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Activity of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *passiflorae* in soils cultivated with monocotyledonous plants[1]

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ABSTRACT

In spite of the importance of passion fruit cultivation in Brazil, this activity has been forced to be itinerant, due to the occurrence of diseases in producing regions, in particular fusariosis. The use of rhizobacteria is a potential control alternative, but other improvements are necessary to guarantee the activity of these bacteria in the field. This study aimed at evaluating the influence of three monocotyledonous groups on the activity of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *passiflorae* (Fop). Rhizobacteria were isolated from passion fruit plants and tested for the production of diffusible and volatile compounds, as well as chitinases that inhibit Fop. From a total of 167 isolates, two produced volatile compounds, 21 diffusible compounds and 32 chitinases. Three compatible combinations of isolates that had at least two action mechanisms against Fop and could grow in a wide pH range were applied in pots with sorghum, millet or maize, in addition to pots left fallow (control), in a greenhouse. The microbial biomass carbon, soil basal respiration, metabolic quotient and activity of the acid phosphatase enzyme were evaluated. The interaction between the cultivated species and rhizobacterial combinations was significant only for the metabolic quotient, whose values tended to be higher under millet cultivation in combination 3 (R77/R95/R104/R120), the most metabolically diversified one. The highest activity of acid phosphatase was also obtained with millet. The results for enzymatic activity and metabolic quotient indicate that millet provides a greater activity of the antagonists in the soil.

KEYWORDS: Microbial activity; passion fruit; millet; biological control.

RESUMO

Atividade de rizobactérias antagonistas a *Fusarium oxysporum* f. sp. *passiflorae* em solos cultivados com monocotiledôneas

Apesar da importância da passicultura no Brasil, essa atividade foi forçada a ser itinerante, devido à ocorrência de doenças nas regiões produtoras, em particular a fusariose. O uso de rizobactérias é uma alternativa de controle potencial, mas outras ações são necessárias para garantir a atividade dessas bactérias no campo. Objetivou-se avaliar a influência de três culturas monocotiledônicas sobre a atividade de rizobactérias antagonistas a *Fusarium oxysporum* f. sp. *passiflorae* (Fop). As rizobactérias foram isoladas de maracujazeiro e testadas para a produção de compostos difusíveis e voláteis, bem como quitinases que inibem Fop. De um total de 167 isolados, dois produziram compostos voláteis, 21 compostos difusíveis e 32 quitinases. Três combinações compatíveis de isolados que possuíam pelo menos dois mecanismos de ação contra Fop, e podiam crescer em ampla faixa de pH, foram aplicadas em vasos com sorgo, milho ou milheto, além de vasos em pousio (controle), em casa-de-vegetação. Avaliaram-se o carbono da biomassa microbiana, respiração basal do solo, quociente metabólico e atividade da enzima fosfatase ácida. A interação entre as espécies cultivadas e as combinações de rizobactérias foi significativa apenas para o quociente metabólico, cujos valores tendem a ser superiores no cultivo de milheto na combinação 3 (R77/R95/R104/R120), a mais diversificada metabolicamente. A mais alta atividade de fosfatase ácida também foi obtida com milheto. Os resultados relativos à atividade enzimática e quociente metabólico indicam que o milheto proporciona maior atividade dos antagonistas no solo.

PALAVRAS-CHAVE: Atividade microbiana; maracujá; milheto; controle biológico.

INTRODUCTION

Brazil is the world's largest producer of passion fruit, and the Bahia state is the leading national producer (Instituto FNP 2016). Although this crop has a great social and economic importance in the country, it has been itinerant, because of various diseases that occur in growing regions (Meletti 2011).

Fusariosis, caused by the soil pathogen *Fusarium oxysporum* f. sp. *passiflorae* Purss, is one of the main diseases affecting passion fruit plants. It causes large losses to growers, due to the death

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of plants, reduction of useful lifetime of plantations and control costs (Silva et al. 2011). The control of this disease is hampered because of the resistance structures produced by the pathogen, which remains in the soil for long periods. Therefore, strategies to control the disease are mainly based on preventive measures (Viana et al. 2003).

Due to the increasing pressure to reduce the use of toxic chemicals, biocontrol has been attracting the attention for preventing or reducing losses caused by pathogens. Biocontrol strategies may also be integrated with other disease management approaches (Lucas 2011). Significant results in controlling fusariosis have been achieved with the use of rhizobacteria (Faheem et al. 2015). However, one of the factors limiting the use of biological control is the low survival and multiplication of biocontrol agents in field conditions (Lucas 2011).

