



Bacterial Consortium and Microbial Metabolites Increase Grain Quality and Soybean Yield

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Abstract

The effects of *Bradyrhizobium* inoculation on soybean growth and productivity are well known, but plant responses to consortia of other beneficial microbes and microbial molecules have not yet been well explored. Therefore, the main aim of this study was to evaluate the effect of different combinations of beneficial bacteria with and without microbial secondary metabolites (MSM) on two soybean cultivars in three cropping seasons under tropical field conditions. The bacterial consortia consisted of *Bradyrhizobium japonicum* (strain SEMIA 5079) plus *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) inoculated with different combinations of *Bacillus subtilis* (strain QST 713), *Azospirillum brasilense* (strains Ab-V5 and Ab-V6), and MSM (metabolites enriched in lipo-chitooligosaccharides (LCOs) extracted from *B. diazoefficiens* (strain USDA 110) and from *Rhizobium tropici* (strain CIAT 889)). Standard inoculation of *Bradyrhizobium* combined with *Azospirillum brasilense* and microbial secondary metabolites increased leaf total N (7.1%), total P (11.1%), and N-ureide (16.5%); nodule number (NN, 26%) and dry weight (NDW, 22%); root (RDW, 15.4%) and shoot dry weight (SDW, 6%); 100-seed weight (3.7%); grain yield (up to 516 kg ha⁻¹); grain crude protein concentration (2.4%); and the agronomic efficiency index (AEI) (11%). Inoculation with bacterial consortia and metabolites increased grain yield and quality, representing a promising technology for sustainable soybean cropping in tropical regions.

Keywords *Azospirillum brasilense* · *Bacillus subtilis* · *Bradyrhizobium diazoefficiens* · *Bradyrhizobium japonicum* · Microbial metabolites · *Rhizobium tropici*

Highlights

- Soybean nodulation and leaf total N, total P, and N-ureide concentrations increased after application of a bacterial consortium and bacterial secondary metabolites.
- Standard inoculation of *Bradyrhizobium* combined with *Azospirillum brasilense* and microbial secondary metabolites increased grain yield by up to 11% and soybean grain quality.
- Inoculation with a bacterial consortium and metabolites can promote sustainable soybean cultivation.

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Abbreviations

PGPR	Plant growth-promoting rhizobacteria
MSM	Microbial secondary metabolites
BNF	Biological nitrogen fixation
AEI	Agronomic efficiency index
SEMIA	Section of Agricultural Microbiology
USDA	United States Department of Agriculture
CIAT	International Center for Tropical Agriculture
CPAC	Embrapa Cerrados
CNPSO	Embrapa Soybean
LCOs	Lipo-chitooligosaccharides
CEC	Cation exchange capacity
MPN	Most probable number
CFU	Colony-forming units
SI	Standard inoculation

1 Introduction

The sustainable development of agricultural ecosystems requires improvements in crop yield and quality. The adoption of sustainable production systems has increased considerably in recent years, driven mainly by society's demand for high-quality food whose production results in low environmental impacts (Santos et al. 2019). Legumes encompass important grain crops, the economically most important being soybean (*Glycine max* [L.] Merrill), which, due to its high protein concentration, is used for animal and human consumption (Sugiyama et al. 2014). In the 2019–2020 cropping season, it is expected that approximately 338 million Mg of soybean grains will be produced, and 123 million ha will be cultivated worldwide (USDA 2020), among which Brazil has been moving towards becoming the largest global producer (Brasil 2019).

Sustainable soybean production in the tropics has been successful mainly due to the inoculation of rhizobial strains that perform the biological nitrogen fixation (BNF) process, providing the nitrogen (N) required for the plants and ensuring high yields without N fertilizer (Cerezini et al. 2016; Moretti et al. 2018; Shi et al. 2019; Vanlauwe et al. 2019). In Brazil, these diazotrophic bacteria are capable not only of supplying up to 300 kg ha⁻¹ of N to the crop but also of releasing 20 to 30 kg ha⁻¹ of N residues into the soil (Hungria et al. 2006; Hungria and Mendes 2015).

Strategies to improve *Bradyrhizobium*-soybean symbiosis and increase the effectiveness of BNF have been extensively surveyed (Hungria et al. 2006; Chibeba et al. 2015; Moretti et al. 2018; Vanlauwe et al. 2019). Among the strategies, the use of a bacterial consortium of *Bradyrhizobium* with plant growth-promoting rhizobacteria (PGPR) has been identified as beneficial for promoting BNF and improving crop performance, resulting in increases in grain yield (Marks et al. 2013, 2015; Moretti et al. 2020). Bacteria belonging to the genus

Azospirillum are the best studied and most widely employed PGPR for agriculture worldwide (Fukami et al. 2018a). In Brazil, *Azospirillum brasilense* strains Ab-V5 and Ab-V6 have been broadly used in commercial inoculants for grain crops, including both legumes and nonlegumes (Hungria et al. 2010, 2016; Fukami et al. 2018a; Santos et al. 2019).

A consortium of rhizobia and *Azospirillum* is feasible to improve grain yield (Hungria et al. 2013; Hungria and Mendes 2015), the tolerance of biotic stresses, usually by improving the plant intrinsic tolerance against pathogens (Bashan and de-Bashan 2010; Cerezini et al. 2016), and attenuate damages caused by abiotic stresses, such as salinity and drought (Fukami et al. 2018a, 2018b). Another important application of microorganisms is as biocontrol agents, which are particularly important when resistance to fungicides has been developed (Leroux et al. 2002; Standish et al. 2015). In this context, *Bacillus subtilis* has been used as a biological fungicide that can induce systemic acquired resistance and release biocide molecules that will provide biocontrol against several plant pathogens (Nicholson 2002; Araújo et al. 2005; Sansinenea and Ortiz 2011).

