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Original article

Diversity of ectomycorrhizal symbionts in a disturbed *Pinus halepensis* plantation in the Mediterranean region

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Abstract – Ectomycorrhizal diversity (ED) associated with *Pinus halepensis* trees was examined 1.5 years after outplanting at a fire-disturbed site of Rieucoulon (Hérault, France). ED analysis was examined on non-inoculated and *Suillus collinitus*-inoculated plants, and on naturally regenerated trees. A total of 461 single ectomycorrhizas was typed using PCR-RFLP analysis and sequencing of the internal transcribed spacer (ITS) of the nuclear rDNA. Twelve ITS RFLP-taxa were detected. The ectomycorrhizal fungus *S. collinitus* (ITS RFLP-taxon 1) was the most abundant (45.8–59.7%) species in the three treatments, suggesting that it is a strong ectomycorrhizal competitor in this site. *S. mediterraneensis* (ITS RFLP-taxon 2) was restricted to control and naturally regenerated trees and was unequally moderate (11.7–31.9%). The remaining below-ground ITS RFLP-taxa were uncommon and rare (0.0–9.6%). The current experimental *P. halepensis* plantation showed a species-poor community dominated by two *Suillus* species. Ecological strategies of these symbionts are discussed.

***Pinus halepensis* M. / plantation / ectomycorrhizal diversity / PCR-RFLP-sequencing / rDNA (ITS)**

Résumé – Diversité ectomycorhizienne dans une plantation à *Pinus halepensis*. La diversité génétique des ectomycorhizes de plants de *P. halepensis* a été examinée une année et demie après introduction dans un site incendié de Rieucoulon (Hérault, France). Cette diversité a été caractérisée à l'aide du polymorphisme de fragments de restriction (RFLP) et du séquençage de l'espaceur interne transcrit (ITS) de l'ADN ribosomal nucléaire. Trois traitements ont été examinés : des plants témoins, des plants mycorhizés avec *Suillus collinitus* et des plants en régénération naturelle. Au total, 461 ectomycorhizes ont été soumises au typage moléculaire. Douze ribotypes d'ITS ont été détectés. *S. collinitus* (ribotype 1) est l'espèce dominante (45,8–59,7 %) dans les trois traitements suggérant une forte capacité de colonisation dans ce site. La présence de *S. mediterraneensis* (ribotype 2) est limitée aux plants témoins et aux autres issus de la régénération naturelle et sa fréquence est modérée (11,7–31,9 %). Les autres symbiotes ectomycorhiziens sont rares (0,0–9,6 %) et leur abondance diffère d'un traitement à l'autre. Cette étude révèle une faible diversité des symbiotes ectomycorhiziens dans la plantation à *P. halepensis*; elle est dominée par deux espèces du genre *Suillus*. Les stratégies écologiques de ces symbiotes sont discutées.

***Pinus halepensis* M. / plantation / diversité des ectomycorhizes / PCR-RFLP-séquençage / ADNr (ITS)**

1. INTRODUCTION

Aleppo or white pine (*Pinus halepensis* Miller) is a common, thermophilous and pioneer forest species in the Mediterranean Basin [9]. It can reconstitute a forest in deteriorated soil in a short time, and can contribute to soil conservation against erosion and to the subsequent establishment of oaks in Mediterranean conditions [1, 9, 16]. Considering these ecologically beneficial features, *P. halepensis* has been effectively used for reforestation and desertification control in harsh Mediterranean environments characterized by drought stress and nutrient deficiency [1, 9, 24]. However, its autecology is dependent on its ability to contract mutualistic associations with ectomycorrhizal fungi. Ectomycorrhizal symbionts are known for their ability to enhance adaptability, growth, mineral nutrition and water absorption of forest trees [22, 25]. Very little is known about *P. halepensis* ectomycorrhizal diversity (ED) in the early stage of forest development in Mediterranean conditions. Many ectomycorrhizal fungi of *P. halepensis* have been identified and characterized *in vitro* in containerized or bioassay mycorrhizal tests or from mature forests [11, 27, 28]. However, the most commonly used and encountered ectomycorrhizal symbiont in association with *P. halepensis* species is *Suillus collinitus* (Fr.) O. Kuntze [11, 15, 27, 28].

