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Original article

Response of soil microbial communities to different management practices in surface soils of a soybean agroecosystem in Argentina

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ABSTRACT

Argentina is the world's third most important soybean producer; hence, there is an urgent need to preserve soil health by applying appropriate agricultural practices to maintain sustainable production in the upcoming years. Because productivity of agricultural systems largely depends on soil microbial processes, the influence of different management strategies on soil microbial community structure was analyzed in a long-term field trial started in 1992. The experimental design was a split-plot arrangement of treatments, consisting in two tillage treatments: zero tillage (ZT) and reduced tillage (RT), in combination with two crop rotation treatments: soybean monoculture (SS) and corn-soybean (CS). Phospholipid fatty acid (PLFA) profiles were used to assess total microbial community structure. Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rRNA were generated to describe the influence of crop practices on fungal communities. Total PLFA content was lowest in soil under reduced tillage and soybean monoculture; therefore the use of reduced tillage-soybean monoculture in agroecosystems might produce important reductions in total microbial biomass. The structure of total microbial communities, as estimated by PLFA, was affected by crop rotation. Moreover, the fungal communities, as estimated by DGGE analysis, were influenced by combined effects of crop rotation and tillage system.

1. Introduction

In Argentina, the agricultural area has expanded in the last years, reaching 27 million hectares in 2006/07, with a record production of almost 85 million tons of grain [32]. Maintaining these high productivity levels requires the incorporation of crop management practices that aim at preserving natural soil richness to improve crop yield, but mainly to conserve the sustainability of a system. A number of alternative tillage practices, cover and rotational crop schemes, and the use of composts and mulches have been promoted in many production systems all over the world [1,8,29]. In Argentina, minimum and no-tillage practices have been widely adopted to reduce soil erosion, increase water use efficiency of summer crops and improve crop productivity [5,35].

It is well accepted that changes in crop management influence soil physical, biological and chemical properties. The effects of management techniques on soil (micro)biota are of great importance, as microorganisms have been identified as the drivers of nutrient turnover in soil and are therefore closely related to soil quality and health [9,11,12]. For example, direct seeding of extensive crops has been found to increase microbial biomass [14,15]. Conversely, soil disturbance by tillage can be a major factor affecting microbial communities and may result in a reduction in soil microbiota due to desiccation, mechanical destruction, soil compaction, reduced pore volume, and disruption of access to food resources [17]. Microbial communities associated with the rhizosphere vary with different plant species [6,18] and crop rotation [23,26]. Similarly, different crops included in a rotation scheme have healthier root systems than plants in a monocultural cropping system because fewer deleterious rhizosphere microorganisms are present in a crop rotation sequence [25]. It has been postulated that soils with high microbial diversity have a higher buffering capacity against changing environmental conditions than soils with low microbial diversity [22,33,34]. However, due to the enormous amount of taxa present in soils, this hypothesis has never been proven and seems difficult to confirm. Therefore, general concepts about the relation between microbial community structure and soil quality have still not been precisely defined. Furthermore, most of the effects described so far were measured in short-term experiments; consequently, in many cases experiments measured the effects of changes in agricultural management and not typical effects of the management technique itself.

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In this work we describe the effects of different management techniques on microbial community structure based on a long-term experiment started in 1992. All the treatments analyzed in this study included soybean, either as a monoculture or in crop rotations.

2. Materials and methods

2.1. Field experiment

The study was performed in a long-term field experiment started in 1995 at the experimental site of EEA INTA Manfredi (Agricultural and Livestock Technology National Institute-INTA) in Córdoba, Argentina. The climate in the region is temperate humid, with little or no water deficit in summer. Mean annual precipitation is 750 mm. Rainfall is mostly (81%) concentrated in spring. The temperature regime is mesothermal, with annual mean values of 16.5 °C. Annual potential evapotranspiration is 901 mm. The relief in the area is moderate to strongly undulating, with up to 2% of slope. The dominant soil type is a typic Haplustoll, clease textural (USDA) silt-loam (12.5% sand, 68.3% silt, 19.2% clay).

