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16S rRNA molecular analysis, phosphorus solubilization, and influence on the growth of soybean (*Glycine max*) and maize (*Zea mays*) by bacteria isolated from the soil

Análise molecular *16S rRNA*, solubilização do fostato e influência no crescimento de soja (*Glycine max*) e milho (*Zea mays*) por bactérias isoladas do solo

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Abstract

Microorganisms play an active role in Phosphorus (P) transformations in the soil, influencing its availability to plants and its flux in nature. The transformations result from the decomposition and mineralization of organic compounds, immobilization in microbiomes, and solubilization of inorganic minerals. The basis of this study was to evaluate 10 bacterial isolates, aiming to characterize them as: growth pattern in TY medium (Tryptone Yeast); growth pattern on NBRIP medium (National Botanical Research Institute's Phosphate); solubilization of calcium hydroxide phosphate ($Ca_5(OH)(PO_4)_3$); pH variation in different media; partial sequencing of the *16S rRNA* gene, and influence of growth, in a greenhouse, for soybean (*Glycine max*) and maize (*Zea mays*) crops. Among the ten isolates, the best P solubilizers were LGA05-V0513, LGA06-V0517 (*Bacillus cereus*), and LGA14-V20J (*Arthrobacter echigonensis*), while the worst one was the isolate LGA08-V20C (*Acinetobacter* sp.). The main isolates that benefited the development of soybean and maize plants belong to the genus *Bacillus*.

Additional keywords: Phosphorus-solubilizing bacteria (PSB), Plant growth-promoting rhizobacteria (PGPR), *Bacillus* spp., pH acidification.

Resumo

Os microrganismos têm participação ativa nas transformações do fósforo (P) no solo, influenciando sua disponibilidade para as plantas e seu fluxo na natureza. As transformações resultam de decomposição e mineralização de compostos orgânicos, imobilização na microbiomassa e solubilização das formas inorgânicas dos minerais. A base desse estudo consistiu em avaliar 10 isolados bacterianos, visando caracterizá-los quanto: padrão de crescimento em meio TY (Tryptone Yeast); padrão de crescimento em meio NBRIP (National Botanical Research Institute's Phosphate); solubilização de fosfato de hidróxido de cálcio

$Ca_5(OH)(PO_4)_3$); variação do pH nos diferentes meios; sequenciamento parcialmente do gene *16S rRNA*, e influência do crescimento, em casa de vegetação, para as culturas da soja (*Glycine max*) e milho (*Zea mays*). Entre os dez isolados, os melhores solubilizadores de P foram LGA05-V0513, LGA06-V0517 (*Bacillus cereus*) e LGA14-V20J (*Arthrobacter echigonensis*), enquanto o pior foi o isolado LGA08-V20C (*Acinetobacter* sp.). Os principais isolados que beneficiaram o desenvolvimento das plantas de soja e milho pertencem ao gênero *Bacillus*.

Palavras-chave adicionais: Bactérias solubilizadoras de fósforo (PSB), Rizobactérias promotoras de crescimento de plantas (PGPR), *Bacillus* spp., Acidificação de pH.

Introduct

Phosphorus (P) is an essential nutrient for plant growth and development, involved in important metabolic pathways such as photosynthesis, biological oxidation, nutrient absorption, and cell division (Mendes et al., 2022). Soils around the world are supplemented with inorganic P as chemical fertilizers to improve agricultural production. Due to the high demand for nutrients, it is important to search for sustainable strategies to alleviate the harmful effects of intensive agriculture practices that use large amounts of P. The microbial solubilization of this fertilizer has been seen as a valuable alternative for the sustainable agricultural system (Nosheen et al., 2021). Worldwide, 99% of phosphate fertilizers are produced from phosphate reserves projecting estimated values close to 71 billion tons of fertilizer long for 80 and 240 years, following current consumption demand. The soybean crop consumes about 7 kg of P_2O_5 to produce 1 ton of grains. For maize crops, to obtain the best productivity it is indicated the use of about 80 kg of P_2O_4 to produce 6 tons of grain (Ameyu & Santos, 2020, Walsh et al., 2023). According to the Brazil-International Production Assessment Division (IPAD), 162 million tons of soybean grains and 137 million tons of maize grains were produced during the 2022/2023 Brazilian harvest (USDA, 2024). Brazil ranks 3rd position in the consumption of fertilizers worldwide presenting a consumption of 7.01 million metric tons of phosphate fertilizer (Statista, 2024).

A sustainable agricultural alternative to minimize the use of this fertilizer would be the application of bacteria that solubilize the phosphate and make it available to plants. It is known that solubilizing microorganisms can convert P-insoluble to P-soluble, through processes that release organic acids, chelation, and ion exchange (Amarasinghe et al., 2022). The development of knowledge on P solubilization leads to the production of plant inoculants based on Phosphorus-solubilizing bacteria (PSB), also named phosphobacteria. Their application became popular in countries such as India and Russia even though their benefits remain dubious concerning its efficacy as an agricultural input. Those bacteria are distributed among diverse genera presenting in soils standing out *Bacillus, Pseudomonas*, and *Agrobacterium* genera. The major mechanism of solubilization consists of the production and liberation of organic acids by bacterial metabolism. Many heterotrophic bacteria produce phytases which are phosphatases able to hydrolyze phosphate (Kour et al., 2020, Vashishth et al., 2023).

