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The ectomycorrhizal symbiosis between *Lactarius deliciosus* and *Pinus sylvestris* in forest soil samples: symbiotic efficiency and development on roots of a rDNA internal transcribed spacer-selected isolate of *L. deliciosus*

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Abstract The effect on plant growth of pre-inoculation of *Pinus sylvestris* with the ectomycorrhizal (ECM) edible basidiomycete *Lactarius deliciosus* (isolate D45) under controlled conditions, and the development on roots of this basidiomycete, were investigated in γ-irradiated and unsterilized containers containing different forest soil cores or a perlite-vermiculite mixture. Five months after planting, *L. deliciosus* mycorrhizal plants exhibited greater growth than the non-mycorrhizal ones in all soil types, i.e. up to a 325% increase in shoot height in the sterilized soils. The experiment demonstrated the dependency of *P. sylvestris* seedlings upon ECM symbiosis for their survival in γ-irradiated, microbiologically disturbed soil samples. Furthermore, in two soils, the growth of *L. deliciosus*-inoculated seedlings was greater in the sterilized soil samples than in the non-sterilized ones, i.e. 46% and 132% increase in shoot height under sterilized soil conditions. In containers randomly sampled from each soil type, the degree of root colonization by the inoculated isolate, calculated as the number of mycorrhizal root tips divided by the total number of root tips ×100, ranged from 80% to 35%. Within the short term, the inoculated isolate developed rapidly on roots, dominated, and hampered ectomycorrhiza formation by various unidentified (but not *Lactarius*) resident ECM fungi in unsterilized soil types. Results indicate that the ECM species *L. deliciosus* is worth investigating to ascertain if other isolates benefit pine growth like the isolate D45, and are therefore also attractive candidates for forestry applications in the Mediterranean area.

Keywords Ectomycorrhiza · Internal transcribed spacer · *Lactarius deliciosus* · Inoculation · Soil bioassay

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Introduction

In temperate forest ecosystems, the ectomycorrhizal (ECM) symbiosis which develops between the roots of host trees and the soil ECM fungi is an important factor for the survival and growth of these trees, as it stimulates their water and nutrient uptake (Smith and Read 1997; Perez-Moreno and Read 2000). Therefore, the controlled mycorrhizal inoculation of seedlings in the nursery usually promotes the establishment of forest plantations, mainly by improving initial seedling growth (Grove and Le Tacon 1993). The symbiotic association allows fruiting of the mycobiont (Godbout and Fortin 1990; Danell and Camacho 1997; Guerin-Laguette et al. 2000; Yamada et al. 2001). Some fungal species may simultaneously stimulate host tree growth and present an additional income for forestry farms through the potential production of edible sporophores. This is the case for the saffron milk-cap, *Lactarius deliciosus* (L.:Fr.) S. F. Gray, an ECM basidiomycete which forms mycorrhizae in nature with a wide range of coniferous species (Trappe 1962). Like most of the basidiomata of the *Dapetes* (Fries) section of *Lactarius*, fruit bodies of *L. deliciosus* are edible and are considered, with those of *L. sanguifluus* (Paulet: Fr.) Fr., as autumn delicacies in continental Europe (Hall and Wang 1998) and in the Mediterranean countries (Bertaux 1962; Hesler and Smith 1960).

In order to develop the use of an ECM species in forestry, a prerequisite step is to set up a reliable method of controlled mycorrhization of trees. The inoculation of pines with *L. deliciosus* has recently been improved, producing seedlings with a high degree of mycorrhization

(i.e. the number of mycorrhizal root tips divided by the total number of root tips×100) which led to the unprecedented fruiting of *L. deliciosus* associated with *Pinus sylvestris* (Scots pine) under controlled soil-less conditions (Guerin-Laguette et al. 2000). However, despite the fact that a high degree of mycorrhization on roots may contribute both to the field performance of trees after outplanting and to the persistence of the associated mycobiont (Marx and Hatchell 1986; Marx et al. 1988; Villeneuve et al. 1991), the ability of the introduced fungal isolate to develop in soil, i.e. "soil adaptedness "(Danielson and Visser 1989; Perrin et al. 1996), and to compete with indigenous mycorrhizal fungi is essential for determining the actual success of inoculation practices (Perry et al. 1987; Grove and Le Tacon 1993). Therefore, there is clearly a need to address experimentally the behaviour of any inoculated isolates and to select those which could be best adapted to a wide range of outplanting conditions (Trappe 1977; Kropp and Langlois 1990). In addition, the symbiotic efficiency of isolates, i.e. the positive effect of inoculation on host tree growth, should be examined under each planting condition.

