



## Effect of fire severity and site slope on diversity and structure of the ectomycorrhizal fungal community associated with post-fire regenerated *Pinus pinaster* Ait. seedlings

Ana Rincón\*, José J. Pueyo

Department of Plant Physiology and Ecology, Instituto de Recursos Naturales, Centro de Ciencias Medioambientales, CSIC, Serrano 115-bis, E-28006 Madrid, Spain

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### ABSTRACT

We investigated the diversity and structure of the ectomycorrhizal (EM) fungal community associated with post-fire regenerated *Pinus pinaster* Ait., and the influence of fire severity and site slope on EM assemblage patterns. Seedlings were sampled in the first autumn and in both spring and autumn of the second growing season after fire, in a total of three samplings. EM percentages per seedling were assessed, morphotypes described, and tentative identification of EM types performed by restriction fragment length polymorphism (RFLP) and sequencing of nrDNA internal transcribed spacer (ITS) region. Seedlings were highly mycorrhizal in all samplings, independently of the factors studied. A total of 45 EM types were identified, and richness and diversity significantly increased from the first to the second autumn after fire. Neither fire severity nor slope had a significant effect on fungal richness and diversity. Overall EM community composition was similar in all samplings, although fire severity, site slope and elapsed time after fire caused evident shifts in presence or in relative frequencies of a number of EM types. No significant effect of fire severity or slope on EM assemblage patterns was detected in the first two samplings after fire. However, a significant effect of fire severity was observed at the end of the second growing season. The harvest of burned wood did not significantly affect EM fungal assemblages although the slope did. We conclude that there was a high potential of active EM inoculum in soil immediately after fire colonizing post-fire natural regenerated *P. pinaster* seedlings with high EM percentages, and that factors defining burn intensity, such as fire severity and topography, directly influenced the species composition and assemblage patterns of EM fungal communities, triggering replacements and succession of EM fungal species.

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### 1. Introduction

Recurring fire is a major disturbance of Mediterranean forest ecosystems (Barbéro et al., 1998). Mediterranean fires are usually highly intense, largely depending on wind speed, temperature, moisture content of fuel, and topography. Mediterranean vegetation has developed evolutionary adaptive strategies against fire, which explains in a great extent the high resilience capacity of Mediterranean forest ecosystems (Buhk et al., 2007; Pausas and Verdú, 2008; Fernandes et al., 2008). However, trends in forest secondary succession beginning after fire depend on multiple factors such as initial plant species composition, fire intensity, seed bank availability, and recovery ability of soil microbial communities (Rodrigo et al., 2004; Hart et al., 2005).

*Pinus pinaster* is one of the most widespread tree species in the Mediterranean basin. Serotiny, bark thickness and early flowering are common adaptive traits of this pine reflecting its evader strategy in relation to fire (Tapias et al., 2004). Mediterranean pines display high genetic variability and phenotypic plasticity, as well as high colonization abilities (Fernandes and Rigolot, 2007; Pausas et al., 2008). But at the same time, natural regeneration and subsequent development of seedlings is highly dependent on both, post-fire environmental conditions (including microclimate and competition with neighbouring plants), and the association with symbiotic ectomycorrhizal (EM) fungi (Rodrigo et al., 2004). Pines are obligatory EM, and the persistence of active EM fungal propagules in soil after fire is crucial for survival and later regeneration of seedlings (Dahlberg et al., 2001).

EM fungi are an integral part of forest function, most notably for their essential role in water and nutrient acquisition by trees (Smith and Read, 1997). Despite their importance in the functioning of forest ecosystems, much is yet to be learnt about how EM fungal communities respond to fire (Bastias et al., 2006). EM fungi

\* Corresponding author. Tel.: +34 917452500; fax: +34 915640800.  
E-mail address: [ana.rincon@ccma.csic.es](mailto:ana.rincon@ccma.csic.es) (A. Rincón).

are immediately affected by fire (loss of vegetation, soil heating, ash deposition, combustion of the organic layer and increased erosion), and later conditioned by alterations in substrate availability, microclimate, and availability of host roots, all of which are key factors influencing EM assemblages (Cairney and Bastias, 2007). Knowing the diversity and structure of EM fungal communities is central for conservation purposes and represents a main prerequisite for understanding the role of EM symbiosis on tree seedling regeneration and secondary succession after fire disturbance (Walker et al., 2005; Cairney and Bastias, 2007). Post-fire dynamics of EM fungal communities have been largely studied in prescribed burnings and wildfires in different forest ecosystems (Stendell et al., 1999; Grogan et al., 2000a; Dahlberg et al., 2001; Tuininga and Dighton, 2004; Bastias et al., 2006), although few studies have been conducted in the Mediterranean basin, where the incidence of wildfires is usually very high (Torres and Honrubia, 1997; De Roman and de Miguel, 2005; Martín-Pinto et al., 2006).

