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Phosphate–solubilizing fungi co–inoculated with *Bradyrhizobium* promote cowpea growth under varying N and P fertilization conditions

Marco Esteban Gudiño–Gomezjurado[1] , Rafael de Almeida Leite[2] , Teotonio Soares de Carvalho[2] , Ludwig Heinrich Pfenning[3] , Fatima Maria de Souza Moreira[2]*

[1]Universidad de Investigación de Tecnología Experimental Yachay – Escuela de Ciencias Biológicas e Ingeniería, C.P. 100119 – Urcuquí, Imbabura – Ecuador.

[2]Universidade Federal de Lavras/Escola de Ciências Agrárias – Depto. de Ciência do Solo – Setor de Biologia, Microbiologia e Processos Biológicos do Solo, C.P. 3037 – 37200-900 – Lavras, MG – Brasil.

[3]Universidade Federal de Lavras Escola de Ciências Agrárias – Depto. Fitopatologia, Lab. de Micologia – Lavras, MG – Brasil.

*Corresponding author <fmoreira@ufla.br>

ABSTRACT: We evaluated the compatibility between two nitrogen–fixing *Bradyrhizobium* inoculant strains and phosphate–solubilizing fungal strains and the effect of co–inoculation of these bacterial and fungal strains on cowpea growth under different N and P conditions. First, the compatibility between *Bradyrhizobium* strains UFLA03–84 and INPA03–11B and fungi *Haematonectria ipomoeae* FSA381, *Eleutherascus lectardii* FSA257a, *Pochonia chlamydosporia* var. *catenulata* FSA109, and *Acremonium polychromum* FSA115 was tested in both solid and liquid media. Cowpea growth and nodulation promotion under two mineral N doses and two P conditions (a low dose of soluble P plus a high dose of $Ca_3(PO_4)_2$ and another condition with a high dose of soluble P) were tested with two N_2 fixing *Bradyrhizobium* strains co–inoculated with each of the P–solubilizing fungal strains FSA109, FSA115, and FSA381. There was compatibility between each fungal strain and the two *Bradyrhizobium* strains, except for FSA257a with either of the bacterial strains in liquid medium. When both mineral N and P were limiting, plants were able to grow and accumulate N and P based on biological N_2 fixation and solubilization of calcium phosphate in the same amount as the mineral N and soluble phosphate. Even when both nutrients were fully available, the type of co–inoculation promoted plant growth and nutrient accumulation. The responses varied in accordance with the co–inoculated strains, the N source, and the P source, reflecting the enormous complexity of the biological interactions between plants and microorganisms, and the nutrient conditions provided by the environment.

Keywords: *Vigna unguiculata* (L.) Walp., P nutrition, rhizobia, fungal strains, plant growth promotion

Introduction

Nitrogen (N) and phosphorus (P) are two elements indispensable to plant development. The main natural means of providing N is through the biological fixation process. Amongst these microorganisms are those belonging to the *Bradyrhizobium* genus (Jordan, 1982), which is considered one of the most abundant in soil samples collected throughout the world (Shah and Subramanian, 2018). As for P, most of the soil P pool is unavailable to plants (Bünemann, 2015). Furthermore, a large amount of the P applied to the soil as fertilizer rapidly becomes unavailable for plant uptake, creating a situation where crop yield may be compromised (Roberts and Johnston, 2015; Zhu et al., 2018). Amongst the soil microorganisms, certain bacterial and fungal genera have the ability to solubilize phosphate from inorganic insoluble forms, mainly through acidification mechanisms (production of low molecular weight organic acids and H^+ extrusion) and chelation of metals previously linked to phosphates (through organic ligands), thereby increasing P availability to plants (Costa et al., 2015; Gadd, 1999; Mendes et al., 2020; Narsian and Patel, 2000). Moreover, few studies have tested the compatibility of different functional microorganisms, especially phosphate–solubilizing fungi and symbiotic nitrogen–fixing bacterial strains to perform together both processes in order to enhance plant growth and nutrition (Abd–Alla and Omar, 2001; Zaidi and Khan, 2006).

