Australian Journal of Crop Science
AJCS
AJCS 18(07):434-441 (2024)
https://doi.org/10.21475/ajcs.24.18.07.pne-170
ISSN:1835-2707

Influence of pre-inoculation of soybean seeds with *Bradyrhizobium* 15 days before sowing

Wilson Story Venancio[1], Eduardo Gilberto Dallago[1], Gislaine Martins Slusarz[1], Ibraian Valério Boratto[1], Vanessa Nathalie Modesto Boratto[1], Ana Carolina Leite[2], Rebeca Cuenca[2], Aline Ratuchne[2]*

[1]CWR Pesquisa Agrícola, Ponta Grossa, PR, Brazil
[2]Nova do Brasil, Londrina, PR, Brazil

***Corresponding author: alineratuchne@gmail.com**

Submitted: 09/04/2024

Revised: 17/07/2024

Accepted: 26/07/2024

Abstract: Nitrogen is an essential nutrient for the soybean cultivation, which can be fully supplied by the biological nitrogen fixation process. This study evaluated the effectiveness of pre-inoculation of soybean seeds with the inoculant Biofix Protec (*Bradyrhizobium diazoefficiens* SEMIA 5080 and *Bradyrhizobium japonicum* SEMIA 5079, 5x10^9 UFC/mL), accompanied by cell protector and chemical treatment. Four trials were done in Brazilian states during the soybean harvest 2021/2022, following a randomized complete block design with six replications. The cultivars used were TMG 7067 IPRO Inox, BRS 245 RR, N 7780 PRO, and Extrema IPRO, chosen according to the characteristics of each region. The treatments were: T1- Control, without inoculation; T2- Fertilization with 200 kg/ha of nitrogen; T3- Inoculation with commercial inoculant (50 mL/50 kg of seeds) on the day of sowing; T4- Biofix Protec (100 mL/50 kg of seeds), cell protector (0.5 mL/kg), Potenzial TS (0.2 mL/kg) and CoMo Platinum (100 mL/ha), all applied 15 days before planting. The results indicated that the application of Biofix Protec 15 days before planting increased the nodulation of the main root and the dry mass of nodules. The pre-inoculation of Biofix Protect, with protective agents and chemical treatments, demonstrated comparable efficacy to the inoculation carried out at the time of sowing and increase in yield between 4.8-11%, compared to the control, being statistically equivalent or superior to the commercial inoculant available in the market, applied on the day of sowing. The use of Biofix Protec favored nodulation and enhanced productivity, being recommended for the treatment of soybean seeds in agricultural production systems.

Keywords: Pre-sowing; Rhizobia; *Glycine max*; symbiosis; Nitrogen fixation; Crop yield.
Abbreviations: CFU_colony-forming unit, BNF_biological nitrogen fixation, N_nitrogen, NNMR_number of nodules on the main root, DMNMR_dry mass of nodules on the main root, FMS_fresh mass of shoots, DMS_dry mass of shoots, GY_grain yield, WTG_ weight of a thousand grains, ATP_adenosine triphosphate, NADPH_reduced nicotinamide adenine dinucleotide phosphate, FCI_Falker chlorophyll index, TGW_thousand grain weight, PNG_percentage of nitrogen in grains.

Introduction

In Brazil, sowing soybeans (*Glycine max* (L.) Merrill) is recommended without nitrogen fertilizers, owing to its biological nitrogen fixation (BNF) character, reducing production costs and increasing grain production (Hungria et al., 2007). Nitrogen (N) is essential for soybeans, needing approximately 80 kg of N to produce 1000 kg of grain (Kaschuk et al., 2016). N sources are nitrogen fertilizers and atmospheric N, available through BNF (Hungria et al., 2001).
BNF involves a symbiosis between legumes like soybeans and nitrogen-fixing rhizobia bacteria (Alves et al., 2003; Sprent et al., 2017). Efficient strains must establish in plant roots for maximum BNF (Bender et al., 2022). Factors influencing plant-rhizobia symbiosis include strain, inoculant dose, co-inoculation, seed treatments, fertilization, and environmental conditions (Obserson et al., 2007; Anghinoni et al., 2017; Hungria et al., 2007; Campo et al., 2009; Zilli et al., 2010).
Some *Bradyrhizobium* strains, like *Bradyrhizobium diazoefficiens* (formerly *B. japonicum*) (Delamuta et al., 2013), are highly efficient and studied. *B. diazoefficiens* also forms nodules on other plants like mung bean and siratro (Sprent et al., 2017; Piromyou et al., 2021).
Inoculating seeds before marketing or sowing (pre-inoculation) has been practiced for decades for crops like soybeans (Deaker et al., 2004; Herridge, 2008; Santos et al., 2019). This provides uniform seed treatment and operational efficiency for large-scale soybean cultivation (Hungria et al., 2020). However, success depends on the survival of bacterial on seeds and storage conditions (Date, 2001).
Soybean seed treatment with fungicides is common to prevent phytopathogens, with 90% of Brazilian soybean seeds treated chemically (Peske and Levien, 2005). However, fungicides can reduce *Bradyrhizobium* populations on seeds due to active ingredients and solvents (Campo et al., 2009). This can lead to fewer and smaller nodules, though some fungicides do not significantly affect nodulation (Andrés et al., 1998; Bikrol et al., 2005).
Using a cell protector in seed treatment can protect bacteria and extend their survival from treatment to planting.

Table 1. Number of nodules on the main root (NNMR), dry mass of nodules on the main root (DMNMR), fresh and dry mass of shoots (FMS and DMS), Falker chlorophyll index (FCI) in the four regions evaluated during 2021/2022 harvest.