Some bacteria tolerate different media conditions offering variable nutrients as carbon and nitrogen sources, besides pH and temperature changes, viewing the production of an efficient biofertilizer. *Pseudomonas libanensis, P. libanensis, Bacillus siamensis, Bacillus sp.* MVY-004 and *B. pumilus* were already tested in different growth conditions for solubilizing iron phosphate and tricalcium phosphate. Among them, *B. pumilus* has proven a better ability to tolerate pH changes due to its metabolic versatility using different carbon sources (Mažylytė et al., 2022, Sanchez-Gonzalez et al., 2022).

Besides their commercial importance, due to the large amount of phosphate fertilizers required for soybean and maize production, this study aimed to evaluate 10 bacterial isolates from the microbial community of soils fertigated with vinasse to characterize them as to (i)

growth curve on TY (Tryptone-Yeast) and NBRIP (National Botanical Research Institute's Phosphate) Broth; (ii) Araxá phosphate effective solubilization capacities and pH profile; (iii) partial sequencing and phenetic classification using 16S ribosomal gene (*16S rRNA*); and (iv) influence on the growth of soybean and maize crops in greenhouse.

Material and method

Origin of the bacterial isolates and growth curve. Ten bacterial isolates from the Laboratory of Applied Genetics (LGA, FCAV/UNESP, Brazil) were used: LGA05-V0513, LGA06-V0517, LGA07-V0508, LGA08-V20C, LGA09-V20L, LGA10-V20I, LGA11-V0522, LGA12-V05D, LGA13-V20F, LGA14-V20J. The strains came from soil samples from the Itaquerê Farm, Santa Fé S.A. Mill (Nova Europa/Brazil). The samples were collected at a depth of 0-20 cm (Omori et al., 2016). The bacterial isolates were obtained after homogenization and serial dilution of 1 g of soil sample in saline solution [NaCl 0.85% (w/v)], at 250 rpm for 30 min at room temperature. Dilutions from 105 to 108 were distributed in PEG medium [composed of (g/L): K_2HPO_4 (0.6), $MgSO_4$ (0.2), NaCl (0.1), yeast extract (1.0), glucose (10.0) and Agar (9.0); pH 6.9], plus cycloheximide (300 µg/mL), and incubated for 15 days at 28 °C. Different colonies were obtained and cultured in PEG broth for 48 h at 28 °C, 120 rpm, and stored at -80 °C in the presence of glycerol [20% (v/v)].

Partial DNA sequencing of the 16S rRNA gene and similarities. Genomic DNA sampled from bacterial strains was obtained based on the method described by Alves et al. (2020). The DNA was resuspended in 100 µL of TE (10:1) pH 8.0, at 4 ºC. After electrophoretic characterization [Agarose 0.8% (w/v)] and DNA quantification in a Nanodrop 1000 Spectrophotometer (ThermoScientific - Uniscience), the DNA samples were stored at -20 ºC. Following DNA purification, 1.5 Kb amplicons were obtained from the *16S rRNA* gene by PCR, to determine the molecular signature of each isolate. The universal primers (oligonucleotides) fD1 (8-27) <5'-AGA GTT TGA TCC TGG CTC AG-3'> and rD1 (1525-1541) <5'-AAG GAG GTG ATC CAG CC-3'> (Weisburg et al., 1991), described for the target regions of Escherichia coli K12, were applied. The amplification reaction consisted of 40,0 ng of target DNA; 7,5 pmol of each universal primer; 1,75 mM of $MgCl_2$; 0,2 mM of triphosphate deoxyribonucleotides (dNTPs); 2,0 ml of 10 X buffer; 1U of the Taq DNA Polymerase (Invitrogen); in a final volume of 20 µL. The amplification program was: 94ºC / 5 min; 35 cycles at 94ºC / 30 s, 56ºC / 40 s, 72ºC / 90 s; 72ºC / 7 min, performed on a PTC-100 ™ programmable thermal controller (MJ Research, Inc.). Each amplicon was purified using the GeneClean Kit (Bio 101) and submitted for DNA sequencing based only on fD1 primer in the ABI 3100 Automated Capillary Sequencer (PerkinElmer). Close to 1300 bp sequence was obtained using that platform. Bacterial affiliations were performed based on partial *16S rRNA* sequences. Sequences prove to be useful from Phred ≥ 20 quality following the nucleotide similarity query against the GenBank (non-redundant) nucleotide database (National Center for Biotechnology Information - NCBI), using the BLASTn tool (Altschul et al., 1990). The parameter of choice was ≥ 92% in BLASTn for the greatest similarities. Sequence alignment was performed using BioEdit Sequence

Alignment Editor v.7.1.9 (Hall, 1999), and a Neighbour-joining tree based on Jukes-Cantor distances between sequences was obtained with 1,000 bootstrap repetitions by MEGA 7.0 (Kumar et al., 2016).

Evaluation of P solubilization on NBRIP medium. For this bioassay, phosphate rock from Araxá/MG (24% P) was used in the form of $Ca_5(OH)(PO4)_3$, based on Inui et al. (2012). The first screening was performed on Phosphate 5 g/L in a solid NBRIP medium. Solubilization capacity was accounted for by the measuring of a clear halo around the colonies and the colony diameter viewed until the 15th day after inoculation. Data was taken and converted into solubilization efficiency (E) classified as low (E < 2), average (2 < E <3), or high (E > 3) (Silva Filho & Vidor, 2000). In addition, subsequent bioassays were accomplished in liquid NBRIP medium starting from 1.5 (DO_{600}) cell suspension. Cellular cultivation and suspension were checked until 120 h. To assess soluble P in each broth, cell cultures were submitted to centrifugation at low speed (420 x g, 2 min at 25 ºC) to sediment insoluble P particles and the supernatant was reserved for further analysis using the molybdate-vanadate method (Malavolta et al., 1989). To evaluate the acidification behavior of bacterial strains in the broth, the pH reading was monitored each 24 hrs. The experiments were performed in triplicate and statistical analysis was executed using GraphPad Prism software (http://www.graphpad.com/scientific-software/prism/).

