Hindawi
Applied and Environmental Soil Science
Volume 2018, Article ID 6027351, 8 pages
https://doi.org/10.1155/2018/6027351

Research Article

Plant Growth Response of *Pinus patula* and *P. maximinoi* Seedlings at Nursery to Three Types of Ectomycorrhizal Inocula

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Received 17 April 2018; Revised 12 August 2018; Accepted 2 September 2018; Published 26 September 2018

Academic Editor: Marco Trevisan

The objective of this study was to assess the response in seedling growth, root colonization, and P content of seedlings of *Pinus maximinoi* and *P. patula* to the inoculation with three types of ectomycorrhizal inocula with three doses (17.5, 35, and 70 kg·m^{-3}) in nursery. The first inoculum was soil from a *Pinus* plantations that contained three ectomycorrhizal fungi (*Amanita muscaria, Amanita* sp., and *Suillus luteus*); the second was a crude inoculum composed by root fragments of *Pinus* seedlings colonized by *S. luteus* suspended in a sterile matrix soil-sand; the third inoculum was a mixture of two ectomycorrhizal fungi *A. muscaria* and *S. luteus* produced under *in vitro* conditions in the potato-dextrose-agar medium. The results showed that the inoculum produced *in vitro* was most effective to promote plant growth and ectomycorrhizal colonization of roots in both plant species. Also, the effects on seedlings were significantly higher with the increase of the doses. In *P. patula* there were not significant effects on foliar P content with type and dose of inocula, whereas in *P. maximinoi* there were interactive effects of both factors. In this case, better results were obtained with the inoculum produced under *in vitro* conditions and with the highest dose.

1. Introduction

The low availability of phosphorus (P) in tropical soils represents one of the main constraints for a successful growth of seedlings in reforestation projects [1]. In the Colombian Andes, where P is strongly fixed by volcanic ash soils [2], reforestation has been mainly performed using exotic coniferous species, more than 50% of them comprises species of *Pinus* genus [3, 4]. Hence, these species must overcome the lack of enough soil available P in order to grow satisfactorily [1].

These plants form a symbiotic association with ectomycorrhizal fungi, which promotes seedling growth and nutrient uptake in P-deficient soils [5, 6]. This strategy is considered a key factor at nursery in ensuring the growth and subsequent establishment of seedlings in the field [7, 8]. The effectiveness to promote plant nutrient uptake and growth depends on ectomycorrhizal propagule-density (EPD) of the inoculum [8–10]. Then, such effectiveness may be controlled by inoculum dose, inoculum type [10–12], and plant-fungus specificity [10, 13, 14]. Inoculation protocols at nursery usually involve a standardized application of some inocula in prescribed doses [15], without discriminating the seedling species. This seems to be the case for both *Pinus maximinoi* and *P. patula*, for which the effectiveness of currently ectomycorrhizal inoculation protocols at nursery remains unknown. This issue is becoming more relevant because both species are planted with increasing frequency worldwide [11, 16–18], representing, for instance, the main pine species for commercial reforestation in highlands of Colombia.

The objective of this work was to evaluate the seedling growth and P uptake responses of seedlings of both *P. maximinoi* and *P. patula* as a function of ectomycorrhizal inoculum dose and three types of inoculum: (i) soil from pine plantations, (ii) crude inoculum composed by plant

root fragments of a cultivated *Pinus* seedlings in a substrate previously inoculated with an ectomycorrhizal fungus, and (iii) *in vitro* inoculum.

2. Materials and Methods

2.1. Site. The study was carried out in Santa Elena, Medellin, Colombia (06°15′26″N, 75°30′08″W), at an altitude of 2500 m. In this area, the mean annual precipitation is 1760 mm, the mean temperature is 15°C, and the relative humidity is above 85% throughout the year. This corresponds to the ecological zone life of lower montane moist forest [19].

