Local diversity of native arbuscular mycorrhizal symbionts differentially affects growth and nutrition of three crop plant species

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Abstract

Intact whole native AMF communities occurring across a 100 m long field were used for the evaluation of plant performance, as determined by the actual fungal species colonizing host roots. The soil from distinct plots within a "hot spot" field was collected to set up 54 experimental units where three different plant species were grown, in order to test whether the whole native AMF communities were able to differentially affect plant growth, to assess the genetic identity of the AMF actually colonizing the tested plants and to analyze their community composition in the different hosts. Molecular analyses revealed that plant growth and nutrition of the crop plants was differentially affected by the diverse native arbuscular mycorrhizal communities colonizing the roots of the three plants, whose

performance varied depending on the identity of plant hosts and fungal symbionts, more than on a rich and diversified AMF community. Such results, improving our understanding of AMF distribution at the local scale, represent a starting point allowing the selection, isolation and characterization of the most efficient AMF assemblages to be used as inoculants in sustainable food production systems.

Keywords: root AMF communities; small scale AMF diversity; small ribosomal subunit (SSU rDNA); plant performance; functional diversity.

Introduction

Arbuscular mycorrhizal fungi (AMF, Glomeromycotina, Spatafora et al. 2016) represent a group of beneficial soil biota which establish root symbioses with the majority of land plants, including the most important food crops. They provide essential ecosystem services, improving plant nutrient availability, soil structure formation, and tolerance to biotic and abiotic stresses, and promote plant diversity (Gianinazzi et al. 2010; Smith and Read 2008). In addition, they enhance the biosynthesis of beneficial phytochemicals in food plants, representing a valuable and innovative tool for the healthy food production chain (Rouphael et al. 2015; Sbrana et al. 2014). After establishing the symbiosis, AMF produce extensive underground extraradical mycelia that spread from the roots into the surrounding soil, and uptake and transport soil nutrients, mainly P and N, to the host plants (Battini et al. 2017; Pepe et al. 2017); in exchange, AMF receive C compounds, which they are unable to synthesize, or to feed off as saprotrophic organisms (Smith and Read 2008). The availability of soil mineral nutrients is also synergistically promoted by a diversified community of beneficial bacteria thriving in association to AMF spores and extraradical mycelium (Agnolucci et al. 2015; Battini et al. 2016; Hildebrandt et al. 2006; Morrison et al. 2017).

AMF are globally distributed, and have been found in the most diverse plant biomes, from grasslands to desert, forest, shrublands, agriculture and wetlands, wherever their host plants were reported to occur (Allen et al. 1995; Oehl et al. 2017; Öpik et al. 2006; Read 1991; Treseder and Cross 2006). Investigations on the distribution of AMF at the global scale revealed its relationship with plant community composition, environmental variables, dispersal ability and geographical distance (Davison et al. 2015; Kivlin et al. 2011; Öpik et al. 2006; Treseder and Cross 2006). Although spatial factors on a broad global scale were considered the main elements affecting AMF distribution (Kivlin et al. 2011; Turrini and Giovannetti 2012), a recent work reported a low level of AMF endemism, with 93% of taxa occurring in multiple continents, suggesting that AMF distribution among sites may be driven by very efficient dispersal vectors, including wind, birds, water and human activities (Davison et al. 2015).

A few studies performed at the regional scale reported that AMF community composition and diversity were not affected by geographical distance (max. 100 km) in apple orchards across Belgium (Van Geel et al. 2015), in coffee plantations across Ethiopia (max. 82 km) (De Beenhouwer et al. 2015) and in *Trifolium repens* and *Lolium perenne* across Ireland (max. 392 km) (Hazard et al. 2013). By contrast, the distribution of AMF species was strongly affected by geographical distance in agricultural soils across England (max. 250 km) and Switzerland (max. 294 km) (Jansa et al. 2014; Van der Gast et al. 2011). A recent molecular study, carried out at a more local scale (max. 27 km), confirmed such findings, as different AMF communities were detected in two geographical locations in South Tyrol (Turrini et al. 2017).

Works focused on AMF genetic diversity and distribution at the local scale showed high differences among AMF genotypes and communities occurring in the same field (Croll et al. 2008; Koch et al. 2004), when separated by a few meters (Stukenbrock and Rosendahl 2005) or even at the sub-meter scale (Mummey and Rillig 2008). Moreover, a high functional diversity was found within AMF species, even among genotypes originating from a single field (Angelard et al. 2010; Croll et al. 2008; Koch et al. 2004; Munkvold et al. 2004).

Knowledge of AMF diversity and community composition at a small scale is relevant not only for improving our understanding of AMF distribution, but also for functional characterization studies of the native AMF communities, in order to isolate and reproduce the most efficient ones to be used as inoculants in sustainable and organic agriculture. Actually, while it has been long known that distinct assemblages of AMF species may occur in different plants (host preference) (Bever et al. 2009; Johnson et al. 2004; Koorem et al. 2017; Rodríguez-Echeverría et al. 2017; Vandenkoornhuyse et al. 2003), and determine plant productivity, after isolation and inoculation in soil microcosms (van der Heijden et al. 1998), only a few studies analyzed the symbiotic performance of whole native AMF communities, utilized as inoculum (Burrows and Pfleger 2002; Moora et al. 2004; Uibopuu et al. 2012).

A previous study identified a site with a very high AMF richness, within the UNESCO Man and Biosphere Reserve, named 'Selva Pisana' (http://www.unesco.org/mabdb/br/brdir/directory/biores.asp?code=ITA+08&mode=all). Such a site, represented by a small field, used periodically for crop production since 1974, but often left uncultivated, is relevant for the study of local AMF diversity, as it has been described as a global "hot spot" of AMF species richness, encompassing 58 different AMF species belonging to 14 genera, the highest number reported so far from a single site (Njeru et al. 2015), and for studies on the symbiotic performance of whole native AMF communities. In the present study, we hypothesized that native AMF communities may show differential symbiotic performance, as determined by the actual fungal species composition occurring in the roots of host plants and by the identity of the plant host. We used the soil from six distinct plots within the "hot spot" field to set up 54 experimental units where three different plant

93 species were grown, *Allium cepa*, *Capsicum annuum* and *Lactuca sativa*. The main aims of this multimodal study
94 were: i) to test whether the whole native AMF communities originating from the six distinct plots were able to
95 differentially affect the growth of the three plant species, ii) to assess the genetic identity of the AMF actually
96 colonizing the tested plants and iii) to analyse their community composition in the different hosts. The results
97 obtained will improve our understanding of AMF distribution at the local scale, that is relevant for functional
98 characterization studies of native AMF communities to be used as inoculants in sustainable food production
99 systems.
100
101 **Materials and Methods**
102 **Field experimental site and soil sampling**
103 The experimental site was located at Interdepartmental Centre for Agri-environmental Research 'Enrico Avanzi'
104 (CIRAA), University of Pisa, S. Piero a Grado, Pisa, Italy (latitude 43° 40′ N, longitude 10° 19′ E), within the
105 UNESCO Man and Biosphere Reserve named 'Selva Pisana'. Since 1974 the field was periodically used for the
106 production of various crops (maize, different horticultural crops, durum wheat, perennial alfalfa) and was also
107 uncultivated for several years (from 1988 to 1997, 2006 to 2011, and 2014 to 2015), allowing the development of
108 spontaneous flora. Since October 2011 to September 2012 the field site was used for cover crops and tomato crop
109 production under the EU-RTD FP7-funded project Strategies for Organic and Low input Integrated Breeding and
110 Management (SOLIBAM 2010-2014) during which the soil was evaluated for AMF occurrence and diversity. In an
111 area of about 2600 m^2, 58 AMF species, belonging to 14 genera were identified by classical morphological spore
112 identification (Njeru et al. 2015).
113 Soil samples were collected from 12 field plots (3x4m each), distributed in an area of 13x114m in spring 2015 (Fig.
114 1). In each plot a "X" shaped sampling pattern was used, in which 5 points were selected, one at the centre of the
115 plot and the other four 1 m far from the first. Soil cores from each point were taken at a depth of 5 to 15 cm, then
116 mixed and labelled as a single sample. Finally soil samples were air dried and then sieved through a 5 mm mesh
117 sized sieve.
118
119 **Soil characterization**
120 ***Soil physical and chemical analyses***
121 Soil samples were analysed for: total N, available P, soil organic matter, $pH_{(H2O)}$, cation exchange capacity, and
122 texture. Total N was evaluated by the macro-Kjeldahl digestion procedure (Bremner and Mulvaney 1982) and
123 available P was determined by colorimetric analysis using the Olsen method (Olsen and Sommers 1982). Organic

matter was determined using the modified Walkley-Black wet combustion method (Nelson and Sommers 1982). Soil pH was measured potentiometrically in a 1:2:5 soil water suspension, the other soil parameters were determined according to the standard methods (Gee and Bauder 1986; Sumner and Miller 1996).

