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Effects of growth-promoting bacteria on soybean root activity, plant development, and yield

Luiz Gustavo Moretti[1,2] | **Carlos A. C. Crusciol**[1] | **Eiko E. Kuramae**[2] | **João W. Bossolani**[1] | **Adônis Moreira**[3] | **Nídia R. Costa**[1] | **Cleiton J. Alves**[1] | **Isabô M. Pascoaloto**[1] | **Artur B. L. Rondina**[3] | **Mariangela Hungria**[3]

[1]Department of Crop Science, São Paulo State University (UNESP), College of Agricultural Sciences, Av. Universitária, 3780, Lageado Experimental Farm, 18610-034, Botucatu, São Paulo State, Brazil

[2]Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), 6708 PB, Wageningen, the Netherlands

[3]Brazilian Agricultural Research Corporation (Embrapa), Embrapa Soybean, Warta District, 86001-970, Londrina, Paraná State, Brazil

Correspondence
Carlos A. C. Crusciol, São Paulo State University (UNESP), College of Agricultural Sciences, Department of Crop Science, Av. Universitária, 3780, Lageado Experimental Farm, 18610-034, Botucatu, São Paulo State, Brazil.
Email: carlos.crusciol@unesp.br

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Abstract

Rhizobia and other plant growth-promoting rhizobacteria (PGPR) have been broadly used as inoculants in agriculture, resulting in morphofunctional improvements in roots and grain yield. This study was carried out during two cropping seasons under field and greenhouse conditions in Brazil to verify the effects of inoculation of two soybean cultivars with PGPR and secondary microbial metabolites (SMMs) on root activity and nodulation, plant development, and grain yield. Inoculation and co-inoculation treatments consisted of *Bradyrhizobium japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 inoculated together, in combination with *Bacillus subtilis* strain QST 713, *Azospirillum brasilense* strains Ab-V5 and Ab-V6, and SMMs extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889. Root systems were evaluated by direct (optical reading) and indirect (rubidium nitrate application, $^{85}RbNO_3$) methods. Increases of up to 1.6% in root diameter (0.01- to 0.5-mm class), 28.5% in length, 19.7% in root volume, 17.8% in root surface area, 29% in the number of nodules, 27.2% in nodule dry weight, 13.5% in root dry weight, and 3.8% in shoot dry weight. Greater exploration and activity within and between rows following inoculation at up to 40 and 10 cm in depth, respectively, were observed in plants co-inoculated with the standard inoculation (only *Bradyrhizobium* spp.) + SMMs + *A. brasilense*, resulting in a yield increase of 485 kg ha^{-1}. The results emphasize the biotechnological potential of using secondary metabolites of rhizobia with inoculants containing rhizobia and PGPR to improve the growth and soybean yield in tropical conditions.

1 | INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the most important Brazilian agricultural commodities and accounts for approximately 13% of the country's exports (Moretti et al., 2018). Soybean has a high N demand, requiring approximately 80 kg of N to produce 1000 kg of grains (Hungria & Mendes, 2015; Hungria, Campo, Mendes, & Graham, 2006). The chemical N fertilizers required for annual soybean production in Brazil would cost approximately US$ 15 billion, making the cultivation of soybean economically unattractive (Hungria & Mendes, 2015; Hungria et al., 2006).

Abbreviations: CEC, cation exchange capacity; CFUs, colony-forming units; LCOs, lipo-chitooligosaccharides; MPN, most probable number; NDW, nodule dry weight; NN, nodule number; PGPR, plant growth-promoting rhizobacteria; RDW, root dry weight; SDW, shoot dry weight; SI, standard inoculation; SMMs, secondary microbial metabolites.

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Biofertilizers are important and low-cost alternative fertilizers to achieve sustainable agricultural production. Rhizobial inoculants have been applied as biofertilizers to legume crops for over 120 years, and inoculants carrying other plant growth-promoting rhizobacteria (PGPR) have been used for over half a century (Ormeño-Orrillo et al., 2012). A broad range of beneficial effects of PGPR have been reported, including biological nitrogen fixation (Ashraf, Rasool, & Mirza, 2011) and the production of compounds such as antimicrobials, exopolysaccharides, lipo-chitooligosaccharides (LCOs) that increase plant growth (Marks, Megías, Nogueira, & Hungria, 2013, 2015).

Secondary metabolites are natural molecules that perform distinct functions in primary metabolism (O'Brien & Wright, 2011). These metabolites are composed of complex organic compounds whose synthesis requires many specific enzymatic reactions (Madigan et al., 2008). Among the most commonly found chemical structures are β-lactam rings, cyclic peptides, nonprotein amino acids, unconverted sugars and nucleosides, unsaturated polyacetylenes, nitrile groups, cyclic triesters, terpenoids, and large macrolide rings (Marks et al., 2013, 2015).

The volume and length of a root system must be developed to optimize the use of the available resources in the soil (Giehl, Gruber, & von Wirén, 2014; Hodge, 2004). Although the root system is under the plant's genetic control, root system growth is affected by chemical, physical, and biological factors in the soil (Giehl et al., 2014; Taylor & Arkin, 1981). It has been widely reported that root system growth can be stimulated by several species of PGPR, however, proper evaluations of this growth are rare. Methods to evaluate root systems vary according to the research aim (Encide-Olibone, Olibone, & Rosolem, 2008; Gockele, Weigelt, Gessler, & Scherer-Lorenzen, 2014; Hoekstra et al., 2014) and can be divided into direct and indirect methods (Böhm, 1979).