Large-scale in situ experiments are essential to assess the ultimate efficiency of ECM isolates (Danielson and Visser 1989). However, many constraints limit the number of field trials, and the development of alternative soil bioassays which could predict the effectiveness of isolates in the field is a challenge in mycorrhizal research (Grove and Le Tacon 1993). In this study, we used a forest soil bioassay approach to analyse the development on roots and the symbiotic efficiency of one isolate of *L. deliciosus* (D45) associated with *P. sylvestris* seedlings. *L. deliciosus* D45 has been selected since its rDNA internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) type (type A) has been shown to be frequent within a population of 46 basidiomata in the south of France, i.e. 76% of the fruit bodies collected displayed the ITS type A (Guerin-Laguette 1998). Furthermore, this type has been identified from field mycorrhizae as well (Guerin-Laguette 1998). In order to simulate the edaphic environment of the field, we used soil cores with limited physical disturbance to their native structure. The cores were γ-irradiated or not, in order to separate the biotic factors, i.e. the activity of soil-colonizing organisms, and the abiotic factors, i.e. the soil physico-chemical properties, of the soil environment (Grove and Le Tacon 1993). A perlite-vermiculite (PV) mixture was used as a reference substrate for the ECM colonization of pines by *L. deliciosus* in nursery growth containers (Guerin-Laguette et al. 2000).

The objectives of this study were to analyse the effect of *L. deliciosus*, isolate D45, on *P. sylvestris* growth in various culture treatments and to check whether the isolate is adapted or not to contrasted soil environments, i.e. to determine its ability to develop new root colonization, in the presence or in the absence of competitive indigenous ECM fungi.

Materials and methods

Soil sample sites and soil core processing

Soil samples were collected in late spring (May and June) in three contrasted forest sites [Dévoluy (D), Ramponenche (R) or Montmirat (M) soils; Table 1] where *L. deliciosus* fruit bodies occur annually. In each site, replicate soil cores were collected within an area <3 m^2 by using cylindrical PVC containers (30 cm height×24 cm diameter) aimed at minimizing the physical disturbance of the natural soils during withdrawal. Core samples were removed by sliding the PVC containers around pre-delimited columns of soil. The soil column was then cut at its base and the bottom of the container was closed with a pierced lid. A drainage cloth was inserted between the soil and the lid.

For sterilization purposes (see also "Treatments and experimental design" section), containers were wrapped in polyethylene film and irradiated at the Gammaster Provence Gamma Irradiation Unit (MIN des Arnavaux, Marseille) during the week following soil removal. Containers were given a minimal dose of 25×10^3 Gy at the centre of the soil samples twice at a 24-h interval.

Treatments and experimental design

The eight soil treatments in PVC containers consisted of three forest soils and one PV mixture (1:1, v/v), hereafter also called soil, which were either sterilized or not by γ-irradiation. For each soil treatment, five replicate containers were planted with three 3-month-old seedlings successfully colonized by *L. deliciosus* D45 and three replicate control containers received three 3-month-old uninoculated, non-mycorrhizal, seedlings (i.e. three seedlings per container and a total of 192 seedlings distributed in 64 containers).

Production of colonized and uninoculated seedlings: symbionts, growth conditions and mycorrhizal status prior to outplanting into containers

Seeds of *P. sylvestris* L. originating from the Bitche-Bout orchard (Allier, central France) were provided by Vilmorin (La Ménitré, France). *L. deliciosus* isolate D45 was obtained by propagating a fruit body collected in the south of France at La Joue-du-Loup (Hautes-Alpes) on BAF agar (1.5%) medium (Moser 1960). The ITS-rDNA polymerase chain reaction (PCR)-RFLP type of *L. deliciosus* D45 was previously identified as type A (Guerin-Laguette 1998). The ITS nucleotide sequence of D45 has been deposited in the GenBank database under the accession no. U80999 (A. Guerin-Laguette, unpublished data).

Seeds were disinfected for 15 min in 30% H_2O_2, rinsed in sterile distilled water, and sown on attapulgite (Oil-Dri, Ga.) previously sterilized for 4 h at 150°C. Seedlings were grown for 1 month with distilled water, and were transferred for 3 weeks into a PVC box equipped with a water-spraying system aimed at enhancing lateral fine root production (see Guerin-Laguette et al. 2000). Seedlings were then individually transferred to growth pouches for 1 month in order to generate *L. deliciosus*-inoculated and uninoculated control seedlings to be outplanted in the containers. Fungal inoculum and mycorrhiza formation were as described in the water treatment used by Guerin-Laguette et al. (2000). No exogenous nutrients were added to the pouches. However, despite rinsing in water, mycelial inoculum may have physically retained some nutrients. Therefore, control pouches (receiving uninoculated seedlings) were fertilized with a membrane-sterilized extract of additional *L. deliciosus* mycelia (Guerin-Laguette et al. 2000). Inoculated and uninoculated pouches were randomly incubated vertically in opaque plastic bags suspended on nylon wires fastened to a wooden frame. They were incubated in a growth chamber under the following environmental conditions: 18/6-h light/dark cycle at 23/18°C, 65% relative humidity, CO_2 concentration of ca. 350 µl/l and photosynthetically active radiation of 200 µmol m^{-2} s^{-1} (400–700 nm). At the time of outplanting into containers, the

Table 1 The three forest soils used in this study

Soil abbreviation	Geographic origin	Sampling site location	Altitude	Dominant *Pinus* species	Parent material	pH[a]	
						A horizon	B horizon
D	Dévoluy range, Hautes-Alpes	44°41′46″N, 5°55′15″E	1,385 m	*P. sylvestris*	Glacial alluvial limestone deposits	5.9	7.5
R	Ramponenche forest, Lozère	44°20′31″N, 3°38′25″E	870 m	*P. nigra*	Colluvial schists	4.5	5.4
M	Montmirat pass, Lozère	44°25′13″N, 3°33′31″E	1,070 m	*P. sylvestris*	Limestones	6.6	7.3

[a] pH measured in water-saturated horizons (Forster 1995)

3-month-old pouch-raised seedlings were healthy, small in size, with juvenile needles only.