Mycorrhizal inoculum potential of the field soil

Mycorrhizal inoculum potential (MIP) bioassay was performed to verify the activity of AMF propagules occurring in the soil of each sample and was assessed using *Cichorium intybus* L. as host plant. Five 50 ml replicate tubes were filled with 40 ml of sampled field soil and sown using the biotest plant. Then they were put in sun-transparent bags and maintained in a growth chamber at 25 °C under a 16/8 h light/dark daily cycle. Four days after germination, plants were thinned to three per tube and harvested 28 days after sowing, by removing roots from soil and washing them with tap water. Roots were then cleared in 10% KOH in a 80 °C water bath for 15 min and stained with Trypan blue in lactic acid (0.05 %) after 10 min in 2 % aqueous HCl. The percentage of AMF colonization was calculated using a dissecting microscope at x25 or x40 magnification and the gridline intersect method (Giovannetti and Mosse 1980).

Evaluation of the symbiotic performance of native AMF communities

On the basis of similarity indices of their soil properties, plots 2, 5, 9, 10, 14 and 22 were selected to set up the microcosm experiments aimed at evaluating the performance of three host plants: *A. cepa* cv. Rossa dolce di Tropea (onion), *C. annuum* cv. Rosso quadrato d'Asti (pepper) and *L. sativa* cv. Foglia di quercia (lettuce) (Fig. 1). Onion, pepper and lettuce were seeded in a sterile calcinated clay (OILDRI Chicago, IL, USA) and grown in a growth chamber at 25 °C under a 16/8 h light/dark daily cycle. Ten days after sowing plantlets were transferred to 8-cell trays (5 cm diameter), which were filled with soil from the 6 field plots selected as described above. Soil from each plot represented a different native AMF treatment, hereafter named with the number of the original soil plot. Three replicate trays of each host plant species were set up for the 6 native AMF treatments. Onion, pepper and lettuce plants were grown in a glasshouse for 8, 5 and 6 weeks, respectively, under ambient natural light and temperature conditions (min T 15-20°C , max T 27-33°C), relative humidity from 50 to 80 % and supplied with tap water as needed. The differential harvest times were chosen on the basis of the length of host life cycles.

At harvest, plants were analysed for shoot dry matter and shoot N and P concentrations (Jones et al. 1991). Roots from the 8 plants of each replicate tray were collected and pooled to assess mycorrhizal root colonization, using the protocol described above for MIP analysis. Aliquots (100 mg) of the pooled roots were collected for DNA extraction and stored at −80 °C.

155

156 **Molecular analyses**

157 The root samples of three replicates of onion, pepper and lettuce from native AMF treatments 2 and 22 were used
158 for DNA extraction (Fig. 1). Genomic DNA was obtained from roots ground in liquid nitrogen by using DNeasy
159 Plant Mini Kit (Qiagen Milan, Italy), and 1 µl of extracted DNA was used as template in PCR reactions. Partial
160 small subunit (SSU) of ribosomal RNA gene fragments were amplified in volumes of 25 µl with 0.125 U of GoTaq
161 G2 Flexi DNA Polymerase (Promega, Milan, Italy), 0.4 µM of each primer (AML1/AML2, Lee et al. 2008), 0.2
162 mM of each dNTP, 1.5 mM of $MgCl_2$ and 1× the manufacturer's reaction buffer. The thermal cycler (Eppendorf
163 Mastercycler personal, Eppendorf, Milan, Italy) was programmed as follows: a manual hot start at 94 °C for 3 min,
164 30 cycles at 94 °C for 30 s, 58 °C for 40 s, 72 °C for 55 s and a final extension step at 72 °C for 10 min. Reactions
165 yields were estimated by using a 1 % agarose gel containing ethidium bromide (0.5 µg ml^{-1}). Wizard® SV Gel and
166 PCR Clean-up system (Promega) was used to purify amplicons from onion, pepper and lettuce roots, which were
167 then ligated into pGem-T Easy vector (Promega) to transform XL10-Gold Ultracompetent *Escherichia coli* cells
168 (Stratagene, La Jolla, CA, USA). The composition of the AM fungal communities was determined using PCR-RFLP
169 screening of clone libraries (AML1/AML2 primers and HinfI and AluI restriction enzymes). Fourty clones per clone
170 library were screened by PCR-RFLP analysis. Plasmids of representative clones of each RFLP pattern in each
171 library were purified by Wizard® Plus SV Minipreps (Promega) and sequenced using T7 vector primers at GATC
172 Biotech (Köln, Germany). The new unique cloned sequences reported in this study have been deposited in the
173 'European Nucleotide Archive' (http://www.ebi.ac.uk/ena) under the accession numbers LT856601-LT856682
174 (study_id PRJEB21051).

175

176 **Bioinformatics**

177 Sequences from *E. coli* libraries were edited in MEGA 6.0 and their similarities were determined using the Basic
178 Local Alignment Search Tool (BLASTn) provided by NCBI. The detection of chimeric sequences was performed
179 using USEARCH 6.0 (http://fungene.cme.msu.edu/FunGenePipeline/chimera_check/form.spr). Sequences were
180 aligned with those corresponding to the closest matches from GenBank as well as with sequences from major clades
181 of Glomeromycotina using MUSCLE as implemented in MEGA6. Phylogenetic trees were inferred by Neighbour-
182 joining analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method.
183 The confidence of branching was assessed using 1000 bootstrap resamplings.

184

185 **Statistical analyses**

Chemical and physical data of soil plots were used to compute Euclidean similarities indices in PAST 3.0 after standardization. Root colonization (after arcsin transformation) and shoot dry weight were analysed by two way ANOVA and means were separated by simple main effect test with Sidak adjustment. Nutrient content data did not fulfill two way ANOVA assumptions and were analyzed by one way ANOVA followed by Tukey HSD for mean separation. Analyses were carried out in IBM SPSS statistics version 24 software (IBM Corporation, Armonk, NY, USA).

Estimates of community diversity were determined as richness (S), bias-corrected Chao1 richness, Shannon (H) and Simpson diversity (1-D) index and evenness (e^H/S). The indices were calculated using PAST 3.0 and 1000 bootstraps were used to determine confidence intervals. Non parametric Kruskall-Wallis analysis was used to determine differences in the diversity indices among AMF communities colonizing the roots of the three different plant species grown in treatments 2 and 22. We determined the rarefaction curves with PAST 3.0 software to estimate whether the number of screened sequenced were sufficient to capture AMF diversity of each host. AMF communities were also evaluated by two ways permutational multivariate analysis of variance (PERMANOVA), to test the effects of soil microcosm and the host plant species, performed in PAST 3.0. Multivariate analyses (PCA and RDA) were performed using Canoco 5.0.

