

1 **Impact of nitrogen fertilization and soil tillage on arbuscular mycorrhizal fungal**
2 **communities in a Mediterranean agroecosystem**

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16 diversity; Trap cultures.

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22 **Abstract**

23 The impact of nitrogen (N) fertilization and tillage on arbuscular mycorrhizal fungi (AMF) was
24 studied in a Mediterranean arable system by combining molecular, biochemical and
25 morphological analyses of field soil and of soil and roots from trap plants grown in microcosm.
26 Canonical correspondence analysis (CCA) of PCR-DGGE banding patterns evidenced marked
27 differences between AMF communities from N-fertilized and unfertilized field plots, which were
28 further differentiated by tillage. N-fertilization was also the main factor affecting AMF
29 communities occurring in *Medicago sativa* trap plant soil and roots. The overall sporulation
30 pattern of the different AMF species showed a predominant effect of tillage, as shown by CCA
31 analysis, which clearly discriminated AMF communities of no-tilled from those of tilled soil.
32 *Funneliformis mosseae* was the predominant species sporulating in tilled soils, while *Glomus*
33 *viscosum* and *Glomus intraradices* prevailed in no-tilled soils. Field glomalin-related soil protein
34 content was reduced by tillage practices. Our multimodal approach, providing data on two main
35 production factors affecting soil AMF communities, may help implementing effective agricultural
36 management strategies able to support the beneficial relationship between crops and AM fungi.

37

38 **1. Introduction**

39 Arbuscular mycorrhizal (AM) fungi (AMF) establish symbiotic associations with most
40 crop plants and play a fundamental role in plant growth, soil fertility and productivity, delivering
41 many essential ecosystem services (Gianinazzi et al., 2010). AM fungal hyphae spread from host
42 roots to the surrounding soil, developing an extensive mycelial network, crucial to the uptake of
43 nutrients, mainly phosphorus (P), nitrogen (N), copper (Cu) and zinc (Zn) (Giovannetti and Avio,
44 2002; Smith and Read, 2008; Blanke et al., 2011). Many AM fungal isolates increase plant
45 tolerance to root pathogens, pests and abiotic stresses, such as drought and salinity (Augé, 2001;
46 Evelin et al., 2009; Sikes et al., 2009) and increase the synthesis of beneficial plant secondary
47 metabolites, thus contributing to the production of safe and high quality food (Ceccarelli et al.,

48 2010; Giovannetti et al., 2012). Moreover, AMF contribute to soil C sequestration and organic
49 matter conservation by means of the extensive mycelial network producing large quantities of a
50 sticky proteinaceous hydrophobic substance, glomalin, that accumulates in soil as glomalin-
51 related soil protein (GRSP) (Rillig and Mummey, 2006; Bedini et al., 2009), and of other
52 recalcitrant polymers, such as chitin and chitosan (Zhu and Miller, 2003; Fortuna et al., 2012).

53 Several studies have demonstrated that different crop management systems involving high
54 intensity of mechanization or high inputs of chemicals may affect AMF species composition or
55 show a negative impact on AMF spore abundance and mycorrhizal colonization, often leading to a
56 reduction of AMF benefits to crop production and soil quality (Douds et al., 1995; Jansa et al.,
57 2002; 2003; Oehl et al., 2004; Castillo et al., 2006; Brito et al., 2012). Indeed, deep ploughing, by
58 disrupting the hyphae of the mycorrhizal network (Kabir, 2005), may differentially affect AMF
59 taxa, which show differential activity and functioning (Klironomos, 2003; Munkvold et al., 2004;
60 Avio et al., 2006). On the other hand, soil chemical fertilization may affect AMF growth and
61 colonization ability by altering the concentration of soil mineral nutrients and shifting the N:P
62 ratio of plant tissues, which in turn may stimulate the growth of AMF populations more adapted to
63 the new nutritional conditions (Johnson et al., 2003; Na Bhadalung et al., 2005; Toljander et al.,
64 2008).

65 The data available on the impact of different levels of tillage and chemical fertilization on
66 AMF community composition and dynamics indicate that such major production factors should be
67 tested in dedicated experimental arable systems, in order to reach a better understanding of the
68 driving forces that shape AM fungal communities and to implement effective agricultural
69 management strategies supporting crop plant-beneficial soil microorganisms.

70 The aim of the present study was to evaluate the impact of N-fertilization and tillage on
71 AMF abundance and diversity, focusing on a long-term experimental site in a Mediterranean
72 arable system. To this aim, we combined molecular, biochemical and morphological analyses to
73 assess: i) AMF diversity in field soil, by means of polymerase chain reaction (PCR)-denaturing

74 gradient gel electrophoresis (DGGE) analysis of 18S rRNA gene fragments, a molecular
75 fingerprinting technique widely used to detect the modifications induced by different factors on
76 soil microbes (Smalla et al., 2001; Castaldini et al., 2005; Oliveira et al., 2009); ii) AMF
77 abundance and diversity, by means of morphological and molecular identification of spores
78 produced in trap plants grown in microcosm, a technique providing newly produced spores
79 suitable for morphological identification (Oehl et al., 2003; Oehl et al., 2004; Yao et al., 2010); iii)
80 AMF diversity in soil and roots of trap plants, by means of PCR-DGGE analysis of 18 S rRNA
81 gene fragments; iv) GRSP content in field and trap cultures soil.

82

83 **2. Materials and methods**

84 *2.1. Study site and soil sampling*

85 The study was conducted at the “Pasquale Rosati” experimental farm near Agugliano,
86 Italy, (43° 32’N, 13° 22’E, 100 m a.s.l., slope 10%). The soil is a calcareic gleyic cambisol almost
87 free of gravel, with a high clay and calcium content. The climate is dry-summer subtropical
88 (Mediterranean), with a mean annual rainfall in the period 1998-2008 of 786 mm. The highest
89 mean monthly temperature (30.6°C) and the lowest precipitation (35 mm) occurred in July. The
90 lowest mean monthly temperature (3.0°C) occurred in January and the highest precipitation (105
91 mm) in September (De Sanctis et al., 2012). The experimental site belong to a long term tillage
92 experiment, established in 1994, with a two year rotation of maize (*Zea mays* L.) and durum
93 wheat (*Triticum durum* L.) since 2002, and designed as a split plot with tillage treatments assigned
94 to the main plots (each 1500 m² in size) and N-fertilization treatments assigned to subplots (each
95 500 m² in size). The experiment was replicated in two blocks with treatments repeated in the same
96 plots every year. In the present study, soil sampling was performed in the subplots treated with no
97 N-fertilization (0) and 90 kg ha⁻¹ N (90) as ammonium nitrate, both in the conventional tillage
98 (CT) and in the no tillage (NT) treatment. CT treatment consisted of ploughing at a depth of 40
99 cm and double harrowing before sowing, whereas NT plots were left undisturbed except for sod

100 seeding, crop residuals and weed chopping and total herbicide spraying prior to seeding. For data
101 on crop yield and soil characterisation, see De Sanctis et al. (2012).

102 After wheat harvest the experimental area was sampled in Autumn 2006 by randomly
103 collecting four 15 cm deep soil cores from each of the eight subplots. The four soil cores were
104 pooled to obtain samples of about 2.0 kg which were air-dried and stored at 4°C until processed.
105 Two hundred grams of each sample were used for GRSP analysis and the remaining soil for
106 establishment of trap cultures. For field soil DNA analysis, soil samples (three replicates) were
107 taken from one subplot of the four relevant treatments, for a total of twelve samples, then stored at
108 -20°C until processed.

