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Eucalyptus growth promotion by endophytic *Bacillus* spp

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ABSTRACT. Clonal eucalyptus plantings have increased in recent years; however, some clones with high production characteristics have vegetative propagation problems because of weak root and aerial development. Endophytic microorganisms live inside healthy plants without causing any damage to their hosts and can be beneficial, acting as plant growth promoters. We isolated endophytic bacteria from eucalyptus plants and evaluated their potential in plant growth promotion of clonal plantlets of *Eucalyptus urophylla* x *E. grandis*, known as the hybrid, *E. urograndis*. Eighteen isolates of *E. urograndis*, clone 4622, were tested for plant growth promotion using the same clone. These isolates were also evaluated for indole acetic acid production and their potential for nitrogen fixation and phosphate solubilization. The isolates were identified by partial sequencing of 16S rRNA. *Bacillus subtilis* was the most prevalent species. Several *Bacillus* species, including *B. licheniformis* and *B. subtilis*, were found for the first time as endophytes of eucalyptus. *Bacillus* sp strain EUCB 10 significantly increased the growth of the root and aerial parts of eucalyptus plantlets

under greenhouse conditions, during the summer and winter seasons.

Key words: Plant-growth promotion; Endophytic microorganism; Rooting; *Eucalyptus*

INTRODUCTION

Endophytes are culturable and non-culturable microorganisms that reside within healthy plants without causing any damage (Azevedo et al., 2000; Azevedo and Araújo, 2007) and, in many cases, may prove beneficial to their host plants. Endophytic microorganisms exhibit agricultural potential as plant growth promoters, mainly by providing beneficial compounds for the plant host (Strobel, 2002; Kuklinsky-Sobral et al., 2004; Berg and Hallmann, 2006; Long et al., 2008). However, the growth-promoting effect of endophytes is dependent on the associated microbial community and the specific plant genotype, which should be investigated in each case (Bruneta et al., 2007).

Clonal-intensive forestry of eucalyptus frequently uses vegetative propagation of superior genetic materials, allowing the optimization of the production of plantlets that retain the favorable characteristics of the plants and avoid seedling variability by open breeding. However, this type of plant propagation poses certain problems, including variable results in the rooting index of cuttings and diseases acquired during this process (Díaz et al., 2009; Lombard et al., 2010).

Many studies have shown evidence of the benefits of endophytic microorganisms in eucalyptus plantlets; for instance, they have been shown to increase the rooting indexes and vegetative biomass or act as biocontrol agents in improving plant resistance when challenged with different pathogens (Mafia et al., 2005; Ferreira et al., 2008; Procópio et al., 2009). The present study involved the isolation of endophytic bacteria from eucalyptus and the evaluation of their potential for root and aerial growth promotion in clonal plantlets of *Eucalyptus urograndis*.

MATERIAL AND METHODS

Bacterial isolation

The endophytic bacterial isolates were obtained according to the method used by Procópio et al. (2009). The *E. urophylla* x *E. grandis* hybrids were located in Guaíba, Rio Grande do Sul State, Brazil (30°13'37.57"S, 51°31'58.31"W). Stem and leaf samples were randomly collected from 3-year-old trees and immediately stored at 4°C. All samples were processed within 24 h. The samples were washed under running tap water and cut into pieces approximately 5 mm in diameter. The fragments were surface-sterilized by dipping them in 70% ethanol for 1 min and 2% sodium hypochlorite for 3 min, and they were rinsed in sterile distilled water 3 times. Aliquots of the water from the final rinsing solutions were plated on the same media used for the fragments (Procópio et al., 2009). Five leaves and stem pieces were placed on each Petri dish containing Trypic Soy agar media amended with 2% polyvinylpyrrolidone. The Petri dishes were incubated at 28°C for 7 days and checked every day. The number of pieces containing bacterial isolates was counted, and the frequen-

cy of endophytic bacteria in the plant fragments was measured by the following formula: the ratio of the number of fragments showing bacterial growth to the total of number of plated fragments. The product of this ratio and 100 was calculated as the percentage of fragments with the bacteria.

