Mycorrhiza (2008) 18:69–77
DOI 10.1007/s00572-007-0160-3

ORIGINAL PAPER

Tracking mycorrhizas and extraradical mycelium of the edible fungus *Lactarius deliciosus* under field competition with *Rhizopogon* spp

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Received: 2 October 2007 / Accepted: 20 December 2007 / Published online: 10 January 2008

Abstract The objective of this study is to evaluate the field persistence of the edible ectomycorrhizal fungus *Lactarius deliciosus* in competition with two ubiquitous soil fungi. Couples of plants inoculated with either *L. deliciosus*, *Rhizopogon roseolus*, or *R. luteolus* were transplanted, 10 cm apart, in two different sites at the following combinations: *L. deliciosus*–*R. roseolus*, *L. deliciosus*–*R. luteolus*, *L. deliciosus*–control (non-inoculated), control–*R. roseolus*, control–*R. luteolus*, and control–control. Eight months after transplantation, root colonization and extraradical soil mycelium for each fungal species were quantified. For mycelium quantification, soil cores equidistant to the two plants in each couple were taken, and total deoxyribonucleic acid (DNA) was extracted. Real-time polymerase chain reaction analysis was performed using specific primers and TaqMan® Minor groove binding (MGB) probes designed in the ribosomal DNA internal transcribed spacer region of each fungal species. Field site significantly influenced persistence of both mycorrhizas and extraradical mycelium of *L. deliciosus*. Extraradical mycelium quantity was positively correlated with the final percentage of ectomycorrhizas for the three fungal species. Different competitive pressure between the two *Rhizopogon* species on *L. deliciosus* persistence was observed, with *R. luteolus* having no effect on *L. deliciosus* survival. Negative correlation between the final percentage of mycorrhizas of *L. deliciosus* and *R. roseolus* was observed. However, no relationship was determined between extraradical mycelia of both fungal species. The results obtained suggest that competition between *L. deliciosus* and *R. roseolus* takes place in the root system, for ectomycorrhiza formation in available roots, rather than in the extraradical phase.

Keywords Real-time PCR · Fungal persistence · Interspecific competition · Extraradical mycelium · *Lactarius deliciosus*

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Introduction

Wild edible fungi are among the most valuable nonwood forest products with high potential for expansion of trade (Arnolds 1995; Boa 2004). Some of the highest appreciated mushrooms are produced by ectomycorrhizal fungi, which form symbiotic associations with roots. This relationship favors water and nutrient uptake in plants and plays an important ecological role in forest ecosystems (Smith and Read 1997; Read and Perez-Moreno 2003). Because symbiotic fungi depend on the host tree for fruiting, cultivation becomes difficult, and harvesting is the most usual way of obtaining this resource (Wang and Hall 2004). On the other hand, it has been demonstrated that harvests of some of the key edible ectomycorrhizal mushrooms have consistently declined over the past century (Arnolds 1995; Hall et al. 2003; Wang and Hall 2004). Habitat degradation, overexploitation of fungal resources, and particular forestry practices are possible causes of this decline (Hall et al. 2003; Molina et al. 2001; Pilz and Molina 2002; Egli et al. 2006; Pilz et al. 2006).

In spite of the difficulties encountered, cultivation of some edible ectomycorrhizal fungi, by inoculating different host plants under controlled conditions, has become an interesting alternative to satisfy the increasing demand and

to promote the conservation of natural fungal populations and ecosystems (Wang and Hall 2004). Special attention has been given to the methods for producing seedlings inoculated with *Tuber* spp. because of the high economic value of their fruit bodies. In addition, the production of plants mycorrhizal with other edible species such as *Lactarius* sp., *Boletus* sp., *Cantharellus* sp., and *Tricholoma* sp. has been attempted with a different degree of success (reviewed in Hall et al. 2003). Given the delay in obtaining the fruit bodies, the plantations with nursery-inoculated seedlings aimed at fungal production should be periodically monitored for the vegetative persistence of the introduced fungus in the field, either in the form of mycorrhizas or extraradical mycelium. Survival and expansion of the inoculated fungus in an experimental plantation can be affected by both abiotic (reviewed in Erland and Taylor 2002) and biotic factors (Hall et al. 2003). Among the last, interactions with native ectomycorrhizal fungi present in the soil may play a key role, as the potential for encounters with mycelia of different taxa is high (Cairney 2005). Root colonization of nursery-inoculated seedlings by native ectomycorrhizal fungi after outplanting has been reported in several studies (Bledsoe et al. 1982; McAfee and Fortin 1985; Villeneuve et al. 1991; Meotto et al. 1999). However, competition studies are scarce in the literature. According to Wu et al. (1999), this fact is probably due to the lack of direct and suitable identification methods. Recent application of molecular techniques has allowed studies suggesting that competition may be an important factor in determining ectomycorrhizal assemblages (Wu et al. 1999; Landeweert et al. 2003b; Lilleskov and Bruns 2003; Mahmood 2003; Kennedy and Bruns 2005; Koide et al. 2005a; Kennedy et al. 2007a, b).

