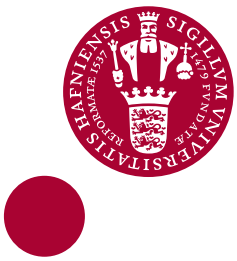

Functional diversity of ectomycorrhizal fungal communities is reduced by trace element contamination

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Title. Functional diversity of ectomycorrhizal fungal communities is reduced by trace element contamination

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Abstract

Trait-based approaches are useful tools to explain ecological assembly rules and ecosystem functioning. However, their use for soil microbiota has not been explored in depth yet. We explored trait-based functional changes of ectomycorrhizal (ECM) fungal communities associated with holm oak (*Quercus ilex* subsp. *ballota*) in a trace element contaminated area. We found a variation in ECM fungal species composition determined by soil C, Ca and trace elements; however, taxonomic diversity was not dependant on contamination level. Mean trait values of ECM fungal communities showed less rhizomorph and emanating hyphae production when increasing contamination, and the community converged towards species developing rhizomorphs less frequently. We suggest that trace elements in soils acted as the main environmental filter of trait diversity of ECM fungal communities. The effect of soil nutrients, i.e. soil C, affected the community mean trait values of emanating hyphae but did not cause a convergence in its distribution.

In summary, we found a reduction in the functional diversity of ECM fungal communities due to trace element contamination with potential to affect ecosystem functioning. This finding supports the potential of trait-based approaches to assess changes in the functional diversity of soil microbial communities.

Key words

Community assembly, Ectomycorrhizal fungi, *Quercus ilex* subsp. *ballota* (holm oak), Trace element contamination, Traits

1. Introduction

Trait-based approaches are excellent tools to disentangle community assembly rules and to link community composition, environmental changes and ecosystem functioning (Díaz & Cabido, 2001; Garnier et al., 2016; Lavorel et al., 2013). The basic principle of trait-based approaches relies on the use of functional traits of organisms, instead of mere species abundance counts, to describe emergent properties of ecosystems (Cadotte et al., 2011). Environmental constraints are known to affect the taxonomic diversity of communities by filtering the species according to their traits -i.e. response traits-, promoting the convergence of species with similar traits, in a process known as environmental filtering (Götzenberg et al., 2012). On the other hand, functional traits that have the potential to change ecosystem functioning are considered effect traits. The degree to which response and effect traits are interrelated determines the possible consequences of environmental filtering (Lavorel and Garnier, 2002).

In plant ecology, the links between plant traits and ecosystem functioning have been widely explored during recent decades (Díaz et al., 2007). Most studies have been focused on aboveground traits (Bardgett et al., 2014; Laliberté, 2016) and only more recently the "hidden" belowground plant functional diversity has started to be addressed (e.g. Bu et al., 2016; de la Riva et al., 2017; Gould et al., 2016). Indeed, the few studies addressing the belowground compartment of plant communities has, ranging from the level of organisms to that of ecosystems, highlighted the methodological potential for explaining ecosystem functioning (e.g. López-García et al., 2014; Mulder et al., 2005; Pelosi et al., 2014; Santorufo et al., 2015). Despite the growing interest, trait-based studies of soil organisms faces important challenges

especially due to the difficulties associated to the direct trait measurements of individual organisms, especially in the case of microbes (see Crowther et al., 2014).

Ectomycorrhizal (ECM) fungi are important components of terrestrial ecosystems: they are symbiotic nutrient suppliers of trees dominating in wide areas of the globe (van der Heijden et al., 2015). Their impact in ecosystems is not only limited to nutrient (mainly N) and water uptake from the soil, but they also participate in aspects of C cycling such as C sequestration (Clemmensen et al., 2013) and organic matter degradation (Tunlid et al., 2017). It has been suggested that their implications for ecosystem processes can be mediated by specific fungal traits which, in turn, are affected by environmental changes (Koide et al., 2014). In particular, the way in which ECM fungal species invest in morphological structures determines the hyphal exploratory capacity. Agerer (2001; 2006) distinguished four broad categories of exploration types: contact, short, medium and long distance, as a function of the morphology and development of emanating hyphae and rhizomorphs, i.e. specialised hyphal cords for long distance transport of water and nutrients, in the soil. The relative abundance of species with different exploration types is determined by the nutrient status of soils (Hobbie and Agerer, 2010; Moeller et al., 2014; Suz et al., 2014). Indeed, fungi exhibiting different exploration types usually harbour different enzyme activities (Tedersoo et al., 2012). Additionally, it has been suggested that ECM exploration type drives long term C sequestration due to differences in biomass production and turnover among them (Clemmensen et al., 2015; Koide et al., 2014). Another relevant trait with implications for ecosystem processes is the melanin content in cell walls, which is considered a protective trait against multiple abiotic stressors (Treseder and Lennon, 2015) such as enzymatic degradation (Rosas and Casadevall, 2000), salinity (Kogej et

al., 2006), water stress (Fernandez and Koide, 2013) and even ionising radiation (Cordero, 2017). Melanin content is inversely related to the decomposition rates of fungal necromass due to its recalcitrant nature (Fernandez and Koide, 2014), and thus it has the potential to influence C storage in soil, acting as an effect trait (Clemmensen et al., 2015). The morphological structure of ECM allows the characterisation of individual root tips that consists of single fungal species. Previous studies have attributed categorical trait information, usually extracted from databases, to each ECM fungal taxa (Aguilar-Trigueros et al., 2014; Kjøller et al., 2012) thereby ignoring the intraspecific variation and plasticity of these traits. As far as we know, only one recent study (Courty et al., 2016) has used direct trait characterisation of individual ECM root tips to develop a trait-based analysis. In that work, the authors demonstrated that extracellular enzyme traits at ECM fungal community level can be driven by the soil nutrient status.

Studies on ECM functional diversity have mainly focused on the impact of soil nutrient status and the natural succession of ECM fungal communities (Clemmensen et al., 2015; Kjøller et al., 2012; Moeller et al., 2014; Suz et al., 2014). However, the effect of trace elements, mainly heavy metals, on ECM fungal community composition and diversity has been scarcely studied and the results are controversial. Hui et al. (2011) and Op De Beeck et al. (2015) did not find any effect of heavy metal contamination on ECM taxonomic diversity but noted a shift in the species composition of their communities. In contrast, Sousa et al. (2014) found both, an effect on community composition and an increase in ECM fungal diversity in Cd-contaminated plots. However, Huang et al. (2012) did not find a clear effect of the contamination neither on community composition nor at the taxonomic richness level. Despite some influences on taxonomic diversity, there exists a gap of knowledge on how such kind of anthropogenic impact

affects the functional diversity of ECM fungal communities. Trace elements are likely to filter against the ECM fungal species spreading more intensively in soils (those producing emanating hyphae and/or rhizomorphs) due to an increased exposure to trace element toxicity (Pawlowska and Charvat, 2004). In addition, increased melanisation of ECM fungal communities would be expected as a consequence of the known protective effect of melanin against heavy metals (Gadd and Rome, 1988; Galli et al., 1994).

Here we determined hyphal exploration types and melanisation level as traits of ECM fungal species, molecularly identified, associated with holm oak (*Quercus ilex* subsp. *ballota*) in a restored trace element contaminated site (Guadiamar River valley, South of Spain). We quantified exploration type by microscopically confirming the presence of emanating hyphae and rhizomorphs on single ECM root tips. Our hypotheses were that: i) higher concentrations of trace element in soil reduce the taxonomic diversity of ECM fungal species and shifts the community composition; ii) there is an effect of trace element contamination on the community mean traits towards shorter exploration types and more melanised fungi; iii) we expect that trace element contamination reduces the trait dispersion in ECM fungal communities, since it acts as a filter of species according to their traits.

2. Material and Methods

2.1. Study area

In 1998 a mine spill contaminated 55 km^2 of the Guadiamar River valley, a traditional mining area in the south of Spain (Grimalt et al., 1999). The spilled acid water and sludge included a variety of trace elements, with high concentrations of several highly toxic heavy metals and

136 metalloids, such as As, Cu, Cd, Hg, Pb, S and Zn (Cabrera et al., 1999). During the following
137 months, the sludge and the upper layers of contaminated soil were mechanically removed, and
138 lime and organic amendments were added to immobilise remaining heavy metals. The stochastic
139 nature of the contamination event and the different broad remediation tasks caused the remaining
140 trace element concentrations in the soil to be unevenly distributed along the river corridor
141 (Burgos et al., 2008; Domínguez et al., 2016). The area was finally remediated and afforested
142 with autochthonous woody plant species, and legally protected as the Guadiamar Green Corridor
143 (Domínguez et al., 2008). Only two patches unaffected by the mine spill were included in the
144 reforestation program and planted with identical vegetation, one in the north of the dam
145 breakdown, to allow connection of the corridor with other natural areas, and one in the south of
146 the corridor, where an entire piece of land was expropriated including contaminated and non-
147 contaminated surface.
148
149 The affected area had two contrasting geologically-based zones (Northern and Southern), that
150 were remediated following the same criteria. Typical bedrock types at the Northern zone are
151 slate and schist, and it is characterised by the presence of naturally acidic soils. This zone
152 comprises the area with the highest soil pollution levels due to its proximity to the mining
153 activities. As a result of the remediation tasks, the soil structure was dramatically affected. The
154 geology at the Southern zone (further than 15 km from the tailings dam) is characterised by the
155 presence of limestone and calcarenite, with associated neutral to calcareous loam soils. Clean-up
156 operations in this zone included the removal of a fine layer of the polluted topsoils, less
157 aggressive in comparison to the clean-up of the Northern zone (Domínguez et al., 2016). Both
158 zones (northern and southern) shared a similar soil texture (see Table S1 for details and soil

