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Seed pre-inoculation with *Bradyrhizobium* as time-optimizing option for large-scale soybean cropping systems

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Abstract

Nitrogen is a key nutrient for soybean cropping and can be fully supplied by the biological nitrogen fixation (BNF) process. Inoculation with elite *Bradyrhizobium* strains greatly improves the supply of N to soybean; however, inoculation of large areas in short-sowing windows delays the sowing process, demanding the development of technologies for pre-inoculation. Here we report the evaluation of a liquid formulation containing cell protectors that proved, in four field experiments located in different edaphoclimatic conditions of Brazil, symbiotic performance comparable to the peat-based inoculant, traditionally considered as the best carrier. The liquid inoculant was also effective when applied to seeds not treated with pesticides 15 days before sowing, providing efficient BNF and time-flexibility to the farmers. Benefits of the liquid inoculant in improving grain yield were confirmed in two areas cropped for the first time and devoid of *Bradyrhizobium*, with an average increase of 89%, and also in two areas traditionally cropped with inoculated soybean, with an average increase of 6.8%, both in comparison to the non-inoculated control without N-fertilizers (NI). It is worth mentioning that, also in comparison to the NI control, the addition of 200 kg N ha^{-1} impacted grain yield in +54% and −1,8%, in new and traditional areas, respectively, indicating that BNF was more effective than the application of N-fertilizer. Large increases in total N content in grains were observed as well in response to the liquid inoculant, on average 47% and 27%, when compared to the NI and NI + N controls, respectively.

1 | INTRODUCTION

The search for sustainable and low-cost technologies to attend the high demands for food by an ever-growing population, based on sustainable models, is pivotal. In this context, microorganisms play important roles, such

Abbreviations: BNF, biological nitrogen fixation; DAE, days after emergence; DAS, days after sowing; GW, grain weight; GY, grain yield; NCG, N concentration in grains; NCS, N content in shoots; NDW, nodule dry weight; NI, non-inoculated; NN, nodule number; SDW, shoot dry weight; T0, time zero, 2 h after inoculation; T15, 15 days after inoculation; TNG, total N in grains; TNS, total N in shoots

as in the soybean [*Glycine max* (L.) Merr.] crop, in which long-term selection programs identified *Bradyrhizobium* spp. elite strains able to fully supply the plant's demand on N via biological nitrogen fixation (BNF), with no need of supplying chemical N-fertilizers (Hungria & Mendes, 2015; Hungria & Nogueira, 2019; Hungria, Campo, Mendes, & Graham, 2006). Estimates of economic savings with this technology in Brazil point out to US$ 15 billion per year (Hungria & Mendes, 2015; Hungria & Nogueira, 2019), in addition to environmental contributions, including decreased emissions of greenhouse gases and lower risk of contamination of surface and groundwater with nitrate (Hungria & Mendes, 2015; Sá et al., 2017). Other soybean producing countries in South America also benefit from inoculants carrying elite nitrogen-fixing *Bradyrhizobium* spp. strains (Chang, Lee, & Hungria, 2015; Hungria et al., 2006). In 2019 the inoculant market in Brazil was estimated at 70 million doses per year, more than 90% for the soybean crop (Santos, Nogueira, & Hungria, 2019), in addition to about 50 million doses commercialized in other South American countries.

Soybean cropping has impressively increased in South America in the last 40 years, e.g. from 6.9 million ha in the late 1970s to 36.9 million in the 2019–2020 crop season in Brazil (Conab, 2020). Agronomic practices had to be adapted to this new scenario, as the sowing window has become very short, especially because of sowing anticipation to deal with the Asian soybean rust (*Phakopsora pachyrhizi*), and also to enable a second crop season, mainly corn (*Zea mays*), by the end of the wet period (Embrapa Soja, 2013). Therefore, among the most frequent demands from farmers, it highlights the search for inoculants that allow cell survival and effectiveness with anticipated inoculation, a practice known as pre-inoculation (Santos et al., 2019).

Some pioneer studies have shown the feasibility of inoculation of soybean and other legumes some days prior to sowing (Deaker, Roughley, & Kennedy, 2004; Herridge, 2008; Peres, Suhet, & Vargas, 1986). However, cell survival can be much lower than inoculation at the sowing time (Hungria, Loureiro, Mendes, Campo, & Graham, 2005; Lupwayi, Clayton, & Rice, 2006). The long-term viability of cell survival on inoculated seeds depends on adhesives and cell protectors in the formulation (Hungria et al., 2005; Santos et al., 2019; Santos, Hungria, & Nogueira, 2017). Nowadays, some inoculants commercialized in Brazil and in other South American countries have been used for pre-inoculation from few (5) to several (60) days (Anguinoni et al., 2017; Araujo et al., 2017; Machineski, Scaramal, Matos, Machineski, & Colozzi Filho, 2018; Silva et al., 2018; Zilli, Campo, & Hungria, 2010); however, in general, the number of surviving cells at sowing time is far below the minimum to provide early and effective nodulation, and might impair the nodulation in areas submitted to abiotic stresses, especially drought.

Core Ideas

- Soybean can benefit from inoculation with elite Bradyrhizobium strains.
- Large-scale soybean cropping and short-sowing window require innovation.
- A liquid inoculant, as effective as peat inoculant, was developed.
- Pre-inoculation was feasible 15 days before sowing.
- Time-flexibility makes inoculation easier for the farmers.

Pre-inoculation of soybean seeds for as long as possible periods, keeping the bacterial viability, allows the farmers to dedicate to the sowing operation, without the need to deal with the daily inoculation, which is usually time-consuming and manpower-demanding. Therefore, there is a demand for liquid inoculants easy to apply, and with guaranteed effectiveness when seeds are pre-inoculated. Here we report the results obtained with a liquid inoculant developed for pre-inoculation for a period of 15 days, and evaluated in four sites in Brazil, comprising both traditionally cropped areas with established populations of bradyrhizobia, and new-challenging areas cropped for the first time and devoid of soybean bradyrhizobia.

2 | MATERIALS AND METHODS

2.1 | Laboratory experiments

2.1.1 | Strains and development of the inoculant formulation

The *Bradyrhizobium* spp. strains used in this study are approved by the Ministry of Agriculture, Livestock and Food Supply (MAPA) in Brazil for the use in commercial inoculants for the soybean crop: *Bradyrhizobium japonicum* SEMIA 5079 (= CPAC 15, = CNPSo 07) and *Bradyrhizobium diazoefficiens* SEMIA 5080 (= CPAC 7, = CNPSo 06) (Mapa, 2011). The strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil, official germplasm bank of MAPA.

