SHORT COMMUNICATION

SANITATION TRIALS FOR THE PRODUCTION OF VIRUS-FREE FIG STOCKS

M. Chiumenti[1], A. Campanale[2], G. Bottalico[1], A. Minafra[2], A. De Stradis[2], V. Savino[1] and G.P. Martelli[1,2]

[1]*Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi "Aldo Moro", Via Amendola 165/A, 70126 Bari, Italy*
[2]*Istituto di Virologia Vegetale del CNR, UOS Bari, Via Amendola 165/A, 70126 Bari, Italy*

SUMMARY

Heat therapy, meristem tip culture *in vitro* and a combination of both techniques were used for obtaining fig (*Ficus carica*) plants free from some viruses associated with fig mosaic, a disease with a worldwide distribution. Source plants were two field-grown adult fig accessions from a germplasm repository of the University of Bari (Italy) that showed severe and mild symptoms of mosaic, respectively, and two symptomless fig seedlings grown under screen. Adult plants were infected by *Fig mosaic virus* (FMV), Fig latent virus 1 (FLV-1) and Fig badnavirus 1 (FBV-1). Seedlings were infected by FLV-1 and FBV-1. Progeny of explants subjected to meristem tip culture were still infected by FMV (93.8% elimination). This virus, however, was eradicated (100% sanitation) by shoot tip culture combined with heat therapy, or *in vitro* heat therapy. High sanitation rates from FLV-1 (81 to 100%) were also registered with all sanitation procedures employed, such as *in vitro* heat therapy alone (two cycles) or combined with tissue culture. By contrast, the DNA virus FBV-1 resisted all attempts of elimination, a behaviour that confirms indirectly its hypothesized integration in the fig genome.

Key words: fig mosaic disease, heat therapy, tissue culture, sanitation, molecular detection, diagnosis.

Fig (*Ficus carica*), a fruit tree widely grown in the Mediterranean basin, is affected to a very large extent by a disease known as "mosaic" (FMD). This disease, first reported from California (Condit and Horne, 1933), is now kown to have a worldwide distribution. FMD is a complex disorder (reviewed by Martelli, 2011) with which eight viruses of different taxonomic position are associated (Table 1). *Fig mosaic virus* (FMV) is the agent that occurs in symptomatic plants more often than any of the other fig-infecting RNA viruses, and is the major incitant of mosaic. The role in symptom induction of other viruses, such as the putative closterovirids Fig leaf mottle-associated virus 1 (FLMaV-1), Fig leaf mottle-associated virus 2 (FLMaV-2), Fig mild mottle-associated virus (FMMaV) (Elbeaino *et al*., 2006, 2007, 2010), and the putative marafivirus Fig fleck-associated virus (FFkaV) (Elbeaino *et al.,* 2011a), has not been ascertained. None of the aforementioned viruses is transmitted through seeds, contrarily to Fig latent virus 1 (FLV-1) (Gattoni *et al.,* 2009) and Fig badnavirus 1 (FBV-1), the only DNA virus identified in fig so far (Laney *et al*., 2012; Minafra *et al*., 2012), both of which are vertically transmitted to seedlings, in which they do not induce symptoms.

Although no estimates of the economic impact of FMD are available, the notion that severely affected trees are less productive than those with milder symptoms and suffer premature fruit abscission has been taken as an indication that FMD can have a detrimental effect on the crop. Thus, attempts to produce FMD-free trees were initiated in the mid 1960s, using heat therapy or *in vitro* meristem tip culture (Martelli, 2011 and references therein). The results of these trials were encouraging in that sanitized plantlets did not show symptoms. In no case, however, the type and number of viruses present in the plants submitted to sanitation had been determined prior to and after

Table 1. Molecularly characterized viruses found in mosaic-affected fig trees.