159 classification). Climatic conditions are typical of a Mediterranean area with mild rainy winters
160 and warm dry summers. Average annual temperature is 19º C (minimum monthly mean of 9º C
161 in January, and maximum of 27º C in July) and annual average rainfall is 484 mm which define a
162 potential vegetation dominated by sclerophyllous Mediterranean forests with the ectomycorrhizal
163 holm oak (*Quercus ilex* subsp. *ballota*) as the most representative species. The area covered by
164 the toxic flood was agricultural, however patches of agro-forest (*Quercus ilex*) and natural
165 Mediterranean vegetation were closely distributed along the corridor ranging from hundreds of
166 meters to one km maximum distance.
167
168 *2.2. Sample design, collection and processing*
169 Four different areas were sampled: two acidic in the Northern zone, one affected by the mine
170 spill and the other unaffected, and two calcareous in the Southern zone, also affected and
171 unaffected by the mine spill (Supporting Information Fig. S1). The choice of these four sampling
172 sites made possible to construct a gradient of contamination availability due to different exposure
173 to contamination and the variability across sites (dependent on the original soil nature -slightly
174 acidic vs. calcic-), that makes harmful effects of contamination vary (as shown by Domínguez et
175 al. 2017). The selection of sites were also hampered by the low availability of sites in which
176 enough trees got established and had a similar spatial distribution (tree mortality rates were high
177 the first two years after plantation, see Domínguez et al., 2010).
178
179 Our sampling was focused in sampling and characterizing individual holm oaks due to its
180 constant presence all along the corridor and its representativeness of this dry Mediterranean
181 region. All trees had been planted at the same time and from similar seed provenance. Keeping

the host species constant, we could focus on the soil variability across the studied area, thus excluding other confounding factors such as plant host identity and age (Albornoz et al., 2016; Davey et al., 2015). Ten trees were randomly selected in each site (Supporting Information Fig. S1 and Table S1 for geographical coordinates). In April 2016, roots of trees were sampled by carefully tracing them from the stem of the tree in the four cardinal directions and ca. 200 g root material was collected from each direction, i.e. subsamples. Soil samples (0-20 cm depth) were taken with an auger from the four directions under each tree canopy projection, and were pooled to a total of 500 g to make a composite sample per tree.

2.3. Soil analyses

All soil samples were air-dried and sieved to <2 mm for physico-chemical analysis. Soil pH was measured in a 1:2.5 soil-water suspension after shaking for 30 min. Total C and N content was determined using a Flash HT Plus elemental analyser. Carbonate content was measured by the manometric method (Demolon and Leroux, 1952); soil organic C was then calculated as the difference between total C and the C contained in carbonates. Ammonium and nitrate were extracted by 1M KCl and determined by multiparametric Bran-Luebbe autoanalyser (Maynard et al., 2007). Olsen method (Olsen et al., 1954) was used for available P estimation in neutral and basic soils and Bray method was used in acidic soils (Bray et al., 1945). Available K, Ca and Mg were extracted with 1 M ammonium acetate and determined by atomic absorption spectrophotometry. Sulphur and pseudo-total trace element concentrations in soil samples (ground to <60 μm) were determined by digestion with aqua regia (1:3 v/v conc. HNO_3/HCl) in a Digiprep MS block digestor (SPS Science) equipped with a temperature-time programmable

204 controller and polypropylene digestion tubes. Trace elements in extracts were determined by
205 ICP-OES.
206
207 *2.4. Mycorrhizal determinations*
208 The seven longest root fragments in each of the four subsamples were selected to make a
209 composite sample of 28 fragments per tree. The extreme left mycorrhizal root tip of each root
210 fragment was photographed for further trait quantification (Supporting Information Methods S1)
211 and a small portion of each individual root tip was cut and immersed separately into 10 µl of
212 Extraction Solution (Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) for subsequent
213 molecular identification. Photographs of individual root tips were used to record the
214 presence/absence of emanating hyphae and rhizomorphs in each root tip. The colour of root tips
215 was assessed in the CMYB scale using ColorPick v. 3.0 (http://www.iconico.com/colorpic/; see
216 detailed description of methodology Supporting Information Methods S1) and the black colour
217 content annotated for each root tip (ranging from 0 to 1). The darkness of the root tips, or the
218 content in black colour, is directly related with the melanin content of fungi in accordance with
219 classical visual criteria used to differentiate between melanised and non-melanised fungi (e.g.
220 Fernandez et al., 2016). When applying our colorimetric approach to the photographs published
221 by Fernandez and Koide (2014), we found a high correlation between black colour and the
222 melanin contents quantified in that publication (Supporting Information Methods S1).
223
224 *2.5. Molecular analyses*
225 Tubes containing individual root tips and Extraction Solution were subjected to a heat shock
226 (95ºC for 10 min, 20ºC for 10 min) followed by the addition of 10 µl of Dilution Solution

(Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) and frozen until PCR setup. PCR amplification was carried out using 0.55 µl of DNA template with a Illustra PureTaq Ready-To-Go bead (GE Healthcare UK Limited, Buckinghamshire, UK) and 0.8 µM of primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) in a final volume of 25 µl. The thermocycling program was as follows: 3 min initial denaturation at 94°C; 35 cycles of 30 s denaturation at 94°C, 35 s annealing at 53°C and 1 min elongation (increased in 5 s each cycle) at 72°C; and a 4 min final elongation (as described by Suz et al., 2014). PCR products were purified using MEGAquick-spin (Intron Biotechnology, South Korea) and Sanger sequenced in the Unidad de Genómica y Síntesis de DNA, Instituto de Biomedicina y Parasitología López Neyra, CSIC (Granada, Spain). Sequence chromatograms were checked individually and those presenting double peaks, i.e. containing more than one fungal sequence, were discarded. In these cases a new root tip was picked up randomly from the root sample to ensure a minimum number of sequences per root sample. The remaining sequences were blasted against the UNITE database (Koljalg et al., 2005) and those found corresponding to ECM fungi were grouped by genera or family. Sequences in each taxonomical group were aligned separately using MAFFT v. 7 (Katoh and Standley, 2013) and clustered in MOTHUR v. 1.35.1 (Schloss et al., 2009) at a 97% cut-off to delimitated Operational Taxonomic Units (OTU). DNA sequences were compared against the UNITE database (Koljalg et al., 2005) for their taxonomic placement and Species Hypothesis determination. ECM fungal sequences were deposited in GenBank (http://www.ncbi.nlm.nih. gov/genbank/) under accession numbers MG273770-MG274263.

2.6. Data analyses

The whole analysis was based in the use of continuous data coming from the individual characterization of holm oak trees. For a broad characterization of study plots, a principal components analysis was carried out after log-transforming of trace element and soil variables. Differences in abiotic and biotic (i.e. ECM fungal traits) variables across plots were assessed by ANOVA after checking for normality and homoscedasticity. Tukey´s Honest was used as *post hoc* test. Non-normal variables were log or square root transformed. Variables that even when transformed were not normally distributed were analysed by non-parametric Kruskal Wallis test with pairwise Dunn test corrected using Bonferroni as *post hoc*.

The OTU abundance data matrix was constructed based on the number of root tips where each species was identified. A rarefaction analysis was carried out to ensure a high and even coverage of the total diversity of OTUs in each plot. The abundance matrix was Hellinger transformed for subsequent analyses (Legendre and Gallagher, 2001). Species richness (S), Chao1 and Simpson (1-D) indices were calculated as alpha diversity measures.

An OTU × trait matrix was constructed by calculating the frequency of emanating hyphae and rhizomorphs in the total root tips of each ECM fungal OTU. The black colour percentage was used as a proxy of melanin content and its value for each species was calculated as the average of the black component across all identified root tips per each OTU. To scale up from OTU to community level, all these traits were weighted by the relative abundance of each OTU to calculate community-weighted means (CWMs) of mycorrhizal traits for each tree (called fixed trait averages by Lepš et al., 2011).

A Variation Partitioning approach (Legendre and Legendre, 1998) was used to assess the influence of soil variables and trace elements on species (species-based RDA) and trait distribution (CWM-based RDA) (Kleyer et al., 2012). For that, every abiotic variable was log transformed, with the exception of pH, and the Hellinger transformed OTU matrix and the CWM matrix were used as response matrices for the species- and CWM-based RDAs, respectively. A previous selection of variables was carried out by stepwise model building for constrained ordination methods (Blanchet et al., 2008) with backward and forward selection to include important variables only. Since the objective of this analysis was to quantify the relative contribution to OTU and CWM distribution of soil background variables, trace elements and their shared covariation, the approach was applied separately for each group of soil factors (soil background variables and trace elements). For each subset of variables selected by the models, the variance inflation factors (VIF) were calculated (Gross, 2003), and variables above VIF=5 were removed. To control for the effect of spatial distribution of samples, principle coordinates of neighbour matrices (PCNM approach; Borcard and Legendre 2002) were calculated. The resulting PCNM axes were subjected to the same selection as described for soil and trace element variables, and those found to significantly influence the OTU or CWM distribution were selected. Every selected variable, either from soil, trace elements or spatial components, were feed to the variation partitioning analysis. To visualise the identified trends, an RDA ordination was carried out including all selected variables.

To assess the significance of each of the soil background variables and trace elements on fungal trait values, RLQ and fourth-corner analyses were performed (Legendre et al., 1997; Dray and Legendre, 2008). This method directly compares the three matrices: environmental, species

abundance and species traits. Effects were calculated using permutation model #6 with 9999 permutations, which is a combination of models #2 (permutes values of sites) and #4 (permutes values of species) which does not have an inflated type I error (Dray and Legendre, 2008; ter Braak et al., 2012). False discovery rate correction for multiple testing (Benjamini and Yekuteli, 2001) was applied.

In order to obtain insights into the rules governing ECM fungal community assembly, the trait distribution across OTUs in communities was compared with random expectations. For that, standardised effect size of mean pairwise distance (ses.mpd) between OTUs in each community was calculated by using the OTU abundance data matrix and a Euclidean trait distance matrix between OTUs. *Independent swap* algorithm was used to generate null communities (Gotelli, 2000). Ses.mpd varies from -1 to 1, where negative values mean trait convergence and positive values trait divergence. Relationships of ses.mpd with soil factors were checked by Pearson correlation applying a false discovery rate correction for multiple testing (Benjamini & Yekuteli, 2001).

All statistics were carried out in R software v 3.3.2 (R Development Core Team) using *vegan* (Oksanen et al., 2012), *picante* (Kembel et al., 2010) and *ade4* (Dray and Dufour, 2007) packages.

