academicJournals

Vol. 12(1), pp. 6-11, 5 January, 2017
DOI: 10.5897/AJAR2016.11711
Article Number: 32CC5CC62225
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Effects of associated co-inoculation of *Bradyrhizobium japonicum* with *Azospirillum brasilense* on soybean yield and growth

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Received 15 September, 2016; Accepted 31 October, 2016

The continuous development of soybean cultivars aiming at higher yields can result in a demand of N supply higher than the standard inoculation with *Bradyrhizobium* species. In this context, *Azospirillum* species, a nitrogen-fixing microorganism found in the rhizosphere of various plant species, may be studied as a way of providing soybean crop with the part of the N it needs. Employing a completely randomized design with four replicates, this study aimed to evaluate the agronomic performance of two different methods of standard inoculation of *Bradyrhizobium* spp. associated with the co-inoculation with *Azospirillum* spp. The tested treatments consisted of the absence of N fertilizer and inoculant, full N recommendation, two single inoculations with *Bradyrhizobium* spp. (in-furrow and seed-applied) combined with an in-furrow application of *Azospirillum* spp. At R_2 growth stage, the nodule number, nodule dry weight, shoot dry biomass and shoot N content were the variables evaluated, whereas at R_8 the grain yield, thousand seed weight and grain N content were assessed. The results demonstrated that the co-inoculation of *Bradyrhizobium* spp. with *Azospirillum brasilense* is beneficial to soybean yield, mainly using the in-furrow method of application for *Bradyrhizobium* spp., associated to the in-furrow co-inoculation with *Azospirillum* spp.

Key words: *Glycine max*, inoculation, nitrogen content, yield.

INTRODUCTION

Soybean [*Glycine Max* (L.) Merr.] production is among the agricultural activities that has presented the most outstanding economic increase worldwide (Dall'Agnol et al., 2010), reaching a prominent place in the global economy. The crop plays a significant role as feedstock in oil edible production or in providing the raw material needed to obtain grain for animal feeding. According to the United States Department of Agriculture, the global grain production of soybean in 2015/2016 crop season was of 312,672 million of tons, which represent an increase of about 41% comparative to the 2005/2006 crop season (USDA, 2016).

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Nitrogen (N) is an indispensable plant nutrient related to amino acid synthesis, protein production, chlorophyll and enzymes associated with vegetable growth and development (Taiz and Zeiger, 2004; Hawkesford et al., 2012). In soybean, constituting around 1 to 5% of the plant dry matter, N is the second major nutrient demanded by the crop. It is estimated that for 1 ton of grains produced, 80 kg of N are demanded by the crop (Hungria et al., 2007). As an essential element, the N balance affects root and shoot formation and the photosynthesis process, impacting thus on the partitioning of photo assimilates at the entire plant level (Taiz and Zeiger, 2004).

In Brazil, the world's second-largest soybean producer (USDA, 2016), the success of this crop has also been attributed to the benefits obtained from the seed inoculation with *Bradyrhizobium* species, bacteria able to enzymatically reduced the atmospheric N into ammonia, which is then assimilated by plant tissues as nitrogenous compounds. The symbiosis between the bacterium and plants is an association that has been pointed out to be capable in providing crop with the N level that it needs (Hungria et al., 2006; Hungria et al., 2015).

A sprayed application of *Bradyrhizobium* spp. in the planting furrow of soybean cultivation is a consolidated agricultural practice in Brazil that can simultaneously be performed in sowing operation or soil fertilization (Embrapa, 2011). However, the continuous improvement of cultivars aiming at higher yields can result in a demand of N supply higher than the standard inoculation with *Bradyrhizobium* spp. (Hungria et al., 2015).

In this context, *Azospirillum* species, a N-fixing microorganism found in the rhizosphere of various plant species, have been reported as a way of providing crop with the part of the N it needs through biological N fixation (Pedraza et al., 2009; Hungria et al., 2010). Beneficial responses to *Azospirillum* spp. inoculation in maize or wheat have been reported in field conditions (Domingues Neto et al., 2013). For soybean, however, considering the diverse environmental conditions in which the crop is cultivated in Brazil, it needs further studies on the effects of the inoculation of this bacterium (Hungria et al., 2013)

On this point, this study aimed to evaluate the agronomic performance of two inoculation methods (seed and in-furrow) of *Bradyrhizobium* spp. in soybean crop combined with an in-furrow co-inoculation with *Azospirillum* spp., both compared to the standard inoculation technique (single inoculation with of *Bradyrhizobium* spp.) used in the crop management.