To develop a formulation able to maintain bacterial survival for up to 15 days after seed inoculation and keep the efficacy in fixing nitrogen, several cell protectors, polymers and adhesives were evaluated, including clay, coal, phosphates, several sugars, glycerol, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), alginate, xanthans. After several tests evaluating protectors, surfactants and buffers, we came to a formulation that included: citric acid, xanthan gum, non-ionic surfactant, PVA, mono- and dipotassium phosphates, saccharides and polysaccharides. The inoculant received the denomination of NG.

2.1.2 | Concentration, purity and strain identity in the inoculant, and counting of viable *Bradyrhizobium* cells recovered from inoculated seeds

Evaluation of concentration of *Bradyrhizobium* spp. and purity of the inoculants followed the official protocol of MAPA (Mapa, 2010), that consists of the serial dilution in sterile saline solution (0.85% NaCl, w/v) and plate-spreading on modified-YMA (yeast-mannitol-agar) medium with Congo Red (Hungria et al., 2016). According to the Brazilian legislation, rhizobial inoculants must present a concentration of at least 10^9 cells ml^{-1} or g^{-1} for at least six months (Mapa, 2011). The formulation developed confirmed at least 5×10^9 cells ml^{-1} for 12 months.

The identity of the two *Bradyrhizobium* strains in the inoculant was confirmed by the DNA amplification with the BOX-A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) primer (Versalovic, Schneider, de Brujin, & Lupsky, 1994), as described by Chibeba, Kyei-Boahen, Guimarães, Nogueira, and Hungria (2017); identification of strains by BOX-PCR is also required by the Brazilian legislation (Mapa, 2010).

The recovery of viable *Bradyrhizobium* cells from inoculated seeds and counting followed the methodology previously described (Araujo et al., 2017; Penna, Massa, Olivieri, Gutkimd, & Cassán, 2011; Santos et al., 2020), also included in the Brazilian legislation (Mapa, 2010). Five treatments were evaluated, and they are described in detail in the item of field experiments. For the recovery analysis, basically, four groups of 500 g of soybean seeds were inoculated according to each treatment, seeds were homogenized and dried at room temperature for 30 min. Seeds were stored under controlled conditions, in kraft paper bags at 25 ± 2 °C and humidity of 50% ± 5%, according to the time established in each treatment. At each sampling time, four subsamples of 100 seeds were transferred to 250-ml Erlenmeyer flask containing 100 ml of sterile 0.85% (w/v) NaCl saline solution + 0.01% (w/v) Tween 80 solution, following the extraction procedures and spread plating of appropriate dilutions in modified-YM medium (Hungria et al., 2016) containing Congo Red (25 mg L^{-1}) as an indicator, and vancomycin (0.1 g L^{-1}) to inhibit contaminants from seeds. For the laboratory experiment, the five treatments were arranged in a completely randomized design (CRD) with three biological replicates, each one with three technical replicates, i.e., three replicates for each biological replicate.

The same seed treatments were performed for the four field sites, and seeds were stored under the same controlled conditions in all sites, 25 ± 2 °C and humidity of 50 ± 5%. As the conditions were the same, cell recovery was analyzed with the seeds of one of the experiments.

2.2 | Field experiments

2.2.1 | Characterization, establishment, and procedures for the field experiments

Four field experiments were performed in the same crop season, but representing four different edaphoclimatic conditions, consisting of different combinations of climate, soil texture and fertility. The sites represent important soybean cropping areas in Brazil, in the southern (Londrina and Paranavaí, state of Paraná), southeast (Lutécia, state of São Paulo), and northern (Aparecida do Rio Negro, state of Tocantins) regions in Brazil (Figure 1). According to the Köppen's climate classification, the three first sites are *Cfa*, humid subtropical, and the fourth is *Aw*, typical savannah climate (Table 1).

At each site, 40 to 60 days before starting the experiment, 20 soil subsamples were taken at 0–20 cm and 20–40 cm depth layers for chemical and granulometric analyses. Soil chemical analyses (Tables 2 and 3) were performed according to van Raij, Andrade, Cantarella, and Quaggio (2001), and Silva (2009). Granulometric analysis (Table 3) was performed after Donagema, Campos, Calderano, Teixeira, and Viana (2011). Soil total organic C (Table 3) was determined in a vario TOC Cube elemental analyzer (Elementar, Langenselbold, Germany) in air-dried and finely ground (<0.02 mm) soil samples. This method is considered suitable for soils with high Fe content (Segnini et al., 2008). The soybean-nodulating rhizobial populations (Table 3) were assessed only at the 0–20 cm topsoil layer in the same samples used for chemical analysis. The populations were estimated using the most probable number (MPN) method with plant counting (O'Hara, Hungria, Woomer, & Howieson, 2016), using soybean cv. BRS 1010 IPRO to trap rhizobia.

In all sites lime was applied 40 to 60 days before starting the experiment based on the results of chemical analysis, to reach 70% of base saturation in Londrina, 60% in