2.2. Plant Growth Substrate. The substrate was prepared by thoroughly mixing a soil with sand in a ratio of 3 : 1 (v : v). The soil sample was collected from a surface horizon (horizon A, 0–25 cm) of a soil classified as Melanudand (volcanic ash soil), which was under grass coverage (without fertilization) at the time of collection. Soil tests were conducted at the Laboratory of Biogeochemistry in the Universidad Nacional de Colombia (Medellín, Colombia). The results were as follows: soil pH 5.6 (water, 1 : 2); soil organic matter content 164 $g \cdot kg^{-1}$ (Walkley and Black); P 2 $mg \cdot kg^{-1}$ (Bray II); Ca, Mg, and K 2.8, 0.5, and 0.12 $cmol_c \cdot kg^{-1}$, respectively (1 M ammonium acetate). The substrate was sterilized with Basamid (Dazomet: 3, 5-dimethyl-1,3,5-thiadiazinane-2-thione) (200 $g \cdot m^{-3}$). The sterilized substrate was covered with a plastic sheet for two weeks and then aerated for five days. After this time period, the substrate received a commercial fertilizer grade 10-30-10 (3 $kg \cdot m^{-3}$).

2.3. Ectomycorrhizal Inocula Treatments. Three ectomycorrhizal inocula were used in this study: (i) Soil collected from *P. patula* plantations (0–25 cm depth) as suggested by Courty et al. [20]; this inoculum contained roots and ectomycorrhizal propagules of the fungi *Amanita muscaria*, *Amanita* sp., and *Suillus luteus*, which was labeled as *Plantation-Soil inoculum* (PS) and is traditionally used in local nurseries (34 infective propagules per g); this type of inoculum is used worldwide [1, 21, 22]. (ii) The second one was a crude inoculum obtained by mixing fine root fragments and its growing substrate (soil sand), which was previously disinfected with Basamid (200 $g \cdot m^{-3}$), inoculated with a fungal suspension of *Suillus luteus* (5×10^4 $CFU \cdot mL^{-1}$), and then planted with *Pinus patula* seedlings that grew for 6 months, and this inoculum was labeled *Crude inoculum* (CR) and was produced following the protocol described by Chen et al. [10]. (iii) The third inoculum was a mixture of two ectomycorrhizal fungi (*A. muscaria* and *S. luteus*) multiplied under *in vitro* conditions in the autoclaved (120°C, 0.1 MPa, 20 min) potato-dextrose-agar medium for 10 days at 25°C; 5 mL of this broth was mixed with 1 kg of disinfected soil and was named *In Vitro inoculum* (IV). This inoculum contained 156 infective propagules per g (MPN technique) [8].

We employed three doses of each inoculum: 17.5, 35, and 70 $kg \cdot m^{-3}$, respectively. The inoculated substrate was used to fill 16 plastic bags (6 × 12 cm, capacity of 160 cm^3) with 180 g per bag.

2.4. Plant Growth Conditions. Seeds of *P. maximinoi* and *P. patula* were obtained from the Santa Elena nursery (Vereda Mazo, Medellin, Colombia) and germinated in sterile sand for 10 days. Then, the seedlings were transplanted into the plastic bags (one per bag) containing the inoculated substrate. After 30 days, the seedlings were sprayed with the fertilizer Wuxal (20-0-15) on a monthly basis. The nursery growth period lasted 6 months. Plants were grown in an open air environment and exposed to site conditions.

2.5. Experimental Design. Each plant species was used in a separated experiment. A completely randomized experimental design was used; the treatments had a factorial 3 × 3 arrangement, i.e., three types of inocula (PS, CR, and IV) and three inoculum doses (17.5, 35, and 70 $kg \cdot m^{-3}$). Each treatment had 16 replicates.

2.6. Variables. Shoot height (SH) and root collar diameter (RCD) of both plant species was measured fortnightly, starting 30 days after germination. At harvest time (180th day), five seedlings were randomly selected from each treatment to check for root ectomycorrhizal colonization (REC) using the gridline intersection method [23]. For this, the roots were rinsed with tap water to remove substrate residues and then kept in 50% ethanol. Subsequently, 30 fragments (1-cm length) were scattered at random on a 1 × 1 cm grid. The presence or absence of the ectomycorrhizal association in each gridline intercept was registered under a stereomicroscope. Shoot dry weight (SDW), after oven drying plant material (65°C, 72 h), was measured in all replicates. Foliar P content (FPC) was measured following the procedure developed by Habte et al. [24] in 1 cm fragments of young-mature needles taken from the seedlings. To this purpose, fragments of the needles were transferred to test tubes, dried in an oven (at 65°C for 24 h), weighed, and then ashed in a muffle furnace at 500°C for 3 h. Subsequently, the ashes were dissolved with distilled water, and the solution P concentration was determined by the molybdate blue method [25].

