Impact of wildfire frequency on the ectomycorrhizal resistant propagules communities of a
Mediterranean open forest

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Summary

Ectomycorrhizal fungi (ECM), and in particular the spore bank and other resistant propagules, play an important role in secondary succession processes facilitating regeneration following disturbance events. In this study, the unknown effects of wildfire frequency on the resistant propagules communities (RPC) of a Mediterranean open pine forest were examined.

Soil samples were collected in four mountain sites affected by different fire frequencies and used to test ectomycorrhiza development in two hosts, *Pinus pinaster* and *Quercus suber*. RPC were characterized using PCR-sequencing from Ectomycorrhizal root tips.

18 ECM species were detected in the bioassay. The most frequent fungi were *Cenococcum geophilum*, *Inocybe jacobii*, *Thelephora terrestris*, *Tomentella ellisii*, *Rhizopogon luteolus*, and *R. roseolus*. The first four species occurred on both hosts. High-fires frequency promoted species like *Rhizopogon roseolus*, and influenced negatively other species like *R. luteolus*. *Inocybe jacobii* was positively affected by moderate frequency fires, but decreased significantly with recurrent fires. The number of ECM species colonizing *Q. suber* decreased significantly with fire frequency.

- High frequency fires have different effects than moderate frequency fires on the structure, composition, and diversity of ECM communities, and induce alterations on the ECM communities that could compromise the resilience of the ecosystem.

Key-words: resistant propagules communities, fire frequency, bioassay, *Pinus pinaster* (maritime pine), *Quercus suber* (cork oak), Mediterranean ecosystem, succession

Introduction

In the last few decades, the Mediterranean basin region is witnessing a change of the natural fire regime mainly due to an increase of fire frequency and intensity. These alterations can compromise the resilience of ecosystems in highly disturbed areas modifying landscape and vegetation structure (Pausas, 2004) and altering ecosystem functioning (Rodrigo *et al.*, 2004). Many Mediterranean tree species, like pine trees or cork oaks, have functional traits such as thick bark or cork layers, resprouting ability, xerotiny and fire-induced seed germination, that allow them to survive fires. In spite of this, frequent fires can change vegetation from pine forests to shrub dominated areas because the regenerating trees are unable to reach reproductive maturity between successive fires (Vázquez & Moreno, 2001). Pine and oak trees are also obligate ectomycorrhizal (ECM) symbionts that depend on the presence of fungal inoculum for establishment and survival. The spore bank and other resistant propagules of ECM fungi, collectively known as the resistant propagules community (RPC), play an important role in succession processes facilitating regeneration following disturbance events (Taylor & Bruns, 1999). Fungal resistant propagules can successfully colonize poor

1 soil regions, resist periodic droughts, severe temperatures, and other natural stresses (Gupta *et*
 2 *al.*, 2000), and their absence may delay the colonization of disturbed areas by ectomycorrhizal
 3 trees (Dickie & Reich, 2005). Therefore, understanding the effect of fire frequency on ECM
 4 fungi is crucial to understand the dynamics of secondary succession after fire.

5 Most investigations to date have focused on the effects of a single fire event on ECM. The
 6 changes caused by fire on the ECM community depend on both pre-fire ecosystem conditions
 7 and fire characteristics. In areas with high-intensity fires and high tree mortality most ECM
 8 fungi may locally be killed (Dahlberg, 2002). In these situations the organic layer may be
 9 eliminated, soils may be partially sterilized by heat, and deposition of ashes can alter soil pH
 10 and availability of nutrients with potential consequences on the structure and function of the
 11 ECM communities (Grogan *et al.*, 2000). ECM community tends to decrease in complexity
 12 after a stand replacing fire event shifting from a stable community composed by a relatively
 13 large number of species to a more homogeneous and simple community mainly characterized
 14 by previously rare fungi (Visser, 1995; Stendell *et al.*, 1999) such as species of the order
 15 Pezizales and the genus *Rhizopogon* (Baar *et al.*, 1999; Grogan *et al.*, 2000; Smith *et al.*,
 16 2004; Fujimura *et al.*, 2005). The post-fire ECM community composition has a strong
 17 resemblance to the composition of the pre-fire spore bank community which can be caused by
 18 the survival of species present in the RPC (Baar *et al.*, 1999; Taylor & Bruns, 1999). The
 19 RPC of mature pine and fir forests following a fire event is generally represented by a
 20 relatively low number of species. Among these species, *Rhizopogon* spp., *Wilcoxina* spp., and
 21 *Cenococcum geophilum* Fr. are regularly present and evenly distributed across the forest
 22 (Baar *et al.*, 1999; Taylor & Bruns, 1999; Izzo *et al.*, 2006a,b). Some studies have examined
 23 the effects of different fire factors, such as soil drying and heating, on the RPC, and revealed

that different ECM fungi have a differential response to fire-related factors. For example, the frequency of *Rhizopogon olivaceotinctus* A.H. Sm. increased significantly in dried soil (Baar *et al.*, 1999) and with heat treatment at 70-75°C (Izzo *et al.*, 2006a; Peay *et al.*, 2009), while *Wilcoxina* sp., *C. geophilum* (Izzo *et al.*, 2006a), *Laccaria proxima* (Boud.) Pat., and *Tomentella subliacina* (Ellis & Holw.) Wakef. (Peay *et al.*, 2009) showed a decrease in frequency in heat-treated soils. The changes induced by fire on the RPC depend on fire regime and while there are studies that evaluate the effects of fire occurrence and intensity on the RPC, little information appears to exist about the effects of fire frequency on whole ECM communities (Hart *et al.*, 2005; Tuininga & Dighton, 2004; Bastias *et al.*, 2006; Anderson *et al.*, 2007), and, as far as we are aware, no studies are available in relation to the effect of frequent fires on the RPC.