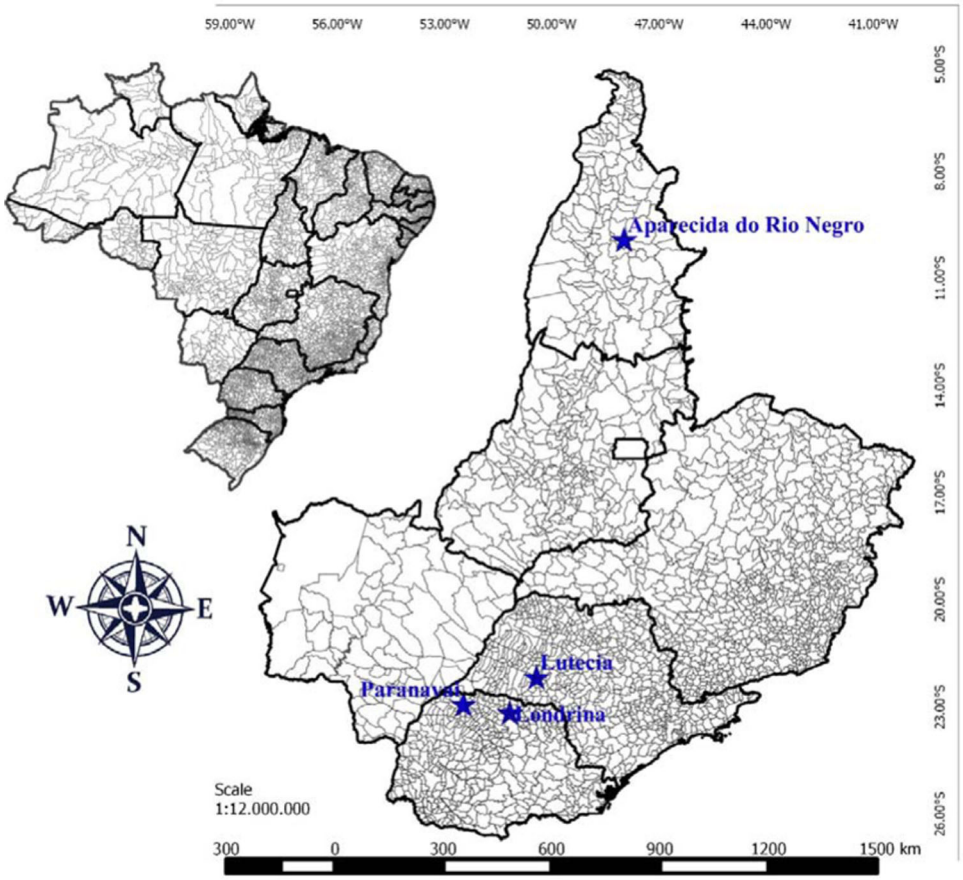

FIGURE 1 Map showing the sites of the four field experiments performed in Brazil

Lutécia, and 50% in Paranavaí and Aparecida do Rio Negro, as recommended for each region, and the amount of lime to be applied was estimated as recommended by Silva (2009). About 30 days before sowing, weed control was done with the application of 2.5 L ha^{-1} of glyphosate. In Aparecida do Rio Negro, the control of invasive plants was complemented with 1.5 L ha^{-1} of 1,1′-dimetil-4,4′-bipiridilio dichloride.

All procedures in the experiments followed the normative for inoculants according to the Brazilian legislation. i.e., the protocols for conduction of field experiments to confirm the agronomic efficiency of microbial inoculants, regulated by specific governmental legislation (Mapa, 2011). The soybean genotypes used in the experiments are shown in Table 4 and they are in the list of recommended genotypes for each of the four sites. Seeds were not treated with fungicides.

The experiments consisted of five treatments: (1) Non-inoculated and non-N-fertilized control (NI); (2) Non-inoculated control receiving 200 kg of N ha^{-1}, 50% at sowing and 50% at the flowering stage (NI+N); the objective of this treatment is to verify if the symbiotic performance is able to fulfill plant N requirements, and the treatment is required by the Brazilian legislation (Mapa, 2011) to prove agronomic efficiency of rhizobia; (3) Peat inoculant, traditionally considered as the best carrier (Hungria et al., 2005), applied at sowing to deliver 1.2 million cells $seed^{-1}$ (Peat); (4) Liquid inoculant NG applied at the sowing (time zero) to deliver 1.2 million cells $seed^{-1}$ [NG (T0)]; (5) Liquid inoculant NG applied to the seeds to deliver 1.2 million cells $seed^{-1}$ 15 days before sowing [NG (T15)].

The experiments were performed in a randomized complete block design (RCBD) with six replicates. Each experimental unit measured at least 4 m by 6 m (Table 4), consisting of 8 lines spaced 0.5 m apart, with lines 1 and 8 considered as borders; spacing between plots of 2 m was maintained to avoid contamination between treatments.

Immediately before sowing, fertilization with 300 kg ha^{-1} of the formulation 00–20–20 (60 kg ha^{-1} of P_2O_5 and 60 kg ha^{-1} of K_2O) was applied in-furrow in all treatments using a no-till sowing machine. In the NI + N treatment, 100 kg of N ha^{-1} (as urea, 450 kg urea ha^{-1}) were applied by surface spreading and slight incorporation. Sowing was manual, with aseptic procedures between treatments. The sowing date, plant density and further information of each site are shown in Table 4.

In the phenological stages V3-V5 (Fehr & Caviness, 1977), Co (2.5 g ha^{-1}) + Mo (20 g ha^{-1}) were leaf-sprayed.

TABLE 1 Geographic coordinates, climatic classification, soil textural class and classification at each site where the field trials were performed

State	Municipality	Coordinates	Altitude	Climate[a]	Average annual temperature	Average annual rainfall	Soil textural class	Soil classification[b]
			m		°C	mm		
Paraná	Paranavaí	22° 57′ 16.82″ S52° 28′ 4.31″ W	446	*Cfa*	20.7	1289	Sand	Typic Acrudox
São Paulo	Lutécia	22° 11′ 45.58″ S,50° 25′ 27.82″ W	541	*Cfa*	20.6	1255	Sandy loam	Typic Acrudox
Paraná	Londrina	23° 11′ 2.75″ S51° 10′ 30.29″ W	550	*Cfa*	20.9	1429	Clay	Rhodic Eutrudox
Tocantins	Aparecida do Rio Negro	09° 57′ 07″ S47° 58′ 19″ W	265	*Aw*	25.7	1619	Clay loam	Typic Acrudox

[a] According to Köppen-Geiger classification; *Cfa*, humid subtropical; *Aw*, tropical with dry season in winter.
[b] According to USDA, Soil Survey Staff.

