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Detection and antibiotic treatment of *Herbaspirillum huttiense* isolated from *in vitro* explants of *Eucalyptus* sp.

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ABSTRACT - In vitro *assays involving explant cultures aiming the establishment of a genetic transformation protocol for the* Eucalyptus grandis vs. E. smithii *hybrid were hampered by the contamination of the explants. One yeast and one bacterium were isolated. The bacterium was identified as* Herbaspirillum huttiense *(Leifson) Ding and Yokota, with which further analyses were carried out and its response to different antibiotics were evaluated. Amongst 34* in vitro *tested antibiotics, 12 inhibited bacterial growth. Sulfadiazine, streptomycin, kanamycin and penicillin were selected for determination of the minimal inhibitory concentration. Sulfadiazine, streptomycin and kanamycin presented inhibition halos at 256 and 512 mg L^{-1}, while penicillin did not display bacteriostatic effects in concentrations up to 1024 mg L^{-1}.*

Key words: *Eucalyptus*, bacterial contamination, endophyte, plant tissue culture.

INTRODUCTION

Plant tissue culture is contributing to the success of clonal propagation, germplasm conservation and genetic transformation of diverse species, including *Eucalyptus* spp. In spite of the success in the regeneration (Lainé and David 1994, Tibok et al. 1995), genetic transformation (Moralejo et al. 1998, González et al. 2002), clonal propagation (Zobayed et al. 2000), and germplasm conservation (Watt et al. 2000) eucalyptus is still considered recalcitrant for *in vitro* cultivation. Main factors that hamper the eucalypt *in vitro* cultivation are the adequacy of the regeneration protocols, genotype x protocol interactions, production of phenolic compounds, the explant oxidation and endophytic contamination of the explants (Watt et al. 1991, Assis and Teixeira 1999, Dias et al. 2001).

Particularly, the endophytic contamination has been a hindrance for *in vitro* cultivation of *Eucalyptus* sp. (Frossard et al. 1977, Watt et al. 1991, Dias et al. 2001). These contaminants have prejudiced the establishment and multiplication of material *in vitro* and ongoing

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experiments of regeneration and genetic transformation.

Bacteria species of the genera *Agrobacterium*, *Alcaligenes*, *Corynebacterium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Xanthomonas* are frequently associated with *in vitro* cultivated tissues (Buckley et al. 1995, Tanprasert and Reed 1998, Reed et al. 1998, Kobayashi and Palumbo 2000). Additionally, direct effects on the *in vitro* cultivated material are observed similarly to Leifert and Waites (1992) who verified significant dry matter reductions in *Delphium* sp. due to *Erwinia carotovora* contamination.

With the objective of solving this problem, this study aimed the isolation, identification and characterization of the response to antibiotics of an isolated bacteria species from *in vitro* cultures of a *E. grandis x E. smithii* hybrid genotype.

MATERIAL AND METHODS

Plant material

In vitro propagated hybrid plants of *E. grandis* Hill ex Maiden and *E. smithii* Baker were acclimated and kept in a greenhouse. After establishing the plants in 10-liter pots filled with soil fumigated with methyl bromide (50 cc m^{-3}), they were regularly pruned every two months serving as stock-plants to harvest nodal segments to be introduced *in vitro*. The terminal shoot portions with 3 to 4 buds were harvested and disinfected for 15 min in 0.5 g l^{-1} benomyl solution. Subsequently, under aseptic conditions in a laminar airflow hood, they were rinsed thrice in distilled autoclaved water, and transferred to a 2.5% (v:v) sodium hypochlorite solution for 15 min and rinsed again.

The material was sectioned so that each explant consisted in a nodal segment with a bud and transferred to establishment medium. This medium consisted in half the concentration of the MS salts (Murashige and Skoog 1962), 15 g L^{-1} sucrose, 15 g L^{-1} glucose, White's vitamins (White 1943), 100 mg L^{-1} i-inositol, 800 mg L^{-1} PVP 40.000 (polyvinilpirrolidone), 0.1 mg L^{-1} of NAA, 0.5 mg L^{-1} of BAP (6-benzylaminopurine), 7 g L^{-1} agar.

