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Switching between monocot and dicot crops in rotation schemes of Argentinean productive fields results in an increment of arbuscular mycorrhizal fungi diversity

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ABSTRACT

Despite the importance of mycorrhizal symbiosis, we understand little how different soil managements affect arbuscular mycorrhizal fungi (AMF) communities. Crop rotation is recommended in sustainable agriculture because of its benefits in soil fertility improvement and positive effect decreasing soil borne diseases incidence and pest abundance. Amplicon sequencing of LSU and SSU rRNA gene fragments was used to analyse AMF diversity in fields from one of the most productive regions in Argentina, which varied in the main class of the plant component included in the crop rotation scheme. The samples encompassed different agricultural settings; one involving only monocot plants in the crop rotation schemes, one including a dicot crop, and the other an alternation and/or a combination of monocot and dicot plant components. We found lower richness and diversity in soils under monocot succession than in a dicot/monocot rotation or consociation. We observed that agricultural management had an influence on beta diversity patterns. Principal coordinate analysis showed that communities from the dicot/monocot rotation or consociation samples clustered together and separated from the monocots samples. These findings suggested that the increment of soil AMF diversity is more dependent on the alternation between monocot and dicot crops than other factors related to the farming systems.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) belonging to the phylum Glomeromycota (Schüßler et al., 2001), together with ectomycorrhizal fungi (EMF) colonize the roots of most land plants, facilitating mineral nutrient uptake from soil in exchange for plant-assimilated carbon (Smith and Read, 2008). AMF are the beneficial microorganisms more often associated to plant roots (Smith and Read, 2008) and represent an important group in ecosystem functioning because of its ubiquity and direct implications in different processes involved in plant-soil interactions. AMF act at different levels, leading to morphological and anatomical changes in host plants such as shoot–root ratio, root tissue structure, chloroplast numbers, lignification enhancement and other effects that are not explicable merely as a simple plant nutrition improvement due to an increased efficiency in nutrient uptake by the root, but to more complex metabolic changes related to the physiological integration of the symbionts (Smith and Smith, 2011).

Different agroecosystems involve a wide range of possibilities in the crops management and therefore the soil. One specific plot could be managed under traditional plowing to no tillage, and from monoculture production to rational crop rotation between species with different root exploration capabilities etc., according to the type of production pursued. It is not surprising that such different agricultural practices could alter AMF communities. Thus, it has been reported that conventional tillage or plowing, breaks and concomitantly reduces the network length of AMF hyphae and root colonization (McGonigle and Miller, 1996). A comparative study showed the effect of conventional tillage and no-till system on the formation of mycorrhizal symbiosis in wheat (Schalamuk et al., 2006). While the effect of tillage with nitrogen (N) fertilization significantly reduced the colonization and the number of infective propagules, no-till practice increased colonization during flowering and grain filling. Soil biodiversity has been recognized as an important component of soil health, potentially enhancing plant

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productivity and ecosystem sustainability (Brussaard et al., 2007; van der Heijden et al., 2008). It is worth mentioning that it is being progressively more accepted that some farming managements have a negative impact on the environment, resulting in a reduced biodiversity (Tilman et al., 2002). In general, agroecosystems showed less AMF diversity along with different other sites in which AMF communities have been analysed (Oehl et al., 2003; Öpik et al., 2006), although specific results might depend on the study location. It is still not well understood if one particular land-use treatment leads to AMF communities that are more similar to natural assemblages. (Verbruggen et al., 2010). AMF usually have broad host ranges, although some preferences or functional diversifications between plants and mycorrhizal fungi have been suggested (Goomaral et al., 2003; Helgason et al., 2002; Vandenkoornhuyse et al., 2003). Moreover, it has been proposed that plant communities' composition and productivity might be in some way dependent on AMF diversity (Klironomos et al., 2000; van der Heijden et al., 2008). In agreement to this fact, Burrows and Pfleger (2002) reported that a rise in the number of plant species correlated to AMFs sporulation increment and to the composition of their communities. However, this apparent selection of AMF species by the plant has not been extensively investigated. In mycorrhiza is easy to imagine that partners, plant and AMF, influence one another and consequently changes in the AMF communities composition could influence the plants community. In a complementary view of the same scenario, it would be foreseeable, that changes in the plant communities have an effect on the AMF community. Variations in fungal population have been reported in agricultural soils under different cropping practices (Johnson et al., 1991). Meanwhile, many studies showed that AMF communities are diversified among different plant species (Bever et al., 1996; Sanders and Fitter, 1992; Vandenkoornhuyse et al., 2002, 2003). Thus, it is feasible that plant community could themselves be a determinant of the mycorrhizal fungi community (Goomaral et al., 2003).

It has been demonstrated that diversifying the crops used in a rotation scheme, increases the taxonomic and functional diversity of soil fungal communities (Larkin and Honeycutt, 2006). Crop rotation has an effect on the microbial activity and on the substrate utilization (Larkin et al., 2010). This is a remarkably significant practice in conventional agriculture because contributes to the improvement of soil quality (Anderson, 2005; Varela et al., 2014), and offers a possibility for management of pathogenic soil-borne fungi and weeds (Chave et al., 2014; Dias et al., 2015; Garrison et al., 2014; Schillinger and Paulitz, 2013). Thus, combining different crops such as cereals with canola (Nelson et al., 2012), or legumes (Blackshaw et al., 2010) can result in an increase of productivity by reducing diseases and weeds incidence.

The application of a well-planned crop rotation scheme implies much less synthetic external inputs and consequently, less environmental impact to manage diverse pests (Davis et al., 2012; Karlen et al., 1994). In the case of weeds management, when a dicot crop is followed by a monocot one, the combination of both chemical and mechanical control can be done more properly (Heap, 2014; Leroux et al., 1996).

Despite the fact that AMF are possibly the most important fungi in terrestrial ecosystems, we understand very little about many aspects of their biology partly because of their asexual, obligate symbiotic and subterranean lifestyle. Nevertheless, traditional culture-independent methods have shed light on some aspect of AMF phylogenetic relationships and diversity. The limitation of these methods is that they are low throughput that renders many taxa undetectable. The massively parallel pyrosequencing enables metagenomic and metagenetic analyses in a way that exceeds the capacity of traditional Sanger sequencing-based approaches by several orders of magnitude (Margulies et al., 2005; Sogin et al., 2006). Pyrosequencing offers great promise in the high-throughput identification of hundreds of samples at a reasonable cost and time consumption (Margulies et al., 2005; Roesch et al., 2007; Sogin et al., 2006). The most frequently used markers are the nuclear ribosomal RNA genes, especially the small subunit rRNA (SSU) (Helgason et al., 1999; Lee et al., 2008; Wubet et al., 2006) and the internal transcribed spacer rRNA region (ITS) including the 5.8S rRNA (Hempel et al., 2007; Sýkorová et al., 2007; Wubet et al.,

Table 1
General characteristics, soil chemical properties and descriptions of the agricultural treatments and crop rotations schemes.

