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SHORT NOTE

Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.)

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Abstract Ectomycorrhizas of *Boletus aereus*, *Boletus edulis*, and *Boletus reticulatus* were synthesized with *Cistus* sp. under laboratory conditions using synthesis tubes filled with a mixture of sterilized peat-vermiculite and nutrient solution. The fungal strains isolated from sporocarps were identified by molecular techniques. The inoculated seedlings were grown for 4–5 months. The ectomycorrhizas formed were described based on standard morphological and anatomical characters. The three ectomycorrhizas described were very similar, with white monopodial-pinnate morphology, a three-layered plectenchymatous mantle on plan view and boletoid rhizomorphs.

Keywords Pure culture synthesis · Ectomycorrhizas · *Boletus* · Anatomy · Morphology · Description · ITS rDNA

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Introduction

Boletus Fr. is a cosmopolitan genus of ectomycorrhizal fungi widely represented in the temperate zones of Northern and Southern Hemispheres. The genus comprises more than 1,000 species with epigeous fructification, inhabiting forests in tropical and mid latitudes. *Boletus* forms ectomycorrhizas with a large number of suitable hosts: Fagales—Fagaceae (*Castanea, Castanopsis, Fagus, Lithocarpus, Quercus*) and Betulaceae (*Carpinus, Corylus, Betula, Ostrya, Populus*); Malvales—Malvaceae (*Tilia*) and Cistaceae (*Cistus*); Malpighiales—Salicaceae (*Salix*); Ericales—Ericaceae (*Arctostaphylos*); and Pinales—Pinaceae (*Abies, Keteleeria, Picea, Pinus, Tsuga*) (Olivier et al. 1997; Águeda et al. 2006; Mello et al. 2006).

The *Boletus edulis* species complex (*B. edulis* Bull. *sensu stricto*, *Boletus aereus* Bull., *Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff.) has great economic importance for its edibility (Singer 1986; Hall et al. 1998) being *B. edulis* a major commercial mushroom consumed worldwide. This fungal species is collected exclusively from the wild (Cannon and Kirk 2007) and no controlled production has been done to date.

Edible mycorrhizal mushrooms are not only a gourmet food but also a source of income for collectors (Wang and Hall 2004). Total annual worldwide consumption of *B. edulis* complex is between 20,000 and 100,000 tons (Hall et al. 1998). Important markets include North America, France, Italy, and Germany (Hall et al. 1998). The estimated annual production of *B. edulis* species complex from the autonomous community of Castilla y León in Spain, is 8,500 tons, worth approximately 38 million Euros (Martínez-Peña et al. 2006-2008).

In some regions of Central Spain (Zamora, León, and Salamanca provinces) with abundant fires and dominated

exclusively by *Cistus ladanifer, B. edulis* sporocarps are regularly observed. Águeda et al. (2006) described field ectomycorrhizas formed by these organisms and sequenced them for taxonomic verification. This fact is important since *Cistus* species occur in degraded areas where economic resources are scarce to maintain human population. The family Cistaceae, with eight genera and almost 200 species (Muñoz and Navarro 1993) is distributed primarily in the temperate areas of Europe and the Mediterranean basin, but is also found in North and South America (López González 2001). The genus *Cistus* is represented in the Iberian Peninsula by 12 shrub species, all occurring during primary succession of tree stands. Cistaceae species are pyrophytic in general, and their germination is benefited by high temperatures where they are adapted to fires in Mediterranean forests (Alonso et al. 1992). *Cistus* species often form pure stands in vast areas heavily subjected to fire and/or grazing because of their ability as early colonizers after disturbance.

Cistus can form both ecto- and arbuscular mycorrhizas (Smith and Read 1997). More than 200 fungal ectomycorrhizal species belonging to 40 genera are reported to associate with *Cistus* (Puppi and Tartaglini 1991; Comandini et al. 2006). Rockroses (*Cistus* and *Helianthemum* sp.) are ecologically important species because they may act as a reservoir of mycorrhizal fungi after a forest disturbance (Torres et al. 1995; Díez 1998).

