

Quantification of soil microorganisms under several cover crops managed with no-tillage system for fifteen years in the Brazilian Cerrado

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Abstract

The natural heterogeneity of soil sustains a dynamic balance between environmental conditions and the residents of the microbiota. In this context, the objective of our work was to identify and quantify distinct groups of microorganisms in winter (2014) and summer (2015), in an Oxisol cultivated for fifteen years with no-tillage system in the Cerrado biome. The experimental design was randomized blocks in a factorial scheme (3x3) with three cover crops (brachiaria, millet and crotalaria) evaluated at three depths (0-0.05, 0.05-0.1 and 0.1-0.2 m), with four replications in both seasons (winter and summer). Endospore-forming bacteria, actinomycetes, yeasts, phosphate solubilizing microorganisms and cellulolytic microorganisms were evaluated. Among the studied cover crops, millet presented superior populations of endospore-forming bacteria in winter, and actinomycetes in summer. The populations of other microorganisms were not different among cover crops in the same season. The population of phosphate solubilizing microorganisms did not differ between seasons, indicating that these microorganisms are less vulnerable to seasonal variations in no-tillage system. In winter, smaller populations of endospore-forming bacteria, yeasts, and actinomycetes were observed with increasing depth in the soil. In summer only the population of endospore-forming bacteria decreased with soil depth. Soil depth did not affect the population of cellulolytic microorganisms under on-tillage system. Our findings suggest that millet is the best fallow cover crop for no-tillage system.

Keywords: actinomycetes, cellulolytic microorganisms, endospore-forming bacteria, phosphate solubilizing microorganisms, yeast.

Abbreviations: CFU _ Colony Forming Units; ANC _ Agar Nutrient Culture; YMA _ Yeast Malt Agar; SCA _ Starch-Casein Agar; ISG _ Inorganic Salts-Glucose; CAA _ Cellulose-Asparagine-Agar; ANAVA _ Analysis of Variance; EB _ endospore-forming bacteria; Y _ Yeast; ACT _ Actinomycetes; PS _ Phosphate-solubilizing microorganism; CEL _ cellulolytic microorganism.

Introduction

Soil is a heterogeneous tridimensional space for growth and development of microorganisms, and a complex environment with a great variety of microbial communities which coexist balanced by a complex equilibrium among species and the environment as well (Paul, 2014). This microbiological variety present in soil is subject to changes in its quantity and diversity throughout the year. Therefore, the identification and quantification of specific groups of microorganisms serve as important biological parameters. They are environmental indicators of ecological stress and the general sanity of the local flora and fauna (Coleman et al., 2004).

The activity of soil microbes is mainly responsible for various processes that recover energy and nutrients in the environment. They are also connected to the maintenance of life in the Earth's Crust (Nair and Ngouajio, 2012). Among these processes are: (i) decomposition of organic matter, (ii) cycling of water and minerals, (iii) fixation of atmospheric nitrogen, and (iv) production of active compounds, which can influence the development of other organisms, besides influencing soil aggregation and the availability of essential mineral elements (Wall et al., 2012). Many of the soil microorganisms are protagonists of several processes that

recycle energy, water, and nutrients in the environment (Brevik et al., 2015).

Among the main factors that alter the dynamic equilibrium established by the microorganisms in an arable soil are: predominant flora and its root system, crop rotation, soil preparation before planting, humidity, temperature, aeration, texture, pH of soil solution, nutrient availability and organic residues (Mathew et al., 2012; Wang et al., 2012; Jacobsen and Hjelmsø, 2014). Consequently, the management of the soil and cover vegetation directly or indirectly alters the quantity, quality and activity of microorganisms, thereby directly affecting agricultural productivity (Treonis et al., 2010; Njira and Nabwami, 2013).

Depending on the activity of a certain microorganisms in soil biological processes, they can be classified into functional groups with different contributions to the bioenergetic cycles in the soil. In this way, the isolation and estimations of soil microorganisms help: (i) study various metabolic pathways, (ii) evaluate the interactions among microorganism, and (iii) indicate the factors that affect the microbiological balance, energy, and nutrient cycling in soil (Inderjit, 2005; Paul, 2014). The different cover crops for

fallow and biomass production in the no-tillage system can promote the development of different microbiological communities. Therefore, the aim of this study was to identify and quantify various groups of microorganisms and compare them during two seasons, in an area under no-tillage system for fifteen years in the Brazilian Cerrado biome.