Plant growth-promoting rhizobacteria (PGPR) potential in the greenhouse. PGPR effect on soybean (M6210) and maize (AS1633) plants was tested. Seeds (COOPERCITRUS Rural Producers Cooperative) were disinfested by immersion in 70% (v/v) ethanol and 4% (v/v) sodium hypochlorite, followed by three washes in sterile distilled water and then sowed in 290 cm3 tube containing vermiculite. A completely randomized design (CRD) was applied (two crops x 10 isolates/inoculations) taken experiments entirely at random being performed in seven replicates using non-inoculated seeds as controls. After germination, buds were inoculated with a suspension of YMA broth containing 109 cells/mL and irrigated with 10 ml of sterile autoclaved GIBSON nutrient solution on the 1st, 7th, and 15th days after germination. Irrigation was performed daily with 25 mL of water per tube. The experiments were finished 35 days after germination and plants were collected, washed, and dried at 60 °C for 72 h. Measurements and weightings were taken for fresh and dried root and shoot parts of each plant in all treatments. Calculations of the dry matter of the plants were accounted for (Shalhevet et al., 1995).

Statistical data analysis. To analyze and correlate quantitative variables, a split-plot design was used testing the isolated factor (10 levels) in the plots and the time factor (1 level) in the subplots, with 03 replications per isolate. The mean square repetition within the isolate was used as a residue to test the effectiveness of each isolate. When significative differences were observed among isolates, in terms of time and interaction (isolate/time), data were compared by the Tukey test at 5% probability. Root, shoot, and dry mass data were subjected to analysis of variance for a completely randomized design with a significance level of 5%. In the multiple comparisons of means, the Tukey test was used (p = 0.05). For both analyses,

the General Linear Models (GLM) procedure in SAS software (SAS 9.1, SAS Institute, Cary NC, USA) was used. was used.

Results

Analysis of bacterial isolates by partial sequencing of the 16S rRNA and taxonomy. The genetic signature based on *16S rRNA* sequences was predominant in Firmicutes, followed by Actinobacteria and Proteobacteria. The three different phyla together illustrated five different species. *Bacillus* was the most abundant genus including six of ten bacterial isolates, mostly *B. thuringiensis* showing the highest identity value for LGA07-V0508. Identity values sloped from 92 – 99% for comparison results obtained from GenBank similarities. The following bacterial isolates presented the same identity: LGA05-V0513 and LGA06-V0517 (*B. cereus*); LGA07-V0508. LGA09-V20L, LGA10-V20I and LGA12-V05D (*B. thuringiensis*); LGA13-V20F and LGA14-V20J (*Arthrobacter echigonensis*). LGA08-V20C (*Acinetobacter* sp.) and LGA11-V0522 (*Chromobacterium violaceum*) were exclusive in Proteobacteria.

Taxonomy identities were distributed in five branches figured out in a dendrogram showing phenetic concordance (Figure 1). The broadest branch includes the six bacterial isolates as a monophyletic group to the *Bacillus* genus. Again, *B. thuringiensis* and *B. cereus* were in agreement with this branch, corroborating the similarities analysis (Table 1). The branches “B” and “C” were coherent with exclusivity shown in the Proteobacteria phylum for two bacterial isolates. Both were monophyletic to *Acinetobacte*r and *Chromobacterium* genera, respectively. Finally, branch “D” clustered the two isolates to *Arthrobacter* and other related genera, such as *Corynebacterium* and *Sinomonas*. This was the branch that represents the bacterial isolates showing the lowest values of identity (Table 1) by similarities analysis.

Table 1 Similarity among partial *16S rRNA* sequences from bacterial isolates based on GenBank, using the BLAST nucleotide tool (BLASTn).

Isolates	Access number	Phylum	Lineage	Identity (%)
LGA05-V0513	KT583479.1	Firmicutes	*Bacillus cereus*	97
LGA06-V0517	KX101236.1	Firmicutes	*Bacillus cereus*	96
LGA07-V0508	KX101236.1	Firmicutes	*Bacillus thuringiensis*	99
LGA08-V20C	KU867576.1	(Gamma) Proteobacteria	*Acinetobacter* sp.	95
LGA09-V20L	KX101236.1	Firmicutes	*Bacillus thuringiensis*	96
LGA10-V20I	AB677944.1	Firmicutes	*Bacillus thuringiensis*	97
LGA11-V0522	KJ634484.1	(Beta) Proteobacteria	*Chromobacterium violaceum*	98
LGA12-V05D	KX101236.1	Firmicutes	*Bacillus thuringiensis*	96
LGA13-V20F	GU326383.1	Actinobacteria	*Arthrobacter echigonensis*	93
LGA14-V20J	GU326383.1	Actinobacteria	*Arthrobacter echigonensis*	92

