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ORIGINAL PAPER

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Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions

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Abstract Different methods to inoculate seedlings of *Pinus pinaster* and *P. sylvestris* with edible *Lactarius* species under standard greenhouse conditions were evaluated. Fungal inoculations were performed both under pure culture synthesis in vitro, followed by transplantation of acclimatized seedlings, and directly in the greenhouse using different techniques for inocula production (mycelial slurries, vegetative inoculum grown in peat-vermiculite and alginate-entrapped mycelium). In vitro inoculations with *L. deliciosus* produced thoroughly colonized seedlings. However, a sharp decrease in mycorrhizal colonization was detected on transplanted seedlings after 4 month's growth in the greenhouse. On the other hand, all the inocula applied directly in the greenhouse, except the alginate-entrapped mycelium, produced a variable number of mycorrhizal seedlings and colonization rates after the first growing season, depending on the plant-fungal combination and the inoculation method. Inoculations with vegetative inocula of the strain 178 of *L. deliciosus* were the most effective in producing mycorrhizal seedlings. All the seedlings inoculated with this strain were colonized although the colonization rates were relatively low. The commercial feasibility of the different inoculation methods for the production of seedlings colonized with edible *Lactarius* species is discussed.

Keywords Edible fungi · Inoculum production · *Lactarius* · Mycorrhizal colonization · *Pinus*

Introduction

The cosmopolitan, ectomycorrhizal fungal genus *Lactarius* has been intensively marketed in many countries of Europe, Asia and northern Africa, especially the choice edible species *Lactarius deliciosus* (L. ex Fr.) S. F. Gray and *Lactarius sanguifluus* (Paulet ex) Fr. (Singer 1986). *Lactarius* forms ectomycorrhizas with a variety of host plants (Trappe 1962; Hutchison 1999). However, the edible species of the section Dapetes form ectomycorrhizas mainly with members of Pinaceae. Host-plant associations of *L. deliciosus* (confirmed by in vitro synthesized mycorrhizas) have been reported for *Picea abies*, *Pinus echinata* , *P. mugo* , *P. nigra* , *P. strobus* , *P. sylvestris* (Trappe 1962), *P. contorta* (Parladé et al. 1996a), *P. halepensis* (Torres and Honrubia 1994), *P. pinaster* (Mousain et al. 1979; Pera and Álvarez 1995), *P. pinea* (Rincón et al. 1999), *P. ponderosa* (Riffle 1973), *P. radiata* (Malajczuk et al. 1982; Parladé et al. 1996a), and *Pseudotsuga menziesii* (Molina and Trappe 1982; Parladé et al. 1996a). Reported mycorrhizal associations with *L. sanguifluus* are restricted to *Pseudotsuga menziesii* (Zak 1969) and *Pinus sylvestris* (Guerin-Laguette et al. 2000).

Observations on fruiting behaviour suggest that most species of *Lactarius* are late-stage ectomycorrhizal colonizers as sporocarps are generally observed in older forest stands (Hutchison 1999). However, other studies report regular collections of *L. deliciosus* in young (0–5 years old) Scotch pine plantations (Bonet 2001). Also, pure culture inoculation studies demonstrate that this fungal species readily colonizes the root system of pines under aseptic conditions. Guerin-Laguette et al. (2000) obtained fruit body primordia of *L. deliciosus* 1 year after inoculation of *Pinus sylvestris* seedlings in growth pouches and then transferred to containers. These results suggest that *L. deliciosus* could be effectively used for controlled mycorrhizal plant production in nurseries as has been successfully done with other ectomycorrhizal fungi. However, data on mycorrhizal inoculation of pines with *L. deliciosus* under nursery conditions are reported only for *P. pinea* (Rincón et al. 1999).

The production of edible ectomycorrhizal fruit bodies from plants inoculated under controlled conditions has been especially developed for truffles (Chevalier and Frochot 1997) and, to a lesser extent, for chanterelles

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(Danell 1997) and *Boletus* (Olivier et al. 1997). Managing mycorrhizal inoculations with edible *Lactarius* to increase forest productivity can be a promising alternative for many Mediterranean forest areas with limited resources. The establishment of plantations with *Lactarius* -inoculated *P. pinaster* seedlings to obtain fruit bodies was first attempted by Poitou et al. (1984). The evolution of these plantations was investigated by Guinberteau et al. (1989) who reported the production of sporocarps in the field after 3 years. However, further attempts to produce quality plants mycorrhizal with *L. deliciosus* under nursery or greenhouse conditions and outplanting field studies are not reported. The objective of the present work is to evaluate and compare different inoculation techniques, applied both under in vitro and in standard greenhouse conditions, to produce pine seedlings colonized with edible *Lactarius* species.

