Endophytic bacteria in seed germination and rooting of Pinus spp.[1]

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ABSTRACT

The inoculation of seeds with associative and growth-promoting bacteria is a prosperous mechanism to achieve high germinability rates and production of well-developed plants, in addition to the aspects related to the rhizogenic process in the clonal propagation of superior genotypes. Consequently, the objective of this work was to isolate endophytic bacteria from *Pinus caribaea* var. hondurensis plant tissues and evaluate its potential as a promoter in seed germination and rooting of *P. taeda* mini-cuttings. Hence, endophytic bacteria were isolated from *Pinus caribaea* var. hondurensis micro-plants grown in vitro and phenotypically characterized. From this collection of formed endophytic isolates, in addition to two *Azospirillum brasilense* commercial strains, seed germination and rooting tests of *Pinus taeda* L. mini-cuttings were established. Bacterial inoculation promoted the germination rate, germination speed and vigor of the seedlings. *A. brasilense* and CNPF 316 promoted an increase in the percentage of rooted mini-cuttings, number and average length of roots. The isolates present characteristics of plant growth-promoting bacteria, as they enhance the development of plant physiological and morphological stages.

Keywords: adventitious rooting; *Azospirillum brasilense*; germination; *Pinus sp.*; plant growth-promoting bacteria.

INTRODUCTION

Pinus spp. makes up 23% of the Brazilian forest cover aimed at silviculture, covering an area of approximately two million hectares. This crop is significantly important in the South region, covering 87% of the cropped area of this conifer in Brazil (IBÁ, 2020). Aiming at the sustainability and profitability of the *Pinus* silvicultural process, it is extremely important to plant forests that achieve high productivity rates, either through improved propagation techniques, improvements in management or the use of plant biotechnology techniques (Jesus *et al.*, 2020; Stadler *et al.*, 2022). Mini-cutting and micro-propagation techniques are constantly applied in plant genetic improvement processes in silvicultural companies as an important tool to improve the seminal and clonal propagation process. However, forest species, such as *Pinus*, present particular issues, such as low rooting rates in cuttings and difficulty in rejuvenating the stock plants (Stadler *et al.*, 2022). As a result, it is necessary to develop new strategies to improve the propagation performance of *Pinus* among the different cloning techniques.

The use of microbial inoculants in *Pinus*, composed of endophytic bacteria and plant growth promoters can be an alternative to propagation difficulties, however it still little researched. In experiments carried out for *Eucalyptus* spp., significant progress related to bacterial application has been observed (Zul *et al.*, 2022). The interest in the application of these microorganisms in agriculture has expanded considerably in recent years. They are consid-

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ered potential substitutes for conventional fertilizers and act benefiting plant growth through symbiotic relationships (Brader *et al.*, 2014; Vandana *et al.*, 2021).

These bacteria responsible for stimulating and benefiting the vegetative growth are called "Plant Growth-Promoting Bacteria" (PGPB). The gains in growth and development are due to several factors, such as the ability to synthesize plant regulators such as indole-3-acetic acid (IAA), considered the main plant regulator involved in the plant/growth-promoting microorganisms association. In addition to phytohormonal synthesis, it also has other mechanisms such as biological fixation of nitrogen, phosphate solubilization, iron chelation, induction of systemic resistance and production of siderophores (Santoyo *et al.*, 2016; Panigrahi *et al.*, 2020; Duarte *et al.*, 2020).

Although the works on the application of bioinoculants in the vegetative propagation of forest species are, for now, still incipient, their use is considered promising for international silviculture.

In this context, the objective of this work was to isolate and phenotypically characterize *Pinus caribaea* var. hondurensis endophytic bacteria, as well as to evaluate the potential of bacteria in seed germination and rooting of *Pinus taeda* L mini-cuttings.

