INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA GOIANO –
CAMPUS RIO VERDE
DIRETORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS
AGRÁRIAS – AGRONOMIA

RESPOSTA DA CULTURA DA SOJA À COINOCULAÇÃO COM *BRADYRHIZOBIUM JAPONICUM* E *PSEUDOMONAS FLUORESCENS*.

Autora: Tatiana Carvalho Faria
Orientador: Prof. Dr. Edson Luiz Souchie

Rio Verde – GO
Dezembro - 2021

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RESUMO

FARIA, TATIANA CARVALHO. Instituto Federal Goiano – Campus Rio Verde – GO, dezembro de 2021. **Formas de inoculação de bactérias promotoras de crescimento de plantas (BPCP) e seu desempenho nos parâmetros agronômicos, nutricionais e em produtividade de soja.** Orientador: Dr. Edson Luiz Souchie. Coorientadores: Dr. Moacir Ribeiro Neto e Dr.Adriano Jakelaitis.

O uso de bactérias promotoras de crescimento de plantas (BPCP), além de ser uma técnica sustentável e econômica, tem mostrado potencial para melhorar a disponibilidade de nutrientes e aumentar a produtividade da soja. O primeiro estudo teve como objetivo avaliar a eficiência agronômica e a solubilização de fósforo por meio de *Bradyrhizobium japonicum* e produto a ser registrado *Pseudomonas fluorescens* (ATCC13525) em soja, na inoculação de sementes e pulverização foliar. Quatro experimentos com soja (safra 2020/21) foram instalados nos seguintes locais do Estado de Goiás: Área Experimental do Instituto Federal Goiano, em Rio Verde, Fazenda Bela Vista, em Indiara, Fazenda Bauzinho, em Rio Verde, e Cachoeira Fazenda, em Doverlândia. O *B. japonicum* foi inoculado na semente de todos os tratamentos. Foram testadas três doses de fertilização fosfatada: 0, 50 e 100% da dose recomendada de P, com e sem *P. fluorescens*, no tratamento das sementes e inoculação por pulverização foliar. O uso de inoculação com *P. fluorescens* e *B. japonicum* aumenta o conteúdo de nitrogênio (N) nos grãos e N. O conteúdo de P na massa seca, grãos e total são aumentados usando *P. fluorescens* e *B. japonicum*, confirmando a capacidade de solubilizar fosfatos. A inoculação com *P. fluorescens* e *B. japonicum* é eficiente para aumentar a massa seca e a produtividade da parte aérea, podendo ser utilizada como tecnologia de manejo sustentável da soja. A pulverização foliar foi mais eficiente do que a inoculação no tratamento de sementes e pode ser usada como modo alternativo de aplicação. Os resultados demonstraram que o produto em teste (*P. fluorescens* - ATCC13525) pode ser utilizado associado a *B. japonicum*, em TS ou spray foliar, resultando em aumento dos parâmetros agronômicos e da produtividade da soja. O segundo estudo avaliou o desempenho de plantas inoculadas e coinoculadas com BPCP, bem como os efeitos da aplicação de nitrogênio (N) e aditivos celulares protetores na produtividade da soja. O experimento ocorreu em duas safras (2018/2019 e 2019/2020) em blocos ao acaso com os seguintes tratamentos (com 4 repetições): T1: testemunha (sem inoculantes ou fertilizantes à base de nitrogênio); T2: 200 kg N ha^{-1}; T3: *Bradyrhizobium japonicum*; T4: *B. japonicum* + aditivo protetor; T5: *Azospirillum brasilense*; T6: *A. brasilense* + aditivo protetor; T7: *Pseudomonas fluorescens*; T8*: P. fluorescens* + aditivo de proteção; T9: *B. japonicum + A. brasilense + P. fluorescens*, e T10*: B. japonicum + A. brasilense + P. fluorescens* + aditivo protetor. Foi avaliada a massa seca da parte aérea; o número e a massa seca dos nódulos; Teor de N, massa seca e grãos; N total e rendimento. Os resultados indicam que o uso de fertilizantes nitrogenados prejudica a nodulação da soja, prejudicando a FBN e não resulta em aumento de produtividade. A inoculação com BPCP é essencial para a assimilação adequada do N pela planta, além de favorecer o aumento da produtividade, ao mesmo tempo que melhora os níveis de N da soja. Os aditivos protetores aplicados aos inoculantes não influenciaram a nodulação e nem o FBN.

PALAVRAS-CHAVES: *Glycine max* L., FBN, solubilização, pulverização foliar

ABSTRACT

FARIA, TATIANA CARVALHO. Instituto Federal Goiano - Campus Rio Verde - GO,

December 2021. **Forms of inoculation of plant growth promoting bacteria (BPCP) and their performance in agronomic, nutritional and soybean yield parameters.** Advisor: Dr. Edson Luiz Souchie. Co-advisors: Dr. Moacir Ribeiro Neto and Dr.Adriano Jakelaitis.

The use of plant growth promoting bacteria (PGPB), in addition to being a sustainable and economical technique, has shown potential to improve nutrient availability and increase soybean yield. The first study aimed to evaluate agronomic efficiency and phosphorus solubilization through *Bradyrhizobium japonicum* and a product to be registered *Pseudomonas fluorescens* (ATCC13525) in soybean, at seed and leaf-spray inoculation. Four experiments with soybean (2020/21 crop) were installed in the following locations in the State of Goiás: Experimental Area of the Goiano Federal Institute, in Rio Verde, Bela Vista Farm, in Indiara, Bauzinho Farm, in Rio Verde, and Cachoeira Farm, in Doverlândia. The *B. japonicum* was inoculated in the seed of all treatments. It was tested three phosphate fertilization doses: 0, 50, and 100% of recommended P dose, with and without *P. fluorescens*, at seed treatment and leaf-spray inoculation. The inoculation with *P. fluorescens* and *B. japonicum* increases nitrogen (N) content in grains and total N. The P content in dry mass, grains and total are increased using *P. fluorescens* and *B. japonicum*, confirming the ability to solubilize phosphates. Inoculation with *P. fluorescens* and *B. japonicum* is efficient for increasing shoot dry mass and productivity, then it can be used as a sustainable soybean management technology. Leaf-spray was more efficient than inoculation in seed treatment and can be used as an alternative mode of application. The results demonstrated that the product under test (*P. fluorescens* - ATCC13525) can be used associated with *B. japonicum*, in ST or leaf-spray, resulting in increases of agronomic parameters and soybean yield. The second study evaluated the performance of plants inoculated and co-inoculated with PGPB, as well as the effects of the application of nitrogen (N) and protective cellular additives on the soybean productivity. The experiment happened in two crop (2018/2019 and 2019/2020) in random blocks with the following treatments (with 4 repetitions): T1: control (no inoculants or nitrogen-based fertilizers); T2: 200 kg N ha^{-1}; T3: *Bradyrhizobium japonicum*; T4: *B. japonicum* + protective additive; T5: *Azospirillum brasilense*; T6: *A. brasilense* + protective additive; T7: *Pseudomonas fluorescens*; T8: *P.*

fluorescens + protective additive; T9: *B. japonicum* + *A. brasilense* + *P. fluorescens*, and T10: *B. japonicum* + *A. brasilense* + *P. fluorescens* + protective additive. It was evaluated the shoot dry mass; the number and dry mass of nodules; N content of dry mass and grain; N total and yield. The results indicate that use of nitrogen fertilizers harm soybean nodulation, harming the BNF and does not result in productivity increases. The inoculation with PGPB is essential for the adequate assimilation of N by the plant, in addition to supporting an increase in productivity, while also improving the N levels of the soybean. The protective additives applied to the inoculants did not influence either nodulation or BNF.

Keywords: *Glycine max* L., BNF, solubilization, spray foliar

1. INTRODUÇÃO

A crescente percepção global da importância dos inoculantes microbianos para promover a produtividade e a sustentabilidade na agricultura, estimula a adoção de bioinsumos pelos agricultores. A utilização de cepas de elite que fixam nitrogênio (N) e outros microrganismos que promovem o crescimento de plantas cria um mercado promissor para os fabricantes de inoculantes. No entanto, a combinação de microrganismos com diferentes necessidades fisiológicas e nutricionais requer desenvolvimento biotecnológico (Garcia et al., 2021)

A soja [*Glycine max* (L.) Merrill] representa a principal safra do Brasil, com área cultivada de 36,8 milhões ha e mais de 120 milhões de toneladas de grãos colhidos (CONAB 2021). Os efeitos da inoculação de *Bradyrhizobium* no crescimento e na produtividade da soja são bem conhecidos, mas as respostas das plantas aos consórcios de outros micróbios benéficos e moléculas microbianas ainda não foram bem explorados.

Tanto bactérias diazotróficas associativas quanto de vida livre têm a capacidade de estimular o crescimento das plantas por mecanismos diretos, como também por meio da fixação biológica de nitrogênio ou produção de fitormônios e de maneira indireta, atuando contra patógenos. Por esse motivo, as bactérias diazotróficas associativas são também consideradas bactérias promotoras do crescimento de plantas (BPCP) e assumem papel importante na interação com raízes de plantas e ciclagem de nutrientes (Moreira et al., 2018).

