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Research Article

Assessment of the Effectiveness of Ectomycorrhizal Inocula to Promote Growth and Root Ectomycorrhizal Colonization in *Pinus patula* Seedlings Using the Most Probable Number Technique

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The aim of this study was to evaluate the response of *Pinus patula* seedlings to two inocula types: soil from a *Pinus* plantation (ES) and an *in vitro* produced inoculum (EM). The most probable number method (MPN) was used to quantify ectomycorrhizal propagule density (EPD) in both inocula in a 7-order dilution series ranging from 10^0 (undiluted inoculum) to 10^{-6} (the most diluted inoculum). The MPN method allowed establishing differences in the number of infective ectomycorrhizal propagules' density (EPD) (ES = 34 per g; EM = 156 per g). The results suggest that the EPD of an inoculum may be a key factor that influences the successfulness of the inoculation. The low EPD of the ES inoculum suggests that soil extracted from forest plantations had very low effectiveness for promoting root colonization and plant growth. In contrast, the high EPD found in the formulated inoculum (EM) reinforced the idea that it is better to use proven high quality inocula for forest nurseries than using soil from a forestry plantation.

1. Introduction

It is broadly recognized that ectomycorrhizal associations play a key role in the nutritional status and growth of forest plants [1–3] and, in some cases, their presence is essential for such plants [2]. One of these plants is *Pinus patula*, a species with great economic importance worldwide [4]. The success of *P. patula* plantations depends on the establishment of ectomycorrhizal associations with certain fungal species [5–7].

The ectomycorrhizal association has been demonstrated as an outstanding symbiotic mechanism for *P. patula* silviculture because (a) it promotes seedling growth and nutrient uptake during the nursery stage [2], (b) it reduces nutrient additions within nursery management programs [8], and (c) it protects the roots against soil-borne pathogens [2, 9].

It has been accepted that the addition of a formulated ectomycorrhizal inoculum for *P. patula* seedlings effectively induces the establishment of ectomycorrhizas features in the roots in comparison with the spontaneous and natural colonization from native propagules [2, 7, 10]. Despite the importance of inoculation for promoting plant growth, little is known about the quality of ectomycorrhizal inocula or inoculum type, which has limited the scale of their use.

We hypothesize that the growth and root ectomycorrhizal colonization of *P. patula* seedlings are affected by ectomycorrhizal propagule density (EPD) and it can be used as an indicator of inoculum quality. From a practical point of view, it will help nursery producers and governmental authorities to regulate inoculum standards to be used. In this study, we propose that the most probable number (MPN) method [11] may be used to quantify the EPD in different inocula capable of promoting *P. patula* seedling growth and root colonization. Furthermore, this method may be used to examine the effectiveness of techniques traditionally used to produce ectomycorrhizal inocula: (i) the extraction of soil from *P. patula* plantations [8, 12, 13] and (ii) *in vitro* production of fungal inoculum [14].

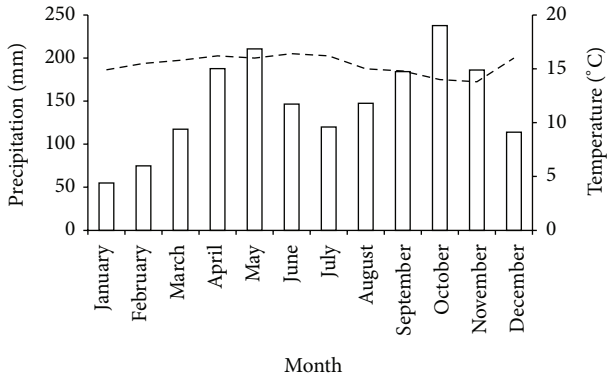

Figure 1: Monthly precipitation and mean temperature values along 2013.

2. Materials and Methods

2.1. Site. The experiment was carried out in Santa Elena, Medellin, Colombia (06°15′25.6″N, 75°30′08.2″W), at an altitude of 2500 m. In this area, the mean annual precipitation is 1760 mm and the mean temperature is 15°C (Figure 1).

2.2. Soil/Substrate. The substrate was obtained by thoroughly mixing a soil with sand (3 : 1, V : V); the soil was collected from a surface horizon (horizon A, 0–25 cm). This soil was classified as Melanudand (volcanic ash soil) and was under grass coverage at the time of collection. Soil tests were conducted at the Biogeochemistry Laboratory at the Universidad Nacional de Colombia at Medellín (soil pH 5.6, organic matter content 16.4%, Bray No. 2-phosphorus 2 mg kg^{-1}, and 1 M ammonium acetate-Ca, -Mg, and -K 2.8, 0.5, and 0.12 $cmol_c$ kg^{-1}, resp.). The substrate was sterilized with Basamid (active ingredient dazomet: 3,5-dimethyl-1,3,5-thiadiazinane-2-thione) with a dose application of 200 g/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 3 kg m^{-3} of a commercial fertilizer grade 10-30-10.