MATERIAL AND METHODS

This experiment was carried out at the Tissue Culture and Transformation and Soil Microbiology Laboratories of Embrapa Forests, located in the municipality of Colombo, Paraná state, Brazil, together with the Macropropagation Laboratory of the Plant Department of the Federal University of Paraná, located in the municipality of Curitiba, Paraná, Brazil.

Isolation and phenotypical characterization of endophytic bacteria

The bacteria were isolated from shoots in the multiplication phase of *Pinus caribaea* var. hondurensis grown in vitro for at least three years and without any apparent contamination (Figure 1A). According to the methodology proposed by Döbereiner *et al.* (1995), 10 g of needles were weighed and superficially disinfected in a laminar flow hood (Figure 1B). Disinfestation occurred by soaking the plant material in 70% ethyl alcohol for 30 seconds and then in a 6% sodium hypochlorite solution for 2 minutes and 30 seconds and abundantly washing in sterile distilled water. Subsequently, the plant material was processed with 90 mL of 0.85% sodium chloride saline solution (Figure 1C). A serial dilution from 10^{-2} to 10^{-7} was performed and, for each concentration, aliquots of 100 µL were inoculated in solid DYG'S culture medium, remaining in a growth oven at 28 ± 2 °C for 10 days.

After the incubation period, phenotypically distinct bacterial colonies were selected and subcultured in the same medium to obtain pure bacterial colonies (Figure 1D, 1E, 1F). After purification, the pure isolates were characterized in terms of shape, elevation, edge, surface, mucus production, transparency and color of bacterial colonies, classified according to the Embrapa Forests Microorganism Collection and stored in solid medium containing mineral oil at room temperature and at – 20 °C and in liquid DYG›S medium with 30% glycerol.

Seed germination tests

Germination tests were performed with seeds from second generation material (2018) acquired from the Westrock company (located in the state of Santa Catarina) and stored in plastic bags at 4 °C. Three germination tests were carried out, in which the seeds subjected to immersion in sterile distilled water for 48 hours under constant stirring (75 rpm) were considered as a control treatment (T1) and subsequently subjected to disinfestation with 70° alcohol for 60 seconds and 3% sodium hypochlorite solution for 15 minutes, followed by six washings with sterile distilled water. A second control treatment (T2) was used, in which the seeds were soaked in hydrogen peroxide (H_2O_2) 40 volumes for 60 minutes, with the purpose of disinfestation and overcoming tegumental dormancy. For the other treatments, the seeds were soaked in H_2O_2 and, after repeated washing with sterile water, the bacterial strains were inoculated, configuring the other treatments.

The bacteria were cultivated in liquid DYG's medium under constant stirring, at 150 rpm, for 24 hours. For inoculation, the seeds were placed in plastic bags, separately for each bacterium, to which aliquots of 500 µL of bacterial suspension were added. Each bag was closed, with a certain volume of air inside, and stirred to spread and homogenize the bacterial cells to the seeds. The seeds were placed in sterile gerboxes boxes (first assay) and in Petri dishes (second and third assays) containing moistened filter paper.

Test 1 used *Azospirillum brasilense* strains 2083 (T3) and 2084 (T4), alone and combined (T5), totaling five treatments with five replications of 40 seeds per experimental unit. For test 2, the seeds were inoculated with 38

