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ORIGINAL PAPER

Field persistence of the edible ectomycorrhizal fungus *Lactarius deliciosus*: effects of inoculation strain, initial colonization level, and site characteristics

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Abstract *Pinus pinea* plants were inoculated with different strains of the edible ectomycorrhizal fungus *Lactarius deliciosus*. The inoculated plants were established in six experimental plantations in two sites located in the Mediterranean area to determine the effect of the initial colonization level and the inoculated strain on fungal persistence in the field. Ectomycorrhizal root colonization was determined at transplantation time and monitored at different times from uprooted plants. Extraradical soil mycelium biomass was determined from soil samples by TaqMan® real-time polymerase chain reaction (PCR). The results obtained indicate that the field site played a decisive role in the persistence of *L. deliciosus* after outplanting. The initial colonization level and the selection of the suitable strain were also significant factors but their effect on the persistence and spread of *L. deliciosus* was conditioned by the physical–chemical and biotic characteristics of the plantation soil and, possibly, by their influence in root growth. Molecular techniques based on real-time PCR allowed a precise quantification of extraradical mycelium of *L. deliciosus* in the field. The technique is promising for non-destructive assessment of fungal persistence since soil mycelium may be a good indicator of root colonization. However, the accuracy of the technique will ultimately depend on the development of appropriate soil sampling methods because of the high variability observed.

Keywords Fungal persistence · Edible fungi · *Lactarius* · Colonization level · Real-time PCR

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Introduction

Natural harvests of some key species of edible fungi as *Tuber* spp. and *Tricholoma matsutake* (Ito et Imai) Singer have declined over the last century (Arnolds 1995; Hall et al. 2003; Wang and Hall 2004). Different studies have tried to elucidate the role of factors such as habitat degradation, overexploitation, and forestry practices in the decline of production of wild edible fungi (Egli et al. 2006; Hall et al. 2003; Molina et al. 2001; Pilz and Molina 2002; Pilz et al. 2006). Despite the progressive decline, the demand for edible fungi continues to increase and this is reflected in the high economic values associated with the commercialization of some species (Arnolds 1995; Boa 2004). Since most appreciated edible fungi are ectomycorrhizal (Hall and Wang 1996, 2001) and play a key role in forest ecosystems (Read and Perez-Moreno 2003; Smith and Read 1997), cultivation could represent a promising alternative to satisfy both the increasing demand of this resource and the conservation of natural ecosystems. Controlled production of edible symbiotic ectomycorrhizal fungi involves the inoculation of plants with a given fungus and the establishment of the plants in experimental plantations aimed at the production of fruit bodies. Special attention has been given to the controlled production of *Tuber* spp. (reviewed in Wang and Hall 2004) and *Terfezia* spp. (Honrubia et al. 2007). The production of other edible fungi like *Lactarius* sp., *Boletus* sp., *Tricholoma* sp., or

Cantharellus sp. has been attempted with a variable degree of success (reviewed in Hall et al. 2003).

Among the key edible ectomycorrhizal fungi, fruit bodies of *Lactarius deliciosus* (L.) Gray are highly regarded in some local European markets and their commercialization provides important economic benefits (Boa 2004; Singer 1986). The establishment of productive plantations of this species could represent a significant income for landowners, especially in Mediterranean areas with limited resources. Under laboratory conditions, the culture of *L. deliciosus* and the production of large amounts of mycelial inoculum have been found to be feasible (Carrillo et al. 2004). The inoculation of different pine species with *L. deliciosus* has also been described (Guerin-Laguette et al. 2000; Parladé et al. 2004) and occasional production of *L. deliciosus* sporocarps in a nursery was reported by Guerin-Laguette et al. (2000) for 20-month-old plants. The only published studies on the establishment of productive plantations with plants inoculated with *L. deliciosus* have been reported by Poitou et al. (1984) and Guinberteau et al. (1989) in France and by Wang and Hall (2002) and Wang et al. (2003) in New Zealand. The production of *L. deliciosus* fruit bodies was recorded after 3 years in the field in the first study and after 18 months in the second one.

Since the production of sporocarps depends on many factors and can take a long time to occur, tracking the vegetative persistence of *L. deliciosus* in experimental plantations is essential to evaluate the viability of the inoculation program. Although the persistence of the inoculated fungus in the form of mycorrhizas and extraradical mycelium does not ensure a regular production of sporocarps, it is necessary to allow for future fruit body production. Field persistence of mycorrhizas can be recorded by identifying and counting short roots from entire plants or soil samples (Brundrett et al. 1996; Parladé et al 1996). In spite of the significant advances in morphological characterization of ectomycorrhizas (Agerer 2006; de Román et al. 2005), their accurate identification is limited to some species. The development and adaptation of molecular techniques to complement morphological data have represented an important step forward in the identification of mycorrhizas in the field (Anderson and Cairney 2004; Di Battista et al. 2002; El Karkouri et al. 2006; Gardes and Bruns 1993; Gardes et al. 1991; Henrion et al. 1992; Horton and Bruns 2001; Martin 2007; Weber et al 2002).