Only mycorrhizal seedlings were planted into containers for the mycorrhiza treatment. The average number of mycorrhizal root tips per pouch-raised seedling was 20–50 at the time of outplanting as found previously by Guerin-Laguette et al. (2000). Seedlings were free of ECM contaminants and control seedlings lacked ectomycorrhizae. No size difference was seen between mycorrhizal and control seedlings at the time of outplanting into containers.

Core planting and growth conditions

In each container, three pouch-raised seedlings were planted at equidistant positions in drilled holes (23 mm diameter). Irradiated and water-saturated B horizon was used to fill in the holes. In all treatments, seedlings were watered immediately following planting. Sterilized and unsterilized containers were incubated in separate growth chambers under the environmental conditions described above. Irradiation is known to produce little disturbance on soils (Forster 1995). However, after exposition of irradiated soils to a non sterile environment, a short-term acceleration in the decomposition of organic matter occurs (Powlson and Jenkinson 1976). In order to limit the effects of decomposition flushes on subsequent pine/*L. deliciosus* cultivation, all soil-containing containers (irradiated or not) were left to stand for 2 months, in their respective growth chambers, prior to seedling planting. During this period, containers were watered daily with 215 ml distilled water. The same watering regime was applied after pine outplanting in soil cores. Seedlings raised in the PV mixture received daily 215 ml of the A+B solution of Nylund and Wallander (1989) diluted 1,000-fold and adjusted to pH 5.7.

Seedling growth, sampling and mycorrhizal assessments

Five months after planting, i.e. seedlings were 8 months old, the effects of mycorrhizal colonization and cultural treatments on pine growth were recorded. The shoot height of seedlings was measured from the cotyledon base to the apical bud. Then, for each cultural treatment, one inoculated and one control container were randomly taken and dismantled for exhaustive root analysis. At the same time, the mycorrhiza development in the remaining containers was observed by sliding out ca. 10 cm of the soil cores from the PVC container, and *L. deliciosus*-like mycorrhizae (one per container) were randomly sampled in the inoculated treatments for molecular analysis. The soil cores were then slid back into the containers. Trees were left to grow outside in a nursery for a further 1 year. The three pine root systems of each dismantled container were carefully separated, cut into 5-cm-long segments and the number of segments counted for each pine. Under a dissecting microscope, three broadly defined morphotypes of root apices were identified: (1) *L. deliciosus*-like mycorrhiza, (2) non *L. deliciosus*-like mycorrhiza (i.e. "spontaneous" mycorrhiza), and (3) non-mycorrhizal tip. *L. deliciosus* mycorrhizae were identified using their distinct morphology: bright orange, thick, fleshy and with a smooth surface, in contrast to both non-mycorrhizal roots and roots mycorrhizal with other fungal species (Guerin-Laguette et al. 2000). Molecular analysis was also performed on randomly sampled *L. deliciosus*-like mycorrhizae (one per container). The three morphotypes were then counted using 25 random segments of 5 cm length. Multiple-branched mycorrhizae ranging from dichotomous to coralloid types were counted as one mycorrhizal tip. The percentages of the three morphotypes described above (expressed against the total amount of root tips, mycorrhizal or not) were then extrapolated to the whole root systems using the number of 5-cm segments per pine. This number was 108.3±15.2 segments (mean±SEM, all treatments mixed). Shoots and roots of the three seedlings analysed per treatment were then dried (65°C, 4 days) and weighed.

DNA confirmation of *L. deliciosus* mycorrhization

DNA was extracted from fresh mycelia or from individual mycorrhizal tips (multiple-branched or not) using the DNeasy plant kit (Qiagen, Courtabœuf, France). A cellophane membrane (Biorad no. 1650963) was used to harvest the mycelium of D45 grown on BAF agar plates. *L. deliciosus*-like mycorrhizal root tips (one per inoculated container, dismantled or not, i.e. 40 mycorrhizae) were soaked for 30 s in H_2O_2 (30%), rinsed in sterilized distilled water and frozen at –80°C prior to DNA extraction. DNA extracts were diluted five- or 25-fold in pure water to be used as a template for PCR using the primer set ITS1/ITS4 (White et al. 1990). Aliquots of 25 µl diluted DNAs were combined with an equal volume of PCR mixture. Final concentrations of the following components in 50 µl reaction mixture were: 50 µM each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, St Quentin en Yvelines, France), 0.2 µM of each primer, 2.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and the appropriate buffer supplied by the manufacturer (GIBCO-BRL). Each PCR reaction was overlaid with one drop of mineral oil (Sigma, St Quentin Fallavier, France). The reactions were run in a programmable heat block MiniCycler PTC-150 (MJ Research, Watertown). Temperature cycling included a progressive extension step exactly as described by Kraigher et al. (1995). A negative control (no DNA template) was used in each experiment to test for the presence of DNA contamination in reaction mixtures. *P. sylvestris* DNA was not amplified using the pair ITS1/ITS4 (data not shown).

