAMP, peach, reverse transcriptases

Introduction

Peach latent mosaic viroid (PLMVd), a member of the family *Avsunviroidae* of the genus *Pelamoviroid* (Navarro and Flores, 1997), is the causal agent of an economically important disease of peach, responsible for reduction of fruit quality, tree vigor, and increased susceptibility to biotic and abiotic stresses. The term latent in the name of PLMVd refers to the observation that the vast majority of natural infections of peach occur without leaf symptoms and the prolonged time required for the onset of symptoms. Therefore, a sensitive, accessible, reliable, cost effective and fast diagnostic method that could contribute to the restriction of viroid spread and the production of healthy and of high quality propagation material is needed. Reverse Transcription-Loop Mediated Isothermal Amplification (RT-LAMP) seems to be a good candidate method for this purpose, since it combines the following characteristics, such as: 1) amplification of nucleic acids under isothermal conditions in the range of 65 ºC, 2) high specificity, 3) high amplification efficiency, 4) easy detection of amplified target DNA. LAMP is a novel nucleic acid amplification method, relative simple, characterized by the use of a DNA polymerase with strand displacement activity and a set of four different primers designed specifically to recognize 6 distinct regions on the target sequence (Nagamine et al., 2002; Mori et al., 2001; Notomi et al., 2000). Boubourakas et al. (2009) developed, for the first time, an RT-LAMP protocol for the detecion of PLMVd. According to the findings of this study, the combination of the OLD1 primer set with the degenerate loop primer, under 62.5 ºC and 0.8 M betaine concentration, led to the detection of PLMVd within almost 30 min, with a detection limit of 10^{-5}.

In the present study we introduced some modifications in RT-LAMP in order to make it simpler and the improved method was used for the detection of PLMVd in several host species in addition to peach.

Materials and methods

Plant material: In the present study, the Greek PLMVd isolate 52, coming from naturally infected peach trees cv. SpringCrest exhibiting the characteristic symptom of fruit cracked sutures, and the Italian isolates P51 and P39 (calico isolate) were used as source of PLMVd material. Leaf and fruit samples of apricot, peach, plum, cultivated and wild pear and quince were also collected from North-Eastern Peloponnesus, Greece. Leaves of healthy peach GF305 were used as negative controls. Also, Italian isolates of *Apple scar skin viroid* (ASSVd), *Pear blister cancer viroid* (PBCVd), *Hop stunt viroid* (HSVd) and *Potato spindle tuber viroid* (PSTVd) were used for specificity evaluation of the method. All the Italian isolates and the healthy seedling material came from the collection of the CRA-Centro di Ricerca per la Pathologia Vegetale in Rome.

RT-LAMP method: The amplification reaction was preformed at 62.5 ºC for 1 h followed by 2 min at 80 ºC using the OLD1 primer set in combination with the degenerate loop set, as described by Boubourakas et al. (2009). The parameters examined during this study were: 1) the type of the template used: total RNA using the protocol described by Rott and Jelkmann (2001) or traces of leaf tissue taken by a sterilized toothpick, and 2) the types of reverse transcriptase: AMV (Promega, Madison, USA) or Thermoscript (Invitrogen, Ltd, Paisley, England, UK). All components were assembled and reactions were preformed either in a Real Time Turbimeter LA200 (Teramecs Co. Ltd, Kyoto, Japan) which measures the turbidity of each reaction mixture in real time or in a PTC-200 DNA Engine Cycler (MJ Research, Waltham, Massachusetts, USA). The amplified products were also visualized with agarose gel electrophoresis (2 %). In addition, the amplified products were digested with *RsaI* (Promega, Madison, USA) that cleaves at the site GT$^{\downarrow}_{\uparrow}$AC within the primer B2c and the digestion products were analysed by electrophoresis. The Loopamp Fluorescent Detection Reagent (Eiken Chemical Co. Ltd., Tochigi, Japan) was added in the reaction mixture to obtain a direct visual observation of the reaction tube under UV light.

Specificity of RT-LAMP: In order to determine the specificity of the method, other viroids such as ASSVd, PBCVd, HSVd and PSTVd were subjected to RT-LAMP using the PLMVd OLD1 and degenerate loop primer sets; and all reactions were analyzed in parallel.

Results and discussion

This RT-LAMP protocol was able to specifically detect various PLMVd peach isolates from Greece and Italy, including an Italian calico isolate. No signal was generated when template from other viroids, ASSVd, HSVd, PBCVd and PSTVd or when extracts from healthy peach plants were subjected to the RT-LAMP assay using the PLMVd specific primer sets (Figure 1). The above results indicate that the amplified products were PLMVd-specific and not the result of cross-contaminations.

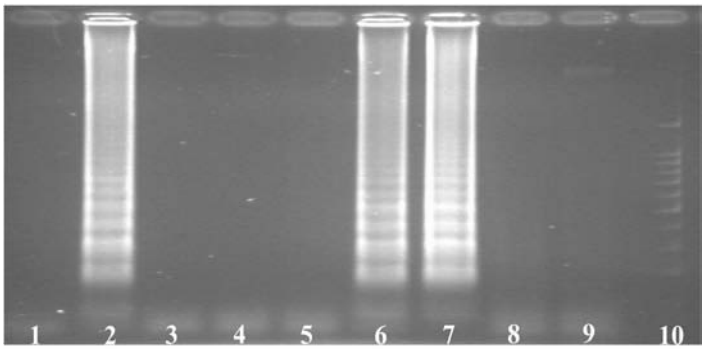

Fig. 1 Gel electrophoresis of RT-LAMP products from various PLMVd isolates and other viroids. Lane 1: water, Lane 2: PLMVd 52 (Greece), lane 3: PBCVd, lane 4: ASSVd, lane 5: HSVd, lane 6: PLMVd P51 (Italy), lane 7: PLMVd P39 calico (Italy), lane 8: PSTVd, lane 9: healthy peach control, lane 10: molecular weight marker (100 bp, New England Biolabs, Hertfordshire, England, UK).

It was shown previously that total RNA extracted using the Rott and Jelkmann (2001) protocol provides a reliable template for RT-LAMP on PLMVd (Boubourakas et al., 2009). In the present study it was proven that traces of leaf tissue, taken by a sterilized toothpick, can be used as template (Figure 2). However, the intensity of the signal obtained was lower than that of using tRNA as template. The fact that the method could be employed using traces of plant tissue results in: a) further reduction of the reaction time, and b) allowing the use of RT-LAMP in the field when the internal fluorescent dye is used (Figure 3).

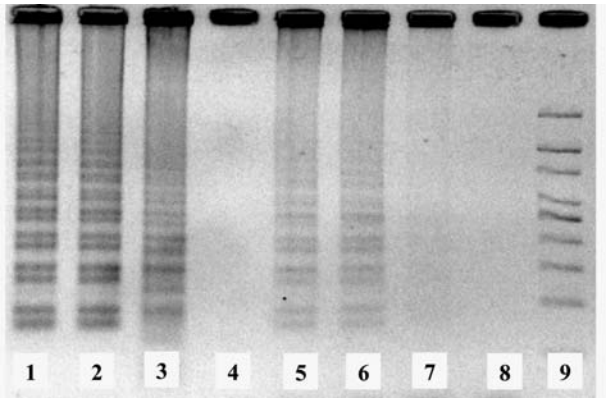

Fig. 2 Gel electrophoresis of RT-LAMP products using AMV (lanes 1-4) or Thermoscript (lanes 5-8); using as template tRNA (lanes 1-2 and 5-6) or traces of leaf tissue taken by a sterilized toothpick (lanes 3 and 7). Lanes 4 and 8: healthy peach control, lane 9: molecular weight marker (100 bp, New England Biolabs, Hertfordshire, England, UK).

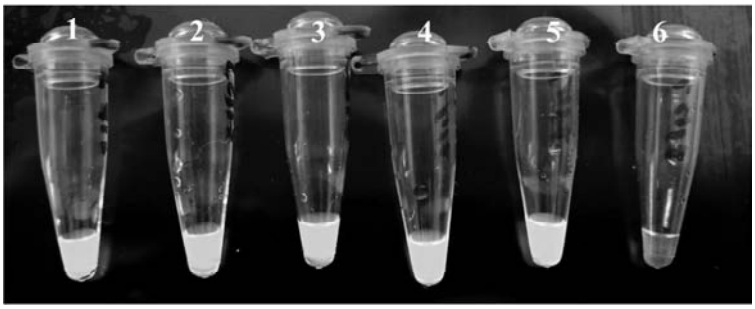

Fig. 3 Detection of PLMVd by RT-LAMP using fluorescent internal dye, on peach (tube 1, 2), on pear (tube 3), on wild pear (tube 4) and on quince (tube 5). Healthy control (tube 6).

When the reverse transcriptases AMV and Thermoscript were compared in RT-LAMP, a less intense, however positive, signal was observed when Thermoscript was used, when either tRNA or traces of plant tissue were used as templates (Figure 2); thus Thermoscript is considered to be less efficient in RT-LAMP.

In order to confirm that the RT-LAMP products have the corresponding sequences of the selected target, a portion of the amplified products is subjected to restriction enzyme analysis (Kubota et al., 2008). Based on the sequence of the expected amplified products and using the NEBcutter V2.0 software (New England BioLabs, Hertfordshire, England, UK, http://tools.neb.com/NEBcutter2/), the *RsaI* restriction enzyme was found to cut the RT-LAMP products in four fragments, namely 131 bp, 171 bp, 198 bp και 225 bp (Figure 4).

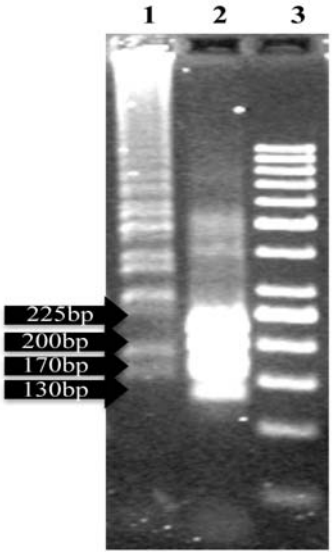

Fig. 4 Restriction enzyme digestion of RT-LAMP products. RT-LAMP products (lane 1) were digested by *RsaI* (lane 2). Lane 3: molecular weight marker (50 bp, New England Biolabs, Hertfordshire, England, UK).

Detection of PLMVd in stone fruit hosts, other than peach, such as plum, or pome fruits is difficult, presumably due to viroid concentration in infected tissue at relative low titers (Flores et al., 1992). Since RT-LAMP has an extremely high efficiency and sensitivity, it proved to be sufficient for the detection of PLMVd in hosts such as plum (2/2), apricot (1/2), pear(1/2), wild pear (2/2) and quince (1/1) (Figure 5).

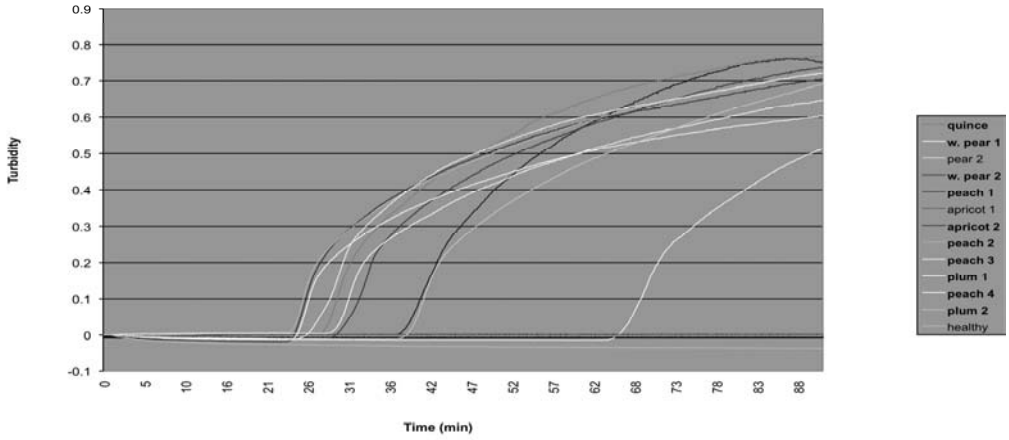

Fig. 5 Real time detection of PLMVd by RT-LAMP assay in several hosts such as apricot, peach, pear, plum, quince and wild pear.

With the results of the present work, we think that the study for the use of RT-LAMP in PLMVd detection has got closed to its end. RT-LAMP method is easy, relatively cheap, fast, extremely sensitive, highly specific, reliable, with its improved version to have the possiblility to be performed in the field.

Acknowledgments

We would like to thank Dr Andreas Voloudakis for creetically reviewing the text.

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Application of scanning electron microscopy for diagnosing phytoplasmas in single and mixed (virus-phytoplasma) infection in Papaya

Lebsky, V.[1]; Poghosyan, A.[1]; Silva-Rosales, L.[2]

[1] Centro de Investigaciones Biologicas del Noroeste (CIBNOR), La Paz, BCS, 23090, Mexico

[2] Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), Campus Guanajuato, Irapuato, Gto 36500, Mexico

Abstract

Phytoplasma and some viruses, *papaya ring spot* (PRSV) and *papaya mosaic* (PapMV) have been reported in papaya, from different Mexican states. Some symptoms of yellow type diseases, such as mosaics, stunting, bunchy top and leaf chlorosis, necrosis and malformations are somewhat similar in appearance, but caused by distinct pathogens. Using a scanning electron microscopy (SEM) technique phytoplasmas were detected in the phloem tissues of field and greenhouse-indexed papaya plants from Baja California Sur (BCS). Samples from 32 local varieties, as well as cv. Maradol, showing numerous symptoms of dieback, mosaics, bunchy top, and yellow crinkle were analyzed. The pathogen was detected in stems, leafstalks, roots, axillary leaflets, leaf veins and flowers. Phytoplasma was also detected in dry and in germinated seeds within the fruit, suggesting seed transmission of the pathogen. Some ultrastructural peculiarities of phytoplasma in infected tissues were also observed. No viral infection with PRSV and PapMV was revealed neither in test-plants nor by molecular techniques. Application of SEM technique for analysis of papaya samples from Veracruz and Irapuato, both from field-grown and mechanically inoculated plants with PRSV and PapMV in various combinations also revealed phytoplasmas in the phloem of most of tested samples. In some cases, along with phytoplasmas, rod-shaped bacteria were distinguished.

Keywords: Papaya, phytoplasma, papaya ringspot virus, papaya mosaic virus, scanning electron microscopy, Mexico.

Introduction

Papaya (*Carica papaya* L.) is an important perennial fruit crop in the tropics and subtropics, and is very susceptible to numerous diseases, probably as a result of extensive monoculture and a narrow gene pool ("The Biology of *Carica papaya* L.", 2008). Phytoplasma and virus-associated papaya maladies are among the more destructive, and there is no strategy for controlling these diseases on a commercial scale. A number of viral diseases have been associated with papaya. Papaya ringspot, caused by *Papaya ringspot virus* (PRSV) significantly reduced crop productivity in Hawaii, the Caribbean, Brazil, Southeast Asia and Australia (Yeh and Gonzalves, 1984; Purciful et al., 1984; Gonzalves, 1998; Golnaraghi and Shnhraeen, 2003; Mowlick et al., 2007). *Papaya mosaic virus* (PMV) is also a serious problem in some countries, such as the USA, Venezuela and Bolivia (Purciful and Heibert, 1971; Rajapakse and Herath, 1981).

Phytoplasma associated diseases of papaya were reported from different papaya producing countries. Papaya dieback (PDB), yellow crinkle (PYC) and mosaic (PM) were recognized in Australia (Gibb et al, 1996, 1998; Elder et al, 2002). Papaya disease, Nivun Haamir (NH), similar to PDB, was reported in Israel (Lju et al., 1996), and attributed to the same taxon as PDB, *Ca. Phytoplasma australiense* (Gera et al., 2005). Phytoplasma associated with bunchy top-like disease (BTS), known in Cuba (Arocha et al, 2006), was recently reported in mixed infection with a potyvirus (Arocha et al., 2009).

Mexico is one of the original centers of papaya cultivation (Nakasone & Paull, 1998), and one of the main papaya producers (Ploetz , 2007). PRSV is considered a very important limiting factor in some regions of Mexico (Treviño, 1980; Teliz et al., 1991; Silva-Rosales et al., 2000). PapMV has been reported in Mexico with low economic impact (Noa-Carrazana and Silva-Rosales, 2001). Some symptoms caused by PRSV and PapMV; stunting, chlorosis, leaf mosaic and distortion and filiform appearance, are common with phytoplasma related papaya symptoms.

In Mexico phytoplasmas associated with papaya infection were first reported in Oaxaca, Central Mexico (Rojas-Martinez et al., 2003). In the Yucatan peninsula Australian Papaya Yellow Crinkle (PYC)-like symptoms were noted in association with detected phytoplasmas (Navarette-Yabur et al., 2003; Moreno-Valenzuela and Navarette-Yabur, 2005). Similar symptoms associated with phytoplasma were recorded in different regions of Baja California Sur (BCS state) (Poghosyan et al., 2004). In 2004, two experimental plots were established in El Centenario and El Comitan, with seeds of 32 local papaya varieties and cv. Maradol. Variability of symptoms was observed in diseased plants, some of which were similar to symptoms reported for different yellow-type diseases. Our objective in this investigation was to analyze the role of phytoplasmas in all types of symptom expression, using disease indexing and an SEM technique.

Additionally, we report the results of phytoplasma detection in papaya samples infected with PRSV and PapMV that were obtained from other regions of Mexico.

Materials and methods

Plant samples from BCS: Samples of apical and axillary leaflets from papaya plants exhibiting different symptoms of presumed phytoplasma infection and asymptomatic samples were taken during field surveys in El Centenario and El Comitan from 2004 through 2007, where experimental fields of papaya were established. Plots were prepared with seeds of 32 local papaya lines and cv. Maradol collected during field surveys in 2002 and 2003. Samples were used for disease indexing under greenhouse conditions and for processing for diagnosis by SEM. For SEM samples other plant organs were also used. Samples of micropropagated papaya were used as a control for SEM. Some weed plants with yellow-type symptoms near papaya plantations and among the trees were also collected and analyzed by SEM. Symptoms were transmitted from the field to greenhouse-grown test plants by grafting. Transmission by dodder (*Cuscuta* spp.) from indexed papaya to Madagascar periwinkle (*Catharanthus roseus*) was conducted in some cases. Samples from some symptomatic plants with leaf distortions of possible viral origin have been sent to CINVESTAV, Irapuato, for PRSV and PapMV- analysis.

Plant samples, Irapuato: Samples of papaya with viral infection were collected from the States of Veracruz (PapMV) and Yucatan (PRSV). The presence of each virus was confirmed by a serological DAS-ELISA test and RT-PCR (Ruiz Castro and Silva Rosales, 1997; Noa-Carrazana and Silva-Rosales, 2001). Then a series of inoculations were performed differently in the greenhouse on papaya test-plants to reproduce symptoms of each virus and to elucidate the symptoms of possible mixed viral infection: single infection with either PapMV or PRSV; mixed simultaneously, PRSV+PapMV, and stepwise, PapMV-PRSV (PapMV first, followed by PRSV after 30 days), and PRSV-PapMV (PRSV first, followed by PapMV after 30 days) (Noa-Carrazana et al., unpublished data). This part of the experiment was conducted at the Virology Laboratory of CINVESTAV in Irapuato. To verify the presence of phytoplasma in samples, including the controls, samples were processed for SEM analysis (up to 70 % ethanol grade) and sent to CIBNOR for further processing and phytoplasma analysis by SEM.

Applied SEM technique: Leaf vines, leafstalks, axillary leaflets, stem, roots, floral parts, and fruits (seeds and plantlets germinated within fruits) from symptomatic, asymptomatic, and control papaya samples were fixed in 2.5 % glutaraldehide dissolved in 0.2 M sodium cacodylate buffer (pH 7.2-7.4) for one day at 4 °C. After rinsing the samples in the same buffer, they were dehydrated in increasing grades of ethanol (30 %, 50 %, 70 %, 95 %,100 %) followed by absolute acetone (or hexamethyl- disilazane), for 20 min in each of these solutions. After dehydration the samples were dried in carbon dioxide (Critical Point Drier, Samdry-PVT-3B), and then attached to SEM stages by double-sided tape. The samples were coated with paladium in an ion sputter (Denton Vacuum, DESK II) and examined in the scanning electron microscope (S-3000N, Hitachi, Japan) at different accelerating voltages (5-20 kV). Samples of wild plants wereprocessed in the same manner and examined by SEM. In the case of infection of papaya with PRSV and PMV, only apical leaves were processed for SEM analysis.

Results

Disease symptoms, BCS: Diverse symptoms of yellow-type diseases were observed in local lines and cv. Maradol papaya in the field. Some plants had stunted growth, with shortened internodes and proliferation of shoots and internodal leaves, while others had a bunchy top appearance;sometimes, thickening of the petioles and formation of tumors on the trunk was noted (Figure 1, G-H). When old leaves abscised, a hole-like “wound” formed on the trunk. Flow of latex was reduced and was either watery or absent, in some lines. Symptoms on flowers were noted as sepal hypertrophy and petal reduction in female flowers and dried or underdeveloped inflorescences of male and hermaphrodite flowers. Fruit formation depended on the growing stage. If infection was late, fruit formation was initially normal, but then ceased. In early infections, the plants formed only 2 or 3 fruits that were deformed and small.

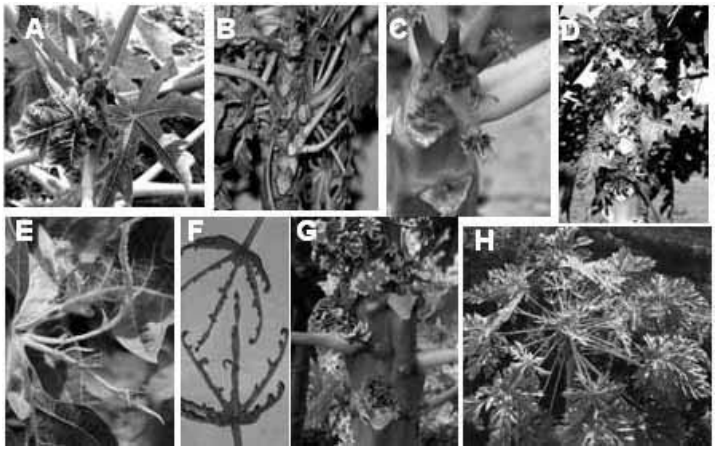

Fig. 1 Symptoms of yellow-type diseases in papaya from BCS: (A) leaf wrinkling; (B) shortening of internodes and proliferation of internodal leaves; (C) button-like leaf reduction; (D) shortening of leafstalks and leaf distortions; (E) filiform leaf structure; (F) claw-like leaf deformation; (G) tumors on trunk; (H) bunchy top.

Many kinds of leaf malformations was observed on plants in different stages of growth, including filiform and claw-like leaf distortion of old leaves, yellowing and wrinkling, severe leaf crinkle and reduction. Small "clawed" or "button-like" bunched leaflets appeared on stem tips, apex, and internodes. When old leaves were falling, very small bunches of distorted leaves with very short leafstalks appeared in the internodes or on the lower parts of the trunk (Fig.1, A-F). Tumors on the trunk turned brown, then necrotic and finally desiccated. When phytoplasma infection was transmitted to papaya test plants by grafting, similar symptoms occurred in the test plants. Disease indexing of symptoms from two papaya local lines having tumors on the trunk did not show this specific symptom on test papaya plants. All diseased test plants died within one year.

Transmission to periwinkle by dodder led to strong interveinal chlorosis, and finally, total necrosis and abscission of leaves and proliferation and necrosis of branches. In some branches only very small leaves survived on the tips, other branches died within one to two years. Formation of flowers was reduced, with malformed and pale rose-colored petals.

Virus symptoms: Symptoms of viral infection in field-grown papaya plants from 15 states of Mexico (not BCS) included yellowing, vein clearing, mosaic, and leaf distortion (Noa –Carranaza et al, unpublished data). Different symptoms were observed in inoculated papaya test-plants, from mild mosaic from infection with only PapMV or PapMV-PRSV, to necrotic lesions and leaf distortion in the case of single infection with PRSV, or mixed infections of PRSV-PapMV or PRSV+PapMV.

SEM analysis: samples from BCS: Phytoplasma cells were detected in the phloem tissue of samples analysed from local papaya lines and the commercial Maradol variety, raised in the field or in the greenhouse (Figure 2).

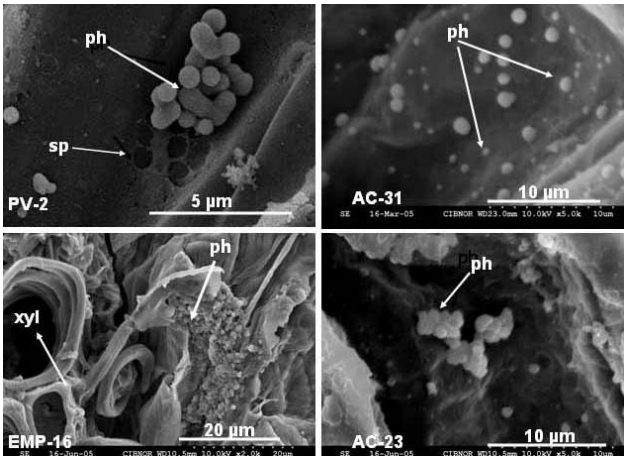

Fig. 2 Scanning electron micrographs of phytoplasmas in phloem tissue of some local papaya lines. (PV-2) and (AC-31), field samples; (EMP-16) and (AC-23), indexed plants. Arrows indicate: ph-phytoplasma cells; sp-sieve pores; xyl- xylem tissue.

The pathogen was detected in different parts of diseased plants: leaves, stems, leafstalks, roots, flowers, fruit and seeds (Figure 3-4). Phytoplasma was not found in symptom-free micropropagated plants, but was however detected in some symptomless field-grown papayas. The concentration of the pathogen in phloem cells depended from the season, plant condition, disease stage and severity. Phytoplasmas were observed as spherical bodies, ranging in size from 500 to 1800 nm. They appear as separate cells or clustered particles in phloem tissue, sometimes observed near or within sieve pores. Some phytoplasma were in the process of binary fusion, or with buds. The fibril structure of thecytoplasm and nucleus with surrounded plastids was distinguishable in host cells in some cases. Uneven distribution of phytoplasmas in sieve tubes was noted.

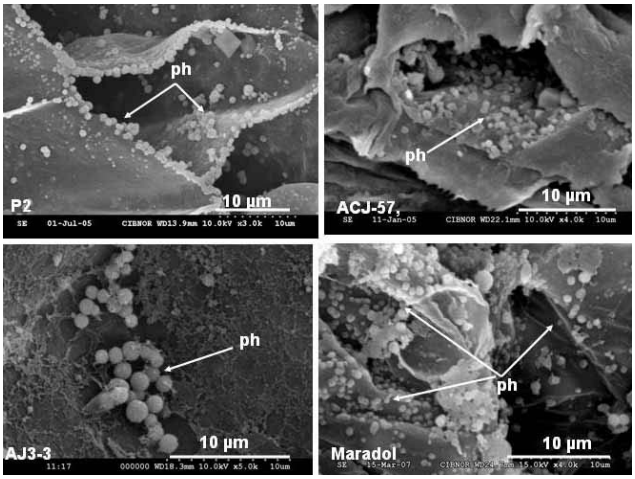

Fig. 3 Phytoplasmas in different organs of diseased local lines and cv. Maradol. (P2), floral bud; (ACJ-57), leaf vine; (AJ3-3), root; (Maradol), leafstalk. Arrows indicate phytoplasma (ph).

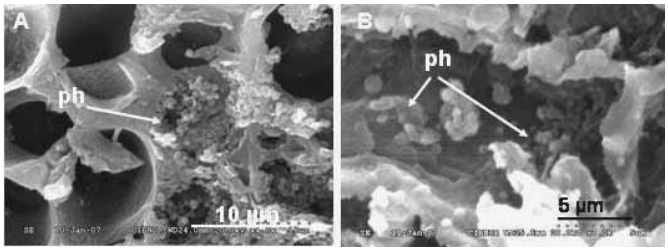

Fig. 4 Phytoplasmas (ph) detected in dry mature seed (A), and in germinated seedling (B).

In some cases the “infected zone” was observed at low magnification in the phloem tissue of a diseased plant, including about 20 neighboring phloem cells in one section plane (Figure 5).

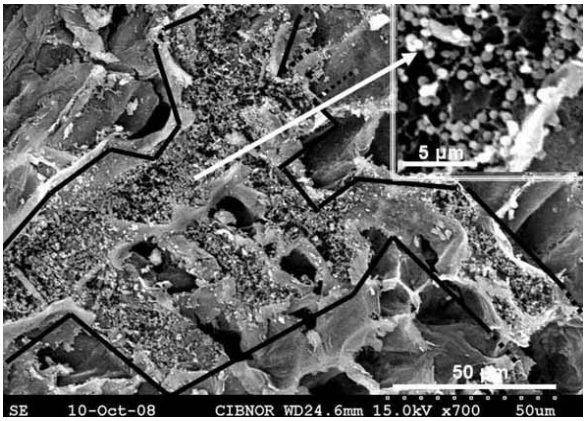

Fig. 5 Distribution of phytoplasmas in phloem of diseased papaya, low Magnification (X700). The “zone” of infection is marked in black. White arrow indicates part of the same image, high magnification (X4.000).

In phloem tissue of papaya with tumor-like structures on the trunkmany rod-shape bacteria were also detected alongside the phytoplasma, (Figure 6). Some ultrastructural features in the diseased host plant phloem tissues were observed: starch granules in phloem parenchyma, many inorganic crystals and some paracrystals.

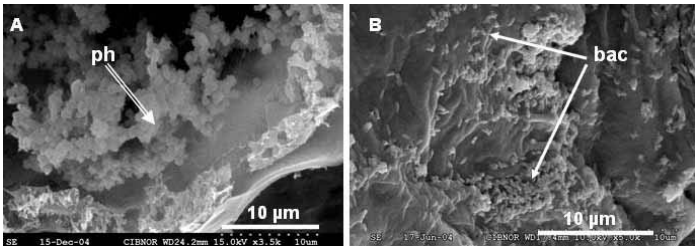

Fig. 6 Trunk section of papaya with tumor. (A). Phytoplasmas (ph), and (B) rod-shaped bacteria (bac).

When symptoms were transmitted by dodder, phytoplasmas were observed in periwinkle and in dodder haustoria (Figure 7).

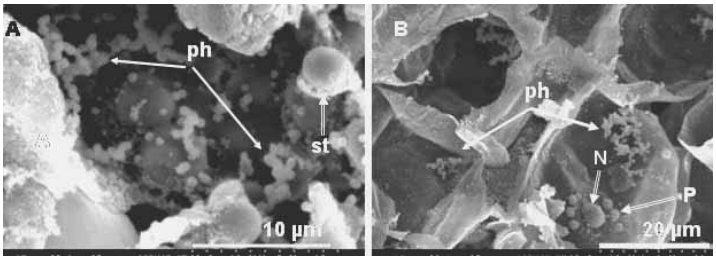

Fig. 7 (A). SEM- image of phytoplasmas transmitted by dodder to periwinkle.(B). Phytoplasmas in dodder haustoria. Indications: ph-phytoplasma; N-nucleus, P-plastids, St-starch granule.

In samples from morning glory (*Convolvulus* spp.) and other symptomatic and asymptomatic weeds, abundant phytoplasma particles were observed in phloem tissue with an average size of 1000nm (Figure 8).

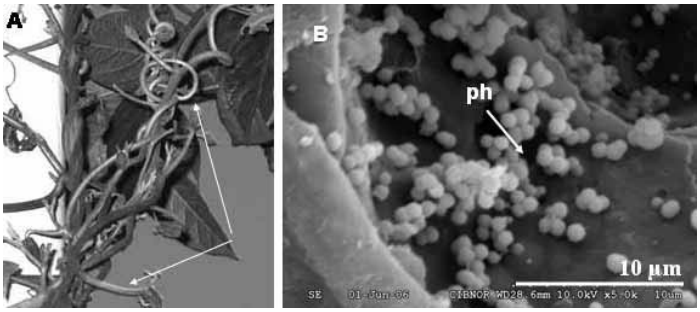

Fig. 8 (A). Morning glory (*Convolvulus* spp.) with symptoms of leaf reduction and distortion (arrows). (B). Phytoplasmas (ph) in phloem of morning glory.

SEM analysis, samples with viral infection: Phytoplasmas were detected in all samples with single (PRSV and PapMV) and mixed (PRSV+PapMV, PRSV-PapMV) viral infection. (Figure 9). Phytoplasmas were more abundant in the samples, inoculated with PRSV only, or simultaneously with PapMV.

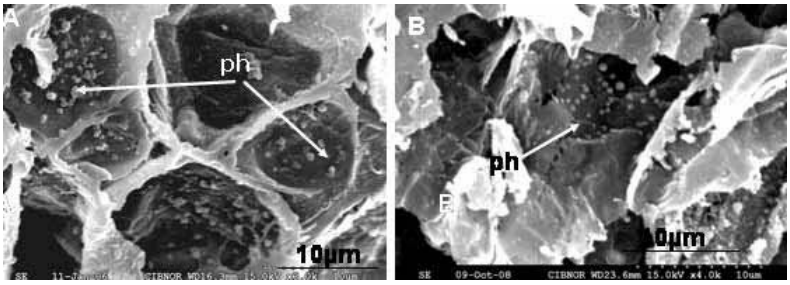

Fig. 9 SEM micrograph of phytoplasmas(ph) in phloem tissue of papaya with PRSV. (A) Field-sample from Veracruz. (B) Test- plant inoculated with PRSV.

A low phytoplasma concentration occurred in samples that contained PapMV only. In the case of mixed PapMV-PRSV (first-PapMV), rod-shaped bacteria but not phytoplasma were detected. Samples without viruses also contained phytoplasmas in phloem tissue.

Discussion

The character of symptom development and plant death observed in distinct papaya local lines was somewhat similar to Australian papaya dieback (PDB), and yellow crinkle (PYC) diseases (Gibb et al., 1996). SEM analysis of the 32 local papaya lines and cv. Maradol revealed phytoplasma in phloem tissue of distinct organs in different growth stages. Among the symptoms in damaged papayas was bunchy top, one of the most frequently reported symptoms among related to yellow-type diseases. This "bunchy" symptom was reported also in Australian PDB disease as an intermediate symptom ("Biology of Papaya", 2008). Bunchy symptoms appeared in our indexed papaya plants two months after grafting from diseased papaya, that did not express this symptom, and phytoplasma cells were observed in phloem tissue of bunched leaves. Button-like and claw-like leaf symptoms at internodes and tips also developed in grafted papaya test-plants, and phytoplasmas were found in their phloem tissues.

Previous to our study, tumors on the trunk of plants had not been reported for any phytoplasma related papaya malady. Phytoplasma cells were observed by SEM both in tumors and tissues of trunk section. Similar tumor-like growths were described within trunks of papaya with an unknown disease reported in the Republic of Congo, but PCR analysis showed the plants to be infected with a potyvirus rather than a phytoplasma (Arocha et al., 2008). On the indexed plants tumors did not develop, but when infection was transmitted from a tumor-bearing papaya to test plants, phytoplasmas were observed in indexed papaya and later, in periwinkle, connected by dodder to this phytoplasma positive papaya plant.

Not all samples collected from symptomatic plants (about 10 % of over 500 analyzed samples) revealed the presence of phytoplasmas in their phloem tissue. This may be related with the mechanisms of phytoplasma movement ("migration") within the plant. The appearance of symptoms, especially in leaves, does not always correlate with phytoplasma presence, and the pathogen in some cases could not be observed (Siddique et al., 1998; Wei et al., 2004). In our experiments with indexed papaya plants, when the temperature in greenhouse was extremely high (>35 °C) or low (<15 °C), phytoplasma was more easily detected in the lower leaves and roots than in the upper parts of symptomatic test plants. These data correlate with phytoplasma distribution in other woody plants (Jiang et al., 2004).

Detection of phytoplasma in mature and germinated seeds within fruit from diseased papaya is of special interest. Vertical transmission of phytoplasma was not recognized earlier, but is now strongly disputed. Phytoplasma DNA was found in embryos from coconut palm with lethal yellowing (LY) disease and maize kernels (Cordova et al. 2003; Jones et al, 2007). Seed transmission of some phytoplasmas into seedlings of alfalfa, tomatoes and oilseed rape, and plantlets of lime was reported (Khan et al., 2002; Botti and Beratccini, 2006). These findings are further supported by a recent report of the possible transmission of ESFY phytoplasma through apricot flowers and seeds (Nečas et al., 2008). No data about possible seed transmission of phytoplasma associated with papaya diseases have been recorded. Detection by SEM of phytoplasma in different papaya organs, including flower parts, plantlets and mature and germinated seeds within the fruit demonstrates that phytoplasma could move from the flower parts to seeds and seedling. Nipah et al. (2007) discussed the possible mechanism of phytoplasma movement to seeds, noting the importance of electron microscopy in the identification of phytoplasmas in seeds. Though further validation with PCR analysis is required in our study of seed transmission, and application of SEM, in some other cases, these findings present new perspectives for studying the epidemiology of diseases associated with phytoplasmas.

The application of both molecular techniques and electron microscopy may be needed to diagnose some complex yellow-type symptoms when disease origin is not clear. With these methods, mixed infection with phytoplasma and viruses was shown in malformed clovers (Franova et al., 2004) and recently demonstrated in papaya with bunchy top symptoms (BTS) in Cuba (Arocha et al., 2009). The presence of rod shaped bacteria along with phytoplasma in phloem tissue of papaya with a tumor on the trunk, suggests a possible mixed infection with two distinct prokaryotic pathogens. The reliability of phytoplasma diagnosis in phloem tissue by SEM technique, was reported and discussed earlier (Poghosyan et.al., 2004; Al-Awadhi et al., 2002).

Diagnosis of phytoplasmas in 32 local papaya lines and cv. Maradol in one state, BCS, and the detection of this pathogen in papaya samples from other Mexican states (Veracruz and Yucatan) with viral infection, was an additional reason for using SEM technique for the diagnosis of phytoplasma.

Future investigations by PCR and other molecular tests could help to characterize phytoplasmas found in diseased papaya plants in BCS. Moreover, studies about the phylogenetic relations between phytoplasmas in papaya from different Mexican states and beyond, could provide more information to better understand mixed infections in papaya.

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New viruses found in fig exhibiting mosaic symptoms

Tzanetakis, I.E.[1]; Laney, A.G.[1]; Keller, K.E.[2]; Martin, R.R.[2]
[1] Department of Plant Pathology, University of Arkansas, Division of Agriculture, Fayetteville, AR, 72701, USA
[2] USDA-ARS Horticultural Crops Research Lab. Corvallis, OR, 97330, USA.

Abstract

Mosaic is the most widespread viral disease of fig, affecting the crop wherever it is grown. The causal agent of the disease was poorly characterized and until recently it was considered a virus-like agent with double membrane bound semispherical bodies transmitted by eriophyid mites. During the molecular characterization of the Fig mosaic virus we discovered two new closteroviruses and a new badnavirus affecting the tree used in our studies. The characterization and presence of the three new viruses in mosaic-affected plants is the subject of this communication.

Keywords: Fig mosaic, Emaravirus, Closterovirus, Badnavirus

Introduction

Fig mosaic (FM) was first discovered in 1933 [4] and has since been found worldwide. The symptoms vary from tree to tree ranging from mild mosaic and ringspots to malformation of leaves and tree decline. Double membrane-bound bodies have been found associated with FM [1, 3], and recently a virus in the genus *Emaravirus*, Fig mosaic virus, was proven to be the causal agent of the disease [7, 8, 13]. Other than FMV several other viruses have been found in FM trees, including clostero-, umbra-, luteo- and cryptic viruses [5, 6, 13]. These discoveries and the symptom range suggested that FM may be caused by the synergistic effects of several viruses when they co-infect the crop. In the plant used in our study we discovered three new viruses, two closteroviruses, provisionally named Arkansas fig closterovirus-1 and -2 (AFCV-1 and 2) and one in the badnavirus named Fig badnavirus-1 (FBV-1). These three viruses are the focus of this communication.

Materials and methods

Mechanical transmission: At least eight plants of the following potential alternative virus hosts species Nicotiana occidentalis, N. tabacum, N. sylvestris, N. rustica, N. benthamiana, *Gomphrena globosa*, Glycine max, Cucumis sativus, Pisum sativum, Vigna unguiculata, Phaseolus vulgaris var. Black Valentine, Spinacia oleracea, Chenopodium *amaranticolor*, and *C. quinoa*, were inoculated with symptomatic fig tissue ground in cold 0.05 M phosphate buffer pH: 7.0. The plants were kept in an insect-free greenhouse under 14 h light/10 h dark regime, then observed for visual symptoms for one month followed by reverse transcription polymerase chain reaction (RT-PCR) tests for each of the three viruses communicated in this report.

DsRNA extraction: The Yoshikawa and Converse [14] and Tzanetakis and Martin [11] dsRNA extraction methods were used on tissue of a severely FM affected fig plant. Only symptomatic tissue was used in the extraction. In the final step of the extraction, nucleic acids were sequestered on glass in the presence of 50 % EtOH/50 % TE solution as described in Tzanetakis et al. [12] and eluted in 100µl TE after 10 min incubation at 70C.

Sequencing: cDNA synthesis was performed using Superscript III reverse transcriptase and a primer with a 6-nucleotide random region and a 16 nt known sequencing, making it essentially a random reverse transcription with the incorporation of known sequence that could be used downstream in a DOP-PCR amplification. After incubation at 50C for 60 min, the reaction was terminated by incubation at 75C for 15 min. The reaction was then digested by RNase H and subjected to PCR using a primer with identical sequence to the known portion of the reverse transcription primer and using 1/10 (v/v) RT reaction in the PCR. The PCR product was either shotgun cloned using TOPO technology (Invitrogen) followed by Sanger sequencing at the University of Arkansas DNA resource center or subjected to Illumina sequencing as described by the manufacturer at the Central Services Laboratory of Oregon State University.

Sequence and phylogenetic analysis: Contigs of Illumina 36 bp single end reads were first constructed using Velvet, Edena, QSRA, SSAKE, and VCAKE algorithms. CodonCode Aligner 3.0 (CodonCode Corporation, Dedham, MA) was then utilized to assemble these five sets of contigs into larger contigs. Those contigs along with sequences obtained through Sanger sequencing were compared with database sequences using BLAST [2]. Regions that had similarities with described viruses were used to develop primers for detection of the new viruses and for closing the sequence gaps that were not obtained through sequencing. Phylogenetic analysis was performed with ClustalW [10] using the neighbor-joining algorithm, Kimura's correction and bootstrap consisting of 1000 pseudoreplicates.

Detection: Primers AFCV1F (5'-CTGTATCTGTCATTACCTCTTCGGG) and
AFCV1R (5'-ATGCTTCCTCGGCTGC);
AFCV2F (5'-GTTCGGAATTAGTTAATAGATACGGTC) and
AFCV2R (5'-ACCCGCTAGAGTAATCAGTCAAAGTT);
FBVF (5'-ACCAGACGGAGGGAAGAAAT) and
FBVR (5'-TCCTTGCCATCGGTTATCTC);
FLMaV-1F (5'-GGGTTGGAGTTCGGG) and
FLMaV-1R (5'-ACGTGTCTGATGGGGA);
FLMaV-2F (5'-ACTTGCGACCCGATAA) and
FLMaV-2R (5'-CCTAGTCGGAGTGGATT)

that amplify a 375, 1671, 474, 580 and 479 base region of the genomes AFCV-1, -2 and FBV-1, Fig leaf mottle associated virus -1 and -2 (FLMaV-1 and -2) respectively were selected for RT-PCR detection. The primers were used on the tree of our study and another 39 FM accessions provided by the fig germplasm repository in Davis, California. The PCR program on an Eppendroff Mastercycler® using Genescript Taq polymerase consisted of denaturation for 2 min a 94C followed by 40 cycles at 94C, 55 C and 72C for 30, 10 and 90 sec respectively and terminated with 10 min incubation at 72C. Several amplicons from each virus were sequenced and all were virus specific, validating the detection protocols.

Results and discussion

Ds RNA extractions from an Arkansas FM tree revealed the presence of several bands ranging from more than ten to less than one kilobase (Figure 1). Shotgun cloning and Illumina sequencing revealed the presence of at least four viruses in the FM tree used in our study. The Arkansas FMV isolate is less than 90 % identical at the nucleotide level (97 % aa identity) to the sequenced European isolate. The AFCV-1 and -2 belong to the *Closterovirus* genus and are more closely related to Fig leaf mottle associated virus-1 than any virus found in the database. The Badnavirus is most closely related to *Citrus yellow mosaic virus* and *Cacao swollen shot virus* (Figure 2).

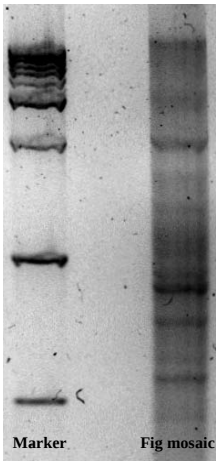

Fig. 1. Double stranded RNA (dsRNA) extraction from fig mosaic diseased plant. Left: 1 kilobase ladder, top band: 10 kilobases, bottom band: 0.5 kilobases; Right: Fig dsRNA.

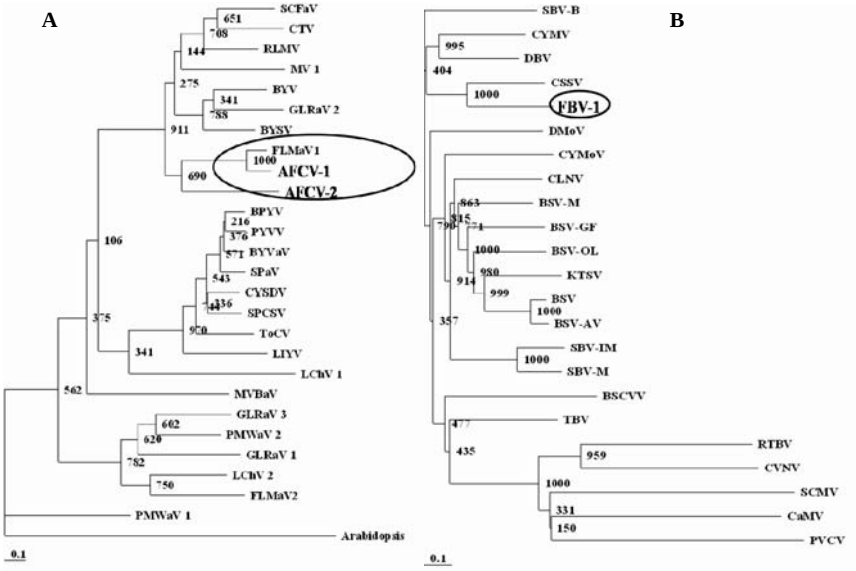

Fig. 2 Phylogenetic analysis of Arkansas fig closteroviruses (A) and Fig badnavirus (B). Bootstrap values presented on the nodes of the trees.

Our survey revealed that AFCV-1 is not a widespread virus in FM germplasm accession unlike AFCV-2 and FBV-1 that were present in several symptomatic trees (Figure 3). The similarity of AFCV-1 to FLMaV-1 led us investigate the possibility that the two virus are actually the same species. None of the 40 plants tested were found infected by FLMaV-1 or -2. Another conclusion of the survey was the presence of the AFCV-2 vector in California given that there are accessions with one of the trees infected and others that are free of the virus, unless there were multiple plants of the same accession brought into the collection. Asymptomatic fig plants were also tested for the presence of the new viruses and one of them was found to be infected with FBV-1, indicating that the virus is asymptomatic on this fig accession.

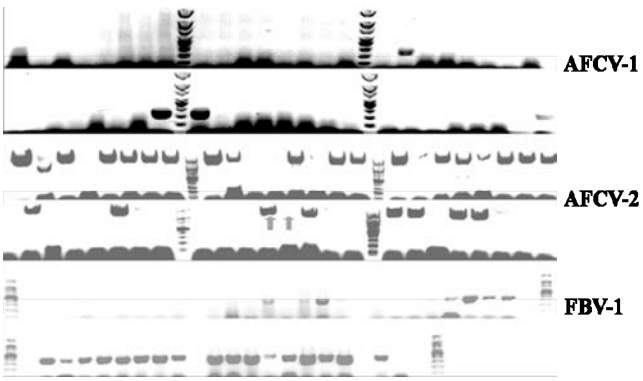

Fig. 3 Detection of Arkansas fig closteroviruses and Fig badnavirus in mosaic accessions from the fig repository in Davis, California. Arrows indicate two trees of a single accession, one infected and one free of Arkansas fig closterovirus-2.

Fourteen plant species of herbaceous indicators were tested for transmission of the three new viruses and *G. max* was found to be an alternative host for AFCV-2 and FBV-1 developing mild mottling symptoms. Although *N. occidentalis*, *N. tabacum*, *N. rustica*, *N. sylvestris*, and *P. sativum* did not develop symptoms, they all tested positive for FBV-1. All positive tests were verified by sequencing the PCR products.

A single FM trees was found infected with at least four viruses: FMV, AFCV-1 and -2 and FBV-1. The diversity of the viruses and their vectors reveal the complexity of the disease and the symptomatology observed in FM trees.

FMV belongs to the genus *Emaravirus* that includes viruses related to tospoviruses and is transmitted by the eriophyid mite *Aceria ficus*. Several closteroviruses belonging to the genera *Closterovirus* and *Ampelovirus*, presumably transmitted by aphids and mealybugs respectively, have been found in fig in Europe, North Africa, the Middle East, and the United States [9, 13]. Badnaviruses are vectored primarily by mealybugs and aphids and thus FBV-1 may share vectors with the closteroviruses that infect the crop.

FM is a disease that is now known to be caused by the emaravirus FMV. The FM symptom diversity suggest that the additional viruses that have been found in FM plants in both the Old and New World may play a significant role on the expression of the disease. It is possible that some of the newly identified viruses share vectors that may account, in addition to the clonal propagation, for the large numbers of viruses that fig harbors. We are working towards further characterization of these new viruses including symptoms in single infections and possible vector species.

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Worldwide diffusion of Fig latent virus 1 in fig accessions and its detection by serological and molecular tools

Gattoni, G.[1]; Minafra, A.[2] ; Castellano, M.A.[1] ; De Stradis, A.[2] ; Boscia, D.[2] ; Elbeaino, T.[3]; Digiaro, M.[3]; Martelli, G.P.[1]
[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi,
Via Amendola 165/A, 70126 Bari, Italy.
[2]Istituto di Virologia Vegetale del CNR, Unità Organizzativa di Bari, c/o DPPMA,
Via Amendola 165/A, 70126 Bari, Italy.
[3]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano, Bari, Italy.

Abstract

A virus with filamentous particles *ca.* 700 nm long, denoted Fig latent virus 1 (FLV-1) is widespread in Apulian (southern Italy) fig orchards, in trees showing or not mosaic symptoms and in symptomless seedlings. The virus was transmitted by sap inoculation to a very restricted range of herbaceous hosts without inducing apparent symptoms and was transmitted through fig seeds to a very high percentage (80 to 100 %). It was successfully purified from root tissues of infected figs. A virus-specific antiserum raised in rabbits, proved useful for its detection in fig leaf dips by immunosorbent electron microscopy (ISEM), Western Blot, dot immuno-binding (DIBA), ELISA. The viral genome structure resembles that of members of the genus *Trichovirus* in the family *Flexiviridae*.

Keywords: fig latent virus, *Trichovirus*, serology, ISEM, Western blot, DIBA, ELISA.

Introduction

The presence of viruses with filamentous particles in figs (*Ficus carica* L.) affected by mosaic disease (FMD) has been reported from several countries (Elbeaino et al., 2006, 2007; Martelli et al., 1993; Grbelja and Eric, 1983; Salomon et al., 2003, 2008; Doi, 1989; Serrano et al. 2004). During a survey for viruses in a fig germplasm collection of the Faculty of Agriculture of the University of Bari (southern Italy), filamentous virus-like particles were very frequently observed in negatively stained leaf dips from a number of fig accessions, regardless of whether they showed mosaic symptoms or not. Similar particles were also found in 1-year-old symptomless seedlings from seeds collected from mosaic-diseased figs (Castellano et al. 2009; Gattoni et al. 2009).

The present report describes further serological detection tools for FLV-1, which, compared with molecular results (Minafra et al. 2009), verify the efficacy of the produced polyclonal antiserum.

Materials and methods

Virus sources: Plant samples used in this study were collected from: (i) an adult mosaic-diseased (accession F5P5) and a symptomless fig tree, both of undetermined variety, from the fig germplasm collection of the University of Bari; (ii) 40 different accessions from the same fig collection showing or not mosaic symptoms; (iii) 22 plants from different geographical origin: Greece (3), Bosnia-Herzegovina (1), Montenegro (2), Hungary (1), Portugal (1), France (2), England (1), California (1), Mexico (1), and South Africa (9); (iv) symptomless seedlings grown from F5P5 seeds under greenhouse.

Antiserum production and serological detection: The virus was purified from roots of infected *Ficus Carica* L. by a method slightly modified from that used for grapevine filamentous viruses (Boscia et al. 1993). Partially purified virus preparations containing ca. 0.9 mg nucleoprotein, mixed 1:1 with Freund's incomplete adjuvant were injected subcutaneously to a New Zealand white rabbit. Boost injections were delivered intramuscularly three times, 10 days apart from one another. Bleedings were four, beginning one week after the last boost injection. The antiserum was absorbed twice with extracts from healthy fig roots to eliminate antibodies to plant components. The rough titre was determined by decorating virus particles (Milne, 1993) with progressive antiserum dilutions.

Western Blot: Leaf tissues (0.1 g) from accession F5P5 and virus-free sources were extracted in 1 ml of denaturing buffer (Berger et al., 1989) and aliquots equivalent to 10 mg were fractionated in SDS-polyacrylamide gel electrophoresis. Likewise, partially purified virus preparations were treated in denaturing buffer. Blots were treated overnight at 4 °C with the antiserum diluted 1:500 in TBS-blocking buffer and were developed by NBT-BCIP solution (Sigma-Aldrich, USA).

DIBA: For a rapid immunoenzymatic coat protein detection of FLV-1, 0.1g of leaf tissues were homogenized in 5 vol TBS buffer, centrifuged 2 min at 5000rpm and 20µl of supernatant was spotted on PVDF membranes. Membranes were incubated in blocking solution (5 % dry milk, 1 % BSA and 0.05 % Tween-20 in TBS) for 2 h. The membrane was washed in TBS 0.05 % Tween-20, and incubated overnight at 4 °C, with an antiserum dilution 1:1000 in blocking solution. The antiserum has been previously pre-adsorbed with healthy fig extracts as described by Da Rocha et al. (1986). The membrane was washed and incubated 30 min at room temperature in blocking solution containing an anti-rabbit AP-conjugated antibody (1:2500; Sigma) and finally developed by NBT+BCIP.

ELISA: The polyclonal antiserum produced was used for the FLV-1 detection in plant tissue extracts by DAS-ELISA (Clark and Adams, 1977). Wells were coated with the purified IgG of the produced antiserum at 37 °C for 2 h. Plates were washed three times, loaded with 100 µl per well of extracted samples and incubated overnight at 4°C. Conjugated antibodies were loaded in the wells (100µl per well) and incubated at 37 °C for 2 h, then finally washed and revealed with p-nitrophenylphosphate substrate.

Electron microscopy: Immunosorbent electron microscopy (ISEM) assays were done as described by Milne (1993), using leaf dips in 2 % uranyl acetate. Decoration was with antisera to FLV-1 diluted 1:20. For thin sectioning, tissue fragments were excised from leaves of an infected symptomless seedling, a symptomless adult fig tree, and from accession F5P5. All samples were processed according to standard procedures (Martelli and Russo, 1984).

Molecular detection: For diagnostic purposes, besides the virus-specific antiserum, the primers CPtr1 and CPtr2 (Gattoni et al. 2009) were used in RT-PCR on silica-extracted TNAs template (Foissac et al. 2001) from tissue of a number of different fig trees from the above mentioned collection. Amplicons (389 bp) were resolved in agarose gels stained by ethidium bromide.

Results

Although partially purified preparations contained a substantial amount of plant contaminants, virus particles showing a distinct cross banding were plentiful. Most of the particles were fragmented, so their size was determined from leaf dip preparations. The most frequent length of some 60 particles measured was *ca.* 700 nm.

The antiserum had a titre of 1:160. It clearly decorated homologous virions (De Stradis et al. 2007; Gattoni et al. 2009). Western blots of extracts from F5P5 leaf tissue and partially purified virus preparation resolved a major band with mol. wt. of *ca.* 45 kDa, which was recognized by the antiserum to FLV-1 and was absent in leaf extracts from virus-free sources (not shown). An evident recognition of the capsid protein of FLV-1 by the produced antiserum was obtained in DIBA assays, using bleed-3 antiserum diluted 1:1000. FLV-1 was detected from leaf extracts of infected plants (Figure 1). No signal was detected from extracts of healthy tissue.

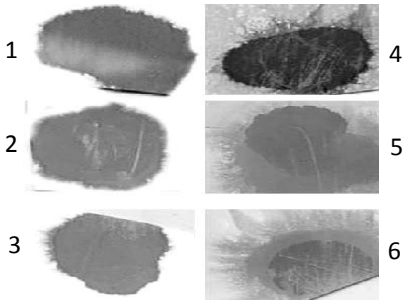

Fig. 1 Results of DIBA assay, using antiserum absorbed twice with extracts from healthy fig roots, obtained from bleed-3 diluted 1:1000, on the following samples: 1. Bosnia, healthy control; 2. Bari, healthy control; 3 seedling healthy control; 4. Montenegro infected sample; 5. F5P5 infected accession; 6. F17P2 infected accession.

The results of DAS-ELISA on roots extracts from samples already tested in RT-PCR assay, using IgG purified from four different bleeds, showed that the IgG obtained from bleed-3 diluted 1:1000 gave the optimal binding specificity (Figure 2), and confirmed the molecular assays. For leaves extracts, IgG diluted 1:500 gave a more specific result. Probably the higher sensitivity of FLV-1 antiserum for the roots extract could be due to the higher concentration of viral particle in this tissue.

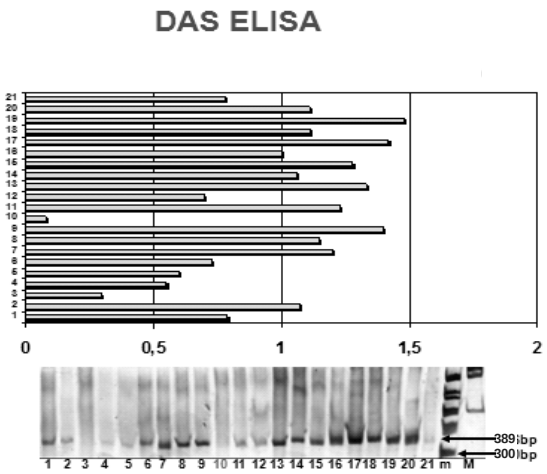

Fig. 2 Comparison of molecular (RT-PCR) and serological (ELISA) detection obtained on 21 samples. Sample 10 is the negative (virus-free) control.

Reliable detection of FLV-1 was obtained all year round by RT-PCR of silica-extracted TNAs from leaf tissues or cortical scraping using the CPtr1/CPtr2 primers that amplify a 389 bp segment of the CP gene. A survey for the preliminary assessment of the incidence of FLV-1 infections and the association of this virus with symptoms, showed that FLV-1 infects a high percentage (68 %) of the 40 different cultivars tested from the fig germplasm collection of the University of Bari and was detected also in fig accessions from Europe, Africa and America (Castellano et al., 2007). However, its presence does not seem to be associated with mosaic or other symptoms for the virus was detected in more than 40 % symptomless trees and in the totality of 10 symptomless seedlings grown from seeds of accession F5P5 (Table 1).

Tab. 1 Incidence of FLV-1 in italian and foreign samples.

	Tested samples (n.)	**PCR positive (n.)**	**Infected samples (%)**
Fig with mosaic symptoms	57	46	81
Fig without mosaic symptoms	31	14	45
Total	88	60	68

The transmission of FLV-1 through seeds, was investigated on batches of seedlings of different age and geographical origin by ISEM through the virus-specific antiserum (Castellano et al. 2009). FLV-1 was detected in all groups of seedlings, the great majority of which were symptomless and the infection rate ranged from 73 to 100 % (average 92 %). Positive RT-PCR responses were obtained using the FLV-1-specific primers designed in the CP gene. Since fig is not propagated through seeds, the epidemiological significance of seed infection would be negligible.

Discussion

In conclusion, a filamentous virus was isolated in fig trees and seedling showing or not mosaic symptoms and its detection has been successfully surveyed by different methodology. The high seeds transmission rate that differentiates FLV-1 from most of the other seed-transmitted plant viruses, and from the extant trichoviruses none of which is apparently transmitted though seeds, call for further investigations for a better definition of the taxonomic status of this virus. However, the availability of sensitive and specific diagnostic tools allows a careful detection of FLV-1 in certification programs to be run in future for the crop.

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Molecular characterisation of viruses from Kiwifruit

Pearson, M.N.[1]; Cohen, D.[2]; Chavan, R.[1]; Blouin, A.G.[2]; Cowell, S.J.[1]
[1] School of Biological Sciences, The University of Auckland, Auckland, New Zealand.
[2] The New Zealand Institute for Plant and Food Research Ltd., Auckland, New Zealand.

Abstract

In 2003 Apple stem grooving virus was discovered in Actinidia accessions from China, being held in quarantine in Auckland. Subsequent examination of kiwifruit germplasm from the same source has detected several additional viruses, including a ~300 nm rigid rod related to Ribgrass mosaic virus (Tobamovirus), a 700-750 nm flexuous virus related to Citrus leaf blotch virus (Flexiviridae) and a novel vitivirus. Currently these viruses have not been reported from commercial kiwifruit crops in New Zealand or elsewhere. The biological properties of the viruses from kiwifruit and their phylogenetic relationships with similar viruses from other plants will be described, and the possible implications for the international movement of Actinidia germplasm are discussed.

Introduction

Although kiwifruit has been grown commercially since the 1930's (Ferguson and Bollard, 1990) until recently very little has been published on viral diseases. In 1983 in New Zealand, local lesions, of presumed viral origin, were observed on C*henopodium quinoa* mechanically inoculated with leaf extracts from imported *Actinidia* germplasm (Gary Wood, DSIR, pers. comm.). Other reports include a sap transmissible (presumed) virus disease of *Actinidia* in China (Lin and Gao, 1995) and a report of viral-like symptoms in in *A. deliciosa* grafted to *A. polygama* rootstocks in Japan (Nitta and Ogasawara, 1997). The first definitive identification of a virus from *Actinidia* was *Apple stem grooving virus* (ASGV) in germplasm of Chinese origin being held in quarantine in New Zealand (Clover et al., 2003). Following the discovery of ASGV we examined the quarantine material for further viruses.

Given the lack of information on viruses of Actinidia and the absence of any closely related crops, initial screening of Actinidia germplasm used non-specific virus detection methods such as symptoms in Actinidia itself, mechanical transmission to herbaceous indicators and electron microscopy (EM). Where particle morphology could be determined by EM specific PCR tests for likely families or genera were performed. Where no virus particles were seen and/or subsequent test did not provide an identification, we adopted an approach involving mass spectrometry of potential viral proteins from the presumed virus infected plants.

Materials and methods

Source plants: Woody cuttings of Actinidia accessions from Shaanxi province, China, were grafted onto healthy rootstock of A. chinensis cv. Hort 16A in post-entry quarantine in New Zealand. In addition, samples of Actinidia glaucophylla, A guilinensis and A fortunatii were obtained from Plant and Food Research germplasm collections.

Sap transmission to herbaceous indicators: Leaf tissue (1–2 g) from both young and mature leaves of Actinidia was homogenized using a pestle and mortar in 4 ml 0.1 M phosphate buffer, pH 7.5 (Sweet 1975) containing 5 % polyvinylpyrrolidone and 0.12 % sodium sulphite. The homogenate was mechanically inoculated to 3-4 leaf seedlings of *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. occidentalis* 37B, and *Phaseolus vulgaris* cv. 'The Prince'. The inoculated plants were grown in a greenhouse at 20–22 °C for up to 6 weeks and observed for viral symptoms.

Transmission electron microscopy (TEM): Leaf extracts in 0.01 M Sørensen's phosphate buffer, pH 7 were negatively stained with 2 % (w/v) aqueous potassium phosphotungstate, pH 7 and observed using a Phillips Tecnai 12 electron microscope (FEI, Eindhoven, Netherlands). Particle lengths were determined using the microscope's internal calibration and by comparison to Tobacco mosaic virus.

Enzyme-linked immunosorbent assay (ELISA): Leaf samples from Actinidia and herbaceous indicators with were tested for Alfalfa mosaic virus (AMV) and Cucumber mosaic virus using antisera from Bioreba (CMV) and Prime Diagnostics (CMV), according to the manufacturer's instructions.

RT-PCR and sequencing: RNA was extracted from 100 mg leaf samples of Actinidia and indicators using an RNeasy® Plant mini kit (Qiagen) with the lysis buffer modification of MacKenzie et al. (1997) or a SpectrumTM (Sigma-Aldrich) kit, according to the manufacturer's protocol. RT was performed using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol using anchored oligo(d)T primers or virus

specific reverse primers. PCR amplification was performed with either specific or generic primers, Platinum® Taq polymerase (Invitrogen) and 2.5 mM dNTPs (Invitrogen) according to the manufacturer's instructions. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. DNA was purified using 'Perfectprep Gel Cleanup' purification kit (Eppendorf,

Hamburg, Germany), and sequences cloned in E. coli using pGEMT easy vector (Promega, Madison, WI, USA). Plasmids were purified using a FastPlasmid®Mini kit (Eppendorf, Hamburg,Germany) and the inserts sequenced with an ABI PRISM automated DNA sequencer (University of Auckland, New Zealand).

Sequence analysis: Consensus nucleotide sequences were compiled using Sequencher 4.5 (Gene Codes Corporation, Michigan 48108, USA) and translated into amino acid sequence using BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008). Sequence comparison and phylogenetic analyses were conducted using BLAST (Altschul et al. 1997; Schäffer et al. 2001) and MEGA version 3.1 (Kumar et al. 2004). Neighbour joining trees were constructed with Poisson corrected amino acid distances and pairwise gap deletion options and 50,000 bootstrap replicates. Consensus sequences were analysed for recombination events using RDP3 Beta 24-Recombination (Martin et al. 2005).

Virus partial purification and protein separation: Virus isolates were maintained in Nicotiana occidentalis and virions were partially purified from approximately 1 g of plant tissue using a protocol based on that of Lane (http://lclane.net/minipur.html). Denatured total soluble protein (15 µg) from the partially purified extracts was separated on a 12 % polyacrylamide gel (NuPAGE® Novex Bis-Tris Gels, Invitrogen) and molecular weights determined against Benchmark (Invitrogen) protein standards. Gels were stained with Coomassie blue G250 (SimplyBlue™ SafeStain, Invitrogen) following the manufacturers' instructions.

Tryptic digestion of proteins and mass spectrometry: Protein bands were excised from stained gels and digested with trypsin as described by Blouin et al. (2009). The resulting peptides (10 µl sample) were subjected to liquid chromatography electrospray tandem MS using a Finnigan™ LTQ-FT™ mass spectrometer coupled to a Surveyor HPLC system (ThermoFisher Scientific. Resulting peptide tandem (MS/MS) data were analyzed using TurboSEQUEST from the BioWorks v3.3 Suite (Thermo Fisher), against a virus database extracted from the National Centre for Biotechnology Information (NCBI) GenPep database (March 2008) containing non-human virus protein sequences (107,951 sequence entries). All resulting matched peptides were then confirmed by visual examination of the individual spectra.

Results

Infections in herbaceous hosts and electron microscopy: Mechanical inoculations to herbaceous indicators from both symptomatic and asymptomatic Actindia resulted in a range of virus-like symptoms, including mottles, mosaics, ring spots, vein clearing, and leaf distortion. EM examination of negatively stained leaf extracts from the herbaceous hosts revealed the presence of both ~300nm rigid rods and ~700-750 nm flexuous rods in several samples. However, virus particles were not observed in all symptomatic samples, suggesting the likely presence of other, less easily detectable particle types.

PCR and sequencing using generic and virus specific primers: Based on the particle morphology and size we tested for tobamoviruses (~300 nm rigid rod) using the Tob Uni-1 and Tob Uni-2 primers of Letschert et al. (2002) and carlaviruses (610-700nm flexuous rods) using Agdia carlavirus group PCR mix (Agdia Inc, Elkhart, USA). The Tob Uni-1 and Tob Uni-2 primers yielded a ~650bp product and the sequence confirmed this was a tobamovirus. Further PCR and sequencing, using primers of Zhu et al (2001) plus primers designed using alignments of 10 closely related tobamoviruses confirmed the virus as belonging to sub-group 3 of the genus Tobamovirus (Figure 1), most closely related to the R14 strain of Ribgrass mosaic virus (Heinze et al., 2006; Chavan et al., 2009).

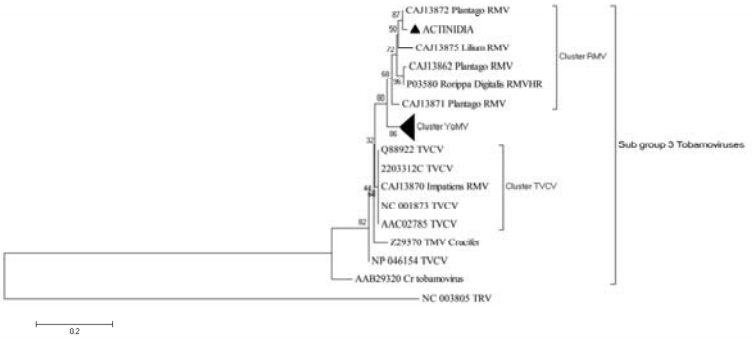

Fig. 1 Eighbour joining tree showing phylogenetic relationship of of putative coat protein gene amino acid sequence of *Actinidia* tobamovirus with CP aa sequences of sub group 3 tobamoviruses.

A ~300bp product was generated from the presumed carlavirus but the sequence was found to be most closely related to Citrus leaf blotch virus (CLBV) (Flexiviridae) rather than to carlaviruses. Subsequent sequencing using primers based on the published coat protein gene sequences of CLBV plus conserved regions from trichovirus, carlavirus, potexvirus, and vitivirus, confirmed this relationship (Table 1).

Tab. 1 Nucleotide identity of the partial replicase, complete movement and coat proteins and partial 3' UTR of a flexivirus from *Actinidia chinensis* with representative members of the *Flexiviridae*.

Virus	GenBank Accession No	% nucleotide identity
Citrus leaf blotch virus [Citrivirus]	NC_003877.1	80%
Citrus leaf blotch virus NZ_G18 [Citrivirus]	EU857539.1	80%
Citrus leaf blotch virus_G78 [Citrivirus]	EU857540.1	79%
Dweet mottle virus_932 [Citrivirus]	FJ009367.1	80%
Apple stem grooving virus [Capillovirus]	D14995.2	15%
Apple chlorotic leaf spot virus [Trichovirus]	M58152.1	16%
Apple stem pitting virus [Foveavirus]	D21829.1	14%
Grapevine virus A [Vitivirus]	AF007415.2	15%
Indian citrus ringspot virus [Mandarivirus]	AF406744.1	12%
Hop mosaic virus [Carlavirus]	NC_010538.1	15%
Shallot virus X [Allexivirus]	M97264.1	14%

Protein sequences from mass spectrometry: Where no particles were seen by EM, and consequently there was no indication of which viruses to test for, protein profiles from infected indicators were examined for proteins not present in healthy plants. Several of these were sequenced by mass spectrometry together with a sample known to contain the CLBV-like virus. In addition to the expected CLBV proteins indicative of the presence of Alfalfa mosaic virus (AMV) and Cucumber mosaic virus (CMV) and an apparently novel vitivirus were identified. The identity of AMV was confirmed from three Actinidia species by ELISA and by PCR using primers designed to amplify the CP and MP (Table 2). The Actinidia AMV coat protein and movement protein sequences showed >96 % nucleotide identity with each other and < 98% nucleotide identity with other AMV isolates (Table 2). Peptides from the vitivirus showed closest identity to Grapevine virus B (GVB) and subsequent sequencing showed 66 % and 69 % to the RdRp and 60 % and 69 % to the CP of GVA and GVB, respectively (Table 3). The CMV isolates were confirmed by ELISA but are yet to be sequenced.

Tab. 2 Nucleotide identity (%) of movement protein and coat protein sequences of *Actinidia* isolates of *Alfalfa mosaic virus* (AMV) with other isolates of AMV. Coat protein identity above the diagonal (italicized), movement protein below the diagonal.

	A. glaucophylla	***A. guilinensis***	***A. fortunatii***	**X00819**	**AF015717**	**M59241**	**AB126031**
A. glaucophylla		96.0	98.4	93.5	95.4	94.2	93.7
A. guilinensis	96.0		96.5	94.2	98.4	95.4	94.5
A. fortunatii	98.6	96.2		94.0	95.8	94.7	94.2
X00819	95.1	94.7	95.1		93.9	97.7	97.2
AF015717	96.2	98.0	96.4	94.0		94.4	93.5
M59241	95.0	94.4	94.7	97.2	94.9		97.7
AB126031	94.2	93.0	93.9	96.2	93.5	95.4	

Tab. 3 Amino acid identity (%) of *Actinidia* vitivirus with other vitiviruses.

Virus	ORF 1			ORF 2	ORF 3	ORF 4	ORF 5
	MeTr 336aa	Hel no data	RdRp 310aa	? 219aa	MP 297aa	CP 198aa	NB 105aa
Grapevine virus A	63	-	66	22	42	60	45
Grapevine virus B	63	-	69	17	38	69	11
Grapevine virus E	43	-	60	19	34	42	17
Mint virus 2	-	-	72	19	36	61	35

Discussion

Since 2003 six different viruses have been identified in kiwifruit germplasm that originated in China. The first was clearly an isolate of ASGV (Clover et al., 2003). The nucleotide sequence (~2900nt of 3' end) of the Actinidia isolate differs by ~15 % from both citrus and apple strains, which differ from each other by approximately the same amount. It is not known whether the Actinidia isolate of ASGV infects citrus and apple and vice versa.

The second virus to be described was a tobamovirus (Pearson et al, 2007) which clusters with cruciferous infecting isolates in sub-group three of the tobamoviruses (Chavan et al., 2009). Based on the coat protein and movement protein genes it is most similar to Ribgrass mosaic virus (RMV), a widely distributed virus. The host range of the Actinidia isolate has not been determined but RMV isolates have been found in a wide range of species, including common vegetables.

The Actinidia flexivirus sequences showed greatest identity with CLBV, the single member of the (proposed) genus Citrivirus (Mayo & Haenni, 2006). However, the Actinidia isolates may be sufficiently different to represent a distinct strain or species, but there are currently no guidelines for species differentiation within the genus Citrivirus (Fauquet et al., 2005). The host range of the Acitinidia isolate has not been determined but citrus isolates of CLBV are only known to infect citrus, are not mechanically transmitted in the field and have no known insect vectors. The vitivirus is most closely related to GVA and GVB but based on ICTV criteria (Fauquet et al., 2005) is sufficiently different to be considered a novel species. As with the other viruses isolated from Actinidia little is known about the host range of this virus but vitiviruses such as GVA and GVB typically have restricted host ranges. The other two viruses identified from Actinidia, AMV and CMV, both have very wide host ranges and are readily transmitted by a range of polyphagous aphid species. Since the sequences of the Acitinidia isolates show high similarity to isolates from other hosts it is quite likely that these viruses have moved into Actinidia from other hosts.

All six of the viruses were detected during routine screening of germplasm lines and to date no viral diseases have been reported from commercial crops in New Zealand. There have been reports of viral-like diseases in Actinidia from China and Japan but no specific virus identifications have been published and the impact of these is unknown. Although the Actinidia viruses detected so far appear to belong to previously known virus genera and species, some may be sufficiently different to be considered as distinct species or strains. Since the viruses were detected in individual germplasm accessions and have not been assessed in replicated infection trials very little is known about cultivar susceptibility and the effects, if any, on plant productivity. With traditional, long established crops the detection of viruses has mostly been driven by the presence of distinct disease symptoms in the crop and characterisation has advanced along with development of new technologies. The situation for kiwi fruit is quite different since we have detected several viruses in imported germplasm but so far have not observed any viral disease problems in commercial crops. Thus, modern molecular techniques have allowed us to detect and identify viruses but tell us little about pathogenicity and potential host range. This poses problems when comparing the virus status of kiwifruit in different countries, since in the absence of obvious disease problems in commercial crops researchers and government agencies are unlikely to actively look for viruses.

Since we have not observed any viral disease problems in commercial kiwifruit orchards in New Zealand the current focus is to provide high health material for breeding programmes. Since we have found viruses in material originating from China it is likely that these or other viruses are also present in germplasm collections in other countries. Consequently we would encourage growers and researchers in China and other kiwifruit growing countries to investigate the presence of viruses in order to: (a) understand the full range of kiwi fruit viruses, (b) determine what risk these may pose to the industry, and (c) take necessary action to mitigate any potential virus problems.

Acknowlegements

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Towards dissecting the structural determinant of *Peach latent mosaic viroid* inducing mosaic symptoms

Delgado, S.[1]; Navarro, B.[2]; Minoia, S.[2]; Gentit, P.[3]; Di Serio, F.[2]; Flores, R.[1]
[1]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain
[2]Istituto di Virologia Vegetale del CNR and Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università di Bari , 70126 Bari, Italy
[3] Centre Technique Interprofessionel de Fruits et Légumes de Lanxade, 24130 Prigonrieux, France.

Abstract

Most isolates of *Peach latent mosaic viroid* (PLMVd) do not incite foliar symptoms, but a few number of isolates cause peach mosaic (PM) or peach calico (PC), an extreme albino phenotype. The PC determinant has been previously mapped at an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop but the PM determinant, which is not associated with a specific insertion, remains unidentified and could reside in one or more domains of the branched conformation proposed for PLMVd. To tackle this problem we have selected for further dissection one variant (GDS6), recovered from a typical PM isolate (GDS), which is very infectious and elicits consistently a characteristic PM. We have initially focused on G337, a position that appears associated with PM in multiple alignments that include GDS6 and other PM-inducing and latent variants. To determine the role of G337 in infectivity and symptoms, GF-305 peach seedlings were inoculated with *in vitro* transcripts of recombinant plasmids containing dimeric tandem inserts of PLMVd-cDNAs with all possible changes at this position introduced by site-directed mutagenesis. Deletion of G337 abolished infectivity, while substitutions by A, C or U incited, in most inoculated plants, PM symptoms. Cloning and sequencing showed that the A substitution at position 337 was preserved in the progeny or reverted to G, while C or U substitutions at this position were not stable and reverted to A or G in the progenies. Extending this approach to additional nucleotides of loop A, or of other PLMVd domains, may provide hints in identifying the determinant of PM.

Keywords: Viroids, Pathogenesis, Peach disease

Introduction

Peach latent mosaic viroid (PLMVd) (Hernández and Flores, 1992; Flores et al., 2006), is the type species of the genus *Pelamoviroid* within the family *Avsunviroidae* (Flores et al., 2005). Members of this family do not have a central conserved region, but are able to form hammerhead ribozymes in both polarity strands that mediate self-cleavage of the replicative intermediates generated through a symmetric rolling-circle mechanism (Flores et al., 2000). There is direct evidence indicating that replication and accumulation occurs in the chloroplast in two members of this family, *Avocado sunblotch viroid* (ASBVd) (Bonfiglioli et al., 1994; Lima et al., 1994; Navarro et al., 1999) and PLMVd (Bussière et al., 1999), a property that is presumably shared by the other members of the family *Avsunviroidae*.

PLMVd infection of peach cultivars grown under field conditions, and of the peach indicator GF-305 grown in greenhouse, may induce a wide variety of leaf symptoms and, accordingly, isolates are classified into three types: i) latent, the most frequent as reflected in the name of the disease (Desvignes, 1976; Desvignes 1980; Desvignes 1986), ii) peach mosaic (PM) or peach blotch of variable severity, and iii) peach calico (PC), an extreme chlorosis that completely covers the leaf area. Previous results have shown that the structural determinant of PC maps at an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop (Malfitano et al., 2003; Rodio et al., 2006; Rodio et al., 2007). However, the molecular determinant of PM remains unidentified because, not being associated with a specific insertion, it could reside in one or more domains of the branched PLMVd conformation. Moreover, molecular characterization of latent and PM-inducing PLMVd isolates has revealed that they are formed by complex populations of variants (Ambrós et al., 1998), and bioassays on GF-305 peach seedlings of some individual variants have shown that the biological properties of PLMVd isolates depend on the complexity of their populations and on the presence of specific variants (Ambrós et al., 1999). Therefore, mapping the structural determinant of PLMVd inducing PM may be considerably more complicated than in the case of PC. Here, we report our first attempts in this mapping.

Material and methods

Infectivity bioassays: Inoculations were performed by slashing the stems of GF-305 peach seedlings, which were kept in a greenhouse and periodically examined for symptom expression 2-3 months postinoculation (Ambrós et al., 1999). Infection of GF-305 peach seedlings was additionally tested by dot-blot hybridization of nucleic acid preparations with a radioactive full-length PLMVd-cRNA probe, and by RT-PCR with a pair of adjacent primers (RF-43 and RF-44) of sense and antisense polarity derived from a region with low variability (Ambrós et al., 1998).

Inocula: The recombinant plasmid pGDS6, containing a head-to-tail dimeric full-length cDNA insert of the GDS6 variant (Ambrós et al., 1999), served as starting material for generating by site-directed mutagenesis (Byrappa et al., 1995) plasmids pGDS6Δ337, pGDS6A337, pGDS6C337 and pGDS6U337, wherein the G337 nucleotide was deleted or substituted in both cDNA copies by A, C or T, respectively. *In vitro* transcripts from these plasmids, synthesized as reported previously (Hernández and Flores, 1992), were used as inocula.

Progeny characterization and sequence analysis: RT-PCR products were fractionated by electrophoresis in polyacrylamide gels (5 %) and, after ethidium bromide staining, the PLMVd-cDNAs of the expected size were eluted and cloned into a plasmid vector. The sequence of the corresponding inserts were automatically sequenced.

Results and discussion

Our working hypothesis is that the molecular determinant for PC may map at the same loop (loop A) at which the 12-13 nt insertion characterictic of PC has been reported before (Malfitano et al., 2003). As an experimental system to start testing this hypothesis we have selected the PLMVd variant GDS6, recovered from a typical PM isolate (GDS), because it is very infectious and incites consistently a characteristic PM (Ambrós et al., 1998; Ambrós et al., 1999). Multiple alignments of previously characterized PLMVd variants inducing latent and PM phenotypes, including GDS6 (Ambrós et al., 1998; Ambrós et al., 1999), suggest that nucleotide G337 of variant GDS6, located in the loop A (Figure 1), is associated with PM.

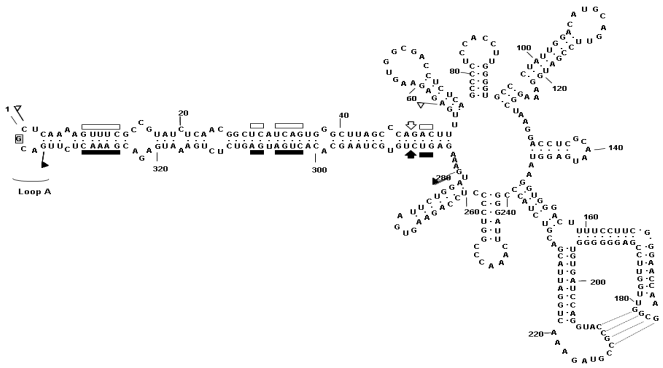

Fig. 1 Proposed secondary structure of PLMVd variant GDS6 (Ambrós et al., 1998). Sequences involved in forming the hammerhead structures are flanked by flags, conserved nucleotides present in most natural hammerhead structures are indicated by bars, and self-cleavage sites are marked by arrows; solid and open symbols refer to plus and minus polarities, respectively. Nucleotides involved in a pseudoknot between positions 176 to 179 and 209 to 212 (Bussière et al., 2000) are denoted by broken lines. The G337 residue within the loop A is boxed and with a grey background.

To examine this possibility we generated the recombinant plasmids pGDS6Δ337, pGDS6A337, pGDS6C337 and pGDS6U337, containing head-to-tail dimeric full-length cDNAs in which the G337 nucleotide was deleted or substituted by A, C or T, respectively (Table 1). The infectivity and pathogenicity of the resulting GDS6-mutated variants was tested by slash inoculation of the corresponding *in vitro* transcripts into blocks of eight GF-305 seedlings. Symptom observation and dot-blot hybridization and RT-PCR analyses revealed that deletion of G337 abolished infectivity of the GDS6Δ337 transcript, while substitutions by A, C or U had moderate effects, with most plants showing clear PM symptoms although less severe than the GDS6 wild-type control. Cloning and sequencing of the PLMVd progeny accumulating in seedlings inoculated with variant GDS6A337 showed that it was composed by

variants characterized by having a G or an A residue at position 337, which are the nucleotides found at this same position in all the natural variants characterized previously from the GDS isolate (Ambrós et al., 1998; Ambrós et al., 1999) (Table 1). In contrast, the progenies resulting from variants GDS6C337 and GDS6U337 did not preserve the artificially-introduced C337 and U337 nucleotides, respectively (Table 1).

Tab. 1 Nucleotide at position 337 in the progeny of the mutated variants with respect to the parental GDS6 variant (with a G at this position).

Variant	Sequenced clones	A337	G337	C337	U337
GDS6*	8	2	6	0	0
GDS6Δ337	NI	-	-	-	-
GDS6U337	9	3	6	0	0
GDS6A337	20	10	10	0	0
GDS6C337	10	4	6	0	0

*Data from Ambrós et al. (1999); NI: not infectious variant

Instead, in both cases they were substituted by G or A as in the progeny resulting from variant GDS6A337. Additionally, other point mutations with respect to the parental inoculated transcripts were detected in two positions (but not in another two) of the loop A in the characterized progeny variants (Figure 2). However, these substitutions have been already reported in the progeny of the natural GDS6 variant (Ambrós et al., 1999), and no specific correlation with induction of PM could be established in the present study. As expected, a relatively high sequence variability distributed throughout the viroid genome at positions similar to those previously reported for the progeny of the natural GDS6 variant (Ambrós et al., 1999), was also noticed (data not shown).

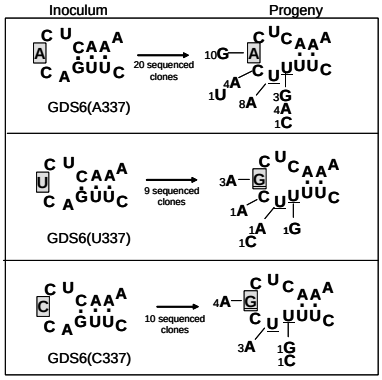

Fig. 2 Schematic representation of the loop A and the adjacent stem of the GDS6-mutated infectious variants (left) and of their respective progeny variants (right). The nucleotide at the position 337 is boxed and with a grey background. The number of variants sequenced in the progeny of each infectious variant is indicated below the arrows. The most abundant point mutations in progeny variants are underlined, and the number of clones in the progeny in which additional mutations were found, as well as their location, are also indicated.

Our results show that a deletion at position 337 of variant GDS6 abolish infectivity, most likely because a nucleotide at this position is critical for a minimal replication threshold that might facilitate, due the high mutation rate observed in some chloroplastic viroids (Gago et al., 2009), incorporation of a 1-nt insertion restoring the original size of loop A. In line with this view, the three substitutions at the same position would allow replication above the threshold and ultimately lead to selection of a purine. Because of the natural reversion of substitutions at position 337, it is not possible to establish a direct correlation between PM and the G337 residue in the GDS6 variant. However, further experiments extending this approach to additional nucleotides of loop A, or of other PLMVd domains, may provide hints to identify the molecular determinant of PM.

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Variability assessment and construction of infectious clone of Indian *Apple Scar Skin Viroid*

Walia, Y.; Kumar, Y.; Rana, T.; Ram, R.; Hallan, V.; Zaidi, A.A.
Plant Virology Lab, Institute of Himalayan Bioresource Technology, CSIR, Palampur, 176061, Himachal Pradesh, India.

Abstract

Apple scar skin viroid (ASSVd) is widely distributed and economically important pome-fruit infecting viroid belonging to the genus *Apscaviroid*. It causes huge economic losses to the apple industry. Apple fruits with dappling, scarring, cracking and deformation symptoms were noticed during survey of apple growing regions of Himachal Pradesh, India. ASSVd was detected from four isolates showing dappled fruits. Molecular characterization of the viroid was done. Ten clones each from five isolates were sequenced out of which seven new sequence variants of ASSVd were found. Four of the clones were 330 nucleotides (nt) long and the other eight had an additional nucleotide. The clones showed significant sequence variability (94-100%) with each other. Variability was more common in the pathogenic domain of the viroid genome. Present isolates grouped with some Chinese and Korean isolates in phylogenetic analysis. The study reports seven new sequence variants of ASSVd and also gives a first molecular evidence of a viroid infection (ASSVd) in apple from India. Infectious clone of ASSVd were constructed for *in vitro* mutagenic studies.

Keywords: *Apple scar skin viroid*, cloning, DNA sequencing, phylogenetic analysis

Introduction

Viroid genome consists of single stranded covalently close circular, non-coding, non-encapsidated RNA. Without encoding capacity, the viroid RNA genome and its replication intermediates interact directly with host components for nearly all aspects of infection process, including replication, intercellular movement, intercellular movement, systemic movement and pathogenecity. They exist as rod-shaped structures in their secondary state (Diener, 2001). Viroids have been grouped into two families, *Pospoviroidae* (nucleus replicating) and *Avsunviroidae* (chloroplast replicating) based on their mode of replication (Flores et al., 2005). ASSVd is the type species of genus *Apscaviroid*, family *Pospiviroidae* (Hashimoto et al., 1987).

Apple is one of the major fruit crops of India and cultivated in the northern states (Himachal Pradesh, Jammu and Kashmir, Uttarakhand) and some north-eastern states (Arunachal Pradesh, Sikkim and Nagaland). Apple can be infected with viroids viz. ASSVd, *Apple fruit crinkle viroid* (AFCVd) and *Apple Dimple fruit viroid* (ADFVd) (Hashimoto and Koganezawa, 1982; Di Serio et al., 2002; Koganezawa, 2001).

These viroids cause severe symptoms on apple fruits which include color dappling, cracking, scarring and distortion depending upon the cultivar (Koganezawa, 2001) rendering it totally unmarketable. Three other diseases, dapple apple (Hadidi et al., 1990), pear rusty skin (Chen et al., 1987) and Japanese pear fruit dimple (Osaki et al., 1996) have been attributed to ASSVd molecular variants (Zhu et al., 1995). In India, this viroid has already been reported based on symptomatology and PAGE assays (Handa et al., 1998). In the present communication ASSVd is detected and molecularly characterized from apple bark tissue from Northern India. Apple plants were found to be infected with complex mixture of sequence variants of ASSVd. To the best of our knowledge this is the first molecular report of a viroid infection in apple from India. Infectious clones of the ASSVd were constructed to study infectivity and host pathogen interactions.

Materials and methods

Molecular characterization of ASSVd: Surveys were conducted in different apple growing regions of Himachal Pradesh. Forty- five fruit and bark samples of the symptomatic apple plants were collected and analyzed for the presence of ASSVd. Total RNA was extracted using RNeasy® Plant Mini kit (Qiagen, Germany) with slight modification in the extraction buffer. From the total RNA, RT-PCR was performed on standard conditions using ASSVd specific primer pair (cASSVD and hASSVD; Di Serio et al., 2002). For first strand cDNA synthesis, a reaction mixture of 25µL was made using 7µL RNA, 5µL M-MLV reverse transcription buffer (5x, USB), 1µL (200ng/µL) primer (down primer), 1.5µL dNTP mix (40mM), 0.5µL (200 U/µL) M-MLV reverse transcriptase (USB) and 0.1µL (40U/µL) RNase inhibitor. The RT reaction was incubated at 37 ºC for 75 min and then at 70 ºC for 5 min. PCR was carried out in 0.2ml thin walled tubes in an automated thermocycler (Applied Biosystems, USA). PCR reaction mixture

consisted of 10µL of cDNA, 5µL 10x Taq DNA polymerase buffer (Genei, India), 2µL of dNTP mix (10mM), 1µL (200ng/µL) each of downstream and upstream primers and 0.5µL Taq DNA polymerase (Genei, India).

PCR products were run on 1 % agarose gel, stained with ethidium bromide (1µg/ml) and visualized under UV transilluminator. PCR products were purified from the gel using GenElute Gel Extraction Kit (Sigma, USA) and the eluted products were cloned into pGEM®-T Easy vector (Promega, USA). Recombinant plasmids were purified using GenElute Plasmid Miniprep Kit (Sigma, USA) and sequenced with an automated DNA sequencer (ABI PRISM® 3130xl Genetic Analyzer) using ABI prism Big Dye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems), sequencing both strands. Sequences were submitted to EMBL database and accession numbers AM993159, AM993160, FM178283, FM178284, FM178285, FM208138, FM208139, FM208140, FM208141, FM208142, FN547406 and FN547407 were obtained. Sequences were analyzed with the help of Basic local alignment search tool (BLAST; Altschul et al., 1990). Multiple alignments were carried out with the help of MultAlin software available online at

http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html (Corpet, 1988).

For phylogenetic analysis the accession numbers of different ASSVd sequences taken from GeneBank are: EU031496, EU031489, AF421195, X17696, EU031464, DQ362906, M36646, GQ249350, GQ249349 and FJ974063. An isolate of *Apple dimple fruit viroid* (EF088665) reported from Italy, was taken as an out-group. Origin of these isolates has been shown in the phylogenetic tree. The modified ClustalW program (Chenna et al., 2003) was used (www.ddbj.nig.ac.jp) with 1000 bootstrap replicates and phylogenetic tree was viewed using TreeExplorer software (Kumar et al., 1994) downloaded from the site http://www.megasoftware.net/

Construction of Infectious Clones: Infectious ASSVd clones were constructed by dimerizing of the ASSVd sequence. Primers were designed from the unique *Sal*I restriction site region of ASSVd sequence at nucleotide position 87. Homologous primer with the *Sal*I site (87-106 nts, ASSVdICh; GTCGACGAAGGCCGGTGAGAA), and complementary primer (87-67 nt, ASSVdICc; CGTCGACGACGACAGGTGAGTT) was used for amplifying the viroid and then the same homologous primer in combination with complementary primer without the *Sal*I restriction site (81-62 nt, ASSVdIC1; GACGACAGGGTGAGTTCCTTC) was used for genome amplification. Both of the amplified products were cloned into pGEM®-T Easy vector (Promega, USA) and sequenced. Recombinant plasmid containing the insert amplified by primer pair ASSVdICh and ASSVdC1 were digested with *Sal*I restriction enzyme, purified using Phenol: Chloroform: Isoamyl alcohal (25:24:1) solution and ligated using T4 DNA Ligase (Fermentas). The ligated product was transformed into *E.coli* DH5α strain. Recombinant plasmids were purified using GenElute Plasmid Miniprep Kit (Sigma, USA) and sequenced. Finally, plasmid containing the dimer sequence was cloned into binary vector pCambia-1300 and transformed into agro bacterium GV3101 strain.

Results and discussion

Expected sized amplicons of ~330 bp were obtained in five isolates from three apple varieties viz. Royal delicious (four isolates), Gold Spur (one isolate) and Red Chief (one isolate). Ten randomly selected cDNA clones were sequenced from each positive sample and identified as ASSVd in BLAST search. The sequences showed 94-100 % similarity with each other. Seven clones were found to be new sequence variants of ASSVd. Two clones (AM993159 and AM993160) showed 100% similarity to a Chinese isolate (EU031496) whereas eight clones (FM178283, FM178284, FM208139, FM208140, FM208141, FM208142, FN547406 and FN547407) showed 99% similarity to a Korean isolate (AF421195). Remaining two clones (FM178285 and FM208138) were more similar to the Chinese (EU031455) and Japanese isolate (M36646). The Korean isolate is 331 nt long and differs from the Japanese isolates in its 'G' insertion between the nucleotide 133-134 (Lee et al., 2001).

However all sequences reported in this communication had the 'G' insertion, though four of them are 330 nt and other eight are 331 nt long. Eight 331 nt long ASSVd clones contain a 'T' insertion between the 220-221 nt which lacks in the other four ASSVd clones. Sequences of the present clones were aligned with the ASSVd sequences reported from other locations (Figure 1). Variability was identified at 25 positions out of 310 (excluding primer sequences), which were more common in the 5'-end of the putative pathogenicity domain. This suggested that ASSVd is quite variable in its sequence in this domain, which might be responsible for different symptom expressions in different plants.

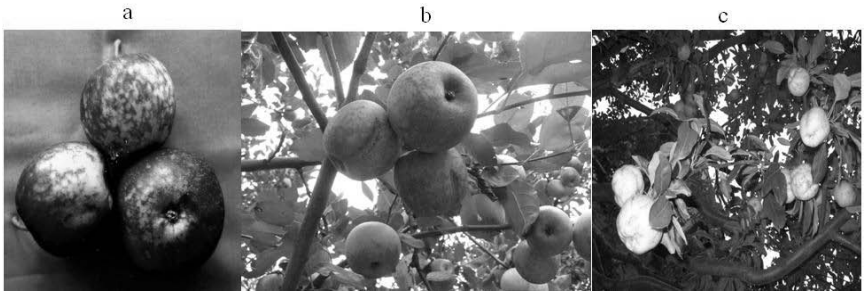

Fig. 1 a) Dappling symptoms on apple fruits, b) Scarring of apple fruit skin c) Deformation of the apple fruit.

In the phylogenetic analysis (Figure 2) our clones grouped with Chinese, Japanese, Korean isolates, which suggests that these have a geographical relationship, as all these countries are part of Asian continent and possibly there could be an exchange of material among them. Interestingly, AM993159 and AM993160 clustered with a Chinese isolate (EU031496) reported from apricot, which suggests their close relationship.

Greece isolates from cherry, wild apple and pear also showed close resemblance with our isolates. All other isolates clustered with different apple isolates reported from China, Korea and Japan (Figure 3).

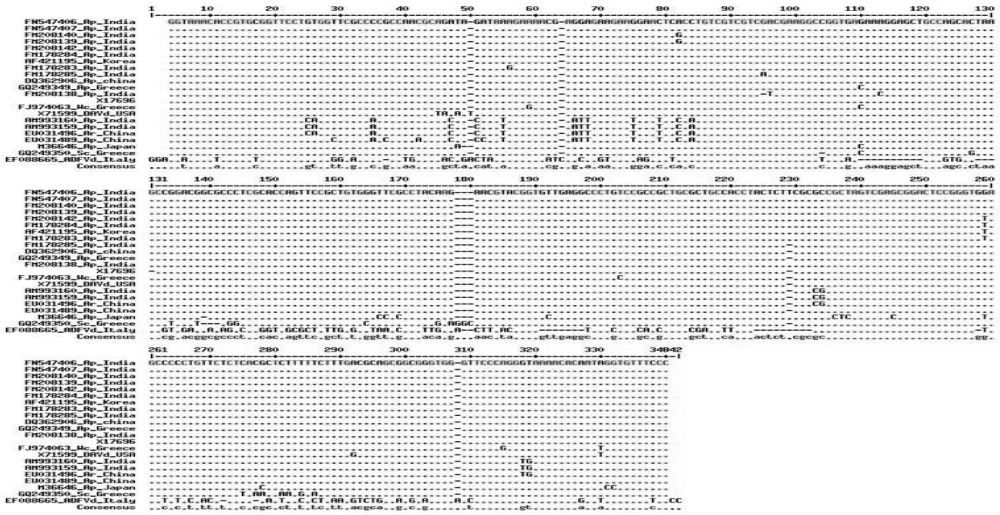

Fig. 2 Multiple alignment of the present isolates showing variability in first 90 nucleotide part and the central conserved region.

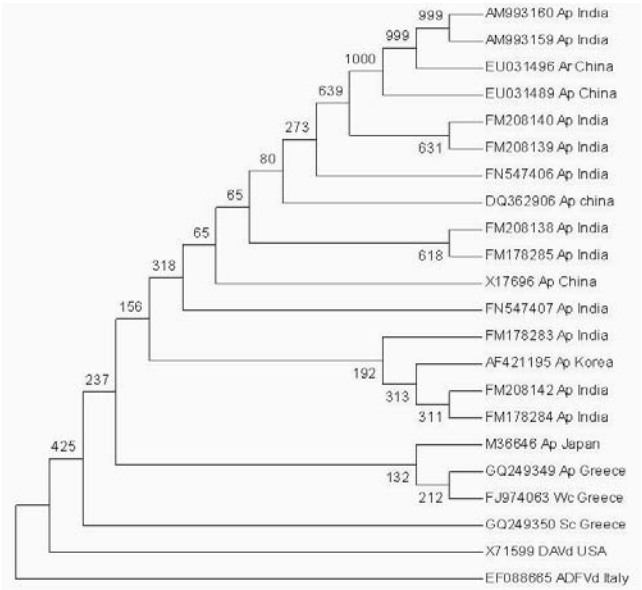

Fig. 3 Phylogenetic tree showing evolutionary relationship of the present isolates with other ASSVd isolates taken for study. AM993159 and AM993160 show close relationship with some Chinese isolates reported from apricot. Other clones cluster with different apple isolates reported from China, Korea, Greece and Japan. Numbers at the nodes of the branches indicate bootstrap values out of 1000 replicates (In percent). *Apple dimple fruit viroid* sequence (EF088665) reported from Italy, was taken as an out-group. Ap: Apple; Ar: Apricot; wc: Wild Cherry; wa: Wild Apple.

The dimer of ASSVd was constructed successfully and experiments involving the agroinoculation of these constructs into different hosts are under process. All five domains of the viroids of family *Pospoviroidae* can cause symptom alterations in the host. Mutational analysis of *Potato spindle tuber viroid* and construction of intra-specific chimeras have shown that sequences within at least three of these domains (i.e. the left terminal loop, pathogenecity domain and variable domain/right terminal loop) play important roles in modulating symptom expression (Visvader et al., 1986; Sano et al., 1992) and mutations in the loop E of the CCR of the *Pospoviroids* suggested that CCR can also play role in the symptom development (Qi and Ding, 2003). ASSVd causes symptoms of dappling (new apple cultivars) as well as scarring (old apple cultivars). Symptoms depend upon the cultivars (Desvignes et al., 1999) and probably on the viroid variants infecting the cultivars. Thus mutational studies with ASSVd may help us to predict which particular nucleotides could be responsible for the specific symptoms on apple fruit. The study will make a base for viroid research of temperate fruits in India. To the best of our knowledge this is the first molecular evidence of viroid infection on apple in India.

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The molecular characterization of HSVd isolates associated with dapple fruit and fruit rugosity in plum seedlings suggests a possible role of breeding in viroid dissemination

Luigi, M.[1]; Faggioli, F.[1]; Barba, M.[1]; Giunchedi, L.[2]
[1]CRA-Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
[2]Dipartimento di Scienze e Tecnologie Agroambientali - Università di Bologna

Abstract

In a wide range of hosts, the infection caused by *Hop stunt viroid* (HSVd) appears to be latent, whereas in some others it is frequently pathogenic. In this work, the presence of HSVd has been found to be associated with symptoms of dapple fruit and fruit rugosity in plum seedlings obtained from cross breeding for quality. Symptomatic and symptomless plum seedling samples have been analyzed for the presence of the principal stone fruit viroids and viruses. HSVd was found in all symptomatic samples, whereas no other viruses or viroids were found in the analyzed samples with the exception of ACLSV, which was detected rarely. The RNAs of all HSVd isolates have been cloned and sequenced. The sequence analysis showed a high percentage of homology among the isolates, making it possible to hypothesize a potential unique origin of the infection. For this purpose, those plants used in breeding as pollen donors have been analyzed. The results showed that the same HSVd isolate was also present in the parental plants, both in the leaves and pollen, suggesting a possible role of breeding in the dissemination of the viroid.

Keywords: plum, seedlings, fruit rugosity, dapple fruit, HSVd, pollen

Introduction

Viroids are subviral pathogens consisting of a circular single strand of RNA that ranges in size from 240 to 400 nucleotides. Their genome is highly structured without any detectable messenger activity so they are host-dependant in their life-cycle (Diener, 1991). Viroids are classified into two families: *Pospiviroidae* and *Avsunviroidae* (Flores et al., 1998). *Hop stunt viroid* (HSVd) belongs to the *Hostuviroid* genus, itself belonging to the *Pospiviridae* family, with a size that ranges between 294-303 nt. HSVd has been found in a wide variety of hosts: hop, cucumber, grapevine, citrus, plum, peach and pear (Shikata, 1990) as well as apricot and almond (Astruct et al., 1996, Canizares et al, 1999). In some species, like grapevine, the infection seems to be latent (Shikata, 1990; Polivka et al., 1996). Contrastingly, in some others, like hop, citrus, plum, peach and apricot, certain variant sequences can cause symptoms like stunting, dapple fruit, fruit rugosity and cachexia (Diener et al., 1988; Sano et al., 1989, Shikata, 1990, Ragozzino et al., 2002 , Amari et al., 2007).

The variants in plum and peach induce the dapple fruit disease that was first discovered in Japan in 1986 on *Prunus salicina* 'Taiyo' (Terai, 1985). Symptoms of dapple fruit disease are restricted to the fruit and they vary according to species (peach or plum) and to cultivars. Generally, it induces chlorotic blotches and spots on the skin of the fruit, and the surface of the fruits also becomes irregular (Sano et al., 1989). In Italy, HSVd was reported on symptomless stone fruit samples by Loreti *et al.* (1998). Subsequently, dapple fruit disease was reported by Ragozzino et al. (2002) in Japanese plum 'Sorriso di Primavera' and 'Florentia'.

In this work, plum seedlings, obtained from cross breeding for quality and showing dapple fruit and fruit rugosity symptoms (Figure 1), have been analyzed for the presence of viroids (HSVd and *Peach latent mosaic viroid* – PLMVd) and viruses (*Plum pox virus* – PPV, *Apple chlorotic leaf spot virus* – ACLSV, *Prunus dwarf virus* – PDV, *Prunus necrotic virus* – PNRSV, *Strawberry latent ring spot virus* – SLRSV, *Cherry leaf roll virus* – CLRV).

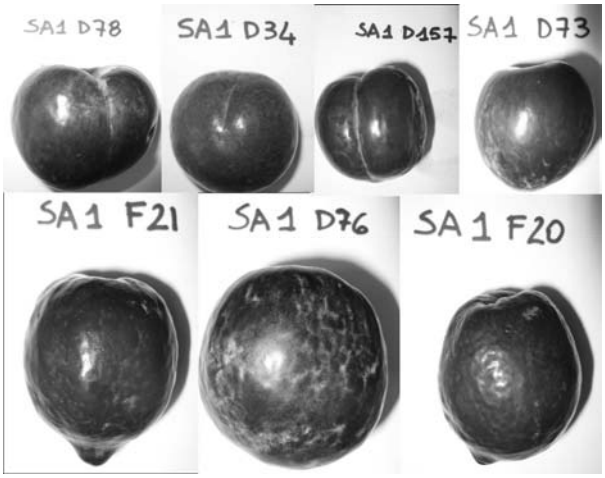

Fig. 1 Fruits from plum seedlings showing dapple fruit and rugosity symptoms

Materials and methods

Source of material: Plum seedlings (4 years old), obtained from cross breeding for quality, grown in an experimental orchard in Emilia Romagna region (Northern Italy) were used as source material. More specifically, symptomatic fruits were collected from 20 seedlings; 10 non-symptomatic fruits from seedlings located in the same orchard were also analyzed as negative control samples. HSVd-infected and healthy GF 305 were used as controls. Parental pollen donor plants ('Black sunrise' and 'Black glow'), used for breeding, were also analyzed for the presence of HSVd.

RNA target preparation and pathogen detection: For the detection of viroids (HSVd and PLMVd), total nucleic acids (TNA) were extracted from fruit skin according to the protocol established by Faggioli et al, (2001). TNA was finally eluted in 100 µL of DEPC water and analyzed following the two step/one tube RT-PCR protocol described by Ragozzino et al, (2002), using specific primer pairs (Loreti et al., 1999; Astruc et al., 1996). For the viruses detection (PPV, ACLSV, PDV, PNRSV, SLRSV, CLRV), 0.1 g of leaf sample was powdered in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The RNA was finally eluted with 50 µL of RNase-free water and analyzed following the one-step RT-PCR protocol described by Pasquini et al. (1999), using specific primer pairs (Wetzel et al., 1991; Candresse et al., 1995; Faggioli et al., 2005; MacKenzie et al., 1997; Spiegel et al., 1997). All amplified products were analyzed using electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

Pollen isolation and extraction: Closed flowers were collected and the pollen grains mechanically separated from anthers and stored in a Petri dish. To gain evidence about the presence of the viroid inside or in the surface of the pollen grains, total RNA was extracted from 20 mg of pollen and analysed for the presence of HSVd following a modified protocol by Aparicio *et al*. (1999). Briefly, 20 mg of pollen was suspended in 900 µl of 0.2 M Tris-HCl pH 8.2, 17.5 µl of 5M NaCl, 8 µl of 10% Triton X-100 and 2 µl of 2-mercaptoethanol, vortexed for 1 min and centrifuged at 3,000 rpm for 5 min. This procedure was repeated three times. Aliquots from the three supernatants and washed homogenized pollen were phenol-extracted and the aqueous phase ethanol-precipitated and resuspended in sterile water. Supernatants and triturated pollen were analysed for the presence of HSVd by RT-PCR.

Cloning and sequence analysis: All the HSVd amplified products were purified and cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA). Obtained sequences from the recombinant plasmids were multiple aligned using the Clustal W program and compared with the HSVd isolates retrieved from the Gene Bank database.

Results

Fruits from symptomatic (dapple fruit and fruit rugosity symptoms) and asymptomatic plum seedlings were analyzed by RT-PCR to test for the presence of the main viroids and viruses which affect stone fruit trees. Negative results were obtained for PLMVd and all viruses analyzed with the exception of ACLSV, which was detected rarely (data not shown). HSVd was isolated from all symptomatic samples, whereas no HSVd RNA was detected in asymptomatic seedlings. The sequencing of the cloned HSVd isolates showed a high homology (99-100 %) among the clones, with the exception of the clone F20.2 (92 %). The length of all the HSVd variants was of 296 nt (297 for the clone F20.2) and were identical to the already characterized asymptomatic plum isolates PL 278 from Turkey (Gazel et al., 2008, accession number EF523829), whereas no specific similarities were identified when comparing the HSVd plum seedlings isolates with the isolates previously found to be associated with symptoms in stone fruits.

The parental pollen donor plants 'Black sunrise' and 'Black glow' were infected with the same HSVd isolate of the fruit seedlings. The pollen collected from the parental plants also resulted positive to the test for the presence of HSVd, both inside and outside the granules.

Discussion

HSVd has been constantly found to be associated with plum seedlings showing symptoms of dapple fruit and fruit rugosity. Both these symptoms were previously associated with the HSVd infection: dapple fruit in plum and peach in Japan, Italy and China (Sano et al., 1989, Ragozzino et al., 2002; Zhou et al., 2006) and fruit rugosity in apricot in Spain (Amari et al., 2007). Our results showed that also fruit rugosity, in addition to the dapple fruit symptom, seems to be associated with HSVd in plum. The molecular characterization of the HSVd variant isolated from the symptomatic tree does not show any peculiarity. The comparison of the plum seedling isolates to the isolates previously reported in the literature in symptomatic stone fruit trees, does not highlight any typical polymorphism that could be associated with the symptoms. The symptom expression does not seem to be correlated with any peculiar nucleotide sequence of HSVd, since the most closely related HSVd isolate was an isolate identified in an asymptomatic plum in Turkey. Probably, symptom expression could be dependent on the cultivar's response.

Almost all HSVd plum seedling isolates showed a high percentage of homology (99-100 %), making it possible to hypothesize a potentially unique origin of the infection. For this reason, the parental pollen donor plants used for breeding ('Black Sunrise' and 'Black Glow') have been analyzed and found infected with the same HSVd isolate of the progeny. This result highlighted a possible role of pollen in viroid transmission. Flowers from the infected plants were collected and the pollen isolated and analyzed. The analysis performed on the pollen collected from the parental plants confirmed the hypothesis, with HSVd being found on and inside the pollen grains.

Infected pollen has been shown to play a key role in both seed and plant-to-plant transmission of several viroids, (i.e. *Potato spindle tuber viroid*- Singh et al., 1992; *Coleus blumei viroid* - Singh et al., 1991; grapevine viroids - Wah and Symons, 1997; *Avocado sunblotch viroid* - Allen et al., 1981; *Hop stunt viroid* cucumber pale fruit strain - Kryczynski et al., 1988; *Peach latent mosaic viroid* – Barba et al., 2007). The evidence of the presence of the same HSVd isolate in the parental pollen donor plants, in the pollen and in the obtained seedlings suggests a possible role of breeding in HSVd dissemination through infected seeds, even though other biological factors such as the possible transmission by thrips or other flower-working arthropods have to be taken into consideration for plant-to-plant transmission.

Studies are under way to investigate the role of infected pollen in HSVd transmission in plum, as well as to reproduce the symptomatology in healthy plum by experimental infection with the HSVd plum seedling isolate.

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Two novel variants of hop stunt viroid associated with yellow corky vein disease of sweet orange and split bark disorder of sweet lime

Bagherian, S.A.A.; Izadpanah, K.
Plant Virology Research Center, College of Agriculture, Shiraz University, Shiraz, Iran

Abstract

Yellow corky vein was reported as a graft-transmissible disease of lime in India. It was attributed to infection by hop stunt viroid (HSVd) and citrus exocortis viroid (CEVd). Recently similar symptoms have been observed in Washington navel orange in Jahrom and Darab in the Fars province of Iran. It is characterized by yellowing and suberization of veins followed by tree decline. Sweet lime split bark is another disorder of increasing importance in the Fars province. It is characterized by cracks in the bark of the main stem which may spread to branches of the tree. Since these symptoms resembled those of certain viroids, a study was undertaken to determine possible association of viroids with the disorders. Reverse transcription polymerase chain reaction (RT-PCR) followed by cloning and sequencing of PCR products and dot-blot hybridization were used to identify the viroids associated with the diseases. Comparison of molecular properties (nucleotide composition, primary and secondary structures, molecular weights, phylogenetic relationships and percent nucleotide similarity and difference) of viroid variants were carried out. It was found that a novel variant of hop stunt viroid (HSVd-sycv) was associated with yellow corky vein disease of Washington navel and another new variant (HSVd-sb) with split bark disorder of sweet lime. No other viroids were constantly detected. HSVd-sycv was closely related to noncachexia variant of hop stunt viroid (HSVd-cit) but only with 93.7% homology with HSVd-lycv. It differed in a single nucleotide from HSVd-cit, in the variable domain in the so-called "cachexia expression motif". HSVd-sb had only 94.8% homology with a noncachexia variant of hop stunt viroid (CVd-IIa-117) which causes mild bark-cracking symptoms on Pomeroy trifoliate orange rootstocks. According to the performed molecular comparisons, HSVd-sb differed from CVd-IIa-117 in "cachexia expression motif" and probably severe cracks induced by HSVd-sb occurred because of variation in this motif.

Keyword: HSVd, Sweet lime split bark, Sweet orange yellow corky vein, Citrus viroids, Viroid phylogeny.

Introduction

Citrus viroids are classified into four genera, all belonging to the family *Pospiviroidae.* (Flores et al., 2005; Ito et al., 2001). *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd, formerly called CVd-II) and *Citrus viroid IV* (CVd-IV) are assigned to the genera *Pospiviroid*, *Hostuviroid*, and *Cocadviroid*, respectively (Ito et al., 2000; Ito et al., 2001; Flores et al., 1998). The genus *Apscaviroid* includes *Citrus bent leaf viroid* (CBLVd, including *Citrus viroid-I-LSS*), *Citrus viroid-III* (CVd-III), *Citrus viroid-V* (CVd-V) and *Citrus viroid-OS* (CVd-OS) (Barbosa et al., 2005). While some of these citrus viroids are apparently harmless to citrus, others may cause important diseases such as exocortis (Semancik and Weathers, 1972) and cachexia (Diener et al., 1988; Semancik et al., 1988).

Citrus strains of HSVd, in addition to causing cachexia, have been implicated in other diseases including yellow corky vein of Kagzi lime (*Citrus aurantifolia*), reported from India (Roy and Ramachandran, 2003). The disease was characterized by yellow spots on leaf lamina, which soon spread along the mid and lateral veins. The veins turned rough on underside and developed corky tissues. A yield loss of 51.3–60.4 % was reported from Assam (Azad, 1993). HSVd (HSVd-lycv) and CEVd were associated with the disease (Roy and Ramachandran, 2006).

In recent years, a disease with specific symptoms of yellow corky vein has emerged in navel oranges in the Fars province of Iran (Figure 1, top). These symptoms are often associated with declining of affected trees. It has become of concern to the growers as it appears to be spreading from tree to tree.

Split bark is a disorder first described in declining sweet lime (*Citrus limettoides*) trees from Iran (Izadpanah, 1983). The disorder is characterized by cracks in bark of the stem, which spreads along main branches. The affected tree shows retarded growth and without any symptoms in fruits and leaves. Split bark disorder is becoming important in sweet lime which is a commercially important variety in Iran (Figure 1, bottom).

Fig. 1 Yellow corky vein symptoms on Washington navel sweet orange leaves (top) and split bark symptoms on the main trunk of sweet lime (bottom).

In the present study we report two novel variants of hop stunt viroid each associated with yellow corky vein disease of sweet orange or split bark disorder of sweet lime.

Materials and methods

Plant samples and Extraction of nucleic acids: Leaves of affected trees showing yellow corky vein and split bark symptoms were collected in 2007 and 2008 from Jahrom and Darab in the Fars province of Iran. Extraction of nucleic acids from samples was carried out by standard viroid extraction method (Semancik et al., 1975) designed to yield high viroid titers. The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris–HCl; 10 mM KCl; 0.1 mM MgCl2; pH 7.4). A citrus sample known to be infected with HSVd was used as positive control. The negative control consisted of leaf samples of a non-affected tree.

RT-PCR, cloning and sequencing of the amplified fragments: Reverse transcription polymerase chain reaction (RT-PCR) to detect CEVd, CBLVd, HSVd, CVd-III, CVd-IV and CVd-V were carried out following the method described by Bernad and Duran-Vila (2006). It involved the use of primers designed to amplify the full length of each target viroid (Table 1). Electrophoretic analysis in 1 % agarose gels was used to confirm the synthesis of a DNA product of the expected size. The PCR-amplified products were ligated into the vector pTZ57R/T (Fermentas) and the recombinant plasmids were used to transform DH5α *E. coli* cells. Plasmids from transformed cells were purified using the High Pure Plasmid Extraction Kit (Roche). PCR analysis was used to demonstrate the presence of correct insert.

Tab. 1 Primers used in RT-PCR to amplify citrus viroids.

Viroid	Direction	Primer Sequence	Reference
CEVd[a]	Reverse	5′-CCGGGGATCCCTGAAGGA-3′	Gross et al., 1982
	Forward	5′-GGAAACCTGGAGGAAGTCG-3′	
HSVd	Reverse	5′-GGGGCTCCTTTCTCAGGTAAGTC-3′	Sano et al., 1988
	Forward	5′-GGGGCAACTCTTCTCAGAATCC-3′	
CBLVd	Reverse	5′-TTCGTCGACGACGACCAGTC-3′	Ashulin et al., 1991
	Forward	5′-GGCTCGTCAGCTGCGGAGGT-3′	
CVd-III	Reverse	5′-TTCGTCGACGACGACAGGTA-3′	Bernad and Duran-Vila, 2006
	Forward	5′-GGCAGCTAAGTTGGTGACGC-3′	
CVd-IV	Reverse	5′-GGGGATCCCTCTTCAGGT-3′	Bernad and Duran-Vila, 2006
	Forward	5′-GGGGAAATCTCTTCAGAC-3′	
CVd-V	Reverse	5′-GGAACCACAAGGTTGTTCAC-3′	Serra et al., 2007
	Forward	5′-TGTGGGTCACCCCGCCCC-3′	

[a] CEVd = Citrus exocortis viroid, CBLVd = Citrus bent leaf viroid, HSVd = Hop stunt viroid, CVd-III = Citrus viroid III, CVd-IV = Citrus viroid IV, CVd-V = Citrus viroid V.

Detection of viroids by dot-blot hybridization: Full-length cDNAs of CEVd, CBLVd, HSVd, CVd-III, CVd-IV and CVd-V were cloned into plasmid vector pTZ57R/T (Fermentas). DIG-labeled cDNA probes were prepared from these plasmid DNAs according to the method described by Li et al. (1995). Dot-blot hybridization followed the method of Li et al. (1995) with slight modification. Hybridization was carried out at 50 ^{0}C and the membranes were washed at 60^{0}C. The signals were detected by a chromogenic assay.

Sequence analysis and prediction of RNA secondary structure: Eight independent clones from each disease were sequenced in both directions (Macrogen Inc., Seoul, South Korea). Sequence data were compiled, analyzed and compared with those available in GenBank, using NCBI/BLAST, to search for related sequences. Alignment of sequences was performed using the Vector NTI 9 software package (InforMax, Bethesda, MD). Comparison of nucleotide composition was performed employing the BioEdit (version 5.0.9) Sequence Alignment Editor program (Hall, 1999). Phylogenetic analysis was carried out using DNAMAN software (version 4.0.1.1) and a tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on 10 000 replicates. Genetic distances were calculated using the MegAlign program in the DNASTAR software package (Madison, WI). The most stable secondary structure analysis was obtained with the RNAstructure software (version 4.6). The nucleotide sequence data reported in this paper were submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

Results and discussion

Detection of viroids by RT-PCR: The PCR amplification with HSVd specific primers produced an amplicon of ~300 bp, as expected (Fig. 2, left). Nonspecific bands were seldom observed, and no fragments were detected from samples used as healthy controls (Figure 2).

CBLVd, CVd-IV and CVd-V were not detected in symptomatic trees by RT-PCR and dot-blot hybridization. CEVd and CVd-III were detected only rarely in some symptomatic trees (data not shown). However, CEVd has been reported in Kagzi lime with yellow corky vein disease (Roy and Ramachandran, 2006).

The nucleotide sequence analyses of HSVd from yellow corky vein affected sweet orange trees (HSVd-sycv) and split bark affected sweet lime trees (HSVd-sb) showed that those were new variants, similar to HSVd-lycv. The differences between the HSVd-sycv and HSVd-lycv are shown in Figure 4, top.

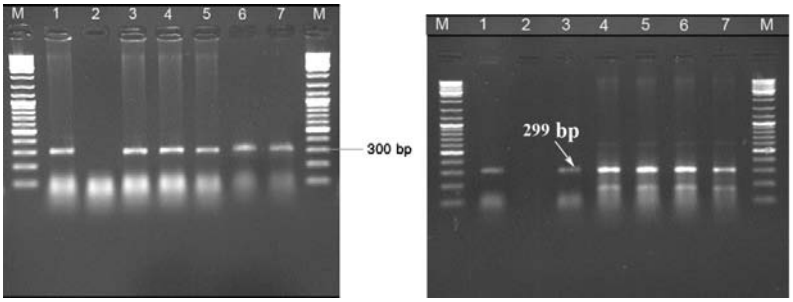

Fig. 2 Electrophoresis pattern of RT-PCR products with sweet orange yellow corky vein (left) or sweet lime split bark affected (right) samples and specific hop stunt viroid primer pair: (1) Positive HSVd cDNA, (2) healthy citrus control, (3-7) sweet orange yellow corky vein or sweet lime split bark samples, (M) 100-bp DNA ladder.

Detection of viroids by dot-blot hybridization: All yellow corky vein and split bark samples showed a strong positive hybridization reaction against a DIG-labeled HSVd-cDNA probe (Figure 3). Dot-blot hybridization clearly detected the viroid in infected trees. The healthy control sample showed no reaction. No signal was obtained with CEVd, CBLVd, CVd-III, CVd-IV and CVd-V probes (data not shown). Neither RT-PCR nor dot-blot hybridization could detect HSVd-sycv and HSVd-sb in five samples of other citrus variants, which appeared healthy or had symptoms other than yellow corky vein or split bark, although other viroids could be detected occasionally (data not shown).

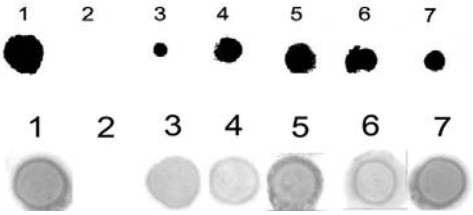

Fig. 3 Dot-blot hybridization assay of sweet orange yellow corky vein (top) and sweet lime split bark affected (bottom) samples for HSVd cDNAs with specific Dig-labled probes. (1) Known HSVd cDNA, (2) Healthy control, (3-7) Samples from trees affected by yellow corky vein (top) or split bark (bottom) diseases.

Sequence analysis and prediction of RNA secondary structure: HSVd-sycv sequence [accession no. FJ465506] was 93.7 % homologous with HSVd-lycv while HSVd-sb sequence [accession no. FJ465507] was 94.8 % homologous with CVd-IIa-117 (Palacio-Bielsa et al., 2004), which according to the criteria of the International Committee for Taxonomy of Viruses (ICTV), are variants of HSVd rather than novel species (Flores et al., 2005).

Figure 4 illustrates the primary structures and alignments for maximum homology of HSVd-sycv, HSVd-sb and some other variants of HSVd. It shows that HSVd-sycv differs from HSVd-cit8 in a single nucleotide in the so-called “cachexia expression motif” in the variable domain. The HSVd-cit8 induces no symptoms in St. Michael orange-Wakayama trees in Japan (Ito et al., 2002). The “cachexia expression motif” plays a major role in inciting cachexia symptoms, and that changes within this motif affect symptom severity and may even suppress symptom expression. The lack of pathogenicity of HSVd-cit8, and association of symptoms with HSVd-sycv support the low flexibility of this motif (Serra et al., 2008).