Treatments	Agricultural managements	Crop rotation schemes						Soil characteristics		
		Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	pH	OC (%)[a]	EP (ppm)[b]
Monocots	RG3	*Allium cepa* Tillage	*Allium cepa*	*Lolium perenne* No-till	*Lolium perenne*	*Lolium perenne*	*Allium cepa* Tillage	7.8	1.6	48.4
	Agropyron	*Thinopyrum ponticum* No-till	*Thinopyrum ponticum*	*Thinopyrum ponticum*	*Thinopyrum ponticum*	*Thinopyrum ponticum*	*Allium cepa* Tillage	8.0	1.8	40.2
	GM	*Setaria italica* Tillage	*Allium cepa*	*Setaria italica*	*Allium cepa*	*Setaria italica*	*Allium cepa*	7.9	1.0	43.9
Monocot/dicot alternation	Grain	*Helianthus annuus* Tillage	*Triticum aestivum*	*Helianthus annuus*	*Triticum aestivum*	*Helianthus annuus*	*Allium cepa*	8.0	1.7	39.2
	RM	*Helianthus annuus* Tillage	*Triticum aestivum* No-till	*Medicago sativa–Thinopyrum ponticum*	*Medicago sativa–Thinopyrum ponticum*	*Medicago sativa–Thinopyrum ponticum*	*Allium cepa* Tillage	7.7	2.3	18.8
	Alfa3	*Medicago sativa* No-till	*Medicago sativa* Tillage	*Medicago sativa*	*Allium cepa*	*Allium cepa*	*Allium cepa*	7.7	2.1	23.9
Dicots	Alfa5	*Medicago sativa* No-till	*Medicago sativa*	*Medicago sativa*	*Medicago sativa*	*Medicago sativa*	*Allium cepa* Tillage	7.7	2.1	19.9

[a] OC, percentage of organic content in the soil samples as determined by the Walkley and Black method.
[b] EP, extractable phosphorous in the soil samples.

2006). However, many molecular analyses are biased, as some of the primers used detect only parts of the community and the level of taxonomic resolution in most cases is uncertain. Species-level community analyses based on rRNA should be feasible (Gamper et al., 2009; Stockinger et al., 2009), but no single molecular marker or DNA barcode is yet suitable for species-level resolution of all AMF. Accurate sequence classification is a critical step for the identification of microbial taxa and for the evaluation of a fungal community diversity and ecology (Blackwell, 2011). Most AMF biodiversity data have been obtained by analyzing the SSU rRNA gene, providing the principal sequence data set for phylogenetic studies but not necessarily the most accurate one (da Silva et al., 2006; Krüger et al., 2009; Sharmah et al., 2010). It has been reported that the D2 region of the large subunit rRNA (LSU) is more variable across different fungal taxa and, consequently, the taxonomic precision achieved by pyrosequencing this region could be quite high (Liu et al., 2012). Moreover, other previous reports are in accordance with this finding (Gollotte et al., 2004; Mummey and Rillig, 2007; Verbruggen et al., 2010). On the other hand, in most preceding research studies the LSU-based mycorrhizal diversity has been assessed by employing traditional molecular methods such as Sanger sequencing of gene libraries or fingerprinting techniques but no by 454 pyrosequencing (Mummey and Rillig, 2007; Stockinger et al., 2010; Verbruggen et al., 2010).

In this study, we examined the AMF diversity and community structure in soils from agricultural fields, which varied in the main class of the plant component included in the crop rotation scheme. We collected soil samples from a field trial located in one of the most productive agricultural regions of Argentina, which encompassed different agricultural settings; one involving only monocot plants in the crop rotation schemes, one including a dicot crop, and the other an alternation and/or a combination of monocot and dicot plant components. From these samples, we generated amplicon libraries of the SSU and D2 LSU rRNA gene regions that were subsequently, sequenced using 454 FLX pyrosequencing. We hypothesized that alternation in the class of the plant components (mono/dicot) in a rotation scheme has an impact in the variability of AMF communities.

Understanding how the AMF community varies according to changes in the plant communities could provide further insight into the influence and relationship that plant communities have on fungal soil diversity in cropping systems.

2. Materials and methods

2.1. Experimental design, sampling and DNA extraction

Soil samples were collected in December 2009 from an experimental field at Hilario Ascasubi Agricultural Experimental Station from INTA, Buenos Aires province, Argentina (39°23′57.15″S; 62°37′30.64″W). The study region, known as Valle Bonaerense del Río Colorado, is the major onion productive region of Argentina. The climate is temperate semiarid with average annual precipitation of 450 mm and mean annual temperatures of 7.5 °C and 22 °C. The soil was classified as an *Entic Hapludol* and had a sandy loam texture (67% sand, 17.5% silt, 15.5% clay) and the main chemical properties were determined by standard methods (Bray and Kurtz, 1945; Walkley and Black, 1934) and are shown in Table 1. The experimental site has been used for studying the effect that different rotations and green manures have on productivity and quality of the onion. Agricultural managements were classified into three general treatments according to the class of the plant components including in the rotation schemes; in that way treatments were defined as monocot, monocot/dicot alternation and dicot. The seven different types of management under analysis encompassed different long-term crop rotation practices (Table 1). The rotation schemes intended the management of onion (*Allium cepa* L.) diseases caused by soil borne fungi, specifically *Fusarium oxysporum* and *Setophoma terrestris*, and control of annual and perennial weeds. Each rotation or soil treatment includes non-host plant species for the soil borne fungal pathogens mentioned above. The switching between monocot and dicot crops was conceived as a way to efficiently control weeds and to contribute to soil fertility and structure, features also taken into account with the incorporation of green manure (GM) and the inclusion of periods of no-till (RG3 and RM). Each rotation scheme has been conducted for six years. The sampling was performed at the end of the crop rotation cycles. The soil chemical properties of the sites under study are presented in Table 1. The field trial was established following a complete randomized block design with four repetitions and experimental units of 15 m × 15 m (plots). Three treatments were defined according to the main class of plant component included in the crop rotation: monocot, monocot/dicot alternation and dicot. Treatments consisted in plots under seven different agricultural managements: RG3, *Agropyron* and GM for monocot; RM, Grain and Alfa3 for monocot/dicot alternation, and Alfa5 for dicot (Table 1). Samples were taken as duplicated for each management (RG3, *Agropyron*, GM, RM, Grain, Alfa3 and Alfa5). Each sample was collected as a composite of fifteen-soil core randomly selected and uniformly distributed subsamples (5 cm in diameter and 10–12 cm in depth). Composite samples were homogenized in the field and brought to the laboratory at 4 °C. Samples were sieved through 2 mm mesh to remove roots and plant detritus, and stored at −20 °C until processing. Total soil microbial DNA was extracted from 0.5 g of soil by using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. To avoid PCR inhibitors, a clean-up was performed by centrifugation of DNA extracted through a column containing polyvinyl polypyrrolidone (PVPP) (Arbeli and Fuentes, 2007). The eluted DNA samples were stored at – 20 °C.