Besides the mycorrhizas, extraradical mycelium plays a key role in the mycorrhizal symbiosis (Guidot et al. 2003; Landeweert et al. 2003a, b; Simard and Durall 2004; Simard et al. 2002; Smith and Read 1997), but it still remains as the least understood phase due to the difficulties of studying its distribution, dynamics, and function in the soil (Anderson and Cairney 2007; Horton and Bruns 2001; Leake et al. 2004; Read 1992). The use of molecular techniques such as real-time polymerase chain reaction (PCR) (Heid et al. 1996; Schena et al. 2004) has significantly improved the study of extraradical fungal mycelium. In addition, mycelium quantification allows non-destructive tracking of the fungus in the field since there is no need to uproot the entire plant to obtain soil samples. The quantification of *L. deliciosus* extraradical mycelium present in the soil by TaqMan® real-time PCR has been optimized by Parladé et al. (2007) and Hortal et al. (2008), who proposed a methodology to establish a direct relationship between fluorescence values and mycelium biomass, thus avoiding the conversion of DNA quantities in units of biomass. Mycelium quantification of ectomycorrhizal fungi by real-time PCR has also been performed by Landeweert et al. (2003b), Raidl et al. (2005), Schubert et al. (2003), and Suz et al. (2008).

Many factors such as the initial root colonization level (Wang and Hall 2004), the site characteristics, and the competitive pressure of native fungi (Perrin et al. 1996) can affect the persistence of the inoculated fungal strain once established in the field. In this study, we report the results obtained from six experimental plantations established in two sites with *Pinus pinea* plants inoculated with different strains of *L. deliciosus*. Two hypotheses were tested. First, we hypothesized that higher root colonization level at the transplanting time would result in higher and longer persistence of the fungus in the field and, second, we hypothesized that different *L. deliciosus* strains would show differences in fungal persistence due to different colonization ability or site adaptation.

Material and methods

Plant inoculations

Seven *L. deliciosus* strains from the Institut de Recerca i Tecnologia Agroalimentaries (IRTA) ectomycorrhiza (ECM) Culture Collection were used as inoculum in the different experiments (Table 1). All the isolates were previously tested for ectomycorrhiza formation with *Pinus pinaster* under pure culture synthesis (Hortal 2008). Vegetative inoculum of each strain was prepared in a peat–vermiculite matrix according to Marx and Bryan (1975) modified as described in Parladé et al. (2004). After 10 weeks of incubation at 25°C, the inoculum of each fungal strain was mixed at the proportion 1:10 (*v*:*v*) with an autoclaved (120°C, 60') substrate composed of equal volumes of Floratorf peat (Floragard, Oldenburg, Germany) and vermiculite grade 2 (Asfaltex, Barcelona, Spain). Forest pot trays (Vivers La Fageda, Santa Pau, Girona, Spain), with 50 cells of 300-cm^3 capacity each, were filled with the

Table 1 *L. deliciosus* strains from the IRTA ECM Culture Collection used as inoculum in the different experiments

Strain	Associated species	Collection date	Locality (province)
178	*P. pinea* L.	October 1994	Cabrils (Barcelona)
217	*P. pinea*	November 1994	La Bisbal (Girona)
312	*P. pinaster* Ait.	October 2000	Sta. Coloma Farners (Girona)
313	*P. pinea*	October 2000	Sta. Coloma Farners (Girona)
330	*P. halepensis* Mill.	November 2003	Pierola (Barcelona)
332	*P. sylvestris* L.	October 2003	Berga (Barcelona)
335	*P. sylvestris*	September 2004	Saldes (Barcelona)

Pure cultures were obtained from sporocarps collected in different locations of Catalonia (NE Spain). Date of collection, locality, and associated *Pinus* species are indicated

inoculated substrate. Two *P. pinea* L. seeds from the coastal Mediterranean area of NE Spain were placed in each cell and thinned to one per container after germination. Control, non-inoculated, plants were also produced.

Both inoculated and non-inoculated plants were maintained in a greenhouse under automatized daily irrigation. Plants were fertilized every 15 days with Universol NPK 18+11+18+2.1 MgO (Scotts, Heerlen, Holland) plus micronutrients (Fetrilon 13 and Hortrilon, Compo, Barcelona, Spain). Each plant received 3.24 mg N, 1.98 mg P, 3.24 mg K, 0.38 mg Mg, 0.35 mg Fe, 0.06 mg Mn, 0.06 mg Cu, 0.01 mg Zn, 0.01 mg B, and 0.01 mg Mo in each fertilization.

The plants were planted into two types of field plots regarding plantation spacing: *plots* with a 2×2 m framework and *microplots* with 1×1 m. Plants in plots were maintained for a longer period of time than in microplots. Microplots, on the other hand, allowed for comparisons over more homogeneous conditions. The initial root colonization level of the plants established in plots (Can Badia 1 and 2, see Table 2) was estimated by counting at least 200 tips of a sample of ten plants from each inoculated lot as described in Parladé et al. (1996). The plants established in microplots (Micro 05, Micro 06, Micro 07C, and Micro 07B, see Table 2) were individually assessed before transplantation by determining the *L. deliciosus* mycorrhizal coverage of each plant (Hortal et al. 2008). This method allows non-destructive characterization of the initial *L. deliciosus* root colonization by image analysis of digital pictures of the plug. Briefly, the method consisted of pulling out the plant from the container and taking a digital picture of the apparently most colonized side of the plug. The obtained image was analyzed with the software Adobe Photoshop Elements using the "magic wand" tool with a color code adjusted in each experiment and corresponding to the color of a mature *L. deliciosus* mycorrhiza. Mycorrhizal coverage was defined as the number of pixels corresponding to the mycorrhizas divided by the total number of pixels of the analyzed area. The equation defined in Hortal et al.