The experiment was designed as a split-plot arrangement of treatments (four replicates each). Each plot was 35 × 110 m in size. The treatments included rotations with RR soybean (*Glycine max*) and corn (*Zea mays*): soybean monoculture (SS) and corn-soybean (CS). Both treatments were subjected to zero tillage (ZT) and reduced tillage (RT) (disc harrow). In ZT treatment, crops were planted using a planter with a single coulter to cut through crop residues and loosen the soil, the only soil disturbance being due to planting. Reduce tillage treatment was conducted with a machine with straight shanks and twisted sweeps. Tillage depth in reduce tillage treatment was 22–25 cm.

Soybean was seeded on December 25, 2006, and harvested on April 23, 2007. Seeding rate was 25 seeds m^{-1}. Row width was 52 cm. All plots were treated with glyphosate (48% i.a., 3l ha^{-1}) before planting. During the vegetation period glyphosate or atrazine (48% i.a., 3l ha^{-1}) was applied, depending on the weeds present.

2.2. Soil sampling

Sampling was conducted 10 days before harvest for each plot. Samples were taken from 0 to 5 cm soil, because cell density is generally greater in surface soils when compared to subsurface soils [24]. In addition, surface soils will contain phototrophic microorganisms that will not be present at lower soil horizons [24]. Rhizosphere samples were randomly collected near the roots of the plants using soil cores. Six subsamples per plot were taken and homogenized. Samples from different plots under the same treatment were treated as independent replicates. The samples were placed in plastic bags and kept cool until processing at the laboratory (within 1 h). In the laboratory, soil samples were sieved (4-mm mesh) and stored at −20 °C for further analysis.

2.3. Phospholipid fatty acid analysis

Lipid analysis was based on Zelles et al. [38]. Aliquots equivalent to 10 g dry weight were extracted with 125 ml methanol, 63 ml chloroform and 50 ml phosphate buffer (0.05 M, pH 7). After 2 h of horizontal shaking, 63 ml water and 63 ml chloroform were added for phase separation. After 24 h the water phase was removed and discarded. The total lipid extract was separated into neutral lipids, glycolipids and phospholipids on a silica-bonded phase column (SPE-SI 2 g/12 ml; Bond Elut, Analytical Chem International, CA, USA). The phospholipid fraction was further separated into saturated (SATFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids [38] to facilitate identification of fatty acids as well as to obtain a good baseline separation of peaks. PLFAs were analyzed as fatty acid methyl esters (FAMEs) on a gas chromatograph/mass spectrometry system (5973MSD GC/MS Agilent Technologies, Palo Alto, USA). FAMEs were separated on a polar column (BPX-70, SGE GmbH, Griesheim, Germany, 60 m × 0.25 mm × 0.25 μm, coated with 70% of cyanopropyl polysilphenylene-siloxane). The mass spectra of the individual FAME were identified by comparison with established fatty acid libraries (Solvit, CH 6500 – Luzern, Switzerland) using MSD Chemstation (Version D.02.00.237). Prior to measurements, an internal standard (nonadecanoic acid methyl ester) was added to calculate absolute amounts of FAMEs.

Standard nomenclature was used [16], where the number before the colon represents the number of C-atoms and the number after the colon represents the number of double bonds and their location (ω). The prefixes "cy", "i" and "a" indicate cyclopropyl-groups, and iso- and anteiso- branching, respectively. "Br" indicates methyl-branching at an unknown position. Cis ("c") and trans ("t") configuration in double bonds are indicated after the location.

Total values of PLFA were obtained employing an internal standard 19:0. The individual fatty acids are expressed as nmol%, i.e., the sum of all the absolute amounts of fatty acids is 100%. This calculation is made to standardize the amounts of individual PLFAs (nmol g^{-1} soil), so that the differences observed between them are not simply due to a difference in the amount of total PLFAs.

