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ORIGINAL PAPER

Inoculation of *Pinus halepensis* Mill. with selected ectomycorrhizal fungi improves seedling establishment 2 years after planting in a degraded gypsum soil

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Abstract Vegetative inoculum of *Amanita ovoidea* (Bull.) Link and three isolates of *Suillus collinitus* (Fr.) Kuntze, as well as spore inoculum of *Rhizopogon roseolus* (Corda) Th. M. Fr. and *S. collinitus*, were evaluated for the production of *Pinus halepensis* Mill. in nursery and for the establishment of seedlings in a degraded gypsum soil. In nursery, most of the fungi significantly improved the height of seedlings and modified the accumulation of nutrients in needles. The percentage of ectomycorrhizas (ESR) per seedling ranged from 25 to 78%, depending on the fungi. One and 2 years after planting in the field, the survival of seedlings was significantly improved by inoculation with two isolates of *S. collinitus* and with spores of the same fungus. Inoculation with *A. ovoidea* had no significant effect on seedling survival, whilst *R. roseolus* caused a significant mortality of seedlings. Seedling height was significantly improved by inoculation with all fungi except *R. roseolus* and isolate CCMA-1 of *S. collinitus*. One year after planting, mycorrhization of control seedlings was negligible, and percentages of ESR were under 38% for the rest of treatments. In spring of the second year, seedlings in all treatments, including the control, became highly mycorrhizal (60–77% of ESR). Low ectomycorrhizal diversity (five morphotypes described) and seasonal variation on morphotype composition were detected 2 years after plantation. From a perspective of soil restoration management under limiting environmental conditions, nursery inoculation with selected fungi can be a key advantage for tree seedlings to surmount the initial transplant stress, assuring their establishment in the field. Our results emphasise the importance of selecting compatible fungal–host species combinations for nursery inoculation and sources of inoculum adapted to the environmental conditions of the transplantation site.

Keywords Ectomycorrhizas · Afforestation · Soil restoration · Nursery · *Pinus halepensis*

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Introduction

Pinus halepensis is one of the most representative tree species of the Mediterranean Basin. In Spain, this pine has often been used in afforestation and reforestation programmes destined to prevent soil degradation and losses by erosion in semi-arid areas, where gypsum soils are particularly abundant (Maestre and Cortina 2004; Dana and Mota 2006). Physical and chemical characteristics of gypsum soils, such as low water retention, formation of hard surface crusts, poor structure, high infiltration and ion washing and low availability of nutrients, are usually constrictive for the development of trees (Guerrero Campo et al. 1999; Palacio et al. 2007). Together with soil properties, the performance of reforestation tasks in Mediterranean semi-arid areas are frequently limited by the climatic conditions characterised by prolonged dry periods, high temperatures and rainfalls concentrated into a few months. Many studies have focused on the optimisation of nursery practises to produce high-quality seedlings and of site preparation techniques to improve reforestation in Mediterranean semi-arid zones (Querejeta et al. 1998; García et al. 2000; Clemente et al. 2004; Caravaca et al. 2005; Barberá et al. 2005; Rincón et al. 2006).

Inoculation with selected ectomycorrhizal (EM) fungi has often been identified as a promising nursery cultural practise to improve the quality of the seedling stock and its performance after out-planting (Cordell et al. 1987; Le Tacon et al. 1992; Brundrett et al. 1996). EM fungi usually improve the uptake and transfer of nutrients and water to the host plant, decreasing moisture and nutritional stresses, especially under unfavourable environmental conditions (Smith and Read 1997; Simard et al. 2002). In addition, the EM mantle acts as a physical barrier avoiding water losses and root desiccation (Smith and Read 1997). On the other hand, in semi-arid areas, the optimal time for mycelial growth and spore germination is usually shortened, minimising the opportunities for immediate root colonisation by native mycorrhizal fungal propagules (Mousain et al. 1994; Argillier et al. 1997). Under these circumstances, controlled nursery inoculation with suitable EM fungi can be an important initial advantage for the successful establishment of out-planted seedlings.

The host–fungus symbiotic compatibility, the ease of inoculum production and the fungal ecological adaptability to the transplantation site are some important criteria to select EM fungi for nursery mycorrhization programmes (Trappe 1977; Brundrett et al. 1996). *Suillus collinitus* has been the most frequent fungus of choice for the production of *P. halepensis* in nurseries (Torres and Honrubia 1994; Honrubia 2000; González-Ochoa et al. 2003; El Karkouri et al. 2006; Rincón et al. 2006). This fungal species is commonly found in natural forests of *P. halepensis* producing abundant fruitbodies, which makes it easy to obtain spore inoculum for a large-scale application in nursery (Dahlberg and Finlay 1999). Moreover, inoculation with *S. collinitus* has been demonstrated to improve the settlement and performance of *P. halepensis* plantations (Roldán and Albaladejo 1994; Querejeta et al. 1998). Other fungal species like *Pisolithus tinctorius* (Pers.) Coker & Couch, and *Rhizopogon roseolus* (Corda) Th. M. Fr. have also been used as inoculants for producing *P. halepensis* in nursery and tested in field trials (Roldán and Albaladejo 1994; Roldán et al. 1996; Querejeta et al. 1998; Parladé et al. 2004).

