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Interaction between Thermotolerant Coliforms and Rhizobacteria in Soil Fertilized with Treated Domestic Wastewater

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ABSTRACT: Studies on the survival of pathogenic microorganisms in the soil after use of wastewater for fertilization of agricultural crops report the effects of moisture, pH, organic matter, and soil temperature on microorganisms. There are few studies that assess the survival of these microorganisms in the rhizosphere of plants fertilized with wastewater. Thus, the aim of this study was to quantify the number of fecal coliforms and rhizobacteria (fluorescent *Pseudomonas* spp., *Bacillus* spp,) in the rhizosphere of winter and summer crops fertilized with wastewater. In the experiment, we used 20 plots, and each plot occupied an area of 200 m². The treatments used in the winter crop consisted of uncultivated plots and single crops of wheat, triticale, black bean, and intercropped black bean/wheat. In the summer season, we used uncultivated plots and single crops of corn, sunflower, bean, and intercropped bean/corn. The experiment was conducted in a randomized block design with five treatments and four replications. Soil samples from the rhizosphere for microbiological analyses were collected at the flowering stage of the crops at a depth of 0.00-0.20 m. Plants stimulated fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the rhizosphere, with average scores of 7.9 and 6.9 log CFU g^{-1} of dry soil, respectively, whereas in bare soil, these scores were 6.7 and 5.8 log CFU g^{-1} of dry soil for these rhizobacteria groups. However, this stimulating effect was not seen for fecal coliforms, which had an average score of 31.7×10^3 MPN g^{-1} of dry soil in the uncultivated area and 20.0×10^3 MPN g^{-1} of dry soil in crop areas. Overall, the numbers of rhizobacteria colonies in the rhizosphere soil under intercropping were higher than those observed in the rhizosphere soils of single winter and summer crops. Therefore, the presence of plants enhances the development of rhizobacteria and changes the balance among the species of microorganisms in the soil microbial community fertilized with wastewater.

Keywords: rhizosphere, reclaimed water, wastewater.

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INTRODUCTION

Adding treated wastewater to agricultural areas has provided benefits: partial substitution of chemical fertilizers, increases in yield, and savings in the amount of water directed to irrigation. In addition, it improves soil physical and biological conditions through the addition of organic matter, and, at the same time, solves the problem of final disposal of the wastewater (Medeiros et al., 2005; Fonseca et al., 2007). In spite of these benefits, care must be exercised in agricultural use because, if, on the one hand, the chemical characteristics of wastewater are favorable to reutilization, on the other hand, it should not be forgotten that even when treated, wastewater still contains pathogenic microorganisms, and continuous use may contaminate the soil with these microorganisms (Hespanhol, 2009).

Several studies have shown that the survival of pathogenic microorganisms in the soil after the application of wastewater is conditioned on variations in soil conditions, such as soil moisture, pH, solar radiation, texture, temperature, and organic matter content (Santos, 2004; Soares et al., 2005; Guber et al., 2007; Bech et al., 2010; Souza et al., 2011). However, in addition to environmental conditioning of the soil, it is important to note that plants exert a decisive influence on the number and diversity of microbes due to the zone of influence of roots because the application of wastewater will stimulate root development, increase the activity of microorganisms, and modify their community in the rhizosphere (Herman et al., 2006; Nakatani et al., 2008).

The rhizosphere environment is quite complex, due to the release of exudates, secretion, mucilages, and cell lysates that contain various organic substrates, vitamins, and hormones. These compounds, after being released by the roots, are used by microorganisms, causing what is known as the rhizospheric effect, which is an increase in the activity and change in the microbial community in the rhizosphere (Cardoso and Nogueira, 2007).

