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BIOTECHNOLOGICAL POTENTIAL OF GROWTH-PROMOTING BACTERIA AND MICROBIAL METABOLITES IN SOYBEAN CROP

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I dedicate this thesis to my parents Anilson and Suzi, and to my grandfathers Laurindo and Laudelina, and Arnóbio and Maria (*in memory*).

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“Ama e faz o que quiseres.

Se calares, calarás com amor;

se gritares, gritarás com amor;

se corrigires, corrigirás com amor;

se perdoares, perdoarás com amor.

Se tiveres o amor enraizado em ti,

nenhuma coisa senão o amor serão os teus frutos”.

Santo Agostinho

ABSTRACT

The adoption of sustainable production systems has increased considerably in recent years, driven mainly by societal demand for the production of high-quality food under low environmental impact. For soybean (*Glycine max* [L.] Merrill), a broad range of beneficial effects of bacteria have been reported, including biological nitrogen fixation (BNF), plant pathogen biocontrol, plant growth development, and the production of compounds such as lipochitooligosaccharides (LCOs), which activate several physiological and biochemical mechanisms. Rhizobial inoculants and plant growth-promoting bacteria (PGPB) have been widely applied as biofertilizers to legume crops, but plant responses to consortia of other beneficial microbes and microbial molecules have not been comprehensively explored. Therefore, in this study we evaluated the effects of co-inoculating N_2-fixing *Bradyrhizobium japonicum* (strain SEMIA 5079) and *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) with or without foliar application of plant growth-promoting *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) and the biocontrol agent *Bacillus subtilis* (strain QST 713) and seed treatment with microbial secondary metabolites (MSM, rhizobial metabolites enriched in LCOs) on soybean. Experiments were conducted during two growing seasons under greenhouse conditions and three growing seasons under tropical field conditions with a short dry spell at the Lageado Experimental Farm of São Paulo State University (UNESP), Botucatu, São Paulo State, Brazil. We evaluated root system development (directly by optical reading and indirectly by applying rubidium nitrate, $^{85}RbNO_3$), nutritional status, N-ureides, photosynthetic pigments, gas exchange parameters, antioxidant enzyme activities, proline, nodulation, plant growth development, agronomic efficiency index, and soybean grain yield and quality. Combining the beneficial bacterial consortium comprising *Bradyrhizobium* spp. and *A. brasilense* strains with MSM application helped alleviate soybean water stress under field conditions, promoting nodulation, N-ureide content, root and shoot development, photosynthetic activity, the production of photoassimilates and, ultimately, grain filling. Taken together, the results suggest that this combination is an important biotechnological strategy to achieve sustainable food production and security.

Keywords: Antioxidant metabolism. *Azospirillum brasilense*. *Bacillus subtilis*. Biological nitrogen fixation. *Bradyrhizobium diazoefficiens*. *Bradyrhizobium japonicum. Glycine max. Rhizobium tropici.*

RESUMO

A adoção de sistemas de produção mais sustentáveis aumentaram consideravelmente nos últimos anos, impulsionados principalmente pela demanda da sociedade por alimentos com alta qualidade, cuja produção resulta em baixos impactos ambientais. Uma ampla gama de efeitos benéficos por bactérias é relatada na cultura da soja (*Glycine max* [L.] Merrill), incluindo a fixação biológica de nitrogênio (FBN), o biocontrole de patógenos, o desenvolvimento e crescimento, e a produção de compostos como lipo-chitooligossacarídeos (LCOs), ativando vários mecanismos fisiológicos e bioquímicos. Inoculantes rizobianos e bactérias promotoras de crescimento de plantas (BPCP) têm sido amplamente aplicados como biofertilizantes em cultivos de leguminosas, mas as respostas das plantas à consórcios com outros microrganismos benéficos e metabólitos microbianos secundários ainda não são bem explorados. Portanto, neste estudo avaliamos os efeitos da coinoculação por fixadores de N_2 - *Bradyrhizobium japonicum* (estirpe SEMIA 5079) e *Bradyrhizobium diazoefficiens* (estipe SEMIA 5080), com ou sem aplicação foliar do promotor de crescimento de plantas - *Azospirillum brasilense* (estipes Ab-V5 e Ab -V6), e o agente de biocontrole - *Bacillus subtilis* (estirpe QST 713), e com ou sem tratamento de sementes com metabólitos microbianos secundários (MMS, metabólitos rizobianos enriquecidos em lipo-chitooligossacarídeos (LCOs)) na soja durante duas safras em condições de casa de vegetação, e três safras sob condições de campo tropical com curto período de estiagem na Fazenda Experimental Lageado da Universidade Estadual Paulista (UNESP), Botucatu, Estado de São Paulo, Brasil. Neste estudo, avaliamos o desenvolvimento do sistema radicular (por método direto - leitura óptica e método indireto - aplicação de nitrato de rubídio, $^{85}RbNO_3$), estado nutricional, N-ureídeos, pigmentos fotossintéticos, trocas gasosas, atividades de enzimas antioxidantes, teor de prolina, nodulação, desenvolvimento e crescimento das plantas, índice de eficiência agronômica, rendimento e qualidade de grãos de soja. O consórcio bacteriano benéfico compreendendo *Bradyrhizobium* spp. e *A. brasilense* combinado com aplicação de MMS auxiliam no alívio do estresse hídrico da soja em condições de campo, garantindo nodulação, teor de N-ureídeos, desenvolvimento radicular e da parte aérea, melhora a atividade fotossintética da planta e a produção de fotoassimilados e, por fim, o enchimento dos grãos. Tomados em conjunto, os resultados representam uma estratégia biotecnológica importante para alcançar a produção e segurança alimentar sustentável

Palavras-chave: *Azospirillum brasilense*. *Bacillus subtilis*. *Bradyrhizobium diazoefficiens*. *Bradyrhizobium japonicum.* Fixação biológica de nitrogênio. *Glycine max.* Metabolismo antioxidante. *Rhizobium tropici.*

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SUMMARY

GENERAL INTRODUCTION

Symbiosis, like most pathogenic interactions, is characterized by complexity and specificity. Symbiotic processes are of enormous importance for global agricultural productivity and as models for fundamental studies of plant-microorganism interactions (MORETTI et al., 2018). A physiological understanding of the process of biological nitrogen fixation (BNF) and the factors that control it is of paramount importance for both researchers and farmers because such knowledge can support adjustments in crop management to increase nitrogen (N) use efficiency and crop productivity (FUKAMI et al., 2018).

In Brazil, BNF has been successfully exploited through the use of inoculants containing *Bradyrhizobium* spp. to meet the N requirements of soybean cultivation, generating savings for the country of approximately 15 billion dollars annually (HUNGRIA et al., 2015). These diazotrophic bacteria reduce atmospheric nitrogen (N_2) to ammonia (NH_3) through the nitrogenase enzyme complex and proliferate in a wide range of environments with different lifestyles. Depending on their strategies in agricultural systems, diazotrophic bacteria can be classified as symbiotic, associative or free-living (HUNGRIA et al., 2006).

BNF requires abundant energy, and therefore the agronomic significance of free-living N fixers is limited compared with symbiotic fixers. Free-living N fixers colonize plant surfaces and can even invade intercellular spaces but do not form specialized structures and cannot directly transfer NH_3 to the plant (KASCHUK et al., 2010). Thus, it has been suggested that the growth-promoting effects of free-living bacteria could be improved by mechanisms that do not involve BNF (CEREZINI et al., 2016). The entire symbiotic bacterium-plant association process begins with an intense exchange of molecular signals between the organisms involved. The host plant secretes chemotactic substances (sugars, amino acids and carboxylic acids), which stimulate the multiplication of bacteria in the rhizosphere and promote the adherence of rhizobia to the roots of plants (MORETTI et al., 2020).

Many microorganisms synthesize compounds that differ in chemical structure from those found in primary metabolism and that are not essential to the growth and reproduction of the organism (MARKS et al., 2015). This secondary metabolism comprises natural adaptation molecules that are involved in different functions than primary metabolism. The chemical structures of secondary metabolites include β-

lactams, cyclic peptides, non-protein amino acids, sugars and unusual nucleosides, unsaturated polyacetylenes, nitrile groups, cyclic triesters, terpenoids, and large rings of macrolides (MARKS et al., 2013). Despite not playing key roles in growth and development, microbial secondary metabolites are essential tools for communication and the establishment of rhizobia-legume symbiosis and function as antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, substrates or ligands for enzymes and antagonist and agonist receptors, biocontrol agents, and plant growth-promoting molecules (GOUGH; JACQUET, 2013).

We hypothesized that beneficial microbes and their metabolites increase soil exploration by the root system, photosynthetic activity, and plant nutrition and development and alleviate water stress in soybean by increasing the intrinsic tolerance of soybean plants to abiotic stresses, thereby improving yield. To test this hypothesis, we evaluated root system activity and development, photosynthetic pigments, gas exchange parameters, antioxidant enzyme activities, proline content, nodulation, plant growth development and grain yield in soybean inoculated with beneficial bacterial consortia (N_2-fixing *Bradyrhizobium* spp. with different combinations of *Azospirillum brasilense* strains, *Bacillus subtilis*, and rhizobial metabolites) during two growing seasons under greenhouse conditions and three growing seasons under tropical field conditions.

CHAPTER 1
EFFECTS OF GROWTH-PROMOTING BACTERIA ON SOYBEAN ROOT ACTIVITY, PLANT DEVELOPMENT, AND YIELD

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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%.

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.

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.

1.1 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).

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.

1.2 MATERIALS AND METHODS

Experimental site

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 Köppen's classification (Alvares, Stape, Sentelhas, Gonçalves, & Sparovek, 2013), the climate is Cwa, which corresponds to subtropical 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 15.3ºC, with an average of 20.7ºC. Average annual rainfall is approximately 1360 mm (Unicamp, 2019). Climatological data during the experiments are presented in Figure 1.

