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Special Supplement: Bamboo

In vitro establishment of *Bambusa oldhamii* Munro from field-grown matrices and molecular identification of endophytic bacteria[1]

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ABSTRACT

In plant micropropagation, the establishment stage is difficult, due to the presence of microorganisms in tissues from field-grown matrices, especially for bamboo. This study aimed to establish an efficient asepsis protocol for *Bambusa oldhamii* explants from field plants, as well as to carry out the molecular identification of a possible endophytic bacterial isolate. The explants were exposed to 70 % alcohol, 1 % sodium hypochlorite (NaOCl), 0.1 % mercuric chloride ($HgCl_2$), thiophanate-methyl (Cercobin®) and chlorhexidine digluconate (2 % Riohex®) in different combinations, and introduced into Murashige and Skoog culture medium (solid or liquid), supplemented or not with 4 mL L^{-1} of Plant Preservative Mixture (PPM™), totaling seven treatments. The asepsis and immersion of the explants in the liquid culture medium containing 4 mL L^{-1} of PPM™ visually inhibited the bacterial and fungal growth, allowed the development of shoots with a mean length of 2.2 cm and posterior subcultures, being the best treatment used for the *in vitro* establishment of *B. oldhamii*. The molecular identification of an endophytic bacterium performed by 16S rDNA sequencing allowed to identify the bacterial isolate as *Ralstonia* sp., with 100 % of similarity, and the phylogenetic analysis grouped it with *Ralstonia pickettii.* In addition, the bacterial isolate showed to be sensitive to 4 mL L^{-1} of PPM™ by the minimum inhibitory concentration test.

KEYWORDS: *Ralstonia*, bamboo, micropropagation.

RESUMO

Estabelecimento *in vitro* de *Bambusa oldhamii* Munro a partir de matrizes cultivadas a campo e identificação molecular de bactéria endofítica

Na micropropagação de plantas, a etapa de estabelecimento é dificultada pela presença de micro-organismos em tecidos oriundos de matrizes do campo, especialmente para bambu. Objetivou-se estabelecer um protocolo eficiente de assepsia de explantes de *Bambusa oldhamii* oriundos de plantas em campo e efetuar a identificação molecular de um possível isolado bacteriano endofítico. Os explantes foram expostos subsequentemente a álcool 70 %, hipoclorito de sódio 1 % (NaOCl), cloreto de mercúrio 0,1 % ($HgCl_2$), tiofanato-metílico (Cercobin®) e digliconato de clorexidina (Riohex® 2 %), em diferentes combinações, e introduzidos em meio Murashige and Skoog (sólido ou líquido), acrescido ou não de 4 mL L^{-1} de Plant Preservative Mixture (PPM™), em sete tratamentos. A assepsia e imersão dos explantes no meio de cultura líquido contendo 4 mL L^{-1} de PPM™ inibiram visualmente o crescimento de bactérias e fungos, permitiram o desenvolvimento de brotações com comprimento médio de 2,2 cm e posteriores subcultivos, sendo o melhor tratamento usado para o estabelecimento *in vitro* de *B. oldhamii*. A identificação molecular da bactéria por meio do sequenciamento do 16S rDNA permitiu identificar o isolado bacteriano como *Ralstonia* sp., com 100 % de similaridade, e as análises filogenéticas o agruparam com *Ralstonia pickettii.* Além disso, a bactéria se mostrou sensível a 4 mL L^{-1} de PPM™ pelo teste de concentração mínima inibitória.

PALAVRAS-CHAVE: *Ralstonia*, bambu, micropropagação.

INTRODUCTION

Bamboos are grasses of the Poaceae family, Bambusoideae subfamily, which includes about 1,575 species distributed mainly in tropical and subtropical countries (Singh et al. 2013). Presenting fast growth and perennial leaves, bamboos are widely used as renewable resources for various commercial purposes, such as the construction industry, cellulose and paper industry, textiles, food and bioenergy (Sandhu et al. 2017). *Bambusa oldhamii* Munro is a cultivated bamboo, native to Taiwan (Hsu et al.

