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Common environmental factors explain both ectomycorrhizal species diversity and pine regeneration variability in a post-fire Mediterranean forest

Erika Buscardo · Helena Freitas · João Santos Pereira · Paolo De Angelis

Abstract Natural seedling regeneration and establishment after stand replacing wildfires is influenced by a series of environmental and biological constraints. In this study, we characterized the diversity and structure of the ectomycorrhizal (ECM) fungal community associated with post-fire naturally regenerated maritime pine saplings, and individuate the environmental factors responsible for fungal species distribution. We also identify the main environmental factors responsible for maritime pine regeneration variability and assessed the relation between saplings performance and ECM fungal diversity indices. Fungal species were identified by direct sequencing of internal transcribed spacer regions. Five years after the disturbance event, a total of 30 taxa colonized the pine saplings. The ECM fungal community was dominated by ruderal species of the genus *Rhizopogon* (present in almost half of the samples). Almost one third of the identified ECM fungal species belonged to the family *Thelephoraceae*. Typical k-selected species like *Amanita pantherina*, *Boletus aestivalis*, *Lactarius chrysorrheus*, and *Russula densifolia* were found on pine saplings collected in proximity of unburnt pine trees, in correspondence with low erosion extents. Pine regeneration varied throughout the study areas and was enhanced at higher elevations, in correspondence with moderate slopes, shallower soils, and a reduced cover of ericaceous shrubs and bare ground. These conditions were found in close proximity to patches of pine trees that survived the disturbance event and were previously characterized by a higher pre-fire pine biomass. Even though no correlations were found between saplings performance and ECM fungal diversity indices, common environmental factors (i.e., ericaceous shrub cover, extent of erosion, slope, and soil depth) were responsible for shaping the ECM fungal distribution and for describing most of the explained regeneration variability.

Keywords Ectomycorrhizal fungi · *Pinus pinaster* (maritime pine) · Wildfire · Regeneration variability · Mediterranean ecosystem · Succession

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Introduction

Wildfires represent a major form of disturbance of Mediterranean ecosystems and influence their dynamics and structure with changes in above and below-ground communities, functions, and processes (Naveh 1975; Trabaud 1987; Neary et al. 1999; Certini 2005).

The success of *Pinus pinaster* Ait. as a colonizer of disturbed soils may be attributed to adaptive responses that allow survival after low intensity fires, such as thick protective bark and reproduction processes that facilitate recovery after stand replacement fire from seeds stored in serotinous cones (Fernandes and Rigolot 2007). However, the occurrence of repeated high-intensity fires is changing the dynamics of these pine stands with a regression of the

vegetation structure and a delay of the succession processes due to the invasion of shrubs of the Mediterranean macchia. Under these unfavorable circumstances, even species that usually regenerate well after fire show irregular or low regeneration (Lloret 1998), and a better understanding of the changing regeneration processes is needed in order to promote the establishment and survival of pine seedlings. Pine regeneration is known to go on for more than 2 years after fire (Luis-Calabuig et al. 2002), and subsequent development of seedlings depends not exclusively on post-fire environmental factors (Pausas et al. 2004) but also on seedlings association with symbiotic ECM fungi (Taylor and Bruns 1999). Pine trees are indeed ecologically obligate mycorrhizal symbionts that depend on the presence of fungal inoculum for establishment and survival (Smith and Read 1997).

The diversity and composition of ECM fungal communities are driven by mechanisms that involve the interactions between disturbance, ECM fungal colonization potential, species competition, and resource partitioning (Bruns 1995). Post-fire ECM fungal communities resemble the composition of pre-fire resistant propagule communities (Baar et al. 1999; Taylor and Bruns 1999). However, new fungal species may colonize the areas following the disturbance event, benefitting from the release of competition induced by fire (Buscardo et al. 2010). Maritime pine is compatible with many different ECM fungi with broad host range (Pera and Alvarez 1995), and the presence of residual trees and shrubs after a wildfire could also play an important role by providing available fungal networks to adjacent regenerating seedlings (Perry et al. 1989; Cline et al. 2005). The understorey of Mediterranean pine forests are commonly colonized by both ECM and ericoid mycorrhizal shrubs species and the presence on shrub roots of mycorrhizal fungal species able to colonize both shrubs and pine could represent a source of inoculum for the newly regenerated seedlings and promote their survival and establishment (Horton et al. 1999; Nara and Hogetsu 2004; Richard et al. 2009). Nevertheless, in certain conditions, shrubs could also represent a threat to pine regeneration and compete with natural regenerated seedlings for light and moisture (O'Brian et al. 2007). The importance of post-disturbance interactions between early colonizing plants and plants that are established concurrently or at later stages is well recognized (Pugnaire et al. 1996; Nara and Hogetsu 2004), but how environmental constraints affect pine regeneration, ECM fungal communities, and the interaction between them is poorly understood in wildfire prone forest ecosystems.

