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Short communication

Attempts to eliminate *Candidatus* phytoplasma phoenicium from infected Lebanese almond varieties by tissue culture techniques combined or not with thermotherapy

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Abstract

Elimination of *Candidatus* phytoplasma phoenicium from two infected Lebanese varieties of almond by using different tissue culture techniques is reported. Except for the oxytetracycline therapy which totally inhibited the development of explants, stem cutting cultures associated with thermotherapy, shoot tip cultures associated or not with thermotherapy, and shoot tip micrografting were all suitable, either for shoot regeneration or for elimination of phytoplasma from the two varieties. However, stem cutting culture coupled with thermotherapy seemed to be the most effective for regeneration of phytoplasma-free plantlets.

Phytoplasmas are bacteria lacking a cell wall, and affecting a wide range of plants (Marwitz, 1990). In Europe, several decline diseases associated with phytoplasmas have been described in stone fruit species (Dosba et al., 1991; Poggi Pollini et al., 1995). Recently, a lethal disease of almond trees was reported from Lebanon. The disease is characterized by the development of conspicuous witches' brooms and leaf yellowing, leading to tree death within a few years after appearance of the first symptoms (Abou Jawdah et al., 2002; Choueiri et al., 2001). A phytoplasma was found to be associated with the disease. The phytoplasma was characterized as 'Candidatus Phytoplasma phoenicium' (Verdin et al., 2003) and shown to belong to the Pigeon Pea Witches' Broom (PPWB) phytoplasma group as defined by Schneider et al. (1995) or in the 16S rIX-A group as defined by Lee et al. (1998). The same phytoplasma produces also almond tree witches' broom disease in Iran (Bové et al., 1999).

Tissue culture techniques have been successfully employed for the elimination of viruses from plants by shoot tip culture alone or in combination with thermotherapy (Faccioli, 2001). Stem cutting culture coupled to thermotherapy and/or chemotherapy has also been used with some success as an alternative to shoot tip culture (Sanchez et al., 1991; Faccioli and Colalongo, 2002). Micrografting of shoot tips has been successfully employed for producing virus-free plants in citrus (Navarro et al., 1975, Navarro and Juarez, 1977; Nicoli, 1985), peach (Mosella et al., 1980), cherry (Deogratias et al., 1986) and grapevine (Cupidi and Barba, 1993). For elimination of phytoplasmas, only a few techniques have been reported: hot water treatment successfully used on grapevine scions (Bianco et al., 2000; Tassart-Subirats et al., 2003), and oxytetracycline-containing growth medium for pear (Davies and Clark, 1994). Also, shoot tip grafting has been used with success to eliminate the sieve tube restricted huanglongbing

liberibacter from citrus (Navarro et al., 1991). In the present paper, we report several procedures for producing phytoplasma-free plants of Lebanese almond varieties.

Symptomatic trees of two local varieties of almond, Halwani and Khachabi, from Bekaa valley and proved to be infected with *Candidatus* Phytoplasma phoenicium as described previously (Verdin et al., 2003), were used in this study. Young shoots from the current season's growth were surface sterilized with a 2% solution of Na-hypochloride for 20 min, soaked three times in sterile distilled water, and blotted on filter paper. Single-node stem cuttings (1 cm in length) and shoot tips (0.4–0.5 mm diam) were excised in a laminar flow cabinet, and used as explants according to treatments. The growth medium was MS (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} BAP (6-benzylaminopurine), 0.5 mg l^{-1} IBA (3-indolylbutyric acid), 0.1 mg l^{-1} GA_3 (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 artificial light and 8 h darkness at 25 ± 2 °C, except for cultures exposed to thermotherapy.

