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Mulching and Cover Crops Effects on the Soil and Rhizosphere-associated Bacterial Communities in Field Experiment

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ABSTRACT

Agricultural sustainability is closely related with the efficient use of natural resources, which are primarily transformed by the action of microorganisms. Soil microorganisms are usually used as early indicators of soil quality since they rapidly respond to changes in soil management. A field experiment was carried out aiming to evaluate the effect of siratro (*Macroptylium atropurpureum*), bahiagrass (*Paspalum notatum*) and mulching on the bacterial communities of bulk soil and rhizoplane of siratro and bahiagrass. DNA was extracted directly from soil samples and from bacterial cells of siratro and bahiagrass rhizoplane and analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). Results showed that bacterial communities were affected by both types of cover crop (siratro, bahiagrass and mulch) and the evaluated compartment (soil and rhizoplane). However, the greatest similarity (76%) was observed between bacterial communities of the samples under mulch and bahiagrass rhizoplane. The cluster analysis based on operational taxonomic units (OTU) showed that rare bands were preferentially related to mulch treatment. The diversity of bacterial community of the mulch treatment was 19% and 36% greater than the bacterial communities of siratro and bahiagrass, respectively, as revealed by Shannon-Weaver index. Besides, bacterial community diversity of the soil was 12% greater than that of the rhizoplane. These results indicate a clear effect of the rhizoplane on the selection of the bacterial community, leading to lower diversity index as compared with mulch samples.

Keywords: Bahiagrass, PCR-DGGE, Similarity, Siratro.

INTRODUCTION

Sustainable production systems have been proposed as a strategy to minimize environmental impacts caused by agriculture. According to Altieri (2002), these production systems are based on diversified crops, ecological soil management and pests bio-control, resulting in improved food production with focus on the biodiversity preservation without risks to consumer health (Welch and Graham, 1999).

Agricultural sustainability is based on conservative practices, as well as efficient use of natural resources. Thus, it is possible to infer that biological processes taking place in the soil-plant system (mediated by microorganisms) constitute the basis for agroecological farming (Faria and Franco, 2002). Besides, microorganisms and/or microbial attributes can be used as indicators of environmental impacts caused by agricultural practices since they rapidly respond to crop rotation and tillage management practices (Ferreira *et al*., 2010).

Microorganisms are found in the soil in amounts of 10^9 cells per gram of soil (Torsvik *et al*., 1994), however, only a small

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fraction of these microorganisms are able to grow on the conventional culture medium. Classical studies of microbial diversity, based on morpho-physiological characteristics, can only be applied to culturable microorganisms, which represent only 1 to 10% of the total soil microorganisms (Cowan, 2000).

Cultivation-independent methodologies applied to the study of microbial community diversity are based on the DNA extraction directly from soil samples, instead of the isolation and cultivation of the microorganisms. Torsvik *et al*. (1994) reported that, for the same soil sample, these methodologies can reveal a genetic diversity 200 times greater than the cultivation-dependent methods.

Microbial diversity has been studied through molecular methods, using molecular techniques such as random amplified polymorphic DNA (RAPD) (Abriouel *et al*., 2008), amplified ribosomal DNA restriction analysis (ARDRA) (Viti and Giovannetti, 2005), terminal restriction fragment length polymorphism (T-RFLP) (Hartmann *et al*., 2005), denaturing gradient gel electrophoresis (DGGE) (Ferreira *et al*., 2009a), temperature gradient gel electrophoresis (TGGE) (Molina-Muñoz *et al*., 2009), and single-strand conformation polymorphism (SSCP) (Schmalenberger *et al*., 2008).

DGGE has been used as a common method to assess the effect of different treatments on the soil microbial community. Examples of these treatments include plant development stage and cultivars (Ferreira *et al*., 2008), use of pesticides (Ferreira *et al*., 2009a), soil pollution with oil (Duarte *et al*., 2001), organic composts (Ferreira *et al*., 2009b) and genetically modified plants (Knupp *et al*., 2009).