Bradyrhizobium é um dos gêneros que abrigam espécies de bactérias que fazem a fixação biológica de nitrogênio (FBN) e vivem em simbiose com vegetais superiores. Atualmente, no Brasil, as estirpes bacterianas recomendadas para a inoculação da soja são SEMIA 5079 e SEMIA 5080, da espécie *Bradyrhizobium japonicum*, e SEMIA 587 e SEMIA 5019, pertencentes à espécie *Bradyrhizobium elkanii* (Hungria & Nogueira, 2019).

O gênero *Azospirillum* tem capacidade de fixar nitrogênio da atmosfera, mas seu benefício maior é a capacidade de sintetizar fitormônios que influenciam diretamente no crescimento de plantas e indiretamente por mecanismos de tolerância a estresses abióticos. Ao contrário de *Bradyrhizobium*, que em geral "quanto mais, melhor", no caso de *Azospirillum*, um potente produtor do fitormônio ácido indol acético, a dose deve ser exatamente a recomendada, caso contrário poderá haver inibição do crescimento da soja. (Fukami et al., 2018)

O gênero *Pseudomonas* contribui com a nutrição mineral da planta, através da solubilização de fosfatos e síntese de sideróforos. Além disso, a promoção do crescimento por *Pseudomonas* spp também é favorecida pela síntese da ACC-deaminase, que crava o precursor do etileno em plantas mais altas (Hungria et al., 2021)

Essas bactérias podem ser inoculadas separadas ou em associação, técnica que se denomina coinoculação. Na coinoculação são combinadas cepas ou espécies atuando em diferentes processos microbianos, de forma que os benefícios combinados de cada uma resultem em maiores rendimentos. Por exemplo, a combinação entre microrganismos cujos processos principais são FBN (*Bradyrhizobium* spp., *Rhizobium* spp.) com aqueles que produzem fitormônios (*Azospirillum* spp., *Pseudomonas* spp.), solubilizam fosfatos e promovem controle biológico (*Bacillus* spp., *Pseudomonas* spp.) (Santos et al. 2019).

O inoculante contém células vivas que têm a viabilidade prejudicada por fatores como mistura com agrotóxicos, exposição a altas temperaturas ou luz solar e desidratação (Rodrigues et al., 2020). Boas práticas de inoculação garantem a sobrevivência bacteriana e fornecem o N necessário para atingir altos patamares produtivos de soja (Hungria & Nogueira 2019).

A aplicação via foliar pode ser usada como alternativa para evitar o contato das bactérias com produtos químicos (Gautam et.al., 2021). Além disso, o desenvolvimento de tecnologias, como a adição de protetores de inoculante, garante o êxito da inoculação durante a prática do tratamento das sementes com defensivos agrícolas (Araújo et al., 2017).

O nitrogênio (N) é o nutriente requerido em maior quantidade pela cultura da soja. Para cada 1000 kg de grãos são necessários, aproximadamente 80 kg de N. A FBN é a principal fonte de N para a cultura da soja e pode fornecer todo o N que a soja necessita (Moretti et al., 2020; Hungria et al., 2015).

Existe grande discussão sobre os possíveis benefícios do uso de N mineral na soja, porém a maioria dos resultados obtidos em condições de campo demonstram que a aplicação de N, na semeadura ou em cobertura via solo e/ou foliar, não traz resultados significativos de produtividade (Oliveira Junior et al., 2015).

O fósforo é um nutriente com baixa mobilidade e se concentra nas camadas superficiais do solo. O principal processo de contato desse nutriente com as raízes é pela difusão (Guimarães et al., 2021).

A acidificação da rizosfera é um importante mecanismo pelo qual o fósforo é solubilizado a partir de fontes minerais indisponíveis, além de contribuir para a atividade de algumas das enzimas fosfato-solubilizadoras (Rathinasabapathi et al., 2018). Os microrganismos do solo fazem essa acidificação através da via periplasmática e a glicose desidrogenase e o glicosconato desidrogenase na membrana oxidam a glicose a compostos ácidos liberados pelas plantas e encontrados na rizosfera, resultando em pH inferior do ambiente (Buch et al., 2008). A produção de ácido glucônico e glicerolphosphodiesterases por bactérias *Pseudomonas* spp também é importante para solubilizar o fosfato de fontes indisponíveis (De Werra et al., 2019; Lidbury et al., 2017).

Esses benefícios provenientes das BPCP aliados com a diminuição do uso de adubação química, sustentabilidade e redução de custos, tornam o uso dessa tecnologia atrativa e indispensável no manejo da cultura da soja.

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2. OBJETIVOS

2.1 Geral

Avaliar as formas de inoculação de bactérias promotoras de crescimento de plantas (BPCP) e seu desempenho nos parâmetros agronômicos, nutricionais e em produtividade de soja.

2.2 Específicos

Avaliar a eficiência agronômica e a solubilização do fósforo através do B*radyrhizobium japonicum* e do produto a ser registrado *Pseudomonas fluorescens* (ATCC13525) em soja, na inoculação de sementes e spray de folha.

Avaliar o desempenho das plantas inoculadas e coinoculadas com bactérias promotoras do crescimento vegetal BPCV, bem como os efeitos da aplicação de nitrogênio (N) e aditivos protetores celulares na produtividade da soja.

CAPÍTULO I

Agronomic efficiency and phosphate solubilization of *Pseudomonas fluorescens* and *Bradyrhizobium japonicum* in leaf-spray inoculation and seed treatment in soybean.

(Normas de acordo com a revista Journal of Agricutural Science. Submetido em novembro de 2021)

Abstract

The use of plant growth promoting bacteria (PGPB) that can solubilize phosphorus (P) has shown potential to improve nutrient availability in many crops such as soybean. This research aimed to evaluate agronomic efficiency and phosphorus solubilization through *Bradyrhizobium japonicum* and product to be registered *Pseudomonas fluorescens* (ATCC13525) in soybean, at seed and leaf-spray inoculation. Four experiments with soybean (2020/21 crop) were installed in the following locations in the State of Goiás: Experimental Area of the Goiano Federal Institute, in Rio Verde, Bela Vista Farm, in Indiara, Bauzinho Farm, in Rio Verde, and Cachoeira Farm, in Doverlândia. The *B. japonicum* was inoculated in the seed of all treatments. It was tested three phosphate fertilization doses: 0, 50, and 100% recommended P dose, with and without *P. fluorescens*, at seed treatment and leaf-spray inoculation. The use of inoculation with *P. fluorescens* and *B. japonicum* increases nitrogen (N) content in grains and total N. The P content in dry mass, grains and total are increased using *P. fluorescens* and *B. japonicum*, confirming the ability to solubilize phosphates. Inoculation with *P. fluorescens* and *B. japonicum* is efficient for increasing shoot dry mass and productivity, can be used as a sustainable soybean management technology. Leaf-spray was more efficient than inoculation in seed treatment and can be used as an alternative mode of application. The results demonstrated that the product under test (*P. fluorescens* - ATCC13525) can be used associated with *B. japonicum*, in ST or leaf-spray, resulting in increases of agronomic parameters and soybean yield.

Keywords: *Glycine max* L., P fertilization, solubilization, yield

3.1 Introduction

Humanity has always been concerned about food production to attend the increasing population and, for a long time, the solution was to expand agriculture to new areas. However, this scenario has changed in recent decades, first due to limitations of unexplored cultivable land, but also reinforced by the development of new technologies that allow higher yields, in addition to increasing environmental concerns, leading to agricultural practices aiming at achieving sustainable production. In this context, microbial inoculants with Plants Growth Promotion Bacterias (PGPB) have received increasing attention, gaining prominence and market scale in agriculture (Santos et al., 2019).

In the last decade, the use of inoculants containing microorganisms of “different type” has expanded. The idea is of combining strains or species acting in different microbial processes, so that the combined benefits of each one would result in higher benefits and yields. Examples of mixed inoculant are those combining microorganisms whose major processes are Biological Nitrogen Fixation - BNF (*Bradyrhizobium* spp., *Rhizobium* spp.) and phytohormone production (*Azospirillum* spp., *Pseudomonas* spp.), solubilization of phosphate (*Bacillus* spp.), or biological control (*Pseudomonas* spp., *Bacillus* spp.). If the microorganisms cannot be combined in a single product, they are manufactured separately and the bags containing each one is sold in the same package (Santos et al., 2019).

The success of microbial inoculation depends on the inoculation method, inoculum density, in addition to pH, temperature and soil moisture (Lopes et al., 2021). Although seed inoculation is the most used method, there are some limiting factors that can rapidly reduce the inoculum density or its ability to colonize the host plant, such as chemical treatments and allelochemical compounds produced in the germination of some species (Goutam, 2021; Lopes et al., 2021). Differences in compatibility between pesticides and inoculants depend on their active ingredient, formulation, time of application and period of contact with live microorganisms; however, in general, they have a high impact on cell survival and metabolism, affecting the microbial contribution to plant growth (Santos et al., 2021).

Besides phytohormones synthesis, beneficial properties associated with PGPB include BNF, phosphate and potassium solubilization, production of siderophores, detoxification of heavymetals, induction of plant systemic tolerance to abiotic and biotic stresses, production of hydrolytic enzymes, and production of exopolysaccharides (Vishwakarma et al., 2020). Such properties have been reported in several microorganisms, and the most cited carrying one or more of these properties are *Azospirillum* spp. (Cassán et al., 2021), *Pseudomonas* spp. (Zang et al., 2018; Sandini et al., 2019), and *Bacillus* spp. (Ribeiro et al., 2018).

