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Biotechnological potential of growth-promoting bacteria in cotton (*Gossypium hirsutum* L.) crop

Potencial biotecnológico de bactérias promotoras de crescimento na cultura de algodão (*Gossypium hirsutum* L.)

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ABSTRACT

Studies involving plant growth-promoting bacteria are attracting increasing attention in the agricultural sector due to their potential to improve growth and production, and to protect plants from biotic and abiotic stresses. The present study aimed to evaluate the effects of three species of plant growth-promoting bacteria (*Bacillus subtilis*, *Priestia megaterium,* and *Priestia aryabhattai*) on the growth and morphological and biochemical aspects of *Gossypium hirsutum* L. (cotton) seedlings. The experiment was conducted in a greenhouse with four treatments (one control and three inoculations) and five replications per treatment. The seeds were inoculated by immersion in bacterial suspensions (10^9 CFU/mL) and then sown in pots. The plants were monitored for 60 days. During collection, the plants were measured for the fresh mass of roots and shoots, the height of the shoots, stem diameter, and number of leaves. Leaf samples were collected and used for biochemical analyses. The results obtained showed that seeds treated with *P. aryabhattai* had significant improvements in the parameters of fresh mass, plant height, stem diameter, and number of leaves, and in the contents of chlorophyll (*a*, *b,* and total), nitrogen, and proteins concerning plants in the control treatment. Plants treated with *P. megaterium* also achieved improvements in fresh mass, stem diameter, nitrogen, and protein contents. These results indicate the potential of these plant growth-promoting bacteria for use in cotton crops and can be employed in the preparation of biostimulants and biofertilizers.

Keywords: plant growth-promoting bacteria; biostimulants and biofertilizers; *Bacillus subtilis; Priestia megaterium; Priestia aryabhattai*.

RESUMO

Estudos envolvendo bactérias promotoras de crescimento de plantas vêm chamando cada vez mais atenção no setor agrícola, devido ao seu potencial para melhorar o crescimento, produção e proteger as plantas dos estresses bióticos e abióticos. O presente estudo teve como objetivo avaliar o efeito de três espécies de bactérias promotoras de crescimento de plantas (*Bacillus subtilis*, *Priestia megaterium* e *Priestia aryabhattai*) no crescimento e nos aspectos morfológicos e bioquímicos de plântulas de *Gossypium hirsutum* L. (algodão). O experimento foi conduzido em casa de vegetação com quatro tratamentos (um controle e três inoculações) e cinco repetições por tratamento. As sementes foram inoculadas por imersão em suspensões bacterianas (10^9 UFC/mL) e então semeadas em vasos. As plantas foram acompanhadas por 60 dias. Na coleta, as plantas foram mensuradas quanto à massa fresca das raízes e da parte aérea, à altura da parte aérea, ao diâmetro do caule e número de folhas. Amostras foliares foram submetidas às análises bioquímicas. Os resultados obtidos mostraram que sementes tratadas com *P. aryabhattai* tiveram melhorias significativas nos parâmetros de massa fresca, altura da planta, diâmetro do caule e número de folhas e nos teores de clorofila (*a*, *b* e total), nitrogênio e proteínas em relação às plantas do tratamento controle. Plantas tratadas com *P. megaterium* também obtiveram melhorias na massa fresca, no diâmetro do caule e nos teores de nitrogênio e proteínas. Esses resultados indicam um potencial dessas bactérias promotoras de crescimento de plantas para utilização em culturas do algodão, podendo ser empregadas na elaboração de bioestimulantes e biofertilizantes.

Palavras-chave: bactérias promotoras de crescimento de plantas; bioestimulantes e biofertilizantes; *Bacillus subtilis; Priestia megaterium; Priestia aryabhattai.*

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Introduction

Bacteria are one of the most diverse and dominant groups of microorganisms present in the soil. Their diverse metabolism and ability to use a wide range of substances as sources of nutrients and energy make bacteria important partners in interactions with plants (Oleńska et al., 2020). These beneficial interactions include nutrient supply, growth stimulation, phytohormone production, pathogen biocontrol, soil structure improvement, bioaccumulation of inorganic compounds, and bioremediation of biotic and abiotic stresses (Singh et al., 2017).

Bacteria that directly or indirectly benefit plant growth are commonly referred to as plant growth-promoting bacteria (PGPB). PGPB have attracted particular attention in the agricultural sector due to their potential to replace or reduce the use of toxic agrochemicals and mitigate their harmful effects on the environment and human health. In the last two decades, the terms biofertilizer or bioinoculant have been commonly used as a result of the progress made in studies on the association between microorganisms and plants (Basu et al., 2021).