109

110 2.2. Trap cultures and spore analysis

111 Each soil sample was mixed, 1:1 by volume, with Terragreen (calcined attapulgite clay, Oil
112 Dri, Chicago, IL), and poured into four 750 cm³ plastic pots, two for each of the two trap plant
113 species utilized, *Z. mays* and *Medicago sativa* L. Plants were grown in glasshouse, under ambient
114 natural light and temperature conditions and supplied with tap water as needed. In addition, they
115 received weekly fertilization with half strength Hoagland's solution (10 mL per pot). After six
116 months' growth, three soil samples (10 g each) were collected from each pot and processed. AMF
117 spores and sporocarps were extracted by wet-sieving and decanting, using a set of nested sieves,
118 down to a mesh size of 50 µm (Gerdemann and Nicolson, 1963), then flushed into Petri dishes
119 and examined under a dissecting microscope (Wild, Leica, Milano, Italy). The spores were
120 separated into groups, according to their morphology. Spores were isolated by using capillary
121 pipettes, mounted on microscope slides in polyvinyl alcohol lacto-glycerol (PVLG) and in PVLG
122 + Melzer's reagent (1:1, v:v) and examined under a Polyvar light microscope (Reichert-Young,
123 Vienna, Austria). Qualitative spore traits (spore shape, colour and size, spore wall structure and
124 shape, colour and size of the subtending hypha) were examined on at least 50 spores for each
125 morphotype. Morphotype identifications were based on original descriptions and current species

126 descriptions available online (International Culture Collection of (Vesicular) Arbuscular
127 Mycorrhizal Fungi [[http://invam.caf.wvu.edu/fungi/taxonomy/ speciesID.htm](http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm)]; Prof. Janusz
128 Blaszkowski website at Szczecin University [<http://www.zor.zut.edu.pl/Glomeromycota/>]). Since
129 important changes of AMF nomenclature have been recently proposed by different authors (Oehl
130 et al., 2011; Krüger et al., 2012), with some taxa inconsistently named, we utilized the new
131 binomials for consistent names and maintained the previous ones for the others.

132 After sixteen months' growth, three soil samples were collected from each pot and
133 processed as described above, with the aim of retrieving a higher number of species (Oehl et al.,
134 2009). The data reported are from such a sampling.

135

136 *2.3. Field soil DNA extraction*

137 DNA extraction was performed on 500 mg of each field soil sample, with the FastDNA[®]
138 Spin Kit for Soil (MP Biomedicals, Solon, OH) according to manufacturer's instructions, with
139 minor modifications: a double homogenization in the FastPrep[®] Instrument (MP Biomedicals) for
140 30 s at a speed setting of 6.0 and 25 s at a speed setting of 6.5, and a final resuspension in 100 µL
141 of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8). The DNA was then purified with the DNA
142 Clean Up Spin Kit (GENOMED GmbH, Löhne, Germany), according to manufacturer's
143 instructions.

144

145 *2.4. DNA extraction from roots and soil of trap cultures*

146 Soil and roots of *Z. mays* and *M. sativa* were collected from trap cultures six months after
147 establishment. Three samples of roots (100 mg) and soil (500 mg) were utilized for each plant
148 species and treatment. Root DNA was extracted in liquid nitrogen using DNeasy Plant Mini Kit
149 (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. Soil DNA was
150 extracted as described above.

151

152 2.5. DNA extraction from spores

153 Intact, healthy spores belonging to the following morphospecies were isolated from trap
154 cultures six months after establishment and utilized for DNA extraction: a) *Glomus viscosum*
155 T.H.Nicolson (pools of spores); b) *Glomus intraradices* N.C.Schenk & G.S.Sm. (pools of spores);
156 c) *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A.Schüssler (single spores and
157 sporocarps). Spores and sporocarps were manually collected with a capillary pipette under the
158 dissecting microscope and cleaned by sonication (120 s) in a B-1210 cleaner (Branson
159 Ultrasonics, Soest, NL). After three rinses in sterile distilled water (SDW), spores and sporocarps
160 were surface sterilized with 2% Chloramine T supplemented with streptomycin ($400 \mu\text{g mL}^{-1}$) for
161 20 min and rinsed five times in SDW. Spore clusters, spores and sporocarps were selected under
162 the dissecting microscope and transferred in Eppendorf tubes before DNA extraction (Redecker et
163 al., 1997).

164

165 2.6. DNA amplification

166 Aliquots of soil DNA (50 ng) were used to amplify the V3-V4 region of 18S rDNA using
167 the universal eukaryotic NS31GC primer (Kowalchuk et al., 2002) and the AM1 primer (Helgason
168 et al., 1998) in a 50 μL PCR mix consisting of 250 μM each primer, 250 μM each dNTP, 1.5 mM
169 MgCl_2 , 1x Buffer (67 mM tris-HCl pH 8.8; 16.6 mM $(\text{NH}_4)_2\text{SO}_4$; 0.01 % Tween-20) and 2.5 U of
170 Taq DNA Polymerase (Polymed, Firenze, Italy). The reaction was performed in a iCycler thermal
171 cycler (Bio-Rad Laboratories Inc., Hercules, CA) with a protocol consisting of an initial cycle of
172 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 62.3°C for 45 s and 72°C for 60 s, and a
173 final extension step at 72°C for 7 min. Each sample was amplified three times and the amplicons
174 were pooled together before DGGE analysis.

175 Root and fungal spore DNA amplifications were performed in the same conditions, except
176 for the starting material (25 ng), and for annealing time of spore samples (60 s).

177

178 *2.7. Double Gradient DGGE analysis of AMF communities*

179 The analysis was performed with the INGENYphorU[®] system (Ingeny International BV,
180 Goes, The Netherlands) on a 5 to 6% polyacrylamide gel (acrylamide/bis 37.5:1), under
181 denaturation conditions (urea, 7 M; 40% formamide with a denaturing gradient ranging from 25 to
182 50%); the gels were run in 1x TAE buffer at 75 V for 17 h at 60 °C and were stained with 14 mL
183 of 1x TAE containing 1.4 µL of SYBR[®] Gold (Molecular Probes, Inc., Eugene, OR) (dilution
184 1:10,000) for 30 min in the dark. Visualization and digital pictures were performed with a
185 ChemiDoc System (Bio-Rad Laboratories). Using electrophoretic patterns, a matrix of the
186 presence and absence of bands was obtained by GelCompar II 4.6 software (Applied Maths NV,
187 Sint-Martens-Latem, Belgium).

188

189 *2.8. Cloning and Sequencing of 18S rDNA fragments*

190 Selected PCR-DGGE bands pertaining to spores or roots samples were excised from the
191 gel, resuspended in 30 µL of sterile TE and stored at -30°C. The DNAs extracted from the DGGE
192 bands were re-amplified with the primers NS31GC and AM1 and the PCR products were loaded
193 onto a new DGGE gel to ensure the purity of each single band. The amplicons were then cloned
194 into a pCR[®]4-TOPO[®] vector using TOPO TA Cloning[®] kit for Sequencing (Invitrogen
195 Corporation, Carlsbad, CA) and sequenced using the M13 primer.

196 Sequencing was carried out at the C.I.B.I.A.C.I. (University of Florence) using the ABI
197 PRISM[®] BigDye[®] Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA)
198 according to the manufacturer's recommendations. The parameters for cycle sequencing in the
199 thermocycler Primus 96 plus (MWG Biotech, Ebersberg, D) were 18 s delay at 96°C, followed by
200 25 cycles with 18 s at 96°C, 5 s at 50°C and 4 min at 60°C. Electrophoresis was performed on an
201 ABI Prism 310 CE system (Applied Biosystems).

202

203 *2.9. Phylogenetic analysis*

204 Sequences were entered in the BLASTn program of National Center for Biotechnology
205 Information GenBank database (<http://www.ncbi.nlm.nih.gov/>) to search for closely related
206 sequences. Before phylogenetic analysis, sequences were screened with Chimera Check version
207 2.7 (Cole et al., 2003) (<http://rdp.cme.msu.edu>) and aligned with ClustalW program (Chenna et
208 al., 2003), using Glomeromycota sequences available in GenBank. The phylogenetic tree was
209 inferred by neighbour joining (NJ) method using Kimura 2-parameter in TREECON for Windows
210 software (Van de Peer and De Wachter, 1994). The confidence of branching was assessed using
211 1000 bootstrap resamplings. The sequences were deposited at EMBL Nucleotide Sequence
212 Database (www.ebi.ac.uk/embl/) under the accession numbers HE806381-HE806417.

213

214 *2.10. GRSP analyses of field and trap culture soil*

215 GRSP was extracted from soil using the procedures described by Wright and Upadhyaya (1996)
216 for easily extractable (EE-GRSP) and total (T-GRSP) GRSP. EE-GRSP analyses were carried out
217 on field soil and on six months' old trap cultures. Briefly, EE-GRSP was extracted from 1 g of 2
218 mm-sieved soil with 8 mL of a 20 mM citrate solution, pH 7.0, by autoclaving at 121 °C for 30
219 min. T-GRSP was extracted from 1 g of 2 mm-sieved soil samples, by repeated cycles with 50
220 mM citrate, pH 8.0, by autoclaving at 121 °C for 60 min. Extractions of samples continued until
221 the supernatant content of GRSP was under method detection limits (2 mg mL⁻¹). Supernatants
222 from each cycle were collected after centrifugation at 10,000 g for 10 min to pellet soil particles,
223 pooled and stored at 4 °C until analysed. Protein content was determined by Bradford assay
224 (Sigma-Aldrich, Inc.) with bovine serum albumin as the standard. Each determination was
225 repeated three times.

