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Original article

Dominance of the mycorrhizal fungus *Rhizopogon rubescens* in a plantation of *Pinus pinea* seedlings inoculated with *Suillus collinitus*

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Abstract – We examined the below-ground mycorrhizal diversity of *P. pinea* seedlings inoculated with *S. collinitus* six years after outplanting in a disturbed site, located at La Petite-Camargue (Gard, France). This was performed using the polymerase chain reaction (PCR), the restriction fragment length polymorphism (RFLP) and the sequencing of the nuclear ribosomal internal transcribed spacer (ITS). Seven and one plants corresponding, respectively, to inoculated and non-inoculated (control) treatments were chosen randomly. Examinations were carried out directly from ectomycorrhizae. A total number of 233 root tips was examined. Five ITS RFLP taxa were detected. The ITS taxon I corresponded to the ectomycorrhizal species *Rhizopogon rubescens*. This fungus was abundant (55%) in the *P. pinea* ectomycorrhizal community. The other ITS taxa were rare and remained unidentified. The current *P. pinea* plantation showed a restricted diversity of the ectomycorrhizal community which is typical of ectomycorrhizal communities in young plantations established in disturbed stands.

***Pinus pinea* L. / plantation / ectomycorrhizal diversity / PCR-RFLP / rDNA (ITS)**

Résumé – Dominance de l'espèce mycorhizienne *R. rubescens* dans une plantation à *Pinus pinea*. La diversité ectomycorhizienne de plants de *P. pinea*, inoculés par *S. collinitus*, a été examinée six années après transplantation dans un site perturbé de La Petite-Camargue (Gard, France). L'identification de cette diversité fongique a été effectuée à partir de l'espaceur interne transcrit (ITS) de l'acide désoxyribonucléique (ADN) nucléaire et ribosomal. Deux marqueurs moléculaires dérivés de la réaction de polymérase en chaîne (PCR) ont été utilisés : le polymorphisme de fragments de restriction (RFLP) et le séquençage des produits d'amplification de l'ADN. Sept et un plants correspondant, respectivement, aux plants inoculés et non inoculés (témoin) ont été choisis au hasard. Le typage moléculaire a été réalisé directement à partir des ectomycorhizes. Deux cent trente trois racines courtes mycorhizées ou non ont été examinées. Cinq types d'ITS RFLP ont été détectés. Le type I correspond à l'espèce ectomycorhizienne *Rhizopogon rubescens*. Cette dernière, est dominante (55 %) dans la communauté ectomycorhizienne de cette plantation. Les autres types d'ITS RFLP ont été rares et restent non identifiés. La plantation de *P. pinea* a montré une faible diversité ectomycorhizienne qui est typique des communautés ectomycorhiziennes des jeunes plantations établies dans des sites perturbés.

***Pinus pinea* L. / plantation / diversité ectomycorhizienne / PCR-RFLP / ADNr (ITS)**

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1. INTRODUCTION

In natural ecosystems, roots of forest trees live in association with below-ground soil-born fungal communities and populations forming symbiotic structures called ectomycorrhizae (ECM) [11]. The ectomycorrhizal mycelium of these symbiotic organs grows and develops or not fruit bodies above the ground. The ectomycorrhizal symbiosis is known to improve the mineral nutrition, the growth and the adaptation of forest trees. Ectomycorrhizal fungi have thus been exploited in many reforestation programmes (for a review see [17, 18]). However, knowledge of the structure and the composition of the ectomycorrhizal fungal communities (EFCs) of the young plantations is still limited [10, 22]. In addition, comparing to the number of recent reports performed on the analysis of the EFCs from mature forests [e.g. 4, 5, 8, 13, 14, 23, 24], information on the EFCs in the young plantations and in the young stands [3, 24] and their relationships with those of the mature forests are rare.

In the Mediterranean region, the deterioration of forests and its consequences on soil conservation and the stress accompanying the outplanting of young forest trees are often related to the drastic edaphic and climatic conditions. Indeed, for example in the region of "La Petite-Camargue" (Gard, France), the Brasinvert's domain of approximately 200 hectares of calcareous arenosol was subjected to ecological disturbances (violent storms and drought stress) before 1983, leading to the death of 90% of mature *Pinus pinea* trees between 1983 and 1986 [2]. After that disturbance, an experimental plantation was established in the domain in 1990 with nursery-non-inoculated and– inoculated with *S. collinitus Pinus pinea* seedlings [2].

