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GROWTH PROMOTION OF GENETICALLY IMPROVED *Pinus taeda* SEEDLINGS BY INOCULATION WITH SPECIES OF *Bacillus*.

KONDO, Y. R.; PRIMON, A. P.; FIOREZE, A. C. da C. L.; CRUZ, S. P. da. Growth promotion of genetically improved *Pinus Taeda* seedlings by inoculation with species of *Bacillus*. **CERNE**, v. 26,n. 4, p.456-463, 2020.

HIGHLIGHTS

B. amyloliquefaciens at sowing increased shoot dry mass of 2.0-generation plants in 28%.

B. amyloliquefaciens at post-emergence improved by 9.8% height of 1.5-generation plants.

Bacillus amyloliquefaciens at post-emergence raised quality index of *Pinus taeda* by 30%.

Distinct inoculation methods are recommended for 1.5 and 2.0-generation *Pinus* seedlings.

Keywords:
Bacillus amyloliquefaciens
Bacillus subtilis
Genetic improvement
Pine trees
Rhizobacteria

Abstract

This study was carried on evaluating effects of inoculation with *Bacillus* species and generations of genetically improved seeds on *Pinus taeda* growth in nursery conditions. Two experiments, based on distinct inoculation methods (sowing and post-emergence) were performed under a 3 x 2 factorial, completely randomized block design (3 levels of inoculation and 2 levels of genetic improvement of *Pinus taeda*). Plant height and diameter were measured at different stages along seedling development. At harvest, root volume, root fresh and dry mass, shoot fresh and dry mass and quality index were also estimated. Data were submitted to a two-way analysis of variance ($p<0.05$) and Tukey´s test was used to separate means ($p<0.05$). Data from experiment with inoculation at sowing showed that *Bacillus amyloliquefaciens* improved plant height (20%) and shoot dry mass (28%) of 2.0 generation seedlings compared to control plants. Regarding post-emergence inoculation, plants from 1.5 generation presented more pronounced effects of *Bacillus amyloliquefaciens*. Overall, inoculated seedlings were 9.8% taller and produced 60% more root dry mass than non-inoculated plants. Regardless of seedling generation, *B. amyloliquefaciens* increased Dickson Quality Index by 30%. These results suggest that each generation of genetically improved *P. taeda* has different growth responses to *B. amyloliquefaciens*. Methods of inoculation, combined with level of genetic improvement, must also be considered to obtain the best benefit to seedling development when using this rhizobacterium. Based on the current findings, inoculation with *B. amyloliquefaciens* should be incorporated as a silvicultural practice to improve growth of *P. taeda* under nursery conditions.

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INTRODUCTION

Most recent statistics indicate that forest products exhibited, between 2017 and 2018, the highest growth in the past 70 years (FAO, 2019). Moreover, reports show a world production of 3,971 million m^3 of roundwood in 2018, and this average has risen annually since 1980 (FAO, 2020). Americas are the top roundwood producers, holding 28.2% of this value, and Brazil is currently the fourth largest manufacturer (FAO, 2020). This increased demand for wood requires cultivation of fast-growing tree species such as *Eucalyptus* and *Pinus*. In Brazil, 1.6 million hectares of land are currently cultivated with *Pinus* species, mainly *Pinus taeda* and *Pinus elliotti* (IBA, 2019).

Successful wood production in the field demands extensive research programs on genetic improvement of *Pinus* spp., which have been developed since 1950 in Brazil (Paludzyszyn Filho et al., 2002). Most of genetic improvement is based on properties such as wood volume, stem shape and resistance to abiotic stresses (Coutinho et al., 2017, Medeiros and Florindo, 2017; Hayatgheibi et al., 2019). Seeds from improved genotypes are then commercialized and used to obtain seedlings in nursery conditions, which will then be transferred to the field to establish *Pinus* plantations.