Identification of bacterial isolates

Genomic DNA was extracted from the isolated endophytic bacteria by using the following protocol. Two milliliter of overnight liquid bacterial culture was centrifuged for 5 min at 12,000 *g* and resuspended in 500 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), recentrifuged, decanted, and resuspended in 500 µL TE buffer plus, 0.5 g glass beads (0.1 mm in diameter) and 15 µL 20% sodium dodecyl sulfate. The cells were homogenized for 1 min in a bead beater (Braun cell homogenizer; B. Braun, Germany). Tris-buffered phenol (500 µL) was added, and the solution was mixed well and centrifuged for 5 min at 12,000 *g*. The aqueous phase was extracted once with phenol-chloroform (1:1) and once with chloroform, and the DNA was precipitated with NaCl (0.1, v/v) and isopropanol (0.6, v/v) (3 min at room temperature) and then collected by centrifugation (10 min at 12,000 *g*). The pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µL Milli-Q water. The total DNA was analyzed by electrophoresis by spotting 4 µL of the mixture onto 1% agarose gel and visualized under ultraviolet light after staining with 1% ethidium bromide in 1X TAE buffer (40 mM Tris-acetate; 1 mM EDTA), according to the method described by Sambrook et al. (1989).

The diversity of the culturable bacterial isolates was studied by partial sequencing of the 16S rDNA gene. PCR was conducted in a 50-µL reaction volume containing 0.5-10 µg DNA, 0.2 µM of each primer (R1378: 5'-CGGTGTGTACAAGGCCCGGGAACG-3' and PO27F: 5'-GAGAGTTTGATCCTGGCTCAG-3'), 0.2 mM of each dNTP, 3.75 mM $MgCl_2$, 0.05 U Taq DNA polymerase (Invitrogen, Brazil) in 20 mM Tris-HCl, pH 8.3, and 50 mM KCl. The amplification protocol consisted of an initial step at 94°C for 4 min, followed by 35 amplification cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The 16S rRNA gene PCR products were purified by the polyethylene glycol method described by Lis (1980) and sequenced at Instituto do Genoma Humano (USP, São Paulo, Brazil). The sequences were evaluated in BLAST-N (Altschul et al., 1990) against the database of the GenBank Development [National Center for Biotechnology Information website and RDPQuery (Dyszynski and Sheldon, 2011)].

Phylogenetic relationships among the sequences of the isolates and the reference-species were inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the two-parameter method (Kimura, 1980). Both analyses were performed using the MEGA 4 program (Tamura et al., 2007).

Indole acetic acid (IAA) production

IAA production was evaluated using the Patten and Glick (2002) method. First, the bacterial isolates were inoculated in 10% Tryptone Soy broth medium supplemented with L-tryptophan (5 mM) and incubated at 28°C for 24 h in the dark. Triplicate cultures were then

centrifuged (5 min, 7000 *g*, at room temperature), and 1.5 mL Salkowski reagent (Patten and Glick, 2002) was added to 1.5 mL of the supernatant. This mixture was incubated for 20 min in the dark at room temperature and analyzed using a spectrophotometer (520 nm; Ultrospec 3000, Amersham-Pharmacia Biotech). The absorbance values obtained were interpolated in a standard curve to determine the IAA concentration ($r^2 = 0.9867$).

Phosphate solubilization

The ability of the bacterial strains to solubilize inorganic phosphate was evidenced by a halo obtained after cultivation of the culture medium supplemented with $Ca_3(PO_4)_2$ according to the method described by Nautiyal (1999). The evaluation of the presence of a halo around the bacterial colony was observed after 7 days of incubation at 28°C.

Biological fixation of atmospheric nitrogen

The ability to fix atmospheric nitrogen was evaluated according to the method described by Döbereiner et al. (1995). The isolates were inoculated in tubes containing 10 mL semi-solid NFb medium (5 g/L malic acid; 0.5 g/L K_2HPO_4; 0.2 g/L $MgSO_4.7H_2O$; 0.1 g/L NaCl; 0.01 g/L $CaCl_2.2H_2O$; 4 mL 1.64% Fe-EDTA); 2 mL 0.5% bromothymol blue; 2 mL micronutrients (0.2 g/L $Na_2MoO_4.2H_2O$; 0.235 g/L $MnSO_4.H_2O$; 0.28 g/L H_3BO_3; 0.008 g/L $CuSO_4.5H_2O$), and 1.75 g/L agar. Bacterial growth was evaluated after 72 h of incubation at 28°C in the dark. The formation of a growth disc in the culture medium indicated atmospheric nitrogen fixation by the bacterial isolates.