2.4. Analyses of the fungal population structure

To generate fungal fingerprints, RNA extraction and cDNA synthesis from soil were performed as described by Aneja [2], as follows. Nucleic acid extraction was performed using the method of DNA and RNA coextraction described by Griffiths [19] with a few modifications. Prior to nucleic acid extraction, water was treated overnight with 0.1% diethyl pyrocarbonate (DEPC) and all solutions were prepared in DEPC-treated water. Also, the glassware was baked at 180 °C for 4 h. The method principally involved bead beating and solvent extraction of the nucleic acids. To prevent mRNA degradation, all incubations were performed on ice. To obtain pure RNA, DNA was removed from RNA by treatment with DNase (1 U μl^{-1}; RNase free, Promega) according to the manufacturer's instructions. The cDNA synthesis was performed with RNA which was reverse transcribed using the Omniscript RT Kit (Qiagen, Hilden, Germany). A total of 2 μl of total-RNA was added to an 18-μl RT mixture containing 20 pmol of random hexamers (RT mixture prepared following the manufacturer's instructions). The reaction was incubated at 37 °C for 90 min. The enzyme was inactivated by heating at 93 °C for 5 min followed by rapid cooling on ice. The synthesized cDNA was stored at −20 °C.

The amounts of DNA extracted from the soil ranged from 82 ng DNA per gram dry weight of soil to 105 ng DNA per gram dry weight of soil, a concentration that was quantified using a Nano-drop spectrophotometer (Thermo Scientific, USA) at 260 nm.

Primer pair NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and NS2-GC (5′-GGC TGC TGG CAC CAG ACT TGC-3′) at an annealing temperature of 52 °C was used in the reaction [37]. For PCR reaction, 1 μl of synthesized cDNA was added to 48 μl PCR reaction mix, which consists of 5 μl of 10× reaction buffer, 5 μl of 3% BSA, 2.5 μl of dimethysulfoxide, 5 μl of 25 mM $MgCl_2$, 5 μl of 2 mM of deoxyribonucleoside triphosphate mixture (dNTP) and 1 μl of 10 μM of each primer. The reaction involved a hot start at 95 °C for 10 min, followed by addition of 2.5 U *Taq* DNA Polymerase (Invitrogen, Karlsruhe, Germany). The cycling parameters were 94 °C for 1 min, annealing for 1 min and 72 °C for 1 min, for 30 cycles, followed by a final extension at 72 °C for 10 min. RT-PCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany).

DNase-treated nucleic acids, without being reverse transcribed, were used as controls in RT PCR to check for residual DNA in RNA preparations (data not shown).

Denaturing gradient gel electrophoresis (DGGE) was used to resolve the PCR products obtained from the different treatments using polyacrylamide gels (6%, ratio of acrylamide to bisacrylamide 37:1) with a gradient of 35–45%. A 100% denaturant is defined as 7 M urea plus 40% formamide. Each lane of the gels was loaded with appropriate volumes containing about 2 μg purified RT-PCR products, measured by absorbance at 260 nm. The gels were run at 50 V for 17 h at 60 °C using the D-Code system (Bio-Rad, Munich, Germany). After electrophoresis, gels were silver-stained using the protocol described by Heukeshoven [21], and then scanned using HP Scanjet 7400c.

2.5. Statistical analyses

The statistical analyses were performed with INFOSTAT/Professional v. 2007 (National University of Córdoba, Argentina). Individual PLFAs and PLFA clusters were analyzed by two-way MANOVA to determine treatment effects on PLFA composition in the soil. Correlation analysis was performed on PLFAs data, PC1 and PC2 ($P \leq 0.05$), to identify those PLFAs whose gradients were represented by PCs 1 and 2. The banding patterns of DGGE profiles were analyzed using GelCompar II Software (Applied Maths, Kortrijk, Belgium). Each gel contained several marker lanes for references purposes. For analysis of DGGE fingerprints, a binary matrix showing presence (1) and absence (0) of DGGE bands was used. DGGE and PLFA data were then analyzed by principal component analysis. LSD was employed for testing post-hoc analysis.

3. Results

3.1. Microbial community structure based on PLFA analysis

Microbial community structure as estimated by PLFA profiles is shown in Table 1. Overall, the lowest PLFA content was detected in soils treated by reduced tillage and soybean monoculture and the highest one in the reduced tillage and corn-soybean treatment. A similar trend was also found for the zero tillage samples with higher values in the corn-soybean treatment samples than in the soybean monoculture samples. Two-way MANOVA for individual PLFA showed a significant effect of both tillage and crop rotation for several PLFAs. In addition, the evaluation of the interaction of tillage × rotation treatments showed significantly higher representation in the 16 individual PLFAs (Table 1).