The rhizospheric community consists of microorganisms with different types of metabolisms and mechanisms of antagonistic action, with potential for suppressing the pathogenic microorganisms in the rhizospheric soil (Coelho et al., 2007). Among the rhizobacteria, studies have reported the effectiveness of fluorescent *Pseudomonas* spp. because they are capable of producing compounds that can suppress the development of pathogenic microorganisms in the rhizosphere and Bacillus spp. by producing antibiotics that inhibit the growth and development of microbial cells (Botelho and Mendonça-Hagler, 2006; Santos et al., 2006; Kavino et al., 2010; Marroni and Germani, 2011). Due to these characteristics, fluorescent *Pseudomonas* spp. and *Bacillus* spp. are used as indicators to measure the soil's ability to suppress pathogenic soil microorganisms and plant roots (Compant et al., 2005; Botelho and Mendonça-Hagler, 2006; Cipriano et al., 2013).

Therefore, the quantification of thermotolerant rhizobacteria and coliforms present in the rhizosphere of crops fertilized with wastewater can provide information on the influence of the rhizosphere on the survival of pathogenic microorganisms, contributing to a better understanding of wastewater application practices and management in agricultural areas.

The hypothesis of this study was that application of wastewater may stimulate the development of fluorescent *Pseudomonas* spp. and *Bacillus* spp., and the presence of these rhizobacteria may inhibit the growth of thermotolerant coliforms in the rhizosphere. The aim of the present study was to quantify the number of fluorescent *Pseudomonas* spp., *Bacillus* spp., and thermotolerant coliforms in the rhizosphere of winter and summer agricultural crops fertilized with wastewater.

MATERIALS AND METHODS

The study was conducted in the experimental field of the Department of Agricultural Sciences of the University of Taubaté (23° 02' 34" S and 45° 31' 02" W) in Taubaté, in the eastern part of the state of São Paulo, in 2009, 2010, and 2011 in a soil classified as a

Latossolo Vermelho-Amarelo (Embrapa, 2006) or Ferralsol (IUSS Working Group WRB, 2015), whose chemical properties in the 0.00-0.20 m layer are pH($CaCl_2$) 4.5; organic matter (OM) 21 g dm^{-3}; P 10 mg dm^{-3}; K 1.0 $mmol_c$ dm^{-3}; Ca 29 $mmol_c$ dm^{-3}; Mg 12 $mmol_c$ dm^{-3}; H+Al 28.6 $mmol_c$ dm^{-3}; CEC 70.4 $mmol_c$ dm^{-3}; S 41.7 $mmol_c$ dm^{-3}; and V 59.3.

The local climate, according to the Köppen (Köppen, 1948) classification system, is of the Cwa type (sub-tropical), with a rainy summer and average annual rainfall of 1,300 mm.

Wastewater from a wastewater treatment plant (WTP) with wetlands planted to *Typha* spp. had the following composition: pH 7.2; Sodium adsorption ratio (SAR) 6.1; Electrical conductivity (EC) 0.93 dS m^{-1}; Biological oxygen demand (BOD) 37.66 mg L^{-1}; Cl 2.18 mg L^{-1}; N-total 54.8 mg L^{-1}; NH_3-N 48.12 mg L^{-1}; NO_3-N 0.91 mg L^{-1}; P 9.66 mg L^{-1}; and thermotolerant coliforms 4000 MPN 1 L^{-1}.

The experimental area, of 5,488 m^2, was plowed and 4 Mg ha^{-1} of dolomitic limestone was applied. Subsequently, disking was performed to 0.20 m of soil depth. After disking, the soil of the experimental area was leveled to demarcate the experimental units. The experimental plots of 200 m^2 (10 m wide × 20 m long) were distributed in a randomized block experimental design with four blocks and five treatments, thus making for a total of 20 experimental plots separated by 3-m width pathways.

In 2009, 2010, and 2011, wheat (*Triticum aestivum* L.), triticale (*Triticale* Wittmack), black bean (*Phaseolus vulgaris* L.), and black bean intercropped with wheat were grown in the winter season. In the summer crop, corn (*Zea mays* L.), sunflower (*Helianthus annus* L.), bean (*Phaseolus vulgaris* L.), and bean intercropped with corn were grown.