Within this group of bacteria, some species stand out based on their potential to produce many essential products for the food, pharmaceutical, environmental, and agricultural industries. The *Bacillus subtilis*, *Priestia* (*Bacillus*) *megaterium* and *Priestia* (*Bacillus*) *aryabhattai* species can produce a wide range of biologically active molecules that are useful for promoting plant growth and improving biochemical parameters, showing great potential for being marketed as biofertilizers and bioinoculants (Nascimento et al., 2020; Sun et al., 2020; Sultana et al., 2021).

Cotton (*Gossypium* sp.) is an important agricultural crop in the world. Its cultivation extends across five continents, covering an area of more than 30 million hectares (Khan et al., 2020). However, cotton production has raised environmental concerns due to the high consumption of agrochemicals (Gedik and Avinc, 2020). Therefore, sustainable alternatives for maintaining cotton productivity are important, and the use of PGPB has shown promising in the processing of this crop. Strains of *B. subtilis* and *P. aryabhattai* have already been reported as cotton growth promoters, improving vegetative and reproductive parameters of plants in experiments carried out in Pakistan (Ahmad et al., 2023). Bataeva et al. (2020) also observed positive effects of using *P. megaterium* on the growth and productivity of cotton crops in the Astrakhan region of Russia.

In the Brazilian semi-arid region, the use of PGPB seeks to introduce a sustainable perspective to agricultural activities in the region, where cotton cultivation stands out. Brazil is the third largest producer and second leading exporter of cotton worldwide and the Northeast region is the second largest cotton producer in the country, contributing with 26% of total production (Alves et al., 2019). The advancement of the agricultural sector in this region brings inherent concerns related to soil degradation and the risks that toxic agrochemicals cause to one's health. Sustainable alternatives in this sector are urgently essential for conserving biodiversity and maintaining people's health. Therefore, this study aimed to evaluate the efficiency of three bacterial species (*B. subtilis*, *P. megaterium,* and *P. aryabhattai*) on the growth and biochemical parameters of cotton plants (*Gossypium hirsutum* L.).

Materials and Methods

Seeds and bacterial strains

The cotton seeds (*G. hirsutum* L.; BRS 286), used in the experiment had no previous treatment and were kindly provided by cotton seed producer Josefa Maria Francieli da Silva, located in Crato (CE), Brazil. The species *Bacillus subtilis* (EKO700), *Priestia* (*Bacillus*) *megaterium* (EKO701), and *Priestia* (*Bacillus*) *aryabhattai* (EKO703) were kindly provided by Ekoa Life Sciences in Brasília (DF), Brazil.

Preparing the bacterial inoculum and inoculating the seeds

To inoculate the seeds, the bacterial strains were activated and grown separately in nutrient broth at 30°C for 48 hours. All suspensions were then adjusted to 10^9 CFU/mL. Before inoculation, the seeds were rinsed in 2% sodium hypochlorite (w/v) for three minutes, followed by three washes with distilled water to remove the residual sodium hypochlorite. The seeds were subsequently inoculated by immersing in Erlenmeyer flasks containing suspensions of each bacteria adjusted to 10^9 CFU/mL and left to stir in a rotary shaker (Cientec, Model CT-713R, Brazil) at 130 rpm for 30 minutes.

Setup of the experiment in the greenhouse

Four treatments were established: a control (non-inoculated seeds); treatment I - with seeds inoculated with *B. subtilis*; treatment II - with seeds inoculated with *P. megaterium*; and treatment III — with seeds inoculated with *P. aryabhattai*. After inoculation, the seeds were taken to the greenhouse and then sown in 30 × 20 cm polyethylene bags containing a mixture of fine soil, plant substrate, and organic fertilizer in a ratio of 3.5:1.5:1, totaling 5 kg of soil in each replication. The chemical analysis of the soil used in the experiment is shown in Table 1. Five replicates were established for each treatment and five seeds were sown in each polyethylene bag (replicate). The experimental design was entirely randomized. Four seedlings already emerged were removed seven days after sowing (DAS), keeping one plant per bag (experimental unit). All treatments received daily irrigation standardized by the substrate's field capacity. The height of each plant was measured every seven days, and the experiment was conducted until 60 DAS.

Morphometric analysis

At 60 DAS, plants were collected and measurements were taken of the height of each plant, the diameter of the stem (with a caliper 2 cm above the ground), the number of leaves, the fresh mass of the aerial part and root, and the total fresh mass were measured. Leaf samples from each treatment replication were also collected, crushed in liquid nitrogen, and stored in a freezer (-18°C) until biochemical analysis.