Among the increasing number of edible ectomycorrhizal fungi that can be cultivated, *Lactarius deliciosus* (L.) Gray is a highly appreciated species in local European markets, and its trade has become an important business in many countries (Singer 1986; Boa 2004). Experimental conditions to obtain pines mycorrhizal with *L. deliciosus* have been optimized by Guerin-Laguette et al. (2000) and Parladé et al. (2004). Molecular techniques have been developed for specific and intraspecific identification of mycorrhizas and extraradical mycelium of *L. deliciosus* (Hortal et al. 2006) as well as for mycelium quantification (Parladé et al. 2007).

The establishment of experimental plantations with trees inoculated with *L. deliciosus* is a promising alternative for a sustainable exploitation of abandoned agricultural land. In these disturbed sites, *Rhizopogon* species are commonly found as indigenous fungi (McAfee and Fortin 1985) and may play an important competition role because of its soil colonization ability (Agerer 2001; Leake et al. 2004). In a previous study, we analyzed the relationship between *L. deliciosus* and *R. roseolus* (Corda) Th. Fr. in an interspecific competition experiment established under greenhouse conditions (Parladé et al. 2007). Optimization of real-time polymerase chain reaction (PCR) allowed us to specifically detect and quantify *L. deliciosus* extraradical mycelium in two soils. The objective of the present study is to determine the field persistence of different strains of *L. deliciosus* under competition with two *Rhizopogon* species in two different experimental plantations. The evolution of mycorrhizal colonization and extraradical mycelium has been quantitatively evaluated for all the fungal species in both field sites applying both morphological and molecular techniques. The results are discussed as to their value in assessing fungal persistence in experimental field plantations aimed at producing edible fruit bodies of *L. deliciosus*.

Materials and methods

Seedling inoculations

Three *L. deliciosus* strains 178, 312, and 330 (IRTA ECM Culture Collection) were preselected for their in vitro colonization ability in pure culture synthesis. Pure cultures were isolated from sporocarps collected in October 1994 in Cabrils (Barcelona) under *Pinus pinea* L. for strain 178, in October 2000 in Santa Coloma de Farners (Girona) under *Pinus pinaster* Ait. for strain 312, and in November 2003 in Pierola (Barcelona) under *Pinus halepensis* Mill. for 330. Vegetative inocula of the three strains were prepared 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 min) 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 inoculated substrate. Two *P. pinea* L. seeds (lot 1581 origin ES06 coastal Catalonia, Ministerio de Medio Ambiente D.G. Biodiversidad, collection year 1994–1995) were placed in each cell in May 2005 and thinned to one per container after germination.

Spore inoculum of *Rhizopogon roseolus* and *R. luteolus* Fr. were obtained from sporocarps collected in October 2004 in Tortosa (Tarragona, Spain) under *P. sylvestris* L. and in Massanes (Girona, Spain) under *P. pinaster*, respectively. Water suspensions of spores from air-dried sporocarps of each fungal species were prepared in May 2005 as described in Parladé et al. (1996) and inoculated at the rate of 10^7 spores per seedling to 1-month-old *P. pinea* seedlings produced as described above. Control, noninoculated seedlings were also produced.