The varieties sown in the winter crop were wheat, 385-Mojave-S1; triticale, 6-Pardal-S1; and black bean, Una-S1. In the summer crop, the varieties were corn, 3090-S1; sunflower, Irama-S1; and bean, Formoso-S1. Wheat, triticale, and corn seeds were sown at a spacing of 0.20 m between rows and 20 seeds per linear meter; bean and sunflower seeds were sown at a spacing of 0.40 m between rows and 10 seeds per linear meter.

The amount of 380 m^3 ha^{-1} of wastewater applied to the soil was calculated in accordance with the N available in the composition of the wastewater, based on the amount of 100 kg ha^{-1} of N to meet the needs of the agricultural crops (Cetesb, 1999). The amount was divided into two surface applications of 190 m^3 ha^{-1} at 10 and 40 days after sowing. Wastewater was applied in a uniform manner using a plastic hose of 63 mm diameter in all plots. Phosphate and K fertilization was carried out 15 days before sowing, and the amounts applied were: 70 kg ha^{-1} P_2O_5 and 60 kg ha^{-1} K_2O for wheat and triticale, 50 kg ha^{-1} P_2O_5 and 80 kg ha^{-1} K_2O for sunflower, 80 kg ha^{-1} P_2O_5 and 50 kg ha^{-1} K_2O for corn, and 80 kg ha^{-1} P_2O_5 and 60 kg ha^{-1} K_2O for bean.

The treatments used for the winter crop were uncultivated plots and single crops of wheat, triticale, black bean, and intercropped black bean/wheat. For the summer crop, uncultivated plots were used and single crops of corn, sunflower, bean, and intercropped bean/corn. Rhizospheric soil samples were collected in the cultivated plots and soil samples were collected in the uncultivated plots. Soil samples were collected at the beginning of flowering; 20 simple soil samples from each plot composed a composite sample. In the plot without planting, samples were collected from the 0.00-0.20 m depth with the aid of a Dutch auger and this was designated soil. In the cultivated plots, 20 whole plants were collected, with soil adhering to the roots. After collection, the manual separation of the soil aggregates adhering to the roots of the plants were separated manually, and this was designated “rhizospheric soil”.

For quantification of fluorescent *Pseudomonas* spp, *Bacillus* spp., and thermotolerant coliforms, 10 g of soil and rhizospheric soil were weighed and placed in vials containing 90 mL of sterile 0.01 mol L^{-1} $MgSO_4.7$ H_2O. The mixture was then stirred for 30 min stirring in a mechanical stirrer. Subsequently, serial dilutions by a factor 10 were prepared from the suspension inside the vial.

In the bacterial count of the fluorescent group of the genus *Pseudomonas*, 0.1 mL aliquots of each dilution were transferred to Petri dishes with culture medium B from King et al. (1954) and scattered with the aid of a Drigalski loop, in triplicate. Afterwards, the Petri dishes were kept in a greenhouse for growth of the colonies at the temperature of 28-30 °C, for 72 h. After the incubation period, colonies that fluoresced under a near ultraviolet light wavelength (King et al., 1954) were counted as bacteria of the *Pseudomonas* genus.

The procedure used for counting *Bacillus* spp was initially similar to that of the fluorescent *Pseudomonas* spp. However, the serial dilutions were first placed in a water bath at 80 °C for 20 min, aiming to select the bacteria of this genus (Bettiol, 1995). The dilutions were then cooled to room temperature and the 0.1 mL aliquots of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions, with three replicates, were transferred and distributed with the aid of the Drigalski loop onto the medium. The PDA culture (potato, 200 g; dextrose, 20 g; agar, 20 g; and water qs to 1 L) was contained inside the Petri dish. Subsequently, the Petri dishes were placed in a greenhouse with a temperature set at 28 °C for 72 h and *Bacillus* colonies were counted in the PDA medium.

