Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Phosphate solubilization capacity by bacteria in soybean crops

Capacidade de solubilização de fosfato por bactérias na cultura da soja

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Abstract

The availability of phosphorus (P) to plants is reduced by its binding to solid mineral and soil organic particles. However, phosphate-solubilizing microorganisms can increase nutrient availability by secreting extracellular enzymes such as fosfatases. The objective was to evaluate the phosphate solubilization capacity of bacteria coinoculated in soybean crops. Soybean plants were grown in nutrient solution in a combination of treatments of bacteria inoculated into the seeds [*Bradyrhizobium japonicum* (Brady); Brady + *Pseudomonas fluorescens* (Pf); Brady + *Bacillus megaterium* (Bm) + *Bacillus subtilis* (Bs); and Brady + Bs] with doses of P. In the laboratory, another experiment was conducted to evaluate the phosphate solubilization capacity in the NBRIP solid culture medium by the bacteria Bs, Bs+Bm, Pf and the isolate of *Pseudomonas* spp. The root dry mass increased by 201.26% with Brady+Bs coinoculation. Considering the phosphorus content in the aerial part of plant content for a dose of 40 kg ha^{-1} of $NH_4H_2PO_4$, with *B. japonicum* + *P. fluorescens* resulted in an increase of 26.7% in relation to the control. The use of *B. japonicum* + *B. megaterium*/B. *subtilis* increased the phosphorus concentration in the shoot plant by 22.5%. In the laboratory solubilization test, the bacteria *B. subtilis* showed a medium phosphate solubilization index, making this bacterium an alternative for better use of this nutrient in the soil.

Keywords: *Bradyrhizobium japonicum*, *Pseudomonas fluorescens*, *Bacillus subtilis*.

Resumo

A disponibilidade de fósforo (P) para as plantas é reduzida pela sua ligação a minerais sólidos e partículas orgânicas do solo. No entanto, os microrganismos solubilizadores de fosfato podem aumentar a disponibilidade de nutrientes através da secreção de enzimas extracelulares, como as catalases. O objetivo deste estudo foi avaliar a capacidade de solubilização de fosfato de bactérias coinoculadas na cultura da soja. Plantas de soja foram cultivadas em solução nutritiva em uma combinação de tratamentos de bactérias inoculadas nas sementes [Bradyrhizobium japonicum (Brady); Brady + Pseudomonas fluorescens (Pf); Brady + Bacillus megaterium (Bm) + Bacillus subtilis (Bs); e Brady + Bs] com doses de P. Em laboratório, outro experimento foi conduzido para avaliar a capacidade de solubilização de fosfato no meio de cultura sólido NBRIP pelas bactérias Bs, Bs+Bm, Pf e pelo isolado de Pseudomonas spp. Houve aumento no teor de P nas folhas de acordo com as doses de fósforo. A massa seca da raiz aumentou 201,26% com a coinoculação Brady+Bs. Considerando o teor de fósforo na parte aérea da planta para a dose de 40 kg ha^{-1} de $NH_4H_2PO_4$, o tratamento com B. japonicum + P. fluorescens resultou em aumento de 26,7% em relação ao tratamento controle. O uso de B. japonicum + B. megaterium/B.subtilis aumentou a concentração de fósforo na parte aérea em 22,5%. No teste de solubilização em laboratório, a bactéria Bacillus subtilis apresentou médio índice de solubilização de fosfato, tornando esta bactéria uma alternativa para melhor aproveitamento deste nutriente no solo.

Palavras-chave: *Bradyrhizobium japonicum*, *Pseudomonas fluorescens*, *Bacillus subtilis*.

1. Introduction

Phosphorus (P) is an essential element for plants as it participates in the flow of cellular energy and in the composition of several macromolecules. P is part of the structure of nucleotides and phospholipids and participates in the processes of photosynthesis, respiration, energy storage and transfer, cell division and growth, etc (Barra et al., 2019). Therefore, its availability in the soil influences plant growth and development (Li et al., 2017; Lu et al., 2016). The main source of P for agricultural soils is phosphate fertilizers obtained from the mining of phosphate rocks, which are finite and have a high extraction cost, which tends to increase the price of these fertilizers (Wu et al., 2023).

