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Chemical seed treatment, inoculation, and co-inoculation of winter bean combined with nitrogen doses

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ABSTRACT: Faced with the contrasting results of inoculation and co-inoculation in the bean crop, an experiment was carried out in pots, with the objective of evaluating the performance of the bean plant in treatments inoculated or co-inoculated with *Rhizobium tropici* and *Azospirillum brasilense*, via seeds, with or without chemical treatment with fungicide and insecticide, combined with 4 top-dressing nitrogen doses (0, 30, 60, and 120 kg ha^{-1}), in a 'Latossolo Vermelho-Amarelo distrófico arenoso' soil collected from the surface layer from 0 to 0.20 m depth. The results of the Scott Knott test and regression showed that co-inoculation via seeds did not alter vegetative growth, components, and grain yield. However, without chemical treatment, co-inoculation increased plant root volume. Root dry matter production was higher when only *A. brasilense* was used and when combined with fungicide, or with insecticide and *R. tropici*. While the combination of fungicide, insecticide and *A. brasilense* increased the dry matter production of shoots and grains per plant. Even with inoculation or co-inoculation, there was a linear response of the common bean to top-dressing fertilization up to 120 kg ha^{-1} of N in vegetative and root growth, pod production and grain per pod.

Key words: *Azospirillum brasilense*; fungicide; insecticide; *Phaseolus vulgaris*; *Rhizobium tropici*

Tratamento químico de sementes, inoculação e coinoculação do feijão de inverno combinados com doses de nitrogênio

RESUMO: Frente aos contrastes de resultados de inoculação e coinoculação na cultura do feijão, realizou-se experimento, em vasos, com objetivo de avaliar o desempenho do feijoeiro em tratamentos inoculados ou coinoculados com *Rhizobium tropici* e *Azospirillum brasilense*, via sementes, com ou sem tratamento químico com fungicida e inseticida, combinados com 4 doses de nitrogênio em cobertura (0, 30, 60 e 120 kg ha^{-1}), em um Latossolo Vermelho-Amarelo distrófico arenoso coletado da camada superficial de 0 a 0,20 m de profundidade. Os resultados do teste de Scott Knott e de regressão, demonstraram que a coinoculação via sementes, não alterou o crescimento vegetativo, componentes e produção de grãos. Entretanto, sem tratamento químico, a coinoculação aumentou o volume radicular das plantas. A produção de matéria seca de raízes foi maior quando utilizado somente *A. brasilense* e quando combinados ao fungicida, ou ao inseticida e ao *R. tropici*. Enquanto, a combinação de fungicida, inseticida e *A. brasilense* incrementou a produção de matéria seca da parte aérea e de grãos por planta. Mesmo com inoculação ou coinoculação, houve resposta linear do feijoeiro à adubação de cobertura até 120 kg ha^{-1} de N no crescimento vegetativo, de raízes, produção de vagens e grãos por vagem.

Palavras-chave: *Azospirillum brasilense*; fungicida; inseticida; *Phaseolus vulgaris*; *Rhizobium tropici*

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Introduction

Brazil is the third largest bean producer in the world (FAO, 2021) and each crop year has three growing seasons, which in 2020/21 resulted in the production of 3.1 million tons of the grain, of which 0.8 million tons were grown in the winter season, with the highest average national productivity (Conab, 2020). The culture has special relevance, since the grain is one of the most important sources of vegetable protein for humans (Kläsener et al., 2019), because in the composition of the grain there is a high content of proteins, which are related to nitrogen, which is the nutrient most absorbed and extracted by the bean (Leal et al., 2019).

Due to the high financial and environmental costs of nitrogen fertilizers (Capristo et al., 2020; Tocheto & Boiago, 2020), inoculation and co-inoculation techniques gain attention in research, however, the results are discrepant in the measurement of the technology, with negative, no effect or positive effect on bean productivity (Gitti et al., 2012; Mellini et al., 2020).

As an alternative to reduce or even replace nitrogen fertilization of the crop, an increase in biological nitrogen fixation has been sought through inoculation with bacteria of the genus *Rhizobium* and/or co-inoculation with bacteria of the genus *Azospirillum*, since these can influence the increase in the root system and volume of explored soil, with an increase in nodulation, efficiency of absorption of water and nutrients and, consequently, in grain yield (Riggs et al., 2001; Hungria et al., 2013).

Among the various bacteria capable of establishing symbiosis with common bean, *R. tropici* is the most adapted and recommended species for tropical acidic soils, being able to nodulate with a variety of legumes (Hungria et al., 2000; Cerro et al., 2015; Cerro et al., 2017).

In Brazil, according to the Ministério da Agricultura, Pecuária e Abastecimento (MAPA), for commercial rhizobium based inoculants the requirement is the presence of at least 1×10^9 CFU g^{-1} or mL^{-1} of the commercial product, while for commercial inoculants containing *A. brasilense*, the selected and approved strains are AbV5 and AbV6, with a legal requirement of at least 1×10^8 CFU g^{-1} or mL^{-1} of the commercial product (Brasil, 2011).