226

227 *2.11. Statistical Analysis*

228 Data of spore counts and GRSP concentrations were analysed on IBM SPSS 19.0 software
229 (SPSS Inc., Chicago, IL). The GLM Univariate procedure was utilized to investigate the effects of

230 tillage management, fertilization levels, and host plants in trap cultures, as fixed factors, and their
231 interactions, with block as random factor. Canonical correspondence analysis (CCA) was
232 performed by using PAST 1.99 software (Hammer et al., 2001), on the presence /absence matrix
233 based on DGGE banding pattern and on spore numbers after logarithmic transformation.
234 Permutation test ($n = 1000$) was performed by using PAST software.

235

236

237 **3. Results**

238 *3.1. PCR-DGGE analyses of AM fungal diversity in field soil and trap cultures*

239 CCA revealed a significant effect of N-fertilization on AMF communities of the field plots
240 ($P = 0.007$). The first canonical axis explained 86.2% of the cumulative variance of PCR-DGGE
241 banding patterns data, and the second one explained the remaining 13.8% (Fig. 1). CCA showed
242 an additional effect of tillage on AMF community diversity (Fig. 1).

243 PCR-DGGE profiles of AMF communities occurring in the soil of trap cultures from
244 different treatments were always more dissimilar than profiles from the same treatment (Fig. 2).
245 CCA suggests a separation of AMF soil communities of *M. sativa* and *Z. mays* trap plants, though
246 not statistically significant ($P = 0.172$) (Fig. 3).

247 N-fertilization was the main factor affecting AMF communities occurring in *M. sativa* trap
248 plants, as revealed by CCA of the relevant PCR-DGGE profiles, showing a clear-cut separation
249 between patterns obtained from N-fertilized and unfertilized trap soil ($P = 0.009$) (Fig. 4 A). A
250 minor effect of tillage treatments was found (Fig. 4 A). The first canonical axis explained 90.1%
251 of the cumulative variance of PCR-DGGE banding patterns data, and the second one explained
252 the remaining 9.9% (Fig. 4 A). Consistent results were obtained by CCA of AMF communities
253 occurring in *M. sativa* roots ($P = 0.002$) (Fig. 4 B).

254

255 *3.2. Analyses of DNA sequences*

256 NS31-GC/AM1 amplicons obtained from plant roots, spores and sporocarps of trap
257 cultures of unfertilized plots generated multiple PCR-DGGE bands, which, after excision from the
258 gel, cloning and sequencing, yielded a total of 37 sequences with high similarity (98-100%
259 identity) to those of Glomeromycota, after BLASTn searches in GenBank databases. Only two
260 sequences matched with Ascomycota sequences.

261 PCR-DGGE bands obtained from *M. sativa* and *Z. mays* roots yielded 20 sequences which
262 grouped into four Glomeromycota sequence types, showing identities with sequences of both
263 cultured and uncultured AMF deposited in GenBank databases. In particular, we recovered two
264 sequence types, clustering with sequences of *F. mosseae* (Ag1 sequence type) and *G.*
265 *intraradices*/*Glomus fasciculatum* (Thaxt.) Gerd. & Trappe/*Glomus irregulare* Błaszk., Wubet,
266 Renker & Buscot group, hereafter *G. intraradices* (Ag3 sequence type) (Fig. 5). Two other
267 sequence types, Ag4 and Ag5, which matched (99% identity) with sequences of uncultured
268 *Glomus* species already present in GenBank were found (Table 1). Ag1 and Ag3 sequences were
269 retrieved from all *Z. mays* and *M. sativa* roots, with the exception of *M. sativa* roots of NT0 trap
270 cultures, where Ag1 was absent. Ag5 sequences were retrieved from all trap cultures roots, while
271 Ag4 sequence type were found only in *M. sativa* roots, irrespective of the treatment (Fig. 5). No
272 sequences of *G. viscosum* were retrieved from trap plant roots.

273 Blast and phylogenetic analyses of sequences derived from the amplification of spores and
274 sporocarps lead to the identification of three separate clusters, Ag1 (11 sequences), Ag2 (4
275 sequences) and Ag3 (2 sequences), corresponding to *F. mosseae*, *G. viscosum* and *G. intraradices*,
276 respectively (Fig. 5, Table 1).

277

278 3.3. Abundance and diversity of AMF spores produced in trap cultures

279 The numbers of AMF spores produced in trap cultures were consistently decreased by
280 tillage in both *M. sativa* and *Z. mays* host plants (Fig. 6), ranging from 35 to 130 and from 3 to 34
281 per 10 g of soil, in no-tilled and tilled soil, respectively. AMF spore number was also affected by

282 trap plant species, while a strong interaction ($P < 0.001$) was found between host plant species and
283 tillage/fertilization treatments. Therefore, distinct statistical analyses were performed for each host
284 plant, which showed that in *M. sativa* spore production was marginally affected by tillage and
285 fertilization treatments, while in *Z. mays* tillage significantly decreased sporulation (Table 2).
286 Moreover, an interaction between fertilization and tillage was detected ($P = 0.01$).

287 The overall sporulation pattern of the different AMF species showed a predominant effect
288 of tillage, as compared with that of host and fertilization treatments, as revealed by CCA ($P =$
289 0.032). The first canonical axis explained 95.9% of the cumulative variance of PCR-DGGE
290 banding patterns data, and the second one explained the remaining 4.1% (Fig. 7). *F. mosseae* was
291 the predominant species sporulating in tilled soils, while *G. viscosum* and *G. intraradices*
292 prevailed in no-tilled soils (Fig. 8). Interestingly, *G. intraradices* spores were not retrieved from
293 all tilled treatments. A low number of *Funneliformis geosporus* spores (T.H. Nicolson & Gerd.) C.
294 Walker & A. Schüssler was retrieved only from *M. sativa* traps (Fig. 8).

295 With *M. sativa* as host plant, the number of *G. viscosum* spores was significantly lower in
296 trap cultures from tilled than from no-tilled soils ($P = 0.01$), while *F. mosseae* spore number
297 significantly decreased in fertilized soil ($P = 0.001$). With *Z. mays* as host plant, only *G. viscosum*
298 spore number was significantly affected by fertilization treatments ($P = 0.011$), with a strong
299 fertilization by tillage interaction ($P = 0.002$), as a results of its high sporulation in fertilized and
300 NT plots.

301

302 3.4. GRSP content in field soil and in trap cultures

303 Both T- and EE-GRSP concentrations were larger in no-tilled than in tilled field soil, and
304 correlated well each other (Pearson correlation = 0.823; $P < 0.001$). T-GRSP content was
305 significantly affected by tillage ($P = 0.023$), and was about 36.1% larger in NT than in CT plots
306 (Fig. 9). On the other hand, fertilization did not affect GRSP content ($P = 0.132$ and 0.082 ,

307 respectively for T-GRSP and EE-GRSP). No differences in GRSP content of trap culture soil were
308 found.

309

310 **4. Discussion**

311 This is the first multimodal study of the effects of N-fertilization and tillage on AMF communities
312 in arable soils, which combined molecular and biochemical analyses of field soil and molecular,
313 biochemical and morphological analyses of soil and roots from trap cultures established in
314 microcosm. PCR-DGGE banding patterns evidenced marked differences between AMF
315 communities characterising both N-fertilized and unfertilized, and no-tilled and tilled field plots.
316 A predominant effect of tillage was shown by the overall sporulation pattern of the different AMF
317 species and by field glomalin-related soil protein.

318

319 *4.1. PCR-DGGE pattern analysis of AM fungal diversity in field soil and trap cultures*

320 CCA of PCR-DGGE profiles clearly discriminated AMF communities characterising N-
321 fertilized and unfertilized field soils. Such data were confirmed by CCA of PCR-DGGE profiles
322 from roots and soil of *M. sativa* trap plants, which evidenced different AMF community structure
323 composition between the two treatments, in contrast with other findings obtained either from
324 spores or from root DNA sequences (Jansa et al., 2002; Sýkorová et al., 2007).

