



The effects of fire severity on ectomycorrhizal colonization and morphometric features in *Pinus pinaster* Ait. seedlings

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Abstract

Aim of the study: Mycorrhizal fungi in Mediterranean forests play a key role in the complex process of recovery after wildfires. A broader understanding of an important pyrophytic species as *Pinus pinaster* and its fungal symbionts is thus necessary for forest restoration purposes. This study aims to assess the effects of ectomycorrhizal symbiosis on maritime pine seedlings and how fire severity affects fungal colonization ability.

Area of study: Central Spain, in a Mediterranean region typically affected by wildfires dominated by *Pinus pinaster*, a species adapted to fire disturbance.

Material and Methods: We studied *P. pinaster* root apices from seedlings grown in soils collected one year after fire in undisturbed sites, sites moderately affected by fire and sites highly affected by fire. Natural ectomycorrhization was observed at the whole root system level as well as at two root vertical sections (0-10 cm and 10-20 cm). We also measured several morphometric traits (tap root length, shoot length, dry biomass of shoots and root/shoot ratio), which were used to test the influence of fire severity and soil chemistry upon them.

Main results: Ectomycorrhizal colonization in undisturbed soils for total and separated root vertical sections was higher than in soils that had been affected by fire to some degree. Inversely, seedling vegetative size increased according to fire severity.

Research highlights: Fire severity affected soil properties and mycorrhizal colonization one year after occurrence, thus affecting plant development. These findings can contribute to a better knowledge of the factors mediating successful establishment of *P. pinaster* in Mediterranean forests after wildfires.

Keywords: Wildfire; mycorrhizal fungi; Maritime pine; bioassay.

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Introduction

Wildfires constitute the main source of disturbance in Mediterranean forest ecosystems (Gassibe *et al.*, 2011), strongly affecting soil fungi and plant communities (Dahlberg *et al.*, 2001, Carney & Bastias, 2007, Rincón & Pueyo, 2010). This comes both as a direct result of the high temperatures reached during a forest fire, but also indirectly through changes to soil chemical and physical properties, like a pH increase, enhanced hydrophobicity or changes of available nutrients (Certini, 2005).

Ectomycorrhizal fungi (ECM) play an important role in the colonization of new areas of land or in the

restoration of the vegetative community following a disturbance (Claridge *et al.*, 2009, Rincón & Pueyo, 2010). Indeed, survival of tree seedlings strongly depends on the rapid formation of an efficient root system, determined by the development of mycorrhizal symbiosis but also by favourable ecological conditions (Jackson *et al.*, 2007, Palfner *et al.*, 2008). ECM help their plant symbionts by improving water and nutrient uptake from the soil, carbohydrate distribution and production of plant growth regulators and avoiding water losses and root desiccation (Rincón *et al.*, 2007). All this contributes to prevent environmental stress caused by drought (Scattolin

et al., 2014), pathogenic agents (Martín-Pinto *et al.*, 2006a) or heavy metal pollution.

Then, how does fire affect the ability of soil fungi to establish new mycorrhizal symbiosis with emerging seedlings, might help us to understand forest recovery after fire. ECM inoculum may survive in infected roots, as sclerotia, or as resistant spores (Horton *et al.*, 1998, Baar *et al.*, 1999), likely coming from deep soil horizons (Claridge *et al.*, 2009). Abundant research has shown that ECM can be particularly active soon after wildfire (Hart *et al.*, 2005, Jiménez-Esquilín *et al.*, 2007, Claridge *et al.*, 2009). For example, a study in a *Pinus muricata* D. Don forest in California (Baar *et al.*, 1999) showed that ectomycorrhizae were formed within six months after a stand-replacing wild fire.

But however present and active in forest soils after fire, ECM fungal communities do suffer consequences from forest fires. Many studies report large shifts in species abundances, decreases in diversity, large losses in ECM sporocarp production, and even complete loss of ectomycorrhizal species after severe fires (Grogan *et al.*, 2000, Treseder *et al.*, 2004, Martín-Pinto *et al.*, 2006b, Gassibe *et al.*, 2011). For example, Dahlberg *et al.* (2001) established a link between different fire severities and survival of mycorrhizal flora, after four months of prescribed burning in a Scandinavian boreal forest. Mycorrhizae were absent from high intensity burning treatments, while in mildly burned treatments part of the existing mycorrhizae were kept in the deeper mineral soil layers. Thus, soil horizon also mediates the impact of fires on ECM fungi (Bastias *et al.*, 2006, Jiménez-Esquilín *et al.*, 2007, Ponzetti *et al.*, 2007, Kipfer *et al.*, 2010). This is evident as heat is greater closest to the surface, and it is within the top few centimeters of soil where the majority of fungal communities are located (Dahlberg *et al.*, 2001, Cairney & Bastias 2007, Kipfer *et al.*, 2010).

After a forest fire, the extent of the decrease in species richness (Smith *et al.*, 2005, Hernández-Rodríguez *et al.*, 2013) and relative abundance (Jonsson *et al.*, 1999) and its further impact on forest regeneration depend on many factors such as variations in the intensity/severity of the fire, time elapsed after fire, the fire return interval and soil depth (Cairney & Bastias, 2007, Turrión *et al.*, 2012). However, while the direct effect of fire is mainly destructive for existing fungal communities, at the same time fire also provides large inputs to new communities, mainly competition-free resources and substrates for fungi and plants to use (Penttilä & Kotiranta, 1996).

