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

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

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21

24 **Abstract**

25 Trait-based approaches are useful tools to explain ecological assembly rules and ecosystem

26 functioning. However, their use for soil microbiota has not been explored in depth yet. We

27 explored trait-based functional changes of ectomycorrhizal (ECM) fungal communities

28 associated with holm oak (*Quercus ilex* subsp. *ballota*) in a trace element contaminated area.

29 We found a variation in ECM fungal species composition determined by soil C, Ca and trace

30 elements; however, taxonomic diversity was not dependant on contamination level. Mean trait

31 values of ECM fungal communities showed less rhizomorph and emanating hyphae production

32 when increasing contamination, and the community converged towards species developing

33 rhizomorphs less frequently. We suggest that trace elements in soils acted as the main

34 environmental filter of trait diversity of ECM fungal communities. The effect of soil nutrients,

35 i.e. soil C, affected the community mean trait values of emanating hyphae but did not cause a

36 convergence in its distribution.

37 In summary, we found a reduction in the functional diversity of ECM fungal communities due to

38 trace element contamination with potential to affect ecosystem functioning. This finding supports

39 the potential of trait-based approaches to assess changes in the functional diversity of soil

40 microbial communities.

41

45 **1. Introduction**

46 Trait-based approaches are excellent tools to disentangle community assembly rules and to link

47 community composition, environmental changes and ecosystem functioning (Díaz & Cabido,

48 2001; Garnier et al., 2016; Lavorel et al., 2013). The basic principle of trait-based approaches

49 relies on the use of functional traits of organisms, instead of mere species abundance counts, to

50 describe emergent properties of ecosystems (Cadotte et al., 2011). Environmental constraints are

51 known to affect the taxonomic diversity of communities by filtering the species according to

52 their traits -i.e. response traits-, promoting the convergence of species with similar traits, in a

53 process known as environmental filtering (Götzenberg et al., 2012). On the other hand,

54 functional traits that have the potential to change ecosystem functioning are considered effect

55 traits. The degree to which response and effect traits are interrelated determines the possible

56 consequences of environmental filtering (Lavorel and Garnier, 2002).

57

58 In plant ecology, the links between plant traits and ecosystem functioning have been widely

59 explored during recent decades (Díaz et al., 2007). Most studies have been focused on

60 aboveground traits (Bardgett et al., 2014; Laliberté, 2016) and only more recently the "hidden"

61 belowground plant functional diversity has started to be addressed (e.g. Bu et al., 2016; de la

62 Riva et al., 2017; Gould et al., 2016). Indeed, the few studies addressing the belowground

63 compartment of plant communities has, ranging from the level of organisms to that of

64 ecosystems, highlighted the methodological potential for explaining ecosystem functioning (e.g.

65 López-García et al., 2014; Mulder et al., 2005; Pelosi et al., 2014; Santorufo et al., 2015).

66 Despite the growing interest, trait-based studies of soil organisms faces important challenges

67   especially due to the difficulties associated to the direct trait measurements of individual

68   organisms, especially in the case of microbes (see Crowther et al., 2014).

69

70   Ectomycorrhizal (ECM) fungi are important components of terrestrial ecosystems: they are

71   symbiotic nutrient suppliers of trees dominating in wide areas of the globe (van der Heijden et

72   al., 2015). Their impact in ecosystems is not only limited to nutrient (mainly N) and water uptake

73   from the soil, but they also participate in aspects of C cycling such as C sequestration

74   (Clemmensen et al., 2013) and organic matter degradation (Tunlid et al., 2017). It has been

75   suggested that their implications for ecosystem processes can be mediated by specific fungal

76   traits which, in turn, are affected by environmental changes (Koide et al., 2014). In particular, the

77   way in which ECM fungal species invest in morphological structures determines the hyphal

78   exploratory capacity. Agerer (2001; 2006) distinguished four broad categories of exploration

79   types: contact, short, medium and long distance, as a function of the morphology and

80   development of emanating hyphae and rhizomorphs, i.e. specialised hyphal cords for long

81   distance transport of water and nutrients, in the soil. The relative abundance of species with

82   different exploration types is determined by the nutrient status of soils (Hobbie and Agerer,

83   2010; Moeller et al., 2014; Suz et al., 2014). Indeed, fungi exhibiting different exploration types

84   usually harbour different enzyme activities (Tedersoo et al., 2012). Additionally, it has been

85   suggested that ECM exploration type drives long term C sequestration due to differences in

86   biomass production and turnover among them (Clemmensen et al., 2015; Koide et al., 2014).

87   Another relevant trait with implications for ecosystem processes is the melanin content in cell

88   walls, which is considered a protective trait against multiple abiotic stressors (Treseder and

89   Lennon, 2015) such as enzymatic degradation (Rosas and Casadevall, 2000), salinity (Kogej et

90   al., 2006), water stress (Fernandez and Koide, 2013) and even ionising radiation (Cordero,

91   2017). Melanin content is inversely related to the decomposition rates of fungal necromass due

92   to its recalcitrant nature (Fernandez and Koide, 2014), and thus it has the potential to influence C

93   storage in soil, acting as an effect trait (Clemmensen et al., 2015). The morphological structure

94   of ECM allows the characterisation of individual root tips that consists of single fungal species.

95   Previous studies have attributed categorical trait information, usually extracted from databases,

96   to each ECM fungal taxa (Aguilar-Trigueros et al., 2014; Kjøller et al., 2012) thereby ignoring

97   the intraspecific variation and plasticity of these traits. As far as we know, only one recent study

98   (Courty et al., 2016) has used direct trait characterisation of individual ECM root tips to develop

99   a trait-based analysis. In that work, the authors demonstrated that extracellular enzyme traits at

100   ECM fungal community level can be driven by the soil nutrient status.

101

102   Studies on ECM functional diversity have mainly focused on the impact of soil nutrient status

103   and the natural succession of ECM fungal communities (Clemmensen et al., 2015; Kjøller et al.,

104   2012; Moeller et al., 2014; Suz et al., 2014). However, the effect of trace elements, mainly heavy

105   metals, on ECM fungal community composition and diversity has been scarcely studied and the

106   results are controversial. Hui et al. (2011) and Op De Beeck et al. (2015) did not find any effect

107   of heavy metal contamination on ECM taxonomic diversity but noted a shift in the species

108   composition of their communities. In contrast, Sousa et al. (2014) found both, an effect on

109   community composition and an increase in ECM fungal diversity in Cd-contaminated plots.