From the perspective of sustainable crop production systems, it is essential to amend organic matter and to maintain soil cover by using different plants, as well as mulching process. The objectives of the present study were to evaluate the effect of siratro (*Macroptylium atropurpureum*), bahiagrass (*Paspalum notatum*), and mulching on the structure and diversity of the soil bacterial community and associated with siratro and bahiagrass rhizoplane.

MATERIAL AND METHODS

Study Area

This study was carried out at the Experimental Station in Agroecology of the Embrapa Rice and Beans Research Center, in Santo Antônio de Goiás county-GO, Brazil (16º 28' S and 49º 17' W, altitude 823 m). According to the Köppen classification, the climate is Aw, tropical savanna, megathermic. The rainfall regime is well defined, with a wet season from October to March and a dry season from May to September, and an annual average rainfall of about 1,460 mm (Silva *et al*., 2002).

The soil of the experimental site was a clay loam Oxisol with sand: 410 g kg^{-1}, silt: 270 g kg^{-1}, and clay: 320 g kg^{-1}. The chemical characteristics of the top layer (20 cm) were: 2.01% of organic matter, 5.8 mg kg^{-1} of phosphorus, 145.9 mg kg^{-1} of potassium, 720 mg kg^{-1} of calcium, 144 mg kg^{-1} of magnesium and pH 6.2 in water (1:2.5). The original vegetation was characteristic of the Brazilian Cerrado (Savanna).

Soil and Rhizoplane Sampling

Samples were taken on December 2008 from a common bean (*Phaseolus vulgaris* L.) experimental station that had three soil covering treatments at the sampling time: siratro, bahiagrass and mulch – residues of bahiagrass. The experiment was carried out in a randomized block design with three replicates. Soil samples for bacterial soil community analysis were taken from 0-10 cm depth between interlines of siratro and bahiagrass and in the area of mulching, while for the rhizoplane-associated bacterial community analysis, the samples collected

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contained the root systems of siratro and bahiagrass. All samples were placed inside icebox and kept cooled until analyses. The sampled treatments and abbreviations used on the samples description are shown in Table 1.

The experiment was carried out in the period comprising the summer season, from December 2007 to February 2008, when climate conditions were relatively constant over the entire period (Table 2).

Bacterial Cells Extraction from Siratro and Bahiagrass Rhizoplane

Roots of the plants (siratro and bahiagrass) collected from the experimental site were gently separated from the bulk soil. The roots were carefully washed with autoclaved saline solution (NaCl 0.85%), in order to eliminate the excess of rhizospheric soil, and were placed on autoclaved absorbent paper to remove the excess of water. Then, 10 g of washed roots was placed in a 150 ml Erlenmeyer flask containing 90 ml of sterile saline solution. Samples were shaken at 150 rpm for 30 min, and an aliquot of 5 ml was transferred to a 15 ml falcon tube, followed by centrifugation at 9,000 *g* for 30 min at 4ºC. The supernatant was discarded, and the pellet was rinsed with 1 ml of sterile water and vortexed. The bacterial cells suspension was transferred to a 1.5 ml microtube and centrifuged as described above for 15 min (Schwieger and Tebbe, 1998). The supernatant was discarded and the pellet was stored at -20°C until DNA extraction.

DNA Extraction

DNA extraction of bacterial cells obtained from siratro and bahiagrass rhizoplane and directly from soil samples was performed by using MoBio isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturers' instructions. For the DNA extraction of bacterial cells obtained from siratro and bahiagrass rhizoplanes, the pellets obtained in the previous step were used after rinsing with 0.5 ml of sterile water, while for the DNA extraction directly from the soil samples, 0.5 g of each bulk soil sample was used.

PCR-DGGE Conditions

PCR amplification was carried out on a final volume of 50 µl, containing 1.5 µl of DNA template, PCR buffer (10 mM), $MgCl_2$ (3.5 mM), dNTP (0.2 µM of each), *Taq* DNA polymerase (Invitrogen) (0.7 U) and

Table 1. Description of samples used for DNA extraction and PCR-DGGE assay according to their different soil covering.