Pinus pinaster Ait. is a common widespread conifer species in Western Mediterranean countries, whose forests typically suffer from frequent forest fires. Varying fire regimes have even selected for different fire-related traits across *P. pinaster* populations, like bark

thickness, serotiny (Tapias *et al.*, 2004) and precocity (Santos-del-Blanco *et al.*, 2012). *P. pinaster* is also an obligate mutualist with ectomycorrhizal fungi and normal growth does not occur without them (Smith & Read 1997, Read, 1998). Given its ecological relevance and intimate relationship between forest fires and fungi, *P. pinaster* has been the focus of several studies in Mediterranean areas on how does fire affect fungal communities, as seen by the decrease in richness, diversity and production of ECM sporocarps as a product of wildfires (Martín-Pinto *et al.*, 2006b, Gassibe *et al.*, 2011). Also several studies (Buscardo *et al.*, 2010, 2011, Rincón & Pueyo 2010, Rincón *et al.* 2014) have found that wildfires influence the structure of ectomycorrhizal fungal communities associated with *P. pinaster*, showing that some fungal groups are potentially fire-adapted (Rincón *et al.*, 2014). These findings highlight the interest to deepen our knowledge on the colonization of *P. pinaster* seedlings by ECM fungi after wildfires. In this framework, it is particularly interesting to learn about the interrelationships between changes in soil properties, host plant development and associated belowground fungal communities, all of it impacting biodiversity and ecosystem services (Buscardo *et al.*, 2015).

A devastating crown-fire occurred in Central Spain in 2008 providing the opportunity to increase further this knowledge. Here, we looked at how vegetative traits of *P. pinaster* seedlings differ when they are grown in soils exposed to increasing fire severity, having as a consequence different biochemical properties and likely different potential for ECM colonization. We performed a bioassay experiment under greenhouse controlled conditions where we analyzed i) seedling vegetative traits and ii) ECM colonization at two different root vertical sections. We hypothesised that ECM-colonization would be highest in undisturbed soils and at upper root sections, and lowest in highly disturbed soils. We also expected colonization rate to be correlated with seedling performance.

Materials and methods

Study site

Our study was performed in Central Spain, in the Autonomous Community of Castilla y León, the third European region in size and one of the most strongly damaged by wildfires. According to the European Forest Fire Information System (EFFIS), 1996 fires occurred in the region during 2008 affecting 152.64 km² (Quintano *et al.*, 2011). The fire season occurs during the period of June-September, corresponding to the warm to hot and dry summer, typical of Mediterranean climate.

The study was carried out in Honrubia de la Cuesta (northern part of Segovia province), which is a Mediterranean ecosystem dominated by *Pinus pinaster* plantations established by the Spanish Forest Services in previously deforested areas (440901-443169 longitude-UTM, 4592 704-4590583 latitude-UTM, 750-880 m.a.s.l.). Here, a large wildfire burned 1200 ha of forest and canopies in August 2008 at which pine trees were about 40 years old. This site has a supra-Mediterranean climate with 3 months of dry season in the summer, a mean annual rainfall of 480-500 mm and mean temperatures ranging from 8 to 13 °C. The warmest month is July and the coldest January. These data were provided by the closest meteorological station (Linares del Arroyo 41° 31' 40'' N, 3° 32' 72'' W) located 15 km from the study area.

This area is composed of Paleozoic metamorphic rocks, dominated by Ordovician and Silurian shales (Barrenechea & Rodas, 1992). The soil is classified as Inceptisol suborder Xerept (Alvarez *et al.*, 1993).

In the forest understory, sparse individuals of *Cytisus scoparius* (L.) Link, *Quercus faginea* Lam and *Quercus ilex* (L.) ssp. *ballota* (Desf.) Samp. were found. A number of species have been identified during mushroom forays in the area (pers. obs.). Ectomycorrhizal *Collybia* sp., *Cortinarius cinnamomeus* (L.) Fr., *Cystoderma amianthinum* (Scop.) Fayod, *Hebeloma mesophaeum* (Pers.) Qué., *Inocybe* sp., *Laccaria laccata* (Scop.) Cooke, *Rhizopogon luteolus* Fr., *Tricholoma scalpturatum* (Fr.) Qué., and saprophytic *Astraeus*

hygrometricus, *Baeospora myosura* (Fr.) Singer, *Collybia butyracea* (Bull.) P. Kumm., *Hemimycena* sp., *Hygrophorus gliocyclus* Fr., *Mycena pura* (Pers.) P. Kumm., *Mycena pura* ssp. *lutea* (Gillet) Arnolds, were observed at sites not affected by fire. While *Galerina* sp., *Gerronema* sp., *Omphalina* sp., *Pholiota carbonaria* (Fr.) Sing., were found in places moderately affected by fire and only pyrophilic *Pholiota carbonaria* was found at those sites fully impacted by the fire. No ECM species were found to form sporocarps in areas affected by fire.

