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Growth promotion mediated by endophytic fungi in cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids

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Eucalyptus cultivation has expanded considerably in Brazilian systems, leading to the current search for technologies to optimize growing conditions and the production of seedlings in nurseries. Based on the understanding that the development of tree species such as *Eucalyptus* sp. can be influenced by endophytic fungi that act directly as plant growth-promoting species, cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids grown from minicuttings we stimulated with three species of endophytic fungi and the effects of inoculation on seedling growth was evaluated. Strains of *Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp. were forced to colonize the root system of the plants, which were continuously maintained under protected cultivation. Inoculation of the symbionts had positive effects on stem length, stem diameter and the fresh and dry biomass of the treated plants. Non-inoculated plants presented a shorter stem length than the plants treated with any of the endophytic species. The cloned seedlings inoculated with *Trichoderma* sp. exhibited the greatest stem measurements at 120 days after transplanting. The seedlings inoculated with *Fusarium* sp. displayed a greater number of leaves than the other seedlings as well as greater amounts of fresh and dry biomass. The authors also conducted quarterly evaluations of the increment in seedling growth promoted by the inoculants, which were more effective in the early stages, up to 60 days after transplanting.

Key words: Inoculants, microorganisms, minicutting, tree species.

INTRODUCTION

Many microorganisms, especially those associated with roots, have historically demonstrated the ability to

promote plant growth and productivity (Chang et al., 1986; Kloepper et al., 1988). The numerous effects of

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such plant-microorganism associations include an increased N₂ fixation capacity (Kuss et al., 2007; Lammel et al., 2013; Richardson et al., 2009) and the provision of solubilized nutrients, such as phosphates (Anzuay et al., 2013), where the solubilizing ability of many fungi has been associated with the release of organic acids and a decrease in pH. Gains in plant growth have also been related to facilitated absorption of iron promoted by certain microorganisms (Pii et al., 2015; Sadeghi et al., 2012) that synthesize siderophores (low molecular weight iron-chelating molecules) (Lemanceau et al., 2009; Miethke and Marahiel, 2007). Siderophores act as solubilizing agents of iron from minerals or organic compounds under iron-limited conditions (Rajkumar et al., 2010). The efficiency of siderophores from *Trichoderma asperellum* in donating Fe to plants grown under iron deficiency was demonstrated by De Santiago et al. (2009).

In addition to all of processes described above, some fungi have demonstrated the ability to synthesize phytohormones that are directly associated with plant development, such as auxins and gibberellins. In a study conducted in *Arabidopsis thaliana*, Contreras-Cornejo et al. (2011) found that *Trichoderma virens* produced compounds related to auxin, such as indole-3-acetic acid, indole-3-acetaldehyde and indole-3-ethanol. This phytohormone is known to act in the division and cell elongation process, since it activates enzymes that act on components of the bonds between cell wall cellulose microfibrils, increasing their plasticity (Castro et al., 2001). Consequently, water enters the cells more easily and rapidly increases their size (Taghavi et al., 2009).

There are few studies demonstrating the involvement of endophytes in promoting the growth of perennial plants such as those of the genus *Eucalyptus*. Cultivation of *Eucalyptus* has increased considerably in recent decades, associated with the use of various technologies and information on the edaphoclimatic diversity of cultivation areas (Gonçalves et al., 2014). Large forest stands of *Eucalyptus*, which is wood intended mainly for the civil construction and furniture industry, are distributed across all regions of Brazil, accounting for approximately 70% of the total planted forest area (ABRAF, 2013). Although, vegetative propagation of superior genotypes is employed in intensive clonal *Eucalyptus* forestry, this type of reproduction causes a number of problems, including variable results for the rooting index of cuttings and diseases acquired during this process (Díaz et al., 2009; Lombard et al., 2010). In this context, all technologies that optimize the growing conditions and production of ministumps directly favor the productive capacity of a nursery. Various agricultural strategies can be employed to improve the production of seedlings, including the use of bio-fertilizers, which are prepared organominerals rich in bacteria (Mehnaz, 2014; Sharma and Chaubey, 2015). Currently, the most widespread bacterial fertilizers are produced based on

