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ORIGINAL PAPER

Molecular characterization and evaluation of mycorrhizal capacity of *Suillus* isolates from Central Spain for the selection of fungal inoculants

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Abstract *Suillus* fungal specimens of pine forests from a Mediterranean area of central Spain (Madrid region) were studied based on molecular and physiological analysis of sporocarps to obtain fungal native inocula to produce mycorrhizal *Pinus halepensis* Miller in nursery. Variation within the internal transcribed spacer (ITS) region of the ribosomal RNA genes of *Suillus* was examined by restriction fragment length polymorphism (RFLP) and direct sequencing of polymerase chain reaction products. Ribosomal DNA (rDNA) spacers were amplified from pure cultures obtained from fruit bodies of a range of *Suillus* species: *Suillus bellinii* (Inzenga) Watling, *Suillus bovinus* (Pers.) Kuntze, *Suillus collinitus* (Fr.) Kuntze, *Suillus granulatus* (L.) Snell, *Suillus mediterraneensis* (Jacquet. & Blum) Redeuil, *Suillus luteus* L. (Gray), and *Suillus variegatus* (Sw.) Kuntze. Interspecific variation in the length and number of restriction sites of the amplified ITS region was observed. This variation was confirmed by sequencing, which allowed us to identify some isolates. This is the first time that the ITS sequence of *S. mediterraneensis* is completely described. No intraspecific rDNA variation was observed within isolates of *S. collinitus*, *S. mediterraneensis*, and *S. luteus*. The phylogenetic analysis established the close relationship among these Mediterranean fungal species. As a further step to characterize the different isolates and to understand the relation between genetic and functional diversity, some physiological variables were evaluated. Intraspecific variation in axenic fungal growth and in mycorrhizal capacities was detected, especially within *S. collinitus* isolates. The fungal isolates stimulated the growth of *P. halepensis* in different rates. These studies indicated that ITS analysis, in conjunction with mycorrhizal tests, provides suitable combined tools for the analysis of *Suillus* spp. in a small geographic area for selecting isolates with final afforestation purposes.

Keywords Ectomycorrhizal inocula · ITS · Mediterranean area · Mycorrhization · *Pinus halepensis* · *Suillus*

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Introduction

Ectomycorrhizal (ECM) fungi have great value in reforestation practices mainly by facilitating the establishment of tree plantations in degraded zones (Smith and Read 1997). These fungi can be considered as one of the metabolically more active components of forest ecosystems, being especially relevant for the assimilation/uptake of limiting nutrients such as phosphorus and nitrogen. Before the afforestation of a defined area, it is very important to identify and select the native ECM fungi to produce effective inocula for suitable tree establishment in the field. ECM fungi exist as a complex of ectomycorrhizas, extraradical mycelia, and sporocarps. Sporocarp surveys allow us to identify the species level, in most cases, and the isolation of pure cultures, which can be employed as inoculants of plant seedlings before outplanting into the same area (e.g., Rincón et al. 2001).

Suillus species present a high degree of host specificity toward conifers and their distribution coincides with the

natural distribution of Pinaceae (Dahlberg and Finlay 1999). Surveys in pine forests of Madrid region have confirmed that *Suillus* sporocarps are among the prominent fruiting ECM fungal species in Mediterranean pine forests (Rincón et al. 2006). In contrast to other ECM species, the mycelia of *Suillus* are usually easy to culture and this advantage was exploited in different studies, from ECM in vitro synthesis trials to fungal population dynamics (Manian et al. 2001; Rincón et al. 2005; Wu et al. 2000).

Different attributes of ECM fungi such as morphology, growth rates, and mycorrhizal ability may be modified in a natural environment (González-Ochoa et al. 2003; Jacobson and Miller 1992; Sen 1990) and this is especially the case of *Suillus* spp., which exhibit a considerable intraspecific variation for a broad range of physiological traits (Cazzoli 2002; Dahlberg and Finlay 1999). These features can differentiate isolates within the same species and could be used to select isolates for inocula production in nursery (Brundrett et al. 1996; Trappe 1977). The aspect of ECM fungal host specificity could indicate coadaptation of the fungus to certain soil and environmental conditions (Cairney 1999; Graham and Miller 2005).

In studies of fungal ecology, the application of molecular methods for identification of ECM fungi has complemented classical methods where only a part of the ECM fungal taxa could be identified using sporocarps (reviewed in Graham and Miller 2005). Different investigations focusing on *Suillus* species have provided a phylogenetic framework for biogeographical analyses and demonstrated the advantage of using the nuclear ribosomal RNA region, which spans the two internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) sequences to diagnose and infer phylogenetic relationships at the species level in this genus (Horton 2002; Kretzer et al. 1996; Manian et al. 2001; Wu et al. 2000). Restriction fragment length polymorphism (RFLP) and sequence analysis of the ITS region have proven to be helpful techniques for relating mycorrhizas to sporocarps and successfully monitoring inoculated fungi after outplanting (Horton 2002; El Karkouri et al. 2004).