Virus species	Genus	Reference
Fig mosaic virus (FMV)	*Emaravirus*	Elbeaino *et al*., 2009
Fig leaf mottle associated virus 1 (FLMaV-1)[a]	*Closterovirus*	Elbeaino *et al.,* 2006
Fig leaf mottle associated virus 2 (FLMaV-2)	*Ampelovirus*	Elbeaino *et al.,* 2007
Fig mild mottle-associated virus (FMMaV)	*Closterovirus*	Elbeaino *et al.,* 2010
Fig fleck-associated virus (FFkaV)	*Maculavirus*	Elbeaino *et al.,* 2011a
Fig latent virus 1 (FLV-1)	*Trichovirus*	Gattoni *et al.,* 2009
Fig cryptic virus (FCrV)	*Alphacryptovirus*	Elbeaino *et al.,* 2011b
Fig badnavirus 1 (FBV-1)	*Badnavirus*	Laney *et al*., 2012

[a]Viruses with non italicized names are still tentative species in the genus

Corresponding author: A. Minafra
Fax: +39.080.5442911
E-mail: a.minafra@ba.ivv.cnr.it

the treatment. Thus, as reported in the present paper, trials were carried out on a limited number of fig accession for devising efficient protocols for the elimination of some molecularly identifiable fig-infecting viruses, based on heat therapy and/or *in vitro* meristem tip culture.

To this aim, two adult fig accessions from the germplasm plot of the University of Bari showing severe (*f9-p5*) and mild (*Mo-1*) mosaic symptoms, respectively, and two symptomless 2-year-old screenhouse-grown seedlings from field accessions *f5p5* and *p8* were chosen for sanitation trials in spring 2010 and tested by RT-PCR (primers in Table 2) for the presence of FMV, FLV-1, FLMaV-1, FLMaV-2, the most frequently encountered viruses in the area. Results disclosed that all these plants hosted FLV-1, FMV was present only in the two symptomatic adult trees (*f9-p5* and *Mo-1*), whereas FLMaV-1 and FLMaV-2 were absent (Table 3). When in 2012 Fig badnavirus 1 (FBV-1) was discovered (Laney *et al*., 2012), the four source plants were tested and found positive also for the presence of this virus.

Apical or axillary buds excised from 1-year-old cuttings of all accessions were surface-sterilized by exposure to 70% ethanol for 30 sec followed by 0.05% mercuric chloride for 10 min and rinsing with sterile distilled water. After removal of the scales, the buds were plated in medium 1A [Quorin and Lepoivre (1980), modified] and kept in a growth chamber at 24°C with a 16 h light/8 h dark photoperiod and a light intensity of 67.5 microeinsteins per square meter per second to stimulate sprouting. Small shoots were excised, subcultured at least twice at 3-week intervals in fresh medium 1A, and used for explant recovery.

Sanitation treatments consisted of: (i) *in vitro* shoot tip culture. Explants (0.3-0.5 mm) excised under a laminar-flow hood were placed in glass vessels containing medium 1A and transferred after about one month, first to fresh 1A then, after 30 additional days, to medium DKW (Driver and Kuniyuki, 1980); (ii) *in vitro* heat therapy. *In vitro*-grown sprouts *ca.* 2 cm in size were maintained for 35 days at 38°C under the aforementioned lighting conditions. Then, two types of explants were excised: shoot tips 5-10 mm long and meristem tips 0.3-0.5 mm in size. Longer explants (5-10 mm) were multiplied in DKW medium, then individually transferred to medium 1A deprived of hormones for rooting. The smaller explants (0.3-0.5 mm) were first plated in hormone-containing medium 1A for elongation, then moved to the hormone-free medium 1A for rooting; (iii) *in vitro*-grown sprouts from which meristem tips had been excised were exposed to a second 35-day cycle of heat treatment at 38°C, after which their tips (5-10 mm) were cut and allowed to grow and proliferate in standard medium 1A. Shoots 2-3 cm in size were then individually transferred to hormone-free 1A for rooting. All rooted plantlets were transferred to Jiffy pots and covered with a plastic film for 4 weeks, then transplanted in plastic pots in a insect-proof glasshouse for acclimatization.