2.2. Amplification of SSU and D2 LSU rRNA regions and pyrosequencing

Since it has been reported that the LSU primers FLR3/FLR4 could exclude from the amplification some AMF lineages such as *Paraglomus* (Krüger et al., 2009), we performed in parallel pyrosequencing and analysis of both, SSU and D2 LSU rRNA fragments. Three rounds of PCR amplification were carried out to generate SSU and D2 LSU amplicon libraries. For SSU, the first

Table 2
Primers used in this work.

Primer	Nucleotide sequence (5′-3′)	rRNA gene region	Source
LR1	GCATATCAATAAGCGGAGGA	LSU	Van Tuinen et al. (1998)
NDL22	TGGTCCGTGTTTCAAGACG		
FLR3*tag*[a]	CACGACGTTGTAAAACGACTTGAAAGGGAAACGATTGAAGT	D2 LSU	Modified from Gollotte et al. (2004)
FLR4*tag*[a]	CAGGAAACAGCTATGACCTACGTCAACATCCTTAACGAA		

[a] The *tag* sequences includes 454 sequencing (Roche, Life Science) adapters and was added to the original primer sequences to allow the matching of the primers that carry the different 10 nucleotide-length Multiplex Identifiers (MIDs) necessary for sample identification.

reaction was performed using DNA extract as template and the primer pair GEOA2 and GEO11 (Schwarzott and Schüßler, 2001). The second PCR round was performed by using appropriate dilutions of GEOA2/GEO11 amplicons as template and the primers FA.AMV4.5N-key/RB.AMDG-key, modified from (Lumini et al., 2009), to amply an internal fragment of about 300 bp of the Glomeromycota SSU region (Table 2). For D2 LSU, the first round of amplification was done by using the primers LR1/NDL22 (van Tuinen et al., 1998) and DNA extract as template in order to amplify a fragment of approximately 780 bp corresponding to the 5′-end of LSU rRNA gene from fungi in general (Table 2). The second PCR round was carried out by using the primer pair FLR3tag/FLR4tag, modified from Gollotte et al. (2004) (Table 2) and employing appropriate dilutions of LR1/NDL22 amplicons as template. These primers amplify a fragment of about 450 bp, internal to the LR1/NDL22 fragment and matching exclusively the D2 domain of the LSU rRNA gene of AMF. The amplicons obtained were purified and then a third cycle of amplification was carried out in order to add to the SSU and D2 LSU amplicons 10 nucleotide-length multiplex identifiers (MIDs) necessary for Roche 454 sequencing identification of each one of the samples. A 20 μl reaction mix contained 1X Phusion GC Buffer, 200 μM dNTPs, 1 unit of Phusion High-Fidelity DNA polymerase (New England Biolab) and 0.5 μm of each primer was used for each amplification cycle. The PCR conditions for the first PCR were: initial denaturation at 98 °C for 3 min, 35 cycles of denaturation a 98 °C for 30 s, annealing at 59 °C for 1 min, elongation at 72 °C for 1 min, and final extension at 72 °C for 5 min. The second and third PCR products were amplified using the following parameters: initial denaturation at 98 °C for 30 s (s), 30 cycles of denaturation a 98 °C for 10 s, annealing at 72 °C for 30 s, elongation at 72 °C for 30 s, and final extension at 72 °C for 7 min. Each of the subsamples was amplified separately, later combined and employed as a representative composite of each sample (two per agricultural management, six and two per treatment). All PCR products were purified by using Illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare), and quantification was performed with NanoDrop (NanoDrop Technologies). The SSU and D2 LSU amplicon libraries were sequenced using 454-FLX-Titanium chemistry (Roche Applied Science) at the INDEAR genome sequencing facility (Rosario, Argentina), according to standard protocols and the manufacturer's instructions and using 1/4 of one Pico Titer Plate in one sequencing run.

2.3. Sequence analysis, alpha and beta diversity analyses

Pyrosequencing reads were processed and analyzed using the QIIME software v1.5.0 as described by Caporaso et al. (2010). A total of 216,447 raw sequences were obtained. Using the script split_libraries.pybarcodes and tags were removed, sequences were denoised using AmpliconNoise 1.25, reads were truncated to remove reverse primer and subsequent sequences, and reads containing any unresolved nucleotide were also eliminated from the dataset. Filter parameters were set to reject reads that had mean quality score < 25, maximum homopolymer run > 6, number of primer mismatches > 0, and read length < 200 bp or >1000 bp. A total of 59,481 and 128,643 filtered sequences were finally obtained for D2 LSU and SSU reads, respectively. By using the script pick_otus.pywe assigned similar sequences to operational taxonomic units (OTUs) by clustering sequences based on 97% similarity threshold according to UClust (Robert Edgar, unpublished, 2009). The most abundant sequences in an OTU cluster was selected as OTU representative sequence (pick_rep_set.py). For taxonomy-based analysis, the RDP Classifier of the Ribosomal Database Project (RDP) was used at a confidence threshold of 80% (Wang et al., 2007) and the alignments were performed by MUSCLE (Edgar, 2004). With the script make_otu_table.py we tabulated the number of times an OTU is found in each sample and added the taxonomic predictions for each OTU. Sequence data normalization was done by calculating multiple and incremental rarefactions on the OTU file to standardize the data obtained from samples with different sequencing efforts (from a minimum value of 500 to reach the top value of the most abundant sample, with a fixed step of 100 and with 10 repetitions for each step, script: multiple_rarefactions.py). The rarefied OTU table was used for the downstream alpha and beta diversity analyses. By using the scriptalpha_diversity.py, we computed measures of alpha diversity

