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Strategies for Improving Cowpea Grain Yield in the Eastern Amazon: Biological Nitrogen Fixation, Phosphorus Nutrition, and Molybdenum Seed Enrichment

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ABSTRACT
This work aimed to evaluate the use of phosphorus (P) fertilizer, rhizobia inoculation, and seed enrichment with molybdenum (Mo) as tools for enhancing the cowpea yield in the Brazilian eastern Amazon. A set of field and greenhouse experiments were carried out in cerrado and upland forest environments. Four levels of P fertilization, eight rhizobia strains, and the application of mineral nitrogen (N) were evaluated for the cultivar BRS Tumucumaque (BR = Brasil e S = Sementes), and two levels of Mo seed enrichment were assessed for the cultivars BRS Tumucumaque and BRS Guariba. Grain yield increased linearly with the level of P applied. Inoculation with the rhizobia strain BR 3267 increased the shoot and nodule mass of the cultivar BRS Tumucumaque. There was no difference in grain yield and grain N accumulation between inoculation with seven rhizobia strains, N mineral fertilization and the control (without inoculation and N fertilization) in cerrado and upland forest environments. Cowpea grain yield and grain N accumulation were higher in the cerrado than in the upland forest environment and when the seeds were enriched with Mo. Phosphorus fertilization and seed enrichment with Mo are efficient tools to enhance cowpea yield in the Brazilian Amazon.

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Cerrado; latosol; upland forest; *Vigna unguiculata*

Introduction

Common bean (*Phaseolus vulgaris* L.) (dry bean) and cowpea (*Vigna unguiculata* (L.) Walp.) (pulse) are frequently consumed as beans in Brazil. According to the Brazilian Institute of Geography and Statistics, 3.39 million tons of beans were produced from 2.78 million ha in 2019, resulting in an average yield of 1,090 kg ha^{-1}. In the Brazilian Amazon region, the common bean is not as widely grown as the cowpea as it is susceptible to root rot disease (Eke et al. 2020), which is prevalent in the Amazon region due to the high rainfall and temperatures. Furthermore, the common bean is less adaptable to the weathered and acidic soils of the region and requires high investment in fertilization practices. According to the Brazilian Agricultural Research Corporation, the cowpea planted area in Brazil was reduced by 200,229 ha, and production increased by 96,879 tons between 2010 and 2020. This increase in production, with simultaneous area reduction was possible through an increase in productivity, which jumped from 320 to 466 kg ha^{-1} in the same period. The increase in productivity resulted from the adoption of several technologies, such as inoculation with efficient rhizobia strains

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(Silva Júnior et al. 2018), using cowpea cultivars with superior performance (Bastos et al. 2011), and inputs to correct soil acidity and nutrient supply (Borges et al. 2021).

Cowpea crops contribute to food security and are a crucial source of income for family farmers' agroecosystems worldwide. Cowpea crops are an essential biofortified food source, are resilient to pathogens, tolerant to drought (Bastos et al. 2011; Munjonji et al. 2018), adapt to low soil pH (Moura et al. 2012; Oliveira et al. 2017), and can form symbiotic relationships with atmospheric nitrogen-fixing rhizobia (Zilli et al. 2011). Among the strategies that have been adopted to achieve the yield potential of cowpea, biological nitrogen (N_2) fixation (BNF) is a low-cost technique that also reduces the environmental impact of applying nitrogen (N) chemical fertilizers. Direct approaches to improve BNF include the selection and validation of elite rhizobia strains (Marinho et al. 2014; Zilli et al. 2011), the application of higher inoculant levels (Silva Júnior et al. 2014), new inoculant formulations and carrier material evaluation (Fernandes Júnior et al. 2009), and selection of plant genotypes responsive to BNF (Abaidoo et al. 2017). The elite rhizobia strains BR 3262, BR 3267, and BR 3299 have been used for cowpea showing satisfactory results in different edaphoclimatic conditions such as humid forest and cerrado environments in Brazil, Ghana, and Mozambique (Boddey et al. 2017; Borges et al. 2021). Strains that are efficient under different environmental conditions are valuable since the industrial process of inoculant production becomes simpler, increasing the probability of distribution and adoption. Additionally, indirect approaches to improve BNF include co-inoculation with mycorrhizal or phosphate – solubilizing fungus (Gudiño–Gomezjurado et al. 2022; Lino et al. 2022) or *Paenibacillus* (Lima et al. 2011; Rodrigues et al. 2013), that has shown a synergistic effect with rhizobia. The application of mineral N (Bandeira et al. 2019), correction of acidity and aluminum (Al^{3+}) soil content (Farias et al. 2016), and the soil's natural deficiency in phosphorus (P), molybdenum (Mo), and zinc (Zn) (Emmanuel et al. 2021; Kyei-Boahen et al. 2017; Melo et al. 2018) has also been evaluated. The management of edaphic limitations systemically affects plant growth with a positive effect on nodulation and BNF.

Cowpea can form nodules with a wide range of soil rhizobia (Sena et al. 2020). The evolutionarily determined cowpea promiscuity represents an obstacle to the introduction of elite rhizobia strains in the field conditions, since there are different levels of symbiotic efficiency and competitiveness among the soil native rhizobia population. (Mbah et al. 2022). In addition, the native soil rhizobia are distributed deep in the soil, delaying rhizobia-cowpea root contact, and rhizobial density is generally insufficient to allow higher nodulation. This obstacle can be aggravated depending on the available soil N content. When seed inoculation is carried out correctly, the nodule occupancy by inoculated rhizobia is high and inoculation has a significant effect on BNF and grain yield, especially when the soil's native rhizobia density is low. Increasing inoculant density can be used to allow fast nodulation and early initiation of the BNF process (Silva Júnior et al. 2014). On the other hand, when cowpea is cultivated with no inoculation, in soils with elevated inefficient rhizobia populations, or in low fertility soils, the contribution of BNF is erratic and low.