P. halepensis contains bio-polymers and essential oils which make this forest tree highly susceptible to fire [9, 20]. However, very little information is available on *P. halepensis* ED following disturbances such as fire. The ability of ectomycorrhizal fungi to survive and resist these disturbances depends on the duration and intensity of the disturbance, and the environmental conditions [6, 18, 28]. For instance, the diversity of *P. halepensis* ectomycorrhizal basidiomycetes and the number

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of *Cenococcum* sclerotia were lower and higher in burned as compared to unburned stands, respectively [28]. Ectomycorrhizal infection via resistant propagules was also demonstrated in both naturally regenerated and mycorrhizal bioassays of *P. muricata* following fire disturbance [5, 26]. Surveys of ED following disturbances in young plantations, in naturally regenerated plants and in mature forests are therefore needed to improve our understanding of the ecological strategy and to design pre-selection of mycorrhizal species for inoculation programs in both nurseries and plantations.

The current investigation examined the ectomycorrhizal diversity (ED) of *P. halepensis* 1.5 years after outplanting at a disturbed experimental site. This was carried out on introduced (control and mycorrhizal) plants and those naturally established. ED analysis was performed directly from below-ground ectomycorrhizae (ECM) using PCR-RFLP and sequencing of nuclear rDNA (ITS).

2. MATERIALS AND METHODS

2.1. The Rieucoulon site

The Rieucoulon site is a mature (20–30 year-old) forest of *Pinus halepensis* located in Prades-le-Lez (Hérault) in the south of France. Plant community of this forest includes shrubs (*Quercus coccifera*, *Juniperus oxycedrus*, *Quercus ilex*, *Thymus vulgaris* and *Rosmarinus officinalis*) and herbaceous plants (*Lavandula latifolia*, *Sanguisorba minor*, *Argyrolobium zanonii*, *Bituminaria bituminosa*, *Aphyllanthes monspeliensis*, *Barlia robertiana*, *Rosa* sp., *Brachypodium* sp., *Phillyrea angustifolia*, *Eryngium campestre*, *Odontites luteus* and *Carex* sp.). In autumn 2000, a survey of a macrofungal *P. halepensis* forest at the Rieucoulon site indicated the presence of *Suillus collinitus*, *S. mediterraneensis*, *Xerocomus subtomentosus*, *Tricholoma fracticum*, *Lactarius sanguifluus*, *L. mairei*, *Cortinarius elegantior* var. *quercilicis*, *Volvariella taylori, Inocybe cervicolor, Russula* sp., *Clitocybe* sp. In 1991, a fire destroyed part of the Rieucoulon forest. The climate is Mediterranean with annual and summer rainfalls of 856 and 122 mm and mean temperatures of 1.5 and 28.6 °C in January and July, respectively.

2.2. *P. halepensis* nursery seedlings

Seedlings were prepared at the Pépinière Forestière de l'État (DDAF, Les Milles, Aix-en-Provence, Bouches-du-Rhône, France). Seeds of *P. halepensis* (provenance: 02-Provence, Vilmorin, France) were disinfected and sowed on March 1996 in a sterilized peat-vermiculite mixture (1:1, v/v) containing milled rock phosphate (1 g per plant) in containers, according to nursery procedures [3]. Seedlings were watered at 4 L/m^2/192 plants/day. They were then fertilised for 10 weeks with a 0.1% nutrient solution (N-P_2O_5-K_2O 12-0-8%, Dynaflor, Sète, France), two weeks after inoculation. Two distinct *P. halepensis* treatments [control seedlings (C) and seedlings inoculated (M) with *S. collinitus* (strain J 3-15-32)] were carried out. Seedlings were inoculated in May 1996 as described by Argillier et al. [4]. All inoculated seedlings showed ectomycorrhizal morphotypes similar to *S. collinitus/P. halepensis* ECM 4 months following inoculation [29]. This identity was confirmed using ITS RFLP analysis (data not shown). By contrast, no ectomycorrhiza was observed in non-inoculated seedlings after visual inspection of *P. halepensis* root systems.

