Plant Cell Culture & Micropropagation

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Use of antimicrobial agents and ultrasound to control endogenous contaminants in the micropropagation of a hybrid clone of *Eucalyptus urophylla* x *E. globulus*

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ABSTRACT

Micropropagation is a technique widely applied aiming at the large-scale multiplication of selected individuals. However, challenges such as endogenous microbial contamination can lead to substantial losses of plant materials. Endophytic contamination consists of resident microorganisms that actively participate in the plant's metabolism, making them difficult to control. In this context, the use of antimicrobial agents in plant tissue culture has increased as an alternative approach to optimize clonal cleaning. Additionally, the application of ultrasound (sonication) may enhance the penetration and effectiveness of these agents within plant tissues. Therefore, the present study aimed to evaluate the effects and efficacy of disinfectant agents combined with ultrasound in controlling endophytic microbiota during the micropropagation of a hybrid clone of *Eucalyptus urophylla* × *Eucalyptus globulus*. Explants were inoculated into JADS culture medium supplemented with two β-lactam antibiotics (Timentin® and Cefotaxime®) and two biocides [PPM® (Plant Preservative Mixture) and FZ (Fungozur B7DM)], combined with three ultrasound exposure times (0, 9, and 18 s), for a total of 15 treatments, including the control. At 15 and 30 days of *in vitro* culture, survival rate (%), contaminated area (mm²), rooting rate (%), height, shoot length (mm), and callus formation (%) were evaluated. The use of FZ or PPM® resulted in lower contamination rates; however, FZ promoted taller and more developed plantlets. The use of ultrasound sonication increased the level of contamination and did not significantly affect explant development. For clonal cleaning of the *Eucalyptus* hybrid, the use of FZ without sonication is recommended, regardless of the duration of *in vitro* culture.

Keywords: Disinfestation, Clonal Cleansing, Endophytic, Biocide, Sonication.

INTRODUCTION

The remarkable success of exotic forest species in Brazil is associated with factors such as well-developed clonal forestry programs, continuously evolving genetic improvement strategies, hybrid production and selection of elite clones, as well as advances in clonal control technologies. Micropropagation is an important technique in clonal forestry, enabling the large-scale production of genetically selected plantlets derived from a single explant, based on the principles of cellular pluripotency (Xavier et al., 2021).

The initial stages of micropropagation include the selection of plant material, disinfestation, and the establishment of explants in an appropriate nutrient medium under aseptic conditions (Micheli et al., 2020). Contamination by microorganisms represents one of the main challenges, limiting the establishment of many cultures under *in vitro* conditions (Leão et al., 2020). Therefore, aseptic conditions are essential, requiring a suitable laboratory environment and cultivation under sterile conditions—through autoclaving or other effective sterilization methods—to ensure the success and maintenance of *in vitro* cultures.

The presence of contaminants in tissue culture represents a major limitation for many species or genetic materials, as the culture medium

https://doi.org/10.46526/pccm.2026.v21.001
Received in November 4, 2025 and approved in April 28, 2026
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also provides a favorable environment for microbial growth due to its high sucrose and nutrient content elevated humidity (Nguyen et al., 2020). Endophytic contaminants are microorganisms that inhabit plants or plant tissues without causing apparent disease symptoms; however, they can reduce the growth and overall health of host plants (Vandana et al., 2024; Polesi, 2020; Tessema & Moges, 2021). Moreover, they are difficult to detect and eliminate.

Nonetheless, the use of chemical agents can be an effective strategy for clonal cleansing of micropropagated plants, aiding in controlling and eliminating endophytic contaminants (Polesi, 2020; Kim et al., 2025). This approach strengthens the *in vitro* preservation of genotypes with valuable superior traits, thereby safely supporting genetic improvement programs and the production of plantlets with high phytosanitary quality (Souza et al., 2023).

The use of antibiotics, bacteriostatics, biocides, and other chemical agents to control endogenous contaminants is employed in tissue culture to inhibit or manage the growth of microorganisms that were not completely eliminated during disinfestation or that remain latent within plant tissues (Gammoudi et al., 2022; Leão et al., 2020). Such agents act efficiently and directly on the contaminating organisms without affecting their host (Dinçer & Dündar, 2023).