Direct methods are correlated with root system biometrics, while indirect methods are based on determining changes in the flow of water or nutrients in the soil profile that influence the distribution and activity of roots (Gockele et al., 2014; Hoekstra et al., 2014). According to Läuchli and Epstein (1970), rubidium (^{85}Rb) is stable isotope analogous to potassium (K^+), exhibits no toxicity or irregular distribution within the plant, and can be used in indirect methods that satisfactorily assess plant root activity (Encide-Olibone et al., 2008; Pivetta, Castoldi, Santos, & Rosolem, 2011). In addition, because ^{85}Rb is not radioactive, there are no ecological risks of its use (Rosolem & Pivetta, 2017). Furthermore, ^{85}Rb can be precisely applied since it is not present in fertilizers and soils or essential to plants. Thus, the objective of the present study was to simultaneously evaluate under greenhouse and in field conditions the effects of single inoculation and co-inoculation of soybean with PGPR and secondary microbial metabolites (SMMs) on root activity, nodulation, plant development, and grain yield.

Core ideas

- Plant growth-promoting rhizobacteria (PGPR) enhance the development and activity of the soybean root system and its ability to exploit the soil.
- Soybean nodulation and plant biomass increased with the use of PGPR and their metabolites.
- Co-inoculation with PGPR increases soybean grain yield by up to 10%.

2 | MATERIALS AND METHODS

This study consisted of two field and two greenhouse experiments carried out during the 2016–2017 and 2017–2018 cropping seasons at Lageado Experimental Farm of São Paulo State University in Botucatu, São Paulo State, Brazil (48°26′W, 22°51′S, 786 m altitude). The soil is a Typic Haplorthox (Soil Survey Staff, 2014) and has a clayey textural class with 602, 117, and 281 g kg^{-1} of clay, sand, and silt, respectively. According to Köeppen's classification (Alvares, Stape, Sentelhas, Gonçalves, & Sparovek, 2013), the climate is Cwa, which corresponds to tropical altitude with a dry winter and a hot, wet summer. The long-term (1956–2019) average annual temperatures are maximum of 26.1°C, minimum of 15.3°C, with an average of 20.7°C. Average annual rainfall is approximately 1360 mm (Unicamp, 2019). Climatological data during the experiments are presented in Figure 1.

Soil chemical attributes (0.0–0.2 m) were determined according to van Raij, Andrade, Cantarella, and Quaggio (2001), and results obtained were: soil organic matter, 26.2 g kg^{-1}; pH ($CaCl_2$), 5.0; P (resin), 59 mg kg^{-1}; K^+, 3.9 $mmol_c$ kg^{-1}; Ca^{2+}, 25 $mmol_c$ kg^{-1}; Mg^{2+}, 15 $mmol_c$ kg^{-1}; $H^+ + Al^{3+}$, 42 $mmol_c$ kg^{-1}; Al^{3+}, 2 $mmol_c$ kg^{-1}; cation exchange capacity (CEC), 86 $mmol_c$ kg^{-1}; S-SO_4^{2-}, 4.9 mg kg^{-1}; Fe^{2+}, 22 mg kg^{-1}; Cu^{2+}, 8.8 mg kg^{-1}; Mn^{2+}, 26.2 mg kg^{-1}; Zn^{2+}, 2 mg kg^{-1}; BO_3^{3-}, 0.4 mg kg^{-1}; base saturation, 51%. Dolomite lime (28% CaO, 18% MgO, w/w) was applied to the soil to raise base saturation to 70%. In addition, autochthonous bacteria population capable of nodulating soybean was estimated by the most probable number (MPN), according to O'Hara, Hungria, Woomer, and Howieson (2016) at 9.32×10^4 cells g^{-1} soil.

2.1 | Treatments and experimental design

A randomized block design using two soybean cultivars, BRS 317 (Embrapa), conventional and with determinate growth

