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Impact of tillage system on arbuscular mycorrhiza fungal communities in the soil under Mediterranean conditions

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ABSTRACT

A more diverse arbuscular mycorrhiza (AM) fungal community should be more versatile and resilient to variation in environmental conditions over space and time. To evaluate the effect of no-till and conventional tillage systems, AM fungal diversity was assessed as part of a long term field experiment by sequencing of DNA, extracted from soil, that encoded the large ribosomal sub-unit and was obtained by nested-PCR. In comparison with no-till, conventional tillage decreased AM fungal diversity by 40%. Differences between treatments in the frequency of the operational taxonomic units (OTUs) present in soil, confirm that AM fungi are differently vulnerable to soil disturbance.

1. Introduction

Arbuscular mycorrhiza fungi (AMF) exhibit considerable functional diversity, at the inter- and intraspecific level (Koide, 2000; Munkvold et al., 2004). There are pronounced plant × fungus interactions (Klironomos et al., 2001; Pivato et al., 2007) together with seasonal variability (Allen et al., 1995; Daniell et al., 2001) and evidence of many synergistic and complementary effects between fungal species that occur together (van Tuinen et al., 1998; Gustafson and Casper, 2006; Jansa et al., 2008). Consequently, diversity among AMF has practical implications for the quantitative outcome of specific symbioses. Depending on the prevailing environmental conditions, a diverse community of AMF would be expected to increase the frequency of successful symbioses.

Douds and Millner (1999) argued that an understanding of the impacts of agronomic practices on communities of AMF would help the development of approaches to ensure the establishment of a symbiosis and contribute to the identification and success of sustainable management options. Any factor causing differential reproduction and survival of AMF would operate as a selective pressure on the composition of the soil population and have consequences for the dynamics and diversity of the fungal community. Therefore knowledge of the different factors influencing the population of AMF has been viewed as essential in any attempt to use them in sustainable agriculture (Bethlenfalvay and Linderman, 1992).

Soil disturbance is one factor which, through disrupting the extraradical mycelium (Jasper et al., 1989; Evans and Miller, 1990) and mixing surface residues into the soil profile (Abbott and Robson, 1991; Kabir et al., 1998), selectively interferes with different AMF, depending on their life and colonising strategies, promoting or impairing specific groups (Abbott et al., 1992; Brundrett et al., 1999; Klironomos and Hart, 2002). *Glomus* spp. are believed to survive perturbations well and hence prevail in highly disturbed agricultural systems (Douds et al., 1995; Dodd et al., 2000). It seems likely that AMF that depend mainly on extraradical mycelium to colonise roots of newly sown plants would be more frequent in no-till systems whereas those relying mostly on spores for colonisation would be less affected by soil disturbance. However, evidence from the literature suggests that this is too simplistic. For example, Jansa et al. (2003) proposed that changes in the community structure (abundance and diversity) of AMF, which colonised maize roots, could result from differences between species of AM fungi in their tolerance to the tillage-induced disruption of the hyphae together with changes in the nutrient content of the soil, the activity of other microbes and changes in weed populations. Nevertheless, a reduction in the diversity of AM fungal communities in roots from distinct agro-ecosystems specifically imposed by conventional tillage has been reported by several studies (Schnoor et al., 2011; Alguacil et al., 2008).

For studies of the diversity of AMF the choice of using soil (Cesaro et al., 2008) or root samples (Clapp et al., 1995; van Tuinen et al., 1998; Gollotte et al., 2004) as the basis of investigation is

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Table 1
Essential chemical characteristics of the soil from the field experiment.

Field plot	Soil layer (cm)	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})	pH (water)	OM (mg g^{-1})
No-till	0–10	59	83	5.3	16
	10–20	25	59	5.7	9
Conv. tillage	0–10	19	59	5.6	10
	10–20	16	63	5.6	10

important because of the possibility that biological bias introduced by the host plant will influence the taxonomic groups present in the roots (Jansa et al., 2002) or their abundance (Pivato et al., 2007). Moreover, to identify AM fungal diversity based on soil analysis has the advantage that all forms of inoculum (spores, extraradical mycelium and colonised root fragments) are likely to be present in the samples, immediate biological bias is avoided and species identification is less dependent on spore formation.