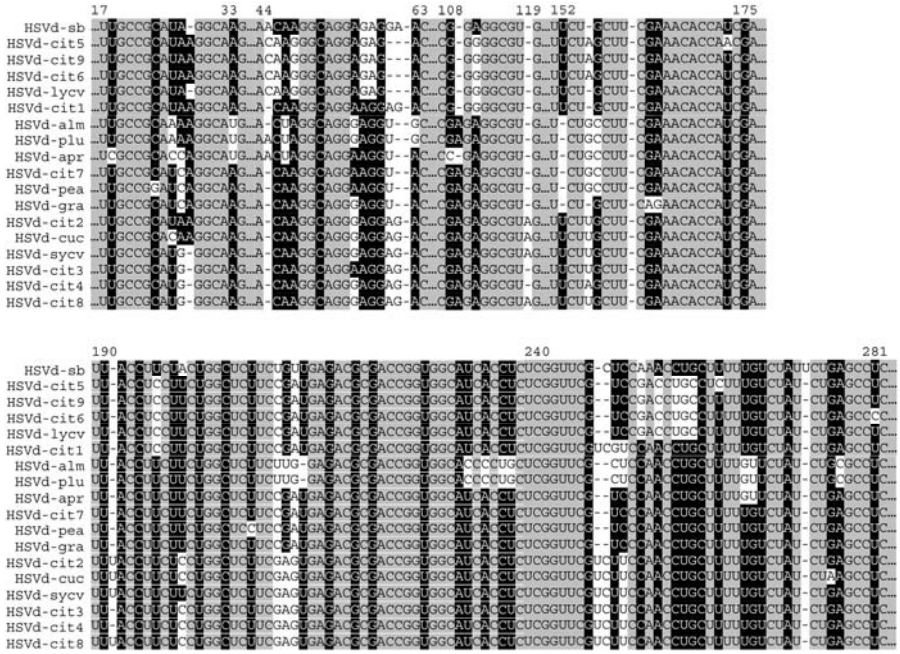

Fig. 4 Primary structures of HSVd variants aligned for maximum homology using Vector NTI program (version 9.0.0). Portions of identical sequences are not shown. Nucleotides**:** Identical sequences, Nucleotides**:** Conserved sequences, -: lack of nucleotide

Figure 4 also shows that HSVd-sb differs from CVd-IIa-117 (HSVd-cit 2) in four nucleotides in "cachexia expression motif". The CVd-IIa-117 is a noncachexia variant of hop stunt viroid that induces mild bark-cracking symptoms on Pomeroy trifoliate orange rootstocks in Japan (Verniere et al., 2006). This difference between HSVd-sb and CVd-IIa-117 further suggests a low flexibility of this motif (Serra et al., 2008). It is yet to be determined whether these diseases are caused solely by HSVd or other factors, including those of host and environment, are involved in symptom expression. However, a single nucleotide change in the genome has been shown to affect the symptom expression (Serra et al., 2008).

Likewise, although viroid interactions are reported to alter plant symptoms (Verniere et al., 2006), none of the common citrus viroids other than HSVd-sycv or HSVd-sb were detected constantly in yellow corky vein or split bark affected plants, respectively. Point mutation experiments and testing the variants on the same host must be carried out to verify the role of nucleotide change in production of specific symptoms.

HSVd-sb is 4 nt shorter than CVd-IIa-117. Percent G+C in CVd-IIa-117 is somewhat higher than that of HSVd-sb. On the other hand, the secondary structure free energy of CVd-IIa-117 is higher than that of HSVd-sb. HSVd-sycv is 7 nt longer than HSVd-lycv. Percent G+C in HSVd-lycv is somewhat higher than that of HSVd-sycv. However, the secondary structure free energy of HSVd-sycv is higher than that of HSVd-lycv (Figure 5).

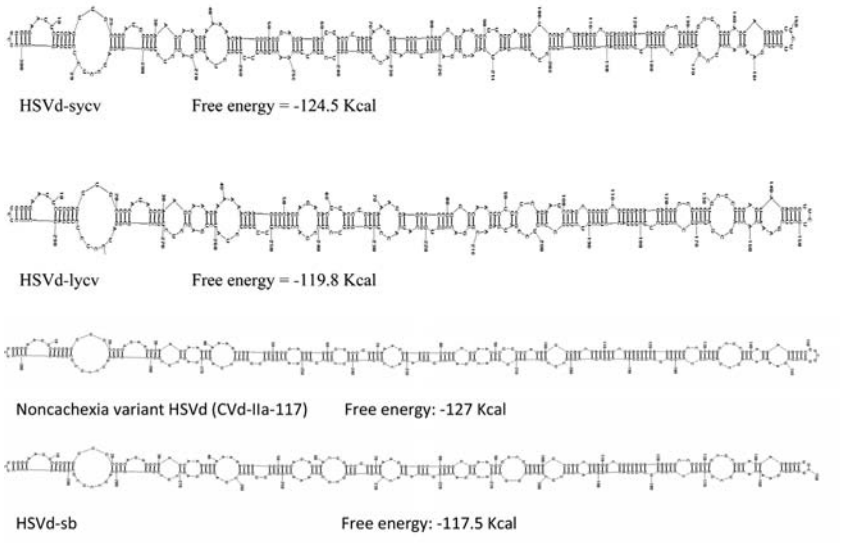

Fig. 5 Comparison of secondary structures and minimum free energy of HSVd-sycv, HSVd-lycv, HSVd-sb and CVd-IIa-117 using RNAstructure software (version 4.6). The sequences are arranged so to obtain a maximum number of base pairs and taking into consideration the sensitivity of specific sites to controlled enzymatic digestions.

Sequence analysis revealed that the HSVd-sb consists of 299 nucleotides [accession no. FJ465507], composed of 63 A, 85 C, 80 G and 71 U residues, thus resulting in a G + C/A + U ratio of 1.23, suggestive of a highly base paired, heat stable molecule, characteristic of viroid-like low molecular weight RNAs. The most stable secondary structures of HSVd-lycv, HSVd-sycv, HSVd-sb and CVd-IIa-117 were a classical rod-like structure and all variants adopted a cruciform structure including various additional small hairpins. Comparison of the secondary structures of these variants indicates their close similarities in the rod-like structures, number of loops and the free energies.

Phylogenetic relationships: Sequence alignment between HSVd-sycv, HSVd-sb and other variants of the HSVd revealed identities of more than 90 %. A consensus phylogenetic tree based on the multiple sequence alignment illustrates the relationship between HSVd-sycv, HSVd-sb and other variants of the HSVd (Figure 6). HSVd-sycv formed a distinct cluster with HSVd-cit8 and HSVd-cuc. In BLAST analysis HSVd-sycv showed nearly 99% sequence identity with cucumber isolate of HSVd (HSVd-cuc) [accession no. X00524] and 99.7 % identity with citrus variant of HSVd from Japan (HSVd-cit8) [accession no. AB054615], but it has comparatively low homology with lime yellow corky vein and other isolates from citrus, grapevine, prune, plum and almond. The HSVd-sycv belongs to the cluster where cucumber and citrus variants of HSVd from Japan (HSVd-cit8) are present; HSVd-cuc is reported to cause cucumber pale fruit disease in Japan (Sano et al., 1984). HSVd-cit8 was reported as a noncachexia variant of HSVd from Japan (Ito et al., 2002).

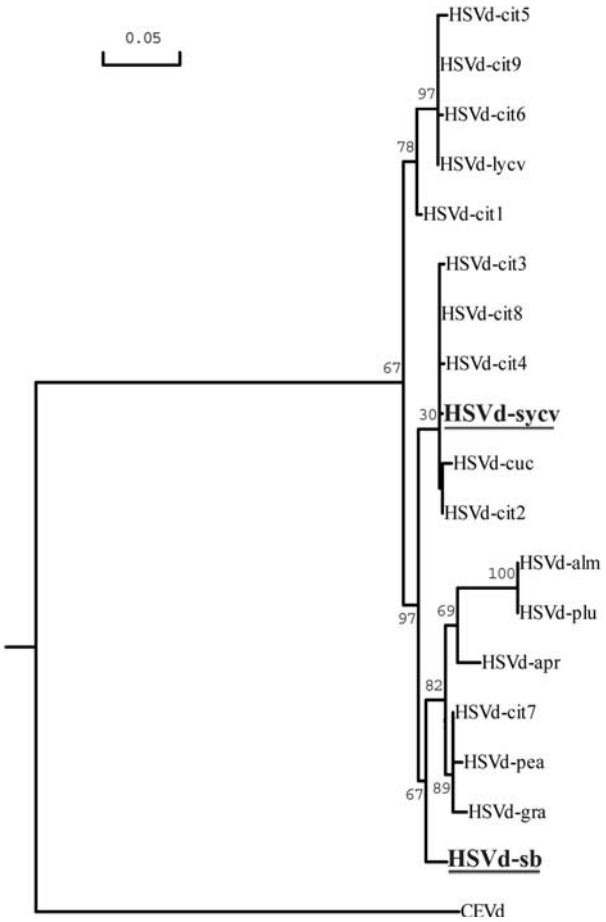

Fig. 6 Phylogram, drawn by neighbor-joining Bootstrap Method in CLUSTAL X (1.81b) software, illustrating phylogenetic relationships based on multiple alignments of the complete sequences of hop stunt viroid variants and the sweet orange yellow corky vein variant and split bark variant of hop stunt viroid (HSVd-sycv and HSVd-sb). See Table 2 for viroid accession numbers. CEVd is used as an out group.

HSVd-sb formed a distinct cluster and most of citrus variant isolates are present in other clusters. In BLAST analysis HSVd-sb showed comparatively low homology with CVd-IIa-117 (inducing mild split bark symptoms) and other isolates from citrus, grapevine, prune, plum and almond. It was also seen that HSVd-sycv and HSVd-sb have distant relationship with variants belonging to other HSVd groups mentioned earlier. Following the nomenclature used to name citrus viroid variants the new viroid variants have been tentatively designated as sweet orange yellow corky vein variant of hop stunt viroid (HSVd-sycv) and sweet lime split bark variant of hop stunt viroid (HSVd-sb).

Tab. 2 Characteristics of HSVd isolates used in this study.

Variant	GenBank accession no.	Host	Reported from	Number of nucleotides
HSVd-sb	FJ465507	Sweet lime	Iran (Fars)	299
HSVd- cit 2 (CVd-IIa-117)	AF213503	Pomeroy trifoliate orange	Japan	303
HSVd-lycv	AJ490824	Kagzi lime	India	295
HSVd-sycv	FJ465506	Sweet orange	Iran (Fars)	302
HSVd- cit 8	AB054615	St. Michael orange-Wakayama	Japan	302
HSVd-cit 3	EF126046	Satsuma	Iran (Mazandaran)	300
HSVd-cit 4	EF186992	Satsuma	Iran (Mazandaran)	300
HSVd-cit 5	AF213494	Citrus	Spain	297
HSVd-cit 6	AF213495	Citrus	Spain	297
HSVd-cit 1	AF131249	Citrus	California	299
HSVd-cit 7	X00009	Citrus	Japan	297
HSVd-gra	M35717	Grapevine	United States and Japan	296
HSVd-cuc	X00524	Cucumber	Japan	303
HSVd-pea	D13765	Peach	Japan	297
HSVd-plu	D13764	Plum and Peach	Japan	297
HSVd-cit 9	AF213491	Citrus	Spain	297
HSVd-apr	AJ297840	Prunus	Spain	297
HSVd-alm	AJ011813	Almond	Spain	296
CEVd (out group)	FJ626865	Citrus	Iran (Fars)	370

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Expression of the coat protein genes of PNRSV and PDV in the synergistic disease peach stunt

Kim, B.T.; Gibson, P.G.; Scott, S.W.
Genetics & Biochemistry,Gwinnett Technical College, Entomology, Soils & Plant Sciences, Clemson University, Bioscience, Lawrenceville, GA 30043, Clemson, SC 29634, USA

Abstract

Simultaneous infections of peach (*Prunus persica* Batsch L.) with the two ilarviruses, Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV), produce a synergistic disease referred to as "peach stunt". Previous work showed significant differences in the expression of the coat protein (CP) genes. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, was observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et al., 2001). This work re-examines and extends these observations using multiplex real-time PCR. Probes to both the plus and minus strands of the RNA 3 of each virus were designed and synthesized. The comparative C_T method ($\Delta\Delta C_T$) was used for relative quantitation of gene expression. A reduction in the amount of (+) strand RNA 3 of PDV observed in the earlier work was not seen using real-time PCR. However, in a time course experiment with samples collected at 14-day intervals for 6 weeks, there was a substantial increase in the concentration of the (+) strand of RNA 3 of PDV after 14 days irrespective of whether the virus was present as a sole infecting agent or as a co-infection with PNRSV. At this same point in time there was a decrease in concentration of (+) of the RNA 3 of PNRSV. By the next sample date the concentrations of the (+) strand of RNA 3 of both viruses had returned to "normal". The results are discussed in relation to the most extensively studied plant viral synergism synergism (PVY and PVX) and to known changes in concentration of PNRSV and PDV based on earlier observations made using ELISA.

Keywords: Peach, Prunus necrotic ringspot virus, Prune dwarf virus, peach stunt disease, molecular interaction, Multiplex real-time PCR.

Introduction

The infection by plants with two or more unrelated viruses/virus-like agents is a common phenomenon in nature particularly in long lived, perennial species such as fruit trees (Nemeth, 1986). Many such infections result in a synergistic interaction that is visibly manifest by a remarkable change in symptom expression, yield loss, and/or host longevity. In addition, in well studied synergies a change in virus accumulation of one of the participant viruses has been documented. Many synergistic interactions involve a member of the potyvirus group and viruses belonging to other families. In most of these interactions, the accumulation level of the potyvirus remains unaltered, while the level of accumulation of the nonpotyvirus increases considerably (Pruss et al., 1997; Vance, 1991).

The most extensively studied synergistic interaction involves Potato virus Y (PVY, the type member of the genus Potyvirus, family *Potyviridae*), and Potato virus X (PVX, the type member of the genus *Potexvirus*, family *Flexiviridae*) in tobacco (Vance, 1991). In plants co-infected with both PVY and PVX, accumulation of PVX in systemically infected leaves increases up to 10-fold compared to equivalent leaves of singly infected plants. The level of PVY is the same irrespective of whether the plant is infected with PVX. In addition, the accumulation of PVX (–) strand RNA was much higher in doubly infected cells than was PVX (+) strand RNA, indicating that PVY induced a change in the regulation of PVX replication (Pruss et al., 1997; Vance, 1991). Moreover, it has since been demonstrated that replication of the potyvirus is not required for potyviral/PVX synergism, as the response is mediated by 5' potyviral sequences comprising P1 and helper component proteinase proteins (P1/HCPro) (Pruss et al., 1997; Vance et al., 1995). P1/HC-Pro also functions as a pathogenicity enhancer and activator of replication of other unrelated viruses such as Cucumber mosaic virus (CMV, the type member of the genus *Cucumovirus*, family *Bromoviridae*). In co-infections, PDV and PNRSV (both members of the genus *Ilarvirus*, family *Bromoviridae*) may interact in either a synergistic or an antagonistic fashion (Cropley, 1968). In peach (*P. persica* L. Batch), infection with both viruses results in a disease known in Australia as peach rosette and decline (Smith and Challen, 1976) and in California as peach stunt disease (Asai and Uyemoto, 1991). Symptoms of the two diseases include stunting, yellowing, and a 2 to 6-fold reduction in fruit yield. In both diseases the effects on growth and yield are typically greater than infections with either virus alone and are greater than the additive effects of infections by individual viruses. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, has been observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et

al., 2001). These results would indicate a significant reduction in the accumulation of PDV in the plant when PNRSV was present but that accumulation of PNRSV is unaffected by the presence of PDV. In this way the effects on the accumulation of PNRSV mimic the effects observed on the accumulation of PVY in the PVY/PVX synergism in tobacco but the effects observed on the accumulation of PDV are in complete contrast to those observed for PVX. However, unlike PVY and PVX, PDV and PNRSV are members of the same viral genus (*Ilarvirus*), both possess a tripartite genome, and neither code for a molecule similar to P1/HCPro. Nor do these two particular ilarviruses possess the ORF found in some other ilarviruses and the cucumoviruses that codes for the 2b protein shown to be involved in the suppression of RNA interference (Lucy et al., 2000). A role that is associated with the HCPro protein of potyviruses. Thus, although the biology of the synergism between PDV and PNRSV is well documented, information on the regulation of the synergism at the molecular level is absent. In this work we attempt to confirm earlier findings concerning the accumulation of the viruses and assess if either virus affects the replication of the other through examining the production of minus strand RNA 3. Unique probes to the CP genes of PDV and PNRSV and to the plus and minus strands of the RNA 3 of each virus were used in one-step, multiplex, real-time PCR to simultaneously detect up to three target RNAs in a single sample.

Materials and methods

Preparation of RNA: Total RNA was extracted from leaves of small trees of the peach cultivar 'Juneprince' growing on the rootstocks, 'Lovell', 'Nemaguard' and 3-17-7 (Guardian®) using a procedure modified from Hughes and Galau (1988). Samples were collected from a single bud at 2-week intervals for a period of 6 weeks beginning as leaves emerged from the dormant buds in the spring. The RNA was extracted from 100 mg samples of leaf tissue and the yield of nucleic acid per sample (µg/µl) determined by UV spectophotometry. Integrity of the sample was confirmed by gel electrophoresis in 2 % agarose gels buffered with TAE and with the sample having been denatured using formamide. Aliquots (5 µl) of each sample containing 190 ng of total RNA were prepared and stored at -80 °C until added to a real-time PCR reaction so as not to subject the RNA sample to cycles of freezing and thawing.

Real-time PCR: Real-time PCR was completed using a QIAGEN QuantiTect Multiplex RT-PCR NR Kit according to the manufacturer's instructions. Probes to plus strand RNA 3 and minus strand RNA 3 of the movement protein gene, and to the coat protein gene (RNA 3, RNA 4) were used to detect the expression of the RNA 3. Probes for PDV were labeled with FAM at the 5' terminus and quenched at the 3' terminus with BHQ 1. Probes for PNRSV were labeled with Quasar 670® at the 5' terminus and quenched at the 3' terminus with BHQ 2 (Biosearch Technologies, Novato, CA). Sequences of primers and probes used in this work are shown in Table 1.

Tab. 1 Sequence of the primer pairs and probes used to detect plus and minus strand RNA 3 and the coat proteins of PDV and PNRSV.

PDV Movement protein	Forward primer	5' AAGCGGCTATCTTCGTTGGAA 3'
Plus strand	Reverse primer	5' GCGTCTACACTTACGGCTGAT 3'
	Probe	5' ATGGCCAAGAGCAGTTCACGCC 3'
PDV Movement protein	Forward primer	5' CCTTTCCACTACTCCCAATTACCA 3'
Minus strand	Reverse primer	5' CCCTTGCTGCTGTAGATGATGTG 3'
	Probe	5' CGCGATTTGGCGAATGTTTGGAGTAT 3'
PNRSV Movement protein	Forward primer	5' CCGACAGGCCGATAAAGTAAAGAAG 3'
Plus strand	Reverse primer	5' CGAGGTCGTTGCTTGAATGATC 3'
	Probe	5' TTACAGATGTGTAGGCCGAGTATTCC 3'
PNRSV Movement protein	Forward primer	5' CCTTCTGTACCTGCCAATATCCTA 3'
Minus strand	Reverse primer	5' TTCCCGATTGCCGAGACAA 3'
	Probe	5' TCGGACCATAGACATCAACACCTTC 3'
PDV Coat protein	Forward primer	5' TGATACCAAGGTRTACGGAATYG 3'
	Reverse primer	5' TGAACTTCCTACGTTGTAGGGGATT 3'
	Probe	5' TCTAYGGACTCATTAAAGGT 3'
PNRSV Coat protein	Forward primer	5' CCKCAGTTGATGGGTCAGAATTT 3'
	Reverse primer	5' CCTTCAAGAACCCCTTCCTAGAC 3'
	Probe	5'CCGAATGAACTCTATGAGTTCGAATGGTTGG 3'

Experimental design and analysis: Experiments were set up in 96-well plates using 5 replicates of 16 treatments arranged in a balanced lattice design (Burrows et al., 1984). The reactions were performed in a Stratagene Mx 3000p real-time thermocycler using ROX as the passive reference dye. CT values for different treatments were analyzed using the GLM procedure of SAS (SAS Institute Inc, Cary, NC, USA) and significant differences among means of treatments identified.

Results

Multiplex real-time PCR was able to detect the segments of the RNA 3 of both PNRSV and PDV to which the probes were designed, specifically, unambiguously, and with equal efficiency in the same sample, thus allowing the comparison of the relative expression of the genomic molecules using the comparative C_T method ($\Delta\Delta C_T$) (Dorak, 2006). Typical results are shown in Table 2. In no instances were the C_T values obtained for a virus in a plant infected with both viruses (PDV + PNRSV) significantly different from the C_T values obtained for the virus (PDV or PNRSV) when infecting the plant alone. The presence of PDV was not detected in total RNA samples collected at the start of this experiment even though the PCR reaction was detecting PDV in positive controls (C_T counts of 11). However, samples collected two weeks into the time course experiment showed levels of PDV (average C_T counts of 16.9) for the CP similar to those detected throughout the remainder of the time-course experiment. The levels of PNRSV detected in these experiments (C_T values 15 – 20.1) were consistent throughout the entire 6 weeks of the experiments. No significant differences between the C_T values detected for plus or minus strands of the RNA 3 of either virus were detected. In non-inoculated/non-infected trees no detectable C_T values were recorded before the maximum number of cycles (45) in an experiment were completed. In preliminary experiments, no detectable C_T values were recorded in non-inoculated/non-infected trees even when the maximum number of cycles used for amplification was increased to 55.

Tab. 2 C_T values recorded for one-step, real-time PCR reactions completed using RNA extracted from samples collected from the scion cultivar 'Juneprince' 2 weeks into the time course experiment. Mean C_T values of 5 replications for each virus rootstock combination are shown. The columns labeled PNRSV/PDV show the C_T values detected for PNRSV and PDV, respectively, in trees in which both viruses were present. Each reaction contained 190 ng of total RNA. ROX dye was used as the passive reference dye. Positive controls using 190 ng of purified virus gave C_T values of 11 or 12.

Mean CT values for minus strand RNA 3

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	20.0	18.8	20.3/17.8	>45
Nemaguard	19.9	18.5	20.6/17.4	>45
Guardian	20.1	17.9	20.2/18.8	>45

Mean CT values for coat protein gene (RNA 3 and RNA 4)

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	15.0	14.9	15.6/14.8	>45
Nemaguard	15.0	18.1	16.2/17.8	>45
Guardian	16.2	17.9	17.2/18.8	>45

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	17.3	19.5	17.3/20.8	>45
Nemaguard	16.2	19.3	17.0/19.0	>45
Guardian	16.1	19.5	16.3/19.8	>45
Guardian	16.2	17.9	17.2/18.8	>45

Discussion

In an initial examination of this synergism (Scott et al., 2001), the presence of PNRSV led to a 17 fold reduction in the accumulation of PDV as detected using northern blots and cRNA probes to the CP gene (RNA 3 and RNA 4). The presence of PDV had no effect on the accumulation of PNRSV. In this respect the absence of changes in the accumulation of PNRSV is similar to that observed for PVY, whereas the reduction in the accumulation of PDV is in complete contrast to the observed effects on PVX. Furthermore it was clear that PVY affected the replication of PVX (Vance, 1991). In this work we were unable to duplicate the reduction in accumulation of PDV achieved earlier. The concentrations of PNRSV were unaffected by the presence of PDV and there were no effects on the replication of either virus as judged by the relative levels of minus strand RNA 3 that were detected for each virus. Although not shown in the data presented, there appears to have been a delay in the replication /accumulation of PDV in buds at the beginning of this experiment.

The major difference between this series of experiments and those completed previously are the genotypes of the material used. In the previous experiment (Scott et al., 2001), the scions 'Garnet Beauty', 'Elberta', and 'GF 305' growing on the rootstocks Lovell and Nemaguard were used. The reduction in accumulation of PDV occurred in 'Elberta' and 'GF305'. Both of these cultivars are recommended as susceptible woody indicators for a number of stone fruit viruses and virus-like diseases providing good symptom expression in the greenhouse in particular. It is possible that the genotypes of these two cultivars are responsible for the reduction in accumulation of PDV. Now that reliable, one-step multiplex real time PCR has been developed for these two viruses, it will be possible to re-examine the original work using 'Elberta' and 'GF305'. The two viruses can be detected simultaneously in a single sample and, within the limitations of current technology, paired comparisons of the accumulation of the plus and minus strands of all three genomic molecules can be completed in the hopes of identifying the molecules that interact in this non potyvirus synergism.

Acknowledgments

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Investigation of virus occurrence in different tissues throughout the year and sequence variability of *Apple stem pitting virus*

Arntjen, A.; Jelkmann, W.
Julius Kühn- Institute, Federal Research Center for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim

Abstract

The occurrence of *Apple stem pitting virus* (ASPV) isolate PB 66 in three different types of tissue of four different apple varieties throughout the year was determined. Reliable virus detection in phloem tissue was observed in all four apple varieties investigated, at all sampling dates during the year. The complete nucleotide sequence of ASPV isolate PB 66 was determined and compared to ASPV isolate PA 66. The isolates show 80 % sequence identity. Comparison of the ASPV PA 66 coat protein amino acids sequence with 16 other ASPV isolates from different hosts revealed an insertion event of 18 amino acids.

Keywords: *Apple stem pitting virus*, Foveavirus, Flexiviridae

Introduction

In commercial apple cultivars infection with latent RNA viruses cause yield losses up to 60%. *Apple stem pitting virus* (ASPV) is one of the most common latent viruses in apple worldwide. It is classified as a *Foveavirus* and belongs to the family *Flexiviridae* (Adams et al., 2004; Martelli and Jelkmann 1998). Viruses belonging to this family have flexuous filamentous virions with a size of 12 -13 nm in diameter and monopartite, positive sense ssRNA genomes with a 3'-polyA tail. ASPV consists of five open reading frames (ORF) of which the first ORF encodes the replication related proteins. ORF 2-4 encode the triple gene block which is necessary for virus movement in the plant. The coat protein is encoded by ORF 5. High sequence variability of different ASPV isolates was reported previously (Yoshikawa et al., 2001).

ASPV is transmitted by grafting, root contact and mechanical inoculation. To avoid spreading of ASPV it is necessary to use virus free material for propagation. For testing/re-testing virus free propagation material it is important to know which tissue of apple trees provides reliable results and if this depends on the sampling time during the year. Investigation of virus occurrence in different tissues throughout the year was done previously for Apple stem grooving virus (Kundu et al., 2003).

In this report we present the results of sequence comparison between the two ASPV isolates PA 66, PB 66 and other isolates in Genbank, also the results of virus detection in different tissues at different times throughout the year.

Material and methods

The ASPV isolate PB 66 was maintained in four different apple varieties (*Malus domesticus* 'Gloster', 'Golden Delicious', 'Cox' and 'Egremont Russet') in the field and in *Nicotiana occidentalis* 'Wheeler' 37B in the greenhouse. For the time course investigation total RNA was extracted at least once a month throughout the year from leaves, phloem from stem tissue, and phloem from root tissue, using the silica capture method (Rott and Jelkmann 2001). cDNA was synthesized from viral RNA template using random hexanucleotide primers (Invitrogen) and M-MuLV reverse transcriptase (Fermentas). Virus detection was performed with the primers described by Menzel et al. (Menzel et al., 2002). For the determination of the sequence of isolate PB 66 the virus RNA was extracted with RNeasy Plant Kit (Qiagen). First strand cDNA synthesis was performed with SuperScript reverse transcriptase II (Invitrogen) and Oligo(dT) Primer (Fermentas). Second strand DNA synthesis was performed with the Phusion Taq- Polymerase (Biocat). Amplification of the 5'- end was done with RACE- PCR. In both cases the amplified products were analyzed in 1% agarose gels in 1x TAE buffer for 50 min at 100V, and stained with EZ Vision (AMRESCO). The PCR products for sequence determination were cloned using the pJet1.2 vector (Fermentas) and sequenced by Seqlab (Göttingen). Alignments and analysis of sequences were performed with Lasergene programs (DNASTAR).

Results

For the investigations on time course studies for virus detection total RNA was extracted 16 times per year from leaves and phloem from stem tissue, and 13 times from the phloem of roots, because the frozen ground prevented extractions from roots in the wintertime. In the apple varieties 'Egremont Russet' and 'Gloster' ASPV could be detected by PCR during the whole year, in all three tissue types (Table 1). In the two apple varieties 'Golden Delicious' and 'Cox' PCR results were positive, except for August in leaves and in December in the phloem from roots. Apart from these exceptions, reliable virus detection was observed in all four apple varieties throughout the year.

Tab. 1 Results of *Apple stem pitting virus* occurrence in four apple varieties in different tissues throughout the year

Apple variety	Tissue	Positive results
Egremont Russet	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13
Golden Delicious	leaf/bud	15/16
	phloem	16/16
	phloem of roots	12/13
Gloster	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13
Cox	leaf/bud	15/16
	phloem	16/16
	phloem of roots	12/13
Golden Delicious	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13

The complete nucleotide sequence of ASPV isolate PB 66 was determined. The molecule is 9363 nucleotides (nt) excluding the polyA- tail and has a GC- content of 43,5 %. It consists of five open reading frames (ORFs). Comparison of isolate PA 66 (Jelkmann 1994) and isolate PB 66 revealed 80 % sequence identity (Table 2).

Tab. 2 Comparison between the genomes of *Apple stem pitting virus* isolates PA 66 and PB 66 (ORF: Open reading frame; nt : nucleotide; aa : amino acid; UTR : untranslated region)

	PA 66		PB 66		nt sequence	amino acid
	nt	aa	nt	aa	identity (in %)	similarity (in %)
genome size	9332	-	9363	-	80	-
5' End UTR	59	-	60	-	96	-
ORF 1	6549	2183	6549	2183	79	90
UTR between ORF 1 and ORF 2	99	-	175	-	76	-
ORF 2	669	223	669	223	83	95
UTR between ORF 2 and 3	1	-	1	-	-	-
ORF 3	360	120	363	121	87	91
overlapping Region ORF 3/ ORF 4	91	-	94	-	-	-
ORF 4	210	70	228	76	85	90
UTR between ORF 4 und ORF 5	88	-	75	-	75	-
ORF 5	1242	414	1188	396	82	81
3' End UTR	132	-	135	-	90	-

The first ORF (M_r 246810) which encodes the replication related proteins is identical in size between both isolates. It has 79 % nt identity and 90 % amino acid (aa) identity. ORF 1 has the lowest nt identity of all coding regions. ORF 2 – 4 encode the triple gene block. ORF 2 (M_r 25242) has 83 % nt identity and 95 % aa identity between these two isolates. The ORF 2 of both isolates encodes a putative protein 223 aa in length. ORF 3 (M_r 12916) is one aa longer in isolate PB 66 and has a sequence identity of 87 % and an aa identity of 91 %. ORF 4 (M_r 8057) has a nt identity of 85 % and an aa identity of 90 %. It is 6 aa longer in isolate PB 66. ORF 5 (M_r 42144) encodes the coat protein and has a nt identity of 82 % and an aa identity of 81 %. It has the lowest aa identity of all ORFs and is 18 aa shorter in isolate PB 66. Figure 1 shows an alignment of 17 ASPV coat protein sequences between the start codon and position 65 related to isolate PA 66. Only isolate PA 66 and the isolate associated with pear vein yellows (Jelkmann, 1994) have an insertion of 18 aa (from pos. 28 to 45). The 5'- end untranslated region (UTR) shows 96 % nt identity and is one nt longer in isolate PB 66. The 3'- end UTR has 90 % sequence identity and is three nt longer in PB 66. The other UTRs between the ORFs show sequence identities under 80 %.

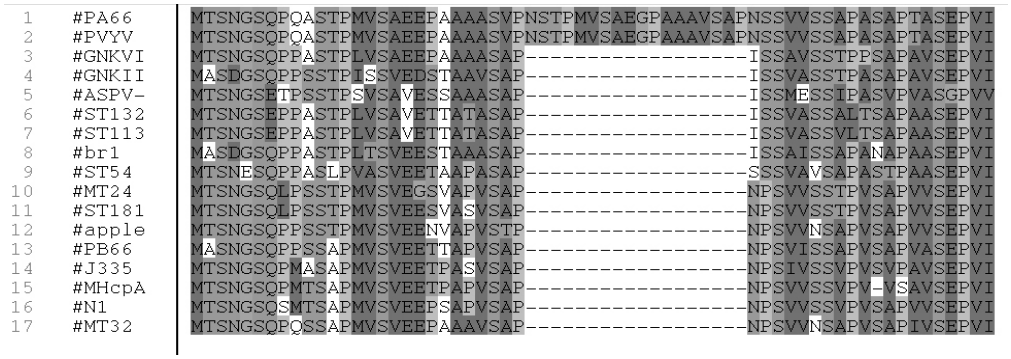

Fig. 1 Aligment with ClustalX of a partial coat protein amino acid sequence of different ASPV isolates. Gaps indicated by tabs.

Discussion

The complete genomic sequence of ASPV isolate PB 66 consists of 5 ORFs with a total length of 9363 nt excluding the polyA-tail. In comparison to isolate PA 66 it is 30 nt longer. Both isolates have a sequence identity of 80 %. The 5'-UTR with 96 % and the 3'- UTR with 90% sequence identity are highly conserved among these two isolates. This underlines their importance for the infectivity of the virus. The first ORF which encodes the replication related proteins shows the lowest nt identity, but has 90 % aa similarity thus demonstrating a high rate of silent mutations. The triple gene block seems to be more conserved among ASPV isolates. The fourth ORF is 6 aa longer in PB 66 than in PA 66. Comparison with the two other complete ASPV isolate sequences from Genbank show a deletion of 5 aa (QGVSV) at the C-terminus in ASPV isolate PA 66 and one additional aa isolate PB 66 (Q). The effect of this sequence variation in the triple gene block protein for virus movement has not yet been investigated. The coat protein shows very low nt identity with 82 % and also low aa identity at 81 %. The isolate PA 66 has an insertion of 18 aa in the coat protein. Comparison with other isolates from the database show that only the isolate PA 66 and a pear vein yellows isolate have the insertion of 18 aa in the coat protein. It was suggested that an insertion in the coat protein sequence can influence the geographical distribution of virus isolates (Viswanathan, Karuppaiah et al. 2009), host specialization of certain isolates (Galipienso et al., 2009) or the virus accumulation in the host plant (Szathmary et al., 2009).

Detection of ASPV is possible during the whole year. In summer the virus titer seems to decrease in the leaves of all four varieties, which was also reported previously for *Apple stem grooving virus* (Kundu et al., 2003). For the detection of ASPV throughout the year phloem is a reliable tissue in all four apple varieties. The detection primers (Menzel et al., 2002) which were used for the investigation on virus occurrence in different tissues are located in the coat protein coding region. In the case of ASPV isolate PB 66 the forward primer has no mismatches or gaps. The reverse primer has a mismatch and a base exchange at the 3'- end. These isolates show high variability in the coat protein coding sequence indicating a possibility that there could be isolates that are not detected with the published detection primers.

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Close similarities between Cherry chlorotic rusty spot disease from Italy and Cherry leaf scorch from Spain.

Barone, M.[1]; Covelli, L.[2]; Di Serio, F.[3]; Carrieri, R.[1]; Garcia Becedas, M.T.[4]; Ragozzino A.[1]; Alioto, D.[1]
[1] Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli, 80055 Portici, Italy
[2] Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain
[3] Istituto di Virologia Vegetale del CNR, 70126 Bari, Italy
[4] Junta de Extremadura, Servicio de sanidad vegetal, 10600 Plasencia, Spain

Abstract

Cherry chlorotic rusty spot (CCRS), a disease affecting sweet and sour cherry in Southern Italy was regularly found associated with an unidentified fungus and with a complex pattern of viral-like double-stranded RNAs as well as with two small circular RNAs (cherry small circular RNAs, cscRNAs). Further studies revealed that i) the ds-RNAs correspond to the genome of different mycoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* and ii) the two viroid-like RNAs consist of two groups of variants with similar sequences but differing in size (394–415 and 372–377 nt for cscRNA1 and cscRNA2, respectively). Here we report that the dsRNAs of *Chrysovirus* and *Partitivirus* have been detected by RT-PCR analysis with CCRS specific primers in nucleic acid preparations from cherry leaves affected by cherry leaf scorch (CLS) in Spain, a disease whose etiological agent is the ascomycetes *Apiognomonia erythrostoma*, order *Diaporthales*. Moreover, Northern-blot hybridization assays showed that a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported in Italy, accumulate in leaves from CLS affected trees in Spain. These data, together with other evidence showing similar symptoms, disease cycle and fungal fructifications in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

Keywords: dsRNAs, cscRNAs, *Apiognomonia erythrostoma*, Diaporthales

Introduction

Cherry chlorotic rusty spot (CCRS), was firstly described affecting sweet cherry trees in Campania (Southern Italy) in 1996 (Di Serio et al., 1996). Two years later, sour cherry plants showing the same disorder were reported in the same area (Di Serio et al., 1998).The disease, similar to Amasya cherry disease (ACD) described in Turkey in 1970 (Blodget et al., 1970; Citir, 1987; Açikgöz et al., 1994; Di Serio et al., 1996; 1998; Coutts et al., 2004; Covelli et al., 2004; 2008; Kozlakidis et al., 2006), causes chlorotic and rusty spots on leaves and deformation, color alteration and premature dropping of fruits (Figure 1).

Fig. 1 Chlorotic rusty spots on leaves affected by CCRS disease.

Since CCRS was found regularly associated to 10-12 double-stranded RNAs (dsRNAs) and to 2 small circular RNAs (cherry small circular RNAs, cscRNAs), a viral agent of unknown nature was initially proposed as a possible causal agent (Di Serio et al., 1996).

However, further studies revealed that:

i) the disease is not graft transmissible to cherry seedlings and peach GF 305 (Alioto et al., 2003);
ii) mycelium-like structures are constantly associated to symptomatic tissues of leaves and fruits (Alioto et al., 2003);
iii) the two cscRNAs consist of two groups of variants with similar sequences but differing in size (394–415 and 372–377nt for cscRNA1 and cscRNA2, respectively) (Di Serio et al., 2006);
iv) The largest dsRNAs correspond to the genome of a putative new species of mycoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* (Covelli et al., 2004; Coutts et al., 2004; Kozlakidis et al., 2006);
v) The smallest dsRNAs do not show sequence similarities neither with CCRS-associated mycoviruses or with the two small circular RNAs or with the sequences deposited in databases, so their nature remains to be clarified (Covelli et al., 2008).

Most of these findings suggested that a fungus could be the etiological agent of the disease but, no fungus was isolated on artificial media and identified. Recently, molecular analysis of 18S fungal ribosomal gene has shown that CCRS symptomatic tissues of infected sour and sweet cherry leaves are closely associated with a fungus belonging to the order *Diaporthales* (Carrieri, 2009). A bibliographic research on *Diaporthales* infecting cherry trees has suggested the involvement of *Apiognomonia erythrostoma* (*Diaporthales*, *Gnomoniaceae*), in etiology of CCRS (Sánchez Sánchez and García Becedas, 2007). This fungus is reported as the agent of a disease named Cherry leaf scorch (CLS) inducing symptoms resembling those of CCRS.

In this paper, we report data showing a close similarity between the Italian CCRS and the Spanish CLS diseases.

Materials and methods

Diseased and healthy sample sources: CLS-affected leaves were collected from sweet cherry (*Prunus avium* L.) trees of the local variety “Ambrunés” from fields of three different areas of Plasencia (Cáceres, Spain). All leaves showed mild or severe chlorotic spots (Figure 2). CCRS-affected leaves, showing the typical symptoms of the disease, were sampled from sour and sweet cherry trees at Ariano Irpino (Avellino, Italy). Healthy leaves were collected from sour and sweet cherry trees in Ariano Irpino and from sweet cherry in Cáceres.

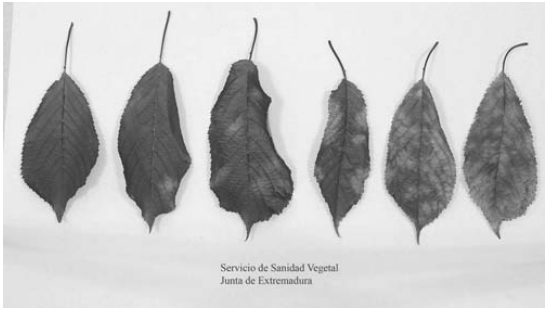

Fig. 2 Chlorotic spots on leaves from Spain affected by CLS disease caused by *Apiognomonia erythrostoma.*

RNA extraction and RT-PCR: Total RNA was extracted from 200-500 mg of healthy and CCRS and CLS infected leaf tissues as described by Foissac et al. (2001). A two-step RT-PCR was performed according to the protocol described by Covelli (2004) and Covelli et al. (2004), with minor modifications, using specific internal primers derived from the *Chrysovirus*-dsRNA4 (RF195/RF270/RF105) *Partitivirus*-dsRNA1 (RF480/RF481). The PCR amplicons, expected to be 180 bp (*Chrysovirus*) and 280 bp (*Partitivirus*) in size, were purified and sequenced (BMR Sequencing Service, Padova, Italy).

Extraction and purification of cscRNAs and Northen blot hybridization: Nucleic acid preparations enriched in viroid-like RNAs were obtained from leaves of healthy and CCRS-affected (*Prunus avium* L. 'La Signora') and CLS-affected (*Prunus avium* L. 'Ambrunés') sweet cherry trees as reported previously (Di Serio et al., 1997). These preparations were

separated by two consecutive electrophoreses under non-denaturing and denaturing conditions, electroblotted to nylon membranes (Amersham), fixed by UV irradiation (Di Serio et al., 1997) and, finally, hybridized with a cscRNA1 specific digoxigenine-labelled riboprobe following the protocol reported by Lolic et al. (2007). The riboprobe was generated by *in vitro* transcription from a linearized plasmid containing the full-length cDNA of cscRNA1 (Di Serio et al, 2006) using a commercial Dig-labelling kit (Roche Diagnostics GmbH, Germany).

Fruiting body observations: To evidence the presence of fungal fruiting bodies or conidia on CCRS infected trees, the symptomatic tissues of infected leaves and fruits were marked. Ten leaves were monthly removed from June to October and examined under a stereomicroscope (WILD Heerbrugg M35, Switzerland) and images were recorded by digital camera. During September and October, marked symptomatic leaves were also collected and placed in terylene mesh bags (20 per bag). Samples were placed on the soil surface or buried at depths of 0.1-0.5 cm and left overwintering under the infected trees. Sufficient bags were included to allow monthly sampling. The leaves were examined for the presence of ascocarps under a stereomicroscope and images were recorded by digital camera.

Microscopy: When conidia and perithecia were firstly observed, squash preparations were made to have an indication of the morphology of conidia and asci and ascospores. Mounts were made in lactophenol Cotton Blue and observed under a Leica DMR optical microscope.

Results and discussion

The RT–PCR amplifications using nucleic acid preparations from symptomatic leaf tissues of CLS affected sweet cherry trees of 'Ambrunés' yielded the expected fragments of 180 and 280 bp (Figure 3), suggesting the presence of mycoviruses of the genera *Chrysovirus* and *Partitivirus* respectively, that was further confirmed by sequence analysis of the RT-PCR amplicons showing high similarity (ranging from 97.3 to 100 % for *Chrysovirus* and from 98.6 to 100 % for *Partitivirus*) with the corresponding genomic sequences of the two mycoviruses constantly associated to CCRS disease. Moreover, in RNA extracts from CLS samples, a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported from CCRS diseased trees, was detected by Northern-blot hybridization assays (data not shown).

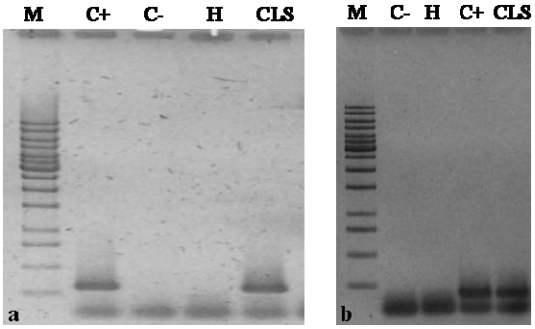

Fig. 3 Analysis by agarose gel electrophoresis of the RT-PCR products from CLS samples (a) *Partitivirus* and (b) *Chrysovirus.* Lane M, DNA marker 1Kb; Lane C+, cherry chlorotic rust spot affected samples; C-, negative control; H, Healthy sweet cherry sample;. CLS, Cherry leaf scorch affected sample.

Finally, orange pycnidia containing β-like conidia (Fig. 4a) were observed on the lower surface and in symptomatic areas of CCRS infected leaves that fell, at the beginning of October. Necked perithecia containing asci with bicellular ascospores (Fig. 4b) were also observed on the lower surface of the fallen leaves on the soil from month of November. These perithecia and pycnidia present the same morphological characters of fruiting bodies produced by *Apiognomonia erythrostoma* on CLS affected leaves.

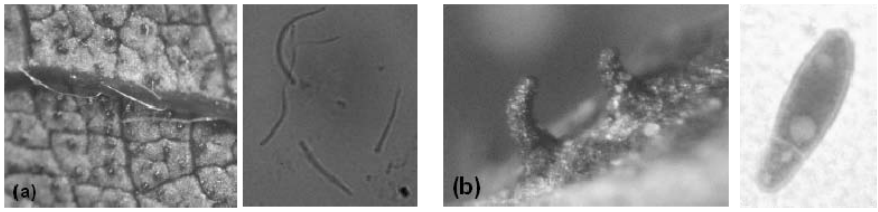

Fig. 4 (a) Picnidia (left) and β-like conidia (right) and (b) perithecia (left) and ascospora (right) observed on CCRS affected leaves.

These data, together with other evidence showing similar symptoms and a similar disease cycle in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

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Widespread occurrence of Tomato ring spot virus in deciduous fruit trees in Iran

Moini, A.A.[1]; Roumi, V.[2]; Masoumi, M.[2]; Izadpanah, K.[2]
[1] Iranian Research Institute of Plant Protection, Agricultural Research Organization, Tehran, Iran
[2] Plant Virology Research Center, College of Agriculture, Shiraz University, Shiraz, Iran

Abstract

Despite a long tradition of fruit-tree growing in all provinces of Iran, information on tree viruses in this country is scant. In the present study, presence of *Tomato ring spot virus* (ToRSV) was surveyed in various woody plants in this country by mechanical inoculation to herbaceous hosts, ELISA using a commercial antiserum, and PCR with specific primers. ToRSV was identified in the following plant-symptom combinations: Walnut with mottling, deformation, necrosis, and yellowing of main veins from Tehran Province; plum with yellowing of main veins, peach with yellowing of major veins and marginal necrosis, and hazelnut with interveinal chlorosis and marginal necrosis from Ardabil Province; apple with yellowing of main veins, mosaic and necrotic lesions, quince with large necrotic spots, and almond with leaf deformation and rosetting from Khorasan Province; and raspberry with marginal necrosis of leaf and necrotic lesions from Mazandaran Province. Mechanical inoculation from walnut, plum, peach, hazelnut, apple, quince, almond, and raspberry to *Nicotiana tabacum* cv. Samsun resulted in systemic infection. The virus isolates induced local lesions, leaf deformation, and necrosis in *N. rustica*, chlorotic local lesions on *Chenopodium quinoa*, and large local lesions on *Gomphrena globosa*. All samples were ELISA positive. PCR with specific primers resulted in the amplification of the expected fragment (490 bp). This study shows extensive occurrence of ToRSV in Iran.

Keywords: *Tomato ringspot virus*, Fruit tree viruses, Iran

Introduction

Tomato ringspot virus (ToRSV) is primarily a pathogen of woody and semi-woody plants and has been shown to cause mild to severe economic loss in many perennial fruit crops including *Malus* species (Stouffer and Uyemoto, 1976) and *Prunus* (Schlocker and Traylor, 1976). It can also be found in herbaceous ornamental and weed species (OEPP/EPPO, 2001), raspberry, grapevine, and dogwood (Rosenberger et al., 1983). The virus is a member of the genus *Nepovirus*, tramssmited by *Xiphinema Americana*, and through seed and pollen (Stace-Smith**,** 1984). ToRSV is widespread in the temperate region of Asia, Europe, and North and South America (OEPP/EPPO, 2005). ToRSV symptoms in peach and almond are pale-green to pale-yellow blotches developing along the main vein or large lateral veins of the leaves (OEPP/EPPO, 2001). ToRSV isolated from lily shows yellow stripe symptoms (Kim and Choi, 1990). It is associated with yellow bud mosaic and stem pitting and decline in peach and other *Prunus* species, brown line in prune, graft union necrosis and decline in apple, ringspot and decline in raspberry, and decline in grapevine (Stace-Smith**,** 1984). In the present study ToRSV was surveyed in various woody plants in Iran by mechanical inoculation to herbaceous hosts, ELISA using a commercial antiserum, and PCR with specific primers.

Materials and methods

Sources of the samples: ToRSV-infected samples were collected from various parts of Iran. Collected samples included, walnut with mottling, deformation, necrosis, and yellowing of major veins from Tehran Province; plum with yellowing of major veins, peach with yellowing of main veins and marginal necrosis, and hazelnut with interveinal chlorosis and marginal necrosis from Ardabil Province; apple with yellowing of major veins, mosaic and necrotic lesions, quince with large necrotic spots, and almond with leaf deformation and rosetting from Khorasan Province; and raspberry with marginal necrosis of leaf and necrotic lesions from Mazandaran Province.

Inoculation: Symptomatic leaves of plants were used for mechanical inoculation experiments. The tissues were homogenized in 5 volumes of 0.01 M phosphate buffer containing 0.01 M sodium sulfite, PH. 7.4. The extracts were rubbed on Carborundum-dusted leaves of *Nicotiana rustica*, *N. tabacum* cv. Samsun, *Gomphrena globosa*, *Chenopodium amaranticolor*, and *C. quinoa*.

Serological tests: A commercial ELISA-kit of ToRSV (Agdia, USA) was used to detect the virus in naturally and experimentally infected plants. DAS-ELISA (Clark and Adams**,**1977) was used throughout the study.

RT-PCR: A pair of ToRSV primers specific for the putative viral polymerase gene (F: 5'- GAC GAA GTT ATC AAT GGC AGC- 3' / R: 5'- TCC GTC CAA TCA CGC GAA TA- 3') (Griesbach, 1995) was used to detect the virus. Total RNA was extracted from infected *N. tabacum* cv. Samsun according to Boom et al. (1990).

Results and discussion

Mechanical inoculation of extracts from walnut, plum, peach, almond, apple, quince, hazelnut, and raspberry to *N. tabacum* cv. Samsun resulted in systemic infection. We found Samsun tobacco a suitable host plant for initial isolation of the virus. However, it took sometimes more than a month for systemic symptoms to develop. The virus isolates induced local lesions, leaf deformation, and necrosis in *N. rustica*, chlorotic local lesions on *Chenopodium quinoa*, and large local lesions on *Gomphrena globosa*. Some infected tobacco (*N. tabacum* cv. Samsun) remained symptomless. ToRSV was identified serologically in walnut, plum, peach, almond, hazelnut, apple, quince, and raspberry. All symptomatic samples of fruit trees were ELISA positive. RT- PCR with ToRSV specific primers resulted in the amplification of the expected fragment (449 bp). However, there were many symptomatic samples which were ELISA positive but failed to show amplification in RT-PCR. This could be due to the presence of inhibitors. ToRSV was present in all Iranian provinces surveyed. It was also detected in many non-woody plants such as tomato and *Physalis* sp. However, it is considered to be economically more important in fruit crops than in other crops (OEPP/EPPO, 2005). The widespread occurrence of and severe symptoms associated with ToRSV in Iran warrants a comprehensive study on its sources and its mode of spread and survival in this country.

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Occurrence of small fruit viruses in Belarus

Valasevich, N.; Kolbanova, E.
Department of Biotechnology, Institute for Fruit Growing, Kovalev str., 2, Samokhvalovichi, 223013, Belarus

Abstract

Epidemiological control of plant virus diseases is necessary for creation of small fruit nurseries and production of berries with stable high yields. Phytosanitary state of *Rubus idaeus* L. and *Ribes* sp. L. was studied in Belarus. The aim of the research was to determine the most widespread small fruit viruses and to select plants that free from all tested viruses for further propagation in vitro as a basis for Nuclear stock. The following viruses were identified: CMV, ApMV, SLRV, RRV, RBDV, ArMV, TBRV and ToRSV. High level of virus infection for small fruit plantations was shown. The most common viruses for all three crops (raspberry, red and black currant) were RRV, SLRV and ArMV. It was noted that infection level of viruses considerably varied from plant cultivars and crops.

Keywords: viruses, raspberry, black currant, red currant, Belarus.

Introduction

Viruses cause pathological changes in small fruits decreasing plant productivity. More than 20 viruses infect *Rubus* worldwide (Diekmann et al., 1994). A number of nepoviruses and *Raspberry bushy dwarf virus* (RBDV) have been detected in raspberry plantations of many countries: UK (Barbara et al., 2001), USA (Ellis et al., 2005), Czech Republic (Špak, 1995) and others. Nepoviruses occurring in *Rubus* are *Arabis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRV), *Raspberry ringspot virus* (RRV), *Tomato black ring virus* (TBRV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV). They are transmitted in nature by nematodes of genera *Longidorus* (RRV, TBRV) and *Xiphinema* (ArMV, SLRV, TRSV, ToRSV) and may cause progressive decline in vigour with symptoms ranging from chlorotic mottling, line-pattern, mosaic, vein yellowing to leaf curling (Diekmann et al., 1994). RBDV, genus *Idaeovirus*, is transmitted by infected pollen and causes yellow disease, crumbly and deformed fruit (Jones et al., 1982).

The most common among graft transmissible viruses of black and red currants are RRV, SLRV, TBRV, ArMV and *Cucumber mosaic virus* (CMV). In Great Britain the following viruses was found: RRV, SLRV, ArMV and CMV (Thresh, 1966), in Germany - RRV, SLRV, ArMV, CMV and *Tobacco mosaic virus* (TMV) (Kleinhempel, 1970), in Finland - TBRV and RRV (Bremer, 1983). Injuriousness of these pathogens can be very significant for red and black currant plants. Productivity reduction of red currant by 32 % was noted after an artificial inoculation by RRV (Kleinhempel, 1970). CMV can reduce growth and productivity of black currant plants by 15 %-20 %. ArMV infection may also cause decreasing of productivity and growth (Thresh, 1966). ToRSV may induce lower fruitage and even death of red currant plants (Hildebrand, 1939).

Epidemiological control of plant virus diseases is necessary for creation of small fruit nurseries and production of berries with stable high yields. We studied the phytosanitary state of *Rubus idaeus* L. and *Ribes* sp. L. in Belarus. The aim of the research was to determine the most widespread small fruit viruses and to select plants that free from all tested viruses for further propagation in vitro as a basis for Nuclear stock.

Material and methods

Objects of the research were commercial cultivars of *Rubus idaeus* L. and *Ribes* sp. L. in Belarus. Occurrence of small fruit viruses was studied by DAS-ELISA (commercial kits of Sanofi Diagnostics Pasteur company). Analysis was carried out in spring time (from middle of May till beginning of June). Leaves were source of plant tissue for test. The following viruses were identified: CMV, ApMV, SLRV, RRV, RBDV, ArMV, TBRV and ToRSV according to the “Statute of fruit plant material production in Belarus”. Automatic rider PR2100 with wavelength 405 nm was used for ELISA results recording.

Results

Raspberry viruses: Raspberry plants were mostly infected by the following viruses: 30.5% (of samples) by RBDV, 29.3 % - by RRV, 26.2 % - by ApMV, 21.9 % - by SLRV, 16.7 % - by ArMV. TBRV, ToRSV and CMV were absent in all tested raspberry plants. At the average 41.5 % of samples was free from tested viruses, 25.6 % of samples was infected by only one virus, 21.9 % - by two viruses, 9.7 % - by tree viruses and 1.2 % of samples contained four viruses simultaneously. Interesting that in samples with single virus infection in 61.9 % of cases RBDV was detected. In samples with two viruses more often was found the following combination of viruses: RBDV+RRV (33.3%) and RRV+SLRV (33.3%) (Table 1).

Tab. 1 Occurrence of graft transmissible viruses in raspberry plantation of the Institute for Fruit Growing

	Percent of infection							
Cultivar	**RRV**	**SLRV**	**RBDV**	**ArMV**	**CMV**	**ApMV**	**TBRV**	**ToRSV**
at the average within 6 cultivars	29.3	21.9	30.5	16.7	0	26.2	0	0
'Alyonushka'	22.7	36.4	4.5	28.6	0	71.4	0	0
'Meteor'	52.6	52.6	21	15.8	0	0	0	0
'Balsam'	0	0	76.5	0	0	5.9	0	0

Occurrence of raspberry viruses considerably varied from plant cultivars. Thus 'Alyonushka' plants were infected by ApMV (71.4 %), SLRV (36.4 %), ArMV (28.6 %), RRV (22.7 %) and RBDV (4.5 %). It was noted that 27 % of tested plants contained simultaneously 2 viruses, 18.2 % of infected plants contained either 1 or 3 viruses. 36.4 % of plants had no one from all tested viruses.

'Meteor' plants were infected basically by the same group of viruses but in other proportion: SLRV (52.6 %), RRV (52.6 %), RBDV (21 %) and ArMV (15.8 %). It was determined that 5.3 % of samples contained only one virus, 26.3 % of samples contained two viruses, 21 % contained tree viruses, and 5.3% contained 4 viruses simultaneously. 42.1 % of given variety samples had no one from all tested viruses. Interesting that in samples with complex infection in 90 % of cases SLRV and RRV viruses were found.

'Balsam' plants were heavily infected by RBDV (76.5 % of samples was positive), and only 5.9 % of plants by ApMV. 23.5 % of samples were free from all tested viruses.

Black currant viruses: High level of infection was detected in black currant collection: RRV (100 %), SLRV (100 %), TBRV (97.5 %) and ArMV (81.8 % of tested samples were infected correspondingly) while CMV infected only 5.8 % of samples. TBRV infection rate of black currant cultivars was 100 % with the exception of 'Katyusha' plants (83.3 % of samples were positive). Rate of ArMV infection have considerably varied among tested cultivars. Thus black currant cultivars 'Cerera', 'Buelorusskaya Sladkaya', 'Partisanka', 'Zagadka', 'Seyanec Golubky', 'Orloviya' were infected by 100%, while 'Pamyat Vaviloba' – by 60 %, 'Katyusha' – by 44.4 % (Table 2).

Tab. 2 Occurrence of graft transmissible viruses in black currant collection planting of the Institute for Fruit Growing

	Percent of infection				
Cultivar	**RRV**	**SLRV**	**TBRV**	**ArMV**	**CMV**
'Cerera'	100	100	100	100	100
'Buelorusskaya Sladkaya'	100	100	100	100	54.5
'Partisanka'	100	100	100	100	0
'Zagadka'	100	100	100	100	4.5
'Katyusha'	100	100	83.3	44.4	0
'Pamyat Vavilova'	100	100	100	60	0
'Seyanec Golubky'	100	100	100	100	0
'Kantata'	100	100	100	0	0
'Orloviya'	100	100	100	100	0

Infection level was no less intensive in propagation plantation of black currant: RRV (100 %), TBRV (93.3 %), SLRV (71.1 %), CMV (62.2 %). TBRV was detected in 90 % of 'Pamyat Vavilova' samples, in 80 % of 'Katyusha' samples, and 100 % infection was presented in cultivars 'Klussonovskaya', 'Cerera', 'Zagadka', 'Kupalinka' and 'Naslednica'. SLRV infection rate vary from 40 % to 100 %. All tested cultivars were infected by CMV. It should be noted that the least CMV infection had cultivars 'Kupalinka' and 'Naslednica' (both 20 %) (Table 3).

Tab. 3 Occurrence of graft transmissible viruses in black currant propagation plantation of the Institute for Fruit Growing

	Percent of infection			
Cultivar	**RRV**	**SLRV**	**TBRV**	**CMV**
'Pamyat Vavilova'	100	40	90	40
'Katyusha'	100	70	80	70
'Klussonovskaya'	100	100	100	100
'Cerera'	100	80	100	100
'Zagadka'	100	80	100	100
'Kupalinka'	100	100	100	20
'Naslednica'	100	60	100	20

Red currant viruses: The most widespread red currant viruses were RRV and TBRV (47.9 % and 34 % of infected plants correspondingly). Infection of cultivars by RRV varied from 5 % ('Rondom') to 100 % ('Fertody'). The highest infection rate of TBRV was noted of cultivars 'Krasnaya Andreychenko' (90 % of infected samples) while in all other cultivars it didn't exceed 35 %. CMV was detected in 2.1 %, ArMV – in 4.3 %, SLRV – in 9.6 % and ToRSV – in 10.6 % of checked samples.

CMV occurred only in "Jonkher Van Tets' plants (14.3 % infected plants). Cultivars 'Fertody' and 'Krasnaya Andreychenko' were affected by ArMV (5 % and 15 % infected samples correspondingly). SLRV was found in 10 % of 'Krasnaya Andreychenko' samples, in 15 % of 'Rondom' samples and in 28.6 % of 'Jonkher Van Tets' samples. ToRSV was detected in 'Fertody', 'Nenaglyadnaya' and 'Jonkher Van Tets' (5 %, 25 % and 28.6 % of infected samples correspondingly) (Table 4).

Tab. 4 Occurrence of graft transmissible viruses in red currant propagation plantation of the Institute for Fruit Growing

	Percent of infection					
Cultivar	**RRV**	**SLRV**	**TBRV**	**ArMV**	**CMV**	**ToRSV**
'Rondom'	5	15	35	0	0	0
'Nenaglyadnaya'	40	0	5	0	0	25
'Jonkher Van Tets'	35.7	28.6	14.3	0	14.3	28.6
'Krasnaya Andreychenko'	55	10	90	15	0	0
'Fertody'	100	0	20	5	0	5

Discussion

The results characterise the epidemiological situation for the most common *Rubus* and *Ribes* viruses in Belarus. High level of virus infection for small fruit plantations was shown. The most common viruses for all three crops (raspberry, red and black currant) were RRV, SLRV and ArMV. It was noted that infection level of viruses considerably varied from plant cultivars and crops. For example, in red currant plants percentage of RRV was the lowest in 'Rondom' (5 %) and the highest in 'Fertody' plants (100 %), in red raspberry the level of the infection varied from 0% for 'Balsam' till 52.6 % for 'Meteor' cultivar while all tested black currant plants contained the virus.

In the issue of conducted research free from tested viruses plants were isolated and used for in vitro propagation. In cases when it was impossible to find virus-free plants plant material was used for chemotherapy experiments *in vitro*.

Knowledge of viruses that infect berries, their distribution and ways of control is important for establishing commercial berry plantations and especially nurseries. Thus, virus diagnostics and epidemiological control of plant virus diseases are necessary for creation of virus-free plants and for the monitoring of propagated certificated planting stock. Production of virus tested and virus free planting stock is one of the scientific priorities in development of small fruit growing in Belarus.

This work was a part of the State Program of Fruit Growing Development with the aim to develop and apply in industry a production system of certified plant material for fruit and berry crops in Belarus.

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Pathogen-derived methods for improving resistance of transgenic plums (*Prunus domestica* L.) for Plum pox virus infection

Dolgov, S.[1]; Mikhaylov, R.[1]; Serova, T.[1,3]; Shulga, O.[2]; Firsov, A.[1]
[1]Branch of Shemyakin Institute of Bioorganic Chemistry RAS, Pushchino, Russia
[2]Institute of Agriculture Biotechnology RAAS, Moscow, Russia
[3]Pushchino State University, Pushchino, Russia

Abstract

Plum pox virus (PPV), the causal agent of Sharka disease, is considered to be one of the most serious pathogens of stone fruits including apricots, plums and peaches. This disease is of particular concern in central and southern Europe, the Mediterranean areas and North America.

The transformation of plum with viral genes, such as coat protein, can provide virus resistant varieties or gene resources for breeding new resistant varieties. In the current study we report the evaluation of two technologies for producing plants resistance to PPV, one based on co-suppression and another on RNA-silencing. Two gene constructs were evaluated; the binary vector pCamPPVcp that contained the selective *hpt* gene and *ppv-cp* gene in sense-orientation (driven by double 35S promoter) and vector pCamPPVRNAi that contained self-complementary fragments of gene *ppv-cp* (698bp) driven by double 35S promoter and the *hpt* and *gus* genes.

The fragments of the *ppv-cp* gene in pCamPPVRNAi were separated by a pdk-intron to produce a “hairpin” RNA structure in antisense-sense orientation. Seven independent transgenic lines with the sense-oriented *ppv-cp* gene and five transgenic lines with inverted repeats of the *ppv-cp* gene fragment were produced. The accumulation of coat protein in five pCamPPVcp lines was confirmed by Western blotting. Transgenic shoots were rooted and acclimatized to the greenhouse. After grafting with PPV infected buds PPV-CP was detected by Western blotting in all control and pCamPPVcp transformed plants whereas no PPV coat protein were observed in samples from plants transformed with the pCamPPVRNAi “hairpin” construct. These preliminary results confirmed the efficiency of the RNAi strategy for producing virus resistant plants in general and PPV resistant stone fruits in particular.

Keywords: RNA interference, PPV, transformation, coat protein, *Prunus domestica*.

Introduction

Stone fruit trees are valued for the quality of their fruits and as sources of hard wood worldwide. Virus and fungus diseases are main the most important pathogens of stone fruits. PPV, the causal agent of Sharka disease, is considered the most important pathogen in peach and nectarine, apricot, plum and cherry world-wide. A critical review of the literature dealing with PPV resistance, suggests that there is no source of high-level resistance or immunity to PPV in *Prunus* that protects trees against all strains of the virus. Considering the severity of the disease, the difficulty in controlling its spread, and the lack of resistant varieties, the need for novel approaches to the development of resistance is apparent.

Modern biotechnology presents new pathogen resistance strategies, especially applicable to virus resistance. The concept of pathogen-derived resistance (PDR) proposed by Sanford and Johnston (1985) has been widely used as a basis for obtaining virus-resistant plants. Since the first success with the tobacco plants transformed with the tobacco mosaic virus (TMV) coat protein (CP) gene (Powell et al., 1986), this strategy, based on transgene produced viral CP, has been the most studied application of PDR and has provided various degrees of protection (delay in the symptom development, partial resistance or immunity) against numerous plant virus groups (for review, see Beachy, 1990; Lomonossoff, 1995). This work led to the notion that the level of virus resistance was directly related to the CP expression level of transgenic lines. Further investigations by several laboratories, however, led to the surprising finding that that some transgenic lines with high virus resistance levels in fact did not express any viral CP. Moreover, the CP RNA level was very low or even not detectable in these resistant plants. Subsequent work clarified this apparent discrepancy of non-expressing transgenic plants with virus resistance. We now know that these transgenic lines were resistant to virus because the expressed CP mRNA triggered post-transcriptional gene silencing (PTGS) and provided RNA-mediated virus resistance by the siRNA pathway.

The siRNA pathway targets double-stranded (ds) RNA for degradation by DICER-like proteins (DCLs) in a sequence-specific manner through the production of siRNA. Whereas DCL2 cleaves dsRNAs from replicating viruses (Xie et al., 2004), DCL3 cleaves dsRNAs derived from endogenous transcripts through the activity of RDR2 and RDR6 (Mourrain

et al., 2000). The siRNAs produced are incorporated into RNA-induced silencing complexes (RISC), which guide cleavage of target RNAs. In RISC, siRNAs mediate sequence-specific binding and cleavage of target RNAs (Baulcombe, 2004). Once cleaved, the RNA is further degraded by exonucleases in the cytoplasm. Alternatively, siRNAs are used as primers for RDR polymerase, using target RNA as a template to generate more dsRNA and produce additional siRNAs. This RDR activity expands the pool of siRNA and amplifies PTGS resulting in more potent silencing activity and effective defense against plant viruses. Thus, in the virus-resistant lines, not only the transgene mRNAs but also the invading viral RNA (with homology to the transgene) was degraded.

In addition to CP mRNA, RNA-mediated virus resistance can be brought about by expression of satellite RNA, defective interfering (DI) RNA or even noncoding regions of viral genome RNAs that compete and interfere with virus replication (Baulcombe, 1996). This type of resistance can also be accomplished by expression of viral sequences in the sense or antisense orientation (Smith et al., 1994; Waterhouse et al., 1998) or in double-stranded forms (Helliwell and Waterhouse, 2003). In all these cases, expression triggers degradation of both the transgene RNA and the corresponding viral RNA via the siRNA pathway.

RNA interference (RNAi) is a potent method requiring only a few double stranded RNA (dsRNA) molecules per cell to silence the viral gene expression. This has made it one of the hottest topics in molecular biology in recent years. Reports from several laboratories have established that the loss of the target mRNA in steady-state accumulation is almost total if the designed transgene construct of the transgenic plant produces the nuclear transcript in the duplex conformation. Recently it was reported that the expression of self-cRNA of PPV under the control of the *rolC* promoter caused degradation of transgenic viral RNA and as a result, systemic disease resistance to challenge inoculums of PPV in transgenic *Nicotiana benthamiana* (Pandolfini et al., 2003). This evidence points out that the production of dsRNA is required to initiate PTGS in plants. Based on this, plants carrying strongly transcribing transgenes in both the sense and antisense orientations are currently being produced that show strong PTGS features (Chuang and Meyerowitz, 2000; Smith et al., 2000).

Materials and methods

Plant culture media and culture conditions: Shoot cultures of plum [*Prunus domestica* (L.)] were established *in vitro* from buds of 4-year-old trees of cultivars "Startovaya". Micro-shoots were maintained *in vitro* on JS proliferation medium (modified QL). JS proliferation medium consisted of salts supplemented with (mg/l) myo-inositol 100; thiamine HCl 0.1; nicotinic acid 0.5; pyridoxine HCl 0.5; glycine 2.0; sucrose 3 % (w/v), 0.7 % agar (w/v). The growth regulators used to plum multiplication were 2 mg/l BAP and 0.1 mg/l IBA. The medium was adjusted to pH 5.8 using 1N KOH and autoclaved for 20 min at 1.4 kg/cm^2 (121 °C). All phytohormones and antibiotics were filter-sterilized and added to autoclaved medium when required. The cultures were grown in the culture room at 24 °C ± 1 °C under a 16/8-h (light/dark) photoperiod with light provided by an equal mixture of warm-white and fluorescent lamps.

The shoot regeneration medium consisted of MS salts supplemented with (in mg/l) myo-inositol 100; thiamine HCl, 0.1; nicotinic acid 0.5; pyridoxine HCl 0.5; glycine 2.0; sucrose 3 % (w/v); agar 0.7 % (w/v). The growth regulators used to induce shoot regeneration from plum leaves were 5 mg/l BAP and 0.5 mg/l IBA. The basal rooting medium was 1/2-strength JS medium with 1 % sucrose, proliferation medium organics and 0.5 mg/l IBA.

Bacterial strains and vectors: For transformation we used the super virulent strain AGL0 (Lazo et al., 1991) with pCamPPVcp containing the *hpt* gene under the cauliflower mosaic virus (CaMV) 35S promoter and the coat protein (CP) gene of plum pox virus (PPV) driven by the double CaMV 35S promoter and with binary vector pCamPPVRNAi containing the *hpt* gene under the duplicated cauliflower mosaic virus 35S promoter (d35S), the *gus*-intron gene under the CaMV 35S promoter and self-complementary fragments of PPV-CP gene under the modified enh35S promoter (Kay et al., 1987), with a duplicated enhancer sequence (Figure 1). The fragments of the PPV-CP gene were separated by a PDK intron (from pHANNIBAL) to produce a "hairpin" RNA (hp-RNA) structure in antisense - sense orientation. The PPV-CP gene fragment contained the PPV-CP gene with the ATG codon with a length of 698 bp. The size of the PPV-CP intron-hairpin-RNA transcripts was 2220 bp.

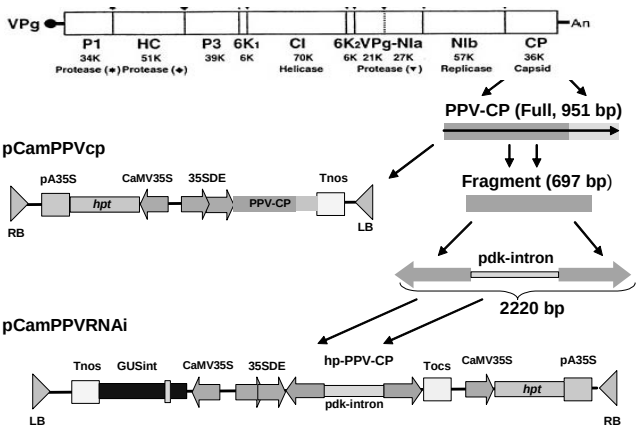

Fig. 1 Vectors construction for transfer fragments of PPV genome to plum trees. See text for details.

Plant transformation: The 1-2 youngest a fully expanded leaves of 5 to 12-week-old in vitro rooted shoots were used for experiments. The leaves were wounded by making cuts perpendicular to the midrib, not reaching the leaf edges. Leaf explants were subjected to auxin shock in liquid MS medium containing 5 mg/l indole-3-acetic acid (IAA) for a period of 5 hours. Explants were then placed in the *Agrobacterium* suspension for 30 minutes. Co-cultivation was carried out for 3 days in darkness with the adaxial side of the leaves in contact with the shoot regeneration medium. The shoot regeneration medium (SRM) consisted of MS salts supplemented with (in mg/l) myo-inositol 100; thiamine HCl 0.1; nicotinic acid 0.5; pyridoxine HCl, 0.5; glycine 2.0; calcium pantothenate 4.0; sucrose 3 % (w/v); agar 0.7 % (w/v). The growth regulators used to induce shoot regeneration from plum leaves were 5 mg/l BAP and 0.5 mg/l IBA. After co-cultivation, the leaves were transferred to the shoot regeneration medium containing 500 mg/l cefotaxime and maintained in the dark at 23 °C ± 2 °C. Immediately after co-cultivation or after 10 days, all leaf explants were transferred to the SRM supplemented selective antibiotic in various concentrations. After 2-2.5 months from the beginning of transformation, all leaf explants were transferred to the shoot elongation medium (SEM) containing 2 mg/l BAP, 0.1 mg/l IBA, 300 mg/l cefotaxime and selective antibiotic, under a 16-h photoperiod. Control explants were treated as described above, with the exception that they were not co-cultivated with *A. tumefaciens*.

Rooting and acclimatization: Three-week-old 2-3 cm long transgenic plum shoots were transferred to basal rooting medium (1/2-strength JS salts) supplemented with 10 g/l sucrose, MS vitamins and 0.5 mg/l IBA, under a 16-h photoperiod. When roots grew to at least 3 cm in length, plants were transferred to the greenhouse.

PCR analysis: The stable integration of the gene cassettes into the genome of plants was confirmed by PCR analysis. PCR was performed in Eppendorff thermocycler gradient. The primers used for amplification of a 951 bp fragment of the *hpt* gene were

5¢-CGACGTCTGTCGAGAAGTTTCTGATC-3¢ and 5¢- GTACTTCTACACAGCCATCGGTCCA-3¢.

The primers used for amplification of a 950-bp fragment of the CP-PPV gene (PPV-1/PPV-2) were

5¢-ATGGCTGACGAAAGAGAAGACGAG-3¢ and 5¢- CTACACTCCCCTCATACCGAGGAG-3¢.

The primers used for amplification of an 880 -bp fragment of the PPV gene in the "harpin" construct (PPVup - ocs-ter) were

5¢-AGACGAGGAGGAAGTTGATG-3¢ and 5¢- ACAATCAGTAAATTGAACGGAG-3¢.

The primers used for amplification of a 740 -bp fragment of the *gus* gene were

5¢-TCGTAATTATGCGGGCAACGTC-3¢ and 5¢- CGAATCCTTTGCCACGCAAG-3¢.

PCR products were separated by electrophoresis in 1.2 % (w/v) agarose-ethidium bromide gels. The absence of *Agrobacerium* contamination was shown by the PCR amplification of a fragment of the *VirB*1 gene.

Histochemical GUS assay: Histochemical GUS assay of plant tissues was performed as described in Jefferson et al. (1987).

Western blot analysis: The leaves of transgenic plum plants were ground in liquid nitrogen. Powdered material was resuspended in four volumes of extraction buffer containing 50 mM Tris-HCl, pH8.0; 10 mM EDTA, pH 8.0; 10 % glycerol (v/v); 30 mM 2-mercaptoethanol; 4 µg/ml aprotinin; 4 µg/ml leupeptin. Total proteins were extracted for 20 min at room temperature, then centrifuged for 10 min at 20 ºC and the supernatant was taken for further analysis. Thirty µl of total protein extract from each transgenic line was separated on 12.5 % SDS-PAGE and transferred onto NC membrane (BioRad, USA) by tank transfer. Western-blot analysis was performed using rabbit polyclonal antibodies to PPV coat protein; the antibody was diluted 1:1000. Anti-rabbit IgG conjugated with fluorescent label CY3 was used as secondary antibody (Amersham, USA; dilution 1:5000). The images of blots were obtained by Variable Mode Imager Typhoon 9200 (Molecular Dynamics, USA) and developed by ImageQuant program (Molecular Dynamics, USA).

Results and discussion

Seven transgenic plants with the PPV-CP gene in sense orientation were obtained after transformation of 524 leaf explants (Table 1). Transformation frequency was 1.3 %. PCR-analysis confirmed the transgenic status of plants by amplification of the predicted fragment for the *hpt* (Figure 2a) and PPV-CP genes (Figure 2b). The accumulation of coat protein was demonstrated by Western blot assay in five of six analyzed lines (Figure 3).

Tab. 1 Results of plum transformation by vector pCamPPVcp contain full length PPV-CP gene.

		Total number of lines			
Variety	**Number of explants**	**Putative**	***hpt* positive (%) transformation**	**Insertion of PPV-CP gene**	**Coat protein expression**
Startovaya	524	7	7 (1.3%)	7	5

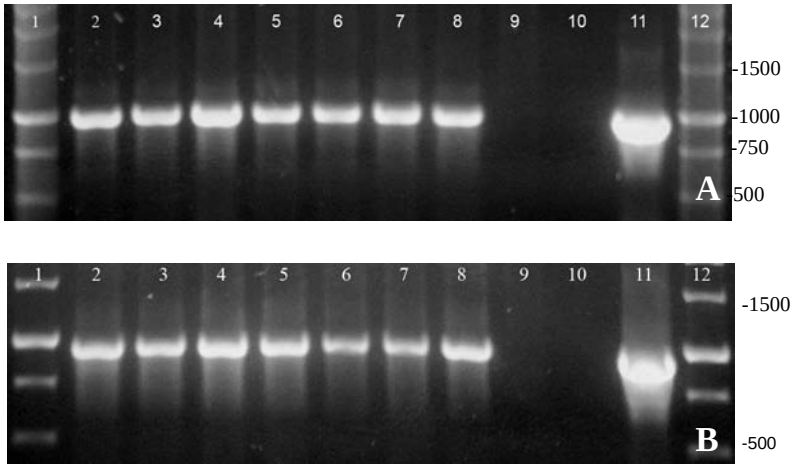

Fig. 2 PCR-analysis of genomic DNA extracted from transgenic "Startovaya" plum plants (pCamPPVcp transformed plants). **A -** agarose gel contains PCR products of the *hpt* gene. Amplification produced an 951 bp product. **B -** agarose gel contains PCR products of the fragment of the promoter and *CP-PPV* gene. Amplification produced a 950 bp product. Lines 1, 12 - molecular weight marker; 2-8- pCamPPVcp transformed plants; 9 – negative control, cv."Startovaya"; 10 – H_2O; 11 – pCamPPVcp.

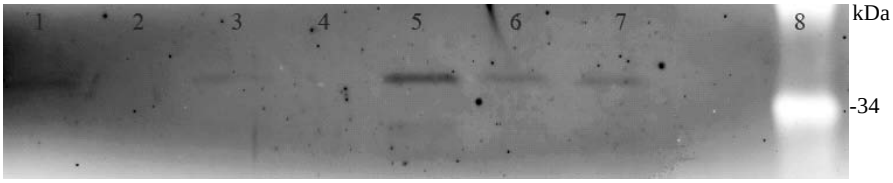

Fig. 3 Western blot assay of transgenic plants var. "Startovaya" (pCamPPVcp) with rabbit polyclonal antibodies against PPV coat protein and anti-rabbit IgG conjugated with fluorescent label CY3. Line 8 – molecular weight marker; 1-3, 5-7 – pCamPPVcp transformed plants; 4 –negative control, cv."Startovaya".

Five independent transgenic PPV-CP intron-hairpin-RNA lines (ihpRNA) were produced (Table 2). All transgenic plants were selected by on 5 mg/l hygromycin. Transformation frequency in this experiment was lower than in the previous experiments (1.1 % against 1.8-2.2 %) with the *gfp* gene (Mikhaylov et al. 2007) or *PPV-CP* gene (Mikhailov et al. 2006) probably due to the large size of the T-DNA locus (about 10,000 bp). PCR-analysis confirmed the transgenic status of plants by amplification of the predicted fragment of the *hpt* (Figure 4a) and "hairpin"-*PPV-CP* genes (Figure 4b,c).

Tab. 2 Results of plum transformation by vector pCamPPVRNAi transformed contain self-complementary fragments of PPV-CP gene.

Cultivar	Infected explants	Hyg-resistant shoots no. (%)	GUS-positive regenerants	PCR-positive		
				hpt	gus	PPV-CP
Startovaya	452	5 (1,1)	3	5	4	5

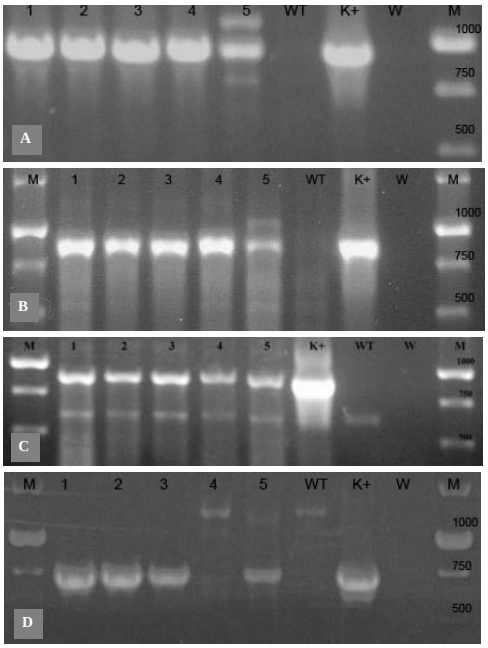

Fig. 4 PCR analysis of genomic DNA extracted from transgenic "Startovaya" plum plants (pCamPPVRNAi transformed plant). Agarose gel contains PCR products of the *hpt* (A), *PPV-CP* left (**B**), right arms (C) and *gus* (D) gene. Amplification produced a 951, 880, 880 and 740 bp product (respectively). Lines 1, 10 - molecular weight marker; 2-6 – transgenic lines; 7 – negative control, cv."Startovaya"; 8- pCamPPVRNAi; 9 – H_2O.

GUS gene expression was detected in leaves of 3 of 5 transgenic lines (fig.4d). One transgenic line (N4) had no *gus* gene; line N3 contained a transgene insertion (Figure 4d), but the protein was not expressed. Expression of GUS was detectable in all tissues, whereas no blue staining was observed in non-transformed plantlets (Figure 5).

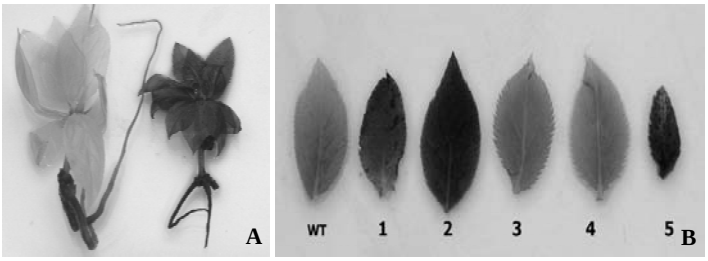

Fig. 5 Histochemical GUS analysis of plants (A) and leaves (B) of control (wt) and transgenic (1-5) plum plants cv. Startovaya. Vector pCamPPVRNAi. Scale bar on each picture represents 10 mm.

The rooted 2-month-old shoots were placed directly into a greenhouse, bypassing a stage of adaptation in a culture room. Rooted shoots had a 70 % rate of survival and successfully grew in the greenhouse. Ten plants of each transgenic line transformed by coat protein and "hairpin" construct genes and ten control plants were infected with PPV in September 2008 by grafting buds from plum shoots with Sharka symptoms. In the next spring they were cut above the grafts. Shoots that developed were tested for PPV by Western blotting (Figure 6). In all control and *ppv-cp* transformed plants PPV coat protein (39K) was detected (Figure6A,B), whereas no PPV coat protein was observed in samples from plants transformed with the "hairpin" construct (Figure 6C).

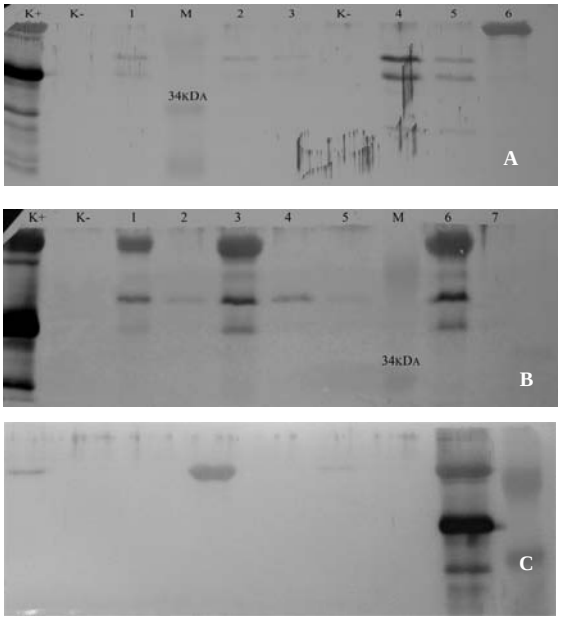

Fig. 6 Western blot assay of plums var. "Startovaya" infected by PPV with polyclonal antibodies against PPV coat protein (Loewe). **A**- nontransgenic plants lines 1-6; **B** - plants transformed by PPV coat protein gene 1-7; **C** - plants transformed by hairpin construct-lines 1-6, K- (noninfected plant) – line 7, K+ (infected plant) – line 8, size marker line - 9.

The PPV-CP gene has been transferred into the hypocotyls of the plum (*P. domestica*) (Scorza et al., 1994; Petri et al., 2008). These authors have shown that one transgenic plum line, C5, is highly resistant to PPV and remained so for over 5 years in greenhouse tests (Ravelonandro et al., 1997, 1998). Long-term field tests in Europe have confirmed that C5 is highly resistant to PPV (Malinowski et al., 1998).

Molecular analyses of the C5 transgenic clone have shown a high level of transgene transcription in the nucleus, low levels of transgenic mRNA in the cytoplasm, a complex multicopy transgene insertion with partial insert copies, transgene methylation and no detectable protein, all characteristics typical of a post-transcriptional gene silencing (PTGS) mechanism, where the expression of a transgene induces the plant to degrade RNA of the same sequence, including any RNA from an infecting virus (Scorza et al., 1994, 2001). This was initially demonstrated through nuclear run-on analysis, confirming that mRNA from the PPV coat protein is produced but does not accumulate in the plant cells (Scorza et al., 2001). The PTGS mechanism of resistance in C5 was further confirmed by the detection of short interfering RNA (siRNA) homologous to the PPV sequences (Hily et al., 2005). While the short (22nt) siRNA production has been detected in non transformed PPV-susceptible plums upon inoculation with PPV, the resistant C5 produces both the short (22nt) and the long (25-26nt) species of siRNA. This finding has lead to the suggestion that the high level virus resistance in the transgenic C5 is connected with the production of long-sized class of siRNA (Hily at al., 2005). Interestingly, the presence of the short siRNA found naturally in plum suggests that gene silencing is a natural system of reaction to PPV infection in plum. But resistant transgenic lines such as C5 developed from seedling transformation could be used only as the resistant parents in long term breeding programs. With generation cycles ranging from 3 to 6 years for *Prunus* species (Sherman and Lyrene, 1983), the time necessary to incorporate a high level of resistance to PPV plus incorporate a high levels of fruit quality, yield potential, cold-tolerance, and resistance to other diseases can be greater than the lifetime of a breeder. We also show that resistance to plum pox virus (PPV) can be induced in transgenic plants using a vector (pCamPPVRNAi) that generates PPV-CP homologous RNAi (fig.1).

Our preliminary results confirmed the efficiency of RNA strategy for protecting plants from virus attack in general, and for stone fruits from PPV particular. Our work also extends transgenic RNAi-based PPV resistance to an established plum cultivar.

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Hairpin Plum pox virus coat protein (hpPPV-CP) structure in 'HoneySweet' C5 plum provides PPV resistance when genetically engineered into plum (*Prunus domestica*) seedlings

Scorza, R.[1]; Georgi, L.[1,2]; Callahan, A.[1]; Petri, C.[1,3]; Hily, J.-M.[1,4]; Dardick, C.[1]; Damsteegt, V.[5]; Ravelonandro, M.[6]
[1] USDA-ARS-Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430 USA
[2] Department of Genetics, Biochemistry and Life Science Studies, Clemson University, Clemson, SC 29634 USA
[3] CEBAS-CSIC. Departamento de Mejora Vegetal. Grupo de Biotecnología de Frutales. P.O. Box 164, 30100 Murcia, Spain
[4] New York State Agricultural Experimental Station, Cornell University, 630 West North Street, Geneva, NY 14456 USA
[5] USDA-ARS-FDWSRU, Ft. Detrick, MD 21702 USA
[6] INRA-1090 Unité Mixte de Recherches Génomique, Diversité, Pouvoir Pathogène, 33883 Villenave d'Ornon, France.

Abstract

The genetically engineered plum 'HoneySweet' (aka C5) has proven to be highly resistant to *Plum pox virus* (PPV) for over 10 years in field trials. The original vector used for transformation to develop 'HoneySweet' carried a single sense sequence of the full length PPV coat protein (*ppv-cp*) gene, yet DNA blot analyses indicated that there was an inserted copy of the *ppv-cp* that appeared to be an inverted repeat structure. Since the resistance mechanism of 'HoneySweet' was found to be based on post-transcriptional gene silencing (PTGS), it was hypothesized that the inverted repeat structure conferred the resistance to PPV in 'HoneySweet'. Sequencing of the transgene insertions confirmed the presence of an inverted repeat of the PPV-CP sequence. We hypothesized that transcription from this structure produced a hairpin (hp) RNA that was responsible for PTGS of the transgene and the destruction of PPV viral RNA resulting in the high level of resistance to PPV infection. In order to confirm this hypothesis the hpPPV-CP insert was cloned from 'HoneySweet' and transferred into 'Bluebyrd' plum seedlings through *Agrobacterium tumefaciens* transformation of hypocotyl slices. The introduced DNA contained the CP inverted repeat flanked by 35S promoters on either end. Transgenic plum plants containing single or multiple copies of this hp insert were inoculated with PPV D isolated from Pennsylvania, USA. PPV infection was evaluated through three cycles of cold-induced dormancy (CID) by symptom expression and by two or more ELISA and PCR tests. Of the 18 plants evaluated, eight were always virus-free, five occasionally had weak or moderate infections, and five plants were clearly infected in multiple tests. While all plants of some clones were virus-free others had a mix of uninfected and mildly infected plants of the same clone. Most of the resistant plants contained a single copy of the hp construct. These data strongly support the hypothesis that the hp structure of the *PPV-CP* insert in 'HoneySweet' plum can confer PPV resistance.

Keywords: breeding, gene silencing, Rosaceae, sharka

Introduction

The earliest reports of gene silencing involved the transformation of petunia with the chalcone synthase gene for the purpose of increasing purple flower color. Unexpectedly, activity of the native chalcone synthase gene was shut down and white or partially white flowers resulted (Napoli et al, 1990). This homology-related gene silencing phenomenon appeared to be RNA-related and was shown to be triggered by double stranded (ds) RNA (Hannon 2002; Kooter et al., 1999; Matzke et al., 2001; Vaucheret et al., 2001; Waterhouse et al., 2001). RNA silencing participates in the regulation of endogenous gene expression in developmental processes and serves as a component of the protective mechanism against mobile genetic elements, such as transposons and viruses (Voinnet, 2005). A key component of gene silencing is short interfering RNA (siRNA). siRNAs are derived from the cleavage of dsRNA by the Dicer enzyme a member of the RNase II family that specifically cleaves dsRNA. ds RNAs are cleaved into RNA duplexes of 21 to 28 nucleotides (Hamilton and Balcombe, 1999; Berstein et al, 2001; Elbashier et al, 2001; Baulcombe 2004). These siRNAs, corresponding to both sense and antisense strands, guide a multi-subunit ribonuclease, the RNA-induced silencing complex (RISC), and ensure that it specifically degrades RNAs that share sequence similarity with the dsRNA. Inverted repeats of the target gene have been shown to efficiently trigger silencing (Stam et al., 1997) presumably through the generation of dsRNAs. Self-complementary hairpin-RNA (hpRNA) constructs induce a high level of PTGS in transgenic plants (Wesley et al., 2001). The presence of an intron between the two complementary regions enhances silencing efficiency (Smith et al., 2000).

Specifically for resistance to PPV Pandolfini et al. (2003) showed that the expression of hpRNA containing a *PPV* sequence conferred systemic resistance to PPV but did not prevent local infection when introduced into *N. benthamiana* under the control of the *rolC* promoter. Di Nicola-Negri et al. (2005) reported that more than 90 % of transgenic *N. benthamiana* lines were resistant to the virus when engineered with hairpin constructs using *PPV-P1* and *PPV-Hc-Pro* gene sequences under *35S*-Cauliflower mosaic virus (CaMV) promoter. Hily et al (2007) tested four ihpRNA gene constructs of the PPV-CP gene and showed that full-length (1 kb) and 213 bp ihpRNA PPV-CP sequences could induce siRNA production, gene silencing, and PPV resistance.

The clearest case of gene silencing based PPV resistance has been reported in plum in a clone that was originally transformed with a sense construct of the PPV-CP gene (Scorza et al, 1994; Ravelonandro et al, 1997). The high level of resistance of the C5 clone (cv. 'HoneySweet') has been demonstrated for over 10 years in field tests in 4 countries (Hily et al, 2004; Malinowski et al, 2006; Zagrai et al., 2008). The complex insertion of the sense PPV-CP gene (Scorza et al., 1994) appeared to include adjacent complementary copies of the PPV-CP gene which would produce hpRNA (Figure 1). In this report we confirm the presence of the predicted rearranged PPV-CP (hp) sequence through sequencing. We show the hp nature of the insert and through cloning of the insert and transformation into plum we demonstrate that the *PPV-CP* hp sequence from 'HoneySweet' plum provides PPV resistance.

Materials and methods

BAC library construction followed the procedures of Georgi et al. (2002). High-molecular-weight DNA was extracted in solution from leaves of transgenic plum C5.

Hybridization: Probes were made from PCR fragments representing PPV-CP and labeled with α^{32}P dCTP by random priming. Each BAC clone from the 'HoneySweet' library was double spotted on a membrane and hybridized with the probe. Hybridization signals were detected autoradiographically.

BAC DNA extraction: BAC DNA was extracted by a modified alkaline-lysis procedure. Larger-scale BAC DNA extractions (50–500 ml culture volume) were additionally purified on cesium chloride density gradients using a Beckman TL100 ultracentrifuge.

Confirmation of selected clones: BAC DNA prepared from positive clones was digested with *Eco*RI, *Bam*HI or *Hin*dIII, electrophoresed on 0.8 % SeaKem LE agarose and stained with ethidium bromide. Southern transfer of the DNA to Hybond N+ membranes was performed using the manufacturer's Alkaline Transfer Protocol. Southern blots were hybridized with appropriate probes.

Subcloning and sequencing: For sequencing, BAC DNA was digested with *Sau*3AI, *Eco*RI, *Bam*HI or *Hin*dIII, and the resulting fragments were ligated into pUC19 or pBluescript and transformed into *Escherichia coli* strain DH5α by calcium/heat shock (Sambrook et al 1989). Clones were individually grown and plasmid DNA for sequencing was prepared using a protocol similar to that used for BAC DNA. Subclones were sequenced using ABI's Dyedeoxy terminator cycle sequencing kit and an ABI377 DNA sequencer. Sequences were assembled using Sequencher 4.2 software (GeneCodes Corp.).

Plasmid construction: A 3.1 kb *Hin*dIII fragment from a BAC subclone was ligated into pBINPLUS/ARS a pBIN19-based plant transformation plasmid (van Engelen et al., 1995). Sequence 1738 consisted of the sense and antisense complementary PPV-CP sequences and their respective 35S promoters (Figure 1). *Agrobacterium tumefaciens* EHA 105 was electrotransformed with this plasmid yielding the strain WV1738.

Fig. 1 The PPV-CP hairpin insert cloned out of C5 plum and used to produce new transgenic plum clones. (35S = cauliflower mosaic virus promoter; CP = the Plum pox virus coat protein gene; CP-3' = the 3' an incomplete untranslated end of CP; 3'-CP = a longer incomplete 3' untranslated end of CP.)

Plum transformation and transgenic plant production: Seed hypocotyls of 'Bluebyrd' (Scorza and Fogle, 1999), a PPV susceptible plum variety, were used as explants for transformation using the protocol of Petri et al. (2008). Plum plants that regenerated shoots and rooted in vitro under kanamycin selection were transferred to a greenhouse. DNA blotting following the procedures of Petri et al. (2008) was used to confirm integration of the insert into the plant genome and to estimate transgene copy number in each transgenic line.

Evaluation of PPV infection: The PPV virus source for aphid inoculation and PPV infection evaluation were as described in Hily et al. (2007). Briefly, aphids were starved for 30 min and then allowed to feed on detached, highly symptomatic leaves of GF305 peach seedlings for virus acquisition. Infected leaves with aphids were then placed onto plum test plants in cages which included transformed and non-transformed susceptible plum seedlings. Aphids were allowed to move from peach leaf pieces to plum leaves over a 24-hour inoculation access period (IAP) and inoculations were repeated up to 3 times if aphid numbers were low. Plants were sprayed with an appropriate insecticide.

Inoculated plants were placed under natural daylight conditions supplemented with 400-W lamps to provide a 15-h photoperiod. Temperature was maintained at 24 °C. All plants were assayed by ELISA and any plants testing negative for two consecutive assays were re-inoculated as above. All plants then were exposed to cold-induced dormancy (CID) at 4-5 °C for 60 days. Plants were removed from the CID treatment, allowed to re-flush in a warm greenhouse, and re-evaluated for infection.

PPV infection of test plants was evaluated by visual observation of symptoms and by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Leaf samples ranged from 0.25 to 0.5 g, and were ground in PBS (Phosphate Buffered Saline) -Tween ELISA buffer at a 1:10 ratio (w:v). Leaf extract samples were centrifuged for 5 min at 1200 xG (Beckman GPR centrifuge; Beckman Instruments, Palo Alto, CA). A one ml sample of the supernatant was collected and then processed according to the manufacturer's protocol (REAL® Kit, Durviz S.L.U., Paterna, Spain). Plant samples with ELISA values three times greater than the background were considered positive.

Results

Sequence analyses of 'HoneySweet': One BAC clone contained sequence that consisted of *ppv-cp* and *35S-35S* promoter but did not have any of the other sequence from the vector including the marker *uidA* gene or NPTII. Sequence analysis determined that this sequence represented the postulated inverted repeat sequence. The *35S-35S* promoter sequence was followed by the cp sequence and an incomplete 3' untranslated end, then the inverse of an incomplete but longer stretch of the 3' untranslated end, the coding sequence of the cp and the *35S-35S* promoter sequence (Figure 1). This structure was inserted into plum DNA.

Transfer of ppv-cp inverted repeat: A 3.1 kb fragment was transferred to plum seedlings through *Agrobacterium* mediated transformation. Eight distinct clones containing the C5 insert (*C5IN*) were selected, and DNA blotting revealed that C5IN copy number ranged from 1 to 5 in regenerated clones.

PPV Resistance: Eight unique transgenic clones were evaluated. Five of the eight unique clones were vegetatively multiplied through self-rooting to produce a total of 19 plants that along with control plants (non-transgenic and C5) that were inoculated with PPV. Over the course of three consecutive cycles each cycle consisting of a growth period and a CID period the plants were monitored for symptoms and periodically tested during the growing period for the presence of the virus by ELISA, PCR and by quantitative real-time PCR to detect very low levels of infection. Of 18 plants evaluated, eight plants had no detectable levels of virus, five plants on occasion had weak or moderate levels of infection, and five plants were clearly infected in multiple tests (Table 1).

Tab. 1 Results of multiple evaluations of PPV infection of transgenic plum plants containing the PPV-CP hairpin insert from plum clone C5. Inoculations were aphid mediated with the PPV-D serotype from Pennsylvania USA. All clones contain the same construct but may have single or multiple copy inserts (ND = no data).

Clone/plant	# C5 inserts	Symptoms	ELISA 1	ELISA 2	PCR 1	PCR 2	PCR 3
D/1	1	-	-	-	-	-	-
D/2	1	-	-	-	-	-	-
F/1	1	-	-	-	-	-	ND
F/2	1	-	-	-	-	-	ND
F/3	1	-	-	-	-	ND	ND
G/1	1	-	-	-	-	+	+
G/2	1	-	-	-	-	ND	ND
G/3	1	-	-	+/-	-	ND	ND
G/4	1	-	+/-	+/-	-	ND	ND
K/1	2	-	-	-	-	-	ND
A/1	2	++	+	+	-	++	+
L/1	2-3	+	+	+	-	ND	ND
L/2	2-3	-	-	+	-	++	++
L/3	2-3	+	+	+	-	++	++
L/4	2-3	-	-	+	+	-	ND
E/1	4	-	+	+	-	++	++
H/1	>5	-	-	-	-	-	-
H/2	>5	+	-	+	-	ND	ND

Where multiple plants of a single clone were tested, all the plants for two clones, D and F were resistant while in G and H some plants tested completely negative and some gave a weak positive reaction. Several plants in clones A, E, and L gave strong positive reactions in some tests, generally PCR. There were few other strong positive reactions. Non-transformed control plants were clearly infected and strongly positive early in the inoculation tests. The original C5 clone was clearly resistant.

Discussion

C5 plum originated from transformation with a sense PPV-CP construct. This clone was the only highly resistant clone produced using that particular PPV-CP construct. All other clones were susceptible. C5 contained a duplicated and rearranged transgene insert (Scorza et al., 1994). It was suspected that one component of the insert in C5 was a tandem duplication of the CP gene and this component may have been responsible, at least in part, for resistance. Through the construction of BAC libraries of C5 plum, followed by gene cloning and sequencing we isolated the PPV-CP hairpin structure from C5. This structure was then engineered into a plant transformation vector and used to develop new C5 insert (C5IN) transgenic plants. Upon inoculation with PPV these plants were generally resistant to PPV indicating that the PPV-CP hp portion of the transgene insert in C5 is, if not solely, at least one of the components of the C5 transgene insert responsible for PPV resistance in this clone. Variability in resistance when using hp constructs is common and the basis for this variability is not known. Although all of the clones that were clearly susceptible to PPV in this trial were multicopy clones, other authors have found either no correlation between hp insert copy number and resistance (Hily et al, 2007) or a positive correlation between resistance and copy number (Kalantidis et al., 2002). Smith et al. (2000) found that many of the virus "immune" plants developed from hp transformants contained single copy inserts.

The confirmation of a PPV-CP hairpin insert in the PPV resistant plum clone C5 provides conclusive evidence of the mechanism for resistance. Further, almost 20 years of work with the C5 clone including over 10 years of field testing of resistance in four countries demonstrated the stability, efficacy and safety of hp gene silencing for PPV resistance in plum (Capote et al., 2008; Fuchs et al., 2007; Hily et al., 2004, 2005, 2007; Kundu et al., 2008; Malinowski et al., 2006; Polak et al., 2005, 2008; Ravelonandro et al., 1994, 1997, 1998a,b,c, 2001, 2002a,b, 2004; Scorza et al., 1994, 1998, 2001a,b, 2007; Scorza and Ravelonandro, 2006; Zagrai et al., 2007, 2008). Current efforts with C5 ('HoneySweet') are focused on the deregulation of this cultivar (Scorza et al., 2007) in order to make it available to breeders and growers who face the serious threat of PPV.

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The hypersensitivity resistance of european plum to the Plum pox virus and its potential impact on the epidemiology of the virus

Neumüller, M.[1]; Hartmann, W.[2]; Petruschke, M.[3]; Treutter, D.[1]
[1] Technische Universität München, Center of Life and Food Science Weihenstephan, Unit of Fruit Science, Dürnast 2, D-85350 Freising, Germany
[2] University of Hohenheim, Institute of Special Crop Cultivation and Crop Physiology (370), Unit of Fruit Science, Emil-Wolff-Straße 25, D-70599 Stuttgart, Germany
[3] Landwirtschaftliches Technologiezentrum Augustenberg, Außenstelle Stuttgart, Reinsburgstr. 107, D-70197 Stuttgart, Germany

Abstract

Since the detection of the Plum pox virus (PPV) different strategies for Sharka containment were developed. One of the most important one is the breeding of resistant cultivars. Other than in *Prunus persica* and in *Prunus armeniaca*, in *Prunus domestica* a type of natural resistance was detected which seems to be able to prevent the spread of PPV both over long and short distances. Therefore, this type of Sharka resistance which is based on a hypersensitive response and has been stable for more than 20 years is suggested to have the most beneficial impact on the epidemiology of the virus compared to other mechanisms of resistance or tolerance.

Keywords: Sharka containment, *Prunus domestica*, plum breeding

Introduction

Already in 1935, Atanasoff stated that it should be possible to obtain cultivars of woody hosts of *Plum pox virus* (PPV) such as *Prunus domestica*, *P. persica* and *P. armeniaca* which are tolerant or resistant to the virus. In the meantime, great efforts were undertaken worldwide in developing strategies to reduce the impact of the Sharka disease on stone fruit production. Following the terminology introduced by Cooper and Jones (1983), both tolerance and resistance can reduce the economic effect of the virus disease.

During the second half of the 20th century, extensive breeding programs on European plum were established to obtain cultivars resistant and/or tolerant to the disease. The degree of resistance can only be determined by analyzing the virus titer in the plant tissue whereas the degree of tolerance can be defined by rating the visible symptoms and yield reduction of infected versus non-infected trees. Methods for determining the viral concentration have only been available since the development of ELISA tests but it took years after the first report (Dounin and Minoiu, 1968) until these and other methods (e. g. methods based on the detection of viral nucleotide sequence such as quantitative real time PCR (Olmos et al., 2006)) were made generally available. Therefore, during the first decades after detection of PPV, breeding strategies focused on developing tolerant varieties which only show mild symptoms but get infected by PPV. On the one hand, their use allowed the production of European plum to be maintained in eastern and central European countries which are strongly affected by PPV. On the other hand, their use contributed to a large extent to the spread of PPV over long distances via latently infected plant material which resulted in the current situation that PPV is present in all continents.

Breeding for resistance to PPV follows two strategies: Pathogen derived resistance by creation of genetically modified organisms and natural resistance obtained and transferred by cross pollination. Pathogen derived resistance uses viral genomic sequences which are introduced into the plum genome. Double-stranded (ds) replication structures of the viral genome, oder viral RNA transcribed by RDR6 to ds RNA triggers a defense mechanism called RNA interference which rersults in the sequence-specific degradation of viral RNA und thus in resistance for the respective virus. It was shown that the resistance of the transgenic 'C5'-hybrid is based on that phenomenon (Ravelonandro et al., 1993; Scorza et al., 1994; Ravelonandro et al., 1998; Hily et al., 2004b). Many working groups are trying to create transgenic PPV resistant *Prunus* genotypes based on the RNA-silencing mechanism. In recent years, PPV-specific Hairpin RNA constructs have been used for triggering the silencing mechanism in annual and woody PPV hosts (Pandolfini et al., 2003; Hily et al., 2007; Scorza, 2007; Tian et al., 2008). However, the degree of resistance of transgene plants varies. 'C5'-hybrid seems to withstand natural aphid inoculations in the field, but when inoculated with budsticks or in the case of natural infections of the non-transgenic rootstock, PPV symptoms could be detected in leaves, and the presence of the virus verified (Hily et al., 2004a; Malinowski et al., 2004; Malinowski et al., 2006). Thus, this kind of resistance allows to decrease the spread of PPV over short distances (by aphids) but not over long distances by latently infected plant material.

Natural Sharka resistance was found in *P. domestica* genotypes which show a hypersensitive response to PPV in the so-called 'K4'-hybrid (Kegler et al., 1983; Kegler et al., 1985; Bivol et al., 1987; Kegler et al., 1991; Kegler and Hartmann, 1998; Grüntzig et al., 2001; Kegler et al., 2001). In a twelve year long trial, trees of 'K4' remained free from PPV in the field (Kegler et al., 2002). However, the kind of hypersensitivity resistance found by these authors was shown to be overcome by a specific isolate called PPV-CG. Later, a second genetic source of hypersensitivity resistance was detected in the variety 'Jojo' (Hartmann, 1998). It was shown that this genotype elicited the hypersensitive response also against the PPV-GC isolate. Up to now, no isolate was found to break the hypersensitivity resistance of 'Jojo' and its hypersensitive relatives (Kegler et al., 2001; Neumüller et al., 2006). Its phenotypic and genetic background was described (Neumüller and Hartmann, 2008; Hartmann and Neumüller, 2009). This study deals with assessing the epidemiological impact of hypersensitivity resistance based on a long term experimental study in the field and on the synopsis of long term observations in plum orchards throughout Germany under natural inoculation conditions.

Material and methods

In 1989, eight one year old trees of *Prunus domestica* 'Jojo' grafted onto the rootstock 'GF 655/2' were planted into an experimental plot at Weil der Stadt, Germany. The trees were surrounded by PPV infected European plum trees of susceptible genotypes. Four of the trees were twice inoculated with a PPV-D isolate present in orchards in Southwest-Germany by chip budding (in 1990 and in 2003). Each time, four inoculation chips were grafted onto young shoots of the plants. The other four trees were not artificially inoculated. The natural infection pressure by aphids is high in the experimental orchard. Sensitive cultivars such as 'Common Prune' got infected not later than in the third year.

During the first five years annually, afterwards biannually, the presence of PPV was tested by ELISA (using 5B-IVIA antibodies) and RT-PCR (using the protocol given by Wetzel et al. (1991)) methods (EPPO, 2004) using leaves collected in June. Visual inspections for Sharka symptoms took place annually in June. In 2003, the 'GF 655/2'-rootstocks of the four 'Jojo' trees which were artificially inoculated with PPV in the second year were inoculated by chip budding by grafting suckers. Leaves of the rootstock suckers were taken in 2004 and 2006 for testing by ELISA and RT-PCR. In spring 2004, four budsticks of each 'Jojo' tree were taken for inoculation of indicator plants *Prunus tomentosa* which were observed for two years after grafting.

Results

No PPV symptoms could be detected on the leaves and fruits of 'Jojo' trees. No leaf sample tested positivel either with ELISA or RT-PCR-test during the whole period. In 80 % of the trees of PPV susceptible culitvars surrounding the 'Jojo' trees PPV could be detected. None of the *Prunus tomentosa* trees grafted with 'Jojo' budsticks developed PPV symptoms. PPV was detected in the susceptible rootstocks of all 'Jojo' trees growing at Weil der Stadt both in 2004 and 2006. This means that the rootstocks which were not artificially inoculated got infected by aphids landing on suckers.

During a period of 20 years, the 'Jojo' variety could neither naturally nor artificially be infected in the field by PPV. This is longer than today's plum orchards are cultivated. Trees with hypersensitivity resistance were the only trees which remained free from PPV. Quantitatively resistant genotypes such as 'Cacanska najbolja' got infected but displayed only a few symptoms.

Several hundred hectares of 'Jojo' orchards are grown in Europe the,oldest being 15 years old. In none of these orchards, could PPV infections of 'Jojo' trees be observed. Even if the rootstock got infected by PPV, the trees did not die off. The few tree losses which could be observed were usually due to *Pseudomonas* infections on the stem.

Discussion

The use of genotypes with a sufficiently high degree of hypersensitivity resistance against PPV such as 'Jojo' interrupts the cycle of PPV spread both over short and long distances. Hypersensitive plants do not get infected by aphid inoculation. As shown by Kegler (1994) and confirmed by Hartmann and Petruschke (2000), hypersensitive genotypes die off a few weeks after being grafted onto PPV infected rootstocks. Therefore, only trees free from PPV can leave the nursery. Up to now, the hypersensitivity resistance is the only resistance mechanism which is able to prevent the spread of PPV when the resistant genotype is grafted onto a latently infected rootstock.

Interestingly, there is a difference in the behavior of 'Jojo' trees growing on PPV infected rootstocks: If a budstick of 'Jojo' is grafted onto a PPV infected rootstock, the 'Jojo' scion will die off within a few days or weeks after budbreak. If the tree is older and the rootstock gets infected by inoculation of suckers the 'Jojo' trees grow normally without any symptoms of hypersensitivity. PPV cannot be detected neither by ELISA, RT-PCR nor biological indexing. At least for

young trees it is known that the transport of PPV through a hypersensitive interstem between the susceptible rootstock to the susceptible scion part is possible. This means that the virus is able to move through the vessel system (phloem and maybe xylem) of hypersensitive genotypes, but it is not replicating in the hypersensitive vessel tissue. We do not yet know whether this movement is able in full-grown trees as well. However, there is, up to now and with currently available detection methods, no proof of the presence of PPV in vessels of hypersensitive genotypes.

The different reaction of young and old trees of hypersensitive genotypes when growing on PPV infected rootstocks remains somehow mysterious. From the practical point of view it is a good situation: 'Jojo' trees are *per se* free from PPV when being planted in the field. If some years later, the rootstock of the 'Jojo' trees gets infected by aphids the tree remains unaffected and does not suffer from any kind of hypersensitive response. The grower does not lose any trees.

As already mentioned by Kegler et al. (2002) hypersensitive genotypes can stand the natural PPV inoculation pressure but not excessive PPV inoculation pressure after artificial inoculation. As shown by Hartmann and Petruschke (2002) the ratio between tree size and amount of inoculums can influence the testing results, e. g. if a young 'Jojo' tree gets inoculated by a high number of PPV infected chips or budsticks the 'Jojo' tree can die off partially or completely. With a full-grown tree, this is no longer possible. The influence of the inoculation method such as time of inoculation and amount of inoculums plays an important role in testing for PPV resistance as mentioned by Neumüller (2005) and Rubio et al. (2009).

Via latently infected plant material, PPV spreads over long distances. The use of scion and rootstock varieties which do not express clear PPV symptoms but which can get infected by PPV (even if the viral concentration is low) must be avoided in the future. This is not only true for *Prunus domestica* but for all other PPV hosts. If, in a species, no completely resistant genotype is available, it is better to use varieties which clearly show symptoms on the leaf because infected trees can easily be detected and removed. Only in regions where PPV is prevalent can the use of varieties, which show no clear symptoms but can contain the virus, be justified, but nurseries will have difficulties to keep their plantations free from PPV infections. The advantage of hypersensitivity resistance over all other known resistance mechanisms is that it withstands natural inoculation pressure (aphids) completely, i. e. the plant remains healthy. It dies off only in the case of high inoculation pressure which can just be the case under artificial inoculation conditions. The resistance has been stable for 20 years. Thus, the use of hypersensitive genotypes is the most effective method to prevent the spread of PPV over long and short distances and may become an important tool for influencing the epidemiology of PPV. In the future, the use of rootstocks with hypersensitivity resistance and interspecific hybridization could make the resistance trait available for other PPV hosts such as Japanese plum, apricot and peach (Neumüller et al., 2009).

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Natural deletion is not unique in the coat protein (CP) of recombinant *Plum pox virus* (PPV) isolates in Hungary

Szathmáry, E.; Palkovics, L.
Corvinus University of Budapest, Faculty of Horticultural Science, Department of Plant Pathology, H-1118 Budapest, Ménesi Road 44., Hungary

Abstract

Three *Plum pox virus* (PPV) isolates (Soskut1, Godollo2, Szigetcsep1), collected from apricot (*Prunus armeniaca* L.) trees in Hungary in 2008, were characterized in this study by sequence analysis of the RT-PCR amplified 3' part of the viral genome spanning the 3' part of the NIb gene the complete CP gene and the 3'UTR [3'NIb–CP–3'UTR] and also by restriction analysis of the PCR products derived from the 3' part of the P3, the complete 6K1 and the 5' end of the CI genes [3'P3–6K1–5'CI]. Phylogenetic analysis of the 3'NIb–5'CP region showed that one isolate (Godollo2) could be classified as a member of the PPV-Rec group, while the other two (Soskut1 and Szigetcsep1) belonged to PPV-D isolates. In the case of the recombinant Godollo2 isolate a 33-nucleotide (nt) in frame natural deletion was detected in the 5' part of the CP gene during the sequence analysis of the cDNA fragment corresponding to the 3'NIb–CP–3'UTR region. Currently we have reported on another Hungarian PPV-Rec isolate (PPV-B1298) collected from plum that also had a shorter CP gene bearing a much larger 135-nt in frame natural deletion at a similar position to that of the Godollo2. The PPV-D type Soskut1 isolate showed an atypical restriction pattern in the 3'P3–6K1–5'CI region using *Eco*RI and *Dde*I endonucleases, respectively. Nucleotide sequence analysis of this region indicated that its unusual pattern is as a result of a point mutation affecting the *EcoRI* restriction site.

Keywords: *Plum pox virus*, PPV, natural CP deletion mutant, *Eco*RI restriction site

Introduction

Plum pox virus (PPV, the genus *Potyvirus*, the family *Potyviridae*) is the most important viral pathogen of *Prunus* trees in Europe. It causes serious symptoms and substantial yield losses (Németh, 1986). It was first recorded in Bulgaria around 1915 (Atanasoff, 1932), and since then it has rapidly spread throughout Europe and nowadays is present almost all over the world (Wetzel et al., 1991; Roy and Smith, 1994; Thakur et al., 1994, Milius, 1999; Thompson et al., 2001; Dal Zotto et al., 2006).

PPV has a single-stranded plus-sense genomic RNA, about 10 kilobases long. The RNA genome consists of a single open reading frame encoding a large polyprotein that is subsequently processed into functional viral proteins by viral proteinases (Urcuqui-Inchima et al., 2001). Recently a new potyviral protein has been discovered which is not part of the polyprotein (Chung et al., 2008). The RNA genome carries a virus encoded protein (the viral protein genome-linked, VPg) covalently bound to the 5' terminus and a poly(A) tail at the 3' end (Urcuqui-Inchima et al., 2001).

PPV has been classified into seven groups of isolates PPV-M, PPV-D, PPV-Rec, PPV-EA, PPV-C, PPV-W and PPV-T according to their serological and molecular features (Candresse et al., 1998; Glasa et al., 2004; James et al., 2003; Serçe et al., 2009). PPV-Rec group contains viral isolates that emerged from a natural recombination between PPV-D and PPV-M isolates with a recombination breakpoint located in the nuclear inclusion b (NIb) gene. Later on another recombination point was detected in the third protein (P3) gene (Glasa et al., 2004). A more recently identified group of closely related PPV isolates was PPV-T which is characterized by a unique recombination point in the helper component protease (HC-Pro) gene around nucleotide position 1566 (Serçe et al., 2009).

The present study reported on two PPV isolates possessing unusual molecular characteristics. One of them (Godollo2) has an 11-amino acid (aa) deletion in the N-terminal (Nt) part of the coat protein (CP) and the other (Soskut1) possesses an atypical restriction typing characteristic in the genomic region corresponding to the 3' part of the P3, the complete 6K1 and the 5' part of the cylindrical inclusion (CI) genes [3'P3–6K1–5'CI].

Material and methods

Leaf samples showing typical symptoms of PPV infection were collected from different apricot (*Prunus armeniaca* L.) varieties from three different locations in Hungary (Sóskút, Gödöllő and Szigetcsép) in 2008. Total nucleic acids were extracted from systemically infected leaves by the method of White and Kaper (1989). Partial molecular characterization was done by RT-PCR for amplification of (i) the 3' part of the NIb gene, the complete CP gene and the 3' untranslated region (3'UTR) [3'NIb–CP–3'UTR] using primers PolyT2 and Poty7941 (Salamon and Palkovics,

2005) and (ii) the 3'P3–6K1–5'CI region using the PCI/PP3 set of primers (Glasa et al., 2002). Purified PCR products were sequenced after cloning to pGEM-T Easy vector and the PCI/PP3 amplicons were subjected to restriction analysis using *EcoR*I and *Dde*I D-type sequence specific endonucleases (Glasa et al., 2002).

Obtained nucleotide (nt) and deduced amino acid sequences were compared to other PPV sequences available in GenBank database overlapping the genomic region examined. Sequence comparisons and phylogenetic analysis were performed using the neighbor-joining method of the MEGA 3.1 software with 1000 bootstrap replicates (Kumar et al., 2004).

Results

Three PPV isolates were investigated in this study (Table 1). CPs of all three isolates started with alanine and the DAG aa motif associated with aphid transmission (Atreya et al., 1990) found in all sequences at the Nt end of the protein. CPs of the Soskut1 and the Szigetcsep1 isolates were identical in size (330-aa residues), while the CP of the Godollo2 isolate was shorter. In the case of the Godollo2 in frame natural deletion was detected in the CP gene during the sequence analysis of the RT-PCR amplified cDNA fragment corresponding to the 3'NIb–CP–3'UTR region. The Godollo2 isolate had a 33-nt deletion at the 5' end of the CP gene, which corresponds to an 11-aa deletion in the Nt region of the CP downstream to the DAG motif (Figure 1).

Tab. 1 Original hosts and specific molecular groups of PPV isolates investigated in this study and their GenBank accession numbers

Isolates	Localities	Original hosts	Groups	Accession numbers
PPV-Soskut1	Sóskút	*P. armeniaca* 'Bergeron'	PPV-D	FN179152[a], FN179155[b]
PPV-Godollo2	Gödöllő	*P. armeniaca*	PPV-Rec	FN179153[a]
PPV-Szigetcsep1	Szigetcsép	*P. armeniaca* hybrid 10/7	PPV-D	FN179154[a]

a: 3'NIb–CP–3'UTR, b: 3'P3–6K1–5'CI

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            1                                                                92
Soskut1     ADEREDEEEVDAGKPIVVTAPAATSPILQPPPVIQPAPRATAPMFNPIFTPATTQPATTPVSRVSGPQLQTFGTHGNEDASPSNSNALVTTN
Szigetcsep1 ADEREDEEEVDAGKPIVVTAPAATSPILQPPPVIQPAPRTTAPMFNPIFTPATTQPATKPVPRVSGPQLQTFGTYGNEDASPSNSNALVNTN
Godollo2    ADEKEDDEEVDAGKPTVVTAPAATVATTQPAPVIQPAIQTTTPMFNPIFTPATTQPAT----------QSFGVYGNEGASPSTSNTLVNTG
B1298       ADEKEDDEEVDAGKPPVVTAPAATVATTQPAPVIQPAIQTTT------------------------------------------LVNTG
NAT         ADEREDEEEVDA--------------LQPPPVIQPAPRTTAPMLNPIFTPATTQPATKPVSQVSGPQLQTFGTYSHEDASPSNSNALVNTN
SH          ADEREDEEEVDA--------------ILQPPPVIQPAPRTTAPMFNPIFTPATTQPATKPVSQVSGPQLQTFGTYGNEDASPSNSNALVNTN
KAZ         ADEREDEEEVDAGKPIVVTAPAATSPILQP--VIQPAPRTTAPMFNPIFTPATTQPATKPVSQVSGPQLQTFGTHGNEDASPSNSNALVNTN
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Fig. 1 Multiple amino acid alignment of the Nt CP region of PPV isolates. The DAG motif is underlined. Soskut1 (Acc. No.: FN179152), Szigetcsep1 (Acc. No.: FN179154), Godollo2 (Acc. No.: FN179153), B1298 (Acc. No.: AM184114), NAT (Acc. No.: D13751), SH (Acc. No.: X81073), KAZ (Acc. No.: AY591253).

Nucleotide and deduced amino acid sequences of the CP regions of Soskut1 and Szigetcsep1 Hungarian apricot isolates were most identical to PPV-D isolates, while Godollo2 showed the highest sequence similarities to isolates having M-type CP region (members of the PPV-M and the PPV-Rec groups), and it was the most similar to PPV-Rec isolates. It is known that recombination causes changes in the sequence of the CP, or in the CP gene, but the 3' recombination point is located upstream to the CP coding region, at the 3' end of the NIb gene (Glasa et al., 2004).

To examine the recombinant nature of these three Hungarian PPV isolates phylogenetic analysis was performed using nt sequence data corresponding to the 3' end of the NIb and the 5' end of the CP genes [3'NIb–5'CP, nt 8050–8902]. The phylogenetic tree clearly showed the clustering of the Godollo2 isolate with some previously characterized recombinant (PPV-Rec) isolates, while the Soskut1 and the Szigetcsep1 clustered with PPV-D isolates (Figure 2).

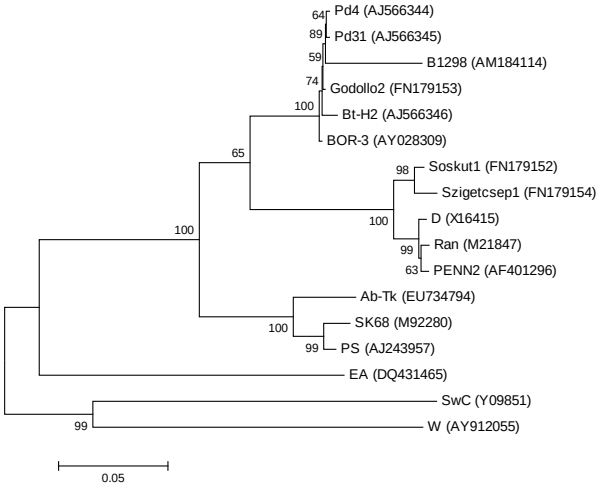

Fig. 2 Phylogenetic tree of PPV isolates based on nucleotide sequence data corresponding to the 3'NIb–5'CP (nt 8050–8902) genomic region. Bootstrap values are presented next to tree nodes. The scale bar represents 0.05 substitutions per site. Accession number of PPV isolate is in brackets after the name of isolate.

As an additional step for molecular characterization of the three Hungarian PPV isolates the 3'P3–6K1–5'CI (nt 2976–3696) region were also investigated. The RT-PCR amplified products derived from this genomic region were subjected to restriction analysis using *Eco*RI and *Dde*I endonucleases, respectively, in order to chracterize this region. These restriction enzymes cleave cDNA fragments amplified from isolates having a D-type genome in the analysed region (members of PPV-D and PPV-Rec groups), while do not recognize cDNAs derived from isolates having an M-type genome (PPV-M isolates) in the 3'P3–6K1–5'CI region (Glasa et al., 2002). The *Dde*I enzyme could cleave cDNA fragments amplified from all three Hungarian PPV isolates, while *Eco*RI could only cut PCR products derived from the PPV-Rec type Godollo2 and the PPV-D type Szigetcsep1, but could not cleave cDNA obtained from the PPV-D type Soskut1 isolate similar to the control PPV-M type sequence. Thus, the RFLP analyses using D-type sequence-specific enzymes resulted in an atypical restriction pattern in the case of the Soskut1 isolate. The PCR fragment could be recognized only by *Dde*I, but not by *Eco*RI, although this isolate was determined as PPV-D type according to the 3'NIb–5'CP region. Such unusual typing behaviour could arise from point mutations affecting the recognition site but may also indicate a possible recombination event between PPV-D and PPV-M isolates. Sequence analysis revealed that the sixth base of the *Eco*RI site (3410 nt position, GAATT**C**>GAATT**T**) has been changed from C to T. Phylogenetic analysis performed using nucleotide sequences of PPV isolates corresponding to the 3'P3–6K1–5'CI region showed that despite the lack of the *Eco*RI restriction site the Soskut1 isolate has a D-type genome in the 3'P3–6K1–5'CI region (data not shown).

