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Inoculation with *Bradyrhizobium* sp. and *Azospirillum brasilense* Associated with Application of Cobalt and Molybdenum on Nutrition and Soybean Yield

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Abstract

Considering the main current limitations and potential of biological fixation of N_2 (BNF) in soybean crop and benefits attributed to various crops by inoculation with *Azospirillum brasilense* (diazotrophic bacteria with free life), with emphasis on larger development of the root system and consequently greater absorption of water and nutrients, we can infer that co-inoculation with both microorganisms of *Bradyrhizobium* sp. and *A. brasilense* can improve the crop performance in an approach that meets the current demands of agricultural, economic, and environmental sustainability. Thus, important researches are needed to evaluate the nutritional status, production components, and the soybean yield affected by cobalt and molybdenum application mode and co-inoculating seeds with bradyrhizobia and *A. brasilense*. We found that seed inoculated with *A. brasilense* and application of cobalt and molybdenum provided higher N concentration in leaf and mass of 100 grains, with a positive impact on the grain yield of soybean, with an increase of 1007 kg ha^{-1} of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia). This research demonstrated that co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt and molybdenum is beneficial for nutrition and soybean yields.

Keywords: co-inoculation, diazotrophic bacteria, mineral fertilization, nutritional status, foliar diagnosis

1. Introduction

The soybean is the major source of vegetable protein, an essential component in the production of animal feed, in addition to increasing use for human consumption [1].

This explains why the soybean plant is very demanding on nitrogen (N). It is estimated that 80 kg of N is needed to produce 1000 kg of soybean grains. Therefore, to obtain high yields, the biological fixation of N_2 (BNF) should be as efficient as possible [2–7].

The process of BNF in Brazil is responsible for nitrogen accumulated by plants; it represents about 200 kg ha^{-1} N [8], which is no longer applied via mineral fertilizers. It reduces the cost of production [9].

In addition, the use of selected and efficient bradyrhizobia inoculant and cobalt (Co) and molybdenum (Mo) nutrition contributes decisively in the BNF [10]. Cobalt and molybdenum are essential for BNF [11]. The first B12 vitamin is essential for the processing of BNF and other parts of the molybdoenzymes, used in absorption and metabolism of nitrogen [12]. The application of Mo and especially Mo + Co increases BNF [13].

In Brazil, soybean generally responds positively to fertilization with Mo in soils of low fertility and in fertile soils depleted of Mo due to long-term cropping. The micronutrient can be supplied by seed treatment. However, the toxicity of Mo sources to *Bradyrhizobium* strains applied to seed as inoculant has been observed resulting in bacterial death and reductions in nodulation, N_2 fixation, and grain yield [14].

Considering the main current limitations and potential of BNF in soybean crop and benefits attributed to various crops by inoculation with *Azospirillum brasilense* (diazotrophic bacteria with free life), with emphasis on larger development of the root system and consequently greater absorption of water and nutrients, we can infer that co-inoculation with both the microorganisms can improve the crop performance in an approach that meets the current demands of agricultural, economic, and environmental sustainability [15].

Bacteria promoters of plant growth (BPPG) correspond to a group of beneficial microorganisms to plants due to the ability to colonize the surface of roots, rhizosphere, phyllosphere, and internal plant tissues [16, 17]. The BPPG can stimulate plant growth in several ways. The most relevant are BNF capacity [18], increase in nitrate reductase activity when the BPPG grows endophytically plants [19], production of hormones such as auxins, cytokinins, gibberellins, and ethylene, and a variety of other molecules [20], phosphate solubilization [21], and act as biological control agent of pathogens [22]. In general, it is believed that the benefit of BPPG to plant growth is caused by a combination of all these mechanisms [23].

A. brasilense can act in relations between rhizobia and legumes, promoting increases in plant growth, grain yield, and total nitrogen biologically fixed as well as improvements in nitrogen use by plant through symbiosis with rhizobia [24].

Based on the above information and the lack of research about the interaction between co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt

and molybdenum in soybean crop, researches to evaluate the nutritional status, production components, and the soybean yield affected by cobalt and molybdenum application mode and co-inoculating seeds with bradyrhizobia and *A. brasilense* are important.

2. Materials and methods

The experiment was conducted in the 2014/2015 season in an experimental area that belongs to the UNESP Engineering Faculty located in Selvíria, MS/Brazil, with the following geographical coordinates, 20°22′S and 51°22′W and an altitude of 335 m. The experimental area soil was classified as Distroferric Red Oxisol with clay texture (the granulometric analysis indicated values of particle size of 420, 50 kg^{-1}, and 530 g of sand, silt, and clay, respectively), according to Embrapa (2013) [25], which has been cultivated with annual cultures over 27 years, with the last 10 years in the direct tillage system. Before soybean sowing, corn was cultivated in the area. The annual average temperature was 23.5°C, the annual average pluvial precipitation was 1370 mm, and the annual average relative air humidity was between 70% and 80%.