MATERIALS AND METHODS

The experiment was carried out during the soybean crop year of 2015/2016 at the Iguatemi Research Station (FEI) of the State University of Maringá (UEM), in Maringá, in northwestern Paraná State, located at latitude 23°25' south and longitude 51°57' west of Greenwich and with an average altitude of 540 m. The region's climate and soil are, respectively, classified as Cfa just as Köppen classification (Caviglione et al., 2000) and Typical Red Dystrophic Argisol according to the Brazilian Classification System (Embrapa, 2013).

Experiment installation and conduction

Under tillage conservation system, sowing took place on October 21st 2015 in a completely randomized design with four replicates. The plots comprised of eight rows of 5 × 0.45 m apart; however, the harvesting area of each experimental unit consisted of only 10.8 m², since the lateral rows and the end boundaries of the central portion were not considered as a way of minimizing the border effect described in Petersen (1994).

BMX Potência RR, an indeterminate growth habit cultivar classified as semi-early for the maturation cycle (relative maturity groups 6.7), was employed in the experiment. Previously sowing and inoculation, seeds were treated with an insecticide/fungicide product based on pyraclostrobin + thiophanate-methyl + fipronil (Standak Top®, 200 ml 100 kg^{-1} of seeds). Sowing was then performed by uniformly distributing 12 viable seeds per linear meter. Apart from the N supply and the inoculation with *Bradyrhizobium japonicum* (standard inoculation), soil correction and fertilization, weed control as well as pest and disease management were all conducted as recommended by the Brazilian Agricultural Research Corporation (Embrapa, 2011).

Inoculants and inoculation methods

The strains AbV5 and AbV6 at the concentration of 2×10^8 CFU ml^{-1} (Masterfix Gramíneas®) were used as source of *Azospirillum brasilense*, whereas a blend of the strains Semia5019 and Semia5079 at 5×10^9 CFU ml^{-1} (Masterfix®) were the source of *B. japonicum*. Both inoculants were liquid commercial and the strains used were found to be the most effective in biological N fixation (Hungria et al., 2010).

The methods of inoculation consisted either of mixing the products with the seeds or of applying them in the planting furrow prior sowing (Table 1). In the first method, after inoculation, seeds were left to dry in the shade for a period no longer than 2 h, while in the second one, the inoculants was applied via a CO_2 propelled backpack sprayer (with 2 bar constant pressure and a flow of 0.35 L min^{-1}) equipped with a pipe containing one XR 110 02 nozzle (type TeeJet Series), which provided a spray volume of 100 L ha^{-1} working at a height of 50 cm from the target and at a speed of 1 m s^{-1}, reaching a 50 cm wide strip of application.

A detailed scheme of the treatments describing the doses and the inoculation methods used is shown in Table 1. Two controls treatments were performed: the first consisted purely of a non-inoculated treatment (T1), whereas the second was a non-inoculation control that received N fertilizer (T2). Using urea (46% of N) as source, N fertilizer was applied at 200 kg ha^{-1}, split into two applications of 50% each, one at sowing and the other at the R_2 (55 days after sowing) growth stage (Hungria et al., 2015). The other treatments consisted of either inoculation of *B. japonicum* (standard inoculation) or of co-inoculation of *B. japonicum* with *A. brasilense*. While *B. japonicum* followed an in-furrow (T5 and T6) or seed application (T3 and T4), *A. brasilense* was directly sprayed in the sowing furrow (T4 and T6) just as performed in Hungria et al. (2015).

Agronomic characteristics and yield evaluation

At the R_2 growth stage (55 days after sowing), ten plants were randomly collected from the harvesting area of each plot in order to

Table 1. Scheme of the treatments describing the N fertilizer level, the control treatments, the doses and the inoculation methods of *B. japonicum* associated, or not, with the co-inoculation with *A. brasilense* (Maringá, Paraná, Brazil, 2015/2016).

