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Elimination of in vitro bacterial contaminants in shoot cultures of 'MRS 2/5' plum hybrid by the use of *Melia azedarach* extracts

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Abstract The antimicrobial activity of leaf and callus extracts of *Melia azedarach* was tested on in vitro shoot cultures of the peach rootstoch 'MRS 2/5' (*Prunus cerasifera* × *Prunus spinosa*) that were heavily contaminated with *Sphingomonas paucimobilis* (Sp) and *Bacillus circulans* (Bc). The extracts were filter-sterilised and added at 0%, 1%, 5%, 10% and 20% to a modified Murashige and Skoog proliferation medium previously autoclave-sterilised. Up to about 17% shoots died with 10–20% extract, except for Sp-contaminated shoots, whose survival was reduced to 50% after treatment with 20% extract. No shoots died with 1% to 5% supplement. The undiluted leaf extract showed bactericidal activity on plated Sp and Bc isolates. The homogenates of shoots randomly collected from treated cultures were processed for bacterial colony counting. Thus the 10% supplement was the best treatment for ridding Bc-contaminated cultures of bacteria (although 5% had a similar bactericidal effect), and allowing shoot growth and proliferation comparable to controls at the fifth subculture on a standard medium, while 20% extract was needed to eliminate Sp, and could induce higher growth and proliferation rates in surviving shoots than in untreated cultures. Callus extract was ineffective. The bactericidal activity of the leaf extract seemed attributable to a synergistic effect of azadirachtin with other unidentified compounds present in the extract.

Keywords *Bacillus circulans* · Bactericidal activity · Micropropagation · Persian lilac · Plant extracts · *Sphingomonas paucimobilis*

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Introduction

Bacterial contaminations can be a great problem for in vitro plant tissue culture; their influence on shoot growth can range from no apparent effect to inhibition, and they can even cause great losses of plants, increasing the production costs in commercial laboratories. Bacterial strains that are naturally not

pathogenic to plants may become harmful to them under in vitro culture conditions. Contaminations of in vitro plant material may derive from deficient surface-sterilisation, be internal to the tissue (Kulkarni et al. 2007), or also come from the laboratory environment, including strains of human origin (Leifert et al. 1994). Bacteria can produce phytotoxic metabolites, modify the medium pH, nutrient uptake (Leifert et al. 1994) and the gaseous microenvironment (Marino et al. 1996). Antibiotic treatments are the most widely used method to eliminate bacterial contaminations (Cassels 1997; Kulkarni et al. 2007); however, antibiotics are not always effective against different bacterial strains, sometimes phytotoxic to plant tissues, and can lead to the selection of resistant strains after their permanent presence in culture (Cassels 1997; Falkiner 1997; Reed et al. 1998).

Over the last 20 years, a more responsible attitude to environmental preservation has increased interest for natural compounds to be used in organic and integrated pest and disease control (Marangoni et al. 2004). Derivatives from the tropical plant *Azadirachta indica* (neem tree) and other species belonging to the *Meliaceae* family have shown insecticidal (Mansour et al. 1997; Di Ilio et al. 1999) and antimicrobial activity (Cantrell et al. 1999; Coventry and Allan 2001). Extract activities of *Meliaceae* spp. have been reported to be related mainly to azadirachtin, and also to other terpenoids and limonoids (Bohnenstengel et al. 1999; Cantrell et al. 1999; Coventry and Allan 2001). In particular, the extracts of fruits, seeds, leaves and in vitro-cultured tissues of *Melia azedarach* (Persian lilac) have shown biocide activity against insects (Banchio et al. 2003; Hammad and McAuslane 2006; Hammad et al. 2001; Nathan 2006) and phytopathogenic fungi (Sharma 1995; Carpinella et al. 2003). *Melia azedarach* is well-adapted to the climate of Mediterranean countries, where it is grown as an ornamental plant in gardens and parks. It is generally propagated by seeds and cuttings, and can be also multiplied in vitro from different explants of seedlings and mature plants (Shahzad and Siddiqui 2001; Vila et al. 2003, 2005). These findings have acted as a stimulus for the present research in order to investigate the antimicrobial activity of *M. azedarach* extracts against two bacterial strains contaminants of in vitro shoot cultures of the plum hybrid 'MRS 2/5'.