The symbiotic interaction between rhizobia and host legumes to establish the BNF process involves an intense exchange of signals between partners. One of these signals is created by lipo-chitooligosaccharides (LCOs), also known as nodulation (*Nod*) factors (Lerouge et al. 1990), which are secondary metabolites essential for communication and establishment of rhizobia-legume symbiosis (Cullimore et al. 2001; Gough 2003). The structural arrangement of LCOs is diverse, with up to 60 known structures (D'Haese and Holsters 2002), and is dependent on the bacterial species and the environmental conditions (del Cerro et al. 2015). Although microbial secondary metabolites (MSM) do not act directly on the growth and development of the host plant, there are reports showing that they can stimulate symbiosis and promote plant growth (Dardanelli et al. 2008; Marks et al. 2013, 2015). However, to date, the effect of beneficial microbes combined with BNF and biological pathogen control properties as well as MSM under field conditions has not received proper attention.

The use of a bacterial consortium with different beneficial properties and microbial metabolites acting in different biological processes may represent a simple, inexpensive, and sustainable strategy to improve plant performance, quality, and yield. We hypothesized that this practice could increase not only soybean yield but also improve grain quality by increasing the crude protein and oil in grains. Therefore, in this study, we determined the effect of a bacterial consortium consisting of nitrogen-fixing *Bradyrhizobium* in different combinations with plant growth-promoting *Azospirillum brasilense*, the biocontrol agent *Bacillus subtilis*, and MSM (rhizobial metabolites enriched in lipo-chitooligosaccharides, LCOs) on soybean growth, nutrient uptake, and grain yield and quality under tropical field conditions.

2 Materials and Methods

2.1 Site Description

The study consisted of three field experiments carried out under rainfed conditions during the 2016–2017, 2017–2018, and 2018–2019 cropping seasons, at the Lageado Experimental Farm of São Paulo State University in Botucatu, São Paulo State, Brazil (48° 26' W, 22° 51' S, 786 m altitude) (Supplementary Figure 1). The soil is classified as clayey textural class, kaolinitic, thermic Typic Haplorthox (Soil Survey Staff 2014). According to Köppen's classification (Alvares et al. 2013), the climate is Cwa, which corresponds to a humid subtropical zone, with dry winter and hot summer. The long-term (1956–2020) average annual temperatures are 26.1 °C maximum and 15.3 °C minimum, with a 20.7 °C average. The average annual rainfall is approximately 1360 mm (CEPAGRI 2020). Climatological data during the experiments are presented in Fig. 1.

The physical-chemical and biological properties (0.00–0.20-m depth) are shown in Table 1. The physical attributes were determined according to Donagema et al. (2017) and the chemical properties were determined according to van Raij et al. (2001). The autochthonous bacterial population capable of soybean nodulation was estimated by the most probable number (MPN) using soybean plants, according to O'Hara et al. (2016). Dolomitic lime (28% of calcium oxide—CaO, 18% of magnesium oxide—MgO, and 81% of calcium carbonate equivalents—%E_{CaCO₃}) was applied 60 days prior to installing the experiment to increase the base saturation in the topsoil (0.00–0.20-m depth) to 70%, according to the methodology of Quaggio and van Raij (1997).

2.2 Experimental Design and Treatments

A randomized complete block design using two soybean growth types, conventional cultivar BRS 317 (Embrapa) with a determinate growth type and transgenic cultivar TMG 1264 RR (Tropical Breeding & Genetics) with an indeterminate growth type, and eight bacterial consortium treatments with four replicates was employed during three cropping seasons.

The inoculation treatments were as follows: (i) standard inoculation (SI) with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) via seed; (ii) SI via seed + the application of MSM extracted from *B. diazoefficiens* (strain USDA 110) + *Rhizobium tropici* (strain CIAT 889) via seed; (iii) SI via seed + MSM via seed + foliar-spray inoculation of soybean plants at the V₃ stage (Fehr and Caviness 1977) with *Bacillus subtilis* (strain QST 713); (iv) SI via seed + MSM via seed + foliar-spray inoculation of soybean plants at the V₄ stage (Fehr and Caviness 1977) with *Azospirillum brasilense* (strains Ab-V5+ Ab-V6); (v) SI via seed + MSM via seed + foliar-spray

inoculation of soybean plants at the V₃ stage with *B. subtilis* + foliar-spray inoculation of soybean plants at the V₄ stage with *A. brasilense*; (vi) SI via seed + foliar-spray inoculation of soybean plants at the V₃ stage with *B. subtilis*; (vii) SI via seed + foliar-spray inoculation of soybean plants at the V₄ stage with *A. brasilense*; and (viii) SI via seed + foliar-spray inoculation of soybean plants at the V₃ stage with *B. subtilis* + foliar-spray inoculation of soybean plants at the V₄ stage with *A. brasilense*.

2.3 Microbial Inoculants and Secondary Metabolites

Liquid inoculants containing *B. japonicum* strain SEMIA 5079 (=CPAC 15, =CNPSO 07) and *B. diazoefficiens* strain SEMIA 5080 (=CPAC 7, =CNPSO 06) 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. The MSM enriched in lipo-chitooligosaccharides (LCOs) were extracted from *R. tropici* strain CIAT 899 (= CNPSO 103, = SEMIA 4077) and *B. diazoefficiens* strain USDA 110 (=CNPSO 56) and produced as described before (Marks et al. 2013, 2015) by Embrapa Soybean. Prior to sowing, lyophilized metabolites were resuspended in a mixture of acetonitrile and water (20%) as previously described (Marks et al. 2015). The concentration was adjusted to 1.0 mL L⁻¹, corresponding to approximately 10⁻⁸ M, and applied in a volume of 200 mL per 50 kg of seeds.