Cowpea (*Vigna ungiculata* [L.] Walp) is a legume species cultivated in several tropical and subtropical regions throughout the world. In Brazil, cowpea is an important species in terms of food security, even though it is mainly cultivated with few technological inputs and in soils with low natural fertility (Soares et al., 2006). This plant species benefits greatly from biological nitrogen fixation in symbiosis with strains of the *Bradyrhizobium* genus (Costa et al., 2011; Costa et al., 2019; Guimarães et al., 2012; Rufini et al., 2014; Silva et al., 2012) and it already benefits from inoculant strains approved by the Brazilian Ministry of Agriculture for production purposes. Since many of the Brazilian soils in which cowpea is cultivated have low P conditions as well, it could also benefit from phosphate solubilization performed by microorganisms. Therefore, the aim of this study was to evaluate compatibility between *Bradyrhizobium* inoculant strains and phosphate–solubilizing fungal strains, and evaluate the effects of co–inoculation of both bacterial and fungal strains on cowpea growth under different N and P conditions.

Materials and Methods

Two experiments were carried out to evaluate compatibility between fungal and bacterial strains

and their efficacy in promoting cowpea growth. The *Bradyrhizobium* strains UFLA 03–84 and INPA 03–11B were chosen on account of their abilities to promote cowpea growth under field conditions (Lacerda et al., 2004; Soares et al., 2006) and their approval by the Brazilian Ministry of Agriculture as official inoculants for cowpea. The INPA 03–11B strain is classified as *Bradyrhizobium elkanii* (Guimarães et al., 2015), and the UFLA 03–84 strain was recently classified as *Bradyrhizobium viridifuturi* symbiovar tropici (Costa et al., 2019). Both strains were grown in 79 medium (Fred and Waksman, 1928) with the following components: yeast extract at 0.4 g L^{-1}, mannitol, 10 g L^{-1}; K$_2$HPO$_4$ (10 %), 1 mL L^{-1}; KH$_2$PO$_4$ (10 %), 4 mL L^{-1}; MgSO$_4$.7H$_2$O (10 %), 2 mL L^{-1}; NaCl (10 %), 1 mL L^{-1}; bromothymol blue (0.5 %) in 0.2 N KOH, 5 mL L^{-1}; and agar, 15 g L^{-1}. Strains were incubated for seven days at 28 °C. Both INPA 03–11B and UFLA 03–84 had been previously tested for *in vitro* P solubilization. UFLA 03–84 is able to solubilize CaHPO$_4$ and Al(H$_2$PO$_4$)$_3$ in solid medium but is unable to solubilize inorganic P in liquid medium. In contrast, the INPA 03–11B strain was not able to solubilize the P sources tested (Marra et al., 2011; Marra et al., 2012).

Saprophytic Ascomycota fungal strains were chosen on the basis of their calcium phosphate solubilization index in solid Pikovskaya agar medium: *Haematonectria ipomoeae* FSA381 (= CML 3249), *Eleutherascus lectardii* FSA257a, and *Pochonia chlamydosporia* var. *catenulata* FSA109 (= CML 3250). A fourth strain (*Acremonium polychromum* FSA115) was chosen as a negative control, due to its inability to solubilize calcium phosphate in the same medium (Gudiño–Gomezjurado et al., 2014). These fungi were previously isolated from different land uses in the Brazilian Amazon region. The fungal strains were grown for seven days at 25 °C in 2 % malt extract agar medium (MEA) containing malt extract at 20 g L^{-1} and agar at 20 g L^{-1}.

Compatibility between bacteria and fungi

An antagonism assay to evaluate the compatibility between bacterial and fungal strains prior to inoculation on plants was performed in both solid and liquid 79 media. Each bacterial strain was inoculated into the solid medium with two perpendicular streaks, forming a cross (Sadfi et al., 2001). Four 5-mm disks cut from the fungi in the MEA medium were then placed between the bacterial streaks. The distance of each streak to the disks was 2.5 cm. The plates were incubated for 5 days at 25 °C. The percentage of compatibility between the two microorganisms after the incubation period was calculated using the Whipps formula (Whipps, 1987): (R1–R2)/R1 × 100, in which R1 is the radial growth of the fungi (mm) in the opposite direction to bacterial growth, and R2 is the radial growth of the fungi (mm) in the direction of bacterial growth. Growth inhibition (GI) was determined on a 0 to 3 scale as follows: 0 = no inhibition (compatible microorganisms); 1 = 1 % to 25 %

growth inhibition; 2 = 26 % to 50 % growth inhibition; and 3 = 51 % to 75 % growth inhibition. Four replicates were tested.