Materials and methods

Plant material

'MRS 2/5' (*Prunus cerasifera* × *Prunus spinosa*) was selected as a suitable rootstock for peach in waterlogged and calcareous soils (Loreti 2005). It can be easily multiplied through in vitro shoot tip culture (Morini and Perrone 2006). Mother shoot cultures used for the present trials were supplied by a commercial laboratory, and maintained (subcultures at 4 weeks) on a proliferation medium (MRS-P) with the following composition: Murashige and Skoog (1962) macro and microelements, and (μM): 555 *myo*-inositol, 2.96 thiamine hydrochloride, 8.1 nicotinic acid, 4.9 pyridoxine hydrochloride, 26.6 glycine, 2.2 6-benzyladenine (BA), and (w/v) 3% sucrose and 0.65% 'type A' agar; the pH was adjusted to 5.6–5.7 by the addition of 0.1–1 N KOH before autoclaving at 120°C (110 kPa) for 20 min. All compounds were from Sigma (Sigma-Aldrich Ltd., Milan, Italy), except for commercial sucrose. Standard growth conditions were 22±2°C and a 16 h photoperiod at 30 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR, supplied by Osram L 18W/20 cool-white lamps; Osram Co., Munich, Germany) at the jar surface.

Melia shoot cultures were established from uninodal shoot segments of a single adult plant, and multiplied on a medium similar to MRS-P, but supplemented with 4.4 (μM BA); pH 5.8 (MA-P). Rooting of shoots was induced on a medium with half MS salt strength and 2.5 μM IBA (Shahzad and Siddiqui 2001). Plantlets were established to greenhouse conditions after about 4 weeks, and finally transferred to pots.

Melia callus cultures were obtained from 2 mm-thick internode slices of in vitro grown shoots on a medium like MA-P, except for reduction to one half of nicotinic acid and pyridoxine hydrochloride concentrations, and addition of 19.7 μM IBA (Hammad et al. 2001). Callus was grown at 24±1° C in darkness in 90 mm Petri dishes (about 1 g per dish) sealed with double-layered strips of polyvinyl chloride (PVC) transparent food film; sub-cultures were made at 4-week intervals. Extracts were prepared from callus and from leaves of plants obtained through in vitro micropropagation, as reported below.

Bacterial strains

The bacterial strains were previously isolated from apricot shoot cultures, identified (API test) as the Gram-negative *Sphingomonas paucimobilis* (Yabuuchi et al. 1990; previously named *Pseudomonas paucimobilis*, Holmes et al. 1977), Sp, and the Gram-positive *Bacillus circulans*, Bc, and stored at −135°C (Marino et al. 1996). The isolate Sp was cultured twice for optimal growth in tryptone soya broth (Oxoid CM0129); Bc was cultured twice in nutrient broth no. 2 (Oxoid CM0067). Both bacterial strains were incubated 20–24 h at 30°C under gentle shaking. Suspensions were prepared from cultures in the exponential growth phase and used for the following trials.

Evaluation of bacterial contamination effects on 'MRS 2/5' shoots

Since bacterial contaminations can affect the growth of different plants in different ways (Marino et al. 1996, 2003), Sp and Bc effects on 'MRS 2/5' cultures were evaluated before testing the antimicrobial activity of *M. azedarach* extracts. Shoots were dissected from mother cultures free of bacteria, their bases dipped singly for a few seconds in each Sp or Bc suspension (Marino et al. 2003), and transferred to MRS-P proliferation medium. Four weeks later, shoot clusters were cut into newly proliferated shoots, that were transferred to fresh medium and further subcultured at 4-week intervals in order to allow bacterial spread. At the end of the fourth and sixth subculture, single shoots (about 15 mm long) were randomly dissected from each culture type (i.e., controls, free of cultivable bacteria, and cultures contaminated with Sp or Bc). Some of the shoots were transferred to fresh proliferation medium (five shoots per 500 ml glass jar each filled with 45 ml of MRS-P), and compared after 4 weeks for their weight and proliferation (shoot number per initial explant) and length of main shoot. Other shoots were transferred singly to tubes (26 mm diam closed with Kaputs; Bellco Ltd., Vineland, NJ, USA) each filled with 10 ml of rooting medium (MRS-R) similar to MRS-P, except for half MS salt strength, lack of BA and the addition of 2.5 μM indolebutyric acid (IBA). These were left in darkness during the first week after transplanting, and compared for their shoot length, root number and length after an additional 3 weeks under standard growth conditions. At each subculture (fourth or sixth), three jars (replicates) per culture type were used for comparisons in the proliferation medium, while 20 shoots per culture type were used for rooting tests. Ex vitro plant establishment in the greenhouse was obtained in peat (Satum-2 Media Tor, MedioVeneta S.r.l., Treviso, Italy), and plant height and root weight were measured after 4 weeks.

