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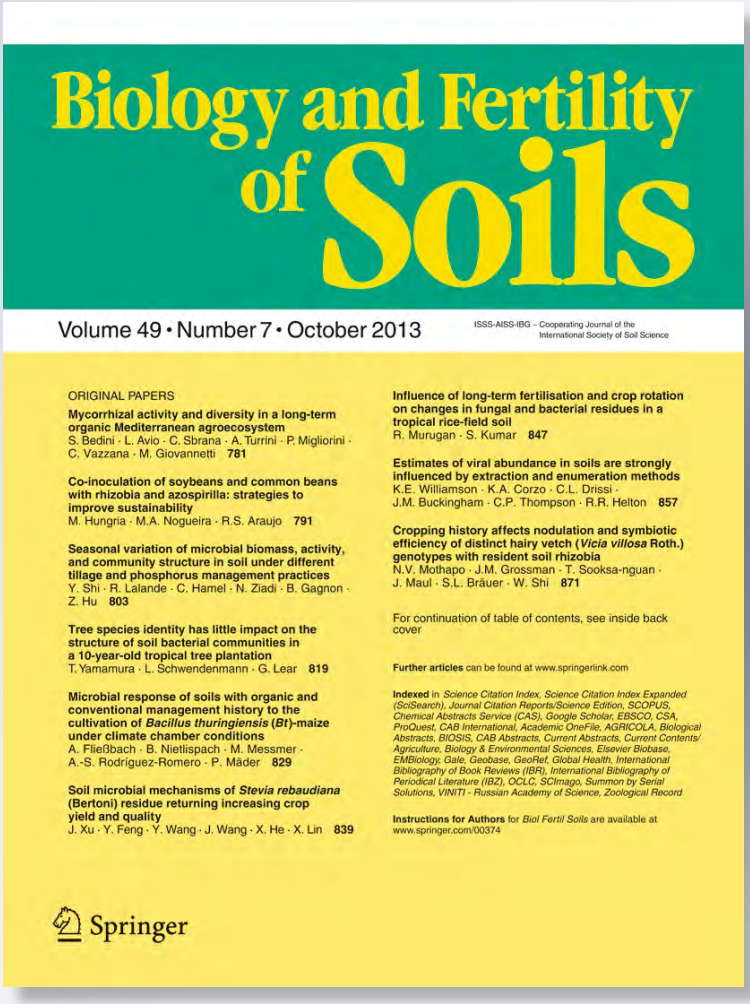


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ORIGINAL PAPER

# Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability

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**Abstract** Plant–microorganism associations have long been studied, but their exploitation in agriculture partially or fully replacing chemical fertilizers is still modest. In this study, we evaluated the combined action of rhizobial and plant growth-promoting rhizobacteria inoculants on the yields of soybean and common bean. Seed inoculation with rhizobia ($1.2\times10^6$ cells seed$^{-1}$) was compared to co-inoculation with *Azospirillum brasilense* in-furrow (different doses) or on seeds ($1.2\times10^5$ cells seed$^{-1}$) in nine field experiments. The best in-furrow inoculant dose was $2.5\times10^5$ cells of *A. brasilense* seed$^{-1}$ for both crops. Inoculation with *Bradyrhizobium japonicum* increased soybean yield by an average 222 kgha$^{-1}$ (8.4 %), and co-inoculation with *A. brasilense* in-furrow by an average 427 kgha$^{-1}$ (16.1 %); inoculation always improved nodulation. Seed co-inoculation with both microorganisms resulted in a mean yield increase of 420 kgha$^{-1}$ (14.1 %) in soybean relative to the non-inoculated control. For common bean, seed inoculation with *Rhizobium tropici* increased yield by 98 kgha$^{-1}$ (8.3 %), while co-inoculation with *A. brasilense* in-furrow resulted in the impressive increase of 285 kgha$^{-1}$ (19.6 %). The cheaper, more sustainable inoculated treatment produced yields equivalent to the more expensive non-inoculated + N-fertilizer treatment. The results confirm the feasibility of using rhizobia and azospirilla as inoculants in a broad range of agricultural systems, replacing expensive and environmentally unfriendly N-fertilizers.

**Keywords** BNF · *Bradyrhizobium* · *Azospirillum* · *Rhizobium* · Co-inoculation · *Glycine max* · PGPR · *Phaseolus vulgaris*

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## Introduction

Many legumes can establish a symbiotic partnership with specific bacteria collectively called rhizobia, which possess an enzymatic complex denominated dinitrogenase that enables them to capture atmospheric nitrogen and reduce it to ammonia, followed by the incorporation into nitrogenous forms that can be assimilated by the host plant. Brazil has been considered as a model country in the application of the benefits of biological nitrogen fixation, especially for the use of elite strains of *Bradyrhizobium japonicum*/*Bradyrhizobium elkanii* in soybean [*Glycine max* (L.) Merr.], fulfilling the plant's demand on N (Hungria et al. 2005, 2006a, b).

Another group of beneficial soil microorganisms is represented by associative bacteria capable of promoting plant growth by means of several biological processes (Chaparro et al. 2012), including the production of several plant growth hormones as auxins (Ashraf et al. 2011; Tien et al. 1979), gibberelins (Bottini et al. 1989), cytokinins (Strzelczyk et al. 1994; Tien et al. 1979), ethylene (Strzelczyk et al. 1994), the capacity to induce plant resistance to diseases and stresses (Wang et al. 2009), to solubilize phosphate (Rodriguez et al. 2004), and also biological nitrogen fixation (Ashraf et al. 2011; Döbereiner and Pedrosa 1987). Among these bacteria

those belonging to the genus *Azospirillum* are certainly the most studied and employed as inoculants worldwide, including Brazil (Hungria et al. 2010).

Soybean is a key cash crop in Brazil and the fixation of as much as 300 kg of N $ha^{-1}$ in addition to the release, in the soil, of 20–30 kgN $ha^{-1}$ for the following crop are current estimates (Hungria et al. 2005, 2006a, b). Continuous research has been performed to guarantee the benefits to more productive cultivars. However, the contribution of the biological process is threatened by global weather changes, with increasing periods of drought and high temperatures, and Brazil is not an exception (Zullo et al. 2008). Environmental stresses help to increase the susceptibility to diseases, leading to seed treatment with a variety of fungicides and insecticides that are currently used in more than 90 % of the soybean cropped in Brazil and that can be highly deleterious to the rhizobia (Campo et al. 2009). Any factor that reduces nodulation may result in decreased $N_2$ fixation, leading to the search for strategies to minimize impacts of seed treatments with fungicides and insecticides, such as the inoculation in-furrow (Campo et al. 2010).