Because of inconsistent results or limited scope in the field, biocontrol agents have been employed along with a set of crop management practices (Tarigan et al. 2013). Practices such as intercropping may favor the activity of antagonists by forming an optimal niche for these microorganisms (Pereira & Pinheiro 2012). This effect occurs due to the release of exudates by plants, which attract antagonists and help their proliferation by providing nutrients to them (Richardson et al. 2009).

Besides constituting a source of extra income for growers, monocotyledonous plants can stimulate the activity of antagonists against phytopathogens existing in an area. Therefore, the integration of these species in crop rotation or intercropping systems may reduce the need for inoculation in the soil. Based on this, this study aimed at assessing the effect of planting three monocotyledon crops on the activity of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *passiflorae*, in a greenhouse, since the identification of a monocotyledonous plant that improve the rhizobacteria activity may help to improve integrative control measures (cultural and biological) to reduce the damage caused by fusariosis to passion fruit plants.

MATERIAL AND METHODS

Samples of rhizospheric soil were collected from fields where passion fruit plants showed a good phytosanitary appearance, but in areas with a history of fusariosis, in Livramento de Nossa Senhora (13.673.898S; 41.898.277W), Rio de Contas (14.074.381S; 41.729.755W) and Dom Basílio (14.156.371S; 41.221.802W), all in the Bahia state, Brazil.

To isolate the rhizobacteria, 10 g of soil from each sample were transferred to an Erlenmeyer flask containing 90 mL of a sterilized saline solution (0.85 %), to which two drops of Tween 80 were added. The samples were kept under continuous agitation in a shaker for 30 min, at room temperature. Then the soil suspensions were serially diluted by a factor of 10, up to a dilution of 10^{-6}. Aliquots of 100 μL of the last three serial dilutions were placed in Petri dishes containing agar nutrient, in triplicate, spread with the help of a Drigalski spatula. Then, the dishes were incubated at 28 ºC, for 48 h. The individual colonies, with different morphological characteristics, were isolated and preserved at -80 ºC.

All the rhizobacteria were tested *in vitro* to detect the production of diffusible and volatile compounds and chitinases able to inhibit *Fusarium oxysporum* f. sp. *passiflorae*. In these tests, the phytopathogenic mitosporic isolate 05, from the collection of the Embrapa Mandioca e Fruticultura, in Cruz das Almas, Bahia state, identified by phylogenetic analysis, was used.

To verify the presence of diffusible compounds, mycelial disks of the pathogen, with diameter of 1.0 cm, were transferred to the central part of Petri dishes containing potato-dextrose-agar (PDA) and incubated at room temperature. After 48 h, eight distinct rhizobacteria were seeded on spots equidistant from the center of each dish and incubated at 28 ºC, for 7 days. The control consisted of dishes without the antagonists and the assay was conducted in three replicates, represented by three Petri dishes. The presence of a mycelial growth inhibition halo in three repetitions with each isolate was the criterion to select the rhizobacteria for further analysis (Oliveira 2009).

The production of volatile compounds by the antagonists was checked by the method described by Bharat et al. (1980), which consists of using edge-to-edged paired dishes, starting by spreading 100 μL of each bacterial suspension on nutrient agar medium in one dish, so as to produce a layer of cells on the surface of the medium. After bacterial growth at 25 ºC, for 24 h, a mycelial disk of the pathogen measuring 7.0 mm in diameter was placed on the medium, in the bottom of another dish containing

PDA medium. The lids were then removed and the two paired dishes were sealed with PVC film. The test was performed in duplicate and dishes only containing *Fusarium oxysporum* f. sp. *passiflorae* composed the control. The dishes were incubated at 25 ºC, until the mycelia in the control dish reached a maximum growth. Only the rhizobacteria that reduced the mycelial growth area of the pathogen in two dish pairs were considered.

To detect the production of chitinases, the method adapted from Renwick et al. (1991) was used, where the rhizobacteria were multiplied in mineral medium supplemented with 0.08 % of colloidal chitin as the only carbon source. For this, distinct isolates were placed on the surface of the medium in each dish, after which the dishes were incubated at 28 ºC, for 10 days, with three repetitions for each treatment. After the incubation period, the production of chitinases was confirmed by observation of a transparent halo below the colony, contrasting with the milky aspect of the rest of the medium.