In this context, different antibiotics such as Timentin®, Cefotaxime®, and Carbenicillin have stood out in clonal cleaning during *in vitro* culture. Their addition to the culture medium or immersion of explants in these compounds allows the inhibition of growth or elimination of endophytic bacteria (Kim et al., 2025; Tessema & Moges, 2021). In *Cannabis sativa*, seed germination in a culture medium containing 300 mg L^{-1} of Timentin® enabled complete contamination control without causing damage to seedling development (Kim et al., 2025). In a cherry rootstock hybrid (*Prunus cerasus* L. × *P. canescens* L.), when evaluating the efficacy of eight antibiotics in inhibiting bacterial growth, Cefotaxime provided bacterial control, being second only to tetracycline (Liang et al., 2019).

Despite their potential in several crops, bacteria may remain latently present within plant tissues, and contamination may reappear at later stages, since some compounds act only as growth suppressors and do not promote complete elimination (Miyazaki et al., 2010; Romadanova et al., 2022; Izarra et al., 2020).

In this context, biocides have emerged as an important alternative due to their broad-spectrum nature, resistance to high temperatures, and ability to inhibit essential metabolic processes in microorganisms, such as the Krebs cycle and electron transport. Through diffusion across the cell membrane in bacteria and the cell wall in fungi, these compounds block enzymatic activity, ultimately leading to cell death (Compton & Koch, 2001; Romadanova et al., 2022; Silva et al., 2020; Morley, Kapur & Charlton, 2005).

Thus, isothiazolinone-based biocides, such as FZ (Enzur S/A, Fungozur B7DM: 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) and PPM® (Plant Preservative Mixture; 5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone), have gained prominence in several fields due to their antibacterial, antifungal, and antiviral activity (Silva et al., 2020; Maienfisch & Edmunds, 2017).

In six apple cultivars, the use of culture medium supplemented with PPM® (0.2% v/v) enabled the control of the endophytic bacterium *Bacillus megaterium* without negatively affecting *in vitro* culture growth (Romadanova et al., 2022). In *Casuarina equisetifolia*, decontamination of apical microcuttings in liquid medium under agitation for three days, supplemented with 8 mg L^{-1} of a biocide with the same chemical composition as FZ, followed by transfer to semisolid culture medium containing 6 mg L^{-1} of the same compound, resulted in reduced contamination and high explant survival (Putri et al., 2020).

Additionally, the sonication (ultrasound) technique generates high-frequency acoustic waves that produce physical, mechanical, and chemical effects in solutions and materials (Dobránszki, 2021). Sonication is widely used for contaminant removal in cleaning processes and has proven effective in reducing contamination across various materials (Chong et al., 2021; Shaofeng et al., 2021). In the context of micropropagation, ultrasound may represent an effective approach for eliminating microorganisms and reducing persistent endogenous contamination. Therefore, the combination of ultrasound with antimicrobial agents may enhance complete disinfestation and clonal cleaning of plant material.

Sonication has also been highlighted in the decontamination of plant-derived materials, such as fruits and vegetables, enabling contaminant removal through acoustic cavitation during liquid propagation (Yu et al., 2024; Chong et al., 2021; Shaofeng et al., 2021). This technique presents considerable potential due to its low equipment cost, operational simplicity, high degree of automation, and high cleaning efficiency (Jiang et al., 2020).

This process promotes cuticle removal and the formation of erosions on the cell surface, allowing the penetration of water, nutrients, disinfecting agents, and other compounds into plant tissues (Silva & Dobránszki, 2014). However, excessive exposure may result in cell death and tissue necrosis (Muratova & Papikhin, 2018). Therefore, the application of sonication depends on the cell type and the presence of physical barriers, such as liquid medium or semisolid matrices, as it may cause irreversible cellular damage or induce cell lysis (Rajewska & Mierzwa, 2017; Gopalakrishnan et al., 2022).

Prolonged exposure may play a significant role in the morphogenesis of various plant species, promoting, for instance, callus formation (Király, Farkas & Dobránszki, 2025). In grapevine (*Vitis vinifera* L.), ultrasound application for 120 seconds enhanced somatic embryogenesis traits, including callus formation, size, and mass, as well as increasing embryo germination percentage (Farrokhzad & Rezaei, 2016). In apple, ultrasound treatment for 60 seconds applied to leaf explants promoted callus formation and shoot regeneration (Muratova & Papikhin, 2016). Therefore, for the control of endophytic microorganisms without impairing plant development and growth, shorter exposure periods may be essential.