2.3. Experimental plantation

The experimental plantation is located within the burned area of the Rieucoulon site on a 10% slope (GPS ProXRS Lambert II coordinates, X: 724 084 m, Y: 1 859 144 m; elevation = 85 m). Soil is a mineral calcareous marly type soil without a litter layer. In 1995, rare and scattered old *P. halepensis* trees, and naturally-regenerated *P. halepensis* and *Quercus* spp. seedlings were found. The soil was ploughed to a depth of 80 cm in October 1995. The C and M *P. halepensis* seedlings were introduced in three plots (I, II and III) in December 1996. Each plot was heterogeneous and contained both C and M treatments. Seedlings were planted out in lines 4.5 m apart. Each line corresponded to C or M seedlings. Within each line, they were 2.5 m from each other. No old trees, and no visible naturally-regenerated *P. halepensis* and *Quercus* spp. seedlings were found at the time of planting.

2.4. Sampling plants, roots and ECM

Seedlings (51 C and 46 M) of the largest plot II (72 m × 45 m) were considered for sampling and DNA typing. Since fruit body surveys, investigated in Autumn 1997 and Spring 1998, did not reveal the presence of ectomycorrhizal sporophores, *P. halepensis* ED was examined directly from ECM. The introduced C (6%) and M (20%) plants were examined in Spring (April–June 1998). At the same time, three naturally regenerated seedlings (R), less than 1.5 years of age, were also collected and their ectomycorrhiza analyzed. These were located between lines of C and M seedlings and were considered as a third "treatment". The soil and roots were carefully removed at 5–30 cm depth [12]. Roots (1–5 per plant) were randomly chosen and immediately examined or stored at +4 °C for 1–4 days for further analysis. Single ECM (3–29 per root) were randomly chosen and they corresponded to the highest number of young ECM observed on the excised roots. A total of 461 single ECM from C, M and R plants were excised, washed with H_2O_2 (20 s) followed by immediate rinsing (three times) with autoclaved H_2O. They were then stored in Eppendorf tubes at –70 °C for DNA extraction and molecular analysis.

2.5. Molecular analysis

Total DNA was extracted from mycelia, fruit bodies and single ECM using the DNeasy Plant Mini Kit according to the manufacturer's recommendations (QIAgen S.A.). The nuclear rDNA internal transcribed spacer (ITS, 3'end of 18S + ITS1 + 5.8S + ITS2 + 5'end of 25S) was amplified by PCR using ITS1 and ITS4 primers [17, 31]. PCR amplification was carried out using a PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA) [13]. Negative controls (no DNA template) were included in all PCR experiments to check for DNA contamination of reaction mixtures. For RFLP analysis, 10 µL aliquots of ITS products were mixed with 1.5 µL of the React mix, containing 5 units each of *Hin*fI, *Msp*I or *Taq*I restriction endonucleases (Gibco BRL, Life Technologies), and adjusted to 15 µL with deionized water according to the manufacturer's recommendations. The amplified products and restriction fragments (RFLPs) were electrophoresed on 1.5% and on 3% regular (Sigma) and Nusieve (FMC) agarose gels, respectively, stained with ethidium bromide and photographed using the Oncor-Appligene Imager 2.02. Digested pUCBM21 DNA (molecular weight marker VIII, Boehringer Mannheim) was used as a size standard. Sizes of PCR and RFLP fragments were determined using the DNAFRAG v. 3.03 program (National Research Council of Canada). The sequencing reactions were performed on ITS of *S. collinitus* mycelium (strain J.3.15.32) and on 14 ECM randomly chosen from each ITS RFLP-taxon. The double stranded ITS products were then purified using the QIAquick PCR purification Kit (QIAgen) in accordance with the manufacturer's instructions. Both strands were sequenced separately using the BigDye Terminator Cycle Sequencing Kit, the AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster, City, CA, USA) and ITS1 and ITS4 primers. Sequencing products were analysed using the automated ABI PRISM 310 DNA Genetic Analyser (Perkin Elmer-Applied Biosystems) at the DNA Sequencing

Table I. List and origins of ectomycorrhizal references used in this study.

