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CROP PRODUCTION

Plant growth-promoting rhizobacteria isolated from cultivated soils using *Glycine max* L. plants as bait

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ABSTRACT: The current production of major crops, such as *Glycine max* L., has become increasingly adept on the use of bio-inputs, such as application of plant growth-promoting inocula. However, the evaluation of the potential of bacterial isolates from soils with different time histories of agricultural use are still scarce, and methods for isolation and testing of multifunctional microorganisms need to be continuously innovated and improved. Thus, we used *G. max* bait plants to attract rhizobacteria from soils obtained from three areas with different time histories of soybean cultivation. We evaluated the influence of the management and characteristics of soils on the phosphate-solubilizing bacterial population. We then tested the multifunctional potential of the isolated rhizobacteria for calcium phosphate ($CaHPO_4$) and iron phosphate ($FePO_4$) solubilization, considering their potential as microbial inoculants in the future. The use of bait plants enabled the isolation of 139 phosphate solubilizing rhizobacteria, including four strains with multifunctional potential. The largest number of solubilizing bacteria was obtained from the interaction of bait plants with soil from an area with a history of soybean cultivation for thirty consecutive years. The high concentration of P, Fe, and K found in the soil were associated with this occurrence. In the in vitro tests, the pH values of the culture media had a moderate negative relationship with the amounts of P made available by the isolates, indicating that other processes besides the availability of organic acids, may underlie the solubilizing action of the isolates. The rhizobacteria SAF9 (*Brevibacillus* sp.), SAF11 (*Brevibacillus* sp.), BRC11 (*Pseudomonas fluorescens),* and SAC36 (*Bacillus velezensis*) stood out as multifunctional and are indicated within a perspective of obtaining bioinoculants to promote plant growth directly, indirectly, or synergistically, contributing to increase the range of bio-inputs for soybean cultivation and more sustainable agricultural practices.
Key words: bioinoculants, phosphate solubilization, multifunctional microorganisms, phytohormones, siderophores.

Rizobactérias promotoras de crescimento de plantas isoladas de solos cultivados usando plantas *Glycine max* L. como isca

RESUMO: A produção atual de grandes culturas, como *Glycine max* L. tem se mostrado cada vez mais adepta da utilização de bioinsumos, como a aplicação de inóculos promotores do crescimento vegetal. No entanto, avaliações do potencial de isolados bacterianos de solos com diferentes históricos de tempo de uso agrícola ainda são escassos e os métodos para isolamento e testagem de micro-organismos multifuncionais precisam ser continuamente inovados e melhorados. Assim, usamos plantas iscas de *G. max* para atrair rizobactérias de solos obtidos de três áreas com diferentes históricos de cultivo de soja. Avaliamos a influência do manejo e das características dos solos sobre a população de bactérias solubilizadoras de fosfato. Em seguida, testamos o potencial multifuncional das rizobactérias isoladas para solubilização de fosfato de cálcio ($CaHPO_4$) e fosfato de ferro ($FePO_4$), considerando seu potencial como inoculantes microbianos no futuro. O uso de plantas iscas permitiu o isolamento de 139 rizobactérias solubilizadoras de fosfato, incluindo quatro cepas com potencial multifuncional. O maior número de bactérias solubilizadoras foi obtido a partir da interação das plantas-isca com o solo de uma área com histórico de cultivo de soja por trinta anos consecutivos. Características como a alta concentração de P, Fe e K neste solo, estiveram associadas a esta ocorrência. Nos testes *in vitro*, os valores de pH dos meios de cultivo se correlacionaram negativamente, mas de forma moderada com as quantidades de P disponibilizados pelos isolados, indicando que outros processos, além da disponibilização de ácidos orgânicos podem estar subjacentes à ação solubilizadora dos isolados. As rizobactérias SAF9 (*Brevibacillus* sp.), SAF11 (*Brevibacillus* sp.), BRC11 (*Pseudomonas fluorescens*) e SAC36 (*Bacillus velezensis*) se destacaram como multifuncionais e são indicadas dentro de uma perspectiva de obtenção de bioinoculantes para promoção do crescimento vegetal de forma direta, indireta ou sinérgica, contribuindo para aumentar a gama de bioinsumos destinados à cultura da soja e para práticas agrícolas mais sustentáveis.
Palavras-chave: bioinoculantes, solubilização de fosfatos, micro-organismos multifuncionais, fitormônios, sideróforos.

INTRODUCTION

Soybean (*Glycine max* L.) production is among the most important economic activities in the world, promoting the development of many countries, including Brazil. With a record production estimated at 125,6 Mtons, Brazil is the world's largest soybean producer for 2021/22 (CONAB, 2022). The implementation of management systems that involve soil correction and conservation practices, in areas

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where annual crops are grown, has contributed to Brazil reaching this production level (LATHUILLIÈRE et al., 2017). Soil use and management practices can significantly affect microbial and enzymatic activities due to fertilizer application (SPAGNOLETTI et al., 2017) and soil preparation (PEIGNÉ et al., 2018).

Phosphorus (P) is one of the essential macronutrients for plant growth, and its bioavailability in the soil can influence crop productivity (ZHU et al., 2018). Different strategies have been developed to improve P supply to crops, including using soil microorganisms that participate in soil P transformations (BARGAZ et al., 2021; ESTRADA-BONILLA et al., 2021; LUCERO et al., 2021).

Nevertheless, in addition to phosphate solubilization, multifunctional microorganisms can benefit plant growth and crop yield through various mechanisms, including nitrogen fixation; ammonia production; synthesis of siderophores and growth-inducing hormones, such as auxins, gibberellins, and cytokinins; control of phytopathogens by antibiosis or synthesis of a 1-aminocyclopropane-1-carboxylic acid deaminase, which increases plant growth under stress conditions; and improving plant resistance to heavy metal toxicity (ALORI et al., 2017; NOR, 2020; RAWAT et al., 2021; RIAZ et al., 2021). Rhizosphere inhabiting bacteria, which beneficially influence plant growth, are known as plant growth-promoting rhizobacteria (PGPR) (ALORI et al., 2017). The PGPR have been commonly used as biofertilizers in agricultural systems, and research has shown that using them results in a 50%–70% increase in crop yields (MA et al., 2018; COMPANT et al., 2019). Engineering the rhizosphere with these PGPRs has wide application and interest not only in crop fertilization but also in the development of sustainable and environmentally friendly agriculture (HAKIM et al., 2021). This happens because these rhizobacteria can act as biofertilizers, increasing plant access to nutrients already available in the soil, reducing the need to apply nitrogen or phosphate chemical fertilizers (RAI et al., 2020).

Rascovan et al., (2016) reported a wide variety of bacteria, including *Enterobacter spp*., *Pseudomonas spp.*, *Paraburkholderia spp*., and *Bacillus spp*. in soybean roots. These exhibited important growth promotion traits such as phosphate solubilization, nitrogen fixation, indole acetic acid (IAA) production, improvements in nutrient uptake, and tolerance to salt and water stress in vitro. These in vitro bacterial assays have been widely adopted to aid in decision-making on efficient bacterial isolates formulated as potent bioinoculants for crops (ELHAISSOUFI et al., 2020). However, much information about the ecological conditions of the growing and developing environment of the rhizospheric community still needs to be incorporated into the tests. For example; although, agricultural management is vital in defining the composition of the symbiotic microbiota, experiments evaluating the potential of bacterial isolates from soils with different time histories of agricultural use are scarce. In addition, methods for isolating and testing multifunctional microorganisms from the rhizosphere or plant tissues need to be continuously innovated and improved (REIS et al., 2021), so that increasingly effective bio-inputs can be introduced to the market.