Quality and health of seedlings is a key factor to effective adaption and growth of plants in the field. Along with genetically improvement, some techniques have been explored to increase quality of *Pinus* seedlings, such as alternative substrates, fertilizers, growth stimulators and inoculation (Dominguez-Nunes et al., 2015; Madrid-Aispuro et al., 2020; Ostroshenko and Poleschuk, 2020). Among those, inoculation with plant growth-promoting bacteria is likely the least explored in Brazil, despite its proved benefit in other countries (Probanza et al., 2001; 2002; Heredia-Acuna et al., 2019).

Inoculation of forest species, mainly those from *Pinus* genus, has been proved to be a valuable method in silvicultural practices worldwide. For instance, nursery production of *P. pinaster* seedlings can be done without addition of fertilizer if inoculation is performed with mixed inoculum of certain ectomycorrhizal fungi (Sousa et al., 2012a). Also, the ectomycorrhizal fungus *Suillus bovinus* enhances shoot development of in *Pinus pinaster* up to 30% when plants are grown in soil contaminated with cadmium (Sousa et al., 2012b). Combined inoculation with *Tuber* and *Pseudomonas* increases *Pinus halepensis* shoot biomass in 105% and root biomass in 70%, compared to absence of inoculation (Dominguez et al., 2012).

A few preliminary studies in Brazil about inoculation of *P. taeda* with rhizobacteria highlight potential benefits of this technique for seedlings in nursery conditions. For instance, Brunetta et al. (2010) verified height increments from 10 to 16% when *P. taeda* was grown in substrate containing species of *Bacillus*. Shoot dry mass was also improved by 23%. Santos et al. (2018) reported that substrate inoculation with *Bacillus subtilis* resulted in 33% more shoot dry mass of *Pinus taeda*.

Hence, findings indicate inoculation of *P. taeda* as a technique to improve seedling quality and support faster growth and biomass production, what is a desirable trait for transplanting to the field. However, no study has investigated how different genetically improved generations of *Pinus* could respond to plant growth-promoting rhizobacteria. This is a fundamental aspect that must be understood in order to transfer results from basic to applied research, and also to recommend use of inoculants in forestry crops. Thus, the aim of this study was to evaluate effects of *Bacillus* species and generations of genetically improved seeds on development of *P. taeda* seedlings under nursery conditions.

Material and Methods

This study was carried out in nursery conditions of "Primon Mudas Florestais", located in Curitibanos – SC, Brazil (27°16'60"S, 50°35'7"W). Climate is classified as Cfb, a humid temperate climate with moderately hot summer, according to Köppen´s classification.

Two experiments were established based on distinct inoculation methods: in the substrate at sowing (Experiment 1) and in the substrate at post-emergence (Experiment 2). The first factor was inoculation in three levels (control, *Bacillus subtilis* and *Bacillus amyloliquefaciens*). The second factor was genetically improvement of *Pinus taeda* seedlings in 2 levels (1.5 and second generation). Fifty replicates were established in Experiment 1 and twenty-five replicates were established in Experiment 2. Seeds from 1.5 and 2.0 generation of genetically improvement of *P. taeda* seeds were tested. 1.5 generation seeds were obtained from a first-generation orchard after progeny test and selection, while second-generations seeds were obtained from a 2.0 generation orchard before progeny test and selection (MSU, 2017). Genetic improvement was conducted by Westrock Company based on phenotypic traits related to increased tree volume. Seeds used from both generations were obtained from different individuals in the orchard.

In both experiments, a substrate for plants from Carolina Soil Company was used, which is the standard material used to obtain *Pinus* seedlings in most nurseries of Santa Catarina. Seed dormancy was broken by immersion in water for 24 hours and exposure to 5 °C for 50 days, as established by protocols of Ministry of Agriculture, Livestock

and Food Supply in Brazil (MAPA, 2009). Pre-sterilized 50 cm^3 plastic pots were used in both experiments, which are standard for *Pinus* seedling production.