PALMEIRA-PR											
N°	Treatments	NNMR[1]		DMNMR[1] mg		FMS[1] g		DMS[1] g		FCI[1]	
1	Control	22.17	ab	61.22	A	29.40	b	5.18	b	437.97	b
2	N200	15.06	b	32.06	B	33.39	a	6.42	a	489.93	a
3	Commercial inoculant	26.28	a	66.78	A	29.78	b	5.75	ab	476.57	ab
4	Biofix Protec	23.61	a	64.83	A	32.59	a	5.83	ab	476.10	ab
	C.V. (%)[2]	35.76		12.15		7.68		18.44		7.95	
	ITAPIRA-SP										
N°	Treatments	NNMR[1]		DMNMR[1] mg		FMS[1] g		DMS[1] g		FCI[1]	
1	Control	6.42	a	97.72	b	26.85	b	6.15	b	427.30	b
2	N200	5.83	ab	54.56	c	32.82	a	7.77	a	495.70	a
3	Commercial inoculant	5.75	ab	171.67	A	31.75	a	7.61	a	500.20	a
4	Biofix Protec	5.18	b	170.72	A	31.22	a	7.47	a	493.37	a
	C.V. (%)[2]	18.44		13.74		10.25		6.66		6.52	
	ARAGUARI-MG										
N°	Treatments	NNMR[1]		DMNMR[1] mg		FMS[1] g		DMS[1] g		FCI[1]	
1	Control	11.61	d	58.24	d	49.41	b	10.77	a	410.87	b
2	N200	27.28	c	98.72	c	56.80	a	11.88	a	468.27	a
3	Commercial inoculant	77.39	a	288.00	A	54.80	a	11.32	a	454.73	a
4	Biofix Protec	69.72	b	272.06	b	53.41	ab	11.10	a	448.13	a
	C.V. (%)[2]	15.33		5.46		9.25		10.69		6.36	
	CATALÃO-GO										
N°	Treatments	NNMR[1]		DMNMR[1] mg		FMS[1] g		DMS[1] g		FCI[1]	
1	Control	6.83	d	99.54	c	54.63	b	10.75	c	297.53	b
2	N200	21.67	c	128.04	b	61.00	a	13.95	a	328.10	a
3	Commercial inoculant	43.06	b	192.67	A	58.15	a	12.87	ab	315.17	a
4	Biofix Protec	48.17	a	192.47	A	59.94	a	12.18	b	321.60	a
	C.V. (%)[2]	11.93		6.13		4.74		9.33		4.88	

Means followed by the same letter in the column do not differ from each other using the Duncan test at 10% probability.
Coefficient of variation in percentage.

Experiments in Brazil showed soybeans could be inoculated up to five days before sowing (Campo and Hungria, 2007), and with a protectant, it may be extended up to 30 (Araujo et al., 2017) or 60 days (Machineski et al., 2018).

Araujo et al. (2017) demonstrated that pre-treatment with inoculant, selected fungicides, and insecticide was viable when combined with a microbial protectant, allowing inoculation days before planting. Seed producers are adopting industrial seed treatment, offering pre-treated seeds with insecticides, fungicides, micronutrients, and *Bradyrhizobium*, streamlining the sowing process (Brzezinski et al., 2015).

Results

Symbiotic efficiency

The effectiveness of treatments based on the number of nodules on the main root (NNMR), dry mass of nodules on the main root (DMNR), fresh and dry mass of the shoot (FMS and DMS), and the Falker chlorophyll index (FCI) in four regions (Table 1).

In Palmeira-PR, Biofix Protec (T4) applied 15 days before planting resulted in 23.61 nodules and 64.83 mg in the main root, statistically superior to nitrogen fertilization (T2) and similar to the market inoculant (T3) and control (T1). T4 significantly differed from T1 and T3 in FMS, but was equal to T2. No significant variations were observed in DMS and FCI for T4 compared to other treatments.

In Araguari-MG, the commercial product (T3) had higher averages of 77.39 nodules and 288.0 mg DMNR compared to other treatments. Biofix Protec (T4) also showed significant increases compared to T2 and T1. FMS did not significantly vary among T4, T3, and T2, but T3 and T2 differed from T1. No significant differences were observed in DMAP and FCI among T4, T3, and T2, but they were superior to T1.

In Catalão-GO, Biofix Protec (T4) increased nodulation, with 48.17 nodules on the main root, statistically higher than other treatments. Both T4 and T3 significantly differed from T1 and T2 in DMNR. FMS and FCI were statistically equal for T3, T4, and T2, but differed from T1. DMS showed statistical differences for T3 and T4 compared to T1, but T4 did not differ from T3. Productivity results, relative gain, thousand grain weight (TGW), nitrogen content, and total N content in grains are shown in Table 2. In Palmeira-PR, Biofix Protec (T4) resulted in 3546.13 kg.ha^{-1}, an 8.5% yield gain, statistically superior to T1 and T3 but not different from T2. No significant differences were found in TGW, with N content ranging from 5.43% to 5.71%.

In Itapira-SP, Biofix Protec (T4) and the commercial product (T3) were equal, with gains of 11% and 12%, respectively, significantly differing from T2 and T1. TGW varied from 130.53 to 136.47 grams, with Biofix Protec (T4) resulting in 5.48% N in grains and 212.51 kg.ha^{-1} of total N, significantly different from other treatments.

In Araguari-MG, the inoculated treatments (T3 and T4) achieved a 7% production gain over T1, with no statistical differences between pre-inoculated (T4) and inoculation at sowing (T3), or T2, which showed a 6% yield gain. No significant differences in TGW were observed. N content in grains varied from 5.40% to 5.53%, with T3, T4, and T2 equal to each other but higher than T1.

Table 2. Product factors (productivity and weight of a thousand grains – TGW), relative production gain in percentage, percentage of nitrogen in grains and total nitrogen content in the different regions evaluated during 2021/2022 agricultural year.