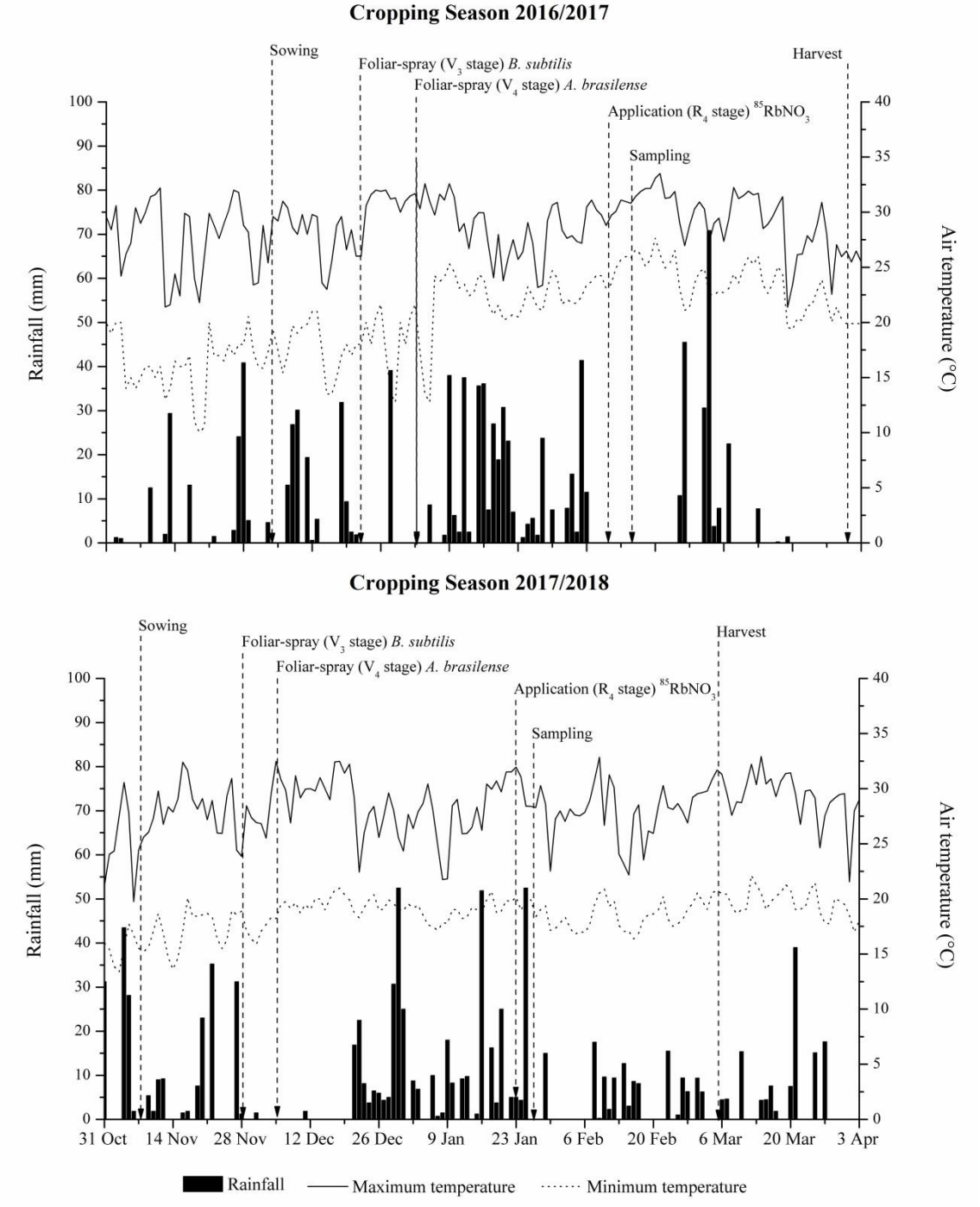

Figure 1. Rainfall and maximum and minimum air temperatures during the experimental period. Botucatu, São Paulo State, Brazil, 2016–2018.

Soil chemical attributes (0.0 - 0.2 m) were determined according to van Raij et al. (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: 22 mg kg^{-1}, Cu: 8.8 mg kg^{-1}, Mn: 26.2 mg kg^{-1}, Zn: 2 mg kg^{-1}, B: 0.4 mg kg^{-1}, base saturation: 51%. Dolomit 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.

Treatments and experimental design

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

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.

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 andWinRHIZO 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.

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 werefertilized 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 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).

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 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 $p \leq .05$.

1.3 RESULTS

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.

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.

Factors	Diameter class (mm)			Length	Volume	Surface Area
	0.01-0.5	0.51-2.0	2.1-5.0			
Inoculation (I†)		%		m	cm^3	m^2
SI	88.3 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 (C)						
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 (S)						
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)*						
I	0.031	0.027	0.523	0.001	0.023	0.016
C	0.165	0.672	0.165	0.753	0.875	0.759
S	0.463	0.172	0.156	0.042	0.027	0.032
I*C	0.263	0.356	0.562	0.187	0.986	0.193
I*S	0.165	0.465	0.243	0.180	0.129	0.908
C*S	0.352	0.543	0.145	0.962	0.435	0.130
I*C*S	0.742	0.476	0.132	0.874	0.223	0.962

†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. ‡Means followed by different letters differ from each other by LSD test at $p \leq 0.05$.

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 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**	**NDW**	**RDW**	**SDW**
Inoculation (I†)	n° plant^{-1}	mg plant^{-1}	g plant^{-1}	
SI	62 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 (C)				
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 (S)				
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)*				
I	0.035	0.001	0.013	0.001
C	0.287	0.642	0.552	0.852
S	0.623	0.764	0.047	0.023
I*C	0.821	0.121	0.651	0.412
I*S	0.283	0.875	0.172	0.155
C*S	0.387	0.275	0.422	0.290
I*C*S	0.287	0.287	0.575	0.187

†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. ‡Means followed by different letters differ from each other by LSD test at $p \leq 0.05$.

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.

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. $^{85}RbNO_3$ was applied within and between rows at five depths. Botucatu, São Paulo State, Brazil, 2016/2018.

Factors	**Within rows** (depth-cm)					GY
	5	10	20	40	60	
Cultivar (C)			mg kg^{-1}			kg ha^{-1}
BRS 317	238 a‡	203 a	157 a	109 a	72 a	4985 a
TMG 1264 RR	242 a	210 a	165 a	111 a	75 a	5018 a
Cropping Season (S)						
2016/2017	152 b	138 b	113 b	92 b	73 a	4730 b
2017/2018	307 a	271 a	204 a	123 a	81 a	5285 a
ANOVA *(F probability)*						
Inoculation (I†)	0.001	0.021	0.001	0.041	0.621	0.001
C	0.123	0.963	0.144	0.237	0.875	0.175
S	0.001	0.001	0.001	0.001	0.271	0.001
I*C	0.857	0.832	0.473	0.764	0.232	0.532
I*S	0.287	0.923	0.233	0.754	0.113	0.242
C*S	0.623	0.875	0.290	0.986	0.673	0.321
I*C*S	0.986	0.348	0.924	0.187	0.230	0.128
	Between rows (depth-cm)					
Cultivar (C)						
BRS 317	102 a	84 a	70 a	50 a	50 a	-
TMG 1264 RR	106 a	87 a	73 a	51 a	53 a	-
Cropping Season (S)						
2016/2017	88 b	65 b	58 b	44 b	40 a	-
2017/2018	121 a	102 a	95 a	57 a	65 a	-
ANOVA *(F probability)*						
Inoculation (I†)	0.001	0.001	0.231	0.134	0.852	-
C	0.243	0.234	0.823	0.423	0.870	-
S	0.001	0.001	0.001	0.001	0.242	-
I*C	0.242	0.240	0.473	0.633	0.875	-
I*S	0.209	0.852	0.391	0.253	0.249	-
C*S	0.234	0.352	0.677	0.342	0.210	-
I*C*S	0.932	0.652	0.862	0.173	0.633	-

†= Means for inoculation treatments were presented in graphic form.

‡Means followed by different letters differ from each other by LSD test at $p \leq 0.05$.

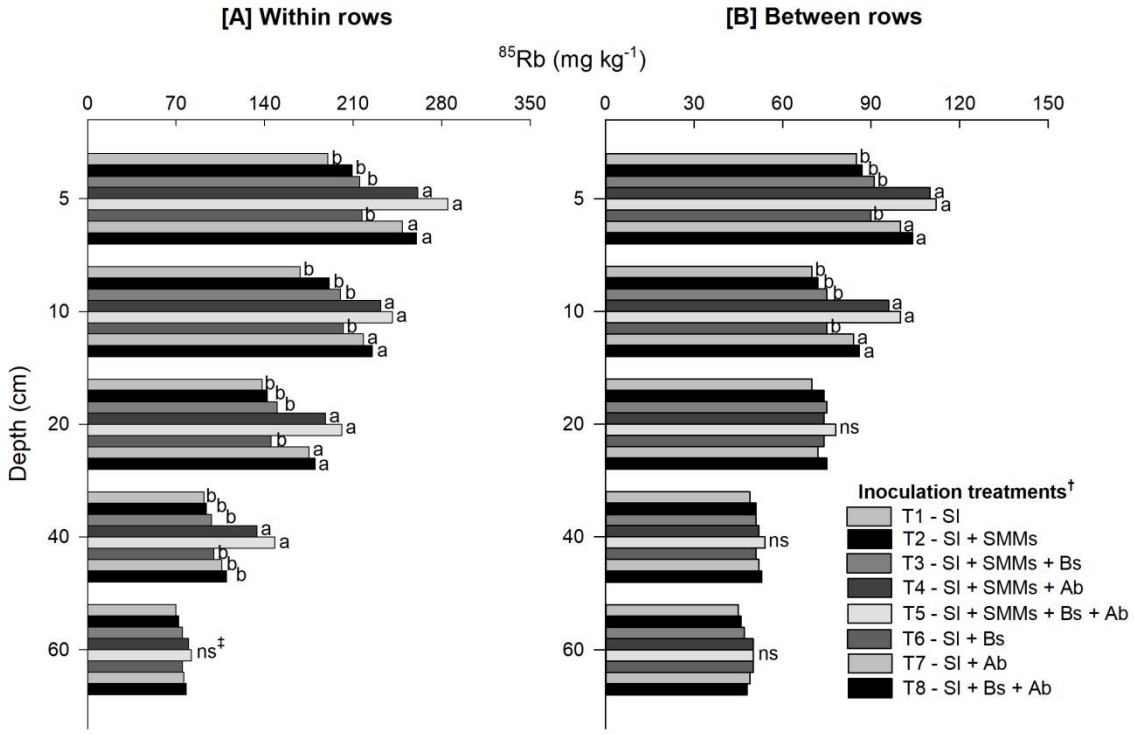

Figure 2. Rubidium (^{85}Rb) concentrations in the shoots of two soybean cultivars that received different inoculation treatments in the field during two cropping seasons. (A) Application of $^{85}RbNO_3$ within rows. (B) Application of $^{85}RbNO_3$ between rows.

† 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 $p \leq .05$

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.

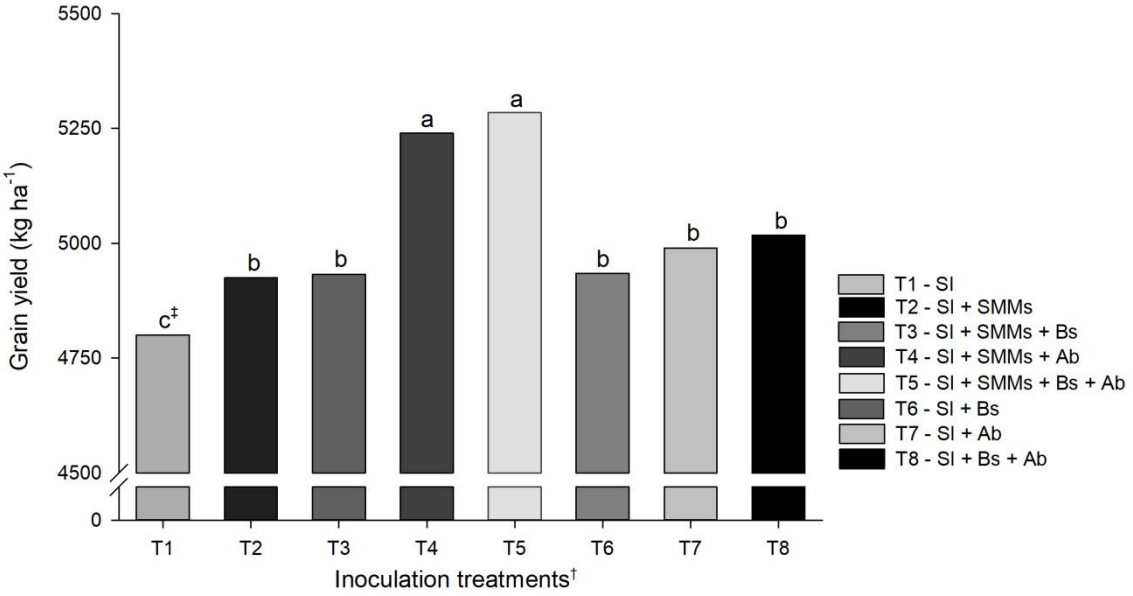

Figure 3. Average grain yield of two soybean cultivars that received different inoculation treatments in the field during two cropping seasons.

