TÜBİTAK

Research Article

Turk J Agric For
33 (2009) 469-476
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doi:10.3906/tar-0807-20

Putative agents of fig mosaic disease in Turkey

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Received: 17.07.2008

Abstract: Leaves from 58 fig tree accessions showing different severity of mosaic and necrotic symptoms were collected in 2 different regions of Turkey and analysed by transmission electron microscope (TEM) and RT-PCR to determine putative causal agents of fig mosaic disease (FMD). Ultrastructural studies showed that double membrane-bound bodies (DMBs) were mainly observed in the parenchyma and mesophyll cells of infected leaves of the cultivars Göklop and Morgüz and some seedlings showing only mosaic symptoms. These bodies were surrounded by a fibrillar matrix in most of the infected cells. Long, flexuous rod-shaped virus-like particles (LFPs) were also observed in vascular tissues of the Bursa Siyahı, Sarılop, Sarızeybek, and Yediveren cultivars, as well as seedlings that exhibited mosaic and necrotic symptoms together. Isometric virus-like particles (IVLPs) were mainly observed in mesophyll parenchyma cells of cv. Yeşilgüz, which showed very mild mosaic symptoms. Some phytoplasma-like bodies, as well as mosaic symptoms, were also observed in the phloem tissues of cv. Morgüz, Göklop, and Yediveren. These kinds of particles were not observed in the healthy plant leaves. RT-PCR analyses revealed that a PCR product of 3.0 kbp was obtained from virus-purified RNA of cvs. Bursa Siyahı, and Sarılop and an unknown cultivar (seedling) using degenerate fig-associated primers. When primers specific for FMD agent in Spain were used, a DNA fragment of 750 bp was amplified from cvs. Göklop and Yeşilgüz. However, no product was obtained by using DC-random primers.

Key words: Fig mosaic disease, electron microscope, PCR, Turkey

Türkiye'de incir mozaik hastalığının olası etmenleri

Özet: Türkiye'nin iki farklı bölgesinden toplanan ve farklı şiddetlerde mozaik ve nekrotik simptomlar gösteren 58 incir ağacından toplanan yapraklar, incir mozaik hastalığının olası etmenlerini saptamak amacıyla transmision elektron mikroskop çalışmaları ve RT-PCR yöntemi ile analiz edilmiştir. Ultrayapısal çalışmalar sonucu sadece mozaik simptomu gösteren Göklop, Morgüz çeşitleri ile bazı çöğürlerin infekteli yapraklarının parankima ve mezofil hücrelerinde çift membranla çevrelenmiş yapılar gözlenmiştir. İnfekteli hücrelerin çoğunda bu yapılar fibrilar matriks ile çevrelenmiştir. Bursa Siyahı, Sarılop, Sarızeybek, Yediveren çeşitleri ile bazı çöğürlerin iletim demetlerinde gözlenen uzun, kıvrımlı çubuk şeklindeki virus benzeri partiküller ise mozaik ve nekrotik simptomların birlikte görüldüğü durumlarda gözlenmiştir. Orta derecede mozaik simptomları gösteren Yeşilgüz çeşidinin mezofil parankima hücrelerinde ise izometrik şekilli virus benzeri yapılar saptanmıştır. Mozaik simptomu gösteren Morgüz, Göklop ve Yediveren gibi çeşitlerin floeminde bazı fitoplazma benzeri yapılar gözlenmiştir. Söz konusu bu yapıların sağlıklı bitki yapraklarında bulunmadıkları saptanmıştır. RT-PCR analizlerinde incir mozaik ile ilişkili dejenere primerler kullanıldığında infekteli örneklerden Bursa Siyahı, Sarılop ve çeşidi bilinmeyen çöğürlerden saflaştırılan RNA'larda 3,0 kb PCR ürünü saptanmıştır. İspanya'daki incir mozaik hastalığı etmenlerine karşı üretilen spesifik primerler kullanıldığında ise Göklop ve Yeşilgüz çeşitlerinde yaklaşık 750 bp büyüklüğünde bir DNA fragmenti saptanmış ancak DC-Random primerleri ile hiç bir ürün elde edilememiştir.

