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Increase in yield, leaf nutrient, and profitability of soybean co-inoculated with Bacillus strains and Arbuscular mycorrhizal fungi

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ABSTRACT: Microorganisms in the soil and rhizosphere can release part of the total phosphorus in the soil through solubilization, mineralization, and an increase of the root absorption surface. The ability of phosphate solubilizing bacteria and mycorrhizal fungi to promote higher yield and profitability in co-inoculated soybean was investigated. For this purpose, field and greenhouse experiments were conducted in the years 2020 and 2021 in Brazil. In the field, the first factor was composed of microorganism application on soybean (simple inoculation with *Bradyrhizobium;* co-inoculation with *Bacillus* strains; co-inoculation with arbuscular mycorrhiza), and the second factor consisted of the application or not of phosphate fertilizer (0 and 100 kg ha^{-1} of P_2O_5). In the greenhouse, treatments of the first factor were maintained with 50 % of the phosphate fertilization and one treatment added (standard inoculation with 100 % of the fertilization). Plant growth, roots, nodules, leaf nutrition, yield, and profitability were evaluated. In 2020, co-inoculation increased plant height, the number of pods, grains, and profitability index. The co-inoculation with *Bacillus* strains and arbuscular mycorrhiza promoted yield increase only associated with phosphate fertilization, by 813 and 761 kg ha^{-1} compared to standard inoculation, respectively. In 2021, there were increases for pods, grains, yield, gross profit, net income, and profitability index. Co-inoculation with *Bacillus* strains and arbuscular mycorrhiza promoted increased soybean yield and profitability, confirming itself as a sustainable technology for Brazilian soybean fields.

Keywords: *Glycine max*, *Bacillus megaterium*, *Bacillus subtilis*, *Rhizophagus intraradices.*

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INTRODUCTION

Soybean (*Glycine max* [L.] Merril) is a crop of global importance and one of the most widely cultivated worldwide, with dual suitability as a source of protein and oil, used for human and animal consumption and industrial purposes (Singh, 2010). The 2019/2020 world soybean crop occupied a planted area of 122.647 million hectares, with global production of 337.298 megatonnes (USDA, 2020).

Brazil is the world's largest producer of this oilseed, with a planted area of 39.1 million hectares in 2021 and 138.1 megatonnes harvested in the same year (Conab, 2022). Part of the success of Brazilian soybeans is due to the economy of not using nitrogen fertilizers (Döbereiner, 1997; Hungria et al., 2006). The nitrogen needs of soybean are met by symbiosis with elite strains of *Bradyrhizobium*, applied through seed inoculation during sowing (Rodrigues et al., 2020).

Despite the cost reduction by the association soybean-*Bradyrhizobium*, soybean production systems of high technological level are highly dependent on chemical inputs, such as insecticides, fungicides, herbicides, and mineral fertilizers that increase production costs and cause greater ecological impact when used in excess (Sousa et al., 2020). Among mineral fertilizers, the need for phosphate fertilizers is one of the most common in Brazilian tropical soils (Withers et al., 2018). As most of these soils are highly fixative of phosphorus (P), high applications of phosphate fertilizers to the soil, beyond that required for crop supply, are required to overcome the rapid immobilization of inorganic-P that occurs in highly weathered soils (Roy et al., 2016; Withers et al., 2018). The P present in these soils is only partially available to plants due to the high adsorption of this element and the formation of Iron/Aluminum phosphates in acid soils (Leite et al., 2017; Ribeiro et al., 2018).

Besides fertilization, the only possible way to increase P-available for a plant is microbial solubilization and mineralization of P (Alori et al., 2017). In nature, a great diversity of microorganisms in the soil and rhizosphere, called Phosphorus Solubilizing Microorganisms (PSM), can release part of the total P from the soil through solubilization and mineralization (Bhattacharyya and Jha, 2012; Alori et al., 2017). Arbuscular mycorrhizal fungi collect and transfer water and minerals such as phosphate to their host (Marquer et al., 2019).

The main mechanisms of P solubilization employed by PSM are a release of chelating or dissolving mineral compounds (organic acid anions, siderophores, protons, hydroxyl ions, and CO_2); release of extracellular enzymes (non-specific acid phosphatases, phytases, phosphonatases, and C-P lyases) and release of P during substrate degradation (exopolysaccharides) (Yi et al., 2008; Sharma et al., 2013). Moreover, microorganisms in the presence of labile carbon, immobilize P quickly and, later, after their death, make it available to plants (Sharma et al., 2013).

Studies have shown the ability of benefic microorganisms to promote plant development, specifically *Pseudomonas* in wheat and soybean (Afzal et al., 2010; Karimzadeh et al., 2020; Shirmohammadi et al., 2020), *Bacillus* strains on millet, corn and oil palm (Ribeiro et al., 2018; Lima et al., 2020, Sousa et al., 2020), *Trichoderma* in soybean (Bononi et al., 2020), arbuscular mycorrhiza in rice, corn and tomato (Zhang et al., 2016; Saia et al., 2020), among others. Also, these microorganisms, in most cases, are of the "multifunctional" type and can bring benefits to plants through multiple mechanisms of direct and indirect stimulation (Sobral et al., 2018; Silva et al., 2020), with the capacity to fix atmospheric nitrogen (Döbereiner, 1997), potassium solubilization (Basak et al., 2022), siderophore production (Yadav et al., 2020) and mitigation of biotic and abiotic stresses (Leite et al., 2019; Wang et al., 2020; Oliveira et al., 2021).

In this perspective, several studies have sought to associate soybean inoculation with other beneficial microorganisms through co-inoculation (Hungria et al., 2013;

Bononi et al., 2020; Rondina et al., 2020). Co-inoculation involves the addition of more than one beneficial microorganism, each of which acts in different microbial processes to maximize plant development (Filipini et al., 2020). Therefore, we hypothesized that the adoption of soybean co-inoculation with beneficial microorganisms can improve crop yields and increase profitability. So, the objective was to evaluate the co-inoculation of soybean with *Bacillus* strains and arbuscular mycorrhiza with two levels of phosphate fertilization.

MATERIALS AND METHODS

For the investigation of this study, field and greenhouse experiments were conducted during the 2020 and 2021 agricultural years in the municipalities of Paragominas and Belém, both in Pará State.

Field experiment

Study site and experimental design

Experiments were developed under field conditions during 2020 and 2021, on a farm in the municipality of Paragominas, Pará State, Brazil (Figure 1). The region is classified as an Amazon biome, with climate “Aw” with a transition to “Am”, according to the Köppen International Classification system (Alvares et al., 2013). The soil of the experimental area has a very clayey texture (Table 1), being classified as a *Latossolo Vermelho* (Santos et al., 2018), which corresponds to an Oxisol (Soil Survey Staff, 2014).