Brazil's most representative soil types are the heavily weathered Latossolo (Oxisol) and Argissolo (Argisol), naturally deficient in Mo and P. Mo, is an essential constituent of the enzymes nitrogenase and nitrate reductase and plays a vital role in plant N metabolism. Legume species have a higher Mo demand when dependent on BNF. Previous studies have demonstrated that Mo application improves BNF, N use efficiency, and grain yield in common beans, soybeans, and peanuts (Almeida, Araujo, and Alves 2013; Campo, Araujo, and Hungria 2009; Chagas et al. 2010; Crusciol et al. 2019; Pacheco et al. 2012). However, the effect of Mo on cowpea BNF is still poorly understood. In the same way, P deficiency negatively affects BNF (Araújo, Plassard, and Drevon 2008) since N_2 fixation and N assimilation are energy-consuming processes that depend on the energy status of the nodules. Legume species possess various strategies for P acquisition, such as more extensive root growth, modified root architecture, symbioses with mycorrhizal fungi, and exudation of phosphatases and organic acids into the rhizosphere. However, these strategies represent an energy cost too.

Cowpea BNF is affected by biotic and abiotic factors and their interactions, making its optimization complex. However, cowpea BNF is vital to reduce the difference between farmers' average productivity and the crop's potential, especially in agroecosystems with low input and resilience like the Amazon. This study hypothesized that P supplementation, inoculation with elite rhizobia strains, and seed enrichment with Mo systemically affect cowpea growth and improve BNF and grain yield. Then, the objective of this work was to evaluate the P fertilization, inoculation with rhizobia, and Mo seed enrichment as strategies to increase the cowpea grain yield under two edaphoclimatic conditions in the Brazilian eastern Amazon.

Materials and methods

Description of experimental areas

Field experiments were carried out in upland forest (Mazagão, AP) and cerrado (Macapá, AP) environments. The experimental areas are owned by Embrapa Amapá, and the soils in the experimental areas are Latossolo Amarelo, according to the Brazilian soil classification system (Santos et al. 2018), i.e., an Oxisol. In the upland forest environment, according to the Köppen-Geiger classification, the climate is of the Am type, with an average annual temperature of 27.3ºC and an average annual rainfall of 2,410 mm. In the cerrado environment, according to the Köppen-Geiger classification, the climate is of the Ami type, with an average annual temperature of 26.3ºC and an average annual rainfall of 2,475 mm. Two well-defined climatic seasons are observed in both areas. The first, between December and July, is characterized as rainy (winter), where 90% of annual precipitation occurs. The second, between August and November, is characterized as dry (summer), where 10% of annual precipitation occurs, associated with high temperature and low relative humidity (Tavares 2014). The seeds were sown in periods of low climate risk, between 20th May and 10th June of each year.

Experimental designs and treatments

To evaluate cowpea response to phosphorus levels a three-crop field experiment was carried out between 2012 and 2014, in an upland forest environment, in the experimental area of Embrapa Amapá in the municipality of Mazagão (0º07'19.2"S and 51º17'57.4" W). A randomized block design was used with three replications. The treatments consisted of four levels of P fertilizer (0, 40, 80, and 120 kg ha^{-1} of P_2O_5 as triple superphosphate), applied at sowing. Each plot contained six 5 m lines, spaced at a distance of 0.5 m, with five seeds of the cultivar BRS Tumucumaque (BR = Brasil e S = Sementes), sown per meter. The soil of the area (pH 4.9, O.M. 19,7 g kg^{-1}; P 2 mg dm^{-3}; K 23 mg dm^{-3}; Ca^{2+} + Mg^{2+} 1.2 cmolc dm^{-3}; Al^{3+} 1.1 cmolc dm^{-3}; H^{+}+ Al^{3+} 5.0 cmolc dm^{-3}; SB 1.3 cmolc dm^{-3}, texture loam, sand 441 g kg^{-1}, silt 318 g kg^{-1} and clay 241 g kg^{-1}, Oxisol) was in fallow. In 2012, sixty days before sowing (DBS), the soil was treated with 2,700 kg ha^{-1} of limestone to increase base saturation to 60%. Every plot received 70 kg ha^{-1} of K_2O as potassium chloride (50% at sowing and 50% at cover 25 days after sowing (DAS)), and 25 kg ha^{-1} of micronutrient fertilizer FTE BR12 (at sowing). The plots were identifiable for the duration of the experiment, and the fertilization process was replicated in 2013 and 2014, with each plot receiving the same treatment as before.

To evaluate cowpea response to phosphorus levels and N sources, a greenhouse experiment was carried out in 2015, at Embrapa Amapá in the Macapá municipality (00°00'46.2"S and 51°05'00.8" W). A randomized block design was used with four replications in a 4 × 3 × 3 factorial design. The treatments consisted of four levels of P fertilizer (0, 40, 80, and 120 kg ha^{-1} of P_2O_5 as triple superphosphate), three N sources (inoculation with strain BR 3267, application of mineral N, control (without inoculation and N application)), and three soils. Two of the soils originated from the cerrado environment (Soil 1 - pH 5.1, O.M. 13.8 g kg^{-1}, P 2 mg dm^{-3}, K 8 mg dm^{-3}, Ca^{2+} + Mg^{2+} .7 cmolc dm^{-3}, Al^{3+} .6 cmolc dm^{3}, H^{+} + Al^{3+} 4.6 cmolc dm^{-3}, SB 0.7 cmolc dm^{-3}, texture loamy sand, sand 833 g kg^{-1}, silt 67 g kg^{-1} and clay 100 g kg^{-1}, Oxisol; Soil 2 - pH 5.1, O.M. 13.5 g kg^{-1}, P 1 mg dm^{-3}, K 12 mg dm^{-3}, Ca^{2+} + Mg^{2+} .6 cmolc dm^{-3}, Al^{3+} .6 cmolc dm^{-3}, H^{+} + Al^{3+} 4.4 cmolc dm^{-3}, SB 0.6 cmolc dm^{-3}, texture sandy loam, sand 753 g kg^{-1}, silt 80 g kg^{-1} and clay