The results of the *Pseudomonas* spp. and *Bacillus* spp. were expressed as the number of colony forming units per gram of dry soil (CFU g^{-1} dry soil). To do so, the dilutions that had from 30 to 300 colonies were selected counting and the gram of dry soil was obtained after drying the moist soil in a laboratory oven (105 °C) until constant weight.

To quantify thermotolerant coliforms, 1 mL aliquots of vials containing soil samples mixed with 90 mL of saline solution were diluted by 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} and, for each dilution, five tubes containing 10 mL of Sodium Lauryl Sulfate (LST) broth were used with inverted Durham tubes, which were subsequently incubated at 35 to 37 °C for 24 h. Tubes showing gas formation in the LST broth were considered positive. With the use of a platinum loop, an aliquot of the positive tubes was withdrawn with LST broth and seeded in tubes containing 5 mL of Brilliant Green Bile Broth 2 % (BGB), and the Durham tubes were inverted for total coliform growth. In a second step, BGB positive tubes were transferred to tubes containing *Escherichia coli* (EC) broth, a confirmatory medium for thermotolerant coliforms, and left in a water bath from 44.5 to 45 °C for 24 h. From the reading of the combination of the positive tubes (with gas production inside the Durham tubes) and the negative tubes (without gas production), thermotolerant coliforms were counted, and the result was expressed in the most probable number (MPN) of thermotolerant coliforms g^{-1} of soil (APHA, 2005).

The results of fluorescent *Pseudomonas* spp. and *Bacillus* spp. were transformed by log(X); then, together with the results of thermotolerant coliforms, they were subjected to Anova (Test F) and, in cases where F was significant at the 5 % level, the Tukey test was performed at 5 % for comparison of means.

RESULTS AND DISCUSSION

Rhizobacteria

The mean results of quantification of fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the soil without crops and in the rhizospheric soil of the winter and summer crops, fertilized with residual water, are shown in figures 1 and 2.

The fluorescent *Pseudomonas* spp. community varied significantly in the rhizospheric soil of the agricultural crops compared to the soil without cultivation (Figure 1). The lowest value of 4.8 log CFU g^{-1} of soil of fluorescent *Pseudomonas* spp. was observed in the uncultivated soil in 2010, and the highest value of 7.9 log CFU g^{-1} of soil was observed in the rhizosphere of the intercropped black bean/wheat in 2009. Furthermore, the occurrence of fluorescent *Pseudomonas* spp. remained predominant in the rhizospheres of the intercropped black bean/wheat and in the single crops of black bean, triticale, and wheat during the three years of winter cultivation.

In the same period, the number of *Bacillus* spp. exhibited a significant increase in the rhizospheric soil of the cultivated plots compared to the soil of the plot without crops (Figure 1). The average numbers of *Bacillus* spp. ranged from 5.5 log CFU g^{-1} of soil in