In the context of micropropagation, ultrasound can be an effective technique for eliminating microorganisms and reducing persistent endogenous contamination. Thus, combining ultrasound with antimicrobial agents may enhance complete disinfestation and clonal cleaning of plant material. The present study aimed to evaluate the effects and efficacy of antimicrobial agents and ultrasound in controlling endophytic contamination during the micropropagation of a hybrid clone of *Eucalyptus urophylla* × *E. globulus*, seeking to improve the *in vitro* propagation process by enhancing microbial contamination control and increasing the quality of the propagated material.

MATERIAL AND METHODS

The experiment was conducted at the Tissue Culture Laboratory II (LCT II) of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) at the Federal University of Viçosa, in Viçosa, Minas Gerais state, Brazil.

The plant material exhibiting endophytic contamination (explants) was obtained from an *in vitro* cultivated hybrid clone of *Eucalyptus urophylla* × *E. globulus* maintained in the Germplasm Bank of LCT II, at the 58th subculture (30-day subculture interval). The explants were propagated in test tubes (25 × 150 mm) containing 10 mL of JADS culture medium (Correia et al., 1995), supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 800 mg L^{-1} PVP (polyvinylpyrrolidone), 0.3 mg L^{-1} BAP (6-benzylaminopurine), and 0.01 mg L^{-1} NAA (α-naphthaleneacetic acid), solidified with 5.5 g L^{-1} agar (PhytoTech Labs - Lenexa, KS, US). The culture medium was adjusted to pH 5.8 before adding agar, then autoclaved for 20 minutes at 121 °C, and pressure at 1.0 Kgf cm^{-2}.

The explants used for the experiment were standardized to 1.0 cm in length, containing at least two pairs of fully expanded leaves and approximately three lateral shoots measuring about 0.5 cm in length, with no callus formation at the base. Two antibiotics were evaluated, Timentin® [PhytoTech Labs -Lenexa, KS, US; Ticarcillin Disodium Salt/ Potassium Clavulanate mixture (15:1)] and Cefotaxime® (PhytoTech Labs - Lenexa, KS, US; Sodium Salt, Potency: 948 μg mg^{-1}), at concentrations of 400 μg mL^{-1} and 250 μg mL^{-1}, respectively, along with two biocides: (i) PPM® (Plant Preservative Mixture – Plant Cell Technology, Inc., Washington, DC, US; 5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone) at a concentration of 2 mL L^{-1}; and (ii) FZ (Enzur S/A - Montevideo, Uruguay, Fungozur B7DM: 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) at a concentration of 0.5 mL L^{-1}.

The explants were placed completely submerged in sterile 50 mL Falcon tubes, each containing 10 mL of liquid JADS culture medium supplemented with the antimicrobial agent corresponding to the treatment. Subsequently, sonication pulses of 9 and 18 seconds were applied using an ultrasonic cleaner (B1210E-Mt Bransonic Ultrasonic Cleaner, Branson Ultrasonics Corp., Brookfield, CT, USA) at 80 W power and 47 kHz ± 6%. After sonication, the explants were inoculated onto semisolid JADS culture medium under the previously described culture conditions.

The explants were then isolated and placed in test tubes containing the previously described gelified JADS culture medium, supplemented with the antimicrobial agents. The experiment was maintained for 30 days in a growth chamber at 25 ± 2 °C with an irradiance of 60 µmol m^{-2} s^{-1} provided by fluorescent lamps and a 16 h photoperiod. After explant inoculation, the tubes were sealed with polypropylene caps. At 15 and 30 days, the following parameters were evaluated: survival rate (%), contaminated area (mm^2), rooting rate (%), callus formation (%), shoot length (cm), and lateral shoot length (mm).

The experiment was conducted in a completely randomized design (CRD) using a 5 × 3 factorial scheme, consisting of four antimicrobial agents (Timentin®, Cefotaxime®, PPM®, and FZ) plus a control, combined with three sonication durations (0, 9, and 18 s) (Table 1). Sixteen replicates were used, with each experimental unit consisting of a single plant.

Table 1: Schematic of the treatments, combining antimicrobial agent and sonication time. s = seconds.