In the case of common bean (*Phaseolus vulgaris* L.)—a cheap source of protein for the world's increasing population—there are still concerns about the capacity of biological nitrogen fixation to support plant growth and crop yield. Limitations to the symbiosis is attributed to plant breeding focused on N-fertilizer, highly competitive but poorly effective indigenous population of rhizobia in soil, and high susceptibility to diseases and environmental stresses (Graham 1981; Hungria and Vargas 2000). However, increases in nodulation, nitrogen fixation and grain yield have been observed when selected elite strains are used as inoculants (Hungria et al. 2000, 2003).

Considering the main limitations to the biological $N_2$ fixation with soybeans and common beans inoculated with rhizobia and the benefits to crop growth attributed to *Azospirillum*, co-inoculation with both microorganisms might improve plant's performance. This approach is current with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al. 2012). Despite reports on the benefits of co-inoculation of rhizobia and azospirilla, very few field experiments have been performed with rhizobia and azospirilla or other plant growth-promoting rhizobacteria (PGPR), and none under the Brazilian conditions.

In this study, we report the results from experiments performed during three crop seasons with co-inoculation of soybean and common bean with rhizobia and azospirilla. Co-inoculation of microorganisms with different functions represents a simple, cheap but innovative tactic that is still far from being adopted in large-scale agriculture, and the results described here encourage the use of this microbial approach in the tropics.

## Materials and methods

### Sites description

Nine experiments were conducted with soybean (*G. max* (L.) Merr.) and common bean (*P. vulgaris* L.) for three consecutive crop seasons (Table 1). In all crop seasons, experiments were established in two ecosystems of the State of Paraná, southern Brazil, in the districts of Londrina and Ponta Grossa. The experimental station of Embrapa Soja, Londrina (23°11′S, 51°11′W) is at 620 m of altitude and the climate is classified as Cfa (Köppen's classification); the trials were performed on an oxisol, Latossolo Vermelho Eutroférrico (Rhodic Eutrudox). In Ponta Grossa (25°13′ S and 50°1′ W) the altitude is 880 m, climate is classified as Cfb and the trials were performed at the Service of Production of Basic Seeds of Embrapa, on an oxisol, Latossolo Vermelho Distrófico (Typic Hapludox). The experiments were performed in these two sites, but in each season it was in a different area of the same site.

In each site, at the onset of experimentation, 20 soil subsamples (0–20 cm) were taken to evaluate soil chemical properties and soil granulometry. Chemical analysis followed basic procedures (Klute 1986; Sparks 1996), as described before (Hungria et al. 2006b). Before being analyzed, soil samples were dried (60 °C for 48 h) and sieved (2-mm). Soil pH was determined in 0.01 M $CaCl_2$ (1:2.5; soil/solution), after agitation for 1 h. Exchangeable Ca, Mg and Al were determined in the extract obtained with 1 mol $L^{-1}$ KCl (1:10; soil/solution) after agitation for 10 min. P and K contents were evaluated in the Mehlich-1 (0.05 mol $L^{-1}$ HCl+0.0125 mol $L^{-1}$ $H_2SO_4$) extract (1:10; soil/solution) after agitation for 10 min. Aluminum was determined by titration with 0.015 Mo $L^{-1}$ standardized NaOH, using bromothymol blue as indicator. Concentrations of Ca and Mg were determined in an atomic absorption spectrophotometer, K in a flame photometer and P by colorimetry, using the molybdenum-blue method and ascorbic acid as reducing agent. Carbon was determined by the oxidation of dichromate. Soil characteristics before sowing at each site are shown in Table 2.

The rhizobial populations at each site were estimated using the most probable number technique (Vincent 1970) and statistical tables with soybean plants of cultivar BRS 133 and common bean of cultivar IPR-Colibri. Rhizobial populations at each site are shown in Table 1.

### Field management

About 50 days before starting the experiment, soil pH values were determined at each site and lime was applied to alleviate acidity. The amount of applied lime was estimated for a saturation of bases of 70 % to increase the pH to approximately 5.5. Thirty days before sowing glyphosate was applied (1.5 L $ha^{-1}$).

**Table 1** Locations, seasons, dates, plant species, cultivars, and naturalized rhizobial populations at the sites where field experiments were performed

| Season | Crop | Site | Cultivar | Sowing | Harvest | Harvested area[a] | Rhizobial cells (CFU $g^{-1}$ soil)[b] |
|---|---|---|---|---|---|---|---|
| Summer season 2009/2010 | Soybean | Londrina | BRS 133 | 11/06/2009 | 03/09/2010 | 8 $m^2$ | $3.57\times10^3$ |
| | Soybean | Ponta Grossa | BRS 133 | 11/19/2009 | 04/08/2010 | 8 $m^2$ | $4.27\times10^3$ |
| | Common bean | Londrina | IPR-Colibri | 11/04/2009 | 01/14/2010 | 6 $m^2$ | $3.57\times10^3$ |
| | Common bean | Ponta Grossa | IPR-Colibri | 12/02/2009 | 02/23/2010 | 6 $m^2$ | $3.57\times10^3$ |
| Dry season 2010 | Common bean | Londrina | IPR-Colibri | 02/20/2010 | 05/30/2010 | 6 $m^2$ | $3.14\times10^4$ |
| | Common bean | Ponta Grossa | IPR-Colibri | 02/26/2010 | 06/07/2010 | 7.2 $m^2$ | $3.14\times10^4$ |
| Summer season 2010/2011 | Soybean | Londrina | BRS 133 | 11/04/2010 | 03/21/2011 | 8 $m^2$ | $1.79\times10^4$ |
| | Soybean | Ponta Grossa | BRS 133 | 11/25/2010 | 04/13/2011 | 8 $m^2$ | $2.14\times10^4$ |
| | Common bean | Londrina | IAPAR 81 | 11/04/2010 | 02/07/2011 | 8 $m^2$ | $3.38\times10^4$ |

[a] Plot sizes varied from 4×6 (24 $m^2$) to 5.5×6 (33 $m^2$)

[b] Estimated by the most probable number (MPN) method (Vincent 1970) using soybean and common bean as trap plants

For both soybean and common bean, 300 kg $ha^{-1}$ of N–P–K (0–28–20) were applied in furrow immediately before sowing, as recommended for the soybean crop in Brazil. No N-fertilizer was applied, except for the N-fertilizer control plots, as detailed below. Also, for both crops, at V4 stage of soybean [four nodes on the main stem with fully developed leaves, beginning with the unifoliolated node (Fehr and Caviness 1977)] and 25 days after emergence (DAE) for common bean, plants received 20 g $ha^{-1}$ of Mo (as $Na_2MoO_4{\cdot}2H_2O$) and 2 g $ha^{-1}$ of Co (as $CoCl_2{\cdot}6H_2O$) as a foliar spray.