For foliar spraying at the V₃ soybean phenological stage (Fehr and Caviness 1977), 3 L of inoculant containing *B. subtilis* strain QST 713 at a concentration of 1×10^9 CFUs mL⁻¹ was diluted in 200 L ha⁻¹ in water. For foliar spraying at the V₄ soybean phenological stage (Fehr and Caviness 1977), 300 mL of inoculant containing *A. brasilense* strain Ab-V5 (=CNPSO 2083) and strain Ab-V6 (=CNPSO 2084) at a concentration of 2×10^8 CFUs mL⁻¹ was diluted in a total volume of 150 L ha⁻¹ in water. Foliar sprays containing the two *A. brasilense* strains and *B. subtilis* were always applied late in the afternoon (5:00 pm).

2.4 Agronomic Practices and Measures

In the three growing seasons, soybeans were sown after black oats (*Avena strigosa* Schreb.) that had been cropped in the winter as a mulch crop in a no-till system, providing an average of 4.5 Mg ha⁻¹ straw in a dry land area (without irrigation). The treatments were applied to the same plots in all growing seasons. Each plot consisted of 10 rows that covered an area of 45 m² (10 m × 0.45 m). Plots were separated by 0.5-m-wide rows and 1.5-m-wide terraces to avoid cross-contamination from surface runoff containing bacteria or fertilizers that may occur as a consequence of heavy rainfall. In the three growing seasons, all plots were fertilized with 00-20-

Fig. 1 Rainfall and maximum and minimum air temperatures during the experimental period (2016–2019)

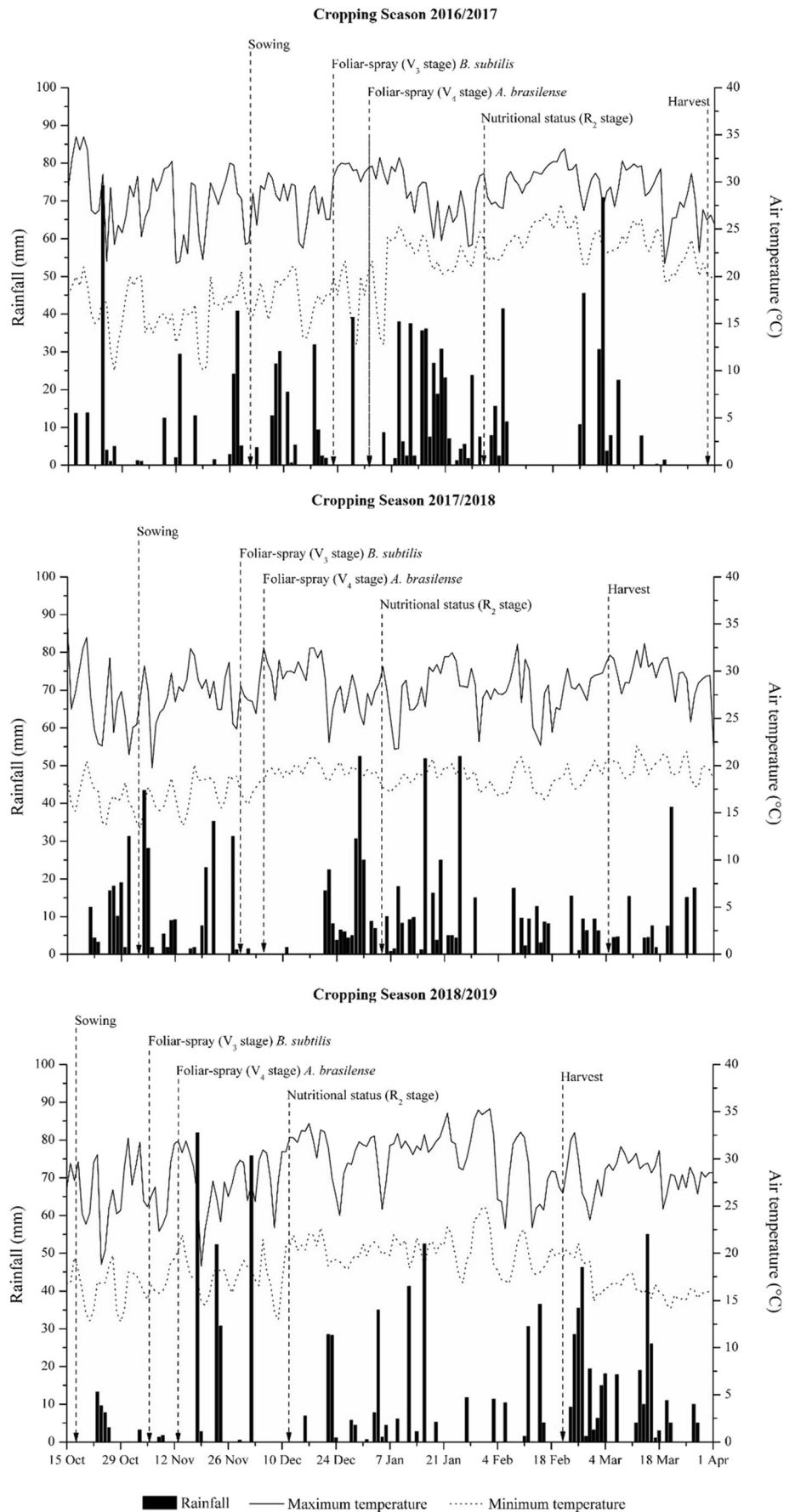


Table 1 Physical-chemical and biological attributes (0.00–0.20-m depth) before sowing the experiment. Botucatu, São Paulo, Brazil, 2016