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Received: August 22, 2024 – Accepted: May 27, 2025
Editor: Takako Matsumura Tundisi

Presently, Brazil stands as the fourth-largest global consumer of fertilizers, as indicated by the International Fertilizer Association (Brasil, 2022). Phosphate fertilizers and soil amendments are employed intensively in Brazilian agriculture, the country consumed respectively 11,8 million and 33,0 million tons of phosphate fertilizers and limestone (IPNI, 2017). In 2020, Brazil imported 73% of its phosphorus (P) required for agricultural purposes, amounting to 4,8 million tons of monoammonium phosphate (MAP), equivalent to 2,3 million tons of phosphorus oxide (P_2O_5), as reported (GlobalFert, 2021). This scenario underscores Brazil's dependence on the importation of phosphorus. More than 40 countries extract phosphate rock from phosphate deposits. China, Morocco, the United States, and Russia stand as the top four phosphate producers, accounting for approximately 79% of the total global production (Ruan et al., 2019).

Most P in the soil is unavailable due to adsorption to colloids and soil organic matter, fixation to primary and secondary minerals, or immobilization in soil biota. An alternative to increasing P availability in the soil is the use of solubilizing microorganisms. Plant growth-promoting bacteria (PGPB) can solubilize calcium, iron, aluminum and magnesium phosphates, which results in increased P availability to plants (Cruz et al., 2022; Ferraro et al., 2023). The use of GPB in hydroponic systems tends to have similar results in sand tests, which in general where these bacteria are used tend to increase phosphorus solubilization compared to the control without microorganisms (Qin et al., 2011).

Several mechanisms are used by these microorganisms to solubilize phosphates, such as the production of siderophores, organic acids, and secretion of extracellular enzymes called phosphatases (Prabhu et al., 2019). PGPB (Plant Growth-Promoting Bacteria) associate with plants and inhabit the rhizosphere zone, playing various roles in regulation, growth promotion, and development of plants. These bacteria utilize root exudates from plants to fuel their metabolic processes (Kumar et al., 2024). For the process, PGPB use specific attributes such as chemotaxis, lipopolysaccharides, and exopolysaccharides, and cell motility is enabled by structures such as fimbriae and pili (Yadav and Chandra, 2020).

The bacteria *Bacillus megaterium*, *B. subtilis* and *Pseudomonas fluorescens* have been frequently researched as potential phosphate solubilizers (Aberathna et al., 2022). In a study carried out with different isolates of B. subtilis, Chagas Junior et al. (2022) found that isolates Bs01, Bs02, and Bs04 obtained higher phosphate concentrations than the control, with a solubilization percentage that ranged from 350 to 7100% on the sixth day of growth. The greater acquisition of P by crops is mainly due to the increase in the root system and the solubilization of P present in the rhizosphere (Sousa et al., 2021). In studies with bacteria of the genus Bacillus, they demonstrated the capacity to reduce phosphate fertilization by 33 to 50% in corn, soybean, and rice (Granada et al., 2018; Oliveira-Paiva et al., 2020).

However, it is necessary to significantly increase research efforts on this topic. The essentiality of P for agriculture is unquestionable, however the increasing costs of phosphate fertilizers for importing countries have become one of the major problems of modern agriculture. Increasing the supply of inoculants containing microorganisms efficient in phosphate solubilization can contribute to better use of P that is fixed in the soil, reducing the amounts of P added annually by phosphate fertilizers. The prospecting and selection of phosphate-solubilizing microorganisms are fundamental steps for the development of new inoculants. In this sense, the objective was the phosphate solubilization capacity of bacteria coinoculated in soybean crops.