The experimental design was carried out in a randomized blocks with six treatments and four replications. The treatments were as follows: (1) control (without soybean inoculation with *A. brasilense* and without application of cobalt and molybdenum); (2) cobalt and molybdenum application on the seed with commercial product (15% Mo (195 g L^{-1}) and 1.5% Co (19.5 g L^{-1})) at a dose of 150 ml ha^{-1}, based on Sfredo et al. (2010) [10] recommendation; (3) seed inoculated with *A. brasilense* at a dose of 200 ml ha^{-1} (strains Abv5 Abv6 with guaranteed 2×10^8 colonies forming units (CFU) per ml) and application of cobalt and molybdenum in the abovementioned dose; (4) leaf application of *A. brasilense* in the V3 stage of soybeans, in the abovementioned dose; (5) leaf application of cobalt and molybdenum in the V3 stage in the aforementioned dose; and (6) leaf application of cobalt and molybdenum along with foliar inoculation with *A. brasilense* in V3 stage in the aforementioned doses.

In all treatments, the inoculation with Rhizobium was performed in seeds at a dose of 200 ml ha^{-1} (strains: SEMIA 5019 (*B. elkanii*) and SEMIA 5079 (*B. japonicum*) with 5×10^9 guarantee of viable cells per ml). Each plot consisted of seven lines of 5-m soybean, spaced by 0.45 m, totaling 15.75 m^2. Useful area of the plot was considered to be the three central lines, discounting the simple surround, totalling effective sampling area of 6.75 m^2 per plot.

Chemical properties of the soil in the tillable layer were determined before 2014, before the soybean experiment began. The methods proposed by Raij et al. [26] provided the following results: 10 mg dm^{-3} of P (resin), 5 mg dm^{-3} of S-SO$_4$, 22 g dm^{-3} of organic matter (OM), pH(CaCl$_2$) of 5.3, 2.4 mmol$_c$ dm^{-3} of K$^+$, 21.0 mmol$_c$ dm^{-3} of Ca^{2+}, 18.0 mmol$_c$ dm^{-3} of Mg^{2+}, 28.0 mmol$_c$ dm^{-3} of H+Al, 3.2 mg dm^{-3} of Cu, 22.0 mg dm^{-3} of Fe, 24.2 mg dm^{-3} of Mn, 1.2 mg dm^{-3} of Zn (diethylenetriaminepentaacetic acid (DTPA)), 0.16 mg dm^{-3} of B (hot water), and 60% base saturation. Based on soil analysis and soybean crop fertilization recommendation [27], the fertilization was done in the seed furrows with 96 kg P$_2$O$_5$ ha^{-1} (in the form of triple superphosphate) and 70 kg ha^{-1} K$_2$O (in the form of potassium chloride).

The seeds were treated with the fungicide Thiram + Carbendazim at a dosage of 30 + 70 g active ingredient (a.i.) per 100 kg seed, respectively, after drying the seeds, and were inoculated with Rhizobium, and depending on the treatment the seed was inoculated with *A. brasilense* just before soybean planting in the shade. We used the soybean cultivar BMX Power RR, with a spacing of 0.45 m between lines, with 17 seeds per meter.

The experiments were conducted in a no-tillage system. The area was irrigated by a central pivot sprinkler system when necessary. The water coverage was 14 mm over a period of around 72 h. The control of weeds, pests, and diseases prevention was carried out when necessary in soybean crop. The plants were harvested 120 days after soybean emergence.

Concentrations of N, P, K, Ca, Mg, S, Cu, Fe, Mn, and Zn were measured in soybean plant leaves. The third upper trifoliate leaves (30 plants) in the flowering soybean plants (R2 stage) were collected according to the methodology described in Ambrosano et al. [27]. The determination of nutrients was carried out as described by Malavolta [28]. The leaf chlorophyll index (LCI) was determined indirectly after application of the treatments and when the plants were in the flowering (R2 stage), in 10 plants per plot through readings in the third upper trifoliate leaves, using a digital chlorophyll CFL 1030 Falker (Falker Agricultural Automation, Porto Alegre, Brazil).

The leaf area of 10 leaves per plot was measured using the software ImageJ 1:45 (2011), according to the methodology described by Bauermann [29]. At the time of harvest, 10 soybean plants representing were collected for counting the number of grains per pod, grains per plant, and mass of 100 grains. The mass was determined on a precision scale of 0.01 g and corrected for 13% moisture (wet basis). The soybean was harvested from the plants in the useful area of each plot and grain yield was calculated after mechanical threshing. Data were transformed into kg ha^{-1} and corrected for 13% moisture (wet basis). The results of all the evaluations were subjected to analysis of variance and the Tukey test at 5% probability to compare the averages of treatments, using the Sisvar program.