rhizobacteria (Dias et al., 2012; Melo et al., 2012; Vitorazi Filho et al., 2012). For species of the genus *Eucalyptus*, studies have related the incorporation of rhizobacteria in the substrate with increased rooting of minicuttings, growth after rooting and gains in dry mass (Alfenas et al., 2009; Díaz et al., 2009; Mafia et al., 2009), and these effects are significant when compared with those caused by the treatment of cuttings with indole butyric acid (IBA) (Teixeira et al., 2007). It is also known that there are many environmental and genetically effects such as nursery practice and seed source in tree seedling growth (Dilaver et al., 2015; Yazici et al., 2011; Yazici and Babalik, 2011, 2016).

Since eucalyptus plantations are located in low-fertility soils in Brazil (Gama-Rodrigues et al., 2005), studies that take into account fertilization and nutrient cycling techniques are important for increasing and maintaining forest production. Thus, this study evaluated the effect of inoculation with three endophytic fungi (*Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp.) on the growth of cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids grown from minicuttings.

MATERIALS AND METHODS

Acquisition of plant material

The plant material was obtained via the cloning of *E. grandis* x *E. urophylla* hybrids grown from minicuttings. Shoots were collected from a clonal minigarden grown in a sand bed and were approximately one year old. The shoots were maintained in Tri-Mix substrate for the adaptive period of two weeks in a protected environment. To set up the experiment, the seedlings were transferred to 3.5-L pots containing Tri-Mix that had previously been sterilized in an autoclave at 121°C (Figure 1A and D).

Acquisition of fungal isolates

Endophytic isolates were previously obtained from the roots of *Hyptis marruboides*, a medicinal plant native to the Cerrado (Brazilian savanna) (Vitorino et al., 2012). These isolates were maintained in flasks containing nutrient agar culture medium and stored in the culture collection of the Laboratory of Agricultural Microbiology of the Federal Institute of Goiás (IF Goiano), Rio Verde Campus. The isolates were activated in PDA (infusion of 200 g potato, 20 g dextrose, 15 g agar and water up to 1000 mL) (Figure 1C). Mycelial disks of approximately 0.5 cm in diameter were removed from the established colonies and carefully distributed inside the planting holes, where they were placed in contact with the root system of the seedlings at the time of transplantation to the plastic pots (Figure 1D).

Three species of endophytic fungi were tested (*Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp.), where the control treatment consisted of plants free of microorganism inoculation. The experimental design was completely randomized, and the four treatments (the three fungi and a control) were analyzed in seven replicates. Growth evaluations of stem length (SL), stem diameter (SD), number of leaves (NL), root length (RL), fresh biomass (FB), dry biomass (DB) and leaf area (LA) were performed. The biometric analyses were conducted using a caliper and an analytical balance. To calculate the leaf area, photographs were taken with a digital