To evaluate the compatibility in liquid medium between the bacterial strains and the fungi *Eleutherascus lectardii* FSA257a, *Pochonia chlamydosporia* var. *catenulata* FSA109, *Haematonectria ipomoeae* FSA381, and *Acremonium polychromum* FSA115, 1 mL of 10^9 conidia of each fungal strain, inoculated in MEA medium, was incubated with 1 mL of 10^9 CFU mL^{-1} of each bacterial strain in 200 mL of 79 medium. Growth inhibition was assessed by observing the presence of both microorganisms in the microscope, every two days, over 10 days of cultivation at 25 °C and 120 rpm. As growth control, each of these microorganisms was inoculated in 79 medium separately under the same conditions. Four replicates were tested in this evaluation as well.

Growth and nodulation promotion in cowpea plants

A Leonard jar experiment was conducted in a completely randomized design in a 7 × 2 × 2 factorial arrangement to test bacterial ability to provide N from biological N$_2$ fixation (BNF) and fungal ability to solubilize insoluble phosphate thus providing P. The first factor was the co-inoculation of each of the two *Bradyrhizobium* strains UFLA 03–84 and INPA 03–11B combined with each of three fungal strains FSA 109, FSA 115 and FSA 381, chosen from the previous experiment, and a control without inoculation. The second factor was fertilization with a high dose of insoluble phosphorus (HPins) or a high dose of soluble phosphorus (HPs) treatment. Finally, the third factor was fertilization with either a low dose of nitrogen (LN) or a high dose of nitrogen (HN) treatment. The rationale was that on low available P and/or N, microbial interaction with the plants would be stimulated to provide the limiting nutrients via phosphate solubilization and/or biological nitrogen fixation. It is well known that high mineral N concentrations inhibit BNF and low mineral N concentrations stimulate it.

The composition of the treatments, was based on a Hoagland and Arnon solution (Hoagland and Arnon, 1950). The HPins received 10 % of the original soluble P dose (3.1 mg P mL^{-1}) plus 50 mg P mL^{-1} as Ca$_3$(PO$_4$)$_2$, which is insoluble, and HPs received a full soluble P dose from the original solution (31 mg P mL^{-1}). Since phosphate solubilization provides P at a lower rate than P provided by acidified phosphate fertilizers, an initial small dose of soluble P was provided to meet the initial requirements of plants and a higher dose of insoluble P was added to the HPins treatments. The same pattern was used when planning the N treatments: the LN treatments received 10 % of the N dose of the nutrient solution, resulting in 21 mg N mL^{-1} and the treatments with the HN dose received the full amount of the N dose (210 mg N mL^{-1}). The nutrient solution was changed every week and 0.5 L was applied to each jar according to the respective treatments.

In total 28 treatments were the result of the combination of the factors, and 24 of them received microbial co–inoculation. Three replicates were carried out for each treatment.

As regards mineral fertilization, the combination of the factors resulted in the following treatments and doses: 1) low N, low soluble P and high insoluble P [21 mg L^{-1} N + 3.1 mg L^{-1} P + 50 mg L^{-1} P–Ca$_3$(PO$_4$)$_2$, designated as LN + HPins]; 2) low N, high soluble P [21 mg L^{-1} N + 31 mg L^{-1} P, designated as LN + HPs]; 3) high N, low soluble P and high insoluble P [210 mg L^{-1} N + 3.1 mg L^{-1} P + 50 mg L^{-1} P–Ca$_3$(PO$_4$)$_2$, designated as HN + HPins]; and 4) high N, high soluble P [210 mg L^{-1} N + 31 mg L^{-1} P, designated as HN + HPs]. The control treatment received the nutrient solution with a full amount of every element. Plants showed no symptoms of any nutrient deficiency during the experiment.

The *Bradyrhizobium* strains were incubated in 79 medium at 25 °C for four days until reaching 10^9 CFU mL^{-1}. The fungal inoculum was prepared as follows: 5 mm disks of fungal growth were suspended in a sterile 1:1 solution of 0.1 % (v/v) Tween 80 and 0.85 % (w/v) NaCl (Sheng et al., 2011). From this suspension, a serial dilution was applied to the rate of 10^8 conidia mL^{-1}, verified in a Neubauer chamber.

Cowpea seeds, cv. Gurgueia BR17, were surface sterilized in 95 % ethanol for 1 min and in 5 % NaClO for 2 min and washed thoroughly in sterile distilled water until the NaClO residues had been removed. The seeds were then germinated in sterilized dishes with moistened cotton and filter paper for 72 h at 28 °C. After germination, four seeds were sown in sterilized Leonard jars containing 500 cm^3 of a mixture of sand and vermiculite (2:1). Each seed received a treatment of 10^9 CFU of the corresponding bacterial strain and 10^8 conidia of each fungal strain, according to the combination. All four plants were kept up to 12 days and then thinned down to one plant. The surface of the jar was covered with a mixture of sand, paraffin, and chloroform 100:0.01:10 (w:w:v) to avoid contamination and water loss (Vincent, 1970).