325 Our findings on N-fertilization are in agreement with a previous DGGE-based study
326 showing differences in the community composition of AMF colonizing the roots of *Festuca*
327 *pratensis* and *Achillea millefolium* in a Swedish grazed grassland along a gradient of soil N and P
328 concentration (Santos et al., 2006). Other studies, performed on AMF spores, indirectly evidenced
329 that AMF may be affected by the use of chemical fertilizers: for example Oehl et al. (2004)
330 showed that organic farming, where the use of chemical fertilizers is not allowed, promoted
331 higher AM fungal diversity and abundance than conventional agriculture, whilst other authors

332 found a lower AMF diversity and abundance in N fertilised agroecosystems (Egerton-Warburton
333 and Allen, 2000).

334 CCA of PCR-DGGE profiles further differentiated AMF communities of no-tilled and
335 tilled field soil, supporting recent data obtained in long-term experiments in temperate regions
336 (Mirás-Avalos et al., 2011; Mathew et al., 2012). The effects of tillage treatments on AMF
337 communities observed in field soil were confirmed by CCA of PCR-DGGE profiles from roots
338 and soil of *M. sativa* and *Z. mays* trap plants.

339 In this work, CCA clearly separated AMF soil communities of *M. sativa* and *Z. mays* trap
340 plants and evidenced a major effect of N-fertilization on AMF occurring in the soil and in the
341 roots of *M. sativa* plants, suggesting that the responses of AMF to different agronomical
342 treatments may depend also by host plant taxon or its nutritional status (Giovannetti et al., 1988;
343 Egerton-Warburton and Allen, 2000). Indeed, Oliveira et al. (2009) showed that tropical maize
344 genotypes contrasting for phosphorus efficiency had a greater influence on AMF rhizosphere
345 community than the level of P in the soil.

346

347 4.2. Abundance and diversity of AMF spores as revealed by morphological and molecular analyses

348 Our data evidenced that tillage treatments decreased the number of AMF spores produced
349 in trap cultures of the two host plants *M. sativa* and *Z. mays*, and showed a major effect on the
350 overall sporulation pattern of *F. mosseae*, which predominated in tilled soils, and of *G. viscosum*
351 and *G. intraradices*, which prevailed in no-tilled soils. Such findings obtained in microcosm
352 suggest that tilled soils maintain the relevant qualities affecting AMF communities even when
353 subsequently cultivated without tilling (Johnson et al., 1991).

354 Our findings support previous studies showing that intense tillage and high-input
355 conventional farming negatively affect AMF abundance and community composition, involving a
356 reduction of AMF species not belonging to the genus formerly described as *Glomus* (Jansa et al.,
357 2002; Oehl et al., 2004). The prevalence of *F. mosseae* in microcosms from deeply ploughed soil

358 is a strong indication of its resilience, which could be ascribed to its ability to re-establish a
359 functional mycorrhizal network by means of anastomosis after hyphal disruption caused by tillage
360 (Giovannetti et al., 1999; Giovannetti et al., 2001; Sbrana et al., 2011). Indeed, in an arable site
361 92% of DNA sequences amplified from mycorrhizal roots were assigned to *G. mosseae*, which
362 represented only 10% of sequences in a nearby woodland (Helgason et al., 1998; Daniell et al.,
363 2001).

364 Here we detected only spore morphotypes belonging to the genus formerly described as
365 *Glomus* (*F. mosseae*, *G. intraradices*, *G. viscosum*, *F. geosporum*), consistent with data reporting
366 the prevalent occurrence of species of the genus *Glomus* in intensively managed agroecosystems
367 (Land & Schönbeck, 1991; Blaszowski, 1993; Kurle & Pflieger, 1996; Franke-Snyder et al.,
368 2001; Bedini et al., 2007). On the other hand, our data reinforce previous observations indicating
369 the rarity or absence of Glomeromycota genera other than *Glomus* in arable fields, compared with
370 natural sites, such as woodland and sand dunes (Koske and Walker, 1986; Helgason et al., 1998;
371 Daniell et al., 2001; Turrini et al., 2008; Turrini and Giovannetti, 2012). The AMF species
372 described from our site and from different agricultural soils worldwide, defined as ‘typical AMF
373 of arable lands’ or AMF ‘generalists’ (Oehl et al., 2003), have been presumed to adapt and thrive
374 in heavily tilled soils, as a result of their ability to sporulate quickly and massively (Daniell et al.,
375 2001; Jansa et al., 2008).

376 The low AMF diversity detected in our work is in agreement with other results obtained in
377 agricultural soils in both temperate (Daniell et al., 2001; Jansa et al., 2002; Jansa et al., 2003) and
378 Mediterranean or subarid climate (Calvente et al., 2004; Alguacil et al., 2011), although higher
379 numbers of AMF species were also reported (Ellis et al., 1992; Oehl et al., 2004). In addition, the
380 high clay content of our experimental soil could represent an environmental factor limiting AMF
381 species richness (Mathimaran et al., 2005). It is interesting to note that the retrieval of a high
382 number of sporulating morphotypes was boosted by the use of additional hosts and long culture
383 periods (Oehl et al., 2004; Oehl et al., 2009).

384 DNA sequences obtained from PCR-DGGE bands of *M. sativa* and *Z. mays* roots and from
385 DNA spore PCR-amplification consistently identified two AMF species, *F. mosseae* and *G.*
386 *intraradices*, while no sequences of *G. viscosum* were retrieved from trap plant roots. This could
387 be the result a poor competitive ability of *G. viscosum* compared with other AMF, in particular *F.*
388 *mosseae*, which is an early colonizer (Jansa et al., 2008; Oehl et al., 2010). A similar discrepancy
389 among AMF communities obtained from extraradical mycelium, spores and roots was previously
390 observed in vineyards (Schreiner and Mihara, 2009) and in a grassland soil (Hempel et al., 2007).

391 Here, two sequence types retrieved from plant roots, Ag4 and Ag5, did not match with any
392 sequence obtained from spores formed in soil. Indeed, many DNA sequences of AMF obtained
393 from environmental samples (soil or roots) deposited in public databases do not find any match
394 with those originating from morphologically described spores. For example, some *Glomus* species
395 rarely sporulating in the field have recently been described using spores produced only in trap
396 cultures (Blaszkowski et al., 2009a; 2009b; 2010), showing that this method can provide optimal
397 conditions for the completion of life cycle of peculiar AMF (Stutz and Morton, 1996; Oehl et al.,
398 2004), in particular when long periods of cultivation are utilised (Oehl et al., 2003; Yao et al.,
399 2010). Interestingly, Ag4 and Ag5 sequences matched well with database sequences obtained from
400 vine roots in northwestern Italy (Balestrini et al., 2010).

401

402 4.3. GRSP content in field and trap culture soil

403 The higher content of GRSP in no-tilled compared with tilled field soil suggests either the
404 occurrence of higher density of AMF in plots under no tillage management or a difference in AMF
405 community composition leading to the production of larger amounts of GRSP (Lovelock et al.,
406 2004; Bedini et al., 2009). Alternatively, changes in GRSP contents among treatments may
407 represent the result of different rates of GRSP turnover. However, our results support previous
408 data on the negative impact of intensively managed crops on GRSP content (Bedini et al., 2007;
409 Roldán et al., 2007; Spohn and Giani, 2010). N-fertilization did not affect GRSP content in our

410 field experiments, consistently with previous reports from forest and arable soils (Wuest et al.,
411 2005; Antibus et al., 2006), but in contrast with other data obtained in crop and grassland soils
412 (Wilson et al., 2009; Wu et al., 2011). No differences in GRSP content of trap culture soil were
413 found, probably as a result of glomalin production during the growth of mycorrhizal trap plants,
414 buffering the differences detected in the original field soil.