Species of *Bacillus* were provided as liquid inoculants by Total Bio (Curitiba – PR, Brazil). Concentration of *B. subtilis* inoculum was $1.87x10^8$ CFU mL^{-1}, whereas in *B. amyloliquefaciens* it was of $6.6x10^8$ CFU mL^{-1}. Neither inoculant is currently commercially registered or recommended for *Pinus taeda* by the Ministry of Agriculture, Livestock, and Supply – MAPA. Both inoculants were diluted in water and volume was standardized in order to apply $13.5x10^6$ CFU per gram of substrate regardless of inoculation method. Therefore, bacteria concentration per gram the substrate was the same across experiments and species used.

Experiment 1: Inoculation at sowing

Inoculation was performed in the substrate, placed in large plastic trays. A mixture of inoculant and water was added to the substrate and homogenization was thoroughly executed. In the control treatment, water was added to the substrate. Plastic pots were then filled with substrate. At sowing, three seeds were placed in each pot at a depth of three centimeters. After emergence, only one seedling was kept so each pot containing a plant was considered an experimental unit. Plants were maintained for 150 days under standard nursery conditions. Inoculation at sowing was tested because improved growth and quality of seedlings could translate into less time in nursery conditions. Better developed plants are more likely to be early selected to market sell and generate faster economic turnover.

Plant height and diameter were measured at 120 and 150 days after sowing. At 150 days, evaluations included fresh and dry shoot mass, fresh and dry root mass, root volume and Dickson Quality Index - DQI (Dickson et al., 1960).

First, shoots were separated from roots by cutting at soil level. Both fresh roots and shoots were weighed. Roots were placed in a graduate cylinder containing water to estimate root volume by water displacement (Rossielo et al., 1995). Roots and shoots were then placed in paper bags and dried at 65 °C until constant mass, when values of dry mass of shoot and roots were obtained. DQI was calculated according to Dickson et al. (1960).

Experiment 2: Inoculation at post-emergence.

Inoculation was performed at post-emergence, at 60 days after sowing. All plants used for this experiment had a standard height of 13.0 cm. At this time, seedlings are closer to being commercialized and inoculation could improve the growth of plants before going to the field.

Inoculants were applied by pipetting at the substrate surface close to the plant stem. Non-inoculated plants received water in an equivalent volume. Plants were then kept for additional 90 days under standard nursery conditions. Hence, the complete cultivation time was 150 days (60 days before inoculation and 90 days after inoculation).

Statistical analysis was conducted with the software R Core Team (2013). Data were first verified for fulfillment of ANOVA assumptions. Then, a two-way analysis of variance ($p<0.05$) was executed. When significant effects were found, either from interaction or from a single factor, means were analyzed by the Tukey test ($p<0.05$).

RESULTS

Inoculation at sowing

Stem diameter values from 120 days after sowing were log-transformed so that normality was achieved. This trait affected by genetic breeding ($Pr>Fc=0.0085$), and it was higher in 1.5 generation (1.31cm) than in 2.0 generation (1.21cm).

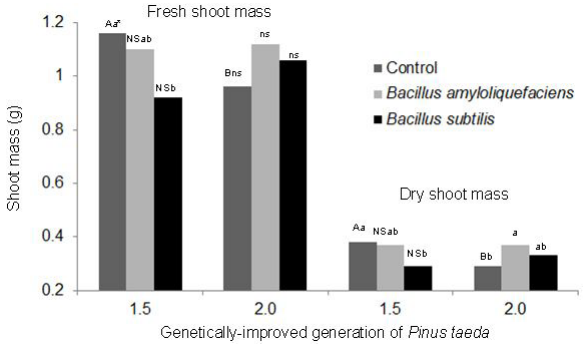
Plant height was affected by interaction between both factors at 150 days after sowing ($Pr>Fc=0.0074$). Plants from 2.0 generation seeds when inoculated with *Bacillus amyloliquefaciens* were 2.63 cm taller than non-inoculated plants (Table 1). Height of non-inoculated 1.5 generation seedlings (15.78 cm) was very similar to height of 2.0 generation seedlings inoculated with *B. amyloliquefaciens* (15.60 cm), as shown in Table 1.