The objectives of this study were to (1) to characterize the ECM fungal assemblage on 135 maritime pine saplings and individuate the environmental factors responsible for ECM fungal diversity 5 years after a stand replacing wildfire and (2) to assess the variability of maritime pine regeneration in relation to ECM fungal diversity indices and a series of environmental factors including topographic variables and microsite conditions.

Materials and methods

Study site

Sampling was performed in a maritime pine open forest at S. Romolo (Imperia, Liguria), in the north-east of Italy (43º51' N, 7º44' W). In 2003, this area was affected by a severe wildfire that spread over approximately 350 ha. The vegetation covering the area previous to the disturbance event was characterized by an uneven-aged maritime pine forest (dominant trees with 35–40 cm diameter) with sporadic occurrence of broadleaved species like holly oak (*Quercus ilex* L.), downy oak (*Quercus pubescens* Willd.), and chestnut (*Castanea sativa* Mill.). The understorey was represented by typical post-fire resprouters and seeders species such as tree heather (*Erica arborea*), Mediterranean strawberry tree (*Arbutus unedo* L.), common heather (*Calluna vulgaris* L.), spiny broom (*Calicotome spinosa* L.), sage leaf rockrose (*Cistus salvifolius* L.), Montpellier rockrose (*Cistus monspeliensis* L.), and hairybroom (*Cytisus villosus* Pourr.).

The rugged topography of the study area and the presence of high-speed winds during the wildfire thwarted the operations of fire extinguishment. Only few patches of pine trees survived erratically on upper slope positions. Fire-killed trees were not harvested, and 5 years after the disturbance event, understorey shrub species and naturally regenerated pine saplings were colonizing the area.

Soils, shallow to moderately deep, are classified as eutric regosols and leptosols and lay on the top portion of flysh characterized by the presence of the "Arenarie di Bordighera" formation (Sagri 1980). The region is characterized by a Mediterranean climate with cool, wet winters, and warm, dry summers.

Data sampling procedure

Nine 10×10-m^2 plots were delimited in the burnt area in April 2008. The plots were chosen trying to encompass the variability of the area in terms of biotic and abiotic conditions. In every 10×10-m^2 plot, three 2×2-m^2 subplots were randomly delimitated in an imaginary quadrate made by 25 2×2-m^2 subplots. Within each 10×10-m^2 plot average height and percent cover of vegetation in each of several layers/growth-form categories were recorded: broadleaved trees, maritime pine saplings, shrubs, field layer (herbaceous vegetation), and ground layer (bryophytes).

Percent cover of bare soil or rock, percent cover of litter, and percent cover of trunks and branches were also recorded. For every 2×2-m² subplot, height and percentage cover of each of the five vegetation layers listed above were recorded as well as cover and height of ericaceous shrubs. In each subplot, pine saplings were counted, measured to the nearest centimeter, and classified into three groups according to height (Table 1).

For each plot, the following variables were recorded: slope, aspect (degrees), elevation, topographic position (five-point scale: from 1, gully to 5 ridge), soil depth, extent of erosion (three-point scale: from 0, no signs of erosion to 3 evident and widespread signs of erosion), distance (three-point scale: from 1, under 50 m to 3 over 100 m), and position (degrees) of the unburned maritime pine patches and pine pre-fire basal area (Table 1). Soil depth was measured using a manual soil auger. Aspect and position of the unburned pine patches were transformed to a linear scale (Beers et al. 1966).

To characterize the ECM fungal community associated with pine saplings, five individuals were randomly sampled in every subplot resulting in a total of 135 samples. After removing litter soil samples, blocks of approximately 12× 12 cm² surface and 20 cm depth were carefully excavated around each sapling using a spade and a sharp knife. A total of 135 samples were collected.

Pine roots were carefully washed over a 2-mm mesh sieve, analyzed under a dissecting microscope, and considered to be ECM if root hair were absent, if they appeared swollen, and in presence of hyphae or hyphal sheath. For each soil sample, all pine mycorrhizal roots were sorted into morphotypes based on their color, size, texture, emanating hyphae and rhizomorphs, and distinct branching morphology (Agerer 1991). The root present in 21 out of the 135

Table 1 Predictors and dependent variables used in the statistical analysis recorded at plot (P, 10×10-m²) and subplot (S, 2×2-m²) level