Experimental design and field sampling

Three different sites were chosen within our study area according to the degree of damage caused by fire on vegetation and soil (Figure 1). Fire severity and soil damage were classified following Rincón & Pueyo (2010) and Vega *et al.* 2013 criteria: control, unburned site (hereafter UB) was established in an adjacent *P. pinaster* forest unaffected by fire at least in the preceding 40 years (dominant trees of approximately 40-cm diameter). Moderate fire severity site (hereafter MFS) had all pine crowns and upper barks burned. Here, the soil organic matter was not consumed and the ground surface remained intact after the fire. The soil was darkened and water repellent. The high fire severity site (hereafter HFS) had pines, canopy and understory litter totally burned and the entire humic soil organic layer

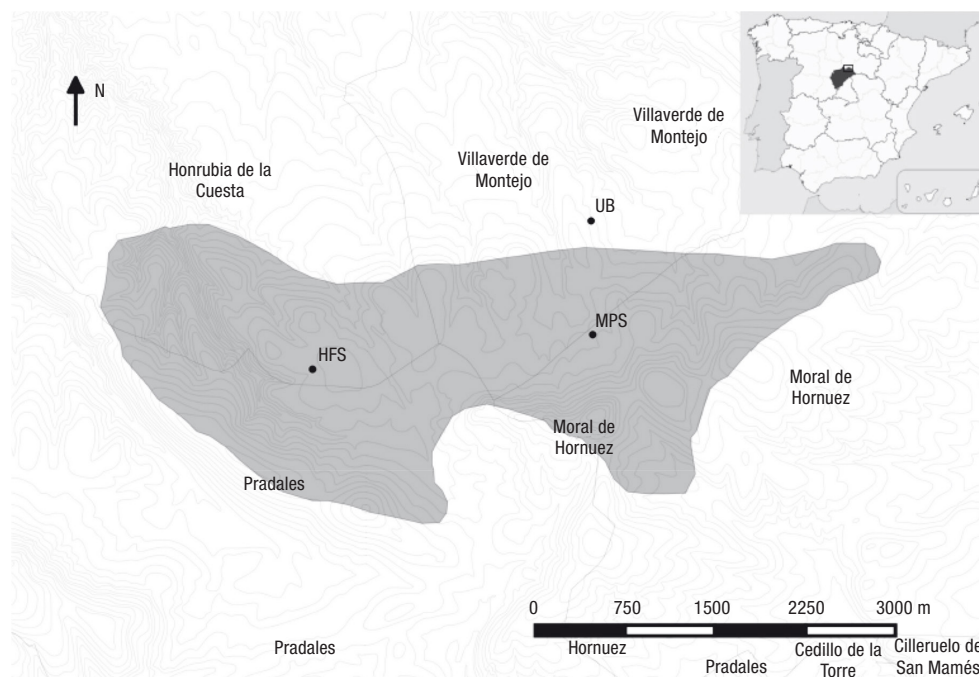


Figure 1. Study area location, with the zone affected by fire in grey. Black circles indicate the sampling sites where the soil blocks were taken. Sampling points within sites were at least 100 m apart.

consumed. For HFS, the loss of soil structure was very evident and the rootlets were consumed. We devoted our greatest effort to select sites differing in fire severity but similar in terms of vegetation and local topography, aiming to avoid obvious major variation between sampling sites (Dias *et al.*, 2010). That is, we aimed to select sites such that observed differences between them could be linked to differences in fire intensity.

In mid-June 2009, uniform areas were sampled within the three above mentioned sites. A total of 15 intact soil blocks (22 x 22 x 20 cm), five per site, were extracted randomly with a minimum spacing of 100 m with a metal cube with a sharpened edge at HSF, MSF and adjacent UB sites. This minimum distance between sampling points was chosen in order to ensure low autocorrelation and provide estimates as independent from each other as possible (Lilleskov *et al.*, 2004, Dias *et al.*, 2010, Buscardo *et al.*, 2015). Soil blocks were then placed into square plastic containers of the same dimensions and taken to the greenhouse facilities of the Forest Engineering Faculty, Palencia Campus, University of Valladolid. Nine evenly spaced soil samples, three from each of the previously sampled sites were also extracted using a cylindrical (2 cm radius, 20 cm deep, 250 cm³) soil borer (Taylor, 2002) for chemical analysis. The edaphic parameters analyzed included pH, organic matter, total nitrogen, phosphorous and potassium (N–P–K), sodium, magnesium, calcium, cation exchange capacity (CEC) and conductivity (Cond) (see details in Table 1).

Greenhouses were naturally lit, with controlled temperature (15–20°C) and humidity, maintaining the natural soil at field capacity. *P. pinaster* seeds (375 in total) were provided by a local nursery (Viveros Fuente Amarga, Cabezón de Pisuerga, Valladolid, Spain), these were surface sterilized with 30% hydrogen peroxide for 30 min and washed three times with sterile water. Twenty five seeds were sown in each container following a square grid, ensuring a homogenous distribution. Pots were tap-watered daily and no nutrients were added. Four months after sowing, five randomly selected seedlings from each pot (75 in total) were extracted from the center of each container thus avoiding border effects.