110   However, Huang et al. (2012) did not find a clear effect of the contamination neither on

111   community composition nor at the taxonomic richness level. Despite some influences on

112   taxonomic diversity, there exists a gap of knowledge on how such kind of anthropogenic impact

113 affects the functional diversity of ECM fungal communities. Trace elements are likely to filter

114 against the ECM fungal species spreading more intensively in soils (those producing emanating

115 hyphae and/or rhizomorphs) due to an increased exposure to trace element toxicity (Pawlowska

116 and Charvat, 2004). In addition, increased melanisation of ECM fungal communities would be

117 expected as a consequence of the known protective effect of melanin against heavy metals (Gadd

118 and Rome, 1988; Galli et al., 1994).

119

120 Here we determined hyphal exploration types and melanisation level as traits of ECM fungal

121 species, molecularly identified, associated with holm oak (*Quercus ilex* subsp. *ballota*) in a

122 restored trace element contaminated site (Guadiamar River valley, South of Spain). We

123 quantified exploration type by microscopically confirming the presence of emanating hyphae and

124 rhizomorphs on single ECM root tips. Our hypotheses were that: i) higher concentrations of trace

125 element in soil reduce the taxonomic diversity of ECM fungal species and shifts the community

126 composition; ii) there is an effect of trace element contamination on the community mean traits

127 towards shorter exploration types and more melanised fungi; iii) we expect that trace element

128 contamination reduces the trait dispersion in ECM fungal communities, since it acts as a filter of

129 species according to their traits.

130

131 **2. Material and Methods**

132 *2.1. Study area*

133 In 1998 a mine spill contaminated 55 km^2 of the Guadiamar River valley, a traditional mining

134 area in the south of Spain (Grimalt et al., 1999). The spilled acid water and sludge included a

135 variety of trace elements, with high concentrations of several highly toxic heavy metals and

6

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

182   the host species constant, we could focus on the soil variability across the studied area, thus

183   excluding other confounding factors such as plant host identity and age (Albornoz et al., 2016;

184   Davey et al., 2015). Ten trees were randomly selected in each site (Supporting Information Fig.

185   S1 and Table S1 for geographical coordinates). In April 2016, roots of trees were sampled by

186   carefully tracing them from the stem of the tree in the four cardinal directions and ca. 200 g root

187   material was collected from each direction, i.e. subsamples. Soil samples (0-20 cm depth) were

188   taken with an auger from the four directions under each tree canopy projection, and were pooled

189   to a total of 500 g to make a composite sample per tree.

190

191   *2.3. Soil analyses*

192   All soil samples were air-dried and sieved to <2 mm for physico-chemical analysis. Soil pH was

193   measured in a 1:2.5 soil-water suspension after shaking for 30 min. Total C and N content was

194   determined using a Flash HT Plus elemental analyser. Carbonate content was measured by the

195   manometric method (Demolon and Leroux, 1952); soil organic C was then calculated as the

196   difference between total C and the C contained in carbonates. Ammonium and nitrate were

197   extracted by 1M KCl and determined by multiparametric Bran-Luebbe autoanalyser (Maynard et

198   al., 2007). Olsen method (Olsen et al., 1954) was used for available P estimation in neutral and

199   basic soils and Bray method was used in acidic soils (Bray et al., 1945). Available K, Ca and Mg

200   were extracted with 1 M ammonium acetate and determined by atomic absorption

201   spectrophotometry. Sulphur and pseudo-total trace element concentrations in soil samples

202   (ground to <60 μm) were determined by digestion with aqua regia (1:3 v/v conc. HNO_3/HCl) in a

203   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

227   (Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) and frozen until PCR setup. PCR

228   amplification was carried out using 0.55 µl of DNA template with a Illustra PureTaq Ready-To-

229   Go bead (GE Healthcare UK Limited, Buckinghamshire, UK) and 0.8 µM of primers ITS1F

230   (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) in a final volume of 25 µl. The

231   thermocycling program was as follows: 3 min initial denaturation at 94°C; 35 cycles of 30 s

232   denaturation at 94°C, 35 s annealing at 53°C and 1 min elongation (increased in 5 s each cycle)

233   at 72°C; and a 4 min final elongation (as described by Suz et al., 2014). PCR products were

234   purified using MEGAquick-spin (Intron Biotechnology, South Korea) and Sanger sequenced in

235   the Unidad de Genómica y Síntesis de DNA, Instituto de Biomedicina y Parasitología López

236   Neyra, CSIC (Granada, Spain). Sequence chromatograms were checked individually and those

237   presenting double peaks, i.e. containing more than one fungal sequence, were discarded. In these

238   cases a new root tip was picked up randomly from the root sample to ensure a minimum number

239   of sequences per root sample. The remaining sequences were blasted against the UNITE

240   database (Koljalg et al., 2005) and those found corresponding to ECM fungi were grouped by

241   genera or family. Sequences in each taxonomical group were aligned separately using MAFFT v.

242   7 (Katoh and Standley, 2013) and clustered in MOTHUR v. 1.35.1 (Schloss et al., 2009) at a

243   97% cut-off to delimitated Operational Taxonomic Units (OTU). DNA sequences were

244   compared against the UNITE database (Koljalg et al., 2005) for their taxonomic placement and

245   Species Hypothesis determination. ECM fungal sequences were deposited in GenBank

246   (http://www.ncbi.nlm.nih. gov/genbank/) under accession numbers MG273770-MG274263.

247

248   *2.6. Data analyses*

11

249 The whole analysis was based in the use of continuous data coming from the individual

250 characterization of holm oak trees. For a broad characterization of study plots, a principal

251 components analysis was carried out after log-transforming of trace element and soil variables.