The aim of this work was to evaluate different fungi for the production of mycorrhizal *P. halepensis* seedlings in nursery and to check the influence of inoculation on seedling survival and development after planting in a degraded gypsum soil. The mycorrhizal status of seedlings and the EM morphotype composition have been described and monitored for 2 years in the field.

Materials and methods

Fungal material and seedling inoculation

Isolates CCMA-1, CCMA-5 and CCMA-24 of the EM fungus *S. collinitus* and isolate CCMA-44 of *A. ovoidea* were obtained from sporocarps collected in forest of *P. halepensis* at different locations of southeastern Madrid (Spain; Table 1). Pure cultures were grown in solid modified Melin–Norkrans (MMN) medium (Marx, 1969) at 25°C for 1 month. Mycelial inoculum of each isolate was prepared as previously described (Rincón et al. 2001). Briefly, mycelial suspensions of each fungus were obtained by growing in liquid MMN medium (five plugs per 10 ml) at 25°C for 4 weeks. Once grown, 40–60 ml of each fungal suspension were transferred to 1-l bottles containing an autoclaved mixture of peat–vermiculite (50–550 ml) moistened with 350 ml of liquid MMN medium (glucose reduced to 2.5 g l^{-1}). The inocula were incubated at 25°C in the dark for 1 month before use.

Sporocarps of *S. collinitus* were collected under *P. halepensis* plantations close to the studied site in Rivas-Vaciamadrid (southeastern Madrid, Spain; Table 1). Sporocarps of *R. roseolus* were collected in a *Pinus pinea* L. forest at Pelayos de la Presa, southwestern Madrid (Spain; Table 1). After collection, basidiomata were dried at 40°C

Table 1 Fungi used in this study

Fungal species	Isolate number	Herbarium number[a]	Associated tree species	Collection date	Location[b]
Amanita ovoidea	CCMA-44	Hccma-44	*P. halepensis*	14/11/2002	Morata de Tajuña
Rhizopogon roseolus	CCMA-50	Hccma-50/Hspo-50	*P. pinea Quercus ilex*	20/11/2003	Pelayos de la Presa
Suillus collinitus	CCMA-1	Hccma-1	*P. halepensis*	20/11/2000	Rivas Vaciamadrid
Suillus collinitus	CCMA-5	Hccma-5	*P. halepensis*	17/10/2002	Belmonte del Tajo
Suillus collinitus	CCMA-24	Hccma-24	*P. halepensis*	30/10/2002	San Martín de la Vega
Suillus collinitus	CCMA-80	Hccma-80/Hspo-80	*P. halepensis*	26/11/2003	Rivas Vaciamadrid

[a] Voucher herbarium specimens are deposited at IRN-CCMA-CSIC laboratory. *Hccma* A single dried sporocarp from which a pure culture was isolated; *Hspo* a collection of various dried sporocarps of the same fungus to use as spore inoculum
[b] All locations are sited in the province of Madrid.

for 72 h and kept at room temperature for further use (voucher samples were kept at the herbarium of the IRN-CCMA-CSIC). To obtain the spore inoculum, dry mature hymenia (in the case of *S. collinitus*) or entire sporocarps (in the case of *R. roseolus*) were re-hydrated overnight in autoclaved distilled water and blended at low speed until the spores were suspended (Castellano et al. 1985). Initial spore concentration was assessed with a haematocytometer, and the bulk spore suspension was diluted in water to obtain a target suspension of 10^6 spores ml^{-1}.

P. halepensis seedlings were grown in 165-ml containers (Ray Leach C-10 "Cone-tainers"™, Stuewe & Sons, USA) filled with a mixture of soil/vermiculite (1:1, v/v). Soil, collected from the site of the future plantation, was homogenised and autoclaved at 120°C for 40 min before mixing with the autoclaved vermiculite (Termita®, grade 2, Asflatex S.A., Spain). Three *P. halepensis* seeds were sowed per container and thinned to one after germination.

Vegetative inocula of CCMA-1, CCMA-5, CCMA-24 and CCMA-44 were independently applied at the time of sowing and mixed with the substrate at a dose of 1:10 (v/v, inoculum:substrate). Spore inoculum of *S. collinitus* or *R. roseolus* was separately applied to 1-month-old seedlings, adding 10 ml of the target spore suspension at a final inoculum concentration of 10^7 spores per seedling, in both cases. A seventh treatment consisted of control (non-inoculated) seedlings. A total of 100 seedlings were produced per treatment.

Seedlings were fertilised every 2 weeks with 10 ml per seedling of a solution containing 16-5-25 Peter's fertiliser (Scott, Spain; 2.3 g l^{-1}) and the micronutrients preparations Fetrilon (0.12 g l^{-1}) and Hortrilon (0.28 g l^{-1}; Basf, Spain).