For soybean, fungicide (fluquinconazole, 50 g a.i. $ha^{-1}$) against soybean rust (*Phakopsora pachyrhizi*) was applied in the second year, after flowering. For the common bean, insecticide against *Elasmopalpus lignosellus* was applied, as methyl-carbamate at a rate of 1.50 L 100 kg $seed^{-1}$.

The cultivars used in each experiment are shown in Table 1.

## Inoculants and experimental design

According to the Brazilian legislation, inoculants containing rhizobia must carry at least $10^9$ cells $g^{-1}$ or $mL^{-1}$ of inoculant, and for *Azospirillum*, a new product in the market, $10^8$ cells $g^{-1}$ or $mL^{-1}$. In the last 10 years, legal requirements for rhizobia in inoculants increased from $10^8$ to $10^9$ cells, as the industrial technology has considerably improved; therefore, increases in the concentration of cells for *Azospirillum*-based inoculants are also expected. The technical recommendation for both the soybean and common bean crops is that the dose of inoculant must supply 1.2 million cells $seed^{-1}$. When in-furrow inoculation replaces seed inoculation, the concentration should be increased to at least 2.5 million cells $seed^{-1}$. Considering actual legislation and expectations for the next years, we have decided to work with estimates of number of cells $seed^{-1}$. Therefore, for this study, rhizobial inoculation consisted of seed inoculation: for soybean, with *Bradyrhizobium japonicum* commercial strains SEMIA 5079 and SEMIA 5080 (in Brazil, soybean inoculants carry two strains and this combination represents more than 80 % of the commercial inoculants sold in country), and for common bean, with *Rhizobium tropici* strain SEMIA 4080 (=PRF 81), always supplied at $1.2\times10^6$ cells $seed^{-1}$ (common bean commercial inoculants in Brazil carry only one strain). For *Azospirillum brasilense*, the commercial strains

**Table 2** Chemical properties of the soils (0–20 cm) performed before sowing

| | | | pH | H+Al cmolc/$dm^3$ | Al | P mg/$dm^3$ | K cmolc/$dm^3$ | C g/$dm^3$ | Ca+Mg | SB[a] | CEC[a] | BS % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Summer (rainy) season 2009/2010 | Londrina | Soybean/common bean | 5.72 | 3.01 | 0.02 | 6.48 | 0.58 | 19.71 | 9.00 | 9.59 | 12.60 | 76.07 |
| | Ponta Grossa | Soybean/common bean | 5.25 | 3.94 | 0.00 | 3.36 | 0.22 | 20.85 | 5.54 | 5.76 | 9.71 | 59.39 |
| Dry season 2010 | Londrina | Common bean | 5.55 | 3.05 | 0.03 | 6.33 | 0.59 | 20.01 | 9.02 | 9.61 | 12.66 | 75.71 |
| | Ponta Grossa | Common bean | 5.22 | 3.34 | 0.00 | 4.01 | 0.44 | 21.12 | 6.64 | 7.08 | 10.42 | 67.95 |
| Summer (rainy) season 2010/2011 | Londrina | Soybean/common bean | 5.35 | 4.18 | 0.05 | 7.77 | 0.43 | 21.68 | 5.62 | 6.05 | 10.23 | 59.11 |
| | Ponta Grossa | Soybean/common bean | 4.89 | 4.75 | 0.17 | 2.83 | 0.23 | 25.73 | 4.85 | 5.08 | 9.82 | 68.84 |

[a] *SB* sum of bases; *CEC* cation exchange capacity; *BS* bases saturation=((K+Ca+Mg)/$T_{cec}$)×100, where $T_{cec}$ = K+Ca+Mg+total acidity at pH 7.0 (H+Al)

Granulometric properties in each site were (in gram per kilogram): Londrina: 710 (clay), 82 (silt), 208 (sand); Ponta Grossa: 238 (clay), 30 (silt), 732 (sand)

Ab-V5 and Ab-V6 were used (combination representing 100 % of the inoculants used in Brazil), and seed inoculation was applied at $1.2\times10^5$ cells seed$^{-1}$ (legal requirement is ten times lower than for rhizobia). For the in-furrow inoculation with *Azospirillum*, doses were of $2.5\times10^5$ cells seed$^{-1}$, $5\times10^5$ cells seed$^{-1}$, and $7.5\times10^5$ cells seed$^{-1}$ of commercial inoculant.

Every experiment included two controls, non-inoculated control (NI), and non-inoculated control receiving N-fertilizer (NI+N). For soybean, 200 kgN ha$^{-1}$ were applied as urea (46.6 %N), split-applied with 50 % applied at sowing and 50 % at the R2 stage (open flower at one of the two uppermost nodes on the main stem with a fully developed leaf) (Fehr and Caviness 1977). For common bean, plants received 20 kgN ha$^{-1}$ split-applied as urea at sowing and 60 kgN ha$^{-1}$ split-applied at 25 DAE. All experiments also included one treatment inoculated exclusively with seed-applied rhizobia (I Brady, I Rhizo). Finally, different concentrations of *Azospirillum* (Azo) were applied in-furrow or on seeds, with or without co-inoculation with rhizobia. Treatments with *Azospirillum* differed from 1 year to the other because they were redesigned according to the results obtained in the previous year.

Soybean was always cropped in the summer (rainy) season. Six treatments were included in the first crop season: (1) NI; (2) NI+N; (3) I Brady; (4) I Brady + Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (5) I Brady+Azo in-furrow $5\times10^5$ cells seed$^{-1}$; (6) I Brady+Azo in-furrow $7.5\times10^5$ cells seed$^{-1}$. The best results were achieved with I Brady+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$, so in the second year this treatment was tested in addition to the application on the seeds, also with six treatments: (1) NI; (2) NI+N; (3) I Brady; (4) I Brady+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (5) NI+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (6) I Brady+Azo on seeds $1.2\times10^5$ cells seed$^{-1}$. In the third year, there was no need of confirming the results with Azo applied in-furrow, therefore the performance of seed co-inoculation was verified, with only four treatments: (1) NI; (2) NI+N; (3) I Brady; (6) I Brady+Azo on seeds $1.2\times10^5$ cells seed$^{-1}$.

For common bean, seven treatments were included in the first cropping (summer crop, or rainy season) and in the second cropping (dry season): (1) NI; (2) NI+N; (3) I Rhizo; (4) I Rhizo+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (5) NI+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (6) I Rhizo+Azo in-furrow $5\times10^5$ cells seed$^{-1}$; (7) NI+Azo in-furrow $5\times10^5$ cells seed$^{-1}$. The best results were also obtained with co-inoculation and *Azospirillum* in-furrow at a concentration of $2.5\times10^5$ cells seed$^{-1}$, thus in the third cropping season, seed co-inoculation was also tested, with six treatments: (1) NI; (2) NI+N; (3) I Rhizo; (4) I Rhizo+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (5) NI+Azo in-furrow $2.5\times10^5$ cells seed$^{-1}$; (6) I Rhizo+Azo $1.2\times10^5$ cells seed$^{-1}$.