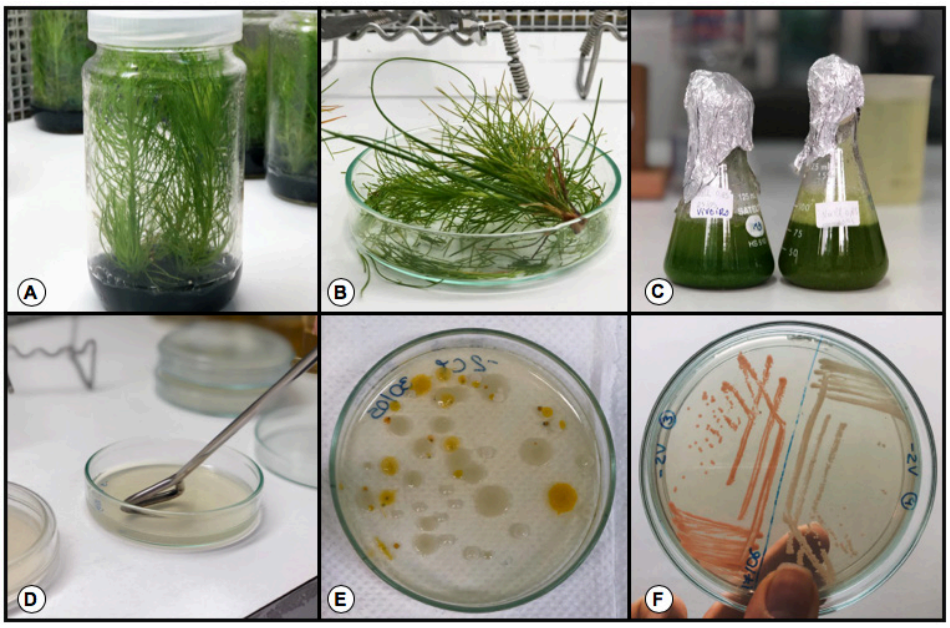

Figure 1: A – *Pinus caribaea* var. hondurensis microplant in vitro grown; B – Separation of the shootings for miling; C – shootings processed with 90 mL of 0.85% sodium chloride saline solution; D – sample inoculation in DYG's medium; E –Endophytic bacterial colonies growth; F – Pure bacterial isolates.

endophytic bacterial isolates (CNPF 293, 294, 295, 296, 297, 298, 299, 300, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330 and 331), totaling 40 treatments with 4 replications of 10 seeds per experimental unit. Regarding test 3, 11 endophytic bacterial isolates were selected (CNPF 297, 300, 303, 304, 305, 307, 311, 312, 314, 317, 321), according to the best results obtained in germination experiments previously carried out, resulting in 13 treatments with 4 replications of 10 seeds per sample unit.

The tests were kept in a growth room at 23 ± 2 °C, with a photoperiod of 16 hours and controlled humidity. The number of germinated seeds was counted daily and, after 45 days, the percentage of germinated seeds (radicle larger than 2 mm), non-germinated seeds and seeds with incomplete germination (radicle protrusion with abnormal development), mean germination time index (MGT) (Labouriau, 1983), germination speed index (GSI) (Maguire, 1962) and relative germination frequency (Fr) (Labouriau & Valadares, 1976) were evaluated.

The germination percentage variables were transformed for statistical analysis purposes. Data followed a completely randomized design (CRD) and were submitted to Bartlett's test and analysis of variance (ANOVA). The means were compared using the test of Tukey at the 5% probability level, using the Assistat 7.7 software (Silva & Azevedo, 2016).

Mini-cuttings tests

Two rooting trials of *P. taeda* L. mini-cuttings were carried out in duplicate in a greenhouse with intermittent mist from 6 a.m. to 10 p.m., using 30 seconds of irrigation every 15 minutes, about 80% RH and temperature of 25 ± 2 °C.

As mini-cutting donors, three-year-old *P. taeda* L. mini-strains were used, maintained in a channel system with washed sand and fertilization with a nutrient solution in a semi-hydroponic system. The mini-cuttings were produced with 6 cm in length, keeping 1/3 of the needles in the upper portion, with a bevel cut in the basal region and a straight cut above the last apical bud. Planting took place in tubes with a capacity of 53 cm^3, filled with fine-grained vermiculite and commercial substrate Tropstrato® at 1:1 proportion. In both tests, the control treatments consisted of soaking the mini-cutting bases in distilled water.

