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Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
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Assessment of mycorrhizal colonisation and soil nutrients in unmanaged fire-impacted soils from two target restoration sites

J. M. Dias[1,2], R. S. Oliveira[1], A. R. Franco[1], K. Ritz[2], N. Nunan[3] and P. M. L. Castro[1]*

[1] *CBQF/Escola Superior de Biotecnologia. Universidade Católica Portuguesa. Rua Dr. António Bernardino de Almeida. 4200-072 Porto. Portugal*
[2] *National Soil Resources Institute. Cranfield University. Bedfordshire MK43 0AL. United Kingdom*
[3] *CNRS. Laboratoire BioEMCo, UMR7618. Bâtiment EGER. Aile B. Campus AgroParisTech. F-78850 Thiveval-Grignon. France*

Abstract

The mycorrhizal colonisation of plants grown in unmanaged soils from two restoration sites with a fire history in Northern Portugal was evaluated from the perspective of supporting restoration programmes. To promote restoration of original tree stands, *Quercus ilex* L. and *Pinus pinaster* Ait. were used as target species on two sites, denoted Site 1 and 2 respectively. The aim of the study was to assess whether mycorrhizal propagules that survived fire episodes could serve as *in situ* inoculum sources, and to analyse the spatial distribution of soil nutrients and mycorrhizal parameters. In a laboratory bioassay, *P. pinaster* and *Q. ilex* seedlings were grown on soils from the target sites and root colonisation by ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi was determined. The ECM root colonisation levels found indicated that soil from Site 2 contained sufficient ECM propagules to serve as a primary source of inoculum for *P. pinaster*. The low levels of ECM and AM colonisation obtained on the roots of plants grown in soil from Site 1 indicated that the existing mycorrhizal propagules might be insufficient for effective root colonisation of *Q. ilex*. Different ECM morphotypes were found in plants grown in soil from the two sites. At Site 2 mycorrhizal parameters were found to be spatially structured, with significant differences in ECM colonisation and soil P concentrations between regions of either side of an existing watercourse. The spatial distribution of mycorrhizal propagules was related to edaphic parameters (total C and extractable P), and correlations between soil nutrients and mycorrhizal parameters were found.

Additional key words: arbuscular mycorrhiza, ectomycorrhiza, *Pinus pinaster*, post fire, *Quercus ilex*, spatial variation, soil restoration.

Resumen

Evaluación de la colonización micorrícica y de los nutrientes del suelo en dos zonas de restauración afectadas por fuego

Con el fin de dar soporte a un programa de restauración de dos zonas afectadas por un historial de fuego en el norte de Portugal se evaluó la colonización micorrícica de las plantas de cada zona. Se utilizaron *Quercus ilex* L. y *Pinus pinaster* Ait. para promover la restauración de las especies originales de las dos zonas seleccionadas (zona 1 y zona 2). El objetivo de este estudio fue evaluar si los propágulos de los hongos formadores de micorrizas, que habían logrado sobrevivir a los episodios de fuego, podían servir como fuente de inóculo *in situ* y analizar la distribución de los nutrientes del suelo y los parámetros micorrícicos. Se determinó la colonización por hongos ectomicorrícicos (ECM) y por hongos formadores de micorrizas arbusculares (MA) en las raíces de plántulas de *P. pinaster* y *Q. ilex* cultivadas en los suelos seleccionados. Los niveles de colonización ECM observados en los suelos de la zona 2 indicaron que la cantidad de propágulos infectivos era suficiente como para servir de fuente primaria de inóculo para *P. pinaster*. Sin embargo, en la zona 1 se encontraron niveles muy bajos de colonización ECM y MA lo que indicaba que no había suficiente cantidad de propágulos para colonizar eficientemente las plántulas de *Q. ilex*. Se encontraron diferentes morfotipos de ECM en las plantas cultivadas en las distintas zonas. En la zona 2 se encontraron diferencias

* Corresponding author: plcastro@esb.ucp.pt
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significativas en la colonización ECM y en la concentración de P en el suelo de las regiones situadas a ambos lados del curso del agua. Se encontró una distribución espacial de los propágulos de hongos formadores de micorrizas relacionada con parámetros edáficos (C total y P extraíble) y se encontró correlación entre los nutrientes del suelo y los parámetros micorrícicos.