252 Differences in abiotic and biotic (i.e. ECM fungal traits) variables across plots were assessed by

253 ANOVA after checking for normality and homoscedasticity. Tukey´s Honest was used as *post*

254 *hoc* test. Non-normal variables were log or square root transformed. Variables that even when

255 transformed were not normally distributed were analysed by non-parametric Kruskal Wallis test

256 with pairwise Dunn test corrected using Bonferroni as *post hoc*.

257

258 The OTU abundance data matrix was constructed based on the number of root tips where each

259 species was identified. A rarefaction analysis was carried out to ensure a high and even coverage

260 of the total diversity of OTUs in each plot. The abundance matrix was Hellinger transformed for

261 subsequent analyses (Legendre and Gallagher, 2001). Species richness (S), Chao1 and Simpson

262 (1-D) indices were calculated as alpha diversity measures.

263

264 An OTU × trait matrix was constructed by calculating the frequency of emanating hyphae and

265 rhizomorphs in the total root tips of each ECM fungal OTU. The black colour percentage was

266 used as a proxy of melanin content and its value for each species was calculated as the average of

267 the black component across all identified root tips per each OTU. To scale up from OTU to

268 community level, all these traits were weighted by the relative abundance of each OTU to

269 calculate community-weighted means (CWMs) of mycorrhizal traits for each tree (called fixed

270 trait averages by Lepš et al., 2011).

271

272   A Variation Partitioning approach (Legendre and Legendre, 1998) was used to assess the

273   influence of soil variables and trace elements on species (species-based RDA) and trait

274   distribution (CWM-based RDA) (Kleyer et al., 2012). For that, every abiotic variable was log

275   transformed, with the exception of pH, and the Hellinger transformed OTU matrix and the CWM

276   matrix were used as response matrices for the species- and CWM-based RDAs, respectively. A

277   previous selection of variables was carried out by stepwise model building for constrained

278   ordination methods (Blanchet et al., 2008) with backward and forward selection to include

279   important variables only. Since the objective of this analysis was to quantify the relative

280   contribution to OTU and CWM distribution of soil background variables, trace elements and

281   their shared covariation, the approach was applied separately for each group of soil factors (soil

282   background variables and trace elements). For each subset of variables selected by the models,

283   the variance inflation factors (VIF) were calculated (Gross, 2003), and variables above VIF=5

284   were removed. To control for the effect of spatial distribution of samples, principle coordinates

285   of neighbour matrices (PCNM approach; Borcard and Legendre 2002) were calculated. The

286   resulting PCNM axes were subjected to the same selection as described for soil and trace

287   element variables, and those found to significantly influence the OTU or CWM distribution were

288   selected. Every selected variable, either from soil, trace elements or spatial components, were

289   feed to the variation partitioning analysis. To visualise the identified trends, an RDA ordination

290   was carried out including all selected variables.

291

292   To assess the significance of each of the soil background variables and trace elements on fungal

293   trait values, RLQ and fourth-corner analyses were performed (Legendre et al., 1997; Dray and

294   Legendre, 2008). This method directly compares the three matrices: environmental, species

295 abundance and species traits. Effects were calculated using permutation model #6 with 9999

296 permutations, which is a combination of models #2 (permutes values of sites) and #4 (permutes

297 values of species) which does not have an inflated type I error (Dray and Legendre, 2008; ter

298 Braak et al., 2012). False discovery rate correction for multiple testing (Benjamini and Yekuteli,

299 2001) was applied.

300

301 In order to obtain insights into the rules governing ECM fungal community assembly, the trait

302 distribution across OTUs in communities was compared with random expectations. For that,

303 standardised effect size of mean pairwise distance (ses.mpd) between OTUs in each community

304 was calculated by using the OTU abundance data matrix and a Euclidean trait distance matrix

305 between OTUs. *Independent swap* algorithm was used to generate null communities (Gotelli,

306 2000). Ses.mpd varies from -1 to 1, where negative values mean trait convergence and positive

307 values trait divergence. Relationships of ses.mpd with soil factors were checked by Pearson

308 correlation applying a false discovery rate correction for multiple testing (Benjamini & Yekuteli,

309 2001).

310

311 All statistics were carried out in R software v 3.3.2 (R Development Core Team) using *vegan*

312 (Oksanen et al., 2012), *picante* (Kembel et al., 2010) and *ade4* (Dray and Dufour, 2007)

313 packages.

314

315 **3. Results**

316 *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

340 (%): 66.6 to 100; rhizomorphs (%): 0 to 66.6; melanisation (%): 64.6 to 72.9). OTUs belonging

341 to the other two dominant families were highly variable in terms of emanating hyphae and

342 rhizomorphs (ranging 0 % to 100 %), while melanisation spanned in the range between 70 % and

343 94.7 %. The two most dominant species (*H. cavipes* and *T. terrestris*) had similar rhizomorph

344 frequency and melanisation (around 12 % and 68 %, respectively), but *H. cavipes* showed

345 emanating hyphae more frequently (95.1 %) than *T. terrestris* (88.5 %). The trait ranges

346 exhibited by the detected ECM fungal species were congruent with the available descriptions of

347 species and genera (Deemy database, see Supporting Information Table S3 for a comparison).

348

349 *3.3. Effect of abiotic variables on ECM fungal community composition*

350 According to the selected RDA models, the variables that best explained ECM fungal

351 community variability (OTU matrix) were available Ca, organic C and total C among soil

352 background variables, and Cu, Ni, S and Zn among trace elements (Fig. 2a; Supporting

353 Information Table S4). Sulphur was removed from the subsequent analysis due to a high VIF

354 result. Two PCNM axes were found to influence OTU community composition. The variation

355 partitioning approach revealed that soil background variables and their covariation with trace

356 element explained 8.36 and 0.55 %, respectively; meanwhile trace elements alone explained 3.82

357 % of variation in the model (Fig. 2a, pie chart). The spatial distribution of ECM communities

358 explained a 2.06 % alone, and shared 2.86 % with soil background and trace element variables.