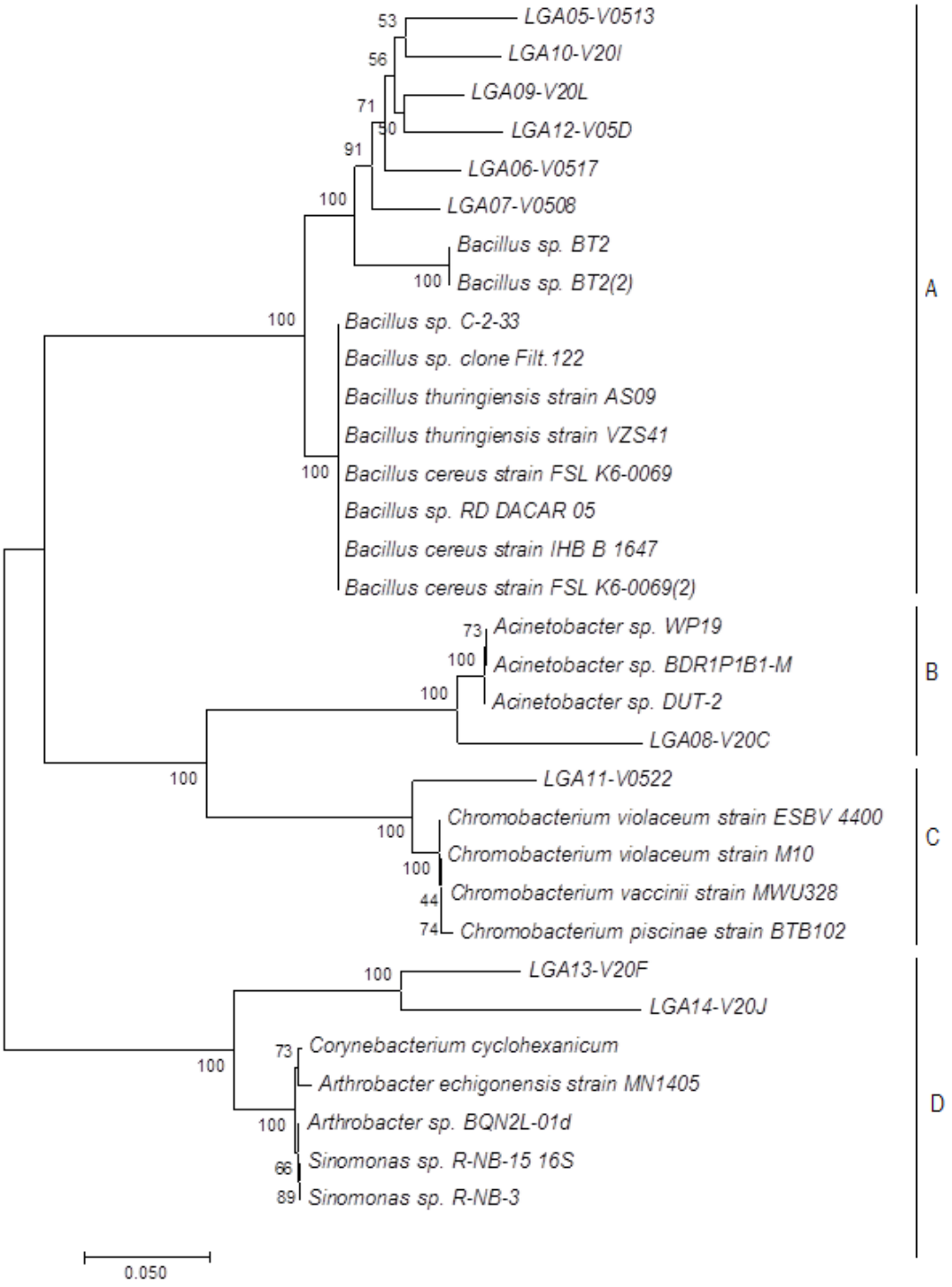

Figure 1 Dendrogram characterized by the *16S rRNA* genetic marker using the Neighbor-Joining method with 1,000 bootstrap repetitions, showing the taxonomic positions of bacterial isolates related to sequences from databases.

Evaluation of P solubilization on NBRIP medium. From ten isolates tested, six showed high solubilization rates fifteen days after inoculation based on determined rate E > 3 (Table 2). These data were superior to those previously reported for many sugarcane rhizobacteria including standard positive controls of *Burkholderia cepacia* and *B. ferrariae* (Inui et al., 2012),

and also for those reported for traditional *Pseudomonas* and *Bacillus* isolates (Nautiyal, 1999). Isolates LGA05-V0513, LGA06-V0517, LGA11-V0522, LGA13-V20F, and LGA14-V20J had E values over 6.00, higher than traditional positive controls. LGA07-V0508, LGA09-V20L, and LGA10-V20I had the average ability on NBRIP medium, and only LGA08-V20C were considered inferior or classified as low capacity for phosphate solubilization.

Table 2 Solubilization ability evaluated on NBRIP medium.

Isolates	**E = (Ø halo/Ø colony) mm***
LGA05-V0513	6.84
LGA06-V0517	6.78
LGA07-V0508	2.83
LGA08-V20C	1.00
LGA09-V20L	2.66
LGA10-V20I	2.80
LGA11-V0522	6.90
LGA12-V05D	5.30
LGA13-V20F	6.48
LGA14-V20J	6.20
*B. cepacia***	4.31
*B. ferrariae***	5.31

*Efficiency of P solubilization up to 15 days after inoculation.

**Standard controls.

According to P solubilization on NBRIP broth during the period of cultivation, the ten isolates could be clustered into three groups. The most efficient solubilizers were LGA11-V0522, LGA13-V20F, and LGA14-V20J, solubilizing more than 32 mg/mL of P in 120 hours of cultivation. LGA05-V0513, LGA06-V0517, LGA12-V05D, LGA09-V20L, LGA07-V0508, and LGA10-V20I had rates varying from 21 to 31 mg/mL of P in 120 hours of cultivation. Finally, LGA08-V20C showed the lowest solubilization conditioned by 16 mg/mL during cultivation (Figure 2). When acidification capacity was accessed, all isolates tended to acidify NBRIP broth during growth showing pH values rate from 4.9 to 6.3. The higher and faster acidifiers were LGA07-V0508 and LGA14-V20J. and the lower one was LGA08-V20C. Interestingly, LGA06-V0513 and LGA06-V0517 quickly acidify the broth at the period of 24 – 48 h but after that had an increase of pH again, culminating with the high values at 120 h compared to all isolates.

