

Co-Inoculation of Soybean with *Bradyrhizobium* and *Azospirillum* Promotes Early Nodulation

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Abstract

Soybean inoculation with elite strains of *Bradyrhizobium* to improve nodulation, N₂ fixation, and grain yield is well established worldwide. However, when grown in soils where N is deficient, soybean undergoes an initial phase of N starvation that may last up to 20 days after seedling germination due to the lack of synchronism between the phase when seed N reserves are exhausted and the moment when plants begin to benefit from the nitrogen fixed by the bacteria. Practices that promote early nodulation may play a key role in reducing the N starvation period. *Azospirillum* is a plant growth promoting rhizobacteria (PGPR) that can stimulate root hair formation and root growth, creating more sites for early root infection and nodule formation by N₂-fixing *Bradyrhizobium* spp. In this study, the effects of co-inoculating soybeans with *Bradyrhizobium* spp. and *Azospirillum brasilense* on nodulation precocity and N₂ fixation were evaluated under greenhouse and field conditions. Nodule number and dry weight, as well as plant and root dry weight and N accumulated in shoots at 15, 18, 21, 24 and 30 days after emergence (DAE) were evaluated in response to inoculation with *Bradyrhizobium* spp. alone or when co-inoculated with *Azospirillum* sp. In the greenhouse, co-inoculated plants nodulated precociously as indicated by a significant increase ($p < 0.05$) in nodule biomass observed at (include) 21 DAE. More pronounced effects of co-inoculation were observed in the field as early as 18 DAE, suggesting that the presence of *Azospirillum* helps plants to overcome environmental stresses.

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Keywords

Biological Nitrogen Fixation, Inoculation, Soybean, Symbiosis

1. Introduction

The practice of seed inoculation with nitrogen-fixing bacteria with the objective of increasing soybean [*Glycine max* (L.) Merr.] yield has long been successfully established worldwide. In Brazil, 100% of the areas grown with soybeans today have been inoculated at least once, and about 60% of these areas are re-inoculated every year [1] [2]. However, frequent climatic limitations and increased incidence of pests and diseases at sowing, among other factors, may reduce soybean nodulation, thus compromising nitrogen (N) supply to the crop.

When grown in soils where N is deficient, even when effective rhizobia are present, legumes may undergo an initial phase of N starvation that may last up to 15 to 20 days after seedling emergence [3]-[5]. This is due to the absence of synchronism between the phase when seed N reserves are exhausted and the moment when plants begin to benefit from the nitrogen fixed by the bacteria in the recently formed root nodules [3] [4] [6]. Therefore, any practice that promotes earlier nodulation may help to reduce the N starvation period.

Besides rhizobia, another group of beneficial soil microorganisms contains associative plant growth promoting rhizobacteria (PGPR) [7] which have also been exploited in agriculture with remarkable success. PGPR perform an array of biological processes that are beneficial to plants, including the production of plant growth hormones [8] [9], such as auxins [10] [11], gibberellins [12], and cytokinins [11] [13]. Other PGPR attributes include inducing plant resistance to stresses and diseases [14], solubilization of phosphates [15]-[18], and atmospheric N₂ fixation [19].

Among the PGPR, the genus *Azospirillum* has long been employed in plant inoculants worldwide, and, more recently, in Brazil too [20]. A clear result of the action of *Azospirillum* spp. is an increase in the production of root hairs [21] [22] and in root growth, benefiting plants with better absorption of water and nutrients [23]. The co-inoculation of soybeans with *Azospirillum* and *Bradyrhizobium* is known to result in increased N₂ fixation and grain yield [24]-[27].

Given that the formation of legume root nodules is initiated in root hairs [28], it is possible that co-inoculation with *Azospirillum* and *Bradyrhizobium* may result in earlier nodule formation in soybean due to the increased number of root hairs available to be infected by rhizobia. However, early nodulation is difficult to evaluate in the field because of the presence of previously established populations of both bacteria genera. Therefore, the objective of this study was to evaluate, under controlled conditions in the greenhouse, the effect of co-inoculation of soybean grown in sterile substrate with both *Azospirillum brasilense* and *Bradyrhizobium* spp., on the precocity of nodulation. The results obtained under the greenhouse were then validated in field conditions.

