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

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Keywords

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Abstract

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

1. Introduction

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

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

2. Materials and methods

2.1. Study site and soil sampling

The study was conducted at the "Pasquale Rosati" experimental farm near Agugliano, Italy, (43° 32'N, 13° 22'E, 100 m a.s.l., slope 10%). The soil is a calcaric gleyic cambisol almost free of gravel, with a high clay and calcium content. The climate is dry-summer subtropical (Mediterranean), with a mean annual rainfall in the period 1998-2008 of 786 mm. The highest mean monthly temperature (30.6°C) and the lowest precipitation (35 mm) occurred in July. The lowest mean monthly temperature (3.0°C) occurred in January and the highest precipitation (105 mm) in September (De Sanctis et al., 2012). The experimental site belong to a long term tillage experiment, established in 1994, with a two year rotation of maize (*Zea mays* L.) and durum wheat (*Triticum durum* L.) since 2002, and designed as a split plot with tillage treatments assigned to the main plots (each 1500 m^2 in size) and N-fertilization treatments assigned to subplots (each 500 m^2 in size). The experiment was replicated in two blocks with treatments repeated in the same plots every year. In the present study, soil sampling was performed in the subplots treated with no N-fertilization (0) and 90 kg ha^{-1} N (90) as ammonium nitrate, both in the conventional tillage (CT) and in the no tillage (NT) treatment. CT treatment consisted of ploughing at a depth of 40 cm and double harrowing before sowing, whereas NT plots were left undisturbed except for sod

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

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

2.2. Trap cultures and spore analysis

Each soil sample was mixed, 1:1 by volume, with Terragreen (calcined attapulgite clay, Oil Dri, Chicago, IL), and poured into four 750 cm^3 plastic pots, two for each of the two trap plant species utilized, *Z. mays* and *Medicago sativa* L. Plants were grown in glasshouse, under ambient natural light and temperature conditions and supplied with tap water as needed. In addition, they received weekly fertilization with half strength Hoagland's solution (10 mL per pot). After six months' growth, three soil samples (10 g each) were collected from each pot and processed. AMF spores and sporocarps were extracted by wet-sieving and decanting, using a set of nested sieves, down to a mesh size of 50 μm (Gerdemann and Nicolson, 1963), then flushed into Petri dishes and examined under a dissecting microscope (Wild, Leica, Milano, Italy). The spores were separated into groups, according to their morphology. Spores were isolated by using capillary pipettes, mounted on microscope slides in polyvinyl alcohol lacto-glycerol (PVLG) and in PVLG + Melzer's reagent (1:1, v:v) and examined under a Polyvar light microscope (Reichert-Young, Vienna, Austria). Qualitative spore traits (spore shape, colour and size, spore wall structure and shape, colour and size of the subtending hypha) were examined on at least 50 spores for each morphotype. Morphotype identifications were based on original descriptions and current species

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

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

2.3. Field soil DNA extraction

DNA extraction was performed on 500 mg of each field soil sample, with the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH) according to manufacturer's instructions, with minor modifications: a double homogenization in the FastPrep® Instrument (MP Biomedicals) for 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 of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8). The DNA was then purified with the DNA Clean Up Spin Kit (GENOMED GmbH, Löhne, Germany), according to manufacturer's instructions.

2.4. DNA extraction from roots and soil of trap cultures

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

2.5. DNA extraction from spores

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

2.6. DNA amplification

Aliquots of soil DNA (50 ng) were used to amplify the V3-V4 region of 18S rDNA using the universal eukaryotic NS31GC primer (Kowalchuk et al., 2002) and the AM1 primer (Helgason et al., 1998) in a 50 µL PCR mix consisting of 250 µM each primer, 250 µM each dNTP, 1.5 mM $MgCl_2$, 1x Buffer (67 mM tris-HCl ph 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.01 % Tween-20) and 2.5 U of Taq DNA Polymerase (Polymed, Firenze, Italy). The reaction was performed in a iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) with a protocol consisting of an initial cycle of 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 final extension step at 72°C for 7 min. Each sample was amplified three times and the amplicons were pooled together before DGGE analysis.

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

2.7. Double Gradient DGGE analysis of AMF communities

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

2.8. Cloning and Sequencing of 18S rDNA fragments

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

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

2.9. Phylogenetic analysis

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

2.10. GRSP analyses of field and trap culture soil

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

2.11. Statistical Analysis

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

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

3. Results

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

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

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

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

3.2. Analyses of DNA sequences

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

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

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

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

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

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

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

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

3.4. GRSP content in field soil and in trap cultures

Both T- and EE-GRSP concentrations were larger in no-tilled than in tilled field soil, and correlated well each other (Pearson correlation = 0.823; $P < 0.001$). T-GRSP content was significantly affected by tillage ($P = 0.023$), and was about 36.1% larger in NT than in CT plots (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

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

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

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

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

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

Our findings support previous studies showing that intense tillage and high-input conventional farming negatively affect AMF abundance and community composition, involving a reduction of AMF species not belonging to the genus formerly described as *Glomus* (Jansa et al., 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; Blaszkowski, 1993; Kurle & Pfleger, 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).