Mycorrhizal status and morphometric measurements

After extraction from the containers, each plant was rinsed with tap water in a plastic tray and gently shaken to soften and remove adhering soil. Tap root length and shoot length were measured with a ruler to the nearest millimeter (Palfner *et al.*, 2008). The root

system of each plant was then divided into two vertical sections, from 0 to 10 cm and from 10 to 20 cm from the plant root collar (Anderson *et al.*, 2007).

All root tips were classified morphologically as mycorrhizal vs. non-mycorrhizal or “non vital” (Agerer, 1991, 1987–2002, de Román & de Miguel, 2005, Scattolin *et al.*, 2008), using a Leica M3 dissecting microscope and a 15x magnification stereomicroscope. The percentage of mycorrhization per site, overall and at each of the two vertical sections, was calculated by dividing the number of mycorrhizal root tips by the total number of root tips (Brundrett *et al.*, 1996). Shoot and root dry biomass were determined after drying the plant material at 70°C for 48 h. in a drying oven (Sousa *et al.*, 2011). Root/shoot ratio was derived from these measurements (Palfner *et al.*, 2008).

Statistical analysis

One and two-way repeated measures ANOVA were applied to explore the influence that different fire damage levels (UB, MSF and HFS) and root section parts (upper and lower) had on the response variables (morphometric traits, mycorrhizal colonization and soil chemical characteristics). Non-independence of errors between seedlings grown in the same pot was accounted within the model. Post-hoc tests ($P > 0.05$) (LSD for plant morphometric traits and percentage of mycorrhization and Tukey test for soil chemical variables) were applied to explore the differences between fire intensity levels. Normality of data was checked with a Kolmogorov-Smirnov test and we applied log transformations when necessary, namely for some soil nutrients. We used STATISTICA '08 Edition software to perform these statistical analyses (StatSoft Inc. 1984–2008).

Finally, we carried out a DCA (Detrended Correspondent Analysis) with our complete dataset. The longest gradient length, indicative of how heterogeneous our data are, was below 3.0 standard deviations. Then, a Principal Component Analysis (PCA) was performed based on Pearson product-moment correlation coefficients (R Development Core Team, 2011). Fire severity parameters were coded as 1 (UB), 2 (MSF) and 3 (HSF). Only those variables showing a relatively strong relationship to the first two PCA axes (vector length greater than 0.5 units) are shown.

Results

Plant development

Mortality rates were low and did not affect more than three plants per container. Seedlings grown in high

Table 1. Soil chemistry parameters (mean \pm SE) of the 2009 at three sampling sites in a *Pinus pinaster* forest in Central Spain, one year after a wildfire. UB: soils not affected by fire; MFS, soils moderately affected by fire; HFS, soils highly affected by fire. (n=9, three soil samples per site)

| Sites | N ¹ | P ² | K ³ | pH ⁴ | MO ⁵ | Na ⁶ | Mg ⁷ | Ca ⁸ | Cond ⁹ | CEC ¹⁰ |
|-------|--------------------|-------------------|---------------------|-------------------|-------------------|---------------------|-------------------|-------------------|-------------------|--------------------|
| UB | 0.10 \pm 0.004 a | 4.40 \pm 1.30 b | 115.00 \pm 6.75 b | 6.04 \pm 0.12 c | 1.37 \pm 0.11 a | 0.016 \pm 0.004 a | 0.87 \pm 0.05 a | 4.08 \pm 0.38 a | 0.04 \pm 0.02 a | 11.35 \pm 0.69 a |
| MFS | 0.05 \pm 0.004 b | 6.06 \pm 1.30 a | 168.00 \pm 6.75 a | 7.30 \pm 0.12 a | 0.91 \pm 0.11 b | 0.020 \pm 0.004 a | 0.60 \pm 0.05 b | 3.07 \pm 0.38 b | 0.06 \pm 0.02 a | 8.23 \pm 0.69 b |
| HFS | 0.05 \pm 0.004 b | 7.66 \pm 1.30 a | 115.33 \pm 6.75 b | 6.83 \pm 0.12 b | 1.05 \pm 0.11 b | 0.020 \pm 0.004 a | 0.59 \pm 0.05 b | 2.65 \pm 0.38 b | 0.06 \pm 0.02 a | 6.88 \pm 0.69 b |

Soil characteristics and their respective quantification methodology (upper numbers) with different letter indicate significant differences at $P < 0.05$ after Tukey post-hoc test.