Variable description	Units/classes	Plot level	Mean(SE)	Min	Max
Predictors					
Elevation (ELE)	m (a.s.l.)	P	884(22)	807	975
Aspect (ASP)	0 to 2	P	0.8(0.21)	0.1	2
Slope (SLO)	%	P	22.1(2.53)	12	33
Soil depth (SoilD)	m	P	55.6(9.30)	20	100
Broadleaved tree cover (TreeC)	%	S/P	4.7(0.74)/2.1(1.06)	0	20
Broadleaved tree height (TreeH)	m	S/P	1.7(0.65)/0.4(0.13)	0	2.3
Shrub cover (ShrbC)	%	S/P	34.3(4.21)/31.7(3.40)	10	90
Shrub height (ShrbH)	m	S/P	0.7(0.04)/0.8(0.07)	0.3	1.2
Ericaceous shrub cover (EriC)	%	S	15.2(2.11)	0	43
Herbaceous species cover (HerbC)	%	S/P	5.7(4.58)/9.44(2.11)	0	20
Herbaceous species height (HerbH)	m	S/P	0.2(0.15)/0.2(0.03)	0	0.8
Ground cover (bryophytes) (GrndC)	%	S/P	0.1(0.03)/2.11(0.55)	0	0.5
Leaf litter cover (LittC)	%	S/P	49.3(3.70)/33.9(7.44)	20	90
Trunks and branches cover (TrBrC)	%	S/P	9.9(1.59)/9.9(1.7)	0.5	30
Bare ground cover (BarGC)	%	S/P	15.7(2.73)/30.0(5.00)	3	60
Pine pre-fire basal area (PreBA)	m²/plot	P	0.3(0.15)	0.14	0.62
Distance from unburnt pine patches (DiUB)	<50 m (1), 50–100 m (2), >100 m (3)	P	1.8(0.36)	1	4
Extent of erosion (ERO)	0, 1, 2	P	1.6(0.29)	0	2
Position of unburnt pine patches (PosUB)	0 to 2	P	0.3(0.06)	0.3	0.8
Topographic position (ToPos)	Gully (1), bottom slope (2), mid-slope (3), upper slope (4), ridge (5)	P	2.4(0.37)	1	4
Dependent variables					
Pine saplings cover (SapC)	%	S/P	10.1(1.42)/10.9(2.81)	3	25
Pine saplings height (SapH)	m	S/P	0.5(0.04)/0.5(0.06)	0.2	1
Density of saplings with height between 1 and 40 cm (Class 1)	Individuals/m²	S	5.6(3.74)	0	12
Density of saplings with height between 41 and 100 cm (Class 2)	Individuals/m²	S	8.6(7.28)	0	27
Density of saplings with height between 101 and 200 cm (Class 3)	Individuals/m²	S	2.9(5.92)	0	28
Total sapling density (TDen)	Individuals/m²	S	17.1(12.14)	4	56

samples did not contain ECM root tips and were therefore discarded. For each seedling, an average of four tips (ranging from one up to six) for each morphotype were individually sampled. A total of approximately 970 root tips were placed in Eppendorf tubes and washed in sterile water before molecular analyses.

DNA extraction, PCR, and sequencing

Total DNA was extracted with the C-TAB method [100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% cetyl-trimethyl-ammonium-bromide] from 220 samples. The internal transcribed spacer (ITS) region was amplified with the primer combination ITS1-F and ITS4 (White et al. 1990; Gardes and Bruns 1993) and succeeded for 204 samples (92.7 %). PCR was performed with ready-to-use PCR reaction mixture ImmoMix™ (Bioline) in 25 μl volume with the following cycling parameters: a denaturation step at 95°C for 7 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. Amplification products were ran by gel electrophoresis in 1.2% agarose gel. The DNA was stained with ethidium bromide, and single PCR products were purified from gel with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Both strands of the purified PCR products were sequenced separately by Macrogen laboratories. DNA sequences were manually checked and edited if necessary using the BioEdit software version 7.0.9.0 (Hall 1999), and consensus sequences from the two strands of the ITS nrDNA of each isolate were compared with sequences in the UNITE (Kõljalg et al. 2005) and INSD (International Nucleotide Sequence Database) online nucleotides databases, using the BLASTn algorithm (Altschul et al. 1997). A 97% sequence similarity cut-off was used for taxa identification.

Statistical analysis

Species accumulation curve with 95% confidence intervals was generated for the study site using the program EstimateS version 8 (Colwell 2006).

The data on ECM fungi were analyzed using Canonical Correspondence Analysis (CCA) (ter Braak 1986) employing species data frequency in 2×2-m^2 subplots. Forward selection was used to select significant explanatory variables and only those significant at the $p > 0.05$ level were included. Ordination analyses were performed using CANOCO version 4.5 (ter Braak and Smilauer 2002). Relative abundance was calculated for each morphotype on each sapling as the number of ECM root tips of that morphotype divided by the total number of ECM root tips on the considered pine sapling. Species richness and Shannon diversity index (H'; Pielou 1975) were calculated at the subplot level, and

correlations between diversity indices and environmental variables and between environmental variables themselves were analyzed using Pearson's and Spearman's rho methods with SPSS version 17.0 (SPSS Inc., Chicago, USA).