Altogether, comparing P solubilization on solid and liquid NBRIP and to acidification ability, our data corroborate the same tendency observed for all bacterial isolates tested. LGA05-V0513, LGA06-V0517, and LGA14-V20J should be considered as the best P solubilizers acting by acidification, while LGA10-V20I and LGA08-V20C were the worst ones.

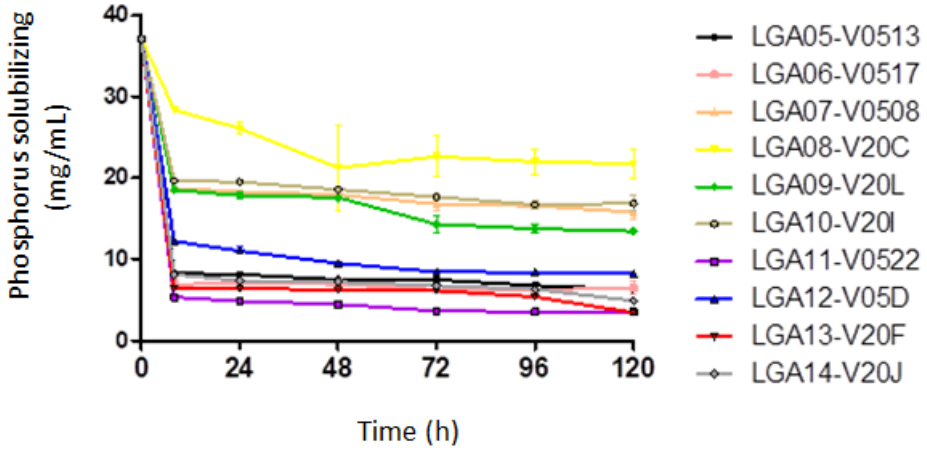

Figure 2 Solubilization ability on NBRIP medium during bacterial isolate growth, expressed by P remaining in the broth.

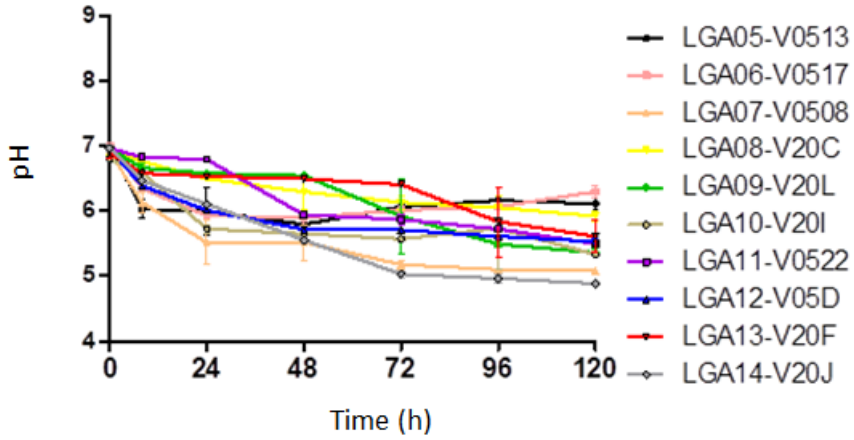

Figure 3 Acidification ability on NBRIP broth during bacterial isolate growth, expressed in pH values.

The relationship among P solubilization, bacterial growth (D.O. at 600 nm), and pH (Table 3) corroborates data for the isolates' efficiency and allows us to consider that media acidification is not directly linked to increased solubilization. Not all isolates presenting lower pH at the end of the cultivation period were the ones that most solubilized P. The growth rate of isolates also indicates that not always the higher optical density is related to the best P solubilization, as in the case of LGA07-V0508 and LGA11-V0522. In this case, it is possible to consider that those isolates could be able to produce large amounts of solubilizing substances (Table 3).

Table 3 Relationship among bacterial growth, pH, and P solubilization after 120 hs of bacterial growth.

Isolates	**D.O.$_{600}$**	**pH**	**P solubilization (mg/mL)**
LGA05-V0513	1.61 ± 0.01 ^{b}	6.11 ± 0.05 ^{b}	30.96 ± 0.03 ^{a}
LGA06-V0517	1.64 ± 0.02 ^{a}	6.33 ± 0.06 ^{a}	30.58 ± 0.44 ^{a}
LGA07-V0508	0.26 ± 0.01 ^{ef}	5.10 ± 0 ^{efj}	36.26 ± 0.17 ^{a}
LGA08-V20C	1.30 ± 0.03 ^{h}	5.73 ± 0 ^{beghi}	15.45 ± 0.95 ^{b}
LGA09-V20L	0.63 ± 0.01 ^{h}	5.39 ± 0 ^{cfg}	23.28 ± 0.06 ^{a}
LGA10-V20I	0.31 ± 0.06 ^{cfh}	5.27 ± 0.02 ^{ceghi}	20.45 ± 0.32 ^{a}
LGA11-V0522	0.30 ± 0 ^{ce}	5.49 ± 0 ^{def}	33.81 ± 0.10 ^{a}
LGA12-V05D	0.90 ± 0 ^{i}	5.53 ± 0.03 ^{def}	28.89 ± 0.13 ^{a}
LGA13-V20F	1.03 ± 0.03 ^{g}	5.51 ± 0.06 ^{def}	33.65 ± 0.07 ^{a}
LGA14-V20J	0.53 ± 0.02 ^{de}	4.88 ± 0 ^{c}	32.19 ± 0.02 ^{a}

In each column, averages followed by the same letter do not differ by the 5% Tukey test.