Discussion

On the basis of the sequence similarities of the CP region and phylogenetic analyses generated using nucleotide sequences corresponding to the 3'NIb–5'CP (nt 8050–8902) genomic region, one isolate (Godollo2) out of the three belonged to the PPV-Rec group, while the remaining two (Soskut1, Szigetcsep1) could be classified as members of the PPV-D group.

The atypical typing property of the Soskut1 isolate in the 3'P3–6K1–5'CI region resulted from a point mutation in the *Eco*RI cleavage site as compared to D-type sequences, the unusual restriction pattern was not as a consequence of a recombination event in this region. Previously we have reported on a Bulgarian isolate (PPV-Troy6) collected from plum that showed also an abnormal typing property in the 3'P3–6K1–5'CI region (Szathmáry et al., 2009b). In that case the PCR product could only be cleaved by *Eco*RI, but not by *Dde*I, although this isolate was determined as a member of PPV-Rec group according to the 3'NIb–5'CP region. In the case of the PPV-Troy6 a point mutation (A3102 to C3102, CTN**A**G>CTN**C**G) was also responsible for the lack of the *Dde*I cleavage site. Our earlier and present study indicates

some limitation of restriction enzyme mapping for proper classification of PPV isolates. The accurate identification of specific PPV isolate groups can only be achieve using different methods (restriction analysis, sequence analysis) targeting the same genomic region or by parallel investigation of different genomic regions.

In the case of the Godollo2 Hungarian PPV-Rec isolate in frame deletion was detected in the CP gene during the sequence analysis of the RT-PCR amplified cDNA fragment corresponding to this region. Currently we have reported on another Hungarian PPV-Rec isolate (PPV-B1298) collected from plum bearing a much larger, 135-nt (45-aa) in frame natural deletion at a similar position to that of Godollo2 (Szathmáry et al., 2009a). There are only three other known examples for natural CP deletion mutant PPV isolates (PPV-NAT, PPV-SH, PPV-KAZ) (Maiss et al., 1989; Deborré et al., 1995; Spiegel et al., 2004). In all cases, deletions were located in the N-terminal (Nt), hypervariable region of the CP similar to Godollo2. It is known that deletions in the CPs of PPV-NAT and PPV-SH isolates affect the DAG motif, while this motif in the CPs of PPV-B1298 and PPV-Godollo2 isolates are not affected by the deletions.

Different forms of ELISA technique are widely used in almost all quarantine laboratories worldwide for the detection of plant viruses for decades:ELISA based on the detection of the viral CP using antibodies produced usually against the Nt region. Thus, a deletion affecting the Nt domain should have a significant role in virus detection as we currently showed in the case of PPV-B1298 isolate (Szathmáry et al., 2009a). Occurrence of deletions in the highly immunogenic regions could result in a failure of detection in spite of using well-characterized and widely used serological diagnostic reagents for plant virus identification, which could be dangerous with regard to the safe detection of PPV infection.

Our present study and previous data suggest that the presence of a natural deletion in the N-terminal part of the CP in the Hungarian recombinant PPV population is not unique.

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Tracking *Plum pox virus* in Chile throughout the year by three different methods and molecular characterization of Chilean isolates

Fiore, N.[1]; Araya, C.[1]; Zamorano, A.[1]; González, F.[1]; Mora, R.[1]; Sánchez-Navarro, J.[2]; Pallás, V.[2]; Rosales, I.M.[1]
[1] Fundación Facultad de Ciencias Agronómicas de la Universidad de Chile, Unidad de Biotecnología, Instituto de Investigaciones Agropecuarias (INIA), Santiago, Chile. Av. Santa Rosa 11610, La Pintana, Santiago, Chile.
[2] Instituto de Biología Molecular y Celular de Plantas, CSIC-UPV, CPI 8E, C/Ingeniero Fausto Elio s/n, 46022 Valencia, España.

Abstract

During 2007, a survey was performed to detect and identify *Plum pox virus* (PPV) in Chilean stone fruit commercial orchards. A total of 1396 trees were analyzed and 45 (3.22 %) of them resulted positive. A fragment of 467 bp, corresponding to the replicase-coat protein (Nib-CP) region from the virus genome, was amplified and the the sequences obtained permitted the characterization of all isolates as PPV-D type, confirming that, so far, this is the only serotype present in Chile. To optimize virus detection, 27 PPV-positive trees were selected and sampled monthly from December 2006 until December 2007, collecting plant tissues available at the time of sampling (leaves, cuttings, buds and flowers). Each sample was analyzed by three different techniques: DASI-ELISA, RT-PCR and non-isotopic molecular hybridization (MH). The results showed that RT-PCR was more sensitive for detection in all months excepting January 2007, when the three techniques showed the same sensitivity. In general, MH showed a better sensitivity compared with DASI-ELISA. The best plant materials for analysis were: leaves, in February, March, September and October; phloem from cuttings in June; buds in July, and flowers in August.

Keywords: PPV, detection, phylogeny, sampling.

Introduction

Plum pox virus (PPV) has been previously reported in Chile (Acuña, 1993), and its presence is related exclusively with *Dideron* strain (Reyes et al., 2003). Since 1994, the Chilean phytosanitary service (Servicio Agrícola y Ganadero - SAG) has been forcing nurseries to analyze propagating material in order to avoid virus dissemination, which mainly affect apricots, plums and peaches, by decreasing marketable production. PPV detection techniques are based on the biological indexing, serological tests and PCR-based methods (EPPO, 2004). The selected period for PPV analysis has been concentrated in spring since good results have been obtained with the different detection techniques (Olmos et al., 2007).

However, the possibility to perform several surveys throughout the year could significantly improve the certification and clean-stock programs. Limits to detection are posed by the erratic distribution of viruses within the plants, the type and age of tissue used for analysis, the season, and the environmental factors, which influence virus concentration. A study was conducted to evaluate how some of these factors influence the virus detection. Ability to determine virus presence by DASI-ELISA, non-isotopic molecular hybridization (MH) and RT-PCR was investigated on a monthly basis for different kind of tissues throughout 13 months, to establish which tissue and method is the best to use at a certain time of the year. In addition, a short survey was performed in the most important stone fruit growing zone in the country, in order to characterize PPV isolates found.

Materials and methods

During the southern hemisphere spring (September and October) of 2007, several Chilean stone fruit commercial orchards of different sizes were visited to assess their sanitary status concerning PPV in the most important fruit growing regions: Valparaiso (V), Metropolitana de Santiago (RM) and Libertador General Bernardo O'Higgins (VI). A total of 1396 trees (180, 606 and 610 from V, RM and VI regions, respectively) were sampled during the whole survey period, their geographical position was established by the GPS system and the precise coordinates of sampled plants within the orchards were recorded to facilitate identification if further sampling was required. Three samples were randomly collected in each orchard. Leaf samples (20 per tree) were collected and transferred by a tissue printing procedure (Más and Pallás, 1995; Amari et al., 2001) onto nylon membranes. PPV detection was carried out by non-isotopic molecular hybridization (MH) using a riboprobe labeled with digoxigenin and designed to hybridize the region coding for coat protein (Herranz et al., 2005). Approximately 15 % of negative and all positive samples obtained by tissue printing were tested again by RT-PCR. Total nucleic acids (TNA) extraction was carried out using a silica capture method (MacKenzie et al., 1997; Malinovski, 1997). Fifteen µl aliquots of TNA were primed with using DNA

random hexanucleotides (Roche, Switzerland) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV-RT, Promega, USA). DNA amplification was done using Invitrogen Taq DNA polymerase (Brazil) and the PPV universal primers P1/P2 (Wetzel et al., 1991).

All positive samples were analyzed to determine the strain, using specific primers for PPV-M, PPV-D, PPV-Rec, PPV-C and PPV-EA (Candresse et al., 1998; Szemes et al., 2001; Šubr et al., 2004). A fragment of 467 bp corresponding to the replicase-coat protein (Nib-CP) region from the virus genome was amplified from positive samples by using the primers P4b and P3D (Candresse et al., 1998). Fragments were directly sequenced in both directions by the dideoxynucleotide chain-termination method in an automated sequencer (ABI 3100 Genetic Analyzer; Perkin Elmer Applied Biosystem) using the primers employed for the amplification. The sequences were then aligned with the BLAST-N tool for local alignment of nucleotide sequences (version Blast N 2.2.12). The publicly available sequences of PPV strains were downloaded from TreeBase (University of Buffalo, NY, USA) and compared with the Chilean ones. A total of 30 sequences were aligned (Table 1) using the BioEdit and CLUSTAL X programs (Thompson et al., 1997; Hall, 1999); then a phylogenetic tree was constructed using the maximal parsimony algorithm of MEGA version 2.1 (Kumar et al., 2001). The statistical significance of the nodes was conferred by bootstrap analysis of 10000 pseudoreplicates.

Tab. 1. List of the isolates obtained in this work and others used as reference. [a]Serbia and Montenegro. [b]Bosnia and Herzegovina. [c] Sweet cherry.

Sequence code	Source	Origin	Strain	Accession number
CL 812 Pch	Peach cv. Loadell	Chile	D	This work
CL 803 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 811 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 816 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 790 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 795 Apr	Apricot cv. Castelbrite	Chile	D	This work
CL 814 Plm	Plum cv. D'Agen	Chile	D	This work
CL 885 Plm	Plum cv. Black Beaut	Chile	D	This work
CL 65 Ne	Nectarine cv. unknown	Chile	D	This work
CL 64 Pch	Peach cv.unknown	Chile	D	This work
CL 70 Pch	Peach cv.unknown	Chile	D	This work
CL 68 Pch	Peach cv.unknown	Chile	D	This work
CL 63 Pch	Peach cv. Nemaguard	Chile	D	This work
Cdn1 Plm	Plum cv. Italian plum	Canada	D	AY953261
Chile116 Plm	Plum cv.unknown	Chile	D	AF440743
NAT	Prunus sp.	Germany	D	NC_001445
Chile20 Pch	Peach cv. GF677	Chile	D	AF440745
Chile114 Apr	Apricot cv. unknown	Chile	D	AF440742
Chile31 Pch	Peach cv. unknown	Chile	D	AF440746
Chile12 Plm	Plum cv.Marianna 2624	Chile	D	AF440744
Chile112 Pch	Peach cv. unknown	Chile	D	AF440741
Abricotier Turquie-M	Apricot cv. unknown	Turquie	M	AY677115
PS-M	Prunus sp.	S. and M.[a]	M	AJ243957
Bos64Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749998
Bos257Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749997
Bos148Pl-Rec Plm	Plum cv. unknown	B. and H.[b]	Rec	AJ749996
Pakistan-Rec Apr	Apricot cv unknown	Pakistan	Rec	DQ422148
Pd31-M Plm	Plum cv. unknown	Hungary	M	AJ566345
PPV-El Amar Apr	Apricot cv.unknown	Egypt	El Amar	DQ431465
PPV-C SwC	Cherry[c] cv. unknown	Italy	C	Y09851

To optimize virus detection, 27 PPV-positive trees were selected in the Metropolitana region: 16 apricots cv. Castelbrite, 1 peach cv. Loadell, 1 peach cv. Jungerman and 2 plums cv. D'Agen from the locality of Calera de Tango; 7 plums cv. Marianna 2624 from the locality of La Pintana. All the plants were positive to PPV by RT-PCR but only one (apricot cv. Castelbrite) presented symptoms at time of the study. The sampling period was from December 2006 until December 2007, collecting plant tissues available at each sampling time: leaves were sampled from December 2006 to April 2007 and from September 2007 to December 2007; cuttings from April to August 2007; buds from May to August 2007 and flowers only in August. Each sample was analyzed by RT-PCR (with P1/P2 primers) and MH as described, and DASI-ELISA (EPPO, 2004) using the kit "Realisa Reforzado" (REAL, Spain).

Results

A total of 1396 trees were analyzed by tissue printing of which 24 were positive to PPV (1.72 %). All positive plants were found in the VI (17 out of 610) and Metropolitana (7 out of 606) regions, which have the highest number of hectares planted to stone fruits. To evaluate the reliability of these results all positive samples plus 15 % of MH-negative ones were analyzed by RT-PCR. A total of 45 positive samples were obtained, including the 24 MH-positive samples, representing 3.22 % of the plants analyzed by both techniques. Regarding the three production areas the percentage of infected plants varied between 4.4 (27 out of 610), 2.6 (16 out of 606) and 1.1 (2 out of 180) for the VI, RM and V regions, respectively. The analysis of positive samples using specific primers revealed that all PPV isolates were classified as PPV-D variants. To further characterize the PPV variant present in the infected samples, we performed a phylogenetic analysis using representative sequences of the different PPV isolates present in the database and the nucleotide sequence of the PPV-D specific 467 bp amplified fragments (Figure 1).

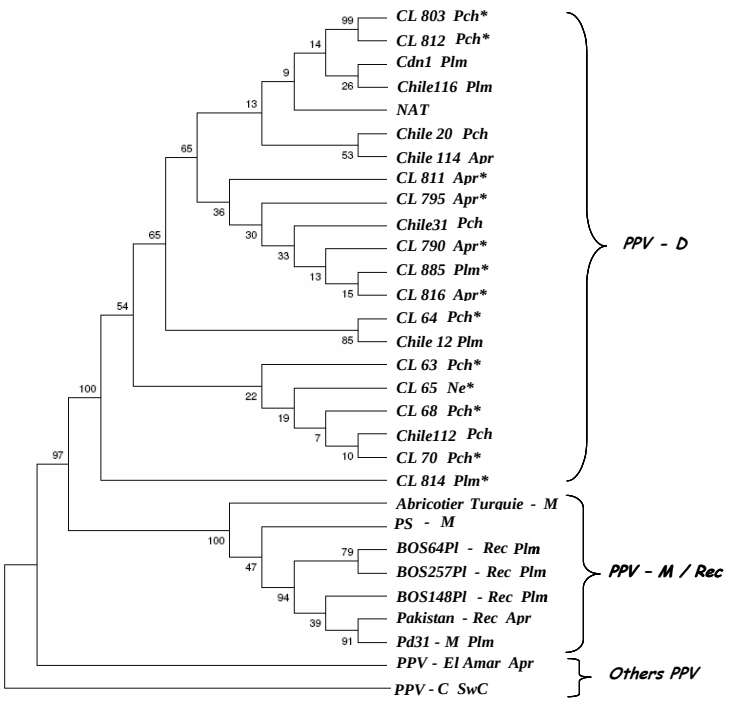

Fig. 1 Phylogenetic tree obtained with nucleotide sequences of the genomic region Nib-CP (467 bp). *Some of Chilean PPV isolates found during the survey. All the Chilean isolates clustered together with the PPV-D variant, confirming the results obtained with RT-PCR using strain specific primers.

Next, we decided to analyze different aspects affecting the detection of PPV such as the period of the year, which plant tissue to select and the detection method used. For this purpose we selected 27 PPV-infected trees (obtained from the previous surveys) that were subjected to a monthly periodical analysis during one year and using three different detection techniques: DASI-ELISA, MH and RT-PCR (Table 2).

Tab. 2 Comparison of techniques for *Plum pox virus* detection. [a]Best materials and results; [b]Second best materials and results; (+/t): Number of positives versus number of tested samples.

Month	Type of analyzed samples				Results (+/t) ELISA	MH	RT-PCR
Dec '06	Leaves[a]				1/27	3/27	16/27
Jan '07	Leaves				1/27	1/27	1/27
Feb '07	Leaves[a]				1/27	1/27	23/27
Mar '07	Leaves[a]				1/27	1/27	22/27
Apr '07	Leaves[a]	Phloem			1/27	1/27	9/27
May '07		Phloem[b]	Buds[a]		1/27	1/27	4/27
Jun '07		Phloem[a]	Buds[b]		2/27	2/27	19/27
Jul '07		Phloem	Buds[a]		1/27	6/27	25/27
Aug '07		Phloem[b]	Buds	Flowers[a]	1/27	6/27	26/27
Sep '07	Leaves[a]				1/27	16/27	24/27
Oct '07	Leaves[a]				2/27	5/27	20/27
Nov '07	Leaves[a]				2/27	2/27	16/27
Dec '07	Leaves[a]				2/27	2/27	14/27

The most sensitive method was the RT-PCR, as expected, allowing the detection of 96 % of the infected trees (26 positives out 27 samples in August), followed with the MH and ELISA with the 59.2 % (16 positive out 27 samples in September) and 7.4 % (2 positives out 27 in October), respectively. The best period to analyze PPV varied between the three techniques. For DASI-ELISA we obtained the best results between October and November, meanwhile for MH we observed good results between July and October, September being the best month. In the case of RT-PCR, we observed two different periods: one between July and September with an average detection of 92.6 % and another one between February and March with an average detection of 83.3 %. Only in January, was the sensitivity the same for all techniques, representing 3.7 % of the infected samples. The best plant material for sampling was: leaves in February, March, September and October; phloem scrapings from cuttings in June; buds in July and flowers in August (Table 2).

Discussion

In the present study we have analyzed the prevalence of PPV in the three main stone fruit production areas of Chile. The analysis of 1396 trees by MH revealed a prevalence of 1.72 %, a percentage that increased to 3.22 % when we analyzed the 15 % of MH-negative samples by RT-PCR technique. However, if we only consider the results obtained from the analysis of the 15 % of MH-negative samples by the RT-PCR, the PPV prevalence could reach 10.24 % (21 positive samples out 205). Regarding the three production areas, the prevalence of PPV ranged between 4.4 % in the VI region, followed by the RM and V areas with a prevalence of the 2.6 % and 1.1 %, respectively. In addition, we observed that all characterized PPV isolates were assigned to PPV-D by using specific strain primers or by phylogenetic analysis using the nucleotide sequence obtained from the specific amplified PCR product. These results indicate that, so far, the only PPV variant present in Chile is PPV-D. A similar observation has been reported for other North and South America PPV isolates (Damsteegt et al., 2001; Reyes et al., 2003).

In a second step, we analyzed the critical aspects that could influence routine PPV detection such as the plant tissue selected, the period of the year or the detection technique. For this purpose, we analyzed a collection of 27 PPV infected trees during one year using three different techniques. The direct comparison between the three detection methods revealed that RT-PCR was the most sensitive technique followed by MH and DASI-ELISA, detecting 96, 59.2 and 7.4 % of infected trees. Similar results have been described previously for the detection of other plant viruses, although the differences between MH and ELISA were considerably smaller (Sánchez-Navarro et al., 1998; Myrta et al., 2003; Alfaro et al., 2009). In addition, the different detection percentages obtained with RT-PCR and DASI-ELISA (96 % vs 7.4 %) are not in agreement with the results presented by Olmos et al. (2007) using the same validated ELISA kit and a collection of 205 infected trees (100 % vs 98.5 %). Since both analyses utilized the same antisera, the discrepancy observed could be attributed to the sensitivity obtained for the RT-PCR. Thus, both analyses differed in the RNA extraction procedure in which the silica chemical method (herein) gave a better detection limit for the analysis of stone fruits tissue than the Qiagen procedure (Sánchez-Navarro et al., 2005). However, we can not discard that the discrepancy could be attributed as well to the DASI-ELISA method. Thus, while we observed one period during the year that showed the high percentage of infected plants for both MH and RT-PCR methods corresponding probably to the high titre of the virus, the amount of positive samples detected by DASI-ELISA was always the same in one or two trees. This could be explained by considering that the DASI-ELISA was not sensitive to the accumulation of PPV and thus, some PPV strains would not have been detected. In this sense, we are now analyzing the CP gene of some PPV-infected trees that were negative by DASI-ELISA to look for putative amino acids changes. The results obtained with

RT-PCR, showed we were not able to detect the virus 100 % in infected trees, a fact which could be attributed to the erratic distribution of the virus and the lack of symptoms.

During December 2006 and 2007 the highest temperatures of the year were recorded. It is very probable that this has influenced the bad results of the RT-PCR in January, in which only one sample out of 27 was positive. These results suggested that the use of RT-PCR should be considered for stone fruit nursery routine control of PPV in Chile, taking into account two major sampling windows during the year: the first, from July to September (from the end of winter to early spring); the second with slightly lower sensitivity, during the months of February and March (end of summer). MH is shown as a clear alternative to the serological test for large-scale surveys, but only during September. This study allowed the optimization of PPV detection in Chile.

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Identification of host genes potentially implicated in the *Malus pumila* and 'Candidatus *Phytoplasma mali*' interactions

Aldaghi, M.[1,2]; Massart, S.[2]; Bertaccini, A.[3]; Lepoivre, P.[2]
[1] Jouybar Road (toward Larim), Agricultural and Natural Resources Research Center of Mazandaran, P.O. Box 48175-556, Sari, Iran
[2] Plant Pathology Unit, Gembloux Agricultural University (FUSAGx), Passage des déportés 2, 5030 Gembloux, Belgium
[3] DiSTA, Patologia vegetale, *Alma Mater Studiorum*, Università di Bologna, viale Fanin 42, 40127 Bologna, Italy

Abstract

Two'Candidatus *Phytoplasma mali*' strains (AP and AT), were studied in experimentally infected apple trees to analyze transcriptional profiles during interaction with phytoplasmas. Three groups of sample combinations were employed: healthy - infected, symptomatic - non-symptomatic, and AP-infected - AT-infected sample. The majority of genes were differently expressed between healthy and infected samples. Changes in gene expression involved a wide spectrum of biological functions, including processes of metabolism, cell defence, photosynthesis, transport, transcription, signal transduction and protein synthesis. The possible effect of phytoplasma infection on these processes and their relationships with disease development, symptom appearance and possible plant defence system is discussed.

Keywords: Apple, phytoplasmas, 'Ca. *P. mali*', gene expression, transcriptome.

Introduction

Phytoplasmas are cell wall-less and phloem-restricted plant pathogenic bacteria. They are known to be associated with diseases in several hundred plant species and have been shown to be transmitted in a propagative manner by sap-sucking insect vectors (Aldaghi et al., 2005). Apple proliferation (AP) is one of the most serious phytoplasma diseases of apple trees in Europe: it is caused by 'Candidatus *Phytoplasma mali*', belonging to the apple proliferation group; this disease causes considerable economic losses mainly by decreasing the size and quality of fruits (Frisinghelli et al., 2000).

Little is known about the genes involved in the phytoplasma-plant host interaction. There is only some information about the increase of phenolic compounds and hydrogen peroxide in host plants infected by phytoplasmas (Musetti et al., 2000; 2004; Junqueira et al., 2004). For more than 10 years, scientists worked toward the development and improvement of methods to study gene expression regulation. Transcriptome analysis is a common way of discovering differences in gene expression because regulation of gene activity occurs primarily at a transcription level. Whether particular genes are over- or underexpressed when comparing diseased with normal tissue provides information with respect to the understanding of the mechanisms of the disease. To date, a number of methods have been successfully developed to identify differential gene expression in various biological systems, including DDRT-PCR, cDNA-AFLP and microarray (Frolov et al., 2003; Venkatesh et al., 2005). cDNA-AFLP is a comprehensive transcript profiling methodology (Donson et al., 2002) for genome-wide expression analysis that does not require any prior knowledge of gene sequences. This PCR-based technique combines the feature of high-throughput with a high sensitivity and specificity, allowing detection of rarely expressed genes and distinguishing between homologous genes (Reijans et al., 2003). The aim of the present research is to study the gene expression differentially regulated by phytoplasma in infected host plant (apple) during compatible interaction with 'Ca. *P. mali*'.

Materials and methods

Biological materials and RNA extraction: Apple AP-infected scions by two different strains of 'Ca. *P. mali*' (AP-N17 and AT2-SO8D) were grafted on healthy apple trees (MM106) in an insect-proof greenhouse. Fifty mg of whole plant tissues from healthy, AP-symptomatic, AP-nonsymptomatic and AT2-symptomatic samples of apple trees maintained in a greenhouse were used for RNA extraction, ground with liquid N_2, and processed with Invisorb® spin plant RNA mini kit (Invitek GmbH, Berlin, Germany) following the manufacturer's instructions. DNA-free™ kit (Ambion Inc., Austin, Texas, USA) was used to eliminate genomic DNA carry-over in RNA samples, according to the manufacturer's instructions.

Transcriptional profile: Double-stranded cDNA was synthesized from 5-10 µg of total RNA according to the instructions for the Superscript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) using oligo dT_{12-18}-contaning primers. Double-stranded cDNA was then incubated with *Eco*RI and *Mse*I for 2 h at 37 °C, according to the instructions of the AFLP® Analysis System I (Invitrogen, Carlsbad, CA, USA). The products were subjected to pre-amplification with combinations of *Eco* and *Mse* primers. After dilution of the PCR fragments, selective amplifications were carried out with combinations of an *Eco* primer and an *Mse* primer containing two or three selective bases at the 3' end following the instructions of the AFLP® Analysis System I kit. Amplification products were separated in a vertical denaturing polyacrylamide gel (6 %).

Interesting cDNA-AFLP fragments were recovered as described previously (Dellagi et al., 2000; Campalans et al., 2001) and amplified with the selective primers used to generate the corresponding cDNA-AFLP profile. The purified cDNAs were directly processed to a sequencing reaction, and fragments without clear alignment in direct sequencing were cloned. Alignment and homology of obtained sequences was then carried out, and the identified protein sequences used in queries against the UniProt database (http://www.expasy.org/cgi-bin/sprot-search-de).

Evaluation of gene differentially expression by real-time RT-PCR: Actin, GAPDH, ef1-α and 18S rRNA housekeeping genes were selected as reference genes to normalize the result of real-time RT-PCR. The expression stability for these reference genes was evaluated using the geNorm software program (Vandesompele et al., 2002b).

After new RNA extraction for each sample type (healthy, symptomatic and non-symptomatic infected plants) and elimination of DNA carry-over, cDNA synthesis was carried out on approximately 1.5 µg of the total RNA solution with the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{20}$ primers. Specific primers were designed for all sequenced cDNAs (Primer Express V.5, PE Applied Biosystems, Forster City, USA). Real-time PCR quantification of fragments was performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, USA) in 25 µl volumes containing 1× qPCR MasterMix plus for SYBR green I, 200 µM of each primer, and 5 µl of cDNA (0.5 ng/µl). The thermal cycle consisted of 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

PCR efficiency for each primer pair were determined according to Ramakers et al. (2003) due to $E=10^{slope}$ formula. Expression levels were determined as the number of cycles (C_t) needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Walker, 2002). For each repetition of samples in every run, the level of gene expression was normalized to that of GAPDH and actin, separately, by means of "C_t of target – C_t of reference gene" formula namely ΔC_t. Individual ΔC_t values were further subjected to the SAS V9.1 (SAS Institute Inc., Cary, NC, USA) by means of three-way analysis of variance (completely hierarchical) model using multiple comparison of means. Statistical significance level was 0.05. The relative expression ratio (R) of each target gene between infected (symptomatic or non-symptomatic) and healthy samples or between symptomatic and non-symptomatic samples was calculated by means of the $\Delta\Delta C_t$ method described by Applied Biosystems ($R = 2^{-\Delta\Delta Ct}$). To test the similarity of two methods (cDNA-AFLP and real-time RT-PCR), the Chi-Square test of independence (SAS V9.1) was performed on their data.

Results

Transcriptional profile: The 20 primer pairs used in cDNA-AFLP for cDNA amplification amplified ~8,000 fragments. Of these, 491 (6.1 %) transcript derived fragments (TDF) were differentially expressed among healthy, symptomatic and non-symptomatic infected samples (Figure 1). The fragments differentially expressed were classified into 3 groups (healthy versus infected samples; symptomatic versus non-symptomatic; and AP- versus AT2-infected sample) (Table 1). 34.2 % of the differentially expressed bands were common among the three groups of samples tested and were differentially regulated in the three groups under comparison. Interestingly, more than 50 % of the fragments were differentially regulated between symptomatic and non-symptomatic samples. The majority of fragments (95.3 %) were differently expressed between healthy and infected samples. Among 491 TDFs, 66 bands that clearly showed differences between two or more samples were selected for further analyses. After isolation from gel and re-amplification, the sequences of 45 out of 66 fragments were obtained by direct or cloning-sequencing and 27 of them showed significant similarities to different plant genes present in the databases, while 18 had no matches (novel genes). With the information from UniProt Knowledgebase, putative functions were assigned to 18 out of 27 fragments (others were related to unknown or hypothetical proteins).

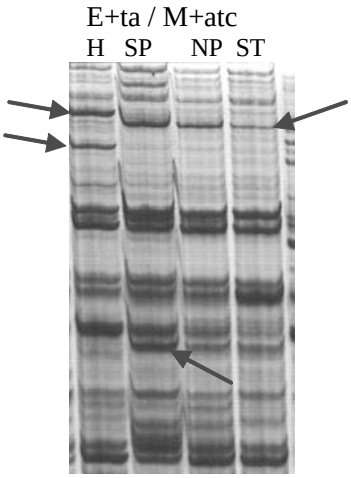

Fig. 1 A profile of cDNA-AFLP obtained by a primer pair [*Eco* (E) / *Mse* (M) primers + added nucleotides] for healthy (H), symptomatic AP-infected (SP), non-symptomatic AP-infected (NP) and symptomatic AT2-infected (ST) samples. Arrows show bands differentially regulated among samples.

Tab. 1 Comparison of cDNA-AFLP gene expression between different combinations of samples, and number of fragments for which the expression patterns were confirmed by real-time RT-PCR.

cDNA-AFLP gene expression comparison between	**Number of fragments differentially expressed**
Healthy sample and infected samples (Group 1)	468 (95.3 %)*
Symptomatic and non-symptomatic samples (group 2)	252 (51.3 %)*
AP-infected sample and AT2-infected sample (group 3)	149 (30.3 %)*
Confirmation of cDNA-AFLP data by real-time PCR	**Number of confirmed fragments**
Fragments confirmed by both GAPDH and actin	11 among 18 known genes
Fragments confirmed by both GAPDH and actin	7 among 25 unknown, hypothetical or novel genes
Fragments confirmed only by GAPDH	6 among 25 unknown, hypothetical or novel genes
Total confirmation	24 fragments out of 43 (56%)

*: percentage of differentially expressed genes in each group to total differentially expressed genes (491).

Comparison of cDNA-AFLP and real-time RT-PCR results: By geNorm analysis, GAPDH and actin were respectively evaluated as the most suitable reference genes to normalize the amounts of starting cDNA in real-time RT-PCR; so, GAPDH was chosen as the principal reference gene and actin as the second in line with the cDNA-AFLP results. Among the sequenced 45 TDFs, no primer combination was designed for two fragments (novel genes), and real-time RT-PCR analyses were carried out for 43 remaining TDFs identified by cDNA-AFLP. For the same PCR run, efficiencies of amplification obtained for normalization genes and each interested genes were never significantly different ($P<0.05$); making the interpreted normalized expression ratios reliable and accurate. In real-time RT-PCR, statistical analyses comparing ΔC_t values showed that using GAPDH and actin, respectively, 27 and 28 out of 43 TDFs were significantly ($P<0.05$) differentially expressed between healthy, symptomatic and/or non-symptomatic samples. Also, the Chi-Square test of independence showed 60 and 42 % of concordance between the two methods (cDNA-AFLP and real-time RT-PCR) by GAPDH and actin, respectively (data not shown). On the other hand, among 18 differentially expressed genes with known function, real-time RT-PCR confirmed the expression pattern of eleven TDFs by both reference genes. Besides, seven, and six TDFs among 25 unknown, hypothetical or novel genes (without similarity) confirmed their deregulated expression profile, respectively, by both reference genes and only GAPDH (Table 1).

Discussion

Using the cDNA-AFLP technique, a series of plant genes whose expression is altered during phytoplasma infection was identified. Some of differentially-expressed fragments did not show any similarity with sequences in the databases, representing therefore potential novel proteins important in interaction that may be related to specific plant responses to

phytoplasmas. Based on the function of each identified gene, a relationship between these genes and their role in pathogen-host interaction is proposed. In the model, the identified genes are classified into three groups to interpret the role of identified genes in symptom expression and regulation of plant responses to phytoplasma infection:

i) Genes related to photosynthesis pathways. Two genes related to photosystems I and II and a gene coding for fructose-1,6-bisphosphatase that were down-regulated only in the symptomatic part of plants were characterized. Underexpression of these photosynthesis genes is probably due to carbohydrate accumulation in infected (symptomatic) leaves (Maust et al., 2003). So, 'Ca. *P. mali*' infection has a detrimental effect on photosynthesis, and the deregulation of key genes in photosynthesis could contribute to symptom expression.

ii) Genes involved in symptom expression. Down-regulation of cell wall-associated hydrolase induces proliferation symptoms since this enzyme is involved in cell wall disassembly; necessary for elongation (Hernandez-Nistal et al., 2006). Moreover, the down-regulation of auxin efflux carrier in this study causes accumulation of auxin in certain cells. The auxins have been implicated in growth, morphology (Brown et al., 2001), and also in apical dominance (Hoshi et al., 2009). So, under-expression of an auxin-transport related gene within MM106 plants inhibits apical dominance, consequently, inducing specific phytoplasma symptoms (proliferation). The only gene which was over-expressed in this group was the one coding for no apical meristem (NAM). Up-regulation of this protein in symptomatic parts of infected plants induces adventitious shoots and proliferation symptoms. On the other hand, the family to which this protein belongs is induced by auxin (Ooka et al., 2003). This means that the accumulation of auxin in certain cells increases the expression of NAM in the same cells. Together, these gene deregulations stimulate the appearance of the specific symptoms of apple proliferation disease.

iii) Genes involved in plant defence mechanisms. Anthranilate hydroxycinnamoyl benzoyltransferase catalyses the first committed reaction of phytoalexin biosynthesis (Yang et al., 1997). Consequently, down-regulation of this enzyme is responsible for the reduction of phytoalexins and high phytoplasma titres in this susceptible host. Also, under-expression of universal stress protein (Usp) increases susceptibility of the host to phytoplasmas as stress agents. On the other hand, the expression of cellular retinaldehyde-binding protein with a potential antimicrobial activity (Molina et al., 1993) or protection property against stresses (Kearns et al., 1998) is reduced in the infected MM106 apples. Together, the plant defence mechanism is thus repressed and higher susceptibility of the host (MM106) and increased multiplication of pathogen occurs.

Taken together, cDNA-AFLP analysis showed that the plant gene expression is modulated in apple in response to phytoplasma colonisation. These results provide a valuable first step towards the understanding of the 'Ca. *P. mali*'–apple interaction. Except for the effect of phytoplasma on photosynthesis, other pathways identified in the current study are presented for first time as target pathways of phytoplasma infection in plants. However, the expression level of the identified genes must be compared in susceptible and resistant (or tolerant) varieties or genotypes in order to find molecular markers and genes important in resistance or tolerance.

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In vitro screening of interspecific hybrids (*Malus* spp.) for resistance to apple proliferation

Bisognin, C.[1]; Ciccotti, A.M.[1]; Salvadori, A.[1]; Jarausch, W.[2]; Grando, M.S.[1]
[1] Fondazione Edmund Mach, IASMA Research Centre - Via Mach 1, 38010 San Michele all'Adige (TN), Italy
[2] AlPlanta – Institute for Plant Research, RLP AgroScience, Breitenweg 71, 65435 Neustadt/W., Germany

Abstract

A breeding programme was set up six years ago in Trentino as part of the Project "Scopazzi del Melo - Apple Proliferation" (SMAP) in order to obtain AP resistant apple rootstocks.

Twenty-six hybrids generated from the crossings (*Malus sieboldii*, second generation, x Malus domestica) were micropropagated and studied in standardised conditions. An *in vitro* screening system for AP resistance previously set for the parents of the crosses was adopted and modified. Specific symptoms of the disease, as well as height and basal proliferation of the shoot and size of the leaves, were recorded *in vitro* at 3 months post-inoculation. At the same time, phytoplasma concentration was determined in the whole shoot by quantitative RT-PCR. An *in vitro* disease index taking into account all the above-mentioned parameters was developed.

Each healthy genotype was graft-inoculated in triplicate with two different phytoplasma strains after plant rooting and acclimatisation. Phenotype and phytoplasma titre were evaluated in the roots the year after infection.

Preliminary results indicated that the resistance trait segregates in the progenies. The resistant genotypes had lower phytoplasma concentrations than the susceptible controls, did not show AP-specific symptoms and their growth was not affected by infection. By comparing the resistant behaviour of the same genotypes, the *in vitro* screening allows for a quick selection of genotypes that are worth evaluating in the field for agronomic traits.

Keywords: Apple Proliferation, *Malus sieboldii*, resistance screening, quantitative real-time PCR, disease index.

Introduction

Apple proliferation (AP) is one of the most serious phytoplasmoses in Europe, causing considerable economic loss (Kunze 1989). As there is no cure for phytoplasma infection, the most promising approach to combating AP appears to be the use of resistant plant material. Natural resistance to AP has been discovered only in wild, apomictic *Malus* species, namely *M. sieboldii*. Crosses of these wild *Malus* species with *M. domestica* have been carried out in the past and attempts have been made to exploit this resistance in apple rootstocks (Seemüller et al., 1992). Studies on the colonisation behaviour of phytoplasmas in apple have revealed that they are eliminated once a year in the aerial parts of the tree during phloem renewal in late winter/early spring. Field trials over one year have further demonstrated that the use of resistant rootstocks can prevent the disease and is sufficient to establish field resistance in the whole tree (Bisognin et al., 2008a). Resistance to AP in the field has been classified as reduced phytoplasma titre in the roots and absence of symptoms in the aerial parts of the tree. In the terminology proposed by Cooper & Jones (1983) the tested genotypes were resistant to 'Candidatus *Phytoplasma mali*' and tolerant to the disease. Unfortunately, all the *M. sieboldii* hybrids examined turned out to be too vigorous to be used as rootstocks in modern apple culture.

A breeding programme was set up six years ago in Trentino as part of the Project "Scopazzi del Melo - Apple Proliferation" (SMAP) aimed at developing a rootstock comparable to the standard stock, cv. M9, and having features of both dwarfing and resistance to AP. Several different cross combinations have been made between apomictic-resistant accessions and susceptible *M.* x *domestica* parents in order to find the best selection (Bisognin et al., 2009). Resistance screening of the progeny is currently carried out by graft inoculation of the seedlings which are then observed in the nursery for symptom expression over a period of 2-5 years. As an alternative to this time-consuming and labour-intensive screening, an *in vitro* resistance screening system based on micrografting has been developed (Bisognin et al., 2008b). It allows response to infection in resistant selections to be analysed and compared with infection in susceptible controls under standardised conditions.

The objective of the present study was to find an *in vitro* disease index to apply to the progenies obtained from the breeding programme in order to select the resistant genotypes, thus speeding up the procedure in the field. Twenty-six hybrids were infected *in vitro* by micrografting. Phenotypic data and phytoplasma titre were compared with the resistant parent *M. sieboldii*. Three hybrids were identified as resistant and were reinoculated in controlled conditions to verify resistance to different strains of the pathogen.

Materials and methods

The *in vitro* resistance screening system: The screening system, previously set up in the parents of the crosses (Bisognin et al., 2008b), was adopted to test twenty-six hybrids generated from the crossings *Malus sieboldii*, second generation, x Malus domestica. The system requires the establishment of *in vitro* shoot cultures of the genotype and the maintenance of 'Ca. *P. mali*' in micropropagated apple. Healthy *in vitro* shoot cultures of the progenies were successfully established and propagated in order to test susceptible (Golden Delicious, M9) and resistant genotypes (*M. sieboldii*, D2212, H0909), as well as infected cultures of cv. Golden Delicious (Ciccotti et al., 2008). Hybrids were inoculated by micrografting with infected shoots of cv. Golden Delicious used as graft tips. Micrografting was carried out in 10 repetitions for each genotype. Graft contact was maintained for 1.5 months. Only grafts where a good phloem connection between the two scions was established were considered successful grafts, and these were then subcultured and analysed for the presence of phytoplasma. The twenty-six hybrids were also studied in controlled conditions. *Ex vitro* healthy genotypes were graft-inoculated in triplicate with two different phytoplasma strains after plant rooting and acclimatisation. PM6 was the strain used for the *in vitro* screening, while PM11 is a local 'Ca. *P. mali*' strain found in commercial orchards in Trentino. Phenotype and phytoplasma titre of the roots were evaluated the year after infection.

In vitro phenotype index: Specific symptoms, i.e. enlarged stipules and/or witches' brooms, in the *in vitro* shoots were recorded at three months post-inoculation (m.p.i.). The incidence of these symptoms in all repetitions allowed three classes of varying intensity to be determined (Table 1).

The height of *in vitro* shoots was measured at three m.p.i and at five m.p.i. Each genotype was evaluated by comparing the height of the taller shoot of the healthy vs. the infected *in vitro* plant. Significant differences were found with analysis of variance (ANOVA) and these were subsequently compared using Fisher's least significant difference (LSD). Two intensity classes were thus determined. At the same time, basal shoot proliferation was measured in healthy and infected genotypes maintained under equivalent cultivation conditions. Significant differences were found with analysis of variance (ANOVA) and these were subsequently compared using Fisher's least significant difference (LSD). A phenotype index for each genotype was determined as the sum of the number of intensity classes found for each of the above-mentioned three parameters, as shown in Table 1 and Table 2.

Tab. 1 Specific symptoms considered in determining the *in vitro* phenotype index.

Parameter	Incidence %	Intensity classes
SYMPTOMS		
no symptoms	0 %	0
stipules -	< 50 %	0
stipules	= 50 %	1
stipules +	> 50%	2
witches' brooms	< 50 %	1
witches' brooms +	> 50%	2

Tab. 2 Growing parameters considered in determining the *in vitro* phenotype index.

Parameter	*p* value*	Intensity classes
Height		
infected vs healthy	$p > 0.05$	0
infected vs healthy	$p < 0.05$	1
Basal shoot proliferation		
infected vs healthy	$p > 0.05$	0
infected vs healthy	$p < 0.05$	1

* *p* value determined by analysis of variance (ANOVA).

Concentration index: Total DNA was extracted according to Doyle and Doyle (1990) from 0.1 - 0.5 g of *in vitro* plant material or from *ex vitro* root material. Direct PCR was carried out with specific primers fAT - rAS (Smart et al., 1996) to detect infected plants.

A real-time PCR assay based on the method published by Baric and Dalla Via (2004) was applied at three m.p.i. to quantify 'Ca. *P. mali*' in the inoculated and infected plants using the automated LightCycler® 480 apparatus (Roche). A multiplex qPCR was performed simultaneously by amplifying a fragment of the 16SrRNA gene of 'Ca. *P. mali*' with the *Malus* chloroplast gene coding for tRNA leucine as housekeeping gene. Absolute quantity of phytoplasma DNA was determined by comparison with a standard curve based on serial dilutions of a plasmid containing a fragment of the 16SrRNA gene from 'Ca. *P. mali*'. The relative presence of 'Ca. *P. mali*' in the plant material was finally expressed as Genome Unit (GU) of phytoplasma per nanogram of plant DNA. Phytoplasma concentration of *M. sieboldii* was taken

as the resistance reference. The concentration index of each hybrid was obtained by estimating its phytoplasma titre in relation to *Malus sieboldii* phytoplasma concentration.

Results and discussion

As in the field, infected *M.* x *domestica* genotypes were severely affected by the disease *in vitro*,showing stunted growth with proliferation of shoots and enlarged stipules and/or witches' brooms. A phenotype index based on parameters of symptoms, height and basal shoot proliferation was developed in the parents (Table 3) and applied to the hybrids in this study (tab. 4). Leaf area was found to have no correlation with AP susceptibility. The highly resistant parents of the crosses were almost unaffected by the disease both *in vitro* and *in vivo*. In the *in vitro* system two threshold values of resistance were found. The absence or slight presence of symptoms corresponding to a phenotype index ≤ 2 and a concurrent concentration index of ≤ 1.5 in the parents allowed a given genotype to be considered resistant (table 3). In this study eleven out of twenty-six genotypes showed an *in vitro* phenotype index ≤ 2, while eight out of the twenty-six hybrids analysed had a concentration index of ≤ 1.5. Only three genotypes attained both resistance threshold values. Screening in the field confirmed the *in vitro* results (data for one of these are still being processed). As in the field, a clear correlation between a higher concentration of phytoplasma and a presence of witches' brooms was found (Bisognin et al., 2008a).

Tab. 3 *In vitro* evaluation of resistance to AP in parents inoculated with PM6 strain. Phenotype index is the sum of intensity classes of the three parameters considered in tab.1 – 2 for each genotype. Concentration index is the ratio of mean phytoplasma concentration of genotype per ng of host DNA to the mean concentration of strain PM6 per ng of *M.sieboldii* DNA

Genotype	Phenotype index	Concentration index
M. sieboldii	1	1.00
D2212	0	0.60
H0909	2	1.45
Golden delicious	4	2.80
M9	3	1.60

All genotypes were also tested in controlled conditions and their resistant behaviour was confirmed. Evaluation of visual symptoms alone was not enough to classify a genotype. Many hybrids showed only mild symptoms *in vivo* but investigation of the root system showed a wide variation in phytoplasma concentration. Quantification in the roots became essential in order to screen all genotypes displaying an uninfected phenotype after 2 years.

Comparing the behaviour of genotypes in the two systems (*in vitro* and in controlled conditions) showed that the *in vitro* procedure gave an advantage of at least one year in the breeding programme timetable. Susceptible genotypes can be promptly eliminated from the screening and only resistant ones can then be submitted to further assessment, such as agronomic evaluation and resistance to a wide spectrum of pathogens.

Conclusions

A reduced phytoplasma concentration and the absence or slight expression of disease symptoms are necessary and sufficient for assessing resistance to AP in the plants. The results obtained indicate that resistance to 'Candidatus *Phytoplasma mali*' can be observed *in vitro* in a similar way to *in vivo*, thus speeding up the resistance screening procedure. The *in vitro* disease index developed here should help to reduce to one year the time of screening it takes to select susceptible genotypes and carry out experimental trials.

The method is currently employed for testing several individual genotypes of different breeding progenies. Moreover, the *in vitro* system offers the possibility of studying specific host-pathogen interactions in standardised conditions and of simultaneously evaluating the virulence of different strains.

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Experimental transmission trials by *Cacopsylla pyri*, collected from pear decline infected orchards in Turkey

Çağlayan, K., Gazel, M., Ulubaş Serçe, Ç., Can, F.
Mustafa Kemal University, Agriculture Faculty, Plant Protection Department, Antakya-Hatay, Turkey

Abstract

A study was carried out on the experimental transmission efficiency of the Pear Decline (PD) phytoplasma by *Cacopsylla pyri* (L.), collected from naturally infected orchards from Bursa and non-infected orchard from Hatay province of Turkey. *C. pyri* adults captured from infected orchards were directly transmitted to healthy periwinkle plants (*Catharanthus roseus*) whereas the second group firstly fed on infected pear for two weeks and then transferred to periwinkles. Groups of five psyllids per plant were used for transmissison tests and the study was replicated three times. The presence of 'Candidatus *Phytoplasma pyri*' in psyllids and *C. roseus* plants was analysed by nested PCR using P1/P7 and U3/U5 primer pairs.. Although *C. pyri* has limited host range, they were able to survive up to 20 days on periwinkles. Insects collected from Bursa province survived 16-20 days whereas second group from Hatay were survived 7-12 days on periwinkles. Symptoms consist of a yellowing or clearing of the veins in newly infected leaves and shortening of the internodes of the main stem. They also remain stunted and flowers were small. According to the RFLP analysis of Bursa samples, the experimental infection rate of periwinkle plants and psyllids was 33.3 % and 16.6 %, respectively. No infected periwinkle was found in second group but psyllids were 33.3 % infected. Transmission trials under controlled conditions showed the capability of *C. pyri* to transmit PD from infected pears to healthy periwinkles and confirmed as vector of Ca. *P. pyri* in Turkey.

Keywords: Candidatus *Phtoplasma pyri*, pear psyllid, transmission efficiency

Introduction

Pear decline caused by 'Candidatus *Phytoplasma pyri*' (Seemüller and Schneider, 2004) is widespread in many pear-growing countries including Turkey. The first suspicious and common symptoms of PD was observed on cv. 'Deveci' in Bursa province of Turkey in 2005 and then the disease was confirmed by PCR and RFLP analyses (Ulubas Serce et al. 2006). This phytoplasma belongs to the Apple proliferation group (16SrX) (Seemüller et al. 1998), and transmitted by pear psyllids (*Cacopsylla pyricola, C. pyrisuga, C. pyri*). In North America and England the known vector is *Cacopsylla pyricola* (Foerster) but in other part of Europe *Cacopsylla pyri* (L.) has been found as main vector (Carraro et al., 2001; Garcia-Chapa et al., 2005). Transmission of PD by *C. pyri* has already been demonstrated in Italy (Carraro et al., 1998) and France (Lemoine, 1984), suggesting that this psyllid is probably the most important vector in the Mediterranean area. Although transmission capability has not yet been evaluated , *C. pyri* is also most common psyllid in pear orchards in Spain (Garcia-Chapa et al., 2005) In Turkey, *C. pyri* is the predominant psylla on pear trees (Gençer, 1999) and gave 3 to 4 generations a year (Kovanci et al., 2000). Naturally infected psyllids, captured from infected pear orchards, were already reported (Ulubas Serce et al., 2006) but its capability to transmit Ca. *P. pyri* has not been investigated yet.

Present paper describes experimental transmission possibility of Ca. *P. pyri* using *C. pyri* which were collected from naturally infected orchards, to periwinkle plants.

Materials and methods

Field studies: In December 2007 two commercial plots of pear cv. 'Deveci' located in Bursa (B1 and B2) and one plot of cv. 'Santa Maria' in Antakya (A) provinces of Turkey were selected. PD symptoms and presence of C. *pyri* had been previously recorded in plot B1 and B2 but no PD symptoms were observed in plot A despite previous report on presence of the disease in that province (Sertkaya et al., 2005). The incidence of the disease in three plots was evaluated and 10 % of the total pear trees were randomly selected and tested by nested PCR. The psyllids were also captured in December by shaking insects onto an underlying net. Twenty individual insects from each plot were also analyzed for the presence of PD .

Experimental transmission of PD by *C. pyri* on periwinkle plants: All the transmission experiments were carried out in an environmentally controlled growth room at 25±1 °C with supplementary light and 16-h days. In December 2007, adult *C. pyri* were captured and then 3 groups of psyllids, each of 5 individuals, were transferred to healthy periwinkle seedlings. Psyllids captured from plot A were firstly fed on PD infected pear plant for 2 weeks and then transferred to periwinkles. All test plants were covered individually with a plastic-screen cage (Figure 1). Another group of three healthy periwinkle plants were used as negative controls. Longevity of the insects and symptom expression were observed and died psyllids were immediately analyzed for the presence of PD phytoplasma (Garcia-Chapa et al., 2003).

Fig. 1 Test plants covered individually with a plastic-screen cage (on left), *Cacopsylla pyri* feeding on periwinkle plant (on right).

Testing for the presence of phytoplasmas in test plants and in psyllids: All test plants and individual psyllids were tested by nested PCR. The first amplification was with the universal primers P1/P7 (Lee ve ark., 1992). FU5/rU3 amplicons of nested PCR were digested with *Ssp*I and *RsaI* at 37 °C fallowing the manufacturer's instructions (MBI Fermentas, Germany). Digested products were analyzed by electrophoresis using 2 % agarose gel and stained with ethidium bromide, DNA bands were photographed under UV light. PD, Apple proliferation (AP) and European Stone Fruit Yellows (ESFY) infected periwinkle plants were kindly supplied by Dr. Foissac-INRA, France and used as positive controls.

Results

Field studies: PCR analyses of two commercial plots of pear tree cv. 'Deveci' in Bursa (B1 and B2) showed that the incidence of PD infected trees was 60 % and 65 %, respectively whereas no infection was recorded in plot A. Analyses of 20 field collected psyllids evaluated by nested PCR showed that 2 and 5 psyllids from plot B1 and B2 were found infected by PD, respectively but no infected psyllid was found in Antakya province.

Experimental transmission of PD by *C. pyri* on periwinkle plants: Although *C. pyri* has limited host range, they were able to survive up to 20 days on periwinkles (Table 1). Insects collected from Bursa province survived 16-20 days whereas insects from Antakya were survived 7-12 days on periwinkles. First symptoms were observed 4 months after exposure on test plants. Symptoms consist of a yellowing or clearing of the veins in newly infected leaves and shortening of the internodes of the main stem. They also remain stunted and flowers were small (Figure 2). Two periwinkle plants from plot B1 and B2 showed phytoplasma-like symptoms whereas no symptomatic plant was found in plot A. According to the RFLP analysis of Bursa samples, infection rate of periwinkle plants and psyllids was 33.3 % and 16.6 %, respectively. No infected periwinkle was found in second group but psyllids were 33.3 % infected.

Tab. 1 Survival of *Cacopsylla pyri* L. on periwinkle plants

Location and number of test plants		Survival (days)
Bursa 1 (B1)	B1-1	19
	B1-2	16
	B1-3	20
Bursa 2 (B2)	B2-1	12
	B2-2	12
	B2-3	17
Antakya (A)	A1	7
	A2	12
	A3	7

Fig. 2 Symptoms of stunting, shortening of the internodes of the main stem and small flowers of experimentally infected periwinkle (on right) and healthy control (on left).

Testing for the presence of phytoplasmas in test plants and in psyllids: Using the primer pair FU5/rU3, PD-phytoplasma DNA was amplified from positive controls-that is PD, AP and ESFY infected periwinkle plants as well as from test plants and individual psyllids used in the trials. After digestion with *Ssp*I and *RsaI*, the restriction products obtained from all samples showed the same restriction profiles, by which three different phytoplasmas could be, distinguished (Figure 3).

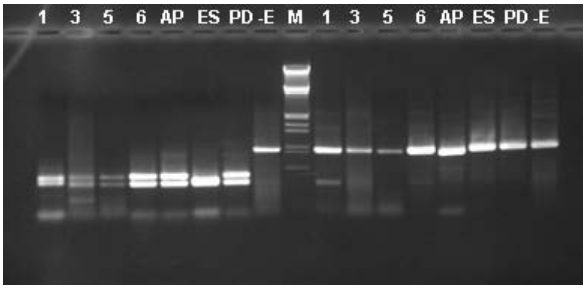

Fig. 3 Restriction products of Ca. *Phytoplasma pyri* DNA after digestion with *Ssp*I and *RsaI*, respectively. 1, 3, 5 and 6 represent infected periwinkle plants. Positive controls: AP (apple proliferation), ES (European stone fruit yellows), PD (pear decline). –E: negative control without enzyme.

Discussion

Pear decline is a destructive disease that occurs in Europe, North America and wherever the domestic European pear (*Pyrus communis* L.) is grown (Davies et al., 1992; Garcia-Chapa et al., 2003). In the last 5 to 6 years rapid spread of PD disease in Bursa province of Turkey represents a serious outbreak with high level of infection. Previous studies in this province showed that out of the 116 tested pear samples, 52.58 % were found infected by PD (Gazel et al. 2007). In this study similar results were obtained and 60 to 65 % infection rate was recorded in randomly tested pear trees from which psyllids were captured for transmission trials. The psyllids, collected from two different infected orchards of Bursa province were also found infected by PD (7 infected out of 40). Two periwinkle plants out of 6 were experimentally infected by *C. pyri,* collected from Bursa province where PD is very common. According to these results, the detection of same RFLP pattern for pear, psyllid and periwinkle confirm that *C. pyri* is an active vector of PD agent in that province. However in plot A no naturally infected psyllids were found and PD was only detected in one insect but none of the periwinkle plants. This data showed us that *C. pyri,* collected from plot A may be also potential vector candidate for this province because it can acquire phytoplasma from the infected pear tree but not able to transmit to periwinkle for this experiment. It might be due to using limited number of insects and periwinkles. Since *C. roseus* does not seem to be a good host for pear decline transmission, it also might be necessary to use pear seedlings for transmission experiments (Avinent et al., 1997). Because of PD is difficult to reproduce experimentally with other psyllid species (Davies et al., 1992), new laboratory transmission trials with insects fed on infected trees should be performed in future.

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Analysis of the acquisition and multiplication efficiency of different strains of Ca. *Phytoplasma mali* by the vector *Cacopsylla picta*

Jarausch, B.; Fuchs, A.; König, D.; Krczal, G.; Jarausch, W.
RLP AgroScience GmbH, AlPlanta, Institute for Plant Research, Neustadt, Germany

Abstract

Based on previous observations during long-term acquisition and transmission trials, studies were carried out under standardized conditions in order to analyse the acquisition and multiplication efficiencies of different strains of Candidatus *Phytoplasma mali* by different developmental stages of *Cacopsylla picta*. The acquisition of Ca. *P. mali* from micropropagated plants infected with different strains was tested for nymphs, larval stages and new adults of *C. picta*. When born on infected plants a nearly 100% acquisition was achieved for all strains of Ca. *P. mali* by *C. picta*. Differences in acquisition efficiency were observed for new generation adults which acquired the phytoplasma as imagines. The multiplication efficiency of the different Ca. *P. mali* strains inside the insects was analysed by quantitative real-time PCR. Significant differences in the capacity of the different strains to colonise the insect were found. Despite high acquisition rates only few subsequent transmission events to healthy test plants could be recorded.

Introduction

For several years now increased efforts have been undertaken to characterize different strains of Ca. *P. mali* by molecular means and to elucidate the impact of strain variability on the plant-pathogen interaction. Using PCR-RFLP analysis of an important number of isolates of Ca. *P. mali* from different European apple growing regions three dominant subtypes could be described: AP, AT-1 and AT-2 (Jarausch et al., 2000). Recently, the complete genome of Ca. *P. mali* strain AT has been sequenced (Kube et al., 2008). For a couple of years, strains with different biological properties (virulent/avirulent) have been differentiated in plants (Seemüller & Schneider, 2007) and an increasing genetic variability was currently discovered by SSCP analysis of the *hfl*B gene within strains or isolates of Ca. *P. mali* (Schneider et al., 2009). However, the biological meaning of the strain variability and especially its role in the pathogen-vector-interaction are still unknown.

Ca. *P. mali* is transmitted under natural conditions by two psyllid species: *Cacopsylla picta* (Frisinghelli et al., 2000; Jarausch et al., 2003) and *Cacopsylla melanoneura* (Tedeschi et al., 2002). In most apple growing regions *C. picta* is the most efficient vector (Jarausch et al., 2007). *C. melanoneura* is even regarded as non-vector in Germany (Mayer et al., 2009).

This study was therefore initiated to obtain the first indications on the influence of the different strains of Ca. *P. mali* on its interaction with the insect vector *C. picta*.

Materials and methods

All studies were conducted with homogeneous micropropageted plant material transferred *ex vitro* into the greenhouse. Healthy plants of *M.* x *domestica* cultivar 'Golden Delicious' as well as 'Golden Delicious' plants infected with Ca. *P. mali* strains PM4, PM5, PM6, PM 7, PM 9 and PM19 were used. Micropropagation, *in vitro* rooting and acclimatisation was done as previously described (Jarausch et al., 1996; Bisognin et al., 2008; Ciccotti et al., 2008). All *ex vitro* plants used were of similar age and stage. Ca. *P. mali* strains were those described by Schneider et al. (2009).

Insect material originated from rearings of *C. picta* on healthy or infected plants. All trials were conducted in greenhouse chambers or climatic cabinets under controlled conditions. Transmission trials with new adults were conducted according to Jarausch et al. (2004). The acquisition efficiency of different strains of Ca. *P. mali* by its vector *C. picta* was analysed in the following versions:

- acquisition feeding of fully developed new imagines from healthy rearings.
- acquisition feeding of larval stages obtained from healthy rearings.
- acquisition feeding during the complete larval development until emergence of new adults (born on infected plants).

Total DNA from individual insects as well as from plants was extracted using the CTAB method described in Jarausch et al. (2004). PCR amplification of phytoplasma 16S rDNA and of a non-ribosomal fragment was carried out following the protocols reported in Jarausch et al. (1994; 2004). Samples with positive signals were quantified for the phytoplasma titer by real-time PCR using the SYBR green method (Jarausch et al., 2004b).

Statistical analysis was done with R statistical software (R development core team, 2009) applying non-parametric tests to the data.

Results and discussion

The acquisition efficiency of the different developmental stages of *Cacopsylla picta* was highest when the individuals were born on infected plants thus reaching an acquisition rate of about 100 % for almost all strains of Ca. *P. mali.* In this variant the specimens could acquire the agent from the inoculum source plant during their whole larval cycle. When the insects were only allowed to acquire the agent as new adult a consistent percentage of about 10 % of the specimens became infected in 2006 and 2007, respectively. This indicates that the fully developed individuals of the new generation do not feed constantly on apple anymore but already prepare for migration to their overwintering host plants. The acquisition of Ca. *P. mali* was also less efficient by individuals which were transferred to infected plants as larval stages (L1-L5). These stages yielded intermediate acquisition rates. Although the larval stages should still feed intensively they might be affected by the technical transfer conditions where the stylet can be damaged.

A detailed analysis of the acquisition capacity of new imagines of *C. picta* showed that strain PM 6 was acquired most efficiently after an acquisition period of 7 days compared to all other strains tested. Interestingly, regardless of the strain, no significant increase of the phytoplasma concentration - as determined by quantitative real-time PCR in single individuals - could be observed after an acquisition feeding of 7 days and subsequent latency period compared to individuals tested after 2 or 4 days acquisition only. This indicates that no significant multiplication occurred in new adults during the latency period and might explain the low transmission rate to healthy plants. However, apart from the main portion of individuals which showed statistically no significant multiplication some individuals reached a high phytoplasma concentration and could be evaluated as potentially infectious.

By analysing the multiplication efficiency of the different strains in the new adults a clear trend became obvious: PM6 strain had a significantly higher multiplicationefficiency when compared to PM5 while the other strains reacted intermediately. Amazingly, the same trend was observed when analyzing the multiplication efficiency of the different strains inside individuals which were born on infected plants. In this case a significant difference in multiplication efficiency was obtained in the order PM6>PM4>PM5. Due to the more important number of individuals tested this result strongly confirms that the PM6 strain of Ca. *P. mali* was the most efficiently acquired, multiplied and transmitted by all developmental stages of *C. picta.* Surprisingly, the analysis of the phytoplasma concentration in the inoculum plants gave exactly the opposite trend with preference for PM4 and low multiplication of PM6 (Bisognin et al., 2008). These results indicate that there is a difference in the multiplication capacity of the various strains of Ca. *P. mali* within the insect and plant milieu.

Conclusions

The study showed that acquisition and transmission with new generation adults of *C. picta* is not efficient in the same season. The acquisition efficiency was highest when individuals were born on infected plants but the subsequent transmission was not efficient. The most reliable and only measurable parameter is the multiplication efficiency once the phytoplasma has been acquired. All data together reveal significant differences among the Ca. *P. mali* strains in colonising their insect vector. The multiplication efficiency of the strains in insects is different from that in plants which may indicate the existence of various interaction mechanisms.

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Molecular characterization of 'Candidatus *Phytoplasma mali*' strains in outbreaks of apple proliferation in north eastern Italy, Hungary, and Serbia

Paltrinieri, S.; Duduk, B.; Dal Molin, F.[2]; Mori, N.[3]; Comerlati, G.[2]; Bertaccini, A.
[1] DiSTA, Patologia vegetale, Alma Mater Studiorum, Università di Bologna, viale Fanin 42, 40127 Bologna, Italy
[2] Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia
[3] Servizio Fitosanitario Regione Veneto, viale dell'Agricoltura 1a 37060 Buttapietra (VR), Italy
[4] Dipartimento di Agronomia Ambientale e Produzioni vegetali - sez. Entomologia, Università di Padova, viale dell'Università 16, 35020 Legnaro (PD), Italy

Abstract

During 2005-2008 apple plants of different varieties showing proliferation symptoms were observed in diverse areas of north eastern Italy, Hungary and Serbia. PCR/RFLP analyses showed that all the samples were infected with 'Candidatus *Phytoplasma mali*'. In the 16S plus spacer region two phytoplasma profiles (P-I and P-II) were distinguished. P-I profile was detected in reference strains AP, AT1, AT2, in samples from Serbia, and in the majority of samples from Trentino; the P-II profile was prevalent in samples from Veneto; both profiles were identified in samples from Hungary, in some cases together in single samples. The analyses of rpl22-s3 genes allow the identification, in all the samples showing a P-I profile, the presence of phytoplasmas belonging to rpX-A subgroup, while in the samples showing a P-II profile it was possible to distinguish the other three reported rpX subgroups. In the majority of samples from the Veneto region phytoplasmas belonging to rpX-D subgroup were identified, while rpX-B and rpX-C subgroups were identified only in a few samples from Trentino and Veneto regions, respectively. Further RFLP analyses on AP13/AP10 amplicons differentiate among strains belonging to the rpX-A subgroup: the samples from Serbia show AP profiles, while those from Trentino show AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified.

Keywords: Apple, 'Candidatus *Phytoplasma mali*', phytoplasma strains, PCR/RFLP analyses, epidemiology

Introduction

Apple Proliferation (AP) is a phytoplasma disease found only in Europe, reported for the first time in Veneto in the fifties (Rui, 1950).Its major impact on agriculture is that the infected plants continue to vegetate producing small and unmarketable fruits. Affected apple cultivars are almost all those present in the main apple growing areas of Europe (Refatti and Ciferri, 1954; Bovey, 1961; Brcak et al., 1972; Minoiu and Craciun, 1983; Bliefernicht and Krczal, 1995, Marcone et al., 1996a), like Golden Delicious, and Renetta of Canada grafted on different rootstocks. Recently the disease was also reported in Hungary (Del Serrone et al., 1998) and in Serbia (Duduk et al., 2008). AP is one of the most important phytoplasma diseases of apple, affecting almost all cultivars, reducing size (by about 50 %), weight (by 63-74 %) and quality of fruit, as well as reducing tree vigour and increasing susceptibility of the plants to powdery mildew.

Apple is the main host of 'Candidatus *Phytoplasma mali*', the agent of AP (Seemüller and Schneider, 2004). The disease can be observed on cultivars or on rootstocks, as well as on wild and ornamental Malus. 'Ca. *P. mali*' was found in hazelnut (*Corylus* spp.) (Marcone et al., 1996b), cherry (*Prunus avium*), apricot (*P. armeniaca*) and plum (*P. domestica*) (Mehle et al., 2007).

Two insect species, *Cacopsylla melanoneura* and *C. picta*, have been reported as AP vectors in Italy (Frisinghelli et al., 2000; Tedeschi et al., 2002). Whereas *C. picta* appears to be the main vector in Germany (Jarausch et al., 2003; Mayer at al., 2008). Another leafhopper, *Fieberiella florii* Stal (Homoptera: Cicadellidae), has been implicated as a vector of AP in Germany (Krczal et al., 1989) and recently, also in Italy (Tedeschi and Alma, 2006).

Although in Europe AP disease affects most or all varieties of apple trees, it is caused by a relatively homogeneous pathogen in which strains or subtypes were identified by PCR/RFLP of AP10 and rpS3 gene (Jarausch et al., 2000; Martini et al., 2008).

To further evaluate the possibility to correlate molecular polymorphism with geographical distribution of AP strains three regions of the AP genome; the 16S rDNA, spacer region and beginning of 23S (Khan et al., 2002; Casati et al., 2007), the ribosomal protein (rp) gene sequences *rpl22* and *rps3*, and the nitroreductase gene (Martini et al., 2008; Bertaccini et al., 2008; Jarausch et al., 2000; 2004) were studied in selected strains from different geographic areas.

Material and methods

During 2005-2008 apple plants belonging to diverse varieties and showing proliferation symptoms were observed in different areas of northeastern Italy, Hungary and Serbia. Selected plants were employed for sampling (Table 1) in particular: six from Hungary collected in a small field destroyed by AP, near to the Austrian border (samples H-1 trough H-6). Two from Serbia (RS-135 and RS-151) collected in Bela Crkva where the disease was only observed in a few plants. Fourteen samples were collected in Italy, three in different areas of the Trentino region (I-TN1 to I-TN3) where the disease is epidemically present for more than 15 years, and another 11 samples from Veneto region (Table 1) in areas where the disease was observed in 2007. As reference strains the three AP strains reported in the literature as AP-15, AT-1 and AT-2 were employed; AT-2 was a strain isolated from Golden Delicious apple kindly provided by S. Grando (E. Mach Foundation, S. Michele all'Adige, TN; Italy) while AP-15, AT-1 are from the phytoplasma collection at DiSTA (Bertaccini, 2003).

Tab. 1 Results of RFLP analyses and the diverse phytoplasma genomic sequences to characterize AP strains from different geographic origin.

Strain	Primers F1/B6			Primers AP13/AP10			Primers rpAP15f/rpAP15r	
acronyms	HpaII	FauI	Group	RcaI	HincII	Group	AluI	Group
H-1	-	-	nd	A	A	AT2	A	rpX-A
H-2	A+B	A	PI+PII	-	-	nd	A	rpX-A
H-3	B	A	PII	B	B	AP	A	rpX-A
H-4	A+B	A	PI+PII	B	A	AT1	A	rpX-A
H-5	A+B	A	PI+PII	B	B	AP	A	rpX-A
H-6	A	A	PI	B	B	AP	A	rpX-A
RS-135	A	A	PI	B	B	AP	A	rpX-A
RS-151	A	A	PI	B	B	AP	A	rpX-A
I-VE11	B	A	PII	-	-	nd	C	rpX-C
I-VE12	A	A	PI	-	-	nd	A	rpX-A
I-VE14	A	A	PI	A	A	AT2	A	rpX-A
I-VE16	B	A	PII	B	A	AT1	C	rpX-C
I-VE22	B	A	PII	-	-	nd	D	rpX-D
I-VE27	B	A	PII	B	A	AT1	D	rpX-D
I-VE28	B	A	PII	-	-	nd	D	rpX-D
I-VE30	B	A	PII	-	-	nd	D	rpX-D
I-VE31	B	A	PII	-	-	nd	D	rpX-D
I-VE32	B	A	PII	-	-	nd	D	rpX-D
I-VE34	B	A	PII	-	-	nd	D	rpX-D
I-TN1	A	A	PI	A	A	AT2	A	rpX-A
I-TN2	A	A	PI	-	-	nd	A	rpX-A
I-TN3	B	A	PII	-	B	nd	B	rpX-B
AP-15	A	A	PI	B	B	AP	A	rpX-A
AT-1	A	A	PI	B	A	AT1	B	rpX-B
AT-2	A	A	PI	A	A	AT2	A	rpX-A

nd, group not determined

Leaves and young apple shoots were collected from June to October and nucleic acid was extracted from fresh leaf midribs and phloem by a chloroform/phenol procedure (Prince et al., 1993) or by a CTAB procedure (Angelini et al., 2001).

16S ribosomal DNA plus spacer region: Direct PCR amplification with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider et al., 1995), amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed. Nested PCR amplification was carried out on P1/P7 amplicons diluted 1:30 in sterile distilled water with R16F2/R2 (Lee et al., 1995) and F1/B6 (Davis and Lee, 1993; Padovan et al., 1995) primer pairs. Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of *Taq* polymerase, 0.2 mM dNTPs, 1.5 mM MgCl2 and 0.4 µM each primer. PCR conditions were: 35 cycles (Biometra, Uno Thermoblock, Gottingen, Germany as thermal cycler), 1 min (2 min for the first cycle) denaturation step at 94 °C, 2 min for annealing at 50 °C and 3 min (10 min for the last cycle) for primer extension at 72 °C. Samples with the reaction mixture lacking a DNA template were included in each experiment as negative controls. PCR products were subjected to electrophoresis in a 1 % agarose gel and visualized by staining with ethidium bromide and UV illumination. Three µl of PCR product was digested using *Ssp*I and *Rsa*I restriction enzymes for R16F2/R16R2 amplicons following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). Further strain characterization was carried out using RFLP analyses with

*Hpa*II and *Fau*I restriction enzymes on F1/B6 amplicons. The comparison of restriction patterns obtained with those of control strains was carried out after electrophoresis through a 5 % polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Ribosomal protein (rp) gene sequences *rpl22* and *rps3*: *RpS3* gene was amplified in direct PCR reactions using primers rpAP15f/rpAP15r. This detection method is specific for 'Ca. *P. mali*' and can distinguish up to four different RFLP-subtypes (rpX-A, B, C and D) (Martini et al., 2008). PCR conditions were as follows: initial denaturation for 2 min at 94 °C; 40 cycles (1 min at 94 °C, 45 s at 55 °C and 90 s at 72 °C); final extension for 8 min at 72 °C. Negative control and visualization of results were carried out as described above. Three µl of PCR products were digested using *Alu*I restriction enzyme following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then observed as described above.

Non ribosomal DNA fragment: For the amplification of the non-ribosomal DNA fragment (nitroreductase–like gene, Jarausch et al., 2000) a semi-nested PCR assay was employed using as a template the PCR product of the initial amplification diluted 1:30 with primers AP8/AP10 followed by primers AP13/AP10. The PCR cycle was as follows: 95°C for 1 min, followed by 45 cycles with 94 °C for 1 min, 53 °C for 45 sec, 72 °C for 1.30 min, the extension of last cycle was at 72 °C for 8 min. Three µl of PCR products were digested using enzymes *Rca*I and *Hinc*I following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then compared as described above.

Results

Nested PCR amplification with R16F2/R2 primers followed by RFLP analyses with *Rsa*I and *Ssp*I allow to confirm that all samples analyzed were infected with '*Ca*. P. mali' (data not shown). Also primers F1/B6 amplified all tested samples in nested PCR assays and RFLP analyses on these amplicons distinguished two phytoplasma profiles (P-I and P-II) (Figure 1). P-I profile was detected in reference strains AP, AT1, AT2, in samples from Serbia, in one sample from Hungary, in two out of the three samples from Trentino, and in two out of the 11 samples from Veneto. The P-II profile was detected in the majority of samples from Veneto and in samples from Hungary, however the majority of the latter samples show both profiles together (Table 1).

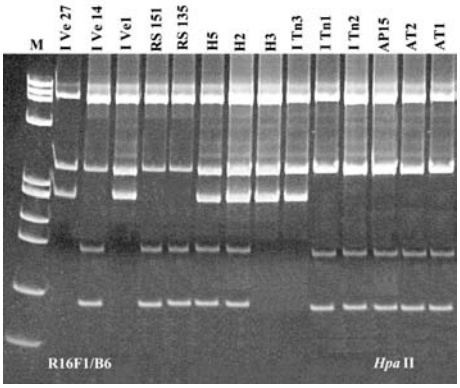

Fig. 1 Polyacrylamide gel showing RFLP profiles with *Hpa*II of nested-PCR amplicons amplified from selected samples from Serbia, Hungary and Italy (see list in Table 1) with primers F1/B6. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1,353; 1078; 872; 603; 310; 281; 271; 234; and 194.

The RFLP analyses of *rpl22-s3* genes allowed identification, in all the samples showing P-I profile the presence, of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish the other described rp subgroups. The rpX-B and rpX-C subgroups were identified in one of the samples from Trentino and in the two samples from Veneto respectively, while in the majority of samples from Veneto subgroup rpX-D was identified (Figure 2, and Table 1).

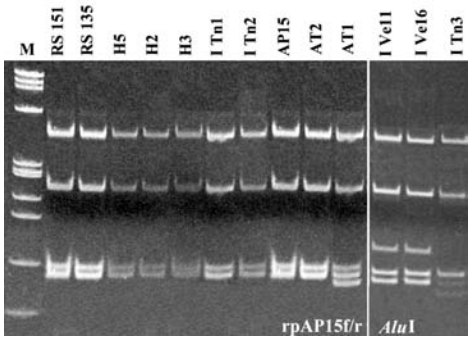

Fig. 2 Polyacrylamide gel electrophoresis of RFLP results from selected positive samples (see Table 1) obtained with *Alu*I restriction enzyme on amplicons from rp primers. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194.

Further RFLP characterization on AP13/AP10 amplicons differentiates among strains belonging to rpX-A subgroup: the two samples from Serbia showed AP profiles, while those from Trentino and the only positive among the two samples from Veneto showed AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was confirmed without the rp strain being identified. (Table 1).

Discussion

The combined use of the three molecular markers employed in this study allows the differentiation of '*Ca*. P. mali' strains according to geographical and, in some cases, also with epidemic distribution. The polymorphism detected in the 16S ribosomal region plus spacer region was shown to be related to a restriction site located inside the spacer region in agreement with a recent finding (Casati et al., 2009), therefore not relevant to phytoplasma classification but reliable for AP strain characterization. It was shown that in the Trentino areas where the disease is at epidemic levels, these three molecular markers show a high homogeneity in their RFLP profiles (Bertaccini et al., 2008) indicating a possible link of the epidemic to one phytoplasma strain. Strain variability was detected in samples from Veneto, and the presence of mixed AP strains was observed in the Hungarian samples. Strains showing rpX-D profile were found in several apple growing areas of Veneto region, surrounding areas where the disease is starting to show epidemic tendencies. The combined use of these molecular markers allows differentiating 'Ca. *P. mali*' strains according to their geographical and epidemic distribution.

In several orchards of Veneto vector monitoring by yellow sticky traps was carried out and *C. melanoneura* was consistently detected, while *F. florii* was erratically found, and only one specimen of *C. picta* was captured. Work is in progress to further detect and differentiate these phytoplasma strains in insect vectors and alternative host plants and relate these findings to the epidemiology of the phytoplasma diseases.

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Breeding of rootstocks resistant to apple proliferation disease

Seemüller, E.[1]; Bisognin, C.[2]; Grando, M.S.[2]; Schneider, B.[1]; Velasco, R.[2]; Jarausch, W.[3]
[1] Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany
[2] Fondazione Edmund Mach – IASMA, San Michele all'Adige (TN), Italy
[3] RLP AgroScience, AlPlanta – Institute for Plant Research, Neustadt/W., Germany

Abstract

Apple proliferation (AP) is caused by the wall-less bacterium Candidatus *Phytoplasma mali* and is spread by psyllids. Previous work indicated that, due to the colonization behavior of the causal agent, the disease can be controlled by the use of resistant rootstocks. However, extensive screening revealed no satisfactory resistance in established rootstocks. In contrast, substantial levels of resistance were identified in experimental rootstocks derived from crosses of the apomictic species *Malus sieboldii* and genotypes of *M.* x *domestica* and *M.* x *purpurea*. However, trees on these hybrids are more vigorous and less productive than trees on standard stock M 9. For this reason, a program was initiated to reduce vigor and improve yield by crossing and backcrossing *M. sieboldii* and its apomictic hybrids with M 9 and other dwarfing stocks. From 2001 through 2006 a total of 36 crosses were made. However, only 23 progenies consisted of a substantial number of seedlings while the other crosses largely failed due to pollen incompatibility. The 3,500 seedlings obtained were DNA-typed using codominant SSR markers to distinguish apomicts and recombinants in the progenies. A total of 1,800 seedlings consisting of all recombinants and a representative number of apomicts were screened for AP resistance by graft inoculation followed by observation in the nursery and under commercial growing conditions. Several progenies showed a good inheritance of resistance. In two of them (4608 x M 9 and D2212 x M 9) more than 50% of the individuals never developed symptoms.

Keywords: Candidatus *Phytoplasma mali*, *Malus sieboldii*, SSR analysis

Introduction

Apple proliferation (AP) is caused by the wall-less bacterium Candidatus *Phytoplasma mali*, and is a serious disease in several major apple-growing areas of western and central Europe. The disease is difficult to control. The most often recommended means, phytosanitary measures and insecticide treatments against the insect vectors, are often not fully satisfactory. The most promising approach to control AP would be the use of resistant plants.

Previous work revealed that phytoplasma colonization in the aerial parts of the tree is subject to seasonal fluctuation. Ca. *P. mali* is eliminated in the top during winter and cannot be detected by fluorescence microscopy in early spring. Transmission by grafting aerial tissue was not possible in this period (Seemüller et al., 1984). Elimination of the pathogen in the stem is due to the degeneration of phloem sieve tubes the functional status of which the pathogen is depending on. Overwintering occurs in the roots where intact sieve tubes and phytoplasmas are present throughout the year (Schaper and Seemüller, 1982). From the roots the stem may be recolonized in spring when new phloem is being formed (Schaper and Seemüller, 1984). This fluctuation in the colonization pattern of the stem has been confirmed recently for Northern Italian (Trentino) conditions (Pedrazzoli et al., 2008). The overwintering of the pathogen in the roots has lead to the presumption that growing scion cultivars on resistant rootstocks can prevent the disease or reduce their impact.

There was little information on the influence of apple rootstocks on the occurrence of AP. Resistance screening of *Malus* x *domestica* rootstocks revealed that there is no satisfactory resistance in the established stocks. However, natural resistance to AP was detected in experimental apomictic rootstocks with *M. sieboldii* in their parentage (Seemüller et al., 1992). These rootstocks, which develop seeds that are genetically identical to the mother, have been developed in the 1950s and 1960s for easy propagation by seeds, virus-free plants, better anchorage and higher resistance to some fungal and bacterial diseases such as crown rot, apple scab, powdery mildew and fire blight (Schmidt, 1988). However, these apomictic rootstocks did not succeed in European apple cultivation because the trees grown on them were mostly more vigorous and less productive than trees on standard stock M 9. Year long field trials carried out with these *M. sieboldii*–derived rootstocks under natural and experimental infection conditions confirmed the resistance which is characterized by the absence of symptoms and a highly reduced concentration of the pathogen in the plant. These field trials demonstrated the feasibility of the adopted resistance strategy by preventing the development of aerial symptoms including undersized fruits in susceptible cultivars (Bisognin et al., 2009; Seemüller et al., 2008) .

Based on these findings a breeding program was started in 2001 which aims to improve the agronomic value of the *M. sieboldii*–derived rootstocks.

Material and methods

Parentage and ploidy levels of AP-resistant apomictic *M. sieboldii* hybrids used in the crosses made from 2001-2006 are listed in Table 1. Details on pollination, seedling growing and microsatellite or simple sequence repeat (SSR) analysis to identify recombinant seedlings, were as described by Bisognin et al. (Bisognin et al., 2009). Recombinant genotypes were screened by graft-inoculating potted seedlings in the first or second year of growth using scions from Golden Delicious trees showing witches'-broom symptoms. Evaluation of resistance was carried out in a three-step procedure. Following inoculation in July or August, the plants were kept in pots until the spring of the following year. In this period, highly susceptible plants declined or were severely stunted. Such plants were discarded whereas the lightly affected and unaffected plants were transplanted to the nursery where they were observed for two or three years. Plants that never showed symptoms or recovered from disease in the nursery phase were considered to be resistant and were transplanted at standard spacing for further evaluation for resistance and pomological traits under commercial growing conditions. Quantitative real-time PCR for Ca. *P. mali* quantification was carried out as described (Seemüller and Schneider, 2007).

Tab. 1 Parentage and ploidy level of AP-resistant apomictic *M. sieboldii* hybrids used in breeding from 2001-2006

***M. sieboldii* Hybrid**	**Parentage**	**Ploidy level**
4551	*M.* x *domestica* cv Laxton's Superb x *M. sieboldii*	3n
4608	*M. purpurea* cv Eleyi x *M. sieboldii*	3n
C1907	4608, open pollinated	4n
D2118	4556, open pollinated	4n
D2212	(*M.* x *domestica* cv Laxton's Superb x *M. sieboldii*), open pollinated	4n
Gi477/4	4808, open pollinated	4n
H0801	(*M.* x *domestica* cv Laxton's Superb x *M. sieboldii*) x M 9	4n
H0909	(*M.* x *domestica* cv Laxton's Superb x *M. sieboldii*) x M 9	4n

Results and discussion

Breeding data and SSR categories: From 2001 through 2006 a total of 36 crosses were made. The AP-resistant parents *M. sieboldii* and the F_1 and F_2 hybrids of *M. sieboldii* listed in Table 1 were crossed mainly with M 9, occasionally with other dwarfing rootstocks P 22, M 27 and Supporter 1, or with the scion cultivar Gala in order to reduce vigor and improve productivity. Of the crosses made, 23 yielded a satisfactory number of seedlings. In contrast, crosses with the triploid *M. sieboldii* hybrids 4608 and 4551 as male parents resulted in few or no seeds due to unsuitable pollen properties. Also, tetraploid apomicts such as *M. sieboldii* and hybrid D2212 were often poor pollinators for M 9 and other *M.* x *domestica*-based genotypes. For these reasons, the resistant apomicts were preferentially used as seed parents.

As expected for apomictic seed parents, SSR analysis revealed that the majority of the plants of most progenies was genetically identical to the mother. However, apomixis is not obligate in *Rosaceae* and additional fertilization following autopollination and cross pollination is possible. Crosses of triploid apomictic seed parents 4608 and 4551 with a diploid pollen parent, such as M 9, resulted in two categories of seedlings, namely motherlike plants and plants having the unreduced maternal genome combined with a recombinant set of alleles from the male parent (in the following referred to as 'hybrid I'). A higher variability in the progeny was obtained in crosses of tetraploid apomicts with M 9 or other diploid *M. x domestica* genotypes as pollen parents. In such cases, two additional combinatorial categories occurred that included full recombinants (recombination of the maternal and paternal genotype, referred to as 'hybrid II') and plants derived from autopollination. Thus, such combinations were preferred in the more recent crosses because they appeared to offer better chances to obtain suitable genotypes for commercial apple growing. The breeding products obtained up to the crosses made in 2006 sum up to a total of more than 3,500 plants. Major successful crosses were 4608 x M 9, 4551 x M 9, H0909 x M 9, H0909 x Supporter 1, D2212 x M 9, Gala x *M. sieboldii*, D2118 x M 9, C1907 x M 9 and H0801 x M 9. In 2007 and 2008 the breeding program was continued by using genotypes derived from the previous cross 4608 x M 9.

Evaluation of resistance: Resistance of the progenies obtained from the resistant apomicts was evaluated by rating the various disease symptoms and accumulating the annual values over the entire observation period (Seemüller et al., 2008). This procedure leads to a cumulative disease index (CDI) for each seedling or each progeny. Low CDI values associated with a high percentage of plants that never showed symptoms or only temporarily mild symptoms were only shown by the progenies of the 4608 x M 9 and D2212 x M 9 crosses. Progenies obtained from crosses of other resistant apomicts as seed parents with *M. x domestica* genotypes showed distinctly higher disease values. These higher CDI

values might be at least partially due to the high sensitivity of some genotypes to latent apple viruses (Seemüller et al., 2008).

Preliminary data from the orchard-scale screening are only available for the progenies of the crosses made in 2001 and 2002. From these progenies, a total of 254 trees mostly grown on recombinant rootstocks were transplanted from the nursery to the orchard in 2005 and 2006, respectively. Most of these trees did not develop any symptoms or developed only temporarily mild symptoms such as foliar reddening. Only 9 % of the trees showed moderate symptoms, mainly reduced vigor in combination with foliar reddening. In contrast, similarly inoculated control trees on M 9 exhibited permanently severe symptoms including undersized fruits and strongly reduced vigor. Trees on resistant rootstocks varied considerably in yield and vigor. Phytoplasma concentration in trees on resistant and susceptible rootstocks was determined in progenies of the cross H0909 x M 9 during the screening period in the nursery. RT-PCR analysis of inoculated trees revealed that the titer in the roots of moderately to severely affected trees (CDI ≤1) is 15 to 23 times higher than in trees showing no or mild symptoms (CDI >1). In the shoots the differences are in the range of 60 in the two symptom categories. These findings are in agreement with the previous finding that the phytoplasma concentration in trees on resistant, *M. sieboldii*-based rootstocks is lower than in trees on susceptible stocks (Bisognin et al., 2008).

Conclusions

In this breeding project *M. sieboldii* and several apomictic *M. sieboldii*-derived hybrids were crossed with *M.* x *domestica*-based genotypes, mainly rootstock M 9, in order to reduce vigor and improve yield of trees on apomicts. Screening of this material by graft inoculation revealed segregation of the resistance trait. In crosses of resistant apomicts that were tolerant to latent apple viruses more than 50 % of the recombinant progeny showed a high level of AP resistance. A considerable number of AP-resistant and virus-tolerant seedlings were also obtained from virus-sensitive apomictic parents. Promising seedlings are being screened for pomological suitability under commercial growing conditions. In these field tests, a considerable variability in vigor and productivity within recombinant progeny plants are being observed. The inheritance of AP resistance and the variability in vigor and productivity offer a good chance to select AP-resistant stocks with suitable pomological properties.

Acknowledgement

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Influence of *Apple stem grooving virus* on *Malus sieboldii*-derived apple proliferation resistant rootstocks

Liebenberg, A.; Wetzel, T.; Kappis, A.; Herdemertens M.; Krczal, G.; Jarausch, W.
RLP AgroScience, AlPlanta – Institute for Plant Research, Breitenweg 71, 67435 Neustadt, Germany

Abstract

Apple stem grooving virus (ASGV, Capillovirus) is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *Malus sieboldii*. Recently, *M. sieboldii* and its hybrids have gained new interest in Europe as they confer resistance to apple proliferation (AP) disease. A new breeding program aiming to develop AP-resistant rootstocks of agronomic value for modern apple culture, reported unexpected tree decline which was to be associated with ASGV. As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV associated with tree decline was cloned and sequenced. Sequence comparisons with available ASGV isolates revealed two regions of high variability in the genome. The genetic variability of additional isolates from Germany and other countries were collected and the variable areas characterised. In addition ASGV was successfully maintained in micropropagated apple trees and could be transmitted by *in vitro* grafting to various genotypes, making it possible to study *in vitro* the effect of the virus and virus/phytoplama combination on *M. sieboldii*-derived genotypes.

Keywords: Latent apple viruses, Candidatus *Phytoplasma mali*, micropropagation, *in vitro* grafting, genetic variability.

Introduction

Apple proliferation (AP) disease is one of the most devastating fruit tree diseases in Europe. The disease is caused by Candidatus *Phytoplasma mali* and causes significant economic loss by rendering the fruit unmarketable. Symptoms include “witches brooms”, enlarged stipules, growth reduction and undersized fruits (Seemüller et al., 2008). The planting of healthy material and elimination of diseased trees are not sufficient in controlling the spread of the disease because of the difficulty controlling the two psyllid vectors, *Cacopsylla picta* and *C. melanoneura* (Frisinghelli et al., 2000; Tedeschi and Alma 2004). The breeding of apple proliferation resistant rootstocks is considered to be the most promising solution to control apple proliferation disease. Natural resistance was discovered in the wild *Malus* species *M. sieboldii* making it a prime candidate for breeding experiments to develop apple proliferation resistant rootstocks (Kartte and Seemüller, 1988, 1991; Jarausch et al., 2008; Seemüller et al., 1992, 2007). During the screening of *M. sieboldii* and its hybrids for AP-resistance, unexpected tree decline was observed and found to be associated with latent apple viruses (Seemüller et al., 2008). An AP-resistance screening system has also been established *in vitro* (Bisognin et al., 2008) which is based on *in vitro* graft inoculation of *Ca.* P. mali (Jarausch et al., 1999). After the report of Seemüller et al. (2008) about virus-associated tree decline in the breeding progeny a similar decline could be observed in the *in vit*ro resistance screening. In this case the decline was associated with *Apple stem grooving virus* (ASGV) alone.

ASGV is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *M. sieboldii* (Yanase, 1974; 1981). *Citrus tatter leaf virus*, a strain of ASGV, causes bud union incompatibility and necrosis when grafted on sensitive citrus material (Calavan et al., 1963; Miyakawa and Matsui, 1977; Miyakawa and Ito, 2000). ASGV has a positive sense single stranded genome consisting of 6496bp (excluding the poly A tail) containing two overlapping open reading frames (ORF’s) (Yoshikawa & Takahashi, 1988; Yanase et al., 1990; Yoshikawa et al., 1992), with two areas of high variability,V1 from amino acid (aa) 530-570 and V2 from aa 1583-1868 (Tatineni et al., 2009). The natural transmission is unknown. It is transmitted through grafting of infected material.

The objective of this study is to understand the influence of latent viruses on phytoplasma resistant genotypes. We established an *in vitro* test system to better understand the virus and the virus isolates associated with the rapid decline in phytoplasma resistant plants.

Materials and methods

Healthy, ASGV and/or Ca. *P. mali* infected Malus shoot cultures were propagated in a growth chamber on modified Murashige & Skoog medium as described (Jarausch et al., 1996, 1999; Ciccotti et al., 2008). The plantlets were subcultured every 6 to 8 weeks. ASGV and/or Ca. *P. mali* infected lines were developed by grafting ASGV and/or Ca. *P. mali* infected material on healthy Malus plantlets (Jarausch et al., 1999). Healthy genotypes were *M.* x *domestica* cv. Golden Delicious and *Malus sieboldii.* The graft contact was maintained for 6-8 weeks and then, graft tips were removed and tested for infection. Total nucleic acid from the inoculated rootstocks was extracted using the CTAB method described in Jarausch et al. (2004) and tested with RT-PCR and PCR using pathogen specific primers for ASGV and Ca. *P. mali*, respectively (Massart et al., 2008; Jarausch et al., 1994).

The complete genome of a German isolate of ASGV was amplified cloned and sequenced according to standard procedures. ASGV variability analysis was performed on isolates received form Germany, Canada, France, Australia and Austria. The two variable regions were amplified and the generated fragments cloned and sequenced. BioEdit (Ver. 7.0.4) (Hall, 1999) was used to perform sequence editing and compilation. Generated ASGV nucleotide sequences were compared to ASGV sequences downloaded from GenBank, using the ClustalW (Ver. 1.4) alignment function embedded in the BioEdit software.

Results

As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV (ASGV-AC) associated with tree decline was cloned and sequenced. The 6496bp (excluding the poly A tail) generated ASGV-AC sequence showed 80.6-82.5 % identities when compared to available ASGV sequences from GenBank. Sequence comparisons also revealed two regions of high variability in the genome, consistent with the findings of Tatineni et al., (2009). The genetic variability of additional isolates from Germany, Canada, France, Australia and Austria were collected and the two variable areas characterised. Phylogenetic analysis showed that all the studied German isolates grouped together.

Tissue culture lines of *M.* x *domestica* cv. Rubinette infected with ASGV or Ca. *P. mali* have been established previously in the lab. In this work, repeated molecular analysis of the viral infection status has been used to select and maintain homogenous culture lines infected with the specific ASGV-AC isolate. This culture line was used to successfully transmit ASGV by *in vitro* grafting to *M. sieboldii* and *M.* x *domestica* cv. Golden Delicious yielding an overall transmission rate of 32 %. These grafting experiments were performed to reproduce *in vitro* the putative hypersensitive reaction of *M. sieboldii* to ASGV in comparison to the reaction of the virus tolerant cultivar Golden Delicious. And indeed, *M. sieboldii* showed a high incidence of graft union necrosis when grafted with ASGV infected material. No necrosis was observed on the Golden Delicious controls. These data are preliminary and need confirmation, but they show that the *in vitro* system can be used to study the interactions associated with the rapid decline observed in *M. sieboldii* derived AP-resistant genotypes.

Conclusion

Tissue culture lines were developed and established making it possible to reproduce symptom expression observed in the field for the system ASGV – Ca. *P. mali – M. sieboldii*. The tissue culture system allows us to monitor and enhance symptom expression under controlled conditions. As the interaction between virus and phytoplasma is poorly understood, this system provides a means to analyse the effect of these two pathogens on each other's titres, symptom expression and transmission capabilities. To understand these interactions more knowledge is required on the different Ca. *P. mali* strains, virus genome functions and variability and host reactions to the pathogens. The German ASGV-AC isolate was successfully cloned and sequenced and will be used to construct an infectious clone.

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Infection rates of natural psyllid populations with 'Candidatus *Phytoplasma mali*' in South Tyrol (Northern Italy)

Baric, S.; Öttl, S.; Dalla Via, J.
Research Centre for Agriculture and Forestry Laimburg, I-39040 Auer/Ora (BZ), Italy

Abstract

Apple proliferation is a severe disease of apple trees spreading in many European apple growing areas. It is caused by 'Candidatus *Phytoplasma mali*' that was shown to be transmitted through infected grafting material, via natural root grafts and by sap-sucking insects. Two psyllid species, *Cacopsylla picta* and *C. melanoneura*, that are recognised as the vectors of the disease, occur in orchards of South Tyrol (Northern Italy). The aim of this study was to assess the infection rates of natural populations of these insect species with 'Ca. *P. mali*'. Two additional psyllid species (*C. mali* and *Trioza urticae*), which are frequent in some apple orchards of South Tyrol, were also investigated. A total of 801 specimens from 18 orchards was analysed using a real-time PCR procedure. While no specimen of *T. urticae* was found to be infected with 'Ca. *P. mali*', the mean infection rate of *C. melanoneura* and *C. mali* was below 1 %. The highest infection rate was found for *C. picta*, with a mean value of 11 % and peaking at 33%. Based on these results, it can be concluded that *C. picta* plays the major role as the vector of apple proliferation in South Tyrol.

Keywords: apple proliferation, *Cacopsylla mali*, *Cacopsylla melanoneura*, *Cacopsylla picta*, pathogen transmission, *Trioza urticae*

Introduction

Apple proliferation (AP), a disease of apple trees caused by 'Candidatus *Phytoplasma mali*' (Seemüller and Schneider 2004), has been spreading over the last decade in many European apple growing areas (Frisinghelli et al. 2000; Tedeschi et al. 2003; Carraro et al. 2008; Mayer et al. 2009). Apart from proliferation of auxiliary shoots (witches' brooms), enlarged stipules, chlorosis, yellowing or early leaf reddening, the pathogen can induce symptoms of economic relevance such as decreased size, quality and overall yield of fruit (Kartte and Seemüller 1988). The AP phytoplasma was shown to be transmitted through grafting of infected propagation material (Kartte and Seemüller 1988), via natural root grafts (Baric et al. 2008; Ciccotti et al. 2008) and by sap-sucking insects. So far, two psyllid species, *Cacopsylla picta* and *C. melanoneura*, were identified as the vectors of AP phytoplasma (Frisinghelli et al. 2000; Tedeschi and Alma 2004). In addition, the leafhopper *Fieberiella flori* was suggested as a further vector of this disease (Krczal et al. 1989; Tedeschi and Alma 2006). Since there is no therapy available to cure infected trees, the only possibility to control the disease is to prevent it from spreading by planting healthy material, uprooting diseased plants and vector control.

In order to propose a strategy for insect vector control in the Autonomous Province of South Tyrol (Northern Italy), the psyllid fauna present in the apple orchards was monitored during the vegetation period of 2006 (Walch 2006). The study revealed the presence of 13 psyllid species: *C. melanoneura*, *C. picta*, *C. mali*, *C. pyri*, *C. pyricola*, *C. brunneipennis*, *C. nigrita*, *C. affinis*, *C. crataegi*, *C. pruni*, *Psylla alni*, *Trioza urticae* and *Bactericera albiventris* (Walch 2006), though at different frequencies. The highest number of collected individuals was assigned to two species, *C. melanoneura* (80 %) and *C. picta* (6 %), while the occurrence of *C. mali* was notable in abandoned orchards with up to 8.9 individuals per branch (Walch 2006). The aim of the present study was to assess the infection rates with 'Ca. *P. mali*' of the most common psyllid species in South Tyrolean orchards in order to provide additional data about their potential role for the propagation of AP in this region.

Material and methods

Psyllids were captured by beating tray sampling in orchards distributed all over the apple production area of South Tyrol from March until June 2006 (Walch 2006). After species determination, insects were preserved in absolute ethanol. 801 specimens comprising four different species from 18 AP affected apple orchards were selected for DNA analysis (Table 1 and Figure 1). Preference was given to samples with higher specimen numbers to more reliably estimate the proportion of AP infected psyllids per orchard. The orchards considered in the study were mainly managed according to the principles of integrated production, while one orchard was organic and four abandoned. Nucleic acid was isolated from individual insects by following the procedure of Marzachì et al. (1998). Each DNA isolate was analysed in duplicate applying a highly sensitive TaqMan real-time PCR approach for the specific detection of AP phytoplasma (Baric and Dalla Via 2004; Baric et al. 2006).

Tab. 1 Number of specimens of four psyllid species from different localities in South Tyrol (Northern Italy) tested for the presence of AP phytoplasma by real-time PCR

		Cacopsylla picta		*Cacopsylla melanoneura*		*Cacopsylla mali*		*Trioza urticae*	
	Apple orchard locality	**N analysed**	**N AP positive (%)**	**N analysed**	**N AP positive (%)**	**N analysed**	**N AP positive (%)**	**N analysed**	**N AP positive**
1	Mals/Malles	n.p.		47	0	n.p.		n.p.	
2	Glurns/Glorenza	n.p.		31	0	2	0	n.p.	
3	Latsch/Laces	14	0	24	1 (4.2)	n.p.		n.p.	
4	Naturns/Naturno[a]	18	1 (5.6)	113	1 (0.9)	n.p.		n.p.	
5	Saltaus/Saltusio	2	0	n.p.		n.p.		n.p.	
6	Schenna/Scena[a]	n.p.		n.p.	.	113	0	n.p.	
7	Tscherms/Cermes-A[a]	15	1 (6.7)	84	0	111	2 (1.8)	n.p.	
8	Tscherms/Cermes-B	27	9 (33.3)	n.p.		n.p.		n.p.	
9	Tscherms/Cermes-C	16	1 (6.3)	n.p.		n.p.		n.p.	
10	Tscherms/Cermes-D	3	0	n.p.		n.p.		n.p.	
11	Lana-A	12	0	n.p.		n.p.		n.p.	
12	Lana-B	58	7 (12.1)	5	0	n.p.		n.p.	
13	Lana-C	5	0	n.p.		n.p.		n.p.	
14	Tisens/Tesimo	28	2 (7.1)	n.p.		n.p.		n.p.	
15	Nals/Nalles[b]	10	2 (20.0)	8	0	n.p.		n.p.	
16	Eppan/Appiano-A	n.p.		31	0	n.p.		n.p.	
17	Eppan/Appiano-B	n.p.		3	0	n.p.		n.p.	
18	Salurn/Salorno[a]	n.p.		n.a.		n.p.		21	0
	Total	208	23 (11.1)	346	2 (0.6)	226	2 (0.9)	21	0

n.p., no specimens present in the beating tray sample; n.a., not analysed because of small sample size
[a] abandoned orchard; [b] organically managed orchar

Fig. 1 Map of South Tyrol (Northern Italy) showing the locations where psyllid samples were collected. The number of orchards sampled at each location is given in the circles.

Results

The AP phytoplasma was detected in 27 out of the 801 specimens tested with the real-time PCR assay (see Table 1). The highest number of infected individuals (N = 23) was found for *C. picta*, the infection rates in the seven positively tested orchards ranging from 5.6 to 33.3 % (Table 1). Orchards lacking infected *C. picta* specimens were generally represented by low sample numbers. Two *C. melanoneura* individuals, each from a different orchard, were tested AP

positive, resulting in infection rates of 0.9 and 4.2 %. The same number of AP positive individuals was found for *C. mali* which was particularly abundant in two of the three sampling sites where this species was present (Table 1). Both infected specimens originated from the same orchard (Tscherms/Cermes-A). *Trioza urticae* was found in higher numbers in a single orchard in the very south of the investigation area and none of the 21 individuals analysed was found to carry 'Ca. *P. mali'* (Table 1).

Discussion

Apple trees with symptoms of AP have been observed in the intensive orchards of South Tyrol since 1998. In the first years, however, the disease was mainly confined to orchards situated on the slopes of the low mountain range (Österreicher and Thomann 2003). The highest number of AP symptomatic apple trees, amounting to 520,000, was ultimately reached in 2006 (Mair 2009). Although by that time the disease had spread all over South Tyrol, the hot-spot was located in the district of Burggrafenamt/Burgraviato, where in some orchards more than 30% of the apple trees showed pronounced AP symptoms (Österreicher and Unterthurner 2006).

The recent explosive outbreak of the disease in South Tyrol could be related to the occurrence of *C. picta* in this area. This species was first noticed in 2004 in a single orchard (Wolf and Zelger 2006) and has since been observed in the entire apple production area except in the district of Eisacktal/Valle Isarco (Unterthurner and Österreicher 2008). Again, the highest number of orchards harbouring *C. picta* was located in the district of Burggrafenamt/Burgraviato (Wolf and Zelger 2006; Unterthurner and Österreicher 2008), even if the maximum density did not exceed 1.8 individuals per branch (Walch 2006).

Cacopsylla picta had been proven as an efficient vector of 'Ca. *P. mali*' in several transmission trials (Frisinghelli et al. 2000; Jarausch et al. 2003; Seemüller et al. 2004; Carraro et al. 2008). Furthermore, various studies have demonstrated that a high percentage of individuals occurring in apple orchards can carry the AP phytoplasma and the average natural infection rate of 11.1 % in South Tyrol is absolutely comparable with the results from Germany, Northern France and Switzerland (Jarausch et al. 2007) as well as the neighbouring Italian Region of Trentino (Cainelli et al. 2004). In contrast, the mean infection rate of *C. melanoneura* was almost 20-fold lower (0.6 %). Even though this species was shown to be able to acquire 'Ca. *P. mali*' (Pedrazolli et al. 2007), extensive transmission experiments performed with *C. melanoneura* in Germany (Mayer et al. 2009) and South Tyrol (Wolf et al. 2003) failed to infect healthy test plants, while in Trentino the pathogen was successfully transmitted in only one of the 278 trials performed over a six-year period (Mattedi et al. 2008). In northeastern Italy, however, *C. melanoneura* is considered the main vector of 'Ca. *P. mali*' (Tedeschi and Alma 2004), since its transmission to healthy test plants was successful with naturally and experimentally infected specimens. In addition, the pathogen was commonly detected in natural populations of overwintered adults with maximum infection rates of 3.5 % (Tedeschi et al. 2003). The differences in vectoring ability found for *C. melanoneura* in different studies may be caused by the existence of distinct populations varying in their capacity to acquire and transmit the AP phytoplasma (Mayer et al. 2009). On the other hand, 'Ca. *P. mali*' was found to be genetically highly variable (Schneider and Seemüller 2009) and one could speculate that particular strains may be transmitted by *C. melanoneura* while others may be vectored by *C. picta*. However, this speculation needs to be further investigated by typing 'Ca. *P. mali*' isolates from different geographic regions and vector populations.

Low natural infection rates with 'Ca. *P. mali*' of 0.9 % were determined for *Cacopsylla mali*. This species is known to occur abundantly in untreated and abandoned apple orchards (Seemüller et al. 2004), which was also the case in South Tyrol (Walch 2006). While *C. mali* was shown to carry the pathogen, it has not been confirmed as a vector of 'Ca. *P. mali*', although transmission experiments involved a large number of individuals (Seemüller et al. 2004). Our findings seem to confirm that the ability of Ca. *P. mali* to acquire the phytoplasma is poor, since all the specimens tested were collected from an area with a high number of AP-affected apple trees.

Based on the relatively high infection rates of natural *C. picta* populations, the frequent occurrence of this species in many commercial apple orchards and its proven vectoring ability, we conclude that this species carries the largest risk for the spread of 'Ca. P. mali' in South Tyrol. Therefore, management strategies for vector control should focus on this species. However, further research on insect vectors is necessary to explain the dissemination of AP in areas where this species has so far never been observed.

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Comparison of European stone fruit yellows phytoplasma strains differing in virulence by multi-gene sequence analyses

Marcone, C.[1]; Schneider, B.[2]; Seemüller, E.[2]
[1] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Via Ponte Don Melillo, I-84084 Fisciano (Salerno), Italy
[2] Julius Kuehn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Strasse 101, D-69221 Dossenheim, Germany

Abstract

Twenty strains of the ESFY phytoplasma, which on the basis of graft-inoculation experiments greatly differ in aggressiveness, were examined by sequence analyses of several PCR-amplified non-ribosomal genes in order to identify molecular markers linked to virulence. These strains, which were maintained in *P. insititia* rootstock St. Julien GF 655/2 were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and RFLP analyses of PCR-amplified rDNA. Also, the virulent ESFY strains maintained in periwinkle, namely GSFY1, GSFY2 and ESFY1, as well as an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in recovered apricot trees in France and used there as a cross protecting agent, were included in the work for comparison. For PCR amplification, primers were designed from a number of genes distributed over the chromosome of the closely related apple proliferation phytoplasma strain AT. Visible PCR products were only obtained with primer pairs derived from the *tuf* gene which encodes the elongation factor Tu (EF-Tu), *rps*C (*rps*3) gene encoding the ribosomal protein S3, *tly*C gene which encodes a hemolysin known as a membrane-damaging agent and important virulence factor of many bacteria, the *imp* and *fol* genes encoding an immunodominant membrane protein and an enzyme involved in the folate biosynthesis, respectively. Nucleotide sequence comparisons revealed that the highest genomic variability occurred within the *imp* gene sequence with dissimilarity values ranging from 0.2 to 4.6%. For the remaining genes, the strains examined proved to be identical or nearly identical. Within the *tuf* gene, an extra *Taq*I site known to occur in strain GSFY1 was not identified in other strains. The genetic differences observed among the strains examined are neither suitable markers for strain differentiation nor linked to pathological traits.

Keywords: European stone fruit yellows, strain virulence, 16SrX group, *tly*C gene, *Prunus* spp.

Introduction

The European stone fruit yellows (ESFY) agent 'Candidatus *Phytoplasma prunorum*' is an important prokaryotic pathogen that infects most or all kinds of stone fruits in Europe and is known to cause apricot chlorotic leaf roll of apricot (*Prunus armeniaca*), leptonecrosis and decline of Japanese plum (*P. salicina*), yellows and decline diseases of peach (*P. persica*), Molières disease of sweet cherry (*P. avium*) and European plum (*P. domestica*), and other diseases that include those affecting almond (*P. dulcis*) and flowering cherry (*P. serrulata*) (Lorenz et al., 1994). This organism is closely related to important fruit trees pathogens like apple proliferation (AP), pear decline (PD) and peach yellow leaf roll (PYLR) agents. Together they form a distinct phylogenetic cluster, the AP- or 16SrX group (Seemüller et al., 1998; IRPCM, 2004). Previous work has shown that strains of the ESFY phytoplasma greatly differed in virulence when examined by graft inoculation of trees on peach, peach hybrid GF 677 and *Prunus* 'Marianna' GF 8/1 rootstocks. While some strains were nearly avirulent or weakly virulent and induced only mild foliar symptoms and slightly reduced vigor but no mortality, others were highly virulent and caused severe symptoms and a high mortality rate of affected trees (Kison and Seemüller, 2001). However, the strains showing pathological differences were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) as well as Southern blot hybridization using probes suitable for the differentiation of the fruit tree phytoplasmas of the AP group (Kison and Seemüller, 2001; Seemüller and Schneider, 2004). Knowledge of genes mediating pathogenicity and virulence is urgently needed for insights into phytoplasma pathology and for studying plant resistance against phytoplasmas. Thus, twenty differently virulent strains of the ESFY phytoplasma were examined by analysing the sequence of several PCR-amplified non-ribosomal genes in order to attempt to identifymolecular markers linked to virulence.

Materials and methods

Phytoplasma Sources. Twenty strains of the ESFY phytoplasma differing in virulence that were maintained in *P. insititia* rootstock St. Julien GF 655/2 in the experimental field at institute in Dossenheim, were examined (Table 1). Also, the virulent ESFY phytoplasma strains maintained in *Catharanthus roseus* (periwinkle), namely GSFY1, GSFY2 and ESFY1 (Marcone and Seemüller, 2001) as well as four isolates (F1, F2, F3 and F4) of an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in a recovered apricot tree in France and used there as a cross protecting agent to control the apricot chlorotic leaf roll disease (Morvan et al., 1986; Castelain et al., 1997), were included in the work for comparison. The latter isolates are also indistinguishable from severe strains of ESFY phytoplasma on the basis of RFLP analysis of PCR-amplified rDNA (unpublished data). DNA samples from strain AT of the AP phytoplasma were also included in this study. DNA Isolation. From trees either petioles, midribs, or phloem tissue from stem portions and roots, approximately 3.0 cm in diameter, were used. Phloem tissue was prepared as described (Ahrens and Seemüller, 1994). Young shoots including leaves were taken from periwinkle. DNA was isolated from approximately 1.0 g of fresh tissue using a phytoplasma-enrichment procedure as described previously (Ahrens and Seemüller, 1992).

Tab. 1 German strains of the ESFY phytoplasma maintained in stone fruit genotypes in the field and examined in this study.

Strain designation	Designation[a]	Virulence	Vigor reduction
G1*		+	Moderate
G2	Peach 4	(+)	No
G3	Peach 2	(+)	No
G4	Peach 3	+	Moderate
G5	Almond 6	(+)	No
G6	Almond 1	++	Severe
G7	Almond 7	+	Moderate
G8	Almond 4	+	Moderate
G9	Almond 3	++	Severe
G10	Almond 8	(+)	No
G11*		+	Moderate
G12	Apricot 2	(+)	No
G13	Apricot 1	++	Severe
G14*		(+)	No
G15	Apricot 3	(+)	No
G16	Apricot 4	(+)	No
G17	Japanese plum	++	Severe
Gapr1*		++	Severe
Gapr2*		++	Severe
Gapr3*		+	Moderate

[a] Strain designation given by Kison and Seemüller (2001); * Not clearly described.

Primers and PCR amplification. For PCR amplification of ESFY fragments, primers were derived from 'Candidatus *Phytoplasma mali*' strain AT genes listed in Table 2 for which the complete sequence is available (Kube et al., 2008). Amplification was performed in 25-µl reactions containing 0.5 µM of each primer, 0.1 mM each dNTPs, 0.6 U of heat-stable polymerase (Invitrogen), and 1x polymerase buffer. The reaction was subjected to 35 cycles in a thermal cycler (Stratagene) at the following parameters: 95 ºC for 1 min, 50 ºC (48 °C for primers fFol/rFol, and 52 °C for primers fTlyC/rTlyC) for 1 min, and 70ºC for 1 min (10 min for the final cycle). Five microliters of PCR product were analyzed by electrophoresis in a 1.5 % horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in the presence of 0.5 µg/ml ethidium bromide. DNA bands were visualized using a UV transilluminator.

Tab. 2 Oligonucleotide primers used in this study.

Primer	Primer sequence (5'-3')	Sense	Target (gene)
fTuf	GCA AAT GGA CGC TGG TAT TT	Forward	Tuf
rTuf	ACA TTA TAG AAT GGT TAA ATA AGC	Reverse	Tuf
fRpsC	TTG GGA TTC TAA ATG GTT TGC	Forward	RpsC
rRpsC	TTT CGC CTG GTA AAA CAT CA	Reverse	RpsC
fTlyC	CGG TTT AAT GGT TCC TTT CG	Forward	TlyC
rTlyC	CCT GAT AAA ACA AAT AAA TGC CAA A	Reverse	TlyC
fImp	CAA ATG ATA AAG CTG ATC AA	Forward	Imp
rImp	CAC ATC CTT TGT TTA AAA ATT TTA T	Reverse	Imp
fFol	TAA TAT GCT TCC TTG GCA TT	Forward	Fol
rFol	CAA CAA AAA TTA ATT CGG GAT A	Reverse	Fol

Sequence analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and then sequenced either directly or cloned prior to sequencing. For cloning, DNA fragments were ligated into plasmid vector pGEM-T (Promega) and recombinant plasmids used to transform *Escherichia coli* strain DH5α. Sequencing of both strands was performed using a dideoxy chain termination chemistry. Primers for direct sequencing of PCR products were the same as for PCR amplification whereas the standard primers pUC/M13 forward and pUC/M13 reverse were used for sequencing the cloned fragments. Sequence alignments were performed by using CLUSTAL, version 5, with LaserGene software (DNASTAR).

Results and discussion

With PCR assays, visible PCR products from all strains tested were only obtained with the primer pairs fTuf/rTuf, fRpsC/rRpsC, fTlyC/rTlyC, fImp/rImp and fFol/rFol. Primers fTuf/rTuf derived from the *tuf* gene encodes the elongation factor Tu (EF-Tu) and mediates the transport of aminoacyl-tRNA to the codon recognition site of ribosomes; primers fRpsC/rRpsC derived from the *rps*C (*rps*3) gene encoding the ribosomal protein S3 is an essential component of ribosomes; fTlyC/rTlyC were from *tly*C gene which encodes a hemolysin, a membrane-damaging agent which has been implicated as a virulence factor for a variety of human pathogens, mainly Gram-positive bacteria (Radulovic et al., 1999); and, fImp/rImp and fFol/rFol from the *imp* and *fol* genes encoding an immunodominant membrane protein and an enzyme essential for folate biosynthesis, respectively. The sizes of the amplified PCR fragments were identical to those obtained from strain AT. Other selected primers failed to amplify the target DNA from ESFY phytoplasma strains at different annealing temperatures. A representative number of amplimers from strains of each virulence category was sequenced directly or after cloning.

Nucleotide sequence comparisons revealed that the highest genomic variability occurred in the *imp* gene with similarity values ranging from 95.4 (GSFY1 *versus* G17) to 99.8 % (F1 *versus* G17). The highest sequence similarity value was shared by two differently virulent strains, the avirulent strain F1 and the severe strain G17, whereas the greatest dissimilarity occurred between two strains of the same virulence category, namely GSFY1 and G17, both virulent. For *tuf* and *tly*C genes, the strains examined proved to be identical or nearly identical with similarity values between 99.7 and 99.9 %, and 99.8 and 100 %, respectively. No dissimilarities were observed in both *rps*C and *fol* genes. Within the *imp* gene, the polymorphisms observed in the sequence of a given strain were usually not shared by the strains of the same virulence category. For instance, the polymorphism at position 641 where T was replaced by a C residue, in the mild strain G3, did not occur in other mild or avirulent strains such as F1 and G1. Similar results were obtained for *tly*C and *tuf* genes. In the *tly*C gene, the polymorphism at position 50 where G was replaced by an A residue occurred in both virulent and avirulent strains. In the *tuf* gene, the substitution of C with an A residue in the virulent strain GSFY2 at position 840, did not occur in the other virulent strains GSFY1, G17, ESFY1 and G13. Also, within the *tuf* gene, the presence of an additional *Taq*I restriction site, in the virulent strain GSFY1 following position 429, due to a substitution of T with a C residue, was not identified for the other strains. This additional *Taq*I restriction site, which had already known to occur in the strain GSFY1, is responsible for differences in restriction profiles as confirmed by RFLP analysis of PCR-amplified *tuf* gene sequences (Marcone et al., 2002).

Results of the present study confirm previous findings that ESFY phytoplasma strains showing pathological differences are relatively homogenous at the level of the molecular markers so far examined. Also, the genetic differences observed among the strains examined, mainly those occurring in the *imp* and *tuf* genes are neither suitable markers for strain differentiation nor linked to pathological traits. Thus, further comparisons of DNA fragments from avirulent and virulent strains are needed to identify genes mediating pathogenicity or virulence. The most promising approach would be the sequence comparison of the entire chromosome from an avirulent and severe strain.

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Hypo- and hyper-virulence in apricot trees infected by European stone fruit yellows

Ermacora, P.; Loi, N.; Ferrini, F.; Loschi, A.; Martini, M.; Osler, R.; Carraro, L.
Dipartimento Biologia e Protezione delle Piante (DiPi), University of Udine, Via delle Scienze 208, 33100 Udine, Italy

Abstract

An apricot orchard, located in an area of north eastern Italy under serious pressure from European stone fruit yellows (ESFY) infection, has been monitored since the year it was planted (1990). During this time, most of the trees displayed symptoms or were shown by PCR analyses to be infected. Two groups of apricot trees were particularly interesting: some trees were asymptomatically infected while others recovered from the symptoms but not from the pathogen. In order to isolate those strains of the phytoplasma characterised by varying virulence, each of the two groups was used as mother plants and propagated. The new trees were used to constitute experimental orchards, where they were observed for the presence of symptoms and in part were tested by PCR, starting in 2003. The results obtained confirmed the presence of strains of the pathogen characterised by varying virulence. The strains originally present in infected apricot trees which recovered from the symptoms of ESFY were seen to be hypovirulent; none of the propagated infected trees ever showed symptoms of the disease. Surprisingly, the strains present in asymptomatic apricot mother plants were hypervirulent and the propagated trees always displayed severe symptoms. In the propagated trees, the transmission of the pathogen was higher in the hypervirulent strains than in the hypovirulent ones. A graft transmission trial carried out in the greenhouse using some of the identified hypo- and hypervirulent strains, confirmed the results obtained in open field. Real time PCR analyses showed that in the trees infected by hypovirulent strains the colonisation of the pathogen was lower than in those infected by the hypervirulent strains. It is possible to affirm that the hypovirulent strains were present in those mother plants which had originally recovered. The research will continue with the aim of verifying the possibility of cross protection among the identified hypo- and hypervirulent strains.

Keywords: 'Candidatus *Phytoplasma prunorum*', real-time PCR, *Prunus*

Introduction

European stone fruit yellows (ESFY), a phytoplasma disease caused by 'Candidatus *Phytoplasma prunorum*' ('Ca. *P. prunorum*') (Seemüller and Schneider, 2004) and spread by *Cacopsylla pruni* (Carraro et al., 1998) is present in several European countries and was previously reported in the Friuli Venezia Giulia region (North East Italy) on several cultivated and spontaneous *Prunus* species (Carraro et al., 2002). Among the cultivated *Prunus* species, *Prunus salicina* and *Prunus armeniaca* are the most frequently affected by ESFY in this region and ESFY frequently became the most relevant phytosanitary problem for these crops, thus reducing the orchard's productive lifespan in economically sustainable terms. Typical ESFY symptoms are: breaking dormancy in winter, yellowing leaves and leaf roll in summer, decline and dieback. Recovery from symptoms was reported but not as commonly as is described in other crops like grapevines and apple-trees (Musetti et al., 2005).

Evidence of variability in virulence between 'Ca. *P. prunorum*' isolates have been reported (Cornaggia et al., 1995; Kison and Seemüller, 2001) and new tools have recently been developed with the aim of obtaining molecular markers for the characterization of the strains according their virulence (Danet et al., 2008; Martini et al., this issue).

The aim of this work was to identify 'Ca. *P. prunorum*' hypovirulent strains and investigate the stability of the hypovirulence in apricot trees in field conditions, and with artificial infection. The final goal of this research was to investigate the feasibility of applying cross protection based on mild strains to reduce losses in commercial orchards.

Material and methods

Field trials: An apricot orchard, cv. 'Reale d'Imola' grafted onto myrobalan rootstocks, which was established in 1990 in an area with a high risk of ESFY infection near Cividale del Friuli (Udine, Italy), was monitored at least twice a year for the expression of ESFY symptoms and samples were collected for molecular analyses in order to confirm the presence of 'Ca. *P. prunorum*'. Two groups of apricot trees were particularly interesting: some trees were asymptomatically infected while others recovered from the symptoms but not from the pathogen. In order to isolate those strains of the phytoplasma characterised by varying virulence, each of the two groups was used as mother plants and propagated. Buds were collected in August 2001 from apricot trees and chip-grafted onto virus-free myrobalan rootstocks. Part of the propagation material underwent heat treatment and buds thus treated were grafted onto virus-free myrobalans.

A total of more than 500 new trees were used to constitute experimental orchards, where they were visually inspected twice a year for the presence of symptoms and in part were tested by PCR, starting in 2003. Healthy controls (virus-free certified 'Reale d'Imola' trees) were planted in the same orchards in order to understand natural ESFY diffusion. Plants were divided into four classes according to symptom severity: class 0 = no symptoms; class 1 = faint symptoms only during the vegetative season, but without a reduction in growth; class 2 = dormancy break, yellowing, leaf roll, no growth reduction, class 3 = dormancy break, yellowing, leaf roll, growth reduction and severe decline.

Greenhouse and screenhouse trials: In July 2006, based on the behaviour of the plants in experimental orchards, two isolates from consistently asymptomatic plants but which gave heavy symptoms in the progeny (hyper-virulent), and two isolates from recovered plants that gave asymptomatic progeny (hypo-virulent) were inoculated by grafting onto sixty seedlings of peach, apricot and myrobalan. As inoculum, 3 apricot buds for plant were used and seedlings were grown in a greenhouse and monitored for graft survival and symptom expression. Leaf samples for molecular analyses were collected in June and September 2007.

The above mentioned ESFY isolates were also used as a source of inoculum for an experimental trial in an insect-proof screenhouse. In August 2007, four groups of ten cv 'Reale d'Imola' apricot trees, each two years old, were grafted with six buds from infected trees. During March 2008 bud survival was recorded; trees were inspected for symptom expression and molecular analyses were carried out in 2008 and 2009.

Molecular analyses: Total DNA from 0.7g apricot midribs was extracted by a slightly modified CTAB method as described by Doyle and Doyle(1990). A nested PCR protocol with P1/P7 and f01/r01 primers was applied in order to detect the presence of 'Ca *P. prunorum*'. Amplified products were visualized by agarose gel electrophoresis and then stained with ethidium bromide. A PCR/RFLP protocol, based on *ace*F genes was adopted to investigate genetic variability of 'Ca. *P. prunorum*' (Danet et al., 2008; Martini et al., this issue). Quantitative detection of phytoplasma, based on a real-time PCR protocol, was performed on some samples following the protocol described by Martini et al., (2008). The plants selected for quantitative experiments were 7 apricots with no ESFY symptoms and 7 apricots with severe ESFY symtpoms.

'Ca. *P. prunorum*' was quantified by SYBR® Green I real-time PCR as the number of 'Ca. *P. prunorum*' genome units (GU)/ng of plant DNA (Marzachì and Bosco, 2005). Ribosomal protein (rp) gene *rpl22* was used as the target for amplification of 'Ca. *P. prunorum*' with primer pair rpLNS2f/rpLNS2r2; whereas the 18S rDNA was chosen as the target for the amplification of plant DNA (Christensen et al., 2004). Standard curves, PCR reactions and cycling conditions were performed as previously described (Martini et al., 2007). The data were analysed using one-directional ANOVA and Student's t-test.

Results

Field trials: After 10 years of observations apricot trees from the original orchard were divided into classes according symptom severity; among them, two groups were considered for further investigation: trees which had never displayed symptoms and trees which recovered. All the trees belonging to the two groups tested positive in PCR.

In the established experimental orchards, according to the adopted clustering method for symptom intensity, the mean score for trees from recovered buds was 0.02. Surprisingly, the strains present in symptom-free apricot mother plants were hypervirulent and the infected trees propagated always showed severe symptoms; over the 5-years period the mean score for symptom intensity was 1.09. The score for ESFY symptoms on control trees naturally infected during the same period was 1.10. Mean graft transmissibility of 'Ca *P. prunorum*' strains from recovered trees to progeny was 10 % while from the symptom-free trees the figure was 84 %. Five years after planting, the natural rate of infection on healthy controls was 73 %; 56 % of the progeny from recovered trees were infected, while the progeny from the symptom-free trees were 100% infected.