Plantation establishment

Eighteen-month-old nursery seedlings were transplanted into an unstructured gypsum soil destined for the creation of an artificial forest park in Rivas-Vaciamadrid (southeastern Madrid, Spain), in November 2004. Soils in this site contained large crystals of gypsum and calcite, and they have been previously classified as Leptosols and Calcic Regosols (FAO 1989; Román et al. 2003). Soil chemical characteristics were: pH 7.7, Organic matter 4.3 g kg^{-1}, C 2.5 g kg^{-1}, N 0.3 g kg^{-1}, SO_4 180 g kg^{-1}, CO_3 270 g $kg^{-1}$and Ca 28 g kg^{-1}, P_2O_5 90 mg kg^{-1}, K 210 mg kg^{-1}, Mg 120 mg kg^{-1}, Mn 36 mg kg^{-1}.

Soil preparation consisted of a superficial tillage done 1 month before planting. Seven inoculation treatments were established: (1–4) vegetative inoculum (CCMA-1, CCMA-5, CCMA-24) and spore inoculum of *S. collinitus* (Sc-spores), (5) vegetative inoculum of *A. ovoidea* (CCMA-44), (6) spore inoculum of *R. roseolus* (Ros-spores) and (7) control non-inoculated seedlings. The seedlings were manually planted in a completely randomised design considering a row of 15 seedlings from each treatment as the experimental unit with five replicates per treatment. The plantation framework was set to 2.5×2.5 m.

Content of nutrients, field monitoring and measured parameters

Before planting, a sample of 15 seedlings per treatment was randomly harvested to estimate the percentage of EM short roots (ESR; see methodology below) and the content of nutrients in needles. Once dehydrated (60°C, 72 h), needles of three seedlings of the same treatment were grouped as a replicate and ground to a particle size less than 2 mm (in total, five replicates per treatment). After digestion of needles by nitric–perchloric acid (5:3), percentage of N was determined by the Kjeldahl method (Isaac and Kerber 1971). The contents of P, K, Mg and Mn were estimated by inductively coupled plasma spectrometry (Optima 4300DV, Perkin-Elmer, UK).

Plant height was measured before planting and every year thereafter for 2 years after plantation in the field. Seedling survival was determined each year after transplanting, for 2 years. One and two samplings were carried out in 2005 (autumn) and 2006 (spring and autumn), respectively. At each sampling, four to five plants per treatment were randomly harvested for EM examination. In total, 24, 26 and 24 seedlings were harvested in autumn 2005, spring 2006 and autumn 2006, respectively. No seedlings were harvested from the Ros-spores treatment.

Ectomycorrhizal assessment

At each sampling, entire roots were separated from shoots, washed free of substrate and cut into 2-cm segments. The percentage of ESR was assessed under the stereo-microscope by counting all single root tips. These were classified as mycorrhizal or non-mycorrhizal according to the presence or absence of fungal mantle and mycelium and to the lack or presence of root hairs, respectively. In each treatment, EM tips were classified by morphotypes based on characteristics of their mantle and extra-matrical mycelium (branching, surface colour, texture, emanating hyphae and rhizomorphs; Agerer 1987–1998, 1995). For each root system, five EM tips of each morphotype were individually sampled and stored at −80°C until used for molecular analyses. EM morphotype composition was described as a relative frequency (expressed as percentage), defined as the absolute frequency of an individual morphotype (the number of

samples in which a morphotype occurred divided by the total number of samples) divided by the sum of absolute frequencies of all morphotypes (Gardes and Bruns 1996; Buée et al. 2005). The relative abundance of ESR of a given morphotype (expressed as percentage) was the estimated number of tips of this morphotype divided by the estimated total number of tips of all morphotypes assessed for all the plants collected at each sampling time (Gardes and Bruns 1996).

Molecular analysis

Deoxyribonucleic acid (DNA) from EM tips was extracted and purified as described by Gardes and Bruns (1993). The internal transcribed spacer region (ITS) of the ribosomal DNA was amplified by polymerase chain reaction (PCR) using the primers ITS1F and ITS4 under the PCR conditions previously described (White et al. 1990). The PCR products were digested by the endonucleases *Hinf*I, *Alu*I and *Taq*I (New England Biolabs, UK), and the restriction patterns (restriction fragment length polymorphisms, RFLPs) were analysed by electrophoresis in 2.5% low-fusion-point agarose gel (MS4, Pronadisa, Spain) in Tris–acetate–ethylenediamine tetraacetic acid buffer. The presence or absence of the restriction fragments obtained with each enzyme was scored for all isolates, and a binary 1/0 matrix was generated for estimating genetic relationships among samples. One representative sample among those sharing an identical RFLP type was chosen for sequencing. The PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, USA). Sequencing reactions were performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Kit (Applied Biosystems, UK) using the primers ITS5, ITS2, ITS3 and ITS4 (White et al. 1990) and analysed on an automatic sequencer ABI PRISM 3730 (Applied Biosystems). For identification, sequences were compared with those of sporophore vouchers and ectomycorrhizas by BLAST searching in the GenBank database.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse data with the SPSS 14.0 software package. Field data of ESR percentages were analysed by two-factor ANOVA taking into account the factors (1) inoculation treatment and (2) time of sampling. Before performing the statistical analysis, percentages of ESR were arc-sin transformed to get homogeneous variances. In all ANOVA analyses, differences among treatments were separated by the least significant difference test ($P \leq 0.05$).