Soil covering	Sampled compartment	Replicate	Abbreviation
Siratro	Soil	1	SirSoil1
Siratro	Soil	2	SirSoil2
Siratro	Soil	3	SirSoil3
Siratro	Rhizoplane	1	SirRhiz1
Siratro	Rhizoplane	2	SirRhiz2
Siratro	Rhizoplane	3	SirRhiz3
Bahiagrass	Soil	1	BahSoil1
Bahiagrass	Soil	2	BahSoil2
Bahiagrass	Soil	3	BahSoil3
Bahiagrass	Rhizoplane	1	BahRhiz1
Bahiagrass	Rhizoplane	2	BahRhiz2
Bahiagrass	Rhizoplane	3	BahRhiz3
Mulch	Soil	1	Mulch1
Mulch	Soil	2	Mulch2
Mulch	Soil	3	Mulch3

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Table 2.Values of some climatic parameters during the experimental period.

	Mean temperature (ºC)		Mean humidity (%)	Total precipitation (mm)	Total evapotraspiration (mm)	Solar radiation (h day^{-1})
	Maximum	Minimum				
Dec 2007	29.4	19.5	82	207.3	114.3	5.3
Jan 2008	28.6	19.4	85	347.8	161.6	5.0
Feb 2008	29.1	19.4	87	292.3	127.9	4.5

Source: Meteorological station of the Embrapa Rice and Beans, County of Santo Antônio de Goiás, Goiás, Brazil.

the bacterial primers GC-968f and 1401r (0.2 µM of each) (Muyzer *et al*., 1993), spanning the region roughly between nucleotides 968 and 1401 of the 16S subunit ribosomal DNA (16S rDNA), which includes the variable regions V6–V8, resulting in amplicons of about 500 bp. These primers and PCR program were described by Gelsomino *et al*. (1999).

The best denaturing conditions for the samples were determined according to Ferreira *et al*. (2009a). Depending on the efficiency of PCR amplification, 12 to 20 µl of the mixed amplified products were loaded in a denaturing polyacrylamide gel 6% (N-acrylamide, N`-methylbisacrylamide, 37:1) in 0.5X TAE buffer (Tris-base, 20 mM pH 7.8; sodium acetate 10 mM and Na-EDTA, 0.5 mM). Electrophoresis was carried out in a *Dcode*^{TM} Detection Mutation System (Bio-Rad) under constant voltage (120 V; 60ºC; 16 h). The gels were stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes) in 1x TAE for 30 minutes and visualized under UV light in an IMAGO (B and L) photo-documentation system.

Data Analyses

The presence or absence of bands on the PCR-DGGE profiles of bacterial communities of soil and siratro and bahiagrass rhizoplanes were used on the composition of the binary matrix according to Kozdrój and Elsas (2001). The binary matrix used on the clustering analysis was performed using the Simple Matching (SM) index, that assigns equal weights for the presence or absence of the characteristic (Valentin, 1995) and Unweighted Pair Group Method - UPGMA as grouping method. Clustering analyses were performed on the basis of the distribution of bands in each treatment and considering each band as an Operational Taxonomic Unit (OTU), which makes possible an easier viewing of the distribution of common and rare bands throughout the treatments. Dendrograms were performed by the computational program NTSYSpc version 2.10t (Rohlf, 2002). Binary matrix was also used to compute the Shannon-Weaver diversity index (Shannon and Weaver, 1949).

RESULTS

PCR-DGGE Fingerprinting of Soil and Rhizoplane-associated Bacterial Community

The PCR-DGGE analysis showed clear and distinguishable profiles, representing the bacterial communities under different treatments (Figure 1). Some bands were preferentially associated with a specific soil covering treatment, as indicated by "*r*". In contrast, some bands were common to all treatments, independent of the soil covering treatment, as indicated by "*c*", suggesting that these bands may represent a well established group or groups of bacteria and, hence, were not disturbed by the soil covering treatments.