PCR analyses on propagated trees at planting time showed the success of heat treatment in the eradication of 'Ca. *P. prunorum*' from infected material. Comparison between heat-treated and untreated material 5 years after planting showed that 77 % of trees obtained from recovered and heat treated material were PCR positive; while 56% of trees obtained from recovered un treated buds were infected.

Greenhouse and screenhouse trials: A graft transmission trial carried out in the greenhouse using some of the identified hypo- and hypervirulent strains, confirmed the results obtained in the open field. The mean transmissibility was 61 % for virulent and 35 % for hypovirulent strains. Among the PCR positive plants grafted with virulent strains 73 % of the trees showed symptoms (100 % of apricot and peach trees, 0 % of myrobalans). The effects of virulent strains on susceptible host apricot and peach trees were stunting, yellowing and dieback. Among the trees which tested positive

for inoculation with hypovirulent strains, only 8% displayed symptoms (17 % apricot, 0 % peach and myrobalans). Symptoms of hypo-virulent strains on apricot were faint leaf-roll and yellowing.

Results from apricot trees artificial infected in a screenhouse highlight the presence of strains with varying degrees of virulence (Figure 1). The first symptoms induced by virulent strains inoculated in August 2007 were dormancy break in February 2008 and severe leaf roll and decline in May-June of the same year.

Fig. 1 Comparison between the effects of strains of 'Ca. *P. prunorum*' in artificial infections on apricot trees in a screenhouse. In the middle a group of trees inoculated with a hyper-virulent strain; on the left and right trees inoculated with a hypo-virulent strain.

Molecular analyses: For quantification of 'Ca. *P. prunorum*' infection level in the tested plant, 18S rDNA served to normalize the data. Slopes of standard curves for quantification of '*Ca.* P. prunorum' DNA (diluted in 20 ng/µl of total DNA from healthy apricot) and quantification of apricot plant DNA indicated PCR efficiencies close to 100 %. Infection levels in symptomatic apricots ranged from 74.53 to $1.25*10^4$ GU of 'Ca. *P. prunorum*'/ng of plant DNA (Figure 2); whereas the infection level in asymptomatic apricots ranged from 32.52 to $1.23x10^3$ GU of 'Ca. *P. prunorum*'/ng of plant DNA (Figure 2). The average infection rate of 'Ca. *P. prunorum*' in symptomatic and asymptomatic apricots was respectively $5.42x10^3$ and $4.66x10^2$ GU of 'Ca. *P. prunorum*'/ng of plant DNA. These results indicated that between the two groups of plants the infection level of '*Ca.* P. prunorum' was higher in the symptomatic plants and more than 10 times different. The ANOVA test demonstrated that the observed divergence was statistically significant for α=0.01.

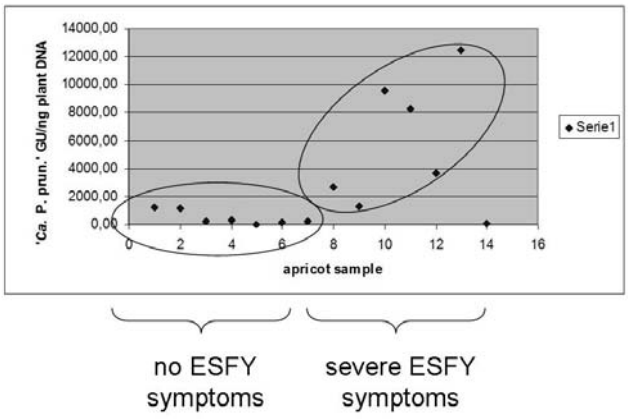

Fig. 2 Quantification of 'Ca. *P. prunorum*' GU/ng plant DNA using SYBR Green I real-time PCR assays in two groups of apricot plants showing respectively no symptoms and severe ESFY symptoms.

Discussion

Previous reports of the presence of 'Ca. *P. prunorum*' strains with varying virulence on apricot and peach trees was confirmed in this study. In particular, by means of long-term monitoring of an apricot orchard in an area with high natural pressure of ESFY infection, it was possible to identify apricot trees that never displayed symptoms but tested positive by PCR analyses. This research focused on two groups of apricot trees: one that never showed symptoms but tested positive in PCR analyses and another group of trees which recovered from the symptoms but not from the pathogen. By artificial infection we demonstrated that in the first case the characteristic of asymptomaticity was plant-related: in fact, progeny obtained from the symptom-free trees themselves displayed symptoms, and artificial infection of several *Prunus* spp. confirm the virulence of this strains, which are also highly transmissible by grafting. By contrast, strains isolated from recovered trees never induced symptoms in the progeny, even in greenhouse test trees, and are only weakly transmissible by grafting. The heat-treated material seemed to be characterized by higher susceptibility to ESFY infection than the un treated material in the first years after planting, (data not shown) but the percentage of infected plants in the groups of apricot trees become similar after 5 years from planting.

A Real-time PCR quantification method was applied so as to better understand the correlations between phytoplasma concentration and symptom intensity. First results seem to correlate low phytoplasma concentration and an absence of symptoms for apricot trees infected with mild strains; strains that induce severe symptoms, on the other hand, seem to be able to achieve high concentrations in the host. The high percentage of symptoms on plants used as negative controls reflects the high disease pressure in the test area, and probably, the greater fitness of virulent strains in these conditions.

Further investigation is needed to clarify the stability of hypovirulence on trees and the interaction between hypo- and hypervirulent strains in mixed infections in order to utilize mild strains for ESFY management.

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Tomato ringspot nepovirus (ToRSV) in wild blackberry (*Rubus fruticosus* L.) in Hatay province of Turkey

Sertkaya, G.
University of Mustafa Kemal, Faculty of Agriculture, Department of Plant Protection, 31034 Antakya-Hatay, Turkey

Abstract

During observations of virus-like symptoms in wild blackberry (*Rubus fruticosus* L., Rosaceae) some stunted plants growing in the border of stone-fruit orchards in Hatay were found showing severe yellow blotching and deformity of the leaves. Samples (shoots and leaves) were collected in September 2008 and May 2009 from wild blackberry plants growing at the border of apricot orchards and neighboring stone fruit nurseries in Hatay province in Eastern Mediterranean Region of Turkey. Each of 12 wild blackberry samples taken from 7 symptomatic and 5 symptomless plants were tested for virus by mechanical inoculation of sap to herbaceous plants. Sap was inoculated on *Chenopodium amaranthicolor, C. quinoa, Cucumis sativus, Gombhrena globosa* L., *Nicotiana benthamiana, N. clevelandii, N. glutinosa, Phaseolus vulgaris* L. and *Vigna unguiculata* L. Sap from six symptomatic plants induced symptoms of necrotic or chlorotic lesions, and ringspot on test plants. No symptoms were induced in the test plants inoculated by sap from symptomless blackberry plants. A sap-transmissible virus was obtained from all symptomatic plants and later identified as *Tomato ringspot nepovirus* (ToRSV) by enzyme-linked immunosorbent assay-ELISA. Thus, results of biological indexing were also confirmed by serological assays (ELISA). Cuttings of symptomatic plants were rooted in pots and kept in an insect-proof growing room for symptom observations and testing. Investigations on the other viruses in wild and cultivated *Rubus* spp. and its vector/s are still in progress. Further studies are necessary to investigate the distribution and natural transmission of the main virus diseases in cultivated *Rubus* spp. in particular because of the economic importance of *Rubus* cultivation and the recent increase in new commercial plantings in Hatay. This work represents the first report of ToRSV in wild blackberry (*R. fruticosus*) in Turkey.

Keywords: Bioassay, Blackberry, ELISA, *Rubus*, ToRSV, virus

Introduction

Blackberries have been reported to be infected by several viruses (Spak,1995; Martin et al., 2004; Tzanetakis and Martin, 2004; Tzanetakis et al., 2008). *Tomato ringspot virus* (ToRSV) is one of the more important viruses causing diseases in *Rubus* spp. ToRSV is a distinctive member of the genus *Nepovirus* (*Comoviridae*) (Stace-Smith, 1996). The virus is found in woody and semi-woody hosts, but it can also be found in herbaceous ornamental and weed species. ToRSV has a similar host range to *Tobacco ringspot nepovirus* (TRSV) (OEPP/EPPO, 2001). It also causes infection in main fruit crops including *Prunus* spp.which are important in the Eastern Mediterranean Region of Turkey. Blackberries (*Rubus* sp.) are also becoming an economically and socially important crop in Hatay province which is located in the same region.

Although Rubus stunt disease in *Rubus* spp was reported for the first time in Turkey (Sertkaya et al., 2004), there is lack of knowledge about virus diseases of blackberries in Turkey. The aim of this study is to investigate *Tomato ringspot nepovirus* (ToRSV) on wild blackberry (*Rubus fruticosus* L., Rosaceae) in the Hatay province of Turkey.

Material and methods

Field observations were made for the identification of symptoms on plants growing in the border of commercial stone fruit orchards in the Hatay province of the Eastern Mediterranean Region of Turkey in September 2008 and May 2009. In order to determine the presence of *Tomato ringspot nepovirus* (ToRSV) on wild blackberry (*Rubus fruticosus* L.), visual inspections were made. A total of 12 shoot and leaf samples were collected from 7 plants exhibiting severe symptoms and 5 asymptomatic plants.

Shoot and leaves samples were collected from the same plants and assayed with sap inoculations and the Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) in both autumn and spring. Mechanical transmission of the virus was carried out with the herbaceous test plants *Chenopodium amaranthicolor, C. quinoa, Cucumis sativus, Gombhrena globosa, Nicotiana benthamiana, N. clevelandii, N. glutinosa, Phaseolus vulgaris* L. and *Vigna unguiculata* L. Young blackberry leaves were homogenized in 0.1 M phosphate buffer (pH 7.2) containing 2% nicotine in a pestle and mortar, and the sap extracts rub-inoculated onto Celite-dusted leaves of the herbaceous indicator plants. Four plants from each of the herbaceous indicator species were mechanically inoculated with the sap of a single blackberry sample. All samples taken from wild blackberry and inoculated test plants were tested for the presence of ToRSV by *ELISA* as described by Clark and Adams (1977). *Antiserum* kits from *Bioreba AG* (Switzerland) were used in standard DAS-*ELISA.* Each group including four indicator plants of each species used for testing for each sample in Bioassays were accepted as one sample for ELISA.

Small cuttings were excised from investigated symptomatic plants,rooted in pots containing a peat:turf (1:1) mixture and kept in insect-proof growing room at 25°C±2 with a16:8 h photoperiod (day:night) in the autumn of 2008 for symptom observation and indexing.

Results and discussion

During the field inspections, some virus-like symptoms were observed on wild blackberry plants growing in the border of stone fruit orchards in Hatay. Suspicious blackberry plants mainly exhibited symptoms possibly related to disease in *Rubus* spp. caused by *Tomato ringspot nepovirus* (ToRSV) such as stunting, deformity of the leaves, severe yellow blotching or chlorosis, and at the end of autumn the chlorotic areas became an intense yellow (Figure 1). ToRSV can be transmitted by nematode vector, *Xiphinema spp.* (Brown, 1989).

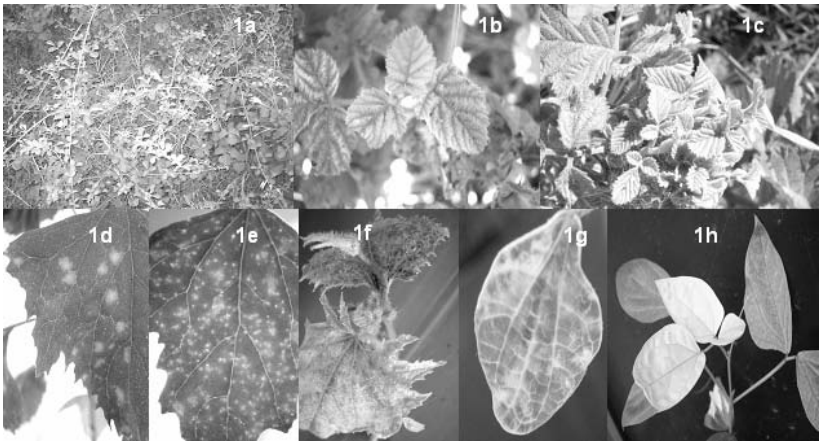

Figure 1 Symptoms on plants infected by *Tomato ringspot virus* (ToRSV); Wild blackberry: stunting, deformity of the leaves, severe yellow blotching or chlorosis on *Rubus fruticosus* L. (1a-c), Test plants: Chlorotic local lesions were developed on test plants, *C. amaranticolor* (1d), *C. quinoa*(1e), *Cucumis sativus* (1f), *P. vulgaris* (1g) and *V. unguiculata* (1h), in two weeks after sap inoculations.

However, no obvious symptoms of ToRSV were observed on peach plants in orchards close to sampled wild blackberry plants. A total of 12 samples taken from 7 symptomatic and 5 asymptomatic plants were tested for virus by mechanical inoculation of sap to herbaceous test plants. Chlorotic local lesions developed in *C. amaranticolor, C. quinoa, P. vulgaris* and *V. unguiculata* test plants two weeks after sap inoculation: necrotic lesions in *C. quinoa,* mosaic in *C. sativus*, and rugosity in *P. vulgaris* (Figure 1 and Table 1).

Table 1 Symptomatology of host plants mechanically inoculated by sap of wild blackberry (*Rubus fruticosus* L., Rosaceae)

Indicator Plants	Symptoms
Family: Amranthaceae	
Gomphrena globosa L.	0
Family:Chenopodiaceae	
Chenopodium amaranticolor Coste and Reyn	C. L.L.
Chenopodium quinoa Wild	C. L.L., (or N.L.L.)
Family:Cucurbitaceae	
Cucumis sativus L. cv. Cemre F1	M, Cl
Family: Fabacceae	
Phaseolus vulgaris L.	C.L.L. (or Ru.)
Vigna unguiculata L.	C.L.L
Family: Solannceae	
Nicotiana benthamiana L., *Nicotiana clevelandii* L.	0
Nicotiana glutinosa L.	0

C.L.L.=Chlorotic Local Lesion, Cl.=Chlorosis, Ru.=Rugosity, M=Mosaic, N.L.L.=Necrotic, 0=No symptoms.

Sap from six symptomatic wild blackberry plants induced symptoms of chlorotic or necrotic lesions, rugosity on test plants. No symptoms were induced in the test plants inoculated by sap from one symptomatic and five symptomless blackberry plants. Serological tests are necessary to identify specifically the reactions produced on indicator plants. However, leaf samples collected from the all inoculated test plants including asymptomatic ones were also analysed by DAS-ELISA. A sap-transmissible virus was identified as *Tomato ringspot virus* (ToRSV) in six out of seven symptomatic plants, but not from five symptomless plants, by sap transmission and ELISA tests. The results were confirmed by re-testing in May 2009. One symptomatic sample might have been infected with different isolates of ToRSV or another virus which causes similar symptoms on the blackberry plants.

Seventeen of 25 cuttings survived and developed symptoms of stunting, chlorosis and deformity of the leaves on new shoots in May 2009. Of 11 rooted cuttings that were detected by ELISA, 8 were infected with ToRSV. ELISA results confirmed the presence of ToRSV in wild *Rubus* spp. in our region.

Investigations into the distribution and transmission of ToRSV on cultivated *Rubus* spp. by grafting and detection of potential vector nematode species such as *Xiphinema* spp. around the roots of symptomatic plants are in progress. Further studies are also necessary to investigate the present status of virus diseases in cultivated *Rubus* spp. in Turkey. Further studies are necessary to determine the distribution and natural transmission of the main virus diseases in cultivated *Rubus* spp. especially due to the economic importance of rubus cultivation in Hatay.

This work represents the first report of ToRSV in wild blackberry (*R. fruticosus*) in Turkey.

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Detection of *Blueberry red ringspot virus* in highbush blueberry cv. 'Coville' in Slovenia

Mavrič Pleško, I., Viršček Marn, M., Koron, D.
Agricultural Institute of Slovenia, Hacquetova 17, SI-1000 Ljubljana, Slovenia. Email: irena.mavric@kis.si

Abstract

Blueberry red ringspot virus (BRRSV) infects blueberries and is present in USA. It causes red ringspots or red blotches on one year old stems or older. In mid- to late summer reddish-brown spots develop on older leaves. In some blueberry cultivars also fruit symptoms, circular areas of light colour and/or fruit deformations, can be seen. In spring 2008 BRRSV was detected in symptomatic bark from blueberry cv. 'Coville' showing typical BRRSV symptoms. The obtained PCR product was sequenced and the identity of the virus confirmed. To our knowledge this was the first finding of BRRSV in Slovenia.

Keywords: BRRSV, Vaccinium, PCR, detection

Introduction

Blueberry red ringspot virus (BRRSV) is a member of genus *Soymovirus* in the family Caulimoviridae. It is known to infect *Vaccinium corymbosum*, *V. formosum*, *V. australe* and probably *V. macrocarpon*. Many blueberry cultivars are sensitive to BRRSV, like 'Blueray', 'Bluetta', 'Coville', 'Earlyblue' and others. Cv. 'Bluecrop' is reported to be field-resistant and cv. 'Jersey' is field-immune. BRRSV causes red ringspots or red blotches on older stems. In mid- to late summer reddish-brown spots develop on older leaves. The spots are prominent on the upper surface of the leaf. Similar symptoms can be caused by powdery mildew (*Microsphaera alni* var. *Vaccinii*) except that leaf spots are prominent on both sides of the leaf. In some cultivars fruit symptoms, circular areas of light colour and/or fruit deformations can be seen. The disease can significantly reduce yield.

The disease is present in USA. Paulechova (1972) has reported the occurrence of red ringspot in former Czechoslovakia on wild *Vaccinium myrtillus*. She did not detect the virus but only showed that powdery mildew was not present. The identity of BRRSV in the Czech Republic was confirmed in 2009. In 2009 BRRSV was reported also in Japan (Isogai et al., 2009).

On one plant of highbush blueberry in an introduction plantation at Brdo pri Lukovici, symptoms indicating BRRSV infection were observed. Red rings appeared on some of the stems and also red rings or spots were observed on some leaves. At the harvest time spots of a light colour were observed in ripening fruits. The aim of our work was to introduce PCR for virus identification into our laboratory and to confirm BRRSV infection of symptomatic blueberry.

Material and methods

In spring 2008 young non-symptomatic leaves and bark from symptomatic blueberry cv. 'Coville' were sampled for virus identification. DNA was isolated from two samples of young leaves and one sample of symptomatic bark tissue using DNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The quality and quantity of isolated DNA was checked on an agarose gel. Serial 10-fold dilutions in water were prepared from isolated DNA and plant DNA control PCR using primers Gd1 and Berg54 was performed (Ward, 2007). Primers RR13 and RR14 (Glasheen et al., 2002) were used in subsequent PCR assays on diluted DNA to detect the virus. Amplified products were analysed on 1% agarose gels and stained with ethidium bromide. An amplification product of the expected size, approximately 490 bp, was obtained and subsequently sequenced (Macrogen, Korea) to confirm it represented sequence of BRRSV.

Results and discussion

For undiluted DNA only one sample (symptomatic bark) produced the PCR product of expected size. However with a dilution 1/10 two samples yielded a product, and with a dilution of 1/100 and 1/1000 all three samples produced the amplification product of expected size. This indicates the presence of PCR inhibitors in isolated DNA. In subsequent BRRSV specific PCR assays we used the same dilutions and quantities of isolated DNA as in the plant DNA control PCR. Only DNA isolated from symptomatic bark produced a PCR product. With undiluted DNA and dilutions of 1/10 and 1/100 we obtained the PCR product of expected size. With a dilution of 1/1000 no PCR product was observed.

A BRRSV specific amplification product of approximately 490 bp was sequenced and the infection of blueberry plant with BRRSV confirmed. To our knowledge this was a first finding of BRRSV in Slovenia.

BRRSV is known to be present in blueberries in the USA and can cause symptoms on some of the cultivars. It was first described in New Jersey in 1950 and is still a problem there. In recent years it has been reported in Arkansas, Michigan, Connecticut, Massachusetts, New York, North Carolina and Oregon (Martin et al., 2009).

In Slovenia, the symptoms of BRRSV were observed on one plant in a plantation over 20 years old. No symptoms were observed on other plants in the same plantation. No virus was detected in plants growing adjacent to the infected plant. This result indicates that the virus is not spreading in our conditions, which is similar to observations in Michigan and Oregon, where the virus does not appear to spread (Martin et al., 2009).

Using our method, the virus could be detected only in symptomatic tissue and not in young leaves, which are recommended for sampling and testing in New Zealand (Ward, 2007). In future work we plan to determine the reliability of BRRSV detection throughout the growing season in different symptomatic and asymptomatic plant tissues to optimize the ability to detect this virus and provide more accurate information for growers and extension services.

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Comparison of *Raspberry bushy dwarf virus* isolates from Hungary and Slovenia

Virščck Marn, M.[1], Mavrič Pleško, I.[1], Goršek, J.[1], Nyerges, K.[2], Lázár, J.[3], Tökés, Á.[4]
[1] Agricultural Institute of Slovenia, Hacquetova 17, 1001 Ljubljana, Slovenia. Email: mojcavm@kis.si
[2] Agricultural Office of County Fejér, Plant Protection and Soil Conservation Directorate, Ország út 23, 2481 Velence, Hungary;
[3] Research Institute for Viticulture and Enology, Miklóstelep, Urihegy 5/A, 6000 Kecskemét, Hungary;
[4] Central Agricultural Office, Keleti Károly u. 24, 1024 Budapest, Hungary;

Abstract

In 2006 and 2007 samples of grapevine and *Rubus* species were collected and analysed by DAS-ELISA to survey the presence of *Raspberry bushy dwarf virus* (RBDV) in Slovenia and Hungary. Seven varieties of raspberry from one Hungarian collection orchard were found to be infected. In Slovenia the presence of RBDV was confirmed only in three samples of wild *Rubus*. None of the 133 samples from different locations in Hungary proved to be infected with RBDV, although this virus is found to be widely distributed in grapevine in neighbouring Slovenia. Serological characterisation with three monoclonal antibodies (R2, R5 and D1) was performed on positive samples. Selected positive samples were partially sequenced. The results of serological and molecular analyses were compared with the analyses of raspberry and grapevine isolates obtained in Slovenia from other projects and published RBDV sequences from the GeneBank database to study the variability among hosts and locations. Isolates from grapevine grouped separately from the black raspberry isolate and all the red raspberry isolates. RBDV isolates from Hungarian samples formed a subgroup within red and black raspberry group.

Keywords: RBDV, variability, *Rubus*, raspberry, grapevine, sequences, monoclonal antibodies

Introduction

Raspberry bushy dwarf virus is found to infect *Rubus* species worldwide and can cause serious damage in certain varieties. In 2003 this virus was reported to infect grapevine, too. This was the first report of RBDV naturally infecting a host outside the genus *Rubus* (Mavrič et al. 2003). Later RBDV was found to be widespread in Slovenia on numerous white and red grapevine varieties (Virščck Marn and Mavrič 2006, Mavrič Pleško et al. 2009). Within the framework of a bilateral project between the Republic of Hungary and the Republic of Slovenia entitled “Study of *Raspberry bushy dwarf virus* (RBDV) infection in grapevine and *Rubus* plantations”, grapevine and wild and cultivated *Rubus* samples were collected in Hungary and Slovenia to survey the presence of this virus on host plants in both countries and to study serological and genetic differences among hosts and locations. Since until then RBDV had only been found in Slovenia and on a limited number of grafts in Serbia (Paunović, personal communication), the survey of grapevine in Hungary was of special interest.

Material and methods

In Hungary 52 leaf samples of grapevine (17 different varieties) were collected in five different vineyards in surrounding areas of Nagyréde and Kecskemét in May 2007. At the same time 49 leaf samples of raspberry (varieties ‘Autumn Bliss', 'Blissy' - a Hungaran selection of 'Autumn Bliss', 'Chilliwack', 'Comox', ‘Fertödi Kétszertermő', 'Fertődi Vénusz', 'Fertődi Zamatos', 'Glen Ample', 'Glen Moy', 'Golden Bliss', 'Malling Exploit', 'Nootka', ‘Summit', 'Tulameen', three samples of unknown raspberry variety, one *Rubus* hybrid and one wild *Rubus* plant) were taken at seven locations around Nagyréde and Pölöske. In November 2008, 81 wood samples of grapevine were collected from 19 grapevine varieties typically grown in Hungarian vine growing regions (Transdanubian North Balaton – Badacsony; North Hungary Bükk Mountains - Eger; North Hungary Hegyalja - Tokaj). In Slovenia 18 samples of eight raspberry varieties (‘Autumn Bliss', 'Glen Ample', 'Himbotop', 'Loch Ness', 'Meeker', 'Polka', 'Tulameen', and 'Willamette') were taken from orchards of the Posavje region in October 2007. Wild *Rubus* was collected in the Gorenjska and the Notranjska region in September 2008. Altogether 43 samples were taken from 14 locations.

Collected samples were tested for the presence of RBDV by DAS-ELISA (Loewe Biochemica or Bioreba AG) according to manufacturer’s instructions, except that only 100 µl per well was used. Absorbance was read at 405 nm in a Sunrise Remote Control Reader (TECAN Austria GmbH). Samples were considered positive when the mean absorbance value of a sample after three hours exceeded the threshold. The threshold was set as at least two times the mean absorbance value of healthy controls. All of the positive samples from Hungary and some samples of RBDV infected grapevine, red raspberry and wild *Rubus* from Slovenia (collected in the frame of other projects) were tested

with three monoclonal antibodies R2, R5 and D1 (R. R. Martin, ADA-ARS, Corvallis OR, USA) to differentiate isolates in TAS-ELISA using the protocol described by Mavrič Pleško et al. (2009). Samples of raspberries from Slovenia and the wild *Rubus* sample were collected in 2005 and were kept as freeze dried material at -20°C. These samples were diluted with DAS-ELISA extraction buffer in a 1:200 ratio.

IC RT-PCR with primer pairs CPUP and RNA12 for the coat protein and MPUP and MPLO for the movement protein (Mavrič Pleško et al. 2009) were performed for three positive raspberry samples from Hungary. The amplicons were purified and cloned into pGEM-T easy vector (Promega) according to the manufacturer's instructions. Transformed colonies were selected by blue/white screening and subsequent PCR. Plasmids were isolated from selected colonies and sent for sequencing (Macrogen, Korea). Analysed sequences (BioEdit version 7.0.5.3, Hall 1999) and their deduced amino acid sequences were compared with sequences of RBDV published in the GeneBank database (http://www.ncbi.nlm.nih.gov/). Phylogenetic analyses were conducted using MEGA 4 programme (Tamura et al. 2007). Phylogenetic trees were constructed using neighbour-joining method and bootstrap analysis with 1000 replications.

Results

None of the 133 samples of numerous grapevine varieties taken in several Hungarian grapevine growing regions proved to be positive for RBDV. Seven raspberry varieties from a collection in Pölöske ('Autumn Bliss', 'Blissy', 'Comox', 'Glen Moy', 'Golden Bliss', 'Summit' and 'Tulameen') were found to be infected with RBDV. In Slovenia the presence of RBDV was confirmed only in three out of 43 samples of wild *Rubus* collected from woods. All of the tested RBDV positive raspberry samples with the exception of one freeze dried sample of variety 'Willamette' tested positive for all three isolate groups. Variety 'Blissy' from Hungary and variety 'Fall Gold' from Slovenia showed a quick reaction with all three monoclonal antibodies whereas the rest of raspberry samples reacted slowly with D1. Grapevine isolates and the freeze dried sample of raspberry variety 'Willamette' reacted only with R2 and R5 (Fig. 1).

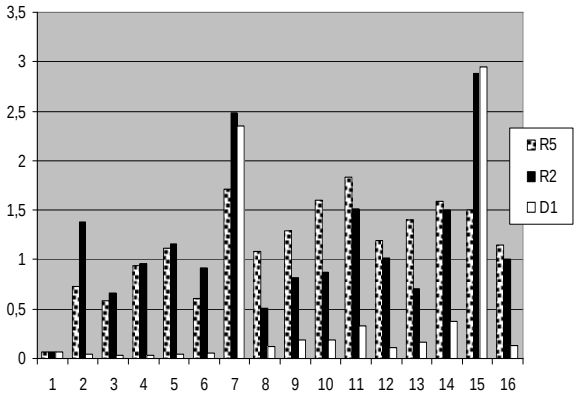

Fig. 1 Results of TAS-ELISA (absorbance was read at 405 nm after 3 hours) for monoclonal antibodies R5, R2 and D1 from October 2007: 1 = negative control x 2; 2 = grapevine variety 'Chardonnay' (Slovenia, 2007); 3 = grapevine variety 'Zweigeld' (Slovenia, 2007); 4 = grapevine variety 'White Riesling' (Slovenia, 2007); 5 = grapevine variety 'Furmint' (Slovenia, 2007); 6 = red raspberry variety "Willamette' (Slovenia, 2005, freeze dried); **7 =** red raspberry variety 'Fall Gold' (Slovenia, 2005, freeze dried); 8 = wild *Rubus* (Slovenia, 2005, freeze dried); 9 = red raspberry variety 'Meeker' (Slovenia, 2005, freeze dried); 10 = red raspberry variety 'Autumn Bliss' (Hungary, 2007); 11 = red raspberry variety 'Summit' (Hungary, 2007); 12 = red raspberry variety 'Comox' (Hungary, 2007); 13 = red raspberry variety 'Golden Bliss' (Hungary, 2007); 14 = red raspberry variety 'Tulameen' (Hungary, 2007); 15 = red raspberry variety 'Blissy' (Hungary, 2007); 16 = red raspberry variety 'Glen Moy' (Hungary, 2007).

Phylogenetic analyses performed with nucleotide sequences (not shown) and deduced amino acid sequences of coat protein (Fig. 2) and movement protein (Fig. 3) showed three major groups of isolates. *R. multibracteatus* grouped separately and was genetically most distant from other isolates. Isolates from grapevine formed a separate group. The black raspberry isolate and all the red raspberry isolates formed the third group, within which Hungarian ones clustered together in a subgroup**.**

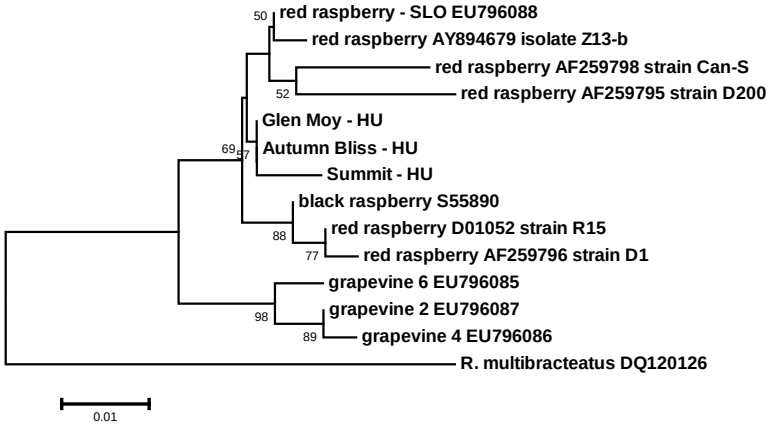

Fig. 2 Polygenetic tree based on the coat protein amino acid sequences

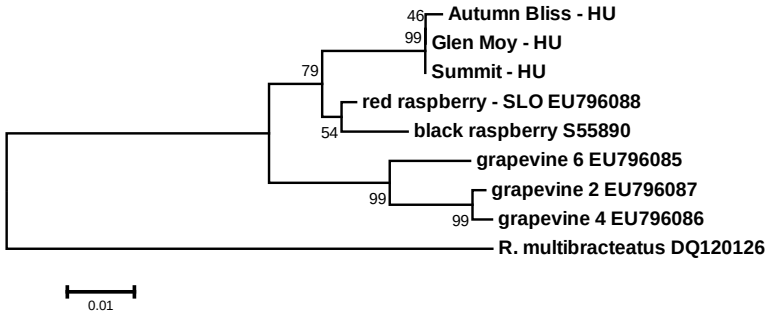

Fig. 3 Polygenetic tree based on the movement protein amino acid sequences

Discussion

RBDV infection was not found in grapevine in Hungary. This is rather surprising considering the abundant presence of this virus in Slovenian vineyards, especially in the NE Slovenia towards the Hungarian border (Viršček Marn and Mavrič, 2006; Mavrič Pleško et al. 2009), and the fact than many of the sampled varieties are grown both in Hungary and Slovenia. The exchange of plant material between the two countries is not very frequent, which could explain the absence of RBDV infection in Hungary. In continuation of our work we will therefore survey Hungarian vineyards close to the border with Slovenia where plant material exchange is probably more common.

In *Rubus* RBDV is distributed naturally by pollen to progeny and pollinated plant. In grapevine all the 390 tested seedlings grown from seeds collected on the RBDV infected grapevine were free from RBDV, which indicates that the virus is not transmitted or transmitted at low efficiency in grapevine (Mavrič Pleško et al. 2009). RBDV was detected by nested RT-PCR in *Longidorus juvenilis* nematodes from the soil in the vineyard infected with this virus. Since viruses can be detected in nematodes also if these are not their vectors, the possible role of *L. juvenilis* in RBDV transmission is still under investigation at the Agricultural Institute of Slovenia (Mavrič Pleško et al. 2009). The spread of viruses by nematodes is rather limited, so RBDV was probably distributed within Slovenia with grapevine propagation material, either rootstocks, scion material or both, but its origin is still unknown.

All the grapevine varieties reacted with monoclonal antibodies R5 and R2 but not with D1 (Fig. 1). A sample of the raspberry variety ‘Willamette’, collected in 2005 and stored as a freeze dried material, showed the same reaction as grapevine, but the results of testing with D1 monoclonal antibodies were closer to the threshold as in grapevine and the

long storage might have deteriorated the material. The analysis will have to be repeated to confirm or reject the finding that red raspberries can react the same way as grapevine with the three monoclonal antibodies. Two of the studied red raspberry samples showed high absorbance values (at 405 nm) for all three monoclonal antibodies whereas the others had high values for R5 and R2, but reacted slowly with D1. Isolates with different reactions to D1 were found in the same collection orchard, so two or more isolates must have been introduced in the collection with planting material of different varieties. There are also small genetic differences among the tree sequenced Hungarian varieties, which could be the consequence of a different origin of infected planted material or/and due to mutations occurring in the field. Nevertheless Hungarian isolates proved to be very similar to each other and formed a subgroup within the group of red and black raspberry isolates. Grapevine isolates formed a separate group. *R. multibracteatus* isolate proved to be genetically more distant. The infection of this plant species was reported by Chamberlain et al. in 2003 on a plant from China. The *R. multibracteatus* isolate reacted only with monoclonal antibodies R5 and R2 and not with D1 (Chamberlain et al. 2003) and was thus serologically similar to grapevine isolates. Serological and genetic data about RBDV are still scarce and more data is needed to understand the variability and epidemiology of this virus. In the laboratory of the Agricultural Institute of Slovenia work to obtain more sequences of raspberry, wild *Rubus* and grapevine isolates is in progress.

Acknowledgements

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Occurrence of small fruit viruses in Belarus

Valasevich, N., Kolbanova, E.
Department of Biotechnology, Institute for Fruit Growing, Kovalev str., 2, Samokhvalovichi, 223013, Belarus.
Email: natallia.valasevich@rambler.ru; kolbanova@tut.by

Abstract

Epidemiological control of plant virus diseases is necessary for creation of small fruit nurseries and production of berries with stable high yields. Phytosanitary state of *Rubus idaeus* L. and *Ribes sp*. L. was studied in Belarus. The aim of the research was to determine the most widespread small fruit viruses and to select plants that free from all tested viruses for further propagation in vitro as a basis for Nuclear stock. The following viruses were identified: CMV, ApMV, SLRV, RRV, RBDV, ArMV, TBRV and ToRSV. High level of virus infection for small fruit plantations was shown. The most common viruses for all three crops (raspberry, red and black currant) were RRV, SLRV and ArMV. It was noted that infection level of viruses considerably varied from plant cultivars and crops.

Keywords: viruses, raspberry, black currant, red currant, Belarus.

Introduction

Viruses cause pathological changes in small fruits decreasing plant productivity. More than 20 viruses infect *Rubus* worldwide (Diekmann *et al*., 1994). A number of nepoviruses and *Raspberry bushy dwarf virus* (RBDV) have been detected in raspberry plantations of many countries: UK (Barbara *et al.*, 2001), USA (Ellis *et al.*, 2005), Czech Republic (Špak, 1995) and others. Nepoviruses occurring in *Rubus* are *Arabis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRV), *Raspberry ringspot virus* (RRV), *Tomato black ring virus* (TBRV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV). They are transmitted in nature by nematodes of genera *Longidorus* (RRV, TBRV) and *Xiphinema* (ArMV, SLRV, TRSV, ToRSV) and may cause progressive decline in vigour with symptoms ranging from chlorotic mottling, line-pattern, mosaic, vein yellowing to leaf curling (Diekmann *et al.*, 1994). RBDV, genus *Idaeovirus*, is transmitted by infected pollen and causes yellow disease, crumbly and deformed fruit (Jones *et al.*, 1982).

The most common among graft transmissible viruses of black and red currants are RRV, SLRV, TBRV, ArMV and *Cucumber mosaic virus* (CMV). In Great Britain the following viruses was found: RRV, SLRV, ArMV and CMV (Thresh, 1966), in Germany - RRV, SLRV, ArMV, CMV and *Tobacco mosaic virus* (TMV) (Kleinhempel, 1970), in Finland - TBRV and RRV (Bremer, 1983). Injuriousness of these pathogens can be very significant for red and black currant plants. Productivity reduction of red currant by 32% was noted after an artificial inoculation by RRV (Kleinhempel, 1970). CMV can reduce growth and productivity of black currant plants by 15%-20%. ArMV infection may also cause decreasing of productivity and growth (Thresh, 1966). ToRSV may induce lower fruitage and even death of red currant plants (Hildebrand, 1939).

Epidemiological control of plant virus diseases is necessary for creation of small fruit nurseries and production of berries with stable high yields. We studied the phytosanitary state of *Rubus idaeus* L. and *Ribes sp*. L. in Belarus. The aim of the research was to determine the most widespread small fruit viruses and to select plants that free from all tested viruses for further propagation in vitro as a basis for Nuclear stock.

Material and methods

Objects of the research were commercial cultivars of *Rubus idaeus* L. and *Ribes sp*. L. in Belarus. Occurrence of small fruit viruses was studied by DAS-ELISA (commercial kits of Sanofi Diagnostics Pasteur company). Analysis was carried out in spring time (from middle of May till beginning of June). Leaves were source of plant tissue for test. The following viruses were identified: CMV, ApMV, SLRV, RRV, RBDV, ArMV, TBRV and ToRSV according to the "Statute of fruit plant material production in Belarus". Automatic rider PR2100 with wavelength 405 nm was used for ELISA results recording.

Results

Raspberry viruses: Raspberry plants were mostly infected by the following viruses: 30.5% (of samples) by RBDV, 29.3% - by RRV, 26.2% - by ApMV, 21.9% - by SLRV, 16.7% - by ArMV. TBRV, ToRSV and CMV were absent in all tested raspberry plants. At the average 41.5% of samples was free from tested viruses, 25.6% of samples was infected by only one virus, 21.9% - by two viruses, 9.7% - by tree viruses and 1.2% of samples contained four viruses

simultaneously. Interesting that in samples with single virus infection in 61.9% of cases RBDV was detected. In samples with two viruses more often was found the following combination of viruses: RBDV+RRV (33.3%) and RRV+SLRV (33.3%) (Table 1).

Tab. 1 Occurrence of graft transmissible viruses in raspberry plantation of the Institute for Fruit Growing

	Percent of infection							
Cultivar	**RRV**	**SLRV**	**RBDV**	**ArMV**	**CMV**	**ApMV**	**TBRV**	**ToRSV**
at the average within 6 cultivars	29.3	21.9	30.5	16.7	0	26.2	0	0
'Alyonushka'	22.7	36.4	4.5	28.6	0	71.4	0	0
'Meteor'	52.6	52.6	21	15.8	0	0	0	0
'Balsam'	0	0	76.5	0	0	5.9	0	0

Occurrence of raspberry viruses considerably varied from plant cultivars. Thus 'Alyonushka' plants were infected by ApMV (71.4%), SLRV (36.4%), ArMV (28.6%), RRV (22.7%) and RBDV (4.5%). It was noted that 27% of tested plants contained simultaneously 2 viruses, 18.2% of infected plants contained either 1 or 3 viruses. 36.4% of plants had no one from all tested viruses.

'Meteor' plants were infected basically by the same group of viruses but in other proportion: SLRV (52.6%), RRV (52.6%), RBDV (21%) and ArMV (15.8%). It was determined that 5.3% of samples contained only one virus, 26.3% of samples contained two viruses, 21% contained tree viruses, and 5.3% contained 4 viruses simultaneously. 42.1% of given variety samples had no one from all tested viruses. Interesting that in samples with complex infection in 90% of cases SLRV and RRV viruses were found.

'Balsam' plants were heavily infected by RBDV (76.5% of samples was positive), and only 5.9% of plants by ApMV. 23.5% of samples were free from all tested viruses.

Black currant viruses: High level of infection was detected in black currant collection: RRV (100%), SLRV (100%), TBRV (97.5%) and ArMV (81.8% of tested samples were infected correspondingly) while CMV infected only 5.8% of samples. TBRV infection rate of black currant cultivars was 100% with the exception of 'Katyusha' plants (83.3% of samples were positive). Rate of ArMV infection have considerably varied among tested cultivars. Thus black currant cultivars 'Cerera', 'Buelorusskaya Sladkaya', 'Partisanka', 'Zagadka', 'Seyanec Golubky', 'Orloviya' were infected by 100%, while 'Pamyat Vaviloba' – by 60%, 'Katyusha' – by 44.4% (Table 2).

Tab. 2 Occurrence of graft transmissible viruses in black currant collection planting of the Institute for Fruit Growing

	Percent of infection				
Cultivar	**RRV**	**SLRV**	**TBRV**	**ArMV**	**CMV**
'Cerera'	100	100	100	100	100
'Buelorusskaya Sladkaya'	100	100	100	100	54.5
'Partisanka'	100	100	100	100	0
'Zagadka'	100	100	100	100	4.5
'Katyusha'	100	100	83.3	44.4	0
'Pamyat Vavilova'	100	100	100	60	0
'Seyanec Golubky'	100	100	100	100	0
'Kantata'	100	100	100	0	0
'Orloviya'	100	100	100	100	0

Infection level was no less intensive in propagation plantation of black currant: RRV (100%), TBRV (93.3%), SLRV (71.1%), CMV (62.2%). TBRV was detected in 90% of 'Pamyat Vavilova' samples, in 80% of 'Katyusha' samples, and 100% infection was presented in cultivars 'Klussonovskaya', 'Cerera', 'Zagadka', 'Kupalinka' and 'Naslednica'. SLRV infection rate vary from 40% to 100%. All tested cultivars were infected by CMV. It should be noted that the least CMV infection had cultivars 'Kupalinka' and 'Naslednica' (both 20%) (Table 3).

Tab. 3 Occurrence of graft transmissible viruses in black currant propagation plantation of the Institute for Fruit Growing

	Percent of infection			
Cultivar	**RRV**	**SLRV**	**TBRV**	**CMV**
'Pamyat Vavilova'	100	40	90	40
'Katyusha'	100	70	80	70
'Klussonovskaya'	100	100	100	100
'Cerera'	100	80	100	100
'Zagadka'	100	80	100	100
'Kupalinka'	100	100	100	20
'Naslednica'	100	60	100	20

Red currant viruses: The most widespread red currant viruses were RRV and TBRV (47.9% and 34% of infected plants correspondingly). Infection of cultivars by RRV varied from 5% ('Rondom') to 100% ('Fertody'). The highest infection rate of TBRV was noted of cultivars 'Krasnaya Andreychenko' (90% of infected samples) while in all other cultivars it didn't exceed 35%. CMV was detected in 2.1%, ArMV – in 4.3%, SLRV – in 9.6% and ToRSV – in 10.6% of checked samples.

CMV occurred only in "Jonkher Van Tets' plants (14.3% infected plants). Cultivars 'Fertody' and 'Krasnaya Andreychenko' were affected by ArMV (5% and 15% infected samples correspondingly). SLRV was found in 10% of 'Krasnaya Andreychenko' samples, in 15% of 'Rondom' samples and in 28.6% of 'Jonkher Van Tets' samples. ToRSV was detected in 'Fertody', 'Nenaglyadnaya' and 'Jonkher Van Tets' (5%, 25% and 28.6% of infected samples correspondingly) (table 4).

Tab. 4 Occurrence of graft transmissible viruses in red currant propagation plantation of the Institute for Fruit Growing

	Percent of infection					
Cultivar	**RRV**	**SLRV**	**TBRV**	**ArMV**	**CMV**	**ToRSV**
'Rondom'	5	15	35	0	0	0
'Nenaglyadnaya'	40	0	5	0	0	25
'Jonkher Van Tets'	35.7	28.6	14.3	0	14.3	28.6
'Krasnaya Andreychenko'	55	10	90	15	0	0
'Fertody'	100	0	20	5	0	5

Discussion

The results characterise the epidemiological situation for the most common *Rubus* and *Ribes* viruses in Belarus. High level of virus infection for small fruit plantations was shown. The most common viruses for all three crops (raspberry, red and black currant) were RRV, SLRV and ArMV. It was noted that infection level of viruses considerably varied from plant cultivars and crops. For example, in red currant plants percentage of RRV was the lowest in 'Rondom' (5%) and the highest in 'Fertody' plants (100%), in red raspberry the level of the infection varied from 0% for 'Balsam' till 52.6% for 'Meteor' cultivar while all tested black currant plants contained the virus.

In the issue of conducted research free from tested viruses plants were isolated and used for in vitro propagation. In cases when it was impossible to find virus-free plants plant material was used for chemotherapy experiments *in vitro*.

Knowledge of viruses that infect berries, their distribution and ways of control is important for establishing commercial berry plantations and especially nurseries. Thus, virus diagnostics and epidemiological control of plant virus diseases are necessary for creation of virus-free plants and for the monitoring of propagated certificated planting stock. Production of virus tested and virus free planting stock is one of the scientific priorities in development of small fruit growing in Belarus.

This work was a part of the State Program of Fruit Growing Development with the aim to develop and apply in industry a production system of certified plant material for fruit and berry crops in Belarus.

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Characterisation of mixed virus infections in *Ribes* species in Switzerland

Besse, S., Gugerli, P., Ramel, M.-E., Balmelli, C.
Agroscope Changins-Wädenswil Research Station ACW, Virology-Bacteriology, CH-1260 Nyon 1, Switzerland.
Email: sebastien.besse@acw.admin.ch

Abstract

Various virus disease-like symptoms are frequently observed in *Ribes* sp. in Switzerland but the aetiology remains poorly documented, although a number of viruses infecting *Ribes* sp. were described elsewhere. Therefore, symptomatic and apparently healthy plants from diverse origins were analysed by electron microscopy (EM), immuno-precipitation electron microscopy (IPEM), Western blot and (RT-)PCR. By EM, at least four different particle types, often in combination, were observed. (1) Bacilliform particles were typical for the *Badnavirus* genus with dimensions of 145 x 28 nm. This virus was identified by PCR as the *Gooseberry vein banding associated virus* (GVBaV). (2) Filamentous particles were mainly observed on black currants with downward rolling of leaves with interveinal reddening during summer and fall. We tentatively named this unknown virus Blackcurrant leafroll-associated virus 1 (BCLRaV-1). In phylogenetic analysis of HSP70h nucleotide sequences, BCLRaV-1 felt in the *Closterovirus* genus. In Western blot analysis, one dominant protein with an estimated molecular weight of about 28 kDa was detectable.

The virus was shown to be different from the *Raspberry mottle closterovirus* (RMoV) by IPEM and RT-PCR. (3) RT-PCR and sequencing of products also clearly demonstrated the presence in our *Ribes* samples of *Rubus chlorotic mottle virus* (RuCMV), a *Sobemovirus* recently described in Scotland. This finding correlates with the presence of the 30 nm diameter particles observed by EM. (4) A further structure with isometrical particles of 60 nm could not yet be attributed to a particular genus. Altogether, our data suggest the presence of multiple virus infections in *Ribes* sp. in Switzerland and emphasize the need for an efficient sanitary selection process.

Keywords: *Ribes* sp., *Gooseberry vein banding associated virus* (GVBaV), *Blackcurrant leafroll-associated virus* 1 (BCLRaV-1), *Rubus chlorotic mottle virus* (RuCMV)

Introduction

Over the past few years, symptoms of reversion, leafroll, vein clearing, defoliation, fruit failure, reduction of fruit calibre have been observed in *Ribes* sp. in Switzerland. So far, the sanitary status of commercial *Ribes* sp. in Switzerland has never been assessed. In this preliminary work, we focused on some accessions of *Ribes* sp. with symptoms of leafroll and early defoliation (Fig. 1). To our knowledge, such disorders have not yet been reported in *Ribes* species. In an attempt to determine if these disorders were associated with a viral agent, we analysed a few accessions by electron microscopy (EM), immuno-precipitation electron microscopy (IPEM), Western blot and RT-PCR.

Fig. 1 Left: leafroll symptoms characterised by the downward rolling of leaves with interveinal reddening in summer and fall. Right: early defoliation of a black currant bush.

Material and methods

All *Ribes* and *Rubus* sp. used in this study were from the reference collection of Agroscope ACW. Viral nucleoproteins were purified from leaves as previously published (Gugerli, Brugger et al. 1984). Purified extracts were observed by electron microscopy (EM) in negative contrast with phosphotungstic acid according to Bovey (1971). Mechanical inoculation to herbaceous hosts was performed in phosphate buffer 0.02 M + 0.01 M sodium diethyldithiocarbamate pH 7.6. The production of antiserum, purification of immunoglobulins, IPEM, electrophoresis and Western blot analysis were essentially done as described elsewhere (Gugerli 1986).

Leaf samples were tested by double-antibody-sandwich ELISA (DAS-ELISA) according to Gugerli (1986) with commercial kits from BIOREBA AG (Reinach, Switzerland) as follows: *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Strawberry latent ringspot virus* (SLRSV) and *Raspberry ringspot virus*-cherry strain (RpRSV-ch). Extraction of viral RNA or DNA was performed according to Rütsche (2008), whereby the DNeasy® Plant Mini Kit (QIAGEN, Switzerland) was used for DNA purification. RT-PCR protocols and primers' sequences for the detection of *Gooseberry vein banding associated virus* (GVBaV) (Jones, Mcgavin et al. 2001), *Raspberry mottle virus* (RMoV) (Tzanetakis, Halgren et al. 2007), *Rubus chlorotic mottle virus* (RuCMV) (Mcgavin and Macfarlane 2009) and HSP70h gene of the members of the *Closteroviridae* family (Dovas and Katis 2003) are described in the corresponding publication.

Detection of *Blackcurrant reversion virus* (BRV) was performed according to Lemmetty et al. (1998) with the primers P1/ P2 and P5/ P6, however without the immunocapture step. Amplification products were purified with the QIAquick PCR Purification Kit (QIAGEN, Switzerland) according to the manufacturer's instructions. DNA sequencing was performed by FASTERIS SA (Geneva, Switzerland). Oligonucleotide primers for the specific detection of the filamentous virus observed in currants were: Cass_Fw 5'-TCCTACCAGACGCTTC-3' and Cass_Rv2 5'-AGTGCGCTGTATTGTG-3'; Clost1Fw 5'-CTCATCTCGGGACA-3' and Clost2Rv 5'-ACAGAGCATACGAC-3'. RT-PCR setup and thermal conditions were described by Rütsche (2008) with an annealing temperature of 55°C. Alignments were performed in MacClade (Maddison and Maddison 2003) and ambiguously aligned regions were excluded from phylogenetic analyses. Searches for the most parsimonious tree(s) were conducted in PAUPv.4* (Swofford 2003) and used 500 RAS searches, with MAXTREE=unlimited and TBR branch swapping. Branch support was estimated based on 500 boostrap (BS) replicates, with the same settings as for the best tree(s) searches.

Results

Electron microscopy analysis: During the last years, several small fruits accessions (mainly *Ribes* sp. and *Rubus* sp.) were collected from various sources and maintained as references in the field or in greenhouse at Agroscope, Nyon. EM of purified extracts from selected symptomatic and apparently healthy *Ribes* sp. accessions (Table 1) disclosed the presence of at least four different particle types Fig. 2), often observed in combination. Bacilliform particles measured about 145 x 28 nm and were mainly observed in red currants samples.

Filamentous particles had an approximate dominant maximum length of 1500 nm based on 129 measures (BC No3SB 28074) and were observed in leafroll-affected *Ribes* sp. (downward rolling of leaves with interveinal reddening in summer and fall with symptoms on black currants being more pronounced than on red currants). At least two types of isometrical particles were observed: ~30 nm particles that were observed consistently in three accessions (Table 1) and ~60 nm particles that appeared more common. A significant variability was noticed with bigger spherical particles size, whereby diameters ranged from 40 to 80 nm, disclosing the possible presence of other particle types.

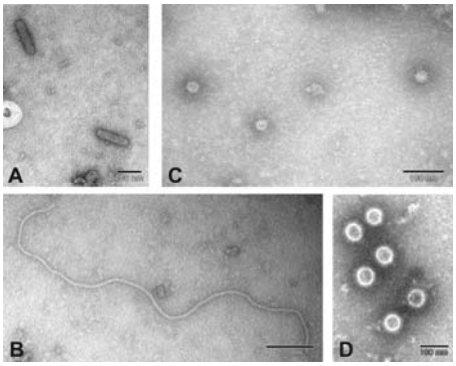

Fig. 2 Virus particles observed in EM from various *Ribes* sp. accessions. (A) Bacilliform particles. (B) Filamentous particles. (C) ~30 nm isometrical particles. (D) ~60 nm isometrical particles.

Tab. 1 EM analysis of symptomatic and apparently healthy *Ribes* accessions. +: virus particles observed in every extracts analysed; (+): virus particles observed in some extracts analysed.

		Particle types			
Accession	**Symptoms**	**Bacilliform**	**Filamentous**	**Spherical (~60 nm)**	**Spherical (~30 nm)**
Red currant No2SB 27986	Vein clearing and leafroll	+	+		
Red currant No3SB 28005	Vein clearing and leafroll	+	+	+	(+)
Red currant TE 27966	Vein clearing and leafroll	+	+		(+)
Red currant 0g 28650	No symptom			+	+
Black currant Titania 8/6 28636	Defoliation and leafroll		+		+
Black currant Titania 8/8 28640	Defoliation and leafroll		+	+	+
Black currant TE 27940	No symptom	(+)		+	
Black currant Titania 7/13 28119	Strong defoliation	(+)		+	
Black currant No3SB 28074	Strong leafroll	(+)	+	+	

Serological and molecular characterization of filamentous particles: Two antisera were produced, the first against the filamentous virus observed in currants (As 83) and the second against RMoV (As 160). Immunoglobulins were purified by DEAE chromatography and subsequently used in IPEM and Western blot. In IPEM, As 83 aggregated filamentous particles of all *Ribes* accessions tested so far (Fig. 3), whereas it did not react with RMoV virions. Furthermore, as 160 did not cross-react with filamentous virions observed in currants. Hence, these results indicated that these two viruses were not serologically related. In Western blot analysis, the estimated capsid protein molecular weight of the filamentous virus observed in currants was about 28 kDa (Fig. 3).

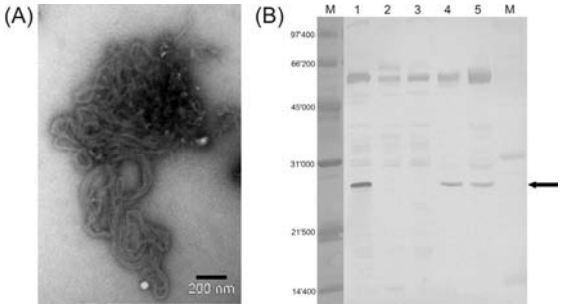

Fig. 3 Serological detection with As 83 of the filamentous virus observed in currants. (A) Decoration and aggregation of filamentous virions (BC No3SB 28074) by IPEM. (B) Staining of the ~28 kDa capsid protein by Western blot analysis (black arrow). M: marker (Da); 1) BC No3SB 28074; 2) BC Titania 7/13 28119; 3) RC 0g 28650; 4) RC TE 27966; 5) RC No2SB 27986.

Partial HSP70h genes of the filamentous virus identified in the accessions BC No3SB 28074 and RC TE 27966 were sequenced using the method developed by Dovas and Katis (2003). The generated nucleotide fragments diverged from 26% (sequence similarity of 74%), suggesting the existence of molecular variants (hereafter referred to as the BC and RC variants). In the maximum parsimony (MP) phylogenetic analysis of HSP70h partial nucleotide sequences, the two variants clustered together (BS = 91%) within the genus *Closterovirus* (BS = 90%). Based on nucleotide sequence information, an RT-PCR procedure for the variant-specific detection of the filamentous virus observed in currants was developed (Fig. 4).

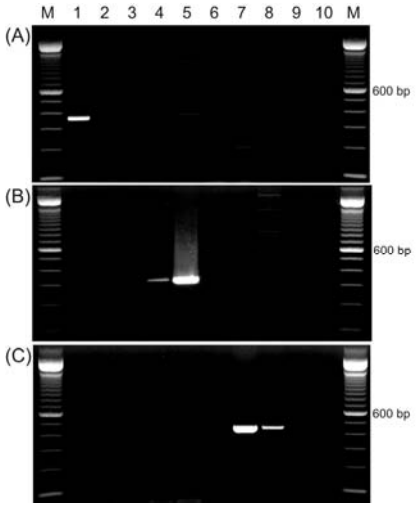

Fig. 4 RT-PCR detection of the filamentous virus observed in currants (A and B) and RMoV (C). (A) Detection of the BC variant (Cass_Fw/ Rv2; 365 bp). (B) Detection of the RC variant (Clost1Fw/ 2Rv; 324 bp). (C) Detection of RMoV according to Tzanetakis *et al.* (2007) (primers CPhF/ CPhR; 452 bp). M : 100 bp marker (bp) ; 1) BC No3SB 28074; 2) BC Titania 7/13 28119; 3) BC TE 27940; 4) RC No2SB 27986; 5) RC TE 27966; 6) RC 0g 28650; 7) Raspberry 5eL RMoV reference; 8) Raspberry 25081 RMoV reference; 9) Raspberry 12205; 10) H_2O.

Despite several attempts, the filamentous virus observed in currants could not be transmitted by mechanical sap inoculation to *Nicotiana benthamiana*.

Evidence of infection by the *Gooseberry vein banding associated virus* and the *Rubus chlorotic mottle virus*: Bacilliform particles were typical for the *Badnavirus* genus. Following the PCR protocol of Jones et al. (2001), this virus was identified as *Gooseberry vein banding associated virus* (GVBaV). Sequencing of the amplicon generated by the primers GVB1 for/ rev showed a similarity of 97% between the original sequence (AF298883) and those obtained from our red currant isolates (RC TE 27966; RC No3SB 28005). GVBaV-infection in some of the black currant accessions was also confirmed by PCR and sequencing (BC No3SB 28074; BC Titania 7/13 28119).

Following the RT-PCR procedure of McGavin and MacFarlane (2009), we clearly demonstrated the presence in our *Ribes* samples of *Rubus chlorotic mottle virus* (RuCMV), a *Sobemovirus* recently described in Scotland. A similarity of 91% was obtained between the sequenced RT-PCR product (primer 1082/ 1083) generated from the accession RC 0g 28650 and the reference sequence AM940437. This finding correlates with the presence of the 30 nm diameter particles observed by EM. Selected accessions analysed by EM (Table 1) tested negative for ArMV, TBRV, RpRSV, SLRSV in DAS-ELISA and BRV by RT-PCR.

Discussion

Closterovirus-like particles have been observed 12 years ago in *Ribes* samples (Roberts and Jones 1997). However, these particles were neither characterised nor associated with particular symptoms. In this work, EM analysis of nine reference accessions suggested an association between leafroll symptoms and the presence of a filamentous virus.

Therefore, we tentatively propose the name of Blackcurrant leafroll-associated virus 1 (BCLRaV-1) for this new virus. BCLRaV-1 was shown to be different from the RMoV by IPEM and RT-PCR. BCLRaV-1 virions have an approximate maximum length of 1500 nm and a capsid protein molecular weight of about 28 kDa. In phylogenetic analysis of partial HSP70h nucleotide sequences, BCLRaV-1 fell in the *Closterovirus* genus. Interestingly, we were able to distinguish two molecular BCLRaV-1 variants. Closteroviruses are known to display a high genetic variability (Maliogka, Dovas et al. 2008) with populations composed of variants or isolates with different biological properties (Meng, Li et al. 2005;Kong, Rubio et al. 2000;Beuve, Sempe et al. 2007). The question of considering the molecular variants of BCLRaV-1 described here as two variants of the same species or different virus species remains open, until their biological and molecular properties have been further analysed.

Symptoms of defoliation seems not to be associated with a particular virus or a complex of viruses and are possibly caused by physiological problems rather than having a viral aetiology. GVBaV was shown to be abundant in extracts of red currant and may be responsible for the vein clearing symptoms observed on three red currant accessions (Table 1) (Jones, Mcgavin, Geering, and Lockhart 2001). Furthermore, we report RuCMV infection of *Ribes* samples. RuCMV was isolated from a bramble with chlorotic mottle leaf symptoms and was shown to infect raspberry (Mcgavin and Macfarlane 2009). Its symptomatology on *Ribes* sp. needs however to be determined. Finally, we observed by EM in most of our samples spherical particles with a diameter of about 60 nm of an other uncharacterized entity.

This preliminary work pointed out the presence of multiple virus infections in *Ribes* sp. in Switzerland and emphasizes the need for an efficient sanitary selection process. A prerequisite is however the development of reliable and affordable diagnostic reagents such as monoclonal antibodies. The development of such diagnostic tools will not only be of interest for sanitary selection but they will also help us to pursue the study of *Ribes* viruses that frequently occur in mixed infection.

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Transient expression of the coat protein of *Apple chlorotic leaf spot virus* inhibits the viral RNA accumulation in *Nicotiana occidentalis*

Yaegashi, H.[1], Yoshikawa, N.[2]
[1] Plant Pathology Research Team, Apple Research Station, National Institute of Fruit Tree Science, National Agricultural and Food Research Organization (NARO), Morioka 020-0123, Japan
[2] Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

Abstract

The coat protein of *Apple chlorotic leaf spot virus* (ACLSV-CP) plays a crucial role in infectivity and efficient viral RNA accumulation in host cells (J. en. Virol, 88, 2007). In this study, the effect of ACLSV-CP on viral RNA accumulation in *Nicotiana occidentalis* was investigated. The CP, CPm40 (an amino acid (aa) substitution of Ala to Ser at aa position 40), CPm75 (a substitution of Phe to Tyr at aa position 75), and CPm40m75 (two aa substitutions at positions 40 and 75) of ACLSV (P205) were transiently expressed in *N. occidentalis* leaves by agroinfiltration. Immunoblot analysis showed that CP and CPm40m75 accumulated in infiltrated tissues, in contrast to CPm40 and CPm75 which were not detected, suggesting that the stable accumulation of CP is important for effective viral RNA accumulation. However, co-agroinfiltration of an infectious ACLSV cDNA clone (pBICLSF) or pBICLSF-based CP mutants (pBICLCPm40, pBICLCPm75, and pBICLCPm40m75) with a vector expressing CP (pBE2113-CP) showed no viral genomic RNA accumulations were found in any leaves infiltrated with these constructs. The inhibition of ACLSV-RNA accumulation was found only in leaves co-expressed with CP protein, but not with a frame-shift mutant of CP, a movement protein (P50), and a frame-shift mutant of P50.

Keyword: *Apple chlorotic leaf spot virus*, coat protein, protein stability, coat protein mediated resistance (CP-MR), agroinfiltration

Introduction

Apple chlorotic leaf spot virus (ACLSV) is distributed world-wide and is known to infect *Rosaceae* fruit tree species, including apple, peach, pear, plum, cherry, and apricot (Lister, 1970; Martelli et al., 1994; Yoshikawa, 2001). ACLSV is one of the causative agents of apple top-working disease in Japan (Yanase, 1973). The virus is classified into the *Trichovirus* genus, *Flexiviridae* family, and has filamentous particles approximately 600–700 nm in length, which contain a single-stranded, plus-sense RNA and multiple copies of a single coat protein of 21 KDa (CP) (Yoshikawa & Takahashi, 1988). Complete nucleotide sequences have been reported for isolates P863 and PBM1 from plum, the BAL1 from cherry, and P205, A4, B6, and MO5 from apple (German et al., 1990 & 1997; Sato *et al*., 1993; Yaegashi et al., 2007b). The genome of an apple isolate of ACLSV (P-205) consists of 7552 nucleotide (nt) and encodes three genes, including a 216 KDa replication-associated protein (Rep), a 50 KDa movement protein (P50), and a CP (Sato et al., 1993).

Based on the phylogenic analysis of CP amino acid sequences from ACLSV isolates from apple trees in Japan, we showed that ACLSV isolates are separated into two major clusters in which five amino acids at positions 40, 59, 75, 130, and 184 were highly conserved into Ala^{40}-Val^{59}-Phe^{75}-Ser^{130}-Met^{184} or Ser^{40}-Leu^{59}-Tyr^{75}-Thr^{130}-Leu^{184} within each cluster. Furthermore, we showed that the combinations of two amino acids at the positions 40 and 75 are crucial for effective virus replication in host plant cells, suggesting that ACLSV-CP plays important roles for effective viral replication, in addition to virion assembly (Yaegashi et al., 2007b).

In this study, we examined the stability of mutant CP with an amino acid substitution (CPm40; Ala to Ser at position 40, CPm75; Phe to Tyr at position 75; which is fatal to viral infectivity and replication) by agroinfiltration. The results showed the marked reduction of protein stability of CPm40 and CPm75, indicating that the stable accumulation of CP is important for effective viral RNA accumulation. On the other hand, we also showed that transient expression of ACLSV-CP inhibits viral RNA accumulation in cells. Our data showed that there are two conflicting roles of CP related to the ACLSV replication cycle.

Materials and methods

Plasmid: The binary plasmid, pBE2113-P35T (empty vector), pBE2113-CP (an expression vector for wild-type CP), pBE2113-FSCP (an expression vector for a frame-shift mutant of CP mRNA), pBE2113-P50 (an expression vector for P50), and pBE2113-FSP50 (an expression vector for a frame-shift mutant of P50 mRNA) were described previously (Yaegashi et al., 2007a; Yoshikawa et al., 2000). To construct binary vectors for expression of CPm40 (an amino acid (aa) substitution of Ala to Ser at aa position 40), CPm75 (a substitution of Phe to Tyr at aa position 75), and CPm40m75 (two aa substitutions at positions 40 and 75), each cDNA were amplified from pCPm40, pCPm75, or pCPm40m75 (Yaegashi et al., 2007b) by polymerase chain reaction (PCR) with KOD plus (TOYOBO) and two primers; ACCPBam (+)(5'-CGCGGATCCATGGCGGCGGCAGTGCTGAAC-3', *Bam*HI site is underlined) and ACCP(-) (5'-ACTAAACGCAAAGATCAG-3'). These PCR products were double-digested with *Bam*HI and *Sac*I, and replaced with a corresponding region of pBE2113-CP. The resulting plasmids were denoted as pBE2113-CPm40, pBE2113-CPm75, or pBE2113-CPm40m75, respectively. The cDNA clones of ACLSV-P205 (pBICLSF) and its mutants (pBICL⊿Rep, pBICLCPstop, pBICLCPm40, pBICLCPm75, pBICLCPm40m75) were described previously (Yaegashi et al., 2007b). All constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 by a freeze-thaw method.

Agroinfiltration: The agroinfiltration method was described previously (Yaegashi et al., 2007a & b). The 5th and 6th leaves of the 7-8 leaf stage *Nicotiana occidentalis* plants were used for agroinfiltration. The agrobacteria suspensions were prepared at OD_{600}=2.0. When two agrobacteria containing two different constructs were co-infiltrated, equal volumes of each suspension were mixed prior to infiltration. The infiltrated plants were kept at 25 ℃.

Immunoblot analysis: Total protein samples from *N. occidentalis* leaves were electrophoresed in a 12.5% polyacrylamide-SDS gel and transferred electrophoretically to a PVDF membrane (Millipore). The membrane was incubated with an antiserum against ACLSV particles, followed by an anti-rabbit IgG (H&L)-alkaline phosphatase linked antibody (Cell Signaling) and immersed in development solution containing a Fast Red TR salt (Sigma) and a naphthol AS-MX phosphate (Sigma).

Northern blot analysis: For Northern blot analysis, denatured RNAs were separated on the 1% agarose gel containing 6 % formaldehyde and transferred to Hybond N+ membrane (GE healthcare). After UV-crosslinking, the membrane was hybridized with a digoxigenin (DIG) labeled RNA probe complementary to plus-strand RNA containing the CP coding region (nt positions 6888 to 7552). The hybridized membrane was immunodetected with an anti-DIG Fab fragment coupled to alkaline phosphatase (Roche), and visualized with a chemiluminescent substrate, CDP-star (GE Healthcare) on X-ray films.

Results and discussion

Stable accumulation of CP may be important for viral RNA accumulation: We already showed that an amino acid substitution of Ala to Ser at aa position 40 or Phe to Tyr at aa position 75 is fatal to viral infectivity and effective RNA replication (Yaegashi et al., 2007b). To analyze whether these amino acid substitutions have an effect on the stability of CP mutants, CP, CPm40, CPm75, and CPm40m75 (two aa substitutions at positions 40 and 75) were transiently expressed in *N. occidentalis* leaves by agroinfiltration with agrobacteria carrying pBE2113-CP, pBE2113-CPm40, pBE2113-CPm75, and pBE2113-CPm40m75, respectively (Fig. 1A).

To analyze the accumulation level of CP and CP-mRNA, infiltrated leaves were collected at 24, 48, or 72 days post infiltration (dpif). Immunoblot analysis showed that both CP and CPm40m75 accumulated in infiltrated tissues at 24, 48, or 72 dpif, in contrast to proteins of CPm40 and CPm75 which were not detected (Fig. 1B, top panel). On the other hand, northern blot analysis showed that there was no obvious difference in the accumulation levels of CP-mRNA among CP, CPm40, CPm75, and CPm40m75 (Fig. 1B, bottom panel).

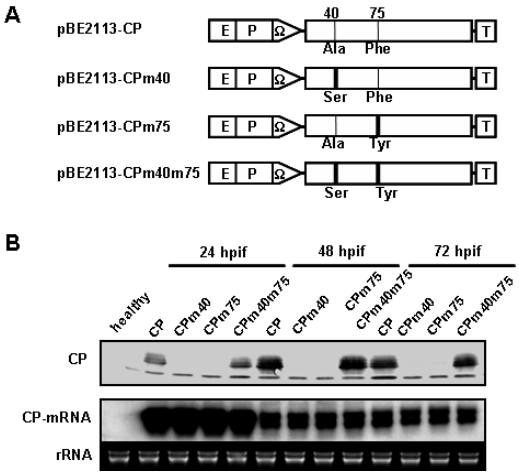

Fig. 1 Transient expression of mutant CPs in *Nicotiana occidentalis* by agroinfiltration. (A) Schematic representation of Ti plasmid constructs expressing CP and mutant CPs (CPm40, CPm75, CPm40m75). The pentagon labeled E, P, and Ω represents the transcriptional enhancer, 35S promoter of *Cauliflower mosaic virus* (CaMV), and the translational enhancer of the *Tobacco mosaic virus* 5'untranslated region, respectively. The box labeled T represents the nopaline synthase terminator. Bold lines in the CP boxes indicate the position of amino acid substitutions from Ala to Ser at 40 or Phe to Tyr at 75. (B) Immunoblot analysis of mutant CPs (Top panel) and northern blot analysis of CP-mRNA (bottom panel) in *N. occidentalis* leaves infiltrated with agrobacteria carrying a vector shown in (A) at 24, 48, 72 hours post infiltration (hpif). Ethidium bromide stainings of rRNA are shown as a loading control for northern blot analysis.

These results indicated that an amino acid substitution at aa position 40 or at aa position 75 reduces CP stability in *N. occidentalis*, and that the stable accumulation of CP is important for effective viral RNA replication and accumulation. It is not clear why CPm40 and CPm75 could not accumulate in *N. occidentalis* cells. The chemical structures of Ala and Phe are similar to those of Ser and Tyr, respectively, although the latter have no hydroxyl group in a side chain. Possibly, both CPm40 and CPm70 may not be able to form a highly stable structure in plant cells.

Transient expression of CP inhibits viral RNA accumulation: The results described above suggested that the reduction of viral replication of pBICLCPm40 and pBICLCPm75 in host cells may be due to instability of mutant CPs. Thus, we speculated that the stable accumulation of CP may enable pBICLCPm40 and pBICLCPm75 to replicate effectively in host cells. To test this hypothesis, agrobacteria containing a plasmid vector expressing CP (pBE2113-CP) and each pBICLSF based mutant shown in Fig. 2A were co-infiltrated into leaves of *N. occidentalis*. Northern blot analysis showed that viral genomic RNA did not accumulate in leaves infiltrated with a mixture of agrobacteria carrying pBE2113-CP and pBICL⁄rep, pBICLCPstop, pBICLCPm40, or PBICLCPm75 at 4 dpif (Fig. 2B). Similar results were obtained from northern blot analysis of viral genomic RNA in leaves co-infiltrated with a mixture of agrobacteria carrying pBE2113-P35T or pBE2113-P50 and each of the pBICLSF-based mutants (data not shown). These results indicated that the transient expression of CP could not complement the replication of pBICLCPm40 and pBICLCPm75. Intriguingly, when the leaves were co-infiltrated with a mixture of agrobacteria carrying pBE2113-CP and pBICLSF or pBICLCPm40m75, the accumulation levels of viral genomic RNA from pBICLSF and pBICLCPm40m75 were lower than that in leaves co-infiltrated with a mixture of agrobacteria carrying pBE2113-P35T and pBICLSF (Fig. 2B). The result supports a previous report showing that CP-expressing transgenic *N. occidentalis* was resistant to ACLSV (Yoshikawa et al., 2000). In the present study, we used an agroinfiltration method for viral inoculation which allowed us to analyze the RNA accumulation level in one cell (Voinnet et al., 2000; Yaegashi et al., 2007b). Therefore, we think the inhibitory effects of CP on ACLSV infection might be accounted for by the reduction of viral RNA accumulation in a cell.

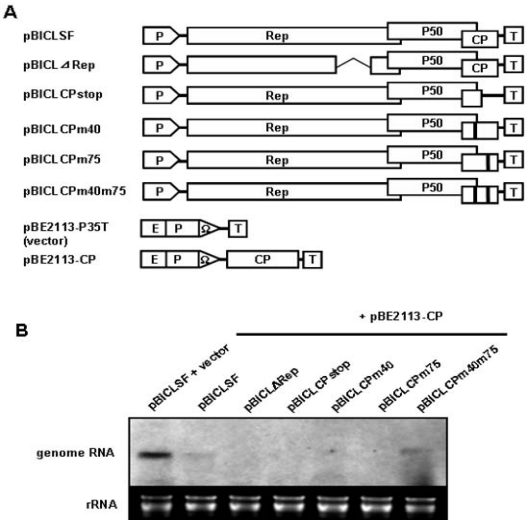

Fig. 2 The effect of transient expression of CP on accumulation of viral RNA from pBICLSF-based CP mutants. (A) Schematic representation of pBICLSF-based mutants and transient expression vector used for co-agroinfiltration. Bold lines in the CP box of pBICLCPm40, pBICLCPm75, and pBICLCPm40m75 indicate the amino acid substitutions at positions 40 (Ala to Ser) and 75 (Phe to Tyr). The representations of the pentagon labeled E, P, and Ω and the box labeled T are shown in the legend of Fig. 1. Rep, 216 KDa replication associated protein; P50, 50 KDa movement protein; CP, coat protein. (B) Northern blot analysis of viral genomic RNA extracted from leaves infiltrated with a mixture of agrobacteria carrying pBICLSF and pBE2113-P35T (vector), or pBICLSF-based mutants and pBE2113-CP at 4 days post infiltration. Ethidium bromide staining of rRNA is shown as a loading control.

Inhibition of viral genomic RNA accumulation by CP expression: To test whether the inhibitory effect of transient expression of CP on viral genomic RNA accumulation is mediated by a protein or mRNA, pBICLSF and each vector expressing CP, a frame-shift mutant of CP (FSCP), P50, or a frame-shift mutant of P50 (FSP50) shown in Fig. 3A was co-infiltrated into leaves of *N. occidentalis*.

Infiltrated leaves were collected at 4 dpif and an accumulation of viral genomic RNA was analyzed by northern blot analysis. The result showed that the accumulation level of viral genomic RNA in leaves co-infiltrated with pBICLSF and CP was lower than that in leaves co-infiltrated with pBICLSF and FSCP, P50, or FSP50 (Fig. 3B), indicating that the suppression of RNA accumulation is mediated by expression of CP, not by mRNA. It has been reported that transgenic plants expressing viral CP confer resistance to a homologous virus, referred to as CP-mediated resistance (CP-MR; Beachy, 1999). Several mechanisms of CP-MR are proposed, i.e., expression of CP interferes with disassembly of the challenge virus because transgenic plants expressing CP of *Tobacco mosaic virus* (TMV) are resistant to TMV particle inoculation, but not to TMV RNA inoculation (Register & Beachy, 1988; Bendahmane & Beachy, 1999). In the case of *Potato virus X* (PVX), PVX CP interacts with the origin of assembly and restricts replication, or it interferes with translation of the replicase because transgenic plants expressing CP are resistant to inoculation with both PVX particles and PVX RNA (Hemenway et al. 1998; Spillane et al. 1997).

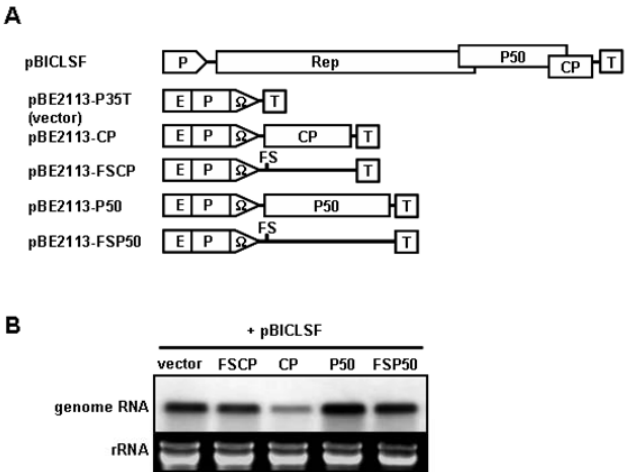

Fig. 3 Suppression of viral RNA accumulation by transient expression of CP. (A) Schematic representation of pBICLSF and transient expression vector used for co-agroinfiltration. The representation of the pentagon labeled E, P, and Ω and the box labeled T are shown in the legend of Fig. 1. (B) Northern blot analysis of viral genomic RNA extracted from leaves infiltrated with a mixture of agrobacteria carrying pBICLSF and each transient expression vector shown in (A) at 4 days post infiltration. Ethidium bromide staining of rRNA is shown as a loading control.

In this study, we show two conflicting sets of data; (1) stable accumulation of CP is important for effective viral genomic RNA accumulation, and (2) transient expression of CP inhibits viral genomic RNA accumulation. We think that ACLSV replication may be regulated by the level of CP accumulation and/or the timing of CP expression. Viral CP is known to have many functions including viral replication, symptom modulation, cell-to-cell movement, systemic spread, and suppression of RNA silencing, in addition to virion formation (Callaway *et al*., 2001; Thomas *et al*., 2003; Lu *et al*., 2004). Understanding the function(s) of ACLSV-CP in viral replication will elucidate the actual mechanism of positive and negative effects of CP on viral genomic RNA accumulation.

Acknowledgements

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Highly efficient inoculation method of apple viruses to apple seedlings

Yamagishi, N., Sasaki, S., Yoshikawa, N.
Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Iwate, Morioka 020-8550, Japan

Abstract

Virus inoculation to original plants is an important step in research for many reasons. For example, it is used to satisfy Koch's postulates, to test resistance to viruses in breeding programs, and to analyze gene function by virus vectors etc. However, it is generally difficult to inoculate viruses to woody fruit trees like apple, and an efficient inoculation method has not been developed thus far. In this study, we showed that a biolistic inoculation of total RNAs from infected tissues or virus RNAs resulted in a high infection rate in apple seedlings. Total RNAs extracted from *Chenopodium quinoa* leaves infected with *Apple latent spherical virus* (ALSV) or *Apple chlorotic leaf spot virus* (ACLSV) and ALSV- RNAs from purified virus were biolistically inoculated to the cotyledons of apple seedlings by a Helios Gene Gun system (BIO-RAD) or a PDS-1000/He Particle Delivery System (BIO-RAD). Analysis of true leaves 2-4 weeks after inoculation by Northern blot hybridization, RT-PCR, or ELISA showed that 36 out of 38 plants (95%) inoculated with total RNAs from ALSV-infected tissues, 39 out of 41 plants (95%) inoculated with ALSV-RNAs, and 6 out of 7 plants (86%) inoculated with total RNAs from ACLSV-infected tissues were infected with each virus. Thus, the biolistic inoculation of total RNAs from infected tissues or virus RNAs from purified virus to apple seedlings is found to be an efficient inoculation method of apple viruses. We think that the method can be applied to other virus-fruit tree combinations.

Keywords: ALSV, ACLSV, biolistic inoculation, apple, cotyledon

Introduction

Virus inoculation to an original plant is an important step to establish the viral etiology of a disease, to test resistance to viruses in a breeding program, and to analyze gene function by virus vectors etc. In woody fruit trees like apple, it is generally difficult to inoculate viruses by conventional inoculation methods, and an efficient inoculation method has not been developed thus far.

Recently, we reported that *Apple latent spherical virus* (ALSV)-based vectors could be used for reliable and effective VIGS and expression of foreign proteins in a broad range of plants (Li et al. 2004; Igarashi et al. 2009). However, the infection efficiency of ALSV inoculation to apple trees by conventional methods had been poor (Ito et al. 1992; Li et al. 2004). Thus, it is necessary to establish an effective inoculation method to analyze gene function of apple by using the ALSV vectors. In this study, we have established a highly efficient inoculation method of apple viruses to apple seedlings by biolistic inoculation.

Materials and methods

Viruses: ALSV and ACLSV (P205) were used in this study (Li et al. 2000, 2004; Sato et al., 1993).

Apple plants: Apple seeds were germinated at 4°C, sown in soil, and grown in a growth chamber (25°C, 16:8 light:dark photoperiod). Germinated apple seeds before being sown in soil were also used for biolistic inoculation.

Inocula: For rubbing and stem slashing inoculation, ALSV was purified from infected *C. quinoa* leaves by homogenizing with 0.1M Tris buffer (pH7.8), clarification by bentonite, precipitation with PEG6000, and sucrose density gradient centrifugation as described previously (Li et al. 2000).

For biolistic inoculation by particle bombardment, total RNAs were extracted from ALSV- or ACLSV- infected *C. quinoa* leaves by Tri reagent (Sigma) or Tripure isolation reagent (Roche Applied science) as recommended by the instruction manuals. The RNA samples were re-extracted by phenol/chloroform and then precipitated with ethanol. ALSV-RNAs were extracted from purified virus preparations by Tripure isolation reagent (Roche Applied science) as recommended by the instruction manual or by phenol/chloroform and then precipitated with ethanol.

Plant inoculation: For rub inoculation, the opened and expanded cotyledons of apple seedlings were dusted with 600 mesh carborundum and then rubbed with purified virus (1-6 μg/μl). For stem slashing, apple seedlings were grown to the 11-20 true leaf stages, and their stems were inoculated by cross-cutting with blades dipped in purified virus (1-6 μg/μl). Biolistic inoculation was performed by using a Helios Gene Gun system (BIO-RAD) or a PDS-1000/He Particle Delivery System (BIO-RAD). Microcarriers (gold particles) coated with total or virus RNAs were prepared as described previously (Yamagishi et al., 2006) with some modifications. For inoculation of total RNAs by a Helios Gene Gun system, microcarriers were prepared in the same amounts as those for total RNAs and gold particles which were approximately 5.0 μg and 0.3 mg per shot. For inoculation of virus RNAs by a Helios Gene Gun system and a PDS-1000/He Particle Delivery System, the amounts of virus RNAs and gold particles were approximately 3.0 μg and 0.4mg (0.6 μm in diameter) per shot, respectively.

The cotyledons of germinated seeds (Fig.1A) were bombarded with gold particles coated with total or virus RNAs at a pressure of 250 to 320 psi (Helios Gene Gun system) or 1100 psi (PDS-1000/He Particle Delivery System) using helium gas. Cotyledons of the apple seedling were bombarded with 3 to 4 shots (Helios Gene Gun system) or 2 shots (PDS-1000/He Particle Delivery System) per cotyledon. After particle bombardment, germinated seeds were sprayed with water and placed on a KIMWIPE (Crecia) soaked with water to retain humidity in the petri dish. The petri dish was then covered and placed at 4°C in the dark for 1-2 days. Then, the petri dish was wrapped with paper to lower light intensity, and placed into a growth chamber (25°C, 16:8 light:dark photoperiod) for 1day. The next day, the paper was removed and the apple seedlings were grown under the same conditions. When the apple seedlings grew up to the cover of the petri dish, the cover was removed from the petri dish. Then, the petri dish was placed into a large plastic case containing a paper towel soaked with water. When the second true leaf developed, the seedlings were planted in soil and acclimatized to the open air. In another procedure, apple seedlings were grown to the 2-19 true leaf stages (Fig.1B), and total RNAs were bombarded onto the leaves with 1-3 shots per leaf.

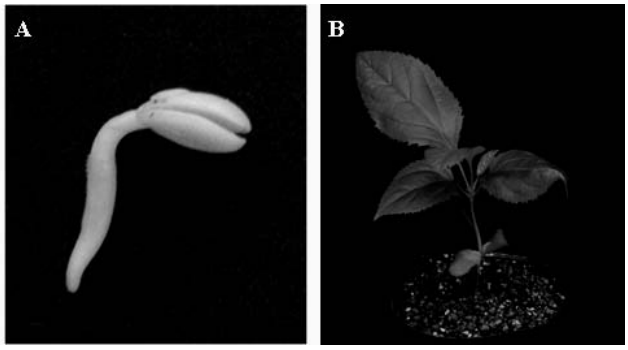

Fig. 1 Plant materials for biolistic inoculation. A, A germinated apple seed in which the seed coat was removed. Total RNAs or virus RNAs were bombarded to the cotyledons of this stage. B, An apple seedling at true leaf stage. Total RNAs from infected tissues were bombarded to the true leaves.

Analyses of inoculated plants: The infection efficiency was determined by Northern blot analysis, ELISA, and RT-PCR 2-4 weeks after inoculation as follows: total RNAs from inoculated apple seedlings were prepared according to Gasic et al. (2004) and Northern blot analysis was conducted as described previously (Yamagishi et al. 2009). For RT-PCR, first-strand cDNA was synthesized from total RNA using an oligo (dT) primer and Superscript reverse transcriptase (Promega). PCR amplification was performed using the following primer pairs: R2ALS1363+ (5'-GCGAGGCACTCCTTA -3'; homologous to positions 1362-1376 of ALSV RNA2) and R2ALS1551- (5'-GCAAGGTGGTCGTGA -3'; complementary to positions 1524-1510 of ALSV RNA2). The ELISA procedure used was the direct method of the conventional double antibody sandwich.

Results and discussion

The infection efficiency of viruses to apple seedlings by three inoculation methods is shown in Table 1. By rubbing and stem slashing inoculation, no virus infection was found. Although ALSV was previously shown to infect apples by rubbing and stem slashing inoculations (Ito et al., 1992; Li et al., 2004), these inoculation methods had a low level of efficiency. On the other hand, when total RNAs and virus RNAs were inoculated to the cotyledons of germinated seeds by particle bombardment, 36 out of 38 plants (95%) inoculated with total RNAs and 39 out of 41 plants (95%) inoculated with virus RNAs were found to be infected with ALSV. Similarly, 6 out of 7 plants (86%) were infected with ACLSV by the same methods. Our results showed that biolistic inoculation with RNAs resulted in high infection efficiency. In particular, it is thought that biolistic inoculation by using a Helios Gene Gun system with total RNAs from infected tissues is an attractive inoculation method, because total RNAs from infected tissue were sufficient for highly efficient inoculation. Furthermore, ALSV could be detected from the first true leaf which was developed just above the bombarded cotyledons (Fig. 2).

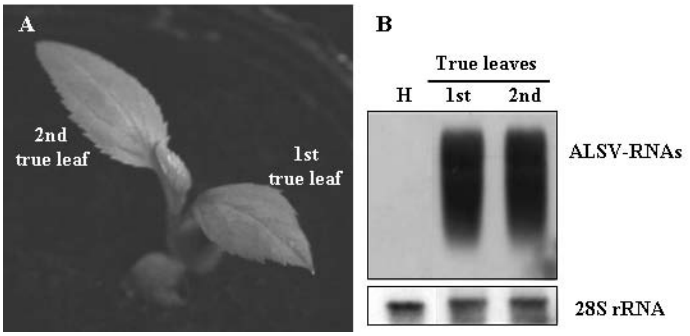

Fig. 2 Systemic infection of ALSV at the early growth stage of an apple seedling in which total RNAs were inoculated to cotyledons by biolistic inoculation. A, The apple seedling (17dpi) which was inoculated to total RNAs from infected tissues to the cotyledons in the stage of Figure 1A. B, Detection of ALSV-RNA from 1st and 2nd true leaves of apple seedling. H, healthy leaf sample.

Our results suggest that the cotyledons of germinated seeds were very sensitive for virus inoculation because biolistic inoculations to the true leaves of apple seedlings resulted in a low infection rate (Table 1).

Tab. 1 Infection efficiency of viruses to apple seedlings by three inoculation methods

Inoculation Methods	Virus	Inocula	Inoculation sites	No. of infected / Inoculated plants
Rubbing	ALSV	Purified virus	Cotyledons	0/17 (0%)
Stem slashing	ALSV	Purified virus	Stem	0/39 (0%)
Biolistic inoculation	ALSV	Total RNAs	True leaves	2/48 (4.2%)
	ALSV	Total RNAs	Cotyledons*	36/38 (95%)
	ALSV	Virus RNAs	Cotyledons*	39/41 (95%)
	ACLSV	Total RNAs	Cotyledons*	6/7 (86%)

*Cotyledons of germinated seeds

In conclusion, biolistic inoculation to germinated apple seeds is an efficient inoculation method of apple viruses. The method achieves high and reproducible infection efficiency and could be applied to other virus -fruit tree combinations.

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Nucleotide analysis of pome fruit virus isolates detected in apple and pear samples from Italy and India

Ferretti, L.[1], Hallan, V.[2], Rana, T.[2], Ram, R.[2], Dhir, S.[2], Negi, A.[2], Lakshmi, V.[2], Thockchom, T.[2], Zaidi, A.A.[2], Barba, M.[1]
[1] C.R.A. - Centro di Ricerca per la Patologia Vegetale, Via C. G. Bertero, 22, 00156 Rome, Italy.
Email: luca.ferretti@entecra.it
[2] Virology Lab, Institute of Himalayan Bioresource Technology (CSIR), Palampur-176061, HP, India

Abstract

In the framework of a joint research project between Italy and India field surveys were done in different pear and apple growing areas of North of India and Central and Southern Italy. Samples were collected from plants belonging to common and local varieties and molecularly analyzed for the detection of the main pome fruit viruses (*Apple stem pitting virus*, *Apple stem groving virus*, *Apple chlorotic leaf spot virus*, *Apple mosaic virus*) by using harmonized diagnostic protocols.

The sequence homology was evaluated and a phylogenetic tree was built, on the basis of which, the Indian isolate of ASGV showed maximum sequence identity at a nucleotide level to Italian isolates when analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarly, a maximum identity, ranging from 90-93%, was found for the Italian isolates of ASPV and pear and apple isolates from Poland, while a sequence homology ranging from 83 to86% was observed within the Indian isolates of ASPV. Multiple alignment of the Indian pome ACLSV-isolates indicate maximum variability in the middle portion while the first 140 nucleotides are maximally conserved and shared a percent identity at nucleotide level of 86-100% with the Italian isolates.

The ApMV Indian isolates showed maximum (92-99%) sequence homology to the Korean isolate (AY125977) from apple. However, a comparison with other isolates from different host plant species revealed a clustering of Indian isolates with a Czech isolate from pear and a sequence homology of 84 to 98%. Phylogenetic analysis showed that sequence variability was independent to the geographical origin or the host for all the investigated viruses.

Keywords: ACLSV, ASPV, ASGV, ApMV, sequences analysis, Italy, India.

Introduction

Among the temperate fruit crops, apple and pear are widespread species of relevant economic importance in many countries all over the world. In India apple is one of the major cash crops especially in the North Western Himalayan region (states of Himachal Pradesh, Jammu & Kashmir and Uttarakhand), where the local economy is largely dependant on apple cultivation. In Europe, apple and pear crops are particularly important and Italy is the main producing country.

Pome fruit are affected by a number of diseases, including those of viral etiology. Particularly, *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*), *Apple mosaic virus* (ApMV, *Ilarvirus*) and *Apple stem grooving* virus (ASGV, *Capillivirus*) are common pathogens in pome fruit trees. These viruses frequently occur in mixed infections and can significantly reduce the fruit yield and quality (Posnette et al., 1963; Desvignes, 1999).

In the framework of a joint research project between Italy and India, a molecular investigation on pome fruit viruses from these two countries was carried out in order to evaluate possible genomic diversity and phylogenetic relationships among isolates of different geographical origin. In this work we report the results of the nucleotide sequence analysis performed on the gene encoding for the capsid protein (CP) of ACLSV, ASPV and ASGV isolates from Italy and India and the comparison with other isolates retrieved in GenBanik[J1].

(http://www.ncbi.nlm.nih.gov/Genbank/).

A nucleotide analysis of ApMV isolates from India is also reported.

Material and methods

Source of isolates: A total of 12 Italian isolates of ACLSV (4), ASPV (5) and ASGV (3) identified in apple and pear trees from the regions of Latium and Sicily (Central and Southern Italy) were molecularly analyzed and compared with 19 Indian isolates of ACLSV (16), ASPV (2) and ASGV (1) identified in apple, pear and quince trees from the pome

fruit growing belts of Himachal Pradesh (North Western India). Seven ApMV isolates from India was also included in the analysis. No ApMV isolates were found in the samples collected from theItalian regions. Analyzed isolates are listed in table 1.

RNA extraction and cDNA synthesis and amplification: Total RNA was extracted from phloem tissue by use of the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacture's instructions with some modifications. For cDNA synthesis and amplification, specific primer pairs amplifying fragments of the complete or partial coat protein region of each virus were used: ACLSV, ASPV and ASGV primers as described by Menzel et al. (2002) were used in Italian and Indian (ASPV) labs; degenerate primers amplifying the complete coat protein and part of 3'UTR of ACLSV (Rana et al., 2008a) and the ASGV primer pairs 6396R/5641F (MacKenzie et al., 1997) were used in the Indian lab; finally, the primer pair PAPMCP3/PAPMCP5 (Choi et al., 2003) was used in the Indian lab for cDNA synthesis and amplification of ApMV. Synthesis of cDNA and amplification were performed as detailed in Rana et al., (2008b) at standardized temperatures of annealing for each virus specific primers.

Cloning of cDNA products, sequencing and sequence analysis: Amplified cDNA fragments of the analyzed isolates were cloned into pGEM T-Easy vector as directed by the supplier (Promega, USA) and then sequenced. A BLAST search was performed to verify the correspondence of the cloned amplicons with the investigated viruses. One recombinant plasmid containing cDNA was selected for each isolate for sequence comparisons. For each virus, a nucleotide sequence analysis was then carried out by generating a multiple alignment of both Indian and Italian isolate sequences using Clustal[J2] (Larkin et al., 2007). Finally, in order to investigate the phylogenetic relationships among isolates of different geographical origin, nucleotide sequences of Indian and Italian isolates were compared to corresponding regions of ACLSV, ApMV, ASPV, ASGV isolates worldwide identified in pome fruits downloaded from the NCBI database[J3] (http://www.ncbi.nlm.nih.gov/sites/genome). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al., 2007).

Tab. 1 List of the Indian and Italian virus isolates used in the nucleotide sequence analyses.

Virus	Isolate	Country	Host	Accession no.
ACLSV	India1	India	Apple	AM494505
	India2	India	Apple	AM494506
	India3	India	Apple	AM494507
	India4	India	Apple	AM494508
	India5	India	Apple	AM494509
	India6	India	Apple	AM494510
	India7	India	Apple	AM494511
	India8	India	Apple	AM494512
	India9	India	Apple	AM494513
	India10	India	Apple	AM494514
	India11	India	Apple	AM408891
	India12	India	Apple	AM409322
	India13	India	Apple	AM709776
	India14	India	Apple	AM709777
	India17	India	Pear	AM882704
	India21	India	Quince	AM498049
	ACLSV_MLO13P	Italy	Apple	
	ACLSV_PRO37P	Italy	Pear	
	ACLSV_RM22	Italy	Pear	
	ACLSV_VT2	Italy	Pear	
ASPV	Solan-SD3	India	Apple	FM863704
	Solan-SD4	India	Apple	FM863705
	ASPV_10M	Italy	Apple	
	ASPV_RM3	Italy	Apple	
	ASPV_VT36	Italy	Apple	
	ASPV_RM22	Italy	Pear	
	ASPV_VT2	Italy	Pear	
ASGV	India1	India	Apple	FM204881
	ASGV_VT36	Italy	Apple	
	ASGV_VT31	Italy	Apple	
	ASGV_10M	Italy	Apple	
ApMV	India1	India	Apple	FM178274
	India2	India	Apple	FJ429311
	India3	India	Apple	FJ429309
	India4	India	Apple	FN435317
	India5	India	Apple	FN435316
	India6	India	Apple	FN435315
	India7	India	Apple	FN43514

Results

Apple stem grooving virus: A high degree of identity (99%) at the nucleotide level was shown for the Italian isolates. Comparison with the Indian isolate showed an identity of 98% (Tab. 2). Moreover, the Indian isolate had the most identical sequence when Italian isolates were analyzed by BLAST. A clear clustering of Indian and Italian isolates into a closely related group was revealed by the phylogenetic analysis (Fig. 1). The ASGV-apple isolate from India shared a sequence identity of 87% with another ASGV Indian strain (FM393044) from pear (data not shown).

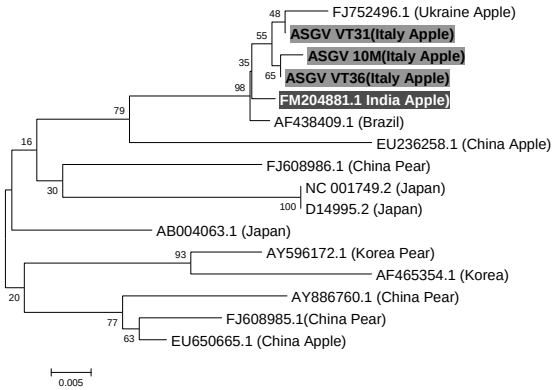

Fig. 1 Phylogenetic relationships among ASGV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Tab. 2 Nucleotide sequence identity (%) among Italian and Indian ASGV isolates.

	ASGV 10M	ASGV VT36	ASGV VT31	FM204881
ASGV 10M	-	99	99	98
ASGV VT36		-	99	98
ASGV VT31			-	98
FM204881				-

Apple stem pitting virus: An identity percentage ranging from 91 to94 was shared among the Italian isolates, while a sequence homology ranging from 83 to 86% was observed in comparison with the Indian ones (Tab. 3). Maximum identity ranging from 90 to93% was shown for the Italian isolates with pear and apple isolates from Poland (data not shown) when blasted with the ASPV-CP sequences from database. Phylogenetic analysis performed on all the apple and pear ASPV-CP isolates identified worldwide showed the presence of two main groups of isolates, reported as I and II: the Italian and Indian isolates clustered into the same group I, but with a high level of divergence clustering in two possible different sub-clusters (Fig. 2).

Tab. 3 Nucleotide sequence identity (%) among Italian and Indian ASPV isolates.

	ASPV 10M	ASPV RM3	ASPV VT36	ASPV RM22	ASPV VT2	FM863704	FM863705
ASPV 10M	-	91	93	92	93	83	83
ASPV RM3		-	94	91	92	84	84
ASPV VT36			-	92	92	86	86
ASPV RM22				-	94	84	84
ASPV VT2					-	86	86
FM863704						-	100
FM863705							-

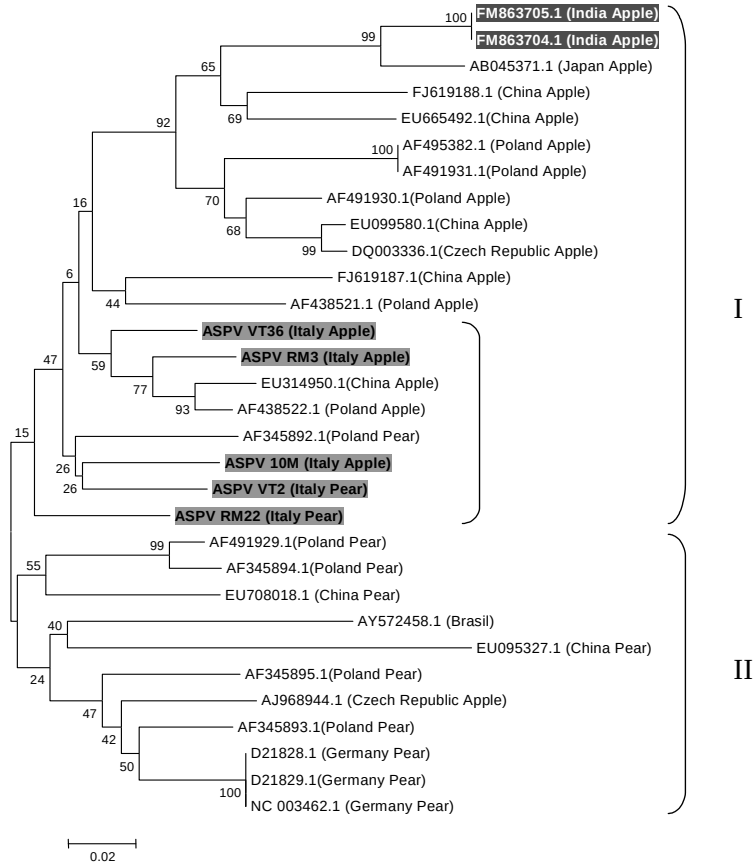

Fig. 2 Phylogenetic relationships among ASPV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Apple chlorotic leaf spot virus: The 20 ACLSV-apple isolates (16 Indian and 4 Italian) shared a percent identity at nucleotide level ranged from 86 to100. When compared among themselves the Italian isolates showed a sequence identity of 88-96%, while Indian isolates shared 82-93% (data not shown). An Indian ACLSV-quince isolate had a nucleotide sequence identity of 84% with a partial ACLSV-CP from Greece. A multiple alignment of the Indian pome ACLSV-isolates indicates maximum variability in the middle portion, while the first 140 nucleotides are maximally conserved. According with the classification proposed by Yaegashi et al. (2007), based on changes of the five highly conserved amino acids at positions 40, 59, 75, 130 and 184, India 2, 5, 6, 8, 9, 10,13, 17 and Italian MLO13 P, RM22 isolates resulted as 'B6 type', while India 1, 3, 4, 7, 11, 12, 14, 21 and Italian PRO37P, VT2 isolates as 'P205 type'. In a phylogenetic analysis no clustering on basis of location or host was evident (Fig. 3).

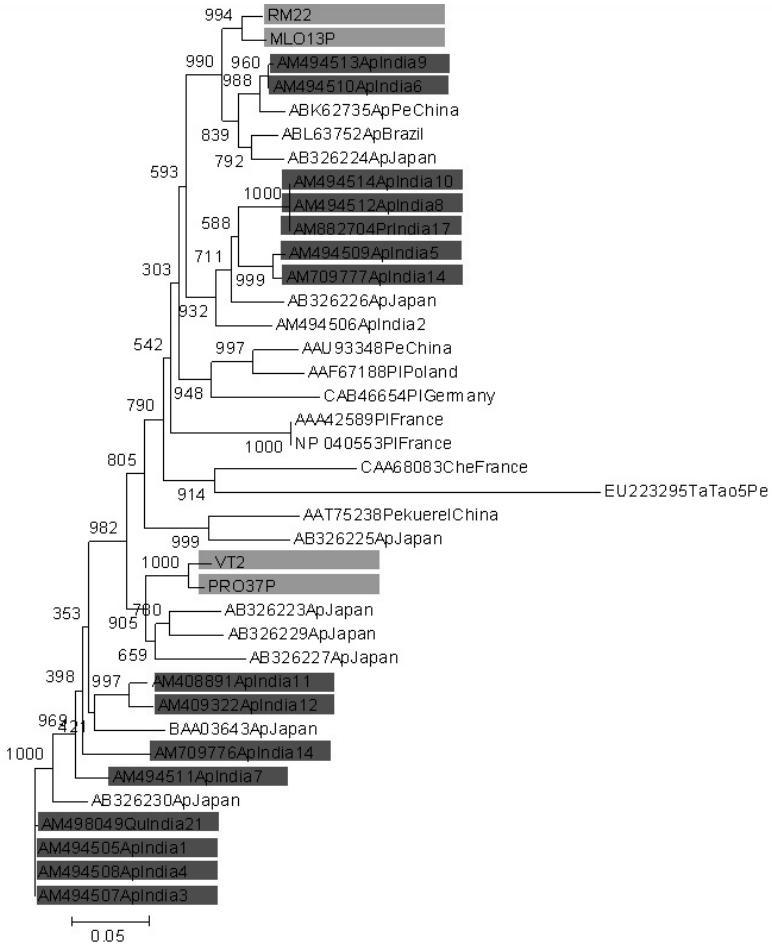

Fig. 3 Phylogenetic relationships among ACLSV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Apple mosaic virus: All the Indian sequences shared 92-99% sequence identity at a nucleotide level when compared among themselves and 59-60% with the PNRSV-apple isolate from India, used as an outgroup. The ApMV apple isolates India1 (FM178274), India2 (FJ429311), India3 (FJ429309), India4 (FN435317), India5 (FN435316), India6 (FN435315), India7 (FN43514) showed maximum (92-99%) sequence identity to the Korean isolate (AY125977) from apple. However, comparison with other isolates, characterized from different host plant species, revealed clustering of Indian isolates with a Czech isolate from pear and a sequence identity ranging from 84 to 98% was observed (data not shown). No clear clustering of ApMV-CP isolates from apple and other hosts was revealed by phylogenetic analysis (Fig. 4).

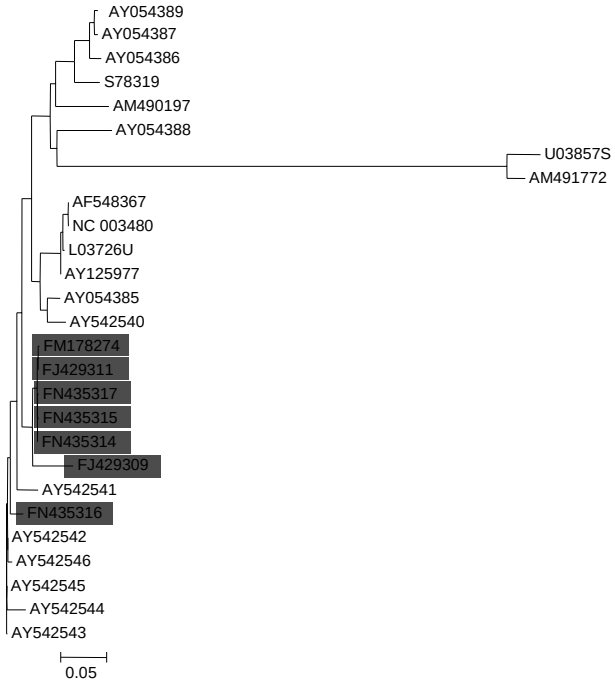

Fig. 4 Phylogenetic relationships among ApMV isolates from different hosts worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red the Indian isolates.

Discussion

On the basis of the obtained results new and improved knowledge on the genomic and phylogenetic relationship of pome fruit viruses has been obtained. In particular, this study provided further knowledge about the genetic diversity of ASPV and ASGV, especially in Italy, from where little information was available.

The nucleotide analysis performed on the investigated viruses confirmed the high level of molecular variability among different isolates of ACLSV (Candresse et al., 1995; Pasquini et al., 1998; Krizbai et al., 2001; Al Rwahnih et al., 2004) and ASPV (Nemchinov et al., 1998) both in Italian and Indian isolates. In contrast, a very high degree of similarity among Indian and Italian isolates was observed for ASGV, confirming the low coat protein gene variability among isolates from distinct regions, previously ascertained by other authors (Nickel et al., 2001). The phylogenetic analysis generated using the nucleotide sequences of virus isolates identified worldwide showed that the sequence variability was generally independent of the geographical origin of the isolates. This is probably due to the exchange of propagative plant material that currently occurs between different countries. Only for ASPV a possible correlation with the geographical origin resulted evident between Italian and Indian isolates, that clustered in two distinct sub-clusters. Nevertheless, more ASPV isolates from these countries should be investigated to confirm this evidence. No correlation based on host plant was shown by the phylogenetic analyses for all the investigated viruses. In particular, no clear clustering of ApMV-CP isolates of apple and other hosts was found, as previously observed by Lee et al. (2002), so classifying into subgroups need further characterization and analysis. The nucleotide sequences of two ASPV-CP (FM863705, FM863704) and three ApMV-CP (FJ429311, FJ429309, FM178274) Indian isolates were submitted to the GenBank database.

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Detection of *Pear Vein Yellows Disease* caused by *Apple stem pitting virus* (ASPV) in Hatay province of Turkey

Sertkaya, G.
University of Mustafa Kemal, Faculty of Agriculture, Department of Plant Protection, 31034 Antakya-Hatay, Turkey

Abstract

Pear vein yellows disease (PVYD) caused by *Apple stem pitting virus* (ASPV) was studied in 9 pear orchards and 3 nurseries in Hatay province of Turkey. A survey was carried out to inspect the symptoms of PVYD on pear (*Pyrus communis*). Leaf symptoms consist of yellow vein banding, reddening and flecking along the veins that were observed during the late spring to winter (dropping leaves). ApMv, ACLSV which are important viruses of pome fruits were also investigated on pear orchards and several quince (*Pyrus cydonia*) trees in pear orchards. The shoot and leaf samples were taken randomly from inspected trees in orchards and seedlings in nurseries for ApMv, ACLSV and for ASPV in April of 2008 and 2009. A total of 20 pear samples from 15 symptomatic and 5 asymptomatic young trees of local pear cultivars ('Mustafa Bey' and 'Ankara' cvs.) and 6 from quince (unknown cultivar) were collected. All samples were detected for the presence of the viruses by ELISA. Fifteen samples (ten samples selected from symptomatic plants and 5 samples from asymptomatic trees) were also tested for the viruses by Bioassay-sap inoculation. Sap extracts were mechanically inoculated on some indicator plants (*Chenopodium amaranthicolor, C. quinoa, Cucumis sativus, Gompherena globulosa, Nicotiana benthamiana, N. glutinosa, N. occidentalis*). Although, mild symptoms including vein clearing and leaf necrosis were observed on *N. glutinosa, and N. occidentalis* test plants, in general, no symptoms associated with the investigated viruses appeared on test plants. Some samples from local pear cultivars in Hatay were found to be infected with PVYD by serological tests. ASPV was found to be present in 60% of the ELISA-tested samples in 2008. This preliminary study demonstrated that a high rate of ASPV infection was present for local pear cvs. in the province. ApMV, ACLSV infections were not tested in detected samples.

Keywords: ELISA, Ilarvirus, pear, pome fruit, test plant

Introduction

Due to its geographical position, many horticultural crops can be grown in Turkey and the northern part of Turkey, called Black Sea Region, is one of the main genetic origins of several fruits such as apple, pear, sweet cherry, walnut, hazelnut and chestnut.

The main viruses affecting pome fruit trees are *Apple stem pitting foveavirus* (ASPV), *Apple stem grooving capillovirus* (ASGV), *Apple chlorotic leaf spot tricovirus* (ACLSV) and *Apple mosaic ilarvirus* (ApMV). These viruses occur frequently in mixed infections and cause significant yield reduction (Posnette et al., 1963 and Desvignes, 1999). ASGV is a member of the genus *Capillovirus* and is widespread in rosaceous fruit trees, particularly species of *Malus* and *Pyrus*. ApMV induces bright yellow patterns on leaves, while ASPV, ASGV and ACLSV are associated with latent infections (Németh, 1986). Different combinations of mixed infections of viruses were identified in samples of infected apple, pear and quince trees from different geographical regions in Turkey (Akbaş and İlhan, 2006; Birişik et al., 2006; Çağlayan et al., 2006; Ulubaş Serçe and Ertunç, 2006), and pear decline (PD, 16SrX-C) phytoplasma was first reported in Hatay,Turkey (Sertkaya et al., 2005).

Material and methods

Virus-like symptoms were observed on pear during field inspections in recently established pear orchards of local cultivars in the Hatay province of Eastern Mediterranean Region of Turkey in the summer of 2007. A survey was carried out to inspect the symptoms of PVYD on pear (*Pyrus communis*) trees in the Hatay province of Turkey in 2008 and 2009. For further examination of suspicious plants, samples from 20 symptomatic pear trees (3-4 years-old) of local cultivars ('Mustafa Bey' and 'Ankara' cvs.) were collected in 9 production orchards and 3 nurseries in the early spring of 2008 and 2009. Becausemany viruses can be symptomless in pome fruits, 5 samples from asymptomatic plants and 6 quince (*Pyrus cydonia*) samples from an unknown cultivar were also randomly selected. A total of 20 pear and 6 quince samples were investigated for ACLSV, ASGV and ASPV infection by ELISA (Clark and Adams, 1977). Reagents, obtained from Bioreba AG (Switzerland) were used according to the manufacturere's double antibody sandwich protocol. Plates were coated with IgG followed by incubation with a mixture of sample and alkaline phosphatase-labelled IgG. 4-Nitrophenyl phosphate substrate was used. Negative controls (healthy apple extracts) were

included in eight wells of each ELISA plate and samples were considered positive if they reached twice the Optical density (OD) of the average of the controls. Preliminary ELISAs were carried out in April 2008 and in May 2009.

Sap extracts of fifteen samples selected from symptomatic plants were mechanically inoculated on some indicator plants, *Chenopodium amaranthicolor, C. quinoa, Cucumis sativus, Gomphrena globulosa, Nicotiana benthamiana, N. glutinosa, N. occidentalis*. Young pear leaves were homogenized in 0.1 M phosphate buffer (pH 7.2) containing 2% nicotine in a pestle and mortar, and the sap extracts inoculated onto Celite-dusted leaves of herbaceous virus indicator plants. Four plants from each of the herbaceous indicator species were mechanically inoculated with the sap of a pear sample. Inoculated test plants were kept in an insect-proof growing room at 25°C±2 and 16:8 h photoperiod (day:night) for symptom observations.

Results and discussion

Observations of symptom expression were made during spring to autmn by repeated surveys in an attempt to associate the results of laboratory tests with field symptoms. More than 100 young trees were inspected in 9 commercial orchards and 3 nurseries. Leaf symptoms consisting of yellow vein banding, reddening and spotting or flecking along the veins and poor growth, were only observed on trees (3-4 years-old) in the orchards from spring to winter (due to leaf fall) of 2008 and spring of 2009. No obvious symptoms were seen on trees except for severe vein clearing on the leaves. Mild yellow spots were also observed on young leaves of a few pear trees (Figure 1).

Fig. 1 Vein yellowing and yellow spot symptoms of Pear vein yellows disease caused by *Apple stem pitting foveavirus* (ASPV) on naturally infected local pear cv. 'Mustafa Bey' in Hatay.

Serological tests in the first year showed that 60% of the total samples tested were infected by ASPV. Twelve of 20 pear samples were positive for ASPV by ELISA in 2008. However, four of them gave a negative serological result for the virus in 2009. In both years, young leaves taken from vegetative buds in spring gave the best results with highest readings for ASPV in ELISA. The results of field surveys and serological assays revealed that ASPV is one of the common agents of pear disease in Hatay. The evidence of the presence of ASPV have been proved in local cultivars of pear and quince in Hatay by serological assays repeated several times in both years. A high incidence of ASPV was also reported in different studies carried out on apple by molecular methods in Turkey (Birişik et al., 2006; Çağlayan et al., 2006). According to results of Kirby et al., (2001), the IC-PCR results were confirmed but two of the 22 infected trees were negative by ELISA. ApMv and ACLSV which are known as important viruses of pome fruits, were not found in the tested samples probably due to the limited number of quince and pear samples investigated in this study. All of these samples were mechanically inoculated to a standard series of herbaceous plants. Some *N. glutinosa, N. occidentalis* test plants inoculated with extracts from symptomatic pear plants showed mild vein clearing three to four weeks after inoculation. However, these plants did not test positive for ASPV in ELISA. In general, no symptoms associated with the investigated viruses appeared on test plants up to six weeks after sap inoculation. ApMV or ACLSV were not recovered by sap transmission tests. Therefore, serological test results were confirmed with biological assays for ApMV and ACLSV. ELISA which is comonly used for virus detection is a fast and simple assay with a relatively low cost, however it becomes unreliable for the detection of woody plant viruses during the summer due to a decrease in virus concentration. In several cases it is reliable only during a short period (maximum two months after bud break in spring). Although, some laboratory methods include ELISA as rapid and cheap laboratory assay for detecting ASGV, IC-PCR was reported to be most reliable for ASGV detection compared to the slow and expensive bioassays (Fuchs,

1980; Kirby et al., 2001). In preliminary results of Paunović and Jevremović (2006), in contrast to apples, detection of ASPV was not possible in young leaves nor in the flower petals of pear isolates.

The number of orchards has increased recently in high plateaus of the region. Because of the use of propagation material taken from plants in commercial orchards which are possibly infected with common virus/es, the main virus diseases, such as PVYD, spread from infected seedlings to new plantations in the region. The use of virus-free certified material for the establishment of new plantations is the main measure to prevent the spread of these viral diseases in orchards. Further detailed investigations are necessary, since ASPV is a major latent virus of pome fruits. It will also be necessary to investigate by advanced techniques local pear cultivars and other pome fruits grown in our region for the presence of the most important viruses.

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Determination of the effects of *Apple stem grooving virus* on some commercial apple cultivars

Birişik, N.[1], Baloğlu, S.[2]
[1] Plant Protection Research Institute. Adana, Turkey, nevzatbir@yahoo.com
[2] Plant Protection Department, University of Cukurova, Adana, Turkey. Email: saba@cu.edu.tr

Abstract

Studies to determine the effects of *Apple stem grooving Capillovirus* (ASGV) on external and physical characteristic of some commercial apple (*Malus dometsica* Borkh.) cultivars were carried out in the Adana Plant Protection Research Institute's screen house facility in Turkey during 2006-2008. The selected cultivars for this aim were 'Jersey Mac', 'Fuji', 'Golden Delicious', 'Summer Red', 'Granny Smith', 'Vista Bella', 'Galaxy Gala' and 'Starking'. The selection of the cultivars was based on their common use by growers in the country. All cultivars were grafted on M9 rootstock and potted in the screen house. Turkish io-50 ASGV isolate, which had been obtained from previous works from an 'Anna' apple tree, was used for inoculation by chip budding, and the success of inoculation was confirmed by DAS-ELISA. The trial was evaluated two years after inoculation, based on six external and two physical parameters of the inoculated trees. The results demonstrated that ASGV has no statistically important effects on tree length, number of the branches, average and total length of the branches, and leaf dry matter. However, ASGV decreased the trunk diameter by about 18%, and the woody dry matter in a statistically significant rate, whereas the angle of the branches from the trunk increased on average about 41% by ASGV infection. The cultivars reacted differently to virus inoculation and stem grooving symptoms were observed on some tested cultivars.

Keywords: ASGV, apple, cultivar, effectivness.

Introduction

Pome fruits are grown in nearly all agricultural areas of Turkey. Among the pome fruits, apple is the most important in the country with a yearly production of about 2.7 million tonnes. Several virus diseases of fruits have been reported on apple in different regions and on different cultivars. *Apple stem grooving* (ASGV) is known as one of the widest distributed latent pome fruit virus in all apple growing areas (Khan and Dijikstra, 2006). This virus has been reported in Turkey and is considered to exist in all the main apple production areas of Turkey, due to the use of infected rootstocks (Birisik et al, 2008*)*. ASGV is a member of *Capillovirus* genus from the *Flexiviridae* family (Anonymous, 2005). It has a ssRNA genome of 6496 nucleotides, with particles of 600-700 nm in length (Hirata et al, 2003). Research has demonstrated the economical importance of virus diseases even when they are mostly latent on commercial apple varieties with ASGV infection resulting in 23.4 % less growth and 13.7 % reduction in trunk diameter (Maxim et al, 2004). Virus diseases can be a considerable problem in intensive production systems and with correlating high temperature and fast grooving of sapling.

Materials and methods

Inoculum source: For the inoculation of the tested varieties, an ASGV isolate (io-50) from an infected 'Anna' apple in Adana region was used. This isolate has been detected and reported as Turkish ASGV isolate from previous research (Birişik et. al 2008).

Cultivar selection: The selection of cultivars from early, fall and winter type apples was made on the basis of their frequency in Turkey. 'Jersey Mac', 'Vista Bella' and 'Summer Red' are considered summer type apples, 'Galaxy Gala', 'Fuji' as fall harvest type apples and 'Granny Smith', 'Starking' and 'Golden' as winter type varieties.

Inoculation: Virus tested saplings were obtained from the Eğirdir Horticultural Research Institute (Isparta, Turkey) and inoculated by chip budding with ASGV according to Boscia et al., (1999) in June 2006. For each variety four inoculated and two healthy plants were used in the experiment.

Confirmation: DAS-ELISA tests were performed for the detection of ASGV one year after inoculation in order to understand the status of ASGV presence in inoculated trees (Clark and Adams, 1977). Before the final evaluation of the experiment, RT-PCR was used as a confirmatory test. For the RT-PCR assay total RNA was extracted from healthy and infected tissues using the Promega SV total RNA kit as described by suppler with slight modifications. The primers used in RT-PCR assays are the same as those reported by James, (1999) and Kummert et, al., (1998).

Evaluation of the experiment: The data were collected from the experiment after two vegetative years by means of measuring listed parameters for all plants. The counting of plant dry weight was done according to Walsh and Beaton (1973). All obtained data were processed in the SPSS statistical program.

Morphological characters:	**Physical characters:**
1. Length of trees.	1. Dry weight of the leaf tissues
2. Number of branches	2. Dry weight of the woody tissues
3. Average branch length	
4. Total branch length	
5. Trunk diameter	
6. Average branch angle	

Results

ASGV inoculation was made in May 2006, and plants were controlled for infection by ELISA and RT-PCR. In the latter, two different DNA bands (574 and 499 bp) were obtained , as expected size for the primers pairs used(Fig. 1). Data obtained after two years is presented in table 1. Results showed that; statistically ASGV has an effect on trunk diameter, branch angel and dry weight of woody tissues. Other characteristics of the inoculated plant were not different in comparison with healthy control plants. The selected apple cultivars did not react differently to ASGV inoculation based on their harvesting time.

The average trunk diameter of infected plants was calculated as 14.86 cm while control plants were 17.95 cm (Fig. 2). However the average branch angle of the inoculated trees was 66.5º, compared to the negative controls of 47.0º (Fig. 3). Moreover the dry weight of the woody tissue was 5.88 gr. for ASGV infected trees, and 5,99 gr. for control plants (Table 1). Obtained data were processed in the SPSS statistics program and are shown in Fig. 4. During the evaluation of the experiment stem grooving symptoms, which are characteristic for ASGV, have been observed on 'Starking' trees (Fig.6).

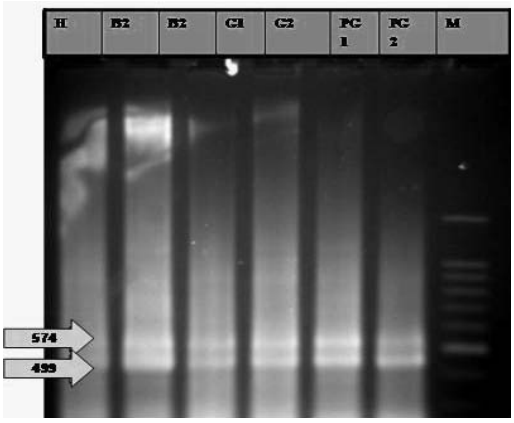

Fig. 1 RT-PCR results; ASGV-2/U 499 bp. (James, 1999) ASGV-4F/574 bp. (Kummert et. al1998)

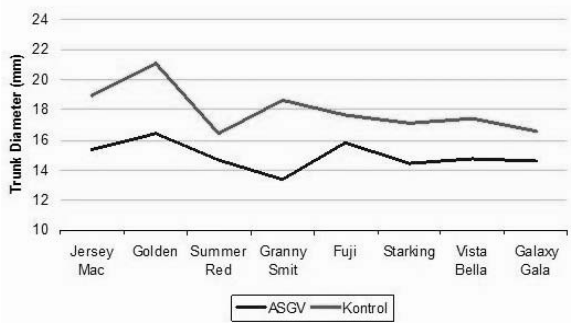

Fig. 2 Comparison of ASGV infected trees trunk diameter with control.

Tab. 1 Average of measured parameters for ASGV infected and control plants in the experiment.

	Characters															
Para-meters	**Jersey Mac**		**Golden**		**Summer Red**		**Granny Smith**		**Fuji**		**Starking**		**Vista Bella**		**Galaxy Gala**	
	ASGV	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**	**ASGV**	**Cont.**
Tree length (cm)	152	140	181	188	172	193	191	159	159	161	119	119	160	180	175	169
Number of branches	12,0	10,5	15,2	13,5	9,2	15,5	9,7	12,5	15,0	16,0	19,0	11,5	12,0	9,0	17,2	15,5
Total length of the branches (cm)	274	245	678	593	408	316	380	359	503	589	118	103	301	394	482	495
A. Length of the branches (cm)	24,2	23,8	45,5	44,6	46,5	20,5	41,8	16,9	33,7	36,8	6,3	9,0	25,2	44,3	28,2	32,2
Trunk diameter	15,3	18,9	16,3	21,0	14,6	16,4	13,3	18,6	15,8	17,6	14,4	17,1	14,7	17,4	14,5	16,6
A. angle of the branches	55,5	44,0	64,5	47,0	71,2	48,5	61,0	45,5	60,7	45,5	50,0	47,0	66,2	49,5	63,0	49,0
Dry weight of leaf tissue (gr)	4,15	4,01	4,03	4,13	3,85	3,78	4,27	4,44	4,39	4,37	4,36	4,57	4,14	4,41	4,20	4,29
Dry weight of woody tissue	5,81	5,94	5,81	5,93	5,87	5,99	5,81	5,94	5,91	5,98	6,05	6,16	5,97	6,09	5,84	5,95

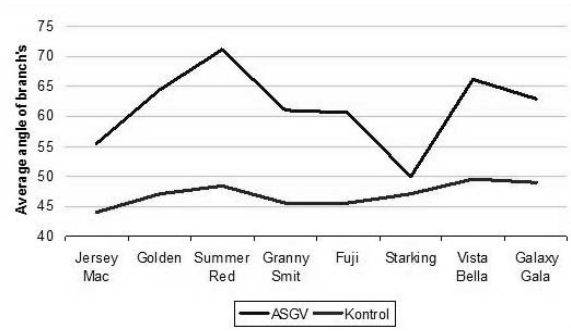

Fig. 3 Comparison of branches angle of ASGV infected trees with control.

