Morphophysiological aspects of bean plants cultivated with natural reactive phosphate and solubilizing and growth promoting microorganisms

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Abstract. Beans (Phaseolus vulgaris L.) are an economically important crop, being part of the daily meal of a large part of the Brazilian population. One of the most common problems in bean cultivation is the low availability and low mobility of phosphorus. Among the strategies to improve the acquisition of phosphorus from the soil, the association of plants with microorganisms that promote growth and/or phosphate solubilizers stands out, since the efficiency of phosphate fertilizers depends directly on the microbial action in their cycle. Thus, the objective of this study was to evaluate physiological, morphological and biochemical characteristics in bean plants cultivated with the presence or absence of reactive natural phosphate associated or not with seed inoculation with different efficient microorganisms. The experiment was carried out in a greenhouse with a completely randomized design, consisting of eight treatments and four replications. The treatments were composed of: i) control, and application of ii) Azospirillum brasilense, iii) Bacillus subtilis and Bacillus megaterium, iv) efficient microorganisms (ME), v) reactive natural phosphate (RNP), vi) RNP + A. brasilense, and vii) RNP + Bacillus, and viii) RNP + ME. Gas exchange and material collection to determine height, stem diameter, dry matter, acid phosphatase activity and P content were performed 55 days after sowing. The treatment RNP + A. brasilense and other microorganisms contributed to a higher photosynthetic rate and transpiration in bean plants. The treatments RNP + ME and RNP + Bacillus promoted greater plant height and stem diameter, respectively. The shoot dry matter was higher in the RNP + ME treatment, in relation to the other treatments. Acid phosphatase activity was higher in the area of bean plants exposed to reactive natural phosphate, and in the roots of plants exposed to treatment with species of the genus Bacillus. It was possible to verify that the application of microorganisms with natural phosphate, despite not causing an increase in the P content of the shoot, favored the conditions of growth and production of bean plants.

Keywords: Phosphorus, microorganisms, photosynthesis, Phaseolus vulgaris.
Introduction

Beans are a legume that stands out for being an important source of protein, phosphorus, iron, vitamin B1 and fiber in human food (HEINEMANN, 2009). Brazil is among the world's largest producers of beans (FAOSTAT, 2022), with production of 2,856.1 thousand t in the 2020/21 harvest (CONAB, 2022). Brazil has adequate climate and soil conditions for the cultivation of this crop (COÊLHO, 2018), in addition to the technological advances that have contributed to the development of agriculture. Also, Elias et al. (2012) reported that around 65% of the national bean production comes from family farming, and the southern region of Brazil extends its cultivation in three annual harvests between August and April. The same authors highlighted that this crop is demanding in macro and micronutrients, and that phosphorus is directly associated with bean yield.

Although production conditions are potentially favorable, crops with inadequate soil management, by disregarding sustainable ecological bases, have resulted in the degradation of more than two billion hectares of soil worldwide (UNEP, 2000). The current agricultural production model with intensive use of inputs disregards the exploitation of biological components of the soil and nutrient cycling (COLA, 2012).

Phosphorus (P) availability in soil is naturally low and, in degraded areas, this condition can become even more critical. In this context, the adoption of production systems using low-solubility phosphorus sources associated with the use of phosphorus-solubilizing microorganisms can contribute to environmental sustainability and improve the productive potential of the crop. Phosphorus is present in the soil solution in the forms of orthophosphoric acid (H₃PO₄), phosphoric acid (H₂PO₄⁻), and phosphate (PO₄³⁻), and the concentrations of these anions are pH dependent (MENDES et al., 2003). In the solid phase, phosphorus can be in organic and mineral form. Deficiency of this nutrient can impair respiration, photosynthesis and generate the accumulation of carbohydrates, in addition to affecting the synthesis of nucleic acids and proteins, inducing the accumulation of soluble/nitrogenated compounds in plant tissue (RAVEN, 2013; MENG et al., 2021) and reduced plant growth (SILVA et al., 2014).

