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RESEARCH PAPERS

Four viruses infecting figs in Western Saudi Arabia

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Summary. Many diseases are compromising fig production in Saudi Arabia and in particular those caused by viruses. RT-PCR assays were conducted on 80 samples collected from four fig-growing provinces in the West Mecca region of Saudi Arabia, including the Fatima, Khulais, Rabigh and Alshifa valleys. Samples consisted of leaf tissues taken from caprifig and common fig trees. The presence of *Fig mosaic virus* (FMV), Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2) and Fig mild mottle-associated virus (FMMaV) was assessed from the samples. RT-PCR results showed that all four viruses were present in the surveyed areas with different proportions of infection. Incidence was 69% of samples, with a peak of 80%, from the Alshifa and Fatima valleys, 60% from Rabigh and 55% from Khulais valley. FLMaV-1 was the prevailing virus (55% of samples), followed by FMV (34%), whereas FLMaV-2 (11% of samples) and FMMaV (6%) were less common. Most of the mosaic symptoms observed in surveyed fig orchards occurred with the presence of FMV. However, many other symptoms remained unexplained because of the arduous task of determining the involvement of other fig-infecting viruses with mosaic disease. This is the first report of FMMaV and FLMaV-2 in Saudi Arabia, and of FMV and FLMaV-1 in western Saudi Arabia. The virus status of this crop is probably compromised and a sanitation programme is required to produce healthy plant material in Saudi Arabia.

Key words: *Ficus carica*, mosaic disease, virus detection, RT-PCR, electron microscopy.

Abbreviation. (FLMaV-1) Fig leaf mottle-associated virus 1; (FLMaV-2) Fig leaf mottle-associated virus 2; (FMMaV) Fig mild mottle-associated virus; (FMV) *Fig mosaic virus*.

Introduction

Common fig (*Ficus carica* L.), family Moraceae, is one of the longest cultivated fruit trees in the Mediterranean region (Storey, 1976), and is very important in Saudi Arabia (Alhudaib, 2012). Fig fruits are consumed fresh or dry, and are recognized for their mild laxative activity (Baraket *et al*., 2009).

Among the recorded diseases of fig crops, fig mosaic disease (FMD) is the most serious, and remains a critical constraint facing fig production and germplasm exchange. The first report on FMD was by Swingle in 1928 (Alfieri, 1967), but the first informative study was by Condit and Horne (1933). In general, mosaic-diseased trees show a wide range of symptoms, mainly on the leaves, in the form of mosaic-like discolorations, various patterns of chlorotic mottling and blotching, vein banding, vein clearing, chlorotic or necrotic ringspot and line patterns. Some plants may display apical rosetting of the leaves and reduced vigour. FMD is transmitted experimentally by grafting to fig and to other members of the family Moraceae, primarily in the genus *Ficus* (16 different species), plus *Cudrania tricuspidata* and *Morus indica* which are the only two known experimental hosts of different genera (Martelli, 2009).

Natural transmission of FMD-causing elements occurs by the eriophyid mite *Aceria ficus* (Flock and Wallace, 1955), and no seed transmission has been recorded (Martelli *et al.*, 1993; Açikgös and Döken, 2003). The aetiology of the disease has remained uncertain for a long time, even though filamen-

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tous and isometric virus particles were occasionally observed in ultra thin-sections from symptomatic fig leaves tissues from the United Kingdom, Italy, Spain, Croatia, Japan, Turkey and Portugal (Grbelja and Eric, 1983; Doi, 1989; Nolasco and Sequeira, 1991; Martelli *et al*., 1993; Açikgös and Döken, 2003; Serrano *et al*., 2004). A turning point regarding the aetiology of FMD was in 2009 with the discovery of *Fig mosaic virus* (FMV), recently classified as a member of the genus *Emaravirus*, which was found to be the causal agent of the disease (Elbeaino *et al*., 2009a; 2009b).

The first molecular information regarding natural virus infection of fig was for two members of the *Closteroviridae,* Fig leaf mottle-associated virus 1 (FLMaV-1) and Fig leaf mottle-associated virus 2 (FLMaV-2), which were detected in fig trees showing FMD symptoms in Italy and Algeria, respectively (Elbeaino *et al*., 2006; 2007). Later, the records of fig-infecting viruses rapidly increased and additional viruses joined the list, including Fig mild mottle-associated virus (FMMaV), Fig cryptic virus (FCV), Fig latent virus 1 (FLV-1) and Fig fleck-associated virus (FFkaV) (Elbeaino *et al*., 2010; 2011a; 2011b; Gattoni *et al*., 2009). Contemporarily, partial or complete nucleotide sequences of other viruses probably belonging to the *Partitiviridae* (Luteovirus-like) and *Caulimoviridae* (Badnavirus-like) families were also found in diseased fig plants (Walia *et al*., 2009; Tzanetakis and Martin, 2010).