Table 1 – Chemical analysis of the soil used in the experiment.

Sample	pH	Ca	Mg	Al	Na	K	P	O.C.	O.M.	H+Al
	1:2.5	------------cmol$_c$/dm^3------------					mg/dm^3	---g/kg---		cmol$_c$/dm^3
Substrate	5.85	3.2	2.1	0.15	0.4	0.09	43.83	18.33	31.60	4.20

pH: potential of hydrogen; Ca: calcium; Mg: magnesium; Al: aluminum; Na: sodium; K: potassium; P: phosphorus; O.C.: organic carbon; O.M.: organic matter; H+Al: potential acidity.

Biochemical analysis

To carry out the biochemical analyses, leaf samples crushed in liquid nitrogen were macerated in 80% ethanol (v/v) and centrifuged at 5,000 rpm for 10 minutes at room temperature to obtain the ethanolic extract. From this extract, the concentrations of chlorophyll (*a* and *b*) and carotenoids were quantified using the spectrophotometric method of Arnon (1949) and Lichtenthaler and Wellburn (1983), respectively. The measurements were taken at wavelengths of 645 nm, 663 nm, and 470 nm, respectively, and the results were expressed in mg/g of fresh mass (FM). Soluble proteins were quantified by the spectrophotometric method of Bradford (1976) using a standard curve with bovine serum albumin, measured at 595 nm and the results were expressed in mg/g of FM.

To determine the total nitrogen content, dry leaf samples were digested in sulphuric acid and measured according to the colorimetric method proposed by Baethgen and Alley (1989). Spectrophotometric readings were taken at a wavelength of 650 nm and the results were expressed in mg/g dry mass (DM). For the phosphorus and potassium determinations, dried leaf samples were extracted with hydrochloric acid. Phosphorus measurements were made using the vanadate-molybdate spectrophotometric method (670 nm), and potassium measurements were made using flame photometry (Miyazawa et al., 2009).

Statistical analysis

All the results were subjected to the homoscedasticity test (Levene) and the normality test (Shapiro-Wilk). For data that proved to be consistent, analysis of variance (ANOVA) was applied, and a posteriori Duncan's mean comparison test was used to detect significant differences between treatments at a 5% significance level. For data that was not normal, the Kruskal-Wallis test was applied, followed by the Dunn's test adjusted by Bonferroni at a 5% significance level. A principal component analysis (PCA) was applied to all the data obtained to determine the association and correlation between the treatments and the parameters analyzed. A permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis index was used to confirm the groups formed in the PCA. A Pearson correlation analysis was conducted to verify correlations between the parameters analyzed (at a significance level of 5%). The univariate statistical analysis was performed in the RStudio software (version 2022.07.2+576) and the multivariate analysis was carried out in the Past software (version 4.14).

Results and Discussion

Vegetative growth

The accumulation of biomass assessed by quantifying the FM of the cotton plants collected at 60 DAS showed significantly higher values in the individuals treated with *P. aryabhattai* in relation to other treatments, especially compared with the control treatment. A significantly greater investment in root growth was observed in plants inoculated with *P. aryabhattai*, with values 94.2% higher than non-inoculated plants (Figure 1A). The FM of the aerial part showed a higher increase in plants treated with *P. aryabhattai* (52.5%) and *P. megaterium* (48.6%) than non-inoculated plants (Figure 1A). Furthermore, plants treated with *P. aryabhattai* showed 49.3% higher values of total FM compared to the group of plants without inoculation (Figure 1B). There was also a significant increase in the height of the plants treated with *P. aryabhattai*, which was 15.7% higher than the plants in the control group (Figure 1C) and in the stem diameter, which was 15.5% greater (Figure 1D). The number of leaves was also greater (36.5%) in plants treated with *P. aryabhattai* than in the control treatment (Figure 1E).

The positive effects of *P. aryabhattai* and *P. megaterium* on the biomass of cotton plants can be attributed to several interconnected factors, such as higher nutrient availability and accumulation, higher efficiency in plant primary metabolism and phytohormone production, and better plant acclimatization to the environment (Mendes et al. 2020; Munns et al. 2020). The ability of PGPB to promote growth and increase biomass in plants is well known and has already been reported in many economically important plant crops. Previous studies have reported positive effects on sugarcane, corn, and soybean growth promoted by species of the genera *Bacillus* and *Priestia* (Antunes et al., 2017; Breedt et al., 2017; Bavaresco et al., 2020).