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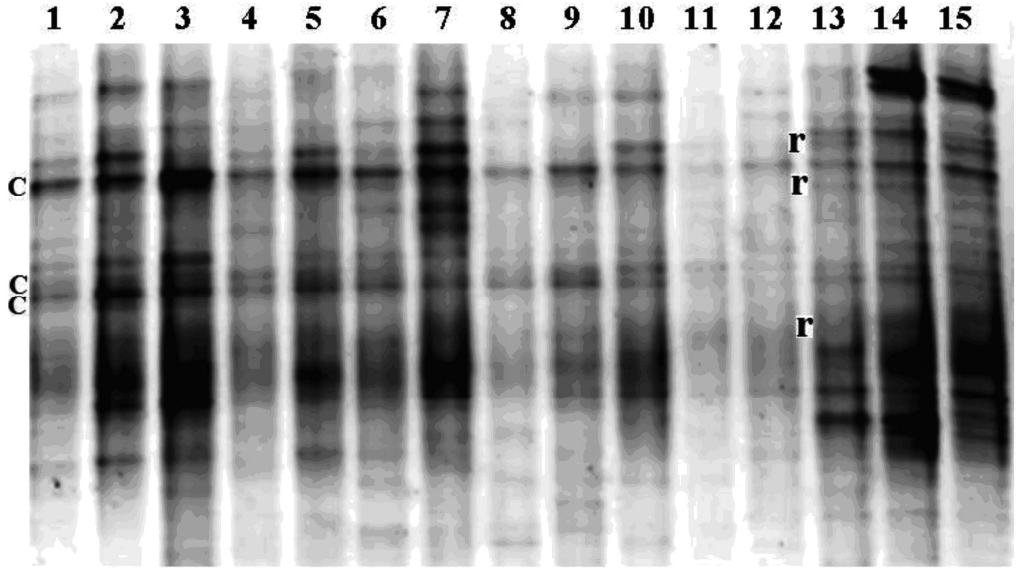

Figure 1. PCR-DGGE fingerprinting of bacterial communities of soil and siratro and bahiagrass rhizosphere. "c"-common bands, "r"-rare bands Lanes 1 to 3-bacterial communities of the soil cultivated with siratro (SirSoil1, SirSoil2 and SirSoil3) lanes 4 to 6- bacterial communities of the rhizosphere of siratro (SirRhiz1, SirRhiz2 and SirRhiz3) lanes 7 to 9- bacterial communities of the soil cultivated with bahiagrass (BahSoil1, BahSoil2 and BahSoil3) lanes 10 to 12 bacterial communities of the rhizosphere of bahiagrass(BahRhiz1, BahRhiz2 and BahRhiz3) lanes 13 to 15- bacterial communities of the soil under mulching treatment (Mulch1, Mulch2 and Mulch3).

Dendrogram Analysis of Soil and Rhizoplane-associated Bacterial Community

Based on the PCR-DGGE profiles, dendrogram representing the bacterial communities of different soil covering and compartment was drawn (Figure 2). All the treatments showed at least 70% of similarity. However, at 80% similarity (dashed line) treatments and replicates grouped in five distinct clusters. Bacterial communities of the soil samples under mulch covering (Mulch1, Mulch2 and Mulch3) had formed a cluster with 94% of similarity and showed about 74% of similarity with most treatments under bahiagrass (Cluster C2). Clusters C3, C4, and C5 separate bacterial communities from soil under bahiagrass and siratro and rhizoplane of siratro, respectively.

Similarity Analysis Based on Operational Taxonomic Unit

After the identification of all OTU's and the samples in which they appeared, a similarity dendrogram was performed by SM/UPGMA. As shown in Figure 3, it is possible to distinguish different OTUs clustering at 100% similarity.

Shannon-Weaver Diversity Index

Binary matrix used for the dedrogram analysis was also used in the calculations of Shannon-Weaver diversity index shown in Table 3. The soil under mulch treatment showed the highest index value and bahiagrass rhizoplane the lowest one. Also, the rhizoplane of both bahiagrass and siratro showed lower diversity index as compared to their respective soils. Under mulch