Palabras clave adicionales: ectomicorrizas, micorrizas arbusculares, *Pinus pinaster, Quercus ilex,* restauración de suelos.

Introduction

The forest area in Portugal occupies 3.4 million ha, consisting of a mixture of tree species, which in decreasing order of abundance are *P. pinaster* Ait, *Eucalyptus globulus* Labill., *Quercus suber* L., *Q. ilex* L. and, with a smaller representation, other coniferous and broadleaf species. In Portugal conditions are favourable for forest fires to occur (IFFN, 2005) and according to the European Forest Fires Information System (EFFIS), the burned area to mid-2004 was approximately 94,000 ha, with 35,000 ha being forestland including forest stands and shrub land. Forest fire normally causes degradation of plant and soil microbial communities, contributing to a reduction in the density of fungal propagules (Rashid *et al.*, 1997; Stendell *et al.*, 1999; Cairney and Bastias, 2007). However, the magnitude of such negative impacts will depend on many factors such as the intensity of the fire, the amount of available fuel, the soil moisture at the time of the episode and the type of ground cover (Torres and Honrubia, 1997). Due to the negative effects of fire in plant communities and soil structure, there is an urgent need to improve restoration practices. These practices should be implemented together with an environmentally-beneficial soil restoration and conservation strategy.

Mycorrhizas are the most widespread terrestrial symbiotic associations between plants and fungi (Smith and Read, 2008) and occur at the level of roots via morphological and anatomical modifications. This relationship is known to improve plant health by increasing nutrient and water uptake, alleviating environmental stresses and enhancing plant resistance against disease (Quoreshi *et al.*, 2008; Smith and Read, 2008; Rivero *et al.*, 2009). The importance of these mutualistic associations has gained significant interest due to increasing costs of irrigation and synthetic fertilisers (McKane and Kandel, 1996; Rincon *et al.*, 2007). The natural soil biota, which includes mycorrhizal fungi, is a very important biotechnological resource and its application represents a step forward in the use of sustainable techniques for forestry production. The most common types of mycorrhizas in nature are arbuscular mycorrhizas and ectomycorrhizas. Members of the Pinaceae are known to be obligatory with respect to ectomycorrhizal (ECM) associations (Bidartondo *et al.*, 2001). *Quercus* spp. are usually considered to be ECM, nonetheless arbuscular mycorrhizal (AM) colonisation has been found in both seedling and mature *Quercus* spp. (Ian *et al.*, 2001). Different types of mycorrhizas often co-occur in the same ecosystem (Moyersoen *et al.*, 1998) forming dual mycorrhizal associations, however, ECM fungi are apparently capable of displacing AM fungi (Lodge and Wentworth, 1990). A variety of processes may lead to species-specific spatial patterns in ECM populations, including variation in rates of genet growth (if patches are made up of one individual), internal structure of genets, or patterns of intraspecific establishment or survival that lead to clusters of individuals (Lilleskov, 2004). Relatively little is known about how mycorrhizal communities are structured spatially (Taylor, 2002) and it is therefore relevant to evaluate how individuals and communities show different spatial distributions.

The aims of this study were to assess the mycorrhizal root colonisation of plants grown in unmanaged soils from two target restoration sites with a fire history, using qualitative and quantitative approaches and to characterise the spatial variation of the mycorrhizal propagules. We hypothesised that there would be spatially-related associations between soil nutrients and mycorrhizal parameters. *Quercus ilex* and *P. pinaster* were used as target plants on laboratory bioassay studies using soils derived from two sites, since these species were extant at the sites prior to fire events.

Abbreviations used: AM (arbuscular mycorrhizal), ECM (ectomycorrhizal), EFFIS (European Forest Fires Information System), OM (Organic matter).

