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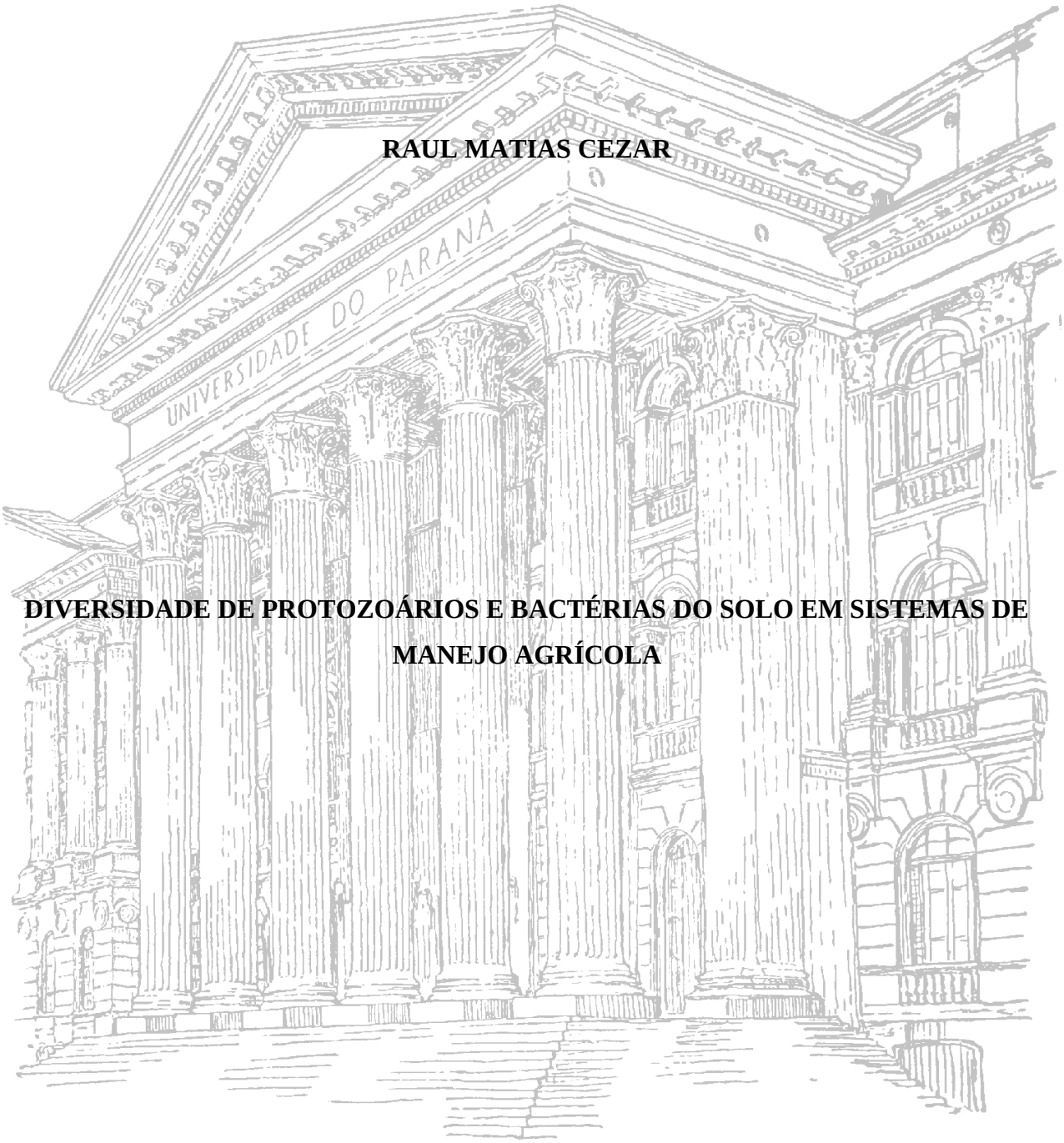

RAUL MATIAS CEZAR

DIVERSIDADE DE PROTOZOÁRIOS E BACTÉRIAS DO SOLO EM SISTEMAS DE MANEJO AGRÍCOLA

CURITIBA
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Tese apresentada ao Programa de Pós-Graduação em Ciência do Solo, Área de Concentração Solo e Ambiente, do Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciência do Solo.

Orientadora: Profa. Dra. Fabiane Machado Vezzani

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Mestrado e Doutorado

PARECER

A Banca Examinadora designada para avaliar a defesa da Tese de Doutorado de **Raul Matias Cezar** intitulada: **"Diversidade de protozoários e bactérias do solo em sistemas de manejo agrícola",** do Programa de Pós-Graduação em Ciência do Solo do Setor de Ciências Agrárias da Universidade Federal do Paraná, após análise do texto e arguição do candidato, emitem parecer pela **"APROVAÇÃO"** da referida Tese. O candidato atende assim um dos requisitos para a obtenção do título de **Doutor em Ciência do Solo - Área de Concentração Solo e Ambiente.**

Secretaria do Programa de Pós-Graduação em Ciência do Solo, em Curitiba, 17 de fevereiro de 2017.

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À minha esposa Melina e
à minha pequena princesa por do sol Alice,
dedico.

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Para Alice:
“Mas te vejo e sinto
O brilho desse olhar
Que me acalma
E me traz força pra encarar tudo”
(Tiago Iorc)

SUMÁRIO

DIVERSIDADE DE PROTOZOÁRIOS E BACTÉRIAS DO SOLO EM SISTEMAS DE MANEJO AGRÍCOLA

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RESUMO GERAL

As interações tróficas são essenciais para o fluxo de nutrientes no solo, as quais são dependentes de plantas, protozoários, fungos e bactérias. Nesse sentido, os 5 capítulos desta tese tem o objetivo de estudar as interações entre a comunidade microbiana do solo em sistemas agrícolas. O primeiro capítulo é uma revisão sobre a regulação das funções bacterianas alteradas pela predação dos protozoários edáficos. O efeito dos protozoários edáficos está além de uma simples predação das células bacterianas. Os protozoários interagem em diversos caminhos com as células microbianas, potencializando suas importantes funções. O segundo artigo tem o objetivo de verificar o efeito da sucessão de culturas (S – trigo - soja) e rotação de culturas (R – ervilhaca – milho – aveia – soja – trigo – soja) na comunidade microbiana do solo. A rotação de culturas aumentou a frequência relativa de bactérias gram-positivas, micorriza arbuscular e amebas em microagregados, enquanto a sucessão de culturas aumentou os flagelados em macroagregado. O efeito dos sitemas de culturas está relacionado com a adição de matéria seca com alta relação C/N em rotação de culturas, aumentado processos conservativos no solo. Os mesmos sistemas de culturas citados anteriormente foram usados para um terceiro artigo, com o objetivo de investigar o efeito dos sistemas de culturas na comunidade bacteriana do solo em macro e microagregado. Os sistemas de culturas não afetaram a diversidade de bactérias, entretanto a estrutura da comunidade mudou entre tratamentos. A sucessão de culturas aumentou a frequência do filo *Spirochaetes* e a acidobactéria *Holophagae*, enquanto a rotação de culturas aumantou a frequência de *Chloracidobacteria*. A bactéria *Holophagae* é uma bactéria copiotrófica, apta em consumir carbono lábil, enquanto a *Chloracidobactéria* é uma bactéria oligotrófica, vivendo em nichos oligotróficos do solo. Dessa forma, a rotação de culturas revelou ser um sistema conservativo de carbono. O quarto artigo apresentado nessa tese teve o objetivo de investigar o efeito de duas formas de adição de carbono na comunidade de protozoários edáficos. Adição via raiz e cobertura morta na cultura do milho. Os protozoários

foram coletados nas profundidades 0-10 cm; 40 – 50 cm; 60 – 70 cm em julho e setembro. Os tratamentos, produndidades e épocas afetaram os flagelados Glissomonad, *Spumella* e as amebas Acanthapodiais e Eruptives. Todos morfotipos de protozoários decresceram em profundidade devido ao menor concentração de carbono e nitrogênio em profundidade. Apesar disso, os índices ecológicos não variaram entre os tratamentos. Dessa forma, a adição de carbono na forma de resíduos de culturas ou raiz apenas alterarou a abundância e pequenos flagelados e pequenas amebas em época com maior umidade no solo. O quinto capítulo apresenta o objetivo de avaliar o efeito da inoculação da *Acanthamoeba castellanii* no crescimento de *Arabdopsis*, a qual apresentou o dobro de volume radicular com a inoculação de *Acanthamoeba castellanii*. Como conclusão, temos que o sistema de rotação de culturas alterarou a distribuição de protozoários e bactérias em micro e macroagregados, além de conservar importantes funções da comunidade microbiana do solo. *Acanthamoeba castellanii* aumentou o volume radicular de *Arabdopsis*, podendo ser uma importante alternativa como parte de estratégias de inoculação em plantas.

Key-words: Ecologia do solo. Comuinidade microbiana. Protozoários do solo. Crescimento de planta.

DIVERSITY OF SOIL PROTOZOA AND SOIL BACTERIA OF SOIL UNDER AGRICULTURE CROP SYSTEM

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Co- Advisor: Profa. Dra. Glaciela Kaschuk

GENERAL ABSTRACT

The trophic interactions are essential to nutrient flow in soil, which are dependent of soil protozoa, fungi and bacteria. In this sense, five papers are presented in this thesis to study the interactions between microbial communities in crop land. The first work is a review about the regulation of soil bacteria function provide by soil protozoa grazing. The protozoa interact in different ways with microbial cells, potentiating important functions of bacterial colonies. The second article has the aim of verify the effect of crop succession (S - wheat-Soybean) and crop rotation (R-Vetch – Maize – Oat – Soybean – Wheat – Soybean) on soil microbial community. The crop rotation increased the relative frequence of gram-positive bacteria, arbuscular mycorrhiza fungi and soil amoebas in microaggregate, while the crop succession increased the falgellates in macroaggregate. The crop system effect is related to addition to high dry matter production with high ratio C/N in crop rotation, increasing the conservative process in soil. The same crop system cited above was used in a third article with the aim of investigate the effect of crop system on soil bacterial community in macro and microaggregate of soil. The crop system did not affect the diversity of bacteria, however the community structure changed through the crop system. The crop succession increased only the phyla *Spirochaetes* and the acidobacterium *Holophagae*, while the crop rotation increased the acidobacterium *Chloracidobacteria*. The *Holophagae* is a copiotrophic bacterium able in consume labile carbon. While the Chloracidobacteria is an oligotrophic bacteria able living in olitrophic niches of soil. Thus, the crop rotation selected the oligotrophic bacterium, revealing to be a conservative system. In addition to effect of crop system, the fourth article has the aim of investigate the effect of two ways of input of organic carbon in the soil (growthing plant or mulch) on structure of soil protozoa community. The treatment used was green plants (P); only mulch (L); and fallow (F), as a control, in three depths: 0 at 10 cm; 40 at 50 cm; 60 at 70 cm in July and September. The treatments, depths and seasons affected the Glissomonad, Spumella flagellates and Acanthapodial, Eruptive amoebas. All morphotypes of the soil

protozoa decreased in depth, it could be related to the soil carbon and nitrogen decrease. Despite these effects, the ecological indexes did not vary between the treatments. Thus, the adding carbon in the form of crop residues or by root presence did not change the diversity and number of total soil protozoa, but increased the abundance of small flagellates and small amoebae in soil surface on season with higher water content. The chapter five has the aim of evaluate the effect of inoculation of *Acanthamoeba castellanii* in *Arabdopsis* growth, which increased two fold the root volume in compare with control. Thus, the crop rotation changes the soil protozoa distribution in micro and macroaggregate. In addition, the crop rotations conserve important functions of soil microbial community. The *Acanthamoeba castellanii* increased the root volume of *Arabdopsis*, which this inoculation can be an alternative to promote plant growth.

Key-words: Soil ecology. Microbial community. Soil protozoa. Plant growth.

INTRODUÇÃO GERAL

Evidência recente sugere que a maior parte da estrutura da cadeia alimentar do solo depende mais da adição de carbono via exsudação radicular do que via cobertura morta (Bonkowski et al., 2009). A exsudação radicular e os restos de culturas adicionados sobre o solo são partes de dois nichos edáficos designados respectivamente de rizosfera e detritosfera (Beare et al., 1995). Tanto as carcterísticas da rizosfera quanto as da detritosfera serão dependentes da presença das plantas no sistema agrícola. As diferentes espécies de plantas diferem na qualidade e quantidade de carbono disponível para biomassa microbiana (Wardle et al., 2004), e portanto, essas diferenças irão selecionar a população microbiana apta para decompor diferentes compostos orgânicos gerados à partir de cada espécie vegetal presente.

Portanto, as carcterísticas da rizosfera e da detritosfera estão ligadas à espécie de planta presente no sistema de culturas. Plantas que geram uma cobertura morta mais lignificada, com maiores relçãoes C/N favorecem bactérias aptas a consumir carbono mais recalcitrante, como as Acidobactérias oligotróficas (Ward et al., 2009). Por outro lado, a rizosfera selecioana bactérias através da exudação de compostos lábeis pela raiz (Sun et al., 2014).

Além do efeito da espécie vegetal presente no sistema agrícola na composição da comunidade microbiana do solo, outros fatores bióticos também agem como selecionadores de espécies microbianas edáficas. A predação exercida pelos protozoários do solo é um exemplo. Os protozoários são os principais predadores de bactérias no ambiente adáfico (Bonkowski, 2002), e apresentam preferencias por bactérias Gram-negativas (Khan et al., 2014). Essa preferência alimentar é devido à ausência de membranas de lipossácarideos e peptidioglicanos na parede celular dessas bactérias, tornado-as mais digestivas para os protozoáriios (Alsam et al., 2006). Por esse motivo os protozoários podem regular a população bacteriana do solo. Entretanto, o efeito de predação realizado pelos protozoários é dependente do contato direto com as células bacterianas no espaço poroso edáfico (Hattori, 1994). Para isso, a estrutura do solo apresenta papel fundamental na conexão entre os espaços porosos habitáveis, onde microporos são habitados por bactérias e inacessíveis para os protozoários (Ranjard e Richaume, 2001).

Dessa forma, fatores “microbióticos” interagem com os fatores abióticos no solo, resultando em uma distribuição heterogênea da comunidade microbiana dentro dos agregados. Essa distribuição está ligada ao crescimento vegetal, pois as plantas apresentam diferentes morfologias radiculares, e por esse motivo cada espécie de planta irá distribuir os hotspots

para o crescimento da população microbiana diferentemente entre agregados do solo. Além do efeito das plantas na diversidade de mocroorganismos do solo, há também a remineralização de nutrientes proporcionado pelos protozoários edáficos (Bonkowski, 2002). Esta remineralização de nutrientes aumenta a disponibilidade de nitrogênio para o crescimento radicular (Røon et al., 2001). Assim, a diversidade de plantas em sistemas de rotação aumentará a diversidade de nichos do solo, aumentando a diversidade interação entre protozoários e bactérias edáficas e inteferindo na diversidade dos microorganismos em microescala. Isso é devido ao efeito de diferentes sistemas radiculares e diferentes coberturas mortas sobre o solo ao longo de um sistema de rotação de culturas.

Dessa forma, o objetivo geral da tese é avaliar a diversidade de protozoários e de bactérias em sistemas de cultura agrícola, apresentando a hipótese gereal de que o histórico de diversidade de plantas utilizados ao longo do sistema de rotação de culturas aumenta a diversidade microbiana do solo em microescala, e que o efeito da presença de raiz é diferente do efeito da cobertura morta na diversidade de protozoários edáficos.

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1. CHAPTER I. THE SOIL PROTOZOA AS REGULATOR OF MICROBIAL FUNCTIONS

ABSTRACT

The plants begin almost all the biochemical process in the soil, due the photosynthesis process. Part of this carbon is release in root exudation, selecting the soil microbial community and constructing the soil macroaggregate. The interaction between the microbial community result in a flow of carbon and nutrients in micro soil food web, which is compost by plant, bacteria, fungi and protozoa. The relationship of predation, symbiosis, and mutualism affect in different forms the plant development. In this scenario the soil protozoa act as regulator of microbial functions by grazing on soil microorganisms. This regulation is related to selective grazing of soil protozoa on soil bacteria, potentiating important functions of bacterial colonies in soil. However, the effect of soil protozoa is neglected in studies of microbial ecology. Thus, it is necessary the understanding of the diversity of interaction in soil integrating the soil protozoa. Thereby, this review clusters the principals groups of microbial food web and the interrelationship with soil protozoa.

Key-words: Soil ecology; Microorganisms; Nutrients cycling.

1.1. INTRODUCTION

The protozoa are eukaryotic microorganisms belonging to Protist kingdom, which are heterogeneous and polyphyletic group with diverse form of life classified mainly by the locomotive mode, the ciliates, flagellates, amoebas and the sessile sporozoa. However, the classification of protozoa through of locomotive mode is inappropriate due the diversity of protozoa that exist in each group. Thus, there are four main phyla of protozoas.

The Cercozoa comprises the majority of zooflagellates bacteriophages of fresh-water, sea and the soil (Cavalier-Smith and, 2003); the Amoebozoa that comprises the major groups of amoeboids eukaryotic, subdivides in two subphylum Lobosa and Canosa (Cavalier-Smith et al., 2016); the Ciliophora are ciliates represented by subphylum Mesodiniea Postciliodesmatophora and Intramacronucleata (Gao et al., 2016); the Myzozoa phylum is related to two different protozoa, the dinoflagellate and the sporozoas (Cavalier-Smith and Chao, 2004).

The protozoa are aquatic organisms, which can be parasite, predator of microorganisms and decomposer, controlling the bacteria population. They ingest the food through the food vacuole that conjugate with the lysosomes. The action of lysosomic acids and hydrolytic enzymes digest the food inside of food vacuole, and then the indigested particles are conjugated with residual vesicle before to release through of membrane in a process called exocytose. The sequence of steps of digestion of protozoa did not change along with phyla, but the time of process, and the trait of each step will depend of each species of protozoa and of the traits of environment.

The evolutionary process that allowed the adaptation of protozoa in the soil was the small size, and the capacity of explore the microporous. In addition, soil protozoa present physiological and morphological changes that protect them from extreme fluctuations of moisture, pH, food resources, which is linked with the encystation. Thus, the soil protozoa have all traits of a great predator of soil microrganisms, and this predation is related with the main ecological function of protozoa in soil that is control the bacterial community, cycling of nutrients, contributing to plant development.