Results

Analyses of soil samples

Soil physical and chemical characteristics

Soil physical and chemical analyses showed that the experimental site had a sandy loam soil texture with low available P (Olsen) values (Table 1). A low degree of variability was detected among the different plots; for example, N concentration ranged in most plots from 1.55‰ to 1.91‰, showing the lowest (1.32‰) and highest (2.23‰) values in plots 17 and 21, respectively. Organic matter ranged from 2.5% to 2.8% in the majority of plots, with minimum and maximum of 1.63% and 3.01%, in plots 17 and 5, respectively. However, Euclidean similarity indices of soil properties ranged from 0.84, in the case of the plot pair showing the highest similarity (9 vs. 14), to 7.07, in the case of the plot pair with the lowest similarity (17 vs. 21). In order to select the most similar plots, in terms of physical and chemical soil properties, we sorted all the pairwise comparison by similarity, and selected five pairs (2/5, 9/10, 9/14, 10/14, 2/22) with similarity values ranging from 0.84 to 1.82, involving 6 out of 12 field plots. The soil from the 6 selected field plots was used to set up the native AMF treatments for the plant performance experiment.

MIP bioassay

217 The analysis of the activity of AMF soil propagules, as assessed by the MIP bioassay, showed no significant
218 differences among the soil originating from all 12 plots ($F_{11, 33}$, P=0.81). The percentage of mycorrhizal root length
219 of biotest plants (*C. intybus*) varied from 37.7 ± 2.9 to 55.7 ± 3.7, showing a good level of AMF activity in all plots.
220
221 **Plant performance in microcosm experiment**
222 The percentage of colonized root length was affected by host plant species and native AMF treatments and their
223 interaction (Table 2). Colonization levels in onion, the most mycotrophic plant of the experiment, were significantly
224 higher than those detected in lettuce and pepper (Table 3).
225 Significantly different growth responses among native AMF treatments were observed within each of the
226 three plant species (Table 2). Lettuce plants grown in treatment 22 showed the highest shoot dry weight (SDW),
227 while the lowest SDW value was observed in treatment 2 (Table 3). Consistently with the previous results, the
228 highest and lowest SDW values of pepper plants were in treatments 22 and 2, respectively. Statistically significant
229 differences were observed also in the growth of onion plants, with the highest and lowest biomass in treatment 5 and
230 2 (Tables 2, 3).
231 Native AMF treatments produced significant differences in N shoot content of lettuce and pepper (Table 2).
232 The highest N contents were detected in all host plants growing in treatment 22, while the lowest contents were
233 found in treatment 2 (lettuce and onion), and in treatment 14 (pepper) (Table 3).
234 Statistically significant differences in P content, among native AMF treatments, were detected in lettuce
235 and pepper (Table 2). The lowest values were shown by plants grown in treatment 2, while the highest occurred in
236 treatments 22, and 5, depending on the plant species (Table3).
237 In order to select the root samples for molecular analyses, we evaluated plant responses, in terms of plant
238 growth and nutrient uptake, obtained in the different treatments. To this aim, the selected five treatment pairs
239 associated with the most similar soil in terms of physical and chemical properties, were compared for their plant
240 performance. A radar graph, obtained by adding the relative increases in SDW, N and P content values of each pair
241 for the three plant species, allowed the detection of treatments 2 and 22 as the pair showing the largest plant
242 differential performance (Fig. 2a). The principal component analysis (PCA) performed using the same plant
243 response variables supported the selection of treatments 2 and 22 for molecular analyses (Fig. 2b).
244
245
246 **Molecular identification of native AMF communities**

The DNA extracted from onion, lettuce and pepper roots of treatments 2 and 22 was successfully amplified using the primer pair AML1/AML2, obtaining a fragment of the expected size (~800 bp). A total of 720 clones from the 18 clone libraries were examined for the presence of the insert (40 clones/library) and 707 positive clones were screened by RFLP analysis, obtaining 19 RFLP groups. For each RFLP group, clones of the different libraries were sequenced accounting for a total of 214 sequences. BLAST analyses showed that all the sequences had a high similarity (97-100% identity) to glomeromycotan sequences. No chimeric sequences were found. All non-redundant sequences from the 18 clone libraries (82 out of 214) and 25 references from GenBank were used for neighbour-joining phylogenetic analyses (Fig. 3). After RFLPs, BLASTn and phylogenetic analyses, the sequences were grouped into 14 OTUs supported by a bootstrap value >84%. Of these, 10 belonged to the family Glomeraceae, one to Gigasporaceae, one to Claroideoglomeraceae and two to Paraglomeraceae. Among Glomeraceae, five genera (*Dominikia*, *Funneliformis, Rhizoglomus*, *Sclerocystis*, *Septoglomus*) were retrieved. *Racocetra*, *Claroideoglomus* and *Paraglomus* were the genera found for Gigasporaceae, Claroideoglomeraceae and Paraglomeraceae, respectively. The genus *Rhizoglomus* was the most abundant, accounting for 84% of the total sequences, followed by *Septoglomus* (6.1%), *Claroideoglomus* (3.5%), *Funneliformis* (2.8%) and *Paraglomus* (2.0%). *Dominikia*, *Sclerocystis* and *Racocetra* sequences corresponded to less than 1 % of total sequences.

Within the genus *Rhizoglomus*, one OTU (Rh3, 11.2% of the total sequences) was identified as *Rhizoglomus irregulare* (synonym *Rhizophagus irregularis*, basionym *Glomus irregulare*); the remaining three OTUs (Rh1, Rh2, Rh4) represented sequences of uncultured species (Table 4). Rh1 and Rh2 were the most abundant sequences, accounting for 54.5% and 16.1% of the total, respectively. One OTU (Fun1, 2.4% of total sequences) in the genus *Funneliformis* was identified as *Funneliformis mosseae* (Table 4), while sequences grouped in the OTU Fun2 (0.4% of total sequences) were close to *Funneliformis geosporus* (99% similarity) (Fig. 3). The remaining OTUs within Glomeraceae (Glo, Scle, Sept1 and Sept2) matched to sequences of either known (Dom, *Dominikia iranica* basionym *Glomus iranicum*), or unknown species (*Sclerocystis* sp., *Septoglomus* sp.). Sequences grouped in the OTU Clar showed a high similarity with *Claroideoglomus etunicatum* (Table 4), forming a unique clade together with the sequences of such species in the phylogenetic analysis (Fig. 3). OTUs Rac and Par2 matched sequences of *Racocetra fulgida* and *Paraglomus laccatum* (Table 4), and Par1 sequences showed high homology with sequences of uncultured *Paraglomus* species. MaarjAM database (http://maarjam.botany.ut.ee/, accessed on February 2017) was used to confirm the assignment of detected OTUs to sequences of Glomeromycotina. The number of analysed sequences was generally sufficient to capture the AMF diversity in the roots of most host plants as shown by rarefaction analyses, since the curves almost reached the asymptote (Online Resource 1).

AMF root community composition as affected by native AMF treatment and host plant species

The composition of AMF root communities differed significantly, when considering both the two native AMF treatments (2 and 22, P<0.001), the host plants (*A. cepa*, *C. annuus* and *L. sativa*, P<0.001), and the interaction between native AMF and host plants (P=0.024) as revealed by two-ways PERMANOVA analysis. We detected 6 and 13 OTUs in the roots of plants from treatments 2 and 22, respectively (Fig. 4). All the OTUs in treatment 2 were found also in 22, except one (Rac), matching *R. fulgida* sequences, but their frequencies were very different between the two treatments. This trend was confirmed by diversity indices values, which showed significant differences in richness (S), Chao and Shannon (H) diversity values related to the AMF communities occurring in the roots of all the host plants from treatment 2, compared with 22 (Table 5). Interestingly, in onion and lettuce, Simpson (1-D) index was significantly higher in AMF treatment 22 than in 2. Actually, root AMF communities found in treatment 2 were similar for all diversity parameters, while those found in treatment 22 showed a clear difference between pepper and onion and lettuce.

Overall, in treatment 2, Rh1 was highly dominant, (83% of all sequences), while the OTUs Rh2, Sept2, Fun1 (*F. mosseae*), Clar (*C. etunicatum*) and Rac (*R. fulgida*) were found at very low percentages (8.7%, 3.7%, 3.7%, 0.6%, 0.3%, respectively). Interestingly, lettuce hosted all six OTUs, while onion missed Clar and Rac, and pepper harbored only Rh2 in association with Rh1 (Fig. 4).