In general, roots are more susceptible to PGPB effects because in real growing conditions the plant's root system is colonized, which leads to better root development and its metabolites influence several other important factors for plant growth and yield (Bavaresco et al., 2020). It has already been reported that the increase in root biomass promoted by PGPB occurs mainly due to an increase in the number of root hairs, which are extremely important for the absorption of water and nutrients (Sousa et al., 2021). It is possible that the PGPB in question employed a combination of direct and indirect mechanisms to increase root growth, such as the synthesis of indoleacetic acid, a phytohormone directly related to cell elongation, and phosphate solubilization (Mendes et al., 2020).

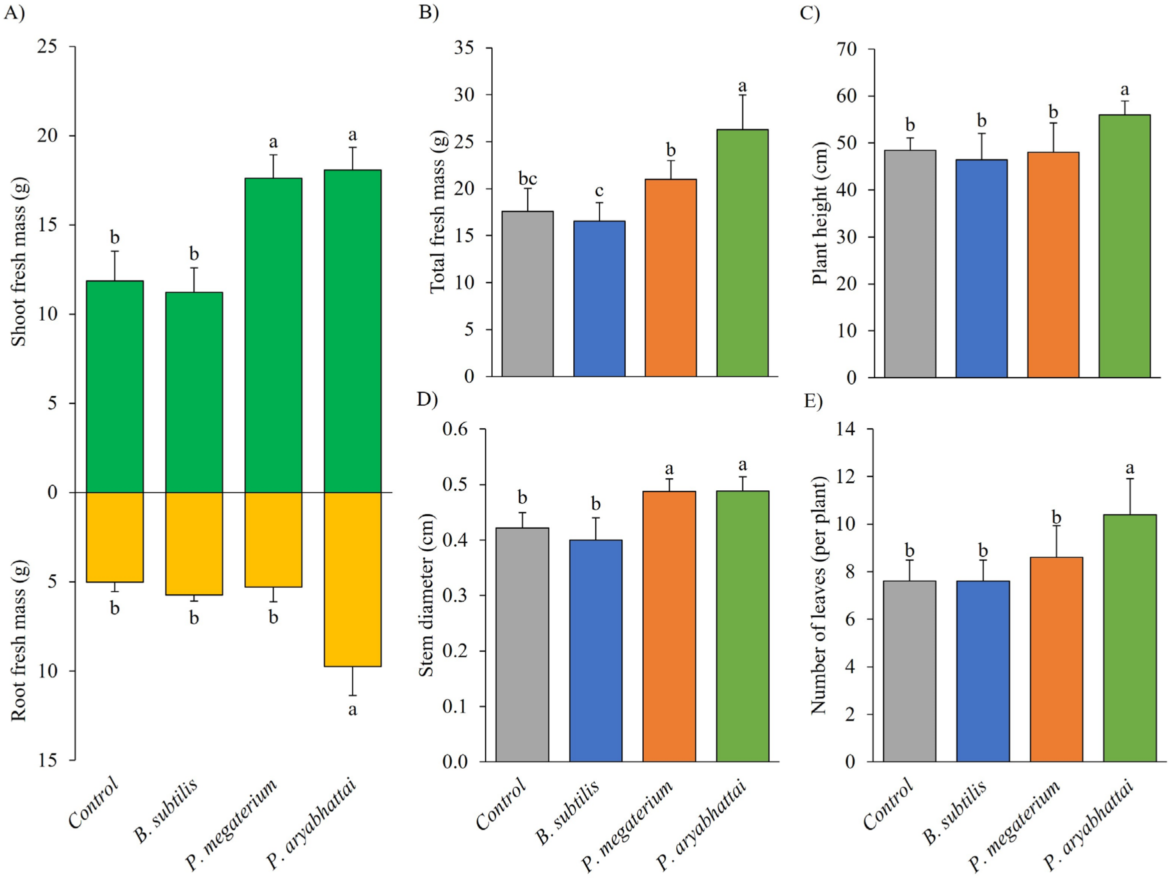


Figure 1 – Root and shoot fresh mass (A), total fresh mass (B), plant height (C), stem diameter (D), and number of leaves (E) in *G. hirsutum* plants 60 days after sowing. Different letters represent significant differences between treatments using Duncan's test (p<0.05).

Increases in shoot height and stem diameter may also be related to the production of phytohormones. Tahir et al. (2017) reported that PGPB can induce changes in the gene expression of phytohormone metabolism in plant tissues, thus increasing the endogenous levels of these hormones and influencing plant growth responses. Stem diameter is related to plant support and to water and nutrient transportation processes. The translocation of photosynthates, for example, occurs through phloem and it is necessary to maintain plant growth. Therefore, thicker stems in the early stages of the plant enable more efficient transportation of these photoassimilates, resulting in higher plant growth (Lemoine et al., 2013).

