DETECTION OF *FIG MOSAIC VIRUS* IN VIRULIFEROUS ERIOPHYID MITE *ACERIA FICUS*

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SUMMARY

Fig leaves showing typical fig mosaic symptoms on cv. Bursa siyahı (donor plant) were cut under a stereo microscope into small pieces each hosting 10 putatively viruliferous eriophyid mites (*Aceria ficus* Cotte) and placed directly on the top leaves of healthy *Cucumis sativus*, *Chenopodium quinoa. C. amaranticolor*, *Nicotiana occidentalis*, *Catharanthus roseus*, *Fraxinus excelsior* plants, and fig seedlings. Donor and test plants were analyzed by electron microscopy, RT-PCR and sequencing, whereas the mites (ErMs) underwent molecular assays using *Fig mosaic virus* (FMV)-specific primers. Mite-infested leaves of fig seedlings and *C. roseus* showed small yellowish spots after 10 days and 6 weeks, respectively, whereas no symptoms were observed in other test or control plants for three months. Electron microscopy observations showed the occurrence of double membrane bodies (DMBs) in the palisade cells of donor and mite-inoculated fig plants, but not in *C. roseus.* However, 302 bp RT-PCR products specific to FMV were amplified from donor and inoculated figs, *C. roseus* and ErMs. Nucleotide identity with the sequence of the FMV isolate in GenBank (accession No. AM941711.6) was 87%, 89% and 87% for donor plant (JQ708183), inoculated fig seedlings (JQ708184) and *C. roseus* (JQ408437, JQ408438), respectively. The sequences obtained from ErMs (JQ408432, JQ408436) showed 87% and 88% nucleotide identity with the reference FMV isolate, respectively. When dsRNA extracts were analyzed to confirm virus presence in inoculated periwinkles, a complex dsRNA profile was obtained, suggestive of infection by a multipartite virus or by multiple viruses. Sequence from RT-PCR amplicons of dsRNA (JX040436) showed 88% identity with those the reference FMV isolate (AM941716.1) and the donor plant (JQ708183). According to these results, Madagascar periwinkle (*C. roseus*) can be retained as a new experimental host for FMV and *A. ficus* appears to be able to transmit FMV from fig to periwinkle plants.

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Key words: Fig mosaic disease, *Aceria ficus*, herbaceous and woody indicators, electron microscopy, RT-PCR, sequencing.

INTRODUCTION

Fig mosaic disease (FMD) was first described in 1933 from California (Condit and Horne, 1933) and it was suspected that the eriophyid mite *Aceria ficus* (Cotte) might transmit the causal agent due to its widespread occurrence on figs in that region. Although this fig mite was considered the probable vector of FMD, no evidence was provided until 1955 (Flock and Wallace, 1955). These authors observed that feeding injury by *A. ficus* might cause early symptoms that could be confused with a virus infection. To differentiate between the two disorders, eggs from a virus-free colony were transferred to one group of healthy seedlings and infective eriophyid mites to another group. Virus-free mites caused leaf distortion, chlorosis and russetting which were distinguishable from fig mosaic symptoms that appeared on plants fed on by the infective mites (Oldfield, 1970). This experiment showed that the FM agent(s) was not transmitted through the egg of *A. ficus.* Flock and Wallace (1955) demonstrated that mosaic symptoms persisted on figs in the absence of *A. ficus* by treating infested cuttings with sulphur to kill the mites. Since all stages and both sexes of this mite were found throughout the year (Baker, 1939), it has the potential for vectoring the FM agent(s) rapidly in the field. FMD has been reported only from species of the family Moraceae up to now. The host range of the FMD agent includes different *Ficus* species as reported by Condit and Horne (1933) and Burnett (1962), who also showed *Ficus diversifolia* Blume and *Cudranea tricuspidata* to be a reliable indicator plants for FMD. Vashisth and Nagaich (1965) showed that it also infects mulberry (*Morus indica*).