3. Results

3.1. Soil abiotic factors

317 The two sites affected by the mine spill (CN and CS) showed significantly higher values of most
318 of the measured pseudo-total trace element concentrations (As, Cd, Cu, Pb, S and Zn) in relation
319 to the non-affected sites (UN and US) (Table 1, Fig. 1). However, when looking at other soil
320 variables, the sites from the northern zone (CN and UN) had relatively similar values of pH, NH_4
321 and total N - more acidic and N-rich -, than those from the southern zone, CS and US (Table 1,
322 Fig. 1).
323
324 *3.2. ECM fungal community composition, taxonomic and functional diversity*
325 From a total of 1,120 sampled root tips, 494 produced successful PCR amplifications and were
326 identified as ECM fungal species. They were classified into 55 different OTUs belonging to 14
327 families and 19 genera (Supporting Information Table S2). There were two species which
328 dominated the communities: *Hebeloma cavipes* and *Thelephora terrestris*, representing 16.4%
329 and 12.3% of sequences, respectively. Most of the species occurred on less than two trees
330 (Supporting Information Table S2). Rarefaction analysis showed that for each site, most of the
331 OTU richness was recorded (Supporting Information Fig. S2). The mean number of ECM fungal
332 species per tree was 3.8, the estimated Chao richness was 4.9 species per tree, and the Simpson
333 dominance index averaged 0.6. For the three diversity measures there were no significant
334 differences between sites or contamination levels.
335
336 The frequencies of emanating hyphae and rhizomorphs across OTUs ranged from 0 to 100 %,
337 and melanisation from 64 to 94.7 % (Supporting Information Table S3). Among the three most
338 abundant families (Cortinariaceae, Russulaceae and Thelephoraceae), OTUs in the
339 Cortinariaceae family showed the lower variability in the three studied traits (emanating hyphae

(%): 66.6 to 100; rhizomorphs (%): 0 to 66.6; melanisation (%): 64.6 to 72.9). OTUs belonging to the other two dominant families were highly variable in terms of emanating hyphae and rhizomorphs (ranging 0 % to 100 %), while melanisation spanned in the range between 70 % and 94.7 %. The two most dominant species (*H. cavipes* and *T. terrestris*) had similar rhizomorph frequency and melanisation (around 12 % and 68 %, respectively), but *H. cavipes* showed emanating hyphae more frequently (95.1 %) than *T. terrestris* (88.5 %). The trait ranges exhibited by the detected ECM fungal species were congruent with the available descriptions of species and genera (Deemy database, see Supporting Information Table S3 for a comparison).

3.3. Effect of abiotic variables on ECM fungal community composition

According to the selected RDA models, the variables that best explained ECM fungal community variability (OTU matrix) were available Ca, organic C and total C among soil background variables, and Cu, Ni, S and Zn among trace elements (Fig. 2a; Supporting Information Table S4). Sulphur was removed from the subsequent analysis due to a high VIF result. Two PCNM axes were found to influence OTU community composition. The variation partitioning approach revealed that soil background variables and their covariation with trace element explained 8.36 and 0.55 %, respectively; meanwhile trace elements alone explained 3.82 % of variation in the model (Fig. 2a, pie chart). The spatial distribution of ECM communities explained a 2.06 % alone, and shared 2.86 % with soil background and trace element variables. There was no sign of collinearity between variables in the variation partitioning analysis. The two most abundant species, *H. cavipes* and *T. terrestris*, showed contrasting patterns regarding the trace element and Ca gradients, respectively, in the RDA ordination (Fig. 2a). *H. cavipes*

seemed to be related to lower concentrations of Cu, Zn and total C, and higher concentrations of Ni. *T. terrestris* appeared to be related with lower concentrations of Ca, as shown in Fig. 2a.

3.4. Effect of abiotic variables on ECM fungal community traits

The RLQ analysis showed a significant effect of the abiotic environment on the community composition by an interaction with species traits (model #2, $P = 0.006$; model #4, $P < 0.001$). Significant negative interactions were found between CWM of emanating hyphae and rhizomorphs and some trace elements and total C (displayed in Table 2). On the other hand, melanisation significantly interacted with $CaCO_3$.

The soil background variables that best explained CWM traits distribution included $CaCO_3$, total C, organic C and available P (Fig. 2b; Supporting Information Table S4), however, total C was removed from subsequent analysis due to a high VIF. On the other hand, among the trace elements, As, Cd and Cu best explained the variation of fungal community traits. Cu was finally removed due to a high VIF. No spatial variables (PCNM axes) were found to significantly explain any variation in trait distribution and were not included in the variation partition analysis. When partitioning the variation into trace element and soil background variables, trace elements explained 15.46% of the total variation, soil background 7.54% and their covariation 6.59% of the trait variability (Fig. 2b, pie chart). In agreement with the fourth corner analysis (Table 2), emanating hyphae and rhizomorphs appeared negatively related to trace element concentrations and organic C. Meanwhile, melanisation and $CaCO_3$ showed a clear positive covariation (Fig. 2b).

385 The analysis of trait distribution (ses.mpd values) across sites showed no differences among
386 them. The correlation of ses.mpd values of fungal traits with the selected variables in the RDA
387 models (As, Cd, $CaCO_3$, organic C and available P) showed that rhizomorph ses.mpd negatively
388 correlates with Cd concentration (Table 3), which means that the ECM species in communities
389 became more similar with increasing Cd concentration. No other significant correlations were
390 found, however emanating hyphae ses.mpd showed a similar magnitude in its positive
391 correlation coefficient with Cd (Table 3).
392

393 **4. Discussion**

394 Overall, our trait-based approach proved to be a highly useful tool to quantify potential effects of
395 an environmental disturbance on the functional diversity of natural microbial communities.
396 Firstly, because our trait measurements were consistent with the previous descriptions of species,
397 but because, in addition to the reliability, it allows for a numeric quantification of exploration-
398 type related traits and melanisation degree which was lacking in previous categorical
399 classifications. Furthermore, the analyses showed, as expected, an effect of trace element
400 contamination on the functional traits of ECM fungal communities.
401

402 *4.1. Effect of contamination on ECM fungal community diversity and structure*

403 Soil trace element contamination had no effect on ECM fungal richness. This fact has to be
404 discussed due to the inconsistency of previous results. Some authors did find a negative impact
405 of heavy metal contamination on ECM fungal diversity (Huang et al., 2014; Ruotsalanien et al.,
406 2009; Staudenrausch et al., 2005). In contrast, other studies missed such an effect, in agreement
407 with our results (see Hui et al., 2011; Op de Beeck et al., 2015). In our case, the relatively young

age of the trees, all of them planted only 17 years ago, could increase the chances that stochastic effects, i.e. priority effects, were acting on the community assembly of the ECM fungal communities. This fact would explain two results: on one hand, the relatively low ECM species richness (average of 3.8 species per tree) in comparison with previous studies in near mature Mediterranean forests (evergreen *Quercus suber*) which averaged 6.3 species per tree (Aponte et al., 2010). This trend is in agreement with the known increase in ECM species richness during ecosystem development as observed by Visser (1995) or Wallander et al. (2010). On the other hand, the effect of soil background variables and trace element contamination on the ECM fungal community composition was relatively low (a small percentage of variation in species composition was explained by these variables). This is consistent with a primary successional scenario where stochastic processes such as dispersal and/or priority effects drive the community assembly (Jumponen, 2003; Kennedy et al., 2009; Peay and Bruns, 2014) and thus blur the deterministic effects caused by soil factors, i.e. the proportion of community composition explained by the soil environment or its effect on species richness. Indeed, although low as well, a certain proportion of the variation of the OTU community composition was found to depend on the spatial position, which is a sign of a stochastic process influencing community assembly. On the other hand, other environmental factors not measured in this study, such as the relative influence of the seasonal river floods on different sites, could be responsible for the proportion of unexplained variance in community composition.

Despite the variance explained by soil factors being limited, soil background variables and trace elements explained a similar proportion of the variation in species composition. Previous studies of ECM fungal communities have shown the important influence of nutrient-related variables,

such as total C or organic C in soil, in the determination of ECM fungal community composition (Twieg et al., 2009). In our study, the two most frequent ECM species, *H. cavipes* and *T. terrestris*, were related to two independent abiotic gradients: *H. cavipes* to a trace element concentration gradient, and *T. terrestris* to a gradient in Ca concentration (likely related to the $CaCO_3$ and pH). This fact would explain why the variance in community composition was equally explained for each group of variables, as each of these groups explains the presence of one of the two most abundant ECM fungal species. Indeed, this result resembles the results by Op de Beeck et al. (2015) who also found that communities of ECM fungi were driven according to two environmental gradients: one responding to heavy metal contamination levels and the other driven by Fe, Mn, Mg and K.

4.2. Effect of contamination on mean fungal trait values

The effect of contamination was visible both in terms of the mean trait values of communities and the trait similarity across species in communities. Both rhizomorph and emanating hyphae frequency were found to be negatively associated with the concentration of some trace elements, which indicates a suppressive effect of the contamination on extramatrical mycelium growth. This effect has previously been found in controlled experiments, and varies across ECM fungal species (Qi et al., 2016). At the same time, the recorded patterns for the exploration-type related traits, particularly the relationship between emanating hyphae and total C, are also highly congruent with the known variation of exploration type in response to changes in N sources in the soil, i.e. a change from inorganic to organic N sources will reduce the development of extramatrical mycelium (Hobbie and Agerer, 2010; Lilleskov et al., 2002; 2011). Previous studies have pointed out the capacity of melanin to biosorb Cu and reduce its environmental

toxicity (Gadd and Rome, 1988), and that dark Ascomycota species usually are more resistant to heavy metal contamination than Basidiomycota (Likar and Regvar, 2013). The hypothesis that the degree of melanisation would increase with heavy metal concentration has to be rejected for this dataset since we did not record an increase in the black colour of ECM fungi present in contaminated sites compared with non-contaminated ones. The relationship between black colour of ECM fungal species and $CaCO_3$ could be the result of other biochemical interactions since melanin seems to be involved in the Ca^{2+} regulation of the cells (Bush et al., 2007). In the present study, the variation in CWM fungal traits explained by trace element concentrations doubled the variation explained by soil background variables. These effects were also independent of the spatial distribution of the samples, excluding any potential site effect in the results. This fact, together with the smaller overall variance explained in the case of the OTU matrix, highlights the interest of this trait-based approach to explain the consequences of trace element contamination on ECM fungal communities.