Material and methods

Sites description and soil sampling

The study sites were located on a mountainous region within the Serra da Cabreira, Northern Portugal (40°40'12" N, 08°3'33" W), consisting mainly of low granite mountains with steep valleys and mild climate (mean annual temperature 13 to 15°C; mean annual precipitation 1820 mm). Two contrasting sites were selected on the basis of their past fire history and their target tree species for restoration. Site 1 (860 m asl) had 4 ha targeted for restoration using *Q. ilex* and had the last fire episode in 1975, while Site 2 (830 m asl) had 5 ha targeted for restoration using *P. pinaster* and had the last fire episode in 2000. Both sites had scarce vegetation with patchily distributed herbaceous plant species such as *Pteridium aquilinum*, *Rubus* spp. and *Agrostis* spp. At Site 1, a few *Quercus* spp. were present, whereas at Site 2 some *P. pinaster* plants were identified. A uniform area was sampled at both sites, according to presence of vegetation and local topography and avoiding obvious major variation between sample points (*e.g.* emergent rock, erosion points). Thirty soil samples were collected from each site on a regular grid pattern for chemical analyses and for use in a laboratory bioassay (Fig. 1). The sampling design consisted of a regularly spaced grid (30 m average) of 6 × 5 samples on Site 1. At Site 2, the design included a 6 × 2 grid at north east and a 6 × 3 grid at south west of a small existing watercourse, with an average spacing of 50 m. Soils were air-dried before the determination of their chemical properties. For loss-on-ignition, soils were oven-dried at 40°C overnight.

Laboratory bioassay

According to the restoration scheme, *Q. ilex* and *P. pinaster* seeds were sown in soil from Site 1 and 2, respectively. Soil collected from each sampling point was homogenised and divided into three cells of labelled containers (CETAP, António Matos Lda, Espinho, Portugal). Trays with 20 cells (800 mL each) were used for *Q. ilex*, while trays with 40 cells (210 mL each) were used for *P. pinaster*. Controls were also established in