Table 2 Outline of the six experimental plantations established to determine the effects of initial colonization level and inoculation strain on the persistence of the mycorrhizal symbiosis and growth of *P. pinea* plants inoculated with different strains of *L. deliciosus*

Effect determination	Plot name	Experimental design[a]	Site	Inoculation treatments[b]	Plantation date	Sampling time (months)	Measured variables
Initial colonization level	Can Badia 2	RBD (plot)	Cabrils	312 (high), 312 (medium)	12-2004	01-2006 (13) 01-2007 (25) 11-2007 (35)	%M, ERM, GRW
	Micro 05	CRD (microplot)	Cabrils	312 (high), 312 (medium)	05-2005	02-2006 (9) 12-2006 (19) 05-2007 (24)	%M, GRW
Inoculated strain	Can Badia 1	CRD (plot)	Cabrils	178, 312	12-2002	03-2004 (15) 01-2005 (25)	%M, ERM, GRW
	Micro 06	CRD (microplot)	Cabrils	312, 313, 330, 332, 335	04-2006	12-2006 (8) 06-2007 (14)	%M, ERM, GRW
	Micro 07 C	CRD (microplot)	Cabrils	312, 313, 330, 217	04-2007	10-2007 (6)	%M, GRW
	Micro 07 B	CRD (microplot)	La Bisbal	312, 313, 330, 217, 178	03-2007	10-2007 (7) 04-2008 (13)	%M, ERM, GRW

RBD randomized block design, *CRD* completely randomized design, *%M* percentage of mycorrhizas, *ERM* quantity of extraradical mycelium present in the soil (see "Material and methods"), *GRW* plant growth (height and diameter)

[a] Plot and microplot refer to the plantation framework (see "Material and methods")

[b] Number of the inoculated *L. deliciosus* strain (initial colonization level). See Table 1 for strain data

(2008) relating mycorrhizal coverage and real percentage of mycorrhizas (calculated by direct root counting) was used to estimate the percentage of mycorrhizas of each plant before its establishment in the microplots.

Experimental layout

The effects of the initial colonization level and the inoculated strain in the field persistence of *L. deliciosus* were evaluated in the different experimental plantations. The outline of the established plots and microplots is shown in Table 2. In the experiments designed to evaluate the effect of the initial colonization level, plants were classified into two groups before outplanting according to the visual estimation of the level of *L. deliciosus* mycorrhizas (high or medium). Initial mycorrhizal colonization of each group was further determined as described above.

Experimental plantations were set up in either a completely randomized design or a randomized block design (see Table 2) where trees were the replicates for within-plot analyses. Plots and microplots were established on flat ground in two different sites with a Mediterranean climate: Cabrils (Barcelona, Spain) and La Bisbal (Girona, Spain). The Cabrils site is located at an elevation of 82 m at 41.518° latitude and 2.378° longitude. Soil from the Cabrils location has a sandy loam texture; pH 8.1; EC 0.15 dS/m; OM 1.09%; N (nitric) 6 ppm; P Olsen 28 ppm; K 51 ppm; Mg 107 ppm; Ca 2,107 ppm; Na 43 ppm; CIC 6.2 meq per 100 g. The La Bisbal site is located at an elevation of 28 m at 41.973° latitude and 3.035° longitude. La Bisbal soil has a clayey loam texture; pH 7.7; EC 0.1 dS/m; OM 1.45%; N (nitric) 5 ppm; P Olsen 8 ppm; K 72 ppm; Mg 137 ppm; Ca 1,083 ppm; Na 37 ppm; CIC 6.9 meq per 100 g. Superficial tilling was performed in each site before plantation establishment. No maintenance treatments were applied after plantation. Mean monthly temperature and accumulated precipitation data for each site during the experiments are represented in Fig. 1.

Field tracking of *L. deliciosus* colonization

After outplanting, field tracking of mycorrhizas and extraradical mycelium of *L. deliciosus* was performed by morphological and molecular techniques. Percentages of mycorrhizas were established by direct short root counting from uprooted plants while biomass of extraradical mycelium was quantified by TaqMan® real-time PCR. Root colonization of a minimum of six plants per treatment was determined at each sampling time in all the plantations (see Table 2). The identity of mycorrhizas of *L. deliciosus* was verified by DNA extraction from representative tips followed by specific PCR with primers internal transcribed spacer (ITS)1-LDITS2R (Hortal et al. 2006). The presence of other ectomycorrhizal fungi in the root system was also quantified. Molecular identification of these native fungi was attempted by DNA extraction of representative mycorrhizas, amplification of the ITS region with primers ITS1–ITS4, sequencing of the amplified fragment, and searches for highly similar sequences in the GenBank database.