In test 1, the mini-cutting bases were soaked in hydroalcoholic solutions (50%) of indole-3-butyric acid (IBA) at concentrations of 2000, 4000 and 6000 mg L^{-1} for 10 seconds, as well as in bacterial solutions of the

Azospirillum brasilense strains 2083 and 2084, both isolated and associated with each other, for 60 seconds. In all, seven treatments were counted with four replications and 20 mini-cuttings per experimental unit. For test 2, 27 endophytic bacterial isolates were used (CNPF 293, 295, 296, 298, 304, 305, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 328 and 331), each containing an individualized strain. In the substrate, close to the base of each mini-cutting, 1000 uL of bacterial solutions were inoculated, accounting for a total of 28 treatments with 4 repetitions of 10 mini-cuttings per sampling unit.

After 120 days, the following were counted: the percentage of rooted mini-cuttings (%MC), number of roots per mini-cutting (NR), average length of the three largest roots per mini-cutting (ML), percentage of mini-cuttings with callus (%MC), live mini-cuttings (%LM), dead mini-cuttings (%DM), mini-cuttings which maintained the initial needles (%MIN) and with new shoots (%MS).

The mini-cutting tests followed a completely randomized design (CRD) and the data were analyzed using the Assistat 7.7 software (Silva & Azevedo, 2016). The Anova and Tukey tests for comparing means were performed at 5% probability.

RESULTS AND DISCUSSION

Isolation and phenotypical characterization of endophytic bacteria

Thirty-nine endophytic isolates were isolated from *P. caribaea* var. hondurensis, which according to Liotti *et al.* (2018), grinding provided a greater isolation of endophytic microorganisms in relation to the fragmentation method. Also, according to Ishizawa *et al.* (2017), this method allows for a more pronounced expression of the endophytic microbiome as the internal tissues are more exposed.

The variations regarding the morphology of bacterial colonies are shown in Table 1. The vast majority had a circular shape (74.36%), convex elevation (35.9%), with smooth or nodulated margins (41.03%), smooth surface (66.67%), moderate production of mucus (35.9%), with opaque character (64.10%) and a prevailing yellow color (48.72%).

Table 1: Morphological characteristics of colonies of endophytic bacterial isolates from Pinus caribaea var. hondurensis micro-plants

Morphological traits		Percentage (%)
Colony form	Circular	74.36
	Punctiform	7.69
	Irregular	17.95
Colony elevation	Flat	12.82
	Convex	35.90
	Umbonate	23.08
	Protuberant	2.56
	Raised	25.64
Colony margin	Smooth	41.03
	Undulate	41.03
	Lobate	17.94
Colony surface	Smooth	17.95
	Rough	66.67
	Papilarry	15.38
Mucus production	Scarce	33.33
	Poor	30.77
	Moderate	35.90
Mucus trait	Opaque	64.10
	Translucent	35.90
Color of the colony	White	5.13
	Beige	38.46
	Yellow	48.72
	Pink	7.69

Seed germination tests

It was observed that hydrogen peroxide acted efficiently in breaking tegumental dormancy, which increased germinal development and significantly reduced germination time, as was also observed in a work by Sharma *et al.* (2020) and Quisen & Degenhardt-Goldbach (2020). In the absence of hydrogen peroxide, lower values of germinated seeds were observed in the period of 45 days. This solution has oxidant activity that suppresses the activity of germination inhibitors in the seed coat, promotes gas exchange, increases the rate of oxidative respiration and contributes to a more effective seed imbibition (Wojtyla *et al.*, 2016). Also, hydrogen peroxide is a very efficient disinfectant and is routinely used in the scarification of agricultural seeds (Amjad *et al.*, 2004; Çavusoglu & Kabar, 2010).

In the germination experiments with bacterial isolates, treatments with immersion in distilled water were disregarded from the statistical analyses as a complete absence of germination was found. This result indicates that immersion in water for 48 hours and storage at low temperatures was not effective in overcoming the tegumental dormancy of *Pinus taeda* L.