2.7. Data Analysis. Data were analyzed using ANOVA (*F*-test) and the multiple range test of Duncan for mean separation (*t*-test); both were conducted with a significance level ($P \leq 0.05$). The tests were performed with the software R (R Studio 0.98.501).

3. Results and Discussion

The treatments had significant effects ($P \leq 0.05$) on the variables studied (Table 1); in *P. patula*, there were only simple effects due to each individual factor (inoculum type and inoculum dose) without interaction between them. In the case of *P. maximinoi*, there were significant effects of inoculum type and inoculum dose on SH and SDW, but on RCD only the inoculum type had significant effects.

Table 1: *P* values of the ANOVAs for shoot height (SH), root collar diameter (RCD), shoot dry weight (SDW), root ectomycorrhizal colonization (REC), and foliar P content (FPC) of *P. patula* and *P. maximinoi* seedlings under the different treatments tested.

Source	REC	SH	RCD	SDW	FPC
P. patula					
Inoculum type (T)	<0.001	<0.001	<0.001	<0.001	0.082
Inoculum dose (D)	<0.001	<0.001	<0.001	<0.001	0.057
T × D	0.204	0.382	0.057	0.290	0.950
P. maximinoi					
Inoculum type (T)	<0.001	<0.001	<0.001	<0.001	<0.001
Inoculum dose (D)	<0.001	<0.022	<0.070	<0.001	0.041
T × D	<0.001	0.903	0.844	0.919	0.003