¹ Total Nitrogen (%); modified Kjeldahl

² Assimilable Phosphorus (mg/kg); Olsen

³ Assimilable Potassium (mg/kg); atomic emission spectrometry

⁴ pH; Ph-Meter (1:2, 5)

⁵ Oxidizable Organic Matter (%); Walkley-Black

⁶ Assimilable Sodium (meq/100g); atomic emission spectrometry

⁷ Assimilable Magnesium (meq/100g); atomic absorption spectroscopy

⁸ Assimilable Calcium (meq/100g); atomic absorption spectroscopy

⁹ Soil Conductivity (mS/cm); conductivity meter

¹⁰ Cation Exchange Capacity (meq/100g)

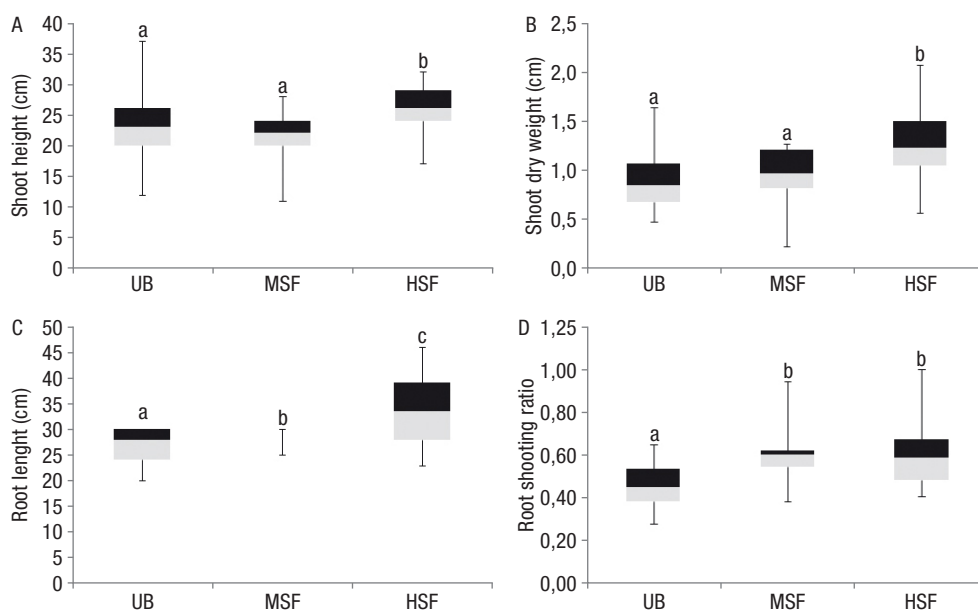


Figure 2. Morphometric traits of *P. pinaster* seedlings grown in a bioassay with forest soils taken from Unburned (UB), Moderate severity fire (MSF) and High severity fire (HSF) sites after a forest fire in Central Spain (Honrubia de la Cuesta, Segovia); **A** shoot height; **B** shoot dry weight; **C** tap root length; **D** root/shoot ratio; box plots showing mean values (n=75) and 5th and 95th percentile. Box plots marked with different letters differed significantly according to LSD Fisher test ($P < 0.05$).

severity fire soils (HSF) had heavier and longer shoots ($P < 0.01$ and $P=0.030$, respectively; Figure 2) and longer roots ($P=0.004$) than those grown in moderate severity fire (MSF) and unburned (UB) soils (Figure 2). In turn, root length from MSF sites was also longer than that from UB sites ($P=0.042$). Root/shoot ratio was highest for HSF and MSF sites and lowest for UB samples (HSF vs MSF $P=0.798$; HSF vs UB $P=0.0000$; MSF vs UB $P=0.0001$) (Figure 2).

Mycorrhization and soil chemistry

The proportion of total mycorrhization from both post-fire sites was significantly lower than that from undisturbed soils (HSF, $P=0.006$; MSF $P=0.005$). Regarding the two vertical sections of the root system, higher levels of mycorrhization ($P < 0.01$) were observed in the upper (0-10cm) section compared to the lower one (>10 cm) for all three sites (Figure 3). At the upper

root section, we observed a higher colonization rate in UB sites when compared to MSF ($P=0.012$) and HSF ($P=0.044$) sites (Figure 3). The same trend was followed by colonization in the lower root section, but in this case no differences were observed between seedlings from MSF soils compared to the other two sites. Regarding soil chemical characteristics, the main differences between sites were a lower N, K and organic matter content and a higher pH and P content in burnt soils (Table 1).

Relationships among plant development, mycorrhization and soil chemistry

The PCA grouped samples from HFS and MFS sites in the positive area of Axis 1 whereas UB ones were located in the negative area (Figure 4). The two axes explained 68.85% of the variation present in the samples (48.47% axis 1 and 20.38% axis 2). Unburned soil samples appeared associated with higher values of organic matter, N, Ca and cation exchange capacity (CEC), but lower values of pH, Mg, Na and conductivity. The opposite was true for both burned (HFS and MFS) sites (Table 1, Table 2). Regarding seedling vegetative traits, the PCA revealed a positive correlation between shoot biomass with P and fire severity. Axis 1 also reflected a relation between higher levels of mycorrhization (total, 0-10 cm and 10-20 cm) with the unburned sites.