All these functions are controlled by plants. Because the plants begin amost all process of carbon fixation due to photosynthesis, regulating the bacterial community. In addition the plants, the soil protozoa also control the microorganisms functions through the grazing, and the aim of this review is present the importance of this grazing to soil ecology, with the hypothesis: i) the soil protozoa changes de bacterial functions in the soil; ii) the soil protozoa

improves some functions of soil bacteria; iii) the presence of plants modifies the interaction between soil protozoa and bacteria.

1.2. HOW PROTOZOA INTERACT WITH BACTERIA IN THE SOIL

The main interactions between soil protozoa and soil bacteria are linked with direct grazing of soil bacteria by soil heterotrophic protozoa. The grazing of soil protozoa on the soil bacteria reduces the soil microbial biomass (Clarholm, 1981; Frey et al., 2001; Roon et al., 2002; Rosenberg et al., 2009). Indeed, the grazing on the bacteria biomass present ecological relevance for three reasons: (i) the predatory activities of protozoa maintain a high proportion of young bacteria, due to the protozoa prevent the accumulation of senescent cells (Griffiths, 1994); (ii) the consumption of bacteria by protists maintain the optimal level of oxygen for nitrogen fixation by N fixing bacteria, because the soil protozoa reduce the microbial biomass; (iii) the protozoa excrete substances that stimulate the microbial activity (Pussard et al., 1994), mainly the nitrogen.

All effects of grazing by soil protozoa increase the N mineralization (Griffiths, 1989; Krome et al., 2009). The N mineralization by soil protozoa is related to higher ratio C/N of protozoa than bacterial cell. Thus, about 70 % of N is released in the form of ammonium (NH_4^+) readily available for the plants (Krome et al., 2009). The grazing of bacteria by soil protozoa is responsible for recycling 80 kg de N ha^{-1} per year (Bouwman and Zwart, 1994). Generally the inoculation of protozoa plus bacteria in soil increases the uptake of N by plant (Ekelund et al., 2009; Koler et al., 2013) and root growth (Ekelund et al., 2009). It is not clear why the soil protozoa stimulate the plant growth, because the presence of soil protozoa in rhizosphere also increases the root biomass and the root branch with finer roots (Bonkowski, 2002), revealing some hormonal effects. The effect of soil protozoa in plant development can be due the change of auxin distribution in plant (Krome et al., 2010). The soil protozoa can produce auxin (Ienne et al., 20014) or the soil protozoa can select the producing bacteria auxin in rhizosphere soil (Bonkowsli and Brandt, 2002). For this reason, the relationship predator/prey of protozoa and bacteria is essential for decrease the use of fertilization, which is a prerequisite to achieve the sustainability in the agricultural land (Bonkowski et al., 2000).

The soil protozoa control the functions of soil bacteria mainly by changing of microbial community. The function of soil bacteria which are changed by soil protozoa are methanotrphy (Dunfield et al., 2007), biological nitrogen fixation, nitrification, decomposition of xenobiotics (Souza et al., 2013; Shivlata and Satyanarayana, 2015), plant growth

promoteion (Le et al., 2016), and antibiotic production (Shivlata and Satyanarayana, 2015). The *Acanthamoeba castelanni* for example, increased the density of Verrucomicrobia, Planctomycetes, Actinobacteria and decreasing the Proteobacteria population in rhizosphere of *Arabidopsis thaliana* (Rosenberg et al., 2009). It is assumed that increase of Verrucomicrobia in soil lead to improve of methane oxidation (methanotrophy) (Dunfield et al., 2007), which is an important function to reduce emission of greenhouse gases, and the increase of Panctomycetes can improve the oxidation of ammonia in soil (nitrification) (Fuerst and Sagulenko, 2011). The increase of Actinobacteria improves the soil macroaggregation due growth of hyphal threads of Actinobacteria (Keendy, 1999). In addition, the Actinobacteria produces antibiotic substances antagonist to inhibit the vegetative growth of plant pathogen (El-Tarabily et al., 2006).

Thus, the grazing of soil protozoa improves important functions of soil bacteria, mainly by change the bacterial population. The change of bacteria community proportionate by the protozoa is related to specific food preference of the soil protozoa (Khan et al., 2014), which can be explained by production of pheromones/terpenes by the bacteria, by the presence of functional chemoreceptors in protozoa membrane, by the size and morphological characteristics of the prey and by the biochemical properties of the bacterial surface or elements such as capsule, lipopolysaccharide outer membrane proteins and peptideoglycans (Alsam et al., 2006). The traits of cell wall of bacteria also induce the survival of bacterial cell in food vacuole of soil amoeba (Karás et al., 2015). The capacity of *Mesorhizobium loti* in survive of digestion process of *A. castellanii* is due the presence of lipid 27-hydroxyoctacosanoic acid (27-OH-28:0) in cell wall of bacteria indigestible for amoeba, and the bacterial cells that survived to the digestion process increased the nodule-forming capacity in *Lotus corniculatus* (Karás et al., 2015).

The surviving of bacteria in the food vacuole of protozoa facilitates the genetic transference between bacteria and soil protozoa (Clarke et al., 2013; Schulz et al., 2014; Zeybek and Binay, 2014). The genetic transference called horizontal gene transfers (HGT) is the transfer of genetic information between distantly organisms (Keeling and Palmer, 2008). Despite of the HGT is very rare in recent times, the genetic study of *Acanthamoeba castellani* revealed that 450 genes to have arisen through HGT, which confer to *A. castellenii* several bacterial functions (Clarke et al., 2013). The flagellate protozoa that live in leaf surface, the trypanosomatid (*P. serpens*), acquired the ipdC gene from donor phytobacteria, conferring to

P. serpens to descarboxylate indole-3-pyruvic acid (IPyA) and to proceed the biosynthesis of IAA (Ienne et al., 2014).

Thus, the biochemical interactions between soil protozoa and bacteria exercise positive functions to soil ecology in agricultural lands. The interactions described above are essential for good soil function, mainly by functional resilience of soil. The soil protozoa can exercise similar functions of the bacteria or improve some bacterial functions. However, the effects of interaction between soil bacteria and protozoa are dependent of specie of plant present in agricultural land. Thus, the choice of crop system will determine what interactions will occur in soil.

1.3. HOW THE PLANTS AFFECT THE SOIL PROTOZOA INTERACTIONS

The photosynthesis is responsible for the main step of food web, because the plants are producers that provide carbon and energy to all the trophic levels. The soil carbon is available in specific niches of soil, as the detritusphere formed by crop mulching and rhizosphere formed by soil adhered to the roots (Beare et al., 1995). This means that the composition of plants in the ecosystem is important to create hotspots of the microbial activity into the soil (Kuzyakov and Blagodatskaya, 2015). The carbon available to soil food web arises through of photosynthetic organisms, which capture solar energy and form ATP and NADH to synthesise organic compounds.

Thus, the carbon and energy from the plant in crop land will be available in form of mulch and root exudates (Wardle et al., 2006; Shi et al., 2011; Saar et al., 2016). The mulch will select the microbial community due the differences in nutrients contents in the plant tissue (Carrilo et al., 2016). The mulch with high C/N ratio increase the bacteria able in consume recalcitrant carbon like the gram-positive bacteria (Fierer et al., 2007), decreasing the soil protozoa population, while the fungi population increase in mulch with lower C/N ratio (Carrilo et al., 2016). The plants also select microbial community by root exudate (Ling et al., 2011; Zhang et al., 2014), which represent about 20 % of C fixed in the photosynthetic processes (Nguyen, 2003). Regarding the chemical composition of root deposits, substances are found such as carbohydrates, amino acids, organic acids, sterols, vitamins, purines, flavonoids, lignins, anthocyanins, enzymes and proteins arising from the primary or secondary plant metabolism (Jones et al., 2009). However, the organic acids are the main modulator of microbial community (Shi et al., 2011). The addition of quinic acid, lactic acid and maleic acid in microcosms experiment resulted in greater change in the bacterial

community than the single sugar (glucose, sucrose and fructose) (Shi et al., 2011). It is evident that plant community will affect the kind of carbon which will be available to bacterial consumption in the form of litter or of root exudation.

The rhizosphere soil is inhabited mainly by gram-negative bacteria (Prashar et al., 2014), due the ability of that bacteria to use labile carbon as food source (Fierer et al., 2007). The protozoa prefer the gram-negative bacteria as food resource (Khan et al., 2014). Thus, the root exudation indirectly regulates the soil protozoa community in rhizosphere soil, by regulate the soil bacteria community. However, it is not only the quantity and the lability of carbon that will regulate the bacteria and soil protozoa community, the locality of carbon resources inside of soil porous also has an effect in the soil process that regulate the microbial population. The organization of the macroggregate built by the root and fungi mycelium (Tisdall and Oades, 1982) connects the all soil porosity, and distributes the carbon in the macroaggregate. The distribution of soil C regulates the decomposition of soil organic matter. In addition, the connection of soil porosity provided by root facilitate the direct contact of soil protozoa with bacterial colonies, which is essential to promote effective control of bacterial community (Hattori et al., 1994), promoting plant growth indirectly by stimulate the grazing of soil protozoa.

Other indirect effect of plants is related with the mycorhizal fungi population. The obligate symbiotic life style of arbuscular mycorrhizal (AM) fungi (Vos et al., 2012) became this fungi dependent of presence of plants in the system. The mycorrhizal fungi change the plant exudation and consequently alter the structure of bacterial community in the rhizosphere, due to the change in the quality and quantity of rhizodeposition (Marschner et al., 1997; Wamberg et al., 2003; Vestergard et al., 2007; Vos et al., 2012). However, all these effects are dependent of the others levels trophic into the soil food web, which can improve the soil protozoa functions.

The symbiosis mycorrhizal fungi decrease the root exudation that in turn decrease the number of protozoa in rhizosphere soil (Timonen et al., 2004). However, the protozoa increase the N uptake by AM (Koller et al., 2013b), revealing a synergistic effect between mycorhizal fungi and soil protozoa on the plant development (Bonkowski et al., 2001). The effect of mycorrhizal fungi also is related to exudation of amino acids, organic acids and polysaccharides by the AM fungi (Toljander et al., 2006), including the glomalin exudation, which increase the soil macroaggregation (Rillig and Steinberg, 2002). In addition, the fungal hyphae live about 5 to 6 days in the soil (Stadoon et al., 2003). The decomposition of dead

hyphae contribute for hotspot of microbial development (Toljander et al., 2003), which is important for food source to soil protozoa. Thus, the fungi also can select the bacterial population in soil in macro and microaggregate. Therefore, the interaction between fungi, protozoa and bacteria in the soil must be improved to accelerate the process linked with aggregation and biochemical cycling.

The improved of all these interaction can be achieved using the plant with different ecological functions. The crop rotation or consortiums of plants are alternatives, integrating all microbial population, due to increase of diversity and richness of plants. The plant diversity increases the mycorhizal fungi, promoting the macroaggregation through interactions between soil protozoa, fungi and bacteria, which are essential for the sustainability in the agricultural fields. Thus, the knowledge about these interactions must be improved to reduce the use of mineral fertilizers and predict how the ecological threats could damage soil ecological functions (Trap et al., 2016). The complexity of all interactions cited above will preserve the self-regulation of agroecosystem. The self-regulation of the agroecosystem is connected with the micro food web of soil, which soil protozoa, bacteria and fungi are part.

There is also a direct interaction between fungi and soil protozoa. The immense potential enzymatic repertoire such as chitinases was detected in the genome of *A. castellanii* (Anderson et al., 2005; Clarke et al., 2013). For this reason, the *A. castellanii* is able to digest fungi yeast, decreasing about 30 % of growth of fungi *S. cerevisiae* in the soil (Geisen et al., 2016). On the other hand, some fungi produce toxins that can protect them from grazing in a specific manner (Geisen et al., 2016) and decrease the abundance of some soil protozoa.

Thereby, soil protozoa present key role in moulder the microbial community structure. The effect of soil protist in the bacteria population has close relationship with plant diversity. If the plant produce mulch with low C/N ratio, the fungi will dominate the microbial community (Wardle et al., 2004), leading to increase the mycophagous protozoa (Anderson and McGuire, 2013), decreasing the mineralization of nutrients. Soil fungi present higher ratio C/N than bacteria (Carrillo et al., 2016; Kooijman et al., 2016). Consequently the mycophagous protozoa will immobilize the N arising from fungi due to the similarity between ratio C/N of fungi and soil protozoa. The similarity of C/N ratio between the soil food web components increases the competition between plant and protozoa for nutrients. In the soil with low N content, the presence of protozoa in the rhizosphere lead the plant allocates more

carbon in root tissue (Koller et al., 2013a). The increase of allocation of C in root tissue is a plant strategy to increase nitrogen uptake (Krome et al., 2009).

The fungi present longer life cycle and occupy different niches in the soil than bacteria, resulting in an association with slow and conservative nutrient cycling (Wardle et al, 2004; Kooijman et al., 2016). Consequently the fungi present different ecological functions into the soil, due to the biochemical apparatus able in degrade recalcitrant organic compounds (de Boer et al., 2005; Wang et al., 2010) and colonize microenvironment with higher acidity, such as some areas of rhizosphere richer in organic acids, and degrading parts of plant with higher lignin content, where the bacteria has low development. In this scenario, the soil food web is fungal-based energy channel, which is related with the lower value nutritional of litter in this ecosystem with infertile soil (Wardle et al., 2004).

Thus, the soil protozoa are important link between soil fungi and soil bacteria in infertile system. The fungi present slower development than bacteria, therefore the presence of fungi is related with environments with slow turnover. The soil protozoa can improve the bacterial functions in this kind of environment, including synergetic effect with AM and to improve plant development, considering the effect of AM mycorrhiza in soil structure. It is evident that the soil protozoa are important connection between soil bacteria and soil fungi, mainly due the resilience of functions in soil with low fertility.

1.4. CONCLUSION

We have the soil protozoa as a connection of soil food web, regulating the bacterial and fungi functions. For this reason, the positive effect of soil protozoa in soil microbial biomass should not be seen as a villain against of the positive effect of functions of soil bacteria and soil fungi. Thus, it is necessary integrate the soil protozoa in studies of bacterial ecology, aiming mainly to explain the effect of crop system in soil ecology. Because it is impossible to think in separate the interactions between microbial live in soil. The soil protozoa have a key role in the soil ecological process, which is linked with the grazing on the microbial biomass. This grazing maintaining the stability of microbial functions by selective power, or by own execute of some similar functions of soil bacteria.

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2. CHAPTER II. ECOLOGY OF SOIL MICROBIAL COMMUNITY IN CROP ROTATION AND CROP SUCCESSION UNDER NO TILLAGE.

ABSTRACT

The soil microbial community is essential for the organic matter decomposition and nutrient cycling. These processes are affecting by soil aggregation and by plant present in crop system. Thus the aim of this work is analyse the soil microbial community in crop succession (S - wheat-Soybean) and crop rotation (R-Vetch – Maize – Oat – Soybean – Wheat – Soybean) under no tillage system, in macro and microaggregate. The experimental design was the randomized block with 4 replicates. The soil was taken with the two trenches in depth of 5 to 10 cm. The soil was fractionated with the actual field moisture using a sieve with mesh size opening of 0.25 mm separating aggregates into two classes: macroaggregates (> 0.25 mm) and microaggregates (< 0.25 mm). All analyses were realized in macro and microaggregate. The soil chemical properties and soil texture was analysed with the aim of correlation with soil microbial community. The soil protozoa were extracted by liquid aliquot method and identified using an optical microscope with 40x amplification capacity. The bacterial and fungi population was analysed by PLFA method. The crop succession presented a relative frequency of 33 % of Cercomonad against 19 % of Acanthapodial amoeba, while the crop rotation presented 44 % of Acanthapodial amoeba and 13 % of Cercomonad. This change of soil protozoa population along with crop systems was accompanied by significant increase of mycorrhiza and gram-positive bacteria in microaggregate of crop rotation. These increases probably are due to the addition to high dry matter production with high ratio C/N in crop rotation. These results show that the crop rotation increase the conservative process in soil, due the increase of Gram+ bacteria, mycorrhiza and amoebas in microaggreggate of crop rotation.

Key-words: Soil amoeba. Soil bacteria. Diversity index. Aggregates. Crop system.

2.1. INTRODUCTION

The soil harbours immense inorganic, organic, and biological constituents hierarchically organized in biogeochemical interfaces (Hanzel et al., 2013), resulting in a flow of elements in the ecosystem. The biogeochemical interface is distributed heterogeneously into the soil aggregates, and it is associated with the root development. The roots involve the microaggregate in macroaggregate (Tisdall and Oades, 1982), realising exudates into the soil (Helgason et al., 2010). These exudates are amino acids, organic acids and polysaccharides (Toljander et al., 2006), which contribute to generate hotspot for microbial development (Kuzyakov and Blagodatskaya, 2015).

The aggregate is the primary structure organizational for distribution of soil microbial community (Kim et al., 2008). The distribution of microbial community in soil aggregates suffers direct impact of presence of plants. This is due each plant differ in quantity of nutrients in your tissue (Wardle et al., 2004; Sun et al., 2016), and it present difference in root exudate composition, which have selective power of shift the microbial communities in rhizosphere (Ling et al., 2011; Zhang et al., 2014). For this reason, the differences among plant species in agricultural crop systems might be key drivers the spatial patterning of soil organisms in local scale (Ettema and wardle, 2002).

The use of plant richness must be used to attain the sustainability in agriculture. The crop rotation is an alternative. Govaerts et al. (2007) working with only maize and maize-wheat succession found higher catabolic diversity in maize-wheat succession. The authors attribute this result to the differences in rhizosphere traits and the difference in the chemical composition of crop residues between the plants. This difference is directly linked with the kind of carbon that will be available in soil food web. The presence of corn residues with higher C:N ratio, for example, increase the macroaggregation due the improvement of fungal development (Wang et al., 2010), while the presence of roots tissue with more N, as leguminous plants increase the bacterial development (Saar et al., 2016).

The shift of microbial community is directly linked with carbon cycling (Malik et al., 2016). The higher lability of carbon in soil favours the copiotrophic bacteria (Gupta, 2000; Chodak et al., 2015), that possess ‘r’ life style (Gupta, 2000). On the other hand, the oligotrophic bacteria, like Gram-positive (Fierer et al., 2007), possess genetics apparatus able in decompose recalcitrant organic matter with low N content (Malik et al., 2016). Thereby, there is a relation between these groups of bacteria and crop residue in soil. This change of bacteria community causes a cascade effect in soil food web, mainly regarding of soil

protozoa population that are main predators of bacteria in soil food web (Bonkowski, 2002). The cascade effect is related to feeding preference of soil protozoa (Khan et al., 2014), which also change de bacterial population and suffer direct effect of soil chemical properties.

