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SHORT NOTE

Removal of viruses from Lebanese fig varieties using tissue culture and thermotherapy

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Summary. Two Lebanese fig accessions of local varieties (*Biadi* and *Aswad*), infected by Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2) and *Fig mosaic virus* (FMV), were subjected to tissue culture and thermotherapy for producing virus-free plant material. The virus status of all progeny explants was assayed by RT-PCR using viruses-specific primers. The shoot tip culture technique was reliable for elimination of from 60 to 100% of fig viruses. However, stem cutting culture coupled with thermotherapy was the most effective for shoot regeneration (40% of reactive explants), while elimination of the three viruses was possible even though with lower rates of removal (from zero to 81%) were achieved. This study has indicated that FLMaV-2 is more susceptible to thermotherapy than FLMaV-1 and FMV.

Key words: *Ficus carica*, shoot tip culture, thermotherapy, RT-PCR.

Introduction

In recent years the number of characterized fig viruses has seen a sharp increase mainly due to the advent of molecular virus detection techniques. The current list of fig-infecting viruses comprises eight entities that belong to different taxonomic positions. These are: *Closteroviridae*; Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), and Fig mild mottle-associated virus (FMMaV); *Emaravirus*; *Fig mosaic virus* (FMV): *Partitiviridae*; *Fig cryptic virus* (FCV): *Tymoviridae*; Fig fleck-associated virus (FFkaV); *Flexiviridae*; Fig latent virus 1 (FLV-1); and *Caulimoviridae*; *Fig badnavirus 1* (FBV-1) (Elbeaino *et al*., 2006, 2007, 2009a, 2009b, 2010, 2011; Gattoni *et al.*, 2009; Laney *et al*., 2012). All of these viruses have been detected in mosaic-affected fig trees, and have been partially or completely characterized. Of all the recorded diseases associated with fig crops, fig mosaic disease (FMD) is the most serious virus pathogen, and remains a critical pathological constraint facing fig production and germplasm exchange.

The etiology of FMD has been hindered for decades (Swingle, 1928; Condit and Horne, 1933), until the discovery of FMV that was verified as the cause of the disease (Elbeaino *et al.*, 2009a, 2011). In general, mosaic-diseased fig trees show a wide range of symptoms, mainly on leaves, as discolorations, yellowing, various patterns of chlorotic mottling and blotching, vein banding, vein clearing, chlorotic or necrotic ringspot and line patterns, variegation and mosaic (Condit and Horne, 1933; Flock and Wallace, 1955; Bradfute *et al.*, 1970; Plavšic and Milicic, 1980; Appiano *et al.*, 1990; Martelli *et al.*, 1993; Elbeaino *et al.*, 2009a). However, the virus infections associated with FMD are varied, and several viruses have been identified from figs with different mosaic-like symptoms. Nevertheless, the roles of particular viruses as

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causes of FMD have not been established, except for FMV (Elbeaino *et al.*, 2009a). It cannot be excluded that FMD is a complex disease with which several fig-infecting viruses could be involved (Martelli, 2011).

In Lebanon, fig is grown on a limited area 1,713 ha (Anonymous, 2010), and is considered as one of the oldest fruit crops in the country. The wide range of symptoms, resembling those typical of FMD, observed on different varieties, incited a screening for three viruses, i.e. FLMaV-1, FLMaV-2 and FMV that were all found to be present in figs throughout the country (Elbeaino *et al.*, 2012). Although the economic incidence of FMD on the Lebanese fig culture has not been determined, there is strong demand from nurseries and national phytosanitary institutions for virus-free material for the new plantations. Tissue culture techniques are likely to provide methods for production of virus-free fig planting material. Shoot tip culture, combined or not with thermotherapy, has been widely used for the elimination of viruses from plants (Stretch and Scott, 1977; Faccioli, 2001; Chalak *et al*., 2013). Recently, these techniques have also been assessed on figs to remove FLV-1 and FMV from fig material infected with these viruses (Sahraroo *et al.*, 2009, Chiumenti *et al*., 2013).

This paper reports the efficiency of using tissue culture and thermotherapy for eliminating FLMaV-1, FLMaV-2 and FMV from two Lebanese fig varieties.

Materials and methods

Two field-grown fig trees of local varieties designated as *Biadi* and *Aswad*, and found to be infected with FLMaV-1, FLMaV-2 and FMV (Elbeaino *et al*., 2012), were used as virus-infected source material. During spring 2012, young shoots were picked from the trees and surface sterilized with a 0.1% solution of $HgCl_2$ for 7 min, soaked three times in sterile distilled water, and dried on a filter paper. Single-node stem cuttings (1 cm in length) and shoot tips (0.4–0.5 mm in diameter) were excised in a laminar flow cabinet and used as explants. The growth medium adopted was MS (Murashige and Skoog, 1962) supplemented with 5 mg L^{-1} of 6-benzylaminopurine, 1 mg L^{-1} 3-indolylbutyric acid and 1 mg L^{-1} gibberellic acid, solidified with 0.8% Bacto Difco agar, and autoclaved at 118°C for 20 min. All cultures were placed in a growth cabinet with a photoperiod of 16 h of artificial light and 8 h of darkness at 25 ± 2°C, except for cultures exposed to thermotherapy.