TABLE 1 Interaction effects of generations and inoculation treatments on height (centimeters) of *P. taeda* seedlings under nursery conditions at 150 days after sowing. Curitibanos – SC, Brazil.

Generation/ Inoculation treatment	Control	*Bacillus amyloliquefaciens*	*Bacillus subtilis*
1.5	$15.78\pm2.7^{BAns*}$	14.76 ± 3.29^{NS}	14.58 ± 2.92^{NS}
2.0	12.97 ± 3.29^{Bb}	15.60 ± 2.99^{a}	13.41 ± 3.67^{b}

* Means followed by distinct capital letters (within columns) and lower-case letters (within lines) are statistically different according to Tukey´s test ($p<0.05$). ns = no significant difference among means within lines; NS = no significant difference between means within columns.

A significant interaction effect was observed on fresh shoot mass ($Pr>Fc= 0.0285$) and dry shoot mass ($Pr>Fc=0.0226$). The highest mean of fresh shoot mass was observed in 1.5 generation for non-inoculated seeds (Figure 1). However, shoot dry mass was increased by 28% when second-generation seeds were inoculated with *Bacillus amyloliquefaciens* (Figure 1). Inoculation with this rhizobacterium resulted in 0.37g of dry mass. It is similar to the observed in 1.5 generation for non-inoculated seeds (0.38g).



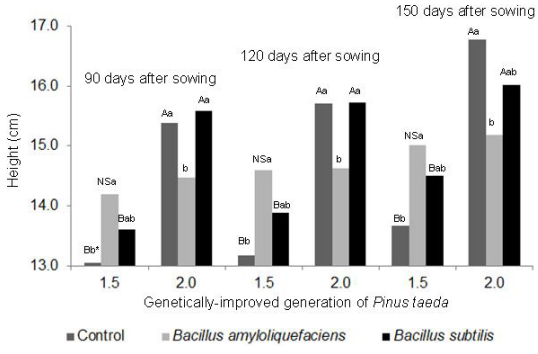
* Means followed by distinct capital letters (distinct generations with the same inoculation treatment) and lower-case letters (distinct inoculation treatments in the same generation) are statistically different according to Tukey´s test ($p<0.05$). NS = no significant difference between distinct generations with the same inoculation treatment; ns = no significant difference among distinct inoculation treatments in the same generation.

FIGURE 1 Interaction effects of generations and inoculation treatments on fresh and dry shoot mass of *Pinus taeda* seedlings under nursery conditions. Data obtained at 150 days after sowing. Curitibanos – SC, Brazil.

Neither Dickson quality index of root volume was affected by any studied factor (inoculation or genetic improvement. The quality index and root volume averages were 0.06 and 96.1mL, respectively.

Inoculation at post-emergence.

A significant interaction effect was verified on plant height throughout all sampling dates: 90 days after sowing (Pr>Fc=0.00003), 120 days after sowing (Pr>Fc=0.00002) and 150 days after sowing (Pr>Fc=0.00002). Plants obtained from 1.5 generation seeds showed means of height that were 8.7%, 10.8% and 9.8% higher with *B. amyloliquefaciens* at 90, 120 and 150 days after sowing, correspondingly (Figure 2).



* Means followed by distinct capital letters (distinct generations with the same inoculation treatment) and lower-case letters (distinct inoculation treatments in the same generation) are statistically different according to Tukey´s test ($p<0.05$). NS = no significant difference between distinct generations with the same inoculation treatment; ns = no significant difference among distinct inoculation treatments in the same generation.