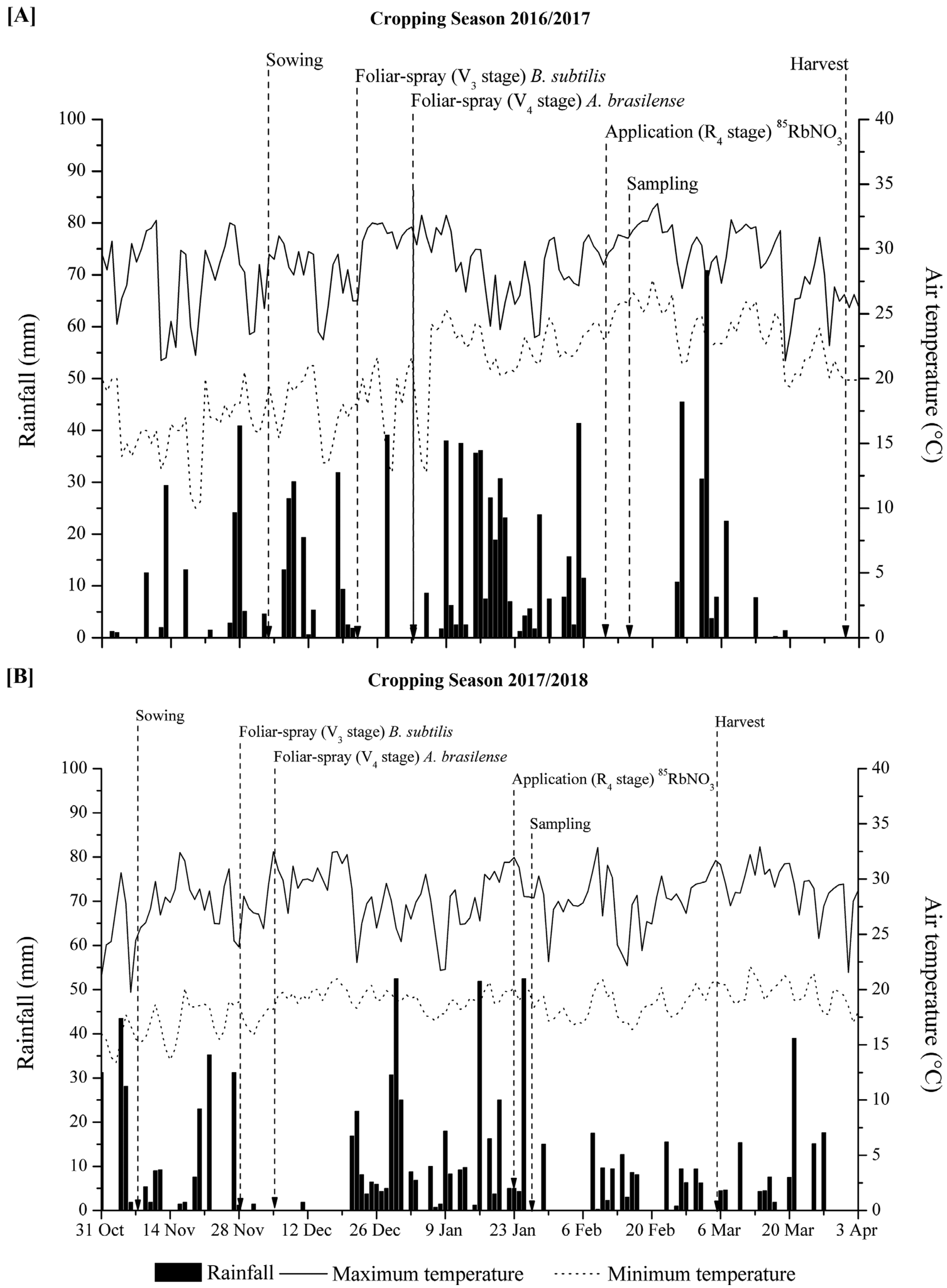

FIGURE 1 Rainfall and maximum and minimum air temperatures during the experimental period. Botucatu, São Paulo State, Brazil, 2016–2018

type, and TMG 1264 RR (Tropical Breeding & Genetics), transgenic and with indeterminate growth type, and eight inoculation treatments with four replicates were employed in both seasons.

The inoculation treatments were as follows: (i) standard inoculation (SI) with *Bradyrhizobium japonicum* (SEMIA 5079) + *B. diazoefficiens* (SEMIA 5080) via seed; (ii) SI + the application of SMMs extracted from USDA 110 + CIAT 889 via seed; (iii) SI + SMMs + foliar-spray inoculation of soybean plants at the V_3 stage (Fehr & Caviness, 1977) with *Bacillus subtilis* (QST 713); (iv) SI + SMMs + foliar-spray inoculation of soybean plants at the V_4 stage with *Azospirillum brasilense* (Ab-V5 + Ab-V6); (v) SI + SMMs + *B. subtilis* + *A. brasilense*; (vi) SI + *B. subtilis*; (vii) SI + *A. brasilense*; and (viii) SI + *B. subtilis*. + *A. brasilense*.

Foliar-sprays with *A. brasilense* and *B. subtilis* were carried out according to the recommendations of the companies that sold the inoculants. These products are registered in the Ministry of Agriculture, Livestock, and Food Supply of Brazil (MAPA).

2.2 | Microbial inoculants and secondary metabolites

Liquid inoculants contained *B. japonicum* and *B. diazoefficiens* strains SEMIA 5079 (= CPAC 15) and SEMIA 5080 (= CPAC 7), respectively, which is the most common combination of commercial inoculants used in Brazil (Hungria, Nogueira, & Araujo, 2013), were prepared at a concentration of 7×10^9 colony forming units (CFUs) ml^{-1} and applied to provide 1.2×10^6 cells $seed^{-1}$. A commercial inoculant containing *B. subtilis* strain QST 713 at a concentration of 1×10^9 CFUs ml^{-1} was applied as a foliar-spray at a rate of 3 L ha^{-1} in a final volume of 200 L ha^{-1} of water. A commercial inoculant containing *A. brasilense* strains Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084) at a concentration of 2×10^8 CFUs ml^{-1} was applied as foliar-spray at a rate of 300 ml ha^{-1} in a final volume of 150 L ha^{-1} of water. Secondary microbial metabolites enriched in LCOs were extracted from *Rhizobium tropici* strain CIAT 899 (= CNPSo 103, = SEMIA 4077) and *B. diazoefficiens* USDA 110 (= CNPSo 56), were produced at a concentration of 1.0 ml L^{-1} by Embrapa Soybean, as described previously (Marks et al., 2013), and applied in a volume of 200 ml per 50 kg of seeds.