Double membrane-bound bodies (DMBs) were reported to be associated with FMD by Bradfute *et al.* (1970) and, very recently, a virus, *Fig mosaic virus* (FMV), genus *Emaravirus,* was shown to be the causal agent of FMD (Walia *et al.*, 2009; Elbeaino *et al.*, 2009). Little is known about the mechanism of transmission of

FMV by *A. ficus*. Proesler (1969, 1972) showed that FMV is transmissible by a single eriophyid mite from infected to virus-free fig seedlings and that the minimum feeding time for acquisition of the virus by *A. ficus* was 5 min.

All published reports suggest that fig trees may be infected with a complex of viruses (reviewed by Martelli, 2011). However, which virus is effectively transmitted by *A. ficus* to different hosts has not yet been determined. In this study, experimental transmission of FMV to some herbaceous and woody hosts through presumably viruliferous *A. ficus* was carried out and the presence of FMV in the eriophyid body was investigated.

MATERIALS AND METHODS

Plants and eriophyid mites (ErMs). A fig tree of cv. Bursa siyahı from the University collection orchard located in the Hatay province of Turkey, which exhibited clear-cut FMD symptoms (Fig. 1A), was used as the main source for presumably viruliferous *A. ficus* in transmission trials with *A. ficus* (Fig. 2). *Cucumis sativus*, *Chenopodium quinoa, C. amaranticolor, Nicotiana occidentalis*, *Catharanthus roseus* (Madagascar periwinkle), *Fraxinus excelsior* (European ash) and *Ficus carica* cv. Bursa siyahı seedlings were used for transmission trials. All test plants were kept in a growth chamber at 25°C for a 16 h day period and at 20°C for a 8 h night period, with a constant 70% relative humidity.

Transmission tests. Symptomatic leaves that hosted putatively viruliferous ErMs from the field were examined under a binocular microscope, then cut into small pieces (each piece hosting 10 ErMs), and placed on young leaves of the above listed hosts. The experiment was replicated five times, so that 50 ErMs were used to transmit FMV to five plants of each test species. Inoculated test plants were held in the laboratory for 1 day prior to transferring to the growth chamber for symptom observations. When the first symptoms appeared, 10 ErMs were collected for molecular analyses and the plants were sprayed with Hexythiazox (50 ml/100 litre). Symptomless test plants were not sprayed. All plants were observed regularly for symptoms and were analyzed by EM and RT-PCR; all amplicons obtained were custom sequenced.

Transmission electron microscopy (TEM). Leaves for TEM observations excised from the symptomatic donor fig, the fig seedlings and periwinkles exposed to ErMs, were fixed immediately in 3% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) and kept overnight at 4°C. The samples were washed in the same buffer,