Among the edible mycorrhizal mushrooms, only *Tuber melanosporum* Vittad. and *Tuber uncinatum* Chatin have been cultivated commercially (Wang and Hall 2004).

Also, some success has been achieved with *Lactarius deliciosus* (L.) Gray, *Lyophyllum shimeji* (Kawam.) Hongo, *Tuber borchii* Vittad., and *Rhizopogon roseolus* (Corda) Th. Fr. (Wang and Hall 2004). Few attempts to produce edible sporocarps using a Cistaceae host have been reported, most of them involving *T. melanosporum* and *Cistus* sp. (Chevalier et al. 1975; Díez et al. 1994; Fontana and Giovanetti 1978; Giovannetti and Fontana 1982; Roth-Bejerano et al. 2003; Wenkart et al. 2001), or *Terfezia claveryi* Chatin and *Helianthemum* sp (Morte et al. 2004).

Ectomycorrhizal synthesis experiments are useful to determine fungus–plant host compatibility and for morphological and physiological research (Giomaro et al. 2005). In this paper, this technique has been used to test the ability of the *B. edulis* species complex to form ectomycorrhizas with *Cistus* sp. under controlled conditions as well as provide detailed anatomical descriptions of the formed ectomycorrhizas. This research is part of a project aimed at promoting shrub inoculations for the production of edible mycorrhizal fungi (Martínez-Peña et al. 2007).

Materials and methods

Fungal isolates

Fungal isolates of *Boletus aereus, B. edulis, B. reticulatus*, and *B. pinophilus* were obtained from sporocarps collected in northern Spain (Table 1). Isolations were made by explants from sporocarp tissues plated on modified Melin–Norkrans agar culture medium (MMN) (Marx 1969) or biotin-aneurin-folic acid agar culture medium (BAF) (Oort 1981) and maintained by transferring to fresh media every 3 months.

The identification of the fungal species was confirmed by molecular analysis of the internal transcribed spacer (ITS) of the nuclear rDNA region (Leonardi et al. 2005).

Amplicons obtained with the ITS1/ITS4B primers (Gardes and Bruns 1993) from each fungal isolate were purified and sequenced in both directions. The ITS sequences obtained were compared with sequences deposited in GenBank to confirm their taxonomic identification, and submitted to the GenBank databases under accession numbers reported in Table 1.

Pure culture synthesis procedures

Cistus albidus L. and *C. ladanifer* L. seeds were rinsed in 90°C water for 30 min, maintained in 30% sodium hypochlorite for 10 min, and washed in sterile distilled water. Disinfected seeds were placed on BAF agar Petri dishes and stratified for 2–3 weeks at 4°C. Plates were then placed at room temperature for seed germination (20–23°C). After 2 weeks of incubation, seeds showing contamination were discarded.

Table 1 Fungal isolates used in the ectomycorrhizal synthesis

Species	Site (region)	Host	Strain code	GenBank code
Boletus aereus	Osor (Cat)	*Castanea sativa*	393 IRTA	EU554663
Boletus edulis	Aránser (Cat)	*Pinus uncinata*	375 IRTA	EU554664
Boletus reticulatus	Ocenilla (CyL)	*Quercus pyrenaica*	1054 DIEFV	EU554661
Boletus pinophilus	La Póveda (CyL)	*Pinus sylvestris*	1082 DIEFV	EU554662

Cat Cataluña, *CyL* Castilla y León

Aseptically germinated seedlings (radicle 1–2 cm long) were transferred into ectomycorrhizas synthesis tubes (Molina 1979) filled with a sterilized mixture of 10 ml peat, 110 ml vermiculite and 60 ml BAF nutrient solution modified reducing the glucose to 20 g/l. The synthesis tubes were inoculated with 10 ml of a mycelium culture of either *B. aereus, B. edulis, B. reticulatus*, and *B. pinophilus* grown in BAF liquid medium. All the fungi were tested with *C. albidus* and *C. ladanifer* with four replicates for each fungus–host combination. Synthesis tubes with the inoculated seedlings were grown for 4–5 months at 20–25°C under fluorescent lights (150 μmol s^{-1} m^{-2} [400–700 nm], 16 h/day). At the end of the growing period, seedlings were removed from the synthesis tubes and root systems washed and examined for ectomycorrhizal formation.