The annual contrast in the soil environment associated with the Mediterranean climate might cause a diverse AM fungal community to be an important factor in the resilience of agricultural systems and possible performance of crops. We hypothesise that in the longer term crop production using no-till will enhance soil AM fungal diversity compared with conventional tillage. To evaluate the effect of the tillage system on the soil AM fungal community structure under Mediterranean conditions, an approach based on nested PCR was applied to soil samples from a long term study of no-till and conventional tillage systems, conducted in the Alentejo Province of southern Portugal.

2. Materials and methods

AMF propagules, mycelium, colonised root fragments and spores, and spore densities are known to exhibit a strong spatial structuring at small scales (Bever et al., 2001; Wolfe et al., 2007). Consequently, designing sampling procedures for evaluating diversity, when AMF distribution is spatially heterogeneous, is a complex problem. The results of Whitcomb and Stutz (2007) clearly indicate that sampling intensity and strategy can affect perceptions of AMF community structure. However in an agricultural field the patchy distribution is reduced, in part, as the same host plant is dominant across a field and the pattern of cropping rows helps to determine the location of plant roots.

The study was conducted under field conditions at the Revilheira farm, Alentejo (38°28′N 7°28′W). This site has been used since 1995 as part of a large research program on soil tillage with different crop rotations. Two tillage systems were compared: conventional tillage (mouldboard plough followed by a disk harrow) (CT) and no-till, direct seeding (NT). The rotation was wheat-triticale-sunflower. The soil was a Luvissol and the chemical characteristics of the 0–10 cm and 10–20 cm soil layers for the two tillage treatments are given in Table 1.

2.1. Sampling procedure

Soil samples were taken in the wheat field after harvest in June 2004. Two adjacent tillage plots were selected to reduce soil (physical and chemical characteristics) and field (slope, shade) variability. One plot had been cultivated by conventional tillage (mouldboard ploughed and then disk harrowed to form the seedbed) and was designated as the disturbed (D) treatment. The other plot, which had not been tilled for the last 9 years, was identified as the undisturbed (U) treatment. From each plot (6 × 17 m), 10 soil cores of approximately 200 mL (0–10 cm) were taken at random across the plot and then mixed to produce a single composite sample. This soil was sieved and very carefully homogenised and a sample was kept at 4 °C until used.

2.2. Laboratory procedures for OTUs definition

Three 200 mg sub-samples of the composite sample from each field plot were used to isolate total DNA according to Martin-Laurent et al. (2001). Next, 1 μL of the purified DNA and a 1/10 dilution was used to perform the first PCR amplification with the eukaryotic specific primers LR1 and NDL22 (van Tuinen et al., 1998) designed to border the D1 and D2 variable domains of the 5′ end of the large ribosomal subunit encoding gene. Reactions were performed in a final volume of 20 μL containing 2 μL 10× PCR buffer with 1.5 mM $MgCl_2$ (Qbiogen), 2.5 mM dNTP, 10 μM of each primer, 0.5 U per 100 μL of *Taq* polymerase (Qbiogen) and an aliquot (1 μL) of soil DNA in ultra-pure water all overlaid with mineral oil. Amplification was performed in a thermal cycler (Biometra T3000) programmed as follows: initial denaturation cycle at 93 °C (3 min), annealing at 56 °C (1 min), extension at 72 °C (1 min) followed by 29 cycles of denaturation at 93 °C (1 min), annealing at 56 °C (1 min) and extension at 72 °C (1 min). The last cycle was followed by a final extension at 72 °C for 5 min. For this PCR 1 μL/reaction of the T4 bacteriophage gene 32 product (T4 gp 32) (Q-Bio Gene) was added.

The amplification product was diluted 1/500 and used as template for the second PCR with the primers LR1 and the AMF specific primer FRL4 (Gollotte et al., 2004). The PCR conditions were similar except that the number of cycles was 28 and the annealing temperature was 60 °C.

The second PCR products were separated on a 1.4% agarose gel in TAE buffer (40 mM Tris, pH 7.8, 20 mM acetic acid and 2 mM EDTA) and visualised under UV light after ethidium bromide staining (50 μg L^{-1}).

The product of the second PCR was pooled together and cloned using a TOPO TA Cloning® kit for sequencing (ref. 45-0030 or 45-0641, Invitrogen). The selection of clones to be analysed further was made according to the number of base pairs in the second PCR product, using only inserts ranging between 650 and 750 bp; the expected size for AMF. As a result 48 positive clones (white colonies) were analysed by PCR, using the LR1-FLR4 primers. These clones were multiplied in liquid LB medium (ref. 12780-052 Invitrogen) with ampicillin (0.5 μg mL^{-1}) and the plasmid purified using kit (Nucleo Spin® Plasmid, Macherey-Nagel). The plasmids were sent for sequencing by MWG (Germany).