2.3 | Greenhouse experiments

Differences in soybean nodulation due to inoculation with *Bradyrhizobium* strains are difficult to confirm in soils with indigenous or naturalized populations of compatible bradyrhizobia, which is the case for most soils in Brazil cropped with soybeans that previously underwent massive inoculation. Therefore, an experiment was performed under greenhouse conditions in 15-L pots using sterilized substrate composed of a mixture of sand and crushed coal (1:1, v/v). Nutrients were supplied in the form of 200 mg kg^{-1} of P, 150 mg kg^{-1} of K, 30 mg kg^{-1} of S, 5 mg kg^{-1} of Zn, 4 mg kg^{-1} of Mn, 3 mg kg^{-1} of Ca, 2.5 mg kg^{-1} of Mg, 2 mg kg^{-1} of Fe, 1.5 mg kg^{-1} of B, and 1 mg kg^{-1} of Cu according the recommendations from Moreira, Moraes, Souza, and Bruno (2016).

The seeds were treated with fungicides (carboxin + thiram, 100 g + 100 g a.i. per 100 kg of seeds) prior to inoculation and sowing. Seed inoculation was performed 1 h before sowing by evenly coating the seeds with the appropriate amounts of inoculants. Five seeds were sown per pot, and the seedlings were thinned to three plants 10 d after sowing. Four reference pots were weighed every four days, and distilled sterile water was added as needed to return the water potential to -0.01 MPa. To carry out foliar-spray inoculations, an aerograph atomizer was employed to mimic the action of a spraying apparatus. The soil surface was covered with aluminum foil to prevent the inoculant from reaching it.

Plants at the R_2 stage (Fehr & Caviness, 1977) were harvested and separated into shoots and roots. The roots were washed to remove substrate particles. Nodules were removed from the roots, oven-dried at 65°C for 72 h, counted, and weighed. The root samples were collected according to the methodology described by Costa et al. (2000), for further assessment of total root length, root surface area, root volume, and root diameter by scanning using an HP Scanjet 4c/T scanner and WinRHIZO Reg 3.8b-1993-1997 (Regent Instruments, Sainte-Foy, Canada) software. The entire root system was oven-dried at 65°C for 72 h and weighed.

2.4 | Field experiments

In both agricultural years, soybeans were sown after black oats (*Avena strigosa* Schreb.) that had been cropped in the winter as a mulching crop for a no-till system, providing an average of 4.5 Mg ha^{-1} straw in a dry land area (no irrigation). The treatments were applied to the same plots in both growing seasons. Field plots consisted of 10 rows that were 10-m long and spaced 0.45 m apart, leading to a total plot area of 45 m^2. Plots were separated by 0.5-m wide rows and 1.5-m wide terraces to avoid cross-contamination from surface run-off containing bacteria or fertilizers that may occur as a consequence of heavy rainfall. In both seasons, all plots were fertilized with 300 kg ha^{-1} of the 00–20–20, N–P_2O_5–K_2O formulation. The treatments and seed inoculations in both field and greenhouse experiments were identical. Foliar-spray inoculations were performed by a tractor-mounted sprayer. In all treatments, foliar-spray containing 20 g ha^{-1} of Mo (as $Na_2MoO_4 \cdot 2H_2O$) and 2 g ha^{-1} of Co (as $CoCl_2 \cdot 6H_2O$) was

TABLE 1 Root diameter by class, length, volume and surface area per plant of two soybean cultivars that received different inoculation treatments during two cropping seasons. Plants were grown in greenhouse and harvested at R_2 stage. Botucatu, São Paulo State, Brazil, 2016–2018

	Diameter class (mm)					
	0.01–0.5	0.51–2.0	2.1–5.0	Length	Volume	Surface Area
Factors	%			m	cm^3	m^2
Inoculation (In[a])						
SI	88.3 b[b]	11.4 a	0.3 a	50.2 c	34.5 b	15.2 b
SI + SMMs	88.4 b	11.3 a	0.3 a	56.8 b	36.3 b	16.4 a
SI + SMMs + *B. subtilis* (Bs)	88.4 b	11.4 a	0.2 a	56.5 b	37.1 b	16.4 a
SI + SMMs + *A. brasilense* (Ab)	89.6 a	10.1 b	0.3 a	62.3 a	40.2 a	17.6 a
SI + SMMs + Bs + Ab	89.7 a	10.1 b	0.2 a	64.5 a	41.3 a	17.9 a
SI + Bs	88.5 b	11.2 a	0.3 a	55.5 b	36.0 b	16.4 a
SI + Ab	89.0 a	10.7 a	0.3 a	58.7 b	37.8 b	16.8 a
SI + Bs + Ab	89.1 a	10.7 a	0.2 a	59.3 b	38.0 b	17.2 a
Cultivar (Cv)						
BRS 317	88.8 a	10.9 a	0.3 a	56.2 a	38.4 a	16.6 a
TMG 1264 RR	89.0 a	10.8 a	0.2 a	54.9 a	38.7 a	16.6 a
Cropping season (CS)						
2016–2017	88.9 a	10.8 a	0.3 a	51.4 b	32.8 b	15.3 b
2017–2018	89.0 a	10.7 a	0.3 a	60.2 a	38.6 a	18.0 a
ANOVA (*F* probability)						
In	.031	.027	.523	.001	.023	.016
Cv	.165	.672	.165	.753	.875	.759
CS	.463	.172	.156	.042	.027	.032
In × Cv	.263	.356	.562	.187	.986	.193
In × CS	.165	.465	.243	.180	.129	.908
Cv × CS	.352	.543	.145	.962	.435	.130
In × Cv × CS	.742	.476	.132	.874	.223	.962