2.3. Fungal Inocula Treatments. Two ectomycorrhizal inocula were used in this study. The first inoculum corresponded to an undetermined mixture of two ectomycorrhizal species (*Amanita muscaria* and *Suillus luteus*), which was multiplied under *in vitro* conditions. The culture medium potato-dextrose-agar (15 g per L) used was previously autoclaved (120°C, 0.1 MPa, 20 min); after a period of incubation of 10 days at 25°C, 5 mL of this broth was mixed with 1 kg of autoclaved soil and was named *ectomycorrhizal mixture* (EM). The second inoculum was obtained from a soil collected from the A horizon (0–25 cm) in a *P. patula* plantation [9]; besides soil it contained roots and ectomycorrhizal propagules of the fungi *Amanita muscaria*, *Amanita* sp., and *Suillus luteus,* which have been observed around the trees of *P. patula* roots; this inoculum was labeled *ectomycorrhizal soil* (ES) and its collection was accomplished by excavating the soil surface and putting it into sterile containers for a week until the inoculation time. This latter inoculum is traditionally used in local nurseries.

2.4. MPN Technique. We used the most probable number (MPN) technique, which is based on the presence or absence of particular features linked to the target microorganism effects in a serial dilution of infected media [15]. In this case, the attribute selected was the presence or absence of ectomycorrhizal structures in the roots of *P. patula* seedlings, as described by Dames et al. [16]. We diluted 320 g of the inoculum (one part) in 2880 g (nine parts) for obtaining 3200 g of the first dilution; it represented a 10^{-1} dilution and was used for 16 seedlings in bags with capacity for 180 g (=3200 g). Subsequently, the second dilution (10^{-2}) was prepared from the 10^{-1} dilution keeping the same proportions. The procedure was repeated until obtaining the 10^{-6} dilution. As a control treatment, we also used undiluted inocula as a growth substrate (without dilution, named 10^0). This substrate of each dilution was used to fill 16 plastic bags (6 × 12 cm, 160 cm^3), with 180 g per bag.

2.5. Plant Growth Conditions. Certified seeds of *P. patula*, obtained from the Santa Elena nursery (Vereda Mazo, Medellin, Colombia), were germinated in sterile sand for 10 days. At this moment the seedlings were transplanted into plastic bags (one per bag) containing inoculated substrate. After 30 days, the seedlings were sprayed monthly with the fertilizer Wuxal (20-0-15). The nursery growth period lasted from February to June 2013 (five months).

2.6. Experimental Design. We used a completely randomized experimental design. All seedlings occupied a surface area of 8 m^2 and were exposed to the same weather conditions without any gradient of moisture, slope, shade, and wind. Treatments had a factorial 2 × 7 arrangement, that is, two inocula (EM, ES) and seven dilution series (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Each treatment had 16 replicates.

2.7. Variables. We measured plant height in all replicates at harvest time (150 days after germination). We also measured root ectomycorrhizal colonization using the gridline intersection method as described by Brundrett et al. [17]. The resulting data were used to estimate the MPN of ectomycorrhizal propagules density (EPD) using the probabilistic table developed by Cochran [11]. Thus, from each treatment group, only five seedlings were randomly selected to check for ectomycorrhizal colonization as indicated in the method. Root collar diameter and shoot dry weight (after oven-drying the plant material at 65°C, 72 h) were measured in all replicates.

2.8. Data Analysis. Data were analyzed using ANOVA and the LSD mean separation test. Both tests were conducted with a significance level (P) ≤ 0.005. The tests were performed with the software R (R Studio 0.98.501) [18].

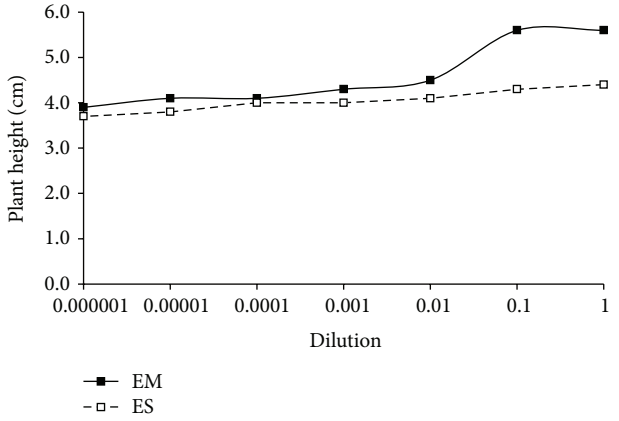

Figure 2: Plant height of *P. patula* seedlings as a function of EM and ES inoculation at each level of serial dilution.