3. Results and discussion

The seed inoculated with *A. brasilense* and the application of cobalt and molybdenum provided higher N concentration in the leaf, significantly differing from the control only inoculated with Rhizobium (**Table 1**), indicating that the biological nitrogen fixation was potentiated by these treatments. On average, the leaf N concentrations, which are considered suitable, were shown to be 40–54 g kg^{-1} of dry matter (D.M.), according to Ambrosano et al. [27]. However, the co-inoculation with *A. brasilense* associated with cobalt and molybdenum led to the higher leaf N concentration than the concentrations considered suitable, regardless of modes of application and inoculation.

Increases in total nitrogen biologically fixed by plant through symbiosis with rhizobia, associated with *A. brasilense,* have also been reported in other researches [24, 15]. However, Zuffo et al. [30] observed that the use of *A. brasilense* alone or in co-inoculation with *B. japonicum* does not have significant effect on leaf N concentration.

The treatments in this research provided similar leaf concentrations of P, K, Ca, and S (**Table 1**). However, there was a higher concentration of Mg in the leaves when Co and Mo and *A. brasilense* were applied in the leaves, although leaf Mg concentration did not differ significantly between most other treatments. It is worth noting that the foliar concentrations of P, K, Ca, Mg, and S (**Table 1**) were within the ranges of 2.5–5.0, 17–25, 4–20, 3.0–10.0, and 2.1–4.0 g kg^{-1} D.M., respectively, which were recommended by Ambrosano et al. [27] as the ideal.

The leaf chlorophyll index (LCI) and leaf Fe and Cu concentrations were not affected by treatments (**Table 2**). This can be explained by adequate leaf N concentrations obtained for soybean crop. Zuffo et al. [30] also observed that the use of *A. brasilense* alone or in co-inoculation with *B. japonicum* does not have significant effect on LCI.

The results are different from those found by other authors using corn plants, who found that the LCI was higher in the treatments with diazotrophs than in the treatments without inoculation. Corn plants that were inoculated with *A. brasilense* had greater LCI than those that were not inoculated, in two crop seasons [31]. Kappes et al. [32] and Quadros et al. [33] found that plants inoculated with *A. brasilense* had improved LCI. These divergent results can be explained by the fact that *A. brasilense* increases reductase activity of nitrate when they grow endophytically plants [19], a fact of minor importance for soybean plant that spends more metabolic energy (ATP) to biologically fix N$_2$ in the root nodules in relation to nitrate reduction assimilation.

Leaf application of Co and Mo and foliar inoculation with *A. brasilense* provided the largest concentration of Mn in the leaves (**Table 2**), while only the foliar inoculation with *A. brasilense* stood out with the highest Zn concentration in leaf. However, averages of leaf concentrations of Cu, Fe, Mn, and Zn were also suitable as described by Ambrosano et al. [27], being 10–30, 50–350, 20–100, and 20–50 mg kg^{-1} D.M., respectively.

Treatments	N	P	K	Ca	Mg	S
	----------------------------------- g kg^{-1} -----------------------------------					
Control	48.65 b	4.01 a	19.12 a	9.70 a	5.67 ab	3.06 a
Co, Mo seed	50.91 ab	3.75 a	19.98 a	8.94 a	4.55 b	3.08 a
Co, Mo + Azos seed	56.21 a	4.16 a	20.42 a	8.64 a	4.73 ab	3.16 a
Azos foliar	49.00 b	4.75 a	19.92 a	9.51 a	5.63 ab	3.36 a
Co, Mo foliar	55.95 a	4.01 a	18.38 a	8.74 a	5.32 ab	3.35 a
Co, Mo + Azos leaf	54.30 ab	4.28 a	18.70 a	8.99 a	5.83 a	3.64 a
Overall average	52.50	4.16	19.42	9.09	5.29	3.28
CV (%)	4.34	9.42	5.42	8.75	7.77	12.73
LSD (5%)	6.46	1.11	2.99	2.26	1.17	1.18

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 1. Leaf concentrations of Cu, Fe, Mn, and Zn of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

Treatments	LCI	Cu	Fe	Mn	Zn
			------------------------- mg kg^{-1} -------------------------		
Control	43.79 a	9.00 a	156.33 a	90.00 ab	47.67 b
Co, Mo seed	44.12 a	8.33 a	163.33 a	71.00 b	50.00 ab
Co, Mo + Azos seed	44.52 a	8.67 a	189.67 a	70.33 b	47.67 b
Azos foliar	43.56 a	10.33 a	212.33 a	85.00 ab	53.33 a
Co, Mo foliar	44.23 a	9.33 a	191.00 a	84.67 ab	46.00 b
Co, Mo + Azos leaf	44.15 a	10.67 a	184.00 a	97.67 a	50.00 ab
Overall average	44.06	9.39	182.78	83.11	49.11
CV (%)	3.11	9.46	27.89	11.06	3.89
LSD (5%)	1.55	2.52	144.60	26.08	5.42