P. aryabhattai was reported as a plant growth promoter for the first time by Lee et al. (2012) who identified growth promotion in *Xanthium italicum* caused by different bacterial strains of this species, mainly due to phosphate solubilization and phytohormone production. Recent studies have reported that strains of *P. aryabhattai* can act on various plant growth mechanisms, such as nitrogen fixation, indoleacetic acid production, phosphate solubilization, siderophore production, 1-aminocyclopropane-1-carboxylate deaminase activity, and exopolysaccharide production (Farahat et al., 2020). In addition, as it is a halotolerant bacterium, *P. aryabhattai* is also capable of acting to alleviate salt stress (Sultana et al., 2021). Strains of *P. megaterium* have also been reported as plant growth promoters, improving various morphological aspects, participating in the solubilization of nutrients (such as phosphorus, potassium, and zinc) and in the production of phytohormones (Nascimento et al., 2020; Miljaković et al., 2022). In the present study, it was possible to observe that the inoculation of cotton plants with *P. aryabhattai* and *P. megaterium* resulted in significant increases in the vegetative growth parameters of this crop. This corroborates the results found by Bataeva et al. (2022) who observed the potential of these PGPB for promoting growth and improving cotton crop yield.

Photosynthetic pigments

The treatment of cotton plants with PGPB significantly affected the concentration of photosynthetic pigments. The contents of chlorophylls *a*, *b*, and total were higher (16.3, 17.2, and 16.5%, respectively) in plants inoculated with *P. aryabhattai* than in non-inoculated plants (Figure 2A). The carotenoid content (Figure 2B) and the chlorophyll *a*:chlorophyll *b* ratio (Figure 2C) showed no significant differences between treatments.

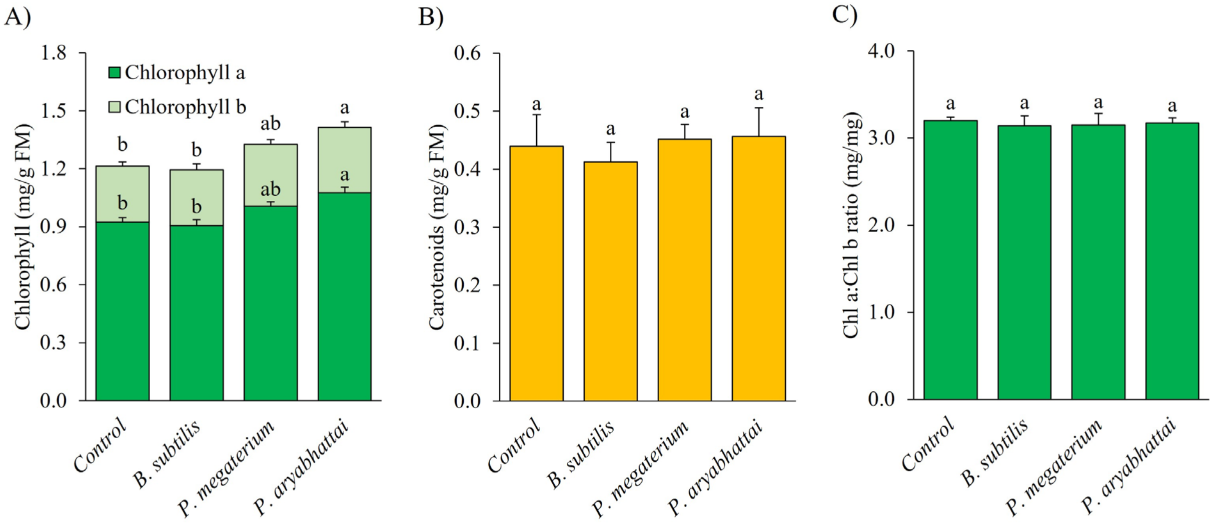


Figure 2 – Chlorophyll *a*, *b* and total contents (A), carotenoids (B) and chlorophyll *a*:chlorophyll *b* ratio (C) in *G. hirsutum* plants 60 days after sowing. Different letters represent significant differences between treatments according to Duncan's test ($p<0.05$).

