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Resource use and management

Sympatric species develop more efficient ectomycorrhizae in the *Pinus-Laccaria* symbiosis

Las especies simpátricas desarrollan ectomicorrizas más eficientes en la simbiosis Pinus-Laccaria

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Abstract

The mycorrhizal symbiosis is optimal when the plant and the fungi are sympatric. However, in forest plantations the inoculum typically belongs to exotic or allopatric fungi. In this paper, the efficiency of mycorrhization was determined by evaluating the effect of 2 sympatric fungi species (*Laccaria trichodermophora* and *L. bicolor s.l.*) and 2 allopatric (*L. laccata* var. *laccata* and *L. vinaceobrunnea*) on the growth and nutrient contents of *Pinus montezumae*. We also tested the effect of the mycorrhizal helper bacteria *Pseudomonas fluorescens* (Pf_Ag001). After 1 year of growth, we evaluated the mycorrhization percentage, plant height, diameter at root collar, dry weight and nutrient contents (N, P, K) of aerial part and roots. The mycorrhization percentage varied from 93.5% to 98.5%. The treatments that showed higher efficiency (biomass accumulation and K contents) were those inoculated with sympatric species. All *Laccaria* treatments, either in the presence or absence of the bacteria, showed a better response compared to not inoculated controls. This work demonstrates the significance of using inocula of sympatric species as these are genetically predisposed to associate with their hosts, naturally adapted to the local environmental and edaphic conditions compared with those of allopatric origin.

Keywords: Ectomycorrhizal inoculum; Exotic; *Laccaria laccata*; *Laccaria trichodermophora*; *Laccaria vinaceobrunnea*; *Laccaria bicolor*; *Pseudomonas fluorescens*; *Pinus montezumae*

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Resumen

La simbiosis micorrízica es óptima cuando la planta y los hongos son simpátricos. Sin embargo, en las plantaciones forestales típicamente se usa inóculo de hongos exóticos. En este trabajo, la eficacia de la micorrización se determinó mediante la evaluación del efecto de 2 especies de hongos simpátricas (*Laccaria trichodermophora* y *L. bicolor s.l.*) y 2 alopátricas (*L. laccata* var. *laccata* y *L. vinaceobrunnea*) en el crecimiento y contenido de nutrientes de *Pinus montezumae*. También evaluamos el efecto de la bacteria ayudadora de micorrizas *Pseudomonas fluorescens* (Pf_Ag001). Después de 1 año de crecimiento, evaluamos el porcentaje de micorrización, la altura de la planta, el diámetro en el collar de la raíz, el peso seco y el contenido de nutrientes (N, P, K). El porcentaje de micorrización varió de 93.5% a 98.5%. Los tratamientos que mostraron una mayor eficiencia fueron los inoculados con especies simpátricas. Todos los tratamientos con *Laccaria*, en presencia o ausencia de bacterias, mostraron una mejor respuesta en comparación con los controles no inoculados. Este trabajo demuestra la importancia de usar inóculos de especies simpátricas ya que están genéticamente predispuestas a asociarse con sus hospedadores y están naturalmente adaptadas a las condiciones ambientales y edáficas locales.

Palabras clave: Inóculo ectomicorrízico; Exótico; *Laccaria laccata*; *Laccaria trichodermophora*; *Laccaria vinaceobrunnea*; *Laccaria bicolor*; *Pseudomonas fluorescens*; *Pinus montezumae*

Introduction

The ectomycorrhizal symbiosis between fungi and trees or shrubs, both Gymnosperms and Angiosperms, mainly occurs in temperate and boreal zones. This symbiosis is ecologically relevant due to the impact on the structure, composition, and functioning of plant communities (Pérez-Moreno & Read, 2004; Smith & Read, 2008; Umbanhowar & McCann, 2005). Mycorrhized seedlings have advantages over non mycorrhized ones since fungi improve their nutritional status, water absorption, drought and disease resistance enhancing plant growth and fitness (Barroetaveña & Rajchenberg, 2003; Bonfante & Genre, 2010; Pérez-Moreno & Read, 2000).