The experimental design was in randomized blocks in a 3 × 2 factorial scheme, with four repetitions. The first factor was composed of microorganism application: simple inoculation with *Bradyrhizobium* (standard inoculation); co-inoculation with *Bacillus* strains (*B. subitilis* and *B. megaterium*); co-inoculation with arbuscular mycorrhiza (*Rhizophagus intraradices*). The second factor was composed of the application or not of phosphate fertilizer (0 and 100 kg ha^{-1} of P_2O_5, with MAP), applied at the time of planting. All treatments received inoculation with *Bradyrhizobium* [strains SEMIA 5019 (*Bradyrhizobium elkanii*) and SEMIA 5079 (*Bradyrhizobium japonicum*)] strains at a concentration of 5×10^9 colony forming units (CFUs) mL^{-1}, applied at a dose of 300 mL diluted in 70 L ha^{-1} of water.

Bacillus strains isolated from the rhizosphere and leaf endosphere of tropical corn genotypes, composed of elite strains from Embrapa Corn and Sorghum, CNPMS B2084

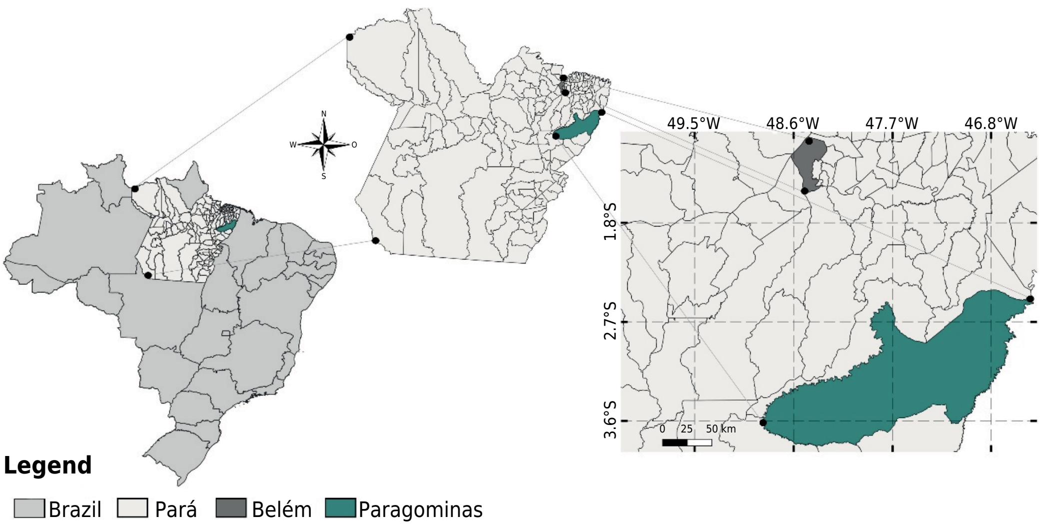

Figure 1. Geographic location of the experimental fields (Paragominas-PA) and greenhouse (Belém-PA).

of *Bacillus subitilis* and CNPMS B119 of *Bacillus megaterium* [molecular identification described in Sousa et al. (2020)] were applied at a concentration of 4 × 10^9 CFU mL^{-1} at a dose of 100 mL ha^{-1} [commercial register No. PR 000497-9.000045 in the Brazilian Ministry of Agriculture (MAPA)]. The inoculant with arbuscular mycorrhiza (commercial product under registration no. 2290210000-0 in MAPA) and composed of propagules of *Rhizophagus intraradices* was applied at a concentration of 20,800 propagules g^{-1} and a dose of 120 g ha^{-1}. All treatments were applied in the furrow at a dose of 70 L ha^{-1} of solution with rhizobia strains.

The sowing occurred on January 20, 2020, for the first year, and on January 30, 2021, for the second year. The experimental plot consisted of 12 planting lines (spaced at 0.5 m) with a length of 25 m. The experiment was grown with soybean hybrid M8644IPRO at a sowing rate of 11 seeds m^{-1}, performed with a Stara model seeding machine (Victoria 4050) with six lines, speed of 6 km h^{-1}, with inoculation, co-inoculation, and phosphate fertilization via furrow. Potassium fertilization (150 kg ha^{-1} of K_2O) was carried out before planting via Potassium Chloride fertilizer. Cultural practices were carried out according to the recommendations for soybean cultivation, and applications of insecticides, herbicides, and fungicides registered for pest and disease control were made when necessary (Seixas et al., 2020). Harvesting was performed on May 11, 2020, and May 24, 2021, respectively, for the first and second years, by harvesting the central rows in each plot. During the experimental period, temperature and precipitation data were collected with a mobile station (Figure 2).

Table 1. Chemical and physical properties of the soil (layer 0.00-020 m) of fields experiment

Soil property	2020	2021	Soil property	2020	2021
			Fields		
pH($CaCl_2$)	5.80	5.60	Manganese (mg kg^{-1})	5.20	7.10
Organic matter (dag kg^{-1})	3.26	3.96	Zinc (mg kg^{-1})	1.20	0.70
Phosphorus (mg kg^{-1})	21.40	10.00	Exchangeable aluminum ($cmol_c$ kg^{-1})	0.00	0.00
Potassium (mg kg^{-1})	98.30	236.50	Potential acidity (H+Al) ($cmol_c$ kg^{-1})	2.10	2.90
Calcium ($cmol_c$ kg^{-1})	4.42	4.85	Sum of bases (Ca+Mg+K) ($cmol_c$/kg)	6.30	7.60
Magnesium ($cmol_c$ kg^{-1})	1.63	2.10	Cation Exchange Capacity ($cmol_c$/kg)	8.40	10.50
Sulfur (mg kg^{-1})	7.39	13.00	Base saturation (V%)	75.00	72.00
Boron (mg kg^{-1})	0.45	0.43	Sand (g kg^{-1})	105	123
Copper (mg kg^{-1})	0.70	0.20	Silt (g kg^{-1})	50	232
Iron (mg kg^{-1})	36.00	41.00	Clay (g kg^{-1})	845	645
			Greenhouse		
pH($CaCl_2$)		5.60	Manganese (mg kg^{-1})		19.50
Organic matter (dag kg^{-1})		3.07	Zinc (mg kg^{-1})		0.80
Phosphorus (mg kg^{-1})		10.30	Exchangeable aluminum ($cmol_c$ kg^{-1})		0.00
Potassium (mg kg^{-1})		118.01	Potential acidity (H+Al) ($cmol_c$ kg^{-1})		3.30
Calcium ($cmol_c$ kg^{-1})		4.58	Sum of bases (Ca+Mg+K) ($cmol_c$ kg^{-1})		6.30
Magnesium ($cmol_c$ kg^{-1})		1.45	Cation Exchange Capacity ($cmol_c$ kg^{-1})		9.60
Sulfur (mg kg^{-1})		25.00	Base saturation (V%)		65.90
Boron (mg kg^{-1})		0.27	Sand (g kg^{-1})		110
Copper (mg kg^{-1})		0.20	Silt (g kg^{-1})		208
Iron (mg kg^{-1})		73.00	Clay (g kg^{-1})		682

K, Cu, Fe, Mn and Zn: extraction with Mehlich-1; P available in resins; Exchangeable Ca, Mg and Al: extraction with KCl 1 mol L^{-1}; H + Al: extraction with calcium acetate; S: extractor $Ca(H_2PO_4)_2$ 0.01 mol L^{-1}; B: extractor $BaCl_2$ 0.125 % hot water.