The inoculation of seeds with *A. brasilense* strains 2083 and 2084 in test 1, resulted in high germination averages, a reduction in the average germination time and a rise in germination speed (Table 2). The positive effects of *A. brasilense* inoculation can be attributed, mainly, to the capacity of synthesis of plant hormones, as reported by Dartora *et al.* (2013). Several strains of *A. brasilense* have been used as bioinoculants and phytostimulators in agricultural crops to increase productivity and, in a sustainable way, replace chemical agents with natural compounds with similar effectiveness, as observed in corn (*Zea mays* L.), soybean (*Glycine max* L.) and wheat (*Triticum* spp.) (Cassán *et al.*, 2009; Dartora *et al.*, 2013).

As for the other strains, CNPF 300, 304, 305, 309, 311, 320 and 328 stood out. These strains constituted efficient inoculum in increasing germinability, increasing germination speed and decreasing the average germination time, whose mean ranged from 90% to 95% (Figure 2). Despite the statistical equality among treatments, the inoculated seedlings had visual gains in seedling growth and vigor, as well as a greater number of secondary roots, in relation to the control treatment with immersion in hydrogen peroxide (Figure 3).

Marques *et al.* (2014) found that inoculation with endophytic bacteria provided symbolic increases in the germination of *Eucalyptus urophylla*. For *P. taeda* L., Santos *et al.* (2018) reported that inoculation with *Bacillus* promoted satisfactory effects on seed root development. It should be observed that the success of the germination process is due not only to auxinic synthesis, but mainly to the presence of gibberellic acid in plant tissues. Experiments on microbial synthesis of gibberellins were not performed in this work.

Mini-cuttings tests

The stimulus to the formation and growth of adventitious roots comes from the interaction between internal and external factors, such as treatment with exogenous auxins or other compounds that act to promote rhizogenesis. Since the effect of microbial IAA is similar to that promoted by plant regulators, the use of microorganisms in agricultural crops aims to increase the productivity of plantations in a sustainable way, as discussed by Mariosa *et al.* (2017) and Duarte *et al.* (2020).

Table 2: Germinated seeds (%GER), non-germinated seeds (%NGER), incomplete-germinated seeds (%GERINC), mean germination time (MGT) and germination speed index (GSI) of *Pinus taeda* L. seeds inoculated with *Azospirillum brasilense* and hydrogen peroxide (H_2O_2)

Treatments	%GER	%NGER	%GERINC	MGT	GSI
H_2O	13.0 b	85.0 a	2.0 ab	33.46 a	0.16 b
H_2O_2	92.0 a	6.5 b	1.5 b	12.08 b	3.38 a
H_2O_2 + 2083	88.0 a	10.5 b	1.5 b	11.81 b	3.31 a
H_2O_2 + 2084	80.0 a	9.5 b	10.5 a	11.74 b	2.95 a
H_2O_2 + 2083 + 2084	88.0 a	7.0 b	5.0 ab	11.41 b	3.37 a

Treatments: H_2O (water soaking); H_2O_2 (soaking in hydrogen peroxide); H_2O_2 and *A. brasilense* 2083 inoculation; H_2O_2 and *A. brasilense* 2084 inoculation; H_2O_2 and *A. brasilense* 2083 and *A. brasilense* 2084 inoculation. Means followed by the same letter in the columns are not statistical different by the test of Tukey at 5% probability.