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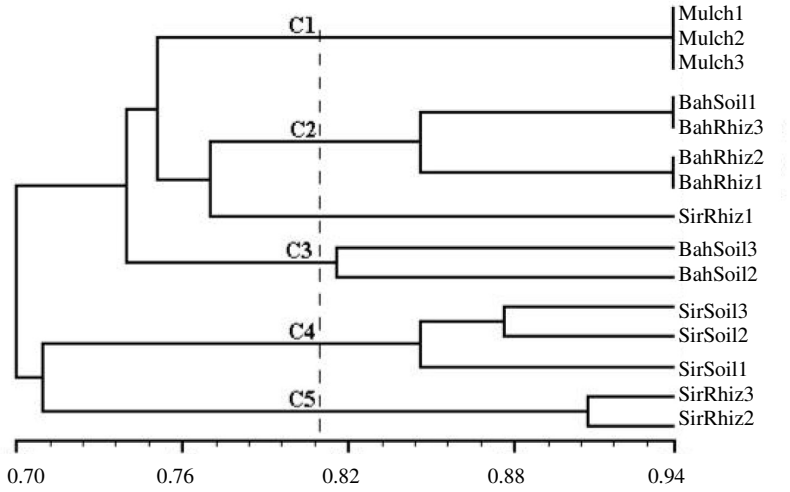

Figure2. Similarity dendrogram representing the bacterial communities of soil and Siratro and bahiagrass rhizosphere costructed with data from the matrix generated by the Jaccard similarity coefficent, using UPGMA grouping method. bacterial communities of the soil under siratro (SirSoil1, SirSoil2 and SirSoil3), bacterial communities of the rhizosphere of siratro (SirRhiz1, SirRhiz2 and SirRhiz3), bacterial communities of the soil under bahiagrass (BahSoil1, BahSoil2 and BahSoil3), bacterial communities of the rhizosphere of bahiagrass(BahRhiz1, BahRhiz2 and BahRhiz3), bacterial communities of the soil under mulching treatment (Mulch1, Mulch2 and Mulch3).

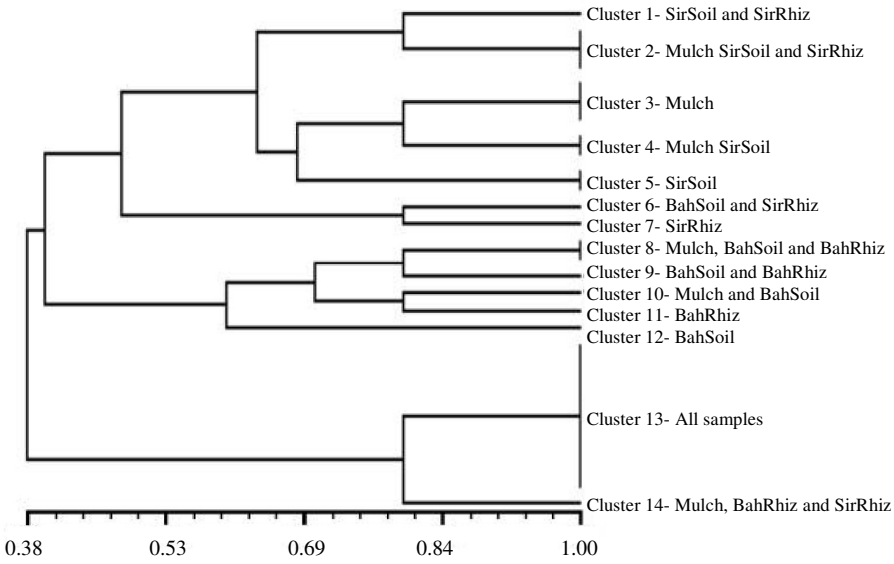

Figure3. Similarity dendrogram constructed with data from the matrix generated by the Jaccard similarity coefficent, using UPGMA grouping method, representing the clustering of the Operational Taxonomic Unit and treatments were they appeared. bacterial communities of the soil under siratro (SirSoil1, SirSoil2 and SirSoil3), bacterial communities of the rhizosphere of siratro (SirRhiz1, SirRhiz2 and SirRhiz3), bacterial communities of the soil under bahiagrass (BahSoil1, BahSoil2 and BahSoil3), bacterial communities of the rhizosphere of bahiagrass(BahRhiz1, BahRhiz2 and BahRhiz3), bacterial communities of the soil under mulching treatment (Mulch1, Mulch2 and Mulch3).