Treatments	N dose (kg ha^{-1})	*Bradyrhizobium japonicum*		*Azospirillum brasilense*	
		Doses* (ml)	Inoculation method	Doses* (ml)	Inoculation method
T1	0	0	-	0	-
T2	200	0	-	0	-
T3	0	200	Seeds	0	-
T4	0	200	Seeds	200	Furrow**
T5	0	600	Furrow**	0	-
T6	0	600	Furrow**	200	Furrow**

*ml 100 kg^{-1}seed or ml ha^{-1} according to the method. ** Total volume of 100 L ha^{-1}.

evaluate the nodule number (NN), the nodule dry weight (NDW), as well as the shoot dry biomass (SDB) and shoot N content (SNC). On the other hand, the grain yield (GY), the thousand seed weight (TSW) and the grain N content (GNC) were assessed at R_8 (135 days after sowing), which is the complete physiological maturity of the cultivar (Veloso et al., 2006; Hungria et al., 2006).

Nodule number (NN) and nodule dry weight (NDW)

The ten samples were collected in each plot using a shovel to excavate a block of soil of 0.40 × 0.40 m by 0.20 m deep. In the laboratory, nodules were removed from roots and then counted and eventually dried in a forced-air oven at 65 ± 2°C for 72 h (Brasil, 2009).

Shoot dry biomass (SDB) and shoot N content (SNC)

Once collected, the plants were placed in paper bags, identified and dried in a forced-air oven at 65 ± 2°C for 72 h (Brasil, 2009) and eventually weighted. The average SDB data were expressed in g $plant^{-1}$. Dried shoots were then ground (18 mesh) and subjected to sulfuric digestion to determine total SNC as documented in AOAC (2000) with the modifications proposed by Vitti et al. (2001), SDB data were expressed in grams per plant (g pl^{-1}), while SNC as percent of N content in the dry matter.

Grain yield

From five to eight days after the complete physiological maturity (R_8 stage), harvest was manually performed collecting all the plants of the evaluated area of each plot. Pods were mechanically threshed and then impurities were removed in order to obtain a very clean lot. After proper moisture content adjustment of 13% (Brasil, 2009), the cleaned grains were weighed and the average data were converted into kg ha^{-1}.

Thousand seed weigh (TSW)

After the moisture adjustment described earlier, it was determined by weighing 8 subsamples of 100 seeds for each field plot, with an analytical scale accurate to 1 mg. For all plots, the coefficient of variation was less than four, and the results were multiplied by 10 (Brasil, 2009).

Grain N content (GNC)

From the cleaned seeds portion, it was determined through the method of sulfuric acid digestion using a micro Kjeldahl distillation apparatus, as described in AOAC (2000) with the modification pointed out in Vitti et al. (2001). GNC data were expressed as percent of dry matter.

Statistical analysis

All analyses were performed using the statistical software Sisvar (Ferreira, 2011). The data were submitted to the Shapiro-Wilk test ($p<0.10$) and Levene test ($p<0.10$) to verify the normal distribution and homoscedasticity, respectively. Each variable was subjected to analysis of variance at 10% probability and when it was significant, the means were compared by Fishers' protected t-test LSD (Least Significant Difference) ($p\leq0.10$) according to Banzatto and Kronka (2008).

RESULTS AND DISCUSSION

The results obtained in the crop season of 2015/2016 for the soybean nodulation and growth, the total N accumulated in shoots and grains as well as, the results of the yield compounds are shown in Table 2. All the variables showed statistically significant at 10%, indicating thus that the two tested inoculation methods of *B. japonicum* associated to an in-furrow co-inoculation with *A. brasilense* significantly affected the crop.