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

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

4.3. GRSP content in field and trap culture soil

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

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

4.4 Concluding remarks

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

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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)	*Funneliformis mosseae*
Ag2	AJ505813 (99)	*Glomus viscosum*
Ag3	AJ536822 (99)	*Glomus intraradices*
Ag4	GU353916 (99)	Uncultured *Glomus* sp.
Ag5	GU353731 (99)	Uncultured *Glomus* sp.

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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*

	Medicago sativa				*Zea mays*			
Source of variation	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		

693

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

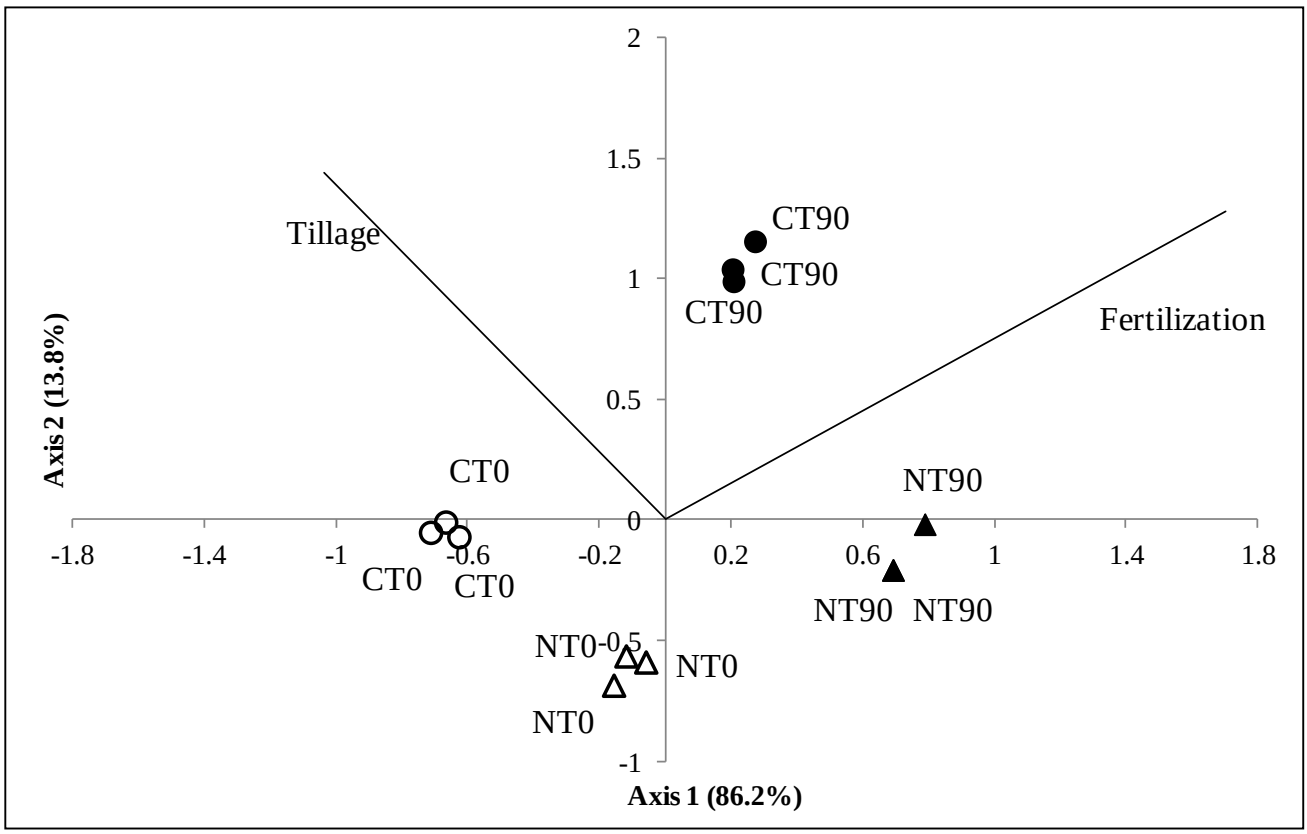
2
1.5
1
0.5
0
-0.5
-1
-1.8
-1.4
-1
-0.6
-0.2
0.2
0.6
1
1.4
1.8
Axis 2 (13.8%)
Axis 1 (86.2%)
Tillage
Fertilization
CT90
CT90
CT90
CT0
CT0
CT0
NT90
NT90
NT90
NT0
NT0
NT0