Fire effects on soil living organisms, including EM fungi, vary between different sections of the same fire, mainly owing to differences in fire severity and topography (Certini, 2005). Moreover, removal of burned wood using hard mechanized equipment is a common post-fire management practice that can directly influence soil properties and EM fungi, especially in areas of pronounced slope. Our study was conducted in a burned *P. pinaster* forest stand sited on a steep hill, in which different levels of fire severity and slope could be distinguished. We firstly hypothesized that EM colonization of post-fire regenerated seedlings as well as EM fungal diversity will be negatively affected by fire, and secondly that factors such as fire severity and site slope will alter diversity and assemblage patterns of the EM fungal community triggering replacements in EM species. Our main objectives were (i) to assess the EM colonization level of naturally regenerated *P. pinaster* one and two years after fire and characterize the species composition of the EM community associated with seedlings; and (ii) to determine the effect of fire severity and site slope, as well as burned wood removal, on EM fungal diversity and EM assemblage patterns. Results obtained will provide important background information about the role of EM fungal communities on regeneration and secondary succession after fire.

## 2. Materials and methods

### 2.1. Site description and experimental design

The study was conducted in a *P. pinaster* forest located at “El Rodenal” in Guadalajara, Spain. The climate is Mediterranean with cool, wet winters and hot, dry summers. In July 2005, a major wildfire occurred in the zone resulting in approximately 10,000 ha of *P. pinaster* forest and canopies burned. An area of 8 ha containing burned and unburned surface was selected for this study. The site had NE exposure, 1300 m elevation and a pronounced slope. The burned wood was harvested one year after fire (October 2006), using ground-based mechanized equipment. A retention patch of trees representative of the original stand was left in place and used as control.

The selected area was divided into four transects (60 m × 280 m) separated by 20 m and orientated cross-slope according to different fire severities, and three transversal transects (60 m × 300 m) separated by 50 m, according to slope (Vega et al., 2009). Fire severity was classed as: (F1) unburned (a patch of trees representative of the original stand with few crowns little affected by fire), (F2) intermediate fire severity with all pine crowns and upper barks burned, and (F3) high fire severity with pines, canopy and under storey litter totally burned and the entire humic soil organic layer consumed. One year after fire, all the burned wood was harvested in transects

F2 and F3, and half of the trees were also harvested in F1 in order to assess the effect of wood removal (new treatment named F1c). Slope was classed as low 5% (T1), intermediate 12% (T2) and high 19% (T3). A total of 12 quadrants were delimited ((three fire severity levels + one wood removal treatment) × three slope levels).

### 2.2. Seedling sampling and ectomycorrhizal morphotyping

High germination of *P. pinaster* seeds was observed in the studied area after fire (Vega et al., 2009). The first growing season after fire, in October 2006 (Au06), a preliminary sampling of three *P. pinaster* seedlings per quadrant was carried out. Two years after fire, in May and November 2007 (Sp07 and Au07, respectively), two subsequent samplings of five to six seedlings per quadrant were carried out (the F1c treatment included). Seedlings, separated by a minimum of 10 m, were randomly harvested within each quadrant digging out as much as their root system as possible to a depth of 20–30 cm. Each seedling was carefully removed and bagged with the roots and surrounding soil as intact as possible and once in the lab, stored at 4 °C until processed (not longer than two weeks).

Roots were separated from shoots, washed free of substrate and cut into 2 cm segments. Total percentage of ectomycorrhizas (number of mycorrhizal tips/total number of root tips) was assessed under the stereomicroscope by counting all individual root tips per seedling. Root tips were classified as mycorrhizal or non-mycorrhizal based on the presence or absence of fungal mantle and mycelium, and to the lack or presence of root hairs, respectively. Dark tips lacking turgidity were counted as non-mycorrhizal for the estimation of EM colonization. EM tips of each seedling were classified by morphotypes based on characteristics of their mantle and extra-matrical mycelium (branching, surface colour, texture, emanating hyphae and rhizomorphs; Agerer, 1987–1998, 1995). Relative abundance of each morphotype within a seedling was determined as the number of mycorrhizal tips of this morphotype divided by the total number of root tips. When mycorrhizae were not evident, cross-sections were made and examined under the microscope to verify the presence of the Hartig net. For each seedling, two to five EM root tips of each morphotype were individually sampled, the excess of water retired, and stored at –80 °C for further analyses.

### 2.3. Molecular analysis of EM root tips

To identify each morphotype in a molecular basis, the internal transcribed spacer region (ITS) of nuclear ribosomal DNA was analyzed by combining restriction fragment length polymorphism (RFLP) and sequencing analyses. DNA was extracted and purified as described by Gardes and Bruns (1993). PCR amplifications were done with the primer pair ITS-1F/ITS-4 or ITS4B under the PCR conditions previously described (White et al., 1990; Gardes and Bruns, 1993). Negative controls without DNA were performed to detect possible contaminations. PCR products were digested by the endonucleases *Hinf*I, *Msp*I and *Taq*I (NewEngland Biolabs) and the restriction patterns (RFLPs) were analyzed by electrophoresis (5 V/cm) in 2.5% low fusion point agarose (MS4, Pronadisa, Spain) gels (~50 bp resolution) in TAE buffer. Samples were scored for the presence/absence of the fragments obtained by each endonuclease and a binary matrix was generated. Jaccard similarity indices were calculated for each pair of isolates, and a dendrogram was constructed by the un-weighted pair group method of analysis (UPGMA) using the SPSS v 15.0 software. Samples were distributed within clusters on the basis of their RFLP patterns which allowed verifying the previous classification made by morphological features and defining definitive morphotypes since no attempt to match morphotypes between seedlings had

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