However, the outcome of the mycorrhizal symbiosis varies from positive to neutral (even negative) depending on the plant species, the species of fungus and their origin, as well as the soil fertility (Barroetaveña et al., 2016; Umbanhowar & McCann, 2005). The origin of the participants, i.e., whether they are sympatric or allopatric, is particularly important because it determines its natural predisposition to establish the symbiosis. In previous studies with arbuscular mycorrhizal fungi, the results indicate significant effects in the local adaptation when the tests have included sympatric plants and fungi, instead of allopatric combinations (Hoeksema et al., 2010; Klironomos, 2003; Rúa et al., 2016). The ectomycorrhizal symbiosis with native species has also shown better local adaptations, mostly reflected in terms of plant growth and colonization (Carrasco-Hernández et al., 2010, 2011; Carrera-Nieve & López-Ríos, 2004; Cuevas-Rangel, 1979; Martínez-Reyes et al., 2012; Méndez-Neri et al., 2011; Perea-Estrada, 2009; Quoreshi et al., 2009; Valdés et al., 1983, 2009).

Species of the genus *Laccaria* (Berk & Bromme) are among the main ectomycorrhizal fungi used around the world (Kropp & Mueller, 1999). *Laccaria* species are habitat pioneers and they have been used as model species in the study of ectomycorrhizal symbiosis (Khasa et al., 2009; Pera & Parladé, 2005; Quoreshi et al., 2008; Trappe, 1977; Wadud et al., 2008, 2014). Additionally, the publication of the genome sequence of the ectomycorrhizal fungus, *L. bicolor* (Martin & Selosse, 2008), was the foundation of subsequent studies of the ectomycorrhizal interaction at genomic level (Larsen et al., 2011). Species of this genus have been used to perform mycorrhization on different tree genera such as *Pinus*, *Pseudotsuga*, *Betula*, *Quercus*, among others (Dixon & Johnson, 1992; Gibson & Deacon, 1988; Mortier et al., 1988; Onwuchekwa et al., 2014; Parladé & Álvarez, 1993; Sudhakara & Natarajan, 1997; Zadworny et al., 2004). Even though all species of the genus are considered good mycorrhizal candidates, the former statement is not always accurate at the species level because the symbiosis is highly specific (Kropp & Mueller, 1999; Molina et al., 1992; Perea-Estrada et al., 2009; Wilson et al., 2017). Therefore, the adaptation to local conditions reflects evolutionary processes in the plant-fungal symbiosis process under specific environmental conditions within the geographic distribution of both symbionts (Hoeksema et al., 2010).

Species of *Laccaria* that have been used in mycorrhization processes are *L. laccata s.l.*, *L. bicolor*, *L. amethystina*, *L. proxima*, and *L. trichodermophora* in association with *Pinus* as *P. ayacahuite*, *P. banksiana*, *P. contorta*, *P. densiflora*, *P. douglasiana*, *P. greggii*, *P. michoacana*, *P. montezumae*, *P. oaxacana*, *P. patula*, *P. pinaster*, *P. pinea*, *P. pseudostrobus*, *P. radiata*, *P. rudis*, and *P. sylvestris* (Carrasco-Hernández et al., 2010, 2011; Carrera-Nieve & López-Ríos, 2004; Chapela et al., 2001; Galindo-Flores et al., 2015; Hynson et al., 2013; Martínez-Reyes et al., 2012; Méndez-Neri et al., 2011; Onwuchekwa et al., 2014; Parladé & Álvarez, 1993; Pera

& Parladé, 2005; Perea-Estrada et al., 2009; Perrin et al., 1997; Quoreshi et al., 2009; Sudhakara & Nataranja, 1997; Teramoto et al., 2012; Valdés et al., 2006; Zadworny et al., 2004). However, in previous works, the efficiency of sympatric versus allopatric species with *Pinus* hosts has not been experimentally tested.