In the work described here, we have used a combination of ITS–RFLP and sequence analysis to study the genetic variation within pure isolates of *Suillus* spp. that are representatives of different pine forests in a Mediterranean region of central Spain, and performed a phylogenetic analysis to determine their relationship. As a further step to characterize the different isolates, their growth was evaluated, the in vitro mycorrhizal capacity of the different isolates was measured, and their benefits for the growth of a typical Mediterranean pine species (*Pinus halepensis* Miller) were determined. The overall aim of such analyses is to select isolates that are best adapted to the environmental conditions of the specific geographic area to produce fungal inoculants and obtain nursery mycorrhizal *P. halepensis* seedlings for afforestation.

Materials and methods

Fungal isolates

Fruit bodies of all *Suillus* species listed in Table 1 were collected in young pine forests of the Madrid region in autumn 2002 and 2003. Pure cultures were obtained from sporocarp tissue and grown on modified Melin–Norkrans medium (MMN) agar (Marx 1969). Dry specimens (45°C for 3 days) and pure cultures were deposited in the ECM fungi collection of Centro de Ciencias Medioambientales (CCMA-CSIC). The isolate CCMA-02, employed as the reference strain, was obtained from the Departamento de Protección Vegetal, Instituto para la Investigación y Technología Agroalimentaria collection (Cabrils, Barcelona, Spain).

DNA extraction

Fungal DNA was isolated from pure cultures grown on MMN agar using a rapid extraction procedure. Briefly, a maximum of 20–40 mg mycelium removed from agar were frozen with liquid nitrogen and genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) protocol according to Lanfranco et al. (1998). The genomic DNA was further purified by treatment with RNAse A and proteinase K (Ruiz-Díez et al. 1997). Concentration and integrity of the DNA samples was analyzed by electrophoresis through 0.8% agarose gels, staining with ethidium bromide, and by comparison with known amounts of phage lambda DNA (Sambrook et al. 1989).

PCR amplification

Fungal rDNA was amplified by polymerase chain reaction (PCR) as previously described (White et al. 1990) with modifications. The primers used were ITS5 (White et al. 1990) and ITS4B (Gardes and Bruns 1993) and PCR was carried out in a volume of 50 µl containing 3 mM $MgCl_2$, 50 mM KCl, 15 mM Tris–HCl (pH 8.0), 200 µM each of diethylnitrophenyl thiophosphate (Applied Biosystems, Roche), 0.5 µM of each primer, 1.25 U of *AmpliTaq* Gold DNA polymerase (Applied Biosystems), and 0.5–1 ng of genomic DNA. Amplification was performed in a thermal cycler (PCR Express, Hybaid, UK) with parameters of a initial denaturation of 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 2 min, annealing at 54°C for 30 s, and extension at 72°C for 2 min, with final extension at 72°C for 10 min at the end

of the amplification. Negative controls without DNA were performed in each experiment. The PCR products were analyzed by electrophoresis on 0.8% agarose gels (Sambrook et al. 1989).

Restriction analysis

The restriction endonucleases *Alu*I, *Hin*fI, and *Taq*I (GibcoBRL) were used in separate digestion reactions with PCR-amplified rDNA fragments from isolates listed in Table 1. A 15-µl portion of DNA from amplification reactions was digested according to the manufacturer's recommendations (GibcoBRL). The digested DNA was electrophoresed in 3% agarose MS-4 plus 0.3% agarose D-1 low EEOO (both from Pronadisa) in 1×TAE buffer [0.1 M Tris–HCl, 12.5 mM sodium acetate, 1 mM EDTA (pH 8.1)] for 5 h at 40 V.

DNA sequencing

Before sequencing, the amplified ITS–rDNA complex (ITSI–5.8S–ITSII) from each of the *Suillus* isolates (Table 1) was purified with EZNA CYCLE-PURE kit (Omega). Sequences of PCR products were obtained with an ABI PRISM 3700 (Applied Biosystems) sequencer using the *Taq* Dyedeoxi Terminator cycle systems in the Automatic Sequencing Service of Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas (Madrid). Two different PCR products from each fungus were sequenced to confirm the sequence. All products were sequenced using each of the primers ITS5 and ITS4B. In some cases, it was necessary to employ internal primer (White et al. 1990) ITS2 and its reverse complement ITS3 to complete the sequence.

Table 1 Isolates and molecular analyses of the different *Suillus* used in this study