4.3. Ecological processes driving ECM fungal community assembly

The trait dispersion of species within communities was driven mainly by soil contamination and not by the nutrient status of the soil. The increase in Cd concentrations made species in ECM fungal communities become more similar in terms of presence of rhizomorphs. This reveals the potential environmental filtering that heavy metal contamination can have on the trait composition of ECM fungal communities. While species richness was similar across the studied sites, the increase in trait convergence indicates a reduction in the functional diversity of the community (Bässler et al., 2015) in response to soil contamination. Although we also found an average reduction in the emanating hyphae with increasing contamination levels, the tendency,

marginally significant, with increasing contamination was a divergence in the frequency of emanating hyphae produced by species in the same community. This is not in agreement with an environmental filtering, as suggested for rhizomorphs, but could indicate that competition between species is selecting species that differ in this trait. This could be explained by an interaction between the two traits: once the community has been filtered according to the production of rhizomorphs, the remaining subset of species is selected against biotic interactions, i.e. competition, as observed for example by Ingram and Shurin (2009).

The consequences of the reduction in the functional diversity of ECM fungal communities for plant and ecosystem functioning might depend on the specific traits affected. For ECM fungi it is known that the decomposition rate of their biomass is very dependent on melanin content and hyphal architecture (i.e. hydrophobic rhizomorphs versus hydrophilic feeder hyphae, Fernandez et al., 2016), which thus influences C storage in soil (Clemmensen et al., 2015). Additionally, these two traits also have an important role in water stress alleviation for plants (Fernandez and Koide, 2013), which may have important consequences for host fitness, particularly in Mediterranean environments.

4.4. Conclusions

In this study, we demonstrated that ECM functional traits correlated better with soil contamination than fungal taxonomic diversity or community structure. Thus, adding trait-based approaches to the description of ECM fungal communities facilitates a better understanding of the potential consequences of environmental degradation on ecosystem functioning. The often contradictory results of the effect of environmental impact on ECM fungal communities at the

500 species level, both in terms of community compositions and taxonomic diversity, can be
501 overcome by these functional approaches. However, more research is needed to show how the
502 community trait changes influences the functionality of ecosystems.
503

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514 **Competing interest statement**

515 The authors declare no competing interests.

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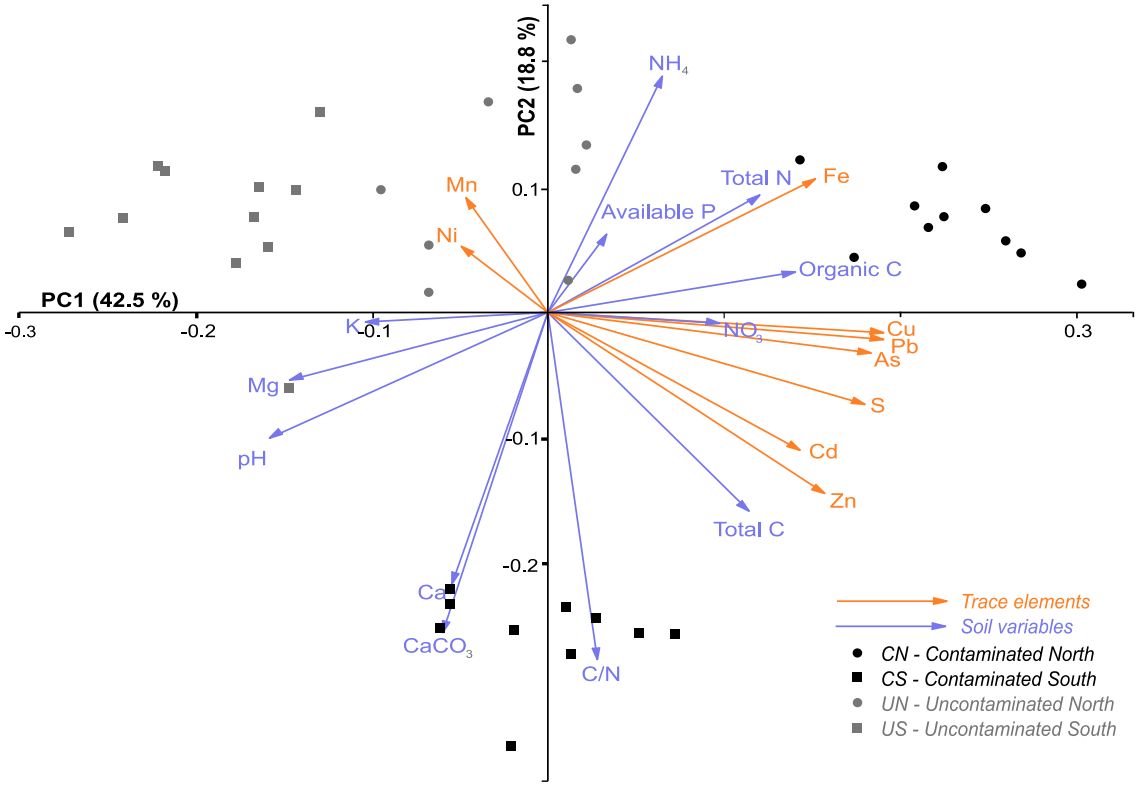
794 **Fig. 1**. Principal component analysis (PCA) of soil variables and trace elements in four locations
795 across the Guadiamar river valley (SW Spain) which differ in exposure to contamination by trace
796 elements and inherent soil background variables.



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801 **Fig. 2**. Redundancy analysis triplots of ectomycorrhizal (ECM) fungal communities driven by
802 trace element contamination and soil background variables in the Guadiamar river valley (SW
803 Spain). a) Species-based redundancy analysis (triplot) and variation partitioning analysis (pie
804 chart). Species present in less than 5% have not been represented. b) Trait Community Weighted
805 Mean (CWM)-based redundancy analysis (triplot) and variation partitioning analysis (pie chart).
806 The mean frequency of emanating hyphae, rhizomorphs and melanization (as a function of the
807 black color component) of ECM fungal communities are included in the analysis.

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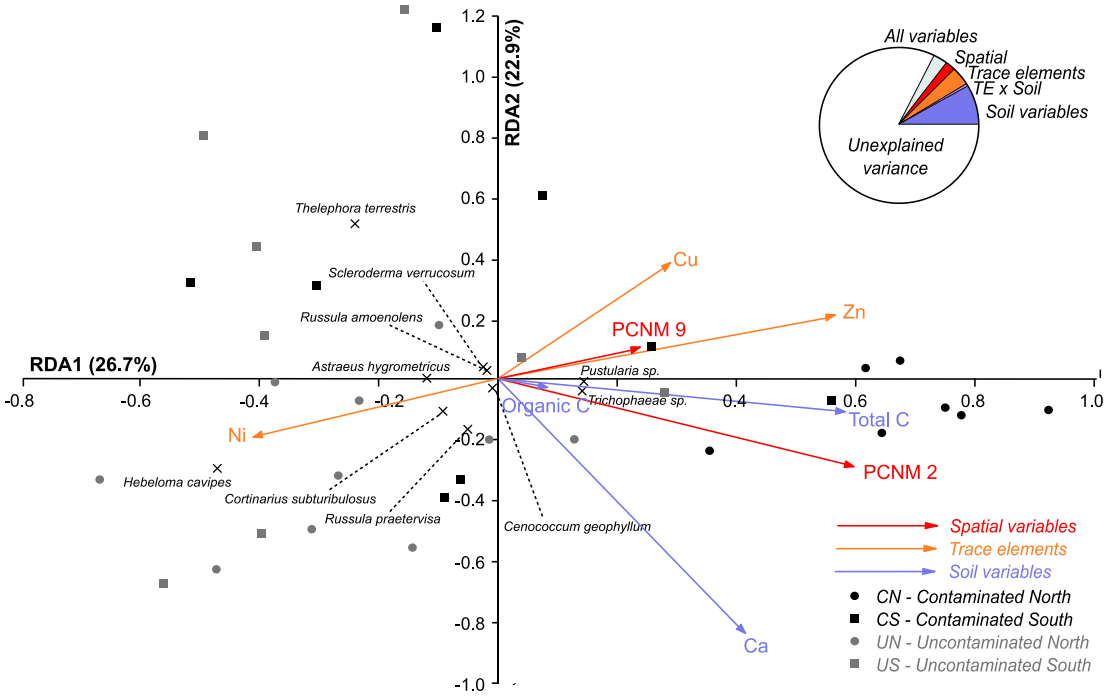
a) Species-based redundancy analysis (triplot) and variation partitioning analysis (pie chart)



b) Trait Community Weighted Mean (CWM)-based redundancy analysis (triplot) and variation partitioning analysis (pie chart)

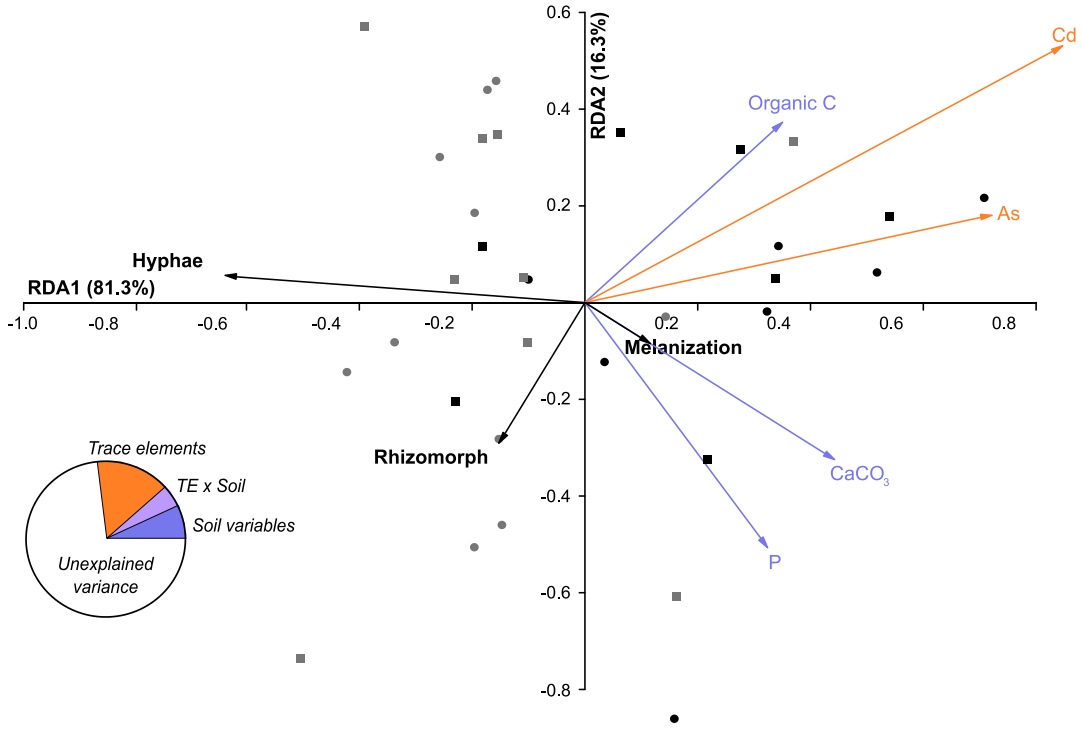


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Table 1. Mean values of soil variables (±SE) in the two studied plots affected and the two unaffected by the toxic mine spill of Guadiamar river (SW Spain). Contaminated north (CN) and south (CS), uncontaminated north (UN) and south (US). ANOVA analysis is displayed in last two columns (F and *P*). Means not sharing a letter in common differ significantly according to the Tukey´s Honest *post hoc*.