1. Received: Jun. 25, 2018. Accepted: Sep. 10, 2018. Published: Mar. 28, 2019. DOI: 10.1590/1983-40632019v4953673.
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2000), that has an outstanding drought resistance. This species produces edible shoots and woody culms constituting 75 % of the total plant biomass (Sanquetta et al. 2015).

Bamboos are propagated by seeds and also vegetatively by rhizomes and culm cuttings (Singh et al. 2013), what is impracticable for a large-scale production of seedlings (> 500,000 plants per year), due to the expensive and laborious nature of the process (Sandhu et al. 2017). Seminiferous propagation is also difficult, due to the long flowering cycles, low seed viability, gregarious plant behavior and formation of highly heterogeneous seedlings (Azzini et al. 1982, Singh et al. 2013). Tissue culture is therefore an excellent alternative for the large-scale production of seedlings (Gielis & Oprins 2002).

Micropropagation of *B. oldhamii* from nodal segments of young plants (one year old) was reported by Thiruvengadam et al. (2011). However, no micropropagation protocol from adult plant explants has yet been reported for this species (Mudoi et al. 2013). Endogenous contamination is one of the main challenges for plant tissue introduction *in vitro*, especially when using explants directly from field conditions (Gielis et al. 2001, Oprins et al. 2004). This type of explant presents large intercellular spaces and cavities, which allow contaminating agents, such as bacteria and fungi, to settle deeply, being difficult to eliminate by disinfection procedures (Sandhu et al. 2017). The biocidal agent Plant Preservative Mixture (PPM™) has been recommended for curbing bacteria and fungal growth *in vitro* (Plant Cell Technologies 2018). The proper concentration of PPM™ has to be tested for each plant tissue culture, as it can be different depending on the species, and its use may have negative effects on the growth and development of plant tissues (Huh et al. 2015).

The development of protocols for the *in vitro* establishment of adult plants is necessary to clone superior genotypes with desirable characteristics. Therefore, the present study aimed to establish a protocol for efficient surface sterilization of primary shoots from adult plants of *B. oldhamii* for *in vitro* establishment and to characterize the associated bacterium at a molecular level.

MATERIAL AND METHODS

Primary shoots of several *B. oldhamii* unmanaged adult clusters (nine years old) were collected at the experimental station of the Universidade Federal do Paraná, in Pinhais, Paraná state, Brazil (25º23'11.8"S and 49º07'33.9"W), in December 2017, from experimental planting (Mognon 2015). Shoots of 2-7 mm diameter had their apex excised, were surface disinfected with cotton soaked in 70 % alcohol and cut in nodal segments of 3 cm in length, containing one node each. These segments were subsequently exposed to seven different combinations of disinfectant agents (Figure 1). These agents and concentrations are: 70 % alcohol (30 s), 1 % sodium hypochlorite (NaOCl) (10 min), 0.1 % mercuric