After 45 days, plants were harvested. Shoot dry weight (SDW), nodule dry weight (NDW), and whole plant dry weight (PDW) were measured. In addition, the number of nodules (NN) was determined. Shoot nitrogen accumulation (SNA) was determined by the Kjeldahl method (Fawcett, 1954) and shoot phosphorus accumulation (SPA) was according to Malavolta et al. (1997).

Statistical analysis

Treatments were compared by analysis of variance and grouped by the Scott–Knott test ($p < 0.05$). The number of nodules was transformed by the formula $(x + 0.5)^{0.5}$. All data were analyzed in the R environment (R Core Team, version 4.0.4) and the R Studio platform (RStudio, version 1.4.1106) using the *agricolae* and *ExpDes* packages.

Results

Compatibility between bacteria and fungi

The controls with each microbial strain incubated separately showed growth for all strains, as expected. The compatibility test between each fungal strain and each of the two *Bradyrhizobium* strains ensured full compatibility between them, except for the *Eleutherascus lectardii* FSA257a fungus in 79 liquid medium with both *Bradyrhizobium* strains. The other three fungal strains grew in the presence of the two bacterial strains, confirmed by microscope observation. The presence of more than 100 bacterial cells and mycelia or fungal spores was considered positive compatibility.

Growth and nodulation promotion and N and P accumulation of cowpea plants

There was significant interaction ($p < 0.01$) between the three factors (inoculation, P condition, and N condition) for all variables. All non–inoculated plants did not nodulated regardless N and P conditions, indicating that there was no contamination in the experiment. Conversely, all inoculated plants in both P and N factors had nodules.

In general, inoculation (factor 1) promoted an increase in all variables studied in relation to the control without inoculation under both P and N conditions (factors 2 and 3, respectively), as expected. Under the low N and insoluble P condition, all fungal strains co–inoculated with the *Bradyrhizobium* strains INPA 03–11B and UFLA 03–84, except INPA 03–11B + FSA 381, resulted in higher plant (PDW) and shoot dry weight (SDW) than the control without inoculation (Figure 1 and Figure 2). When the condition changed to a higher availability of P (LN + HPs), the co–inoculation with INPA 03–11B + FSA 109 increased plant weight. Under the high level of N treatment, UFLA 03–84 + FSA 115 and INPA 03–11B + FSA 115 were the best co–inoculations.

Nodule dry weight (NDW) under the low N and insoluble P treatment was higher for the *Bradyrhizobium* strain INPA 03–11B co–inoculated with the fungal strains FSA 109 and FSA 115 and also for the co–inoculation UFLA 03–84 + FSA 115 (Figure 3). However, with a low N dose and under both P conditions, the UFLA 03–84 + FSA 381 combination accumulated more N in the shoot (SNA) even though it did not have the highest nodulation or nodule weight (Figure 3, Figure 4 and Figure 5). Under the high N condition, the co–inoculations associated with insoluble P did not differ in their NDW, but some of them accumulated more N, probably as a response to differences in nodule number (Figure 4). When both nutrients were fully available (HN + HPs), nodulation and shoot N accumulation (SNA) generally increased with inoculation, with a few exceptions.
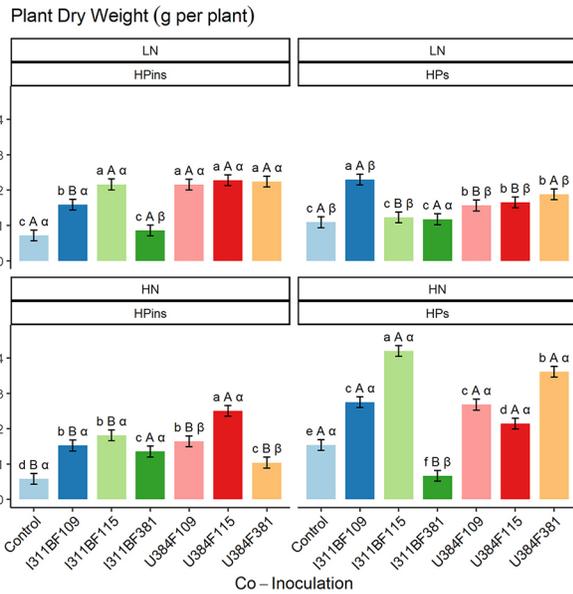
Figure 1 – Plant dry weight (PDW) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the FSA 115 (F115) strain, in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

Figure 2 – Shoot dry weight (SDW) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the FSA 115 (F115) strain, in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

There was a difference in P content in the plants depending on the type of co–inoculation (Figure 6). Most treatments had a shoot P accumulation (SPA) higher than the non–inoculated control under the low N and high insoluble P conditions (LN + HPins). Under the high N condition, the co-inoculations had no effect on SPA when P was insoluble (HPins), but when it was added as soluble P (HPs), only the INPA 03–11B + FSA 115 co-inoculation was superior to the control.

