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Intraspecific variability of *Lactarius deliciosus* isolates: colonization ability and survival after cold storage

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Abstract Intraspecific variability in root colonization, extraradical growth pattern, and survival after cold storage of *Lactarius deliciosus* isolates was determined in pure culture conditions using *Pinus pinaster* as a host plant. The ectomycorrhizal ability of *L. deliciosus* at 30, 45, and 60 days from inoculation was highly variable among isolates and was negatively correlated to the age of the culture (time elapsed from isolation). The formation of rhizomorphs was related to colonization ability, but no relationship was found between colonization and formation of extraradical mycelium. The final colonization achieved at 60 days from inoculation was not related to the tree species under which the sporocarps were collected. However, isolates from sporocarps collected under *P. pinaster* colonized more rapidly the seedlings than those collected under other pine species. The climatic range of the sporocarps from which the isolates were obtained (maritime vs. continental) was not related to the formation of mycorrhizas at 60 days from inoculation. However, isolates from sporocarps collected from a maritime climate area colonized more rapidly the *P. pinaster* seedlings than those collected from a continental zone. Tolerance to cold water storage of *L. deliciosus* was also isolate dependent. Growth revival in agar was obtained from most of the isolates after 28 months of cold storage at 4°C, but only 10 out of 29 isolates showed unaffected growth. The ITS rDNA alignment of all the *L. deliciosus* isolates showed a low variability with identities over 99%. Most of the variation was detected in the ITS1 region and consisted in single nucleotide changes and/or punctual indel mutations. The number of base differences per sequence from averaging over all sequence pairs was 1.329, which is in the low range when compared with other ectomycorrhizal species. No ITS pattern due to geographical origin of the isolates could be discerned.

Keywords *Lactarius deliciosus* · Isolate · Root colonization · Intraspecific variation · Cold storage

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Introduction

Ecophysiological variability within ectomycorrhizal fungal species has been reported for different parameters, e.g., the ability of using organic nitrogen in *Hebeloma cylindrosporum* (Guidot et al. 2005), the tolerance to toxic elements, enzymatic activities, and phytohormone production in *Pisolithus tinctorius* (Thompson and Medve 1984; Ho 1987), the growth response to pH *in vitro* in *Laccaria laccata* and *Cenococcum geophilum* (Hung and Trappe 1983), or the colonization ability in *Rhizopogon* spp. (Molina and Trappe 1994). However, few comparative studies have been conducted over a range of isolates belonging to a single ECM fungal species. Cairney (1999) reviewed the literature in which five or more isolates were screened for variation in one or more parameters finding high isolate variation in most of the 39 references surveyed to date.

Ecotypic variation of fungal isolates, together with the provenance of the tree seed, have to be taken into account

in the selection processes aimed at obtaining superior isolates for controlled inoculations (Trappe 1977; Brundrett et al. 1996). The candidate fungi should exhibit the physiological capacity to form abundant ectomycorrhizas on the desired hosts (Marx et al. 1992), the ability to grow in pure culture, and be able to withstand biological manipulation and storage maintaining genetic stability (Marx et al. 1982, 1984, 1992; Richter 2008). In some cases, these characteristics have been found to be as variable among isolates as they are among different fungal species (Trappe 1977; Cairney 1999).

The selection of isolates of mycorrhizal fungi for controlled inoculations also involves the development of tracking methods to determine the persistence of the inoculated fungi after transplantation of the mycorrhizal plants in the field. The identification of ectomycorrhizal fungi at species level in different stages of the symbiosis can be performed by molecular techniques based on the amplification of the internal transcribed spacer (ITS) regions in the ribosomal genes (rDNA) (Bruns et al. 1991; Horton and Bruns 2001; Gomes et al. 2002; Horton 2002; Bridge et al. 2005). However, intraspecific differences in the ITS rDNA region show limited variability (Nilsson et al. 2008) which restrict the detection at isolate level. These differences can be detected by sequencing or by using high resolution techniques for DNA analysis as single-strand conformation polymorphism (SSCP) (Hortal et al. 2006).