Partial least squares (PLS) regression analysis was used to examine the influence of environmental variables and ECM fungal diversity indices on regeneration variability (i.e., different growth of pine saplings and post-fire saplings density) at plot and subplot level. The number of pine saplings recorded at subplot level was transformed into pine density (individuals/square meters), which was then normalized using a logarithmic transformation. Prior to calculations, the data were centered and scaled to unit variance to give all variables the same relative importance. The significance of components for the models was determined by uncertainty tests carried out within a full cross-validation. PLS was carried out using the Unscrambler 9.8 software package (CAMO).

Results

Structure and diversity of the ECM fungal community

A total of 30 taxa were detected on maritime pine saplings root system (Table 2). Three of them were considered fungal root inhabiting taxa (i.e., *Phialocephala fortinii* C.J.K. Wang and H.E. Wilcox, *Oidiodendron maius* G.L. Barron, and an uncultured *Lachnum*). Another species, *Chroogomphus rutilus* (Schaeff.) O.K. Mill., was detected on ECM root tips of both *Rhizopogon roseolus* (Corda) Th. Fr. and *Rhizopogon luteolus* Fr. and Nordholm. Even though members of the *Chroogomphus* genus have been thought to be ECM with various species of pine, there are now evidences that suggest that these species act as parasites on the fungal host, the plant host, or both (Olsson et al. 2000; Agerer 1990). For this reason, *C. rutilus* and the other non-ECM fungal root inhabiting taxa were considered to be supplementary species in the ordination analyses. The species accumulation curve increased with the number of 2×2-m^2 subplots without reaching a plateau (Fig. 1), indicating insufficient sampling effort.

Pine saplings were colonized on average by two ECM fungal taxa (ranging from one to five). The ECM fungal community was dominated by Basidiomycota with only three species belonging to the Ascomycota (i.e., *Coniochaetaceae* sp., *Elaphomyces muricatus* Fr. 1829, and the uncultured Ascomycotina). More than one third of the ECM fungi occurred only in a single sample. Approximately 30% of the ECM fungal species found on pine saplings belonged to the *Thelephoraceae*. *Rhizopogon* was the most frequent genus and appeared in 49.6% of the samples, followed by the uncultured *Thelephoraceae* 2 (5.6%), *Russula densifolia* Secr. ex Gillet (4.8%), and *Boletus aestivalis* (Paulet) Fr.

Table 2 Database matches of ITS sequences obtained from ECM fungi and fungal root inhabiting taxa, colonizing maritime pine saplings 5 years after a stand replacing wildfire

Accession number	Type	Best blast hit	No. of colonized seedlings	BLAST expected value	% Similarity/bp
HM545718	*Amanita*	AB080776 *Amanita pantherina*	1	0.0	100%/689
HM545720	Bolete 1	DQ131609 *Boletus aestivalis*	5	0.0	99%/777
HM545722	*Chroogomphus*	UDB001529 *Chroogomphus rutilus*	20	0.0	99%/683
HM545723	*Coniochaetaceae*	EU082785 *Coniochaetaceae* sp.	1	0.0	91%/578
HM545724	*Elaphomyces*	UDB000093 *Elaphomyces muricatus*	2	0.0	99%/638
HM545725	*Hydnellum*	AY569027 *Hydnellum cyanopodium*	2	e^{-102}	96%/214
HM545727	*Lactarius* 1	AM930241 *Lactarius chrysorrheus*	4	0.0	99%/702
HM545728	*Lactarius* 2	AF249283 *Lactarius deliciosus*	3	0.0	99%/692
HM545721	Bolete 2	GU222293 *Octaviania tasmanica*	2	e^{-155}	91%/469
HM545729	*Oidiodendron*	AF062800 *Oidiodendron maius*[a]	7	0.0	99%/528
HM545730	*Phialocephala*	EU882733 *Phialocephala fortinii*[a]	4	0.0	99%/534
HM545731	*Rhizopogon* 1	GQ267481 *Rhizopogon luteolus*	16	0.0	99%/823
HM545732	*Rhizopogon* 2	HM036649 *Rhizopogon roseolus*	55	0.0	99%/672
HM545733	*Russula*	UDB001626 *Russula densifolia*	6	0.0	100%/663
HM545734	*Suillus*	AY898621 *Suillus bellinii*	1	0.0	99%/617
HM545735	*Thelephora*	GQ267490 *Thelephora terrestris*	3	0.0	98%/638
HM545736	*Tomentella* 1	UDB001656 *Tomentella badia*	1	0.0	99%/624
HM545737	*Tomentella* 2	UDB000219 *Tomentella ellisii*	3	0.0	99%/582
HM545740	*Tomentella* 5	AY641459 *Tomentellopsis submollis*	2	0.0	96%/681
HM545719	*Amphinema*	FJ210728 uncultured *Amphinema*	1	0.0	98%/568
HM545741	Asomycotina	EU557319 uncultured Ascomycotina	2	0.0	99%/540
HM545742	*Atheliaceae*	EU557321 uncultured Atheliaceae	1	0.0	99%/383
HM545744	uncultured ECM fungus	DQ054568 uncultured ECM fungus	1	0.0	100%/662
HM545726	*Lachnum*	FJ440910 uncultured *Lachnum*[a]	1	0.0	98%/494
HM545747	*Tricholomataceae*	FJ816733 uncultured *Mycena*	4	0.0	97%/561
HM545745	*Sebacina*	FN393153 uncultured *Sebacina*	1	0.0	96%/624
HM545743	*Thelephoraceae* 1	AJ972894 uncultured *Thelephoraceae* 1	2	0.0	99%/474
HM545746	*Thelephoraceae* 2	FJ013055 uncultured *Thelephoraceae* 2	7	0.0	100%/686
HM545738	*Tomentella* 3	AM161537 uncultured *Tomentella* 1	1	0.0	99%/641
HM545739	*Tomentella* 4	FJ013054 uncultured *Tomentella* 2	1	0.0	96%/692