Fig. 2

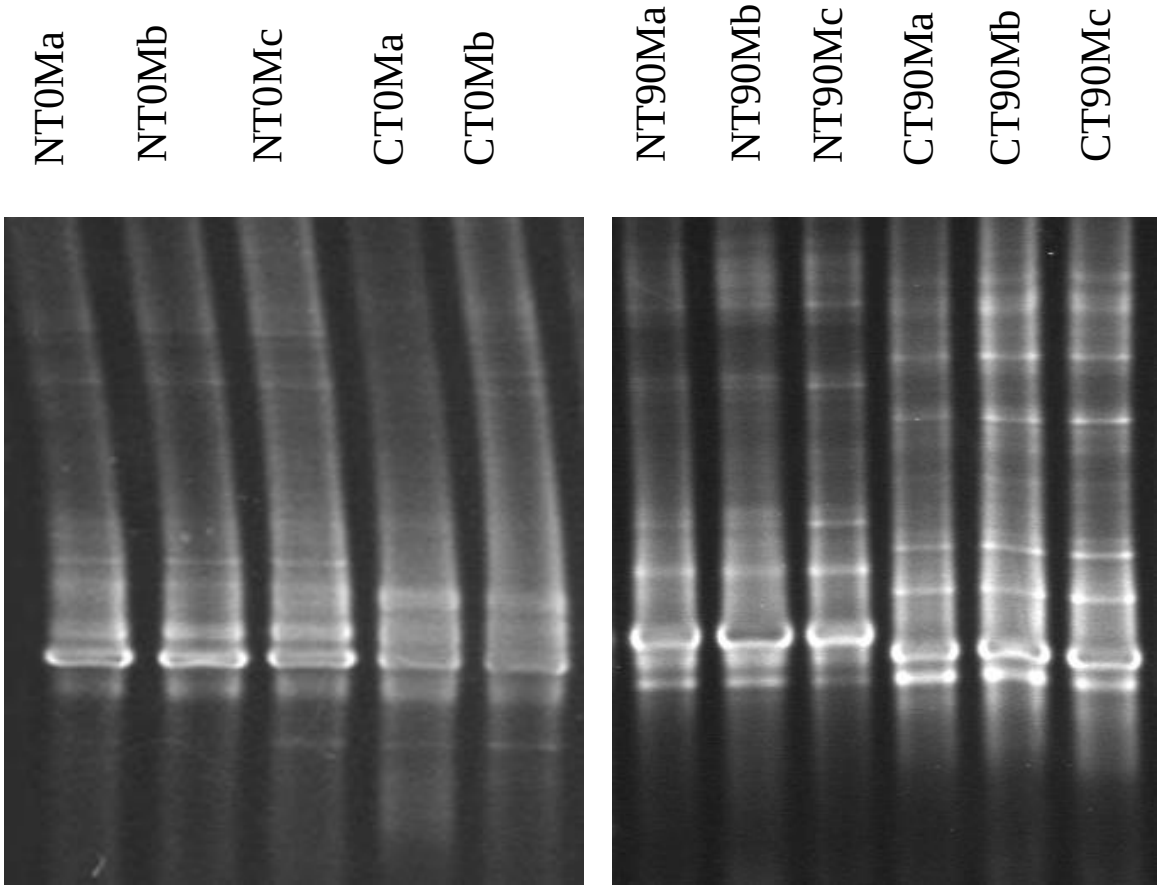
NT0Ma
NT0Mb
NT0Mc
CT0Ma
CT0Mb
NT90Ma
NT90Mb
NT90Mc
CT90Ma
CT90Mb
CT90Mc