Soil chemical attributes	Unit	Value	
Clay	g kg ⁻¹	502 ± 10 ^a	
Sand	g kg ⁻¹	117 ± 6	
Silt	g kg ⁻¹	281 ± 8	
Bulk density	g cm ⁻³	1.19 ± 0.1	
pH (CaCl ₂)	–	5.10 ± 0.1	
TOC ^b	g kg ⁻¹	15.2 ± 0.3	
Total N	g kg ⁻¹	1.00 ± 0.1	
P-available (Mehlich 1)	mg kg ⁻¹	57.0 ± 3.1	
Exchangeable	Ca ²⁺ (resin)	mmol _c kg ⁻¹	25.0 ± 1.2
	Mg ²⁺ (resin)	mmol _c kg ⁻¹	15.0 ± 0.9
	K ⁺ (resin)	mmol _c kg ⁻¹	3.90 ± 0.3
	Al ³⁺ (KCl)	mmol _c kg ⁻¹	2.00 ± 0.2
H + Al	mmol _c kg ⁻¹	42.0 ± 1.8	
S-SO ₄ ²⁻ (Ca(H ₂ PO ₄) ₂)	mg kg ⁻¹	4.90 ± 0.3	
B (Hot water)	mg kg ⁻¹	0.40 ± 0.1	
Cu (DTPA)	mg kg ⁻¹	8.80 ± 0.5	
Fe (DTPA)	mg kg ⁻¹	22.0 ± 1.3	
Mn (DTPA)	mg kg ⁻¹	26.2 ± 1.1	
Zn (DTPA)	mg kg ⁻¹	2.10 ± 0.3	
BS ^c	%	51.0 ± 1.9	
CEC ^d (pH 7.0)	mmol _c kg ⁻¹	86.0 ± 2.4	
MPN ^e	CFU ^f g ⁻¹	9.32 × 10 ⁴	

^a Means ± SE (standard error); ^b TOC, total organic carbon; ^c BS, base saturation; ^d CEC, cation exchange capacity; ^e MPN, most probable number; ^f CFU, colony-forming units

20 of N-P₂O₅-K₂O at 300 kg ha⁻¹. The seeds were treated with fungicides (carboxin + thiram at 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 amount of inoculant. Foliar-spray inoculations were performed by a tractor-mounted sprayer. In all treatments, foliar spray containing 20 g ha⁻¹ Mo (as Na₂MoO₄·2H₂O) and 2 g ha⁻¹ Co (as CoCl₂·6H₂O) was applied to plants at the V₄ stage (Fehr and Caviness 1977). Phytosanitary treatments were carried out according to the needs of and recommendations for the soybean crop (Embrapa 2013).

Plant nutritional status was evaluated at the R₂ phenological stage (Fehr and Caviness 1977) by collecting the third fully developed leaf and its petiole from 30 plants in each plot, according to Ambrosano et al. (1997). The material was used to determine the N, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), and boron (B) concentrations according to the methodology described by Malavolta et al. (1997). Additionally, at the full flowering (R₂) stage, dry leaves with petioles were used for the determination of the N-ureide

(allantoin and allantoic acids) concentration as described by Herridge and Giller (2016). Additionally, at the R₂ stage, five plants were harvested per plot and separated into shoots and roots according to the methodology described by Hungria et al. (2006). Roots were washed to remove substrate particles, and nodules were removed. Shoots, roots, and nodules were oven dried at 65 °C for 72 h, and from this material, the nodule number (NN), nodule dry weight (NDW), root dry weight (RDW), and shoot dry weight (SDW) were determined.

At physiological maturity (R₈) (Fehr and Caviness 1977), 15 m² of plants from the central part of each plot was collected to estimate final population of plants, plant height, position of insertion of the first pod, numbers of branches and pods per plant, number of grains per pod, grain yield (13% moisture base), and 100-grain weight (13% moisture base). The agronomic efficiency index (AEI) was calculated according to the methodology described by Moreira et al. (2014). The AEI was determined using Eq. (1), where Y1 is the crop yield with SI and Y2 is the crop yield with the corresponding bacterial consortium:

$$\text{Agronomic efficiency index, \%} = \frac{Y2 \times 100}{Y1} \quad (1)$$

The ether extract (EE) concentration from the grains was based on the AOAC official method 920.39—diethyl ether, traditional Soxhlet extraction, and the crude protein (CP) concentration was estimated using the AOAC official method 2001.11 (AOAC 2019). For the CP, the N-concentration was multiplied by a factor 6.25. The N-concentration from the grains samples was extracted using H₂SO₄, and the concentration was determined using the Kjeldahl distillation method (Singh et al. 2020). The CP was calculated using Eq. (2):

$$\text{Crude Protein, \%} = \% \text{Kjeldahl N} \times 6.25 \quad (2)$$

2.5 Statistical Analysis

The data were initially analyzed using the Shapiro-Wilk’s test (Shapiro and Wilk 1965) for normality and Levene’s homoscedasticity test (Levene 1960), both at 0.05 probability ($p < 0.05$), based on the UNIVARIATE procedure of SAS version 9.4 (SAS Institute 2015). The data were also tested for sphericity by the Bartlett’s test (Tobias and Carlson 1969) using FACTOR procedure of SAS version 9.4 (SAS Institute 2015). The results indicated that all data were distributed normally ($W \geq 0.90$) and no sphericity. All data were then analyzed using the linear mixed effect model by PROC MIXED procedure of SAS and Satterthwaite approximation to determine the denominator 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 were separated using the probability of differences option (PDIFF). The means were compared using the LSD test. The main factor and interactive effects were considered statistically significant at $p \leq 0.05$.