Results and discussion

Endospore-forming bacteria

Estimates of the number of colony forming units (CFU) of endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilizing microorganisms and cellulolytic microorganisms per gram of soil under brachiaria, crotalaria or millet cover crops, for both seasons, are shown in Table 1.

There are no significant differences among the studied cover crops for the microorganism populations in summer. However, in winter the area covered with millet had a higher endospore-forming bacteria population. A larger quantity of endospore-forming bacteria is expected to occur in more stressful situations, such as in water-stress conditions of the Cerrado biome which trigger the formation of endospores, the dormant structures of bacteria (Giri et al., 2005).

The spore-forming bacteria are taxonomic and physiologically diverse microorganisms, including different genus (*Agrobacterium*, *Bacillus*, *Clostridium*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Xanthomonas spp*), which have the ability to form endospores. The heterotrophic habit of these prokaryotic microorganisms suggests that they play an important role in the carbon cycle as: (i) fixers and denitrifiers of nitrogen, (ii) manganese reducers, and (iii) sulfur and organic matter oxidizers. Spore-forming bacteria are also important in the flow of other nutrients in the soil environment. Many of these bacteria are also effective in degrading cellulose, hemicellulose and pectins. They also present chitinolytic activity which enables the degradation of fungal cell walls and insect exoskeletons (Mandic-Mulec and Prosser, 2011).

Other endospore-forming bacteria are capable of associating with plant roots and fixing atmospheric nitrogen (diazotrophic bacteria), which is then partially directed to the plant (Ormeño-Orrillo et al., 2013). Mandic-Mulec and Prosser (2011) have also observed that root exudates of millet varieties were able to stimulate the proliferation of *Azospirillum spp.* rhizobacteria, which are endophytic bacteria able to assimilate atmospheric nitrogen. This diversity of important functions associated with spore-forming bacteria demonstrates how this group of microorganisms is vital to recycle biomass and energy in the soil ecosystem.

Between the evaluated seasons (winter and summer), the population of spore-forming bacteria did not change in the areas covered with brachiaria or crotalaria. However, winter was more favorable to the development of spore-forming bacteria under millet cover crop. The millet root system is deep and abundant (Rosolem et al., 2001) with high nutrient cycling potential. It offers considerable amounts of organic matter added to soil layers after the plants complete their cycle in winter. All this plant material becomes a substrate for endospore-forming bacteria.

Actinomycetes bacteria

The actinomycetes is a group composed exclusively by aerobic and Gram-positive bacteria, belonging to the

Actinobacteria phylum, which is one of the largest taxonomic units within the Bacteria Domain (Ventura et al., 2007). No differences were observed among the cover crops in winter for the population of these bacteria. Regarding the seasons, summer was more favorable to the development of actinomycetes under all cover crops when compared to winter. Among the cover crops evaluated in summer, millet presented higher population of actinomycetes. The abundant millet root system allows better oxygenation of the soil layers, especially in a rainy summer, making this condition more suitable to the development of actinomycetes in areas where millet is cultivated. Among the actinomycetes bacteria, the *Streptomyces* genus is the most abundant. These microorganisms are capable of forming filamentous branches, similar to fungal hyphae, and reproduce asexually. These bacteria are ubiquitous in soils recognized for producing several antibiotics and degrading resilient compounds in the environment such as cellulose and chitin (Sharman, 2014). A regular characteristic of this group is the production of *phytase* type enzymes which hydrolyze phytate, an organic form of phosphorus not available to plants without the presence of hydrolyzing enzymes as those produced by actinomycete bacteria (Ghorbani-Nasrabadi et al., 2012).

Yeasts

In both seasons no differences in yeast population were observed among the evaluated cover crops. However, winter was more favorable to the development of yeasts, presenting a population about 144% bigger than in summer. Yeasts are associated with cycling of various mineral nutrients and energy in the soil, and also contribute to preserve soil structure (Botha, 2011).

Phosphate solubilizing microorganism

The phosphate solubilizing microorganisms did not differ between the evaluated seasons. The phosphate solubilizing microorganism community is from different origins (e.g. fungi, yeast, bacteria, actinomycetes) what allows these microorganisms to be less vulnerable to cover crop variations.

Cellulolytic microorganisms

While winter conditions damaged the population of cellulolytic microorganisms under brachiaria and crotalaria cover crops, there was no significant change under millet.