Extract preparation from *M. azedarach* tissues

Callus was sub-cultured for about one year to constant growth before samples were collected, weighed and stored at −20°C prior to being used to prepare extracts. Frozen samples of callus from three successive subcultures were mixed, homogenised in a mortar with distilled water (144 g fresh weight, FW, corresponding to about 7.5 g dry weight, DW, in 30 ml water), covered with Parafilm™, left for 48 h at room temperature and centrifuged 10 min at 1,000×*g*. The supernatant was filtered through decreasing pore sizes (Whatman nos. 41 and 2 paper filters, then a 0.45-μm Millipore membrane; Millipore, Milan, Italy), and its pH measured. Finally, the extract was filter-sterilised (0.22 μm Millipore membrane) and stored at −20°C prior to being tested for its activity.

Completely expanded apical leaves were collected in late Summer from potted in vitro propagated plants 2 years after establishment; 40 g FW (about 12 g DW) leaves were homogenised in 100 ml distilled water, and processed as callus samples. The same callus and leaf extracts were used for all trials.

Extract characterisation

Chromatographic separation was carried out by HPLC using a modified method of Sidhu et al. (2004). Analyses were performed on a Shimadzu liquid chromatograph equipped with two LC-10 ADvp pumps, a SPD-M10A diode array detector and a Rheodyne 7125 sample injector fitted with a 20 μl loop. Shimadzu LCMS solution software was used for data management. A Discovery C_{18} (250×4.6 mm i.d., 5 μm particles size, Supelco) analytical column was used for analysis. Mobile phase consisted of an isocratic mixture of acetonitrile:water (50:50) at a flow rate of 1.0 ml min^{-1}. Leaf and callus extracts were qualitatively compared to each other, and with retention times of standards of azadirachtin, nimbin

and salannin (Trifolio-M GmbH, Lahnau, Germany) as methanolic solutions. Azadirachtin, nimbin and salannin have been reported as major metabolites with biocide activity in extracts of 'neem' seeds and kernels and 'neem' oil in organic solvents, but information on these compounds in leaf and callus aqueous extracts is extremely scarce (Abou-Fakhr Hammad et al. 2001).

Determination of the in vitro antimicrobial activity of the extracts on bacterial isolates

The antimicrobial activities of leaf and callus aqueous extracts were determined, and compared to azadirachtin, by a well diffusion assay after Coventry and Allan (2001). Plate count agar (Merck, KGaA, Darmstadt, Germany) was seeded with late exponential cultures of the test organisms (final population 10^6 ml^{-1}) and allowed to set in 90 mm Petri dishes. Four 7 mm diam wells per dish were cut aseptically from the medium; three wells were filled with 50 µl each of callus or leaf extract, or azadirachtin (10 mg 500 μl^{-1} ethanol), and one with the control solution. Distilled water (pH 6.5 and 4.5) and absolute ethanol were used as controls, respectively, for callus and leaf extract, and azadirachtin. Plates were incubated aerobically for 24–48 h at 30°C. The diameter of the inhibition zone (reduced growth/no growth), including the diameter of the well, was taken as a measure of the activity of each extract, and azadirachtin; three separate Petri dishes were used for each activity test against Bc or Sp.

Evaluation of short- and long-term effects of the leaf extract on growth of 'MRS 2/5' shoots

This trial aimed to determine the highest extract doses that allowed satisfactory shoot growth, to be used to counteract bacterial spread in 'MRS 2/5' cultures. Shoots of each culture type (i.e., shoots contaminated with Sp or Bc, and controls) that had been subcultured for about 1 year on MRS-P medium were used. The filter-sterilised leaf extract was added at 0%, 1%, 5%, 10% and 20% to the MRS-P medium that had been previously autoclave-sterilised (20 min at 120°C, 110 kPa) and cooled to about 50°C to prevent denaturation of any thermolabile compounds. Higher extract levels did not allow shoot survival in previous trials. In the present trial, shoots were singly transferred to tubes (26 mm diam, closed with Kaputs, Bellco Ltd., Vineland, NJ, USA) each filled with 10 ml of different media, and randomly placed in the growth room under standard growth conditions. Four weeks later, survival rates and weights were determined for each culture type on three shoots per extract treatment. The experiment was repeated 8 weeks later with shoots that had been processed for two additional subcultures on standard MRS-P medium.