Edible ectomycorrhizal fungi are a major economic forest resource which provides not only gourmet food but also a livelihood for many people who collect mushrooms from natural habitats (Boa 2004; Yun and Hall 2004; Martínez de Aragón et al. 2007). Most of the supplies of edible fungi come from natural forests, where intensive harvesting has caused the decline of some key species (Arnold 1991; Hall and Wang 2002; Hall et al. 2003). Thus, cultivation of edible ectomycorrhizal mushrooms has been carried out but with limited results (Yun and Hall 2004). The cultivation of epigeous edible ectomycorrhizal fungi has been less successful than the culture of truffles. To date, only sporocarps of *Lactarius deliciosus* and *Lyophyllum shimeji* have been obtained from inoculated plants (Yun and Hall 2004). *L. deliciosus* can be easily isolated and adequately formulated for nursery inoculation of plants to produce edible sporocarps (Parladé et al. 2004).

A range of isolates of the edible ectomycorrhizal fungus *L. deliciosus* have been compared to determine intraspecific variability in root colonization, mycelia and rhizomorph growth pattern, and survival after cold storage under standardized conditions. The number and location of base differences within the ITS rDNA regions have been identified to determine the possibility of tracking the isolates after inoculation by molecular methods. The design of standardized screening procedures *in vitro* for the selection of isolates of *L. deliciosus* and the methods for determining their persistence in inoculated plants after field transplantation are discussed.

Materials and methods

Fungal isolates

The cultures of *L. deliciosus* (L. ex Fr.) S.F. Gray were obtained from sporocarp isolation (except isolate 347 which was obtained from an ectomycorrhiza) in BAF agar medium (Oort 1981). Data on sporocarp collections are shown in Table 1. The isolates were maintained in BAF agar medium at 4°C with transfers every 3 months onto fresh medium at 25°C until regrowth. The extraction of DNA from each isolate was carried out with the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR amplification of the rDNA ITS region was performed with the universal primers ITS1 and ITS4 (White et al. 1990). The amplified DNA was purified with the High Pure PCR Product Purification (Roche) and sequenced in a 3730 DNA Analyzer (Applied Biosystems). The consensus sequences for each isolate were deposited in the GenBank with accession numbers included in Table 1.

Pairwise analyses of 31 high-quality sequences of *L. deliciosus* isolates were conducted in MEGA4 (Tamura et al. 2007) to determine the average number of base differences in the ITS1-5.8S-ITS2 rDNA region. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option in the MEGA4 software). Standard error estimate (SE) was obtained by a bootstrap procedure (1,025 replicates).

Colonization ability

Pure culture glass synthesis tubes were prepared according to Molina (1979) modified by using BAF liquid solution, instead of MMN, and adding 15 g glucose/l. The tubes were autoclaved for 45 min at 120°C. Seeds of *Pinus pinaster* (lot A11300299 Llevant Region, collection date 1999 from the Seed Bank of the Generalitat Valenciana, Spain) were surface sterilized for 45 min in 30% hydrogen peroxide and then rinsed twice with sterile distilled water. *P. pinaster* was chosen as a model plant over other pine species because of the plant's good growth and root formation under pure culture synthesis and the availability of genetically homogeneous seeds.

The disinfected seeds were pregerminated in agar plates, then placed in the synthesis tubes (one per tube) and grown for 1 month. Contaminant-free tubes were then inoculated with liquid suspensions of homogenized 30-day-old colo-

Table 1 Collection data of the *Lactarius deliciosus* isolates inoculated in the pure culture synthesis experiment

Isolate number[a]	Associated tree	Age of isolate (days)	Location (province)	Climate range[b]	GenBank accession
120	*Pinus pinaster*	6,717	Girona	Maritime	DQ116894
178	*Pinus pinea*	4,546	Barcelona	Maritime	DQ116888
217	*Pinus pinea*	4,523	Girona	Maritime	DQ116895
274	*Pinus pinea*	4,184	Girona	Maritime	DQ116889/90
312	*Pinus pinaster*	2,357	Girona	Maritime	DQ116886
313	*Pinus pinea*	2,357	Girona	Maritime	DQ116887
330	*Pinus halepensis*	1,225	Barcelona	Maritime	DQ116898
331	*Pinus sylvestris*	1,274	Tarragona	Maritime	DQ116899
332	*Pinus sylvestris*	1,274	Barcelona	Continental	DQ116900
334	*Pinus sylvestris*	924	Barcelona	Continental	DQ116901
335	*Pinus sylvestris*	924	Barcelona	Continental	DQ116902
336	*Pinus sylvestris*	908	Barcelona	Maritime	DQ116903
337	*Pinus sylvestris*	908	Barcelona	Maritime	DQ116904
347	*Pinus pinaster*	754	Girona	Maritime	EU423912
352	*Pinus sylvestris*	582	Lleida	Continental	n.a.
358	*Pinus* sp.	547	Barcelona	Continental	n.a.
361	*Pinus uncinata*	540	Andorra	Continental	EU423915
362	*Pinus pinaster*	512	Girona	Maritime	EU423916
364	*Pinus* sp.	507	Girona	Maritime	n.a.
366	*Pinus halepensis*	504	Barcelona	Continental	EU423917
378	*Pinus sylvestris*	183	Lleida	Continental	EU423920
381	*Pinus pinaster*	170	Girona	Maritime	HQ328776
383	*Pinus pinaster*	151	Girona	Maritime	EU423922
892	*Pinus sylvestris*	1,655	Soria	Continental	DQ116892
894	*Pinus sylvestris*	1,654	Soria	Continental	DQ116891