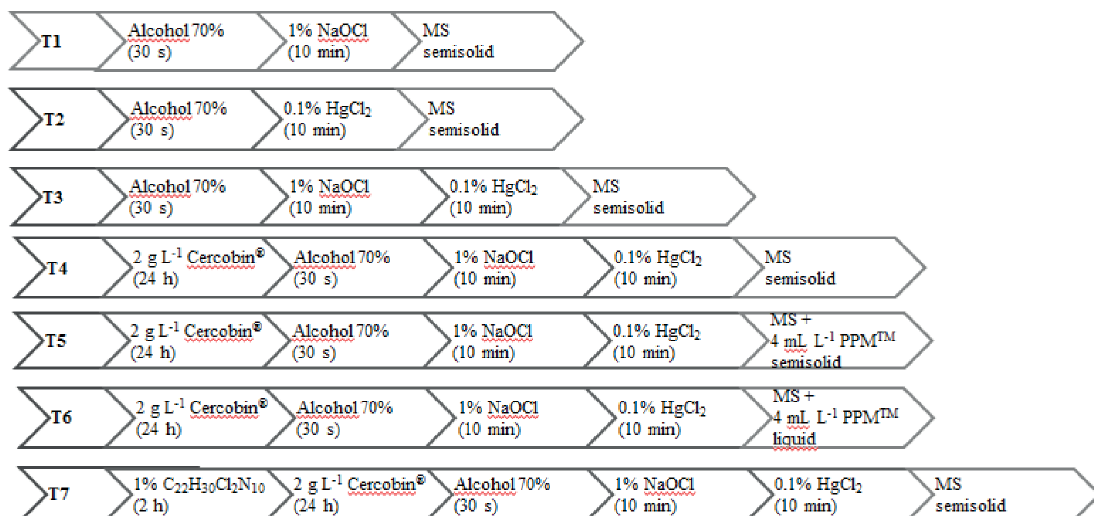

Figure 1. Treatments used for the surface sterilization of *Bambusa oldhamii* nodal segments. MS: Murashige and Skoog culture medium.

chloride ($HgCl_2$) (10 min), 2 g L^{-1} of thiophanate methyl (24 h) and 1 % chlorhexidine digluconate ($C_{22}H_{30}Cl_2N_{10}$) (2 h). After the application of each one of the seven treatments, the explants were rinsed three times with sterile distilled water, after immersion in the germicidal solutions. The exposition times and concentrations had been determined in previous studies. The culture medium was composed of micronutrients and vitamins, according to the original classic formulation of Murashige & Skoog (1962), 30 g L^{-1} of sucrose, and treatments T5 and T6 were supplemented with 4 mL L^{-1} of PPM™. The pH was adjusted to 5.8, and the culture medium gelled with 6 g L^{-1} of agar (Synth®), except for the treatment T6 (liquid medium). Thereafter, 10 mL of culture medium were poured into test tubes (25 mm x 150 mm) and the tubes autoclaved at 120 ºC, for 20 min.

The experimental units were arranged in a completely randomized design, consisting of seven treatments, with four replicates of six test tubes each. After 21 days in the culture medium, the explants were evaluated according to the percentage of explants with bacterial, fungal and total microbial growth, shoot development, average number of shoots per explant and length of newly formed shoots. The data were transformed and subjected to the Bartlett's test, analysis of variance and, when significant, compared by the Tukey test at 5 % of probability, with the Assistat® software (Silva & Azevedo 2016).

In order to validate the efficiency of the disinfection treatments, 100 mg of shoots of visually aseptic explants cultured *in vitro*, selected from each treatment, were ground separately, solubilized in 0.85 % NaCl salt solution and kept refrigerated for 24 h. Then, aliquots of 1 mL were inoculated into Luria-Bertani (LB) solid culture medium (Sambrook et al. 1989), incubated at 29 ºC for 24 h, and bacterial growth was evaluated. The isolates obtained were plated in nutrient agar medium, incubated at 29 ºC for 24 h, and then purified by streaking.

The extraction of bacterial genomic DNA was performed with an UltraClean® Microbial DNA Isolation kit (MoBio®), and the extracted DNA was qualitative and quantitatively evaluated by electrophoresis on 1 % agarose gel and by a Nanodrop 1000 spectrophotometer, respectively.

The polymerase chain reaction (PCR) of the 16S rDNA was performed using the oligonucleotides 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5'GGTTACCTTGTTACGACTT3'). The PCR was performed with 20 ng of DNA sample, 1U of Taq DNA polymerase, 1.5 mM of $MgCl_2$, 1X PCR buffer, 250 μM of dNTPs and 0.5 μM of oligonucleotides (Invitrogen®), in a final volume of 25 μL. The PCR program was carried out with an initial step of denaturation at 95 ºC, for 2 min, followed by 35 cycles of 95 ºC for 40 s, 55 ºC for 2 min and 72 ºC for 1 min, and a final cycle of 72 ºC for 10 min (Klindworth et al. 2013). The PCR reactions were purified in Sephadex G-50 fine (GE Healthcare), and both the amplification and purification products were evaluated by electrophoresis on 1.6 % agarose gel.