Both inoculated and noninoculated seedlings 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 seedling 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 colonization level of 15 plants inoculated with each of the three strains of *L. deliciosus* was individually determined in March 2006 by the nondestructive system proposed in Parladé et al. (2007) with some modifications. Briefly, the method consisted of pulling out the plant and taking a digital picture of the apparently most colonized side of the plug. The obtained image was analyzed with the software Photoshop Elements v. 2.0, using the magic wand tool with the color code F29F46 (corresponding to a mature *L. deliciosus* mycorrhiza) and tolerance set at 70. The number of pixels corresponding to the mycorrhizas divided by the total number of pixels of the analyzed area was defined as the mycorrhizal coverage. We just analyzed the apparently most colonized side of the plug because we observed a strong significant correlation between values of mycorrhizal coverage obtained from the analysis of this side and those obtained by calculating the mean coverage of the four sides of the plug (data not shown). To relate the obtained values of mycorrhizal coverage with the real percentage of mycorrhizas, a correlation analysis was performed between the mycorrhizal coverage of 40 *L. deliciosus*-inoculated plants and the percentage of root colonization measured by effectively counting mycorrhizal root tips after washing the root system of the same plants.

The mean percentage of mycorrhizal short roots in *Rhizopogon* spp.-inoculated seedlings was determined in March 2006 by counting at least 200 tips of a sample of six seedlings from each inoculated lot.

Experimental layout

The experiment was repeated in two different sites with a Mediterranean climate: La Bisbal (Girona, Spain) and Cabrils (Barcelona, Spain). Soil from the La Bisbal location has a clayey loam texture, pH 7.7, electrical conductivity (EC) 0.1 dS/m, organic matter (OM) 1.45%; N (nitric) 5 ppm, P Olsen 8 ppm, K 72 ppm, Mg 137 ppm, Ca 1083 ppm, Na 37 ppm, and cation interchange capacity (CIC) 6.9 meq/100 g. 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 2107 ppm, Na 43 ppm, and CIC 6.2 meq/100 g. Before plantation establishment, superficial tilling was performed in each site.

In March 2006, couples of 1-year-old *P. pinea* plants from different inoculation treatments were transplanted, 10 cm apart, at the following combinations: *L. deliciosus*–*R. roseolus*, *L. deliciosus*–*R. luteolus*, *L. deliciosus*–control (noninoculated), control–*R. roseolus*, control–*R. luteolus*, and control–control with five replicates each. Combinations included the plants inoculated with the three *L. deliciosus* strains (previously characterized for mycorrhizal colonization as described above) giving a total of 120 transplanted plants (60 couples) in each site. The transplanted plants (couples) were randomly distributed in each site in a plantation framework of 1 × 1 m and maintained for 8 months from March to November 2006. From now on, when we refer to the "accompanying plant" (mycorrhizal with *R. roseolus*, *R. luteolus*, or control), we mean the one in front of the *L. deliciosus*-inoculated plant in each couple.

No maintenance treatments were applied after plantation. The accumulated precipitation and mean temperature during the growing period were, respectively, 225 mm and 19.2°C in Cabrils and 378 mm and 19°C in La Bisbal.

Quantification of fungal colonization in the field

At the end of the experiment, root colonization of the 120 plants in each field site was determined by direct short-root counting. Extraradical soil mycelium for each fungal species was quantified by real-time PCR. Soil samples were obtained from three soil cylinders taken in the mid-area equidistant to the two plants of each couple using a 3-cm-diameter soil auger. Soil from 0 to 10-cm depth of the three cylinders of each couple was mixed and sieved through a 2-mm mesh. A subsample of this homogenized soil was taken and stored at −20°C until processing.

Deoxyribonucleic acid (DNA) extraction from the soil samples was performed with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) from 0.25 g soil per sample according to the manufacturer's instructions. Special care was taken to remove any root fragment from the sample with the aid of a stereomicroscope.

Real-time PCR from soil samples was carried out in an ABI PRISM®7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using specific primers and probes for each fungal species. Specific primers and TaqMan® MGB probe for *L. deliciosus* were the same as described in Parladé et al. (2007). Specific primers and TaqMan®-MGB probes for *R. roseolus* and *R. luteolus* were designed in the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region of each fungal species with the Primer Express 2.0 software (Applied Biosystems). Primers and probe for *R. roseolus* were FWD-Rro (TCGACTTTGCGCGACAAG), RVS-Rro (CATGCGCTTCAGCAAACG), and STQ-Rro (FAM-ATCATTATCACGCCGAAAG-MGB). Primers and probe

for *R. luteolus* were FWD-Rlu (TCTAGGATTCGA GCTCTCCTGAA), RVS-Rlu (CCTTATCGCACAA AGTCGAAAGT), and STQ-Rlu (NED-CCCGCAAGCCA ATGT-MGB). A search for highly similar sequences (megablast) was performed in the GenBank database to test for specificity of the designed oligonucleotides.