All the cultures were isolated from sporocarps except isolate no. 347 which was obtained from an ectomycorrhiza

n.a. not available; sequences with double signal (probably heterozygotic dikaryons)

[a] Isolates deposited in the IRTA–ECM culture collection

[b] Refers to overall climatic influence in the collection area

nies of *L. deliciosus* as described in Parladé et al. (2004). Each tube received 5 ml of the homogenate equivalent to approximately 30 mg of mycelium (fresh weight). A total of 450 tubes were prepared (18 for each of the 25 isolates). The tubes were maintained at 25°C in a growth chamber with a 16-h photoperiod under fluorescent light (135 μmol s^{-1} m^{-2}) distributed in a completely randomized design.

Evaluation of ectomycorrhizas formation was carried out at 30, 45, and 60 days from inoculation by counting the total number of colonized roots formed by each isolate. Six replicates (tubes) were evaluated for each isolate. The presence of extraradical mycelium, formation of rhizomorphs, and plant growth (height and diameter) were also recorded at 60 days from inoculation. Isolates covering more than half of the tube surface with white extraradical mycelium were compared with isolates with low or no visible external mycelium at the end of the experiment. Isolates with orange rhizomorphs clearly visible through the tube surface and isolates which did not produce them were also compared.

Differences in root colonization due to isolate, associated tree, climatic range of the sporocarps, and *in vitro* extraradical mycelium pattern were determined by analysis of variance. The relationship between time in culture and mycorrhizal ability of the different isolates was determined by the Pearson correlation analysis. Statistical analyses were performed with JMP®, version 7 (SAS Institute Inc., Cary, NC, 1989–2007).

Survival to cold storage

Fifteen mycelium disks (5 mm diameter), taken with a cork borer from 30-day-old agar colonies growing at 25°C, of 29 isolates of *L. deliciosus* (Table 4) were placed in screw-cap 13×100 mm Pyrex glass tubes containing 3 ml of sterile

distilled water. The tubes were maintained at 4°C in the dark and fungal viability was determined after 7, 12, and 28 months by placing four disks from each tube onto agar plates with BAF medium. Six additional isolates of *Lactarius sanguifluus* were included for comparison. Growth revival after 15 days was measured for each storage period. Further observation at 30 and 45 days was also conducted to detect delayed growth revival before discarding the plates.

Results

After 60 days, the *P. pinaster* seedlings growing in the synthesis tubes were, on average, 5.97±0.11 standard error (SE) cm high (measured from the point of insertion of the cotyledon to the terminal bud) and 1.51±0.02 (SE) mm stem diameter. The formation of ectomycorrhizas showed a high variability, from no mycorrhiza formation in two isolates (894 and 274) to 670 colonized short roots in a single tube at 60 days from inoculation (isolate 378). Total number of mycorrhizas formed by each isolate at 30, 45, and 60 days from inoculation are shown in Fig. 1. The factors "isolate", "time from inoculation", and their interaction were significant ($P<0.0001$). In most of the tubes, ectomycorrhizas were already detected in the first observation at 30 days from inoculation, although in a low number. After 45 days, some isolates (330, 381, 383, and 352) stood out from the rest forming, on average, over 150 colonized short roots. At 60 days, 23 out of 25 isolates formed ectomycorrhizas. Isolates 330, 364, 892, 120, and 334 did not increase the colonization from 45 to 60 days after inoculation. Significant differences ($P<0.05$) among groups of isolates at the end of the experiment are represented in Fig. 1.