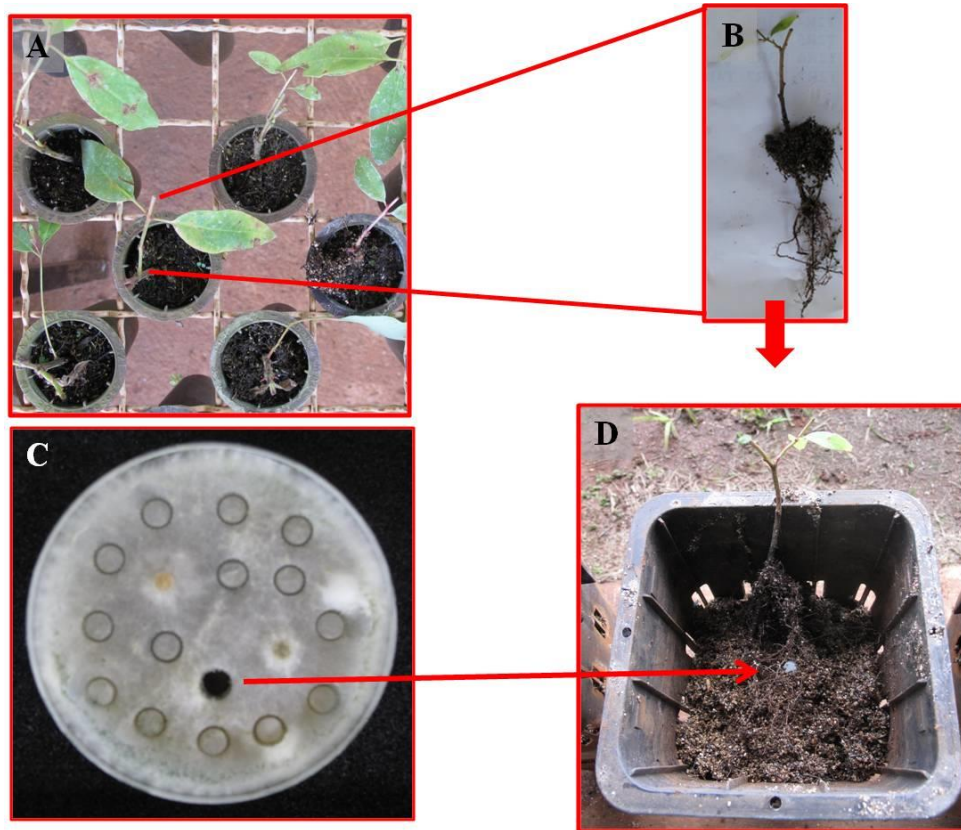


Figure 1. Cloned seedlings of *E. grandis* x *E. urophylla* in the adaptive period (A); seedling to be transplanted (B); Petri dish containing *Fusarium* sp., highlighting the removal of the mycelial discs (C) and mycelial disc inside the planting hole (D).

camera using a reference for size comparison. A white piece of paper with centimeter markings was employed as the reference, upon which the leaves of the plants were placed to take the photographs. The photographs were then analyzed using the software Sigma Scan Pro- V.S.O, Jandel Scientific.

The quarterly values for stem length and stem diameter were used to determine the percentage growth increase of the seedlings via the following equation:

$$x = \frac{100(b - a)}{a}$$

Where, b = value of the variable on the assessment day; a = value of the variable in the previous assessment.

RL, FB and LA were measured only at 120 days, and mean DB values were obtained after six days of drying in a forced air oven at 65°C, when the samples reached constant weight. The growth data were compared with the values recorded for the seedlings on the day of transplantation. The means for the different treatments were subjected to analysis of variance (ANOVA) using the F-test, and when significant differences were found, the means corresponding to FB, DB and LA were compared using Tukey's test at the 5% probability level, while the means for SL, SD and NL were compared through regression analysis. The data for SL obtained from the inoculation treatments were compared with the control treatment using a t test (inoculated plants x non-inoculated plants) for the different evaluation times. Statistical tests were performed with the aid of the R statistical package (R Core Team, 2016).

RESULTS

When evaluating the capacity of the three endophytic fungal isolates to promote the growth of cloned *E. grandis* x *E. urophylla* seedlings, analysis of variance of the means for the biometric variables revealed the significance of the treatments for SL, SD, FB and DB, while no difference was detected between the inoculation treatments and the control for LA. Additionally, differences were observed in relation to the evaluation times (0, 30, 60, 90 and 120 days after transplanting) for SL, SD and NL. The interaction between the different types of fungi used for inoculation and the evaluation times was not significant. This finding indicates independence of these variables, according to the F-test (Table 1).

For SL, the regression analysis was significant for time ($R^2 = 95.1$) and treatment ($R^2 = 30.5$). Breakdown of the time variable within the different treatments revealed significance for this growth parameter only at 90 and 120 days after transplanting ($p = 0.0003$ and $p = 0.0000$, respectively). For SD, the regression was also significant for time ($R^2 = 81.3$) and treatment ($R^2 = 20.1$), where the breakdown was significant at 30, 60 and 90 days ($p = 0.0005$, 0.0103 and 0.0001 , respectively). Regarding NL,

Table 1. Summary of ANOVA results for the biometric measurements obtained from cloned seedlings of *E. grandis* x *E. urophylla* hybrids, grown from minicuttings and inoculated with endophytic microorganisms.