On the contrary, in treatment 22, we found a more homogeneous distribution of OTUs, as Rh1, Rh2, Rh3 occurred each in the range 22.5% - 25.4% of total sequence number. In treatment 22, onion and lettuce hosted the highest amounts of OTUs (12 and 11, respectively), while only 7 OTUs were detected in pepper roots, confirming the low species richness obtained in treatment 2 in this host plant (Fig. 4). Rh1 and Rh2 sequences were highly dominant in pepper (37.9% and 55.2%, respectively), while their frequencies were 17.8 and 10.2%, and 20.5 and 6%, in lettuce and onion, respectively. Other OTUs found in lettuce (Rh3, Rh4 and Clar) and in onion (Rh3, Sept2 and Clar) occurred with higher frequencies than in pepper. Moreover the AMF community composition change can be ascribed to the occurrence of further OTUs in lettuce and onion (Fun1, Fun2, Glo, Par1, Par2, Scle and Sept1), together representing 8.8% and 18.8% of the total sequences (Fig. 4).

Multivariate RDA analysis showed that 51.2% of the total variance (I and II axes) of AMF communities occurring in roots of onion, pepper and lettuce from treatments 2 and 22 could be explained by native AMF treatments and host species (Fig. 5). Moreover, Monte Carlo permutation tests on RDA confirmed that such variables significantly affected the composition of root AMF communities (P=0.002).

Discussion

309 This work shows that diverse whole native arbuscular mycorrhizal communities, occurring across a 100 m long
310 field, differentially affected growth and nutrition of three crop plant species, *A. cepa*, *C. annuum* and *L. sativa*. Our
311 data revealed that plant performance varied depending on the identity of host plants and fungal symbionts, more
312 than on a rich and diversified native AMF community.
313
314 **Plant performance in the different whole native AMF community treatments**
315 The six different native AMF treatments showed variable plant performances in the three host species. Treatment 22
316 produced, in most cases, the highest plant SDW, N and P content, compared with the other five native AMF
317 treatments. In particular, it significantly enhanced pepper and lettuce SDW, by 57 and 119%, respectively, compared
318 with treatment 2. Treatment 22 was the most efficient in N shoot uptake, although the increases in N levels varied in
319 the different plant species (18, 37 and 98% in onion, pepper and lettuce, respectively), compared with treatment 2. P
320 contents followed the same trend, with variable increases depending on the identity of the host plant. Differential
321 plant responses to AMF (functional host preference, see Walder and van der Heijden 2015) may be explained by the
322 fact that during plant/fungal interactions plant gene expression is modulated depending on the plant and fungal
323 genotypes involved in the symbiosis (Feddermann et al. 2008; Hohnjec et al. 2005; Massoumou et al. 2007).
324 Here, intact whole native AMF communities were used for the evaluation of plant performance. Previous
325 works carried out experiments with diluted or modified native communities sampled from natural soils and utilized
326 as inocula (Burrows and Pfleger 2002; Ji et al. 2010; Moora et al. 2004; Uibopuu et al. 2012), while most studies
327 employed single isolated species or artificially assembled AMF communities (Gustafson and Casper 2006; Jansa et
328 al. 2008; Munkvold et al. 2004; van der Heijden et al. 1998). In this work, six selected native AMF treatments
329 showed variable effects on growth and nutrition of the diverse host plants, in agreement with other studies, where
330 different native AMF communities collected from grassland and forest or from young and old forest in Estonia,
331 produced differential plant performance in rare and common *Pulsatilla* species or in different plant species typical of
332 the Koeru boreonemoral forest (Moora et al. 2004; Uibopuu et al. 2012). Other authors showed that spores
333 originating from native AMF communities collected from serpentine and prairie grassland and used as inoculum
334 differed functionally, depending on their local host-soil environment (Ji et al. 2010). Interestingly our results were
335 obtained using native AMF originating from sampling points within a single field, in a very short distance, whereas
336 the communities analysed in the previous papers belonged to distant sites and to different biomes. Here, in order to
337 assess the relationship between plant performance and the native AMF communities originating from the diverse
338 plots and actually colonizing the three host plants, further molecular analyses were performed.
339

Molecular identification of native AMF communities

Overall, we detected 14 OTUs in the roots of host plants, a number similar to that found in maize roots growing in the same area (Turrini et al. 2016) and in other Mediterranean agroecosystems (Brito et al. 2012; Cesaro et al. 2008; Pivato et al. 2007). The detection of sequences of *C. etunicatum*, *F. geosporus*, *F. mosseae*, *P. laccatum*, *R. fulgida* and *R. irregulare*, whose spores were identified also by Njeru et al. (2015) in the same field site using a morphological approach, confirmed their occurrence in this AMF hot spot within the Biosphere Reserve "Selva Pisana". Although molecular analyses of whole roots showed a lower number of fungal phylotypes compared with laser microdissected arbusculated cells (Berruti et al. 2013), our work allowed the detection of additional ribotypes corresponding to uncultured species, confirming that studies exclusively based on spore morphological characterization are not sufficient to describe the whole biodiversity of AMF (Kivlin et al. 2011). Indeed, the number and diversity of spores occurring in soil are not always correlated with AMF actively colonizing roots (Oehl et al. 2005), as some AMF may occur in the soil only in the form of hyphae and colonized roots rather than spores (Abbott and Gazey 1994). Moreover, morphological identification of spores may be either difficult for their partial degradation or parasitization, or impossible as sporulation is seasonal-dependent in some AMF species (Helgason et al. 2002, Oehl et al. 2005). Actually, spore production was reported to depend on fungal physiological parameters and environmental conditions (Redecker 2002; Redecker et al. 2003). Our data suggest that a multidisciplinary approach is the best strategy for a complete assessment of AMF biodiversity in natural and agro-ecosystems (Oehl et al. 2010; Redecker et al. 2003).

Here, we retrieved sequences of *C. etunicatum*, *F. mosseae* and *R. irregulare*, considered generalist fast root and soil colonizers, frequently found in arable soils (Oehl et al. 2003, 2005). Interestingly, we detected also sequences of the rare species *R. fulgida*, which was reported to occur in coastal sand dunes not far from our experimental field (Błaszkowski et al 2004; Turrini et al. 2008). The finding of both generalist and rare species and of both cultured and uncultured taxa may be related to the climate of the Mediterranean Basin (where temperate and sub-tropical species co-occur), and also to the diversified land use history of the site during the past 40 years (Njeru et al. 2015). The area was occasionally used for crop production, but it was also left unploughed, allowing the development of a rich and diversified AMF community. Such findings agree with the results of a recent meta-analysis (Ohsowski et al. 2014) on the occurrence of cultured and uncultured Glomeromycotina in different habitats worldwide, reporting a greater association of cultured AM fungal taxa with human-impacted habitat and cultivated plants when compared to uncultured AMF, usually occurring in natural habitats and wild plants. In this perspective, the study site within the Mediterranean Basin, which is considered a hyper-hotspot of biodiversity (Myers et al. 2000), assumes a great importance for AMF biodiversity conservation.

AMF root community composition as affected by native AMF treatment and host plant species

The roots of plants growing in treatment 22 showed a more diversified AMF community, compared with treatment 2, where only one dominant species was detected, OTU Rh1, which represented 81-86% of the analyzed clones from the three plants of treatment 2. Thus, assuming the high frequency of such sequences as prognostic of a main role of this OTU, the lower plant performance reported in treatment 2, compared with treatment 22, could be ascribed to its low efficiency, irrespective of host plant identity. On the other hand, it can be speculated that in pepper the better performance in treatment 22 could be the result of the activity of a single distinct AMF OTU, as Rh2 dominance on Rh1 may have changed the final outcome of the symbiotic relationship. In lettuce and onion, hosting a richer AMF community, plant performance cannot be clearly ascribed to a single OTU, as an interaction among the different OTUs may have occurred. However, such diverse AMF assemblages in treatment 22 produced a better plant performance, compared with treatment 2, only in lettuce, confirming that AMF species identity may be more important than community diversity (Njeru et al. 2017; Vogelsang et al. 2006). Indeed, the occurrence of a diversified and rich AMF community has long been known to enhance plant growth and nutrition by different mechanisms, i.e by functional complementarity (Koide 2000; Maherali and Klironomos 2007), by buffering negative effects of either unfavorable abiotic and biotic stresses (Pringle and Bever 2002) or inefficient AMF species, which may persist in host roots within a heterogeneous AMF community (Hart et al. 2013).