[a]Inoculation treatments: SI, standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 on seeds; SMMs, application of secondary microbial metabolites extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889 on seeds; *B. subtilis*, foliar-spray inoculation with *Bacillus subtilis* strain QST 713 at V_3 stage; *A. brasilense*, foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V_4 stage.
[b]Means followed by different letters differ from each other by least significant difference test at $p \leq .05$.

applied to plants at the V_4 stage (Fehr & Caviness, 1977). Phytosanitary treatments were carried out according to need and recommendations for the soybean crop (Embrapa, 2013).

Soybean root activity was evaluated as described by Encide-Olibone et al. (2008). When soybean plants were at the R_4 stage (Fehr & Caviness, 1977), 4 ml of rubidium nitrate ($^{85}RbNO_3$) at a concentration of 1.0 mol L^{-1} was applied to the soil 0.0 and 0.22 m from the plant and at depths of 5, 10, 20, 40 and 60 cm using a graduated syringe. To perform these applications, the soil was drilled with cylindrical rods 12 mm in diameter, and three replicates were performed for each depth and location. Four days after $^{85}RbNO_3$ application, plant shoots were collected and oven-dried at 60°C for 72 h. In addition, one plant that did not receive the marker was collected per plot to be used as a background control. The samples were ground and digested with nitro-perchloric acid, followed by ^{85}Rb determination by atomic absorption (AOAC, 1990).

When the soybean plants were at physiological maturity—the R_8 stage (Fehr & Caviness, 1977)—samples were collected in 15 m^2 from the center of each plot to estimate the grain yield in kg ha^{-1} (on a 13% moisture base).

2.5 | Statistical analyses

All data were initially tested for normality using the Shapiro–Wilk test (Shapiro & Wilk, 1965) from the UNIVARIATE procedure of SAS version 9.3 (SAS Institute, 2015), and the results indicated that all data were distributed normally ($W \geq .90$). The assumption of the homogeneity of variances was tested using Levene's test for residual errors. When variances could not be considered homogeneous ($p \leq .10$), Welch's F-test was performed to determine the overall significance of the statistic of interest.

The data were then analyzed using the MIXED procedure of SAS and the Satterthwaite approximation to determine

TABLE 2 Nodule number (NN), nodule dry weight (NDW), root dry weight (RDW) and shoot dry weight (SDW) of two soybean cultivars that received different inoculation treatments during two cropping seasons. Plants were grown in greenhouse and harvested at R_2 stage. Botucatu, São Paulo State, Brazil, 2016/2018

Factors	NN n° plant^{-1}	NDW mg plant^{-1}	RDW g plant^{-1}	SDW
Inoculation (In[a])				
SI	62 b[b]	250 b	7.4 b	18.6 b
SI + SMMs	67 b	266 b	7.5 b	18.7 b
SI + SMMs + *B. subtilis* (Bs)	69 b	277 b	7.7 b	18.7 b
SI + SMMs + *A. brasilense* (Ab)	77 a	308 a	8.3 a	19.2 a
SI + SMMs + Bs + Ab	80 a	318 a	8.4 a	19.3 a
SI + Bs	65 b	262 b	7.4 b	18.7 b
SI + Ab	75 a	305 a	7.7 b	18.7 b
SI + Bs + Ab	77 a	310 a	7.8 b	18.8 b
Cultivar (Cv)				
BRS 317	67 a	265 a	7.7 a	18.7 a
TMG 1264 RR	71 a	272 a	7.8 a	18.9 a
Cropping season (CS)				
2016–2017	69 a	278 a	7.3 b	18.5 b
2017–2018	73 a	290 a	7.9 a	19.2 a
ANOVA (*F* probability)				
In	.035	.001	.013	.001
Cv	.287	.642	.552	.852
CS	.623	.764	.047	.023
In × Cv	.821	.121	.651	.412
In × CS	.283	.875	.172	.155
Cv × CS	.387	.275	.422	.290
In × Cv × CS	.287	.287	.575	.187

[a]Inoculation treatments: SI, standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 on seeds; SMMs, application of secondary microbial metabolites extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889 on seeds; *B. subtilis*, foliar-spray inoculation with *Bacillus subtilis* strain QST 713 at V_3 stage; *A. brasilense*, foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V_4 stage.
[b]Means followed by different letters differ from each other by least significant difference test at $p \leq .05$.

the degrees of freedom for the tests of fixed effects. Blocks and block interactions were considered random effects. Inoculations, cultivars, cropping seasons, and their interactions were considered fixed effects. The results are reported as the least square means and separated using the probability of differences option (PDIFF). The means were compared using Fisher's protected Least Significant Difference test. The main factor and interaction effects were considered statistically significant at $p \leq .05$.