Treatments	Sonication time (s)	Antimicrobial agent
1	0	Without antimicrobial agent
2	0	Timentin®
3	0	Cefotaxime®
4	0	PPM®
5	0	FZ
6	9	Without antimicrobial agent
7	9	Timentin®
8	9	Cefotaxime®
9	9	PPM®
10	9	FZ
11	18	Without antimicrobial agent
12	18	Timentin®
13	18	Cefotaxime®
14	18	PPM®
15	18	FZ

The contaminated area was determined using the formula for the area of an ellipse, as contamination typically occurs in an irregular circular shape. Using a caliper, the length (A) and width (B) of the contamination were measured to determine the diameters. These measurements were then converted to radii and applied to the formula: Area = Radius (A) × Radius (B) × π.

To identify the bacterial genus present, 20 samples were collected from the *in vitro* plants and macerated in 1 mL of 1X PBS buffer. The resulting suspension was streaked onto Petri dishes containing 30 mL of LB (Luria Bertani) (Bertani, 1952) culture medium and incubated overnight at 28 °C in a BOD incubator to obtain pure colonies. Total DNA was extracted from the samples using the Wizard® Genomic - Madison, WI, US - DNA Purification Kit according to the manufacturer's protocol. The DNA was diluted to 15 ng µL^{-1} for PCR (Polymerase Chain Reaction) preparation. The PCR product was purified, and the bacteria were identified at the genus level.

The data were processed using the GENES software (Globule version), subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test at 5% significance. For survival, rooting, and callus formation, the data were expressed as percentages relative to the absolute values.

RESULTS AND DISCUSSION

PCR analysis indicated that the microorganism present in the micropropagated cultures of the *Eucalyptus urophylla* × *E. globulus* clone belonged to the genus *Bacillus* sp. The use of different chemical agents for controlling endophytic contamination proved effective, with FZ application without sonication being particularly notable in cultures up to 15-d-old. However, no statistical differences were observed when ultrasound was combined with antibiotics. Statistically significant differences were detected for the evaluated traits of plant height and shoot development.

At 15 days of *in vitro* culture, the contaminated area differed significantly among treatments (Figure 1A). In the absence of sonication (0 s), treatments containing PPM® and FZ showed the lowest mean contaminated areas, indicating reduced contamination. At sonication times of 9 and 18 s, the lowest contamination levels were observed in treatments with Timentin®, PPM®, and FZ.

Treatments with FZ and PPM® stood out across all three sonication durations, showing lower contamination rates than the other disinfectant agents. Moreover, the absence of sonication (0 s) under these conditions allowed better control of *Bacillus* sp., reducing the contaminated area. In other words, when antimicrobial agents are applied, avoiding sonication is the most effective strategy for controlling contamination. The ultrasound technique provides relevant benefits for *in vitro* culture, including the control of bacterial and fungal contamination, as well as promoting callus development and shoot and root production (Király, Farkas & Dobránszki, 2025). However, its use generally optimizes plant tissue sterilization protocols and is more effective when combined with antibiotics, biocides, disinfectants, and detergents (Yu et al., 2024).

At 30 days of *in vitro* culture, the contaminated area also showed significant differences at the 5% level (Figure 1B); however, the disinfectant agents differed only under the 9 and 18 s sonication conditions. Likewise, at 9 s of sonication, PPM® and FZ resulted in lower mean contamination levels than the other antimicrobial agents. For the 18 s sonication treatments, only FZ showed a significant difference, exhibiting the lowest contamination.

The similarity between the agents PPM® and FZ is due to the fact that both are biocides with similar active ingredients. Several studies have employed PPM across various cultures, including in the micropropagation of *Colubrina glandulosa*, where its use promoted higher survival rates of nodal segments (Hass et al., 2022). Similar results have been observed in the micropropagation of various native woody species (Silva et al., 2025), as well as in introduced species; for example, Grimaldi and Bastos (2022) demonstrated its efficiency as an anticontamination agent in *Pyrus communis*. Thus, the use of biocides such as PPM® and FZ has proven effective for disinfestation and for controlling endogenous bacterial contamination. Their incorporation into culture media offers advantages, including the potential replacement of antibiotics, and they are heat-stable, allowing autoclaving without loss of biocidal properties (Romadanova et al., 2022).

Biocides differ from antibiotics in that they are generally less specific and, therefore, less likely to induce bacterial resistance (Jones & Joshi, 2021). According to Ventola (2015), antimicrobial resistance is a growing global problem, and the search for new antimicrobial agents is a significant area of interest in microbiological research.