With this in mind, and also in view of the market need for PGPRs specifically designed for soybean cultivation, we decided to test the technique of using *G. max* bait plants to attract and symbiotically facilitate the colonization of the resident microbiota in soils from three areas with different time histories of soybean cultivation. The use of bait plants remains disused in bioassays with symbiotic microorganisms. However, bait plants have been used to detect the presence of phytopathogens in possibly contaminated soils and for studies of mycorrhizal and endophytic fungal community composition in soil samples (NARISAWA et al., 2007; SÝKOROVÁ et al., 2007; AGUSTÍ-BRISACH et al., 2013). Nevertheless, we were interested in selecting multifunctional bacterial inocula for growth promotion in soybean crops, focusing on phosphate solubilization. Furthermore, we wanted to verify whether different management patterns and soil characteristics could interfere with the functionality of rhizobacteria. We hypothesized that the different phosphate-solubilizing bacterial isolates can be obtained, from soils from the three areas with different time histories of soybean cultivation, through rhizospheric attraction with bait-plants and they could possess other critical functional traits as well.

MATERIALS AND METHODS

Soil sampling and establishment of Glycine max bait plants

The root colonizing bacteria of *G. max* bait plants were evaluated. Rhizospheric soil obtained from 10 samples, from a depth of 0–20 cm, were collected from three agricultural production areas located in the municipality of Indiara, southwest of the state of Goiás, Brazil. These areas were chosen for presenting different time histories for soybean cultivation: area 1 was used to cultivate soybean for 30 consecutive

years (17º 9' 29.63" S, 50º 0' 31.36" W); area 2 was used to cultivate soybean for 15 years (17º 9' 27.82" S, 50º 0' 29.38" W); and area 3 was cultivated for only 1 year (17º 10' 0.02" S, 50º 0' 25.50" W). Soil was collected directly from the rhizosphere of G. max plants in the reproductive stage. The plants as well as the soil adhered to the roots were collected with a manual shovel. The plants were discarded and the soil stored for later use. The soil was sampled from Latosol (SANTOS et al., 2018), which was developed initially under Cerrado vegetation. Approximately 24 kg of soil was collected per area and used to establish *G. max* plants that served as bait for the colonization of symbiotic rhizobacteria. Additionally, three soil samples from depths of 0–20 cm were obtained from each area for chemical and granulometric characterization (Table 1).

To establish the bait plants, three 8 L capacity pots were prepared, and 10 soybean seeds of cultivar M7110 IPRO were sown in each pot. When the plants reached the R1 vegetative stage, manual thinning was performed, leaving only 3 plants per pot. The plants were maintained in a protected environment and irrigated according to the field capacity of each pot.

Isolation and selection of $CaHPO_4$ and $FePO_4$ solubilizing rhizobacteria associated with Glycine max bait plants

When the bait plants reached the V3 stage, the roots of the three plants grown in each pot were sampled. These roots were placed in a plastic container, properly labeled, refrigerated, and transported to the Agricultural Microbiology Laboratory of the Federal Goiano Institute Rio Verde campus, where the rhizobacteria were isolated.

The ability to solubilize calcium phosphate ($CaHPO_4$) and iron phosphate ($FePO_4$) was used as an initial criterion for the isolation of rhizobacteria. This is due to the high P content observed in areas 1 and 2; additionally, the Ca content and high Fe content in the soil of the three areas would tend to immobilize P. Furthermore, rhizosphere bacteria tend to express functional traits directly related to the supply of nutrients to plants (MARASCO et al., 2013).

For the qualitative isolation, 10 g of randomly chosen root fragments were shaken (NT712 shaker incubator, Nova Técnica®) in sterile peptone water containing Tween 80 (0.1%) for 30 min at 70 rpm at 25 °C. Then, the supernatant was serially diluted to 10^{-5} in saline solution (NaCl 0.85 %). Aliquots of 1 mL from the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were seeded using the pour plate technique in GELP culture medium (glucose 10 g; peptone 5 g; yeast extract 0.05 g, and agar 15 g L^{-1}) and added to 25 mL of $CaCl_2$ (10%) and 12.5 mL of K_2HPO_4 (10%), forming an inorganic phosphate precipitate, $CaHPO_4$ (10%) (SYLVESTER-BRADLEY et al., 1982). The formation of a transparent halo confirmed the ability of rhizobacteria to solubilize $CaHPO_4$ in contrast to the opaque culture medium around the bacterial colony (SOUCHIE & ABBOUD, 2007).

Iron phosphate ($FePO_4$) solubilization was evaluated in modified basal medium (sucrose 10 g; NaCl 0.1 g; $MgSO_4$ 0.5 g; yeast extract 0.2 g; NH_4Cl 0.5 g; $MnSO._4H_2O$ 0.1 g; $FePO_4$ 2 g L^{-1})

Table 1 - Chemical and granulometric characterization of soils sampled in three *Glycine max* L. cultivation areas in southwest Goiás, Brazil. These areas present different time histories for soybean cultivation. area 1 - soybean cultivation for 30 consecutive years; area 2 – soyabean cultivation for 15 years; Area 3 – soyabean cultivation for 1 year.

Attributes	Area 1	Area 2	Area 3
pH ($CaCl_2$)	5.6	6.1	5.5
Ca (cmolc dm^{-3})	3.8	4.6	4.6
Mg (cmolc dm^{-3})	1.1	1.5	1.1
Al (cmolc dm^{-3})	0.0	0.0	0.0
H+Al (cmolc dm^{-3})	3.4	2.5	4.3
K (cmolc dm^{-3})	1.5	1.3	0.5
P (mg dm^{-3})	29.6	30.0	10.7
Fe (mg dm^{-3})	21.9	22.5	15.1
OM (g dm^{-3})	22.4	23.7	23.7
Sand (g Kg^{-1})	61.0	50.0	66.0
Silt (g Kg^{-1})	15.0	8.0	12.0
Clay (g Kg^{-1})	24.0	42.0	22.0

with bromocresol green dye (0.5%) (GADAGI & SA, 2002). Rhizobacteria solubilizing iron phosphate were recognized by their ability to change the blue color of the medium to a colorless state due to the secretion of organic acids (GADAGI & SA, 2002).

The tests were conducted in triplicate, and the growth of microorganisms and the appearance of solubilization halos of $CaHPO_4$ and $FePO_4$ were evaluated on the fourth day after plating. The solubilizing rhizobacteria colonies were isolated, purified, and stored.

The isolates were purified using streak seeding technique in nutrient agar (NA) (meat extract 3 g; peptone 5 g; and agar 25 g L^{-1}). The isolated colonies were transferred to penicillin flasks containing NA and stored. Every 45 days, the strains were reactivated and again stored. One replicate of each isolate was stored in an ultra-freezer at an average temperature of -80 ºC.