FIGURE 2 Interaction effects of generations and inoculation treatments on height of *P. taeda* seedlings (centimeters) under nursery conditions at 90, 120 and 150 days after sowing. Curitibanos – SC, Brazil.

Data on stem diameter at 90 days after sowing were log-transformed so that normality was achieved. A single effect of seed generation was observed throughout the entire experiment, and highest means were achieved with 1.5 generation seeds (Table 2).

TABLE 2 Effect of generations of genetic improvement on stem diameter (millimeters) of *Pinus taeda* seedlings under nursery conditions at 90, 120 and 150 days after sowing (DAS). Curitibanos – SC, Brazil.

Generation	90DAS	120DAS	150DAS
1.5	$2.66\pm0.49^{A*}$	2.75 ± 0.51^{A}	2.98 ± 0.56^{A}
2.0	2.19 ± 0.26^{B}	2.46 ± 0.27^{B}	2.83 ± 0.33^{B}

* Means followed by distinct capital letters (within columns) are statistically different according to Tukey´s test ($p<0.05$).

Shoot fresh and dry mass, root volume, as well as root fresh mass, were not affected by any experimental factor (data not shown). However, an interaction effect was observed on root dry mass (Pr>Fc=0. 03458). Plants obtained from 1.5 generation seeds produced 60% more root mass when inoculated with *Bacillus amyloliquefaciens* (Table 3).

TABLE 3 Interaction effects of generations and inoculation treatments on root dry mass (expressed in grams) of *Pinus taeda* seedlings under nursery conditions at 150 days after sowing. Curitibanos – SC, Brazil.

Generation/ Inoculation treatment	Control	Bacillus amyloliquefaciens	Bacillus subtilis
1.5	$0.22\pm0.11^{NSb*}$	0.35 ± 0.17^{NSa}	0.28 ± 0.11^{NSab}
2.0	0.29 ± 0.11^{ns}	0.29 ± 0.13	0.27 ± 0.15

* Means followed by lower-case letters (within lines) are statistically different according to Tukey´s test ($p<0.05$). ns = no significant difference among means within lines; NS = no significant difference between means within columns.

An inoculation effect was expressed on Dickson quality index (Pr>Fc=0. 0.01647). Inoculation with *B. amyloliquefaciens* improved this parameter by 30% over control plants, regardless of seed generation (Table 4).

TABLE 4 Effect of inoculation treatments on Dickson Quality Index (DQI) of *Pinus taeda* seedlings under nursery conditions. Data obtained at 150 days after sowing. Curitibanos – SC, Brazil.

Inoculation treatment	DQI
Control	$0.10\pm0.04^{B*}$
Bacillus amyloliquefaciens	0.13 ± 0.06^{A}
Bacillus subtilis	0.11 ± 0.04^{AB}

* Means followed by distinct capital letters (within columns) are statistically different according to Tukey´s test ($p<0.05$).

DISCUSSION

Results from both experiments showed that more pronounced inoculation effects were achieved

with *B. amyloliquefaciens* rather than *Bacillus subtilis*, regardless of seed generation. *B. amyloliquefaciens* has been evidenced to greatly improve growth and yield of agricultural crops such as soybean and corn (Masciarelli et al., 2014; Kim et al., 2017, Marag and Suman, 2018). Therefore, data from the current study suggest one more species of *Bacillus* (*B. amyloliquefaciens*) that may be further explored in silviculture of *P. taeda*.

Overall, *Bacillus* species are fundamentally known to promote plant growth by production of biofilms and action against root pathogens (Chen et al., 2016; Altaf et al., 2017; Zaccardelli et al., 2020). However, a broader range of plant growth-promoting mechanisms has been suggested, especially, for *B. amyloliquefaciens*. Shahzadi et al. (2019) reported that this species can degrade long chained hydrocarbons, producing small metabolites. Other enzymes described to occur in *B. amyloliquefaciens* strains include chitinases (Wang et al., 2002), proteases (Lee et al., 2010) and laccases (Pan et al., 2011). This, in fact, may help plant growth by accelerating decomposition of organic residues (even the more chemically complex ones) and nutrient mineralization. Lipase, starch hydrolysis and nitrate reduction activities reported in *B. amyloliquefaciens* (Shahzadi et al., 2019) are also properties that may improve soil fertility. Therefore, plant nutrition may be increased, reflecting on enhanced plant height and biomass production.