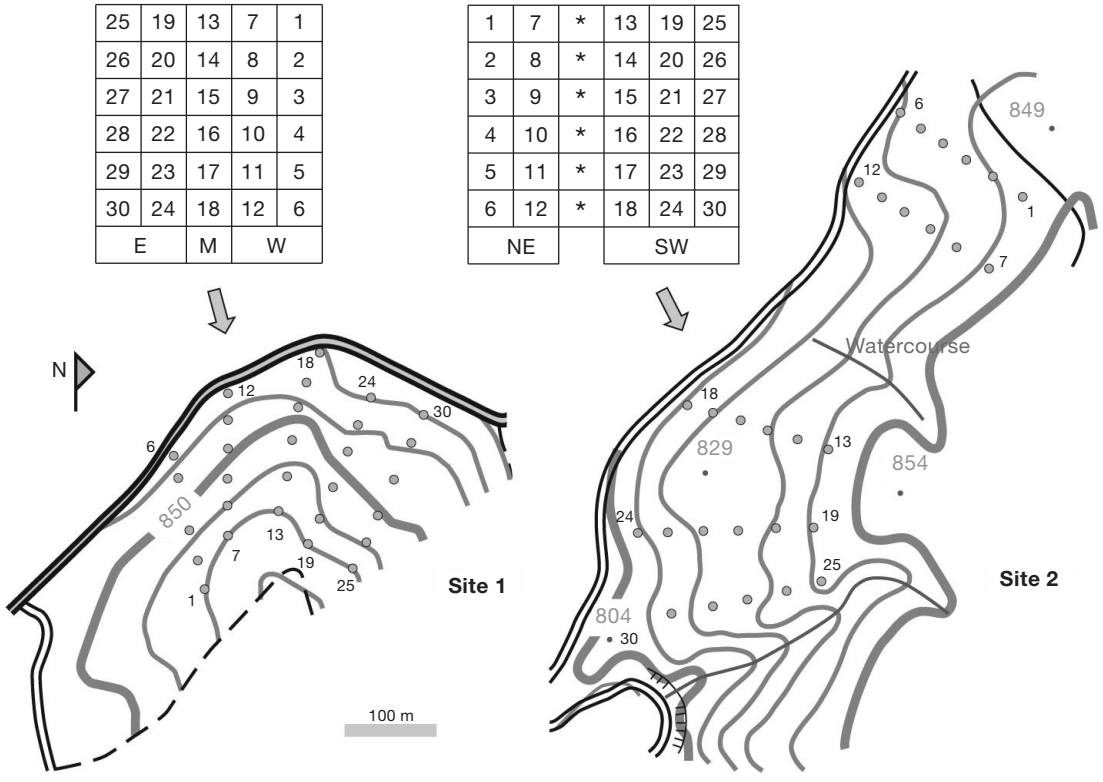

Figure 1. Sampling design showing location of sampling points. Box arrow represents viewpoint for posting plots. Insets show framework for posting plots with associated sample numbers.

order to check for possible laboratory, glasshouse or aerial mycorrhizal root contamination. For that purpose, soil collected from the 30 sampling points of Site 1 was mixed and autoclaved twice at 121°C for 80 min on two consecutive days. The autoclaved soil was placed into 15 cells of a tray. The same procedure was followed for controls from Site 2. Twenty millilitres of a soil suspension (prepared by shaking 100 g of unsterilised soil in 1 litre of sterilised deionised water for 2 h and followed by filtration (Whatman No. 1) were added to each cell to replace soil microbial populations. Seeds of *P. pinaster* were rinsed overnight in running tap water, surface sterilised with 0.5% (v/v) NaOCl for 15 min and rinsed three times with autoclaved deionised water. Seeds of *Q. ilex w*ere stratified at 4°C for 6 months, rinsed with tap water, surface sterilised with 0.5% (v/v) NaOCl for 10 min and washed three times with autoclaved deionised water. Each cell with soil from Site 1 received two seeds of *Q. ilex*, while each cell with soil from Site 2 received two seeds of *P. pinaster*. If both seeds successfully germinated in the same cell, one was eliminated so that only one plant per cell was grown. Plants were grown in a greenhouse (temperature and relative humidity varied from 7 to 45°C and from 12 to 90%, respectively) at a forest nursery. *Quercus ilex* and *P. pinaster* were harvested after 5 and 4 months of growth, respectively. *Quercus ilex* plants were analysed for AM and ECM colonisation, whereas *P. pinaster* plants were analysed for ECM colonisation.

Mycorrhizal analysis

The intact root system was separated from the block of soil by immersing the roots in a beaker of water and gently agitating them to remove the attached soil. To analyse the ECM colonisation, roots were separated from shoots, placed in Petri dishes with water and maintained at 4°C until analysis. Afterwards, roots were cleaned individually, while inspecting them visually under a dissecting microscope (Olympus SZ60, Japan). Ectomycorrhizas were identified using a dissecting microscope with a black microscope base and a direct source of light at a magnification of 22x. Unstained ECM roots were distinguished from each other by differences in morphological characteristics. The degree of ECM colonisation was assessed by counting mycorrhizal root tips using the gridline intersection method (Giovanneti and Mosse, 1980). The number of ECM tips, total number of tips and total number of intersections was counted to assess the percentage of ECM colonisation and the number of ECM root tips per metre of fine root. Representative ECM morphotypes were characterised on the basis of type of ramification, shape of unramified ends, presence of rhizomorphs, colour, size and features of mantle surface (Agerer, 1998). To assess the AM root colonisation, fresh root samples were cleared and stained with trypan blue as described in Oliveira *et al.* (2005), placed in Petri dishes and stored in 50% glycerol for further analysis. The degree of AM colonisation was assessed by counting mycorrhizal roots under a dissecting microscope (Olympus SZ60, Japan) using the gridline intersection method.

Soil chemical analysis

The Egner-Riehm method was used to determine extractable P concentration (Riehm, 1958) and the Kjeldhal method was used to determine the total N concentration (MAFF ADAS, 1986). Organic matter (OM) was measured by loss-on-ignition (3 h at 450°C) and pH was measured in water according to the British Standard Methods (1990). The organic C content was calculated by considering 58% (w/w) of OM mass present as C.

