Long-term soil alteration in historical charcoal hearths affects *Tuber melanosporum* mycorrhizal development and environmental conditions for fruiting

Sergi Garcia-Barreda[1,2*], Sara Molina-Grau[3], Ricardo Forcadell[3], Sergio Sánchez[1], Santiago Reyna[4]

[1] Unidad de Recursos Forestales, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Avda. Montañana 930, 50059 Zaragoza, Spain

[2] Centro de Investigación y Experimentación en Truficultura de la Diputación de Huesca, Polígono Fabardo s/n, 22430 Graus, Spain

[3] Fundación Centro de Estudios Ambientales del Mediterráneo, C/ Charles Darwin 14 Parque Tecnológico, 46980 Paterna, Spain

[4] ETS Ingeniería Agronómica y del Medio Natural, Universitat Politècnica de València, Camino de Vera s/n, 46021 Valencia, Spain

* Corresponding author. Tel.: (+34) 976716300, fax: (+34) 976716335, e-mail: sergigarciabarreda@gmail.com

Acknowledgements

This work was funded by INIA (Government of Spain) project PET2007-13-C07-04. We would like to thank Dr Cristina Gimeno (Fundación CEAM) for her advice with CO_2 efflux measures.

Abstract

Abandoned charcoal hearths constitute a very particular habitat for spontaneous fruiting of *Tuber melanosporum*, leading some harvesters to hypothesise that the fungus could benefit from the alterations that these soils underwent. However ecological mechanisms involved in this relation are not fully elucidated yet. As a first step to understand it, influence of long-term soil alteration on the symbiotic stage of *T. melanosporum* and on selected soil properties considered key to fruiting was assessed by conducting a greenhouse bioassay and a field observational study. In the bioassay, percent root colonisation and relative abundance of *T. melanosporum* were significantly lower in hearth than in control soils. Hearth soils showed significantly lower resistance to penetration, larger temperature fluctuation, reduced plant cover and reduced herbaceous root abundance. The results do not support the hypothesis that soil from historical charcoal hearths currently enhances development of *T. melanosporum* mycorrhizas. However, whether this is due to increased infectivity of native ectomycorrhizal communities or to worse conditions for development of *T. melanosporum* mycorrhizas remains unresolved. Native ectomycorrhizal communities in hearths showed altered composition, although not a clear change in infectivity or richness. Direction of change in hearth soil properties is compared to alteration occurring in soils spontaneously producing *T. melanosporum*. The interest of these changes to improve *T. melanosporum* fruiting in plantations is discussed.

Keywords: *Tuber melanosporum*; charcoal hearth; ectomycorrhizal infectivity; soil heating; soil structure; soil temperature

Introduction

The European black truffle (*Tuber melanosporum* Vittad.) is a commercially harvested, ectomycorrhizal (EM) fungus. It grows wild in Mediterranean calcareous *Quercus* forests. Its cultivation, although not completely domesticated yet, has advanced greatly in recent decades thanks to studies of its ecology in the wild, development of mycorrhizal seedlings and molecular studies of its biology (Chevalier and Grente 1979; Garcia-Barreda et al. 2007; Le Tacon et al. 2016).

Historical charcoal hearths constitute a very particular soil habitat due to degree and persistence of soil alteration (Mikan and Abrams 1995; Hardy et al. 2016). During a 1998 census of wild *truffières* in a 1400 ha forest in El Toro (eastern Spain), six out of 98 productive *truffières* were located on abandoned charcoal hearths, with local harvesters reporting that it was not an exceptional case. Soil from hearths was held in high regard by local harvesters on the basis of their empirical experience introducing it in truffle holes in plantations (Reyna and Colinas 2007), although without any scientific design or monitoring. This atypical environment for truffle fruiting poses the questions of which ecological factors are involved and whether they have practical interest for truffle cultivation (Reyna and De Miguel 2007).

Production of wood charcoal is an historical use of Mediterranean *Quercus* forests. Traditional charcoal making involved creating a mound kiln by piling wood on circular levelled platforms (hereafter called "hearths") generally set up inside forest areas. Piles were covered with soil and carbonised by slow combustion. Wood carbonisation usually took 3-10 days, with temperature reaching up to 400-500ºC. This process was usually repeated on the same hearth several times a year over many years.

Some of the factors that have been claimed to contribute to *T. melanosporum* fruiting in abandoned charcoal hearths are: (a) long-lasting change in light conditions due to canopy

openings created, (b) decrease of native EM populations caused by soil heating and wood distillates, (c) long-lasting alteration of soil properties caused by heating and incorporated charcoal particles, and (d) spore dispersal agents such as wild boars being attracted to hearths by wood distillates (Reyna and De Miguel 2007).

As a first step to understand the relation between historical charcoal hearths and *T. melanosporum*, we assess the influence of long-term soil alteration on the symbiotic stage of *T. melanosporum* and on selected soil properties that are generally considered key to fruiting. We conducted a bioassay to investigate the ability of *T. melanosporum* to develop mycorrhizas in these soils and to compete against native EM fungi. We also characterised the degree of alteration of selected physical and biological soil properties and discuss their role in the ecology of the fungus.

Materials and methods

The study was conducted in *T. melanosporum*-producing areas of Teruel and Castelló provinces (eastern Spain). Three *Quercus ilex-Juniperus thurifera* forests were used as experimental blocks: Albarracín (40º28'N, 1º28'W), Formiche (40º18'N, 0º51'W) and El Toro (39º57'N, 0º44'W). They all had produced charcoal with traditional earthmound kilns for decades, and in all cases production was abandoned during the first half of the 20th century.