The ectomycorrhizal symbiosis is a tripartite partnership, where mycorrhizal helper bacteria (MHB) promote host colonization and enhance the symbiosis function (Aspray et al., 2013; Garbaye, 1994; Frey-Klett et al., 1999; Vik et al., 2013). There are MHB included in the genera *Enterobacter, Paenibacillus, Pseudomonas, Burkholderia, Rhodococcus* and *Streptomyces* (Kumari et al., 2013). The MHB can promote mycorrhization in the bacteria-fungus-plant interaction. However, in general the MHB favor the phase of pre-infection as they favour spore germination, mycelia growth through the soil, as well as an increase in the root susceptibility to mycorrhizal colonization. These effects have been demonstrated for *Pseudomonas fluorescens* in *L. laccata* and *L. bicolor* (Deveau et al., 2007, 2010; Duponnois & Garbaye, 1991; Frey-Klett et al., 1999).

With the aim of producing native ectomycorrhizal inocula suitable for forest plants adapted to local conditions we selected the Trans-Mexican Volcanic Belt (TMVB) as a study model. The TMVB is around 1,000 km length with irregular amplitudes ranging between 80 and 230 km. This mountain range is recognized as a center of diversification, endemism and biogeographic transition for a variety of taxa, making it one of the most heterogeneous and complex biogeographic provinces (Flores-Villela & Canseco-Márquez, 2007; Morrone, 2010; Morrone & Escalante, 2002). *Pinus* is the most diverse ectomycorrhizal host worldwide. Mexico is an important diversification center for *Pinus* Lamb. with 47 species, from which 50% are distributed in the TMVB (Farjon, 1996; Farjon & Styles, 1997).

To test the hypothesis that the ectomycorrhiza becomes more efficient (in terms of plant growth and nutrient content) when a sympatric relationship between fungi and plants exists, we evaluated the mycorrhization effect on *P. montezumae* with 2 sympatric fungi (*L. trichodermophora* and *L. bicolor s.l.*) and 2 allopatric ones (*L. laccata* var. *laccata* and *L. vinaceobrunnea*). We also tested the effect of *P. fluorescens* on the mycorrhization by the 4 fungal species.

Materials and methods

One of the most important forest trees in the TMVB is *Pinus montezumae* Lamb. This species is naturally distributed between 2,000 and 3,200 m asl forming large woodland areas in the National Parks. Four *Laccaria* species that produce sporomes in great abundance were selected (Garibay-Orijel et al., 2009; Montoya et al., 2005): *Laccaria trichodermophora* and *L. bicolor s.l.* that are sympatric with *P. montezumae*, and *L. laccata* var. *laccata* and *L. vinaceobrunnea* that do not share the same habitat with this host.

Fruitbodies of *L. trichodermophora* and *L. bicolor s.l.* were collected from the Malinche National Park in the State of Tlaxcala. There, the average altitude is 3,200 m, climate is temperate sub humid with annual average temperature of 15.3 °C and an average rainfall range between 600 to 800 mm. The main vegetation are conifer forests dominated by *P. montezumae*, *P. teocote*, *P. hartwegii*, and *Abies religiosa* (Castillo-Guevara et al., 2012; Montoya et al., 2012). Fruitbodies of *L. laccata* var. *laccata* and *L. vinaceobrunnea,* were obtained from Ixtlán de Juárez, at the Sierra Norte in the State of Oaxaca. In this area, the average elevation is 2,470 m, the predominant climate is temperate humid with annual average temperature of 15 °C and average rainfall ranging between 1,000 and 1,300 mm. The main vegetation is constituted by mixed temperate *Pinus-Quercus* forests dominated by *P. patula*, *P. oaxacana*, and *P. douglasiana* (UNFOSTI, 2012, Valdés et al., 2006).

Pileus from fruitbodies were dried at 35 °C and manually grinded to obtain the inoculum. To know the concentration of spores in the inoculum of each species, we performed triplicate counts in a Neubauer chamber. We also conducted spore viability tests following the protocol of Moreno-Martínez (1984) and Santiago-Martínez et al. (2003) using 1.0% of tetrazolium buffer. We prepared 1 L of 1.0% 2, 3, 5 trifeniltetrazolium chloride in a buffer solution. We mixed 400 mL of KH_2PO_4 and 600 mL of NaH_2PO_4-H_2O solution; we added 10 g of tetrazolium salt, adjusted to pH 6 with KOH. In 1.5 mL Eppendorf tubes we placed a sample of each inoculum, we re-suspended them for 1 min with a vortex and incubated them for 30 min at room temperature. We then counted the total number of metabolically active spores in a Neubauer Chamber.