Plant growth promotion assay

Eight isolates were selected for *in vivo* assays on the basis of the results obtained from *in vitro* assays of the production of IAA and their ability to make nitrogen available through biological fixation and phosphate solubilization; the selected isolates were *Bacillus* spp EUCB1, 2, 10, 13, 21, 25, 26, and 28 and *Pseudomonas* sp EUCB 3 was used as the negative control.

Twenty-five *E. urograndis* cuttings per isolate were immersed for 1 h in the bacterial suspension (10^3 CFU/mL) and were planted in burned rice coat and vermiculite (1:1) substrate, according to the method described by Procópio (2004). The rooting percentage, the lengths of the root and aerial part, and dried weight were evaluated. The experiments were conducted in the summer of 2008. In the same year, the most promising isolate was tested again in the winter season, and the seedling quality index, proposed by Dickson et al. (1960), was calculated.

Statistical analysis

Data analysis was performed with the SPSS 16.0 package. A completely random design was used for all the assays. The values showed the percentage, which were then arcosen transformed by $\sqrt{X}/100$ to normalize the data. All data were analyzed for significance ($P > 0.05$) using Tukey and Scott-Knott tests.

RESULTS AND DISCUSSION

Differences between the tissues in the number of isolates per fragment were determined. The percentage of endophytic bacteria isolated from the stems was 10.6% and that from leaf fragments, was 1.19%. No bacteria were observed on the plates used to confirm superficial disinfection.

Studies showing the spatial distribution of endophytic fungi inside the plants are common (Verma et al., 2007), and the preferences of the fungi for host, tissue, and light, among other factors, have been verified (Unterseher et al., 2007). These preferences may differ even in different areas of the same tissue (Rodriguez et al., 2009). A similar phenomenon is possibly true for endophytic bacteria. Some *Eucalyptus* species seem to be more thickly colonized by endophytic bacteria than others, as verified by Procópio et al. (2009). The authors also observed a low-isolation frequency in a different clone of *E. urophylla* x *E. grandis* hybrid, suggesting that these differences occur due to a variation in the phenolic compounds and essential oils among *Eucalyptus* species.

The bacterial isolates obtained in the present study were identified using the partial sequence of 16S rDNA analyses. The majority of the isolates were not identified at the species level: 61% *Bacillus* sp, followed by *Bacillus subtilis* (16%). One strain was identified as *Bacillus pumilus* (EUCB 1); another, as *Bacillus licheniformis* (EUCB 5), and a third, as *Pseudomonas* sp (EUCB 3), which was the only one isolated from leaves (Table 1). The genus *Bacillus* was also the main group found by Ferreira et al. (2008) who isolated endophytic bacteria from eucalyptus seeds. Moreover, *Pseudomonas* and *Bacillus* spp are found as endophytes in several other plant species (Chanway, 1998; Araújo et al., 2002; Nunes, 2004).

Table 1. Identification of endophytic bacteria from stems of *Eucalyptus urograndis* hybrid.

Isolates	Identification	Similarity (%)	Bacterial species (%)
EUCB 3*	*Pseudomonas* sp	89	4.76
EUCB 1	*Bacillus pumilus*	96	4.76
EUCB 2	*Bacillus* sp	97	60.1
EUCB 4	*Bacillus* sp	98	
EUCB 6	*Bacillus* sp	100	
EUCB 10	*Bacillus* sp	96	
EUCB 13	*Bacillus* sp	98	
EUCB 14	*Bacillus* sp	91	
EUCB 15	*Bacillus* sp	88	
EUCB 21	*Bacillus* sp	98	
EUCB 24	*Bacillus* sp	98	
EUCB 25	*Bacillus* sp	98	
EUCB 27	*Bacillus* sp	98	
EUCB 28	*Bacillus* sp	96	
EUCB 7	*Bacillus subtilis*	96	16.6
EUCB 8	*Bacillus subtilis*	92	
EUCB 26	*Bacillus subtilis*	98	
EUCB 5	*Bacillus licheniformis*	98	4.76

*EUCB 3 was the only bacterium isolated from eucalyptus leaves.