In order to evaluate the impact of soil alteration on development of *T. melanosporum* mycorrhizas, a bioassay was conducted in which 72 seedlings were planted in a full factorial design with three replicates per treatment. The predictor variables were experimental block, land use (historical charcoal hearth, control non-hearth soil from a nearby forest opening of similar size), heat treatment of the soil (microwaves, no treatment) and inoculation with *T. melanosporum* (inoculated, not inoculated). In each block one hearth was used as soil source,

selecting hearths in which no truffle fruiting was reported by local harvesters to minimise the confounding effects of non-controlled (and likely high) levels of native *T. melanosporum* inoculum (Table S1). Seedling mortality and dieback reduced sample size to 54.

In each hearth and the corresponding control area, topsoil (0-20 cm) from three different points was collected and mixed. Depth of soil sampling corresponded to the thoroughly altered soil layer of selected hearths. Soil was collected in the centre of hearth platforms and control forest openings, with equal distances to the nearest surrounding tree.

Quercus ilex L seedlings were transplanted into Quick-pot® 0.65 l containers filled with a soil:perlite mix (19:1, v/v) within ten days after soil collection from the field, in April. Microwaves were applied the day before bioassay set-up, with a domestic oven (nominal maximum power 700 W, frequency 2.45 GHz, 300 seconds). The soil was put in a layer 3.5 cm depth, reaching 90-95ºC at 1.7 cm depth. The inoculation treatment was applied during the bioassay set-up. Previously sliced, dried and homogenised sporocarps of *T. melanosporum* were mixed with talcum powder. The inoculation was performed by root-powdering with 2.0 g fresh *T. melanosporum* per seedling. The containers were watered to saturation every 2-3 days and kept outdoors to avoid glasshouse-adapted EM fungi.

The mycorrhizal status of seedlings was assessed ten months after the set-up (February-March). In each seedling a sample with 9% of the substrate volume (57 ml) was taken, consisting of three subsamples: the depth of the container was divided into three equal parts and in the centre of each third a horizontal core across the container was taken. The EM tips were sorted in morphotypes (Agerer 2002). Morphotype frequency was defined as the proportion of samples in which a particular morphotype was found, root colonisation was defined as the proportion of root tips colonised by a particular morphotype, and relative abundance was defined as the proportion of EM tips colonised by a particular morphotype. Morphotype richness was defined as the number of morphotypes present in a sample.

Variables with normal distribution were analysed through conventional ANOVA. Main effects and two-way interactions were examined and the model with lower Akaike information criterion (AIC) was selected. When model assumptions were violated, the response variable was transformed. Transformed variables are presented in their original scale after being transformed back by applying the inverse transformation to means and limits of confidence intervals (CI). Binary response variables were analysed through logistic regression. In order to characterise soil alteration, six hearths were selected in each experimental block. Currently productive areas were excluded to avoid differences caused by truffle harvesting, trampling and soil tillage. Hearth surface ranged 16-89 m^2 (mean: 46 m^2) and depth of the thoroughly altered soil layer ranged 10-30 cm (mean: 18 cm). In each hearth, a set of soil properties related to air and water circulation, temperature regime, pH, microbial activity and plant community (Table S2) were measured both within the hearth (in the centre of platform) and in nearby forest openings of similar size used as control (in areas with distance to surrounding forests equal to those of hearth centre, in order to minimise differences in light conditions). Soil properties were assessed through conventional ANOVA except for variables involving repeated measures (temperature range and CO_2 efflux) that were analysed through linear mixed models.

Results

In bioassay seedlings, percent root colonisation by the inoculated *T. melanosporum* was significantly lower in charcoal hearth than control soils. It was also significantly affected by the interaction between heat treatment and block, with two blocks showing higher percent root colonisation in microwave-treated than non-treated soils and the remaining block not showing significant differences. Relative abundance of *T. melanosporum* was significantly affected by the interaction between land use and block and the interaction between heat

treatment and block. In two blocks hearth soils showed significantly lower relative abundance than control soils, with the remaining block not showing significant differences. In two blocks microwave-treated soils showed significantly higher relative abundance, with the remaining block not showing significant differences (Table 1, Table S4). No *T. melanosporum* mycorrhizas were found in non-inoculated seedlings.

Percent root colonisation by native EM communities was significantly affected by heat treatment and the interaction between land use and inoculation (Table S4). In hearth soils there were no significant differences between non-inoculated (mean: 26.7%, 95% CI: 17.6-35.8) and inoculated seedlings (mean: 30.9%, 95% CI: 21.8-40.0), whereas in non-hearth soils inoculated seedlings (mean: 11.0%, 95% CI: 1.9-20.1) showed significantly lower percent root colonisation than non-inoculated seedlings (mean: 32.7%, 95% CI: 24.2-41.1). The native EM community showed significantly lower colonisation in microwave-treated (mean: 20.1%, 95% CI: 14.2-26.0) than non-treated soils (mean:33.0%, 95% CI: 26.1-39.8).

Richness of native EM morphotypes in seedling roots was significantly affected by the interaction between land use and heat treatment, with microwave-treated soils showing significantly lower richness than non-treated soils in both hearth and control soils. Richness was also affected by the interaction between inoculation and heat treatment, with microwave-treated soils showing significantly lower richness than non-treated soils in both inoculated and non-inoculated seedlings (Table 2, Table S4). Length of fine roots was significantly affected by the interaction between land use and heat treatment, indicating that the effect of microwaves was significantly more positive in non-hearth than in hearth soils, and by the interaction between inoculation and heat treatment, indicating that the effect of microwaves was significantly more positive in inoculated than in non-inoculated seedlings (Table 2, Table S4).