Factor 2 (P fertilization) in relation to factors 1 (inoculation) and 3 (N fertilization) also showed interaction. All plants showed the same amount or more of PDW and SDW under both low N conditions (with high insoluble P and high soluble P doses), except for inoculation with INPA 03–11B + FSA 109 (Figure 1 and Figure 2). In contrast, with a high mineral N dose, plants developed more when they received a high concentration of soluble P, except for INPA 03–11B + FSA 381 and UFLA 03–84 + FSA 115.

NDW and SNA increased in plants fertilized with a high soluble dose of P, especially associated with the high dose of mineral N (Figure 3 and Figure 5). Plants co–inoculated with INPA 03–11B + FSA 115 and UFLA
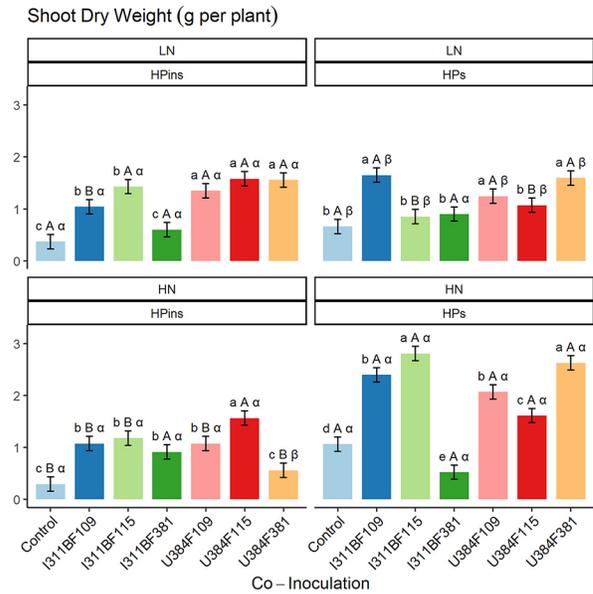
03–84 + FSA 381 had comparable NDW under both P sources with low N doses. N accumulation followed the same pattern of response under both P conditions associated with the low N dose. However, when N was fully provided, plants that also received soluble P accumulated more N. For the number of nodules, in general, plants fertilized with insoluble P produced more nodules than plants receiving soluble P. (Figure 4). P accumulation was generally higher under the soluble P condition than under the insoluble P condition for all inoculation treatments and N conditions, except for INPA 03–11B + FSA 115 associated with a low N dose, which was similar under both P conditions (Figure 6).

The effect of N fertilization was highly dependent on inoculation and P fertilization conditions for both PDW and SDW (Figure 1 and Figure 2). In most cases, when insoluble P was used, inoculated treatments under low N resulted in similar or higher PDW and SDW relative to their counterparts under a low N dose than under a high N dose. Within the soluble P condition, plant growth was generally lower under the low N condition. When a low dose of N was provided, nodule weight and N accumulation were similar or
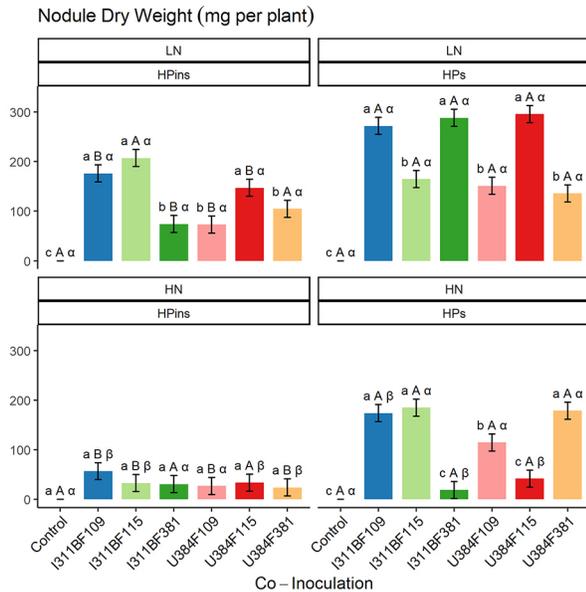
Figure 3 – Nodule dry weight (NDW) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the strain FSA 115 (F115), in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