EUCB 10, 13, 15, 25, 26, and 28 belonged to the same clade of *B. subtilis* reference strains. Similarly, the isolates EUC 8, 14, and 24 showed a high similarity with *B. pumilus* reference strains (Figure 1). Our dendrogram suggests a possible identification of the isolates described as *Bacillus* sp by the NCBI BlastN analysis. Chun and Bae (2000) suggest the

phylogentic analysis of *Bacillus* taxa using the *gyr*A gene instead of 16S rDNA. The *Bacillus* classification system has been frequently modified, complicating its correct identification.

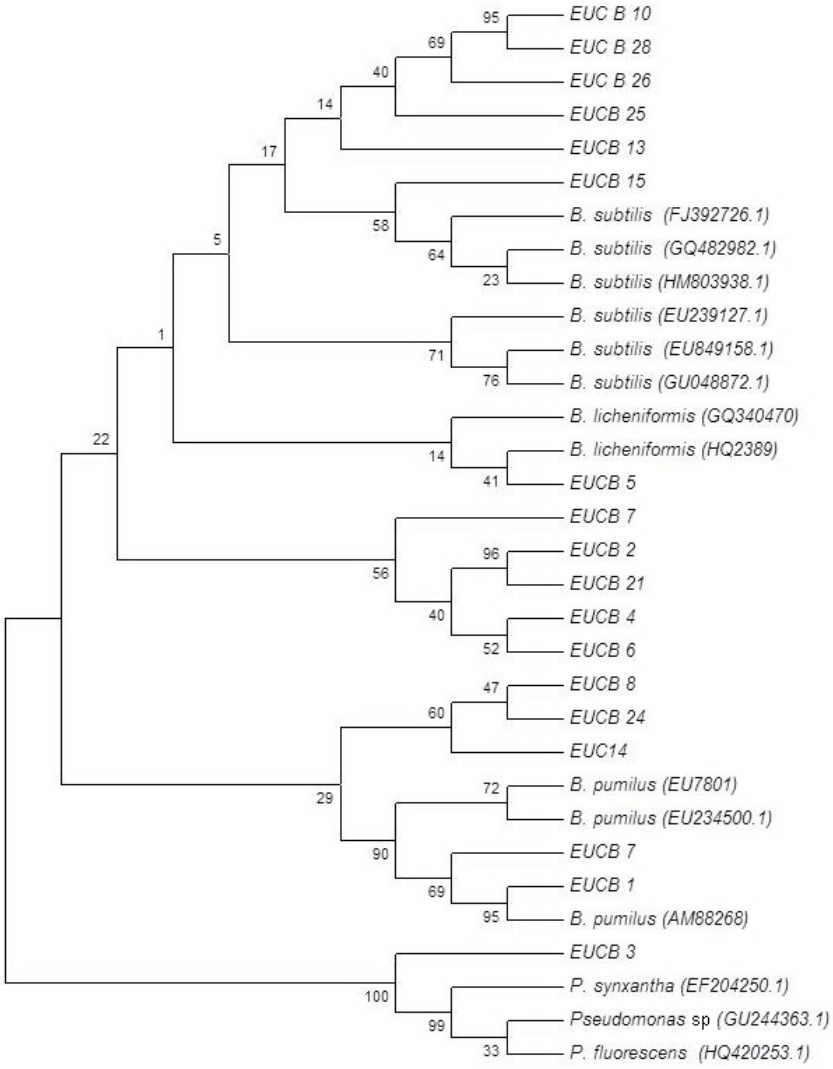

Figure 1. Phylogenetic dendrogram of endophytic strain from *Eucalyptus urograndis* plantlets and selected reference sequences. Sequences of >1500 nucleotides 16S rDNA were aligned using the Clustal W and phylogenic tree constructed using neighbor joining (MEGA 4.1). Sequence references exported from Gene Bank (NCBI) are indicated by reference numbers (1000 replicates). The scale bar indicates 0.02 inferred nucleotide substitutions per position.