Factors such as competition between bacteria, the recent use of more specialized strains, different levels of compatibility with fungicides and insecticides, different forms of application, among others, may be related to the variability in the results of inoculation and co-inoculation in the bean crop, since they involve aspects of a physical, chemical, and biological nature, in the complex dynamics of nitrogen in the soil (Braccini et al., 2016; Silva et al., 2020; Tocheto & Boiago, 2020).

Therefore, this study aimed to characterize some of these aspects by evaluating the performance of winter bean and the possible increase in plant growth and grain yield in treatments inoculated or co-inoculated via seeds with *R. tropici* and *A. brasilense*, with and without chemical treatment with fungicide and/or insecticide, combined with four different doses of nitrogen in coverage, in sandy soil.

Materials and Methods

The experiment was conducted between June and September 2020, in pots, under open conditions, in the municipality of Ilha Solteira, São Paulo, Brazil. The climate of the region, according to the Köppen classification, is of the Aw type, defined as tropical humid with rainy season in summer and dry season in winter.

To fill the pots, soil was collected from the surface layer from 0 to 0.20 m deep, from a 'Latossolo Vermelho-Amarelo distrófico arenoso', according to the Brazilian Soil Classification System (Santos et al., 2018) occupied by brachiaria grass pasture.

For initial soil characterization, 10 single samples were sampled to form a composite sample. Subsequently, it was air-dried, disintegrated and passed through a sieve with a mesh size of 2 mm, constituting Fine Air-Dried Soil (FADS) of which granulometric (Teixeira et al., 2017) and chemical (Raij et al., 2001) analyzes were carried out, respectively, in the Soil Physics and Fertility Laboratories of the Faculty of Engineering of Ilha Solteira.

As for the granulometric analysis, the soil contained 126.0, 3.0, and 871.0 g kg^{-1} of clay, silt, and total sand, respectively. The chemical attributes of the soil in the 0 to 0.20 m depth layer were: phosphorus (P resin) = 1 mg dm^{-3}, organic matter = 15 g dm^{-3}, pH ($CaCl_2$) = 4,5; potassium (K) = 0.4 $mmol_c$ dm^{-3}; calcium (Ca) = 8 $mmol_c$ dm^{-3}; magnesium (Mg) = 6 $mmol_c$ dm^{-3}; potential acidity (H + Al) = 13 $mmol_c$ dm^{-3}; aluminum (Al) = 2 $mmol_c$ dm^{-3}; sum of bases (SB) = 14.4 $mmol_c$ dm^{-3}, sulphur ($S\text{-}SO_4$) = 3 mg dm^{-3}; cation exchange capacity (CEC) = 27.4 $mmol_c$ dm^{-3}; base saturation (V) = 53%; aluminum saturation (m) = 12%; boron (B) = 0.06 mg dm^{-3}; copper (Cu) = 0.2 mg dm^{-3}; iron (Fe) = 18 mg dm^{-3}; manganese (Mn) = 3.4 mg dm^{-3}; and, zinc (Zn) = 0.2 mg dm^{-3}.

In each pot, containing at its base two perforations for drainage, 7 kg of soil sieved in 40 mm mesh were placed and based on the results of the soil analysis, the calculation of the need for lime was performed by Equation 1:

$$\mathrm{LR} = \mathrm{CEC}\frac{(\mathrm{V2} - \mathrm{V1})}{10 \times \mathrm{RTNP}} \quad (1)$$

where: LR - limestone requirement (Mg ha^{-1}); CEC - cation exchange capacity ($mmol_c$ dm^{-3}); V2 - desired base saturation for the crop (70%); V1 - base saturation found in the soil analysis; and, RTNP - relative total neutralizing power of limestone.

Thus, for the correction of soil acidity, 2 g of dolomitic limestone of PRNT 86 per pot, equivalent to 0.54 Mg ha^{-1}, was applied and incorporated into the soil. An incubation period of 30 days was used, with the soils in the pots being constantly moistened in order to favor the reaction of the lime in the soil.

For sowing fertilization, the mixture of fertilizers with the soil was individualized for each pot, using 2.1 g of the 05-25-25 formulation + boron (0.5%) and 0.16 g of zinc sulfate as nutrient sources. Top-dressing was carried out at 36 days after sowing, at the stage of emission of the first flower of the bean, with 0, 0.23, 0.46, or 0.92 g of Excellen (N) per pot, corresponding to 0, 30, 60, and 120 kg of nitrogen per hectare, according to the dose proposed for each treatment, beyond the addition of 0.44 g of potassium chloride in all pots.

The experimental design adopted was entirely randomized, in a factorial scheme with 28 treatments, combining 7 seed treatments with 4 levels of nitrogen in coverage, with 4 replications, totaling 112 plots. Seed treatments consisted of fungicide (F), insecticide (I), *R. tropici* inoculation (R), and *A. brasilense* inoculation (A). While for the nitrogen top-dressing levels, four different doses were used, corresponding to 0, 30, 60, and 120 kg of N per hectare.