At 15 days of *in vitro* culture, there was a statistically significant difference at the 5% level for the height of plantlets of the *Eucalyptus urophylla* × *E. globulus* clone (Figure 1C). When 9 s of sonication was combination with the biocide FZ, the lowest mean height was observed (9.3 mm). For 18 s of sonication, Timentin® treatment was the only one to show a significant difference, with the highest mean height (11.5 mm). When evaluating the growth of the *Eucalyptus* clone under control conditions across different sonication times, no statistically significant differences were observed, indicating that ultrasound does not affect the development of this genetic material.

At 30 days of *in vitro* culture (Figure 1D), without ultrasound, the application of FZ resulted in the highest mean height (12.7 mm). For 9 s of sonication, the control and Cefotaxime® treatments showed the highest mean heights (12.4 and 12.8 mm, respectively). For 18 s of sonication, the PPM® treatment exhibited the lowest mean height (9.9 mm).

Regarding shoot elongation, no statistically significant differences were observed among the disinfectants at 0 and 18 s of sonication (Figure 1E). Therefore, it can be inferred that the mere addition of these agents to the culture medium does not affect shoot formation. However, at 9 s of sonication, the highest mean number of shoots was observed in the control treatment, without disinfectant agents.

At 30 days of culture, the use of disinfectants resulted in greater shoot lengths, with only the control differing from the others (Figure 1F). When subjected to 9 s of sonication, the PPM® and FZ treatments showed the lowest mean shoot lengths (11.7 and 12.2 mm, respectively), while at 18 seconds of sonication, no significant differences were observed among the treatments.

Regarding survival rate, at 15 days of in vitro culture, no significant differences were observed among treatments, with mean values above 93.7% (Figure 1G). However, at 30 days, two treatments exhibited survival rates below 93.7%. The Timentin® treatment combined with 9 s of sonication showed a survival rate of 87.5%, whereas the FZ treatment combined with 18 s of sonication resulted in a survival rate of 56.2%, which was significantly lower than the other treatments (Figure 1H).

