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Alternative methods of soybean inoculation to overcome adverse conditions at sowing

Mariangela Hungria[1]*, Marco Antonio Nogueira[1] and Ricardo Silva Araujo[2]

[1]Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, Paraná, Brazil.
[2]Total Biotecnologia Indústria e Comércio Ltda. Rua Emílio Romani, 1190, CIC, 81460-020, Curitiba, Paraná, Brazil.

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Soybean growth in Brazil relies solely on biological fixation for nitrogen nutrition, However, the effective establishment of the symbiosis between plants and elite strains of *Bradyrhizobium* is jeopardized by current agricultural practices, such as seed treatment with pesticides that can be toxic to the bacteria. In addition, global climatic changes have altered temperature and rainfall patterns, which, in turn, may affect the early stages of the symbiosis and, consequently, nodulation, N2 fixation, and yield, especially when drought and high temperatures occur right after sowing. New technologies to improve nodulation and N_2 fixation must be developed. In this study, we evaluated the effects of spraying diluted inoculants towards the seeds at sowing, or on the soil-root interface after seedling emergence on attributes relative to soybean N_2 fixation and yield. Field experiments were set up at different locations, in a randomized block design according to standard Brazilian protocols. Inoculant application in the soil resulted in benefits for both nodulation and yield when plants faced adverse conditions at the initial stages of growth, and the inclusion of *Azospirillum* in co-inoculation with *Bradyrhizobium* also helped plants bypass initial adverse situations. The results also revealed that when adverse situations to nodulation occur, it may be possible to perform corrective inoculation by spraying diluted inoculant at sowing or after seedling emergence, even though some degree of yield loss may be expected. However, more information is necessary to establish inoculation frames.

Key words: Spray-inoculation, plant growth-promoting rhizobacteria (PGPR), *Azospirillum*, *Bradyrhizobium.*

INTRODUCTION

Many legumes can establish symbiotic relationships with specific soil bacteria collectively referred as rhizobia, which possess the dinitrogenase enzyme complex capable of capturing atmospheric nitrogen (N_2) and fixing it into ammonium, which is incorporated into carbon skeletons to form nitrogenous organic acids that can be readily assimilated by plants (Ormeño-Orrillo et al., 2013). Brazil stands as a model country in benefiting from biological N_2 fixation (BNF), especially from the inoculation of soybeans [*Glycine max* (L.) Merr.] with elite strains of the genus *Bradyrhizobium*, a symbiotic combination capable of fully supply the crop demand for nitrogen (Hungria et al., 2005a, 2006a,b; Hungria and Mendes, 2014). Another group of beneficial soil microorganisms comprises plant growth-promoting rhizobacteria (PGPR). These may produce plant growth

*Corresponding author. E-mail: mariangela.hungria@embrapa.br, Tel: (+55) 43-33716206. Fax: (+55) 43-33716100.

hormones (auxins, giberellins, cytokinines, and ethylene), induce the plant’s systemic resistance to diseases and or stresses, act as biocontrol agents, and solubilize phosphates, besides performing non-symbiotic biological N_2 fixation (Hartmann and Zimmer, 1994; Compant et al., 2005; Cassán et al., 2008; Bassan et al., 2012; Bhattacharyya and Jha, 2012; Fibach-Paldi et al., 2012). Bacteria belonging to the genus *Azospirillum* are the best studied and most employed worldwide as PGPR inoculants for agriculture (Okon and Labandera-Gonzalez, 1994; Bashan and Holguin, 1997), including Brazil (Hungria et al., 2010). Co-inoculation of rhizobia and *Azospirillum* results not only in soybean grain yield increase (Hungria et al., 2013), but also in pathogen control as satisfactory as the action of fungicides (Cassán et al., 2008).

The soybean crop is of utmost economic importance for Brazil. The fixation of over 300 kg of N per hectare, in every cropping season, and the delivery, to the soil, of about 20 to 30 kg of N per hectare, which remain available for the following crop, are certainly key elements for the success of soybean in the country (Hungria et al., 2005a, 2006a, b; Hungria and Mendes, 2014). However, more investigation is necessary to extend these benefits to the new soybean cultivars, as well as to make them compatible with modern soil and crop management techniques (Hungria and Mendes, 2014).

The global climatic changes also threaten the contribution of BNF to agriculture, as longer periods of drought and high temperatures have become more frequent, and Brazil is not an exception (Zullu Jr et al., 2008). Environmental stresses have marked negative effects on nodulation and BNF (Hungria and Franco, 1993; Hungria and Vargas 2000; Hungria and Kaschuk, 2014). Moreover, climatic changes increase plant susceptibility to diseases and pests, demanding more intensive seed treatment with chemicals that may be highly toxic to rhizobia, such as fungicides, insecticides, and nematicides; the use of pesticides is a common practice for over 90% of the soybeans grown in Brazil (Campo et al., 2009).