Six bean seeds (cv. BRS Estilo) were used in each pot, sown on June 12, 2020, in the shade and treated according to each treatment (fungicide, insecticide, inoculation, and co-inoculation). The order of application of seed treatments was: fungicide (F), followed by insecticide (I), then the inoculant based on *R. tropici* (R), and finally the inoculant based on *A. brasilense* (A). In treatments where not all inputs were used, the same order of application was respected.

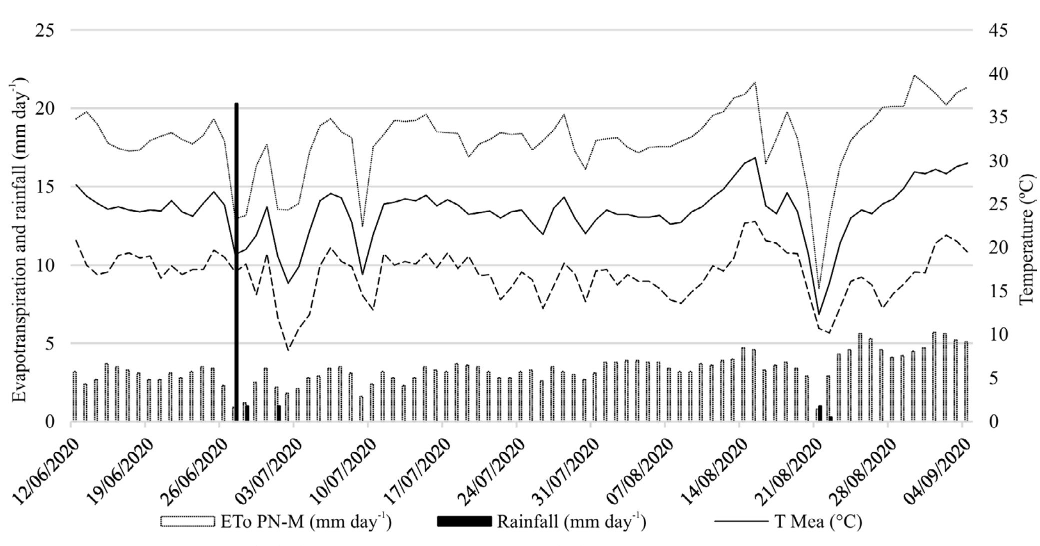
The treatments were prepared in 7 plastic cups, containing 50.35 g of seeds each cup, an average of 200 seeds per cup, with a time interval between the addition of one commercial product and another, so as to allow the next product to be applied only after the previous one had dried.

In the fungicide treatments, 0.15 mL (300 mL per 100 kg of seeds) of the fungicide Carbendazim + Tiram, of systemic and contact action, of the chemical groups Benzimidazole and Dimethyldithiocarbamate, were used in a concentrated suspension (CS) formulation. Classified as category 5 in the toxicological classification (unlikely to cause acute harm) and class II in the classification of the potential for environmental hazard (product very hazardous to the environment), indicated for bean cultivation exclusively in the form of seed treatment against anthracnose (*Colletotrichum lindemuthianum*) and root rot (*Rhizoctonia solani*).

The insecticide was the concentrated suspension (CS) formulation of Imidacloprid 150 g L^{-1} + Thiodicarb 450 g L^{-1}, specific for seed treatment. This product has systemic action by the chemical group of neonicotinoids (Imidacloprid) and contact and ingestion by the chemical group methylcarbamate oxime (Thiodicarb) with a recommended dose of 1 L for every 100 kg of seeds. In the treatments tested here, 0.50 mL of the commercial product was applied to each cup containing 50.35 g of seeds. The product is classified as category 3 in the toxicological classification (moderately toxic product) and class II in the classification of environmental hazard potential (product very dangerous to the environment). This insecticide is indicated for the control of pests such as *Bemisia tabaci*, *Diabrotica speciosa*, and *Empoasca kraemeri* in the bean crop.

For inoculation with *R. tropici*, a peat inoculant was applied with the strain SEMIA 4080 containing 2×10^9 colony forming units (CFU) g^{-1}, using 0.20 g in each cup with 50.35 g of seeds, referring to the recommended dose of 200 g of the inoculant for every 50 kg of seeds. For *A. brasilense*, strains AbV5 and AbV6 were applied, with a guarantee of 2×10^8 CFU mL^{-1} or g^{-1}, using a syringe to administer 0.20 mL in each cup with 50.35 g of seeds, referring to the recommended dose of 200 mL of the liquid inoculant for every 50 kg of seeds. Both commercial products are registered with the Ministério da Agricultura, Pecuária e Abastecimento (MAPA).

The climatic data for the period of the experiment were collected by the agrometeorological station of the Hydraulics and Irrigation area of the Faculty of Engineering of Ilha Solteira - UNESP, located in the municipality of Ilha Solteira, SP, Brazil (Figure 1).



Source: http://clima.feis.unesp.br.