Extraradical soil mycelium was quantified by real-time PCR in the experiments and sampling times indicated in Table 2. Soil samples were obtained from four soil cylinders taken at 10 cm of the plant stem following two perpendicular lines in the four directions. Soil from 0- to 10-cm depth of the four cylinders was mixed, dried at 30°C to eliminate the excess of water, and sieved through 2-mm mesh to avoid the presence of roots in the soil sample. Additional rhizospheric soil in contact with the root system was obtained by gently shaking the roots of the sampled plants in a plastic bag. A subsample of each homogenized soil sample was taken and stored at −20°C until processing.

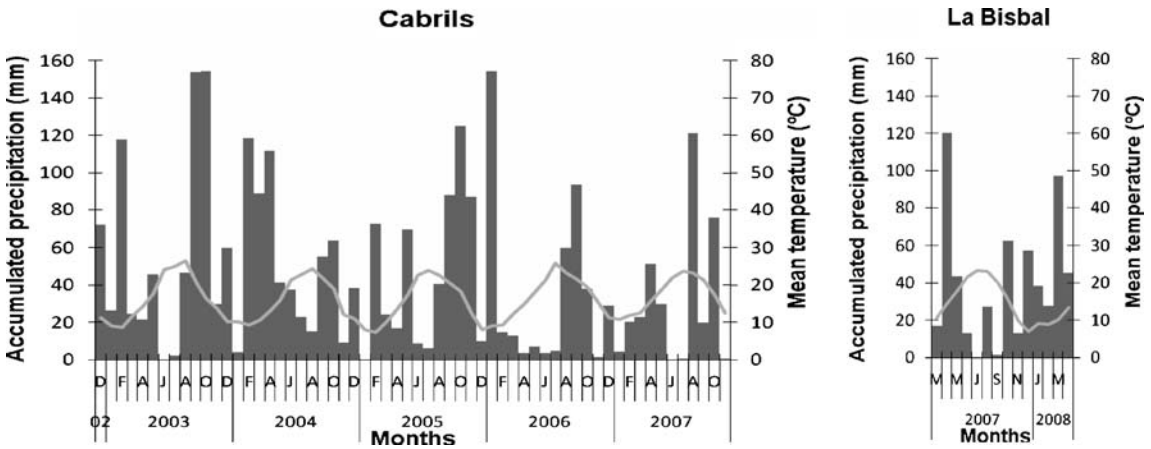

Fig. 1 Mean monthly temperature (*line*) and accumulated precipitation (*bars*) in Cabrils and La Bisbal sites during the experiments. See Table 2 for details on the date of establishment and sampling schedule for each of the experimental plantations

DNA extraction from the soil samples was performed with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) from 0.25 g of soil per sample according to the manufacturer's instructions. Real-time PCR was carried out in an ABI PRISM®7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the specific primers and TaqMan®-MGB probe for *L. deliciosus* described in Parladé et al. (2007). Real-time PCR reactions were performed with the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions with a final reaction volume of 25 µl. Working concentrations were 800 nM for the primers and 100 nM for the TaqMan® probe (Parladé et al. 2007). Five microliters of a 1:5 dilution of DNA extracted from soil samples were added as a template for each reaction. Three replicates per sample were prepared. Standard curves for mycelium quantification were generated for each type of soil using known amounts of mycelium from 1-month-old colonies of *L. deliciosus* as described in Parladé et al. (2007). Briefly, 6 mg of the target mycelium growing on a cellophane sheet on agarified biotin aneurin folic acid medium (Oort 1981) were weighted with a precision balance and then added to 0.25 g of each soil (previously checked for the absence of inoculum of *L. deliciosus* with specific primers). DNA was then extracted from the whole sample and real-time PCR of serial tenfold dilutions was performed. Ct values (cycle number at which the fluorescence emission exceeds a fixed threshold established in the exponential phase of the amplification curve) for each dilution were plotted against the logarithm of the corresponding amount of mycelium to generate the standard curves for each experiment. Quantification of mycelium biomass of the different soil samples was determined by interpolation of their Ct values in the corresponding standard curve.

Plant growth

Plant height and diameter were measured at the beginning of the experiment and at each sampling time in all the plantations (see calendar in Table 2). Plant volume (V) was considered as a growth parameter because of its close relationship with plant biomass in pines (Byrne and Wentworth 1988). Plant volume was calculated using the formula V=1/3 basal area×height ($V=1/3\pi r^2 h$).

Statistical analyses

Percentage of mycorrhizas, extraradical mycelium biomass, and plant growth (height and diameter) were analyzed by two-way fixed-factor analysis of variance (ANOVA) with inoculation treatment and time as factors. The effect of the experimental site was also analyzed by ANOVA. Differences between means were determined by Tukey's test. Linear relationships between fungal colonization parameters were evaluated by Pearson correlation coefficient analyses. Variables were transformed when necessary to fit the analysis requirements. Results were considered significant at $P<0.05$. All the statistical analyses were performed with the SAS® Enterprise Guide software v 2.0.0.417 (SAS Institute Inc., Cary, NC, USA).