Materials and methods

Seeds of *Pinus pinaster* and *P. sylvestris* used in all the experiments were collected in 2000 and 1999, respectively, from forests of the Spanish Central Range. Pre-germination treatment consisted of 24 h immersion in cold water. Four fungal strains belonging to the species *L. deliciosus* and *L. sanguifluus* isolated from sporocarps collected in different locations of Catalonia (north eastern Spain) were used for inocula production (Table 1). Fungal strains were stored at 4°C in agar slants and transferred every 3 months to fresh BAF media prepared as described by Oort (1981). A total of four parallel inoculation experiments were conducted throughout the same growing season. However, as will be noted below, the timing of inoculation varied according to the protocols defined for each inoculation method. Growth conditions in the greenhouse were the same for all the plants so the experiments could be compared.

In vitro inoculations and plant acclimatization

P. pinaster and *P. sylvestris* seeds were surface-sterilized with H_2O_2 for 30 min and aseptically germinated in glass vials containing 1% malt agar to remove contaminants. Non-contaminated, germinated seeds (2–3 cm radicle) were transferred to glass synthesis tubes filled with a mixture of peat, vermiculite and nutrient solution in the proportions described by Molina (1979). The nutrient solution consisted of BAF liquid medium modified with 20 g glucose l^{-1}. For inoculum preparation, *L. deliciosus* 217 was transferred to BAF agar plates and incubated at 25°C for 1 month. Three colonies of *L. deliciosus* of approximately 4 cm in diameter were homogenized in 70 ml sterile distilled water with a Waring blender to obtain 0.005 g mycelium (dry weight) ml^{-1} suspension. Fifty tubes of each plant species were inoculated with 5 ml mycelial suspension in April 2001. The tubes were randomly placed in a growth chamber and incubated at 25°C with a 16-h photoperiod under fluorescent light (135 μmol s^{-1} m^{-2}). After 12 weeks, all the seedlings were removed from the tubes, their roots cleaned under running water and assessed for ectomycorrhiza colonization as described in Parladé et al. (1996b). Seedlings were then transplanted to 150-cc Forest-Pot containers (Vivers La Fageda, Santa Pau, Girona) filled with a (1:1, v:v) sterilized mixture of Floratorf peat (Floragard, Oldenburg, Germany) and horticultural grade 2 vermiculite (Asfaltex, Barcelona). The transplanted seedlings were moved to the greenhouse in covered trays with regulated openings (mini-serre BHR; Puteaux, France) to keep moisture high for acclimatization. After 6 days, the containers were removed from the trays and maintained under regular greenhouse conditions (see below).

Greenhouse inoculations

Mycelial slurries

A total of 120 seedlings of both *P. pinaster* and *P. sylvestris* were grown in 150-cc Forest-Pot containers, filled with the plant-growth substrate [1:1 (v:v) mixture of peat and vermiculite]. Each seedling was inoculated in April 2001 at emergence (15 days after sowing) with 5 ml of a mycelial suspension of *L. deliciosus* 217 as described above (0.025 g dry weight mycelium $plant^{-1}$) injected in the rhizosphere zone. The same amount of control, non-inoculated, seedlings of each tree species were also produced.

Vegetative inoculum

Two-litre glass flasks were filled with a peat-vermiculite substrate moistened with a nutrient solution as described by Marx and Bryan (1975) but with a double amount of peat and a modified BAF liquid medium (20 g glucose l^{-1}). Starting cultures of the four fungal strains were prepared by blending 1-month-old colonies in sterile distilled water at two colonies per 100 ml water. An inoculum of each fungus was prepared by adding 10 ml of the blended mycelial suspension to each autoclaved flask. Inoculum flasks were incubated at 25°C in the dark for 12 weeks. In April 2001, the fungal inoculum was thoroughly mixed with the plant-growth substrate at a ratio of 1:20 (inoculum:substrate, v:v). Forest-Pot containers (150 cc) were filled with the inoculated substrate and seeded with *P. pinaster* and *P. sylvestris* seeds. A total of 120 plants were produced for each plant-fungal combination including control, non-inoculated, seedlings.