Three sanitation trials were conducted. These were: (i) direct culture of stem cuttings (6 mm) without thermotherapy, (ii) stem cutting culture combined to thermotherapy (35 ± 1°C, for 30 d), and (iii) shoot tip (0.6 mm) culture without thermotherapy. The new shoots regenerated from the explants of the different trials were sub-cultured for 2 months under the culture conditions described above.

The virus status of all regenerated shoots was tested using RT-PCR to evaluate for the presence of FLMaV-1, FLMaV-2 and FMV. Total nucleic acids in each sample were extracted from 100 mg tissue from leaf veins, 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 pH 5 and 2.5% w/v PVP-40), captured with silica particles, according to the methods of Foissac *et al.* (2001).

Eight to 10 μL of total nucleic acid extract were mixed with 0.5 μL of random hexamer primers (Boehringer) (0.5 μg μL^{-1}), denatured at 94°C for 5 min and quickly chilled in ice. Reverse transcription reaction was done for 1 h at 39°C by adding 4 μL RT 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.

PCR amplifications were performed by using three couple of virus-specific primers designed on the HSP70 gene sequences of FLMaV-1 (N17s: 5′-CGTGGCTGATGCAAAGTTTA-3′; N17a: 5′-GTTAACGCATGCTTCCATGA-3′), FLMaV-2 (F3s: 5′-GAACAGTGCCTATCAGTTTGATTTG-3′; F3a: 5′-TCCCACCTCCTGCGAAGCTAGAGAA-3′) and on the RNA-dependent RNA polymerase gene of FMV (E5s: 5′-CGGTAGCAAATGGAATGAAA-3′; E5a: 5′-AACACTGTTTTTGCGATTGG-3′), which amplified DNA fragments of 352 bp, 360 bp and 300 bp, respectively (Elbeaino *et al*., 2006; 2007; 2009a). Reverse-transcribed mixture (2.5 μL) was submitted to PCR amplification in 2.5 μL of 10× Taq polymerase buffer (Promega), with a final concentration of 1.5 mM $MgCl_2$ for a total volume of 25 μL. PCR products were analyzed by agarose gel electrophoresis (1.2%) in 1× TBE buffer (Sambrook *et al*., 1989), stained with "GelRed Dye" (Biotium) and visualized under a UV transilluminator.

Results and discussion

A large number of explants from both fig varieties (*Biadi* and *Aswad*) did not survive because of an early oxidation problem. However, all of the surviving explants produced regenerated shoots. Table 1 indicates the numbers of shoots regenerated from the explants in different trials, as well as the proportions of shoots free of viruses as determined by RT-PCR. Regeneration ability was twice as great for stem cutting explants (39–42%) than for shoot tip explants (16–17%) (Figure 1), with poor survival mostly resulting from the oxidation problem. There was no significant difference in survival between the two varieties. The thermotherapy did not affect the stem cuttings responses.

The RT-PCR test based on the sequencing of gene HSP70 clearly showed the expected DNA fragments of 352 pb, 360 bp and 300 bp, respectively, for the positive control samples of FLMaV-1, FLMaV-2 and FMV (Figure 2). This test indicated different virus elimination rates ranging from zero to 100% according to the virus, the treatment and the fig variety (Table 1, Figure 2). For *Biadi* infected with FLMaV-1, elimination rates were 36% via stem cutting culture submitted to thermotherapy and 66% via shoot tip culture. When infected with FLMaV-2, *Biadi* elimination rates were 81% via stem cutting culture submitted to thermotherapy and reached 100% after shoot tip culture. For *Aswad* infected with FLMaV-1, elimination rates did not exceed 60% even after shoot tip culture, and were 70 to 73% for this variety infected with FLMaV-2. For FMV, elimination rates from shoot tip cultures were 80% from *Aswad* and 86% from *Biadi* (Figure 1b). Only 60% of *Aswad* shoots deriving from stem cutting culture with thermotherapy, and 72% of *Biadi* shoots, were free of FMV. The use of shoot tip culture without thermotherapy has always been adopted (Stretch and Scott, 1977; Faccioli, 2001; Chalak *et al*., 2013), although with a limited extent, depending on the virus (and phytoplasma) type to be eliminated and on the availability of plant material. When combined to thermotherapy this method can oxidize and/or compromise the regeneration of explants. However, in this study the virus elimination rates obtained using this method was in harmony with those reported in the literature for other plant pathogens (Faccioli, 2001; Chalak *et al*., 2004, 2013; Špak *et al*., 2014).

Most of the sprouts first produced from infected buds grew more slowly than those from virus-free buds. No symptoms of mottled and deformed leaves were noticed. At the end of the second subculture, proliferation ability was estimated to be an average of two new sprouts per bud.