Any factor that reduces the population of inoculum rhizobia on the seeds and, consequently, nodulation, may decrease the contribution of BNF. Therefore, scientists must develop new technologies that minimize the negative impacts of seed treatment with chemicals on inoculated rhizobia. One technology is in-furrow delivery of *Bradyrhizobium* inoculants. This technology has long been proposed (Brockwell et al., 1988) as an effective possibility to release soybean rhizobia into the soil. This alternative is currently under successful utilization, although still by few farmers in Brazil, as long as larger doses of inoculant than those recommended for seed inoculation are employed (Vieira Neto et al., 2008; Campo et al., 2010).

Another palliative technology, especially in cases of failure or limitations to seed inoculation, is spray inoculation, which can promote, at least, partial recovery of nodulation and BNF (Zilli et al., 2008). However, this technology must be analyzed very carefully, especially if performed after seedling emergence. The successful establishment of the legume-rhizobia symbiosis starts with an intricate exchange of molecular signals between the partners (Hungria et al., 1996; Hungria and Stacey, 1997; Geurts and Bisseling, 2002; Desbrosses and Stougaard, 2011), triggered by seed germination, resulting in root hair deformations that are visible right after exposure of bradyrhizobia to soybean root exudates (Hungria et al., 1996). As new root segments are only transiently susceptible to rhizobial infection (Bhuvaneswari et al., 1981), delayed contact between the bacteria and the roots may result in poor nodulation and BNF. Therefore, it is essential to gain information about doses and timeframe for delayed inoculation, as well as about when such practice would be viable.

The increasing use of pesticides for soybean seed treatment at sowing jeopardizes the maintenance of adequate numbers of viable rhizobial cells on the seed surface (Campo et al., 2009). This situation is even more critical in the case of sowing pre-inoculated seeds, a practice that is becoming more common and popular in South America (Hungria and Mendes, 2014). The objectives of this study were to evaluate soybean spray inoculation as an alternative to traditional seed or in-furrow inoculation, and investigate the effects of co-inoculation with *Azospirillum* on soybean growth and yield.

MATERIALS AND METHODS

Site description and procedures before sowing

Four field experiments were conducted in the 2012/2013 cropping season. Geographic information about each experimental location is presented in Table 1.

At each location, 20 soil subsamples were collected at 0-20 cm soil layer about 40 days before experimental setup. Subsamples were combined and one composite sample from each location was analyzed for chemical, granulometry and microbiological characteristics. For chemical analysis (Pavan et al., 1992), samples were oven-dried (60°C, 48 h) and sieved (2 mm). Soil pH was determined in 0.01 M $CaCl_2$ (1:2.5; soil:solution) after 1 h shaking. Ca, Mg, and Al contents were determined in the extract obtained with 1 M KCl (1:10; soil:solution) after 10 min shaking. P and K contents were determined en Mehlich-1 extract (0.05 M HCl + 0.0125 M H_2SO_4; 1:10 soil:solution) after shaking for 10 min. Al was determined by titration with 0.015 N NaOH, with bromthymol blue as indicator. Ca and Mg concentrations were determined in an atomic absorption spectrophotometer, K in a flame photometer, and P by colorimetry, by the molybdenum blue/ascorbic acid method. C was determined by dichromate oxidation. Soil chemical characteristics are presented in Table 2.

Soil granulometry at each experimental site was determined according to Embrapa (1997), and soil rhizobial populations were estimated by the plant-infection most probable number technique (Vincent 1970), with soybean cultivar BMX Potência RR as the trapping host, using statistical tables based on Andrade and

Table 1. Geographic and climatic information about the locations where experiments were conducted.

Location	Coordinates[a]	Altitude (m)	Climatic Classification[b]
Rio Verde	17°47' S; 50°54' W	730	Aw
Cachoeira Dourada	18°29' S; 49°28' W	450	Aw
Luiz Eduardo Magalhães	12°05' S; 45°48' W	720	Aw
Ponta Grossa	25°05′ S; 50°09′ W	950	Cfa

[a] Latitude and longitude; [b] According to the Köppen-Geiger system of classification.

Table 2. Chemical characteristics of the soils (0-20 cm) at the locations where experiments were conducted. All analyses were performed before sowing.

Location	pH	Al	H + Al	K	Ca	Mg	P	C	B	S	Sum of bases	V	Zn	Cu	Mn	Fe
	$CaCl_2$	$cmol_c\ dm^{-3}$					$g\ dm^{-3}$		$mg\ dm^{-3}$		$cmol_c\ dm^{-3}$	%	$mg\ dm^{-3}$			
Rio Verde	5.14	0.00	3.64	0.80	1.65	1.78	9.56	22.55	0.29	5.87	4.23	53.75	2.95	2.21	115.85	32.42
Cachoeira Dourada	5.40	0.00	3.07	0.37	3.55	1.73	1.71	18.55	0.20	7.23	5.65	64.79	1.41	8.27	146.84	38.09
Luiz Eduardo Magalhães	5.57	0.00	1.03	0.03	2.94	0.77	10.36	5.72	0.05	1.68	3.74	78.41	0.24	0.25	4.57	49.99
Ponta Grossa	4.60	0.26	7.89	0.15	2.02	1.30	0.80	30.50	0.30	5.80	3.47	30.55	1.10	1.40	41.00	56.00

Table 3. Soil granulometry and soybean rhizobial population of the soils at the locations where experiments were conducted.