2. Materials and Methods

2.1. Experimental Site

Both the greenhouse and field experiments were conducted during the summer season of 2012/2013, at the experimental station of Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Soja (Embrapa Soja). The station is located in Londrina (23° 11'S, 51° 11'W, 620 m altitude, Köpen-Geiger climate type Cfa), in the State of Paraná, Brazil.

The field trial was performed in an oxisol (Latossolo Vermelho Eutroférico, Brazilian classification; Rhodic Eutrudox, American classification). At the beginning of the experiment, twenty soil subsamples (0 - 20 cm) were taken to evaluate soil chemical properties and soil granulometry, as described before [29]. Soil chemical properties, granulometry and bacterial population are shown in **Table 1**.

2.2. Bacterial Inoculants and Plant Cultivars Employed

Inoculants employed in this study were made of strains SEMIA 5079 (=CPAC 15) of *Bradyrhizobium japonicum*, SEMIA 5080 (=CPAC 7) of *B. diazoefficiens*, both at 2×10^9 cells·mL⁻¹, and Ab-V5 and Ab-V6 of *Azos-*

pirillum brasilense, both at 1×10^8 cells·mL⁻¹. All strains are used for commercial production of inoculants in Brazil. Inoculation treatments consisted of 1) a combination of both strains of *Bradyrhizobium*, without *Azospirillum*; and 2) a combination of inoculants containing the two strains of *Bradyrhizobium* and with the two strains of *Azospirillum*. For all experiments, the commercial soybean cultivar BRS 295RR, well adapted to the conditions of Londrina, was the host of choice.

2.3. Greenhouse Experiment

Seeds were surface disinfested by immersion in 80% ethanol for two to three minutes, followed by immersion in a 10% sodium hypochlorite (NaClO) solution for three to four minutes and then washed with sterile distilled water several times to remove all traces of hypochlorite [30].

Treatments consisted of the inoculation with 1) 1.2×10^6 cells seed⁻¹ of *Bradyrhizobium* spp. and 2) 1.2×10^6 cell seed⁻¹ of *Bradyrhizobium* spp. + 1.2×10^5 cells seed⁻¹ of *Azospirillum brasilense*. Seed inoculation was accomplished by mixing seeds and inoculants and leaving the mixtures in contact for half an hour before sowing.

After inoculation, four seeds were sown in each of the pre-sterilized modified Leonard jars [30] containing a 1:1 (v:v) mixture of sand and vermiculite and N-free nutrient solution with pH adjusted to 6.6 - 6.8 [31]. Seven days after sowing seedlings were thinned to two per jar. All along the experiment, jars received sterile N-free nutrient solution as needed.

Temperature and relative humidity of the air inside the greenhouse, and temperature at 2 - 3 cm inside the substrate in the Leonard jars were measured in the morning and afternoon throughout the duration of the experiment. Daily average air temperatures at 9 a.m. and 3 p.m. were $26.0^\circ\text{C} \pm 1.7^\circ\text{C}$ and $33.5^\circ\text{C} \pm 1.6^\circ\text{C}$, respectively. Daily average relative humidity of the air inside the greenhouse at 9 a.m. and 3 p.m. were $66.4\% \pm 13.7\%$ and $52.5\% \pm 7.9\%$, respectively. Daily average temperatures of the substrate in the jars at 9 a.m. and 3 p.m. were $26.4^\circ\text{C} \pm 1.7^\circ\text{C}$ and $30.4^\circ\text{C} \pm 3.0^\circ\text{C}$, respectively.

The experiment was conducted under a completely randomized design with a 2×5 factorial, being two inoculants, *Bradyrhizobium* spp. and *Bradyrhizobium* spp. + *A. brasilense*, and five sampling times, 15, 18, 21, 24, and 30 days after emergence (DAE), with four replicates.

2.4. Field Experiment

Before sowing the experiment, soil rhizobial population was estimated (**Table 1**) by the most-probable-number (MPN) technique using cultivar BRS 295RR as the trap host as described before [30]. For the estimation of the populations of free-living diazotrophic bacteria, counts were made in semi-solid NFB medium [32]. Soil pH (**Table 1**) was previously corrected to reach 5.5 - 5.8 at sowing. One week before sowing fertilizer was applied at a rate of $300 \text{ kg}\cdot\text{ha}^{-1}$ of a 0-28-20 NPK; no N fertilizer was applied. The same treatments and concentrations of cells per seed of the greenhouse experiment were tested in the field experiment. Seed inoculation was performed by mixing both inoculants with the seeds and allowing them to dry in the shade for 1 h. Seeds were sown in 10 m-long rows, with eight rows per replicate and distance between rows was of 50 cm. Plots were separated by 1.5 m-wide terraces to avoid cross contamination.