For soybean, liquid inoculants were used, adding the estimated amounts to deliver $1.2\times10^6$ cells seed$^{-1}$, vigorously mixing with the seeds and allowed to dry for 2 h under shade. Best results with common bean have been achieved with peat inoculants; therefore, for this legume, peat inoculant was used, added with a 10 % commercial sugarcane sugar solution to increase adhesiveness (at a rate of 200 mL of sugar solution 50 kg seeds$^{-1}$), and inoculant to deliver $1.2\times10^6$ cells seed$^{-1}$. *Azospirillum* was supplied as liquid inoculant and the procedure was the same as for soybean. For in-furrow inoculation, the inoculant was mixed with 200 L ha$^{-1}$ of water and delivered in-furrow.

All experiments were assigned to a completely randomized block design with six replicates. Plot sizes varied from 4×6 m (24 m$^2$) to 5.5×6 m (33 m$^2$) and at all sites the plots were separated by rows of at least 0.5 m and small terraces of at least 1.5 m to prevent contamination by superficial run-off containing bacteria or fertilizer, caused by heavy rains that often occur in the summer season. Sowing and harvesting dates are shown in Table 1. Plant density was about 300,000 plants ha$^{-1}$ for soybean and about 240,000 plants ha$^{-1}$ for common bean.

## Plant sampling, harvesting, and analyses

At the V4 stage of soybean (Fehr and Caviness 1977), and at 25 DAE for common bean, six plants were randomly collected per plot (avoiding rows established for grain yields) for evaluation of nodulation (nodule number and dry weight per plant). This early evaluation of nodulation is able to identify effects of the inoculation, as late nodules are also formed by the indigenous soil rhizobial population. Another harvest was performed at R2 stage of soybean and at 38 DAE (flowering) for common bean for evaluation of plant growth and total N in shoots.

In the laboratory, shoots were separated from roots and the latter were carefully washed and placed in an air-forced dryer at 65 °C until constant weight was obtained (approximately 72 h). Nodules were removed from roots and dried again, for determination of nodulation (nodule number and dry weight). Shoot dry weight was evaluated, as well as shoot N content by the Kjeldahl digestion method, by using a Tecator automatic N analyzer.

Grain yield at physiological maturity was determined by harvesting a central area of each plot, and the harvested areas are shown in Table 1. Grains were cleaned and weighed and values were corrected to 13 % moisture content, after determination of the humidity in a grain moisture tester.

## Statistical analyses

Data from each experiment were first submitted to tests of normality and homogeneity of variances for each variable and then to analysis of variance (ANOVA). When confirming a statistically significant value in the $F$ test ($p\leq0.05$), a post hoc test (Duncan's multiple-range test at $p\leq0.05$) was used as a multiple comparison procedure (SAS Institute 2001).

## Results

In both Londrina and Ponta Grossa, the soils had a high population of naturalized soybean bradyrhizobia (Table 1), and although no differences on nodulation were observed at the V4 stage, seed inoculation with *B. japonicum*, with or without in-furrow inoculation with *A. brasilense* at the concentration of $2.5\times10^5$ cells seed$^{-1}$ improved nodule number and dry weight, particularly in Ponta Grossa (Table 3). In the second year, the differences in Ponta Grossa were outstanding, with a 3.5-fold increase in nodule dry weight (Table 4). As expected, nodulation always decreased with the application of N-fertilizer (Tables 3 and 4). At the R2 stage, in both places and years, again the best strategy for higher production of plant biomass and N accumulation in tissues was of seed inoculation with *B. japonicum* and of co-inoculation with the lower concentration of *Azospirillum*, with results comparable to the treatment receiving a high level of N-fertilizer (Tables 3 and 4).

In the first year in Londrina, when compared to the non-inoculated control, soybean yields increased by 214 kg ha$^{-1}$ (8 %) with seed inoculation with *B. japonicum*, and by 296 kg ha$^{-1}$ (11.1 %) when co-inoculated with *A. brasilense* in-furrow at the concentration of $2.5\times10^5$ cells seed$^{-1}$ (Table 3). Similar and yet more expressive results were obtained in Ponta Grossa, with yield increases of 244 kg ha$^{-1}$ (12.3 %) and 520 kg ha$^{-1}$ (26.3 %) for inoculation and co-inoculation, respectively, relative to the non-inoculated control. Interestingly, crop performance was not improved by further increases in the concentration of *A. brasilense* applied in-furrow, and yields decreased in these treatments in both Londrina and Ponta Grossa. Finally, it is worth mentioning that in both localities, the application of 200 kg of N ha$^{-1}$ brought no yield benefits in comparison to the co-inoculation (Table 3). Co-inoculation improved yield by 82–276 kg ha$^{-1}$ (2.9–12.4 %) (2009/2010) and 140–323 kg ha$^{-1}$ (9.2–4.9 %) (2010/2011) when compared with the inoculation with *B. japonicum* alone.

After defining the best concentration of *A. brasilense* to be applied in-furrow in the first year, the good performance of co-inoculation with *B. japonicum* on seeds and $2.5\times10^5$ cells seed$^{-1}$ of *A. brasilense* in-furrow was confirmed in the second year in both Londrina and Ponta Grossa (Table 4). It should be remembered that the experiments were performed in different plots. Increases of 475 kg ha$^{-1}$ (14.1 %) and 418 kg ha$^{-1}$ (16 %) in Londrina and Ponta Grossa were observed, respectively, when compared to the non-inoculated control. Interestingly, yields were also statistically higher than in the treatment inoculated exclusively with *B. japonicum*, and also when compared with *A. brasilense* applied in-furrow without seed inoculation with *B. japonicum*. Finally, yield increases were also observed in a new treatment included in the second year, consisting of seed co-inoculation with both *B. japonicum* ($1.2\times10^6$ cells seed$^{-1}$) and *A. brasilense* ($1.2\times10^5$ cells seed$^{-1}$). In comparison to the non-inoculated control, seed co-inoculation resulted in yield increases of 395 (11.7 %) and 445 (17.1 %) kg ha$^{-1}$ in