These interactions are also connected with soil chemical matrix. The high pH and presence of aluminium into the soil increase the ratio fungi:bacteria (Wardle et al., 2004). The fungi have genetic traits that allow the survival in microenvironment with higher acidity, stating that the chemical properties of soil influence the food web structure. The change of phytosociological traits causes change in soil chemical properties (Rannjan et al., 2015) consequently the structure of bacterial population also change. Thus, all biological interactions suffer effect of vegetable composition in agroecosystem, affecting the integration between crop rotation, soil fungi, soil bacteria and soil protozoa between aggregates.

The integration between soil microorganisms is important for sustainability inagricultural land. While the fungi increase the macroaggregation and distribute the bacterial community into the aggregate (Ruamps et al., 2011), the soil protozoa use the connection between the soil porosity to eat the bacterial community favouring the nutrients cycling (Jentschke et al., 1995; Koller et al., 2013), shifting the distribution of microbial community between soil aggregates. This is key for structure of soil microbial community. Thus, the aim of this work was to verify the effect of crop rotation on soil microbial community and its distribution within aggregates. We hypothetize that crop rotation will increase the diversity of soil protozoa community and the crop system will change distribution of microbial community in microscale, this mean in macro and microaggregate.

2.2. **MATERIALS AND METHODS**

2.2.1. Experimental area, experimental design and treatments

The experimental area is localized in the Centro de Experimentação para Assistência e Divulgação Técnica Agropecuária da Fundação ABC, in the city of Ponta Grossa, with the geographical location 25°00’35” S and 50°09’16” W and the altitude of 890 m. The climate is classified as Cfb (humid subtropical, mesothermal) according to Köppen. The experimental area was assembled 27 years ago, in an experimental design of randomized block with two treatments: Crop succession (S) with *Triticum aestivum* and *Glycine max* respectively in winter and summer, and crop rotation (R) with *Vicia sativa – Zea mays – Avena sativa – Glycine max – Triticum aestivum – Glycine max,* where the *Vicia sativa, Avena sativa* and *Triticum aestivum* was used as winter crop and, *Zea mays* and *Glycine max* as crop summer.

The soil was classified as Oxisol with 419, 133, 449 g kg^{-1} respectively of clay, silt and sand. The predominant mineral in clay fraction are kaolinite and hematite (Winck et al., 2014), with flat slope. It was used 400 kg ha^{-1} of formulation 00-20-20 (NPK) and 50 kg of KCl ha^{-1} for soybean fertilization in 2014. It was used 400 kg ha^{-1} of formulation 14-34-00 (NPK) and 50 kg of KCl ha^{-1} for soybean fertilization in 2015. The fertilization in wheat was applied 221 kg ha^{-1} of formulation 14-34-00 (NPK) and 250 kg ha^{-1} of urea and 100 kg of KCl ha^{-1}. In the rotation treatment the Vetch and Oat were unfertilized.

2.2.2. Soil sampling and aggregate distribution

The soil was taken with the opening of two trenches in each replicate with the aid of spatulas in depth of 5 to 10 cm, in September 2015. The soil aggregates was fractionated with the actual field moisture using a series of 2 sieves with 125 mm of diameter and mesh opening of size of 0.25 mm. Thus, was obtained macroaggregate in succession and rotation treatments and microaggregate in succession and rotation treatments with a total of 32 samples.

2.2.3. Soil chemical properties

The chemical properties was analysed in macroaggregate and microaggregate. The macroaggregate samples were sieved through screens with mesh of 2 mm, afterwards the samples was dry before the analyses. The pH was evaluated in water and in $CaCl_2$. The nutrients calcium (Ca^{2+}), magnesium (Mg^{2+}) and aluminium (Al^{3+}) was extracted with a solution of KCl 1 mol L^{-1}. The nutrients Ca^{2+} and Mg^{2+} were determined by Atomic absorption spectrophotometer, and the Al^{3+} was determinate by titration in NaOH. The potassium (K^{+}) and phosphorus (P) was extracted with mehlich solution, the determination of K^{+} and P was respectively by flame emission spectrometer and spectrophotometry. Total soil nitrogen (TN) were determinated in macroaggregate and microaggregate by dry combustion analyser elementary Vario El III – Elementar®.

2.2.4. Soil protozoa extraction

The soil protozoa were quantified for employed the liquid aliquot method (LAM) according to Butler and Rogerson (1995) in macro and microaggregate. Briefly, it was used the soil dilution of 1:225. 1 g of soil (macroaggregate and microaggregate) was shaken horizontally with one litter oat grass medium (OG), to dilute and separate the protozoa from

soil particles. After this, 10 mL this soil suspension was add in 30 mL of OG medium to stimulate the bacterial growth, and then 20 µL aliquots were filled the 96 well plate for visualization at microscopy. To account the number of protozoa was necessary check the water content, weighing 1 g of wet soil, and after dried in a kiln at 110 °C during 24 hours, and weighed again, to verify the dry weigh.

2.2.5. Protozoa identification

The culture medium to identify protozoa was stored at 20 °C until the check. It is necessary check the plates two times, because the soil protozoa community change with the time. Thus, the plates were checked the first time after 7 days of extraction and the second check was realized 21 days after the extraction of soil protozoa, using an optical microscope with 40 x amplification capacity. The soil protozoa were identifies in morphotypes, according with Smirnov and Brown (2004), Smirnov et al. (2011) and Jeuck and Arndt (2013). For calculate the number of soil protozoa was using the equation:

Number of soil protozoa = I/U (225000/[144x5])

Where I is the number of well which there is presence of protozoa; U is water content in g/g.

The number of each morphotype of soil protozoa was using for calculate the ecological index. The ecological index was: Shannon Index (H' =-Σ(pi log pi)) (H'), Simpson Index (e = H/log S) (D) and Pielou Index (Is = Σ pi^2) (e).

Where pi = Ni / N and ni = number of individuals of the species i, N = the total number of individuals in the sample and S is the number of species.

2.2.6. PLFA extraction and purification

It was used 2 g of soil (macroaggregate and microaggregate) to extract the lipids with a mixture of chloroform, methanol and aqueous citrate buffer (Bligh and Dyer reagent) (Frostegård et al., 1993). The organic, lipid-containing phase is collected and the lipids are separated into neutral, glyco- and phospholipid fractions using silicic acid columns. The phospholipids are then converted to their methyl-esters by alkaline methanolysis and detected by gas chromatography. The PLFAs i15:0, a15:0, i16:0 and i17:0, a17:0 were used as indicative for Gram-positive bacteria (Gram+), and cy17:0 and cy19:0 for Gram-negative bacteria (Gram-). The PLFA Me16:010, Me17:010 and Me18:010 were indicative of actinobacteria, and the PLFA 16:1w5c is indicative of arbuscular mycorhizal fungi (AMF).

2.2.7. Statistical analysis

The data normality for chemical properties and PLFA data was verified by Shapiro-Wilk test. Afterwards, it was realized the variance analysis (ANOVA) to verify the interactions between treatments and aggregates. The significant results ($p < 0.05$) were submitted on test Tukey at 5 % of probability using the R-Studio®. The soil protozoa morphotypes was submitted to the variance analysis (ANOVA) using the Quasi-poisson distribution. The significant results ($p < 0.05$) were submitted on test Tukey at 5 % of probability using the R-Studio®. It was realized the Redundancy analyses (RDA) with the Canoco for Windows 4.5, using the number of soil protozoa morphotype data as variable response and soil chemical properties, soil bacteria and mycorrhiza as explicative variable. The variables Al^{3+} (Aluminium), Ca^{2+} (Calcium), Mg^{2+} (Magnesium), K^{+} (Potassium) and V % (Base saturation) was not use to RDA analyse due the high colinearity verify by high inflation factor value in RDA.

2.3. RESULTS

2.3.1. Redundancy analysis (RDA) of soil protozoa community

The Redundancy analysis of soil protozoa community demonstrates a clear separation between macroaggregate of the crop succession and microaggregate of the crop rotation (Figure 1). The separation was oriented significantly by Actinobacteria and aluminium saturation (m %) in the axis 1 ($p < 0.05$), explaining 35.3 % of all data variability. The axis 1 was responsible by grouping of succession treatment in macroaggregate together with the flagellates Cercomonads, *Spumella* sp., Euglenozoa, total flagellates and richness of soil protozoa. In opposite way, the carbon was contrary to abundance of soil protozoa in axis 1, separating the flagellates and the richness of microaggregates. The axis 2 explains 20.3 %, orienting the Heterobosea, Acanthapodial, Branched amoebas by higher presence of AMF and soil water content. Contrary, the TN in macroaggregate favoured the flagellates. The soil pH, sum of bases (SB), cation exchange capacity (CEC) presented lower influence in soil protozoa community.

2.3.2. Soil chemical properties

The RDA demonstrated the lower direct effect of pH in soil protozoa community. However, the pH affected directly others chemical properties like Al^{+3} ($r = -0.88$; $p < 0.01$). The Al^{3+} was significantly higher in crop sucession independently of aggregates (Table 1).

Despite of increase of acidity components in crop sucession, all bases analysed was not affect by crop systems ($p > 0.05$), reflecting in absence of crop systems effect on the base saturation (V %). The phosphorus (P) was higher in microaggregate ($p < 0.05$). Though the carbon (C) was significantly higher in microaggregate in succession and rotation treatments, total nitrogen (TN) and soil moisture were not affected by crop management neither by aggregates.

	Axis 1	Axis 2
Gram-positive	**-0.12**	**-0.19**
Gram-negative	**-0.31**	**-0.05**
Actinobacteria	**-0.37**	**0.24**
AMF	**0.15**	**-0.40**
pH $CaCl_2$	**0.21**	**-0.12**
P	**0.07**	**-0.01**
C	**0.56**	**0.09**
SB	**-0.04**	**0.29**
CEC	**-0.05**	**0.22**
m %	**-0.31**	**0.24**
Soil moisture	**0.40**	**-0.52**

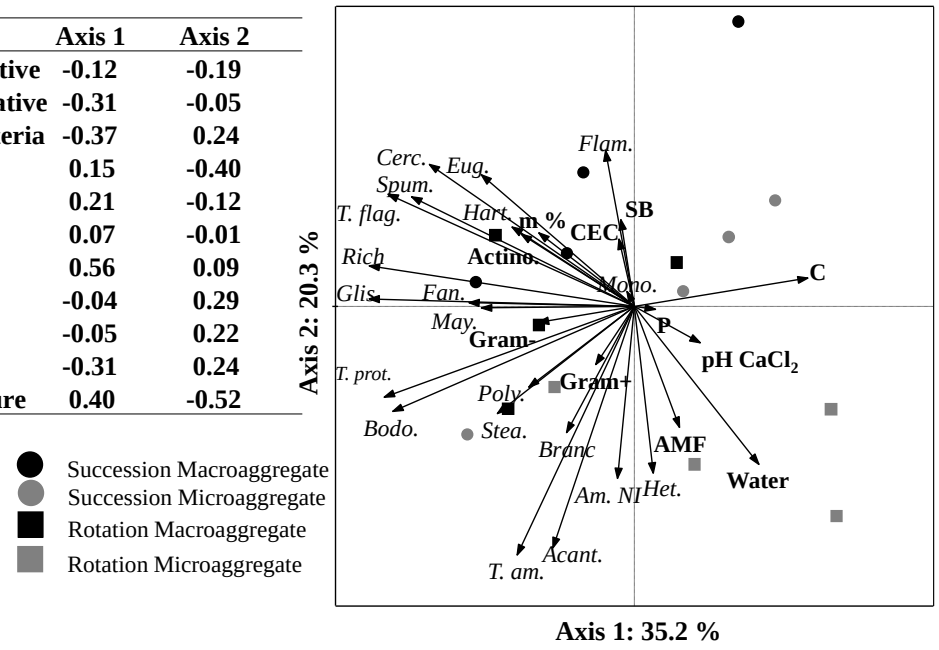

Figure 1. Redundancy analysis (RDA) using soil protozoa morphotype as dependent variables and phospholipids fatty acids and soil chemical properties as the environmental variables. Cerc.: Cercomonad; Spum.: *Spumella*; Eug.: Euglenozoa; Glis.: Glissomonad; Bodo.: Bodonids; Hart.: Hartmenella; Flam.: Flamellian; Stea.: Stenamoeba; Poly.: Polytactic amoeba; Mono.: Monopodial amoeba; Branc.: Branched amoeba; Acant. Acanthapodial amoeba: Het: Heterolobosea; May: Mayorellian; Fan: Fan-shaped; Am. NI: Amoeba not identified; T. Flag.: Total flagellate: T. am.: Total amoeba: Rich: Richness of soil protozoa; AMF: Arbuscular mycorrhizal fungi; Actino.: Actinobacteria; Gram-.: Gram-negative bacteria; Gram+.: Gram-positive bacteria; CEC: Soil cation exchange capacity; m %: Soil Aluminium saturation; P: Soil Phosphor; C: Soil Carbon. (Monte Carlo test $p < 0.05$).

2.3.3. Phospholipids fatty acids of soil bacteria

The macroaggregate presented the higher dominance of Actinobacteria ($p < 0.01$) (Figure 2) independently of crop system, which was the biological property that most affected positively the soil flagellates community ($r = 0.67$; $p < 0.05$). The Gram+ bacteria were higher

in rotation system in microaggregate ($p < 0.05$), while the Gram- bacteria were not affected by crop systems niether by the aggregates. The Gram+/Gram- ratio in rotation system was 9.50 significantly higher than 1.65 in succession treatment ($p < 0.01$) with treatment:aggregate interaction ($p < 0.01$), where the microaggregate presented higher Gram+/Gram- ratio than macroaggregate in rotation treatment. The ratio Gram+/Gram- reflect in the dominance of Gram+ bacteria in rotation treatment (Figure 2). The rotation presented 5.35 nmol g^{-1} dry soil of phospholipid fatty acid indicative of AMF in microaggregate, significantly higher than 3.05 nmol g^{-1} dry soil in the macroaggregate of succession treatment ($p < 0.05$).

Table 1. Chemical properties of soil under crop succession and crop rotation in microaggregates and macroaggregates in no till of Ponta Grossa, Paraná, Brazil.

Chemical properties	Succession Macroaggregate	Rotation Macroaggregate	Succession Microaggregate	Rotation Microaggregate	HSD	CV
pH $CaCl_2$	4.40	4.60	4.40	4.60	0,17	3.56
Al ($cmol_c\ dm^{-3}$)	0.35	0.20	0.40	0.15	0.15	51.42
H+Al ($cmol_c\ dm^{-3}$)	8.43	6.70	9.10	8.10	1.34	15.29
Ca ($cmol_c\ dm^{-3}$)	2.20	2.60	2.80	1.30	0.56	23.13
Mg ($cmol_c\ dm^{-3}$)	0.85	0.98	1.03	1.33	0.31	27.55
K ($cmol_c\ dm^{-3}$)	0.40	0.39	0.44	0.42	0.17	37.25
SB ($cmol_c\ dm^{-3}$)	3.50	4.00	4.30	3.10	0.86	21.38
CEC ($cmol_c\ dm^{-3}$)	11.90	10.70	13.40	11.20	1.04	17.07
P ($mg\ dm^{-3}$)	50.10	36.00	96.70	58.40	31.69	48.23
V %	29.70	37.70	32.10	27.50	7.60	4.38
m %	10.00	4.90	9.40	4.70	4.42	56.30
C ($g\ dm^{-3}$)	24.00	20.50	27.80	27.80	4.18	15.33

Value in parentheses represent the Honest Significant Difference (HSD); CV: Coefficient of variation; CEC: cation exchange capacity; SB: sum of bases; C: organic carbon.

2.3.4. Soil protozoa and ecological index

The number of total protozoa and morphotypes did not differ significantly between crop systems ($p > 0.05$) neither between class of aggregates ($p > 0.05$) (Table 2), with 25,769 individuals g^{-1} soil collected distributes in 13 morphotypes in macroaggregate under crop Succession and 23,925 individuals g^{-1} collected in soil distributes in 12 morphotypes in macroaggregate under crop rotation treatment. The total protozoa collected in microaggregate were respectively 18,089 and 19,171 individuals g^{-1} soil in crop succession and crop rotation treatments. The number of flagellates did not differ between treatments. However, the number

of flagellates differed between aggregates ($p < 0.01$), presenting means value of 13,373 individual g^{-1} soil in macroaggregate, significantly higher than 5,843 individual g^{-1} soil in microaggregate. The total of amoebas were not affected by crop systems or by aggregates ($p > 0.05$), which only the amoebas Acanthapodial and Heteroloboseae presented higher population in crop rotation ($p < 0.05$) independently of the class of aggregates. The increase of Acanthapodial and Heteroloboseae amoebas in microaggregate of crop rotation favoured the increase of frequency of the amoebas in rotation treatment (Figure 3b).

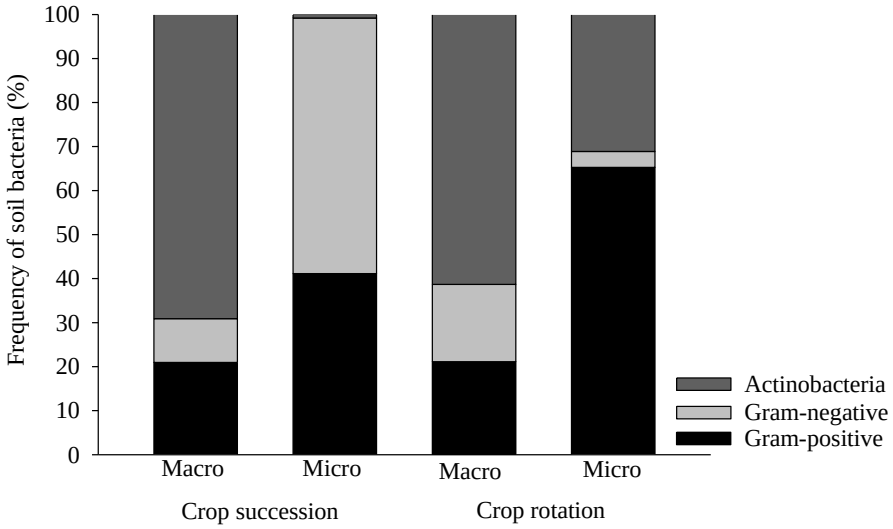

Figure 2. Frequency of the phospholipid fatty acids indicative of Actinobacteria, Gram-positive and Gram-negative bacteria under crop succession and crop rotation in macroaggregates (Macro) and microaggregates (Micro).