Real-time PCR reactions were performed with the TaqMan® Universal Master Mix (Applied Biosystems) according to the manufacturer's instructions for a final reaction volume of 25 μl. Working concentrations were 800 nM for primers and 100 nM for probes of *L. deliciosus* (already optimized in Parladé et al. 2007) and 200 nM for probes of both *Rhizopogon* species. Five microliters of a 1:5 dilution of DNA extracted from soil samples were added as a template. Three replicates per sample were prepared. Reactions were run in simplex (i.e., with primers and probe of one species each time) because we found problems with the detection of *L. deliciosus* in multiplex reactions (i.e., with primers and probes for different species in the same reaction tube). Although duplex reactions for *L. deliciosus* and *R. roseolus* were optimized to quantify both fungal species at the same time, it was observed that in situations in which the proportion of *R. roseolus* was 100-fold greater than *L. deliciosus*, the latter species was detected in simplex but not in duplex. Considering that *R. roseolus* is a common fungus in the field and has a long-distance exploration strategy while *L. deliciosus* has a contact exploration strategy (Agerer 2001), reactions were all run in simplex to avoid false negatives for *L. deliciosus*.

Standard curves for mycelium quantification were generated for each fungal species in each type of soil using known amounts of mycelium from active-growing colonies as described in Parladé et al. (2007) for *L. deliciosus*. Briefly, 6mg of the target mycelium growing on a cellophane sheet on agarified biotin-aneurin-folic acid (BAF) medium (Oort 1981) were added to 0.25 g of each soil (previously checked for the absence of inoculum of the same species), 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 standard curves. Quantification of mycelium biomass of the target soil samples was determined by interpolation of the Ct values of each sample in the corresponding standard curve.

Plant growth

Plant height and diameter of *L. deliciosus*-inoculated and control (noninoculated) plants were measured at the beginning and at the end of the experiment.

Statistical analyses

Percentages of mycorrhizas and growth (height and diameter) of *L. deliciosus*-inoculated plants and extraradical mycelium biomass of each fungal species were analyzed by three-way fixed factor analysis of variance with site (La Bisbal or Cabrils), competition (*R. roseolus*, *R. luteolus*, or control), and *L. deliciosus* strain (178, 312, or 330) as factors. Initial mycorrhizal coverage and initial height and diameter were included as covariates in *L. deliciosus* colonization and growth analyses, respectively. 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. All the statistical analyses were performed with the SAS® Enterprise Guide software v 2.0.0.417 (SAS Institute, Cary, NC).

Results

Mycorrhizal colonization

Linear correlation between the percentage of mycorrhizas (determined by short-root counting) and mycorrhizal coverage (calculated by image analysis) of *L. deliciosus*-inoculated plants was significant ($y = 14.40x + 39.52$, $R = 0.70$, $P < 0.0001$). According to the obtained equation, the inferred initial mycorrhizal colonization of the transplanted plants was 62.8 ± 2.1% (mean ± standard error) for strain 178, 72.0 ± 3.4% for strain 312, and 74.6 ± 3.0% for strain 330. Root colonization percentage for transplanted plants inoculated with *R. roseolus* and *R. luteolus* was 16.2 ± 2.0 and 63.5 ± 10.1%, respectively.

Eight months after field transplantation, the mean percentage of *L. deliciosus* mycorrhizas in *L. deliciosus*-inoculated plants was significantly higher in the La Bisbal than in the Cabrils site (Tables 1 and 2). No significant effects of the initial coverage, inoculated strain, or competition with *R. roseolus* or *R. luteolus* were detected. Cross-colonization of the accompanying plant by *L. deliciosus* occurred only once in the Cabrils site and in 20 out of 44 couples of plants in the La Bisbal site. The mean percentage of *L. deliciosus* mycorrhizas in the accompanying plants established in the La Bisbal site was significantly higher in the control, noninoculated than in *R. luteolus*-inoculated plants (12.1 ± 3.7 and 0.8 ± 0.5, respectively).