Taking into account all the above, the main objective of this study was to assess the effect of fire frequency on the composition of the RPC in areas where the vegetation has changed from a mature maritime pine open forest (not affected by fire) to a shrubland (high frequency fire) with a transition phase characterized by young pine trees (moderate frequency fire). The RPC present in each soil was analysed by colonization assays using cork oak and maritime pine seedlings as potential hosts.

We hypothesized that (1) recurrent fires and the subsequent alterations of the vegetation structure from pine forest to shrubland affects negatively the ectomycorrhizal RPC and (2) that the influence of fire frequency on ectomycorrhizal RPC varies depending on the host, affecting the potential establishment of different host seedlings.

Materials and Methods

Study sites and sampling

Sampling was conducted in June 2007 in two areas in Central Portugal: Isna de Oleiros (39°51' N, 7°51' W, altitude 750-850 m) and Alvito da Beira, (39°48' N, 7°49' W, altitude 500-600 m). Four soil sampling sites were selected in these areas affected by different fire frequency: an unburnt control site (UB), a site that have been burnt once in 2003 (B, moderate frequency fire), and two sites that have been burnt twice in the last 15 years and present different shrub cover (B1 and B2, high frequency fire). The region is characterized by a Mediterranean climate with hot dry summers and cool wet winters, and is covered mainly by *Pinus pinaster* Aiton.

The unburnt (UB) and burnt (B) sites were selected in Isna de Oleiros. The unburnt site was characterized by an uneven aged-maritime pine open forest (dominant trees with 40-45 cm diameter), unaffected by fire in the preceding 40 years. This site had an understory shrub community dominated by *Erica* species, *Calluna vulgaris* (L.) Hull, and *Pterospartum tridentatum* (L.) Willk. in Willk. & Lange. Site B, in which wildfire occur once in the last 40 years, was separated from UB by a road, and showed a vigorous natural pine regeneration associated with the same shrubby species present in site UB. The area of Alvito da Beira, located approximately 5 km from Isna de Oleiros, has been affected by two high intensity fires in the last 15 years, being the most recent in 2003. The last fire has changed radically the vegetation cover from a maritime pine young forest to a shrubby area. The two selected

sampling sites were dominated either by *Cistus ladanifer* L. (B1) or by *P. tridentatum* and *Erica* spp. (B2).

In June 2007, three 10 x 10 m² plots were delimited along a belt of approximately 600 m, spaced equally in each of the four sites. Within each plot, nine soil samples were taken at a depth of approximately 20 cm using a shovel. The environmental conditions at the time of sampling, high temperatures and summer drought, likely eliminated non-resistant fungal species. In order to select only resistant propagules, soil samples were further air-dried for 4 weeks. Soils belonging to the same site were then pooled, sieved through a 2 mm sieve, and stored at 4°C.

Preparation of bioassays

Bioassays were performed with maritime pine (*P. pinaster*) and cork oak (*Quercus suber* L.) seedlings. *Pinus pinaster* represents the dominant species in the area and is known to be an early colonizer after disturbance events. *Quercus suber* is occasionally present in the region and it occurs in the unburnt forest of Isna de Oleiros as naturally regenerated seedlings.

In August 2007 the soil of each site was mixed with autoclaved coarse sand to help improving the drainage (1:1) and used to set up the bioassay. To check for external contamination, negative controls were prepared with autoclaved coarse sand and autoclaved soil from each site. Soil-sand mixtures were put in 300 ml pots previously disinfected in 10% bleach for 30 min and soaked in tap water for another 30 min. Pine seeds were surface sterilized in 80% bleach and soaked in running water for 48 h. Oak acorns were immersed in tap water for two days. Afterwards, the acorns that presented a 3 mm germinated radicle were selected,

sterilized in 80% bleach, and soaked in running tap water for 10 min. For both hosts, 15 replicate pots and 15 control pots were planted for each site (240 pots in total). Three seeds (for *P. pinaster*) or an acorn (for *Q. suber*) were sown in each pot. After emergence of pine seedlings, only one was allowed to develop. The experiment was conducted in a growth chamber under controlled conditions, 14/10 h of light/darkness at 24°C/18°C. Seedlings were watered twice a week with tap water. To avoid cross-contamination between treatments pots were grouped by site and single trays were separated from each other with unbending plastic sheets.