We acquired the MHB (strain *P. fluorescens* Pf_Ag001) from BIOqualitum that sells it under the tradename BactoCROP guaranteeing minimum concentration as 100 millions of bacteria per gram.

Seeds of *P. montezumae* were collected from the surroundings of the Iztaccíhuatl Volcano in the State of Mexico located in the TMVB. They were surface-sterilized with hydrogen peroxide (H_2O_2) 30% and 20 mL of Tween-20 in 500 mL distiled water, and subsequently washed with running water and placed 24 h in water for pre-germination. We used a substrate composed of a 1:1 mixture of peat and agrolite and 134 mL containers. Peat was sterilized with 50 kiloGrays of Gamma radiation at the Institute of Nuclear Sciences, UNAM,

as it has been shown it contains ectomycorrhizal fungi spores resistant to pasteurization (Ángeles-Argáiz et al., 2016). At the beginning of the experiment, each plant was inoculated with 10^7 spores placed in water solution added to the substrate. Bacteria treatments included 0.1 g of BactoCROP per container, diluted with the fungal inoculum. All plants remained 365 days in the greenhouse without any fertilization and watered with tap water every third day up to the saturation point; all treatments were randomly rotated every week.

The experimental design included 2 factors: *(1)* the ectomycorrhizal fungal species, including 4 levels (*L. trichodermophora, L. bicolor s.l., L. laccata* var. *laccata* and *L. vinaceobrunnea*); *(2)* the bacterial inoculum, including 2 levels (presence or absence of *P. fluorescens*). We also included a treatment only with bacterial inoculum and a negative control without inoculum. In total, the experiment had 10 treatments, with 13 seedlings each, comprising a total of 130 experimental units, each one constituted by one plant.

After a year of growth, we measured each plant height from the root collar and the root collar diameter (RCD); subsequently plants were dehydrated at 80 °C in an oven during 48 h to evaluate the dried shoots and roots weight. We determined total P, N and K content of the aerial and root parts for 5 randomly selected pines by treatment. Phosphorus was determined by colorimetry, N by wet digestion (Bremner, 1975), and K by flame photometry ammonium acetate extraction (Chapman & Parker, 1986).

The mycorrhization percentage in the root system for each seedling was randomly calculated. We divided the root system in 3 equal fractions (upper, middle and lower) and randomly selected 4 secondary roots per fraction, thus twelve secondary roots per plant were analyzed (Carrasco-Hernández et al., 2011), counting the number mycorrhizal and non-mycorrhizal root tips in each under a stereoscopic microscope.

Evaluation of the mycorrhization effect and promotion by MHB on each response variable was conducted using two-factor variance analysis. When significant differences were obtained, we looked for the homogeneous groups by Tukey tests with the software Statistica 8 (StatSoft ver.2008).

To describe the ectomycorrhizae morphology, we conducted the characterization of structures as proposed by Agerer (1987-2002). Colors were recorded according to Kornerup and Wanscher (1978). Photographs were taken with the aid of a multifocal automatic microscope (Leica Z16 APOA) with an 8 mega-pixel camera (Leica DFC490); 3D images were assembled in Leica application systems V4.3.0. Scanning electron photographs were taken with a scanning electron microscope (JEOL JSM-5310LV); anatomic characteristics were photographed with an Olympus BX51 microscope.

Results

The percentage of mycorrhization varied from 93.5% to 98.5%, we did not find significant differences between sympatric and allopatric species. However, we found significant differences ($F = 55.95$, $p < 0.0001$) between all

Table 1

Growth, biomass accumulation and mycorrhization of *Pinus montezumae* inoculated with different *Laccaria* species and *P. fluorescens*.