Results

Nursery seedling growth, ectomycorrhizal colonisation and content of nutrients in needles

Eighteen months after growing in nursery, seedling height was significantly improved by inoculation with most of the fungi tested (Fig. 1a). The exceptions were isolate CCMA-1 of *S. collinitus*, which had no effect, and *R. roseolus*, which significantly reduced the growth of seedlings with respect to the control (Fig. 1a). Percentages of ESR ranged from 25 to 78%, depending on the inoculation treatment (Fig. 1b). Nursery-spontaneous mycorrhization of control seedlings was negligible (Fig. 1b).

After growing in nursery, the content of nutrients in needles of seedlings was modified by inoculation, and variable results were obtained depending on the fungus and the nutrient (Table 2). Compared with the control treatment, the content of N in needles was significantly increased by inoculation with spores of *S. collinitus*, whereas the rest of

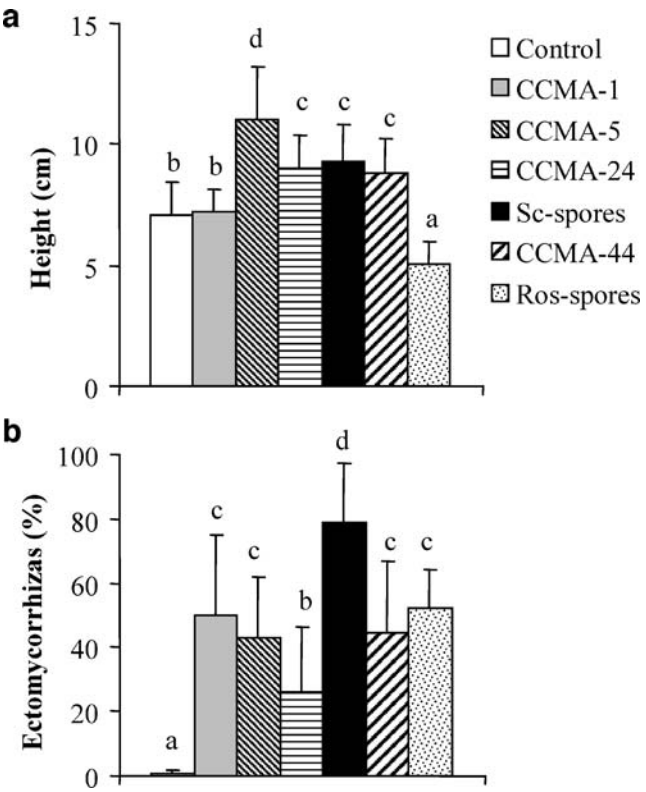

Fig. 1 Effect of inoculation with different ectomycorrhizal fungi on the height (**a**) and the percentage of ectomycorrhizas (**b**) of *P. halepensis* seedlings, after 18 months growth in a nursery. *CCMA-1*, *CCMA-5* and *CCMA-24* Different isolates of *S. collinitus* applied as vegetative inocula, *Sc-spores* spore inoculum of *S. collinitus*, *CCMA-44* vegetative inoculum of *A. ovoidea*, *Ros-spores* spore inoculum of *R. roseolus*, *Control* non-inoculated seedlings. Data are mean±SD. Different letters denote significant differences among inoculation treatments according to the LSD test ($P \leq 0.05$)

Table 2 Effect of inoculation with different ectomycorrhizal fungi on the content of nutrients in needles of *P. halepensis* seedlings, after 18 months growth in a nursery

	N (%)		P (g/kg)		K (g/kg)		Mg (g/kg)		Mn (mg/kg)	
Control	1.3±0.3	ab	0.5±0.1	bc	5.1±0.5	ab	1.5±0.4	a	34.0±1.4	e
CCMA-1	1.1±0.1	ab	0.4±0.1	ab	5.8±0.8	bc	2.8±0.2	d	17.4±2.1	cd
CCMA-5	1.3±0.1	b	0.8±0.1	de	6.4±0.1	c	2.1±0.2	bc	10.2±1.3	a
CCMA-24	1.0±0.1	a	0.7±0.2	cd	6.0±1.3	bc	2.5±0.4	d	15.5±4.0	bc
Sc-spores	1.7±0.2	c	0.7±0.2	cd	5.8±0.9	bc	2.0±0.2	b	17.8±5.1	cd
CCMA-44	1.2±0.1	ab	0.9±0.1	e	6.9±1.2	c	2.4±0.3	cd	12.4±1.5	ab
Ros-spores	1.4±0.1	b	0.3±0.1	a	4.5±0.7	a	1.7±0.4	ab	20.2±4.7	d