Figure 4 – Number of nodules (NN) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the FSA 115 (F115) strain, in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

higher under most treatments than under the high N condition (Figure 3 and Figure 5). Plants co–inoculated with INPA 03–11B + FSA 115 and UFLA 03–84 + FSA 381 accumulated more N in the shoots when N came from biological N$_2$ fixation, i.e. plants fertilized with a low N dose. All plants fertilized with high amounts of N and soluble P had higher SNA. There was no difference in the number of nodules for most plants under either high or low mineral N doses (Figure 4).

Plants fertilized with insoluble P accumulated the same or higher amount of shoot P when N was provided at a low dose than with the high N dose (Figure 6). However, under the condition of both the high N and soluble P doses, P accumulation was superior. Plants co–inoculated with INPA 03–11B + FSA 115 were able to accumulate more SPA when P was insoluble (HPins). Under soluble P (HPs), plants co–inoculated with INPA 03–11B + FSA 381 accumulated more P under LN than the corresponding inoculation under HN. However, the other variables for growth and nodulation show that the plants co–inoculated with INPA 03–11B + FSA 381 under the HN + HPs condition did not develop well, and this development appears to be consistent within the replicates.
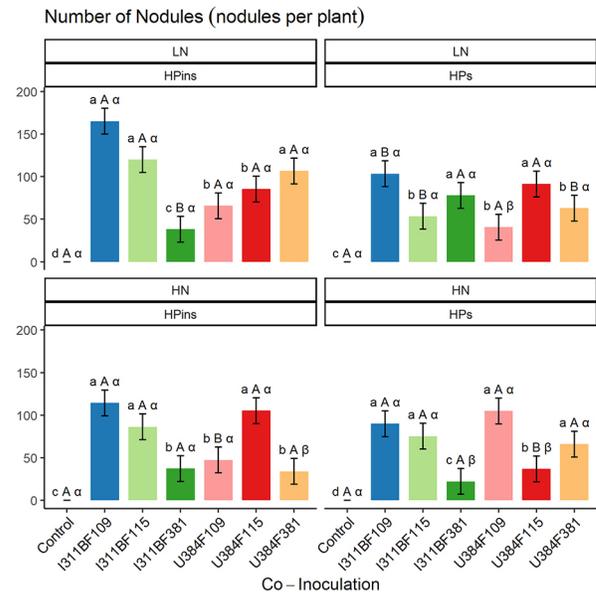
Discussion

Overall, co–inoculation of plants with INPA 03–11B + FSA 115 and all three fungal co–inoculations with UFLA 03–84 promoted plant growth when the plants were fertilized with low N (LN) and insoluble P doses (HPins). This indicates that it is possible to use a low soluble source of phosphate to increase plant growth and nutrient accumulation. In this experiment, BNF associated with insoluble P played a key role in promoting plant growth, providing enough N to ensure the growth of plants to the same extent as their counterparts with mineral N.

The ability of phosphate–solubilizing fungi to stimulate plant growth and the ability of nodule–forming bacteria to fix nitrogen varied according to the solubilization of insoluble P, as previously reported (Adb–Alla and Omar, 2001). Although most papers report *Aspergillus* and *Penicillium* strains as P–solubilizers, other fungal genera also have this ability (Gudiño–Gomezjurado et al., 2014). *Acremonium polychromum* FSA 115, which was planned as a negative control for P solubilization, promoted plant growth and P acquisition when inoculated into cowpea plants under an insoluble P condition. In
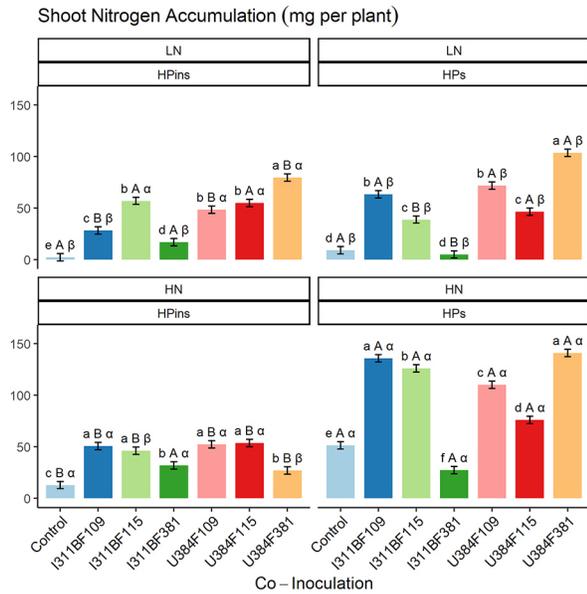
Figure 5 – Shoot nitrogen accumulation (SNA) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the FSA 115 (F115) strain, in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