BNF is the main source of nitrogen (N) for soybean crop. Bacteria of the genus *Bradyrhizobium* infect the roots of the plant via the root, forming the nodules. BNF can provide all the N that soybeans need. Soybean is the ideal crop in which to explore biological nitrogen fixation (BNF) as inoculation of the seed with efficient *Bradyrhizobium* strains provides rates of fixed atmospheric nitrogen (N_2) greater than 80% (Alves et al. 2006; Hungria et al. 2006) and high grain yield (Kaschuk et al. 2016; Moretti et al. 2020). In terms of nitrogen (N) fertilizer equivalents, the economic savings achieved using BNF in Brazil exceed 12 Tg of mineral N per year, worth over US$ 13 billion (Hungria & Mendes 2015; Santos et al. 2019).

The genus *Pseudomonas* comprises a taxon capable of using a wide variety of simple or complex organic compounds. Consequently, they are distributed by soils and water, being important as plant pathogens, animals, and humans, with some strains related to the promotion of plant growth and biocontrol of phytopathogens (Zago et al.,2000).

It has been shown that plant growth-promoting bacteria like *Pseudomonas* can solubilize phosphorus (P) and make it more available to plants (Rathinasabapathi et al., 2018).

Fernandez et al. (2012) evaluated *Pseudomonas* strains using an assay for phosphorus solubilization from tricalcium phosphate in buffered liquid media. Isolates that displayed a high degree of phosphorus solubilization also exhibited acid and alkaline phosphatase activities, extracellular protease, and hydrogen cyanide production, the last two traits being recognized for biocontrol of pathogenic microbes.

Acidification of the rhizosphere is a major mechanism by which phosphorus is solubilized from unavailable mineral sources, can also improve the activity of some of the phosphate-solubilizing enzymes (Rathinasabapathi et al., 2018). Microbes use the carbon sources from the plant roots and the soil environment. Glucose excreted from the roots can be taken up by the bacteria, which was converted to glucose 6-phosphate in the cytosol. However, this process requires ATP. Under phosphatelimiting conditions, the bacteria instead use the periplasmic pathway where glucose dehydrogenase and gluconate dehydrogenase in the membrane oxidize the glucose to acidic compounds, resulting in lower pH of the environment (Buch et al., 2008).

De Werra et al. (2009) made deletion mutants of *Pseudomonas fluorescens* CH0 lacking either glucose dehydrogenase (gcd) or gluconate dehydrogenase (gad) or both. It was proved that gluconic acid production was important for solubilizing phosphate from unavailable sources.

Phytate (i.e., myo-inositol 1,2,3,4,5,6-hexakisphosphate) is made in plants as a storage form of phosphorus. This is also the most abundant organic form of phosphorus in the soil (Turner et al., 2002). There are multiple taxa of microbes that can mineralize phytic acid and make the phosphorus available including soil isolates of *Pseudomonas* spp. (Sun et al., 2017). Cho et al. (2003, 2005) has identified a phytase of histidine acid phosphatase family from P. syringae MOK1. Beta-propeller phytase in rhizosphere *Pseudomonas* sp. has been well-characterized also (Shen et al., 2016). Recent research has uncovered that P*seudomonas* and other soil bacteria also secrete glycerolphosphodiesterases that could degrade phospholipids, another organic source of phosphorus in the soil (Lidbury et al., 2017).

Information on the solubilization of iron and aluminum phosphates is scarce, although they are the predominant forms of phosphates (Raij, 1991). Furthermore, there are differences in the capacity and solubilization potential of microorganisms. The specific microorganism can solubilize only Ca-P, while others solubilize Al-P and Fe-P, and it should be considered that microorganisms can solubilize these phosphates in different intensities (Doyle et al., 1990; Silva Filho & Vidor, 2000).

Therefore, the use of bacterial inoculants is an alternative for the better utilization of phosphorus applied via fertilization and immobilized in the soil, besides promoting plant growth and development, resulting in higher yields.

Thus, the present research aimed to evaluate agronomic efficiency and phosphorus solubilization through *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* (product to be registered) in soybean, in seed treatment and leaf-spray inoculation.

3.2 Materials and Methods

3.2.1 Location

Four experiments with soybean (crop 2020/21) were installed in the following locations in the State of Goiás: a) Experimental area of the Federal Institute of Goiano campus Rio Verde, located at latitude 17º 47' 53" S, longitude 50º 55' 41" W (Red Oxisol) and altitude 744 m, in Rio Verde; b) Bela Vista Farm, located at latitude 17º 11’ 04" S, longitude 49º 59’ 04" W (Yellow Oxisol) and altitude 586m, in Indiara; c) Bauzinho Farm, located at latitude 18º 00' 26" S; longitude 50º 31' 02" W (Red Oxisol) and altitude: 620 m, in Rio Verde, and d) Cachoeira Farm, located at latitude 16º 42' 21" S; longitude 52º 13’ 51” W (Yellow Oxisol) and altitude: 623m, in Doverlândia.

The region's climate, according to the Köppen-Geiger classification, is classified as Aw (tropical climate with dry season). The test sites were sampled 60 days in advance for the evaluation of physical and chemical characteristics. The Table 1 shows the chemical and granulometric characterization of the of each area. The population of native rhizobia in the soil in each area was estimated by counting in Petri dishes. The methodologies followed are described in Normative Instruction nº 30 of MAPA (Ministério da Agricultura, pecuária e abastecimento) (MAPA, 2010).

The planting date was between the 2nd and 5th of November 2020. The rainfall during the period was 833 mm in the IF Goiano (Rio Verde); 768 mm at Fazenda Bela Vista (Indiara); 852 mm at Fazenda Bauzinho (Rio Verde) and 791 mm at Fazenda Cachoeira (Doverlândia).

3.2.2 Experimental design and treatments

The cultivar used was Bônus® (8579 RSF IPro), planting density of 280,000 seeds ha^{-1}. In all locations, the predecessor crop was maize and the product Standak Top®: insecticide Fipronil from the pyrazole group, the fungicides Pyrclostrobin from the strugirulin group, and Methyl Thiophanate from the benzimidazole group (2mL kg $seeds^{-1}$) was used in the seed treatment. After treatment of the seeds, they were dried in the shade for 4 hours and then received the inoculation treatments.

The research was installed in a randomized complete block design with 10 treatments and six replications. The plots were 6 m long x 4 m wide, totaling 24 m^2, separated by 1 m wide corridors. The results were subjected to analysis of variance, and average were separated by the Tukey test (5%) using Sisvar® software (Ferreira, 2019).

In all treatments, the inoculant Atmo® was used in seed treatment (ST), with strains of *Bradyrhizobium japonicum* (SEMIA 5079 and 5080; 5.0 x 10^9 CFU mL-1), 100 mL 50 kg of $seeds^{-1}$. As standard inoculant of *Pseudomonas fluorescens*, the product Fertibio Phospro® (ATCC13525; 2.0 x 10^8 CFU mL^{-1}) was used, 50 ml 50 kg of seeds-1. The inoculant evaluated was *Pseudomonas fluorescens* (ATCC13525; 2.0 x 10^8 CFU mL^{-1}), 100 ml 50 kg of seeds-1, in seed treatment (ST) and 200 mL ha^{-1} in leaf-spray inoculation (V3-V4).

All plots were fertilized with KCl (150 kg ha^{-1}; 00:00:52). The treatments consisted of different doses of phosphate fertilization, three levels being tested: 0, 50 and 100% of the recommended phosphorus dose, with and without the inoculant with *P. fluorescens* via seed treatment and leaf-spray inoculation. The recommended dose of phosphorus for the four areas was 80 kg ha-1 of P_2O_5 or the equivalent of 400 kg ha^{-1} of super simple fertilizer (Table 2).

3.2.3 Analyzed Parameters

At 35 days after emergence (DAE), the following parameters were evaluated: shoot dry mass and N and P contents and number and dry mass of nodules. Five plants were collected from the central area of the second row of each plot. At harvest, shoot dry mass, grain yield (13% moisture), N and P contents, and total N and P in grains were evaluated, and the 8 central lines of each plot were harvested.

3.3 Results

In relation to the **number of nodules,** in IF Goiano, the Control 100%P treatment had a higher value than *B. japonicum+ P. fluorescens* 100%P. In **dry mass of nodules** at Bela Vista Farm, the treatment *B. japonicum+ P. fluorescens* without P in ST had greater mass than all controls (Table 3).

In **shoot dry mass,** treatments with *B. japonicum+ P. fluorescens* without P were greater than the control without P, regardless of the form of application (Table 3). Bela Vista Farm the treatments *B. japonicum+ P. fluorescens* via the foliar were higher than those on ST, regardless of the dose of P. At Cachoeira Farm, the treatment *B. japonicum+ P. fluorescens* foliar, without P, had higher shoot dry mass than in ST without P. At Bauzinho Farm, treatments *B. japonicum+ P. fluorescens* 50%P, regardless of application, also had higher dry mass than the control 50%P. The treatments *B. japonicum+ P. fluorescens* 50% P, regardless of the form of application, obtained greater aerial part dry mass than the standard treatment *B. japonicum* + Fertibio Phospro® ST 50%P (Table 3).

There was a difference **in N content in dry mass** only in IF Goiano where the treatment *B. japonicum+ P. fluorescens* without P, regardless of the form of application, resulted in higher values than the control (Table 4).