PALMEIRA-PR								
Treatments	Productivity kg/ha		Relative gain	TGW g		Nitrogen (B.U) %	Total N in grains kg/ha	
Control	3267.28	c	0.0	180.29	a	5.57	181.99	c
N200	3561.31	a	9.0	183.23	a	5.43	193.38	b
Commercial inoculant	3462.39	b	6.0	181.20	a	5.71	197.70	a
BiofixProtec	3546.13	a	8.5	184.14	a	5.55	196.81	ab
C.V. (%)	1.89			2.26			1.89	
ITAPIRA-SP								
Treatments	Produtivity kg/ha		Relative gain	TGW g		Nitrogen (B.U) %	Total N in grains kg/ha	
Control	3493.23	c	0	130.53	b	5.65	197.35	d
N200	3699.70	b	6	131.05	b	5.56	205.68	c
Commercial inoculant	3913.12	a	12	136.47	a	5.34	208.95	b
BiofixProtec	3877.88	a	11	134.89	ab	5.48	212.51	a
C.V. (%)	1.49			3.30			1.50	
ARAGUARI-MG								
Treatments	Produtivity kg/ha		Relative gain	TGW g		Nitrogen (B.U) %	Total N in grains kg/ha	
Control	3915.00	b	0	153.55	a	5.53	216.50	b
N200	4139.83	a	6	155.45	a	5.51	228.11	a
Commercial inoculant	4205.75	a	7	155.41	a	5.40	227.11	a
BiofixProtec	4185.45	a	7	155.78	a	5.52	231.04	a
C.V. (%)	1.69			1.84			1.69	
CATALÃO-GO								
Treatments	Produtivity kg/ha		Relative gain	TGW g		Nitrogen (B.U) %	Total N in grains kg/ha	
Witness	4147.60	b	0.0	172.68	a	5.36	222.31	b
N200	4349.22	a	4.9	175.87	a	5.43	236.16	a
Commercial inoculant	4345.26	a	4.8	176.20	a	5.49	238.56	a
BiofixProtec	4347.93	a	4.8	173.96	a	5.33	231.75	a
C.V. (%)	3.37			2.31			3.37	

1. Means followed by the same letter in the column do not differ from each other using the Duncan test at 10% probability.
2. Variation coefficient in percentage.

In Catalão-GO, productivity was equal for T4, T3, and T2, with gains of 4.8% to 4.9%, higher than T1. All treatments were equal in TGW, but T3, T4, and T2 showed significant differences in total N content compared to T1, with averages ranging from 231.75 to 238.56 kg.ha^{-1}.

Discussion

In the four different edaphoclimatic regions, nodule formation on main roots varied by location. Initial rhizobia population assessments in Palmeira-PR and Itapira-SP influenced nodulation in control treatments (T1) (without inoculation). Inoculation is crucial in first-year soybean fields or recently uncultivated legumes due to low N_2-fixing bacteria. Even in well-cultivated areas, inoculation via seeds or sowing furrow remains beneficial, with an average annual yield increase of 8% from *Bradyrhizobium* inoculation (Hungria et al., 2007).

In Brazil, re-inoculation increased soybean yield even in soils with high *Bradyrhizobium* cell counts, as shown in Cerrados and Paraná experiments (Vargas et al., 1994). In Araguari-MG and Catalão-GO, no rhizobia were found despite a history of soybean rotation. T1 (control without inoculation) showed less nodulation and lower dry mass, while seed inoculation treatments showed efficient nodulation.

Based on this context, in Palmeira-PR the treatment with Biofix Protec (T4) was significantly equal to the commercial product inoculated on the day of sowing (T3) and statistically superior to the nitrogen treatment (T2). The same was observed in Itapira-SP, where the average number of nodules did not show significant variations with the use of Biofix Protec (T4) compared to the commercial product (T3).

In the Araguari-MG region, the number of nodules for Biofix Protec (T4) was lower than the commercial product (T3), while, statistically higher than T2 and T1. In Catalão-GO, the results for Biofix Protec in pre-inoculation (T4) were highly efficient, significantly differentiating from the other treatments used. These results directly reflected the final yield of the crop, as according to Câmara (2000), soybean plants with a large number of nodules at flowering present sufficient conditions to obtain high levels of fixed nitrogen and, consequently, high grain yield.

In Palmeira-PR, Araguari-MG, and Catalão-GO, nodule numbers aligned with the literature: 4-8 nodules per plant 10-15 days after emergence and 15-30 nodules at flowering (Vargas and Hungria, 1997). Ribeiro Neto et al. (2018) also found that inoculants and bacterial protective additives with early inoculation led to 27% more nodules on the main root at 45 days compared to non-inoculated controls.

In Itapira-SP, the average number of nodules was lower than that found in other trials. However, the application of Biofix Protec (T4) did not statistically differ from the commercial product (T3). It is known that crop nodulation may vary according to soil pH, vegetative stage and environmental conditions in which the plants are located. According to Silva et al. (2002), to have an efficient development of bacteria, the soil must have a pH of 6.5, with the soil pH in Itapira-SP being around 5.8.

Table 3. Description of biological products, additives, protectors and fertilizer, classes, active ingredients and doses used in soybean cultivation.

	Commercial product	Class	Active ingredient	Dose
1	Commercial inoculant	Inoculant (Control)	*B. japonicum* (Semia 5079 and 5080) $5x10^{12}$ $CFU.mL^{-1}$	$1.0\ mL.kg^{-1}$
2	Biofix Protec	Inoculant (evaluated)	*B. diazoefficiens* (Semia 5080) and *B. japonicum* (Semia 5079) 5.10^9 $CFU.mL^{-1}$	$2.0\ mL.kg^{-1}$
3	Potenzial TS	Mixed mineral fertilizer	Mo 3.8%	$0.4\ mL.kg^{-1}$
4	Protetor Protec	Polyvinyl adhesive	-	$0.5\ mL.kg^{-1}$
5	Maxim XL Cruiser 350 FS	Fungicide Insecticide	Metalaxil-M 10 $g.L^{-1}$ + Fludioxonil 25 $g.L^{-1}$ Tiametoxan 350 $g.L^{-1}$	$1.0\ mL.kg^{-1}$ $2.0\ mL.kg^{-1}$
6	CoMo Platinum[1]	Liquid fertilizer	-	$100\ mL.ha^{-1}$

1. Foliar fertilizer applied at V3/V4 stage.

In general, in all studied places, the Biofix Protec inoculant (T4) showed potential for use in soybean crops in pre-treatment up to 15 days before sowing, where it directly influenced the nodulation of the crop.