	Contaminated plots		Uncontaminated plots		ANOVA	
Soil variables	**CN**	**CS**	**UN**	**US**	F	*P* values
pH	4.84±0.23**c**	6.97±0.15**a**	6.26±0.13**b**	7.33±0.03**a**	51.48	**<0.001**
Ca (mg kg^{-1})	1,890±270**b**	4,890±90**a**	2,190±520**b**	3,240±410**b**	13.39	**<0.001**
K (mg kg^{-1})	139.16±18.33**b**	212.01±12.71**ab**	286.11±27.93**a**	235.92±18.39**a**	9.25	**<0.001**
Mg (mg kg^{-1})	97.02±8.27**c**	193.21±5.76**b**	203.99±29.82**b**	289.54±29.20**a**	15.68	**<0.001**
P (mg kg^{-1})	12.72±1.28	8.12±0.88	10.38±1.71	17.17±4.75	0.72	0.547
$CaCO_3$ (%)	0.55±0.06**c**	8.13±0.38**a**	1.20±0.13**b**	1.41±0.24**b**	133.1	**<0.001**
NH_4(mg kg^{-1})	4.77±0.34**a**	2.87±0.17**b**	5.07±0.57**a**	3.49±0.28**b**	10.55	**<0.001**
NO_3 (mg kg^{-1})	4.78±1.35**a**	2.49±0.45**a**	2.64±0.47**a**	1.21±0.19**b**	4.27	**0.011**
Total C (%)	1.72±0.16**b**	2.04±0.08**a**	1.56±0.13**b**	1.02±0.09**c**	11.93	**<0.001**
Total N (%)	0.16±0.02**a**	0.11±0.00**b**	0.15±0.01**a**	0.10±0.01**b**	10.65	**<0.001**
Total Trace Element						
As (mg kg^{-1})	161.83±21.71**a**	40.39±4.98**b**	18.03±1.27**c**	13.52±1.09**c**	97.26	**<0.001**
Cd (mg kg^{-1})	0.68±0.11**a**	0.67±0.07**a**	0.21±0.03**b**	0.02±0.01**c**	43.62	**<0.001**
Cu (mg kg^{-1})	192.55±7.82**a**	58.15±5.70**b**	40.54±4.46**b**	18.69±1.72**c**	211	**<0.001**
Fe (mg g^{-1})	40.48±2.14**a**	21.97±0.57**c**	27.52±0.93**ab**	22.80±1.50**bc**	27.57	**<0.001**
Mn (mg kg^{-1})	391.53±39.47**b**	414.78±15.41**b**	851.88±29.62**a**	486.40±47.77**b**	37.09	**<0.001**
Ni (mg kg^{-1})	13.01±0.70**b**	14.60±0.44**b**	21.69±1.04**a**	15.73±1.23**b**	17.44	**<0.001**
Pb (mg kg^{-1})	274.40±37.54**a**	76.66±8.26**b**	57.57±6.87**b**	19.82±1.14**c**	89.89	**<0.001**
S (mg g^{-1})	3.12±0.41**a**	0.71±0.09**b**	0.17±0.02**c**	0.10±0.01**c**	123.3	**<0.001**
Zn (mg kg^{-1})	228.99±29.61**a**	229.65±21.54**a**	96.93±9.25**b**	44.43±3.71**c**	66.39	**<0.001**

817 **Table 2**. Results of the fourth corner analysis of the relationships between ectomycorrhizal
818 fungal traits and soil factors in the Guadiamar river valley (SW Spain). The r values shown
819 indicate the strength of the interactions. Bold letter: P<0.10; *: *P*<0.05.

	Hyphae	Rhizomorph	Melanization
As	**-0.30***	-0.13	0.10
Cd	**-0.33***	**-0.27**	0.08
Cu	**-0.25**	-0.17	-0.06
Fe	-0.08	0.00	-0.26
Mn	0.14	0.10	-0.28
Ni	0.14	0.17	-0.27
Pb	**-0.28**	-0.19	-0.01
S	**-0.33***	-0.16	0.09
Zn	**-0.35***	-0.16	0.12
pH	0.06	0.12	0.25
$CaCO_3$	-0.19	0.00	**0.41***
K	0.05	0.03	0.13
Ca	-0.13	0.12	0.31
Mg	0.08	0.20	0.14
Total C	**-0.31***	-0.14	0.14
Organic C	-0.14	-0.12	-0.12
C/N	-0.13	-0.14	0.02
Total N	-0.12	-0.09	-0.18
NH_4	0.07	0.00	-0.18
NO_3	-0.23	**-0.25**	0.13
P	-0.12	0.16	-0.02

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822 **Table3**. Pearson correlation coefficients between trait distribution of ectomycorrhizal fungal
823 communities, as standardized effect size of mean pairwise distances of communities for each
824 fungal trait, and trace element concentrations and soil variables in the Guadiamar river valley
825 (SW Spain). Only the selected soil variables in the best trait-based RDA model were included. *:
826 $P<0.05$

	Emanating hyphae (ses.mpd)	**Rhizomorph (ses.mpd)**	**Melanization (ses.mpd)**
As	0.183	-0.250	-0.420
Cd	0.427	**-0.482***	-0.331
$CaCO_3$	0.104	0.111	0.095
Organic C	0.181	-0.222	-0.396
Available P	0.349	0.360	0.126

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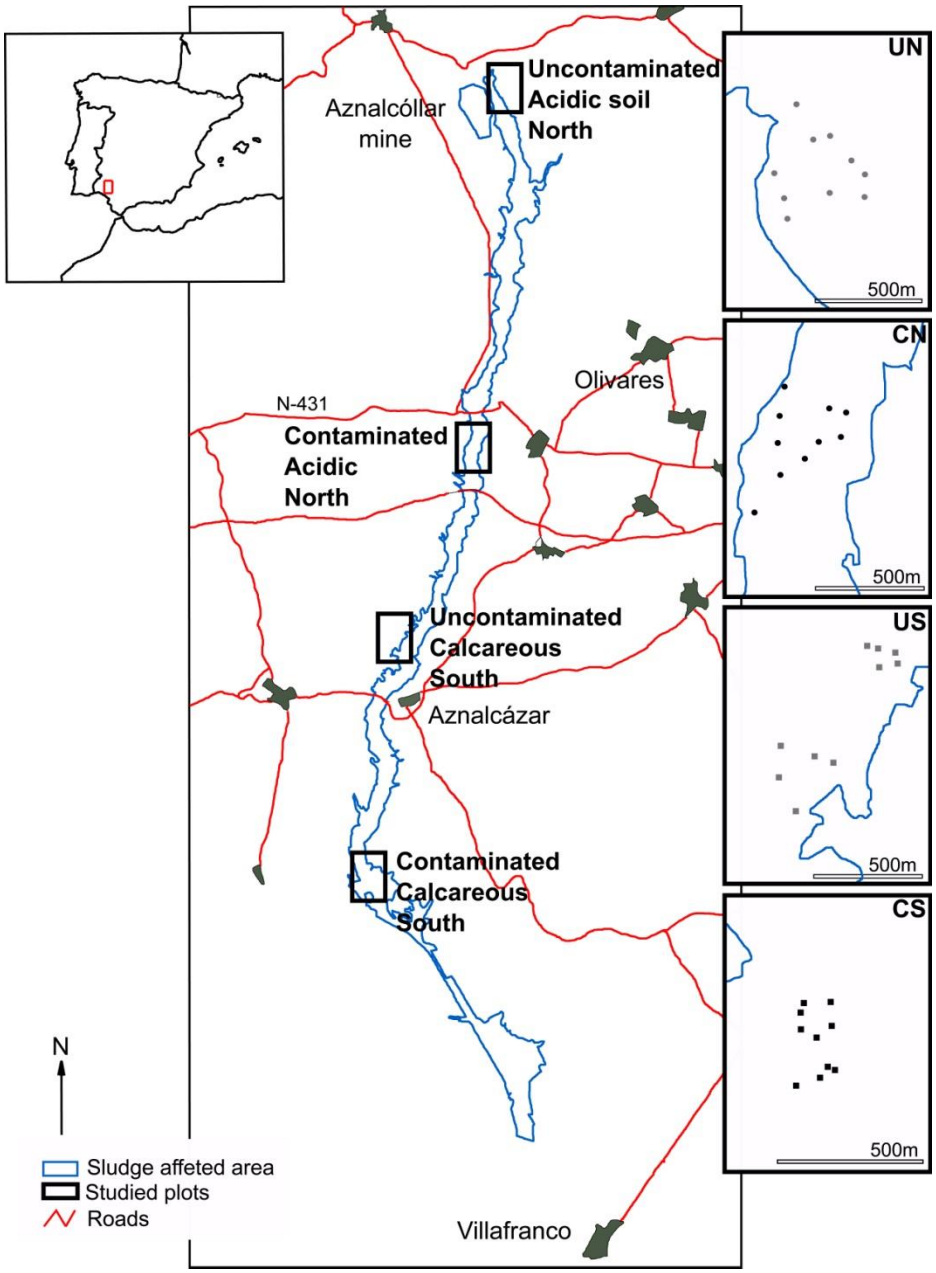
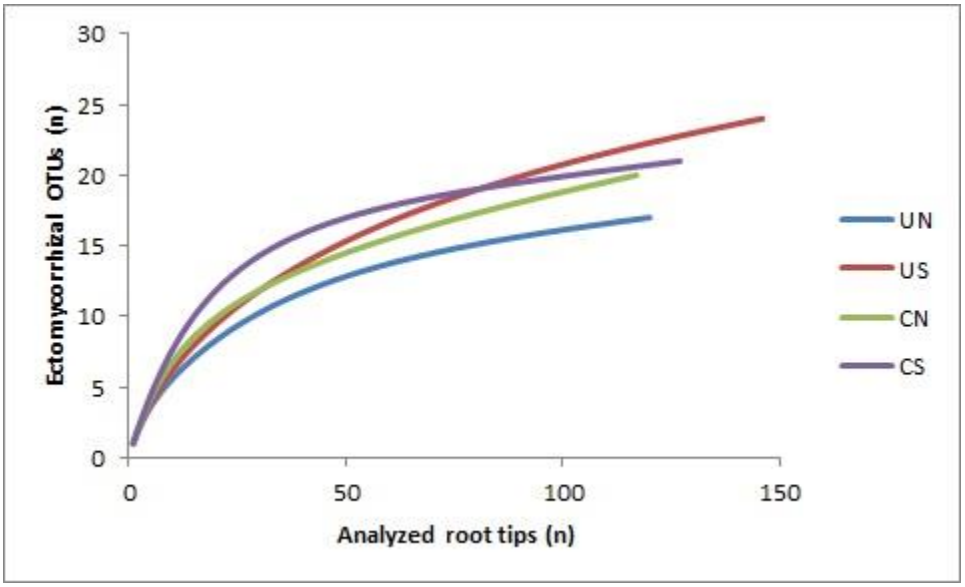
Supporting Information