Soil depth and microorganisms

Estimates of the number of CFU of endospore-forming bacteria, yeast, actinomyces bacteria, phosphate solubilizing, and cellulolytic microorganisms per gram of soil at different depths are presented in Table 2. In winter smaller populations of endospore-forming bacteria, yeast, and actinomycetes bacteria were observed with increasing depth in the soil. In summer this difference was observed only for endospore-forming bacteria in the superficial soil layer (0-0.05 m).

The population of phosphate solubilizing microorganisms was reduced in the top 0.05 m layer in winter. This difference was not observed in the summer. The depth, however, was not a factor that affected the population of cellulolytic microorganisms. Between the studied seasons, there were significant reductions in the populations of endospore-forming bacteria and yeast in summer in the top soil layers.

Table 1. Number of unit forming colonies of endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilising and cellulolytic microorganisms in a sandy clay loam Oxisol under various cover crops, in winter and summer.

Season	Coverage	EB	Y	ACT	PS	CEL
	×10 ³ CFU g ⁻¹				
2014						
Winter	Brachiaria	1075.0 bA ¹	140.8 aA	666.7 aB	50.0 aA	2.3 aB
	Crotalaria	916.7 bA	132.5 aA	716.7 aB	75.0 aA	3.4 aB
	Millet	1500.0 aA	145.0 aA	800.0 aB	45.8 aA	3.5 aA
	CV%	41.73	48.48	35.67	65.38	76.95
2015						
Summer	Brachiaria	808.3 aA	54.2 aB	1066.7 bA	71.7 aA	4.0 aA
	Crotalaria	958.3 aA	58.3 aB	1058.3 bA	84.2 aA	5.0 aA
	Millet	816.7 aB	59.2 aB	1208.3 aA	69.2 aA	4.5 aA
	CV%	22.09	15.93	8.64	28.12	26.80

¹ = Averages followed by different lowercase letters in the column differ covers in each season, and capital letters in the column differ covers between seasons by Tukey's test at 5% significance level. EB: Endospore-forming bacteria; Y: Yeast; ACT: Actinomycetes; PS: Phosphate-solubilizing microorganisms; CEL: cellulolytic microorganisms.

Table 2. Number of unit forming colonies of endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilizing microorganisms and cellulolytic microorganisms at three depths in a sandy clay loam Oxisol under various cover crops, in winter and summer.

Season	Depth	EB	Y	ACT	PS	CEL
	m×10 ³ UFC g ⁻¹				
2014						
Winter	0.00-0.05	1700.0 aA ¹	210.0 aA	950.0 aB	34.2 bA	3.6 aA
	0.05-0.10	975.0 bA	145.8 bA	808.3 aB	72.5 aA	2.3 aB
	0.10-0.20	816.7 bA	62.5 cA	425.0 bB	64.2 abA	3.3 aA
	CV%	45.60	54.43	37.32	68.81	107.32
2015						
Summer	0.00-0.05	1308.3 aB	80.8 aB	1150.0 aA	70.8 aA	5.0 aA
	0.05-0.10	775.0 bA	48.3 aB	1108.3 aA	91.7 aA	4.4 aA
	0.10-0.20	500.0 bA	42.5 aA	1075.0 aA	62.5 aA	4.1 aA
	CV%	21.10	17.59	8.28	24.97	24.23

¹ = Averages followed by different lowercase letters in the column differ depths in each season, and capital letters in the column differ depths between seasons by Tukey's test at 5% significance level. EB: Endospore-forming bacteria; Y: Yeast; ACT: Actinomycetes; PS: Phosphate-solubilizing microorganisms; CEL: cellulolytic microorganisms.

However, summer was a favorable season for the development of actinomycetes at all depths. The phosphate solubilizing and cellulolytic microorganisms were mostly stable among depths for both seasons.