Statistical analysis

Differences between sites and between regions either side of the water course at Site 2 were analysed using *t*-tests ($\alpha = 0.05$). One-way ANOVA ($\alpha = 0.05$) was used to assess differences between samples in the same field. Spatial analysis was also performed using the Mantel test by comparing a distance matrix in mycorrhizal colonisation or chemical properties with a matrix of spatial distances among samples. This produced a normalised Mantel statistic (r_M) that is bounded between –1 and +1 and behaves like a correlation coefficient (Legendre and Legendre, 1998). It should be noted, however, that Mantel statistics are often small, even when highly statistically significant. The significance of the relationship was determined by permutation of the first of the two distance matrices (999 permutations). A significant r_M meant that there was a relationship between physical distance and the variable analysed (*i.e.* spatial structure). When spatial trends were identified, the data were analysed in more detail using *t*-tests.

Results

Soil chemical properties

At Site 1 the soil pH was 4.4 ± 0.02 and at Site 2 was 4.3 ± 0.05. Total N concentration and total organic C showed a 4-fold and 5-fold variation between samples at Sites 1 and 2, respectively, being on average 0.63 ± 0.04 % (w/w) and 11.5 ± 0.60 % (w/w) at Site 1 and 0.40 ± 0.03 % (w/w) and 9.3 ± 0.63 % (w/w) at Site 2, respectively. The total organic C content was high at both sites with Site 2 showing a significantly higher C:N ratio (24 ± 0.76 compared to 19 ± 1.02, $P < 0.001$) and Site 1 showing a significantly higher total organic C ($P < 0.05$). Extractable P values were very low at Site 2 (3.1 ± 0.39 mg P kg^{-1} soil), considering the values adopted for agricultural crops in Portugal (LQARS, 2000). At Site 1 soil P concentration was low (8.2 ± 2.06 mg P kg^{-1} soil) but significantly higher ($P < 0.05$) than at Site 2. A higher P concentration was however found at Site 1 in sample point 15, being 65.3 mg P kg^{-1} soil. Extensive spatial variation in chemical properties was found across each site, with no simple or distinct patterning apparent for the majority of properties. However, the P levels in Site 2 showed a slight, but significant spatial structure at NE and SW of the watercourse ($r_M = 0.13$, $P = 0.022$), with higher values at the NE side (Fig. 2).

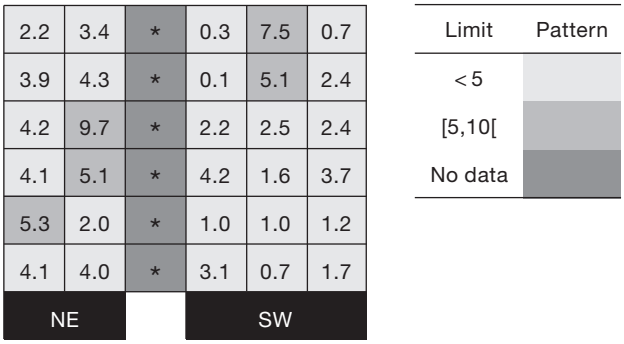

Figure 2. Spatial patterns for extractable phosphorus (mg P kg^{-1} soil) at Site 2 (NE and SW side of small watercourse represented by cells marked with as asterisk). Mean values: 4.4 ± 0.6 mg P kg^{-1} (NE) and 2.3 ± 0.4 mg P kg^{-1} (SW).