Anahtar sözcükler: İncir Mozaik Hastalığı, elektron mikroskobu, PCR, Türkiye

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Introduction

The first known reports of fig mosaic disease (FMD) were made by Condit in 1922 and Swingle in 1928 (cited in Alfieri, 1967), but the first critical study was conducted by Condit and Horne (1933). The disease has been widespread in several fig growing countries, including Turkey (Ülkümen et al., 1948; Özalp and Heper, 1972). Although it was not sap- or seed-transmissible (Martelli et al., 1993; Elbeaino et al., 2006), successful transmission of the disease by an eriophyid mite, *Aceria ficus* Cotte, has been reported by Flock and Wallace (1955). The aetiology of the disease is still uncertain. The disease was thought to be of viral origin until ultrastructural observations revealed the occurrence of intracytoplasmic enveloped spherical bodies in infected fig cells (Bradfute et al., 1970; Plavsic and Milicic, 1980; Appiano et al., 1990). In the following years, the agents of the disease were called "disease-associated bodies" (DABs), which differed in shape and size (Martelli et al., 1993). Putative potyviruses were reported from Croatia (Grbelja, 1983), and then the pathogen was assumed as a member of the *Potyviridae* family by Brunt et al. (1996). Some double membrane-bound bodies (DMBs) and rod-shaped virus particles (720 nm in size) showing a tail of 230, which might responsible for the disease, were described as possible agents of the disease by a Spanish scientist (Serrano et al., 2004). Double stranded RNAs (dsRNA) with a size ranging from 0.6 to 6.6 kb were obtained from infected trees in Turkey (Açıkgöz and Döken, 2003). Recently Elbeaino et al. (2006) showed that figs host a putative closterovirus species, for which the name of *Fig leaf mottle-associated virus* (FLMaV) is proposed. Then the same agent was reported from Tunisia (Nahdi et al., 2006). A second member of the *Closteroviridae* from the mosaic-diseased fig was identified and named *Fig leaf mottle-associated virus 2* (FLMaV-2). Although FMD spans a wide range of leaf symptoms, the agent-symptom relationship is still uncertain. In this study this relationship was investigated and the putative agents of fig mosaic disease in Turkey were reported.

Materials and methods

Plant samples

Fig plantations in Hatay and Bursa provinces, located in the East Mediterranean and Marmara regions of Turkey, respectively, were randomly surveyed during 2006 and 2007. A total of 58 infected leaf samples were collected from symptomatic trees of the most common local cultivars, such as Göklop, Sarılop, Bursa Siyahı, Yediveren, Morgüz, Yeşilgüz, Sarı zeybek, and some seedlings that grow naturally for TEM studies. For molecular studies, 19 different samples were collected and lyophilised (Table 1).

Transmission electron microscopy (TEM)

Samples for TEM were excised from infected fig leaves, fixed immediately in a solution of 3% glutaraldehyde in 50 mM phosphate buffer (pH 7.2), and kept overnight at 4 °C. The samples were then washed in the same buffer and post-fixed in 1% osmium tetroxide (OsO_4) in the same buffer for 2 h at room temperature. Following osmium tetroxide fixation, samples were dehydrated in a graded series of increasing acetone concentrations. Dehydrated

Table 1. Plant samples used for molecular testing.