The top layers of the soil profile are usually more profuse in microbiological activity and regularly present high content of organic material and big community of saprophytic microorganisms (bacteria and fungi). However, despite the fact that this soil layer is more affected by the dry weather in winter in Cerrado, only the phosphate solubilizing microorganisms presented reduced population comparing to deeper layers. This result indicates that these microorganisms, which release phosphorus from soil minerals, are more sensitive to season than to cover crop species. The CFU were found regularly in the upper soil layers. This fact is mainly due to the higher amount of organic materials (e.g. cellulolytic compounds) and residues of mineral fertilizers (e.g. phosphate fertilizers) from the no-tillage system, where no soil disturbances permits the formation of layers with different conditions in soil profile (Mathew et al., 2012). With regard to the occurrence of the five groups of microorganisms for the studied depths, our results do not differ from the information found in the literature (Buscot and Varna, 2005; Ngosong et al., 2010; Njira and Nabwami, 2013; Gougoulis et al., 2014; Paul, 2014; Bhattarai, 2015). In a similar study in the same experimental area, comparing areas under no-tillage and conventional tillage Angelini et al. (2012) observed that in conventional tillage the occurrence of species of arbuscular mycorrhizal fungi was lower when compared to areas covered with millet or brachiaria in no-tillage system after corn and soybeans. That highlights how the diversity of crops

and the lack of soil disturbance promote diversity and proliferation of microorganisms in the soil.

Distribution of microorganisms in soil layers

The uniform distribution of microorganisms in the upper layers of soil is commonly observed in cropping systems with soil disturbance (Treonis et al., 2010). However, this uniformity was also observed under the no-till system, suggesting that the fifteen years of maintaining the crop residue on the soil favored the uniform distribution of the evaluated microorganisms, especially in summer. It was expected that the population of these microorganisms would follow a significant gradient of occurrence in the initial 0.2 meters of a no-tilled soil, following the gradient of organic matter and nutrients, which are formed when the soil remains for many years without aggressive plowing. Estimates of the presence and microbial activity provide useful data about the biological properties of the soils, and can be used to evaluate the effects of applied management and of different types of crops conducted throughout the year (Jordan et al., 1995). The understanding of the benefits provided by certain soil microorganisms to crops, as well as the understanding of their biology and dynamics throughout the seasons is important to define better soil and cover crop managements. The findings of the present study suggest that the dynamics of soil microbial communities during the dry winter or rainy summer is not a function solely determined by the cover crop. The results found here are also a reflection of other prevailing environmental conditions such as the physical and chemical characteristics of the soil (Bossio et al., 2005), and the amount of available organic matter (Nair and Ngouajio, 2012). Among the brachiaria, crotalaria or millet cover crops,

we suggest the use of millet during the fallow period since the soil population of endospore-forming bacteria and the actinomycetes under this cover crop were superior in winter and summer, respectively. These microorganisms are known as cyclers of nutrients in soil system and can help improve soil stability and productivity for following crops. This study, conducted in winter and following summer, allowed us better understand the dynamics of large soil microbiological groups on an area in the Cerrado biome which was under no-till system for fifteen years.

Materials and Methods

Site description

The studies were conducted at the Instituto Federal do Triângulo Mineiro (IFTM) in Uberaba, Minas Gerais state, located at 19°39'19''S and 47°57'27''W, at 795 m of altitude, in an area with no-tillage system from 2000 to 2015. The experimental area was about 1000 m².

Soil type

The soil was classified as Oxisol, with medium texture (EMBRAPA, 2013), in a soft wavy relief. The soil presented the following characteristics in the layer of 0-0.2 m: 210 g kg⁻¹ of clay; 710 g kg⁻¹ of sand; 80 g kg⁻¹ of silt; pH (CaCl₂) 5.5; 76 mg dm⁻³ of P (Resin); 0.2 cmol_c dm⁻³ of K; 2.2 cmol_c dm⁻³ of Ca; 1 cmol_c dm⁻³ of Mg; 1.7 mmol_c dm⁻³ of H + Al, and 3.27% of organic matter.

Local climate

The climate is classified as Aw (tropical hot, dry season in winter), according to Köppen (1948). During the two weeks preceding the soil sample collection, in the winter of 2014, the average daytime temperature ranged from 27°C to 34°C, with 8 mm of accumulated rainfall and always low air relative humidity (RH<30%). In the summer of 2015, the average daytime temperature ranged from 22°C to 27°C, with 97 mm of accumulated rainfall and relative humidity of the air regularly higher than 60% (INMET, 2015).

Cover crops

Grain crops as soybeans, corn or beans were always sown in early November of each year, with corn being eventually sown, especially when the preceding crop was a legume (soybeans or beans). Brachiaria (*Urochloa brizantha* cv. *Marandu*), crotalaria (*Crotalaria juncea* L.) or millet (*Pennisetum glaucum* (L.) R. Brown) were always grown as cover crops on the same experimental plots (65 m²) before or after each crop. Fertilization and management of pests, diseases and weeds were performed as needed and recommended for each crop.