Morphological description of ectomycorrhizas

Ectomycorrhizal roots and rhizomorphs were carefully examined with the aid of a stereomicroscope, fixed in FAA (Agerer 1986) and stored as voucher specimens in the Dpto. Inv. Exp. For. Valonsadero (Soria, Spain). The general methodology and terminology for characterizing the ectomycorrhizas follows Agerer (1987-2006, 1991) and Agerer and Rambold (2004-2008). For the observation of the mantle, the ectomycorrhizas were grated with the peeling technique (Agerer 1991). Mantle and rhizomorph preparations of fresh ectomycorrhizas were fixed on slides with lactic acid for microscope observation.

Results

B. aereus, B. edulis, and *B. reticulatus* formed ectomycorrhizas with *C. ladanifer* and *C. albidus*, whereas *B. pinophilus* did not form ectomycorrhizas with either *Cistus* host (Table 2).

Table 2 Formation of ectomycorrhizas in each host–fungus combination

Fungal species	Strain code	Plant species	
		Cistus albidus	*Cistus ladanifer*
Boletus aereus	393 IRTA	++++	++++
Boletus edulis	375 IRTA	++	++++
Boletus reticulatus	1054 DIEFV	++++	++
Boletus pinophilus	1082 DIEFV	–	–

Four replicates were tested for each combination. The crosses indicates the number of replicates in which ectomycorrhizal formation was detected

– No mycorrhizal formation in any replicate

Mycorrhizal structures of each fungal species were identical in both *Cistus* species as it has been described for other host–fungus combinations (Agerer and Rambold 2004-2008). Consequently, only one complete description is given for each fungal species indicating the host plant taken into account in each case. Herbarium codes for the described mycorrhizas were: VALONSADERO-MYCORRHIZA 049 for *B. aereus* and *C. ladanifer* (from strain 393 IRTA), VALONSADERO-MYCORRHIZA 047 for *B. edulis* and *C. albidus* (from strain 375 IRTA), and VALONSADERO-MYCORRHIZA 045 for *B. reticulatus* and *C. albidus* ectomycorrhizas (from strain 1054 valonsadero).

B. aereus + *C. ladanifer*

Mantle type: plectenchymatous, colorless, clamps lacking, outer mantle layer with ring-like arrangement of hyphal bundles, Type A (Fig. 1b); middle mantle layer hyphae arrangement without pattern; inner mantle layer hyphae arrangement with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: up to 15 mm, highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arranged; forming nodia at branching points, clamps absent, colorless, without short inflated cells. Peripheral hyphae similar to mantle cystidia, colorless, smooth (Fig. 1c).

Cystidia: awl-shaped, bristle-like, present on outer mantle layer and on rhizomorphs; ramification presence-position absent or proximal, monopodial or bifurcate; branches ramification absent; septa: present, simple, septa number 2–3; surface smooth.

Emanating hyphae: clamps lacking, straight, colorless; ramification approximately 90°, adjacent to septum, one side-branch at septum; cells even or slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system solitary, monopodial-pyramidal or irregularly pinnate; unramified ends straight, not inflated, cylindrical, yellowish; mantle surface smooth and glistening, loosely woolly or forming rings (reticulate) (Fig. 1a). Emanating hyphae present, infrequent, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points, connection to mantle kind distinct, origin location proximal, surface smooth or hairy.