The present investigation examined the diversity of below-ground *P. pinea* EFC six years after outplanting in this disturbed site. This was performed using PCR-RFLP and sequencing of the rDNA ITS directly from the ectomycorrhizal root tips.

2. MATERIALS AND METHODS

2.1. The experimental plantation of *P. pinea*

The description of the experimental *P. pinea* plantation in the Brasinvert's domain (latitude: 43° 28' 12"; longitude: 4° 18' 52"; altitude: 1 m) was detailed by [2]. Two treatments were carried out: seedlings inoculated with *S. collinitus* (J 3.15.2) and non-inoculated (= control) seedlings. This fungus was collected under a 20-year-old *P. pinea* plantation in a calcareous arenosol site at "La Grande-Motte" (south of France) in 1985. It was also distinguished from other *Suillus* spp. [16] and from other *S. collinitus* strains [6] using ITS sequence and isozyme analysis, respectively. Each treatment was subjected to a fertilization with a liquid fertilizer (formula 8-6.5-13, Dynaflor®, Sète, France) diluted at 0.5% (D), a fertilization with the same solution at 0.1% (D/5) and no fertilization (NF) in the nursery. The inoculated and the control *P. pinea* seedlings were introduced in three randomized complete plots (I, II and III) in the plantation in January 1990. Only 55 plants of plot III (36 m × 24 m) inoculated with *S. collinitus* (J 3.15.2) and the corresponding 14 control plants, all fertilized with the D/5 solution, were considered for further sampling and DNA typing. The inoculated and the control treatments were distributed in three and two lines of plants, respectively. The two treatments, the lines and the plants were 9, 3 and 1.5 m apart, respectively.

2.2. Sampling plants, roots and ECM

Soil was carefully removed starting near the base of the plant stem until the roots appear. Digging was then carefully continued in a centrifugal direction to the end of the long roots of 0.97– 2.3 m of length. No abundant roots and young mycorrhizal morphotypes were observed during root and ECM surveys. Two types of ECM were found: young ECM with well developed mantle and old ECM associated to dried black roots and having either no mantle or a naturally damaged mantle. A total of eight plants corresponding to 12.7% and 7% of respectively inoculated and control treatments were examined randomly (*table I*). The inoculated treatment was examined in both Spring and Autumn 1996 (i.e. six years after outplanting), while control treatment was examined in Spring of the same year. Nearly all the roots (4 to 9 per plant) and all the young ECM observed were sampled, while the old ECM were chosen randomly. Both roots and ECM were found at 10–30 cm of soil depth. However, prior to excising the root tips, the root systems were carefully washed to remove most of the adhering soil substrates. A total of 233 ECM (9 to 53 per plant) were taken (140 young ECM, 91 old ECM and two short roots – which seemed non-mycorrhizal) using a binocular microscope (*table I*). They were then washed once with H_2O_2 during 30 seconds and then three times with autoclaved H_2O, and stored at –70 °C for further DNA

Table I. Number of plants and ECM examined and results of the successful PCR-ITS amplification and ITS-RFLP types obtained in the *P. pinea* plantation.

Seasons	Total of			PCR of Y. ECM				ITS-RFLP types				
& plants	ECM	[O.	Y.]	T	[s	d	t]	I	II	III	IV	V
Spring												
C1/L2	28[1]	[6	20]	12	[12	0	0]	0	0	7	5	0
I2/L2	22	[19	3]	3	[1	2	0]	1	0	0	0	2
Autumn												
I1/L1	20	[0	20]	17	[17	0	0]	11	0	6	0	0
I2/L1	35	[13	22]	21	[8	12	1]	0	10	0	0	11
I3/L2	21	[12	9]	9	[0	9	0]	9	0	0	0	0
I4/L2	9	[5	4]	0	[0	0	0]	0	0	0	0	0
I5/L2	45	[23	22]	17	[3	14	0]	17	0	0	0	0
I6/L2	53	[13	40]	39	[0	39	0]	39	0	0	0	0
Total	233	[91	140]	118	[41	76	1]	77	10	13	5	13

ECM: ectomycorrhizae. O.: old. Y.: young.
C and I: control and inoculated plants, respectively, with their corresponding numbers and lines (L) in the plot.
T [s, d, t]: total of successful PCR amplifications [single, double, triple amplified DNA bands].
[1] Two short roots which seemed non-ectomycorrhizal were included.

extraction and molecular analysis of the *P. pinea* EFC. The sampling approach described above presents three advantages: the plants were not completely removed from the soil, the plantation was less damaged, and links between the roots and the plants were ascertained.