359 There was no sign of collinearity between variables in the variation partitioning analysis. The

360 two most abundant species, *H. cavipes* and *T. terrestris*, showed contrasting patterns regarding

361 the trace element and Ca gradients, respectively, in the RDA ordination (Fig. 2a). *H. cavipes*

16

362   seemed to be related to lower concentrations of Cu, Zn and total C, and higher concentrations of

363   Ni. *T. terrestris* appeared to be related with lower concentrations of Ca, as shown in Fig. 2a.

364

365   *3.4. Effect of abiotic variables on ECM fungal community traits*

366   The RLQ analysis showed a significant effect of the abiotic environment on the community

367   composition by an interaction with species traits (model #2, $P = 0.006$; model #4, $P < 0.001$).

368   Significant negative interactions were found between CWM of emanating hyphae and

369   rhizomorphs and some trace elements and total C (displayed in Table 2). On the other hand,

370   melanisation significantly interacted with $CaCO_3$.

371

372   The soil background variables that best explained CWM traits distribution included $CaCO_3$, total

373   C, organic C and available P (Fig. 2b; Supporting Information Table S4), however, total C was

374   removed from subsequent analysis due to a high VIF. On the other hand, among the trace

375   elements, As, Cd and Cu best explained the variation of fungal community traits. Cu was finally

376   removed due to a high VIF. No spatial variables (PCNM axes) were found to significantly

377   explain any variation in trait distribution and were not included in the variation partition analysis.

378   When partitioning the variation into trace element and soil background variables, trace elements

379   explained 15.46% of the total variation, soil background 7.54% and their covariation 6.59% of

380   the trait variability (Fig. 2b, pie chart). In agreement with the fourth corner analysis (Table 2),

381   emanating hyphae and rhizomorphs appeared negatively related to trace element concentrations

382   and organic C. Meanwhile, melanisation and $CaCO_3$ showed a clear positive covariation (Fig.

383   2b).

384

17

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, CaCO₃, 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

408   age of the trees, all of them planted only 17 years ago, could increase the chances that stochastic

409   effects, i.e. priority effects, were acting on the community assembly of the ECM fungal

410   communities. This fact would explain two results: on one hand, the relatively low ECM species

411   richness (average of 3.8 species per tree) in comparison with previous studies in near mature

412   Mediterranean forests (evergreen *Quercus suber*) which averaged 6.3 species per tree (Aponte et

413   al., 2010). This trend is in agreement with the known increase in ECM species richness during

414   ecosystem development as observed by Visser (1995) or Wallander et al. (2010). On the other

415   hand, the effect of soil background variables and trace element contamination on the ECM

416   fungal community composition was relatively low (a small percentage of variation in species

417   composition was explained by these variables). This is consistent with a primary successional

418   scenario where stochastic processes such as dispersal and/or priority effects drive the community

419   assembly (Jumponen, 2003; Kennedy et al., 2009; Peay and Bruns, 2014) and thus blur the

420   deterministic effects caused by soil factors, i.e. the proportion of community composition

421   explained by the soil environment or its effect on species richness. Indeed, although low as well,

422   a certain proportion of the variation of the OTU community composition was found to depend on

423   the spatial position, which is a sign of a stochastic process influencing community assembly. On

424   the other hand, other environmental factors not measured in this study, such as the relative

425   influence of the seasonal river floods on different sites, could be responsible for the proportion of

426   unexplained variance in community composition.

427

428   Despite the variance explained by soil factors being limited, soil background variables and trace

429   elements explained a similar proportion of the variation in species composition. Previous studies

430   of ECM fungal communities have shown the important influence of nutrient-related variables,

431   such as total C or organic C in soil, in the determination of ECM fungal community composition

432   (Twieg et al., 2009). In our study, the two most frequent ECM species, *H. cavipes* and *T.*

433   *terrestris*, were related to two independent abiotic gradients: *H. cavipes* to a trace element

434   concentration gradient, and *T. terrestris* to a gradient in Ca concentration (likely related to the

435     $CaCO_3$ and pH). This fact would explain why the variance in community composition was

436   equally explained for each group of variables, as each of these groups explains the presence of

437   one of the two most abundant ECM fungal species. Indeed, this result resembles the results by

438   Op de Beeck et al. (2015) who also found that communities of ECM fungi were driven according

439   to two environmental gradients: one responding to heavy metal contamination levels and the

440   other driven by Fe, Mn, Mg and K.

441

442   *4.2. Effect of contamination on mean fungal trait values*

443   The effect of contamination was visible both in terms of the mean trait values of communities

444   and the trait similarity across species in communities. Both rhizomorph and emanating hyphae

445   frequency were found to be negatively associated with the concentration of some trace elements,

446   which indicates a suppressive effect of the contamination on extramatrical mycelium growth.

447   This effect has previously been found in controlled experiments, and varies across ECM fungal

448   species (Qi et al., 2016). At the same time, the recorded patterns for the exploration-type related

449   traits, particularly the relationship between emanating hyphae and total C, are also highly

450   congruent with the known variation of exploration type in response to changes in N sources in

451   the soil, i.e. a change from inorganic to organic N sources will reduce the development of

452   extramatrical mycelium (Hobbie and Agerer, 2010; Lilleskov et al., 2002; 2011). Previous

453   studies have pointed out the capacity of melanin to biosorb Cu and reduce its environmental

454   toxicity (Gadd and Rome, 1988), and that dark Ascomycota species usually are more resistant to

455   heavy metal contamination than Basidiomycota (Likar and Regvar, 2013). The hypothesis that

456   the degree of melanisation would increase with heavy metal concentration has to be rejected for

457   this dataset since we did not record an increase in the black colour of ECM fungi present in

458   contaminated sites compared with non-contaminated ones. The relationship between black

459   colour of ECM fungal species and $CaCO_3$ could be the result of other biochemical interactions

460   since melanin seems to be involved in the Ca^{2+} regulation of the cells (Bush et al., 2007). In the

461   present study, the variation in CWM fungal traits explained by trace element concentrations

462   doubled the variation explained by soil background variables. These effects were also

463   independent of the spatial distribution of the samples, excluding any potential site effect in the

464   results. This fact, together with the smaller overall variance explained in the case of the OTU

465   matrix, highlights the interest of this trait-based approach to explain the consequences of trace

466   element contamination on ECM fungal communities.