Nodule mass, a key factor for BNF, was statistically higher in inoculated treatments (T3 and T4) compared to the nitrogen treatment (T2) across all regions. In Itapira-SP, Araguari-MG, and Catalão-GO, nodule mass was also higher than in the absence of inoculation (T1). This aligns with Döbereiner (1966), that identified nodule mass as a key indicator of effective symbiosis. Hungria et al. (2001) reported that plants with 15-30 nodules must have a nodule mass of 100-200 mg at flowering to meet nitrogen demands and achieve high productivity.

Soybeans obtain nitrogen from fertilizers and atmospheric N via biological nitrogen fixation (BNF) (Hungria et al., 2007; Hungria et al., 2015). In Brazil, due to BNF's efficiency, inoculating seeds with *Bradyrhizobium* bacteria eliminates the need for nitrogen fertilizers (Hungria et al., 2017).

Nitrogen application (T2) significantly reduced the number and mass of taproot nodules in all studied regions. Similar results have been observed by other authors, showing that nitrogen fertilization in legumes adversely affects BNF by decreasing nodular respiration and limiting carbohydrates in nodule metabolism (Silva et al., 2011).

According to Hungria et al. (2001), it is not necessary to carry out this fertilization method for the crop, as long as it has been inoculated with bacteria that favor BNF. Hungria et al. (2007), investigated the effect of different types of inoculation on soybean crops, and showed that inoculation alone presents more satisfactory results when combined with fertilization with nitrogen fertilizers. According to Fagan et al. (2007), soybean plants subjected to nitrogen fertilization have a significant reduction in the development of nodules, as regards the data from this study.

The chlorophyll content in Biofix Protec pre-inoculated treatments (T4) showed no significant differences compared to the commercial product (T3) and nitrogen fertilization (T2) across all regions evaluated. However, it was higher than the control (T1) in Itapira-SP, Araguari-MG, and Catalão-GO. Chlorophyll content is crucial for assessing plant nutrition and can serve as an indicator for nitrogen levels in crops (Argenta et al., 2001).

The importance of plant nodulation is emphasized by Vollmann et al. (2011), which showed a high relationship with increased chlorophyll content in leaves, where there is usually a correlation between chlorophyll and nitrogen content in plants (Argenta et al., 2001). Pereira et al. (2010) pointed out that this assessment is valid to attest agronomic efficiency of diazotrophic bacteria. In Palmeira-PR, Biofix Protec (T4) significantly differed from the commercial product (T3) in fresh aerial mass. In Itapira-SP and Catalão-GO, T4 increased both fresh and dry aerial mass compared to the control (T1), similar to T2. Li and Alexander (1988) observed a 23% increase using *B. japonicum* treatment compared to the control.

For grain productivity, all inoculated treatments (T3 and T4) and nitrogen treatment (T2) significantly outperformed the control (T1), with yield increases ranging from 4.8% to 12.0%. In Palmeira-PR, pre-sowing Biofix Protec (T4) application resulted in 3546.13 $kg.ha^{-1}$, an 8.5% yield gain compared to the commercial product (T3). Hungria et al. (2007) also reported an 8% productivity increase with *Bradyrhizobium*-inoculated soybean seeds, offering a favorable return on inoculation costs.

In other regions, Biofix Protec (T4) showed comparable productivity to the commercial product (T3). However, in Itapira-SP, T4 significantly outperformed the nitrogen treatment (T2) by approximately 5% in crop yield. Several studies demonstrated that inoculation can achieve high soybean yields, potentially reducing or eliminating the need for nitrogen fertilizers (Hungria et al., 2007; Manchineski et al., 2018).

The thousand grain weight (TGW) is crucial in soybean yield. However, no significant differences were observed among treatments in the trial across four regions. Toni et al. (2018) also reported no statistical differences in TGW among soybean varieties inoculated with *Bradyrhizobium japonicum.*

Nitrogen content in grains influences their physiological and nutritional quality. In Palmeira-PR, Biofix Protec pre-inoculation (T4) significantly increased grain nitrogen content compared to the control (T1), though results did not differ from other treatments. In Itapira-SP, T4 showed statistically superior nitrogen accumulation in grains compared to other treatments. In Araguari-MG and Catalão-GO, T2, T3, and T4 showed no significant differences among them but were all superior to T1.

Inoculation with *Bradyrhizobium* species has economic and environmental benefits but involves labor and time for sowing-day inoculation (Hungria et al., 2017). Pre-inoculation or early inoculation of *Bradyrhizobium* strains is recommended to mitigate the challenges, since the industrial seed treatments do not compromise inoculant viability. Few studies have evaluated the association of pre-inoculation with industrial seed treatments (Anghinoni et al., 2017; Manchineski et al., 2018). Further research is needed to determine the optimal pre-sowing period that maintains bacterial viability, plant nodulation, and soybean productivity (Pereira et al., 2010; Zilli et al., 2010).

Table 4. Cultural treatments in each experimental region. 2021/2022 growing season.