Alginate-entrapped inoculum

Liquid cultures of the four fungal strains were grown in modified BAF liquid medium and maintained for 3 weeks under static incubation at 25°C in the dark. The mycelium produced by each strain was weighed, gently blended in sterile water and added to 2-l flasks containing 1 l autoclaved 2% sodium alginate solution (Sigma, Steinheim, Germany; viscosity approximately 250 cps at 25°C) at 2.5 g fresh weight mycelium l^{-1} alginate solution (Mortier et al. 1988). Flasks were gently shaken and the mycelial suspension was dropped into a 0.3 M sterile $CaCl_2$ solution to entrap the mycelium particles into polymerized alginate beads. The beads were cured in the $CaCl_2$ solution for 1 h and then rinsed with abundant sterile distilled water. Mycelium viability was assessed by plating 50 beads for each fungal strain in BAF agar and checking for mycelial growth. In March 2001, the inoculum of each *Lactarius* strain was mixed with the plant-growth substrate at a

Table 1 Collection data of the fungal strains of *Lactarius* spp. used in the inoculation experiments

Fungal species	Strain no.	Collection date	Location (Spanish province)	Associated host plant
L. deliciosus	178	October 1994	Cabrils (Barcelona)	*Pinus pinea*
L. deliciosus	217	November 1994	Fitor (Girona)	*P. pinea*
L. sanguifluus	261	October 1995	St Feliu Buixalleu (Girona)	*Pinus radiata*
L. sanguifluus	263	October 1995	Fitor (Girona)	*Pinus pinaster*

Table 2 Mean growth parameters and mycorrhizal colonization of *P. pinaster* and *P. sylvestris* seedlings formerly inoculated in vitro with *L. deliciosus* 217 and transplanted into containers under greenhouse conditions

Plant species	Assessment time	Height (cm)	Diameter (mm)	Dry weight (g)		% Colonized plants	% Plant colonization
				Shoot	Root		
P. pinaster	Initial[a]	5.1	–	–	–	95	63
	Final[b]	13.1	2.9	1.40	0.83	49	13*
P. sylvestris	Initial	2.0	–	–	–	94	63
	Final	3.5	2.2	0.49	0.58	2	1*

*$P<0.05$ (for the same plant species, where *final* percentages are significantly different from *initial* ones)
[a] At transplantation (12 weeks after inoculation)
[b] At harvest (4 months after transplantation)

ratio of 1:20 (v:v, inoculum:substrate). Forest-Pot containers (150 cc) were filled with the inoculated substrates and seeded with *P. pinaster* and *P. sylvestris* seeds. Each plant received approximately 0.025 g (dry weight) of the corresponding fungal strain. A total of 120 plants were produced for each plant-fungal combination including control, non-inoculated, seedlings.

Experimental layout and mycorrhizal assessment

The four inoculation experiments were arranged in a completely randomized design on the greenhouse benches. The seedlings were irrigated daily and fertilized every 15 days with soluble NPK Peters professional conifer grower 20+7+19 (Scott, Tarragona, Spain) plus micronutrients (Fetrilon 13 and Hortrilon; BASF, Barcelona). Each plant received at each fertilization 3.6 mg N (2.1 mg NO_3^-, 1.26 mg NH_3, 0.24 mg urea), 1.26 mg P, 3.42 mg K, 0.35 mg Fe, 0.07 mg Mg, 0.06 mg Mn, 0.06 mg Cu, 0.01 mg Zn, 0.01 mg B, and 0.01 mg Mo. In November and December 2001, seedling growth (height, root-collar diameter and dry weight) and mycorrhizal colonization for each inoculation treatment were assessed either from all the seedlings (in vitro inoculation experiment) or from 25 seedlings taken randomly (greenhouse inoculation experiments). The relationship between the initial and final colonization level in the acclimatized seedlings inoculated in vitro was analysed by Pearson product moment correlation analysis ($P<0.05$) using the Statgraphics Plus 5.0 statistical package. Growth and colonization data at the end of the experiments were analysed by ANOVA and means compared by Tukey's test ($P<0.05$). Colonization percentages were arcsin transformed before performing the analyses.

