Introduction of certification program in production of plum planting material

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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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