The unsaturated, branched, and hydroxylated PLFA clusters, together with 10 individual PLFAs, were significant for the tillage system. However, the effect of tillage was not significant for total PLFA. This indicates a clear influence of the rotation treatment on microbial biomass, with no effect of tillage systems on this parameter. PCA of PLFA profiles revealed a shift in microbial community structure with different tillage and rotation managements (Fig. 1). PC1 explained 37% of variance, whereas PC2 explained 19%, for a cumulative total of 56%. A clear separation was found for zero tillage-soybean monoculture and reduced tillage-soybean monoculture samples, and also for zero tillage-corn soybean and reduced tillage-corn soybean samples, with the exception of one outlier sample. Loadings along PC1 were negatively correlated with saturated (11:0, 13:0, 14:0, and 16:0) and branched (a15:0, i16:0, and i17:0) fatty acids, whereas PC2 was mostly weighted by the fatty acids 18:2, 16:0 2OH, 17:0, 12:0 2OH, cy17:0 and i17:0 (Fig. 1b, Table 2).

Branched PLFAs were influenced by the different tillage practices; those lipids (a15:O, i16:O, i17:O) are indicative of Gram-positive bacteria.

Table 1
Mole percentage of phospholipids fatty acids profiles of different tillage and crop rotation systems.

Individual PLFAs	RT-SS	RT-CS	ZT-SS	ZT-CS	LSD	Till.	Rot.	TillxRot
11:0	0.35	0.29	0.40	0.25	0.07	NS	**	*
10:0 2OH	0.45	0.43	0.55	0.41	0.07	NS	**	*
12:0	20.10	19.81	15.35	23.17	4.82	NS	*	*
13:0	0.01	0.07	0.11	0.05	0.05	NS	NS	NS
12:0 2OH	0.61	0.52	0.65	0.43	0.14	NS	**	NS
12:0 3OH	0.31	0.30	0.38	0.27	0.10	NS	NS	NS
14:0	1.70	1.63	2.27	1.60	0.28	*	**	**
i15:0	1.95	1.82	2.82	1.56	0.83	NS	*	NS
a15:0	1.10	0.99	1.50	1.08	0.24	*	**	*
15:0	0.51	0.80	0.55	0.67	0.37	NS	NS	NS
14:0 2OH	1.06	1.10	1.40	0.53	0.47	NS	*	*
14:0 3OH	0.76	1.05	1.21	1.12	0.18	**	NS	*
i16:0	2.02	1.42	2.72	1.94	0.68	*	**	*
16:1ω9c	2.45	2.49	3.38	2.87	0.59	**	NS	*
16:0	18.79	17.75	21.46	18.93	2.28	*	*	*
i17:0	0.78	0.74	1.23	0.88	0.27	**	*	*
cy17:0	0.79	0.76	1.59	1.18	0.64	*	NS	*
17:0	0.89	0.57	1.08	1.06	0.60	NS	NS	NS
16:0 2OH	0.59	0.77	0.74	0.96	0.10	***	***	*
18:2	4.59	3.65	4.19	3.25	2.24	NS	NS	NS
18:1ω9c	7.27	7.60	7.16	6.80	1.53	NS	NS	NS
18:1ω7c	4.79	8.66	3.46	3.54	3.30	*	NS	*
18:0	20.34	17.58	18.51	17.64	1.60	NS	**	*
cy19:0	5.52	5.84	4.67	6.97	1.18	NS	**	*
20:0	2.27	3.38	2.62	2.86	1.10	NS	NS	NS
PLFA clusters								
Saturated	64.97	61.88	62.35	66.22	2.92	NS	NS	*
Unsaturated	19.11	22.39	18.19	16.46	3.48	*	NS	*
Branched	5.85	4.97	8.28	5.45	1.12	**	***	*
Hydroxylated	3.78	4.17	4.93	3.73	0.49	*	*	**
Cyclopropyl	6.30	6.60	6.26	8.15	1.45	NS	*	*
Total PLFAs (nmol g^{-1} soil)	158.66	197.42	174.00	190.04	20.6	NS	**	NS

Abbreviations: RT = reduced tillage, ZT = zero tillage, SS = soybean monoculture and CS = corn/soybean rotation, LSD = least significant difference, NS = not significant at $P \leq 0.05$, * = significant at $P \leq 0.05$, ** = significant at $P \leq 0.01$ and *** = significant at $P \leq 0.001$.