Figure 1. Climatic data of Ilha Solteira, SP, Brazil, during the experiment period: evapotranspiration by Class A Tank and Penman Monteith (mm day^{-1}), rainfall (mm day^{-1}), mean, minimum, and maximum temperature (°C). Ilha Solteira, SP, Brazil, 2020.

Irrigation throughout the crop cycle was carried out based on readings from soil moisture meter equipment - PCR, manually applying the required amounts of water.

At stage V1, the stage of emergence of bean seedlings, thinning was performed, keeping only two plants per pot. At R5, when more than 50% of the plants were in flower, plant height and chlorophyll index in the third trefoil of each plant were measured using a digital chlorophyll meter (Falker). These trifolia were collected for N content analysis (Malavolta et al., 1997) and due to the amount of leaf mass required for this analysis, the trifolia of the plots were joined, which did not generate repetitions for N content analysis, and only characterization by treatment.

At harvest, the number of pods per plant, grains per pod, and grain yield per plant were evaluated. Plants were separated into aerial part and roots, for determination of root length (graduated ruler), root volume (water displacement method in beaker), fresh and dry mass of aerial part and roots (dried at 65 °C for 72 horus in forced air circulation oven).

The results were entered into the statistical program Sisvar (Ferreira, 2011) and submitted to analysis of variance at 5% probability, and the means were compared by the method of Scott & Knott (1974), and for the doses of N in coverage by polynomial regression, adopting the significant models of greater R^2.

Results and Discussion

The foliar nitrogen contents in winter bean submitted to the different seed treatments and nitrogen doses (0, 30, 60 and 120 kg ha^{-1} of N) applied in top-dressing (Table 1), are according to Ambrosano et al. (2022), within the adequate range for the crop, except in the control treatment without top-dressing.

Table 1. Foliar nitrogen contents (g kg^{-1}) in winter bean, subjected to different seed treatments and N top-dressing doses, in sandy soil. Ilha Solteira, SP, Brazil, 2020.

Treatment	Nitrogen doses (kg ha^{-1})			
	0	30	60	120
C (1)	28.9	37.4	32.2	36.0
R (2)	37.5	37.1	32.3	36.3
F+I+R (3)	36.1	30.9	34.2	33.3
A (4)	38.6	32.5	34.9	36.5
F+I+A (5)	39.6	33.9	36.9	34.6
R+A (6)	37.2	36.6	34.4	35.4
F+I+R+A (7)	32.6	34.3	33.5	33.6

C - Control; F - Fungicide; I - Insecticide; R - *Rhizobium tropici*; A - *Azospirillum brasilense*.

There was no interaction effect between seed treatment and nitrogen top-dressing doses on vegetative and root growth, dry matter and grain yield of winter bean (Tables 2 and 3).

Plants showed similar heights in the seed treatments at the time of evaluation at bean flowering (R5) (Table 2). However, there was an increasing linear effect of N doses in coverage (Figure 2A), demonstrating the role of N in the vegetative growth of plants, even when inoculated with diazotrophic bacteria. A better agronomic performance of the crop was also seen by Rosa et al. (2020) with doses of 120 and 240 kg ha^{-1} of N, compared to 0, 30, and 60 kg ha^{-1}.

In the reading of the leaf chlorophyll index (LCI) there was a significant difference between the treatments, with higher values when using *R. tropici* (2), fungicide + insecticide + *R. tropici* (3), and fungicide + insecticide + *A. brasilense* (4), while the control treatment (1), presented the lowest values, demonstrating significant N deficiency for use in the formation of photosynthetic pigment and plant development (Table 2), therefore, with the effect of diazotrophic bacteria applied alone in bean seeds. There was also a positive linear effect of N doses (Figure 2B), due to the increase in

Table 2. Averages of plant height (PH), leaf chlorophyll index (LCI), fresh (FMAP) and dry (DMAP) matter production of aerial part per plant, root volume per plant (RVP), fresh (RFM) and dry (RDM) matter production of roots per plant of winter bean with doses of nitrogen in coverage, in sandy soil. Ilha Solteira, SP, Brazil, 2020.

	PH (cm)	LCI	FMAP (g)	DMAP (g)	RVP (cm^3)	RFM (g)	RDM (g)
Treatment	0.14 ns	0.0001*	0.0001*	0.0001*	0.05*	0.0001*	0.0001*
Dose	0.0001*	0.0001*	0.0001*	0.14 ns	0.0001*	0.0001*	0.0001*
Treatment*Dose	0.39 ns	0.18 ns	0.30 ns	0.34 ns	0.21 ns	0.09 ns	0.12 ns
C (1)	92.09	40.83 c	19.97 c	9.55 c	26.30 b	19.08 b	14.44 b
R (2)	96.84	45.76 a	21.01 c	9.58 c	25.00 b	19.82 b	14.87 b
F+I+R (3)	96.13	45.17 a	24.16 a	10.43 b	25.38 b	23.45 a	17.41 a
A (4)	96.88	44.41 b	20.84 c	9.52 c	28.06 b	24.26 a	17.85 a
F+I+A (5)	105.25	46.67 a	25.70 a	11.64 a	27.59 b	18.94 b	13.91 b
R+A (6)	92.63	44.09 b	22.52 b	10.36 b	31.52 a	21.34 b	15.54 b
F+I+R+A (7)	99.81	43.73 b	22.70 b	10.58 b	27.55 b	20.44 b	15.57 b
Mean	97.09	44.38	22.41	10.24	27.34	21.05	15.66
CV (%)	14.29	5.25	13.54	15.72	21.37	13.90	15.05
0	88.75	41.97	19.88	9.64	23.70	15.58	12.66
30	97.73	43.48	22.15	10.29	26.73	20.44	16.10
60	98.95	45.04	23.25	10.49	29.20	20.76	14.67
120	102.93	47.03	24.37	10.54	29.76	27.40	19.20