Sequencing reactions were performed using a Big Dye Terminator Cycle Sequencing v 3.1 (Thermo Fisher Scientific) kit, according to the manufacturer's instructions, and sequenced in an ABI PRISM® 3700 DNA sequencer (Gomes et al. 2016). The sequences were analyzed and edited using the Bioedit version 7.0 software (Hall 1999) and subjected to BLAST against the National Center for Biotechnology Information (NCBI) database, for characterization at genus level. Thereafter, the sequences were aligned with MAFFT version 7 (Katoh et al. 2017) and subjected to phylogenetic analyzes using the MEGA7 software (Kumar et al. 2016) for Maximum Likelihood Estimation, with 1,000 bootstraps implemented using the evolutionary model of Kimura-2-parameters (1980).

The isolates were inoculated in Nutrient Broth (NB) medium (Neder 1992) and placed in a shaker at 120 rpm, for 24 h. After the growth period, a bacterial suspension of 1.5 x 10^6 cells mL^{-1} was prepared, according to the standards of the McFarland scale. Then, 100 μL of the bacterial suspension were inoculated in nutrient agar medium supplemented with 1 mL L^{-1}, 2 mL L^{-1}, 4 mL L^{-1}, 6 mL L^{-1} or 8 mL L^{-1} of PPM™ and incubated at 29 ºC, for 24 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of PPM™ that inhibited the bacterial growth.

RESULTS AND DISCUSSION

During the *in vitro* establishment, part of the *B. oldhamii* explants indicated contamination with different types of bacteria and fungi, what caused the death of the plant material (Figure 2). However, the reduction of the visual contamination rate was obtained with the use of $HgCl_2$ (T2) and the addition of PPM™ to the culture medium (T5 and T6) (Table 1).

After 21 days in culture, 56 % of the explants showed one shoot, on average, without significant differences among the treatments. The lowest rates of microbial growth were observed in the explants from the T2, T5 and T6 treatments, without significant differences between them. The explants from T5 and T6 formed larger new shoots, when compared to T2 (Table 1 and Figure 3).

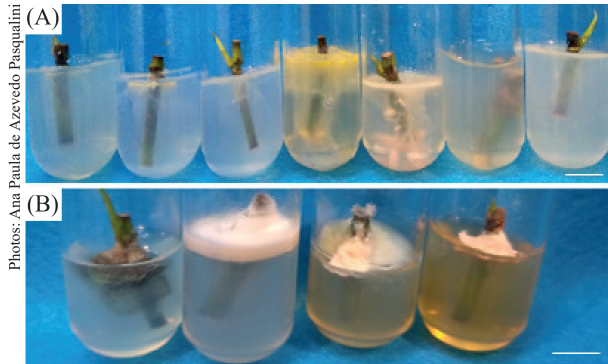

Figure 2. Explants of *Bambusa oldhamii* after 21 days in culture indicating bacterial (A) and fungal growth (B), after different disinfection treatments. Bar: 1 cm.

For T2 (Figure 3A), the total microbial contamination was 12 % and the shoot mean length was 0.8 cm (Table 1), being considered efficient for contamination control. The use of 0.1 % $HgCl_2$ for 10 min indicated a toxic effect on plant tissue, and the shoots were shorter, if compared to treatments T5 and T6 (Table 1). The same treatment, combined with antibiotics and fungicides, was efficient in the initial phase of the *in vitro* culture of *Bambusa balcooa*, where contamination rates were reduced by 80 % (Ray et al. 2017). Our results are in agreement with those of these authors, with a reduction of 88 % in the total microbial contamination (data not shown).