The experimental design was the same of the greenhouse experiment, but with six replicates. Five plant samples were taken from each of the six replicates at 15, 18, 21, 24 and 30 DAE, avoiding the two external lines.

Average maximum and minimum temperatures during the experiment were 28.8°C and 19.2°C , respectively, and accumulated rainfall was 202.4 mm well distributed in the 30 days of the experiment.

Table 1. Soil chemical properties, granulometry and population of soybean bradyrhizobia and free-living diazotrophic bacteria at the 0 - 20 cm layer of the field site in Londrina. Analyses performed before sowing.

pH (CaCl ₂)	H + Al	K	Ca + Mg	P	C	SB ^a	BS ^b	Granulometry			<i>Bradyrhizobium</i> ^c	Diazotrophic ^d
								cmol _c ·dm ⁻³	mg·dm ⁻³	g·dm ⁻³		
5.35	5.8	0.43	5.62	7.77	21.68	6.05	59.11	71.0	8.2	20.8	1.79×10^4	9.0×10^6

^aSB, sum of bases; ^bBS, bases saturation = $[(K + Ca + Mg)/T_{\text{ec}}] \times 100$, where $T_{\text{ec}} = K + Ca + Mg + \text{total acidity at pH 7.0 (H + Al)}$; ^cEstimated by the most probable number (MPN) method [30] using soybean as trap plants; CFU, colony forming units; ^dEstimated by dilutions and counts in semi-solid NFB medium [32].

2.5. Evaluation of Nodulation, Dry Matter Accumulation and N Content in the Shoots

From the greenhouse experiment, at each sampling date, all plants from four replicates were cut at the cotyledonary node, separating roots and shoots. Shoots were stored in paper bags and dried in the oven (approximately 50°C, 72 h) until constant weight was obtained. Roots were carefully removed from the jars to avoid nodule loss and were rinsed over a sieve. The roots and all the nodules collected were dried until constant weight was obtained. After drying, nodules were removed from the roots and allowed to dry further. Nodules were then counted and dry nodules and shoots were weighed.

From the field, five plants were randomly taken from each plot, digging carefully around the roots with a straight shovel. Shoots were separated from the roots at the cotyledonary node. Excess soil was removed from the roots over a sieve in order to avoid nodule loss. All other procedures were performed as in the greenhouse experiment.

After weighing the material from the greenhouse and the field, shoots were ground (18 mesh) and employed for determination of N content by the salicylate green spectrophotometric method [33], with readings taken at 697 nm.

2.6. Statistical Analyses

Data were analyzed with SISVAR [34] statistical package. Data were also statistically analyzed by analysis of variance (ANOVA) preceded by verification of normality of residues and variance homogeneity [35].

3. Results and Discussion

In the greenhouse, plants responded positively but not significantly to the dual inoculation with *Bradyrhizobium* and *Azospirillum* when nodule number (NN), root (RDW) and shoot (SDW) dry weight were analyzed (Table 2). However, a significant increase ($p < 0.05$) in nodule dry weight (NDW) and precocity of nodulation was observed in response to co-inoculation at 21 DAE, as well as total N in shoots (TNS) at 24 DAE (Table 2).

Correlation coefficients among all variables analyzed are presented in Table 3. Although a highly significant ($r = 0.92$, $p < 0.001$) correlation between NN and NDW was observed, NDW was more strongly correlated with SDW ($r = 0.95$, $p < 0.001$). Positive and highly significant ($r = 0.95$, $p < 0.001$) correlation between SDW and TNS was also observed.

Highly significant correlation between NDW and TNS has been reported previously on soybean both under greenhouse and field conditions [36]-[40]. Correlation coefficients between NDW and TNS obtained in this study are similar to those reported from soybean experiments grown in sterile substrate under greenhouse controlled conditions (means of three strains of *Bradyrhizobium* on 152 cultivars, $r = 0.80$, $p < 0.001$ [36]) and in pots containing non-sterile soil [means of 152 cultivars, $r = 0.697$, $p < 0.001$ [38]]. Furthermore, it has been demonstrated that NDW is the best variable to evaluate biological N₂ fixation in the field [40], since it is highly correlated with SDW and TNS, which was confirmed in this study. It is also important to mention the high correlation between SDW and TNS obtained in our study, emphasizing that under N-limiting conditions the simple measurement of SDW could be used to assess nitrogen fixation, eliminating the need to determine TNS [36] [38]-[41].