Plant growth-promoting rhizobacteria (PGPR) potential in the greenhouse. The response of soybean and maize plants to inoculation of bacterial isolates was investigated under greenhouse conditions. According to the results observed after soybean plant treatments, it can be concluded that some bacterial isolates influenced all parameters analyzed (Table 4). LGA06-V0517, LGA09-V20L and LGA13-V20F were those that better affected the growth of soybean roots, followed by isolates LGA08-V20C, while for shoot growth stood out isolates LGA07-V0508, LGA08-V20C, LGA09-V20L, LGA10-V20I, LGA13-V20F and LGA14-V20J. The soybean dry matter was significantly increased by treatments with LGA08-V20C, LGA10-V20I, LGA13-V20F, LGA11-V0522, and LGA12-V05D (Table 4). For maize plants, none of the ten isolates promoted significant shoot growth but isolates LGA05-V0513, LGA06-V0517and LGA09-V20L were positive for root growth. Regarding maize dry matter, the improvement of this parameter was associated with isolates LGA05-V0513, LGA08-V20C, LGA09-V20L, and LGA10-V20I (Table 5).

Table 4 Soybean inoculation and growth influence by soil bacterial isolates.

Treatment	Root (cm)	Shoot (cm)	Dry Matter (%)
Without inoculation	4,67 ± 1,03 [a]	16,67 ± 3,50 [a]	7,63 ± 3,9 [a]
LGA05-V0513	5,6 ± 1,14 [ab]	18,6 ± 1,52 [a]	7,08 ± 1,27 [a]
LGA06-V0517	10,67 ± 1,53 [c]	18,00 ±1,73 [a]	6,46 ± 2,46 [a]
LGA07-V0508	6,25 ± 2,02 [abd]	21,25 ± 2,63 [b]	8,41 ± 1,76 [a]
LGA08-V20C	5,00 ± 1,73 [bde]	16,67 ± 4,02 [bc]	12,76 ± 6,27 [b]
LGA09-V20L	9,67± 1,53 [cdf]	22,00 ± 1,73 [bd]	6,98 ± 1,73 [a]
LGA10-V20I	5,25 ± 2,00 [g]	16,25 ± 4,79 [bde]	10,59 ± 2,68 [b]
LGA11-V0522	4,80 ± 2,95 [hd]	14,80 ± 2,95 [acde]	6,14 ± 4,09 [bc]
LGA12-V05D	6,50 ± 1,00 [h]	19,25 ± 1,71 [abde]	8,88 ± 4,14 [b]
LGA13-V20F	9,67 ± 2,31 [ic]	22,67 ± 1,53 [f]	7,61 ± 3,59 [a]
LGA14-V20J	7,75 ± 3,40 [adefghi]	16,75 ± 6,65 [bfg]	7,85 ± 2,95 [a]

In each column, averages followed by the same letter do not differ by the 5% Tukey test.

Table 5 Maize inoculation and growth influence by soil bacterial isolates.

Treatment	Root (cm)	Shoot (cm)	Dry Matter (%)
Without inoculation	20,79 ± 2,75 [a]	34,30 ± 4,95 [a]	14,10 ± 1,14 [a]
LGA05-V0513	21,00 ± 6,03 [b]	33,00 ± 2,76 [a]	14,00 ± 2,06 [b]
LGA06-V0517	19,67 ± 5,72 [bc]	32,83 ± 2,32 [a]	13,03 ± 1,70 [abc]
LGA07-V0508	24,83 ± 5,00 [abcd]	35,17 ± 4,22 [ab]	11,43 ± 2,49 [abcd]
LGA08-V20C	22,67 ± 4,46 [abcd]	34,50 ± 4,14 [ab]	13,73 ± 1,82 [bc]
LGA09-V20L	22,67 ± 5,54 [bcd]	38,33 ± 2,58 [abc]	12,07 ± 4,81 [e]
LGA10-V20I	25,20 ± 6,30 [abcde]	34,40 ± 1,82 [abc]	14,34 ± 1,92 [bcde]
LGA11-V0522	24,33 ± 5,05 [abcde]	34,50 ± 3,62 [abc]	14,11 ± 0,70 [abcde]
LGA12-V05D	23,67 ± 3,83 [abcde]	37,17 ± 5,49 [abcd]	13,53 ± 1,61 [abcde]
LGA13-V20F	22,33 ± 2,80 [abcde]	38,83 ± 2,56 [abcd]	13,73 ± 0,59 [abcde]
LGA14-V20J	22,00 ± 2,10 [abcde]	33,17 ± 2,32 [abcd]	13,88 ± 1,35 [abcde]

In each column, averages followed by the same letter do not differ by the 5% Tukey test.

Discussion

Firmicutes and Proteobacteria are among several bacterial phyla related to P cycling and contribution to plant nutrition. The main PSB genera include strains of *Acinetobacter*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Escherichia*, *Herbaspirillum*, *Pseudomonas*, *Pantoea*, and *Rhizobium*, among others (Hegyi et al., 2021). The three predominant phyla in our analysis, Firmicutes, Actinobacteria, and Proteobacteria, were reported to increase their abundance in soils impacted by high doses of phosphate fertilization. Bacteria from these groups grow rapidly when there is greater availability of nutrients in the environment (Campolino et al., 2023).