Nodule number (NN) and nodule dry weight (NDW)

Regardless of the application method, all treatments in which the standard inoculation (*B. japonicum*), associated or not to the co-inoculation (*B. japonicum* + *A. brasilense*), showed higher values for NN and NDW than the non-inoculated treatments (T1 and T2) at the R_2 physiological stage (55 days after sowing). On this point (Table 2), the in-furrow inoculation with *B. japonicum* associated to the in-furrow application of *A. brasilense* provided significantly superior results among the tested

Table 2. Nodule number (NN), nodule dry weight (NDW), shoot dry biomass (SDB) and shoot N content (SNC) at R_2stage, as well, the grain yield (GY), the thousand seed weight (TSW) and grain N content (GNC) at$R_8$of the soybean cultivar BMX Potência RR in response to standard inoculation (*B. japonicum*) and to the co-inoculation (*B. japonicum +A. brasilense)* in Maringá, Brazil, crop season 2015/2016.

Treatments	NN (No. pl^{-1})	NDW (g pl^{-1})	SDB (g pl^{-1})	SNC (%)	TSW (g)	GNC (%)	GY (kg ha^{-1})
T1	49.50^{e}	0.49^{e}	9.58^{e}	6.62^{f}	120.48^{f}	7.85^{d}	1843.62^{e}
T2	54.75^{e}	0.59^{d}	12.97^{d}	7.21^{e}	131.06^{e}	8.51^{c}	2196.44^{d}
T3	61.92^{d}	0.66^{c}	15.01^{c}	7.56^{d}	137.88^{d}	8.75^{c}	2333.16^{d}
T4	74.50^{c}	0.69^{c}	15.85^{c}	7.92^{c}	140.72^{c}	9.13^{b}	$2.579.44^{c}$
T5	95.83^{b}	0.76^{b}	16.82^{b}	8.36^{b}	143.95^{b}	9.39^{b}	2788.45^{b}
T6	103.00^{a}	0.95^{a}	26.63^{a}	9.37^{a}	150.31^{a}	9.97^{a}	3354.58^{a}
Means	73.25	0.69	16.14	7.84	137.4	8.93	2515.95
C.V. (%)	4.88	7.32	15.72	2.76	2.78	2.78	4.13

Within columns, means followed by same letter do not significantly differ from each other ($p < 0.10$ LSD).

treatments.

As expected, the lowest NN and NDW values were observed in treatments T1 and T2. Fact that can be explained by the absence of the standard inoculation, indicating thus that the supply of N for soybean crop is needless when the strains Semia 5019 and Semia 5079 of *B. japonicum* are used prior sowing (Mendes et al., 2003; Zilli et al., 2006).

Regarding the NDW, T3 (single seed inoculation) and T4 (co-inoculation) did not differ significantly from each other, which means that the in-furrow co-inoculation with *A. brasilense* had no influence on this nodulation response. Further, in the presence of *A*. *brasilense* (T4), higher NN value was observed. A contrasting finding was pointed out in Hungria et al. (2015), in which the co-inoculation did not increase the NN comparative to standard inoculation; however, no incompatibility between the tested inoculants was found when the same method of application was employed.

Hungria et al. (2007) affirmed that at R_2 growth stage, a NN ranging from 15 to 30 nodules by plant and a NDW ranging from 0.10 to 0.20 g pl^{-1} are the adequate nodulation parameters in which the bacteria are able to supply the full N demand of the crop. However, as pointed out in Hungria et al. (2013), the higher results found in the present study may be due to the enrichment of soil bradyrhizobial population as the field area used has been continuously cultivated with inoculated soybean.

Shoot dry biomass (SDB) and shoot N content (SNC)

The SDB was significantly increased by the in-furrow inoculation of *B. japonicum*, relative to the non-inoculated controls (T1 and T2) or to the seed-inoculated treatments (T3 and T4). The same performance was found for SNC.

Further, once co-inoculated with *A. brasilense*, the treatments T4 and T6 provided equal or superior SNC and SDB values than those in which the single inoculation was performed, regardless of the method of application (Table 2). In this context, Hungria et al. (2015) summarized that it is unclear whether for soybean the benefits of co-inoculation of *B. japonicum* with *A. brasilense* are merely due to an intensification of N_2 fixation rates, or if other any secondary factor is involved. On the other hand, for maize, Domingues Neto et al. (2013) found an increase in the dry biomass of both the shoots and roots as a result of foliar application of *Azospirillum* spp. Bashan and Dubrovsky (1996) proposed that by affecting root roles, *Azospirillum* spp. participates in the partitioning of the carbon and minerals at the entire plant level. Later, Bashan et al. (2004) pointed that the positive response of dry matter accumulation to *A. brasilense* inoculation could be attributed to the phytohormones excreted by this bacterium.