467

468   *4.3. Ecological processes driving ECM fungal community assembly*

469   The trait dispersion of species within communities was driven mainly by soil contamination and

470   not by the nutrient status of the soil. The increase in Cd concentrations made species in ECM

471   fungal communities become more similar in terms of presence of rhizomorphs. This reveals the

472   potential environmental filtering that heavy metal contamination can have on the trait

473   composition of ECM fungal communities. While species richness was similar across the studied

474   sites, the increase in trait convergence indicates a reduction in the functional diversity of the

475   community (Bässler et al., 2015) in response to soil contamination. Although we also found an

476   average reduction in the emanating hyphae with increasing contamination levels, the tendency,

477   marginally significant, with increasing contamination was a divergence in the frequency of

478   emanating hyphae produced by species in the same community. This is not in agreement with an

479   environmental filtering, as suggested for rhizomorphs, but could indicate that competition

480   between species is selecting species that differ in this trait. This could be explained by an

481   interaction between the two traits: once the community has been filtered according to the

482   production of rhizomorphs, the remaining subset of species is selected against biotic interactions,

483   i.e. competition, as observed for example by Ingram and Shurin (2009).

484

485   The consequences of the reduction in the functional diversity of ECM fungal communities for

486   plant and ecosystem functioning might depend on the specific traits affected. For ECM fungi it is

487   known that the decomposition rate of their biomass is very dependent on melanin content and

488   hyphal architecture (i.e. hydrophobic rhizomorphs versus hydrophilic feeder hyphae, Fernandez

489   et al., 2016), which thus influences C storage in soil (Clemmensen et al., 2015). Additionally,

490   these two traits also have an important role in water stress alleviation for plants (Fernandez and

491   Koide, 2013), which may have important consequences for host fitness, particularly in

492   Mediterranean environments.

493

494   *4.4. Conclusions*

495   In this study, we demonstrated that ECM functional traits correlated better with soil

496   contamination than fungal taxonomic diversity or community structure. Thus, adding trait-based

497   approaches to the description of ECM fungal communities facilitates a better understanding of

498   the potential consequences of environmental degradation on ecosystem functioning. The often

499   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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513

Competing interest statement

515   The authors declare no competing interests.

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793

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.
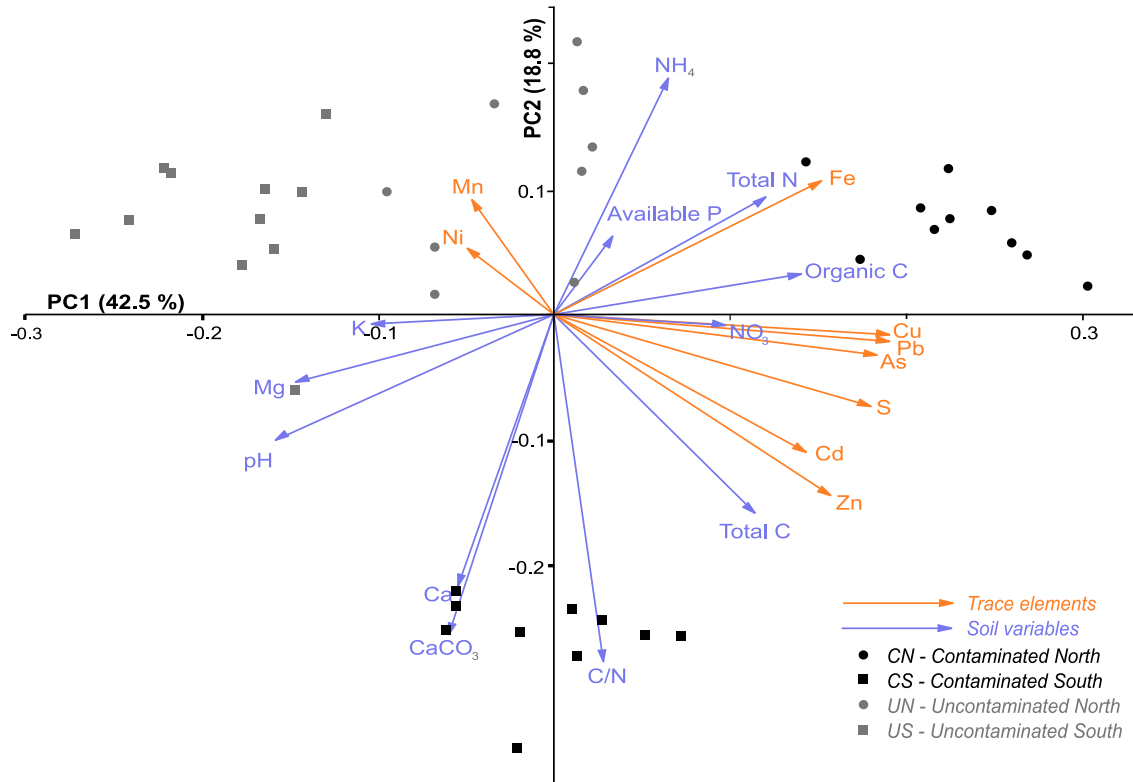
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801 **Fig. 2**. Redundancy analysis triplots of ectomycorrhizal (ECM) fungal communities driven by

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.