C - Control; F - Fungicide; I - Insecticide; R - *Rhizobium tropici*; A - *Azospirillum brasilense*. Means followed by different letters in the column differ by the Scott-Knott test ($p < 0.05$).

Table 3. Averages of number of pods per plant (NPP), grains per plant (NGP), grains per pod (NGPo) and grain mass per plant at 13% moisture (GM) of winter bean with doses of nitrogen in coverage, in sandy soil. Ilha Solteira, SP, Brazil, 2020.

	NPP	NGP	NGPo	GM (g)
Treatment	0.22 ns	0.29 ns	0.68 ns	0.005 *
Dose	0.002 *	0.68 ns	0.001 *	0.92 ns
Treatment*Dose	0.55 ns	0.22 ns	0.75 ns	0.21 ns
C (1)	6.16	25.22	4.13	5.28 b
R (2)	6.53	24.63	3.87	5.12 b
F+I+R (3)	6.97	25.63	3.81	5.36 b
A (4)	6.63	24.25	3.71	5.10 b
F+I+A (5)	7.44	28.41	3.83	6.14 a
R+A (6)	6.69	24.75	3.76	5.28 b
F+I+R+A (7)	6.69	25.78	4.02	5.41 b
Mean	6.73	25.52	3.88	5.38
CV (%)	19.77	19.40	19.03	17.91
0	6.07	25.18	4.25	5.34
30	6.79	25.29	3.79	5.48
60	7.13	25.11	3.57	5.39
120	6.93	26.52	3.89	5.31

C - Control; F - Fungicide; I - Insecticide; R - *Rhizobium tropici*; A - *Azospirillum brasilense*. Means followed by different letters in the column differ by the Scott-Knott test ($p < 0.05$).

constitutive N of the chlorophyll molecule. For Viçosi & Pelá (2020), in plants with and without *R. tropici*, the increase in the dose of nitrogen in coverage (0, 30, 60, 90, and 120 kg ha^{-1}) increased the content of the nutrient in the aerial part, but reduced the efficiency of its use, as well as the root dry mass and the number of nodules.

The fresh and dry matter of aerial part differed according to the use of seed treatments. The use of fungicide + insecticide + *A. brasilense* (5), stood out with higher values than the others, followed by treatments 3 (fungicide + insecticide + *R. tropici*), 7 (fungicide + insecticide + *R. tropici* + *A. brasilense*), and 6 (*R. tropici* + *A. brasilense*), also indicating a lower development of aerial part in the treatments with only *R. tropici* (2), only with *A. brasilense* (4), and in the control (1) (Table 2).

Therefore, the effect as growth-promoting bacteria, and increase in plant dry matter production, was more evident in the inoculation of the bacteria with the seed treatment (fungicide + insecticide) or in the co-inoculation of both, regardless if in seeds treated with the pesticides. There was also a significant positive linear effect of N doses in the increase in fresh matter of aerial part of the plants (Figure 2C) and also a linear increase in the dry matter of aerial part (Figure 2D).

Regarding root volume, the treatment with the combination of the two bacteria, *R. tropici* and *A. brasilense* (6), was superior to the others (Table 2). There was also a significant and positive adjustment in plant root volume due to N top-dressing doses, indicating a linear increase in root system development (Figure 3A).

According to Riggs et al. (2001), the beneficial effects of *Azospirillum* on plants can be characterized as increased density and length of absorbing root hairs; increases in the rate of lateral root emergence and root surface volume; alteration of root respiration and activities of enzymes of the glycolytic pathway and the tricarboxylic acid cycle; nitrite production; increased nutrient uptake and molecular signals that interfere with plant metabolism, including the contribution to the plant associated with fixed N. However,