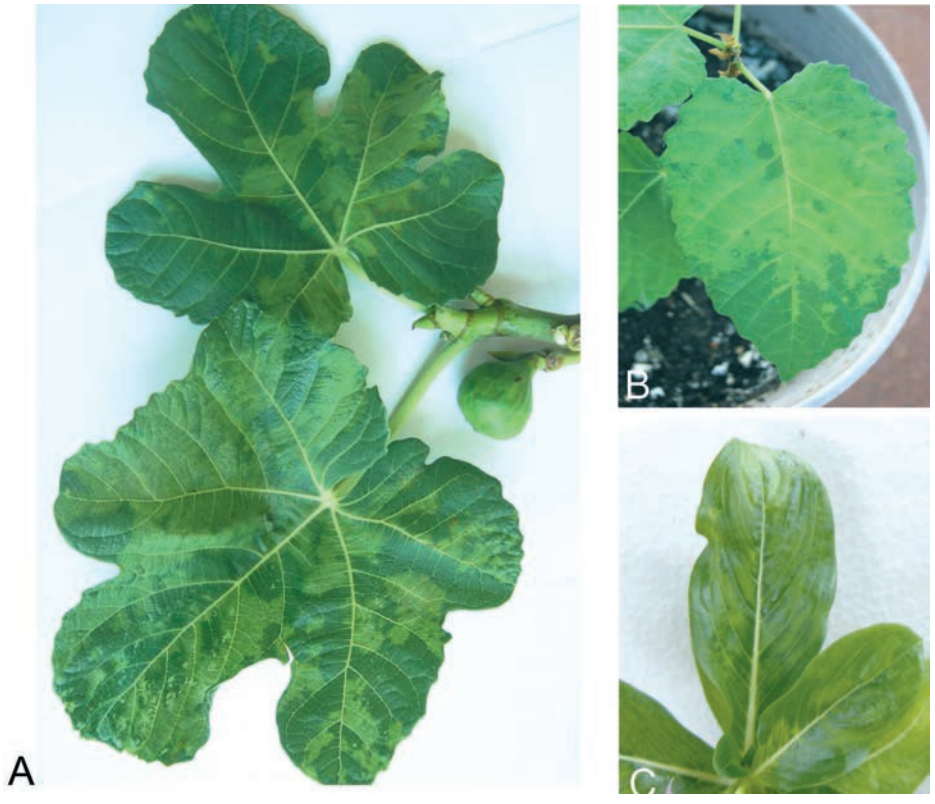

Fig. 1. A. Mosaic symptoms on both leaves and fruit of donor fig plant, cv. Bursa siyahı B. Symptoms of *Fig mosaic virus* on inoculated fig seedlings using viruliferous *Aceria ficus*. C. Mottling of the leaves of *Catharanthus roseus* plant exposec to viruliferous *A. ficus*.

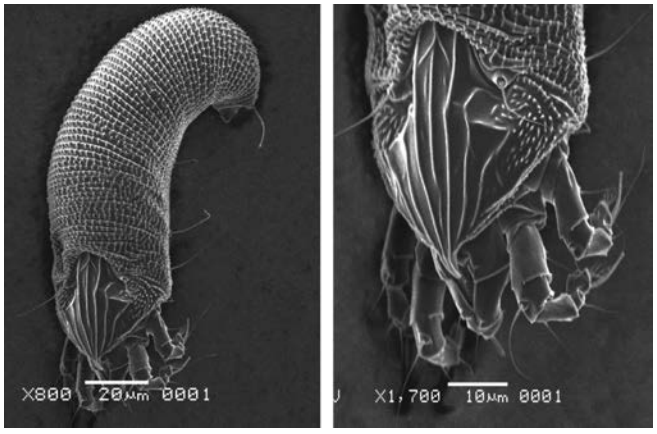

Fig. 2. Dorsal view of *Aceria ficus* under scanning electron microscope (X800, left) and head of *A. ficus* (X1700, right).

post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature then dehydrated in a graded series of increasing acetone concentrations. Dehydrated samples were subsequently embedded in Epon-Araldite mixture as described (Medina *et al.*, 2003). Ultrathin sections (70-90 nm) were cut with an Ultracut E microtome (Reichert, UK) using glass or diamond knives (Diatome, Switzerland). The sections were then routinely mounted for staining on formvar-coated 200 mesh copper grids (Aldrich, UK). Sections with silver-gold interference colour were stained in drops of 4.5% uranyl acetate, washed in distilled water and further stained in drops of Reynold's lead citrate (Roland and Vian, 1991). All sections were viewed with a Zeiss-910 TEM at an accelerating voltage of 75 kV.