Location	Soil granulometry (%)			No. of rhizobia g^{-1} soil
	Clay	Silt	Sand	
Rio Verde	36.35	9.55	54.10	< 10
Cachoeira Dourada	57.75	18.20	24.05	< 10
Luiz Eduardo Magalhães	13.55	1.00	88.45	< 10
Ponta Grossa	58.45	15.70	25.85	1×10^2

Hamakawa (1994). Soil granulometry and rhizobial populations at each location are shown in Table 3.

Lime was applied to the soil at each location about 50 days before sowing. The amounts of lime to be applied were determined on the basis of soil base saturation as specified by Embrapa Soja (2011), so as to obtain 70%. Still before sowing, all sites received 300 kg ha^{-1} of N-P-K (0-28-20) fertilizer, applied in-furrow. No N fertilizer was applied, except where specified (in the control with N-fertilizer).

Treatments, inoculation and field management

The experimental protocol adopted for the experiments reported here followed the guidelines established by the Ministry of Agriculture, Livestock and Food Supply (MAPA) in all tests of agronomic efficiency of new products or technologies making use of biological nitrogen fixation with legumes (MAPA, 2011). All experiments had 12 treatments, and treatments 1, 2, and 3 are mandatory in Brazil, as controls required by guidelines mentioned above. The treatments were:

Treatment 1 (T1) = Non-inoculated control;
T2 = T1 + 200 kg N ha^{-1} (100 kg N ha^{-1} at sowing + 100 kg N ha^{-1} as side dressing around 35 days after seedling emergence);
T3 = Standard peat-based *Bradyrhizobium* inoculant applied to seeds at sowing to provide 1.2×10^6 cells $seed^{-1}$ (1 dose);
T4 = Liquid *Bradyrhizobium* inoculant applied to seeds at sowing (1 dose);
T5 = Liquid *Bradyrhizobium* inoculant applied in-furrow at sowing (3 doses);
T6 = Liquid *Bradyrhizobium* inoculant sprayed close to the sowing line at sowing (3 doses);
T7 = T6, but with 5 doses;
T8 = Three doses of liquid *Bradyrhiobium* inoculant + two doses of liquid *Azospirillum* inoculant, sprayed close to the sowing line at sowing;
T9 = T8, but with five doses of liquid *Bradyrhizobium* inoculant + two doses of liquid *Azospirillum* inoculant;
T10 = Liquid *Bradyrhizobium* inoculant sprayed towards the root/stem interface region between VC and V1 (3 doses);
T11 = T 10, but with 5 doses;
T12 = T10, but with 10 doses

All bacterial inoculants employed in this study were analyzed for purity and cell concentration. Inoculants with *Bradyrhizobium* were counted by spread-plating on yeast extract-manitol agar with Congo red (Vincent, 1970), while inoculants containing *Azospirillum* were counted by spread-plating on RC agar medium (Cassán et al., 2010). In all cases, bacterial colony morphology was compared to expected patterns to confirm the absence of contaminants. The results of cell concentrations and purity of the inoculants are presented in Table 4.

Standard peat-based inoculant was prepared at a concentration of 5×10^9 cells g^{-1}, and contained the commercial strains *B.*

Table 4. Composition, concentration[a], and purity[b] of the inoculants used in the experiments.

Inoculant	Bacterial species	Strains	Concentration[a]	Contaminants[b]
Standard peat-based	*B. japonicum/B. diazoefficiens*	5079 + 5080	4.14×10^9	Absent
Standard liquid	*B. japonicum/B. diazoefficiens*	5079 + 5080	7.33×10^9	Absent
Standard liquid	*A. brasilense*	Ab-V5 + Ab-V6	2.97×10^8	Absent

[a] Number of colony forming units (CFU) per g or mL of product. [b] Characterized as presence (present) or absence (absent) of detectable contaminants at the 10^5 dilution of the products spread on plates with appropriate media.

Table 5. Agronomic information about the experiments.