Approximately 60% of the plants were colonized by *R. roseolus* at the end of the experiment, regardless of the inoculation treatment, because of the presence of natural inoculum in both sites. Confirmation of the identity of the

Table 1 Multifactor ANOVA table for mycorrhizal colonization of *L. deliciosus*-inoculated plants, 8 months after transplantation

Factor (source)	Percentage of mycorrhizas		
	L. deliciosus	*R. roseolus*	*R. luteolus*
P value			
Site	*0.0007*	*0.0073*	*0.0008*
Competition	0.6615	0.2664	Nonevaluated
Strain	0.1124	0.2934	0.3194
Coverage	0.1699	–	–
Degrees of freedom			
Model	35	17	5
Error	51	69	23
Corrected total	86	86	28

P values in italics are significant (<0.05). Coverage was included in the model as a covariate in the *L. deliciosus* colonization analysis. No significant interactions were detected. Data were either angular (for percentage of mycorrhizas) or $\log^{+1}$ (for *L. deliciosus* coverage) transformed for the analysis.

native fungal contaminant was established by blasting the sequence of the rDNA ITS region in the GenBank database. The percentage of *R. roseolus* mycorrhizas in the *L. deliciosus*-inoculated plants was higher in the Cabrils than in the La Bisbal site (Tables 1 and 2), whereas no differences between sites were detected in control plants (mean = 17.9 ± 4.6%). The percentage of *R. roseolus* mycorrhizas in the accompanying plants was not significantly different between sites (mean = 18.5 ± 2.2%).

No *R. luteolus* mycorrhizas were detected in control plants established in any of the two field sites. The mean percentage of *R. luteolus* mycorrhizas detected in *L. deliciosus*-inoculated plants was significantly higher in the Cabrils than in the La Bisbal site (Tables 1 and 2). The percentage of *R. luteolus* mycorrhizas in the accompanying plants inoculated with this fungal species was not significantly different between sites (mean = 30.4 ± 4.0%).

Table 2 Mean percentage of mycorrhizas of *L. deliciosus*-inoculated plants 8 months after transplantation for those factors showing a significant effect by ANOVA analysis (see Table 1)

Fungal species	Site	Percent mycorrhizas	
L. deliciosus	La Bisbal	37.11	a
	Cabrils	8.27	b
R. roseolus	La Bisbal	5.73	b
	Cabrils	20.15	a
R. luteolus	La Bisbal	1.78	b
	Cabrils	32.23	a

Different letters within fungal species show significant differences between means by Tukey's test ($P < 0.05$).

Quantification of extraradical soil mycelium by real-time PCR

Standard curves for mycelium quantification of each fungal species in both soils were defined. Extraradical soil mycelium of *L. deliciosus* was successfully detected in half of the La Bisbal soil samples, while it was only detected in 4 of the 43 analyzed soil samples from the Cabrils site. Consequently, the factor "site" was excluded from the model, and the analysis of *L. deliciosus* extraradical mycelium was performed only with data from the La Bisbal site. Figure 1 shows the standard curves for the three fungal species in La Bisbal soil. Mean *L. deliciosus* mycelium biomass in the La Bisbal site was 3.3 ± 1.5 μg mycelium/g soil. No significant effect of the inoculated strain or competition with *R. roseolus* or *R. luteolus* was detected. No *L. deliciosus* mycelium was detected in control samples.

Mean extraradical mycelium biomass of *Rhizopogon* spp. in the La Bisbal site was 42.0 ± 20.3 μg mycelium/g soil for *R. roseolus* and 32.3 ± 17.4 μg mycelium/g soil for *R. luteolus*. The biomass of extraradical *Rhizopogon* spp. mycelium was not significantly affected by any of the analyzed factors.