In terms of grain N and Total N, the results were higher in treatments with B. *japonicum+ P. fluorescens* without P, regardless of the form of application, when compared to the control without P, reaching 47.5 kg and 32 kg of Total N more when applied foliar and ST respectively (Table 4). The treatments *B. japonicum+ P. fluorescens*, foliar and ST with 50% P had Total N greater than the Control 50%. At Cachoeira Farm, the treatment B. *japonicum+ P. fluorescens*, foliar and TS, 100%, had higher Total N than the 100% control. When the application was foliar, in the treatment *B. japonicum+ P. fluorescens* without P, the results of Total N also exceeded the control with 50% P. Regarding the type of application, in the areas

IF Goiano and Bela Vista Farm, when the treatment *B. japonicum+ P. fluorescens* without P, was foliar, the results of total N were higher (Table 4).

Regarding **P content in mass, grains and Total P,** the results were higher in the treatments *B. japonicum+ P. fluorescens* without P and with 50% foliar P and ST when compared to the control without P, with the exception Bela Vista and Cachoeira Farm where only *B. japonicum+ P. fluorescens* without P and foliar had higher P content by mass than the control (Table 5). The *B. japonicum+ P. fluorescens* treatment, 100% P, foliar and ST in IF Goiano had higher P contents in grains and Total P than in the 100% P control. At Bauzinho Farm this treatment, foliar and ST, had higher Total P value when applied foliarly compared to 100% P control, while at Fazenda Bela Vista it presented higher P content in grains and higher Total P, both foliar and ST and higher P content in mass when applied foliarly, compared to 100% control. At Cachoeira Farm, the treatment *B. japonicum+ P. fluorescens*, 100% P, presented higher Total P when applied via the leaves, compared to the 100%P control (Table 5).

Regarding the form of application of the inoculated treatments, the treatments with foliar application without P presented higher values of the analyzed P variables, in all areas, in relation to the ST without P; except for Bauzinho Farm where there was no difference. At Bela Vista Farm, the treatment with foliar application 50% P had a Total P value greater than the 50% ST. The standard treatment *B. japonicum* + Fertibio Phospro® TS 50%P obtained higher Total P compared to the treatment *B. japonicum* + *P. fluorescens* TS 50%P, at Bela Vista Farm (Table 5).

The treatments *B. japonicum* + *P. fluorescens* foliar without P and with 50% P produced on average 409 kg ha-1 and 247 kg ha-1 more than controls without P and with 50% respectively (Table 6). In IF Goiano and Cachoeira Farm, the treatment *B. japonicum* + *P. fluorescens* foliar with 100%P produced an average of 286 kg ha-1 than the 100%P control. Regarding the form of application, the treatment *B. japonicum* + *P. fluorescens* foliar without P had better performance than the ST with P, except at Bauzinho Farm. At Cachoeira Farm, *B. japonicum* + 100%P foliar *P. fluorescens* was better than ST100% (Table 6).

3.4. Discussion

The present experiment showed that treatments with application of *B. japonicum* + *P. fluorescens* increased shoot dry mass, nitrogen and phosphorus concentration in the plant, grains and, consequently, yield.

The use of PGPB positively influences the absorption of nutrients that is linked to total dry matter production and nutrient concentration in the plant. The amounts of nutrients exported are directly proportional to the productivity and concentration of nutrients in the grains (Oliveira Júnior et al., 2020). Guimarães et al. (2021) reported that the inoculation of *P. fluorescens* in soybean increased morphometric parameters reflecting dry mass gain, and higher nutrient content (NPK) of plant tissues.

In the present study, the N content in grains and total N were higher in treatments with *B. japonicum* + *P. fluorescens* (Table 4). P. fluorescens contributes to BNF indirectly, improving root architecture and nodule formation by *Bradyrhizobium* sp. The proposed mechanisms by which PGPB can improve the nodular activity of *Rhizobium* are: production of binding proteins in the cell membrane (Burns et al., 1981), production of antimicrobial agents (Li & Alexander, 1988), stimulation of root colonization by mycorrhizal fungi, which result in changes in root morphology (Meyer & Linderman, 1986).

Sandini et al. 2019, demonstrated that, inoculation P. *fluorescens* in Maize, promoted plant growth and yield at both levels, increasing plant biomass accumulation by 24 and 20%, relative to the non-inoculated control, at standard or high levels, respectively. The grain yield increased by 29 and 31%, relative to the non-inoculated control, under standard and high levels of technology, respectively. Under both situation, plant growth and grain yield improved by *P. fluorescens* was equivalent to the application of 100% of the recommended N fertilizer, even when the amount of N fertilizer applied to the crop was reduced by 25%, without compromising yield.

In the present study, the p content in shoot dry mass, in grains and total P in all areas, were higher in treatments with *B. japonicum* + *P. fluorescens* regardless of the P_2O_5 dose when compared with the controls (Table 5). This suggests that the inoculation of PGPB improves phosphorus availability and, consequently, reduces the need for phosphate fertilization.

Bashan and Bashan (2004) said that increased biomass and P contents in plants are indicative of the effect of phosphate solubilization that can result in plant growth and development. It has been shown that plant growth-promoting bacteria like *Pseudomonas* can solubilize P and make it more available to plants (Chien et al., 2011).

P. fluorescens is highly positive in the synthesis of siderophores, phosphate solubilization (CaHPO4 2H2O), and of 1-aminocyclopropane-1- carboxylate (ACC) deaminase (Hungria et al., 2021). Glick *et al*. (1998) proposed that PGPB strains possessing ACC-deaminase can hydrolyze ACC, the precursor of ethylene in higher plants, producing α-ketobutyrate and ammonia which, in turn, can promote plant growth. Recent research has uncovered that *Pseudomonas* and other soil bacteria also secrete glycerolphosphodiesterases that could degrade phospholipids, another organic source of phosphorus in the soil (Lidbury et al., 2017).

Guimarães et al. (2021) inoculated *P. fluorescens* in ST soybean, without P and with 50% and 100% of the recommended dose and found that this bacterium promotes growth and productivity gains, related to its phosphate solubilization potential, with half of the recommended dose of phosphate fertilizer.

It comes to the form of inoculation, in most of the parameters evaluated, the leaf-spray inoculation treatments obtained superior results in relation to Inoculation ST. The treatment *B. japonicum* + *P. fluorescens* with 100% of phosphate fertilization, presented yield averages higher than the average of Brazilian productivity (3,523 kg/ha) and of the state of Goiás (3,714 kg/ha) (Table 6) (CONAB, 2021).

The leaf inoculation method is an alternative for seeds with chemical treatment that end up reducing bacterial survival (Puente et al., 2018). In this Standak ® Top, composed of two fungicides and an insecticide, was the product used for seed treatment. Rodrigues et al. (2020) report this product affects the survival of *B. japonicum* and, mainly, of *B. elkanii* cells, with a drastic decrease verified after 7 days of contact (Rodrigues et al., 2020). Pre-inoculated soybean seeds are with Standak® Top for up to 90 days, often showing zero recovery of rhizobia cells from the seeds (Hungria & Nogueira, 2019).

The leaf spray inoculation was beneficial when using *Azospirillum brasilense* in sorrh (Nakao et al., 2014), soybean (Puente et al., 2018) and corn (Fukami et al., 2017; Galindo et al., 2020; Fukami et al. 2016; Barbosa et al. 2021). Machado et al. (2020) report that when applying *Bacillus subitils* leaf route in corn, higher height parameters and significant productivity increase. Positive results were also obtained with leaf inoculation of *Azospirillum brasilense* and *Pseudomonas fluorescens* in brachiaria pastures (Hungria et al., 2021).

The results of the present study demonstrated that the product under test (P. fluorescens - ATCC13525) can be used associated with *B. japonicum*, in ST or leaf-spray, resulting in increases of agronomic parameters and soybean yield. Results compatible with the product containing the same strain of *P. fluorescens*, already registered and marketed: Fertibio Phospro®, were obtained, conferring recommendation for use and registration of the technology in soybean crop.

3.5. Conclusion

The use of inoculation with *Pseudomonas fluorescens* and *Bradyrhizobium japonicum* increases n content in grains and total N, indicating that *P. fluorescens* collaborates with *B. japonicum* facilitating BNF.

The p content in dry mass, grains and total are increased using *P. fluorescens* and *B. japonicum*, confirming the ability to solubilize phosphates, facilitating their absorption by plants and making possible reduction of the use of phosphate fertilization in soybean crop.

Inoculation with *P. fluorescens* and *B. japonicum* is efficient for increasing shoot dry mass and productivity, confirming its ability to promote plant growth, and can be used as a sustainable soybean management technology

Leaf-spray was more efficient than inoculation in seed treatment and can be used as an alternative mode of application.

The results of the present study demonstrated that the product under test (*P. fluorescens* - ATCC13525) can be used associated with *B. japonicum*, in ST or leaf-spray, resulting in increases of agronomic parameters and soybean yield.

Authors contributions

The authors contributed equally to the research data analysis, modelling, and manuscript writing.

Data Availability Statement

Data supporting this study will be shared upon reasonable request to the author for correspondence.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. The soil's chemical and granulometric characteristics in four locations in the state of Goiás, Brazil (crop 2020/21).