a) Species-based redundancy analysis (triplot) and variation partitioning analysis (pie chart)
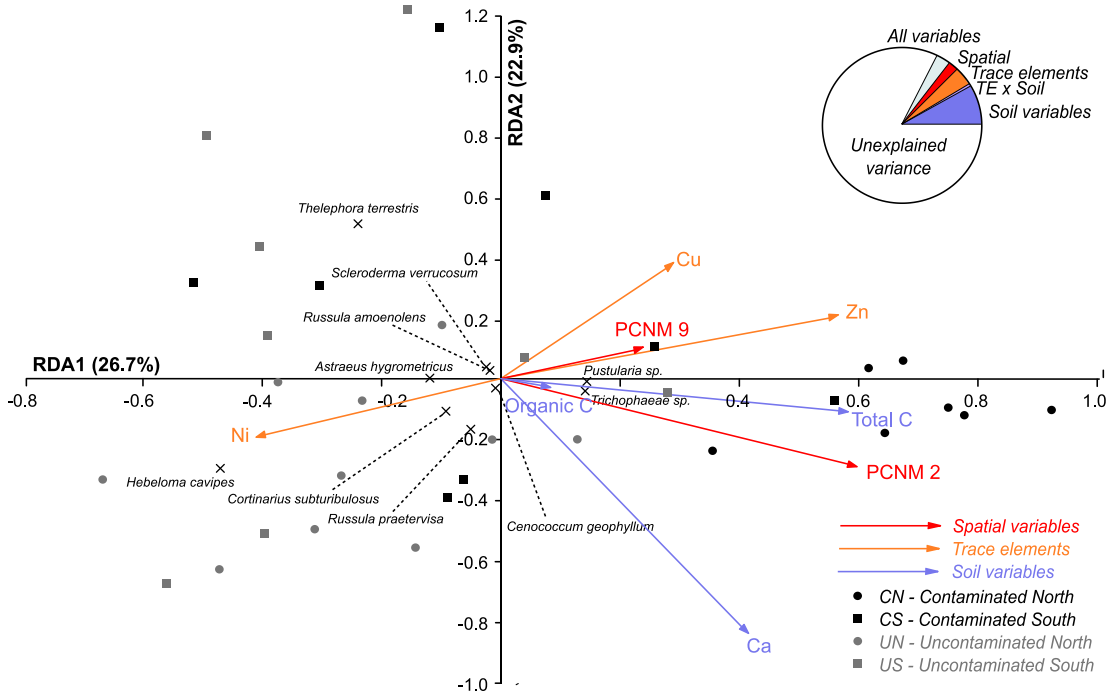
b) Trait Community Weighted Mean (CWM)-based redundancy analysis (triplot) and variation partitioning analysis (pie chart)
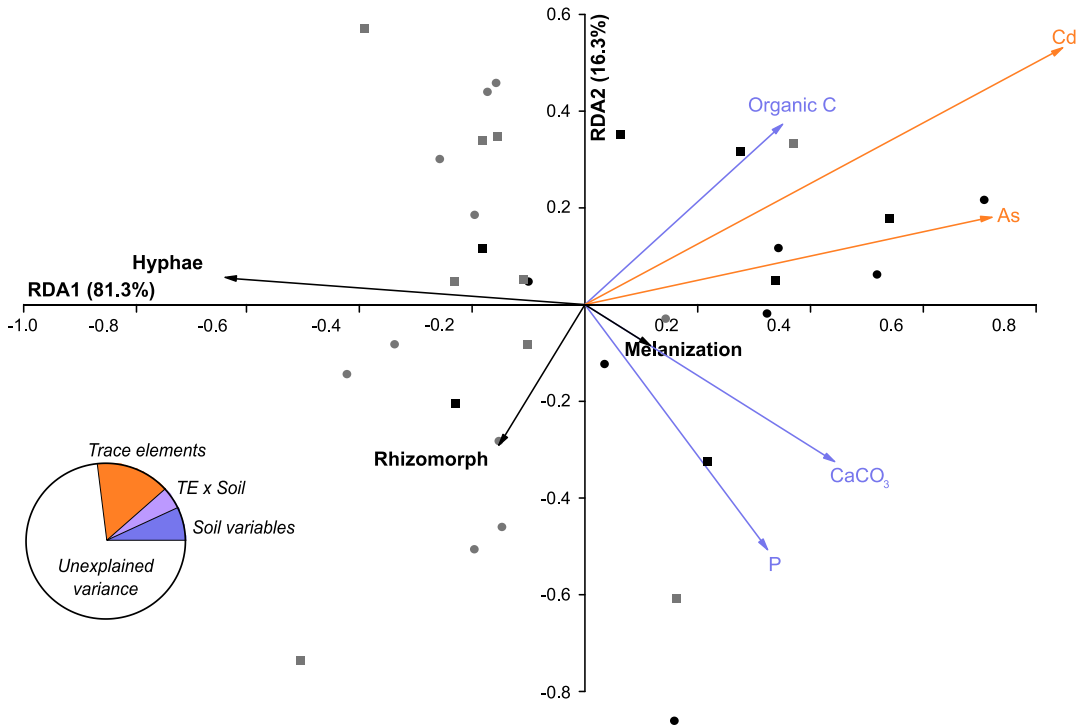
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810 **Table 1**. Mean values of soil variables (±SE) in the two studied plots affected and the two

811 unaffected by the toxic mine spill of Guadiamar river (SW Spain). Contaminated north (CN) and

812 south (CS), uncontaminated north (UN) and south (US). ANOVA analysis is displayed in last

813 two columns (F and *P*). Means not sharing a letter in common differ significantly according to

814 the Tukey´s Honest *post hoc*.

815
816

| Soil variables | Contaminated plots | | Uncontaminated plots | | ANOVA | |
	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

820

821

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

827

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829   **Supporting Information**