Fungal taxa	Strains	Authors and years of isolations	Geographical origins	Associated forest trees
Suillus collinitus (Fr.) O. Kuntze	Sc6*	El Karkouri K. (2000)	Rieucoulon (Hérault)	*P. halepensis* M.
	Sc7*			
	Sc8*			
	J 3-15-35*	Conventi S. (1998)	Lauret (Hérault)	
	J 3-15-2*	Mousain D. (1991)	La Grande-Motte (Hérault)	*P. pinea* L.
	J 3-15-32*	(1995)	Nîmes (Gard)	*P. halepensis* M.
	J 3-15-24*	Mauré L. (1991)	La Grande-Motte (Hérault)	*P. pinea* L.
S. mediterraneensis (Jacq. & Blum) R.	Sm1**	El Karkouri K. (2000)	Rieucoulon (Hérault)	*P. halepensis* M.
	Sm2**			
	Sm3**			
	Sm4*			
	Sm11*			
	Sm12*			
Xerocomus subtomentosus (L. :Fr.) Quélet	Xst1**			
	Xst2**			
	Xst4**			
S. bovinus (L. :Fr.) O. Kuntze	ECM51***	El Karkouri K. (1998)	Nursery (Bouches-du-Rhône)	*P. nigra* A. ssp. *nigra*
	ECM57***			
S. variegatus (Sw. :Fr.) O. Kuntze	ECM31***			
	ECM30***			
Rhizopogon rubescens (Corda) Th. Fr.	B.S.1**			*P. nigra* A. ssp. *salzmannii*
	B.S.2**			
	R 19-1*	Tillard P. (1990)		*P. nigra* A.
Thelephora terrestris Fr.:Fr.	T 20-1*		n.d.	n.d.
Cenococcum geophilum Fr.	Cg Nancy*	Fienema (1988)	Nancy (Meurthe-et-Moselle)	*Tilia* sp.
	Cg SIV*	Kiffer (1974)	Nancy (Meurthe-et-Moselle)	*Picea* sp.

*, ** and ***: mycelium, fruit body and ectomycorrhizae respectively. *P.*: *Pinus*; n.d.: not determined.

Facilities of INRA-Nancy (France). The sequencing data were edited using the Sequencher (Genes Codes Corporation, Ann Arbor, MI, USA) for Macintosh computers.

2.6. Molecular identification and frequency of ECM

Each distinct "ITS RFLP-type" shared by ECM was named "ITS RFLP-taxon". To identify these taxa, ITS RFLP patterns and sequences were, respectively, compared with our ITS RFLP-types of identified ectomycorrhizal fruit bodies and mycelia (Tab. I) [12, 18, 23] and with GenBank ITS sequences using the Blastn program (National Center for Biotechnology Information) [2]. Sequences of RFLP-taxa are available in the EMBL database. The relative abundance of ITS RFLP-taxa was calculated by dividing the number of ECM of each ITS RFLP-taxon by the total number of the ECM typed in each treatment.

3. RESULTS

PCR-RFLP analysis was performed on 461 single ECM collected from C, M and R *P. halepensis* seedlings. A high percentage (97.2%) of ITS amplifications was successful indicating that the QIAgen spin column provides clean DNA with low or no inhibitors. In total, 359 (77.9%) ECM showed a single amplified ITS product (570–700 bp in size) and interpretable RFLP patterns (Tab. II). In contrast, 89 (19.3%) and 13 (2.8%) ECM showed double ITS amplifications with non-interpretable RFLP patterns and no PCR amplification, respectively (Tab. II).

Twelve distinct ITS RFLP-taxa were found using *Hin*fI, *Msp*I and *Taq*I restriction enzymes (Tab. II). ITS RFLP-taxon 1 matched the ITS RFLP-pattern of known *S. collinitus* fruitbodies and mycelia (For an example, see Fig. 1, Tab. II) [8]. *S. collinitus* species was the most common and dominant (51.1, 59.7 and 45.8%) symbiont found on *P. halepensis* in the three treatments (Fig. 1, Tab. II). ITS RFLP-taxon 2 matched the ITS RFLP-pattern of identified *S. mediterraneensis* fruit bodies and mycelia (Tab. II). ITS sequence of *S. mediterraneensis* (EMBL ac. # AJ410860) was very similar (94–96% of sequence similarities) to ITS sequences of other *Suillus* species. This species abundance ranged between 12 and 32% of ECM tips and it was restricted to C and R *P. halepensis* treatments, respectively.