Fig. 3

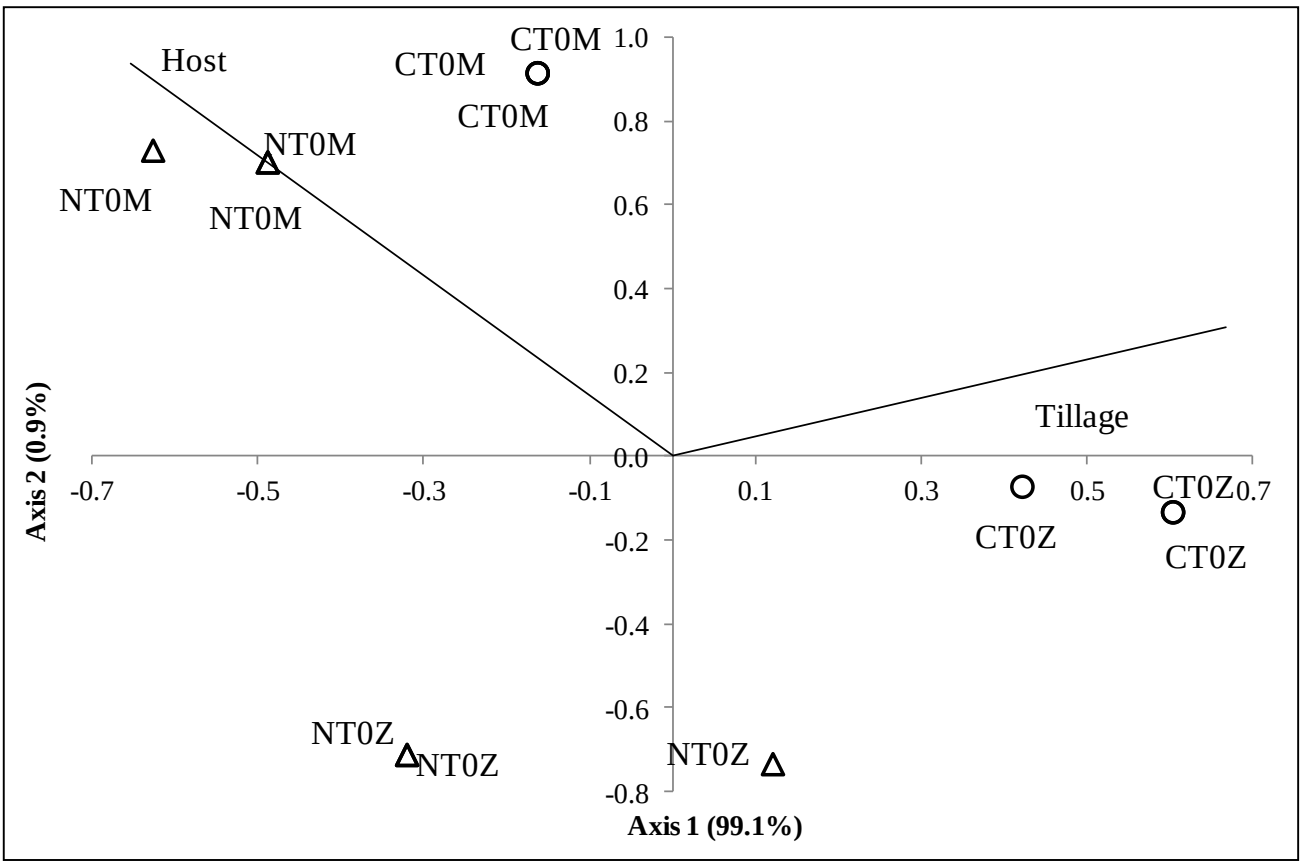
Host
Tillage
CT0M
CT0M
CT0M
NT0M
NT0M
NT0M
CT0Z
CT0Z
CT0Z
NT0Z
NT0Z
NT0Z
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.7
-0.5
-0.3
-0.1
0.1
0.3
0.5
0.7
Axis 2 (0.9%)
Axis 1 (99.1%)