In recent years, poor growth of fig trees, scant yields and low quality of fruit have been common complaints from growers in the Mecca regions of Saudi Arabia. A wide range of foliar symptoms resembling those of FMD were commonly observed in fig tree orchards. These have included diverse patterns of chlorosis, mosaic, mottling, vein banding and clearing, yellowing, chlorotic ringspot and blotching, leaf deformation and blistering, leaf curling and puckering.

This situation prompted an investigation to study the virus status of fig with a special regard to FLMaV-1, FLMaV-2, FMMaV and FMV, likely involved in the FMD-like symptoms observed in the fig growing areas of the Mecca region. Detection of these four viruses was carried out using RT-PCR, and electron microscopy observations were applied to FMV-infected plant tissues. This paper reports results from this study.

Materials and methods

Field survey and plant material

A survey was conducted during February 2015 and focused both on symptomless and FMD-symptomatic trees. Eighty leaf samples, were collected from naturally infected fig trees of two main fig types, caprifig and common fig, including the cultivars Black Mission, Brown Turkey and Brunswick. All the samples were collected from four fig growing provinces of the West Mecca region, including Fatima valley, Khulais valley, Rabigh valley and Alshifa valley.

Extraction of total nucleic acids

Total nucleic acids (TNAs) were extracted from tissues of leaf veins or cortical scrapings (100 mg), from asymptomatic fig plants and plants showing mosaic-like symptoms. Plant tissue samples were each macerated in 1 mL grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc and 2.5% w/v PVP-40), recovered with a silica-capture procedure (Foissac *et al*., 2001), and stored at –20°C until used.

Synthesis of cDNA

Ten microliters of each TNA extract (1 μg) were mixed with 1 μL (0.25 μg) of random hexamer primers, (Boehringer Mannheim, GbmH), denatured at 95°C for 5 min and quickly chilled on ice. Reverse-transcription was done for 1 h at 39°C by adding 4 μl M-MLV buffer 5× (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 2 μL of 10 mM DTT, 0.5 μL of 10 mM dNTPs, and 200 units of *Moloney Murine Leukaemia virus* (M-MLV) reverse transcriptase enzyme (Bethesda Research Laboratories) in a final volume of 20 μL.

RT-PCR, cloning and sequencing

The detection of FLMaV-1, FLMaV-2, FMMaV and FMV was conducted with RT-PCR using four sets of specific primers (Table 1), the nucleotide sequences and use-conditions of which were previously described (Elbeaino *et al*., 2006; 2007; 2009a; 2010). Briefly, 2.5 μL of reverse-transcribed TNA mixture

Table 1. RT-PCR specific primers of four fig-infecting viruses (FLMaV-1, FLMaV-2, FMMaV and FMV).

Virus	Sequence (5′ to 3′)		Amplicon (bp)	Reference
FLMaV-1	FLMaV1-s	CGTGGCTGATGCAAAGTTTA	352	Elbeaino *et al*., 2006
	FLMaV1-a	GTTAACGCATGCTTCCATGA		
FLMaV-2	FLMaV2-s	GAACAGTGCCTATCAGTTTGATTTG	360	Elbeaino *et al*., 2007
	FLMaV2-a	TCCCACCTCCTGCGAAGCTAGAGAA		
FMV	FMV-s	CGGTAGCAAATGGAATGAAA	302	Elbeaino *et al*., 2009
	FMV-a	AACACTGTTTTTGCGATTGG		
FMMaV	FMMaV-s	AAGGGGAATCTACAAGGGTCG	311	Elbeaino *et al*., 2010
	FMMaV-a	TATTACGCGCTTGAGGATTGC		

was used for amplification with the addition of 2.5 µL of 10× *Taq* polymerase buffer (Promega Corporation), with a final concentration of 1.5 mM $MgCl_2$ for 25 µL as total volume. The RT-PCR products were stained with gel star (Lonza) and analyzed on 1% agarose gels in 0.5× TBE buffer (Sambrook *et al*., 1989), then visualized by UV illumination using the Gel Documentation System (Gel Doc 2000, Bio-Rad). Fragments were sized using a 100 bp marker.