2. Materials and Methods

2.1. Plant cultivation

The experiment was conducted in a completely randomized design, with three replications in a bifactorial design (4x4), with the first factor (*Bradyrhizobium japonicum*) (Brady), Brady + *Pseudomonas fluorescens*, Brady + *Bacillus megaterium* + *Bacillus subtilis* and Brady + *Bacillus subtilis* in combination with a second factor with four doses of phosphorus (0, 40, 80 and 120 kg/ha $NH_4H_2PO_4$), totaling 16 treatments. The soybean seeds used were the NS5959 IPRO cultivar and were inoculated with four commercial inoculants. The "Brady" inoculant was composed of *Bradyrhizobium japonicum* strains SEMIA 5079 and SEMIA 5080 (7×10^9 UFC mL^{-1}). The "Pf" inoculant was composed of *Pseudomonas fluorescens* CCTB03 at a concentration of 1×10^9 UFC mL^{-1}. The "BmBs" inoculant was composed of *Bacillus megaterium* (BRM 119) + *B. subtilis* (BRM 2084) both at concentrations of 1×10^9 UFC mL^{-1}. The "Bsu" inoculant was composed of *B. subtilis* CCTB04 at a concentration of 1×10^9 UFC mL^{-1}.

Cell counting was performed by serial dilution and plating on nutrient agar culture medium, as described below. The inoculants were combined as follows: Brady + Pf; Brady + Bm + Bs; Brady + Bsu and Brady. All inoculants were used at a dose of 100 mL for 50 kg of seeds. The inoculated seeds were sown in trays with commercial substrate and kept for two weeks in a greenhouse with daily irrigation. Subsequently, four seedlings were transplanted into the hydroponic system, consisting of an eight-liter container with a nutrient solution from Hoaglang and Arnon (1950). The concentrations of P in the nutrient solution were 0; 23.01; 57.52 and 138.04 mg L^{-1}. The total concentrations (mg L^{-1}) of macronutrients in the nutrient solution were: 0.435 of KNO_3, 0.432 of $Ca(NO_3)_2$, 0.246 of $MgSO_4$, 0.136 of KH_2PO_4 and 0.230 of $NH_4H_2PO_4$. pH was maintained at 7.0 by frequent corrections with 1M NaOH 0.1 mL L^{-1} of the micronutrient stock solution composed of (mg L^{-1}) $CuSO_4 5H_2O$ (0.031), $MnCl_2 4H_2O$ (0.103), $ZnSO_4 7H_2O$ (0.149), $NiSO_4 6H_2O$ (0.012), H_2MnO_4 (0.024) and H_3BO_3 (0.401). Iron was added separately as iron ethylenediaminetetraacetic acid (Fe-EDTA) (1 mg L^{-1} of the nutrient solution).

The plants were grown in the greenhouse until the R2 stage (full flowering), from 10/02/2020 to 19/03/2020. The plants were grown in a greenhouse until the R2 stage (full flowering), from 10/02/2020 to 19/03/2020. The variables analyzed were P content in aerial part (PPA) and root dry mass (SARM). To determine the P content, 35 trifoliate leaves were collected per treatment before harvest, dried in forced air at 60°C until constant mass, weighed, and subjected to P evaluation. The determination of P in the leaf tissue was performed by spectrophotometry from an aliquot extracted from the ammonium molybdate and amino naphtholsulfonic acid solution. The dry mass of the roots of the four plants per repetition was determined after drying as described above.