Phylogenetic analysis based on the partial sequencing of the *16S rRNA* gene classified all isolates into four different genera, predominantly *Bacillus* which covered six of the ten isolates classified. *Bacillus* is a broad genus containing in its subdivision 632 child taxa, 426 species names have synonyms and only 109 have child taxa with a validly published and correct name according to List of Prokaryotic Names with Standing in Nomenclature (LPSN). The type species is *B. subtilis* (Ehrenberg 1835) Cohn 1872. The genus *Arthrobacter* encompassed two of the isolates analyzed. Of the 150 child taxa of its subdivision, 70 correspond to child taxa with a validly published and correct name, being the species type *A. globiformis* corrig. (Conn 1928) Conn and Dimmick 1947 (Skerman et al., 1980, Parte et al., 2020).

The taxa *Acinetobacter* and *Chromobacterium*, respectively, marked only one isolate in this study. *Acinetobacter* presents *A, calcoaceticus* (Beijerinck 1911) Baumann et al. 1968 as a type species and contains 82 child taxa with a validly published and correct name of a total of 111 classified. *Chromobacterium* consists of a genus with fewer representatives and 14 child taxa with a validly published and correct name of a total of 18. The type species is *C. violaceum* Bergonzini 1880 (Skerman et al., 1980; Parte et al., 2020).

B. thuringiensis (LGA07-V0508, LGA09-V20L, LGA10-V20I, and LGA12-V05D) has insecticidal properties based on the production of pore-forming proteins in the intestines of its insects and host larvae. These entomopathogenic toxins can be classified as Cry and Cyt and have a long history of success as bioinsecticides applied in the biological control of insect pests in crops (Bravo et al., 2011). These spore-forming bacteria also have Vegetative insecticidal protein (Vip) during their vegetative growth phase. Vip proteins have been seen as a second generation of insecticide proteins that can be used alone or in synergism with Cry proteins in pest management that already have resistance to biological control (Gupta et al, 2021). Although they did not stand out as better, the isolates belonging to this classification (*B. thuringiensis*) showed potential to act as PSB. LGA09-V20L and LGA10-V20I still showed an important role in corn and soybean development, respectively. LGA12-V05D affected the growth of soybean roots.

B. cereus (LGA05-V0513 and LGA06-V0517) is a gram-positive aerobic or facultatively anaerobic spore-forming rod producer of emetic toxin and enterotoxin, frequently associated with food poisoning (Tuipulotu et al., 2021). *B. cereus* group includes other closely related phylogenetically *Bacillus* species, including pathogenic and non-pathogenic species of wide ecological diversity, being the best-studied *B. anthracis*, *B. cereus*, and *B. thuringiensis* (Ehling-Schulz et al., 2019). It is known that for this group a better discriminatory analysis is important and necessary, knowing that species in this group cannot be classified only in the sequences of the *16S rRNA* gene. The bacterial isolates belonging to this classification were the ones that stood out as the efficiency in P solubilizing and the LGA05-V0513 showed an important role in the increment of corn plants, particularly related to dry mass.

A. echigonensis (LGA13-V20F and LGA14-V20J) had proposed taxonomic reclassification within the genus *Sinomonas* [*S. echigonensis*, comb. nov.], due to the similarity

based on the composition of the main fatty acids, polar lipids, and cell wall amino acids (Zhou et al., 2012). *Arthrobacter* consists of the most divergent heterotrophic bacterial group of actinobacteria. Recently it has been shown that an isolate corresponding to *A. echigonensis* presented a subtle sub-lethality effect on the larval weight of *Spodoptera frugiperda* (Miranda et al., 2023), consisting of an alternative for possible biological control based on bacteria non-sporulating. Bacterial isolates closely associated with genera *Sinomonas* strain Cw 108 (I.4.2) and *Arthrobacter* (I.5.3.) through the analysis of diversity using sequences *16S rRNA* showed positive properties as PGPR acting on the growth of roots and shots of the bok choy seedlings (Agustiyan et al., 2021). *Arthobacter* species acting as PGPR in several plants such as maize, pea, rice, and soybean were reported due to their properties such as potassium and phosphate solubilization, nitrogen fixation, and indole acetic acid synthesis (Su et al., 2024). Both isolates were excellent PSB, particularly LGA14-V20J which was additionally an important acidifier of the medium. Additionally, they contributed to better performance in the growth of soybean stem roots.

Acinetobacter sp. (LGA08-V20C) corresponds to gram-negative bacteria easily isolated from the rhizosphere of many plants, playing the role of PGPR in hosts such as tomatoes, canola, soybeans, and beans, due to important roles in the production of gibberellin, siderophore, IAA, biosurfactants/bioemulsifiers, and antibiotics, as well as solubilization of zinc, potassium, and phosphate (Sun et al., 2024). Species of *Acinetobacter* have also been shown to have potential in the bioremediation of different compounds (Xia et al., 2020, Yin et al., 2020, Abdulmalik et al., 2023). Although this isolate was considered the worst PSB among the ten isolates analyzed in this study, LGA08-V20C contributes to the increase of soybean in all parameters analyzed, especially dry mass.

C. violaceum (LGA11-V0522) consists of a pathogenic gram-negative bacterium that rarely causes diseases in humans. However, tropical and subtropical isolates from this genus have been associated with infections considered rapid and lethal in humans and other mammals. It produces the purple pigment violacein and several types of antibiotics which are active against amoebae, trypanosomes, and gram-positive and gram-negative bacteria. Violacein was detected in several environments and may be related to the wide diffusion of the bacteria in its habitat (Alisjahbana et al., 2021). *C. violaceum* may have an antagonistic effect on PGPR species, such as *Azospirillum brasilense* Sp7, *Rhizobium* UPMR1102, and *B. sphaericus* UPMB10 involving quorum sensing mechanism. In this case, it can be a threat to the use of PGPG in agriculture (Loke & Saud, 2021). In this study, LGA11-V0522 showed considerable potential in P solubilization and contributed significantly to root growth and dry mass of soybean plants.