Production and growth

Plant height was measured with a graduated ruler from the ground to the top of the plant. The height of insertion of the first pod was measured from the ground to the insertion of the first pod. The number of pods, grains, and branches is made by manual counting on all plants in one linear meter. For 1000-grains weight, 100 grains were counted manually and weighed on an analytical balance (humidity set at 13 %), and then extrapolated to 1000 grains. Yield consisted of manual harvesting and mechanical threshing of all plants in 5 m^2 in the center of the plot, followed by weighing on balance (humidity set at 13 %) and the values converted into kg ha^{-1}.

Foliar nutrients

The 3rd leaf with petiole was collected from 25 plants in each plot during full flowering [phenological stage R2 (Fehr and Caviness, 1977)]. Samples were identified, cleaned, and placed in paper bags and stored in Drying Incubator with Air Circulation (55 °C) until constant weight. Dried samples were ground in a stainless-steel knife mill (Willey type, 20 mesh sieve) and stored. The contents of macronutrients (N, P, K, Ca, Mg and S) and micronutrients (B, Cu, Fe, Mn, Zn, and Mo) were evaluated according to the Manual of Chemical Analysis of Plants (Silva, 2009). The N content by the sulfur digestion method and determination in a Kjeldahl distiller. Phosphorus, K, Ca, Mg, S, Cu, Fe, Mn, Mo, and Zn by nitroperchloric digestion and determination in ICP-AES (Inductively coupled plasma atomic emission spectroscopy). Boron content was determined by dry digestion and determination on ICP-AES.

Greenhouse

An experiment was conducted under greenhouse conditions at the Federal Rural University of the Amazon, Belém-PA, Brazil (Figure 1), to evaluate soybean co-inoculated biomass, nodulation, and roots. Soybean plants (cv. M8644IPRO) were grown in pots (6 dm^{-3}) filled with a very clayey Oxisol collected from an adjacent area where the field experiments were conducted (Table 1).

The experimental was a completely randomized design, with four treatments and four repetitions. The treatments were: simple inoculation with *Bradyrhizobium* (standard inoculation) + 100 % of fertilization (I100); standard inoculation + 50 % of fertilization (I50); co-inoculation with *Bacillus* strains + 50 % of the fertilizer (BS): co-inoculation

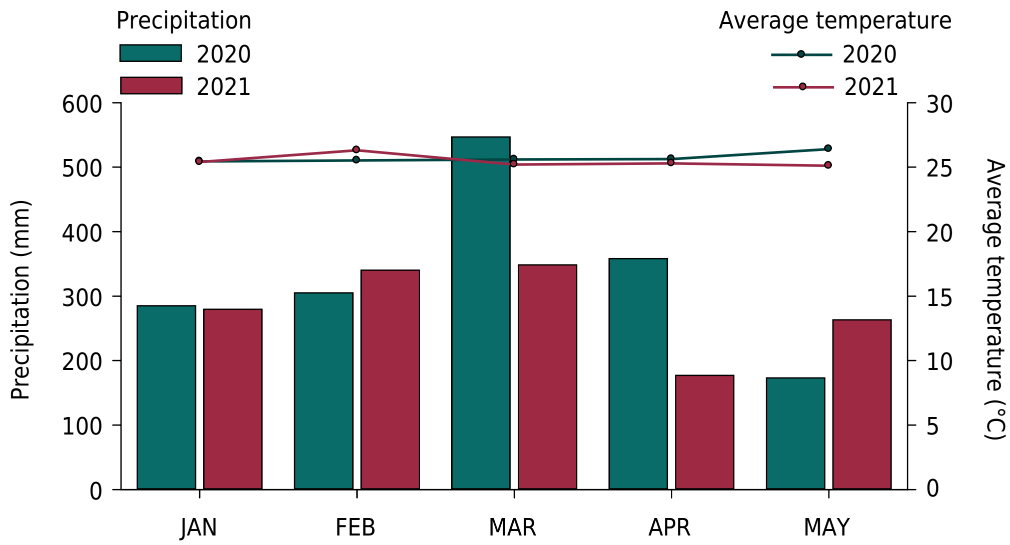

Figure 2. Average precipitation and temperature data of the experimental area during 2020 and 2021.

with arbuscular mycorrhiza + 50 % of the fertilization (AM). The concentrations of microorganisms are similar to those described in the field experiment.

On May 27, 2021, sowing occurred with three seeds per pot, thinning to one plant per pot after emergence. Prior to sowing, the soil of the pots was fertilized at a dose of 100 kg ha^{-1} of P_2O_5 (100 % fertilization); 30 kg ha^{-1} of K_2O and 30 kg ha^{-1} of micronutrient source (Molybdenum - 0.1 %; Boron - 1.8 %; Copper - 0.8 %; Manganese – 2 %; Zinc – 7 %). The values expressed in kg ha^{-1} were calculated for the volume of soil in the pots, considering soil density of 1 Mg m^{-3}. During the experimental period, the soil was maintained at 45 % field capacity.

The evaluation of the plants occurred 25 days after sowing, during the phenological stage V4 (Fehr and Caviness, 1977). On the third fully expanded leaflet, counted from the apex, three measurements were taken to determine the chlorophyll index, with chlorophyll meter SPAD 502 Plus (Konica Minolta, Japan). At the end of the experiment, plants were carefully collected from the pots and separated into shoots and roots. Roots were washed and separated from root nodules. Root volume was determined through the displacement of water in a graduated cylinder after immersion (Rondina et al., 2020). Root nodules were counted manually. Shoot, roots, and nodules were packed in paper bags and stored in a drying incubator with air circulation (55 °C) until reaching a constant weight.

Economic analysis

For the economic analysis of the use of soybean co-inoculation technology, the production cost methodology proposed by “National Supply Company” was used (Conab, 2010). The data referring to variable costs were obtained in the property where the field experiments were conducted, while the values of depreciation (machinery, implements, and rural buildings), other fixed costs (maintenance, social charges, and insurance fixed asset), and factor income (expected return on fixed capital and land use) were used according to Conab estimates for the crop at a high technological level in the years 2020 and 2021.

To determine the variable costs, defrayal costs were used (operation with machinery, labor, administrator, seeds, planting fertilizers, foliar fertilizers, inoculants, adjuvants), pesticides (herbicides, insecticides, and fungicides), financial and other expenses (external transport, administrative costs, storage and special contribution for rural social security). For fixed costs, we have determined depreciations (machinery and rural buildings) and other fixed costs. The operating cost was composed of the sum of fixed and variable costs. Total cost is composed of operating cost, plus factor income.

The financial indicators gross profit (GP) (yield in 60 kg bags multiplied by average selling price), operating profit (equation 1), net income (equation 2), and profitability index (equation 3), were determined according to Martin et al. (1998).

$$OP = GP - OC \quad \text{Eq. 1}$$

$$NI = GP - TC \quad \text{Eq. 2}$$

$$PI = \left(\frac{OP}{GP}\right) \times 100 \quad \text{Eq. 3}$$

in which: OP is the operating profit; OC is the operational cost; NI is the net income; TC is the total cost; and PI is the profitability index.

The average price of the soybean bag (60 kg) was established according to Cepea (2020, 2021) at the harvest date of the experiments. The amounts were converted from reais (Brazilian currency) to dollars according to the exchange rate on the harvest date.