TABLE 2 Chemical properties of the soils in the experimental sites at the 0–20 and 20–40 cm layers before sowing

Municipality	Depth	pH ($CaCl_2$)	pH (H_2O)	Potential acidity (H + Al)	H^+	Ca^{+2}	Mg^{2+}	K^+	Na^+	P (Mehlich 1)	P (Mehlich 3)	Cu^{2+}	Fe^{2+}	Mn^{2+}	Zn^{2+}
					$cm_c\ dm^{-3}$					$mg\ dm^{-3}$			$mg\ dm^{-3}$		
Paranavaí	00–20	5.2	5.8	2.4	2.38	1.00	0.57	0.17	0.01	19.2	27.0	1.10	124	165	5.19
	20–40	4.8	5.5	2.5	2.52	0.73	0.43	0.10	0.02	4.0	7.8	1.09	129	158	8.39
Lutécia	00–20	4.7	5.4	2.8	2.78	0.85	0.41	0.07	0.01	5.2	7.1	0.88	130	69	1.01
	20–40	4.2	5.0	3.0	2.99	0.79	0.48	0.04	0.01	4.4	5.6	0.91	154	62	1.31
Londrina	00–20	5.2	5.8	5.3	5.31	3.75	2.00	0.84	0.01	22.5	13.8	12.93	101	445	2.04
	20–40	5.1	5.7	5.4	5.35	3.22	1.73	0.37	0.01	11.4	6.5	11.19	99	316	1.28
Aparecida do	00–20	5.7	6.4	1.8	1.8	2.90	1.20	0.40	0.01	18.5	nd[a]	0.7	59	6.50	4.60
Rio Negro	20–40	4.9	5.6	2.5	2.5	1.20	0.60	0.20	0.01	7.0	nd	nd	nd	nd	nd

[a] nd, not determined

TABLE 3 Chemical properties, granulometric fractions, and soybean-nodulating rhizobial population at the experimental field sites before sowing

Municipality	Depth	CEC pH 7.0	CEC Effective	Saturation of bases	C	Granulometric fractions Clay	Granulometric fractions Silt	Granulometric fractions Sand	Soybean-nodulating rhizobia
		$cmol_c\ dm^{-3}$		%	$g\ dm^{-3}$	%			rhizobia g^{-1}
Paranavaí	00–20	4.13	1.75	42	4.50	6.50	2.85	90.65	zero
	20–40	3.80	1.28	33	2.36	8.50	2.10	89.40	nd[a]
Lutécia	0–20	4.12	1.34	32	4.14	11.95	1.10	86.95	7.36×10^1
	20–40	4.31	1.32	30	4.07	9.95	1.00	89.05	nd
Londrina	00–20	11.91	6.60	55	19.10	75.25	18.50	6.25	1.469×10^4
	20–40	10.68	5.33	50	13.94	80.10	14.10	5.80	nd
Aparecida do	00–20	6.20	4.50	73	15.60	36.50	24.50	39.00	4.621×10^5
Rio Negro	20–40	4.40	1.90	45	10.40	27.00	17.50	55.50	nd

[a]nd, not determined

At the phenological state R1 (Fehr & Caviness, 1977) the NI + N treatment received the second dose of N, of 100 kg of N ha^{-1} as urea, spread on the surface as topdressing.

All cultural and phytosanitary procedures followed the recommendations for the soybean crop in Brazil (Embrapa Soja, 2013).

2.2.2 | Plant sampling, analyses, and grain yield

Between 42–44 days after sowing (DAS), corresponding to 35-36 days after emergence (DAE), when the plants were at the V5 developmental stage (Fehr & Caviness, 1977), five plants per plot were randomly collected from the second and the sixth rows for assessment of nodulation (number and mass), shoot dry mass, N concentration and contents in the shoots. Plants were carefully collected with the aid of a straight shovel, forming a square next to the roots, and collecting nodules that might have fallen.

In the laboratory, the shoots were separated from roots at the cotyledonary node, and roots were washed carefully, collecting any falling nodule, and oven-dried at 50 °C until constant mass (approximately 72 h). The nodules were removed from the roots, counted, dried again and weighed to obtain nodule dry weight. Shoot N concentration was determined in sulfuric digests by the green salicylate method (Feigl & Anger, 1972). Total N in shoots was obtained by multiplying the shoot N concentration by the shoot dry weight.

At physiological maturity, plants were harvested in the central area of each plot for estimating the grain yield; harvest dates and size of the areas harvested are shown in Table 4. Grains were cleaned, weighed, grain moisture determined in a moister meter, and the mass was corrected to 13% moisture. The N concentration in grains was also determined as for shoots. Total N in grains (kg ha^{-1}) was obtained by multiplying the N concentration in grains by grain yield.

2.3 | Statistical analysis

The laboratory experiment consisted of five treatments for the evaluation of *Bradyrhizobium* cell recovery from the seeds, and the results were submitted to the analysis of variance (ANOVA) at 5% of significance, considering a completely randomized design (CRD). After the initial analysis, the non-inoculated treatments, with and without N-fertilizer were not considered, as there were close to zero bacteria in both treatments. The means were then compared by the Dunnett's test (bilateral), comparing each treatment with the control, represented by the peat (Peat)

TABLE 4 Agronomic information about the field experiments performed in the 2017–2018 cropping season

Municipality	Soybean cultivar	Sowing	Density of plants (plants ha^{-1})	Vegetative harvest	Days at the vegetative harvest	Grain harvest	Total area	Useful central area harvested to estimate yield
Paranavaí	BRS 1010 IPRO[a]	1 Nov 2017	333,333	14 Dec 2017	43 DAS,[c] 35 DAE,[c] V5[d]	5 Mar 2018	24 m^2	6.75 m^2
Lutécia	BRS 1010 IPRO	7 Nov 2017	333,333	20 Dec 2017	42 DAS, 35 DAE,	8 Mar 2018	25.2 m^2	6.75 m^2
Londrina	BRS 1010 IPRO	15 Nov 2017	240,000	27 Dec 2017	42 DAS, 35 DAE, V5	24 Mar 2018	24 m^2	8.0 m^2
Aparecida do Rio Negro	BRS 8980 IPRO[b]	28 Nov 2017	240,000	12 Jan 2018	44 DAS, 36 DAE, V5	4 Apr 2018	24 m^2	8.0 m^2

[a] Indetermined growth, group of maturity 6.4.
[b] Indetermined, group of maturity 7.9.
[c] DAS, days after sowing; DAE, days after emergence.
[d] V5 stage of soybean development, according Fehr and Caviness (1977).

inoculation supplying 1.2 million cells seed^{-1} at time zero (T0, 2 h after inoculation), using a 95% confidence interval. Statistical analysis was performed using the Statistica software version 7.0 (StatSoft Inc., 2004).

The data of each of the four field experiments performed with a randomized complete block design (RCBD) with six replicates were individually submitted to tests of normality and homogeneity of variances. ANOVA was then performed using the Statistica software version 7.0 (StatSoft Inc., 2004). When the "F" test was significant at 5%, the means were compared by Duncan's test at 5% of significance.