Data are mean±SD. For each nutrient, different letters denote significant differences among inoculation treatments according to the LSD test ($P \leq 0.05$). Isolates *CCMA-1*, *CCMA-5* and *CCMA-24* Vegetative inoculum of *Suillus collinitus*; *Sc-spores* spore inoculum of *S. collinitus*, Isolate *CCMA-44* vegetative inoculum of *Amanita ovoidea*, *Ros-spores* spore inoculum of *Rhizopogon roseolus*

the fungi did not affect this nutrient (Table 2). The contents of P and K were significantly increased by inoculation with CCMA-5 and CCMA-44, and the content of P was significantly lowered in seedlings inoculated with spores of *R. roseolus* (Table 2). Inoculation with all fungi (except Ros-spores) significantly increased the content of Mg in needles. In contrast, the content of Mn was significantly reduced in all inoculated seedlings compared to the control ones (Table 2).

Field seedling survival

One year after planting in the field, seedling survival ranged from 16 to 77% depending on the inoculation treatment, and it was maintained, at approximately the same rate in each treatment, 2 years after plantation (Fig. 2a). Seedling survival was significantly improved by inoculation with isolates CCMA-5 and CCMA-24 of *S. collinitus* and with spores of the same fungus (Sc-spores; Fig. 2a). Isolate CCMA-44 of *A. ovoidea* had no significant effect on seedling survival, whilst inoculation with *R. roseolus* significantly reduced this parameter compared with the control treatment (Fig. 2a).

Inoculation with CCMA-5, CCMA-24 and Sc-spores represented a significant gain in seedling survival of 41–63% with respect to the control, 1 and 2 years after planting in the field (Fig. 2b). Inoculation with *A. ovoidea* CCMA-44 ameliorated, although not significantly, the seedling survival by 21–28% with respect to the control seedlings (Fig. 2b). On the other hand, a reduction, although not significant, of 13% on survival gain with respect to the control was recorded for inoculation with isolate CCMA-1 of *S. collinitus*. Furthermore, a drastic and significant mortality of seedlings was produced by inoculation with *R. roseolus* (a decrease of 66–76% on survival gain with respect to the control, after 1 or 2 years, respectively, Fig. 2b). Because of the high mortality of the Ros-spores treatment, it was not considered for subsequent field analyses.

Field seedling height

Inoculation with isolates CCMA-5 and CCMA-24 of *S. collinitus* and with spores of the same fungus significantly improved the height of seedlings 1 and 2 years after plantation in the field (Fig. 3). A significant effect on

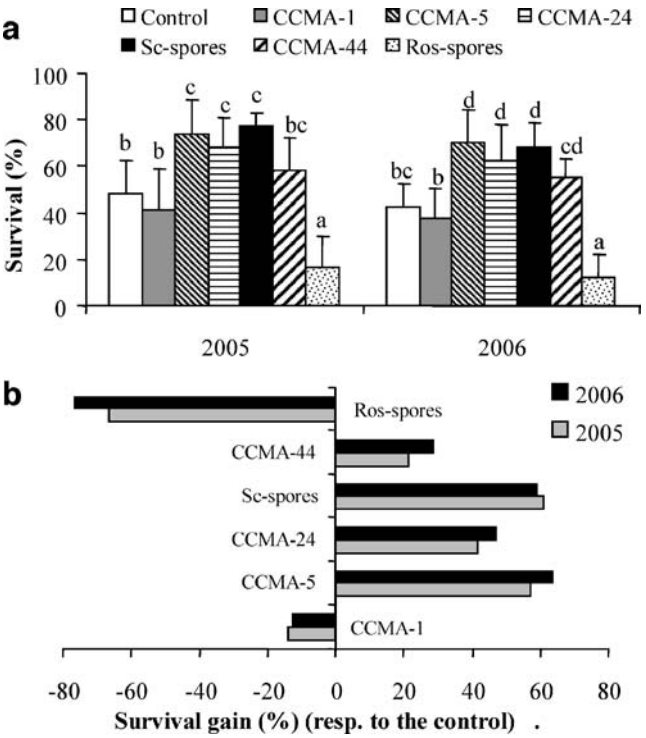

Fig. 2 Field survival of *P. halepensis* seedlings inoculated with different ectomycorrhizal fungi (**a**) and survival gain with respect to the control seedlings (**b**), 1 and 2 years after transplanting to a degraded gypsum soil. Data are mean±SD. In each year (**a**), different letters denote significant differences among inoculation treatments according to the LSD test ($P \leq 0.05$). *CCMA-1*, *CCMA-5* and *CCMA-24* Different isolates of *S. collinitus* applied as vegetative inocula, *Sc-spores* spore inoculum of *S. collinitus*, *CCMA-44* vegetative inoculum of *A. ovoidea*, *Ros-spores* spore inoculum of *R. roseolus*, *Control* non-inoculated seedlings