Statistical analysis

Initially, data were analyzed for normality (Shapiro-Wilk) and homoscedasticity (Bartlett's test). Subsequently, they were submitted to variance analysis by the F test and, when significant, to the Tukey test ($p \leq 0.05$) for field experiments and Duncan test ($p \leq 0.05$) for greenhouse experiment. Statistical tests were performed using the RStudio® language. The graphs were plotted using the SigmaPlot® version 10 program and the geographic location map was made using the QGIS Desktop® program.

RESULTS

Soybean growth and yield in the field

In 2020, there was an interaction ($p<0.05$) between the factors (co-inoculation × phosphate fertilization) for plant height, first pod insertion, 1000-grains weight, and grain yield (Figure 3). The number of pods and grains differed ($p<0.05$) between treatments but without interaction between the factors.

Plants inoculated only with *Bradyrhizobium* (standard inoculation) and without application of phosphate fertilizer showed greater height (55 cm) compared to co-inoculation with arbuscular mycorrhiza, but significantly similar to plants co-inoculated with *Bacillus* strains (51 cm) (Figure 3a). First pod insertion was higher for standard inoculation compared to the other treatments (Figure 3b).

Co-inoculation with arbuscular mycorrhiza showed a higher ($p<0.05$) number of pods (88 pods) compared to standard inoculation (79 pods), but significantly similar to co-inoculation with *Bacillus* strains (82 pods) (Figure 3c). Co-inoculation with arbuscular mycorrhiza provided an increase of 11.1 and 10.8 % in the number of grains per plant, compared to standard inoculation and co-inoculation with *Bacillus* strains, respectively, regardless of phosphate fertilization (Figure 3d).

With fertilizer supply, co-inoculation promoted higher grain yields, with increases of 813 and 761 kg ha^{-1} compared to standard inoculation, respectively for co-inoculation with *Bacillus* strains and arbuscular mycorrhiza (Figure 3h).

In 2021, there was an interaction ($p<0.05$) between the factors for plant height and reproductive branches (Figure 4). The first pod insertion, number of pods, grains, grains per pod, 1000-grains weight, and grain yield differed among treatments, but without interaction between the factors.

Plants co-inoculated with arbuscular mycorrhiza had a higher number of pods (74 pods per plant) compared to standard inoculation (62 pods per plant), but similar to co-inoculation with *Bacillus* strains (73 pods per plant) (Figure 4c). For the number of grains, the co-inoculation with *Bacillus* strains was significantly higher than standard inoculation (14.8 %). Co-inoculation with mycorrhiza showed an intermediate value, not differing from the other treatments (Figure 4d).

The highest grain yield was found in plants that received co-inoculation with *Bacillus* strains (4787 kg ha^{-1}) and with arbuscular mycorrhiza (5013 kg ha^{-1}) compared to standard inoculation (4379 kg ha^{-1}), regardless of whether or not phosphate fertilization was provided (Figure 4h).

Foliar content of macro and micronutrients in the field

In 2020, leaf nutrient content differed significantly ($p<0.05$) for macronutrients P, K, and Mg and micronutrients B, Cu, Fe, Mn, and Mo (Figure 5). Foliar K content was higher for plants co-inoculated with arbuscular mycorrhiza (25.5 g kg^{-1}) compared to standard inoculation (22.0 g kg^{-1}) and *Bacillus* strains (21.3 g kg^{-1}) regardless of application or not

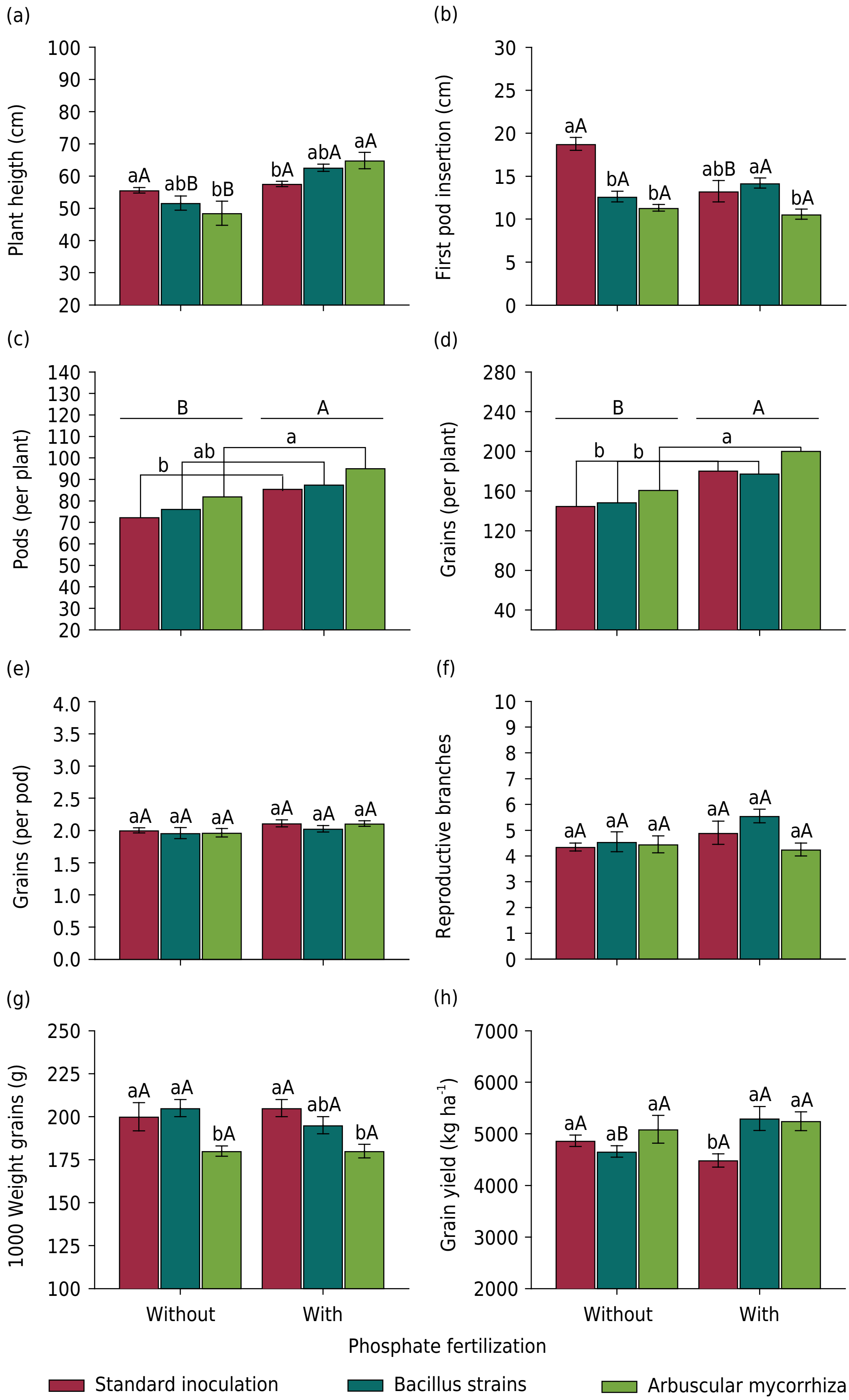

Figure 3. Plant height (a), first pod insertion (b), pods (c), grains (d), grains per pod (e), reproductive branches (f), 1000-grains weight (g) and yield (h) of soybean plants under standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza in 2020; values are averages of four repetitions. Means followed by the same letter, upper case for co-inoculation and lower case for fertilization, do not differ significantly by the Tukey test at 5 % probability.

