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MICROPROPAGATION

Endophytic bacteria from *Ilex paraguariensis* shoot cultures: localization, characterization, and response to isothiazolone biocides

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Abstract Nodal segment explants of *Ilex paraguariensis*, collected from greenhouse-grown plants, were found to contain endophytic bacteria. After culturing in bioreactors, 16 rRNA gene analyses and analytical profile index biochemical tests were used to identify these bacteria as *Stenotrophomonas malthophilia*. The presence of bacterial cells in the intercellular spaces of stem cortical parenchyma was detected in histological sections by scanning electron microscopy. A range of commercial isothiazolone biocides were tested for their ability to repress the growth of Gram-negative bacteria grown in liquid media during the micropropagation phase. The addition of 0.75 $ml l^{-1}$ Delcide™ TG (5-chloro-2-methyl-4-isothiazolin-3-one + 2-methyl-4-isothiazolin-3-one, 1.05% and 0.45%, respectively) to the culture media resulted in 100% visibly clean cultures, with no suppression of shoot growth.

Keywords Bioreactors · Contamination · 16S rDNA analysis · Woody plant species · Micropropagation

Introduction

Endophytic contamination is a serious problem for the *in vitro* vegetative propagation of woody plant species and is one of the main factors that may restrain the use of temporal immersion systems. To eliminate bacteria, antibiotics are either added to the culture media (Reed et al. 1998) or used in the preparation of solutions for soaking plant material at the beginning of *in vitro* culture (Leifert et al. 1991; Luna et al. 2008). Unfortunately, antibiotics often exert only a bacteriostatic effect, leading to selection of resistant strains and inducing phytotoxic effects in plant tissues (Leifert et al. 1991; Falkiner 1997).

Another approach to controlling contamination in explant tissues that does not lead to the formation of resistant mutants uses the Plant Preservative Mixture (PPM™, Plant Cell Technology, Inc., Washington, DC). This mixture is a combination of two broad-spectrum industrial isothiazolone biocides called methylisothiazolone (MIT) and chloromethylisothiazolone (CMIT) (Miyazaki et al. 2010). However, the constant culture of plant materials on media containing PPM™ may limit the growth of shoots due to phytotoxicity. In fact, PPM™ sensitivity has been demonstrated in seedlings of *Arabidopsis thaliana*, leaf explants of *Chrysanthemum*, and bryophyte protonemata (George and Tripepi 2001; Paul et al. 2001; Rowntree 2006). In addition, our previous results showed early defoliation and severe growth restriction in nodal segment explants of *Ilex dumosa* subjected to temporal immersion (Luna et al. 2008).

Other commercial isothiazolones that contain both MIT and CMIT in different ratios have been used at very low concentrations to preserve paint as well as woody, cosmetic, and body care products (Ballantyne and Jordan 2004) against bacteria, fungi, and yeast. CMIT is the most effective biocide and is 100- to 1,000-fold more active than MIT. CMIT is also very soluble in water (Collier et al. 1991). Isothiazolone biocides have a complex mechanism of action that involves rapid growth inhibition followed by a loss of viability. These biocides attack the central metabolic pathways of microbial cells (oxygen consumption and ATP synthesis) by inhibiting several key enzymes involved in the tricarboxylic acid cycle and energy generation (Williams 2007).

The aims of this study were to detect, identify, and characterize the endophytic bacteria associated with *Ilex paraguariensis* (a woody plant species) and explore the

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feasibility of using commercial isothiazolone biocides to control proliferation of such bacteria during the multiplication phase of micropropagation. Using light microscopy, the colonization of shoots by these plant-associated bacteria was examined in detail. To the best of our knowledge, this is the first detailed ultrastructural study of naturally occurring endophytic bacteria in *I. paraguariensis*. This is also the first report of successful efforts to control the growth of these endophytes *in vitro*.