The calcium phosphate and iron phosphate solubilization by the isolated rhizobacteria were also quantitatively evaluated in a liquid medium. Bacterial cultures established in nutrient agar (NA) (meat extract 3 g and peptone 5 g L^{-1}) were standardized with OD_{600} (optical density at 600 nm) of 0.2, by dilution with saline solution (0.85%). Subsequently, 1 mL of the standardized cultures were inoculated in triplicate into penicillin flasks containing 9 mL of GL (glucose 10 g and yeast extract 2 g) culture medium, supplemented separately with two phosphate sources: 5 g L^{-1} calcium phosphate ($CaHPO_4$) or 2 g L^{-1} iron phosphate ($FePO_4$). The pH of the medium was adjusted to 6.5, and the cultures were kept under constant agitation at 90 rpm for 72 h at 28 ºC. As a control, GL medium with each phosphate source, without inoculum, was used. After this growth period, the pH of the cultures was measured, and the amount of inorganic P was determined by the colorimetric method described by (MURPHY & RILEY, 1962). Phosphate solubilization was estimated using the standard curve equation. The amount of solubilized P was obtained by subtracting the amount of soluble P observed in the inoculated sample from that in the corresponding control sample.

The characterization of the solubilization capacity by the isolates was based on three levels: weak, when the microorganism solubilized between 0.0 to 1.9 mg L^{-1} of P; medium, when the microorganism solubilized between a 2.0 to 5.9 mg L^{-1} of P; and strong, when the microorganism solubilized between 6.0 to 12. 2 mg L^{-1} of P. From this classification, the 10 bacterial isolates, which showed higher potential for phosphate solubilization by this test, regardless of the study area, were selected and evaluated for multifunctional potential.

Multifunctional potential of rhizobacteria isolated and selected from Glycine max bait plants

Synthesis of indol-3-acetic acid (IAA)

The production of IAA by rhizobacteria was quantified by the colorimetric method described by GORDON & WEBER (1951). The bacterial isolates were inoculated in 5 mL of nutrient broth medium and incubated at 30 ºC for 24 h under constant agitation (90 rpm). After this growth period, all bacterial samples had their OD_{600} adjusted to 0.2. Subsequently, they were inoculated in triplicate in nutrient broth supplemented with 50 µL of tryptophan and incubated at 30 ºC in the dark for 72 h at 90 rpm. Then, 2 mL of the supernatant was centrifuged (centrifuge TDL80-2B, Centrobio®) at 16,000 x *g* for 5 min at 4 °C and 150 µL of the supernatant was transferred to a microplate, and 150 µL of Salkowski's Reagent ($FeCl_3$ 1.875 g; H_2O 100 mL and H_2SO_4 150 mL) was added. After 20 min of incubation in the dark, the absorbance of samples was read at 530 nm using a spectrophotometer (VersaMax™ Microplate Reader, Molecular Devices). As a negative control, only nutrient broth supplemented with tryptophan and added Salkowski Reagent was used. Indol-3-acetic acid synthesis was quantified using an equation obtained by a standard curve.

Synthesis of gibberellic acid (GA_3)

The bacterial cultures obtained in nutrient broth were standardized with OD_{600} of 0.2 by dilution with saline solution (0.85%) and inoculated in triplicate in flasks containing Mueller-Hinton broth (Oxoid, United Kingdom®) (meat extract 2.0 g; acid hydrolyzed casein (casein peptone) 17.5 g; and starch 1.5 g L^{-1}; pH 7,0± 0.2). Each flask was inoculated with 10% of the overnight grown culture of each strain and incubated at 30 °C for 72 h under stirring (150 rpm). The cultures were centrifuged at 10,000 x *g* for 5 min at 4 °C; the supernatant was collected and kept at 4 °C for subsequent determination of GA_3 concentrations.

Gibberellic acid quantification was determined based on the method described (ABOU-ALY et al., 2019): 1.0 mL of concentrated HCl and 1.0 mL of Folin Ciocalteau reagent (Dynamics®) were added to 1.0 mL of the bacterial supernatant in transparent test tubes. Distilled water was then added to reach the final volume of 6 mL. The mixture was boiled in a water bath for 5 min, and after cooling, the intensity of the bluish-green color produced was measured at 760 nm using a digital spectrophotometer

(UV/VIS 5100, Tecnal®). The synthesis of GA_3 by the isolates was also quantified using an equation obtained from a standard curve.

Production of siderophores

The production of siderophores was evaluated using the universal methodology adapted by SCHWYN & NEILANDS (1987). The bacterial isolates were previously grown in soybean trypto-casein (TSA) culture medium (pancreatic casein digest 15 g; soybean digestive enzyme 5 g; NaCl 5 g; and agar 15 g L^{-1}; pH 7.3 ± 0.2) at a concentration of 4 g L^{-1}, and incubated in an oven at 28 ºC, for 72 h. The cell suspension was then centrifuged at 16,000 x *g* for 10 min, 1 mL of the supernatant was transferred to test tubes, and then 1 mL of chrome azurol S indicator solution (CAS) was added. The conversion of the blue color of the CAS solution in the supernatant to yellow-orange within 15 min indicated that the isolate were capable of producing siderophores.

Antibiosis to the phytopathogens Fusarium sp. and Sclerotinia sclerotiorum

According to dual culture methodology, the rhizobacteria were tested for antagonism against the phytopathogens *Fusarium* sp. and *Sclerotinia sclerotiorum* (MEW & ROSALES 1986). In plates containing potato dextrose agar (BDA) medium (dextrose 20 g; potato 200 g; and agar 15 g L^{-1}), a mycelial disc of 5 mm diameter containing each phytopathogen was inoculated at equidistant points at one end of the plate and the other end, a bacterial culture streak was then made across the length of the plate. Plates containing only the phytopathogen were considered as the control treatment. The plates were incubated at 28 °C for the period necessary for the mycelium of the phytopathogen in the control treatment to cover the entire culture medium (3 to 7 days). The zone of inhibition caused by the different rhizosphere bacteria was evaluated during this period. The test was carried out in triplicate and assessed visually. For this, the diameter of each fungus was measured with a pachymeter, and the zone of inhibition, formed by bacterial production of suppressive compounds, was verified. The percentage of suppression for each treatment was calculated using the relative index (RI):

IR (%) = (RC-RX) / RC×100, where:

RC = colony radius of the pathogen in the control treatment

RX = radius of the pathogen colony paired with the rhizospheric isolate

Molecular identification of multifunctional rhizobacteria

Only bacterial strains that showed multifunctional potential were identified. The identification was made through partial sequencing of the 16S rDNA gene, using primers 27F, and 1492R (WEISBURG et al., 1991). Sequencing was performed by the Sanger method, using the Big Dye Kit in the ABI3100 Applied Biosystem. The procedures were performed at the Instituto Biológico in the state of São Paulo. The sequences obtained were compared with known sequences in the GenBank database (http://www.ncbi.nlm.nih.gov), using a similarity search via Blastn (ALTSCHUL et al. 1990) and considering homology greater than 99.8 %.