Soil Sampling

Samples were collected at depths of 0-0.05, 0.05-0.1 and 0.1-0.2 m at the beginning of winter after corn harvest (June, 2014), and in late summer (February, 2015) before sowing the next corn crop. From each plot 500 grams of soil were taken in each layer, always removing the layer of non-degraded crop residues present on the soil surface and avoiding contamination of the lower layers as much as possible.

Isolating and counting the microorganisms

To identify the microorganisms, we transferred 10 g of soil (20 mesh) into Erlenmeyer (250 mL) with 90 mL of distilled water, followed by automatic shaking (200 rpm) for five minutes (shaker TE-085). From the resulting solution (dilution 10⁻¹), dilutions 10⁻² and 10⁻³ were performed. The selective medium was inoculated with 0.1 mL of the appropriate dilution for the isolation methodology (Valarini, 2000), for each microorganism group (endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilizers and cellulolytic microorganisms). The suspension was spread evenly over the solidified medium in the Petri dish with the aid of the Drigalski spatula. The dishes were placed in an oven at 25 °C in an inverted position – varying the time of culture according to the methodology of each microorganism group. The evaluation of each Petri dish was done by counting the number of colony forming units (CFU).

Selective culture media

Selective culture media were used for each evaluated microorganism (endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilizing and cellulolytic microorganisms), according to methodology proposed by Atlas (2010). To determine the endospore-forming bacteria we used the agar nutrient culture (ANC). The 10⁻³ dilution underwent thermal shock in water bath at 85°C for 15 minutes before plating so that only endospore-forming bacteria resistant to heat treatment would remain. After 24 hours we observed and counted opaque colonies with a smooth, flat surface, determining the number of colony forming units per gram of soil. For yeasts, we used the yeast malt agar (YMA) medium, with added antibiotics: chloramphenicol (0.1 g L⁻¹), and tetracycline (0.1 g L⁻¹). The 10⁻² dilution was plated and after 24 hours we proceeded to the CFU count. To determine the actinomycetes, the culture medium was a starch-casein agar (SCA). The 10⁻³ dilution underwent thermal shock in water bath at 50°C for 10 minutes, before plating. The CFU counts occurred six days after plating. Compressed and dull colonies were observed. For phosphate solubilizing microorganisms, the culture medium was the inorganic salts-glucose (ISG) with soil extract. The soil extract was obtained using 1 kg of forest soil autoclaved for 30 minutes with 1 L of distilled water. The solution was filtered, completed to obtain 1 L, and adjusted to pH 7.2. This time the 10⁻² dilution was used. After seven days CFU which formed a clear halo of solubilization of inorganic phosphate from the culture medium were counted. In order to determine the cellulolytic microorganisms the cellulose-asparagine-agar culture (CAA) was used. A solution of 0.5% of Triton X-100 (10%) was added to reduce the size of the fungal colonies facilitating the visualization of cellulose degradation halo. The 10⁻¹ dilution was used and the count of CFU occurred 15 days after plating.

Experimental design and statistical analysis

The experimental design was randomized blocks in a factorial scheme (3x3). We evaluated three cover crops (brachiaria, crotalaria, or millet) at three soil depths (0-0.05, 0.05-0.1 and 0.1-0.2 m) for five groups of microorganisms (endospore-forming bacteria, yeasts, actinomycetes, phosphate solubilizing and cellulolytic microorganisms), with 4 replications (n = 180 plots). Each plot was represented by two Petri dishes, totaling 360 plates assessed per season (winter and summer). To meet the basic assumptions of analysis of variance model (ANAVA), the amount of spore-

forming bacteria, yeasts, actinomycetes, phosphate solubilizing and cellulolytic microorganisms per gram of soil was turned into square root (x) or $\log(x + 10)$ when necessary ($p < 0.01$). After ANAVA results, the treatment averages and seasons were compared by Tukey test at 0.05% of significance using the *GENES* analytical software (Cruz, 2013).

Conclusion

The Cerrado environment in winter was less favorable for the development of actinomycetes, while summer was less favorable for yeast development under all cover crops. Brachiaria and crotalaria cover crops did not vary the populations of the evaluated soil microorganisms for any of the studied periods. The millet presented superior population of endospore-forming bacteria in winter, and superior population of actinomycetes bacteria in summer.

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