Suillus species	Isolate (CCMA-number)	*Pinus* host species	Collection forests[a]	ITS-RFLP		ITS accession number (GenBank)
				Pattern[b]	genotype	
S. granulatus	0–2	*P. sylvestris*	Barcelona	aaa	1	AY898617
S. collinitus	1	*P. halepensis*	S–E	abb	2	AY935515
S. collinitus	5	*P. halepensis*	S	abb	2	DQ440567
S. luteus	14	*P. pinaster*	N–W	aac	3	AY898618
S. bellinii	22	*P. pinaster*	S–W	aac	3	AY898621
S. collinitus	24	*P. halepensis*	S–E	abb	2	AY935519
S. mediterraneensis	26	*P. halepensis*	S–E	aca	4	AY935512
S. mediterraneensis	27	*P. halepensis*	S–E	aca	4	AY935513
S. luteus	35	*P. sylvestris*	N	aac	3	AY898619
S. luteus	37	*P. pinaster*	N	aac	3	DQ440568
S. collinitus	46	*P. halepensis*	S–E	abb	2	AY935516
S. luteus	57	*P. pinaster*	N	aac	3	AY898620
S. variegatus	58	*P. sylvestris*	C	bdd	5	AY898622
S. bovinus	67	*P. sylvestris*	C	bed	6	AY898623
S. collinitus	73	*P. halepensis*	S–E	abb	2	DQ440569
S. collinitus	75	*P. halepensis*	S–E	abb	2	DQ440570
S. collinitus	77	*P. halepensis*	S–E	abb	2	DQ440571
S. collinitus	79	*P. halepensis*	S–E	abb	2	AY935517
S. collinitus	80	*P. halepensis*	S–E	abb	2	AY935518

[a] All isolates with the exception of CCMA-02 were collected in the Madrid region.

[b] Three letters were arbitrarily assigned to represent specific fingerprinting pattern obtained from RFLP analysis of ITS rDNA digested with restriction endonucleases *Alu*I, *Hin*fI, and *Taq*I, respectively. Different numbers were assigned to represent each RFLP group.

C Center, *E* east, *N* north, *S* south, and *W*=west

Analysis of DNA sequences

Sequence similarities were identified by pairwise alignments employing the Clustal W program, and were analyzed by comparison with those in databases using the Basic Local Alignment Search Tool program (GenBank). Similarity of ITS sequences was calculated with Clustal W multiple alignment (http://npsa-pbil.ibcp.fr/). Phylogenetic analyses were performed using the TREECON program, version 1.3b and the phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei 1987) based on the two parameter distance model of Kimura (1980). To assess the relative support for each clade, bootstrap values were calculated from 1,000 replicated analysis.

Evaluation of fungal growth

The growth of each isolate was measured in axenic conditions using MMN agar as culture medium and four replicates per isolate. Briefly, fungal plugs were grown on a cellophane sheet, in 90-mm Petri dishes at 25°C, in the dark. Fungal radial growth (mean of the four values for each colony) and dry weight were recorded after 4 weeks.

In vitro mycorrhizal synthesis

The growth of *P. halepensis* inoculated with the different *Suillus* isolates was performed as previously described (Rincón et al. 2005). Briefly, *P. halepensis* seeds were surface sterilized in 33% (v:v) H_2O_2 for 35 min and rinsed several times with sterile distilled water. Thereafter, they were sown in 1.5% water–agar plates. Tubes (100 cm^3) filled with peat, vermiculite 1:10 (v:v) and 25 ml of liquid MMN (glucose reduced to 2.5 g l^{-1}), were inoculated with liquid cultures of each *Suillus* isolate listed in Table 1. After 1 month when the fungus had colonized the substrate, the pregerminated seedlings (1- to 2-cm roots) were transferred into the tubes and the roots were protected from direct light by wrapping the bottom half of the tubes with aluminum foil. Four replicates were established per each fungal isolate and four seedlings without fungus as controls. Plants were incubated in a growth chamber (15 h photoperiod, 250 μmol photon m^{-2} s^{-1}, day/night temperature of 25/20°C) for 3 months.

Mycorrhizal percentages (ECM short roots to total number of short roots) were counted under the stereomicroscope. To quantify the influence of mycorrhization on pine growth, the number of lateral roots, root length, number of needles, and epicotyl length all seedlings were measured. Seedling shoots were oven-dried to determine shoot dry weight.

Statistical analysis

Data were analyzed by one-way ANOVA and differences among treatment means were separated by the least significant difference (LSD) test ($P \leq 0.05$) (Steel and Torrie 1980). When it was necessary, data were log-transformed to meet the assumptions of normality and homogeneity of variance. Percentages of mycorrhizas were arcsine-transformed before ANOVA.

Results and discussion

Molecular analysis of *Suillus* isolates

PCR products from the ITS region consisted of a single band of an approximate size of 800 bp for all the *Suillus* isolates. Isolates identification was confirmed by greater than 98% sequence similarity with closely related fungal specimens (Kretzer et al. 1996; Manian et al. 2001). To our knowledge, this is the first time that the ITS sequence of *S. mediterraneensis* is entirely described. Accession numbers for sequences deposited in the GenBank database are given in Table 1. Sequence analysis allowed proper identification of some isolates, which had been previously misclassified by morphological methods as *Suillus* spp. or as erroneous *Suillus* species. For example, CCMA-14 was identified as *S. luteus* (with 99% homology), CCMA-22 as *S. bellinii* (99% homology), and CCMA-46 as *S. collinitus* (98–100% homology). Restriction enzyme digests of the ITS–rDNA products for 19 *Suillus* isolates (Table 1) with *Alu*I, *Hin*fI, and *Taq*I gave a combination of patterns for the three enzymes, which separated the isolates into six different groups (Table 1). When comparing DNA sequences and RFLPs (Table 1), the *Suillus* spp. could be reliably distinguished by RFLP with the exception of *S. bellinii* (CCMA-22), which gave a similar overall pattern to *S. luteus* (Table 1).