The effect of the associated pine species and climatic range of the sporocarps from which the isolates were obtained (maritime vs. continental) on the colonization ability of the different isolates is shown in Table 2. The associated tree species were not related to the final colonization at 60 days. However, isolates from sporocarps collected under *P. pinaster* colonized the seedlings more rapidly than those collected under other pine species. Similarly, the climatic range of the sporocarps from which the isolates were obtained was not related to the formation of mycorrhizas at 60 days from inoculation, but isolates from sporocarps collected in maritime climate colonized more rapidly the *P. pinaster* seedlings than those collected under continental climate.

All the isolates grew similarly in the agar plates after 30 days at 25°C. Inside the synthesis tubes, however, the growth pattern showed differences among isolates. Twelve isolates formed abundant external mycelium (178, 217, 312, 334, 335, 336, 337, 347, 352, 366, 892, and 894) whereas 13 isolates formed abundant rhizomorphs (334, 336, 347, 366, 330, 334, 336, 337, 352, 362, 366, 378, and 381). The differences in ectomycorrhiza formation according to the mycelial growth pattern are shown in Table 3. The formation of ectomycorrhizas was not significantly different between isolates showing different amounts of

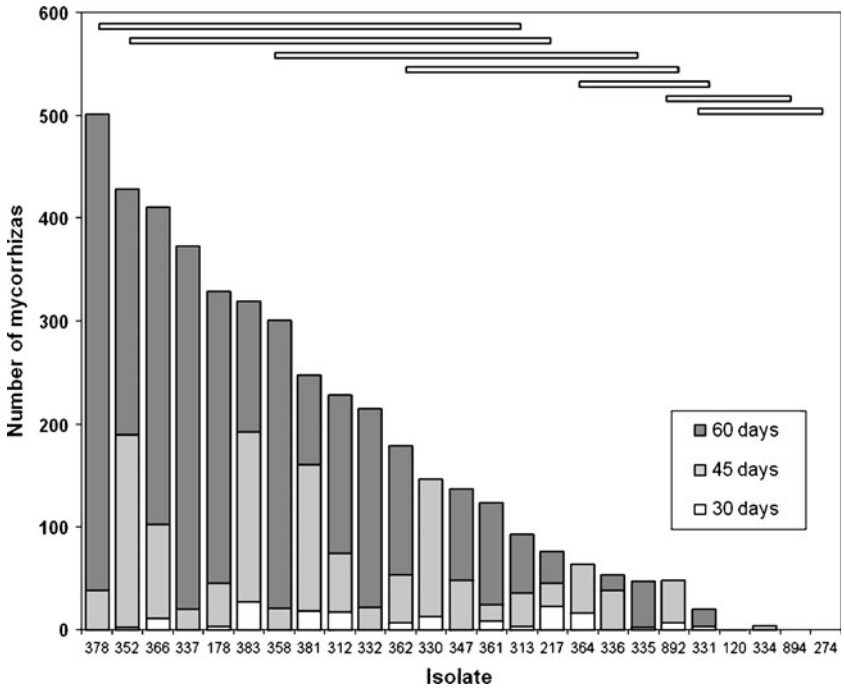

Fig. 1 Number of *L. deliciosus* mycorrhizas formed in synthesis tubes after 30, 45, and 60 days of incubation. The results are the mean of six replicates for each isolate. Groups of isolates under *overlapping horizontal bars* in the top of the graph are not different by Tukey's test at 60 days from inoculation ($P<0.05$)

Table 2 Mean number of *Lactarius deliciosus* ectomycorrhizas formed by different isolates in pure culture synthesis with *Pinus pinaster* according to their origin (associated tree species and climatic range of the sporocarps from which isolations were made)

	Time from inoculation		
	30 days	45 days	60 days
Associated species[a]			
Pinus pinaster (6)[b]	18 a	113 a	236 a
Pinus pinea (4)	11 a	56 b	177 a
Pinus sylvestris (10)	2 b	23 b	172 a
Climatic range			
Maritime (15)	13 a	80 a	187 a
Continental (10)	2 b	29 b	197 a

Six replicates per isolate were considered. Values within each analysis sharing the same letter are not different by Tukey's test ($P<0.05$)

[a] Pine species with less than three associated isolates were not considered for the analysis

[b] Number of *L. deliciosus* isolates included in each group

extraradical mycelium. On the other hand, the isolates showing rhizomorphs in the synthesis tubes formed significantly more ectomycorrhizas.