Variable	F			CV (%)
	Treatment	Time	Treatment X time	
Stem length	21.153*	15.559*	1.317	4.38**
Stem diameter	12.768*	10.830*	0.447	4.28
Number of leaves	2.479	11.411*	0.084	7.37
Root length	1.025	-	-	6.65
Fresh biomass	3.986*	-	-	6.61
Dry biomass	4.732*	-	-	6.22
Leaf area	1.828	-	-	7.26

*Significant according to the F-test at 5% probability; **data transformed into \sqrt{x} .

the regression analysis was significant for treatment ($R^2 = 86.6$) and the interaction, where the breakdown showed significance for all time periods evaluated ($p = 0.0093$, $p = 0.0067$, $p = 0.0072$ and $p = 0.0006$).

The behavior of the different variables as a function of the treatments is shown in Figure 2. A linear effect was observed (a typical demonstration of a temporal increase in SL growth) in all treatments. For SD, linearity was also obtained for the data corresponding to the plants inoculated with *Papulaspora* sp. and *Trichoderma* sp.; however, a quadratic effect on the growth of the control plants as well as those inoculated with *Fusarium* sp. was detected. A quadratic effect was also found for the variable NL in all the treatments as well as the control.

The plants inoculated with the tested endophytic fungi exhibited better stem growth when compared with the control plants (Figure 2A), where the plants inoculated with *Trichoderma* sp. presented the greatest measurements at the end of the evaluation period. The results of the *t* test (Table 2) for SL indicated significant growth of plants inoculated with the endophytes when compared with non-inoculated plants, confirming the benefit of the plant-endophyte association as well as the growth-promoting character of the evaluated species.

The cloned seedlings inoculated with *Fusarium* sp. exhibited more leaves than the other seedlings (Figure 2C) and showed a trend towards high FB and DB values (Table 3).

When evaluating the quarterly increase in the SL of cloned of *E. grandis* x *E. urophylla* seedlings, the highest percentages were obtained 60 days after transplanting (DAT) in association with the endophytes *Fusarium* sp. and *Papulaspora* sp. (Figure 3). Therefore, these fungi (especially *Fusarium* sp.) were effective in stimulating the cloned seedlings in the early stage of their growth. There have been few studies associating the genus *Papulaspora* with growth promotion, and the endophytes of this genus are best known for the biosynthesis of enzymes (Tuppad and Shishupala, 2014) and other metabolites with antimicrobial (Ramos et al., 2010) and/or

cytotoxic (Gallo et al., 2014) activity. With respect to SD, the largest increases were also obtained in the early stages, where at 60 DAT, the plants inoculated with the fungus *Trichoderma* sp. exhibited a considerable increase in SD growth; however, in the final assessment at 120 DAT, the plants inoculated with *Papulaspora* sp. exhibited a greater increase in SD development than the plants inoculated with the other fungi (Figure 3).

DISCUSSION

Endophytic microorganisms are currently being evaluated as growth promoters for use in the production of seedlings of various species. It has been suggested that endophytic fungi can act symbiotically with plants, increasing the absorption of nutrients as well as the response to pathogens and stress conditions, which translates into gains in growth (Mandyam and Jumpponen, 2014). In this study, a linear temporal increase was observed for SL in all treatments. In contrast, for the variable NL, the control plants and those subjected to the inoculation treatments exhibited quadratic behavior. The same pattern was detected in the control plants and those inoculated with *Fusarium* sp. with respect to SD. This observation may be explained by a reduction in the values of these variables that occurred at the final evaluation times, possibly related to exhaustion of the nutritional content of the available substrate, or even an absence of symbiotic microflora in the autoclaved substrate, specifically in the case of the control plants for the latter scenario. Symbionts mediate the transfer of nutrients from the soil to plants (Barretti et al., 2008; Behie and Bidochka, 2014), and the presence of endophytic fungi has been linked to mechanisms such as the mineralization of available organic matter (Van Hecke et al., 2005), alteration of the chemical and biological properties of the soil and changes in hydraulic characteristics and aggregate stability (Hosseini et al., 2015). Filamentous fungi, such as those used in this