Local	pH water	P	K	Fe	Mn	Zn	Ca	Mg	Al	Organic matter	Saturation by bases	Areia	Silte	Argila	Native rhizobia*
		------------- mg dm^{-3} --------------					-- $cmol_c$ dm^{-3} --			g dm^{-3}	------------------ % ------------------				UFC g $soil^{-1}$
IF Goiano campus Rio Verde	6,1	15,2	255	9,0	60,2	6,0	4,4	1,3	0,03	34,9	55,6	36,2	17,4	46,4	4,8 x 10^3
Bela Vista Farm, Indiara	6,3	29,6	138	22,5	38,2	3,0	3,8	1,1	0,00	22,4	65	61	15	24	2,6 x 10^4
Bauzinho Farm, Rio Verde	6,1	22,3	302	13,8	22,6	4,0	3,5	0,92	0,02	28,6	58	30	14	56	3,2 x 10^3
Cachoeira Farm, Doverlândia	6,2	18,5	280	21,3	18,6	3,5	2,8	0,88	0,03	21,8	61	46	18	36	4,1 x 10^3

Extractors: Mehlich 1 (P, K, Fe, Zn e Mn); KCl 1 N (Ca, Mg e Al); * Analysis performed 48h before planting.

Table 2. Inoculation and fertilization treatments were used in soybean experiments in four locations in Goiás, Brazil (crop 2020/21).

Nº	Tratamento	P fertilization
1	*B. japonicum* (Control)	0%
2	*B. japonicum* (Control)	50%
3	*B. japonicum* (Control)	100%
4	*B. japonicum* + *P. fluorescens* ST	0%
5	*B. japonicum* + *P. fluorescens* ST	50%
6	*B. japonicum* + *P. fluorescens* ST	100%
7	*B. japonicum* + *P. fluorescens* leaf-spray	0%
8	*B. japonicum* + *P. fluorescens* leaf-spray	50%
9	*B. japonicum* + *P. fluorescens* leaf-spray	100%

10	*B. japonicum* + Fertibio Phospro® ST	50%

*** According to the soil analysis, the doses of P should correspond to 0, 50, and 100% of the recommendation for soybean cultivation.

Table 3. Number of nodules, shoot dry mass of nodules, dry mass of aerial part per plant evaluation, in soybean inoculated with *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* and phosphate fertilization (crop 2021/21).

		---------------IF Goiano----------- -			------Bauzinho Farm------			-------- Bela Vista Farm-------			------------ Cachoeira Farm--------		
Treatments	P Fertilization	Nº nodules plant^{-1}	Dry mass nodules (mg plant^{-1})	Shoot dry mass (g plant^{-1})	Nº nodules plant^{-1}	Dry mass nodules (mg plant^{-1})	Shoot dry mass (g plant^{-1})	Nº nodules plant^{-1}	Dry mass nodules (mg plant^{-1})	Shoot dry mass (g plant^{-1})	Nº nodules plant^{-1}	Dry mass nodules (mg plant^{-1})	Shoot dry mass (g plant^{-1})
B. japonicum (Control)	0%	19,7 bc	116,36 a	82,67 b	20,65 a	125,23 a	101,71 c	21,83 a	115,28 c	90,26 e	16,3 b	115,97 a	89,25 d
B. japonicum (Control)	50%	19,9 bc	117,79 a	99,78 ab	21,55 a	122,32 a	116,30 b	22,88 a	116,57 bc	100,90 d	17,51 ab	119,73 a	101,58 c
B. japonicum (Control)	100%	24,1 a	118,58 a	99,41 ab	20,61 a	124,34 a	125,67 ab	22,93 a	116,38 bc	109,70 c	19,63 a	117,38 a	110,36 bc
B. japonicum + *P. fluorescens* ST	0%	18,5 c	117,82 a	101,95 a	20,93 a	123,74 a	125,36 ab	22,68 a	118,48 a	118,05 b	17,5 ab	119,51 a	105,01 c
B. japonicum + *P. fluorescens* ST	50%	20,3 abc	120,13 a	107,95 a	22,18 a	123,66 a	129,11 a	23,16 a	117,27 ab	120,30 b	17,61 ab	117,50 a	118,86 ab
B. japonicum + *P. fluorescens* ST	100%	20,5 abc	118,38 a	110,24 a	19,51 a	124,92 a	128,49 ab	23,01 a	117,34 ab	120,98 b	18,28 ab	117,33 a	120,28 ab
B. japonicum + *P. fluorescens* leaf-spray	0%	21,7 abc	117,95 a	108,15 a	21,83 a	125,30 a	134,35 a	22,06 a	118,33 ab	124,18 a	18,36 ab	117,31 a	120,12 ab
B. japonicum + *P. fluorescens* leaf-spray	50%	23,3 ab	119,47 a	110,07 a	22,40 a	124,78 a	131,97 a	22,96 a	117,41 ab	129,77 a	16,45 b	118,82 a	118,86 ab
B. japonicum + *P. fluorescens* leaf-spray	100%	19,2 c	118,64 a	110,16 a	22,81 a	124,49 a	128,93 a	23,08 a	118,20 ab	130,92 a	17,65 ab	118,28 a	127,66 ab
B. japonicum + Fertibio Phospro® ST	50%	20,8 abc	119,48 a	115,61 a	21,30 a	124,38 a	131,18 a	23,01 a	117,64 ab	131,54 a	17,51 ab	117,50 a	106,22 c
CV(%)		6,09	0,88	3,28	8,88	1,66	2,36	9,64	2,47	2,69	8,56	2,27	2,54

* Means followed by the same letter in the column are not different according to the comparison of Tukey's test (5%).

Table 4. Nitrogen evaluation in soybean inoculated with *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* and phosphate fertilization (crop 2021/21).

		-----------------IF Goiano------------			--------Bauzinho Farm------			-------- Bela Vista Farm-------			------------ Cachoeira Farm--------		
Treatments	P Fertilization	N contents dry mass (g kg^{-1})	N contents grains (%)	Total N (kg ha^{-1})	N contents dry mass (g kg^{-1})	N contents grains (%)	Total N (kg ha^{-1})	N contents dry mass (g kg^{-1})	N contents grains (%)	Total N (kg ha^{-1})	N contents dry mass (g kg^{-1})	N contents grains (%)	Total N (kg ha^{-1})
B. japonicum (Control)	0%	61,73 b	5,3 c	143,2 e	76,97 a	5,63 b	151,55 d	67,82 a	6,05 c	161,48 d	65,87 b	5,88 c	177,33 f
B. japonicum (Control)	50%	64,34 ab	6,0 b	182,9 d	75,97 a	6,23 a	188,95 d	68,22 a	6,25 ab	180,55 cd	67,41 ab	6,2 b	192,99 ef
B. japonicum (Control)	100%	64,61 ab	6,3 ab	246,8 ab	76,00 a	6,26 a	253,80 a	67,24 a	6,26 ab	261,22 a	66,59 ab	6,26 b	242,21 b
B. japonicum + *P. fluorescens* ST	0%	65,75 a	6,4 a	183,0 d	76,62 a	6,30 a	186,08 c	68,00 a	6,33 ab	181,11 cd	68,03 ab	6,35 ab	199,16 de
B. japonicum + *P. fluorescens* ST	50%	66,66 a	6,3 ab	200,4 c	77,13 a	6,38 a	201,86 bc	66,94 a	6,36 a	189,58 bc	69,15 ab	6,61 a	212,67 cd
B. japonicum + *P. fluorescens* ST	100%	64,68 ab	6,4 a	258,4 ab	77,25 a	6,26 a	262,91 a	67,17 a	6,41 a	271,67 a	67,88 ab	6,56 a	266,28 a
B. japonicum + *P. fluorescens* leaf-spray	0%	65,97 a	6,4 a	201,8 c	77,67 a	6,36 a	199,51 bc	68,05 a	6,51 a	207,73 b	68,11 ab	6,40 ab	213,31 cd
B. japonicum + *P. fluorescens* leaf-spray	50%	66,44 a	6,4 ab	210,8 c	76,40 a	6,33 a	209,57 b	67,60 a	6,33 ab	206,63 b	69,53 a	6,48 ab	219,04 c
B. japonicum + *P. fluorescens* leaf-spray	100%	64,88 ab	6,4 ab	265,4 a	76,62 a	6,26 a	271,09 a	67,88 a	6,45 a	271,67 a	68,45 ab	6,56 a	280,73 a
B. japonicum + Fertibio Phospro® ST	50%	65,33 ab	6,3 ab	197,0 cd	76,15 a	6,33 a	199,99 bc	68,33 a	6,45 a	202,41 b	68,77 ab	6,38 ab	209,10 cd
CV(%)		2,26	2,51	4,93	1,98	2,13	4,55	2,96	3,30	3,93	2,76	2,42	3,78

* Means followed by the same letter in the column are not different according to the comparison of Tukey's test (5%).

Table 5. Phosphorus evaluation in soybean inoculated with *B. japonicum* and *P. fluorescens* and Phosphate Fertilization (crop 2021/21).