All ecological indexes of soil protozoa were not affected by crop system ($p > 0.05$) and by class of aggregates ($p > 0.05$) (Table 2). Nevertheless, the crop systems and classes of aggregates changed the structure of the community of soil protozoa. The dominance of soil flagellate was 33 % of Cercomonad and 12 % of *Spumella* sp. in macroaggregate of crop succession (Figure 3a). The relative frequency of flagellates was 59 %, 42 %, 49 % and 23 % respectively in macroaggregate and microaggregate of crop succession and macroaggregate and microaggregate of crop rotation (Figure 3b). The amoebas increased in the opposite order with the relative frequency of 77 % of amoebas in microaggregate of crop rotation (Figure 3b). The amoebas were dominated by presence of 44 % of Acanthapodial morphotype in microaggregate of crop rotation, followed by 15 % of Branched amoeba. It is important to note that the C was negatively correlated with total soil protozoa ($r = -0.67$; $p < 0.05$).

2.4. DISCUSSION

The crop system changed the distribution of the soil protozoa community, mainly by to alter the abundance between amoebas and flagellates among the systems. The flagellates were more abundant in the macroaggregate of the crop succession, contrary of the amoebas that occupied the microaggregates of the crop rotation. This difference between flagellates and amoebas probably is due to the difference in feeding strategies of soil protozoa (Boenigk and Arndt, 2000; Boenigk and Arndt, 2002; Rønn et al., 2012). The flagellates possess raptorial feeding or interception feeding mode (Boenigk and Arndt, 2002), permitting high mobility and capture of free suspension bacterial cells (Boenigk and Arndt, 2000; Rønn et al., 2012) in the macroporous full of water among the microaggregates, it means, into the magroaggregates. The amoebas possess the feeding strategy called grasping, feeding on attached bacteria (Ekelund and Patterson, 1997; Rønn et al., 2012) in microaggregates.

Table 2. Ecological indexes of soil protozoa of soil under crop succession and crop rotation in microaggregates and macroaggregates in no till of Ponta Grossa, Paraná, Brazil.

Ecological indexes	Succession Macroaggregate	Rotation	Succession Microaggregate	Rotation
Richness	13 ns	12	11	9
Number of individuals	25,769 ns	23,924	18,088	19,171
H'	2.00 ns	1.89	1.93	1.65
D	0.80 ns	0.80	0.81	0.72
E	0.78 ns	0.78	0.83	0.75

Richness: number of order; *H'*: Shannon index; D: Simpson dominance index; Pielou evenness index; ns: not significant according to the Tukey test at 5 % probability.

The major effect of clay particles on bacteria is the adhesion of bacterial cells on the charges of the clay minerals (Mueller, 2015), which facilitate the grasping of amoeba in microaggregate. The porous with diameters between 0.75 and 2.0 µm in clay soil prevent the access of soil protozoa to bacterial colonies (Rutherford and Juma,1992). Despite this, the Acanthapodial amoeba possess a pseudopodium less than 2 µm (Darbyshire, 2005), allowing these amoebas to eat the attached bacterial cells. In addition, the Acanthapodial amoeba as *Acanthamoeba castlannii* can survive in environment under microaerophilic conditions (Clarke et al., 2013) within soil microaggregate (Gupta and Germida, 2015). These

differences of feeding strategies largely regulate and differentiate the ecological niches exploited by amoebas compared to heterotrophic flagellates (Bischoff and Horvarh, 2011).

The heterotrophic flagellates as Cercomonads are highly mobile (Rønn et al., 2012), allowing the exploration of different soil niches with low Al^{3+} in macroaggregate in crop succession. The Actinobacteria favoured the niche exploration of soil flagellates, because the kind of growth of hyphae threads of Actinobacteria has key role in macroaggregation construction (Kennedy, 1999). In opposite way, the amoebas are slower than flagellates and suffer more the negative effect of Al^{3+}, which decrease the feeding ability of soil amoebas (Amaroli, 2015). This explains the increase of amoebas in the crop rotation, especially in microaggregate where the aluminium content was lower than succession system.

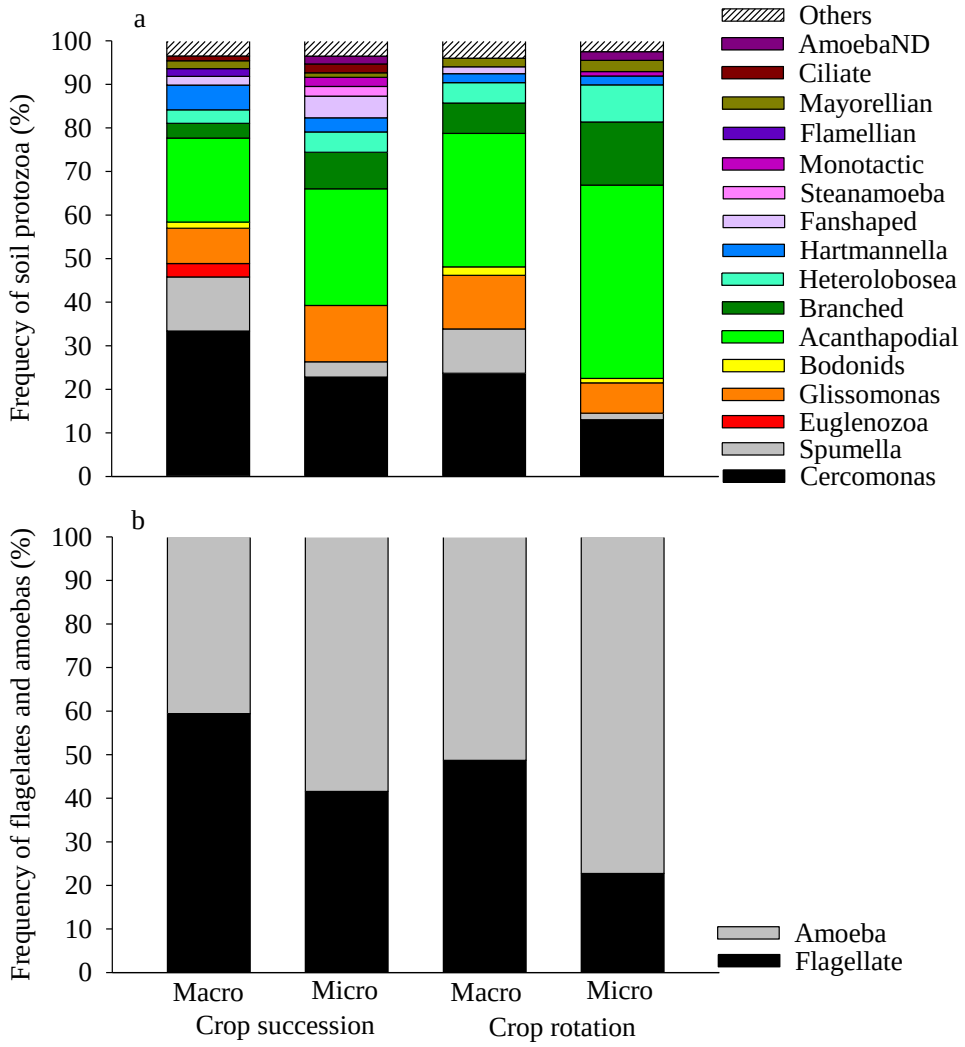

Figure 3. Frequency of soil protozoa (a) and relative frequency of soil amoeba and soil flagellate (b) under crop succession and crop rotation in macroaggregates (Macro) and microaggregates (Micro).

The decrease of Al^{3+} content in soil under crop rotation can be due to the higher presence of arbuscular mycorrhiza (AMF), because the AMF has high capacity of Al^{3+} immobilization in its cell wall (Yang and Goulart, 2000; Aguilera et al., 2011), decreasing the availability of Al^{3+} in soil solution in the crop rotation. Probably, the improvement of soil chemical properties in crop rotation increased the development of the higher ecological interactions between the soil organisms. Since several authors report that the richness of plants in the crop land increase the number of AMF in soil (Mathimaran et al., 2007; Tahat et al., 2008; Tiemann et al., 2015). Possibly, the obligate symbiotic life style of AMF (Vos et al., 2012) became these fungi highly dependent of species present in crop management (Douds and Milner, 1999). The maize and oat plants are crops that present up to 76 % (Tahat et al., 2008) and 88 % of mycorrhizal colonization (Manns et al., 2009), respectively. Therefore, the use of maize and oat in crop rotation ensure the high abundance of AMF in soil, especially in microaggregate due to the capacity of these fungi to explore smaller pores in soil inaccessible to the roots (Ruamps et al., 2011). The presence of AMF is important to increase the macroaggregation (Tisdall and Oades, 1982), mainly by exudation of glomalin (Rilling and Steinberg, 2002) and amino acids, organic acids and polysaccharides (Toljander et al., 2006), primordial for hotspots for biological activity. Thus, the AMF is an important connection of soil food web, especially by action of physical properties, characterizing the crop rotation as a conservationist crop management.

In the same way, the higher presence of Gram+ bacteria also characterizes the rotation system as conservative system. The Gram+ bacteria are more efficient in use the soil carbon (Fierer et al., 2007), and it is able to consume the carbon more recalcitrant. The maize produces high amounts of residue in compare with soybean (Ding et al., 2011) and with higher C/N ratio (Manns et al., 2009), leading to increase the bacteria decomposer of recalcitrant organic matter. However, the higher dominance of Gram+ bacteria was in the microaggregate of the crop rotation. The microaggregates present the organic matter older and more microbially processed than macroaggregates (Six et al., 2004; Tiemann et al., 2015). For this reason, the microaggregate offer habitat for oligotrophic organisms specialized in decomposing recalcitrant substrates, supporting slower turnover rates (Ruamps et al., 2011; Gupta and Germida, 2015), as the Gram+ bacteria (Gupta, 2000; Fierer et al., 2007), explaining their higher presence in microaggregate (Figure 2).

The decrease of the relative frequency of Gram- bacteria in crop rotation was accompanied with the increase of the frequency of soil amoebas. This increase is due the

selective grazing of amoebas, which change the bacterial community (Rosenberg et al 2009). The *Acanthamoeba castellanii* graze preferably gram- bacteria (Khan et al., 2014). Probably, this food preference helps to increase the frequency of gram+ in microaggregate. The preference is due to the absence of lipopolysaccharide outer membrane proteins and peptideoglycans of the bacterial surface in gram- bacteria (Alsam et al., 2006).

Thus, the conservative process in crop rotation is related to presence of AMF, Gram+ bacteria and amoebas. The junction of microaggregates by fungal hyphae connects the micropores to facilitate the direct contact of soil protozoa with bacterial colonies, which is essential to promote effective control of bacterial community and cycling of nutrients (Hattori et al., 1994). The soil *Acanthamoeba* ate preferentially Gram- bacteria (Khan et al., 2014), increasing the relative frequency of Gram+ bacteria. Thereby, all these microorganisms are linked to sustainability of crop system in no till.

2.5. CONCLUSION

The crop management affected the microbial community and their distribution in the soil aggregates classes. Although several works affirm that soil protozoa present ubiquitous distribution in the soil, we demonstrate that the soil protozoa community change in micro scale. This change was oriented mainly by crop system. The crop rotation did not increase the soil protozoa diversity. However, the change of community structure changed, indicating that the redundancy functional of soil protozoa ensure the resilience of functions in soil ecosystems. The changes occur mainly in microaggregate and they are linked with conservative process, which they are related with presence of AMF, amoeba, and Gram+ bacteria, improving the stability of soil system due their slower turnover rates.

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3. CHAPTER III. BACTERIAL COMMUNITY IN CROP SYSTEM ON SOIL UNDER NO TILL

ABSTRACT

The plants have capacity to affect the structure of the microbial community in the soil, mainly by difference between species on root exudates and chemical composition of mulch between species. We work with the hypothesis of that the different plants used in crop rotation interfere on distribution of soil bacterial community and that interference is different along with the aggregates classes of diameter. We investigate the bacterial community in the soil macro and microaggregate classes of the two crop systems of South of Brazil: crop succession (S – *Triticum aestivum– Glycine max*) and crop rotation (R – *Vicia sativa – Zea mays – Avena sativa – Glycine max – Triticum aestivum – Glycine max*) in an experimental design of randomized block with 4 replicates. The soil chemical properties were verified to relate with the bacterial community, which was performed by PCR (polymerase chain reaction) by Illumina MiSeq Platform. The crop system did not affect the richness of soil bacteria. However the crop system change the community structure in soil. The *Gemmatimonadetes* and *Verrucomicrobia* phyla were associated with the Ca), magnesium (Mg) and phosphorus (P) content and sum of base (SB) of soil in crop rotation in the redundancy analysis (RDA). The dominant phylum was *Proteobacteria* (31 %), *Acidobacteria* (22 %), *Actinobacteria* (10 %) and *Gemmatimonadetes* (7 %) regardless crop system and aggregate class. However, the crop succession increased only the phyla *Spirochaetes* and the acidobacterium *Holophagae*, while the crop rotation increased the acidobacterium *Chloracidobacteria*. The *Holophagae* is a copiotrophic bacterium able in consume labile carbon, and it is found in rhizosphere soil. While the *Chloracidobacteria* is an oligotrophic bacteria able in consume recalcitrant carbon, living in olitrophic niches of soil. Thus, the crop rotation regardless of the aggregates classes selected the oligotrophic bacterium *Chloracidobacteria*, *Verrucomicrobia* and *Gemmatimonadetes*, revealing to be a conservative system.

Key-words: Bacterial ecology. Crop system. No tillage. Soil aggregates.

3.1. INTRODUCTION

The practice of no till in the agriculture is characterized by increase of crop residues on the soil surface (Lal, 2007; Figuerola et al., 2015), reducing the soil organic matter decomposition, improving soil sequestration of carbon (Six et al., 2000) and increasing of soil aggregate stability (Tivet et al., 2013). The increase of the aggregate stability ensure microsites for microbial activity (Helgason et al., 2010), and the connectivity between the microbial colonies in pores space of soil (Vos et al., 2013). The edaphic traits provide by no till system affect the composition of microbial community and the biological processes of soil (Gupta and Germida, 2015), which are substantial for biogeochemical interfaces (Hanzel et al., 2013).

In addition to the good interferences of no tillage in the biological activity are the presence of plants that influence the soil biological processes (Ling et al., 2011; Shi et al., 2012; Zhang et al., 2014). The different root exudate of plants (Zhang et al, 2014) and nutritional composition of mulch (Wardle et al., 2004; Scharroba et al., 2012; Duarte et al., 2013) are the main driving of soil microbial community, controlling the functions of bacteria community like methanotrphy (Dunfield et al., 2007), biological nitrogen fixation, nitrification, decomposition of xenobiotics (Souza et al., 2013; Shivlata and Satyanarayana, 2015), plant growth promoter (Le et al., 2016), antibiotic production (Shivlata and Satyanarayana, 2015). Thus, the change of plant species over time in crop rotation can be used to manage of the soil biological functions.

The crop rotation also changes the chemical properties (Ranjan et al., 2015) and the soil physical properties concomitantly with biological properties. Thus, there is an interaction between chemical, physical and biological soil properties. The carbon more recalcitrant favour the *Acidobacteria* (Ward et al., 2009), while the *Protobacteria* is favoured in soil with organic matter with higher lability (Shi et al., 2011). In addition, the decomposition of soil carbon (C) is not only related with the chemical composition of organic matter. The distribution of C into the aggregate can protect the C against the microbial attack, even when this organic matter is richer in nutrients. Thereby, the aggregation would change the bacterial community by two reasons, i) would increase the habitable porous space and; ii) would increase the protection of carbon in microaggregate (Gupta and Gemida, 2015).

The accessible C in microaggregate is generally older and more microbially processed than in macroaggregate (Tiemann et al., 2015). These differences will change the microbial communities in macro and microaggregate (Tiemann et al., 2015) in different crop systems, because different species of plant present differ in morphological traits of roots. The

differences in roots traits modify the distribution of microbial community between aggregates, because the root acts connecting the microaggregates in macroaggregates (Tisdall and Oades, 1982). In addition, the plant exudates are released in the microaggregate, influencing the biological activities and the formation and stabilization of the aggregates (Rasse et al., 2005; Helgason et al., 2010). However, all these effects will depend of which plants are present in crop system, because each plant drives the interactions with soil microbial communities in the different form. Furthermore, the analysis at the aggregate is important to elucidate what are the biotic and abiotic factors that control the soil bacteria diversity (Mummey et al., 2004).

Thus, the increase of knowledge in the dynamic of biogeochemical interfaces in micro scale allows advances in to direct new forms of soil management. The aim of this study was investigate the effect of crop system on soil bacterial community distribution in soil macro and microaggregate, with the hypothesis that the crop rotation present richness bacteria species than crop succession.

3.2. **MATERIAL AND METHODS**

3.2.1. Experimental area and experimental design

The experimental area was localized in the Centro de Experimentação para Assistência e Divulgação Técnica Agropecuária da Fundação ABC, in the Ponta Grossa city, state of Paraná (25°00'35" S and 50°09'16" N), with the altitude of 890 m. The clime is classified by Köppen as humid subtropical, mesothermal (Cfb). The experimental area was assembled in 1989, in an experimental design of randomized block with 4 replicates. The treatments were: Crop succession (S) with *Triticum aestivum* and *Glycine max* respectively in winter and summer, and crop rotation (R) with *Vicia sativa – Zea mays – Avena sativa – Glycine max – Triticum aestivum – Glycine max* where the *Vicia sativa*, *Avena sativa* and *Triticum aestivum* as winter crop and, *Zea mays* and *Glycine max* as crop summer.

The soil is classified as Oxisol. The soil textural analyses revealed 419, 133, 449 g kg^{-1} respectively of clay, silt and sand using the densimeter method. The predominant mineral in clay fraction are kaolinite and hematite (Winck et al., 2014), with flat slope. It was used 400 kg ha^{-1} of formulation 00-20-20 (NPK) and 50 kg of KCl ha^{-1} for soybean fertilization in 2014. The fertilization in wheat was applied 221 kg ha^{-1} of formulation 14-34-00 (NPK) and 250 kg ha^{-1} of urea and100 kg of KCl ha^{-1}. The vetch and oat were unfertilized in the rotation treatment (R).

3.2.2. Soil sample and aggregate distribution

The soil samples were taken with the opening of two minitrenches with the aid of spatulas in depth of 5 to 10 cm. The soil was collected in March 2014, the soybean was the predecessor culture in this period. The soil aggregates was fractionated with the actual field moisture using a sieves with 125 mm of diameter and mesh opening of size of 0.25 mm. Thus, it was obtained in rotation system the macroaggregate (RMAC) and microaggregate (RMIC) and in succession system the macroaggregate (SMAC) and microaggregate (SMIC). The both aggregate classes were kept at -80 °C prior to DNA extraction.