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Table 3. Shannon-Weaver diversity index of rhizoplane and soil bacterial communities calculated on the basis of binary matrix.

Bahiagrass		Siratro		Mulch
Rhizosphere	Soil	Rhizoplane	Soil	
1.63	1.87	1.92	2.10	2.39

treatment, the diversity of bacterial community was 19% and 36% greater than under siratro and bahiagrass, respectively. Besides, bacterial community of the soil was 12% greater than the bacterial community of the rhizoplane.

DISCUSSION

PCR-DGGE Fingerprinting of Soil and Rhizoplane-associated Bacterial Community

Fingerprinting analysis showed the presence of common bands on all treatments, which means that different bacterial groups are capable of growing under different conditions of soil and/or plant management (Figure 1). Studying bulk soil, rhizospheric soil, and rhizoplane-endorhizosphere of *Trifolium repens* and *Lolium perenne*, Marilley and Aragno (1999) reported that bulk soil was mostly colonized by gram-positive bacterial species, while in the rhizoplane-endorhizosphere and rhizospheric, *Pseudomonas* bacterial group was prevalent.

Ferreira *et al*. (2008, 2009a; b) reported that some bacterial communities were not affected by different crop cultivars, pesticide treatments, or organic composts, which characterizes them as generalist group since they are able to grow under different environmental conditions. However, the same authors pointed out the effect of these treatments on some specific bacterial communities as a result of their selection driven mainly by particular conditions found in the soil zone under influence of the plant roots.

Our findings and results of the cited literature show a strong evidence of the influence exerted by rhizoplane on bacterial community, leading to the selection of bacterial groups most adapted to those conditions.

Dendrogram Analysis of Soil and Rhizoplane-associated Bacterial Community

Clustering analysis revealed at least 70% of similarity among bacterial communities (Figure 2). However, compared to the other treatments, mulching process possibly had provided particular conditions for the growth of the soil bacterial community and with this agricultural practice many organic compounds might have been released in the soil and used by the soil bacterial population as nutrient source during organic matter transformation. (Rethemeye *et al*., 2005). Thus, the continuous decomposition of the mulch releases organic compounds into the soil at different rates and these compounds play an important role in the selection of the bacterial community associated with this treatment.

The bacterial communities associated with bahiagrass rhizoplane and present in the soil under bahiagrass were grouped in two different clusters (C2 and C3, respectively) with 73% of similarity, while the bacterial communities present in the soil under siratro and associated with the siratro rhizoplane formed two different clusters (C4 and C5, respectively) with 71% of similarity. In these cases, the remarkable effect of the rhizoplane on the selection of the bacterial communities is observed under either treatments of siratro and bahiagrass, since plant rhizoplane and soil are very distinct habitats and, thus, they trigger different mechanisms for the soil microorganisms selection. These findings

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corroborate with the published reports in the literature in which different plants and/or cultivars of the same plant specie can provide different compounds through root exudation (Grayer *et al*., 2004; Kato-Noguchi and Ino, 2005) that play important role in the selection of bacterial communities (Ferreira *et al*., 2008).

Similarity Analysis Based on Operational Taxonomic Unit

Among the several features attributed to molecular tools for the study of microorganisms, there is the possibility of using banding profile for the calculation of diversity indexes (Coutinho *et al*. 1999; Kennedy, 1999). For these calculations, each band was considered an Operational Taxonomic Unit - OTU (Coutinho *et al*., 1999).

Similarity analysis of OTU showed the formation of 14 clusters, grouping different soil covering and compartment treatments (Figure 3). However, what stands out in this dendrogram is the cluster 13 that gathered about 1/3 of the OTUs, which occurred in all samples and, also, OTUs clusters exclusive of some samples. In this case, the cluster 3 under mulch treatment showed three OTUs not found in the other treatments. Therefore, this is the evidence that distinct bacterial groups were present under this treatment as compared to the other treatments. Their presence could be explained by the higher input of organic matter into the soil, thereby favoring the development of ecological niches for those bacteria (Rogers and Tate III, 2001).