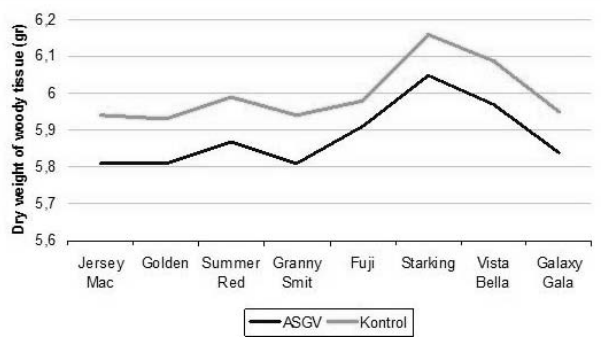

Fig. 4 Comparison of dry weight of ASGV infected woody tissues with control.

Fig. 5 ASGV infected Summer Red trees (right) have larger branch angle than healthycontrol (left).

Fig. 6 Stem grooving symptom observed on Starking.

Discussion

This study demonstrated that ASGV infection decreases the trunk diameter by about 18%, the dry weight of woody tissues by 2%, and increases the average branch angle to around 41%. Trunk diameter is an indicator for plant health: if the trunk growth is less vigorous compared to non-inoculated plants, then ASGV has a serious effect on tree size, a situation that will result in serious yield loss. Among the tested varieties 'Granny Smith' (39,8%) and 'Golden' (28,8%) were the most effected varieties in decreasing size of trunk diameter. These varieties are still popular in Turkey. The results of this study showed that ASGV infection has serious affect on plant dry weight. This fact could be very important in high density apple production systems using dwarfed trees because in these systems fruited branches are not as thick as classical trees. In case of ASGV infection branches could break at high yield. For woody dry weight, there were no big differences between the varieties. 'Granny Smith' and 'Jersey Mac' were most affected by 2.1% on average. Moreover ASGV infection increased the angle of the branches of all varieties but mostly 'Summer Red', ''Vista Bella' and 'Golden' with 46.8%, 37.2% and 34.6% on average, respectively (fig.5). This reaction might be due to shortage of dry material in woody tissues. As a result, trees could not be strong enough to carry long branches. ASGV infection induced stem grooving symptoms on 'Starking' which is not known as susceptible varieties to ASGV infection.

This result indicates that ASGV can induce some typical symptoms on many apple varieties in the appropriate growing conditions.

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Pome fruit viruses in Bosnia and Herzegovina

Lolić, B.[1,3], Matić, S.[2], Đurić, G.[3], Hassan, M.[4], Di Serio, F.[2], Myrta, A.[5]
[1] Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy
[2] Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy
3 Faculty of Agriculture, Bulevar vojvode Petra Bojovića 1A, 78000 Banja Luka, Bosnia and Herzegovina
4 PlantPathology Division, Faculty of Agriculture, Fayoum University, Egypt
5 Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA) Italy

Abstract

During autumn 2005 and summer 2006, field surveys were carried out to assess the sanitary status of pome fruit trees in Bosnia and Herzegovina. Inspections were done in the main pome fruit growing areas including 10 orchards, 2 nurseries and one varietal collection. A total of 65 apple and 50 pear cultivars were tested by biological indexing for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). The average infection level was 81%. Both species showed a similar infection rate (83% for apple and 78% for pear). The most frequent viruses of apple were ACLSV (72%) and ASPV (69%), and of pear ASGV (69%) and ACLSV (64%). The same samples were also tested by ELISA, with a lower virus detection rate compared to the biological indexing. Multiplex RT-PCR results of 20 randomly selected apple cultivars were in line with biological indexing. Results of our surveys report for the first time the presence of ACLSV, ASPV, ASGV and ApMV on pome fruits in Bosnia and Herzegovina.

Keywords: *Malus*, biological indexing, ELISA, multiplex RT-PCR, sanitary status

Introduction

Pome fruit growing in Bosnia and Herzegovina is practiced mainly in the northern part of the country. Among the pome fruits, apple and pear are the most important crops. There are 8,000 ha of cultivated apple and 2,600 ha of pear (Anonymous, 2002). The most popular cultivars are 'Idared', 'Golden Delicious', 'Granny Smith' and 'Jonagold', grafted mostly on the rootstock MM106 and less on M9. The leading pear cv. is 'William' and common rootstocks for pear are quince and pear seedlings. Pome fruit viruses are frequently latent and therefore easily spread by propagation material. Latent viruses are more widespread than the others (Hadidi, et al., 2003). The main viruses affecting pome fruit trees are *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). These viruses occur frequently in mixed infections and can cause significant yield reduction (Posnette et al., 1963; Desvignes, 1999). This study, the results of which are presented here, set out to survey for the presence of pome fruit viruses, for the first time, in Bosnia and Herzegovina.

Materials and methods

Surveys for symptoms and sample collections were done in the main pome fruit growing regions. Samples were collected from 10 commercial orchards, 2 nurseries and one varietal collection. The visited commercial orchards were of different sizes and mostly young, though a few older orchards were also visited. The varietal collection was mid-aged, but not properly managed, in particular for pest control practices. Most of the visited pome fruit trees did not show any symptoms and that is in line with previous reports on the latency of pome fruit viruses (Desvignes, 1999). However, in some orchards, leaf symptoms of yellow mosaic on apple cvs. 'Idared', 'Jonagold', 'Granny Smith' and 'Melrose', associated with the presence of ApMV, and vein yellowing on pear cultivars: 'Mindjusica', 'Kolacusa' and 'Sarajka', associated with a strain of ASPV, were observed. Bud-sticks of 30 to 40 cm long from one-year old shoots were taken from 115 trees of pome fruits (65 apple and 50 pear les, each of them a different cultivar) during autumn 2005, whereas symptom observation was done during spring-summer 2006. Collected budwoods were grafted onto one–year old virus-free seedlings of indicators of apple (Spy 227, R 12740 7A and 'Virginia Crab') and pear (*Pyronia veitchii*, LA 62 and 'Virginia Crab'). The entire collection was double chip-bud grafted using three indicator replicates per sample. Seven to ten days later, the rootstock was cut off 1-2 cm above the upper bud graft to force growth of the indicator. All inoculated plants were maintained in the acclimatized greenhouse at 20-24ºC and constant light. Grafting of indicator plants was done during December 2005. The first observation for symptoms was made 4 months after inoculation. Two more observations were done in May and June 2006.

All samples were tested by DAS-ELISA (Clark and Adams, 1977) for ApMV, ASPV and ASGV, and DAS-simultaneous ELISA (Flegg and Clark, 1979) for ACLSV. Serological reagents were provided by commercially kits (Loewe, Germany). For ASPV, ELISA was performed with Bioreba commercial kit (Switzerland). ELISA was done using leaf extracts from grafted indicator plants. Multiplex RT-PCR, for the simultaneous detection of ACLSV, ASPV, ASGV and ApMV, was done as described by Hassan et al. (2005). Total nucleic acid (TNA) extraction was according to Foissac et al. (2001). The PCR mixture contained a cocktail of five primer pairs: 1 μM for each ACLSV primer (Menzel et al., 2002), 0.8 μM for each ASPV primer (Menzel et al., 2002), 0.6 μM for each ASGV primer (Menzel et al., 2002), 0.4 μM for each ApMV primer (Hassan et al., 2005) and 0.25 μM for each internal control (*nad5*) primer (Menzel et al., 2002).

Results

- *R 12740 7A* - chlorotic spots and leaf deformation (small, sickle-shaped leaves) were seen, similar to those caused by ACLSV. Dwarfing of indicators grafted with apple cvs. 'Golden Delicious', 'Florina', 'Srcika' and 'Pinova' was also present. A total of 44 plants were infected out of 61 tested (infection rate 72%) for ACLSV (Table 1).
- *Spy 227* - epinasty of the leaves, showing within 2-3 months, was associated with the presence of ASPV. A total of 45 plants out of 65 tested (69%) were found infected with ASPV (Table 1).
- *Virginia Crab* – a necrotic line at graft union was associated with ASGV. A total of 10 plants, out of 30 tested (33%), were found infected by ASGV (Table 1). The results of this test were not satisfactory because a limited number of tests were considered reliable, due to the low graft take and the short observation period of 6 months after grafting. Biological testing of ASGV gives more reliable results in the open field in 2-3 years (Boscia et al., 1999).
- *LA 62* - chlorotic spots on the leaves were associated with the presence of ACLSV. Considered as sensitive indicator for the virus (Boscia et al., 1999), a total of 32 LA62 plants out of 50 tested (64%), were found to be infected (Table 1).
- *Pyronia veitchii* - epinasty and vein yellowing on the leaves were associated with the presence of PVYV (ASPV). A total of 11 plants were found infected out of 50 tested (22%) (Table 1).
- *V. Crab* - greenhouse tests indicated an infection rate of 69% by ASGV, due to 11 plants infected out of 16 tested. Numerous indicator plants (34 cultivars) were eliminated as non-tested (NT) due to the unsuccessful graft take (Table 1).

ELISA showed to be less reliable than biological indexing, which could be due to the host species, low virus titer, inhibitory effects of polysaccharides and phenolic compounds, as previously reported (Desvignes et al., 1992; Boscia et al., 1999; Kinard et al., 1996).

Tab. 1 Detected viruses by biological indexing and ELISA in apple and pear

	ACLSV		ASPV/PVYV		ASGV		ApMV	
Samples	**Index* (I/T)**	**ELISA (I/T)**	**Index** (I/T)**	**ELISA (I/T)**	**Index*** (I/T)**	**ELISA (I/T)**	**Field Sympt (I/T)**	**ELISA (I/T)**
Apple cultivars	44/61	13/61	45/65	9/65	10/30	1/30	4/65	0/65
Pears cultivars	32/50	12/50	11/50	6/50	11/16	1/16	0/50	0/50
Total	76/111	25/111	56/115	15/115	21/46	2/46	4/115	0/115

I: Infected; T: Tested; Woody indicators for apple: * R12 (ACLSV); **Spy 227 (ASPV); *** V. Crab (ASGV);
Woody indicators for pear: * LA62 (ACLSV); ***Pyronia veitchii* (PVYV); *** V. Crab (ASGV)

Thirty-five (30% of 115 samples) tested positive for at least with one virus(ACLSV, ASPV and ASGV). ApMV was not found (Table 1). Twenty randomly selected apple cultivars were tested by multiplex RT-PCR which detected the presence of all four viruses in apple (ACLSV, ASPV, ASGV and ApMV), significantly reducing the time required for detection. Amplified products of TNA extractions from leaves of indicator plants, showed clear bands of the expected size. Multiplex RT-PCR proved more sensitive (infection rate higher with 50%) as compared with ELISA. Interestingly, multiplex RT-PCR results were generally in line with those of biological indexing. Both techniques showed similar levels of infection rates for: ACLSV, ASPV and ASGV (data not shown).

Discussion

This is the first extensive survey on the sanitary status of pome fruit trees in Bosnia and Herzegovina including 115 cultivars: 65 apples and 50 pears. The present study showed that the local pome fruit industry in the country is affected by the presence and high incidence of apple and pear viruses. The presence of 4 viruses (ACLSV, ASPV, ASGV and

ApMV) was detected. The most frequent virus was ACLSV in apple and ASGV in pear. Latent infection by pome fruit viruses and their presence in nurseries present a threat to pome fruit production increasing the difficulty of their future control. To our knowledge, this is the first report of ACLSV, ASPV, ASGV and ApMV on pome fruits in Bosnia and Herzegovina.

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Detection and identification of *Apple stem pitting virus* and *Apple stem grooving virus* affecting apple and pear trees in Egypt

Youssef, S.A.[1], Moawad, S.M.[2], Nosseir, F.M.[3], Shalaby, A.A.[1]
[1] Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
[2] Central Administration of Seed Test and Certification (CASC), Giza, Egypt
[3] Central Administration for Foreign Agriculture Relations, Dokki, Giza, Egypt

Abstract

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) are economically important and infect either individually or in mixed infection commercial apple and pear cultivars causing yield loses. Young green bud and/or base of petiole were collected from naturally infected apple and pear trees from different locations in Egypt. Both viruses were detected frequently in apple and pear samples. A total of 420 trees from 9 different orchards were tested using one-step RT-PCR; 13% and 17% of these samples were infected with ASPV and ASGV, respectively. Mixed infection with both viruses occurred in 4% of the tested trees. ELISA was reliable for detection of ASGV but not ASPV. Total RNA for one-step RT-PCR was isolated from 100 mg fresh affected apple and pear leaf tissue using Qiagen RNeasy plant mini-kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. The one step-RT-PCR method was performed using ASPV and ASGV-specific primers for each virus. A 316 bp fragment for ASPV and 524bp fragment for ASGV were amplified and detected by gel electrophoresis analysis which indicated the presence of ASPV and ASGV in affected apple and pear cultivars. Southern blot hybridization of the amplified products to digoxigenin (DIG)-labeled cDNA probe for ASPV or ASGV confirmed the results obtained by electrophoresis analysis. No product was detected in amplified extracts of uninfected apple and pear samples. The detection of ASPV and ASGV by one step-RT-PCR assay was successful and appears useful for testing pome fruit germplasm in quarantine and budwood in certification programs.

Keywords: apple and pear, ASPV, ASGV, virus detection, One step RT-PCR, Southern blot hybridization

Introduction

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) belong to the genera *Foveavirus and Capillovirus,* respectively. Both viruses have elongated filamentous particles and are latent in most commercial apple cultivars, graft transmissible, world-wide distributed in pome fruit trees, and often occur in mixed infection (Nemeth, 1986; Kummert et al., 1998; Kundu, 2003a). ASGV symptoms are expressed when an infected apple cultivar is grafted on a sensitive rootstock such as *Malus pumila* cv. Virginia crab. ASPV-infected plants grafted on the sensitive apple rootstock "Spy 227" cause epinasty and decline and stem pitting. The virus also causes stem pitting of Virginia crab. ASPV may cause characteristic symptoms on some susceptible apple cultivars. Also, it causes different diseases in pear such as pear vein yellows and pear stony pit. Biological indexing by grafting on woody indicators constitutes the baseline test for certification of fruit tree planting material, but it is cumbersome, lengthy and expensive to perform (time, space, and qualified manpower required) and does not respond to the actual constraint of production of certified material in a world where volume, distances, and rapidity of the exchange of material have increased dramatically (Kummert et al., 1998). Under these conditions, reliable and rapid detection protocols for latent viruses is thus important in the implementation of sanitary control of propagative material of fruit trees. Enzyme linked immunosorbent assay (ELISA)-based detection is routinely used for the detection of ASGV and ASPV (Fuchs, 1981; Kummert et al., 1998; Gugerli and Ramel, 2004) and several commercial kits are available, but ELISA is not sensitive enough to detect low concentration of these viruses in infected trees during summer months or in dormant tissues. The molecular amplification-based assay, reverse-transcription polymerase chain reaction (RT-PCR) has contributed to increase the detection sensitivity providing a means for detecting viruses in woody plants throughout the year, even during seasons of low concentration. Moreover, PCR primers are more easily produced than the virus-specific antiserum needed for ELISA. This paper presents results obtained for detection of ASGV and ASPV infection in apple and pear orchards in different locations in Egypt.

Materials and methods

Source of Samples: Leaf samples were collected randomly from different cultivars grown in commercial orchards during June and July of 2008 for ASGV and ASPV, respectively. Totally 420 samples were collected; 224 apple samples were collected from 2 verities (Anna, Dourest gold) and 196 pear samples were collected from 3 verities

(Lecont, Hod and Florida home). The samples were collected from 9 commercial orchards located in 4 Governorates in Egypt.

ELISA: All collected samples were subjected to DAS-ELISA (Clark and Adams,1977) using the ASGV detection kit of Loewe Biochemica (GmbH,Germany), and ASPV detection kit of Bioreba (AG,Switzerland) following recommendations of the manufacturer.

Total RNA extraction: One hundred mg of fresh apple or pear leaves were ground under liquid nitrogen to a fine powder using a mortar and pastel, then applied for RNA extraction using Qiagen RNeasy Plant Mini Kit (Qiagen) according to the instruction manual.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR): RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Inc.). The master mix typically contained all the components required for RT-PCR except the RNA template. The mix prepared in a thin-walled 0.2 µl PCR tube, by combining 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 2.0 µl of dNTP mix (containing 10mM of each dNTP), 1µl of each viral and complimentary primers (Table 1) to a final concentration of 0.6µM, 2.0 µl of QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStartTaq™ DNA Polymerase). To this mix 5 µl of total RNA were added and RNase-free water to a final volume of 50 µl. First strand cDNA synthesis was done using the following parameters: 50ºC for 30 min at 1 cycle. Second strand cDNA synthesis and PCR amplification were done in the same tube using DNA Thermal Cycler (DNA Engine, Bio-Rad) with the following parameters: 95ºC for 15 min at 1 cycle to activate HotStarTaq DNA Polymerase and to inactivate simultaneously the reverse transcriptases. The amplification parameters: denaturation at 94ºC for 30s, primer annealing at 54ºC (ASPV) and 60ºC (ASGV) for 45s, and extension at 72ºC for 60s, for 35 cycles with a final extension at 72ºC for 7 min.

Tab. 1 DNA primers used for reverse transcription-polymerase chain reaction (RT-PCR) amplification of ASGV or ASPV

Virus	Primer	Sequence	(bp)	Reference
ASGV	C6396	5'-CTGCAAGACCGCGACCAAGTTT-3'	524	Serghini et al., 1990
	H5873	5'-CCCGCTGTTGGATTTGATACACCTC-3'		
ASPV	C8849	5'-TGCCTCAAAGTACACCCCTCAGT-3'	316	Jelkmann, 1994
	H8534	5'-CGCCAAGAAATGCCACAGC-3'		

Electrophoresis Analysis: Five-microliter aliquots of RT-PCR product were analyzed on 1.5 % agarose gels (6 x 8 cm), in TBE buffer (89 mM Tris-HCL, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volts. 100bp DNA molecular weight markers were used to determine the size of RT-PCR products. Gels were stained with ethidium bromide 10 µg/ml and visualized by UV illumination (Bio-Rad) (Sambrook et al., 1989). About 75 min were required for running the agarose gels and staining with ethidium bromide.

Probe Preparation and Southern Blot Hybridization Analysis of PCR-Products: PCR products for ASPV and ASGV were used as the template to synthesize a cDNA probes. Digoxigenin-ll-dUTP (Dig-ll-dUTP), (Boehringer Mannheim, Indianapolis, IN) was incorporated into the newly synthesized cDNA during 35 cycles of PCR with melting, annealing, and extension as described above. The cDNA was amplified in a Bio-Rad Engine, Thermal Cycler (Bio-Rad). For each reaction final concentrations were 0.2 µM of each primer, 10 mM dATP, dCTP, dGTP; 0.65 mM dTTP; 0.35 mM Dig-ll-dUTP labelled and 1.2 U *Taq* DNA polymerase (Promega Corporation, Madison, WI) in 50 µ1 containing 200 ng target cDNA. The expected molecular weight of the PCR-amplified product was confirmed by agarose gel electrophoresis as mentioned above (Sambrook et al., 1989). For Southern hybridization analysis (Southern, 1975), agarose gel containing separated cDNA fragments were soaked twice in 0.5M NaOH/1.5 M NaCl for 10 min each to denature the DNA, the denatured agarose gel was placed on a Saran Wrap covered glass plate and overlaid with the positively charged Hybond N+ membrane, 3 layers of Whatman 3MM paper, followed by 2 cm thick paper towels or blotting paper and glass plate. All were cut to the gel size. This was covered with Saran Wrap to prevent dehydration; a 250 g weight was placed on top of the covered gel and left for 6 h to overnight to allow transfer the DNA from the gel to the membrane. Pre-hybridization, hybridization and immunological detection were carried out using the "Genius II DNA Labeling and Detection Kit" (Boehringer Mannheim, IN) according to the manufacturer's recommendation.

Results and discussion

ELISA and RT-PCR: ELISA absorbance values for ASGV detection allowed the differentiation between positive and negative samples. ELISA-ASPV detection, however, yielded not always clear results and some of the results were difficult to interpret. Moreover, the background values were high and it was not possible to clearly distinguish in some samples virus-positive from virus-negative. The RNA Extraction protocol described in this paper has facilitated the routine RT-PCR detection of a number of different plant viral pathogens in their woody hosts. The method was based

on the commercially available RNase kit and was superior to other methods, in that it mitigated the effects of phenolic compounds and other inhibitory substances, avoided the use of organic solvents and phenol, and was extremely rapid. A modification of the manufacturer's tissue lysis buffer, which included addition of PVP and pH buffering to 5.0 with 0.2 M sodium acetate, was instrumental in improving the reproducibility of RNA extractions from diverse host species (Mackenzie et al., 1997). High-quality RNA suitable for use in One-step RT-PCR could be obtained from apple and pear allowing detection of ASGV and ASPV infection in these hosts (Table 2).

Tab. 2 Incidence of ASGV and ASPV among pome fruit samples from 9 commercial orchards in Egypt.

			Infected trees		
Species	**Variety**	**Tested trees**	**ASGV**	**ASPV**	**Mixed infection**
	Anna	200	47	30	11
Apple	Dorset Gold	24	3	00	00
Pear	Le Conte	100	12	20	4
	Hood	65	3	5	2
	Florida Home	31	7	00	00
Total		420	72 (17%)	55(13%)	17 (4%)

One-step RT-PCR was elaborated using primers targeting amplification a part of the coat protein of ASGV or ASPV genome. Primer pairs specifically detected ASGV or ASPV are presented (Fig.1 and Fig.2).

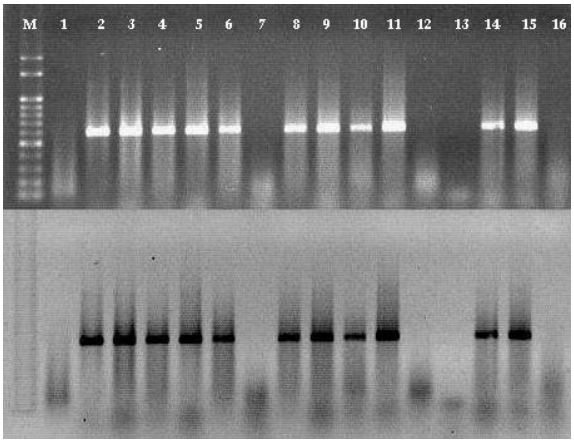

Fig. 1 **a)** Agarose gel electrophoresis of RT-PCR products of different *Apple stem grooving virus* (ASGV)-infected apple and pear trees. Lane M, 100 bp DNA molecular weight marker; Lanes 1, 7,12 and 13 non-infected apple and pear samples; lane 16 negative control; the remaining lanes represent some infected samples from apple and pear trees. **b)** An autoradiograph of Southern blot hybridization of a DIG-11-labeled ASGV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees. A DNA fragment of the expected size (524 bp) from infected apple and pear leaves hybridized to ASGV- specific probe. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (lanes 1,7,12,13 and 16) .

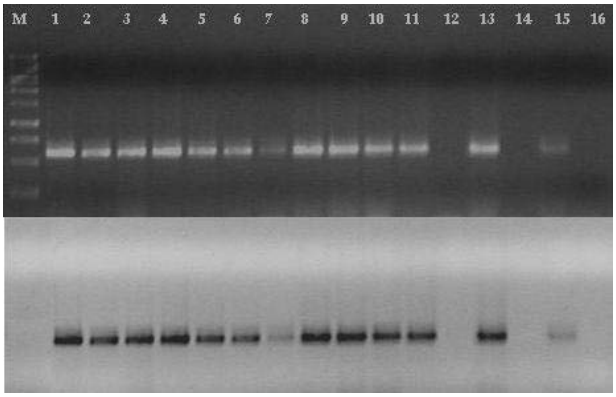

Fig. 2 **a)** Agarose gel electrophoresis of RT-PCR products of different *Apple stem petting virus* (ASPV)-infected apple and pear trees. Lane M, DNA molecular weight marker ; Lanes 12 and 14 non-infected apple and pear samples; lane 16 negative control; the remaining lanes represent some infected samples from apple and pear trees. **b)** Autoradiograph of Southern blot hybridization of a DIG-11-labeled ASPV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees. A DNA fragment of the expected size (316 bp) from infected apple and pear leaves hybridize to the ASPV specific probe. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (lane 12, 14 and 16).

One–step RT-PCR confers a highly sensitive tool for the detection of these viruses, in which the entire reaction is carried out in a single tube, thus the risk of contamination, is significantly lower (Kundu, 2003b).

Autoradiographs were obtained for Southern blots of hybridization DIG-11-labeled ASGV or ASPV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees from different commercial orchards in Egypt. A DNA fragment of the expected size (524 bp for ASGV) from infected apple and pear leaves hybridized to ASGV specific probe (Fig. 1b). Another DNA fragment (316 bp) hybridized to ASPV specific probe (Fig. 2b). These fragments were similar in size to those amplified from ASGV and ASPV infected apple and pear samples. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (Fig. 1, lanes 1,7,12,13 and 16) for ASGV and (Fig. 2, lanes 12,14 and 16) for ASPV.

For many viruses such as ASGV and ASPV that cause significant diseases in *Malus* and *Pyrus* species, the most commonly used diagnostic methods rely on biological indexing by either woody or herbaceous indicator plants (Leone and Lindner, 1995). While serological reagents are available for these viruses, the use of ELISA for routine screening of viruses has been limited to their detection in herbaceous plants, or to specific times of the year from young leaf tissue or flower blossoms. It is anticipated that the RT-PCR method described in this paper will facilitate the development of rapid and sensitive diagnostic techniques that can be used year-round for specific detection of these and other important viral pathogens directly in their woody hosts.

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The first survey of pome fruit viruses in Morocco

Afechtal, M.[1], Djelouah, K.[1], D'Onghia, A.M.[2]
[1] Institut National de la Recherche Agronomique, Centre de Kénitra, Maroc.
[2] Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy

Abstract

Considering the limited information on the presence and incidence of pome fruit virus and viroid diseases in Morocco, a preliminary assessment of the presence of pome fruit viruses in Morocco was carried out. Twenty orchards and nurseries were surveyed in the regions of Midelt, Meknès and Azilal. A total of 100 samples (apples and pears) were collected and tested. Biological indexing was made in a acclimatised greenhouse using the following indicators: *Malus pumila* cvs. ʻSpy 227', ʻRadiant' and ʻR 12740 7A', and *Pyrus communis* cv. ʻLA/62'. All samples were also tested by ELISA for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV). The prevailing viruses infecting apple were ACLSV (71%) and ASPV (58%), whereas ASGV was found in 12 tested trees. The same viruses were present, but less frequently, in pear: ACLSV (61%), *Pear* Vein Yellows Virus (PVYV) (25%) and ASGV (18%). Only four apple trees were found to be infected by ApMV. Additional RT-PCR testing confirmed the high incidence of ACLSV and ASPV.

This was the first report of the presence of pome fruit viruses in Morocco, indicating the high infection rate worsened by the recent report of the presence of fire blight (*Erwinia amylovora*) in the country. Moreover, a total of 168 apples and 81 pears were sampled and tested for pome fruit viroids *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) by tissue printing hybridization. No viroids were detected.

Keywords: pome fruit, viruses, viroids, Morocco, ELISA, Tissue printing hybridization, PCR.

Introduction

Pome fruit crops are mainly grown in the regions; Khénifra-Midelt, the Middle and High Atlas (Azrou-Ifrane), the Saïs plain (Meknès-Fès), Haouz Marrakech and Gharb. Among all rosaceous fruit trees, apple ranks first in terms of production (372,400 t) and second in terms of surface (25,700 ha) after almond (MADRPM, 2002). The area occupied by fruit tree nurseries covers 120 ha, which allows an annual production from 600,000 to 700,000 plants (all fruit species included); however most of the nurseries do not have an appropriate infrastructure for producing certified trees. No sanitary selection has ever been done in the country for pome fruit crops, whereas the sanitary status of the stone fruit industry was recently assessed by Bouani et al. (2004).

Materials and methods

Field Survey: Field inspections and sample collection were carried out in November-December 2006 from a total of 277 trees grown in commercial orchards and nurseries located in Meknès, Midelt, and Azilal regions. Inspected trees varied in age and were almost all imported cultivars in addition to two local cultivars, one of apple and one of pear. Leaf samples collected from different parts of the canopy of the trees were used for printing leaf petioles onto nylon membranes to perform viroid detection. One-year-old bud sticks, 20-30 cm in length, from 70 apple and 30 pear trees were collected (Table 1), labelled and stored at 4°C for about two months and then used for woody indexing. Visual inspection of specific symptoms of virus and viroid infections was also carried out during field surveys.

Tab. 1 Crops, locations, cultivars and number of trees sampled during the survey.

Crop	Location	Cultivar	Sample	
			Budsticks	Leaves
Apple	Meknès	Galaxy	4	29
		Golden Smoothee	9	39
		Crips Pink	4	30
		Mondial Gala	5	10
		Fuji	5	10
		Red Chief	5	10
		Golden Delicious	3	10
	Midelt	Starking Delicious	5	10
		Golden Delicious	3	10
		Royal Gala	5	10
		Delicious	3	-
		Golden	3	-
		Stark	3	-
		Ramboothee	5	-
	Azilal	Stark	1	-
		Golden Delicious	1	-
		Local variety (Lhlou)	6	-
Pear	Meknès	William Rouge	5	25
		William Blanc	6	10
		Cascade	6	15
		Dr Jules Guyot	7	31
	Azilal	Local variety	6	-
Total	-	-	100	249

Biological indexing assay: The collected dormant cuttings were individually tested on woody indicators in three replicates and kept under a screenhouse for nine months of observations. Apple samples were tested on Malus pumila cvs. 'R 12740 7A', 'Spy 227' and 'Radiant', and pear samples on Pyrus communis cv. 'LA/62'. The indicator plants were double chip-budded, positive and negative controls were included in the testing of each pathogen. Visual observations were made weekly from April to June, after the appearance of the first symptoms

ELISA: All collected samples were tested by DAS-ELISA (Clark and Adams, 1977) for the detection of the Apple stem pitting virus (ASPV) and the Apple mosaic virus (ApMV), and by simultaneous (cocktail) DAS-ELISA (Flegg and Clark, 1979) for the detection of the Apple chlorotic leaf spot virus (ACLSV) and the Apple stem grooving virus (ASGV). ELISA was done using extracts from young leaves of the indicator 'R 12740 7A' for apple and of 'LA/62' for pear. Serological reagents were commercial kits produced by Loewe (Germany) and Bioreba (Switzerland).

RT-PCR: A limited number of representative samples (12 apples and 3 pears) were also tested by RT-PCR for the presence of ACLSV and ASPV. Total RNA purification from leaves was carried out using RNeasy Plant Mini Kit, according to the manufacturer's instructions (Qiagen S.p.A, Italy). Complementary DNA was synthesized using total RNA as a template. 6 µl of total RNA were mixed with 1 µl/µg of antisense primer (Table 2) in a final volume of 8.6 µl, denatured for 5 min at 95°C, and then put for 5 min on ice. Reverse transcription was done using 4 µl 5X of AMV buffer (Promega), 1 µl of 10 mM dNTPs (each) (Roche), 0.4 µl 100 U/µl AMV (*Avian myeloblastosis virus*) reverse transcriptase in a final volume of 20 µl. The synthesis was carried out at 39°C for 1h.

Tab. 2 Sequences, positions and expected size of RT-PCR product for each primer as reported by Menzel *et al.*, (2002).

Virus	Primer sequence in 5'-3' orientation	Primer position	Product size
ACLSV	TTCATGGAAAGACAGGGGCAA	6860-6880	677 bp
	AAGTCTACAGGCTATTTATTATAAGTCTAA	7507-7536	
ASPV	ATGTCTGGAACCTCATGCTGCAA	8869-8895	370 bp
	TTGGGATCAACTTTACTAAAAAGCATAA	9211-9238	

The PCR reaction mixture contained 5 µl of cDNA, 5 µl 10X PCR buffer (provided with the hotStart *Taq* polymerase, QIAGEN), 0.5 µl of $MgCl_2$ (25 mmol/L), 1 µl dNTP mixture (each dNTP 10 mmol/l), 1 µl of 10 µM each complementary and homologous DNA primers (Table 2), and 0.4 µl HotStart *Taq* polymerase (5U/ µl, QIAGEN). The cycling parameters were: activation of the HotStart *Taq* polymerase at 95°C for 15 min followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. The final extension step was

at 72°C for 7 min. PCR products were separated by electrophoresis in 2% agarose gels in TAE buffer, stained with ethidium bromide, and visualized under UV light.

Tissue printing hybridization: In total, 249 leaf samples (168 apples and 81 pears) were tested by tissue printing hybridization (TPH) for the presence of *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) as recently reported by Lolic et al., (2007). Imprints of a fresh cut end of the leaf petiole were carried out onto a nylon membrane; the imprinted membranes were covered with a plastic envelope and exposed to ultraviolet light for 2-3 min to fix the nucleic acid. The DIG-labelled riboprobes were synthesized as suggested by the manufacturer's instructions (Roche Diagnostics) using T3 or T7 RNA polymerase and recombinant plasmids containing monomeric ASSVd, ADFVd and PBCVd cDNA insert that were linearized with appropriate restriction enzymes. The pre-hybridization step was done at 68°C by using the pre-hybridisation solution (DIG Easy Hyb granules substrate, Roche), then the specific synthesized cRNA probe was added and the hybridization step was performed overnight. After washing with SSC solution containing SDS, the membranes were treated with RNase A (1 µg/ml), then incubated with antidigoxigenin antibody (Roche Diagnostics) and the chemiluminescent substrate CDP-Star.

Results

Field surveys: During the field surveys, almost 300 apple and pear trees were individually inspected in commercial orchards and nurseries located in the regions of Mèknes, Midelet and Azilal. No symptoms were observed in the surveyed orchards and nurseries, which is in line with previous reports on pome fruit viruses that are normally latent (Desvignes, 1999).

Biological indexing: The 'SPY 227' indicator within 2-3 months from graft-inoculation showed symptoms associated with ASPV infections consisting of epinasty of leaves, with downward curled blades; chlorotic spots were also observed in this indicator and were associated with ACLSV, while characteristic symptoms associated with ASPV were also observed in 'Radiant' indicator that showed epinasty of leaves. The chlorotic spots and leaf deformation (sickle-shaped leaf) that appeared on the 'R 12740 7A' apple indicator were associated with the presence of ACLSV, whereas some inoculated apple indicator plants showed a sudden decline and died. The pear indicator 'LA/62' developed rings and mottling on the leaves, which were associated with the presence of ACLSV.

The biological indexing evidenced a first attempt on the sanitary status of the tested trees; in fact, a total of 51 out of 70 tested apple plants proved to be infected by ACLSV (infection rate of 72.8%). 42 out of 70 apple samples were proved to be infected with ASPV. For pear, 50% of the tested plants were proved to be infected by ACLSV (Table 3).

Tab. 3 Viruses of pome fruits detected by biological indexing on woody indicators in Morocco.

	ACLSV R12 740 7A / LA 62		ASPV SPY 227 / Radiant	
Species	**Infected/Tested**	**Infection rate (%)**	**Infected/Tested**	**Infection rate (%)**
Apple	51/70	72.8	42	60
Pear	15/30	50	nt	-

nt: not tested

ELISA tests: The ELISA tests showed for ACLSV a 37.1% infection rate in apple, whereas the infection by this virus was higher in pear (63.3%). ASPV was detected in 25.7% of the tested apple samples and Pear vein yellows virus (PVYV) was detected in 26.6% of tested pear samples. Regarding ASGV, it was found in 11.4% of the apple and in 20% of pear samples. As for ApMV, 4 apples were positive (5.7% incidence) but no pear was infected (Table 4). ELISA kits failed to detect some viruses which were already detected by previous biological indexing, probably, due to the low virus titer or to the presence of inhibitors such as polysaccharides, or phenolic compounds in tissue extracts from woody plants as reported by Kinard et al. (1999).

Tab. 4 Viruses of pome fruits detected by ELISA.

	Samples			Viruses detected			
Species	**Tested**	**Infected**	**Infection rate (%)**	**ACLSV**	**ASPV/PVYV**	**ASGV**	**ApMV**
Apple	70	42	60	26	18	8	4
Pear	30	21	70	19	8	6	0
Total	100	63		45	26	14	4

RT-PCR: A limited number of representative samples (12 apples and 3 pears) were also tested by RT-PCR for the presence of ACLSV and ASPV. Out of the twelve tested apple cultivars, eight were infected by ACLSV and six were infected by ASPV. As for pear, two out of the three tested cultivars were infected by ACLSV and ASPV. RT-PCR assays were in line with those of biological indexing and both techniques were more sensitive than ELISA. Therefore,

for large scale virus surveys of pome fruits, it is necessary to integrate different diagnostic techniques in order to obtain more accurate results.

Tissue printing hybridization: A total of 168 apple trees and 81 pear trees were sampled and their leaves were collected from different parts of the canopy and used for imprinting petioles onto nylon membranes. All the trees were assayed by TPH for ASSVd, ADFVd and PBCVd infections. No hybridization signals were detected in the membranes for any of the three viroids on the tested trees (data not shown).

Discussion

The sanitary status of pome fruit crops in Morocco was preliminarily evaluated, showing a relatively high rate of virus infection. The presence of four viruses (ACLSV, ASPV, ASGV and ApMV) was detected and the most frequent virus was ACLSV (66%).The infection rate for ACLSV detected by woody indicators on 'R 12740 7A' was 72.8%, much higher than that for ELISA (37.1%), but the woody indicators were less sensitive than RT-PCR (91.66%). In general, ELISA was less reliable than biological indexing in the greenhouse. This low reliability of ELISA for pome fruits has already been reported (Desviges et al., 1992; Nemichinov et al., 1995; Boscia et al., 1999). The large scale survey on pome fruit viroids (ASSVd, ADFVd and PBCVd) by tissue printing hybridization (TPH) did not reveal their presence in the tested samples from Morocco. It was concluded that the three viroids do not occur in Morocco, or at least not in the surveyed areas and orchards.

Recent data obtained in other countries where no sanitary selection has ever been carried out in pome fruits showed a high virus infection level (Myrta et al., 2004, Ismaeil et al., 2006, Lolic., 2006). This study showed that virus infection is widespread in the Moroccan pome fruit industry. The degraded sanitary status of pome fruit trees calls for the enforcement of a national certification program for the propagating material of pome fruit and for measures to prevent the importation of virus-infected material into Morocco.

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Evaluation of the presence and symptomology of viruses in commercial quince orchards in Turkey

Birişik, N.[1], Baloğlu, S.[2]
[1] Plant Protection Research Institute. Adana, Turkey, nevzatbir@yahoo.com
[2] Plant Protection Department, University of Cukurova, Adana, Turkey. Email: saba@cu.edu.tr

Abstract

Turkey is the biggest quince (*Cydonia oblonga* Mill.) producer country in the world with a production of about 120.000 tons/year. Virus diseases *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) *Apple mosaic virus* (ApMV) and *Apple chlorotic leaf spot vius* (ACLSV) are known as viral pathogens that can affect quality and quantity of quince production. This study was carried out in the Mediterranean region of Turkey between 2006 and 2008. The study was based on a survey with symptomatological observations and the detection of viruses by DAS-ELISA and/or RT-PCR techniques. During the survey, 33 commercial orchards in five different counties were visited and 115 samples were collected and examined. Laboratory results showed that 27.82% of the samples were infected by either single or mixed infection of any tested viruses. Single infection of ASPV, ACLSV and ASGV were found in 12.17%, 5.21% and 2.60% of the samples, respectively, while mixed infections of ASPV+ASGV, ASPV+ACLSV, and ASPV+ASGV+ACLSV were in 2.60%, 3.47% and 1.73% of the samples, respectively.ApMV was not found in any tested samples.

Infected trees were marked and observed monthly during the whole vegetative period for two years. The observed symptoms were evaluated in accordance with the laboratory results. During the study; leaf mosaics, leaf deformation, fruit malformation, gummy fruit, dwarfing of the tree, bud-union abnormalities and trunk deformations were observed.

Keywords: Quince, ApMV, ACLSV, ASPV, ASGV, Turkey

Introduction

Quince is one of the most important pome fruits in Turkey, and is produced in all regions of the country. Turkey is the biggest quince (*Cydonia oblonga*) producer in the world with a production of about 120.000 tons/year (Anonymous, 2009). Several virus diseases are known to be present in quince (Waterworth, 1989). Among those virus diseases *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) *Apple mosaic virus* (ApMV) and *Apple chlorotic leaf spot vius* (ACLSV) are known as viral pathogens that can affect quality and quantity of quince production (Nemeth, 1986). ACLSV has been reported in quince from Turkey (Akbaş and İlhan, D.; 2008.) and Greece, and ASPV from former Yugoslavia according the research activity (Paunovic and Rankovic, 1998). This study was carried out in the Mediterranean region of Turkey between 2006 and 2008 in five different counties where quince production is high. The study was based on a survey with symptomatological observations and the detection of viruses by DAS-ELISA and multiplex RT-PCR.

Materials and methods

Survey: The survey carried out in commercial quince orchards of Adana, Niğde, Mersin, Kahramanmaraş, and Osmaniye provinces. Sampling was done according to Bora and Karaca (1970) and the number of samples collected from orchards was based on the size of the orchards. Three samples were taken from orchards smaller than 0.5 ha, four samples from 0.5-1 ha, and six samples from orchards bigger than 1 ha. Sampling was done in spring and fall during the vegetative period collecting one year old branches from selected trees.

Observations: All quince trees in the surveyed area were checked by visual observation for any kind of virus related symptoms during the whole vegetative period for two years. The visual observation results were compared with the laboratory tests.

DAS-ELISA: DAS-ELISA was performed according to Clark and Adams, (1977). Leaf and bark tissues were used in the assay.

Multiplex RT-PCR: Total RNA was extracted from healthy and infected tissues using the Promega SV total RNA kit as described by supplier. The primers for ASPV, ACLSV and ASGV were as previously described by Menzel et al., (2003) and for ApMV by Hassan et al. (2005). The multiplex RT-PCR assays were performed as reported by Hassan et al. (2005).

Results

During the survey, 33 commercial orchards in five different counties were visited and 115 samples were collected and examined. Laboratory results showed that 27.82% of the samples were either singly or mixed infected by any of the tested viruses. The single infection rate (20.00%) was higher than mixed infections (7.82%). Single infection of ASPV, ACLSV and ASGV were found in 12.17%, 5.21% and 2.60% of the samples, respectively, while mixed infections of

ASPV+ASGV, ASPV+ACLSV, and ASPV+ASGV+ACLSV

were in 2.60%, 3.47% and 1.73% of the samples, respectively.ApMV was not found in any tested samples. Infected trees were marked and observed monthly during the whole vegetative period for two years. The observed symptoms were evaluated in comparison with the laboratory results. During the study; leaf mosaics, leaf deformation, fruit malformation, gummy fruit, dwarfing of the tree, bud-union abnormalities and trunk deformations were observed.

ELISA: According to the ELISA results; there was no ApMV infection, whereas ASPV is the dominant virus in quince orchards, and mixed virus infections werefound only in the Kahramanmaraş region. Consequently 32 of the 115 collected samples were found infected with one or more viruses.(Table 1).

Tab. 1 ELISA results for quince samples collected during the survey.

			Single infection				Mixed infection			% of infection
Name of province	**Num. of orchards**	**Num. of samples**	**ApMV**	**ACLSV**	**ASPV**	**ASGV**	**ACLSV ASPV**	**ASPV ASGV**	**ACLSV ASPV ASGV**	
K. maraş	17	67	-	2	14	3	4	3	2	41,79
Adana	5	15	-	-	-	-	-	-	-	-
Niğde	4	12	-	2	-	-	-	-	-	16,66
Mersin	4	12	-	1	-	-	-	-	-	8,33
Osmaniye	3	9	-	1	-	-	-	-	-	11,11
Total	33	115	0	6	14	3	4	3	2	32
% infection			-	5,21	12,17	2,60	3,47	2,60	1,73	27,82
%single & mixed infection				% 20				% 7,82		

RT-PCR: RT-PCR tests confirmed the presence of ASPV, ASGV and ACLSV detected previously by ELISA. The image of DNA bands for ACLSV (712 bp), ASPV (414 bp) and ASGV (300) bp) is showed in Figure 1.

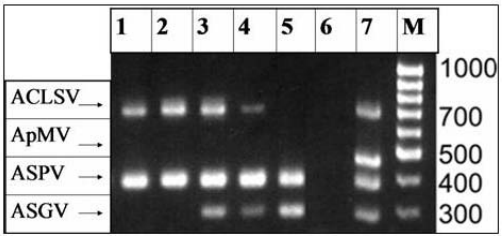

Fig. 1 Representative results obtained by multiplex RT-PCR assay for four viruses in quince samples. Lanes 1 to 5 are quince sample infected with different virus combinations. Lanes 6 is healthy quince and line 7 is positive virus controls.

Symptomology: Visual observations of infected quince trees showed that; ASPV and ASGV infections resulted in severe leaf deformation (Fig. 2), mosaic (Fig. 3) and over-growth of the trunk (Fig. 8). Single ASPV infection cause gummy tissues on the surface of the fruit, gritty and gummy tissues around the seed, fruit malformation and trunk flexion (Fig. 4, 5, 6, 7).

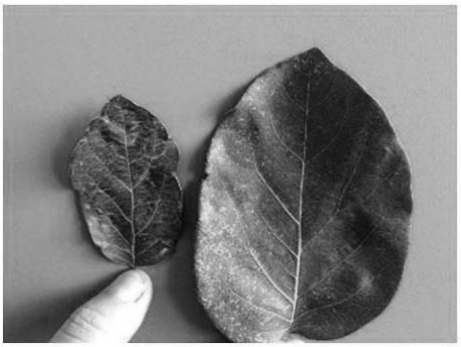

Fig. 2 Mixed infection causes small and deformed leaves. These leaves also have some mosaic symptoms in a short period of spring.

Fig. 3 Some infected trees gives normal size leaves but the mosaic symptoms remain for a long period till mid July.

Fig. 4 ASPV infection causes gummy tissues on the surface of the fruit, and fruit has a rusty appearance.

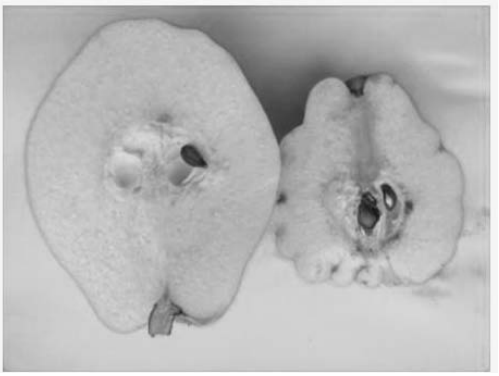

Fig. 5 The tissue around the seed became very hard and gummy with ASPV infection.

Fig. 6 ASPV+ASGV infection cause very severe fruit malformation. Those fruits were very hard to cut and unpleasant.

Fig. 7 Trunk flexion observed only on ASPV infected trees.

Fig. 8 Bud-union oversize symptoms have been observed on trees infected with both ACLSV+ASPV.

Discussion

This study showed that ACLSV, ASPV and ASGV viruses are present in commercial quince orchards in Mediterranean region of Turkey. There was no ApMV found neither by RT-PCR nor by ELISA in collected samples from commercial quince orchards. Different types of symptoms were observed on virus infected trees and the observed symptoms were evaluated in comparison with the laboratory results. During the study; leaf mosaics, leaf deformation, fruit malformation, gummy fruit, dwarfing of the tree, gritty tissues in the fruit, bud-union abnormalities and trunk deformations were observed assumed to be the result of virus infection. Symptoms were more severe in the case of mixed infection, and more than 25% of the fruits of infected trees were not marketable. All symptoms were observed on a local quince variety that known as 'Ekmek'. This study showed that virus diseases have an important impact on quince production because, due to virus infection, fruits are becoming unmarketable and trees are becoming more weak and amorphous. Results showed that for quince production more severe damage occurs for ASPV+ACLSV mixed infection. Because of severe fruit symptoms and leaf misshapenness productive trees are losing their ability to bear fruit. It is thought that virus infections of quince are caused by infected plant propagating material. Because the production material which is used for planting quince orchards is not certified, and growers are used to using cuttings from their orchards to produce new production materials.

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The occurrence of *Ilarviruses* in Latvian fruit orchards

Pūpola, N., Kāle, A., Jundzis, M., Moročko-Bičevska, I.
Latvia State Institute of Fruit-Growing, Graudu str. 1, Dobele, LV-3701, Latvia. Email: neda.pupola@lvai.lv

Abstract

In order to study the occurrence of ilarviruses in fruit orchards in Latvia the samples from apple, pear and *Prunus* trees from commercial orchards and varietal collections were collected during spring 2007 and 2008. Polyclonal antibodies were used for DAS ELISA test for large-scale screening. In Total 890 samples from apple, 252 samples from pear and 655 samples from *Prunus* spp. were tested for the occurrence of fruit tree ilarviruses – ApMV, PDV and PNRSV. The screening results showed that all tested ilarviruses were present in the fruit orchards. ApMV was detected in 2.1% of the tested apple samples and in 1.8% of the plum samples, but it was not detected in pears. In *Prunus* spp. PNRSV was detected in 13.6% and PDV in 11.6% of samples. Mixed infections of ilarviruses were detected in 4% of the tested *Prunus* spp. samples of which 2% was PNRSV in combination with PDV. The plant samples from apple and pear trees were tested for ApMV infection also by RT-PCR and compared with data obtained by DAS ELISA. The RT-PCR results showed that 22% and 20.2% of tested apple and pear trees are infected with ApMV, respectively. The occurrence of ilarviruses in the tested plants varied greatly among the cultivars. The commonly grown cultivars such as 'Ausma', 'Rubin', 'Kursa', 'Bere Kievskaya', 'Perdrigon', 'Mirabelle de Nancy' and 'Skoroplodnaya' were highly infected. Since a certification system for planting material is not established in the country, there is very high risk for continuous spread of these viruses in the orchards. Similarly, as showed for ApMV, also PNRSV and PDV possibly are more widely spread than detected with preliminary screening by DAS ELISA. The study should be continued and other test methods, such as RT-PCR, used. The obtained data indicate a great need for the establishment of a certification system for fruit tree propagation.

Keywords: ApMV, apple, ELISA, PDV, pear, PNRSV, *Prunus*, RT-PCR

Introduction

Apples, pears and plums are important horticultural crops in Latvia, occupying around 85% of commercial fruit orchard area. Stone and pome fruit trees are affected by a large number of viruses, which cause significant economic losses (Saade et al., 2000). *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV) are worldwide pathogens of stone and pome fruit trees and they often occur in mixed infections (Vaškova et al., 2001). Symptoms of infection may not be visible or can appear as leaf chlorosis and reduction in growth (Scott et al., 2001). PDV and PNRSV may also cause necrotic spots and shot-holes in some *Prunus* species. On plums, PDV causes rosetting of the internodes, stunting and malformation of leaves (Ogawa et al., 1995). PNRSV on plums induces chlorotic rings or line patterns on the foliage, which later become necrotic and drop off, developing the shot-hole effect (Diekmann & Putter, 1996). ApMV is named after the disease it causes in apple, the first host in which it was described, although at least 65 other plant species are susceptible hosts of ApMV, including pears and plums (Petrzik & Lenz, 2002). ApMV induces pale yellow to cream-colored areas on leaves of infected apple trees and line patterns or bands along major veins on plums, but pears are mostly symptomless (Jones et al., 1990; Petrzik, 2005). All three viruses are transmitted by graft, pollen and seed, except ApMV for which only graft transmission is known. They also have similar biological properties that contribute to their wide distribution (Vaškova et al., 2000).

The use of healthy plant material is a requirement to prevent virus spread in woody crops. In this context certification schemes worldwide are being established with the objective of identifying healthy sources for propagation (Massart et al., 2008). The certification program for planting material has not been established in Latvia yet. Therefore the risk that viruses have spread uncontrolled in fruit orchards with infected planting material and by natural transmission is very high. Previous studies on occurrence of viruses in pome fruits in Latvia were carried out in 1980s and were based only on visual observations and biological indexing. The occurrence of viruses in stone fruits has not been studied before in Latvia. During the last decade new commercial orchards have been widely planted, and the assortment of cultivars and rootstocks have changed greatly. Nowadays data are not available about the spread of viral diseases in fruit orchards, including such important pathogens as ApMV, PNRSV and PDV. The aim of this research was to determine the occurrence of ApMV, PNRSV and PDV in stone and pome fruit orchards in Latvia.

Materials and methods

In total 50 apple, 36 pear and 28 plum commercial orchards and varietal collections were surveyed during spring 2007 and 2008. In total 890 samples from different apple (*Malus domestica* Borkh.), 252 samples from different European pear (*Pyrus communis* L.) and 655 samples from several European plum (*Prunus domestica* L.), cherry plum (*Prunus cerasifera* Ehrh.), *Prunus* hybrids and other *Prunus* spp. genotypes were collected. Leaves were sampled randomly from symptomless and symptomatic trees from all cultivars, which were present in the surveyed orchard. Ten fully expanded leaves were collected around the canopy of each individual tree from the middle of each scaffold branch, according to EPPO standards (EPPO, 2004). The samples were transported to the laboratory in an ice bag, immediately used for analyses or frozen in liquid nitrogen and stored at -80°C.

For the large-scale screening and detection of ApMV, PDV and PNRSV in plant material, commercially available double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) kit (Bioreba AG, Switzerland) was used according to the manufacturer's instructions with some modifications. The coating and conjugate conditions were changed from the manufacturer's standard procedure of a 4 h incubation at 30°C to an overnight incubation in the refrigerator at 4 – 6 °C. The absorbance was read at 405/492 nm with dual filter microplate reader Asys Expert 96 (Hitech, Austria) after 30 min, 1 h and 2 h of incubation. A "cut-off" value was calculated according to the manufacturer's technical information (Bioreba AG, Switzerland).

The samples from apple and pear trees were tested for ApMV infection with reverse transcription polymerase chain reaction (RT-PCR). The same leaf samples were used for RT-PCR and DAS ELISA. For total RNA isolation frozen leaf tissues were ground into a fine powder in liquid nitrogen. The extraction of RNA was carried out with the RNeasy Plant Mini kit (Qiagen AG, Germany) following the manufacturer's recommendations. Lysis buffer RLT was used for apple and RLC buffer for pear leaf tissues. The quantity and quality of the RNA was measured using a spectrophotometer NanoDropR ND-1000 (Thermo Scientific, USA). RNA was stored at -20°C and for long-term storage at -80°C.

RT-PCR assays were carried out with OneStep RT-PCR kit (Qiagen AG, Germany) following the manufacturer instructions. ApMV coat protein specific primers and *nad5* primers as an internal control were used (Hassan et al., 2006; Menzel et al., 2002) at concentrations 0.4 µM for each ApMV and 0.25 µM for each *nad5* primer. The primers' sequences are shown in Table 1. RT-PCR was carried out in a thermocycler Mastercycler® ep (Eppendorf AG, Germany) at the following cycling conditions: reverse transcription step 30 min at 50ºC, activation of the hotstart Taq polymerase at 95ºC for 15 min, followed by 40 cycles of: 30 s at 94ºC, 45 s at 55ºC, 2 min at 72ºC and a final extension step at 72ºC for 10 min (Hassan *et al*., 2006). PCR products were separated by electrophoresis in 2% agarose gels in TAE buffer, stained with ethidium bromide, and visualized under UV light. The occurrence of viruses was calculated as a percentage of positive samples from totally tested samples.

Tab. 1 Primer sequences for detection of ApMV and internal control

Primer	Primer sequences	Product size	References
ApMV	F 5' CGTAGAGGAGGACAGCTTGG3'	450 bp	Hassan et al., 2006
	R 5' CCGGTGGTAACTCACTCGTT 3'		
nad5	F 5' GATGCTTCTTGGGGCTTCTTGTT 3'	181 bp	Menzel et al., 2002
	R 5' CTCCAGTCACCAACATTGGCATAA 3'		

Results

The occurrence of ApMV in apple and pear orchards: In the orchard surveys no obvious symptoms were observed on fruit trees except some leaf mosaic on apple trees. With the DAS ELISA test 2.1% of samples from apple trees were positive for ApMV, but all samples from pears were negative. Duplex RT-PCR resulted in the amplification of a viral DNA fragment of 450 bp in length and and internal control of 181 bp (Figure 1). RT-PCR results showed high rates of ApMV infection of pome fruit trees while DAS ELISA gave fewer positive results (Table 2). Among orchards the occurrence of ApMV varied from 0% to 75%. Among different apple and pear cultivars a high variability in the occurrence of the virus was observed. Almost all commonly grown cultivars were infected with ApMV. Among those, the Latvian apple cultivar 'Ausma' and the cultivar 'Rubin' (originating from Kazakhstan) were highly infected;62.5 % and 77.1 % of plant samples, respectively. On these two cultivars obvious symptoms of ApMV, such as, pale yellow to cream-colored areas on leaves, were also observed in the orchards. In other apple cultivars the number of infected samples with ApMV ranged from 0% to 38.5 %. Pear cultivars 'Kursa' and 'Bere Kievskaya' had the highest number of infected trees; 40 % and 50 % of plant samples, respectively. In other pear cultivars the occurrence of ApMV ranged

from 0% to 33.3%. All tested samples from the common apple cultivars ‘Orlik’ and ‘Alro’ and common pear cultivars ‘Clapp’s Favourite’, ‘Conference’ and ’Talsu Skaistule’ were negative for ApMV.

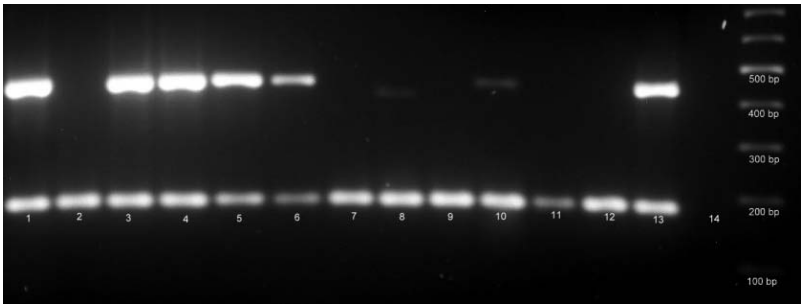

Fig. 1 Gel electrophoresis showing RT-PCR results: apple samples in lane 1-6; pear samples in lane 7-12; positive control in lane 13; water control in lane 14

Tab. 2 The occurrence of ilarviruses in fruit trees and orchards (%)

Fruit trees	ApMV DAS ELISA		ApMV RT-PCR		PNRSV DAS ELISA		PDV DAS ELISA	
	Samples	Orchards	Samples	Orchards	Samples	Orchards	Samples	Orchards
Apples	2.1	14	22.0	90	nt*	nt	nt	nt
Pears	0	0	20.2	55.6	nt	nt	nt	nt
Plums	1.8	28.6	nt	nt	13.6	67.9	11.6	78.6

* nt – not tested.

The occurrence of ApMV, PNRSV and PDV in plum orchards: In the orchard surveys no obvious symptoms were observed on plum trees, except some necrotic spots and shot-holes on plum leaves. The obtained data showed that ilarviruses are widespread:in only two plum orchards were no viruses detected. The occurrence of ilarviruses varied greatly among fruit tree species and orchards (Table 2). The occurrence with ApMV was rather low in all inspected orchards. It ranged from 0% to 16.7 % while occurrence of PNRSV and PDV was from 0% to 85.7% in individual plum orchards. Mixed infections with two or three viruses were detected in 4% of tested plum leaf samples. Mostly mixed infections with PDV and PNRSV were detected and only a few samples were infected with all three *Ilarviruses*.

Among different *Prunus* spp. genotypes a high variability in the occurrence of the viruses was observed (Table 3). The occurrence of ApMV was rather low in all common *Prunus* cultivars. It ranged from 0% to 15%. The *Prunus* hybrid 'Skoroplodnaya' showed a high occurrence of PDV (67.7%) but in other *Prunus* cultivars it ranged from 0% to 30.8 %. A high occurrence PNRSV was observed in several *Prunus* cultivars, such as ‘Perdrigon’ (46.7%), ‘Mirabelle de Nancy’ (44.4%), and ‘Reine-Claude d'Oullins’ (45%). In other *Prunus* cultivars the occurrence of PNRSV ranged from 0% to 25%. All samples of cultivars ‘Jubileum’, ‘Tragedy’, and ‘Stanley’ were negative to all tested *Ilarviruses*.

Tab. 3 Occurrence of ilarviruses in common *Prunus* cultivar (%)

***Prunus* cultivars**	**ApMV**	**PDV**	**PNRSV**
‘Experimentalfältets Sviskon’	0	25	13
‘Jubileum’	0	0	0
‘Julius’	0	11	0
‘Kubanskaja Kometa’	5	31	6
‘Latvijas Dzeltenā Olplūme’	4	7	11
‘Lāse’	0	0	7
‘Mirabelle de Nancy’	0	0	44
‘Perdrigon’	7	7	47
‘Prince of Wales’	0	19	25
‘Reine-Claude d' Oullins’	15	10	45
‘Reine-Claude Verte’	14	0	14
‘Skoroplodnaya'	3	68	13
‘Stanley’	0	0	0
‘Tragedy’	0	0	0
‘Victoria’	0	4	9

Discussion

The research presented here demonstrated that ApMV, PNRSV and PDV are widespread in pome and stone fruit tree orchards in Latvia and most of the commonly grown cultivars are infected. In individual orchards the high occurrence of *Ilarviruses* could be due to the preference of cultivars for commercial growing. The most common apple, pear and *Prunus* cultivars are highly infected with ApMV, PDV and PNRSV, which indicates that infected planting material was used in propagation, because only graft-transmission for ApMV is known, and the natural spread of PNRSV and PDV is usually slow (Diekmann & Putter, 1996).

ApMV was detected in apple and plum trees, but not in pear samples with the DAS ELISA test indicating possible false negatives also for other viruses tested in this study. Low sensitivity of the ELISA test and ApMV high sensitivity to temperature fluctuation result in false negatives as has been demonstrated in other studies (Kobylko et al., 2005). In other studies it has been shown that ApMV occurrence in pears is about 77% (Petrzik, 2005). In this research we found that the occurrence of ApMV at 20%. As weak DNA bands were obtained from pear sample PCR productsit might indicate that ApMV is present in pear trees at very low concentrations and therefore was not detectable with the ELISA test. Although the high sequence correlation among apple and pear ApMV isolates has been reported (Petrzik, 2005), the obtained weak DNA bands from pear samples may also indicate that the primers, which were used in this study might be less specific for Latvian pear isolates. Furthermore, in other research where the same primers were used, ApMV was not detected in pears (Hassan et al., 2006). To test this hypothesis and confirm ApMV occurrence in pears the study should be continued with another primers and comparisons of sequences from PCR products should be done.

The obtained results confirmed the presence of ApMV, PNRSV and PDV in Latvia and showed that these viruses are widespread in plum orchards. Although in other countries in Europe ApMV is more common on *Prunu*s than on *Malus* (Desvignes, 1999), in this research ApMV was more widespread on *Malus* than on *Prunus*. To exactly determine ApMV occurrence in plum orchards, it would be necessary to test plum samples with a more sensitive method such as RT-PCR or immunocapture RT-PCR. In other studies in Europe the occurrence of PDV has been demonstrated to range from 0.4% to 47% and PNRSV from 5.6% to 46% (Massart et al., 2008), which corresponds to the data obtained in this study. Out of the three ilarviruses, PNRSV, due to its modes of transmission, is more widespread in plum orchards than PDV and ApMV, as has also been demonstrated in this study. Some experimental evidence suggest that thrips are also involved in PNRSV natural transmission (Shiel & Berger, 2000). Although in other studies PNRSV, PDV and ApMV have been demonstrated to occur often in mixed infections (Scott et al., 2001; Petrzik & Lenz, 2002), in this study mixed infections in plum trees were detected only in a few cases. Desvignes (1999) reported that PNRSV and PDV concentrations are high and constant in plant tissues and various virus strains can be easily detected by ELISA. However, false negatives may occur with ELISA and are less likely when using molecular techniques.

The obtained results showed that ApMV, PDV and PNRSV are spread in fruit orchards and most of the commonly grown cultivars are infected. Since a certification system for planting material is not established in the country, there is very high risk for continuous spread of these viruses in the orchards. Similarly, as shown for ApMV, it is possible thatPNRSV and PDV are more widespread than detected with preliminary screening with DAS ELISA. This study should be continued and other test methods, such as RT-PCR, used. The obtained data indicate a great need for the establishment of a certification system for fruit tree propagation.

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Occurrence of *Little cherry virus*-1 on *Prunus* species in the State of Baden-Württemberg, Germany

Schröder, M., Petruschke, M.
Landwirtschaftliches Technologiezentrum Augustenberg, Außenstelle Stuttgart, Reinsburgstraße 107, 70197 Stuttgart

Abstract

A survey on *Little cherry virus*-1 (LChV-1) on several *Prunus* species has been performed at four different sites in the State of Baden-Württemberg (BW) between 2003 to 2006. These included a state-run growing site for prebasic and basic material, two commercial nurseries for certified scion or rootstock production and an orchard for cultivar verification testing. A total of 63 varieties of sweet, sour and ornamental cherries belonging to four *Prunus* species (*P. avium* L., *P. cerasus* L., *P. serrulata* Lindl., *P. subhirtella* Miq.) as well as six types of *Prunus*-rootstocks were tested. Ten of the 44 *P. avium* and one of the two *P. serrulata* varieties were partly or totally infected, whereas the *P. cerasus* and *P. subhirtella* varieties and the *Prunus*-rootstocks gave negative results. None of the infected plants showed distinctive disease symptoms.

Dispersal of LChV-1 from the infested *P. avium* trees was not detected in the orchard for cultivar verification testing after a period of five years. A natural dispersal from varieties infected for about 10 years in a nursery for scion production to adjacent healthy varieties was observed only in single cases. There was no indication of any involvement of animal vectors.

Testing of randomly sampled material from some trees of *P. avium* and *P. serrulata* for scion production proved a homogenous distribution of the virus in shoots in autumn.

At the moment studies are conducted to verify the responses of young trees of the sweet cherry variety 'Regina' to experimental inoculation with either LChV-1 or LChV-2 or a mixture of both viruses. First year results indicate that - in complete contrast to LChV-2 - no adverse effects of LChV-1 on the fruit yield, single fruit weight, fruit size and trunk circumference were observed. In mixed infections, LChV-1 seems to attenuate the adverse effects of LChV-2 on fruit yield and trunk circumference.

Keywords: *Little cherry virus*-1, *Little cherry virus*-2, Baden-Württemberg, *Prunus* species, varieties, rootstocks, certification, dispersal, distribution, effects

Introduction

Observations on the presence of Little Cherry Disease symptoms in German cherry fruit production orchards were already reported in the 1980s (Büttner and Graf, 1995). Two different virus types were detected through detailed genome analysis relating the disease to either LChV-1, LChV-2 or a mixture of both (Rott and Jelkmann, 2001). Until a few years ago there was no indication of the presence of Little Cherry Disease in the commercial cherry tree production of Baden-Württemberg. Particularly with regard to certified propagation material there was no justified reason to presume any infestation, since the legally regulated testing for certification with the common indicators did not produce any disease signs. Only after the adoption of the PCR method as a specific technique for LChV-1 and LChV-2 detection and random testing of samples from basic material of some sweet cherry varieties was LChV-1 infection confirmed. Thereafter, the entire stock of prebasic and basic material of sweet and sour cherry in BW were tested, as well as *Prunus*-stocks of other institutions involved in the regional certification system (one nursery for scion and one nursery for rootstock production and an orchard for cultivar verification testing).

Since there is little information on the distribution of LChV-1 in the tree, or the effects of the disease on the pomological properties of cherries e.g. yield, fruit weight, studies were initiated.

Materials and methods

The presence of LChV-1 in varieties of different *Prunus* species was examined in leaf samples (commonly 3-5 leaves/tree as mixed sample). The number of tested trees per variety varied, due to availability, between 2 and ca. 100. For virus detection in rootstocks, rows were divided in sections of typically 20 m. Within one section 20 leaves were sampled and pooled. The number of replicates per type varied between 14 and 27. For the assessment of virus distribution in individual shoots 15 random shoot samples were taken from mid to late October from about 10 years old trees of the varieties 'Hedelfinger Riesenkirsche, type Froschmaul' (*P. avium* L.) and 'Amanogawa' *(P. subhirtella*

Miq.) (3 replicates/variety). The shoots were divided in three sections of equal length. From each part three leaves or buds (if leaves were unavailable) were taken, pooled and analysed.

For comparing the effects of a LChV-1 infection with those of either LChV-2 or a mixed infection, the rootstock 'Gisela 5' was grafted with the sweet cherry variety 'Regina' and inoculated by chip budding (5 replicates/treatment) in 2004. The LChV-1 and LChV-2 inoculum was obtained from the sweet cherry variety 'Kassins Frühe Herzkirsche' (basic material site nearby Stuttgart) and 'Regina' (commercial orchard near Lake Constance), respectively. The infection with either the single viruses or their combination was confirmed by PCR. Fruit yield and further parameters were measured in 2008 for the first time. The single fruit weight and size (diameter) was determined as an average of 10 fruits/tree, respectively. The trunk circumference was measured 30 cm above the grafting point.

For the detection of LChV-1 and -2 total nucleic acid was extracted from leaves or buds using the silica capture method as described by Menzel (2003). RT-PCR for LChV-1 and LChV-2 was performed with primer pairs LCV1 U 16390 / LCV1 L 16809 and LCV2UP2 / LCV2LO2, respectively, according to Rott und Jelkmann (2001) and later extended with primers LCH1_7634F / LCH1_7942R and LCH2_01F / LCH2_03R according to Jelkmann et al. (2008).

Results

Occurrence of LChV-1 in *Prunus* species and varieties: Out of 44 tested varieties of *P. avium* 10 tested positive for LChV-1 (Tab.1). Mostly, all tested trees of a variety proved to be virus positive, in few cases only single trees of a variety were infested. In one case ('Hedelfinger Riesenkirsche, type Froschmaul') a section of trees within an infested row tested negative. The majority of the diseased varieties were identified as being old varieties, e.g. 'Burlat', 'Büttners Rote Knorpelkirsche', 'Große Schwarze Knorpelkirsche' and 'Kassins Frühe Herzkirsche'. Among the remaining *Prunus* species only the variety 'Amanogawa' (*P. serrulata*) tested LChV-1 positive. All six types of *Prunus* rootstocks ('Colt', 'Gisela 3', 'Gisela 5', 'Gisela 6', 'F12/1', 'Piku 4') belonging to different certification categories (prebasic, basic, certified mother plants) were tested virus negative. A part of the samples (scions and rootstocks) was also tested for LChV-2 but there was no evidence for the presence of the virus.

Tab. 1 Number of varieties / types of *Prunus* species from several sites (certified commercial scion and rootstock production, state-run facility for prebasic and basic material, state orchard for cultivar verification testing) found to be LChV-1 infested between 2003 and 2006

Species	No. varieties/types totally tested	LChV-1-positive
P. avium	44	10
P. cerasus	16	0
P. subhirtella	1	0
P. serrulata	2	1
Prunus rootstocks	6	0

None of the virus positive trees showed obvious symptoms indicating a virus infection with LChV-1. The motherplants of some of the varieties were previously submitted to thermotherapy for virus elimination and failed to produce any indication of LChV-1 infestation in subsequent tests with the standard indicators 'Sam' and 'Canindex'. Nevertheless, these varieties proved later to be LChV-1 positive by PCR.

In an orchard for cultivar verification testing comprising about 80 *P. avium* and *P. cerasus* trees (44 varieties), 10 trees belonging to 5 varieties were LChV-1 positive. Diseased trees of the same variety were adjacent but the varieties were randomly distributed in the plot. At the time of planting it was unknown that the trees were already diseased, however backtracking to the origin of the planting material revealed that the mother plants were already LChV-1 infected. The distance between single trees within a row was 2.5 m and between rows 4 m. However, a spread of the virus was not observed after a period of five years. Similar observations were made in a plantation for scion production comprising different varieties grafted on *P. avium* F12/1. Also in this case, LChV-1 infested varieties were placed side-by-side with healthy varieties over a period of 10 years and the infection was, except very few cases, restricted to the originally diseased plants. In the latter site the distance between the trees was 0.8 m and 2.75 m between the rows. Also here the status of virus infection was unknown at the time of planting, but infested varieties could be traced back to the infected mother plants of basic material.

Virus distribution within shoots: Examination of scion producing trees of the varieties 'Hedelfinger Riesenkirsche , type Froschmaul' *(P. avium)* and ‚Amanogawa' *(P. serrulata)* revealed that all samples, except an indistinct single case, were PCR positive at the time of sampling. The virus was present in leaves and buds.

Effects of virus infection: Four years after inoculation of the sweet cherry variety 'Regina' with LChV-1 no disease symptoms nor any negative effects on the pomological parameters measured were observed (Tab. 2). In contrast, LChV-2-infection induced significant reduction in fruit yield (-74%), single fruit weight (-44%), fruit size (-23%) and trunk circumference (-46%). In addition, typical symptoms like a reddish-brownish colouring of intercostal fields (in 2009 appearing since July) were recorded. An infection with a mixture of both viruses had intermediate effects on fruit yield and trunk circumference compared to those of LChV-2 alone. However, single fruit weight and fruit size were comparable to LChV-2 infected trees.

Tab. 2 Recorded effects of LChV-1, LChV-2 and a mixture of both on the sweet cherry variety 'Regina' four years after experimental inoculation of trees (first year results).

	Fruit yield/tree (g)	Single fruit weight (g)	Fruit size (mm diameter)	Trunk circum-ference (cm)
Control	950	11.1	25.0	18.0
LChV-1	1215	11.8	25.9	20.7
LChV-2	250	7.3	19.3	9.7
LChV-1+ 2	685	6.5	19.6	15.0

Discussion

The detection of LChV-1 in certified propagation material of various varieties of two *Prunus* species in the State of Baden-Württemberg (BW) was very surprising, since all mother trees were virus tested at earlier occasions. The expression of the typical symptoms, as described in the literature for the Little Cherry Disease, is therefore apparently primarily related to the infection by LChV-2. Symptoms of LChV-1 infection are reported to be milder or even latent (Jelkmann and Eastwell, 2009). The LChV-1-isolate(s) present in BW is obviously not detected when the standard indicators (‚Sam', 'Canindex') are used nor could it be eliminated by standard thermotherapy for cherry viruses. Only the use of the PCR assays made its detection possible. As there is no known vector for the transmission of LChV-1, it can be assumed that the dispersal of the virus is via vegetative propagated tree material. Since many diseased varieties are growing already for more than 30 years in the virus-tested cherry stands, it can be assumed that the LChV-1 virus does exist in the State of BW the same time. The locally certified rootstock stands, however, are free of LChV-1 since no proof of infection was obtained until now. In Poland, however, there is evidence for LChV-1 in the rootstock cultivar ‚Gisela' (Komorowska und Cieślińska, 2004). The fact that within five years, no dispersal of LChV-1 in the orchard for cultivar verification testing was observed, can be explained by a lack of vector transmissibility or the absence of a potential vector. The observed cases of virus transmission to neighbouring healthy varieties in a scion producing nursery after a 10 years period can be probably attributed to root transmission. The narrow tree distance and growth properties of the used rootstocks of *P. avium* F12/1 strongly support root expansions into the adjacent rhizosphere.

A homogeneous distribution pattern of the virus within the aerial parts of trees for scion production can be assumed at least for two varieties in autumn due to positive testing of all sampled shoots. Because of the periodical strong pruning of trees in spring, virus particles are likely to move apically due to shoot re-growth thereby reaching a homogeneous distribution in autumn or possibly earlier. The risk of failing to detect the virus when mixed samples are collected in autumn is most likely very low.

The preliminary results of the experimental infection trials using the sweet cherry variety 'Regina' with LChV-1 showed that the infection had no effects on the pomological parameters studied. Therefore, it is likely that the isolate of LChV-1 used for inoculation, contrary to LChV-2, is only weakly virulent or avirulent. Surprisingly, the infection with a mixture of the two viruses seems to attenuate the effects of LChV-2 on fruit yield and trunk circumference. This trial is ongoing and hopefully results in the years to come contribute to a further understanding of the interaction.

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Transmission of *Little cherry virus* -1 (LChV-1) by *Cuscuta europea* to herbaceous host plants

Jelkmann, W., Hergenhahn, F., Berwarth, C.
Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany. Email: Wilhelm.Jelkmann@jki.bund.de

Abstract

Little cherry virus -1 (LChV-1) was transmitted from infected *Prunus avium* F12 rootstocks by *Cuscuta europea* to *Nicotiana occidentalis* '37B'. Transmission of the virus was confirmed by RT-PCR analysis of total nucleic acid extracts from dodder and *N. occidentalis*. Symptoms consisted of curled leaves, reddening of leaf margins and veins, and plant decline. In parallel attempts virus transmission was not successful for LChV-2. Propagation of LChV-1 by mechanical transmission on *N. occidentalis* failed, however the virus was transferred serially by grafting.

Introduction

Little cherry disease has been associated with two different long flexuous filamentous viruses of the family Closteroviridae. *Little cherry virus* -1 (LChV-1) is an unassigned member in the family while *Little cherry virus* -2 (LChV-2) has been assigned to the genus *Ampelovirus*. Both viruses have been characterized at the molecular level (Rott and Jelkmann 2005;Jelkmann, Fechtner et al. 1997).

The viruses can be found individually and in mixed infections. The disease is distributed worldwide in ornamental and sweet cherry and has a great impact on fruit quality of infected trees. Symptoms of infection consist of small angular and pointed fruit that do not ripen fully and are imperfectly coloured. Fruit have reduced sweetness and are unsuitable for consumption. The disease is readily graft–transmissible from cherry to cherry. There is no known vector associated with LChV-1, however, LChV-2 is transmitted by the apple mealybug (*Phenacoccus aceris*). Both viruses can be detected by RT-PCR and woody indexing on sensitive indicator plants (Jelkmann and Eastwell 2010). The host range of the viruses is limited to species within the genus *Prunus*. In addition to cherry, LChV-1 was identified in plum, peach and almond (Matic, Minafra et al. 2009b).

A recent report described the transmission of *Grapevine leafroll associated virus* -7 (GLRaV-7) from a grapevine accession to *Tetragonia expansa* and to *Nicotiana occidentalis* by different *Cuscuta* species (Mikona and Jelkmann 2010). Although this method had been used for many virus transmission experiments in past decades (Hosford 1967) no reports of closterovirus transmission from woody plants to herbaceous hosts were found in the literature. In order to evaluate potential herbaceous host plants for LChV-1 and LChV-2 a similar experimental setup was investigated as used for GLRaV-7 transmission.

Materials and methods

Transmission experiments. *Cuscuta europea* L. (greater dodder) seeds were germinated on moist filter paper or soil and transferred to healthy *N. occidentalis*. After three weeks of growth on healthy tobacco the dodder shoots were connected to *Prunus avium* F12 rootstock plants infected with LChV-1 or LChV-2. Experiments were done in an insect-proof greenhouse at temperatures between 20° and 30°C. After three months the parasite was removed completely from the virus donor and acceptor and put on young tobacco plants for maintenance. During this time the first virus detections were carried out. Virus detection. Total plant nucleic acid was extracted from cherry and tobacco leaves using a silica capture protocol (Rott and Jelkmann 2001). LChV-1 and -2 detection by RT-PCR was done as previously described (Jelkmann, Leible et al. 2008).

Results and discussion

In order to identify alternative hosts the use of *C. europea* as a vector was investigated in transmission trials (Fig. 1, 2). LChV-1 and -2 were graft inoculated onto *Prunus avium* F12 rootstocks and parasited by *C. europea*. Healthy *N. occidentalis* '37B' served as receptor host plant and could be infected systemically with LChV-1. Transmissions were done in the greenhouse over a period of up to 6 month. Virus detection in *Cuscuta* and *N. occidentalis* tissue was confirmed by RT-PCR. Attempts at propagation of LChV-1 by mechanical transmission to *N. occidentalis* failed, however the virus was serially transferred by grafting. This result is in accordance with our observations for GLRaV-7,

the most closely related virus of LChV-1 (Mikona and Jelkmann 2010). Virus transmission was not successful for LChV-2 under the same circumstances.

Fig. 1 and 2 Transmission experiment with *Little cherry virus* -1 (LChV-1) infected *Prunus avium* F12 rootstock plants, parasitic *Cusucta europea* acting as a vector, and healthy *Nicotiana occidentalis* 37B.

Symptoms on *N. occidentalis* '37B' consisted of curled leaves, reddening of leaf margins and veins, and decline. The severity of reduction in vegetative growth, and the survival of plants up to 6 months depended on the virus isolate.

After our recent reports for GLRaV-7 (Mikona and Jelkmann 2010), this is the second time that a virus in the family *Closteroviridae* was successfully transferred to a herbaceous plant by dodder. Transmissions of members of the family *Closteroviridae* by dodder have been reported earlier, however with the natural host as acceptor plant (Nariani and Raychaudhuri 1970;Fuchs and Reiss 1954). These results will allow attempts at virus purification from a non-woody host for the purpose of producing antibodies suitable for sensitive virus detection in ELISA. Antisera for LChV-1 produced from purified virus preparations, bacterially expressed protein, or a DNA vector that expressed the cloned coat protein (CP) gene *in vivo* have been described (Matic, Minafra et al. 2009a;KeimKonrad and Jelkmann 1996), however, the sensitivity was not suitable for virus detection in ELISA. Attempts will be continued to identify a herbaceous host for LChV-2.

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First occurence of *Cherry virus a* (cva) in the Czech Republic

Grimová, L.[1], Zouhar, M.[1], Ryšánek, P.[1], Drabešová, J.[1], Mazáková, J.[1], Paprštein, F.[2]
[1] Department of Plant Protection, Czech University of Life Sciences Prague, Kamycká 129, Praha 6, Suchdol, Czech Republic. Email: lgrimova@seznam.cz
[2] Research and Breeding Institute of Pomology Holovousy Ltd., Holovousy 1, Hořice, Czech Republic

Abstract

A preliminary survey on *Cherry virus A* (CVA) has been performed in the Czech Republic in 2008-2009, including a germplasm collection, various growing areas and nurseries. 200 sweet and sour cherry leaf samples (*Prunus avium*, *P. cerasus*) were collected and tested by optimized RT-PCR using a new set of primers CVAZR2/CVAZF2. The 405 bp CVA-specific amplicon was obtained from two sweet cherry trees, namely cv. H 15/31 from Holovousy germplasm collection (originally from Romania) and the seedling *P. avium* from the nursery SEMPRA Turnov. To confirm RT-PCR results, CVA amplification products were directly sequenced. To our knowledge, this is the first report of CVA in the Czech Republic.

Keywords: CVA, sweet and sour cherries, RT-PCR.

Introduction

Sweet and sour cherries are important and widely grown fruits in the Czech Republic, producing 16 701 and 13 818 tons of fruit per year, respectively (Anonymous, 2008). A wide range of viruses and virus-like diseases is known to occur in cherry trees (Gilmer et al., 1976; Németh, 1986). In this region, the most common viruses include *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Cherry leaf roll virus* (CLRV) (Polák, 2007). *Cherry virus A* (CVA), a definitive species in the genus *Capillovirus*, family *Flexiviridae*, is another, recently discovered virus attacking stone fruit trees. CVA was first reported in *P. cerasus* in Germany (Jelkmann, 1995). CVA infection is not limited to cherry, the virus was also found in apricot and peach. The virus does not appear to cause any obvious symptoms in the plants, but when combined with other viruses it may affect the severity of symptoms, or it may have some influence on graft incompatibility in susceptible combinations of scion and rootstock (James and Jelkmann, 1998). CVA is widely distributed in Europe, North America and Japan (Eastwell & Bernady, 1998; James & Jelkmann, 1998; Isogai *et al.*, 2004; Komorowska & Cieślińska, 2004). Despite the importance of cherry production, no information is available about the occurrence and the potential incidence of CVA in the Czech Republic. Thus, a survey was done during the growing seasons of 2008-2009 encompassing different regions and germplasm collections, where sweet and sour cherries a were grown. The information obtained was needed as the first step toward the search for control strategies of virus diseases in cherry trees.

Materials and methods

RT-PCR detection: Sweet and sour cherry plant material, infected by CVA, was received from B. Komorowska, Research Institute of Pomology and Floriculture, Skierniewice, Poland. Three methods were used for RNA extraction, namely phenol-chloroform extraction (Robinson, 1992), isolation with Qiagen Rneasy Plant Minikit according to the manufacturer's instructions and nucleic acid extraction using the modified silica capture method (Rott and Jelkmann, 2001). RT-PCR tests were done using the new set of oligonucleotides,

CVAZR2 [5´-ACCTTTGGAACAAACGATGC-3´] and

CVAZF2 [5´- CAAGAATCCAGGGGCCTACT-3´],

amplifying a fragment of the virus coat protein (CP) and 3´Non-coding region (6891 to7295 nt, 405 bp). Cycling parameters were as follows: denaturation at 94ºC for 30 sec, annealing at 52ºC for 30 sec., and extension at 72ºC for 1 min., totalling 35 cycles, with a final extension at 72°C for 7 min. Healthy cherry trees were used as negative controls. Products of amplification were stained by silver after polyacrylamide gel electrophoresis.

CVA monitoring: Using optimized RT-PCR method, a preliminary survey of the CVA occurence was done in cooperation with Research and Breeding Institute of Pomology Holovousy Ltd. and with cherry growers from various growing areas in the Czech Republic during summer 2008-2009. In order to check the presence and distribution of CVA, leaf samples from 200 cherry trees were collected randomly for RT-PCR analysis. Acquired positive PCR amplicons were directly sequenced in both orientations (Biogen), sequence data were assembled with the BioEdit 7.0.9 program (Ibis Biosciences, USA) and compared with database sequences using BLAST on the NCBI Web server.

Results

RT-PCR detection: From the three methods of RNA extractions the modified silica capture was only suitable technique. The PCR method was very reliable as there were no problems with the identification of positive samples according to the presence of a specific bands, which were completely absent in negative controls.

CVA monitoring: Of the 200 samples tested, 2 sweet cherries, namely *P. avium* 'H 15/31' from Holovousy germplasm collection (originally from Romania) and seedling *P. avium* from nursery SEMPRA Turnov, were positive for CVA in RT-PCR (Fig. 1). These infections were not associated with any particular symptoms. Sequences of 407 bp of CP amplicons obtained from PCR analysis were deposited in the EBI website with the accession numbers FN547890 (sequence code 09HOLche) and FN547891 (sequence code 09TURche). The alignment indicated that the nucleotide sequence of cv. H 15/31 and *P. avium* isolate were closely related to the published sequences of CVA (EMB Accession No. X82547.1) and had 98 % and 92 %, homology to the corresponding region, respectively. To our knowledge, this is the first report of CVA in the Czech Republic.

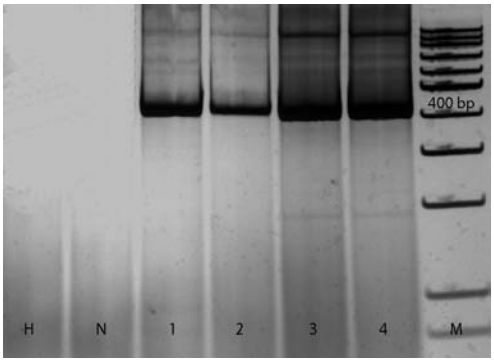

Fig. 1 CVA detection by RT-PCR in cherry trees. Lane M: DNA marker (Mass ruler low range, Fermentas); H: water control; N: healthy control; 1: cherry cultivar H 15/31, Holovousy; 2:seedling *P. avium*, Turnov; 3, 4: CVA positive control (Poland). Polyacryl-amid gel stained by silver.

Discussion

A wide distribution of CVA in cherry sources was reported from Germany (James & Jelkmann, 1998) and Poland (Komorowska & Cieślińska, 2004), the neighbouring countries of the Czech Republic. Therefore, the occurence of CVA was in all likelihood expected in the region and this assumption was partially confirmed by our studies. However, the low incidence of the virus did not correspond to the situation in other European countries. One of the potential explanations of such a small amount of acquired positive samples can be an utilization of unsuitable sets of primers. CVA diversity based on the sequence of an internal fragment of the RdRp (the PDO fragment) have shown the existence of five phylogenetic groups, with up to 19% genetic divergence (Marais et al., 2008). Nevertheless, the primer set used in the present work was designed in the N-terminal portion of the gene, comprising the relatively conserved 3´end of the CP gene and a part of the 3´NTR, allowing the detection of all the CVA isolates tested in previous studies (Svanella-Dumas et al. 2009).

In order to investigate whether this situation stems from a low incidence of the pathogen in the region or from the use of unsuitable diagnostic methods, further work should be done using other polyvalent assays for the efficient detection of all isolates of CVA.

Acknowledgements

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Occurrence of *Prunus necrotic ringspot virus* and *Prune dwarf virus* in wild cherries in the locality velehrad (South Moravia, Czech Republic)

Navrátil, M., Šafářová, D.
Faculty of Science, Palacký University in Olomouc, Šlechtitelů 11, 783 71 Olomouc-Holice, Czech Republic.
Email: milan.navratil@upol.cz

Abstract

The occurrence and spatial distribution of PDV and PNRSV in a seed wild cherry (*Prunus avium*) orchard were studied during the period 1996 - 1999. Each year any newly infected trees were immediately removed. The cumulative infection rate of PDV-positive trees reached 4.7% and number of new infections per year was 1.2 %, on average. Although the number of centers was found to be decreasing (from 22 in 1996 to 10 in 1999), eradication of PDV was not achieved. Only one case of PNRSV infection was found in 1997.

Keywords: PDV, PNRSV, ELISA, epidemiology

Introduction

Prune dwarf virus (PDV) and *Prunus necrotic ringspot virus* (PNRSV) represent important pathogens, affecting stone fruit trees production. Both viruses are considered as the causal agent of serious viral diseases of both sweet and sour cherries in the Czech Republic. During the period of former Czechoslovakia, the occurrence and distribution of PDV and PNRSV was monitored by Blattný (1958), Králiková (1959, 1960, 1962), and Paulechová and Baumgartnerová (1970). These research papers indicated that the distribution of both viruses was relatively wide. The highest occurrence of these viruses was observed on plums and both sour and sweet cherries. Sporadic occurrences were also noted on peaches and apricots. Recently, Polák (2007) studied the distribution of PDV and PNRSV on road-border trees of plum, myrobalan, sweet and sour cherry, as well as blackthorn in the Czech Republic. The occurrence of both viruses was confirmed in all tested tree species. In this study, we summarize the results of monitoring the incidence of the PDV and PNRSV, as well as their spatial distribution in a wild cherry orchard over a four-year period; plus the probable effects of the eradication of infected trees.

Material and methods

During the period 1996 – 1999, a seed orchard of wild cherry (*Prunus avium* L.) located in Velehrad (South Moravia, Czech Republic) was examined for both the *Prunus necrotic ringspot virus* and *Prune dwarf virus*, and their occurrence and distribution were monitored. This twenty year-old orchard was represented by 930 trees spaced at 7 x 7 meters. Samples of the leaves or flowers from each tree were collected from the beginning of May through the beginning of June. Representative samples, all done in two repetitions, were prepared from 20 leaves or flowers collected around the crown. ELISA detection kits from Bioreba AG were used to detect both viruses, according to the manufacturer's instructions.

The percentage of infected trees was noted and the spatial pattern of diseased trees within the orchard was visualized using contour plotting, implemented in NCSS 2001 software package (Statistical Solution Ltd. Cork, Ireland).

Results and discussion

Twenty-year old wild cherries were visually inspected repeatedly for the presence of virus infections, until 1995. All trees manifesting symptoms of either PDV or PNRSV infections (mosaics on leaves and reduced growth) were immediately eliminated. During this period, a total of 156 trees (16.8%) died or were cut-down. During the subsequent four-year period, the presence of these viruses within the orchard was screened by ELISA. Infection by PDV was detected every year, but only one case of tree infection by PNRSV was noted in 1997. The symptoms of PDV infection on the trees were very weak; with only a few of them showing a mild yellow mosaic or chlorotic-necrotic ringspots on the leaves, sporadically. All trees that tested positive were removed in the same season, in order to reduce the inoculum potential within the plot. The spatial distribution of PDV during the period 1996-1999 is illustrated on the contour map (Fig. 1). The ELISA test confirmed the occurrence of PDV at limited focal points (centres). Newly PDV-infected trees were only found in the vicinity of trees that were previously positive. Although the number of centers of infection steadily decreased, from 22 in 1996 to 10 in 1999, eradication of PDV was not achieved.

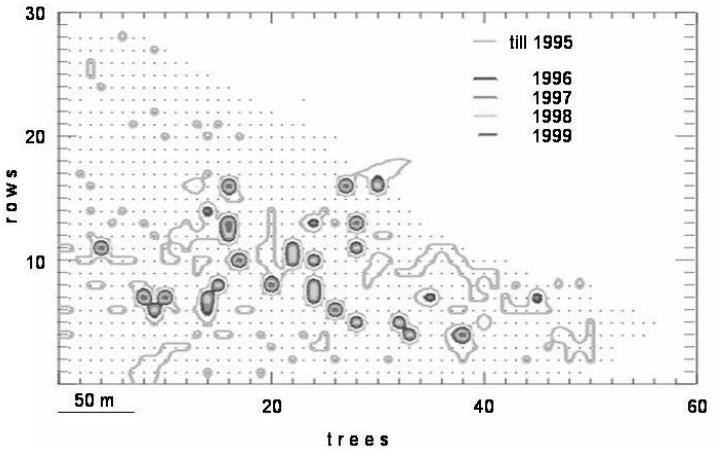

Fig. 1 Distribution of PDV-infected wild cherry trees within the orchard.

The result of repeated viral assays (Fig. 2) clearly indicates that during each vegetative season additional cherry trees had become infected, and/or a latent infection was present. In the seed orchard studied, the cumulative infection rate reached 4.7 % PDV-positive trees within the four-year period. The number of new infections per year was 1.2%, on average. Surprisingly, PNRSV was practically undetected during the monitoring period; which indicates the absence of PNRSV infection resources. Only a few authors have studied the progress of PDV infections within cherry orchards. Gerginova (1981) noted the increasing incidence of PNRSV and PDV (from 8.2% to 68.2% and from 2.1% to 35.3%, respectively) in a cherry orchard during a 7-year period. Andersone et al. (2002) reported 4% PDV and 13% PNRSV naturally infected trees, after two years of growing virus-free trees within their experimental plot. The spread of PDV in our study was lower, and with less intensity. This could be due to the absence of virus resources and thus a decrease of infection pressure, due to the elimination of infected trees.

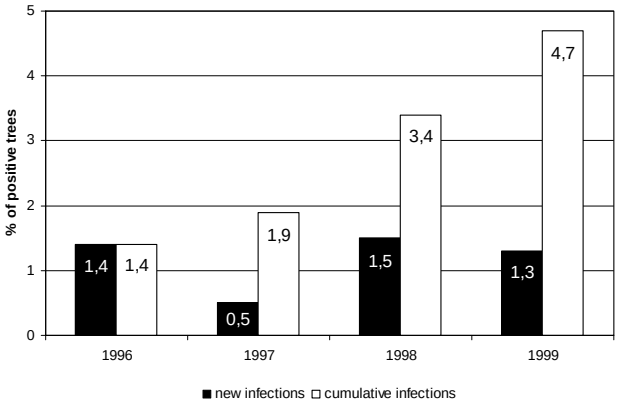

Fig. 2 Percent of infections of PDV in the wild cherry orchard in the period 1996-1999.

Acknowledgements

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Identification of Ilarviruses in almond and cherry fruit trees using nested PCR assays

Maliogka, V.I.[1], Charou, A.[1], Efthimiou, K.[1], Katsiani, A.T.[1], Chatzivassiliou, E.K.[2], Katis, N.I.[1]
[1] Plant Pathology Lab, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[2] Democritus University of Thrace, Department of Agricultural development, 193 Pantazidou St., 68200-N. Orestiada, Greece

Abstract

In this study nested PCR assays have been developed for the detection of *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) modifying a previously reported assay for the generic detection of ilarviruses. In all cases one generic upstream primer was used along with a virus-specific downstream primer in respective nested PCR assays. The application of the same thermocycling profile allowed all amplifications to run in parallel. *Ilarvirus* isolates from different hosts were used for the evaluation of the detection range of the assays, which were afterwards applied for screening almond and cherry plant material. In almond trees the incidence of PNRSV and PDV was 41% and 21.5%, respectively. In cherry orchards the opposite was observed with PDV (56.6%) being the prevalent virus followed by PNRSV (19.4%). Mixed infections with both viruses were also encountered in approximately 10 and 17% of cherry and almond trees, respectively. ApMV was not detected in any of the samples tested. This is the first extensive survey conducted in Greece in order to monitor the distribution of these viruses using molecular assays.

Keywords: *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Apple mosaic virus*, cherry, almond, nested PCR

Introduction

Stone fruits are susceptible to many virus associated diseases (Nemeth, 1986). Some of the most important viruses of cherry and almond belong to the genus *Ilarvirus and* exist either in single or in mixed infections within the trees (Nemeth, 1986). Vegetative propagation and grafting are mainly responsible for their wide dissemination and the production of certified propagating material is the most effective way for their control. However, an important step in the process of controlling fruit tree viruses is the application of sensitive and reliable molecular diagnostic techniques. A nested PCR assay has been recently developed for the generic detection of ilarviruses amplifying a 371 bp RdRp fragment (Maliogka et al., 2007). Using this method a survey was conducted on a number of almond and cherry trees in Greece and revealed high rates of *Ilarvirus*-related infections. In order to further identify the viral agents involved in these infections the nested PCR step of the generic assay was modified so as to specifically detect three of the most widespread ilarviruses of stone fruits namely, *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). The developed assays were applied for screening almond and cherry orchards from different geographic districts so as to provide further insight on the distribution and relative importance of PDV, PNRSV and ApMV on these plant species in Greece.

Materials and methods

Virus isolates: PDV, PNRSV and ApMV isolates from different hosts (cherry, almond, apple, rose, plum, and apricot) were used for the evaluation of the detection range of the herein developed assays.

RT-PCR: The first generic RT-PCR took place as reported earlier (Maliogka et al., 2007), using 2 µl of total RNA (Rott & Jelkmann, 2001) as template.

Virus-specific nested PCR assays: For the detection of each virus, specific downstream primers (Fig. 1) were designed from conserved RdRp regions, after a proper alignment of homologous nucleotide sequences available in the genebank and/or determined herein, and used along with the generic upstream "Ilarpolsequp"

5'-TCGAMRTTYGAYAARTCGCA-3' (Maliogka et al., 2007)

in respective nested PCR assays. The reaction mixture contained in all cases 1µl of the first RT-PCR, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 3% DMSO, 0.2 mM of each dNTP, 1 unit Dynazyme II™ DNA polymerase (Finnzymes), 1 µM of "Ilarpolsequp" and depending on the specificity of the detection 0.2 µM from each of the PDV, PNRSV or ApMV downstream primers. The same thermal profile was applied for all reactions which consisted of a 3 min incubation at 94 °C followed by 40 cycles at 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 20 sec and a final extension step of 72 °C for 2 min.

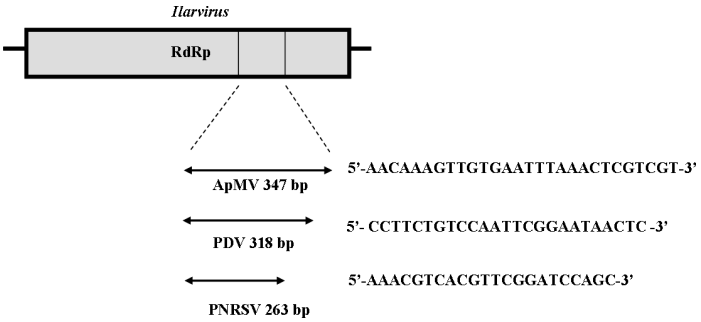

Fig. 1 Position of the PDV, PNRSV and ApMV specific downstream primers on the RdRp gene and size of the nested PCR products.

Plant material: A total of 265 almond and 196 cherry samples were collected in a random way from different districts of Greece (Table 1) during 2005-2007 and tested using the herein developed nested PCR assays.

Tab. 1 Sampling of Plant Material and incidence of PDV and PNRSV

Area surveyed	No. of samples collected	PDV positive samples	PNRSV positive samples
Almond			
Larissa	75	18	40
Thessaloniki	22	0	0
Magnesia	42	15	19
Ioannina	10	1	1
Pieria	38	6	8
Kavala	19	3	19
Evros	19	0	2
Ilia	3	0	0
Serres	37	14	20
Total	265	57 (21.5%)	109 (41%)
Cherry			
Imathia	37	11	5
Pella	19	12	4
Pieria	52	26	16
Kavala	3	2	2
Thessaloniki	22	18	4
Evros	19	17	4
Ioannina	19	3	0
Grevena	3	3	3
Komotini	22	19	0
Total	196	111 (56.6%)	38 (19.4%)

Sequence analysis: PDV and PNRSV amplicons were directly sequenced using "Ilarpolsequp" and the virus specific downstream primers. The obtained sequences were compared after clipping the primer-binding regions with homologous sequences of other PDV and PNRSV isolates already deposited in the genebank.

Results

Evaluation of the assays: All PDV, PNRSV and ApMV isolates tested, originating from different hosts, were successfully amplified using the developed assays (Fig. 2). No amplicon was obtained in each reaction from the isolates of the other two viruses or the healthy controls.

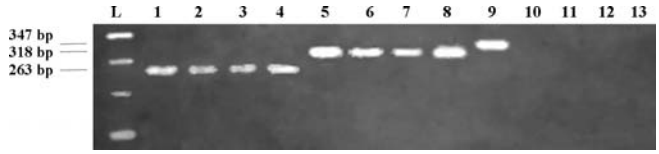

Fig. 2 Agarose gel electrophoretic analysis of the nested PCR products for the detection of PDV, PNRSV and ApMV. Lane 1: PNRSV infected cherry, lane 2: PNRSV infected almond, lane 3: PNRSV infected rose, lane 4: PNRSV infected apricot, lanes 5-6: PDV infected cherries, lane 7: PDV infected almond, lane 8: PDV infected plum, lane 9: ApMV infected apple, lanes 10-13: healthy cherry, almond, plum and apricot, respectively, lane L: 100 bp DNA ladder.

Incidence of PDV, PNRSV and ApMV: A high incidence of PDV and PNRSV was encountered on both plant species tested (Table 1). In almond trees PNRSV was identified in 41% of the analysed samples and it was prevalent in the areas of Larissa, Magnesia and Serres. PDV was identified at lower rates (21.5%) mainly in the areas of Larissa, Magnesia, Kavala and Serres. Both viruses were detected, though at lower rates (10%), in wild almonds (region of Ioannina, Table 1). In cherry orchards the opposite was observed with PDV (56.6%) being the prevalent virus followed by PNRSV (19.4%). PDV exhibited a higher infection rate in the regions of Komotini, Evros and Thessaloniki, whereas PNRSV was mainly found in Pieria. Mixed infections with both viruses were also encountered in approximately 10 and 17% of cherry and almond trees, respectively. ApMV was not detected in any of the samples tested.

Sequence analysis: Sequencing of an almond isolate from PNRSV and an almond and cherry isolate from PDV confirmed the specificity of the assays. The almond partial RdRp sequence of PNRSV was 99% identical in nucleotides with that of an apricot isolate (Acc. No. AM412232). The homologous sequences from the almond and cherry isolates of PDV showed 91 and 99% nucleotide identities, respectively with that of an already published Greek cherry isolate (Acc. No. AM412231). The sequences determined herein were deposited in the EMBL-EBI database under the accession no. FN556183, FN556184 and FN556185.

Discussion

In this study nested PCR assays were developed for the specific detection of three major stone fruit ilarviruses, namely PDV, PNRSV and ApMV. Various isolates of the three viruses originating from different host plants were used for the evaluation of the specificity and detection range of the assays while the application of the same thermocycling profile enabled all amplifications to run in parallel. The assays were successfully applied for screening almond and cherry plant material. The results indicated high infection rates with PDV and PNRSV while ApMV was not detected. The low sanitary status of cherry and almond trees calls for the implementation of certification programs in the production of their propagating material in Greece in order to prevent further spread of the viruses. To our knowledge, this is the first extensive survey conducted in Greece for monitoring the distribution of PDV, PNRSV and ApMV in almond and cherry trees using molecular assays. The sequence information that exists so far in the databases for PDV, PNRSV and ApMV comes mainly from the coat protein gene of the viruses. Thus, the herein developed assays can be applied for the enrichment of sequence data on the RdRp gene from different isolates of the three viruses, which would in turn contribute to future molecular variability studies. Finally, these methods could be used in combination with the generic PCR previously reported for ilarviruses (Maliogka et al., 2007) for the identification of genetically distant PDV, PNRSV and ApMV variants or even putatively new *ilarvirus* species.

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Effects associated with graft-transmissible agents found in the peach variety 'Ta Tao 5'

Gibson, P.G.[1], Reighard, G.[2], Marini, D.[3], Scott, S.W.[4]
[1] Gwinnett Technical College, 5150 Sugarloaf Parkway, Lawrenceville, GA, 30043 USA
[2] Department of Horticulture, Clemson University, Clemson, SC 29634
[3] P.O. Box 78, Gral. San Martin (5570) Mendoza, Argentina
[4] Department of Entomology, Soils and Plant Sciences, Clemson University, Clemson, SC 29634

Abstract

The peach variety 'Ta Tao 5' is host to at least three graft-transmissible agents. Extraction and characterization of these agents indicates that they are variants of *Peach latent mosaic viroid* (PLMVd), *Apple chlorotic leaf spot virus* (ACLSV) and a previously uncharacterized Foveavirus referred to as Asian prunus virus 1 (APV1). Each of these agents are utilized separately, in defined combinations and in concert in a field study designed to identify their graft-transmissibility and consequent contribution to phenological changes in the peach varieties 'Springprince' and 'Juneprince.' Field data record variations in bloom date, vegetative growth, and fruiting in both varieties tested. Further, such phenological variation associated with 'Ta Tao 5' differs significantly from artificial combinations of inoculants. The use of 'Ta Tao 5' as an inoculant source to manipulate growth and development of peach trees is unique when compared with other sources.

Keywords: *Prunus persica*, stone fruit, bloom delay, CGRMV, RT-PCR, *trichovirus*

Introduction

The presence of two or more infectious agents has been associated with unique phenological developments in peach (Gibson and Reighard, 2002; Stubbs and Smith, 1971; Scott et al., 2001). *Peach Latent Mosaic Viroid* (PLMVd) is often associated with a delay in bloom, reduced vegetative vigor, and higher fruiting efficiency (Nemeth, 1986; Desvignes et al., 1996; Gibson et al., 2001). In peach, *Apple Chlorotic Leaf Spot Virus* (ACLSV) produces dark green sunken spots or wavy lines on peach leaves, hence the name of the disease; peach dark-green sunken mottle (Nemeth, 1986). Additionally, ACLSV often causes incompatibilities in some peach cultivars (Llacer and Cambra, 1975). *Cherry Green Ring Mottle Virus* (CGRMV) is latent in peach (Parker et al., 1976). Some infectious agents act synergistically within the host to produce novel symptoms. *Prune Dwarf Virus* (PDV) and *Prunus Necrotic Ringspot Virus* (PNRSV) inoculated singly and in combination produce different symptoms in peach (Stubbs and Smith, 1971; Scott et al., 2001).

The peach variety 'Ta Tao 5' is host to at least three graft-transmissible agents. These include isolates of ACLSV; GenBank accession number EU223295 (Marini et al., 2008) and Asian Prunus Virus 1; GenBank accession number FJ824737 (Marini et al., 2009). The PLMVd isolate aligns closely with other variants, but does not contain the 11 nt insertion typically associated with peach calico (Marini, 2007).

Materials and methods

Virus-indexed trees of the peach cultivar 'Springprince' grafted onto Guardian® rootstock were planted January 2005 in a high-density, Y-trained orchard system with 1.8 m spacing in the row and 5.5 m between rows. Trees were inoculated on March 11, 2005 with chip buds to initiate treatments. One control and eleven treatments consisted of two isolates of ACLSV, an unknown high-chill peach (acronym PK), PLMVd, ACLSV & PLMVd, PK & ACLSV, PLMVd & ACLSV, PLMVd & PK, ACLSV & PLMVd & CGRMV, the peach cultivar 'Ta Tao 5,' and PK & ACLSV & PLMVd. Each of the treatments and the control consisted of 4-tree plots randomly assigned within each block and replicated 3 times.

Virus-indexed trees of the peach cultivar 'Juneprince' grafted onto Guardian® rootstock were planted January 2006 in a high-density, Y-trained orchard system with 1.8 m spacing in the row and 5.5 m between rows. Trees were inoculated on September 29, 2006 with chip buds to initiate treatments. One control and seven treatments consisted of 'Ta Tao 5,' ACLSV, PK, PLMVd, Heat-treated 'Ta Tao 5,' ACLSV & PLMVd, Heat-Treated 'Ta Tao 5' & ACLSV. Each of the treatments and the control consisted of 5-tree plots randomly assigned within each block and replicated 3 times.

Treatment verification: Total RNA was extracted from newly emerged peach shoots from all 'Springprince' trees in spring 2006, 2008, and 2009, and from all 'Juneprince' trees in spring 2009; using a modified procedure of Hughes and Galau (1988) (Sara Spiegel, The Volcani Center, Israel, personal communication). The amount of RNA in each sample was measured by recording the absorbance at 260 nm and calculating the concentration using an extinction coefficient

of 25 $(mg/ml)^{-1}cm^{-1}$ (Noordam, 1973). The QIAGEN OneStep™ RT-PCR kit (QIAGEN Inc., Valencia, CA) was used to perform detections of ACLSV, APV1, PLMVd and CGRMV, according to the manufacturer's instructions. Treatments were verified by analysis of RT-PCR fragments.

Field data: Leaf abscission (leaf drop) was recorded by observing the date on which 10%, 50% or 90% defoliation occurred in autumn. Trunk cross-sectional area was calculated from stem caliper measured at the trunk base with a Digimatic Caliper (Mitutoyo Corporation, Japan). Spring bloom was recorded by observing the date on which 10%, 50%, and 90% (full bloom) of flowers were open during spring. Harvest data consisted of individual fruit weight (average of 10 fruit per tree), percent soluble solids as measured with a MT-032ATC Refractometer (International Ripening Company, Norfolk, VA), total number of fruit prior to picking and total weight of fruit. Fruiting efficiency was calculated by dividing total fruit weight by trunk cross-sectional area. Puncture pressure (*i.e.*, firmness) of the fruit was determined with a McCormick FT 327 Penetrometer (International Ripening Company, Norfolk, VA) using a 0.8 cm diameter plunger tip. The skin was sliced off each cheek face perpendicular to the suture of 5 fruit per tree and the mean pressure calculated. Pruning was recorded as the time required to prune each tree and the weight of the clippings removed. Photosynthetically active radiation (par) was recorded 1 m above the ground as an average between one measure perpendicular and one measure parallel to the row between 11:00 AM and 1:00 PM on a clear day in $\mu mol \cdot m^{-2} \cdot s^{-1}$ using a line quantum sensor (LI-191SA; LI-COR, Lincoln, Nebr, USA). All data was analyzed by ANOVA with significance determined using *F* ratios as calculated using mean square across blocks or treatments divided by mean square error.

Results

'Springprince': 'Springprince' trees inoculated with 'Ta Tao 5' were the only treatment exhibiting a striking reduction in trunk cross-sectional area increase for the growing seasons 2006 and 2007, but none of the treatments were significantly different during the 2008 growing season (see Table 1). Leaf drop occurred significantly, 2 to 4 days earlier on 'Ta Tao 5' inoculated trees, but were sometimes similar on other treatments. Pruning data through three growing seasons indicate that 'Ta Tao 5' inoculated trees were significantly less vigorous. Surprisingly, a few other treatments were significantly more vigorous through three growing seasons when compared with the controls. A similar pattern is demonstrated by the light penetration data where significantly more light penetrated the canopy of 'Ta Tao 5' inoculated trees in 2007 and 2009, yet all other treatments exhibit less light penetration during the same years.

Bloom date is the most significant variation recorded in the 'Springprince' trees during the period 2006 to 2009 (see Figure 1). The trees inoculated with 'Ta Tao 5' bloom 1 to 4 days later when compared with all the other treatments and control with the exception of PLMVd inoculated treatments in 2007 which bloomed 3 days later when compared with the control.

Fig. 1 Springprince Bloom 2008. Key left to right: Ta Tao 5/ACLSV, Ta Tao 5/PK/ACLSV, Ta Tao 5, Ta Tao 5, Ta Tao 5/PK, PK, ACLSV, PLMVd/ACLSV, ACLSV, PK/ACLSV, PLMVd/ACLSV/CGRMV, and non-inoculated control. (photo by Dr. Simon Scott).

Fruiting characters measured across the 2006 to 2009 seasons exhibit unremarkable variation with few notable exceptions. These are a significant reduction of total harvest in 'Ta Tao 5' and PLMVd inoculated trees when compared with controls or PK inoculated trees in 2009. Additionally, all treatments had significantly firmer fruit in all 4 seasons when compared with the controls except PK inoculated trees in 2006 and 2007 and PLMVd inoculated in 2006.

'Juneprince': The 'Juneprince' trial demonstrated generally unremarkable results across all measured growth and fruiting characters amongst all treatments (see Table 2). Growth rates, winter and summer pruning requirements, and light penetration were exceptionally uniform for all treatments. Most treatments had higher summer clipping weights when compared with the controls in 2007, 2008 and 2009. Leaf drop occurred 2 to 3 days earlier on the 'Ta Tao 5' inoculated trees when compared with all other treatments in 2008. Fruiting efficiency, firmness, fruit count, soluble solids, and individual fruit weight were quite similar in 2008 and 2009. Surprisingly, total harvest was significantly higher for all treatments except PLMVd when compared with controls. Unlike the 'Springprince' trial, bloom date varied less than 2 days in 2008 and not at all in 2007 and 2009.

Tab. 1 Field data for 'Springprince' cultivar.

Agent\trtmt	Units	Control	Ta Tao 5	PK1	ACLSV\PLMVd	α=0.05
08-'09 TCA increase	cm^2	19.1	15.21	17.02	16.57	
07-'08 TCA increase	cm^2	20.13	13.39	17.53	21.41	3.38E-05
06-'07 TCA increase	cm^2	18.12	13.94	20.9	20.77	4.86E-05
08 Leaf drop	JD	310.4	308.1	308.2	308.9	2.36E-09
07 Leaf drop	JD	316.4	312.2	314.5	316.8	2.63E-05
06 Leaf drop	JD	322.7	320.5	325.2	327.1	4.14E-05
09 Winter prune time	m	2.75	2.082	2.716	2.836	4.71E-04
08 Winter prune time	m	1.9	1.387	2.096	1.841	1.26E-08
09 Winter clipping weight	kg	3.2	2.328	3.1	3.204	2.14E-03
08 Winter clipping weight	kg	2.71	2.032	2.664	2.753	2.46E-04
09 Summer prune time	m	1.824	1.525	1.584	1.747	
08 Summer prune time	m	2.326	1.941	2.01	2.624	2.10E-05
07 Summer prune time	m	3.995	3.634	3.92	3.843	
09 Summer clipping weight	kg	3.65	3.017	2.93	3.557	3.95E-02
08 Summer clipping weight	kg	4.575	3.441	3.76	4.832	5.33E-04
07 Summer clipping weight	kg	6.169	5.43	6.275	7.171	2.39E-02
09 Light penetration	par	112.4	111.4	50.94	59.03	4.05E-02
08 Light penetration	par	113.5	135.7	104.3	106.4	
07 Light penetration	par	349.2	361.7	218.2	282	2.77E-02
09 Bloom date - 90%	JD	70.11	71.13	70.6	70.5	4.30E-06
08 Bloom date - 90%	JD	76.06	79.27	76.6	76.64	1.09E-18
07 Bloom date - 90%	JD	76.67	80.67	78.5	79.71	5.71E-11
06 Bloom date - 90%	JD	70.11	70.83	70.6	70.43	5.16E-03
09 Fruiting efficiency	kg/cm^2	0.158	0.113	0.132	0.108	2.00E-03
08 Fruiting efficiency	kg/cm^2	0.101	0.137	0.11	0.089	6.00E-03
09 Puncture pressure	g	7.176	9.835	9.815	9.768	3.00E-04
08 Puncture pressure	g	8.853	10.71	9.815	9.768	1.67E-07
07 Puncture pressure	g	8.285	9.901	8.505	9.463	4.82E-06
06 Puncture pressure	g	8.07	9.855	7.841	8.134	4.30E-05
09 Fruit count	no.	75.27	42.88	65.35	51.81	5.79E-07
08 Fruit count	no.	42.68	45.72	45.93	36.11	
09 Soluble solids	%	8.706	9.278	9.236	9.037	1.60E-02
08 Soluble solids	%	9.856	10.03	10.24	10.43	
07 Soluble solids	%	11.8	12.03	11.74	11.91	
06 Soluble solids	%	9.356	9.25	10.12	9.643	
09 Fruit weight - individual	kg	0.164	0.163	0.155	0.165	
08 Fruit weight - individual	kg	0.137	0.136	0.139	0.151	1.60E-04
09 Total harvest	kg	12.17	6.9	10.02	8.601	7.10E-08
08 Total harvest	kg	5.885	6.239	6.398	5.465	

Tab. 2 Field data for 'Juneprince' cultivar

Measure	Units	Ta Tao 5	PK	HT TT5	ACLSV	HT TT5 & ACLSV	PLMVd	PLMVd & ACLSV	Control	*α=0.05*
08-'09 TCA increase	cm^2	17.04	23.704	23.87	25.2	21.7	18.092	26.48	22.06	
09 Winter prune time	m	1.824	2.2033	2.194	2.292	1.951	2.0844	2.178	1.97	
08 Winter prune time	m	1.617	2.2167	1.97	1.55	1.31	1.2633	1.45	1.74	3.37E-07
09 Winter clipping we	kg	2.339	3.1333	2.789	3.317	2.489	2.2778	3.011	2.428	3.44E-02
08 Winter clipping we	kg	1.043	1.1967	1.05	0.947	0.72	0.6067	0.85	0.96	1.69E-05
09 Summer prune tim	m	2.293	2.4967	2.847	2.58	2.737	2.02	2.44	2.357	5.47E-08
08 Summer prune tim	m	1.483	1.8767	2.067	2.057	1.967	1.7833	2.11	1.467	6.74E-03
07 Summer prune tim	m	1.667	2.58	2.303	3.203	3.47	2.1756	2.546	3.219	4.17E-05
09 Summer clipping w	kg	3.667	4.5833	4.072	5.25	4.1	3.3944	5.211	3.278	8.98E-05
08 Summer clipping w	kg	2.917	4.5667	3.46	3.877	2.823	2.94	4.133	2.41	6.27E-08
07 Summer clipping w	kg	3.944	5.9222	5.15	6.156	4.661	4.3444	5.956	4.178	3.48E-03
09 Light penetration	par	65.66	57.074	62.29	39.77	71	55.82	25.42	95.09	
08 Light penetration	par	268.4	146.11	145.2	148.8	155.9	222.22	149.2	266.2	
09 Bloom date - 90%	JD	70	69.333	69	69	69.33	69.667	69	69.33	3.49E-08
08 Bloom date - 90%	JD	76	75.444	76	74	76	74.667	74.22	73.89	1.83E-14
07 Bloom date - 90%	JD	72.89	74.556	72.78	72.89	72.56	73.556	73.44	73.33	1.93E-03
08 leaf drop	JD	315.8	317.78	318.1	320.7	317.4	318.11	317.9	319.6	1.74E-03
09 Fruiting efficiency	kg/cm	0.182	0.1185	0.146	0.128	0.165	0.159	0.127	0.146	
08 Fruiting efficiency	kg/cm	0.096	0.082	0.13	0.076	0.127	0.0765	0.088	0.064	1.60E-09
09 Puncture pressure	kg	4.948	4.5533	4.668	4.311	4.339	4.3344	4.644	4.172	3.65E-03
09 Fruit count	no.	52.6	41.603	53.71	44.49	53.92	43.037	46.99	45.32	
08 Fruit count	no.	35.11	37	54.67	33	51.22	27.111	36.44	25.33	1.53E-11
09 Soluble solids	%	11.1	11.36	11.01	11.06	11.35	11.351	11.43	11.8	
09 Fruit weight - indiv	kg	0.175	0.1897	0.173	0.189	0.171	0.1974	0.18	0.181	2.18E-03
08 Fruit weight - indiv	kg	0.097	0.1021	0.097	0.096	0.087	0.1	0.094	0.087	
09 Total Harvest	kg	9.024	7.8608	9.269	8.305	9.228	8.3871	8.457	8.304	
08 Total Harvest	kg	3.549	3.7522	5.279	3.036	4.373	2.7533	3.451	2.249	5.21E-11

Discussion

Graft-transmissible agents utilized in this study are stable and detectable by RT-PCR in 'Springprince' and 'Juneprince' cultivars of peach. Phenological affects are measurable and minimal in both cultivars from 2006 to 2009. Bloom delay is desirable to avoid the risks of late spring frosts which frequently occur in the southeastern United States. Additionally, vegetative growth reduction is desirable to reduce labor requirements. This study suggests there are accelerations in vegetative growth and fruiting associated with some combinations of graft-transmissible agents. Further study is required to determine which agents or combination of agents is responsible for such increases in growth and development.

Most notable from the study is the uniqueness of 'Ta Tao 5' inoculation. Artificial combinations of PLMVd, ACLSV and APV1 failed to reproduce the effects found with 'Ta Tao 5' inoculation. Possible explanations include: PLMVd, ACLSV, and APV1 in 'Ta Tao 5' are sufficiently variable from other isolates to result in unique effects; there are additional, yet to be discovered agents present; or, genetic factors occur between the agents and 'Ta Tao 5.' Further molecular characterization and continued field studies are required.

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Assessment of the main stone fruit viruses and viroids in Algeria

Meziani, S.[1,3], Rouag, N.[2], Milano, R.[3], Kheddam, M.[1], Djelouah, K.[3]
[1] Centre National de Contrôle et de Certification des Semences et Plants (CNCC), Alger, Algérie
[2] UFASetif, Faculté des Sciences, Département d'Agronomie Sétif, Algérie
[3] Istituto Agronomico Mediterraneo di Bari (IAMB), Italy

Abstract

In order to improve the sanitary status of the propagating material of stone fruits, a field survey was conducted to assess the main viruses and viroids affecting stone fruits in selected growing areas and their distribution on the collected material by using serological and molecular detection methods.

Serological assays were carried out to detect *Plum pox virus* (PPV), *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV), Apple mosaic virus (ApMV) and *Apple chlorotic leaf spot virus* (ACLSV). Moreover, tissue-print hybridization was performed to detect *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd).

Among nearly 2000 trees tested, no PPV infection was detected, while 14% of them positively reacted to at least one virus. The highest infection rate (18%) was reported in both nurseries and commercial orchards. PNRSV was the most detected virus (9%), followed by ApMV (3%) and PDV (1.5%). Cherry was the most infected species (20%). As for viroids, a high infection rate was recorded for PLMVd (9%) and HSVd (5%); the highest infection rate was reported in mother blocks and varietal collections.

Keywords: Algeria, *Prunus*, virus, viroids, ELISA, tissue-print hybridization, sanitary status.

Introduction

The Algerian stone fruit industry accounts for a production of about 257,848 tons covering a total surface area of 165,490 ha (MADR, 2006). The creation of new stone fruit orchards in Algeria is always based on the use of standard propagation material that is often not certified, thus causing a major risk of establishment of virus and viroid diseases according to their mode of transmission by grafting.

Different surveys were conducted on stone fruit species in Algeria for sanitary assessment, mainly based on visual observations. *Prune dwarf virus* (PDV), *Prunus necrotic ring spot virus* (PNRSV) *Apple chlorotic leaf spot virus* (ACLSV) (Aouane, 2003) and *Peach latent mosaic viroid* (PLMVd) (Torres et al., 2004) were hence reported. Another recent study, performed in Eastern Algeria, allowed the detection of the major viruses cited above and, for the first time, *Hop stunt viroid* (HSVd) (Rouag et al., 2008). This study was undertaken to assess the presence of the main viruses (PPV, PNRSV, PDV, ApMV and ACLSV) and viroids (PLMVd and HSVd) affecting stone fruits in selected growing areas and to verify their distribution by using serological and molecular detection methods.

Materials and methods

Survey and collection of samples: A survey was carried out over the last three years (2006-2008) in the main Algerian stone fruit growing areas: Algiers, Blida, Medea, Skikda, Constantine, Ain Temouchent, Sétif, Bordj Bou Arreidj, Mila, M'sila and Batna (Fig. 1). One thousand seven hundred fifteen samples were collected in spring and autumn and tested by ELISA for virus detection, while direct tissue print hybridisation was performed for viroid detection. Besides the 574 samples collected from commercial orchards, 553 were taken from mother blocks, 354 from variety collections and 227 from nurseries (Tab. 1). Sampling was performed on all the surveyed trees grown in mother blocks and under the screenhouse, while, for variety collections, sampling was hierarchically carried out on selected trees from each variety. In commercial orchards, samples were randomly collected on the basis of field observations; on each tree four shoots were taken from the tree quadrant.

Fig. 1 Geographical distribution of monitored areas.

Tab. 1 Samples collected from tree sources.

	N° of collected samples				
Species	**Nurseries**	**Mother blocks**	**Commercial orchards**	**Varietal collections**	**Total**
Apricot	57	55	180	65	357
Peach	34	153	137	94	418
Plum	24	171	125	21	341
Almond	37	59	57	163	316
Cherry	49	105	75	18	247
Other Prunus spp	26	10	0	0	36
Total	227	553	574	354	1715

In general, sample collection was performed on European plum (*Prunus domestica*), peach (*Prunus persica*), apricot (*Prunus armeniaca*), almond (*Prunus amygdalis*), sweet cherry (*Prunus avium*), sour cherry (*Prunus cerasus*) and Myrobalan (*Prunus cerasifera*) trees.