415

416 *4.4 Concluding remarks*

417 A comprehensive and exhaustive evaluation of changes in AMF community diversity produced by
418 anthropogenic and environmental variables may be difficult to accomplish utilising singular
419 approaches. Actually, morphological analyses based on spores collected in the field may miss non
420 sporulating species or those represented by old and parasitized spores, while root DNA analyses
421 may reveal only the amplifiable DNA, representing a subset of AM fungal communities
422 colonizing the sampled roots, which may differ from those detected in rhizosphere or bulk soil
423 and from those described using morphological analyses as well (Hempel et al., 2007; Cesaro et
424 al., 2008; Mirás-Avalos et al., 2011). In this work we used a multimodal approach to reach a
425 thorough view of the impact of two major production factors on AMF populations, by combining
426 morphological and molecular analyses of field soil and of soil and roots from trap cultures
427 established in microcosm. Our PCR-DGGE data show that repeated N application is a stronger
428 driving force in shaping native AMF communities, compared with tillage, which represents the
429 major factor affecting the composition and abundance of sporulating taxa, as revealed by
430 morphological analysis. The availability of native AMF isolates obtained from trap plants will
431 allow further investigations aimed at elucidating the specific functional role played by single
432 components of AMF communities thriving in differently managed agroecosystems. Such findings
433 may help implementing effective agricultural management strategies able to support the beneficial
434 relationship between crops and native AM symbionts.

435

436

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441

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688

689 **Table 1.** Nuclear SSU rDNA sequence types obtained from clones of DGGE excised bands of
690 *Medicago sativa* and *Zea mays* roots and spores of AMF produced in trap cultures.

Sequence types	Identity (%)	Taxonomic affiliation
Ag1	NG017178 (100)	<i>Funneliformis mosseae</i>
Ag2	AJ505813 (99)	<i>Glomus viscosum</i>
Ag3	AJ536822 (99)	<i>Glomus intraradices</i>
Ag4	GU353916 (99)	Uncultured <i>Glomus</i> sp.
Ag5	GU353731 (99)	Uncultured <i>Glomus</i> sp.

691
692

Table 2. Results of split plot analysis of the effects of tillage and fertilization on total spore number of AMF produced in trap cultures after sixteenth months' growth on *Medicago sativa* and *Zea mays*

Source of variation	<i>Medicago sativa</i>				<i>Zea mays</i>			
	df	MS	F	P value	df	MS	F	P value
block	1	0.472	8.829	0.207	1	0.308	10.956	0.187
tillage	1	4.271	79.960	0.071	1	10.541	375.204	0.033
error-main	1	0.053			1	0.028		
fertilization	1	1.105	17.490	0.053	1	0.018	0.645	0.506
tillage x fertilization	1	0.156	2.470	0.257	1	2.848	102.535	0.010
error-subplots	2	0.063			2	0.028		

694 Figures legends

695 **Fig. 1.** Canonical correspondence analysis (CCA) biplot of V3-V4 region of nuclear 18S rDNA
696 PCR-DGGE fragments from three replicates of field soil from conventionally tilled (CT) and no
697 tilled (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90, CT90) Kg ha⁻¹ N.

698 **Fig. 2.** PCR-DGGE profiles of V3-V4 region of nuclear 18S rDNA fragments from roots of
699 *Medicago sativa* (M) trap cultures from conventionally tilled (CT) and no tilled (NT) plots
700 fertilized with 0 (NT0, CT0) or 90 (NT90, CT90) Kg ha⁻¹ N.

701 **Fig. 3.** Canonical correspondence analysis (CCA) biplot of V3-V4 region of nuclear 18S rDNA
702 PCR-DGGE fragments from three replicates of trap culture soil from unfertilized no tilled (NT0)
703 and conventionally tilled (CT0) plots, with *Medicago sativa* (M) and *Zea mays* (Z) as host plants.

704 **Fig. 4.** Canonical correspondence analysis (CCA) biplot of V3-V4 region of nuclear 18S rDNA
705 PCR-DGGE fragments from (A) soil and (B) roots of *Medicago sativa* (M) trap cultures from
706 conventionally tilled (CT) and no tilled (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90,
707 CT90) Kg ha⁻¹ N.

708 **Fig. 5.** Neighbour-joining phylogenetic tree of glomeromycotan sequences derived from PCR-
709 DGGE bands obtained from *Medicago sativa* and *Zea mays* trap plants. The analysis is based on
710 V3-V4 region of nuclear 18S rDNA sequences, and the tree is rooted with a reference sequence of
711 *Geosiphon pyriformis* (X86686). Bootstrap values (>70%) were determined for neighbour joining
712 (1000 resamplings). Different sequence types are indicated in brackets: Ag1, Agugliano1; Ag2,
713 Agugliano2; Ag3, Agugliano3; Ag4, Agugliano 4; Ag5, Agugliano5. Sequences obtained in the
714 present study are shown in bold with their accession numbers (HE806381-HE806417) followed
715 by their DNA source (spores, sporocarps, roots) and treatment (trap cultures from conventionally
716 tilled (CT) and no tilled (NT) plots fertilized with 0 (NT0, CT0) Kg ha⁻¹ N, with *Medicago sativa*
717 (M) or *Zea mays* (Z) as trap plant.

718 **Fig. 6.** Total AMF spore density in trap cultures from conventionally tilled (CT) and no tilled
719 (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90, CT90) Kg ha⁻¹ N, and with *Medicago sativa*

720 (M) or *Zea mays* (Z) as trap plant, after sixteen months' growth. Error bars refer to standard error
721 of the means (n = 2).

722 **Fig. 7.** Canonical correspondence analysis (CCA) biplot of AMF spore species composition, after
723 sixteen months' growth, across all trap cultures obtained from conventionally tilled (CT) and no
724 tilled (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90, CT90) Kg ha⁻¹ N, and with *Medicago*
725 *sativa* (M) or *Zea mays* (Z) as trap plant.

726 **Fig. 8.** Relative abundance of AMF spore by species, in trap cultures from conventionally tilled
727 (CT) and no tilled (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90, CT90) Kg ha⁻¹ N, and
728 with *Medicago sativa* (M) or *Zea mays* (Z) as trap plant. *Funneliformis mosseae* (dark grey),
729 *Glomus viscosum* (blank), *Glomus intraradices* (light grey), *Funneliformis geosporus* (black).

730 **Fig. 9.** Concentration of total glomalin-related soil protein (T-GRSP) in field soil of
731 conventionally tilled (CT) and no tilled (NT) plots fertilized with 0 (NT0, CT0) or 90 (NT90,
732 CT90) Kg ha⁻¹ N. Error bars refer to standard error of the means (n = 3).