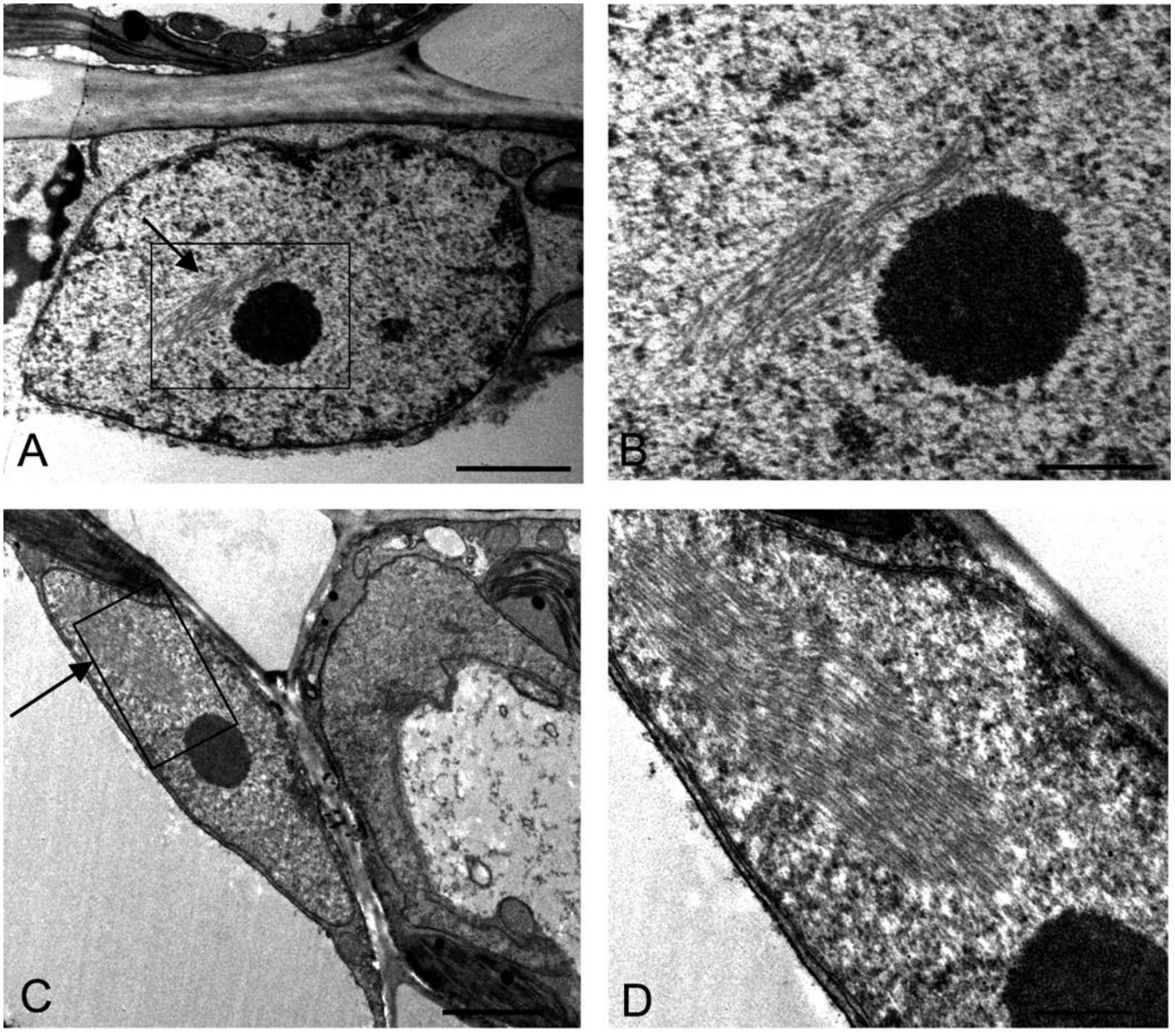

Fig. 3. Ultrathin section of FMV-infected periwinkle leaf. A. Nucleus of a mesophyl cell showing a bundle of filamentous virus-like particles (arrow) next to the nucleolus. B. Close up of boxed area in A. C. Bundle sheeth cell showing a group of filamentous virus-like particles (arrow) in the nucleus. D. Close-up of boxed area in C (Bars: A and C = 5 µm, B and D = 1.6 µm).