Figure 6 – Shoot phosphorus accumulation (SPA) of cowpea plants co–inoculated with the nitrogen–fixing *Bradyrhizobium* strains INPA 03–11B (I311B) and UFLA 03–84 (U384) and the phosphate solubilizing fungal strains FSA 109 (F109) and FSA 381 (F381), and the strain FSA 115 (F115) strain, in low nitrogen (LN) and high nitrogen (HN) fertilization, and high insoluble phosphorus (HPins) and high soluble phosphorus (HPs) condition. Each color represents the same co–inoculation. The lowercase letters compare co–inoculations under the same N and P conditions. The uppercase letters compare both P conditions under the same N condition. The Greek letters compare both N conditions under the same P condition. Means were grouped by the Scott–Knott test ($p < 0.05$).

most cases, co–inoculation led to favorable responses in the form of promotion of plant growth and in N and P nutrition in cowpea plants. It is noteworthy that the INPA 03–11B + FSA 381 co-inoculation did not perform very well, and for most variables, plant response was equal to and sometimes worse than the non–inoculated control. It is possible that a negative interaction resulted from the co-inoculation between the phosphate–solubilizing fungus and the nitrogen–fixing bacterial strain, as previously reported (Adb–Alla and Omar, 2001), even though we did not find growth inhibition in both solid and liquid media for this combination. In contrast, plants inoculated with UFLA 03–84 + FSA 381 had better responses for the variables, especially for nutrient accumulation under low N and P conditions.

The ability of the two *Bradyrhizobium* strains, INPA 03–11B and UFLA 03–84, to provide N efficiently for cowpea under field conditions with low available N has already been described (Lacerda et al., 2004; Soares et al., 2006). Next, we provide evidence of the nitrogen–fixing ability of these strains in association with growth–promoting fungi that are able to solubilize P. Under conditions of low available N, plants with the
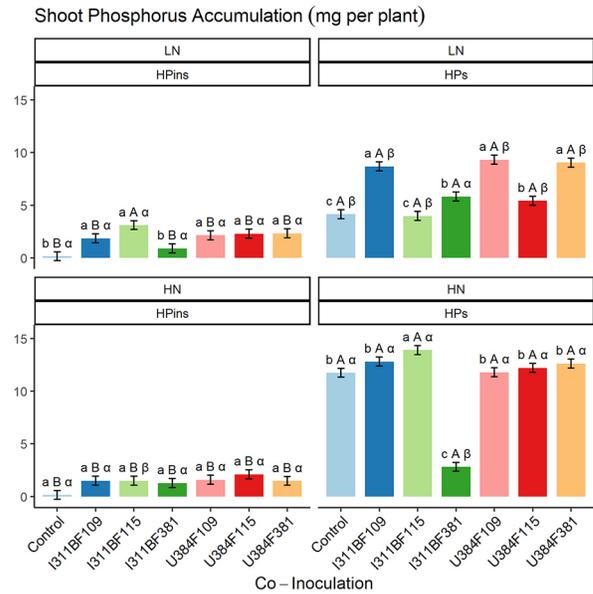
UFLA 03–84 strain co-inoculated with *Haematonectria ipomoeae* FSA 381 accumulated more nitrogen (SNA) than the other co-inoculations, regardless of the source of P. Although there was no difference in SDW compared to other treatments, it is noteworthy that the plant co-inoculated with UFLA 03–84 + FSA 381 grew as much as the others, but with a higher N and P nutritional status under LN + HPins. INPA 03–11B + FSA 115 and UFLA 03–84 + FSA 381 were also able to provide more N from BNF than from mineral N.

Both *Bradyrhizobium* strains, INPA 03–11B and UFLA 03–84, plus FSA 115 were able to accumulate N based on BNF and insoluble P source. However, even though the amount of N accumulated from the high soluble P dose (HPs) for these co-inoculations was not the most expressive compared to the other co-inoculations, it reveals the ability of the combinations INPA 03–11B and UFLA 03–84 plus FSA 115 to perform well when P and N conditions are limiting. This ability was also reflected in plant growth, as plants inoculated with these combinations (LN + HPins) had more PDW and SDW than plants under low N and soluble P conditions. The *Acremonium* genus produces several

secondary metabolites, which may also be involved in plant growth and nutrition.