		-----------------IF Goiano------------			--------Bauzinho Farm------			-------- Bela Vista Farm-------			------------ Cachoeira Farm--------		
Treatments	P Fertilization	P contents dry mass (g kg^{-1})	P contents grains (%)	Total P (kg ha^{-1})	P contents dry mass (g kg^{-1})	P contents grains (%)	Total P (kg ha^{-1})	P contents dry mass (g kg^{-1})	P contents grains (%)	Total P (kg ha^{-1})	P contents dry mass (g kg^{-1})	P contents grains (%)	Total P (kg ha^{-1})
B. japonicum (Control)	0%	3,99 c	0,50 d	13,4 f	4,22 c	0,53 c	14,25 e	3,80 e	0,76 cd	20,20 fg	3,72 c	0,82 cd	25,07 ef
B. japonicum (Control)	50%	4,32 b	0,66 d	20,2 ef	4,81 b	0,66 c	20,20 e	4,16 d	0,66 d	19,13 g	4,12 b	0,66 d	20,64 f
B. japonicum (Control)	100%	4,80 a	1,02 c	40,1 d	5,32 a	1,09 b	44,40 bcd	4,80 bc	1,15 c	48,01 cd	4,78 a	1,17 bc	45,41 bcd
B. japonicum + *P. fluorescens* ST	0%	4,38 b	0,98 c	28,2 e	5,28 a	1,14 b	33,79 d	4,62 c	1,08 bc	31,03 ef	4,32 b	1,17 bc	36,81 de
B. japonicum + *P. fluorescens* ST	50%	4,83 a	1,31 b	41,8 cd	5,32 a	1,31 ab	41,67 cd	4,96 ab	1,31 ab	39,29 de	4,69 a	1,31 ab	42,37 cd
B. japonicum + *P. fluorescens* ST	100%	4,93 a	1,41 ab	56,9 ab	5,24 a	1,28 ab	53,97 ab	5,09 ab	1,41 ab	60,67 ab	4,72 a	1,41 ab	57,60 ab
B. japonicum + *P. fluorescens* leaf-spray	0%	4,92 a	1,61 a	50,8 bc	5,42 a	1,31 ab	41,24 cd	5,06 ab	1,66 a	53,16 bc	4,74 a	1,61 a	53,96 bc
B. japonicum + *P. fluorescens* leaf-spray	50%	4,98 a	1,51 ab	50,3 bc	5,24 a	1,53 a	50,80 bc	5,09 ab	1,60 a	52,21 bc	4,84 a	1,51 ab	51,18 bc
B. japonicum + *P. fluorescens* leaf-spray	100%	5,00 a	1,53 ab	64,1 a	5,34 a	1,35 ab	58,44 a	5,12 a	1,55 a	65,29 a	4,79 a	1,53 ab	65,47 a
B. japonicum + Fertibio Phospro® ST	50%	4,97 a	1,53 ab	48,0 bc	5,22 a	1,31 ab	41,66 cd	5,09 ab	1,60 a	50,14 bc	4,79 a	1,53 ab	50,21 bc
CV(%)		3,28	14,55	13,56	2,36	13,10	14,19	2,69	12,53	12,34	2,54	16,22	16,12

* Means followed by the same letter in the column are not different according to the comparison of Tukey's test (5%).

Table 6. Yield (kg ha^{-1}) evaluation in soybean inoculated with *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* and phosphate fertilization (crop 2021/21).

Treatments	P Fertilization	IF Goiano	Bauzinho Farm	Bela Vista Farm	Cachoeira Farm	Average
B. japonicum (Control)	0%	2685 e	2694 d	2680 e	3015 e	2.766
B. japonicum (Control)	50%	3050 d	3030 bc	2889 de	3111 de	3.020
B. japonicum (Control)	100%	3928 b	4052 a	4168 a	3864 b	4.004
B. japonicum + *P. fluorescens* ST	0%	2853 e	2954 cd	2859 de	3137 de	3.200
B. japonicum + *P. fluorescens* ST	50%	3182 cd	3162 bc	2981 bcd	3214 cd	3.318
B. japonicum + *P. fluorescens* ST	100%	4018 ab	4195 a	4283 a	4055 b	4.248
B. japonicum + *P. fluorescens* leaf-spray	0%	3145 cd	3134 bc	3188 bc	3333 c	3.175
B. japonicum + *P. fluorescens* leaf-spray	50%	3322 c	3309 b	3262 b	3378 c	3.267
B. japonicum + *P. fluorescens* leaf-spray	100%	4180 a	4326 a	4212 a	4274 a	4.290
B. japonicum + Fertibio Phospro® ST	50%	3128 d	3158 bc	3139 bc	3275 cd	2.939
CV(%)		4,76	4,65	2,99	2,91	

* Means followed by the same letter in the column are not different according to the comparison of Tukey's test (5%)

CAPÍTULO II

Inoculation and Co-inoculation of plant growth promoting bacteria (PGPB) with additive protective and nitrogen fertilization in soybean

(Normas de acordo com a revista Funcional Plant Biology. Submetido em outubro de 2021)

Abstract. The soil is a dynamic biological system, which is the principal reservoir of biological diversity. Given this, the importance of recent research on the micro-organisms found in the soil has increased progressively, in terms of both its scientific value and economic benefits. The present study evaluated the performance of plants inoculated and co-inoculated with PGPB, as well as the effects of the application of nitrogen (N) and protective cellular additives on the productivity of the soybean. The experiment happened in two crop (2018/2019 and 2019/2020) in random blocks with the following treatments (with 4 repetitions): T1: control (no inoculants or nitrogen-based fertilizers); T2: 200 kg N ha^{-1}; T3: *Bradyrhizobium japonicum*; T4: *B. japonicum* + protective additive; T5: *Azospirillum brasilense*; T6: *A. brasilense* + protective additive; T7: *Pseudomonas fluorescens*; T8: *P. fluorescens* + protective additive; T9: *B. japonicum* + *A. brasilense* + *P. fluorescens,* and T10: *B. japonicum* + *A. brasilense* + *P. fluorescens* + protective additive. It was evaluated the shoot dry mass; the number and dry mass of nodules; N content dry mass and grain; N total and yield. The results indicate that use of nitrogen fertilizers harm soybean nodulation, harming the BNF and does not result in productivity increases. The inoculation with PGPB is essential for the adequate assimilation of N by the plant, in addition to supporting an increase in productivity, while also improving the

N levels of the soybean. The protective additives applied to the inoculants did not influence either nodulation or BNF.

Key words: reinoculation, *Glycine max* (L.) Merrill, spray foliar

4.1 Introduction

The search for sustainable and low-cost technologies to attend the high demands for food by an ever-growing population, based on sustainable models, is pivotal. In this context, microorganisms play important roles, such *Bradyrhizobium* spp. able to fully supply the soybean's demand on N via biological nitrogen fixation (BNF), with no need of supplying chemical N-fertilizers (Hungria & Mendes, 2015; Hungria & Nogueira, 2019;).

The cultivation of the soybean (*Glycine max* L. Merrill) demands high levels of nitrogen (N) due to the protein content – approximately 40% – of its seeds. This nitrogen is obtained from the soil through the mineralization of organic matter, but primarily through the biological nitrogen fixation (BNF) by diazotrophic bacteria that extract the nitrogen from the atmosphere (N_2) and reduce it to the ammoniacal form in the root nodules, from where it is translocated and converted into amino acids and proteins (Nogueira & Hungria 2014; Moretti et al., 2020; Santos et al., 2019).

The inoculation of soybean with nitrogen-fixing bacteria may reduce either partially or even completely the need for the application of nitrogen-based fertilizers. The potential economic, social, and environmental benefits of the use of these inoculants can be enormous, including saving on the use of mineral nitrogen fertilizers, more productive crops, and the preservation of the microbiota of the soil, as well as minimizing environmental impacts and reducing production costs (Hungria et al. 2015a).

The application of nitrogen-based fertilizer did not improve the performance of the plants, which indicates that the fertilization of soybean with nitrogen is disadvantageous. An excess of mineral N may deform the absorbent trichomes of the root and affect the adhesion of the rhizobium and the formation of the infective cordon (Cassetari et al., 2016; Santos et al., 2019; Santos et al., 2021). In addition, the ammonia produced by the nitrogenase inside the nodule has a self-regulating effect derived from the enzymatic activity itself. Additional quantities of ureia and nitrates may interrupt this self-regulatory effect, given that they are transformed into ammonia inside the cells, affecting the production of nodules (Câmara 2014).

Both associative and free-living diazotrophic bacteria can stimulate plant growth through direct mechanisms, such as BNF and the production of phytohormones, as well as indirectly, by providing a defense against pathogens. For this reason, associative diazotrophic bacteria are also known as plant growth-promoting rhizobacteria (PGPB) and play a fundamentally important role in the interaction between plant roots and nutrient cycling (Vishwakarma et al., 2020; Cardoso & Andreonte, 2016).

Co-inoculation with PGPB is a biotechnological tool that can be used to maximize the productivity of the soybean while avoiding the need for industrialized nitrogen-based fertilizers (Hungria et al. 2015b; Garcia et al., 2021). The idea is of combining strains or species acting in different microbial processes, so that the combined benefits of each one would result in higher benefits and yields. Examples of mixed inoculant are those combining microorganisms whose major processes are BNF (*Bradyrhizobium spp., Rhizobium spp.)* and phytohormone production (*Azospirillum spp., Pseudomonas spp*.), solubilization of phosphate (*Bacillus spp*.), or biological control (*Pseudomonas spp., Bacillus spp*.) (Santos et al. 2019).

The inoculant contains live cells, and is often exposed to inadequate handling procedures, such as being mixed with pesticides, exposed to high temperatures or sunlight, and dehydration, which can all provoke mortality (Rodrigues et al., 2020).