Chlorophyll concentration is an important physiological parameter, as it is directly related to the photosynthetic rate and, consequently, to plant growth. Chlorophyll *a* is the most relevant pigment in the photosynthesis process and an increase in its content in the leaves may indicate that the plant is receiving a higher supply of nitrogen, the main nutrient required for the synthesis of the chlorophyll molecule (Costa Neto et al., 2017). Chlorophyll *b* acts as an accessory pigment, helping to extend the range of light used in the photosynthesis process and, consequently, improving the photosynthetic rate and increasing the plant's growth and productivity (Peres et al., 2016). Furthermore, carotenoids also act as an accessory pigment, as they are associated with many of the proteins present in the photosynthetic system, besides helping to capture light at different wavelengths, and have an important function as an antioxidant preventing damage caused by the oxidative effect of excess light on chlorophyll molecules (Taiz and Zeiger, 2017).

The significant increase in chlorophyll *a* and *b* content in plants inoculated with *P. aryabhattai* suggests the ability of this PGPB to increase the assimilation of essential nutrients such as nitrogen, enhance the activity of electron transporters related to photosynthesis, and improve stomatal conductance (Lima et al., 2019; Silva et al., 2019). The lack of a significant increase in carotenoid content in the inoculated plants may be attributed to their exposure to the same conditions in the greenhouse. Carotenoids accumulate in plants under different abiotic stresses; in the absence of stressful conditions, plants tend to invest in the production of chlorophylls (Swapnil et al., 2021).

Macronutrients and proteins

Concerning the main plant macronutrients (nitrogen, phosphorus, and potassium), only nitrogen content was significantly affected by bacterial inoculation in cotton plants. Higher values were observed in the three treatments with PGPB compared to the plants of the control treatment, with *P. aryabhattai*, *P. megaterium,* and *B. subtilis* being responsible for an increase of 270.9, 248.9, and 243.1%, respectively, in nitrogen content compared to non-inoculated plants (Figure 3A).

After carbon, nitrogen is the most abundant element in plants and one of the fundamental nutrients in the formation of proteins, nucleic acids, chlorophylls, and hormones (Taiz and Zeiger, 2017). Nitrogen is directly related to the growth, metabolism and productivity of a vegetable crop and is required in large quantities by plants. To avoid losses and enhance production, the agricultural sector uses high doses of nitrogen fertilizers. However, the intensive use of these fertilizers has caused pollution, acidification, and soil degradation, resulting in losses both for the environment and for crop productivity itself (Aquino et al., 2021). In this respect, the action of PGPB in making nitrogen available to the plant is relevant to agriculture, given that these bacteria are capable of positively affecting nitrogen metabolism in plants; this benefits a chain of important mechanisms, improving nitrogen absorption by increasing root biomass, acting in the biosynthesis of phytohormones, increasing the photosynthetic rate and translocation of photosynthates, and assisting in the production of macromolecules such as proteins (Bloom, 2015; Zeffa et al., 2019).

The results of the soluble proteins quantification showed that the plants treated with *P. aryabhattai* and *P. megaterium* were able to produce higher amounts of these molecules (13.8 and 20.4%, respectively), compared to the plants of the control treatment (Figure 3D). Proteins are among the most abundant molecules in plants, performing a variety of functions, and are involved in various aspects of plant metabolism, including acclimatization and adaptation mechanisms to stress conditions (Santos et al., 2018). The amount of protein that a plant has is directly associated with the amount of nitrogen it can assimilate (Soumare et al., 2020). In the present study, the amount of total protein and the nitrogen content were positively correlated in plants treated with *P. aryabhattai* and *P. megaterium*, showing a higher efficiency in nitrogen metabolism.