Twelve EM morphotypes were found in the bioassay, although only seven appeared in five or more samples (Table S3). Two of them (types *Pisolithus* and complex *Tuber albidum*) showed significantly lower occurrence in hearth than control soils, whereas type unidentified 2 showed lower occurrence in hearth soils only for one block and type unidentified 1 showed lower occurrence in hearths only for non-inoculated seedlings. Three morphotypes (type *Genea*, unidentified 3 and *Tomentella galzinii*) showed significantly higher occurrence in hearth soils for at least one block, while not presenting significantly lower occurrence in hearth soils for any block (Tables S4-S7).

Most soil properties measured were significantly altered in hearths when compared to non-hearth forest openings. Resistance to penetration, infiltration capacity, chroma and value were lower, whereas daily temperature range at 10 cm depth was higher. pH was also lower, but the change can barely be considered ecologically meaningful. Waterdrop penetration test values were always very low, indicating that hydrophobicity was not present in any sample. No significant effect on soil CO_2 efflux was found (Table 3; Tables S8-S10).

The plant community in the studied soils was dominated by herbs and subshrubs (Tables S12, S13). Plant cover, plant richness and root biomass of herbs and subshrub were significantly lower in hearth than non-hearth openings (Table 3; Table S8). Only the occurrence of *Thymus vulgaris* L and *Brachypodium retusum* (Pers.) Beauv. were consistently altered across blocks, with both species showing significantly lower occurrence in hearths (Tables S8, S11-S13).

The studied soils frequently supported roots from trees surrounding hearths or forest openings. Biomass of *Juniperus* fine roots was not significantly affected in hearths, whereas *Quercus* fine root biomass doubled in hearths, with no significant difference in specific root length (SRL: ratio fine root length to dry weight; Table 3; Table S8).

Discussion

Our results do not support the hypothesis that soil from historical charcoal hearths currently enhances development of *T. melanosporum* mycorrhizas. Reduced root colonisation by *T. melanosporum* points to lower soil receptiveness to *T. melanosporum* EM development. Reduced relative abundance in two of the three experimental blocks points to lower competitiveness against at least some native EM communities. Lower receptiveness and competitiveness is supported by the fact that in hearth soils root colonisation by the native EM community was not decreased by inoculation with *T. melanosporum*, whereas in non-hearth soils inoculated seedlings showed reduced colonisation by native EM communities. This situation could be related to a higher infectivity of native EM communities or to alteration of soil properties hampering *T. melanosporum* EM development. Thus, even if repeated soil heating associated with historic charcoal making most likely decreased soil EM infectivity, the hypothesised positive effect on *T. melanosporum* competitiveness was transitory or offset by edaphic factors.

The facts that (a) infectivity of EM communities of non-inoculated hearth soils was not significantly lower than that of non-inoculated non-hearth soils and (b) richness of morphotypes in hearth soils with no microwave treatment was not significantly lower than that of the corresponding non-hearth soils, despite the altered composition of the EM community, suggest that some fungi found propitious environmental conditions in hearths, with types unidentified 3, *Genea* and *T. galzinii* being likely candidates in our soils. The two former were not negatively affected by microwaves (type *Genea* was affected by the interaction between microwaves and inoculation, but in non-inoculated seedlings microwaves treatment did not show reduced occurrence of type *Genea*). Conversely, microwaves showed a negative effect on three of the four morphotypes that were negatively affected by hearth soils in at least some situations (types *Pisolithus*, unidentified 1 and unidentified 2).

The studied hearth soils consistently showed reduced resistance to penetration, larger temperature fluctuation and reduced occurrence of understorey species. These modifications are generally associated with *T. melanosporum* fruiting and could be supporting fruiting in abandoned charcoal hearths.

Resistance to penetration occurs as a result of soil compaction, which is commonly lower in highly productive *truffières* than in surrounding soils (Rebière 1981). Lulli et al. (1999) and Jaillard et al. (2014) showed this was related to changes in soil structure and improved air and water circulation. Its consequences on soil resistance to root and sporocarp growth are obvious. The reduced resistance to penetration of our hearth soils parallels reduced bulk density and increased porosity found by Mikan and Abrams (1995) in North American hearths.

Suppression of understorey is typical in *T. melanosporum*-producing soils as a consequence of fungal activity and is generally associated with changes in plant community composition (Ricard et al. 2003). According to Bragato (2014), this in turn induces changes in soil physical properties such as aggregate size, soil temperature range and water and air circulation. The herb and subshrub community in the studied hearths suffered an apparently similar suppression process, whichever the mechanisms involved, even including negative effects on species typically impaired in Spanish truffle-producing soils such as *T. vulgaris* and *B. retusum* (Garcia-Barreda et al. 2007).

Reduced infiltration capacity in our hearth soils apparently challenges the view that air and water circulation is higher in hearths. However, it could simply reflect a drastic change in soil surface characteristics, with lower soil aggregation and reduced abundance of shallow herbaceous roots hampering water transmission through soil surface (Horton 1941).

Soils that warm up easily, such as those with southern aspect or reduced canopy cover, are propitious for *T. melanosporum* fruiting (Olivier et al. 2012). This could be related to the

drastic change in soil environmental situation needed to induce fruiting, according to Pacioni et al. (2014), although it could also be related to reinforced freeze-thaw cycles reducing soil compaction (Bragato 2014). In our hearth soils increased temperature range was related to changes in soil colour, which are very likely linked to addition of charcoal fragments and reduced surface albedo (Criscuoli et al. 2014). However enhanced air circulation could also be partly responsible for increased temperature fluctuation.

The pH is a central parameter in truffle cultivation as an indicator of soil alkalinity, which in *T. melanosporum* soils generally correlates with soil carbonates and organic matter (Jaillard et al. 2014). In soils with low base saturation, charcoal making generally increases soil pH and alkalinity (Mikan and Abrams 1995). However in the studied hearths and in most truffle soils the exchange complex is usually saturated (Jaillard et al. 2014). Thus the scarce long-term effect of charcoal making on soil pH (once the short-term effect of ash incorporation has decayed) is not surprising.