Adequate inoculation practices ensure the survival of the bacteria and the assimilation of the N necessary for high levels of soybean production, while avoiding high production costs and impacts on the environment and human health (Saturno et al. 2017; Santos et al., 2021; Lopes et al., 2021). The development of complementary technologies, such as the addition of protective substances to the inoculant, may also improve the results of the inoculation when the seeds are also exposed to industrial pesticides (Araújo et al. 2017; Lopes et al., 2021; Santos et al., 2021).

The study's purpose was to evaluate the performance of PGPB inoculation and co-inoculation with the use of a protective additive, on agronomic parameters and nitrogen assimilation by the soybean plant.

4.2 Materials and Methods

The experiment was conducted in the field, in two crops (2018/2019 and 2019/2020), in the Experimental area of the Federal Institute of Goiano campus Rio Verde, located at latitude 17º 47' 53" S, longitude 50º 55' 41" W and altitude 744 m, in Rio Verde. The region’s climate is of the *Aw* (tropical) type in the Köppen classification system, with a dry season between May and September and rains between October and April.

The region's climate, according to the Köppen-Geiger classification, is classified as Aw (tropical climate with dry season). The soil is classified as typical Red Dystrophic Argisol (Embrapa 2013). The test sites were sampled 60 days in advance for the evaluation of physical and chemical characteristics. The Table 1 shows the area chemical and granulometric characterization. The population of native rhizobia in the soil in each area was estimated by counting in Petri dishes. The methodologies followed are described in Normative Instruction nº 30 of MAPA (Ministério da Agricultura, pecuária e abastecimento) (MAPA, 2010).

The experimental design was based on random blocks, with 10 treatments and 4 repetitions. T1: control (no inoculants or nitrogen-based fertilizers); T2: 200 kg N ha^{-1}; T3: *Bradyrhizobium japonicum*; T4: *B. japonicum* + protective additive; T5: *Azospirillum brasilense*; T6: *A. brasilense* + protective additive; T7: *Pseudomonas fluorescens*; T8: *P. fluorescens* + protective additive; T9: *B. japonicum* + *A. brasilense* + *P. fluorescens*, and T10: *B. japonicum* + *A. brasilense* + *P. fluorescens* + protective additive. The plots consisted of 5 lines of 5m length, spaced in 0.5m, totaling 40 plots of 25 m^2 each. The two central lines were considered as useful area.

Seed of the Guaiá 7487 RR® soybean cultivar was planted 20th November 2018, at a density of 420.000 plants ha^{-1}, fertilized at 350 kg ha^{-1} (02:20:18). The product Standak Top®: insecticide Fipronil from the pyrazole group, the fungicides Pyrclostrobin from the strugirulin group, and Methyl Thiophanate from the benzimidazole group (2mL kg $seeds^{-1}$) was used in the seed treatment. After treatment of the seeds, they were dried in the shade for 4 hours and then received the inoculation treatments. Except for the control, the seeds of all treatments were inoculated with the inoculant Atmo® in seed treatment (ST), with strains of *Bradyrhizobium* *japonicum* (SEMIA 5079 and 5080; 5.0 x 10^9 CFU mL^{-1}), 100 mL 50 kg of $seeds^{-1}$ + protective cellular additive (Synflex Microquímica®) (100 mL 50 kg^{-1} of seed).

The plants were inoculated with bacterial isolates by foliar spray at the V2 stage, spray volume 100 L ha^{-1}, using a CO_2-powered backpack sprayer operating at a constant pressure of 2 BAR (or 29 PSi), with a discharge of 0.35 L min^{-1}, equipped with a lance-type sprayer with a fan-shaped Teejet XR 110 02 tip. The N-based treatment involved the application of 200 kg N ha^{-1}, with ureia as the cover, at the V4 stage.

Foliar inoculation dose was 600 mL ha^{-1} ($7x10^9$ UFC.mL^{-1}) for *Bradyrhizobium*, 300 mL ha^{-1} ($2x10^8$ UFC mL^{-1}) for *Azospirillum*, and 300 mL ha^{-1} ($1x10^8$ UFC mL^{-1}) for *Pseudomonas*, with 200 mL ha^{-1} being applied in the case of the protective additive (Synflex® Microquímica).

At 35 days after emergence (DAE), five plants were collected at random from each plot to determine the number and dry mass of nodules, the shoot dry mass and N content dry mass. For this, and shoots plants were separated carefully, washed, and dried in a stove at 65ºC for 72 h. The nodules were then removed from the roots and dried for a further 72 h before being counted and weighed. The leaves were ground in a Wiley-type mill for the evaluation of the dry mass of the aerial portion, and the subsequent measurement of the level of N.

After maturation plants, the plots were harvested manually and the pods were deseeded in a stationary threshing machine, cleaned using sieves, dried under natural conditions and stored in paper bags. Humidity was standardized to 13% prior to weighing for the calculation of productivity (Silva, 2009). The N levels of the aerial portion of the plant and the seed were determined using the Kjeldahl method (Liao, 1981). The data were analysis using an Analysis of Variance, with the means being compared using the Scott-Knott test (5%). These analyses were run in the SISVAR statistical package (Ferreira 2011).

4.3 Results

Regarding the number of nodules per plant, in both crops, the inoculated and co-inoculated treatments had higher values than the N-fertilized which even had the lowest number of nodules (Table 2). In 2018/2019, the control had a lower number than the inoculated and co-inoculated treatments. In the 2018/2019 crop, the treatments *P.fluorescens* and *B. japonicum*, both with protective additive, had a higher amount of nodules than the corresponding treatments without additive. In the 2019/2020 crop, *P.fluorescens* without additive showed higher values than with the protective additive . In the 2019/2020 harvest, the co-inoculated treatment without additive was better than the same with protective additive (Table 2).

In both crops, the co-inoculated treatments had a greater number of nodules than the control, N-Fertilized. In the 2018/2019 crops, co-inoculates were better than *P. fluorescens*, *A. brasilense* with and without protective additive, and B. japonicum. In the 2019/2020 harvest, the co-inoculated treatment, without additive, was better than *P.fluorescens* with protective additive (Table 2).

In relation to the dry mass of nodules, in both crops, the N-fertilized treatment had lower mass than the other treatments. In 2018/2019 the inoculated and co-inoculated treatments, except for the co-inoculated + protective additive and *P. fluorescens* + additive that were equal to the control, obtained the best results (Table 2).

In shoot dry mass, in 2018/2019, the control and N-fertilized obtained lower values than the other treatments. In 2019/2020, the inoculated and co-inoculated treatments, except for *B. japonicum* and *A. brasilense*, both with protective additive, obtained the highest shoot dry mass results. There was no difference regarding the use of protective additive or co-inoculation technology (Table 2).

In both crops, the control and N-fertilized obtained N content dry mass and N content in grains and total N, lower than the other treatments (Table 3). In the 2018/2019 crop, the control had the lowest value of total N; the co-inoculated and *A. brasilense* + protective additive treatments obtained the highest values (Table 4). In both crops there was no difference in relation to the use of protective additive and in relation to co-inoculation, except for the 2018/2019 crop where the co-inoculated treatments were better than *P. fluorescens*, *A. brasilense*, both without additive and *B. japonicum* with and without additive (Table 3).

The control and N-fertilized showed lower yields than inoculated and co-inoculated treatments (Table 4). In 2018/2019 crop the N-fertilized had lower productivity than the control. The *A. brasilense* treatment with additive showed higher yields than the one without additive protection. *P. fluorescens* with additive had higher productivity than without additive. There was no difference in the coinoculation technology with the inoculation (Table 4).

4.4 Discussion

In general, the control and N-fertilized treatments presented the worst performance in variables analyzed. The control plants were not inoculated with bacteria and only received the base fertilization (02-20-18), highlighting the need for the establishment of an effective symbiosis with diazotrophic bacteria to ensure the satisfaction of the nitrogen demands of the plant.

The inoculation of the soybean, on its own, results in a productivity equivalent to that obtained by fertilization with 200 kg N ha^{-1} (Cassetari et al. 2016). However, given the extreme efficiency of the BFN system, which provides 70–95% of the N requirements of the plant, and its low cost, there is no obvious reason to recommend the application of nitrogenated fertilizers to soybean plantations (Câmara 2014).

Kaschuk et al. (2016) showed that inoculation with *Bradyrhizobium* was sufficient to supply all the N needed by the soybean plant, avoiding the need for nitrogen-based fertilizers, which may, in fact, impact the BNF by the bacteria by deforming the absorbent trichomes, and modifying the adhesion of the rhizobium and the formation of the infective cordon.

Saturno et al. (2017) tested different doses and application routes for mineral N in soybean, and found the use of nitrogen fertilization harms the BNF, and there was no improvement in the production of seed. Hungria et al. (2006) and Gautan. (2021) observed that the larger the amount of mineral N applied, the greater the negative effect on the formation of nodules, corroborating this study where the number of dry mass of nodules were lower in the treatment with N-fertilized.

The inoculation and co-inoculation resulted in an increase in the N levels of both the shoot dry mass and the seeds (Table 2). Puente *et al*. (2018) found that the levels of N and the protein content of the seeds were higher in plants sprayed with *Azospirillum.* Zarei *et al*. (2014) reported that the inoculation of the soybean with a combination of *Bacillus* and *Pseudomonas* increased productivity, and the levels of N and protein in the seeds.