The ITS region is generally polymorphic between species and intraspecific variability is infrequent in fungi (Horton 2002; Leonardi et al. 2005; Manian et al. 2001; Mello et al. 2005; Ruiz-Díez and Martínez-Súarez 1999). The sequenced isolates with the same RFLP pattern showed identical or almost identical nucleotide composition. A similarity percentage of 99.35% (607 conserved positions out of 611 alignment length) was found within isolates of *S. collinitus*. The two isolates of *S. mediterraneensis* were identical with 99.74% homology (378 conserved positions out of 379 alignment length). The alignment of *S. luteus* sequences did not show any polymorphism (99.83% homology: 603 conserved positions out of 604 alignment length). These facts contrast with previous reports that found intraspecific variability among isolates of *S. luteus*

based on similar molecular approaches (Kretzer et al. 1996; Manian et al. 2001) and analyzing *Suillus* species from different European and American locations. On the other hand, species-specific conservation of ITS–RFLP types was found in strains of distinct *Suillus* spp. (*Suillus albidipes* (Peck) Singer, *Suillus brevipes* (Peck) Kunt, *Suillus tomentosus* (Kauffman) Singer, and *Suillus umbonatus* (Dick & Snell) collected at a local scale (Horton 2002). The *Suillus* strains characterized in the present study came from different pine forests of the Madrid region and were isolated over various years, suggesting a conserved regional distribution of each *Suillus* spp. and probably a clonal origin of the different fruit bodies isolated over a relatively limited area. As the identification of fungal individual is a main goal of population genetics, the next objective of our work will be the search of new molecular markers, which could delineate the *Suillus* populations within each particular species in small regions.

The neighbor-joining tree method, which represents the best estimate for studying the phylogeny within *Suillus* spp. (Kretzer et al. 1996), was used to construct a phylogenetic tree (Fig. 1) with sequences of the entire ITS complex. Available sequences from fruit bodies of different *Suillus* spp. from European locations were included. Sequences were aligned in all possible pairwise combinations. Reanalysis of the data set with a high gap penalty did not significantly alter the tree topology. The phylogenetic analysis showed that the 25 *Suillus* isolates were grouped into nine clades, as supported by the 100% bootstrap values. The maximum parsimony analysis yielded a similar topology (data not shown). The analysis demonstrated that the different *Suillus* spp. isolated in Madrid region clustered properly with other European examples of the same species (Fig. 1). Different isolates of the same species grouped together irrespective of their origin, confirming the low variability found in the ITS sequences. The *Suillus* spp. were divided into two clearly distinguished clusters, reinforced by high bootstrap values (100%). *Suillus* spp. described as more typical of the Mediterranean area (*S. bellinii*, *S. collinitus*, *S. luteus*, and *S. mediterraneensis*) clustered closely together. *S. mediterraneensis*, which was only described, to date, in Italy, France, and Spain (Cazzoli 2002; Moreno et al. 1995), stood apart within this cluster (supported with 80% bootstrap). Species belonging to the