Characterization of the seedling ECM community

Pine seedlings were harvested after 6 months in February 2008, while oak seedlings were left to grow for 6 more months. A total of 96 pine and 94 oak seedlings survived until the end of the experiment and were harvested. Mycorrhizal root tips were morphotyped under a dissecting microscope and percentage of root colonization was recorded for each morphotype. Morphotypes from each seedling were placed and conserved in separate eppendorf tubes in 300 µl CTAB buffer, which was removed before the molecular analysis. Total DNA was extracted with a modification of the Qiagen DNeasy[®] Plant Mini kit protocol (QIAGEN, Valencia, California, USA). Root tips were ground with eppendorf pestles in AP1 buffer and incubated overnight at 60°C. The internal transcribed spacer (ITS) region was amplified with the primer combination ITS1-F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) that targets both ascomycotina and basidiomycotina. PCR were performed with PuReTaq[™] Ready-To-Go[™] PCR Beads (GE Healthcare UK Limited, Buckinghamshire, UK) in 25 µl

1 volume with the following cycling parameters: an initial denaturation step at 94°C for 5 min,
2 followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and
3 extension at 72°C for 1 min and 30 s, followed by 33 cycles of denaturation at 94°C for 30 s,
4 annealing at 48°C for 30 s and extension at 72°C for 1 min and 30s, with a final extension step
5 at 72°C for 10 min. Amplification products were separated by gel electrophoresis in 2%
6 agarose gel in 1xTAE buffer and visualized using SYBR green. Prior to sequencing, the
7 amplification products were cleaned using QIAquick gel PCR purification kit (QIAGEN).
8 Both strands of the purified PCR products were sequenced separately using primers
9 mentioned above at Secugen S.L. (Madrid, Spain).
10 Sequencher™ 4.2 (Gene Codes Corporations, Ann Arbor, Michigan, USA) was used to
11 identify the consensus sequence from the two strands of the ITS nrDNA of each isolate. Using
12 the BLASTn algorithm (Altschul *et al.*, 1997), the consensus sequences were compared with
13 sequences in the UNITE (Kõljalg *et al.*, 2005) and INSD (International Nucleotide Sequence
14 Database) online nucleotides databases. The new consensus sequences have been lodged in
15 the EMLN-EBI database with the accession numbers indicated in Table 1.

17 Data analysis

19 Correspondence analyses (CA) were performed to analyze the ectomycorrhizal assemblage on
20 seedlings using CANOCO version 4.5 (Microcomputer Power, Ithaca, NY, USA). ECM
21 presence/absence data were used considering each seedling as an individual sample.
22 The potential effects of fire frequency on the percentage of seedling ECM colonization was
23 tested using one-way analysis of variance (ANOVA), followed by Tukey HSD test. Changes

in the ECM species colonizing seedlings in the four treatments were tested using both frequency, based on the presence/absence of morphotypes on the seedlings, and abundance (percentage of colonization of the species *i* on the seedling *j* multiplied by percentage of total ectomycorrhization of the seedling *j* divided by 100). Since both principles of normality and variance homogeneity could not be assumed for the data set, non-parametric Kruskal-Wallis and Mann-Whitney U-tests were used to compare species frequency and abundance in the four sites. Species that occurred too infrequently to apply statistical tests were left out. The following diversity indices were calculated for each site: species richness, Shannon diversity index (Pielou 1975), and the evenness index (Smith & Wilson 1996). Non-parametric Kruskal-Wallis and Mann-Whitney U-test were used to test for differences in the diversity indices between different treatments. Statistical testing was carried out using SPSS version 17.0 (SPSS Inc., Chicago, USA).

Results

No ectomycorrhizal colonization was detected on control seedlings grown in sterile soil. Of the 251 sampled root tips, 206 were analysed with molecular techniques. Due to the high frequency of root samples colonized by *C. geophilum* (57 seedlings) and the fact that this species is morphologically easily identified, molecular techniques were applied only to 12 samples colonized by this fungal species. 181 out of 206 analyzed samples (88 %) were identified using PCR-sequencing, which rendered a total of 18 different species observed on the bioassay seedlings (Table 1). The most commonly found fungal species were *C.*

1 *geophilum*, *Thelephora terrestris* Ehrh., *Tomentella ellisii* (Sacc.) Jülich & Stalpers, *Inocybe*
 2 *jacobi* Kühner, and two *Rhizopogon* species. Only four of the 18 fungal species, (*C.*
 3 *geophilum*, *T. terrestris*, *I. jacobi*, and *T. ellisii*), were found in both host trees (Table 2). Both
 4 moderate and high fire frequency affected the ECM colonization of pine and oak seedlings, in
 5 terms of species composition, frequency and abundance.

6 The correspondence analysis separated the ectomycorrhizal assemblages of all seedlings
 7 along axis 2 into two groups that corresponded to the two different hosts (Fig. 1). However,
 8 pine and oak seedlings grown on soil from site B were grouped mainly on the upper left hand
 9 side of the ordination diagram showing that moderate fire frequency reduced the number of
 10 host specific ECM species present in the unburnt site, leading to a greater similarity of ECM
 11 assemblages in the two hosts. High fire frequency led, however, to a divergence of the ECM
 12 communities present in oak and pine seedlings.

14 *ECM species colonizing Pinus pinaster*

15 ECM colonization of pine seedlings did not differ significantly between treatments ranging
 16 from 55 ± 7.34 % (mean $\pm$ S.E.) in the unburnt soil to 64.2 ± 9.94 % in the site B2 (Table 2).
 17 For pine-tree, the number of ECM species was higher on seedlings grown in the soil from the
 18 high frequency fire site (eight) than on those grown in unburnt or moderate frequency fire
 19 soils (Table 2). However, no significant differences were found on ECM richness, diversity or
 20 evenness among treatments (Table 2). *Cenococcum geophilum* and both *Rhizopogon* species
 21 were present on seedlings grown in all four sampled soils. Species like *I. jacobi* and *T.*
 22 *terrestris* were found only in the burnt soils. *Wilcoxina mikolae* (Chin S. Yang & H.E.