Total RNA extraction, RT-PCR and nucleotide sequence analysis. Total RNAs were extracted from leaves of donor fig, inoculated test plants and healthy controls using the RNeasy kit (Qiagen, Germany) following the manufacturer's instructions. RNA aliquots of 50 ng were used as template for RT-PCR. Ten ErMs were collected from the symptomatic donor plant and inoculated fig seedlings after first symptom appearance and tested individually. Each ErM was placed onto a cellulose membrane (Roche, Switzerland), crushed and the membrane was transferred to an eppendorf tube. Fifty µl of 0.5% Tween X100 were added to membranes and incubated at 95°C for 10 min. After vortexing and centrifugation, 5 µl of the mixture were used as template for RT-PCR.

One-step RT-PCR protocol was used in a 25 µl reaction volume containing template, 2.5 µl of 10X PCR buffer, 1.1 µl of 25 mM $MgCl_2$, 1.25 µl of 0.1 M DTT (dithiothreitol), 0.5 µl of 10 mM dNTPs, 1.25 µl of FMV-specific primer set (10 µM) 5'-CGGTAGCAAATGGAATGAAA-3' and 5'-AACACTGTTTTTGCGATTGG-3', 0.25 µl of Taq polymerase (5 U/µl; Fermentas, Canada), and 0.035 µl of *Moloney murine leukemia virus* (MoMLV) reverse transcriptase (200 units/µl; Fermentas, Canada). Reactions were performed at one cycle of 42°C for 30 min; 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and a final 72°C extension for 10 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels in Tris-Acetate-EDTA buffer, stained with ethidium bromide and visualized under UV light.

Sequence analysis. Selected PCR products were sequenced using an AB1373 Automated Sequencer at Iontek (Istanbul, Turkey). Sequences were compared those retrieved form GenBank using BLAST programs (NCBI), and utilized for constructing phylogenetic trees using the neighbor-joining method with the Mega5 program (Tamura *et al.*, 2011). Bootstrap analyses with 1000 replicates were performed. The FMV sequence AM941711 was used as reference.

Double-stranded RNA extraction, cDNA synthesis and PCR. Symptomatic leaves from inoculated periwinkle seedlings were used for dsRNA extraction. Leaf samples (24 g) were ground in liquid nitrogen. The dsRNAs were recovered using 2 cycles of CF-11 (Whatman, England) column chromatography (Walia *et al.*, 2009). After ethanol precipitation, dsRNA pellets were dissolved in 10 µl of TE buffer and subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized under UV light.

cDNAs were synthesized using 0.2 µg/µl random hexamer primers and 200 ng dsRNA as template. After incubation at 95°C for 5 min, 1 µl of MoMLV reverse transcriptase (200 U/µl; Fermentas, Canada), 4 µl of 5X reverse transcriptase buffer, 2 µl of 1 M dithiothreitol (DTT) and 0.5 µl of 10 mM dNTP added to eppendorf tubes and incubated at 42°C for 60 min. Following incubation at 70°C for 10 min, synthesized cDNAs were recovered and PCR was carried out with 2 µl of cDNA, 0.5 µl of 200 µM dNTP, 1 µl of 25 mM $MgCl_2$, 2.5 µl of 5X PCR buffer and 0.5 µl of 10 µM of each FMV specific primer set with 0.25 µl of 5 U/µl Taq DNA polymerase (Promega, USA). The mix was incubated at 94°C for 2 min and submitted to 35 amplification cycles (30 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C). Final PCR products were visualized under UV light after electrophoresis on ethidium bromide-stained 1% agarose gels.

RESULTS AND DISCUSSION

Symptomatological observations of inoculated plants. All fig seedlings and three *C. roseus* seedlings out of five exposed to eriophyide mites showed mosaic, vein clearing and small yellowish spots after 10 days and 6 weeks, respectively (Fig. 1B and C). When symptomatic periwinkle shoots were grafted onto healthy periwinkles by chip-budding, similar symptoms appeared within two months, demonstrating that the symptoms were due to virus infection rather than to damage from mite feeding. No symptoms were observed in other test or control plants for one year. Similar transmission experiments from fig to periwinkle using viruliferous *A. ficus* had been done by Credi (1998) who reported the appearance of virus-like symptoms on a periwinkle plant after an incubation period of 40 days. This plant showed chlorotic spotting, mosaic and yellowing of the leaves and malformation of the laminae.