Pairwise correlations between the age of the culture (days from isolation) and the formation of ectomycorrhizas resulted in a significant negative relationship at 45 and 60 days from inoculation [correlation coefficient=−0.54 (P=0.009) and −0.54 (P=0.010), respectively]. No significant relationship was found at 30 days from inoculation.

Growth revival of different *L. deliciosus* isolates stored in cold water was similar in almost all of them after 7 and 12 months (Table 4). A higher variability was observed after 28 months of storage, with three out of 29 isolates with no growth revival and 10 isolates showing consistent growth in all the plugs. A lower growth revival was detected in the rest of isolates after 15 days of incubation. The isolates of *L. sanguifluus* included here for comparison purposes showed a similar variation pattern as *L. deliciosus*, but a sharper decline was noted after 28 months in cold storage.

Table 3 Number of *Lactarius deliciosus* ectomycorrhizas formed by 25 isolates according to their growth pattern in the synthesis tubes at 60 days after inoculation

Growth pattern	Mycorrhizas
External mycelium	
Scarce/none	137 a
Medium	202 a
Abundant	179 a
Rhizomorphs	
Yes	242 a
No	123 b

Six replicates were considered for each isolate (N=150). Number of mycorrhizas sharing the same letter within each analysis are not different by Tukey's test ($P<0.05$)

The sequence alignment of the ITS rDNA of all the *L. deliciosus* isolates showed a low variability, with identities over 99%. Most of the variation was detected in the ITS1 region and consisted in single nucleotide changes in positions 13, 16, 62, 77, 78, 148, 192, and 196, and indel mutations of one base in positions 16, 189, and 196 (all the positions are referred to the GenBank entries; see Table 1 for reference numbers). In the ITS2 region, single nucleotide changes were detected in position 630. The positions 16 and 62 showed the highest variability. ITS fragments of some of the dikaryotic isolates were difficult to sequence without cloning because of heterozygosis in the indel mutations. Cloning was carried out previously for isolate 274 showing the indel mutation in position 16 of one of the two nuclei (Hortal et al. 2006). The number of base differences (d) per ITS1-5.8S-ITS2 rDNA sequence (647 bp) from averaging over all sequence pairs was 1.329 (SE=0.420), giving a mean variation of 0.20%.

Discussion

L. deliciosus has been associated with a range of conifer species (Trappe 1962; Singer 1986). However, further studies on taxonomy and phylogeny in *Lactarius* sect. Deliciosi using both molecular and morphological characters concluded that *L. deliciosus* characteristically associates with *Pinus* species in Europe (Nuytinck and Verbeken 2005, 2007). Pure culture synthesis experiments demonstrated the potential colonization ability of *L. deliciosus* with a range of European, American and Neotropical pine species (Pera and Álvarez 1995; Parladé et al. 1996; Díaz et al. 2007).

Intraspecific variation in the colonization ability of different ectomycorrhizal fungi, including three strains of *L. deliciosus*, was previously reported by Pera and Álvarez (1995) in *P. pinaster* growing in pure culture. The present study shows the high variability in the colonization potential of a number of isolates ranging from 5 to 220 months old. Some isolates used in former studies, in similar experimental conditions, showed a loss of colonization ability as observed in the oldest isolate (no. 120) (Pera and Álvarez 1995; Parladé et al. 1996). The significant negative relationship between the colonization ability and the time in culture since isolation is relevant for the maintenance of the symbiotic potential of superior strains for inoculation purposes (Marx and Daniel 1976; Thomson et al. 1993).

Table 4 Viability (growth revival in agar) of *Lactarius* isolates after different periods of cold storage in sterile water (4°C)