[a] Root inhabiting taxa

1838 (4%). *R. roseolus* was the most abundant species with a colonizing root rate between 50% and 100% of the total root ectomycorrhization, in half of the samples.

Environmental variables and ECM fungal community

Five variables were able to significantly explain the variation in ECM fungal species frequency (Fig. 2): broad-leaved tree height (TreeH), extent of erosion (ERO), the interaction term ericaceous shrub cover (EriC)×soil depth (SoilD), trunks and branches cover (TrBrC), and slope (SLO). The first two axes (eigenvalues 0.615 and 0.489) explained respectively 10.3% and 8.2% of the total variability in the species data (total inertia, 5.975; explained variability, 34.66%). Axis 1 was correlated with TrBrC and

TreeH, and axis 2 was most closely associated with EriC× SoilD and SLO.

Species that are known to be more associated with mature forests such as *Amanita pantherina* (DC.) Krombh., *B. aestivalis*, *Lactarius chrysorrheus* Fr., and *Suillus bellinii* (Inzenga) Watling were found in the lower left-hand side of the ordination diagram in correspondence with a decreasing erosion extent (axes 1 and 2). Species belonging to the *Thelephoraceae* presented a scattered distribution, and non-ECM fungal roots inhabiting taxa were located close to the center of ordination space, together with *R. roseolus* and *R. luteolus*.

Species richness (mean ± SE, 2.67±0.38,) and Shannon diversity index (0.75±0.12) were negatively correlated with the distance from the unburnt pine patches (DisUB), ERO,

Fig. 1 Species accumulation (rarefaction) curve and confidence intervals (sobs 95%) of ectomycorrhizal fungi colonizing maritime pine saplings 5 years after a stand replacing wildfire

and shrub height (ShrbH), and positively correlated with pine pre-fire basal area (PreBA; Table 3). To avoid an excess of redundant data, only significant correlation between variables that were considered to be important for the purpose of the study were shown (Table 4). Elevation (ELE) was negatively correlated with SoilD, ERO, and EriC. SoilD was positively correlated with DiUB, EriC, and shrub cover (ShrbC), and was negatively correlated with PreBA. PreBA was also negatively correlated with DiUB, ERO, ShrbC, and EriC. DiUB was positively correlated with ERO, ShrbC, and ShrbH.

Environmental variables/ECM fungal community
and regeneration variability

Several regression analyses (PLS-R) were carried out, including the data set of explanatory variables and the ECM fungal diversity indices and different combinations of response variables both at plot (2×2 m^2) and subplot (10×10 m^2) level. Three PLS-R performed with data recorded in the 2×2 m^2 subplots provided significant components: a first regression (two components, $p < 0.05$) between pine saplings height (SapHt) and a set of four environmental variables (RMSE=0.09; R^2=0.62), a second (two components, $p < 0.05$) between the density of saplings belonging to Class 2 and a set of seven environmental variables (RMSE= 0.17; R^2=0.57), and a third (two components, $p < 0.05$) between total sapling density (TDen) and a set of six environmental variables (RMSE=0.12; R^2=0.72). SapHt was positively correlated with ELE and negatively correlated with slope (SLO), EriC×SoilD, and bare ground cover (BarGC; Fig. 3a); Class 2 was positively correlated with ELE and DiUB and negatively correlated with SLO, SoilD, EriC, BarGC, and leaf litter cover (LittC; Fig. 3b), and TDen

was positively correlated with ELE and negatively correlated with SLO, SoilD, ShrbH, BarGC, and LittC (Fig. 3c). No correlations were found between sapling performance and ECM fungal diversity indices (data not shown).