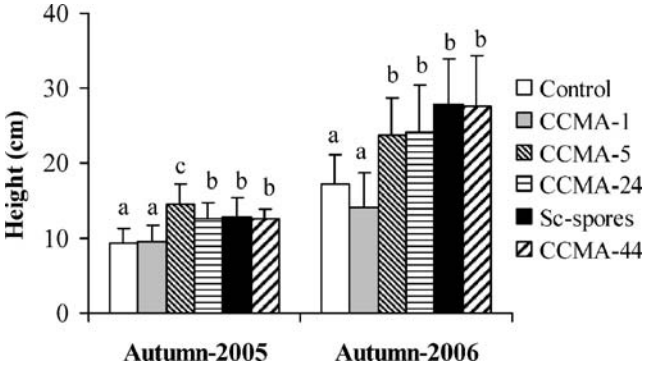

Fig. 3 Effect of inoculation with different ectomycorrhizal fungi on seedling height 1 and 2 years after planting in a degraded gypsum soil. Data are mean±SD. Different letters denote significant differences among inoculation treatments according to the LSD test ($P \leq 0.05$). *CCMA-1*, *CCMA-5* and *CCMA-24* Different isolates of *S. collinitus* applied as vegetative inocula, *Sc-spores* spore inoculum of *S. collinitus*, *CCMA-44* vegetative inoculum of *A. ovoidea*, *Ros-spores* spore inoculum of *R. roseolus*, *Control* non-inoculated seedlings

seedling height was also obtained with isolate CCMA-44 of *A. ovoidea*, whilst inoculation with isolate CCMA-1 of *S. collinitus* had no effect on this parameter (Fig. 3).

Ectomycorrhizal colonisation of seedlings in the field

The factor 'inoculation treatment' had no significant effect on the percentage of ESR of seedlings ($P=0.395$; Fig. 4a), whereas the 'time of sampling' had a significant effect ($P=0.000$; Fig. 4b). Significant interactions between factors were not detected ($P=0.193$). One year after plantation in the field, the percentages of ESR were low in all treatments (between 13 and 38%), and control seedlings were scarcely colonised by native EM fungi (1,2%). However, 2 years after planting, seedlings in all treatments (even the control) showed significantly higher percentages of ESR (Fig. 4b). Seasonal variation of ESR of *P. halepensis* seedlings showed significantly higher levels in spring (Fig. 4b).

Ectomycorrhizal morphotypes

Only five EM morphotypes were described for all the different field samplings, and their identity was estimated by sequence comparison with closely related sequences in the Genbank database (Table 3). Types I and V matched with unknown ascomycetes, type II with *S. collinitus*, type III with *Tomentella* sp. and type IV with *Geopora* sp (Table 3). None of the morphotypes described was coincident with characteristics describing *A. ovoidea*.

Because no significant differences on ESR as a result of the inoculation treatment were observed (Fig. 4a), the relative frequency and the relative abundance of ESR of each morphotype were calculated taking into account all the seedlings at each sampling time. In autumn of 2005, the fungi corresponding to types I and IV showed the highest relative frequencies on *P. halepensis* seedlings, followed by type II (Table 4). Types III and V were scarcely representative, being only found on one seedling out of the 24 sampled (Table 4). The relative abundance of ESR formed by types I, II and IV was between 21 and 35% (Table 4). Type V was only found in the sampling of autumn 2005. In spring of 2006, type II corresponding to *S. collinitus* was the most frequent fungal morphotype found in association with *P. halepensis* seedlings, followed by type I. The relative abundance of ESR for types II and I was of 51.9 and 23.5%, respectively, whereas it was under 13% for types III and IV (Table 4). In autumn of 2006, Types II and IV were the most representative fungal morphotypes forming ectomycorrhizas with *P. halepensis* seedlings, followed by types I and III. The relative abundance of ESR was between 21 and 32% for all the morphotypes (Table 4).

Discussion

In nursery, the height of *P. halepensis* seedlings was significantly enhanced by inoculation with almost all the fungi tested. When having an effect, inoculation increased