**Table 3** Effects of inoculation with *Bradyrhizobium* and *Azospirillum* on nodulation (nodule number, *NN*; and nodule dry weight, *NDW*) at V4 stage, plant growth (shoot dry weight, SWD) and N accumulation in shoots (total N in shoots, TNS) at the R2 stage and grain yield at the final harvest of soybean. Experiments performed in oxisols of Londrina and Ponta Grossa, in the crop season of 2009/2010

| Treatment[a] | Londrina | | | | | Ponta Grossa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | V4 | | R2 | | Harvest | V4 | | R2 | | Harvest |
| | NN (n° pl$^{-1}$) | NDW (mg pl$^{-1}$) | SDW (g pl$^{-1}$) | TNS (mg N pl$^{-1}$) | Yield (kg ha$^{-1}$) | NN (no pl$^{-1}$) | NDW (mg pl$^{-1}$) | SDW (g pl$^{-1}$) | TNS (mg N pl$^{-1}$) | Yield (kg ha$^{-1}$) |
| NI | 20.7 a[b] | 101 a | 3.26 a | 95 b | 2663 c | 28.8 a | 106 a | 5.13 b | 144 b | 1976 c |
| NI+N | 13.9 b | 33 b | 3.79 a | 119 ab | 2881 b | 26.0 a | 53 b | 6.57 a | 212 a | 2305 ab |
| I (Brady seed) | 21.1 a | 102 a | 3.78 a | 118 ab | 2877 b | 32.7 a | 125 a | 6.56 a | 210 a | 2220 b |
| I+Azo furrow $2.5\times10^5$ cells seed$^{-1}$ | 22.7 a | 108 a | 3.94 a | 135 a | 2959 a | 32.8 a | 121 a | 6.50 a | 214 a | 2496 a |
| I+Azo furrow $5\times10^5$ cells seed$^{-1}$ | 19.1 a | 100 a | 3.52 a | 113 ab | 2843 b | 29.1 a | 106 a | 5.55 ab | 166 ab | 2321 ab |
| I+Azo furrow $7.5\times10^5$ cells seed$^{-1}$ | 21.9 a | 96 a | 3.33 a | 111 ab | 2813 b | 28.8 a | 101 a | 5.57 ab | 170 ab | 2217 bc |

Scale of Fehr and Caviness (1977)

[a] *NI* non-inoculated and without N-fertilizer; *NI+N* non-inoculated with 200 kg of N ha$^{-1}$, split twice, at sowing and R2; *I Brady* seed inoculation with *Bradyrhizobium*, $1.2\times10^6$ cells seed$^{-1}$; *Azo* inoculation with *Azospirillum* in furrow, with different concentrations

[b] Means ($n=6$) from a same column followed by different letters are significantly different ($p\leq0.05$, Duncan's test)

**Table 4** Effects of inoculation with *Bradyrhizobium* and *Azospirillum* on nodulation (nodule number, NN; and nodule dry weight, NDW) at V4 stages, plant growth (shoot dry weight, SWD) and N accumulation in shoots (total N in shoots, TNS) at the R2 stage and grain yield at the final harvest of soybean. Experiments performed in oxisols of Londrina and Ponta Grossa, in the crop season of 2010/2011

| Treatment[a] | Londrina | | | | | Ponta Grossa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | V4 | | R2 | | Harvest | V4 | | R2 | | Harvest |
| | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) |
| NI | 32.1 a[b] | 112a | 3.35 b | 107 b | 3360 c | 20.2 b | 56 b | 4.98 b | 145 b | 2599 c |
| NI+N | 18.9 b | 24 b | 4.01 a | 134 a | 3760 a | 17.7 b | 44 b | 6.88 a | 223 a | 3069 a |
| I (Brady seed) | 33.2 a | 123 a | 4.08 a | 136 a | 3512 b | 42.1 a | 195 a | 6.96 a | 188 ab | 2877 bc |
| I+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 41.3 a | 136 a | 4.21 a | 149 a | 3835 a | 45.7 a | 212 a | 6.99 a | 234 a | 3017 a |
| NI+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 21.1 b | 133 a | 3.61 ab | 116 b | 3446 bc | 20.1 b | 66 b | 5.15 b | 154 b | 2873 bc |
| I+Azo seed $1.2\times10^5$ cells $seed^{-1}$ | 35.6 a | 120 a | 3.88 a | 129 ab | 3755 a | 38.8 a | 181 a | 6.55 a | 218 a | 3044 a |

Scale of Fehr and Caviness (1977)

[a] *NI* non-inoculated and without N-fertilizer; *NI+N* non-inoculated with 200 kg of N $ha^{-1}$, split twice, at sowing and R2; *I Brady* seed inoculation with *Bradyrhizobium*, $1.2\times10^6$ cells $seed^{-1}$; *Azo* inoculation with *Azospirillum* on seeds or in furrow, with different concentrations

[b] Means ($n=6$) from a same column followed by different letters are significantly different ($p\leq0.05$, Duncan's test)

Londrina and Ponta Grossa, respectively, and of 243 (6.9 %) and 167 (5.8 %) kg $ha^{-1}$ in comparison to the seed inoculation exclusively with *B. japonicum* (Table 4).

Considering the treatments that were included in the four field experiments performed with soybean, annual seed inoculation with *B. japonicum* resulted in a mean grain yield increase of 222 kg$ha^{-1}$, or 8.4 %, whereas co-inoculation with *A. brasilense* in-furrow applied at a concentration of $2.5\times10^5$ cells $seed^{-1}$ increased grain yield by an average 427 kg $ha^{-1}$, or 16.1 % (Fig. 1). Increases in yield due to the co-inoculation with *Azospirillum* were statistically higher

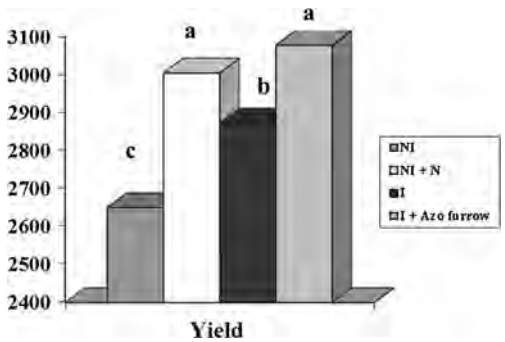


**Fig. 1** Effects of seed inoculation with *Bradyrhizobium* and in-furrow inoculation with *Azospirillum* on soybean grain yield (in kilogram per hectare). Treatments are represented by NI (non-inoculated); NI+N (non-inoculated+200 kg of N $ha^{-1}$, 50 % at sowing and 50 % in R2); I Brady, seed inoculation with *Bradyrhizobium*, $1.2\times10^6$ cells $seed^{-1}$; Azo, inoculation with *Azospirillum* in furrow, at a concentration of $2.5\times10^5$ cells $seed^{-1}$. Data represent the means of four field experiments, each with six replicates, performed in two sites (Londrina and Ponta Grossa) for two crop seasons (2009/2010 and 2010/2011). *Different letters* indicate statistical difference ($p\leq0.05$, Duncan's test)

than the inoculation exclusively with *B. japonicum* (Fig. 1). Considering the two experiments performed in the second year, seed co-inoculation with both microorganisms resulted in an increase of 420 kg $ha^{-1}$ (14.1 %) in comparison to the non-inoculated control and of 205 kg $ha^{-1}$ (6.4 %) in comparison to the seed inoculation exclusively with *B. japonicum* (Table 4).