Later, the material was recultivated in multiplication medium consisting in MS salts, vitamins TL (2.5 mg L^{-1} nicotinic acid, 10 mg L^{-1} thiamine, 1.2 mg L^{-1} pyridoxine), 100 mg L^{-1} i-inositol, 800 mg L^{-1} PVP 40.000, 15 g L^{-1} sucrose, 15 g L^{-1} glucose, 0.1 mg L^{-1} of NAA, and 0.15 mg L^{-1} of BAP, 7 g L^{-1} agar. In the experiments involving the adventitious shoot regeneration, leaves of the material were aseptically removed and transferred to induction medium, which differs from the multiplication medium in the regulators 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ (1-phenyl-3-(1, 2, 3-thidiazol-5-yl) urea).

Bacteria isolation

Mucous exudate of the explants was collected and transferred to liquid regeneration medium without addition of growth regulators. Thereafter, it was sown onto solid medium from which isolates of independent colonies were identified and subjected to antibiograms.

Pathogenicity test

A bacteria culture multiplied in solid medium 523 (Kado and Heskett 1970) for 24 h was used to prepare a cell suspension in 0.85% NaCl salt solution adjusted to $DO_{l=550}$=0.1. Three plants of each one of the species *Lycopersicon esculentum* Mill., *Pipper nigrum* L., *Coffea arabica* L. and *Nicotina tabacum* L., containing 4 to 6 pairs of leaves each were inoculated by injection into two leaves and maintained at 25 ºC for a photoperiod of 12 h. Six-months-old *E. grandis* x *E. smithii* plants kept in a greenhouse were inoculated by the same method. The hypersensitivity reaction (HR)-indicator plants were evaluated for 72 h, while the eucalyptus was evaluated on the tenth day after inoculation.

Bacteria identification

Biochemical and Gram tests

Hugh and Leifson's Gram tests and use of asparagine were performed according to Schaad et al. (2001). The bacteria was identified by a Biolog microplate system (GN MicroPlate). For this purpose, a culture obtained from an isolated colony was multiplied in BUGM medium (Biolog Universal Growth Medium) and after 24 h the cells were resuspended in 0.85% NaCl salt solution. The turbidity of the suspension was adjusted to 63% transmittance of which 150 mL were poured into each microplate cavity and incubated at 28 ºC. The measurements were performed with an ELISA reader at 560nm and the results analyzed by the program MICROLOG TM4.01B

Cloning and sequencing of rDNA16S

The rDNA16S of the bacteria was PCR-amplified with the primers R1- AGA GTT TGA TCC TGG CTC AG and R2 – AAG GAG GTG ATC CAG CCG CA (Weisburg et al. 1991). The reaction mix, with a final volume of 25 µL, consisted of 100 ng genomic DNA of the bacteria, 20pM of each primer, 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, and one Taq polimerase unit. The PCR product was subjected to electrophoresis in 1% agarose gel in TAE (1X) buffer for 2 h at 100 volts. The gel was stained with 0.5 µg mL^{-1} ethidium bromide and visualized

under UV light. The bands were eluted and purified with kit QIAquick (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The fragments were cloned in pGEMT and sequenced in an ABI Prism™ DNA sequencer (Perkin-Elmer, UK). The partial gene sequence was compared to the GenBank data base. The phylogenetic tree was constructed based on the distance matrix estimated by the Neighbor-Joining method (Saitou and Nei 1987).

Antibiotics evaluation

Antibiograms

Paper discs (0.5 cm diameter) with different antibiotics were used in the antibiograms (Table 1) by the double-layer technique (Romeiro 2001). A layer of agar - water (2%) was poured onto Petri dishes and then a second layer of semi-solid medium consisting of: 10 g L^{-1} sucrose, 8 g L^{-1} hydrolyzed casein, 4 g L^{-1} yeast extract, 2 g L^{-1} KH_2PO_4, 350 mg L^{-1} $MgSO_4$, and 0.85% agar containing 1% (v:v) bacteria suspension ($DO_{l=600}$=0.4). The discs containing the antibiotics were placed on top of the second layer and the dishes incubated for 24 h at 28 °C without light. The formation of inhibition halos was then evaluated.