2.3. DNA extraction and PCR amplification

Total DNA was extracted from the fresh vegetative mycelia and from the single ECM using the CTAB protocol [7, 12]. 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 [25]. The amplification reaction consisted of a total volume of 50 µL. The first 25 µL corresponded to the diluted total DNA (1/100, 1/125 or 1/250) of the mycelium, or (1/2.5, 1/5, 1/10 and 1/15) of the root tips. The second volume corresponded to a PCR mixture adjusted to 25 µL with deionized water (MilliQ). The reagents of the PCR reaction and their final concentrations were: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% W-1 (GibcoBRL, Life Technologies), 200 mM each of ultrapure dATP, dCTP, dGTP and dTTP (Pharmacia Biotech), 0.2 mM each of the two primers (Eurogentec, Belgium) and 1.75 units *Taq* DNA polymerase (GibcoBRL, Life Technologies) [12]. The PCR cycles were ensued according to [7] using a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA, USA). Negative controls (no DNA template) were made in all PCR experiments to check DNA contamination of reaction mixtures. The pUCBM21 DNA (molecular weight marker VIII, Boehringer Mannheim), cleaved with *Hpa*II and *Dra*I plus *Hin*dIII was used as size standards. Size of the PCR and RFLP fragments were determined using the ImageMaster 1D Gel Analysis (v 3.0) programme (Amersham Pharmacia Biotech). For RFLP analysis, the non reproducible bands with size lower than 67 bp were not considered.

2.4. RFLP analysis

Ten microliters of ITS products were mixed with 1.5 µL of the React mix, containing 5 units each of *Hin*fI, *Alu*I, *Msp*I, *Cfo*I, and *Rsa*I (GibcoBRL, Life Technologies), and adjusted to 15 µL with deionized water according to the manufacturer's recommendations. The amplified products and the restriction fragments (RFLPs) were electrophoresed on 1.5% and on 2% high

resolution agarose gel (Sigma), respectively, stained with ethidium bromide, and photographed under ultraviolet light using the Imager 2.02, a system including a micro-computer incorporating an image processing software v. 2.02 related to an UV cabinet by a CDD camera (Oncor-Appligene).

2.5. ITS sequencing

The sequencing reactions were performed on the amplified ITS of mycelia and some representative ECM (No. 125, 18, TQ & TU) showing single ITS products. The double stranded ITS products were then purified using the QIAquick PCR purification Kit (Quiagen) in accordance with the manufacturer's instructions. Both strands were sequenced separately using the BigDye Terminator Cycle Sequencing Kit, the AmpliTaq DNA Polymerase FS (Perkin Elmer Applied Biosystems, Foster, City, CA, USA) and the ITS1 or ITS4 primers. Sequencing products were analysed using the automated ABI PRISM 310 DNA Genetic Analyser (Perkin Elmer Applied Biosystems) at the DNA Sequencing Facilities of INRA-Nancy (France). The sequencing data were edited using Sequencher (Genes Codes Corporation, Ann Arbor, MI, USA) for Macintosh computers.

2.6. Molecular identification of ECM

Each "ITS RFLP-type" corresponding to a pool of ECM was named "ITS RFLP-taxon". To identify these taxa, the ITS RFLP patterns and the ITS sequences were compared with referenced ITS RFLP patterns of ectomycorrhizal species, and with GenBank ITS sequence database, respectively. ITS sequences were then deposited in the GenBank database. The determination of the taxa having the closest sequence was performed by DNA sequence comparison using the Blastn program at the National Center for Biotechnology Information.

3. RESULTS

Amplification yield of the fungal ITS of the 140 young ECM was high (84%) (*table I*). In contrast, the old ECM and the short roots of *P. pinea* did not produce any PCR products. The lack of PCR products with the old ECM likely resulted from the presence of enzyme inhibitors and/or to the poor quality of these ECM (dry roots without mantle or with a naturally damaged mantle). Therefore, only young ECM were considered in the subsequent calculations. The amplification products of the young ECM showed either single (29%), double (54%) or triple (approx. 1%) ITS bands, suggesting the presence of fungal contaminants in most mycorrhizal roots. The additional bands showed a low intensity and thus did not affect the analysis. The absence of a common ITS product in the 233 mycorrhizal root tips indicated that there was no amplification of the *P. pinea* ITS.