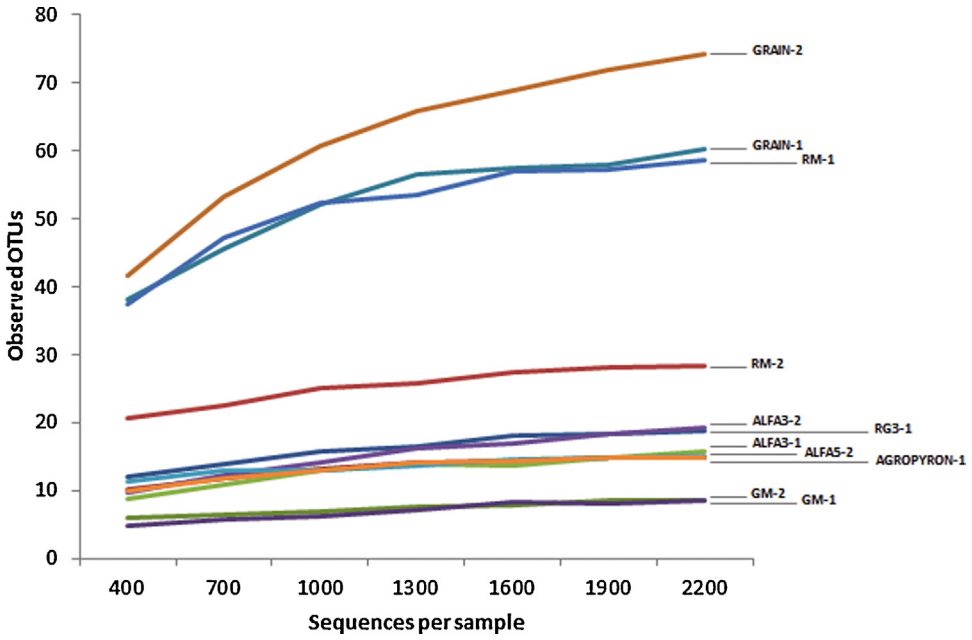

Fig. 1. Sampling effort curves depicting the rate of discovery of AMF OTUs (LSU-based data, 97% of similarity level) at the sampling sites. The rarefaction curves show the number of AMF OTUs as a function of the number of sequences per sample as explained in Section 2. The asymptote of the rarefaction curves reveals that the AMF diversity coverage was sufficient for most of the samples analysed.

such as Shannon, Chao 1, Simpson's dominance index and equitability based on the OTU picker data normalized to an equal number of sequences per sample. To evaluate the differences between AMF communities we calculated unweighted Unifrac and Bray–Curtis dissimilarity matrices (script:beta_diversity.py, and using as input the rarefied OTU file) and performed principal coordinate analysis (PCoA) to compare AMF diversity across the treatments (script:principal_coordinates.py). *Jackknife* replicates were utilized to estimate the uncertainty in PCoA plots (100 permutations,jackknifed_beta_diversity.py). All statistical analyses were performed using QIIME. Analysis of similarities (ANOSIM) and Adonis were applied to compare pyrosequencing derived data communities among agricultural managements (compare_categories.py). ANOSIM is a non-parametric (permutation-based) test that is similar to nonmetric multidimensional scaling (NMDS) ordination in that it uses the rank order of dissimilarity values (from a distance matrix) across metadata categories (Lekberg et al., 2014). Adonis, is a non-parametric statistical method that takes a QIIME distance matrix file such as a UniFrac distance matrix, a mapping file, and a category in the mapping file to determine sample grouping from. It computes an R^2 value (effect size), which shows the percentage of variation explained by the supplied mapping file category as well as a P value to determine the statistical significance (http://qiime.org/tutorials). Statistical analysis using ANOVA: in addition to ANOSIM and Adonis calculations, we applied one-way ANOVA to further determine how much variation in the Shannon AMF diversity index among the samples is explained by differences in the application of tillage in each treatment (see Table 1). All statistical significance and comparisons were performed by using Infostat software (Di Rienzo et al., 2003). Sequence data was deposited in the BioProject/NCBI database, Accession: PRJNA239364.

3. Results

3.1. AMF diversity in soils under monocot, monocot/dicot alternation and dicot treatments

We assessed and compared the composition of AMF communities present in soil samples derived from a field trial established to study the effect that different agricultural practices have on productivity and quality of the onion culture.