Results

Seedlings inoculated under in vitro conditions showed abundant mycorrhizas of *L. deliciosus* 217 in almost all the inoculated tubes after 12 weeks (Table 2). *Lactarius* mycorrhizas could be clearly identified after visual examination of the root system, especially of the lower third. Colonized roots had a typical smooth surface with a dichotomous to profusely branched pattern and showed a typical bright orange colour at maturity, turning brown to dark violet when ageing, with abundant mycelial strands. All the plants were well acclimatized and reached a normal size for 1+0 nursery plants after 4 months in the greenhouse, according to local requirements (Peñuelas-Rubira 1993). At harvest, the percentage of colonized plants was reduced approximately to 50% in *P. pinaster* and to 2% in *P. sylvestris*. Also, the colonization level of mycorrhizal plants was significantly reduced (Table 2). The correlation between colonization percentages at transplantation and at harvest in all the *P. pinaster* plants

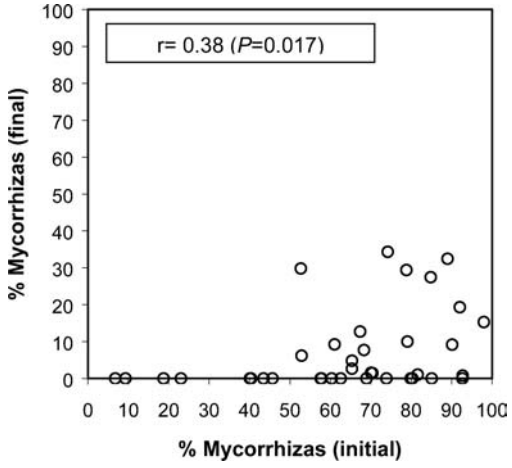

Fig. 1 Correlation between initial and final mycorrhizal colonization of *Pinus pinaster* seedlings inoculated in vitro and transplanted later into containers under greenhouse conditions. Correlation coefficient (r) and the P -value for the estimated correlation are shown

was statistically significant (P <0.05). Only seedlings having a >50% initial colonization level at transplantation maintained *L. deliciosus* mycorrhizas at the end of the experiment (Fig. 1).

Injection of mycelial slurries under greenhouse conditions, conducted with the same inoculum dose as used in the in vitro tests, produced only a limited number of mycorrhizal plants of both plant species. However, their colonization percentage was greater than that obtained in the acclimatization experiment (Table 3).

Vegetative inoculum grown in peat-vermiculite was effective for the production of colonized plants with all the *Lactarius* species tested. Nevertheless, the seedling colonization level was very variable depending on the fungal species, strain and host plant involved. Strain 178 of *L. deliciosus* readily colonized all the inoculated seedlings of both plant species whereas strain 217 of *L. deliciosus* was less effective and only 59% of the inoculated seedlings of *P. pinaster* and 33% of those of *P. sylvestris* became mycorrhizal (Table 4). The percentage of mycorrhizas of *P. pinaster* and *P. sylvestris* showed similar values for both fungal strains. *L. sanguifluus* mycorrhizas were detected in a low percentage of

Table 3 Mean growth parameters and mycorrhizal colonization of *P. pinaster* and *P. sylvestris* seedlings inoculated with a mycelial suspension of *L. deliciosus* 217

Plant species	Inoculation treatment	Height (cm)	Diameter (mm)	Dry weight (g)		% Colonized plants	% Plant colonization[a]
				Shoot	Root		
P. pinaster	Control	8.2	2.4	1.27	1.22	0	0
	L. deliciosus	9.3	2.3	1.15	1.86*	24	36±18
P. sylvestris	Control	3.4	2.2	0.51	0.78	0	0
	L. deliciosus	4.4	2.3	0.47	0.86	10	18±0

*$P<0.05$ where values are significantly different from the respective control values
[a] Means±SE

Table 4 Mean growth parameters and mycorrhizal colonization of *P. pinaster* and *P. sylvestris* seedlings inoculated with vegetative inocula of four edible *Lactarius* strains grown in peat:vermiculite