B. edulis + *C. albidus*

Mantle type: plectenchymatous, colorless, clamps lacking; outer mantle layer ring-like arrangement of hyphal bundles, Type A (Fig. 1e); middle mantle layer hyphae arrangement

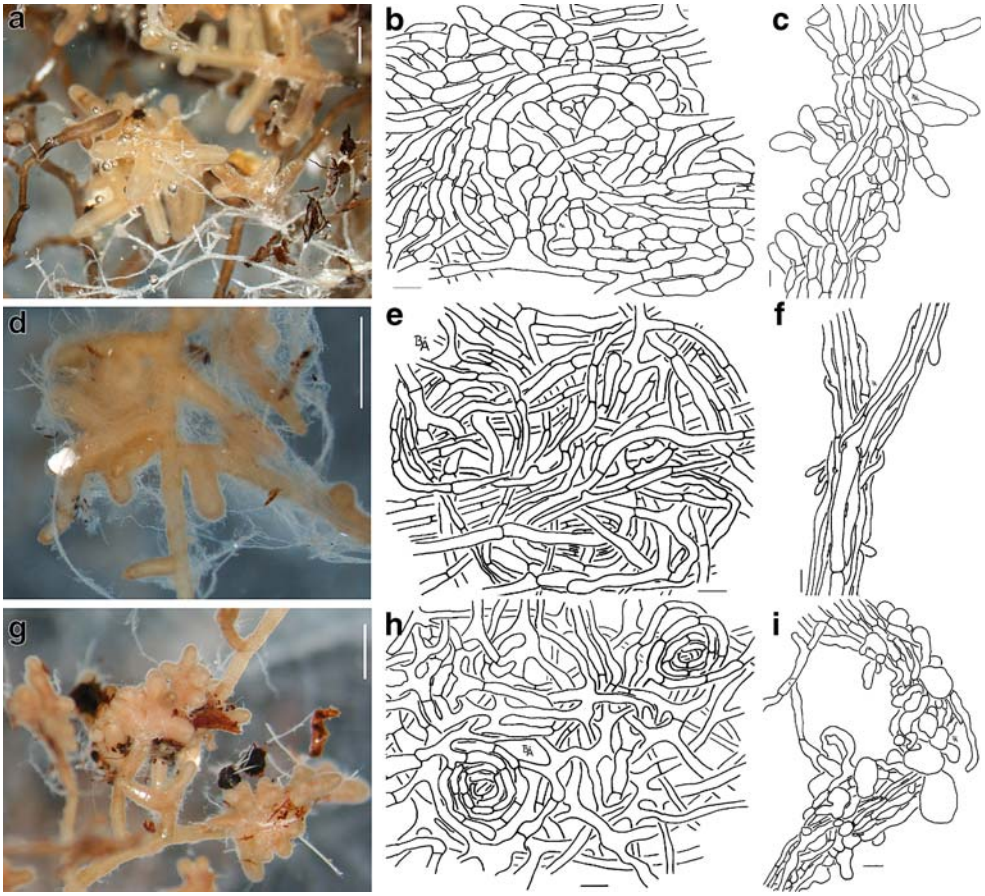

Fig. 1 Morphological and anatomical characters of the ectomycorrhizas obtained in pure culture synthesis. **a** Ectomycorrhizas and rhizomorphs of *Boletus aereus* and *Cistus ladanifer*. Bar=10 mm. **b** Outer mantle layer of *Boletus aereus* and *Cistus ladanifer*. Bar=10 µm. **c** Surface of rhizomorph with cystidia of *Boletus aereus* and *Cistus ladanifer*. Bar=10 µm. **d** Ectomycorrhizas and rhizomorphs of *Boletus edulis* and *Cistus albidus*. Bar=10 mm. **e** Outer mantle layer of *Boletus edulis* and *Cistus albidus*. Bar=10 µm. **f** Rhizomorph with vessel-like hyphae of *Boletus edulis* and *Cistus albidus*. Bar=10 µm. **g** Ectomycorrhizas and rhizomorphs of *Boletus reticulatus* and *Cistus albidus*. Bar=10 mm. **h** Middle mantle layer of *Boletus reticulatus* and *Cistus albidus*. Bar=10 µm. **i** Surface of rhizomorph with cystidia of *Boletus reticulatus* and *Cistus albidus*. Bars=10 µm

ring-like; inner mantle layer hyphae arrangement with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arranged; forming nodia at branching points, clamps absent, colorless, without short inflated cells (Fig. 1f).

Cystidia: lacking.