The PCR reaction products were digested with *Cfo*I (GIBCO-BRL) and the restriction fragments were separated by electrophoresis on 3% high resolution (Sigma) agarose gels. Horizontal electrophoreses were carried out in 0.5× TBE buffer (44.5 mM TRIS base, 44.5 mM boric acid, 1.25 mM EDTA) for 3–4 h at 4 V cm^{-1}. The gels were stained with ethidium bromide and photographed under UV light with an imager video system (The Imager, version 2.03; Appligene).

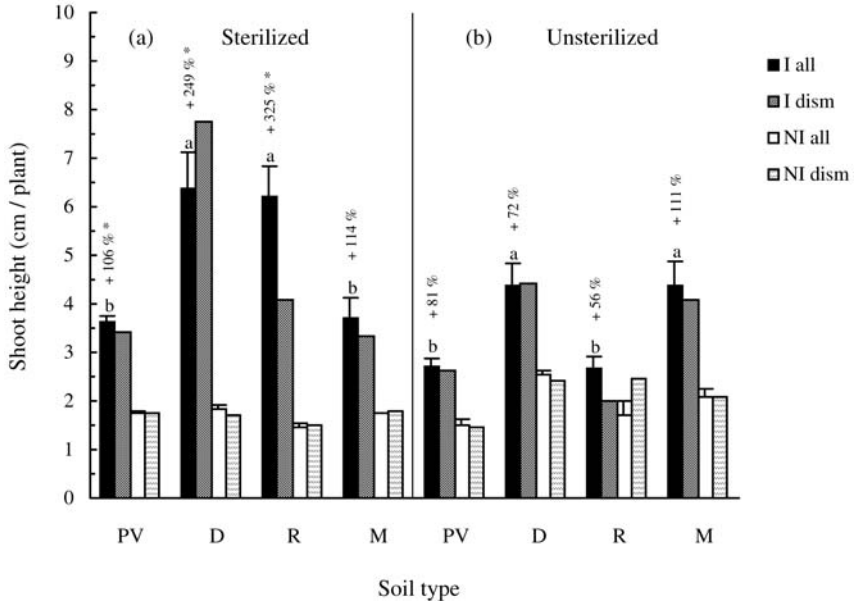

Fig. 1a, b Shoot height of *Pinus sylvestris* seedlings previously inoculated (*I*) or non-inoculated (*NI*) in growth pouches with *Lactarius deliciosus* D45 and then cultivated for 5 months in different soil types. *Bars* indicate SEM. For all soil types and for all containers (i.e. dismantled or not), growth stimulation due to inoculation with *L. deliciosus* D45 was significant (Student *t*-test, $P<0.05$) except for the dismantled containers of the unsterilized Ramponenche soil. In a given treatment (**a** or **b**), *different letters* indicate a significant effect of soil type on the growth of *L. deliciosus*-inoculated pines (Tukey-Kramer tests, $P<0.05$). *Percentages* indicate the shoot height increase of inoculated compared to uninoculated seedlings. *Indicates a significant height difference of inoculated seedlings between a sterilized vs. an unsterilized soil type (Tukey-Kramer tests, $P<0.05$). *PV* Perlite-vermiculite, *D* Dévoluy soil, *R* Ramponenche soil, *M* Montmirat soil, *all* shoot height average of blocks of three pines from replicate containers (I, $n=5$ containers; NI, $n=3$ containers), *dism* shoot height average of block of three pines from the dismantled containers ($n=1$ container)

Statistical analyses

In a given cultural treatment, the effects of *L. deliciosus*-pouch mycorrhization on shoot height, dry weight, and on spontaneous mycorrhizal colonization of seedlings (for the unsterilized soil samples) were analysed using Student's *t*-test. Within each disinfection treatment (i.e. irradiated containers or not), the effects of the different soil types on pine shoot height and on mycorrhization proportion of pines (by *L. deliciosus* or by spontaneous mycorrhizal fungi) were subjected to multiple comparisons (one-way ANOVA, Tukey-Kramer tests).

Then, the effects of soil type, inoculation, and sterilization on shoot height ($n=5$ blocks of three inoculated pines, $n=3$ blocks of three non-inoculated pines) were tested in fitted least square models (multiple regression), using nested crossed designs. The same method was used to analyse the effects of soil type and sterilization on the development of the inoculation isolate ($n=3$ pines), and the effects of soil type and inoculation on the development of indigenous mycorrhizae (forest soils only) in the unsterilized containers ($n=3$ pines). All computations were carried out using JMP 4.0.2 (SAS Institute) for Macintosh.