Figure S1 Map of the study area and location of plots.



Supporting Information

Figure S2 Rarefaction analysis of OTU distribution in the analyzed ectomycorrhizal root tips from Guadiamar river valley (SW Spain). Contaminated North (CN), Contaminated South (CS), Uncontaminated North (UN), Uncontaminated South (US).



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Supporting Information

Table S1. Overall distribution of texture components in the sampled plots (data from Domínguez pers. comm.) and soil type classification (according to Clemente et al. 2000) in the four sample sites in Guadiamar river valley (SW Spain). Geographic locations of specific sampled trees. Contaminated north (CN) and south (CS), uncontaminated north (UN) and south (US).

	CN	CS	UN	US
Coarse sand (%)	30.1	24.5	31.6	24.8
Fine sand (%)	21.6	15.2	16.2	27.2
Silt (%)	27.8	33.3	31.2	24.1
Clay (%)	20.4	27	21.1	22.7
Soil type	Typic/Aquic Xerofluvent	Aquic Haploxeralf	Typic Xerofluvent	Typic Rhodoxeralf/ Typic Haploxeralf
UTM coordinates of sampled trees				
	37.386733,-6.226050	37.242796,-6.262997	37.501699,-6.223200	37.326128,-6.254079
	37.385683,-6.226283	37.242426,-6.263540	37.500837,-6.222986	37.326017,-6.253461
	37.384788,-6.228140	37.243197,-6.264157	37.501934,-6.220785	37.325835,-6.252575
	37.385500,-6.227400	37.242692,-6.264152	37.501747,-6.218971	37.325364,-6.252395
	37.387800,-6.229283	37.241216,-6.263381	37.502676,-6.218921	37.321194,-6.255822
	37.386588,-6.229400	37.240979,-6.264334	37.503267,-6.219647	37.321470,-6.256862
	37.385405,-6.229497	37.241460,-6.262851	37.504298,-6.220750	37.321916,-6.258642
	37.384155,-6.229326	37.241546,-6.263120	37.504149,-6.221652	37.320483,-6.258804
	37.382667,-6.229817	37.243488,-6.264055	37.505631,-6.222518	37.319079,-6.257846

Reference

Clemente, L., Cabrera, F., García, L.V., Cara, J.S. 2000. Reconocimiento de suelos y estudio de su contaminación por metales pesados en el valle del Guadiamar. Edafología, 7-3, 337-349.

852 **Supporting Information**

853 **Table S2.** Species list found in the study (Guadiamar river valley, SW Spain). Number of root tips
854 identified in each plot (Contaminated North - CN, Contaminated South - CS, Uncontaminated North -
855 UN, Uncontaminated South - US). Number of trees in which they were detected (Occurence). Blast
856 results against the UNITE database and Species Hypothesis (SH) (only matches higher than 97% are
857 shown).

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859

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
Ascomycota										
	Gloniaceae	*Cenococcum geophyllum*			4	10	3	Uncultured ectomycorrhiza (*Cenococcum geophilum*) (AY299214)	99	SH214459.07FU
	Pyronemataceae	*Geopora cervina*		9			2	Uncultured *Geopora* (GU327416)	99	SH213655.07FU
	Pyronemataceae	*Geopora* sp.		1			1	*Geopora* sp. (UDB011007)	97	SH213666.07FU
	Pezizaceae	*Peziza michelii*		5		1	3	*Peziza michelii* (JF908553)	98	SH218195.07FU
	Pezizaceae	*Peziza* sp.	1				1	*Peziza* sp. (KP311474)	99	SH189857.07FU
	Pyronemataceae	*Pustularia* sp.	3	8		2	4	Uncultured Ascomycete (EU557319)	99	SH222141.07FU
	Pyronemataceae	Pyronemataceae sp. 1			1		1	Uncultured fungus (JF927116)	93	SH213666.07FU
	Pyronemataceae	Pyronemataceae sp. 2		4			1	Uncultured fungus (KM247654)	99	-
	Pyronemataceae	Pyronemataceae sp. 3				4	1	Uncultured ectomycorrhizal fungus (FJ008039)	99	SH025866.07FU
	Pyronemataceae	*Trichophaeae* sp.	2	7		4	5	Uncultured Pyronemataceae sp. (HM370456)	97	SH215396.07FU
	Tuberaceae	*Tuber oligospermum*				1	1	*Tuber oligospermum* (FM205504)	97	SH188863.07FU
	Tuberaceae	*Tuber* sp. 1		1			2	*Tuber* sp. (KC517481)	95	-
	Tuberaceae	*Tuber* sp. 2		3			1	Uncultured *Tuber* (HQ204754)	96	-
	Tuberaceae	*Tuberaceae* sp. 1	1		7		1	Uncultured ectomycorrhizal fungus (HM057200)	92	SH185378.07FU
Basidiomycota										
	Diplocystidiaceae	*Astraeus hygrometricus*	3		5	1	4	*Astraeus hygrometricus* (HG000293)	99	SH190454.07FU
	Cortinariaceae	*Cortinarius belleri*			5		2	*Cortinarius belleri* (AY669685)	99	SH188471.07FU
	Cortinariaceae	*Cortinarius subbalaustinus*				6	3	Uncultured *Cortinarius* (GU246986)	99	SH188517.07FU
Phylum	**Family**	**Species**	**CN**	**CS**	**UN**	**US**	**Occurrence**	**Closest match (Acc. No.)**	**Identity (%)**	**Species Hypothesis (UNITE)**
	Cortinariaceae	*Cortinarius*			3	24	5	Uncultured mycorrhizal fungus (FJ897182)	100	SH188545.07FU

		subturibulosus								
	Entolomataceae	*Entoloma inusitatum*	7				3	Uncultured Entolomaceae (FJ210729)	99	SH181020.07FU
	Cortinariaceae	*Hebeloma cavipes*	10		26	45	19	*Hebeloma cavipes* (KT225477)	100	SH215994.07FU
	Cortinariaceae	*Hebeloma cistophilum*				3	1	Uncultured fungus clone (HQ625447)	99	SH218875.07FU
	Strophariaceae	*Hymenogaster griseus*		1			1	*Hymenogaster griseus* (AF325636)	99	SH218859.07FU
	Inocybaceae	*Inocybe curvipes*	1		3		3	Inocybe cf. curvipes (KT275613)	97	SH201231.07FU
	Inocybaceae	*Inocybe griseovelata*		6			2	Inocybe griseovelata (JF908237)	97	SH176687.07FU
	Inocybaceae	*Inocybe jacobi*	1				1	Inocybe jacobi (HQ604812)	99	SH211892.07FU
	Inocybaceae	*Inocybe praetervisa*				1	1	Inocybe sp. (KM576438)	98	SH212066.07FU
	Inocybaceae	*Inocybe squamata*				1	1	*Inocybe squamata* (FJ904136)	99	SH222043.07FU
	Hydnangiaceae	*Laccaria laccata*	4		3		3	*Laccaria laccata* (KM067883)	100	SH220959.07FU
	Russulaceae	*Lactarius* sp. 1	1				1	*Lactarius atlanticus* (KR025612)	96	-
	Russulaceae	*Lactarius* sp. 2	1				1	*Lactarius atlanticus* (KP420216)	95	-
	Paxillaceae	*Melanogaster vittadinii*				1	1	Melanogaster vittadinii (AJ555525)	97	SH182656.07FU
	Sclerodermataceae	*Pisolithus arhizus*			1		1	*Pisolithus arhizus* (FR748128)	98	SH177625.07FU
	Sclerodermataceae	*Pisolithus tinctorius*			5	3	2	*Pisolithus tinctorius* (HE578142)	99	SH177623.07FU
	Russulaceae	*Russula amoenolens*	19		1	2	5	Russulaceae (KT334781)	99	SH220816.07FU
	Russulaceae	*Russula ilicis*			9		1	Uncultured Russulaceae (HQ330996)	99	SH180269.07FU
	Russulaceae	*Russula insignis*		9			3	*Russula insignis* (AY061700)	98	SH220848.07FU
	Russulaceae	*Russula praetervisa*	10	5	2	16	5	Uncultured *Russula* (FR852096)	97	SH202443.07FU
Phylum	**Family**	**Species**	**CN**	**CS**	**UN**	**US**	**Occurrence**	**Closest match (Acc. No.)**	**Identity (%)**	**Species Hypothesis (UNITE)**