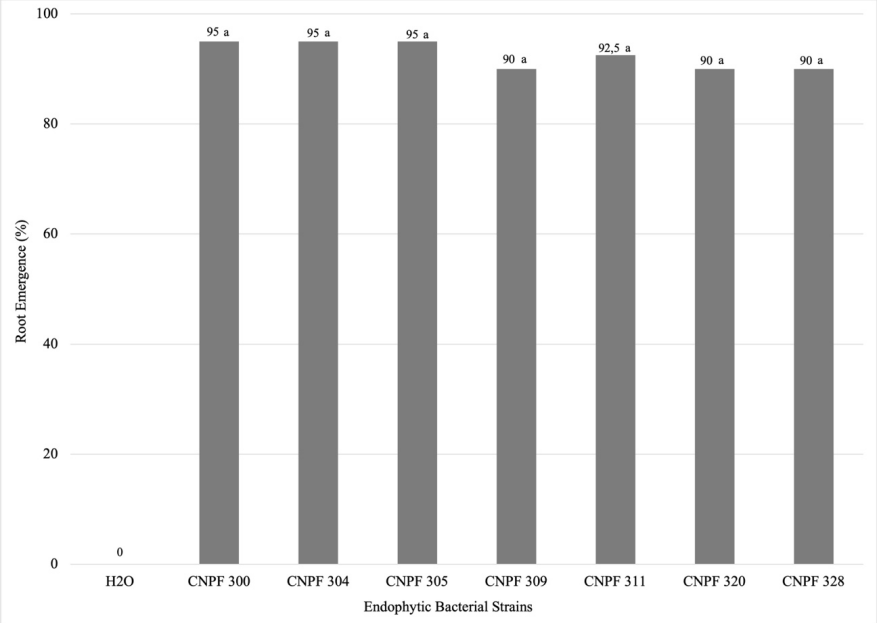

Figure 2: Percentage of root emergence of *P. taeda* L. seeds after inoculation with hydrogen peroxide (H_2O_2) and bacterial isolates CNPF 300, 304, 305, 309, 311, 320 and 328. Means followed by the same letter in the bars are not statistical different by the test of Tukey at 5% probability.

Rooting rates of *P. taeda* L. mini-cuttings significantly increased when inoculated with *A. brasilense* strains 2083 and 2084 as the percentages were higher than the control and IBA treatments (Figure 4). On the other hand, the absence of *A. brasilense* and IBA resulted in a higher mortality of plant propagules, indicating that the auxinic action is beneficial to the rhizogenesis, as observed in the control treatment.

Similar results were observed for the average number (NR) and average length of roots (RL), which determine the quality of the formed root, as well as for new shoots (%MB) (Table 3). The coinoculation of strains 2083 and 2084 resulted in a numerical reduction in root length, indicating that both strains together may have caused a greater auxin synthesis, at a toxic level, and compromised root growth. The treatments with the highest %MIN values

Figure 3: *Pinus taeda* L. seedlings. A – treatment with hydrogen peroxide (H_2O_2); B – treatment with hydrogen peroxide (H_2O_2) + CNPF 300; C – treatment with hydrogen peroxide (H_2O_2) + CNPF 328.