In order to evaluate possible carry-over effects of the extract on re-growth capacity of plants, and to allow spread of bacteria still present in plant tissues, surviving shoots of each experiment were repeatedly subcultured on MRS-P proliferation medium. Cultures that showed visible contaminations at the fourth subculture after treatment were discarded. Otherwise, shoots were randomly chosen from cultures apparently still free of bacteria, singly transferred to the same medium in tubes, randomly placed in the growth chamber under standard growth conditions, and compared for weight and proliferation after 4 weeks; fifteen shoots per treatment/culture type combination were used each time Shoot samples were randomly collected from the same cultures at the end of subculture and processed for bacterial colony counting, as reported below, in order to evaluate the antimicrobial activity of the leaf extract on contaminated plant tissues. A 3-week test was previously shown effective to reveal the presence of bacteria in shoots that had been repeatedly subcultured after contamination (Marino et al. 1998); a 4-week interval, instead, allows optimal shoot growth, while longer subculture times can cause senescence symptoms in the cultures.

Determination of viability of shoot bacterial contaminants

Shoot samples were homogenised in an Omni mixer with a MS salt solution, pH 5.7 (1 g plant tissue in 10 ml solution). Living cell count was taken by tenfold serial dilutions of homogenates in physiological solutions, plating 1 ml on Merck plate count agar (pH 7.0; Marino et al. 2003). For each culture type, the bacteria colony forming units (cfu) g^{-1} fresh tissue were recorded after 24–48 h at 30°C, based on three replicates per extract treatment, but plates were held for five additional days to ensure detection of slow-growing eventually still viable bacterial cells (Reed et al. 1995). The undiluted homogenates were also plated on the same medium in order to exclude the presence of viable bacteria.

Data analysis

Data of weight, proliferation and length, and root number and length were statistically analysed by analysis of variance (ANOVA) in a completely randomised experimental design, except data of weight of shoots at the end of the leaf extract treatment; these were analysed in a factorial design. Means for treatments were separated by the Student Newman Keuls test (SNK, $P \leq 0.05$). The data of cfu were simply processed for descriptive statistics, and are reported in tables as means± standard errors (SE).

Results

Effect of bacteria on shoot and root growth

Table 1 shows data of shoots transplanted from the sixth to the seventh subculture after bacterial inoculation, as no negative symptoms could be previously found in Bc-contaminated shoots. Shoot weight and proliferation were respectively reduced with Bc to about 40% and 20% with respect to controls, although these differences were not significant; root growth was also slightly reduced. However, Sp significantly reduced shoot weight and proliferation on MRS-P medium, and shoot and root development in the rooting phase (Table 1) already evident at the fifth subculture (data not reported). Reduced in vitro growth of Sp-contaminated cultures also negatively affected successive plant development (3.2 cm plant height, 0.2 g FW root weight) with respect to controls (4.9 cm and 0.38 g, respectively, significant at $P \leq 0.05$, SNK) during their acclimatisation in the greenhouse.

Extract characterisation

The HPLC chromatograms (within 0 and 15 min of retention time, RT) of the aqueous extracts of leaves and callus (diluted 100-fold with respect to the initial concentration), and of azadirachtin (10 ppm in methanol) are shown in Fig. 1. No significant difference was found between the two extracts at 1:100 dilution, although the more complex shape of the peak in leaves than in the callus before 4 min of RT suggested the presence of other compounds. When the extract samples were further (ten times, inset in Fig. 1) diluted, some unidentified peaks were found within 4 min of RT in leaves, while a single peak was still detectable in the callus. Both chromatograms of leaves and callus extracts showed a peak that matched the RT of azadirachtin, while nimbin and salannin (standard RTs were 16 and 16.6 min, respectively) were not found. No or hardly no detectable peaks were found from 18 min onwards. The chromatogram plot of fresh samples was comparable to frozen extracts that had been stored for approximately 6 months (not shown).