Minimum Inhibitory Concentration (MIC)

Sulfadiazine, streptomycin, kanamycin, and penicillin were selected for an MIC determination. The antibiotics were diluted to 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 mg L^{-1} and then 10 µL of each concentration were applied to filter paper discs (Whatman nr. 1) of 0.5 cm diameter. Subsequently, the discs were placed over the medium containing bacterial suspension, as described for the antibiograms. Four replications of each concentration of each antibiotic were carried out. Agar portions from the region of the inhibition halo corresponding to a higher concentration of each antibiotic were transferred to liquid medium and incubated at 28 °C for 24 h. After the incubation, the optic density of the samples was measured in a spectrophotometer ($DO_{\lambda=600}$) and an aliquot was streaked onto dishes containing solid medium, which were incubated again under the above described conditions. The data were subjected to analysis of variance (ANOVA) and the regression equations obtained.

RESULTS AND DISCUSSION

The explants of the hybrid eucalypt presented exudation of microorganisms in multiplication (Figure 1A) and in regeneration experiments (Figure 1B). Along with the release of phenolic compounds, this microorganism contamination is associated with the difficulty of regeneration of the material. Although the eucalypt

Table 1. Relation of the antibiotics and substances with antibacterial activity tested for the control of the endophytic bacteria, concentration/content of the substance on the plates and mean diameter of the inhibition halo

Antibiotics	Content	Mean halo diameter	Antibiotics	Content	Mean halo diameter
	µg $disc^{-1}$	cm		µg $disc^{-1}$	cm
amoxicillin	10	0.0	lincomycin	2	0.0
ampicillin	10	0.0	nalidixic acid	30	0.0
bacitracina	10	0.0	neomycin	30	0.0
carbenicilline	100	0.0	netilmycin	30	1.6
cefadroxil	30	0.0	nitrofurantoin	300	2.5
cephalexin	30	0.0	amicacin (novamycin)	30	1.2
cefalotin	30	0.0	oxacillin	5	0.0
cefotaxime	30	1.7	oxdinic acid	30	0.0
cefoxitin	30	0.0	penicillin	10	1.5
ceftriaxone	30	0.0	polymyxin	300	0.0
chloramphenicol	30	0.0	rifampicin	5	0.0
clindamycin (dalacin)	2	0.0	sulfazotrine	25	2.9
erithromycin	15	0.0	sulfadiazine	300	2.0
streptomycin	10	1.1	sulfonamide	300	3.5
phosphomycin	50	0.0	tetracycline	30	0.0
gentamycin	10	1.0	tobramycin	10	1.4
kanamycin	30	1.3	vancomycin	300	0.0

explants presented oxidation, the contamination seemed to aggravate this picture (Figure 1B). The effective sterilization of the instruments is however equally important in view of the existence of bacteria isolates able to survive immersion in alcohol and heat exposure, and that losses of up to 100% can be observed in material of recently introduced perennial species (Skirvin et al. 1999). These observations are in agreement with Leifert et al. (1989), who emphasized the possibility that contaminants would lead to the death of *in vitro* cultivated material. Besides, the stimulation of the phenolic compound production through injuries (George and Sherrington 1984) may affect the morphogenesis (Durand-Cresswell and Nitsch 1977, Gill and Gill 1994).

In spite of the elimination of the material contaminated during the introduction of the *in vitro* explants, microbial contamination was observed (Figure 1A). This fact can be attributed to the absence of macroscopic symptoms or latent stages of the endophytic microorganisms of the explants (Leifert et al. 1991, Kobayashi and Palumbo 2000).