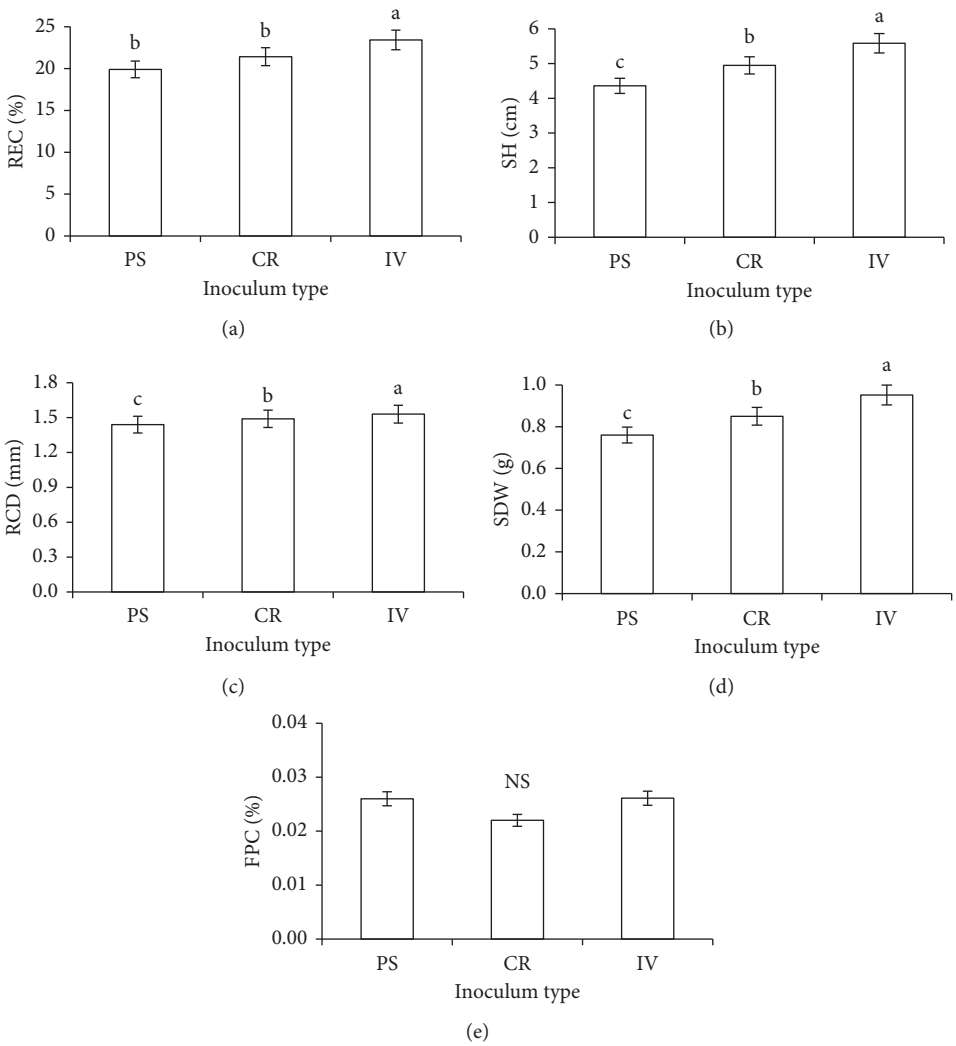

Figure 1: (a) Root ectomycorrhizal colonization (REC), (b) shoot height (SH), (c) root collar diameter (RCD), (d) shoot dry weight (SDW), and (e) foliar P content (FPC) of *P. patula* seedlings as a function of inoculum type. Bars indicate standard error. Columns with different letters indicate significant difference ($P \leq 0.05$) according to the multiple range test of Duncan. NS = not significant.

In addition, REC and FPC were significantly affected by the interaction inoculum type × inoculum dose.

3.1. P. patula. For instance, the REC of seedlings of *P. patula* inoculated with PS, CR, and IV inocula was 19.9, 21.4, and 23.4%, respectively; the last one was significantly higher than the other two (Figure 1). Also, the inoculum dose of 70 kg·m^{-3} exhibited a significantly higher REC (23.6%) than the other doses (21.3 and 19.18% for 35 and 17.5 kg·m^{-3}, respectively), which were not significantly different to each other (Figure 2).

Likewise, the SH of the seedlings was 4.36, 4.95, and 5.59 cm with PS, CR, and IV inocula, respectively, which