3.2.3. DNA extraction and amplification

The DNA extraction was done in macro and microaggregate using an Ultra Clean soil DNA kit (MoBio Laboratories) following the instructions of the manufacturer. Briefly, 0,25 g of soil samples were suspended in a detergent solution to weaken the cell of microorganisms (Solution of. Afterwards, the soil was stirred with glass tiny crystals to cause the cell lysis exposing the genetic material. The 16s rRNA gene was amplified with 200 nM of primer 515F (GTGYCAGCMGCCGCGGTAA) and 200 nM of primer 806R (GGACTACNVGGGTWTCTAAT) for the bacteria domain.

The PCR (polymerase chain reaction) was performed by Illumina MiSeq Platform that measures the fluorescence of labelled nucleotides in duplicate. Briefly, the PCR was performed with 0.025 U of Taq per mL (MBI Fermentas, Hanover, Md.), 5 % acetamide, 1.5 mM Mg^{2+}, 220 mM deoxynucleoside triphosphates (dNTP), 0.2 mg mL^{-1} bovine serum albumin. The PCR conditions used were 95 °C denaturation for 30 s, 55° C annealing for 1 min, and 72 °C extension for 2 min with a final extension of 5 min for a total of 45 rounds of amplification. The relative frequency of bacteria was calculated using 42,224 sequences as the cutting line, due to these was the lowest number of sequences obtained in one of samples in duplicate.

3.2.4. Soil chemical properties

The soil chemical properties was analysed in macroagregate and microaggregate. The macroaggregate samples were sieved through greed with mesh of 2 mm, afterwards the samples were dry before to analysis. The pH was evaluated in water and in $CaCl_2$. The nutrients calcium (Ca^{2+}), magnesium (Mg^{2+}) and aluminium (Al^{3+}) was extracted with a solution of KCl 1 mol L^{-1}. The nutrients Ca^{2+} and Mg^{2+} were determined by atomic absorption

spectrophotometer, and the Al^{3+} was determinate by titration in NaOH. The potassium (K^+) and phosphorus (P) was extracted with mehlich solution, the determination of K^+ and P were respectively by flame emission spectrometer and spectrophotometry. The total soil nitrogen (TN) was determinate by dry combustion analyser elementary Vario El III – Elementar®.

3.2.5. Statistical analyses

The statistical analyses was performed with factorial treatments x aggregates. The data normality for chemical properties and textural was verified by Shapiro-Wilk test, afterwards, it was realized the variance analysis (ANOVA) to verify the interactions between treatments and aggregates. The significant results ($p < 0.05$) were submitted on test Tukey at 5% of probability using the R-Studio®. It was realized the redundancy analysis (RDA) using the Canoco for Windows 4.5, with the significance of RDA tested by Monte Carlo test. The RDA was performed using the bacterial frequency as variable response and chemical properties as explicative variable. The alpha diversity of soil bacteria is the diversity into the each soil sample, which was estimated by Chao1 richness estimators calculated by Qiime (Quantitative Insights Into Microbial Ecology), which is an open resource for bioinformatics.

3.3. RESULTS

3.3.1. General analyses of bacterial sequences

A total of 2,955,998 sequences were performed, identifying 6,359 operate taxonomic units (OTUs). These OTUs were classified in 30 bacteria phyla. The rarefactions curves were close to the saturation, suggesting the microbial communities in crop systems can be well characterized in this study.

3.3.2. Soil chemical properties

The pH and acidity components of the soil (Table 1) were not affected by crop systems. However, the content of the bases of soil (Ca, Mg, K) and the sum of bases (SB) were significantly higher in the rotation system (Table 1). Despite the increase of bases in the rotation system, the cation exchange capacity (CEC), base saturation (V %), aluminium saturation (m %) did not differ across the crop systems.

3.3.3. Redundancy analysis (RDA) of soil bacterial sequences

The redundancy analysis of bacterial sequences (Figure 2) reveals that the crop systems separated some phyla of soil bacteria due the differences in soil chemical properties (p = 0,10). The axis 1 explains 32.3 % of bacterial distributions in the soil, governed mainly by soil P and K contents. The axis 2 explains 24.4 % of all data variability and was oriented by P, Ca and Mg content in the soil. The crop systems and aggregate classes were separate along of axis 2, basically due to soil P, Ca Mg contents and CEC, providing higher frequencies of Verrucomicrobia and Gemmatimonadetes while the pH affected positively the Firmicutes, Proteobacteria, Elusimicorbia.

Table 1. Soil chemical properties under crop succession and crop rotation in the micro and macroaggregates classes in no tillage system in Ponta Grossa, Paraná, Brazil.

	p value crop system	p value aggregates	SMAC	SMIC	RMAC	RMIC
pH $CaCl_2$ ($cmol_c\ dm^{-3}$)	0.62	0.55	4.45	4.37	4.44	4.45
H+Al ($cmol_c\ dm^{-3}$)	0.32	0.22	7.40	8.10	7.50	7.65
Al ($cmol_c\ dm^{-3}$)	0.19	0.90	0.25	0.25	0.19	0.19
K ($cmol_c\ dm^{-3}$)	0.002	0.33	0.45	0.57	0.26	0.29
Ca ($cmol_c\ dm^{-3}$)	0.01	0.06	2.43	2.75	2.80	3.10
Mg ($cmol_c\ dm^{-3}$)	0.004	0.01	0.75	0.90	1.03	1.23
C ($g\ dm^{-3}$)	0.50	0.34	21.93	27.85	21.25	23.03
SB ($cmol_c\ dm^{-3}$)	0.05	0.003	3.63	4.22	4.09	4.61
CEC ($cmol_c\ dm^{-3}$)	0.55	0.008	11.03	12.32	11.59	12.27
P ($mg\ dm^{-3}$)	0.19	0.98	25.75	30.88	23.06	27.98
m %	0.12	0,54	6.55	5.68	4.45	4.10
V %	0.14	0.18	33.00	34.24	35.30	37.63
TN	0.74	0.18	0.20	0.19	0.20	0.18

SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation; CEC: cation exchange capacity; SB: sum of bases; C: organic carbon; TN: Total nitrogen.

3.3.4. Community structure of soil bacteria

The community structure of soil bacteria in the crop systems was dominated by 31 % of *Proteobacteria* phylum, followed for *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes* with the relative frequency of 22 %, 10 %, 7 %, respectively (Table 2). These phyla more frequents were not differ across the crop systems. Exception to *Spirochaetes*, which presented an average of 0.12 % in succession system against 0.07 % in rotation system (p = 0.06). The

only genus present of *Spirochaetes* in this study was *Spirochaetaceae*. Interestingly, the *Gemmatimonadetes* was different between aggregate classes, regardless crop system, presenting an average of 7.61 % of relative frequency in microaggregate.

The *Proteobacteria* community was dominated by 14 % of *Alphaproteobacteria*, followed by 6 % of *Deltaproteobacteria*, 6 % of *Betaproteobacteria* and 3 % of *Gammaproteobacteria* without difference between the crop system (Table 3). The dominant order of *Proteobacteria* was *Rhizobiales* with an average frequency of 7 %. The dominant orders to the *Acidobacteria* were *Acidobacteria-6*, *Acidobacteriia* and *DA052* with frequencies respectively of 6 %, 5 % and 4 % (Table 4). The less frequent orders of the *Acidobacteria* were different across the crop systems. The *Sva0725* order presented 0.14 % of frequency in crop rotation, significantly higher that 0.08 % in crop succession (p = 0.09). The same way, the frequency of the *Chloracidobacteria* was significantly higher in crop rotation (p = 0.03). Whilst the frequency of the *Holophagae* was significantly higher in crop succession (p = 0.02).

	Axis 1	Axis 2
pH $CaCl_2$	0.19	-0.30
Al	-0.14	0.05
P	-0.30	0.38
K	-0.31	-0.17
Ca	0.14	0.30
Mg	0.25	0.40
C	-0.23	-0.00
CEC	-0.29	0.37

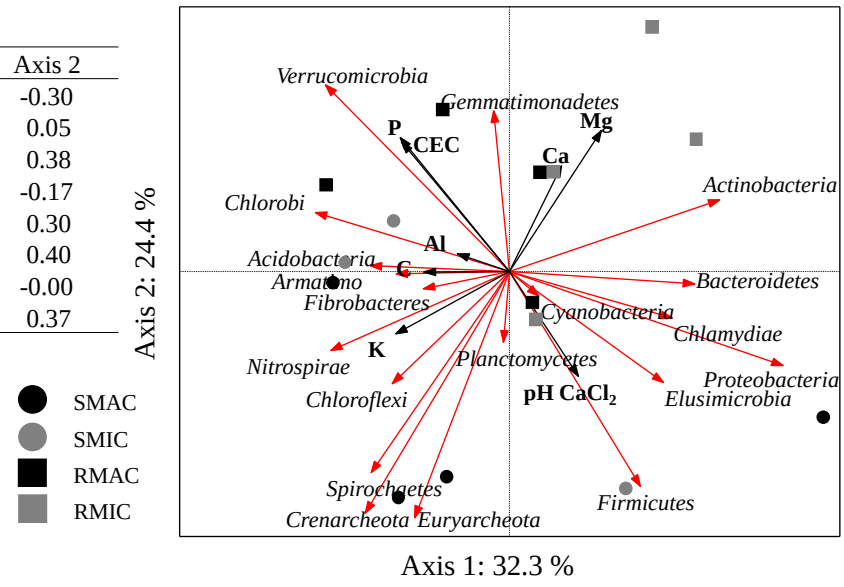

Figure 1. Redundancy analysis (RDA), using soil bacterial phyla as dependent variables and soil chemical properties as environmental variables. SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation; Black arrow: environmental variable; Red arrow: bacterial phyla (Monte Carlo test: p = 0,10)

The *Actinobacteria* was the third phylum more abundant, regardless of the crop systems. The main order of this phylum was *Thermoleophilia*, with frequency average of 4 % (Table 5). The phylum *Gemmatimonadetes* presented only two orders, the *Gemm 1* and *Gemmatimonadetes*. The relative frequency of *Gemm 1* was 6 % in the microaggregate class, higher significantly than the macroaggregate class which had 4.9 % of the relative frequency ($p = 0.06$).

3.3.5. Alpha diversity of soil bacteria

The alpha diversity of soil bacteria was measured by Chao 1 index was not different between crop systems ($p = 0.97$) and aggregates classes ($p = 0.29$). The Chao 1 index was 11220.08, 13939.52, 12886.36 and 12359.62 respectively to the SMAC, SMIC, RMAC and RMIC. There was interaction between crop systems and aggregates classes (treatment:aggregate, $p = 0.04$). The diversity of soil bacteria in microaggregate of the crop succession was higher than macroaggregate in the rotation system.

Table 2. Frequency of bacterial phyla under crop succession and crop rotation in microaggregates and macroaggregates in no tillage system in Ponta Grossa, Paraná, Brazil.

Phyla	SMAC	SMIC	RMAC	RMIC	Phyla	SMAC	SMIC	RMAC	RMIC
	%					%			
Euryarchaeota	0.33	0.35	0.14	0.11	*GN04*	0.00	0.03	0.00	0.00
AD3	2.26	1.80	1.84	1.91	*Gemmat.*	6.25	7.47	6.84	7.73
Acidobacteria	22.64	22.55	22.56	22.85	*Nitrospirae*	2.89	2.63	2.89	2.83
Actinobacteria	9.63	10.05	9.89	10.90	*OD1*	0.10	0.10	0.16	0.14
Armatimonad.	0.54	0.55	0.54	0.51	*OP11*	0.00	0.02	0.00	0.00
Bacteroidetes	1.73	1.72	1.99	1.76	*OP3*	0.00	0.02	0.03	0.01
Chlamydiae	0.23	0.23	0.20	0.21	*Planctom.*	3.14	3.58	3.38	3.09
Chlorobi	0.15	0.17	0.14	0.15	*Proteobac.*	31.91	30.53	31.33	31.36
Chloroflexi	5.81	5.72	5.49	5.58	*Spiroch.**	0.13	0.12	0.09	0.05
Cyanobacteria	0.11	0.10	1.05	0.10	*TM6*	0.16	0.18	0.15	0.14
Elusimicrobia	0.44	0.47	0.44	0.41	*TM7*	0.03	0.03	0.04	0.04
FCPU426	0.01	0.02	0.06	0.04	*Verrucom.*	4.68	4.68	4.99	4.80
Fibrobacteres	0.04	0.00	0.03	0.00	*WPS-2*	0.10	0.05	0.11	0.10
Firmicutes	0.50	0.42	0.35	0.39	*WS3*	3.79	4.32	3.49	3.10
GAL15	0.34	0.15	0.18	0.18					

SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation; *Armatimonad.*: *Armatimonadets*; *Gemmat.*: *Gemmatimonadetes*; *Planctom.*:

Planctomycetes; *Proteobac.*: *Proteobacteria*; *Spiroch.: Spirochaetes; Verrumcom.*: *Verrucomicrobia*; * ($p < 0,05$).

Table 3. Frequency of *Proteobacteria* order under crop succession and crop rotation in microaggregates and macroaggregates in no tillage system in Ponta Grossa, Paraná, Brazil.

Order of *Proteobacteria*	p value crop system	p value aggregate	SMAC	SMIC	RMAC	RMIC
			%			
Alphaproteobacteria	0.95	0.49	14.86	14.13	14.21	14.06
Betaproteobacteria	0.16	0.58	6.5	5.82	6.79	6.93
Deltaproteobacteria	0.51	0.76	7.1	7.06	6.68	6.95
Gammaproteobacteria	0.41	0.38	3.4	2.93	3.61	3.38

SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation.

Table 4. . Frequency of *Acidobacteria* order under crop succession and crop rotation in microaggregates and macroaggregates in no tillage system in Ponta Grossa, Paraná, Brazil.

Order of *Acidobacteria*	p value crop system	p value aggregate	SMAC	SMIC	RMAC	RMIC
			%			
Acidobacteria-5	0.91	0.17	0.51	0.50	0.58	0.43
Acidobacteria-6	0.75	0.43	5.53	7.14	6.79	6.34
Acidobacteriia	1.00	0.83	5.48	4.88	5.08	5.29
BPC102	0.23	1.00	0.00	0.00	0.01	0.00
DA052	0.29	0.64	5.10	4.14	3.70	4.00
EC1113	0.45	1.00	0.03	0.00	0.00	0.01
Holophagae	0.02	0.54	0.16	0.13	0.05	0.04
PAUC37f	0.78	0.55	0.36	0.29	0.33	0.26
RB25	1.00	0.57	0.06	0.10	0.11	0.05
S035	0.26	0.70	0.00	0.01	0.03	0.03
Solibacteres	0.96	0.49	2.80	2.71	2.56	2.98
Sva0725	0.09	0.18	0.05	0.11	0.13	0.15
TM1	0.14	0.28	0.15	0.09	0.08	0.08
Chloracidobacteria	0.03	0.33	1.20	1.62	1.85	1.76
iii1-8	0.01	0.77	0.64	0.64	0.75	0.78

SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation.

Table 5. Frequency of order of soil *Actinobacteria* in succession and rotation treatment, in macroaggregate and in microaggregate of soil under no tillage.

Order of *Actinobacteria*	p value crop system	p value aggregate	SMAC	SMIC	RMAC	RMIC
			%			
Acidimicrobiia	0.61	0.73	1.64	1.60	1.61	1.73
Actinobacteria	0.32	0.59	3.21	3.50	3.65	3.98
MB-A2-108	0.58	0.42	0.83	0.68	0.81	0.74
Thermoleophilia	0.15	0.18	3.50	3.80	3.83	4.44

SMAC: macroggregate in the crop succession system; SMIC: microaggregate in the crop succession; RMAC: macroaggregate in the crop rotation; RMIC: microaggregate in the crop rotation.

3.4. DISCUSSION

The RDA analysis revealed that the crop rotation was associated with increase of *Actinobacteria*, *Gemmatimonadetes* and *Verrucomicrobia*. Despite of some exceptions, the majority of *Actinobacteria*, *Gemmatimonadetes* and *Verrucomicrobia* genus are oligotrophic bacteria (Bergmann et al., 2011; Zeng at al., 2015; Shivlata and Satyanarayana, 2015). It means that this bacteria are more efficient in consume the soil carbon, releasing less carbon to atmosphere and with slower development than copiotrophic bacteria (Fierer et al., 2007). Thus, the crop rotation is favouring the conservative process into the soil, which the crop succession is not able to execute. The association between *Verrucomicrobia* and crop rotation is important for ecological process into the soil, because the genera of the *Verrucomicrobia* present in this work were *Methylacidiphilae*, which use the monoxygenase enzymes to proceed the methanotrophy and survive in environment with low carbon available (Dunfield et al., 2007), important for decrease the methane gas production.

It is important to salient that the crop rotation in no till is primordial for higher soil stability due to the increase of community of microorganisms with capacity of decrease CO_2 emission (Sun et al., 2016) and to conserve the soil carbon. Thereby, the crop rotation can be used for improve the conservative process in no till by preservation of oligotrophic niches. The *Verrucomicrobia* is an optional or compulsory anaerobic phylum with reduced growth in oxygenic environment (Chin et al., 2011). The presence of *Verrucomicrobia* phylum became the crop rotation the system able in reduce the greenhouse gas emission, because the oligotrophic bacteria in soil is related to decrease the carbon mineralization, while the

copiotrophic bacteria are less efficient in carbon utilization and release more CO_2 (Fierer et al., 2007).

The alpha diversity in the macroaggregate in the crop rotation was higher than in the microaggregate, differently of the crop succession that the alpha diversity was higher in the microaggregate. Probably, the difference between morphological attributes of the crops used in rotation and succession systems changed the distribution of bacterial diversity. The roots are responsible by distribution of the biological activity in the soil aggregates (Helgason et al., 2010). Probably the fasciculated roots present in maize and oat plants in rotation treatments lead the diversity of bacteria to macroaggregate.

The bacterial community was dominated by *Proteobacteria, Acidobacteria*, *Actinobacteria* and *Gemmatimonadetes*. Several studies reported the *Proteobacteria*, *Acidobacteria, Actinobacteria* and *Gemmatimonadetes* as the phyla more dominants in soil (Figuerola et al., 2015; Chodak et al., 2015; Noyce et al., 2016). The higher presence of *Proteobacteria* is related with the high metabolic diversity of this phylum (Chodak et al., 2015). The higher metabolic diversity lead to *Proteobacteria* phylum inhabits niches with different traits, explaining the absence of significant difference between the crop systems in *Proteobacteria* community. The both crop system were dominated by the order *Rhizobialis* of the *Proteobacteria*, which encompasses numerous symbiotic nitrogen-fixing bacteria as *Bradyrhizobium* and *Mesorhizobium* (Souza et al., 2013). The absence of the difference in the relative frequency of the *Rhizobialis* order between the crop systems was due to the inoculation of *Bradyrhizobium* in soybean as previous crop in both systems.