Wilcox) Chin S. Yang & Korf was not found in site B and the uncultured Ascomycotina was present only in sites UB and PB2.

Rhizopogon luteolus Fr. & Nordholm frequency and abundance decreased significantly in the high frequency fire sites when compared with UB and B (Fig. 2a,b; Table 3). A complete opposite pattern was found for *Rhizopogon roseolus* (Corda) Th. Fr. that was favoured by fire, and even more by its recurrence. *Inocybe jacobii* appeared only in the burnt sites showing a statistically significant increase between UB and B sites, and a decrease from B to B2. Significant differences in the frequency and abundance of *C. geophilum* were observed only between UB and B sites with a decrease of the species after the disturbance event. *Thelephora terrestris* seemed to be positively affected by fire, increasing from UB to B, while it appeared to suffer with fire frequency decreasing both in frequency and abundance in the high frequency fire sites. The uncultured ascomycete was found only in the unburnt site and at low frequencies in the high frequency fire site, B2, appearing to be sensitive to both fire disturbance and recurrence. Another member of the resistant propagules community encountered in the study area was *W. mikolae* that profit from high fire frequency in the site dominated by *C. ladanifer*, with a significant increase in frequency between UB and B1 and B1 and B2. Frequency and abundance of *W. mikolae* in B2 was significantly lower than in B1. The ECM communities of pine seedlings grown in UB and B soils were different although there was a slight overlap between both groups (Fig. 3a). The uncultured Ascomycotina and Basidiomycotina, were associated with seedlings grown on unburnt soil, while *I. jacobii* and *T. terrestris*, characterized the seedlings from the moderate fire frequency site. The samples from sites B1 and B2 were mainly grouped together in the left hand side of the ordination plot

in correspondence with *W. mikolae* and *R. roseolus*, and showed a greater similarity with the samples from site B than from the unburnt site.

ECM species colonizing Quercus suber

Oak root colonization ranged between 25.5 ± 6.9 % in B2 and 59.1 ± 6.8 % in B1 (Table 2) being the difference significant (test $p = 0.02$) only between B1 and B2.

The number of ECM species found on oak seedlings decreased in soils from burnt sites, both with high and moderated frequency fire (Table 2). Nine different ECM fungi were detected on seedlings grown in the unburnt soil, while only two ECM fungi were found in the soil from the high frequency fire site B1 (Table 2). Seven out of the eleven ECM species found on oak plants colonized only one single seedling (Table 2).

ECM species richness on oak seedlings was severely affected by fire frequency decreasing from 2.60 ± 0.267 in the unburnt soil to lower values in high frequency fire sites (B1: 2.00 ± 0.00 , $p = 0.050$; B2: 1.64 ± 0.152 , $p = 0.010$). Shannon diversity and evenness were significantly lower on soil B2 than in the remaining treatments (Diversity, UB $p = 0.001$; B $p = 0.007$; B1 $p = 0.003$; Evenness, UB $p = 0.013$; B1 $p = 0.003$) (Table 2).

Cenococcum geophilum was the only species colonizing seedlings grown in soils of all four sites. Six species were present exclusively in the unburnt soils. *Laccaria laccata* (Scop.) Cooke, *Laccaria bicolor* (Maire) P.D. Orton, *Scleroderma cepa* Pers., *Scleroderma leave* Lloyd, *Scleroderma polyrhizum* (J.F. Gmel.) Pers., and *Lactarius hepaticus* Plowr. colonized only oak seedlings. *Laccaria bicolor* was found exclusively in the unburnt site while *L. laccata* occurred only in the moderate frequency fire site. However, due to the high similarity in the ITS sequences of both species these two isolates might belong to the same species. The

frequency of *C. geophilum* increased from UB and B to B1, and was higher in B1 than in B2 indicating a positive effect of high frequency fire in the area dominated by *C. ladanifer* (Fig. 4a). *Tomentella ellisii* was completely absent in UB and B sites and showed a significant positive response to high frequency fire, especially in the *C. ladanifer* dominated area. The response to fire recurrence of *I. jacobi* on oak seedlings was analogous to what was found for pine (Fig. 4a,b, Table 4). This species was favoured by moderate frequency fire (site B) but decreased significantly with high frequency fire in both B1 and B2. *Thelephora terrestris*, which did not colonize pine roots at all in UB plots, was the most abundant species on oak root tips grown in the unburnt soil. Its response to fire in oak showed a completely opposite behaviour than in pine, decreasing significantly in all burnt sites (Fig. 4a,b).

The ECM assemblages on oak seedlings were clustered into two distinct groups belonging to UB and B (Fig. 3b). The seedlings grown on the unburnt soil were characterized by *Scleroderma* sp., *Lactarius hepaticus* Plowr., and *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew, while those grown on moderate fire frequency soil were found in association with *I. jacobi*. All oak seedlings grown on soil B1 were colonized by *C. geophilum* and *T. ellisii*, which results in a single symbol in the diagram. The CA placed the samples from oak seedlings grown on soil B2 scattered along axis 1 and overlapped with B1 and UB.