Leifert et al. (1991) pointed out that the success of the treatment with antibiotics can only be safely predicted after the isolation, identification and sensitivity test of the contaminants. Isolated colonies of the microorganisms present in the exudate of *in vitro* cultivated *Eucalyptus* sp. were therefore obtained, in which the presence of one yeast and one bacteria species was verified. In the present study, the bacterial isolate was identified and its response to antibiotics characterized aiming the control of this contamination in regeneration and genetic transformation protocols of this *E. grandis* x *E. smithii* hybrid genotype. The identification of the yeast is under way. Bacteria and yeasts were also found in *in vitro* cultivated tissues of species such as *Hemerocallis* and *Choisya* (Leifert et al. 1991), *Mentha* sp. (Reed et al. 1995), *Fragaria* sp. (Tanprasert and Reed 1998), *Corylus avellana* and *C. contorta* (Reed et al. 1998), and *Citrus* (Niedz and Bausher 2002) among others.

The bacteria cells had a rod shape, gram negative reaction (Figure 1D), characterized as strict aerobes, and used asparagine as sole carbon source. The bacteria colonies are cream-colored and smooth in medium 523, after 72 h they are 0.5 (0.3-0.77) mm long (Figure 1C), while the cells measure 1.05 (0.89-1.35) μm x 0.24 μm (Figure 1D).

According to the MICROLOG ™4.01B data base, the result of the BIOLOG test indicated *Herbaspirillum huttiense* (Leifson 1962) Ding and Yokota (2004). The identification based on rDNA16S gene sequence resulted in the same species when comparing the partial sequences obtained with 94% identity to the sequences of the *H. huttiense* rDNA16S, isolate ATCC 14670T, access number AB021366 (Figure 2). This bacteria was denominated *Pseudomonas huttiensis* (Figure 2) until its recent reclassification to *Herbaspirillum* genera (Ding and Yokota 2004), which was adopted in this work. This fact, besides the endophyte condition, enlarge the interaction possibilities of the isolate studied with eucalypt plants.

The bacteria did not induce hypersensitivity response in *Lycopersicon esculentum*, *Pipper nigrum*, *Coffea arabica* and *Nicotina tabacum*, after 24 h of incubation, while it induced chlorosis and necrosis in tobacco after 72 h (Figure 1E). Inoculation on the *Eucalyptus* sp. clone from which it had been isolated provoked neither leaf spot symptoms nor exudation at the inoculation point. Elbeltagy et al. (2001) observed that *Herbaspirillum* B501, a genetically modified isolate harboring the *gfp* gene, colonized preferentially the intercellular spaces of vegetative organs, being not found in rice vascular tissues. Similar behavior may be found with the *H. huttiense* isolated from eucalypt.