It has already been reported that FMD is transmissible by *A. ficus* from infected to FMD-free fig seedlings (Proesler, 1969, 1972; Çaglayan *et al.,* 2009) so that our data confirm its transmission from fig to periwinkle plants.

EM observations of experimentally inoculated plants. DMBs were only observed in palisade cells of mite-inoculated fig seedlings and donor plant, confirming the results of a previous study (Caglayan *et al.*, 2010). Association of DMBs with FMD was originally observed by Bradfute *et al.* (1970) and later described in more detail (Martelli *et al.*, 1993; Serrano *et al.*, 2004). Due to their presence in field- and experimentally-infected fig trees, it has been assumed that DMBs are significantly involved in the aetiology of FMD. In this study DMBs were not observed in the cells of symptomatic periwinkle plants but bundles filamentous virus-like particles were seen the in nuclei of mesophyll cells (Fig. 3). These virus-like particles are still under investigation. Although DMBs were not observed in periwin-

kle cells, some of the plants exposed to *A. ficus* proved to be infected by FMV according to the outcome of RT-PCR runs. It may be possible that FMV particles are less concentrated in periwinkles than in fig, thus escaping EM visualization.

RT-PCR, dsRNA and nucleotide sequence analysis. Based on RT-PCR assays, the donor plant (cv. Bursa siyahı), all mite-inoculated fig seedlings and three *C. roseus* seedlings were shown to be infected by FMV. This virus was also detected in 8 and 7 individual mites collected from the donor plant and the inoculated fig seedlings, respectively. PCR amplification resulted in amplicons of the expected size (302 bp) (Fig. 4) whose sequence identity with the reference FMV isolate from GenBank (accession No. AM941711) was 87%, 89% and 87% for the donor plant (JQ708183), inoculated fig seedlings (JQ708184) and *C. roseus* (JQ408437, JQ408438), respectively. The sequences obtained from mites (JQ408432, JQ408436) also showed 87% and 88% identity at the nucleotide level with the sequence of the reference FMV isolate. Based on these results, Madagascar periwinkle (*C. roseus*) can be retained as a likely novel experimental host for FMV and *A. ficus* is confirmed as a vector capable of trasmitting FMV from fig to periwinkle as well as from fig to fig. This is the first report of detection of FMV in ErMs and periwinkle plants.

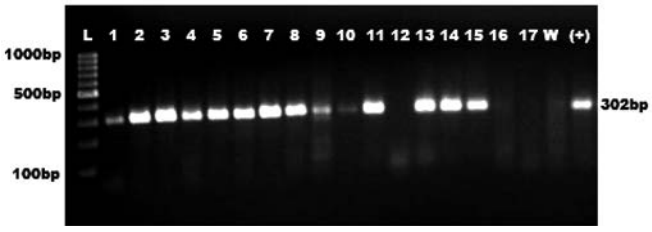

Fig. 4. RT-PCR amplifications from tested fig, periwinkles and eriophyid mites using FMV-RdRp- specific primers. Lane L, 100 bp ladder; lane 1, donor fig plant cv. Bursa siyahı; lanes 2-6, inoculated figs; lanes 7-9, inoculated periwinkles; lanes 10-12, eriophyid mites fed on and collected from donor plants; lane 13, inoculated periwinkle (dsRNA used as template)**;** lanes 14-17, eriophyid mites collected from inoculated figs; lane W**,** water control; lane **(+),** positive control.