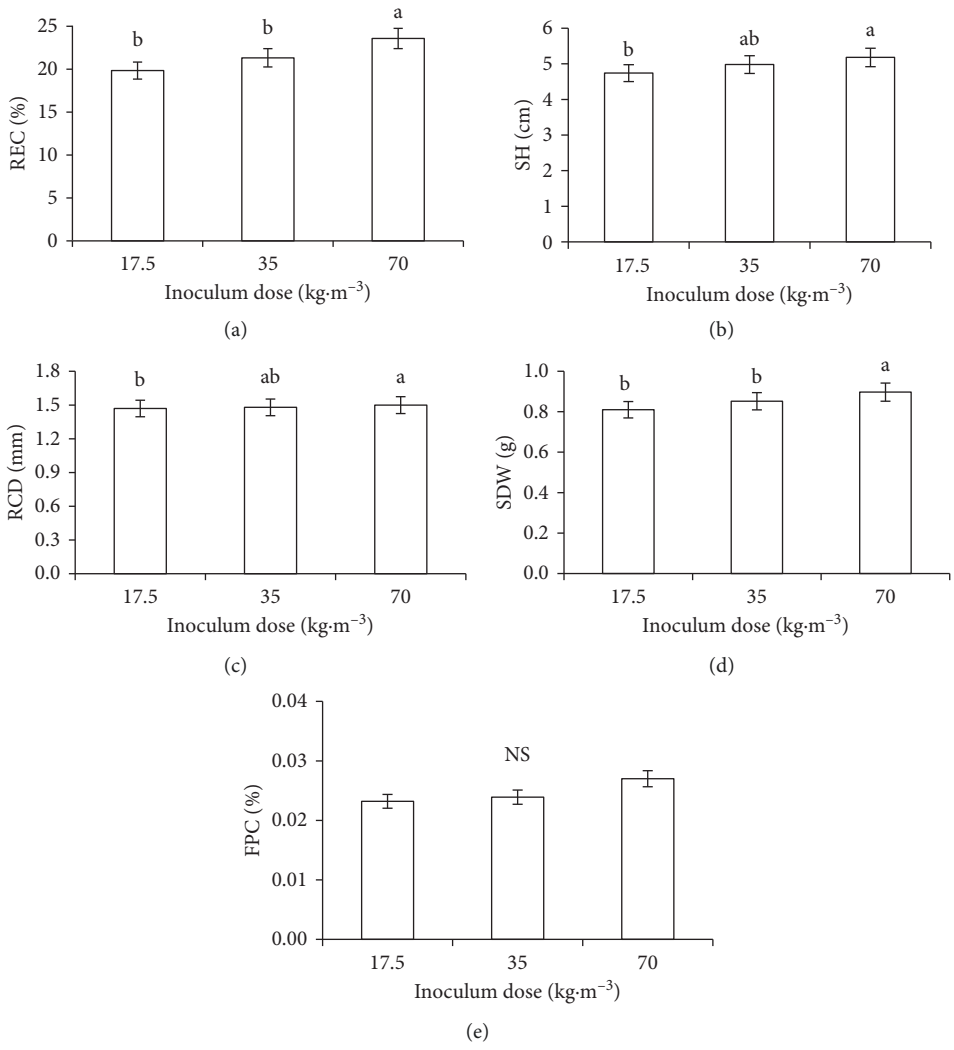

Figure 2: (a) Root ectomycorrhizal colonization (REC), (b) shoot height (SH), (c) root collar diameter (RCD), (d) shoot dry weight (SDW), and (e) foliar P content (FPC) of *P. patula* seedlings as a function of inoculum dose. Bars indicate standard error. Columns with different letters indicate significant difference ($P \leq 0.05$) according to the multiple range test of Duncan. NS = not significant.

were significantly different among them. The seedlings with dose of 17.5 kg m^{-3} had an SH of 4.74 cm, and this was not different from those with the dose of 35 kg·m^{-3} (4.98 cm), but it was different from those with the highest dose (5.18 cm) (Figure 2).

The RCD had a similar pattern, and the values were 1.44, 1.49, and 1.53 mm with PS, CR, and IV inocula, respectively, which had significant differences among them (Figure 1). The seedlings with the inoculum dose of 17.5 kg·m^{-3} had a RCD of 1.47 mm, and this was not different from those with the dose of 35 kg·m^{-3} (1.48 mm), but it was different from those with the dose of 70 kg m^{-3} (1.50 mm) (Figure 2). Also, there were significant differences among the means of SDW as the inoculum type varied: it was 0.76, 0.85, and 0.95 g per plant when the inoculation was with PS, CR, and IV, respectively (Figure 1). The SDW was significantly higher with the highest dose (0.90 g per plant) than the other two doses (0.81 and 0.85 g with 17.5 and 35 kg·m^{-3}, respectively), which did not differ from each other (Figure 2). In contrast, there were no significant differences in the values of FPC with the inocula used, and they ranged between 0.022 and 0.026%.

3.2. P. maximinoi. As mentioned above, there were significant effects of inoculum type and inoculum dose on SH and SDW of *P. maximinoi*; however, in the RCD only, the inoculum type had significant effects (Table 1). Thus, the SH of the seedlings of *P. maximinoi* was 7.81, 9.48, and 10.43 cm with PS, CR, and IV inocula, respectively, which were significantly different among them (Figure 3). On the other hand, the seedlings with the dose of 17.5 kg m^{-3} had an SH of 9.08 cm, and this was not different from those with the dose of 35 kg·m^{-3} (9.11 cm), but it was significantly different from those with the highest dose (9.53 cm) (Figure 4).