For molecular tests, total nucleic acids (TNA) were extracted from 150 mg of leaf tissue (Foissac *et al*., 2001) and stored at -20°C until use. For RNA viruses (FMV and FLV-1) 0.5 µg TNA were denatured by boiling at 95°C for 5 min, then reverse-transcribed with random primers and M-MLV reverse transcriptase (Life Technologies, USA) for 1 h at 42°C (Minafra and Hadidi, 1994). As mentioned, virus-specific primer pairs were used for PCR analyses (Table 2).

Standard RT-PCR (Minafra and Hadidi, 1994) was used for FMV amplification and the products visualized by electrophoresis in 1.2% agarose gel stained with Gel Red (Biotium, USA). Real time RT-PCR was used for FLV-1, utilizing the Brilliant II SYBR Green QPCR kit (Agilent, USA) and a primer concentration of 200 nM. The reaction was run for 10 min at 95°C, followed by 40 cycles of 20 sec at 95°C, 40 sec at 56°C in a CFX96 thermal cycler (BioRad, USA). PCR amplification for FBV-1 was done directly on total nucleic acid extracts without reverse transcription. Efficiency and sensitivity of real-time PCR assays were determined using plasmid DNA containing the cloned target sequence (replicase domain) which, from a starting amount of 1 µg was tenfold diluted in cDNA synthesized from 250 ng TNA from FLV-1-free seedlings. All standard curves were generated using two replicates of each dilution in two independent assays. Melting analysis was done with CFX Manager software (BioRad, USA) increasing the temperature from 65 to 95°C with 0.5°C increment every 5 sec and plate reading at every step. The 125 bp-long amplicon showed a specific melting peak temperature at 79±0.5°C. The correct size of the amplicons was verified by electrophoresis and some PCR products were sequenced to prove their specificity. Furthermore, using a quantity of 1.2 ng/µl cDNA from infected plants per reaction, variations in *Ct* values from 22.41 to 31.76 were observed. A similar range was found (21 to 32 *Ct*) when 1 ng to 10 pg of plasmid DNA were mixed to healthy cDNA before PCR reaction.

All fig accessions were readily propagated *in vitro* although most of the sprouts first pushed from buds explanted from mosaic-diseased plants had mottled and deformed leaves and grew more slowly than the symptomless explants from seedlings. As to the media used for *in vitro* culture, 1A proved most suitable for the early culturing phase (meristem tips reached 5 mm within 40-60 days) whereas DKW was most effective in the proliferation phase (up to 4-5 axillary shoots were produced in each subculture every three weeks).

The *in vitro* behaviour of accessions during sanitation treatments can be summarized as follows: (i) *in vitro* shoot tip culture. Smaller explants (*ca.* 0.3 mm) often necrotized. However, surviving small explants and the larger ones (up to 0.5 mm) developed sprouts three to 5 weeks after plating; (ii) *in vitro* heat therapy was well tolerated by all explants, except for those of accession *f5p5* that were pale green and grew slowly; (iii) shoot tip culture

Table 2. PCR primers used in the present study.

Virus	Primer	Sequence	Amplicon size	References	Accession Number
FLV-1	FFup	5'-CGCTTTGCCCCAATGTGCAGAT - 3'	125 bp	Gattoni *et al.*, 2009; modified	FN377573
	FFrev25	5'- TARTCDGATTCHACRCACAGGTC - 3'			
FMV	BB42 up	5'- TGGCAGATTCAAGGATAATGG - 3'	218 bp	Elbeaino *et al.*, 2009	AM941711
	BB42 down	5'- TGGGACATTCTTGTGTCAGG - 3'			
FLMaV-1	N17s	5'- CGTGGCTGATGCAAAGTTTA - 3'	350 bp	Elbeaino *et al.*, 2006	AM279677
	N17a	5'- GTTAACGCATGCTTCCATGA -3'			
FLMaV-2	F3s	5'-GAACAGTGCCTATCAGTTTGATTTG-3'	360 bp	Elbeaino *et al.*, 2007	AM286422
	F3a	5'-TCCCACCTCCTGCGAAGCTAGAGAA-3'			
FBV-1	P1-s	5'-GCTGATCACAAGAGGCATGA-3'	214 bp	Minafra *et al.*, 2012	NC017830
	P1- as	5'-TCCTTGTTTCCACGTTCCTT-3'			

Table 3. Sanitary status of field-grown adult fig accesions (*f9p5* and *Mo-1*) and greenhouse-grown seedlings (*f5p5* and *p8*) subjected to sanitation trials, as determined by PCR.