Sample no.	Origin	Cultivar name
1	Antakya-Campus	Seedling
2	Bursa-1	Bursa Siyahı
3	Bursa-2	Bursa Siyahı
4	Bursa-3	Bursa Siyahı
5	Bursa-4	Bursa Siyahı
6	Bursa-5	Bursa Siyahı
7	Bursa-6	Bursa Siyahı
8	Bursa-7	Bursa Siyahı
9	Antakya-Merkez	Seedling
10	Antakya-Merkez	Seedling
11	Antakya-Merkez	Seedling
12	Antakya-Merkez	Seedling
13	Antakya-Dörtyol	Bursa Siyahı
14	Antakya-Dörtyol	Mor Güz
15	Antakya-Dörtyol	Sarılop
16	Antakya-Dörtyol	Göklop
17	Antakya-Dörtyol	Sarı Zeybek
18	Antakya-Dörtyol	Sarı İncir
19	Antakya-Dörtyol	Yeşil Güz

samples were subsequently embedded in an Epon araldite mixture, as described earlier (Medina et al., 2003; Soylu et al., 2005). Ultra-thin sections (70-90 nm) were cut with an Ultracut E microtome (Reichert, Milton Keynes, UK) using glass or diamond knives (Diatome, Bienne, Switzerland). The sections were then routinely mounted for staining on formvar-coated, 200 mesh copper grids (Aldrich, Dorset, UK). Grid-mounted sections with silver-gold interference colour were stained in drops of 4.5% uranyl acetate. After treatments, grids were washed in distilled water and further stained in drops of Reynold's lead citrate (Roland and Vian, 1991). All sections were viewed using Zeiss-910 TEM at an accelerating voltage of 75 kV.

Nucleic acid extraction and primer selection for RT-PCR

RNA isolation was performed from partially purified virus preparations, using sucrose gradients essentially as described by Wang et al. (1992) and Trizol LS reagent (Invitrogen Life Technologies). In order to amplify the viral genomic RNA, RT-PCRs were carried out in conjunction with 3 different sets of primers, 1 set amplified the 3' terminal of viral RNA of Potyviridae members (Chen et al., 2001), fig specific primers (Achon et al., 2003), and the others with random degenerate deca-primers (DC) (Comeau, et al., 2004). The PCR products were verified in 1% agarose gels as previously described (Achon et al., 2003).

Results

Field and TEM observations

The most common symptoms observed on leaves were distinctly yellow mosaic spots, contrasting with normal green colour of the foliage. The margins of the yellow spots blended gradually from a light yellow colour into the dark green of healthy tissue. Later in the season, rust-coloured band developed along the border of the mosaic spots, apparently caused by the death of epidermal or sub-epidermal cells in some cultivars. Deformed leaves sometimes occurred on the same twig with normal leaves. Mosaic and necrotic spots on the fruit were very similar to those observed on leaves, but less conspicuous (Figure 1).

When the relationships of symptom-TEM observations were studied, some correlations were found (Table 2). Double membrane-bound bodies (DMBs) surrounded by a fibrial matrix were extensively observed in leaves showing only mosaic spots on cvs. Göklop and Morgüz and seedlings. Long, flexuous, rod-shaped, virus-like particles (LFPs) were mostly found in necrotic tissues of Bursa Siyahı, Sarılop, Sarızeybek, and Yediveren cultivars, as well as seedlings (Figure 2).

In addition to DMBs and LFPs, isometric virus-like particles (IVLPs) were also found in mesophyl parenchyma cells of fig leaves of cv. Yesilgüz. Some phytoplasma- like bodies were also observed in phloem tissues of the Morgüz, Göklop, and Yediveren cultivars, which showed mosaic symptoms. None of these structures was found in the healthy control leaf materials (Figure 3).

RT-PCR analysis

We detected the expected 3.0 kbp amplicon in cvs. Bursa Siyahı (samples 3 and 6) and Sarılop (sample 15), and some seedlings (samples 10 and 11) using degenerate fig-associated primers and the amplicon of 750 bp in cvs. Göklop and Yeşilgüz, using fig specific primers (Figure 4). No specific PCR products were detected in any of the samples by using DC-random primers (Figure 5). Samples labelled 3, 6, and 15 were collected from commercially produced cultivars. Two other samples (labelled 10 and 11) were collected from seedlings of unknown cultivar(s).