Three microliters of PCR products were ligated to pGEM-T Easy Vector (50 ng/µL) following the manufacturer's instructions (Promega), and then subcloned into *Escherichia coli* DH5α cells. Plasmids were extracted from bacterial cells by the boiling method and further purified using polyethylene glycol as described in Sambrook *et al.* (1989). Plasmids containing PCR amplicons of expected sizes (Table 1) were subjected to automated bidirectional sequencing using virus-specific sense and antisense primers (PRIMM). Nucleotide and protein sequence homologies were analyzed using the online BLASTn analysis software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Electron microscopy

For ultrathin sections, fragments from veins and mesophyll tissues of the discolored areas of infected young leaves were processed according to standard procedures (Martelli and Russo, 1984). Ultrathin sections were stained and observed using a JOEL-JEA100 CX electron microscope.

Results

Virus detection and sequence analyses

Symptoms on fig trees observed in the visited fields varied from leaf discoloration to yellowing, and when these symptoms were particularly obvious, various forms of leaf deformation and puckering were also observed. All of these symptoms were typical of diseases caused by viruses (Figure 1).

RT-PCR assays of samples yielded four DNA amplicons of sizes, 352 bp, 360 bp, 311 bp and 302 bp, typical, respectively, FLMaV-1, FLMaV-2, FMMaV and FMV (Figure 2). The viral nature of these amplicons was verified by sequencing four different RT-PCR positive samples randomly chosen for each virus (16 clones in total). BLASTn analyses showed that sequences of FMMaV were 100% identical to the Italian isolate "Cal1" (Genbank accession number FJ611959), whereas similarities of FLMaV-1 (88–99%), FMV (84-99%) and FLMaV-2 (90–99%) were diverse with different levels of variation. The greatest identity (99–100%) for all sequenced viruses was found with the Italian (Genbank accession numbers AM113547, AM941711) and Algerian (Genbank accession number AM286422) isolates. However, no sequence variation (intravariability) was found among the four sequenced isolates for each virus. The sequences of the Western Saudi Arabia isolates were deposited in the Genbank database under the following accession numbers: FLMaV-1, LN873219; FLMaV-2, LN873220; and FMV, LN873221.

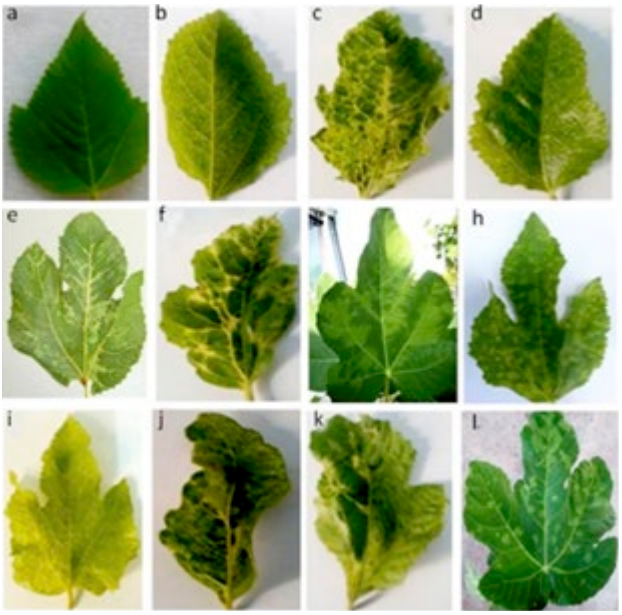

Figure 1. Mosaic-like symptoms on leaves of different diseased fig plants observed in orchards from Western Saudi Arabia, showing a range of foliar discoloration and malformation. (a) asymptomatic, (b) chlorosis, (c) mosaic, (d) mottling, (e) vein banding, (f) vein feathering, (g) vein clearing, (h) chlorotic blotching, (i) yellowing, (j) leaf deformation, (k) leaf blistering, (l) chlorotic ring spot.