$HgCl_2$ is an inorganic mercury salt soluble in water that shows permeability through tissues due to its liposolubility feature, which facilitates its penetration into the intracellular spaces (Micaroni et al. 2000). In plants, the toxicity of mercury is related to its ability

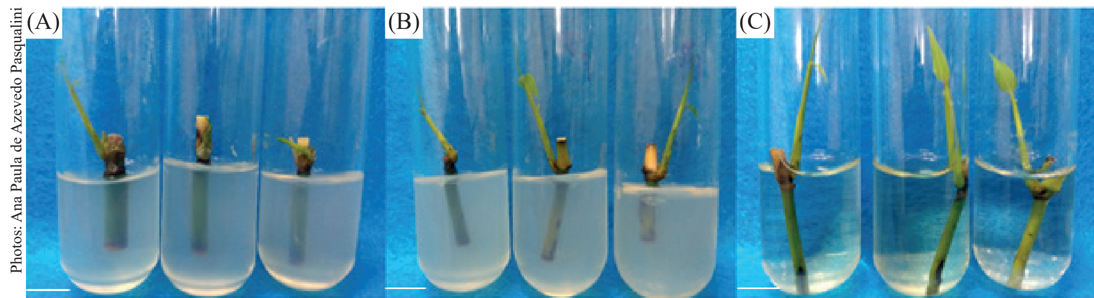

Figure 3. Explants of *Bambusa oldhamii* visually free of bacteria and/or fungi after 21 days in culture. Bar: 1 cm. A) T2: immersion in 70 % alcohol for 30 s and 0.1 % $HgCl_2$ for 10 min; B and C) T5 and T6: immersion in 2 g L^{-1} of thiophanate methyl for 24 h, 70 % alcohol for 30 s, 0.1 % $HgCl_2$ for 10 min and 1 % NaOCl for 10 min. T2 in Murashige and Skoog (MS) culture medium; T5 and T6 in MS medium plus 4 mL L^{-1} of PPM™; T6 liquid; and the others semisolid.

Table 1. Bacterial, fungal and total microbial growth and length of new shoots during the *in vitro* establishment of *Bambusa oldhamii* nodal segments, after disinfection treatments.

Treatments	Bacterial growth (%)	Fungal growth (%)	Total microbial growth (%)	Length of new shoots (cm)
T1	17 A	46 B	63 BC	1.4 AB
T2	8 A	4 A	12 A	0.8 B
T3	8 A	25 AB	33 AB	1.4 AB
T4	58 B	0 A	58 BC	1.1 B
T5	0 A	13 A	13 A	2.3 A
T6	4 A	0 A	4 A	2.2 A
T7	75 B	8 A	83 C	1.4 AB
CV (%)	6.72	6.73	6.83	8.69

* T1: immersion in 70 % alcohol for 30 s and 1 % NaOCl for 10 min; T2: immersion in 70 % alcohol for 30 s and 0.1 % $HgCl_2$ for 10 min; T3: immersion in 70 % alcohol for 30 s, 0.1 % $HgCl_2$ for 10 min and 1 % NaOCl for 10 min; T4, T5 and T6: immersion in 2 g L^{-1} of thiophanate methyl for 24 h, 70 % alcohol for 30 s, 0.1 % $HgCl_2$ for 10 min and 1 % NaOCl for 10 min; T7: immersion in 1 % chlorhexidine digluconate for 2 h, 2 g L^{-1} of thiophanate methyl for 24 h, 70 % alcohol for 30 s, 0.1 % $HgCl_2$ for 10 min and 1 % NaOCl for 10 min. The treatments T1, T2, T3, T4 and T7 were established in Murashige and Skoog medium (MS); T5 and T6 in MS plus 4 mL L^{-1} of PPM™; T6 in liquid MS; and the others were semisolid. ** Means followed by the same letter do not differ by the Tukey test at 5 % of probability.

to replace magnesium in the chlorophyll molecule, directly affecting photosynthesis. $HgCl_2$ may also inhibit the water transport in the transcellular pathway, since many aquaporins are sensitive to this heavy metal (Patra & Sharma 2000). It is important to note that, due to their toxicity and accumulation in the food chain, mercury residues must be properly packed and sent to recycling companies licensed by the competent environmental agencies, for a correct disposal.