Linear relationships between fungal colonization parameters

Eight months after transplantation, fungal colonization parameters were analyzed for correlation. Extraradical *L. deliciosus* mycelium biomass was positively correlated with the mycorrhizal percentage of *L. deliciosus* in the *L. deliciosus*-inoculated plants in the La Bisbal site (Table 3). The colonization percentages of *R. roseolus* in the *L. deliciosus*-inoculated plants were positively correlated with those of the accompanying plants in both sites and with the extraradical mycelium of *R. roseolus* in the La Bisbal site (Table 3). The presence of *R. luteolus* mycorrhizas in the accompanying plants inoculated with this fungal species was correlated with the extraradical mycelium of *R. luteolus* in the La Bisbal site and with the *R. luteolus* mycorrhizas in *L. deliciosus*-inoculated plants in the Cabrils site (Table 3).

Percentages of *L. deliciosus* and *R. roseolus* mycorrhizas in *L. deliciosus*-inoculated plants were negatively correlated to each other in both field sites (Table 4). Furthermore, the mycorrhizal colonization of *L. deliciosus* in *L. deliciosus*-inoculated plants was negatively correlated with the mycorrhizas of *R. roseolus* in the accompanying plants in the Cabrils site (Table 4). Extraradical *L. deliciosus* mycelium biomass was negatively correlated with the percentage of mycorrhizas of *R. roseolus* in the *L. deliciosus*-inoculated plants in the La Bisbal site (Table 4). No significant

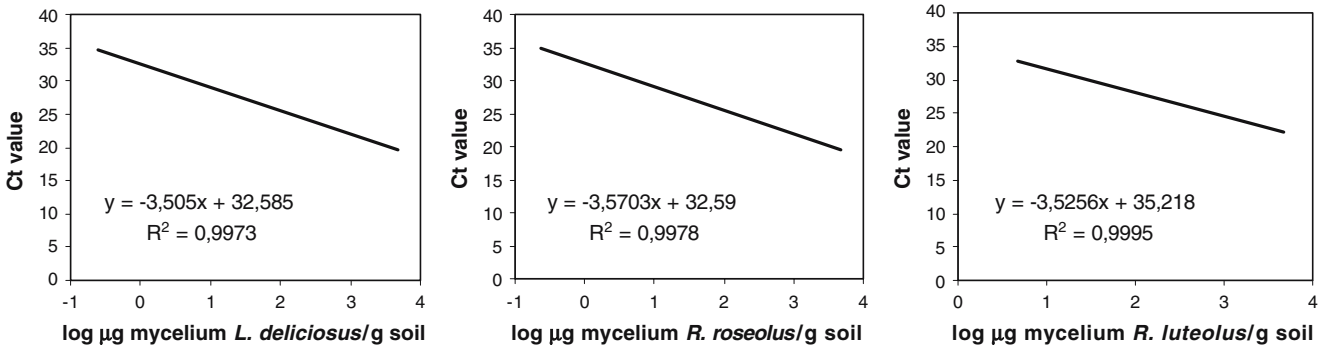

Fig. 1 Standard curves obtained for mycelium quantification of each fungal species in La Bisbal soil by real-time PCR. Curves were generated by plotting the obtained Ct values against the logarithm of known amounts of the target mycelium added to La Bisbal soil. The slope of the standard curves indicates that the mean efficiency of the real-time PCR was 92% ($E=(10^{-1/\text{slope}}-1)\times 100$)

relationships between fungal parameters of *L. deliciosus* and *R. luteolus* were detected.

Plant growth

At transplantation, *L. deliciosus*-inoculated plants averaged 35.1 ± 0.5 cm in height and 4.5 ± 0.04 mm in diameter. Eight months after transplantation, the average height of *L. deliciosus*-inoculated plants was 38.7 ± 0.5 cm, and the average diameter was 8.2 ± 0.3 mm. The final diameter was significantly higher in plants transplanted in the Cabrils (10.8 ± 0.2 mm) than in the La Bisbal site (5.7 ± 0.06 mm), while no significant differences because of competition, *L. deliciosus* strain, or initial diameter were detected. Final height was only significantly affected by initial height (covariable). No significant differences in height or diameter were detected between *L. deliciosus*-inoculated plants and control plants (noninoculated).