For common bean in the rainy season, seed inoculation with *R. tropici* and co-inoculation in-furrow with *Azospirillum* at a concentration of $2.5\times10^5$ cells $seed^{-1}$ resulted, at 25 DAE, in higher nodulation in both Londrina and Ponta Grossa (Table 5). Interestingly, in Ponta Grossa, nodulation increase was also observed by inoculation exclusively with *Azospirillum* in-furrow. At 38 DAE, the largest plant biomass and total N in shoots was also obtained by the co-inoculation ($2.5\times10^5$ cells $seed^{-1}$). An increase of 147 kg $ha^{-1}$—statistically significant compared to the non-inoculated control with an indigenous population of more than $10^3$ cells $g^{-1}$ soil—was observed in Londrina with the seed inoculation with *R. tropici*, but the difference was not statistically significant in Ponta Grossa (Table 5). However, co-inoculation with *A. brasilense* in-furrow ($2.5\times10^5$ cells $seed^{-1}$) significantly increased the grain yield in Londrina and Ponta Grossa, by 383 and 432 kg $ha^{-1}$, respectively, in comparison to the non-inoculated control, and by 236 and 323 kg $ha^{-1}$, respectively, in comparison to the inoculation with only *R. tropici*. It is interesting to observe that grain yield of the inoculation treatment which promoted the highest results was higher, although not significantly, than when 80 kg $ha^{-1}$ of N, as chemical fertilizer, was applied, either in Londrina or Ponta Grossa (Table 5).

**Table 5** Effects of inoculation with *Rhizobium* and *Azospirillum* on nodulation (nodule number, NN; and nodule dry weight, NDW) at 25 (early vegetative) days after emergence (DAE), plant growth (shoot dry weight, SWD) and N accumulation in shoots (total N in shoots, TNS) at 38 DAE (flowering) and grain yield at the final harvest of common bean. Experiments were performed in oxisols of Londrina and Ponta Grossa in the rainy season of 2009/2010

| Treatment[a] | Londrina | | | | | Ponta Grossa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 25 DAE | | 38 DAE | | Harvest | 25 DAE | | 38 DAE | | Harvest |
| | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) |
| NI | 0.8 b[b] | 1.8 d | 1.69 b | 34 a | 915 c | 13.0 c | 10.6 b | 4.80 a | 102 c | 1434 b |
| NI+N | 2.8 b | 1.2 d | 1.98 ab | 45 a | 1176 ab | 18.0 c | 20.7 b | 5.06 a | 119 bc | 1739 ab |
| I (Rhizo seed) | 18.2 a | 20.2 a | 1.67 b | 37 a | 1062 b | 34.1 ab | 43.8 a | 5.16 a | 143 ab | 1543 b |
| I+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 16.4 a | 19.0 a | 2.38 a | 54 a | 1298 a | 48.2 a | 41.9 a | 5.27 a | 151 a | 1866 a |
| NI+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 2.07 b | 1.9 d | 1.99 ab | 44 a | 983 bc | 14.6 c | 20.3 b | 4.87 a | 114 bc | 1709 ab |
| I+Azo furrow $5\times10^5$ cells $seed^{-1}$ | 13.4 a | 12.9 b | 1.86 ab | 42 a | 1077 b | 49.8 a | 41.1 a | 5.12 a | 130 abc | 1584 ab |
| NI+Azo furrow $5\times10^5$ cells $seed^{-1}$ | 6.6 b | 7.8 c | 1.64 b | 36 a | 933 bc | 24.1 bc | 28.1 ab | 4.82 a | 112 c | 1484 b |

[a] *NI* non-inoculated and without N-fertilizer; *NI+N* non-inoculated with 20 kg N $ha^{-1}$, split-applied as urea at sowing and 60 kg of N split-applied at 25 DAE; *I Rhizo* seed inoculation with *Rhizobium*, $1.2\times10^6$ cells $seed^{-1}$; *Azo* inoculation with *Azospirillum* in furrow, with different concentrations

[b] Means ($n=6$) from a same column followed by different letters are significantly different ($p\leq0.05$, Duncan's test)

In the dry season of 2010, the results were similar to those obtained in the rainy season, but with lower yields in Ponta Grossa, due to a long drought period (Table 6). Again, increases in nodulation were observed in both Londrina and Ponta Grossa. In Londrina, co-inoculation with *A. brasilense* increased the grain yield by 270 kg $ha^{-1}$ compared to the non-inoculated control, while in Ponta Grossa the difference was 237 kg $ha^{-1}$. Again, no benefits by increasing

**Table 6** Effects of inoculation with *Rhizobium* and *Azospirillum* on nodulation (nodule number, NN; and nodule dry weight, NDW) at 25 (early vegetative) and 38 (flowering) days after emergence (DAE), plant growth (shoot dry weight, SWD) and N accumulation in shoots (total N in shoots, TNS) at 38 DAE and grain yield at the final harvest of common bean. Experiments were performed in oxisols of Londrina and Ponta Grossa, in the dry season of 2010

| Treatment[a] | Londrina | | | | | Ponta Grossa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 25 DAE | | 38 DAE | | Harvest | 25 DAE | | 38 DAE | | Harvest |
| | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) | NN (n° $pl^{-1}$) | NDW (mg $pl^{-1}$) | SDW (g $pl^{-1}$) | TNS (mg N $pl^{-1}$) | Yield (kg $ha^{-1}$) |
| NI | 13.1 b[b] | 15.8 b | 1.22 b | 22.3 b | 980 c | 8.9 b | 9.2 b | 1.01 b | 21.7 b | 551 b |
| NI+N | 6.9 b | 5.1 c | 2.05 a | 46.1 a | 1298 a | 7.0 b | 5.1 b | 2.02 a | 43.2 a | 808 a |
| I (Rhizo seed) | 30.2 a | 35.3 a | 2.18 a | 42.7 a | 1150 bc | 21.2 a | 25.2 a | 1.88 a | 41.3 a | 615 b |
| I+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 35.5 a | 39.2 a | 2.22 a | 48.2 a | 1250 a | 29.9 a | 30.4 a | 2.12 a | 42.9 a | 788 a |
| NI+Azo furrow $2.5\times10^5$ cells $seed^{-1}$ | 15.6 b | 19.7 b | 1.30 b | 28.1 b | 972 c | 10.1 b | 11.1 b | 1.22 b | 24.6 b | 638 b |
| I+Azo furrow $5\times10^5$ cells $seed^{-1}$ | 29.7 a | 36.3 a | 1.89 ab | 38.9 ab | 1170 b | 23.2 a | 18.2 ab | 1.34 b | 27.2 b | 784 a |
| NI+Azo furrow $5\times10^5$ cells $seed^{-1}$ | 12,8 b | 18.9 b | 1.33 b | 29.0 b | 970 c | 9.9 b | 10.1 b | 1.02 b | 21.1 b | 551 b |