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 2. Leaf chlorophyll index (LCI) and leaf concentrations of Cu, Fe, Mn, and Zn of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

The leaf area of soybean was greater in treatment with the application of *A. brasilense*, differing significantly from the application of Co and Mo in the seed (**Table 3**). This bacterium can influence the plant growth by producing auxins, gibberellins, and cytokinins, which provide improved root growth [34] and consequently greater absorption of water and nutrients [22], resulting in more vigorous and productive plant [35, 36].

The control treatment provided greater number of grains per pod, and the number of grains per pod did not differ between treatment with Co and Mo of the leaf and treatment of inoculation of the seed with *A. brasilense* and seed application of Co and Mo. The number of grains per plant showed no difference between treatments. These explain why the smaller mass of 100 grains was obtained for control and leaf application of Co and Mo, in other words, there was less filling grain due to the higher number of seeds per pod.

Seed inoculated with *A. brasilense* and seed application of Co and Mo provided higher mass of 100 grains and grains yield of soybean, with an increase of 1007 kg ha^{-1} of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia), corroborating with Hungria et al. [15] showing that co-inoculation with *A. brasilense* increased yield of soybeans in 16.1% compared to isolated use of *Bradyrhizobium* strains.

These results may be due to several mechanisms, which are the anticipation in the BNF of the nodes, an increase in the dry weight of nodes, promoting the occurrence of nodulation heterologous through the increased formation of hair root and secondary roots, an increase in infection sites, inhibition of plant pathogens and production of phytohormones and influences in the partition of dry matter between the roots and shoots [24]. Yet, pondering Hungria et al. [15], these results caused by co-inoculation bacteria promoters of plant growth and Rhizobia appear to be

Treatments	Leaf area (cm^2)	Grains per pod	Grains per plant	Mass of 100 grains (g)	Grains yield (kg ha^{-1})
Control	64.05 ab	3.00 a	176.30 a	14.68 b	5550 b
Co, Mo seed	62.90 b	2.55 b	145.20 a	15.88 ab	6083 ab
Co, Mo + Azos seed	68.35 ab	2.78 ab	155.67 a	16.10 a	6557 a
Azos foliar	77.80 a	2.65 b	156.90 a	14.80 ab	5355 b
Co, Mo foliar	69.75 ab	2.80 ab	185.07 a	14.60 b	5685 ab
Co, Mo + Azos leaf	67.50 ab	2.65 b	138.47 a	14.88 ab	5602 ab
Overall average	68.39	2.74	159.60	15.15	5805
CV (%)	4.89	4.87	14.61	3.76	7.27
LSD (5%)	14.26	0.31	66.14	1.31	970

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 3. Leaf area, grain per pod, grains per plant, mass of 100 grains, and grains yield of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

under the influence of specific signals among bacterial genotypes involved and the genotype of the host plant. It is important to do more related studies on the response of the co-inoculation depending on the genotypes, aiming at the development of more responsive genotypes.

In an important research by Campos et al. [13], they concluded that there are no Mo and Co effects on nodulation in soil with established *Bradyrhizobium* population, soil application of Mo and Co does not supply the Mo and Co necessary to the plant and to the BNF, application of Mo and especially that of Mo and Co increase BNF, as in the present study, application of Mo and Co on leaves has the same effect on BNF as seed applications, as in the present study, and seeds with high concentration of Mo show higher BNF than those with low Mo contents, as in the present study. Also, Hungria et al. [37] reported increases in grains yield of soybean (20%) by application of Mo and Co associated with *Bradyrhizobium* compared to treatment inoculated with *Bradyrhizobium*.

4. Final consideration

Leaf application of Co and Mo and foliar inoculation with *A. brasilense* provided the largest concentration of Mg and Mn in the leaves, while only the foliar inoculation with *A. brasilense* stood out with the highest Zn concentration in leaf and leaf area.

Seed inoculated with *A. brasilense* and seed application of Co and Mo provided higher N concentration in leaf and mass of 100 grains, with a positive impact on the grain yield of soybean, with an increase of 1007 kg ha^{-1} of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia).

This research demonstrated that co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt and molybdenum is beneficial for nutrition and soybean yields. Therefore, as inoculation with *A. brasilense* is a low-cost technique, easy to apply and use, non-polluting, and the technique falling within the desired sustainable context at present, the trend is that this technology be increasingly used in soybean crop.

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