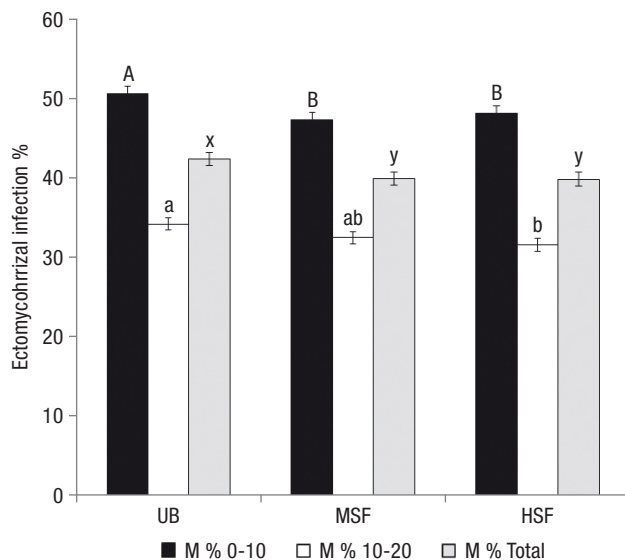


Figure 3. Percentage of ectomycorrhization observed on *P. pinaster* seedlings ($n=75$) grown on Unburned (UB), Moderate severity fire (MSF) and High severity fire (HSF) forest soils taken after a forest fire in Central Spain (Honrubia de la Cuesta, Segovia). Black bars, upper root section (0-10 cm); white bars, lower root section (10-20 cm); grey bars, total mycorrhization. Columns marked with different letters differed significantly according to LSD Fisher tests ($P < 0.05$).

Discussion

We found a higher mycorrhization rate for seedlings grown in unburned soils across all root sections, thus supporting our prior expectations on the negative relationship between fire severity and ECM propagules. Nonetheless, higher mycorrhization rates were unexpectedly not correlated with seedling size. In the following sections we discuss our results in the light of plant, fungi and soil features as well as their interactions, notwithstanding potentially confounding factors.

Plant development

Seedlings in the high severity fire soil showed higher shoot height, root length and root/shoot ratio and also more than 10% of the shoot biomass of those growing in unburned soil. Similar biometric results have been found by Pausas *et al.* (2003) in *Pinus halepensis* Mill. seedlings in eastern Iberian Peninsula under three fire severity classes, where seedlings from sites most affected by fire grew significantly more. Those differences may lie in the changes in soil chemistry caused by different fire severities and leading to higher fertility (see below) (Pausas *et al.*, 2003).

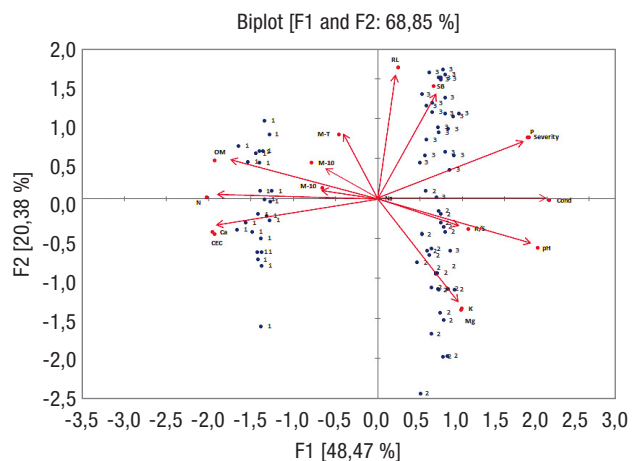


Figure 4. Ordination diagram of Principal Component Analysis (PCA) performed on mycorrhizal presence on and vegetative traits of bioassay *Pinus pinaster* seedlings ($n=75$) as well as soil chemical properties. Soils were sampled after a forest fire at 1, Unburned (UB); 2, Moderate severity fire (MSF) and 3, High severity fire (HSF) sites. The Axis 1 (F1) and Axis 2 (values in brackets) explain the percentage variation of the samples according to (normalized) principal component scores. Arrows indicate the projection of a multidimensional set of axes onto a two-dimensional plane. These arrows are standardized to be of unit length, projected onto a plane. The ones that lie most closely within that plane appear longer than the ones that are more orthogonal to it. Ectomycorrhization % (M-T: Total; M-10: 0-10 cm; M-20: 10-20 cm); soil chemical characteristics (see Table 1); morphometric traits (SB: shoot biomass in g; RL: root length in cm; R/S: root to shoot ratio).

Table 2. Pearson's correlation coefficients between ECM colonization, soil and morphometric variables affected by fire severity