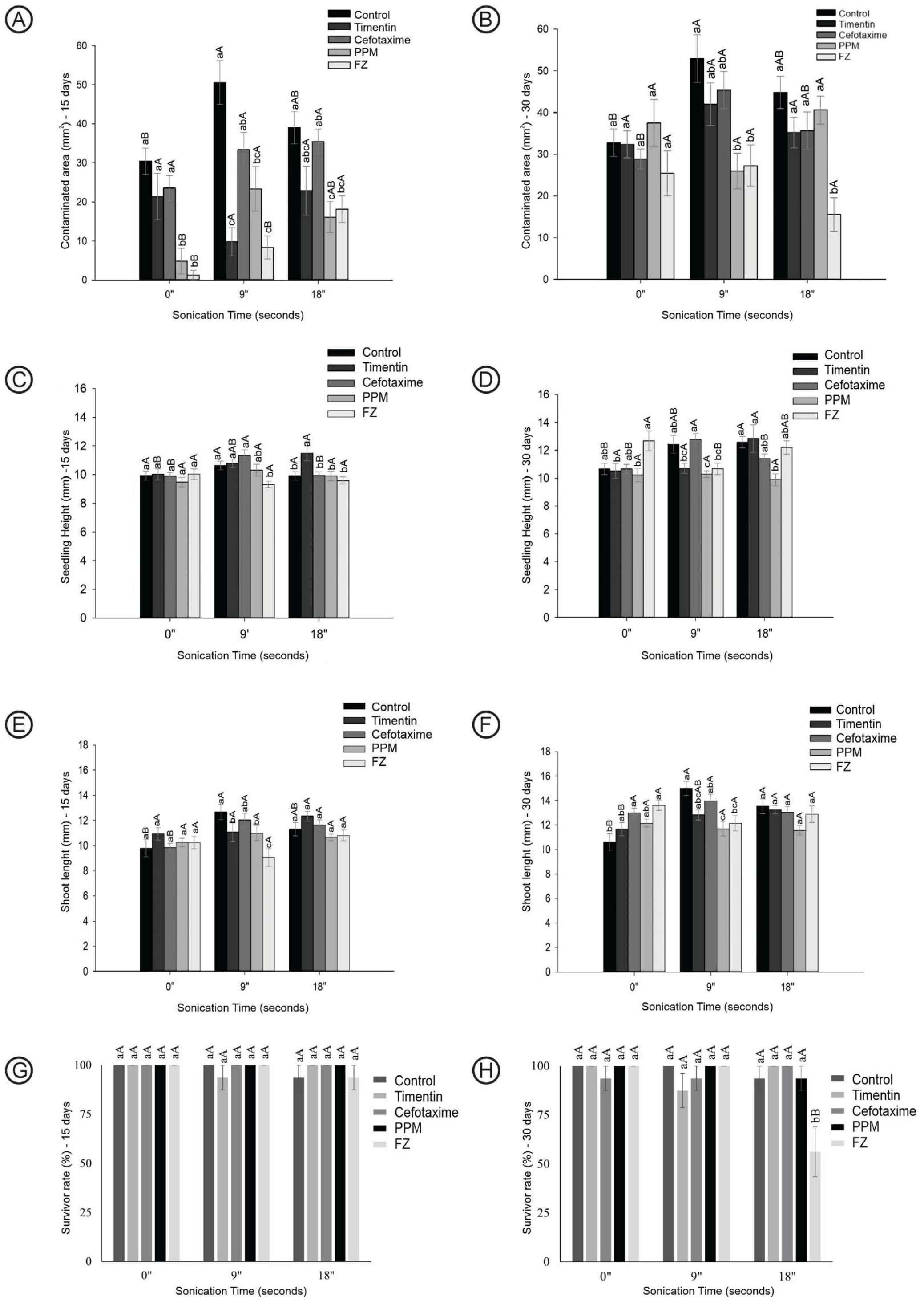

Figure 1: Effect of disinfectant agents at different sonication times on the clonal cleaning of *Eucalyptus urophylla* × *E. globulus*. Contaminated area at 15 days of culture (A) and 30 days of culture (B); seedling height at 15 days (C) and 30 days (D); shoot length at 15 days (E) and 30 days (F); and survival rate at 15 days (G) and 30 days (H). Identical lowercase letters indicate no significant differences among disinfectant agents at each sonication time, according to Tukey's test at the 5% significance level. Identical uppercase letters indicate no significant differences among sonication times for each disinfectant agent, according to Tukey's test at the 5% significance level.

Regarding callus formation and rooting rates, the data did not meet the assumptions of normality. Callus formation occurred in 100% of the explants, whereas root formation was absent under all evaluated conditions at both 15 and 30 days of *in vitro* culture. This outcome was expected, given the absence of rooting-inducing treatments, highlighting the importance of prior clonal cleaning. Although callus formation was observed, the lack of root development indicates the need to explore alternative strategies or to supplement the culture medium with rooting-promoting substances to enhance this process, particularly because the studied genetic material shows difficulty in adventitious rooting due to its woody hybrid nature. Adequate rooting is essential for successful plant micropropagation, as it ensures the production of healthy and vigorous plantlets. Furthermore, the success of micropropagation is closely associated with effective clonal cleaning procedures that enable the production of pathogen-free plants.

The results of this study showed that, in the absence of sonication, FZ was significantly more effective. When sonication was applied for 9 s, the absence of disinfectant agents or the use of cefotaxime appeared to be more suitable, whereas for 18 s, the control treatment or the use of Timentin® was recommended. These findings suggest that sonication did not enhance the antimicrobial activity of the agents in controlling endophytic contamination and raise questions regarding the usefulness and necessity of this procedure during plant micropropagation.

CONCLUSIONS

In the micropropagation of the *Eucalyptus urophylla* × *E. globulus* clone, the use of FZ without sonication is recommended, regardless of the *in vitro* culture duration, as it reduces contamination rates without negatively affecting plant development. The application of disinfectant agents and sonication did not impact the micropropagation of the clone. The microorganism present in the micropropagated cultures of this clone was identified as an endophytic *Bacillus* sp. Overall, the use of biocides such as PPM® and FZ proved promising for controlling endophytic contamination, offering an alternative to antibiotics. However, further research is needed to improve the rooting process of explants and to better investigate the role of ultrasound in the efficacy of antimicrobial agents during plant micropropagation.

ACKNOWLEDGMENTS

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES – Finance Code 001), the Research Support Foundation of the State of Minas Gerais (FAPEMIG; grant no. APQ-01433-22), the National Research Council for Scientific and Technological Development (CNPq) and the Forest Research System (SIF) for financial support. The authors also thank Department of Forest Engineering, and Tissue Culture Laboratory II, and Forest Pathology Laboratory, both of the Institute of Biotechnology Applied to Agriculture at the Federal University of Viçosa (UFV).

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