The high-throughput sequencing of D2 LSU rRNA gene fragment yielded 70,138 raw sequences. For one of the two replicates of the *Agropyron* and Alfa5 managements the number of reads obtained was not adequate and thus they were excluded from further analysis. So as to reduce the overestimation of rare phylotypes, a quality filtering of the sequences' dataset was performed by QIIME. Furthermore, clustering and diversity estimates were carried out at a genetic divergence of ≥3% (Kunin et al., 2010). Denoising of each sequences subset was executed to avoid overestimation of operational taxonomic units (OTUs) and diversity. As a result, 59,481 high-quality reads were achieved and used for further data analysis. For the LSU-based data subset the number of sequences per samples ranged from 2137 to 6514 with a mean sequence length of 387.4 bp (SD 75.61). At this depth of sequencing, we have surveyed almost the full extent of taxonomic diversity within individual soils at the 97% similarity level of taxonomic resolution. This is evidenced by the trends of the rarefaction curves for the samples (Fig. 1). The results showed that the coverage was adequate for most samples as most accumulation curves reached an asymptote. Comparison of rarefaction analysis with the number of OTUs determined by the Chao 1 richness estimator revealed that 94.5% of the expected richness was covered by the surveying effort (Table 3). Clustering of D2 LSU pyrotags at similarity levels of 97% resulted in 405 OTUs. This value was reduced to 185 after eliminating singletons. The great majority of the OTUs were related to the genus *Funneliformis* and *Glomus* (Table 4,Fig. 2). It has been reported that by using the couple of primers FLR3/FLR4 it is possible to exclude some AMF groups such as *Paraglomus* (Krüger et al., 2009). To avoid this, we generated in parallel amplicon libraries of SSU rRNA gene region and subsequently sequenced them by pyrosequencing. We obtained 146,309 raw sequences and 128,643 reads after the quality filtering. The number of sequences per samples for the SSU-based data subset ranged from 2961 to 22,083 with a mean length of 300.1 bp (SD 35.25). One of the replicates of the *Agropyron* and one of the Alfa5 managements did not yield enough number of reads and were excluded from the analysis. Although we observed a lower percentage of reads belonging to the phylum Glomeromycota and higher biases to other taxonomical groups (data not shown), the analysis of the SSU pyrotags revealed similar results to the D2 LSU approach and we were able to detect members within the genus *Gigaspora*, *Scutellospora*, *Acaulospora*, *Entrophospora*, *Diversispora*, *Paraglomus* (Table 5, Fig. 2). We observed no significant differences in the community's membership among the soil treatments. The genus *Glomus*, *Funneliformis* and *Rhizophagus* were dominant in soil samples from monocot/dicot alternation and dicot treatments. The samples from RG3, *Agropyron* and GM showed a more variable composition though a higher proportion of genus *Glomus* and/or *Funneliformis* was also found. The Shannon diversity (H′), Simpson's dominance and equitability indices were also computed for all samples (Table 6). At a genetic distance of 3%, the Shannon index ranged from 0.17 to 4.02. The lowest values were observed for GM (0.17 and 0.54), *Agropyron* (0.54), and Alfa3 (0.55 and 0.56). We found the highest Shannon values for RM and Grain treatments, indicating an increasing in the AMF diversity. In general, we observed higher dominance values and lower equitability indices in GM, Alfa3, *Agropyron* and RG3 in contrast to the lower dominance and higher equitability found for RM and Grain samples. Taking into account richness and diversity values, these results would indicate that the monocot/dicot alternation management applied in RM and Grain favors the establishment of a more diverse and even AMF community. The high mean number of observed OTUs in Grain and RM was accompanied by a more equal distribution of their members, as revealed by the high dominance and low equitability indices (Table 3). ANOVA analysis of alpha diversity revealed no significant differences between monocot samples (GM, RG3, *Agropyron*) or between monocot/dicot rotation (Grain) and monocot/dicot consociation (RM). Significance was observed when comparing overall monocot samples with those including a monocot and a dicot plant species ($R^2 = 0.98$, $P = 0.001$, Table 7). No significance was obtained by analyzing the variable "tillage" (Table 7).

Table 3
Chao1 richness estimator and AMF OTUs prediction (LSU-based data, 97% similarity level).

Samples	Chao1	Observed OTUs	
		Total	Glomeromycota
RG3-1	19.00	19	19
RG3-2	15.50	15	15
Agropyron-1	21.00	16	12
GM-1	10.00	9	9
GM-2	10.00	9	9
Grain-1	63.00	61	61
Grain-2	82.50	76	75
RM-1	59.00	59	59
RM-2	32.00	29	29
Alfa3-1	27.50	20	15
Alfa3-2	19.20	19	19
Alfa5-1	19.00	16	15

Table 4
LSU sequences analysis. OTUs found at 97% similarity level and divided according to their taxonomy.

Samples	Glomeraceae					Claroideoglomeraceae	Gigasporaceae
	Glomus	*Funneliformis*	*Rhizophagus*	*Septoglomus*	*Sclerocystis*	*Claroideoglomus*	*Gigaspora*
RG3-1	5	14	0	0	0	0	0
RG3-2	11	4	0	0	0	0	0
Agropyron-1	6	3	1	1	0	1	0
GM-1	6	0	2	0	0	1	0
GM-2	3	4	1	0	0	1	0
GRAIN-1	24	25	12	0	0	0	0
GRAIN-2	19	35	16	0	0	5	0
RM-1	14	32	8	0	2	3	0
RM-2	13	7	5	0	1	2	0
Alfa3-1	2	13	0	0	0	0	0
Alfa3-2	1	15	1	1	0	0	1
Alfa5-1	1	14	0	0	0	0	0

3.2. AMF beta diversity

To compare AMF communities among the different management types, we performed principal coordinate analysis (PCoA) based on UniFrac distance measures (UU) and Bray–Curtis distances (BC) by using the QIIME pipeline. We performed either unweighted UniFrac, using only presence–absence information, or Bray–Curtis which takes into account the relative abundance of