	FMD symptoms	FMV	FLV-1	FLMaV-1	FLMaV-2	FBV-1
f9p5	Strong	+	+	–	–	+
Mo-1	Mild	+	+	–	–	+
f5p5 (s)	Absent	–	+	–	–	+
p8 (s)	Absent	–	+	–	–	+

Table 4. PCR analysis of sanitized fig plantles. Testing for *Fig mosaic virus* (FMV) and Fig latent virus 1 (FLV-1) was carried out three and 15 months after acclimatization. A single test for Fig badnavirus 1 (FBV-1) was carried out about 2 years after acclimatization.

Accessions	*In vitro* shoot tip culture		*In vitro* heat therapy		Shoot tip culture after heat therapy		Two cycles of heat therapy		
	FMV	FLV-1	FMV	FLV-1	FMV	FLV-1	FMV	FLV-1	FBV-1
f9p5	0/3	(0)2/4*	0/5	(0)2/5*	/	/	0/5	0/5	27/27
Mo-1	(0)1/4*	0/4	0/4	0/4	/	/	(0)1/5*	0/5	26/26
f5p5 (s)	0/5	1/5	0/0	0/3	0/2	0/2	/	/	17/17
p8 (s)	0/4	0/4	0/4	0/4	0/3	0/3	/	/	20/20
Total	1/16	3/16	0/16	2/16	0/5	0/5	1/10	0/10	94/94
Sanitation rate (%)	93.8	81.3	100	87.5	100	100	90	100	0

* Some apparently virus-free (0) plantles tested three months after acclimatization, proved to be infected when PCR was repeated 15 months after acclimatization. (s) = seedling

associated with heat therapy. Nearly 80% of the meristem tips excised after one or two successive cycles of heat treatment were successfully established in culture, growing into shoots that were rooted.

Regardless of the type of treatment they had been subjected to, acclimatized plantlets, grew vigorously showing no symptoms.

Since fig accessions submitted to sanitation procedures were infected by FMV and FLV-1 all sanitized plantlets were checked only for the presence these viruses three and 15 months after acclimatization. In autumn 2012, all acclimatized plants were also checked once for the presence of FBV-1. The first PCR round disclosed that all sanitized plantlets were apparently free from FMV and FLV-1 but, when the test was repeated a year later, four false FLV-1 negatives were discovered in accession *f9p5* and two false FMV negatives in accession *Mo-1*. The totality of the tested plants was still infected by FBV-1 (Table 4)

As shown in Table 4, FLV-1 could not be totally removed when explants were subjected to meristem tip culture or to a single cycle of *in vitro* heat therapy (81.3 and 87.5% successful elimination, respectively) but was eradicated (100% sanitation) by protocols encompassing shoot tip culture combined with heat therapy, or two heat therapy cycles. Very high sanitation rates (90-100%) from FMV were also obtained with all sanitation procedures adopted, with special reference to *in vitro* heat therapy alone or combined with tissue culture. By contrast, FBV-1 resisted all sanitation attempts. The recalcitrant behaviour of this DNA virus can be taken an indirect confirmation of its hypothesized integration in the host genome (Laney *et al.,* 1992).

Results of the present investigation, are largely in agreement with those obtained in the past with heat therapy and tissue culture (Sahraroo *et al.,* 2009; Martelli, 2011 and references therein), but their outcome is now supported by the use of efficient detection techniques for the assessment of the health status of sanitized plants. It seems therefore reasonable to conclude that notwithstanding the objectively small number of explants recovered from the four fig accesions that underwent sanitation and grew into acclimatized plants, efficient procedures have been developed also for reliable testing and more extensive applications against other fig-infecting viruses.

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