830   **Figure S1** Map of the study area and location of plots.
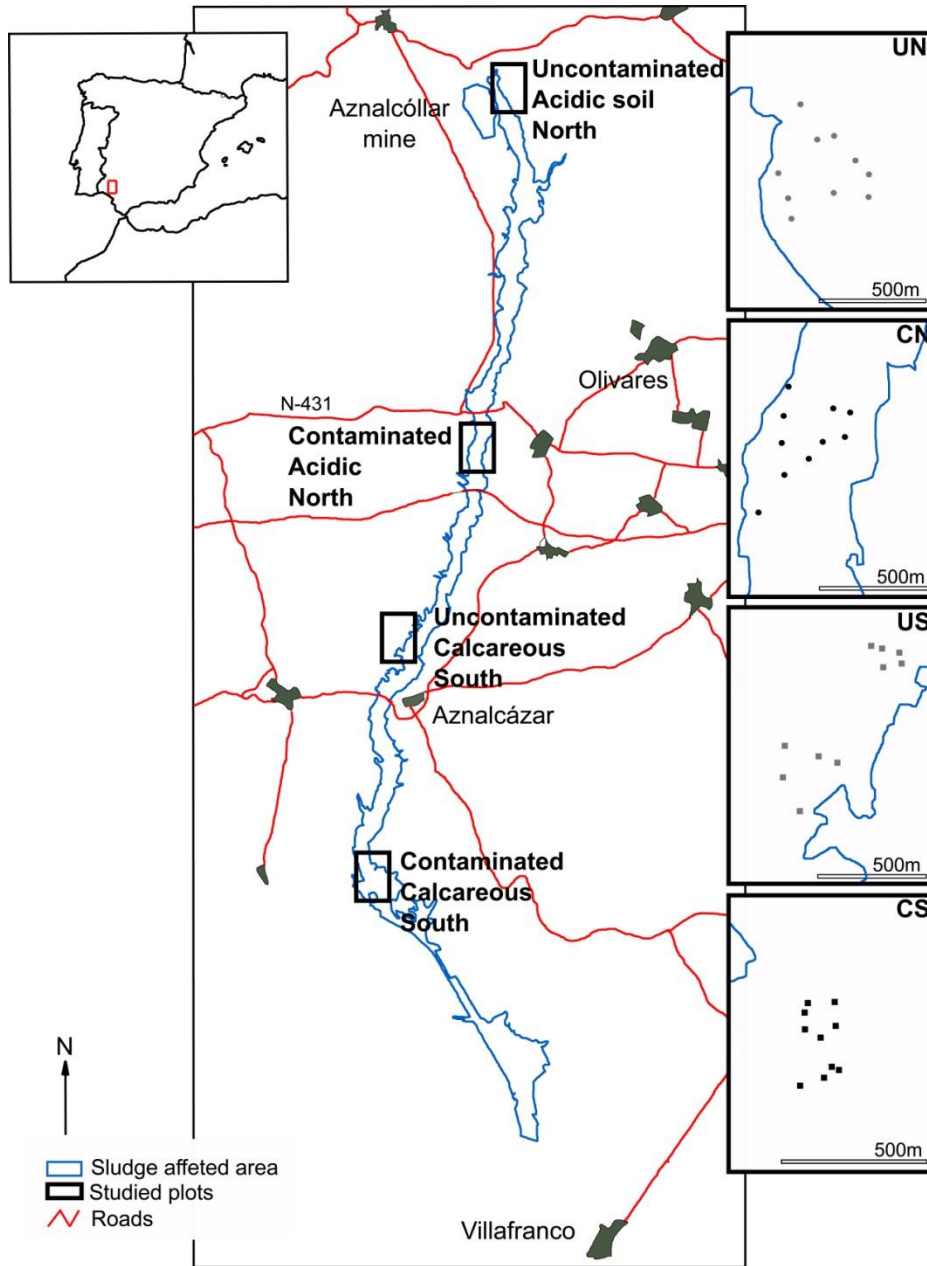
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833   **Supporting Information**

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

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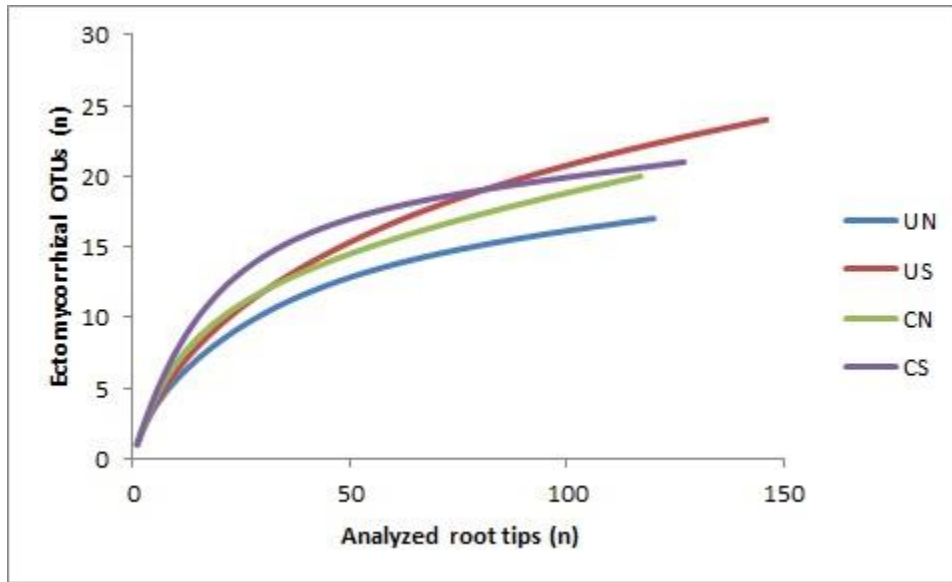
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841 **Supporting Information**

842 **Table S1.** Overall distribution of texture components in the sampled plots (data from Domínguez pers.
843 comm.) and soil type classification (according to Clemente et al. 2000) in the four sample sites in
844 Guadiamar river valley (SW Spain). Geographic locations of specific sampled trees. Contaminated north
845 (CN) and south (CS), uncontaminated north (UN) and south (US).
846

|	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

847

848 **Reference**

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

851

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).

858

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

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
	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

Family	Species						Reference	%	SH code
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

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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.

| Phylum | Family | Species | n | Experimental observations | | | Deemy database | | |
				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

Phylum	Family	Species	n	Experimental observations			Deemy database		
				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

Phylum	Family	Species	n	Experimental observations			Deemy database		
				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

Phylum	Family	Species	n	Experimental observations			Deemy database		
				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

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	Df	AIC	F	Pr(>F)
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 .