Results

Effect of the initial colonization level (plot Can Badia 2 and microplot Micro 05)

The two groups of plants established in plot Can Badia 2 (initial colonization medium and high) had significantly different mycorrhizal colonization at plantation ($P<0.0001$). After outplanting, no significant differences in the percentage of mycorrhizas due to the initial colonization level ($P=0.0810$) or the sampling time ($P=0.4248$) were detected (Fig. 2). In addition, no interaction between factors was observed ($P=0.2035$). Although the two-way ANOVA showed no significant effect of the initial colonization level, separate analysis for the two sampling times revealed significant differences between the two initial colonization groups at 25 months after outplanting (Fig. 2) with a better persistence of mycorrhizas in plants with the highest initial colonization level.

Plants transplanted at Micro 05 were also visually classified into two colonization groups (medium and high) according to the mycorrhizas observed in the plug after pulling out the plants from the containers. However, further image analysis of the mycorrhizal coverage showed no significant differences between the two groups at the transplanting time (Fig. 2). Once in the field, a significant decrease of the percentage of mycorrhizas with the sampling time was detected ($P<0.0001$). No significant effect of the initial colonization level ($P=0.9247$) and no interaction between factors ($P=0.7165$) were observed (Fig. 2).

A standard curve ($y=-3.5448x+34.193$) was defined for the quantification of *L. deliciosus* extraradical mycelium in the soil of the Can Badia 2 plantation by real-time PCR. Extraradical mycelium was only detected in the rhizospheric soil attached to the root system, while no mycelium was detected in the soil samples collected at 10 cm from the plant stem. No significant differences in the quantity of *L. deliciosus* rhizospheric mycelium due to the initial colonization level of the plants ($P=0.8418$) or the sampling time ($P=0.7494$) were detected. The interaction between factors was marginally significant ($P=0.0567$) suggesting that the quantity of mycelium detected in the rhizospheric soil of plants with a high initial colonization level had a tendency

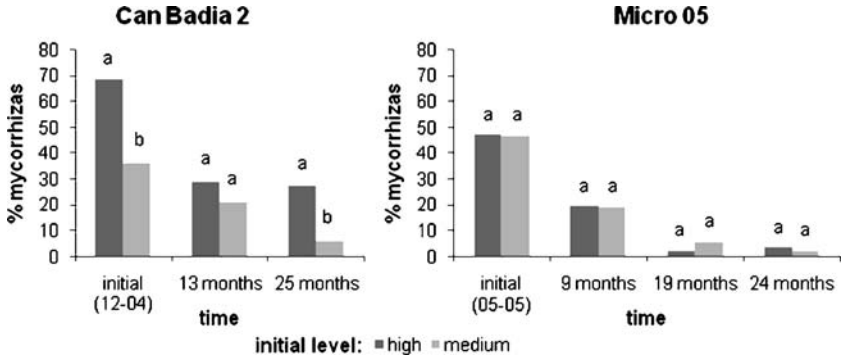

Fig. 2 Evolution of the percentage of mycorrhizas of *L. deliciosus* strain 312 in inoculated *P. pinea* plants transplanted in two experimental plantations (Can Badia 2 and Micro 05) established at the Cabrils site. Plants were classified into two groups according to the visual estimation of the level of mycorrhizas of *L. deliciosus* (high or medium) before outplanting. The initial colonization percentages of each group were established by further examination of the root system (see "Material and methods" for details). Different *letters* indicate significant differences in the percentage of mycorrhizas between plants in each colonization group (high or medium) within each sampling time. Date of establishment of the plantation (month-year) is indicated

to increase (32.2 μg mycelium per gram of soil after 13 months in the field and 87.7 μg mycelium per gram of soil after 25 months) whereas the rhizospheric mycelium in plants with a medium initial colonization level tended to decrease (125.4 μg mycelium per gram of soil after 13 months in the field and 34.0 μg mycelium per gram of soil after 25 months). A significant correlation between the quantity of rhizospheric mycelium and the percentage of mycorrhizas was detected (R=0.6368; P=0.0080). The *L. deliciosus* extraradical mycelium in Micro 05 was not quantified because of the low values of mycorrhizal colonization detected in this plot after each sampling.

Effect of the inoculation strain (plot Can Badia 1 and microplots Micro 06, 07B, and 07C)

The initial mean percentage of *L. deliciosus* mycorrhizas in the plants transplanted at Can Badia 1 was not significantly different between strains (36.9% for strain 178 and 48.8% for strain 312). Once established in the field, *L. deliciosus* mycorrhizas were not detected in any of the plants inoculated with strain 178 at 15 and 25 months after transplantation. On the other hand, the mycorrhizas of *L. deliciosus* in plants inoculated with the strain 312 persisted with a mean colonization percentage of 28.8% after 15 months in the field and of 12.2% after 25 months. No significant effect of the sampling time (P=0.5229) was detected.

The initial percentage of *L. deliciosus* mycorrhizas in plants transplanted at Micro 06 inferred by image analysis of the mycorrhizal coverage showed significant differences between strains (P<0.0001; Fig. 3). This effect was also significant after 8 months in the field (P=0.0042; Fig. 3). No mycorrhizas of any strains in this microplot were detected in the sampling done 14 months after outplanting. Approximately one third of the plants were colonized by the native ectomycorrhizal fungus *Rhizopogon roseolus* (Corda in Sturm) Th. M. Fr. at both sampling times. The percentage of *R. roseolus* mycorrhizas in the plants colonized by this fungus was significantly higher (P<0.0001) after 14 months (78.5%) than after 8 months (36.6%) in the field.