†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 LSD test at $p \leq 0.05$.

1.4 DISCUSSION AND CONCLUSIONS

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 out growth 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 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, 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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REFERENCES

Alvares, C. A., Stape, J. L., Sentelhas, P. C., Gonçalves, J. L. M., & Sparovek, G. (2013). Köppen's climate classification map for Brazil. Meteorologische Zeitschrift, 22, 711–728. https://doi.org/10.1127/0941-2948/2013/0507

AOAC – Association of Official Analytical Chemists. (1990). Official methods of analysis (15th ed., p. 771).Washington, DC: Association of Official Analytical Chemists.

Ashraf, M. A., Rasool, M., & Mirza, M. S. (2011). Nitrogen fixation and indole acetic acid production potential of bacteria isolated from rhizosphere of sugarcane (*Saccharum officinarum* L.). Advanced Biomedical Research, 5, 348–355.

Berendsen, R. L., Verk, M. C. V., Stringlis, I. A., Zamioudis, C., Tommassen, J., Pieterse, C. M. J., & Bakker, P. A. H. M. (2015). Unearthing the genomes of plant-beneficial *Pseudomonas* model strains WCS358, WCS374 and WCS417. BMC Genomics [Electronic Resource], 16, 539. https://doi.org/10.1186/s12864-015-1632-z

Bhattacharjee, J., Taylor, J. P. Jr., Smith, L. M., & Spence, L. E. (2008). The importance of soil characteristics in determining survival of first-year cotton wood seedlings in altered riparian habitats. Restoration Ecology, 16, 563–571. https://doi.org/10.1111/j.1526-100X.2007.00328.x

Bohm, W. (1979). Methods of studying root system (p. 188). Berlin: Springer-Verlag.

Brencic, A., & Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiology and Molecular Biology Reviews, 69, 155–194. https://doi.org/10.1128/MMBR.69.1.155-194.2005

Costa, C., Dwyer, L. M., Hamilton, R. I., Hamel, C., Nantais, L., & Smith, D. L. (2000). A sampling method for measurement of large root systems with scanner-based image analysis. Agronomy Journal, 92, 621–627. https://doi.org/10.2134/agronj2000.924621x

Downie, J. A. (2010). The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiology Reviews, 34, 150–170. https://doi.org/10.1111/j.1574-6976.2009.00205.x

Embrapa. (2013). Technology of soybean yield in central region of Brazil [In Portuguese] (p. 265). Londrina, PR, Brazil: Embrapa Soja.

Encide-Olibone, A. P., Olibone, D., & Rosolem, C. A. (2008). Atividade radicular da soja: Definição de um método. The Revista Brasileira de Ciência do Solo, 32, 899–903. https://doi.org/10.1590/S0100-06832008000200044

Fehr, W. R., & Caviness, C. E. 1977. Stages of soybean development. Special Report 80. Iowa State Univ., Ames.

Giehl, R. F. H., Gruber, B. D., & von Wirén, N. (2014). It's time to make changes: Modulation of root system architecture by nutrient signals. Journal of Experimental Botany, 65, 769–778. https://doi.org/10.1093/jxb/ert421

Gockele, A., Weigelt, A., Gessler, A., & Scherer-Lorenzen, M. (2014). Quantifying resource use complementarity in grassland species: A comparison of different nutrient tracers. Pedobiologia, 57, 251–256. https://doi.org/10.1016/j.pedobi.2014.09.001

Gosal, S. K., Kalia, A., Uppal, S. K., Kumar, R.,Walia, S. S., Singh, K., & Singh, H. (2012). Assessing the benefits of *Azotobacter* bacterization in sugarcane: A field appraisal. Sugar Technology, 14, 61–67. https://doi.org/10.1007/s12355-011-0131-z

Hari, K., & Srinivasan, T. R. (2005). Response of sugarcane varieties to application of nitrogen fixing bacteria under different nitrogen levels. Sugar Technology, 7, 28–31. https://doi.org/10.1007/BF02942525

Hodge, A. (2004). The plastic plant: Root responses to heterogeneous supplies of nutrients. New Phytologist, 162, 9–24. https://doi.org/10.1111/j.1469-8137.2004.01015.x

Hoekstra, N. J., Finn, J. A., Buchmann, N., Gockele, A., Landert, L., Prill, N., … Lüscher, A. (2014). Methodological tests of the use of trace elements as tracers to assess root activity. Plant and Soil, 380, 265–283. https://doi.org/10.1007/s11104-014-2048-2

Hungria, M., & Mendes, I. C. (2015). Nitrogen fixation with soybean: The perfect symbiosis? In F. J. de Bruijn (Ed.), Biological nitrogen fixation (pp. 1009–1024). Hoboken, NJ: Wiley & Sons, Inc. https://doi.org/10.1002/9781119053095.ch99

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2013). Co-inoculation of soybeans and common beans with rhizobia and azospirilla: Strategies to improve sustainability. Biology and Fertility of Soils, 49, 791–801. https://doi.org/10.1007/s00374-012-0771-5

Hungria, M., Campo, R. J., Mendes, I. C., & Graham, P. H. (2006). Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: The success of soybean (*Glycine max* L. Merr.) in South America. In R. P. Singh, N. Shankar, & P. K. Jaiwa (Eds.), Nitrogen nutrition and sustainable plant productivity (pp. 43–93). Houston, TX: Studium Press, LLC.

Läuchli, A., & Epstein, L. (1970). Transport of potassium and rubidium in plant roots, the significance of calcium. Plant Physiology, 45, 639–641. https://doi.org/10.1104/pp.45.5.639

Marks, B. B., Megías, M., Nogueira, M. A., & Hungria, M. (2013). Biotechnological potential of rhizobial metabolites to enhance the performance of *Bradyrhizobium* spp. and *Azospirillum brasilense* inoculants with soybean and maize. AMB Express, 3, 21. https://doi.org/10.1186/2191-0855-3-21

Marks, B. B., Megías, M., Nogueira, M. A., & Hungria, M. (2015). Maize growth promotion by inoculation with *Azospirillum brasilense* and metabolites of *Rhizobium tropici* CIAT 899 enriched on lipo-chitooligosaccharides (LCOs). AMB Express, 5, 71. https://doi.org/10.1186/s13568-015-0154-z

Madigan, M. T., Martinko, J. M., Dunlap, P. V., & Clark, D. P. (2008). Brock biology of microorganisms (12th ed.). International Microbiology, 11, 65–73.

Moreira, A., Moraes, L. A. C., Souza, L. G. M., & Bruno, I. P. (2016). Bioavailability of nutrients in seeds from tropical and subtropical soybean varieties. Communications in Soil Science and Plant Analysis, 47, 888–898. https://doi.org/10.1080/00103624.2016.1146899

Moretti, L. G., Lazarini, E., Bossolani, J. W., Parente, T. L., Caioni, S., Araujo, R. S., & Hungria, M. (2018). Can additional inoculations increase soybean nodulation and grain yield? Agronomy Journal, 110, 715–721. https://doi.org/10.2134/agronj2017.09.0540

O'Brien, J., &Wright, G. D. (2011). An ecological perspective of microbial secondary metabolism. Current Opinion in Biotechnology, 22, 552–558. https://doi.org/10.1016/j.copbio.2011.03.010

O'Hara, G. W., Hungria, M., Woomer, P., & Howieson, J. G. (2016). Counting rhizobia. In J. G. Howieson &M. J. Dilworth (Eds.), Working with rhizobia (pp. 109–124). Canberra: ACIAR.

Okumura, K., Goh, T., Toyokura, K., Kasahara, H., Takebayashi, Y., Mimura, T., … Fukaki, H. (2013). GNOM/FEWER ROOTS is Required for the establishment of an auxin response maximum for *Arabidopsis* lateral root initiation. Plant & Cell Physiology, 54, 406–417. https://doi.org/10.1093/pcp/pct018

Oldroyd, G. E., & Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology, 59, 519–546. https://doi.org/10.1146/annurev.arplant.59.032607.092839

Ormeño-Orrillo, E., Menna, P., Almeida, L. G. P., Ollero, F. J., Nicolás, M. F., Rodrigues, E. P., … Vasconcelos, A. T. R. (2012). Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (Phaseolus vulgaris L.). BMC Genomics [Electronic Resource], 13, 735. https://doi.org/10.1186/1471-2164-13-735

Pivetta, L. A., Castoldi, G., Santos, G. P. D., & Rosolem, C. A. (2011). Soybean root growth and activity as affected by the production system. Pesquisa Agropecuária Brasileira, 46, 1547–1554. https://doi.org/10.1590/S0100-204X2011001100017

Rosolem, C. A., & Pivetta, L. A. (2017). Mechanical and biological approaches to alleviate soil compaction in tropical soils: Assessed by root growth and activity (Rb uptake) of soybean and maize grown in rotation with cover crops. Soil Use Manage, 33, 41–152. https://doi.org/10.1111/sum.12313

Santi, C., Bogusz, D., & Franche, C. (2013). Biological nitrogen fixation in non-legume plants. Annals of Botany, 111, 743–767. https://doi.org/10.1093/aob/mct048

SAS Institute. (2015). Procedure guide for personal computers. Version 9.4. Cary, NC: SAS Inst. Schachtman, D. P., & Goodger, J. Q. (2008). Chemical root to shoot signaling under drought. Trends in Plant Science, 13, 281–287. https://doi.org/10.1016/j.tplants.2008.04.003

Schlemper, T. R., Dimitrov, M. R., Silva Gutierrez, F. A., van Veen, J. A., Silveira, A. P. D., & Kuramae, E. E. (2018). Effect of *Burkholderia tropica* and *Herbaspirillum frisingense* strains on sorghum growth is plant genotype dependent. PeerJ, 6, e5346. https://doi.org/10.7717/peerj.5346

Silva, E. A., Damatta, F. M., Ducatti, C., Regazzi, A. J., & Barros, R. S. (2004). Seasonal changes in vegetative growth and photosynthesis of Arabica coffee trees. Field Crops Research, 89, 349–357. https://doi.org/10.1016/j.fcr.2004.02.010

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete
samples). Biometrika, 52, 591–611. https://doi.org/10.1093/biomet/52.3-4.591

Soil Survey Staff. (2014). Keys to soil taxonomy. Washington, DC: US Gov. Print. Office.