Results

Pine growth and root colonization by *L. deliciosus* D45 in the sterilized containers

In a given soil type/inoculation treatment, qualitative and quantitative growth responses of pines among replicate containers were quite homogeneous. The averages of the morphometric measurements made on all pines (15 or nine pines for the inoculated or control containers, respectively) or on the three pines of the corresponding dismantled container were therefore often similar (Fig. 1a). The growth of inoculated seedlings was greatly superior to that of control pines, irrespective of the soil type (Figs. 1a, 2a). The greatest increase in pine shoot height was observed in the D and R soils (Fig. 1a) with a 249% and 325% increase, respectively. In the PV treatment, fertilized uninoculated control seedlings grew little but did not show any signs of nutrient deficiency. In the three forest soils, control pines grew very little and showed signs of severe nutrient deficiency (Fig. 2a). Control pines of the undismantled forest soil containers died ca. 7 months after planting. In the M soil, inoculated seedlings showed moderate signs of physiological stress (i.e. yellow needle ends, data not shown) and grew less in comparison with seedlings grown in D and R soils (Fig. 1a).

In the dismantled containers for each soil type, the dry weight of inoculated pines was significantly higher than that of control pines (Fig. 3a) and reflected quite well the shoot growth-rate data. The dry weight of control seedlings was greater in the PV mixture than in any of the soil samples (Student *t*-test, $P<0.05$). Except for traces of contamination with *Cenococcum* sp., which were observed in the R soil, all ECM roots which were found on the inoculated root systems of both undismantled and dismantled containers were of the *L. deliciosus*-like morphotype, irrespective of the soil type. After *Cfo*I

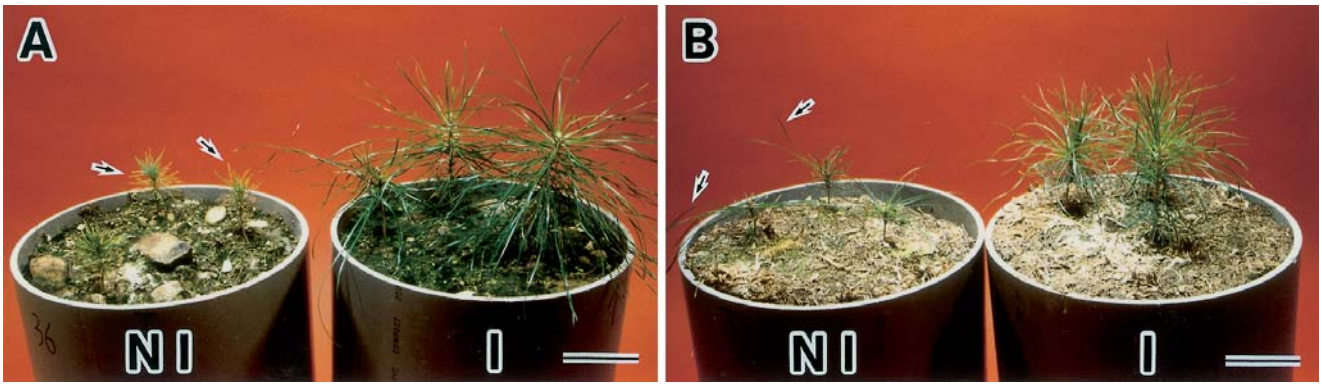

Fig. 2a, b Positive effect of mycorrhization by *L. deliciosus* D45 on *P. sylvestris* growth in soil D. *Bars*=6 cm. **a** Sterilized soil; note the withering of the uninoculated seedlings showing yellowish needles (*arrows*). **b** Unsterilized soil; note the long green needles (*arrows*) produced by the uninoculated seedlings which were healthy and were shown to be colonized by resident ectomycorrhizal (ECM) fungi. For other abbreviations, see Fig. 1

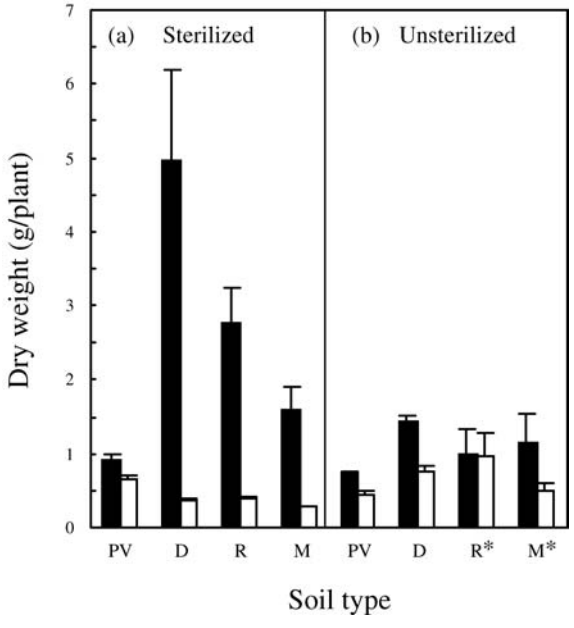

Fig. 3 Total dry weight of *P. sylvestris* seedlings inoculated (*filled columns*) or non inoculated (*unfilled columns*) with *L. deliciosus* D45 grown in different soil types (dismantled containers). *Bars* indicate SEM, n=3. In each soil type the growth stimulation due to *L. deliciosus* D45 was found to be significant (Student t-test, $P<0.05$), except when indicated (R^*, M^*). For abbreviations, see Fig. 1