The rhizobacteria that produced a positive result in at least two of the three tests (eight isolates) were submitted to mutual antibiosis and growth tests at different pH values. The aim of the mutual antibiosis test was to determine the compatibility between the rhizobacteria, and it was conducted for all pairs of isolates in nutrient agar medium, by the overlay method adapted from Romeiro (2007). All the combinations were tested, so that each isolate was paired with all the others, with seven repetitions, in a dish with seven isolates. The evaluation was performed at one, two and five days after adding the overlay with the challenge isolate, by observing the presence or absence of a halo indicating growth inhibition of the challenged isolate.

The rhizobacteria growth was assessed at different pH levels by transferring disks from the rhizobacterial cultures with 5 mm diameter to nutrient agar medium with pH values adjusted to 5.0, 5.5, 6.0 or 6.5. For the control, the isolates were cultivated on the same medium, with pH adjusted to 7.0. HCl (1.0 %) was used to adjust the pH values below 7.0. The dishes were incubated in a growth chamber at 28 ºC, for 7 days, with daily observation of rhizobacterial growth. Growth was considered to have occurred in colonies whose diameter doubled.

The activity of the seven rhizobacterial isolates that performed best in the previous tests was checked in combination with other isolates, in soil cultivated with three monocotyledonous plants (maize 'AL Bandeirante', millet 'BRS 1504' and sorghum 'BRS 304'), as well as in soil without cultivation (fallow). The experiment was conducted in a greenhouse of the Embrapa Mandioca e Fruticultura.

The experimental design was completely randomized, in a 4 x 3 factorial scheme [three monocotyledonous species (millet, maize and sorghum) plus the control (fallow) and three combinations of rhizobacteria], with six replications, for a total of 72 experimental plots. Each plot consisted of a pot with one plant. The controls consisted of pots containing only soil and pots that received only the rhizobacterial suspension.

The following criteria were established to choose the combinations of rhizobacteria: a) all isolates in each combination had to present at least two antagonism mechanisms; b) all combinations had to have at least one isolate with three mechanisms; c) the isolates within each combination could not inhibit any of the other isolates; d) all the isolates had to grow in a broad range of pH values (5.0-7.0) up to the third day of incubation. This led to the formation of the following combinations: 1) R76/R77; 2) R77/R97/R116; 3) R77/R95/R104/R120.

The rhizobacterial suspensions were prepared at 24 h after the start of growth in nutrient agar medium at 28 ºC. The concentration of each suspension was adjusted with a spectrophotometer to A_{540} = 0.2, equivalent to 10^8 CFU mL^{-1}. The combinations were obtained from the suspensions of isolates prepared individually and the concentrations were adjusted for a subsequent mixture in equal volumes.

The seedlings of the three monocotyledonous species [maize (*Zea mays*), sorghum (*Sorghum bicolor*) and millet (*Pennisetum glaucum*)] were grown from seeds purchased at a nursery. These seeds were sown in trays containing sterilized soil in the greenhouse and watered every day with distilled and autoclaved water. At 30 days after sowing, the seedlings were transplanted to the definitive pots, at which time 100 mL of each rhizobacterial suspension was applied in each pot. After transplantation, the seedlings were watered on alternate days with distilled and autoclaved water (200 mL pot^{-1}).

Soil samples were collected at a depth of 0-10 cm near the roots, at two intervals, at 47 and 63 days after the application of rhizobacteria, to evaluate the following biological attributes: microbial biomass carbon, soil basal respiration, metabolic

quotient (qCO_2) and acid phosphatase activity. This last parameter was only analyzed after 63 days. The biological attributes were analyzed at the Embrapa Mandioca e Fruticultura.

Before the analytic tests, the soil samples were sifted (2 mm mesh), moistened and pre-incubated at 25 ºC, in a BOD chamber, for two days. The results of all the analyses are expressed based on the soil dry weight, determined by deducting the water content of each soil sample, measured by the percentage difference in weight of 10 g of each soil sample before and after drying in an oven at 100 ºC, for 24 h.

The microbial biomass carbon was extracted by the irradiation-extraction method described by Ferreira et al. (1999) and quantified according to Bartlett & Ross (1988), considering a correction factor kc = 0.33 (Sparling & West 1988). The soil basal respiration was determined by an adaptation of the fumigation-incubation method proposed by Jenkinson & Powlson (1976), based on the CO_2 released from non-irradiated samples incubated for 10 days and captured in NaOH. The qCO_2 was obtained from the ratio between basal respiration and microbial biomass carbon (Anderson & Domsch 1993) and expressed in mg C-CO_2 kg^{-1} of microbial biomass carbon h^{-1}. Finally, the acid phosphatase activity was evaluated by the method proposed by Tabatabai & Bremner (1969), without employing toluene to inhibit the microbial activity during the analysis process. The activity of this enzyme was expressed in µg *p*-nitrophenol cm^{-3} of soil h^{-1}.