Suzaki, T., Ito, M., & Kawaguchi, M. (2013). Genetic basis of cytokinin and auxin functions during root nodule development. Frontiers in Plant Science, 4, 42. https://doi.org/10.3389/fpls.2013.00042

Taylor, D., & Arkin, G. F. (1981). Root zone modification fundamentals and alternatives. In G. F. Arkin & H.M. Taylor (Eds.), Modifying the root environment to reduce crop stress (pp. 3–16). ASAE: St. Joseph.

Unicamp. (2019). Center of Meteorological and Climatic Research Applied to Agriculture. Botucatu: Municipalities climate of São Paulo State. Retrieved from www.cpa.unicamp.br/outras-informacoes/ clima_muni_086.html#

van Raij, B., Andrade, J. C., Cantarella, H., & Quaggio, J. A. (2001). Chemical analysis to evaluate soil fertility in tropical soil [In Portuguese] (p. 285). Campinas, SP, Brazil: Instituto Agronômico de Campinas.

Verbon, E. H., & Liberman, L. M. (2016). Beneficial microbes affect endogenous mechanisms controlling root development. Trends in Plant Science, 21, 218–229. https://doi.org/10.1016/j.tplants.2016.01.013

Walker, T. S., Bais, H. P., Grotewold, E., & Vivanco, J. M. (2003). Root exudation and rhizosphere biology. Plant Physiology, 132, 44–51. https://doi.org/10.1104/pp.102.01966

CHAPTER 2
BACTERIAL CONSORTIUM AND MICROBIAL METABOLITES INCREASE GRAIN QUALITY AND SOYBEAN YIELD

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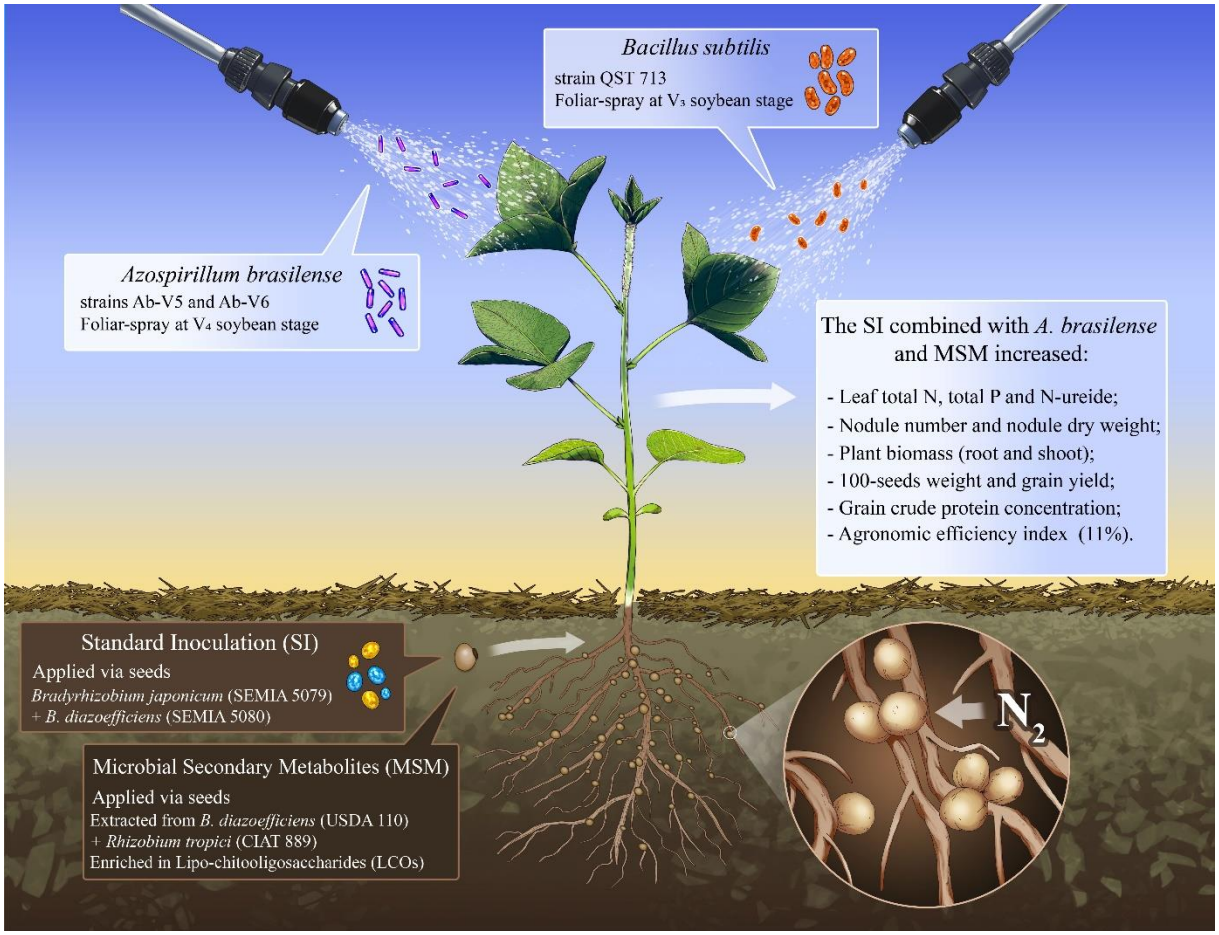

Figure 1. Graphic abstract

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.

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.

ABSTRACT

Purpose: 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. **Methods:** 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)). **Results:** 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%). **Conclusions**: 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*.

2.1 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, b). 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'Haeze 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 consortia 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.

2.2 MATERIALS AND METHODS

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 (48º26'W, 22º51'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 1,360 mm (CEPAGRI 2020). Climatological data during the experiments are presented in Figure 2.

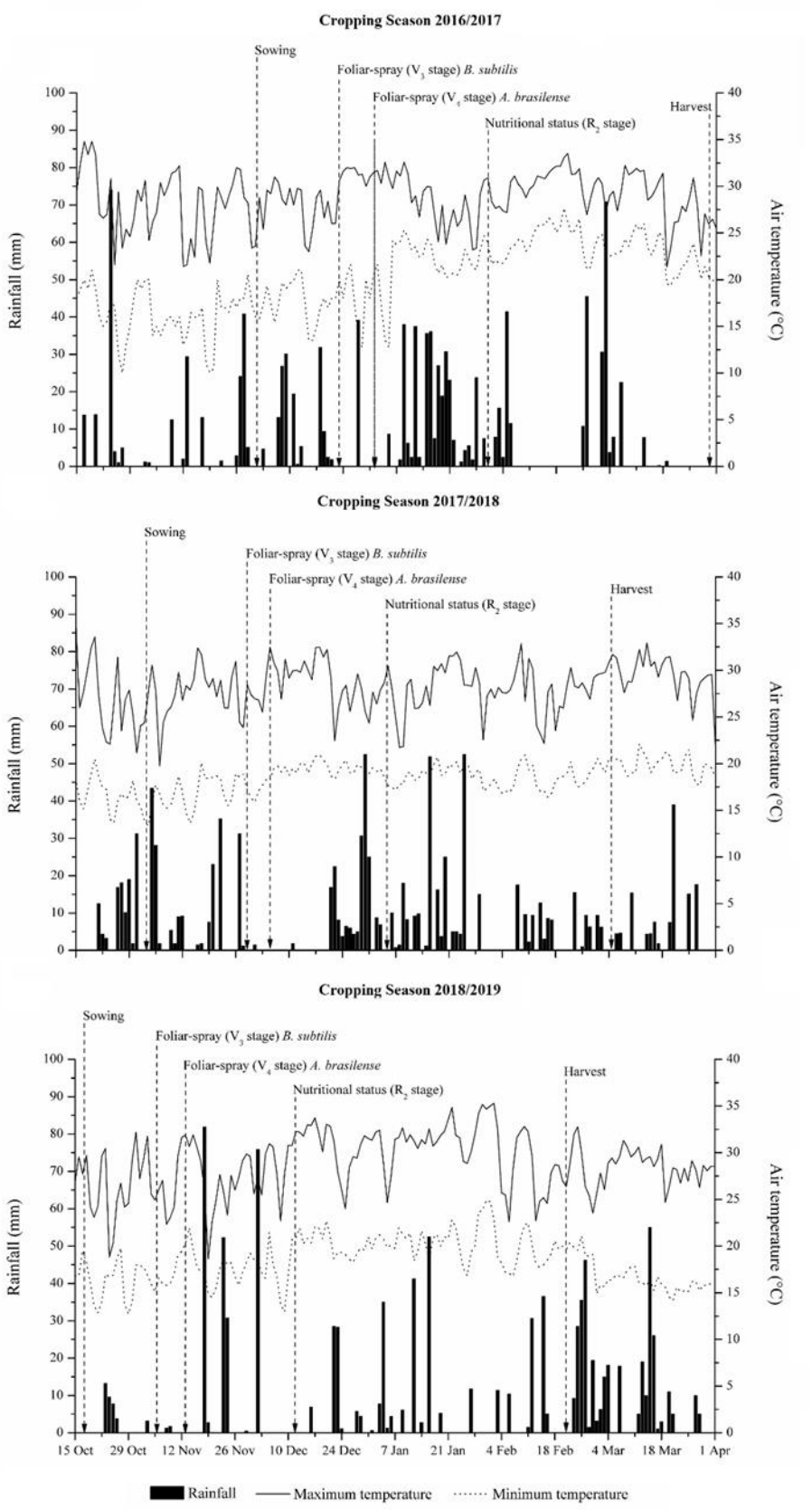

Figure 2. Rainfall and maximum and minimum air temperatures during the experimental period (2016–2019).

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 et al. (1997).

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

Soil chemical atributes		Unit	Value
Clay		g kg^{-1}	602 ± 10[a]
Sand		g kg^{-1}	117 ± 6
Silt		g kg^{-1}	281 ± 8
Bulk density		g cm^{-3}	1.19 ± 0.1
pH (CaCl2)		–	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-} ($Ca(H2PO4)2$)		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

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 V_3 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 V_4 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 V_3 stage with *B. subtilis* + foliar-spray inoculation of soybean plants at the V_4 stage with *A. brasilense*; (*vi*) SI via seed + foliar-spray inoculation of soybean plants at the V_3 stage with *B. subtilis*; (*vii*) SI via seed + foliar-spray inoculation of soybean plants at the V_4 stage with *A. brasilense*; and (*viii*) SI via seed + foliar-spray inoculation of soybean plants at the V_3 stage with *B. subtilis* + foliar-spray inoculation of soybean plants at the V_4 stage with *A. brasilense*.

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 V_3 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 V_4 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).

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 m^2 (10 m x 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 00-20-20 of N-P_2O_5-K_2O 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 $Na_2MoO_4.2H_2O$) and 2 g ha^{-1} Co (as $CoCl_2.6H_2O$) was applied to plants at the V_4 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 R_2 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 (R_2) 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 R_2 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 (R_8) (Fehr and Caviness 1977), 15 m^2 of plants from the central part of each plot were 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 = crop yield with SI and Y2 = crop yield with the corresponding bacterial consortium:

$$\text{Agronomic efficiency index}, \% = \frac{\text{Y2 x } 100}{\text{Y1}}$$

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 H_2SO_4, and the concentration was determined using the Kjeldahl distillation method (Singh et al. 2020). The CP was calculated using Eq. (2):

$$\text{Crude Protein}, \% = \% \text{ Kjeldahl N x } 6.25$$

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 $p \leq 0.05$.