Municipality	Pre-treatment date 15 days	Sowing date	Harvest date	Variety	Density (stes.ha^{m})	Spacing (cm)	Predecesso culture	Rotation with soybean	Base fertilization	
									Source	Doses (kg.ha^{-1})
Palmeira - PR	12/02/2021	12/17/2021	05/17/2022	TMG 7067 IPRO Inox	311.111	45	Wheat	Yes	Amino acids + 00-00-60	450 + 60
Itapira - SP	02/07/2022	02/21/2022	06/02/2022	BRS 245 RR	280.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106
Araguari - MG	02/09/2022	02/24/2022	06/17/2022	N 7780 PRO	300.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106
Catalão - GO	01/25/2022	02/09/2022	06/14/2022	Extrema IPRO	300.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106

The use of seed protectors is also crucial for the success of pre-inoculation, as they ensure the survival of cells for a longer period in seeds treated with insecticides and/or fungicides (Silva et al., 2018).

The results from Biofix Protec inoculation 15 days before sowing in the four regions align with previous trials. Studies by Zilli et al. (2010) found that pre-inoculation did not affect analyzed parameters for up to five days, while others extended this period to 30 days with a protectant (Araujo et al., 2017) and up to 60 days with additional treatments (Manchineski et al., 2018). Silva et al. (2018) demonstrated that pre-inoculating soybean seeds with fungicides and insecticides 10 days before sowing enhanced nodulation, plant development, and grain productivity comparably to standard inoculants.

Pereira et al. (2010), treated soybean seeds with carbendazim+thiram or thiabendazole+thiram fungicides, with or without polymer, and did find any effect on nodule establishment and development, when seeds were inoculated with *Bradyrhizobium*.

Similarly, Manchineski et al. (2018) found that pre-inoculated soybean seeds with a cell protectant maintained viable bacterial inoculant for up to 60 days without negatively impacting soybean productivity. Hungria et al. (2020) also demonstrated that liquid inoculants containing *Bradyrhizobium*, applied 15 days before sowing, effectively enhanced Biological Nitrogen Fixation (BNF) and improved grain productivity.

Manchineski et al. (2022) found that treating soybean seeds with Standak Top or Maxim XL+Cruiser and pre-inoculating them with liquid *Bradyrhizobium* inoculant 25-30 days before sowing did not reduce nodulation, biological nitrogen fixation, or productivity compared to standard inoculation across most study áreas. Anghinoni et al. (2017) also discovered that soybean seeds treated with the fungicide fludioxonil and the insecticide thiamethoxam can be inoculated and stored up to 10 days before sowing without adversely affecting grain productivity.

Moretti et al. (2018) demonstrated a 27% increase in grain yield with seed inoculation compared to uninoculated controls. Sei et al. (2018), evaluatined soybean seed inoculation with a commercial biological protector, and observed statistically higher productivity compared to control treatments and standard inoculation (without protector), with increases of 32% and 15%, respectively.

Materials and methods

Product description and doses

The products used in field tests are described in Table 3. Nitrogen fertilization was carried out in T2, consisting of the application of urea (46-00-00) in sufficient quantity to offset the recommendation of 200 kg.ha^{-1} of nitrogen, applied in two stages: 50% at planting and 50% in R1, in the Itapira-SP, Araguari-MG and Catalão-GO trials, and for the Palmeira-PR trial the second application was carried out 35 days after emergence.

The inoculation of T3 consisted of the application of a commercial inoculant available on the market, formulated with the bacteria *Bradyrhizobium japonicum* strains 5079 and 5080, following the technical recommendation on the label.

The inoculant tested in T4, Biofix Protec, is a liquid inoculant made of the bacteria *Bradyrhizobium diazoefficiens* - SEMIA 5080 and *Bradyrhizobium japonicum* - SEMIA 5079, at a final concentration of 5x10^{9} UFC/mL.

Field test methodology

During the 2021/2022 agricultural harvest, trials were installed in four municipalities representing soybean cultivation in the central-west, southeast and south regions of Brazil (described in Supplementary Material). The trials were conducted in different edaphoclimatic regions to assess the product's effectiveness across varying soil types and climatic conditions. In Palmeira, PR, the soil was Alitic Haplic Cambisol with silty clay loam texture, under a Cfb climatic classification. Itapira, SP, featured Dystrophic Red-Yellow Latosol with clay texture, classified as Cfa. Araguari, MG, had Dystrophic Red Latosol with sandy clay texture, categorized as Aw. Catalão, GO, had Dystrophic Haplic Cambisol with loamy sand texture, also classified as Aw. The trials were conducted in different edaphoclimatic regions to evaluate the efficiency of the product across varying soil types and climatic characteristics.

The design used was randomized complete block design, with 6 replications. The distribution of test locations can be found in Figure 1. The size of the experimental field was 24.8 m^{2} (5 lines x 11 meters) with a useful area of 12.2 m^{2} (3 lines x 9 meters). The initial rhizobia concentration in the soil was determined using the analytical method approved by Normative Instruction DAS/MAP 30/2010 of 11/12/2010, published in the D.O.U. of 11/17/2010. It consists of inoculating serial dilutions of the samples, in specific test plants (soybeans), grown under aseptic conditions, evaluating the formation of nodules (NMP Technique – Most Probable Number).

The management and fertilization used in soybean cultivation during the trials was carried out in order to meet the cultivation needs for each region (Table 4). Other cultural treatments, such as applications of herbicides, fungicides and insecticides, were carried out according to the needs of the crop (Seixas et al., 2020).

Table 5. Description of each treatment performed on soybean seeds. 2021/2022 growing season.

Evaluated treatments	Inoculant	Additive protectors[1]	Chemical treatment[2]	Pre-treatment days	Base fertilization ($kg.ha^{-1}$)
Treatment 1	-	-	Yes	-	-
Treatment 2	-	-	Yes	-	200
Treatment 3	Commercial inoculant	No	Yes	0	-
Treatment 4	Biofix Protec	Yes	Yes	15	-

1- Protectors, additives and fertilizers: Mixed mineral fertilizer Potenzial TS and Ultra protector.