Fig. 4

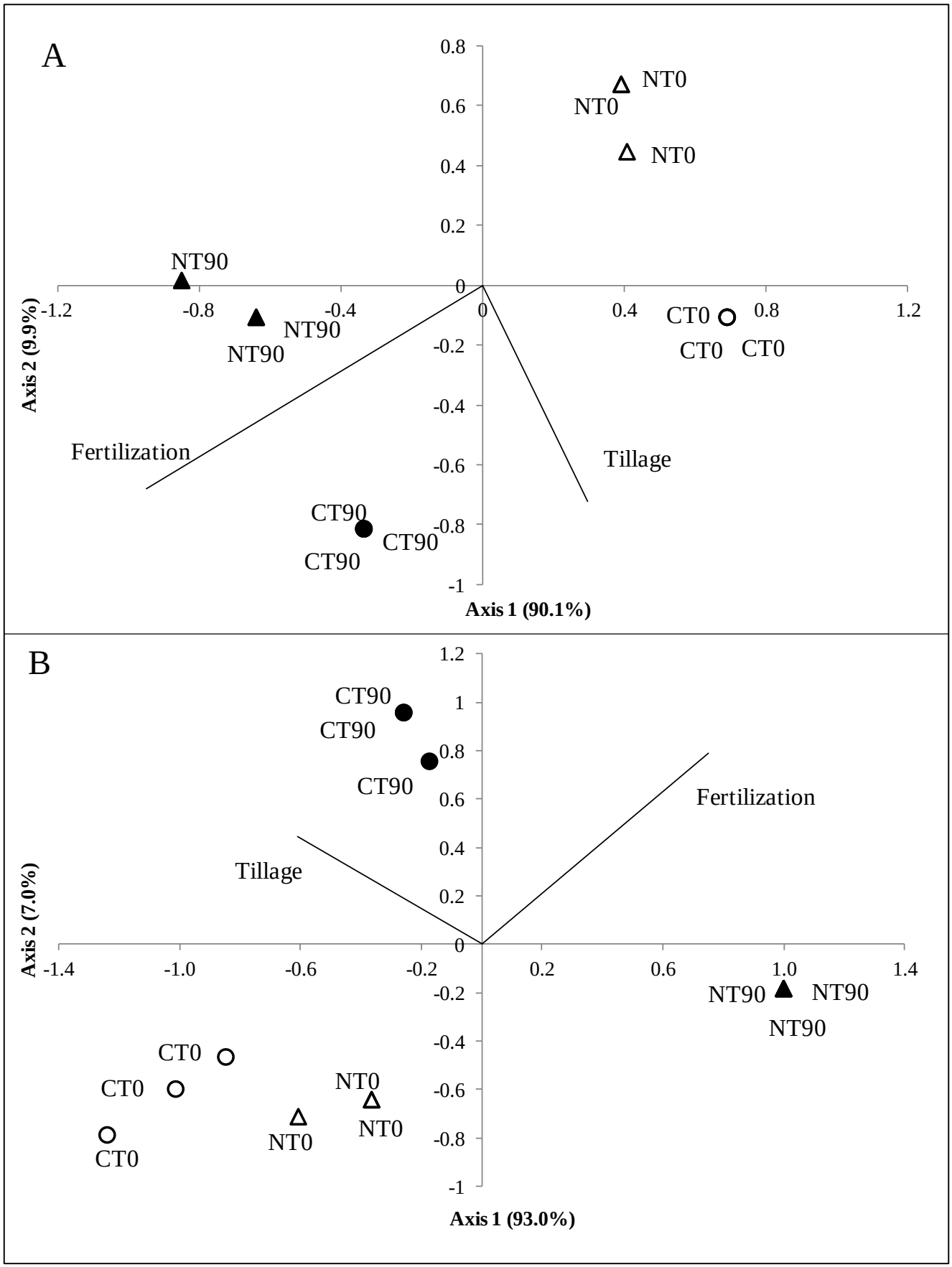
A
NT0
NT0
NT0
NT90
NT90
NT90
CT0
CT0
CT0
CT90
CT90
CT90
Fertilization
Tillage
Axis 2 (9.9%)
Axis 1 (90.1%)
B
CT90
CT90
CT90
Fertilization
Tillage
NT90
NT90
NT90
CT0
CT0
CT0
NT0
NT0
NT0
Axis 2 (7.0%)
Axis 1 (93.0%)