The pH values measured for callus and leaf extracts were 6.2 and 4.5, respectively. Thus, sterile distilled water with pH 6.5 and 4.5 (the latter adjusted with 0.1 N KOH) were used as control solutions to be compared respectively to callus and leaf extracts in the test of activity against plated Sp and Bc. Since

Table 1 Growth, proliferation and root development of bacteria-contaminated 'MRS 2/5' shoots and shoots free of viable bacteria (controls)

	Proliferation phase			Rooting phase		
	Shoot weight[a] (g)	Shoot length[a] (mm)	Proliferation[a]	Shoot length[b] (mm)	Root number[b]	Root length[b] (mm)
Controls	0.41 a	22.8	6.7 a	38.1 a	4.0 a	27.7 a
Bc-contaminated	0.25 ab	22.0	5.6 a	34.9 a	3.7 a	24.7 a
Sp-contaminated	0.20 b	21.4	3.4 b	21.2 b	0.7 b	6.4 b
Significance	$P \leq 0.05$	ns	$P \leq 0.05$	$P \leq 0.01$	$P \leq 0.01$	$P \leq 0.01$

Means followed by different letters are statistically different at $P \leq 0.05$ (SNK).

ns not significant

[a] Values are means of three jars (replicates) with five shoots each.

[b] Means refer to 20 shoots per culture type grown singly in tubes.

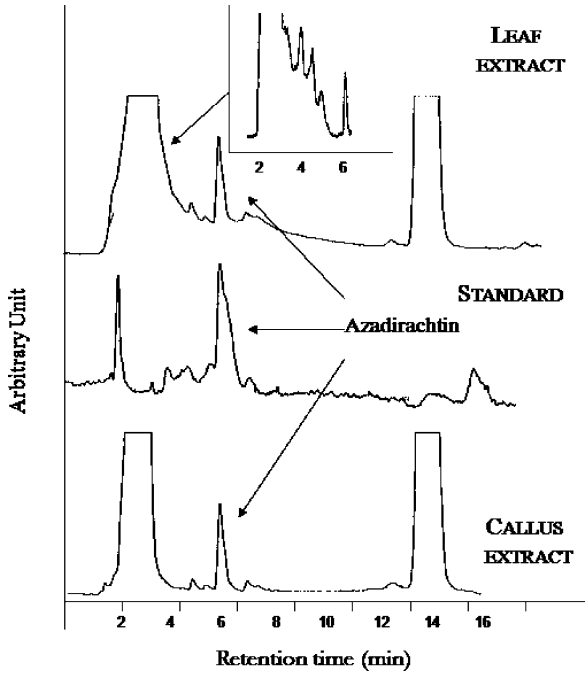

Fig. 1 The HPLC chromatograms (within 0 and 15 min of retention time, RT) of leaf (*top*) and callus (*bottom*) aqueous extracts (both diluted 100-fold with respect to the initial concentration; further ten times dilution, *inset*) of *M. azedarach*, and of azadirachtin (10 ppm in methanol, *middle*)

azadirachtin was found in both extracts, it was also tested against the two bacterial strains. It was dissolved in ethanol, as it is hardly soluble in water; thus ethanol was used as the control for azadirachtin.

In vitro antimicrobial activity of the extracts against bacterial isolates

In each Petri dish, both Sp (Fig. 2a) and Bc (Fig. 2b) strains were strongly affected by the leaf extract, showing a diameter of growth inhibition ranging from 14 to 17 mm, and from 12 to 15 mm, respectively. The antimicrobial activity was preserved after several months of extract storage at −20°C (data not shown). Growth of bacteria was stimulated around the inhibition zones (Fig. 2a and b), possibly because of an increase in available nutrients from the zone of no growth (Visser et al. 1986). No inhibition or stimulation zones could be detected around the wells with water used as a control (Fig. 2a and b). Callus extract was ineffective, although previous trials had shown its bacteriostatic activity against *Kocuria kristinae* (Gaggia et al. personal communication). Thus, only the leaf