Mycorrhizal colonisation

The ECM colonisation was higher in samples from Site 2. The percentage of ECM colonisation varied between 0 and 3% in samples from Site 1 and between 10 and 57% in samples from Site 2. The number of ECM root tips per metre of fine root ranged from 0 to 17 in samples from Site 1, and from 3 to 22 in samples from Site 2. There was no ECM colonisation on control samples. The spatial analysis showed that there was no distinct patterning for ECM colonisation at Site 1. However, at Site 2, spatial structure was identified in the percentage of ECM colonisation ($r_M = 0.2$, $P < 0.01$) and in the number of ECM root tips per metre of fine root ($r_M = 0.18$, $P = 0.01$), with higher values found at the NE region (Fig. 3). Additionally there was no evidence of spatial structure within both regions. Different ECM morphotypes were found in plants grown in soil from the two sites. For Site 2, a higher number of morphotypes were found (18 types at Site 2 and 10 types at Site 1). More common morphotypes were those with a simpler morphology, such as Morphotypes A and B at Site 1 (Table 1) and Morphotypes K and L at Site 2 (Table 2). Morphotypes at Site 2 also showed higher diversity in morphological characteristics than at Site 1, with some presenting rhizomorphs and bigger diffe-

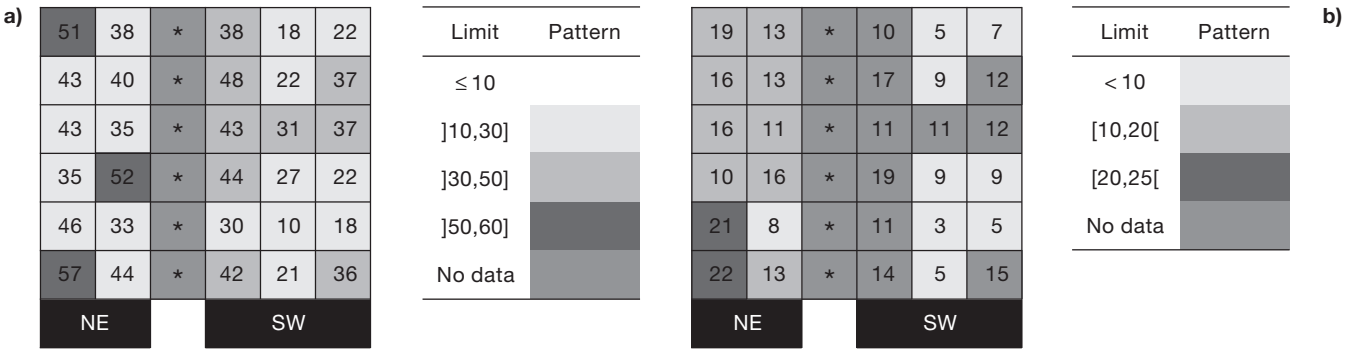

Figure 3. Spatial patterns for ectomycorrhizal (ECM) parameters at Site 2 (NE and SW side of small watercourse represented by cells marked with as asterisk). a) Mean percentage of ECM colonisation: 43 ± 2.1% (NE) and 30 ± 2.6% (SW). b) Mean number of ECM root tips per metre of fine root: 15 ± 1.2 (NE) and 10 ± 1.0 (SW).

Table 1. Morphological characteristics and location of ectomycorrhizal morphotypes identified in the roots of *Quercus ilex* grown in soil samples from Site 1

Code	Location[a]	Type of ramification[b]	Shape of unramified ends	Colour	Size	Mantle-surface
A		S	Straight	White	Medium	Grainy
B		S	Bent	White	Variable	Grainy
C		S	Straight	Orange	Small	Smooth
D		MPI	Straight	Brown	Medium	Smooth
E		S/MPy	Straight	Yellow	Variable	Smooth
F		IPD	Bent	White	Large	Grainy
G		S	Straight	Brown	Variable	Smooth
H		D	Bent/straight	White	Small	Smooth
I		S	Straight	Yellow	Medium	Smooth
J		S	Bent/straight	White	Medium	Cottony

[a] Black cells show location, white cells show sampling points where morphotype was not present, grey cells show sampling points where data is not available. [b] MPy: monopodial-pyramidal. IPD: irregularly-pinnate dichotomous-like. D: dichotomous. MPI: monopodial pinnate. S: simple (unramified).

rences in features of the mantle-surface. Spatial distribution was assessed for each morphotype in terms of number of morphotypes at each sample point. The number of morphotypes per sample point varied from 0 to 4 at Site 1 and from 1 to 7 at Site 2. However, no simple or distinct pattern was found for either each morphotype or number of morphotypes per sample point at both sites. Arbuscular mycorrhizal colonisation in *Q. ilex* (Site 1), estimated as a percentage of root colonised, ranged from 0 to 20%. There was no AM colonisation on control samples. A spatial analysis showed that there was a significant difference ($P < 0.05$) in the percentage of AM colonisation at the Eastern, middle and Western side of the field and a higher degree of colonisation was found at the Eastern side (Fig. 4).