The data from each evaluation were submitted to variance analysis and the means of plants and combinations of rhizobacteria were compared by the Tukey test, in both cases at 5 %, using the Sisvar statistical software (Ferreira 2014).

RESULTS AND DISCUSSION

A total of 167 rhizobacteria were isolated, of which 21 produced diffusible compounds, 32 chitinases and 2 volatile compounds (Table 1). Besides the production of antibiotics, the production of chitinases is a desirable trait in a biocontrol agent, because these enzymes degrade chitin, provoking lysis of the phytopathogenic fungal cells (Faheem et al. 2015). The production of volatile compounds, in turn, is advantageous, because those compounds reach the target pathogen over both short and long distances, leading to their inhibition (Yuan et al. 2012).

The mutual antibiosis test was performed with the isolates R76, R77, R89, R95, R97, R104, R116 and R120, because these presented at least two antagonistic mechanisms to determine the

Table 1. Production of volatile and diffusible antimicrobial compounds and chitinases by rhizobacteria isolated from passion fruit plants.

Isolates	Diffusible compounds	Volatile compounds	Chitinases
R2	-	-	+
R3	-	-	+
R4	-	-	+
R6	-	-	+
R8	-	-	+
R11	-	-	+
R12	-	-	+
R13	-	-	+
R14	-	-	+
R19	-	-	+
R20	-	-	+
R23	-	-	+
R30	-	-	+
R38	-	-	+
R41	-	-	+
R56	-	-	+
R64	-	-	+
R75	+	-	-
R76	+	+	+
R77	+	+	+
R78	+	-	-
R80	-	-	+
R82	-	-	+
R86	+	-	-
R89	+	-	+
R90	-	-	+
R94	+	-	-
R95	+	-	+
R96	+	-	-
R97	+	-	+
R98	-	-	+
R102	+	-	-
R103	+	-	-
R104	+	-	+
R116	+	-	+
R117	+	-	-
R119	+	-	-
R120	+	-	+
R127	+	-	-
R134	+	-	-
R148	-	-	+
R149	-	-	+
R150	-	-	+
R151	+	-	-
R152	+	-	-

* (-) no inhibition; (+) inhibition.

compatibility between these rhizobacteria. Isolate R76 inhibited the growth of all the other isolates, except for R77, which, in turn, only inhibited R89 (Table 2). The other rhizobacteria were not mutually sensitive, enabling the formation of combinations among them without any restriction. The interest in using combinations of biocontrol agents is due to the possibility of obtaining benefits such as a greater colonization of the rhizosphere and a better adaptation to different soil conditions (Thilagavathi et al. 2007).

Another desirable trait of a biocontrol agent is its ability to grow under a wide range of environmental conditions. A greater flexibility, in terms of environmental adaptability, means that the agent has a better chance of being employed to develop a biological product (Longa et al. 2008). In Brazil, since the great majority of soils is acidic, pH testing is an important step in the selection of candidate agents for the biocontrol of soil pathogens. In this study, all eight rhizobacterial isolates selected showed growth in the pH range tested (5.0-7.0) at the third day of incubation.

In the greenhouse tests, after the first evaluation interval (47 days), no significant differences were found between the species cultivated and the different combinations of rhizobacteria evaluated, regarding the microbial biomass carbon, soil basal respiration and qCO_2 values, as well as for the interaction between the plants and combinations. However, after the second interval (63 days), significant differences were observed between the species cultivated, regarding the microbial biomass carbon, soil basal respiration, *q*CO2 and acid phosphatase activity values. The variance analysis revealed that the interaction between the species cultivated and the combinations of rhizobacteria was significant ($p < 0.05$) only for the attribute qCO_2.

The highest values of microbial biomass carbon in the soil, with respect to the monocotyledonous species, were found in the fallow soil and the soil cultivated with sorghum (Figure 1). Higher microbial biomass carbon values are related to larger input of carbon by rhizodeposition and decomposition of phytomass (Carneiro et al. 2008). However, high microbial biomass carbon values may also be found in soils without crops, as observed in this study, probably due to the absence of competition between microorganisms and plants for nutrients in the soil (Richardson et al. 2009).