Fig. 5

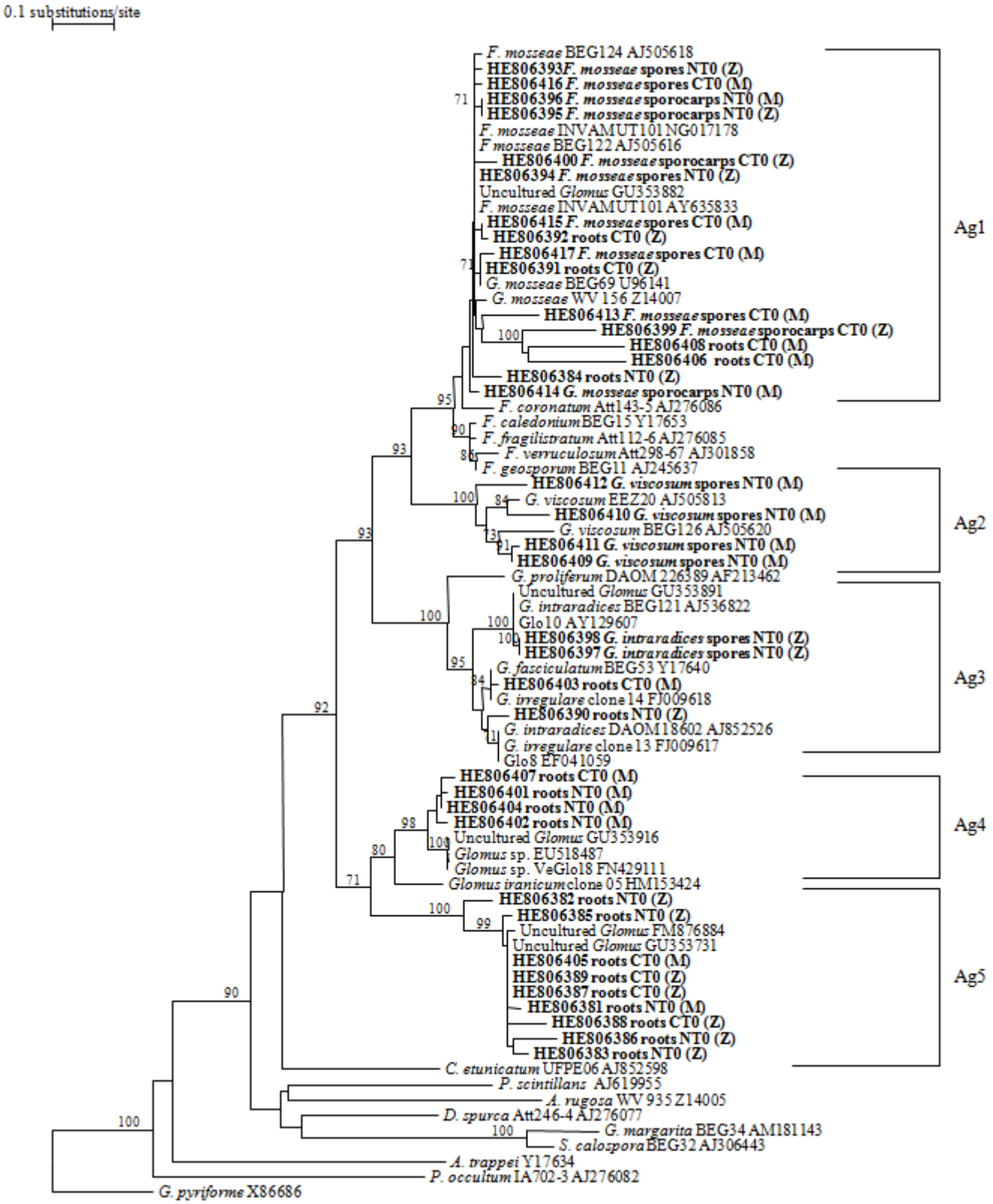
0.1 substitutions/site
F. mosseae BEG124 AJ505618
HE806393 F. mosseae spores NT0 (Z)
HE806416 F. mosseae spores CT0 (M)
HE806396 F. mosseae sporocarps NT0 (M)
HE806395 F. mosseae sporocarps NT0 (Z)
F. mosseae INVAMUT101 NG017178
F mosseae BEG122 AJ505616
HE806400 F. mosseae sporocarps CT0 (Z)
HE806394 F. mosseae spores NT0 (Z)
Uncultured Glomus GU353882
F. mosseae INVAMUT101 AY635833
HE806415 F. mosseae spores CT0 (M)
HE806392 roots CT0 (Z)
HE806417 F. mosseae spores CT0 (M)
HE806391 roots CT0 (Z)
G. mosseae BEG69 U96141
G. mosseae WV156 Z14007
HE806413 F. mosseae spores CT0 (M)
HE806399 F. mosseae sporocarps CT0 (Z)
HE806408 roots CT0 (M)
HE806406 roots CT0 (M)
HE806384 roots NT0 (Z)
HE806414 G. mosseae sporocarps NT0 (M)
F. coronatum Att143-5 AJ276086
F. caledonium BEG15 Y17653
F. fragilistratum Att112-6 AJ276085
F. verruculosum Att298-67 AJ301858
F. geosporum BEG11 AJ245637
HE806412 G. viscosum spores NT0 (M)
G. viscosum EEZ20 AJ505813
HE806410 G. viscosum spores NT0 (M)
G. viscosum BEG126 AJ505620
HE806411 G. viscosum spores NT0 (M)
HE806409 G. viscosum spores NT0 (M)
G. proliferum DAOM226589 AF213462
Uncultured Glomus GU353891
G. intraradices BEG121 AJ536822
Glo10 AY129607
HE806398 G. intraradices spores NT0 (Z)
HE806397 G. intraradices spores NT0 (Z)
G. fasciculatum BEG53 Y17640
HE806403 roots CT0 (M)
G. irregulare clone14 FJ009618
HE806390 roots NT0 (Z)
G. intraradices DAOM18602 AJ852526
G. irregulare clone13 FJ009617
Glo8 EF041059
HE806407 roots CT0 (M)
HE806401 roots NT0 (M)
HE806404 roots NT0 (M)
HE806402 roots NT0 (M)
Uncultured Glomus GU353916
Glomus sp. EU518487
Glomus sp. VeGlo18 FN429111
Glomus iranicum clone05 HM153424
HE806382 roots NT0 (Z)
HE806385 roots NT0 (Z)
Uncultured Glomus FM876884
Uncultured Glomus GU353731
HE806405 roots CT0 (M)
HE806389 roots CT0 (Z)
HE806387 roots CT0 (Z)
HE806381 roots NT0 (M)
HE806388 roots CT0 (Z)
HE806386 roots NT0 (Z)
HE806383 roots NT0 (Z)
C. etunicatum UFPE06 AJ852598
P. scintillans AJ619955
A. rugosa WV935 Z14005
D. spurca Att246-4 AJ276077
G. margarita BEG34 AM181143
S. calospora BEG32 AJ306443
A. trappei Y17634
P. occultum IA702-3 AJ276082
G. pyriforme X86686
Ag1
Ag2
Ag3
Ag4
Ag5