3 Results

The shoot N, P, and N-ureides concentrations significantly increased in all plants co-inoculated with *Bradyrhizobium* spp. strains SEMIA 5079 and SEMIA 5080 and *A. brasilense* strains Ab-V5 and Ab-V6, while the K, Ca, Mg, S, Cu, Fe, Zn, Mn, and B in shoots concentrations were not affected by any treatment (Table 2). The concentrations of all nutrients were adequate for soybean, according to the concentrations proposed by TPS (2013). Plants co-inoculated with *Bradyrhizobium* and *A. brasilense* plus MSM resulted in increases of up to 26% in nodule number (NN), 22% in nodule dry weight (NDW), 15.4% in root dry weight (RDW), and 6% in shoot dry weight (SDW) when compared to the standard inoculation (SI) exclusively with *Bradyrhizobium* (Table 3).

At physiological maturity, positive effects on plant height were observed in plants co-inoculated with *Bradyrhizobium* and *A. brasilense* and in the number of pods of plants that were co-inoculated and received rhizobial metabolites (SI+ MSM + *A. brasilense*). Overall, significant effects were not observed in the final population of plants (mean = 286,500 plant ha⁻¹), the position of insertion of the first pod (mean = 13 cm), the number of branches per plant (mean = 3), or the number of grains per pod (mean = 2.2) (data not shown).

Again, positive effects on the 100-grain weight and grain yield were observed in plants inoculated with SI+ MSM + *A. brasilense* strains. In addition, there was an increase of up to 3.7% for the 100-seed weight and 2.4% for the crude protein concentrations, promoting an increase of up to 516 kg ha⁻¹ of grain and 11% of AEI when compared to plants inoculated only with *Bradyrhizobium* (Fig. 2). However, no treatment differences were found for the ether extract concentrations in seeds.

It is worth mentioning that in the 2017–2018 cropping season, higher averages were obtained for most parameters, possibly due to the more favorable climatic conditions during this season (Fig. 2); nevertheless, no interaction with the factors (cultivar or inoculation) was observed. Additionally, it is important to emphasize that plants inoculated with *B. subtilis* may not have demonstrated their full potential because during the three cropping seasons; the soybean crops had no suppression in relation to a high infestation of pests and disease inoculum.

4 Discussion

This study aimed to determine the effects of a new generation of inoculants containing mixes of bacteria contributing to different processes and of bacterial metabolites on soybean plant growth and grain yield and quality to select a bacterial consortium able to improve sustainability and decrease the environmental impacts caused by N fertilization and pesticides.

Although it has been reported that the response to bacterial inoculants may vary with plant genotype (Wani et al. 1985; Penot et al. 1992), one reason for the variation is that the growth habit may influence the source-sink relationship of the plants, mainly because of the different hormonal balances and permanence of the alteration in the activity regime of the photoassimilate source (Taiz et al. 2017). In addition, changes in plant C allocation in association with BNF have been reported in several studies (Santachiara et al. 2017; Tamagno et al. 2018), where the soybean growth types influence not only phenology but also growth and allocation of biomass and N. However, in our study, the tested cultivars, one with a determinate growth habit and nontransgenic and another that was indeterminate and transgenic, showed similar responses to the treatments used. Kaschuk et al. (2016) also did not detect differences in symbiotic performance and grain yield of soybean with different growth habits, while Hungria et al. (2014) observed in a comparison of several parental and nearly isogenic transgenic soybean tolerant to glyphosate that although the transgenic trait negatively affected some BNF variables, over a 3-year period, these effects had no significant impact on soybean grain yield.

The results obtained revealed important increases in leaf total N, total P, and N-ureide concentrations in plants inoculated with SI + MSM + *A. brasilense*. The higher P uptake is possibly due to the greater development of the soybean root system after inoculation. In a previous study, Moretti et al. (2020) reported the capacity of the *Azospirillum* strains Ab-V5 and Ab-V6 to promote greater uptake capacity of soybean plants due to the greater development of the root system, such as length, volume, surface area, and smaller diameter of roots (0.01–0.5 mm). D'Angioli et al. (2017) reported positive correlations between the P supply and the exudation of carboxylate in a corn root system stimulated by *A. brasilense* strains Ab-V5 and Ab-V6, which was correlated with greater length and root area. This indicates positive feedback in which the inoculation of *A. brasilense* stimulates root carboxylate exudation, influencing the microbial community of the rhizosphere.