Even when plants had access to high mineral N availability, nodulation was not entirely compromised, but the response appears to be linked to the combination of co–inoculation and the source of P. Previous studies have showed an increase in nodulation, nitrogenase activity, and N content in *Glycine max* in the presence of an insoluble P source and N–NO$_3$ in soil, after co–inoculation with *Bradyrhizobium* SEMIA 5019 and *Penicillium* sp. (Seneviratne and Jayasinghearachchi, 2005; Seneviratne et al., 2010). The plants co–inoculated with INPA 03–11B + FSA 115 and UFLA 03–84 + FSA 381 accumulated as much NDW with insoluble P as with soluble P under LN, suggesting that, even with the presence of HPins, insoluble P was not limiting enough to halt nodule growth. Under the same condition, LN + HPins, the number of nodules was also not limited by the presence of insoluble P compared with the soluble P source. Overall, the data showed that plants inoculated with the proper combination of nitrogen–fixing rhizobia and phosphate–solubilizing fungal strains were able to nodulate well even in the presence of an insoluble P source.

Plant acquisition of P in the condition of insoluble P with the fungal strain FSA 115 of *Acremonium polychromum* and the two *Bradyrhizobium* strains, INPA 03–11B and UFLA 03–84, indicated by P accumulation in the shoots (SPA), suggests the ability of this fungal strain to solubilize calcium phosphate, even though this strain did not solubilize calcium phosphate in Pikovskaya solid medium (Gudiño–Gomezjurado et al., 2014). In fact, INPA 03–11B + FSA 115 in the insoluble P condition were able to provide as much plant P as under the soluble P condition with low N. In co–inoculations with the INPA 03–11B strain, phosphate–solubilizing ability can be attributed to the associated fungi due to this rhizobial inability to solubilize phosphate *in vitro* (Marra et al., 2011). The ability of FSA 115 to solubilize phosphate could be derived from the different types of organic acids released during the solubilization process (Mendes et al., 2014; Narsian and Patel, 2000). Similarly, this ability can also vary according to the method and the culture medium (Nautiyal, 1999) and the type and availability of carbohydrates that could be exudated in the rhizosphere (Marra et al., 2019). Considering the conditions of insoluble P availability, only the FSA 381 fungal strain of *Haematonectria ipomoeae* co–inoculated with the strain INPA 03–11B was not able to provide as much P as the other co–inoculations, and accumulated the nutrient in the same amount as in the control.

The P nutritional state of the plants showed that plants benefitting from BNF accumulated as much P from the insoluble P source as the plants fertilized with mineral N, or even more. This means that in regard to P accumulation by the plants under the insoluble P source, BNF had the potential to increase P nutrition as much as the mineral N.

We conclude that, despite the *in vitro* compatibility between fungal and rhizobial strains, responses varied according to the strains co–inoculated, the source of N (mineral or through BNF), and the source of P (soluble or insoluble), as well as the different nutrient conditions provided, reflecting the enormous complexity of the biological interactions between plants and microorganisms. When readily available nutrients were limiting, biological nitrogen fixation and solubilisation of calcium phosphate was high enough to sustain plant growth and N and P accumulation to the same extent as observed under high availability of mineral N and soluble P. Even when both nutrients were fully available, the type of co–inoculation had an effect on plant growth as well as N and P accumulation. Future research is required to assess these *Bradyrhizobium* and phosphate–solubilizing fungal strains on cowpea under field conditions, and thereby test the synergistic effect of these two groups of plant growth–promoters under a more complex edaphic system.

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Authors' Contributions

Conceptualization: Gudiño–Gomezjurado, M.E.; Pfenning, L.H.; Moreira, F.M.S. **Data acquisition**: Gudiño–Gomezjurado, M.E.; Moreira, F.M.S. **Data analysis**: Gudiño–Gomezjurado, M.E.; Leite, R.A.; Carvalho, T.S.; Moreira, F.M.S. **Design of methodology**: Gudiño–Gomezjurado, M.E.; Pfenning, L.H.; Moreira, F.M.S. **Writing and editing**: Gudiño–Gomezjurado, M.E.; Leite, R.A.; Carvalho, T.S.; Moreira, F.M.S.

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