Fig. 6

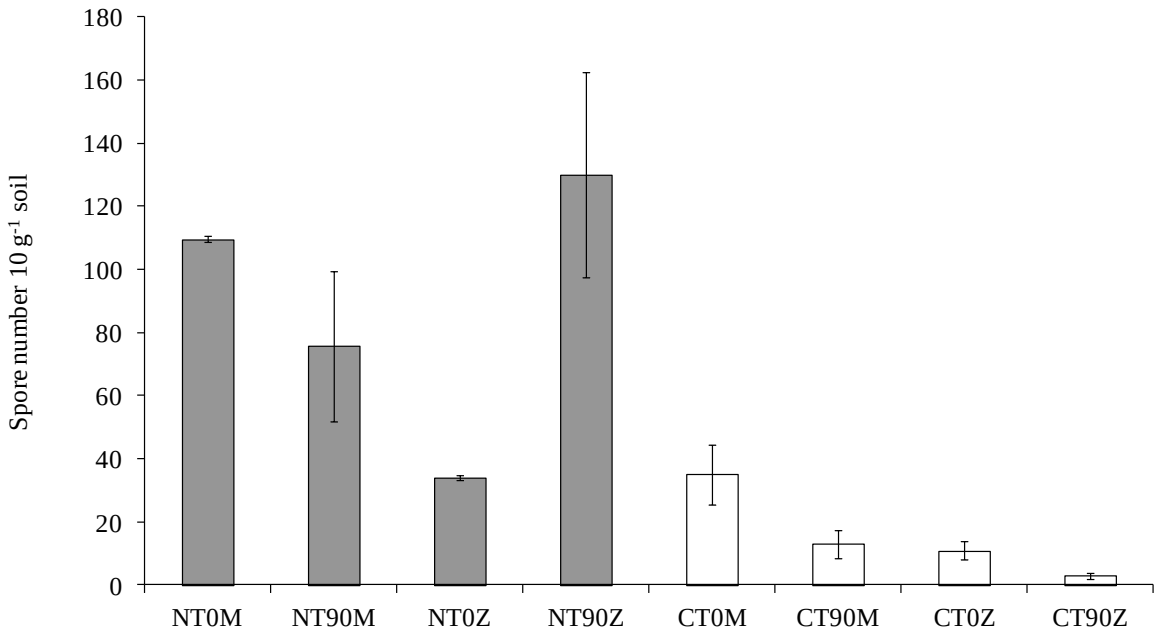
Spore number 10 g-1 soil
180
160
140
120
100
80
60
40
20
0
NT0M
NT90M
NT0Z
NT90Z
CT0M
CT90M
CT0Z
CT90Z

Fig. 7

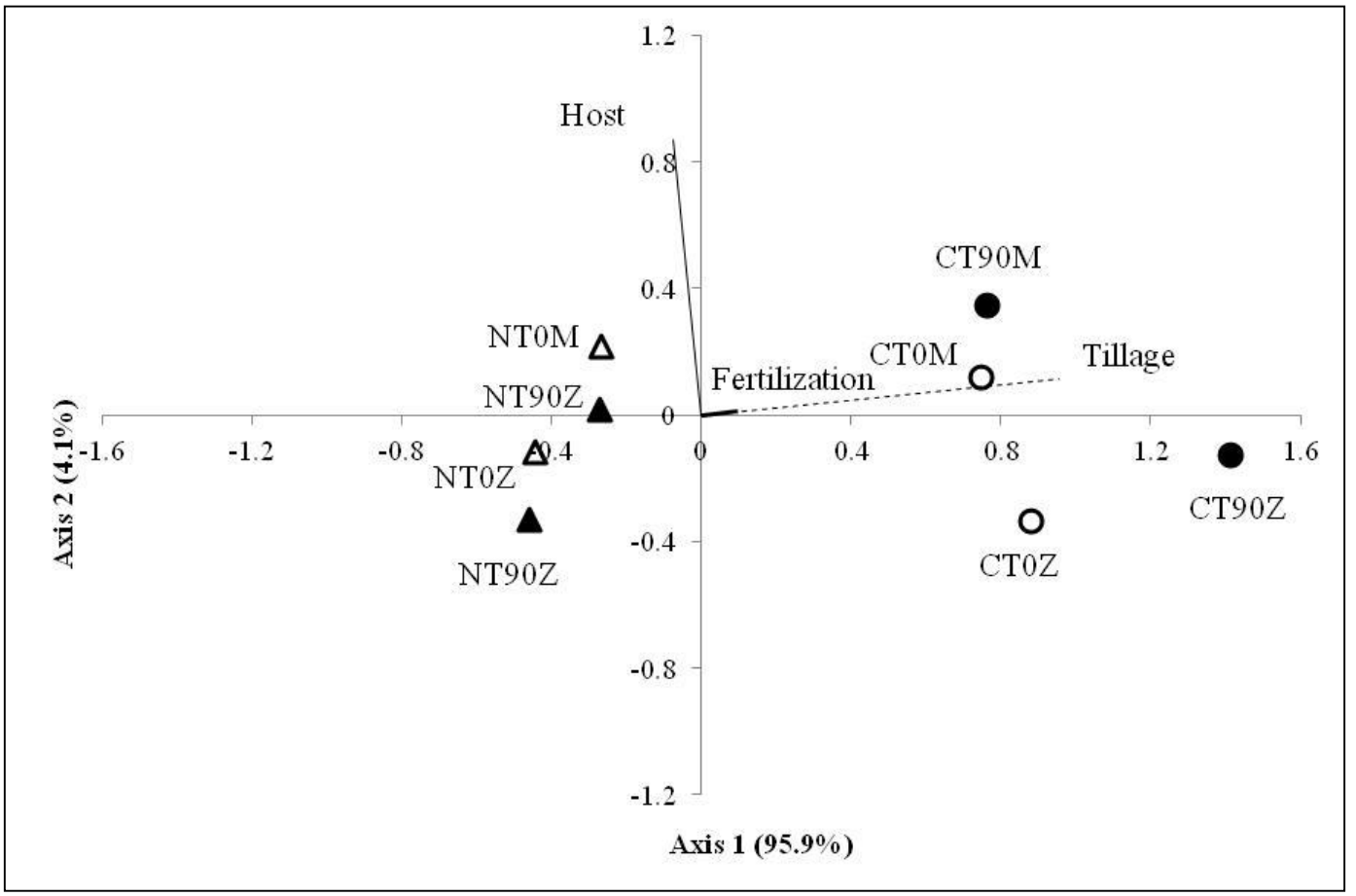
1.2
Host
0.8
CT90M
0.4
NT0M
CT0M
Tillage
Fertilization
NT90Z
0
-1.6
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
NT0Z
CT90Z
-0.4
CT0Z
NT90Z
-0.8
-1.2
Axis 2 (4.1%)
Axis 1 (95.9%)