2.2. Phosphate solubilization

The experiment consisted of a completely randomized design with four treatments (inoculants) and three replications. The treatments were the inoculants Bs+Bm, Pf, Bsu, and *Pseudomonas* spp. (Bac 71) at the concentrations shown in Table 1. The latter consisted of a *Pseudomonas* spp. with cell concentration of 1 x 10^9 CFU mL^{-1}. Bacteria were isolated from commercial products by plating on nutrient agar culture medium (3 g of meat extract, 10 g of peptone, 2.5 g of glucose and 15 g of agar, 1L of distilled water, pH 7.0) and incubation at 28°C in the dark. To evaluate phosphate solubilization, bacteria were cultivated in solid NBRIP culture medium (Nautiyal, 1999), composed of 10 g of glucose; 5 g of ($Ca_5(OH)(PO_4)_3$) (calcium hydroxyapatite); 5 g of $MgCl_2$ $6H_2O$; 0.25 g of $MgSO_4$ $7H_2O$; 0.2 g of KCl; 0.1 g of $(NH_4)_2$ SO_4; 15 g of agar; 1L of distilled water and pH 7.0. The Petri dishes with the NBRIP culture medium were inoculated in their center with a subculture loop, from an isolated colony obtained in the nutrient agar culture medium and incubated at 28°C in the dark. The diameter of the halo and the colony were measured after three and 17 days of incubation, using a digital caliper. The phosphate solubilization index (SI) was calculated by dividing the halo diameter by the colony diameter according to Berraquero et al. (1976), being classified as low (IS < 2), medium (2 ≥ IS ≤ 4) and high (IS > 4).

Table 1. In vitro colony-forming units of phosphate-solubilizing bacteria 12 hours after inoculation, containing the bacteria *Bacillus subtilis*, *Bacillus megaterium* + *Bacilus subtilis*, *Pseudomonsas fluorescens* and BAC 71 in culture medium.

Microrganisms	Colony-forming units /mL
T1 – *Bacillus subtilis*	5.00 x 10^7
T2 – *Pseudomonas fluorescens*	1.14 x 10^8
T3 – *Bacillus subtilis* + *Bacillus megaterium*	1.08 x 10^{11}
T4 – BAC 71	1.21 x 10^8

2.3. Statistical analysis

The data were subjected to compliance with the assumptions of the mathematical model (Steel and Torrie, 1986) and analysis of variance (ANOVA). When significant, regression analysis was performed for P doses. The analyses were carried out using the statistical software Sisvar® 5.6 (Ferreira, 2011). To analyze the phosphorus content, the leaves of all plants in the treatment were used, with no repetitions, which made statistical analysis impossible.

3. Results

For the greenhouse experiment, the relationship between treatments with microorganisms and P doses in the nutrient solution was verified in relation to phosphorus concentration in soybean leaf tissue (Figure 1). When phosphorus is not used in the solution, there is the lowest concentration of P in the leaf tissue for all treatments with microorganisms, with no statistical difference among them. However, when P is introduced into the solution above 23.01 mg L^{-1}, variations in the P content of the leaves were observed. Considering the dose of 23.01 mg L^{-1} of P, the treatment with *B. japonicum* + *P. fluorescens* (T2) obtained an increase of 26.7% in relation to the control treatment and the use of *B. japonicum* + *B. megaterium*/*B.subtilis* resulted in a 22.5% increase in phosphorus concentration in the shoot. When the highest dose of P was used (138.04 mg

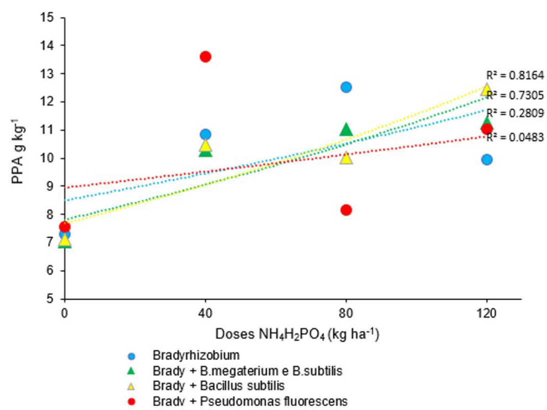

Figure 1. Phosphorus content in the aerial part-PPA (g kg^{-1}), in the different doses of $NH_4H_2PO_4$ (0; 40; 80 and 120 kg ha^{-1}) in relation to the treatments with bacteria in hydroponics.

Table 2. Average of phosphorus content in the aerial part-PPA (g kg^{-1}) at different doses of NH4H2PO4 (0; 40; 80 and 120 kg ha^{-1}) in relation to treatments with bacteria in hydroponics.