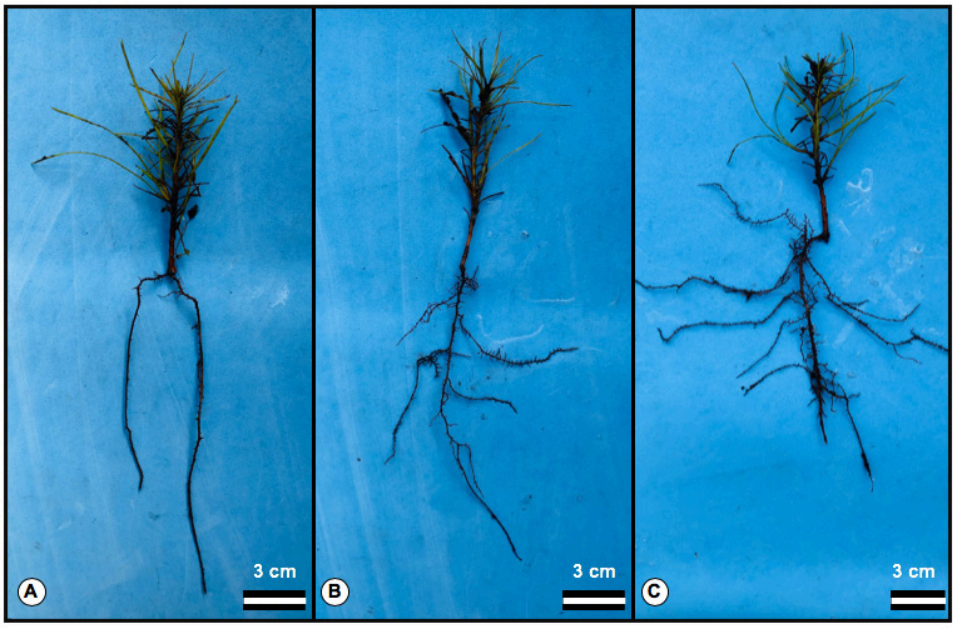

Figure 4: A – Rooted mini-cutting of *Pinus taeda* L. with IBA 4000 mg L^{-1}; B – rooted mini-cutting of Pinus taeda L. inoculated with *Azospirillum brasilense* strain 2083; C – rooted mini-cutting of *Pinus taeda* L. inoculated with *Azospirillum brasilense* strain 2084.

presented the lowest numerical values of rooting, unlike those presented by Duarte *et al.* (2020), who state that the presence of leaves promotes the rhizogenic process by acting in the translocation and supply of carbohydrates and plant hormones.

The use of endophytes exhibited, to a large extent, the increments in the percentage of rooted mini-cuttings, number of adventitious roots and average length of formed roots. The strains CNPF 311 and 316 were considered the most effective in root formation; CNPF 316, 321 and 328, in the amount of roots/mini-cutting and; CNPF 307 and 316, in the average length of roots (Figure 5).

According to Santoyo *et al.* (2016), the use of associative bacteria, endophytic or epiphytic, in plant cloning, as well as synthetic auxins, can provide many benefits to plants, such as increasing the number and length of adventitious and secondary roots, increased leaf area, greater resistance of plants to stresses and consequent decrease in the percentage of plant necrosis. The gains in growth and development provided by endophytic bacteria are related, in their vast majority, to the production of not only auxins, but also gibberellins and cytokinins, which are capable of stimulating root and aerial growth. The beneficial effects caused by Plant Growth-Promoting Bacteria (PGPB) make it possible to replace chemical compounds with bioinoculants made up of this group of microorganisms (Rosa *et al.*, 2018).

In summary, the inoculation of plant tissues with plant growth-promoting bacteria, which have the ability to biosynthesize IAA, can provide a chain of physiological

Table 3: Percentage of rooted mini-cuttings (%RM), average number of roots (RN), average root length (AL), percentage of live (%LM), dead (%DM) mini-cuttings, which maintained the initial needles (%MIN) and with new shoots (%SM) of *Pinus taeda* L. subjected to indole-3-butyric acid (IBA, mg L^{-1}) and *Azospirillum brasilense* (AZO 2083, AZO 2084) commercial strains

Treatment	%RM	RN	AL (cm)	%LM	%DM	%MIN	%SM
Control	5.0^{ns}	1.50^{ns}	3.57^{ns}	31.25^{ns}	23.75^{ns}	38.75 a	26.25^{ns}
IBA 2000	8.75	1.75	5.24	22.50	18.75	38.75 a	25.00
IBA 4000	11.25	2.25	6.30	26.25	13.75	40.00 a	22.50
IBA 6000	8.75	1.75	7.33	16.25	10.00	25.00 ab	15.00
AZO 2083	13.75	3.50	11.15	11.25	7.50	23.75 ab	30.00
AZO 2084	13.75	3.00	10.93	18.75	13.75	16.25 b	27.50
AZO 2083 + 2084	15.00	3.25	6.89	22.5	15.00	7.50 b	33.75

Means followed by the same letters in the columns are not statistical different from each other by the test of Tukey at 5% probability.