When *B. amyloliquefaciens* was used in the substrate, 2.0 generation seedlings produced as much height and shoot dry mass as 1.5 generation, non-inoculated seeds. Thus, inoculation was not a determining factor to early development of 1.5 generation seedlings. On the other hand, 1.5 generation seedlings were more benefited from inoculation at post-emergence, since height and root dry mass were highly increased by *B. amyloliquefaciens*. Yet, Dickson Quality Index of seedlings of both generation plants was improved with this bacterium.

Although no other research work reports the interaction between inoculation and genetic improvement of seeds, it is known that this interaction is controlled by genetic and environmental factors, and other researchers have disclosed distinct benefits of inoculation based on how bacteria are applied. Santos et al. (2018) shown that shoot dry weight of *P. taeda* seedlings was improved only when *B. subtilis* was incorporated to the substrate at sowing. In the control treatment, mean shoot dry weight was 1.51g, and inoculated plants produced 2.01g of shoot biomass.

Similar observations have been made regarding other forestry species. Melo et al. (2012) inoculated *Eucalyptus urophylla* cuttings with *Herbaspirillum seropedicae*. Authors observed the best effect on root fresh mass when bacteria were added to the substrate (328g), compared to inoculation by immersion of cuttings (257g). Sarr et al. (2005) studied six methods to inoculate *Acacia senegal* and *Acacia nilotica*. Inoculation with alginate beads containing a mixture of rhizobial strains promoted the best production of shoot dry weight (2.83g) compared to all other treatments, and also to non-inoculated plants (2.20g). Odee et al. (2002) also evaluated five methods to inoculate *Calliandra calothyrsus*. Highest values of total dry weight of plants were obtained with root collar inoculation immediately after transplanting pre-germinated seedlings. However, inoculation at five days after sowing was the most effective method to improve seedling height. Therefore, previous reports support the fact that diverse growth effects are generated from distinct inoculation methodologies.

Results from both experiments indicate inoculation as a significant practice to improve *P. taeda* growth and biomass production under nursery conditions. However, further research must be developed to deeper understand inoculation benefits and elucidate plant growth-promoting mechanisms of *B. amyloliquefaciens* in association with species of *Pinus*. *B. amyloliquefaciens* may also be an interesting bacterium to inoculate when plants are further transferred to the field. In most places, *Pinus* is usually cultivated in highly degraded soils, with low pH and problems regarding fertility and physical stability. This bacterium species produces exopolysaccharides that are responsible for its acid tolerance (Deka et al., 2019), what may improve rates of survival of the bacteria if inoculated on *Pinus* in field conditions. Furthermore, these exopolysaccharides are also correlated with soil aggregation (Deka et al., 2019), an important aspect to improve soil quality and plant growth.

Also, enzymatic activity of *B. amyloliquefaciens* related to degradation of complex substrates (Wang et al., 2002, Pan et al., 2011; Shahzadi et al.; 2019) may be of extreme value when *Pinus* is introduced in previously-cultivated soils, with higher quantities of acicula, bark and leftover roots, as well as unbalanced C:N ratio. In this situation, bacteria may accelerate degradation of these organic compounds and improve nutrient mineralization, which may have positive effects on plant nutrition, growth, and improved wood production.

CONCLUSIONS

B. amyloliquefaciens should be used in the substrate, at sowing, to inoculate 2.0 generation seeds

of *P. taeda*. Post-emergence inoculation, however, is indicated for 1.5 generation seedlings.

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