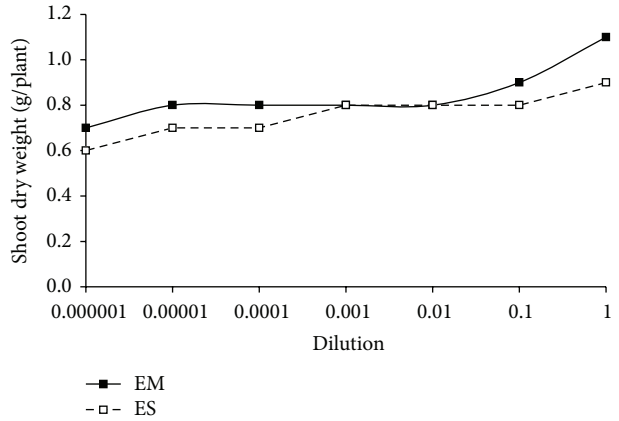

Figure 3: Shoot dry weight of *P. patula* seedlings as a function of EM and ES inoculation at each level of serial dilution.

Table 1: Number of roots (of five) with presence of ectomycorrhizal features and estimated ectomycorrhizal propagule density (EPD) per gram of substrate.

Inoculum	Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	EPD per g
EM	5	5	4	3	3	3	156
ES	4	4	4	4	3	3	34

3. Results

The MPN technique allowed us to separate inoculum based on the estimated EPD. The results indicated that EM and ES had 156 and 34 ectomycorrhizal propagules per gram of inoculum (Table 1). These values will explain the differential effects observed between both inocula on plant performance. In fact, the inoculum type and dilution treatments significantly affected *P. patula* seedling growth and ectomycorrhizal colonization of roots (Table 2). Interactive effects of both factors were detected on plant height and shoot dry weight but not on root collar diameter and root ectomycorrhizal colonization.

Values of all variables showed a tendency to decrease as the dilution increased. For instance, in the case of plant height, values ranged between 3.7 cm (ES inoculum) and 3.9 cm (EM inoculum) with the most diluted treatment (10^{-6} dilution), while those with the least diluted treatment (10^{-1} dilution) had values of 4.3 cm and 5.6 cm, respectively. This represented a relative increase of 16 and 44%, respectively (Table 2, Figure 2). The difference associated with inoculum type was evident in the 10^{-1} dilution and in the undiluted treatment. Thus, at the 10^{-1} dilution seedlings inoculated with EM and ES reached a plant height of 5.6 cm and 4.3 cm, respectively, which means 30% of relative difference. When *P. patula* seedlings grew in the EM and ES undiluted inocula, the plant heights were 5.6 cm and 4.4 cm (27% difference), respectively.

A similar pattern was detected with the shoot dry weights; with the most diluted ES treatment (10^{-6}) the shoot dry weight was 0.6 g per plant, while with the least diluted (10^{-1}) it was 0.9 g per plant (50% of relative increase). With the EM inocula the respective shoot dry weights were 0.7 (10^{-6} dilution) and 1.1 (10^{-1} dilution) g per plant which represent 64% of relative increase (Table 2, Figure 3). In general, the shoot dry weights of seedling inoculated with EM inoculum were higher than with ES inoculum, which was more evident in the 10^{-1} dilution and in the undiluted treatment. Thus, at the 10^{-1} dilution seedlings inoculated with EM and ES reached a shoot dry weight of 0.9 cm and 0.8 g per plant, respectively, which means 13% of relative difference. When *P. patula* seedlings grew in the EM and ES undiluted inocula, the shoot dry weights were 1.1 and 4.4 g per plant (22% difference), respectively. On the other hand, the root collar diameter values were higher for seedlings grown in the most concentrated inocula (10^{-1}) than in the most diluted (10^{-6}) (Figure 4); however, this variable seemed to be less sensitive to treatments than the other variables.

Root ectomycorrhizal colonization presented the highest values with the undiluted inocula of 31.6% with EM inoculum and 23.6% with ES inoculum (Figure 5). All seedlings assessed developed at least an incipient degree of ectomycorrhizal colonization. For both inocula, seedlings grown in the highest dilution (10^{-6}) presented the lowest root ectomycorrhizal colonization, 10.4% with EM inoculum and 15.8% with ES inoculum.