Correlation between properties

Strong positive correlation was found between total N and total C at Site 1 ($r^2 = 0.55$, $P < 0.001$) and Site 2 ($r^2 = 0.89$, $P < 0.001$). No correlation was found between total N and extractable P or total C and extractable P values for both sites. Significant and positive correlations between percentage of ECM colonisation and

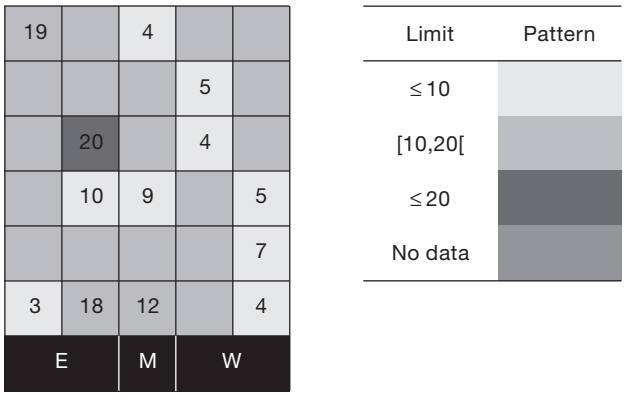

Figure 4. Spatial pattern for mean percentage of arbuscular mycorrhizal colonisation at the East (E), Middle (M) and West (W) side of Site 1. Mean values: 14 ± 3.3% (E), 8 ± 2.4% (M) and 5 ± 0.5% (W).

Table 2. Morphological characteristics and location of ectomycorrhizal morphotypes identified in the roots of *Pinus pinaster* grown in soil samples from Site 2

Code	Location[a]	Type of ramification[b]	Shape of unramified ends	Rhizomorphs	Colour	Size	Mantle-surface
K		S	Straight	Absent	Dark orange	Small	Smooth
L		S	Straight	Orange	Dark orange	Small	Smooth
M		S	Straight	Absent	Brown	Variable	Grainy
N		S	Straight	Absent	Dark brown	Small	Reticulate
O		D	Straight	Absent	Dark orange	Small	Smooth
P		D	Straight	Light orange	Very dark orange	Small	Smooth
Q		D	Straight	Absent	Dark brown	Medium	Short-spiny
R		D	Straight	Absent	Light orange	Medium	Short-spiny
S		S	Straight	Absent	Brown	Long	Smooth
T		D	Bent	Absent	Dark orange	Small	Grainy
U		S	Straight	Absent	Dark red	Medium	Stringy
V		D	Straight	Absent	Light pink	Small	White cottony
W		D	Straight	Absent	Light pink	Medium	Smooth
X		D	Straight	Absent	Dark brown	Medium	Smooth
Y		D	Straight	Absent	Dark orange	Small	Orange cottony
Z		D	Straight	Absent	Orange	Small	White cottony
A1		D	Straight	Absent	Light orange	Medium	Reticulate
B1		D	Straight	Absent	Very light orange	Small	Smooth

[a] Black cells show location, white cells show sampling points where morphotype is not present and grey cells represent sampling points where data is not available. [b] D: dichotomous. S: simple (unramified).

the number of ECM root tips per metre of fine root were found at Site 1 ($r^2 = 0.83$, $P < 0.001$) and at Site 2 ($r^2 = 0.83$, $P < 0.001$). No correlation was found between the percentage of ECM and AM colonisation at Site 1. Significant and positive correlation was found between extractable P and AM colonisation at Site 1 ($r^2 = 0.76$, $P < 0.01$); the percentage of ECM colonisation and the number of ECM root tips per metre of fine root were also found to be positively correlated with extractable P only at Site 2 ($r^2 = 0.68$, $P < 0.001$ and $r^2 = 0.64$,

$P < 0.001$, respectively). At Site 1 a positive correlation was found between the percentage of ECM colonisation and total C ($r^2 = 0.34$, $P < 0.1$). For the number of morphotypes per sample point, no correlation was found with any of the measured chemical properties.