Discussion

The initial percentages of *L. deliciosus* mycorrhizas inferred by image analysis were always greater than 60%, a higher value than the 33% suggested as acceptable for transplantation by some authors (Wang and Hall 2004). Under these experimental conditions, the persistence of mycorrhizas and expansion of extraradical mycelium of *L. deliciosus* were significantly different between the two field sites studied. The final percentage of *L. deliciosus* mycorrhizas was higher in the La Bisbal than in the Cabrils site despite the higher initial colonization in the Cabrils site. The percentage of mycorrhizas of *Rhizopogon* spp. in the *L. deliciosus*-inoculated plants was higher in the Cabrils than in the La Bisbal site, but differences were not significant in control plants. These results suggest that the greater the uncolonized roots remaining on *L. deliciosus*-inoculated plants after the outplanting period, the higher the colonization by *Rhizopogon*

Table 3 Significant Pearson correlation coefficients ($P<0.05$) between fungal colonization parameters for each species in both sites 8 months after transplantation (plant 1: *L. deliciosus*-inoculated plant; plant 2: accompanying plant)

		La Bisbal site		Cabrils site
		Percent mycorrhizas plant 2	Mycelium quantity	Percent mycorrhizas plant 2
		L. deliciosus		*L. deliciosus*
Percent mycorrhizas plant 1	*L. deliciosus*	n.s.	*+0.43*	–
Percent mycorrhizas plant 2		–	n.s.	–
		R. roseolus		*R. roseolus*
Percent mycorrhizas plant 1	*R. roseolus*	*+0.42*	*+0.56*	*+0.82*
Percent mycorrhizas plant 2		–	*+0.75*	–
		R. luteolus		*R. luteolus*
Percent mycorrhizas plant 1	*R. luteolus*	n.s.	n.s.	*+0.74*
Percent mycorrhizas plant 2		–	*+0.77*	–

Data were either angular (% mycorrhizas) or log+1 (mycelium biomass) transformed for the analysis. $N=44$ (La Bisbal) and 43 (Cabrils) for *L. deliciosus* and *R. roseolus*; $N=14$ (La Bisbal) and 15 (Cabrils) for *R. luteolus*.
n.s. Nonsignificant, − nonevaluated

Table 4 Significant Pearson correlation coefficients ($P<0.05$) between fungal colonization parameters of *L. deliciosus* and *Rhizopogon* spp. in La Bisbal and Cabrils sites 8 months after transplantation (plant 1: *L. deliciosus*-inoculated plant; plant 2: accompanying plant)

		La Bisbal site			Cabrils site
		Percent mycorrhizas plant 1 *L. deliciosus*	Percent mycorrhizas plant 2	Mycelium	Percent mycorrhizas plant 1 *L. deliciosus*
Percent mycorrhizas plant 1	*R. roseolus*	*−0.36*	n.s.	*−0.34*	*−0.46*
Percent mycorrhizas plant 2		n.s.	n.s.	n.s.	*−0.35*
Mycelium quantity		n.s.	n.s.	n.s.	–

Correlations between *L. deliciosus* and *R. luteolus* were not significant and are not shown. Data were either angular (% mycorrhizas) or log+1 (mycelium biomass) transformed for the analysis. $N=44$ (La Bisbal) and $N=43$ (Cabrils). Abbreviations as in Table 3

spp., irrespective of the site, *L. deliciosus* strain, or competition treatments.

Real-time PCR after DNA extraction from soil samples has allowed specific detection and quantification of *L. deliciosus*, *R. roseolus*, and *R. luteolus* mycelia in both sites. Sensitivity level was similar for all the standard curves obtaining a Ct value of around 34 in the last dilution for the three fungal species in both soils (detection limit at 0.48 μg mycelium/g soil). In our case study, the soil DNA extraction efficiency for a given species was similar for the two analyzed soils, so standard curves were also similar between sites.

Whereas *Rhizopogon* spp. mycelium was detected in both sites, extraradical *L. deliciosus* mycelium was only detected in La Bisbal soil, and cross-colonization of the accompanying plant was only observed in this site. These field results agree with those obtained by Parladé et al. (2007) in pots under greenhouse conditions with the same two types of soil and suggest that the same fungal species may vary their extraradical mycelium pattern and persistence depending on the soil characteristics or conditions. Genney et al. (2006) also pointed out that particular site conditions are important in determining the distributions of mycorrhizas and extraradical mycelium of ectomycorrhizal fungi in the soil. Cross-colonization of the accompanying plant by *L. deliciosus* in the La Bisbal site was higher in control plants than in those inoculated with *R. luteolus* probably because the latter has less available roots. No differences in *L. deliciosus* mycorrhizas or mycelium extension were detected between the three evaluated strains in the La Bisbal site, probably because of the similar mean initial colonization level.