3 | RESULTS

3.1 | Greenhouse experiments

In the 2017–2018 cropping season, significant increases in root length, volume, and surface area were observed. However, inoculation did not interact with cultivar or cropping season (Table 1). Increases of up to 1.6% in root diameter (class 0.01–0.5 mm), 28.5% in length, and 19.7% in root volume were observed in plants co-inoculated with SI + SMMs + *A. brasilense*, independently of the presence of *B. subtilis*, compared with SI-treated plants (Table 1). All co-inoculated plants were superior in root surface area, with increases of up to 17.8% compared to that in the SI-treated plants.

The nodule number (NN) and nodule dry weight (NDW) were altered in all plants co-inoculated with *A. brasilense* (Table 2) and increased by up to 29% (NN) and 27.2% (NDW), compared with that in the SI-treated plants. The plants co-inoculated with SI + SMMs + *A. brasilense* exhibited increases in root dry weight (RDW) of up to 13.5% and shoot dry weight (SDW) of 3.8% compared with SI-treated plants. Significant differences in the RDW and SDW were observed in the 2017–2018 cropping season (Table 2).

TABLE 3 Rubidium (^{85}Rb) concentrations in shoots and the grain yield (GY) of two soybean cultivars that received different inoculation treatments in the field during two cropping seasons (2016–2017 and 2017–2018). $^{85}RbNO_3$ was applied within and between rows at five depths in Botucatu, São Paulo State, Brazil

	Depth within rows					Depth between rows					
	5 cm	10 cm	20 cm	40 cm	60 cm	5 cm	10 cm	20 cm	40 cm	60 cm	GY
Factors	mg kg^{-1}					mg kg^{-1}					kg ha^{-1}
Cultivar (Cv)											
BRS 317	238 a[a]	203 a	157 a	109 a	72 a	102 a	84 a	70 a	50 a	50 a	4985 a
TMG 1264 RR	242 a	210 a	165 a	111 a	75 a	106 a	87 a	73 a	51 a	53 a	5018 a
Cropping season (CS)											
2016-2017	152 b	138 b	113 b	92 b	73 a	88 b	65 b	58 b	44 b	40 a	4730 b
2017-2018	307 a	271 a	204 a	123 a	81 a	121 a	102 a	95 a	57 a	65 a	5285 a
ANOVA (*F* probability)											
Inoculation (In[b])	.001	.021	.001	.041	.621	.001	.001	.231	.134	.852	.001
Cv	.123	.963	.144	.237	.875	.243	.234	.823	.423	.870	.175
CS	.001	.001	.001	.001	.271	.001	.001	.001	.001	.242	.001
In × Cv	.857	.832	.473	.764	.232	.242	.240	.473	.633	.875	.532
In × CS	.287	.923	.233	.754	.113	.209	.852	.391	.253	.249	.242
Cv × CS	.623	.875	.290	.986	.673	.234	.352	.677	.342	.210	.321
In × Cv × CS	.986	.348	.924	.187	.230	.932	.652	.862	.173	.633	.128

[a]Means followed by different letters differ from each other by least significant difference test at $p \leq .05$.
[b]Means for inoculation treatments were presented in graphic form.

3.2 | Field experiments

In the field experiments (Table 3), all plants co-inoculated with *A. brasilense* exhibited higher shoot ^{85}Rb concentrations when $^{85}RbNO_3$ was applied within rows at depths of 5, 10, and 20 cm and between rows at 5 and 10 cm of depth (Figure 2). When $^{85}RbNO_3$ was applied within rows at depth of 40 cm, an increase in shoot ^{85}Rb concentrations was observed in plants co-inoculated with SI + SMMs + *A. brasilense*. Nevertheless, no significant effects on ^{85}Rb concentrations were observed following its application at a depth of 60 cm within rows and at 20, 40, and 60 cm of depth between rows.

Notably, in the 2017–2018 cropping season, higher average ^{85}Rb concentrations were observed, which was possibly due to more favorable climatic conditions that favored higher ^{85}Rb uptake (Figure 1). However, inoculation did not interact with cultivar or cropping season. In general, plants co-inoculated with SI + SMMs + *A. brasilense* exhibited higher ^{85}Rb uptake and ^{85}Rb concentrations than other treatment groups, indicating the greatest root activity in the soil profile.

Significant differences in grain yield were observed among different treatment groups, and co-inoculation with SI + SMMs + *A. brasilense* had a positive effect on grain yield, with grain yield increase of 485 kg ha^{-1} compared with that in the SI-treated plants (Figure 3). However, inoculation did not interact with cultivar or cropping season.

4 | DISCUSSION

The effects of PGPR and their metabolites on soybean root volume and length allowed us to identify combinations of microorganisms that may affect cell division and differentiation, thus leading to changes in root system architecture. This effect was confirmed by Berendsen et al. (2015) and by Schlemper et al. (2018) in studies on PGPR in *Arabidopsis thaliana* and sorghum (*Sorghum*), respectively. The authors observed an increase in the numbers of both root primordia and lateral roots, indicating that root sites and lateral root outgrowth areas were affected by inoculation. These changes consequently affected nutrient uptake by changing the effective volume of soil explored by the roots (Hodge, 2004; Verbon & Liberman, 2016; Walker, Bais, Grotewold, & Vivanco, 2003).