Discussion

Five years after the fire event, the early successional ECM fungal community that characterized the study area was dominated by *Rhizopogon* spp., which occurred abundant on the root system of almost half of the naturally regenerated pine saplings. Members of the genus *Rhizopogon* are known to be well distributed and persistent in soil as resistant propagules (Baar et al. 1999; Taylor and Bruns 1999; Izzo et al. 2006a,b; Kjøller and Bruns 2003; Buscardo et al. 2010) and are considered to be weak competitors often favored by fire competition release (Bruns et al. 2002; Kennedy et al.

Fig. 2 Results of CCA performed on frequency data of ECM fungal species colonizing maritime pine saplings 5 years after a fire event. Each *circle* represents a 2×2-m^2 subplot. Eigenvalues of axis 1and 2 are 0.615 and 0.489, respectively, and explain 34.66% of the variance of the species data. Species (*plus sign*) abbreviations are: *Amanita pantherina* (*A.p.*), *Boletus aestivalis* (*B.a.*), *Chroogomphus rutilus* (*C.r.*), *Coniochaetaceae* sp. (*C.sp.*), *Elaphomyces muricatus* (*E.m.*), *Hydnellum cyanopodium* (*H.c.*), *Lactarius chrysorrheus* (*L.c.*), *L. deliciosus* (*L.d.*), *Octaviania tasmanica* (*O.t.*), *Oidiodendron maius* (*O.m.*), *Phialocephala fortinii* (*P.f.*), *Rhizopogon luteolus* (*R.l.*), *R. roseolus* (*R.r.*), *Russula densifolia* (*R.d.*), *Suillus bellinii* (*S.b.*), *Thelephora terrestris* (*T.t*), *Tomentella badia* (*T.b.*), *Tomentella ellisii* (*T.e.*), *Tomentellopsis submollis* (*T.s.*), uncultured *Amphinema* (*u.A.*), uncultured Ascomycotina (*u.As.*), uncultured *Atheliaceae* (*u.At.*), uncultured ECM fungus (*u. ECM*), uncultured *Lachnum* (*u.L.*), uncultured *Mycena* (*u.M.*), uncultured *Sebacina* (*u.S.*), uncultured *Thelephoraceae* 1(*u.Th.1*), uncultured *Thelephoraceae* 2 (*u.Th.2*), uncultured *Tomentella* 1 (*u.T.1*), uncultured *Tomentella* 2 (*u.T.2*). Predictors abbreviations are: ericaceous shrub cover (*EriC*), extent of erosion (*ERO*), slope (*SLO*), soil depth (*SoilD*), trunks and branches cover (*TrBrC*), broadleaved tree height (*TreeH*)

Table 3 Pearson and Spearman correlations between ectomycorrhizal diversity indices and environmental variables recorded at the subplot (2× 2-m^2) level: distance from unburnt pine patches (DiUB), extent of erosion (ERO), pine pre-fire basal areas (PreBA), shrub height (ShrbH)

	Species richness	Shannon diversity
DiUB	−0.556**	−0.601**
ERO	−0.466*	−0.476*
PreBA	0.601**	0.629**
ShrbH	−0.389*	−0.432*

*$p<0.05$, **$p<0.01$

2007). Almost one third of the ECM fungi colonizing pine saplings root system belonged to the *Thelephoraceae* that showed a general scattered distribution, while a group of more typical late seral fungi (i.e., *A. pantherina*, *B. aestivalis*, *L. chrysorrheus*, and *R. densifolia*) were found exclusively in uneroded soils.

Pine regeneration in the study area was variable both in terms of density and height ranging from 1 to 14 individuals/m^2 with saplings belonging mainly to Class 2 (2.1 individuals/m^2), and mean values (4.27 individuals/m^2) not very different from the 5-year-old *Pinus halepensis* Mill. woodland in SE Spain (3.6 individuals/m^2; de las Heras et al. 2002). Pine regeneration was enhanced in the study areas at higher elevations, in correspondence with shallower soils, and a reduced cover of ericaceous shrubs and bare ground. These areas were in close proximity to patches of pine trees that survived the disturbance event and were previously characterized by a higher pre-fire pine biomass (measured as pre-fire basal area). In these circumstances, pine trees may have provided a larger canopy seed bank as well as microclimatic conditions that improved seedling establishment. Furthermore, newly regenerated seedlings might have benefitted from an existing ECM fungal

network able to simultaneously colonize and interconnect them with mature pine trees. Although k-selected ECM fungi were found on pine saplings in proximity to residual pine trees patches, they are known to colonize as well understorey species belonging to the genus *Cistus* (Comandini et al. 2006). These fungal species are unable to compete with early seral fungi for colonization of new roots, unless if associated with other living roots (Fleming 1984) or until the size of vegetation patches containing plant hosts is about 5 m^2 (Nara et al. 2003). Even though data on fire severity were not available for the study site, we can presume that this was less pronounced in the proximity of residual pine trees. In these circumstances, late seral ECM fungi could have survived on pine trees and/or shrubs providing an ECM fungal network to newly regenerated pine seedlings.