Six treatments were tested: (i) culture of stem cuttings with and without thermotherapy (35 ± 2 °C) for 30 days; (ii) culture of stem cuttings with oxytetracycline therapy at 50, 100 and 150 μg ml^{-1} added aseptically to the medium after autoclaving; (iii) culture of shoot tips with and without thermotherapy (shoots were submitted or not to 35 ± 2 °C for 30 days before shoot tip collection); (iv) for shoot tip micrografting, the rootstocks used were *in vitro* cuttings of the second subculture of healthy Khachabi cuttings previously introduced *in vitro* on the growth medium described above. Shoot tips excised from infected cuttings of the second subculture were inserted into inverted T cuts on the rootstocks.

Newly developed shoots derived from the different treatments were subcultured for three months on the growth medium and under the growth conditions, as described above. Percentages of explants regenerating new shoots or phytoplasma-free shoots were determined. Mean differences among treatments were evaluated by performing the Duncan test (General Linear Models Procedure, SAS Institute, Cary, NC).

Shoots regenerated from all techniques cited above, except stem cutting culture coupled with oxytetracycline, have been tested by PCR to evaluate the presence of phytoplasmas. Two positive controls were used: infected stem cuttings and infected almond plants maintained in the greenhouse at INRA-Bordeaux. Negative controls were healthy almond plants obtained from seeds and maintained in the greenhouse.

Nucleic acids were extracted from tissue culture plants using the CTAB (cethyl-trimethyl-ammmonium bromide) extraction protocol described by Maixner et al. (1995). Almond phytoplasma specific PCR primers $AlmF_1/AlmR_1$ (Verdin et al., 2003) were used for amplification of ribosomal DNA. PCR was performed for 35 cycles of 92 °C for 45 s, 53 °C for 45 s and 72 °C for 60 s. Each PCR reaction was performed in 0.2 ml PCR tube in a final volume of 40 μl containing 0.35 μmol of each primer, 200 μmol of each dNTP, 2 mM $MgCl_2$, 0.05% W1 detergent, 200 μg ml^{-1} BSA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 2 U *Taq* polymerase (Life Technologies). Two microlitre of CTAB-extracted DNA (250–300 μg ml^{-1}) were used as the template for PCR. PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator.

Table 1 indicates, for the two almond varieties, Halwami and Khachabi, the number of shoots regenerated from the explants as well as the proportion of shoots free of phytoplasmas as determined by PCR. According to the treatments, a certain number of explants did not survive, but all those, which did, produced regenerated shoots.

Thermotherapy did not significantly affect the stem cuttings response. These results are comparable to those obtained from healthy almond explants, but shoots regenerated from infected material were pale green and had slow development. For oxytetracycline therapy, at the antibiotic concentration of 50 μg ml^{-1}, only six new Khachabi shoots and three new Halwani shoots were regenerated 20 days later, but they were yellowish-brown and declined progressively. At the higher antibiotic concentrations, shoot regeneration was totally inhibited. The two almond varieties appeared more sensitive to oxytetracycline therapy than pear varieties, which tolerated concentrations up to 150 μg ml^{-1} (Davies and Clark, 1994).

Table 1. Effects of different tissue culture techniques on eradication of phytoplasma *Candidatus* phoenicium of two infected Lebanese varieties of almond

Technique	Variety	Regenerated shoots/total explants		Phytoplasma free / tested shoots	
		Nb	%	Nb	%
Healthy stem cutting culture	Halwani	53/60	88.3 b*	10/10	100
(negative control)	Khachabi	57/76	75.0 d	10/10	100
Infected stem cutting culture	Halwani	153/174	87.9 b	0/28	0
(positive control)	Khachabi	226/300	75.3 d	0/28	0
Stem cutting culture with	Halwani	181/208	87.0 c	28/28	100
thermotherapy	Khachabi	184/186	98.9 a	28/28	100
Stem cutting culture with oxytetracycline (μg ml^{-1})					
50	Halwani	0/20	0 i	–	–
	Khachab	0/20	0 i	–	–
100	Halwani	0/20	0 i	–	–
	Khachab	0/20	0 i	–	–
150	Halwani	0/20	0 i	–	–
	Khachabi	0/20	0 i	–	–
Shoot tip culture	Halwani	130/270	48.1 f	28/28	100
	Khachabi	127/385	32.9 g	28/28	100
Shoot tip culture with thermotherapy	Halwani	71/100	71.0 e	20/20	100
	Khachabi	75/100	75.0 d	20/20	100
Shoot tip micrografting	Halwani	25/76	32.8 g	25/25	100
	Khachabi	20/63	31.7 h	20/20	100

*Values followed by the same letter(s) are not significantly different ($P = 0.05$).