Origin	Treatment	Height (cm)	Root collar diam (cm)	Root dry weight (mg)	Shoot dry weight (mg)	Total dry weight (mg)	M%
Sympatric	L.b	16.5abc	2.9a	8.7a	20.0a	28.7a	93.5a
	L.b/P.f	16.3c	2.8a	8.0a	20.0a	28.0a	98.4a
	L.t	15.5c	2.5ab	5.8bc	13.5b	19.3b	98.5a
	L.t/P.f	16.7abc	2.7ab	6.2b	12.0b	18.2b	98.5a
Allopatric	L.v/P.f	16.8abc	2.7ab	4.0de	8.0c	12.0c	94.5a
	L.l	16.7abc	2.5ab	4.5cd	7.3c	11.8c	97.6a
	L.v	17.1ab	2.4ab	3.7de	7.0c	10.7c	95.7a
	L.l/P.f	17.9a	2.2ab	4.1de	6.5c	10.6c	97.1a
Controls	C-	16.4abc	2.0ab	4.2de	7.3c	11.5c	0b
	P.f	16.6abc	1.7b	2.6e	3.9d	6.5d	0b

L.t/P.f: *Laccaria trichodermophora* + *Pseudomonas fluorescens*, L.t: *L. trichodermophora*, L.v/P.f: *L. vinaceobrunnea* + *P. fluorescens*, L.v: *L. vinaceobrunnea*, L.l./P.f: *L. laccata* var. *laccata* + *P. fluorescens*, L.l: *L. laccata* var. *laccata*, L.b/P.f: *L. bicolor* + *P. fluorescens*, L.b: *L. bicolor*, C-: no-inoculated negative control, P.f: *P. fluorescens*, root collar diam: root collar diameter, M%: mycorrhization percentage. Different letters show statistically significant differences on post hoc Tukey test ($p < 0.05$). n = 13.

the mycorrhizal treatments and the 2 control treatments (C and C/PF), which did not develop any mycorrhizae (Table 1).

The height of plants was similar among treatments. There were significant differences ($F = 3.17$, $p < 0.002$) only between the treatments of *Laccaria laccata* var. *laccata* with *P. fluorescens* ($\bar{x}$ = 17.9 cm) compared to those of *L. trichodermophora* and *L. bicolor s.l.* with *P. fluorescens* ($\bar{x}$ = 15.5 and 16.3, respectively). The remaining treatments produced heights ranging from 16.3 to 16.8 cm. The root collar diameter (RCD) showed significant differences ($F = 2.49$, $p < 0.012$) between *L. bicolor s.l.* ($\bar{x}$ = 2.9 cm), and *L. bicolor s.l.* with *P. fluorescens* ($\bar{x}$ = 2.8 cm) treatments compared to the control with only *P. fluorescens* ($\bar{x}$ = 1.7 cm). Although the negative control plants were thinner ($\bar{x}$ = 2.0 cm) than treatments inoculated with fungi, differences were not significant (Table 1).

The 3 variables used to evaluate biomass (i.e., root dry weight, shoot dry weight and total dry weight) showed the same trend (Table 1). The best treatments were those inoculated with *L. bicolor* with or without *P. fluorescens* (28.7 and 28.0 mg, respectively), having a significantly greater biomass ($F = 127.33$, $p = 0.0001$) than the rest of the treatments. *Laccaria trichodermophora* treatments (with or without bacteria) had the second best total dry weight (19.3, 18.2 mg respectively) being significantly higher than the allopatric species that did not presented significant differences than the no-inoculated control. The control inoculated only with *P. fluorescens* showed the lowest total dry biomass ($\bar{x}$ = 6.5 mg) (Table 1).

