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.

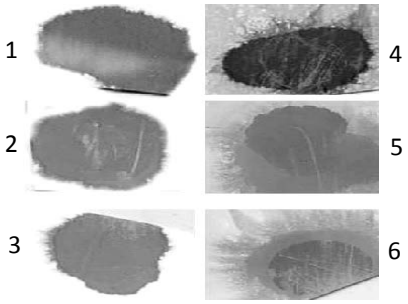

Fig. 1 Results of DIBA assay, using antiserum absorbed twice with extracts from healthy fig roots, obtained from bleed-3 diluted 1:1000, on the following samples: 1. Bosnia, healthy control; 2. Bari, healthy control; 3 seedling healthy control; 4. Montenegro infected sample; 5. F5P5 infected accession;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

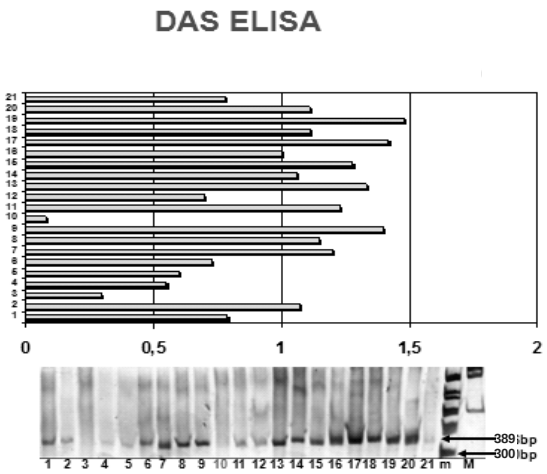

Fig. 2 Comparison of molecular (RT-PCR) and serological (ELISA) detection obtained on 21 samples. Sample 10 is the negative (virus-free) control.

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.

Literature

Berger, P.H.; Hunt, A.G.; Domier, L.L.; Hellmann, G.M.; Stram, Y.; Thornbury, D.W.; Pirone, T.P.; 1989: Expression in transgenic plants of a viral gene product that mediates insect transmission of potyviruses. Proceedings of the National Academy of Sciences USA **86**, 8402-8406.

Boscia, D.; Savino, V.; Minafra, A.; Namba, S.; Elicio, V.; Castellano, M.A.; Gonsalves, D.; Martelli, G.P.; 1993: Properties of a filamentous virus isolated from grapevines affected by corky bark. Archives of Virology **130**, 109-120.

Clark, M.F. and Adams, A.N.; 1977: Characteristics of microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. Journal of General Virology **34**, 475-483.

Castellano, M.A.; De Stradis, A.; Minafra, A.; Boscia, D.; Martelli, G.P.; 2009: Seed transmission of Fig Latent Virus 1. Journal of Plant Pathology **91**, 697-700.

Castellano, M.A.; Gattoni, G.; Minafra, A.; Conti, M.; Martelli, G.P.; 2007: Fig mosaic in Mexico and South Africa. Journal of Plant Pathololog**y 89**, 441-443.

De Stradis, A.; Gattoni, G.; Minafra, A.; Martelli, G.P.; 2007: Fig Mosaic in Corsica. Journal of Plant Pathology **89**, S76

Doi, Y.; 1989: Fig virus S. In: Hull R. Brown; F. Payne C. (Eds): Directory and Dictionary of Animal, Bacterial and Plant Viruses, p.76. McMillan Reference Books, London, UK.

Elbeaino, T.; Digiaro, M.; De Stradis, A.; Martelli, G.P.; 2006: Partial characterization of a closterovirus associated with a chlorotic mottling of fig. Journal of Plant Pathololog**y 88**, 187-192.

Elbeaino, T.; Digiaro, M.; De Stradis, A.; Martelli, G.P.; 2007: Identification of a second member of the family *Closteroviridae* in mosaic-diseased figs. Journal of Plant Pathololog**y 89**, 119-124.

Foissac, X.; Svanella-Dumas, L.; Gentit, P.; Dulucq, M.J.; Candresse, T.; 2001: Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). Acta Horticulturae **550**, 37-43.

Gattoni, G.; A, Minafra; Castellano, M.A.; De Stradis, A.; Boscia, D.; Elbeaino, T.; Digiaro, M.; Martelli, G.P.; 2009: Some properties of Fig Latent Virus 1, a new member of the family *Flexiviridae*. Journal of Plant Pathology **91**, 543-552.

Grbelja, J.; Eric, Z.; 1983: Isolation of a *Potyvirus* from *Ficus carica* L. Acta Botanica Croata **4**, 11-14.

Martelli, G.P.; Russo, M.; 1984: Use of thin sectioning for the visualization and identification of plant virus. Methods in Virology **8**, 143-224.

Martelli, G.P.; Castellano, M.A.; Lafortezza, R.; 1993: An ultrastructural study of fig mosaic. Phytopathologia Mediterranea **32,** 33-43.

Milne, R.G.; 1993: Electron microscopy of *in vitro* preparations. In: Matthews, R.E.F. (Eds.): Diagnosis of Plant Virus Diseases, 215-131. CRC Press, Boca Raton, FL, USA.

Minafra, A.; De Stradis, A.; Clara, M.I.; Martelli, G.P.; 2009: Virus in Mosaic-affected figs in Portugal and the island of Crete. Journal of Plant Pathology **91,** 238.

Salomon, R.; Voronov, N.; Flaishman, M.; 2003: The identification of fig mosaic. Rymovirus, the causative agent of fig mosaic disease, and its elimination from fig. In: 8th International Congress of Plant Pathology, 23.14. Christchurch, New Zealand.

Salomon, R.; Mawassi, M.; Flaishman, M.; 2008: Isolation and characterization of a virus from fig leaves exhibiting mosaic symptoms and development of a sensitive detection procedure for this virus. Acta Horticulturae **798**, 259-262.

Serrano, L.; Ramon, J.; Segarra, J.; Medina, V.; Achon, M.A.; Lopez, M.; Juarez, M.; 2004: New approach in the identification of the causal agent of Fig mosaic disease. Acta Horticulturae **657**, 559-566.