Materials and Methods

Plant material and explant source. Plants of *I. paraguariensis* St. Hil., grown in pots in the greenhouse, were used as the explant source for this study. Explants (1.5–2-cm-long stem segments containing one axillary bud) were collected from young, nonlignified branches, surface sterilized in 70% ethanol for 1 min, followed by treatment with 1.5% NaOCl with 0.1% Triton® X-100 for 30 min, and finally washed with several rinses of sterile distilled water (Luna et al. 2003). Afterward, the explants were cultured in 11-ml glass tubes containing 3-ml autoclaved (1.45 $kg\,cm^{-2}$ for 20 min) original salts and vitamins of Murashige and Skoog (1962) medium (MS) reduced to quarter strength (1/4-strength MS) and 3% sucrose with the pH adjusted to 5.8 prior to the addition of 0.65% agar (A-1296, Sigma-Aldrich Co., St. Louis, MO). The shoots from nodal segments were grown for at least 30 d in a growth room at 27±2°C with a 14-h photoperiod (116 $\mu mol\,m^{-2}s^{-1}$ photosynthetic photon flux density; wavelength, 400–700 nm, from fluorescent lamps) before subculturing in bioreactors.

Isolation and identification of bacterial contaminants. Surface-sterilized *I. paraguariensis* shoot cultures grown in agar-solidified medium for 30 d with no signs of contamination were transferred to the temporal immersion system (Berthouly and Etienne 2005). After 7 d of incubation under controlled environmental conditions, all of the bioreactors showed bacterial contamination. The bacterial suspensions were recovered from contaminated shoot cultures growing *in vitro*, streaked on tryptone soya agar media (Levitt et al. 1955), and incubated in the dark for 7 d at 30°C.

Phylogenetic assignment of isolates was carried out by sequence analysis of the 16S rRNA gene and by analysis of phenotypic traits using the analytical profile index (API) system (BioMérieux SA, Marcy l'Etoile, France). To analyze the 16S rRNA gene, a fragment of approximately 1.5 kb spanning almost the full length of the gene was PCR amplified using the universal primers rD1 (3′-AAGGAGGTGATCCAGCC-5′) for positions 8–27 and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) for positions 1524–1540 of *Escherichia coli* strain K12 (Weisburg et al. 1991). The reactions were performed under the following conditions: one cycle of 95°C for 10 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and one cycle of 72°C for 10 min. PCR products were cloned using the pGEM-T Easy vector (Promega, Madison, WI) and sequenced by Macrogen Inc. (Seoul, Korea). The nucleotide sequences were compared with the database, and pairwise sequence similarities were determined using the EzTaxon server (Chun et al. 2007). The sequence obtained in this study was deposited in the GenBank nucleotide sequence database under accession number EU442188.

The isolates were also characterized using the biochemical test API 20NE for non-Enterobacteriaceae, according to the manufacturer's instructions (BioMérieux). APIWEB software was used to identify the isolates, and the assignment was considered acceptable when a score of at least 80% similarity was obtained (BioMérieux). Finally, the identified isolate was confirmed by PCR amplification with species-specific primers (SS-PCR) following the protocols described by Whitby et al. (2000).

Histological detection of endogenous bacteria. The contaminated stem tissues of *I. paraguariensis* were fixed in an ethanol/formaldehyde/glacial acetic acid [50:10:5% (*v*/*v*), dissolved in distilled water] solution. The samples were dehydrated in an ethanol series and finally placed in acetone. The samples were dried using the critical point technique and coated with gold and palladium (Dawes 1988). A JEOL scanning electron microscope (JLV 5800) operating at 20 kV (JEOL Ltd., Tokyo, Japan) was used to observe and take a photomicrograph of the transversal stem sections.

For tissue homogenization, fresh stems collected from plants growing under greenhouse conditions were surface sterilized in 70% ethanol for 1 min and placed in a sterile syringe for juice extraction. The cell homogenate was collected on 0.22-μm nylon membrane filters (Millipore, Billerica, MA) and processed as described previously.