Microbial biomass and activity have been reported to be lower in truffle-producing soils than in surrounding soils, thus relating to lower free organic matter content (size > 20 μm) and C:N ratios around 10, which are generally considered optimum for truffle cultivation (Ricard et al. 2003). On the other hand, Pacioni et al. (2014) found higher CO_2 efflux inside than outside *T. melanosporum brûlés* during the vegetative period, postulating that the increase was attributable to activity of roots and EM mycelia. In historical hearths, charcoal fragments do not seem to provide habitat for microorganism, although improvement of soil properties may enhance microbial activity (Quilliam et al. 2013). In our hearth soils CO_2 efflux was not significantly altered despite enrichment in charcoal fragments and altered conditions of temperature range, compaction and root biomass of trees and herbs, suggesting trade-offs among these factors. Interestingly the analysis of our soils showed a consistent and marked increase of C:N ratio in hearths, similar to that found by Hardy et al. (2016). In non-hearth

soils such a change would indicate high content of labile, fresh organic matter. In our hearths, it reflects the high C:N ratio of charcoal fragments, whose residence time in soil is much longer than non-hearth soil organic matter (Criscuoli et al. 2014).

Our results suggest that long-term soil alteration in historical charcoal hearths currently does not associate with improved development of *T. melanosporum* mycorrhizas. Whether this is due to increased EM infectivity of these soils linked to changes in the EM community composition or to alteration of soil properties hampering *T. melanosporum* EM development needs to be further evaluated. Although composition of the EM community was altered in hearths, our results do not clearly show a change in EM infectivity or richness of these communities with respect to the corresponding non-hearth soils.

On the other hand, long-lasting alteration of soil properties such as compaction, temperature fluctuation and occurrence of herb and subshrub species are typical features of wild *truffières*, suggesting that hearths feature improved soil environment for *T. melanosporum* fruiting.

After this first approach, further research is needed to understand which of the altered soil properties contribute significantly to improving fruiting. It would be interesting to test biochar in truffle cultivation techniques such as the so called “truffle nests” (Reyna and Colinas 2007), to lengthen lifespan of substrates while avoiding drastic increases of microbial populations and soil hydrophobicity, the latter being a frequent problem with customary peat-based substrates.

References

Agerer R (2002) Colour atlas of Ectomycorrhizae 1st-12th del. Eihorn-Verlag, Berlin

Bragato G (2014) Is truffle *brûle* a case of perturbational niche construction? For Syst 23:349–356. doi: 10.5424/Fs/2014232-04925

Chevalier G, Grente J (1979) Application pratique de la synthèse ectomycorhizienne: production à grande échelle de plants mycorhizés par la truffe. Mushroom Sci 10: 483-505

Criscuoli I, Alberti G, Baronti S, Favilli F, Martinez C, Calzolari C, Pusceddu E, Rumpel C, Viola R, Miglietta F (2014) Carbon sequestration and fertility after centennial time scale incorporation of charcoal into soil. PLoS One 9:1–11. doi: 10.1371/journal.pone.0091114

Garcia-Barreda S, Reyna S, Pérez-Badía R, Rodríguez-Barreal JA, Domínguez-Núñez JA (2007) Ecología de la trufa y las áreas truferas. In: Reyna S (ed) Truficultura: Fundamentos y técnicas. Ed. Mundi-Prensa, pp 153–208

Hardy B, Cornelis JT, Houben D, Lambert R, Dufey JE (2016) The effect of pre-industrial charcoal kilns on chemical properties of forest soil of Wallonia, Belgium. Eur J Soil Sci 67:206–216. doi: 10.1111/ejss.12324

Horton RE (1941) An approach toward a physical interpretation of infiltration-capacity. Soil Sci Soc Am J 5:399–417

Jaillard B, Barry-Etienne D, Colinas C, De Miguel AM, Genola L, Libre A, Neveu P, Oliach D, Saenz W, Saez M, Salducci X, Souche G, Sourzat P, Villeneuve M (2014) Alkalinity and structure of soils determine the truffle production in the Pyrenean Regions. For Syst 23:364–377. doi: 10.5424/fs/2014232-04933

Le Tacon F, Rubini A, Murat C, Riccioni C, Robin C, Belfiori B, Zeller B, De la Varga H, Akroume E, Deveau A, Martin F, Paolocci F (2016) Certainties and uncertainties about

the life cycle of the Périgord black truffle (*Tuber melanosporum* Vittad.). Ann For Sci 73: 105-117 doi: 10.1007/s13595-015-0461-1

Lulli L, Bragato G, Gardin L (1999) Occurrence of *Tuber melanosporum* in relation to soil surface layer properties and soil differentiation. Plant Soil 214:85–92. doi: 10.1023/A:1004602519974

Mikan CJ, Abrams MD (1995) Altered forest composition and soil properties of historic charcoal hearths in southeastern Pennsylvania. Can J For Res 25:687–696

Olivier JM, Savignac JC, Sourzat P (2012) Truffe et trufficulture, 3rd edn. Fanlac, Périgueux, France

Pacioni G, Leonardi M, Di Carlo P, Ranalli D, Zinni A, De Laurentiis G (2014) Instrumental monitoring of the birth and development of truffles in a *Tuber melanosporum* orchard. Mycorrhiza 24:65–72. doi: 10.1007/s00572-014-0561-z

Quilliam RS, Glanville HC, Wade SC, Jones DL (2013) Life in the "charosphere" - Does biochar in agricultural soil provide a significant habitat for microorganisms? Soil Biol Biochem 65:287–293. doi: 10.1016/j.soilbio.2013.06.004