The SDW of *P. maximinoi* seedlings had a similar pattern, and the values were 1.31, 1.57, and 1.72 mm with PS, CR, and IV inocula, respectively, which had significant

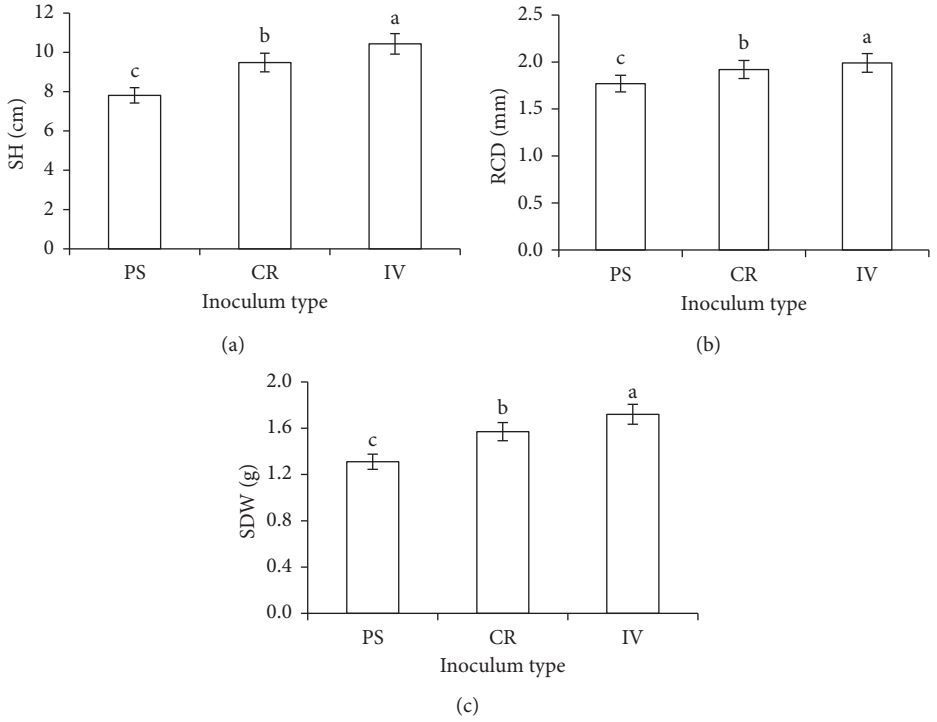

Figure 3: (a) Shoot height (SH), (b) root collar diameter (RCD), and (c) shoot dry weight (SDW) of *P. maximinoi* seedlings as a function of inoculum type. Bars indicate standard error. Columns with different letters indicate significant difference ($P \leq 0.05$) according to the multiple range test of Duncan.

differences among them (Figure 3). The seedlings with the doses of 17.5 and 35 kg·m^{-3} had an SDW of 1.49 mm, and it was different from those with the dose of 70 kg·m^{-3} (1.61 mm) (Figure 4).

On the other hand, the RCD of *P. maximinoi* seedlings was affected only by the inoculum type but not by the inoculum dose. The values of RCD with the inocula PS, CR, and IV were 1.8, 1.9, and 2.0, respectively, which were significantly different among them (Figure 3).

The REC of *P. maximinoi* seedlings was significantly affected by the interaction inoculum type × inoculum dose (Figure 5). The values of REC were significantly higher with the IV inoculum (29–36%) than with the other two inocula (CR: 18–28%, and SP: 18–21%), but with all inocula the values of REC were significantly higher with the dose of 70 kg·m^{-3} than with the other two doses.

Also, there were significant differences in the FPC of *P. maximinoi* seedlings with the interaction inoculum type × inoculum dose (Figure 5). For instance, in the case of the inocula PS and CR, the increase of the inoculum dose decreased the FPC values (PS: from 0.032 to 0.026%; CR: from 0.022 to 0.015%). By contrast, with the IV inoculum the increase of the inoculum dose produced significant increases in the FPC from 0.0255 to 0.036% (Figure 5).