Interestingly, in all plant roots from treatment 22, we found phylogenetically highly related sequences, affiliated to the genus *Rhizoglomus*, although with different frequencies, confirming the highly infective behaviour of *Rhizoglomus* spp. (Alkan et al. 2006; Jansa et al. 2008). Previous works reported that phylogenetic relatedness was positively associated with coexistence in the same root system and, interestingly, it was also positively associated with plant growth (Roger et al. 2013). Though, almost nothing is known about the factors that control AMF coexistence and competition in roots, which are processes depending on spatial scales, ecosystem types, host plant quality and identity (Davison et al. 2016; Knegt et al. 2016).

Our data show that root AMF community composition varied with the identity of the three host plants. Host plant identity has long been known to be one of the most important elements shaping AMF community composition (Gollotte et al. 2004; Helgason et al. 2002; Mummey and Rillig 2008; Scheublin et al. 2004; Sýkorová et al. 2007; Vandenkoornhuyse et al. 2003), more than habitat (Becklin et al. 2012), seasonality (Davison et al. 2011) or agricultural practices (Vályi et al. 2015). Some authors suggested that AMF communities are not random assemblages, but can differentially colonize the roots of ecologically diverse groups of plant species, depending on AMF specific characteristics, i.e. habitat generalist vs. forest specialist AMF (Davison et al. 2011). Recently,

distinct AMF communities were found associated with different plant species within the same mixed cover crops (*V. villosa* and *Trifolium* spp. vs *Avena* sp. and *P. tanacetifolia* and in the successive maize), revealing a strong effect of the host on AMF communities actually occurring in the roots (Turrini et al. 2016).

Conclusions

Molecular analyses of host plant roots revealed that the best plant performance was not necessarily associated with a richer and more diversified whole native AMF community. Indeed, AMF species belonging to Glomeraceae, Acaulosporaceae, Gigasporaceae and Diversisporaceae may differentially activate important core sets of symbiosis-associated genes, i.e. transport, defense, metabolic process (Fedderman et al. 2008; Massoumou et al. 2007), which can be related to the functionality of the symbiosis. It is tempting to speculate that the three host plants assessed in this experimental work may have benefited from the most efficient AMF species combinations in agreement with new insights into AMF/plant interaction and cooperation, suggesting that host plant reward the best fungal symbionts with a larger nutrient transfer (Kiers et al. 2011). Our results on the relationships between plant performance and whole native AMF communities diversity represent a starting point allowing the isolation, characterization and selection of single native AMF species from the "hot spot" site. Such genotypes could be further studied in order to identify the most efficient ones, to be used as single inoculants and/or as assemblages in sustainable food production systems.

Acknowledgments

This work was funded by the University of Pisa through the project "Molecular and functional biodiversity of plant associated microrganisms" and through Fondi di Ateneo and by the National Research Council of Italy. The authors wish to thank Prof. Marco Mazzoncini and Dr. Rosalba Risaliti for their precious help in preserving the field site and determining soil physical and chemical properties.

The authors declare that they have no conflict of interest.

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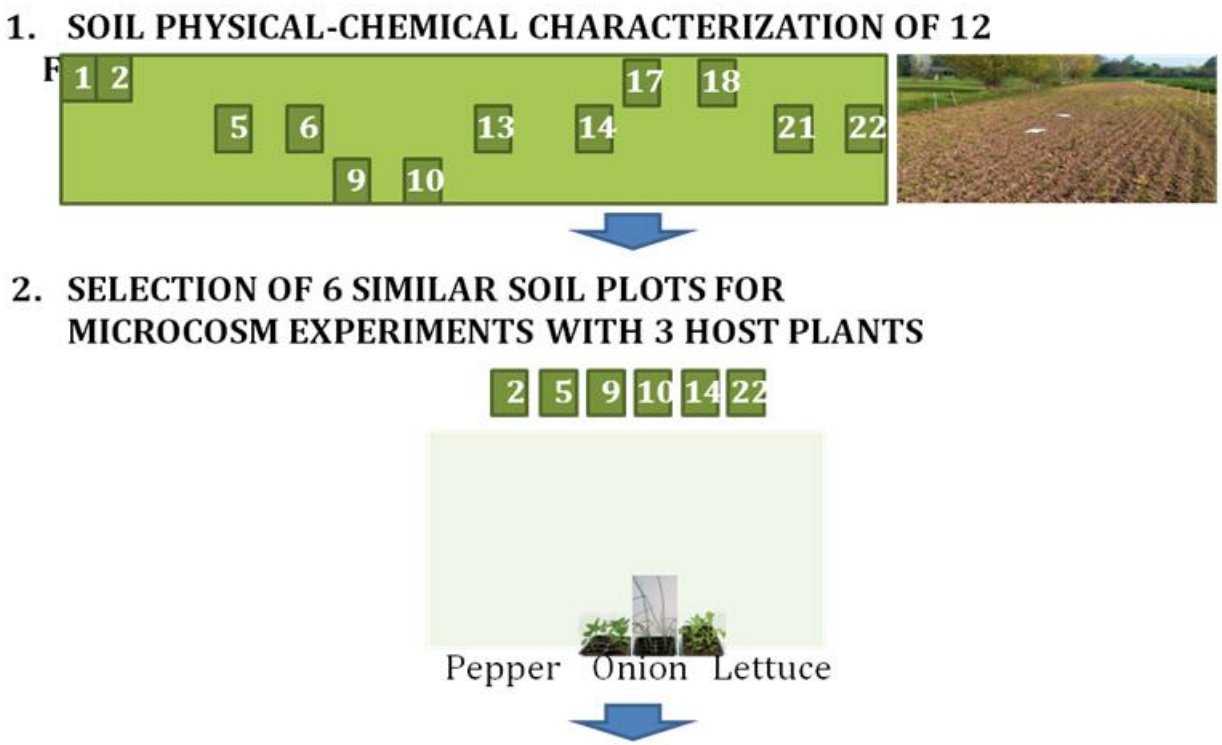
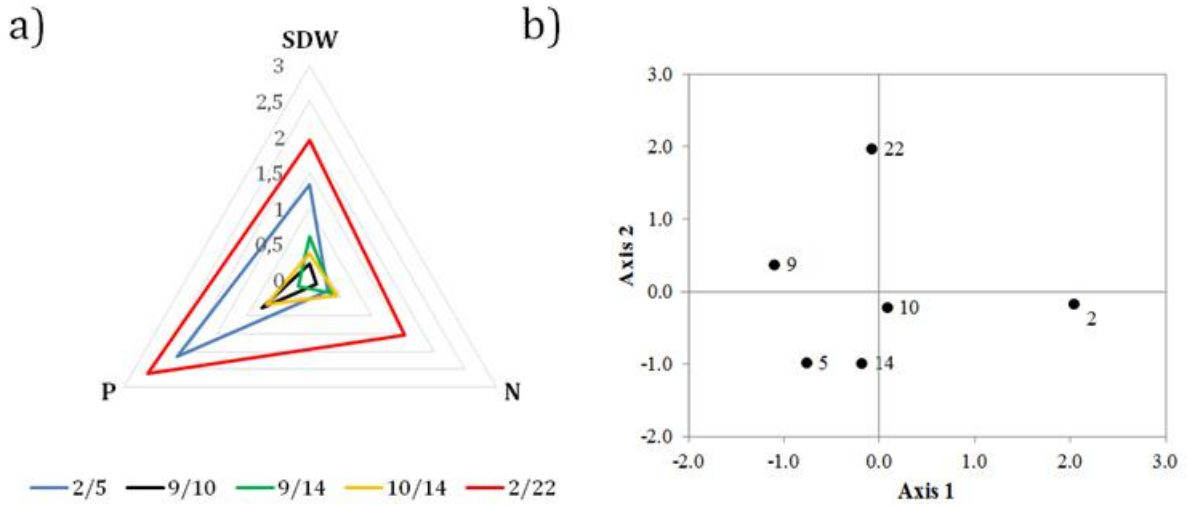