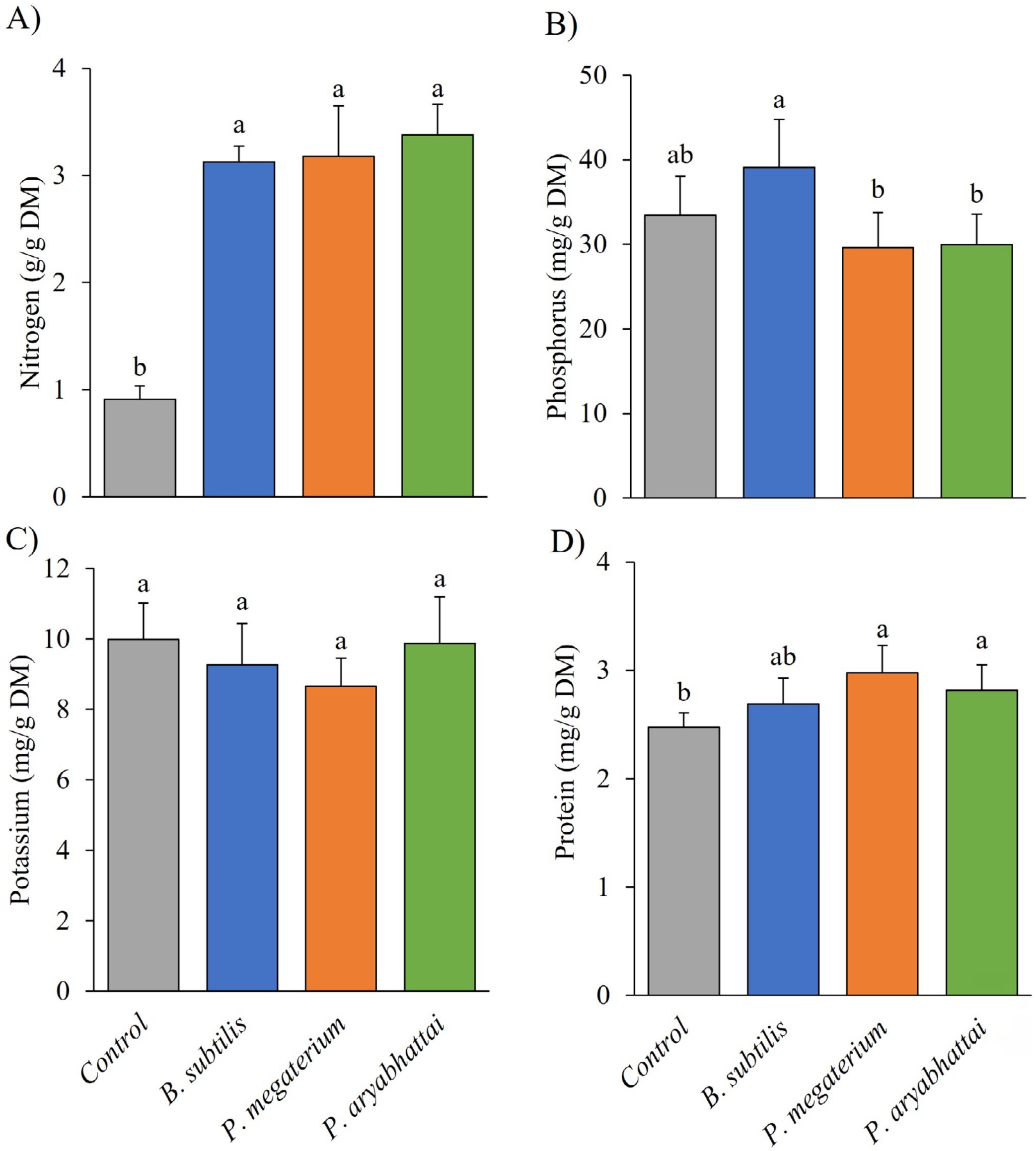
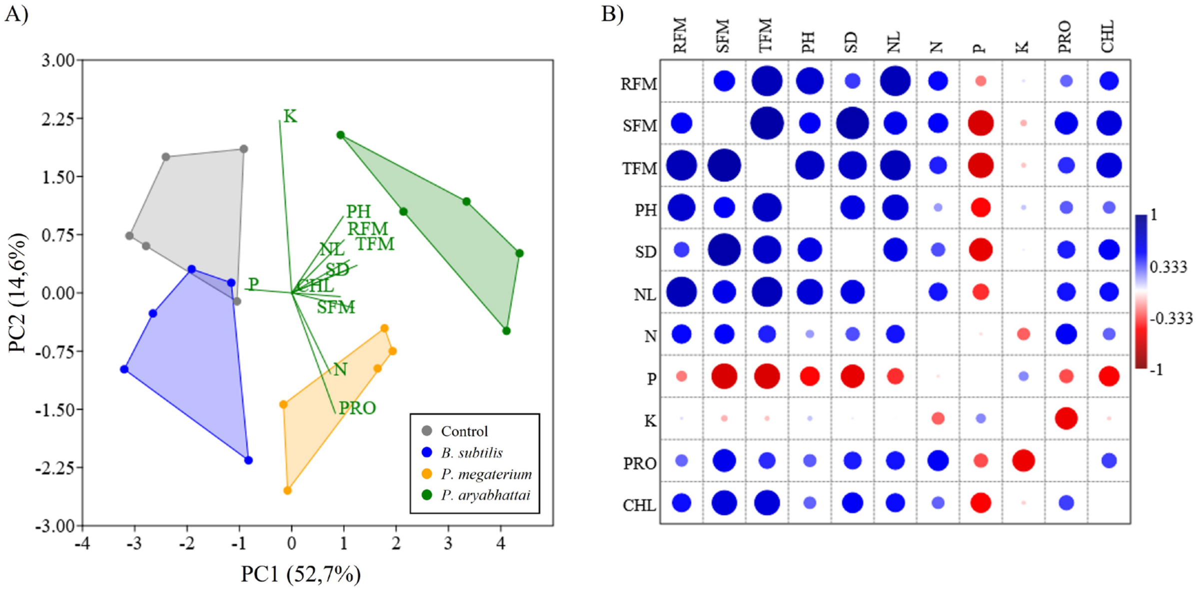


Figure 3 – Nitrogen (A), phosphorus (B), potassium (C), and protein content in *G. hirsutum* plants 60 days after sowing. Different letters represent significant differences between treatments using Duncan's test (p<0.05).