The number of ECM fungal species detected on maritime pine saplings in the present study was comparable to those found on naturally regenerated seedlings of other pine species (Visser 1995; Horton et al. 1998; Stendell et al. 1999; Grogan et al. 2000; Smith et al. 2004; Rincón and Pueyo, 2010). The proximity to patches of pine trees that survived the fire event and a high pre-fire pine basal area appeared to promote both ECM fungal species richness and diversity, which were instead negatively affected by higher degrees of erosion and higher shrub height. Although it has been argued that the association with several ECM fungi should enhance the productivity of young seedlings (Jones et al. 1997), no correlations were found between saplings performance and ECM fungal diversity indices in this study. However, knowledge of diversity in ECM fungi functioning within communities is generally poor, and more research is needed to better understand the effects of ECM fungal diversity over seedling regeneration (Jones et al. 2003).

Shrubs could represent a source of ECM fungal inoculum able to locally influence the ability of pine seedlings to establish after a disturbance event (Perry at

Table 4 Pearson and Spearman correlations between environmental variables at the subplot (2×2-m^2) level: elevation (ELE), soil depth (SoilD), distance from unburnt pine patches (DiUB), extent of erosion (ERO), pine pre-fire basal areas (PreBA), ericaceous shrub cover (EriC), shrub cover (ShrbC), and shrub height (ShrbH)
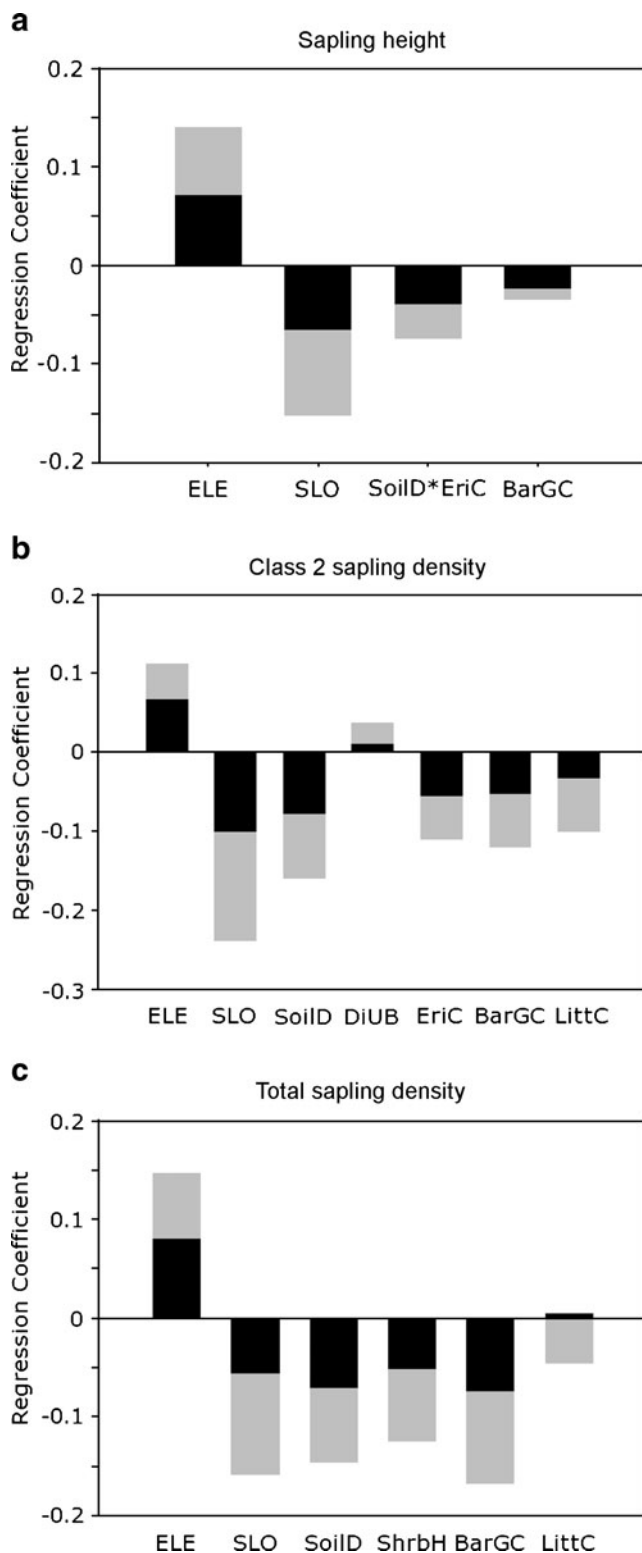
	ELE	SoilD	DiUB	ERO	PreBA	EriC	ShrbC
SoilD	−0.604**						
DiUB	ns	0.581**					
ERO	−0.518**	ns	0.456*				
PreBA	ns	−0.400*	−0.780**	−0.725**			
EriC	−0.401*	0.622**	ns	ns	−0.423*		
ShrbC	ns	0.775**	0.649**	ns	−0.498**	0.700**	
ShrbH	ns	ns	0.408*	ns	ns	ns	−0.409*