3 | RESULTS

3.1 | Inoculants analyses

The inoculants were within the quality limits established by the Brazilian legislation i.e., at least 1×10^9 colony forming units (CFU) per g or mL of inoculant and no contaminants at the dilution 10^{-5} (Mapa, 2011). The concentrations were of 5.0×10^9 CFU g^{-1} in the peat standard inoculant and of 5.6×10^9 CFU mL^{-1} in the NG inoculant. Based on these results, both peat and NG inoculants were applied to deliver 1.2 million cells seed^{-1}. Strains identities were confirmed by BOX-PCR (data not shown).

3.2 | Recovery of viable cells from inoculated seeds

The treatments of seed inoculation consisted of peat inoculation (Peat) at zero time (T0) and the NG liquid inoculant, at T0 and with 15 days of pre-inoculation (T15). At T0, both peat inoculant and the NG inoculant received 1.2 million cells seed^{-1} and resulted in the recovery of 1.6×10^5 CFU seed^{-1} and 2.0×10^5 CFU seed^{-1}, respectively, representing 16% and 20% of the theoretical number of cells applied to the seeds, without differing statistically from each other ($p > .05$). After 15 days of pre-inoculation, the cell recovery from the treatment NG (T15) was of 2.2×10^4 CFU seed^{-1}, and also did not differ from the Peat (T0) or NG (T0). However, it is worth mentioning that, considering the theoretical application of 1.2×10^6 cells seed^{-1}, the recovery rate was low, of less than 2%.

3.3 | Agronomic efficiency of the inoculants

For the field experiments, soils were selected to show different conditions. According to the values estimated for soils growing soybean (Embrapa Soja, 2013), in general, Ca concentrations were low, while P ranged from low (Lutécia) to high (Londrina); Mn concentrations were generally high at all sites (Table 2), as usually found in Brazilian tropical soils. The soil granulometric fractions varied from clayey in Londrina, to sandy in Paranavaí (Table 3). Two out of the four areas had never been cropped with soybean or received inoculant before, showing a low population of soybean-nodulating bradyrhizobia (Paranavaí and Lutécia), while the two other sites had been cropped to inoculated soybean before and showed high populations of naturalized soybean-nodulating bradyhrizobia (Table 3).

The maximum and minimum temperatures during the five months of the experiments in each site are shown in Table 5. Temperatures followed the patterns usually recorded for each site and in general pluviometry was good in all areas. In three sites the treatment with the best performance reached grain yield higher than the average reported for the states in the crop season, of 3508,

TABLE 5 Maximum and minimum temperatures and total precipitation in each month during the soybean growth in the four field sites

State	Municipality	Month	Average Maximum	Average Minimum	Precipitation
			°C		mm
Paraná	Paranavaí[a]	Nov 2017	28.5	18.8	140.4
		Dec 2017	30.1	21.2	233.3
		Jan 2018	31.5	21.2	326.7
		Feb 2018	30.2	19.1	160.0
		Mar 2018	30.9	21.2	150.5
São Paulo	Lutécia[b]	Nov 2017	28.8	17.6	138.8
		Dec 2017	26.9	17.6	129.5
		Jan 2018	28.8	19.3	210.8
		Feb 2018	28.3	18.3	137.5
		Mar 2018	28.0	17.7	96.3
Paraná	Londrina[a]	Nov 2017	29.2	17.3	197.5
		Dec 2017	30.0	19.4	286.0
		Jan 2018	28.2	20.1	408.9
		Feb 2018	27.7	18.4	89.8
		Mar 2018	27.9	20.8	222.9
Tocantins	Aparecida	Nov 2017	33.6	22.6	197.1
	do Rio	Dec 2017	32.4	22.6	368.8
	Negro[b]	Jan 2018	32.3	22.5	273.4
		Feb 2018	31.2	22.2	347.0
		Mar 2018	32.2	22.8	182.0

[a] Data obtained at the station located on the same municipality by the Instituto Agronômico do Paraná (iapar.br/pagina-259.html)
[b] Data obtained at the closest station by the Instituto Nacional de Meteorologia (https://portal.inmet.gov.br/) and Centro Integrado de Informações Agrometeorológicas (https://www.udop.com.br/index.php?item=chuvas)

3546 and 3135 kg ha for Paraná, São Paulo and Tocantins, respectively (Conab, 2020), confirming good climatic conditions. The grain yield was only slightly lower than the average in Paranavaí, also in Paraná, although it was higher than expected for a first-year area of a sandy soil

3.3.1 | Field trials performed in areas cropped for the first time and devoid of soybean-nodulating rhizobia

In Paranavaí, in the evaluation performed at the V5 growth stage, the best nodulation (nodule number, NN) was achieved with peat inoculation at sowing (Peat, T0), followed by the NG (T0), decreasing about three-fold with the pre-inoculation with NG (T15) (Table 6). Concerning the nodule dry weight (NDW), the best performance was also observed with the peat inoculant, and it is worth mentioning that this nodulation was achieved in a sandy soil that had never received any inoculant. Despite the good nodulation in the peat treatment, shoot dry weight (SDW) was higher in the non-inoculated treatment receiving N-fertilizer (NI +N). However, none of the inoculated treatments, Peat, NG (T0) and NG (T15), differed statistically in the total N accumulated in shoots (TNS) from the NI + N treatment.

Despite the initial advantage in the NI + N treatment in Paranavaí, this was not reflected in the physiological maturity, with the highest yields being achieved with the three inoculated treatments, with an emphasis on the NG (T0). Very important, the three inoculated treatments resulted in total N accumulated in grains (TNG) values statistically superior to both the NI + N and the NI control treatments. Considering the average grain yield of the three treatments with inoculation, there was a gain of 402 kg ha^{-1} in comparison to the NI + N treatment. When compared to the NI control, increases with the NG inoculant were of 938 kg ha^{-1} at the sowing (T0) and of 683 kg ha^{-1} with 15 days of pre-inoculation (T15).

The results obtained in the other first-year soybean cropping area, Lutécia, were similar to those observed in Paranavaí (Table 6). Despite the stressful conditions of a very sandy soil, NDW above 90 mg $plant^{-1}$ at the V5 stage was found in all inoculated plants. As also seen in Paranavaí, the highest SDW and TNS at the V5 stage in Lutécia were achieved in the NI + N plants.