The initial percentage of *L. deliciosus* mycorrhizas (inferred by image analysis) was also significantly different (P<0.0001) between inoculated strains in plants transplanted

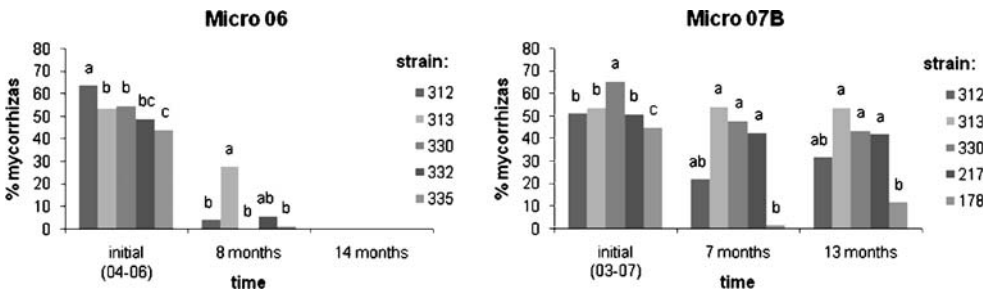

Fig. 3 Evolution of the percentage of mycorrhizas formed by different strains of *Lactarius deliciosus* in inoculated *P. pinea* plants established either at the Cabrils site (Micro 06) or at the La Bisbal site (Micro 07B). The initial colonization percentages of each strain were established by further examination of the root system (see "Material and methods" for details). Different *letters* indicate significant differences in the percentage of mycorrhizas between plants inoculated with different *L. deliciosus* strains within each sampling time. Date of establishment of the plantation (month-year) is indicated

at Micro 07B (Fig. 3). Significant differences in the percentage of *L. deliciosus* mycorrhizas among strains were also detected after outplanting ($P<0.0001$, Fig. 3) while no differences due to the sampling time were observed ($P=0.2448$). No interaction between the two factors was detected ($P=0.6856$). Initial coverage and final percentage of mycorrhizas were significantly correlated ($R=0.3767$; $P=0.0030$). Roots were also colonized by the native fungus *R. roseolus*, in a percentage significantly higher in non-inoculated plants than in plants initially inoculated with *L. deliciosus* ($P=0.0002$). Also, the *R. roseolus* colonization was higher at the second than at the first sampling time ($P=0.0007$). No interaction between factors was detected ($P=0.5879$).

No results were obtained from microplot Micro 07C due to the low survival of the transplanted plants.

Standard curves were defined for the quantification of extraradical *L. deliciosus* mycelium by real-time PCR in each of the three experiments ($y=-3.1207x+33.933$ for Can Badia 1, $y=-3.3115x+33.025$ for Micro 06 and $y=-3.3464x+35.848$ for Micro 07B). In Can Badia 1 and Micro 06 (both in the Cabrils site), *L. deliciosus* mycelium was detected in the rhizospheric soil attached to the root system but not in the soil collected at 10 cm from the plant stem. The mean quantity of extraradical mycelium detected in the rhizospheric soil of Can Badia 1 was 169.9 μg mycelium per gram of soil. In this plot, mycelium was only quantified in the second sampling time and in the soil of plants inoculated with strain 312 because mycorrhizas of strain 178 did not persist in the field. Mean quantity of *L. deliciosus* mycelium in Micro 06 was 0.29 μg mycelium per gram of soil. No differences between strains were detected ($P=0.7056$). In this case, mycelium was only quantified in the first sampling time because of the loss of *L. deliciosus* mycorrhizas in the second one.

In Micro 07B (La Bisbal site), extraradical *L. deliciosus* mycelium was effectively detected in the soil sampled at 10 cm from the plant stem. Significant differences in the quantity of mycelium between inoculated strains ($P=0.0019$) and sampling times ($P=0.0348$) were detected, while no interaction was observed ($P=0.3102$). Mean mycelium quantities for each strain at each sampling time are represented in Fig. 4. Differences between strains in the second sampling were not significant ($P=0.0549$). After 13 months in the field, the correlation between the quantity of *L. deliciosus* mycelium at 10 cm of the plant stem and the percentage of mycorrhizas was not significant ($R=0.3275$; $P=0.0829$).

Effect of the experimental site

Micro 07B (La Bisbal) and Micro 07C (Cabrils) could not be directly compared, as was intended, because of the high mortality registered in the Cabrils site. Although established in different years, the results from Micro 07B were compared with those from Micro 06 for the three *L. deliciosus* strains they had in common. Initial percentages were relatively similar and high in both experiments (Fig. 3). The results from the first sampling (7–8 months after transplantation) showed a higher percentage of *L. deliciosus* mycorrhizas in La Bisbal than in Cabrils site ($P<0.0001$; Fig. 3). Differences were also detected between inoculated strains ($P=0.0017$), with the highest percentages observed for strain 313 in both sites (Fig. 3). No interaction was observed ($P=0.0968$). Results from the second sampling time could not be compared because of the complete loss of mycorrhizas in Micro 06 (Cabrils site)

Extraradical soil mycelium of *L. deliciosus* in the soil samples collected at 10 cm from the plant stem was only detected in La Bisbal site.