These results indicate the efficiency of stem cutting culture coupled with thermotherapy, along with shoot tip culture, as an efficient method for eliminating FLMaV-1, FLMaV-2 and FMV from fig, with virus elimination rates reaching 100%. Additionally, FLMaV-2 was more susceptible to thermotherapy and easier to eradicate than FLMaV-1 and FMV. Our results are largely in agreement with those obtained previously with *in vitro* heat therapy alone or combined with tissue culture, where FLMaV-1, FLMaV-2 and FMV were eliminated after 70 d at 38°C (Saharoo *et al*., 2009; Chiumenti *et al*., 2013). It remains to be confirmed whether the PCR-negative shoots are

Table 1. Numbers of regenerated shoots from two Lebanese fig varieties, and numbers of virus-free explants, for explants produced after application of three different tissue culturing protocols.

Technique	Variety	Regenerated shoots / total explants		FLMaV-1 free / tested shoots		FLMaV-2 free / tested shoots		FMV free / tested shoots	
		Nb	%	Nb	%	Nb	%	Nb	%
Infected stem cutting culture (positive control)	Biadi	83/207	40	0/10	0	0/10	0	0/10	0
	Aswad	44/105	41	0/10	0	0/10	0	0/10	0
Stem cutting culture with thermotherapy	Biadi	52/133	39	4/11	36	9/11	81	8/11	72
	Aswad	34/80	42	0/10	0	7/10	70	6/10	60
Shoot tip culture	Biadi	75/450	16	10/15	66	15/15	100	13/15	86
	Aswad	51/300	17	9/15	60	11/15	73	12/15	80

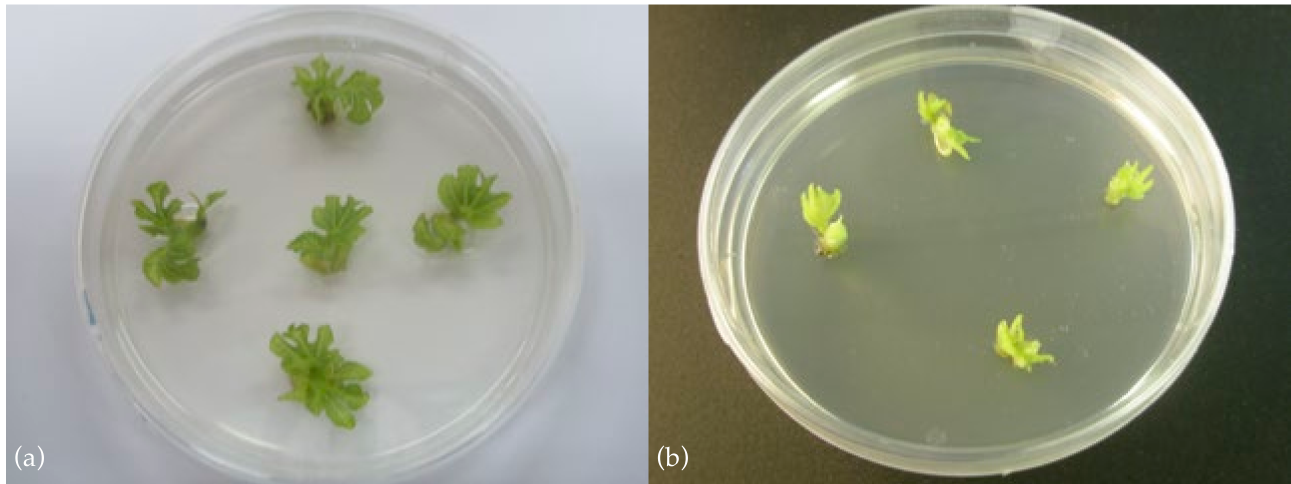

Figure 1. (a) Fig variety *Aswad* regenerated plants from stem cutting culture with thermotherapy. (b) Fig variety *Biadi* regenerated plants from shoot tip culture.

Figure 2. Electropherogram of RT-PCR reactions conducted on several progeny explants generated from tissue culture and thermotherapy techniques. (a): Agarose gel showing healthy (lanes 4, 6, 8) and FLMaV-1-infected explants (lanes 2, 3, 5, 7). (b) Agarose gel showing healthy (lane 7) and FLMaV-2-infected samples (lanes 2-6, 8). (c) Agarose gel showing healthy (lanes 5, 7) and FMV-infected samples (lanes 2-4, 6, 8). Lanes 9 and 10 represent RT-PCR positive and negative controls, respectively, for each virus.

indeed free of all fig mosaic complex viruses and will remain symptomless after hardening. The continued health status of treated plants requires confirmation using efficient virus detection techniques.

This study has demonstrated that stem cutting culture coupled with thermotherapy is an effective method for shoot regeneration with an average of 40% of reactive explants that survived the oxidation phenomenon. This method could become a routine technique for producing FMD-free Lebanese fig varieties, and will also assist with the preservation of fig genetic diversity.

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