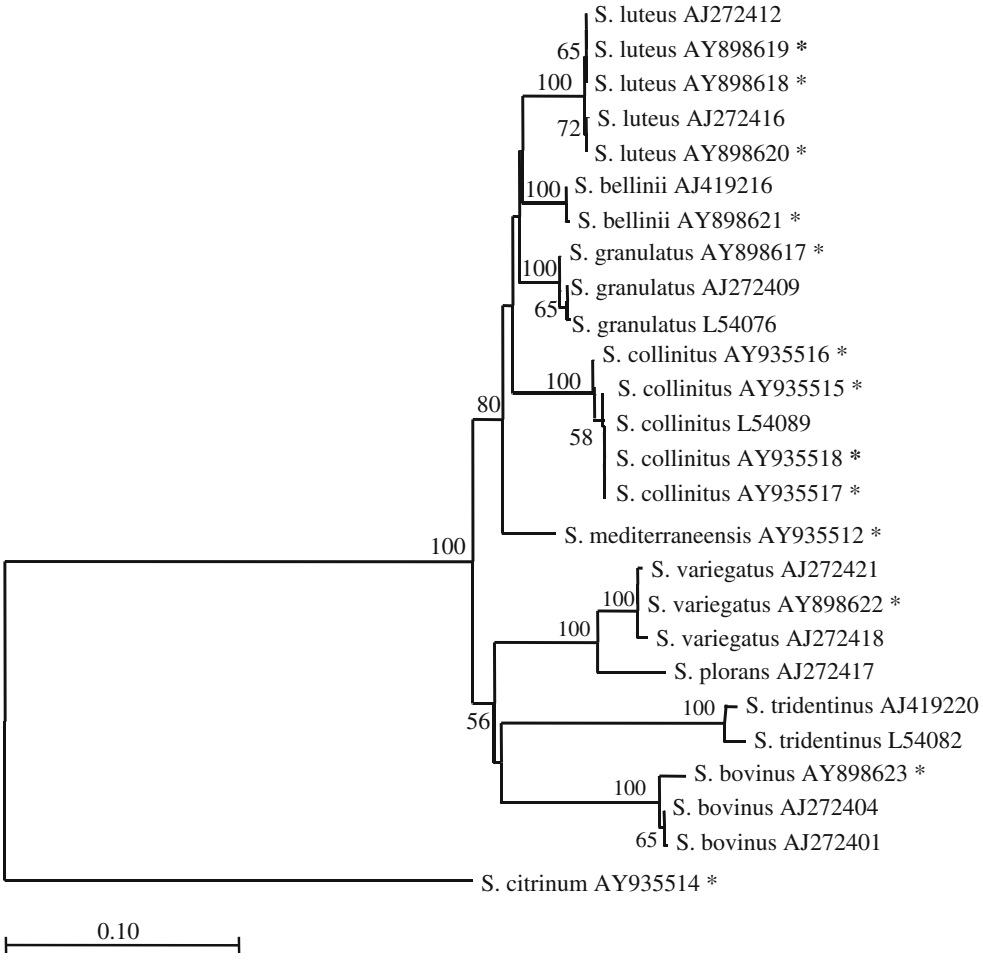

Fig. 1 Phylogenetic tree derived from the neighbor-joining analysis of Madrid region *Suillus* spp. isolates and other European *Suillus* spp. inferred from ITS sequence data. *Numbers above the branches* are bootstrap values based on 1,000 replicates. The scale below the tree measures the distance among sequences. *Scleroderma citrinum* (CCMA-21, AY935514) was used as outgroup (the *asterisk* indicates the sequences obtained in this study)

Table 2 Fungal growth on MMN medium evaluated as the means of mycelium dry weight and colony radius

Suillus species	Isolate (CCMA-number)	Mycelium dry weight (mg)	Colony radius (mm)
S. collinitus	1	19±3.2 bc	22.3±0.7 bc
	5	13±5.0 a	17.1±4.4 a
	24	25±3.7 de	31.2±3.5 e
	46	27.6±1.4 e	37.5±2.9 f
	73	21.4±1.1 bcd	21.7±1.2 bc
	75	22.1±2.7 cd	25.2±0.7 cd
	77	25.8±3.5 de	26.8±2.5 d
	79	19.9±2.8 bc	18.3±3.2 ab
	80	17.5±2.5 ab	21.5±1.8 bc
S. luteus	14	26.6±2.8 a	29.7±3.3 a
	35	26.5±5.4 a	29.4±2.4 a
	37	22.7±2.6 a	40.9±1.3 b
	57	23.2±3.6 a	30.3±1.0 a
S. mediterraneensis	26	25.2±2.7 a	24.3±5.3 a
	27	23.5±0.9 a	32.3±2.0 b

Within each *Suillus* species, means±SD in each column followed by different letters denote significant differences by LSD test ($P \leq 0.05$).

second cluster were *S. variegatus*, *Suillus plorans* (Rolland) Kuntze, *Suillus tridentinus* (Bres.) Singer, and *S. bovinus*. This general division in two major clusters coincides with the description of various European *Suillus* of the same and different species (Manian et al. 2001).