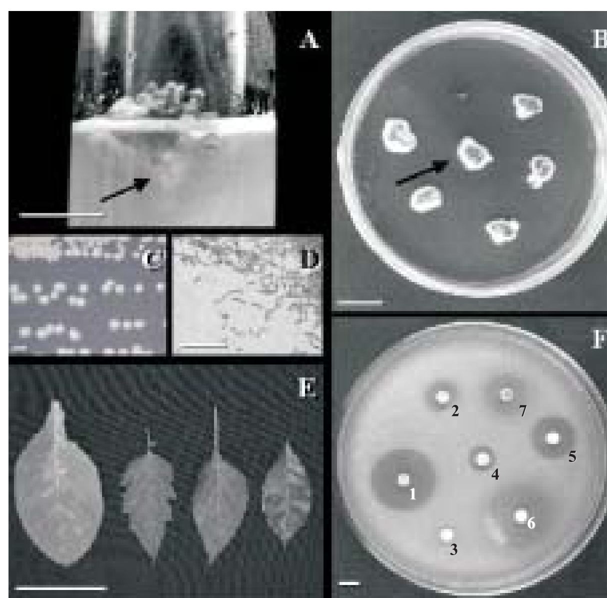

Figure 1. Endophytic contamination in *in vitro* cultivated *Eucalyptus* sp. tissues. **A** - *Eucalyptus* sp. shoot with exudation in multiplication medium (arrow); **B** - Detail of overgrowth of bacteria and/or yeasts in leaf explants in regeneration medium (arrow); **C** - *Herbaspirillum huttiense* colonies in medium 523; **D** - Details of shape and dimensions of the bacteria observed under an optic microscope after stained with safranina; and - Hypersensitivity test for non-host plants (*Nicotina tabacum* L., *Lycopersicon esculentum* Mill., *Pipper nigrum* L., and *Coffea arabica* L.); **F** - Antibiogram realized with the antibiotics nitrofurantoin (1), novamycin (2), oxidinic acid (3), polymixin (4), sulfadiazine (5), sulfonamide (6) and tobramycin (7), details of the inhibition halos Bars: (**A**, **B**, **F**) = 1 cm; (**C**) = 0.1 cm; (**D**) = 10μm; and (**E**) = 10 cm

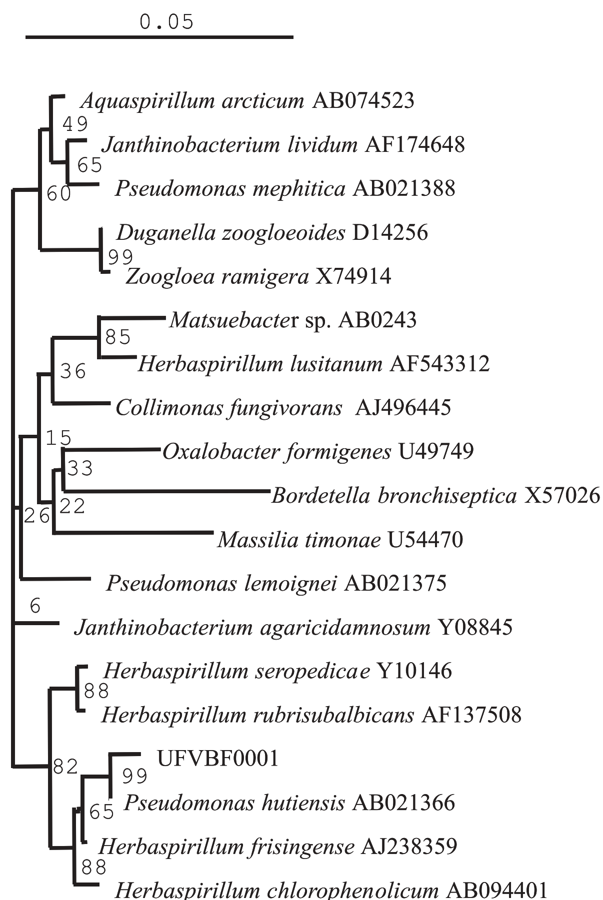

Figure 2. Phylogenetic dendrogram based on the partial sequence of 556 pb of rDNA16S of the bacteria UFVBF0001 showing the correlation with sequences of species from the GenBank-NCBI data base. BOOTSTRAP values computed based on 2000 trees

Although *Herbaspirillum seropedicae* and *H. rubrisubalbicans* cause mottled stripe disease on the sensitive sugarcane variety, *H. seropedicae* was found as an endophyte in ten species of the Gramineae family, whereas *H. rubrisubalbicans* was isolated only form sugar cane. Both were able to fix nitrogen (Baldani et al. 1996). Elbeltagy et al. (2001) also reported evidences of differences in nitrogen fixation depending on the rice species, depending on the variable colonization of the tissues by *Herbaspirillum*.

Other *Pseudomonas* species (Kobayashi and Palumbo 2000) and *Herbaspirillum* were reported as endophytes or diazotrophic endophytes (Sabino et al. 2000) respectively, the occurrence of this species in the present work as endophyte in *Eucalyptus* sp. was not found in the literature. Nevertheless, similarly to the results observed in rice with *Herbaspirillum* (Elbeltagy et al. 2001), the *Herbaspirillum* isolate from eucalypt may result in beneficial responses in eucalypt growth.