3.3. Implications for Practice. Our results showed that both dose and inoculum type were effective in promoting seedling growth of both pine species studied. It is clear that better results were obtained with IV inoculum, followed by CR inoculum and then by PS inoculum in variables of plant growth such as SH, RCD, and SDW. This likely occurred as a result of a higher density of infective propagules in IV inoculum (156 per g), which was four times higher than that in PS inoculum (39 per g) as measured by Restrepo-Llano et al. [8]. This certainly increased the probability of roots to be colonized [26, 27]. For instance, in *P. patula* the value of REC with IV inoculum was 24% and with the other two inocula was 20–21%. In the case of *P. maximinoi*, there were interactive effects, being the REC higher with IV inoculum. However, since seedlings of both species received the same treatments, the differences observed in REC levels may be associated with plant-fungus-specific interactions [10, 11, 28–30]. In general, the effectiveness of these inocula was as follows: IV inoculum > CR inoculum ≥ PS inoculum.

Seedling growth promotion was significantly higher with the increase of the inocula dose in both species. This trend agrees with the results for growth promotion of *P. patula* seedlings reported by Restrepo-Llano et al. [8]. Thus, as the inoculum dose increases, the density of ectomycorrhizal propagules also increases and favors more REC in the seedlings [26, 27, 31]. As a consequence, the probabilities of roots to be colonized may be higher if any factors constraining the root colonization process are absent [14, 15, 31–39]. These results suggest that the inoculum dose employed plays a more important role than normally considered for plant growth promotion [40]. In general, the effectiveness of the inoculum dose was as follows: 70 > 35 ≥ 17.5 kg m^{-3}.

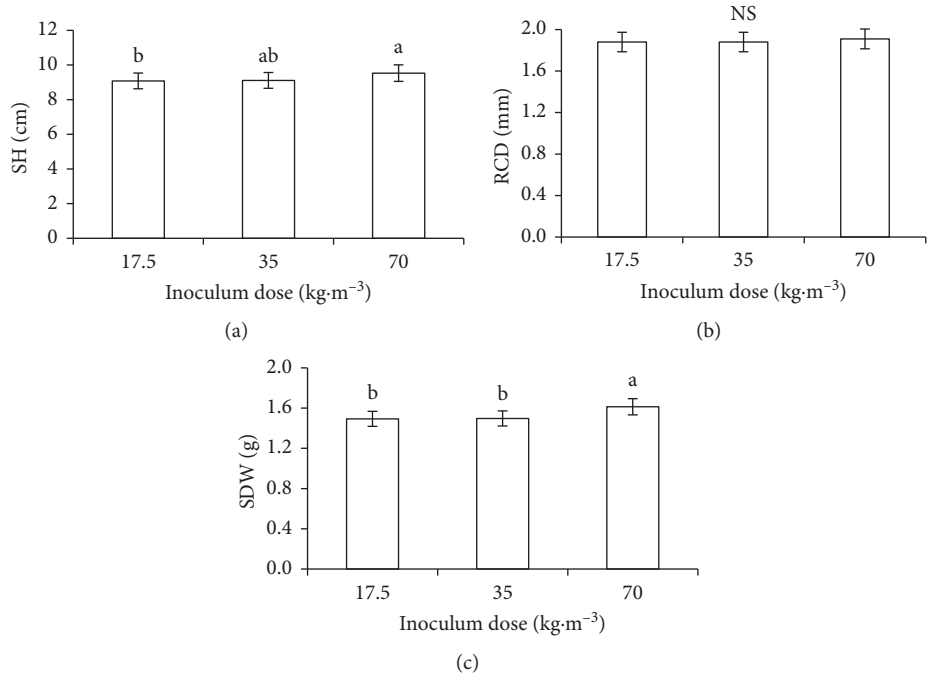

Figure 4: (a) Shoot height (SH), (b) root collar diameter (RCD), and (c) shoot dry weight (SDW) of *P. maximinoi* seedlings as a function of inoculum dose. Bars indicate standard error. Columns with different letters indicate significant difference ($P \leq 0.05$) according to the multiple range test of Duncan.