TABLE 6 Experiments performed in areas cropped for the first time with soybean, in Paranavaí-PR, southern Brazil and Lutécia-SP, southeast Brazil. Nodule number (NN, no. plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), N content in shoots (NCS, g N kg^{-1}), total N accumulation in shoots (TNS, mg N plant^{-1}), N content in grains (NCG, g N kg^{-1}), total N accumulated in grains (TNG, kg ha^{-1}), grain yield (GY, kg ha^{-1}), and grain weight (GW, g 100 seeds^{-1}) of soybean non-inoculated or inoculated with peat or liquid inoculant NG at sowing (T0) or with 15 days of pre-inoculation (T15)

	V5 Stage									Physiological maturity							
	Nodulation				Biomass		N in shoots			N in grains				Yield			
Treatment	NN		NDW		SDW		NCS		TNS	NCG		TNG		GY		GW	
Paranavaí																	
Non-inoculated (NI)	0.07	c[b]	0.5	d	2.75	b	17.1	b	48	b	50.1	b	110	b	2203	c	12.6	ns[b]
NI +N-fertilizer[a]	0.07	c	0.8	d	5.34	a	17.9	b	99	a	41.7	c	107	b	2575	bc	12.8	
Peat (T0)	13.2	a	101.8	a	3.31	b	22.2	a	81	ab	54.0	a	158	a	2905	ab	11.8	
NG (T0)	10.7	a	76.2	b	4.32	ab	20.3	ab	87	ab	53.0	ab	167	a	3141	a	12.2	
NG (T15)	3.7	b	28.9	c	3.41	b	17.3	b	61	ab	50.4	b	146	a	2886	ab	12.0	
	Mean	5.57		41.7		3.83		18.9		75.1		49.8		137.5		2742		12.3
	p value	<.001		<.001		.0156		.0339		.0808		<.001		<.001		<.001		.1076
	CV (%)	51.2		49.7		32.6		15.9		42.8		5.6		14.3		11.6		5.7
Lutécia																	
Non-inoculated (NI)	0	b	0	b	6.71	ab	21.1	ns	134	b	38.4	c	61	c	1602	c	14.6	d
NI +N-fertilizer[a]	0	b	0	b	9.53	a	23.9		221	a	45.4	b	147	b	3290	b	15.7	c
Peat (T0)	9.6	a	94.8	a	4.73	b	27.0		118	b	53.0	a	215	a	4060	a	17.2	b
NG (T0)	8.8	a	91.8	a	5.71	b	21.1		119	b	51.8	a	220	a	4087	a	18.0	a
NG (T15)	8.0	a	92.5	a	6.39	b	22.3		140	b	51.8	a	222	a	4284	a	17.6	ab
	Mean	5.28		55.8		6.6		23.1		147		48.1		173.0		3464		16.7
	p value	<.001		<.001		.0292		.1736		.0092		<.001		<.001		<.001		<.001
	CV (%)	32.2		49.1		36.3		19.8		33.6		4.9		8.4		7.5		3.0

[a] 200 kg of N ha^{-1}, 50% at sowing and 50% at flowering stage.

[b] Means followed by the same letter do not differ from each other by the Duncan test at $p \leq .05$ and, in the case of TNS in Paanavaí at $p \leq .10$; ns, not significant.

The better performance of the NI + N treatment at the vegetative stage in Lutécia did not result in higher grain yield. Higher yields and TNG were achieved in all inoculated plants, and statistically superior to the NI and NI + N treatments (Table 6). Compared to the NI control plants, inoculation with NG (T0) and NG (15) increased grain yield by 2485 and 2682 kg ha^{-1}, respectively. Considering the NI + N plants, the increases were of 797 and 994 kg ha^{-1}, respectively. Increases in both N concentrations in grains (NCG) and TNG were also observed in inoculated plants.

3.3.2 | Field trials performed in areas traditionally cropped with soybean, showing naturalized soybean-nodulating rhizobia

In a traditionally area cropped with inoculated soybean in Londrina, the naturalized soybean-nodulating rhizobial population was estimated at 1.47×10^4 rhizobia g^{-1} (Table 3). Under these conditions, inoculation generally does not improve significantly nodulation, as also observed at the V5 stage in this experiment (Table 7). In this same evaluation, the highest SDW and TNS were verified in the NI + N treatment.

In Londrina, at the physiological maturity, no differences were observed in grain yield; however, both NG (T0) and NG (T15) were highlighted by the highest values of TNG, although not differing statistically from the NI + N treatment (Table 7).

Nodulation was not affected by inoculation in Aparecida do Rio Negro (Table 7), which also contained high population of soybean-nodulating rhizobia in the soil, estimated at 4.62×10^5 rhizobia g^{-1} (Table 3). However, the N-fertilizer decreased NN and especially NDW (Table 7). No statistical differences were verified among the treatments for the other parameters evaluated at the V5 stage.

At the physiological maturity, the highest grain yield was observed in plants inoculated with NG (T0), not differing statistically from plants inoculated with NG (T15), and both were statistically higher than the NI and the NI + N plants (Table 7). In comparison with the NI plants, inoculation with NG (T0) and NG (T15) increased grain yield by 497 and 416 kg ha^{-1}, respectively. Considering the NI + N plants, the increases were of 533 and 452 kg ha^{-1}, respectively. Plants inoculated with NG (T0) and NG (T15) also showed the highest N accumulation in grains (TNG).

4 | DISCUSSION

Despite some variation in supporting rhizobial multiplication, depending on the origin, peat has been considered for decades as the best carrier for inoculants (Balatti & Freire, 1996; Burton, 1984; Hungria et al., 2005; Roughley, 1970). This matrix provides physical protection to the rhizobia, high water holding capacity and nutrients; however, one main limitation relies on the lack of practicality in the inoculant application to the seeds, requiring the use of adhesives (Hungria et al., 2005). Inoculation with peat-based inoculants in large scale soybean areas has been time-consuming and manpower-demanding, resulting in demand by the farmers of inoculants for pre-inoculation.

To attend to this new agricultural reality, liquid inoculants were developed and gained the market in South and North America, based on the easiness of application. Liquid formulations are easier to sterilize, allowing higher purity and cell concentrations. In addition, the inoculants are easy to apply and compatible with mechanized sowing (Bashan, De-Bashan, Prabhu, & Hernandez, 2014; Santos et al., 2019; Singleton, Keyser, & Sande, 2002). Taking Brazil as an example, the first liquid inoculant was registered in 2000 and nowadays 80% of the 70 million doses commercialized yearly are based on liquid carriers, and a similar percentage is reported in Argentina (ANPII, 2019; Santos et al., 2019).