Emanating hyphae: clamps lacking, wavy to slightly tortuous, colorless; ramification Y-shaped, adjacent to septum, one side-branch at septum; cells slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system monopodial-pyramidal or irregularly pinnate, dichotomous-like; unramified ends straight, not inflated, cylindrical, white to yellowish getting more yellow with age; mantle surface shiny and silvery, loosely woolly (Fig. 1d). Emanating hyphae present, abundant, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points, connection to mantle kind distinct, surface smooth or woolly.

B. reticulatus + *C. albidus*

Mantle type: plectenchymatous, colorless, clamps lacking; outer mantle layer with hyphae rather irregularly arranged with no special pattern discernable (Type B); middle mantle layer hyphae arrangement without pattern or ring-like (Fig. 1h); inner mantle layer hyphae arrangement ring-like or with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arranged; forming nodia at branching points, clamps absent, colorless, without short inflated cells, clamps absent. Peripheral hyphae not specialized or roundish, inflated, as short cells, smooth (Fig. 1i).

Cystidia: two different types, type 1 present on outer mantle layer and on rhizomorphs and type 2 present only on rhizomorphs. Type 1 thin-walled, slightly tapering, often rather similar to ends of normal hyphae, ramification presence-position absent or proximal, monopodial or bifurcate, branches ramification absent, septa present, simple, septa number 1–3, surface smooth. Type 2 globular, ramification presence-position absent, septa absent, cell wall color similar to mantle cells, surface smooth.

Emanating hyphae: clamps lacking, wavy or irregularly inflated or even beaded, colorless; ramification acute or Y-shaped, adjacent to septum, one side-branch at septum; cells even or slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system: solitary, monopodial-pinnate or coralloid; unramified ends straight or bent, not inflated, cylindrical, white to yellowish getting more yellow with age; mantle surface shiny and smooth, silvery in some zones, loosely grainy or warty (Fig. 1g). Emanating hyphae present, infrequent, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points; connection to mantle kind distinct, surface: smooth.

Discussion

Of the eight tested combinations, only *B. pinophilus* failed to form ectomycorrhizas with either host, despite the extensive growth of the fungus in the substrate and the adequate root development of both *Cistus* species. Sporocarps of *B. aereus*, *B. edulis*, and *B. reticulatus* are found associated with Cistaceae plants in the Mediterranean region (Oria de Rueda 2007), while sporocarps of *B. pinophilus* are associated to Pinaceae or Fagaceae species. The incompatibility of *B. pinophilus* and *Cistus* spp. must be confirmed or rejected by testing a wider range of fungal strains.

Over the last 40 years many researchers have synthesized ectomycorrhizas of the *B. edulis* species complex on various hosts. Froidevaux and Amiet (1975) synthesized *B. edulis* and *Pinus mugo* Turra ectomycorrhizas; Tozzi et al. (1980) obtained ectomycorrhizas of *B. edulis* and *Quercus pubescens* Willd.; Molina and Trappe (1982a, b) synthesized ectomycorrhizas between *B. edulis* and eight hosts in the genera *Arbutus*, *Arctostaphylos*, *Larix*, *Picea*, *Pinus*, and *Tsuga*; Ceruti et al. (1983-1984) obtained *B. aereus* and *Q. pubescens* ectomycorrhizas; Ceruti et al. (1985) obtained mycorrhizas of *B. aereus* and *Castanea sativa* Mill.; Poitou et al. (1982) synthesized ectomycorrhizas in pure culture between *Pinus radiata* D. Don and *B. edulis* and *B. aereus*; and Duñabeitia et al. (1996) obtained *B. pinophilus* ectomycorrhizas by inoculating *P. radiata* seedlings with a spore suspension of 10^6–10^7 spores per plant.

Seedlings inoculated with *Boletus* species have been outplanted in attempts to promote fruiting of the valuable edible sporocarps. Olivier et al. (1997) describes plantations of *C. sativa* and *Pinus uncinata* Ramond ex DC. inoculated with *B. edulis* and *B. aereus*. Meotto et al. (1999) outplanted *C. sativa* seedlings inoculated with mycelial cultures of *B. edulis*. Unfortunately, sporocarp production has not been reported from either study.