2.3 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).

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	P	K	Ca	Mg	S	Cu	Fe	Zn	Mn	B	U
Inoculation (In[a])	---------- *g kg^{-1}* ----------						---------- *mg kg^{-1}* ----------					*µ mol g^{-1}*
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 + *B. subtilis* (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 + *A. brasilense* (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 *x* Cv	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
In *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cv *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
In *x* Cv *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

[a]Inoculation 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 V_3 stage; *A. brasilense* = foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V_4 stage. [b]The 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 *$p \leq 0.05$ and **$p \leq 0.01$ 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	NDW	RDW	SDW	PH	P	W100	CP	EE
Inoculation (In[a])	*n° plant^{-1}*	*mg plant^{-1}*	*g plant^{-1}*		*cm*	*n° plant^{-1}*	*g*	*g kg^{-1}*	
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 + *B. subtilis* (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 + *A. brasilense* (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 *x* Cv	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
In *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cv *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
In *x* Cv *x* CS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

[a]Inoculation 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 V_3 stage; *Azospirillum brasilense* = foliar-spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at V_4 stage. [b]The 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 *$p \leq 0.05$ and **$p \leq 0.01$ probability.

At physiological maturity, positive effects on plant height were observed in plants coinoculated with *Bradyrhizobium* and *A. brasilense* and in the number of pods of plants that were coinoculated 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* (Figure 3). However, no treatment differences were found for the ether extract concentrations in seeds.

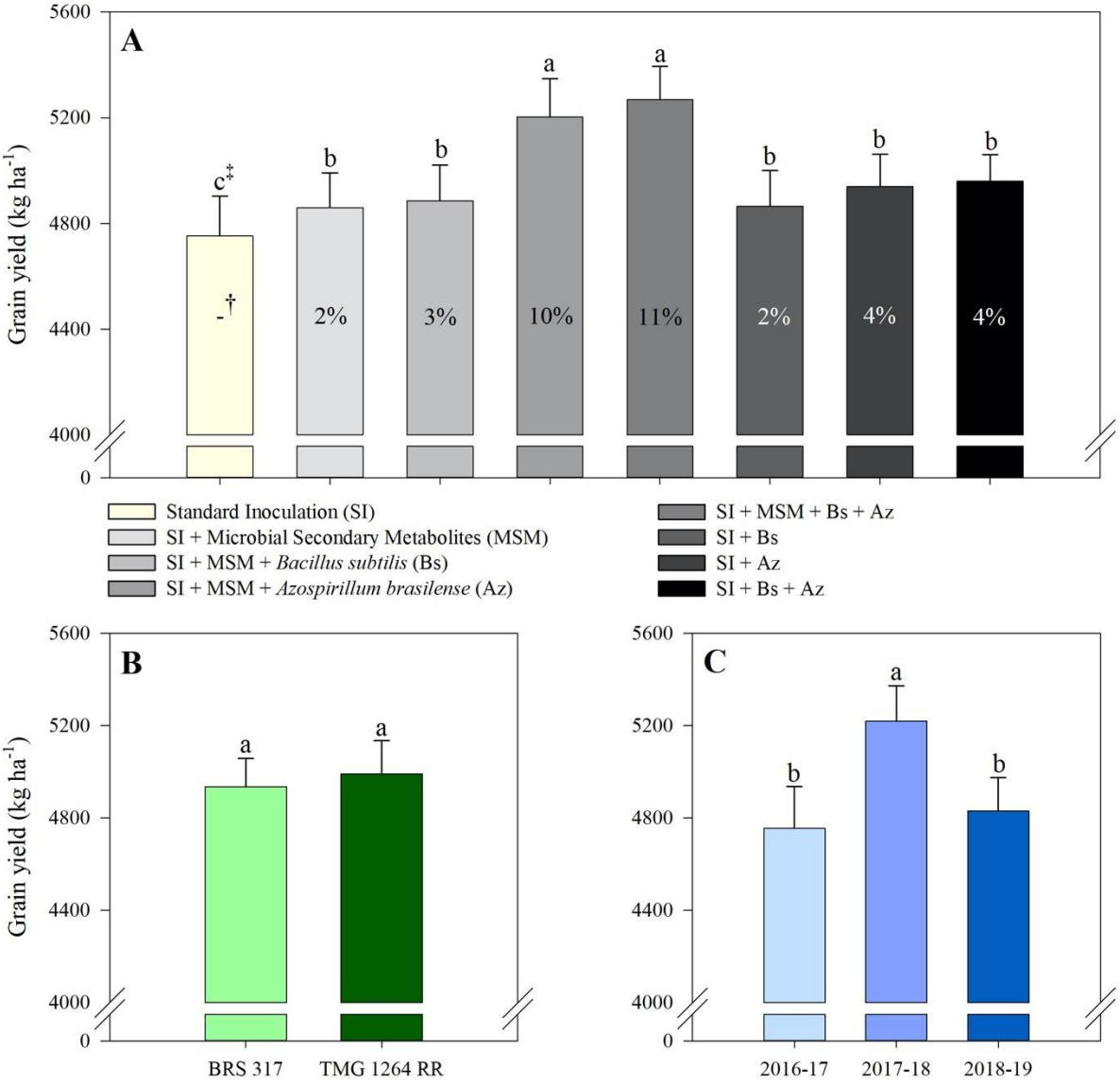

Figure 3. Average soybean grain yield as a function bacterial consortia (A), in two soybean cultivars (B), and in the field during three cropping seasons (C).

†Agronomic efficiency index (AEI). ‡The statisticalmodel 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

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 (Figure 3); 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.

2.4 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 three-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 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, Acunã 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 (Schalaman et al. 1997); (*iii*) As reported by Massoud et al. (2009), a bacterial consortium may promote greater nitrogenase activity and increase 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. 1993), 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, c), 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).

2.5 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 N_2 fixation and grain yield and quality.

2.6 ACKNOWLEDGMENTS

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REFERENCES

Acuña JJ, Marileo LG, Araya MA, Rilling JI, Larama GA, Mora ML, Epstein S, Jorquera MA (2020) In situ cultivation approach to increase the culturable bacterial diversity in the rhizobiome of plants. J. Soil Sci. Plant Nutr.1-16. doi: 10.1007/s42729-020-00222-0

Alvares CA, Stape JL, Sentelhas PC, Moraes G, Leonardo J, Sparovek G (2013) Köppen's climate classification map for Brazil. Meteorol Z. 22:711–728. doi: 10.1127/0941-2948/2013/0507

Ambrosano EJ, Tanaka RT, Mascarenhas HAA, van Raij B, Quaggio JA, Cantarella H (1997) Legumes and oilseeds. In: van Raij B, Cantarella H, Quaggio JA, Furlani AMC (Eds.), Fertilization and liming recommendation for the state of São Paulo (In Portuguese) (pp.187–203), 2nd ed., Instituto Agronômico, Campinas, Brazil. (Boletim Técnico 100).

AOAC (2019) Official methods of analysis of AOAC international. G.W. Latimer Jr. (ed.), 21st ed., AOAC International, Gaithersburg, Maryland.

Araújo FF, Henning AA, Hungria M (2005) Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development. World J. Microbiol. Biotechnol. 21:1637-1642. doi: 10.1007/s11274-005-3621-x

Babalola OO (2010) Beneficial bacteria of agricultural importance. Biotechnol. Lett. 32:1559–1570. doi: 10.1007/s10529-010-0347-0

Baral B, Silva JAT, Izaguirre-Mayoral ML (2016) Early signaling, synthesis, transport and metabolism of ureides. J. Plant Physiol. 193:97–109. doi: 10.1016/j.jplph.2016.01.013

Bashan Y, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. Adv. Agron. 108:77–136. Academic Press. doi: 10.1016/S0065-2113(10)08002-8

Brasil (2019) Agribusiness Projections: Brazil 2018/19 to 2028/29 long term projections (in Portuguese). Agricultural Policy Secretariat. – Brasília: MAPA/ACE. 126p.

CEPAGRI (2020) Center of Meteorological and Climatic Research Applied to Agriculture. Botucatu: Municipalities climate of São Paulo State. Available via: <www.cpa.unicamp.br/outras-informacoes/clima_muni_086.html> Accessed 27 Jan. 2020.

Cerezini P, Kuwano BH, Santos MB, Terassi F, Hungria M, Nogueira MA (2016) Strategies to promote early nodulation in soybean under drought. Field Crop Res. 196:160–167. doi: 10.1016/j.fcr.2016.06.017

Chibeba AM, Guimarães MF, Brito OR, Nogueira MA, Araujo RS, Hungria M (2015) Co-inoculation of soybean with *Bradyrhizobium* and *Azospirillum* promotes early nodulation. Am. J. Plant Sci. 6:1641–1649. doi: 10.4236/ajps.2015.610164

Cullimore JV, Ranjeva R, Bono JJ (2001) Perception of lipo-chitooligosaccharidic Nod factors in legumes. Trends Plant Sci. 6:24–30. doi: 10.1016/S1360-1385(00)01810-0

D'Angioli AM, Viani RAG, Lambers H, Sawaya ACHF, Oliveira RS (2017) Inoculation with *Azospirillum brasilense* (Ab-V4, Ab-V5) increases *Zea mays* root carboxylate-exudation rates, dependent on soil phosphorus supply. Plant Soil 410:499–507. doi: 10.1007/s11104-016-3044-5

D'Haeze W, Holsters M (2002) Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology 12:79R–105R. doi: 10.1093/glycob/12.6.79R

Dardanelli MS, Cordoba FJF, Espuny MR, Carvajal MAR, Díaz MES, Serrano AMG, Okon Y, Megías M (2008) Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress. Soil Biol. Biochem. 40:2713–2721. doi: 10.1016/j.soilbio.2008.06.016

del Cerro P, Rolla-Santos AAP, Gomes DF, Marks BB, Espuny MR, Rodriguez Carvajal MA, Soria-Diaz E, Nakatani AS, Hungria M, Ollero FJ, Megías M (2015) Opening the “black box” of *nodD3*, *nodD4* and *nodD5* genes of *Rhizobium tropici* strain CIAT 899. BMC Genomics 16:864. doi: 10.1186/s12864-015-2033-z

Donagema GK, Viana JHM, Almeida BG, Ruiz HA, Klein VA, Dechen SCF, Fernandes RBA (2017) Granulometric analysis. In: Teixeira PC, Donagema GK, Fontana A, Teixeira WG (Eds.), Soil Analysis Methods Manual (In Portuguese) (pp.95–116), 3rd ed., Embrapa Solos–Documentos (INFOTECA-E).

Embrapa (2013) Technology of soybean yield in central region of Brazil (In Portuguese). Londrina: Embrapa Soja, 265p.

Fehr WR, Caviness CE (1977) Stages of Soybean Development. Iowa State University Cooperative Extension Service, Special Report 80.