The low diversity of the species detected among the bacterial isolates from *E. urophylla* x *E. grandis* does not necessarily represent the actual diversity of endophytic microorganisms associated with *Eucalyptus* spp. The isolation method using plant fragments was ad-

opted to select isolates with faster and easier growth for the purpose of discovering potentially beneficial bacteria for mass use in greenhouses and not the characterization of an endophytic bacterial community associated with the *E. urophylla* x *E. grandis* hybrid.

Ferreira et al. (2008) detected a high frequency of endophytic isolates of the Bacillaceae family, suggesting that this group may be adapted to the interior of eucalyptus plants and reside systemically in this host. However, different species of *Bacillus* apparently have different preferential niches inside the plant host. We isolated *B. subtilis* and *B. licheniformis* from the eucalyptus stem, but this species was not found by Ferreira (2008) as an endophyte of roots or on eucalyptus stems by Procópio et al. (2009). *B. pumilus* was detected as an endophyte in the aerial part of eucalyptus in this study, but only as an endophyte from roots by Ferreira (2008). Interestingly, the same authors did not find *B. pumilus* in the rhizoplane, suggesting that this species could be transmitted by seed, but when evaluating the endophytic communities of eucalyptus seeds from different species they did not find *B. pumilus*. Probably, the stomata and hidathodes serve as a gateway to *B. pumilus* isolates in the aerial part of eucalyptus found in the present study.

From the 18 bacterial isolates obtained in the present study, 7 were selected due to important characteristics pre-assayed *in vitro*; they include EUCB 2, 10, 13 21, 25, 26, and 28. One isolate was selected by neutral activity for use as a positive control (*Pseudomonas* sp EUCB 3) to evaluate the activity of this endophyte in rooting and growth promotion in *E. urophylla* × *E. grandis* hybrid plantlets (Table 2). None of the other isolates showed any related plant growth promotion activity, such as phosphate solubilization, IAA production, or biological nitrogen fixation. From the 18 isolates, only 4 (22%) were able to solubilize phosphate, 7 (38%) showed an ability for biological nitrogen fixation, and 8 (44%) were able to produce IAA (Table 2).

Table 2. Phosphate solubilization, atmospheric nitrogen fixation and indole acetic acid (IAA) production by endophytic bacteria from *Eucalyptus urograndis* hybrid.

Isolates	Phosphate solubilization	Atmospheric nitrogen fixation	IAA (µg/mL)*
EUCB 2	-	+	26.4^{bc}
EUCB 3	-	+	21.7^{abc}
EUCB 10	+	+	24.5^{abc}
EUCB 13	-	-	22.1^{abc}
EUCB 21	-	+	19.9^{abc}
EUCB 25	+	+	23.3^{abc}
EUCB 26	+	+	27.5^{c}
EUCB 28	+	+	17.1^{a}

(-) = no degradation/growth halo production; (+) = degradation/growth halo production. *The values are the average of four replicates. Values in the same column, followed by the same superscript letters did not differ according to the Tukey test (P > 0.05).

During the experiment on plant growth promotion, the responses to bacterial inoculation differed with the isolates tested (Table 3). Among the isolates tested, only EUCB 10 significantly increased the rooting index (96.2%). However, EUCB 21 and 1 showed an increase in the rooting index with 93 and 92.9%, respectively. EUCB 2, 25, 26, and 28 showed a significant negative effect on eucalyptus rooting comparing to the control treatment. Their rooting indexes were 81.2, 77, 79.8, and 75.7%, respectively. We observed that the cuttings treated with EUCB 13 showed increased length of the aerial part, but the greatest activity occurred in the root system. This isolate promoted a significant increase of 24.8% in root length (Table 3).

Table 3. Rooting percentage, dry weight (g) and length (cm) of *Eucalyptus urograndis* hybrid plants inoculated with endophytic bacteria.