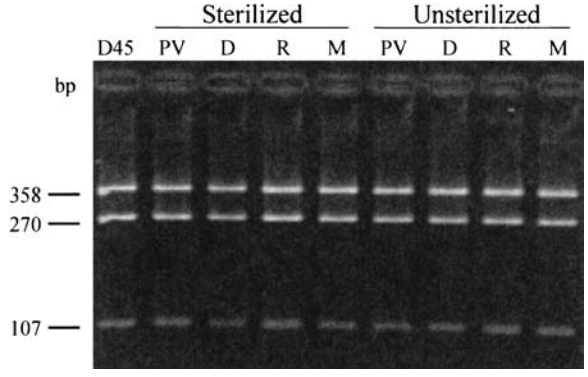

Fig. 4 Characterization by restriction fragment length polymorphism matching of the polymerase chain reaction-amplified internal spacer sequence (ITS) of *L. deliciosus* D45 ectomycorrhizae formed on *P. sylvestris* seedlings in the different soil types (inoculated seedlings only). *D45 Cfo*I restriction pattern of the amplified ITS of *L. deliciosus* isolate D45 in pure culture. *Other lines* correspond to the *Cfo*I restriction patterns of the amplified ITS of individual *L. deliciosus*-like ectomycorrhizae collected each from different soil types. For other abbreviations, see Fig. 1

analysis, all *L. deliciosus*-like mycorrhizae yielded the characteristic ITS-RFLP pattern of isolate D45 (Fig. 4). In most cases, patterns were free of contaminating bands. In the dismantled containers, *L. deliciosus* mycorrhizae were distributed in the upper part of the root systems (4–20 cm from the soil surface). In the R soil, proximal ends (i.e. close to the root axes) of *L. deliciosus* mycorrhizae often turned dark green. Five months after planting, the degree of mycorrhizal colonization of seedlings by *L. deliciosus* D45 ranged between 43% and 80% (Fig. 5a) and the estimated number of *L. deliciosus* mycorrhizae per pine was 2,625±472 (mean±SEM, all treatments). The highest colonization rates were found in the PV mixture and in the R soil (Fig. 5a). No ECM roots were found on the uninoculated control pines, irrespective of the soil type.

Pine growth and root colonization by *L. deliciosus* D45 in the unsterilized containers

Qualitative and quantitative growth responses of pines among replicate containers were homogeneous (Fig. 1b). The growth of inoculated seedlings was significantly superior to that of control pines, including in the R soil, where a small but significant positive effect of inoculation on shoot height was seen (+56%, Fig. 1b). In that soil, however, the dismantled containers were not fully representative of all replicate containers since no significant shoot height difference was seen between inoculated and control pines from these containers (Fig. 1b). Only in unsterilized R soil did such a case occur. The great-

Table 2 Three-way (shoot height, all containers) and two-way (root colonization by *Lactarius deliciosus* D45 and development of indigenous mycorrhizae, dismantled containers) ANOVA. Test of effects based on fitted least squares models

Source of variation	*P>F*		
	Shoot height	Colonization by *L. deliciosus*	Indigenous mycorrhizae
Soil type	0.0057	0.1483	<0.0001
Sterilization	0.0525	0.0385	
Mycorrhization	<0.0001		<0.0001
Mycorrhization and soil type	0.1595		<0.0001
Sterilization and mycorrhization	0.0012		
Soil type and sterilization	0.0276	0.0258	
Soil type, sterilization and mycorrhization	0.0676		

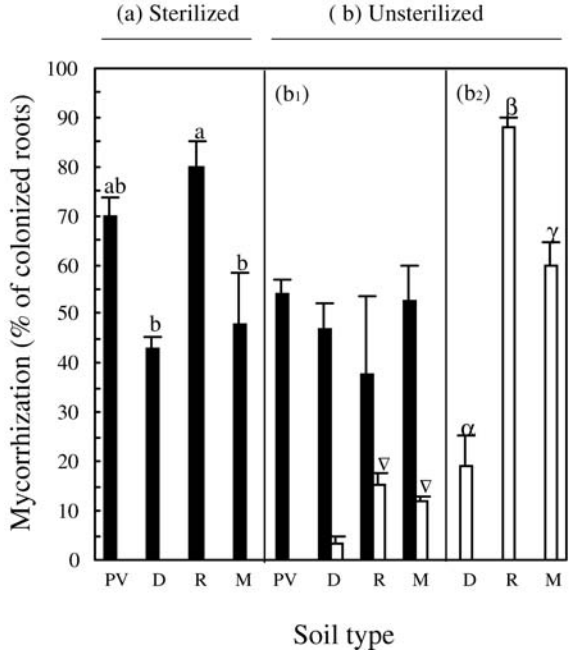

Fig. 5a, b Degree of mycorrhization of *P. sylvestris* seedlings with the inoculated *L. deliciosus* isolate D45 (*filled columns*) and with spontaneously occurring unidentified ECM fungi (*unfilled columns*) in the different soil types from the dismantled containers, 5 months after planting. **a** Sterilized containers, inoculated seedlings; treatments sharing a *common letter* are not significantly different (Tukey-Kramer test, $P<0.05$). **b** Unsterilized containers: inoculated seedlings (*b1*), no significant differences were found (Tukey-Kramer test, $P<0.05$); uninoculated (control) seedlings (*b2*), all treatments are significantly different (α, β, γ, Tukey-Kramer test, $P<0.05$). *Bars* indicate SEM, $n=3$. ∇ In a given soil type, significant reduction of spontaneous ECM colonization of pines by the pre-mycorrhization with *L. deliciosus* (Student *t*-test, $P<0.05$). For other abbreviations, see Figs. 1 and 2