Fukami J, Cerezini P, Hungria M (2018a) *Azospirillum*: benefits that go far beyond biological nitrogen fixation. AMB Express 8:1–12. doi:10.1186/s1356 8-018-0608-1

Fukami J, de la Osa C, Ollero FJ, Megías M, Hungria M (2018b) Co-inoculation of maize with *Azospirillum brasilense* and *Rhizobium tropici* as a strategy to mitigate salinity stress. Funct. Plant Biol. 45:328–339. doi:10.1071/FP171 67

Fukami J, Ollero FJ, de la Osa C, Valderrama-Fernández R, Megías G, Hungria M (2018c) Antioxidant activity and induction of mechanisms of resistance to stresses related to the inoculation with *Azospirillum brasilense.* Arch. Microbiol. 200:1191–1203. doi: 10.1007/s00203-018-1535-x

Gough C (2003) *Rhizobium* symbiosis: insights into Nod factors receptors. Curr. Biol. 13:973–975. doi: 10.1016/j.cub.2003.11.047

Greef JM (1994) Productivity of maize (*Zea mays* L.) in relation to morphological and physiological characteristics under varying amounts of nitrogen supply. J. Agron. Crop Sci. 172:317–326. doi: 10.1111/j.1439-037X.1994.tb00182.x

Herridge D, Giller KE (2016) Measurements of nitrogen fixation. In: Howieson JG. & Dilworth MJ (Eds.), Working with rhizobia (pp. 187–220). Canberra: ACIAR.

Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. Plant Soil 331:413-425. doi: 10.1007/s11104-009-0262-0

Hungria M, Franchini JC, Campo RJ, Crispino CC, Moraes JZ, Sibaldelli RNR, Mendes IC, Arihara J (2006) Nitrogen nutrition of soybean in Brazil: Contributions of

biological N_2 fixation and of N fertilizer to grain yield. Can. J. Plant Sci. 86:927–939. doi: 10.4141/P05-098

Hungria M, Mendes IC (2015) Nitrogen fixation with soybean: The perfect symbiosis? In: F.J. de Bruijn (Ed.), Biological nitrogen fixation (pp. 1009–1024). John Wiley & Sons, Inc., Hoboken, NJ. doi: 10.1002/9781119053095.ch99

Hungria M, Mendes IC, Nakatani AS, Reis-Junior FB, Moraes JZ, Oliveira MC, Fernandes MF (2014) Effects of glyphosate-resistant gene and herbicides on soybean crop: Field trials monitoring biological nitrogen fixation and yield. Field Crops Res. 158:43–54. doi: 10.1016/j.fcr.2013.12.022

Hungria M, Nogueira MA, Araujo RS (2013) Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. Biol. Fertil. Soils 49:791–801. doi: 10.1007/s00374-012-0771-5

Hungria M, Nogueira MA, Araujo RS (2015) Soybean seed co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A new biotechnological tool to improve yield and sustainability. Am. J. Plant Sci. 6:811–817. doi: 10.4236/ajps.2015.66087

Hungria M, Nogueira MA, Araujo RS (2016) Inoculation of *Brachiaria* spp. with the plant growth-promoting bacterium *Azospirillum brasilense*: an environment-friendly component in the reclamation of degraded pastures in the tropics. Agric. Ecosyst. Environ. 221:125–131. doi: 10.1016/j.agee.2016.01.024

Hungria M, Ribeiro RA, Nogueira MA (2018) Draft genome sequences of *Azospirillum brasilense* strains Ab-V5 and Ab-V6, commercially used in inoculants for grasses and legumes in Brazil. Genome Announc. 6:e00393-18. doi: 10.1128/genomeA.00393-18

Kaschuk G, Nogueira MA, de Luca MJ, Hungria M (2016) Response of determinate and indeterminate soybean cultivars to basal and topdressing N fertilization compared to sole inoculation with *Bradyrhizobium*. Field Crops Res. 195:21–27. doi: 10.1016/j.fcr.2016.05.010

Karr-Lilienthal LK, Grieshop CM, Merchen NR, Mahan DC, Fahey GC (2004) Chemical composition and protein quality comparisons of soybeans and soybean meals from five leading soybean-producing countries. J. Agr. Food Chem. 52:6193–6199. doi: 10.1021/jf049795+

Khan W, Prithiviraj B, Smith DL (2008) Nod factor [Nod Bj V ($C_{18:1}$, MeFuc)] and lumichrome enhance photosynthesis and growth of corn and soybean. J. Plant Physiol. 165:1342–1351. doi: 10.1016/j.jplph.2007.11.001

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781–784. doi: 10.1038/344781a0

Leroux P, Fritz R, Debieu D, Albertini C, Lanen C, Bach J, Gredt M, Chapeland F (2002) Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Manag. Sci. 58:876–888. doi: 10.1002/ps.566

Levene H (1960) Robust tests for equality of variances. In: Olkin I, Ghurye SG, Hoeffding W, Madow WG, Mann HB (Eds.), Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling. Stanford University Press. pp. 278–292

Liang Y, Cao Y, Tanaka K, Thibivilliers S, Wan J, Choi J, ho Kang C, Qiu J, Stacey G (2013) Non legumes respond to rhizobial Nod factors by suppressing the innate immune response. Science 341:1384–1387. doi: 10.1126/science.1242736

Lone NA, Mir MR, Khan NA (2005) Effect of gibberellic acid on physiological attributes and yield of mustard (*Brassica juncea* L.). Appl. Biol. Res. 7:24–26.

Malavolta E, Vitti GC, Oliveira SA (1997) Evaluation of plant nutritional status: principles and applications [In Portuguese]. 2nd ed., POTAFÓS, Piracicaba, Brazil. 319p.

Marks BB, Megías M, Nogueira MA, Hungria M (2013) Biotechnological potential of rhizobial metabolites to enhance the performance of *Bradyrhizobium* spp. and *Azospirillum brasilense* inoculants with soybean and maize. AMB Express 3:21. doi: 10.1186/2191-0855-3-21

Marks BB, Megías M, Ollero FJ, Nogueira MA, Araujo RS, Hungria M (2015) Maize growth promotion by inoculation with *Azospirillum brasilense* and metabolites of *Rhizobium tropici* enriched on lipo-chitooligosaccharides (LCOs). AMB Express 5:71. doi: 10.1186/s13568-015-0154-z

Moreira A, Sfredo GJ, Moraes LA, Fageria NK (2014) Agronomic efficiency of two types of lime and phosphate fertilizer sources in Brazilian Cerrado soils cultivated with soybean. Commun. Soil. Sci. Plant Anal. 45:2319–2330. doi:10.1080/00103624.2014.932372

Moretti LG, Crusciol CAC, Kuramae EE, Bossolani JW, Moreira A, Costa NR, Alves CJ, Pascoaloto IM, Rondina ABL, Hungria M (2020) Effects of growth-promoting bacteria on soybean root activity, plant development and yield. Agron. J. 112:418-428. doi: 10.1002/agj2.20010

Moretti LG, Lazarini E, Bossolani JW, Parente TL, Caioni S, Araujo RS, Hungria M (2018) Can additional inoculations increase soybean nodulation and grain yield? Agron. J. 110:715–721. doi: 10.2134/agronj2017.09.0540

Massoud ON, Morsy EM, El-Batanony NH (2009) Field response of snap bean (*Phaseolus vulgaris* L.) to N_2-fixers *Bacillus circulans* and arbuscular mycorrhizal fungi inoculation through accelerating rock phosphate and feldspar weathering. Aust. J. Basic Appl. Sci. 3:844–852.

Nadeem SM, Zahir ZA, Naveed M, Arshad M (2007) Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can. J. Microbiol. 53:1141–1149. doi: 10.1139/W07-081

Nicholson WL (2002) Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59:410–416. doi: 10.1007/s00018-002-8433-7

Nosheen A, Bano A, Yasmin H, Keyani R, Habib R, Shah ST, Naz R (2016) Protein quantity and quality of safflower seed improved by NP fertilizer and rhizobacteria (*Azospirillum* and *Azotobacter* spp.). Front. Plant Sci. 7:104. doi: 10.3389/fpls.2016.00104

O'Hara GW, Hungria M, Woomer P, Howieson JG (2016) Counting rhizobia. In: J.G. Howieson, M.J. Dilworth (Eds.), Working with rhizobia (pp. 109–124). Canberra: ACIAR.

Oliveira GS, Sena PTS, Nascimento TR, Neto RAF, Pereira JRC, Martins LMV, Freitas ADS, Signor D, Fernandes-Júnior PI (2019) Are cowpea-nodulating Bradyrhizobial communities influenced by biochar amendments in soils? Genetic diversity and symbiotic effectiveness assessment of two agricultural soils of Brazilian drylands. J. Soil Sci. Plant Nutr. 1-11. doi: 10.1007/s42729-019-00128-6

Omar MNA, Hhussein KRF, Zaher EA, Abou Zeid MY (2002) Effect of inoculation with *Azospirillum brasilense* on yield and some chemical constituents of rice grain. In: Rice genetic resources and breeding for Europe and other temperate areas: proceedings of Eurorice 2001 Symposium, Cirad, Ird, Ksau, Krasnodar Territory, Vniirisa, September, 3-8, 2001, Krasnodar, Russia. CIRAD, IRD, KSAU, Krasnodar. Montpellier: CIRAD ISBN 2-87614-504-9 Eurorice 2001 Symposium, Krasnodar, Russie, 3 September 2001/8 September 2001.

Ozturk A, Caglar O, Sahin F (2003) Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. J. Soil Sci. Plant Nutr. 166:262–266. doi:10.1002/jpln.200390038

Penot I, Berges N, Guiguene C, Fages J (1992) Characterization of *Azospirillum* associated with maize (*Zea mays* L.) in France using biochemical tests and plasmid profiles. Can. J. Microbiol. 38:798–803. doi: 10.1139/m92-130

Quaggio JA, van Raij B (1997) Soil acidity correction. In: van Raij, B., Cantarella H, Quaggio JA, Furlani AMC (Eds.), Fertilization and liming recommendation for the state of São Paulo (In Portuguese) (pp.14–19). Campinas, SP, Brazil. Instituto Agronômico de Campinas. (Boletim Técnico 100).

Sansinenea E, Ortiz A (2011) Secondary metabolites of soil *Bacillus* spp. Biotechnol. Lett. 33:1523–1538. doi: 10.1007/s10529-011-0617-5

Santachiara G, Borrás L, Rotundo JL (2017) Physiological processes leading to similar yield in contrasting soybean maturity groups. Agron. J. 109:158–167. doi: 10.2134/agronj2016.04.0198

Santos MS, Nogueira MA, Hungria M (2019) Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express 9:205. doi: 0.1186/s13568-019-0932-0

SAS Institute (2015) Procedure guide for personal computers. Version 9.4. Cary, NC: SAS Inst.

Schlaman HR, Gisel AA, Quaedvlieg NE, Bloemberg GV, Lugtenberg BJ, Kijne JW, Potrykus I, Spaink HP, Sautter C (1997) Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting. Development 124:4887–4895.