Location	Cultivar	Sowing	Harvest	Plot size (m^2)	Area for yield evaluation (m^2)
Rio Verde	BMX-Potência (RR)	28/11/2012	19/03/2013	24.3	6
Cachoeira Dourada	BRS-GO-8360 (Conv.)	22/11/2012	22/03/2013	24	5.6
Luiz Eduardo Magalhães	BMX-Potência (RR)	06/12/2013	no harvest	24	6
Ponta Grossa	BMX-Potência (RR)	05/12/2012	08/05/2013	24	6

japonicum SEMIA 5079 (=CPAC 15) and *B. diazoefficiens* SEMIA 5080 (=CPAC 7) (Table 4). The doses of both liquid and peat-based *Bradyrhizobium* inoculants were adjusted to provide 1.2×10^6 viable cells of bradyrhizobia per seed, according to Brazilian regulations (Embrapa, 2011), and the peat-based inoculant was applied to the seeds with 10% sucrose solution to improve adherence, as described before (Hungria et al., 2006b). For *Azospirillum* liquid inoculant, one dose was considered as 1.2×10^5 viable cells per seed (10-fold less than bradyrhizobia)

The non-inoculated control treatment with N fertilizer (T2) received 200 kg N ha^{-1} as urea, split in two broadcast applications of 100 kg N ha^{-1} at sowing, and 100 kg N ha^{-1} as side dressing around 35 days after seedling emergence.

For in-furrow inoculation at sowing, the liquid inoculant was diluted in water to make up a final volume of 150 L ha^{-1}, and the mixture was applied directly over the seeds in the sowing furrow. For spray inoculations, the appropriate amounts of inoculants were mixed with water to make up a final volume of 150 L ha^{-1}, and the mixtures were applied by spraying either towards the sowing line (at sowing), or towards the root/stem interface region between stages VC and V1 (Fehr and Caviness, 1977), both with a coastal sprayer.

Information about cultivars, sowing dates, and sampling dates at each location are shown on Table 5. At all locations, row spacing was 50 cm, with 18 plants m^{-1}, and a final population of approximately 300,000 plants ha^{-1}. All experiments were set in a completely randomized block design with six replicates. Plot sizes varied from 24 m^2 to 24.3 m^2 (Table 5). At all locations the plots were separated by 0.5 m-wide rows and 1.5 m-wide terraces to avoid cross contamination from surface flushes containing bacteria or fertilizers that may occur in consequence of heavy rainfall.

All plants received leaf sprays of Mo (20 g ha^{-1}) and Co (2 g ha^{-1}) at the V4 stage (Fehr and Caviness, 1977). Weeds were controlled with herbicides in all treatments. Glyphosate was employed when the transgenic cultivar was grown, whereas conventional herbicides were employed with the non-transgenic cultivar. Insect control was accomplished by means of biological and chemical insecticides (Embrapa, 2011).

Sampling, harvest and analyses performed

Thirty-five to 50 days after sowing, five plants were collected from each plot for evaluation of nodulation (nodule number and dry weight), plant biomass, and N content and accumulation in shoots. Roots and shoots were separated in the laboratory, carefully washed and oven-dried at 65°C for approximately 72 h. Nodules were then removed from roots and allowed to dry for another 72 h before counting and weighing. Dry shoots were also weighed and then employed for determination of N content and accumulation by the Kjeldahl technique.

At harvest, the central area from each plot (Table 5) was harvested to estimate grain yield. Seeds from the harvested plants were collected, cleaned, weighed, and seed moisture was determined and adjusted to 13%.

All data from each experiment were first tested for normality and for variance homogeneity. If necessary, data were transformed to the square root of (x+1) before analysis of variance (ANOVA) (SAS, 1999). In cases where statistical significance was detected, a *post hoc* test with $p < 0.1$ was performed. For multiple comparisons, Duncan test was employed.

RESULTS

In areas cropped for the first time with soybean, in Rio Verde (Table 6), Cachoeira Dourada (Table 7) and Luiz Eduardo Magalhães (Table 8) nodulation evaluated during vegetative growth was, in general, low, especially when liquid inoculants were employed. These areas suffered from drought immediately after sowing and between sowing and early flowering, which may have reflected negatively on the process of root infection and nodule development.

In Rio Verde the number and dry weight of nodules from plant samples collected at 40 DAS was far superior when peat-based inoculant was employed, compared to the liquid inoculant (Table 6). The application of a triple dose of inoculant in-furrow at sowing also promoted nodule dry weight, even though no significant differences relative to the non-inoculated control were observed

Table 6. Nodule number (NN) and dry weight (NDW), shoot dry weight (SDW), nitrogen content (NC) and total nitrogen accumulated in shoots (TNS) 40 days after sowing, and grain yield at final harvest of the soybean in Rio Verde.