Identification was tested based on the phylogenetic relationship of 16S sequences obtained for the rhizobacteria and sequences from this region available on GenBank for other isolates. These sequences were aligned using CLUSTAL OMEGA software (SIEVERS et al., 2011). The *priors* for the determination of the phylogenetic relationship were obtained by selecting the evolution model of the 16S sequences, using Bayesian Information Criterion (BIC), implemented in the software JMODELTEST 2 (DARRIBA et al., 2012). The model HKY + I + G (-lnL = 6128.5857, wBIC = 0.9483, partition = 010010, K = 38, freqA = 0.2185, freqC = 0.2719, freqG = 0.2795, freqT = 0.2301, kappa = 2.8345, p-inv = 0.0620, gamma shape = 0.4390). Phylogenetic analyses were performed by Bayesian statistics implemented in MRBAYES v.3.2.6 software (RONQUIST et al., 2012). Four independent runs were performed, assigning 10 x 10^6 generations to the chains, sampling the a posteriori probability distribution every 500 generations. Before calculating the consensus tree and to ensure convergence of the chains, the first 2,500 sampled trees were discarded. Subsequently, the recovered phylogeny was tested by the bootstrap method, with 5,000 replications, using the MEGA 7 program (KUMAR et al., 2016). The species *Methylobacterium* sp. was used as an outgroup.

Data analysis

All tests were conducted under an entirely randomized design. The relationships between the characteristics of the sampled soils and the number of isolates solubilizing different rates of P were analyzed together by a correlation matrix and combined in a principal component analysis (PCA). As these variables had different measurement units, a correlation PCA was constructed using standardized data to have a mean of 0 and a standard deviation of 1. The number

of components was chosen as a function of eigenvalues (>1.0) and explained variance (above 80%).

The following data were obtained for the different functional traits evaluated: phosphate solubilization, synthesis of IAA and GA_3, and antibiosis to phytopathogens. An analysis of variance (ANOVA) was conducted to identify significant differences among the isolates. Among significantly different isolate pairs, the Scott-Knott test was used to compare the means at 5% significance. Additionally, the relationship between pH and the amounts of free P obtained by microbial action was evaluated through Pearson's correlation coefficient, and the strength of the relationship was analyzed using the values of r and the significance of the interaction at 5% significance. Alternatively, a correlation PCA was performed, seeking to evaluate the relationship of the isolates with the different functional traits, aiming to identify isolates with multifunctional potential. The number of components was chosen according to the eigenvalues (>1.0) and explained variance (above 80%). All statistical tests were performed in R version 4.0.4 R (CORE TEAM, 2021).

RESULTS

Isolation and selection of rhizobacteria solubilizing $CaHPO_4$ and $FePO_4$

A total of 139 strains of rhizobacteria were obtained from the bait plants, of which 56 were interacting with the roots of plants grown in soil from area 1, 45 strains from area 2, and 38 from area 3. Of the strains isolated from area 1, 32 weakly solubilized $CaHPO_4$, 8 solubilized moderately, and 16 solubilized strongly (Figure 1a). From area 2, while 39 strains were able to weakly solubilize $CaHPO_4$, six solubilized moderately; however, no strain showed potential to strongly solubilize this phosphate source. Of the strains from area 3, 27 solubilized $CaHPO_4$ weakly, four solubilized moderately, and seven strongly. Regarding $FePO_4$, 17 strains isolated from area 1 were able to solubilize this phosphate weakly, while 30 solubilized it moderately, and 9 weakly. In area 2, 12 strains solubilized weakly, 20 solubilized moderately, and 13 solubilized strongly. In area 3, nine of the bacteria solubilized $FePO_4$ weakly, 24 solubilized moderately, and 5 solubilized it strongly (Figure 1b).

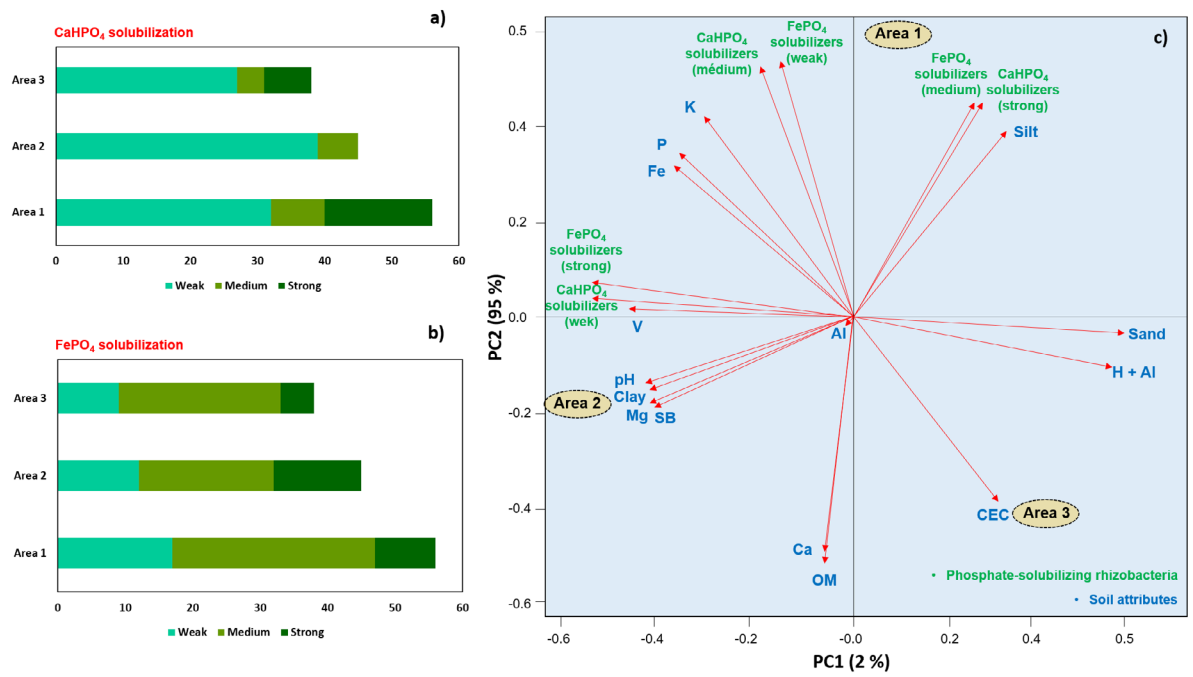

Figure 1 - The number of rhizobacteria solubilizing calcium phosphate ($CaHPO_4$) (a) and iron phosphate ($FePO_4$) (b) and principal component analysis (PCA) of the relationship between soil attributes and the number of rhizobacteria (c) isolated from *Glycine max* L. bait plants grown in soils from three areas with different time histories of soybean cultivation. Area 1 - soybean cultivation for 30 consecutive years; Area 2 - cultivation for 15 years; Area 3 - cultivation for 1 year. OM = organic matter; CEC = cation exchange capacity; SB = sum of bases.

When the relationship between the characteristics of the soil and the solubilizing potential of the strains isolated from the soil from the different areas was analyzed, a relationship was observed between the greatest quantity of isolated phosphate-solubilizing bacteria and the greatest concentrations of P, Fe, K, and silt in the soil from Area 1 (Figure 1c). On the other hand, in the soil from area 3, the greatest CEC, OM, H+ Al, and Ca and sand concentrations were related to the smallest amounts of isolated solubilizing bacteria.

For the two phosphate sources evaluated, negative and significant correlations were observed between the pH values and the amounts of P made available by the rhizobacteria isolated from the soils of the three areas analyzed (Figure 2a and b). These results indicated that the solubilizing potential of the strains is associated with the release of organic acids.