The bacterial consortium of *Bradyrhizobium*, *Azospirillum*, and MSM improved leaf total N by up to 7.1% and N-ureide concentrations by 16.5%. In legumes such as soybean, the majority of N from BNF is transported in the xylem sap as N-ureides that will accumulate in different organ tissues and in different concentrations (Baral et al. 2016), such that their

Table 2 Nutrients and N-ureide (U) concentrations in leaves with petioles of two soybean cultivars that received different bacterial consortia during three cropping seasons (2016–2019), Botucatu, São Paulo, Brazil

Factor	N g kg ⁻¹	P	K	Ca	Mg	S	Cu mg kg ⁻¹	Fe	Zn	Mn	B	U μ mol g ⁻¹
Inoculation (In ^a)												
Standard inoculation (SI)	42.0±0.8 b ^b	2.7±0.1 b	24±0.3	10±0.5	3.5±0.1	2.7±0.2	9.1±0.3	133±2.6	26±1.2	52±1.5	58±1.3	10.3±0.4 b
SI + MSM	42.2±0.8 b	2.7±0.1 b	24±0.2	10±0.4	3.6±0.2	2.8±0.2	8.8±0.3	134±2.9	26±1.2	53±1.3	60±1.6	10.3±0.3 b
SI + MSM + <i>B. subtilis</i> (Bs)	42.2±0.7 b	2.7±0.1 b	24±0.2	11±0.2	3.5±0.1	2.6±0.3	8.9±0.3	136±1.7	26±1.2	52±1.3	59±1.5	10.8±0.4 b
SI + MSM + <i>A. brasilense</i> (Ab)	45.0±0.3 a	3.0±0.1 a	23±0.2	10±0.4	3.7±0.2	3.0±0.1	8.8±0.2	136±2.8	26±1.1	54±1.2	58±1.6	11.9±0.4 a
SI + MSM + Bs + Ab	45.0±0.4 a	3.0±0.1 a	23±0.3	10±0.4	3.5±0.2	3.0±0.1	9.1±0.1	134±1.9	26±1.2	54±1.2	60±1.4	12.0±0.3 a
SI + Bs	42.4±0.8 b	2.7±0.1 b	23±0.2	10±0.5	3.6±0.2	2.8±0.2	8.8±0.3	136±1.7	26±1.1	53±1.3	59±1.7	10.4±0.3 b
SI + Ab	44.8±0.6 a	3.0±0.1 a	23±0.3	11±0.2	3.7±0.2	2.7±0.2	8.8±0.3	135±2.8	26±1.0	54±1.4	59±1.5	11.8±0.3 a
SI + Bs + Ab	44.9±0.7 a	3.0±0.1 a	23±0.2	11±0.2	3.7±0.2	2.7±0.2	9.2±0.1	133±2.6	26±1.2	53±1.3	58±1.6	11.8±0.4 a
Cultivar (Cv)												
BRS 317	43.3±0.6	3.0±0.1	23±0.3	11±0.3	3.8±0.1	2.7±0.2	8.4±0.2	135±2.7	26±1.2	55±1.3	58±1.4	10.6±0.4
TMG 1264 RR	43.5±0.3	2.9±0.1	24±0.3	10±0.4	3.9±0.2	2.8±0.3	8.8±0.3	135±2.6	26±1.1	53±1.2	59±1.3	10.8±0.3
Cropping season (CS)												
2016–2017	43.4±0.3	2.8±0.1	24±0.3	11±0.2	3.7±0.2	2.8±0.2	8.5±0.2	136±2.7	26±1.2	53±1.3	58±1.4	10.4±0.3
2017–2018	43.4±0.5	3.0±0.1	23±0.4	11±0.3	3.8±0.1	2.7±0.3	8.6±0.3	134±2.6	26±1.2	52±1.3	59±1.3	10.6±0.3
2018–2019	43.3±0.3	2.9±0.1	24±0.3	11±0.3	3.6±0.3	2.8±0.2	8.8±0.2	135±2.5	26±1.1	54±1.2	58±1.4	10.2±0.4
ANOVA (F probability)												
In	***	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
Cv	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
In × Cv	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
In × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cv × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
In × Cv × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

^aInoculation treatments: SI = standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 + *B. diazoefficiens* strain SEMIA 5080 inoculated together on seeds; MSM = application of microbial secondary 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 V₃ stage; *A. brasilense* = foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V₄ stage. ^bThe statistical model used was the linear mixed effect (LME). Means ± SE (standard error) followed by the same letter do not differ (*ns*) by the LSD test (Fisher's least significant difference) at **p* ≤ 0.05 and ***p* ≤ 0.01 probability

Table 3 Nodule number (NN), nodule dry weight (NDW), root dry weight (RDW), shoot dry weight (SDW), plant height (PH), pods (P), weight of 100-seeds (W100), crude protein (CP), and ether extract (EE) concentration of two soybean cultivars that received different bacterial consortia during three cropping seasons (2016–2019). Botucatu, São Paulo, Brazil