The order less frequent of the *Acidobacteria* phylum was different across the crop systems. The *Acidobacteria* is an oligotrophic bacteria (Fierer et al., 2007) able to decompose complex plant polymers, and promote the CO oxidation as strategies to optimize the life in a low-carbon environment (Ward et al., 2009). The genome of *Acidobacteria* suggests use of a variability of sources of carbon (Ward et al., 2009). Although the *Acidobacteria* is an oligotrophic bacteria phylum, the order *Holophagae* is an *Acidobacteria* that was found in several rhizosphere soil cultivated with potato and allium in Netherland (Rocha et al., 2010). Thus the *Holophagae* can live in copiotrophic niches. This explains the higher frequency of *Holophagae* in crop succession, due to the higher labile carbon in soil cultivated with soybean (Filho et al., 2004). Differently, the order *Chloracidobacteria* was more frequent in crop rotation. The *Chloracidobacteria* is a microaerophilic, moderately thermophilic, anoxygenic, photoheterotrophic eubacterium (Tank and Bryant, 2015). All physiologic traits of

Chloracidobacteria reveal the capacity of occupy oligotrophic niches in the soil, characterizing the rotation as a conservative management. These results demonstrate how much the crop rotation is important for agriculture sustainability, because the *Chloracidobacteria* have a diversity of metabolism able in conserver the soil carbon, characterizing the crop rotation as a conservative system in compare with crop succession.

The higher frequency of *Gemmatimonadetes* in microaggregate class is related to preference of this phylum for environments with low oxygen like in inner microaggregates (Mummey et al., 2004; DeBruyn et al., 2011; Zeng et al., 2016). The environments with higher oxygen reduce the ability of development of *Gemmatiminadetes* colonies (Zeng at al., 2016). The association in RDA between the *Gemmatimonadetes* and soil Ca, Mg contents is due to the fact that the pH for optimum growth of *Gemmatiminadetes* is about 6.0 to 9.0 (Zeng et al., 2016), being an optimum pH for availability of Ca and Mg in the soil.

In the majority, the phyla of *Actinobacteria* is a gram-positive bacteria that can execute several functions like plant growth promoter (Le et al., 2016), nitrogen fixation, antibiotic production (Shivlata and Satyanarayana, 2015) and improve soil aggregation (Kennedy, 1999). The main order of *Actinobacteria* in this work was the *Thermoleophilia*, which are strictly aerobic and obligate chemiorganotrophic in the nature, important for degradation pollutants and metabolize lethal organic chemicals (Shivlata and Satyanarayana, 2015). Thus, the presence of this order is probably related to the use of pesticides in both crop systems.

The *Spirochaeta* was the single phylum that was different between crop systems, with higher relative frequency in the crop succession. This phylum is monophyletic group of gram-negative bacteria, and it is present in several guts of termites, dipterans and earthworms (Paster et al., 2000). The few studies about the *Spirochaeta* reveal the nitrogen fixing capacity (Liburn et al., 2001), and it is a D-glucose, fructose, maltose, sucrose, starch, D-mannitol consumer (Angelov et al., 2011). The *Spirochaeta* phylum also is a cellulose and hemicellulose consumer, possessing specific enzymes to degrade those molecules (Angelov et al., 2011). Thus, the higher frequency of this phylum in crop succession is probably related to continues soybean year after year, due to the content of hemicellulose in the soybean tissues is about 50 % (Seibel and Beléia 2008).

3.5. CONCLUSION

The results showed that though the crop rotation did not increase the diversity of soil bacteria in comparison with crop succession. The crop rotation regardless of the aggregates classes selected the oligotrophic bacterium *Chloracidobacteria*, *Verrucomicrobia* and *Gemmatimonadetes*, revealing to be a conservative system. Differently, the crop succession increase copiotrophic bacteria that able in consume the labile carbon. Though the no tillage already is a conservationist system, this work reveals the crop rotation improve the sustainability of crop production in this soil management.

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4. CHAPTER IV. THE EFFECT OF GROWTHING PLANTS AND MULCH OF MAIZE (*Zea mays*) IN THE SOIL PROTOZOA COMMUNITY.

ABSTRACT

The soil protozoa occupy an important position in the soil microfood web. The functions of the soil protists will depend of the microbial community structure. The aim of this work was investigate the effect of two ways of input of the organic carbon in the soil (plant or mulch) on structure of soil protozoa community. For that, an experiment was conducted in a corn production field with two treatments: green plants (P); only mulch (L); and fallow (F), as a control, all of them with four replicates. The soil samples were taken in July and September 2013 at each plot with a soil corer, in three depths: 0 at 10 cm (plough layer); 40 at 50 cm (layer with root no ploughed); 60 at 70 cm (deeper soil free root). The soil protozoa were quantified by the liquid aliquot method. The total number of soil protozoa was affected only by depth, with mean value of 9.883 individuals g^{-1} soil distributed in 11 morphotypes. The treatments, depths and seasons affected the *Glissomonad*, *Spumella* flagellates and Acanthapodial, Eruptive amoebas. All morphotypes of the soil protozoa decreased in depth, it could be related to the soil carbon and nitrogen decrease. Despite these effects, the ecological indexes did not vary between the treatments with mean values of 1.95 for the Shannon index, 0.81 for the Simpson index and 0.83 for the Pielou index in the 0 to 10 cm layer. However, only the Shannon and Pielou indexes varied in depth. The change of soil protozoa community occurred in protozoa considered ‘r’ strategist organisms, as small flagellates, which may suffer higher impacts on their population with changes in the environment, mainly the soil moisture, carbon and nitrogen content. Thus, the small soil protozoa are affecting by mulch and plant presence depending of soil moisture.

Key-words: Soil amoeba. Soil flagellate. Diversity index; Seasonality.

4.1. INTRODUCTION

The soil is inhabited by thousands of organisms that interact with each other and participate actively in biogeochemical cycling. The loss of biodiversity due to conventional agricultural practices (Zalasiewicz et al., 2010) decreases carbon uptake, oxygen production and nutrient cycling (Reiss et al., 2009). In this sense, the soil protozoa present a key role in the soil food web, increasing the organic matter decomposition and nutrient cycling due the proportion of nutrient excretion into the soil (Coûteaux and Darbyshire, 1998; Bonkowski et al., 2000; Adl and Gupta, 2006; Krome et al., 2009) and have strong impacts on higher trophic levels in the soil food web (Crotty et al., 2012).

The effects of soil protists are significant, considering the high abundance of protozoa in soil (Finlay et al., 2000). However, data on the taxonomic composition of soil protozoa has been scarce recently (Domonell et al., 2013), because the studies of soil food web has ignored the predatory role of the soil protozoa, mainly the small flagellates and naked amoeba (Geisen et al., 2014). The result is the uncertainty about the real distributions on the protozoa species in the soil (Coûteaux & Darbyshire, 1998; Bates et al., 2013), considering the diversity of functions that these organisms perform in edaphic environment (Cortés-Pérez et al., 2014).

The accumulation of organic matter and presence of roots in the soil surface is responsible by the concentration of soil protozoa functions in the upper layer (Scharroba et al., 2012; Shalinimol et al., 2009). However, the microbial population can decrease or increase depending of the crop residue in soil surface (Carillo et al., 2016). The decrease of C/N ratio in the litter increased the fungal community, and the litter of clover increased the protozoa population (Carrilo et al., 2016). Thus, the crop residue changes fungi/bacteria ratio in the litter (Wardle et al., 2004), shifting the biological traits in soil (Gupta and Germida, 2015).

Concomitantly with mulching, the roots also affect the soil microbial population. The root exudation plays important roles in shaping rhizosphere microbial communities (Shi et al., 2011). In addition, the roots improve the formation of soil macroaggregate (Helgason et al., 2010), which favours the connectivity between soil porous and facilitate the mobility of soil protozoa in the space porous of soil full of water (Hattori et al., 1994). Thus, the presence of roots is essential to soil protozoa activity. The growth of roots in deeper layer of soil creates good conditions for microbial development (Scharroba et al., 2012), being important to niche

differentiation, increasing the diversity of protozoa due to the decrease of predators (Scharroba et al., 2012).

Thereby, the structure of soil food web in soil surface and in deeper layer is linked with presence of roots and mulch in soil. The labile and recalcitrant resources feed bacteria and fungi respectively (Ruess and Ferris, 2004). Thus, the difference in trait of organic matter available in soil food web will change the energetic flow through soil organisms, provoking a cascade effect on soil protozoa community. However, the cascade effect will depend of water regime, because the protozoa are aquatic organisms (Geisen et al., 2014), and the water regime in soil is of a seasonal nature. The aim of this research is investigate the effect of two ways of input of the organic carbon in the soil (plant and mulch) on the structure of soil protozoa community. The hypothesis that the presence of plant will change the protozoa community in soil surface and in deeper layer, due the difference in organic composition between root exudation and plant mulch.

4.2. MATERIALS AND METHODS

4.2.1. Site description and Agriculture management

The experiment was conducted in a crop land with only maize since 2009, in Göttingen city, Germany (51°33'N, 9°53'O). The dominant soil type is Cambisols and Luvisols, with pH of 6.0. The mean annual temperature in the area is 7.9 °C, with the maximum temperature of 21.9 °C in July and the minimum of - 0.9 °C in December. The mean annual precipitation is 54 mm, with the maximum precipitation of 79 mm in June and the minimum of 38 mm in March.

The crop management in April 2012 was performed with application of 4 L ha^{-1} of glyphosate. The filed was tilled with chisel plough to a depth of 12 cm, and the plant used for experiment was maize at density of 11.5 grains m^{-2}. The N fertilizer was performed with 76 kg N ha^{-1} of ammonium nitrate and urea solution, 20 kg N ha^{-1} of ammonium sulphate, and 9 kg N ha^{-1}/111 kg P ha^{-1} of diammoniun phosphate. In April 2013, it was used the same fertilization of 2012, and the maize was sown at density of 8.5 grain m^{-2}.

4.2.2. Experimental design and soil sampling

The experiment was conducted in a factorial design with there treatments, three depths and two seasons, resulting in three factors. The treatments were: plants (P); only mulch (L); and fallow (F) as control, in three depths 0 at 10 cm (plough layer); 40 at 50 cm (layer

rooted zone); 60 at 70 cm (deep root free soil) in all treatments, and two seasons: July and September. The plants treatment presents growing plants of maize, while the mulch treatment received 0.8 kg dry weight m^{-2} of maize shoot (equivalent to 0.35 kg C m^{-2}). The F did not receive plants of maize, however there was presence of spontaneous plants in F treataments.

The treatments had four replicates in 12 plots of five square meters disposed in two rows spaced six meters among them. The soil used for this study was collected with a soil corer with 2.5 of diameter, in July and September to verify the seasonality of data. The samples were stored at 5 °C until the soil protozoa extraction.

4.2.3. Soil Protozoa extraction

The soil protozoa were quantified with the liquid aliquot method (LAM) according to Butler and Rogerson (1995) for all the depths and two seasons. For this, 1 g of soil was shaken horizontally with 225 mL of wheat grass medium (WG) to dilute and separate the protozoa from soil particles. After shaken, 10 mL of soil suspension was add in 30 mL of WG medium to stimulate the bacterial growth, and then 20 µL aliquots were filled the 96 well plate for visualization at microscopy.

To account the number of protozoa was necessary to check the soil water content (Figure 1), weighing 1 g of wet soil and after dried in an oven at 60 °C during 24 hours and weighed again to verify the dry weight. The water content was calculated using the calculus:

$U = (Mw - Md)/Md$

Where Mw is the mass wet soil, Md is the mass dry soil and U is the water content.

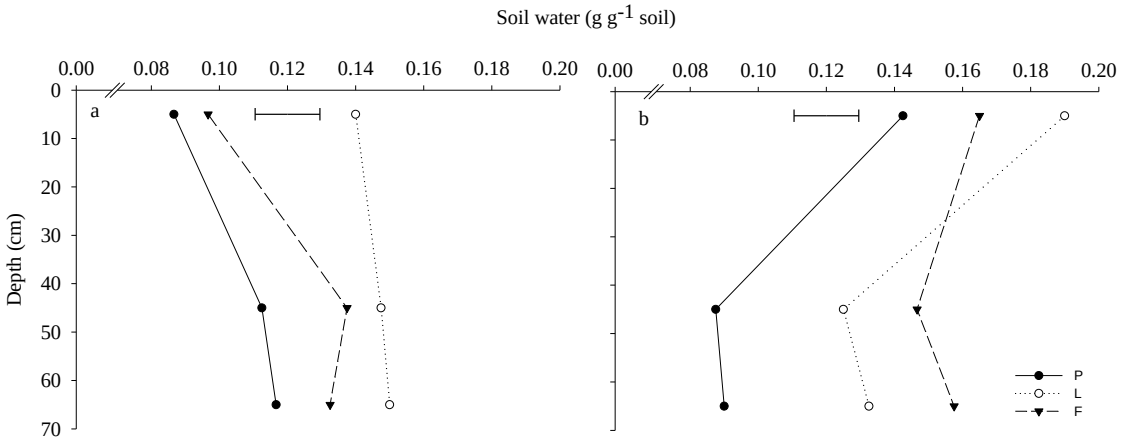

Figure 1. The moisture of samples in the three depths in Plant (P), Litter (L) and Follow (F) treatment in July (a) and September (b). Horizontal bars represent the higher significance difference according to Tukey test with 5% of significance.

4.2.4. Protozoa identification

The culture medium was stored at 7 °C until they were investigated. The plates were checked two times. The first check was 7 days after the extraction and the second check was 21 days after the extraction. This procedure was due the change in the active protozoa community in each well with the time. The optical microscope with 40x amplification was used for check. The soil protozoa were identified by morphotype, according with Smirnov and Brown (2004), Smirnov et al. (2011) and Jeuck and Arndt (2013).

To calculate the number of soil protozoa we used the equation:

Number soil protozoa = I/U (225000/[144x5])

Where I is the number of wells in which there is presence of protozoa; U is dry soil content in g.

The number of each morphotype of soil protozoa was used to calculate the ecological index at each depth. The ecological indexes were: Shannon Index ($H' = -\Sigma(p_i \log p_i)$), Simpson Index ($e = H/\log S$) and Pielou Index ($Is = \Sigma\ p_i^2$).

Where $p_i = N_i / N$ and n_i = number of individuals of the species i, N = the total number of individuals in the sample and S is the number of species.

4.2.5. Soil carbon (C) and soil Nitrogen (N)

The total soil carbon (C) and total soil nitrogen (N) were determinate in all soil samples by dry combustion analyser elementary Vario El III – Elementar® (Table 1).

Table 1. Soil carbon (C) and soil nitrogen (N) in plant (P), litter (L) and fallow (F) treatment in the three depths 0 to 10, 40 to 50 and 60 to 70 cm.

Carbon input	Depth (cm)	July			September		
		C (µg g^{-1} soil)	N (µg g^{-1} soil)	C/N	C (µg g^{-1} soil)	N (µg g^{-1} soil)	C/N
Plant	0-10	537.15	59.53	9.05	575.68	59.78	9.62
	40-50	232.78	28.28	8.22	252.16	29.26	8.58
	60-70	136.27	17.51	7.79	139.23	17.49	7.93
Litter	0-10	587.65	67.31	8.71	563.26	58.94	9.55
	40-50	269.83	34.06	7.87	272.52	33.42	8.11
	60-70	154.91	19.90	7.78	159.86	20.15	7.87
Fallow	0-10	552.60	63.12	8.74	588.79	62.00	9.49
	40-50	221.76	28.03	7.90	300.28	34.08	8.81
	60-70	132.36	17.83	7.39	149.04	17.88	11.54

4.2.6. Statistical analysis

The data normality was verified by Shapiro-Wilk test. The non-normal data was transformed in Log_{10}. The variance analysis (ANOVA) was done using the Quasi-poisson distribution. The significant results ($p < 0.05$) were submitted on test Tukey at 5% of probability using the R-Studio®. It was realized the principal component analysis using the Canoco for Windows 4.5, using the soil protozoa morphotypes as variable response and water content as environmental variable.

4.3. RESULTS

4.3.1. Number of soil protozoa

The number of soil protozoa between periods evaluated was not significantly different ($p > 0.05$). The number of protozoa in the soil also did not vary between treatments ($p > 0.05$), with mean value 9,883 individuals g^{-1} soil dry weight (Figure 2) distributed in 11 morphotypes (Table 2) (mean of two seasons). However, the number of individuals in the deeper layers decreased significantly in all treatments, with no treatment:depth interaction ($p > 0.05$) (Figure 2). Although the richness of morphotypes also decreased significantly along the depth, this decrease was different between the seasons (interaction season:depth) ($p < 0.05$). The 40-50 cm layer and 60-70 cm layer had a higher richness in Sptember relative to July, with no difference between treatments. The total of soil protozoa was correlated positively with soil nitrogen ($p < 0.001$) and soil carbon ($p < 0.001$). The ratio C/N also had a strong positive effect in total of soil protozoa ($p < 0.001$).