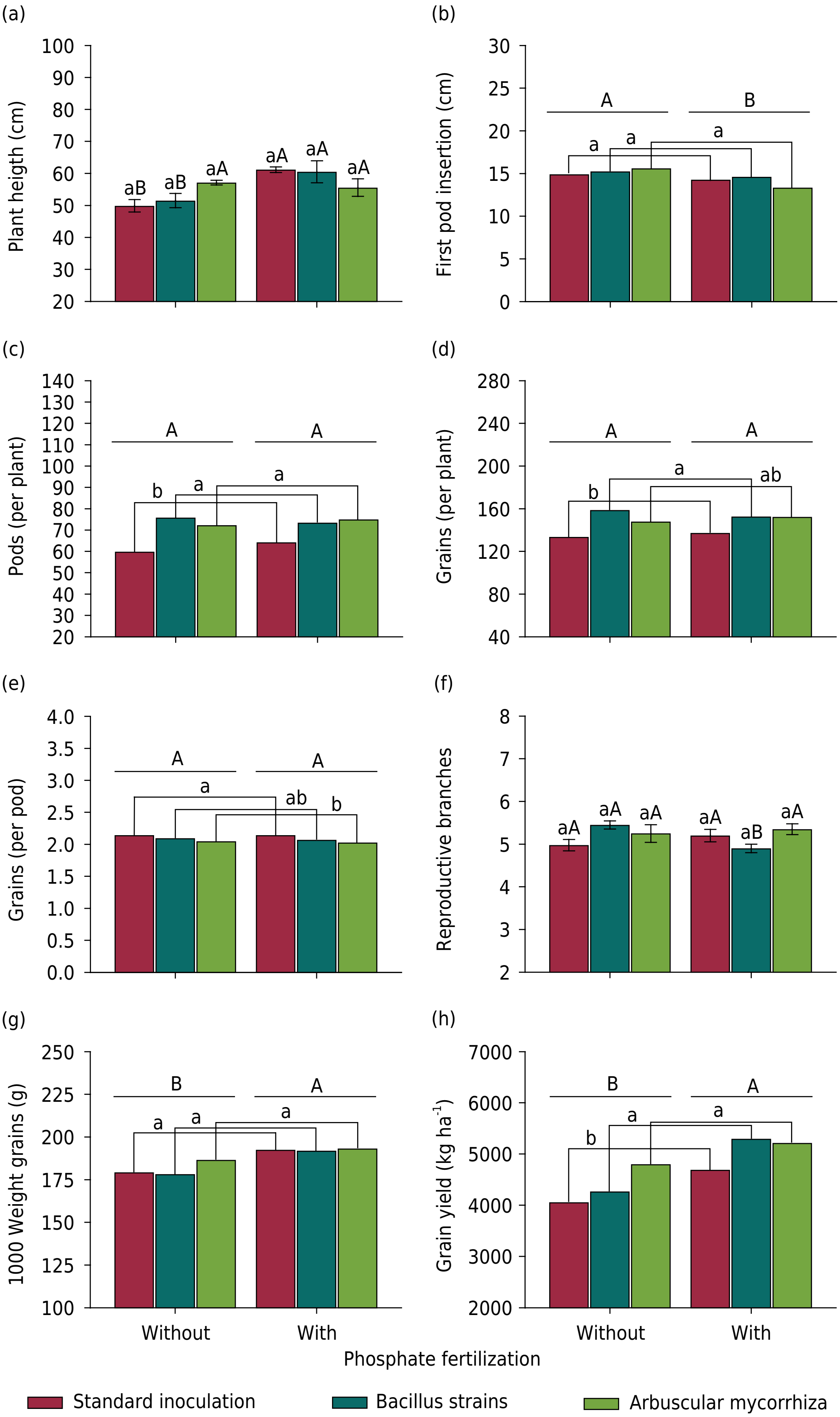

Figure 4. Plant height (a), first pod insertion (b), pods (c), grains (d), grains per pod (e), reproductive branches (f), 1000-grains weight (g) and yield (h) of soybean plants under standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza in 2021; values are averages of four repetitions. Means followed by the same letter, upper case for co-inoculation and lower case for fertilization, do not differ significantly by the Tukey test at 5 % probability.

of phosphate fertilizer (Figure 5c). The Fe-foliar of plants with standard inoculation was significantly higher than co-inoculation with arbuscular mycorrhiza (Figure 5i). Plants co-inoculated with *Bacillus* strains had Mo-foliar content superior to the other treatments, only in the absence of phosphate fertilizer.