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).
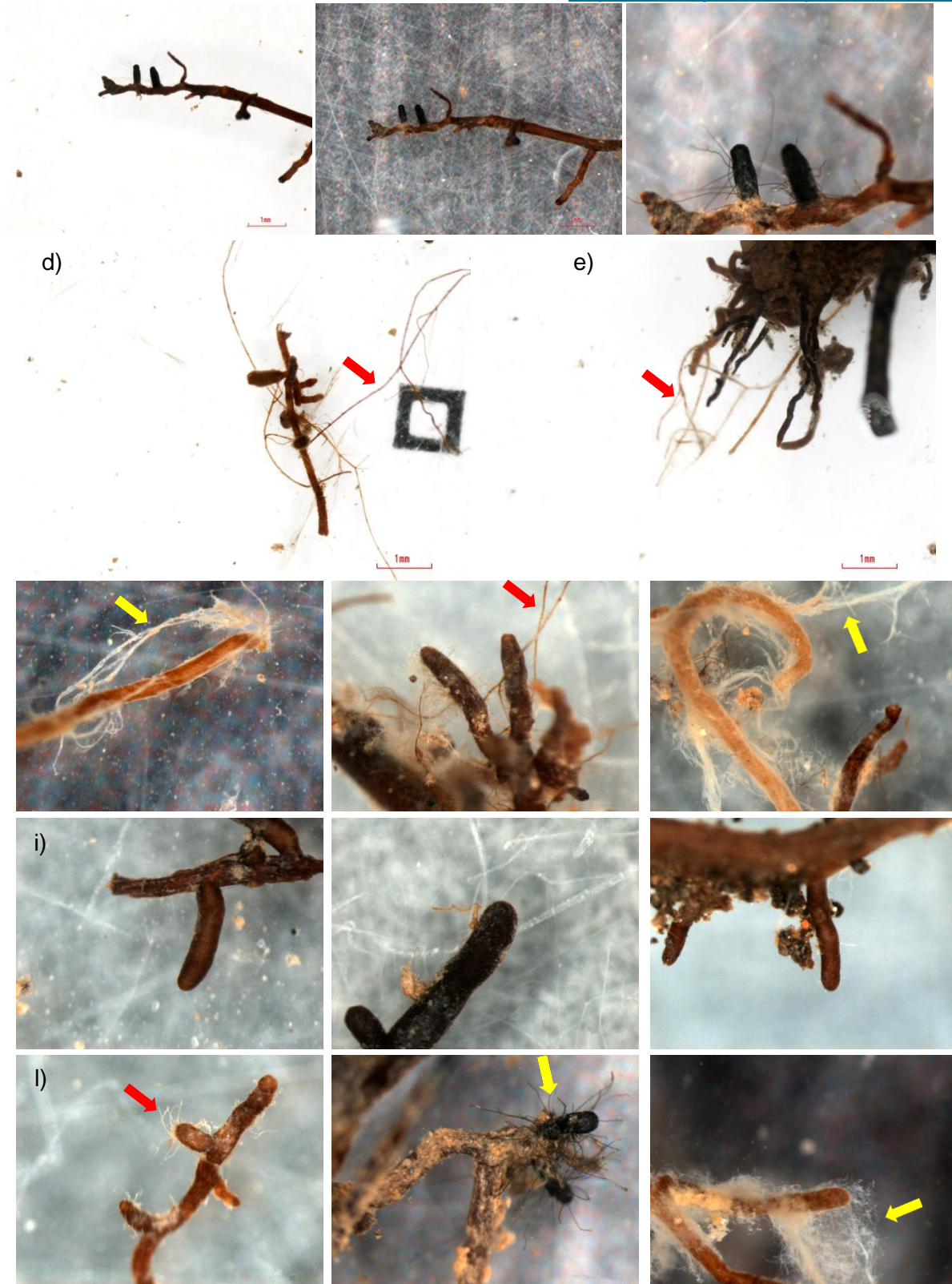
Fig. 1. Examples of photographs showing root tips with different fungal traits. a-c) *Cenococcum geophylum* root tips at 25X magnification (a, b) and 100X magnification (c); d-e) clusters of root tips with associated rhizomorphs (25X magnification); f-h) detailed of root tips showing rhizomorphs (100X 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
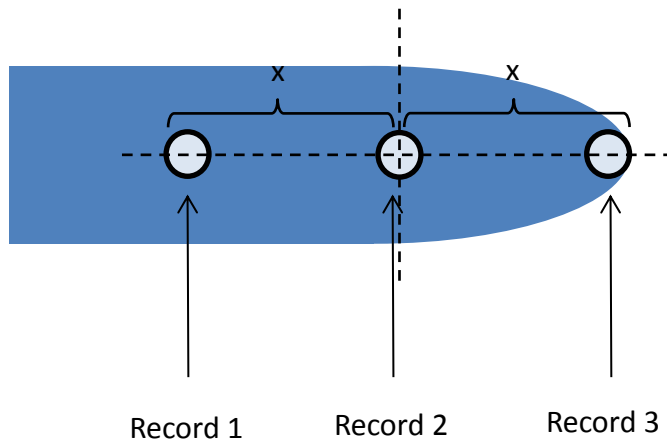number of the three records in each root tip.

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

Record 1   Record 2   Record 3

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).
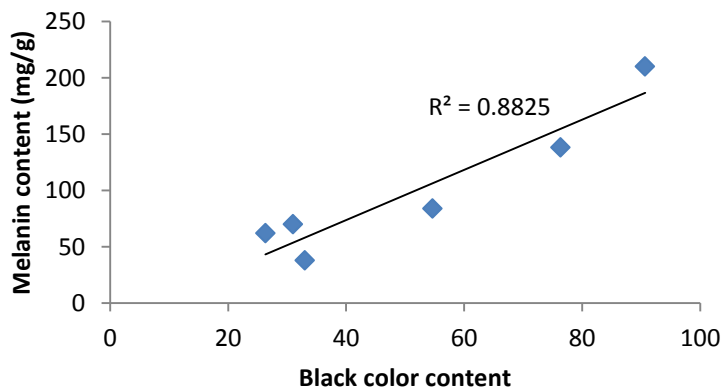

Fig. 3. Relationship between melanin content and black colour of fungal mycelia. The analysis corresponds to the photographs and melanin contents published by Fernandez & Koide (2014).

$R^2 = 0.8825$

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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