The increment of P in the roots promotes a greater exploration of the soil, in order to improve the HPO₄²⁻ uptake (HENDRICKSON et al., 2004). However, uptake is also dependent on the soil's ability to replenish the solution with nutrients (FERNANDES, 2000). Brazilian soils have low cation exchange capacity (CTC) and high ionic adsorption, thus reducing base saturation with increased retention of anions, such as phosphate (PIMENTEL, 2005). Thus, reactive natural phosphate, due to its slow availability and being accepted in organic crops, becomes an economically and environmentally advantageous option.

Microorganisms represent on average about 70% of the living and active fraction of soil organic matter. In agricultural ecosystems, phosphorus requires a symbiotic relationship between the plant and mycorrhizal fungi, the action of phosphorus solubilizing and mineralizing microorganisms and phosphatase producers to become available to plants in the biogeochemical cycle (ALVES, 2003; OLIVEIRA et al., 2021).

Plant growth-promoting bacteria such as Bacillus and Azospirillum can convert insoluble P into soluble forms, which can be taken up by plants (RAMAKRISHNA et al., 2019). In addition, a group of microorganisms, known as efficient microorganisms (EM), have also been used recently as bioinputs in agriculture. EMs are made up of bacteria, fermented products and photosynthetic products of lactic acid, formed from the metabolic activity of fermented foods and bacteria (enzymes and vitamins). The EMs act as plant growth promoters, as they optimize the physiological processes of plants such as photosynthesis, respiration, transpiration and enzymatic systems (ALLAHVERDIYEV et al., 2014). In this way, both growth-promoting bacteria and EM stand out as a sustainable alternative in promoting plant growth, especially in conditions of nutritional deficiency (MA et al., 2019; ZHANG et al., 2019).

Organic production with the use of growth-promoting microorganisms allows the reduction of impacts linked to soil degradation, working from basic ecological principles of agroecosystems such as mineralization and solubilization of phosphates, in order to preserve natural resources in a socially fair way, and economically sustainable (ALTIERI, 1987).

The hypothesis of the present study was that phosphate-solubilizing microorganisms increase P availability in bean plants and improve physiological, morphological and biochemical responses. Thus, this study aimed to evaluate morphophysiological and biochemical characteristics in bean plants cultivated with the presence or absence of reactive natural phosphate associated with or not with seed inoculation with different efficient microorganisms.

Material and Methods

The study was carried out in a greenhouse and in the Entomology and Biochemistry and Soils laboratories at the Federal University of Fronteira Sul (UFFS), Campus Erechim.

Plant material and growth conditions

The Carioca bean cultivar used in this study was obtained from family farmers in the municipality of Cacique Doble, RS, Brazil (SISGEN number A1D7F2E). Three seeds were sown in
Garbin et al. Morphophysiological aspects of bean plants cultivated with natural reactive phosphate and solubilizing and growth promoting microorganisms.

each plastic pot with a capacity of 8 dm³ of soil on 04/06/2021. The soil used as substrate was classified as a typical Aluminoferric Red Latosol (EMBRAPA, 2013) and collected in the experimental area at UFFS, Erechim, RS, Brazil (27.728681° S; 52.285852° W), with the following characteristics: clay content 30%; organic matter 1.1%; pH in water 4.4; phosphorus content 2.7 mg dm⁻³; potassium 28.8 mg dm⁻³; calcium 0.8 cmol dm⁻³; magnesium 0.4 cmol dm⁻³; sulfur 63.3 mg dm⁻³; exchangeable aluminum 12.7 cmolc dm⁻³; zinc 0.3 mg dm⁻³; copper 6.7 mg dm⁻³; manganese 15.0 mg dm⁻³; boron 0.1 mg dm⁻³; CTC at pH 7.0: 14.0; and effective CTC: 4.0.

The soil fertility was corrected 15 days before sowing according to the Rio Grande do Sul Liming and Fertilization Manual (SILVA et al., 2016), based on the results of the chemical analysis. Also, potassium sulfate (60 kg ha⁻¹) and urea (30 kg ha⁻¹) were added in two applications: at sowing and post-emergence of the plants.

From the vegetative stage V2, which occurs 21 days after sowing (DAS), the plants were submitted weekly to applications of Neem oil (8 mL L⁻¹) to control Diabrotica speciosa and the homeopathy Silicea terra 18 CH (1mL L⁻¹). 1) for the Corinthespora cassicola control.