Rebière J (1981) La truffe du Périgord, 2nd edn. Fanlac, Périgueux, France

Reyna S, Colinas C (2007) Truficultura. In: Reyna S (ed) Truficultura: Fundamentos y técnicas. Ed. Mundi-Prensa, Madrid, pp 235–276

Reyna S, De Miguel AM (2007) Las micorrizas. Ciclo biológico de la trufa. In: Reyna S (ed) Truficultura: Fundamentos y técnicas. Ed. Mundi-Prensa, Madrid, pp 106–150

Ricard JM, Bergougnoux F, Chevalier G, Olivier JM, Pargney JC, Sourzat P (2003) La truffe Guide pratique de trufficulture. Ctifl, Paris

Table 1 Effect of land use (LU), heat treatment (H) and block (B) on *T. melanosporum* percent root colonisation and relative abundance (least-square means and 95% confidence interval, n=26). Letters indicate significant differences among groups in pairwise t-test comparisons (α = 0.05). Occurrence of *T. melanosporum* was analysed only in inoculated seedlings because it was not found in any non-inoculated seedling.

			T. melanosporum percent root colonisation (%)[a]	*T. melanosporum* relative abundance (%)
LU×B[b]	Hearth		1.6 (0.5, 3.7)b	
		Albarracín		48.0 (30.4, 67.7)bc
		Formiche		2.7 (0, 20.4)c
		El Toro		8.0 (0, 23.8)c
	Non-hearth		7.8 (3.9, 14.8)a	
		Albarracín		100 (79.6, 100)a
		Formiche		58.6 (42.8, 74.4)ab
		El Toro		15.7 (0, 31.5)c
H×B	No heat treatment			
		Albarracín	0 (0, 3.4)k	0 (0, 25.0)k
		Formiche	0.6 (0, 3.7)k	3.3 (0, 20.9)k
		El Toro	3.3 (0.5, 11.1)jk	21.9 (4.2, 39.6)k
	Microwaves			
		Albarracín	33.0 (12.3, 85.9)i	98.4 (82.6, 100)i
		Formiche	12.9 (4.4, 34.4)ij	58.1 (42.3, 73.9)j
		El Toro	0.5 (0, 2.5)k	5.1 (0, 19.5)k

[a] Variable log-transformed

[b] Since LU×B interaction showed no significant effect on percent root colonisation, only main effects are presented for this response variable

Table 2 Effect of the interactions land use × heat treatment (LU×H) and inoculation × heat treatment (I×H) on fine root length and morphotype richness of bioassay seedlings (least-square means and 95% confidence interval, n=54). Letters indicate significant differences among groups in pairwise t-test comparisons ($\alpha = 0.05$).

		Fine root length (m)[a]	Morphotype richness
LU×H	Hearth		
	No heat treatment	102 (84, 125)a	2.8 (2.4, 3.1)a
	Microwaves	88 (74, 105)ab	0.9 (0.6, 1.3)b
	Non-hearth		
	No heat treatment	72 (58, 87)b	2.4 (2.0, 2.8)a
	Microwaves	96 (82, 113)a	1.0 (0.7, 1.3)b
I×H	Inoculated		
	No heat treatment	82 (67, 102)i	2.2 (1.8, 2.6)i
	Microwaves	104 (88, 123)i	0.8 (0.5, 1.1)j
	Not inoculated		
	No heat treatment	89 (74, 107)i	2.8 (2.5, 3.2)i
	Microwaves	82 (69, 97)i	1.1 (0.8, 1.5)j

[a] Variable log-transformed

Table 3 Soil properties and plant community characteristics in 18 historical charcoal hearths compared to control non-hearth forest openings (least-square means and 95% confidence interval). Asterisks represent significant differences from non-hearth soils according to the ANOVA (*P<0.05; **P<0.01; ***P<0.001). When the interaction between block and land use was significant, asterisks represent differences within blocks.

	Hearth	Non-hearth
Resistance to penetration (Pa)[a]	3.1 (2.7, 3.5)**	4.1 (3.6, 4.7)
Infiltration capacity (l/hour)[a]	85 (56, 130)**	230 (151, 350)
Waterdrop penetration time (sec)	<1	<1
Temperature daily range at 10 cm depth (ºC)	7.2 (2.7, 11.7)*	6.1 (1.6, 10.5)
Chroma (purity of colour)	1.4 (1.1, 1.7)***	3.1 (2.8, 3.4)
Value (lightness of colour)		
Albarracín	3.7 (2.9, 4.4)	3.8 (3.1, 4.6)
Formiche	3.3 (2.6, 4.1)	4.0 (3.3, 4.7)
El Toro	2.4 (1.6, 3.1)**	4.5 (3.8, 5.2)
pH	8.00 (7.92, 8.07)*	8.13 (8.06, 8.20)
CO_2 efflux (g C m^{-2} day^{-1})	0.51 (0.41, 0.61)	0.58 (0.42, 0.74)
Dry weight of *Quercus* fine root (g l^{-1})[a]	0.38 (0.23, 0.58)*	0.17 (0.09, 0.29)
SRL for *Quercus* fine root (m g^{-1})[a]	8.9 (6.5, 12.2)	10.8 (7.7, 15.1)
Dry weight of *Juniperus* fine root (g l^{-1})[a]	0.46 (0.24, 0.82)	0.46 (0.24, 0.83)
Dry weight of herbaceous and subshrub roots (g l^{-1})[a]	0.19 (0.13, 0.29)***	0.90 (0.59, 1.37)
Plant cover (%)	21.2 (13.4, 29.1)***	45.3 (37.5, 53.2)
Plant richness	3.3 (2.6, 4.1)***	6.1 (5.3, 6.8)