Ns not significant

*$p<0.05$, **$p<0.01$

a Sapling height

b Class 2 sapling density

c Total sapling density

mainly dominated by a patchy distribution of heather and rockrose shrubs. In Mediterranean and Boreal forests, where *Ericaceae* and ECM trees occurs in mixed communities, ericoid mycorrhizal endophytes associate as well with ECM root tips (Bergero et al. 2000; Vrålstad et al. 2000). The fungal roots inhabitant taxa found in this study on pine saplings are also common inhabitant of ericaceous shrub roots (Bougoure et al. 2007; Tedersoo et al. 2009), they form mostly non-specific associations with many plant hosts (Chambers et al. 2008), and their occurrence on ECM plants may have an important nutritional significance, either as true mycorrhizal fungi or as root-associated saprotrophic fungi (Bergero et al. 2000). However, there was a negative interaction between pine saplings (both in terms of height and density) and ericaceous shrubs. Shrubs can compete with naturally regenerated pine seedlings for light and moisture (O'Brian et al. 2007), and can affect as well their ECM symbionts. Conifer seedlings are often nutrient stressed and ECM fungal colonization may be reduced in sites that become dominated by ericoid mycorrhizal plants (Handley 1963). Walker et al. (1999) found that total ECM fungal colonization of *Tsuga canadensis* (L.) Carrière seedlings was reduced in thickets of *Rhododendron maximum* L. and that this decline was correlated with a decreased productivity of the seedlings. The tendency towards domination by ericoid mycorrhizal shrubs after fire could lead as well to the ericoid mycorrhizal invasion of previously ECM-dominated fungal communities and consequently delay the succession processes. In the present study, shrubs performance and competition between shrubs and saplings were enhanced in areas characterized by a

al. 1989). Examples of seedling facilitation mediated by shrubs were found in the association of *Pseudotsuga menziesii* with the fungal networks of *Arctostaphylos* (Horton et al. 1999) and between *Q. ilex* and *A. unedo* (Richard et al. 2009). The understorey of the study area is

reduced pre-fire pine basal area, in correspondence with deeper soils. In these conditions, the understorey was dominated by *E. arborea*. This species is a typical post-fire resprouter characterized by populations that are relatively stable for long periods of time, with new seedlings recruitment and consequent population expansion favored by long fire return intervals (De Lillis 1995). Its release by fire from competition with pine for light and growing space (O'Brien et al. 2007), increased its capacity to spread out at expenses of newly regenerated pine seedlings in low density pre-fire pine areas. Analogous results were found in another Mediterranean ecosystems where the dominance of *E. arborea* reduced dramatically the survival of *Q. ilex* seedlings during the summer following germination (Richard et al. 2009), and the high percentages of shrub cover decreased the probability of *Pinus radiata* D. Don seedling presence (O'Brien et al. 2007).

More than 30% of the ECM fungi found on pine saplings in the present study are known to colonize different species of the genus *Cistus* (reviewed by Comandini et al. 2006). However, the potential bridge mediated by *Cistus* spp. between pre-fire ECM fungal communities and emerging seedlings, defined by Perry et al. (1989) as bootstrapping, could have been masked in the present study by *E. arborea*. Its large presence in some areas together with an increased extent of erosion, and soil depth seem to be the main factors responsible for shaping the ECM fungal distribution and for describing with elevation and slope, most of the explained regeneration variability.

Common environmental factors (i.e., ericaceous shrub cover, extent of erosion, slope, and soil depth) were responsible for shaping the ECM fungal distribution and for describing most of the explained regeneration variability. The proportion of variability that remains unexplained could be due to an inadequate sampling effort or the lack of information on other abiotic factors, such as fire characteristics and soil properties. Nevertheless, the results presented in this study are relevant to improve post-fire restoration operations by allowing the identification of areas where poor pine regeneration can be expected. Management to promote maritime pine in declining natural stands should focus on reducing the cover of *E. arborea* and providing seed sources in areas with lower pre-fire pine biomass.

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