The P roots contents did not show significant differences among the treatments ($F = 1.27$, $p > 0.284$). However, both the P content in the shoots ($F = 1.99$, $p < 0.067$) and in the whole plant ($F = 2.18$, $p < 0.044$) showed significant differences and followed a similar trend, with *L. trichodermophora* with *P. fluorescens* always with higher values. Significant differences were observed in the total P content between plants mycorrhized with *L. trichodermophora* with *P. fluorescens* ($\bar{x}$ = 180.3 mg) compared to both *P. fluorescens* control ($\bar{x}$ = 108.4 mg), and the no-inoculated control ($\bar{x}$ = 106.5 mg) treatments. The N content in shoots ($F = 2.48$, $p > 0.024$), roots ($F = 0.67$, $p > 0.728$) and total ($F = 0.93$, $p > 0.513$) parts of the plant did not show any significant differences between treatments and compared to the negative control. This was also true for the K content in the roots ($F = 0.93$, $p > 0.509$). Mycorrhizal plants inoculated with *L. bicolor* showed the highest concentration of K in the shoots ($\bar{x}$ = 34.0 mg), followed by *L. bicolor* with *P. fluorescens* ($\bar{x}$ = 26.6 mg) and *L. trichodermophora* ($\bar{x}$ = 26.1 mg) treatments, showing significant differences ($F = 7.00$, $p = 0.0001$) relative to no-inoculated control ($\bar{x}$ = 14.4 mg). Total K of mycorrhizal plants with *L. bicolor* ($\bar{x}$ = 60.7 mg), *L. bicolor* and *P. fluorescens* ($\bar{x}$ = 51.4 mg), *L. trichodermophora* ($\bar{x}$ = 50.4 mg), *L. trichodermophora* with *P. fluorescens* ($\bar{x}$ = 44.7 mg), and *L. laccata* with *P. fluorescens* ($\bar{x}$ = 43.0 mg) showed higher significant concentrations ($F = 4.76$, $p = 0.0001$) than the negative control ($\bar{x}$ = 32.1 mg) (Table 2).

Table 2

Nutrient contents (mg) in *Pinus montezumae* inoculated with different *Laccaria* species and *P. fluorescens*.

Treatment	Ps	Pr	Pt	Ns	Nr	Nt	Ks	Kr	Kt
L.t/P.f	132.9a	47.4a	180.3a	0.9a	1.0a	1.9a	21.4bc	23.3a	44.7ab
L.t	80.7ab	39.1a	119.8ab	1.2a	0.8a	2.0a	26.1ab	24.3a	50.4ab
L.v/P.f	92.9ab	49.8a	142.7ab	1.1a	1.0a	2.1a	16.6bc	22.0a	38.6bc
L.v	95.5ab	40.0a	135.5ab	1.2a	0.9a	2.1a	18.0bc	22.6a	40.6bc
L.b/P.f	96.3ab	35.4a	131.7ab	1.1a	1.0a	2.1a	26.6ab	24.8a	51.4ab
L.b	95.4ab	44.6a	140.0ab	1.1a	0.8a	1.9a	34.0a	26.7a	60.7a
L.l/P.f	81.3ab	37.3a	118.6ab	0.8a	0.8a	1.6a	17.3bc	25.7a	43.0ab
L.l	106.7ab	33.4a	140.1ab	1.1a	0.8a	1.9a	19.2bc	20.5a	39.7bc
P.f	62.4b	46.0a	108.4b	1.1a	0.9a	2.0a	19.5bc	24.3a	43.8ab
C-	70.0ab	36.5a	106.5b	0.9a	0.7a	1.6a	14.4c	17.7a	32.1c

L.t/P.f: *Laccaria trichodermophora* + *Pseudomonas fluorescens*, L.t: *L. trichodermophora*, L.v/P.f: *L. vinaceobrunnea* + *P. fluorescens*, L.v: *L. vinaceobrunnea*, L.l./P.f: *L. laccata* var. *laccata* + *P. fluorescens*, L.l: *L. laccata* var. *laccata*, L.b/P.f: *L. bicolor* + *P. fluorescens*, L.b: *L. bicolor*, C-: negative control, P.f: *P. fluorescens*, Ps: phosphorous in shoot, Pr: phosphorous in roots, Pt: total phosphorous, Ns: nitrogen in shoot, Nr: nitrogen in roots, Nt: total nitrogen, Ks: potassium in shoot, Kr: potassium in roots, Kt: total potassium. Different letters show statistically significant differences on post hoc Tukey test ($p < 0.05$). n = 5.

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Mycorrhizae morphological description. L. trichodermophora + *P. fluorescens* + *P. montezumae* (Fig. 1A): dichotomous mycorrhizae with lateral branches of the same length, with straight edges and branches. The mantle presented reflective white patches over an orange base, it also showed emerging hyphae varying in quantity at the base and the apex. The base was yellowish brown (5D8 (Kornerup & Wanscher (1978)), the tips and apices were bright orange (5A6). Mantle plectenchymatous with palmate Hartig net widely distributed and with individual hyphae. *L. trichodermophora* + *P. montezumae* (Fig. 1B, C): same as before with 2 main differences: the superficial mantle showed a cottony texture and the mycorrhiza showed a strong orange color (5A8).