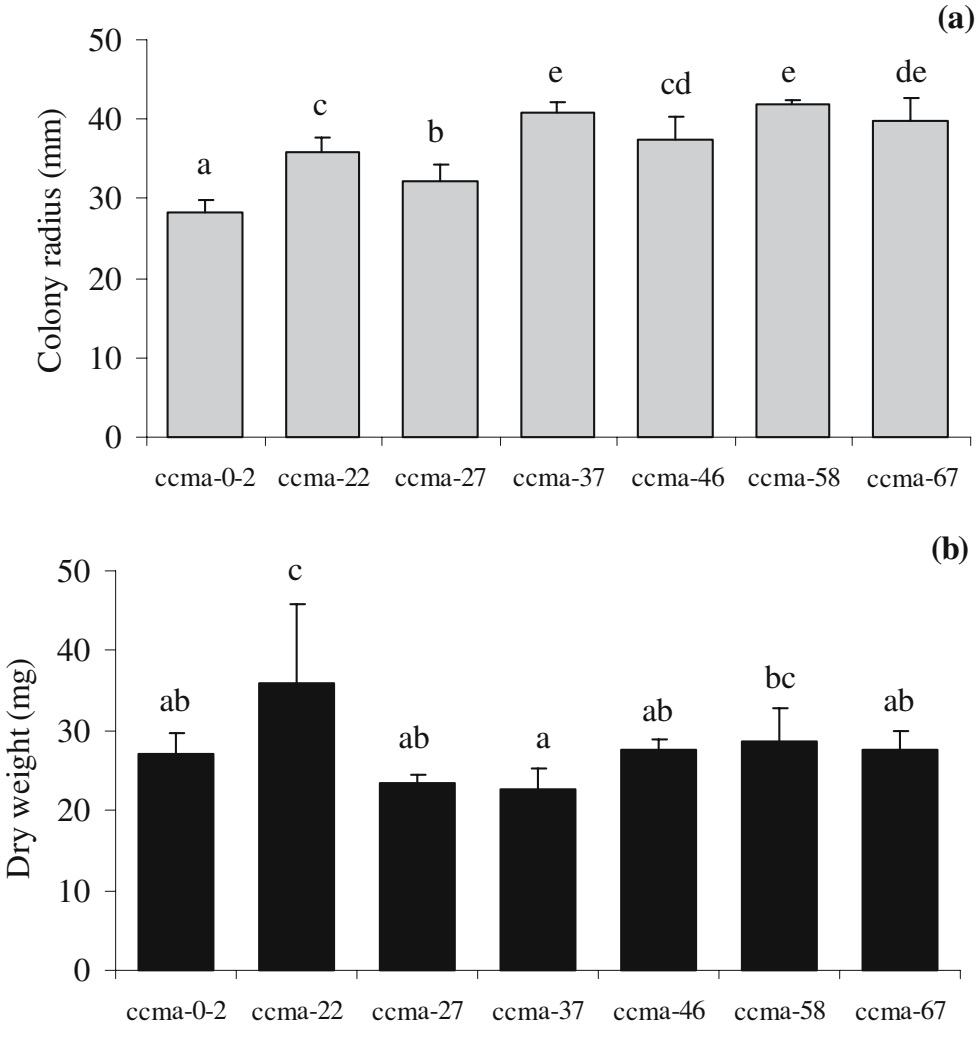

Fig. 2 Growth on MMN medium of different *Suillus* species evaluated as means of colony radius (**a**) and dry weight (**b**). Different *letters* denote significant differences by the LSD test ($P \leq 0.05$). *ccma-02 S. granulatus*, *ccma-22 S. bellinii*, *ccma-27 S. mediterraneensis*, *ccma-37 S. luteus*, *ccma-46 S. collinitus*, *ccma-58 S. variegatus*, and *ccma-67 S. bovinus*

Fungal growth

Significant intraspecific differences in growth within *S. collinitus* isolates were detected (Table 2). Isolate CCMA-46 showed dry weight and radial growth significantly higher than most of the other strains, whereas CCMA-5 showed the lowest values. In general, *S. collinitus* isolates could be divided into two groups (Table 2) with either high growth values (CCMA-24, 46, 75, and 77) or the lowest ones (CCMA-1, 5, 73, 79, and 80). Isolates of *S. luteus* and *S. mediterraneensis* were more homogeneous in growth inside each respective group and no significant intraspecific differences in dry weight were found for these two species (Table 2). CCMA-37 in the case of *S. luteus* and CCMA-27 in the case of *S. mediterraneensis* showed significantly higher radial growth compared with other isolates of the respective species (Table 2).

The ability of fungi to grow fast under in vitro conditions was described as an important criterion of selection (Brundrett et al. 1996; Trappe 1977). Because the general objective of this work was to identify the selection criteria for fungal inoculants, analysis at the species level (interspecific) was performed using the best growing isolate of each *Suillus* species (Fig. 2). Significant differences in radial growth were detected among species (Fig. 2a) and they could be classified into four groups: (1) *S. bovinus* (CCMA-67), *S. luteus* (CCMA-37), and *S. variegatus* (CCMA-58) showing the highest values; (2) *S. bellinii* (CCMA-22) and *S. collinitus* (CCMA-46) with high-intermediate ones; (3) *S. mediterraneensis* (CCMA-27) with intermediate values; and finally (4) *S. granulatus* (CCMA-02) with the lowest values. Concerning dry weight, *S. bellinii* (CCMA-22) and *S. variegatus* (CCMA-58) showed the highest values and *S. collinitus* (CCMA-46), *S. bovinus* (CCMA-67), *S. granulatus* (CCMA-02), *S. mediterraneensis* (CCMA-27), and *S. luteus* (CCMA-37) the lowest ones (Fig. 2b).

All these results revealed high physiological diversity, not only among different *Suillus* species (interspecific) but also within isolates of the same species (intraspecific). This was especially the case for *S. collinitus* isolates, among which CCMA-46 clearly showed higher growth. Similar intraspecific variation was found among populations of *S. bovinus* and *S. variegatus* in other studies (Sen 1990). These analyses were the first step toward selecting isolates with the best in vitro growth for producing laboratory inoculants.

In vitro synthesis of mycorrhizas with *P. halepensis*

Mycorrhizal colonization and *P. halepensis* growth responses varied between some isolates of the same species (Table 3) and among some of the *Suillus* species (Fig. 3). For *S. collinitus* isolates, the percentages of ectomycorrhizas obtained with CCMA-46, 73, and 80 were significantly higher than CCMA-24, whereas intermediate values were obtained for the rest of the isolates. In general, isolate CCMA-46 significantly improved *P. halepensis* growth for almost all variables analyzed (Table 3). When Pearson's correlation analysis (Steel and Torrie 1980) was performed grouping together data of all *S. collinitus* strains, the percentages of mycorrhizas were highly correlated with most of the growth parameters: epicotyl length, 0.424*

Table 3 Growth and mycorrhizal colonization of *P. halepensis* seedlings inoculated in vitro with different isolates of *Suillus* spp.