Tratamentos/Doses	0	40	80	120
Bradyrhizhobium japonicum	7.31	10.86	12.5	9.95
Brady + *B.megaterium* e *B.subtilis*	7.12	10.46	10.04	12.44
Brady + *Bacillus subtilis*	7.05	10.3	11.02	11.27
Brady + *Pseudomonas fluorescens*	7.58	13.62	8.16	11.04

L[-1]), all treatments with *B. japonicum* added with some solubilizing bacteria showed a higher concentration phosphorus content in the aerial part of the plant (Table 2).

Significant differences were observed between treatments, considering root dry mass (Table 3). Among the observed data, the treatments that best fit the regression model are T3 and T4. The treatment using B. japonicum along with B. subtilis showed the best response in terms of root dry mass production with increasing doses of phosphorus. The application of these microorganisms along with B. megaterium also responded positively to the application of $NH_4H_2PO_4$ (Figure 2). The use of growth-promoting microorganisms, such as B. subtilis, B. megaterium, and P. fluorescens, with the nitrogen-fixing B. japonicum, allows for greater root growth, and these bacteria were evaluated for their phosphate solubilization capacity in a future laboratory experiment (Table 4).

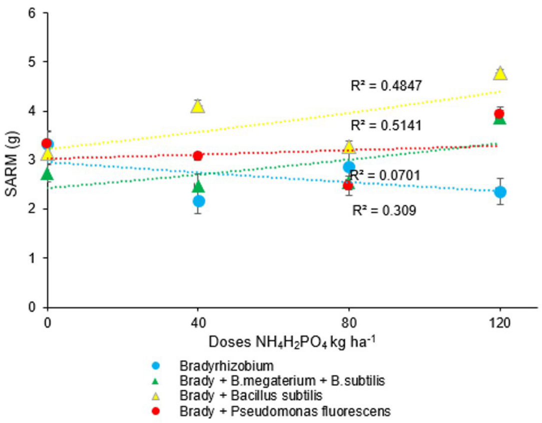

Figure 2. Root dry mass (SARM), at different doses of $NH_4H_2PO_4$ (0; 40; 80 and 120 kg ha[-1]) in relation to treatments with bacteria in hydroponics.

Table 3. Summary of mean squares analysis of variance for the variable root dry mass (SARM).

SV	FD	QM
Treatments	3	2.912**
Block	2	0.063*
Doses	3	2.062**
Treatments x Doses	9	0.969**
Error	30	0.091
Overall average		3.15
CV%		9.60

**, *, significant at 1.5% and not significant respectively, by the F test. Source of variation (SV); freedom degree (FD); coefficient of ariation (CV).

Table 5 shows the results obtained in laboratory solubilization tests. It can be seen that the solubilization index of the isolates varied in relation to the time and the isolate used, presenting values between 0.28 and 2.04.

Treatments with microorganisms B. subtilis + B. megaterium (T2), P. fluorescens (T3) and Pseudomonas spp. BAC 71 (T4) were considered low solubilizers, demonstrating indices between zero and 1.38. The B. subtillis isolate (T1) was considered a medium solubilizer, with indices of 2.04 in the evaluation 17 days after incubation. Considering low and medium solubilization of the microorganisms tested, even so, it can be seen in figure 3 that interaction occurs with the medium forming the solubilization halo.

4. Discussion

The results obtained report the ability of Bacillus bacteria, together with B. japonicum, to utilize the added P. The most efficient way for plants to access P is through the presence of microorganisms that absorb and immobilize phosphorus, gradually releasing it into the soil (Withers et al., 2018). The use of phosphate-solubilizing microorganisms with phosphate fertilizer increases microbial enzymatic activity, as well as nutrient cycling (Cheng et al., 2023). In hydroponic systems, the efficiency of adding Bacillus spp. is reported, improving plant growth, as well as the accumulation of P in plant tissues and the increase of chlorophyll content in the leaves (Cerozi and Fitzsimmons, 2016).