2- The chemical treatments: Fungicide Maxin XL, and Insecticide Cruiser 350 FS (Table 3).

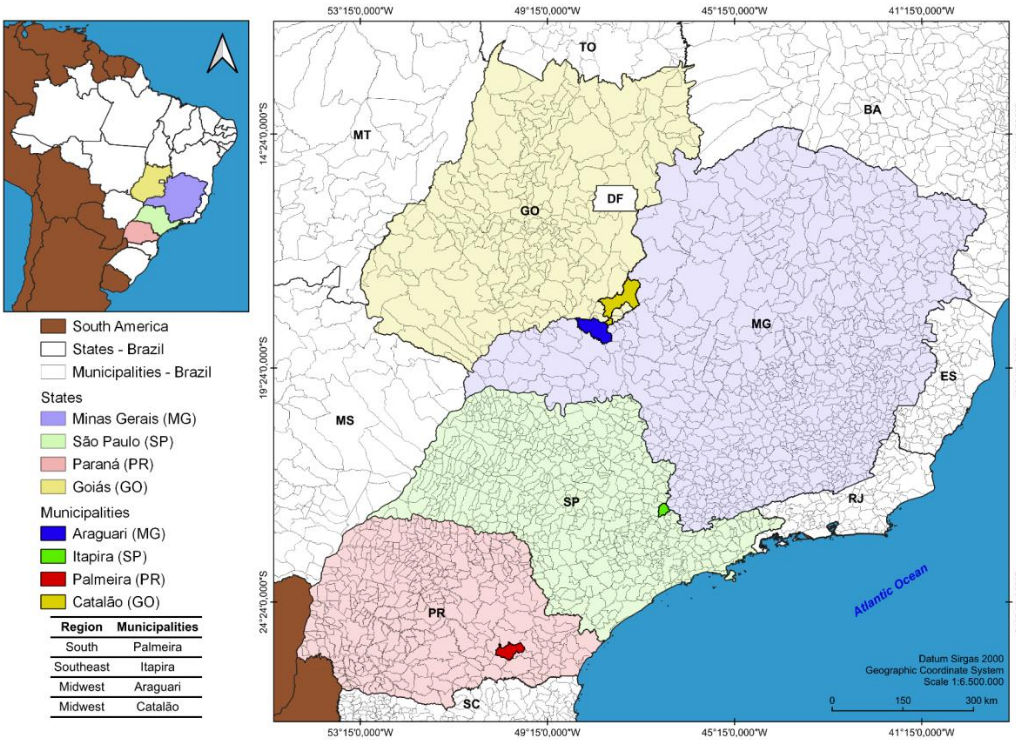

Figure 1: Distribution of testing regions (Venancio et al., 2024).

Treatments

Each trial was carried out with 4 treatments, in the following format:

***Treatment 1**:* Control (no inoculation was carried out, nor nitrogen application);

***Treatment 2**:* Fertilization with 200 $kg.ha^{-1}$ of nitrogen, from which 50% was applied at planting and 50% was applied at flowering;

***Treatment 3**:* Commercial seed inoculation (50 mL/50 kg of seeds), formulated with the bacteria *Bradyrhizobium japonicum* strains SEMIA 5079 and 5080, at concentration of $5x10^9$ CFU mL^{-1}, following the technical recommendation on the label, applied on the sowing day;

***Treatment 4**:* Seed inoculation with Biofix Protec (100 mL/50 kg of seeds), a liquid inoculant made of the bacteria *Bradyrhizobium japonicum* – SEMIA 5079 and *Bradyrhizobium diazoefficiens* – SEMIA 5080, at a final concentration of $5x10^9$ CFU mL^{-1}, combined with the following products: cell protector Protec (0.5 mL/kg), Potenzial TS (0.2 mL/kg), and CoMo Platinum (100 mL/ha), applied 15 days before planting.

A description of treatments is also presented in Table 5.

In T4, Protetor Protec and Potenzial TS mixed mineral fertilizer were also used, treated according to the stipulated pre-treatment time of 15 days, so that the planting of all test treatments occurred within the schedule of each region tested. The volume of syrup did not exceed the recommended 300 mL/50 kg of seeds.

After treatment and complete drying, the seeds were placed in a paper container, stored in a dry place, protected from light, with temperatures between 20 and 24 °C and air humidity below 70%.

Evaluated characters and statistical analysis

Five plants with intact roots were collected from the central area of each plot, immediately before flowering (30 to 40 days after emergence), being evaluated the number of nodules on the main root per plant (NNMR), the dry mass of nodules in the main root (DMNMR) in milligrams per plant (mg $plant^{-1}$), the Falker chlorophyll Index (FCI) for determining the nitrogen (N) content in the shoots, which was determined using a Clorofi Log Falker Chlorophyll Meter, model CFL1030, in 10 leaves of each replicate.

The plants collected were weighed in order to determine the fresh mass of shoots (FMS) and after that, they were dried in an oven at 65 ºC, until they reached constant weight for determination of the dry mass of shoots (DMS). The data was expressed in grams per plant. Grain yield (GY) was expressed in Kg ha^{-1}, and the weight of a thousand grains (WTG) in grams, with data corrected to 13% moisture. The N content in the grains was evaluated by the ABC Foundation laboratory, which used the proposed method by Dumas (AOAC, 2005).

The initial soil concentration of rhizobia was determined by the analytical method approved by the Normative Instruction Nº30/2010 of 12/11/2010 (BRAZIL, Ministry of

Agriculture, Livestock, and Supply of Brazil). It consists of inoculation of serial dilutions, in specific test plants (soybean plants), grown in aseptic conditions to evaluate the formation of nodules (MPN Technique – Most Probable Number).
The results obtained were subjected to analysis of variance using the F test, and the difference between means, when significant, was compared using the Duncan test at a 10% probability level, using the Sasm – Agri software (Canteri et al., 2001).