Virus detection: 1715 samples were tested by Double antibody sandwich-ELISA (DAS-ELISA) as reported by Clark and Adams (1977) for PPV, PNRSV, PDV and ApMV detection and by DAS-simultaneous ELISA (Flegg and Clark, 1979) for ACLSV. The ELISA test was performed by using extracts from young leaves of the collected samples and the serological commercial kits used were purchased from Loewe (Germany). The sample was considered positive if its optical density was three times higher than the negative control.

Viroids detection: Tissue-printing hybridization was carried out for PLMVd and HSVd detection on the 1715 collected samples. From each sample, the petioles of three leaves were printed onto the nylon membrane (Hybond N+, AP Biotech) (Pallás et al., 2003). The membranes imprinted in autumn were stored at 4°C and two weeks later covered with plastic envelope and exposed to UV rays for 2-3 min to fix the nucleic acid.

Hybridization was run using PLMVd and HSVd specific riboprobes labelled with dig-11 dUTP, according to the protocol provided by the Roche Company. For the detection of PLMVd, membranes were hybridized with a specific riboprobes RF43 5'd (CTG GAT CAC ACC CCC CTC GGA ACC AAC CGC T) 3'antisense and RF44 5'd (TGT GAT CCA GGT ACC GCC GTA GAA ACT) 3' sense, amplifying a 337 bp fragment as described by Ambrós *et al*. (1998) and VP19 5'd (GCC CCG GGG CTC CTT TCT CAG GTA AG) 3' antisense and VP20 5'd (CGC CCG GGG CAA CTC TTC TCA GAA TCC) 3' sense, amplifying a 297 bp fragment as described by Astruc *et al*. (1996) for the detection of HSVd.

Results and Discussions

Field observations: During the field survey, more than 3000 trees were visually inspected. Generally speaking, the age of inspected trees varied from 5 to 15 years, except for the varietal collections where the trees were older (more than 25 years old). During the survey, field symptoms were observed in the different species, such as the weak development of the trees, the death of peach and cherry trees, chlorosis, tatter leaves, riddled leaves and fruit cracking (Fig. 2). In some cases, it was impossible to observe field symptoms associated to viruses, taking into account the high infestations of aphids and other pests and pathogens.

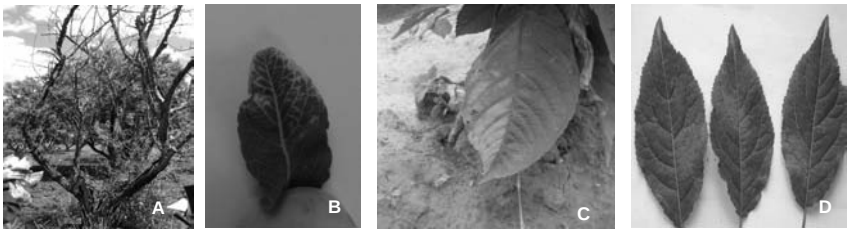

Fig. 2 Main field symptoms observed in the monitored stone fruit growing areas **A**: declining trees; **B**: vein clearing on almond; **C**: chlorosis on cherry; **D**: yellowing on plum.

Virus infection: 248 out of 1715 tested samples reacted positively to at least one virus, showing an infection rate of 14.46%. The highest infection rate was reported on cherry (20.65%) followed by plum (13.78%), peach (12.92%), apricot (11.48%) and almond (11.39%). Interestingly, species used as rootstocks were the most infected ones, where 19 samples out of 36 reacted positively to at least one virus. Ilarviruses (PNRSV, PDV and ApMV) were the most widespread, in particular PNRSV (9%), while ApMV (3%) was detected only in the Eastern area of Algeria. No PPV infected tree was detected from any tested samples (Tab. 2). Nurseries showed the highest level of infection (18.06%), whereas the variety collections (7.06%) displayed the lowest infection rate. The infection rate in mother blocks (12.84%) was relatively high, considering the destination of the propagating material. These results generally match the infection levels observed in other Mediterranean countries (Myrta et al., 2003).

Tab. 2 Distribution of virus infection.

	N° of samples			N° of infections				
Species	**Tested**	**Infected**	**Infection rate (%)**	**PNRSV**	**ApMV**	**PDV**	**ACLSV**	**PPV**
Apricot	357	41	11,48	18	9	8	3	0
Peach	418	54	12,92	40	8	6	0	0
Plum	341	47	13,78	19	16	5	1	0
Almond	316	36	11,39	26	2	3	3	0
Cherry	247	51	20,65	35	10	5	1	0
Other *Prunus*	36	19	52,78	10	7	2	0	0
Total	1715	248	14,46	148	52	29	8	0
Infection rate %	14 %			9 %	3 %	1.5 %	0.5 %	0

Viroid infection: 219 out of 1715 tested samples showed a clear positive reaction, indicating a 12.77% general infection rate. The most infected species were peach (40.19%) followed by apricot (7.28%), cherry (5.26%) and plum (3.52%). Almond and other species used as rootstocks were found free from viroids. The individual viroid incidence reflected a high infection rate for HSVd (5 %) and PLMVd (9%) (Tab. 3). The highest infection rate was reported on mother blocks (18.63%), followed by variety collections (15.82%), commercial orchards (12.02%) and nurseries (6.61%). Similar to virus infection, Algerian viroid infection results match the infection levels observed in European and Mediterranean countries (Torres et al., 2004).

Tab. 3 Results of tissue-printing hybridization on stone fruit viroids in Algeria (2004).

	N° of samples			N° of infection	
Species	**Tested**	**Infected**	**Infection rate (%)**	**HSVd**	**PLMVd**
Apricot	357	26	7,28	11	26
Peach	418	168	40,19	68	108
Plum	341	12	3,52	4	8
Almond	316	0	0,00	0	0
Cherry	247	13	5,26	1	13
Other *Prunus*	36	0	0,00	0	0
Total	1715	219	12,77	84	155
Mean of infection (%)	12,77 %			5 %	9 %

Conclusion

The present study represents the first large-scale survey conducted in the different stone fruit growing areas of Algeria. The survey confirmed the results provided by previous studies (Aouane, 2003; Torres *et al.*, 2004; Rouag et al., 2008) on the presence of fruit tree infectious agents (ACLSV, PNRSV, PDV, HSVd, PLMVd) in Algeria and their wide distribution, while the most important and devastating virus (PPV) was not detected in the collected samples. Therefore, the prompt establishment of effective PPV monitoring is necessary to control the introduction and spread of this pathogen. The large-scale survey allowed us to detect heavy virus and viroid infections, especially in mother blocks that are considered the main source for the distribution of propagating material and can therefore contribute to the rapid and wide dissemination of these agents. It is imperative to continue investigations and analyses to know the real sanitary status with regards to transmissible infections through the propagating material, which may contribute to the success of any certification program for rosaceous stones fruit species.

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Surveying viruses on ornamental trees and shrubs in two Hungarian botanical gardens and an arboretum

Németh, M.[1], Nyerges, K.[2], Hangyál, R.[2], Kósa, G.[3]
[1] Central Agricultural Office, Budaörsi út 141-145., 1118 Budapest, Hungary
[2] Agricultural Office of County Fejér, Plant Protection and Soil Conservation Directorate, Ország út 23., 2481 Velence, Hungary
[3] Institute of Ecology and Botany, Hungarian Academy of Sciences, 2163 Vácrátót, Alkotmány u. 2-4., Hungary

Abstract

In Hungary the most common disease of fruit trees causing the most severe damages is *Plum pox virus* (PPV). This is why it is important to know other woody host plants that can be considered as source of infection. National surveys have been carried out since 2002 for revealing the distribution of PPV on ornamental and wild *Prunus* species.

From 2005 this work has been extended to studying other viruses on other woody plants. In two botanical gardens and an arboretum we selected plants (species and varieties) showing virus symptoms among various thousands of woody plants and submitted them to indexing on woody and herbaceous indicators, as well as to testing with ELISA for the presence of the following 11 viruses occurring on woody plants: PPV, *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Cherry leafroll virus* (CLRV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Apple mosaic virus* (ApMV), *Apple chlorotic leafspot virus* (ACLSV), *Strawberry latent ringspot virus* (SLRV), *Tomato black ring virus* (TBRV) and *Arabis mosaic virus* (ArMV).

Up to now, in 28 plant species and varieties, PPV, PDV, PNRSV, CLRV and ASPV have been detected so far. The presence of PPV was found in 9 species/varieties, such as *Prunus cerasifera* 'Pendula', *P. cerasifera* 'Pissardii', *P. glandulosa*, *P. glandulosa* 'Alba Plena', *P. glandulosa* 'Sinensis', *P. japonica*, *P. sogdiana*, *P. tomentosa* (from Tibet) and *P. x blireana.*

Seventeen species/varieties were found to be infected with PDV: *Lonicera caucasica*, *L. maackii*, *L. sachalinensis*, *Prunus mume* 'Omoi-no-mama', *P. salicina* (from China), *P. spinosa* 'Plena', *P. spinosa* 'Purpurea', *P. serrulata* 'Amanogawa', *P. serrulata* 'Ichiyo', *P. serrulata* 'Pink Perfection', *P. serrulata* 'Taihaku', *P. serrulata* 'Yedo-sakura', *P. subhirtella* 'Plena', *P. tenella*, *P. yedoensis*, *P. yedoensis* 'Moerheimii' and *Syringa yunnanensis.* Certain species/varieties infected with PDV were positive also for CLRV, such as *P. spinosa* 'Purpurea', *P. yedoensis*, *P. yedoensis* 'Moerheimii', *P. subhirtella* 'Plena', *P. serrulata* 'Yedo-sakura' and to ASPV: *P. subhirtella* 'Plena' and *P. serrulata* 'Yedo-sakura'. PNRSV was detected in *P. cerasifera* ' Nigra ' and 'Hollywood'.

The difference of symptoms provoked by PDV on herbaceous hosts suggests that various strains of this virus affect hosts, mainly yellow mottle disease described as a separate strain by Ramaswamy and Posnette on ornamental cherries in 1972. Molecular studies are in progress to confirm the above results.

Keywords: ornamental trees and shrubs, virus symptoms, ELISA, *Prunus*, *Lonicera*, *Syringa*

Introduction

Plum pox virus (PPV) is widespread on stone fruit species causing severe losses in Hungary (Németh, 1986). In the last decades the occurrence of the virus on some ornamental and wild *Prunus* species has also been recorded in Hungary (Salamon and Palkovics, 2002; Sebestyén et al., 2008) as well as in other countries (Labonne et al., 2004; James and Thompson, 2006; Damsteegt et al., 2007). The infection is often symptomless.

In 2002 a several-year-survey was started at the Hungarian Plant Protection and the Soil Conservation Service in order to determine the eventual PPV infection of ornamental *Prunus* species (Sebestyén et al., 2008). From 2005 this work has been extended to studying other viruses on other woody plants. At the same time this work aimed to compile an illustrated diagnostic manual to assist the service staff in inspecting ornamental trees and shrubs. In two botanical gardens and an arboretum, plants (species and varieties) showing virus symptoms among various thousands of woody plants were selected and submitted to indexing on woody and herbaceous indicators, as well as to testing with ELISA for the presence of the 11 viruses (see below) occurring on woody plants.

Materials and methods

Surveys and sampling in propagation sites, such as nuclear stocks, propagation blocks, nurseries, arboretums, public areas including parks and street grown trees, were carried out by plant pathologists and inspectors of the county services in at least one location of each county between 2002 and 2007. Several thousand woody plants were visually checked during 2005 and 2008. Examinations took place three times a year in May, August and early October. The species and varieties showing virus symptoms were submitted to further indexing on woody and herbaceous indicators, as well as to testing with ELISA.

Indexing took place in the testing nursery and the glasshouse of the Agricultural Office of County Fejér, Plant Protection and Soil Conservation Directorate. Virus transmission was performed with double budding and chip budding in the testing nursery from late July to early August. The woody indicators used in indexing were peach seedling GF 305, *Prunus avium* seedling, GF 31 myrobalan hybrid, *P. domestica* 'Italian prune' and *P. serrulata* 'Shirofugen'. In greenhouse indexing, one-year shoots were taken for mechanical transmissions in February. The herbaceous indicators applied were as follows: *Celosia argentea, Chenopodium foetidum, Ch. murale, Ch. quinoa, Cucumis sativus, Cyamopsis tetragonoloba, Momordica balsamina, Nicotiana benthamiana, N. clevelandii, N. langsdorffii, N. tabacum* 'Samsun', *N. tabacum* ' White Burley', *Petunia hybrida, Phaseolus vulgaris, Sesbania exaltata, Solanum sysimbriifolium, Tithonia speciosa, Torenia fournieri* and *Zinnia elegans.*

The leaf samples were tested in duplicates by DAS-ELISA using commercial kits (supplier: BIOREBA for PDV, PNRSV, CLRV, ASPV, ASGV, ApMV, ACLSV, SLRV, TBRV, ArMV and SEDIAG for TBRV) and by DASI-ELISA for PPV (supplier: DURVIZ-IVIA). Optical densities (OD) were recorded at 405 nm after 60 and 90 minutes on Labsystems Multiskan® PLUS reader. Samples with OD double that of the healthy control were considered ELISA-positive.

Tab. 1 Results of biological indexing and ELISA for the ornamental/wild tree and shrub species and cultivars with symptoms collected in two botanical gardens and an arboretum of the Hungarian Academy of Sciences between 2002 and 2008.

		Biological indexing								ELISA				
		Woody host indexing				Herbaceous host indexing								
Species and cultivars	Origin of samples	PPV	PDV	PNRSV	CLRV	PPV	PDV	PNRSV	CLRV	PPV	PDV	PNRSV	CLRV	ASPV
Lonicera caucasica	Vácrátót		X				X				X			
Lonicera maackii	Vácrátót		X				X				X			
Lonicera sachalinensis	Vácrátót		X				X				X			
Prunus cerasifera 'Hollywood'	Budapest			X				X				X		
Prunus cerasifera 'Nigra'	Budapest			X				X				X		
Prunus cerasifera 'Pendula'	Budapest	X				X				X				
Prunus cerasifera 'Pissardii'	Budapest	X				X				X				
Prunus glandulosa	Vácrátót	X				X				X				
Prunus glandulosa 'Alba Plena'	Soroksár	X				X				X				
Prunus glandulosa ' Sinensis'	Vácrátót	X				X				X				
Prunus japonica	Vácrátót	X				X				X				
Prunus mume 'Omoi-no-mama'	Vácrátót		X								X			
Prunus salicina (from China)	Vácrátót		X								X			
Prunus serrulata'Amanogawa'	Vácrátót		X								X			
Prunus serrulata'Ichiyo'	Vácrátót		X								X			
Prunus serrulata'Pink Perfection'	Vácrátót		X				X				X			
Prunus serrulata'Taihaku'	Vácrátót		X				X				X			
Prunus serrulata'Yedo-sakura'	Vácrátót		X		X		X				X		X	X
Prunus sogdiana	Vácrátót	X				X				X				
Prunus spinosa 'Plena'	Vácrátót		X				X				X			
Prunus spinosa 'Purpurea'	Vácrátót		X		X		X				X		X	
Prunus subhirtella 'Plena'	Vácrátót		X		X		X				X		X	X
Prunus tenella	Vácrátót		X				X				X			
Prunus tomentosa (from Tibet)	Vácrátót	X				X				X				
Prunus yedoensis	Vácrátót		X		X		X		X		X		X	
Prunus yedoensis 'Moerheimii'	Vácrátót		X		X		X		X		X		X	
Prunus x blireana	Budapest	X				X				X				
Syringa yunnanensis	Vácrátót		X				X				X			

Results and discussion

During 2002 and 2007, 120 ornamental *Prunus* species and cultivars in arboretums/ botanical gardens (288 samples) and in propagation sites (870 samples) were surveyed and sampled. From the samples found to be infected with PPV by ELISA only 7 trees of ornamental *Prunus* species/cultivars showed symptoms affecting the leaf colour. The most conspicuous symptoms of whitish or light yellow rings of differing size, speckles, line patterns or light green vein banding, were observed on the leaves of *P. glandulosa* 'Sinensis' (Fig. 1). On *P. glandulosa* 'Alba Plena' the symptoms were pale and blurred (Fig. 2), and similarly light were the green discoloured areas along the midribs and the sparse blotches on the leaves of *P. japonica* (Fig. 4). Round blotches associated with the veins or long light green discoloured areas were present on *P. cerasifera* 'Pendula' (Fig. 5). *P. cerasifera* 'Pissardii' and *P. cerasifera* 'Woodii' leaves turned

dark-red or purple, mainly along the main and secondary veins, with a higher intensity on the lower surface. The lower leaves of *Prunus x blireana* exhibited similar symptoms along the main, secondary and tertiary veins. On dark-red leaved trees such as *P. cerasifera* 'Nigra' and *Prunus x davidopersica* 'Atropurpurea' symptoms were not visible (Sebestyén et al., 2008).

Fig. 1 PPV-infected *Prunus glandulosa* 'Sinensis' leaves

Fig. 2 PPV-infected *Prunus glandulosa* 'Alba Plena' leaves

Fig. 3 PPV-infected *Prunus glandulosa* leaves

Fig. 4 PPV-infected *Prunus japonica* leaf

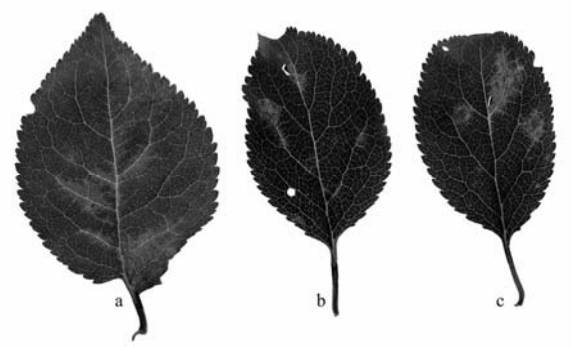

Fig. 5 PPV-infected *Prunus cerasifera* 'Pendula' a) leaf of one-year shoot, b, c) leaves of two-year twig

From 2005 this work has been extended to studying other viruses on other woody plants. Plants (species and varieties) showing virus symptoms among various thousands of woody plants in two botanical gardens and an arboretum were selected and submitted to indexing on woody and herbaceous indicators, as well as to testing with ELISA for the presence of the following 11 viruses occurring on woody plants: PPV, PDV, PNRSV, CLRV, ASPV, ASGV, ApMV, ACLSV, SLRV, TBRV and ArMV. Up to now, among the 28 suspicious species and varieties, PPV, PDV, PNRSV, CLRV and ASPV have been detected so far. In addition to the 9 species/cultivars formerly mentioned as PPV hosts 3 new species/cultivars: *P. glandulosa*, *P. tomentosa* (from Tibet) and *P. sogdiana* were found to be infected by PPV. The symptoms on the leaves of *P. glandulosa* were similar but weaker than those observed on the leaves of *P. glandulosa* 'Sinensis' (Fig. 3). The leave tissue along the secondary midribs of *P. sogdiana* turned to pale green (Fig. 6). *P. tomentosa* (from Tibet) showed distortion and epinasty on the first leaves; later chlorotic spots developed which became necrotic by mid-summer (Fig. 7).

Fig. 6 PPV-infected *Prunus sogdiana* leaf

Fig. 7 PPV-infected *Prunus tomentosa* (from Tibet) leaves

Seventeen species/varieties showing symptoms were found to be infected with PDV. The symptoms observed were the following: *Lonicera caucasica:* pale green spots and tissue clearing along the veins; *L. maackii:* pale green spots; *L. sachalinensis*: large, transparent, light green spots and tissue-clearing along the veins; *Prunus mume* 'Omoi-no-mama': prominent light green rings and tissue clearing along the veins, leave distortion (Fig. 8); *P. salicina* (from China): light green ringspots and tissue-clearing (Fig. 9); *P. spinosa* 'Plena': large, transparent, light green spots on the leaves; *P. spinosa* 'Purpurea' no symptoms on the dark red leaves, but heavy gumming appears on the tree trunk; *P. serrulata* 'Amanogawa', *P. serrulata* 'Ichiyo' (Fig. 10); *P. serrulata* 'Pink Perfection' (Fig. 11); *P. serrulata* 'Taihaku' (Fig. 12) and *P. serrulata* 'Yedo-sakura': oak leave patterns with yellow bands and sparse spots on the leaves; *P. subhirtella* 'Plena': yellow transient spot on the leaves; *P. tenella*: in early springtime transient pale green spots appeared, later and

in most cases it was symptomless - intensive gumming appeared on the twigs of several years old; *P. yedoensis:* prominent, pale green spots and tissue clearing along the midribs, light deformation; *P. yedoensis* 'Moerheimii': conspicuous tissue-clearing mainly along the midribs (Fig. 13); *Syringa yunnanensis*: transient, light green spots on the leaves (Fig. 14).

Fig. 8 PDV-infected *Prunus mume* 'Omoi-no-mama'leaves

Fig. 9 PDV-infected *Prunus salicina* (from China) leaf

Fig. 10 PDV-infected *Prunus serrulata* 'Ichiyo' leaves

Fig. 11 PDV-infected *Prunus serrulata* 'Pink Perfection' leaf

Fig. 12 PDV-infected *Prunus serrulata* 'Taihaku' leaves

Fig. 13 PDV-infected *Prunus yedoensis* 'Moerheimii' leaf

Fig. 14 PDV-infected *Syringa yunnanensis* leaf

Certain species/varieties infected with PDV were positive also for CLRV with ELISA, such as *P. spinosa* 'Purpurea', *P. yedoensis*, *P. yedoensis* 'Moerheimii', *P. subhirtella* 'Plena', *P. serrulata* 'Yedo-sakura' and to ASPV: *P. subhirtella* 'Plena' and *P. serrulata* 'Yedo-sakura'. PNRSV was detected in *P. cerasifera* 'Nigra' and 'Hollywood'. Necrotic flecks and shot-holes were observed on the leaves of infected trees. In the biological indexing the woody indicators and the herbaceous test-plants showed typical symptoms (Németh, 1986) to the viruses detected by ELISA.

The difference of symptoms provoked by PDV on herbaceous hosts suggest that various strains of this virus affect hosts, mainly yellow mottle disease described as a separate strain by Ramaswamy and Posnette on ornamental cherries in 1972.

Conclusions

Based on the six-year study natural PPV infection was found in 12 of the ornamental *Prunus* species/cultivars tested. Only 10 of the 12 naturally PPV-infected *Prunus* species/cultivars/botanical varieties were symptomatic. On infected light red-leaved cultivars symptoms were clearly visible on the lower surface of the leaves, an important consideration for phytosanitary inspectors. On dark red-leaved ornamental *Prunus* cultivars (such as *P. cerasifera* 'Nigra' and *P. x davidopersica* 'Atropurpurea') PPV symptoms could not be recognized even if the virus concentration was high. These latently infected dark red-leaved cultivars can play an important role in the wide distribution of PPV. Such trees in public areas are permanent virus sources. Symptomless infected trees in the vicinity of nuclear stocks and nurseries mean a high risk. Therefore, it is a must for the future to also apply the requirements of the certification scheme for the production of propagating materials to the natural PPV hosts newly identified in this study. Considering the high frequency of latent infections, the use of reliable laboratory methods is essential in regular screening.

During our examinations it was noted that PDV and PPV might cause similar symptoms on the leaves of several *Prunus*, *Lonicera* and *Syringa* species, therefore all the observations should be confirmed by laboratory testing.

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Health status of pome and stone fruit planting material imported to Serbia

Paunović, S., Jevremović, D.
Fruit Research Institute, Kralja Petra I 9, 32000 Čačak, Republic of Serbia. Email: spaunovic@yahoo.com

Abstract

We summarize results of the analysis of pome and stone fruit planting material to be imported to Serbia for the presence of quarantine and economically important viruses and phytoplasmas. The analysis was conducted 2004 – 2009 whereby, in compliance with the phytosanitary law regulations of the Republic of Serbia, officially inspected samples were subjected to the examination. During the period, a total of 325 samples were analyzed, i.e. 89 rootstock samples, 215 samples of different pome and stone fruit varieties, and 21 samples of stone fruit seed. The obtained results reveal that 5 samples were infected with viruses. *Apple chlorotic leafspot virus* was found in one sample of apple budwood, *Prune dwarf virus* was detected in *Prunus avium* L. seedlings, *Plum pox virus* was confirmed in 2 plum budwood samples, and *Apple mosaic virus* was also found in one sample of apple budwood.

Keywords: Pome fruits, stone fruits, planting material, seed, viruses, phytoplasmas.

Introduction

The exchange in fruit propagation material between Republic of Serbia and other countries, mostly European ones, is very dynamic. However, the exchange poses a risk of spreading economically important pathogens through infected propagation material (rootstocks, budwoods or entire plants). The establishment of the National Reference Laboratory, that will take over the responsibility for this type of sample analysis, is currently in progress. All samples of fruit planting material intended for import to Serbia are analyzed for the presence of viruses and phytoplasmas in the virology laboratory of Fruit Research Institute, Čačak. Phytosanitary service and certified laboratories act to prevent the import of infected material into the country. The import procedure includes regular inspection of planting material, both visual and in laboratory, insuring that data written in the phytosanitary certificate are correct. All tests are performed according to plant protection law regulations of the Republic of Serbia. The analysis includes examination for the presence of harmful pathogens, in compliance with Bylaw on the declaration of quarantine list of detrimental organisms (Official Gazette of RS 11/08), Bylaw on health inspections of plant consignments in traffic across the border (Official Gazette of RS, 42/08) and Regulation on sanitary inspection of growing plants and structures intended for the production of seed, replanation and planting material, and sanitary control of seed, replanation and planting material (Official Gazette of SCG 13/03; Official gazette of RS 39/06, 59/06, 115/06 and 119/07).

Material and methods

By the decision of Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, virology laboratory of Fruit Research Institute - Čačak has been officially authorized to analyze fruit propagation material imported to Serbia. Border phytosanitary inspectors are the official representatives, that perform sampling at border crossings, and forward samples to the laboratory for examination. During the six-year period (2004 – 2009), 325 samples of fruit propagation material were analyzed. A total of 89 rootstock samples (*Malus domestica, Pyrus communis, Cydonia oblonga, Prunus cerasifera, P. persica, P. armeniaca, P. avium* and *P. mahaleb*), 215 samples of apple, pear, plum, peach and nectarine, apricot, sweet and sour cherry varieties, and 21 samples of seed (*P. cerasifera, P. persica, P. armeniaca, P. avium, P. mahaleb* and *P. amygdalus*) were laboratory inspected (Tables 1, 2, 3 and 4).

Border inspection of samples need to be rapid, and employed methods sufficiently sensitive. ELISA test is used for routine detection of viruses, whereas PCR test is the most appropriate technique for the detection of phytoplasmas in imported material. The results are reliable and available within 2 – 3 days. Additionally, for all imported stone fruits, one-year supervision is prescribed at the production site.

Depending on fruit species, type of sample, vegetation season, category of planting material and country of origin, laboratory tests were performed for respective viruses (*Plum pox virus* -PPV, *Prune dwarf virus* -PDV, *Prunus necrotic ringspot virus* -PNRSV, *Cherry leaf roll virus* -CLRV, *Arabis mosaic virus* -ArMV, *Strawberry latent ringspot virus* -SLRSV, *Raspberry ringspot virus* -RpRSV, *Tobacco black ring virus* -TBRV, *Tomato ringspot virus* -ToRSV, *Apple mosaic virus* -ApMV, *Apple stem pitting virus* -ASPV, *Apple stem grooving virus* -ASGV and *Apple chlorotic leafspot virus* -ACLSV) (Tables 1, 2, 3 and 4). The viruses were detected by ELISA test (Clark and Adams, 1977) with reagents supplied by BIOREBA AG, Switzerland and LOEWE, Germany. The tests were performed immediately upon the arrival of samples. All the tests were done according to the manufacturers' recommendations. OD values were recorded

on the Multiskan MCC340 ELISA plate reader. On request of a phytosanitary inspector, 4 apple and 5 pear samples were tested for the presence of 'Candidatus *Phytoplasma mali*' and 'Candidatus *Phytoplasma pyri*' respectively. The analyses were done by nested-PCR test. Specific primers for phytoplasmas belonging to the Apple proliferation group were also included in the analyses (Schneider et al., 1995; Lee et al., 1995).

Results

The results of the analysis reveal that plant viruses were detected in 5 samples of tested planting material intended to be imported to Serbia (1.54% of the total number of analyzed samples). One rootstock sample out of 89 tested was found positive in ELISA test. Seedlings of *Prunus avium* L. originating from Hungary were found to be infected with *Prune dwarf virus* (Table 1). *Prune dwarf virus*, spread worldwide, is economically important pathogen of stone fruits. It is a pollen- and seed borne virus, and transmission through infected seed in wild cherry is up to 50% (Desvignes, 1999). As proposed by the EPPO, all categories of plum, peach, apricot and almond planting material are required to be free of this virus (OEPP/EPPO, 2001).

Tab. 1 Number of samples tested and found positive of pome- and stone rootstocks intended to be imported to Serbia, and viruses and phythoplasmas whose presence was tested

Species affiliation of tested rootstock	**Viruses and phytoplasmas whose presence were tested and number of tested samples**										**Total No of tests**	**No of positive samples**
	PPV	**PDV**	**PNRSV**	**ToRSV**	**ApMV**	**ASPV**	**ASGV**	**ACLSV**	**AP**	**PD**		
Malus domestica				4	19	27	27	27	2		34	0
Pyrus communis						4	4	4		4	7	0
Cydonia oblonga				2		2	3	4			9	0
Prunus cerasifera	14	5	6					3			14	0
Prunus persica	2	1									2	0
Prunus armeniaca	1										1	0
Prunus avium	8	5	6		1			2			9	1 PDV
Prunus mahaleb	9	11	12					6			13	0

Out of 46 analyzed samples of different *Prunus domestica* L. varieties, 2 plum samples (budwood) originating from Hungary were found to be infected with *Plum pox virus* (Table 2). *Plum pox virus* is the most detrimental virus of stone fruits causing great losses in sensitive plum, peach and apricot varieties. It is included in the EPPO A2 list (http://www.eppo.org/) and A2 quarantine list of Serbia (Official Gazette of RS 11/08). As stated above for the *Prune dwarf virus*, fruit planting material is required to be free of this virus (EPPO, 2001).

Tab. 2 Number of samples of stone fruit cultivars intended to be imported to Serbia, which were tested and found positive, and viruses whose presence was tested

Species affiliation of cultivars	**Viruses whose presence was tested and number of tested samples**									**Total No of tests**	**No. of positive samples**
	PPV	**PDV**	**PNRSV**	**CLRV**	**ArMV**	**SLRSV**	**RpRSV**	**TBRV**	**ACLSV**		
Prunus domestica	46	6	15						4	46	2 PPV
Prunus persica	34	2	1			1				34	0
Prunus armeniaca	20	8	8							20	0
Prunus avium	12	14	14	2	1	1	1	1		17	0
Prunus cerasus	13	3	3	1						14	0

Sixty six samples of apple (*Malus domestica* L.) varieties were analyzed for the presence of viruses. *Apple mosaic virus* was found in one sample (budwood) imported from Belgium (Table 3). The imported material was characterized as certified. *Apple mosaic virus* causes mosaic symptoms on apple leaves, and yield losses in sensitive cultivars may be up

to 30% (Šutic et al., 1999). Out of 9 samples tested for the presence of phytoplasmas none was found to be infected (Table 1 and 3).

One apple sample (budwood) originating from Italy was infected with *Apple chlorotic leafspot virus* (Table 3). Infection with ACLSV is symptomless in most of commercial apple varieties, occurring frequently with the other apple latent viruses, i.e. *Apple stem pitting virus* and *Apple stem grooving virus*. It is present worldwide and listed in the EPPO certification schemes (EPPO, 2001).

Tab. 3 Number of samples intended to be imported to Serbia which were tested and found positive of pome fruit cultivars (according to species affiliation), and viruses and phythoplasmas whose presence was tested

	Viruses and phytoplasmas whose presence was tested, and number of tested samples							Total No. of tested samples	No. of positive samples
Species	**ToRSV**	**ApMV**	**ASPV**	**ASGV**	**ACLSV**	**AP**	**PD**		
Malus domestica	10	35	40	40	51	2		66	1 ApMV, 1 ACLSV
Pyrus communis			7	6	13		1	18	0

Presence of viruses was not detected in any of the tested samples of stone fruit seeds (Table 4).

Tab. 4 Number of samples tested and found positive of seeds intended to be imported to Serbia, and viruses whose presence was tested

	Viruses whose presence was Tested, and number of tested samples			Total No. of tested samples	No. of positive samples
Species of the tested seed	**PDV**	**PNRSV**	**ToRSV**		
Prunus cerasifera	3	4		4	0
Prunus persica	5	6	2	8	0
Prunus armeniaca		1		1	0
Prunus avium	1	2		2	0
Prunus mahaleb	3	4		4	0
Prunus amygdalus	1	2		2	0

Discussion

According to the laboratory tests, the great majority of the material imported from European countries (98.46%) was free of quarantine and economically important viruses and phytoplasmas. Similarly, performed laboratory tests and results clearly suggest that Phytosanitary service of the Republic of Serbia and certified laboratory are fully capable of identifying whether certified material fulfills strict requirements set by EPPO and National law regulations.

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Investigation on the phytosanitary status of the main stone fruit nurseries and mother plots in Albania

Musa, A.[1], Merkuri, J.[2], Milano, R.[1], Djelouah, K.[1]
[1] Istituto Agronomico Mediterraneo di Bari (IAMB), Italy
[2] Plant Protection Institute, Durres, Albania

Abstract

To assess the virus and viroid infections of the most important stone fruits in Albania, surveys were carried out in nurseries, mother plots and commercial orchards in the main fruit tree-growing areas. The presence of viruses and viroids was assessed by visual inspections and laboratory tests.

During field surveys, more than 5,000 trees were individually inspected for symptoms expression. A total of 749 trees were tested, and shown to be highly infected (27%) by one or more viruses at the same time; in particular, Sharka infection was detected in all the selected areas and in plants of different origin (nurseries: 29%, mother plants: 13%, and commercial orchards: 29%). Infections by *Prunus* necrotic leaf spot virus (PNRSV) and *Apple chlorotic leaf spot virus (*ACLSV) were frequent in peach and plum, while *Prunus* dwarf virus *(*PDV) was more frequent in cherry. Regarding viroids, 740 samples were tested for *Peach latent mosaic viroid* (PLMVd); as for viruses, the infection rate was quite high (23%), particularly on peach (60% of tested samples). This study highlights the quite alarming existing situation, especially for the presence of PPV infection in nurseries. Urgent measures should be taken to avoid a serious crisis and deterioration of the fruit tree industry in Albania.

Keywords: Albania, stone fruits, viruses, viroids, detection, nursery, mother block.

Introduction

In Albania, the stone fruit industry represents a major agricultural activity for both the domestic market and fruit export. In 2007, stone fruit production reached 37,000 tons covering an area of 5,200 Ha (FAOSTAT, 2007). European plum (*Prunus domestica*) and sweet cherry (*P. avium*) are the most planted species, primarily grown on hillsides (Pograde, Tropjojë, Berat and Tirana, Elbasan). Peach, apricot and almond trees are less numerous and confined to the costal areas (Durrës, Fier, Vlorë) (Fig. 1).

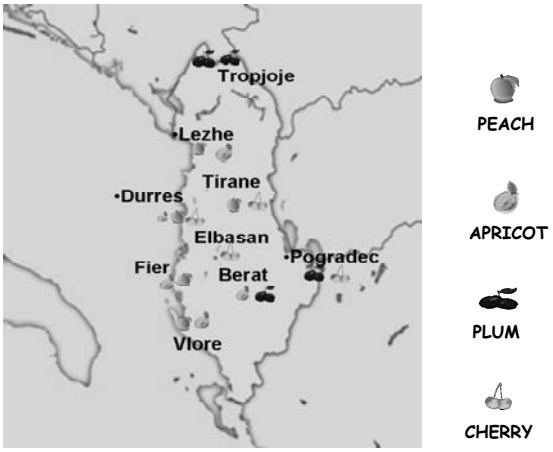

Fig. 1 Location of the monitored stone fruits growing areas, specifying the main cultivated species.

Over the last few years, different surveys have been conducted on stone fruits in Albania in order to assess their sanitary status. The first reports on the presence of virus diseases were only based on the visual observation of symptoms in the open field (Papingji, 1963; 1965). More complete studies including laboratory tests were later performed to determine

the incidence and distribution of viruses, virus-like agents and viroids affecting the stone fruit species (Myrta et al., 1994; 2003; Torres et al., 2004).

Along with the stone fruit industry, Albanian nurseries have rapidly grown in the last two decades; at present, about 35 nurseries are involved in the production of stone fruit plants. Given the poor knowledge on stone fruit virus and virus-like diseases in Albania, monitoring was carried out to evaluate the presence of the main viruses (PPV, PNRSV, ACLSV, PDV) and viroid (PLMVd) in stone fruit nurseries, mother blocks and surrounding orchards.

Materials and methods

Field surveys: Field inspections and sample collection were carried out from early spring to the arrival of high temperatures at the beginning of July, in nine different areas representing some of the main stone fruit-growing areas in Albania: Durrës, Berat, Kavajë, Korçë, Vlorë, Pogradec, Shkodër, Fier and Tirana. Samples were collected from five nurseries including surrounding commercial orchards and one mother block. Nurseries were selected on the basis of their economic importance, the cultivated species, geographical distribution and pedoclimatic conditions. Four out of five inspected nurseries were using their own propagating material from commercial orchards and neighbouring mother plants; one nursery used propagating material imported from Greece. A small number of samples were collected from three commercial orchards which were using propagating material originating from selected nurseries.

For virus diseases, a total of 749 samples were randomly collected: 369 from nurseries, 284 from mother blocks, 96 (including 15 rootstocks) from a commercial orchard close to nurseries. Different species were represented as follows: 273 peaches (P. persica), 262 plums (P. domestica), 107 apricots (P. armeniaca), 107 sweet cherries (P. avium), sour cherries (P. cerasus) and other Prunus species (Tab. 1). Collected samples consisted in leaves, small fruits and flowers. The leaf samples (4–8 per tree) were collected from the four quadrants of the tree.

Tab. 1 LIst of the collected samples**.**

	Peach	**Plum**	**Cherry**	**Apricot**	**Total**
Nurseries	105	152	59	53	369
Mother blocks	100	92	42	50	284
Commercial orchards	68	18	6	4	96
Total	273	262	107	107	749

Serological assay: The double antibody sandwich-ELISA (DAS-ELISA) (Clark and Adams, 1977) was applied to test PPV, PNRSV, PDV; whereas DAS-simultaneous ELISA (Flegg and Clark, 1979) was performed to detect ACLSV, using commercial kits purchased from Loewe Company, Germany. Extracts from young leaves of the tested plants were analysed as reported in the protocol provided by the manufacturer.

Molecular assay: For PLMVd testing, young petioles from different parts of the canopy were taken in spring and summer. Tissue-printing hybridization was carried out to detect PLMVd on 740 samples. From each sample, petioles of three leaves from different parts of the canopy were printed onto a nylon membrane (Hybond N+, AP Biotech). The imprinted membranes were stored at 4°C, later covered with a plastic envelope and exposed to UV light for 2-3 min in order to fix the nucleic acid.

As reported by Pallás et al. (2003), hybridization was carried out using PLMVd-specific riboprobes labelled with dig-11 dUTP, at a concentration of 100 ng/ml according to the protocol provided by Roche Company. The specific riboprobes used were RF43 5'd (CTG GAT CAC ACC CCC CTC GGA ACC AAC CGC T) 3'antisense and RF44 5'd (TGT GAT CCA GGT ACC GCC GTA GAA ACT) 3' sense, amplifying a 337 bp fragment as described by Ambrós et al. (1998).

Results and discussion

During field surveys, more than 5,000 trees were individually inspected for symptom expression. Symptoms induced by specific viruses such as PPV, PNRSV, PDV and ACLSV were difficult to observe, due to the poor growing conditions of the trees, the great variability of varietal responses and to the presence of mixed infections in nurseries, mother blocks and surrounding orchards. Symptoms by Sharka were observed in some surveyed plants of plum, apricot and nectarine. Their severity varied according to the plant species and cultivars.

Several symptoms associated with viral diseases were observed on the monitored plants such as chlorotic patterns and yellowish ring spots in plum; chlorosis of secondary veins in peach; while apricot leaves displayed chlorotic areas and blade deformation, probably associated to PPV infections (Fig. 2).

Fig. 2 Main symptoms observed in the monitored plants: A) Chlorotic ring on plum; B) Chlorosis, yellowing of secondary veins on peach; C) Chlorotic areas on apricot.

Virus detection: Two hundred out of 749 samples analysed by laboratory assays (113 peaches, 60 plums, 15 cherries and 12 apricots) reacted positively to at least one of the viruses tested (Tab. 2). The total infection rate came up to 27%, while the relative species infection rate was the highest in peach (41%), followed by plum (23%), cherry (14%), and apricot (11%). Among the four viruses, PPV infection was the most frequent (20%), followed by PNRSV (12%), ACLSV (4%) and PDV (1%). Taking into account the sample origin, infection in nurseries reached 29%. The most worrying situation was represented by the high percentage of PPV infection in 4 out of 9 nurseries examined, where Sharka was detected in 47% of samples (Fig. 4). As for each species, the highest infection rate in nurseries, was found in peach (67%), followed by plum (32%), cherry (10%) and apricot (17%). Among the samples collected in mother blocks, the total average infection rate was estimated at 13 %. The highest infection rate was found in peach (26%), followed by cherry (12%) and plum (8%), while no apricot mother plant proved to be infected. Among the three viruses present in mother plants, PPV infection was the most frequent (9%), followed by PNRSV (5%) and PDV (1%), considering both single and mixed infection. No infections by ACLSV were found.

Tab. 2 Infected trees detected by ELISA tests

			Single infection				Mixed infection			
Species	**Samples N°**	**Infected samples**	**PPV**	**PNRSV**	**PDV**	**ACLSV**	**PPV PNRSV**	**PPV ACLSV**	**PPV PNRSV PDV**	**PPV PNRSV ACLSV**
Peach	273	113	51	12	0	4	22	2	0	22
Plum	262	60	33	19	0	0	6	0	1	1
Cherry	107	15	0	7	6	2	0	0	0	0
Apricot	107	12	10	1	0	0	1	0	0	0
Total	749	200	94	39	6	6	29	2	1	23

Regarding the surrounding commercial orchards, over a total of 96 samples, 29% were found positive to at least one of the viruses with different levels according to the species. PNRSV and ACLSV infections were frequent in peach and plum, while PDV was more frequent in cherry and PNRSV in plum. Among the 3 viruses found in orchards, PPV infection was the most frequent, with an incidence of 20%, followed by PNRSV (14%) and ACLSV (2%),

Among all the surveyed areas, Vlora had the lowest infection rate (4.9%) and Korça the highest (90%) followed by Tirana (76%), Pogradeci (62%), Durrës (26%), Kavaj (25%) and Shkodër (6.5%).

Viroid detection: PLMVd was detected in 160 peaches (60% Infection rate), 4 apricots (3.7%) and 2 plums (1%). No cherry was found infected by viroids.

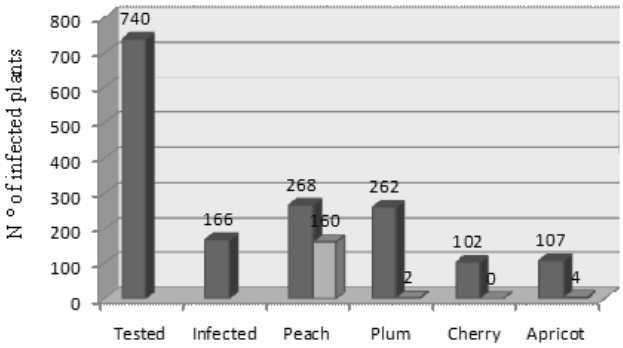

Fig. 3 Relative incidence of PLMVd

Regarding specific cultivars, the highest infection rate was found in the imported peach cvs. Adriana, Early Nectarine, and Nectarine with rates of 95%, 97% and 70% respectively, confirming that peach is the most affected species (Flores et al., 2003) and the high infection rate previously reported in Albania (56%) (Torres et al., 2004).

PLMVd-incidence in peach and apricot was higher than the one found on the occasion of previous monitoring carried out in the Mediterranean area (Torres et al., 2004).

Conclusions

The present study represents the first large-scale survey specifically carried out in stone fruit nurseries in Albania. This survey confirmed the previous studies on the presence of the main fruit tree infectious agents (ACLSV, PNRSV, PDV, HSVd, PLMVd) and their wide distribution, but their presence in nurseries and mother blocks underlines the importance of setting up a well-established and fully implemented certification programme.

Indeed, this survey revealed quite high virus and viroid infection rates, especially in mother blocks that are the source of propagating material and that can contribute to the rapid and wide dissemination of pathogens. Selected plants were highly infected by PPV, particularly peach trees: the virus was detected in all chosen areas and plants of different origin.

The situation is alarming, especially because heavily infected nurseries produce and sell PPV-infected propagating material throughout Albania. This might favour the rapid and wide dissemination of viruses despite both the Albanian law (no. 9362 of 24/03/05) issued under the umbrella of the Plant Protection Service set up to prevent pest introduction and distribution over the national territory and the fact that healthy propagating material is subject to phytosanitary surveillance. Moreover, the general phytosanitary status of propagating material is quite poor, due to the high incidence of other diseases induced by viruses, viroids and other pathogens and pests.

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An investigation on Rose Mosaic Disease of Rose in Hatay-Turkey

Sertkaya, G.
University of Mustafa Kemal, Faculty of Agriculture, Department of Plant Protection, 31034 Antakya-Hatay, Turkey

Abstract

Field inspections were carried out to investigate *Apple mosaic virus* (ApMV), *Arabis mosaic virus* (ArMV) and *Prunus necrotic ringspot virus* (PNRSV) which are associated with rose mosaic disease (RMD) during the years of 2008 and 2009. Characteristic symptoms, including chlorotic line patterns (zigzag pattern), vein-banding and mottles in leaves were observed during spring. Symptoms were also evident during summer on leaves produced until early summer. Flower abnormalities as phyllody were also exhibited during autumn. Distortion and reduction in flower size and early leaf drop have been observed on symptomatic plants in winter. Leaf samples taken from 15 rose plants from 'Rosa hybrida L.' neighboring stone fruit orchards were tested by mechanical inoculation to herbaceous plants and enzyme-linked immunosorbent assay (DAS-ELISA) for the presence of ApMV, ArMV and PNRSV, which are the viruses related to RMD. *Catharanthus roseus* L. G. Don, *Chenopodium amaranthicolor* Coste and Reyn., *C. quinoa* Wild, *Cucumis sativus* L., *Gomphrena globosa* L., *Lagenaria siceraria* (Mol.) Standl, *Nicotiana benthamiana* L., *N. clevelandii* L., *Nicotiana glutinosa* L., *Phaseolus vulgaris* L., *Vigna unguiculata* L. test plants were incubated after mechanical inoculation for symptom appearance at 25°C±2 and 16:8 h photoperiod (day:night) conditions in an insect-proof room. Symptoms including chlorotic local lesions, systemic necrosis, stunting and yellow mottling began to appear on *C. quinoa* and *C. sativus* in 2-3 weeks after sap inoculation. Serological tests of test plants are in progress. The rose plants showing symptoms in home gardens were re-tested for the viruses in spring by ELISA. According to the results of the Bioassay by sap inoculation and ELISA on symptomatic rose plants, the causal agent of RMD is PNRSV. The viruses affecting rose plants spread through cuttings from a diseased plant because new plants are generally produced by the rooting of cuttings in home gardens in Hatay. Further detailed investigations are necessary to find out the causal agent/s of RMD in rose in the region, because infected rose plants can be an important factor in the epidemiology of virus diseases caused by these agents in rose plantations.

Keywords: ApMV, Bioassay, ELISA, Oil Rose, PNRSV, virus

Introduction

Rose mosaic disease (RMD) is the one of most important and widespread virus diseases of rose plants. RMD is caused by infection with any of a number of different viruses. RMD is associated especially with *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), *Arabis mosaic virus* (ArMV) and *Strawberry latent ringspot virus* (SLRV). The most important of these viruses in the United States and United Kingdom is PNRSV, a common disease of stone fruit trees (Thomas; 1981; 1982; 1984; Horst, 1983; Manners, 1985). PNRSV), a member of the genus *Ilarvirus* in the family *Bromoviridae*, occurs worldwide and is a serious pathogen of many plant species, including rose, *Prunus* spp.(Barbara et al., 1978; Barbara,1980; Thomas, 1980; Cambra et al., 1982). The new virus,related most closely to *blackberry chlorotic ringspot virus* was reported to be isolated from rose and is considered a strain of that virus (Tzanetakis et al., 2006).

There has been much opinion and research conducted on the means of transmission of RMD in roses. It was suggested that the Rose mosaic was probably transferred to roses originally from one of the stone fruits, by graftage (Cochran, 1984). It then spread from one rose cultivar to another through infected rootstocks (Manners, 1985). PNRSV and ApMV transmission by seed, pollen, on cutting implements and by root grafting from infected plants to healthy plants has been reported and the results showed that root grafting is involved in the natural spread of the virus in roses (Golino et al., 2005). Rose mosaic viruses cause symptoms on leaves that include ringspots, line patterns (zigzag pattern), mosaics, distortion and puckering. Serological procedures have been used more than other methods for the detection of PNRSV (Mink and Aichele, 1984).

RMD has been shown to cause flower distortion, reduced flower production and flower size, stem caliper at the graft union and reduction in vigor, early autumn leaf drop, lower bush survival rates, increased susceptibility to cold injury and more difficult establishment after transplanting (Cochran, 1972; 1982; 1984; Secor et al., 1977; Thomas, 1982; 1984). The symptoms are highly variable among rose cultivars and are strongly influenced by weather and growing conditions. Infected plants may appear to be quite healthy for much of the year, and any symptoms which do appear may be attributed to other causes, such as spray burn, nutrient deficiencies, high temperature, or poor horticultural

practices. It has been suggested that the "deterioration" which often occurs in rose cultivars several years after their introduction may be a result of virus infection (Allen, 1984).

Material and methods

Leaf samples taken from 15 *Rosa hybrida* plants neighboring stone fruit orchards and showing symptoms associated with virus diseases were tested by Bioassay-sap inoculations on herbaceous plants and enzyme-linked immunosorbent assay (DAS-ELISA) for the presence of ArMV, ApMVand PNRSV in both autumn and spring. For attempted sap inoculations of the viruses to herbaceous test plants, young leaves were homogenized in 0.1 M phosphate buffer (pH 7.2) in a pestle and mortar, and the sap extracts inoculated onto Celite-dusted leaves of herbaceous virus indicator plants: *Catharanthus roseus* L. G. Don, *Chenopodium amaranthicolor* Coste and Reyn., *C. quinoa* Wild, *Cucumis sativus* L., *Gomphrena globosa* L., *Lagenaria siceraria* (Mol.) Standl, *Nicotiana benthamiana* L., *N. clevelandii* L., *Nicotiana glutinosa* L., *Phaseolus vulgaris* L., *Vigna unguiculata* L.. Test plants were incubated after mechanical inoculation for symptom appearance at 25°C±2 and 16:8 h photoperiod (day:night) conditions in an insect-proof room. Four plants from each of the herbaceous indicator species were mechanically inoculated with the sap of a rose sample. All rose samples and inoculated test plants were tested for the presence of the viruses by ELISA as described by Clark and Adams (1977). Antiserum kits from Bioreba AG (Switzerland) were used in standard DAS-ELISA. Four indicator plants of each species used for testing of each samples in Bioassays were pooled together as one samples for ELISA. Four asymptomatic rose seedlings taken from a nursery were also inspected visually as control plants and tested serologically.

Eight to ten single-node cuttings (approximately 15-20 cm long) of symptomatic rose plants were excised. Basal ends of the cuttings were dipped into 0.5% Indole butyric acid (IBA),rooted in pots containing a peat:perlite (1:1) mixture and kept in insect-proof growing room at 25°C±2 and 16:8 h photoperiod (day:night) in the autumn of 2008 for symptom observation. Young shoots grown from the axillary buds of each cutting were tested by ELISA when they were approximately 3 to 5 cm long.

Ten oil rose (*Rosa damascena*) seedlings obtained from a nursery in Isparta province (where the main oil rose production area in the Lakes region of Turkey is) and indexed by grafting and ELISA. Five healthy oil rose plants were graft-inoculated with buds from the PNRSV-rose source in autmn of 2008. The inoculum consisted of three buds per each seedling. Three plants negative for virus by ELISA was used as the healthy control.

Results and discussion

Although, Rose mosaic disease (RMD) is caused by a complex of several viruses in rose plants, PNRSV is the most common agent of RMD (Thomas; 1982; 1984; Horst, 1983). Initially the symptoms were thought to be caused by PNRSV. For this reason, the presence of PNRSV and ArMV were mainly detected in the samples taken from symptomatic rose plants by Bioassay sap inoculations and double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA).

Although symptoms often are evident in spring and early summer but may not be on leaves produced in summer, chlorotic-zigzag or oak leaf patterns, leaf distortion and puckering were noted on rose plants in September and all kinds of symptoms were very abundant during the autumn season of 2008 (Figure 1). However, ringspot patterns and vein clearing symptoms associated with rose mosaic were not observed during field inspections in both years. All of the PNRSV-infected (15) samples taken from young leaves of symptomatic shoots reacted positively for PNRSV by DAS-ELISA and were used in Bioassay sap inoculations. Basal leaves were more symptomatic, but apical leaves gave a more definitive result by ELISA. RMD is known to cause increasing susceptibility to cold injury (Secor et al., 1977), and early autumn leaf drop (Thomas, 1982). Also in the present work, reducing number of leaves and early leaf drop were observed in symptomatic plants compared with asymptomatic ones in the gardens in autmn of 2008. An influence of variety and environmental conditions is suggested. *R. damascena* seedlings obtained from a nursery in Isparta showed no characteristic symptoms related to virus diseases and seemed to be healthy. However, one out of ten *R. damascena* plants was also found to be infected with PNRSV.

Inoculation with extracts from 15 symptomatic *R hybrida* plants and one assymptomatic *R. damascena* seedling mainly produced systemic mosaic, stunting, vein banding on *C. sativus*, chlorotic local lesions on *V. unguiculata*, chlorosis with reducing of the leaves on *C. roseus*, and mosaics and chlorosis on *R. damascena* (Figure 1 and Table 1). These symptoms were generally similar to those that were described previously for these viruses (Boulila and Marrakchi, 2001; Salem et al., 2004; Rakhshandehroo et al., 2006). Only one PNRSV-infected rose sample exhibited symptoms of chlorotic local lesions and top necrosis on three *P. vulgaris* test plants. Except for this sample, no symptoms were observed in *Phaseolus vulgaris* inoculated with extracts from the other 14 rose samples. Single or mixed infections in

combination with ApMV and/or ArMV were not detected by ELISA in this study. According to the results of Rakhshandehroo et al. (2006), mixed infections of PNRSV and ArMV were found in all rose samples tested by sap inoculations and ELISA in Iran. However, PNRSV was reported to be mostly distributed through the red rose varieties (*Rosa × damascena, R. chinensis, R. canina,* and *R. multiflora*) and ArMV was within the white varieties (*R. canina, R. indica,* and *R. multiflora*) by serological tests. A survey for viruses in rose propagated in Europe resulted in the detection of only *Prunus necrotic ringspot virus* (PNRSV) among seven viruses screened by Moury et al. (2001).

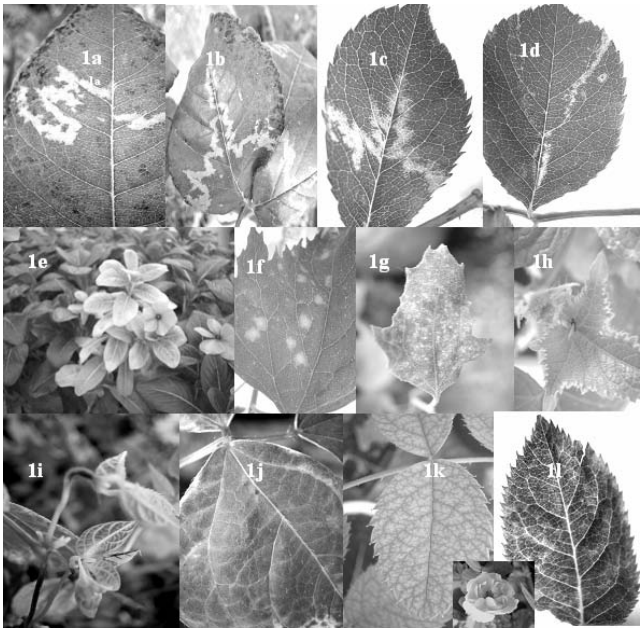

Fig. 1 Symptoms of PNRSV on naturally infected rose and inoculated test plants: 1a-d: Chlorotic zigzag patterns appeared on naturally infected rose leaves, 1e: Severe chlorosis and leaf reducing on *Catharanthus roseus,* 1f-g: Chlorotic spots on *Chenopodium amaranticolor* and *C. quinoa,* 1h: Chlorosis and mosaic symptoms on *Cucumis sativus,* 1i-j: Chlorosis and necrosis on *Phaseolus vulgaris* and *Vigna unguiculata,* 1k-l: Chlorosis and mosaics on artificially infected leaves of *Rosa damascena* (Oil rose)

Using the *P. persica* clone GF 305 it was possible to differentiate rose PNRSV isolates, and the *P. avium* clone F12/1 was also reported to be a new host-plant for differentiating pathogenicity of PNRSV rose isolates ((Moury et al., 2001; Paduch-Cichal et al., 2007). During inspections on new plants in 2009, except leaf deformation and mosaics, no symptom has been observed on rose plants obtained by rooting of cuttings. Further studies are also necessary to investigate the status of other virus diseases of Rose in Turkey. Indexing of PNRSV-infected source plants by using woody indicators such as the *P. persica* clone GF 305 and almond (*P. dulcis*) seedlings are in progress.

Tab. 1 Symptomatology of test plants inoculated with PNRSV mechanically or by tissue grafting

Indicator Plants	Symptoms	ELISA test
Family: Amranthaceae		
Gomphrena globosa L.	0	-
Family: Apocynaceae		
Catharanthus roseus L. G. Don.	Cl, L.R.	-
Family:Chenopodiaceae		
Chenopodium amaranticolor Coste and Reyn.	C.L.L.	+
Chenopodium quinoa Wild	C.L.L.	+
Family:Cucurbitaceae		
Cucumis sativus L. cv. Cemre F1	M, Cl and/or Vb	+
Lagenaria siceraria (Mol.) Standl	0	-
Family: Fabacceae		
Phaseolus vulgaris L.	(C.L.L. and TN)[b]	-
Vigna unguiculata L.	C.L.L	-
Family:Rosacae[a]		
Rosa damascena (Oil rose)	M and Cl	-
Family: Solanmceae		
Nicotiana benthamiana L.	0	-
Nicotiana clevelandii L.	0	-
Nicotiana glutinosa L.	0	-

(+ Positive, - Negative) in ELISA test. C.L.L.=Chlorotic Local Lesion, Cl.=Chlorosis, L.R.=Leaf reducing, M=Mosaic, N.L.L.=Necrotic LL, TN.=Top necrosis, Vb=Vein banding, 0 = no symptoms. [a] : inoculation by grafting, [b]: only for one rose sample infected with PNRSV.

Because the only proven means of transmission of RMD in roses is through vegetative propagation of infected buds, scion or root stocks, the use of clean and virus-tested production material is essential to improve productivity in gardens. The lack of certified virus free plants is one of the main problems in commercial rose production, which includesoil rose, *R. damascena* cultivation in Turkey.

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Agro-ecological incidence and severity of *Pepper veinal mottle virus*, genus Potyvirus, family Potyviridae, on cultivated pepper (*Capsicum annuum* L.) in Nigeria.

Fajinmi, A.A.
Dept. of Crop Protection, COLPLANT. P.M.B.2240 University of Agriculture Abeokuta, Alabata, Ogun State, Nigeria.
Email: ayofaji@yahoo.com

Abstract

A survey for the agro-ecological distribution of *Pepper veinal mottle virus* (PVMV) (Family Potyviridae, genus Potyvirus) its incidence and severity on cultivated pepper was conducted between 2003 to 2005 in six agro-ecological zones in Nigeria, comprising the major pepper producing areas of the humid forest, derived savanna, southern Guinea savanna, mid-altitude, northern Guinea savanna and Sudan savanna The virus was isolated and its physical properties determined.

PVMV was confirmed to be present in cultivated pepper fields in the six agro-ecological zones surveyed but with significant difference in disease incidence and severity within the agro-ecological zones. Out of the three thousand suspected viral infected pepper leaves sample collected from the fields in the six agro-ecological zones, 88% were confirmed to be PVMV positive through the serological test while 12% were found to be negative.

The Electron micrograph photograph showed antiserum decorated PVMV particles, having flexuous filamentous particles of 750nm in length and 10 nm wide. The thermal inactivation point of the virus was 70^{O}C and still infective up to the 12th day, while the dilution end point in which PVMV extract from *Capsicum annuum* L. was still infective was 10^{-4}.

The incidences of PVMV diseases were observed to be high in the derived savanna and the humid forest compared with the other agro-ecological zones. The percentage PVMV disease incidence ranged between 39.14% with 34.48% severity in the Sudan savanna to 50.12% incidence and 43.85% severity in the derived savanna zone.

The high incidence and severity of PVMV in these two agro-ecological zones that are characterized by thick vegetation and warm humid climate, with the presence of many secondary host plants for the virus. suggest that ecological characteristics, climate and vegetation in the different ecological zones appeared to play a major role in determining the incidence and severity of PVMV infection on pepper in the fields.

Keywords: *Pepper veinal mottle virus*, Incidence, Severity, Pepper, Agro-ecological, Zones.

Introduction

Pepper is cultivated principally in southwestern and northern Nigeria between latitude 10^{0}N and 12^{0}3'N in the northern guinea savannah and Sudan ecological zones (Erinle, 1988). However, there is a sizeable production of pepper in the rain forest and derived savannah of southwestern Nigeria (Opoku-Asiama et al., 1987).

Pepper veinal mottle virus (PVMV) was first recognized as a distinct member of a group of viruses which was originally designated the *Potato virus Y* group but was later renamed the *Potyvirus group* (Harrison et al., 1971). PVMV occurs mainly in Africa although; it affects *Capsicum annuum* L. crops in Afghanistan (Lal and Singh, 1988) and India (Nagaraju and Reddy, 1980). The virus probably also occurs in *Capsicum* spp. in Sierra Leone and Zaire, (Huguenot et al., 1996). PVMV has been reported in several West African countries, and in some parts of Nigeria (Alegbejo and Uvah 1987; Fajinmi 2006).

There was a report that a strain of PVMV occurs naturally in *Telfairea occidentalis* (Cucurbitaceae) in Nigeria (Atiri, 1986). Strains of the virus are also experimentally transmissible to at least 35 species of the Solanaceae and to nine species of five other families (Aizoaceae, Amaranthaceae, Apocynaceae, Chenopodiaceae and Rutaceae) (Ladipo and Roberts, 1977, Brunt et al., 1978; Prasada Rao et al., 1979; Igwegbe and Waterworth, 1982).

Symptoms expressed by the leaves of PVMV-infected plants include chlorosis of the veins, followed by systemic interveinal chlorosis, mottle, and small distortion of leaves and at times leaf abscission and fruit distortion occur (Brunt et al., 1978).

There have been reports of one hundred percent losses of marketable pepper fruit due to infection with pepper viruses causing whole field to be abandoned prior to harvest and in some areas making cultivation of pepper to be uneconomical in some parts of Nigeria (Alegbejo and Uvah 1987).

Therefore, this study was targeted at studying the incidence, severity and distribution of *Pepper veinal mottle virus*, genus Potyvirus family Potyviridae in cultivated pepper in the six agro-ecological zones of Nigeria.

Materials and methods

Survey for *Pepper veinal mottle virus*, genus Potyvirus, virus disease incidence and severity was conducted during the 2003, 2004 and 2005 planting seasons in six agro-ecological zones of Nigeria (Humid forest, Derived Savanna, Southern Guinea Savanna, Mid-Altitude, Northern Guinea Savanna and Sudan Savanna). Thirty states within the six-agro ecological zones were surveyed to cover areas where pepper plants were cultivated.

The states were Ogun, Edo, Ondo, Delta, Imo, Abia, Anambra, Cross rivers, Rivers, Akwa Ibom, Bayelsa, Oyo, Osun, Enugu, Benue, Taraba, Kwara, Nassarawa, Plateau, Niger, Gombe, Sokoto, Kaduna, Kebbi, Kano, Borno, Adamawa, Yobe, Bauchi and Ebonyi (Fig 1.)

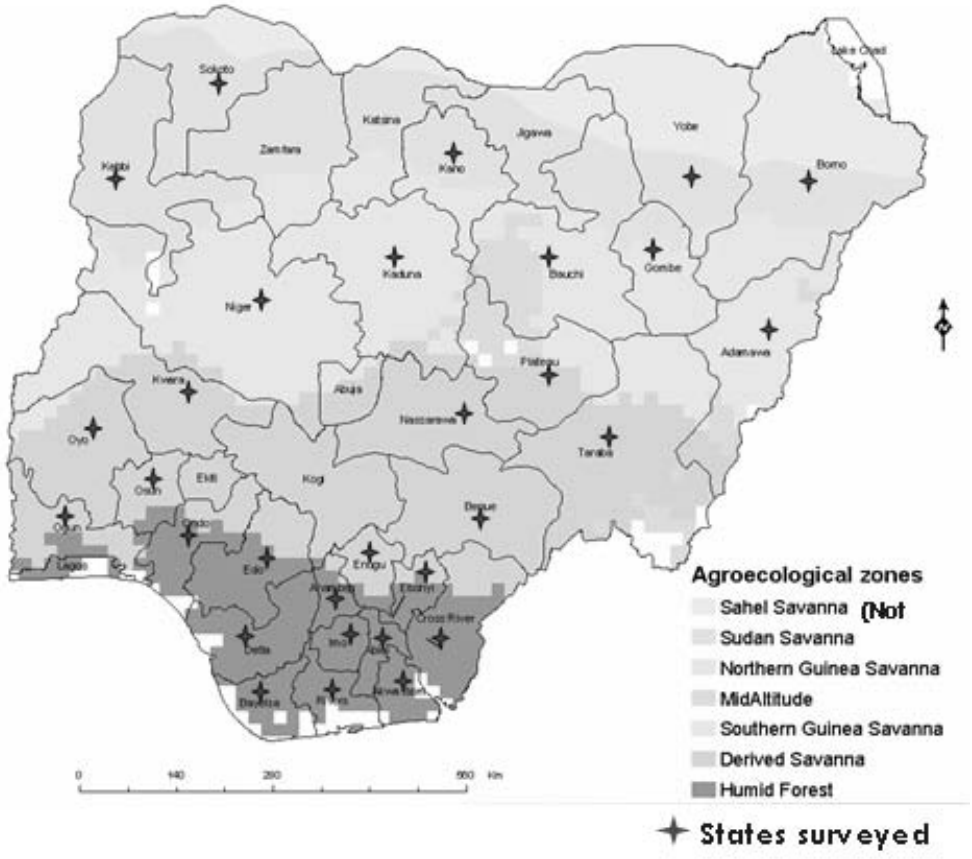

Fig. 1 A map of Nigeria showing the Agro-ecological zones surveyed for the incidence and severity of *Pepper veinal mottle virus* (PVMV) diseases on cultivated pepper *Capsicum* sp. (Source: International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.)

Sampling techniques: Ten cultivated pepper farms of approximately 10 metres by 10 metres were randomly surveyed per state. On each site 10 plants were randomly sampled from the population of plants showing virus-induced symptoms. The symptoms were also scored for severity. Five hundred leaf samples from cultivated pepper plants

showing symptoms of mosaic, chlorosis, yellowing, stunting, mottle, necrosis, leaf deformation and leaf bunching were collected and stored in zip-lock plastic sampling bags per agro-ecological zones. Care was taken to avoid mix up by adequately labelling to reflect location and date of collection with an identifying tag number. All the samples were stored in an ice crest cooler for onward transportation to the virology Laboratory for serological analysis. Percentage virus disease incidence was calculated in each farm plot by counting the number of diseased plants divided by the total number of pepper plants within the farm multiplied by 100.

$$\text{Percentage Disease Incidence} = \frac{N - n \times 100}{N \quad 1}$$

N = Total number of observations
n = Total number of plants with no disease symptoms.

Disease severity index: Virus disease severity was scored in each pepper farm surveyed by using a modified formula-grading scheme from Merritt et al., (1999) and Steel and Torrie, (1980), for disease severity;

1. No disease symptoms
2. Leaf mottling
3. Chlorosis/leaf mottling
4. Stunting/severe mottling / leaf bunching
5. Leaf defoliation.

$$\text{Disease Severity} = \frac{1 \times P_1 + 2 \times P_2 + 3 \times P_3 + 4 \times P_4 + 5 \times P_5 \times 100}{N\,(G - 1) \qquad 1}$$

Where: P_1 P_2 to P_5 = Total number of observed plants in each disease symptoms grading per farm site in each state within the agro ecological zone surveyed.
G = Number of grading = 5
N = Total number of observations

Host range test: Five gram of PVMV infected leaf samples collected from the field was homogenized with 10mls of coating buffer (coating buffer + 2% polyvynyl pyrolidone (PVP): 1.59g of Na_2CO_3, 2.93g of $NaHCO_3$, 0.2g of NaN_3, in 1 litre of distilled water: pH 9.6) with a sterilized pestle and mortar and then filtered through mesh cheesecloth (Green. 1991; Thottappilly, 1992)

The following indicator test plants were inoculated: *Celosia argentia* (L), *Chenopodium amaranticolor*, *Chenopodium murales*, *Chenopodium quinoa*, *Chromolaena odorata*, *Commelina nudiflora* (L), *Cochorus olitorius*, *Lycopersicon esculentum* (MiLL), *Nicotiana clevelandii.* (Gray), *Nicotiana glutinosa* (L), *Nicotiana rustica* (L), *Nicotiana tabacum* (L), *Physalis floridana* (Rydb), *Physalis micrantha* (L), *Talinum triangularae*, *Vinca rosea* (L). Mechanical inoculation was made in a screened greenhouse and insects were controlled by periodic spraying with insecticide (Karate, I.C.I).

Four apical leaves of each of the test plants were mechanically inoculated with the sap extracted from PVMV infected leaf samples. The leaves were dusted with 600-mesh carborundum (Silicon carbide) abrasive and each leaf was inoculated with virus saturated cotton swap renewing the inoculum in the pad frequently whilst supporting the leaf with the other hand. Heavy pressure was avoided and same area was not gone over twice. After inoculation, test plants were rinsed with sterilized water from a squeeze bottle. Hands were washed with detergent between inoculations to prevent contamination. The inoculated plants were kept in a netted cage in the screen house and observed for four weeks for symptom development. Serological test was also carried out on the inoculated plants using Protein-A sandwich Enzyme Linked Immunosorbent Assay (PAS-ELISA) (KPL Technical Guide for ELISA Protocols (on line 1999/2000 edition) and IITA Virology laboratory modified protocols) to confirm the presence of PVMV. The sap from inoculated indicator test plants showing viral symptoms were then re-inoculated mechanically following the same procedure on new set of indicator test plants and healthy 4-week-old pepper plants to confirm infection and symptom expression.

Virus Detection

Protein-a sandwich ELISA (PAS-ELISA): KPL Technical Guide for ELISA Protocols (on line 1999/2000 edition) and IITA Virology laboratory modified protocols for PAS ELISA was also used for the detection of the presence of PVMV, both from the five hundred infected pepper leaf samples collected per agro-ecological zone and samples collected from pepper plants used for the insect transmission test and also from leaf of indicator plants samples used for host range test.

The PVMV antibody used was AAB 328 antiserum diluted in ratio 1:1000 with Phosphate Buffered Saline (PBS-T) (0.05% Tween 20: pH 7.4: 8.0g NaCl, 0.2g KH_2 PO_4, 1.1g Na_2 HPO_4 0.2g KCl, 0.2g NaN_3 in 1 Litre H_2O + 0.5ml Tween 20 (0.05%)) collected from the Virology Laboratory of the International Institute of Tropical Agriculture (IITA) Ibadan.

Virus indexing protocols: One hundred micro liter of protein A at 1µg/ml in coating buffer was dispensed into each well of ELISA plate. The plate was then incubated at 37^{O}C for 2 hours. The plate was washed three times with PBS-T after the incubation period. 100µl of PVMV polyclonal (AAB 328) antiserum diluted 1:1000 in PBS-T was added to each of the ELISA plate and then incubated at 37^{0}C for 2hours. After incubation the ELISA plate was washed three times with PBS-T.

One hundred micro liter of antigen (e.g. sap) ground in PBS-T +2% PVP (Polyvynil pyrolidone) was added into each of the wells of the ELISA plate and incubated overnight at 4^{0}C. The plate was washed three times with PBS-T and 100µl of PVMV polyclonal (AAB 328) antiserum diluted 1:1000 in PBS-T was added into each of the wells. The plate was further incubated at 37^{0}C for 2hours after which it was washed three times with PBS-T. 100µl of protein, A- alkaline phosphatase conjugate diluted 1:1000 in conjugate buffer ($^{1}/_{2}$ PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP + 0.02g NaN_3) was added per well and the plate incubated at 37^{0}C for 2hrs. The plate was washed three times with PBS-T. 200µl of 0.5 -1mg/ml of p-nitrophenyl phosphate substrate in substrate buffer (97ml diethanolamine + 800ml H_2O + 0.2g NaN_3 add HCl to give pH 9.8) was added per well and incubated at room temperature for 30 minutes to one hour.

For all incubations plates were covered with ELISA cover plates to avoid edge effects and to maintain uniform temperature. Healthy pepper plants (*Capsicum* sp.) were used as negative control while PVMV infected *Capsicum* sp were used as positive control.

After one hour the absorbance was measured at 405nm using multiscan ELISA reader. The samples were considered positive when the ELISA reading exceeded that of the healthy control by or was at least twice the reading for the healthy control.

Polymerase chain reaction (PCR)

The total nucleic acid extraction: The total nucleic acid extraction was done by using a modified DNA extraction protocol according to Dellaporta et al. (1983).

Two leaf discs (40mg) of PVMV infected pepper leaf sample were ground with 500µl extraction buffer (100ml of 1M Tris- HCl pH 8.0, 100ml of 0.5M EDTA pH 8.0, 100ml of 5M NaCl made up to 950ml with deionized water, autoclaved for 15minutes, and allowed to cool to approximately 50^{o}C. Then 1% (w/v) Polyvynil pyrolidone (PVP) (40,000MW), dissolved by mixing was added and 700µl β-Mecarpto ethanol was added with Kontes pestle in a microfuge tube).

The extracted sap was poured into 1.5ml Eppendorf tube and 33µl of 20% Sodium dodecyl sulphate (SDS) plus 10mg/ml RNAase (1-2µl) pH 7.2 (100mg RNAase A (pancreatic RNAase) in 10ml 10mM Tris-HCl 15mM NaCl. Heat to 100^{O}C (in a beaker of boiling water) for 15 minutes, cool slowly to room temperature. Dispense aliquot and store at -20 ^{O}C) was added. The sample in the Eppendorf tube was mixed thoroughly for another one minute and then incubated at 65^{o}C for 10 minutes in hot water bath and allowed to cool to room temperature (approximately 2minutes). Ice-cold 160µl of 5M potassium acetate was added and mixed by gently inverting 5-6 times. The sample was then centrifuged at 13,000rpm for 10 minutes. 400µl of the supernatant was carefully transferred to a new 1.5ml Eppendorf tube. Ice-cold 200µl of Isopropanol (-20^{o}C) was added and mixed by gently inverting 8-10 times. It was then kept at 4^{o}C for 15-20minutes after which it was centrifuged at 13,000rpm for 10 minutes to precipitate the nucleic acid. The supernatant was decanted and the last drop of isopropanol was removed by placing the tube face down on paper towel. The nucleic acid was washed by adding 500µl of 70% ethanol and centrifuged at 13,000rpm for 5-10minutes. The ethanol was decanted and the nucleic acid air-dried. The nucleic acid was re-suspended in 500µl high salt TE buffer (50mM Tris-HCl pH 8 / 10mM EDTA, 25ml of Tris-HCl 1M pH 8.0 (121.1g of Tris base, 42ml of HCl), 10ml of EDTA 0.5M pH 8.0 (186.1g of EDTA, 18g of NaOH, 1 litre of de-ionized water, adjust pH to 8.2 and autoclave), make up to 500ml sterilized de-ionized water.). The sample was diluted 200times with the TE buffer and stored at 4^{0}C as stock nucleic acid before amplifying.

Nucleic acid amplification: The nucleic acid was amplified with a primer pair (Gibbs and Mackenzie, 1997) using the PCR machine (Thermal cycler) which has been automated to profile 78 (95^{o}C (Denaturation) 60^{o}C, (Annealing) 72^{o}C (Extension)) (International Institute of Tropical Agriculture (IITA) Ibadan Oyo State Nigeria). The PCR reaction mixture per PCR micro tube was made up of 1.5µl X 10 buffer, 0.9µl $MgCl_2$, 1.2µl 2.5mM Deoxynucleotide

triphosphate (dNTP), 1.0µl Primer PTY Forward, 1.0µl Primer PTY Reverse, 0.16µl Thermus aquaticus (Taq) polymerase, 2.0µl Extracted DNA sample template, 7.24 dH_2O.

Agarose gel electrophoresis

Gel preparation: One percent agarose was dissolved (by heating in mixrowine oven for five minutes at medium setting) in an appropriate volume of Tris-acetate-EDTA buffer (TAE) (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0). When the agarose cooled to touch (about 38^{o}C). It was then poured into the gel tray that has been prefitted with comb. The gel was immersed into the electrophoresis tank containing TAE buffer. The comb was then removed to expose the wells formed.

Loading of sample and running the gel: Loading buffer (7.5µl) (0.25% bromophenol blue, 0.25% Xylene cyanol FF and 30% glycerol in water) was added into each comb well in the gel with the 15µl amplified nucleic acid (1.5µl X10 buffer, 0.9µl $MgCl_2$, 1.2µl 2.5mM Deoxynucleotide triphosphate (dNTP), 1.0µl Primer PTY Forward, 1.0µl Primer PTY Reverse, 0.16µl Thermus aquaticus (Taq) polymerase, 2.0µl Extracted DNA sample template, 7.24 dH_2O) (PCR reaction mixture per PCR micro tube). A standard DNA molecular marker of 1000 base pair (1kilobyte DNA ladder) was used and treated in similar manner. The gel was run at 80-100 volt.

Staining the DNA: The gel was lowered carefully into a solution of 1µl (1%) ethidium bromide to stain the DNA. Thereafter the gel was placed in water to remove excess ethidium bromide. The DNA band in the gel was observed under ultra violet light and its position related to the fractions of the DNA molecular marker used. A Polaroid photograph of the bands in the gel was taken. The expected band size was between 0.6- 1.0 kilobyte.

Electron microscopy: Symptomatic leaf samples of PVMV infected pepper leaf (*Capsicum* sp.) dried with calcium chloride in MacCartney bottles were sent to (Dr. Stephan Winter, Head,) DSMZ Plant Virus Division ^{C}/o Messeweg 11/12 38104 Braunshcweig Germany for the virus isolation, identification and electron microscopy study.

Determination of the stability of the PVMV sap extract in causing infection. The stability of the PVMV sap extracts at different temperatures, and at different dilutions and their longevity invitro were determined using the method as described by Green (1991). Thermal inactivation point. This is the temperature required to completely inactivate the virus in crude sap during a 10- minute exposure (Green, 1991).

Fifteen grams of virus infected leaf sample was homogenized with 30mls of extraction buffer (Phosphate buffer solution: 8.0g of NaCl, 0.2g of KH_2PO_4, 1.1g of Na_2HPO_4, 0.2g of NaN_3, in 1 litre of distilled water and 0.5ml Tween 20 (0.05%) pH 7.8) with a sterilized pestle and mortar and then filtered through mesh cheesecloth. Streptomycin (0.01%) was added to the sap extracted to prevent bacterial contamination. Using a sterilized pipette, 12 screw–capped test tubes were each filled with 2mls of the virus infected sap extract.

Using a sterilized pipette, 2mls of sap extract virus isolate was carefully dispensed into a screw-capped test tube. Each tube was then immersed in a heated shaker water bath for 10 minutes at different temperatures of 35^{0}C to 100^{0}C at 5^{0}C interval. The heated shaker water bath was covered with a transparent glass cover at each temperature level to maintain a uniform temperature within the water bath during the 10 minutes period. After each time limit at each temperature level, the tubes containing the virus sap extract were allowed to cool down to room temperature and then used to inoculate three replicates of five test plant (a 4–week old "Tattasai" pepper) seedlings replicated three times. The test plants were then observed for symptom development between 3 days to 30 days. Serological test using PAS-ELISA was also used to determine the presence of PVMV in inoculated test plants. The temperature range at which virus activity ceased was recorded for the virus isolate. Longevity *in vitro.* This is the length of time the virus is infective in crude sap kept at room temperature [25^{0}C – 27^{0}C].

Three replicates of five test plants (a 4–week old "Tattasai" pepper) replicated three times were inoculated with sap from individual test tubes at different days for 12 days, the test plants were then observed for symptom development between 3 to 30 days from the day of inoculation. Also serological test using PAS-ELISA was used to determine the presence of PVMV in inoculated test plants. The day at which virus activity ceased was recorded for the virus isolate.

Dilution end point: This is the highest dilution of plant sap in which the virus is still infectious. Ten grams of PVMV virus infected leaf sample was homogenized with 20mls of extraction buffer (Phosphate buffer solution: 8.0g of NaCl, 0.2g of KH_2PO_4, 1.1g of Na_2HPO_4, 0.2g of NaN_3, in 1 litre of distilled water and 0.5ml Tween 20 (0.05%) pH 7.8) with a sterilized pestle and mortar and then filtered through mesh cheesecloth.

Serial ten-fold dilutions were then made from this original sap. 2mls of extract was pipetted from the original sap extract into the first of the eight tubes. Using a sterilized pipette, 1.8ml of sterilized distilled water was pipetted into each of the remaining 7 tubes. From the first tube 200µl of the sap was pipetted and transferred to the second tube to

give a dilution of 1/10. From the second tube (1/10 dilution) 200µl of the extract was transferred into the third tube (1/100 dilution) and this was repeated for the remaining tubes up to dilution of 1/10,000,000.

Three replicates of five test plants [4 – week old bell shaped pepper seedlings] replicated three times were inoculated with sap samples for each serial dilution, the test plants were then observed for symptom development between 3 to 30 days from the day of inoculation. Also serological test using PAS-ELISA was used to determine the presence of PVMV in inoculated test plants. The dilution rate at which virus activity ceased was recorded for the virus isolate.

Results

The Incidence and severity of *Pepper veinal mottle virus* diseas: Similar trends were obtained in the results in the years under study with no significant difference. Sixty eight percent of the farms visited were observed to be under sole pepper cropping system while 32% had mixed-cropping system. The pepper plants were either intercropped with maize, cassava, yam or plantain. Mixed pepper cropping system was observed to be mostly practiced in the humid forest and the derived savanna, where 86% of the farmers' grew pepper with other crops on the same parcel of land and 14% of the farmers practiced sole pepper cropping system. But in the southern Guinea savanna, mid-altitude, northern Guinea savanna and Sudan savanna only 10% of the farmers practiced mixed pepper intercrop.

All the farms visited recorded the presence of PVMV. Out of the three thousand suspected viral infected pepper leaves sample collected from the fields in the six agro-ecological zones, 88% were confirmed to be PVMV positive through the serological test while 12% were found to be negative.

All the pepper farms surveyed showed significant difference in PVMV disease incidence and severity within the agro-ecological zones (Table 1). The percentage PVMV disease incidence ranged between 39.14% in the Sudan savanna to 50.12% disease incidence in the derived savanna zone (Table 1). The incidences of virus diseases were observed to be high in the derived savanna and the humid forest compared with the other agro-ecological zones (Table 1). The derived savanna zone recorded a mean percentage PVMV disease incidence (50.12%) and severity (43.85%) while the Sudan savanna zone recorded the lowest disease incidence (39.14%) and severity (34.48%) (Table 1).

Tab. 1 Mean percentage of *Pepper veinal mottle virus* incidence and severity in six agro-ecological zones of Nigeria surveyed

Zones	Mean % Disease Incidence	Mean % Disease Severity
Humid Forest	48.54_{b}	$42.69_{a,b}$
Derived savanna	50.12_{a}	43.85_{a}
Southern Guinea savanna	40.41_{e}	36.88_{c}
Mid Altitude	46.0_{c}	$42.9_{a,b}$
Northern guinea savanna	44.0_{d}	41.30_{b}
Sudan savanna	39.14_{e}	34.48_{d}
Standard error ±	0.56	0.49

Means followed by the same alphabet in each column are not significantly different from each other at 0.05 level.

Viral isolates from the field samples of infected pepper plants collected were able to induce distinct symptoms on inoculated healthy pepper plants and a range of indicator plants used. PVMV induced a systemic leaf mottling on *Nicotiana clevelandii* (Gray), leaf mottling and necrotic spot on *N. benthamiana* and systemic symptoms on *N. rustica*. L. and *N. glutinosa* .L; *Physalis floridana* (Rydb) and *Lycopersicon* spp., On *Physalis micrantha*, PVMV induced premature abscission of leaves, partial defoliation on chronic infected plants (systemic) and local necrotic spot. Local necrotic spot were induced on *Chenopodium amaranticolor* (Costs and Reyn), *C. quinoa*. (Willd), *C. murale* L. and it caused slight leaf mottling of *Chromolina odorata* L. (Table 2).

Tab. 2 Symptoms expression of a host plant range mechanically inoculated with *PVMV*.

	Family	Symptoms
Celosia argentea L.	Amaranthaceae	-
Chenopodium amaranticolor Cost and Reyn.	Chenopodiacea	Nec.
Chenopodium murales L.	Chenopodiaceae	Nec.
Chenopodium quinoa Willd.	Chenopodiaceae	Nec.
Chromolaena odorata (L.) R.M King and H.Rob	Asteraceae	M,
Commelina nudiflora. L.	Commelinaceae	-
Corchorus olitorus L.	Tiliaceae	-
Lycopersicon esculentum Mill., nom.cons.	Solanaceae	S, M
Nicotiana benthamiana L.	Solanaceae	C, Nec., S
Nicotiana clevelandii Gray	Solanaceae	S, V, M,C
Nicotiana glutinosa. L.	Solanaceae	M

	Family	Symptoms
Nicotiana megalosiphon L.	Solanaceae	S, V, M,C
Nicotiana rustica L.	Solanaceae	S, M
Nicotiana tabacu. C.V. xanthi	Solanaceae	-
Petunia hybrida L.	Solanaceae	C, S, M
Physalis floridana Rydb.	Solanaceae	Nec.
Physalis micrantha. L.	Solanaceae	S, M,Nec
Solanum nigrum L.	Solanaceae	Nec.
Talinum triangularie		Nec.
Vinca rosea. L.	Apocynaceae	-

Keys: C = Chlorosis; S = Systemic; V = Vein banding/Vein Clearing; Nec. = Necrotic leision; M = Leaf molting; - = No symptoms;
PVMV - Pepper veinal mottle virus (genus Potyvirus. Family Potyviridae)

Virus detection: All the farms visited recorded the presence of PVMV. Out of the three thousand suspected viral infected pepper leaves sample collected from the fields in the six agro-ecological zones, 88% were confirmed to be PVMV positive through the serological test while 12% were found to be negative. The PVMV antiserum (AAB 328), diluted at ratio of 1:1000 PBS-T was very effective in detecting the PVMV in viral infected leaf samples through PAS-ELISA serological test.

In the Polymerase chain reaction (PCR), there was successful amplification of the PVMV fragment (0.65 kilobytes) using the nucleic acid molecule from PVMV infected leaf samples. Thus the extraction protocol could be considered to be appropriate for the PCR. (Fig. 2). The Electron micrograph photograph showed antiserum decorated PVMV particles, having flexuous filamentous particles of 750nm in length and 10 nm wide (Plate 1).

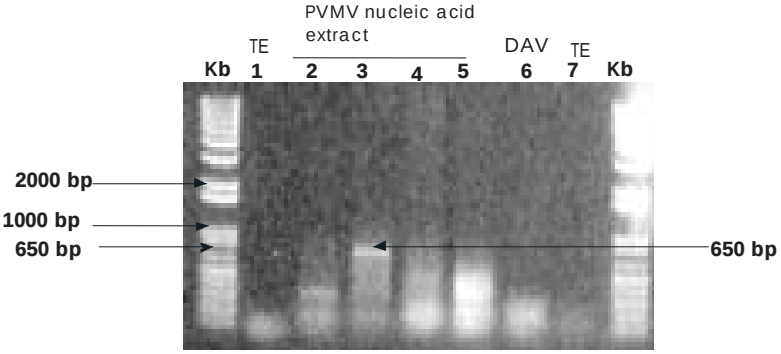

Fig. 2 Polymerase Chain Reaction of total nucleic acids extracted by the modified Dellaporta (1983) extraction method Amplification is 650bp (0.65 kb) nucleic acid of Pepper veinal mottle virus genus Potyvirus Family Potyviridae; Kb = 1kb plus Molecular marker; DAV = Dascoria alata virus.

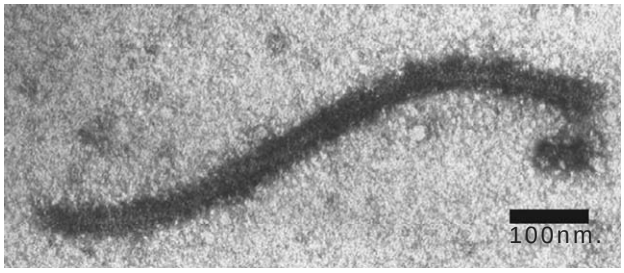

Plate 1 Electron micrograph of a decorated particle of *Pepper veinal mottle virus* isolated from pepper plant infected with PVMV (Courtesy of Dr. Stephan Winter, Head, DSMZ Plant Virus Division Messeweg 11/12 38104 Braunshcweig)

The stability of the *Pepper veinal mottle virus* in sap extract: The thermal inactivation point of PVMV was observed to be 70^{O}C and the virus was still infective up to the 12th day. The dilution end point in which PVMV extract from *Capsicum annuum* L. was still infective was found to be at dilution of 10^{-4}.

Discussion and conclusion: In all the agro-ecological zones surveyed, characteristic symptoms of PVMV disease, mild mottle, mosaic, vein banding, ring spots, various types of necrosis, leaf discoloration, deformation, blistering and severe stunting of the whole plant were observed on pepper plant infected with PVMV but at varied degree of severity which was similar to earlier described symptoms by Atiri (1992).

Climate and vegetation in the different ecological zones appeared to have played a major role in determining the incidence and severity of PVMV infection on pepper in the fields. The high incidence of PVMV especially in the Derived savanna and the Humid forest agro-ecological zones might not be unconnected with the ability of the virus to remain infective for many months in alternative weed host coupled with a good breeding environment for the vectors of the virus that aids effective transmission. These zones are characterized by thick vegetation and warm humid climate (Fajinmi 2006), with the presence of many secondary host plants for the virus. This would have encouraged the rapid multiplication of the aphid and the virus itself and subsequent increase in the efficiency and the ability of the aphid species to successfully transmit the virus non-persistently.

Proximity of pepper plants to certain important weed hosts has contributed greatly to the spread of viral diseases of pepper (Alegbejo 1987). The weeds include *Solanum nigrum, S. gracil, Physalis angulata, Vigna rosea, Vigna sinensis, Commelina nudiflora, Petunia hybrida, Physalis floridana, P. micrantha* and *Solanum incanum* (Alegbejo 1987). The ability of the isolated PVMV strain to cause infection on alternative host plants tested suggest for the adaptive ability of the virus on weed host plants in the two agro-ecological zone characterized by thick vegetation and warm humid climate, with the presence of many secondary host plants for the virus and vectors (Aldyhim, and Khalil. 1993) which aided its infective capability and spread of the virus on pepper plants where its incidence and severity had been observed to be high.

There is every possibility of significant pathogenic variability within the PVMV strain isolated as suggested by Gilbert, et. al., (2005), this might have contributed significantly to the adaptive nature of the virus in these agro-ecological zones. Also the physical properties of the virus that showed that the virus was still capable of causing infection below 65^{O}C confirmed the ability of the virus to be able to survive the climatic condition of both the derived savannah and the humid agro-ecological zones but could not survive very well the climatic conditions of the other agro-ecological zones that are characterized with harsh climatic conditions characterized with high temperatures and sparse vegetation.

Therefore the ability of any virus to adapt, cause severe infection and spread in any given environment depend greatly on the vegetation, climatic and ecological characteristics of such area.

Acknowledgement

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Preliminary results on resistance to PPV-M in *Prunus persica* (L.) Batsch

Casati, P., Bassi, D., Spadone, P., Bianco, P.A.
Di.Pro.Ve., University of Milan, Via Celoria 2, 20133 Milano, Italy

Abstract

Preliminary results of trials evaluating peach cultivar resistance to *Plum pox virus* (PPV) and aphid colonization were obtained. Data after one vegetative cycle since an inoculation assay showed that most of the peach cultivars analyzed were susceptible to PPV-M isolate, used as inoculum source. Also, in our experiments two cultivars, Ambra and Cappucci 18, were found to be tolerant while cultivars Fei Cheng, Harrow Blood, Jing Yu and Rosa Dardi were resistant.

Moreover, to evaluate the possible epidemiological impact of the peach cultivars NJ WEEPING and S6699 resistant to aphid colonization, experimental transmissions were carried out. This was done by using a clonal culture of *Myzus persicae* (Sulzer) as vector and PPV-M isolate as inoculum source under controlled acquisition access period. The results showed that in our conditions aphids were not able to transmitted PPV-M isolate to healthy plants of NJ WEEPING and S6699. Under the same conditions PPV-M was transmitted by aphids from infected to healthy GF305 plants.

Keywords: sharka, peach, ELISA, aphid transmission, virus tolerance

Introduction

Plum pox virus (PPV) is the causal agent of sharka disease, one of the most dangerous diseases of stone fruits and in particular peach. PPV is present in several areas of the world including North America and Asia. In Europe, where sharka was first reported and where it is still spreading, PPV is infecting both fruit trees and wild plant species. In these cases, the application of quarantine measures is time consuming, expensive and not effective, not even when virus-free material is employed for the plantation of new orchards.

A fast spread of the strain PPV-M recently occurred in several areas in Europe and in particular in Italy, where the disease is causing severe losses to the peach crop, and threatens the nursery industry as well. Where sharka is endemic the only sustainable strategy is the employment of resistant cultivars. In the present study, we report preliminary results on the identification of peach cultivars naturally resistant to infections by PPV, or to the colonization by PPV aphid vectors.

Material and methods

Molecular Characterization of PPV Isolates: Total RNA was extracted from symptomatic leaves of 9 naturally infected peach trees using a silica method (MacKenzie et al., 1997). RT-PCR reactions were performed to amplify three different genes (HC-Pro, P3 and CP) for all the PPV isolates investigated.

Amplified fragments were purified and cloned into pCRII vector using the TA cloning kit (Invitrogen). The clones obtained were sequenced and nucleotide sequences were aligned using Bioedit software version 7.0.5.3. CP, HC-Pro and P3 gene sequences were used to construct different phylogenetic trees. Minimum evolution analysis was carried out using the neighbourjoining method and bootstrap replicated 1000 times with the software Treecon version 1.3b. PPV-PS (AJ243957) isolate was used as the outgroup.

Evaluation of Resistance to PPV of Peach Cultivars: Fifteen peach cultivars (Table 1) were tested for PPV resistance by green-grafting on inoculated *P. cerasifera* plants, with two different PPV-M isolates (Fig. 1), in two independent assays. Plants were maintained in a screenhouse during the winter season and transferred to a greenhouse at 22-24°C for PPV symptom observation. At the end of May, leaf samples were collected from analyzed peach cultivars and from *P. cerasifera* for detection of PPV-M by ELISA (Cambra et al., 1994) and RT-PCR (Wetzel et al., 1991) assays.

Tab. 1 Results of resistance trials obtained inoculating fifteen different peach cultivars with PPV-M isolates.

Peach cultivars	*P. cerasifera*		*P. persica*		
	Symptoms	**ELISA**	**Symptoms**	**ELISA**	
AMBRA	+	+	-	+	Tolerant
CAPPUCCI 18	+	+	-	+	Tolerant
Chimarrita	+	+	+	+	Susceptible
Contender (Nct 544)	+	+	+	+	Susceptible
Fei Cheng	+	+	-	-	Resistant
Harrow Blood	+	+	-	-	Resistant
Hardy Red	+	+	+	+	Susceptible
Jing Yu	+	+	-	-	Resistant
Kamarat	+	+	+	+	Susceptible
Maycrest	+	+	+	+	Susceptible
May Fire	+	+	+	+	Susceptible
Nj 307	+	+	+	+	Susceptible
Rosa Dardi	+	+	-	-	Resistant
S 5898:128	+	+	+	+	Susceptible
T 16	+	+	+	+	Susceptible

Fig. 1 Chip budding trials

Peach cultivars were considered to be susceptible when they showed symptoms of the disease, tolerant when symptoms were absent on the scion but ELISA was positive, and resistant when there were neither symptoms nor a reaction in ELISA (Bazzoni et al., 2004).

Aphid Transmission: Aphid transmission experiments were conducted under controlled conditions using a clonal culture of *Myzus persicae* (Sulzer) as a vector and plants of *P. persica* cv. GF305, inoculated with PPV-M, as an inoculum source. The aphids, raised on healthy pea plants, were placed, with the aid of a small brush, onto the leaves of infected *P. persica* GF305 (acquisition period). Later, these aphids were placed onto healthy peach GF305 used as positive control, and onto plants of the two cultivars NJ WEEPING and S6699. Four leaves for every healthy peach tree were used for the inoculation period; in this the branch of leaves was isolated from the remaining part of the plant (Fig. 2).

Fig. 2 Aphid transmission trials with *M. persicae* and two peach cultivars NJ WEEPING and S6699

The plants, after insecticide treatment, were put in a greenhouse under controlled conditions. The occurrence of PPV transmission was diagnosed by symptoms observation, ELISA and RT-PCR assays.

Results and discussion

Molecular Characterization of PPV Isolates: The sequence analyses of 9 PPV isolates confirmed their classification within PPV-M strain and in particular they clustered with PPV-M isolate SK68 (M92280), a sharka strain isolated in Hungary (Fig.3). All isolates have high level of identity with PPV-M SK68 and maintain the DAG and PTK motifs in the CP and HC-Pro genes indicating their aphid transmissibility. These data confirmed previous results reported in Italy (Bianco et al., 2004; Bianco et al., 2005).

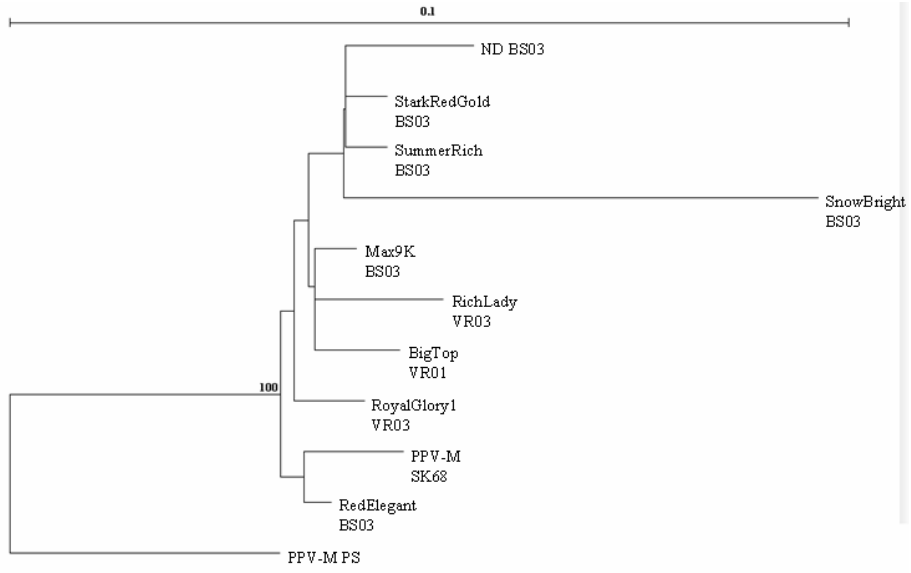

Fig. 3 Phylogenetic tree obtained with Treecon program analyzing the nucleotide sequence of nine PPV-M isolates collected from naturally infected peach.

Two of these viruses, from Snow Bright and Big top cultivars, were used in the assays to evaluate resistance in peach cultivars. Only one PPV-M isolate, from Big Top, was employed for the aphid transmission trials.

Evaluation of Resistance to PPV of Peach Cultivars: The results obtained in the evaluation of sharka resistance of the different peach cultivars are shown in Table 1. Fei Cheng, Harrow Blood, Jing Yu and Rosa Dardi proved to be resistant (no evident symptoms and ELISA negative assays) to two PPV-M isolates after the first cycle of experiments. Harrow Blood was reported as resistant also by Palmisano and colleagues (2008). Ambra and Cappucci 18 were shown to be tolerant (no evident symptoms but with ELISA positive assays) as already reported by Palmisano and colleagues (2008). The remaining cultivars were found to be susceptible to the disease on the basis of their symptom expression and ELISA positive assay.

Aphid Transmission: The transmission experiments confirmed that PPV isolate from Big top cultivar was aphid transmitted from infected to healthy *P. persica* GF305 plants as verified by ELISA and RT-PCR assays. On the contrary, inoculated peach trees of cultivars NJ WEEPING and S6699 were asymptomatic. Moreover, ELISA and RT-PCR, conducted on leaves collected from inoculated plants, were negative indicating that aphids were not able to transmit the virus. Also, samples collected from GF305, used as the rootstock for NJ WEEPING and S6699, were negative in the same analysis and no symptoms were observed. These results would indicate that these two cultivars may be resistant to aphid colonization. In fact other results suggest that these peach cultivars are PPV susceptible. Further experiments will be conducted in order to validate these data with the aim of supplying suitable cultivars for those areas where sharka disease is endemic and no efficient containment measures are available.

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The inheritance of the hypersensitivity resistance of European plum (*Prunus domestica* L.) against the *Plum pox virus*

Lichtenegger, L.[1], Neumüller, M.[1], Treutter, D.[1], Hartmann, W.[2]
[1] Unit of Fruit Science, Centre of Life and Food Science Weihenstephan, Technische Universität München, Dürnast 2, 85354 Freising, Germany
[2] Erikaweg 5, 70794 Bernhausen-Filderstadt, Germany

Abstract

In between 2003 and 2009 more than 500 seedlings have been tested for hypersensitivity resistance against the *Plum pox virus* (PPV), which causes Sharka disease. The seedlings had at least one hypersensitive parent genotype. They were tested for hypersensitivity resistance by double grafting onto PPV infected interstem in the green house. In crossing combinations with two hypersensitive parents the percentage of hypersensitive seedlings was highest. There is also no equal distribution of the genotypes over the individual hypersensitivity classes (HC) in all crossing combinations. The percentage of hypersensitive seedlings strongly depends on the parentage. Furthermore investigations regarding the origin of the hypersensitivity resistance of the cultivar 'Jojo', which is a descendant of a crossing combination from 'Ortenauer' × 'Stanley', were done. It was shown that the cultivar 'Ortenauer' is the donor of the hypersensitivity trait.

Keywords: *Plum pox virus*, hypersensitivity, inheritance, *Prunus domestica* L., resistance

Introduction

Sharka disease has caused big economic losses during the last decades. Large efforts were done to limit the economic impact of the disease by means of resistant genotypes. At present, the most effective resistance mechanism known is the use of hypersensitivity resistance, which was first described in 1991 by Kegler et al. on 'Kischinever Hybride 4', which was not completely resistant against *Plum pox virus*. In 1997 Hartmann also described this type of resistance in crossings of the cultivar 'Ortenauer' with 'Stanley', 'Ruth Gerstetter' and 'Italian Plum'. From these crossings the cultivar 'Jojo', the first completely resistant genotype against the *Plum pox virus*, arose.

According to our present knowledge, the use of hypersensitivity resistance is the most promising mechanism to control the economic impact of Sharka disease in European plum (*Prunus domestica*). In spite of large efforts during the last years, the inheritance of the hypersensitivity trait is not yet fully understood. Therefore, further investigations regarding the heritability of this hypersensitivity resistance are necessary.

Material and methods

500 seedlings originating from crossing combinations between hypersensitive and sensitive, between hypersensitive and hypersensitive, and between hypersensitive and quantitatively resistant genotypes have been tested for hypersensitivity resistance by using a double grafting method described by Kegler et al. 1994. In January budsticks of the seedling genotype to be tested were grafted onto virus free myrobalane seedlings with an infected scion wood of the cultivar 'Katinka' used as an interstem (Fig. 1 A). The 'Katinka' scion woods were cut on frost-free days in December from systemically infected trees in the field. The budsticks and the grafting area were immersed in 70 °C hot Rebwachs WF (Stähler Agrochemie) and afterwards the plants were potted in 5 l containers. Every genotype was tested in three replications. The grafted plants were cultivated in an insect proof greenhouse at a temperature of 15 °C. After budbreak the temperature was raised to 22 °C. Rootstock suckers were removed continously. In order to initiate the growth of the testing genotype, the branches of the 'Katinka' interstem were decapitated with the full development of the third leaf.

Fig. 1 **A + B:** double grafting; white arrow: shoot of a seedling in the testing system; dashed arrow: virus infected 'Katinka' after decapitation; **C:** necrosis on the bark and on the shoot tip of 'Wei 499' ('Ortenauer' × 'Ortenauer'); **D:** strong hypersensitive reaction of a successful grafting of 'Wei 4926' ('Hoh 4941' × 'Hoh 7172'), dying shoot.

A first rating was done after 6 weeks and a second one after 20 weeks. Further more if one could not detect any visible PPV symptoms the virus was serological analyzed by DAS-ELISA with PPV universal antibodies (Bioreba Switzerland) (EPPO 2004).

Results and discussion

After budbreak, hypersensitive genotypes showed necrosis on the leaf blade, the bark and the shoot tip depending on the degree of the hypersensitivity resistance (Fig. 1). The genotypes were grouped to the four classes of hypersensitivity (HC): HC 0 – no, HC 1 – weak, HC 2 – normal, HC 3 – strong hypersensitive response with HC 3. In HC 3, being the highest value, it was reported that plants isolate the virus and rapidly stop its replication within in the plant tissue (Neumüller 2005, Neumüller et al. 2008).

The number of hypersensitive seedlings in some of the investigated progenies is given in Fig. 2. It varies between 0 and 63 %. The highest percentage of hypersensitive seedlings can be observed if both parents are hypersensitive ('Hoh 4517' × 'Jojo', 'Hoh 4517' × 'Hoh 7184'). Having chosen a hypersensitive and a sensitive genotype as a parent genotype, the percentages of hypersensitive seedlings in the progenies varied between 0 and 50 %. In all crossing combinations of a hypersensitive genotype with a genotype which is quantitatively resistant and tolerant (e. g. 'Hoh 2043', 'Mirabelle de Nancy'), less than 15 % hypersensitive seedlings were obtained.

These results are in line with the observations made by Neumüller (2005). There seems to be an oligogenic control for the hypersensitivity trait or the large differences relating to the combining ability of parent genotypes can hardly be explained.

The first genotype with hypersensitivity resistance to the broad range of PPV stains and isolates known in *Prunus domestica*, the cultivar 'Jojo', is a hybrid between the highly sensitive cultivar 'Ortenauer' and the fruit tolerant cultivar 'Stanley' (Hartmann 2002). Hartmann (2002) assumed that the hypersensitivity trait originates from the cultivar 'Ortenauer'.

In order to check this hypothesis descendants of the following crossing combinations were investigated: 'Ortenauer' × 'Ortenauer', 'Stanley' × 'Ortenauer' and 'Stanley' × 'Stanley'. In descendants of 'Stanley' × 'Stanley', no hypersensitive seedlings (HC 2 or 3) could be found. 20 % of the seedlings resulting from 'Stanley' × 'Ortenauer' and 53 % originating from 'Ortenauer' × 'Ortenauer' are strongly hypersensitive. These results show that 'Ortenauer' is the donor of the hypersensitivity resistance to PPV (Fig. 2).

The investigations on the inheritance of hypersensitivity resistance confirm the results described by Neumüller (2005) and Hartmann and Neumüller (2008) and improve our understanding of the inheritance of the resistant trait. The selected genotypes provide a base for further selection and breeding of PPV resistant European plum cultivars.

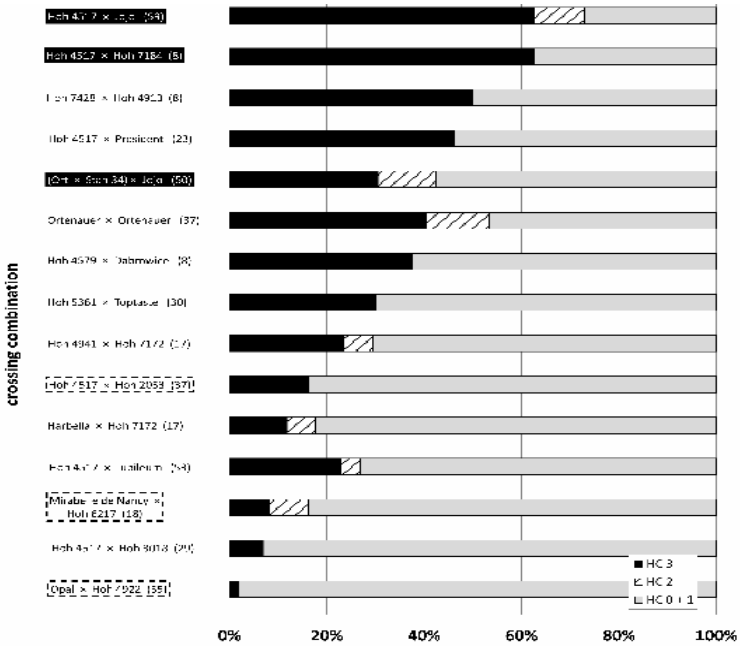

Fig. 2 Percentages of seedlings of some investigated crossing combinations belonging to the different hypersensitivity classes (HC: HC 0 – no, HC 1 – weak, HC 2 – normal, HC 3 – strong hypersensitive response). Crossing combinations of hypersensitive genotypes with hypersensitive ones (marked in black and written in white), hypersensitive with sensitive genotypes and hypersensitive with quantitatively resistant ones (framed with bars). The number in brackets gives the amount of tested seedlings of the respective crossing combination.

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Evaluation of transgenic *Prunus domestica* L., clone C5 resistance to *Plum pox virus**

Jarošová J.[1], Gadiou S.[1], Polák J.[1], Ravelonandro M.[2], Scorza R.[3], Kumar J. K.[1]
[1]Department of Virology, Crop Research Institute, Drnovská 507, 161 06, Czech Republic. Email: jiban@vurv.cz
[2]UMR, GDPP, Virologie, INRA-Bordeaux, Villenave d'Ornon 33883, BP-81 France
[3]USDA-ARS Appalachian Fruit Research Station, 2217 Wiltshire Rd., Kearneysville, West Virginia 25430, USA

Abstract

Plum *pox virus* (PPV) is one of the most devastating diseases of *Prunus* species. Since few sources of resistance to PPV have been identified, transgene-based resistance offers a complementary approach to developing PPV-resistant stone fruit cultivars. C5, a transgenic clone of *Prunus domestica* L., containing the PPV coat protein (CP) gene, has been described as highly resistant to PPV in greenhouse tests, displaying characteristics typical of post-transcriptional gene silencing (PTGS). Moreover, C5 trees exposed to natural aphid vectors in the field remained uninfected after 4 years while susceptible transgenic and untransformed trees developed severe symptoms within the first year. In our study, a high and permanent infection pressure of PPV was provided by bud grafting of inoculum in the field trial of clone C5 conducted in the Czech Republic, in which PPV-infected and healthy control trees were used. Moreover, trees with combined inoculations by PPV, ACLSV and PDV were also used in the trial. The presence of the viruses throughout the tree tissues, the relative titre of the viruses and symptoms on C5 trees have been monitored over the years. The resistance stability of C5 clones under permanent infection pressure is discussed.

Keywords: PPV, C5, resistance, real-time PCR

Introduction

Plum pox potyvirus (PPV) is the causal agent of 'Sharka' disease in *Prunus* species and is considered to be one of the most serious diseases of stone fruit trees in Europe. PPV has recently been detected in North and South America (Gildow et al., 2004). Following the successful transfer of the PPV coat protein (CP) gene to *Prunus domestica* plum (Scorza et al., 1994), the transgenic plum clone C5 has shown to be highly and steadily resistant to PPV infection (Ravelonandro, 1997). The stability of resistance to PPV has also been proved in long term field experiments in Poland, Spain, Romania (Malinowski et al., 2006) and more recently in the Czech Republic (Polak et al., 2008). The PPV resistance in C5 was previously demonstrated to be associated with post-transcriptional gene silencing (PTGS) (Scorza et al., 2001) and RNA silencing associated short interfering RNA duplex (siRNA: 21-22 nt and 25-26 nt) were detected in C5 plant (Hily et al., 2005). It was also demonstrated that long class siRNAs (25-26 nt) mediate the systemic silencing and pay significant role in conferring the resistant phenotype in C5 to PPV infection (Kundu et al., 2008).

Here we have evaluated the field resistance of C5 in a permanent and continual inoculum pressure through challenging the plants with either PPV-Rec solely or in combination of heterologous viruses: *Prune dwarf virus*-PDV and *Apple chlorotic leaf spot virus*-ACLSV by grafting inoculation. The appearance of symptoms was evaluated and the presence of inoculated viruses were tested by RT-PCR using specific primers for PPV-Rec (Subr et al., 2004), PDV and ACLSV (Polak et al., 2008). The PPV concentration in C5 was compared with non-silenced root stock St. Julien by SYBER green based absolute quantification by Real-time RT-PCR.

Material and methods

Virus detection in C5 by RT-PCR: The experiment to evaluate the influence of heterologous viruses: combinations of PPV-Rec together with PDV and ACLSV were graft-inoculated on C5 trees as follows: PPV+PDV, PPV+ACLSV, and PPV+ACLSV+PDV (11 C5 in each combination), and also PPV-Rec as a single infection (9 C5 trees). Leaf samples were collected, grinded in liquid nitrogen and total RNA was isolated by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in (Mekuria et al., 2003). One Step RT-PCR was carried out as in (Kundu, 2003). One Step RT-PCR was performed with the One Step RT-PCR kit (Qiagen) as follows: the One Step RT-PCR mixture containing 5 µl of the 5x Qiagen One Step RT-PCR buffer, 10 nM of each dNTP, 1 µl of the Qiagen One Step RT-PCR enzyme mixture, 1 µl of Q solution, 6 pM of upstream and downstream primers were prepared in microtubes and 1 µl of RNA was added. The mixture was adjusted to 25 µl with RNase-free water. The reaction was carried out in a thermocycler (MJ Research) as follows: a RT step at 50°C for 30 min and an initial PCR activation step at 95°C for 15 min, then 33 cycles of 94°C for 30 s (denaturation), 55-60°C for 45 s (annealing), and 72°C for 80 s (extension). After the last cycle, a final extension step at 72°C for 10 min was added. The PCR products were analyzed in 1% agarose gel electrophoresis, staining was done by SYBR Green (Invitrogen, California, USA).

PPV quantification in C5 by Real-time RT-PCR: Nine C5 transgenic plum trees grown in an experimental orchard were bud-grafted with plum cv. 'St Julien' infected with PPV-Rec strain (Polak et al., 2008). The leave samples from the trees were collected during years 2006, 2007 and 2008. Always, three samples were collected per one tree - leaves of the 'St Julien' infectious bud (the non-transgenic part), leaves of bottom part of the C5 (leaves close to the infectious bud, transgenic part) and leaves of top part of the C5 (far from the infectious bud, transgenic part). The material was grinded in liquid nitrogen and several aliquots of 0.1g were stored at -80°C. Thereafter, total RNA extraction was performed by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in Mekuria et al., 2003. The primer pair specific for

PPV-Rec targeting (Cter) NIb-(Nter) CP 8532-8669 (NCBI Acc. Number AY028309) RecJF:
5' AATGATATTGATGATAGCCTTGAC-3' and RecJR 5'-AGCTGGTTGAGTTGTTGCCAC-3'

amplifying a 138bp product. Specificity of the PPV-Rec primers was checked by Real-time RT-PCR on PPV-D, PPV-M and PPV-Rec isolates (data not shown).

The specific PPV-Rec fragment described above was inserted into the vector pGem-T (Promega Inc.) and cloned into E. coli JM-109. The plasmid was linearized at the Rsa I site and used as target in an in vitro transcription reaction performed with Megascript T7 kit (Ambion Inc., TX) followed by DNase I treatment (DNAfree, Ambion). The amount of RNA was quantified by UV densitometry. Conversion of microgram of single stranded RNA to picomole was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (Nb). The following mathematical formula was applied: pmol of ssRNA= µg (of ssRNA)×(106 pg/1µg)×(1 pmol/340 pg)×(1/Nb). Avogadro constant was used to estimate the number of transcripts (6.023×1023 molecules/mol) (Olmos et al., 2005). Subsequently, ten-fold serial dilutions of the transcripts were prepared and used. Real-time RT-PCR was performed with SYBR Green I, using a 7300 Real-time PCR System (Applied Biosystems, CA, USA) and Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystem, USA) according to the recommendation of the manufacturer.

Results and discussion

Nine C5 plants were inoculated with PPV-Rec. PPV was detected in eight C5 plants, in most of the cases close to the inoculum sources (Table 1). Mild diffuse mosaic symptoms were observed (Figure 1A, B) also in those part of C5 scion. The upper leaves remained symptomless and virus free (except two C5 scions in which PPV were detected by RT-PCR). These results agree with our previous observation that in the permanent inoculum pressure (from non-silenced St. Julien), C5 scions become virus infected and also exhibit symptoms (Kundu et al., 2008). In such circumstances the siRNA mediated silencing system perhaps overwhelmed and blocked the accumulation of long class siRNA (26-27 nt) in C5 (Kundu et al., 2008), which is significant for systemic silencing in plant (Hamilton et al., 2002). ACLSV and PDV viruses were also detected by RT-PCR and results are shown in Table 2. Neither PDV nor ACLSV seemed to have any effect on C5 resistance to PPV infection.

Tab. 1 Number of positives plants during years 2006, 2007 and 2008

	Number of PPV positive/ number of tested plants		
	2006	**2007**	**2008**
Leaves of St Julien	7/8	7/8	7/8
Bottom leaves of C5	8/9	8/9	8/9
Top leaves of C5	0/9	2/9	2/9

Tab. 2 Detection of PPV, PDV and ACLSV during the years 2006, 2007 and 2008

		Number of virus positive/ number of tested plants					
			2007			2008	
		PPV	**PDV**	**ACLSV**	**PPV**	**PDV**	**ACLSV**
Grafting PPV+PDV	C5	4/11	0/11	xxxx	4/11	0/11	xxxx
Grafting PPV+ACLSV	C5	4/11	xxxx	11/11	4/11	xxxx	11/11
Grafting PPV+PDV+ACLSV	C5	5/11	0/11	11/11	5/11	0/11	11/11

PPV and ACLSV were also detected in the each of individual grafted bud for each virus. PDV grafted bud are missing in the plants

The resistance level of C5 to PPV-Rec was furthermore evaluated by Real-time RT-PCR. The field growing C5 plants inoculated with PPV-Rec (from Table 1) were subjected to our analysis. The results of absolute quantification have shown that the average concentration of PPV-Rec in non-silenced root stock (St. Julien) increased gradually over the tested period compared to C5. In C5, the virus concentration remained more or less similar and was slightly increased in 2008 (see Figure 1). The virus concentration in silenced C5 was significantly lower than in the non-silenced St. Julien

(the permanent inoculum sources) (see Table 3). The low level of PPV concentration has again confirmed the stability of resistance in C5 to a Rec isolate in a high and continuous infection pressure in field conditions.

Fig. 1 PPV symptoms in field. **A:** mild diffuse spots in the leaf of C5 inoculated with PPV-Rec. **B:** very mild diffuse spots in the leaf of C5 inoculated with PPV-Rec. (Fifth year after inoculation, July 2007.) **C:** Severe yellow spots and rings in the leaf growing from non-transgenic bud St. Jluien (IB) infected with PPV-Rec.

Tab. 3 PPV copy number in St. Julien versus C5

	Year of the experiment		
	2006	**2007**	**2008**
Bottom leaves of C5	~16 fold	~300 fold	~ 11 fold
Top leaves of C5	~7300 fold	~5500 fold	~ 16 fold

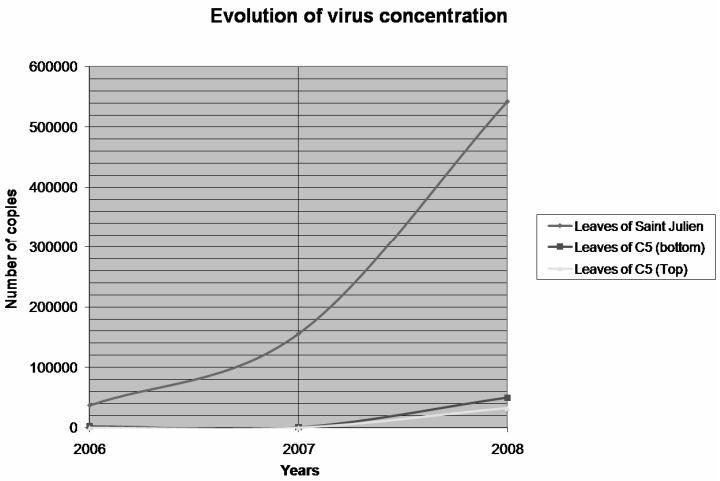

Fig. 2 PPV-rec concentration in C5 plants, detected in 1) PPV-rec inoculum source (St. Julien), 2) Bottom leaves of C5 (close to the inoculm source) and 3) Top leaves of C5. Avarage of eight individual tree ± standard error are given.

In conclusion, C5 plants maintained high level of resistance to PPV-Rec over the six years period after graft inoculation by susceptible st. Julian graft in field conditions. The Sharka symptoms appeared in most of the C5 plants, however, only very mild diffuse spots were observed. PPV concentration in C5 plants was significantly lower than in the non-silenced root stock. In the absence of inoculums pressure, the C5 pant became virus free over the time in field conditions probably due to the active systematic silencing. The above mention results from the field test and previous results from the green house (Kundu et al., 2008) showed that under a high and continual inoculums pressure, the virus can infect the C5 plant at only a very low level and that the active silencing confers the resistance to PPV in this woody perennial.

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Evaluation of different peach genotypes for resistance to *Plum pox virus* strain M: preliminary results

Pasquini, G.[1], Ferretti, L.[1], Gentili, A.[1], Campus, L.[1], Verde, I.[2], Micali, S.[2], Conte, L.[2], Barba, M.[1]
[1] CRA-PAV Centro di ricerca per la Patologia Vegetale Via C.G. Bertero, 22 00156 Roma, Italy. Email: graziella.pasquini@entecra.it
[2] CRA-FRU Centro di Ricerca per la Frutticoltura Via Fioranello, 52 00134 Roma, Italy

Abstract

Different responses to the experimental inoculation with a PPV-M isolate were observed in peach germplasm derived from crosses between *Prunus persica* and peach related species showing resistance to several pathogens. The response evaluation was performed by correlating the phenotypic analysis with serological and molecular tests.

Twenty-one individuals, asymptomatic and with low concentration of the virus inside the leaf tissues, have been selected. This germplasm can be considered 'highly tolerant' or 'resistant' and must be submitted to further investigations.

Keywords: PPV, Prunus persica, P. davidiana, P. ferganensis, tolerance.

Introduction

Plum pox virus (PPV) is the most detrimental stone fruit pathogen, generating significantly high management costs, estimated at over 10.000 million euros during the past 30 years (Cambra et al., 2006b).

The availability of genotypes which are resistant to PPV would represent a starting point to respond to economic, health and environmental problems caused by this pathogen. Some commercial apricot and plum varieties turned out to be resistant or immune to the less severe PPV-D strain. Even some commercial peach varieties resulted to be highly tolerant to the same strain. In peach germplasm, no genes conferring resistance to PPV or tolerance to PPV-M were found. Therefore, we looked for the sources of such resistance and evaluated them within the peach related species. In 1998, the wild *Amigdalus* species *Prunus davidiana*, clone P1908, which originates from China, turned out to be resistant to several pathogens of *Prunus persica*, including PPV (Pascal et al., 1998). His resistance, introduced into the *P. persica* genome using a backcross strategy, was studied on the basis of phenotypic evaluation and mapping of related candidate genes, and displayed a complex pattern of quantitative inheritance (Deqroocq et al., 2005). In this work, the preliminary results of the resistance/tolerance evaluation of different selections derived from crosses between *P. persica* and different parental genomes showing resistance to PPV or to other pathogens are reported.

The evaluation was performed by correlating the phenotypic analysis with the results obtained from different diagnostic tests. The data correlation was done using the classification scale reported in Faggioli et al., (1999), appropriately modified by introducing the real time RT-PCR (rt RT-PCR) analysis (Tab. 1).

Tab. 1 Classification of the plant response by correlation of symptoms expression on rootstock and selections and the different sensitivity of the diagnostic tests.

Phenotypic analysis		Diagnostic test			
Rootstock	**Selection**	**ELISA**	**RT-PCR**	**rt RT-PCR**	**Reaction type**
+	+	+	+	+	sensitive
+	-	+	+	+	tolerant
+	-	-	+	+	highly tolerant
+	-	-	-	+	resistant
-	-	-	-	+	highly resistant
-	-	-	-	-	immune

Materials and methods

Several selections (122), provided by the C.R.A. – Fruit Tree Research Centre of Rome - have been evaluated. All individuals derived from crosses between *P. persica* and different parental genomes showed resistance to either PPV only, or to several pathogens. The sources of resistance were represented by *P. davidiana, P. ferganensis* and some PPV-D tolerant commercial peach varieties crosses with 'Nettarina pendula' (Tab. 2).

Tab. 2 Peach selections assayed to evaluate the resistance/tolerance to PPV-M infection.