3.1. Identification and frequency of the ITS RFLP taxa

The size of the PCR products ranged from 600 to 740 bp. The digestion of the ITS products of the young

Table II. Sizes of the restriction fragments of the amplified ITS of the vegetative mycelia of *Suillus collinitus* and *Rhizopogon rubescens* and the four ITS-types detected in the plantation.

Taxa &	Uncut ITS and ITS-RFLPs (sizes in bp)					
ITS types	ITS	*Hin*fI	*Alu*I	*Msp*I	*Cfo*I*	*Rsa*I*
S. collinitus (J 3.15.2)	698	186/136/114/93/86	635/81	410/154/89	351/332	706
R. rubescens (R 19.1)	726	215/192/126/112	384/258	468/241	363/162/135	733
I	737	212/190/135/112	384/258	468/241	367/165/137	743
II	616	267/208/92	524/65	425/127	301/275/162	621
III	603	264/196/89	508/87	405/115	301/155/115	583
IV	637	270/147	628	216/170/102	207/137/124	488/106

* *Cfo*I and *Rsa*I were used to digest the ITS products of only 4 representative ECM (No. 125, 18, TQ & TU) corresponding to the 4 ITS-RFLP types (I, II, III & IV), respectively. ITS-type V was not determined because it showed restriction digests from a double ITS amplification.

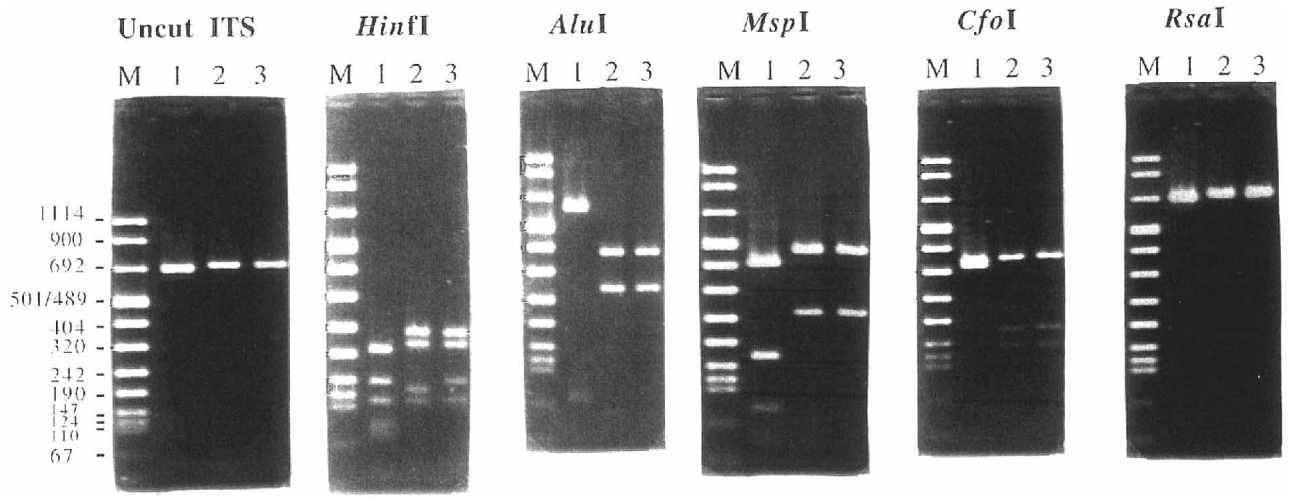

Figure 1. Patterns of the uncut amplified rDNA ITS and the *Hin*fI, *Alu*I and *Msp*I digests of ITS of *S. collinitus* (J 3.15.2) (1), *R. rubescens* (R 19.1) (2) and the abundant ectomycorrhizal ITS RFLP-taxon I (3) of *P. pinea*, six years after outplanting. The *Cfo*I and *Rsa*I were used to digest the ITS of one representative young ECM (No. 125) of the ITS RFLP-taxon I. M: Molecular marker.