Discussion

FMD is widespread in the Mediterranean basin, and different types of symptoms can be commonly seen in infested fig plantations. The cytopathological patterns observed in many studies revealed that disease was caused by double membrane-bound bodies (DMBs), polymorphic particles, or different types of viruses (Bradfute et al., 1970; Martelli et al., 1993; Serrano et al., 2004). The similar structures were also observed in this study. DMBs were surrounded by a fibrillar matrix in most of the infected cells, similar to some animal viruses (Appiano et al., 1990). These bodies were only observed in chlorotic leaves, but never in necrotic tissues. Long, flexuous, rod-shaped, virus-like particles (LFPs) were mainly seen in

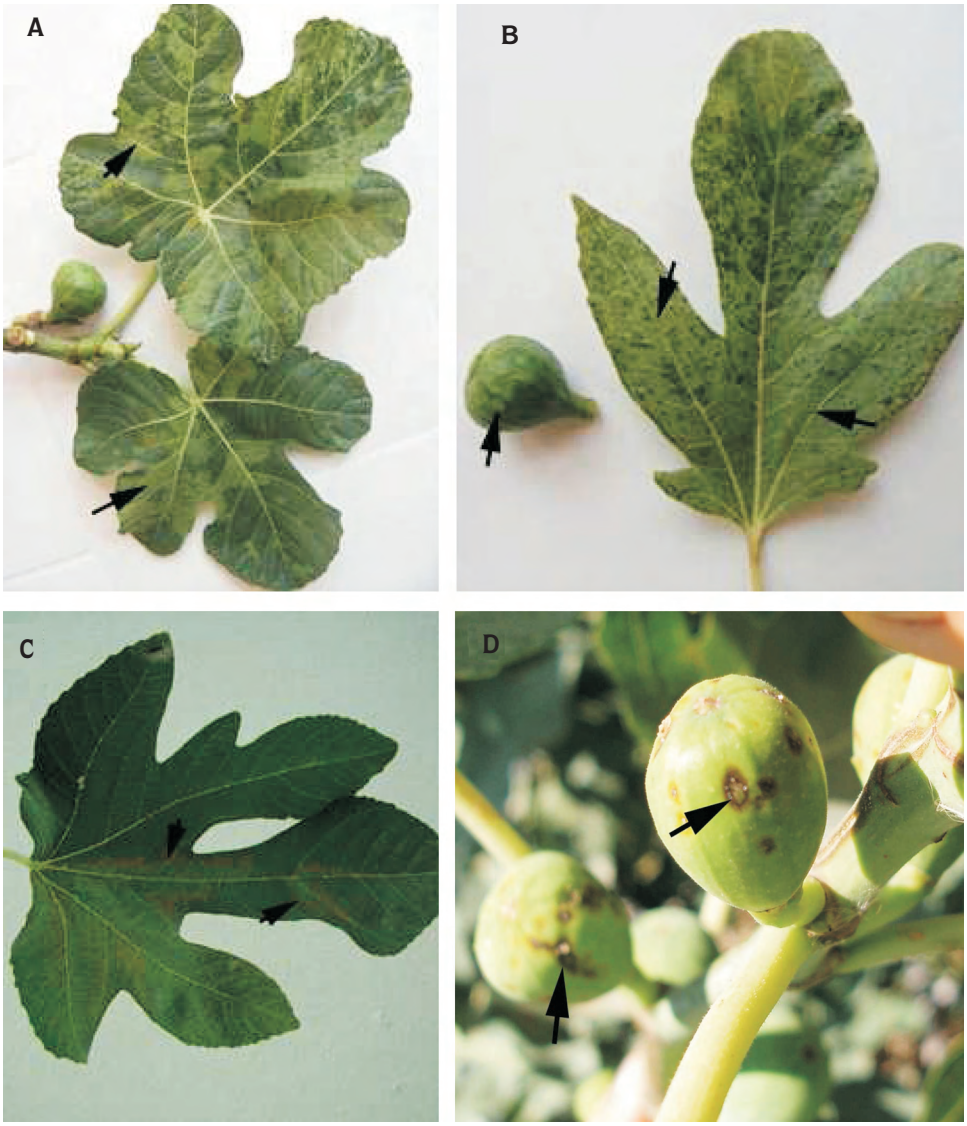

Figure 1. Typical mosaic (**A** and **B**) and necrotic spots (**C** and **D**) on fig leaves and fruits (arrows).

necrotic tissues, but it is still unclear whether or not those necrotic rusty spots were due to LFPs.