733

Fig. 1

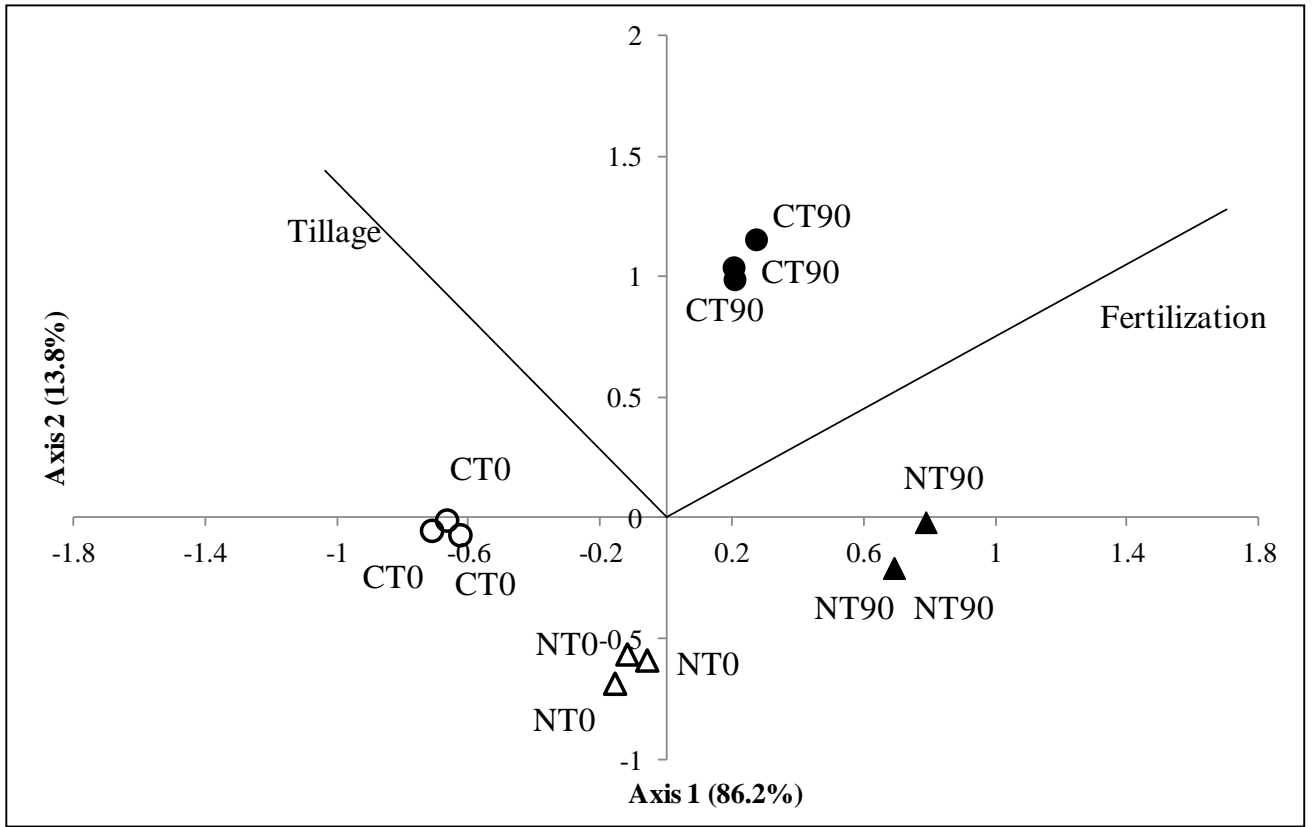


Fig. 2

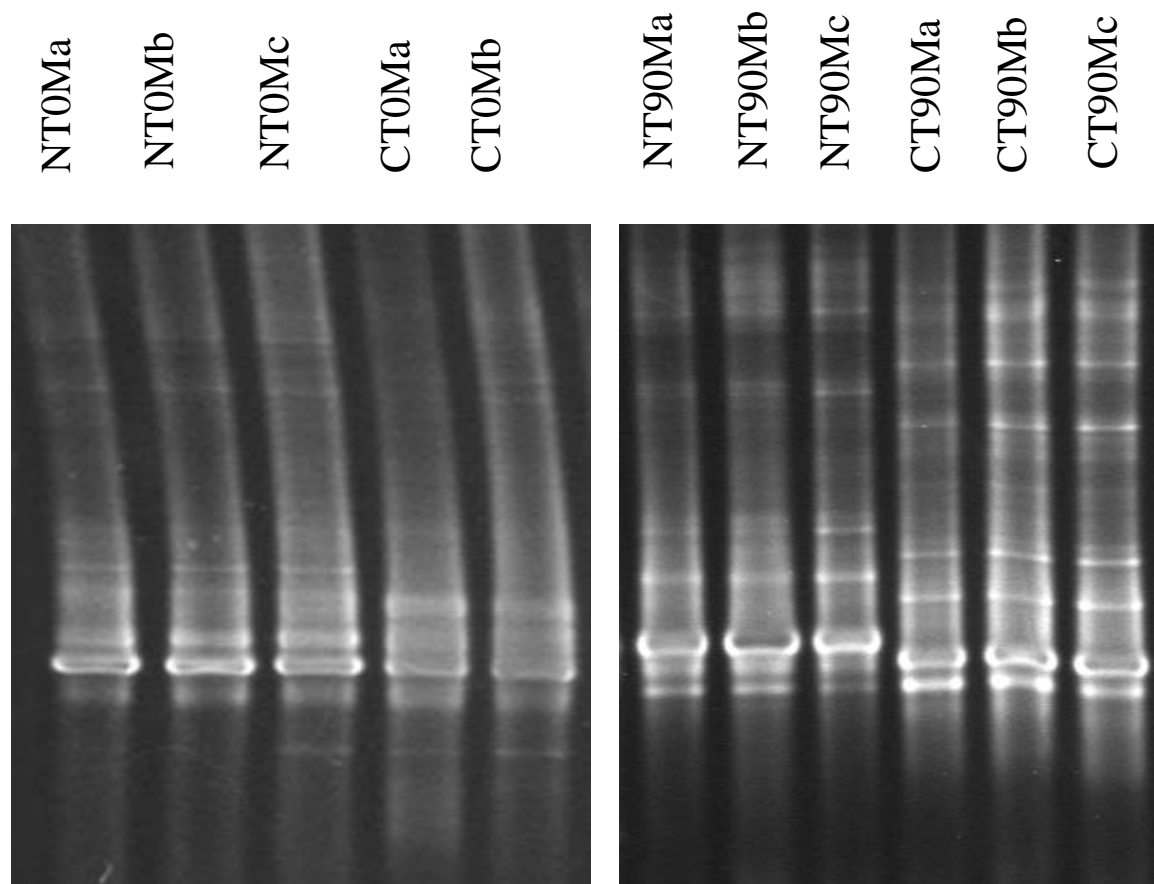


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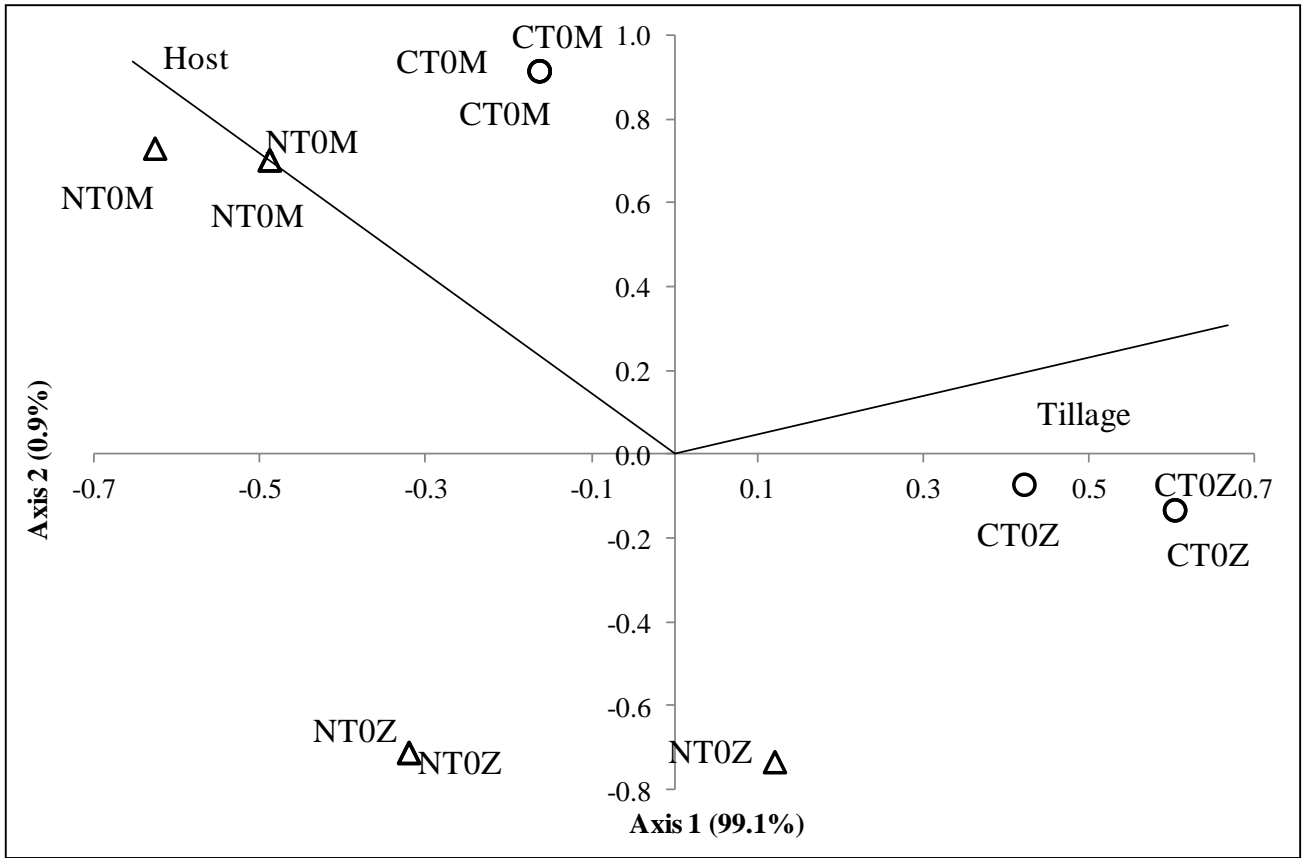