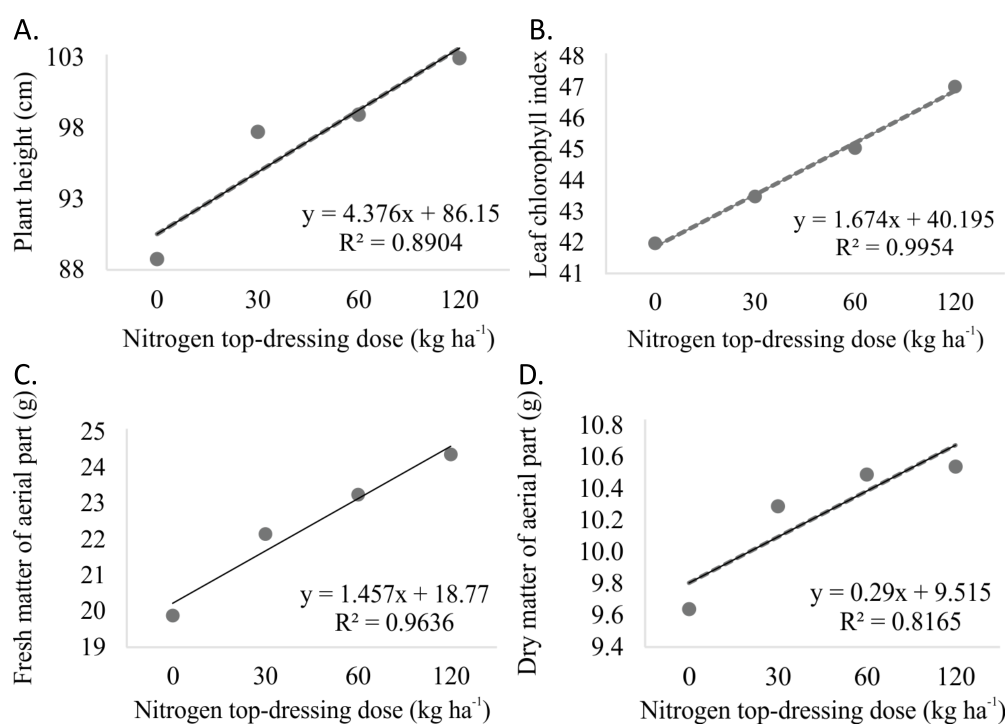


Figure 2. Adjustments of nitrogen doses for plant height (PH), leaf chlorophyll index (LCI), fresh (FMAP) and dry (DMAP) matter production of aerial part of winter bean with nitrogen doses in coverage, in sandy soil. Ilha Solteira, SP, Brazil, 2020.

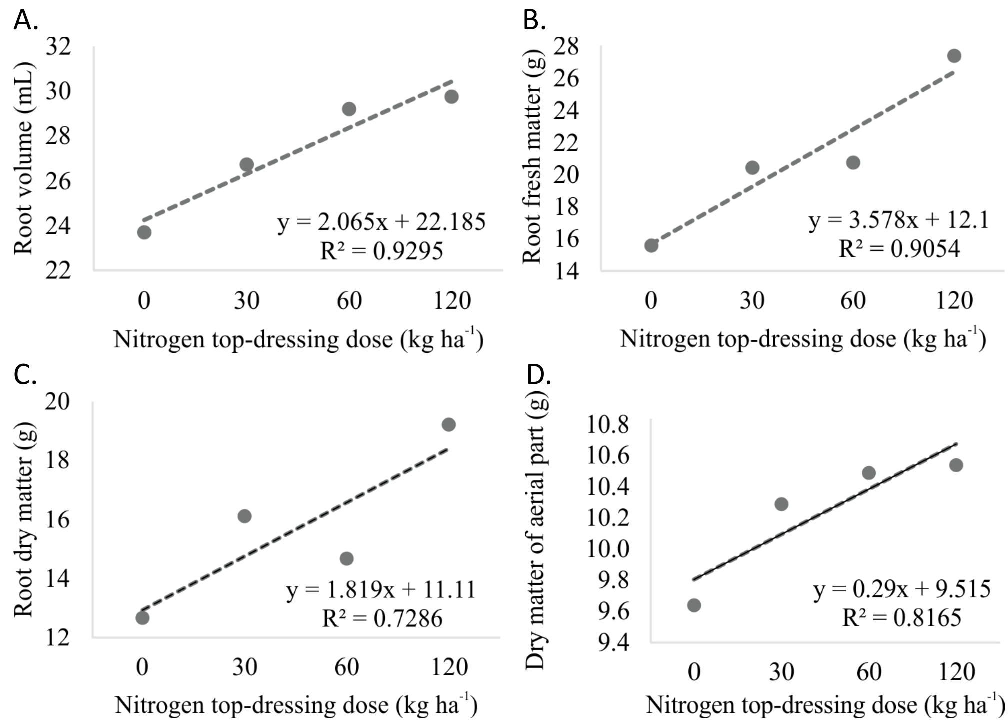


Figure 3. Adjustments of nitrogen doses for root volume per plant (RVP), fresh (RFM) and dry (RDM) matter production of roots and number of pods per plant (NPP) of winter bean with nitrogen doses in top-dressing, in sandy soil. Ilha Solteira, SP, Brazil, 2020.

under the conditions of this experiment, the exclusive use of *A. brasilense* in the seed treatment, or combined with fungicide and insecticide, did not provide an increase in the root volume of the plants (Table 2).