Our molecular analysis showed that at least 7 Turkish samples were infected with the same RNA viral agent associated with FMD from Spain. Recently, some of the members of *Closteroviridae* have been reported from the infected fig trees in Italy. They are called *Fig leaf mottle-associated virus* (FLMaV) and FLMaV-2 (Elbeaino et al., 2006, 2007). The latter was tentatively identified as a putative species of genus *Ampelovirus*. The long, flexuous, rod-shaped, virus-like particles in Turkish fig leaves should be investigated as to whether or not they belong to the family *Closteroviridae* . It was interesting to note that all kinds of putative agents—such as DMBs, LFPs, and agents similar to those observed in Spain—have been found in both commercially produced cultivars and also in seedlings which were found as growing on their own outside of the fig orchards. It was previously reported that FMD can be transmitted by vegetative

Table 2. Interaction between symptoms and electron microscope observation of putative fig mosaic agents in different fig cultivars.

Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Origin	Antakya-Merkez	Antakya-Merkez	Antakya-Merkez	Antakya-Merkez	Antakya-Dörtyol	Antakya-Dörtyol	Antakya-Dörtyol	Antakya-Dörtyol	Antakya-Dörtyol	Antakya-Dörtyol
Cultivar	Seedling-unknown	Seedling-unknown	Seedling-unknown	Seedling-unknown	Bursa siyahi	Morgüz	Sarılop	Göklop	Sarı Zeybek	Yediveren
Macroscopic symptoms										
	Mosaic	Mosaic	Mosaic Necrosis	Mosaic Necrosis	Mosaic Necrosis	Mosaic	Mosaic Necrosis	Mosaic	Mosaic Necrosis	Mosaic Necrosis
Microscopic symptoms										
Osmiophilic bodies in chloroplasts	++	++	+++	+++	++	++	+	+++	++	-
Starch accumulation in chloroplasts	++	+++	-	-	-	+	-	+	+	-
Hypertrophied mitochondria	+	-	±	-	-	-	-	-	-	-
Plasmalemosomes or paramural bodies	-	-	+	-	-	-	-	-	-	-
Necrotic or cells collapsed	-	-	+	+	-	-	-	-	+	-
Amorphous inclusions	-	-	-	-	-	+	-	-	-	-
Granular inclusions in vacuoles	-	-	-	-	-	+	-	+	-	-
Crystals Virus-like particles	-	-	-	-	-	-	-	-	-	-
DMBs(1)	++	+	-	-	-	+	-	+	-	-
Long flexuous particles (LFP)	-	-	+	+	+	-	+	-	+	+
Viroplasms(2) Other	+	-	-	-	-	-	-	-	-	-
Phytoplasms(3)	-	-	-	-	-	+	-	+	-	+
Fungi	-	-	-	-	-	-	+	-	-	-

(1) Double membrane bodies
(2) BYV-type vesicles or aggregates of vesicles
(3) Presence of phytoplasm/bacteria-like structures in vascular tissue