[a] Means ($n=6$) from a same column followed by different letters are significantly different ($p\leq0.05$, Duncan's test)

[b] *NI* non-inoculated and without N-fertilizer; *NI+N* non-inoculated with 20 kg N $ha^{-1}$, split-applied as urea at sowing and 60 kg of N split-applied at 25 DAE; *I Rhizo* seed inoculation with *Rhizobium*, $1.2\times10^6$ cells $seed^{-1}$; *Azo* inoculation with *Azospirillum* in furrow, with different concentrations

the dose of *Azospirillum* to $5\times10^5$ cells to seed$^{-1}$ were observed (Table 6).

A fifth experiment was conducted in the rainy season of 2010/2011 in Londrina, this time only with the best dose of *A. brasilense* established in the previous trials, of $2.5\times10^5$ cells seed$^{-1}$ (in furrow), and including seed co-inoculation, with $1.2\times10^5$ cells seed$^{-1}$ (in seed) of *Azospirillum* (Table 7). Inoculation with either *R. tropici* alone, or co-inoculation with *A. brasilense* in-furrow or on seeds resulted in higher nodulation at 25 DAE, as well as plant biomass and total N accumulated in shoots at 38 DAE. However, no statistical differences were observed in yield of inoculated treatments in comparison to the non-inoculated control, but the co-inoculated treatment with *Azospirillum* in-furrow improved yield by 103 kg ha$^{-1}$ (Table 7).

When a statistical analysis including the five experiments with common bean was performed, seed inoculation with *R. tropici* resulted in an average increase of 98 kg ha$^{-1}$, or 8.3 % when compared to the non-inoculated treatment, with a further increase of 187 kg ha$^{-1}$ (14.7 %) with the co-inoculation of *A. brasilense* in-furrow ($2.5\times10^5$ cells seed$^{-1}$) (Fig. 2). In relation to non-inoculated control, co-inoculation resulted in the impressive increase of 285 kg ha$^{-1}$, or 19.6 % (Fig. 2).

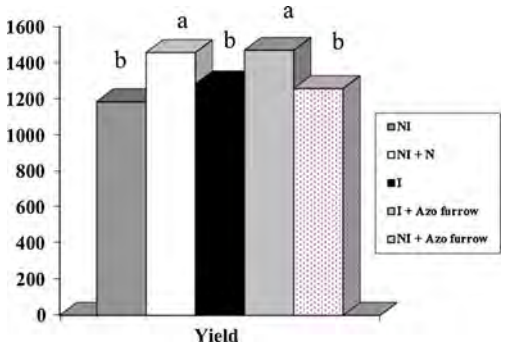


**Fig. 2** Effects of seed inoculation with *Rhizobium* and in-furrow inoculation with *Azospirillum* on common bean grain yield (in kilogram per hectare). Treatments are represented by NI (non-inoculated); NI+N (non-inoculated+20 kg N ha$^{-1}$, split-applied as urea at sowing and 60 kg of N split-applied at 25 DAE); I Rhizo, seed inoculation with *Rhizobium*, $1.2\times10^6$ cells seed$^{-1}$; Azo, inoculation with *Azospirillum* in furrow, at a concentration of $2.5\times10^5$ cells seed$^{-1}$. Data represent the means of five field experiments, each with six replicates, performed in two sites (Londrina and Ponta Grossa) for three crop seasons (2009/2010, 2010 and 2010/2011). *Different letters* indicate statistical difference ($p\leq0.05$, Duncan's test)

## Discussion

The idea of co-inoculating legumes with rhizobia and PGPR has long been pursued. There are reports of experiments performed with alfalfa (*Medicago* sativa L.) (Itzigsohn et al. 1993), common beans (Burdman et al. 1997; Remans et al. 2008), chickpeas (*Cicer arietinum* L.), faba beans (*Vicia faba* L.) (Hamaoui et al. 2001), cowpeas (*Vigna unguiculata* (L.) Walp) (Lima et al. 2011), and lentils (*Lens culinaris* L.) (Kumar and Chandra 2008), among others. However, most studies only addressed early aspects of the development of the symbiotic relationship. In Brazil, very few experiments have been performed under field conditions with co-inoculation of rhizobia and PGPR, including both positive (e.g., *Bacillus subtilis* and *B. japonicum* in soybean) (Araújo and Hungria 1999) and neutral (e.g., *A. brasilense* and *B. japonicum* in soybean) (Bárbaro et al. 2009) responses. Worldwide, there are also very few reports of experiments performed under field conditions with co-inoculation of microorganisms with

**Table 7** Effects of inoculation with *Rhizobium* and *Azospirillum* on nodulation (nodule number, NN; and dry weight, NDW) at 25 (early vegetative) and 38 (flowering) days after emergence (DAE), plant growth (shoot dry weight, SWD) and N accumulation in shoots (total N in shoots, TNS) at 38 DAE and grain yield at the final harvest of common bean. Experiments were performed in oxisols of Londrina, in the rainy season of 2010/2011

| Treatment[a] | Londrina | | | | |
|---|---|---|---|---|---|
| | 25 DAE | | 38 DAE | | Harvest |
| | NN (n° pl$^{-1}$) | NDW (mg pl$^{-1}$) | SDW (g pl$^{-1}$) | TNS (mg N pl$^{-1}$) | Yield (kg ha$^{-1}$) |
| NI | 11.1 b[b] | 9.7 b | 2.29 b | 51.1 b | 2039 b |
| NI+N | 6.8 b | 5.3 b | 2.97 a | 85.2 a | 2259 a |
| I (Rhiz) | 22.3 a | 24.5 a | 2.87 a | 82.9 a | 2040 ab |
| I+Azo furrow $2.5\times10^5$ cells seed$^{-1}$ | 29.6 a | 29.2 a | 2.88 a | 86.1 a | 2142 ab |
| NI+Azo furrow $2.5\times10^5$ cells seed$^{-1}$ | 13.1 b | 11.3 b | 2.39 a | 71.5 ab | 1988 b |
| I+Azo seed $1.2\times10^5$ cells seed$^{-1}$ | 23.4 a | 24.7 a | 2.86 a | 82.6 a | 2018 b |