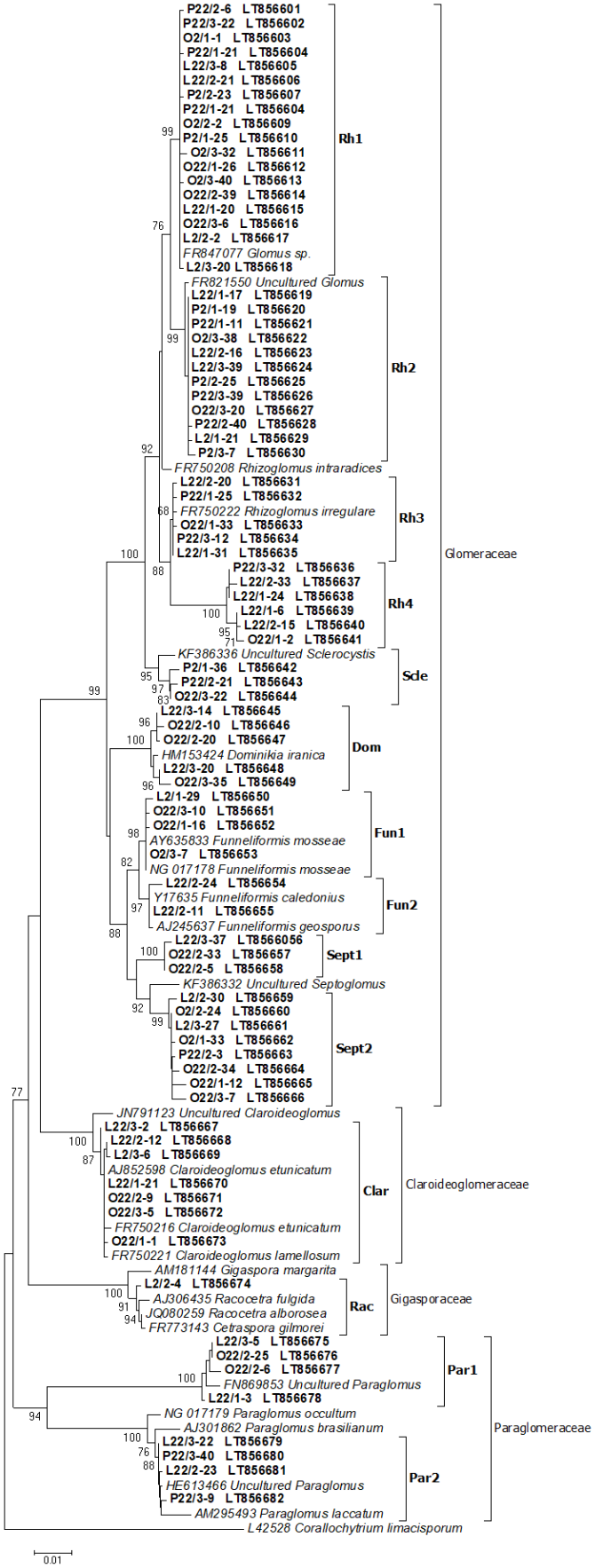
Fig 1 Flow chart showing 1) the experimental field with the 12 plots, characterized for their soil physical and chemical properties; 2) the six soil plots selected for uniform soil characteristics, used as native AMF treatments in microcosm experiments aimed at evaluating plant growth and nutrition of three host plants; 3) the two native AMF treatments, showing the most differential plant performance, selected for molecular analyses of AMF communities occurring in the roots.



642

643 **Fig 2** a) Radar graph representing the relative increases, for onion, pepper and lettuce, in shoot dry weight (SDW),
644 nitrogen (N) and phosphorous (P) content of five selected native AMF treatment pairs; b) results of principal
645 components analysis ordination of native AMF treatments, using scores of growth and nutrition variables for host
646 plants. The first axis explained 37.5%, the second axis 22.1% of variation.

647


P22/2-6 LT856601
P22/3-22 LT856602
O2/1-1 LT856603
P22/1-21 LT856604
L22/3-8 LT856605
L22/2-21 LT856606
P2/2-23 LT856607
P22/1-21 LT856604
O2/2-2 LT856609
P2/1-25 LT856610
O2/3-32 LT856611
O22/1-26 LT856612
O2/3-40 LT856613
O22/2-39 LT856614
L22/1-20 LT856615
O22/3-6 LT856616
L2/2-2 LT856617
FR847077 Glomus sp.
L2/3-20 LT856618
Rh1
FR821550 Uncultured Glomus
L22/1-17 LT856619
P2/1-19 LT856620
P22/1-11 LT856621
O2/3-38 LT856622
L22/2-16 LT856623
L22/3-39 LT856624
P2/2-25 LT856625
P22/3-39 LT856626
O22/3-20 LT856627
P22/2-40 LT856628
L2/1-21 LT856629
P2/3-7 LT856630
Rh2
FR750208 Rhizoglomus intraradices
L22/2-20 LT856631
P22/1-25 LT856632
FR750222 Rhizoglomus irregulare
O22/1-33 LT856633
P22/3-12 LT856634
L22/1-31 LT856635
Rh3
P22/3-32 LT856636
L22/2-33 LT856637
L22/1-24 LT856638
L22/1-6 LT856639
L22/2-15 LT856640
O22/1-2 LT856641
Rh4
KF386336 Uncultured Sclerocystis
P2/1-36 LT856642
P22/2-21 LT856643
O22/3-22 LT856644
Scle
L22/3-14 LT856645
O22/2-10 LT856646
O22/2-20 LT856647
HM153424 Dominikia iranica
L22/3-20 LT856648
O22/3-35 LT856649
Dom
L2/1-29 LT856650
O22/3-10 LT856651
O22/1-16 LT856652
AY635833 Funneliformis mosseae
O2/3-7 LT856653
NG 017178 Funneliformis mosseae
Fun1
L22/2-24 LT856654
Y17635 Funneliformis caledonius
L22/2-11 LT856655
AJ245637 Funneliformis geosporus
Fun2
L22/3-37 LT8566056
O22/2-33 LT856657
O22/2-5 LT856658
Sept1
KF386332 Uncultured Septoglomus
L2/2-30 LT856659
O2/2-24 LT856660
L2/3-27 LT856661
O2/1-33 LT856662
P22/2-3 LT856663
O22/2-34 LT856664
O22/1-12 LT856665
O22/3-7 LT856666
Sept2
Glomeraceae
JN791123 Uncultured Claroideoglomus
L22/3-2 LT856667
L22/2-12 LT856668
L2/3-6 LT856669
AJ852598 Claroideoglomus etunicatum
L22/1-21 LT856670
O22/2-9 LT856671
O22/3-5 LT856672
FR750216 Claroideoglomus etunicatum
O22/1-1 LT856673
FR750221 Claroideoglomus lamellosum
Clar
Claroideoglomeraceae
AM181144 Gigaspora margarita
L2/2-4 LT856674
AJ306435 Racocetra fulgida
JQ080259 Racocetra alborosea
FR773143 Cetraspora gilmorei
Rac
Gigasporaceae
L22/3-5 LT856675
O22/2-25 LT856676
O22/2-6 LT856677
FN869853 Uncultured Paraglomus
L22/1-3 LT856678
Par1
NG 017179 Paraglomus occultum
AJ301862 Paraglomus brasilianum
L22/3-22 LT856679
P22/3-40 LT856680
L22/2-23 LT856681
HE613466 Uncultured Paraglomus
P22/3-9 LT856682
AM295493 Paraglomus laccatum
Par2
Paraglomeraceae
L42528 Corallochytrium limacisporum
0.01