Table 1. Treatments used in the present study, sources of phosphorus and inoculants.

| Treatment         | Phosphorous source            | Seed inoculation                  |
|-------------------|-------------------------------|----------------------------------|
| Control           | -                             | -                                |
| A. brasilense     | -                             | Azospirillum brasilense¹         |
| Bacillus          | -                             | Bacillus subtilis e B. megaterium²|
| EM                | -                             | Efficient microorganisms³        |
| RNP               | Reactive natural phosphate⁴  | -                                |
| RNP + A. brasilense| Reactive natural phosphate⁴  | Azospirillum brasilense¹         |
| RNP + Bacillus    | Reactive natural phosphate⁴  | Bacillus subtilis e B. megaterium²|
| RNP + EM          | Reactive natural phosphate⁴  | Efficient microorganisms³        |

¹Strains ABV5/ABV6 (6 mL Kg⁻¹); ²StrainBHM 2084 (1 mL Kg⁻¹); ³No dilution (1 mL kg⁻¹); ⁴20% total P₂O₅ (140 Kg ha⁻¹)

The bean seeds were inoculated with microorganisms at the sowing time, considering the following doses: Azospirillum brasilense 6 mL Kg⁻¹. Efficient Microorganisms (EM) 1 mL Kg⁻¹, and a mix of Bacillus subtilis and Bacillus megaterium 1 mL Kg⁻¹. Also, a treatment with reactive natural phosphate (RNP) (20% of total P₂O₅; 140 Kg ha⁻¹), and the combination of RNP with the other treatments with microorganisms (A. brasilense, EM and Bacillus) were also carried out, as detailed in table 1.

The experimental design was completely randomized, consisting of eight treatments, with 4 replications.

At 55 DAS, gas exchange was evaluated and then the plants were collected and separated into shoots and roots for biochemical, morphological and nutritional analyses.

Physiological Analysis
Gas exchange evaluations were performed on a fully expanded leaf to determine photosynthetic rate (A, µmol m⁻² s⁻¹), stomatal conductance (gs, mol m⁻¹ s⁻¹), transpiration (E, mol m⁻² s⁻¹), and the relationship between the internal and external CO₂ concentration (Ci/Ca). From these data it was possible to calculate the water use efficiency (WUE = A/E). The evaluations were carried out between 8 and 10 h under photosynthetically active radiation (~1,000 µmol m⁻² s⁻¹), and environmental CO₂ concentration (Ca, ~430 µmol mol⁻¹) and temperature (~20 °C). For these determinations, an infrared gas analyzer (IRGA; LCA PRO Analytical Development Co. Ltd, Hoddesdon, UK) was used.

Morphological Analysis
Plant height was determined by measuring with a ruler. The stem diameter was measured with a digital caliper (Stainless Hardened, Jamarca) at 5 cm from the soil base. Subsequently, the plants were separated into shoots and roots and dried in an oven with forced air circulation at 60°C, until
constant matter. Dry matter was determined using an analytical balance.

**Biochemical Analysis**

To determine the activity of the acid phosphatase enzyme, samples of leaves and roots were collected and immediately frozen in liquid nitrogen, and stored at -20°C until the moment of analysis. Acid phosphatase activity was determined according to Tabaldi et al. (2007). Samples of leaves and roots (~500 mg) were homogenized in 3 mL of citrate solution (100 mM) and centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was collected and homogenized in a reaction solution containing citrate buffer (100 mM, pH 5.5), sodium azide (3.5 mM) and calcium chloride (2.5 mM), and incubated at 35°C for 10 min. The reaction started by the addition of the organic pyrophosphate substrate (PPi, 3.0 mM) and stopped with the addition of trichloroacetic acid (TCA, 5%). Inorganic phosphate was quantified at 360 nm using a spectrophotometer (700 Plus, FEMTO Indústria e Comércio de Instrumentos, São Paulo, Brazil), using malachite green as a colorimetric reagent. Acid phosphatase enzyme activity was determined based on a standard curve of KH$_2$PO$_4$ (10 mM) and expressed as fresh weight.