Discussion

1 Resistant ECM propagules colonizing *P. pinaster* and *Q. suber* seedlings were differently
2 affected depending on the fire regime. High frequency fire had strong effects on the RPC
3 composition. Infrequent species found in the unburnt soil were completely eliminated in the
4 high frequency fire sites, some species were favoured by moderate frequency fire but suffered
5 with frequent fires, and others species positively responded to both fire regimes. The post fire
6 ECM community usually has species in common with the pre fire spore bank (Baar *et al.*,
7 1999). However, other new fungal species might colonize the affected areas after the
8 disturbance event benefitting from the release of competition induced by fire. The resistant
9 propagules assemblage on bioassay seedlings in the present study might therefore be the
10 result of direct effects of fire combined with a posterior spore dispersion phenomenon. Our
11 results show that the direct and indirect effects of fire differed for the several ECM fungal
12 species present in the pre-fire RPC.

13 Fire had a negative effect on *R. luteolus*, which decreased progressively with fire frequency.
14 *Rhizopogon roseolus*, on the contrary, responded positively to the disturbance event
15 increasing both in frequency and abundance in the burnt sites. The colonization frequency by
16 the genus *Rhizopogon* did not show significant differences between the four treatments
17 indicating that the fire induced abundance/frequency changes of *R. roseolus* in one direction
18 was always balanced by the response of *R. luteolus* in the opposite direction. Species of the
19 genus *Rhizopogon* are known to be abundant in soil as fire-resistant spores or sclerotia that
20 represent an important source of inoculum after disturbances (Baar *et al.*, 1999; Taylor &
21 Bruns, 1999; Izzo *et al.*, 2006a,b). They are indeed highly abundant on pine seedlings during
22 post-fire natural regeneration and on bioassay seedlings grown either in soil from mature
23 forest or fire-treated soils (Kjøller & Bruns, 2003; Cline *et al.*, 2005). Post disturbance

seedling colonization is favoured by a combination of traits characteristic of this genus of hypogeous fungi, such as frequent fruiting, efficient dispersal and long-lived spores (Molina *et al.*, 1999). Differences in the ability of resistant propagules to withstand stress (heat and drought), a high density of *Rhizopogon* spores, alteration of the competitive environment by heat treatment, and potential role of heat in the spore activation, could explain the overall shift in species composition after fire disturbance (Izzo *et al.*, 2006a).

Competition between the *Rhizopogon* species found in this study is strongly influenced by the local environment and can be shaped by a series of factors like dispersal limitation and nutrient availability (Kennedy *et al.*, 2007). The different effect of repeated fires on these species suggests that *R. roseolus* has adaptative traits able to grant it the capacity to stand recurrent stressful conditions such as heat and long-term soil drying. These traits result in a significant competitive advantage of *R. roseolus* over *R. luteolus* on highly disturbed areas, being the competition success of *R. luteolus* promoted in late successional forest stages.

Inocybe jacobii colonized both pine and oak seedlings and showed a comparable response to the fire event being apparently favoured by moderate frequency fire. The low frequency of resistant propagules of *I. jacobii* in unburnt sites and its presence in the moderate frequency fire site in approximately 60 % and 90 % of the pine and oak bioassay respectively, could either reveal that this species benefits from moderate frequency fires or be the result of post fire colonization by external spores. Since unburnt and moderated fire frequency sites are close to each other, postfire colonization could have been expected to have occurred in both sites. However, changes in biotic and abiotic variables after the fire event could have created potential niches for airborne spores that favoured their settlement exclusively in the disturbed area. The presence of *I. jacobii* on three pine seedlings and its complete absence on oak root

tips in the high frequency fire sites could also be either attributed to the negative effect of high fire frequency on this species or to a lower rate of colonization by airborne spores compared to the moderate fire frequency site.

In contrast, *T. ellisii* was found exclusively in the high frequency fire sites. This species could have been present at low densities in the area before the disturbance event, and have benefited from the removal of other competitive ECM species by high frequency fire or could have colonized the area by spore dispersal. *Tomentella ellisii* colonized both hosts but while its frequency on pine root tips was very low and limited to the area dominated by *C. ladanifer*, it colonized approximately half of the oak seedlings in both high frequency fire sites. A possible explanation of the different colonizing host behaviour of this species could be the result of interaction among fungal species. The number of ECM colonizing species in the soil from high frequency fire sites was higher for pine ($n = 10$) than oak seedlings ($n = 3$), and their interactions could have led to a process of competition that affected negatively *T. ellisii*.

Similar processes were described for *Rhizopogon occidentalis* Zeller & C. W. Dodge and *Tomentella subliacina* by Lilleskov & Bruns (2003), who showed that, after a fire, *R. occidentalis* rapidly colonise seedling roots from spores, which leads to an early peak in the abundance of this species that is later replaced by *T. subliacina*. The scarce presence of *T. ellisii* on pine roots might be related to the high abundance of *Rhizopogon* species at the time of harvest (6 months). This assumption finds support in the study by Taylor & Bruns (1999) who observed the dominance of *T. subliacina* in the RPC of older *P. muricata* bioassay harvested 15-20 months after planting. The results obtained for oak seedlings, harvested after twelve months, show that *T. ellisii* can establish from spores and slowly colonize oak seedlings.