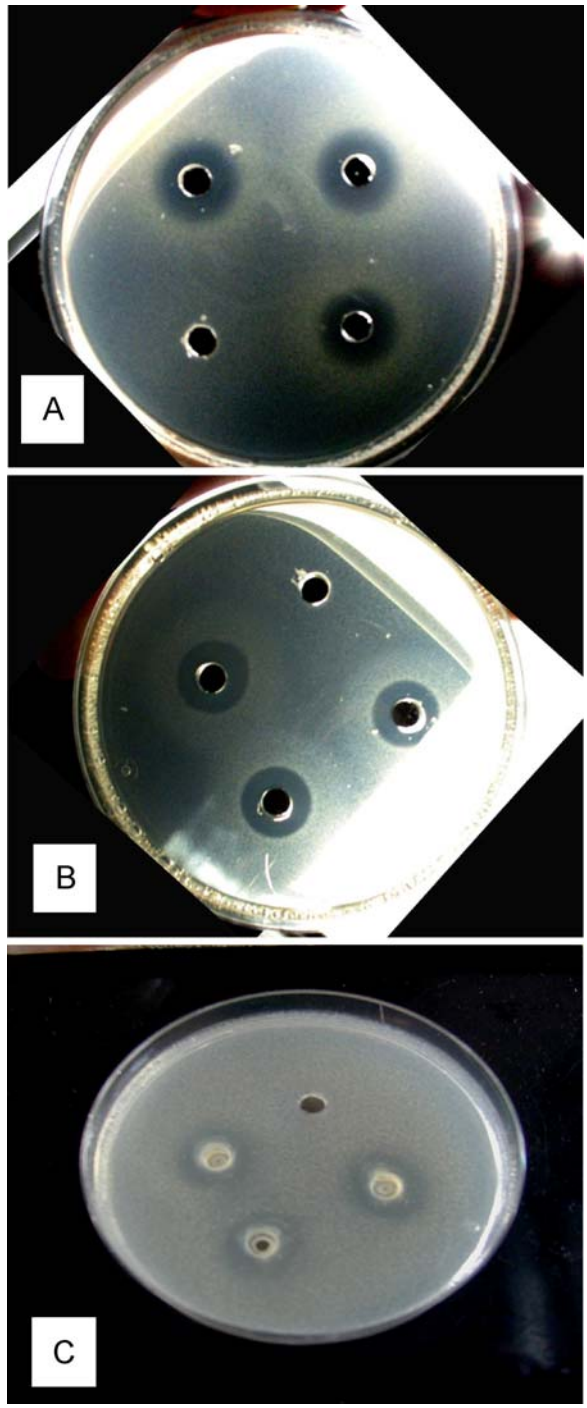

Fig. 2 In vitro antimicrobial activity of the leaf extract of *M. azedarach* and of azadirachtin against bacterial isolates. Bactericidal effect of the leaf extract against **a** *Sp* and **b** *Bc*; **c** bacteriostatic activity of azadirachtin against *Bc*

extract was used in further trials. Azadirachtin had no effect against Sp, and showed only a weak bacteriostatic activity against Bc (Fig. 2c), while absolute ethanol did not affect the growth of either bacterial strain.

Short- and long-term effect of the leaf extract on growth of 'MRS 2/5' shoots

Four weeks after treatment, shoot survival was not affected by extract supplements up to 5%, and even to 10% in controls; otherwise it decreased to about 17% with 10–20% extract, except for Sp-contaminated shoots, i.e., 50% of them died with 20% extract. Data reported in Tables 2 and 3 are means of repeated experiments, as no differences were found between them (ANOVA). Shoot weight gradually decreased in all cultures as the extract amount increased (Table 2). Variable growth of control shoots (free of contaminants) treated with increasing extract supplements is shown also in Fig. 3.

No more shoots died during repeated subcultures on MRS-P medium. At the end of the fifth subculture, the mean weight of control shoots (free of bacteria) previously treated with 20% extract (0.27 g average FW) was still significantly lower than in untreated shoots (0.40 g FW, different at $P \leq 0.05$), while other extract supplements did not show any carry-over effect on the cultures (data not shown in tables). Shoot weight and proliferation of Bc-contaminated shoots were reduced with 1% and 20% extract with respect to untreated cultures, while the 5% and 10% concentrations were not statistically different from controls (Table 3). By contrast, increased growth and proliferation were found even after treatment with 20% extract, and repeated subcultures on standard medium, in shoots previously contaminated with Sp (Table 3). Moreover, after 20% extract treatment, all originally Sp- and Bc-contaminated shoots produced roots on an auxin-enriched medium, and were successfully acclimatised in the greenhouse (data not shown).