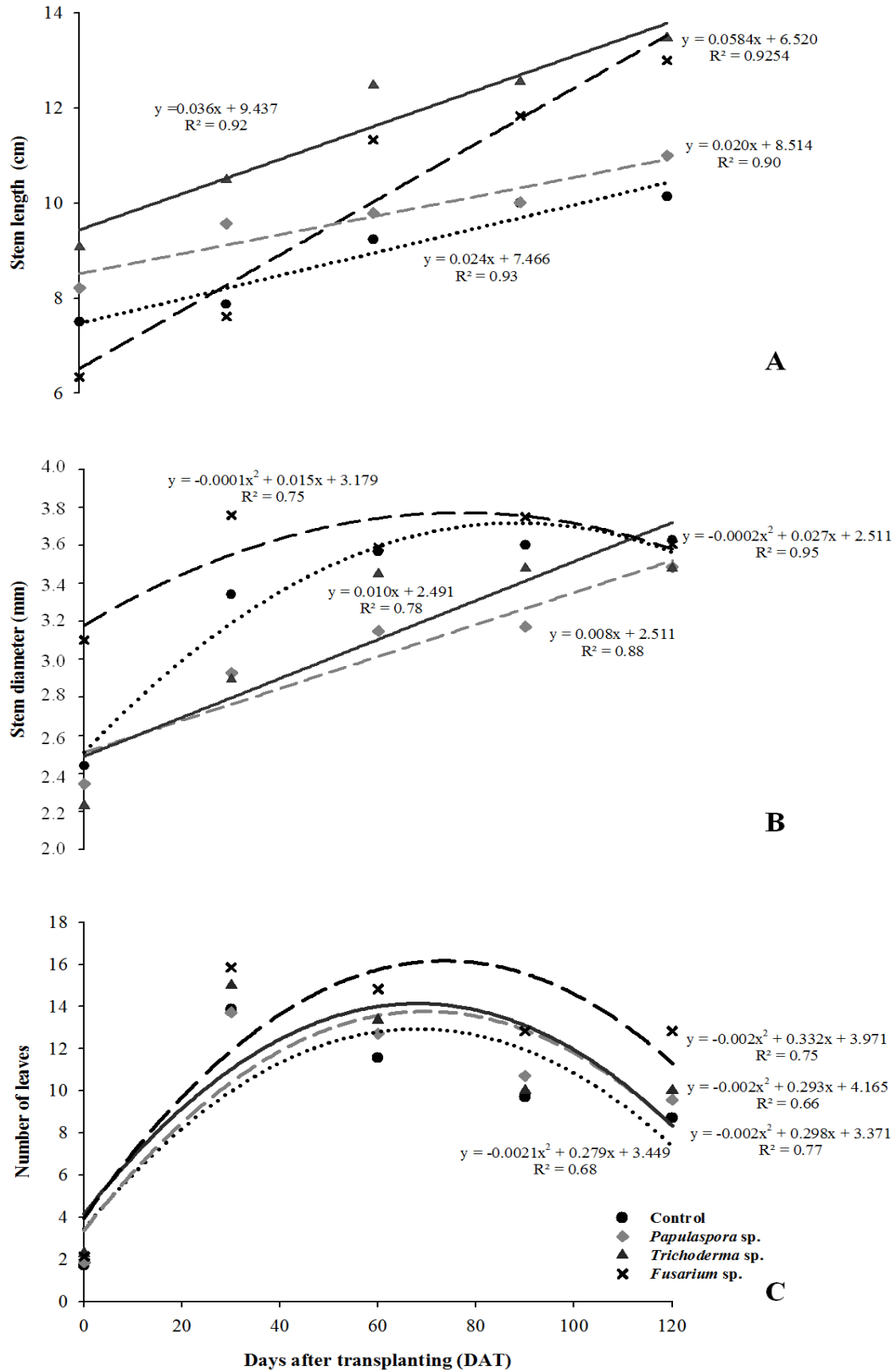


Figure 2. Stem length (A), stem diameter (B) and number of leaves (C) in cloned seedlings of *E. grandis* x *E. urophylla* hybrids grown from minicuttings and inoculated with the endophytes *Papulaspora* sp., *Trichoderma* sp., *Fusarium* sp., assessed at 0, 30, 60, 90 and 120 days after transplanting.