Principal component analysis and correlation between parameters

PCA showed the effects of inoculation on all the morphological and biochemical parameters evaluated. This analysis showed the differences between inoculated and non-inoculated plants in relation to all the parameters assessed and explained 67.3% of the total variation (Figure 4A). The first principal component (PC1) was positively correlated with root fresh mass (RFM), shoot fresh mass (SFM), total fresh mass (TFM), plant height (PH), stem diameter (SD), number of leaves (NL), nitrogen content (N), protein content (PRO), and total chlorophyll content (CHL). Negatively, PC1 correlated only with phosphorus (P) and potassium (K) content. The second principal component (PC2) showed positive correlations with RFM, TFM, PH, SD, NL, P, and K, but negatively with SFM, N, PRO, and CHL.

The PERMANOVA analysis confirmed that the plants treated with *P. aryabhattai* and *P. megaterium* formed groups that were distinct from each other and different from the control group and the group treated with *B. subtilis*, the latter two showing no significant differences between them (Table 2). Pearson's correlation showed that all morphometric variables were positively correlated with each other, except SD and RFM. This analysis also indicated that N was positively correlated with RFM, SFM, and NL. PRO were positively correlated with SFM, SD, NL, and N. In addition, CHL exhibited a positive correlation with RFM, SFM, TFM, SD, and NL (Figure 4B).



RFM: root fresh mass; SFM: shoot fresh mass; TFM: total fresh mass; PH: plant height; SD: stem diameter; NL: number of leaves; N: nitrogen; P: phosphorus; K: potassium; PRO: proteins; CHL: total chlorophyll.

Figure 4 – Principal component analysis showing the distribution of all the parameters analyzed (A) and correlogram based on Pearson's correlation ($p<0.05$) for all the parameters analyzed (B).

Table 2 – Permutational multivariate analysis of variance based on the Bray-Curtis index to confirm the groups formed in the principal component analysis (F=9.566; p=0.0001); p-values for variance between groups (5% significance level).

	Control	*B. subtilis*	*P. megaterium*	*P. aryabhattai*
Control		0.0800	0.0221	0.0092
B. subtilis	0.0800		0.0086	0.0112
P. megaterium	0.0221	0.0086		0.0155
P. aryabhattai	0.0092	0.0112	0.0155	

The formation of a group significantly different from the control group and positively correlated with all the morphometric parameters in the plants treated with *P. aryabhattai* proves the potential of this PGPB to promote vegetative growth in the cotton crop. The positive correlation between the parameters is also important, as it shows a balance in plant growth and in the partitioning of nutrients between plant parts (Bloom, 2015). This combination of evidence confirms the potential of this PGPB to be used as a bioinoculant in agriculture, especially in cotton cultivation. *P. megaterium* also formed a group distinct from the control treatment and positively correlated with growth parameters with the strongest correlation in the plants of this treatment, considering the biochemical parameters, occurring in the N and PRO contents. As N is a key nutrient in PRO formation, it is important that these two parameters exhibit a strong correlation so that the plant also shows increased growth and productivity (Diaz et al., 2019).

Conclusions

The *B. subtilis* strain evaluated in this work did not show positive effects on the growth of *G. hirsutum*, unlike what is reported in the literature for other strains. The PGPB *P. aryabhattai* and *P. megaterium* proved capable of benefiting cotton plants, improving various morphological and biochemical parameters. It is possible to propose that these *Priestia* species have the potential to be used in the formulation of bioinoculants and biostimulants in cotton cultivation and that they constitute an ecologically sustainable alternative to agriculture.

Authors' Contributions:

FARIAS, M.H.F.: formal analysis, investigation, methodology, writing – original draft, writing – review & editing. MELO, A.R.P: investigation, formal analysis. FREITAS, E.M.: conceptualization. LIMA, M.A.B.: resources, conceptualization, writing – review & editing. Silveira, F.A.: conceptualization, writing – review & editing. Galinari, E.: conceptualization, investigation, project administration, writing – review & editing.

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