Conclusions

The Biofix Protec inoculant pre-inoculated 15 days before sowing (T4) in the soybean crop, increased nodulation in the main root and the dry mass of nodules, being, therefore, statistically equal to or greater than the commercial control (T3), in addition to contributing to nitrogen fixation, chlorophyll content, fresh and dry mass of the aerial part, productivity and nitrogen content in grains in most of the different soil and climate regions studied. The yield gains in productivity observed in the treatment with Biofix Protec (T4) ranged from 4.8% to 11.0% in relation to the control (T1), being statistically equal or superior to the commercial standard inoculant (T3), which was inoculated on the day of sowing. Pre-inoculation of the Biofix Protec inoculant 15 days before sowing, using strains of *Bradyrhizobium diazoefficiens* - SEMIA 5080 and *Bradyrhizobium japonicum* - SEMIA 5079 and specific formulation, combined with protective agents and chemical seed treatments, is as effective as the inoculation carried out at the time of sowing, as it did not result in a reduction in plant nodulation, as well as crop productivity. These results confirm the feasibility of effectively implementing pre-treatment of soybean seeds, in producing regions, providing benefits in nodulation and consequently yield, in current agricultural production systems.

References

Alves BJR, Boddey RM, Urquiaga S (2003) The success of BNF in soybean in Brazil. Plant Soil. 252:1-9.

Andrés JA, Correa NS, Rosas SB (1998) Survival and symbiotic properties of *Bradyrhizobium japonicum* in the presence of thiram: isolation of fungicide resistant strains. Biol. Fertil. Soils. 26:141-145.

Anghinoni FG, Braccini AL, Scapim CA, Anghinoni G, Ferri GC, Suzukawa AK, Tonin TA (2017) Pre inoculation with *Bradyrhizobium* ssp. In industrially treated soybean seeds. Agri. Sci. 8:582-590.

AOAC – Association of Official Analytical Chemists. Official methods of Analysis. 18 th ed. Washington, 2005.

Araujo RS, Da Cruz SP, Souchie EL, Martin TN, Nakatani AS, Nogueira MA, Hungria M (2017) Preinoculation of soybean seeds treated with agrichemicals up to 30 days before sowing: technological innovation for large-scale agriculture. Int. J. Microbiol. 2017:1-11.

Argenta G, Silva PRF, Bartolini, CG, Forsthofer EL, Strieder ML (2001) Relação da leitura do clorofilômetro com os teores de clorofila extraível e nitrogênio na folha de milho. Braz. J. Plant Physiol. 13:158-167.

Bender FR, Nagamatsu ST, Delamuta JRM, Ribeiro RA, Nogueira MA, Hungria M (2022) Genetic variation in symbiotic islands of natural variant strains of soybean *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* differing in competitiveness and in the efficiency of nitrogen fixation. Microb. Genom. 22:1-15.

Bikrol A, Saxena N, Singh K (2005) Response of *Glycine max* in relation to nitrogen fixation as influenced by fungicide seed treatment. Afr. J. Biotechnol. 4:667-671.

BRAZIL, Ministry of Agriculture, Livestock, and Supply of Brazil. Normative Instruction n.30, of November 12, 2010.

Brzezinski CR, Henning AA, Abati J Henninf FA, França-Neto JB, Krzyzanowski FC, Zucareli C (2015) Seeds treatment times in the establishment and yield performance of soybean crops. J. Seed Sci. 37:147-153.

Câmara GMS (2014) Fixação biológica de nitrogênio em soja. International Plant Nutrition Institute, Informações Agronômicas.147:1-9.

Campo RJ, Araujo RS, Hungia M (2009) Nitrogen fixation with the soybean crop in Brazil: Compatibility between seed treatment with fungicides and bradyrhizobial inoculants. Symbiosis. 48:154-163.

Campo RJ, Hungria M (2007) Protocolo para análise da qualidade e da eficiência agronômica de Inoculantes, estirpes e outras tecnologias relacionadas ao processo de fixação biológica do nitrogênio em leguminosas. Embrapa Soja. 89-123.

Canteri MG, Althaus RA, Virgens JSF, Giglioti EA, Godoy CV (2001) SASM - Agri: Sistema para análise e separação de médias em experimentos agrícolas pelos métodos Scoft-Knott, Tukey e Duncan. Revista Brasileira de Agrocomputação. 1:18-24.

Date, R.A (2001) Advances in inoculant technology: a brief review. Aust J Exp Agric. 41:321-325.

Deaker R, Roughley RJ, Kennedy IR (2004) Legume seed inoculation technology - a review. Soil Biology & Biochemistry. 36:1275-1288.

Delamuta JRM, Ribeiro RA, Ormeño-Orrillho E, Melo IS, Martínez-Romero E, Hungria M (2013) Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp.. Int J Syst Evol Microbiol. 63: 3342-3351.

Döbereiner J (1966) Evaluation of nitrogen fixation in legumes by the regression of total plant nitrogen with nodule weight. Nat. 210:850-852.

Fagan EB, Medeiros SLP, Mafron PA, Casaroli D, Dourado Neto D, Lier QJ, Santos OS, Muller L (2007) Fisiologia da fixação biológica de nitrogênio em soja-Revisão. Revista da FZVA. 14:89-106.

Herridge DF (2008) Inoculation technology for legumes. In Dilworth MJ, James EK, Sprent JI, Newton WE (ed) Nitrogen-fixing leguminous symbioses. 1rd edn. Dordrecht.

Hungria M, Araujo RS, Silva Junior, EB, Zilli, JE (2017) Inoculum rate effects on the soybean symbiosis in new or old fields under tropical conditions. Agro. J.109:1-7.