Five unmatched RFLP-taxa 3, 7, 9, 11 and 12 (EMBL acs. # AJ410861, AJ410864, AJ410866, AJ410868 and AJ410869) showed 93%, 99%, 95%, 92% and 97% ITS sequence identities with *Tylospora, Tuber, Tomentella*, *Tomentella* and *Tricholoma* species (GenBank acs. # AF052565, AF003918, U83482, U83482, AF241514 and), respectively. The remaining five ITS RFLP-taxa 4, 6, 8 and 10 (EMBL acs. # AJ410862, AJ410863, AJ410865 and AJ410867) and 5 did not show sequence homologies with ITS of any known ectomycorrhizal

Table II. Size of amplified ITS and RFLP fragments and relative abundance of the ectomycorrhizal symbionts found in *P. halepensis* plants.

	Uncut ITS and RFLPs (size in bp)				Relative abundance (%)		
ITS RFLP-taxa	ITS	*Hinf*I	*Msp*I	*Taq*I	C*	M*	R*
S. collinitus	700	202/145/118/86/77/35	408/167/88/49	270/96/85/62	51.1	59.7	45.8
S. mediterraneensis	700	218/135/115/96/77/35	412/239/49	174/93/66	11.7	0.0	31.9
RFLP-taxon 3	600	302/292	600	n.d.	9.6	0.0	0.0
RFLP-taxon 4	611	295/169	251/206/126	n.d.	3.2	0.0	0.0
RFLP-taxon 5	620	327/259/33	484/136	n.d.	0.0	4.4	0.0
RFLP-taxon 6	571	167/156/133/119	188/133/100/40	n.d.	0.0	3.7	0.0
RFLP-taxon 7	651	335/177/75/46	639	292/68	0.0	3.7	0.0
RFLP-taxon 8	651	300/168	259/211/124	n.d.	0.0	2.7	0.0
RFLP-taxon 9	651	312/197/147	647	339/260/60	0.0	1.7	0.0
RFLP-taxon 10	651	316/176/147	445/181	n.d.	0.0	1.4	0.0
RFLP-taxon 11	672	347/326	669	339/260/60	0.0	1.0	0.0
RFLP-taxon 12	672	351/316	669	n.d.	0.0	0.3	0.0
N. i.					19.1	19.7	18.1
N. PCR					5.3	1.7	4.2
Total (number of ECM)					100 (94)	100 (295)	100 (72)

*: Control (C), mycorrhizal (M) and regenerated (R) plants. N. i.: Non-interpretable RFLP patterns; N. PCR: no PCR amplifications; n.d.: not determined.

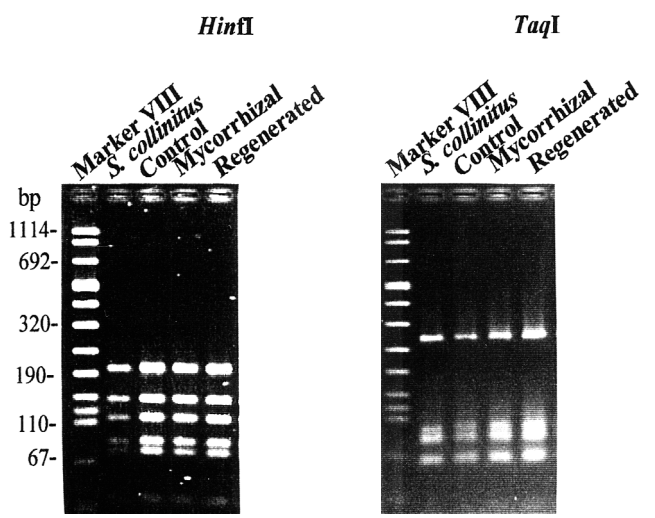

Figure 1. Identification, by *Hinf*I and *Taq*I RFLP analysis of the ITS, of the dominant species *S. collinitus* in *P. halepensis* seedlings 1.5 years after outplanting at the Rieucoulon site. Marker VIII: molecular weight marker. RFLP patterns corresponded to *S. collinitus* mycelia (see Tab. I) and to ECM from the control, mycorrhizal and naturally regenerated treatments.

species or genus in GenBank database or their ITS region could not be sequenced after two to three replicates. All these ten RFLP-taxa were uncommon or rare (0.0–9.6%) on *P. halepensis*.