Although the plants were watered on alternate days, high temperatures were noted in the greenhouse, and the fallow soil, since there was no vegetation to absorb the water, was visibly moister than the cultivated soils. The availability of water in the soil is also a condition that affects the microbial biomass,

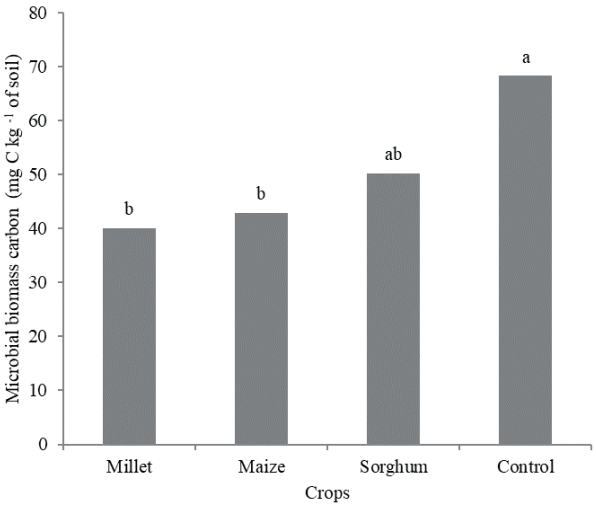

Figure 1. Microbial biomass carbon in soils treated with rhizobacteria and cultivated with monocotyledonous species, after incubation for 63 days.

Table 2. Compatibility of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *Passiflorae*.

Inhibitory isolates	Inhibitory isolates							
	R76	R77	R89	R95	R97	R104	R116	R120
R76		-	-	-	-	-	-	-
R77	-		-	-	-	-	-	-
R89	+	+		-	-	-	-	-
R95	+	-	-		-	-	-	-
R97	+	-	-	-		-	-	-
R104	+	-	-	-	-		-	-
R116	+	-	-	-	-	-		-
R120	+	-	-	-	-	-	-	

* (-) no inhibition; (+) inhibition.

and the growth of microbes not adapted to water deficit tends to decline (Hueso et al. 2012).

Data on biomass carbon, however, are not sufficient to measure the activity of microbial populations, since high quantities of inactive biomass or low quantities of active biomass may occur in the soil (Faleiro et al. 2011). Therefore, basal respiration is another important parameter used to quantify the microbial activity. A higher release of CO_2 indicates a greater microbial activity, due to the deposition of organic residues and large quantities of roots (Silva et al. 2010). In this study, maize and millet provided the highest soil basal respiration values (Figure 2). Therefore, the increase in soil basal respiration, when these plants are grown, is probably associated with the availability of more and/or better quality exudate.

The metabolic (or respiratory) quotient (qCO_2) is a sensitive indicator to estimate the biological activity in a substrate, as well as its quality. The parameter expresses the ratio between the soil basal respiration and soil carbon biomass metrics, revealing the quantity of CO_2 released per unit of biomass (Anderson & Domsch 1993). High qCO_2 values are associated with greater microbial activity and higher CO_2 release per unit of biomass carbon, probably due to the presence of a substrate that is easy to assimilate for microbial development and activity, with lower resistance to microbial attack (Bohm et al. 2007).

The highest qCO_2 values were obtained in the soil cultivated with millet, using rhizobacterial combinations 2 and 3, with a tendency for higher values with the use of combination 3 (R77 + R95 + R104 + R120), which had the largest number of isolates (Figure 3). According to Santos (2014), a microbial community formed by a larger number of isolates is more metabolically diversified. Since each isolate has its own metabolic traits and behaviors, the microbial community will be more able to take advantage of the exudates, what, in turn, favors the microbial activity.

The determination of the enzyme activity in the soil is a way to measure the microbial activity. In this study, the highest levels of acid phosphatase activity were found for cultivation with millet (Figure 4). According to Dotaniya et al. (2014), the acid phosphatase activity varies according to the crop and plant growth periods. They also found the highest acid phosphatase activity with cultivation of millet, in relation to maize and sorghum. The maximum activity with millet was observed at 90 days after sowing. This finding is similar to ours, since our millet plants were transplanted at 30 days after sowing, for a total growth period of 93 days. According to those researchers, these values are related to a higher production of exudates, which stimulate microbial activities and biochemical alterations in the plant system in this period.