Table 2. Summary of the *t* test results for comparisons of the stem growth of cloned seedlings of *E. grandis* x *E. urophylla* hybrids inoculated with endophytic fungi x control seedlings, evaluated at 0, 30, 60, 90 and 120 days after transplanting (DAT).

DAT	<i>t</i>	<i>p</i>
0	-0.475	0.645
30	-2.181	0.046
60	-2.315	0.038
90	-1.372	0.198
120	-2.156	0.040

Table 3. Influence of inoculation with endophytic microorganisms on the mean shoot fresh biomass and dry biomass of cloned *E. grandis* x *E. urophylla* hybrid seedlings grown from minicuttings.

Treatment	Fresh biomass (g)	Dry biomass(g)
Control	9.8 ^b	5.8 ^{b*}
<i>Papulaspora</i> sp.	8.9 ^b	4.8 ^b
<i>Trichoderma</i> sp.	10.3 ^{ab}	5.6 ^b
<i>Fusarium</i> sp.	17.2 ^a	9.5 ^a

*Means followed by the same letter in the columns do not differ according to Tukey's test at 5% probability.

work, release enzymes through their hyphae that interact with the organic matter in the substrate (Chigineva et al., 2011) and transform nutrients, such as nitrogen, into forms that are assimilable by plants (Chen et al., 2013). It is possible that endophytic fungi such as mycorrhizae also alter the permeability of roots, facilitating hydraulic conductivity and the absorption of nutrients, which are fundamental processes for the promotion of growth (Ruiz-Lozano and Azcón, 1995).

The cloned seedlings inoculated with *Fusarium* sp. produced more leaves than the control plants or those inoculated with the other fungal species tested. Endophytes of the genus *Fusarium* have attracted the interest of a large number of researchers, who have reported their ability to synthesize bioactive secondary metabolites, including exopolysaccharides (Mahapatra and Banerjee, 2012), naphthoquinones (Kornsakulkarn et al., 2011) and cytotoxic alkaloids (Musavi et al., 2015; Venugopalan and Srivastava, 2015). When the potential of this symbiont as a growth promoter was evaluated, its action was found to result in the highest FB and DB values in the cloned *E. grandis* x *E. urophylla* seedlings (Table 3). In *Annona squamosa*, a *Fusarium* sp. strain endophytic to *Annona* spp. was also described as a growth promoter due to increase in the DB of the shoots of seedlings cultivated in a greenhouse (de Oliveira Silva et al., 2006). Similar results were observed in seedlings of *Passiflora edulis* f. *flavicarpa* Deg, in which this fungus stood out among other endophytes based on promoting increases in shoot FB (Luz et al., 2006). According to

Magalhães et al. (2003), the dry matter production rate is strongly affected by leaf area, which is influenced by factors such as environment and management. The amount of biomass produced by the plant can therefore be defined by a simple physiological relationship, based on the amount of radiation intercepted and the efficiency of its conversion into dry matter (Charles-Edwards, 1982). Therefore, the increase in the shoot dry biomass of cloned *E. grandis* x *E. urophylla* seedlings may indicate an improvement in the quality of seedlings, which would allow a reduction in the time spent maintaining them in nurseries.