Differences due to the inoculation treatments were more noticeable in the field (Table 4) than in the greenhouse (Table 2), where growth conditions were controlled and optimized. Significant ($p < 0.05$) differences in NN in response to co-inoculation could be observed at 21 and 24 DAE, whereas differences in NDW could be observed as early as 18 DAE till the last evaluation (Table 4). At this early sampling time, the benefits due to the presence of *Azospirillum* under field conditions, where environmental stresses are frequent, were translated into an expressive 90% increase in NDW very early in the growth stage (18 DAE) (Table 4).

The increased nodulation due to co-inoculation reflected in larger ($p < 0.05$) plant biomass (SDW) from 18 to 21 DAE and higher nitrogen fixation, indicated by a greater ($p < 0.05$) TNS from 18 DAE to the last evaluation, at 30 DAE. Considering all five evaluations, co-inoculated plants were 18% superior to those that received only *Bradyrhizobium* inoculation when SDW was analyzed, and 32% superior when TNS was considered (Table 4).

The results from this study are in line with those from previous studies on the benefits of co-inoculation of soybeans with *Bradyrhizobium* spp. and *A. brasilense* on nodule numbers [26] [42]-[44], nodule dry matter [24] [42], and root dry matter [25] [43] [45]. It is possible that the increased nodulation of co-inoculated plants is a

Table 2. Nodule number (NN) and dry weight (NDW), root (RDW) and shoot (SDW) dry weight and total N accumulated in the shoots (TNS) of soybean plants, cultivar BRS 295RR, inoculated with *Bradyrhizobium* alone or co-inoculated with *Bradyrhizobium* and *Azospirillum*, and grown in sterile substrate under controlled greenhouse conditions. Plant samples were harvested at 15, 18, 21, 24 and 30 days after emergence (DAE).

Treatments ¹	Sampling time (DAE)				
	15	18	21	24	30
NN (n° pl ⁻¹)					
Brady	15.5 c ² A	20.7 c A	24.6 c A	36.9 b A	48.6 a A
Brady + Azo	21.1 c A	20.2 c A	30.5 cb A	38.8 ba A	47.0 a A
NDW (mg·pl ⁻¹)					
Brady	64.8 c A	86.3 c A	96.6 c B	161.6 b A	210.6 a A
Brady + Azo	68.9 d A	81.0 d A	120.5 c A	162.8 b A	225.9 a A
RDW (g·pl ⁻¹)					
Brady	0.30 c A	0.35 c A	0.40 c A	0.61 b A	0.79 a A
Brady + Azo	0.30 c A	0.35 cd A	0.45 c A	0.61 b A	0.81 a A
SDW (g·pl ⁻¹)					
Brady	0.54 c A	0.72 c A	0.80 c A	1.56 b A	2.43 a A
Brady + Azo	0.58 d A	0.64 cd A	0.94 c A	1.58 b A	2.49 a A
TNS (mg N pl ⁻¹)					
Brady	16.65 c A	26.60 c A	24.59 c A	51.91 b B	78.99 a A
Brady + Azo	20.83 b A	23.14 b A	30.56 b A	68.52 a A	78.27 a A

¹Brady = *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080; Azo = *A. brasilense* strains Ab-V5 e Ab-V6; ²Data are means of four replicates and when followed by distinct lower case letters on the same line are significantly different; when followed by different uppercase letters on the column, data are significantly different for the variable they represent, within the same sampling time. Mean comparisons performed by Tukey's test at $p < 0.05$.

Table 3. Spearman rank correlation matrix¹ among variables nodule number (NN) and dry weight (NDW), root (RDW) and shoot (SDW) dry weight and total N accumulated in the shoots (TNS) of soybean plants, cultivar BRS 295RR analyzed in the greenhouse experiment.