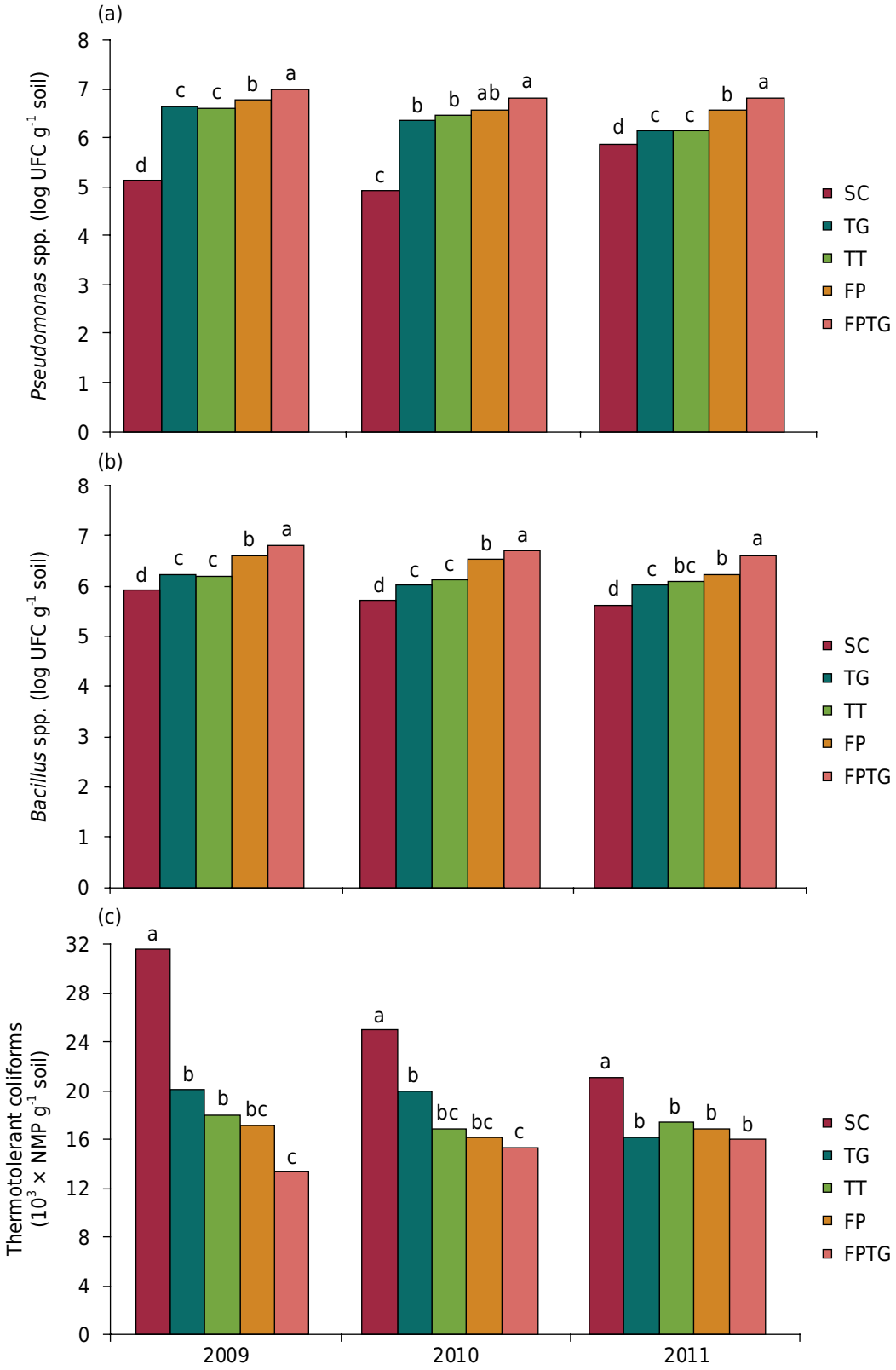

Figure 1. Count of viable cells of fluorescent *Pseudomonas* spp. (a), *Bacillus* spp. (b), and thermotolerant coliforms (c) in the 0.00-0.20 m depth layer of soil without cultivation (SC) and with wheat (TG), triticale (TT), black bean (FP), and intercropped black bean/wheat (FPTG) crops, fertilized with wastewater in the winter season in 2009, 2010, and 2011. Means followed by the same letters do not differ significantly from each other by the Tukey test at 5 %.

the uncultivated plot to 6.7 log CFU g^{-1} of soil in the rhizosphere of the intercropped black bean/wheat. Comparing the number of *Bacillus* spp. in the rhizosphere soil of single crops, it can be seen that the black bean rhizosphere most stimulated the development of *Bacillus* spp., followed by triticale and wheat.