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed following the protocols described by the Clinical and Laboratory Standards Institute (2009). To determine the minimum inhibitory concentrations (MICs) for *Stenotrophomonas maltophilia* populations, suspensions of $5\times10^{5}\,cfu\,ml^{-1}$ of the isolated bacteria were used. A macro dilution method with a final suspension volume of 1 ml was employed, and the bacteria were challenged with various concentrations of the following biocides, each prepared from a solution of 0.25% (*v*/*v*) of each commercial product in Mueller–Hinton (MH) medium: Ipel™ BP15 (Itibanyl Produtos Especiais Ltda., San Pablo, Brazil), Delcide™ TG (Arch Chemical, Buenos Aires, Argentina), Kathon™ CG (Dow Chemical Co., Midland, MI), PPM™ (Plant Cell Technology, Washington, DC), and Preventol™ D7

Table 1. Chemical composition of the commercial biocides added to the basal medium

Trade name	Active ingredients	CMIT/MIT ratio
Ipel™	5-Chloro-2-methyl-4-isothiazolin-3-one (1%) 2-Methyl-4-isothiazolin-3-one (0.4%)	0.25
Delcide™ TG	5-Chloro-2-methyl-4-isothiazolin-3-one (1.05%) 2-Methyl-4-isothiazolin-3-one (0.45%)	2.3
Kathon™	5-Chloro-2-methyl-4-isothiazolin-3-one (1.15%) 2-Methyl-4-isothiazolin-3-one (0.35%)	3.3
Plant Preservative Mixture™ (PPM)	5-Chloro-2-methyl-3(2H)-isothiazolinone (0.1350%) 2-Methyl-3(2H)-isothiazolinone (0.04%)	3.4
Preventol™	5-Chloro-2-methyl-4-isothiazolin-3-one (1.25%) 2-Methyl-4-isothiazolin-3-one (0.25%)	5.0

(Lanxess, Leverkusen, Germany). The cultures were incubated at 35±1°C for 20 h, and each tube was checked for evidence of growth. The MIC for the test compound was defined by the lowest biocide concentration that resulted in zero visible growth. The minimal biocidal concentrations (MBCs) were determined by transferring a 15-µl aliquot from the MIC tubes to MH agar plates. The lowest concentration of biocide in which no survivors were recovered was defined as the MBC. Additionally, the disk agar-diffusion method was employed to determine the susceptibility of microorganisms to antimicrobial agents. Paper disks containing 0.5 µl of each biocide [0.250% (*v*/*v*) of commercial product] were dispensed onto the surface of MH agar plates, and the plates were inoculated with the bacterial isolates and incubated at 35±1°C. After 20 h of incubation, the diameters of the zones of complete inhibition were measured and correlated with the MICs. Data reported are the average of duplicate determinations.

Biocide treatment of plant material. To inhibit the rise of contaminants, the infected explants were transferred into bioreactors (15 explants/recipient) containing 200 ml ¼-strength MS with 3% sucrose and 0.001 to 0.175 $ml l^{-1}$ Ipel™, Delcide™, Kathon™, PPM™, or Preventol™. Each biocide contained different active ingredients (Table 1). Bioreactors containing the culture medium (pH 5.8) were autoclaved at 1.45 $kg cm^{-2}$ for 20 min. All of the biocides were sterilized by filtration (Millipore®, 0.22-µm pore size). For the temporary immersion program, the explants were in contact with the medium for 1 min every 4 h. The cultures were incubated in a growth room at 27±2°C with a 14-h photoperiod (180 $\mu mol m^{-2} s^{-1}$, from fluorescent lamps).

The percentage of establishment, shoot proliferation, and shoot length of the regenerated shoots was recorded after 35 d, at the completion of the experiments. The phytotoxicity of the antibiotics was determined visually by checking for morphological changes, chlorosis, and browning of the