| Variables | M-10 | M-20 | M-T | R/S | SB | SH | RL | Sev | pH | Cond | N | P | OM | Ca | K | Mg | CEC |
|-----------|---------------|---------------|--------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|-----|
| M-10 | 1 | | | | | | | | | | | | | | | | |
| M-20 | 0,291 | 1 | | | | | | | | | | | | | | | |
| M-T | 0,658 | 0,664 | 1 | | | | | | | | | | | | | | |
| R/S | -0,181 | -0,172 | -0,163 | 1 | | | | | | | | | | | | | |
| SB | -0,163 | -0,085 | 0,039 | -0,064 | 1 | | | | | | | | | | | | |
| SH | 0,091 | -0,005 | 0,246 | -0,171 | 0,657 | 1 | | | | | | | | | | | |
| RL | 0,091 | -0,005 | 0,246 | -0,171 | 0,657 | 1,000 | 1 | | | | | | | | | | |
| Sev | -0,237 | -0,271 | -0,054 | 0,434 | 0,430 | 0,245 | 0,245 | 1 | | | | | | | | | |
| pH | -0,323 | -0,204 | -0,210 | 0,440 | 0,124 | -0,033 | -0,033 | 0,616 | 1 | | | | | | | | |
| Cond | -0,317 | -0,259 | -0,159 | 0,486 | 0,283 | 0,096 | 0,096 | 0,866 | 0,927 | 1 | | | | | | | |
| N | 0,317 | 0,259 | 0,159 | -0,486 | -0,283 | -0,096 | -0,096 | -0,866 | -0,927 | -1,000 | 1 | | | | | | |
| P | -0,239 | -0,272 | -0,057 | 0,436 | 0,428 | 0,242 | 0,242 | 1,000 | 0,625 | 0,872 | -0,872 | 1 | | | | | |
| OM | 0,326 | 0,220 | 0,200 | -0,458 | -0,165 | 0,001 | 0,001 | -0,686 | -0,996 | -0,958 | 0,958 | -0,695 | 1 | | | | |
| Ca | 0,283 | 0,275 | 0,106 | -0,474 | -0,376 | -0,183 | -0,183 | -0,972 | -0,785 | -0,960 | 0,960 | -0,974 | 0,839 | 1 | | | |
| K | -0,226 | -0,049 | -0,224 | 0,222 | -0,176 | -0,231 | -0,231 | 0,005 | 0,791 | 0,505 | -0,505 | 0,017 | -0,731 | -0,242 | 1 | | |
| Mg | -0,225 | -0,047 | -0,224 | 0,220 | -0,179 | -0,233 | -0,233 | 0,000 | 0,788 | 0,500 | -0,500 | 0,012 | -0,728 | -0,237 | 1,000 | 1 | |
| CEC | 0,281 | 0,275 | 0,103 | -0,472 | -0,380 | -0,187 | -0,187 | -0,975 | -0,776 | -0,956 | 0,956 | -0,977 | 0,831 | 1,000 | -0,229 | -0,224 | 1 |

Note: significant Pearson correlations are highlighted in black $P < 0.05$. Where M-10: ECM % 0-10 cm; M-20: ECM % 10-20 cm; M-T: ecm % Total; R/S: root to shoot ratio; SB: shoot biomass; SH: shoot height; RL: root length; Sev: Fire Severity; Cond: Soil Conductivity; N: Nitrogen; P: Phosphorus; OM: Organic matter; Ca: Calcium; K: Potassium; Mg: Magnesium; CEC: Cation Exchange Capacity.

Mycorrhization and plant development

Maximum ECM colonization rate was found in soils from unburned sites, although it did not vary significantly among high and moderate severity fire sites. Our results are in agreement with a large body of research on Mediterranean dry forests (de Román & Miguel, 2005, Martín-Pinto *et al.*, 2006b, Buscardo *et al.*, 2010, Hernández-Rodríguez *et al.*, 2013) and specifically with some studies conducted also with *P. pinaster* (Buscardo *et al.*, 2011, Gassibe *et al.*, 2011, Sousa *et al.*, 2011). Nonetheless, the overall evidence in this field cannot be considered as conclusive yet. For example, Rincón & Pueyo (2010) found that fungal richness and colonization of *P. pinaster* seedlings did not depend on fire severity, but on time elapsed after fire. Indeed, time span after fire seems to mediate the evolution of fungal communities, from fire-adapted taxa to later-stage ones (Rincón *et al.* 2014).

Regarding the relationship between vertical root distribution (upper and lower sections) and mycorrhization rate, our results revealed how the upper part of the roots had the highest ECM rates across all fire damage levels (Visser, 1995, Torres & Honrubia, 1997). In fact, differences between upper and lower root parts across fire damage levels were kept constant (Figure 3). This was true also in the site most severely affected by fire, where higher colonization rates would have been expected at the lower (deeper) root parts. This could have come as a result of higher exposure of the top layer to very high temperatures and buffering of the lower layer (Bastias *et al.*, 2006, Kipfer *et al.*, 2010). Our results might indicate a particularly high resistance

of some kind of propagules to fire. Here, molecular taxonomy techniques can reveal the existence of interesting pyrophitic taxa (Rincón *et al.* 2014).

Our experimental design did not allow separating root physiological or anatomical effects from those derived from propagule abundance or soil properties at the two depth levels within sites. For example, seedling root distribution and length varies according to soil depth (Wallander *et al.*, 2004), in this sense post-fire seedlings tend to extend their root system vertically as seen in our results by increasing mainly tap-root length therefore also changing the distribution and structure of the lateral roots (Palfner *et al.*, 2008). Also the mycelium of ECM fungi which is usually most abundant in the superficial organic soil layers in undisturbed ecosystems (Visser, 1995, Neville *et al.*, 2002, Wallander *et al.*, 2004) may influence root morphology and architecture through the formation of short lateral roots and root tips (Ostonen *et al.*, 2009, Kubisch *et al.*, 2015), therefore affecting seedling growth (Jones *et al.*, 2003). There is also evidence of the stratification of fungal communities (Dickie *et al.*, 2002, Rosling *et al.*, 2003; Anderson *et al.*, 2007) between the 0–10 and 10–20 cm sections of soil profile, but is not always the case as reported by Anderson *et al.* (2007) where non stratified homogeneous ECM communities were present within the 20 cm of soil depth after two years after fire.