The use of phosphorus-solubilizing microorganims has demonstrated positive responses in several studies, such as greater plant height, greater grain yield and even greater phosphorus absorption in various crops such as rice (Rawat et al., 2022), common bean (Bedine et al., 2022), corn (You et al., 2020) and soybean (Guimarães et al., 2021). However, the use of PGPB does not always present differences in relation to plant growth and development. Mahanta et al. (2018), used microorganims via soil in wheat and soybean crops and observed that there was no statistical difference between these treatments and the control, however, differences may occur when using doses of P.

Table 4. Average of root dry mass (SARM), at different doses of NH4H2PO4 (0; 40; 80 and 120 kg ha[-1]) in relation to treatments with bacteria in hydroponics.

Treatments/Doses	0	40	80	120
Bradyrhizobium japonicum	3.33a	2.17c	2.86ab	2.37c
Brady + *B.megaterium* e *B.subtilis*	2.73a	2.47bc	2.53b	3.87b
Brady + *Bacillus subtilis*	3.13a	4.1a	3.27a	4.77a
Brady + *Pseudomonas fluorescens*	3.33a	3.07b	2.47b	3.93b

Distinct lowercase letters in the column differ statistically from each other by Tukey's test, at 5% probability of error.

Table 5. Phosphate solubilization index (IS= Halo diameter (mm) / Colony diameter (mm)) by isolates of *Bacillus subtilis*, *Bacillus subtilis/ Bacillus megaterium*, *Pseudomonas fluorescens* and *Pseudomonas* spp. BAC 71, on two evaluation dates.

Microrganisms	*B. subtilis*	*B. subtilis/ B. megaterium*	*P. fluorescens*	*Pseudomonas* spp. Bac71
ø halo (mm) 3 days	11.77	0	23.90	23.85
ø Colony (mm) 3 days	10.38	15.77	19.91	17.43
IS (3 dias)	**1.13**	**0**	**1.20**	**1.37**
ø halo (mm) 17 days	23.84	6.26	30.03	25.22
ø Colony (mm) 17 days	11.67	22.66	23.11	18.33
IS (17 days)	**2.04**	**0.28**	**1.30**	**1.38**

Subtitle: ø = diameter.