Tree species	Inoculation treatment	Height (cm)	Diameter (mm)	Dry weight (g)		% Colonized plants	% Plantcolonization[a]
				Shoot	Root		
Pinus pinaster	Control	12.1	2.3	1.04	0.89	0	0
	L. deliciosus 178	14.3	2.6	1.26	1.18	100	28±5
	L. deliciosus 217	12.0	2.5	1.33	1.18	59	30±6
	L. sanguifluus 261	11.6	2.6	1.42*	1.28*	14	4±2
	L. sanguifluus 263	10.1	2.8 *	1.47*	1.19*	10	1.5±0
Pinus sylvestris	Control	4.3	2.3	0.48	0.75	0	0
	L. deliciosus 178	5.4	2.3	0.60	0.83	100	9±1
	L. deliciosus 217	4.2	2.6	0.63	0.73	33	7±2
	L. sanguifluus 261	5.6 *	2.1	0.62	0.73	14	1±0
	L. sanguifluus 263	4.5	2.3	0.62	0.78	0	0

*$P<0.05$ (in the *same column* data are significantly different from the respective controls; Tukey's test)
[a] Means±SE

the inoculated plants. The colonization percentage for this fungal species was always under 5% (Table 4).

All the alginate inoculum beads of the four fungal strains plated in BAF agar showed mycelial growth. In spite of this, the alginate-entrapped inoculum completely failed in forming mycorrhizas when mixed with the plant-growth substrate.

Seedling growth effects related with mycorrhizal inoculation were not evident. However, a slight, but significant, growth promotion of *P. pinaster* was detected in seedlings inoculated with *L. sanguifluus* (Table 4).

A dispersed but consistent presence of sporocarps of the Discomycete *Sphaerosporella brunnea* (Alb. & Schw.:Fr.) Svrcek & Kubicka (Pezizales) was detected in the substrate of almost all the plants in the experiment. The percentage of root colonization by this contaminant was not systematically assessed but, in most cases, surpassed that of *Lactarius* reaching about 50% of the total short roots assessed. The hyaline to brown branched mycorrhizas formed by *S. brunnea* were easily distinguishable from the *Lactarius*' ones.

Discussion

All the inoculation methods tested, except the alginate-entrapped mycelium, were appropriate for the production of seedlings colonized with *L. deliciosus*. However, the percentage of colonized plants and the degree of colonization observed were highly variable depending on the inoculation method and the plant-fungal strain combination. A sharp decrease in mycorrhizal colonization was detected after transplantation to the greenhouse of well-colonized plants produced in vitro. This could be explained by the poor secondary colonization ability of *Lactarius* compared to the aggressive greenhouse contaminants, which colonized almost all the plants at a higher proportion than *Lactarius*. Only transplanted *P. pinaster* seedlings having an initial mycorrhizal colonization >50% maintained *L. deliciosus* mycorrhizas after the first growing season although the colonization level was significantly reduced. The low secondary colonization of the tested strain agrees with the features stated by Hutchison (1999) who classified *Lactarius* as a late-stage genus. However, the disturbance of the root system of the in vitro inoculated seedlings at the assessment and transplantation stages could also have destroyed part of the mycorrhizal system of the plants, especially the extramatrical mycelium, making secondary colonization more difficult for seedlings with a low number of mycorrhizas. In view of the results obtained and the cost of the manipulation involved in transplantation of colonized seedlings produced in vitro, this method cannot be recommended for commercial production.

The inoculation methods applied directly under greenhouse conditions provided a similar, or even higher, plant colonization rate at harvest than that obtained in the in vitro inoculated, acclimatized plants. Injection of mycelial slurries obtained from blended fungal colonies grown in solid media could be a promising method to produce

mycorrhizal seedlings since the production of enough mycelia and control of contaminants can be easily achieved. Mycelial liquid slurries have been almost exclusively used to inoculate in vitro plants or as starting cultures to inoculate a carrier substrate. Nevertheless, mycelial slurries of different ectomycorrhizal fungi have also been occasionally used in inoculation experiments (Danielson et al. 1984; Boyle et al. 1987; Richter and Bruhn 1989). In this study, however, we have obtained only a low proportion of colonized plants after using this inoculum. Further studies are necessary to determine the influence of the inoculum dosage, mycelial fragmentation and intraspecific variability in the colonization ability of mycelial slurries of *Lactarius* spp. under greenhouse conditions.