648

649 **Fig 3** Neighbour-Joining phylogenetic tree of glomeromycotan sequences derived from host plants growing on the
650 native AMF treatment 2 and 22. Bootstrap values are shown when they exceed 65 % (1000 replications). The
651 analysis is based on partial nuclear small subunit ribosomal RNA gene sequences (SSU; ~800 bp; AML1/AML2
652 fragment) and involved 107 nucleotide sequences. Different sequence types are indicated in brackets and names are
653 reported in Table 4. AMF families are also reported. Sequences obtained in the present study are shown in bold and
654 their accession numbers are prefixed with plant species/native AMF treatment/ clone identifiers (O = onion; L=
655 lattuce; P = pepper; 2 and 22 = native AMF treatments). The tree is rooted with a reference sequence of
656 *Corallochytrium lymacisporum* (L42528).
657

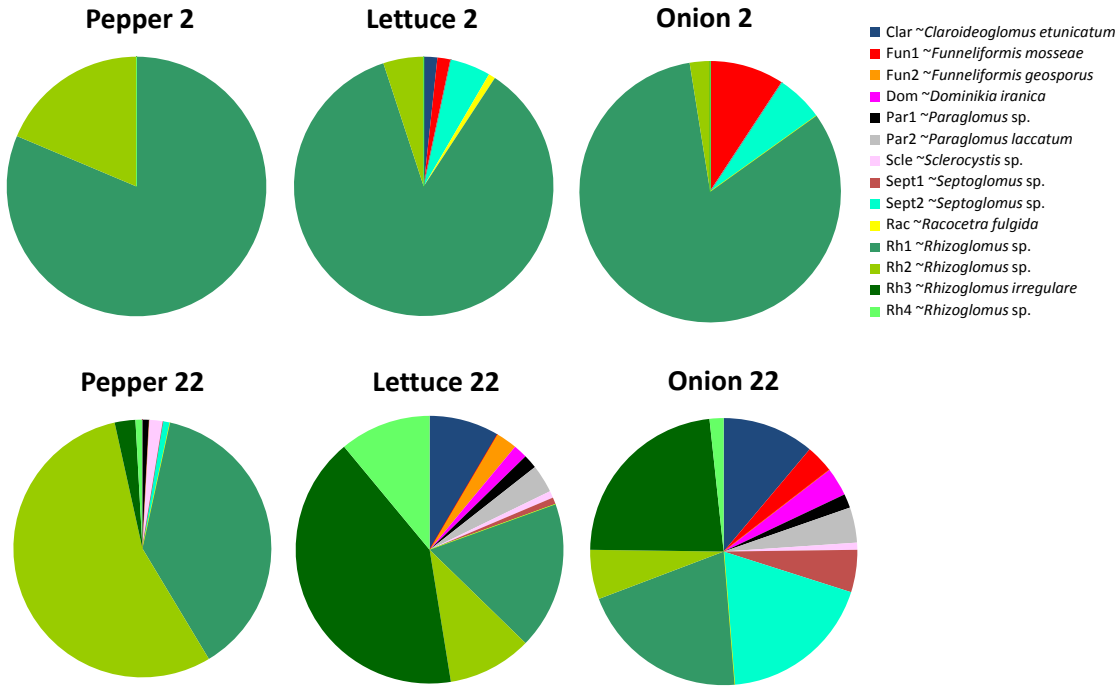
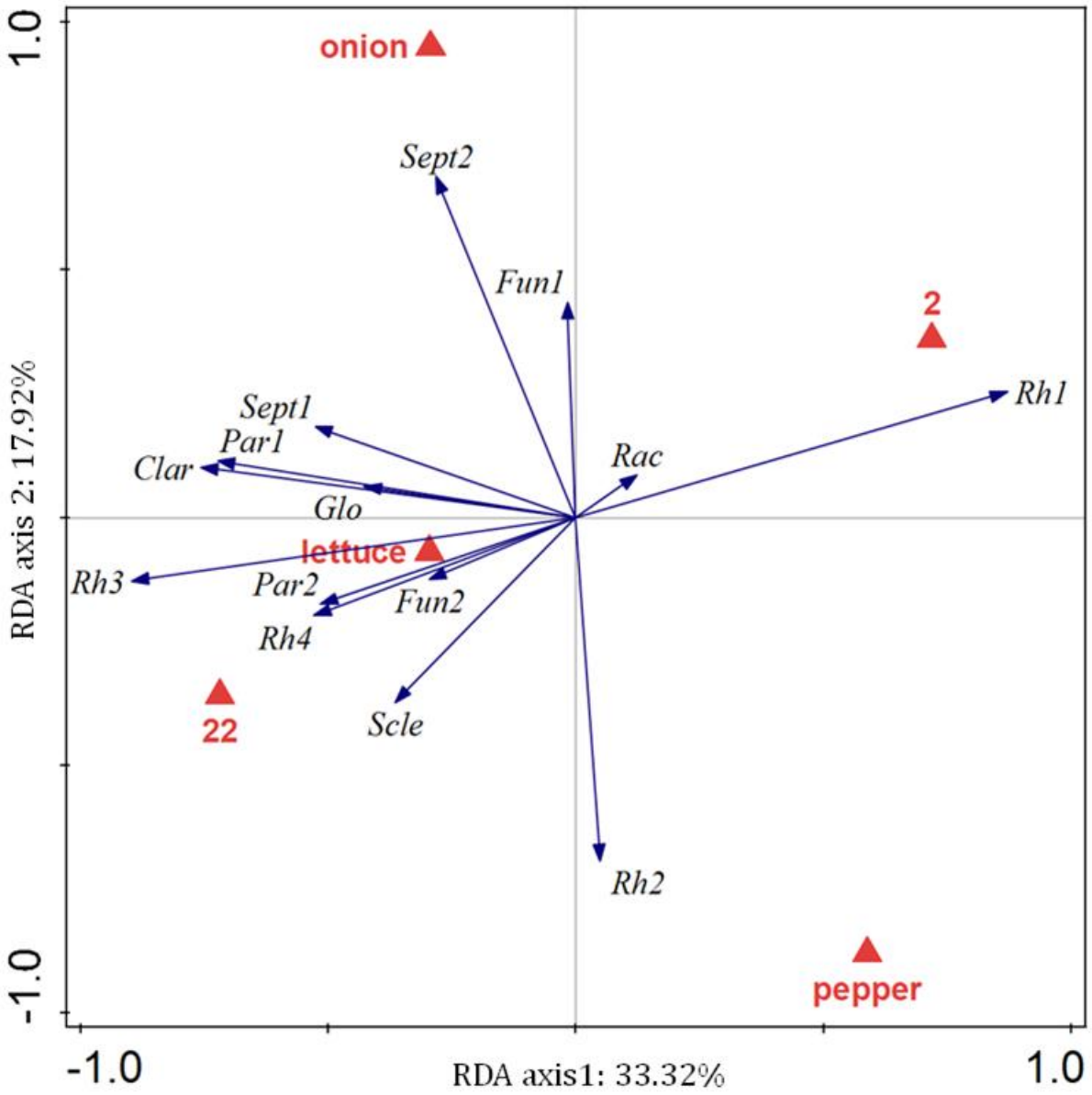


Fig 4 Relative abundance (%) of AMF OTUs detected in the roots of the different host plants (onion, pepper, lettuce), growing in native AMF treatments 2 and 22.



662

663 **Fig 5** Redundancy analysis (RDA) ordination biplot of AMF colonizing the three host species (onion, pepper and
664 lettuce), growing in the AMF soil treatments 2 and 22. The inoculation treatment and the host plants were used as
665 the explanatory variable, and the different OTUs were used as dependent variables. The names of the different
666 OTUs are reported in Table 4. The first and second axes explain 51.2% of total variance.

667

668

669 **Table 1** Descriptive statistics of soil chemical and physical properties of the 12 plots inside the "hot spot" field in

670 the UNESCO Biosphere Reserve.