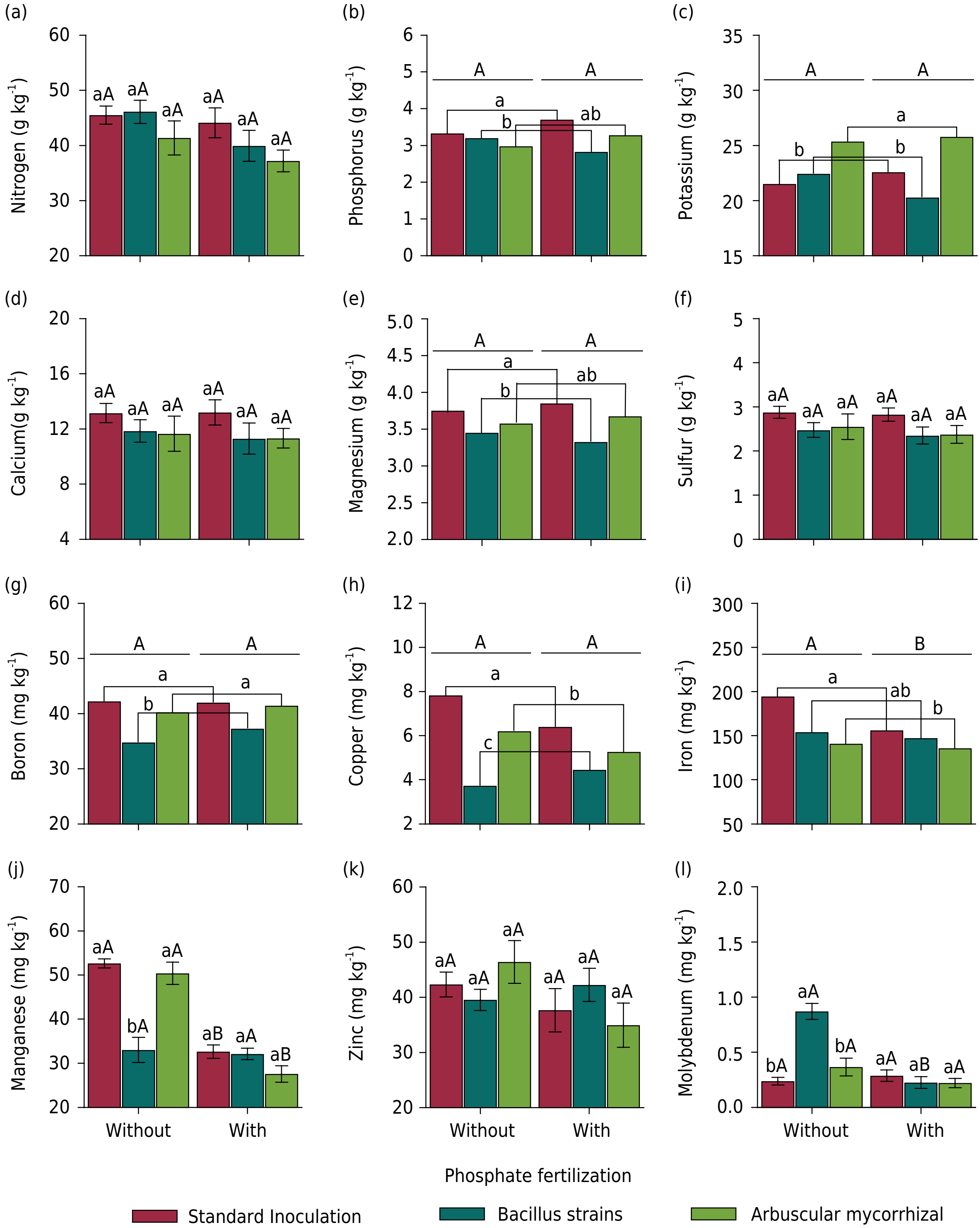

Figure 5. Foliar macronutrient and micronutrient content in soybean leaves under standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza in 2020; values are averages of 4 repetitions; Equal letters, upper case for fertilization and lower case for co-inoculation, do not differ significantly by the Tukey test at 5 % probability.

In 2021, the leaf nutrient content differed significantly ($p<0.05$) for the macronutrients P and Mg and the micronutrients Fe, Mn, and Mo (Figure 6). Plants differed in leaf P content only according to whether or not a phosphate source was supplied (Figure 6b). The Fe-foliar content of plants with standard inoculation was significantly higher than

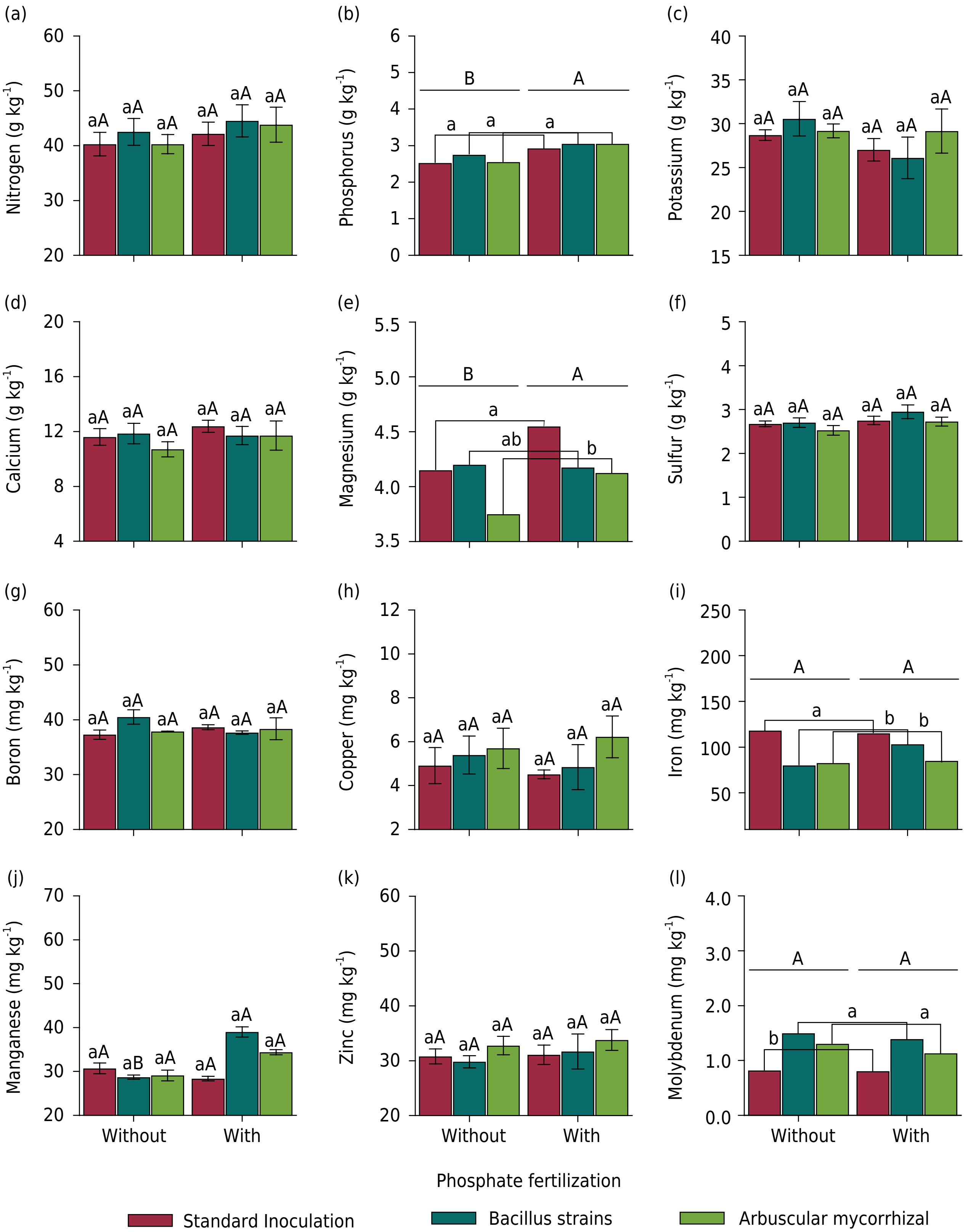

Figure 6. Foliar macronutrient and micronutrient content in soybean leaves under standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza in 2021; values are averages of four repetitions; Equal letters, upper case for fertilization and lower case for co-inoculation, do not differ significantly by the Tukey test at 5 % probability.

co-inoculation with *Bacillus* and arbuscular mycorrhiza strains. For Mo-foliar, *Bacillus* strains (1.4 mg kg^{-1}) and arbuscular mycorrhiza (1.2 mg kg^{-1}) showed higher leaf contents compared to standard inoculation (0.8 mg kg^{-1}).

Profitability of co-inoculation in the field

There was significant interaction ($p<0.05$) among the factors evaluated for the profitability indicators gross profit, net income, and profitability index in 2020. In 2021, the variables showed an independent response between the factors (Table 2).

In 2020, with phosphate fertilizer supply, the gross profit of co-inoculated plants (*Bacillus* strains and arbuscular mycorrhiza) was higher than standard inoculation, and the profitability index of plants with *Bacillus* strains was higher than standard inoculation. In 2021, gross profit, net income, and profitability index of plants co-inoculated with *Bacillus* strains and arbuscular mycorrhiza were higher than standard inoculation, regardless of phosphate fertilizer supply (Table 2). As for the fertilization factor, all profitability indicators were higher ($p<0.05$) in plants that received phosphate fertilizer.