When $CaHPO_4$ solubilization potential was compared among the ten most solubilizing strains, the highest solubilization averages were achieved by strains SAC36, SAC33, SAF11, and SAF9, and, which provided respectively 10.87, 10.87, 10.54, and 12.15 mg L^{-1} of P (Figure 3a). These solubilization levels were proportional to the low pH values observed in the growing media of these isolates, respectively 3.72, 3.72, 3.53, and 4.00 (Figure 3b). For $FePO_4$ solubilization, the best activity was observed in isolates SAC36, SBF3, SAF11, SAF9, BRF28, and BRC9, providing respectively 6.61, 7.62, 7.44, 8.40, 6.32 and 7.34 mg L^{-1} of P (Figure 3a). These solubilization values are related to low pH values mainly for isolates SAC36, SBF3, SAF11, and SAF9, respectively 4.35, 4.34, 4.43, 4.30 (Figure 3b).

Multifunctional potential of phosphate solubilizing rhizobacteria

The rhizobacterium BRC7, despite not solubilizing large amounts of P, proved to be a great producer of IAA, synthesizing 29.68 µg mL^{-1} of the phytohormone. This was followed by the isolates SAC35, SAF11, and SBF3 that synthesized 20.21, 17.00, and 16.13 µg mL^{-1} of IAA, respectively (Figure 4a). Regarding the growth promoter GA_3, the isolates BRC11 and SAF11 synthesized 427.63 and 385.68 µg mL^{-1} of this phytohormone (Figure 4b). The evaluation of the bacterial strains regarding the production of siderophores showed that strains SAC36 and BRC11 produce hydroxamate-type siderophores (indicated by orange color formation). In contrast, strains SAF9, SAC33, and BRC9 produce catechol-type siderophores (indicated by light purple coloration). The other strains evaluated were negative for siderophore production (Figure 5).

All the phosphate solubilizing isolates evaluated presented inhibition rates relative to the

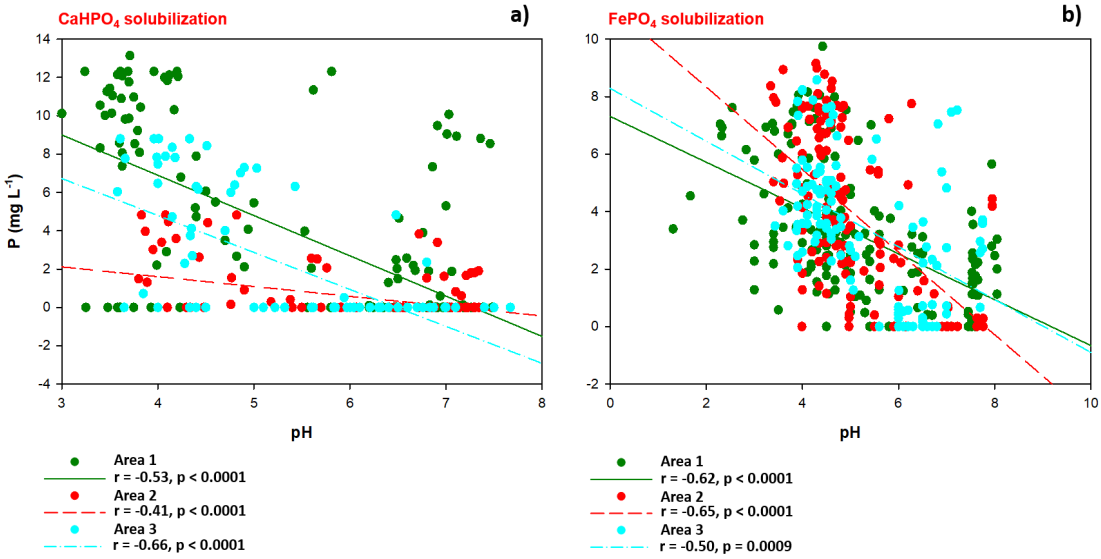

Figure 2 - Correlation between final pH of the culture medium individually supplemented with calcium phosphate ($CaHPO_4$) (a), and iron phosphate ($FePO_4$) (b), and the content of soluble P (mg L^{-1}) made available by rhizobacteria isolated from *Glycine max* L. bait plants cultivated in soils from the three areas with different time histories of soybean cultivation. Area 1 - soybean cultivation for thirty consecutive years; Area 2 - cultivation for fifteen years; Area 3 - cultivation for only one year.

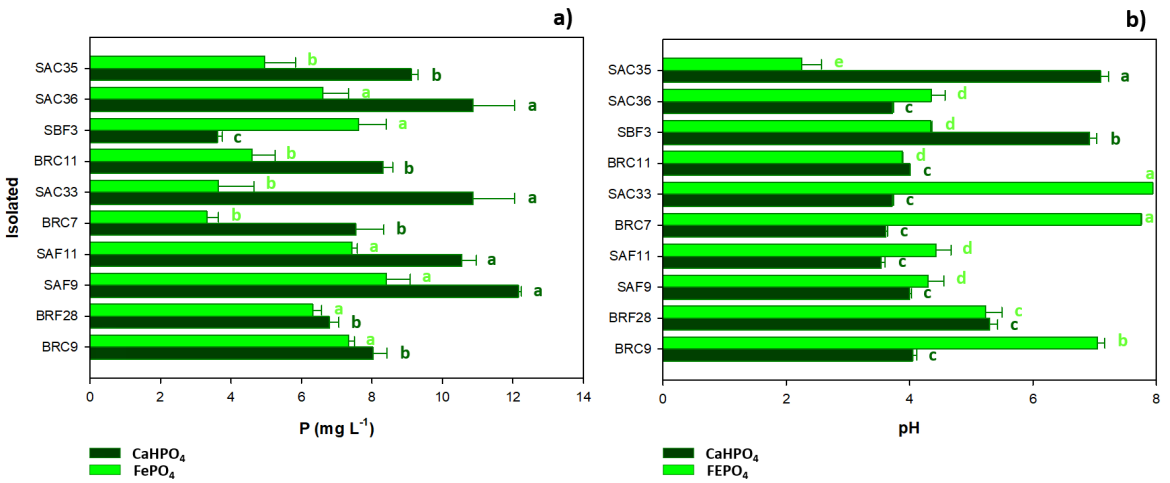

Figure 3 - Calcium phosphate ($CaHPO_4$) and iron phosphate ($FePO_4$) solubilization (a) pH of culture medium (b) observed for phosphate solubilizing rhizobacteria isolated from *Glycine max* L. bait plants cultivated in soils from three areas with different time histories of soybean cultivation. Means followed by the same letter did not differ significantly at $P < 0.05$ (Scott-Knott test).

phytopathogens tested. The highest inhibition activity for *Fusarium* sp. was observed in the strain SAF11 (36.31%; Figure 6a). However, in the case of *Sclerotinia sclerotiorum*, the strains SAF9 and BRC11 showed greater potential for inhibition of the mycelial growth, respectively 51.78% and 43.40% (Figure 6b).

When the phosphate solubilizing rhizobacteria were analyzed together for all functional traits using PCA, four isolates stood out as they were associated with the highest solubilization rates, lowest pH values, highest GA_3 production, and highest relative inhibitions of *Fusarium* sp. and *Sclerotinia sclerotiorum*. These four isolates were SAC36, SAF9, SAF11, and BRC11 (Figure 7).