**Nutritional analysis**

Phosphorus content was determined in the aerial part of bean plants, previously dried and ground, according to the methodology described by Tedesco et al. (1995). Initially, the samples (~0.2 g) were digested with H$_2$O$_2$ (30%) and H$_2$SO$_4$ in a digester block at 160-180°C until the water evaporated. Subsequently, the samples were kept at 350-375°C for 1 h. After cooling the flasks, the material was resuspended in distilled water and stored in a refrigerator. Samples were analysed at 660 nm in a spectrophotometer (Halogen Lamp, Nova).

**Statistical analysis**

Data were submitted for analysis of variance and the means were compared by Tukey's test at 5% probability, using the easyanova package in the R statistical software (version 4.0.4). For the multivariate analysis, the data were transformed by cube root and scaled by centering the mean and analyzed using the MetaboAnalyst software (version 5.0).

**Results and Discussions**

The natural phosphate treatment promoted the highest photosynthetic rate in bean plants, followed by the treatments RNP+A. brasilense, Bacillus, RNP+Bacillus and RNP+EM (Table 2). In general, transpiration was higher in treatments containing RNP, while stomatal conductance, water use efficiency and Ci/Ca ratio did not differ statistically between treatments (Table 2).

| Treatment          | $A$ (µmol m$^{-2}$ s$^{-1}$) | $g_s$ (µmol m$^{-2}$ s$^{-1}$) | $E$ (µmol m$^{-2}$ s$^{-1}$) | WUE | Ci/Ca |
|--------------------|-------------------------------|-------------------------------|-------------------------------|-----|-------|
| Control            | 1.22 b                        | 0.042 a                       | 0.460 b                       | 2.380 a | 0.875 a |
| Azospirillum brasilense | 1.23 b                       | 0.035 a                       | 0.445 b                       | 2.485 a | 0.860 a |
| EM                 | 1.31 b                        | 0.040 a                       | 0.489 ab                      | 2.107 a | 0.869 a |
| Bacillus           | 2.06 ab                       | 0.035 a                       | 0.475 ab                      | 3.055 a | 0.815 a |
| RNP                | 3.61 a                        | 0.045 a                       | 0.630 ab                      | 4.075 a | 0.737 a |
| RNP + Azospirillum brasilense | 2.40 ab                     | 0.048 a                       | 0.705 a                       | 2.424 a | 0.828 a |
| RNP + EM           | 1.78 ab                       | 0.043 a                       | 0.630 ab                      | 2.345 a | 0.831 a |
| RNP + Bacillus     | 1.82 ab                       | 0.035 a                       | 0.553 ab                      | 2.447 a | 0.822 a |

Means of treatment followed by the same letter did not differ by Tukey's test (p<0.05).

In plants, phosphate is a constituent of adenosine triphosphate (ATP), a molecule that represents the metabolic energy of cells and is essential for carrying out processes such as photosynthesis, respiration, glycolysis, starch biosynthesis and ion absorption (HAWKESFORD et al., 2011; VENKLAAS et al., 2012; CARSTENSEN et al., 2018). Despite being an essential nutrient for plants, the concentration of P in the soil solution, at the root-soil interface, is often present at submicromolar levels, with a low rate of diffusion and mobility (JHORI et al., 2015). Thus, plants with adequate phosphorus supply are able to maintain higher photosynthetic rates as a consequence of maintaining ATP production (SILVA et al., 2006; LIN et al., 2009; WARREN, 2011).