1 The overstorey composition of the high frequency fire sites could also explain quantitative
 2 differences in the ECM communities. The survival on shrub roots of mycorrhizal fungal
 3 species able to colonize both shrubs and pine trees could potentially represent a source of
 4 inoculum available for pine seedlings. The bridge between mycorrhizal fungi and pine
 5 seedlings mediated by shrubs known as boot-strapping, plays an important role in the process
 6 of seedlings establishment and its presence offers an important chance for ecosystem recovery
 7 (Perry *et al.*, 1989). Species like *C. geophilum* and *T. ellisii* on oak, and *W. mikolae* on pine
 8 bioassays, were more frequent and abundant in the *C. ladanifer* dominated site (B1) than in
 9 the site dominated by *Erica* spp. and *P. tridentatum* (B2). However, due to the very low
 10 number of species colonizing oak seedlings in these sites, a small oscillation in the abundance
 11 or frequency of one of them in one direction can shift the frequency and abundance of the
 12 others in the opposite direction, highlighting differences that cannot be definitely attributed to
 13 the overstorey composition. The RPC composition was quite similar in the two sites, and
 14 presented the highest number of species colonizing pine seedlings highlighting the importance
 15 of shrubby areas as sources of ectomycorrhizal inoculum, independently, in this specific case,
 16 of the overstorey composition.

17 Previous studies on the RPC associated with other coniferous trees in North America have
 18 found similar results to the ones presented in this study (Baar *et al.*, 1999; Taylor & Bruns,
 19 1999; Izzo *et al.*, 2006a,b). However, information about the RPC colonizing broadleaved
 20 hosts after fire is lacking. The similarity in the ECM assemblage on seedlings between
 21 different sites was greater for oak than pine. Also, species richness and diversity was not
 22 affected by fire frequency on pine bioassays, but it decreased on oak bioassays. Seedlings
 23 grown on soil from high frequency fire sites were colonized only by three not specific ECM

species. These results suggest that recurrent fires can have a more detrimental effect on oak trees than on pine trees. The lost of suitable ECM inoculum in high frequency fire sites could therefore hinder the regeneration of oak forest.

Laccaria, *Scleroderma*, and *Lactarius* species were never found in the high frequency fire sites, and occurred exclusively associated with oak seedlings in soil from unburnt and moderate fire frequency sites. These species are usually frequently observed fruiting in disturbed sites with young trees and shrubs (Danielson, 1984; Comandini *et al.*, 2006). Their absence in the high frequency fire sites and a single presence in the moderate frequency fire site could reveal sensitivity to the harsher environmental conditions of the disturbed sites and in particular to the high frequency fire sites, characterized by patches of vegetation growing on a skeletal soil lacking an organic layer. This hypothesis could be supported by the fact that oak occurred as natural regenerated seedlings punctually distributed, only in the mature unburnt pine stand. The low soil moisture of the high frequency fire sites, which are more exposed to the effects of sun and wind than the other sites could have possibly affected negatively oak trees and their symbionts (Kennedy & Peay, 2007) and favoured more ruderal ECM species associated with pine.

To our knowledge the present work represents the first attempt to assess the effects of fire frequency on the RPC of ectomycorrhizal fungi. Few studies have assessed the effects of fire frequency on the belowground ECM communities. The information available refers to the reduction of ECM biomass following repeated burnings (Hart *et al.*, 2005), to changes on the ECM community structure (Tuininga & Dighton, 2004) or to fire frequency induced shifts on total soil fungal communities (Bastias *et al.*, 2006; Anderson *et al.*, 2007). There is however a lack of information relative to the ecology and the response of ECM species to high frequency

fires. As demonstrated in the current study, high frequency fires can negatively affect species that are known to respond positively to a single fire event and to favour species that would be otherwise uncommon in the unburnt forest, revealing the importance of the fire regime in shaping the general ectomycorrhizal community as well as the more resilient RPC. The alterations induced by high frequency fire on the structure and composition of the aboveground vegetation and on the associated belowground ECM community could compromise the resilience of the ecosystem inverting the succession process towards a degradation cycle.

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1 Figures legends

2

3 Fig. 1. Plot from CA (axes 1 and 2) performed on *Pinus pinaster* (hollow symbols) and
4 *Quercus suber* seedlings (filled symbols). Each point represents a single seedling.

5 Eigenvalues of axis 1 and 2 are 0.631 and 0.591 respectively, and explain 21 % of the
6 variance of species data. Circles indicate unburnt site (UB), squares indicate moderate fire
7 frequency fire site (B), up-triangles indicate high fire frequency site dominated by *Cistus*
8 *ladanifer* (B1), and down-triangles indicate high fire frequency site dominated by ericaceous
9 shrubs and *Pterospartum tridentatum* (B2).

10

11 Fig. 2. Relative frequency (a) and abundance (b) of ECM taxa on pine seedlings grown in
12 unburnt site (UB), moderate fire frequency site (B), high fire frequency site dominated by
13 *Cistus ladanifer* (B1), and high fire frequency site dominated by ericaceous shrubs and
14 *Pterospartum tridentatum* (B2).