Hungria M, Campo RJ, Mendes IC (2001) Fixação biológica de nitrogênio na cultura da soja. Embrapa Soja. 35:1-48.

Hungria M, Campo RJ, Mendes IC (2007) A importância do processo de fixação biológica de nitrogênio para a cultura da soja: Componente essencial para a competitividade do produto brasileiro. Londrina, Embrapa Soja. 1-80.

Hungria M, Mendes IC (2015) Nitrogen fixation with soybean: the perfect symbiosis? In: De Bruijn, (ed) Biological Nitrogen Fixation. Hoboken, Wiley-Blackwell, 1009-1024.

Hungria M, Nogueira MA, Campos LJM, Menna P, Brandi F, Ramos YG (2020) Seed pre-inoculation with *Bradyrhizobium* as time-optimizing option for large-scale soybean cropping systems. J Agron. 112:5222-5236.

Kaschuk G, Nogueira MA, Luca MJ, Hungria M (2016) Response of determinate and indeterminate soybean cultivars to basal and topdressing N fertilization compared to sole inoculation with *Bradyrhizobium*. Field Crops Res. 095:21-27.

Li DM, Alexander A (1988) Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. Plant and Soil. 108:211–219.

Machineski GS, Scaramal AS, Matos MA, Langame JF, Gaiser GG, Machineski O, Colozzi AF (2022) Agronomic Efficiency of Signum Inoculant in Pre-inoculation of Soybean at 35 and 20 Days Before Sowing in Treated Seeds. J. Agri. Sci. 14:141-153.

Machineski GS, Scaramal AS, Matos MA, Machineski O, Colozzi Filho A (2018) Efficiency of pre-inoculation pf soybean with *Bradyrhizobium* up to 60 days before sowing. Afr. J. Agric. Res. 13:1233-1242.

Moretti LG, Lazarini E, Bossolani JW, Parente TL, Caioni S, Araujo RS, Hungria, M (2018) Can additional inoculations increase soybean nodulation and grain yield? Agr. J. 110:715-721.

Obserson A, Nanzer S, Bosshard C, Dubois D, Mader P, Frossard E (2007) Symbiotic N2 fixation by soybean in organic and conventional cropping systems estimated by 15N dilution and 15N natural abundance. Plant Soil. 290, 69–83.

Pereira CE, Moreira FMS, Oliveira JA, Caldeira CM (2010) Compatibility among fungicide treatments on soybean seeds through film coating and inoculation with *Bradyrhizobium* strains. Acta Sci. Agron. 32:1-4.

Peske, S, Levien, A (2005) Demanda de Sementes. In: ABRASEM – Abrasem. Anuário 2005. Tudo começa pelas sementes. 01:10-13.

Piromyou P, Nguyen HP, Songwattana P (2021) The *Bradyrhizobium diazoefficiens* type III effector NopE modulates the regulation of plant hormones towards nodulation in *Vigna radiata*. Sci Rep. 11:1-12.

Ribeiro Neto M, Jakoby ICMC, Buso PHM, Bermudez M, Diaz-Zorita M, Souchie EL (2018) Anticipated inoculation of soybean seeds treated with agrochemicals under brazilian production conditions. Asian J. Microbiol., Biotechnol. Environ. 20:188-199.

Santos MS, Nogueira MA, & Hungria M (2019) Microbial inoculants: Reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express, 9:1-22.

Sei FB, Souchie EL, Ribeiro Neto M, Jakoby, ICM (2018) C. Efeito do tratamento antecipado de sementes de soja com inoculantes e protetor biológico na produtividade em duas safras de cultivo. RELARE. 19:1-5.

Silva AF, Carvalho MAC., Schoninger EL, Monteiro S, Caione G, Santos PA (2011) Doses de inoculante e nitrogênio na semeadura da soja em área de primeiro cultivo. Biosci. 27:404-412.

Silva AK, Silva EE, Farias ENC, Chaves JS, Albuquerque CNB, Cardoso C (2018) Agronomic efficiency of *Bradyrhizobium* pre-inoculation in association with chemical treatment of soybean seeds. Afr. J. Agric. Res. 13:726-732.

Silva EB, Dias MSC, Gonzaga EIC, Santos NM (2002) Estimativa da acidez potencial pelo SMP pH em solos da região norte do estado de Minas Gerais. R. Bras. Ci. Solo. 26:561-565.

Sprent JI, Ardley J, James EK (2017) Biogeography of nodulated legumes and their nitrogen-fixing symbionts. New Phytol. 215:40-56.

Toni RA, Vale WG, Pelozo G, Demarchi J, Vale PACB (2018) Analysis of soybean cultivars in response to different forms of inoculation. Sci. Electron. Arch. 11:1-5.

Vargas MAT, Hungria M (1997) Fixação biológica do N_2 na cultura da soja. Biol. Sol. Cer. 1:297-360.

Vargas MAT, Mendes IC, Suhet AR, Peres JRR (1994) Inoculação de soja em solos de Cerrado com populações estabelecidas de *Bradyrhizobium japonicum* (1994) R Microbiol. 25:245-50.

Venancio WS, Dellago EG, Slusarz GM, Boratto IV, Boratto NM, Leite AC, Cuenca R, Ratuchne A (2024) Impact of pre-inoculation of soybean seeds with *Bradyrhizobium* spp. applied 60 days before sowing. Aust. J. Crop Sci. 18:116-124.

Vollmann J, Walter H, Sato T, Schweiger P (2011) Digital image analysis and chlorophyll metering for phenotyping the effects of nodulation in soybean. Comput Electron Agric. 75:190-195.

Zilli JE, Ribeiro KG, Campo RJ, Hungria M (2010) Influence of fungicide seed treatment on soybean nodulation and grain yield. Ver. Bras. de Cienc. do Solo. 33:917-923.