4. Discussion

The results clearly demonstrated that both factors inoculum type and dilution level had significant effects on *P. patula* seedling growth. The treatments with undiluted inocula were most effective for promoting seedling growth; nevertheless, the most significant growth was still lower than that reported for other species (*P. maximinoii*, *P. oocarpa*, and *P. tecunumanii*) [19, 20].

Root ectomycorrhizal colonization was measurable for all treatments despite the dilution treatment; however, it was incipiently developed with the most diluted treatments. The results of this study support the idea that plant growth is

Table 2: Mean values for plant height, root ectomycorrhizal colonization, root collar diameter, and shoot dry weight in response to interaction between inocula (I) and dilution factors (D). Standard deviations appear between parentheses.

Inoculum	Dilution	Plant height (cm)	Shoot dry weight (g)	Root collar diameter (mm)	Ectomycorrhizal colonization (%)
ES	10^0	4.4 (0.2)	0.9 (0.1)	1.5 (0.0)	23.6 (2.3)
	10^{-1}	4.3 (0.3)	0.8 (0.1)	1.4 (0.0)	22.4 (3.0)
	10^{-2}	4.1 (0.3)	0.8 (0.1)	1.4 (0.0)	21.2 (3.1)
	10^{-3}	4.0 (0.1)	0.8 (0.1)	1.4 (0.0)	19.4 (2.6)
	10^{-4}	4.0 (0.2)	0.7 (0.1)	1.4 (0.0)	17.8 (2.5)
	10^{-5}	3.8 (0.3)	0.7 (0.1)	1.4 (0.0)	16.6 (3.4)
	10^{-6}	3.7 (0.2)	0.6 (0.1)	1.3 (0.0)	15.8 (2.6)
EM	10^0	5.6 (0.9)	1.1 (0.3)	1.5 (0.0)	31.6 (5.1)
	10^{-1}	5.6 (1.0)	0.9 (0.1)	1.5 (0.0)	26.6 (3.5)
	10^{-2}	4.5 (0.5)	0.8 (0.1)	1.5 (0.0)	22.8 (3.7)
	10^{-3}	4.3 (0.3)	0.8 (0.1)	1.4 (0.0)	21.6 (4.4)
	10^{-4}	4.1 (0.5)	0.8 (0.1)	1.4 (0.1)	17.8 (3.4)
	10^{-5}	4.1 (0.6)	0.8 (0.1)	1.4 (0.1)	15.8 (2.7)
	10^{-6}	3.9 (0.7)	0.7 (0.1)	1.4 (0.1)	10.4 (2.9)
ANOVA summary					
Inocula (I)		<0.001	<0.001	<0.001	<0.001
Dilution (D)		<0.001	<0.001	<0.001	<0.001
$I \times D$		<0.0001	<0.01	0.952	0.952
LSD		0.25	0.07	0.03	3.28

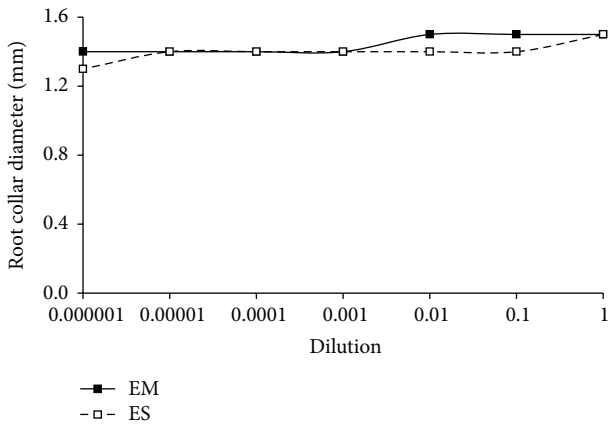

Figure 4: Root collar diameter of *P. patula* seedlings as a function of EM and ES inoculation at each level of serial dilution.