Group	Number of individuals	Parental genome
1	92	F1 hybrids: cv 'Maria Aurelia' x (*P. persica* x *P. davidiana)* (SD45).
2	6	F1 hybrids: *Prunus ferganensis*, IF7310828, F1P72 (ibrido *P. ferganensis* x IF7310828), BC1 19, BC1 25, BC1 61
3	24	*P. persica* (Glohaven, Nectaross, Flavorcrest, O'Henry) x 'Nettarina pendula' clone S2678

All selections were double-grafted in autumn on the 'GF 305' indicator-rootstock and experimentally inoculated the following spring by chip budding. The source of inoculum was a PPV-M isolate (PPV-0019-G), provided by DPPMA-UBA, University of Bari (Italy). The inoculation was performed on 3 repetitions and a non-inoculated plant was kept as the negative control. All the plants were kept in a greenhouse for two years, and both the selection and indicator shoots were left to grow. In order to exclude possible interferences in the resistance/tolerance to PPV-M due to cross-infections by other common pathogens present in peach germplasm, all the selections were assayed in advance with DAS-ELISA and RT-PCR to check for the absence of other viruses (*Apple mosaic virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Apple chlorotic leafspot virus*) and one viroid (*Peach latent mosaic viroid*), respectively. All results were negative: none of the tested pathogens was present (data not shown).

The evaluation of the phenotypic expression of PPV-specific symptoms was performed the following spring, both on the selection and indicator shoots, on the basis of an appropriate scale, based on symptom intensity across the whole plant (Tab. 3).

Tab. 3 Phenotypic scale applied for the evaluation of PPV-specific symptoms on whole plants.

Scale	Symptoms
0	No symptoms
1	Symptoms on 1-3 leaves
2	Symptoms up to 50% of leaves
3	Symptoms on more than 50% of leaves

Serological analyses were performed by TAS-ELISA with the universal MAb 5B (Cambra et al., 2006a). Molecular analyses were done by RT-PCR (Wetzel et al., 1991) and TaqMan real time (rt) RT-PCR (Olmos *et al*., 2005) using, as template, total RNA extracted from leaves by a commercial kit (RNeasy Plant Mini kit, Qiagen - Inc.,Valencia, CA). The symptoms' evaluation and the diagnostic tests were performed every fifteen days during the spring period, and the correlation between phenotypic analysis and the results obtained by the different diagnostic tests was used to classify the PPV-M infection response of the tested germplasm using the scheme reported in Table 1.

Results

Two years after the experimental PPV-M inoculation, the symptom evaluation, performed on the basis of the established scale, revealed that 17.2% (21/122) of the tested selections did not show any symptoms: 13.1% (16/122) of these asymptomatic individuals did not show any symptoms either on the selection or the GF 305 indicator shoots, whereas the remaining ones (5/122) showed symptoms only on the GF 305 indicator rootstock (Tab. 4 and 5).

Tab. 4 Percentages of symptom distribution referred to the established scale, evaluated for each groups of selections.

Symptoms scale	Group 1	Group 2	Group 3	Tot
0	13%	0%	37.5%	17.2%
1	23.9%	83.3%	8.3%	24.6%
2	49%	16.7%	37.5%	44.3%
3	14.1%	0%	16.7%	13.9%
Total of selections	92	6	24	122

Tab. 5 Percentage of symptom expression on the selection and on GF 305 indicator, evaluated for each group.

Symptoms	Group 1	Group 2	Group 3	Tot
On selection and GF 305	82.6%	100,0%	54.2%	77.9%
Only on GF 305	5.4%	0%	0%	4.1%
Only on selection	4.4%	0%	8.3%	4.9%
Asymtomatic	7.6%	0%	37.5%	13.1%
Total of selections	92	6	24	122

Starting in April, all theses were subjected to an ELISA test and all individuals that showed symptoms on the selection shoots were positive and evaluated susceptible (in accordance with the scale reported in Table 1), while all the 21 asymptomatic individuals gave negative results. Nevertheless, the asymptomatic selections showed positive results when assayed using the TaqMan rt RT-PCR (Tab. 6).

Tab. 6 Results of different diagnostics tests, periodically performed to evaluate the temporary evolution of host/virus interaction.

N°	Selection	Symptoms	rt RT-PCR 5/04/09	ELISA 21/04/09	RT-PCR 21/04/09	ELISA 04/05/09	ELISA 26/05/09
1	Dofi 06-08-005	no	15.97	1.7	+	n.t.	n.t.
2	Dofi 06-08-012	only on GF 305	29.18	0.13	-	0.12	0.11
3	Dofi 06-08-023	no	16.22	0.3	+	n.t.	n.t.
4	Dofi 06-08-024	only on GF 305	27.58	0.12	-	0.19	1.58
5	Dofi 06-08-028	only on GF 305	22.55	0.5	+	n.t.	n.t.
6	Dofi 06-08-035	no	30.05	0.13	-	0.10	0.13
7	Dofi 06-08-045	no	29.08	0.13	-	0.12	0.10
8	Dofi 06-08-052	only on GF 305	28.34	0.12	-	0.11	0.10
9	Dofi 06-08-061	no	29.33	0.12	-	0.11	0.12
10	Dofi 06-08-063	no	16.23	2	+	n.t.	n.t.
11	Dofi 06-08-076	only on GF 305	14.65	1.5	+	n.t.	n.t.
12	Dofi 06-08-088	no	26.74	0.14	-	1.09	n.t.
13	394Q-XXXVII 55	no	26.69	0.11	-	0.10	1.68
14	193R-XLIII 127	no	26.99	0.12	-	0.12	0.11
15	394Q-XXXVII 52	no	27.92	0.14	-	0.12	0.14
16	194R-XXXVII 55	no	24.26	0.12	-	1.45	n.t.
17	393Q-XIV 55	no	29.29	0.11	-	0.10	0.14
18	195R-XLIII 123	no	29.6	0.12	-	1.89	n.t.
19	194 Q-XXXIX 100	no	28.18	0.16	-	1.56	n.t.
20	394 Q-XXXVII 54	no	15.42	0.32	+	n.t.	n.t.
21	194 Q-XXXIX 118	no	29.43	0.14	-	0.12	0.14
22	Healthy peach	-	undet	0.11	-	0.12	0.11

ELISA results are reported as absorbance values at 405 nm. Absorbance values two times higher then healthy control were considered positive; RT-PCR results are reported positive when a band of the expected size was observed in 1.2% agarose gel; rt RT-PCR results are reported as Ct values; n.t. = not tested.

ELISA and RT-PCR analyses, used to monitor the evolution of host/virus interaction through time, showed the same sensitivity; therefore, only the ELISA test was periodically performed on the asymptomatic individuals. Some individuals showed positive signals in ELISA, indicating an “in-progress” evolution of the PPV infection and, at the end of May, only nine of theses were confirmed to be negative in serological analysis (Tab.7).

Tab. 7 Ct values obtained in rt RT-PCR performed on GF 305 leaves collected from ELISA negative theses

Selections	Symptoms	Ct values
Dofi 06-08-012	only on GF 305	18.10
Dofi 06-08-035	no	27.09
Dofi 06-08-045	no	32.28
Dofi 06-08-052	only on GF 305	21.02
Dofi 06-08-061	no	undet
394Q-XXXVII 55	no	undet
193R-XLIII 127	no	33.78
394Q-XXXVII 52	no	32.02
194R-XXXVII 55	no	34.14
Healthy	no	undet

In order to verify the translocation of the virus in the indicator rootstocks, an rt RT-PCR was performed on GF 305 leaves from the final nine ELISA-negative individuals, and only two of them resulted negative for the presence of the virus (Table 8).

Tab. 8 Classification of evaluated germplasm response to PPV infection

N°	Group	Selection	Type of reaction
1	1	Dofi 06-08-005	tolerant
2	1	Dofi 06-08-012	resistant
3	1	Dofi 06-08-023	tolerant
4	1	Dofi 06-08-024	tolerant
5	1	Dofi 06-08-028	tolerant
6	1	Dofi 06-08-035	higlhy resistent
7	1	Dofi 06-08-045	higlhy resistent
8	1	Dofi 06-08-052	resistant
9	1	Dofi 06-08-061	higlhy resistent
10	1	Dofi 06-08-063	tolerant
11	1	Dofi 06-08-076	tolerant
12	1	Dofi 06-08-088	tolerant
13	3	394 Q-XXXVII 55	higlhy resistent
14	3	195R -XLIII 127	higlhy resistent
15	3	394Q -XXXVII 52	higlhy resistent
16	3	194R XXXIX 65	tolerant
17	3	393Q XIV 55	higlhy resistent
18	3	195R XLIII 123	tolerant
19	3	194Q XXXIX 100	tolerant
20	3	394Q XXXVII 54	tolerant
21	3	194 Q XXXIX 118	tolerant

Discussion

A high percentage of asymptomatic selections (17.2%) were obtained after two years from the experimental inoculation with the PPV-M strain, indicating an interesting level of resistance/tolerance of the evaluated germplasm. Only the selections derived from the *P. ferganensis* parental genome resulted to be sensitive, as they showed specific symptoms both on the selection and indicator shoots, making it pointless to identify genes conferring resistance or tolerance to PPV-M from this specific germplasm.

At the end of the evaluation tests, nine asymptomatic selections resulted positive only in RT-PCR, indicating the presence of very low concentration of the virus inside the plant tissues. According to the new classification scheme now in use, these selections may be classified as highly resistant germplasm, which renders these results particularly interesting. Moreover, in two individuals, the asymptomatic GF 305 indicator resulted negative in rt RT-PCR analysis for the presence of PPV, confirming that the virus had failed to relocate itself to the lower part of the plant, a result already found in a previous paper (Decrooq et al., 2005). The PPV-M infection response of the remaining asymptomatic individuals ranged from tolerant to resistant (Tab. 8). All these promising individuals have been derived both from 'Maria Aurelia' x SD45 F_1 hybrid (*P. persica* x *P. davidiana*) and from some commercial peach cultivars crossed with weeping peaches (S2678).

Contemporarily to this investigation, a framework molecular map of the evaluated progenies was obtained by genetists (Micali et al., 2009) and further investigation is taking place to implement the results and to correlate the genome mapping with the plants response.

The 21 asymptomatic selections will be kept for one more year in the screenhouse so as to verify the temporal stability of their response to the experimental PPV-M infection. If the resistance/tolerance response will be confirmed, field trials will be established in areas where the disease is endemic, so as to also verify the validity of the evaluated response under the natural inoculum pressure by aphid vectors.

Acknowledgements

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Biolistic transfection of plants by infectious cDNA clones of *Plum pox virus*

Šubr, Z.W., Nagyová, A., Glasa, M.
Institute of Virology, Slovac Academy of Sciences, Dpt. Plant Virology, Dúbravská 9, 84505 Bratislava, Slovakia.
Email: virusubr@savba.sk

Abstract

Plant biolistic transfection by two *Plum pox virus* (PPV) infectious cDNA clones (strains PPV-M and PPV-D) using the gene gun apparatus PDS 1000-He was optimized. *Nicotiana benthamiana* plants were germinated on Petri dishes with MS growth medium. At the age of four weeks the plants were subjected to biolistic transfection and three days later were transplanted into common soil substrate. The plant survival after transplantation was about 70 %, the transfection efficiency was over 80 % (compared to 6 – 10 % efficiency reached by mechanical plant inoculation). The plants showed typical PPV symptoms two weeks post transfection. The virus presence was confirmed by immunoblotting, RT-PCR, as well as by successful transmission by sap to healthy plants. The co-transfection of *N. benthamiana* plants by PPV-M and PPV-D led to mixed infections with PPV-D strongly prevalent. We assumed the properties of cDNA constructs responsible for this behaviour.

Keywords: gene gun, PPV strains, immunoblotting

Introduction

Infectious clones of plant RNA viruses are excellent tools for research of the intracellular infection process (virus-host interactions), as well as the base for preparation of viral vectors for transient expression of exogenous genes in plants. Agroinfection, biolistic transfection, electroporation and mechanic inoculation are the most common methods for introducing foreign DNA into plant cells (Nagyová and Šubr, 2007).

PPV causes detrimental Sharka disease of stone fruit trees in many countries, especially in European and Mediterranean regions. Three of six recognized PPV strains have an economic impact, namely PPV-M, PPV-D and PPV-Rec (Candresse and Cambra, 2006). The relatively recently recognized strain PPV-Rec (evolved by homologous recombination of PPV-D and PPV-M) is commonly spread in central and southern Europe (Glasa et al., 2004). Detailed genomic functional analysis could help elucidate the high fitness of this strain under natural conditions. Therefore in our laboratory attempts are done to prepare an infectious cDNA clone of PPV-Rec. An efficient method of plant transfection is essential for this project. In this work we used infectious clones of PPV-D and PPV-M to optimize a simple biolistic transfection protocol and compare it to mechanical inoculation.

Material and methods

PPV cDNA clones designated pIC PPV-D (isolate Rankovic) and pIC PPV-M (isolate SK68) were kindly provided by Prof. García (CSIC Madrid, Spain) and Prof. Palkovics (CU Budapest, Hungary), respectively (López-Moya and García, 2000; Raughpathy et al., 2006). The plasmids were maintained in *Escherichia coli* DH5α and isolated by alkaline lysis. The *N. benthamiana* plants were transfected mechanically or biolistically using the PDS-1000 He apparatus (Biorad) after DNA binding to the tungsten microparticles M-10 according the manufacturer's recommendation. Four weeks old seedlings of *N. benthamiana* (5 – 6 leaf stage, total leaf surface about 1.5 cm^2 per plant) grown aseptically by two – five on Petri dishes with MS medium were used as target for biolistic transfection. Three days after bombardment the plants were transfered to common soil substrate. The transfection efficiency was evaluated visually (symptom recording) and by immunoblots of crude plant extracts using monoclonal (MAb) or polyclonal antibodies against PPV (Boscia et al., 1997; Šubr and Matisová, 1999). RT-PCR amplifying the NIb-CP viral genome region was performed too. Reverse transcription as described by Glasa et al. (2002), was followed by PCR using strain-unspecific primers

NCuniFor (5'-GAGGCAATTTGTGCTTCAATGG-3') and
NCuniRev (5'-CGCTTAACTCCTTCATACCAAG-3')

under following conditions: 95ºC/5 min, 40 x (95ºC/15 s, 60ºC/30 s, 72ºC/1 min), 72ºC/10 min.

Results and discussion

PDS-1000 He is the only type of biolistic instrument available in Slovakia today. Because its construction with a vacuum box limits target size small plant seedlings arranged on Petri dishes were used. About 70 % of plants survived the subsequent transplanting procedure. Two weeks later typical symptoms of PPV infection were observed (leaf distortion and mosaic). The presence of the virus in systemically infected (not bombarded) leaves was verified by RT-PCR with PPV-specific primers (Fig. 1), and its infectivity was proved by succesful mechanical sap transmission to healthy plants. Results of the immunoblot analyses generally agreed with symptom manifestation.

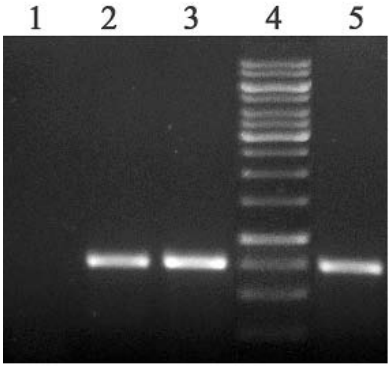

Fig. 1 RT-PCR detection of PPV from biolistically transfected *N. benthamiana*. Lane 1 – negative control (healthy plant), lane 2 – pIC PPV-D-transfected plant, lane 3 – pIC PPV-M-transfected plant, lane 4 – 1 kbp DNA ladder, lane 5 – positive control (PPV-D-infected plant).

The efficiency of biolistic transfection was markedly higher (about 80 % for both clones) compared to mechanical inoculation (6 % for pIC PPV-M, 10 % for pIC PPV-D). We found no influence of helium pressure or target distance from the microparticle source on transfection efficacy (Tab. 1).

Tab. 1 Efficiency of biolistic transfection under various conditions. p – helium pressure, X – distance between the particle source and target tissue, η – transfection efficiency (% of infected plants).

Isolate	p [kPa]	X [cm]	η [%]
SK68	7580	9	73.3
		12	80
	9300	9	81.3
		12	75
Rankovic	7580	9	86.7
		12	75
	9300	9	80
		12	77.8

pIC PPV-D caused slightly more intensive symptoms and it gave a much stronger immunoblot signal than pIC PPV-M. Cotransfection by both infectious clones led to mixed infections with PPV-D being strongly prevalent (Fig. 2). Such difference in capsid protein concentration was not observed with mechanically passaged virus isolates Rankovic and SK68. We suggest different relative infectivity reflected rather properties of constructed cDNA clones than the PPV isolates. Stronger expression could be caused by the doubled 35S promoter in pIC PPV-D. Moreover, absence of the NOS terminator in the pIC PPV-M could lead to the production of longer primary transcripts and problems with their translocation to the cytoplasm where proteosynthesis takes place.

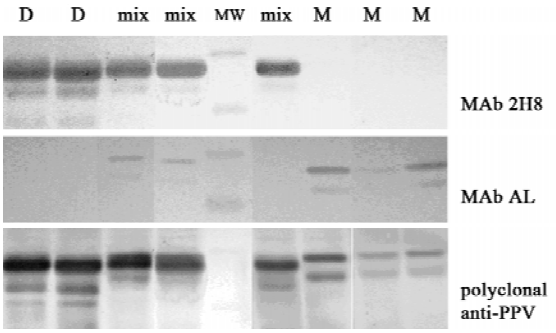

Fig. 2 Immunoblot analysis of *N. benthamiana* transfected with pIC PPV-M, pIC PPV-D and with a mixture of both. MW – molecular weight marker (bands correspond to 47 and 34 kDa); used antibodies are indicated on the right border.

Mechanic inoculation is simple and inexpensive method commonly used for the infection of plants by viruses in laboratory conditions. However, the transfer of isolated DNA into the nucleus without drastically disturbing the cell is statistically improbable using this method. Low transfection efficiency disables to detect changes of infectivity resulted e.g. from directed genome manipulation.

Biolistic techniques are more gentle and facilitate entry into the cell nuclei even in internal tissues. Of the two types of biolistic apparatuses the gun-shaped devices are more flexible and better suited for whole plant transfection. However, PDS-1000 He is the only type of biolistic device available presently in Slovakia. Our optimized protocol enabled a high efficiency of transfection of *N. benthamiana* plants by PPV cDNA clones.

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Typing and distribution of *Plum pox* virus isolates in Romania

Zagrai, I.[1], Zagrai, L.[1], Kelemen, B.[2], Petricele, I.[3], Pamfil, D.[3], Popescu, O.[2], Preda S.[4], Briciu, A.[3]
[1] Fruit Research and Development Station Bistrita, Romania. Email: izagrai@yahoo.com
[2] Babes Bolyai University, Faculty of Biology and Geology, Cluj-Napoca, Romania
[3] University of Agricultural Science and Veterinary Medicine, Cluj-Napoca, Romania
[4] Fruit Research and Development Station Valcea, Romania

Abstract

Plum pox or Sharka, caused by *Plum pox virus* (PPV) is considered the most destructive disease of plum. Although PPV is widespread in all plum growing areas of Romania and causes serious yield losses, little is known about the variability of its isolates at a country level. For this reason, a large-scale study was performed with the aim of obtaining a picture of the prevalence and distribution of PPV strains in plum. During a three year survey, 200 PPV isolates collected from 23 different plum orchards from Transylvania, Moldavia and Muntenia areas were investigated. DAS-ELISA and IC-RT-PCR were used for PPV detection. PPV strains were serologically determined by TAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies. Molecular strain typing was done by IC/RT-PCR targeting three genomic regions corresponding to (Cter)CP, (Cter)NIb/(Nter)CP and CI. RFLP analysis was used to distinguish D and M strains, based on the *Rsa*I polymorphism located in (Cter)CP. To confirm the presence of PPV-Rec strain, 13 PCR products spanning the (Cter)NIb/(Nter)CP were sequenced. Overall results showed that in Romania the predominant strain is PPV-D (73%), followed, with a much lower frequency, by PPV-Rec (14%). Mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent (13%).

Keywords: Romania, PPV strains, DAS/TAS-ELISA, IC/RT-PCR, RFLP, sequencing

Introduction

Plum pox or Sharka is the most devastating disease of stone fruits. The disease is highly detrimental because it reduces the quality of the fruits and causes their premature dropping. (Dunez and Sutic, 1988; Nemeth, 1994). Sharka disease was described for the first time around 1917 in Bulgaria (Atanasoff, 1932). Since then, the disease has progressively spread to a large part of the European continent, around the Mediterranean basin, in Asia (India, China, Pakistan, Kazakhstan and Iran) as well as in America (Chile, Argentina, USA and Canada) (Capote et al., 2006; García and Cambra 2007). Therefore, this disease is among the most significant limiting factors for plum production (Stoev et al., 2004). In Romania, Sharka occurs in all plum growing areas causing serious yield losses especially to sensitive cultivars (Minoiu, 1997; Zagrai et al., 2001).

To control the virus spreading it is important to know the distribution of the virus and the different strains occurring (Pasquini and Barba, 1994). Seven strains of PPV have been reported so far. Two major groups, PPV-D and PPV-M (Kerlan and Dunez, 1976) can be distinguished by strain-specific monoclonal antibodies (Boscia et al., 1997; Cambra et al., 1994), and also by the *Rsa*I polymorphism in the DNA fragment amplified by P1/P2 primer pairs located at the C-terminus of the PPV CP gene (Wetzel et al., 1991a) or by direct IC/RT-PCR typing using PD and PM specific oligonucleotides (Olmos et al., 1997). The third major group was identified and denoted PPV-Rec (Glasa et al, 2002). This natural recombinant between PPV-D and PPV-M was reported in Albania, Bulgaria, Czech Republic, Germany, Hungary, Slovakia (Glasa et al., 2002, 2004), Bosnia and Herzegovina (Matic et al., 2006), Pakistan (Kollerova et al., 2006), Romania (Zagrai et. al., 2006, 2008), Turkey (Candresse et al., 2007) and Canada (Thompson et. al., 2009). Three additional minor PPV groups are represented by geographically limited strains El Amar (PPV-EA) originally isolated from Egypt (Wetzel et al., 1991b), Cherry (PPV-C) isolated from sour cherry in Moldavia (Kalashyan et al., 1994) and from sweet cherry in southern Italy (Crescenzi et al., 1997) and Romania (Maxim et al., 2002a, 2002b), and Winnona (PPV-W) from Canada (James and Varga, 2004). A new PPV strain was recently isolated from apricot in Turkey and called PPV-T (Serce et al., 2009).

The objective of the present study was to provide a picture of the prevalence and distribution of PPV strains occurring in Romania plum orchards.

Materials and methods

Two hundred PPV isolates were collected from 23 different plum orchards from Transylvania, Moldavia and Muntenia areas of Romania. Sampling was initially based on typical PPV symptoms and virus infection was confirmed by serological and molecular testing. Serological diagnosis was made by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum (Bioreba, Switzerland) according to the manufacturer's instructions. Molecular detection was done by IC-RT-PCR using the pair of primers P1/P2 and trapping with the above polyclonal antiserum. Qiagen one-step kit (Qiagen, Germany) was used for RT-PCR.

Serological discrimination was made by TAS-ELISA using the PPV-D and PPV-M specific monoclonal antibodies (Durviz, Spain) according to Cambra et al. (2004). Molecular strain typing was done by IC/RT-PCR targeting three genomic regions corresponding to:

(i) (Cter) CP, using P1/PD and P1/PM pair of primers that distinguish PPV-D and PPV-M, respectively;
(ii) (Cter) NIb/(Nter)CP, using mD5/mM3 pair of primers (Subr et al., 2004) that detect natural recombinants between D and M (PPV-Rec);
(iii) CI, using CIf/CID or CIf/CIM primer sets (Glasa et al., 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter)CP were subjected to RFLP analysis to distinguish D strains from M strains based on the *Rsa*I polymorphism located in this genomic region. To check if the recombination breakpoint position suspected to occur in the (Cter)NIb/ (Nter)CP region corresponds with those previously reported for PPV-Rec, 13 PCR products spanning (Cter) NIb/(Nter)CP region were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA), and then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The samples were run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The alignment of nucleotides was done using the BioEdit package version 5.0.9 (Hall, 1999). Obtained sequences were then compared with those available in GeneBank.

Results and discussion

Similar results were obtained in the differentiation of PPV isolates by TAS-ELISA using D and M monoclonal antibodies and by IC/RT-PCR using PD and PM specific primers (Table 1). All isolates reacted positively to at least one of the two monoclonal antibodies as well as PPV-D or/and PPV-M specific primers. In a few cases, the mixed infection could not be detected by TAS-ELISA. This could be explained by the lower sensitivity of serological techniques in comparison with molecular techniques. RFLP analysis confirmed the IC/RT-PCR results based on the presence of the *Rsa*I polymorphism in the PPV-D strain.

Tab. 1 Serological and molecular differentiation of 200 PPV isolates from Transylvania, Muntenia and Moldavia

			DAS-ELISA			IC/RT-PCR				RFPL				Rate of
Area	No of isolates		PPV poly	PPV-D	PPV-M	P1/P2	P1/PD	P1/PM	mD5/mM3	CIf/CID	CIf/CIM	*Rsa*I	Strain status	infection %
		70	+	+	-	+	+	-		+	-	+	PPV-D	70
Transylvania	100	18	+	-	+	+	-	+	+	+	-	-	PPV-Rec	18
		12	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	12
		34	+	+	-	+	+	-		+	-	+	PPV-D	84
Muntenia	50	4	+	-	+	+	-	+	+	+	-	-	PPV-Rec	12
		12	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	4
		42	+	+	-	+	+	-		+	-	+	PPV-D	68
Moldavia	50	6	+	-	+	+	-	+	+	+	-	-	PPV-Rec	8
		2	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+ PPV-Rec	24

Using the primer pair (mD5/mM3) targeting the (Cter)NIb/(Nter)CP region, it was observed that all PPV isolates typed as PPV-M in (Cter) CP were in fact PPV-Rec. Using specific primers to distinguish the two strains D and M in the CI region only fragments belonging to PPV-D were detected, thus confirming the presence of PPV-Rec. The typing of PPV isolates from Transylvania, Moldavia and Muntenia areas revealed that PPV-D and PPV-Rec occurred in the plum orchards from Romania. In all three areas PPV-D is the prevalent strain. The higher incidence of PPV-D was noticed in Moldavia (84%) and the higher rate of PPV-Rec was recorded in Transylvania (18%). Mixed infections (D+Rec) were more frequent in Muntenia (24 %). Multiple sequence alignment of the 13 PCR products spanning (Cter) NIb/(Nter)CP region showed that the recombination breakpoint is located in the region corresponding to (Cter)NIb at nucleotide position 8450 (Figure 1).

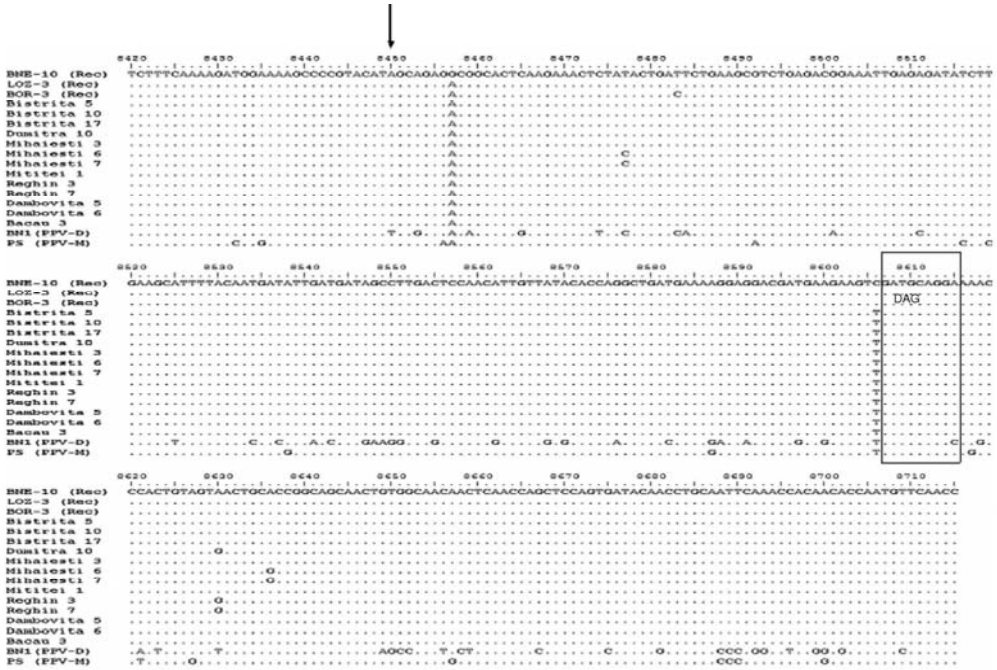

Figure 1 Multiple alignment of recombinant sequences (NIb/CP) of 13 Romanian isolates (Bistrita 5, Bistrita 10, Bistrita 17, Dumitra 10, Mihaiesti 3, Mihaiesti 6, Mihaiesti 7, Mititei 1, Reghin 3, Reghin 7, Dambovita 5, Dambovita 7, Bacau 3) and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR–3 (accession number AY028309)] previously reported.

The DAG motif that is considered as essential for aphid transmission was also present. As expected, this site was located downstream of the recombination breakpoint. Based on a comparative alignment, the sequencing results revealed a high similarity (98%) with different sequences of PPV-Rec available in GeneBank. All these recombinant isolates shared the same recombination breakpoint. Overall results presented in the table 2 showed that in Romania the predominant strain is PPV-D (73%), follow with a much lower frequency by PPV-Rec (14%). Although a big difference between the incidence of PPV-D and PPV-Rec was recorded, our results confirmed that the recombinant strain represents a major PPV group. Mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent (13%).

Tab. 2 Synthesis of serological and molecular differentiation of 200 PPV isolates from Romania

	DAS/TAS-ELISA					IC/RT-PCR				RFLP		
No. of isolates	PPV poly	PPV-D	PPV-M	P1/P2	P1/PD	P1/PM	mD5/ mM3	CIf/ CID	CIf/ CIM	*RsaI*	Strain status	Rate of infection %
146	+	+	-	+	+	-	-	+	-	+	PPV-D	73
28	+	-	+	+	-	+	+	+	-	-	PPV-Rec	14
26	+	+/-	+/-	+	+	+	+	+	-	+	PPV-D+PPV-Rec	13

Conclusions

Both PPV-D and PPV-Rec occurred in plum orchards of Romania. PPV-D is the prevalent strain in all the three plum growing areas and at country level, too. There was a higher incidence of PPV-D in Moldavia and a higher rate of PPV-Rec in Transylvania. The mixed infections (D+Rec) were more frequent in Muntenia.

Acknowledgments

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Preliminary studies on the use of the Cascade Rolling Circle Amplification technique for *Plum pox virus* detection

Hadersdorfer, J.[1], Neumüller, M.[1], Fischer, T.[2], Treutter, D.[1]
[1] Technische Universität München, Center of Life and Food Science Weihenstephan,
Unit of Fruit Science, Dürnast 2, D-85354 Freising, Germany. Email: johannes.hadersdorfer@wzw.tum.de
[2] Ludwig-Maximilians-Universität München, Department of Biology I, Chair of Biochemistry and Physiology of Plants, Großhadenerstr. 2-4, D-82152 Martinsried, Germany

Abstract

Isothermal techniques for the amplification of nucleic acids have emerged in the last years. In contrast to the Polymerase chain reaction (PCR), the most prevalent method to amplify DNA *in vitro*, the reactions can be run at constant temperatures. Specificity and sensitivity are at least as high as that obtained by using PCR and the methods are less time consuming. Therefore, the isothermal amplification of nucleic acids provides a powerful tool for the detection of *Plum pox virus* (PPV), the causal agent of the Sharka disease.

The cascade rolling circle amplification (CRCA), first described by Thomas et al. (1999), is based on the rolling circle mechanism that many viruses use to replicate their genome multiplicatively. Circular Probes, also called Padlock probes (PLP), which arise from the ligation of the terminal region of DNA probes upon side by side hybridization to the target serve as template (Nilsson et al. 1994).

For detecting PPV by CRCA, RNA was extracted and reverse transcribed to cDNA using a PPV specific primer. Several PLPs with varying lengths and sequences complementary region to the cDNA were designed and tested. Furthermore, different pairs of primers for the subsequent amplification were developed. For specific ligation Ampligase and T4 DNA Ligase were tested. In CRCA, two polymerases with strong strand displacement activity were compared: Phi29 DNA Polymerase and *Bst* DNA Polymerase. These enzymes differ in their optimal reaction temperature.

Ligation as well as amplification do occur, but there is high background amplification also in negative and no template controls. Discrimination was possible after a restriction digestion is carried out. As proven by sequencing of reaction products non-specific signals were a result of primer polymerization. Current work focuses on the reduction of the background amplification and improvement of the sensitivity.

Keywords: Cascade Rolling Circle Amplification, CRCA, isothermal amplification of DNA, *Plum pox virus*, *PPV*

Introduction:

Today the Polymerase chain reaction (PCR) is the tool of choice to amplify DNA *in vitro* because of its versatile capabilities in the field of molecular biological research. However, in recent years several isothermal techniques for the amplification of nucleic acids have emerged (Gill and Ghaemi 2008). In PCR, denaturation, priming and elongation are done step-by-step at alternating temperatures whereas in isothermal techniques all steps take place simultaneously avoiding the use of a thermal cycler. Regarding specificity and sensitivity these methods are equal to or even better than PCR. Therefore, the isothermal amplification of nucleic acids should also provide a reliable detection method for *Plum pox virus* (PPV), the causal agent of Sharka disease.

Artificial DNA circles with far less than 100 nucleotides can be enzymatically amplified via the same rolling circle mechanism many viruses use to multiply their genome (Fire et al. 1995, Liu et al. 1996). Such a DNA circle, also called Padlock probe (PLP), can arise from the ligation of the terminal regions of a linear DNA probe upon side by side hybridization to the target (Nilsson et al. 1994). The ligase mediated generation of PLPs combined with rolling circle replication represents a powerful system for the detection of DNA with both high specificity and sensitivity (Banér et al. 1998, Lizardi et al. 1998).

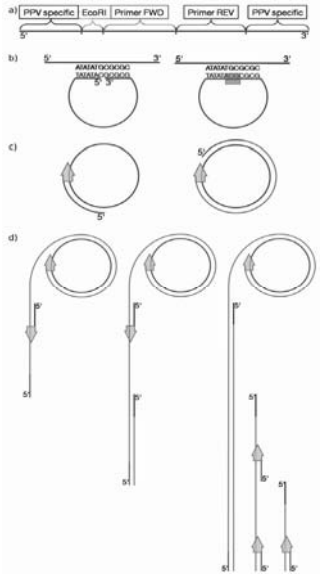

Fig. 1 Cascade rolling circle amplification (CRCA): a) design of a linear DNA probe; b) enzymatic ligation of the PLP upon hybridization to PPV cDNA; c) rolling circle amplification of the circular PLP using a polymerase with strand displacement activity; d) advanced, cascade like amplification.

Thomas et al. (1999) advanced the method with the amplification of the released strand to achieve exponential accumulation of DNA creating the cascade rolling circle amplification (CRCA) (Fig. 1). After gel electrophoresis a ladder like pattern is visible with each step indicating a multimere of the PLP. To verify the use of CRCA for PPV diagnostics preliminary tests were conducted.

Material and methods

For the evaluation of the new PPV detection method based on CRCA PPV free and PPV infected trees of the *Prunus domestica* cultivars `Katinka´ and `Jojo´ were chosen for PPV positive and negative controls, respectively.

RNA was extracted from plum leaves using "Concert™ Plant RNA Reagent" (Invitrogen) according to the manufacturer´s instructions. For the cDNA synthesis carried out by M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) 1 µg total RNA, serving as template, and 25 mM PPV specific primer were filled up to a volume of 17.75 µl by RNase free water and preincubated for 5 min at 70 °C. 5 µl M-MLV reaction buffer, 0.5 mM dNTPs and 200 U of the enzyme were added to obtain a final volume of 25 µl. The incubation time was 1 h starting at 40 °C and raising the temperature to 48 °C after 10 min.

For general detection of PPV the sequences of several isolates representing all PPV subgroups known so far were aligned using ClustalW as hosted on http://www.ebi.ac.uk/Tools/clustalw/index.html. A homologous region located in the coding sequence of the coat protein was selected as the hybridization site of the PLP to the cDNA (Fig. 3).

The PLP consists of two complementary sequences at the terminal regions linked with the restriction site of *EcoR*I, the complementary sequence of Primer FWD and the identical sequence of Primer REV (Fig. 1). These primers were designed to bind exclusively to the PLP and its complement respectively, but not to PPV and known *Prunus* nucleotide sequences as screened by BLAST analysis. 0.5 U Ampligase® Thermostable DNA Ligase (Epicentre™ Biotechnologies) was used to join the 5'- and the 3'-end of the linear PLP after hybridization to PPV cDNA at 64 °C for 15 minutes with three minutes of preincubation at 94 °C in a volume of 15 µl containing Reaction Buffer, 0.12 µM PLP and cDNA. After adding ThermoPol Buffer, 0.2 µM dNTPs, 1 µM of each primer, 0.75 M betaine and 1.6 U *Bst* DNA Polymerase (New England Biolabs (NEB)) to an aliquot of the ligation reaction the CRCA was carried out in 60 minutes at 65 °C. The enzyme was inactivated by heat (20 min at 80 °C). For restriction digestion, *EcoR*I (Fermentas) was used.

Comparison of Ligases: There are a number of different ligases available. To test which DNA joining enzyme provides the optimal basis for the following amplification three further ligases were compared with the standard protocol mentioned above: *Taq* DNA Ligase (New England Biolabs) and 9°N™ DNA Ligase (New England Biolabs) are active at elevated temperatures like Ampligase® Thermostable DNA Ligase, whereas T4 DNA Ligase (New England Biolabs) has its optimum at 37 °C. Results obtained by *Taq* DNA ligase were similar to those by Ampligase® Thermostable DNA Ligase: strong amplification of the positive control, but also background amplification in negative and no template control, so definite discrimination was only possible after restriction digestion. 9°N™ DNA Ligase resulted in a very weak signal. The T4 DNA Ligase produced DNA circles in either case at the same rate indicating an unspecific reaction as a result of the ligation of the PLP without hybridisation to a target (Fig. 2a).

Activity of Polymerases: The second type of enzyme used in this method is the polymerase. The range of polymerases that meet the demand of high strand displacement activity is limited. *Bst* DNA Polymerase was tested against IsoTherm™ DNA Polymerase (Epicentre™ Biotechnologies), DisplaceAce™ DNA Polymerase (Epicentre™ Biotechnologies), both active at 65 °C, and Phi29 DNA Polymerase (New England Biolabs), which exhibits a very high processivity with an optimum at 37 °C. The CRCA performed by the *Bst* DNA Polymerase yielded high amounts of DNA. The incubation time in case of IsoTherm™ and DisplaceAce™ DNA Polymerase was twice as long as that of *Bst* DNA Polymerase, but the intensity of the signal using these thermophilic enzymes was weaker. Phi29 DNA Polymerase did not synthesize any DNA even when using exonuclease resistant primers (Fig. 2b).

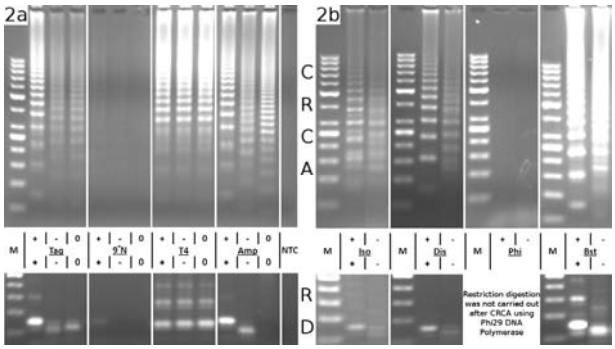

Fig. 2 **a**: CRCA of a DNA probe PLP4 ligated by four different ligases. Taq: *Taq* DNA Ligase, 9°N: 9°N™ DNA Ligase, T4: T4 DNA Ligase, Amp: Ampligase® Thermostable DNA Ligase; +: positive control, -: negative control, 0: no template control, NTC: control without ligation reaction, M: GeneRuler™ 50bp DNA Ladder**;** CRCA: cascade rolling circle amplification, RD: restriction digestion. **2b**: CRCA of the DNA probe PLP4 with four DNA polymerases. Iso: IsoTherm™ DNA Polymerase, Dis: DisplaceAce™ DNA Polymerase, Phi: Phi29 DNA Polymerase, Bst: *Bst* DNA Polymerase; +: positive control, -: negative control, M: GeneRuler™ 50bp DNA Ladder**;** CRCA: cascade rolling circle amplification, RD: restriction digestion.

Probe and Primer Design: Both the primer sequence and the PPV complementary regions of the PLP were varied because of the potential for a high background signal . All designed primers were specific just to the PLP and not to plum or PPV. As proven by the sequencing of reaction products non-specific signals are a result of primer polymerization, which do also occur with two other pairs of primer. Moving the hybridization region (Fig. 3) on the PPV cDNA resulted in a lower signal strength, whereas the variation of the length of the PPV complementary region caused hardly any alternation but did not change the intensity of the background signal either.

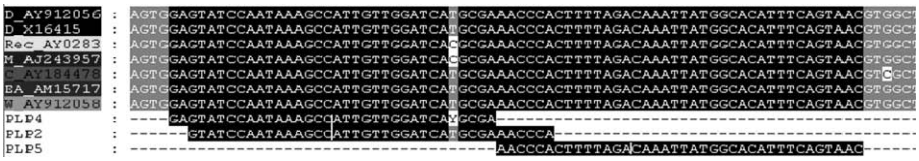

Fig. 3 Alignment of the nucleotide sequence of seven PPV isolates representing all known PPV subgroups and the recognition sites of the tested DNA probes (PLP). Gene bank accession numbers are listed in the picture. The yellow bar indicates the ligation site.

Conclusion

Ligation as well as amplification did occur, but there was high background amplification also in negative and no template controls. Discrimination is possible after restriction digestion is carried out. For establishing a reliable and sensitive PPV detection method the background amplification must be reduced and sensitivity increased.

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Survey on *Plum pox virus* in Norway

Blystad, D.-R.[1], Knudsen, R.[2], Spetz, C.[1], Haugslien, S.[1], Ørstad, K.[1], Cambra, M.[3], Munthe, T.[1]
[1] Norwegian Institute for Agricultural and Environmental Research, Plant Health and Plant Protection Division
[2] Norwegian Food Safety Authority
[3] IVIA, Spain

Abstract

In 1998 *Plum pox virus* (PPV) was detected for the first time in Norway. Virus-like symptoms were observed on several trees in a collection of plum cultivars at Njøs Research Station in the Sogn og Fjordane County in West Norway. The Norwegian Food Safety Authority and the Norwegian Crop Research Institute immediately started surveying other variety collections around the country, nuclear stock material and orchards in all important plum-growing areas. Since 1998 we have surveyed the main part of the commercial plum orchards in Norway. About 75 000 individual trees have been tested. About 1 % of the trees have been found infected by PPV. Only the PPV-D strain has been found. It is suspected that the main infection source was infected plums or apricots imported to Njøs around 1970 or earlier. In most plum orchards in Norway, the spread of PPV by aphids is relatively slow. Therefore, we expect to be able to eradicate PPV from commercial plum orchards in the near future. The eradication work is continuing.

Keywords: *Plum pox virus*, survey

Introduction

We did not think Plum pox virus (PPV) could be a problem in Norway until we detected PPV in a collection of plums at Njøs, Sogn og Fjordane County, in 1998. Almost all virus-like symptoms seen in this collection could be related to PPV and ca 22 % of the trees were infected by PPV. We also found *Apple mosaic virus*, *Prunus necrotic ringspot virus*, *Prune dwarf virus* and *Apple chlortic ringspot virus*, but none of these viruses seemed to be as related to the symptoms as PPV (Blystad et al. 2007). The pattern of distribution of infected trees, including the spread to an orchard of plum seedlings hinted to an insect transmission of PPV. By registration of the spread of virus into a nearby orchard of plum seedlings we also got strong indications for aphid spread on this location. Since then the Norwegian Food Safety Authority and Bioforsk have surveyed and tested samples from all commercial orchards all over Norway. PPV has now been found in all counties with commercial plum production (Blystad & Munthe 2006). Apricot and peach are not produced commercially in Norway, but are grown in private gardens in some of the southern areas of Norway.

PPV is the most damaging plum disease under European conditions. After its first discovery in Bulgaria (Antanassof 1933) it has spread to most European countries and is causing heavy losses in plum and apricot production in several countries (Roy & Smith 1994, Capote et al. 2006). So far there are no reports from other Nordic countries showing an extensive spread of PPV. Single cases have been reported in Denmark, but none from Sweden or Finland (Roy & Smith 1994, Lemmetty 2006)..

This publication gives an overview of the results from the survey for PPV in Norway up to the end of the season of 2008.

Materials and methods

The plum orchards in each district have been chosen according to official overviews over commercial plum orchards and from contact with the local extension officers. Private gardens were included in the survey in 1999, but as we found no infection in private orchards during this first year of the survey, the survey was concentrated on the commercial orchards in the following years.

All plum fields on a selected farm were inspected by officers from the Norwegian Food Safety Authority. From each field 15 trees were selected randomly and one leaf of each tree was collected. Subsequently, 5 leaves of each individual tree were combined into one sample. In addition, any tree showing typical PPV symptoms was sampled individually. If PPV was found in one or more combined samples from a farm, all plum trees on the farm were tested.

All samples were kept cool during storage and transport to the testing lab at Bioforsk. All samples were registered in a database. Detection of PPV was carried out by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using reagents bought from Bioreba and according to the manufacturer's descriptions. Strain identification of PVV was carried out as described in Cambra et al. (2006) and Olmos et al. (2006).

Results

Møre og Romsdal County: In this county altogether 23 farms have been surveyed and PPV has been found on 4 of them. Field observations indicate that PPV has most likely been spread by aphids in these farms. Large portions of the infected orchards have been eradicated to stop the spread. The survey and eradication is still ongoing in this county

Sogn og Fjordane: A total of 344 orchards and private gardens have been surveyed since 1998. PPV has been found on 20 farms (no cases in private gardens). Most infected trees have been of the cultivar 'Mallard'. All cases have been eradicated .The survey and eradication of infected trees have been an effective control of PPV in this county.

Hordaland: PPV has been found in 13 of 217 surveyed farms in Hordaland County. All these cases have been eradicated. The eradication of PPV in this county seems promising and successful.

Rogaland: Since 1998 a total of 50 farms have been surveyed. PPV was found on 9 of them. On 3 of these farms all infected trees were removed in 2000. However, in a subsequent inspection in 2006, newly infected trees were found on three farms. In 2008 no new infections were found. We hope PPV is now eradicated also from this county.

Aust-Agder: In this county PPV has been found in only one small plum orchard and has been eradicated.

Hedmark: Hedmark came relatively late into the survey, but 9 farms with commercial orchards were surveyed in 2007-2008. PPV was found in the orchards of two of the larger farms. The eradication work is ongoing.

Akershus: In this county PPV has been found in just one nursery and has been eradicated.

Buskerud: Altogether 43 farms have been surveyed. PPV was detected on 7 of them. Five of these cases were detected in the period 1998-2006. However, all were eradicated by 2007. In 2008 PPV was found on two new farms.

Vestfold: In this county PPV has been found on 4 of 25 farms surveyed since 1998. Most infected trees have been of the cultivars 'Herman' and 'Victoria'.

Discussion

During the course of this survey (1998-2008), we have found 852 PPV-infected trees among about 75 000 tested trees. In other words, about 1 % of the plum trees have turned out to be infected. In the period from 1998-2008 PPV have been found on 61 farms or nurseries, from which 5 of these were detected in 2007-2008 The eradication work have been successful in important counties such as Hordaland and Sogn and Fjordane. We believe that it is possible to eradicate PPV from Norwegian plum production as there are no wild Prunus-species in Norway functioning as a PPV reservoir for new infections. The peach aphid (*Myzus persicae*) does not survive our winters, sothe most efficient vector for PPV is not important in Norwegian orchards. Our experience from this survey shows the importance of repeated inspections and sampling from infected orchards both to find newly infected trees and to find trees that might have escaped detection in the first inspection. Among the PPV strains occurring most often in *Prunus* spp (PPV-D, PPV-M and PPV-Rec; Candresse & Cambra 2006), the only strain so far detected in Norway is PPV-D.

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Pospiviroidae viroids in naturally infected stone and pome fruits in Greece

Kaponi, M.S.[1], Luigi, M.[2], Barba, M.[2], Kyriakopoulou, P.E.[1]
[1] Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2] CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy

Abstract

Viroid research on pome and stone fruit trees in Greece is important, as it seems that such viroids are widespread in the country and may cause serious diseases. Our research dealt with three *Pospiviroidae* species infecting pome and stone fruit trees, namely *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Hop stunt viroid* (HSVd). Tissue-print hybridization, reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing techniques were successfully used for the detection and identification of these viroids in a large number of pome and stone fruit tree samples from various areas of Greece (Peloponnesus, Macedonia, Thessaly, Attica and Crete). The 58 complete viroid sequences obtained (30 ASSVd, 16 PBCVd and 12 HSVd) were submitted to the GenBank. Our results showed the presence of ASSVd in apple, pear, wild apple (*Malus sylvestris*), wild pear (*Pyrus amygdaliformis*) and sweet cherry; HSVd in apricot, peach, plum, sweet cherry, bullace plum (*Prunus insititia*), apple and wild apple; and PBCVd in pear, wild pear, quince, apple and wild apple. This research confirmed previous findings of infection of Hellenic apple, pear and wild pear with ASSVd, pear, wild pear and quince with PBCVd and apricot with HSVd. Our findings also revealed for the first time the natural mixed infection of apple and wild apple with (ASSVd+PBCVd+HSVd), of apple and pear with (ASSVd+PBCVd), and of wild apple with (ASSVd+HSVd), as well as the natural infection of Hellenic sweet cherry, peach, bullace plum and plum with HSVd. To our knowledge, this is the first published report of detecting HSVd and PBCVd in infected apple and wild apple, and ASSVd in sweet cherry.

Keywords: ASSVd, PBCVd, HSVd, stone fruit, pome fruit, Greece

Introduction

Apple scar skin viroid (ASSVd), *Hop stunt viroid* (HSVd) and *Pear blister canker viroid* (PBCVd) are members of the family *Pospiviroidae* (Flores et al., 2003) and have been reported to infect wild and cultivated pome and stone fruit trees in Greece (Kyriakopoulou and Hadidi 1998; Amari et al. 2000; Kyriakopoulou et al. 2001; Boubourakas *et al*. 2006, 2008). The present study has focused on specifying the host range and geographical extent of viroid infections in Greece and obtaining complete nucleotide sequences of the viroids detected in Rosaceous species from various parts of the country.

Materials and methods

Sample collection: During 2006-2009, 947 field samples of cultivated and wild pome and stone fruit trees with various symptoms (Fig. 1-4) were collected in different regions of Greece (Macedonia, Peloponnesus, Thessaly, Attica and Crete).

Fig. 1 Pear fruit cv 'Kontoula' with ASSVd (scar skin, Argolis, Peloponnesus)

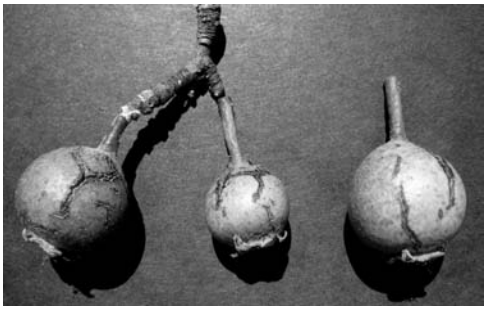

Fig. 2 Wild pear fruit (*Pyrus amygdaliformis*) with ASSVd (scar skin, Achaia, Peloponnesus)

Fig. 3 Pear twig cv 'Kontoula' with PBCVd (pear blister canker, Argolis, Peloponnesus).

Fig. 4 Apple fruit cv. 'Royal Gala' found to be infected with ASSVd, HSVd and PBCVd (red and white dappling on premature fruit, Pella, Macedonia).

Tissue-print hybridization: Tissue-print hybridization was conducted for all samples, using a modified imprint hybridization protocol of Palacio-Bielsa et al. (1999) and DIG-labelled DNA (ASSVd, PBCVd, HSVd) and RNA (ASSVd) probes.

Total RNA extraction and RT-PCR: Total RNA phenol extracts of 120 Rosaceous samples were used in a one tube/two step RT-PCR protocol, employing two different primer pairs per viroid, as described by Faggioli et al. (2001).

Cloning and sequencing: Properly-sized RT-PCR products were either sequenced directly or cloned into pGEM-T and pCR® II plasmid vectors, according to the pGEM-T Easy (Promega, Madison, WI, USA) and TOPO-TA (Invitrogen, Carlsbad, CA, USA) cloning kit instructions, and then sequenced. The sequences obtained were compared with others in the NCBI database and those identified as complete sequence viroid genomes were submitted to the GenBank.

Results

Tissue print hybridization: ASSVd, PBCVd and HSVd were found in 29.3%, 30.1% and 38.9% of field samples tested by tissue print hybridization, respectively. High frequencies were found for: ASSVd in pear and wild pear from Argolis, Achaia and Corinthia (Peloponnesus), in apple and wild apple trees from Pella (Macedonia) and in sweet cherry trees from Florina (Macedonia). PBCVd was found mainly in pear and wild pear trees from Argolis, Achaia and Corinthia, and in quince trees from Argolis; and HSVd in apricot and peach trees from Argolis and Corinthia, as well as in almond and wild almond (Prunus communis) trees from Achaia, Argolis and Arcadia (Peloponnesus) (Table 1a). Mixed infections by 2 or 3 viroids, (ASSVd+PBCVd), (ASSVd+HSVd) or (ASSVd+HSVd+PBCVd) were detected in 63 trees from Argolis, Achaia, Corinthia (Peloponnesus) and Pella (Macedonia) (Table 1b).

Tab. 1a Viroid-positive fruit tree samples in Greece

Viroid	**Tissue print hybridization**			**RT-PCR**			**Sequences**			**Complete sequences**		
Host species	**ASSVd 117/400**	**PBCVd 114/272**	**HSVd 232/596**	**ASSVd 33/120**	**PBCVd 23/120**	**HSVd 39/120**	**ASSVd 44**	**PBCVd 21**	**HSVd 21**	**ASSVd 30**	**PBCVd 16**	**HSVd 12**
Apple	Pella 40%	Pella 26%	Pella 20%	8	7	4	10	7	3	4	7	2
Pear	Argolis 51% Achaia 40%	Argolis 55% Corinthia 36% Achaia 40%		9	8		9	9		5	5	
Wild apple (*Malus sylvestris*)	Pella 77%	Pella 67%	Pella 77%	3	3	3	6	2	5	3	2	3
Wild pear (*Pyrus amygdaliformis*)	Achaia 30%	Argolis 89% Corinthia 71% Achaia 30%		6	4		3	1		2	1	

Viroid	Tissue print hybridization			RT-PCR			Sequences			Complete sequences		
Host species	**ASSVd 117/400**	**PBCVd 114/272**	**HSVd 232/596**	**ASSVd 33/120**	**PBCVd 23/120**	**HSVd 39/120**	**ASSVd 44**	**PBCVd 21**	**HSVd 21**	**ASSVd 30**	**PBCVd 16**	**HSVd 12**
Quince		Argolis 75%		1	1			2			1	
Almond			Argolis 100% Arcadia 60%									
Apricot			Argolis 60% Corinthia 63%			11			1			1
Bullace plum						1			1			
Cherry			Florina 100%	6		11	16		5	16		4
Peach			Argolis 70% Corinthia 52%						5			2
Plum						6			1			
Wild almond (*Prunus communis*)			Achaia 54%			2						
Wild plum (*Prunus spinosa*)			Corinthia 80%									

Tab. 1b Viroid in mixed infectious

Mixed infections	Imprint hybridization						Completely sequenced					
63 Trees	**Apple**	**Wild apple**	**Pear**	**Wild pear**	**Quince**	**Cherry**	**Apple**	**Wild apple**	**Pear**	**Wild pear**	**Quince**	**Cherry**
ASSVd+PBCVd	10		25	10	2		2	1	2			
ASSVd+HSVd		1				6						
ASSVd+PBCVd+HSVd	4	6					1	1				

RT-PCR: RT-PCR amplified products of the expected size were obtained in 70 out of the 120 field samples tested (Table 1a).

Cloning and sequencing: Cloning and sequencing or direct sequencing (using at least 2 different primers) were completed for 39 RT-PCR products from 31 trees (5 apple, 3 wild apple, 5 pear, 3 wild pear, 1 quince, 7 sweet cherry, 3 apricot, 2 peach, 1 bullace plum (Prunus insititia), 1 Japanese plum), resulting in 44 ASSVd, 21 HSVd and 21 PBCVd sequences. Viroids in mixed infections (ASSVd+HSVd+PBCVd) or (ASSVd+PBCVd) were completely sequenced in 7 trees (Table 1b). Fifty-eight complete viroid sequences, 30 ASSVd, 16 PBCVd and 12 HSVd were deposited in the GenBank under the accession numbers FJ974062-FJ974104, EU925587-EU925591, EU978462-EU978464, GQ249347-GQ249350, GQ141739-GQ141740 and FN376408-FN376409.

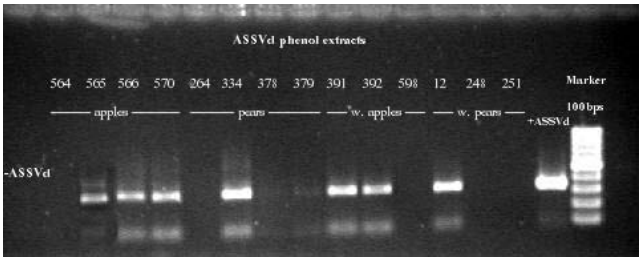

Fig. 5 RT-PCR test on 4 apple, 4 pear, 3 wild apple and 3 wild pear total RNA phenol extracts using ASSVd primers. From left: Lane 1, healthy control; lanes 2-15, pome fruit samples; lane 16, positive control; lane 17, Marker 100 bps (Fermentas, LTU).

Discussion

The data obtained in this study indicate that ASSVd, HSVd and PBCVd, previously reported in Greece, are widely spread in cultivated pome and stone fruit trees, including local varieties, especially in areas of great arboricultural importance, such as Central-Western Macedonia and Peloponnesus, as well as wild pears and wild apples and other wild rosaceous species, in the mountains and foothills of the above areas. Our findings also revealed for the first time the natural mixed infection of apple and wild apple with (ASSVd+HSVd+PBCVd), of apple and pear with (ASSVd+PBCVd), and of wild apple with (ASSVd+HSVd), as well as the natural infection of sweet cherry, plum, bullace plum and peach with HSVd in Greece. To our knowledge, this is the first published report of detecting HSVd and PBCVd in apple and wild apple and ASSVd in sweet cherry (Kaponi et al. 2009).

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Detection by tissue printing hybridization of Pome fruit viroids in the mediterranean basin

Di Serio, F.[1], Afechtal, M.[2], Attard, D.[3], Choueiri, E.[4], Gumus, M.[5], Kaymak, S.[6], Lolić, B.[2], Matić, S.[7], Navarro, B.[1], Yesilcollou, S.[5], Myrta, A.[8]
[1] Istituto di Virologia Vegetale del CNR, Via Amendola 165/A, 70126 Bari, Italy. Email: f.diserio@ba.ivv.cnr.it
[2] Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy
[3] Ministry for Rural Affairs and the Environment, plant biotechnology center, Lija, Malta
[4]Department of Plant Protection, Lebanese Agricultural Research Institute, Tal Amara, P.O. Box 287 Zahlé, Lebanon
[5] Ege University, Department of Plant Protection, Faculty of Agriculture, 35100 Bornova, Izmir, Turkey
[6] Horticultural Research Institute of Eğirdir, 32500 Eğirdir, Isparta, Turkey
[7] Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari, 70126 Bari, Italy
[8] Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA) Italy

Abstract

Available data on the incidence and biodiversity of pome fruit viroids in the Mediterranean basin are limited. Before starting a research survey to fill this gap, a tissue-printing hydridization (TPH) method to detect *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Apple dimple fruit* viroid (ADFVd) has been developed and validated. Afterward, TPH was used in large-scale indexing of pome fruit viroids in Bosnia and Herzegovina, Malta, Lebanon and Turkey. A total of about 1,000 trees was randomly collected and tested. Positive results obtained by TPH were confirmed by at least one additional detection method (RT-PCR and/or Northern-blot hybridization) and viroids were finally identified by sequencing full-length cDNA clones. PBCVd was detected in 13%, 12.4% and 5.4% of the tested pear trees in Bosnia and Herzegovina, Malta and Turkey, respectively, showing a wider diffusion of this viroid than expected. In contrast, ASSVd was never detected and ADFVd was only found in symptomatic trees (cv. Starking Delicious) in Lebanon, confirming a restricted presence of these viroids in the Mediterranean basin. Altogether, these data support the use of TPH as an easy and valuable tool for exploring pome fruit viroid spread.

Keywords: Viroid disease, viroid spread, Pome fruit trees, detection methods, molecular hybridization

Introduction

Pear blister canker viroid (PBCVd) (Hernández et al., 1992) is the causal agent of bark alteration in the pear indicator "A20" (Ambrós et al., 1995). Commercial pear cultivars infected by PBCVd generally do not develop bark symptoms. *Apple dimple fruit viroid* (ADFVd) (Di Serio et al., 1996) may cause a severe fruit disorder in apple trees, characterized by malformed fruits with green scattered depressed spots of 3 to 4 mm in diameter, predominantly around the calyx (Di Serio et al., 2001). Fruits showing similar symptoms or with scar patches on the skin accompanied by a corky texture can also be observed in plants infected *by Apple scar skin viroid* (ASSVd) (Hashimoto and Koganezawa, 1987).

These viroids belong to the genus *Apscaviroid*, family *Pospiviroidae* (Flores et al., 2005). Available data on the incidence and biodiversity of pome fruit viroids in the Mediterranean basin are limited (Flores et al., 2003; Di Serio et al., 2003; Koganezawa et al., 2003). Several efficient methods for detecting pome fruit viroids have been reported previously (Ambros *et al*., 1995; Di Serio et al., 2001; 2002; Ragozzino et al., 2004).

These technologies, based on RT-PCR or on molecular hybridization of labeled probes with plant extracts, need technical expertise for nucleic acid preparations, which is time consuming and relatively expensive. In contrast, tissue printing hybridization (TPH), an alternative detection method based on molecular hybridization, does not require nucleic acid preparation because nucleic acids are applied to the membrane by directly imprinting the fresh plant tissues to be tested. Although TPH has been successfully applied for detecting several viroids infecting fruit trees, such as *Hop stunt viroid* (Astruc et al., 1996; Amari et al., 2001) and *Peach latent mosaic viroid* (Loreti et al., 1999; Torres et al., 2004), the sensitivity of this method may depend on several factors, including the viroid-host combination and the seasonal fluctuation in the viroid titer in the infected plant, as shown by Duran-Vila et al. (1993) in the case of viroids infecting citrus. In the case of pome fruit viroids, detection by TPH of ASSVd in apple plants graft-inoculated with infected material (Podleckis et al., 1993; Hurtt et al., 1996) suggested that this method could be also applied for large-scale indexing of this and other pome fruit viroids, but no data in this respect were available at the beginning of our study. To fill this gap, we developed and validated a tissue-printing hybridization (TPH) method to detect ASSVd, PBCVd and ADFVd (Lolic et al., 2007).

Here we summarize the results obtained in the last few years applying this detection method in large-scale indexing of pome fruit viroids in Bosnia and Herzegovina (Lolic et al., 2007), Malta (Attard et al. 2007), Lebanon (Choueiri et al., 2007), Morocco and Turkey, allowing the identification of several new pome fruit viroid isolates. Our studies show a wider spread of PBCVd and ADFVd in the Mediterranean basin than thought previously and supply additional data on the sequence variability of these viroids**.**

Material and methods

Field surveys and sample collection: Surveys for symptoms and sample collections were made in apple (*Malus pumila* Mill.), pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill.) varietal collections, commercial orchards and nurseries located in Bosnia and Herzegovina (northern and central areas), Malta, Lebanon (northern areas), Morocco (northern areas) and Turkey (western areas). In total, about 1,000 trees were tested for viroid infection (310 samples from Bosnia Herzegovina, 113 from Malta, 264 from Lebanon, 249 from Morocco and 89 from Turkey). Three one-year-old self-rooted apple seedlings of cv. Spy 277 were separately graft-inoculated with ASSVd or PBCVd (kindly supplied by F. Faggioli (CRA, Centro di Ricerca per la Patologia Vegetale, Rome, Italy) and with ADFVd (kindly supplied by A. Ragozzino (Università degli Studi di Napoli, Italy) and grown in pots. One year post-inoculation, these plants were assayed for the respective viroid infection and then used as positive controls in the RT-PCR and molecular hybridization tests.

Detection by molecular hybridization and RT-PCR: Total nucleic acid (TNA) extracts were prepared from 100-200 mg of leaf tissues as reported by Dalmay et al. (1993) and directly used for dotblot (DBH) and Northern blot hybridization experiments or were further purified by a modified silica-gel capture system (Foissac *et al.*, 2001) before performing RT-PCR reactions. Tissue prints were done by pressing fresh cut ends of leaf petioles onto Hybond-N+ (Roche Diagnostics GmbH, Germany) membranes. Labelling of riboprobes, DBH and Northern-blot hybridization experiments were carried out as previously described (Lolic et al., 2007). Detection of ASSVd and ADFVd by RT-PCR was performed as previously reported (Di Serio et al., 2002), whereas PBCVd was detected by RT-PCR following the protocol of Malfitano et al. (2004). PCR-amplified products were analyzed by electrophoresis in 1.2% agarose gels and detected by ethidium bromide staining and irradiation with a UV lamp.

Cloning and sequencing: PBCVd and ADFVd amplified cDNAs of expected sizes from infected pear and apple plants were directly sequenced in both orientations or were eluted from agarose gels and cloned into the pGEM-T-Easy vector (Promega, Madison, WI, USA). Inserts were sequenced automatically (MWG-Biotech, Germany).

Results and discussion

No bark symptom comparable to that reported for PBCVd in the pear indicator “A20” (Ambrós et al., 1995) was observed in the surveyed fields. Although no PBCVd infected pear was identified in Morocco, unexpected high infection rates of 13%, 12.4 and 5.4% were found in Bosnia and Herzegovina, Malta and Turkey, respectively (Table 1). Indeed, out of the 398 pear trees assayed for PBCVd infection by TPH, 35 tested positive. These findings were further confirmed by Northern blot hybridization assays and/or RT-PCR and, for some isolates, by cloning and sequencing of the amplified cDNA products (data not shown). These confirmation tests also showed that some additional pear trees testing positive to TPH were actually not infected, indicating that TPH may occasionally generate a false positive signal.

A careful testing of more than 800 trees for ASSVd and ADFVd was done by TPH in the surveyed regions. ASSVd and ADFVd were insistently searched in the surveyed regions testing by TPH more than 800 trees. ASSVd was not detected in any tested apple tree in Morocco, Lebanon and Bosnia and Herzegovina, suggesting the absence or limited spread of this viroid in such countries and supporting previous indications of the relatively rare incidence of ASSVd in Europe (Koganezawa et al., 2003). Interestingly, ASSVd was not detected in any of the 194 tested pear trees in Morocco and Lebanon. This result partially contrasts with the previous widespread and highly frequent occurrence of this viroid reported in wild and cultivated pear trees in Greece (Kyriakopoulou et al., 2001), suggesting that the incidence of this viroid in pear trees may largely differ among European countries.

Tab. 1 Large scale indexing by TPH of pome fruit viroids in Morocco, Lebanon, Malta, Bosnia and Herzegovina and Turkey.

Viroid / Location	PEAR ASSVd ADFVd	PBCVd	APPLE ADFVd	ASSVd	PBCVd	QUINCE ASSVd ADFVd	PBCVd
Morocco	0/81*	0/81	0/168	0/168	0/168	/	/
Lebanon	/**	/	17/264	0/264	0/264	/	/
Malta	0/113	14/113	/	/	/	/	/
Bosnia & Herz.	/	17/130	0/178	0/178	/	0/2	0/2
Turkey	/	4/74	/	/	/	0/15	1/15
TOTAL	0/194	35/398	17/610	0/610	0/432	0/17	1/17

*Number of positive plants/number of tested plants; ** /, not tested

The identification of ADFVd in cv Starking Delicious in Lebanon is of particular interest because this is the second country in which this viroid has been identified so far. Similar to a previous report from Italy (Di Serio et al., 2001), fruits from the Lebanese infected plants showed typical symptoms of ADFVd infection. It is known that, after experimental inoculation, some apple cultivars (i.e. cv. Golden) may tolerate ADFVd infections without eliciting symptoms (Di Serio et al., 2001). However, this viroid was not found in any of the almost 600 symptomless apple plants assayed in this study, suggesting that the natural diffusion of ADFVd is likely limited at present to symptomatic cultivars and to restricted areas.

Finally, identification of one quince infected by PBCVd is in line with previous data on natural hosts of this viroid (Flores et al., 2003). Altogether these data, show that the spread of PBCVd in the Mediterranean basin is wider than thought before, whereas ADFVd and ASSVd are still rare in this area. Our studies also strongly support the implementation of control measures based on importing viroid-free pome fruit germplasm into European countries and on the use of viroid-indexed mother trees for producing propagation material.

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First report and molecular analysis of *Apple scar skin viroid* in sweet cherry

Kaponi, M.S.[1], Luigi, M.[2], Barba, M.[2], Sano, T.[3], Kyriakopoulou. P.E.[1]
[1] Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2] CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
[3] Phytopathology Laboratoty, Hirosaki University, Japan

Abstract

Apple scar skin viroid (ASSVd) is a serious pathogen of pome fruits. Recently, it has been reported in Chinese apricot and Chinese peach. In the context of our research on fruit tree viroids in Greece, ASSVd was initially detected in a sweet cherry tree cv 'Tragana Edessis' from Florina (Macedonia) by RT-PCR and this finding was confirmed by direct sequencing. This tree is located at the edge of a newly established apple orchard, along with other sweet cherry and wild cherry (*Prunus avium*) trees. In order to verify this interesting finding, we examined for ASSVd four sweet cherry trees, two wild cherry trees and their neighboring apple trees in the same orchard.

The examination was done by imprint hybridization using an ASSVd-specific DIG-labelled probe at stringent hybridization conditions and by RT-PCR using two different ASSVd specific primer pairs. We obtained ASSVd-positive results for all 6 cherry trees. No ASSVd was detected in the apple trees of the orchard. Purified ASSVd-positive RT-PCR products from the cherries were directly sequenced or cloned into the pGEM-T vector and then sequenced. ASSVd sequences were obtained from 5 trees. These sequences are 327-340 nucleotides long and share 96-99% identity with ASSVd isolates from Asian (Indian) apples. These results are similar to our data for other ASSVd variants from cultivated and wild pome fruit trees in Greece.

The cherry ASSVd variants differ from the ASSCS prototype isolate of ASSVd at 18-29 sites. There are 15 nucleotide changes (differences from ASSCS) common to all Hellenic ASSVd variants, including cherry and pome fruit tree variants. There are no cherry-specific nucleotide changes in the ASSVd sequences obtained. To our knowledge, this is the first published report of natural infection of cherry by ASSVd.

Keywords: ASSVd, cherry, molecular analysis, Hellenic sequences

Introduction

Apple scar skin viroid (ASSVd) is the type species of the genus *Apscaviroid* (family *Pospiviroidae*). This 330 nt-long viroid induces serious diseases on pome fruit trees, such as apple scar skin, dapple apple, pear rusty skin and pear dimple fruit in Europe, Asia and North America (Hashimoto and Koganezawa, 1987; Hadidi et al., 1990; Zhu et al., 1995; Osaki et al., 1996; Koganezawa et al., 2003; Kyriakopoulou et al., 2003; Shamloul et al., 2004; Hadidi and Barba, 2010). It has been reported in apple (*Malus domestica*), pear (*Pyrus communis*, *P. pyrifolia*), wild apple (*M. sylvestris*) and wild pear (*P. amygdaliformis*) (Kyriakopoulou and Hadidi 1998; Kyriakopoulou et al. 2001, 2003; Koganezawa *et al.* 2003; Boubourakas et al. 2008). Recently, it was reported in Chinese peach and apricot from Sinkiang (Zhao and Niu 2006, 2008).

Materials and methods

Sampling, extraction, RT-PCR: During a survey for viroids in Greece in 2008, 11 apple and 2 sweet cherry (Prunus avium) samples from a newly established apple orchard in Florina (Macedonia region) were tested by RT-PCR, using ASSVd and HSVd specific primers, respectively. The sweet cherry trees showed mosaic symptoms on leaves and white spots on fruit (Fig. 1-3). After ASSVd-positive results, a second sampling took place in the same orchard; 6 wild and cultivated (cv. 'Tragana Edessis') sweet cherry tree samples, all being at the edge of the orchard, and all their neighboring apple trees were tested for ASSVd, by imprint hybridization using an ASSVd-specific DIG-labelled probe at stringent hybridization conditions (50% formamide, T=60^{o}C).

Five trees were tested by one tube, two step RT-PCR (Faggioli et al. 2001) using two different ASSVd-specific primer pairs (Hadidi and Yang 1990; Di Serio et al. 2002) and another one with one ASSVd-specific primer pair (Di Serio et al. 2002). In addition, 6 apple samples from the orchard were sent to the Phytopathology Laboratory, Hirosaki University (Japan) for further examinations by PAGE and Northern hybridization, using DIG-labelled ASSVd, ADFVd and AFCVd- specific riboprobes.

Fig. 1 – 2 Mosaic symptoms on cherry leaves from trees infected with ASSVd.

Fig. 3 White spots on cherry fruit from a tree cv 'Tragana Edessis' infected with ASSVd.

Cloning and sequencing: RT-PCR products of approximately 330 nt were either directly sequenced or cloned into pGEM-T and pCR® II plasmid vectors, according to the pGEM-T Easy (Promega, Madison, WI, USA) and TOPO-TA (Invitrogen, Carlsbad, CA, USA) cloning kit instructions, and then sequenced. The sequences obtained were compared with others in the NCBI database and those identified as complete sequence viroid genomes were submitted to the GenBank.

Transmissibility tests: Eight cherry rootstocks were bud-grafted with cherry tree buds from the orchard in September 2008 and tested by imprint hybridization and RT-PCR in May 2009.

Results

RT-PCR: The originally tested sweet cherry sample with ASSVd specific primers gave an amplicon of about 330 nt. This amplicon was directly sequenced with both primers of the reaction and found to be 96-97% homologous to ASSVd. ASSVd-positive results were obtained for all 6 sweet cherry trees of the orchard examined (Florina, Macedonia). Neighboring apple samples were not found to be infected by ASSVd or other viroids, using RT-PCR, PAGE and Northern hybridization.

Transmissibility tests: Grafted rootstocks were found positive when tested with ASSVd primers in RT-PCR (Fig.4) and tissue-print hybridization.

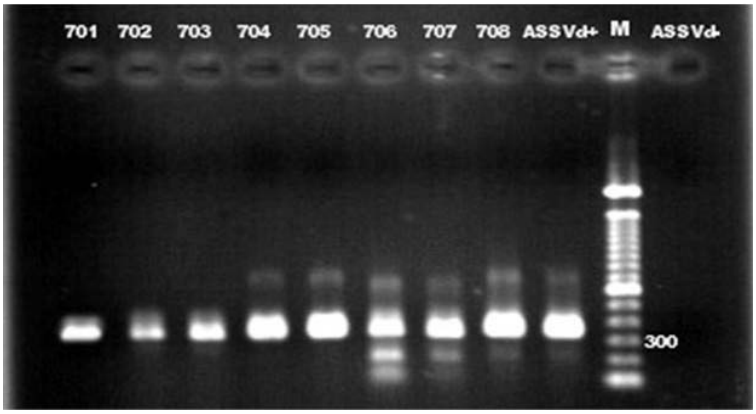

Fig. 4 ASSVd positive RT-PCR products from 8 bud-grafted cherry rootsocks with ASSVd-positive material. From left to right: Lanes 1-8 rootstock samples, lane 9 positive control; lane 10, marker 100 bps (Invitrogen, UK), lane 11 negative control.

Cloning and sequencing: ASSVd-positive results from 5 cherry trees were initially confirmed by direct sequencing, and then by cloning and sequencing, resulting in a total number of about 20 complete ASSVd variants. From these, 16 were deposited in the GenBank under the accession numbers FJ974062-FJ974074, FN376408-FN396409 and GQ249350.

Sequence analysis: Hellenic ASSVd sequences from sweet cherry from clones and directly-sequenced RT-PCR products are 327-340 nt long. They differ from the prototype isolates of ASSVd (ASSCS and Y00435, Hashimoto and Koganezawa 1987) at 4-29 sites. There are 15 nucleotide changes between ASSCS and all Hellenic ASSVd cherry variants. The ASSVd sequences from Hellenic cherry share great homology with all Asian (Indian and Chinese) ASSVd sequences from apple recovered from the NCBI GenBank (93-99% or difference at 1-20 nt) (Fig.5).

In particular, their similarity to the Indian isolates from apple, Y5, Y7 and Y8, is 96-99% (difference at 1-13 nt). They also share a varying homology (91-98%) with Hellenic ASSVd variants from pome fruit trees (difference at 1-30 nt), whereas the heterogeneity among themselves fluctuates between 0 and 10% (0-34 nt).

The secondary structure of cherry ASSVd variants is rod-like (Fig.6).

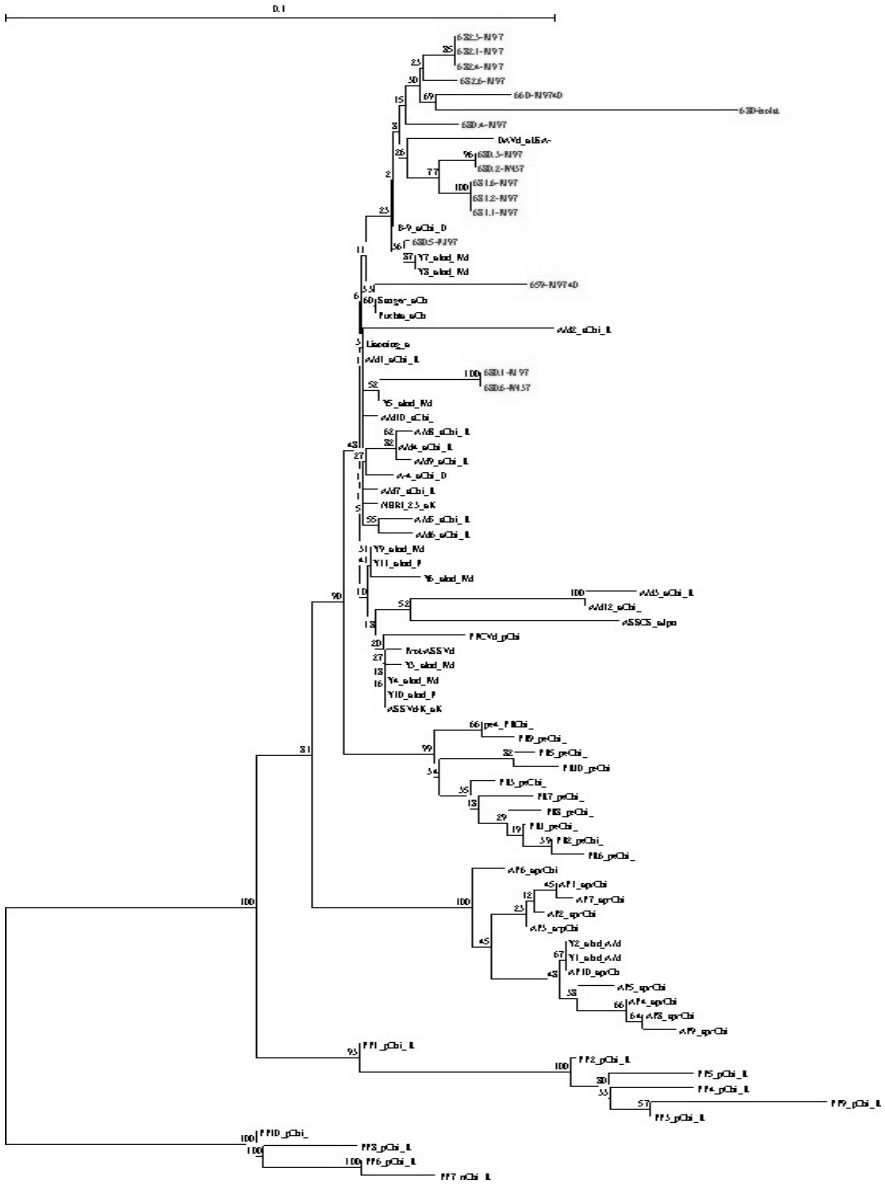

Fig. 5 Phylogenetic tree (neighbour joining analysis) of ASSVd containing the 16 Hellenic cherry sequences (red). Hellenic ASSVd cherry sequences do not form a separate cluster.

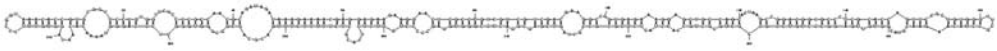

Fig. 6 Secondary structure of the ASSVd clone sequence 680.5 from sweet cherry (MFold).

Discussion

There are 15 nucleotide changes (differences from ASSCS) common to all Hellenic ASSVd variants, including cherry and pome fruit tree variants. There are no cherry-specific nucleotide changes in the ASSVd sequences obtained, therefore these sequences do not form a separate cluster in phylogenetic trees (Fig.5). The sequence variation among sweet cherry ASSVd variants is significant (10%), whereas the overall difference between all cherry sequences and other ASSVd variants does not exceed 10%. The fact that cherry trees harboring ASSVd sequences showed symptoms such as mosaic needs to be examined as to their cause-effect relation. To our knowledge, this is the first published report of detecting ASSVd in naturally infected cherry, including its molecular analysis. Northern blot hybridization analysis is under way in order to have a definitive proof of ASSVd presence in cherry.

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Molecular characterization of Hellenic variants *of Apple scar skin viroid* and *Pear blister canker viroid* in pome fruit trees

Kaponi, M.S.[1], Luigi, M.[2], Barba, M.[2], Kyriakopoulou, P.E.[1]
[1] Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2] CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy

Abstract

Apple scar skin viroid (ASSVd) and *Pear blister canker viroid* (PBCVd) are members of the genus *Apscaviroid* (family *Pospiviroidae*). In order to study the nucleotide sequence and secondary structure of Hellenic variants of these viroids, a large number of collected samples were initially screened by imprint hybridization; then ASSVd and PBCVd positive samples were assayed for the viroids by RT-PCR. Total RNA extracts were reverse-transcribed and amplified by polymerase chain reaction using two different specific primer pairs for each viroid. Purified RT-PCR products were directly sequenced or cloned into the pGEM-T and pCR® II vectors and then sequenced. Fourteen Hellenic full length ASSVd variants from 3 apple, 3 wild apple (*Malus sylvestris*), 1 wild pear (*Pyrus amygdaliformis*) and 3 pear trees are 330-335 nucleotides long. They differ from the reference sequences of ASSVd (ASSCS and Y00435) at 15-29 and 3-36 sites, respectively. Fifteen nucleotide changes (differences from ASSCS) are common among all Hellenic variants. Hellenic ASSVd variants share high identity (97-100%) with ASSVd isolates from Asian apples. Three Hellenic variants, deriving from different hosts and areas, are identical with each other (wild apple and apple from Pella [Macedonia] and pear from Achaia [Peloponnesus]) and with another group of 3 apple variants from China (Liaoning, AM1 and B-9). Sixteen full length Hellenic PBCVd variants from 12 trees (4 apples, 1 wild apple, 5 pears, 1 wild pear and 1 quince) are 314-316 nucleotides long. There are 6-50 nucleotide changes among all Hellenic variants and the prototype PBCVd isolate (NC001830). Twenty-two (22) changes are identical among the majority of the Hellenic variants, regardless of origin, and 28-35 changes occur in PBCVd sequences obtained from apple and wild apple samples. In addition, 2 Hellenic PBCVd variants are 97-98% homologous to some Australian and European (Bosnian) PBCVd pear isolates, whereas the remaining 14 share 86-94% identity with Australian PBCVd isolates from pear, quince and Japanese pear (*Pyrus pyrifolia*). This is the first detailed molecular study of ASSVd and PBCVd in Hellenic cultivated and wild pome fruit trees.

Keywords: ASSVd, PBCVd, pome fruit, molecular characterization

Introduction

Apple scar skin viroid (ASSVd) and *Pear blister canker viroid* (PBCVd) are members of the genus *Apscaviroid* (family *Pospiviroidae*) (Flores et al., 2003b). ASSVd variants are 329-334 nt long (Koganezawa et al. 2003; Kyriakopoulou et al. 2003) and PBCVd variants are 315-316 nt long (Flores et al. 2003a). Both induce serious diseases on pome fruit trees, such as apple scar skin, dapple apple, pear rusty fruit, pear dimple fruit (ASSVd) and pear blister canker (PBCVd) (Koganezawa et al. 2003; Kyriakopoulou *et al*. 2003; Flores et al. 2003).

Fig. 1 ASSVd symptoms on pear cv. 'Kontoula' (Argolis, Peloponnesus)

Fig. 2 PBCVd symptoms on pear cv. 'Kontoula' (Argolis, Peloponnesus)

In Greece, ASSVd and PBCVd have been reported to induce russeting, scarring and cracking on fruit (ASSVd) and blister canker on branches and twigs (PBCVd) of pear (*Pyrus communis*) (Fig. 1-2) and wild pear (*Pyrus amygdaliformis*) (Kyriakopoulou and Hadidi 1998; Kyriakopoulou et al. 2001), whereas ASSVd has also been detected on apple (*Malus domestica* Borkh) and wild apple (*Malus sylvestris*) trees (Boubourakas et al. 2008).

Materials and methods

During 2006-2009, 772 wild and cultivated pome fruit tree samples with various symptoms were collected in the regions of Macedonia, Peloponnesus, Thessaly and Attica. The samples were initially examined by imprint hybridization, using a modified protocol of Palacio-Bielsa et al. (1999) and full-length DIG-labelled probes synthesized in Greece and in Italy by RT-PCR and *in* vitro transcription. In addition, total RNA phenol extracts of 54 samples (8 wild pear, 3 wild apple, 21 pear, 17 apple, 5 quince) were used in a one tube-two step RT-PCR protocol, employing two different primer sets per viroid (Table 1), as described by Faggioli et al. (2001). RT-PCR products of the expected size were either sequenced directly or cloned into pGEM-T and pCR® II plasmid vectors, according to the pGEM-T Easy (PROMEGA, Madison, WI, USA) and TOPO-TA (Invitrogen, Carlsbad, CA, USA) cloning kit instructions, and then sequenced. The sequences obtained were compared with others in the NCBI database and those identified as complete sequence viroid genomes were submitted to the GenBank.

Tab. 1 Primers used for the detection of ASSVd and PBCVd

Primer name	Sequence	Position	Amplicon (nts)
ASSVd1-H[a]	5´-CCGGTGAGAAAGGAGCTGCCAGCAC-3´	98-122	~330
ASSVd1-C[a]	5´-CCTTCGTCGACGACGA-3´	82-97	
ASSVdDSH[b]	5´-CGGTGACAAAGGAGCTGCCAG-3´	98-118	~330
ASSVdDSC[b]	5´-GCCTCCGTCGACGACGACAG-3´	83-102	
PBCVd1-H[c]	5´-TTGCTTGCCTGAGCCTCGTCTTC-3´	167-202	~315
PBCVd1-C[c]	5´-CGCTGGTTTTCTCCAAAGGAGCGATTACTCACA-3´	131-167	
PBCVd3-H[d]	5´-GGAGCGCGCGGCTGTGAGTAATC-3´	121-143	~315
PBCVd3-C[d]	5´-AGCCAGCGCCCAGGCTTCTAGACCCTT-3´	93-120	
PBCVd4-H[e]	5´-GTCTAGAAGCCTGGGCGCTGGCTGG-3´	97-121	~315
PBCVd4-C[e]	5´-CCTTCGTCGACGACGA-3´	80-95	

[a]Hadidi and Yang 1990; [b]Di Serio *et al.* 2002; [c]Loreti *et al.* 1997; [d]Shamloul *et al.* 2002; [e]F. Faggioli

Results and discussion

RT-PCR detected ASSVd infection in 27 pome fruit samples (9 pear, 6 wild pear, 1 quince, 3 wild apple, 8 apple) (Fig. 3) and PBCVd infection in 23 (8 pear, 4 wild pear, 1 quince, 3 wild apple, 7 apple) (Fig. 4). Cloning and sequencing resulted in 44 ASSVd and 21 PBCVd sequences. Complete sequences obtained from 14 ASSVd variants (4 apple, 3 wild apple, 5 pear, 2 wild pear) and 16 PBCVd variants (7 apple, 2 wild apple, 5 pear, 1 wild pear, 1 quince) were deposited in the GenBank under the accession numbers FJ974082-FJ974104, EU978462-EU978464, GQ141739-GQ141740, GQ249347 and GQ249349.

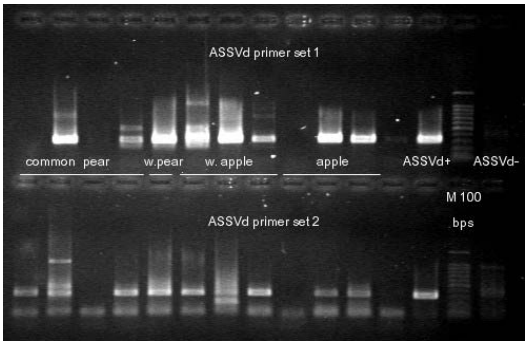

Fig. 3 RT-PCR test on 4 pear, 1 wild pear, 3 wild apple and 3 apple phenol extracts using 2 ASSVd primer sets. From left to right: Lanes 1-11, pome fruit samples; lane 12, cherry sample; lane 13, positive controls; lane 14, marker 100 bps (Fermentas, LTU), lane 15, healthy controls.

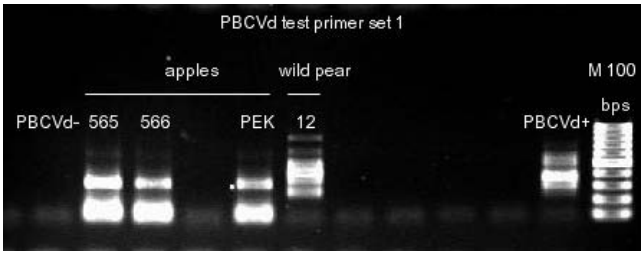

Fig. 4 PBCVd-positive RT-PCR products from apple and wild pear phenol extracts. From left to right: Lane 1, healthy control; lanes 2-9, pome fruit samples; lane 10, positive control; lane 11, marker 100 bps (Fermentas, LTU).

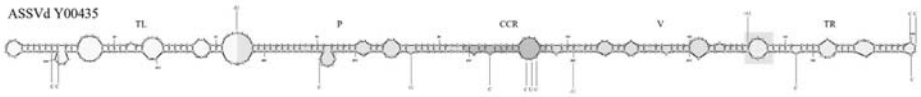

Fig. 5 Secondary structure of the ASSVd Y00435 reference sequence (Hashimoto and Koganezawa 1987). The lines indicate common nucleotide differences of all Hellenic variants from ASSCS (black) and Y00435 (red) reference sequences.

The 14 complete Hellenic ASSVd sequences, 330-335 nucleotides long, differ from the ASSVd reference sequences (ASSCS and Y00435, Hashimoto and Koganezawa 1987) by 15-29 and 3-36 nts, respectively. Considering reference sequence ASSCS, 15 nucleotide changes are identical among all Hellenic variants, spread throughout all regionssharing a very high identity (97-100%) with Asian ASSVd sequences from apple (Indian, Chinese, Korean) (Fig.7).

The variation among themselves is 16% equaling that of the Chinese ASSVd sequences. Three Hellenic variants, from apple (Pella, Macedonia), wild apple (Pella) and pear (Achaia, Peloponnesus), were found to be identical to each other and to another group of 3 apple variants from China (Liaoning, AM1 and B-9) (Fig. 6). The 16 complete Hellenic PBCVd variants are 314-316 nt long. Fourteen of them have an identity of 86-94% with the Australian PBCVd sequences from pear, Japanese pear and quince, and a higher identity (97-98%) with European (mainly Bosnian) and other Australian PBCVd sequences from pear. Hellenic PBCVd sequences show significant variation among themselves (16%), double the variation among all the other known PBCVd sequences (8%) (Fig.8).