Fungal species	Isolate no.	Viability[a]		
		7 months	12 months	28 months
Lactarius deliciosus	120	++++	++++	++++
L. deliciosus	178	++++	++++	+++
L. deliciosus	217	++++	+++	+++
L. deliciosus	274	++++	++++	++++
L. deliciosus	331	++++	+++	++
L. deliciosus	332	++++	++++	++
L. deliciosus	334	++++	++++	++++
L. deliciosus	335	++++	++++	++++
L. deliciosus	336	++++	++++	–
L. deliciosus	337	++++	+++	++
L. deliciosus	347	++++	+++	++
L. deliciosus	352	+++	+++	+
L. deliciosus	353	++++	++++	++++
L. deliciosus	354	++++	+++	++++
L. deliciosus	355	++++	++++	++++
L. deliciosus	356	+++	++++	+
L. deliciosus	358	+++	++	+
L. deliciosus	361	++++	++++	+
L. deliciosus	362	++++	+++	+
L. deliciosus	364	++++	+++	++
L. deliciosus	366	++++	+++	–
L. deliciosus	378	++++	++++	++++
L. deliciosus	381	++++	+++	++++
L. deliciosus	382	++++	+++	++++
L. deliciosus	383	+++	+++	++
L. deliciosus	384	++++	++++	+
L. deliciosus	385	++++	+++	+
L. deliciosus	892	++++	+++	–
L. deliciosus	894	++++	+++	++
Lactarius sanguifluus	261	++++	++++	+
L. sanguifluus	263	+++	+++	+
L. sanguifluus	328	++++	+++	–
L. sanguifluus	329	+++	+++	+
L. sanguifluus	357	+++	–	–
L. sanguifluus	367	+++	++	–

[a] Crosses indicate the number of colonies with fast growth revival (measured at 15 days of incubation at 25°C). − no growth revival of any colony after 30 days

Characteristics of fungi such as pathogenicity, virulence, growth, and colonization rate are known to change over time when mycelium is continually subcultured in agar (Marx et al. 1984; Hung and Molina 1986; Richter et al. 2004). Revival of 77% of the ectomycorrhizal fungi stored in cold sterile distilled water for 20 years has been obtained in a recent study by Richter (2008). Smith et al. (1994) also found a high percentage of growth revival after long-term storage of mycorrhizal fungi in water. These results indicate that cold water storage is a sound method for maintaining fungal collections avoiding subsequent transfers to agar medium and the eventual loss of mycorrhizal ability. Nevertheless, the results obtained for revival of *Lactarius* species stored in cold water are scarce. Richter and Bruhn (1989) revived four out of five *Lactarius* species after different storage periods ranging from 20 to 47 months. They found isolate and species variability in the revival after storage. Only *Lactarius rufus* was tested for a 20 years storage period obtaining 100% revival (Richter 2008). Our results indicate overall growth revival of all the *L. deliciosus* strains after 12 months of conservation. Variability among isolates was detected after 28 months with only 10 out of 29 strains showing growth revival in all the agar disks and 10 strains showing no, or very limited,

growth (one disk revival). In the case of *L. sanguifluus*, growth revival was obtained after 12 months but growth stopped or was very limited after 28 months.

To date, no conservation studies have been done on *L. deliciosus* strains. The results of this study indicate that cold water storage is feasible for maintaining the growth revival of this fungal species up to a year. This can reduce significantly the transfers into fresh agar medium and, possibly, the eventual loss of symbiotic ability.

The artificial growth conditions of the pure culture synthesis experiments can make the results difficult to compare with those obtained under natural conditions in the field (Harley and Smith 1983). Cairney (1999) pointed out the need to critically assess axenic culture-based work for comparative ecological functioning because of the changes in gene expression which may occur during maintenance under laboratory conditions. Also, the presence of glucose in the synthesis tubes upsets the balance of the symbiosis, favoring the fungal colonization (Duddridge and Read 1984; Duddridge 1986). However, the same authors observed a disruption of the host tissue in incompatible associations which was not observed in the mycorrhizas formed in our study. On the other hand, there is a need of establishing comparable standard conditions for testing isolates to be used in controlled inoculations. Moreover, Massicotte et al. (1994) found similar specificity patterns in pure culture and nursery inoculations of different conifers with *Rhizopogon* spp.

Some of the isolates used in this study had been recently tested for persistence in field plantations established with inoculated *P. pinea* seedlings. Hortal et al. (2009) showed that the isolate 330 formed significantly more ectomycorrhizas than isolates 312, 313, 217, and 178 after 1-year growth in the greenhouse. The field persistence of ectomycorrhizas formed by the isolate 330 was also the highest at 7 and 13 months, but differences were only significantly different when compared with isolate 178. This last isolate showed the lowest colonization level after 13 months in the field even if it was a good colonizer in axenic conditions. Isolate 330 was the best colonizer in the greenhouse and maintained good persistence in the field. This isolate reached the maximum amount of colonized short roots just after 45 days of pure culture synthesis. The rest of the isolates tested in the field reached their maximum colonization at the end of the pure culture experiment (60 days). Further research is required to elucidate if the rapid colonization ability, rather than the potential number of roots colonized, can be a competitive advantage in natural conditions.