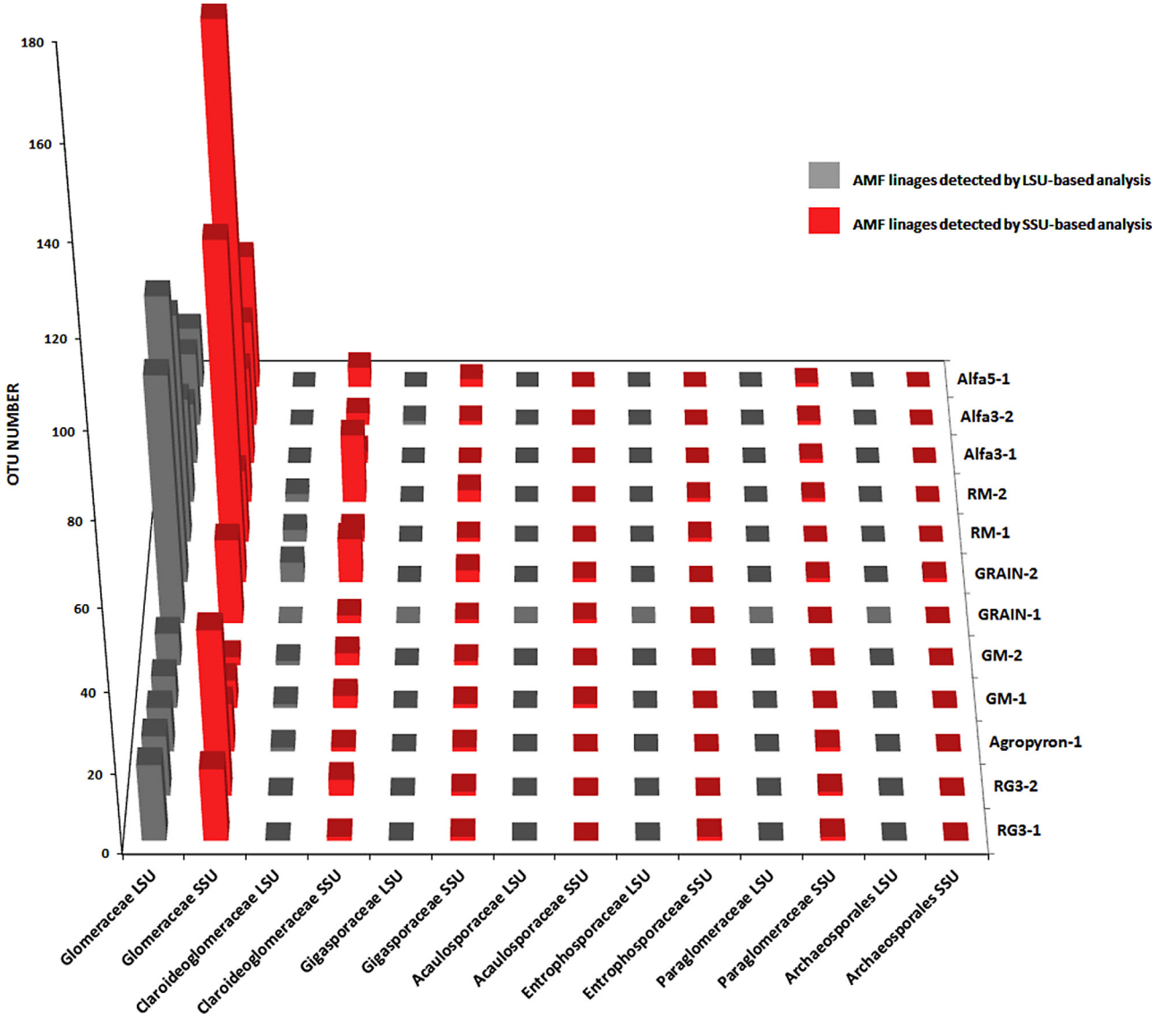

Fig. 2. Graphic display of the AMF taxa profiles as detected by LSU and SSU analysis. In the 3D column chart, bars represent the number of OTUs related to the different AMF families found by clustering the sequences at 97% of similarity level. Grey bars indicate the observed LSU OTUs and red bars the SSU OTUs found in the analysis for each sequence dataset. The samples are represented in the *Z*-axis and the AMF families are indicated in the *X*-axis.

Table 5
SSU sequences analysis. OTUs found at 97% similarity level and divided according to their taxonomy.

Samples	*Glomeraceae*				*Claroideoglomeraceae*	*Gigasporaceae*		*Acaulosporaceae*	*Entrophosporaceae*	*Paraglomeraceae*	*Archaeosporales*
	Glomus	*Funneliformis*	*Rhizophagus*	*Sclerocystis*	*Claroideoglomus*	*Gigaspora*	*Scutellospora*	*Acaulospora*	*Entrophospora*	*Paraglomus*	*Archaeospora*
RG3-1	14	2	1	1	1	0	1	0	1	1	0
RG3-2	34	6	1	0	4	0	1	0	0	1	0
Agropyron-1	7	4	1	0	1	0	1	0	0	1	0
GM-1	2	1	3	1	3	0	1	1	0	0	0
GM-2	2	0	0	0	3	0	1	0	0	0	0
GRAIN-1	10	4	7	0	2	0	1	1	0	0	0
GRAIN-2	45	19	19	0	11	1	2	1	0	1	1
RM-1	16	1	1	0	3	1	0	0	1	0	0
RM-2	93	9	10	3	17	2	1	0	1	1	0
Alfa3-1	5	16	3	0	3	0	0	0	0	1	0
Alfa3-2	4	18	4	0	3	1	0	0	0	1	0
Alfa5-1	9	20	4	0	5	1	1	0	0	1	0

Table 6
Alpha diversity indices (LSU-based data, 97% similarity level).

Samples	Shannon	Simpson's index (dominance)	Equitability
RG3-1	1.29	0.65	0.31
RG3-2	1.39	0.54	0.36
Agropyron-1	0.54	0.87	0.14
GM-1	0.54	0.82	0.17
GM-2	0.17	0.96	0.10
Grain-1	3.39	0.19	0.57
Grain-2	3.83	0.12	0.60
RM-1	4.02	0.10	0.68
RM-2	3.47	0.12	0.71
Alfa3-1	0.55	0.87	0.13
Alfa3-2	0.56	0.86	0.13
Alfa5-1	1.76	0.41	0.44

each individual within the community. Principal coordinate plots based on OTU occurrence showed that AMF communities from monocot/dicot alternation treatments (RM and Grain samples) clustered together and separated from samples of monocot treatments, *Agropyron*, RG3 and GM (Fig. 3A). The first two PcoA axes explained 37.23% of variation among the AMF communities. Visualization of PCoA plots based on Bray–Curtis showed that differences in richness and evenness among the AMF communities were evident in the first two axes, which explained 46.44% of the community composition-soil management relationship (Fig. 3B). Soil samples from RM and Grain clustered separately from RG3 samples. We observed that GM samples were more variable one from each other; one replicate clustered with RM and Grain samples and the other with RG3 and *Agropyron* samples. Statistical analysis showed that samples clustered according to the "rotation scheme" variable (ANOSIM UU $P < 0.01$, Adonis UU $P = 0.001$; ANOSIM BC $P < 0.04$, Adonis UU $P = 0.03$). No significance was obtained by analyzing the variable "tillage". It is noteworthy that Alfa5 (*Medicago sativa,* dicot treatment) sample distributed among the two defined groups in PcoA plots (Fig. 3).

4. Discussion

Several factors such as plant community composition and age, soil chemistry, physical and biological properties, and climate are expected to influence the number of mycorrhizal fungal individuals in a given habitat (Johnson et al., 2012). Crop rotation has been used for centuries but during the 1950s and early 1960s, it was felt that synthetic fertilizers and pesticides could forever replace crop rotation without loss of yield. More recently, that opinion has changed and the current consensus is that crop rotation increases yield and profit and is fundamental in a sustainable agriculture production scheme (Bullock, 1992). In this study, the main hypothesis tested was that variability of AMF communities is influenced by crop rotation schemes that involve a change in the class of the plant components. We analysed soil samples encompassing three treatments defined according to the main class of plant component included in the crop rotation: monocot, monocot/dicot alternation and dicot. Crop rotation is a widely accepted agricultural practice whose main advantage is the improvement in soil quality, both in fertility and structure. Crop rotation has also been reported to have a positive effect on controlling both, plant pathogens and weeds. Therefore, different crop rotation regimes could be proposed in order to reach all these positive effects. Among them, a scheme that considers shifting monocot and dicot crops, or even one scheme that includes a dicot/monocot consociated culture may be regarded as one that promotes a simultaneous enhancement of all aspects mentioned before. From the analysis of the soil samples by 454 pyrosequencing, we obtained 59,481 high quality fungal DNA sequences that

Table 7
Statistical analysis of the Shannon AMF diversity index. The ANOVA analysis of the Shannon index among the samples was applied in order to determine if its variation can be explained by differences in the application of tillage.