Nonetheless, data from our unburned site provide a suitable baseline against which we can compare the two other scenarios. The higher mycorrhization rate in the top layer of UB plant roots might be related to either root physiology or propagule abundance. Lower

mycorrhization rates in MFS and HFS in that same top layer compared to UB plants, is likely due to different soil properties. Under natural conditions, mycorrhization rates in upper layers could be even higher due to mechanisms of fungal colonization such as spore dispersion by wind or rodents, which were suppressed in our experiment. Also, the same rationale applies to the lower layer. Studies similar to ours have also found lower fungal richness (Smith *et al.*, 2004) or different fungal community structures (Bastias *et al.*, 2006) in the upper 10 cm layer of soils variously affected by fire but interestingly, no trend at a depth of 10–20 cm (Bastias *et al.*, 2006).

Considering mycorrhization rates alone, our *P. pinaster* seedlings attained smaller sizes in those soils where mycorrhization was highest i.e. in unburned soils. Nonetheless, given that potential mycorrhizal inoculum was confounded with soil chemistry, direct conclusions cannot be drawn. Inclusion in our study of seedlings grown on sterilized soils from the three studied sites, would have allowed measuring the effect of soil chemistry alone on plant growth. Notwithstanding, a negative effect of mycorrhization on plant growth rates under nursery conditions has been previously reported (Stenström *et al.*, 1985, 1990, Le Tacon *et al.*, 1992), even though global evidence does support the beneficial effect of ECM species on *P. pinaster* plant growth (Sousa *et al.*, 2012).

Relationships among plant development, mycorrhization and soil chemistry

Soil nutrients and plant development

In our study the main differences between sites were a lower N, K and organic matter content and a higher pH and P content in burnt soils. Previous studies had described the influence of fire intensity on soil characteristics and thus indirectly on plant early growth (Ne'eman, 1997, Pausas *et al.*, 2003, Calvo *et al.*, 2013). Thus, decrease in N availability after fire may have been caused by volatilization and mineralization by heating the top soil (Pausas *et al.*, 2003). Reversely, the P content lost by volatilization is usually low and the amount of P available to plants is dependent on ash deposition (Raison *et al.*, 1985, Pausas *et al.*, 2003).

Due to nutrient deposition from ashes, fires can cause a short-term fertilizing effect (Fernandes & Rigolot, 2007, Turrión *et al.*, 2012). This scenario is compatible with our results, where we found a high correlation between seedling size and higher amounts of P (Figure 4), suggesting a causal relationship, although

other variables cannot be ruled out (Pausas *et al.*, 2002, Gray & Dighton, 2009).

Soil nutrients and mycorrhization

In our study, the percentage of ECM colonization was positively correlated with organic matter and N, but negatively with P soil content. Our results are in accordance with those of Dickie *et al.*, (2006) in unburned bioassay soils with *P. sylvestris* L., where seedling ECM colonization was best predicted by humus percentage, N, Ca and clay content and soil cation exchange capacity from 0 to 20 cm depth. Furthermore, other authors noticed that fungal communities can adapt to more nitrogen-rich sites (Toljander *et al.*, 2006, Kranabetter *et al.*, 2009). Also, Sousa *et al.*, (2011) studied 6 month-old *P. pinaster* seedlings grown in burned soils and inoculated with ECM fungi. These authors reported that a low N content was not a factor that limited plant growth, perhaps offset by a higher P content. Indeed increasing P availability has been related to inhibition of fungal ECM colonization (Grant *et al.*, 2005, Smith & Read, 2008) and enhancement of plant growth (Conjeaud *et al.*, 1996).

In our study, mycorrhizal inoculum potential appeared to be affected by soil disturbance caused by fire similar to that reported by Buscardo *et al.* (2010) and Sousa *et al.* (2011), while plant growth increased along a fire severity gradient. This was likely due to post fire nutrient deliverance in the forest soil such as P (Fierro *et al.*, 2007) rather than ECM colonization. As we could not separate fire severity and ECM colonization effects, the latter also may have influenced the uptake of P (de Lucia *et al.*, 1997), but likely to a lesser degree due to the strong fire effect (Turrión *et al.*, 2010). Regardless of the limitations, bioassay studies like ours can be a useful tool when studying ectomycorrhizal infectivity and may be comparable to some field situations (Izzo *et al.*, 2006). Particularly, they can provide insight about background mycorrhization levels in seedlings emerged after a fire and whether artificial mycorrhization can improve plant growth in those situations (Sousa *et al.*, 2011). Also, bioassay studies provide valuable information on seedling performance, which is the main goal of afforestation practices, even more after disturbing events like wildfires.

All this suggests that integrative approaches that combine laboratory and field experiments (Buscardo *et al.*, 2011) are needed to assess ECM functioning in ecosystems dominated by Mediterranean pyrophitic species such as *P. pinaster*, aiming to achieve a successful restoration of Mediterranean forest areas affected by wildfire.

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