Effect of the leaf extract on bacterial spread in shoot cultures

At the end of the fourth subculture, all Sp-contaminated cultures treated with 1% to 10% extract still showed white-yellowish halos at the shoot bases revealing the presence of contaminants, and were discarded. No visible contaminations could be detected after treatment with 20% extract in all originally Bc-contaminated shoots, regardless of treatment. At the fifth subculture, no Bc colonies were found (cfu 0) in homogenates of 'MRS 2/5' sample shoots treated with 5% leaf extract onwards, while 1% supplement caused a ten-fold increase in contamination rates with respect to untreated shoots (Table 3); only 20% supplement was effective in

Table 2 Weight (g) and percentages of dead shoots (in brackets) of bacterial-contaminated 'MRS 2/5' shoots or shoots free of viable bacteria (controls) after 4 weeks in culture on MRS-P medium supplemented with various leaf extract amounts

Extract %	Culture type			
	Controls	Bc-contaminated	Sp-contaminated	Mean
0	0.27	0.25	0.15	0.22 a
1	0.20	0.14	0.12	0.16 b
5	0.14	0.13	0.09	0.12 bc
10	0.10	0.06 (16.7)	0.06 (16.7)	0.07 cd
20	0.05 (16.7)[a]	0.04 (16.7)	0.02 (50)	0.04 d
Mean	0.15 a	0.13 ab	0.09 b	
Significance				
Extract	$P \leq 0.001$			
Culture type	$P \leq 0.01$			
Extract × culture type	ns			

Data are means of two experiments; in each experiment, three shoots grown singly in tubes were used per extract treatment and culture type. Means followed by different letters are statistically different at $P \leq 0.05$ (SNK).

ns not significant

[a] No shoots died if percentage is not reported.

Table 3 Shoot growth, proliferation and colony forming units (cfu) of previously contaminated 'MRS 2/5' shoots at the fifth subculture after treatment with various leaf extract supplements

Extract %	Bc-contaminated			Sp-contaminated		
	Shoot weight (g)[a]	Proliferation[a]	cfu g^{-1} FW[z]	Shoot weight (g)[a]	Proliferation[a]	cfu g^{-1} FW[z]
0	0.41 ab	8.6 ab	$136\pm1\times10^{1}$	0.23 b	2.7 b	$115\pm7\times10^{5}$
1	0.21 c	4.8 c	$146\pm5\times10^{2}$	nd	nd	nd
5	0.28 bc	7.2 b	0	nd	nd	nd
10	0.55 a	10.2 a	0	nd	nd	nd
20	0.16 c	4.1 c	0	0.47 a	9.8 a	0
Significance	$P\leq0.001$	$P\leq0.001$		$P\leq0.05$	$P\leq0.05$	

Data are means of two experiments; in each experiment, 15 shoots grown singly in tubes were used per extract treatment and culture type. Means followed by different letters are statistically different at $P\leq0.05$ (SNK).

nd not determined: shoots showed visible contamination and were discarded.

[a] Mean value±SE

eliminating Sp contamination from shoot cultures (Table 3). The bacterial cell morphology was periodically checked throughout repeated experiments and compared to the original bacterial strains.

Discussion

It is well known that bacterial contaminations are a great problem in mass plant propagation, and can impair the reproducibility of experiments, as they strongly affect the metabolism of plants in culture. Previous trials showed that acetylsalicylic acid, added to culture medium as an alternative treatment to antibiotics, reduced bacterial development in fruit-plant cultures (Marino et al. 1997). Also egg white lysozyme (EWL) had a simple bacteriostatic effect against Sp, while it was effective in eliminating Bc in cultures of quince 'BA 29' and the peach rootstock 'GF 677', without affecting shoot growth (Marino et al. 2003). However, the high cost of EWL does not allow its use in commercial plant mass propagation. Insecticidal (Banchio et al. 2003; Hammad and McAuslane 2006; Hammad et al. 2001; Nathan 2006) and antifungal (Sharma 1995; Carpinella et al. 2003) activities have been described for *M. azedarach* extracts. The present research aims to test the effectiveness of leaf and callus aqueous extracts of *M. azedarach* to control in vitro Sp and Bc contamination in shoot cultures of the peach rootstock 'MRS 2/5'.