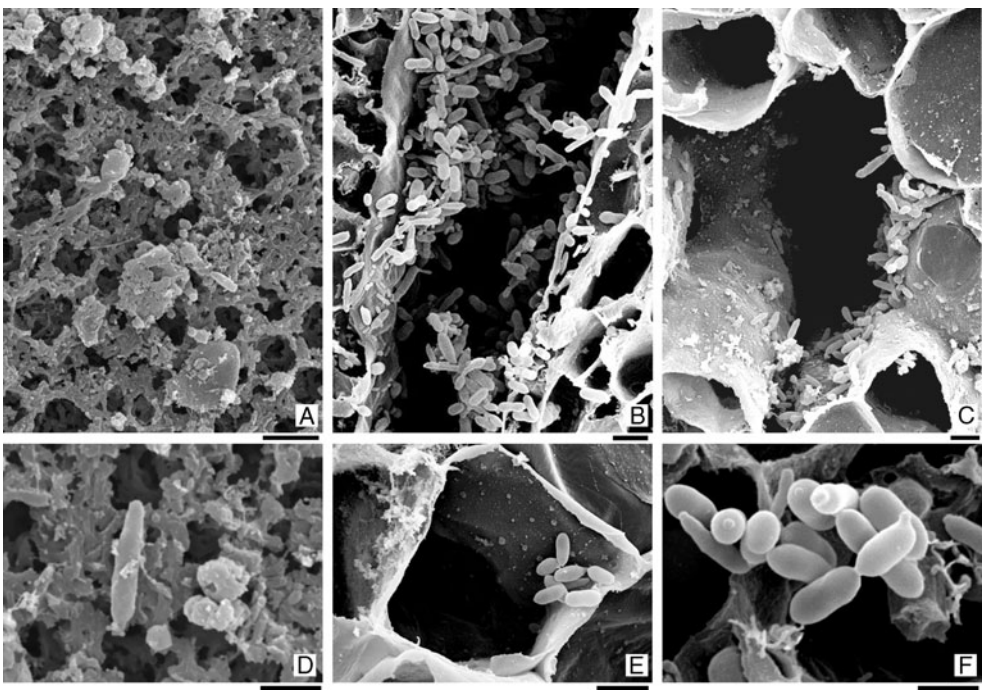

Figure 1. Detection of *S. malthophilia* by scanning electron microscopy in the stem tissues of infected *I. paraguariensis* shoots. (*A*) Bacterial cells from homogenized plant tissue extracted from the mother plants and mounted on membrane filters. (*B*) and (*C*) Bacterial cells in the intercellular spaces of cortex parenchyma cells from control and Delcide-treated shoots, respectively. (*D*) Features of Gram-negative bacilli isolated from homogenized plant tissue. (*E*) Bacterial cells in stem parenchyma cells. (*F*) A small cluster of microorganisms, showing bacterial cell division. *Bars* indicate 5 and 25 µm in *A*, *B*, *C*, *E*, and *D*, *F*, respectively.

explants and/or the regenerated shoots. All of the experiments were run in triplicate for each treatment. Statistical analysis of the data was performed using analysis of variance (GraphPad Software, San Diego, CA) and Tukey tests ($P \leq 0.05$) to compare treatment differences.

Results and Discussion

Detection, isolation, and characterization of bacterial contaminants. Scanning electron microscopy (SEM) of stem tissue homogenate dispersed on a nylon membrane showed a host of cellular organelles and bacterial cells (Fig. 1*A–D*), confirming the presence of an endophytic bacterial population in stock plants. A single type of bacterial colony was detected from infected explants of *I. paraguariensis*. The bacterial contaminant was isolated and identified using API and 16S rDNA analyses. The nearly complete 1.5-kb-long 16S rRNA gene sequence of the isolate showed the 99% sequence similarity to *S. maltophilia* strain 6B2-1 (GenBank accession no. AY445079.1). A high sequence similarity was also found with *S. maltophilia* strain alpha-2 (AB180661.1). The same isolate identification was found using the API 20 NE system (99.7%). Finally, the identification of this bacterium was confirmed by SS-PCR using SM1 and SM4 primers, directed to the 23S rRNA gene.