Molecular identification of multifunctional rhizobacteria

The rhizobacteria that stood out for their multifunctional potential were identified by phylogenetic interaction. The isolate BRC11 was identified as *Pseudomonas fluorescens* upon formation of a stable clade with another isolate of this species (Figure 8). The isolates SAF11 and SAF9 were identified as *Brevibacillus* sp. formed an interactive clade with *Brevibacillus brevis*, *Brevibacillus formosus,* and also *Brevibacillus parabrevis*. The isolate SAC36, however, was identified as *Bacillus velezensis* through a monophyletic clade formation with other isolates of this species. It is also important to highlight that of the four multifunctional isolates that stood out, three were obtained from the interaction of *G. max* plants with soil from area 1 (SAF11, SAF9, and SAC36) and one obtained from this interaction with soil from area 3 (BRC11).

DISCUSSION

Soil attributes of soybean cultivation areas select functional microbial groups

The use of soybean bait plants enabled the isolation of 139 strains of phosphate solubilizing rhizobacteria and four strains with multifunctional potential. These results are superior to those observed by NDUNG'U-MAGIROI et al., (2012) who evaluated a total of 150 isolates from different Kenyan soils cultivated with soybean and observed that only 5% of the total isolates were efficient in solubilizing phosphates.

The highest number of solubilizing bacteria were isolated from the interaction of bait-plants with soil in area 1, with a history of soybean cultivation of 30 consecutive years. The high concentration of P in the soil of this area, associated with the high concentration of Fe, which can immobilize P reserves, stimulates the rhizospheric attraction of phosphate solubilizing bacteria, an important functional trait for the growth and development of plants established in this condition. In fact, the characteristics observed in the soil directly interfere with the functional traits expressed by the resident microbiota (ROCHA et al., 2020). MATTER et al. (2020); however, evaluated the

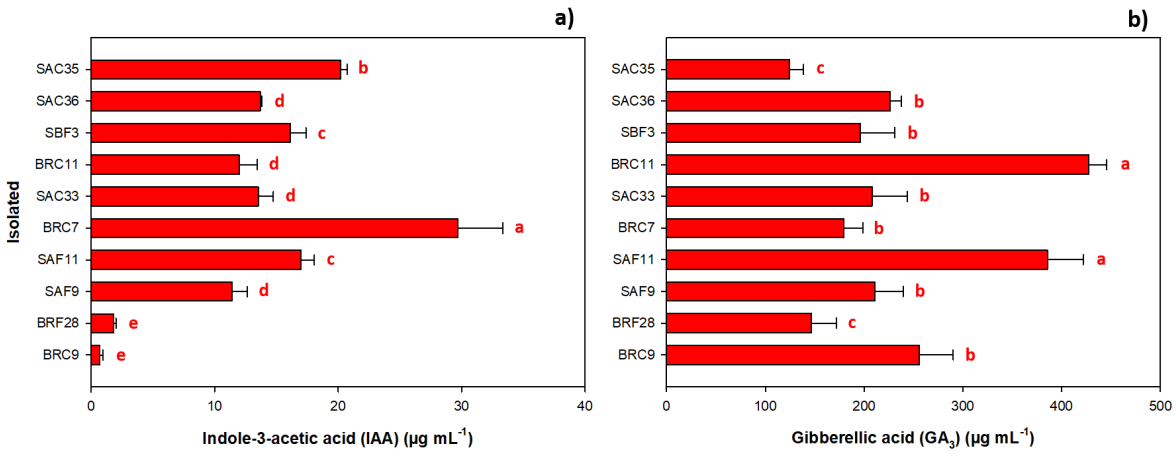

Figure 4 - *In vitro* production of indol-3-acetic acid (IAA) (a) and gibberellic acid (GA_3) (b) observed for phosphate solubilizing rhizobacteria isolated from *Glycine max* L. bait plants cultivated in soils from three areas with different time histories for soybean cultivation. Means followed by the same letter did not differ significantly at $P < 0.05$ (Scott-Knott test).

ability of bacteria to solubilize inorganic phosphates in soils with different fertilization histories and suggested a negative relationship between phosphorus concentrations and the number of BSF in fertilized soils. Our differential results can be explained by the agricultural management of production environments. Many studies suggested that different types of plants and soils select specific microbial communities, influenced in particular by physical properties (texture), soil chemical properties (pH), and nutrient availability (NDUNG'U-MAGIROI et al., 2012; GOSS-SOUZA et al. 2017; ROCHA et al., 2020).

The reduction in pH of the culture medium by the isolates correlates negatively with increasing levels of soluble P. These data corroborated with those observed by other authors (MONROY et al., 2020; SILVA et al., 2021) since medium acidification constitutes an important mechanism associated with P availability (BOLAN et al., 1994; MARDAD et al., 2013; MARRA et al., 2015). The correlations observed; however, were moderate. This is because other mechanisms besides the production of organic acids may be related to the availability of P from insoluble sources. For example, the release of H^+ ions during the assimilation of ammonium (NH_4^+) or by other metabolic reactions such as respiration may trigger the extrusion of protons (ILLMER & SCHINNER, 1995; SHARMA et al., 2013).

Rhizobacteria isolated and selected from Glycine max bait plants are multifunctional and can be indicated as plant growth promoters

The strains SAF09 (*Brevibacillus* sp.), SAF11 (*Brevibacillus* sp.), and SAC36 (*Bacillus velezensis*), isolated from the production environment with a 30-year history of cultivation had solubilization values

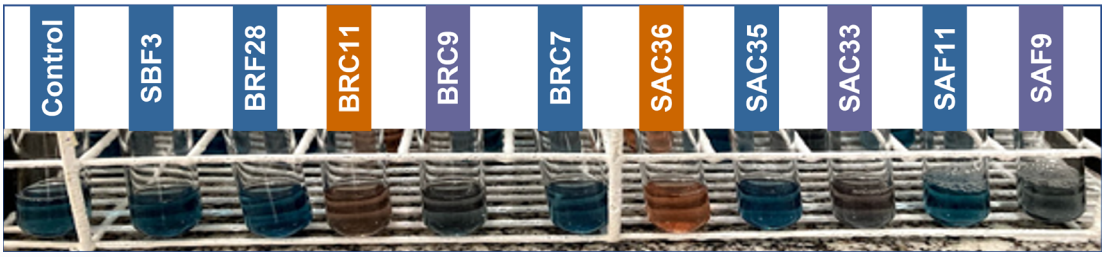

Figure 5 - *In vitro* production of siderophores observed for phosphate solubilizing rhizobacteria isolated from *Glycine max* L. bait plants cultivated in soils from three areas with different time histories for soybean cultivation.