167 g kg^{-1}, Oxisol), and the third from an area of upland forest (Soil 3 - pH 4.6, O.M. 20.5 g kg^{-1}, P 3 mg dm^{-3}, K 12 mg dm^{-3}, Ca^{2+} + Mg^{2+} .6 cmolc dm^{-3}, Al^{3+} 1.4 cmolc dm^{-3}, H^{+}+ Al^{3+} 7.7 cmolc dm^{-3}, SB 0.6 cmolc dm^{-3}, texture sandy clay loam, sand 539 g kg^{-1}, silt 189 g kg^{-1} and clay 272 g kg^{-1}, Oxisol). Limestone was applied to raise the soil pH to 5.8. The liming level for each soil type was determined using a neutralization curve where 0, 500, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 kg ha^{-1} were applied to 0.5 kg of soil in pots, and the soil pH was measured after 90 days (Soil 1 y = 0.81 ln(x) + 0.54, R^2 = 97; Soil 2 y = 0.86 ln(x) + 0.05, R^2 = 98; Soil 3 y = 0.72 ln(x) + 0.34, *R* = 95%). All pots received a level equivalent to 60 kg ha^{-1} of K_2O as potassium chloride and 25 kg ha^{-1} of FTEBR12, which were mixed throughout the soil. Five seeds of the cultivar BRS Tumucumaque were sown per pot, and two plants remained after thinning. At 7 DAS, 0.8 ml of cell culture strain BR 3267 was applied per seedling over the soil in the inoculated pots. Pots with mineral N received 80 kg ha^{-1} of N as urea (50% at sowing and 50% at 25 DAS).

To evaluate cowpea response to N sources a two-environmental conditions field experiment, cerrado and upland forest, was carried out in 2017. A randomized block design with four replications was used, with 10 N sources: seed inoculation with strains BR 3262 (isolated in the southeastern region of Brazil, Atlantic Forest environment); BR 3267, BR 3299, BR 3296 (isolated in northeastern Brazil, semi-arid environment); BR 3351, BR 3315 (isolated in the Amazon region in Manaus); BR 10,654, BR 10,665 (isolated in the Amazon region in Roraima); mineral N fertilization; and control (without inoculation and N application). All strains belonging to the *Bradyrhizobium* genus were obtained from the bacterial collection of Embrapa Agrobiologia. A peat-based inoculant was added at a rate of 250 g 35 kg^{-1} seed with sucrose solution (10% w/v) to increase adherence. The soils showed the following characteristics: pH 5.5, O.M. 14.3 g kg^{-1}, P 7 mg dm^{-3}, K 47 mg dm^{-3}, Ca^{2+} + Mg^{2+} 1.7 cmolc dm^{-3}, Al^{3+} .0 cmolc dm^{-3}, H^{+}+ Al^{3+} 2.0 cmolc dm^{-3}, SB 1.8 cmolc dm^{-3}, texture sandy clay loam, sand 665 g kg^{-1}, silt 104 g kg^{-1} and clay 231 g kg^{-1}, Oxisol (cerrado environment, Macapá); and pH 4.4, O.M. 23.8 g kg^{-1}, P 7 mg dm^{-3}, K 55 mg dm^{-3}, Ca^{2+} + Mg^{2+} .7 cmolc dm^{-3}, Al^{3+} 1.3 cmolc dm^{-3}, H^{+}+ Al^{3+} 6.3 cmolc dm^{-3}, SB 0.8 cmolc dm^{-3}, texture sandy clay, sand 485 g kg^{-1}, silt 139 g kg^{-1} and clay 376 g kg^{-1}, Oxisol (upland forest environment, Mazagão).

To evaluate cowpea response to Mo seed enrichment and N sources, another two-environmental conditions field experiment, cerrado and upland forest, was carried out in 2017. Firstly, seeds enriched with molybdenum via foliar fertilization were produced in 2015 in Embrapa Agrobiologia's experimental field in the municipality of Seropédica, Rio de Janeiro State (22°45'S and 43°40' W; altitude of 26 m). The soil used was characterized as a Argissolo Vermelho-Amarelo according to the Brazilian soil classification system (Santos et al. 2018), i.e., an Typic Hapludult (pH 4.5, P 15 mg dm^{-3}, K 52 mg dm^{-3}, Ca^{2+} + Mg^{2+} 2.2 cmolc dm^{-3}, Al^{3+} .2 cmolc dm^{-3}, SB 2.4 cmolc dm^{-3}, texture sandy clay loam, sand 650 g kg^{-1}, silt 140 g kg^{-1} and clay 210 g kg^{-1}, Typic Hapludult). A randomized block design with five replicates in a 2 × 2 factorial arrangement was used, combining two cowpea cultivars (BRS Tumucumaque and BRS Guariba), and the presence or absence of foliar fertilization with Mo. Each plot contained five 5 m lines spaced at a distance of 0.5 m, with 12 seeds per meter. Each plot received 500 kg ha^{-1} of NPK 4-14-8 at sowing. Mo was applied after flowering of each cultivar, with three foliar sprays at 50, 56, and 68 days after emergence. In each spray, 600 L ha^{-1} of a solution of $(NH_4)6Mo_7O_{24}.4H_2O$ was applied, corresponding to 150 g Mo ha^{-1}. Pods were manually harvested at maturity and threshed. Seed samples were oven-dried at 70 ºC, ground, and analyzed for Mo content using nitro-perchloric digestion and plasma emission spectrometry (ICP-EAS device, Perkin-Elmer) in the Embrapa Solos laboratory, Rio de Janeiro. Seeds from plants that received foliar fertilization had an average Mo concentration of 13.0 and 15.2 μg g^{-1} for cultivars BRS Tumucumaque and BRS Guariba, respectively. The seeds from plants that did not receive foliar fertilization had concentrations below 0.2 μg g^{-1} (the analytical method's detection limit). Then, a randomized block in a 2 × 2 × 3 factorial design with four replications was used to evaluate the effect of seeds enriched with Mo. It combined two cultivars (BRS Tumucumaque and BRS Guariba), seeds enriched or not with Mo, and three N sources (inoculation with strain BR 3267, mineral N fertilization, and control (without inoculation and N application)).

In the field experiments carried out to evaluate cowpea response to inoculation with selected rhizobia strains and enrichment of seeds with molybdenum, the plots contained six 3 m lines at a distance of 0.5 m apart. Every plot received 80 kg ha^{-1} of P_2O_5 as triple superphosphate and 40 kg ha^{-1} of K_2O as potassium chloride at sowing. Plots with N fertilization received 50 kg ha^{-1} of N as urea (30 kg ha^{-1} at sowing and 20 kg ha^{-1} at cover 20 DAS). A peat-based inoculant was added at a rate of 250 g 35 kg^{-1} seed with sucrose solution (10% w/v) to increase adherence.