The vegetative inoculum grown in peat:vermiculite showed the best fungal colonization results. Strain 178 of *L. deliciosus* was the only one to produce 100% mycorrhizal seedlings, in both *P. pinaster* and *P. sylvestris*. This inoculum type has been recognized as the most biologically suitable for producing mycorrhizal seedlings (Marx 1980; Marx and Kenney 1982). Nevertheless, some disadvantages are related with the use of this type of inoculum. *Lactarius* grows slowly in pure culture and substrate colonization takes at least 2 months to be completed. Then, contamination and desiccation are probable and are difficult to manage.

The lack of success with the alginate-entrapped mycelial inoculum is difficult to explain since viability of the mycelium entrapped in the beads was proven to be unaffected. An alginate inoculum can be easily controlled and mechanized and has been effectively used with some fungal species such as *Laccaria bicolor* and *Hebeloma* spp. (Le Tacon et al. 1983; Mauperin et al. 1987; Mortier et al. 1988). Also, alginate inoculum has been used to successfully inoculate *Pseudotsuga menziesii* with entrapped mycelium of *L. bicolor* plus spores of *Rhizopogon subareolatus* in dual inoculations (Parladé et al. 1999). However, attempts to inoculate plants with *Pisolithus tinctorius* entrapped in alginate consistently failed (Pera et al. 1994). Further studies, which consider physical and chemical characteristics of the alginate inoculum and their influence on the colonization ability of the fungus under greenhouse conditions should be undertaken.

Growth of *L. sanguifluus* in agar plates and in a peat:vermiculite substrate was slower than that of *L. deliciosus*. The inoculation with a peat:vermiculite vegetative inoculum resulted in the occasional formation of a low percentage of mycorrhizas with morphological traits similar to those described above for *L. deliciosus*. The formation of ectomycorrhiza with *L. sanguifluus* was also reported by Guerin-Laguette et al. (2000) in a few *P. sylvestris* seedlings under controlled experimental conditions. Zak (1969) reported that *L. sanguifluus* forms mycorrhizas weakly and with great difficulty with pines in pure culture. Their results and those presented here indicate that we are still far from understanding the requirements for producing well-colonized mycorrhizal plants with this fungal species.

A growth effect due to inoculation with *Lactarius* species was not sought in this study but growth variables were measured to examine the appropriate plant development under the experimental greenhouse conditions. As expected for containerized plant production in an artificial substrate and under regular fertilization, no consistent growth differences due to inoculation were found. Additionally, no significant data on the growth performance of outplanted plants following inoculation with *L. deliciosus* are described (Castellano 1996).

From our results and previous studies, it can be concluded that most of the existing inoculation techniques using vegetative inoculum of *Lactarius deliciosus* are appropriate for the production of mycorrhizal seedlings under greenhouse conditions. Nevertheless, the colonization level obtained in the plants produced in the different experiments was variable and relatively low. Screening fungal strains for their aggressiveness under nursery conditions is a prerequisite for the successful commercial application of inoculation techniques. Also, control of environmental parameters and the incidence of fungal contaminants have been found to be critical to improve fungal colonization in *Lactarius* inoculations (Guerin-Laguette et al. 2000). However, these controlled conditions are difficult to obtain in the nursery or greenhouse if a practical inoculation method is to be integrated into the commercial production process. For this, it is critical to know the threshold colonization level required to ensure fungal persistence. In spite of its importance for the economic viability of producing mycorrhizal plants for commercial purposes, this factor has been very little studied and the available data are restricted to *Pisolithus tinctorius* (Marx et al. 1991) and certification criteria for commercial *Tuber* species (Bencivenga et al. 1995). Since commercial nursery conditions are appropriate for the dissemination of a few aggressive ectomycorrhizal fungi (Castellano and Molina 1989) the selection of fungal isolates able to readily colonize the host short roots could be a good strategy (Trappe 1977). Also, further research is needed to optimize the effective inoculation methods with dosage experiments to obtain maximized root colonization, especially if a late-stage fungus like *Lactarius* spp. is to be introduced.

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