Variables	NN	NDW	RDW	SDW	TNS
NN	1.00	0.92	0.93	0.91	0.84
NDW		1.00	0.97	0.95	0.89
RDW			1.00	0.95	0.87
SDW				1.00	0.95
TNS					1.00

¹ $p < 0.001$ for all variables.

response to the alterations caused by *Azospirillum* in root morphology [18] [46], resulting in more nodulation sites. Interestingly, there are also reports that co-inoculation increases nodulation of the root crown and of the tap root [25] [43] [47] [48] forming nodules that are critical to seedling establishment and higher rates of nitrogen fixation [38] [41] [49]. The fact that co-inoculation of legumes with rhizobia and *Azospirillum* results in increased nodulation of the root crown, when compared to the inoculation of rhizobia alone [50] suggests that *Azospirillum* may induce earlier nodulation, since the nodules present in the root crown are the first to be formed and are directly related to the inoculated strains of rhizobia [36] [41] [49] [50]. Therefore, it is possible that the

Table 4. Nodule number (NN) and dry weight (NDW), shoot dry weight (SDW) and total N accumulated in shoots (TNS) of soybean plants, cultivar BRS 295RR, inoculated with *Bradyrhizobium* alone or co-inoculated with *Bradyrhizobium* and *Azospirillum*, and grown under field conditions. Plant samples were taken at 15, 18, 21, 24, and 30 days after emergence (DAE).

Treatments ¹	Sampling time				
	15	18	21	24	30
	NN (n° pl ⁻¹)				
Brady	10.8 c ² A	15.2 bc A	18.3 bc B	20.2 ab B	26.2 a A
Brady + Azo	15.3 b A	20.4 b A	30.5 a A	33.2 a A	35.0 a A
	NDW (mg·pl ⁻¹)				
Brady	31.2 c A	43.2 bc B	45.6 bc B	63.8 ab B	88.2 a B
Brady + Azo	38.4 d A	82.0 c A	115.2 b A	123.4 b A	155.8 a A
	SDW (g·pl ⁻¹)				
Brady	0.46 c A	0.52 c B	1.51 b B	2.03 b A	2.98 a A
Brady + Azo	0.68 c A	1.12 c A	2.21 b A	2.68 b A	3.53 a A
	TNS (mg N pl ⁻¹)				
Brady	13.7 c A	16.6 c B	46.8 c B	71.1 b B	104.3 a B
Brady + Azo	21.4 b A	39.2 b A	79.6 b A	104.5 a A	137.7 a A

¹Brady = *Bradyrhizobium japonicum* strain SEMIA 5079 and *Bradyrhizobium diazoefficiens* strain SEMIA 5080; Azo = *Azospirillum brasilense* strains Ab-V5 and Ab-V6; ²Data are means of six replicates and when followed by distinct lowercase letters on the same line are significantly different; when followed by different uppercase letters on the column, data are significantly different for the variables they represent, within the same sampling time. Mean comparisons performed by Tukey's test at $p < 0.05$.

increased nodulation of co-inoculated plants is a response to the alterations caused by *Azospirillum* in root morphology [18] [46], resulting in more nodulation sites.

It was very important to observe that co-inoculation resulted in earlier nodulation. Precocious nodule formation is very critical for the establishment of the symbiosis and onset of N₂ fixation, particularly in the case of crops with a short growth cycle, as is the case with soybean. The positive effects of co-inoculation were even more evident in the field, where limiting factors that affect nodulation, such as temperature and rainfall frequently result in poor establishment of the symbiosis [51] [52].

Increased plant growth observed in this study in response to inoculation with *Azospirillum* is in agreement with previously reported results [25] [26] [43] [47] and may be attributed to increased availability of N, due to nitrogen fixation [18] and to the presence of hormones that stimulate plant root growth [25] [43] [48] [50] among other factors.

In previous studies done by our research group, we observed that co-inoculation of soybean with *Bradyrhizobium* and *Azospirillum* has resulted in increased grain yield [26] [53], and the technology has been approved and registered for utilization with soybean by the Brazilian regulatory agency. In the present study, we observed that co-inoculation is beneficial throughout the plant's growth cycle, since it favors the precocity of nodulation, a critical step for the establishment of the symbiotic relationship between soybean plants and N₂-fixing bacteria. Earlier nodulation may be particularly important for crops with short growth cycles such as soybean, thus extending and increasing the benefits of inoculation and N₂ fixation.

4. Conclusion

Co-inoculation of soybean with *Bradyrhizobium* spp. and *Azospirillum brasilense* results in earlier nodulation of soybean plants.

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