est increase in pine shoot height, due to *L. deliciosus* inoculation, was observed in the M soil (111%), followed by the PV mixture and the D soil (81% and 72% increase, respectively). Uninoculated control pines were healthy in all unsterilized soils (see Fig. 2b for the Dévoluy soil). No sign of physiological stress was observed in the M soil (data not shown). From all inoculated containers, dismantled or not, *L. deliciosus*-like mycorrhizae appeared dominant on the root systems, irrespective of the soil type. The sampled *L. deliciosus*-like morphotypes yielded the characteristic ITS-*Cfo*I pattern of isolate D45 (Fig. 4).

In the dismantled containers, 5 months following planting, the degree of mycorrhizal colonization by *L. deliciosus* D45 ranged between 38% and 54% (Fig. 5b) and the estimated number of *L. deliciosus* mycorrhizae per pine was 1,412±195 (mean±SEM, all treatments). Besides the *Lactarius* D45 mycorrhizae, several "spontaneous" non-*L. deliciosus*-like mycorrhizae were observed on both inoculated and uninoculated seedlings (Fig. 5b, c). In the dismantled containers, mycorrhizae of white morphotypes were dominant, irrespective of the soil samples. In R and M dismantled soils, the spontaneous ECM colonization of seedlings was significantly reduced by previous inoculation with *L. deliciosus* D45 (Fig. 5b). Using both morphological and molecular analyses, no soil sample-native *L. deliciosus* mycorrhizae could be identified on the uninoculated seedlings. In the PV mixture, no mycorrhizal morphotypes were detected on the uninoculated pines.

Effect tests on height responses or on *L. deliciosus* colonization, and comparisons between the sterilized and unsterilized containers

Mycorrhization and soil type, taken as individual sources of variation, were significant on shoot height (Table 2). Although sterilization by itself was not significant at $P=0.05$ (Table 2), the combination of sterilization and mycorrhization was significant on pine shoot height (Table 2), suggesting that soil type sterilization generally did improve the host growth response to colonization by *L. deliciosus*. Indeed, for the PV, D and R soil types, the positive effect of mycorrhizal colonization on shoot height was significantly higher in sterilized than in unsterilized soils (Figs. 1, 2). On the D and R soils, the shoot height of inoculated pines was 46% and 132% superior in sterilized than in unsterilized soils, respectively. In contrast, for the M soil, shoot height of inoculated pines slightly decreased in the sterilized treatment (Fig. 1). The combination of soil type and mycorrhization on shoot height was not found to be significant (Table 2) suggesting that the effect of controlled inoculation

on this growth parameter did not depend on the soil type. The combination of soil type and sterilization was found to be significant at P=0.05 (Table 2) indicating that the effect of sterilization is likely to depend on the soil biological and biochemical characteristics. The physiological stress of inoculated seedlings observed in the sterilized M soil supports this hypothesis.

Although the effect of soil type alone on the root colonization by *L. deliciosus* D45 was not found to be significant, sterilization alone and the combination of sterilization and soil type had significant effects (Table 2). Indeed, the percentage of *L. deliciosus* colonization tended to increase in the sterilized PV and R soils as compared with these soils unsterilized (Fig. 5). Soil types and pouch-mycorrhization with *L. deliciosus* D45 had highly significant effects on the development of indigenous mycorrhizae (Table 2).

Discussion

This study stressed the tight dependency of 12-week-old *P. sylvestris* seedlings upon ECM symbiosis for their growth and development in γ-irradiated forest soil samples. Only the seedlings previously colonized by *L. deliciosus* survived and developed well after planting in these conditions. On the other hand, control uninoculated seedlings developed normally in similar but non-sterilized soil samples in which they were spontaneously associated with resident, non-*L. deliciosus*, ECM fungi. Therefore, a clear outcome of the present study is that ECM association, either introduced (i.e. *L. deliciosus* D45) or spontaneous, resulted in satisfactory pine growth, irrespective of the soil treatments. Soil perturbation due to γ-irradiation (i.e. the disappearance of native soil microbia followed by development of a new microflora which induced a flush of organic matter decomposition) likely prevented normal functioning of the uninoculated root systems. The perturbation could cause modifications of the root environment such as lack of oxygen or modification of gas circulation. These soil modifications were probably more pronounced in the clayey soil of M. However, mycorrhizal colonization with *L. deliciosus* enabled *P. sylvestris* seedlings to overcome these environmental conditions. The inability of healthy but ectomycorrhiza-free *P. sylvestris* seedlings (originating from the control pouches) to grow after planting in γ-irradiated soil samples supports earlier reports indicating either the seedling death (Rodriguez Barreal et al. 1996) or a severe host tree growth deficiency (Hatch 1936; Gibson 1963; Mikola 1970; Marx 1980) in soils lacking native or compatible ECM fungi.