Harvest, statistical analysis, and access to genetic heritage

In the three-crop field experiment to evaluate cowpea response to phosphorus levels, 25 fully expanded leaves were sampled from each plot 30 DAS, oven-dried at 65°C, then ground and the N concentrations determined using the semi-micro Kjeldahl method. At 60 DAS, pods were harvested from the four central lines of each plot, the pods were threshed, and the grains were weighed to determine grain yield. Analysis of variance (ANOVA) was used to test the cowpea response to phosphorus levels, and when confirming a statistically significant value in the F test ($p \leq .05$), regression analysis was used to evaluate the effect of applied P levels on leaf N concentration, and grain yield. Tukey test, at 5% probability level, was used to evaluate the differences among the crop in the different years.

In the greenhouse experiment to evaluate cowpea response to phosphorus levels and N sources using three soils with different clay contents, plants were harvested at 47 DAS, shoots and root systems were recovered, and nodules detached. Shoot and root nodule were oven-dried at 65°C and weighed. ANOVA followed by regression analysis, or Tukey test were used to evaluate the effect of applied P levels on shoot dry mass (SDM) and nodule dry mass (NDM), and differences among the N sources, respectively.

In the two-environmental conditions field experiments, carried out to evaluate cowpea response to inoculation with selected strains and to evaluate enrichment of seeds with Mo, pods were harvested from the 4 m^2 central area of each plot at 60 DAS, threshed, and the grains weighed to determine grain yield. Grain samples were oven-dried at 65°C and ground, and N concentration was determined using the semi-micro Kjeldahl method. ANOVA followed by the Tukey test was used to evaluate the differences among the N sources, cultivars, seeds enriched with Mo, and environmental conditions. All statistical analysis was conducted using the software Sisvar (Ferreira 2011). The graphs were prepared in the GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, California USA.

The experiments were registered in Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, Brazil's system for management of genetic heritage and associated traditional knowledge, managed by Ministério do Meio Ambiente (Ministry of the Environment), under number A0627F8, as required by Law 13,123/2015 (BRAZIL 2015).

Results

A three-crop field experiment was carried out in 2012, 2013, and 2014 in an upland forest area in the Mazagão municipality, Brazilian eastern Amazon, to determine how cowpea crops respond to P levels in the absence of inoculation and N-mineral fertilization. When no P was applied, grain yield varied between 371 and 612 kg ha^{-1} (Table 1). Grain yield increased significantly in response to increased P levels in 2013 and 2014, but not in the first year, 2012 (Table 1). Although yield at the level of 120 kg ha^{-1} of P_2O_5 was lower than at 80 kg ha^{-1}, a straight-line regression adjusted the response to changing P levels (Table 1). As P levels increased, leaf N concentration reduced, although this relationship (based on the linear adjustment) was only significant in 2013 (Table 2). This is consistent with a dilution effect, whereby enhanced plant biomass, associated with higher P availability, reduces the N concentration in plant tissues. When averaged across the three years, a significant effect of the applied phosphorus levels was observed on grain yield and nitrogen concentration in leaves. When

Table 1. Grain yield (kg ha^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown in a loam oxisol with 241 g kg^{-1} clay, as a function of the increasing levels of P_2O_5 (0, 40, 80, and 120 kg ha^{-1}), in a three-crop field experiment in an upland forest environment.

Levels of P_2O_5 (kg ha^{-1})	Grain yield (kg ha^{-1})			
	2012	2013	2014	Mean
0	425.0	370.8	611.7	469.17
40	658.3	655.8	868.3	727.50
80	979.2	881.3	1027.1	962.50
120	941.7	799.2	915.0	885.25
Regression	$y = \bar{y} = 751.04$	$y = 450.21 + 3.78x$	$y = 695.21 + 2.67x$	$y = 538.61 + 3.71x$
R^2	–	75.63	61.67	77.29
CV (%)	41.32	25.60	12.59	27.50
Mean	751.04 a	676.77 a	855.52 a	761.11

R^2 is the coefficient of determination of the equation, and CV (%) is the ANOVA coefficient of variation. Means followed by different letters differ significantly at the 5% level (Tukey test).

Table 2. Nitrogen (N) concentration in leaves (mg g^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown in a loam oxisol with 241 g kg^{-1} clay, as a function of the increasing levels of P_2O_5 (0, 40, 80, and 120 kg ha^{-1}), in a three-crop field experiment in an upland forest environment.

Levels of P_2O_5 (kg ha^{-1})	Leaf N concentration		
	2012	2013 (mg g^{-1})	Mean
0	40.0	47.2	43.59
40	35.4	42.6	37.29
80	30.8	40.2	35.49
120	33.9	41.4	37.65
Regression	$y = \bar{y} = 3.49$	$y = 44.6–0.04x$	$y = 41.45–0.049x$
R^2	–	38.07	51.74
CV (%)	15.91	54.7	10.62
Mean	3.49 b	4.21 a	38.50

R^2 is the coefficient of determination of the equation, and CV (%) is the ANOVA coefficient of variation. Means followed by different letters differ significantly at the 5% level (Tukey test).

averaged across the applied phosphorus levels, no significant effect of the years was observed on grain yield, but a significant effect on nitrogen concentration in leaves was observed.

As the interactions between P supply, BNF, and cowpea growth remain poorly understood, a greenhouse experiment was carried out to evaluate the effects of P levels and sources of N on the growth and nodulation of cowpea in soils with different clay contents. Shoot dry mass increased in response to P levels and inoculation with strain BR 3267 resulted in higher shoot dry mass than the control (without inoculation or N-mineral application), regardless of soil type (Table 3).

ANOVA did show significant three-way interactions among P levels, soil type and sources of N for cowpea root nodule dry mass. Cowpea nodule dry mass increased linearly in response to increasing applied P levels, regardless of soil type or the N source, with the highest values observed in the 120 kg ha^{-1} of P_2O_5 (Figure 1). Plants inoculated with the strain BR 3267 had more nodulation than control plants in sandy clay loam and sandy loam textures, but not in the soil with the lower clay content. Plants receiving mineral N had the weakest nodulation in all three soils.