The 16 Hellenic variants differ from the reference sequence of PBCVd (Hernadez et al. 1992) at 6-50 positions. Four of these differences (gap-1C, gap-50A, G235C and gap-236U) are common for all Hellenic variants, whereas 22 of them are identical among the majority of the Hellenic variants, regardless of origin. The differences shown by the Hellenic apple and wild apple variants are 28-35 nt, compared to the reference sequence. The Hellenic PBCVd sequences have quasi-linear secondary structures (data not shown).

```
AFCVd_AM1          GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
ASSVd              GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
Liaoning           GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
334-FJ974097       GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
392-FJ974099       GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
565-FJ974098       GGTAAACACCGTGCGGTTCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
                   ************************************************************

AFCVd_AM1          GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
ASSVd              GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
Liaoning           GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
334-FJ974097       GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
392-FJ974099       GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
565-FJ974098       GGAGAAGAAGGAACTCACCTGTCGTCGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
                   ************************************************************

AFCVd_AM1          ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
ASSVd              ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
Liaoning           ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
334-FJ974097       ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
392-FJ974099       ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
565-FJ974098       ACTAAGCCGGACGGCGCCCTCGCACCAGTTCCGCTGTGGGTTCGCCTACAAGAACGTACG
                   ************************************************************

AFCVd_AM1          GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
ASSVd              GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
Liaoning           GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
334-FJ974097       GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
392-FJ974099       GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
565-FJ974098       GTGTTGAGGCCCTGTCCGCCGCTGCGCTGCCACCTACTCTCGCGCCGCTAGTCGAGCGGA
                   ************************************************************

AFCVd_AM1          CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
ASSVd              CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
Liaoning           CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
334-FJ974097       CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
392-FJ974099       CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
565-FJ974098       CTCCGGGTGGAGCCCCCTGTTCTCTCACGCTCTTTTTCTTTGACGCAGCGGCGGGTGGGT
                   ************************************************************

AFCVd_AM1          TCCCAGGGTAAAACACAATAGGTGTTTCCC
ASSVd              TCCCAGGGTAAAACACAATAGGTGTTTCCC
Liaoning           TCCCAGGGTAAAACACAATAGGTGTTTCCC
334-FJ974097       TCCCAGGGTAAAACACAATAGGTGTTTCCC
392-FJ974099       TCCCAGGGTAAAACACAATAGGTGTTTCCC
565-FJ974098       TCCCAGGGTAAAACACAATAGGTGTTTCCC
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Fig. 6 Alignment of 3 Chinese (apple) and 3 Hellenic (334-pear, 392-wild apple, 565-apple) ASSVd sequences with CLUSTAL.

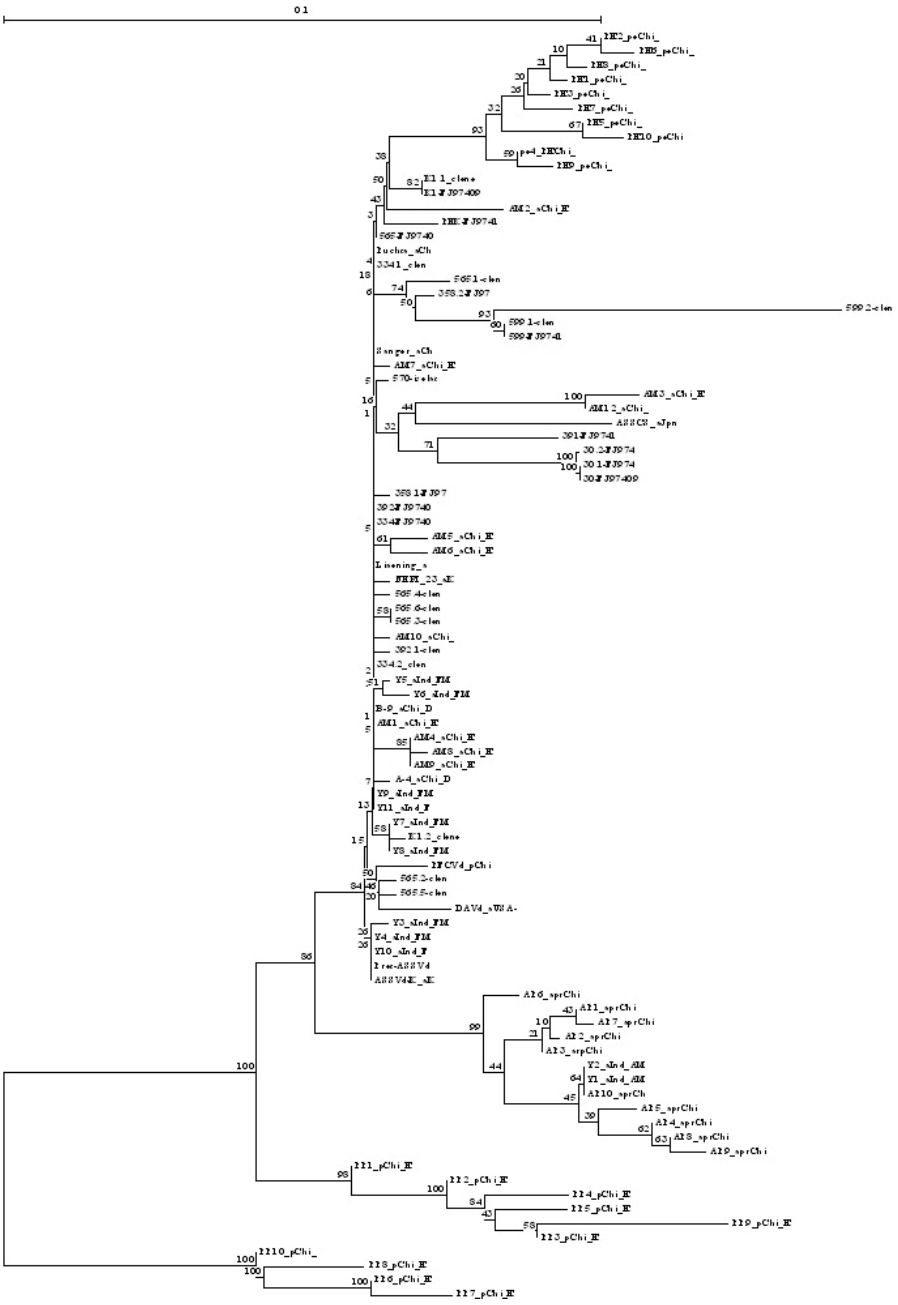

Fig. 7 Phylogenetic diagram of 26 Hellenic ASSVd pome fruit sequences (blue) and other 63 pome fruit sequences (neighbour-joining analysis, bootstrap=100)

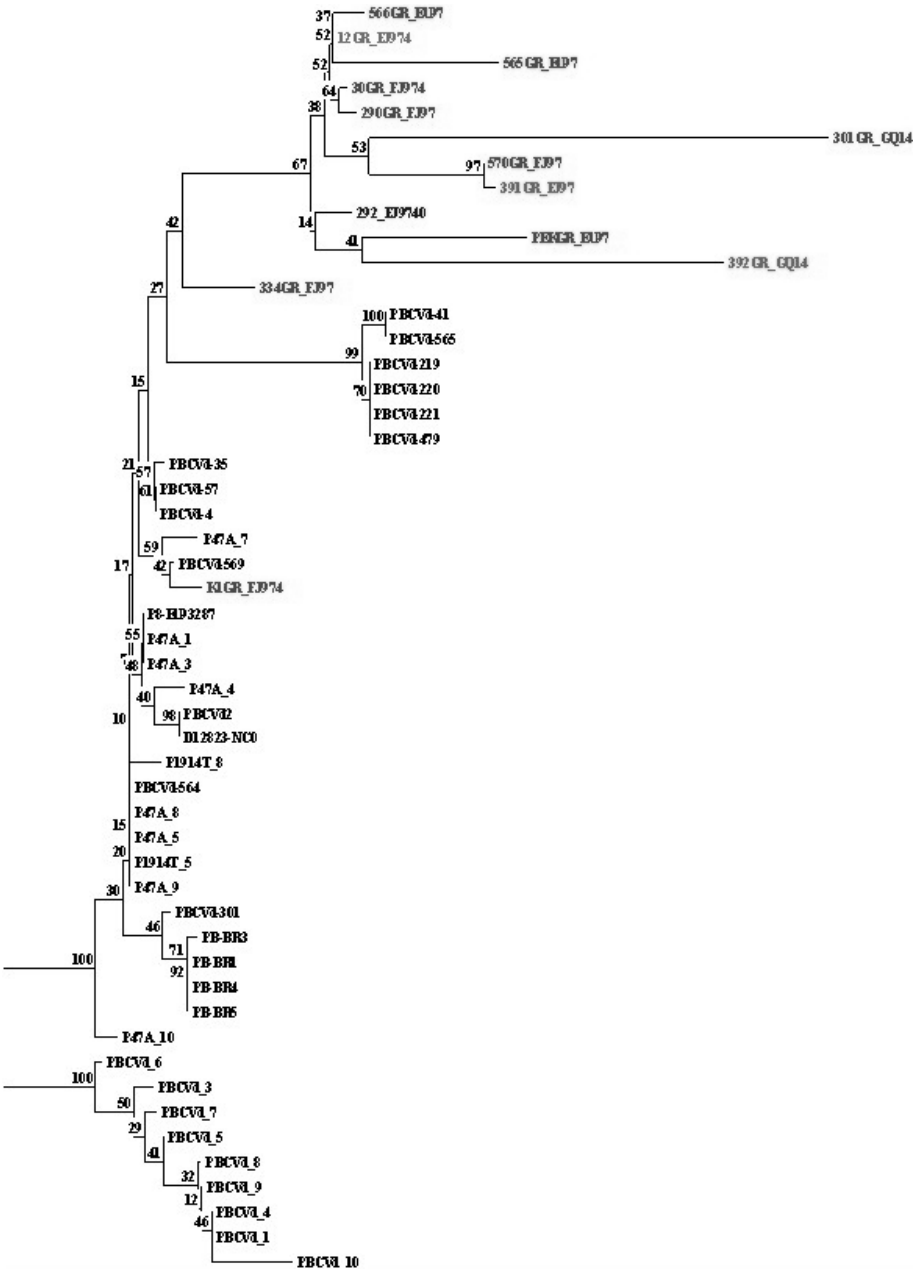

Fig. 8 Phylogenetic diagram of 13 Hellenic PBCVd pome fruit sequences (colored) and other 38 pome fruit sequences (neighbour-joining analysis, bootstrap=100).

This is the first detailed molecular study of ASSVd and PBCVd in Hellenic pome fruit orchards and wild pome fruit trees. The wide host range of ASSVd and PBCVd in Greece includes 5 pome fruit species, 2 of which are wild species of pear and apple, which grow in mountains far away from cultivated crops. The widespread occurrence of these two viroids in local pome fruit varieties as well as in wild species, and the wide variation of their Hellenic sequences, are indications that these viroids are probably native to Greece.

Acknowledgements

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Identification and characterization of *Peach latent mosaic viroid* and *Hop stunt viroid* in different peach cultivars showing dapple fruit, fruit yellow mosaic and cracked suture symptoms

Luigi, M.[1], Faggioli, F.[1], Barba, M.[1], Giunchedi, L.[2]
[1] CRA-Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy. Email: francesco.faggioli@entecra.it
[2] Dipartimento di Scienze e Tecnologie Agroambientali - Università di Bologna, Italy

Abstract

From the early 1990s, a fruit peach syndrome characterized mainly by small discoloured spots (dapple fruit) and/or yellow areas on the skin (yellow mosaic), cracked suture and deformations was identified in most commercial orchards in the Emilia Romagna region (Northern Italy). In the past, *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) have been detected in trees with symptomatic fruits. In order to ascertain the presence and spread of these two viroids, symptomatic fruit samples were collected from five different cultivars: 'Royal Glory', 'Crimson Lady', 'Grenat', 'Diamond Princess' and 'Laura'. Dapple fruit symptoms affected all cultivars, 'Grenat' samples also showed evident yellow mosaic and fruit deformation, and 'Royal Glory' severe cracked sutures. The results showed a large diffusion of the two viroids, mainly in mixed infections. Anvaluation of the role the viroids could play in symptom expression has been complicated by the high number of samples infected by both viroids (60%). Nonetheless, PLMVd was confirmed to be strictly associated with the yellow mosaic, cracked suture and fruit deformation symptoms. The aetiological origin of the dapple fruit disease, however, seems to be more complicated, since in the 'Diamond Princess', only PLMVd has been found to be associated with the symptoms, whereas in all other cultivars, the presence of HSVd could have influenced the symptom expression. Moreover, the molecular characterization of some PLMVd isolates does not show any correlation between nucleotide sequence and symptoms although new PLMVd variants were identified.

Keywords: peach fruit symptoms, PLMVd, HSVd, mixed infection

Introduction

Viroids are pathogens of food, industrial and ornamental plants. Despite their small genome (single strand RNA of 240-400 nucleotides), they can affect a lot of plants causing severe damage. In fruit tree cultivation, the economic impact of viroids could be very important, since fruit quality is the aspect which is mostly affected (Daros et al., 2006). More specifically, two viroids were found on peach cultivars: *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). PLMVd is the causal agent of Peach latent mosaic disease (Flores et al., 1990). The most severe symptoms of PLMVd on the peach fruit are deformation, discolored spot, the presence of cracked sutures and flattened stones. Symptoms induced on foliage are rare. In a few cases a particular albino pattern (peach calico) that covered most of the leaf area was observed. This last symptom was associated to a specific PLMVd variant, characterized by an insertion of 12-13 nucleotides (nts) causing the white coloration (Rodio et al., 2007). PLMVd is a member of the family of *Avsunviroidae* and consists of 335-338 nts for most strains and 347-351 nts for the calico strain (Flores et al., 2003) and an Egyptian strain that does not show any symptoms on the foliage (Hassen et al., 2007).

Dapple fruit symptoms on peach were also associated with the presence of HSVd (Sano et al., 1989; Zhou et al., 2006). HSVd belongs to the genus of *Hostuviroid* within the *Pospiviridae* family and consists of a circular single-strand molecule of RNA with a size that ranges between 294-303 nt.

From the early 1990s, a fruit peach syndrome characterized mainly by discoloured spots (dapple fruit) and/or yellow mosaic, cracked suture and deformations occurred in most commercial orchards in the Emilia Romagna region (Northern Italy) (Albanese et al., 1992). In order to ascertain the presence and the spread of the two viroids in the symptomatic trees, fruit samples have been collected from five peach cultivars: 'Royal Glory', 'Crimson Lady', 'Grenat', 'Diamond Princess' and 'Laura' and molecularly analyzed. Dapple fruit symptoms affected all cultivars (Figure 1), whereas 'Grenat' samples also showed evident yellow mosaic and fruit deformation (Figure 2) and 'Royal Glory' severely cracked sutures.

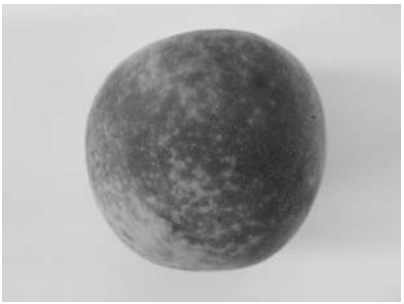

Fig. 1 Fruit of 'Diamond Princess' showing typical dapple fruit symptom

Fig. 2 **'**Grenat' fruits showing dapple fruit, yellow mosaic and fruit deformation symptoms

Materials and methods

Source of material: Symptomatic peach fruits of different cultivars originating from several orchards located in the Emilia Romagna region (Northern Italy) were used as source of materials (Table 1). More specifically, symptomatic fruits have been collected from an average of 20 trees per cultivars. PLMVd-infected, HSVd-infected and healthy GF 305 were used as positive and negative controls.

Tab. 1 List of peach cultivars collected, fruit symptoms observed, viroids detected, sequence analysis and accession numbers of the sequences. The underlined accession numbers refer to isolates showing the new mutations. DF= dapple fruit; CS= cracked suture; YM= yellow mosaic; Def= deformations.

				PLMVd sequence analysis		
Peach cultivar	**Symptoms**	**PLMVd**	**HSVd**	**Length**	**New mutations**	**Accession numbers**
Royal Glory	DF, CS	100%	90%	338-339	G280A	GQ872131- GQ872132- GQ872133
Crimson Lady	DF	70%	100%	339	-	GQ872128- GQ872129- GQ872130
Grenat	DF, YM, Def	90%	30%	337-339	-	GQ872125- GQ872126- GQ872127
Diamond Princess	DF	100%	0%	337-339	C165A	GQ872122-GQ872123- GQ872124
Laura	DF	100%	100%	350	12 nt insertion	GQ872134- GQ872135- GQ872136

RNA target preparation and viroids detection: For the detection of HSVd and PLMVd, total nucleic acids (TNA) were extracted from fruit skin according to the protocol established by Faggioli et al, (2001). TNA was finally eluted in 100 µL of DEPC water and analyzed following a two step/one tube RT-PCR protocol using specific primer pairs (Loreti et al., 1999; Astruc et al., 1996). All amplified products were analyzed using electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

Cloning and sequence analysis: At least three PLMVd positive samples per cultivar were selected for the sequence analysis. Amplified products were purified and cloned into pGEM®-T easy vector (Promega, Madison, WI, USA). Obtained sequences from the recombinant plasmids were multiple aligned using the Clustal W program and compared with the PLMVd isolates retrieved from the GeneBank database. Secondary structures were predicted using the mFold program (Zucker, 1989).

Results and discussion

The results showed a large diffusion of the two viroids in the assayed samples, mainly in mixed infections; more specifically PLMVd was found in 100% of 'Royal Glory', 'Diamond Princess' and 'Laura' samples; in 90% of 'Grenat' samples and in 70% of 'Crimson Lady' samples; HSVd affected 100% of 'Crimson Lady' and 'Laura' samples, 90% of 'Royal Glory' samples and 30% of 'Grenat' samples, whereas it was not found in any 'Diamond Princess' sample (Table 1). The evaluation of the role that the viroids could play in symptoms expression has been complicated by the high number of samples infected by both viroids (60%). Nevertheless, as already reported, PLMVd was confirmed to be strictly associated with yellow mosaic, cracked suture and fruit deformation symptoms. The aetiological origin of dapple fruit disease, however, seems to be less clear, since in 'Diamond Princess' only PLMVd has been found to be associated with the symptoms, whereas in all other cultivars the presence of HSVd could have influenced the symptom expression.

The molecular characterization of the PLMVd isolates does not show any correlation between nucleotide sequence and symptoms, although new variants were identified. In fact, the sequence alignment of our PLMVd isolates with the previously characterized ones revealed the presence of 15 new PLMVd variants (Figure 3): The molecular analysis of the PLMVd clones obtained from the cultivars 'Royal Glory', 'Diamond Princess', 'Grenat' and 'Crimson Lady' did not show any peculiarity with reference to specific symptoms (Figure 3), although variants isolated from the 'Royal Glory' and 'Diamond Princess' showed two mutations that had never been previously described (Table 1). More specifically, the clone RG2 showed a nucleotide change from G to A at position 280 and the clone DP3 showed a nucleotide change from C to A at position 165. However, all the novel variants were clustered in Group III (data not shown). More interesting was the sequencing of clones of the PLMVd isolate detected in the cultivar 'Laura', that showed an insertion of 12 nucleotides in the hammerhead region (Figure 3) Isolates with a similar insertion were previously reported by Hassen et al. (2007), but a comparison of the nucleotide sequences with the 'Laura' isolate highlight these differences. In order to confirm the results, amplification products, obtained with a proof reading *Taq* polymerase, were cloned and twenty clones sequenced. Eighteen out of twenty of the obtained sequences confirmed the presence of the insertion. Clustal W analysis showed some differences between the 18 analysed clones, that are summarized in Table 2. A Blast analysis was performed to understand the origin of this insertion and showed that the most common 12 nt sequence (UUUCGGAAGAAA) has a 100% homology with 12 nt of a previously published PLMVd sequence. The 12 nt are homologous from 113 to 124 nt of the stem 3 of some peach calico sequences published by Rodio et al., 2007. This evidence supports the hypothesis that the insertion could originate from a recombination event between two PLMVd isolates.

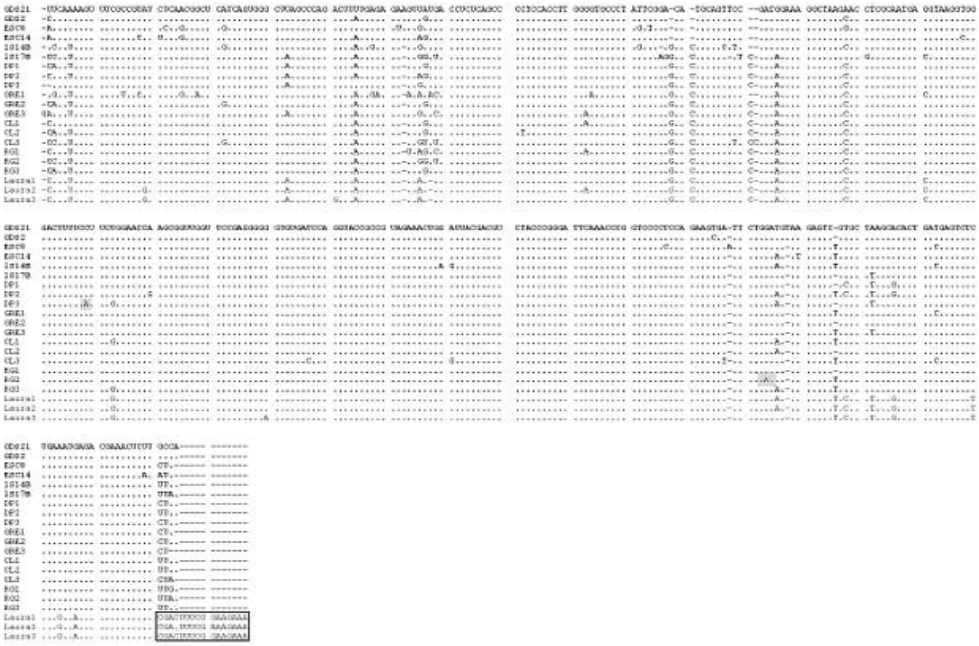

Fig. 3 Sequence alignment of PLMVd clones obtained from peach cultivars showing different symptoms. The reference sequences of group I, II and III are reported in black, the sequence of three clones of cvs Diamond Princess, Grenat, Crimson Lady, and Royal Glory are reported in blue and the PLMVd sequences from cv Laura with the 12 nt insertion are reported in red (highlighted by the red box). In the grey boxes show the two new mutations observed in clones DP3 and RG2

Tab. 2 Variants of the 12 nt insertion among the 18 clones of PLMVd 'Laura' isolate. The changes are underlined

Sequence	Number of clones with the same sequence insertion	Accession numbers
UUUCGGAAGAAA	9/18 (50%)	CQ872134- CQ872135- CQ872140- CQ872143- CQ872146- CQ872148- CQ872149- CQ872150- CQ872151
UUUCGAAAGAAA	5/18 (28%)	CQ872136- CQ872138- CQ872141- CQ872144- CQ872147
UUUCGUAAGAAA	1/18 (5.5%)	CQ872139
UUCCGGAAGAAA	1/18 (5.5%)	CQ872142
UUUCAAAAGAAA	1/18 (5.5%)	CQ872145
UUUCGUUUGGAA	1/18 (5.5%)	CQ872137

The other 'Laura' variants, reported in table 2, seem to be only polymorphisms belonging to the first insertion. In fact, according to the predicted secondary structure analysis, the mutations occurred only in the loop and not in the stem, as happens also for the Egyptian isolates (Figure 4), underlying the importance of the stem-loop secondary structure in the hammerhead region.

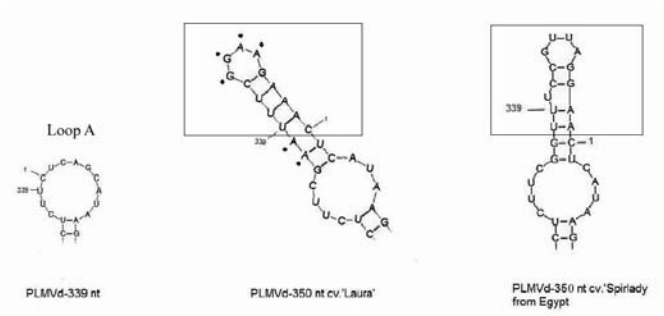

Fig. 4 Predicted secondary structures of the lowest free energy of the loop A of: a typical PLMVd isolate (339 nt), the 'Laura' isolate (350 nt) and the Egyptian isolate cv Spirlady (350 nt). The nucleotides of the characteristic insertion are highlighted in the boxes.

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Assessment of susceptibility to European stone fruit yellows phytoplasma of new plum variety and five rootstock/plum variety combinations

Landi, F.[1], Prandini, A.[1], Paltrinieri, S.[1], Missere, D.[2]., Bertaccini, A.[1]
[1] DiSTA, Patologia vegetale, *Alma Mater Studiorum*, Università di Bologna, viale Fanin 42, 40127 Bologna. Italy
[2] Centro Ricerche Produzioni Vegetali, Via dell'Arrigoni 120, 47522 Cesena (FC) - Italy

Abstract

Two separate experiments were carried out to assess the plum susceptibility to infection by European stone fruit yellows phytoplasmas during a five years period. Commercial varieties/cultivars and new selections grafted on Myrabolan 29C were evaluated in at least two plots of four plants each. Visual inspection and PCR/RFLP identification of phytoplasmas detected an increasing phytoplasma presence in both symptomatic and asymptomatic plants. Eight Japanese plum selections showed ESFY symptoms or pathogen presence in the 50% of the plants and nine selections showed ESFY infection in 20% of the plants. Only nine selections showed absence of both symptoms and pathogen. Although the European selections/cultivars were not symptomatic, plants belonging to six of these cultivars were positive for phytoplasma infection. The evaluation of cultivar/rootstock combinations indicate phytoplasma presence from the first year after plantation on. Two of the rootstocks seem to induce a delay in symptoms appearance and cultivar T.C. Sun resulted to be the most susceptible to the disease independently from the rootstock employed.

Keywords: Japanese plum, European plum, European stone fruit yellows phytoplasmas, resistance, diseases

Introduction

In the last thirty years an increasing presence of European stone fruit yellows phytoplasma (ESFY, '*Candidatus* Phytoplasma prunorum') (Seemüller and Schneider, 2004) on Japanese plum (*Prunus salicina*) was observed in commercial orchards in several European regions (Giunchedi et al., 1978; Desvignes and Cornaggia, 1982; Dosba et al., 1991; Torres et al., 2004). *Prunus* rootstocks (Jarausch et al., 1998) as well as wild *Prunus* species, e.g. *Prunus spinosa* and *P. cerasifera* (Carraro et al., 2002) and cherry (*Prunus avium*) (Paltrinieri et al., 2001) were also reported as infected by these phytoplasmas. In recent years ESFY phytoplasma has been detected in other wild plants such as *Rosa canina*, *Celtis australis*, *Fraxinus excelsior* (Jarausch et al., 2001), and grapevine in Hungary (Varga et al., 2000) and in Serbia (Duduk et al., 2004). The production losses can reach 40% in Japanese plum (Poggi Pollini *et al.*, 1995; Pastore et al., 1999) due to the lack of sanitary control of propagation materials together with the presence of the ESFY-specific vector, *Cacopsylla pruni* (Carraro et al., 1998b) in the orchards.The most evident symptoms on Japanese plum consist of black phloem and leaf malformations such as excessive elongation and upward rolling. European plum cultivars are usually infected only in a latent way (Carraro et al., 1998a). Diseased plants start producing flowers during mild winters, and show then undersized leaves with very little fruit production. The disease usually starts from a branch, affecting within 2-3 years all the plant; infected plants also show abnormal rootstock proliferation. The correct choice of cultivar/rootstock combination that must be tested for resistance/tolerance to the disease is also very important before distributing new cultivars (Desvignes, 1999). During 2003-2008 a research was carried out to assess the ESFY plum susceptibility under natural conditions of disease spreading by visual inspection coupled with molecular analyses.

Material and methods

Two experiments were carried out to verify susceptibility of about 60 plum varieties/cultivars and new selections (A), and of 5 routinely used rootstock/scion combinations (B). The orchards were located in an ESFY severely naturally infected area of Northern Italy (Paltrinieri et al., 2004). Monitoring by visual inspection and PCR/RFLP assays was carried every year out from August to October to verify phytoplasma presence/identity in all plants.

Experiment A: Japanese and European plum varieties and selections grafted into Myrabolan 29C (Table 1 A and B) were evaluated in at least two plots with four plants each.

Experiment B: Three plum commercial cultivars TC Sun, Fortune and Angeleno were grafted on the five rootstocks 'Adesoto 101', 'Ishtara-Ferciana', 'GF 677', 'Montclair-Chanturgue' and 'Myrabolan 29/C'; four plants per each combination per at least two plots were employed.

Tab. 1 A. Results of ESFY phytoplasma identification in Japanese plum cultivars/varieties and selections. B. Results of ESFY phytoplasma identification in European plum varieties/cultivars and selections. In both cases plants were grown in a field located in a severely ESFY naturally infected area.

Japanese plum cultivars and selections		
Table A	**Symptoms**	**n. of ESFY infected plants/n. tested plants**
Anne Gold	yes	2/4
Aphrodite	yes	1/4
Black Glow	yes	1/3
Black Sunrise	yes	3/4
Black Top	no	2/4
Bragialla	no	0/4
Brarossa	no	0/4
Carmen Blu	yes	2/4
Dofi Sandra	yes	0/3
Early Fortune	yes	1/4
Fortune	no	0/4
Gaia	no	1/4
Golden Plumza	yes	1/4
Obilnaja	no	1/4
Red Noble	yes	4/4
Ruby Crunch	no	0/3
Shiro	yes	1/4
Dofi selections (Florence University)		
89.024.004	yes	2/4
89.024.029	no	1/4
89.028.047	no	1/4
89.030.010	no	1/4
89.030.020	no	0/4
89.030.030	yes	1/4
89.030.031	no	0/4
89.036.131	no	0/4
CRA Forli' selections		
IFF on 219	yes	4/4
IFF on 221	yes	4/4
IFF on 260	no	0/4
IFF on 268	no	3/3
IFF on 271	no	0/4

European plum cultivars and selections		
Table B	**Symptoms**	**n. of ESFY infected plants/n. tested plants**
Bellamira	no	0/4
Capitana	no	0/4
Elena	no	0/4
Felsina	no	0/4
Grossa Di Felisio	no	0/4
Jojo	no	0/4
Liablu	no	0/4
Maria Novella	no	0/4
Presenta	no	0/4
President	no	0/4
Rheingold	yes	1/4
Stanley	no	0/4
Tegera	no	0/4
Tipala	no	0/4
Top 2000	no	0/4
Topend Plus	no	0/4
Topfive	no	0/4
Topgigant Plus	no	0/4
Tophit	no	0/4
Tophit Plus	no	0/4
Topking	no	0/4
Topstar Plus	no	0/4
Valcean	yes	3/4
Valerie	yes	1/4
Victory	no	0/4
Agri 2000 selections		
N. 8	no	0/4
N. 10	no	0/4
Hohenheim University selections		
N. 1218	no	0/4
N. 1446	no	0/4
N. 1462	no	0/4
N. 1464	no	0/4
N. 1468	no	0/4
N. 1474	no	1/4
N. 1632	no	0/4
N. 3018	yes	3/4
N. 3217	no	0/4
N. 4913	no	0/4

Molecular analyses: DNA was extracted from fresh leaf midribs and phloem by a chloroform/phenol procedure (Prince et al., 1993). PCR assays were carried out on the nucleic acid samples diluted in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] to give a final concentration of 20 ng per µl, in total 25 µl reaction mixtures under the conditions described by Schaff et al. (1992). Nested PCR reactions were performed under the same conditions, using as template the products of the previous amplification diluted 1: 30 with sterile water. Positive control samples were DNAs extracted from periwinkle plants infected by phytoplasma strains from the micropropagated collection of DiSTA (University of Bologna), in particular GSFY1, GSFY2 (subgroup 16SrX-B), PD (subgroup 16SrX-C) and AP (subgroup 16SrX-A) were employed.

Phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and R16F2/R2 (Lee et al., 1995) amplifying fragments internal to each others in the 16S ribosomal gene were used, respectively in direct and nested PCR. Samples were further tested in nested PCR with primers R16(X)F1/R1 specific for 16SrX (apple proliferation) group (Lee et al., 1994; 1995). Samples with the reaction mixture devoid of DNA template were included in each experiment as negative controls. PCR products were subjected to electrophoresis in a 1% agarose gel and visualised by staining with ethidium bromide and UV illumination.

Three to six µl of PCR products were digested using *Mse*I, *Ssp*I and *Rsa*I restriction enzymes at 37°C for at least 16 hours following the instructions of the manufacturer (Fermentas, Vilinius, Lithuania). The restriction patterns were then compared with those of reference strains after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Results and discussion

Experiment A: After five years 8 Japanese plum selections showed ESFY symptoms or pathogen presence in 50% of the plants and nine selections showed ESFY infection in 20% of the plants. Only 9 selections showed absence of both symptoms and pathogen (Table 1A). None of the European selections/cultivars employed was symptomatic, however plants belonging to 6 cultivars were positive for phytoplasma presence (Table 1B). The comparison of symptom expression and results from PCR/RFLP analyses indicates that selections IFF on 221 and IFF on 219, are very susceptible to ESFY infection; while selection IFF on 268 resulted to be phytoplasma infected even if asymptomatic. A medium level of susceptibility i.e. pathogen or symptom presence starting from the second year after plantation in 50% of the plants, was shown by Anne Gold, Black Sunrise, Black Top, Carmen Blue, Dofi Sandra, Gaia, Obilnaja and Dofi 89.024.004 among the Japanese cultivar and virieties. Less susceptible were Shiro, Black Glow, Dofi 89.030.030, 89.024.029, 89.028.047, 89.030.010, Aphrodite, Early Fortune, and Golden Plumza (one infected plant per cultivar). Varieties Bragialla, Brarossa, Fortune, Ruby Crunch and selections Dofi 80.030.020, 89.030.031, 89.030.131 and IFF on 260 and on 271 were always negative to both symptom presence and molecular analyses. Some European plum cultivars or selections such as Rheingold, Presenta, Valerie, Valcean, 3018 and 1474 were positive to the ESFY presence, however only Rheingold, Valerie, Valcean and selection 3018 showed symptoms possibly related to phytoplasma presence.

Experiment B: The majority of the cultivar/rootstocks combinations showed phytoplasma symptoms and were positive to the PCR/RFLP analyses from the first year after plantation. Two of the rootstocks induced a delay in symptoms appearance indicating some resistance to ESFY in Japanese plum could be present, but only for one-two years after plantation. Intermediate/high susceptibility was detected in varieties grafted on 'GF 677', 'Adesoto 101' and 'Montclair-Chanturgue' showing plants with severe leaf symptoms and reduced growth; while scions grafted on 'Myrabolan 29/C' were efficiently vegetating and growing in spite of the presence of symptoms in the leaves. TC Sun was the most susceptible cultivar to ESFY phytoplasmas in all tested rootstocks; cultivars grafted on 'Ishtara-Ferciana' showed symptoms only after four years from plantation (Figs. 1 and 2) indicating less susceptibility to ESFY infection. The molecular analyses carried out were very important to identify the latently infected combinations that are the most dangerous allowing the pathogen to survive long time in orchards causing recurrent epidemic outbreaks.

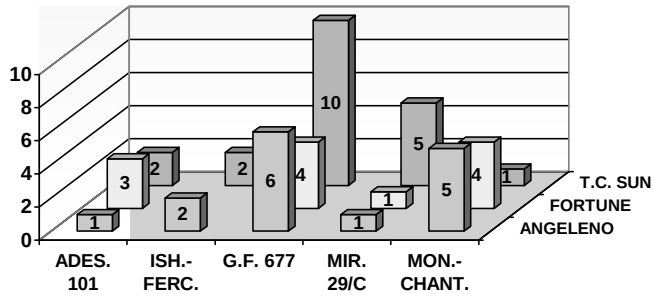

Fig. 1 Number of infected plants for the different cultivar/rootstock combinations

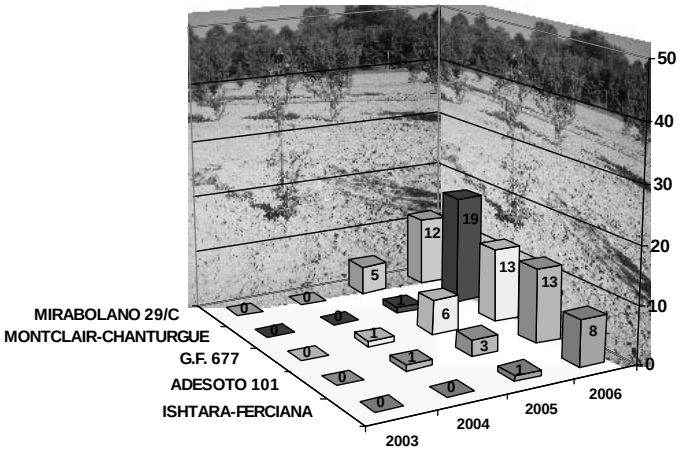

Fig. 2 Number of symptomatic plum plants per year grouped by rootstock.

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Detection and distribution of European stone fruit yellows (ESFY) in apricot cv. 'Bergeron' and epidemiological studies in the province of Trento (Italy)

Poggi Pollini, C.[1], Forno, F.[2], Franchini, S.[3], Gobber, M.[3], Lanzoni, C.[1], Mattedi, L.[2], Miorelli, P.[3], Profaizer, D.[3], Ratti, C.[1]
[1] Dipartimento di Scienze e Tecnologie Agroambientali (DISTA), Alma Mater Studiorum, Bologna, Italy.
Email: carlo.poggipollini@unibo.it
[2] Istituto Agrario S. Michele all'Adige (IASMA), Trento, Italy
[3] IASMA, CAT, Trento, Italy

Abstract

The aim was to investigate the performance of 'Bergeron' on 'Wavit' in 4 experimental fields, in the province of Trento (Italy), where European stone fruit yellows (ESFY) caused by "Candidatus *Phytoplasma prunorum*" has been constantly spreading since 2000.

This included visual inspections for typical symptoms (early bud-break during dormancy and premature leaf-roll) and a highly sensitive Real time-PCR (Rt-PCR) assay. 25 % of the propagation material was checked with this method and found to be healthy, before planting in 2005.

The epidemiology of the disease was also studied by focusing on: the presence of the vector *Cacopsylla pruni* (Scopoli) on conifers, the detection of "Ca. *P. prunorum*" in psyllid eggs and the transmission efficiency at different stages. This was done by exposing apricot trees in 2 locations, during 2 periods from January to July, to the overlapping presence in the orchards of the re-immigrants and the new generation of *C. pruni*.

The results obtained demonstrated that 'Bergeron' seems to be highly susceptible to ESFY: typical bud-break was rarely observed, but up to 20-30% of the plants showed premature leaf-roll, fruit deformation and dieback. *C. pruni* was caught only once on *Picea abies* during winter; "Ca. *P. prunorum*" was found in 4 egg samples from 2 locations and the preliminary results on the exposed trees confirmed that the re-immigrants could be the most efficient vectors at least on apricot.

Keywords: *Prunus armeniaca*, cultivar 'Bergeron', Real time-PCR, "Candidatus *Phytoplasma prunorum*", epidemiology.

Introduction

Surveys have been conducted, since 2004, in different apricot orchards to determine the current status of European Stone Fruit Yellows (ESFY) in the province of Trento (Italy). In this area a constant progression of the disease, caused by "Candidatus *Phytoplasma prunorum*", has been noted in recent years leading to partial or total tree dieback causing major economic losses to growers. To prevent the disease spreading, four experimental orchards of cv. 'Bergeron' grafted on 'Wavit' have been established since 2005. 25% of the propagation material was checked with a highly sensitive Real time-PCR (Rt-PCR) assay, useful for large-scale analyses and found to be "Ca. *P. prunorum*"- free (Pignatta et al., 2006).

Surveys have been conducted in these experimental fields including visual inspections for typical symptoms (early bud-break during dormancy and premature leaf-roll) and Rt-PCR assays. The epidemiology of the disease was also studied by focusing on some points in the life cycle of "Ca. *P. prunorum*" vector, the psyllid *Cacopsylla pruni* (Scopoli), such as the presence of *C. pruni* on conifers during winter, the detection of "Ca. *P. prunorum*" in psyllid eggs, and transmission efficiency at different life stages of the vector.

Material and methods

Field surveys: Visual inspections for typical ESFY-symptoms (early bud-break during dormancy, fruit deformation and premature leaf-roll in summer and autumn) have been performed in the experimental fields at least three times a year since 2006. The presence of *C. pruni* on conifers has been investigated by searching for the insect in different places, especially on *Abies alba* and *Picea abies* (Thebaud et al., 2006), in the province of Trento since winter, 2007. The eggs were carefully removed from the leaf surfaces of apricots and blackthorn (*Prunus spinosa*) with a needle during May, 2008, and, after identification, processed in groups of five.

Detection of "Ca. *P. prunorum*": Samples of trees with typical and dubious symptoms, insects and eggs, were taken and tested for the presence of "Ca. *P. prunorum*" via Rt-PCR. A multiplex procedure was used for simultaneous detection of the pathogen and host DNA, to avoid false negatives due to PCR inhibition, as previously described (Pignatta et al., 2006). Total DNA was extracted from apricot phloem and insects with a phytoplasma enrichment procedure (Marzachì et al., 1999).

Studies on transmission efficiency: The vector transmission efficiency, at different stages of its life, was studied by exposing apricots of the same cultivar, in 2 different locations (Balbido and Calavino) during 2 periods (80 plants in all), to the overlapping presence of re-immigrants (adults that have overwintered) (from 13/3/2007 to 16/5/2007) and the new generation of *C. pruni* in the orchards (from 16/5/2007 to 3/7/2007). All plants were tested individually with Rt-PCR before exposure and found to be phytoplasma-free. After each exposure period, the test plants were treated with insecticide, kept for at least one year in an insect-proof screenhouse, inspected for ESFY-symptoms and finally individually tested with Rt-PCR.

Results

Field surveys and detection of "Ca. *P. prunorum*": The results obtained demonstrated that 'Bergeron' seems to be highly susceptible to ESFY: typical bud-break was rarely observed, but up to 20-30% of the plants showed premature leaf-roll, fruit deformation and dieback causing economic losses (Table 1). Amplification was always obtained from symptomatic plants, but no phytoplasma were found in asymptomatic and healthy apricots kept in an insect-proof screenhouse as healthy controls. Regarding the epidemiological studies, *C. pruni* was caught only once on *P. abies* during winter, 2007, very far from the orchards (Monte Bondone). Moreover, the 4 adults captured (re-immigrants) were individually tested and found not to be infected by "Ca. *P. prunorum*". On the other hand, the phytoplasma was found in 4 egg samples from 2 different locations, on blackthorn and on apricot leaves respectively (Table 2).

Tab. 1 progression of ESFY-infection in 4 experimental fields.

	Location			
	Bleggio Balbido Crosina (170 Trees)	**Bleggio Balbido Farina (172 Trees)**	**Pergine S. Caterina Biasi (200 Trees)**	**Val Di Non Salobbi Pisani (180 Trees)**
Number of trees with symptoms and positive by Rt-PCR (2006-2007)	20 (11.8%)	17 (9.9%)	3 (1.5%)	15 (8.3%)
Number of trees with symptoms and positive by Rt-PCR (2008)	37 (21.8 %)	51 (29.6 %)	9 (4.5 %)	19 (10.5 %)

Tab. 2 results of phytoplasma detection by Rt-PCR on *C. pruni* eggs.

Location*	**Specie**	**Positive/Tested (groups)**
Balbido	*Prunus armeniaca*	1/25**
Calavino	*P. spinosa*	3/25**

*eggs were collected in May, 2008. ** 5 eggs each sample

Table 3 shows the results of Rt-PCR assays on the exposed apricot trees. First typical symptoms were noted at least 15 months after the field exposure.

Tab 3 natural spread of "*Ca*. P. prunorum" in the field.

Location	**Exposure Periods**	**Symptoms Observed**	**Positive/Tested**
Balbido - Crosina	I° : 13/3/07 – 16/5/07	Premature leaf-roll (Sept 08); dieback (Feb 09) (1 plant)	1/20** (5%)
Calavino - Chemelli	I° : 13/3/07 – 16/5/07	Premature leaf-roll (Sept 08); dieback (Feb 09) (1 plant)	1/20** (5%)
Balbido - Crosina	II°: 16/5/07 – 3/7/07	/*	0/20
Calavino – Chemelli	II°: 16/5/07 – 3/7/07	/	0/20

* no symptoms observed. **the 2 symptomatic plants were positive by Rt-PCR.

Discussion

The first aim was to investigate the field performance of the cultivar 'Bergeron' grafted on 'Wavit' in the environmental conditions of the province of Trento, where ESFY has been constantly spreading since 2000. The results obtained

revealed an important annual progression of infected trees (Table 1), expressed by severe foliar and fruit symptoms, decline and total dieback. Latent infections on apparently healthy apricot trees (especially cv. 'Luizet') have been reported in Valais (Western Switzerland) elsewhere (Genini and Ramel, 2004). Our results, however, confirmed a strict association between symptoms - especially premature leaf-roll and dieback, but not typical bud-break that was rarely observed on this cultivar – and the presence of "Ca. *P. prunorum*". A dramatic increase in the disease was noted especially in 2 experimental fields located in Balbido. ESFY spread was, however, lower in the experimental field of Pergine, but this situation could be due to environmental factors which can influence psyllid fitness in different areas.

Rt-PCR tests performed before planting on 25% of propagation material suggested that under our conditions new tree infections are due to the transmission of "Ca. *P. prunorum*" by the vector rather than to contaminated propagation material, as reported in other epidemiological studies (Ramel and Gugerli, 2004). The high proportion of insects and of blackthorn hedges found to be infected by "Ca. *P. prunorum*" in this area (Pignatta *et al*., 2006) and the lack of efficacy of insecticide applications in controlling the disease (Poggi Pollini et al., 2007) justify all efforts to better understand ESFY epidemiology. During this study some insights were gained into the insect overwintering sites that remain unknown. Only once a few adults were captured on *P. abies* at a great distance from the orchards. Large migration movements of this insect have, however., been clearly demonstrated (Sauvion et al., 2007).

"Ca. *P. prunorum*" was found in 4 egg samples from 2 different locations. It should be noted that the proportion of infected eggs is quite substantial, especially on blackthorn (Table 2). This strongly suggests that under our conditions this species could provide an efficient pathogen-source for *C. pruni*. Previous reports indicate that an epidemiological cycle of ESFY can be achieved in blackthorn even in the absence of *Prunus* orchards (Yvon et al., 2004). Moreover, the possibility of transovarial transmission, recently demonstrated for this pathogen (Tedeschi et al., 2006), has important implications for disease management.

The results of Rt-PCR assays on the exposed apricot trees (Table 3) confirmed that the re-immigrants, infected the previous year, can be the most efficient vectors of "Ca. *P. prunorum*"at least on apricot. These data are consistent with the research recently performed on apricot in France that demonstrated how most re-immigrants can be infectious and are able to inoculate susceptible plants when they return and reproduce on *Prunus* (Thebaud et al, 2006).

The potential epidemic threat posed by ESFY to stone fruit orchards is confirmed by the annual increase in the number of infected trees in 4 experimental fields of five-years-old 'Bergeron' on 'Wavit'. Our data suggest that some new, more tolerant, varieties are therefore necessary for apricot orchards in the province of Trento. Further investigation is necessary to complete and clarify the epidemiology of the disease and examine possible control with phytosanitary treatments.

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PCR/RFLP-based method for molecular characterization of 'Candidatus *Phytoplasma prunorum*' strains using the *aceF* gene.

Martini, M.[1], Ferrini, F.[1], Danet, J.-L.[2], Ermacora, P.[1], Sertkaya, G.[3], Delić, D.[4], Loi, N.[1], Foissac, X.[2], Carraro, L.[1]
[1] Department of Biology and Plant Protection, University of Udine, Italy
[2] UMR GDPP, INRA and Université Bordeaux 2, Villenave d'Ornon, France
[3] University of Mustafa Kemal, Faculty of Agriculture, Department of Plant Protection, Antakya, Turkey
[4] University of Banja Luka, Faculty of Agriculture, Banja Luka, Bosnia and Herzegovina

Abstract

New molecular typing tools for phytoplasmas belonging to the 16SrX phytoplasma group have recently been developed based on the non-ribosomal genes *aceF*, *pnp*, *imp*, and *SecY*. In the present work we chose to perform a PCR-RFLP method based on the *aceF* gene. This genetic marker had previously shown high variability among strains of the 16SrX group, moreover, it had allowed for the differentiation of French hypovirulent 'Candidatus *Phytoplasma prunorum*' strains from virulent ones.

Most of the stone fruit samples were collected in north-east Italy, although a few samples from Bosnia and Herzegovina, and Turkey were also included in the work to explore variability. French hypovirulent and virulent strains, one Azerbaijan strain and 'Ca. *P. prunorum*' strains maintained in periwinkles were used as reference strains. Some of the Italian samples were not collected in the field and they became infected by *Cacopsylla pruni* under controlled conditions.

Sequencing of the *aceF* gene was performed on some of the samples tested and based on the alignment, a few restriction enzymes were selected for 'Ca. *P. prunorum*' strain differentiation. Nested PCR was performed using previously developed primers on all samples and RFLP analyses were carried out with *Bpi*I, *Hae*III and *Tsp*509I enzymes. *Bpi*I and *Hae*III enzymes generated two different profiles, one profile was undigested and the second one constituted by two different fragments. The *Tsp*509I enzyme enabled three different pattern types to be distinguished. Combining the results obtained with the three restriction enzymes, it was possible to distinguish between the 'Ca. *P. prunorum*' strains investigated in this study: 6 different RFLP subgroups AceF-A, -B, -C, -D, -E and –F. We confirmed that strains belonging to 4 subgroups, AceF-A, -B, -C and -E were present in north-east Italy, where a large number of the samples were processed. The strains of AceF-A and -E subgroups were the predominant ones (21.6% and 17.0%, respectively) and mixed infections of AceF-A+E subgroups (17.0%), and AceF-B+E (14.8%) subgroups were quite common.

Keywords: phytoplasma, European stone fruit yellows, molecular differentiation, sequencing

Introduction

'Candidatus *Phytoplasma prunorum*' is the causal agent of European stone fruit yellows (ESFY), a quarantine phytoplasma disease mainly present in Europe and also recently reported in Turkey (Sertkaya et al., 2005). European stone fruit yellows have a wide range of host plants among cultivated and wild stone fruits species, which show large differences in terms of symptom expression and susceptibility (Carraro et al., 2002; 2004). *Prunus armeniaca* (apricot) and *P. salicina* (Japanese plum) show a high susceptibility and sensitivity to the disease. 'Ca. *P. prunorum*' is specifically transmitted by the psyllid *Cacopsylla pruni* (Scopoli) (Carraro et al., 1998; 2001) and, together with 'Ca. *P. mali*' and 'Ca. *P. pyri*', belongs to a major phylogenetic group, the apple proliferation (AP) phytoplasma group (16SrX) (Seemüller and Schneider, 2004). Conventional detection of fruit tree phytoplasmas is mainly based on nested PCR using 16S rDNA universal or group specific primer pairs, followed by identification using RFLP analyses. New molecular typing tools for fruit tree phytoplasmas belonging to the 16SrX phytoplasma group have recently been developed based on the non-ribosomal genes *aceF*, *pnp*, *imp*, and *SecY* (Danet et al., 2007; 2008). In the present work we chose to perform a PCR-RFLP method based on the *aceF* gene for differentiation of 'Ca. *P. prunorum*'. This genetic marker showed high variability among strains of the 16SrX group, moreover, it allowed for the differentiation of French hypovirulent 'Ca. *P. prunorum*' strains from the virulent ones (Danet et al., 2008).

Material and methods

Plant material and phytoplasma reference strains: Most of the stone fruit samples were collected in north-east Italy (Friuli Venezia Giulia, FVG) from different locations during the years 2007-2008. Some of Italian samples, with a geographical origin indicated as FVG, Udine, were not collected in the field and they became infected by *C. pruni* under controlled conditions (Table 2).

Samples from Turkey and Bosnia and Herzegovina (BiH) were also included in the work to explore variability (Table 2). One Azerbaijan strain (Azer 10) and some French hypovirulent (PVC-LA8-HypV, B7-HypV) and virulent (G32, Psalor, ECA-M200, ESFY 042-1, ESFY 14-1, ESFY 293-4) strains that were shown to be genetically different in a previous study (Danet et al., 2008) were used as reference strains. Phytoplasma strains maintained in periwinkle LNS2, LNp (= ESFY) and GSFY2 were also used as reference strains in this work.

Nucleic acid extraction and 'Ca. *P. prunorum*' differentiation based on a PCR/RFLP method using the *aceF* gene: Total DNA from periwinkle-maintained phytoplasma reference strains was extracted using the CTAB extraction method (Doyle and Doyle, 1990). Plant total DNA was extracted from stone fruit leaf mid-veins according to a previously recorded protocol (Doyle and Doyle, 1990) that was slightly modified. The presence of phytoplasmas in plant samples was determined by the conventional nested-PCR procedure based on 16S rDNA using P1/P7 or P1/16S-SR primer pairs (Lee et al., 2004) in direct PCR followed by fO1/rO1 primers (Lorenz et al., 1995) in nested PCR. Restriction fragment length polymorphism (RFLP) analyses of fO1/rO1 PCR products were performed with *Ssp*I and *Rsa*I enzymes in order to identify 'Ca. *P. prunorum*' positive samples.

All of the positive samples obtained using the first method were then analysed with the non-ribosomal method based on the *aceF* gene. The *aceF* gene was amplified by nested PCR using the recently published primers AceFf1/AceFr1 followed by AceFf2/AceFr2 (Danet et al., 2008). The amplification protocol was slightly modified from the previously published protocol by Danet *et al.* (2008). Direct and nested PCR were performed as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, and by a final extension step at 72 °C for 8 min. The first amplification products were diluted by 1:30 and dilutions were used as a template in nested PCR. Five microlitres of PCR products were visualized by electrophoresis in 1% agarose gel and stained with GelRed™ (Biotium, Inc., Hayward, CA).

Sequencing of a portion (about 500 bp) of the *aceF* gene was performed on some of the positive samples and the nested-PCR products were purified using a Wizard® SV Gel and the PCR Clean-Up System Kit (Promega, WI, USA). Sequencing was performed with an automated DNA sequencer (ABI Prism Model 3730, Applied Biosystems, CA, USA) at the Genelab (ENEA Casaccia, Rome, Italy) using the forward primer. The obtained a*ceF* gene sequences were aligned using BioEdit v7.0.0 software package (Hall, 1999) and visually inspected.

Based on the alignment, a few restriction enzymes were selected for 'Ca. *P. prunorum*' strain differentiation. RFLP analyses were carried out with *Bpi*I, *Hae*III (Fermentas, Lithuania) and *Tsp*509I (New England BioLabs, USA) enzymes as recommended by the manufacturer to cleave AceFf2/AceFr2 nested-PCR products obtained from the phytoplasma reference strains and all positive field collected samples. The digested products were then separated by electrophoresis through a 10% polyacrylamide gel in 1X TBE (*Tsp*509I digested products) or a 2-3% MS-6 Metagel Agarose (Conda) in 1X TBE (*Bpi*I and *Hae*III digested products).

Results

All of the phytoplasma strains from stone fruit samples showing identical 16S rDNA-based RFLP profiles to 'Ca. *P. prunorum*' reference strains (16SrX-B) were selected for further characterization. Nested-PCR products 797 bp long were obtained using *aceF* gene primers from all selected samples from Italy (FVG) (88 samples), Turkey (6 samples) and BiH (5 samples), and from all used reference strains.

Analysis of the obtained partial *aceF* gene sequences (about 500 bp) enabled four point mutations altering endonuclease restriction sites to be distinguished. The endonucleases whose restriction sites were deleted or created by single base substitutions were *Bpi*I, *Hae*III and *Tsp*509I, and these were used in RFLP analyses of the *aceF* gene sequences for 'Ca. *P. prunorum*' strain differentiation.

The putative restriction sites of *Bbs*I (*Bpi*I), *Hae*III and *Tsp*509I on the *aceF* gene sequences of some representative 'Ca. *P. prunorum*' strains are shown in Figure 1. Actual RFLP pattern types of *Bpi*I, *Hae*III and *Tsp*509I enzymes useful for strain differentiation are illustrated in Figure 2. *Bpi*I and *Hae*III enzymes generated two different profiles, one profile was undigested and the second one constituted by two different fragments. *Tsp*509I enzyme enabled three different pattern types to be distinguished. As shown in Table 1, combining the pattern types obtained with the three restriction enzymes, it was possible to distinguish between the 'Ca. *P. prunorum*' strains investigated in this study: 6 different RFLP subgroups AceF-A, -B, -C, -D, -E and -F.

Tab. 1 Patterns produced by RFLP analyses of *aceF* gene sequences from representative strains of 'Ca. *P. prunorum*'.

		RFLP pattern type with restriction enzyme			
Phytoplasma strain	**Origin**	***Bpi*I**	***Hae*III**	***Tsp*509I**	**AceF-subgroup**
Apricot SP5-36	Italy	1	1	1	A
LNS2 - *C. roseus*	Italy	1	1	2	B
Peach 31	BiH	1	2	1	C
Psalor	France	1	1	3	D
LNp - *C. roseus*	Italy	2	1	2	E
Azer 10	Azerbaijan	2	2	2	F

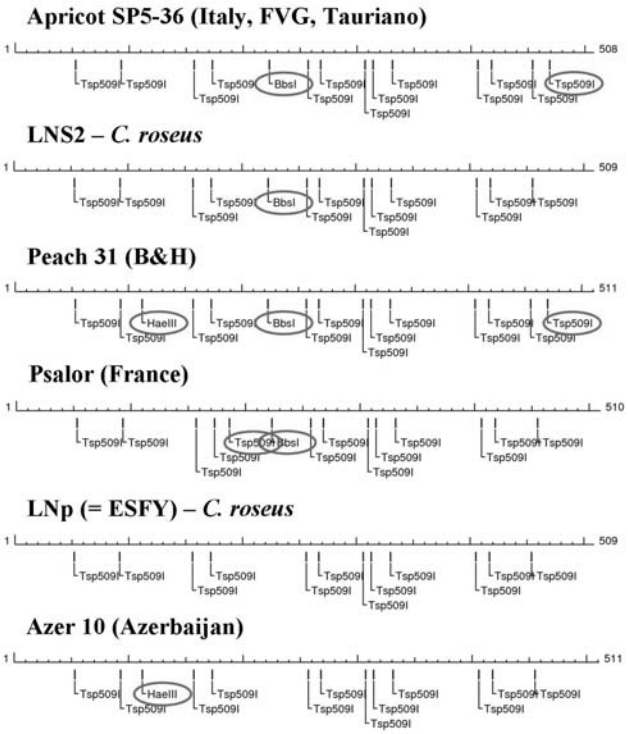

Fig. 1 Putative restriction sites of *Bbs*I (*Bpi*I), *Hae*III and *Tsp*509I enzymes in partial *aceF* gene sequences amplified by nested PCR with primer pair AceFf1/AceFr1 followed by AceFf2/AceFr2 from representative 'Ca. *P. prunorum*' strains.

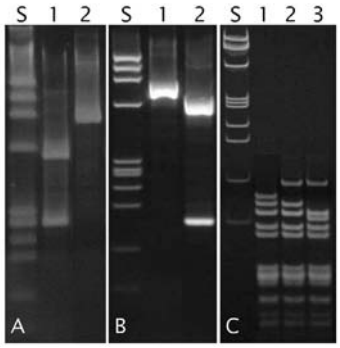

Fig. 2 Actual RFLP pattern types of AceFf2/AceFr2 nested-PCR products digested with restriction enzymes (A) *Bpi*I, (B) *Hae*III and (C) *Tsp*509I. 1, 2, 3: RFLP pattern types. S: Φ174 *Hae*III digested (New England BioLabs, USA).

All of the results obtained by RFLP analyses on field samples from Italy, Turkey and BiH are summarized in Table 2. In north-east Italy, 4 different 'Ca. *P. prunorum*' strains were found to be present belonging to AceF-A, -B, -C, -E subgroups. The 'Ca. *P. prunorum*' strains mostly found in north-east Italy were those belonging to AceF-A (19/88; 21.6%) and -E (15/88; 17.0%) subgroups. Mixed RFLP patterns were quite common, in fact half (44/88; 50.0%) of the analysed samples showed overlapping profiles, especially with *Bpi*I and *Tsp*509I. These results indicated that mixed infections were quite a widespread phenomenon in the orchards that were inspected in north-east Italy. Since a single plant sample could show mixed profiles with more than one enzyme, in order to simplify the interpretation of the RFLP results it was hypothesized that mixed infections are derived from no more than two different strains present at the same time within the plants. The most frequent mixed infections were represented by strains belonging to AceF-A+E subgroups (15/88, 17.0%) and AceF-B+E (13/88, 14.8%) subgroups. Among the few plant samples analysed from Turkey and BiH, 'Ca. *P. prunorum*' strains belonging to the AceF-C subgroup seemed to be as important as the strains of the AceF-A subgroup (Table 2).

Tab. 2 Results obtained by RFLP analyses of *aceF* gene sequences from stone fruits infected with 'Ca. *P. prunorum*' strains and phytoplasma reference strains (in bold) from France, Azerbaijan and those maintained in periwinkles.

Geographical origin	**Infected host/Reference strain**		**AceF-subgroup no. of samples/tested samples**
Italy (FVG, Tauriano)	Apricot		A (4/14), B (1/14), E(1/14), A+B (1/14), A+C (1/14), A+E (3/14), B+E (2/14), C+E (1/14)
Italy (FVG, Gaio)	Apricot		A (7/16), B (2/16), C (2/16), E (4/16), B+E (1/16)
Italy (FVG, Galleriano)	Apricot		A (6/39), B (1/39), C (2/39), E (5/39), A+B (2/39), A+C (5/39), A+E (7/39), B+E (8/39), C+E (3/39)
Italy (FVG, Udine)*	Japanese plum, apricot, *Prunus mahaleb*, *P. cerasifera*, *P. tomentosa*		A (2/19), B (1/19), C (1/19), E (5/19), A+E (5/19), B+E (2/19), C+E (3/19)
Turkey	plum, apricot, almond, peach		A (2/6), C (2/6), A+C (2/6)
BiH	apricot, peach		A (3/5), C (2/5)
France	*Prunus* sp.	G32	B
		Psalor	D
		ECA-M200	B
		ESFY 042-1	B
		ESFY 14-1	B
		ESFY 293-4	B
		PVC-LA8-HypV**	A
		B7-HypV**	A
Azerbaijan	*Prunus* sp. - Azer 10		F
Italy	*C. roseus* - LNp (= ESFY)		E
Germany	*C. roseus* - GSFY2		B
Italy	*C. roseus* - LNS2		B

* Stone fruit trees maintained in controlled conditions under a screenhouse, and exposed to infection by *C. pruni*. ** French 'Ca. *P. prunorum*' hypovirulent strains

Among the phytoplasma reference strains from France, the Psalor strain represented a different subgroup indicated by AceF-D, and the reference strain Azer 10 from Azerbaijan represented another different subgroup indicated by AceF-F (Tables 1 and 2). Using the *Bpi*I enzyme it was possible to differentiate Italian LNp reference strains from the other '*Ca*. P. prunorum' reference strains maintained in the periwinkles.

Discussion

RFLP analyses and sequencing of the 16S rRNA gene and a non-ribosomal gene did not enable differentiation between 'Ca. *P. prunorum*' strains (Jarausch et al., 2000). Recently, genomic variability between 'Ca. *P. prunorum*' strains was shown using a molecular approach based on a multi-locus sequence typing (MLST) strategy (Danet et al., 2007; 2008). One of the four non-ribosomal genetic loci used in the MLST was the *aceF* gene, which was chosen to develop a PCR-RFLP method for strain differentiation because from preliminary results it seemed possible to distinguish hypovirulent strains from virulent ones using this gene (Danet et al., 2008). The PCR-RFLP method based on the *aceF* gene described in this work confirmed the genetic variability among 'Ca. *P. prunorum*' (16SrX-B) strains and distinguished 6 different RFLP AceF-subgroups among the analysed strains.

The results obtained by the RFLP analysis showed that in north-east Italy (FVG), where a large number of samples were processed, it was possible to find a high variability among the strains tested, since four different subgroups were present. It also demonstrated that the strains belonging to AceF-A and -E subgroups were the predominant ones and that mixed infection by the two strains was also quite common together with the mixed infection by strains of AceF-B and -E subgroups. From the results obtained from analysing the samples collected in the screenhouse it appeared that *C. pruni* is able to transmit all of the 'Ca. *P. prunorum*' strains present in north-east Italy. The high percentage of mixed infections could be explained by the presence of several strains in the surveyed locations, high vector population densities and by recurring phytoplama inoculations by the vector year after year.

The French hypovirulent strains were characterized as belonging to the subgroup AceF-A, which is quite a widespread subgroup in north-east Italy and has also been shown to be present in Turkey and BiH. In many cases, this particular type of 'Ca. *P. prunorum*' strain was associated with plants exhibiting clear symptoms of European stone fruit yellows in Italy (FVG), Turkey and BiH. For this reason we cannot confirm that this molecular marker allowed for differentiation between hypovirulent and virulent strains, as it appeared to do in the work by Danet *et al.* (2008). The French strain Psalor and the Azerbaijan strain Azer 10 were found to be molecularly different from all of the other analysed strains, confirming previous published data by Danet et al. (2008). In particular, the strain Azer 10 was shown to be genetically divergent, exhibiting 10 nucleotide substitutions when compared to the reference strain GSFY2.

The molecular method described in this work represents a valid tool in epidemiological studies devoted to elucidate the relationships between plant host/phytoplasma vector. This work can be considered as the first step towards future studies that will be focused on the characterization of 'Ca. *P. prunorum*' strains present in the vectors, and on the biological properties of different strains, such as transmissibility by vectors and virulence.

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Establishment of a quantitative real-time PCR assay for the specific quantification of Ca. *Phytoplasma prunorum* in plants and insects

Jarausch, W., Fuchs, A., Jarausch, B.
RLP AgroScience, AlPlanta – Institute for Plant Research, Breitenweg 71, 67435 Neustadt, Germany

Abstract

A real-time PCR assay for the quantification of Ca. *Phytoplasma prunorum* has been established which combines the specificity of detection with a low cost method of quantitative PCR. The assay uses the specific primers ECA1/ECA2 with a SYBR Green I protocol. A gene fragment of Ca. *P. prunorum* with the target of the primers has been cloned and is used as standard for quantification by the standard curve method. The assay has been successfully applied to measure the concentration of Ca. *P. prunorum* in insects as well as in different kinds of plant samples.

Keywords: European stone fruit yellows, *Cacopsylla pruni*, resistance screening

Introduction

European stone fruit yellows (ESFY) is an economically important decline disease of stone fruits (*Prunus* spp.) in Europe causing the highest losses in apricot, Japanese plum and peach (Lorenz et al., 1994). This quarantine disease is associated with the phloem-limited Candidatus *Phytoplasma prunorum* which is transmitted in nature by the psyllid *Cacopsylla pruni* (Carraro et al., 1998; Jarausch et al., 2001) and by man through dissemination of latently infected planting material or grafting. Ca. *P. prunorum* is closely related to other European fruit tree phytoplasmas like Ca. *Phytoplasma mali*, the agent of apple proliferation disease, and Ca. *Phytoplasma pyri*, the agent of pear decline (Seemüller and Schneider, 2004). Phytoplasma detection is routinely achieved by PCR and primers specific for Ca. *P. prunorum* are available (Jarausch et al., 1998). In recent years, determination of phytoplasma concentration has gained increasing importance for the analysis of the disease spread by the insect vector (Thébaud et al., 2009a) or for the evaluation of resistance *in vivo* (Bisognin et al., 2008a) as well as *in vitro* (Bisognin et al., 2008b). Quantification of the phytoplasma was achieved in these studies by applying real-time PCR and different assays have been published for the quantification of Ca. *P. mali* (Baric and Dalla Via, 2004; Jarausch et al., 2004), Ca. *P. prunorum* (Martini et al., 2007; Thébaud et al., 2009b) or both (Torres et al., 2005).

The objective of this study was to establish a low cost method for the routine quantification of Ca. *P. prunorum* in insects as well as in different kinds of plant samples based on the well established detection primers ECA1/ECA2 (Jarausch et al., 1998).

Material and methods

Total DNA from plant and insect samples was extracted with a modified CTAB-based protocol as described by Maixner et al. (1995). Phloem-preparations were used from field samples for extraction. PCR with primers ECA1/ECA2 was done as described (Jarausch et al., 1998) using *Taq* polymerase furnished by 5 Prime (Germany). PCR product cloning and subsequent sequencing were done according to standard procedures. Real-time PCR was carried out in a MJ Research Chromo4 cycler using white plates (ABgene, UK) and the standard PCR reaction mix supplemented with SYBR Green™ I (1:66000 dilution; AMRESCO, USA) with the Mg2+ concentration adjusted to 3 mM. Primer concentrations were 0.1 µM for primer ECA1 and 1 µM for primer ECA2. The quantification was done by using the standard curve quantification method with a serial dilution of a plasmid preparation containing the ECA1/ECA2 gene fragment. For phytoplasma quantification in insects the plasmid was diluted in total DNA extract of healthy *C. pruni*, to quantify the phytoplasma in plant samples the plasmid was diluted in total DNA extracts obtained from healthy *Prunus* maintained *in vivo* or *in vitro*, respectively. Cycle conditions were for 40 times 15 s at 95°C, 30 s at 54°C, and 30 s at 69°C followed by a plate read. The qPCR was completed by a melting curve analysis.

Results and discussion

A Ca. *Phytoplasma prunorum*-specific PCR product was amplified with primers ECA1/ECA2 from a naturally ESFY-infected apricot from Southwest Germany, cloned and sequenced. The obtained plasmid preparation was used as a standard in a 10-fold dilution series ranging from 10^8 to 10^1 to establish the quantitative PCR (qPCR) assay. A SYBR Green™ I protocol was used as previously published (Jarausch et al., 2004) with one major modification: the use of a commercial qPCR kit was replaced by adding SYBR Green™ I to a normal PCR reaction. Mg2+ concentration was

adjusted to 3mM and the primer concentrations had to be altered to avoid primer dimer formation. The analysis of the standard curve showed that an efficient amplification could be achieved with this protocol (Fig. 1). The qPCR assay was first tested with insect samples and Ca. *P. prunorum* was reliably quantified in samples from field collected individuals of *C. pruni* (Fig. 2). The phytoplasma concentration measured in overwintered re-migrant adults of the vector *Cacopsylla pruni* was in the range of published data (Thébaud et al., 2009a). The test was also successfully applied to quantify the phytoplasma in different micropropagated *Prunus* plants after graft-inoculation *in vitro* as described previously (Jarausch et al., 1999). The assay worked equally well for the quantification of Ca. *P. prunorum* in plant samples obtained from the field. In both kinds of plant samples the phytoplasma concentration was correlated to the fresh weight of plant material used for DNA extraction. In both kinds of tissue comparable phytoplasma concentrations were measured in the range of 10^9 phytoplasma genome copies per g freshweight.

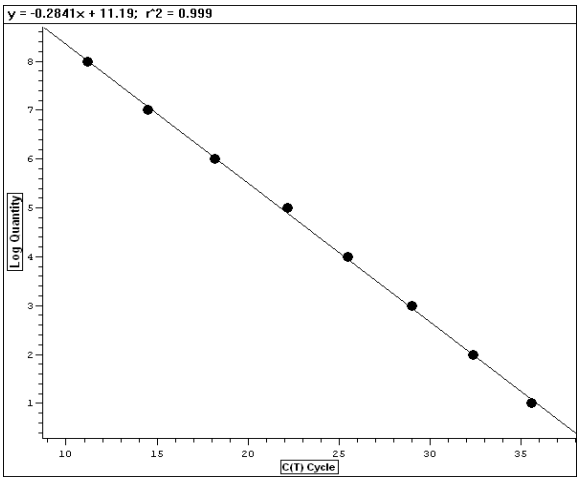

Fig. 1 Standard curve of a serial dilution 10^8 to 10^1 of a plasmid containing the ECA1/ECA2 PCR fragment and amplified in the established qPCR assay; Ct values are plotted against the log plasmid copies.

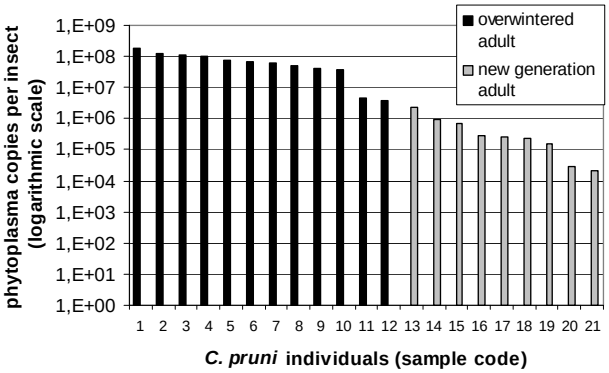

Fig. 2 Quantification of Ca. *P. prunorum* in single adults of *Cacopsylla pruni* at different time points of their life cycle expressed as phytoplasma genome copies per insect.

The established qPCR assay offers the advantage of the use of well established detection primers in combination with a low cost protocol based on SYBR Green™ I technology for the specific quantification of Ca. *Phytoplasma prunorum* in plants and insects. The assay could already be in routinely applied for the determination of the phytoplasma load of

naturally infected vector individuals of *Cacopsylla pruni* as well as for the evaluation of the phytoplasma concentration in inoculated genotypes of *Prunus* species *in vitro* and *in vivo*.

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Evaluation of susceptibility of pear and plum varieties and rootstocks to Ca. *P. pyri* and Ca. *P. prunorum* using Real-Time PCR

Torres, E.[1], Laviña, A.[2], Sabaté, J.[2], Bech, J.[1], Batlle, A.[2]
[1] Laboratori de Sanitat Vegetal. DAR. Generalitat de Catalunya. 08040 Barcelona.
[2] Dpt. Protecció Vegetal. Institut de Recerca i Tecnologia Agroalimentàries. IRTA. 08348 Cabrils, Barcelona.

Abstract

Real-time PCR was used to quantify phytoplasma concentration in fifty inoculated trees from five *Prunus* rootstocks and in forty-eight symptomatic pear and Japanese plum trees from orchards. Seasonal fluctuation of Ca. *P. prunorum* in different *Prunus* rootstocks, over three years, showed that the highest percentage detected by nested-PCR was in the 'Garnem' rootstock on nearly all sampling dates. Intra-varietal differences were also observed. Phytoplasma titer could be estimated by real time PCR in some trees of the rootstocks 'Garnem', 'Barrier', 'GF-677' and 'Marianna', and ranged from $4.7x10^5$ to $3.18x10^9$ phytoplasmas per gram of tissue. Quantification by real-time PCR was not possible in the 'Cadaman' trees analyzed, probably due to a lower phytoplasma titer in this variety. Samples from infected trees from commercial plots had different phytoplasma concentration and detection percentage depending on the variety, both being lower in 'Fortune' and '606' Japanese plum and in 'Blanquilla' pear trees.

Keywords: Candidatus *Phytoplasma pyri*, Candidatus *Phytoplasma prunorum*, real time PCR, detection

Introduction

There are no direct measures of control for diseases caused by phytoplasmas. One way to avoid the damage they produce is to have resistant or tolerant plant material.

The low expression of symptoms with diseases caused by phytoplasmas is often due to the lack of re-infection and therefore to a low concentration of phytoplasma population. This is associated with the difficulty of detecting the phytoplasma in tolerant varieties, even though they may be infected. For the *Prunus* species there are major differences in susceptibility to European stone fruit yellows (ESFY), caused by Candidatus *Phytoplasma prunorum*, with apricot, Japanese plum and peach trees being more susceptible than the *Prunus cerasifera* (Myrabolan) and *Prunus domestica* genotypes.

Many of the peach rootstocks (*Prunus persicae)* and apricot rootstocks (*P. armeniaca*) are extremely sensitive. The rootstocks Marianna GF 8-1 (*P. cerasifera* x *P. munsoniana*) and GF-677 (*P. amygdalus* x *P. persicae)* are highly sensitive. Slightly less sensitive are Myrabolan (*P. cerasifera*) and the hybrid Ishtara (Giunchedi et al., 1982; Desvignes & Cornaggia, 1982). Differences in detection and presence of symptoms have been observed in different varieties of pear infected by Ca. *P. pyri* (Garcia-Chapa et al., 2003a).

The purpose of this work was to apply real-time PCR to quantify phytoplasma concentration in plum and pear trees previously infected by Ca. *P. prunorum* and Ca. *P. pyri* respectively.

Material and methods

Fifty trees of five different rootstocks were inoculated with Ca. *Phytoplasma prunorum*, in February 2006 at the IRTA greenhouse in Cabrils (Spain), to study its seasonal fluctuation over a period of three years. The rootstocks used were 'Garnem' and 'GF 677' (*P. persica* x *Prunus amygdalus*), 'Barrier' and 'Cadaman' (*Prunus persica* x *Prunus davidiana*) and 'Marianna' (*Prunus cerasifera* x *Prunus munsoniana*).

Trees showing symptoms were also selected from orchards of pear and Japanese plum: twenty-four pear trees, cvs 'Blanquilla' and 'Bartlett', and twenty-four plum trees, cvs 'Pioneer', 'Fortune', '606', 'Golden Plum' and 'TC Sun'.

DNA was extracted from fresh material using PGB grinding buffer (Ahrens and Seemüller, 1992) to concentrate phytoplasmas, and E.Z.N.A. Plant MiniPrep Kit (Omega Bio-Tek) following the manufacturer's instructions. The DNA extracted was resuspended in 100µl of elution buffer and stored at -20ºC until use.

For phytoplasma detection, nested-PCR and real-time PCR amplification were used with phytoplasma ribosomal primers. Nested-PCR amplification was using P1 (Deng and Hiruki, 1991) and P7 (Schneider et al., 1995) primers for the first step. The second step was performed with 16Sr-X group specific primers, fO1/ rO1 (Lorenz et al., 1995), amplifying a fragment of about 1050-bp in length. The first amplification, using 5-10ng of DNA, was in a total volume

of 20µl containing the following mixture: 0.250 µM of each universal primer, 250µM dNTPs, 1 unit $100\mu l^{-1}$ Taq DNA polymerase (Promega, Madison, USA) and 1X Taq buffer (Promega, Madison, USA). Two µl of 1:50 dilution of the first amplification product were used for the second step, in a mixture containing the same components, but a different specific primer concentration (0.375µM each). Real-time PCR amplification was according to Torres et al., (2005) using SYBR Green PCR Master Mix (Applied Biosystems) and the universal primer P1 and the specific primer R16(X)F1r. A melting curve was obtained after amplification.

Results

By nested-PCR, Ca. *P. prunorum* phytoplasma was detected at the first sampling date in a high percentage of inoculated trees and in all plum rootstocks except in 'Cadaman', decreasing in subsequent analysis. The highest percentage detected was in the 'Garnem' rootstock on nearly all sampling dates. The highest percentage detected in 'Barrier' and 'Cadaman' were in spring (Figure 1). The detection varied, with the detection in some trees remaining unchanged on the different sampling dates, while that in other trees fluctuated (data not shown). In 'GF-677' and 'Marianna', phytoplasmas were detected in most trees over the three-year period, but in one tree of each rootstock, despite the initial infection, no phytoplasmas were detected from January 2008.

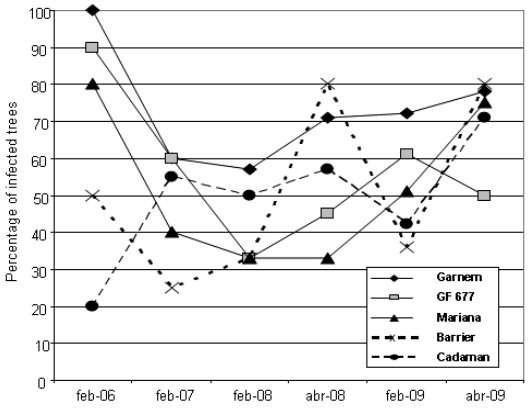

Fig. 1. Evolution of *Ca.* Phytoplasma prunorum detected in fifty inoculated rootstocks, by nested PCR (% of positive trees), over a period of 3 years.

The estimation of phytoplasma titer by real-time PCR, carried out in 2009 in some trees of the rootstocks 'Garnem', 'Barrier', 'GF-677' and 'Marianna', ranged from $4.7x10^5$ to $3.18x10^9$ phytoplasmas per gram of tissue (Table 1). Those trees with high phytoplasma concentration coincide with those that also tested positive in nested PCR analysis on all sampling dates after 2007 (data not shown), and that showed the typical symptoms of off-season growth.

Tab. 1 Percentage of inoculated rootstocks infected with Ca. *Phytoplasma prunorum*, by real-time PCR and average of the estimated number of phytoplasmas.

Rootstock	Cultivar	Real-time PCR detection (%)	Number of phytoplasmas/gr.
P. persica x P. amygdalus	Garnem	33	$2{,}054x10^8$
P. persica x P. davidiana	Barrier	20	$1{,}591x10^7$
P. persica x P. davidiana	Cadaman	0	-
P. persica x P. amygdalus	GF 677	13	$3{,}180x10^9$
P. cerasifera x P. munsoniana	Marianna	13	$4{,}752x10^5$

Although phytoplasmas were detected by nested PCR in 71% of the 'Cadaman' rootstock trees analyzed (Figure 1), quantification by real-time PCR was not possible, probably due to a lower phytoplasma titer in this variety (Table 1). The most susceptible rootstock was 'Garnem', where Ca. *P. prunorum* was detected in a high percentage of trees on all sampling dates, with quantification possible in 33% (Table 1).

The samples of infected plum trees from commercial plots had different phytoplasma concentration depending on the variety (Table 2). The variety 'Pioneer' had the highest phytoplasma concentration. The percentage detected and the estimation of phytoplasma concentration. The percentage detected and the estimation of phytoplasma concentration were lower in 'Fortune' and '606', however, these data were not related in this case with a lower expression of symptoms (Table 2).

Tab. 2 Detection of Ca. *Phytoplasma prunorum* by nested PCR and real-time PCR in symptomatic plums of a commercial plot.

Species	Cultivar	Nested-PCR detection (%)	Real-time PCR detection (%)	Number of phytoplasmas/gr.
Prunus salicina	Pioneer	100	80	$5{,}288x10^6$
Prunus salicina	Fortune	100	40	$4{,}275x10^3$
Prunus salicina	606	100	40	$3{,}552x10^4$
Prunus salicina	TC Sun	66	66	$5{,}725x10^5$
Prunus salicina	Golden Plum	66	66	$1{,}958x10^6$

Infected trees of *Pyrus communis* cv 'Barlett' had a higher phytoplasma concentration than infected 'Blanquilla' trees. Detection was not possible in 'Blanquilla' by real-time PCR, and was only detected in 17% of the trees by nested PCR, despite all trees being infected (Table 3).

Tab. 3 Detection of Ca. *Phytoplasma pyri* by nested PCR and real-time PCR in infected trees.

Species	Cultivar	Nested-PCR (%)	Real-time PCR (%)	Number of phytoplasmas/gr.
Pyrus communis	Barlett	83	67	$2{,}372x10^6$
Pyrus communis	Blanquilla	17	0	-

Discussion

Results of phytoplasma fluctuation in *Prunus* rootstocks indicate that the most susceptible rootstock is 'Garnem'. This is in agreement with other studies that have shown a higher susceptibility of *P.persica* x *P.amygdalus* hybrids (Giunchedi et al., 1982). Intra-varietal differences were also observed, especially in 'GF-677' and 'Marianna' rootstocks, with some trees apparently more resistant to infection than others. This is in agreement with the variability that has been observed within progenies of *Pyrus* taxa (Seemüller et al., 2009).

Quantification by real-time PCR allowed differences in susceptibility among plum and pear tree varieties and rootstocks to be assessed. The quantification of Ca. *P. pyri* in trees of cv 'Bartlett' and 'Blanquilla' confirmed the results obtained previously using nested-PCR for detection (Garcia-Chapa et al., 2003a). In those varieties where phytoplasma is readily detected, such as cv 'Bartlett' and cv. 'Llimonera', the symptoms are much more evident than in cv 'Blanquilla', where it was difficult to detect phytoplasma and there were fewer symptoms (Garcia-Chapa et al., 2003b). The sensitivity of the Japanese plum to the phytoplasma, cited in several studies was also confirmed with real-time PCR

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Molecular characterization of 'Candidatus *Phytoplasma prunorum*' in *Cacopsilla pruni* insect vector

Ferretti, L.[1], Gentili, A.[1], Poggi Pollini, C.[2], Ermacora, P.[3], Pasquini, G.[1]
[1] CRA-PAV Centro di Ricerca per la Patologia Vegetale, Via C.G. Bertero, 22 – I-00156 Rome.
Email: luca.ferretti@entecra.it
[2] Dipartimento di Scienze e Tecnologie Agroambientali (DISTA), Università di Bologna, Viale Fanin 44, I-40127 Bologna;
[3] Dipartimento di Biologia e Protezione delle Piante, Università di Udine, Via delle Scienze 208, I-33100 Udine

Abstract

Recent investigations on molecular characterization of the 'Candidatus *Phytoplasma prunorum*' (16SrX-B subgroup), causal agent of the European Stone Fruit Yellows (ESFY) syndrome, on the non ribosomal *tuf* gene resulted in the finding of two groups of isolates, named 'type a' and 'type b', both with a distinctive geographical distribution in Italian stone fruit growing areas (Ferretti et al., 2007 and 2008). Considering the role of *Cacopsylla pruni* (Scopoli) in the epidemiological cycle of the disease, the presence of the two groups of isolates has also been investigated in infected psyllid individuals from different Italian areas. Both types have been identified in *C. pruni* specimens collected on apricot, plum and wild *Prunus* species, confirming the geographical distribution and the percentages of spread of the two isolates.

Keywords: ESFY, phytoplasma, characterization, *tuf* gene, insect vector

Introduction

'Candidatus *Phytoplasma prunorum*' (16Sr X-B subgroup), the causal agent of the European Stone Fruit Yellows (ESFY) syndrome, is spreading in all Italian stone fruit growing areas. Leaf rolling and discoloration on single branches develop rapidly into leaf chlorosis. Sometimes, necrosis and dieback are observed in the same season. The Psyllid *Cacopsylla pruni* is the specific vector of the phytoplasma. This insect species is characterised by the presence of one generation per year: adults, overwintering on coniferous and other forest plants, migrate at the beginning of spring on the primary host, represented by wild and cultivated *Prunus* spp., where they lay their eggs.

Recently, a molecular investigation revealed the presence of two different groups of isolates of 'Ca. *P. prunorum*', reported as 'type a' and 'type b', on the basis of the molecular variability of the non ribosomal *tuf* gene (Ferretti et al., 2007). Further investigation, carried out on a large number of plant samples coming from infected orchards located in several Italian regions, confirmed the presence of these isolates and their well defined geographical distribution (Ferretti et al., 2008).

In order to also verify this molecular variability in the phytoplasma harboured in the insect vector, individuals of *C. pruni* were collected in several Northern Italian regions, where symptoms resembling the ESFY disease were frequently observed, during the course of recent years, in many stone fruit orchards. The surveys were carried out in orchards in which the distribution of the two isolates has been already defined.

In this paper, the molecular characterization of 'Ca. *P. prunorum*' found in insect samples is reported. The interdependence of isolates identified in insect and plant samples coming from the same growing areas was investigated.

Materials and methods

Individuals of *C. pruni* were captured by means of yellow sticky traps and sweep-net from April to May in ESFY-affected orchards located in several Northern Italian regions (namely Lombardia, Trentino, Veneto and Emilia Romagna), which had been previously surveyed for the detection and characterization of 'Ca. *P. prunorum*'. Insects were collected both from cultivated apricot and peach varieties and wild *Prunus* species (*P. cerasifera* and *P. spinosa*) growing alongside the investigated orchards.

Total DNA was extracted from single specimens according to the procedures described by Marzachì et al., (1998) and submitted to PCR amplification of 16S gene fragments, using the primer pairs P1/P7 (Schneider et al., 1995; Deng and Hiruki, 1991), and fO1/rO1(Lorenz et al., 1995) in direct and nested PCR, respectively. Total DNA from 72 positive individuals was then analyzed by means of specific amplification of non-ribosomal DNA fragments of the phytoplasma *tuf* gene. The primer pairs tuf1f/1r and tuf2f/2r, specifically designed on the *tuf* gene of the 16SrX phytoplasma group, were used in direct and nested PCR, respectively. Amplification was performed as follows: 3 min at 94 °C, 40 cycles of 1 min at 93°C (denaturation), 1 min at 45°C (annealing), 1 min and 30 sec at 72 °C (extension) and a final extension of 15 min at 72°C. Total DNA from 16SrX-A, 16SrX-B and 16SrX-C phytoplasma infected plants and from an healthy apricot were used as controls.

The obtained amplicons were submitted to the RFLP (Restriction Fragments Length Polymorphism) analysis, following digestion with the *Nla*III restriction enzyme. The obtained profiles were analysed after separation by electrophoresis on 5% polyacrilamide gel stained in ethidium bromide and successive visualization under UV light.

Results

Amplification products of the expected size (1120 bp) were obtained in nested PCR with the internal primer pair tuf2f/2r from 66 out of 72 (91.6%) individuals which had previously tested positive by 16S gene molecular analysis (Fig. 1). In many cases, amplicons of the right size were also obtained through direct PCR.

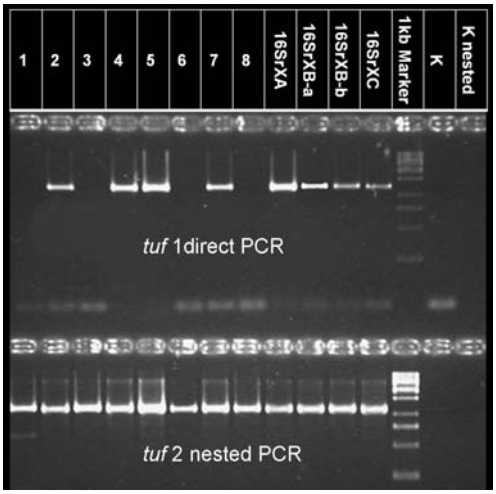

Fig. 1 Agarose gel of direct and nested-PCR on *tuf* gene. Lanes 1-8: insect samples; lanes 9-12 reference strains (16SrX-A, 16SrX-B 'tuf type a', 16SrX-B 'tuf type b', 16 SrX-C); lane 13: 1 kb DNA marker; lanes 14-15: water controls.

RFLP analysis performed on the *tuf* gene amplified fragments showed the presence of two distinct restriction profiles, referable to 'tuf type a' and 'b' (Fig. 2). No mixed infections by either tuf types in single individuals were ever detected in the tested insects (Tab. 1).

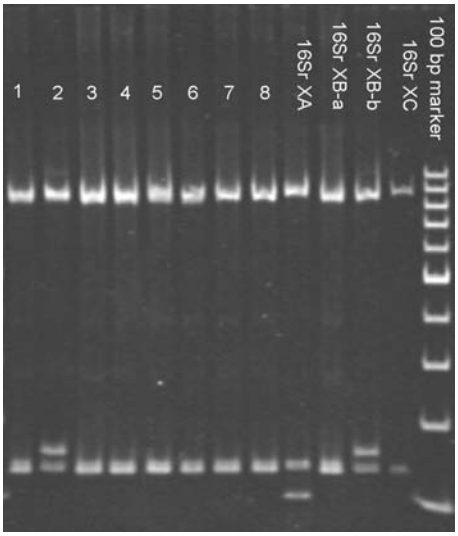

Fig. 2 RFLP analysis of the *tuf* gene amplicons after digestion with *Nla*III restriction enzyme. Lane 1-8: insect samples; lanes 9-12 reference strains (16SrX-A, 16SrX-B 'tuf type a', 16SrX-B 'tuf type b', 16 SrX-C); lane 13: 100 bp DNA marker

Tab. 1 Percentages of distribution of 'tuf type a' and 'b' isolates identified in *C. pruni* individuals collected in four different Italian regions.

			***C. pruni* individuals captured in the investigated orchard**			
Region	**'tuf type' distribution (*) (%)**	**N° orchards**	**Host plant**	**N° positive insects (positive/total)**	**'tuf type' 'a'**	**'b'**
Trentino	a (77.3%); b (22,7%)	5	Apricot: *P. cerasifera*; *P. spinosa*	10/13	8 (80%)	2 (20%)
Friuli-V.G.	a (94,4%); b (5,6%)	1	Apricot	5/6	5 (100%)	-
Lombardia	a (92,3%); b (7,7%)	3	*P. spinosa*	26/26	24 (92,3%)	2 (7,7%)
Emilia-R.	a (100%)	3	*P. spinosa*	25/27	25 (100%)	-
Totale	-	12	-	66/72	62 (96.8%)	4 (3,2%)

(*) Data previously published (Ferretti et al., 2008)

In the 25 *C. pruni* specimen coming from 3 orchards located in Emilia-Romagna and in the 5 insect samples coming from one orchard of the Friuli V.G. region, only the 'tuf type a' was found, whereas on a total of 36 insect individuals from 8 orchards located in Trentino and Lombardia, both tuf types were identified (Tab. 1). In these last two regions, an infection rate of *C. pruni* by 'tuf type a' of 80.0 % and 92.3 % respectively was detected.

Discussion

The primer pairs tuf1f/1r and tuf2f/2r were confirmed to be specific and suitable for the characterization of 'Ca. *P. prunorum*' in insect samples and they also resulted useful for the phytoplasma detection. In fact, the percentage of positive insect specimens was very similar to that obtained in the 16S gene molecular analysis. The PCR/RFLP-based method using the *tuf* gene confirmed the differentiation of the 'Ca. *P. prunorum*' in two distinct types. The same result was observed for phytoplasma harboured from the *C. pruni* specimen, as previously identified in ESFY-affected plants (Ferretti et al., 2007).

Previous studies on the geographical distribution of the two 'tuf types' on infected plants revealed the presence of the only 'type a' in the Emilia-Romagna region, whereas in Friuli V.G., Trentino and Lombardia, both types have been found inside the infected orchards (Ferretti et al., 2008). This geographical distribution of types was also confirmed in the insect specimens, with the exception of the samples coming from the Friuli-V.G. region, where both types were identified on *Prunus* plant species, but only the 'type a' was found in *C. pruni* specimens. Nevertheless, the quality of

the data could have been influenced by the low insect population density recorded during the sampling season, and the subsequent small number of tested insects.

The presence of two distinct isolates of 'Ca. *P. prunorum*', on the basis of the molecular variability of the non ribosomal *tuf* gene, was confirmed at different stages of the phytoplasma epidemiological cycle; the correspondence between the 'tuf types', identified in plants and insect vectors from the same infected areas, enhances the relevance of this molecular characterization as an effective tool for epidemiological investigations.

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Experimental transmission trials by *Cacopsylla pyri*, collected from Pear Decline infected orchards in Turkey

Çağlayan, K., Gazel, M., Ulubaş Serçe, Ç., Can, F.
Mustafa Kemal University, Agriculture Faculty, Plant Protection Department, Antakya-Hatay, Turkey.
Email: kcaglayano@yahoo.com

Abstract

A study was carried out on the experimental transmission efficiency of the Pear Decline (PD) phytoplasma by *Cacopsylla pyri* (L.) C. pyri were collected from naturally infected orchards in Bursa province (Plots B1 and B2) and a non-infected orchard in the Hatay-Antakya province (Plot A) of Turkey. *C. pyri* adults captured from infected orchards were placed directly onto healthy periwinkle plants (*Catharanthus roseus*), whereas the *C. pyri* from plot A were allowed to feed first on infected pear for two weeks, then transferred to healthy periwinkle plants. Groups of five psyllids per plant were used for transmissison tests and the study was replicated three times. The presence of 'Candidatus *Phytoplasma pyri*' in psyllids and *C. roseus* plants was checked by nested PCR using P1/P7 and U3/U5 primer pairs. Although *C. pyri* have a limited host range they were able to survive up to 20 days on periwinkle plants. Insects collected from Bursa province survived 16-20 days whereas insects from Antakya survived 7-12 days on periwinkle plants. Symptoms consisted of yellowing or clearing of the veins in newly infected leaves, and shortening of the internodes of the main stem. The infected plants remained stunted and with small flowers. Results based on the RFLP analysis of infected plants exposed to psyllids from plot B1and B2 indicated that the experimental infection rate of periwinkle plants and psyllids was 33.3% and 16.6%, respectively. No infected periwinkle was found in plants exposed to psyllids from plot A, but the psyllids used for experimental transmission experiments were 33.3 % infected. Transmission trials under controlled conditions showed the capability of *C. pyri* to transmit PD from infected pears to healthy periwinkles and confirmed their potential as vectors of *Ca*. P. pyri in Turkey.

Keywords: Candidatus *Phtoplasma pyri*, pear psyllid, transmission efficiency

Introduction

Pear decline caused by 'Candidatus *Phytoplasma pyri*' (Seemüller and Schneider, 2004) is widespread in many pear-growing countries including Turkey. The first suspicious and common symptoms of PD was observed on cv. 'Deveci' in the Bursa province of Turkey in 2005. The disease was confirmed by PCR and RFLP analyses (Ulubas Serce et al. 2006). This phytoplasma belongs to the Apple proliferation group (16SrX) (Seemüller et al. 1998), and is transmitted by pear psyllids (*Cacopsylla pyricola, C. pyrisuga, C. pyri*). In North America and England the known vector is *Cacopsylla pyricola* (Foerster) but in other parts of Europe *Cacopsylla pyri* (L.) has been found to be the main vector (Carraro et al., 2001; Garcia-Chapa et al., 2005). Transmission of PD by *C. pyri* has been demonstrated in Italy (Carraro et al., 1998) and France (Lemoine, 1984), suggesting that this psyllid is probably the most important vector in the Mediterranean area. Although transmission capability has not yet been evaluated, *C. pyri* is also the most common psyllid in pear orchards in Spain (Garcia-Chapa et al., 2005). In Turkey *C. pyri* is the predominant psylla on pear trees (Gençer, 1999) producing 3 to 4 generations a year (Kovanci et al., 2000). Naturally infected psyllids captured from infected pear orchards have been reported (Ulubas Serce et al., 2006) but its capability to transmit Ca. *P. pyri* has not been demonstrated. The present paper describes experimental transmission of *Ca*. P. pyri to periwinkle plants by naturally infected *C. pyri* collected from infected orchards.

Materials and methods

Field studies: In December 2007 two commercial plots of pear cv. 'Deveci' (B1 and B2) located in Bursa province and one plot of cv. 'Santa Maria' (A) in the Antakya province of Turkey were selected. PD symptoms and the presence of *C. pyri* had been previously recorded in plot B1 and B2, but no PD symptoms were observed in plot A despite a previous report on the presence of the disease in that province (Sertkaya et al., 2005). The incidence of the disease in these three plots was evaluated and 10% of the pear trees were randomly selected and tested by nested PCR. The psyllids were collected in December by shaking insects onto an underlying net. Twenty individual insects from each plot were analyzed for the presence of PD .

Experimental transmission of PD by *C. pyri* on periwinkle plants: All the transmission experiments were carried out in an environmentally controlled growth room at 25±1°C, with supplementary light and 16-h days. In December 2007, adult *C. pyri* were captured and 3 groups of psyllids, each consisting of 5 individuals, were transferred to healthy

periwinkle seedlings. Psyllids captured from plot A were first fed on PD infected pear plant for 2 weeks and then transferred to healthy periwinkle plants. All test plants were covered individually with a plastic-screen cage (Fig. 1). Another group of three healthy periwinkle plants was used as negative control. Survival of the insects and symptom expression were monitored and dead psyllids were analyzed immediately for the presence of PD phytoplasma (Garcia-Chapa et al., 2003).

Fig. 1 Test plants covered individually with a plastic-screen cage (on left), *Cacopsylla pyri* feeding on periwinkle plant (on right).

Testing for the presence of phytoplasmas in test plants and in psyllids: All test plants and individual psyllids were tested by nested PCR. The first amplification was done with the universal primers P1/P7 (Lee et al., 1992). FU5/rU3 amplicons from the nested PCR were digested with *Ssp*I and *RsaI* at 37°C following the manufacturer's instructions (MBI Fermentas, Germany). Digested products were analyzed by electrophoresis using 2% agarose gel, and stained with ethidium bromide. DNA bands were photographed under UV light. PD, Apple proliferation (AP) and European Stone Fruit Yellows (ESFY) infected periwinkle plants used as positive controls were kindly supplied by Dr. Foissac, INRA, France.

Results

Field studies: PCR analyses of trees from the two commercial plots of pear trees cv. 'Deveci' in Bursa (B1 and B2) showed the incidence of PD infected trees with 60% and 65%, respectively. Whereas no infection was detected in plants from plot A. Analyses of 20 field collected psyllids evaluated by nested PCR showed that 2 and 5 psyllids from plot B1 and B2, respectively, were infected by PD. No infected psyllid was found in plot A from the province of Antakya.

Experimental transmission of PD by *C. pyri* on periwinkle plants: Although *C. pyri* have a limited host range, they were able to survive up to 20 days on periwinkles (Table 1). Insects collected from Bursa province survived 16-20 days whereas insects from Antakya survived 7-12 days on periwinkles. The initial symptoms were observed 4 months after exposure of infected psyllids to test plants. Symptoms consisted of yellowing or clearing of the veins in newly infected leaves, and shortening of the internodes of the main stem. The plants remained stunted and flowers were small (Fig.2). Two periwinkle plants exposed to psyllids from plot B1 and B2 showed phytoplasma-like symptoms. No symptomatic plants were obtained using psyllids from plot A. According to the RFLP analysis of the Bursa samples the infection rate of periwinkle plants and psyllids was 33.3% and 16.6%, respectively. No infected periwinkle plants were found among those exposed to psyllids from plot A but the psyllids were 33.3 % infected having acquired the PD phytoplasma after being allowed to feed on infected pear.

Tab. 1 Survival of *Cacopsylla pyri* L. on periwinkle plants

Location and number pf test plants		Survival (days)
	B1-1	19
Bursa 1 (B1	B1-2	16
	B1-3	20
	B2-1	12
Bursa 2 (B2)	B2-2	12
	B2-3	17
	A1	7
Antakya (A)	A2	12
	A3	7

Fig. 2 Symptoms of stunting, shortening of the internodes of the main stem and small flowers of experimentally infected periwinkle (on right) and healthy control (on left) .

Testing for the presence of phytoplasmas in test plants and in psyllids: The primer pair FU5/rU3 was validated by amplification of DNA from the positive controls - that is PD, AP and ESFY infected periwinkle plants as well as from test plants and individual psyllids used in the trials. After digestion with *Ssp*I and *RsaI*, the restriction products obtained from all samples showed the appropriate restriction profiles by which the three different phytoplasmas could be distinguished (Fig. 3).

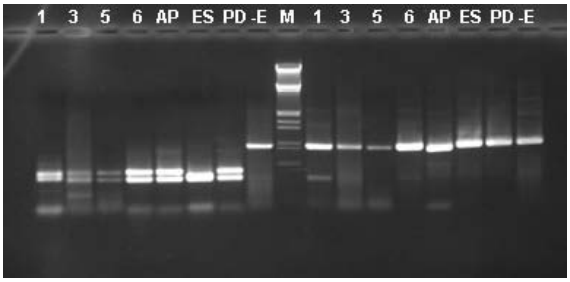

Fig. 3 Restriction products of Ca. *Phytoplasma pyri* DNA after digestion with *Ssp*I and *RsaI*, respectively. 1, 3, 5 and 6 represent infected periwinkle plants. Positive controls: AP (apple proliferation), ES (European stone fruit yellows), PD (pear decline). –E: negative control without enzyme.

Discussion

Pear decline is a destructive disease that occurs in Europe, North America and wherever domestic European pear (*Pyrus communis* L.) is grown (Davies et al., 1992; Garcia-Chapa et al., 2003). In the last 5 to 6 years the rapid spread of PD disease in the Bursa province of Turkey represents a serious outbreak with high levels of infection. Previous studies in this province showed that out of the 116 pear samples tested 52.58% were found to be infected by PD (Gazel et al. 2007). In this study similar results were obtained and 60 to 65% infection rate was recorded in randomly tested pear trees from which psyllids were captured for transmission trials. The psyllids, collected from two different infected orchards of Bursa province were also found infected by PD (7 infected out of 40). Two periwinkle plants out of 6 were experimentally infected by *C. pyri* collected from Bursa province where PD is very common. According to these results, the detection of the same RFLP pattern for pear, psyllid and periwinkle confirm that *C. pyri* is a potential vector of the PD agent in that province. However in plot A no naturally infected psyllids were found. After being allowed to feed on infected pear and based on experimental transmission trials PD was detected in one insect, but in none of the periwinkle plants. This data showed that *C. pyri*, collected from plot A may be a potential vector candidate for this province. It can acquire phytoplasma from infected pear trees, but was not able to transmit to periwinkle in this experiment. This might be due to the use of a limited number of insects and periwinkles. *C. roseus* does not appear to be a good host for pear decline transmission. It might be necessary to use pear seedlings for more reliable transmission

experiments (Avinent and Llacer, 1994). Since the transmission of PD is difficult to reproduce experimentally with other psyllid species (Davies et al., 1992), new laboratory transmission trials with Turkish pear cultivars are under investigation, using *C. pyri* fed on infected pear trees.

Acknowledgements

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Effect of Candidatus *Phytoplasma pyri* infection on fruit quality, total phenolic content and antioxidant capacity of 'Deveci' pear, *Pyrus communis* L.

Ulubaş Serçe, Ç.[1], Gazel, M.[1], Çağlayan, K.[1], Özgen, M.[2]
[1] Mustafa Kemal University, Agricultural Faculty, Plant Protection Dept., Antakya, Hatay, Turkey. Email: culubas@gmail.com
[2] Gaziosmanpaşa University Agricultural Faculty, Horticulture Dept., Taşlıçiftlik, Tokat, Turkey

Abstract

Pear decline is an important threat for Turkish pear production. In this study, we attempt to compare several pomological characteristics, total phenolic content and total antioxidant capacities in Candidatus *Phytoplasma pyri* infected and noninfected 'Deveci' pear from Bursa, Turkey. Based on pear decline symptoms, the fruit samples were taken in October 2008 on harvest maturity from four infected and non-infected trees. Presence of Candidatus *P. pyri* was later confirmed by nested PCR tests. The result indicated that infection significantly reduced fruit size, width, length; and increased pH, color values of a, b and hue. Abortive and healthy seed numbers and weights, soluble solids and acidity did not change significantly. Similarly, the infection did not affect the flesh color. To investigate a possible differential response on skin and flesh of fruits, total phenolic (TP) and total antioxidant capacity (TAC) analyses were conducted on skin and flesh tissues separately. The results indicated that, infected skin tissue had higher total phenolic and total antioxidant capacity for both methods analyzed (TEAC and FRAP). TP content of skin increased from 806 to 923 µg gallic acid equivalents (GAE)/g fresh weight (fw) while TP content of flesh increased from 195 to 249 µg GAE/g fw. TAC also found to be enhanced on infected fruits. On average, non-infected trees had 32.4 and 28.3 µmol TE/g fw for TEAC and FRAP, respectively. Infection increased these averages to 35.4 and 32.3 µmol TE/g fw tabulating 18 and 12% increase in flesh tissue. Similarly, the TEAC and FRAP averages increased from 4.0 to 5.8 and 3.3 to 4.9 µmol TE/g fw, respectively.

Keywords: Abiotic stress, FRAP, pear decline, phytoplasma, TEAC

Introduction

Turkey is an important pear producing country with nearly 350,000 tones yearly production (FAO, 2009). Bursa is the leading province producing approximately 15% of total production. There have been recent surveys to identify phytoplasma diseases of several regions of Turkey (Sertkaya et al., 2005; Ulubaş Serçe et al., 2006, Gazel et al., 2007; Canik and Ertunç, 2007). These surveys revealed a high incidence of "Pear Decline" (PD), caused by 'Candidatus *Phytoplasma pyri*' and suggested that PD is a big threat for pear production in Turkey. The PD presence were first realized by symptoms such as foliar reddening in late summer and fall, leaf roll, leaf curl, poor growth and slow or quick decline (Gazel et al., 2007). The causal agent was then confirmed by PCR. PD is transmitted by pear psylla (*Cacopsylla pyricola*, *C. pyri*). It also can be transmitted by grafting and budding. Decline is much more prevalent on trees with rootstocks of *P. ussuriensis* or *P. pyrifolia* than trees on domestic *P. communis* roots. PD is characterized by two phases; quick decline and slow decline. Trees may wilt, scorch, and die within a few weeks or loose vigor over several seasons during which foliage gets sparse with little or no terminal growth. Abnormal early red leaf coloration and reduced leaf size has been observed on effected trees.

It is known that many plant species increase secondary metabolites especially phenolics by their defense system in response to pathogen attacks (Bennett and Wallsgrow, 1994). Anthocyanine biosynthesis, usually with high antioxidant activity, is also associated with environmental stress including pathogens attacks (Chalker-Scott, 1999). It was recently demonstrated that an environmental stimulus, wounding, resulted in a 60% increase in total phenolic (TP) content and 85% increase in total antioxidant capacity (TAC) in the flesh tissues of wounded purple-colored potatoes when compared to control (Fernando and Cisneros-Zevallos, 2003). In the present study, we attempt to compare fruits from healthy (non-infected) pear trees and Canditatus *P. pyri* infected for several pomological characteristics, TP and TAC.

Materials and methods

Plant materials: A survey study resulted in the identification of numerous pear orchards infected by *Canditatus* P. pyri in Bursa and Yalova provinces of Marmara region in Turkey. The orchard located in Bursa was used in the present study. Seven years old pear trees cv 'Deveci' grafted on BA29 rootstock, four infected and noninfected trees were randomly sampled based on the plant and fruit symptoms. Fruits were harvested when they reached the harvesting

maturity in October. From each experimental tree, three subsamples of 10 fruits were randomly sampled and transferred to the laboratory for analysis. The pomological analysis were completed within 24 hrs.

Pomological analysis: Several pomological characteristics were determined on infected and noninfected fruit samples of 'Deveci' pear. Fruit weight was measured by using a digital balance with a sensitivity of 0.001 g (Scaltec, SPB31). Linear dimensions, length and width of fruits were measured by using a digital caliper gauge with a sensitivity of 0.01 mm. External and internal colors were measured with a Minolta Chroma Meter CR-400 (Minolta-Konica, Japan) having a measuring area of 8 mm in diameter for readings of small samples without cut-off. For the soluble solid contents (SSC), pH and acidity determinations, the samples were homogenized and samples were taken from this slurry. SSC was determined by a digital refractometer (Atago-1, Atago, Japan) at 20 °C. pH was determined by potentiometric measurement at 20 °C with a pH meter. The acidity was determined by titration of 0.1 N NaOH to pH 8.1, expressing malic acid (%).

Confirmation of Canditatus *P. pyri* infection by PCR: To confirm Canditatus *P. pyri* infections on the trees based on plant and fruit characteristics, PCR reactions were carried our according to Ulubaş Serçe et al. (2006). Phytoplasma specific primers P1 (Deng and Hiruki, 1991) and P7 (Smart et al., 1996) followed by R16F2n/R2 universal primers designed to amplify a 1200 bp portion of 16S rRNA gene (Lee et al., 1993) were used.

Sample extraction procedures: A single extraction procedure designed to measure total phenolic content (TP) (Singleton and Rossi, 1965) was used to determine TP and TAC of all samples. Briefly, 100 g samples were homogenized in a blender. A 3 g of aliquot was then transferred to polypropylene tubes and extracted with 15 mL of extraction buffer containing acetone, water, and acetic acid (70:29.5:0.5 v/v) for 24 h. After filtration, samples were concentrated and all laboratory procedures were performed on sub-sample duplicates of each replicate extraction.

Determination of total phenolic content (TP): To determine the amount of total phenolic compounds of the extract, Folin-Ciocalteu's phenol reagent and water 1:1:5 (v/v) were combined and incubated for 8 min followed by the addition of 2.5 mL of 7 % (w/v) sodium carbonate. After 2 h, the absorbance of each sample was measured at 750 nm on a spectrophotometer (Model T60U, PG Instruments). Gallic acid was used as standard. The results are expressed as µg gallic acid equivalent (GAE)/g fw (fresh weight).

Total antioxidant capacity (TAC): TAC was estimated by two standard procedures FRAP and TEAC assays as suggested by Ozgen et al. (2006).

The Ferric Reducing Ability of Plasma (FRAP): FRAP was determined according to the method of Benzie and Strain 1996. Assay was conducted using three aqueous stock solutions containing 0.1 mol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine] acidified with concentrated hydrochloric acid (1000:3.3 v/v), and 20 mmol/L ferric chloride. These solutions were prepared and stored in darkness under refrigeration. Stock solutions were combined (10:1:1 v/v/v) to get the FRAP reagent just prior to analysis. Absorbance at 593 nm was determined after 10 min 3 mL reagent and 50 µL peel and 200 µL fruit flesh extract was mixed. After 10 minutes, the absorbance of the reaction mixture was determined at 593 nm on a UV-VIS spectrophotometer (Model T60U, PG Instruments). The absorbance of each sample was compared with those obtained from the standard curve made from Trolox (10 - 100 µmol/L). Trolox was used as standard and the results are expressed as µmol TE/g fw basis.

Trolox Equivalent Antioxidant Capacity (TEAC): For the standard TEAC assay, ABTS was dissolved in acetate buffer and prepared with potassium persulfate as described in Ozgen et al. (2006). The mixture was diluted in acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm for longer stability (Ozgen et al., 2006). For the spectrophotometric assay, 3 mL of the $ABTS^{+}$ solution and 50 µL peel or 200 µL fruit flesh acetone extract was mixed and incubated in 10 min and the absorbance was determined at 734 nm by a UV-VIS spectrophotometer (Model T60U, PG Instruments). Trolox was used as standard and the results are expressed as µmol trolox equivalent TE/g fw (fresh weight) basis.

Results and discussion

PCR analysis confirmed the Canditatus *Phytoplasma pyri* infections (Figure 1). The phytoplasma specific primers amplified a 1200 bp DNA fragment on four symptomatic trees confirming phytoplasma presence while no amplification products were observed on asymptomatic trees.

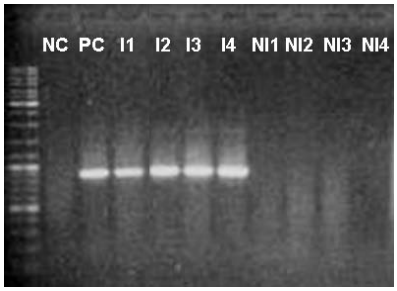

Fig. 1 The confirmation of *Canditatus* Phytoplasma pyri infection on 'Deveci' pear using P1 and P7 primers. NC = negative control, PC = positive control, I = infected, NI = noninfected.

The two fruit groups, infected vs. noninfected, were compared for several pomological characteristics. Fruit size, width, length, pH, a, b and *hue* values of skin color significantly differed between groups (Table 1). Canditatus *Phytoplasma pyri* infection resulted in reductions in fruit size variables. For example, fruit weight, width and length dropped from 939.2 g to 301.2 g; 92.4 to 84.6 mm, and, 90.3 to 81.9 mm, respectively making 31, 9 and 10% reductions. Infection resulted in an increase in pH from 4.0 to 4.3. Canditatus *Phytoplasma pyri* infections also had an effect on skin color; the infected fruits had higher a, b and hue averages for their skin color than those of noninfected fruits. Abortive and healthy seed numbers and weights, SSC and acidity did not change significantly. Similarly, the infection did not affect the flesh color.

Tab. 1 Several pomological characteristics.of noninfected and infected (by *Canditatus* Phytoplasma pyri) of 'Deveci' pear.

Character	Noninfected	Infected	% Change	Significance
Fruit weight (g)	393.2	301.2	-31	0.001
Fruit width (mm)	92.4	84.6	-9	0.002
Fruit length (mm)	90.3	81.9	-10	0.049
Abortive seed no. (no./fruit)	2.56	5.00	49	0.067
Healthy seed no. (no./fruit)	1.19	0.75	-58	0.409
Abortive seed weight (g/fruit)	0.02	0.03	32	0.203
Healthy seed weight (g/fruit)	0.09	0.05	-97	0.203
Soluble solids (%)	13.35	12.38	-8	0.158
Acidity	0.35	0.32	-9	0.341
pH	4.0	4.3	7	0.001
Skin color (L)	74.5	74.8	0	0.688
Skin color (a)	-2.0	-2.8	29	0.036
Skin color (b)	9.8	12.5	22	0.026
Skin color (hue)	10.0	12.8	22	0.026
Skin color (C)	101.4	102.4	1	0.168
Flesh color (L)	62.1	64.9	4	0.211
Flesh color (a)	-13.2	-10.2	-29	0.090
Flesh color (b)	37.9	39.2	3	0.548
Flesh color (hue)	40.4	41.3	2	0.650
Flesh color (C)	105.7	104.1	-2	0.700

Significant comparisons, at 0.05, are bolded.

To investigate a possible differential response on skin and flesh of fruits, TP and TAC of skin and flesh tissues analyzed separately. The results indicated that, as expected, skin tissue had higher TP and TAC for both methods analyzed (Figure 2). The infection increased TP content in both tissues. Skin TP content increased from 806 to 923 µg GAE/g fw while flesh TP content increased from 195 to 249 µg GAE/g fw. The proportion of increase was similar for both tissues (13 and 12%). TEAC and FRAP was also found to be enhanced on infected fruits. On average, noninfected trees had 32.4 and 28.3 µmol TE/g fw for TEAC and FRAP, respectively. Infection raised these averages to 35.4 and 32.3 µmol TE/g fw tabulating 18 and 12% increase. Although TAC was lower in flesh tissue in comparison to skin, the increase by the infection was more profound (32% by both methods). The TEAC and FRAP averages increased from 4.0 to 5.8 and 3.3 to 4.9, respectively.

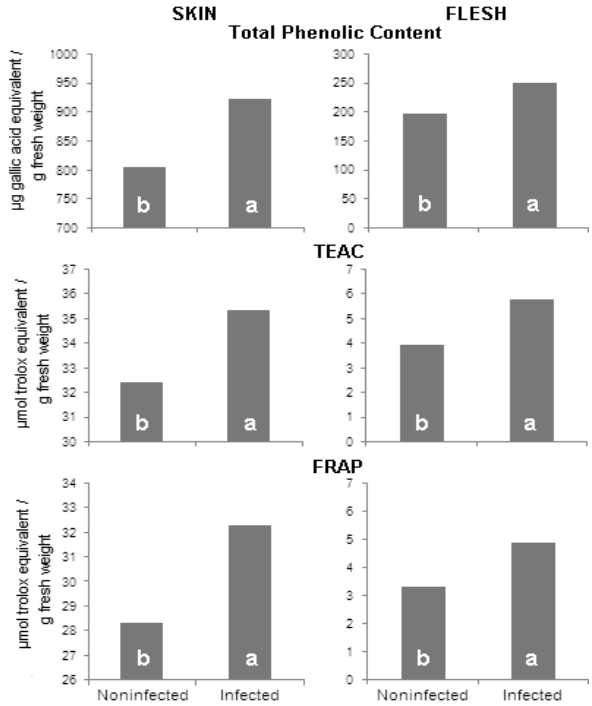

Fig. 2 Total phenolic content and total antioxidant capacity (determined by TEAC, Trolox Equivalent Antioxidant Power, and FRAP, Ferric Reducing Antioxidant Power of skin and flesh tissues of 'Deveci' pear either noninfected or infected by *Canditatus* Phytoplasma pyri. Different letters indicated statistical significance at 0.05.

Pear has many phenolic compounds on their different tissues. For example, Andreotti et al. (2006) determined the several phenolic compounds in leaves of five pear cultivars grown in orchards, glasshouse an *in vitro*. Similar to our findings, they found differential level of several compounds affected by the environment and the age of the leaves. Indeed, specific changes on phenolics by a phytoplasma infection were examined in some of the plant species. For example, Choi et al. (2004) profiled Madagascar periwinkle (*Catharanthus roseus* L. G. Don) infected by 10 types of phytoplasmas, including apple proliferation group 16SrX-A. They identified many metabolites with different levels in phytoplasma-infected and noninfected plants. The infections increased the metabolites of chlorogenic acid, loganic acid, secologanin, and vindoline. avali et al. (2004) reported an elevated total alkaloid concentration in phytoplasma infected plants compared to controls, in particular in vinblastine. These two studies concluded that the biosynthetic pathway of some phenolics is stimulated by the infection with phytoplasmas having an important role on secondary metabolism of the diseased plants, modifying both the total content of alkaloids and their ratio.

The production of defense-related phenolic compounds in response to pathogenic or insect attack was previously described (Vidhyasekaran, 1997; Sadasivam and Thayumanavan 2003). With this in mind, Brandt and Molgaard (2001) argue that these defense-related secondary metabolites may constitute the most significant nutritional advantage of these products. In our study this plant defense reaction caused increase phenolics in both flesh and skin tissue of pear fruits. Since antioxidant activity of fruits closely related to phenolic contents (Ozgen et al., 2006), antioxidant activity of infected fruits was also increased.

Conclusions

In a wide sense phenolics play a role in plant defense against pathogens. We only understand part of the mechanism. Considerably more studies are needed to investigate in detail physiology, biochemistry and biology of this action. Limited data from our study may help future studies to understand and use this natural mechanism to overcome Canditatus *Phytoplasma pyri*.

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Diagnostics of fruit trees phytoplasmas – the importance of latent infections

Mehle, N.[1], Ambrožič Turk, B.[2], Brzin, J.[1], Nikolič, P.[1], Dermastia, M.[1], Boben, J.[1,3], Ravnikar, M.[1]
[1] National Institute of Biology, Department of and Systems Biology, Večna pot 111, 1000 Ljubljana, Slovenia
[2] Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia
[3] Current address: University of Ljubljana, Medical Faculty, Institute of Microbiology and Immunology, Zaloška 4, 1000 Ljubljana, Slovenia

Abstract

In the period 2000-2008 more than 1300 fruit trees from different regions of Slovenia were tested for the quarantine phytoplasmas Apple proliferation (AP), Pear decline (PD), and European stone fruit yellows (ESFY). The majority of samples were collected within systematic official surveys, which was conducted for assessing the presence of these phytoplasma in Slovenia in production and mother plant orchards. Samples were taken from trees with and without expressed symptoms. DNA was extracted from the symptomatic shoots. In addition some roots from asymptomatic trees were sampled for the evaluation of latent infections. The presence of phytoplasmas was analyzed with a nested PCR, RFLP and a real time PCR (Hren et al., 2007). AP, PD and ESFY were confirmed as being present in several areas in Slovenia where fruit trees are cultivated. AP was found not only in apple, but also in stone fruit trees such as cherry, apricot and plum (Mehle et al., 2007). By using highly sensitive diagnostic methods, such as real time PCR, some latent infections were detected and they were confirmed next year also by less sensitive methods.

Keywords: phytoplasma, AP, PD, ESFY, latent infection, fruit trees

Introduction

Phytoplasmas are associated with many important vector borne and graft-transmissible plant diseases. They may affect different host plants including fruit trees. In Europe, the fruit trees of Rosaceae family are seriously affected by phytoplasmas belonging to the apple proliferation group (16SrX group) and causing apple proliferation (AP; 'Candidatus *Phytoplasma mali*'), European stone fruit yellows (ESFY; 'Candidatus *Phytoplasma prunorum*') and pear decline (PD; 'Candidatus *Phytoplasma pyri*') (Seemüller and Schneider, 2004).

The presence of these phytoplasmas was confirmed in several fruit-growing areas of Slovenia (Mehle et al., 2007b). Besides apple trees, AP was shown to be also present in stone fruit trees such as cherry, apricot and plum (Mehle et al., 2007a).

Symptom expression is very variable and many cultivars do not show the distinctive symptoms in the first years of tree development (Lešnik et al., 2007). In addition, it has been assumed that many trees are latently infected and disease symptoms become visible only as a result of special weather conditions or significant changes in the production practices. Such trees may be a hidden source of infection and its early detection and a consequent tree removal is as important as an intensive vector control. This is especially important in newly established orchards in areas with high disease occurrence. The main objective of our study was therefore an implementation of a real-time PCR method, which enabled the detection of the apple proliferation phytoplasma group in asymptomatic trees.

Material and methods

The sampling was done in one production and in one mother plant orchard. The majority of samples were collected within systematic official surveys, conducted to assess the presence of these phytoplasma diseases in Slovenia. Samples were taken from trees with and without expressed symptoms. DNA was isolated from the roots for analyzing latent infections or from symptomatic tree shoots using QuickPickTM Plant DNA kit (Bio-Nobile, Finland) and KingFisher mL (Thermo Scientific,USA) machine (Pirc et al., 2009; Boben et al., 2007).

The presence of phytolasmas was tested using two molecular approaches: a nested PCR followed by the RFLP analyses and a real-time PCR. Initial PCR was performed using the universal phytoplasma P1/P7 (Schneider et al., 1995) and modified primers described by Hren et al. (2007). Nested PCR reactions that followed were done using the AP group specific primers f01/r01 (Lorenz et al., 1995) and using a pair of universal phytoplasma primers U3/U5 (Lorenz et al., 1995). Products were visualized on a 1% agarose gel stained with ethidium bromide. All positive fo1/ro1 PCR products were analyzed for RFLPs (Lorenz et al., 1995) using the restriction enzymes *Ssp*I (Promega, USA) and *Bsa*AI (New England, BioLabs).

A real-time PCR procedure using the universal primers UniRNA as described by Hren et al. (2007) was employed to test the fruit tree samples for the presence of phytoplasma (Boben et al., 2007). A eukaryotic 18S rRNA TaqMan assay (Applied Biosystems, USA) was performed along with the universal testing for the presence of phytoplasmas to evaluate the efficacy of the extraction procedure. All real-time PCR reactions were run in 10 µl reaction volumes under standard conditions on 7900 HT Sequence Detection System (Applied Biosystems, USA). The results of amplifications were analyzed using SDS 2.2 software (Applied Biosystems, USA).

Results

The survey performed in one mother plant orchard revealed that especially young trees did not show typical symptoms and some infected fruit trees were symptomless (Table 1).

Tab. 1 The number of different symptomatic and asymptomatic trees, collected in one mother plant orchard (from 2004 to 2006) together with the percentage of infected trees (positive laboratory result) (Ambrožič Turk et al., 2008).

Fruit species	Symptomatic trees		Asymptomatic trees	
	No. of sampled trees	% of infected trees	No. of sampled trees	% of infected trees
Prunus persica	22	36,4	55	3,6
Prunus armeniaca	14	8,6	6	50,0
Prunus salicina	3	100,0	2	100,0
Prunus domestica	6	66,7	45	48,9
Prunus avium	3	0,0	2	0,0
Total	48	54.2	110	26.4

In autumn 2007, only one out of four real-time PCR positive samples of roots was also positive in a nested PCR analysis. Four months later, the roots of the same pear trees were sampled again. At that time, all previous real-time PCR positive samples were positive by a conventional PCR as well (Table 2).

Tab. 2 The results of an analysis of four pear trees from one plantation. Trees were sampled and analyzed twice (autumn 2007, winter 2008).

Pear	Samples (sampled 11.10.2007)	Real time PCR: phytoplasma	Nested PCR: AP group	Samples (sampled 6.2.2008)	Real time PCR: phytoplasma	Nested PCR: AP group
1	D1052/07	Pos	Neg	D53/08	Pos	Pos
2	D1054/07	Pos	Pos	D54/08	Pos	Pos
3	D1053/07	Pos	Neg	D55/08	Pos	Pos
4	D1051/07	Pos	Neg	D56/08	Neg	Neg

Discusion

The comparison of a real-time PCR with conventional PCR confirmed its higher sensitivity since phytoplasmas were also detected in some samples which were negative according to the nested PCR analysis. The sensitive real-time PCR method has been implemented in our diagnostic scheme for the detection of low concentration of phytoplasmas, which presumably appear in early infected trees and in some asymptomatic trees. However, well defined and accurate sampling of plant material is still a critical part of the detection due to uneven distribution and low concentrations of phytoplasmas in the tree.

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European stone fruit Yellows phytoplasma in Japanese plum and Myrobalan plum in Bosnia and Herzegovina

Delić, D.[1], Mehle, N.[2], Lolić, B.[1], Ravnikar, M.[2], Đurić, G.[1]
[1] University of Banjaluka, Faculty of Agriculture, Bulevar vojvode Petra Bojovića 1A, 78000 Banjaluka, Bosnia and Herzegovina
[2] National Institute of Biology, Department of Biotechnology and Systems Biology, Večna pot 111, 1000-Ljubljana, Slovenia

Abstract

Stone fruits from commercial as well as abandoned orchards were evaluated for European Stone Fruit Yellows phytoplasma (ESFY) presence during 2004-2007 years. Orchards were monitored in western and southern districts of Bosnia and Herzegovina. In the first survey conducted in period of 2004 till 2005 the causal agent of ESFY was identified on peach *(Prunus persica*) and apricot (*Prunus armeniaca*) plants in both surveyed districts. During 2007, a new survey was performed and samples were taken from symptomatic and symptomless plants of European plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), Myrobalan plum (*Prunus cerasifera*) and cherry (*Prunus avium*). Samples were analyzed using real-time PCR and nested PCR approaches. In this extended survey, the presence of ESFY phytoplasma was additionally identified in Japanese plum and myrobalan plum trees.

Keywords: Bosnia and Herzegovina, myrobalan plum, Japanese plum, phytoplasma, ESFY, PCR

Introduction

Stone fruits are traditionally grown in Bosnia and Herzegovina (B&H), among which the main species is European plum. Two types of fruit orchards nowadays exist: old inherited fruit orchards from the previous state farms (many completely abandoned and often with a few scattered trees) which are still the dominant type of orchards and plantings more recently established. For further development of the fruit tree industry and the establishment of a certification program in the last ten years, the sanitary status of fruit trees started to be assessed in detail. The first survey for the presence and distribution of European stone fruit yellows (ESFY; 'Candidatus *Phytoplasma prunorum*') was conducted in period 2004 till 2005. First results on the identification of the ESFY causal agent in peach and apricot trees as well as on vector *Cacopsylla pruni* was reported by Delic et al. (2007). This paper provides a continuation of the ESFY study in B&H.

Materials and methods

Field survey: The survey was organized in the North-Western of the country, an area important for stone fruit production. Field visits and sampling occurred in second part of August 2007. Leaf samples were collected from symptomatic and symptomless European plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), myrobalan plum (*Prunus cerasifera*) and cherry (*Prunus avium*) trees. Our attention was focused on one orchard in the Banjaluka region with Japanese plum and myrobalan plum species showing phytoplasma symptoms. Mid-ribs extracted from five samples (2 Japanese plum, 1 myrobalan plum, 1 European plum and 1 cherry) were analyzed.

Total DNA extraction: Automated DNA extraction procedure was performed (Boben et al., 2007; Pirc et al., 2009). DNA was isolated from 200 mg of homogenized material from fruit tree samples using the QuickPick™ Plant DNA kit (BioNobile, Finland) and KingFisher mL (Thermo Scientific, USA) machine. Final elution was performed in 200 µl of sterile double distilled water.

Molecular analyses: The presence of phytolasma in DNA samples was checked using two molecular approaches: nested PCR followed by RFLP analyses and real-time PCR. PCR was performed using the universal phytoplasma P1/P7 primers (Schneider et al., 1995) slightly modified according to Hren et al. (2007), followed by nested PCR using AP group specific primers f01/r01(Lorenz et al., 1995). Products were visualized on 1% agarose gel, stained with ethidium bromide. All positive fo1/ro1 PCR products were then analysed by RFLP using the restriction enzymes *Ssp*I (Promega, USA) and *Bsa*AI (New England, BioLabs). Real-time PCR assay was done according to the procedure described by Hren et al. (2007) using UniRNA phytoplasma non-specific primers and a TaqMan probe. In the same run, the DNA extraction procedure was checked by using the 18S rRNA TaqMan assay (Applied Biosystem, USA). All real-time PCR reactions were run in 10µl reaction volumes under standard conditions in a 7900 HT Sequence Detection System

(Applied Biosystem, USA). The results of amplification were analyzed using SDS 2.2 software (Applied Biosystem, USA).

Results

Typical symptoms of ESFY were observed on the phytoplasma positive Japanese plum and myrobalan plum. In Japanese plum, symptomatic leaves were rolled longitudinally upwards, and red colored. Red colored leaves as well as red colored leaf veins were found on myrobalan trees. Real–time PCR analyses detected phytoplasmas in two of the five samples tested, one Japanese plum and one myrobalan plum (Table 1). Average Ct values obtained amplifying 18S rRNA confirmed efficacy of the extraction procedure. AP group specific nested PCR products were obtained in the same real-time PCR positive samples. RFLP analyses determined the presence of 'Candidatus *Phytoplasma prunorum*'.

Tab. 1 Nested PCR and real-time PCR results of tested samples

Sample type	Symptoms observed[a]	Nested PCR results	RFLP	Real-time PCR[b] (UniRNA)	Real-time PCR[b] (18S rRNA)
Japanese plum	sl; rl; yl; rv	negative	/	undetermined	19,5
Japanese plum	ur; rl; rv; cl	positive	'Ca. *P. prunorum'*	22,5	15,6
Myrobalan plum	rl; rv;cl	positive	'Ca. *P. prunorum'*	25,9	17,8
European plum	rv; bl; cl	negative	/	undetermined	18,2
Cherry	rv; cl	negative	/	undetermined	16,6

[a]sl-small leaves; ur- longitudinally upward rolled leaves; rl-red colored leaves; rv-red colored leaf veins; yl-yellow colored leaves; cl-crıspy leaves; bl-bronze colored leaves; rv-reduced tree vigor.
[b] Ct are expressed as the mean value from a triplicate

Discussion

Infected Japanese plum and myrobalan plum were young trees of three-four years old. Symptoms observed on Japanese plum were in line with the literature describing ESFY symptoms on this host (Kison and Seemüller, 2001). On the other hand, unusually prominent phytoplasma symptoms were observed on myrobalan plum. Myrobalan plum is usually symptomless or infected trees can showed slight leaf chloroses. However, symptom expression in trees infected with phytoplasma depends on many factors and the most important are host cultivar/rootstock combination and genetic variability among pathogens strains (Kison and Seemüller, 2001). Recently, Martini et al. (2009) characterized 'Candidatus *Phytoplasma prunorum*' strains using *aceF* gene. Results obtained using two restriction enzymes in a PCR/RFLP method differentiated 'Ca. *P. prunorum*' strains into five different RFLP subgroups. Although the symptoms on infected myrobalan plum could be a consequence of other biotic or abiotic factors, it would be interesting to further characterize the present isolates. This limited survey demonstrated the presence of ESFY phytoplasma in Japanese plum and myrobalan plum in Bosnia and Herzegovina. Nevertheless, to have a clear insight into the distribution as well as the strain composition in the country, extensive surveys will need to be done. The distribution of ESFY in different *Prunus* species and varieties still have to be investigated in B&H. As the presence of the vector *Cacopsylla pruni* is confirmed, (Delic et al., 2007), ESFY could be another major disease and threat for the local stone fruit industry in the future.

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Almond witches'-broom phytoplasma (Candidatus *Phytoplasma phoenicium*): a real threat to almond, peach and nectarine.

Abou Jawdah, Y.[1], Abou-Fakhr, E.[1], Sobn, H.[1], Molino Lova, M.[2], Vercesi, A.[2], Bianco, P.A.[2]
[1] Faculty of Agriculture and Food Science , American University of Beirut, Lebanon. Email: abujawy@aub.edu.lb
[2] DI.PRO.VE. Sez. Patologia vegetale, University of Milan, Via Celoria 2, 20133 Milano, Italy.

Introduction

Within less than a decade, Almond witches'-broom (AlmWB) phytoplasma killed over a hundred thousand almond trees in Lebanon (Abou-Jawdah et al., 2002). AlmWB belongs to the pigeon pea witches' broom group (16SrIX), and the scientific name (Candidatus *Phytoplasma phoenicium*) was suggested (Verdin et al., 2003). Grafting experiments revealed that AlmWB may also affect peaches and nectarines (Abou-Jawdah et al, 2003). Later on, a similar disease was reported in Iran (Verdin et al., 2003; Salehi et al., 2006). This disease is still spreading on almond trees to new areas in North Lebanon, but more recently shoot proliferation with succulent small light green leaves were observed on peach and nectarine in South Lebanon, where the disease seemed to be spreading relatively fast. DNA sequencing showed over 99% sequence homology with AlmWB (Abou-Jawdah et al. 2008). This report shows that epidemics of AlmWB may occur also on peach and nectarine under field conditions, and strongly suggests the presence of an efficient vector.

Materials and methods

Surveys on major stone fruit production areas in South Lebanon and in West Bekaa for the presence of proliferation symptoms were conducted in 2008 and 2009. Leaf samples were collected from plants showing proliferation symptoms. DNA extraction from leaf midribs was performed using the modified CTAB protocol as previously described (Zhang et al., 1998; Abou-Jawdah et al., 2002). Samples from different locations were analysed by nested PCR using universal primers (P1/P7 and nested PCR R16F2/R2) as described previously (Gunderson et al. 1996). The amplicons were sequenced and the resulting sequences were subjected to Blast analysis.

Results

In 2008, About 110 nectarine and peach trees in two orchards in the Sarada plain (South Lebanon) showed characteristic symptoms of shoot proliferation; smaller leaves with a light green color (Fig. 1). Nested PCR and sequencing confirmed infection by AlmWB. Farmers eradicated all symptomatic trees.

Fig. 1 Characteristic symptoms of AlmWB on peach and nectarine. A. Shoot proliferation on nectarine, B. Comparison of infected (red arrow) vs. healthy peach tree, C. Close up view: infected nectarine branch (left) vs. healthy one (right)

In 2009, the survey conducted in South Lebanon and West Bekaa showed that only a limited number of peach and nectarine trees are symptomatic; however, they were spread over a large area (Fig. 2).

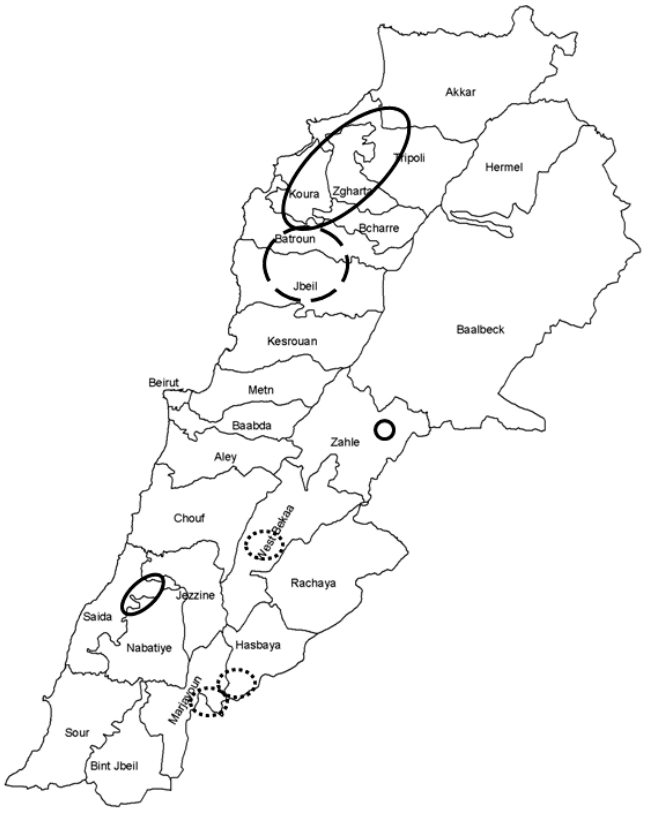

Fig. 2 Map of the AlmWB spread, starting in 2001 (full circles) and surveyed in 2009 (dotted circles); dashed circles show the area of a rapid spread that has not been yet precisely surveyed.

The evolution of symptoms during the season was monitored, showing an earlier flowering and development (Fig. 3A) and later on characteristic shoot proliferation (Fig. 1) and in rare cases witches'-broom symptoms (Fig. 3D). Most infected trees did not set any fruits, but some trees bore a limited number of deformed fruits (Fig. 3C) and abnormal flowers (phyllody) (Fig. 3B). Blast analysis of the 16S rDNA sequences obtained from 15 samples collected from several locations (South and Bekaa) revealed over 99% sequence homology with AlmWB.

Fig. 3 Characteristic symptoms of AlmWB on peach and nectarine. A. Early in the season, infected nectarine tree (red arrow) between healthy ones, B. Phyllody or transformation of flowers into leaf and stem structures of peach, C. Deformed peach fruit, D. Witches'-broom on peach.

In the North the disease on almond is progressing further towards central Lebanon, and has reached the Koura and Jbeil districts, but no surveys have been undertaken yet to monitor precisely the infected locations.

Discussion

AlmWB is a real threat to almond, peach and nectarine, not only in Lebanon, but also worldwide since the disease spreads from sea level to an elevation of about 1000 m. Furthermore, its natural spread on certified imported seedlings, clearly indicates the presence of an efficient vector(s). Further studies are needed on the epidemiology of the disease, the vector(s) involved, alternative hosts, varietal sensitivity/resistance, to decide if almond, peach and nectarine are a dead end host for AlmWB or if they constitute a good source of innoculum. Regional and international cooperation should be initiated and effective legislation and control measures should be implemented to prevent the further spread of AlmWB, which may prove to be more devastating to stone fruit production than *Plum pox virus*.

Aknowledgements

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Results of patch-grafting of tissue infected by 'Candidatus *Phytoplasma pyri*' or by 'Candidatus *Phytoplasma prunorum*', respectively on pear and apricot plants cultivated in pot

Pastore, M.[1], Cardone, A.[2], Catucci, L.[2], Del Vaglio, M.[1], Gervasi, F.[1], Scognamiglio, G.[1], Bertaccini, A.[3]
[1] Unità di Ricerca per la Frutticoltura di Caserta, Council for Research in Agriculture (C.R.A.) via Torrino 3, 81100 Caserta, Italy. Email: Pastore2000@inwind.it
[2] Centro di Ricerca e Sperimentazione in Agricoltura Basile Caramia, Locorotondo, 70010 Bari, Italy
[3] DiSTA, Patologia Vegetale, Alma Mater Studiorum Università di Bologna, viale Fanin 42, 40127 Bologna, Italy

Abstract

Molecular analyses carried out either on the pear varieties 'Conference', 'Comice' and 'William' grafted on different rootstocks or on sixty-eight apricot varieties grafted on Myrobalan, showed the susceptibility of the tested combinations to 'Candidatus *Phytoplasma pyri*', transmitted by *Cacopsylla pyri*, and to 'Candidatus *Phytoplasma prunorum*', transmitted by *Empoasca decedens*, respectively. In order to find pear and/or apricot combinations immune to the associated Phytoplasma, several varieties grafted on new rootstock were tested in the period 2002-2008. 68 pear plants belonging to seven variety/rootstock combinations and 76 apricot plants belonging to seven combinations, all cultivated in pot, in greenhouse covered by anti-aphid tissue, were grafted with patches of infected tissues containing the specific phytoplasmas. Young healthy potted plants belonging to the pear combination 'Comice'/*P. communis* and to the apricot combination 'Palummella'/Myrobalan, both susceptible in open field to the associated phytoplasmas transmitted by the specific vectors, were also used and patch-grafted. Molecular analyses, carried out on nucleic acids extracted from leaf samples, to detect the presence of the pathogens, showed the pear variety 'William' grafted on *Pyrus betulaefolia* to be susceptible to 'Candidatus *Phytoplasma pyri*'. Neither the pear combination 'Comice'/*P. communis* nor the apricot 'Palummella'/Myrobalan 29 C, susceptible, in open field, to the associated phytoplasmas, became infected after patch-grafting under greenhouse conditions. Thus the results show that patch-grafting cannot be utilized in young potted plants for artificial transmission of these two phytoplasmas.

Keywords: Phytoplasmas, source of immunity, variety/rootstock combination, molecular tests, insect proof green-house

Introduction

Previous researches showed that pear varieties 'Conference', 'Comice' and 'William', grafted on different rootstocks, were susceptible to 'Candidatus *Phytoplasma pyri*' transmitted by Cacopsylla pyri (Pastore et al., 1998), and that sixty-eight apricot varieties showed different susceptibility to 'Candidatus *Phytoplasma prunorum*' (Pastore et al., 1995). In order to find a source of immunity to the two phytoplasmas we tested seven new combinations varieties/rootstock of pear and seven new combinations varieties/rootstock of apricot that were patch-grafted by infected tissues containing the specific phytoplasmas were tested. The pear combination 'William'/*Pyrus betulaefolia* and apricot combination 'Palummella'/Myrobalan both susceptible to the specific phytoplasma in open field were also tested.

Materials and methods

Variety/rootstock combinations: The list of pear combinations is reported in Table 1, while the one of apricot combinations is showed in Table 2.

Time schedule and schemes of grafting: The first patch-grafting trial was carried out in Locorotondo (Bari) from July to September 2002 on two pear and six apricot variety/rootstock combinations on young plants cultivated in pots and maintained in a screen-house covered by anti-aphid tissue. The experimental scheme consisted on five plants without graft-inoculation as control, and ten plants for each combination, grafted with patches of shoots derived from infected plants tested by PCR/RFLP analyses.

In the year 2007 the plants were transferred to Caserta, in another greenhouse, covered by aphid-proof tissue, where six pear variety/rootstock combinations and two apricot variety/rootstock combinations were added to the experiment. From June to October 2007 the patch-grafting was carried out on the majority of the plants already grafted in Locorotondo, and on eight out of ten plants belonging to the new combinations (see Table 1 and Table 2).

Tab. 1 Results of patch-grafting of tissues infected by '*Ca.* P. pyri' on pear variety/rootstock combinations.

Variety/rootstock	Grafted July and September 2002	Plants resulted infected 05/03	09/03	10/05	Grafted in June, July and September 2007	Plants resulted infected 09-10/07	07-09/08
Conference/*P. communis*	10	0/10	0/10	0/10	5	Not tested	0/5
Comice/*P. communis* (positive in field 1995)	10	0/10	0/10	0/10	8	Not tested	0/8
Comice/*P. betulaefolia*	-				8	0/8	0/8
Comice/Quince BA 29	-				8	0/8	0/8
William/*P. betulaefolia*	-				8	1/8	1/8
Conference/*P. betulaefolia*	-				8	0/8	0/8
William/Quince BA 29	-				8	0/8	0/8
Conference/Quince BA 29	-				8	0/8	0/8

- not grafted

Tab. 2 Results of patch-grafting of tissues infected by 'Ca. *P. prunorum*' on apricot variety/rootstock combinations.

Variety/Rootstock in pots	Grafted in July and September 2002	Plants resulted infected 05/03	09/03	10/05	Grafted in October 2007	Plants infected in September 08
Tyrinthos/ Myrobalan 29 C	10	0/10	0/10	0/10	5	0/5
Cafona/ Myrobalan 29 C	10	0/10	0/10	0/10	7	0/7
Monaco Bello/ Myrobalan	10	0/10	0/10	0/10	3	0/3
Monaco Bello/MRS 2/5	10	0/10	0/10	0/10	2	0/2
Palummella/Myrobalan 29 C	10	0/10	0/10	0/10	2	0/2
Palummella/ Myrobalan (positive in field 1995)	10	0/10	0/10	0/10	6	0/6
Sancastrese/ Myrobalan	-				8	0/8
Monaco Bello/ Myrobalan	-				8	0/8

-, not grafted

Source of plant tissue infected by phytoplasmas: The pear plant used as source of 'Ca. *P. pyri*' belongs to 'William' grafted on a selection of *P. communis*. It became infected in 1995 by *Cacopsylla pyri* and it is still positive in PCR/RFLP analyses.

The apricot shoots used as source of 'Ca. *P. prunorum'*, in 2002, belonged to 'Harogem', infected by patch-grafting of plum infected tissue (Pastore et al., 2001), while for tests of 2007, shoots were kindly provided by Department of 'Biologia applicata alla difesa delle piante' of the University of Udine.

Molecular tests for phytoplasma detection: To detect the phytoplasmas, molecular tests were carried out on leaf samples collected in May and September 2003, October 2005, from September to October 2007, and from July to September 2008.

Nucleic acids were extracted from leaf samples according to Bosco et al. (2002), while PCR experiments were carried out according to Schaff et al. (1992). Nested PCR reactions were performed under the same conditions using as template the amplicons of the previous reaction diluted 2: 30 with sterile distilled water. Ribosomal general primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995), for direct PCR amplification, primer pair R16F2/R2 (Lee *et al*., 1995), for the first nested PCR reaction, and primer pair R16(X)F1/R1 (Lee et al., 1995), for the second nested PCR amplification, were used. Alternatively primers f01/r01 (Lorenz et al., 1995), for nested PCR, were used after direct amplification with P1/P7 primers. Samples with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. Six µl of each PCR product were subjected to electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination. Three µl of PCR products amplified after first or second nested PCR reaction were digested using endonucleases *Ssp*I and *Rsa*I at 37°C for at least 16 hours following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then compared with those of reference strains after electrophoresis through a 5% poliacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Results and discussion

Only one plant belonging to the combination 'William'/*P. betulaefolia*, sampled after the second grafting, resulted positive in nested PCR reaction with primers f01/r01, but it did not show symptoms of the disease. RFLP analyses of PCR products with restriction enzymes *Ssp*I and *Rsa*I confirmed that phytoplasma infecting this pear belonged to 16SrRNA subgroup X-C, 'Ca. *P. pyri*'. All the other samples were negative independently either from the number of grafting or from the PCR detection system utilized, including the combinations 'Comice'/*P. communis* and

'Palummella'/Myrobalan 29 that in open field resulted susceptible to 'Ca. *P. pyri*' and 'Ca. *P. prunorum*' respectively (see Table 1 and Table 2).

Thus we conclude that the presented experimental approach, the patch-grafting, efficient for transmission of phytoplasma-microorganisms to apricot (*Prunus armeniaca* L.) and Japanese plum (*Prunus salicina* LINDL) trees cultivated in field (Pastore et al., 2001) is not efficient when used on young pear and apricot plants cultivated in pots.

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Evaluation of detection methods for Virus, Viroids and Phytoplasmas affecting pear and apple

Laviña, A., Sabaté, J., Batlle, A.
Dpt. Protecció Vegetal, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Ctra Cabrils s/n. 08348 Cabrils (Barcelona).

Abstract

The RT-PCR technique for the detection of apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV) and pear blister canker viroid (PBCV) was evaluated for health control of fruit plants from nurseries. The technique was evaluated in purified RNA and crude extracts and also in phloem collected in autumn and from young spring shoots. The results obtained for phytoplasma detection with ribosomal and non-ribosomal primers are also presented.

Keywords: Detection, fruit virus, viroids, phytoplasma

Introduction

The virus Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), Apple chlorotic leaf spot virus (ACLSV) and Apple mosaic virus (ApMV), the viroid Pear Blister canker (PBCV) and the phytoplasmas Candidatus *Phytoplasma pyri* (pear decline, PD) and Ca. *P.mali* (apple proliferation, AP), cause significant diseases in *Malus* and *Pyrus* species. The sensitivity of molecular methods to detect these virus and phytoplasmas has been reported by many authors but it is still recommended, that their absence be confirmed by indexing on indicator woody plants.

There is an increasing need for health control in fruit nurseries, due to the large number of diseases caused by viruses, phytoplasmas and other harmful organisms. One explanation why the number of certified plants has not increased, may - depending on the species – be found in the fact that certifying by field indexing can take between 2-4 years: the time needed for symptoms to appear.

In the present work, we established the presence of ASPV, ASGV, ApMV, ACLSV and PBCV by means of RT-PCR and of Ca. *P. pyri* and Ca. *P.mali* by PCR on tree samples from different nurseries and orchards. In addition, the presence of these viruses was evaluated in positive controls of different transmissible diseases, including vein yellows, ring mosaic, red mottle, and russet wart, as was the presence of phytoplasmas in apple samples with chat fruit and rubbery wood symptoms.

Material and methods

Detection of Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus and Apple mosaic virus by RT-PCR: Virus detection was carried out on crude extract and on purified RNA. Total RNA was extracted from different parts of the plant (leaves, petioles and bark) by different extraction methods, using a commercial extraction kit (Rnaeasy Qiagen) according to the manufacturer's instructions. Extraction was performed on young shoots selected in May and from phloem selected in October.

For ASPV, three pairs of specific primers were assayed: those cited by Klerks et al. (2001); those cited in Batlle et al. (2004), and the primers used by Massart et al. (2008). For other viruses, only the primers cited in Massart et al. (2008) were used.

Detection was carried out on samples taken from trees infected with each of these viruses and on hosts of various diseases, such as vein yellows, ring mosaic, red mottle and russet wart. The technique was evaluated in samples from different plant nurseries.

Pear blister canker detection by RT-PCR: The plant material used came from two orchards and positive controls were supplied by the Certification Institute of Zaragoza (Spain). Two pairs of specific primers were assayed: the primer pair cited by Malfitano et al. (2004), and that used by Hassen et al. (2006). The RT-PCR procedure applied was a modified version of the one referred to in Hassen et al. (2006). Four µl of RNA extract was mixed with 0.4 µM complementary primer (Malfitano et al. 2004), heated for 5 min at 100ºC and then immediately chilled on ice. The RT-PCR reaction mixture contained 0.4 µM of homologous primer and the Qiagen reaction mix (5 µl of 5 x RT-PCR buffer, 1 µl dNTPs (0.4 µM final), 1 µl enzyme mix and 5 µl RNase-free water).

Phytoplasma detection by PCR: For phytoplasma detection by PCR, samples were collected between October and December, which has been shown to be the best period for the detection in our climate (Garcia et al. 2003). DNA was isolated from approximately 1.0 g of fresh stems, using the phytoplasma-enrichment procedure of Ahrens and Seemüller (1992). The nested-PCR procedure was used with P1/P7 primers in the first step (Smart et al. 1996) and with specific primers for the apple proliferation group fO1/rO1 (Lorenz et al. 1995) in the second step. The presence of phytoplasmas other than the 16Sr-X or AP group, in those samples giving a negative with the specific primers, was determined using the universal primer pair fU5/rU3 (Lorenz et al. 1995). Samples which tested positive in the nested-PCR assay were further analysed by RFLP. Amplified products (10 µl) obtained with the universal primers fU5/rU3 were digested with Tru9I restriction endonuclease following the manufacturer's instructions (MBI Fermentas, Germany). Amplified products obtained with the fO1/rO1 specific primers were digested as above with Ssp I and Rsa I at 37ºC. Profiles obtained were compared with those established by Seemüller et al. (1998).

In addition, we evaluated primers developed for the amplification of different fragments of the pear decline genome by Garcia et al. (2004): 7 f/r, which amplified fragments of a gene coding for a thymilidate kinase (TMPK), 14f/r for a peptide release (PR), 10 f/r for 50S ribosomal protein L4, 15f/r for DNA polymerase III and 13 f/r (no significant similarity). The primers developed by Danet et al. 2006 that amplify a sequence encoding a surface protein were also evaluated.

Results and discussion

Detection of Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus and Apple mosaic virus by RT-PCR: The best primers for the detection of ASPV, ASGV, ACLSV and ApMV by RT-PCR were those used by Massart et al. (2008). With these primers, it is possible to detect all four viruses, both in purified RNA and in crude extracts, and also in extracts of phloem in autumn and of young shoots in spring. The virus most frequently identified in samples from nurseries was ASPV (Table 1). It was also identified in positive controls for different transmissible diseases: vein yellows, ring mosaic, red mottle, and russet wart. In the latter, ASGV was also detected, indicating that these viruses may be responsible for these diseases either alone, or in synergism with other viruses or pathogenic organisms (Table 2).

Tab. 1 Virus and phytoplasma detection in samples from pear and apple varieties taken from different nurseries

Species	Season	ASPV	ASGV	ApMV	ACLSV	Phytoplasma
Apple, several varieties	Spring	3+/6	0+/6	4+/6	0+/6	0+/6
Pear, several varieties	Spring	3+/6	0+/6	0+/6	0+/6	0+/6
Pear, cv. Conference	Autumn	3+/15	0+/15	0+/15	0+/15	13+PD/15
Pear, several varieties	Winter	0+/5	0+/5	0+/5	0+/5	5+PD/5
Apple, several varieties	Winter	0+/7	0+/7	0+/7	0+/7	3+AP/7

Tab. 2 Virus and phytoplasma detection by RT-PCR and nested PCR in positive controls of different transmissible diseases

Symptom	Virus	Phytoplasma
Ring Mosaic	ASPV	-
Red mottle	ASPV	-
Vein yellows	ASPV	-
Russet wart	ASPV + ASGV	-
Chat fruit	-	positive

Pear blister canker detection by RT-PCR: PBCV was best detected using the primers cited by Malfitano et al. (2004) and the procedure recommended by Hassen et al. (2006). With this method, PBCV was detected in all the infected controls but in no healthy plants.

Phytoplasma detection: For phytoplasma detection in pear and apple trees, both ribosomal and non-ribosomal primers gave good results, the best obtained with PCR using purified DNA and nested PCR with fP1/rP7 primers in the first step and fO1/rO1 primers in the second step. For Ca. *P. pyri* detection, good results were obtained using a single PCR amplification with some of the non-ribosomal primers previously developed (Garcia-Chapa et al. 2004), which amplified small fragments. Nevertheless, more comparative analyses is necessary before these primers can be recommended instead of those normally used. Some of the primers designed for *Ca*. P. pyri sequences also amplified the *Ca*. P. prunorum phytoplasma (European stone fruit yellows, ESFY) (Table 3). Furthermore, the *Imp* primers developed by Danet et al. (2006 and 2008), amplifying a fragment of a gene encoding a membrane surface protein, also gave good results, both in simple and in nested-PCR (Table 3).

Tab. 3 Phytoplasma detection by PCR with different primers

Sample	Phytoplasma detected	Primers						
		13 f/r 197 pb	7f/r 165 pb	14f/r 231 pb	10 f/r 127 pb	15 f/r 264 pb	Imp PD (A)	Imp PD(B)
Pear 1	PD	+	+	+	+	-	-	+
Pear 2	PD	+	+	+	+	-	+	+
Pear 3	PD	+	+	+	+	-	+	+
C. roseus	PD	+	-	-	+	-	+	-
C. roseus	PD	+	-	-	-	-	+	+
Pear 4	PD	+	-	-	+	-	-	-
Pear 5	PD	+	-	-	-	-	-	-
Pear 6	PD	+	-	-	-	-	-	-
Pear 7	PD	+	-	-	+	-	+	+
Prunus cerasifera 1	ESFY	+	+	+	+	-	+	-
Prunus cerasifera 2	ESFY	-	-	+	-	-	-	-

As in the case of virus detection, the presence of phytoplasmas in samples of pear and apple from different plant nurseries was analysed. In contrast to virus detection, the detection of phytoplasmas in crude extracts is very erratic and, on the basis of our results, it cannot be recommended at present. A phytoplasma belonging to the 16Sr-XII group was detected in apple plants with symptoms of chat fruit (Table 2).

Discussion

RT-PCR for the specific detection of ASPV, ASGV, ACLSV, ApMV and PBCV and PCR for specific detection of the phytoplasmas Ca. *P. Pyri* and Ca. *P. mali* can be used throughout the year in woody hosts. RT-PCR appears to be a sensitive method and avoids the need for field indexing of the virus and viroid. It is important to point out that, in samples from many of the pear and apple trees that had supposedly been submitted to a process of sanitary control, virus and phytoplasmas were detected by RT-PCR and PCR. The process may not have been carried out correctly or it is possible that errors may occur when using woody indicators, due to the irregular distribution of phytoplasmas. Results obtained in the detection of Ca. *P. pyri* (PD) and Ca. *P. mali* (AP) show that PCR can be used in certification programs for early detection of these pathogens and that this method is more sensitive than the field indexing technique. There is increased sensitivity when tests are carried out between October and December, which is when phytoplasma accumulation in the phloem is at its peak (Garcia et al. 2003).

The implication of some of the viruses and the phytoplasmas tested in other pear and apple diseases has already been discussed. If this implication could be fully ascertained, the use of RT-PCR to detect viruses and PCR to detect phytoplasmas could significantly reduce the time required for certification.

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Virus and other Graft Transmissible Diseases of Fruit Crops

The papers contained in this volume of the „Julius-Kühn-Archiv" report the Proceedings of the XXIst International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops. All scientists who contributed oral and poster presentations were invited to submit manuscripts for publication.

The contributions cover a wide spectrum of topics of research on viruses, viroids, phytoplasmas and diseases of unknown etiology. The majority of the conference presentations were for historical reasons on pome and stone fruits, as well as on small fruit crops. However, contributions on nuts and subtropical and tropical fruit crops also appear in the program. The topics include characterization of the pathogens, their transmission by vectors, surveys and quarantine aspects, elimination of the agents for certification purposes and effects of the diseases on host plants. A considerable number of presentations are on detection of pathogens using a wide range of modern nucleic acid technologies but also serology and classical biological indexing procedures. In addition, some papers report improvement of crop resistance to pathogens by classical breeding or transgenic approaches.

The papers were reviewed by the organizers of the 2009 Conference, with the support of the Scientific Committee and some professional language editing. Other contributions to the conference not appearing as an article in this volume are available as abstracts for download from the ICVF website.

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Bundesforschungsinstitut für Kulturpflanzen