15

16 Fig. 3. Plot from CAs (axes 1 and 2) performed on *Pinus pinaster* (a) and *Quercus suber* (b)
17 seedlings. Each point represents a single seedling. Eigenvalues of axis 1 and 2 are 0.519 and
18 0.491 respectively, and explain 37.8% of the variance of species data. Hollow circles indicate
19 unburnt site (UB), filled circles indicate moderate fire frequency fire site (B), filled squares
20 indicate high fire frequency site dominated by *Cistus ladanifer* (B1), and hollow squares
21 indicate high fire frequency site dominated by ericaceous shrubs and *Pterospartum*
22 *tridentatum* (B2). Species (crosses) abbreviations are: *Cadophora finlandica* (Ca.f.),
23 *Cenococcum geophilum* (C.g.), *Inocybe jacobii* (I.j.), *Laccaria bicolor* (L.b.), *Laccaria laccata*

(L.l.), *Lactarius hepaticus* (La.h.), *Rhizopogon luteolus* (R.l.), *Rhizopogon roseolus* (R.r.),
Scleroderma cepa (S.c.), *Scleroderma leave* (S.l.), *Scleroderma polyrhizum* (S.p.), *Thelephora*
terrestris (Th.t.), *Tomentella ellisii* (T.e.), uncult. Ascomycotina (uA), uncult. *Cortinarius*
(uC.), uncult. ECM (uE), uncult. Basidiomycotina (uB), *Wilcoxina mikolae* (W.m.).

Fig. 4. Relative frequency (a) and abundance (b) of ECM taxa on oak seedlings grown in
unburnt site (UB), moderate fire frequency site (B), high fire frequency site dominated by
Cistus ladanifer (B1), and high fire frequency site dominated by ericaceous shrubs and
Pterospartum tridentatum (B2).

1 Table 1. Database matches of ITS sequences from ectomycorrhizal fungal taxa.

Morphotype	Host	Accession number	Closest species	% similarity/ bp	BLAST expected value
<i>Cadophora</i> sp.	Q	GQ205351	AF486119 <i>Cadophora finlandica</i>	98%/839	0.0
<i>Cenococcum geophilum</i>	P/Q	GQ205352/GQ205369	AY825508 <i>Cenococcum geophilum</i>	99%/511	0.0
<i>Inocybe</i> sp.	P/Q	GQ205353/GQ205370	AM882710 <i>Inocybe jacobi</i>	100%/505	0.0
<i>Laccaria</i> sp.1	Q	GQ205354	DQ367906 <i>Laccaria bicolor</i>	98%/711	0.0
<i>Laccaria</i> sp.2	Q	GQ205355	EU819477 <i>Laccaria laccata</i>	97%/709	0.0
<i>Lactarius</i> sp.	Q	GQ205356	UDB000861 <i>Lactarius hepaticus</i>	100%/699	0.0
<i>Rhizopogon</i> sp.1	P	GQ205357	UDB001618 <i>Rhizopogon luteolus</i>	100%/786	0.0
<i>Rhizopogon</i> sp.2	P	GQ205358	EU379678 <i>Rhizopogon roseolus</i>	99%/608	0.0
<i>Scleroderma</i> sp.1	Q	GQ205360	EU784412 <i>Scleroderma cepa</i>	99%/715	0.0
<i>Scleroderma</i> sp.2	Q	GQ205359	EU718117 <i>Scleroderma laeve</i>	99%/755	0.0
<i>Scleroderma</i> sp.3	Q	GQ205361	EU718123 <i>Scleroderma polyrhizum</i>	98%/507	0.0
<i>Thelephoraceae</i> 1	P/Q	GQ205362/GQ205371	EU427330 <i>Thelephora terrestris</i>	100%/663	0.0
<i>Thelephoraceae</i> 2	P/Q	GQ205372/GQ205363	UDB000219 <i>Tomentella ellisii</i>	99%/582	0.0
Ascomycotina	P	GQ205364	EU046087 uncult. Ascomycotina	99%/512	0.0
<i>Cortinarius</i> sp.	P	GQ205365	AY634134 uncult. <i>Cortinarius</i>	99%/629	0.0
uncult. ECM	P	GQ205366	EF484935 uncult. ECM fungus	99%/589	0.0
Basidiomycotina	P	GQ205368	FJ013075 uncult. Basidiomycotina	100%/616	0.0
<i>Wilcoxina</i> sp.	P	GQ205367	DQ069000 <i>Wilcoxina mikolae</i>	99%/597	0.0

- 1 Table 2. Occurrence, ECM root colonization (%), species richness, Shannon diversity, and evenness of ECM species on oak (Q) and pine (P) seedlings
- 2 across treatments (UB, unburnt site; B, moderate fire frequency site; B1, high fire frequency site dominated by *Cistus ladanifer*; B2, high fire frequency
- 3 site dominated by ericaceous shrubs and *Pterospartum tridentatum*). Different letters refer to significant differences between treatments.