3.2. Fungal community fingerprints

To characterize fungal communities in the different treatments, 18S rRNA was targeted. The analysis of DGGE profiles showed variation in intensity and number of bands for all soil samples (Fig. 2a). DGGE fingerprints were complex, with the number of bands detected in samples varying between 19 and 28.

The DGGE data for fungi was subjected to PCA. PCA1 and PCA2 accounted for 45% and 27% of the variability, respectively (Fig. 2b). PCA clearly indicated that fungal community structure was influenced by combined effects of cropping sequence and tillage management. The treatments zero tillage-corn soybean and reduced tillage-soybean monoculture were separated from the other treatments, and also a differentiation between zero tillage-soybean monoculture and reduced tillage-corn soybean was possible, with the exception of one outlier sample.

4. Discussion

It is widely accepted that total PLFA concentration is indicative of the total microbial biomass in soil samples [3]. Our results show that the lowest value of total PLFA, was detected in soil under reduced tillage and soybean monoculture. These findings suggest that particularly in the field trial employed, long-term effects of reduced tillage- soybean monoculture can result in an important reduction in total microbial biomass.

Dynamics in microbial community structure was measured by discriminatory PFLA analyses. Overall, the fatty acids found in this

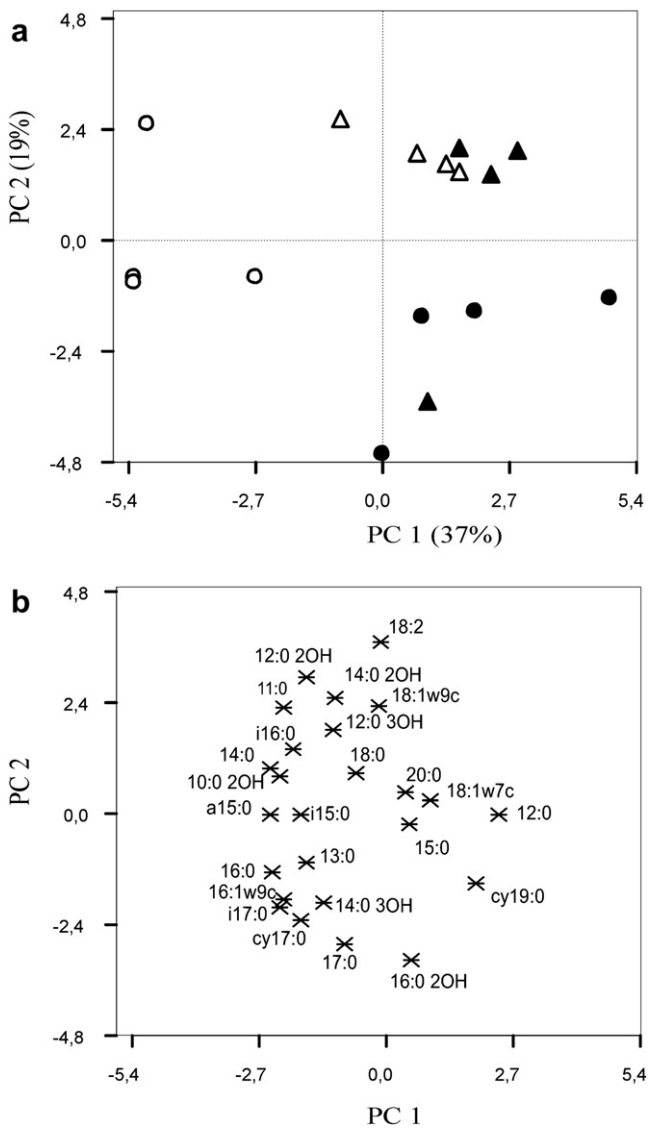

Fig. 1. (a) Principal component analysis plot of microbial communities in soil samples under different tillage and crop rotation systems as determined by bacterial PLFA profiles. The treatments are (△) RT-SS, (▲) RT-CS (○) ZT-SS, (●) ZT-CS, where RT and ZT represent reduced and zero tillage, respectively; SS and CS represent soybean monoculture and corn/soybean rotation, respectively. (b) PCA showing loading values of individual PLFAs and ratios given in Fig. 1(a).