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611. doi: 10.1093/biomet/52.3-4.591

Shi Y, Li J, Wang J, Zhu R, Li S, Li Q, Chen L, Zhu J, Zou J, Wang J, Chang H (2019) Nodulation and genomic capacity of a novel high-latitude *Bradyrhizobium japonicum* HLNEAU001. J. Soil Sci. Plant Nutr. 19:277-89. doi: 10.1007/s42729-019-00027-w

Singh P, Singh RK, Song QQ, Li HB, Yang LT, Li YR (2020) Methods for estimation of nitrogen components in plants and microorganisms. In: Gupta K. (Eds.) Nitrogen Metabolism in Plants. Methods in Molecular Biology, 2057. Humana, New York, NY. doi: 10.1007/978-1-4939-9790-9_10

Soil Survey Staff (2014) Keys to soil taxonomy. 12th ed. USDA-NRCS, Washington, DC.

Standish JR, Tomaso-Peterson M, Allen TW, Sabanadzovic S, Aboughanem Sabanadzovic N (2015) Occurrence of QoI fungicide resistance in *Cercospora sojina* from Mississippi soybean. Plant Dis. 99:1347–1352. doi: 10.1094/PDIS-02-15-0157-RE

Stefan M, Munteanu N, Stoleru V, Mihasan M (2013) Effects of inoculation with plant growth promoting rhizobacteria on photosynthesis, antioxidant status and yield of runner bean. Rom. Biotechnol. Lett. 18:8132–8143.

Sugiyama A, Ueda Y, Zushi T, Takase H, Yazaki K (2014) Changes in the bacterial community of soybean rhizospheres during growth in the field. PLoS One 9:p.e100709. doi: 10.1371/journal.pone.0100709

Taiz L, Zeiger E, Moller IM, Murphy A (2017) Plant Physiology. 6. ed. Porto Alegre: Artmed, 888p.

Tamagno S, Sadras VO, Haegele JW, Armstrong PR, Ciampitti IA (2018) Interplay between nitrogen fertilizer and biological nitrogen fixation in soybean: implications on seed yield and biomass allocation. Sci. Rep. 8:1-11. doi: 10.1038/s41598-018-35672-1

Tobias S, Carlson JE (1969) Brief report: Bartlett's test of sphericity and chance findings in factor analysis. Multivar. Behav. Res. 4:375-377. doi: 10.1207/s15327906mbr0403_8

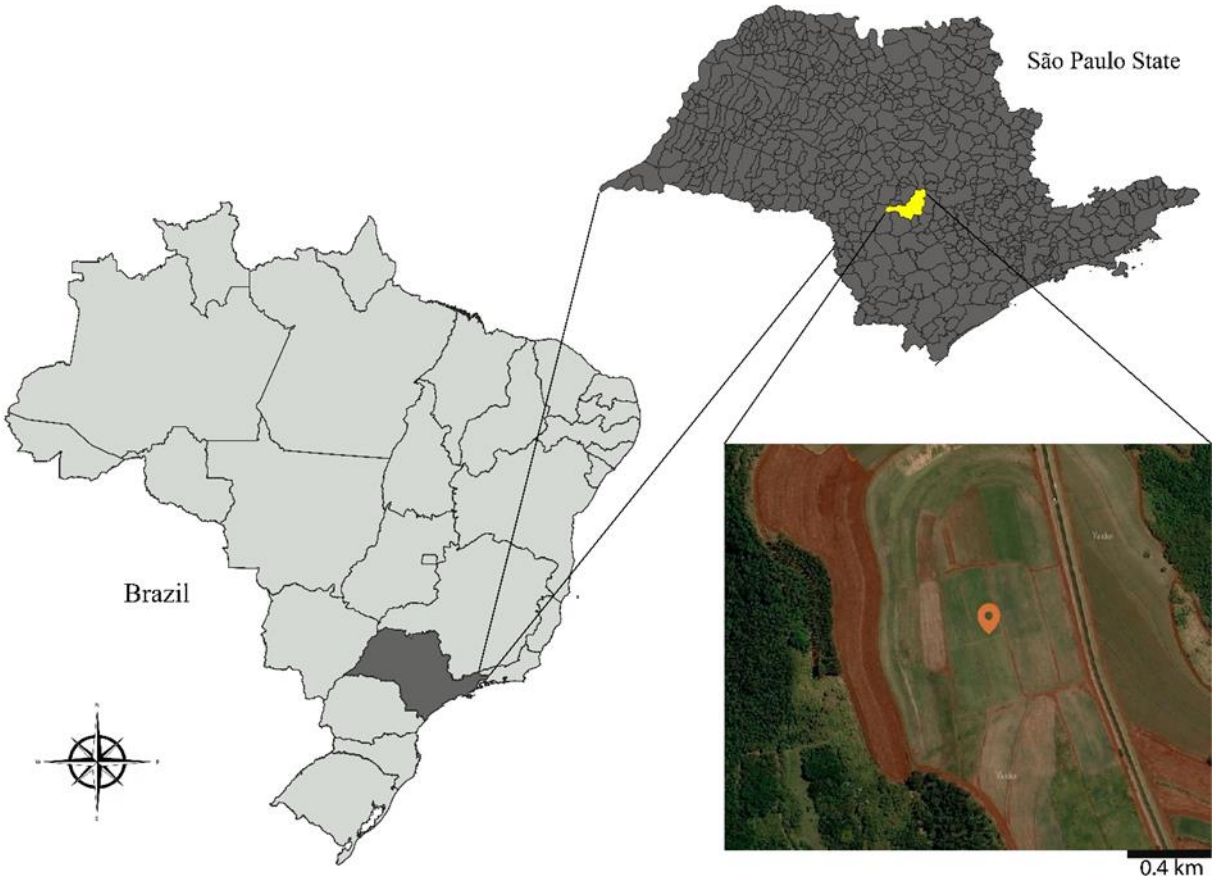
USDA (2020) World agricultural supply and demand estimates. USDA, Washington, DC. Available via: <https://www.usda.gov/oce/commodity/wasde/> Accessed 28 Jan. 2020.

van Raij B, Andrade JC, Cantarella H, Quaggio JA (2001) Chemical analysis to evaluate soil fertility in tropical soil (In Portuguese). Campinas, SP, Brazil. Instituto Agronômico de Campinas. 285p.

Vanlauwe B, Hungria M, Kanampiu F, Giller KE (2019) The role of legumes in the sustainable intensification of African smallholder agriculture: Lessons learnt and challenges for the future. Agric. Ecosyst. Environ. 284:106583. doi:10.1016/j.agee.2019.106583

Wani SP, Chandrapalaih S, Dart PJ (1985) Responses of pearl millet cultivars to inoculation with nitrogen-fixing bacteria. Exp. Agric. 21:175–182. doi: 10.1017/S001447970001245

Supplementary Figure



Supplementary Figure 1. Lageado Experimental Farm – São Paulo State University (UNESP), Botucatu-SP, Brazil. The Field Experiment.

CHAPTER 3
BENEFICIAL MICROBIAL SPECIES AND METABOLITES ALLEVIATE SOYBEAN OXIDATIVE DAMAGE AND INCREASE GRAIN YIELD DURING SHORT DRY SPELLS

Paper in the rules and being processed by the *European Journal of Agronomy* (ISSN 1161-0301)

Highlights

- A bacterial consortium comprising *Bradyrhizobium* spp., *Azospirillum brasilense* strains, and microbial metabolites positively affects photosynthetic pigments and the gas exchange process.
- This beneficial bacterial consortium also circumvents oxidative damage and reduces proline accumulation in soybean leaves.
- An appropriate bacterial consortium can promote soybean resistance to moderate water stress during short dry spells and ensure high grain yield.

Abbreviations

BNF, biological nitrogen fixation;

ROS, reactive oxygen species;

MDA, malondialdehyde;

SOD, superoxide dismutase;

CAT, catalase;

APX, ascorbate peroxidase;

PGPB, plant growth-promoting bacteria;

MSM, microbial secondary metabolites;

LCOs, lipo-chitooligosaccharides;

IAA, indole-3-acetic acid;

ACC deaminase, 1-aminocyclopropane-1-carboxylate deaminase;

A, net photosynthetic rate;

gs, stomatal conductance;

Ci, internal CO_2 concentration in the substomatal chamber;

E, transpiration;

FW, fresh weight;

PH, plant height;

NN, nodule number;

NDW, nodule dry weight;
RDW, root dry weight;
SDW, shoot dry weight;
GY, grain yield.

ABSTRACT

Short dry spells are an important grain yield constraint in tropical regions. Plant growth-promoting bacteria (PGPB) and their metabolites can mitigate the impact of drought stress by promoting changes in plant metabolism, physiology, and biochemistry. However, the effects of PGPB on soybean under drought stress in tropical regions have not been established. Therefore, in this study we evaluated the effects of co-inoculating N_2-fixing *Bradyrhizobium japonicum* (strain SEMIA 5079) and *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) with or without foliar application of plant growth-promoting *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) and the biocontrol agent *Bacillus subtilis* (strain QST 713) and seed treatment with microbial secondary metabolites (MSM, rhizobial metabolites enriched in lipo-chitooligosaccharides (LCOs)) on soybean (*Glycine max* (L.) Merrill) during two growing seasons under tropical field conditions with short dry spells. Photosynthetic pigments, gas exchange parameters, antioxidant enzyme activity and proline concentrations in leaves, nodulation, plant growth development and grain yield were evaluated. The bacterial consortium comprising *Bradyrhizobium* spp., *A. brasilense* strains and MSM application increased the contents of chlorophyll *a* (14.5%), chlorophyll *b* (30.8%), total chlorophyll (17.2%), and total carotenoids (27.3%) compared with *Bradyrhizobium* spp. treatment alone. This consortium also increased the net photosynthetic rate (17.7%), stomatal conductance (56.5%), internal CO_2 concentration in the substomatal chamber (8.3%), and transpiration (44%) compared with plants that received the standard inoculation (*Bradyrhizobium* spp. only), while reducing the leaf contents of hydrogen peroxide (-18.8%) and proline (-29.4%), lipid peroxidation (-15.9%), and the activities of superoxide dismutase (-18.2%), catalase (-21.2%), and ascorbate peroxidase (-19.1%). Taken together, the results indicate that a beneficial bacterial consortium comprising *Bradyrhizobium* spp. and *A. brasilense* strains combined with MSM application can alleviate oxidative damage during dry spells. Furthermore, this consortium improved soybean nodulation, plant growth

development, and grain yield by up to 12.2%, ensuring sustainable yield in tropical regions.

Keywords: Antioxidant metabolism, *Glycine max*, Photosynthesis, Plant growth-promoting bacteria, Water deficit.

3.1 INTRODUCTION

Adverse environmental factors are increasingly impacting agriculture worldwide (FAO, 2019). To guarantee global food security, mechanisms to mitigate the effects of abiotic stresses caused by climate change have been investigated in several crop species (Fukami et al., 2018a; Riar et al., 2018; Cerezini et al., 2019). Soybean (*Glycine max* (L.) Merrill) is an important crop globally because of its use in protein, oil, and biofuel production (Moretti et al., 2020a). In addition, due to its high capacity for biological nitrogen fixation (BNF), soybean plays a key role in agriculture sustainability (Hungria and Mendes, 2015; Ciampitti and Salvagiotti, 2018; Moretti et al., 2018).