Species	P-UB	P-B	P-B1	P-B2	Q-UB	Q-B	Q-B1	Q-B2
<i>Cadophora finlandica</i>					1			
<i>Cenococcum geophilum</i>	11	4	6	6	9	6	11	6
<i>Inocybe jacobii</i>		7	2	1	1	12		
<i>Laccaria bicolor</i>					1			
<i>Laccaria laccata</i>						1		
<i>Lactarius hepaticus</i>					1			
<i>Rhizopogon luteolus</i>	10	10	4	3				
<i>Rhizopogon roseolus</i>	1	4	7	9				
<i>Scleroderma cepa</i>					1			
<i>Scleroderma leave</i>					1			
<i>Scleroderma polyrhizum</i>					2			
<i>Thelephora terrestris</i>		10	2	4	10	6		4
<i>Tomentella ellisii</i>			2				11	8
uncult. Ascomycotina	6			1				
uncult. <i>Cortinarius</i>			1					
uncult. ECM fungus				1				
uncult. Basidiomycotina	1							
<i>Wilcoxina mikolae</i>	1		9	3				
Root colonization (S.E.)	55 (7.34)	59.1 (6.10)	60 (9.51)	64.2 (9.94)	42.5 (6.9) ^{ab}	38.1 (6.0) ^{ab}	59.1 (6.8) ^a	25.5 (6.9) ^b
Species richness (S.E.)	2.50 (0.167)	3.09 (0.315)	2.57 (0.369)	2.22 (0.278)	2.60 (0.267) ^a	1.92 (0.211) ^{ab}	2.00 (0.00) ^b	1.64 (0.152) ^b
Shannon diversity (S.E.)	0.45 (0.112)	0.44 (0.091)	0.40 (0.087)	0.32 (0.102)	0.52 (0.119) ^a	0.38 (0.082) ^a	0.24 (0.058) ^a	0.06 (0.025) ^b
Evenness (S.E.)	0.45 (0.096)	0.41 (0.079)	0.39 (0.072)	0.32 (0.095)	0.44 (0.114) ^a	0.37 (0.105) ^{ab}	0.35 (0.084) ^a	0.08 (0.035) ^b
Total n. of seedlings	12	12	12	12	11	13	11	11

Tab. 3. Results of the non-parametric Kruskal-Wallis test for differences in frequency and abundance on pine seedlings among sites.

	Frequency		Abundance	
	X ²	p-value	X ²	p-value
<i>Cenococcum geophilum</i>	8.87	0.031	7.61	0.050
<i>Inocybe jacobii</i>	14.35	0.002	12.08	0.007
<i>Rhizopogon luteolus</i>	14.18	0.003	12.68	0.005
<i>Rhizopogon roseolus</i>	12.19	0.007	9.26	0.026
<i>Thelephora terrestris</i>	20.56	0.000	16.57	0.001
uncult. Ascomycotina	16.21	0.001	16.81	0.000
<i>Wilcoxina mikolae</i>	20.14	0.000	11.02	0.012

Tab. 4. Results of the non-parametric Kruskal-Wallis test for differences in frequency and abundance on oak seedlings among sites.

	Frequency		Abundance	
	X ²	p-value	X ²	p-value
<i>Cenococcum geophilum</i>	9.91	0.019	16.71	0.001
<i>Inocybe jacobii</i>	36.16	0.000	35.55	0.000
<i>Thelephora terrestris</i>	18.39	0.000	26.75	0.000
<i>Tomentella ellisii</i>	36.20	0.000	34.07	0.000

Fig. 1. Plot from CA (axes 1 and 2) performed on *Pinus pinaster* (hollow symbols) and *Quercus suber* seedlings (filled symbols). Each point represents a single seedling. Eigenvalues of axis 1 and 2 are 0.631 and 0.591 respectively, and explain 21 % of the variance of species data. Circles indicate unburnt site (UB), squares indicate moderate fire frequency fire site (B), up-triangles indicate high fire frequency site dominated by *Cistus ladanifer* (B1), and down-triangles indicate high fire frequency site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).

Fig. 2. Relative frequency (a) and abundance (b) of ECM taxa on pine seedlings grown in unburnt site (UB), moderate fire frequency site (B), high fire frequency site dominated by *Cistus ladanifer* (B1), and high fire frequency site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).

Fig. 3a. Plot from CAs (axes 1 and 2) performed on *Pinus pinaster* (a) and *Quercus suber* (b) seedlings. Each point represents a single seedling. Eigenvalues of axis 1 and 2 are 0.519 and 0.491 respectively, and explain 37.8% of the variance of species data. Hollow circles indicate unburnt site (UB), filled circles indicate moderate fire frequency fire site (B), filled squares indicate high fire frequency site dominated by *Cistus ladanifer* (B1), and hollow squares indicate high fire frequency site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2). Species (crosses) abbreviations are: *Cadophora finlandica* (Ca.f.), *Cenococcum geophilum* (C.g.), *Inocybe jacobii* (I.j.), *Laccaria bicolor* (L.b.), *Laccaria laccata* (L.l.), *Lactarius hepaticus* (La.h.), *Rhizopogon luteolus* (R.l.), *Rhizopogon roseolus* (R.r.), *Scleroderma cepa* (S.c.), *Scleroderma leave* (S.l.), *Scleroderma polyrhizum* (S.p.), *Thelephora terrestris* (Th.t.), *Tomentella ellisii* (T.e.), uncul. Ascomycotina (uA), uncul. Cortinari (uC.), uncul. ECM (uE), uncul. Basidiomycotina (uB), *Wilcoxina mikolae* (W.m.).

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Fig. 4. Relative frequency (a) and abundance (b) of ECM taxa on oak seedlings grown in unburnt site (UB), moderate fire frequency site (B), high fire frequency site dominated by *Cistus ladanifer* (B1), and high fire frequency site dominated by ericaceous shrubs and *Pterospartum tridentatum* (B2).