Treatments [a]	NN plant^{-1} [b]	NDW (mg plant^{-1})	SDW (g plant^{-1})	NC (g kg^{-1})	TNS (mg N plant^{-1})	Yield (kg ha^{-1})
T1	0.5 bc [c]	12.5 c	6.6 a	27.1 d	177 A	2762 ab
T2	0.2 c	2.4 c	6.7 a	31.0 bc	200 A	3035 a
T3	16.1 a	148.7 a	6.6 a	28.2 cd	186 A	3074 a
T4	1.7 bc	21.0 bc	6.6 a	29.1 cd	191 A	2842 ab
T5	2.2 b	37.6 b	6.3 a	28.1 cd	177 A	2705 b
T6	0.3 c	6.7 c	6.2 a	29.3 cd	182 A	2678 b
T7	1.4 bc	12.8 c	6.8 a	33.6 ab	231 A	2698 b
T8	0.8 bc	17.0 bc	6.2 a	34.4 a	215 A	2655 b
T9	0.4 bc	8.0 c	6.0 a	30.7 bc	188 A	2532 b
T10	0.2 c	2.8 c	6.4 a	28.8 cd	182 A	2825 ab
T11	0.9 bc	26.4 bc	6.3 a	33.1 ab	207 A	2507 b
T12	1.0 bc	15.8 bc	5.5 a	33.6 ab	189 A	2824 ab
p value	<0.001	<0.001	0.9841	<0.001	0.7869	0.0369
Mean	2.13	25.97	6.36	30.60	193.7	2761
CV (%)	77.8	85.0	24.3	9.1	26.2	10.6

[a] T1 = Non-inoculated control; T2 = T1 + 200 kg N ha^{-1}; T3 = Standard peat-based *Bradyrhizobium* inoculant applied to seeds at sowing to provide 1.2 x 10^6 cells seed^{-1} (1 dose); T4 = Liquid *Bradyrhizobium* inoculant applied to seeds at sowing (1 dose); T5 = Liquid *Bradyrhizobium* inoculant applied in-furrow at sowing (3 doses); T6 = Liquid *Bradyrhizobium* inoculant sprayed close to the sowing line at sowing (3 doses); T7 = T6, but with 5 doses; T8 = Three doses of *Bradyrhizobium* inoculant + two doses of *Azospirillum* inoculant, sprayed close to the sowing line at sowing; T9 = T8, but with five doses of *Bradyrhizobium* inoculant + two doses of *Azospirillum* inoculant; T10 = Liquid *Bradyrhizobium* inoculant sprayed towards the root/stem interface region between VC and V1 (3 doses); T11 = T 10, but with 5 doses; T12 = T10, but with 10 doses. [b] Analyzed after transformation to square root of (x + 1). [c] Means (n=6) on the same column which are followed by different letters are significantly different ($p \leq 0,10$, Duncan test).

Table 7. Nodule number (NN) and dry weight (NDW), shoot dry weight (SDW), nitrogen content (NC) and total nitrogen accumulated in shoots (TNS) 50 days after sowing, and grain yield at final harvest of the soybean in Cachoeira Dourada.

Treatments [a]	NN plant^{-1} [b]	NDW (mg plant^{-1})	SDW (g plant^{-1})	NC (g kg^{-1})	TNS (mg N plant^{-1})	Yield (kg ha^{-1})
T1	1.5 a [c]	8.4 a	8.6 a	23.0 a	195 a	3000 a
T2	0.9 a	3.2 a	8.9 a	26.6 a	237 a	3054 a
T3	2.7 a	17.7 a	6.7 a	24.1 a	172 a	2543 a
T4	1.1 a	13.6 a	8.1 a	22.4 a	182 a	2563 a
T5	1.5 a	7.6 a	7.7 a	23.3 a	179 a	2880 a
T6	1.1 a	5.8 a	7.1 a	22.5 a	155 a	2662 a
T7	1.0 a	9.9 a	7.3 a	25.0 a	185 a	2499 a
T8	1.4 a	10.1 a	7.0 a	22.7 a	159 a	2792 a
T9	1.1 a	7.8 a	6.3 a	26.2 a	164 a	2767 a
T10	0.8 a	6.6 a	7.3 a	23.4 a	171 a	2991 a
T11	1.3 a	7.9 a	7.1 a	26.4 a	184 a	2726 a
T12	1.7 a	18.3 a	8.6 a	21.6 a	182 a	2826 a
p value	0.2971	0.2523	0.9050	0.6134	0.9346	0.4100
Mean	1.344	9.73	7.55	23.94	180.45	2775
CV (%)	85.4	110.3	38.3	19.3	43.7	12.9

[a] The same as Table 6; [b] Analyzed after transformation to square root of (x + 1); [c] Means (n=6) on the same column which are followed by different letters are significantly different ($p \leq 0,10$, Duncan test).

(Table 6).

A probable more intense effect of nodulation-limiting factors was observed in Cachoeira Dourada, where treatments did not differ from the non-inoculated control when sampled at 50 DAS (Table 7). When ten doses of *Bradyrhizobium*-containing inoculant were sprayed during

Table 8. Nodule number (NN) and dry weight (NDW), shoot dry weight (SDW), nitrogen content (NC) and total nitrogen accumulated in shoots (TNS) 38 days after sowing, and grain yield at final harvest of the soybean in Luiz Eduardo Magalhães.