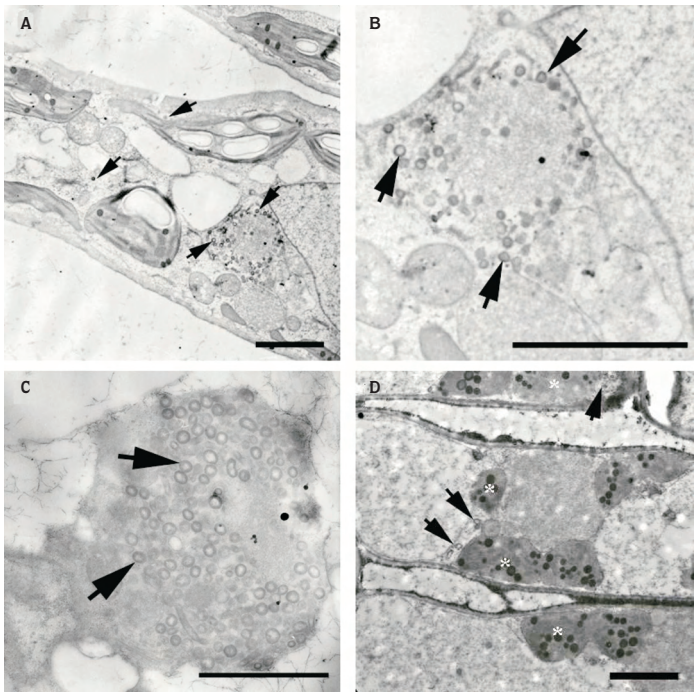

Figure 2. Typical double membrane bodies (DMBs, arrows) associated with fig mosaic disease in cv. Sarı Zeybek (**A, B,** and **C**) and cv. Sarılop **(D)**. In **A**, note that mass of fibrillar material is surrounded by DMBs. **B**, and **C** show appearances of DMBs (arrows) at higher magnifications. **D**, Shows DMBs (arrows) associated with chloroplast (asterisks) in palisade mesophyll cell. Bars, **A**, **B,** and **D** = 2.3 µm; **C** = 0.73 µm.

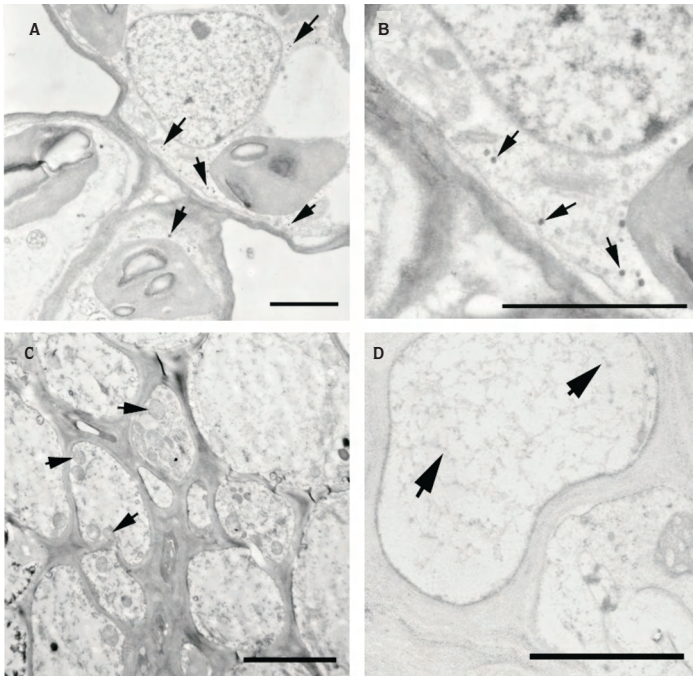

Figure 3. **A** and **B** show isometric virus-like particles (arrows) in mesophyl parenchyma cells of fig leaves of cv. Yeşilgüz. In **C,** Phytoplasms/bacteria-like structures in sieve elements (arrows) in cv. Yediveren. **D**, Shows long flexuous rod-shape virus-like particles (arrows) in vascular tissue in cv. Sarı Zeybek. Bars, **A**, **B,** and **D** = 1.2 µm; **C** = 3.6 µm.