Fig. 4

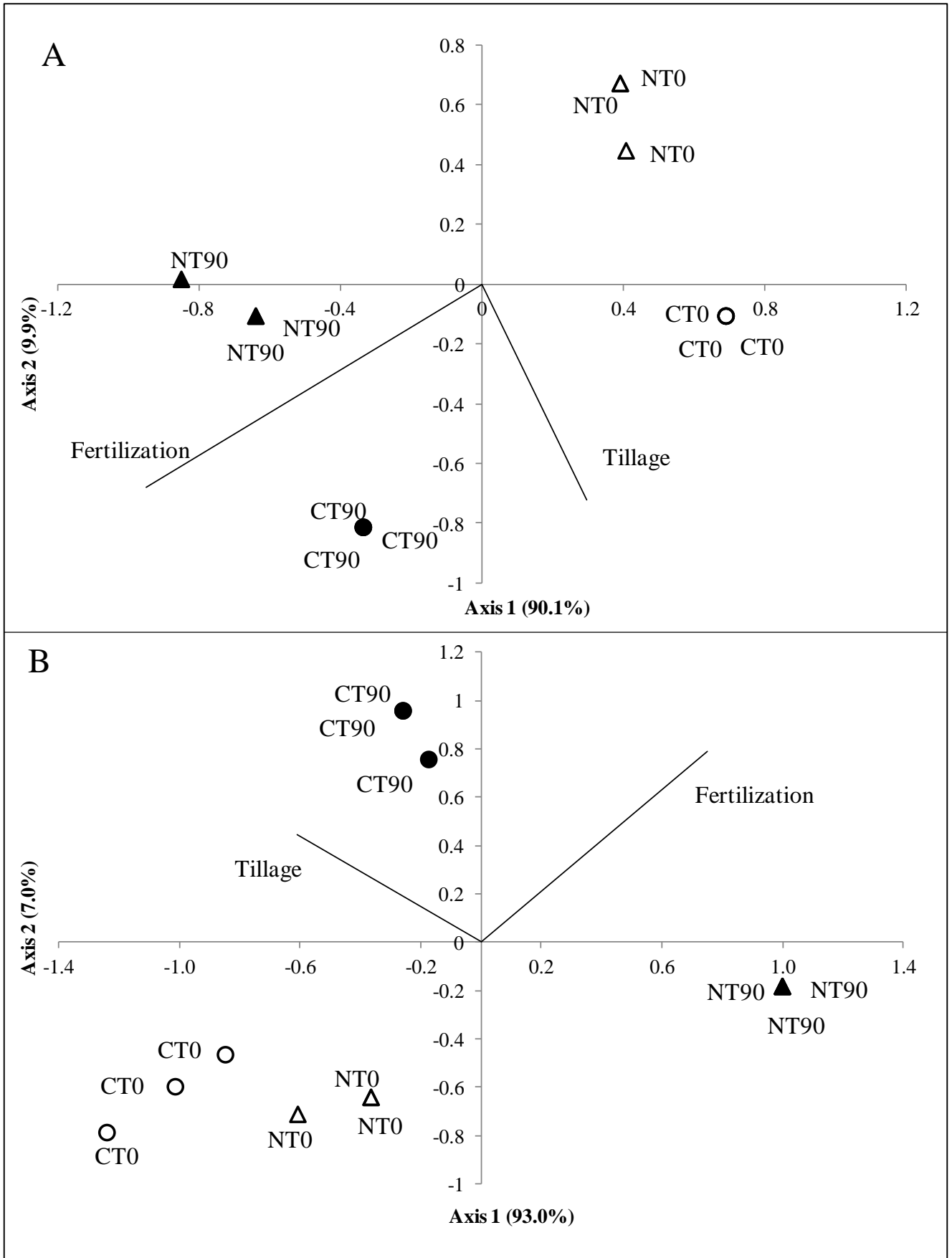


Fig. 5

0.1 substitutions/site

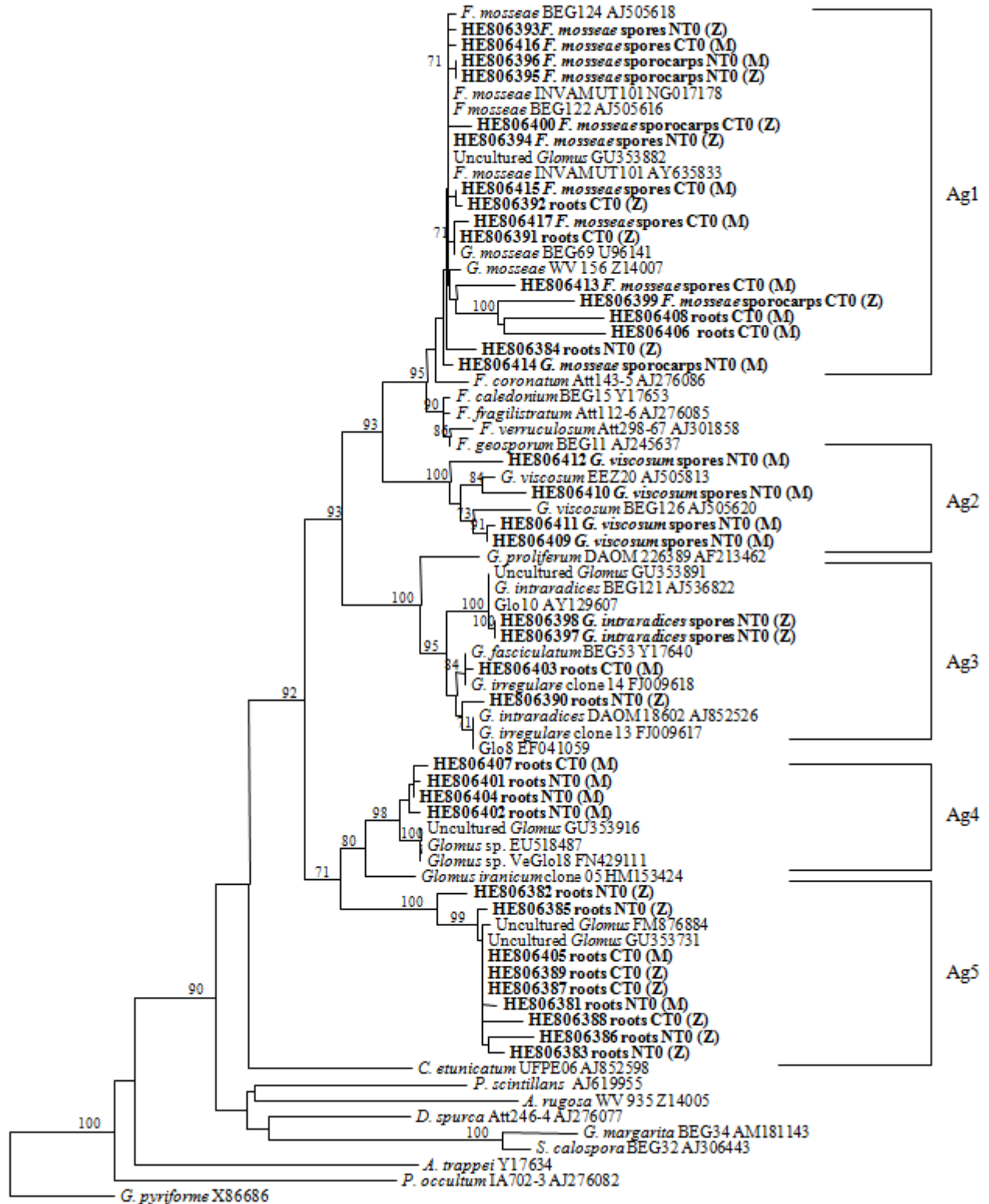


Fig. 6