Description and identification of ectomycorrhizas have evolved greatly following the systematic studies by Agerer (1986, 1987-2006, 1991) and molecular characterization based on DNA analysis (Gardes and Bruns 1993). Although there are some descriptions for *Boletus* mycorrhizas, most are not precise (De Román et al. 2005). The ectomycorrhizas of the three *Boletus* species obtained in this study are very similar and fit well with the characters described in Agerer (2006) for this genus: plectenchymatous mantles from the types A, B, or C, boletoid rhizomorphs with nodes and with or without short inflated cells, emanating hyphae smooth or covered by crystals, clamps lacking, cystidia lacking or with cystidia-like hyphal ends, and white to yellowish hydrophobic ectomycorrhizas. All, *B. aereus*, *B. edulis*, and *B. reticulatus*, form white monopodial-pinnate ectomycorrhizas with three-layered plectenchymatous mantle on plan view and boletoid rhizomorphs. *B. aereus* forms monopodial-pyramidal ectomycorrhizas with ring-like outer mantle layer, and emanating cystidia formed by two or three short cells on the outer mantle layer and rhizomorphs. *B. edulis* has ring-like outer and middle mantle layers, without cystidia and rhizomorphs also without cystidia or globular cells. *B. reticulatus* has ring-like or with broad streaks of parallel hyphae inner mantle layer, emanating cystidia on the outer mantle layer and globular and awl-shaped cystidia on rhizomorphs.

The cystidia of *B. aereus* and *B. reticulatus* ectomycorrhizas are similar to those present on the hymenia of some *Boletus* species, like *B. reticulatus*, *B. edulis*, or *Boletus regius* Krombh., appearing as fusiform or globoid structures (Muñoz 2005). However, it can not be discounted that the formation of these elements, as well as the emanating hyphae on *B. edulis*, could be influenced by the experimental conditions of the synthesis tubes.

The abundance and size of rhizomorphs found in this study, especially for *B. aereus,* conform to those found in the field. In natural conditions, the mycelium of *Boletus* is concentrated as rhizomorphs with a high degree of spatial heterogeneity. However, the type of soil could determine the spread of exploratory elements of the *Boletus* ectomycorrhizas, such as cystidia, rhizomorphs and emanating hyphae, as demonstrated for *Lactarius deliciosus* mycorrhizal seedlings (Hortal et al. 2008).

There are no previously reported descriptions of *B. aereus* ectomycorrhizas. All previous descriptions of *B. edulis* ectomycorrhizas (Ceruti et al. 1987-1988; Garrido 1988; Gronbach 1988; Agerer and Gronbach 1990; Franz and Acker 1995; Palfner 2001; Agerer and Rambold 2004-2008; Águeda et al. 2006) report characters that fit with those we described here. Ceruti et al. (1983-1984), Ceruti et al. (1985), and Garrido (1988) only described the characters of mantle on cross-sections of *B. reticulatus*, so comparison with our study is not possible.

Considering that fungus is the main factor for determining the anatomical structures of ectomycorrhizas, those are identical for the same fungal species regardless of the host. Although morphological characters are mainly driven by the plant genus, some fungi can control, at least partially, the final form (Agerer and Rambold 2004-2008). Both aspects are true for the three described *B. edulis* complex ectomycorrhizas, which show the same structures when associated to *Cistus* as compared to other Pinaceae and angiosperm hosts.

The natural sporocarp production of *B. edulis* in association with *C. ladanifer* (Águeda et al. 2006) offers an alternative economic resource for marginal and inland areas with low incomes. Controlled mycorrhization with *B. edulis* on *Cistus* and outplanting of inoculated seedlings might be a feasible and promising way to exploit this symbiosis providing economic benefits. To accomplish this, further research is needed to determine the appropriate inoculation methods with compatible *Boletus* strains, the persistence of *Boletus* ectomycorrhizas on outplanted, inoculated seedlings, and the factors inducing sporocarp production.

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