Differences among fungal isolates in host specificity within the genus *Pinus* may be due to ecotypic variations as pointed out by Massicotte et al. (1994) for *Rhizopogon* spp. It is possible that habitat differences might be reflected in the colonization patterns, indicating some degree of specialization or host preference among isolates. Malajczuk et al. (1990) found differences in the speed of colonization of *Eucalyptus urophylla* by *Pisolithus tinctorius* related to the geographical origin of the fungus. The isolates from sporocarps collected under *P. pinaster* formed more mycorrhizas than those obtained from sporocarps collected under other pine species at 30 and 45 days after the inoculation. However, differences were not significant at 60 days. A similar pattern was observed in isolates from sporocarps collected in a zone with Mediterranean climate which formed more mycorrhizas on *P. pinaster* than isolates obtained from areas with a continental climate at 30 and 45 days, but no differences were detected at 60 days. This behavior can be of interest when selecting isolates from sporocarps occurring in particular habitats and associated tree species since the colonization speed is a factor which may favor root tip competition in a "priority effect" scenario (Kennedy and Bruns 2005; Kennedy et al. 2009; Kennedy 2010) defined as the advantage which results from an early colonization of the resource which negatively affects the later arrivers. Kennedy and Bruns (2005) reported this effect in dual inoculation experiments with *Rhizopogon* species demonstrating that the more rapid spore germination and root tip colonization of one species resulted in the almost complete exclusion of the slower species. However, other factors should be taken into account as the different microhabitats created by neighboring hosts which may also promote niche specialization not encountered in monoculture experiments (Malajczuk et al. 1990).

The formation of external mycelium and rhizomorphs was variable among isolates at 60 days from inoculation. Isolates forming rhizomorphs also formed more mycorrhizal tips. This fact may be related to the increased secondary colonization due to the faster growth of rhizomorphs. The different ability of ectomycorrhizas to colonize the surrounding media by means of extraradical mycelium and rhizomorphs was pointed out by Agerer (2001) who defined different exploratory types of ectomycorrhizas. The results presented here indicate that this feature also shows intraspecific variation. Timing of colonization seems to be related to root tip competition as suggested by Kennedy et al. (2009). Isolates having more secondary colonization ability by means of rhizomorphs would compete more successfully with native fungi. On the other hand, no relationship was found between the abundance of extraradical mycelium and mycorrhizal colonization, suggesting that the role of extraradical mycelium, being the part of the symbiosis mostly connected to soil surface, could be more related to nutrient acquisition and interplant exchange (Simard et al. 2002; Leake et al. 2004).

Smith et al. (2007) examined intraspecific and intrasporocarp ITS variation by DNA sequencing of both sporocarps and pooled roots from 68 species of ectomycorrhizal fungi collected at a single site in *Quercus* woodland. Although intraspecific ITS variation was generally low (0.16–2.85%, mean=0.74%), it was widespread within this fungal community. In this study, an ITS mean variation of 0.20% was obtained which is in the low range. No ITS pattern due to geographical origin of the isolates could be discerned as found by Nuytinck and Verbeken (2007). Small ITS rDNA differences can be detected by high resolution molecular techniques as SSCP. This technique has been used to detect particular *L. deliciosus* isolates differing by only one nucleotide at different symbiotic stages (Hortal et al. 2006).

In conclusion, the colonization ability of *L. deliciosus* is highly variable among isolates and negatively correlated to the time from isolation. Cold storage could help in maintaining isolate stability. The capacity of secondary colonization and competitivity is higher in isolates showing rhizomorphs. The comparison of the results obtained *in vitro* with those obtained in the field suggests that the persistence of ectomycorrhizas may be more dependent on the colonization speed than on the total colonization level. This colonization speed has been found to be related to the associated species and to the habitat of the sporocarps from where the isolates were obtained. The results obtained in controlled conditions should be interpreted with caution. However, standardized methods allow for comparative studies aimed at selecting isolates for controlled inoculations. Tracking of the isolates in the field based on detection of nucleotide variability in the ITS rDNA region requires high resolution techniques to reveal single nucleotide changes.

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