Shannon-Weaver Index Diversity Index

Differences in diversity indexes were observed for the different treatments; however, the diversity indexes of the rhizoplane of the soil covering treatments were lower than their respective soils (Table 3). This may probably happen as a result of the differentiated composition of the exudates released by the plants roots (Grayer *et al*., 2004; Kato-Noguchi and Ino, 2005), leading to the selection of few groups of microorganisms that are stimulated by these organic substances.

As discussed previously, the soil under mulch treatment showed the highest content of organic matter, with no significant competitive constraint among the microorganisms. This may happen due to continuous addition of organic material that leads to the presence of organic compounds at various stages of decomposition, offering development opportunities to a larger number of microorganisms (Kennedy, 1999; Rogers and Tate III, 2001). In the case of siratro, whose value of the diversity index was higher than in bahiagrass, this phenomenon can also be associated with the large amount of organic matter from leaf fall of this *Leguminosae*, which does not occur so extensively in bahiagrass.

CONCLUSIONS

Bacterial communities are affected by both the type of cover crop (siratro, bahiagrass or mulch) and by the evaluated compartment (soil and rhizoplane). However, the greatest similarity (76%) was observed between samples under mulch and bahiagrass. The cluster analysis based on operational taxonomic units showed that rare bands were preferentially related to the mulch treatment. The diversity of bacterial community of the mulch treatment was 19% and 36% greater than that under siratro and bahiagrass, respectively, as revealed by Shannon-Weaver index. In addition, diversity of the bacterial community of the soil was 12% greater than that of the rhizoplane.

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تاثیر مالچ و گیاهان پوششی روی ریز جانداران خاک و ریشه گاه در مزرعه

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چکیده

پایداری کشاورزی وابستگی زیادی به کار برد کارآمد و درست از منابع طبیعی دارد. این منابع تحت تاثیر ریز جانداران دگرگون می شوند. به همین لحاظ، ریز جانداران خاک غالبا به عنوان نشانگر ها و نمایه های اولیه کیفیت خاک قلمداد می شوند زیرا به سرعت به تغیرات مدیریت خاک واکنش میکنند. به منظور ارزیابی اثر یک گیاه پوششی لگومینه مناطق استوایی به نام سیراترو (*Macroptylium atropurpureum*) و گیاه پوششی باهیگراس (*Paspalum notatum*) روی جامعه ریز جانداران خاک مزرعه و ریز جانداران سطح ریشه ها و مقایسه اثر گیاهان پوششی با اثرمالچ (خاکپوش) روی این

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جانداران، آزمونی در مزرعه اجرا شد. DNA از نمونه های خاک و یاخته های باکتری های سطح ریشه گیاهان پوششی به طور مستقیم استخراج شد و با روش الکترو فورزDGGE واسرشت شد. نتایج نشان داد که نوع گیاه پوششی و مالچ و نیز جایگاه جوامع باکتری (خاک یا سطح ریشه) روی این جوامع اثر داشته است. بیشترین مشابهت (۷۶٪) بین جوامع باکتریایی موجود در نمونه های زیر مالچ و جوامعی که در سطح ریشه گیاه پوششی باهیگراس بودند به دست آمد. تجزیه تحلیل خوشه ای بر مبنای واحدهای تکسونومیکی عملیاتی(operational taxonomic units) حاکی از آن بود که باند های کمیاب (rare bands) به گونه ای ترجیحی به تیمار مالچ مربوط می شدند. تنوع جوامع باکتریایی بر پایه شاخص شانون- ویور (Shannon-Weaver index) در تیمارمالچ به ترتیب ۱۹٪ و ۳۶٪ بیشتر از جوامع مربوط به تیمارگیاه سیراترو و باهیگراس بود. افزون بر این، تنوع جوامع در خاک ۱۲٪ بیشتر از تنوع جوامع در سطح ریشه بود. این نتایج چنین اشاره میکنند که منطقه سطح ریشه تاثیر روشنی بر انتخاب جامعه باکتریایی دارد و در مقایسه با نمونه های تیمار مالچ منجر به شاخص تنوع کوچکتری می شود.

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