Factor	NN no. plant ⁻¹	NDW mg plant ⁻¹	RDW g plant ⁻¹	SDW	PH cm	P no. plant ⁻¹	W100 g	CP g kg ⁻¹	EE
Inoculation (In ^a)									
Standard inoculation (SI)	50 ± 4 b ^b	205 ± 6 b	5.2 ± 0.4 b	18.2 ± 0.4 b	82 ± 3 b	46 ± 3 b	16.4 ± 0.2 b	420 ± 3 b	208 ± 3
SI + MSM	54 ± 2 b	216 ± 7 b	5.2 ± 0.3 b	18.3 ± 0.3 b	82 ± 2 b	46 ± 2 b	16.6 ± 0.1 b	423 ± 2 b	211 ± 2
SI + MSM + <i>B. subtilis</i> (Bs)	54 ± 3 b	215 ± 6 b	5.3 ± 0.3 b	18.4 ± 0.3 b	82 ± 2 b	47 ± 2 b	16.7 ± 0.1 b	424 ± 2 b	212 ± 3
SI + MSM + <i>A. brasilense</i> (Ab)	61 ± 2 a	246 ± 5 a	5.9 ± 0.2 a	19.0 ± 0.2 a	87 ± 1 a	52 ± 2 a	17.0 ± 0.1 a	430 ± 2 a	210 ± 3
SI + MSM + Bs + Ab	63 ± 3 a	250 ± 4 a	6.0 ± 0.2 a	19.3 ± 0.2 a	88 ± 2 a	54 ± 3 a	17.0 ± 0.1 a	430 ± 2 a	210 ± 2
SI + Bs	52 ± 5 b	212 ± 6 b	5.3 ± 0.3 b	18.4 ± 0.2 b	83 ± 2 b	46 ± 2 b	16.4 ± 0.2 b	424 ± 2 b	209 ± 3
SI + Ab	60 ± 2 a	240 ± 6 a	5.8 ± 0.1 a	18.9 ± 0.1 a	87 ± 1 a	47 ± 2 b	16.6 ± 0.1 b	424 ± 2 b	209 ± 3
SI + Bs + Ab	60 ± 2 a	242 ± 5 a	5.8 ± 0.1 a	19.0 ± 0.2 a	87 ± 1 a	47 ± 2 b	16.5 ± 0.1 b	425 ± 2 b	210 ± 2
Cultivar (Cv)									
BRS 317	57 ± 2	230 ± 4	5.5 ± 0.3	18.5 ± 0.3	84 ± 3	49 ± 3	16.6 ± 0.2	423 ± 4	209 ± 4
TMG 1264 RR	55 ± 3	225 ± 5	5.4 ± 0.2	18.9 ± 0.4	86 ± 2	48 ± 3	16.8 ± 0.3	424 ± 3	210 ± 3
Cropping season (CS)									
2016–2017	55 ± 3	227 ± 4	5.6 ± 0.3	18.3 ± 0.4 b	83 ± 2 b	47 ± 2 b	16.3 ± 0.3 b	427 ± 3	210 ± 3
2017–2018	58 ± 3	231 ± 5	5.5 ± 0.3	18.9 ± 0.1 a	87 ± 1 a	52 ± 2 a	16.9 ± 0.1 a	429 ± 4	211 ± 3
2018–2019	56 ± 2	224 ± 5	5.6 ± 0.2	18.4 ± 0.3 b	84 ± 1 b	46 ± 2 b	16.5 ± 0.2 b	423 ± 5	210 ± 4
ANOVA (F probability)									
In	**	**	*	*	*	*	*	*	ns
Cv	ns	ns	ns	ns	ns	ns	ns	ns	ns
CS	ns	ns	ns	*	*	*	*	ns	ns
In × Cv	ns	ns	ns	ns	ns	ns	ns	ns	ns
In × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cv × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns
In × Cv × CS	ns	ns	ns	ns	ns	ns	ns	ns	ns

^aInoculation treatments: SI = standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 + *Bradyrhizobium diazoefficiens* strain SEMIA 5080 inoculated together on seeds; MSM = application of microbial secondary 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 V₃ stage; *Azospirillum brasilense* = foliar-spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at V₄ stage. ^bThe statistical model used was the linear mixed effect (LME). Means ± SE (standard error) followed by the same letter do not differ (*ns*) by the LSD test (Fisher's least significant difference) at **p* ≤ 0.05 and ***p* ≤ 0.01 probability

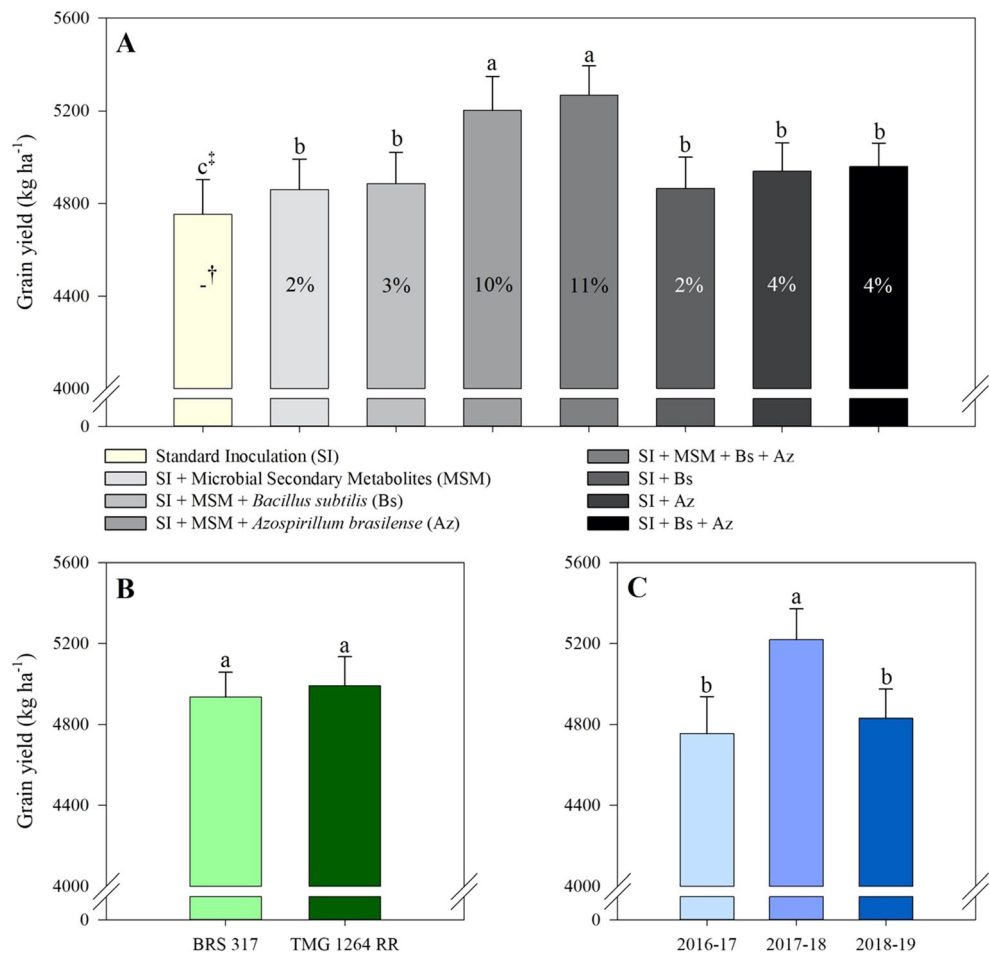
concentration has become a feasible method for the quantification of the contribution of BNF (Herridge and Giller 2016). Since N is a constituent of plant cell components, and responsible for proteins, amino acids, and nucleic acids synthesis, its deficiency limits grain yield; nevertheless, the BNF can provide the N required by legume plants (Taiz et al. 2017; Oliveira et al. 2019; Acuña et al. 2020).