Furthermore, bacteria of the genus Azospirillum are described for promoting plant growth, stimulating the production of phytohormones (auxins) that act on root development, allowing the mineralization of organic phosphate and solubilization of inorganic phosphate. In studies with corn, Azospirillum also contributed to the increase in gas exchange in plants (RODRIGUEZ & FRAGA, 1999).
The natural phosphate associated with EM contributed to the higher plant height and root dry matter in relation to the treatment with natural phosphate plus *A. brasilense* (Table 3). The EMs act by decreasing compaction and increasing soil aggregation/porosity, which directly affects water availability and rooting depth. They also act in the decomposition of organic matter and in the mineralization and availability of nutrients for plants (BONFIM et al., 2011; OLIVEIRA et al., 2021). The synergistic effect of the plant with the microorganisms can be expressed by the plant growth rates, since the increase in the efficiency of phosphorus use indicates the solubilization of aluminum, calcium and iron metal-cation complexes precipitated and fixed in the soil (OLIVEIRA et al., 2019; ESTESAMI, 2020). Most of these effects are due to the presence of arbuscular mycorrhizal fungi, which increase the contact area of plant roots with the soil, allowing greater P absorption (WANG et al., 2012), and, consequently, greater growth and growth, plant productivity, as already described for maize (ALMAGRABI & ABDELMONEIM, 2012), chickpeas (PELLEGRINO & BEDINI, 2014), soybean (OLIVEIRA et al., 2019) and cotton (GAO et al., 2020) plants. In addition, the interaction between bacterial and fungal microorganisms with phosphate sources allowed an increase in the total dry matter of sugarcane and greater efficiency of reactive phosphate (GUMIERE et al., 2019). This occurs when there is a good symbiotic performance of microorganisms with the host plants, as in legumes, root growth is potentiated as the root contact surface increases, thus generating an increase in the bioavailability of nutrients, mainly of phosphorus (TALAAT et al., 2015). The largest stem diameter (Table 3) was observed in bean plants exposed to natural phosphate associated with *B. subtilis* and *B. megaterium*. Species of the genus *Bacillus* are described for being growth-promoting bacteria, promoting an increase in the root surface (SOUZA et al., 2015). In addition, *B. subtilis* and *B. megaterium* act directly on the solubilization of phosphorus and/or the release of soluble phosphates (KALAYU, 2019), in order to increase the efficiency use of this nutrient, which makes them economically important, since it allows reducing the doses of fertilizers used and maintaining crop productivity (ABREU et al., 2017).

The highest activity of acid phosphatase in the aerial part of bean plants was observed in the treatment with RNP, followed by the treatments RNP + *Bacillus*, RNP + EM and RNP + *A. brasilense* (Table 4). The acid phosphatase enzyme acts by hydrolyzing phosphate esters into soluble P, which allows the cycling and availability of this nutrient and the maintenance of metabolic activities in plants (VENEKLAAS, 2012). The availability of P in the medium favors the growth of microorganisms, which, in adequate and not excessive concentration, stimulates soil microorganisms in the production and secretion of phosphatases for greater organic hydrolysis of P (NAHAS, 2015).

**Table 3.** Plant height (PH), stem diameter (SD), shoot dry matter (SDM) and root dry matter (RDM) of bean plants collected 55 days after sowing and cultivated after seed treatment application with *Azospirillum brasilense* (6 mL Kg$^{-1}$), efficient microorganisms (EM; 1mL kg$^{-1}$), *Bacillus subtilis* and *B. megaterium* (1mL Kg$^{-1}$), fertilizer containing reactive natural phosphate (RNP; 140g ha$^{-1}$) and the combination of RNP with the different microorganisms.

| Treatment       | PH (cm plant$^{-1}$) | SD (mm plant$^{-1}$) | SDM (g plant$^{-1}$) | RDM (g plant$^{-1}$) |
|-----------------|-----------------------|----------------------|----------------------|----------------------|
| Control         | 10.33 ab              | 3.36 ab              | 0.282 a              | 0.380 a              |
| *A. brasilense* | 9.63 ab               | 3.16 ab              | 0.376 a              | 0.227 ab             |
| EM              | 10.15 ab              | 3.07 ab              | 0.238 a              | 0.184 b              |
| *Bacillus*      | 9.40 ab               | 3.28 ab              | 0.229 a              | 0.238 ab             |
| RNP             | 10.15 ab              | 3.37 ab              | 0.261 a              | 0.154 b              |
| RNP + *A. brasilense* | 8.98 b                | 2.92 b               | 0.223 a              | 0.268 ab             |
| RNP + EM        | 10.70 a               | 3.08 ab              | 0.297 a              | 0.372 a              |
| RNP + *Bacillus*| 10.00 ab              | 3.60 a               | 0.350 a              | 0.230 ab             |

Means of treatment followed by the same letter did not differ by Tukey’s test (p<0.05).