[a] Variable log-transformed

Electronic Supplementary Material

Table S1 Soils of the experimental blocks selected for the bioassays

	Albarracín hearth	Albarracín control	Formiche hearth	Formiche control	El Toro hearth	El Toro control
Sand (%)	46.9	33.4	82.4	68.3	55.6	53.2
Silt (%)	43.9	49.6	9.4	19.4	31.6	24.8
Clay (%)	9.2	17.0	8.2	12.3	12.8	22.0
Coarse elements (%)	36.6	43.5	26.4	54.2	22.7	14.6
pH (H_2O)	8.21	8.17	8.48	8.37	7.80	7.96
pH (KCl)	7.52	7.48	7.77	7.74	7.15	7.22
Conductivity ($\mu S\ cm^{-1}$)	165.9	170.9	104.1	127.5	153.8	101.4
Organic matter (%)	5.38	5.16	1.75	2.77	9.76	3.23
Total carbonate (%)	63	53	7	14	21	<5
Active carbonate (%)	13	11	2	5	5	<2
N (%)	0.19	0.32	0.07	0.13	0.30	0.20
Fe (ppm)	6582	7970	5416	5871	11219	6393
CEC ($meq\ kg^{-1}$)	241.7	255.5	68.7	104.3	363.3	186.4
P Olsen (ppm)	23.66	11.18	2.18	3.77	7.33	6.88
C:N ratio	16.2	9.5	14.2	11.9	19.0	9.3

Table S2 Methods used for soil determinations

Soil determination	Procedure
Resistance to penetration	Measured with the Scala dynamic cone penetrometer described in Vanags et al. (2004). Calculated as the mean of ten point measurements, separated at least 0.5 m from each other. In each point measurement the hammer was driven into the soil by four hammer blows from 40 cm height.
Infiltration capacity	Following USDA-NRCS (1999), with 15.5 cm diameter collars and 0.5 l water
Waterdrop penetration time	Following DeBano (1981)
Temperature at depth 10 cm	Measured with thermocouple in the morning (10-12 am) and evening (4.30-6.30 pm), in October 2010 and June 2011
Colour	Value and chroma, according to Munsell Colour System
pH	Measured in distilled water (1:2.5 soil/water ratio)
CO_2 efflux	Measured in the morning (10-12 am) and evening (4.30-6.30 pm) in June 2011 with the closed dynamic system EGM-4, SRC-1, PP-Systems, UK, with the dark chamber placed on PVC collars 15.5 cm diameter, 10 cm height, inserted 6 cm into the ground (Casals et al. 2009)
Plant cover, plant richness	Following the point-sampling method, using a 2 x 2 m quadrat centered in the hearth, with a 20 x 20 cm grid, 100 points
Dry weight of roots	Calculated from two cylindrical soil cores 10 cm depth, with a total of 200 ml volume
Fine root length (d < 2 mm)	Following Tennant (1975), using the roots from soil cores

References cited in Table S2

DeBano LF (1981) Water repellent soils: a state-of-the-art. US Department of Agriculture, Forest Service, General Technical Report PSW-46

Casals P, Gimeno C, Carrara A, Lopez-Sangil L, Sanz MJ (2009) Soil CO_2 efflux and extractable organic carbon fractions under simulated precipitation events in a Mediterranean Dehesa. Soil Biol Biochem 41:1915-1922

Tennant D (1975) A test of a modified line intersect method of estimating root length. J Ecol 63:995-1001

USDA-NRCS (1999) Soil quality test kit guide. US Department of Agriculture - Natural Resources Conservation Center, Soil Quality Institute, Ames, IA

Vanags C, Minasny B, McBratney AB (2004) The dynamic penetrometer for assessment of soil mechanical resistance. In: SuperSoil 2004 - 3rd Australian New Zealand Soil Conference, Sydney, 5 – 9 December 2004. University of Sydney (published on CD-rom)

Table S3 Description of the EM morphotypes

Morphotype	Colour	Mantle[a]	Emanating elements[b]
Cenococcum geophilum	Black	PL-type G	H: dark brown, thick-walled, non-ramified, non-clamped
Type *Genea*	Redish brown	PS-type L/K	C: yellowish-reddish, rigid, scarcely ramified, enlarged base, non-clamped
Type *Hebeloma-Cortinarius*	Whitish rose to brown	PL-type B	H: hyaline, ramified, enlarged in the septa, clamped, anastomising R: type A, hyaline, with fan-like connection to the mantle
Type *Pisolithus*	Golden-orange	PL-type B	H: yellowish brown, ramified, sometimes ribbon-like, clamped R: type B, brown, ramified, with inflated cells
Type *Scleroderma*	Pale yellow	PS-type A	H: hyaline-yellowish, ramified, enlarged in the septa, non-clamped R: type D, whitish-yellowish, with knot-like structures in ramifications
Tomentella galzinii	Yellowish to greenish brown	PS-type L	C: bristle-like, enlarged base, yellow below the first septa, clamped H: yellow, ramified, clamped R: type A, yellowish-greenish
Type *Tuber*	Orange to brown	PS-type M	No emanating elements

[a] PL: plectenchymatous, PS: pseudoparenchymatous (Agerer, 1987-2002)

[b] C: cystidia, H: hyphae, R: rhizomorph

Table S3 (continued)