According to Khan et al. (2008), the growth promotion of plants inoculated with MSM could be at least partially related to the fact that LCOs indirectly affect photosynthesis and accelerate growth by stimulating mitotic activity in the meristematic tissue of leaves. It can also be inferred that LCOs promote the suppression of innate immune responses, which possibly facilitates microbial interactions (Liang et al. 2013). Therefore, it is likely that LCOs have a broad spectrum of action in regulating plant

growth, in addition to their primary function in the nodulation of soybean plants.

We hypothesize that the beneficial relationships between strains of *Bradyrhizobium* + strains of *Azospirillum* and MSM observed in this study in the nodulation of soybean plants are promoted by the following facts: (i) *Azospirillum* strains Ab-V5 and Ab-V6 carry *nif* and *fix* genes demonstrated in draft genome sequences by Hungria et al. (2018) and produce high amounts of phytohormones, with an emphasis on indole acetic acid (IAA) (Fukami et al. 2018c); (ii) the LCOs can affect various physiological processes of the host plant, inducing, for example, root hair deformation, expression of host *nod* genes essential for infection, infection thread formation, and cell division in some root cortical cells (Schlaman et al. 1997); (iii) as reported by Massoud et al. (2009), a bacterial consortium may promote greater nitrogenase activity and increase

Fig. 2 Average soybean grain yield as a function bacterial consortia (A), in two soybean cultivars (B), and in the field during three cropping seasons (C). †Agronomic efficiency index (AEI). ‡The statistical model used was the linear mixed effect (LME). Means ± SE (standard error) followed by different letters differ from each other by LSD test (Fisher’s least significant difference) at $p \leq 0.05$. There was no statistical interaction between bacterial consortia, cultivar, or cropping season



the availability of macronutrients, in addition to plant growth, resulting in greater productivity compared to single inoculation.

The grain yield increase with the bacterial consortium and bacterial metabolites when compared to the treatment inoculated exclusively with *Bradyrhizobium* reached 516 kg ha⁻¹, an AEI equivalent of 11%, in agreement with results obtained by Hungria et al. (2013), who observed a similar increase in grain yield when comparing the individual use of *Bradyrhizobium* strains with the use of a bacterial consortium with *A. brasilense*. Marks et al. (2013) obtained an average increase of 4.8% in soybean grain yield with the addition of MSM of *B. diazoefficiens* (USDA 110). Therefore, our study confirms that even higher yields can be obtained if MSM and *A. brasilense* are combined with *Bradyrhizobium*.

Previous reports have also shown that the application of *Azospirillum* influences the crude protein concentration in several crops, such as rice (*Oryza sativa*) (Omar et al. 2002), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) (Ozturk et al. 2003), maize (*Zea mays*) (Nadeem et al. 2007), sunflower (*Helianthus annuus*) (Stefan et al. 2013), and safflower

(*Carthamus tinctorius*) (Nosheen et al. 2016). However, studies evaluating the effect of a bacterial consortium with PGPR and bacterial metabolites on soybean grain quality are still needed.

In our study, the bacterial consortium of *Bradyrhizobium* strains + *A. brasilense* strains with MSM increased crude protein concentration by up to 2.4%. According to Fukami et al. (2018a, 2018c), one main mechanism by which *Azospirillum* promotes plant growth is the synthesis of phytohormones such as auxin, cytokines, and gibberellin, which are closely linked to N signaling. Lone et al. (2005) reported that phytohormones are the main drivers of protein changes and can improve not only yield but also the quality of oilseed crops. Increases in the synthesis of phytohormones may stimulate the biosynthesis of amino acids and the accumulation of protein in grains (Greef 1994). Amino acid synthesis is an important feature of PGPR, and amino acids synthesized by PGPR include glutamic acid, lysine, valine, serine, isoleucine, and leucine (Babalola 2010), which are essential components in the human and animal food base (Karr-Lilienthal et al. 2004).

5 Conclusion

Bacterial consortia with standard inoculation of *Bradyrhizobium* spp. combined with *A. brasilense* and with metabolites of *B. diazoefficiens* and *R. tropici* are agronomically efficient and beneficial for soybean nodulation and nitrogen and phosphorus nutrition, promoting increases in plant growth, grain yield, and protein concentration.

In addition, the results of this study underscore the importance of this strategy, which favors agricultural sustainability, bringing economic and environmental benefits, especially under tropical conditions, where the largest grain production and cultivated area are associated with soybean, culminating in increases N₂ fixation and grain yield and quality.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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