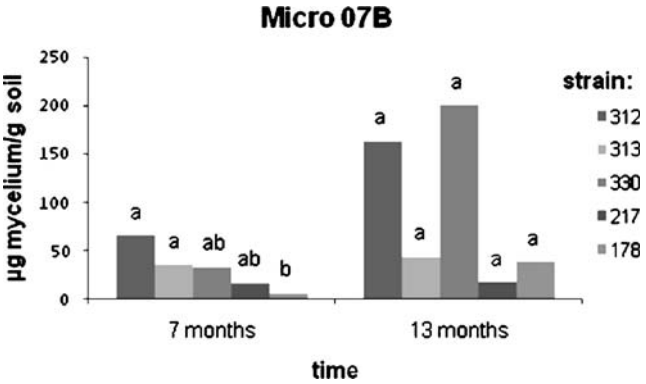

Fig. 4 Quantitative evaluation of extraradical soil mycelium of *L. deliciosus* in the Micro 07B plot (established in La Bisbal site) at 7 and 13 months after plantation. Soil was collected at 10 cm from the stem of each *P. pinea* plant (see "Material and methods" for details). Different *letters* indicate significant differences in the quantity of mycelium between plants inoculated with different *L. deliciosus* strains within each sampling time

Plant growth

No significant differences in growth parameters (either height, diameter, or volume) due to the inoculation treatment were detected in any of the experiments. Mean plant height and diameter of plants at transplantation were 30.1 cm and 4.4 mm, respectively. Mean plant growth in all the plantations established in the Cabrils site was higher than that in La Bisbal site (Fig. 5). However, plant growth within the Cabrils site varied in the different experiments.

Discussion

Controlled inoculations of *P. pinea* seedlings with *L. deliciosus* resulted in mycorrhizal plants with relatively

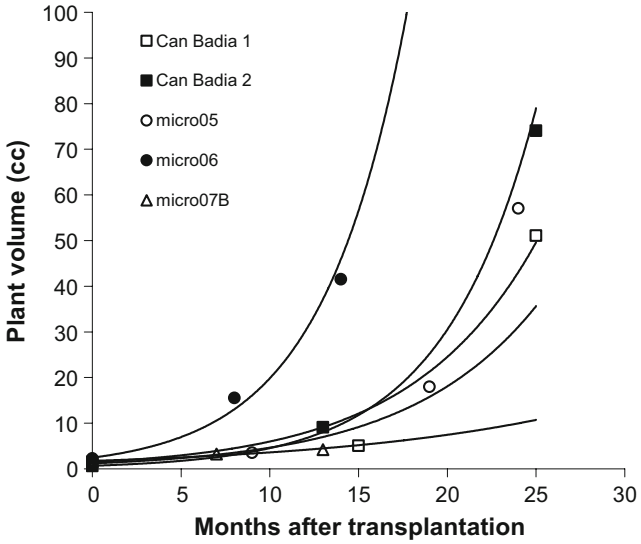

Fig. 5 Growth dynamics of *P. pinea* plants in five experimental plantations established at the Cabrils site (Can Badia 1 and 2, micro 05, and micro 06) and at the La Bisbal site (micro 07B). Plant volume is the mean of all the plants in the site at a given date (see "Material and methods" for calculation procedure). *Lines* are exponential tendence curves

high colonization levels, even for those classified as having a "medium" initial colonization level. All the inoculated plants exceeded the 33% mycorrhizal colonization, which has been suggested as the acceptable minimum by some authors (Wang and Hall 2004). The results from the two experimental plots Can Badia 2 and Micro 05 established in the Cabrils site show a general decrease of fungal colonization with time but suggest a higher persistence of ectomycorrhizas in plants having an initial colonization percentage over 50%. This "threshold" percentage seems closer to that proposed by Marx et al. (1991) for *Pisolithus tinctorius*. However, the low persistence of *L. deliciosus* in the Cabrils soil makes it difficult to generalize the robustness of this effect for a period longer than 25 months. Moreover, the lack of mycelium extension from the rhizospheric area in this soil will probably prevent the secondary colonization of roots.

The rapid decrease in the colonization levels observed in microplot Micro 06 established at Cabrils seems to contradict the aforementioned results since the same *L. deliciosus* strain showed a colonization level well over 50% at the time of transplant. Although we are not sure of the mechanism, the results may have been caused by the faster and higher growth of the plants in this microplot. Faster plant growth would favor the colonization of the root system by native fungi and the eventual displacement of the inoculated *L. deliciosus.* According to McAfee and Fortin (1985), native mycobionts colonize short roots in the region of new root growth whereas inoculated symbionts tend to extend from the already-existing mycorrhizas. This observation is supported by the sudden loss of mycorrhizas in the experiment Micro 06 established at the Cabrils site and the fact that, in other plantations established in the same site with lower plant growth, the mycorrhizas of *L. deliciosus* persisted for a longer period of time.

The better persistence of the strain 312 established at the La Bisbal site, as well as the detection of extraradical mycelium beyond the rhizospheric area closely attached to the roots at La Bisbal only, indicates that the site factors strongly influenced the persistence of the inoculated fungus under the experimental conditions tested. Perrin et al. (1996) found that physical and chemical properties of the soil, as well as the native mycobionts, determine the receptivity of a given soil to ectomycorrhizal colonization. In our study, a positive correlation between initial and final colonization level was observed in the microplot established at the La Bisbal site suggesting that a higher initial colonization level could be related to a higher persistence of the fungus in a soil favorable to *L. deliciosus.* On the other hand, in a non-favorable soil like that at the Cabrils site, the fungus would tend to disappear sooner or later regardless of the initial colonization level.