A two-environmental conditions field experiment was carried out in 2017, comparing the effect of eight elite rhizobia strains and the application of N-mineral on cowpea grown in cerrado and upland forest environments. When strain BR 10,645 was used, cowpea grain yield was lower than those receiving N-mineral, BR 3262 or BR 3296 strains, and control (with no inoculation and N-mineral application) in the cerrado environment. There were no differences in grain yield among plants treated with other N sources (Figure 2). Regardless of the N source, there were no differences in grain yields in the upland forest. Grain N accumulation varied between 36.75 to 46.91 kg ha^{-1} in upland forest and 48.84 to 81.87 kg ha^{-1} in cerrado environments, and no significant difference was observed in grain

Table 3. Shoot dry mass (g pot^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown as a function of the increasing levels of P_2O_5 (0, 40, 80, and 120 kg ha^{-1}), soil clay content (sandy clay loam with 272 g kg^{-1} of clay, sandy loam with 167 g kg^{-1} of clay, loamy sand with 100 g kg^{-1} of clay), and sources of N (inoculation with strain BR 3267, application of mineral N, and control (without inoculation and N application)), in greenhouse conditions.

Levels of P_2O_5	Sandy clay loam	Sandy loam Shoot dry mass (g pot^{-1})	Loamy sand
0	2.25	1.45	2.55
40	3.49	3.80	5.50
80	5.03	5.74	6.30
120	4.93	5.22	6.91
Regression	y = 2.068 + 0.033x	y = 2.487 + 0.024x	y = 3.232 + 0.035x
R^2	79.45	87.76	85.97
N sources	N-Mineral	Control	Strain BR 3267
Mean	3.92 b	4.33 b	5.03 a

R^2 is the coefficient of determination of the equation. Coefficient of variation was 23.94%. Means followed by different letters differ significantly at the 5% level (Tukey test).

N accumulation among the evaluated N source (Figure 2). On the other hand, cowpea grain yield and N accumulation in grains were higher when cowpea was cultivated in the cerrado, rather than in the upland forest (Figure 3).

Seeds of the cultivars BRS Tumucumaque and BRS Guariba, previously enriched or not with Mo, were sown in cerrado and upland forest environments. In this experiment, seed enrichment with Mo, N sources, and environment showed a significant effect (Figure 4). The cultivar had no significant effect (Figure 4a) or interactions among factors. Cowpea grain yield and N accumulation in grains were higher when cowpea was cultivated in the cerrado, using seeds enriched with Mo, and N-mineral as the N source, compared to the upland forest, using seeds not enriched with Mo, and BR 3267 or without inoculation and N application as N source (Figure 4(b–d)).

Discussion

P fertilization positively affects cowpea shoot and grain yield (Emmanuel et al. 2021; Kyei-Boahen et al. 2017; Melo et al. 2018). The present study observed that cowpea grain yield increased linearly with P levels in the second and third crops and averaged across the three crops. Emmanuel et al. (2021) observed that applying the *Bradyrhizobia* inoculant with P and K fertilizer increased cowpea grain yield by more than 200% in Ghana. Grain yield without P was similar to that achieved in low input systems in the Amazon region (Costa 2012). On the other hand, BRS Tumucumaque grain yield did not differ among the years and grain yield at 80 kg ha^{-1} of P_2O_5 varied between 881 and 1027 kg ha^{-1}, highlighting that cowpea can acquire N when P is supplied and that BRS Tumucumaque is stable in different growing seasons, which is an essential characteristic to farmers. The BNF process is energetically costly for the plant, and traits associated with BNF are more responsive to increased P supply than the host plant growth (Abaidoo et al. 2017; Araújo, Plassard, and Drevon 2008; Jemo et al. 2017). This was confirmed by cowpea's intense root nodulation response to increased P levels in the greenhouse trial. N concentration in leaves was evaluated to understand its relationship with cowpea grain yield and its usefulness as a management tool, since it is expensive to collect nodules in the field. It was observed that N concentration in leaves is useful, since it was inversely related to cowpea grain yield.

Isolation, selection, and validation of new rhizobia strains are promising strategies for improving cowpea BNF, shoot, and grain yield (Marinho et al. 2014; Mbah et al. 2022). In this study, it was observed that cowpea grain yield did not differ between the inoculated and control treatments in both strain selection and Mo seed enriched field trials, except for the BR 10,654 strain in the cerrado environment. On the other hand, shoot and root nodule dry mass were improved in the greenhouse trial with BR 3267 inoculation. Several previous studies have demonstrated a positive effect of