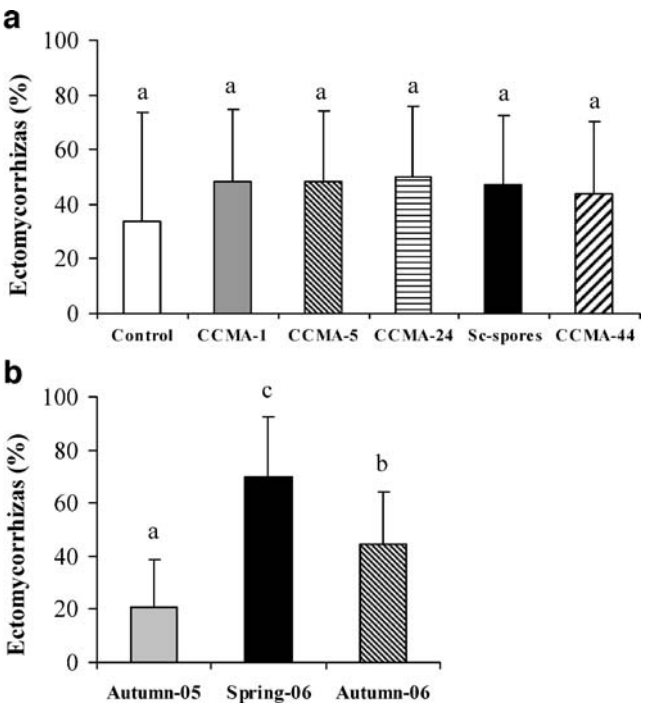

Fig. 4 Effect of the factors 'inoculation treatment' (**a**) and 'season of sampling' (**b**) on the percentage of ectomycorrhizas of *Pinus halepensis* seedlings 1 and 2 years after planting into a degraded gypsum soil. All data are mean±SD. Different letters denote significant differences among treatments according to the LSD test ($P \leq 0.05$). *CCMA-1*, *CCMA-5* and *CCMA-24* Different isolates of *S. collinitus* applied as vegetative inocula, *Sc-spores* spore inoculum of *S. collinitus*, *CCMA-44* vegetative inoculum of *A. ovoidea*, *Ros-spores* spore inoculum of *R. roseolus*, *Control* non-inoculated seedlings

Table 3 Molecular analysis of ectomycorrhizal morphotypes found in association with *P. halepensis* in a young plantation set in a degraded gypsum soil

Morphotype	ITS size (bp)	RFLP fragment size (bp)			Genbank accession number	Closest genbank match	Identity (%)
		*Hinf*I (G/ ANTC)	*Alu*I (AG/ CT)	*Taq*I (T/CGA)			
Type I	608	116/132/150/ 202	158/450	59/90/125/159/ 175	EF484931	AJ410863 Unknown ascomycete	99
Type II	747	75/85/117 149/241	112/635	60/66/81/87/ 96/322	EF484932	AY935515 *Suillus collinitus*	98
Type III	715	180/346	196/ 500	59/308/348	EF484933	AY953422 *Tomentella* sp.	99
Type IV	668	165/200 /295	No cut	59/109/135/312	EF484934	AF387651 *Geopora* sp.	91
Type V	634	69/223/334	No cut	59/241/281	EF484935	AY634115 Unknown ascomycete	81 (4e−128)

the accumulation of nutrients in needles, except for manganese, which was significantly reduced by all fungi. The different foraging demands among fungal species or isolates for hyphal development and EM establishment could explain the variable rates of nutrient transfers to the host (Olsson et al. 2002). Mycorrhization can alter the rhizospheric bacterial community by changing the composition of root exudates, which can then modify the availability of nutrients for the plant (Marschner 1986; Simard et al. 2002). This seems to be particularly the case for manganese availability (Posta et al. 1994; Grayston et al. 1996). Gypsum soils are characterised by the low availability of nutrients such as N and P (Palacio et al. 2007). Most of the fungi tested ameliorated the availability of nutrients for the plant, increasing in some cases the levels of N and P in needles and in all cases the level of Mg. However, plant nutritional benefits because of EM inoculation are usually better evidenced under nutrient-limiting conditions (Smith and Read 1997; Simard et al. 2002), and in our nursery experiment, seedling nutrient availability was assured by fertilisation, supplied at adjusted dosages to optimise the formation of ectomycorrhizas (Rincón et al. 2007).

Two years after transplanting to a degraded gypsum soil, variable effects of inoculation on seedling survival and growth were observed, depending on the fungus. Inoculation with spore and vegetative inocula of *S. collinitus* significantly improved seedling height and survival, except in the case of isolate CCMA-1. High intra-specific variation within *Suillus* strains for different physiological traits, as well as for their effect on plant development, has been previously reported and used as criteria for the selection of fungal isolates (Dahlberg and Finlay 1999; Manian et al. 2001; Ruiz-Díez et al. 2006). When comparing both the vegetative (isolates CCMA-5 and CCMA-24) and the spore inocula of *S. collinitus*, similar results in effectiveness were obtained. From a practical point of view, the ease of spore application makes this kind of inoculum more feasible for its use in large-scale commercial nurseries (Brundrett et al. 1996, 2005).

Inoculation with *A. ovoidea* (CCMA-44) significantly improved the nursery development of seedlings indicating a high compatibility with *P. halepensis*. This growth improvement was maintained after 2 years of seedling establishment in the field, but seedling survival was not significantly enhanced by inoculation with this fungus. *Amanita ovoidea* is a fungal species well-adapted to calcareous soils and usually found in mature forests of *P. halepensis*, probably as a late-stage coloniser. Because no morphotype described in our samplings was coincident

Table 4 Ectomycorrhizal morphotype composition of *Pinus halepensis* seedlings 1 and 2 years after transplanting to a degraded gypsum soil

Morphotype	Autumn, 2005 (n=24)		Spring, 2006 (n=26)		Autumn, 2006 (n=24)	
	RF (%)	RA-ESR (%)	RF (%)	RA-ESR (%)	RF (%)	RA-ESR (%)
Type I	37.5 (9)[a]	21.8	24.4 (10)	23.5	15.6 (5)	25.7
Type II	20.8 (5)	34.5	51.2 (21)	51.9	37.4 (12)	21.8
Type III	4.1 (1)	10.3	12.2 (5)	12.3	12.5 (4)	31.4
Type IV	33.3 (8)	26.4	12.2 (5)	12.3	34.5 (11)	21.1
Type V	4.1 (1)	6.9	0.0 (0)	0	0.0 (0)	0