$HgCl_2$ is often used as a biocidal agent in bamboo explants, with the concentration and time of exposure varing according to the species. Choudhary et al. (2017) tested $HgCl_2$ for disinfection of *Bambusa balcooa* explants prior to their *in vitro* introduction, and obtained the lowest contamination rates and higher survival rates (87 %) with the use of 0.1 % for 6.5 min. The exposure of explants to $HgCl_2$ at this same concentration was efficient for tissue superficial decontamination of *Bambusa tulda* (Mishra et al. 2008) and *Dendrocalamus asper* (Banerjee et al. 2011). Other concentrations of $HgCl_2$ and exposure times have been reported, such as 0.3 %, for 10 min, for *Dendrocalamus giganteus* (Ramanayake & Yakandawala 1997) and 0.1 %, for 20 min, for *Bambusa vulgaris* (Ndiaye et al. 2006).

The T5 treatment was visually effective in controlling bacterial growth, with no contaminated explants (Figure 3B). The success of this treatment in controlling bacterial contamination is apparently not related to the surface disinfection protocol, which was the same used in the T4 treatment, that had a microbial contamination rate of 58 %. Even though disinfection was performed equally to T5, in T4, the microbial contamination did not statistically differ from the treatments that presented the highest rates (T1 and T7). Therefore, the beneficial effect of T5 is related to the supplementation of 4 mL L^{-1} of PPM™ to the semisolid culture medium. However, for Jiménez et al. (2006), the addition of 2 mL L^{-1} of PPM™ into the semisolid culture medium did not enable the *in vitro* contamination to be curbed during the establishment phase of explants of *Guadua angustifolia* from field conditions.

The success in controlling contamination by the presence of PPM™ in T5 is apparently limited, as this biocide only inhibits the bacterial growth in the culture medium. The development of fungal contamination in the node region positioned above the surface of the liquid culture medium continues to be observed, and often culminates in the death of explants after the first 15 days of culture (Figure 4). Torres et al. (2016) also added PPM™ (2 mL L^{-1}) to the semisolid culture medium for the *in vitro* establishment of *Bambusa vulgaris* and did not observe a significant difference between treatments with or without PPM™, in the control of fungal contamination.

In T6 (Figure 3C), 4 % of the explants showed bacterial growth, but no fungal contamination was detected. In this treatment, the explants were subjected to the same treatment as T4 and introduced into liquid culture medium supplemented with 4 mL L^{-1} of PPM™, remaining submerged for 21 days. The complete immersion of the explants allowed the contact by PPM™ with the total surface of the tissue, inhibiting the development of bacteria (4 %) and fungi (0 %) after 21 days of culture (Table 1). This treatment enabled the bud and shoot development, with an average length of 2.2 cm (Table 1) and subsequent subcultures in culture media containing 4 mL L^{-1} of PPM™. It was considered the most effective treatment, suitable for *B. oldhamii in vitro* establishment.

PPM™ is a broad-spectrum biocide/fungicide for use in plant tissue culture (Plant Cell Technologies 2018). Its formula includes the active compounds chloromethylisothiazolone and methylisothiazolone (Huh et al. 2015). Isothiazolinones are electrophilic biocides, which inhibit the activity of dehydrogenase enzymes that act on the Krebs cycle and in the electron transport, preventing metabolic functions, such as cellular respiration and ATP generation (Williams 2007).

Figure 4. Fungal growth (white arrows) in the nodal region of a *Bambusa oldhamii* segment surface sterilized by immersion in 2 g L^{-1} of thiophanate methyl for 24 h, 70 % alcohol for 30 s, 0.1 % $HgCl_2$ for 10 min, 1 % NaOCl for 10 min and cultivated for 21 days in semisolid MS medium supplemented with 4 mL L^{-1} of PPM™. Bar: 1 mm.