Initially, several reports indicated that, in spite of high cell concentration, the liquid inoculants had a poor performance in the field, especially under environmental stressing conditions such as high temperature and drought, that frequently occur in tropical soils, being worse in sandy soils (Hungria et al., 2005). However, formulations have greatly improved, and this was confirmed in our study, as with the NG applied at sowing grain yield was not statistically different from the peat inoculation, also applied at sowing, in three out of the four sites, and was higher in Aparecida do Rio Negro. One major example was verified in Lutécia, as despite the low-fertility and very sandy soil devoid of soybean bradyrhizobia, the NG liquid inoculum promoted 2.5 more grain yield over the non-inoculated control (NI).

Nevertheless, soybean sowing in large areas in Brazil, nowadays 36.9 million ha (Conab, 2020), within a short-sowing window, less than 45 days, has been challenging, and many farmers demand pre-inoculation. The development of liquid inoculants enriched with cell protectors able to maintain rhizobial cells alive for days or weeks after being applied on the seeds can represent a useful strategy for dealing with a short-sowing window and manpower shortage. Here we show the feasibility of a liquid formulation that provides good field performance with 15-days of pre- inoculation, resulting in grain yield statistically similar to both the peat inoculant and the liquid inoculant NG applied at the sowing time in all four field sites. In addition, NG (T15) was similar to the treatment receiving 200 kg ha^{-1} of N-fertilizer in two sites (Paranavaí and Londrina) and superior in the other two sites (Lutécia and

TABLE 7 Experiments performed in areas traditionally cropped with soybean in Londrina-PR, southern Brazil and Aparecida do Rio Negro-TO, northern Brazil. Nodule number (NN, no. plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), N content in shoots (NCS, g N kg^{-1}), total N accumulation in shoots (TNS, mg N plant^{-1}), N content in grains (NCG, g N kg^{-1}), total N accumulated in grains (TNG, kg ha^{-1}). grain yield (GY, kg ha^{-1}), and grain weight (GW, g 100 seeds^{-1}) of soybean non-inoculated or inoculated with peat or liquid inoculant NG at sowing (T0) or with 15 days of pre-inoculation (T15)

	V5 Stage									Physiological maturity							
	Nodulation				Biomass		N in shoots			N in grains				Yield			
Treatment	NN		NDW		SDW		NCS		TNS	NCG		TNG		GY		GW	
Londrina																	
Non-inoculated (NI)	30.0	a[b]	90.4	a	4.85	b	38.5	ns[b]	190	b	44.1	c	186	b	4223	ns	16.6	b
NI +N-fertilizer[a]	20.8	b	32.9	b	6.75	a	42.3		284	a	46.4	bc	195	ab	4123		17.7	a
Peat (T0)	24.5	ab	67.9	a	4.58	b	38.8		175	b	45.8	c	184	b	4025		16.1	b
NG (T0)	28.3	a	85.0	a	5.40	b	40.3		219	b	48.9	ab	212	a	4336		16.6	b
NG (T15)	30.5	a	88.2	a	5.08	b	35.1		176	b	49.4	a	206	a	4181		16.4	b
	Mean	26.8		72.9		5.33		39.0		209		46.9		196.8		4178		16.7
	p-value	.0105		.0056		.0027		.3256		.0057		.0014		.0218		.3832		.0042
	CV (%)	17.9		36.0		16.1		15.0		24.0		4.5		8.0		6.4		3.9
Aparecida do Rio Negro																	
Non-inoculated (NI)	57.8	a	230.0	a	4.63	ns	45.4	ns	210	ns	63.5	ns	187	b	2941	c	12.7	ns
NI +N-fertilizer[a]	33.3	b	76.3	b	4.64		46.6		215		63.6		185	b	2905	c	13.3	
Peat (T0)	59.1	a	269.0	a	5.19		44.3		231		63.4		198	ab	3114	bc	12.9	
NG (T0)	63.5	a	267.0	a	5.74		45.3		258		63.1		217	a	3438	a	13.0	
NG (T15)	52.7	a	240.0	a	6.05		47.1		283		64.4		216	a	3357	ab	13.0	
	Mean	53.3		216		5.25		45.7		240		63.6		200.4		3151.2		13.0
	p-value	.0036		<.001		.3064		.5528		.2887		.3797		.0044		.0032		.7302
	CV (%)	23.0		26.7		26.3		6.9		27.1		1.7		8.1		7.8		5.7

[a] 200 kg of N ha^{-1}, 50% at sowing and 50% at flowering stage.

[b] Means followed by the same letter do not differ from each other by the Duncan test at $p \leq .05$; ns, not significant.

Aparecida do Rio Negro). However, despite the good performance, improvements in the formulation should continue, mainly aiming at increasing the cell survival on the seeds, as the technical recommendation is of recovery of 80,000 to 100,000 CFU seed^{-1} for appropriate symbiotic performance (Hungria & Nogueira, 2019), higher than observed in this study.

In 2020 Brazil became the first world's soybean producer, and along with other South American countries is responsible for 55% of the global production (USDA, 2020). In these countries, most of the N-fertilizers are imported, e.g. 80% in Brazil, very expensive and with a frequent shortage in the market (EPE, 2019). Therefore, to supply the high demand of N of the soybean, estimated at 80 kg of N per 1000 kg of grains, BNF is the unique strategy to achieve economic viability (Hungria & Mendes, 2015; Hungria & Nogueira, 2019). Efforts towards increasing the contribution of the biological process have taken place for decades, one of them to perform several trials to verify the need for annual reinoculation.