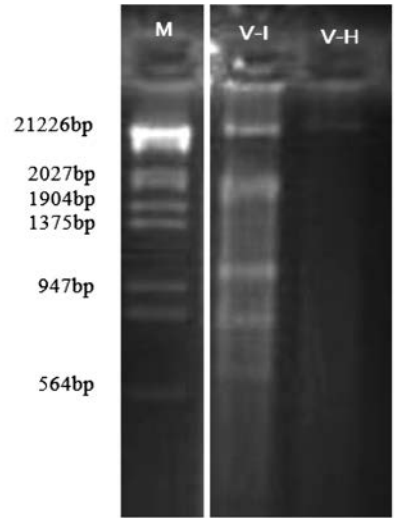

Fig. 5. Electrophoretic pattern of dsRNAs extracted from inoculated periwinkle (lane V-I) and healty periwinkle (lane V-H). M: DNA molecular marker (λ *EcoRI+HindIII*) DNA ladder.

Different dsRNA bands, ranging in size from 600 bp to *ca.* 2 kbp (*ca.* 0.6, 1.1, 1.4, 1.8 and 1.9 kbp.), were recovered from inoculated periwinkle plants (Fig. 5); they represent multipartite or multiple virus incidences. To confirm the presence of FMV, cDNAs were synthesized using dsRNA as template and RT-PCR was conducted using FMV-specific primers. PCR amplification resulted in amplicons of the size (302 bp) expected for FMV (Fig. 5) whose sequence (JX040436) showed 88% identity at the nucleotide level with both the reference FMV isolate and the FMV isolate from the donor cv. Bursa siyahı.

Pairwise comparison of partial nucleotide sequences from different FMV isolates demonstrated a high degree of similarity with the sequence of the FMV reference isolate (not shown). Furthermore, in a phylogenetic tree constructed with the RdRp domain sequence of FMV isolates from donor, five inoculated figs, two periwinkles and five ErMs (2 and 3 ErMs collected from donor and inoculated fig plants) all isolates grouped around the reference FMV isolate into two separate clusters in which sequences from fig, mites and periwin-

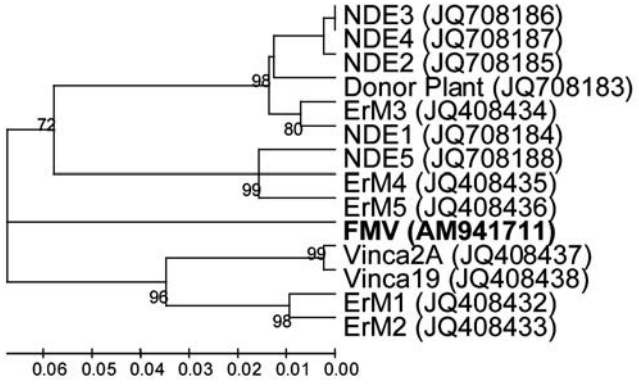

Fig. 6. Phylogenetic tree constructed with RdRp nucleotide sequences of 14 FMV isolates from fig, periwinkle and eriophyid mites. FMV reference isolate (AM941711); donor plant: cv. Bursa siyahı; vinca: periwinkle; NDE1-5: inoculated fig plants; ErM1-2: eriophyid mites collected from donor plant; ErM3-5: eriophyid mites collected from inoculated fig plants. Numbers at the nodes represent the percentages determined by bootstrap analysis with 1000 replicates. Only bootstrap values above 70% are shown. Accession numbers of the sequences used to generate the tree are given in parenthesis.

kle were intermingled without a clear-cut distinction (Fig. 6). This was taken as an indication that the FMV isolate present in the donor cv. Bursa siyahı was acquired from this plant and successfully transmitted to fig seedlings and periwinkles by *A. ficus.* These results are in complete agreement with those recently reported from Italy (Credi *et al.*, 2012).

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REFERENCES

Baker E.W., 1939. The fig mite, *Eriophyes ficus* Cotte and other mites of the fig tree (*Ficus carica* L.). *Bulletin of California Department of Agriculture* **28**: 266-75.