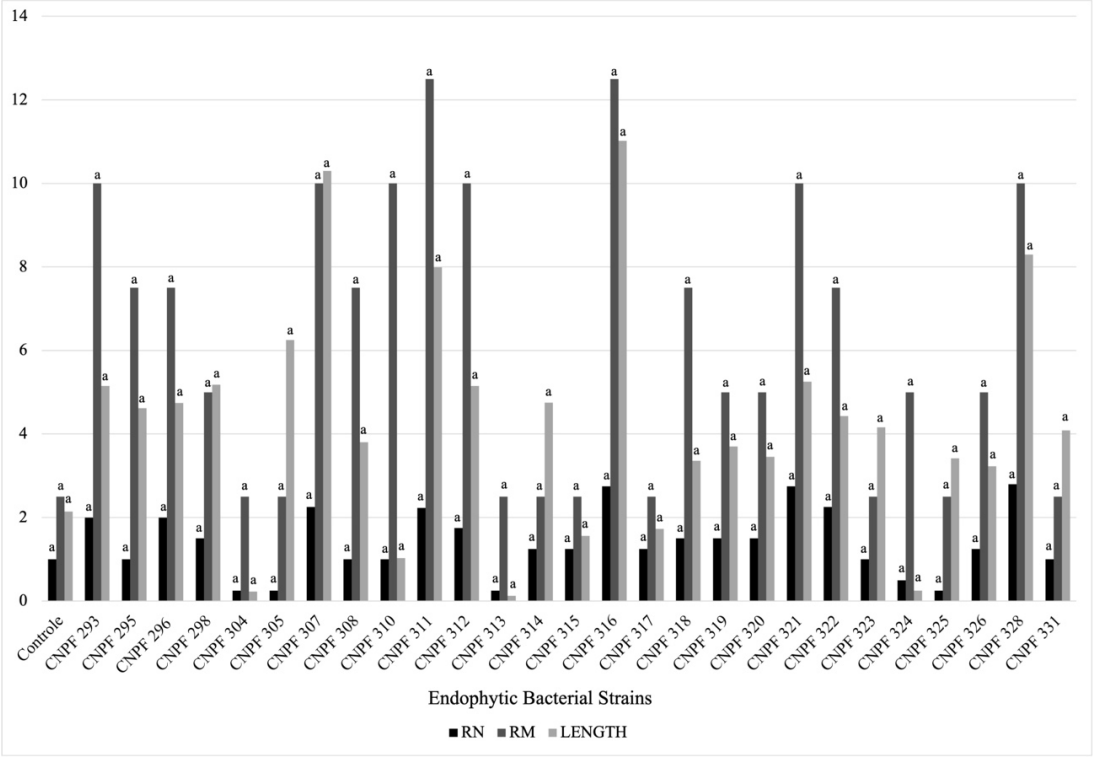

Figure 5: Percentage of rooted mini-cuttings (RM), mean number of roots (RN) and mean root length (LENGTH) in centimeters after inoculation with endophytic isolates of *P. caribaea* var. hondurensis. Means followed by the same letter in the bars are not statistical different by the test of Tukey at 5% probability.

events and benefits in plant development and growth, either under in *in vitro* or in the field conditions. Among these benefits, there is an improvement in rooting rates, an increase in seed germinability and a contribution to the development of more vigorous aerial part and roots (Rosa *et al.*, 2018; Duarte *et al.*, 2020).

A clear growing potential in the use of associative bacteria for the propagation of plant species is observed, which is a promising biological strategy to increase the quality of seedlings, as well as their development and productivity in clonal nurseries.

CONCLUSIONS

1. Hydrogen peroxide is effective in overcoming tegumental dormancy of *P. taeda* L. seeds and in increasing germinability.

2. *A. brasilense* and *P. caribaea* var. hondurensis bacterial isolates have a positive effect on the germination rate and speed of *P. taeda* L. seeds, as well as on the vigor of emerged seedlings.

3. The rooting of *P. taeda* L. mini-cuttings benefits from inoculation with *A. brasilense* and endophytic bacterial isolates. As the promotion of rooting was low, the need to improve this process is highlighted.

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