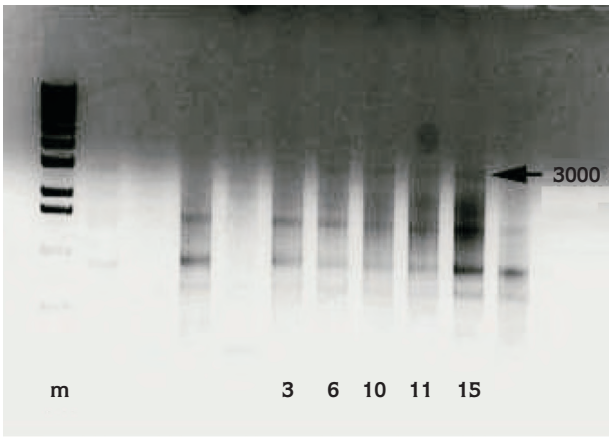

Figure 4. RT-PCR amplifications of a ca. 3000 bp DNA fragment from tissue extracts of different infected fig cultivars, using degenerate fig-associated primers. M: marker, 3 and 6: cv. Bursa siyahı, 10 and 11: seedlings-unknown cultivar, 15: cv. Sarılop.

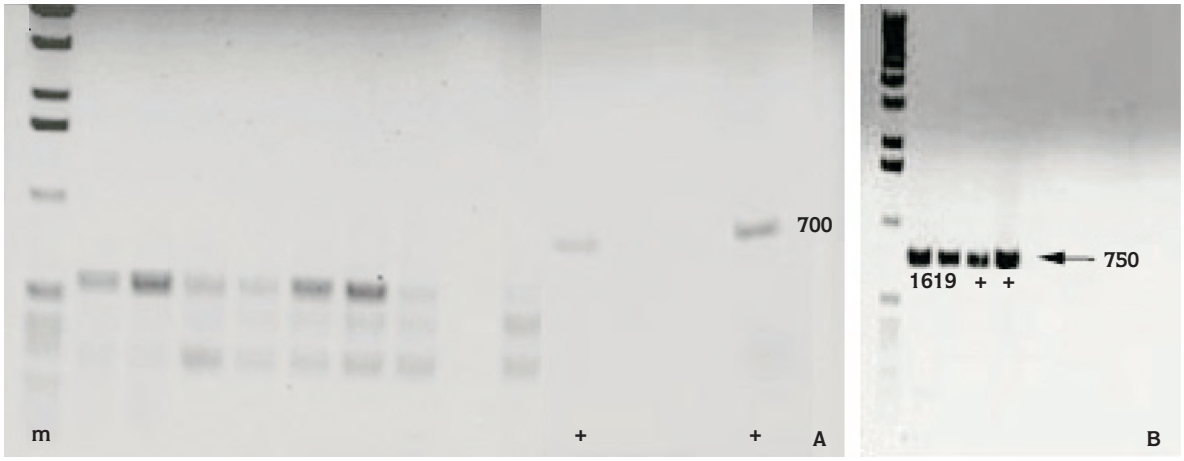

Figure 5. **A**, shows RT-PCR products using DC random primers amplifying 700 bp fragment of fig mosaic disease. Note that the expected cDNA bands were not detected in Turkish fig leaf samples. + indicates samples from infected Spanish fig leaves as positive controls. **B** shows RT-PCR products amplified with specific to fig mosaic disease (750 bp). Expected cDNA bands were detected in samples 16 and 19 from infected Turkish fig leaves.

propagation material and an eriophyd mite, *A. ficus* (Flock and Wallace, 1955), but not by seeds (Martelli et al., 1993). In this case, FMD must have been transmitted into these seedlings by eriophyd mites. In our ongoing studies, this mite transmits FMD agents into healthy fig seedlings (Çağlayan et al., unpublished data).

Despite the fact that some phytoplasma-like bodies were observed in the phloem tissues of field infected fig leaves, it is still uncertain whether they were an accidental companion or the primary agent of the disorder observed on infested fig leaves.

Acknowledgements

This study was supported financially by a grant (TOVAG 106O134) from The Scientific and Technical Research Council of Turkey.

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