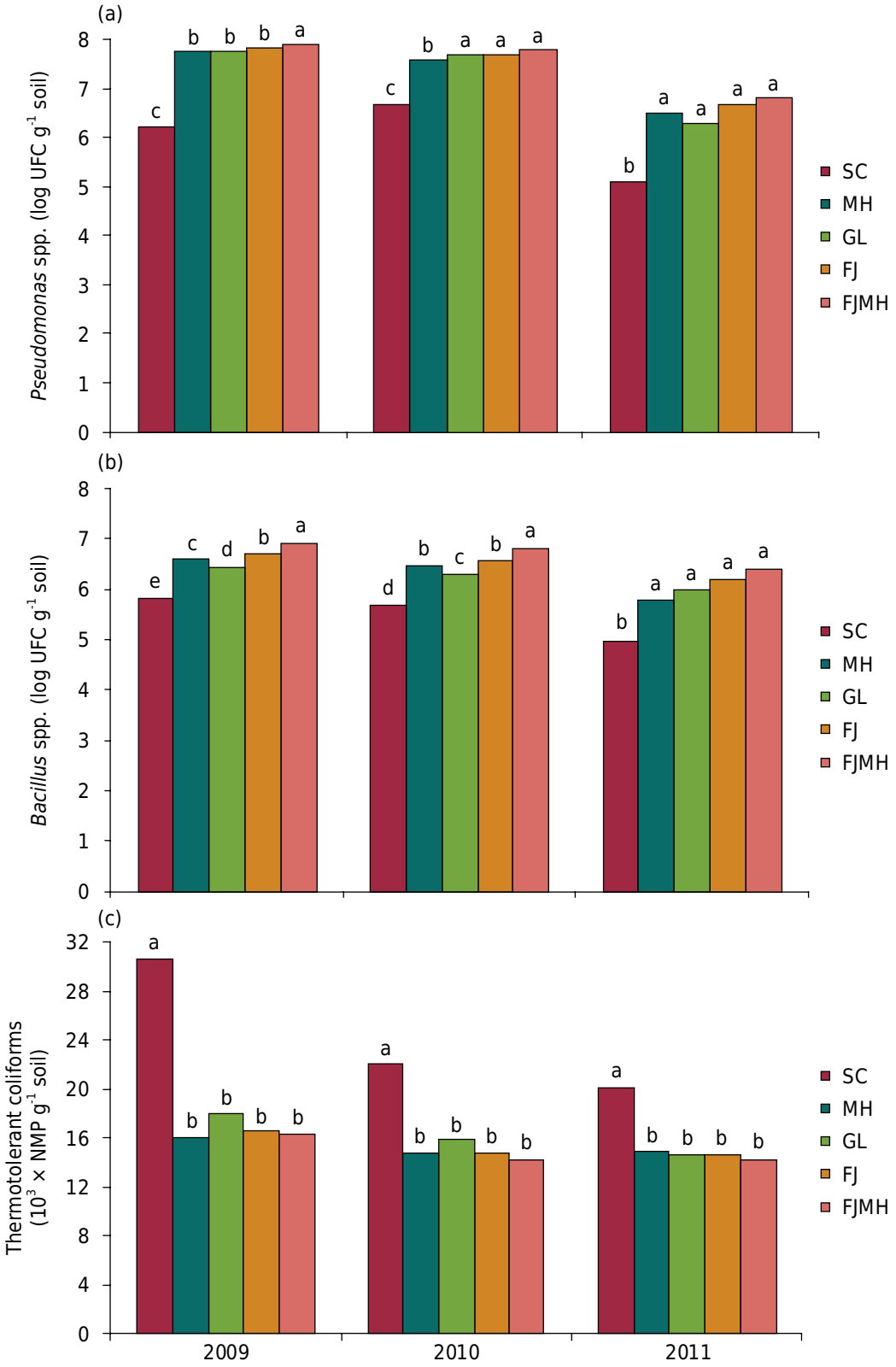

Figure 2. Count of viable cells of fluorescent *Pseudomonas* spp. (a), *Bacillus* spp. (b), and thermotolerant coliforms (c) in the 0.00-0.20 m depth layer of soil without cultivation (SC) and with corn (MH), sunflower (GL), bean (FJ), and intercropped bean/corn (FJMH) crops, fertilized with wastewater in the summer season in 2009, 2010, and 2011. Means followed by the same letters do not differ significantly from each other by the Tukey test at 5 %.