ECM with *Hin*fI, *Alu*I and *Msp*I yielded five ITS RFLP types (I to V) (*table I*). The sizes of the RFLPs, excepted the ITS V where all ECM showed double amplified ITS products, are given in *table II*. Type I was similar to the RFLP pattern of *R. rubescens* (R 19.1) (*table II* and *figure 1*, lanes 2 and 3). The ITS sequences of *R. rubescens* (R 19.1) (EMBL accession # AJ277644) and ECM 125 (type I) (EMBL accession # AJ277645) exhibited 96% and 94% homology with the *R. rubescens* ITS sequence present in the database (GenBank accession # AF158018) [23] (*figure 2*). This species was the dominant (55%) mycorrhizal symbiont on most inoculated plants sampled, at distances ranging from 4 to 21.6 m in the plot. None of the other ITS types (II to V) showed a RFLP pattern similar to ITS RFLP patterns of reference strains which included six *Suillus* species (e.g. *S. collinitus*, *figure 1*), *Thelephora terrestris* and *Cenococcum geophilum*. The ITS sequences of types II (ECM 18), III (ECM TQ) and IV (ECM TU) did not correspond to any ectomycorrhizal fungi in the GenBank ITS sequence database. In contrast to the dominant *R. rubescens* mycorrhizal type, the four other ITS types were detected at a low rate (4 to 9%), in no more than two plants and, at distances of 0.4, 16.4, 0.2 and 2.8 m in the plot, respectively.

4. DISCUSSION

Diversity of below-ground mycorrhizal fungi in a *P. pinea* plantation was examined six years after outplanting seedlings inoculated with *S. collinitus*. This analysis was performed using PCR-RFLP and sequencing of the nuclear rDNA ITS. The absence of visible above-ground fruit bodies, at that time, was consistent with the fact that diversity of below-ground ECM does not necessarily reflect the above-ground fruit body production in mature forests [5, 8]. Five ITS RFLP types were detected on the root system of 6-year-old pines. This low ectomycorrhizal diversity is supported by previous reports which showed that distribution of species-abundance of young *Pinus* trees follow geometric series with poor-species community and few dominant species, while those of mature *Pinus* forests fit log-normal series with a stable and high species diversity and equitability [13, 15, 24]. The poor community in the plantation is also typical of fungal communities developing in severe environmental conditions [19]. The structure and the composition of ectomycorrhizal fungal communities (EFCs) are also known to be influenced by several disturbances such as fire [13], earth-worm activity, N deposition or