Soil property	pH	CEC (cmol kg-1)	Total N (‰)	Organic matter (%)	Olsen P (μg g^{-1})	Clay (%)	Silt (%)	Sand (%)
Mean	7.43	12.71	1.70	2.58	6.13	15.4	16.7	67.9
Median	7.44	13.62	1.64	2.64	6.00	15.4	15.6	69.5
Minimum	6.90	10.15	1.32	1.63	5.11	11.9	13.4	57.5
Maximum	8.02	15.36	2.23	3.01	7.33	20.9	24.9	74.3
SE[a]	0.12	0.55	0.07	0.12	0.22	0.72	1.01	1.42
CV[b]	5.6%	15.0%	13.4%	15.5%	12.3%	16.1%	20.9%	7.2%

671 [a] SE (Standard Error); [b] CV (Coefficient of Variability)

672

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Maximum	8.02	15.36	2.23	3.01	7.33	20.9	24.9	74.3
SE[a]	0.12	0.55	0.07	0.12	0.22	0.72	1.01	1.42
CV[b]	5.6%	15.0%	13.4%	15.5%	12.3%	16.1%	20.9%	7.2%

675 [a] SE (Standard Error); [b] CV (Coefficient of Variability)

676

677 **Table 2** Summary of (a) two way ANOVA testing the effects of native AMF treatments and host species on root
678 colonization and plant growth, and (b) one way ANOVA testing the effects of native AMF treatments on nutrient
679 content of each host species.

a)		**Colonized root length (%)**		**Shoot dry weight (mg plant^{-1})**	
Source of variation	df	F	P	F	P
native AMF treatment	5,36	4.9	0.001	17.2	<0.001
host species	2,36	249.5	<0.001	24.9	<0.001
Interaction	10,36	3.6	0.002	2.9	0.008
b)		**Shoot N content (mg plant^{-1})**		**Shoot P content (mg plant^{-1})**	
	df	F	P	F	P
Allium cepa	5,12	0.725	0.618	1.5	0.273
Capsicum annuum	5,12	12.3	<0.001	4.9	0.011
Lactuca sativa	5,12	10.6	<0.001	10.1	0.001

680

Table 3 Shoot dry matter, nutritional parameters and percentage of mycorrhizal colonization in the roots of *Allium cepa*, *Capsicum annuum* and *Lactuca sativa*, grown in 6 microcosms using soils from different plots of the field site within UNESCO Biosphere Reserve, showing the highest similarity in soil physical and chemical characteristics. In columns, means (± standard error of the mean) within each plant species followed by different lowercase letters are significantly different (P<0.05).

Plant species	**native AMF treatment**	**SDW[a] (mg plant^{-1})**	**Shoot N content (mg plant^{-1})**	**Shoot P content (mg plant^{-1})**	**Colonized root length (%)**
Allium cepa	2	45.7 ± 1.8 bA[b]	0.66 ± 0.02 a	0.12 ± 0.012 a	90.4 ± 2.2 aA
(onion)	5	61.4 ± 7.0 aA	0.72 ± 0.06 a	0.18 ± 0.019 a	86.7 ± 2.6 aA
	9	54.5 ± 1.2 abA	0.68 ± 0.02 a	0.13 ± 0.009 a	92.0 ± 0.6 aA
	10	53.7 ± 4.0 abA	0.72 ± 0.06 a	0.13 ± 0.032 a	89.8 ± 0.8 aA
	14	49.2 ± 3.4 abA	0.68 ± 0.03 a	0.14 ± 0.004 a	86.6 ± 3.9 aA
	22	54.4 ± 4.0 abB	0.78 ± 0.07 a	0.15 ± 0.002 a	90.0 ± 3.6 aA
Capsicum annuum	2	47.5 ± 2.8 cA	0.76 ± 0.04 b	0.07 ±0.007 c	55.6 ± 4.6 abB
(pepper)	5	56.5 ± 2.3 bcA	0.77 ± 0.05 b	0.12 ±0.009 a	66.8 ± 3.3 aB
	9	61.8 ± 3.9 abA	0.76 ± 0.04 b	0.10 ±0.006 abc	38.9 ± 1.3 bB
	10	54.7 ± 3.7 bcA	0.74 ± 0.08 b	0.07 ±0.015 bc	49.7 ± 2.2 abB
	14	49.3 ± 4.0 bcA	0.61 ± 0.03 b	0.09 ±0.006 abc	41.4 ± 4.7 bB
	22	74.5 ± 3.7 aA	1.04 ± 0.04 a	0.11 ±0.004 ab	39.5 ± 1.2 bC
Lactuca sativa	2	25.2 ± 1.0 cB	0.44 ± 0.03 b	0.04 ± 0.003 b	61.6 ± 4.7 aB
(lettuce)	5	45.3 ± 1.7 abB	0.52 ±0.09 b	0.08 ± 0.011 ab	55.7 ± 5.1 abB
	9	51.9 ± 2.6 abA	0.61 ± 0.05 b	0.11 ± 0.010 a	41.2 ± 5.7 bB
	10	48.3 ± 2.4 abA	0.63 ± 0.02 b	0.08 ± 0.003 ab	45.5 ± 5.9 abB
	14	41.5 ± 0.3 bA	0.55 ± 0.03 b	0.10 ± 0.013 a	47.1 ± 2.7 abB
	22	55.3 ± 1.7 aB	0.87 ± 0.02 a	0.11 ± 0.005 a	57.9 ± 2.4 abB

[a] SDW (Shoot Dry Matter)

[b] In columns, means for each native AMF treatment, followed by different uppercase letters, are significantly different (P<0.05).

690 **Table 4** Sequence types of arbuscular mycorrhizal fungi, identified using AML1-AML2 primers pair, in the roots of
691 onion, pepper and lettuce plants from treatments 2 and 22, using native soil collected from two different plots inside
692 the "hot spot" field within the UNESCO Biosphere Reserve "Selva Pisana".

Sequence type	Identity (%)
Rh1	FR821553 (99%)
Rh2	JX296753 (99%)
Rh3	FR750222 (99%)
Rh4	JN791150 (98%)
Scle	KF386336 (98%)
Dom	HM153420 (99%)
Fun1	NG017178 (99%)
Fun2	AJ245637 (99%)
Sept1	KF386332 (99%)
Sept2	FR848639 (98%)
Rac	AJ306435 (100%)
Clar	AJ852598 (99%)
Par1	FN869853 (99%)
Par2	AM295493 (100%)

693 **Table 5** Diversity indices of AMF communities occurring in the roots of different plant species from treatments 2 and 22 (maens± SEM).

Treatment	Plant species	Taxa (S)	Simpson (1-D)	Shannon (H)	Evenness (e^H/S)	Chao
2	*Allium cepa*	2.67 ± 0.54a	0.26 ± 0.13a	0.49 ± 0.24a	0.65 ± 0.03a	2.67 ± 0.54a
	Lactuca sativa	3.00 ± 0.00a	0.25 ± 0.02a	0.47 ± 0.04a	0.54 ± 0.02a	3.33 ± 0.14a
	Capsicum annuum	2.00 ± 0.00a	0.25 ± 0.01a	0.39 ± 0.01a	0.76 ± 0.01a	2.00 ± 0.00a
	P*	0.135	0.874	0.874	0.427	0.118
22	*Allium cepa*	7.67 ± 0.54b	0.82 ± 0.00b	1.83 ± 0.02b	0.82 ± 0.04a	8.00 ± 0.82b
	Lactuca sativa	7.33 ± 0.27b	0.72 ± 0.03b	1.55 ± 0.07b	0.65 ± 0.02a	8.00 ± 0.61b
	Capsicum annuum	4.33 ± 0.27b	0.51 ± 0.02a	0.89 ± 0.03b	0.57 ± 0.02a	5.67 ± 0.95b
	P*	0.051	0.027	0.039	0.039	0.417

694 Values followed by different letters are significantly different (P≤0.05) among plant species within treatments after Kruskal Wallis test.

695 * P represents the significance level of the Kruskal Wallis test within each native AMF treatment.

696