Bradfute O.E., Whitmoyer R.E., Nault R.L., 1970. Ultrastructure of plant leaf tissue infected with mite-borne viral-like particles. *Proceedings of the Electron Microscopy Society of America* **28**: 178-179.

Burnett H.C., 1962. Additional hosts of the fig mosaic virus. *Plant Disease Reporter* **46**: 693.

Çaglayan K., Medina V., Yigit A., Kaya K., Gazel M., Serçe Ç.U., Çalıskan O., 2009. Transmissions of Fig mosaic agents by the eriophyid mite, *Aceria ficus* Cotte (Acari: Eriophyidae). *Journal of Plant Pathology* **91**: 235.

Çaglayan K., Serçe Ç.U., Barutçu E., Kaya K., Medina V., Gazel M., Soylu S., Çalıskan O., 2010. Comparison by sequence-based and electron microscopic analyses of *Fig mosaic virus* isolates obtained from field and experimentally inoculated fig plants. *Plant Disease* **94**:1448-1452.

Condit I.J., Horne W.T., 1933. A mosaic of the fig in California. *Phytopathology* **23**: 887-896.

Credi R., 1998. Mite transmission of the fig mosaic disease agent to periwinkle. *Proceedings 7th International Congress of Plant Pathology*, *Edinburgh, UK* **1**: 13-14.

Credi R., Terlizzi F., Beber R., Poggi Pollini C., Ratti C., 2012. Transmission trials of the fig mosaic disease agent. *22nd International Conference on Virus and other Graft-trasmissibile Diseases of Fruit Crops, Rome, Italy* : 122.

Elbeaino T., Digiaro M., Alabdullah A., De Stradis, A., Minafra A., Mielke N., Castellano M.A., Martelli G.P., 2009. A multipartite single-stranded negative-sense RNA virus is the putative agent of fig mosaic disease. *Journal of General Virology* **90**: 1281-1288.

Elbeaino T., Digiaro M., De Stradis A., Martelli G.P., 2009. Complete nucleotide sequence of four RNA segments of fig mosaic virus. *Archives of Virology* **154**:1719-1727.

Flock R.A., Wallace J.M., 1955. Transmission of fig mosaic by the eriophyid mite *Aceria ficus*. *Phytopathology* **45**: 52-54.

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

Martelli G.P., 2011. Fig mosaic disease and associated pathogens. In: Hadidi A., Barba, M., Candresse, T,. Jelkmann W. (eds). Virus and Virus-like Diseases of Pome and Stone Fruits, pp. 281-287. APS Press, St. Paul, MN, USA

Medina V., Rodrigo G., Tian T., Juarez M., Dolia V.V., Achon M.A., Falk B.W., 2003. Comparative cytopathology of crinivirus infections in different plant hosts. *Annals of Applied Biology* **143**: 99-110.

Oldfield G.N., 1970. Eriophyid mite transmission of plant viruses. *Annual Review of Entomology* **15**: 343-380.

Proeseler G., 1969. Zur Übertragung des Feigenmosaikvirus durch die Gallmilbe *Aceria ficus* Cotte. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **123**: 288-292.

Proeseler G., 1972. Beziehungen zwischen Virus, Vektor und Wirstpflanze am Beispiel des Feigenmosaik-Virus und *Aceria ficus* Cotte (*Eriophyoidea). Acta Phytopathologica Academiae Scientiarum Hungaricae* **7:** 179-106.

Roland J.C., Vian B., 1991. General preparation and staining of thin sections. In: Hall J.L., Hawes C. (eds). Electron Microscopy of Plant Cells, pp.1-66. Academic Press. New York, NY, USA.

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

Vashisth K., Nagaich B.B., 1965. *Morus indica*, an additional host of fig mosaic. *Indian Phytopathology* **18**:315.

Walia J.J., Salem N.W., Falk B.W., 2009. Partial sequence and survey analysis identify a multipartite, negative-sense RNA virus associated with fig mosaic. *Plant Disease* **93**: 4-10.

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