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                          1                                                   50
R. rubescens (AJ277644)   .......... .......... .......... .......... ......GGTT
ITS RFLP type I (AJ277645) .......... .......... .......... .......... ..........
R. rubescens (AF158018)   CGTAGGTGAA CCTGCGGAAG GATCATTAAC GAATATAATT CAGAGGGGCT
                          51                                                  100
R. rubescens (AJ277644)   GACGCTGGCC GAGGAAACGA GGCATGTGCA CGCTCTTCTG TTTTTCATAA
ITS RFLP type I (AJ277645) ..CGCTGGCC TTGGAAACGA GGCATGTGCA CGCTCTTCTG TTTTTCATAA
R. rubescens (AF158018)   GTAGCTGGCC TTGGAAACGA GGCATGTGCA CGCTCTTCTG TTTTTCACAA
                          101                                                 150
R. rubescens (AJ277644)   CTCACCTGTG CACCTAATGT AGGATGCTCC TCTTTCGGGA GGGGGGACCT
ITS RFLP type I (AJ277645) CTCACCTGTG CACCTTATGT AGGATGCTCC TCTTTCGGGA GGGGGGACCT
R. rubescens (AF158018)   CTCACCTGTG CACCTAATGT AGGATGCCTC TCTTTCGGGA GGGGGGACCT
                          151                                                 200
R. rubescens (AJ277644)   ATGTCTTTGT ATAACTCTTC GTGTAGAAAG TCTTAGAATG TTTACTATCA
ITS RFLP type I (AJ277645) ATGTCTTTGT ATAACTCTTC GTGTAGAAAG TCTTAGAATG TTTACTATCA
R. rubescens (AF158018)   ATGTCTTCAT ACGCCTCTTC GTGTAGAAAG TCTTAGAATG TTTACTATCA
                          201                                                 250
R. rubescens (AJ277644)   GAGAGTCGCG ACTTCTAGGA GACGCGAATC TTT.GAGATA AAAGTTA.TT
ITS RFLP type I (AJ277645) GAGAGTCGCG ACTTCTAGGA GACGCGAATC TTCCGAGATA AAAGTTAATT
R. rubescens (AF158018)   GAGAGTCGCG ACTTCTAGGA GACGCGAATC TCT.GAGATA AAAGTTAATT
                          251                                                 300
R. rubescens (AJ277644)   ACAACTTTCA GCAATGGATC TCTTGGCTCT CGCATCGATG AAGAACGCAG
ITS RFLP type I (AJ277645) ACAACTTTCA GCAATGGATC TCTTGGCTCT CGCATCGATG AAGAACGCAG
R. rubescens (AF158018)   ACAACTTTCA GCAATGGATC TCTTGGCTCT CGCATCGATG AAGAACGCAG
                          301                                                 350
R. rubescens (AJ277644)   CGAAAAGCGA TATGTAATGT GAATTGCAGA TCTACAGTGA ATCATCGAAT
ITS RFLP type I (AJ277645) CGAAAAGCGA TATGTAATGT GAATTGCAGA TCTACAGTGA ATCATCGAAT
R. rubescens (AF158018)   CGAAAAGCGA TATGTAATGT GAATTGCAG A TCTACAGTGA ATCATCGAAT
                          351                                                 400
R. rubescens (AJ277644)   CTTTGAACGC ACCTTGCGCT CCTCGGTGTT CCGAGGAGCA TGCCTGTTTG
ITS RFLP type I (AJ277645) CTTTGAACGC ACCTTGCGCT CCTCGGTGTT CCGAGGAGCA TGCCTGTTTG
R. rubescens (AF158018)   CTTTGAACGC ACCTTGCGCT CCTCGGTGTT CCGAGGAGCA TGCCTGTTTG
                          401                                                 450
R. rubescens (AJ277644)   AGTGTCAGTA AATTCTCAAC CCCTCTTGAT TTGTTTCGAG GGGGAGCTTG
ITS RFLP type I (AJ277645) AGTGTCAGTA AATTCTCAAC CCCTCTTG AT TTGTTTCGAG AGGGAGCTTG
R. rubescens (AF158018)   AGTGTCAGTA AATTCTCAAC CCCTCTCGAT TTGTTTCGAG GGGGAGCTTG
                          451                                                 500
R. rubescens (AJ277644)   GATTGTGGGG GCTGCCGGAG ACTAGGACTC G..TCCTTGA CTCGGG.CTC
ITS RFLP type I (AJ277645) GATGGTGGGG GCTGCCG.AC CCTAGGACTT TAATCTTGGA CTCGGGGCTC
R. rubescens (AF158018)   GATAGTGGGG GCTGCCGGAG ACTAGGATTC G..TCCTTGA CTCGGG.CTC
                          501                                                 550
R. rubescens (AJ277644)   TCCTTAAATG CATCGGCTTG CGGTCGACTT TCGACTTTGC GCGACAAGGC
ITS RFLP type I (AJ277645) TCCTTAAATG CATAGGCTTG CGGTCGACTT TCGACTTTGC GCGACAAGGC
R. rubescens (AF158018)   TCCTTAAATG CATCGGCTTG CGGTCGACTT TCGACTTTGC GCGACAAGGC
                          551                                                 600
R. rubescens (AJ277644)   TTTCGGCGTG ATAATGATCG CCGTTCGCTG AAGCGCATGA ATGAAG.GTT
ITS RFLP type I (AJ277645) TTTCGGCGTG ATAATGATCG CCGTTCGCTG AAGCGCATGA ATGAAG.GTT
R. rubescens (AF158018)   TTTCGGCGTG ATAATGATCG CCGTTTGCTG AAGCGCACGA ATGAAATGTT
                          601                                                 650
R. rubescens (AJ277644)   CCGTGCCTCT AATTCGTCGA CTTAGTATCT CTTCCGAGAG AAAACGTCTT
ITS RFLP type I (AJ277645) CCGTGCCTCT AATACGTCGN CTTAGTATCT CTTAGNAGAG AAAACGTCTT
R. rubescens (AF158018)   CCGTGCCTCT AATACGTCGA CTTTT..... .......... ... ATGTCTT
                          651                                                 700
R. rubescens (AJ277644)   CTTCATGAC. .TTTGACCTC AAATCA.... .......... ..........
ITS RFLP type I (AJ277645) CCTTATNAC. .TTTGACCGN AAATCAGGAA G.ACTACCCG CNGACTCAAA
R. rubescens (AF158018)   CCTCATTGAC TTTTGACCTC AAATCAGGTN GGACTACCCG CTNAACTTNA
                          701                      727
R. rubescens (AJ277644)   .......... .......... .......
ITS RFLP type I (AJ277645) .......... .......... .......
R. rubescens (AF158018)   GCATATCAAT GAGCGGANGA AAAGAAA
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Figure 2. Sequence alignment of three *R. rubescens* ITSs written from 5' to 3'. Accession numbers in EMBL database are put in brackets.