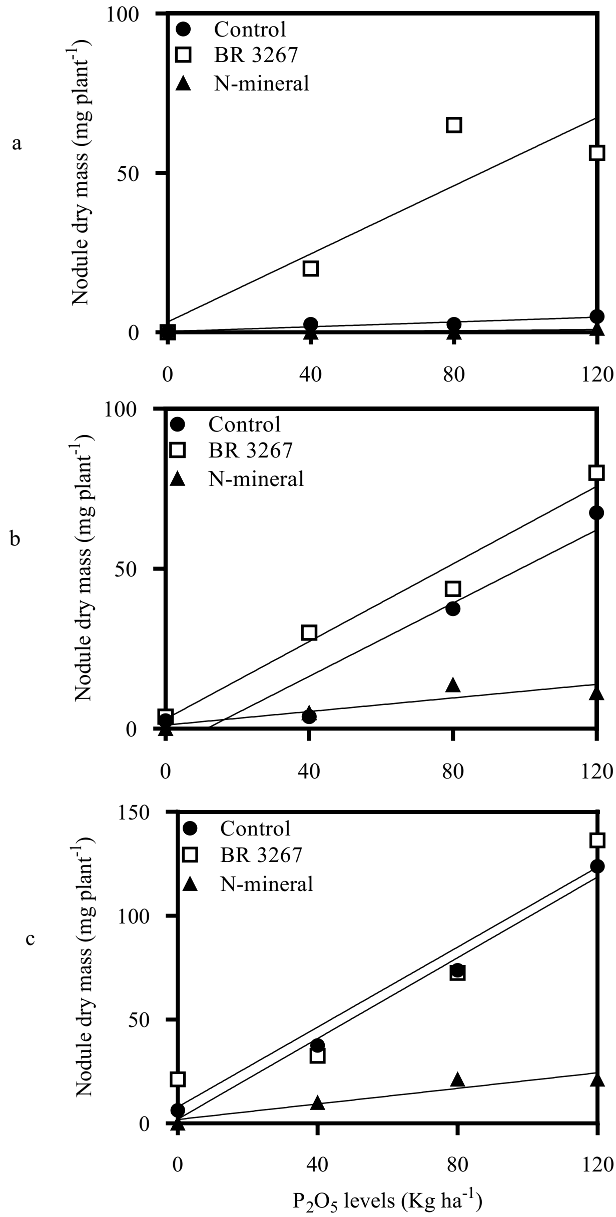

Figure 1. Root nodule dry mass (mg plant^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown as a function of the increasing levels of P_2O_5 (0, 40, 80, and 120 kg ha^{-1}), soil clay content (sandy clay loam with 272 g kg^{-1} clay (a), sandy loam with 167 g kg^{-1} clay (b), loamy sand with 100 g kg^{-1} clay (c)), and sources of N (inoculation with strain BR 3267, application of mineral N, and control (without inoculation and N application)), in greenhouse conditions. (a) sandy clay loam: control $y = 0.0375\times + 0.25$, $R^2 = 90\%$; BR 3267 $y = 0.5344 \times + 3.25$, $R^2 = 82$; N-mineral $y = 0.0094\times - 0.25$, $R^2 = 60$. (b) sandy loam: control $y = 0.5719 \times - 6.5$, $R^2 = 91$; BR 3267 $y = 0.6063 \times + 3$, $R^2 = 97$; N-mineral $y = 0.1063 \times + 1.125$, $R^2 = 78$. (c) loamy sand: control $y = 0.9719 \times + 2$, $R^2 = 99$; BR 3267 $y = 0.9625\times + 7.875$, $R^2 = 91$; N-mineral $y = 0.1875\times + 1.875$, $R^2 = 90$. R^2 is the coefficient of determination of the equation. Coefficient of variation was 59.61%.

inoculation with selected strains on cowpea grain yield. Boddey et al. (2017) observed that the strains BR 3262 and BR 3267 perform well under different edaphoclimatic conditions in Africa. The strain BR 3262 performed better than BR 3299, INPA 03-11B, UFLA 03–84, and a mixed inoculant of the five strains in terms of increasing N derived from biological fixation, N accumulation, and grain yield in

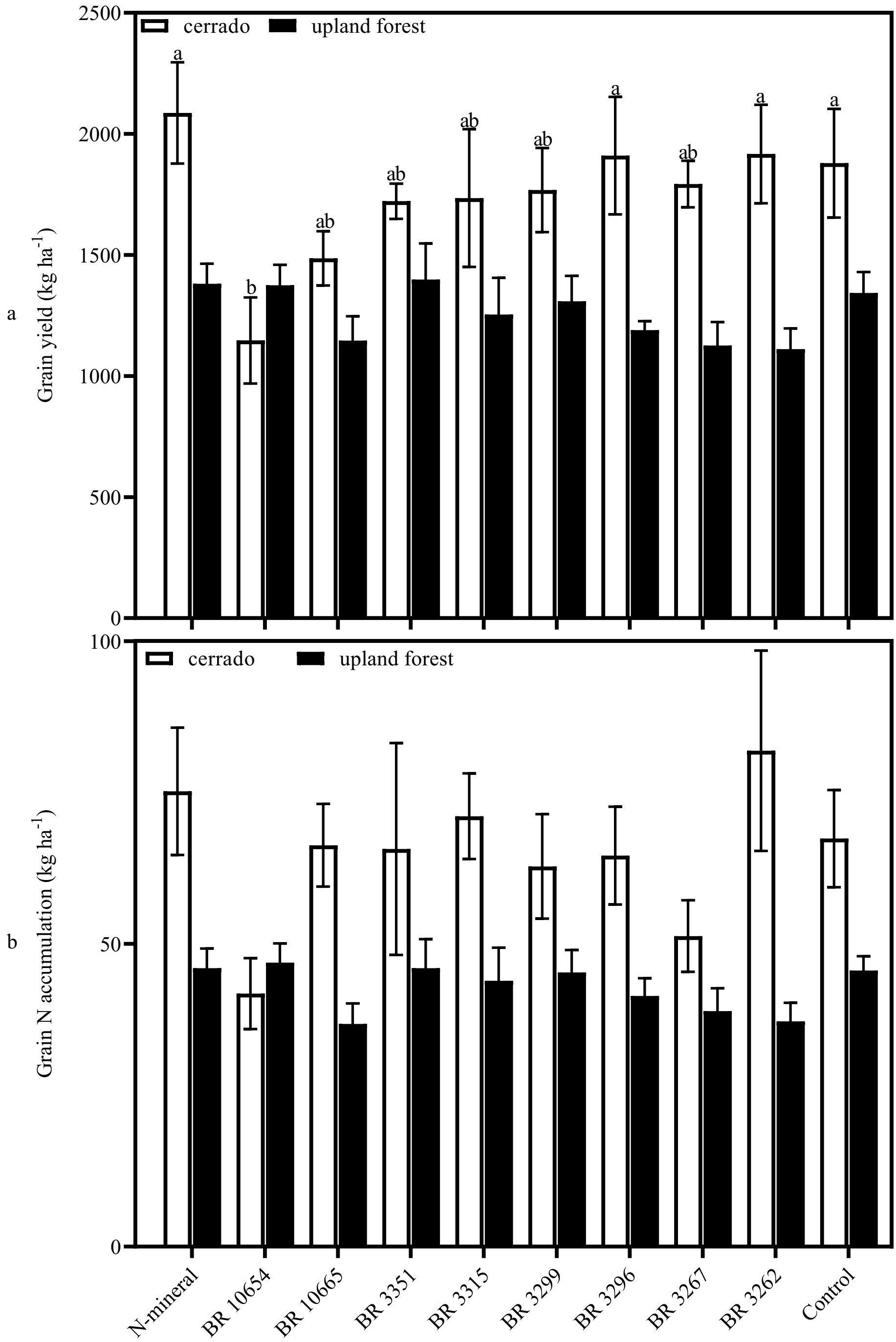

Figure 2. Grain yield (kg ha^{-1}) (a) and accumulation of N in grains (b) (kg ha^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown as a function of the sources of N (inoculation, application of mineral N, and control (without inoculation and N application)), and contrasting environmental conditions (upland forest in a sandy clay oxisol with 376 g kg^{-1} clay, and cerrado in a sandy clay loam oxisol with 231 g kg^{-1} clay). Coefficient of variation 19.07 and 27.62 for grain yield and accumulation of N in grains, respectively. Means followed by different letters within a column differ significantly at the 5% level (tukey test).