Fig. 8

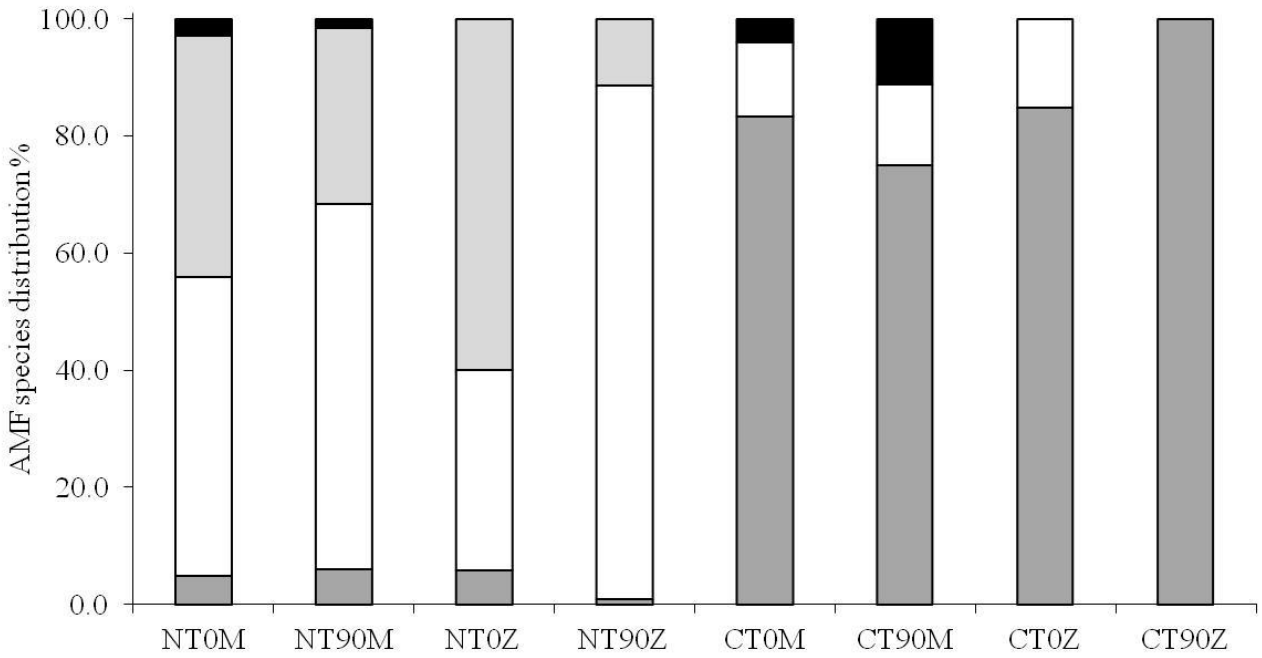
AMF species distribution %
100.0
80.0
60.0
40.0
20.0
0.0
NT0M
NT90M
NT0Z
NT90Z
CT0M
CT90M
CT0Z
CT90Z

Fig. 9