Treatments [a]	NN plant^{-1} [b]		NDW (mg plant^{-1})		SDW (g plant^{-1})		NC (g kg^{-1})		TNS (mg N plant^{-1})		Yield (kg ha^{-1})
T1	3.6	defg [c]	14.7	de	4.6	a	12.4	c	58.1	a	nd [d]
T2	0.4	h	2.5	e	4.6	a	18.4	a	85.9	a	nd
T3	42.1	a	106.2	a	3.5	a	18.0	ab	62.8	a	nd
T4	15.7	b	57.2	b	4.9	a	15.1	c	73.9	a	nd
T5	6.1	cde	33.8	bcd	4.4	a	14.7	c	65.1	a	nd
T6	11.9	bc	40.0	bc	4.4	a	13.2	c	58.0	a	nd
T7	5.7	cdef	22.4	cde	4.3	a	15.4	bc	64.1	a	nd
T8	8.6	bcd	35.5	bcd	4.1	a	18.2	ab	71.3	a	nd
T9	12.1	bc	53.3	b	3.8	a	15.1	c	56.1	a	nd
T10	1.9	efg	11.6	de	4.0	a	13.8	c	56.0	a	nd
T11	1.0	h	5.0	e	4.6	a	18.1	ab	83.8	a	nd
T12	1.3	fg	8.9	e	4.0	a	13.9	c	54.5	a	nd
p value	<0.001		<0.001		0.9515		<0.001		0.3421		-
Mean	9.2		32.6		4.3		15.5		65.8		-
CV (%)	80.8		69.0		34.4		17.6		37.3		-

[a] The same as Table 6. [b] Analyzed after transformation to square root of (x + 1). [c] Means (n=6) on the same column which are followed by different letters are significantly different ($p \leq 0,10$, Duncan test). [d] Yield was not determined (nd) due to drought effects on plant development.

VC-V1, or when seeds received either liquid or peat-based inoculant, there was an improvement in nodule dry weight, but with no statistical difference from the other treatments.

Seed inoculation with peat-based inoculant promoted significantly more nodulation at 38 DAS in Luiz Eduardo Magalhães too (Table 8). In addition, significant gains in nodulation were also obtained by inoculating seeds with liquid inoculant, and by spraying *Bradyrhizobium* alone or in combination with *Azospirillum* in-furrow at sowing, in comparison with the non-inoculated controls (Table 8).

In Ponta Grossa, soybean had been cropped before the experiment, thus the soil had a naturalized population of *Bradyrhizobium* (Table 3). At 35 DAS, no differences in nodule number were observed relative to the non-inoculated control, except when 10 doses of *Bradyrhizobium* inoculant were sprayed in VC-V1, and no differences whatsoever were observed in nodule mass (Table 9). The use of N fertilizer, however, caused a significant decrease in both nodule number and dry weight (Table 9). Negative effects of N fertilizer on nodulation were also observed in the other sites (Tables 6, 8 and 9).

Plant biomass and total N accumulated in shoots were not significantly affected across treatments and locations (Tables 6 to 9), and the differences observed at some locations (Rio Verde, Luiz Eduardo Magalhães, and Ponta Grossa) in N content (%) reflect the dilution caused by variations in plant growth.

No significant differences in grain yield were observed at any of the locations. In Rio Verde, however, the standard practice of seed inoculation with peat-based inoculants resulted in a 292 kg ha^{-1} gain in grain yield (Table 6). N fertilizer had no effect on grain yield either. The same situation was observed in Cachoeira Dourada (Table 7). No grains were produced in Luiz Eduardo Magalhães, where soybean was mostly affected by water deficit. The Ponta Grossa location also suffered the effects of recurrent dry spells, resulting in low yields. However, although not showing statistical difference, in Ponta Grossa the combined inoculation of *Bradyrhizobium* and *Azospirillum* by spraying at sowing (T9) promoted more gain in grain yield than did soil rhizobia (T1) or N fertilizer (T2) (Table 9).

DISCUSSION

The ever-increasing world population and the awareness of potential impacts of human activities on global weather changes demand that agriculture becomes more efficient. Technologies must be developed that guarantee production and food supply, but cause the least, if none, alterations on the natural landscape, and make the area already claimed by agricultural activities more productive. The increasing use of chemicals to protect seeds and seedlings from pests and diseases (Campo et al., 2001, 2009), and the demand for seeds with anticipate inoculation challenge scientists to develop technologies that not only do not affect survival of inoculated bacteria (Ferreira et al., 2011), but also contribute to yield increase.

Fertilization has long been known to increase efficiency in agriculture, as a means to improve plant nutrition. N deficiency is a limiting factor in many places of the world, demanding heavy fertilization, but the supply of N to

Table 9. Nodule number (NN) and dry weight (NDW), shoot dry weight (SDW), nitrogen content (NC) and total nitrogen accumulated in shoots (TNS) 35 days after sowing, and grain yield at final harvest of the soybean in Ponta Grossa.