In our greenhouse experiments, we observed higher mean lengths, volumes, and surface areas, which corroborates Silva, Damatta, Ducatti, Regazzi, and Barros (2004), who found that an increase in root surface occurred with increased root length and volume. Root length is directly related to the root surface; therefore, an increase in root length results in a greater surface area, thus providing better conditions for the uptake of water and nutrients from the soil solution, which can result in higher yield.

The PGPR modified the root system architecture, as described by Hari and Srinivasan (2005) and Gosal et al. (2012). Both studies reported longer root lengths in plants

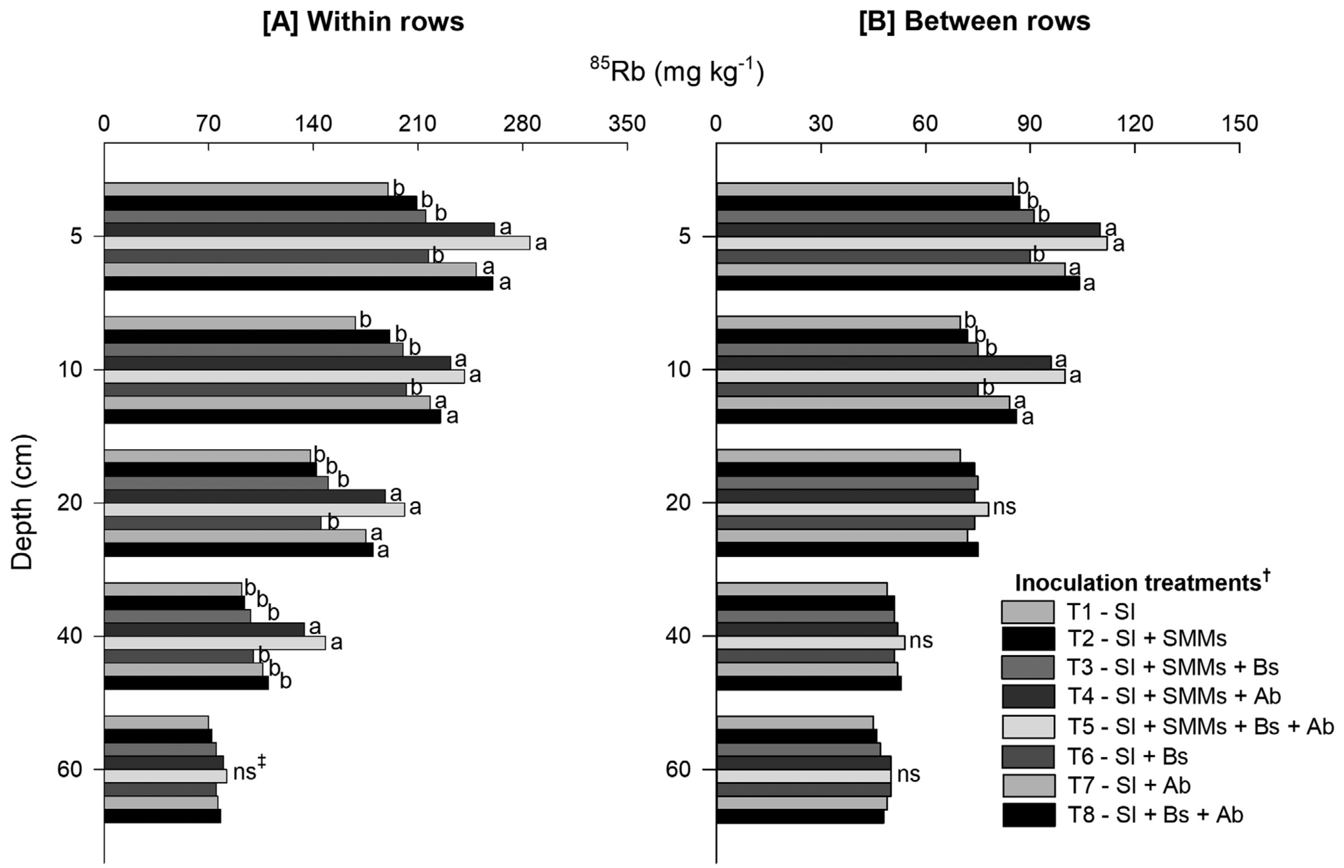

FIGURE 2 Rubidium (^{85}Rb) concentrations in the shoots of two soybean cultivars that received different inoculation treatments in the field during two cropping seasons. (A) Application of $^{85}RbNO_3$ within rows. (B) Application of $^{85}RbNO_3$ between rows. † Inoculation treatments: SI, standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 on seeds; SMMs, application of secondary microbial metabolites extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889 on seeds; Bs, foliar-spray inoculation with *Bacillus subtilis* strain QST 713 at V_3 stage; Ab, foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V_4 stage. ‡ ns, statistically not significant; means followed by different letters differ from each other by least significant difference test at $p \leq .05$

co-inoculated with *Azospirillum* spp. than in plants not inoculated with PGPR, similar to the results of our study. Co-inoculation promoted a thinner root system, which allowed a greater contact surface area with the soil to capture more water and nutrients (Bhattacharjee, Taylor, Smith, & Spence, 2008). According to Okumura et al. (2013) and Santi, Bogusz, and Franche (2013), this change is attributed to the synthesis of phytohormones that alter root morphology and increase lateral roots.