	Russulaceae	*Russula* sp.				1	1	Uncultured *Russula* (KT334781)	95	-
	Sclerodermataceae	*Scleroderma cepa*	4				1	*Scleroderma laeve* (KP004932)	99	SH182463.07FU
	Sclerodermataceae	*Scleroderma meridionale*			1		1	*Scleroderma meridionale* (HF933239)	100	SH186878.07FU
	Sclerodermataceae	*Scleroderma* sp. 1			1		1	Uncultured fungus (FM999606)	95	SH179758.07FU
	Sclerodermataceae	*Scleroderma verrucosum*	13	3		1	6	Uncultured fungus (KM247623)	99	SH182460.07FU
	Thelephoraceae	*Thelephora terrestris*	14		42	5	12	Uncultured *Thelephora terrestris* (KF007266)	99	SH184510.07FU
	Thelephoraceae	*Tomentella castanea*	20				1	*Tomentella* cf. *sublilacina* (KU376404)	100	SH184517.07FU
	Thelephoraceae	*Tomentella ferruginea*		8			1	Uncultured fungus clone (KM247776)	99	SH184518.07FU
	Thelephoraceae	*Tomentella lilacinogrisea*				3	1	Uncultured fungus clone (KF297246)	99	SH178628.07FU
	Thelephoraceae	*Tomentella* sp. 1		1			1	Uncultured fungus clone (KM247736)	99	-
	Thelephoraceae	*Tomentella* sp. 10		1			1	Uncultured *Tomentella* (FJ197002)	96	-
	Thelephoraceae	*Tomentella* sp. 2				7	1	Uncultured fungus clone (KM247732)	99	SH177905.07FU
	Thelephoraceae	*Tomentella* sp. 3				1	1	Uncultured *Tomentella* (FJ210771)	99	SH184642.07FU
	Thelephoraceae	*Tomentella* sp. 4		4			1	Uncultured *Tomentella* (JX630358)	97	SH184626.07FU
	Thelephoraceae	*Tomentella* sp. 5		10			1	Uncultured *Tomentella* (LC013836)	98	-
	Thelephoraceae	*Tomentella* sp. 6	1	15			3	Uncultured fungus (FN397409)	99	SH177879.07FU
	Thelephoraceae	*Tomentella* sp. 8	1	13			2	Uncultured *Tomentella* (FR852207)	99	SH002639.07FU
	Thelephoraceae	*Tomentella* sp. 9				1	1	Uncultured *Tomentella* (KC840637)	99	SH177797.07FU

860

861

Supporting Information

Table S3. Fungal trait measurements in the current study (Guadiamar river valley, SW Spain) and comparison with records of Deemy database (http://www.deemy.de). The experimental observations are expressed in term of frequency (percentage) of number of root tips exhibiting either emanating hyphae or rhizomorphs, and the black color content (0-100) of root tips for melanization. The records of species in this study are compared with the records of the same species in Deemy database (01-10-2017) when available (marked with asterisk). When the species was not recorded in Deemy, records from species of the same genera were displayed. The percentage of records showing different the different categories was shown. NA: absence of data; Distance Exploration types: Contact, Short, Medium mat, Medium fringe and Medium smooth (Agerer 2001, 2006); Emanating hyphae and rhizomorphs: Absent, Infrequent and Abundant. The n column is the number of root tips found for each species.

				Experimental observations			Deemy database		
Phylum	**Family**	**Species**	**n**	**Emanating hyphae**	**Rhizomorphs**	**Melanization**	**Exploration type**	**Emanating hyphae**	**Rhizomorph presence**
Ascomycota									
	Gloniaceae	*Cenococcum geophyllum**	14	100	0	90.5	Short	Abundant	Absent
	Pyronemataceae	*Geopora cervina*	9	11.1	22.2	81.1	NA	NA	NA
	Pyronemataceae	*Geopora* sp.	1	100	0	84	NA	NA	NA
	Pezizaceae	*Peziza michelii*	6	33.3	16.7	82.1	NA	NA	NA
	Pezizaceae	*Peziza* sp.	1	0	0	87.7	NA	NA	NA
	Pyronemataceae	*Pustularia* sp.	13	53.8	0	76.2	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 1	1	0	0	80.3	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 2	4	75	0	85.7	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 3	4	75	0	87.6	NA	NA	NA
	Pyronemataceae	*Trichophaeae* sp.	13	84.6	7.7	83.2	NA	NA	NA
	Tuberaceae	*Tuber oligospermum*	1	100	100	84.7	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6
	Tuberaceae	*Tuber* sp. 1	1	100	0	64	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6

Phylum	Family	Species	n	Experimental observations			Deemy database		
				Emanating hyphae	Rhizomorphs	Melanization	Exploration type	Emanating hyphae	Rhizomorph presence
	Tuberaceae	*Tuber* sp. 2	3	0	0	84.7	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6
	Tuberaceae	*Tuberaceae* sp. 1	8	12.5	0	77	NA	NA	NA
Basidiomycota									
	Diplocystidiaceae	*Astraeus hygrometricus*	9	44.4	44.4	79.3	NA	NA	NA
	Cortinariaceae	*Cortinarius belleri**	5	100	0	64.6	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Cortinariaceae	*Cortinarius subbalaustinus**	6	66.6	66.6	64.8	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Cortinariaceae	*Cortinarius subturibulosus**	27	96.3	25.9	72.9	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Entolomataceae	*Entoloma inusitatum**	7	42.9	0	68	Medium smooth	Abundant 33.3/ Infrequent 33.3/ Absent 33.3	Abundant 33/ Infrequent 66
	Cortinariaceae	*Hebeloma cavipes*	81	95.1	13.6	67.7	Short 87.5/ Medium 12.5	Abundant	Abundant 12.5/ Absent 87.5
	Cortinariaceae	*Hebeloma cistophilum*	3	100	0	69.7	Short 87.5/ Medium 12.5	Abundant	Abundant 12.5/ Absent 87.5
	Strophariaceae	*Hymenogaster griseus*	1	100	0	77.3	NA	NA	NA
	Inocybaceae	*Inocybe curvipes*	4	50	0	64.1	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe griseovelata*	6	66.7	0	71	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe jacobi*	1	100	0	76	Short	Abundant40/ Infrequent 60	Absent

				Experimental observations			Deemy database		
Phylum	**Family**	**Species**	**n**	**Emanating hyphae**	**Rhizomorphs**	**Melanization**	**Exploration type**	**Emanating hyphae**	**Rhizomorph presence**
	Inocybaceae	*Inocybe praetervisa*	1	100	0	93.3	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe squamata*	1	100	0	70.7	Short	Abundant40/ Infrequent 60	Absent
	Hydnangiaceae	*Laccaria laccata*	7	71.4	14.3	71.4	Medium smooth	Abundant 87.5/ Infrequent 62.5	Abundant 12.4/ Infrequent 37.5/ Absent 62.5
	Russulaceae	*Lactarius* sp. 1	1	0	0	79	Contact 35.7/ Medium smooth 64.3	Absent 56.4/ Infrequent 48.7	Abundant 2.4/ Infrequent 64.3/ Absent 33.3
	Russulaceae	*Lactarius* sp. 2	1	100	0	75.3	Contact 35.7/ Medium smooth 64.3	Absent 56.4/ Infrequent 48.7	Abundant 2.4/ Infrequent 64.3/ Absent 33.3
	Paxillaceae	*Melanogaster vittadinii*	1	100	100	85.5	Long	Infrequent	Abundant
	Sclerodermataceae	*Pisolithus arhizus*	1	0	0	77	NA	Infrequent	Abundant 50/ Infrequent 50
	Sclerodermataceae	*Pisolithus tinctorius**	8	75	37.5	78.2	NA	Infrequent	Infrequent
	Russulaceae	*Russula amoenolens**	22	36.4	4.5	70.7	Short 50/ Medium smooth 50	Infrequent	Infrequent
	Russulaceae	*Russula ilicis*	9	55.5	33.3	72.4	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8
	Russulaceae	*Russula insignis**	9	55.5	0	82.1	Short	Infrequent	Absent
	Russulaceae	*Russula praetervisa*	33	44.8	17.2	72.9	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8

				Experimental observations			Deemy database		
Phylum	**Family**	**Species**	**n**	**Emanating hyphae**	**Rhizomorphs**	**Melanization**	**Exploration type**	**Emanating hyphae**	**Rhizomorph presence**
	Russulaceae	*Russula* sp.	1	100	100	71.3	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8
	Sclerodermataceae	*Scleroderma cepa*	4	75	0	71.9	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma meridionale*	1	100	0	72.3	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma* sp. 1	1	100	0	69.7	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma verrucosum*	17	94.1	41.2	73.3	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Thelephoraceae	*Thelephora terrestris**	61	88.5	11.5	69.8	Medium smooth	Infrequent	Abundant 50.0/ Infrequent 50.0
	Thelephoraceae	*Tomentella castanea*	20	25	0	84.2	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella ferruginea*	8	62.5	62.5	86.8	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella lilacinogrisea*	3	100	66.7	83.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 1	1	100	0	86.3	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 10	1	100	100	94.7	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 2	7	57.1	14.3	83.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 3	1	0	0	94.3	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 4	4	100	0	92.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 5	10	100	30	82.4	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 6	16	32.5	6.3	79.7	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1

				Experimental observations			Deemy database		
Phylum	**Family**	**Species**	**n**	**Emanating hyphae**	**Rhizomorphs**	**Melanization**	**Exploration type**	**Emanating hyphae**	**Rhizomorph presence**
	Thelephoraceae	*Tomentella* sp. 8	14	35.7	14.3	90.4	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 9	1	100	0	84	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1

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Supporting Information

Table S4 Forward selection of environmental variables for improving redundancy analysis of factors driving ectomycorrhizal community assembly in the Guadiamar river valley (SW Spain).