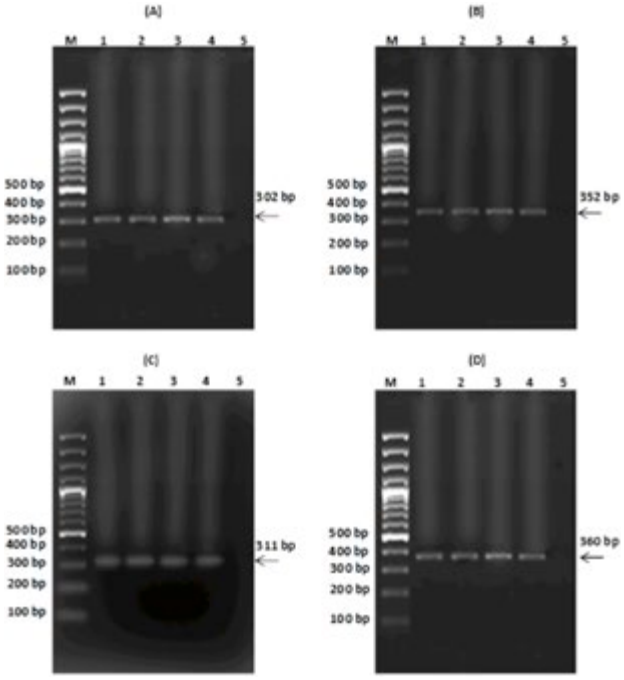

Figure 2. Agarose gel showing PCR amplifications from infected fig plants. (A) FMV, (B) FLMaV-1, (C) FMMaV and (D) FLMaV-2. M indicates the DNA ladder markers, lanes 1-4 are PCR-positive results from infected fig plants, lane 5 is the PCR-negative from asymptomatic fig samples.

From a total of 80 samples, 55 (69%) were infected by at least one virus. FLMaV-1 was the most common virus with an infection rate of 55% (Table 2) and the incidence was particularly high in common fig cultivars grown in Fatima valley (70%) and Alshifa valley (65%). FMV ranked second (34%) and was mostly similarly distributed in all the cultivars and regions (25–45%). FLMaV-2 and FMMaV, although to a lesser extent, were found in all four provinces with respective incidences of 15% and 10% in Khulais valley. In addition, the common fig cultivar Brunswick was the most infected variety, harbouring all viruses assessed, followed by the Black Mission and Brown Turkey (Table 2).

Electron microscopy

Electron micrographs of sectioned cells showed the presence of double-membraned bodies, considered to be FMV particles. These had dimensions of 50-80 nm (Figure 3). These structures were not found in samples that were PCR-negative for FMV. However, no other virus-like particles were observed in sectioned tissues.

Discussion

Our results are in agreement with previous reports from many Mediterranean countries (Elbeaino *et al*., 2006; 2007; 2009b; 2009c; Elbeshehy and Elbeaino, 2011; Alhudaib, 2012). Unlike closterovirus infections, which were frequently detected in symptomatic and asymptomatic fig trees, all FMV PCR-positive samples were correlated to mosaic symptoms in surveyed diseased fig plants. This confirms previous reports on the aetiology of double-membraned bodies in mosaic-affected fig plants (Plavsic and Milicic 1980; Martelli *et al*., 1993; Appiano *et al*., 1995; Castellano *et al*., 2007; Elbeaino *et al*., 2009a; 2009b; Elbeshehy and Elbeaino, 2011; Ishikawa *et al*., 2015). However, many symptoms found in FMV-free plants remained unexplained, because of the ardu-

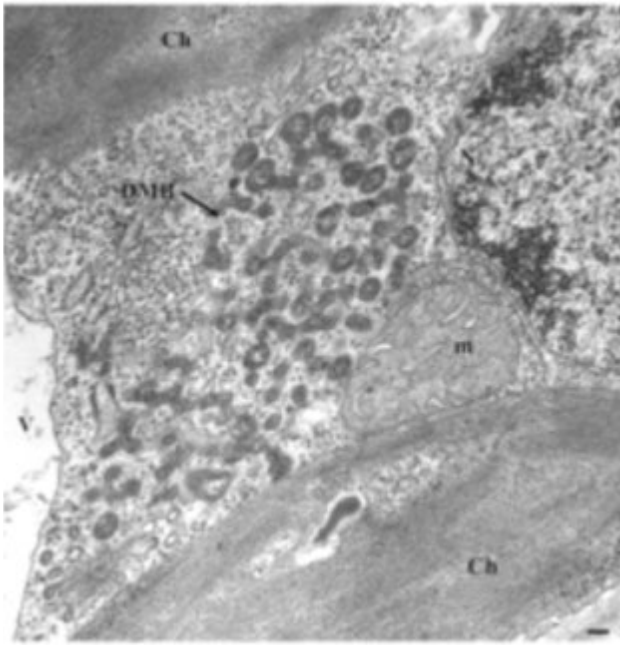

Figure 3. Groups of Double-Membraned Bodies in the cytoplasm of mesophyll cells from a naturally infected fig. Arrows point to the aggregates of convoluted electron-dense double membrane elements. Ch: chloroplast; m: mitochondrion; N: nucleus; V: vacuole. Bar = 100 nm.

ous task of determining the involvement of other fig-infecting viruses with the mosaic disease, and\or of the possible presence of other unknown agents that may have been naturally infecting fig plants.