Thus, microorganisms, in addition to increasing the surface area of the roots by the extension of the root system with the mycorrhizae, they release phytohormones, favoring the displacement of the uptake balance through the transfer of phosphate ions to the soil solution. This increases P mobility, and stimulates metabolic processes related to the P cycle, such as the production of phosphatases, enzymes that hydrolyze organic phosphorus, release organic acids and excrete hydrogen ions (MENDES et al., 2003).

Although the P content in the aerial part of the bean plants did not differ between the treatments evaluated (Table 4), the treatments based on RNP associated with different microorganisms, promoted, in general, higher values for gas exchange and production of dry matter in bean plants. It should also be noted that the genetic potential of cultivars to uptake P depends on factors such as morphology, presence of root hairs, mycorrhizal colonization and association with phosphorus-solubilizing microorganisms, secretion of phosphatase enzymes and high photosynthetic efficiency. This occurs since the responses may vary depending on the genotype used and the soil and climate conditions of cultivation (HUNGRIA et al., 2010).
Table 4. Acid phosphatase activity (APase. U mg\(^{-1}\) protein) in shoot (Sh) and roots (R), and total phosphorus content (P, %) in shoot of bean plants collected 55 days after sowing and cultivated after seed treatment application with *Azospirillum brasilense* (6 mL Kg\(^{-1}\)), efficient microorganisms (EM; 1mL kg\(^{-1}\)), *Bacillus subtilis* and *B. megaterium* (1mL Kg\(^{-1}\)), fertilizer containing reactive natural phosphate (RNP; 140g ha\(^{-1}\)) and the combination of RNP with the different microorganisms.

| Treatment                     | Apase (Sh) | Apase (R) | P   |
|-------------------------------|------------|-----------|-----|
| Control                       | 189.5 c    | 192.0 ab  | 0.236 a |
| *A. brasilense*               | 102.4 d    | 209.3 ab  | 0.211 a |
| EM                            | 116.4 d    | 279.6 ab  | 0.212 a |
| *Bacillus*                    | 143.6 cd   | 312.8 a   | 0.216 a |
| RNP                           | 438.6 a    | 242.4 ab  | 0.222 a |
| RNP + *A. brasilense*         | 337.4 b    | 275.2 ab  | 0.212 a |
| RNP + EM                      | 355.7 b    | 158.7 ab  | 0.224 a |
| RNP + *Bacillus*              | 358.0 b    | 130.1 b   | 0.209 a |

Means of treatment followed by the same letter did not differ by Tukey's test (p<0.05).

Figure 1. Score plot for physiological and morphological traits, and P content of bean plants collected 55 days after sowing and cultivated after seed treatment application with *Azospirillum brasilense* (6 mL Kg\(^{-1}\)), efficient microorganisms (EM; 1mL kg\(^{-1}\)), *Bacillus subtilis* and *B. megaterium* (1mL Kg\(^{-1}\)), fertilizer containing reactive natural phosphate (RNP; 140g ha\(^{-1}\)) and the combination of RNP with the different microorganisms.

Taken the data together, it was possible to verify a clear separation between treatments containing RNP associated with microorganisms and treatments in the absence of RNP, both separate from the control group (Figure 1). This demonstrates that the association of RNP with microorganisms was advantageous for the physiological activities and growth of bean plants.

**Conclusion**

Treatment with reactive natural phosphate associated with *Azospirillum brasilense* and other microorganisms contributed to a higher photosynthetic rate and transpiration in bean plants. The treatments RNP+EM and RNP+*Bacillus* promoted greater plant height and stem diameter, respectively. The shoot dry matter was higher in the RNP+EM treatment, in relation to the other treatments. Acid phosphatase activity was higher in the shoots of bean plants exposed to reactive natural phosphate, and in the roots of plants subjected to treatment with *Bacillus subtilis* and *B. megaterium*.

It was possible to verify that the application together of microorganisms with natural phosphate, despite not causing an increase in the P content of the shoot, favored the conditions of growth and production of bean plants. Thus, it can be concluded that seed inoculation with growth-promoting microorganisms and phosphorus solubilizers, associated with fertilization with reactive natural phosphate, benefits the physiological, biochemical and morphological aspects of bean plants.

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