4. DISCUSSION

Ectomycorrhizal diversity in a fire-disturbed *P. halepensis* plantation was investigated 1.5 years after outplanting *S. collinitus*-inoculated seedlings at the Rieucoulon site. Identification of ECM symbionts was performed using PCR-RFLP and sequencing of the nuclear rDNA (ITS) of single ECM. No ectomycorrhizal fruit bodies were found at the time of the surveys. Twelve distinct ITS RFLP-taxa were identified among a total of 461 ECM typed. This finding indicates that there were remaining resident propagules (e.g. spores, hyphae, old and young roots, rhizomorphs) at the outplanting site after fire. They had probably resisted and survived through successive disturbances (fire, soil ploughing) which took place before pine outplanting, as indicated in other studies [5, 26, 28]. Mycorrhizal roots of resprouting plants, such as *Quercus* spp. and *P. halepensis*, were described to conserve their viability, thus enabling recolonization of introduced *P. halepensis* roots following disturbances [28, 30]. The current results highlighted that ectomycorrhizal fungi perpetuated via the mycelial network in mature forests [19] could do so in disturbed sites through the remaining resistant propagules. The low *P. halepensis* ED observed here is consistent with previous reports which showed that young *Pinus* trees are species-poor communities with few dominant species, while mature *Pinus* forests show stable and high species diversity [10, 12, 14, 18, 21, 30].

The mycorrhizal fungus *S. collinitus* was the dominant symbiont on inoculated seedlings, but also on non-inoculated and naturally regenerated plants. Although ECM of this species was found 1.5 years after outplanting, no epigeous *S. collinitus* fruit bodies were observed either during Autumn 1997 or Spring 1998, thus precluding dispersal via spores. *S. collinitus* therefore seems to propagate via mycelial spread and it appears to be a strong vegetative competitor against other ectomycorrhizal fungi. Abundance of *S. collinitus* in the three treatments indicated that this symbiont was not influenced by the host treatments, soil type (calcareous marly) or site disturbances.

S. collinitus seemed to use some characteristics of combative strategists and others of ruderal species at the disturbed Rieucoulon site. This is corroborated by another study which suggested that the ecological strategies of *S. pungens* and overall *Suillus* spp. combine the two major categories of the R/S/C model [7]. Moreover, the ability of *S. collinitus* species to co-exist in the current young plantation, the Rieucoulon *P. halepensis* forest (see Tab. I) and other mature *P. halepensis* forests [11, 27, 28] supports the hypothesis that this species is a multi-stage ectomycorrhizal fungus. This is similar to *S. brevipes,* but not with *S. tomentosus*, which were described to be multi-stage and late-stage fungi in association with *P. banksiana*, respectively [30].

In contrast to *S. collinitus*, *S. mediterraneensis* was associated exclusively with the C and R plantlets. This suggests that *S. mediterraneensis* was not influenced by both treatments and showed low competitivity against *S. collinitus*. It was, in addition, outcompeted by *S. collinitus* of the M treatment. Detection of *S. mediterraneensis* together with *S. collinitus* in the *P. halepensis* plantation was consistent with the co-existence of both taxa fruit bodies in the mature *P. halepensis* forest of Rieucoulon. These results, combined with the absence of *S. mediterraneensis* fruit bodies in the *P. halepensis* plantation, suggest that the ecological strategy of this species is similar to that of *S. collinitus* and it could also be considered as a multi-stage ectomycorrhizal fungus. The co-existence of both *Suillus* species might have an ecological significance which could be very interesting to determine. The co-existence of both taxa under young and old *P. halepensis* trees supports their pre-selection as potential candidates for mycorrhizal applications with *P. halepensis* trees. In addition, co-inoculation tests with both species should be carried out. On the other hand, the other ectomycorrhizal taxa were uncommon and scarce on *P. halepensis*. They may be poor competitors against both *Suillus* species and/or their colonisation ability may be influenced by other factors of the Rieucoulon plantation.

Results of the present study indicated that *P. halepensis* trees host a diverse below-ground ectomycorrhizal fungi, especially two Boletales species, *S. collinitus* and *S. mediterraneensis*. The survival and adaptation of *P. halepensis* trees on calcareous marly soil may be due to their symbiotic associations with these symbionts. Future investigations on spatio-temporal variations in genetic and functional diversity, with respect to both ECM and potential fruit bodies, will provide a strong ecological background which should enhance management of ectomycorrhizal applications in disturbed Mediterranean stands.

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