The fluorescent *Pseudomonas* spp. community varied significantly in the rhizospheric soil of the agricultural crops compared to the soil without cultivation (Figure 2). The mean number of fluorescent *Pseudomonas* spp. was 7.9 log CFU g^{-1} of soil in the rhizosphere of the intercropped bean/corn in the summer crop of 2009 and 5.1 log CFU g^{-1} of soil in the plot without cultivation in 2011. In general, the number of fluorescent *Pseudomonas* spp. of the rhizosphere soil of the bean/corn consortium exhibited a smaller or equal variation compared to the values determined in the rhizosphere of the single crops of bean, sunflower, and corn.

A significant difference was observed between the treatments of cultivation of agricultural crops and without cultivation (Figure 2). The incidence of *Bacillus* spp. was more accentuated in the plot with intercropped bean/corn in the 2009 and 2010 seasons. Among the single crops, the number was more pronounced in the bean rhizosphere, followed by corn and sunflower. The fluorescent *Pseudomonas* spp. and *Bacillus* spp. communities in the rhizospheric soil of plots cultivated with agricultural crops remained significantly more numerous than those determined in the soil of plots without cultivation in all the years studied (winter/summer) (Figure 2). These results suggest that the presence of the roots of the agricultural plants stimulated the development of fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the rhizosphere because it is known that the roots of plants can exudate many compounds in the form of amino acids, organic acids, and sugars that are used as energy sources and nutrients for the growth and activity of microorganisms in the rhizosphere (Nakatani et al. 2008; Kavino et al., 2010; Marroni and Germani, 2011).

In the rhizosphere of the intercropped treatment, the development of fluorescent *Pseudomonas* spp. and *Bacillus* spp. colonies were favored, both in winter and in summer (Figures 1 and 2). This promotion may be associated with a higher concentration of roots per soil volume because, in this situation, the amount and diversity of root exudates and organic substrates tends to be higher than the amount of exudates and compounds released into the rhizosphere from single crops. Studies carried out by Hungria et al. (1997), analyzing soil extracts under three crop systems, also found a greater number of *Rhizobium tropici* and *Azospirillum lipoferum*, due to the accumulation of different root exudates, such as phenolic compounds, flavonoids, and β-galactosidase, in the soil samples collected in the intercropped bean /corn than in the bean crop alone.

Analyzing the single crops in the winter, the numbers of fluorescent *Pseudomonas* spp. and *Bacillus* spp. were higher in the black bean rhizosphere soil. In the summer, there was no selective stimulation of the rhizospheres of the agricultural crops because the numbers of rhizobacteria were the same, with small variations among the crops. These results suggest that the rhizosphere of black bean, regardless of environment, may have produced some substance beneficial to these bacteria, a substance that is absent or is produced in small quantities in the rhizosphere of wheat, triticale, sunflower, and corn.

Results similar to those seen in the present study were also observed by Lemanceau et al. (1995) when studying the colonization of flax and tomato roots by *Pseudomonas* spp. in the soil; it was observed that most isolates of *Pseudomonas* spp. were associated with flax roots. These data suggest that the plant has a selective influence on the community of fluorescent *Pseudomonas* spp, and this selectivity varies according to the plant species. In this respect, Coelho et al. (2007) found higher numbers of fluorescent *Pseudomonas* spp. in the lettuce rhizosphere compared to the rhizosphere of *Eruca sativa*, *Petroselinum crispim*, and *Ciperus rotundus*. These authors claim that the lettuce plant produced root exudates that stimulated the growth of fluorescent *Pseudomonas* spp.. Variations in root exudation from different plant species growing on the same soil type select different bacterial communities in the rhizosphere since root exudation is known to be responsible for intermediation of molecular signals between plants and microorganisms in the rhizosphere (Wieland et al., 2001, Kowalchuk et al., 2002).