The results of fresh and dry matter of roots (Table 2) were significant among treatments, with the highest values in treatments 3 (fungicide + insecticide + *R. tropici*) and 4 (*A. brasilense*). For this effect on fresh and dry matter of roots, the fundamental role of N applied as top-dressing was also detected, with positive linear fits in sandy soil (Figures 3B and 3C).

The numbers of pods per plant, grains per plant, and grains per pod did not change significantly with the use of the different seed treatments, nor did the treatments interact with the doses of N in coverage (Table 3).

Due to the exclusive effect of N doses, there was a linear increase in the number of pods per plant (Figure 3D). Mellini et al. (2020) in a study with the cultivar BRS Estilo, observed that via seeds, inoculation with *R. tropici*, or with *A. brasilense*, co-inoculation with *R. tropici* + *A. brasilense* and fertilization with 80 kg ha^{-1} of N in coverage, despite significantly increasing the mass of 100 grains, did not provide significant increases in the number of pods per plant, grains per plant, grains per pod, and productivity, in relation to the control. Also for Gitti et al. (2012), seed inoculation with *A. brasilense* did not significantly influence plant development, production components and grain yield in bean cultivars evaluated in winter sowing.

For this variation in results, Andrade et al. (2015) argue that climate is one of the elements that most influence the performance of the bean crop, which is characterized by being very sensitive to extreme environmental factors.

Based on the climatic data of the agrometeorological station located in the municipality of Ilha Solteira, SP, Brazil, referring to the period of conduction of the winter bean in the year 2020 (Figure 1), the means of the minimum, average, and maximum temperatures were considerably high, respectively, of 16.9, 24.1, and 32.4 °C, and those of relative air humidity were 34.9, 59.4, and 86.4%, reaching the minimum value of 14.1%, considered as “alert state” by the Instituto Nacional de Meteorologia - INMET.

Thus, plants may have suffered negative impacts from the combination of high temperatures and very low percentages of relative air humidity recorded during this period. Therefore, although the water demanded during the bean cycle was daily monitored and supplied, the stress generated in plants by high evapotranspiration rates must be considered.

In the productive aspect, treatment 5 (fungicide + insecticide + *A. brasilense*) was the one that stood out, being the only one to present a significant increase in the mass of grains produced per plant (Table 3). There was no interaction between treatments and N doses for grain mass per plant, as well as no significant adjustments for N doses in coverage. Differently, Hungria et al. (2013), in experiments with bean plants carried out for three years of cultivation in two locations in Paraná, Brazil, demonstrated that the co-inoculation of *R. tropici*, via seeds, and *A. brasilense*, via sowing furrow, resulted in an increase in grain yield, on average 19.6% compared to the population of native rhizobia and 14.7% when compared to inoculation exclusively with rhizobia. Therefore, when placed in seed co-inoculation, the bacteria apparently have a competitive effect on the rhizosphere, minimizing the effect on bean grain yield, even with increased root dry matter production (Table 2).

To minimize possible competition between the bacteria in the rhizosphere, studies currently focus on the application of one of them in the seed treatment, usually rhizobium, and the other in the seeding furrow (*Azospirillum*). This technique can make co-inoculation feasible, as evaluated by Tocheto & Boiago (2020) who using the cultivar IPR TUIUIÚ, with *R. tropici* and *A. brasilense* associated, they compared control, co-inoculation via seeds (8 mL kg^{-1}), co-inoculation via sowing furrow (8 mL kg^{-1}), and co-inoculation in the seed and furrow (4 mL kg^{-1} + 4 mL kg^{-1}) and the results showed that the bean plant responded to the practice of co-inoculation via sowing furrow, with an increase in the number of nodules per plant, dry mass of aerial part, number of pods per plant, grains per pod, and yield, with a gain of 540 kg ha^{-1} in relation to the control and 358 kg ha^{-1} in relation to co-inoculation via seeds.

A point of reflection would be the use of treatment 5 (fungicide + insecticide + *A. brasilense*) via seed, with subsequent inoculations or co-inoculations in phenological stages by spraying growth-promoting bacteria in the soil, aiming at a greater production of radicles and formation of new nodules, which could increase the greater absorption of water and efficiency of BNF, resulting in greater productivity of the bean, as already evaluated in the soybean crop (Hungria et al., 2015; Moretti et al., 2018).

Working with doses of 0, 150, 300, 600, and 1200 mL of *R. tropici* inoculant per 50 kg of common bean seeds, Capristo et al. (2020) observed increases in grain yield at doses up to 300 mL, which presented a difference of approximately 620 kg ha^{-1}, compared to the control. Similar to the results obtained here, there were significant differences for green and dry matter of aerial part, plant height, green and dry matter of roots, and root length. However, there was no significant difference in leaf nitrogen, and number of grains per pods.