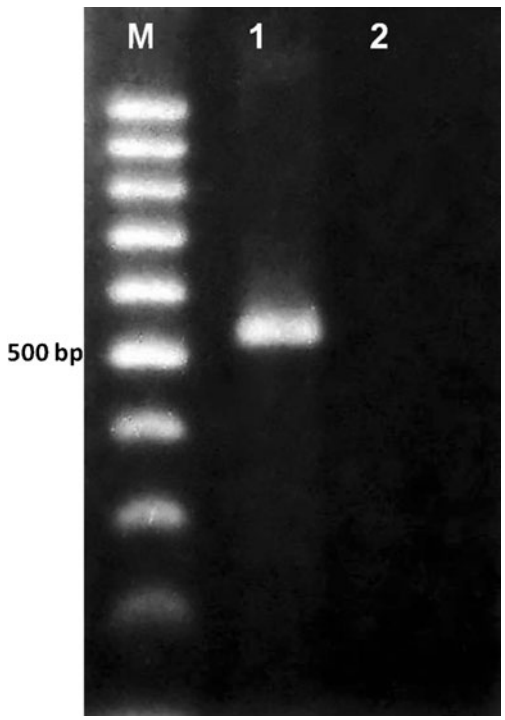

Figure 2. SS-PCR of *S. maltophilia*. *Lane 1* genomic DNA derived from *S. maltophilia* isolate. *Lane 2* negative control. The molecular marker (*M*) is a 100-bp ladder.

Table 2. Sensitivity of *S. maltophilia* to different biocides

Biocide	MIC % *v/v*	MBC % *v/v*
PPM™	0.032	0.063
Ipel™	0.008	0.063
Preventol™	0.002	0.004
Delcide™	0.004	0.008
Kathon™	0.004	0.004

The results represent the mean of two replicates

Positive results were assessed by the amplification of a 531-bp product (Fig. 2).

These results suggest that the isolate can be identified as *S. maltophilia*, a bacterium that is typically associated with plants (Bacon and Hinton 2006; Crossman et al. 2008). The main reservoir for *S. maltophilia* is the rhizosphere, where it has been reported to be associated with a long list of plant species, including orchids, monocots, and dicots (Berg et al. 2010). Recently, we also identified a strain of *S. maltophilia* from the rhizosphere of *I. paraguariensis* plants growing in lateritic soils and determined its *in vitro* phosphate-solubilizing activity (Collavino et al. 2010). Many *S. maltophilia* strains associated with the rhizosphere are able to fix inorganic nitrogen and promote growth of the host plants (Berg et al. 2010). *Stenotrophomonas* strains were also isolated from the root nodules of herbaceous legumes (Kan et al. 2007). Other examples of beneficial modes of interaction with the plant host include the capacity to produce indole-3-acetic acid (Lata et al. 2006; Taghavi et al. 2009), enhance the availability of nutrients

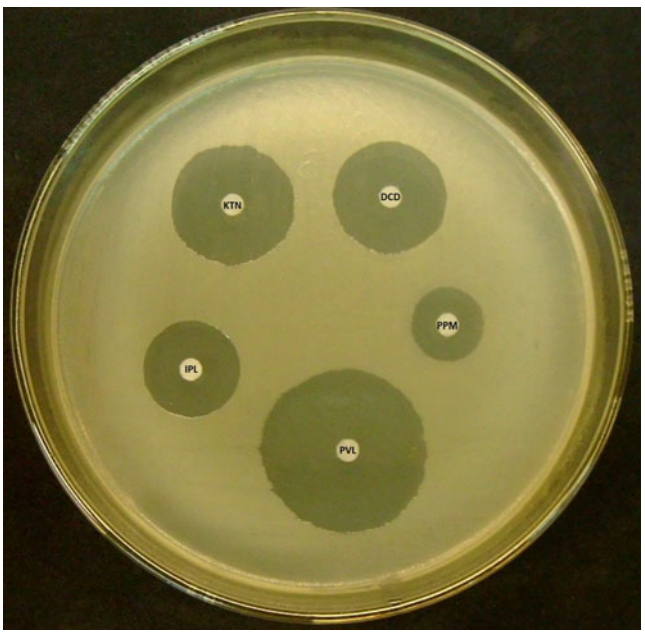

Figure 3. Sensitivity of *S. maltophilia* to Delcide™, Ipel™, Kathon™, *PPM*™, and Preventol™. Active ingredients are described in Table 1.