Among 34 antibiotics tested for the *H. huttiense* isolate from *Eucalyptus* sp., 12 induced inhibition halos (Table 1, Figure 1F). Baldani et al. (1996) observed that *H. seropedicae* was sensitive to chloramphenicol, erythromycin, streptomycin, gentamycin, kanamycin and tetracyclin, and resistant to penicillin. Divergent from these authors' findings, and despite the different results for penicillin in the quantitative and qualitative tests, choramphenical, erythromycin and tetracyclin did not inhibit *H. huttiense* growth in the present work (Table 1).

The addition of antimicrobial substances to the culture medium is an alternative for the control of these contaminations, however, minimal bactericide concentration must be determined and attention to the phytotoxic potential of these products must be paid. Of the antibiotics that presented inhibition halos (Table 1) sulfadiazine, streptomycin, kanamycin, and penicillin were evaluated in different concentrations to determine the minimal inhibitory concentration (Figure 3). Sulfazotrine, streptomycin and kanamycin presented inhibition halos on concentrations between 256 and 512 mg l^{-1}, while penicillin did not present halos at the tested concentrations (Figure 3).

The optic density reading means ($DO_{\lambda=600}$) of the medium inoculated with portions of the inhibition halos (streptomycin=0.0735; kanamycin=0.1088; penicillin=0.0252 and sulfadiazine=0.0191) were lower than the control medium (without antibiotics) inoculated with the bacteria (0.2429). Nevertheless, all dishes sown with the respective mediums presented bacteria colonies, indicating that their activity was only bacteriostatic. Similarly, Reed et al. (1995) and Tanprasert and Reed (1998) observed bacteriostatic and non-bactericide activity of some antibiotics. Likewise, preliminary experiments with the treatment of explants and shoots with antibiotics and fungicides were not effective for the control of endogen contamination (data not shown).

Still, even if the antibiotics had been effective in the elimination of the bacteria, some of the compounds could not be used regarding their phytotoxicity to plant tissue, kanamycin for example. It is known that experiments to determine the dose that would allow the control of the microorganisms and the regeneration of the tissues should be performed considering the possible effects of antibiotics in the morphogenesis of *Eucalyptus* sp. (Picoli et al. 2005). Although adjustments in the regeneration protocol might be necessary, the authors observed that Timentin® and carbenicilline concentrations of up to 600 mg L^{-1} did not affect the development of calli, while 300 mg L^{-1} cefotaxime had a phytotoxic influence.