On the other hand, the regression models adjusted for the attributes plant height, leaf chlorophyll index, production of fresh and dry matter of aerial part, roots, root volume and number of pods in winter bean, as a function of the doses of nitrogen in coverage (Figures 2 and 3), were all linear up to the dose of 120 kg ha^{-1}. While there were no adjustments for the number of grains per plant (NGP), grains per pod (NGP), and grain mass per plant at 13% moisture (GM) (Table 3).

The presence of N in the soil stimulates the development of plants and may interfere in grain yield in different ways, in which low levels of N in the soil can promote the senescence of older leaves and consequently the decay in the photosynthetic rate due to the translocation of N from leaves to grains, thus decreasing the photosynthetic rate for the remaining leaves causing lower grain yield (Portes, 1996), while in the presence of higher doses, better the development of the vegetative part, however with risks of very high doses lead to lower grain production by the preconization of vegetative development and also by the decrease in BNF efficiency (Hungria et al., 2001).

It is observed that attention extends even to the use of rhizobium via seeds, which can be demonstrated by Silva et al. (2020), in a study on the action of fungicides on biological N fixation in bean (BRS Estilo). At the time, it was observed that seeds inoculated with *R. tropici* and not treated with fungicides showed greater potential for efficiency in biological fixation, when compared to seeds inoculated and treated with fungicides. In this case, in addition to the control, which was only inoculated, the treatments consisted of carbendazim + thiram (2 mL kg^{-1}), fludioxonil + metalaxyl (2 mL kg^{-1}), fluquinconazole (3 mL kg^{-1}), thiophanate methyl + fluazinam (1 mL kg^{-1}), and microbiological fungicide *Trichoderma asperellum* (1 g kg^{-1}). Although the total number of nodules was higher with *Trichoderma*, followed by the control and then the commercial fungicides, the highest number of viable nodules was in the control, then *Trichoderma* and then the fungicides. In addition, treatments with chemical fungicides showed an inverse relationship between the total number of nodules produced and the percentage of viable nodules, demonstrating that fungicides can both reduce the amount of nodules on bean roots and make them less effective in biological nitrogen fixation, a fact that aids in explaining the linear effect of N in coverage observed here.

In bean, Filipini et al. (2021) evaluated in a field experiment the association of *R. tropici* and *A. brasilense*, alone and in combination. The bacteria were inoculated on seeds, alone or in combination, associated or not with foliar spraying of *A. brasilense*. The application of *A. brasilense*, in the seed or by foliar spraying, and the inoculation of the seeds with *R. tropici*, had an additive effect, increasing biomass and accumulated nitrogen, thousand grain mass and grain yield.

The effect of inoculation associated with nitrogen fertilization on bean varies with sowing season, climatic conditions, soil and genetic materials, among others. Thus, Viçosi et al. (2020), working with seven pod bean cultivars (Commodore Improved, Contender, Delinel, Jade, Strike, Stringless Green, and Provider) sown with 50 kg ha^{-1} N, 300 kg ha^{-1} P_2O_5 and 100 kg ha^{-1} K_2O, using *R. tropici* (Semia 4077 and Semia 4088), did not observe interference in the vegetative development of the aerial part of the cultivars until the flowering stage. However, the cultivars Delinel, Jade, and Stringless Green stood out in terms of nodulation. Thus the authors concluded that inoculation negatively affected the uptake and content of nitrogen accumulated by the aerial part, but is able to increase the number of nodules, specific nodulation and nitrogen utilization efficiency, and that there is a negative correlation between nodulation and dry mass of aerial part, content and accumulation of nitrogen in the aerial part.

Conclusions

Co-inoculation of *Rhizobium tropici* and *Azospirillum brasilense* via bean seeds in winter cultivation did not increase yield components and grain yield.

Co-inoculation of *Rhizobium tropici* and *Azospirillum brasilense* via seeds without chemical treatment increased the root volume of the plants, without increasing grain yield.

The dry matter production of bean roots was higher when using *Azospirillum brasilense* alone, or when treating seeds with fungicide and insecticide, combined with inoculation with *Rhizobium tropici* in the seeds.

The combination of fungicide and insecticide with the inoculation of bean seeds with *Azospirillum brasilense* increased the production of dry matter of the aerial part and grains per plant.

Winter beans responded linearly in vegetative and root growth, and pod and grain yield per pod, up to the dose of 120 kg ha^{-1} of N in top-dressing, even with seed treatment with fungicide and insecticide, associated with diazotrophic bacteria in inoculation or co-inoculation.

Compliance with Ethical Standards

Author contributions: Conceptualization: DBC, MA; Data curation: DBC, MA, IMDS; Formal analysis: DBC, MA, IMDS; Investigation: DBC, MA, IMDS; PTS, MPBM, IPFS; Methodology: DBC, MA, IMDS; Project administration: DBC; Resources: DBC, MA, IMDS; PTS, MPBM, IPFS; Software: DBC, MA; Supervision: DBC, MA; Validation: DBC, MA, IMDS; PTS, MPBM, IPFS; Visualization: DBC; Writing – original draft: DBC, MA; Writing – review & editing: DBC, MA, IMDS; PTS, MPBM, IPFS.

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