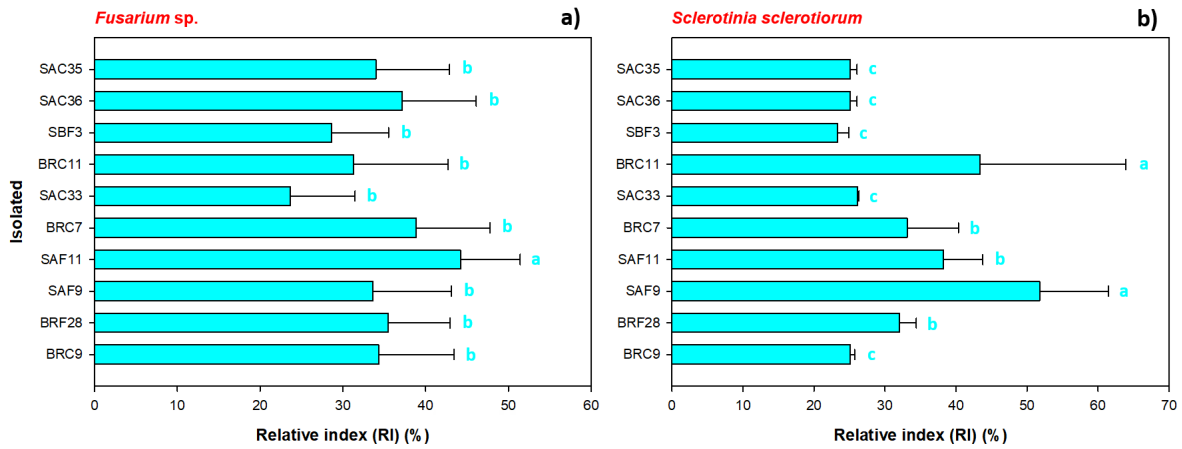

Figure 6 - Relative inhibition (%) of mycelial growth of the phytopathogenic fungi *Fusarium* sp. (a) and *Sclerotinia sclerotiorum* (b) by phosphate solubilizing rhizobacteria isolated from *Glycine max* L. bait plants grown in soils from three areas with different time histories of soybean cultivation. Means followed by the same letter did not differ significantly at $P < 0.05$ (Scott-Knott test).

between 10.54–12.20 mg L^{-1} of P from $CaHPO_4$. These values are in agreement with those observed by MA et al., (2018) when they evaluated rhizobacteria solubilizing $CaHPO_4$ from peanuts, cotton, tobacco, and lettuce, finding an average of 10.17 mg L^{-1} of P solubilized by *Paenibacillus mucilaginosus*, belonging to the same phylum as the bacteria observed in this study. Our isolates; however, are more effective in solubilizing $CaHPO_4$ in vitro than those evaluated by REIS et al., (2021) when investigating the potential of strains of *Bacillus thuringiensis* (SC10), *Paenibacillus alvei (*PA12), and *Bacillus cereus* (SC5) in liquid medium containing $CaHPO_4$, observed 5, 5.62, and 5.13 mg L^{-1} of P solubilization, respectively. However, it should be taken into account that adverse factors such as temperature, pH, and nutritional composition of the culture medium can affect growth and metabolism, promoting variations in phosphate solubilization potential among strains (WALPOLA et al., 2012; YANG et al., 2018; REIS et al., 2021).

The isolates that stood out in terms of multifunctional potential beyond phosphate solubilization were SAF9 (*Brevibacillus* sp.), SAF11 (*Brevibacillus* sp.), SAC36 (*Bacillus velezensis),* and BRC11 (*Pseudomonas* sp.). RASCOVAN et al., (2016) analyzed the microbiomes associated with soybean roots under agricultural field conditions, and observed that 55% of the selected isolates showed in vitro growth promotion activities, among them a high proportion of *Pseudomonas, Bacillus, Variovorax, Burkholderia,* and *Stenotrophomonas* were found. On the other hand, NDUNG'U-MAGIROI et al., (2012) identified 150 isolates from different soils of soybean cultivation in Kenya. Among these isolates, *Bacillus megaterium*, *Bacillus* sp., and *Arthrobacter* sp. were the most abundant and well distributed in these soils.

Bacteria of the genus *Brevibacillus* are known for their wide distribution and production of various enzymes of biological interest (PANDA et al., 2014). Alternatively, the growth-promoting potential of these bacteria has been attested in several works (HOU et al. 2015; NEHRA et al., 2016; WANG et al., 2021). RAY et al., (2020) argued that *Brevibacillus* has high agroecological significance with potential for plant growth-promotion, biocontrol against plant diseases, and for effective soil bioremediation to remove toxic heavy metals from the soil, water, and atmosphere. CHANDEL et al., (2010) attested to the ability of *Brevibacillus brevis* to inhibit the phytopathogen *Fusarium oxysporum* in tomato culture. Similar results were observed for *Brevibacillus* sp. in maize (JOO et al., 2015). The strain *Brevibacillus* sp. (SP-03) isolated from the corn rhizosphere presented characteristics of plant growth promotion, such as N_2 fixation, IAA production, ammonia production, and siderophore production in vitro in the study developed by CHAKRA et al., (2019). In this research, strains SAF9 (*Brevibacillus* sp.) and SAF11 (*Brevibacillus* sp.) solubilized $CaHPO_4$ and $FePO_4$, synthesized IAA, and significantly inhibited the mycelial growth of phytopathogens *S. sclerotiorum* and *Fusarium* sp.

Bacillus velezensis was previously reported as a later heterotypic synonym of *Bacillus*

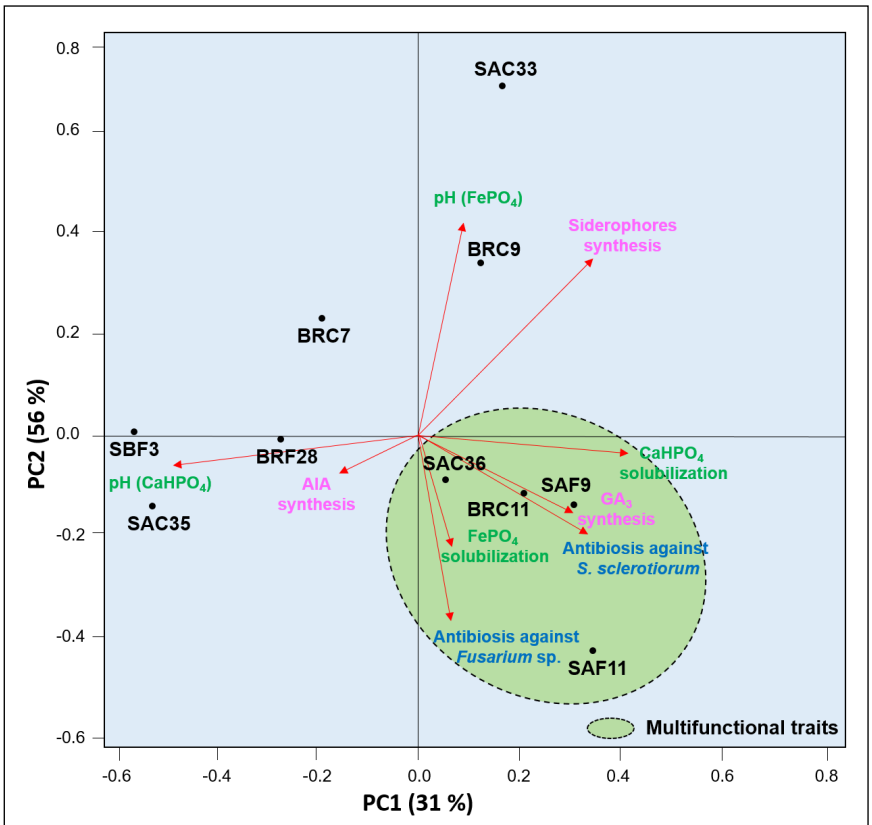

Figure 7 - Projection in the two dimensions of the PCA of the functional traits (solubilization of $CaHPO_4$ and $FePO_4$, pH of $CaHPO_4$, and $FePO_4$ solubilization medium, synthesis of IAA and GA_3, siderophore production and antibiosis to the phytopathogens *Fusarium* sp. and *Sclerotinia sclerotiorum*) evaluated for phosphate solubilizing rhizobacteria isolated from *Glycine max* L. bait plants grown in soils from three areas with different time histories for soybean cultivation.