The recommended concentration of PPM™ in culture medium is 2 mL L^{-1} for woody plants. However, this concentration is variable according to the target species and tissue used. For blueberry, *Ilex paraguariensis* and *Citrus* shoots, the best concentrations of PPM™ are 2.5 mL L^{-1}, 3.5 mL L^{-1} and 4.0 mL L^{-1}, respectively (Plant Cell Technologies 2018). Although literature reports indicate deleterious effects of the use of PPM™ in the culture medium, in the present study, the mean number of shoots per explant cultured on media containing this biocide did not differ from other media, and shoot length was superior, without a phytotoxic effect of PPM™.

The efficiency of the disinfection treatments was confirmed by the absence of bacterium growth in a culture medium inoculated with extracts of *B. oldhamii* shoots from all treatments. After plating, bacterial growth was only observed in T7, showing the efficiency of the other decontamination protocols. Therefore, only the bacterial isolate found in T7 was identified by molecular analyzes. The presence of endophytic bacteria in apparently decontaminated plant material may be explained by the fact that these contaminants are located in the intercellular spaces, what prevents biocide penetration, making it difficult to eradicate endophytic microorganisms (Miyazaki et al. 2010).

In addition, in T7, 75 % of the samples were visually contaminated with bacteria, while in the other treatments the percentage of bacterial contamination was less than 58 % (Table 1). The preliminary immersion of the *B. oldhamii* explants in a 1 % solution of chlorhexidine digluconate for 2 h was inefficient in curbing contamination and inhibited the action of the other biocides included in the decontamination protocol. This fact is due to its incompatibility with chlorides, increasing the percentage of bacterial growth in the culture medium. The chlorhexidine digluconate is an antiseptic, antifungal and bactericidal chemical, less effective with Gram-negative microorganisms (Higioka & Barzotto 2013). The bacteria that colonize the tissues of healthy plants without causing obvious symptoms or damage to the host are defined as endophytic (Bacon & Hinton 2007) and may remain localized at the point of entry or spread throughout the plant (Hallman et al. 1997). These microorganisms have been isolated from species such as oak, pear, beet, corn (Lodewyckx et al. 2002) and also bamboo (Ray et al. 2017).

The isolate obtained in T7 was identified as belonging to the *Ralstonia* genus by the analysis of the 16S rDNA region, with 100 % of similarity. This genus is composed of two complexes, one of them being the *Ralstonia pickettii*, formed by *R. pickettii*, *R. mannitolilytica* and *R. solanacearum*. The other lineage is that of *R. eutropha*, formed by *R. eutropha*, *R. gilardii*, *R. paucula*, *R. oxalatica*, *R. taiwanensis*, *R. basilensis*, *R. metallidurans* and *R. campinensis* (Coenye et al. 2003).

In the phylogenetic tree (Figure 5), the bacterial isolate obtained from T7, referred in this study as