study have been previously reported as being commonly present in soil samples (e.g., [10,30]). Previous studies have reported that soil management strategies may influence PLFA profiles, particularly tillage and crop rotation [13,28]. Different plants in a crop rotation scheme can release specific compounds into the rhizosphere [31,35], which can ultimately lead to plant-associated shifts in the microbial community structure as demonstrated by our PLFA data. Hence, the inclusion of corn in the rotation sequence probably enhanced the variety and amount of soil nutrients, thereby increasing the abundance of microorganisms. In previous studies based on the same trial we reported that soil organic matter content with corn as preceding crop was 20% higher than in soybean monoculture [28]. We also found that the amount of corn residues was double the amount of soybean residues in the field plots evaluated [35], which partly explains the higher soil organic matter content found. According to our findings, corn as preceding crop increased organic matter content through the high C

Table 2
Community PLFAs significantly correlated ($P \leq 0.05$) with Principal Components 1 and 2 of PC plots.

PLFA	Correlated with	
	PC 1	PC 2
11:0	−0.79	NS
10:0 2OH	−0.82	NS
12:0	0.84	NS
13:0	−0.61	NS
12:0 2OH	−0.62	0.65
14:0	−0.89	NS
i15:0	−0.66	NS
a15:0	−0.89	NS
14:0 2OH	NS	0.54
i16:0	−0.72	NS
16:1ω9c	−0.80	NS
16:0	−0.87	NS
i17:0	−0.82	−0.52
cy17:0	−0.65	−0.58
17:0	NS	−0.71
16:0 2OH	NS	−0.79
18:2	NS	0.83
cy19:0	0.68	NS

NS = not significant at $P \leq 0.05$.

contained in corn residues, as a source of nutrients for selected groups of microorganisms [35]. Although some studies found a lower effect of preceding corn on microbial structural diversity [13,20], it should be noted that soil type, climate, or other cultural practices sometimes have greater influence on soil microbial content than the type of residue present in the soil [6].

An influence of the tillage practice was observed also in lipid groups. Branched PLFAs were largely influenced by the different tillage practices; those lipids are indicative of Gram-positive bacteria [25]. These findings are consistent with other studies in which PLFA analysis showed an increase in Gram-positive populations in agricultural soils [4]. Also, the differences in the amount of several other hydroxylated fatty acids (10 2OH, 12:0 3OH, 14:0 3OH, and 16:0 2OH), which are considered indicators for Gram-negative bacteria, separated zero tillage and reduced tillage treatments under soybean monoculture. Unfortunately, in our study PLFA 18:2ω6,9c coeluted with their isomer 18:2ω9,12c. As a consequence, it was not possible to evaluate correlation between 18:2ω6,9 and fungal fingerprints as estimated by RT-PCR DGGE.

Although some of the fatty acids were affected by the tillage practice, total PLFA were not influenced by this practice. Considering that total PLFA content serves as an index of viable microbial biomass, tillage is supposed to cause temporary stress, which limits the ability of soil microbes to assimilate nutrients, and to alter community structure. However, in this work, the negative effect of tillage on total PLFA was not confirmed. The increase in cyclopropyl fatty acids (cy17:0 and cy19:0) is another adaptive mechanism induced by starvation, among other stress factors, with the resultant increase in microbial membrane fluidity. The amount of these fatty acids usually indicates a decline in readily available carbon associated with loss of root proliferation [16]; however, in the present work they were unaffected by the tillage treatment, masking the disruption that tillage is supposed to cause in soil. These results are different from data obtained in the same experimental site the year before [28], in which tillage reduced total PLFA content and conventional tillage produced an increase in branched biomarkers (i15:0 and i16:0), showing a close relationship between the latter practice and those stress indicators. This is confusing in the case of PLFA community structure, which has been shown to be sensitive to site disturbance. In agreement with our findings, other authors [7] reported that depending on the time spanned after tillage, the microbial community of the tilled soil may return to