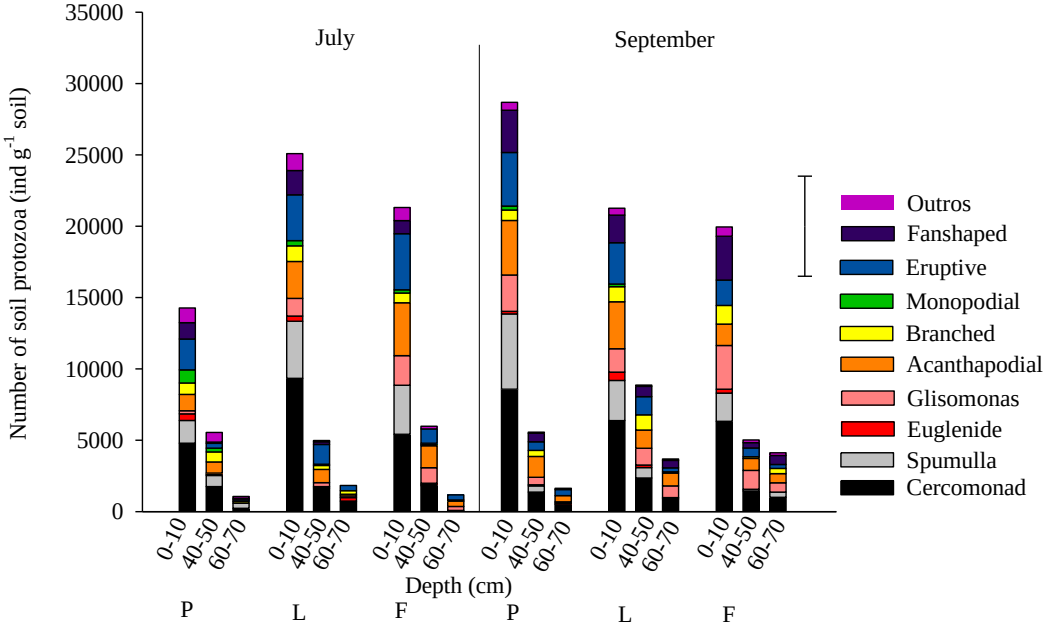

Figure 2. Number of individuals of soil protozoa in plant (P), litter (L) and Fallow (F) treatments in July and September, in layer of 0 to 10, 40 to 50 and 60 to 70 cm. Vertical bar represents the Honest significant difference (HSD) according to the Tukey test at 5 % probability.

4.3.2. The number of flagellates

The most frequent protozoan flagellate at all treatments, depths and two seasons was Cercomonads, followed by *Spumella* sp. and Glissomonad (Figure 2). The Cercomonads and *Euglenideae* varied only by depth ($p < 0.001$), while the *Spumella* sp. and Glissomonad varied between treatments, between seasons and between depth (Figure 5). The *Spumella* sp. presented the interaction treatments:season:depth ($p < 0.001$), and Glissomonad presented the interaction treatment:season ($p < 0.01$).

The Litter treatment affected significantly the number of *Spumella* sp. in July, with higher value in the Litter treatment and Fallow in the layer 0 to 10 cm (Figure 3), while the higher value of *Spumella* sp. in layer of 40 to 50 cm was in the Plant treatment. The L treatment and Fallow did not differ in all deeper layers. The layer 60 to 70 cm was not affected by treatments. In September, the number of *Spumella* sp. decreased significantly in order Plant > Fallow > Litter in layer of 0 to 10 cm. However, the order was opposite in layer of 40 to 50 cm, with a higher number of *Spumella* sp. in the Litter treatment.

In July, the Glissomonad presented the highest value in upper layer of 0 to 10 cm in the Fallow ($p < 0.01$) and the lowest value was in the Plant treatment (Figure 5c). And the layer of 40 to 50 cm presented the higher number of Glissomonad in the Fallow in July. Both treatments Litter and Plant did not differ significantly in the layer 60 to 70 cm in this season. In September, Glissomonad presented the highest number in layer of 0 to 10 in the Fallow and the Plant treatment, and the lowest number was in the Litter treatment (Figure 3d). And the number of Glissomonad did not differ significantly in the layer of 40 to 50 and 60 to 70 cm (Figure 3).

Table 2. Richness of soil protozoa (number of morphotypes) in Plant (P), Litter (L) and Fallow (F) treatments and in the depths 0 to 10, 40 to 50 and 60 to 70 cm.

Depth (cm)	July			September		
	Plant	Litter	Fallow	Plant	Litter	Fallow
0-10	11aA	11aA	10aA	10aA	10aA	9Ab
40-50	6bA	5bA	5bA	6bA	6bB	6bA
60-70	2cA	3cA	2cA	3cB	4cB	4cB

Lower letters compare depths in the same treatments and capital letters compare treatments in different seasons.

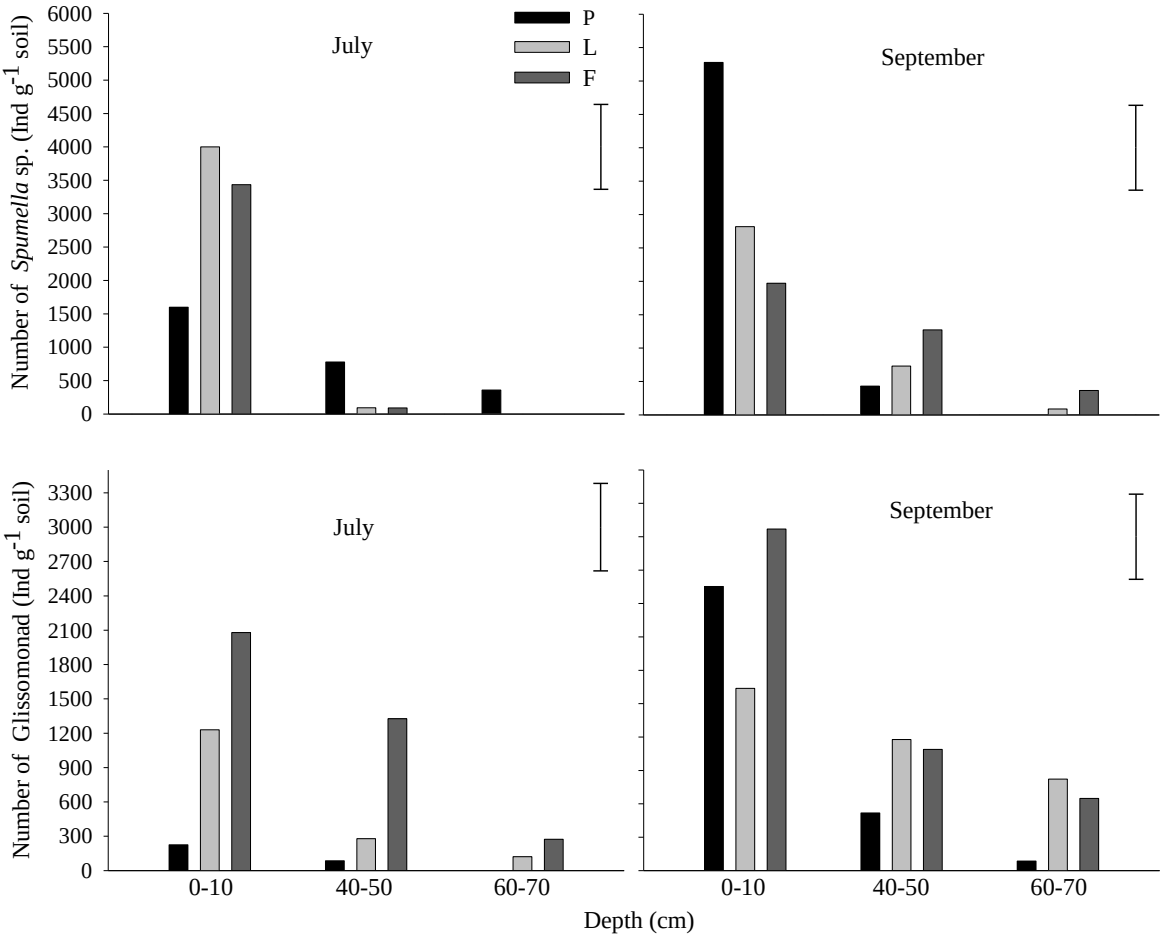

Figure 3. Number of *Spumella* in July and September, number of Glissomonad in July and September in the Plant (P), Litter (L) and Fallow (F) treatments in 0 to 10, 40 to 50 and 60 to 70 cm. Vertical bars represents the Honest significant difference (HSD) according to the Tukey test at 5 % probability.

4.3.3. The number of amoebas

The Eruptive ($p < 0.05$) and Acanthapodial morphotype ($p < 0.01$) were affected by treatments and by seasons ($p < 0.01$). In addition, all morphotypes of amoebae decreased significantly in depth. However, The both morphotypes presented interaction between treatments and seasons (interaction treatments:seasons) ($p < 0.01$). The Eruptive morphotype presented the lowest number in the Plant treatment in July, while the results was opposite in September. In both seasons the layer of 40 to 50 did not suffer the treatment effect, but in general, September had the higher number of Eruptive. The Fallow showed significantly higher value for Acanthapodial amoebae in July in the layer of 0 to 10 cm (Figure 6), while the same treatment presented in September the lowest number of Acanthapodial amoeba.

4.3.4. Ecological index

The ecological index did not vary between treatments ($p > 0.05$) or between seasons ($p>0.05$), despite the treatment and season effects in some morphotypes of soil protozoa. Thus, the results exposed in the table 3 are the averages between the July and September. The three ecological indexes only vary in depth (table 3). The Shannon index and Simpson index decreased significantly from layer of 0 to 10 cm to 60 to 70 cm. The Pielou index did not present a depth effect in the L treatment and F, while in the P treatment the layer of 60 to 70 cm presented the highest Pielou index.

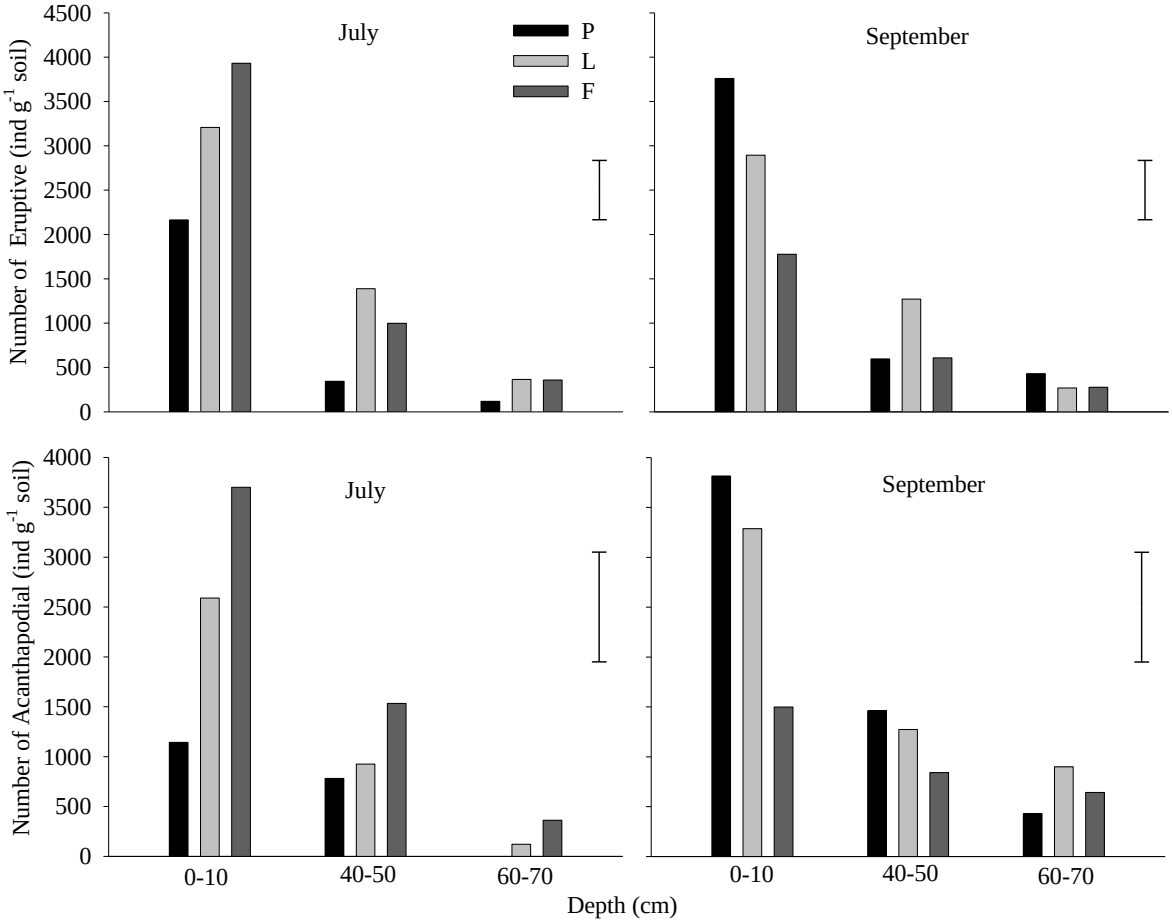

Figure 6. Number of Acanthapodial in July and September, number of Eruptive morphotypes in July and September in Plant (P), Litter (L) and Fallow (F) treatments in layer of 0 to 10, 40 to 50 and 60 to 70 cm. Vertical bars represents the least significant difference (LSD) according to the Tukey test at 5 % probability.

Table 3. Ecological index of soil protozoa in Plant (P), Litter (L) and Fallow (F) treatments in three depths, 0 to 10, 40 to 50 and 60 to 70 cm. Average of the two sampling times (p> 0.05).

Ecological index	Depth (cm)	P	L	F
Shannom index	0-10	2.00 a	1.94 a	1.93 a
	40-50	1.55 b	1.48 b	1.51 b
	60-70	0.89 c	1.10 c	0.93 c
Simpson index	0-10	0.82 a	0.81 a	0.82 a
	40-50	0.73 a	0.71b	0.74 a
	60-70	0.56 b	0.59 c	0.53 b
Pielou index	0-10	0.85 a	0.82 a	0.85 a
	40-50	0.90 a	0.88 a	0.89 a
	60-70	0.96 b	0.81 a	0.81 a

Letters compare depths into the treatment.

4.3.5. Principal Component Analysis

The principal component analysis of July did not reveal the treatment effect, however there was a clear division between the upper and deeper layer. This division was oriented through PC 1, explaining 89.0 % of all data variability, mainly by soil carbon (C), soil nitrogen (N) and ratio C/N of soil (Figure 7a). The PC 2 explained only 3.0 %, oriented by water content. Thus, it is possible to observe the relationship between the upper layer of P treatment and the flagellate *Euglenidae*, *Spumella* and the amoebas Branched, Monopodial, Eruptive and Polytactic while the F treatment was related with flagellates Glissomonad, Cercomonad and the Acanthapodial amoeba. The deeper layers of F treatment were associated with water and Glissomonad morphotype. The Shannon diversity index was associated with soil carbon and nitrogen in this season

The September data also showed division between 0 to 10 cm layer and the deeper layers (Figure 7b), with principal component 1 explaining 76.8 % of variability and principal component 2 explaining only 8.6 %. There was not a grouping in PCA to September season. Differently of July, the water content was more associated with upper layer in P and F treatment, favouring the total flagellate, Glissomonad, *Spumella* sp. and total flagellate. In this season, the C/N ratio favoured the Shannon and Pielou indexes.

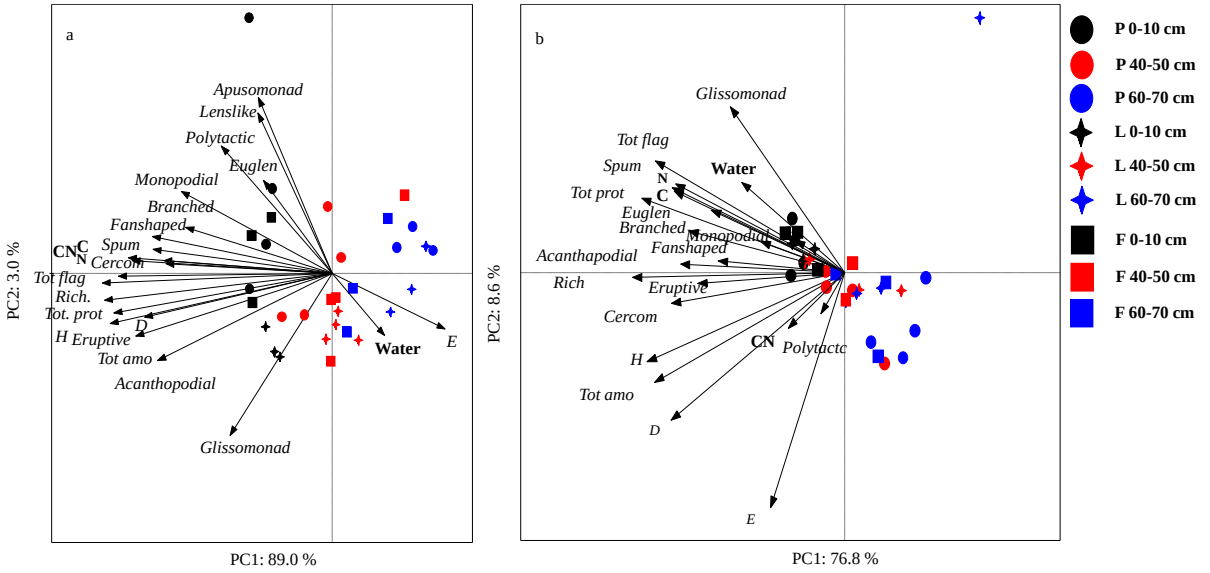

Figure 7. Principal Component Analysis of Plant (P), Litter (L) and Fallow (F) treatments, in the 0 to 10 cm, 40 to 50 cm and 60 to 70 cm of July (a) and September (b). H': Shannon index; D: Simpson Index; E: Equitaility index; C: carbon; N: nitrogen; CN: ratio C/N.; Rich: Richness; Tot prot: Total protozoa; Tot amo: total amoeba; Tot flag: Total Flagellate; Euglen: Euglenidae; Spum: Spumella; Cercom: Cercomonad.

4.4. DISCUSSION

The correlation between soil C and N with total protozoa confirmed previous results, which affirm that the carbon in soil surface favour the microbial development (Zhang et al., 2012; Cezar et al., 2015; Sun et al., 2016). The higher microbial development ensures the food resource for the soil protists in soil surface (Anderson, 1994; Geisen et al., 2016). The soil protozoa present different preferences for food resources, they can be described as fungivores, algivores, protozoan-eaters, yeast-eaters, cyanobacteria-eaters, omnivores, osmothrophs, and species whose feeding preferences are not determined (Cortés-Pérez et al., 2014). This also explains the depth effect of total soil protozoa, which the soil C decreases and caused decline in the all protozoa morphotypes. Some works also demonstrated the decreasing of richness of the soil protozoa in deeper soil layers (Ekelund et al., 2001; Rodriguez-Zaragoza et al., 2005; Scharroba et al., 2012). The PCA confirmed the relationship between soil C, soil N and total protozoa.

Although the soil C and N has strong correlation with total soil protozoa, the PCA showed that when the soil moisture increases in the soil surface in September, the importance of soil C and N in the number of total protozoa decrease. In this case, the water affected more strongly the soil protozoa population, because the protozoans are aquatic organisms

(Bouwman and Zwart, 1994; Novarino et al., 1997; Geisen et al., 2014). This suggests that in soil surface, the higher soil C in the Litter treatment in July ensure higher water content. On the other hand, the Fallow in September presented more C, ensuring the association with the Fallow, soil moisture and flagellates. The flagellates possess raptorial feeding or interception feeding mode (Boenigk and Arndt, 2002), allowing high mobility and capture of free suspension bacterial cells (England et al., 1993; Boenigk and Arndt, 2000; Rønn et al., 2012) in higher soil moisture.