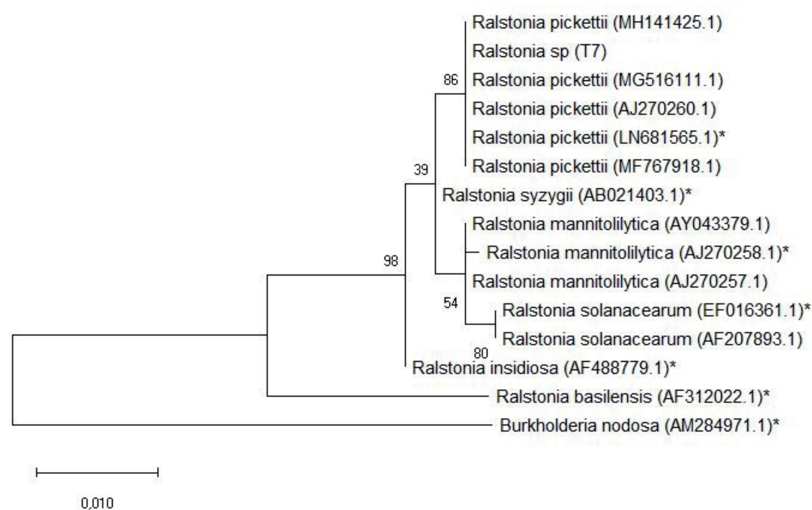

Figure 5. Molecular phylogenetic analysis of *Ralstonia* sp. (T7) obtained from extracts of *Bambusa oldhamii* shoots. The evolutionary history was inferred by using the Maximum Likelihood method, based on the Kimura 2-parameter model. The analysis involved 18 nucleotide polymorphisms. All positions containing gaps and missing data were eliminated. There was a total of 826 positions in the final dataset. * Indicates type sequences.

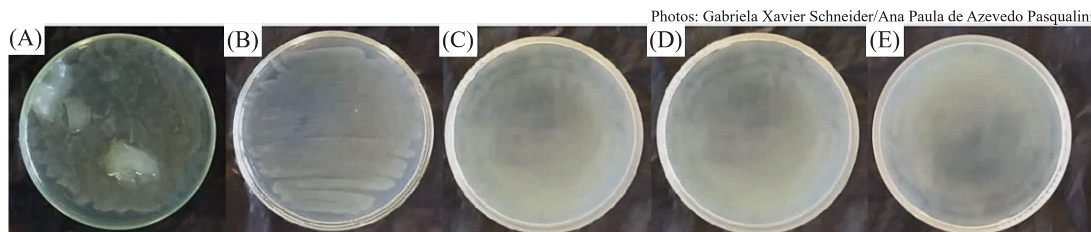

Figure 6. Minimum inhibitory concentration of PPM™ for *Ralstonia* sp. isolated from *Bambusa oldhamii* tissues. A) 1 mL L^{-1}; B) 2 mL L^{-1}; C) 4 mL L^{-1}; D) 6 mL L^{-1}; E) 8 mL L^{-1}.

Ralstonia sp. (T7), was grouped with *Ralstonia pickettii*, indicating a molecular characterization at the species level. *Ralstonia pickettii* is an aerobic bacillus, Gram negative, oxidase-positive, non-fermentative, found in the water and soil (Gilligan et al. 2003). Phylogenetic studies of bacterial isolates also identified a similarity of *Ralstonia pickettii* (NR_114126) with endophytes in sweet sorghum cv. FS501 (Fretes et al. 2018). However, molecular analyses are needed for other gene regions, in order to validate this data.

The isolate was also subjected to the minimum inhibitory concentration test for PPM™ at 1 mL L^{-1}, 2 mL L^{-1}, 4 mL L^{-1}, 6 mL L^{-1} and 8 mL L^{-1}, and 99.9 % of the bacterial population growth was inhibited by concentrations $\geq$ 4 mL L^{-1}.

CONCLUSION

An efficient decontamination method was developed for *Bambusa oldhamii* shoots obtained from primary shoots of adult plants. In the most successful treatment, the explants were completely immersed in liquid MS medium supplemented with 4 mL L^{-1} of PPM™. The success of this protocol for this species is apparently associated with explant submersion in the liquid medium containing a specific concentration of PPM™. In addition, the *B. oldhamii* explants were contaminated by endophytic bacteria phylogenetically grouped with *Ralstonia pickettii*, which were sensitive to 4 mL L^{-1} of PPM™.

ACKNOWLEDGMENTS

The authors thank Dr. M. P. Guerra, for the project initiative "Technologies for the Sustainable Development of the Bamboo Production Chain in Southern Brazil - Subproject: Macro and Micropropagation and *In Vitro* Conservation of Bamboo Germplasm"; Dr. C. R. Sanquetta, for providing the plant material; Dr. V. A. Vicente, for laboratory support; V. Holtman, for the installation of the experiments; E. Bagyary, for editing the manuscript; Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior - Capes (Code 001) and MCTI, for financial support.

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