Table 3. Control of bacterial contamination and phytotoxic effects of different biocides added to the culture medium

Biocide[x]	Dose	Visibly clean cultures (%)	Range of phytotoxicity[y]	Explant death per bioreactor (%)	Mean shoot length of surviving explants (mm)
Control (without biocides)	–	0 c	0	100 c	–
PPM™	50	33±19.2 b c	0.5	83±8.8 c d	12.8±1.1d e
	100	67±19.2 a b	0.5	47±17.6 a b c	16.1±1.0 a
	150	100 a	0.5	10±5.8 a	11.7±0.7 a b
Ipel™	75	100 a	1.0	67±33.3 a b c	16.4±1.9 a
	150	100 a	1.0	100 c	–
Preventol™	75	100 a	0.5	13±13 a	15.0±1.0 a
	150	100 a	1.0	100 c	–
Delcide™	50	100 a	0	10±5.8 a	12.4±1.1 a b
	75	100 a	0	7±3.3 a	13.4±1.6 a b
	100	100 a	0	7±3.3 a	5.3±0.9 b c
	125	100 a	0	10 a	7.1±0.3 b c
	150	100 a	0.5	17±6.7 a b	6.0±1.2 b c
	175	100 a	1.0	10±5.8 a	5.4±0.9 b c
Kathon™	1	100 a	1.0	80±11.5 c	1.3±0.9 c
	2.5	100 a	1.0	100 c	–
	10	100 a	1.0	100 c	–
F		6.99	–	13.38	10.23
P value		<0.0001	–	<0.0001	<0.0001

Mean values in each *column* followed by different *letters* differ by Tukey's least significance range test at 5%

[x]Biocides tested in microliter per liter of the commercial biocide formulations. Values are mean ± SEM; n=45

[y]Phytotoxicity was scored on a semiquantitative basis at the end of the experiment as 1, 0.5, and 0 for high, moderate, and no phytotoxicity, respectively

(Berg et al. 2010), and induce system resistance as antagonistic bacteria for pathogens (Minkwitz and Berg 2001; Whipps 2001).

Determination of bacterial growth inhibition and biocide susceptibility. The relative bacterial susceptibility to the biocides (MICs) indicated that *S. maltophilia* was more susceptible to Preventol™, Delcide™, Kathon™, and Ipel™ (Table 2), given that these biocides caused visible inhibition of bacterial proliferation at a lower concentration [0.002% to 0.008% (*v*/*v*)] than PPM™, which required 0.032% (*v*/*v*) to restrict cellular multiplication. Comparable results were

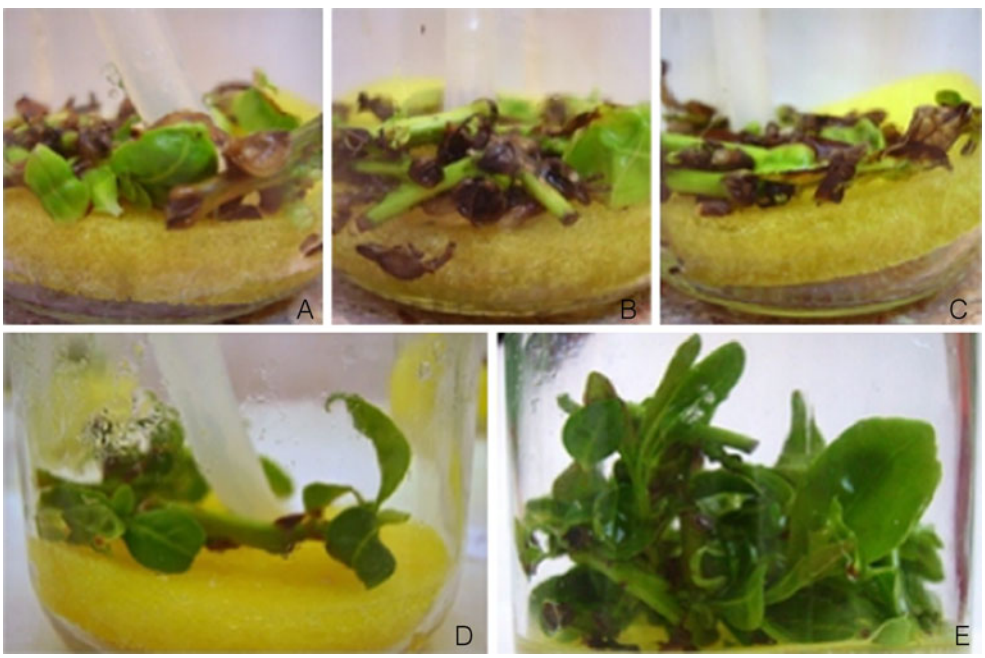

Figure 4. Clean cultures of *I. paraguariensis* treated with Ipel™ (*A*), Preventol™ (*B*), Kathon™ (*C*), PPM™ (*D*), and Delcide™ (*E*).