To our knowledge, the present study describes for the first time a significant positive effect of mycorrhizal association with *L. deliciosus* on the growth of host pine seedlings both in sterilized and unsterilized soils. These results confirm a previously reported beneficial effect of *L. deliciosus* D45 on the growth of 3-month-old *P. sylvestris* seedlings in an artificial substrate (PV), immediately following mycorrhization in growth pouches (Guerin-Laguette et al. 2000). A higher survival rate of *Pinus halepensis* seedlings mycorrhizal with *L. deliciosus*, as well as a greater vitality and growth of the aerial parts, in comparison with uninoculated plants, were previously reported at a field scale (Rodriguez Barreal et al. 1996). However, these effects were not found to be significant. In the present work, the provenance of *P. sylvestris* seedlings (the Bitch-Bout orchard), with a high growth rate, may have contributed to the pronounced effect of mycorrhization on growth. The field performance of *L. deliciosus* D45 on *P. sylvestris* remains also to be demonstrated.

In the present study, the greater mycorrhizal effect in the sterilized containers than in the unsterilized ones was still observed 10 months following planting out of seedlings (data not shown). Separate growth chambers with identical environmental conditions were retained to prevent potential cross-contamination problems. Thus, we cannot exclude that part of the growth differences observed between the sterilized and unsterilized treatments could be explained by slight differences in environmental conditions. However, the growth differences between the two treatments could be due also to: (1) the competition for nutrients with other soil organisms (sensu lato, i.e. fungi, mycorrhizal or not, animals feeding on mycorrhizae, etc.) native to the unsterilized soils, and (2) the lack of significant competition with the soil microorganisms (not ECM as seen from the dismantled containers) which have developed in the γ-irradiated soils. In the unsterilized soils, resident ECM species may have competed with *P. sylvestris* for nutrient acquisition, through their pre-existing mycelial structures rapidly developing ECM connections with roots. Indeed, in the R soil, whose resident ECM flora showed the greatest root colonization potential from the dismantled control container, the effect of *L. deliciosus* on seedling growth was found to be less beneficial than in the other soil treatments (Figs. 1b, 3b). In that soil also, individual replicate containers (like the dismantled ones), could show no significant effect of inoculation. In the field, the effects of *L. deliciosus* inoculation is therefore likely to depend on the mycorrhizal potential of forest stands.

In the dismantled unsterilized containers, *L. deliciosus* D45 showed its ability to develop and to fight replacement by resident ECM species and appeared therefore as an effective competitor. The resistance of *L. deliciosus* to replacement, probably both interactive and non interactive, is likely to be due to the pre-establishment of *L. deliciosus* on short roots during the pouch inoculation stage (Smith and Read 1997) and to its ability to colonize newly formed roots. Consequently, pouch-mycorrhization by *L. deliciosus* strongly limited subsequent colonization by resident ECM fungi, at least in the dismantled containers. A similar phenomenon was observed on a young plantation of Douglas-fir seedlings inoculated or not with *Laccaria bicolor* at the nursery stage (Villeneuve et al. 1991). However, in the R and M soils which showed the greatest pressure of resident ECM fungi (as determined by the de-

gree of mycorrhizal colonization of uninoculated seedlings in unsterilized containers), spontaneous mycorrhization of inoculated seedlings slightly increased. Since competition with indigenous ECM fungi (Villeneuve et al. 1991; Wu et al. 1999), as well as soil specificity (Danielson and Visser 1989), are likely to determine the long-term persistence of any introduced isolate, further bioassay experiments will require a longer cultivation period than in the present study.

The high development on roots of *L. deliciosus* D45 in contrasting soil types support the previous finding that ITS-RFLP type A of isolate D45 was found to be highly dominant both in terms of frequency (76% of the collected basidiomata) and geographic distribution in comparison with two other less common ITS-RFLP types (20% and 4% of the collected basidiomata, respectively) (Guerin-Laguette 1998). On a similar geographic scale, the uneven distribution of different rDNA genotypes of another ECM basidiomycete, *Hebeloma cylindrosporum* Romagnesi, has been reported (Guidot et al. 1999). In subsequent experiments, it would be of particular interest to compare the fate, the competition and the effectiveness of distinct ITS types of *L. deliciosus* in various soil environments.

Evidence was given that: (1) *L. deliciosus* developed intensively after planting out and growing young mycorrhizal seedlings in contrasted soil types, and (2) had a pronounced stimulating effect on pine growth. Together with the former studies (Poitou et al. 1984; Rodriguez Barreal et al. 1996), the present results may encourage Mediterranean foresters to instigate mycorrhizal inoculation programs using *L. deliciosus*, especially in agricultural lands recently abandoned, where the levels of ECM inocula are potentially very low (Boerner et al. 1992).

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