Similarly increased nodulation was attributed by Downie (2010) to the use of SMMs in particular. Such molecules play important roles in several stages of the development of the root nodule symbiosis, including those related to Type III secretion systems and exopolysaccharides. Marks et al. (2013) showed that the best performance was achieved with SMMs addition, which increased the NN and NDW by 21% and 12%, respectively, compared with those of the SI-treated plants. Secondary microbial metabolites may provide an evolutionary advantage for microbe survival in the soil, and they may also help in the establishment of symbiotic partnerships (Brencic & Winans, 2005).

The differences in root activity in the field between the two cropping seasons could be attributed to different climatic conditions. Irregularities in rainfall distribution in the second half of February 2016 may have affected the root-soil system and ^{85}Rb uptake. The accumulated rainfall in the seven days prior to $^{85}RbNO_3$ application in the first cropping season was 52.9 mm, but during the four days required for ^{85}Rb uptake (Encide-Olibone et al., 2008), there was no rain or water supplementation. In contrast, in the second cropping season, 98.1 mm of rainfall accumulated in the seven days prior to $^{85}RbNO_3$ application, and 66.9 mm of rainfall accumulated during the period necessary for ^{85}Rb uptake.

According to Rosolem and Pivetta (2017), root activity reflects a specific time and situation depending on the soil water content. Thus, even with the different climatic conditions between cropping seasons, our study allowed us to understand the effects of PGPR. Even though root length,

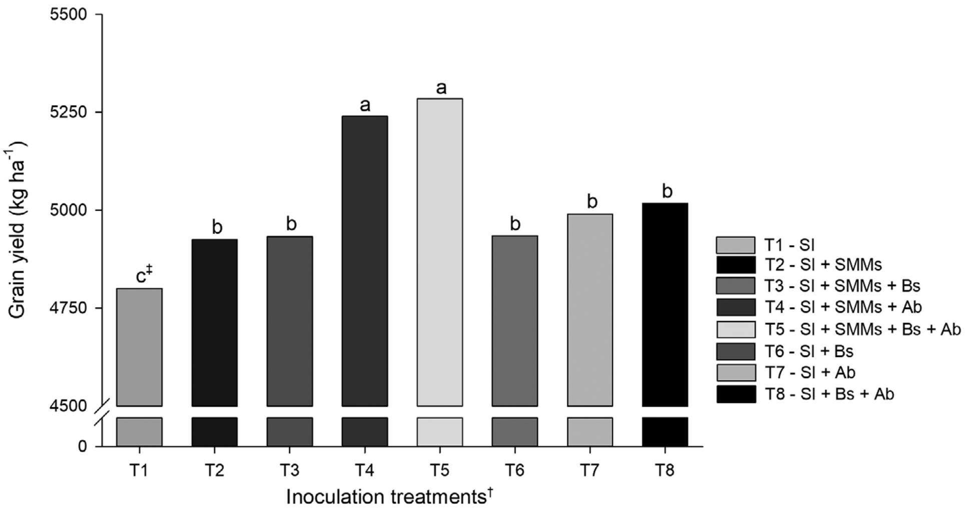

FIGURE 3 Average grain yield of two soybean cultivars that received different inoculation treatments in the field during two cropping seasons. † Inoculation treatments: SI, standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 on seeds; SMMs, application of secondary microbial metabolites extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889 on seeds; Bs, foliar-spray inoculation with *Bacillus subtilis* strain QST 713 at V_3 stage; Ab, foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V_4 stage. ‡ Means followed by different letters differ from each other by least significant difference test at $p \leq .05$

volume, and surface area may be appropriate for biometric studies, the evaluation of root activity is more appropriate when the nutrient uptake is involved (Gockele et al., 2014; Hoekstra et al., 2014).

The increased root activity induced by co-inoculation, especially within rows, is attributed to the stimulation of several physiological processes, including root hair elongation and cell multiplication in root meristems in the host plant (Oldroyd & Downie, 2008; Suzaki, Ito, & Kawaguchi, 2013). According to Schachtman and Goodger (2008), greater exploitation of the soil by the plant is clearly important for its establishment and for the tolerance to biotic and abiotic stresses.

An increase in grain yield by 485 kg ha^{-1} was observed in our experiments in plants co-inoculated with SI + SMMs + *A. brasilense*. This treatment increased the agronomic efficiency by 10% compared to SI-treated plants. This result corroborates Hungria et al. (2013) and Hungria and Mendes (2015), who observed a similar increase in grain yield when comparing the individual use of *Bradyrhizobium* strains and co-inoculation of *Bradyrhizobium* strains with *A. brasilense*, and with Marks et al. (2013), who observed an average increase in yield of 4.8% with the addition of SMMs from *B. diazoefficiens* strain USDA 110. However, our study shows clearly that even higher yields can be obtained if SMMs and *A. brasilense* are added together.

Our results demonstrated the synergistic effects of co-inoculation with rhizobial SMMs together with both rhizobia and PGPR, which promoted greater root development, activity, and nodulation. Co-inoculation was agronomically efficient and beneficial to the crop, which resulted in an increase in grain yield of up to 10% compared to SI-treated plants. Such improvements favor agricultural sustainability, bringing economic, social, and environmental benefits, especially in tropical regions.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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ORCID

Luiz Gustavo Moretti
https://orcid.org/0000-0001-7693-7826
Carlos A. C. Crusciol
https://orcid.org/0000-0003-4673-1071

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