obtained using the disk-diffusion method (Fig. 3). The precise MBCs represented the minimum concentration required to eliminate the ability to recover the test organism, following plating on bacterial culture medium. Preventol™, Delcide™, and Kathon™ showed the highest biocidal activity (lowest MBCs) against pure suspensions of *S. maltophilia*, whereas PPM™ and Ipel™ required 0.063% (*v*/*v*) of the active ingredients to kill the bacteria. Note that the MIC and MBC of Kathon™ are identical [0.004% (*v*/*v*), suggesting higher biocidal activity; in contrast, Ipel™ needed eight times the MIC to kill the strain.

The effect of biocides on the control of contamination and shoot growth during the multiplication phase of micropropagation in bioreactors. Our results show that the micropropagation phase of *I. paraguariensis* under bioreactor conditions requires adding biocides to the culture medium to restrain early bacterial proliferation that competes for nutrient availability and determines the premature browning and death of the shoots (Table 3). All of the isothiazolone biocides tested were able to control bacterial cell proliferation in bioreactor cultures, with 33% to 100% efficacy (Table 3). However, severe damage was evident in the stems and leaves as a result of phytotoxicity. The addition of Ipel™, Preventol™, or Kathon™ led to early defoliation, which caused explant death (Fig. 4*A–C*). Alternatively, treating infected explants with 100–150 $\mu l l^{-1}$ PPM™ for 35 d produced 67–100% visibly clean cultures, but restricted shoot growth and development (Fig. 4*D*). A measurement of an appropriate effectiveness would be the ability of the biocide to control contamination without adversely affecting the vegetative growth of shoots. Use of Delcide™ at low doses was highly effective in preventing bacterial proliferation in plant tissue cultures of *I. paraguariensis*. Addition of 75 $\mu l l^{-1}$ Delcide™ to the culture medium produced 100% perceptibly clean cultures with no visible damage to the explants (no necrosis or leaf malformation), enhanced the shoot growth rate, and improved the quality of several morphological traits in the regenerated shoot; these results demonstrate the considerable advantages of Delcide™ over other treatments (Fig. 4*E*). More than 90% of the explants survived the treatment, produced 11.5±1.2 phytomers per shoot, and retained a leaf area of 112.4± 3.6 mm^2. Using higher biocide concentrations adversely affected the morphology of the shoots, which turned yellow and necrotic. Histological analyses of untreated infected stems revealed an abundant proliferation of bacterial cells in the intercellular spaces of stem cortical parenchyma during the multiplication phase of micropropagation in bioreactors (Fig. 1*B*). Although the SEM examination confirmed that deep-seated endophytic bacteria in stem segments were not wholly eliminated by Delcide™, the addition of 75 $\mu l l^{-1}$ Delcide™ to the culture medium reduced bacterial proliferation (Fig. 1*C*). Occasionally, bacteria were also found in xylem parenchyma cells (Fig. 1*D*) as formations of small clusters of 8–12 (Fig. 1*E*).

The protocol demonstrated that endogenous *S. malthophilia* populations in contaminated tissue cultures of *I. paraguariensis* were controlled successfully with Delcide™. As both isothiazolones, MIT and CMIT, are circulated in the transpiration stream (Miyazaki et al. 2010), contaminating bacterial cells lodged in intercellular spaces may be difficult to eradicate because of their relatively sheltered locations. This protocol resulted in the successful use of specific biocides to eliminate or control contaminating, endogenous microorganisms, for simplified maintenance of plant tissue cultures.

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