Thousand seed weight (TSW) and grain N content (GNC)

As yield or quality components, the TSW and GNC were positively influenced by the inoculation of *B. japonicum* associated, or not to an application of *A. brasilense.* Moreover, treatments submitted to the in-furrow co-inoculation (T4 and T6) performed better than those in which only the standard inoculation was performed (T3 and T5).

In the bibliography, contrasting results have been recorded about the effects of the inoculation with *A. brasilense* on TSW. In this regard, while in Biari et al. (2008), Costa et al. (2015) and Morais et al. (2016) found an increase in seed weigh of maize after inoculation; on the other hand, no significant interaction between inoculation and seed weigh was reported by Novakowiski et al. (2011). Also, Dobbelaere et al. (1999) reported that inoculations with *A. brasilense* did not impact GNC in maize.

Apart from the non-inoculated treatment T1, to which no N fertilizer was supplied, all treatments inoculated with *B. japonicum* (co-inoculated, or not, with *A. brasilense*) showed GNC values within the range considered as suitable for this crop (Martinez et al., 1999), but slightly lower than those reported by Hungria et al. (2015). Therefore, *B. japonicum* was able to provide the full N demand of the crop.

Grain yield (GY)

The in-furrow co-inoculation with *A. brasilense* and *B. japonicum* employed in T6 provided significant yield increase compared to the other treatments, mainly to those non-inoculated (T1 and T2). Interestingly, overall GY revealed the same trend observed earlier in NN, NDW, SDB, SNC, TSW and GNC, in which the highest values were observed in T6 followed in a decreasing order by treatments T5 and T4, with significant differences among them.

Such as observed in this work, Pedraza et al. (2009) and Hungria et al. (2010) found that in non-leguminous, *A. brasilense* promoted increase in the plant growth and yield compounds. Regardless of the co-inoculation, among the two tested standard inoculation of *B. japonicum,* the in-furrow application (T5 and T6) showed superior GY performance than the seed-inoculated treatments (T3 and T4). However, the co-inoculation with *A. brasilense* performed in T4, provided superior NN, SNC, TSW, GNC and GY results than the single seed-inoculated treatment (T3).

Brandão Júnior and Hungria (2000) documented that for *Rhizobium* spp. establishment in the substrate, the minimum concentration of viable bacteria in the inoculant must be hundred times higher than that naturally found in the soil. Based on this finding, it is plausible to suggest that the inoculant doses of *A. brasilense* combined with *B. japonicum* application used in T4 and T6 reached the minimum bacteria concentration able to form a robust soil colony.

As discussed in Araújo et al. (2015), under limited N availability, plants cannot produce sufficient root exudates, which act as sign to influence the ability of *A. brasilense* to colonize soil by horizontal movement or to survive in the rhizosphere (Mark et al., 2005). On this point, based on the understanding of Hallmann et al. (1997) and Saubidet et al. (2002), it is plausible to suggest that *B. japonicum* provided crop with an adequate N supply to sustain its growth and yield and, therefore, the plants were able to provide *A. brasilense* with the needed rate of carbon compounds for its soil establishment.

In the absence of oxygen, *A. brasilense* was found to present higher rate of N fixation (Dobbelaere et al., 1999). Nevertheless, the increase in the growth and yield in co-inoculated treatments of soybean with this bacterium may not only be credited to N biological fixation, since the excretion of hormones from the association plant-bacterium can increase the number and the mean diameter of lateral and adventitious roots (Dobbelaere et al., 2003), which could have intensified the plant nutrient absorption and thus the productivity (Bashan and De-Bashan, 2010).

Conclusion

The results demonstrated that co-inoculation with *B. japonicum* and *A. brasilense* is beneficial to soybean crop. The co-inoculation promoted grain yield increment ranging from 10 to 20% relative to standard inoculation (single application of *B. japonicum*), with the highest value observed using the in-furrow method of application of *B. japonicum* associated to an also in-furrow co-inoculation with *A. brasilense*.

Conflict of Interests

The authors have not declared any conflict of interests.

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