Shannon index analysis of variance								
R^2		P value						
0.98		0.001						
Variable	Mean	Standard error	Significance[c]					
Monocot under till	0.36	0.20	A					
Monocot under not till[a]	0.54	0.28	A		B			
Monocot under not till[b]	1.34	0.20			B		C	
Dicot under not till[b]	1.76	0.28					C	
Monocot/dicot rotation under till	3.61	0.20						D
Monocot/dicot consociation under no till[b]	3.75	0.20						D

[a] No tillage for at least five consecutive years.
[b] No tillage for three consecutive years.
[c] Variables sharing common letters are not significative ($P > 0.05$).

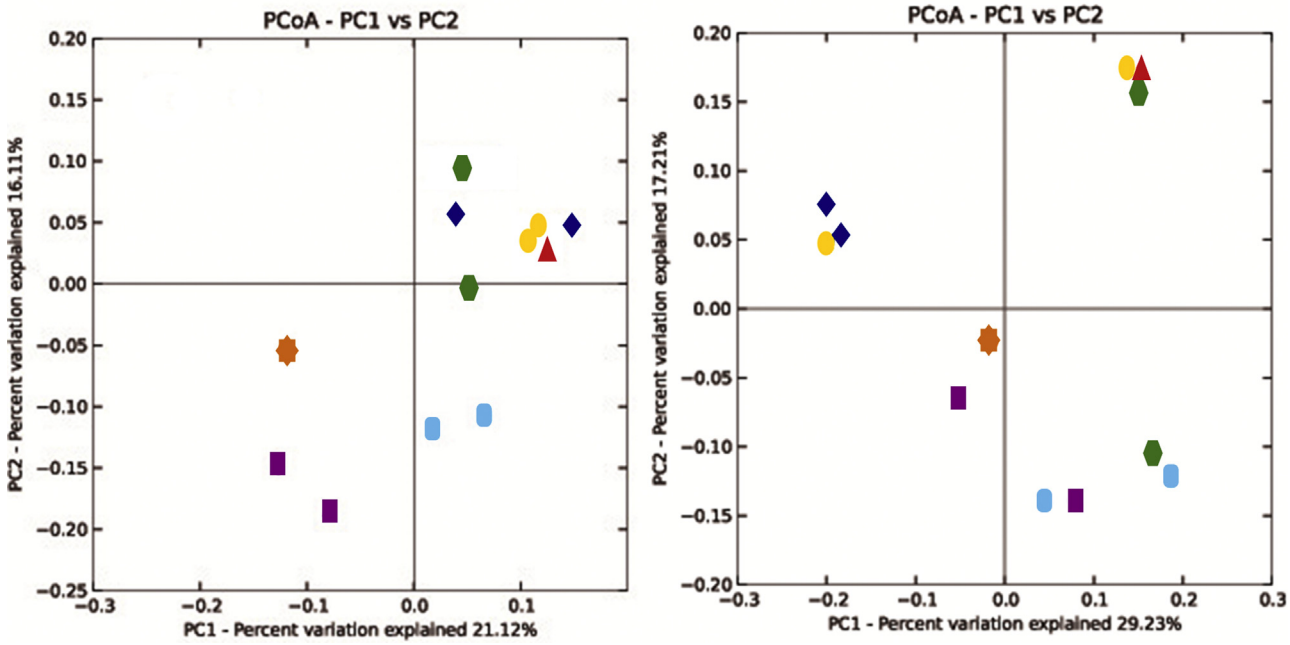

Fig. 3. Principal coordinate analysis (PCoA) plots of AMF communities as affected by crop rotation. According to statistical analysis performed in QIIME, crop rotation was the condition that determines the clustering of the samples. (A) PCoA based on OTU occurrence (unweigthed Unifrac). PCoA axis 1 explains 21.12% and PCoA axis 2 explains 16.11% of variation in AMF community composition. (B) PCoA plots taking into account the relative abundance of each individual within the community (Bray–Curtis). PCoA axis 1 explains 29.23% and PCoA axis 2 explains 17.21% of variation in AMF community composition. Graphic representation of the samples in (A) and (B): Grain, violet rectangle ( ); RM, sky blue rounded rectangle ( ); Alfa3, blue rhombus ( ); RG3, yellow circles ( ); GM, green hexagon ( ), *Agropyron*, red triangle ( ) and Alfa5 orange star ( ) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

were subsequently characterized by the QIIME workflow. The analysis of LSU rRNA allowed us to classify 97.7% of sequences within the phylum Glomeromycota. At this depth of sequencing, we have surveyed almost the full extent of taxonomic diversity within individual soils at the 97% similarity level of taxonomic resolution. Rarefaction analysis revealed that the species accumulation in soils under GM and RG3 (monocot treatments) is slower than in those under Grain or RM rotation schemes (monocot/dicot treatments). By clustering the D2 LSU pyrotags at 97% of similarity, the great majority of the OTUs were related to the genus *Funneliformis* and *Glomus*. A genetic marker such as the nuclear rRNA gene region must facilitate the identification of any mycorrhizal taxa present in a sample allowing discrimination at species-level, with a good separation of closely related species, to serve as a reliable phylogenetic tool. According to previous reports, most data concerning the natural diversity of AMF have been obtained using the SSU region and a number of primers targeting this region have been used in 454 pyrosequencing approaches (Öpik et al., 2006, 2010). Although SSU has been widely used for AMF diversity studies, it has been reported that the D2 region of the large ribosomal subunit of rRNA gene is more variable across different fungal taxa and consequently the taxonomic precision achieved by pyrosequencing this region can be fairly high (Liu et al., 2012). Moreover, previous studies conducted in our laboratory correspond with this finding (Brücher et al., unpublished). Since it has been reported that the LSU primers FLR3/FLR4 could exclude from the amplification some AMF lineages such as *Paraglomus* (Krüger et al., 2009), we performed in parallel pyrosequencing and the analysis of SSU rRNA fragments. This complementary approach allowed us to detect also members of the genus *Gigaspora*, *Scutellospora*, *Acaulospora*, *Entrophospora*, *Diversispora*, *Paraglomus*. Our findings corroborate that the homology between FLR3 and FLR4 and all potential target sequences is not perfect. We found no significant differences in the community's membership among the