Endophytic fungi have only recently been recognized for their importance in improving the overall fitness of host plants. Despite the prevalence and diversity of plant-endophytic fungus associations, studies have sought to document the impact of using these fungi on plants of agronomic or medical interest; however, such research is still in its incipient stages when compared with the number of studies on growth promotion conducted with bacteria, especially rhizobacteria, such as *Rhizobium*, *Pseudomonas*, *Bacillus*, *Azotobacter* and *Azospirillum* (Ahemad and Kibret, 2014; Bashan et al., 2014; Dutta and Khurana, 2015; Egamberdieva and Lugtenberg, 2014; Sharma et al., 2015). Studies using endophytic fungi as growth promoters have thus far been restricted to investigation of their antagonist activity against pathogens (Aktar et al., 2014; Parmar et al., 2015a) and have been much more restrictive than studies conducted with mycorrhizae and non-root rhizosphere fungi. However, new endophytic fungi are frequently isolated and identified as possible inoculants, such as *Penicillium funiculosum*, *Sordariomycetes* sp. and *Fusarium* spp., which were shown to stimulate growth in *Glycine max* L. (Khan et al., 2011), *Oryza sativa* L. (Li et al., 2012) and *Hordeum vulgare* (Maciá-Vicente et al., 2009), respectively.

Fungal strains of the genus *Fusarium* can be found in symbiotic associations with the internal tissues of most plants (Demers et al., 2015; Singh et al., 2015). However, these strains can also act as pathogenic fungi (Sobowale et al., 2005), triggering *Fusarium* wilt, or fusariosis, which is characterized by xylem hypertrophy (Pinto et al., 2010) and wilting followed by death of the affected plants (Costa et al., 2010). The high pathogenicity of *Fusarium* strains in some plants of agronomic interest (Gásperi et al., 2003; Nascimento et al., 2014), together with the great economic losses triggered by fungi of this type in stored fruits (Dantas et al., 2003) or grains (Barros et al., 2005; Ramos et al., 2014), has limited the number of studies attempting to use *Fusarium* strains as species that promote plant growth. Based on the absence of symptoms caused by infestation with pathogenic *Fusarium* strains as well as the growth response observed in the plants evaluated in this study, it can be concluded that the tested *Fusarium* strain is, in accordance with strict criteria, a plant growth-promoting