[a] Means ($n=6$) from a same column followed by different letters are significantly different ($p\leq0.05$, Duncan's test)

[b] *NI* non-inoculated and without N-fertilizer; *NI+N* non-inoculated with 20 kg N ha$^{-1}$, split-applied as urea at sowing and 60 kg of N split-applied at 25 DAE; *I Rhizo* seed inoculation with *Rhizobium*, $1.2\times10^6$ cells seed$^{-1}$; *Azo* inoculation with *Azospirillum* in furrow or on seed

different functions. In our report, the experiments aimed at evaluating nodulation, plant growth and grain yield as the result of co-inoculation, and all were performed at locations which are representative of areas cropped with soybeans and common beans in Brazil. Elite strains used in the experiments have been selected in Brazil for the soybean (Peres et al. 1993), common bean (Hungria et al. 2000) and of *A. brasilense* (Hungria et al. 2010) and compose the commercial inoculants in Brazil for the crops studied.

Crops grown depending on biological nitrogen fixation must be well nodulated by effective bacteria in order to obtain the maximum benefits from the symbiotic association. Since legume nodules are initiated from root hairs, stimulation of root hair production by the activity of azospirilla may result in more nodules on the legume roots. Positive effects of co-inoculation of rhizobia and azospirilla on the number of root hairs and consequently on nodulation were observed in alfafa (Itzigsohn et al. 1993). Burdman et al. (1997) did not measure root hair production, but also observed increased nodulation and nitrogen fixation when common beans were co-inoculated with rhizobia and azospirilla. Remans et al. (2008) determined that the effect of *Azospirillum* co-inoculation depended on the genotype of common bean. Nodulation increase as a result of co-inoculation with *Azospirillum* has also been observed in chickpeas (Hamaoui et al. 2001), galega (*Galega orientalis* Lam.) (Egamberdieva et al. 2010), and lentils (Kumar and Chandra 2008). In our experiments, we did not measure root hair production in response to the presence of *Azospirillum*, but nodulation of both soybeans and beans agree with the above reports of increased nodulation when both inoculants are employed. The improved nodulation observed may have resulted in increased N contents in the shoots and, in turn, with increased grain yields. However, it was interesting to notice that benefits of PGPR were not observed when higher doses of *Azospirillum* were applied in-furrow. PGPR may produce a variety of plant hormones in still not fully understood mechanisms, and in higher concentrations plant hormones, or ethylene, or other compounds may inhibit plant growth (Bhattacharyya and Jha 2012; Martínez-Viveros et al. 2010).

It is not clear, however, if the benefits of inoculating legumes with both rhizobia and azospirilla are only due to increased nodulation and nitrogen fixation. The genus *Azospirillum* belongs to the broad group of PGPR, which may act by different mechanisms, altogether, resulting in stimulus to plant growth. Such types of benefits have been observed for non-legumes, such as bananas (*Musa* spp.) (Baset Mia et al. 2010), where inoculation with PGPR resulted in a synergistic effect on root growth and development, increased yield, improved physical attributes of fruit quality and early flowering initiation. A similar situation was reported with wheat (*Triticum aestivum* L.) co-inoculated with *A. brasilense* and *Rhizobium meliloti*, with improvement not only of yield, but also of grain quality (Askary et al. 2009).

Inoculation with PGPR has also been proven beneficial for legumes. In Egypt, Massoud et al. (2009) observed that a mixture containing arbuscular mycorrhizal fungi, symbiotic *Rhizobium* sp., associative *Azospirillum* sp. and *Bacillus circulans* increased plant height, number of branches, number of nodules per plant and fresh yield of common beans, when compared with control plants. Similar results, but using a mixture of *Rhizobium* and *Pseudomonas* sp. or *Bacillus* sp. resulted in improved shoot dry weight, N and P contents in common bean plants (Stajković et al. 2011). These authors have also observed in vitro phosphate solubilizing activity, indole acetic acid, ammonia, and siderophore production by *Pseudomonas* sp., which altogether could explain common bean growth promotion. Yadegari and Asadi Rahmani (2010) observed that co-inoculation with *Rhizobium* and *P. fluorescens* significantly increased yield and yield components such as the number of pods per plant, number of seeds per pod, weight of 100 seed, mass of seeds per plant, mass of pods per plant, total dry matter at R6, as well as grain yield, and protein content in common beans.

In non-legumes, synergistic effects may also be observed. In tomatoes (*Lycopersicon esculentum* Mill.), for example, inoculation with *Azospirillum* and other nitrogen-fixing PGPR somehow compensates for the yield losses caused by the application of viral satellite RNA to induce systemic resistance and protect plants from damage by virulent strains of cucumber mosaic virus (Dashti et al. 2007). These findings demonstrate that *Azospirillum* contributes to improve plant growth and crop yield by an array of mechanisms, and may explain the results we obtained in our field experiments.

In our experiments, for soybean crop and in general for common bean, no benefits in grain yields were observed when N-fertilizer was applied. Even though responses to mineral N-fertilizer may be sometimes observed, especially for the common bean crop (e.g. Vargas et al. 2000), the cost/benefit relationship must be taken into account. In Brazil, where about 70 % of the N-fertilizers are imported, the costs of applying mineral N are very high and a similar situation is found in several other countries. Microbial inoculants are very cheap and considering only the soybean crop, estimates are that Brazil saves about US$ 7 billion year$^{-1}$ with biological nitrogen fixation. Furthermore, environmental costs of N-fertilizers have to be considered, since the use of 100 kg of N-fertilizer corresponds to about 950 kg of $CO_2$ equivalent. Therefore, the use of effective nitrogen-fixers and PGPR microorganisms in agriculture is economically feasible and environmentally sound. Yield increases in association with microbial inoculation will, therefore, represent both a high cash gain for the farmer and contribute to governmental commitments of decreasing the greenhouse gases emissions. The results reported in this paper corroborate the recommendation of seed

inoculation (and re-inoculation every year) for both soybean and common bean at sowing with elite strains of *Bradyrhizobium* and *Rhizobium*, respectively, and with additional gains when using *A. brasilense* in co-inoculation in-furrow. Considering all field trials, co-inoculation of rhizobia on seeds and *A. brasilense* in-furrow resulted in outstanding increases in grain yield, on average 16.1 % for soybean and 19.6 % for common bean in comparison to the naturalized/indigenous rhizobial population, and of 7.1 and 14.7 %, respectively, in comparison to the inoculation exclusively with rhizobia.

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