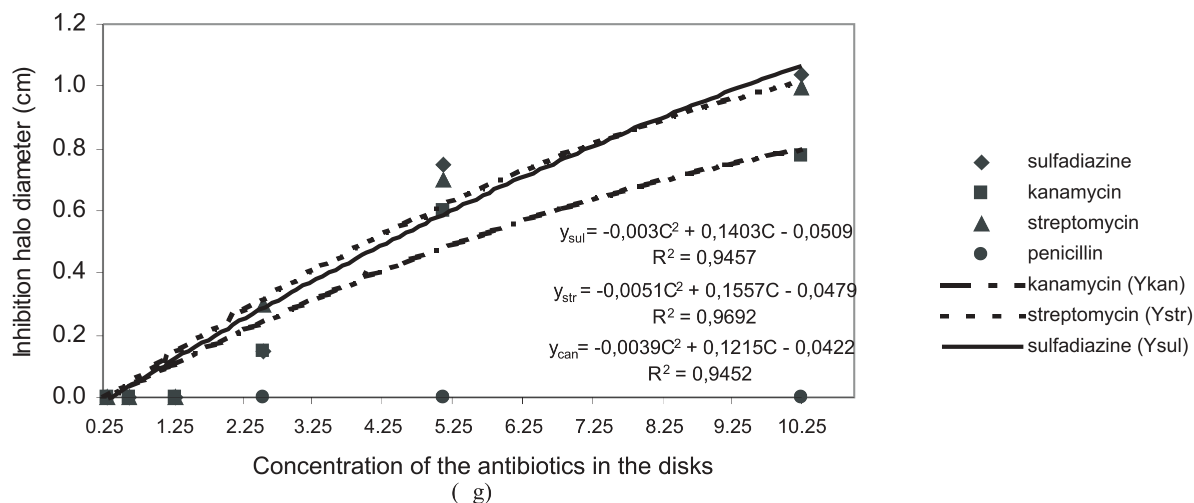

Figure 3. Growth inhibition of *Herbaspirillum huttiense* under increasing concentrations of sulfadiazine, streptomycin, kanamycin, and penicillin. 10 µL of antibiotic solution were poured to each 0.5 cm paper disc

An alternative to overcome this difficulty is the alteration of the pH of the culture medium. Buckley et al. (1995) observed that the sensitivity of *Xanthomonas* sp. and *Agrobacterium* sp. isolates to antibiotics varied according to the pH, being inhibited at pH 7.5. The influence of the pH on the sensitivity to antibiotics was also observed by Leifert and Waites (1992) and Niedz and Bausher (2002). Another option for the control of the endophytic is the treatment of plant tissues with warm water (42-45 ºC), effective in the reduction of endogen contamination of *Lilium* sp. and *Acer* sp. (Langens-Gerrits et al. 1998).

Reed et al. (1998) and Tanprasert and Reed (1998) verified that individually tested antibiotics were inefficient for the majority of the isolates of bacteria under study, however, the mixture of Timentin®, streptomycin and gentamycin was not phytotoxic and presented promising results. Leifert et al. (1991) also verified that the use of antibiotic combinations was effective in the control of contaminants of plant tissues cultivated *in vitro*. Phillips et al. (1981) verified that 50 µg mL^{-1} of rifampicin were sufficient for the control of bacteria present in *Helianthus tuberosus*. Other antibiotics such as nalidixic acid, phosphomycin, streptomycin, penicillin, and chloramphenicol were inefficient against bacteria contaminations of this same species.

In spite of the bacteriostatic activity of the individual antibiotics, experiments with mixtures of these substances are under way in our laboratory. Other perspectives are the use of PPM™ (Plant Preservative Mixture,) substances as well as the alteration of the medium pH value together with the use of antibiotics for the control of this contamination. Observations regarding the phytotoxicity and MIC should also be realized, in view of the costs of the antibiotics treatment and the manageability and reduced risk of the selection of isolates of resistant microorganisms. Although the presence of *H. huttiense* in eucalypt is a difficulty to the hybrid eucalypt *in vitro* culture, its isolation from eucalypt opens perspectives such as nitrogen fixation in a wood plant. Nevertheless, additional studies are necessary to confirm this hypothesis.

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Detecção e tratamento com antibióticos de *herbaspirillum huttiense* isolada de explantes de *eucalyptus sp. in vitro*

RESUMO - *Experimentos de cultura de tecidos vegetais visando a obtenção de um protocolo de transformação genética de um híbrido de* Eucalyptus grandis vs. E. smithii *têm sido dificultados por contaminações presentes nos explantes. Foram isolados uma levedura e uma bactéria dos explantes. A bactéria foi identificada como* Herbaspirillum huttiense *(Leifson) Ding e Yokota, com a qual foi avaliada quanto a sensibilidade a antibióticos. Dentre 34 antibióticos testados* in vitro *no controle de seu crescimento, 12 induziram halos de inibição. Sulfadiazina, estreptomicina, canamicina e penicilina foram selecionados para determinação da concentração inibitória mínima. Sulfadiazina, estreptomicina e canamicina induziram halos de inibição entre as concentrações de 256 e 512 mg L^{-1}, enquanto penicilina não apresentou efeito bacteriostático em concentrações até 1024 mg L^{-1}.*

Palavras-chave*:* *Eucalyptus*, contaminação bacteriana, endófita, cultura de tecidos vegetais.

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