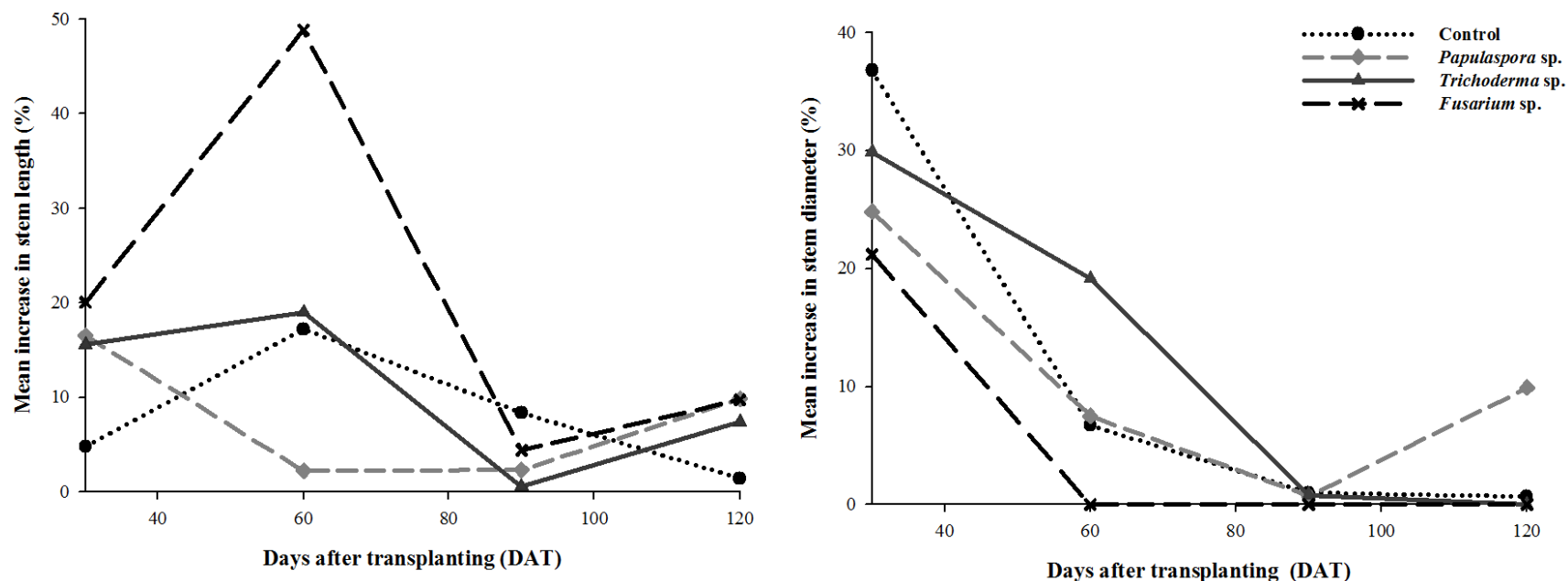


Figure 3. Quarterly increase in the stem length and stem diameter of cloned seedlings of *E. grandis* x *E. urophylla* hybrids grown from minicuttings inoculated with the endophytes *Papulaspora* sp., *Trichoderma* sp. and *Fusarium* sp.

endophytic strain. Many strains of the genus *Fusarium* and other endophytic fungi can produce phytohormones (Nassar et al., 2005) that directly influence plant development. It is well established that some *Fusarium* strains are capable of producing the plant hormone gibberellin (GA) (Troncoso et al., 2010; Tudzynski, 2005). Working with strains of *Fusarium proliferatum* that are symbiotic with orchids, Tsavkelova et al. (2008) identified an isolate that is a significant producer of GAs, and strains symbiotic with *Physalis alkekengi* have been shown to synthesize a wide range of GAs, including GA₃ (Rim et al., 2005). GAs are ubiquitous substances that elicit several important metabolic functions during plant growth (Bomke et al., 2008), which could potentially

represent the means by which *Fusarium* sp. stimulated the cloned *E. grandis* x *E. urophylla* seedlings, promoting their growth.

The plants inoculated with *Trichoderma* sp. exhibited the greatest mean SL at the end of the evaluation period. In *E. urograndis*, Paz et al. (2012) tested the EUCB 10 strain of the endophytic bacterium *Bacillus* sp., which was also shown to be efficient in promoting height gains in the evaluated plants, in addition to increasing the dry mass of the roots and shoots. With respect to the increases in stem length and diameter, the seedlings subjected to the isolate tested in the present study responded positively only in the early stages of development (60 DAT). Many studies have associated *Trichoderma* sp. with

growth promotion, though this effect is almost always related to the ability of these fungi to act as an antagonist of plant pathogens (Druzhinima et al., 2011; Kumar et al., 2015; Parmar et al., 2015b), which was not the case in the present study. However, strains of *Trichoderma* used in biocontrol may stabilize in the rhizosphere, subsequently stimulating plant growth and eliciting plant defense reactions against pathogens (Harman et al., 2011). *Trichoderma* spp. have been shown to induce growth and control *Sclerotinia sclerotiorum* in seedlings of the bean plant cv. Carioca grown in nurseries (Aguiar et al., 2012). However, in seedlings of *Eucalyptus benthamii*, it was indicated that this fungus shows antagonistic potential for the biological control of

Botrytis cinerea (Sbravatti Júnior et al., 2013). Research involving species of this genus as well as other fungi that are considered to be potential plant growth promoters is still in the exploratory stages, and studies associating endophytic microorganisms with *Eucalyptus* species have been conducted with the sole intention of isolation and characterization (Kaewkla and Franco, 2011; Taylor et al., 2009). Therefore, a greater number of studies will be required to improve the understanding and application of these microorganisms in agriculture on a large scale as well as in other areas that rely on biotechnological advances.

Conclusions

From the study, it can be concluded that: 1. plants inoculated with endophytic fungi exhibited better stem growth than that of control plants; 2. cloned *E. grandis* x *E. urophylla* seedlings inoculated with *Fusarium* sp. presented a greater number of leaves as well as higher FB and DB values when compared with the other plants; 3. endophytic fungi were effective in stimulating the growth of cloned *E. grandis* x *E. urophylla* seedlings in the early development stage (60 DAT).

Conflict of interests

The authors have not declared any conflict of interests.

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