Thermotolerant coliforms

A significant difference in the number of thermotolerant coliforms can be observed among the treatments (Figure 1). The highest numbers of thermotolerant coliforms were found in the soil samples from the treatment without cultivation. In this treatment, the density of thermotolerant coliforms varied from 31.7, 25.0, and 21.0 × 10^3 MPN g^{-1} of soil in 2009, 2010, and 2011 crop years, respectively. However, the numbers of thermotolerant coliforms show a tendency of reduction in the rhizospheric soil of the plots with (13.3, 15.3 and 16.0 × 10^3 MPN g^{-1} of soil) were found in the rhizosphere soil of the intercropping with black bean/wheat, 2009, 2010 and 2011, respectively.

A significant difference in the number of thermotolerant coliforms can be seen among the treatments (Figure 2). The highest values (30.5, 22.0, and 20.0 × 10^3 MPN g^{-1} of soil) were found in the soil samples collected in the non-cultivated plots, with a significant reduction in the rhizospheric soil of plots cultivated with corn (16.0, 14.7, and 14.8 × 10^3 MPN g^{-1} of soil), sunflower (18.0, 15.8, and 14.6 × 10^3 MPN g^{-1} of soil), bean (16.6, 14.7, and 14.8 × 10^3 MPN g^{-1} of soil), and intercropped bean/corn (16.3, 14.2, and 14.2 × 10^3 MPN g^{-1} of soil) over the crop seasons (in 2009, 2010, and 2011, respectively).

The trend observed in reduction of thermotolerant coliforms in the soil of plots with winter and summer agricultural crops may be associated with the high density of the colonies of fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the rhizospheric soil compared to the numbers of these rhizobacteria in the soil without cultivation. These results suggest that the presence of these rhizobacteria inhibit the growth of thermotolerant coliforms in the rhizospheric soil. It is known that these rhizobacteria are capable of producing metabolites, such as hydrogen cyanides, siderophores, extracellular lytic enzymes, biosurfactants, and antibiotics, which inhibit the growth and development of microbial cells in the rhizosphere (Compant et al., 2005; Botelho and Mendonça-Hagler, 2006; Avelar et al., 2014). Therefore, it can be affirmed that after establishing themselves in the root system, the colonies of fluorescent *Pseudomonas* spp. and *Bacillus* spp. began to compete for space and nutrients, thus limiting the reproduction of thermotolerant coliforms in the rhizosphere of the winter and summer agricultural crops. As a consequence, several studies have reported these mechanisms of antagonistic action of *Pseudomonas* spp. and *Bacillus* spp. for suppression of phytopathogens in the rhizosphere of agricultural crops (Araújo et al., 2002, Egamberdiyeva, 2007; Cipriano et al., 2013, Ludwig et al., 2013).

CONCLUSIONS

The rhizobacteria numbers were higher in the rhizosphere of the cultivated plots than in the plots without cultivation, as the presence of roots of the summer and winter crops stimulated the development of fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the rhizospheric soil of the cultivated plots. Root exudates were probably used as energy sources and nutrients for the growth and multiplication of rhizobacteria in the rhizosphere.

The presence of fluorescent *Pseudomonas* spp. and *Bacillus* spp. in the rhizosphere of agricultural crops may have caused competition for space and carbon and nutrient resources, thus inhibiting the reproduction of thermotolerant coliforms in the rhizosphere of the cultivated plots.

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