All sequences were checked through a BLASTN analysis (Altschul et al., 1997) for the presence of chimeric sequences, generated during this procedure, and then aligned with ClustalW. Only four chimeric clones were detected and withdrawn from the analysis.

The sequences were aligned with MAFFT and the alignment was optimised manually using the Se-Al v 2.0 software (University Oxford). Phylogenetic analyses were performed using the neighbour joining (NJ) algorithm included in the ClustalW program, using *Mortierella multidivaricata* as an out-group. Positions with gaps were ignored and the reliability of the internal branches of the NJ tree was assessed using the bootstrap method with 1000 replicates. Tree files were drawn using njplot (http://biom3.univ-lyon1.fr) and the sequences grouped together in OTUs on the bootstrap values, with a threshold of more than 970‰.

Fig. 1. Neighbour-joining tree representing AM fungal sequences from undisturbed (in green) soil and from disturbed soil (in brown), obtained by nested PCR, in comparison with known sequences. In red are branches with bootstrap values > 970%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Rarefaction curves were constructed with the freeware program Analytical Rarefaction 1.3 (www.uga.edu~strata/software) to determine whether the number of sequences tested sufficiently represents Glomeromycota diversity in the studied soils (Fig. 3). An ANOVA was preformed to compare the two rarefaction curves using MSTAT-C (version 1.42, Michigan State University) statistical package (Table 2).

3. Results

Using the results from both analyses a total of 83 sequences of good quality were obtained, 36 from the undisturbed treatment and 47 from the disturbed soil Fig. 1. The number of operational taxonomic units (OTUs) in the different clusters is reported in Fig. 2. With the exception of one ribotype, identified as a *Scutellospora*, all the other OTUs belonged to the Glomineae, and mainly to Glomaceae. Four OTUs could be identified at the species level, namely *Glomus mosseae*, *G. irregulare*, *G. claroideum-etunicatum* and *G. occultum basionym* of *Paraglomus occultum*. According to the data obtained only two of the OTUs found (*G. mosseae*, *G. irregulare*) were present in both soil types, six could only be found in undisturbed soil and three only in disturbed soil. The AM fungal diversity was higher in undisturbed soil, where eight different OTUs were identified,

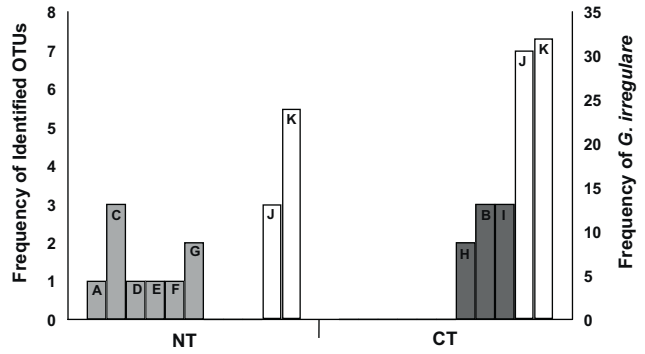

Fig. 2. Identified operational taxonomic units (OTUs) in the two tillage treatments (A – Glomus I; B – Glomus II; C – Glomus III; D – Glomus IV; E – Glomus V; F – Glomus VI; G – Scutellospora; H – *G. cloroidium*; I – *G. ocultum*; J – *G. mosseae*; K – *G. irregulare*). Light grey columns – OTUs found only in the no-tillage treatment; dark grey columns – OTUs found only in the conventional tillage treatment; white columns – OTUs found in both tillage treatments.

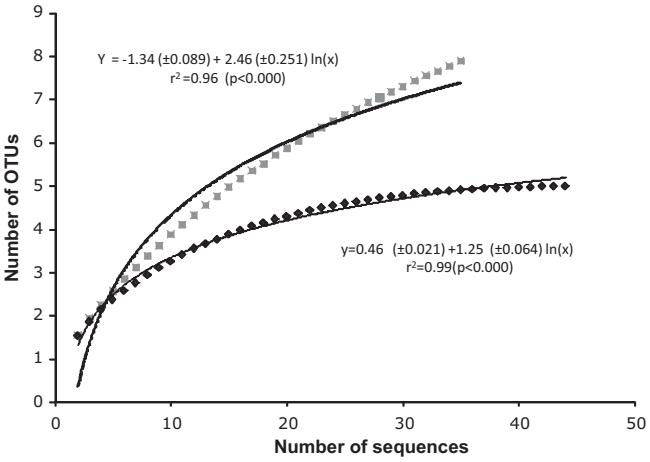

Fig. 3. Rarefaction curve including the data of both years and all the ribotypes identified in no-tillage (grey) and conventional tillage (black) treatments.

when compared to disturbed soil with only five OTUs recognised.