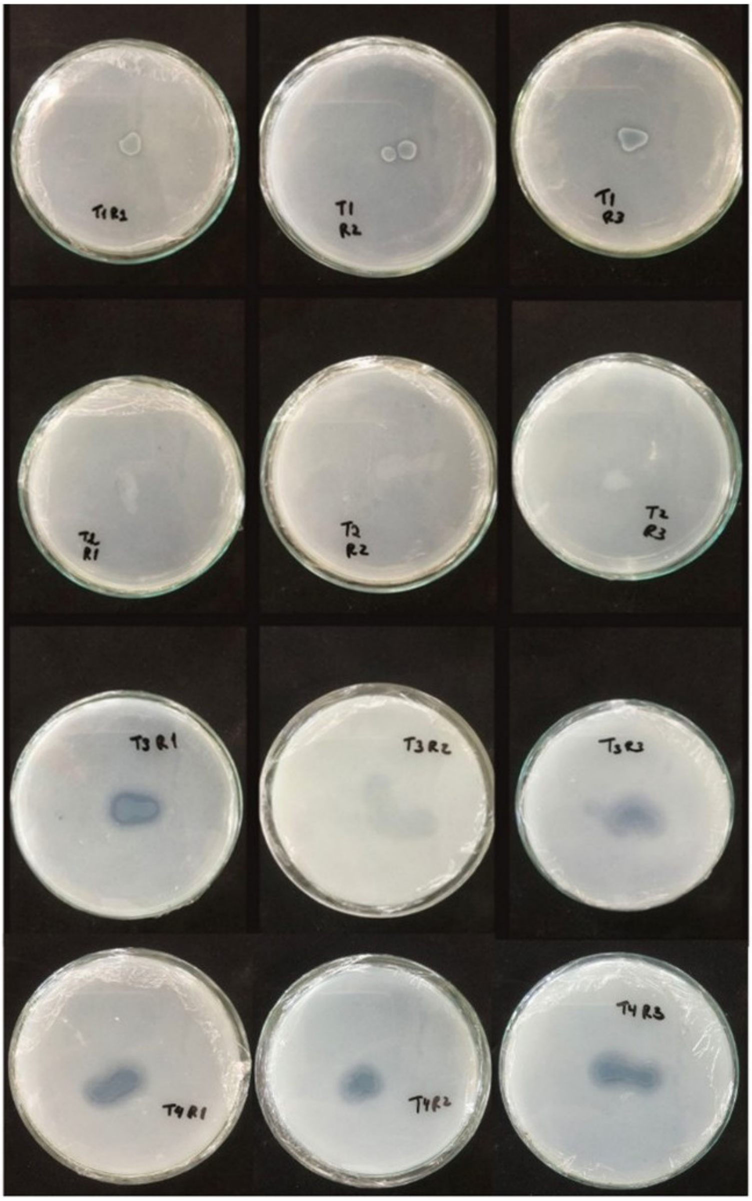

Figure 3. Phosphorus-solubilizing bacteria in culture medium forming a solubilization halo at 17 days.

The use of *B. subtilis* with a reduction in the recommended dose of P, in the case of corn, increased the chlorophyll content and consequently the production of dry mass in the crop. *Bacillus subtilis* can excrete phytohormones such as 3-acetic acid, in addition to stimulating mitotic sites in roots, favoring root growth (Silva et al., 2022).

The use of microorganisms of the genus *Pseudomonas* has been studied as beneficial microorganisms, which promote plant growth, increase plant tolerance to diseases, and also increase the availability of nutrients in the soil (Chandra et al., 2020). The response to the use of PGPB microorganisms is greater in restricted and adverse environmental conditions, especially when it comes to nutritional and water stress (Silva and Pires, 2017).

There is a very intense interaction around the roots with exudates, microorganisms and soil (Vives-Peris et al., 2018). Many researchers have already proven the benefits of microorganisms, whether through the production of root exudates, better phytosanitary condition, cost reduction, greater productivity, among other benefits (Meena et al., 2017). However, the efficiency of the microorganisms used depends on factors such as availability of water, oxygen, temperature and adequate pH (Martin et al., 2023).

The solubilization capacity of some bacteria such as the *Bacillus* genus, they synthesize root hormones such as indoleacetic acid (IAA), which increases the root area, allowing greater exploration of the roots, and consequently greater absorption of water and nutrients (Babalola, 2010). However, other genera such as *Pseudomonas*, *Bradyrhizobium* and *Azospirillum*, induce the promotion of root hairs by stimulating cell multiplication (Bulgarelli et al., 2013). The production of siderophores and organic acids are examples of these mechanisms, which result in increased secretion of enzymes (Rana et al., 2020; Puri et al., 2020). Bacteria of the genus *Bacillus* have been the most researched as phosphate solubilizers. In this research, the bacterium *B. subtilis* in isolation was the one with a medium solubilization capacity (2.04), whereas the other bacteria (*B. subtilis* with *B. megaterium* and *P. fluorescens*) had a low solubilization capacity (Berraquero et al., 1976).

Bashan et al. (2012) reported that for a bacteria to be considered a solubilizer, it needs to, in addition to solubilizing tricalcium phosphate, also solubilize iron and/or aluminum phosphate. As future perspectives, these microorganisms can be used in bioinputs in isolation or formulated, contributing to the sustainability and profitability of agricultural production.

5. Conclusion

The use of the bacteria B. subtilis, B. megaterium and P. fluorescens increased the concentration of phosphorus in soybean leaves and also increased the dry mass of soybean roots when the doses of P in the nutrient solution were increased. The bacterium B. subtilis was the one with the highest phosphate solubilization rate, which is considered an alternative to increase the availability of phosphorus in the soil.

Acknowledgements

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Processes 312480/2020-2), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil - Finance code 001, and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS – Processes 22/2551-0001644-8) for granting scholarships to the authors. To the scholarship students and volunteers for helping in data collection.

Data Availability Statement

All datasets supporting the findings of this study have been published within the article itself.

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