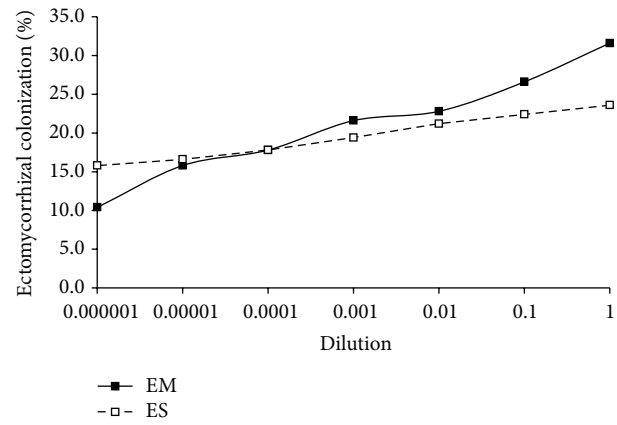

Figure 5: Root ectomycorrhizal colonization of *P. patula* seedlings for EM and ES inocula at each level of serial dilution.

dependent on root ectomycorrhizal colonization, as reported for species such as *P. pinaster* [8], *P. pinea* [21], and *P. thunbergii* [22], and the values for ectomycorrhizal colonization are comparable with those reported [19, 20]. The differences in root ectomycorrhizal colonization levels seem to be in turn determined by the EPD level of the inocula tested (ES inoculum = 34 per g and EM inoculum = 156 per g). However, it is worth mentioning that the ectomycorrhizal colonization may also vary with soil nutrient availability (particularly P), fertilization practices in nurseries, and fungicide application, among other conditions [23–25].

Our findings indicate that the MPN technique was useful for determining the amount of ectomycorrhizal propagules in each inoculum and plant growth responses. The results of this method indicated that the best inoculum was EM, which was produced under *in vitro* conditions [14]. In contrast, the ES inoculum had a low EPD value and was a lower quality inoculum source. This is significant because inoculation with a high EPD (156) inoculum during the nursery stage can yield a satisfactory *P. patula* seedling quality, but with a low EPD (34) inoculum plant performance may be constrained; in fact, the results of plant growth with ES inoculum were even lower

than those of EM-inoculated seedlings. In the case of ES inoculum, it appears that soil substrates extracted from forest plantations are usually low-density inocula. Thus, the EPD values of inocula dictated root ectomycorrhizal colonization trends; in fact, the values with undiluted inocula (10^0) were of 31.6% (EM) and 23.6% (ES).

Independently of several factors affecting ectomycorrhizal establishment [27] as well as colonization by opportunistic ectomycorrhizal fungi commonly present in forestry nurseries [21, 28, 29], our results reinforce the idea that the achievement of high values for root ectomycorrhizal colonization strongly depends on the inoculum EPD [21]. Despite the fact that all seedlings exhibited root ectomycorrhizal colonization, the values observed were lower than those reported in other studies with *Pinus* species. For instance, Sousa et al. [8] reported colonization values ranging from 40 to 70% in the roots of *P. pinaster* seedlings inoculated with mixed and individual fungi and Rincón et al. [21] reported that *P. pinea* seedlings growing in peat/vermiculite substrates reached higher values for root ectomycorrhizal colonization when inoculated with *Rhizopogon luteolus* (>80%; 2×10^7 spores mL^{-1}) and *Scleroderma verrucosum* (~57%; 16×10^8 spores g^{-1}). Castrillón et al. [20] reported with mixed inocula values of root ectomycorrhizal colonization in seedlings of *P. oocarpa* of 43.8% with *Suillus luteus* + *Amanita* sp., 39.9% for *P. tecunumanii* with *S. luteus* + *A. muscaria,* and 38.1% for *P. patula* with *S. luteus* + *Amanita* sp. Reports suggest that EPD-rich inocula are most efficient at promoting colonization in *Pinus* seedling roots and may achieve values for root ectomycorrhizal colonization reaching 50% or higher values [21, 30, 31].

Meanwhile, another key factor that may affect root colonization is a low compatibility between plant and fungus species [5, 24, 26, 32–35]. The effectiveness and specificity of *P. patula*-fungus relationships have been little studied and their role in plant growth remains unclear. Future research must be conducted to test this as well as to determine the effectiveness of the combined use of ectomycorrhizal fungi and other plant promoting-growth microorganisms.

In summary, the results suggest that the MPN method is a valuable tool for assessing the effectiveness of inocula at promoting root colonization in *P. patula* seedlings and promoting plant growth during the nursery stage. This method allowed us to identify inocula into different quality categories, taking into account the EPD value. Our results suggest that the EPD value of an inoculum is a key factor in root colonization. The low EPD value present in the ES inoculum indicates that the extraction of soil from forest plantations may be considered as an ineffective practice for promoting both root colonization and plant growth. On the other hand, the high EPD obtained from the EM inoculum reinforced the thesis of using a high quality inoculum for forest nurseries. From the practical point of view, the MPN technique provides a simple tool to identify the effectiveness of a potential source of ectomycorrhizal inoculum. This may be used by ectomycorrhizal inoculum producers, nursery seedling producers, and governmental agencies that regulate the quality of inocula.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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