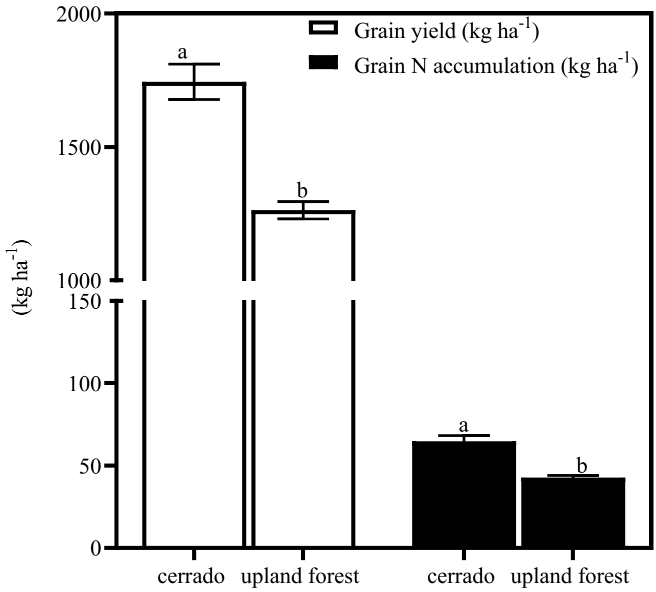

Figure 3. Grain yield (kg ha^{-1}) and accumulation of N in grains (kg ha^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque cultivar grown in contrasting environmental conditions (upland forest in a sandy clay oxisol with 376 g kg^{-1} clay, and cerrado in a sandy clay loam oxisol with 231 g kg^{-1} clay) using different nitrogen sources. Coefficient of variation 19.07 and 27.62 for grain yield and accumulation of N in grains, respectively. Means followed by different letters within a column differ significantly at the 5% level (tukey test).

the cultivar BRS Guariba in a cerrado environment in the State of Mato Grosso (Silva Júnior et al. 2018). BR 3262 inoculation promoted grain yield of cultivars BRS Pujante and BRS Carijó and BR 3267 promoted grain yield of cultivar BRS Acauã more than the other strains evaluated (Marinho et al. 2014). There was no difference in grain yield for these three cultivars between inoculation and 80 kg N ha^{-1} (Marinho et al. 2014). Therefore, efforts to improve cowpea BNF in the Amapá edaphoclimatic conditions can promote significant increases in cowpea grain yield and should continue. In Amapá, 90% of annual precipitation occurs between December and July, and the sowing of cowpea is carried out from the second half of May. It is reasonable to infer that rainfall before sowing should allow native rhizobia population growth, promoting high nodulation in the control treatment and obstacles to introducing selected strains.

Mo is highly relevant in the N metabolism of legume crops relying on BNF. In the present study, it was observed that sowing cowpea seeds enriched with Mo improved grain yield and grain N accumulation, regardless of cultivar, N source, and environmental condition. Recently, Barbosa et al. (2021) observed that cowpea seed Mo content did not affect cowpea yield when urea was the N source. On the other hand, sowing seeds enriched with Mo improved grain yield by 42% and 70%, in 2017 and 2018, respectively, when BR 3262 strain or non-inoculation were used as N sources in sub-humid tropical regions of Brazil. Common bean plants originating from seeds enriched with Mo had increased nodule dry mass and nitrogenase activity, increased nitrate reductase activity (Almeida, Araujo, and Alves 2013) and improved grain yield and N accumulation in grains (Pacheco et al. 2012). Increased grain yield has also been reported for soybeans grown from seeds enriched with Mo (Campo, Araujo, and Hungria 2009) and for peanuts that received a foliar application of Mo (Crusciol et al. 2019). Therefore, enriching seeds with Mo appears to be a valuable tool for improving cowpea performance, especially under low input conditions. When the supplies of P and Mo are adequate, N availability may limit grain yield. Thus, the selection of efficient strains and co-inoculation with other growth-promoting microorganisms can cumulatively act to increase cowpea BNF and yield.