RF Relative frequency (%), *RA-ESR* relative abundance of ectomycorrhizal short roots
[a] The number of plants in which a morphotype occurred is indicated in parenthesis.

with *A. ovoidea*, it could have been rapidly replaced by more opportunistic fungi after out-planting in the degraded gypsum soil. To our knowledge, this is the first time that vegetative inoculum of *A. ovoidea* has been tested for producing mycorrhizal *P. halepensis* seedlings in a nursery.

R. roseolus limited the development of *P. halepensis* in nursery and caused a drastic mortality of seedlings after transplanting to the field. This fungus has been repeatedly demonstrated to be highly efficient for nursery production and reforestation of other Mediterranean pine species such as *P. pinea* and *Pinus pinaster* Ait. (Parladé et al. 1996, 2004; Rincón et al. 2001, 2005). Similarly, to what happened in our study, nursery inoculation of *P. halepensis* with *R. roselus* has been reported to be ineffective for promoting seedling growth and nutrient accumulation (Roldán and Albaladejo 1994; Torres and Honrubia 1994). Neutral effects of inoculation with *R. roseolus* on *P. halepensis* development have also been detailed in afforestation trials (Roldán and Albaladejo 1994; Parladé et al. 2004). In our study, the high mortality caused by this treatment in the field could be due to an excessive cost to the seedlings for the maintenance of their fungal partner. Because spores of *R. roseolus* were obtained from an acidic soil in a *P. pinea* forest, the ecological adaptability of this fungus could have been minimised in gypsum soil.

Taken together, these results emphasise the importance of selecting compatible fungal–host species combinations for nursery inoculation programmes, as well as using sources of fungal inocula that are adapted to the environmental conditions of the future transplantation sites.

In the nursery, the highest percentages of ESR were obtained with spore inoculum of *S. collinitus*, and one of the lowest rates was obtained with vegetative inoculum of isolate CCMA-24 of the same fungus. However, in both cases, significant increases in seedling growth and survival were obtained after transplanting to the field. These results, together with those obtained with *R. roseolus* (53% ESR and a drastic field mortality of seedlings), indicated a low relationship between the initial percentages of ESR and the fungal effect on plant development, at least under the experimental conditions of our study. The minimal level of ESR necessary to ensure an effect on plant development in the field has been poorly studied, and it seems to be highly dependent on the environmental characteristics of the transplantation site and on the fungal–host species combination (Marx 1991; Smith and Read 1997).

One year after planting, low ESR percentages were obtained in all treatments, and control seedlings showed negligible colonisation by native fungi. For most conifers, the development of new short roots, which are susceptible to fungal colonization, is minimal during the first year of plantation, a period that is usually coincident with the maximum level of transplant shock for seedlings (Grossnickle 2005; Parladé et al. 2004). During this period, previous nursery inoculation with EM can be crucial for seedlings to limit nutritional and moisture stresses and overcome the transplant stress. During the second year, seedlings were highly mycorrhizal in all treatments, and certain seasonality could be observed, with the ERS being higher in spring, when a greater root development for *P. halepensis* can be expected.

Only five RFLP types were described in our field surveys, one identified as *S. collinitus*, another as *Tomentella* sp. and the rest as probable ascomycetous fungi (two unknown and one identified as *Geopora* sp.). Low EM diversity has been reported in young plantations of *P. halepensis* (El Karkouri et al. 2004), and ascomycetous fungi are frequently found as earlier colonisers in young conifer plantations and disturbed sites (El Karkouri et al. 2004; Fujimura et al. 2005; Thedesshoo et al. 2006). Two years after transplanting, all seedlings, included the control ones, were colonised by native EM fungi, with *S. collinitus* and *Geopora* sp. being the most representative morphotypes in all treatments. The high potential of native *Suillus* soil inoculum could be due to the proximity of a *P. halepensis* forest in the zone and to the high dispersal ability of *Suillus* species by the production of airborne spores (Dahlberg and Finlay 1999). Thereafter, meiotic sporulation and mating could result in the substitution of the introduced inoculant strains by more competitive ones (Selosse et al. 1999). We are currently conducting additional molecular analyses to verify the persistence in the field of the introduced *S. collinitus* isolates. In a similar study, El Karkouri et al (2006) demonstrated by molecular fingerprinting analysis the persistence of an introduced *S. collinitus* strain in a *P. halepensis* plantation after 4 years.

From a perspective of soil restoration management under limiting environmental conditions, nursery inoculation with selected EM fungi can be considered a powerful tool for minimising the seedling losses caused by the transplant stress and for assuring their later development in the field. Ideally, the selection of EM inoculants should be guided by searching for fungi with consistent abilities for initial root colonisation, benefits to the host and ecological adaptability to the transplantation site.

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