The main differences between the two soils tested in this study are the texture (clay loam at La Bisbal and sandy loam at Cabrils) and their P content (higher at Cabrils than at La Bisbal). These differences probably contributed to the higher plant growth at Cabrils than at La Bisbal and affected fungal secondary colonization as suggested above. Differences in growth among plantations within the Cabrils site could not have been caused by physical–chemical differences or by climatic variation (the plantation Micro 06 with exceptionally high growth was established in the driest and hottest spring along the experimental period considered in this study). The higher growth of plants established in the Micro 06 plantation could be due to a high heterogeneity in soil depth at the Cabrils site (probably because of the patchy agricultural history of the site) and the corresponding increase in root development in the areas with deeper soils. In fact, entire plants from Micro 06 plantation were very difficult to uproot after 14 months in the field due to the deep and thick root development. At both sites, the high percentages of mycorrhizas of the native ectomycorrhizal fungus *R. roseolus* were related with low or zero percentages of *L. deliciosus* in the same root, in concordance with the results obtained in a previous experiment analyzing the relationships between these two fungal species (Hortal et al. 2008). Given that the competitive pressure (mainly by *R. roseolus*) is similar in both sites as recently observed in Hortal et al. (2008) under the same conditions, the differences in *L. deliciosus* receptivity could be explained either by some soil properties unfavorable to *L. deliciosus* development at the Cabrils site, by a higher growth of the root system in

this sandy and more fertilized soil, or by a combination of the two factors.

Differences in the field persistence of mycorrhizas between strains were detected in the three established plots in both sites. The mycorrhizas formed by the strain 178 at the La Bisbal site decreased significantly with time, whereas the other strains maintained their colonization levels close to the initial ones after 13 months in the field. In addition, the same strain completely disappeared at the Cabrils site. A general increase in the amount of extraradical mycelium with time was detected at the La Bisbal site. However, the differences between strains in the second sampling time were only marginally significant (P=0.055), probably because of the high variability observed. Possible DNA amplification from spores was discarded in both soils because no *L. deliciosus* DNA was detected in the samples corresponding to control non-inoculated plants. These results support the importance of taking into account some factors, such as the strain colonization ability, which may be related to the persistence of the fungus in the field in controlled inoculations (Erland and Taylor 2002; Perrin et al. 1996; Rincón et al. 1999; Trappe 1977).

The correlation between the quantity of extraradical mycelium and the percentage of mycorrhizas at the La Bisbal site was not significant. Extraradical mycelium and mycorrhizas could be providing different information on fungal persistence, as suggested by Horton and Bruns (2001), Koide et al. (2005b), KjØller (2006), and Peintner et al. (2007). KjØller (2006) found no correspondence between *Lactarius* spp. mycorrhizal tips and mycelium abundance whereas Landeweert et al. (2005) found a high correspondence between the two analyses. Koide et al. (2005b) suggested the combination of the two approaches when characterizing the dynamics of mycorrhizal communities. The absence of correlation could also be due to the high variability of the data obtained in our soil sampling procedure. Extraradical mycelium has a patchy distribution in the soil as a response to the physical and chemical heterogeneity (Cairney 2005). A positive correlation between the two fungal parameters was observed when quantifying the rhizospheric mycelium (a more homogeneous material directly attached to the root system) in Can Badia 2. Further research is needed to develop an appropriate method of soil sampling for the accurate quantification of extraradical mycelium.

The differences observed in the spread of *L. deliciosus* extraradical mycelium in the two soils tested could be related to the growth of the host plant, as pointed out above for mycorrhizas, or to physical–chemical characteristics of the soil. Korkama et al. (2007) showed that the structure of the ectomycorrhizal mycelial community was related to the tree vigor in *Picea abies*, with fast-growing clones supporting fungal species with high mycelial production. A limited mycelium extension could be expected for the genus *Lactarius*, with a contact exploration type (Agerer 2001). However, other molecular studies have shown that mycelium of *Russula* sp. and *Lactarius* sp. (also with contact exploration types) can be found out away from rhizospheric soil (Chen and Cairney 2002; Dickie et al. 2002; Genney et al. 2006; Hortal et al. 2008; Koide et al. 2005a; Landeweert et al. 2003a, 2005; Smit et al. 2003).

In conclusion, the results obtained show that the field site plays a decisive role in the persistence of *L. deliciosus* after outplanting. The initial colonization level and the selection of the suitable strain are also significant factors but their effect on the persistence and spread of mycorrhizas and extraradical mycelium of *L. deliciosus* may be secondary to the characteristics of the plantation site and how they influence root growth. Molecular techniques based on real-time PCR allowed non-destructive quantification of extraradical mycelium of *L. deliciosus* in the field. These techniques are promising for non-destructive assessment of fungal persistence since soil mycelium may be a good indicator of root colonization. However, the accuracy of the technique will ultimately depend on the development of the appropriate sampling methods.

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