Morphotype	Colour	Mantle[a]	Emanating elements[b]
Complex *Tuber albidum*	Yellow to brown	PS-type M	C: bristle-like, hyaline to pale yellow, thin, sometimes geniculate base
Tuber melanosporum	Orange to brown	PS-type M	C: yellowish-reddish, right angle-ramified, non-clamped
Unidentified 1	Whitish	PL-PS, type H	C: hyaline, long, thin, non-ramified, zig-zag shaped, non-septate
Unidentified 2	Greyish to whitish brown	Type H in young mycorrhizas, type P in the older, gelatinous matrix	H: scarce, hyaline, wide, covered by crystals, Y-shaped ramification, enlarged in or between the septa, sometimes with ring-like shapes, non-clamped, anastomising
Unidentified 3	Pale yellow to whitish	Type A, hyphae constricted at septum, gelatinous matrix	H: hyaline, scarcely ramified, covered by sticky matrix, scarcely septate, non-clamped
Unidentified 4	Pale yellow	Type H, gelatinous matrix	C: hyaline, long, rigid, non-ramified, with clamped and non-clamped septa H: hyaline-yellowish, scarcely ramified, ring-like shapes, clamped

[a] PL: plectenchymatous, PS: pseudoparenchymatous (Agerer, 1987-2002)

[b] C: cystidia, H: hyphae, R: rhizomorph

Table S4 Results of selected ANOVA models for bioassay seedling characteristics and *T. melanosporum* occurrence (F-value and model P-value, NI: variable not included in the selected model). PRC: percent root colonisation.

	Fine root length[a]	PRC by EM native fungi	Morphotype richness	PRC by *T. melanosporum*[a]	*T. melanosporum* relative abund.	PRC by type unidentified 3[a]	PRC by type *Genea*
Land use	1.2 (0.29)	2.1 (0.15)	2.2 (0.15)	11.0 (0.004)	30.2 (<0.001)	62.9 (<0.001)	10.0 (0.003)
Heat treatment	0.7 (0.40)	7.9 (0.007)	83.6 (<0.001)	6.0 (0.026)	20.6 (<0.001)	7.6 (<0.001)	1.8 (0.18)
Inoculation	1.0 (0.32)	4.6 (0.037)	11.6 (0.001)	-[b]	-[b]	NI	<0.1 (0.83)
Block	3.1 (0.054)	2.3 (0.11)	16.7 (<0.001)	6.2 (0.010)	25.4 (<0.001)	17.5 (<0.001)	10.1 (<0.001)
LU×H	6.6 (0.014)	NI	4.7 (0.036)	<0.1 (0.99)	0.7 (0.42)	NI	NI
LU×I	NI	7.9 (0.007)	NI	-	-	NI	NI
LU×B	NI	NI	1.9 (0.16)	2.1 (0.16)	4.5 (0.028)	7.9 (0.001)	15.2 (<0.001)
H×I	5.2 (0.028)	NI	4.1 (0.048)	-	-	NI	16.8 (<0.001)
H×B	NI	NI	NI	11.9 (<0.001)	22.1 (<0.001)	4.6 (0.015)	NI
I×B	2.9 (0.068)	NI	NI	-	-	NI	NI

[a] Variable log-transformed

[b] Occurrence of *T. melanosporum* was analysed only in inoculated seedlings.

Table S5 Effect of land use (LU), heat treatment (H), inoculation (I) and block (B) on percent root colonisation of types *Genea* and unidentified 3 (least-square means and 95% confidence interval, n = 54). Letters indicate significant differences among groups in pairwise t-test comparisons (α = 0.05). A: Albarracín, F: Formiche, T: El Toro, I: inoculated, ni: not inoculated.

	Unidentified 3[a]	Type *Genea*
LU×B		
Hearth	A: 0.6 (0, 1.8)c	A: 19.9 (15.2, 24.6)a
	F: 23.1 (13.3, 39.5)a	F: 0c
	T: 6.0 (3.2, 10.8)b	T: 4.8 (0.4, 9.3)b
Non-hearth	A: 0 (0, 0.7)c	A: 0c
	F: 0.7 (0.01, 1.9)c	F: 0c
	T:0 (0, 0.6)c	T:7.6 (3.4, 11.8)b
H×B, H×I		
No heat treatment	A: 0.7 (0, 1.9)bc	ni: 3.7 (0, 7.4)b
	F: 2.3 (0.8, 4.9)ab	I: 12.0 (7.8, 16.2)a
	T: 0.5 (0, 1.5)bc	
Microwaves	A: 0c	ni: 7.5 (4.0, 11.0)ab
	F: 8.0 (4.5, 13.7)a	I: 1.1 (0, 4.5)b
	T: 2.8 (1.4, 5.0)ab	

[a] Variable log-transformed

Table S6 Results of selected logistic regressions for the frequency of appearance of native EM morphotypes appearing in five or more samples (deviance and chi-square P-value, NI: variable not included in the selected model).

	Type *Pisolithus*	Complex *T. albidum*	Unidentified 2	Unidentified 1	*Tomentella galzinii*
Land use	12.0 (<0.001)	12.6 (<0.001)	0.4 (0.50)	1.7 (0.20)	8.8 (0.003)
Heat treatment	8.4 (0.004)	NI	16.3 (<0.001)	12.5 (<0.001)	14.6 (<0.001)
Inoculation	5.2 (0.022)	NI	1.3 (0.25)	<0.1 (0.91)	3.8 (0.051)
Block	10.6 (0.005)	8.1 (0.017)	0.2 (0.91)	18.6 (<0.001)	6.2 (0.046)
LU×H	NI	NI	NI	NI	NI
LU×I	3.5 (0.062)	NI	NI	6.0 (0.014)	NI
LU×B	NI	NI	14.4 (<0.001)	NI	NI
H×I	NI	NI	NI	NI	NI
H×B	NI	NI	NI	NI	NI
I×B	NI	NI	9.8 (0.007)	NI	NI

Table S7 Effect of land use, heat treatment and inoculation on appearance frequency of EM morphotypes present in five or more samples (predicted means). Letters indicate significant differences among treatments according to the logistic regression (Table S6) and pairwise t-tests (α = 0.05). A: Albarracín, F: Formiche, T: El Toro, I: inoculated, ni: not inoculated.