In the rarefaction curves the number of sequences analysed were plotted against the cumulative number of OTUs (Fig. 3), and the differences between the two equations were highly significant ($p < 0.0001$) as presented in Table 2. Despite the fact that most of the OTUs groups exclusively detected in no-till (Glomus I, IV, V and VI) are poorly represented (Fig. 2), the number of analysed sequences was larger than the one suggested by Pivato et al. (2007) as adequate for characterisation of the diversity of AMF in soil. Moreover, the rarefaction curves indicate that for conventional tillage the plateau had been reached. Consequently, if more sequences were analysed the diversity of AMF would only show an increase for no-till. Therefore, we consider that the data presented support the hypothesis that the diversity of AMF was greater under no-till.

Table 2
Analyses of variance to compare the differences between the two equations of the rarefaction curves presented in Fig. 3.

Source	DF	Mean square	*F* value	Prob.
Between equations	2	32.040	468.81	<0.0001
Differences in level	1	48.016	172.22	<0.0001
Error	76	21.190		
Differences in angle	1	16.064	235.05	<0.0001
Error	75	5.126		

4. Discussion

Blast searches in the GeneBank database showed that all sequences obtained in this study belonged to the Glomeromycota, confirming the specificity of the LR1-FLR4 primer pair for detection of Glomeromycota. The detection of *Scutellospora* and *Paraglomus*, confirmed that all the Glomeromycota families are amplified (Mummey and Rillig, 2007). Of the 83 sequences analysed, 36 were from undisturbed soil and 47 were from disturbed soil, each of which exceeds the minimum number of 30–35 identified by Pivato et al. (2007) as being required to describe the diversity of AMF in a field experiment and also greater than the number of sequences analysed by Schnoor et al. (2011). Differences between treatments in the frequency of the OTUs present in soil confirm that AMF are differently vulnerable to soil disturbance. This appears to be true, both in terms of the community structure, as the same OTUs show different frequencies depending on the level of soil disturbance, and in terms of diversity, given that the OTUs present in the undisturbed soil were not always the same as those isolated from disturbed soil (Fig. 2).

Under both experimental treatments, the OTU corresponding to cluster that included the ubiquitous *G. irregulare*, was clearly the most abundant found relative to other isolates from the Glomaceae. OTU within this taxonomic group provide evidence of high molecular diversity. Our findings are consistent with those of Mathimaran et al. (2005), who found that spores of *G. irregulare* were dominant in a soil community associated with wheat as host plant.

Diversity of the OTUs found under no-till was greater than that in disturbed soil, consistent with the view that species richness can be reduced by intensive tillage (Jansa et al., 2003; Alguacil et al., 2008; Schnoor et al., 2011). Although the frequency of some OTUs found in our study was small, this might be due to the use of soil instead of root samples. Pivato et al. (2007), studying the OTUs frequency in soil and roots, found significantly more present in root samples. Our results demonstrate an effect of tillage on the AMF community in soil irrespective of the host plant. Under mixed farming systems or crop rotation the enhanced diversity of AMF in soil could provide an advantage. An interesting investigation from an agronomic perspective would be to analyse the effect of tillage on the AM fungal community structure using mixed or sequential (crop rotation) host crops.

Belowground diversity is an essential component of ecosystem health (Bever et al., 2001) More diverse AMF communities would be able to exhibit greater versatility and resilience in response to variation of environmental conditions over space and time. By favouring diversity of AMF, management practices, such as no-till, have the possibility of encouraging mycorrhiza as the possible combinations of host plant and AMF are greater and, depending on eventual biotic or abiotic stresses, the chances of having a functional symbiosis are greater.

5. Conclusion

This study confirms that the use of soil samples is an appropriate tool to investigate the effect of production techniques on AMF biodiversity. The enhanced diversity observed under no-till suggests that this tillage system has the potential to enhance the sustainable arable production systems. Our results validate the use of a minimum of 35 sequences to characterise AMF diversity under arable cropping systems.

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