Fig. 3 Growth of 'MRS 2/5' control shoots, free of viable bacteria, 4 weeks after treatment with the aqueous leaf extract of *M. azedarach*. From *left* to *right*: MRS-P medium supplemented with 0%, 1%, 5%, 10%, and 20% extract

Since timing and rates of bacterial spread, and effect on behaviour of plant in vitro cultures during repeated subcultures are influenced by the composition of medium (Leifert et al. 1994) and plant species in culture (Marino et al. 1998), the effect of Sp and Bc on 'MRS 2/5' shoots was investigated before testing the biological activity of the extracts. Thus, Bc was found to be almost non-injurious even about 1 year after inoculation, while Sp negatively affected shoot growth and rooting a few subcultures after inoculation. However, shoot weights of controls and of both contaminated cultures were gradually lowered in similar ways, as leaf extract (40 g FW 100 ml^{-1} water) supplements were increased. These results partially agree with those of kiwifruit shoots treated with leaf

extract (100 g plant FW l^{-1} water) of *Amaranthus retroflexus*. In this case, although very low extract supplements could enhance shoot and/or root growth respectively on cytokinin-enriched and hormone-free medium, 20% supplement strongly reduced both, and this suggests the presence of growth inhibitors in the extract (Marino et al. 2004). Repeated shoot subculturing after treatment aimed to evaluate their re-growth capacity, and also to allow spread of bacteria still present on/inside shoots before tissue indexing. Previous trials, in fact, showed that bacterial contaminations variously increased with repeated subcultures in different fruit plants grown in proliferation media not specific to bacteria. In particular, Sp contamination rates increased about five and ten times from the second to the fifth subculture in quince and apricot shoots, respectively, and bacterial colonies could be found in the latter cultures not only on the shoot surface but also inside the xylem, using scanning electron microscopy (SEM) at the third week after transplanting (Marino et al. 1998). Moreover, it is well known that some bacteria must be present in high numbers to be tested by culture indexing procedures (Leifert et al. 1994). Differing from other treatments, 20% extract heavily reduced survival of Sp-contaminated shoots; however, at the fifth subculture on standard medium (Table 3), these shoots had growth and proliferation rates, respectively, comparable and even >50% higher than untreated cultures originally free of bacterial contaminants (controls, Table 1). Moreover, they did not reveal any residual bacterial contamination, and subsequently easily produced self-rooted plants. Although a strong growth inhibition of both plated Bc and Sp isolates was found for the undiluted leaf extract, responses of shoots to treatments with variable extract supplements were different according to bacterial strains: i.e., a 20% supplement was necessary to eliminate Sp, while even 5% was enough for Bc. Moreover, ten-fold higher numbers of Bc colonies were surprisingly present in shoots treated with 1% extract than in untreated cultures. This last result might be tentatively explained by the low antimicrobial activity of 1% extract supplement, and the presence of growth inhibitors in the extract (shoot weight and proliferation were lower than in untreated shoots, Table 3) that reduced shoot growth, and consequently favoured bacterial spread inside plant tissues.

Data on treatments to shoots and on HPLC analysis showed that the antimicrobial activity of the leaf extract was preserved after several months of storage at −20°C; this is of great interest as it allows the use of natural extracts throughout the year. Since azadirachtin had just a weak bacteriostatic activity against Bc, and callus extract was ineffective on both plated Bc and Sp isolates, the effects of the leaf extract do not seem attributable to azadirachtin alone. The presence of some unidentified peaks, before 4 min of RT in the leaf extract, and not in the callus, suggests that a mixture of compounds with appropriate concentrations can have a higher activity than individual compounds. This is also supported by data on reduced activity of purified compounds with respect to the original mixture of tetranotriterpenoids in the antifungal fraction of neem oil (Coventry and Allan 2001).

In conclusion, although optimal extract supplements should be determined for each bacterial strain and, probably, for different plant species and phases of in vitro culture, the present results suggest that treatments with aqueous extracts of *M. azedarach* leaves may be an alternative method to antibiotics to counteract bacterial spread in shoot cultures, and have low cost and impact on the environment, and reduced toxicity to plants in culture compared to extracts in organic solvents. Moreover they can have bactericidal effects after a single application. Most antibiotics instead have only a bacteriostatic activity, and their successful application can be obtained only through long-lasting procedures, such as periodic subculturing of plant tissues in further cycles on antibiotic-enriched media. On the other hand, the production of bioactive compounds from in vivo-grown plants and in vitro-cultured tissues (i.e., callus) of *M. azedarach* still needs to be quantitatively and qualitatively determined for better employment of these extracts.

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