Figure 1. Morphology of mycorrhizae formed between: *Pinus montezumae*, *Laccaria* spp. and *Pseudomonas fluorescens*. A: *L. trichodermophora* + *P. fluorescens* + *P. montezumae*; B-C: *L. trichodermophora* + *P. montezumae*; D: *L. laccata* var. *laccata* + *P. fluorescens* + *P. montezumae*; E-F: *L. laccata* var. *laccata* + *P. montezumae*; G: *L. vinaceobrunnea* + *P. fluorescens* + *P. montezumae*; H-I: *L. vinaceobrunnea* + *P. montezumae*; J: *L. bicolor s.l.* + *P. fluorescens* + *P. montezumae*; K-L: *L. bicolor s.l* + *P. montezumae*; M: *P. montezumae* + *P. fluorescens*; N-O: *P. montezumae* non mycorrhizal roots.

L. laccata var. *laccata* + *P. fluorescens* + *P. montezumae* (Fig. 1D): dichotomous mycorrhizae with lateral branches of the same length golden yellow (5B7), with straight edges and branches. Cotton-like superficial mantle, with emerging hyphae in some parts, surrounding the apices. Mantle plectenchymatous with anastomosed hyphae in the middle parts. *L. laccata* var. *laccata* + *P. montezumae* (Fig. 1E, F): same as before with 2 main differences: it showed abundant emerging hyphae from all the mycorrhiza, with a fan-like shape and orange (5B8) mantle.

L. vinaceobrunnea + *P. fluorescens* + *P. montezumae* (Fig. 1G): dichotomous mycorrhizae with lateral branches of the same length, with straight edges and branches. Apex orange (5A6), the rest of the mycorrhiza was brownish yellow (5E8). Cottony superficial mantle with emerging hyphae in certain parts; apex is mantle-free. Mantle pseudoparenchymatous, with palmate Hartig net widely distributed and showing individual hyphae. *L. vinaceobrunnea* + *P. montezumae* (Fig. 1H, I): same as before with 2 main differences: it presented constrictions at the base of the branch, in the middle and before reaching the apices and the whole mycorrhiza was orange brown (6C8).

L. bicolor s.l. + *P. fluorescens* + *P. montezumae* (Fig. 1J): dichotomous mycorrhizae with side branches of the same length; straight edges and branches, reddish brown (7E8). Cotton-like superficial mantle with rarely emanating hyphae. Mantle pseudoparenchymatous with palmate Hartig net widely distributed. *L. bicolor s.l* + *P. montezumae* (Fig. 1K-L): same as before with one main difference: the mycorrhiza was orange (5B8).

Roots of *P. montezumae* + *P. fluorescens* (Fig. 1M) and roots of *P. montezumae* (Fig. 1N-O): roots lack mantle and showed root hairs without superficial or intraradical hyphae.

Discussion

All the inoculated plants developed mycorrhizas and mycorrhization percentages in all treatments were high, greater than 93.5%. This is explained by the fact that the 4 *Laccaria* species used are ectomycorrhizal pine symbionts and all are native from Mexican forests as also is *P. montezumae*. As we will discuss later, the main differences found between sympatric and allopatric species are not evident in their ability to colonize the roots, but in their effect to improve the symbiosis efficiency.

The mycorrhiza helper bacteria *P. fluorescens* did not improve the mycorrhization percentage of any of the *Laccaria* species. This contrasts with previous reported positive effects of *P. fluorescens* in mycorrhization percentage (Frey-Klett et al., 1999) and increase in root biomass of *L. laccata* (Duponnois & Garbaye, 1991). *Pseudomonas fluorescens* comprises a complex of genetic species with around 50% of genomic divergence between strains. In consequence, it might be expected that different strains exhibit a diverse spectrum of genetic traits involved in multi-trophic interactions with plants and other microbes (Loper et al., 2012). As is has been recently shown by Barragán-Soriano et al. (2018), MHB do increase the growth and physiological quality of *P. montezumae*, so further research is needed to find compatible strains of MHB-sympatric *Laccaria*- and *P. montezumae*.