Correlation results suggest no extraradical interaction among mycelium of the three fungal species but, again, competition for available roots. Although prior establishment of a given fungal species on a root system probably increases competitiveness with regard to native fungi (Selosse et al. 1998), *L. deliciosus* colonization parameters were negatively correlated with the percentage of *R. roseolus* mycorrhizas in both sites. This result suggests that negative interaction between these two fungal species occurs when they compete for available roots. No significant relationships between *L. deliciosus* and *R. luteolus* were detected in any field site. Because final percentages of mycorrhizas in the accompanying plants were similar between both *Rhizopogon* species in the two field sites, the results suggest a different competitive pressure by the two *Rhizopogon* species on *L. deliciosus* persistence. However, the presence of natural inoculum of *R. roseolus* in the soil could overestimate the competitive ability of this fungal species, which cannot be resolved with the current experimental design. No extraradical mycelium correlations were observed. Furthermore, no growth inhibition in agar culture was observed in dual cultures of *L. deliciosus* and *Rhizopogon* spp. (data not shown). In contrast, inhibition of mycelial extension of *Pisolithus tinctorius* (Pers.) Coker and Couch by the unidentified ectomycorrhizal fungus Tanashi 01 was detected in Wu et al. (1999). Although in our case study, no direct inhibition has been observed, differences in mycelial exploration ability clearly make *R. roseolus* a better competitor in front of *L. deliciosus*, in a similar way as suggested for *R. salebrosus* A.H. Sm. and *R. occidentalis* Zeller and C.W. Dodge by Kennedy et al. (2007a). The coexistence of *R. roseolus* and *L. deliciosus* as mycelia and segregation as mycorrhizas agree with Genney et al. (2006) for *Cortinarius* sp. and *L. rufus* (Scop.) Fr., suggesting competition for root tips by the two fungi.

The observed mycelium patterns for the studied fungi mostly agree with classification in exploration types defined by Agerer (2001), but the detection of extraradical mycelium of *L. deliciosus* in La Bisbal soil may not be an expected result because this fungal species has been classified as a contact exploration type. However, molecular detection of *Russula* sp. and *Lactarius* sp. extraradical mycelia has also been reported in other soil studies (Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003a, 2005; Smit et al. 2003; Koide et al. 2005a; Genney et al. 2006). Distinction between spores and mycelium DNA in soil extracts is not possible as pointed out in several studies (Dickie et al. 2002; Guidot et al. 2003;

Kennedy and Bruns 2005; Koide et al. 2005a, b, Landeweert et al. 2005). In our work, we can rule out the amplification of DNA from spores for *L. deliciosus* and *R. luteolus* because these fungal species were not detected in the soil taken from the control treatment (in between two noninoculated plants). We cannot completely exclude DNA amplification from spores of *R. roseolus* because of the presence of the natural inoculum in both sites. However, no sporocarps were observed when sampling was done.

In conclusion, the field site is the factor that significantly influenced the persistence of the vegetative structures of *L. deliciosus*. A positive correlation between mycelium biomass and percentage of mycorrhizas observed for the three fungal species in the La Bisbal site makes mycelium biomass a good indicator of root colonization for these fungal species under the experimental conditions established. This is an interesting result to be applied in the management of productive plantations in the field because it allows for a nondestructive sampling when evaluating fungal persistence. On the other hand, in our case study, extraradical mycelium quantification does not constitute a good approach for studying interspecific fungal interactions because competition takes place in the root system.

Acknowledgments The authors wish to acknowledge the financial support provided by the Instituto Nacional de Investigaciones Agroalimentarias (INIA, Spain), project RTA04-029, and the European Regional Development Fund. The work is part of the doctorate of S. Hortal financed by the Departament d'Educació i Universitats de la Generalitat de Catalunya and the European Social Fund. Technical advice of Ramón Seminago and Amaya Amador from the Unitat de Genòmica, Serveis Cientificotècnics, Universitat de Barcelona, is appreciated.

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