Pioneer studies in the USA have shown that in soils with as little as 10 cells g^{-1} there would be no response to inoculation (Thies, Singleton, & Bohlool, 1991; Thies, Woomer, & Singleton, 1995). Contrarily, the average increase in grain yield confirmed in more than 100 field trials in Brazil performed in areas with established populations of soybean-nodulating *Bradyrhizobium*, of 10^3 to up to 10^6 cells g^{-1} is of 8% with annual inoculation (Hungria & Mendes, 2015; Hungria & Nogueira, 2019; Hungria et al., 2006). Amazingly, in South America, BNF in soybean supplies the N needed for yield and also enriches the soil with remaining N of plant residues (Hungria & Mendes, 2015; Hungria et al., 2006). Conversely, in Midwest USA the soybean crop has supposed to reduce the soil organic matter levels (Córdova et al., 2019). Positive responses to annual reinoculation have been also reported in Argentina (Hungria et al., 2006; Leggett et al., 2017) and in other South American countries. Differences between countries might explain the estimates that 80% of the Brazilian and Argentinian farmers adopt annual reinoculation, in contrast to 15% of the North American (Chang et al., 2015; Leggett et al., 2017). Another recent report from the USA indicated lack of response to reinoculation (Carciochi et al., 2019); however, in this study, a commercial inoculant was used and there was no check on the cell concentration before sowing, and one may be surprised by the low quality of some commercial inoculants. As high yields and seed protein in the USA seem limited by N that cannot be supplied by the naturalized bradyrhizobia population (La Menza et al., 2020; La Menza, Monzon, Specht, & Grassini, 2017), although edaphoclimatic conditions and soil fertility in the USA are usually very different from those of South America, it seems interesting to suggest the strategy of annual reinoculation to increase yields and protein content in grains. Indeed, Leggett et al. (2017) have recently shown that inoculation may also lead to yield increases in the USA.

The capacity of BNF in soybean-bradyrhizobia symbiosis in Brazil was confirmed in all four field sites. Elite rhizobial strains used in commercial inoculants (Hungria & Mendes, 2015; Hungria et al., 2006) were applied at the concentration of 1.2×10^6 cells seed^{-1}, the minimum dose recommended in the country (Hungria, Araujo, Silva Júnior, & Zilli, 2017). In areas cropped for the first time and void of soybean bradyrhizobia, in Paranavaí and Lutécia, even under very harsh conditions of sandy and low-fertility soils, the biological process supported well plant growth, and considering both sites, mean increases in grain yield in relation to the NI control were of 83% (PI T0), 90% (NG T0) and 88% (NG T15), indicating an average gain of 89% with the NG liquid inoculant. Significant increases, although of lower magnitude, were obtained with the use of the NG inoculant in the two areas traditionally cropped with the legume and showing naturalized soybean-nodulating rhizobial population, on average by 8.5% when applied at sowing and by 5.2% with 15 days of anticipation, representing an average gain of 6.8%, whereas no increases were verified with the addition of N-fertilizer.

Our results also highlight the non-economic return of the application of N-fertilizers, as the inoculation treatments always resulted in higher grain yield increases. Therefore, care should be taken in the analysis of studies preconizing that soybean responds to N-fertilizer, but that do not include a proper control with an inoculation treatment (La Menza et al., 2020). In addition, the negative impact of N-fertilizer on the contribution of the BNF has been broadly reported (e.g. Hungria & Mendes, 2015; Hungria et al., 2006); in a recent meta-analysis of field experiments, the average decrease due to the N-fertilizer was estimated at 44% (Santachiara, Salvagiotti, & Rotundo, 2019). However, we should mention that in our study none of the seeds was treated with pesticides. Soybean seed treatment with pesticides can greatly impair nodulation and nitrogen fixation, especially when in contact with the cells for long periods, as in pre-inoculated seeds (Campo, Araujo, & Hungria, 2009; Hungria & Mendes, 2015; Hungria & Nogueira, 2019). Although in Brazil more than 90% of the soybean seeds are treated with pesticides, our main goal was to evaluate the survival and efficacy of the liquid inoculant with pre-inoculation, and the next step should be to extend this evaluation to seeds treated with pesticides.

Not less important, decreases in protein contents reported in soybean grains have affected industrial processing that requires minimal concentration for economic viability. There is evidence that the N from the BNF is more easily translocated to the grains than the N coming from

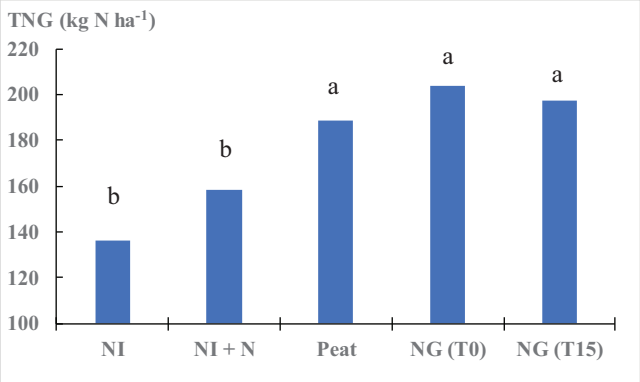

FIGURE 2 Total N accumulated in grains (TNG) of soybean non-inoculated without (NI) or with N-fertilizer (NI + N, 200 kg of N ha^{-1}), or inoculated with peat inoculant at sowing (T0), or liquid inoculant NG at sowing (T0) or 15 days before sowing (T15). Both peat and liquid inoculants were applied to supply 1.2×10^6 cells $seed^{-1}$. Data represent the means of four field experiments, with six replicates per treatment per site. Bars sharing the same letter do not differ statistically ($p \leq .05$, Duncant's test)

the soil or the N-fertilizers (e.g. Hungria & Neves, 1987; Kaschuk et al., 2010). In our study, N contents in grains of plants inoculated with NG (T15) were statistically higher than the NI + N plants in three out of the four experiments. Inoculation greatly increased total N in grains, and both NG (T0) and NG (T15) resulted in higher accumulation of N in grains in all four experiments when compared with both NI and NI + N plants. Considering the average of all three inoculants in all four experiments (Figure 2), inoculation increased total N in grains by 45% and 24%, when compared to the NI and NI + N plants, respectively; considering only the NG liquid inoculant (T0 and T15) the increases were of 47 and 27%, respectively. These results highlight the large contribution of BNF to improve the protein content in soybean grains.

In conclusion, our results highlight the economic and environmental importance of the BNF with the soybean crop in Brazil, that may be extended to other producing countries. The development of inoculants for pre-inoculation is feasible and can bring many benefits to the farmers, giving the flexibility to organize the sowing operation and increasing the use of inoculants.

DATA AVAILABILITY STATEMENT

All datasets generated or analyzed during this study are included in the manuscript, and complementary dataset will be available upon request to the corresponding author.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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