Although there was decrease of soil protozoa through the depth, some protozoas morphotypes dominated the population in 40 to 50 and 60 to 70 layers. Finlay et al. (2001) affirmed that protozoans could be accidentally transported there along with percolating water, influencing the microbial process in subsurface sites. This could have happened mainly with Glissomonad and *Spumella* sp. in the Litter treatment of July, where the Glissomonad was more numerous in surface layer and 40 to 50 layer.

Regarding these flagellates, the higher presence of Cercomonad, *Spumella* sp. and Glissomonad in all treatments indicates that the trophic structure of soil environment was simplified in the crop field. These flagellates are known as heterotrophic bacterivores nanoflagellates (Howe et al., 2011) with faster life cycles and they are very responsive to environment changes (Boenigk and Arndt, 2002). In addition, the abundance of soil protozoa is inversely correlated with organism size (Finlay and Fenchel, 2001), which explain the higher abundance of these small flagellates. Nonetheless, only *Spumella* sp. and Glissomonad was affected by treatments and by seasons. The Cercomonad is generally much larger than Glissomonad and *Spumella* sp., with more metabolic cells and greater intra-clonal variability in morphology and behaviour (Bass et al., 2009). Thus, Cercomonad can adapt to changes faster than other flagellates, which may explain the absence of a treatment effect in Cercomonad.

In general, the increase of soil moisture favoured the Glissomonad and *Spumella* sp. with presence of plant. The Plant treatment increased the soil C in September, this may have been due the higher root activity in soil with higher water availability, increasing the bacterial activity in the rhizosphere and diversifying the food resources, considering that these heterotrophic flagellates are mainly bacterivores (Boenigk and Arndt, 2002; Howe et al., 2011). In addition, the population of small flagellates increase together with bacterial colonies in soil (Rodriguez-Zaragoza et al., 2005), swimming within the soil pores. This facilitates the predation of these protozoas in porous filled with water (Rutherford and Juma, 1992). In July,

the Litter treatment and Fallow presented the highest number of *Spumella* and *Glissomonad* relative to Plant treatment. In this season, the Litter treatment presented higher water, C and N content, which explain these results. Despite the Fallow possess low water content, this treatment presented the lower C/N ratio, which can explain the higher number of *Spumella* and *Glissomonad* relative to Plant treatment in this season. Thus, the seasonality has effect to the soil flagellates in addition to treatment effect.

Similar to flagellates, the presence of plants affected negatively the Acanthapodial and Eruptive amoebas in July. Differently of September, the plants affected positively these amoebas. This result is also linked with higher water content in September in Plant treatment, since the increase of water content in Plant treatment in September may enhance the root activity (Rodriguez Zaragoza et al., 2005), favouring the Acanthapodial and Eruptive amoebas that are important members of the rhizosphere soil (Jentschke et al., 1995; Kreuzer et al., 2006). The *Acanthamoeba castellanii* for example, can survive in environment under microaerophilic conditions like the rhizosphere (Clarke et al., 2013). Furthermore, the flows of water in soil favour the distribution of protozoa between aggregates, becoming the amoebas more activate for predation (Kuikman et al., 1990).

Regarding the Litter treatment, the presence of mulch is recognized as improve the water retention in soil. Thus, the water not varies between seasons in this treatment, ensuring the number constant of Acanthapodial and Eruptive amoebas between seasons in Litter treatment. Moreover, the litter addition (Litter) ensures the survival of fungi in soil surface (Scharroba et al. 2012), that in turn increase the food resource to these amoebas that also are fungivorous (Geisen et al., 2016). In contrast, the lower water availability in Fallow treatment increased the number of these morphotypes in July. The Fallow can maintain a diversity of resources for active microbial community in this season, serving as food resources for growth of these amoebae. The treatments affect indirectly the amoebas by regulate the water content into the soil, mainly the amoebas that respond more strongly the variation of water than flagellates (Geisen et al., 2014).

The treatments did not affect the ecological indexes. This indicates that the micro foodweb structure was preserved across the treatments, which show the importance of this organism in the ecosystem (Clarholm, 2005), allowing the preservation of the functions of soil protozoa in the soil. The lowest value for the ecological indexes in the deeper layer is due to dominance of small flagellates like Cercomonad and Glissomonad, the principal components analysis show a clear association between the Glissomonad and deeper layer in

July. These flagellate are cosmopolitan and highly adaptable, permitting them to exist in extreme environments (Atkins et al., 2000), like the deeper layers of soil.

4.5. CONCLUSION

The adding carbon in the form of crop residues or by root presence did not change the diversity and number of total soil protozoa, but increased the abundance of small flagellates like *Spumella* sp., Glissomonad and small amoebae like Acanthapodial and Eruptive in soil surface on season with higher water content. The results indicate that in crop land, the soil protozoa community is linked with abiotic factors. Thus, the kind of carbon input in the soil interacts with soil protozoa according with abiotic factors as soil moisture. The soil depth has a great negative effect on the soil protozoa diversity and abundance, but maintains some active populations like Glissomonad in the deeper layer.

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5. CHAPTER V. THE *Acanthamoeba castellanii* PROMOTE PLANT GROWTH.

ABSTRACT

The protozoa are an active organism in rhizosphere soil, affecting the microorganism population and root morphololgy. The direct effect in plant development by amoeba is scarce in literature. The aim of this work was verify the direct effect of protozoa in plant development. Thereby, the direct impact of amoeba on plant performance was investigated in a laboratory experiment. Seeds of *Arabidopsis thaliana* were grown in microcosm chambers with defaunated sand with naked amoebae (*Acanthamoeba* sp.) and without protozoa for three weeks. Total length (cm), total surface area (cm^2), average diameter (mm), total volume (cm^3), number of tips and number of forks of roots were evaluated, as well as shoot and roots biomass. The carbon (C) and nitrogen content (N) in tissues were analysed with Flash 2000 CHNS/O© analysers. The amoeba treatment increased the root volume (43 %), the root diameter (12 %), the root surface area (34 %), the numbers of forks (30 %) and the numbers of tips (18 %). Though, only the increases of root volume and diameter were significant. The alterations in root morphology may have been due to the increase of respiration into the microcosms or release of fitohormone by amoeba, once there was not nutritional effect in plant. This release is a strategy for increase the feeding resources from amoeba. However, comparing with control treatment, the protozoa increased the content of carbon in 12 % and nitrogen in 2 % in the roots. The increase of the ratio C/N in the tissue roots with protozoa indicated that the plant allocated more C in the root than N, aiming to improve the uptake of nutrients. The amoeba did not affect the shoot growth, although these alterations in the root morphology are important for better development of plant in natural conditions. In conclusion, soil amoeba affects directly the root morphology, without change the carbon and nitrogen content in the plant.

Key-words: Rhyzosphere. Root volume. Nutrient cycling.

The soil protozoa proliferate in the rhizosphere (Jentschke et al., 1995; Kreuzer et al., 2006), and their presence is often associated with greater root development. It is estimated that the number of soil protozoa in rhizosphere soil is two fold higher than bulk soil (Rouatt et al., 1960). The substances found in rhizosphere like carbohydrates, amino acids, organic acids, sterols, vitamins, purines, flavonoids, lignins, anthocyanins (Badri and Vivanco, 2009; Jones et al., 2009), favours the abundance of *Proteobacteri*a in rhizosphere soil (Mendes et al., 2013), which is an important food resource for soil protozoa (Bonkowski et al., 2004)

The grazing activity of protozoa increase the N mineralization (Clarholm, 1985), leading to improve the plant development (Jentschke et al., 1995; Bonkowski, 2002) and increase of the nitrate production in soil inoculated with protozoa. (Bonkowski et al., 2000). However, the plant development is not always explained by N mineralization. It is well documented in the literature the increase of root length, root volume, numbers of tips and numbers of forks proportionate by soil protozoa (Jentschke et al., 1995; Bonkowski and Brandt, 2002; Krome et al., 2010). The presence of soil amoeba selects groups of bacteria growth promoting of plants in rhizosphere (Bonkowski and Brandt, 2002). However, the effect of amoeba in plant growth can be due the change of distribution of auxin in the plant (Krome et al., 2010). Therefore, there was not in literature a consensus about the real reason of effect of the soil protozoa in plant development.

Thus, to verify if soil protozoa affect the plant development, we setup an experiment to test whether protozoa stimulate plant growth on a more direct way. The hypothesis of this work is that the presence of only soil protozoa in rhizosphere will increase root volume, root length, carbon (C) and nitrogen (N) in plant. For that, we chose two molecular model organisms: *Acanthamoeba castellanii*, a protozoa widely distributed in soil, freshwater and marine environments (Anderson et al., 2005) and *Arabidopsis thaliana*. The *Acanthamoeba castellanii* is a naked amoeba that possess adaptability to various environmental niches, due the diversity metabolic of this amoeba (Clarke et al., 2013), allowing the use of a diversity of food resources.

Thereby, the direct impact of amoeba on plant performance was investigated in a laboratory experiment. The amoebas used for inoculation were isolated from woodland soil in Göttinger, Germany (Bonkowski & Brandt, 2002) and they were cultivated axenically in Peptone Glucose Yeast medium (PGY medium – 2 % peptone, 1 % glucose and 0.5 % yeast extract). The inoculum was prepared by centrifugal washing 4 times (800 rpm for 5 minutes at 2 °C) in nutrient mineral solution (NMS), containing 1.650 mg $(NH_4)(NO_3)$, 0.006 mg

H_3BO_3, 0.3322 mg $CaCl_2$, 0.0000250 mg $CoCl_2$, 0.0000250 mg $CuSO_4.5H_2O$, 0.03726 mg $C_{10}H_{14}N_2Na_2O_8.2H_2O$, 0.02780 mg $FeO_4S.7H_2O$, 0.002 mg $C_2H_5NO_2$, 0.1807 mg $MgSO_4$, 0.0169 mg $MnSO_4$, 0.1 mg $C_6H_{12}O_6$, 0.0005 mg $C_6H_5NO_2$, 0.00083 mg KI, 1.9 mg KNO_3, 0.17 mg KH_2PO_4, 0.0005 mg $C_8H_{11}NO_3$, 0.000250 mg $Na_2MoO_4.2H_2O$, 0.0001 mg $C_{12}H_{17}ClN_4OS.HC$ and 0.0086 mg $ZnSO_4.7H_2O$.

The sterilized seeds of *Arabidopsis thaliana* were grown in microcosm chambers with 12/12 h day/night and 24/18 °C day/night, with light intensity of 160 µm s^{-1} and 70 % of relative humidity in agar layer during one week before the inoculation. Then 24 plants were transferred to magenta jars containing 200 g of sterilized sand, which 12 plants of *Acanthamoeba* treatment received 70.000 amoebas in 1.125 mL of NMS as inoculum, while the control treatment received 1.125 mL of only NMS.

Total length (cm), total surface area (cm^2), average diameter (mm), total volume (cm^3), number of tips and number of forks of roots were scanned and estimated by WinRhizo© software. Shoot and root carbon (C) and nitrogen content (N) in tissues were analysed with Flash 2000 CHNS/O© analysers.

The results evidenced that protozoa stimulated *A. thaliana* growth (Table 1). Inoculation of *A. castellanii* increased root length, volume and surface area in the larger diameter classes of *Arabidopsis thaliana* (Table 1), although it did not affect the smaller ones. The presence of amoeba resulted in two-fold increase in the total root volume, and the increases in the number of tips and forks was lesser extended (Table 1). The stimulation of root growth by inoculation was accompanied by increase of C and N content in root. The increase of C and N in root leads to decrease C/N ratio of shoot and to increase the C/N ratio in roots (Table 2). Despite these results, the treatments did not affect the total N and total C in the plant.

The mechanisms for such stimulation are unclear. There are two possible explanations for the change in root traits. The first explanation can be related to metabolic activity of *A. castellanii* in microcosms, increasing the flow of CO_2 into the microcosms. The elevated atmospheric CO_2 change the distribution of C and N in the plant (Pendall et al., 2004; Nie et al., 2013). Nie et al. (2013) did not find shift in N and C content in the plant, but detected decrease of N and increase of C in the root in plants with CO_2 enriched atmospheric. This kind of distribution of C and N in the plant is connected with genetics expression that coding transmembrane proteins responsible by efflux of auxin (PIN) in *Arabidopsis thaliana* (Gutiérrez et al., 2007), accelerating the distribution of auxin into the roots. This is

responsible by the increase in root length and root volume, which may be a strategy of plant to acquire nutrients from the soil in higher atmospheric CO_2 (Bader et al., 2009; Kim et al., 2015).

Table 1. Effect of *Acanthamoeba castellanii* on the distribution of length, volume and surface area root of *Arabidopsis thaliana* in three diameter classes of roots.

Diameter classes	Root length		Root volume		Surface área	
Cm	Control cm	Acanthamoeba	Control cm^3	Acanthamoeba	Control cm^2	Acanthamoeba
0-0.2	19.2	23.9	0.0017	0.0020	0.61	0.72
0.2-0.4	2.3	4.6 *	0.0012*	0.0024*	0.20	0.34
0.4-0.6	0.2	0.6 *	0.0003	0.0008	0.03 *	0.08 *
0.6-0.8	0.2	0.6 **	0.00019**	0.00045**	0.02 *	0.05 *

ns: not significant; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Table 2. Effect of *Acanthamoeba castellanii* in total volume, number of forks, number of tips, N and C content in root and shoot of *Arabidopsis thaliana*.

Treat	Total volume (cm^3)	Number of tips	Number of forks	N shoot (mg)	N root (mg)	C shoot (mg)	C root (mg)	C/N Shoot (mg)	C/N Root (mg)
Control	0.004*	166	186	0.22	0.06***	1.18	0.63*	5.88***	10.19***
Acanth.	0.008	203	264	0.22	0.07	1.21	0.82	5.28	11.49

Acanth.: Acanthamoeba; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

The second explanation about the change in root traits is the evidence that *A. castellanii* is able in produce auxin. The genes responsible for the biosynthesis of tryptophan and other genes connected with biosynthesis of auxin were identified in this amoeba (Anderson et al., 2005; Clarke et al., 2013). Possibly, the ability of *A. castellani* to harbour several endosymbionts bacteria into the food vacuole allows higher frequency of horizontal gene transfers (HGT) (Pak & Jeon, 1997). HGT refers to the movement of genetic information across normal mating barriers, between more or less distantly related organisms (Kelling and Palmer, 2008). The bacteria can live into the nucleus of amoeba and then this niche would facilitate the HGT during the mytoses process (Schulz et al., 2014). Thus, the auxin producers genes in amoeba may have been acquired by horizontal gene transfers of endosymbionts like several gram-negative bacteria (Zeybek & Binay, 2014).

The results support our hypothesis that only the presence of protozoa changes the root morphology, increasing the root volume and the root diameter and increasing the

allocation of C and N in the root. This is a significant finding. Before we thought that mainly bacteria and fungi (usually associated with mycorrhizae) have co-evolved with plants, however our results suggests that this eukaryotic group of microorganisms also play important role on plant growth. The perspective in the interaction between plants and microorganisms show us unknown microorganisms interfaces, which make us thought that the soil protozoa can be used as inoculants together with other microorganisms to increase the complexity of interactions together with roots. This complexity in inoculation is a strategy for recover the sustainability in crop land, decreasing the use of fertilization.

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CONCLUSÃO GERAL

A presence de plantas no sistema agrícola é primordial para estrutura da cadeia alimentar edáfica. Embora os sistemas de culturas e a presença de plantas de milho não ter afetado a diversidade microbiana, a estrutura da comunidade mudou. Dessa forma, deve-se utilizar as plantas como estratégia para integrar todas as funções microbianas do solo. Pois as mudanças na comunidade microbiana neste trabalho revelam a alta resiliência dos processos microbianos no solo, as quais estão ligados com a nova estabilidade de vida edáfica devido às mudanças em fatorea abióticos.

A abilidade de manejar o solo pelo uso de diferentes plantas é uma boa estratégia para alcançar a sustentabilidade na agricultura. As plantas selecionam o caminho de toda cadeia alimentar edáfica. A rotação de culturas aumentou a abundância de microorganismos relacionados com a conservação de carbono no solo, como por exemplo bactérias Gram+, FMA e amebas. Nesse sentido, o sequenciamento de DNA também mostrou aumento de bactérias do solo relacionado com processos conservativos em rotação de culturas. Dessa forma, a microcadeia alimentar do solo em rotação de culturas foi caracterizado pela presença de nichos oligotróficos. Entretanto, o efeito da adição de carbono via mulch ou planta em crescimento na comunidade de protozoários é dependente da estação.

A escolha de palntas para compor o sistema de culturas deve ser usada para selecionar as funções microbianas, incluindo as funções dos protozoários edáficos, tendo em vista o positivo efeito da inoculação de *Acanthamoeba castellanii* no desenvolvimento de *Arabdopsis thaliana*. Se por um lado as plantas selecionam a população microbiana devido às características da rizosfera e da cobertura morta, por outro os protozoários edáficos também selecionam as funções bacterinas pelo predação seletiva. Então, há duas forças seletivas da comunidade bacteriana no ambiente edáfico. Isso significa que os protozoários edáficos necessitam ser incluídos nos estudos da microcadeia alimentar, devido ao seu efetio na comunidade bacteriana e no desenvolvimento das plantas.

APENDICE

Co-autores dos artigos

Capítulo 1 – THE SOIL PROTOZOA AS REGULATOR OF MICROBIAL FUNCTIONS

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Capítulo 2 – ECOLOGY OF SOIL MICROBIAL COMMUNITY IN CROP ROTATION AND CROP SUCCESSION UNDER NO TILLAGE.

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- Realização do método PLFA foi realizado na Universidade de Colônia na Alemanha.

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Capítulo 3 - BACTERIAL COMMUNITY IN CROP SYSTEM ON SOIL UNDER NO TILL

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Capítulo 4 - THE EFFECT OF GROWTHING PLANTS AND MULCH OF MAIZE (Zea mays) IN THE SOIL PROTOZOA COMMUNITY

Esse capítulo não será publicado como artigo, pois faz parte de um estudo em conjunto da Universidade de Colônia com a Universidade de Göttingen. Neste caso, eu serei co-autor do artigo gerado do conjunto de dados desse trabalho.

Capítulo 5 - THE *Acanthamoeba castellanii* PROMOTE PLANT GROWTH.

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