Isolates	Rooting (%)*	Dry weight (g)*				Length (cm)	
		Aerial part		Root system		Aerial part	Root system
		30 days	80 days	30 days	80 days		
Control	89.4^{b}	3.29^{b}	7.35^{a}	0.59^{b}	2.90^{a}	14.8^{ab}	12.66^{a}
EUCB 1	92.9^{b}	3.59^{b}	5.18^{b}	0.73^{a}	2.19^{b}	14.05^{abcd}	13.68^{ab}
EUCB 2	81.2^{c}	3.41^{b}	5.24^{b}	0.54^{b}	2.13^{b}	13.72^{bcd}	13.36^{ab}
EUCB 3	91^{b}	2.82^{c}	2.50^{c}	0.58^{a}	1.38^{c}	13.3^{e}	12.74^{a}
EUCB 10	96.2^{a}	3.29^{b}	7.70^{a}	0.73^{a}	3.03^{a}	13.96^{abcd}	13.19^{ab}
EUCB 13	90^{b}	4.13^{a}	6.12^{a}	0.80^{a}	2.76^{a}	15.47^{a}	15.8^{b}
EUCB 21	93^{b}	3.41^{b}	5.80^{b}	0.71^{a}	2.92^{a}	14.51^{abc}	15.8^{b}
EUCB 25	77^{c}	3.43^{b}	7.26^{a}	0.87^{a}	2.55^{a}	13.1^{bcde}	14.5^{ab}
EUCB 26	79.8^{c}	2.59^{c}	1.96^{c}	0.43^{b}	0.63^{c}	12.4^{de}	12.7^{a}
EUCB 28	75.7^{c}	2.68^{c}	3.91^{b}	0.54^{b}	1.61^{c}	12.9^{cde}	12.6^{a}

*The values are the average of 25 replicates per isolate. Values in the same column, followed by the same superscript letters did not differ according to the Tukey test ($P > 0.05$).

Plant growth promotion by the *Bacillus* species has already been described by other authors (Wang et al., 2009; Dias et al., 2009). The action is more evident in the root system, which is probably due to auxin production by bacterial isolates. Shi et al. (2009) found a positive correlation between IAA and the length and fresh weight of sugar beet roots, with the optimal concentration ranging between 31.23 and 39.12 µg/mL IAA, in culture. However, the strain EUCB 26, which showed a high production of IAA, had a negative effect on eucalyptus rooting as well as a negative effect on the weight and length of the plant, thereby suggesting a negative correlation between this isolate and the eucalyptus plant. Dias et al. (2009) also verified negative correlations between *Bacillus* endophytic bacteria and host plants in strawberries.

Plant growth promotion by endophytic bacteria could be mediated by different mechanisms, such as the production of phytohormones and siderophores; the biological fixation of nitrogen; phosphate solubilization, or enzymatic activity, such as ethylene suppression by aminocyclopropane-1-carboxylic acid deaminase (Berg and Hallmann, 2006).

Among the isolates tested, EUCB 10, 25, 26, and 28 could solubilize phosphate and had the ability for the biological fixation of nitrogen (Table 2). However, these effects could not be correlated with an increase in the plant's length and dry weight, except in the case of EUCB 10, which significantly promoted the rooting as well as the increase in the radicular biomass. On the basis of the results obtained in the summer, EUCB 10 was selected as the most promising plant growth promoter and was re-evaluated during the winter season.

Many characteristics of the plantlets were evaluated, and one index of plantlet quality (Dickson et al., 1960) was applied for better assessment of the effects of EUCB 10 on plant growth promotion in eucalyptus. Again, EUCB 10 showed a significantly positive effect on inoculated plants (index of 0.231 against 0.171 in control plants; $P \leq 0.05$). Analysis of the individual parameters confirmed the significant positive effects of the EUCB 10 on eucalyptus plants, but no effect was noted on the plant's height (Table 4).

From the results of this study, we concluded that the endophytic bacteria from the *E. urophylla* x *E. grandis* hybrid are promising microorganisms for promoting rooting and plant growth among clonal plantlets. However, further evaluation of the commercial application of the effectiveness of these strains and the designing of an inoculating formulation and application must be conducted under field conditions.

Table 4. Plant growth promotion of *Eucalyptus urograndis* plantlets inoculated with the endophytic bacterial isolate EUCB 10.

Characteristics	Treatment*	
	Control	EUCB 10
Height (cm)	29.64[a]	29.94[a]
Dried weight of aerial part (g)	1.19[a]	1.48[b]
Dried weight of radicular system (g)	0.57[a]	0.751[b]
Diameter of stem (mm)	3.59[a]	3.86[b]
Number of leaves	9.58[a]	10.68[b]

*The values are the average of 25 replicates. Values in the same row, followed by the same superscript letters did not differ by the Tukey test ($P > 0.05$).

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