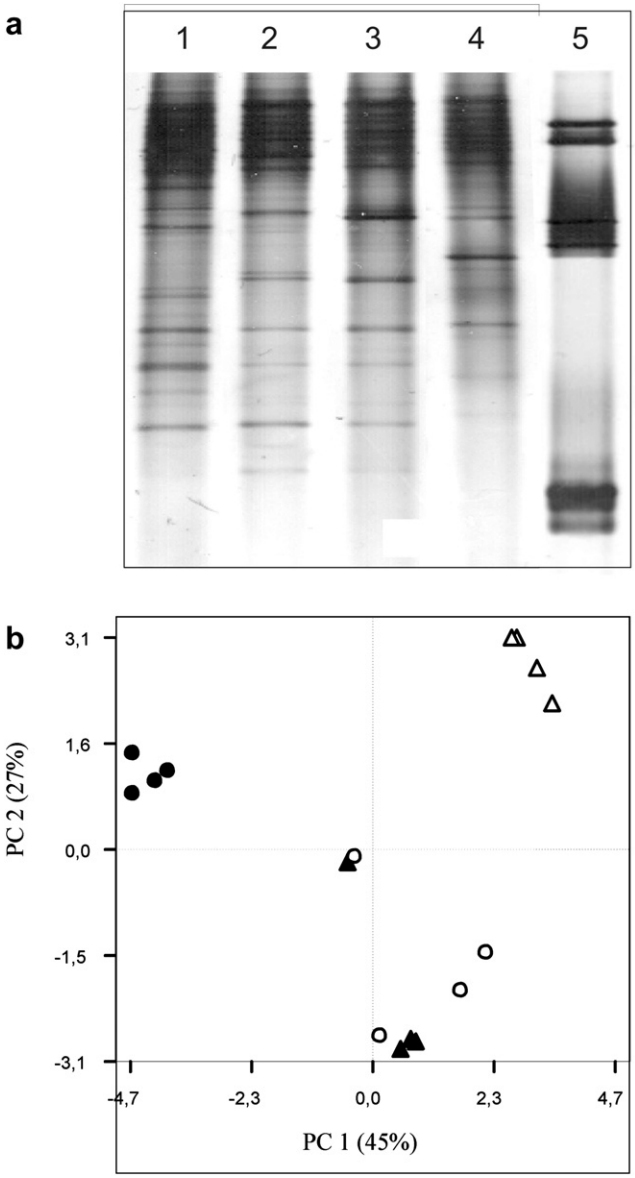

Fig. 2. (a) Representative DGGE profiles of fungal RT-PCR amplified 18S rRNA sequences from soil samples under different tillage and crop rotation systems. Lane 1: ZT-CS, lane 2: ZT-SS, lane 3: RT-CS, lane 4: RT-SS, lane 5: ladder. (b) Principal component analysis plot as determined by fungal PCR-DGGE profiles. The treatments are (△) RT-SS, (▲) RT-CS (○) ZT-SS, (●) ZT-CS, where RT and ZT represent reduced and zero tillage, respectively; SS and CS represent soybean monoculture and corn-soybean rotation, respectively.

a composition similar to that of intact soil. Particularly, the phospholipid fatty acid 19:0 cy can decline after tillage, reaching values of up to half of those obtained in no tilled soil. This stresses the importance of sampling time in drawing conclusions related to the effect of tillage on PLFA, even for a single trial, in different years.

Moreover, it is well known that the composition of microbial communities varies at large spatial scales because of environmental heterogeneity driven by factors such as soil texture, climate, plant community composition, availability of labile carbon and soil pH [27]. This might also explain why tillage did not affect total PLFA, together with the lack of consistence among different years of study. Furthermore, the presence of outlier samples also confirms this assumption, showing that geographic conditions and climate occurring within small areas determine the general characteristics of microbial populations within fields, while fine-scale spatial and temporal factors generate localized microbial heterogeneity [36].

In conclusion, data from this study suggest that crop rotation can increase total microbial communities, as estimated by PLFA, and fungal communities as measured by DGGE. Tillage can also affect fungal communities measured by DGGE, and is supposed to influence total microbial biomass as determined by PLFA; in some cases, however, this effect may be masked by environmental heterogeneity as they can occur on agricultural fields.

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