Treatments [a]	NN $plant^{-1}$ [b]		NDW (mg $plant^{-1}$)		SDW (g $plant^{-1}$)		NC (g kg^{-1})		TNS (mg N $plant^{-1}$)		Yield (kg ha^{-1})	
T1	26.3	a [c]	55.9	a	0.9	a	34.6	A	29.7	a	1796	a
T2	5.6	c	7.6	b	0.9	a	34.8	a	28.8	a	1881	a
T3	24.2	a	53.8	a	1.0	a	31.3	b	32.9	a	1685	a
T4	23.9	a	51.7	a	1.0	a	32.3	ab	30.9	a	1741	a
T5	23.3	a	50.9	a	0.8	a	34.6	a	26.9	a	1858	a
T6	22.2	a	55.5	a	0.9	a	31.7	b	29.7	a	1607	a
T7	27.6	a	39.3	a	0.8	a	32.5	ab	24.5	a	1759	a
T8	27.1	a	53.2	a	0.7	a	31.1	b	22.7	a	1844	a
T9	21.3	ab	53.2	a	0.7	a	32.7	ab	22.0	a	2088	a
T10	21.4	ab	44.9	a	2.6	a	34.6	a	92.9	a	1771	a
T11	23.0	a	53.6	a	0.8	a	32.3	ab	26.4	a	1759	a
T12	15.5	b	40.2	a	0.9	a	32.5	ab	28.0	a	1643	a
p value	<0.001		0.001		0.4273		0.0334		0.3954		0.6172	
Mean	21.8		44.2		1.00		32.9		33.0		1786	
CV (%)	26.3		41.2		127.2		7.0		137.1		19.1	

[a] The same as Table 6; [b] Analyzed after transformation to square root of (x + 1); [c] Means (n=6) on the same column which are followed by different letters are significantly different ($p \leq 0{,}10$, Duncan test).

plants can be increased by using BNF, especially when legumes are the main crop or take part in rotations with cereals (Ormeño-Orrillo et al., 2013). Successful soybean crops in Brazil rely solely on BNF as N source, but yield gains are still possible by the development of new cultivars and of technologies which increase either nodulation (number and mass) or its efficiency (Hungria et al., 2006a, b; Hungria and Mendes, 2014). The inoculation of a combination containing the traditional soybean BNF partner, *Bradyrhizobium*, and the plant growth-promoting *Azospirillum* may improve such benefits (Hungria et al., 2013), so that we tested this approach for soybean inoculation in comparison with traditional seed coating with peat-based or liquid inoculants.

Three of our experiments were carried in central Brazil (Rio Verde, Cachoeira Dourada and Luiz Eduardo Magalhães) which were severely affected by drought before and right after sowing. The hydric stress at sowing and at such an early growth stage affects root infection and nodule formation, and does not favor survival of inoculated rhizobia in the soil. In addition, all were first crop areas, and no naturalized population of rhizobia was present to guarantee a secondary nodulation after stress cessation. This explains why so few nodules were observed at the three locations. Indeed, hydric and thermic stresses are amongst the most limiting factors to BNF in the tropics, seriously affecting stages such as root infection, nodule formation and N fixation (Hungria and Franco, 1993; Hungria and Vargas, 2000; Hungria and Kaschuk, 2014).

Although without statistical difference, in Rio Verde the application of triple doses of the inoculant in-furrow at sowing, in comparison to seed inoculation, resulted in positive effects and, in addition to previous reports (Vieira Neto et al., 2008; Campo et al., 2010), reinforces its usefulness as an alternative inoculation procedure. Indeed, this alternative inoculation method has been recommended and used by Brazilian farmers, especially when pesticides are used in seed treatment in areas without naturalized bradyrhizobia population (Embrapa, 2011).

In our studies, we dedicated more attention to nodulation parameters because it has been shown that especially nodule dry weight is most well-correlated to symbiotic behavior and performance of soybean-*Brayrhizobium* associations in the field (Souza et al., 2008a, b). Nodulation results from the three locations, all first crop areas and under hydric stress, confirmed the superiority of peat-based inoculants under adverse conditions, evidencing the protective effect of peat (Hungria et al., 2005b). These results highlight that although the liquid inoculant market represents most of the commercialized products, e.g. about 80% of the 27 million doses for the soybean crop in Brazil in the last crop season, there is still much to be improved in liquid formulations to achieve the high and secure standards of using peat inoculants. Noteworthy is to reanalyze pioneer studies and to observe that the same observations and concerns date from decades ago (e.g. Burton and Curley, 1965; Burton, 1975). For example, Burton and Curley (1965) report that higher soybean yields and superior nodulation were obtained when peat-base inoculum was used, in comparison to liquid inoculum containing 2.5 times as many rhizobia. The authors speculate that the superiority of peat-inoculant could be because of sheltering rhizobia from toxic substances, or to some protective action of peat after the seeds are in the soil (Burton and Curley, 1965). Almost half a century later, we are still not aware of the peat properties which lead to its superior performance, such that the technology can be transferred to liquid inoculants.