amyloliquefaciens (WANG et al., 2008); however, genomic analyses have shown that it is not a synonym *B. amyloliquefaciens*. Instead, they proposed that *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *Plantarum,* and *B. oryzicola* should be reclassified as posterior heterotypic synonyms of *B. velezensis* (DUNLAP et al., 2016). *Bacillus oryzicola* is a recently described species isolated as an endophyte from rice (*Oryza sativa*). The strain has been shown to have an antagonistic activity to phytopathogens in greenhouse trials, and the 16S rRNA gene was reported to have 99. 7% sequence similarity with *Bacillus siamensis* and *B. methylotrophicus* (CHUNG et al., 2015) which are both known for their antagonism to phytopathogens and for promoting plant growth (MADHAIYAN et al., 2010; MA et al., 2013). ADENIJI et al., (2019) highlighted the potential of *B. velezensis* for agricultural use; YE et al., (2018), as well as FAN et al., (2018), signaled its potential for biocontrol of phytopathogens. The SAC36 strain of *B. velezensis* isolated in this work inhibited 37% of the mycelial growth of *Fusarium* sp. and 25% of the growth of *S. sclerotiorum*, in addition to synthesizing siderophores and solubilizing $CaHPO_4$ and $FePO_4$. According to RABBEE et al., (2019), *B. velezensis* possesses specific groups of genes related to the biosynthesis of secondary metabolites, which play significant roles in pathogen suppression and plant growth promotion. More specifically, *B. velezensis* exhibits a high genetic ability to synthesize cyclic lipopeptides (i.e., surfactin, bacillomycin-D, fengycin, and bacillibactin) and polyketides (i.e., macrolactin, bacillaene, and difficidin). Secondary metabolites produced by *B. velezensis* can also trigger induced systemic resistance in plants, a process by which plants defend themselves against recurrent attacks by virulent microorganisms.

Conversely, bacteria of the genus *Pseudomonas* are frequently reported as phosphate

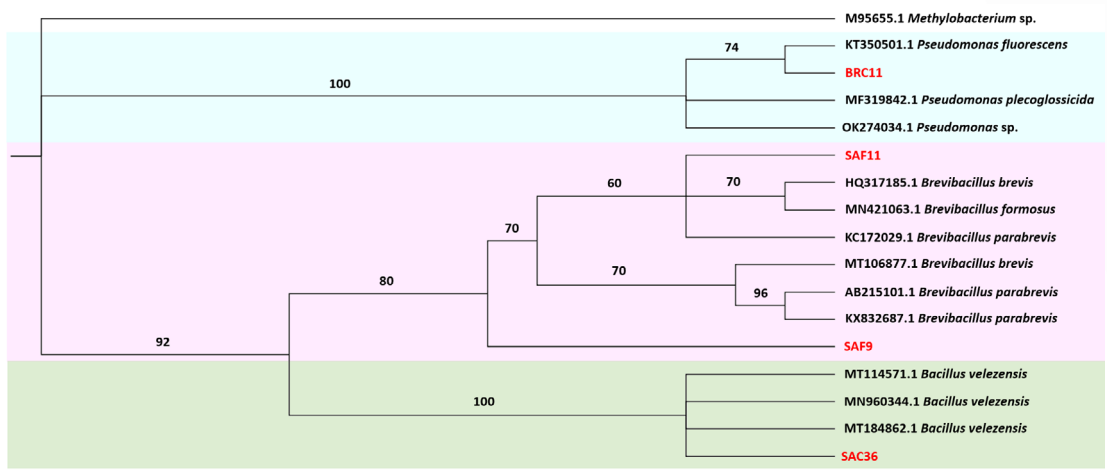

Figure 8 - Relationship and classification of multifunctional rhizobacteria isolated from *Glycine max* L. bait plants grown in soils originating from three areas with different time histories of soybean cultivation. Colors represent differentially established clades. Values are the a posteriori probability of the nodes.

solubilizers (ROSAS et al., 2006; VYAS & GULATI, 2009; PAUL & SINHA., 2017). In addition, research shows that *Pseudomonas* can fix nitrogen, synthesize IAA, and control plant pathogens (PATTEN & GLICK, 2002; LI et al., 2017; MELIANI et al., 2017). In this study, we identified *Pseudomonas fluorescens* strain BRC11 as a significant producer of gibberellin, carboxylate-type siderophores, and inhibitors of phytopathogens, as it reduced mycelial growth of *Fusarium* sp. and *S. sclerotiorum* by 43.40% and 31.30%. PARK et al., (2015) suggest that *P. fluorescens* promotes plant growth by synthesizing volatile organic compounds, including 13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene. These compounds play essential roles in modulating growth and inducing pathogens' systemic resistance (ISR). *Pseudomonas* spp. are responsible for the natural immunity of some plants to soil-borne pathogens. They suppress the growth of pathogenic microorganisms by producing antibiotics, bacteriocins, siderophores, hydrolytic enzymes such as β-1,3-glucanase and chitinases, and other antimicrobial and resistance response metabolites such as phytoalexins (CHOI, 2008; DAVID et al., 2018; PRABHUKARTHIKEYAN et al., 2018; SHAHID et al., 2021). *Pseudomonas fluorescens* synthesizes a pyrroloquinoline quinone that acts as an important plant growth-promoting factor. Thus, this rhizobacterium possesses several characteristics that confer biocontrol activity and promote plant growth (SURESH et al., 2021). It proliferates in vitro and can be mass-produced, rapidly utilizes seed and root exudates, colonizes and multiplies in the rhizosphere and spermosphere environments, and contributes to improving soil enzyme activity (DAVID et al., 2018; SIPAHUTAR et al., 2018).

CONCLUSIONS

Overall, this study demonstrated the efficiency of using *G. max* bait plants in attracting and facilitating colonization by multifunctional rhizobacteria. Furthermore, we demonstrated that soil characteristics are essential to define the resident microbiota, so management is important in defining the microbial composition of soils. Soils from areas with anthropic management conducted for more than 30 years showed a higher concentration of rhizobacteria solubilizing phosphates compared to soils from areas with half the management time or only one year of cultivation.

Through the results obtained for SAF9 (*Brevibacillus* sp.) and SAF11 (*Brevibacillus* sp.), this work attests to the growth-promoting potential of bacteria of the genus *Brevibacillus*. We also confirmed the already reported potential of *P. fluorescens* and *B. velezensis* to interact symbiotically with the plant rhizosphere.

Our results also confirmed the hypothesis that different bacterial isolates obtained from rhizosphere constructed using soils from the three areas with different time histories for soybean cultivation possess essential functional traits that classify them as plant growth promoters. These rhizobacteria can

be essential within the perspective of obtaining bio-inputs for plant growth promotion in a direct, indirect, or synergistic way, contributing to increasing the range of bioinoculants aimed at soybean cultivation and for more sustainable agricultural practices.

This study provided considerable information on rhizospheric microorganisms from soybean cultivation areas located in the Cerrado biome. The findings in this study will support future research to better understand how environmental factors affect the functional characteristics of the different microbial groups sampled in this region.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHOR'S CONTRIBUTIONS

DAFF, LCV and ELS conceived and designed research. DAFF, CFS and NTT conducted measurements. CFS and MVAV contributed new methods in analysis. DAFF analyzed data. DAFF and LCV wrote the manuscript. All authors read and approved the manuscript. Funding acquisition is connected to the two supervisors, LCV and ELS.

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