Species-based redundancy model

Trace elements	Df	AIC	F	Pr(>F)
S	1	-3.6989	2.3380	0.005 **
Ni	1	-4.3694	1.7278	0.005 **
Zn	1	-3.8652	2.1855	0.005 **
Cu	1	-3.2891	2.7170	0.005 **
Soil Background Variables				
Ca	1	-4.1287	2.9858	0.005 **
Organic C	1	-4.6259	2.5075	0.005 **
Total C	1	-4.0746	3.0383	0.005 **

CWM-based redundancy model

Trace elements				
Cu	1	200.87	6.8637	0.010 **
As	1	201.55	7.6055	0.010 **
Cd	1	203.38	9.7030	0.005 **
Soil Background Variables				
CaCO3	1	199.88	6.0131	0.010 **
Organic C	1	202.55	8.8758	0.005 **
Total C	1	204.94	11.6547	0.005 **
P	1	196.62	2.8140	0.090 .

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Supporting Information

Supporting Information Methods S1.

ECM fungal trait determinations

The seven longest root fragments were selected from each root subsample. This made a total of 28 root fragments per tree. Root tips were selected randomly by choosing the extreme left of each root fragment. Each root tip was photographed in triplicates with a digital camera (Nikon DS-Fi1) fitted on a dissecting microscope. Two general pictures (25X magnification) on white and black background, and one detailed picture (100X magnification) on black background were taken, keeping light conditions at maximum and photograph exposition at 1/10s for the general pictures and 1/4s for the detailed one (Fig. 1a-c). Three fungal traits – rhizomorphs, emanating hyphae and melanisation – were measured, as follows.

Rhizomorphs

The presence of rhizomorphs was recorded in the 25X magnified photographs. The presence of rhizomorphs was recorded for a root tip if a rhizomorph emerging from the cluster to which the selected root tip belongs was found (Fig. 1d-h). This procedure was chosen because rhizomorphs are less frequent than individual emanating hypha in a random root tip; however, individual root tips often are part of a bigger cluster of root tips of the same individual fungus.

Emanating hyphae

Emanating hyphae was determined at 100X magnification on black background photographs. The presence of emanating hyphae was recorded when hyphae appeared continuous and homogeneously distributed in the root tip surface (Fig. 1 l-n). However, when only individual, isolated, hyphae appeared, root tips were scored as having no emanating hyphae (Fig. 1 i-k).

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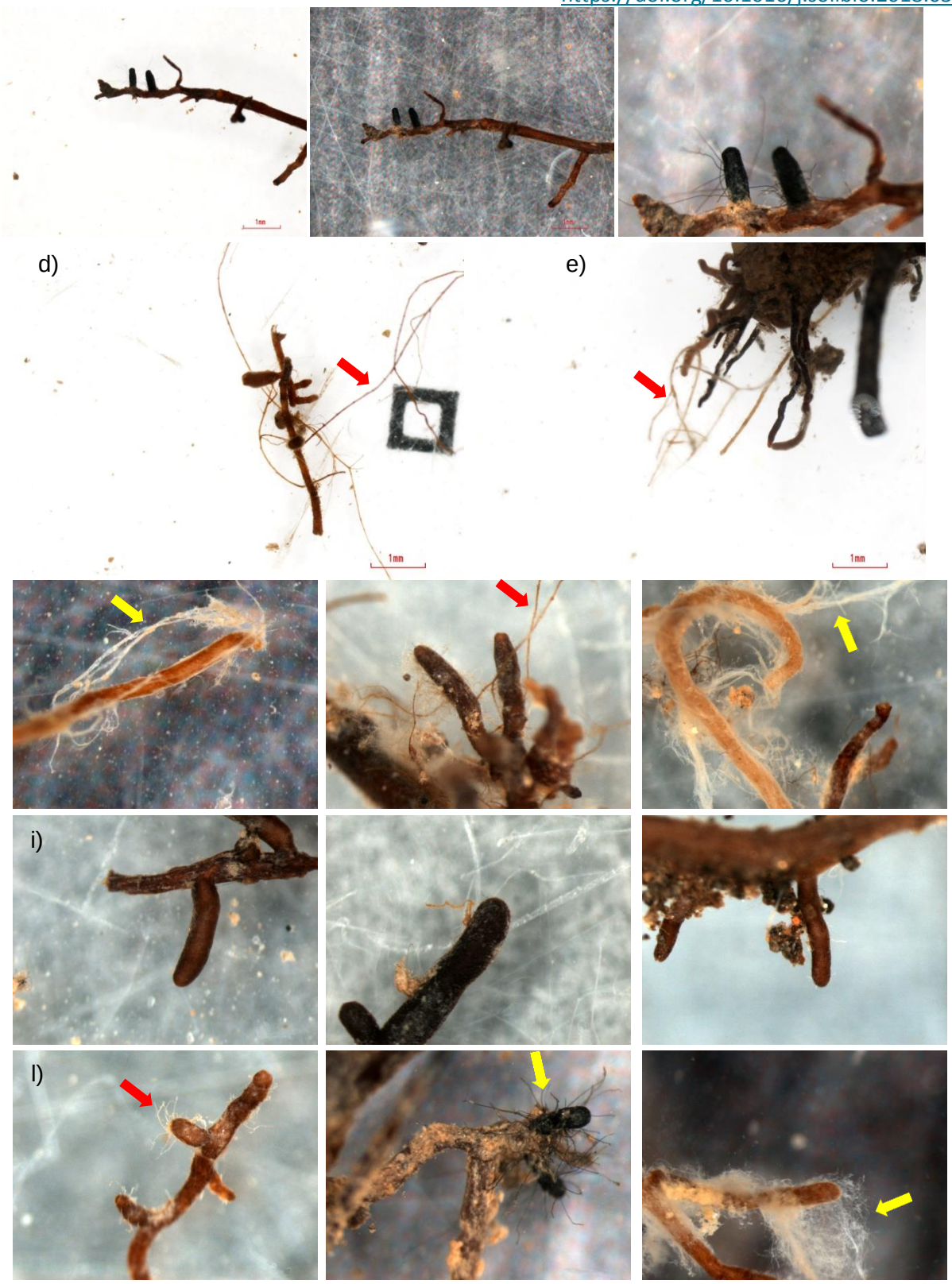

Fig. 1. Examples of photographs showing root tips with different fungal traits. a-c) *Cenococcum geophylum* root tips at 25X magnification (a, b) and 100X magnification (c); d-e) clusters of root tips with associated rhizomorphs (25X magnification); f-h) detailed of root tips showing rhizomorphs (100X magnification); i-k) root tips with no emanating hyphae (100X magnification); l-n) root tips showing different morphologies of emanating hyphae (100X magnification). The contrast of these pictures has been automatically improved to facilitate the visibility of fungal structures in this slide.

Melanisation

The colour of root tips was assessed with the CMYB scale using ColorPick v. 3.0 (http://www.iconico.com/colorpic/). The CMYB scale decomposes colours in cyan, magenta, yellow and black components. Hence, the black colour content is annotated ranging from 0, when completely white, to 1, when completely black. Three locations per root tip were selected (as shown in Fig. 2) and the content in black annotated by clicking with the mouse. The final colour of a root tip was the average number of the three records in each root tip.

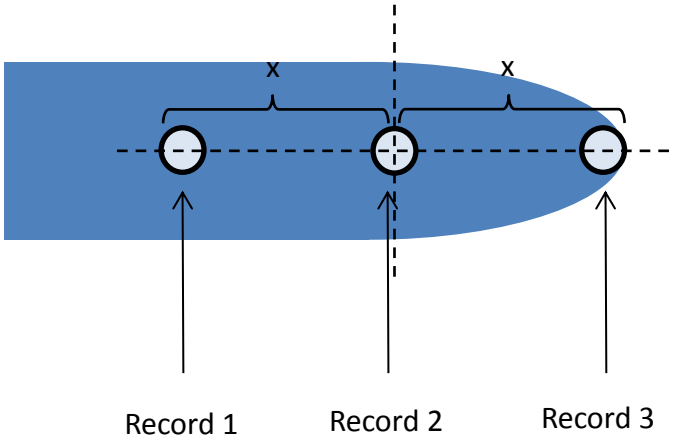

Fig. 2. Schematic diagram of the location of the three points for colour recording in ECM root tips.

The darkness of the root tips, or the content in black colour, is directly related with the melanin content of fungi, in accordance with classical visual criteria used to differentiate between melanised and non-melanised fungi (Fernández *et al*., 2016). Chand *et al*. (2014), for instance, classified fungi as white, mixed and black, and found that the melanin content was related to this classification. We applied our colorimetric approach to the photographs published by Fernandez & Koide (2014) by recording the colour in three random locations of each photograph. We found a good correlation between black colour and melanin contents measured in that publication (Fig. 3).

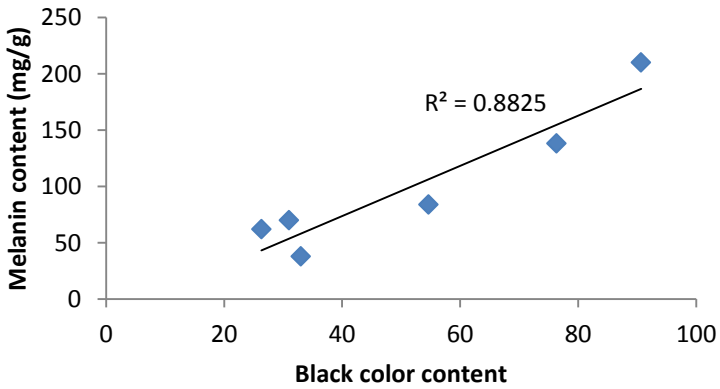

Fig. 3. Relationship between melanin content and black colour of fungal mycelia. The analysis corresponds to the photographs and melanin contents published by Fernandez & Koide (2014).

Calculation of species trait values

The frequency of emanating hyphae and rhizomorphs of each ectomycorrhizal fungal species was calculated as the proportion of root tips showing those traits in the whole study. Thus:

$$Trait\ value = \frac{n_i}{N_i}$$

where n_i is the number of root tips with either emanating hyphae or rhizomorphs of the i-th species and N_i is the total number of root tips belonging to i-th species in the whole study. It resembles the fixed trait value described in Lepš *et al*. (2011) which is independent from the habitat conditions where the species is found.

Melanisation was calculated as the mean value of black colour content across all root tips belonging to a species. Thus:

$$Melanisation = \frac{\sum_{j=1}^{N_i} black_{ij}}{N_i}$$

where $black_{ij}$ is the colour content of i-th species in j-th root tip and N_i is the total number of root tips belonging to i-th species in the whole study. It is the fixed trait value described by Lepš *et al*. (2011).

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