Shoot tip cultures have shown an oxidation problem resulting in relatively low rates of survival. Shoot tips taken on thermotherapy treated shoots had attenuated oxidation and showed a survival rate twice as high. Shoot tip micrografting was the most difficult technique to apply to the almond material, mainly because the bark thinness of the cuttings serving as root-stocks made it delicate to incise an inverted T and perform the micrografting. For shoot tips that escaped this problem, their development started about 12 days after micrografting. Four weeks later, only 31.7% of Khachabi graft tips and 32.8% of Halwani tips were recovered. The PCR test clearly showed that all tested shoots regenerated by different techniques were phytoplasma free, whereas the expected DNA fragment of 1.4 kbp was amplified from the positive control samples (Figure 1).

These results indicate the efficiency of the different tissue culture techniques, except the treatment associating stem culture to oxytetracycline therapy, for phytoplasma eradication of infected almond varieties. Even though these results are encouraging, it remains to be assessed whether the PCR-negative shoots are indeed phytoplasma-free, and will remain symptomless. In addition, attempts to graft-inoculate the almond phytoplasma from the PCR-negative shoots to almond and GF 305 peach seedlings should give negative results. Among the different techniques assayed in this study, tissue stem cutting culture coupled with thermotherapy seemed to be the most practical and successful way for regenerating phytoplasma-free shoots (high rate of shoot regeneration varying from 87 to 98.9% with 100% PCR-negative shoots). It could become a routine technique for producing phytoplasma-free Lebanese almond varieties, in addition to maintaining genetic diversity.

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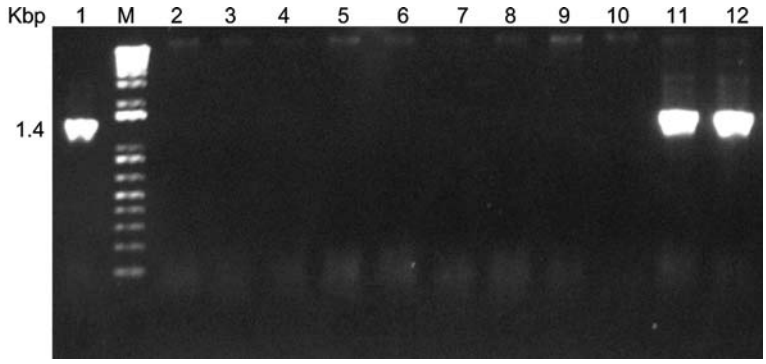

Figure 1. Agarose gel electrophoresis of DNA amplified in PCR primed with primers $AlmF_1/AlmR_1$ for detection of *Candidatus* Phytoplasma phoenicium in shoots derived from tissue culture treatments. Lane 1: positive control from infected almond plant maintained in the greenhouse at INRA Bordeaux. M: 1 kb molecular size marker. Lane 2: negative control from a healthy almond seedling maintained in the greenhouse at INRA Bordeaux. Lanes 3–4: respectively Halwani and Khachabi shoots derived from stem cutting culture with thermotherapy. Lanes 5–6: respectively Halwani and Khachabi shoots derived from shoot tip culture. Lanes 7–8: respectively Halwani and Khachabi shoots derived from shoot tip culture with thermotherapy. Lanes 9–10: respectively Halwani and Khachabi shoots derived from shoot tip micrografting. Lanes 11–12: respectively Halwani and Khachabi shoots derived from stem cutting culture.

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