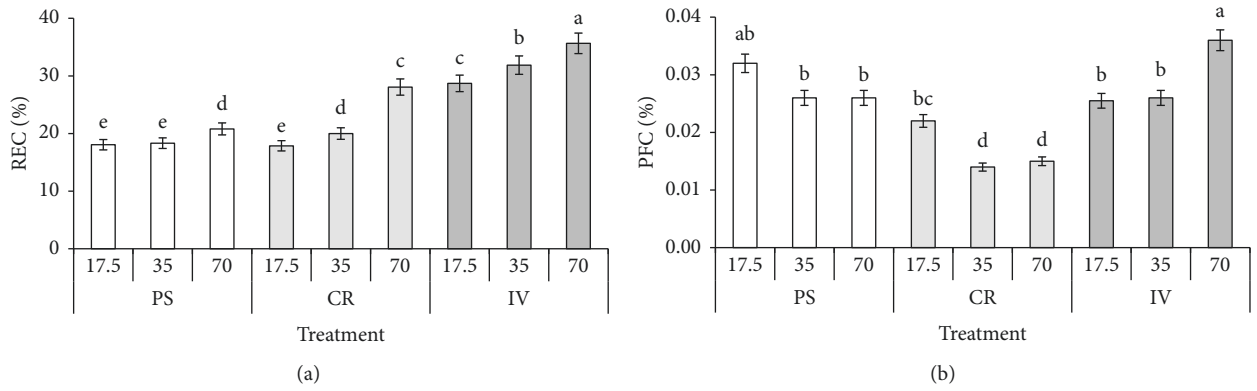

Figure 5: (a) Root ectomycorrhizal colonization (REC) and (b) foliar P content (FPC) of *P. maximinoi* seedlings as a function of the interaction inoculum type × inoculum dose. Bars indicate standard error. Columns with different letters indicate significant difference ($P \leq 0.05$) according to the multiple range test of Duncan.

Surprisingly, in *P. patula* the foliar P concentration was not significantly affected by dose and inoculum type, whereas in *P. maximinoi* there were interactive effects. This may be due to a dilution effect that masked the increase in seedling P uptake or intrinsic differences in growth rates between both species [4, 41, 42]. Note that *P. maximinoi* exhibited higher growth values than *P. patula*, which perhaps limited the plant P uptake in the latter. Although *P. maximinoi* showed a tendency to increase its growth with the application of higher doses of PS and PR inocula, its foliar concentrations of P decreased (Figures 4 and 5). It may be due also to a dilution effect [43–47]. By contrast, the increase in the IV inoculum dose produced a significantly higher foliar P concentration (Figure 5), which suggests that the effect was consistently better with this inoculum. This is also supported by the REC values in this plant species, which trended to be higher with IV inoculum than with the other two. Other studies carried out with *Pinus* species have reported that growth promotion was associated with high levels of ectomycorrhizal root colonization [1, 14, 31, 40, 48].

In this study, the highest doses of inoculation appeared to be most effective for promoting the seedling growth and ectomycorrhizal root colonization of both *P. maximinoi* and *P. patula*. The IV inoculum was more effective than PS and CR inocula for promoting plant growth and colonization in both species, suggesting that the number of infective

propagule in the inoculum should be considered for better effects.

From a practical point of view, the results indicate that the use of soil from pine plantations as a source of ectomycorrhizal inoculum is not the best alternative, as reported earlier by Castrillón et al. [4]. This has been widely used in Colombian forest nurseries. In addition, the removal of soil from these sites produces negative environmental impacts. On the other hand, the use of *in vitro* ectomycorrhizal inoculum seems to be more effective, and also its use would reduce the negative impacts of soil-inoculum extraction. Also, the use of *in vitro* inoculum is a more feasible alternative as a source of inoculum, particularly during the drier seasons when the fruit bodies of ectomycorrhizal fungi are scarce.

Data Availability

The data used to support the findings of our study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors thank D. Carvajal and Vivero Santa Elena at Medellin for technical support and provide facilities and materials. This work was supported by the Departamento Administrativo de Ciencia, Tecnología e Innovación (Colciencias, Young Scientists Program, 2012), and Gobernación de Antioquia (Knowledge Generation Program, 2013).

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