In the present study, the co-inoculation of the plants rendered satisfactory results indicating these species can be used effectively in combination. Even so, the results did not indicate any improvements in productivity in comparison with the inoculation of single bacteria, which implies that co-inoculation would only be worthwhile if it were economically viable (Shneider et al. 2017; Galindo et al. 2018; Galindo et al. 2020).

The Yield was higher in the treatments with inoculation and co-inoculation of PGPB, when compared to the control. This shows that these bacteria can be used separately or combined to increase productivity).

Moretti et al. (2018) found that the inoculation of the seeds *Pseudomonas fluorescens* increased seed production by 28% and 27% in two successive crops. A few experimental studies have shown that the co-inoculation of the soybean seed with *Azospirillum* and *Bradyrhizobium* can increase the productivity of the plant (Cerezini et al. (2016), Hungria et al. 2015b; Chibeba et al. 2015; Fukami et al. 2018; Moretti et al., 2018; Moretti et al., 2020). Zarei et al. (2014) also reported that co-inoculation of the soybean with *Bacillus* and *Pseudomonas* increased the concentrations of nitrogen and protein in the seeds.

Regarding the use of protective additives, in this study, in general, the results did not show considerable differences. Marks *et al.* (2013) evaluated the effects of cellular additives on the survival of *Bradyrhizobium* in inoculated soybean seed treated with different fungicides and found that the number of cells was smaller in the treatments without the additive. However, the additive did not contribute to the survival of the micro-organisms when combined with some brands of fungicide, which indicates the influence of the chemical composition of the fungicide.

Alcântara Neto et al. (2014) observed that the use of a protective additive associated with a difenoconazole-based fungicide contributed to the survival of inoculated *Bradyrhizobium* and nodulation in the soybean. However, negative results were obtained for fungicides based on other compounds, indicating that the use of additives with some active ingredients may maximize the toxicity of the fungicide to the bacteria.

Araújo et al. (2017) tested the use of protectors in the pre-inoculation of soybean seeds together with the treatment of the seeds with pesticides and found that the bacteria survived for up to 30 days following inoculation, with no negative effects on nodulation, the fixation of N_2 or the productivity of the plants. Stecca *et al*. (2019) inoculated soybean seeds coated with osmoprotector in soils of varying pH. The seeds inoculated on the fourth and seventh days prior to planting were 8.3–10.8% more productive than the treatments without osmoprotector, in the case of the NA 5909RG cultivar, in soil at pH 5.3.

4.5 Conclusions

The use of nitrogen fertilizers harm soybean nodulation, harming the BNF and does not result in productivity increases. The inoculation with PGPB is essential for the adequate assimilation of N by the plant, in addition to supporting an increase in productivity, while also improving the N levels of the soybean. The protective additives applied to the inoculants did not influence either nodulation or BNF.

Authors contributions

The authors contributed equally to the research data analysis, modelling, and manuscript writing.

Data Availability Statement

Data supporting this study will be shared upon reasonable request to the author for correspondence.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. The soil's chemical and granulometric characteristics at IF Goiano, Rio Verde, Goiás, Brazil (2019).

Local	pH water	P	K	Fe	Mn	Zn	Ca	Mg	Al	Organic matter	Saturation by bases	Areia	Silte	Argila	Native rhizobia*
		------------- mg dm^{-3} -------------					-- $cmol_c$ dm^{-3} --			g dm^{-3}	----------------- % -----------------				UFC g $soil^{-1}$
IF Goiano campus Rio Verde	6,1	15,2	255	9,0	60,2	6,0	4,4	1,3	0,03	34,9	55,6	36,2	17,4	46,4	4,8 x 10^3

Extractors: Mehlich 1 (P, K, Fe, Zn e Mn); KCl 1 N (Ca, Mg e Al); * Analysis performed 48h before planting.

Table 2. Number of nodules and the dry mass of the nodules and shoot dry mass of the experimental soybean plants raised in the field in 2018/2019 and 2019/2020 in Rio Verde, Goiás, Brazil.

Treatments	**No nodules (plant $^{-1}$)**		**Dry mass nodules (mg $plant^{-1}$)**		**Shoot dry (g $plant^{-1}$)**	
	2018/2019	**2019/2020**	**2018/2019**	**2019/2020**	**2018/2019**	**2019/2020**
Control	21,8 c	18,1 b	56,0 b	108,6 b	1,3 c	1,1 b
200 kg N ha^{-1}	8,8 d	8,6 c	51,0 b	51,6 c	2,6 b	1,3 b
B. japonicum	26,4 b	27,8 a	113,2 a	172,1 a	3,6 a	2,5 a
B. japonicum+additive	35,3 a	23,7 a	120,0 a	164,8 a	3,8 a	1,6 b
A. brasilense	29,4 b	33,0 a	113,5 a	197,3 a	3,5 a	1,9 a
A. brasilense+additive	29,5 b	27,7 a	111,0 a	182,0 a	3,7 a	1,7 b
P. fluorescens	30,6 b	24,3 a	116,5 a	180,6 a	4,0 a	2,5 a
P. fluorescens+additive	40,9 a	15,6 b	120,5 a	142,2 b	3,5 a	2,2 a
B.japonicum+A.brasilense+P.fluorescens	37,6 a	23,2 a	124,0 a	183,6 a	3,7 a	1,8 a
B.japonicum+A.brasilense+P.fluorescens	38,4 a	17,7 b	116,5 a	126,1 b	3,8 a	1,9 a
CV (%)	14,2	22,3	22,7	23,7	13,1	26,6

Means values followed by different letters are significantly different based on the Scott-Knott test (5%).

Table 3. The N contents dry mass and grains, Total N of the experimental soybean plants raised in the field in 2018/2019 and 2019/2020 in Rio Verde, Goiás, Brazil.

Treatments	N contents dry mass ($g\ Kg^{-1}$)		N contents grains (%)		Total N (kg ha-1)	
	2018/2019	**2019/2020**	**2018/2019**	**2019/2020**	**2018/2019**	**2019/2020**
Control	33,7 b	38,3 b	37,9 b	35,3 b	123,9 c	125,8 b
200 kg N ha^{-1}	33,8 b	38,6 b	38,9 b	36,4 b	116,8 d	129,9 b
B. japonicum	35,6 a	42,0 a	50,4 a	45,4 a	191,1 b	172,1 a
B. japonicum+additive	35,6 a	42,1 a	51,1 a	42,4 a	202,7 b	165,1 a
A. brasilense	36,0 a	42,9 a	51,1 a	44,3 a	203,3 b	173,0 a
*A. brasilense+*additive	35,2 a	43,3 a	50,9 a	46,4 a	210,3 a	184,8 a
P. fluorescens	35,7 a	43,2 a	51,2 a	45,8 a	199,3 b	182,6 a
*P. fluorescens+*additive	35,6 a	44,3 a	50,3 a	47,2 a	208,5 a	189,9 a
B.japonicum+A.brasilense+P.fluorescens	35,9 a	44,5 a	51,1 a	46,3 a	212,6 a	189,6 a
B.japonicum+A.brasilense+P.fluorescens	35,6 a	45,4 a	50,6 a	48,1 a	210,7 a	197,9 a
CV (%)	1,5	4,1	7,8	7,0	2,56	4,89

Means values followed by different letters are significantly different based on the Scott-Knott test (5%).

Table 4. Yield (Kg ha^{-1}) of the experimental soybean plants raised in the field in 2018/2019 and 2019/2020 in Rio Verde, Goiás, Brazil.

Treatments	Crop	
	-----	**-----2019/2020-----**
Control	3.187 c	2.925 b
200 kg N ha^{-1}	3.004 d	2.977 b
B. japonicum	3.788 b	3.786 a
B. japonicum+additive	3.965 b	3.885 a
A. brasilense	3.973 b	3.914 a
A. brasilense+additive	4.131 a	3.980 a
P. fluorescens	3.889 b	3.976 a
P. fluorescens+additive	4.145 a	4.022 a
B.japonicum+A.brasilense+P.fluorescens	4.158 a	4.100 a
B.japonicum+A.brasilense+P.fluorescens	4.163 a	4.105 a
CV (%)	2,00	4,22

Means values followed by different letters are significantly different based on the Scott-Knott test (5%).

CONCLUSÃO GERAL

A inoculação de *Pseudomonas fluorescens* e *Bradyrhizobium japonicum* contribui para a assimilação de nitrogênio e fósforo, além de incrementar a produtividade. *P. fluorescens* tem a capacidade de solubilizar fosfatos, facilitando sua absorção pelas plantas e possibilitando a redução do uso da fertilização fosfato na cultura da soja.

A aplicação via foliar foi mais eficiente do que a inoculação no tratamento de sementes e pode ser usado como um modo alternativo de aplicação.

O uso de fertilizantes nitrogenados prejudica a nodulação da soja, prejudicando a FBN e não resulta em aumento de produtividade. A inoculação com PGPB é essencial para a adequada assimilação de N pela planta, além de apoiar aumento na produtividade, ao mesmo tempo em que melhora os níveis N da soja. Os aditivos protetores aplicados aos inoculantes não influenciaram nem a nodulação nem o BNF.

Os benefícios provenientes das BPCP aliados com a diminuição do uso de adubação química, sustentabilidade e redução de custos, tornam o uso dessa tecnologia atrativa e indispensável no manejo da cultura da soja.