Discussion

The acidic soils studied had a relatively high organic C content and low nutrient levels. These are chemical characteristics normally found on impoverished lands, which would be expected for such mountainous lands with a history of exposure to fires (Neary *et al.*, 1999). The C:N ratio at Site 2 (more recently exposed to fire) was significantly different and higher than at Site 1, probably due to the upward movement of organic compounds caused by the transfer of heat downwards after the fire event (DeBano, 2000). The positive correlations between total N and total organic C contents suggest common microscale variability for these chemical properties (Ritz *et al.*, 2004). High levels of spatial heterogeneity in soil chemical properties were found, confirming what was reported for other ecosystems (Jackson and Caldwell, 1993; Gross *et al.*, 1995; Ritz *et al.*, 2004). The differences observed in P levels NE and SW of the small watercourse at Site 2 (Fig. 2) were probably related with hydrological characteristics of the watershed.

A significant correlation between the percentage of ECM colonisation and the number of ECM root tips per metre of fine root was found at both sites. Such correlations were previously reported (Baxter *et al.*, 1999) and suggest a common origin of microscale variability of the named parameters. At Site 2, spatial structure was identified in the percentage of ECM colonisation and in the number of ECM root tips per metre of fine root, showing significant differences between regions of either side of the watercourse. Considering the lack of spatial distribution at each side of the stream in isolation, it appears that the identified spatial structure is related to the differences between each side of the watercourse, therefore suggesting that the hydrological features are influencing soil microbiological properties. Qualitative analysis demonstrated that different ECM morphotypes occurred at both sites.

Arbuscular mycorrhizal colonisation of *Q. ilex* was low and the patterning at Eastern, middle and Western side of Site 1 suggests an influence of soil exposure to sun. For example, a greater sun exposure on one side of the field could have altered soil structure and temperature, therefore influencing mycorrhizal propagules. However, the AM spatial structure was not confirmed when the Mantel test was used (results were not significant). Usually, an inhibition of AM fungal colonisation occurs with increasing soil P availability (Smith and Read, 2008); however, in the present study P concentration varied positively with the percentage of AM colonisation at Site 1. Since extractable P was found to be very low, it is possible that the extractable P values were not high enough to cause inhibition of AM fungal colonisation of *Q. ilex*.

The sampling scheme adopted was designed to provide a basic framework to assess spatial variation at the study sites. Other geostatistical approaches can be used to assess spatial structure in soil properties (Webster and Oliver, 2001), but they require larger numbers of samples (*i.e.* of the order of several hundreds). However, the data here presented can be used to infer that an appropriate starting scale for more thorough studies of spatial structure at the sites would be one that matches the basic grid size used (*i.e.* around 40 m), since there was a high variance at this scale for most properties.

The ECM root colonisation levels found indicated that soil from Site 2 contains ECM propagules to serve as primary source of inoculum for *P. pinaster*. The low levels of ECM and AM colonisation obtained on the roots of plants grown in soil from Site 1 indicated that the existing mycorrhizal propagules might be insufficient for effective colonisation of *Q. ilex*. Positive correlations were found between soil nutrients and mycorrhizal colonisation: extractable P and AM colonisation at Site 1, extractable P and both, the percentage of ECM colonisation and the number of ECM root tips per metre of fine root at Site 2 and the percentage of ECM colonisation and total C at Site 1.

The results from this study show that due to their spatial variation, mycorrhizal propagules that survive fire episodes are not always effective in serving as *in situ* mycorrhizal inoculum source. Therefore, assessment of the mycorrhizal colonisation potential of unmanaged fire-impacted sites should be conducted before restoration practices are implemented.

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