Table 2. Soybean crop profitability as a function of the standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza during the years 2020 and 2021

P-Fertilization	Farm standard	*Bacillus* strains	Arbuscular mycorrhiza	Average
		2020		
		Gross profit (US$ ha^{-1})		
Without	1529±35 Aa	1463±35 Ba	1599±84 Aa	1530
With	1409±40 Ab	1664±73 Aa	1648±57 Aa	1574
Average	1469	1564	1623	CV (%) = 7.05
		Net income (US$ ha^{-1})		
Without	1018±35 Aa	933±35 Aa	1057±84 Aa	1003
With	836±40 Ba	1073±73 Aa	1045±57 Aa	985
Average	927	1003	1051	CV (%) = 11.01
		Profitability index (%)		
Without	69±0.69 Aa	66±0.83 Aa	68±1.52 Aa	68
With	62±1.09 Bb	68±1.45 Aa	66±1.17Aab	65
Average	65	66	67	CV (%) = 3.37
		2021		
		Gross profit (US$ ha^{-1})		
Without	2208±10	2391±54	2612±73	2404 B
With	2552±56	2881±135	2837±112	2757 A
Average	2380 b	2636 a	2725 a	CV (%) = 5.53
		Net income (US$ ha^{-1})		
Without	1379±10	1539±54	1745±73	1554 B
With	1647±56	1954±135	1895±112	1832 A
Average	1513 b	1747 a	1820 a	CV (%) = 8.43
		Profitability index (%)		
Without	63±0.17	65±0.79	67±0.87	65 B
With	65±0.76	68±1.49	67±1.27	67 A
Average	64 b	67 a	67 a	CV (%) = 2.49

Without: unapplied phosphate fertilizer; with: applied phosphate fertilizer; CV (%): coefficient of variation; Price of the soybean bag: 18.85 US$ (Cepea in 11/05/2020); 32.61 US$ (Cepea in 21/06/2021); Values are averages of 4 repetitions; Equal letters, upper case in the columns and lower case in the rows, do not differ significantly by Tukey test at 5 % probability.

Table 3. Chlorophyll content, shoot, number of nodules, nodule dry weight, root volume and root dry weight of soybean plants under standard inoculation, co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza in greenhouse

Treatment	Chlorophyll content	Shoot dry weight	Root volume	Root dry weight	Nodules	Nodules dry weight
		g	cm^3	mg		mg
Inoculation + 100 %	35.82±0.15	3.23±0.45	6.87±0.42 a	416.10±30.73	23.00±3.34	4.98±0.62
Inoculation + 50 %	35.27±0.53	3.07±0.49	5.37±0.23 bc	380.35±12.75	24.15±2.83	5.17±0.76
Bacillus strains + 50 %	33.82±0.84	1.84±0.16	4.85±0.42 c	325.00±33.38	22.25±3.70	3.44±0.81
Arbuscular micorhiza + 50 %	35.20±0.59	2.51±0.19	5.92±0.14 ab	342.80±23.54	21.25±1.79	4.37±0.29
CV (%)	3.11	27.18	11.56	14.39	26.52	29.24
p-value	≥0.1140	≥0.0724	≤0.0063	≥0.1219	≥0.9100	≥0.3058

100 %: total application of the recommended phosphate fertilizer; 50 %: application equivalent to 50 % of the recommended.; Values are averages of four repetitions; Distinct letters in the column indicate significant difference by Duncan test at 5 % probability.

Greenhouse

The root volume of plants with standard inoculation +100 % of fertilization was higher ($p<0.05$) than plants with standard inoculation +50 % of fertilization and plants co-inoculated with *Bacillus* strains +50 % of fertilization, but similar to plants co-inoculated with arbuscular mycorrhiza +50 % of the phosphate fertilization (Table 3).

There was no significant difference ($p>0.05$) among treatments for the variables SPAD (mean = 35), shoot dry mass (mean = 2.66 g), number of nodules (mean = 23 nodules per plant), nodule dry mass (mean = 4.5 mg) and root dry mass (mean = 366 mg).

DISCUSSION

Agriculture has been adopting increasingly sustainable practices, such as the use of microorganisms capable of partially making available the P retained in the soil (Withers et al., 2018; Fatima et al., 2021). Our study presents results from field and greenhouse experiments of soybean co-inoculated with mycorrhiza and phosphate-solubilizing bacteria as a way to increase the efficiency or supplement phosphate fertilization.

Co-inoculation promoted a higher number of pods per plant, with increases of 3.7 and 12.2 % in 2020 and 20.4 and 18.7 % in 2021, respectively for co-inoculation with *Bacillus* strains and co-inoculation with arbuscular mycorrhiza compared to standard inoculation (Figures 3c and 4c). This increase in pod production is possibly related to the capacity of these microorganisms to produce phytohormones and stimulate their synthesis by the plants. Among these phytohormones, cytokinin is considered one of the main hormones related to increased pod production (Carlson et al., 1987).

In 2020, plants under standard inoculation showed no increase in grain yield with the use of phosphate fertilizer (Figure 3h). Possibly, with an adequate level of available-P already present in the soil (Table 1), the plants did not respond to phosphate fertilization. Mariussi et al. (2019), when evaluating the application of phosphate fertilizer to soybean, reported that plants grown in areas with a high level of P-available in the soil have no response in productivity by phosphate fertilizer. These authors recommend that in areas with adequate P levels in the soil, only replacement fertilization of the P exported through the grains should be done. When considering that 10 kg of P_2O_5 is exported for every 1000 kg of grain produced (Câmara, 2015), in the 2020 season, 48 kg ha^{-1} of P_2O_5

were exported. In this perspective, with phosphate fertilizer supply, the practice of plant co-inoculation promoted an increase of 18 and 17 % in the yield in 2020, respectively, for *Bacillus* strains and arbuscular mycorrhiza compared to standard inoculation.

Evaluating the performance of corn inoculated with *Bacillus* strains, among them those that we evaluated in our experiment, Sousa et al. (2020) reported the capacity of these bacteria to produce indol-3-acetic acid (IAA), in addition to other mechanisms of solubilization and growth promotion, already reported in other studies (Oliveira et al., 2009; Ribeiro et al., 2018).

In field experiments with phosphate solubilizing microorganisms and phosphate fertilization on wheat yield, Shirmohammadi et al. (2020) stated that the best results occur when phosphate fertilization is associated with microorganisms. In corn inoculated with *Bacillus* strains, Sousa et al. (2020) stated that the results are better with the association of phosphate fertilization since the bacteria enhance the effect of phosphate fertilizer. Our results indicated higher productivity when co-inoculation was associated with phosphate fertilization, especially for *Bacillus* strains.

The benefit of plant growth-promoting bacteria is not only the release of P to the plant but also through the production of plant growth-promoting substances such as IAA, gibberellins, and cytokinin, which can increase productivity (Mahanta et al., 2014). Moreover, the ability of phosphate solubilizing bacteria to supply P to plants is limited to the complexity of the soil-plant system, where the compounds released by these bacteria to solubilize phosphate are rapidly degraded or the solubilized phosphate is fixed again before root uptake.