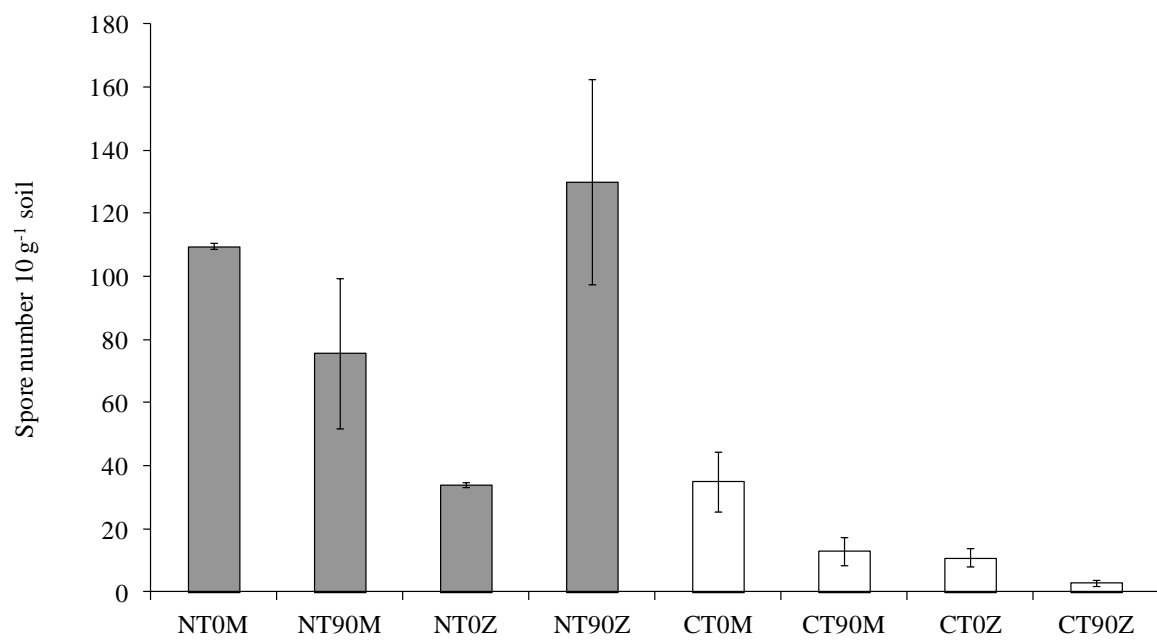


Fig. 7

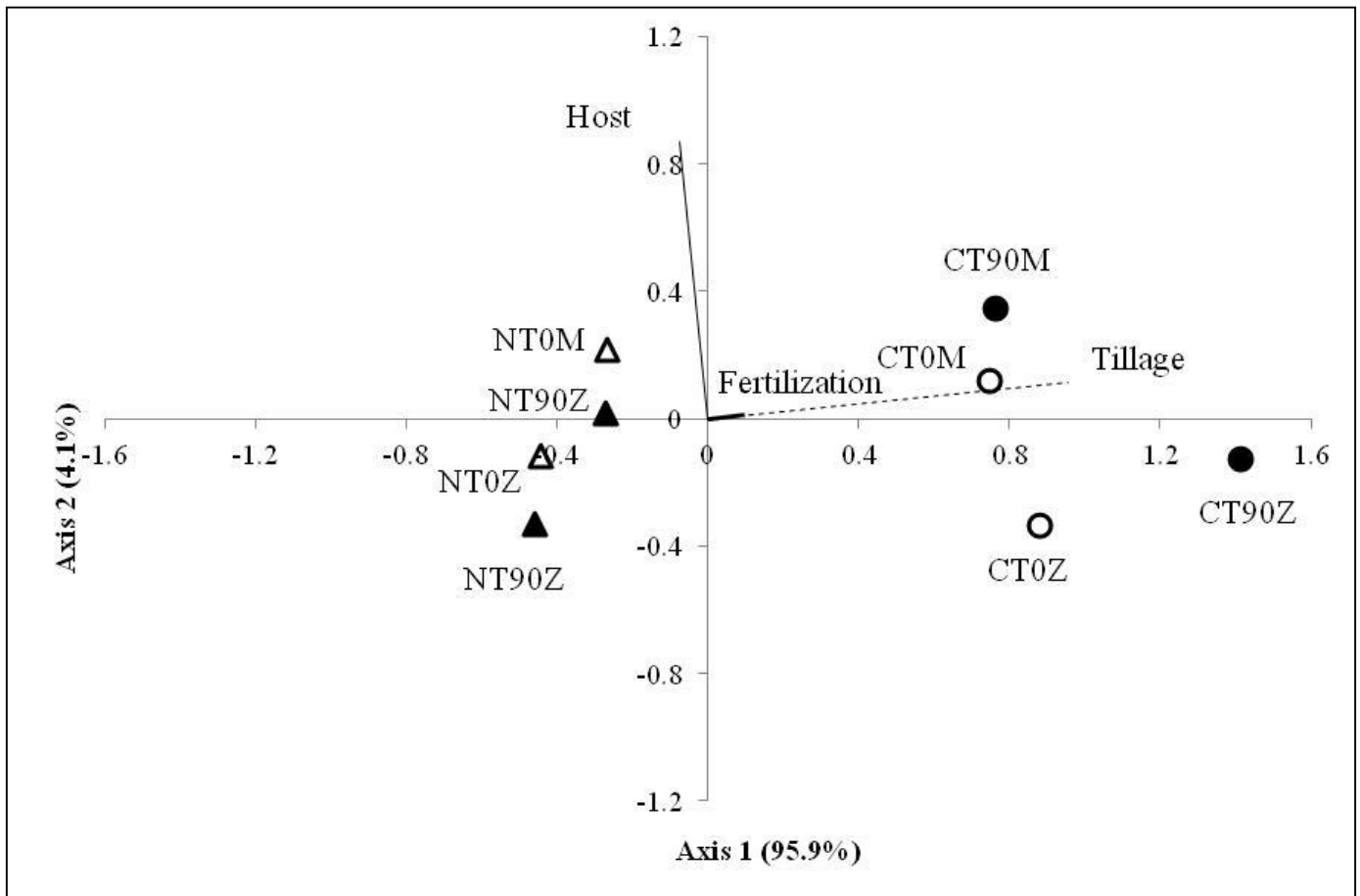


Fig. 8

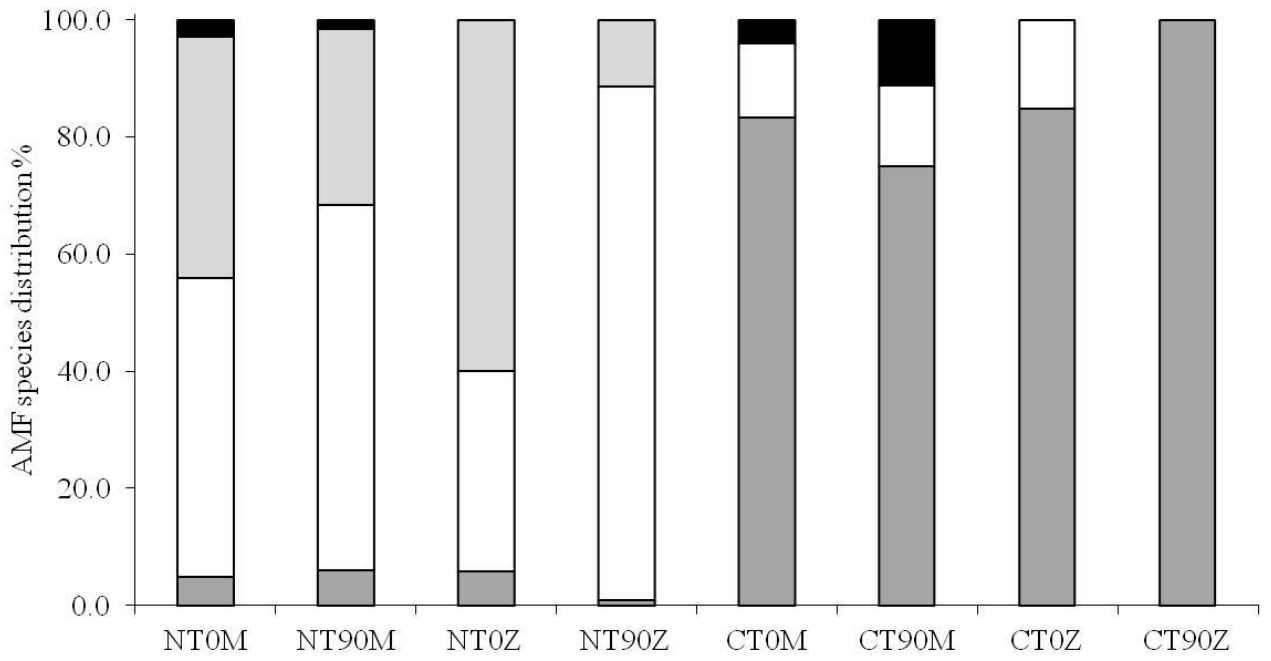


Fig. 9