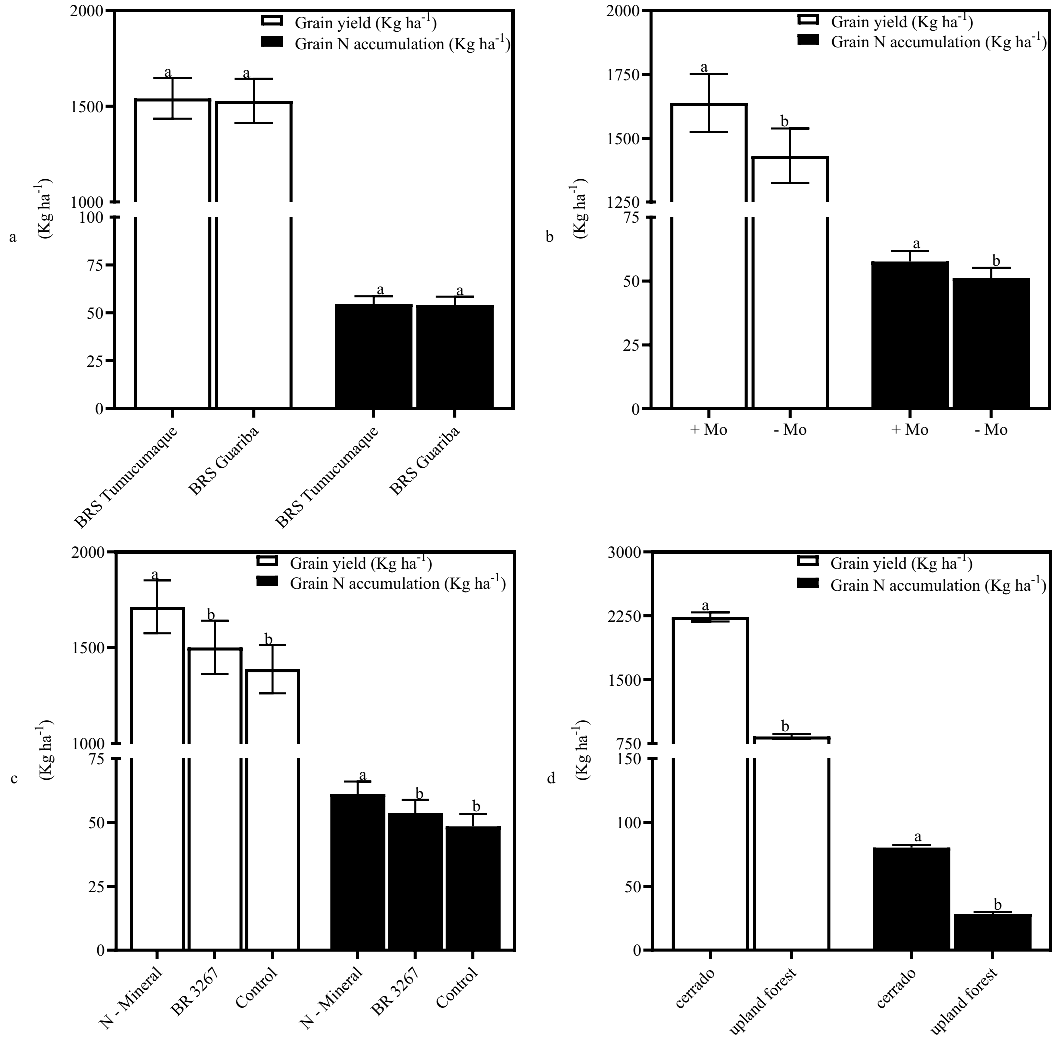

Figure 4. Grain yield (kg ha^{-1}) and accumulation of N in grains (kg ha^{-1}) of cowpea (Vigna unguiculata) BRS tumucumaque and BRS guariba cultivars (a) grown as a function of the seeds enriched or not with Mo (+Mo and − Mo, respectively); (b) sources of N (inoculation with strain BR 3267, application of mineral N, and control without inoculation and N application); (c) contrasting environment conditions (upland forest in a sandy clay oxisol with 376 g kg^{-1} clay, and cerrado in a sandy clay loam oxisol with 231 g kg^{-1} clay); (d) coefficient of variation 14.74 and 17.16 for grain yield and accumulation of N in grains, respectively. Means followed by different letters within a column differ significantly at the 5% level (tukey test).

Weathered clayey soils adsorb most of the P applied as fertilizer, reducing its availability to plants and consequently affecting cowpea BNF, growth and yield (Abaidoo et al. 2017; Kyei-Boahen et al. 2017). In the present study, considering the data from the experiment of N sources and from the experiment of N sources, cultivars, and seed Mo contents the cowpea grain yield was 38% and 168% higher in the cerrado than in the upland forest environment, respectively. This study found that soil from the forest environment had higher levels of clay, Al^{+3}, and organic matter than the cerrado soils. Furthermore, based on the neutralization curves generated during the greenhouse experiment, soil from the forest environment was more resistant to changes in pH (buffer power) than soils from the cerrado. These results are significant for Amazon. Since the productive potential of cerrado areas in the Amazon biome is significantly higher than that of forest areas, this should be considered when vegetation suppression is necessary for expanding cultivated areas.

The use of seeds enriched with Mo, P application and rhizobia inoculation systematically affect the cowpea metabolism. In this context, further studies by nuclear magnetic resonance and mass spectrometry can allow the metabolomics and proteomic mapping and possible paths to manipulate cowpea to optimize BNF. Finally, cowpea is grown under very different conditions in Brazil, and the symbiotic promiscuity of this species requires combined strategies for improving the contribution of BNF. In the eastern Brazilian Amazon, cowpea is cultivated predominantly by family farmers that still use itinerant agriculture practices, using fire to prepare the sowing area. In the present study, when cowpea was grown in the same plot for three consecutive years, providing 40 kg ha^{-1} of P_2O_5 increased grain yield by 233, 285, and 257 kg ha^{-1} in the first, second, and third years, respectively, compared to no P fertilization. This process of intensifying land use by building-up soil fertility may replace itinerant subsistence crops and may also help to reduce gas emissions from biomass burning. Sowing seeds enriched with Mo via foliar fertilization is a low-cost technique that increases cowpea yields in upland forest and cerrado environments. Many bean farmers in Brazil use grains from previous crops as seeds. The production of the cowpea seeds enriched with Mo would require specific cultivation areas and structures for seed storage. Alternatively, these seeds could be produced by associations, cooperatives, or public institutions for subsequent supply to farmers.

Conclusions

This work improves our understanding of the relationship between phosphorus supply, seed enrichment with molybdenum, and nitrogen acquisition by cowpea plants. We have demonstrated that: (i) shoot and grain yield of the cowpea cultivar BRS Tumucumaque responds linearly to increased phosphorus fertilization; (ii) rhizobia inoculation improves growth and nodulation of cowpea cultivar BRS Tumucumaque in greenhouse conditions, without affecting grain yield in the field; (iii) sowing seeds enriched with molybdenum increases grain yield and nitrogen accumulation in grains of cowpea cultivars BRS Tumucumaque and BRS Guariba, regardless of the nitrogen source, in cerrado and upland forest environments of the eastern Amazon, and (iv) grain yield and nitrogen accumulation in grains are higher when cowpea is cultivated in a cerrado environment, regardless of the cultivar, N source, and seed Mo content.

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Disclosure statement

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