Similar to our results, Braga Junior et al. (2017) did not observe an increase in the number and dry weight of nodules of soybean associated with *Bacillus* strains. On the other hand, as observed by chlorophyll content, leaf N content, nodule number, and nodule dry weight, these co-inoculated microorganisms do not seem to have negatively affected the symbiosis of rhizobia with soybean.

Molybdenum foliar content was also altered as a function of co-inoculation practice. In 2020, only the co-inoculation with *Bacillus* strains promoted adequate micronutrient levels for plants (Figure 6). In 2021, *Bacillus* strains and arbuscular mycorrhiza had higher Mo foliar contents than standard inoculation. Possibly, there is a relationship between better Mo nutrition with reduced Fe contents, since Mo ions can be adsorbed to Fe oxides in the soil (Marcondes and Caires, 2005) and co-inoculation seems to have interfered in some way with Fe uptake by the plant.

Even though 70-90 % of terrestrial plant species possess the capacity to form symbiotic interactions with mycorrhiza, common agricultural practices, such as intense soil disturbance and pesticide use, reduce or eliminate the population of these fungi in the soil (Parniske, 2008; Taiz et al., 2017). Co-inoculation of soybean with arbuscular mycorrhiza can increase the population of these fungi in the soil and promote benefits to plants, such as higher productivity found in this study. The success of the symbiosis of these partners (plants-mycorrhizal fungi) occurs through the exchange of molecular signals, such as strigolactone, a phytohormone produced by plants that can stimulate growth and architecture of the plant, tolerance to environmental conditions, and promoter of symbiosis with mycorrhizae (Mishra et al., 2017; Marquer et al., 2019). Additionally, mycorrhizal fungi produce diffusible signals, including chitin oligomers and lipocytooligosaccharides, along with phytohormone-like compounds that stimulate plant root branching, increasing its surface area and range, facilitating P-uptake, a nutrient that is poorly mobile in soil (Maillet et al., 2011; Genre et al., 2013; Marquer et al., 2019).

No increase in P content in soybean leaves was found in 2020 and 2021 (Figure 5). According to Nagahashi et al. (1996), the number of branches and hyphal length, and germinating spores of AM were inhibited by high concentrations of P. Luthfiana et al.

(2021) state that the amount and type of metabolites exuded by arbuscular mycorrhiza (among them amino acids, sugars, and organic acids) are modified as a function of higher P. In fact, it may have occurred in our experiment since the experimental field sown in the 2020 crop had a high level of P in the soil (Table 1). Another hypothesis for the non-addition of P content in leaves of co-inoculated plants is the dilution of leaf contents due to the higher productivity of the co-inoculated plants, a more plausible hypothesis, since the levels of P in the soil of the experimental field evaluated in 2021 was not so high and, even so, there was no response in the content of P.

The enhanced K nutrition in plants interacting with arbuscular mycorrhiza may be due to the increased expression and activity of plant K^+ transport systems and the presence of efficient transporters in the extraradicular mycelium extending through the soil (Casieri et al., 2013). Studies on the detection of genes involved in nutrient transport during arbuscular mycorrhizal symbiosis in *Lotus japonicus* revealed a K^+ transporter (TM0445.37) belonging to the "K^+ uptake permease" (KUP) family confirmed to be forty-four-fold up-regulated in mycorrhizal roots (Guether et al., 2009). Liu et al. (2019) state that the potassium transporter SIHAK10 is involved in mycorrhizal potassium uptake.

In an extensive review on mycorrhizal associations in K nutrition of plants, Garcia and Zimmermann (2014) listed some crops that had increased K contents in their tissues by association with AM, namely corn (*Zea mays*), lettuce (*Lactuca sativa*) and wheat (*Triticum aestivum*). Other studies found K accumulation in plant tissues of tomatoes (Liu et al., 2019), *Coriandrum sativum* (Oliveira et al., 2016), and Pelargonium (Perner et al., 2007) in association with mycorrhizae. Garcia and Zimmermann (2014) reveal studies that identified a strong accumulation of K in spores and hyphae of AM. Our results corroborate these authors and add the association soybean - *Rhizophagus intraradices* to the list of crops with increased K.

In a two-years study, co-inoculated plants showed lower leaf Fe content compared to standard inoculation, being -7 and -15 % in 2020 and -21 and -28 % in 2021, respectively for *Bacillus* strains and arbuscular mycorrhiza. Beneficial microorganisms are recognized as a strategy to improve Fe nutrition in plants by producing siderophores and complexing the nutrient (Hider and Kong, 2010; Lurthy et al., 2020). We emphasize that the foliar levels of Fe were adequate in all treatments and the content present in the soil was above that adequate for soybean (Câmara, 2015). Therefore, these microorganisms may act not only in the absorption but also in regulating adequate levels of Fe for the plants. This statement is plausible since microorganisms have been reported to alleviate Fe stress in wheat by positively regulating genes encoded by ferritin in roots, which is important for maintaining Fe homeostasis (Sun et al., 2017).

Even though plants did not differ significantly in root dry weight, the root volume differed between treatments (Table 3). Plants with standard inoculation +100 % phosphate fertilization had higher root volume than standard inoculation +50 % fertilization and co-inoculation of *Bacillus* strains +50 % fertilization, but similar to co-inoculation with arbuscular mycorrhiza. This behavior may be related to greater production of fine roots by plants co-inoculated with arbuscular mycorrhiza since these roots would be unrepresentative when evaluating the dry weight of the roots. In addition, root weight is not an indication of increased absorptive capacity by the root system, and a drastic change in root architecture can occur without a difference in root weight (Mahanta et al., 2014).

Co-inoculated plants had an increase in gross profit of 18 and 17 % in 2020 (associated with phosphate fertilization) and 10.7 and 14.5 % in 2021 (regardless of phosphate fertilization), respectively for *Bacillus* strains and arbuscular mycorrhiza compared to standard inoculation (Table 2). This result occurred due to the higher productivity achieved by the co-inoculation of plants (Figure 3h), as this is essential to ensure good profitability for the producer (Duete et al., 2009). Galindo et al. (2018) emphasize that investments in practices that promote greater productivity in soybean, such as co-inoculation, can promote greater gross profit for the producer.

Evaluating the economic viability of soybean cultivars co-inoculated with *A. brasilense*, Galindo et al. (2018) observed higher crop profitability when co-inoculated. These authors also emphasize that the potential of co-inoculation in the nutrition and yield of soybean is high, together with the fact that it is a technique with low cost and investment, easy to apply, and non-polluting.

CONCLUSIONS

Co-inoculation of soybean plants with *Bacillus* strains or arbuscular mycorrhiza promotes yield increase. However, co-inoculation is more successful when associated with phosphate fertilizers. Therefore, in further studies, we indicate to research co-inoculation associated with doses of phosphorus source, for reduction and not substitution of phosphate fertilizer. There was an increase in the foliar content of K and Mo by the practice of co-inoculation. The co-inoculation of soybean provided better economic profitability for the activity.

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