When the same treatments were evaluated in Ponta Grossa in a soil with naturalized population of *Bradyrhizobium* established by previous inoculations and soybean cropping, no differences were observed in nodulation. Even though the crop was subjected to some degree of water stress, soil bradyrhizobia were able to nodulate plants satisfactorily. A decrease in nodulation was observed only when inoculation, even with a high dose of *Bradyrhizobium*, was performed after seedlings had emerged. In this case nodulation was probably hampered by the transient susceptibility of roots to infection (Bhuvaneswari et al., 1981). It is well known that the first steps of the symbiosis rely on the existence of molecules present in seed exudates, responsible for turning on important genes in both partners (Hungria et al., 1996; Hungria and Stacey, 1997; Geurts and Bisseling, 2002; Desbrosses and Stougaard, 2011). Therefore, it is crucial that rhizobia be in contact with the plant roots at the right place, at the appropriate time.

Experiments performed in first crop locations such as in Rio Verde, Cachoeira Dourada and Luiz Eduardo Magalhães allow us to identify and confirm positive effects of new products and technologies, which may not be observed when there is a naturalized population of rhizobia in the soil. Indeed, the importance of evaluating strains, inoculants and inoculation in soils void of compatible rhizobia has also been long recognized (Burton and Curley, 1965) and our study reinforces this importance. However, in such areas very frequently no differences are detected in parameters such as plant biomass and N accumulation in the shoots. This might be attributed to the intense mineralization of organic N in these areas, which are generally covered with grass pastures or forests prior to experiment set up. Such process may be able to supply almost all N plants demand during a first year crop.

Due to the water stresses that affected negatively all experiments, no treatment improved grain yield significantly. In Rio Verde, however, considerable increase (292 kg ha^{-1} = ca. 5 bags) was observed when peat-based inoculant was applied to seeds, relative to non-inoculation. It is worth mentioning that no effect of N fertilizer on yield was observed, and plants responded better to inoculation, with an emphasis on peat inoculants, than to mineral N fertilization. These findings support the statement that under the experimental conditions at Rio Verde, the BNF accomplished by the bradyrhizobia is able to supply all N demanded by the soybean crop.

No significant differences among treatments were observed in Cachoeira Dourada either, and no positive effects of N fertilizer occurred. In Ponta Grossa periods of drought occurred recurrently during the plant growth cycle, also affecting grain yield. It is well known that the occurrence of adverse conditions to both plant and bacteria at sowing or during early stages of the plant developmental cycle will reflect on yield. In this case, the negative effect of adverse conditions affected nodulation, which, in turn, was defective and could not supply proper amounts of N to reach higher yields.

The availability of technologies that alleviate adverse conditions for plants and bacteria at early stages of the growth cycle would be welcome. For example, placement of rhizobia in the soil nearby the seed could avoid contact between the sensible bacteria and the pesticides, micronutrients and other chemical used in seed treatment, especially if drougth occurs. Inoculants could also be sprayed at sowing, or later on after seedling emergence. However, the delay between sowing and spray inoculation may affect nodulation, especially if inoculated bacteria reach infection sites on the roots after they are no longer susceptible to infection (Bhuvaneswari et al., 1981). Zilli et al. (2008) have demonstrated that spray inoculation up to 18 days after sowing can partially promote recovery of soybean nodulation and grain yield, but longer delays may seriously compromise the crop.

Alternatively, the inclusion of one or more plant growth-promoting species in the inoculant or in the soil might help plants bypass the initial negative effects, form more nodules, and increase yield. In our experiments, the benefits of including *A. brasilense* strains produced good results. In Ponta Grossa, when five doses of *Bradyrhizobium* inoculant were combined with two doses of *Azospirillum* and applied by spraying at sowing, yield was superior, although not significantly, than that of the non-inoculated (nodulated by soil rhizobia), and of the non-inoculated + N (200 kg ha^{-1}) controls. These findings agree with recent reports (Hungria et al., 2013) of increased yield resulting from combined inoculation of soybeans with *Bradyrhizobium* (seeds) and *Azospirillum* (in-furrow) in soils containing naturalized rhizobia.

Even though our results have confirmed previous reports that peat-based inoculants are by far superior to liquid formulations (e.g. Campo et al., 2001), especially in first year crops or when situations adverse to nodulation establishment occur, it has been shown that in some situations it may be possible to remedy inoculation by spraying diluted inoculant after sowing or after seedling emergence, even though some degree of yield compromise may be expected. However, more information is necessary in order to establish inoculation frames so as to take the most benefits from the new technology.

Conflict of Interest

The authors have not declared any conflict of interest.

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