The outcome of this preliminary study extends knowledge of the spread of fig viruses in Saudi Arabia, particularly in the Mecca region, for which no previous information was available. In particular, the virus sequences detected showed high similarity levels (99–100%) and phylogenetic relatedness with viruses from Italy and Algeria (Elbeaino *et al*., 2006; 2007; 2009c). This probably originates from exchange of infected plant material within the Mediterranean region. This is the first report on the presence of FMMaV and FLMaV-2 in Saudi Arabia, and of FMV and FLMaV-1 in western Saudi Arabia. Although this assessment was limited to 80 fig trees, the results obtained clearly indicate how the sanitary status of fig crops has deteriorated in these regions (69% of sampled plant had virus infections). Particularly worrying is the prevalence of FMV, since this virus has proved to be the unique agent closely correlated with the FMD (Elbeaino *et al*., 2009a; 2009b). The prevalence of FMV was not a surprise considering that it spreads through infected propagating material (cuttings and grafting), and natural vectors (eriophyid mites). The several FMV-infected samples found in association with most of the mosaic symptoms in fig orchards further confirms previous reports about FMV. The greatest level of infection in the assayed samples was attributed to FLMaV-1 and not to FMV. This result is not in agreement with other reports on the occurrence of fig virus infections where FMV has always been found to be the most widespread in mosaic diseased fig orchards (Castellano *et al*., 2007; Caglar *et al*., 2011; Shahmirzaie *et al*., 2012; El Air *et al*., 2015).

The incidence of FLMaV-1 was particularly high, when compared with that reported from fig orchards of many surrounding countries, including Iran (11%), Syria (4%), Tunisia (10%) and Lebanon (15%) (Elbeaino *et al*., 2012; Shahmirzaie *et al*., 2012; El Air *et al*., 2015). The prevalence of this virus prompts the necessity to monitor the presence of mealybugs, known as closterovirid vectors, that may be contributing to dissemination of FLMaV-1 in the orchards.

The knowledge on virus diseases of fig in the Mecca region of Saudi Arabia should prompt suitable sanitary selection, sanitation and certification programmes for the production of healthy fig propagating plant material in this country.

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Table 2. Incidences of FMV, FLMaV-1, FLMaV-2 and FMMaV infections from samples from four fig-growing provinces of Western Saudi Arabia, and different fig cultivars, as determined by RT-PCR assays.

Province			Tested trees No.	Infected trees		FMV		FLMaV-1		FLMaV-2		FMMaV	
				No.	%	No.	%	No.	%	No.	%	No.	%
Fatima valley	Capri		5	2	40	1	20	2	40	-	00	-	00
	Common	Black M.	2	1	50	1	50	1	50	-	00	-	00
		Brown T.	5	5	100	2	40	4	80	-	00	-	00
		Brunswick	8	8	100	4	50	7	87.5	2	35	1	12.5
		Total Common	15	14	93	7	46.67	12	80	2	13.33	1	6.67
	Total (Capri & Common)		20	16	80	8	40	14	70	2	10	1	5
Khulais valley	Capri		15	7	46.67	3	20	5	33.33	1	6.67	1	6.67
	Common	Black M.	1	-	00	-	00	-	00	-	00	-	00
		Brown T.	1	1	100	1	100	1	100	1	100	-	00
		Brunswick	3	3	100	1	33.33	3	100	1	33.33	1	33.33
		Total Common	5	4	80	2	40	4	80	2	40	1	20
	Total (Capri & Common)		20	11	55	5	25	9	45	3	15	2	10
Rabigh valley	Capri		18	10	55.56	4	22.22	7	38.89	1	5.56	-	00
	Common	Black M.	-	-	00	-	00	-	00	-	00	-	00
		Brown T.	1	1	100	-	00	-	00	-	00	1	100
		Brunswick	1	1	100	1	100	1	100	1	100	-	00
		Total Common	2	2	100	1	50	1	50	1	50	1	50
	Total (Capri & Common)		20	12	60	5	25	8	45	2	10	1	5
Alshifa valley	Capri		3	1	33.33	1	33.33	1	33.33	-	00	-	00
	Common	Black M.	5	3	60	1	20	1	20	-	00	-	00
		Brown T.	5	5	100	2	40	4	80	1	20	-	00
		Brunswick	7	7	100	5	71.43	7	100	1	14.29	1	14.29
		Total Common	17	15	88.24	8	47.06	12	70.59	2	11.77	1	5.88
	Total (Capri & Common)		20	16	80	9	45	13	65	2	10	1	5
Total	80			55	68.75	27	33.75	44	55	9	11.25	5	6.25
Mean infection rate	68.75					33.75		55.00		11.25		6.25	

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