Treatment (CCMA-number)	Mycorrhizas (% short roots)	Lateral roots (number)	Root length (cm)	Needles (number)	Shoot dry weight (mg)	Epicotyl length (cm)
S. collinitus						
1	42 ab	32 a	15.3 ab	45 a	33.2 ab	5.6 a
5	47 ab	21 a	20.5 b	98 bc	41.4 ab	5.2 a
24	29 a	33 a	15.0 a	57 a	28.7 a	5.1 a
46	77 b	50 a	20.3 b	118 c	49.9 b	6.8 b
73	73 b	38 a	22.6 b	51 a	35 ab	5.4 a
75	55 ab	67 a	22.1 b	53 a	28.7 a	4.9 a
77	44 ab	23 a	21.5 b	48 a	33.4 ab	5.5 a
79	35 ab	23 a	19.8 b	86 b	32.9 a	5 a
80	72 b	34 a	18.2 ab	92 b	41.5 ab	5.5 a
S. luteus						
14	18 a	27 ab	21.1 a	87 a	36.1 a	4.3 a
35	36 ab	32 ab	16.6 a	88 a	59.5 a	5.9 b
37	27 ab	15 a	15.7 a	76 a	36.1 a	6.4 b
57	61 b	58 b	17.6 a	64 a	37.4 a	5.8 ab
S. mediterraneensis						
26	85 a	81 a	20.6 a	71 a	40.9 a	5.6 a
27	56 a	44 a	23.7 a	69 a	39.3 a	5.3 a

In each column and within each *Suillus* species, values followed by different letters denote significant differences by LSD test ($P \leq 0.05$).