As observed in this study, the significantly higher metabolic quotient values in the soil

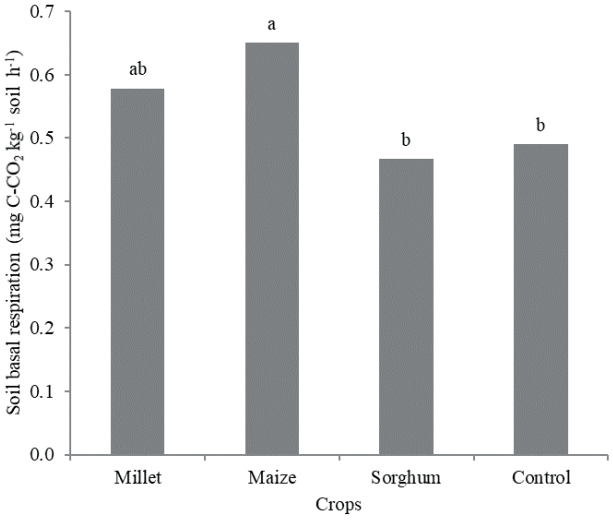

Figure 2. Soil basal respiration in soils treated with rhizobacteria and cultivated with monocotyledonous species, after incubation for 63 days.

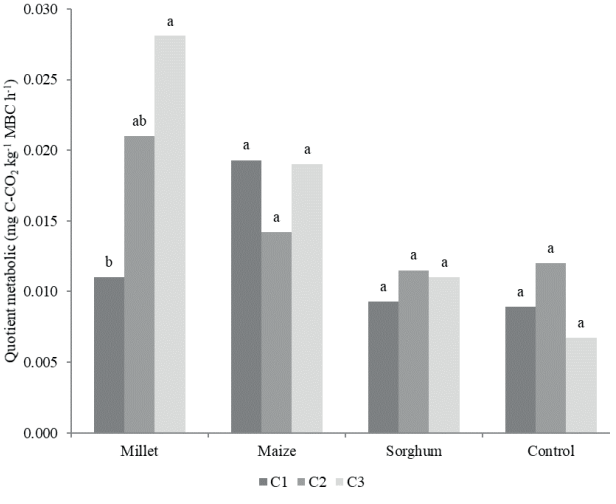

Figure 3. Average metabolic quotient values, as a function of the crops and combinations of rhizobacteria (C1, C2, and C3), after incubation for 63 days. MBC: microbial biomass carbon.

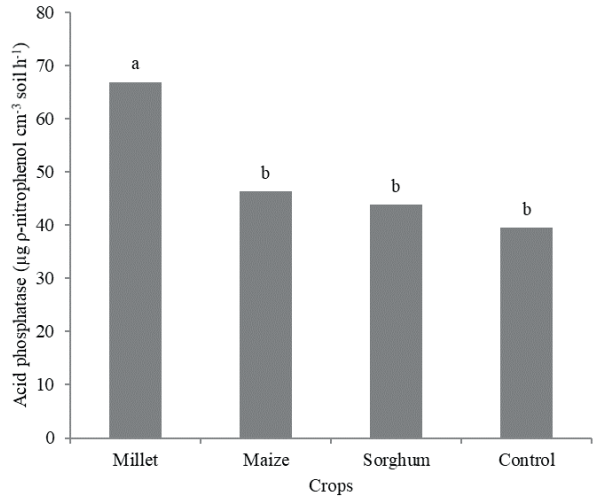

Figure 4. Acid phosphatase activity in soils treated with rhizobacteria and cultivated with monocotyledonous species.

cultivated with millet, along with the rhizobacterial combination 3 (R77 + R95 + R104 + R120), indicate a greater activity of these microorganisms under this culture, thus suggesting an easier assimilation of the exudates released by millet by these rhizobacteria. The greater acid phosphatase activity found after 63 days, in turn, adds a further evidence to the higher microbial activity in the soil cultivated with millet. This activity may be related to alterations of the plants phenological state.

The identification of treatments with a greater activity of the rhizobacterial antagonists may lead to a more effective biocontrol of fusariosis in passion fruit plants, especially in intercropping systems using millet. This knowledge may improve the passion fruit cultivation by reducing the damage caused by fusariosis and providing an extra source of income from millet. However, it is necessary to conduct further research, including *in vivo* tests, to evaluate this possibility in the field.

CONCLUSIONS

1. The cultivation of millet stimulates the combinations of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *passiflorae* in the soil;
2. The combination of four rhizobacteria isolates antagonistic to *F. oxysporum* f. sp. *passiflorae* in soil cultivated with millet causes a significant increase in the metabolic quotient.

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