On the other hand, we demonstrate the efficiency of peat sterilization with Gamma rays, since both the negative control and the treatment with only *P. fluorescens* showed no mycorrhizae. The former means that we managed to eliminate the viability of resistant ectomycorrhizal fungi spores present in the peat (Ángeles-Argáiz et al., 2016).

Overall, we did not find significant differences in growth parameters, although pines mycorrhized with allopatric species were little higher and smaller root collar diameter than those plants mycorrhized with sympatric species. The most important differences occurred in total biomass accumulation, as both sympatric species (*L. bicolor* followed by *L. trichodermophora*) promoted the enhancement of plant biomass (Table 1). Also, the increase in biomass accumulation in these treatments was independent of the presence of *P. fluorescens*. By comparison, allopatric species did not show any increase in biomass accumulation compared to the negative control.

Regarding nutrient contents, we did not find a significant relationship between the plants N content, in any part of the plant, and the mycorrhization treatments. We also did not find differences in P and K content in the roots between treatments. However, plants mycorrhized with the sympatric species *L. trichodermophora* with *P. fluorescens* were the unique treatment with significant higher P concentration in the total plant than the no-inoculated controls. Similarly, treatments with sympatric species, especially *L. bicolor*, showed a higher K concentration of shoot and total plant.

Our experimental data confirm the hypothesis that the sympatric mycorrhizal species are more efficient to accumulate biomass and nutrients (K) in the host plant. This was shown in an artificial substrate were nutrients came form organic matter (peat). However, an enhanced effect of the ability of sympatric fungi should be expected if this symbiosis was grown in the natural soils where it develops. The capacity of mycorrhizas to exploit and transfer soil nutrients to plants is related with ecological adaptations as particular soil ecotypes (Hoeksema et al., 2010; Klironomos, 2003; Rúa et al., 2016).

The mycorrhizae morphology in the presence of *P. fluorescens* showed differences, the mantle shape and general coloring, in contrast with the mycorrhizae synthetized without bacteria. In the case of *L. trichodermophora*, the mantle shape was cottony with strong orange color; whereas, in the presence of *P. fluorescens*, the mycorrhiza had few hyphae with pale orange color. *L. vinaceobrunnea* presented a reduced mantle of yellow-orange coloring; whereas in the presence of bacteria the mycorrhiza had a cottony mantle pale orange in color at the base. While branching type of this symbiotic partners is similar to previous descriptions of *L. bicolor s.l.* with *P. pseudostrobus* in the absence of *P. fluorescens*; they are differences in the color from the brown color previously reported (Carrasco-Hernández et al., 2010; Santiago-Martínez et al., 2003). The mycorrhiza of *L. trichodermophora* matches the type of branching but not in the coloring (strong orange), which is different from the pale yellow mycorrhizas reported previously (Galindo-Flores et al., 2015). Consistency in the branching pattern is a main feature that characterizes the *Pinus-Laccaria* association (Agerer, 1987, 2002), while differences in color may be due to particular metabolic pathways related with the specific species association and also the maturing of the symbiosis.

The widespread use of *Laccaria* species in mycorrhization is explained because they are pioneers with the ability to colonize a variety of important forest trees (Bois & Coughlan, 2009; Fortin & Lamhamedi, 2009; Parent & Moutoglis, 2009; Quoreshi et al., 2009; Sudhakara & Natarajan, 1997). However, in this study, we have shown that the plant compatibility with its ectomycorrhizal fungus is differential, even though with phylogenetically related (within the same genus) fungal species. The fungus ability to colonize and remain in the host roots is evidenced through physiological and morphological responses of the plant (Karst et al., 2014; Onwuchekwa et al., 2014; Perrin et al., 1997; Quoreshi et al., 2008). However, the ectomycorrhizal efficiency depends on the origin of the participants in the symbiosis. The local adaptation potential of sympatric species plays a fundamental role in the successful development in the field (Rúa et al., 2016). Therefore, when selecting ectomycorrhizal fungi inoculum to grow forest plants, we should prioritize sympatric species to increase survival and growth success of plants and to ensure minimal disruption and disturbance of the natural communities.

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