	Type *Pisolithus*	Complex *Tuber albidum*	Unidentified 2	Unidentified 1	*Tomentella galzinii*
Land use					
Hearth	0.08b	0b	A: 0b F: 0.25a T: 0.11ab	ni: 0b I: 0.15ab	0.19a
Nonhearth	0.43a	0.29a	A: 0.33a F: 0b T: 0.20ab	ni: 0.20a I: 0.15ab	0b
Heat treatment					
No heat treatment	0.39i	0.15	0.35i	0.26i	0.22i
Microwaves	0.16j	0.15	0j	0.03j	0j

Table S7 (continued)

	Type *Pisolithus*	Complex *Tuber albidum*	Unidentified 2	Unidentified 1	*Tomentella galzinii*
Inoculation					
Not inoculated	0.36p	0.13	A: 0.27pq F: 0q T: 0.33p	-[a]	0.11
Inoculated	0.15q	0.16	A: 0q F: 0.22pq T: 0q	-[a]	0.08

[a] Interaction effect shown in upper rows.

Table S8 Results of the ANOVAs of soil properties and plant community characteristics (F-value and model P-value). Specific soil cover was analysed only in plant species with appearance frequency higher than 0.33

	Block	Land use	Block × Land use
Resistance to penetration[a]	6.5 (0.004)	9.9 (0.003)	0.4 (0.70)
Infiltration capacity[a]	5.0 (0.013)	11.6 (0.002)	<0.1 (0.92)
Chroma	1.4 (0.25)	66.3 (<0.001)	0.9 (0.42)
Value	0.4 (0.65)	11.3 (0.002)	4.1 (0.027)
pH	10.2 (<0.001)	7.4 (0.010)	0.9 (0.42)
Dry weight of *Quercus* fine root[a]	6.3 (0.005)	5.2 (0.030)	1.6 (0.23)
SRL for *Quercus* fine root[a]	8.8 (0.001)	0.7 (0.41)	0.8 (0.45)
Dry weight of *Juniperus* fine root[a]	19.1 (<0.001)	<0.01 (0.98)	0.4 (0.70)
Dry weight of herbaceous and subshrub roots[a]	1.3 (0.30)	28.9 (>0.001)	1.4 (0.27)
Plant cover	2.0 (0.16)	19.5 (<0.001)	2.0 (0.15)
Plant richness	4.8 (0.015)	28.6 (<0.001)	1.7 (0.21)
Soil cover by *Festuca ovina*[a]	4.0 (0.028)	0.2 (0.70)	4.1 (0.027)
Soil cover by *Thymus vulgaris*[a]	4.2 (0.024)	6.0 (0.020)	0.2 (0.84)
Soil cover by *Helianthemum apenninum*	1.4 (0.26)	1.4 (0.24)	0.5 (0.64)
Soil cover by *Helianthemum marifolium*[a]	2.9 (0.072)	0.2 (0.65)	3.9 (0.030)

[a] Variable log-transformed

Table S9 Results of the linear mixed model for daily temperature range

	F-value (model P-value)
Land use	4.0 (0.050)
Season	12.4 (0.001)
Land use × Season	0.08 (0.77)

Table S10 Results of the linear mixed model for CO_2 efflux

	F-value (model P-value)
Land use	1.0 (0.33)
Time of day	0.4 (0.55)
Land use × Time of day	1.3 (0.25)

Table S11 Results of logistic regressions for the frequency of appearance of plant species that were present in at least five samples (deviance and chi-square P-value)

	Block	Land use	Block × Land use
Stipa gr. *offneri*	3.4 (0.19)	0.1 (0.70)	9.3 (0.010)
Brachypodium retusum	6.7 (0.035)	5.5 (0.019)	2.6 (0.28)
Odontites luteus	0.5 (0.78)	0.2 (0.63)	1.2 (0.54)

Table S12 Effect of land use on percent soil cover (%) of plant species with appearance frequency higher than 0.33 (least-square means and 95% confidence interval). Asterisks represent significant differences from control soils according to the ANOVA (*P<0.05; **P<0.01; ***P<0.001, Table S8). When the interaction between block and land use is significant, values for each block are depicted and asterisks represent differences within blocks. A: Albarracín, F: Formiche, T: El Toro.

	Hearth	Non-hearth
Festuca ovina[a]	A: 2.5 (0.6, 6.4)*	A: 9.5 (3.9, 21.4)
	F: 2.5 (0.7, 6.5)	F: 0.3 (0, 1.8)
	T: 1.7 (0.3, 4.8)	T: 2.5 (0.6, 6.4)
Thymus vulgaris[a]	0.7 (0.1, 1.5)*	2.3 (1.2, 3.9)
Helianthemum apenninum	1.1 (0.2, 2.0)	1.9 (0.9, 2.8)
Helianthemum marifolium[a]	A: 0 (0, 1.1)	A: 2.5 (0.6, 6.4)
	F: 4.5 (1.6, 10.7)	F: 1.4 (0.1, 4.1)
	T: 0.6 (0, 2.3)	T: 0.6 (0, 2.3)

[a] Variable log-transformed

Table S13 Predicted appearance frequency of plant species appearing in at least five samples. Asterisks represent significant differences from control soils according to the logistic regression (Table S11) and pairwise t-tests (*P<0.05; **P<0.01; ***P<0.001). When the interaction between block and land use is significant, asterisks represent differences within blocks. A: Albarracín, F: Formiche, T: El Toro.

	Hearth	Non-hearth
Stipa gr. *offneri*	A: 0.16*	A: 0.83
	F: 0.33	F: 0.17
	T: 0.33*	T: 0
Brachypodium retusum	0.06*	0.33
Odontites luteus	0.11	0.17