soil treatments. *Glomus*, *Funneliformis* and *Rhizophagus* were the dominant genus in Grain and RM samples (monocot/dicot treatments). The soil samples from RG3 and GM (monocot treatments) showed a more variable composition though a higher proportion of genus *Glomus* and *Funneliformis* was also observed. The analysis of the diversity revealed the lowest values for Shannon index in samples from GM (0.17 and 0.54) and RG3 (1.29 and 1.39), both representatives of monocot-involving rotation schemes. The highest Shannon values were found for RM and Grain samples, indicating an increment in the AMF diversity in soils under rotations that switch monocot and dicot crops. Dominance and equitability indices were in accordance with these findings. These results would point out that the agricultural practice applied in monocot/dicot rotation treatment favours a more diverse and even AMF community. From the beta diversity analysis applied to compare AMF communities among the different management types we could observed that AMF communities from RM and Grain soils clustered together and separated from RG3 and GM samples in PCoA plots based in OTU occurrence. Visualization of PCoA plots based on Bray–Curtis showed that differences in richness and evenness among the AMF communities were evident. Soil samples from RM and Grain treatments clustered separately from RG3 samples. These results could be indicating that the applied agricultural management defines the differences observed in the AMF communities, and that soil harbours similar communities according to the crop rotation scheme (monocots succession, dicot/monocot rotation and dicot/monocot consociation). We found that, regardless of whether the soil is subjected to tillage or not, the class of plant utilized in the crop rotation scheme, monocot/dicot, may have an influence in the AMF community in soil. In accordance with our findings, results from Brücher and coworkers (PhD thesis, unpublished) showed that the AMF diversity was high in agricultural soils subjected to continuous monocot/dicot crop rotation (basically, wheat or other monocot winter cover crop/soybean/maize, at least for 10 years). Those soil samples were taken from different geographical locations in Argentina, specifically from fields situated in Monte Buey at Córdoba Province (32°58′14″ S; 62°27′06″W) and Viale (31°52′59.6″S; 59°40′07″W) at Entre Ríos Province. This analysis revealed that the Shannon values ranged from 3 to 3.93. Even though additional work is required, our findings indicated that differences in AMF communities were defined by the applied agricultural management practices. These results suggested that soils harboured similar fungal communities according to the crop rotation scheme (monocots succession, dicot succession, dicot/monocot rotation and dicot/monocot consociation). There are many differences between monocots and dicots especially at the embryo structure, number of flower parts, major leaf veins and stem vascular structure among some others. It has also been reported that differences between both root systems, adventitious roots versus roots developed from radicle, have a strong influence in the mycorrhizal colonization (Weishampel and Bedford, 2006). These potential variations in colonization could have a correlation in the AMF soil community surrounding the roots. Considering that plant diversity influences AMF diversity (Johnson et al., 2004), it has been pointed out that it may be complex to separate direct effects from those inherent factors in agricultural management such as high-nutrient and pesticide input, soil disturbance (An et al., 1993; Hijri et al., 2006; Oehl et al., 2003). However, in the crop systems studied here, crops are strategically rotated so that the management of plant diseases caused by soil borne fungi and control of annual and perennial weeds could be achieved in the more sustainable possible manner. Moreover, the alternation between monocot and dicot crops, as a change in the class of plant component, was conceived to contribute to several aspects of the agricultural system such as soil fertility, soil structure and control of weeds among other aspects.

It is known that different plant species are favored in different degrees by the mycorrhizal symbiosis (Scheublin et al., 2007). It has also been reported that AMF have the ability to change the distribution of nutrients between plants which in turn leads to changes in levels of competitiveness among the plant population (Simard et al., 2003). In this work, *M. sativa* has been included as the main dicot in the rotation schemes. *M. sativa* is a legume and this kind of plants are often highly dependent on the AMF as providers of extra phosphorous (P) that is required for nitrogen (N) fixation. At low levels of P, N fixation can be partially or completely inhibited in the absence of mycorrhizae (Azcón et al., 1991). Moreover, it has been proposed that monocots are able to transport oxygen to their roots more effectively than dicots (Cornwell et al., 2001), and additional oxygen in the rhizosphere promotes phosphorus mineralization and availability. Here, we have found a slight increment in AMF richness between monocot and dicot soil samples, being the higher diversity indices observed in the case of the monocot/dicot consociation or alteration treatments. Thus, we can hypothesize that the ability to procure one or more nutrients might prompt an AMF to reach the vicinity of the plant or, alternatively, some AMF might be attracted by the relatively high nitrogen concentration that can be found in legume nodules. In treatments including both a monocot and a dicot plant, the AMF diversity increment might result from the combination of the nutrient attraction effect of the dicot component plus the phosphorous availability improvement effect of the monocot plant. It is noteworthy that Alfa3 treatment, although is supposed to be a monocot/dicot alternation shows levels of biodiversity similar to those of monocots monoculture and beta diversity values that fall close to those of monocots. A very plausible explanation for this situation is that an alternation of three years of a dicot (*M. sativa*) and three years of a monocot (*Allium cepa*) is closer to a monoculture than to a monocot/dicot alternation. This observation becomes stronger if one considers that samples were taken after three years of a monocot culture (*Allium cepa*). This is not the case of the other two monocot/dicot treatments. In Grain treatment, the alternation was *Helianthus annuus*/*Triticum aestivum* for five years. Finally, after the last sunflower crop, samples were taken after *A. cepa*. RM treatment shows during years 1 and 2 an alternation *H. annuus*/*T. aestivum* and then three years of a consociated crop of *M. sativa* and *Thinopyrum ponticum* and finally *A. cepa*. It is worth mentioning that in this study the dicot treatment was selected based on the class of plant (*H. annuus* and *M. sativa*) independently of being a legume or not. Consequently, it could be said that, independently of the application of tillage, it is the switching between monocots and dicots and not the type of dicot (legume or not legumes) what is responsible for the increment of AMF diversity.

5. Conclusions

Crop rotation is a widely accepted agricultural practice whose main advantage is the improvement in soil quality, both in fertility and structure. Crop rotation has also been reported to have a positive effect on controlling both, plant pathogens and weeds. Therefore, different crop rotation regimes could be proposed in order to reach all these positive effects. Among them, a scheme that considers shifting annually monocot and dicot crops, or even one scheme that includes a dicot/monocot consociated culture may be regarded as one that promotes a simultaneous enhancement of all aspects mentioned before. The switching between monocots and dicots could force natural mechanisms that lead to the rise of AMF biodiversity. Although additional work is required, our findings might be regarded as an initial step toward the

comprehension of the potential role of AMF in agricultural and environmental sustainability in Argentina.

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