occurrence of heavy metals [4]. In the present site, the death of 90% of mature *P. pinea* trees, the lack of soil ploughing and the high soil salinity revealed by the presence of *Salicornia* sp. [2], before and/or after outplanting, probably affected the survival of resident mycorrhizal fungi and the colonization of the roots with diverse mycorrhizal propagules. These factors in combination with the severe conditions in the plot, suggested by the presence of large number of either dried or dead ECM, may have limited the mycelial extension (ECM at a depth of 10–30 cm) up to the first soil horizon O [2] and influenced the development of fruit bodies, at the sixth year of planting. In contrast, temporal ectomycorrhizal surveys carried out under inoculated *P. pinaster* seedlings showed the presence of ectomycorrhizal fruit bodies during ten years of planting [9, 10]. However, the number and the identity of these ectomycorrhizal species were not high (1–4 species) and not identical each year. This suggests that the development of fruit bodies and the survival of resident ectomycorrhizal species may be subjected to distinct influences each year under the experimental plantation.

The mycorrhizal fungus *R. rubescens* (ITS RFLP type I) was the dominant taxon in the *P. pinea* EFC. Similarly, some *Rhizopogon* species were also found to dominate the ectomycorrhizal communities of Douglas fir and pine seedlings grown on disturbed forest soils [20], 1-year-old *P. muricata* seedlings after fire [3] and the resistant propagules community (RPC) in young *P. muricata* bioassay seedlings [23]. Propagules of some *Rhizopogon* species were described to be tolerant to drought stress [20], to resist to fires [3] and to have persistent propagules which respond rapidly to disturbance and increased drastically in colonization success [23]. These data support the fact that *R. rubescens* was able to cope with the various disturbances cited above in the *P. pinea* plantation and thus competed well against the *S. collinitus* inoculant. Its massive presence on *P. pinea* roots therefore suggests that this species followed similar ecological strategy observed for other *Rhizopogon* species [3, 23] and these abilities may be a characteristic of the genus *Rhizopogon*. On the other hand, 20 and 80% of single *R. rubescens* ECM were detected alone or with one additional fungal contaminant, respectively. This result suggests that *R. rubescens* species might be in successively phases of competition or interaction with other unidentified saprophytic, pathogenic or symbiotic fungus. Indeed, other reports showed various states of composite ECM e.g. between *Rhizopogon* or *Suillus* spp. and *Chroogomphus* spp. under *Pinus* species [1, 26].

In contrast to *R. rubescens* species, *S. collinitus*, which was inoculated on *P. pinea* seedlings, was not detected six years after outplanting. The absence of *S. collinitus* ECM explained necessarily the absence of its fruit bodies in the plantation, at the time of surveys. However, the absence of *S. granulatus* and *Lactarius deliciosus* fruit bodies in a plantation of *P. pinaster* inoculated with these species did not necessarily indicate their exclusion [9,10], since no fruit body and ECM relationships were investigated using molecular tools. In our study, the exclusion of the inoculant was unexpected for the following reasons. The *S. collinitus* species is a common symbiotic partner of the *P. pinea* species, in the Mediterranean region [6, 21]. It was, in addition, collected under a 20-year-old *P. pinea* plantation on a calcareous arenosol and was introduced into similar edaphic conditions. Our results, suggest therefore that the ecological conditions cited above might have affected the adaptation of *S. collinitus* species in the plot and its competitive ability against *R. rubescens*. If *Laccaria bicolor* S238N is a competitive fungus which persisted 10 years after outplanting Douglas-fir seedlings [22], the *S. collinitus* inoculant seems to be very sensitive to changing sites. More knowledge of the ecological strategy of this model species under young Mediterranean plantations should therefore be investigated. This should contribute to the determination in which appropriate habitat *S. collinitus* species would be competitive, dominant and efficient.

The current investigation suggests that the analysis of ectomycorrhizal communities in the young plantations provides valuable information on the structure and the composition of the ectomycorrhizal communities and on the ecological strategy of their members. These could improve the management of the plantations in Mediterranean ecosystems.

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