The best P solubilization potentials were also associated with higher acidification capacity, such as those observed for isolates LGA07-V0508 and LGA14-V20J. However, among the bacterial isolates that contributed to the increase of soybean or corn plants, with possible performance as PGPR, not all were excellent P or acidifiers in the NBRIP medium. PGPRs improve the increment of plants acting directly or indirectly, by other functional

mechanisms that are not always the solubilization of P. As examples of direct mechanisms, we can mention the fixation of atmospheric nitrogen and the secretion of phytohormones such as auxins, cytokines, and gibberellins that regulate plant growth. Indirect mechanisms include the production of diversified antimicrobials, Induced systemic Resistance (ISR), production of siderophores, and regulation of stress conditions (Etesami & Adl, 2020, Rehman et al., 2021). The isolates LGA05-V0513 and LGA06-V0517 tended to make the medium less acidic and solubilized similar amounts of phosphate, both in the solid and liquid medium, justifying their comparison to *B. cereus*. However, the results of the greenhouse experiments showed differences in the growth of plant parameters. The isolate LGA06-V0517 promoted better growth for the soybean roots in an average of 11 cm, while the LGA05-V0513 was the microorganism that had one of the lowest results for soybean dry matter. Some references suggest the influence on plant growth through B. cereus (Sebastian et al., 2021). What can be concluded from the tests performed, solubilization of P, and influence of greenhouse growth is that the isolates act as PSB solubilizers and even modestly induce plant growth.

The isolate LGA11- V0522 was determined as one of the best PSB, presenting a solubilization ratio of 6.9 and a solubilizing of 33.7 µg/ml. The pH at the end of the cultivation period ranged from basic (8.2) to acidic (5.5), suggesting that acid production is responsible for the high solubilization rate. Results in the greenhouse showed that there is a good influence on soybeans. Zhou et al. (2016) isolated a lineage of *Chromobacterium* from rice (*Oryza sativa*) roots, suggesting that the genus may be associated with symbiosis with grass culture. However, few articles relate *Chromobacterium* spp. as P-solubilizing PGPR.

The greenhouse tests with soybean and corn plants allowed us to determine more clearly the characteristics of each isolate as to the benefits for acting as PGPR, possibly as potential PSB. The influence on the growth of soybean plants occurred particularly in the root growth parameter. For maize plants, both root growth and dry mass increment were the best parameters influenced. In turn, the proliferation of adventitious roots and dispersion of lateral roots (Zutter et al., 2022) are both increments generated by PGPRs and are responses of the plant itself to increase exudation of attractive organic compounds for soil microorganisms, besides being an investment for P absorption. In maize plants, the efficiency in the use of P is much more associated with the ability of the plant to acquire this nutrient from the soil than with its ability to use it more efficiently (Chen et al., 2022).

Additionally, it is known that there are different mechanisms of solubilization to release P bound to clay, colloids, and soil organic matter, among them solubilization of inorganic P, organic P mineralization, production of siderophores and exopolysaccharides. Solubilization of insoluble P can be given by the release of inorganic/organic acids, protons to reduce pH, siderophores to complex P and iron (P-Fe), and phytase-like acid phosphatases that assist in the release of P and its availability (Patel & Goswami, 2020, Rawat et al., 2021, Amarasinghe et al., 2022).

An indirect mechanism acting on P solubilization consists of exopolysaccharide secretion and biofilm formation. Increased production of exopolysaccharide along with biofilm formation

by bacterial isolates under P-limiting conditions has been observed indicating that the more condensed biofilm structure can release sufficient amounts of soluble P by these bacteria. The biofilm protects the microbial community in its interior preventing the influx of undesirable agents to its interior and assisting in the sequestration of toxic metal ions in the rhizosphere, also protecting the plant from this stress (Lucero et al., 2020, Mandal, 2021).

In some countries, particularly in Southwest China, intercropping or crop rotation between soybean and corn is common. This management increases the activity of acid phosphatase in the soil and the availability of P, improving soil fertility and crop yield. Rates higher than 84.6% in the use and use of phosphate fertilizers were observed in this planting system. PSB belonging to different genera could promote the absorption of insoluble P and promote the growth of plants in different cultures (Song et al., 2020, Song et al., 2022).

The evaluation of the P solubilization profile and the influence on soybean and corn growth in the greenhouse, by the ten soil bacterial isolates from fertilized soil with vinasse, allow us to characterize each isolate and their possible biotechnological and agricultural applications. Its possible use as commercial isolates applied in crops will depend on field experiments in extended versions until the harvest period.

Conclusion

In this study, a total of ten bacterial isolates were partially characterized as belonging to the species *Bacillus cereus, Bacillus thuringiensis*, *Acinetobacter* sp., *Chromobacterium violaceum*, and *Arthrobacter echigonensis*. Most of the identified PSB isolates with good improvement on soybean and maize plants belong to the genus *Bacillus*, while *Acinetobacter* sp. showed the worst performance. The isolates LGA08-V20C and LGA10-V20I stood out for soybean whereas the isolates LGA05-V0513 and LGA09-V20L were better for maize. These bacterial isolates resist changes in growth conditions and could be used as possible PGPR biofertilizers for crops in the future.

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