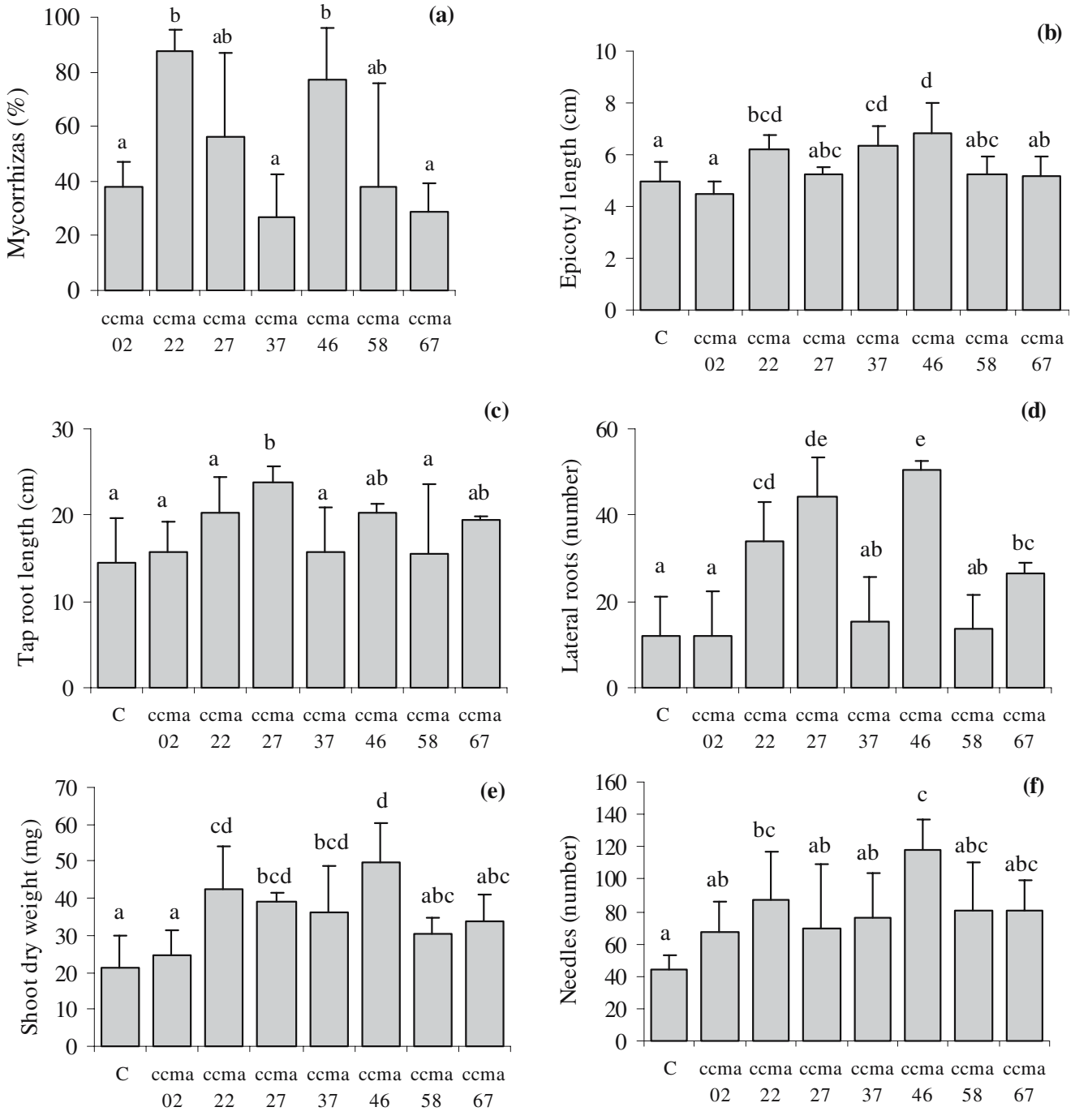

Fig. 3 Growth and mycorrhizal colonization of *P. halepensis* inoculated with different species of *Suillus*: **a** percent of mycorrhizal roots, **b** epicotyl length, **c** taproot length, **d** number of lateral roots, **e** shoot dry weight, and **f** number of needles. For each parameter, different *letters* denote significant differences by the LSD test ($P \leq 0.05$). *C* Control noninoculated seedlings, *ccma-02 S. granulatus*, *ccma-22 S. bellinii*; *ccma-27 S. mediterraneensis*, *ccma-37 S. luteus*, *ccma-46 S. collinitus*, *ccma-58 S. variegatus*, and *ccma-67 S. bovinus*

($P<0.05$); tap root length, 0.532** ($P<0.01$); needle number, 0.284 (NS); shoot dry weight, 0.666** ($P<0.01$); root dry weight, 0.694** ($P<0.01$); and lateral root number, 0.693** ($P<0.01$). Thus, pine growth enhancement due to *S. collinitus* isolate CCMA-46 (results obtained by ANOVA) could be related to the high mycorrhizal rates obtained with the same isolate. In the case of *S. luteus*, CCMA-57 formed significantly more mycorrhizas than CCMA-14, and intermediate values were obtained with CCMA-35 and CCMA-37. Isolates CCMA-35 and CCMA-37 significantly improved epicotyl length of the *P. halepensis* seedlings, whereas isolate

CCMA-57 significantly improved the number of lateral roots (Table 3). The ECM development obtained with both *S. mediterraneensis* isolates was similar and no differences between isolates were found for any of the pine growth parameters studied (Table 3).

The *Suillus* spp. previously selected for in vitro growth analysis were also evaluated for interspecific effects on *P. halepensis* growth. Three groups could be clearly distinguished by their mycorrhizal capacity among the different *Suillus* species: (1) *S. bellinii* and *S. collinitus* gave the highest mycorrhizal development, (2) *S. mediterraneensis* was intermediate, and (3) the remaining species showed the lowest values (Fig. 3). Concerning *P. halepensis* growth, *S. collinitus*, *S. bellinii*, and *S. mediterraneensis* significantly increased most variables in inoculated compared with noninoculated seedlings. *S. luteus* significantly increased shoot weight and epicotyl length, whereas *S. granulatus*, *S. bovinus*, and *S. variegatus* did not significantly affect seedling growth compared with controls. Although these results were obtained under in vitro conditions, they could suggest a certain degree of "physiological specificity" between three of the *Suillus* spp. (*S. collinitus*, *S. bellinii*, and *S. mediterraneensis*) and *P. halepensis*.

In summary, *S. collinitus*, *S. bellinii*, and *S. mediterraneensis* were the species that formed more mycorrhizas and also increased *P. halepensis* growth. The *S. collinitus* isolate CCMA-46 enhanced *P. halepensis* growth the most, and could therefore be a good candidate for afforestation purposes. *Suillus collinitus*, *S. bellinii*, and *S. mediterraneensis* are among Mediterranean species that were previously described for their high mycorrhizal capacity in nurseries with *P. halepensis* (González-Ochoa et al. 2003), a pine species that was extensively employed for restoration in semiarid Mediterranean areas (Maestre and Cortina 2004). The fact that three *Suillus* spp. (*S. collinitus*, *S. bellinii*, and *S. mediterraneensis*) were most abundant in young forests of *P. halepensis* is in agreement with other studies reporting the occurrence of abundant fruit bodies of few fungal species in introduced pine plantations (Dustan et al. 1998). *S. collinitus*, *S. bellinii*, and *S. mediterraneensis* share the same ecological range, i.e., Mediterranean pine groves on calcareous soil (Moreno et al. 1995), and it is likely that these fungal species were introduced into the Madrid region as a consequence of the recent reforestations with *P. halepensis* on calcareous soils.

In conclusion, the detailed ITS sequence analysis of *Suillus* spp. found in pine forests of the Madrid region has clarified the phylogenetic relationship among these taxa and provided the possibility of developing ITS-specific primers to each *Suillus* species, which will be useful in identifying mycorrhizas to monitor inoculated fungi in tree plantations.

The present study also highlights the importance of performing physiological studies before selecting fungal isolates to use as inoculants for nursery production of mycorrhizal plants to be used for afforestation purposes in a defined area. It is especially important that introduced fungi have high competition abilities. Selection of native fungi, which are better adapted to the local environmental conditions (Requena et al. 2001) will reduce problems associated with species introduction into novel habitats.

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