Soybean grain yield is affected by different environmental conditions, especially pluvial precipitation and temperature (Chen and Wiatrak, 2010). Variations in the climate characteristics of tropical regions used for cropping (Alvares et al., 2013; Cunningham, 2020) result in differences in the intensity, frequency, and timing of water supply (Hu and Wiatrak, 2012; Battisti and Sentelhas, 2014). Water deficit is a major limiting factor of soybean yield and can be mitigated at least partially by using cultivars with appropriate physiological traits and crop management (Battisti and Sentelhas, 2017).

Several mechanisms of tolerance and resistance can be adopted by plants to deal with biotic and abiotic stresses. Among them, the major mechanism is to reduce the accumulation of reactive oxygen species (ROS) in plant tissues (Reis et al., 2017; Santos et al., 2017). ROS are byproducts of several metabolic pathways in various organelles, including chloroplasts, mitochondria, and peroxisomes (Gill and Tuteja, 2010). ROS include free radicals resulting from the metabolism of oxygen (O_2), such as superoxide radicals (O^{2-}s), hydroxyl radicals (OH^-s), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In plants, oxidative stress may be relieved by antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC: 1.11.1.6), and ascorbate peroxidase (APX; EC: 1.11.1.11). SOD, which dismutates O_2

radicals ($^1O_2$) in the first excited electronic state to H_2O_2, is the first line of defense against ROS (Gill and Tuteja, 2010); H_2O_2 can then be detoxified to H_2O by CAT and APX through the conversion of H_2O_2 to H_2O and O_2 (Alam and Ghosh, 2018).

Stomatal and osmotic adjustments can occur through several mechanisms and channels. Inorganic ions such as sodium (Na^+), potassium (K^+), and chloride (Cl^-) drive most of the osmotic potential in many plant species, while sugars and amino acids, especially proline, are the main osmoregulators in vascular plants (Alexieva et al., 2001; Ueda et al., 2001). Oxidative damage caused by excess ROS affects cellular components, resulting in membrane disruption via lipid peroxidation (Santos et al., 2017). However, the accumulation of solutes such as proline can protect cells against increased ROS levels (Juge et al., 2012; Mauad et al., 2016). Increased proline biosynthesis in plant tissues contributes to enzymatic stabilization and/or osmoregulation in response to abiotic stress (e.g., salinity, drought, heavy metals, or extreme temperature) (Torello and Rice, 1986; Abbasi et al., 2013; Mauad et al., 2016).

Plant-growth promoting bacteria (PGPB) and their metabolites, e.g., lipo-chitooligosaccharides (LCOs) and lipopolysaccharides (LPS), may play a strategic role in alleviating the deleterious effects of ROS through the production of various phytohormones, such as ethylene (Glick, 2012), cytokinins (Tien et al., 1979), gibberellins (Bottini et al., 1989), abscisic acid (Cohen et al., 2008), salicylic acid (Sahoo et al., 2014), indole-3-acetic acid (IAA), and jasmonic acid (Spaepen and Vanderleyden, 2015), among others (Fukami et al., 2018a), leading to the activation of physiological and biochemical mechanisms that confer plant tolerance to abiotic and biotic stresses (Fukami et al., 2018b,c).

Phytohormones produced by PGPB regulate processes and signaling networks involved in plant responses that ultimately lead to increased grain yields (Moretti et al., 2020a,b), including antioxidant metabolism (Fukami et al., 2017), exopolysaccharide production (Sandhya et al., 2010), osmotic adjustment (Cerezini et al., 2014), heavy metal mitigation (Bashan and de-Bashan, 2010), plant pathogen biocontrol (Nicholson, 2002), and induced systemic tolerance (Pozo et al., 2008). However, the effects of beneficial microbes and microbial metabolites on soybean under water stress are poorly understood.

We hypothesized that beneficial microbes and their metabolites can alleviate water stress in soybean by increasing the intrinsic tolerance of soybean plants to abiotic stresses, thereby improving yield. To test this hypothesis, we evaluated

photosynthetic pigments, gas exchange parameters, antioxidant enzyme activities, proline content, nodulation, plant growth development and grain yield in soybean inoculated with beneficial bacterial consortia (N_2-fixing *Bradyrhizobium* spp. with different combinations of *Azospirillum brasilense* strains, *Bacillus subtilis*, and rhizobial metabolites) during two growing seasons under tropical field conditions with short dry spells during the plant reproductive stage.

3.2 MATERIALS AND METHODS

Site Description

Two field experiments under rainfed conditions were carried out in the 2016–2017 and 2017–2018 growing seasons with soybean at the Lageado Experimental Farm of São Paulo State University (UNESP), located in the municipality of Botucatu in the southeastern region of São Paulo State, Brazil (48°26'W, 22°51'S, elevation of 786 m above sea level). According to the Brazilian soil classification system (Santos et al., 2018), the soil is classified as a Latosol, which corresponds to the classifications of Ferralsol (FAO, 2006) and Oxisol (Soil Survey Staff, 2014). According to the Köppen-Geiger climatic classification system (Alvares et al., 2013), the region has a mesothermic climate (Cwa), that is, a humid subtropical climate with dry winters and hot summers. The average rainfall is 1,360 mm $year^{-1}$, and the mean annual air temperature is 20.7°C (50-year average) (Unicamp, 2020). Climatological data for the study period were collected from meteorological stations located near the experimental location. The characterization of short dry spells in southeastern Brazil during the monsoon season (from October to April) were comprehensively established by Cunningham (2020) based on an analysis of a historical series of 37 austral summer seasons (from 1979 to 2016).

Soil texture (Donagema et al., 2017) and chemical (van Raij et al., 2001) properties at a depth of 0.00–0.20 m were determined prior to the establishment of the experiment and are presented in Table S1. The quantification of the autochthonous bacterial population capable of nodulating soybean was estimated by the most probable number (MPN) for soybean plants according to O'Hara et al. (2016).

Liming to increase the base saturation (BS) of the topsoil (0.00–0.20-m depth) to 70% was conducted approximately 60 days prior to the beginning of the experiment

using dolomitic limestone [$CaMg(CO_3)_2$] (280 g kg^{-1} of calcium oxide—CaO, 180 g kg^{-1} of magnesium oxide—MgO, and 81% of calcium carbonate equivalents—$\%E_{CaCO3}$) according to the methodology described by Quaggio and van Raij (1997).

Experimental Design and Treatments

A randomized complete block (RCB) design involving a 4 (inoculant treatments) × 2 (metabolite applications) × 2 (growing seasons) factorial scheme was used with four replicates.

The inoculation treatments factor was represented by (*i*) standard inoculation (SI) with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) via seeds at sowing; (*ii*) SI + foliar spraying with *Bacillus subtilis* (Bs) (strain QST 713) at the V_3 growth stage (Fehr and Caviness, 1977); (*iii*) SI + foliar spraying with *A. brasilense* (Ab) (strains Ab-V5 and Ab-V6) at the V_4 growth stage; (*iv*) SI + foliar spraying with Bs at V_3 + foliar spraying with Ab at V_4. The metabolite applications factor was represented by the presence or absence of application of microbial secondary metabolites of *B. diazoefficiens* USDA 110 + *Rhizobium tropici* CIAT 899. Finally, the field experiments were conducted in the growing seasons of 2016–2017 and 2017–2018.

Microbial Inoculants and Metabolites

The standard inoculation (SI) was carried out with liquid inoculants containing *B. japonicum* strain SEMIA 5079 (= CPAC 15, = CNPSo 07) and *B. diazoefficiens* strain SEMIA 5080 (= CPAC 7, = CNPSo 06) at a total concentration of 7×10^9 cells mL^{-1} and applied to provide 1.2×10^6 cells $seed^{-1}$. SI is a common practice in soybean cultivation in Brazil (Hungria et al. 2006). The Brazilian strain selection program for natural variants of *B. japonicum* and *B. diazoefficiens* adapted to environmental conditions was detailed previously by Hungria and Vargas (2000), and the strains used in this study are representative of the majority of strains applied to soybean-cropped areas in tropical regions.

The microbial secondary metabolites (MSM) were extracted from *R. tropici* strain CIAT 899 (= CNPSo 103, = SEMIA 4077) and *B. diazoefficiens* strain USDA 110 (= CNPSo 56), which are enriched in lipo-chitooligosaccharides (LCOs), according to

Marks et al. (2013, 2015). The MSM was lyophilized and resuspended in a mixture of acetonitrile and water (20%; w/v) (Marks et al., 2015) prior to soybean sowing. The MSM concentration was adjusted to 1.0 mL L^{-1}, which corresponds to approximately 10^{-8} M, and applied at rate of 4 mL kg^{-1} of seeds.

Foliar inoculation (by foliar spraying) of *B. subtilis* strain QST 713 (1×10^9 cells mL^{-1}) was performed at the V_3 soybean phenological stage (Fehr and Caviness, 1977) by applying 3 L of the inoculant in 200 L of water to 1 ha. Foliar spraying of *A. brasilense* strains Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084) at a concentration of 2×10^8 cells mL^{-1} was carried out at the V_4 soybean phenological stage (Fehr and Caviness, 1977) by applying 300 mL of inoculant in 150 L water to 1 ha.

Foliar spraying with *A. brasilense* strains and *B. subtilis* was carried out according to the technical recommendations of the manufacturers and was always performed in the late afternoon (5:00 pm). These products are registered with the Ministry of Agriculture, Livestock, and Food Supply of Brazil. The *Bradyrhizobium* and *A. brasilense* strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soybean" (WFCC Collection # 1213, WDCM Collection # 1054), and the *B. subtilis* strain was developed by Bayer CropScience (European Union, Reg. (EC) No. 839/2008)).

Crop Management Practices History

Prior to soybean sowing (cultivar TMG 1264 RR; Tropical Breeding & Genetics), the experimental area was cultivated with black oats (*Avena strigosa* Schreb.) to provide straw for the no-tillage system. Each plot consisted of 10 rows spaced 0.45 m apart. The plots were separated by 0.5-m-wide rows and 1.5-m-wide terraces to avoid cross-contamination (bacteria and fertilizer) from rainfall surface runoff. The soybean plants were fertilized with 300 kg ha^{-1} of 00–20–20 (60 kg ha^{-1} of P_2O_5 and 60 kg ha^{-1} of K_2O) in both growing seasons. Seeds were treated with fungicides (carboxin + thiram at 100 g + 100 g a.i. 100 kg^{-1} seeds) prior to inoculation and sowing. Seed inoculation was performed 1 h before sowing. Foliar inoculations were carried out with a tractor-mounted sprayer. At phenological stage V_4 (Fehr and Caviness, 1977), all treatments received a foliar application of 20 g ha^{-1} of molybdenum (Mo) (as $Na_2MoO_4.2H_2O$) and 2 g ha^{-1} of cobalt (Co) (as $CoCl_2.\ 6H_2O$), which are required to maximize BNF with

soybean (Embrapa, 2020). Phytosanitary treatments were carried according to the recommendations of Embrapa (2020) when necessary.

Sampling, Measurements and Data Analyses

Soil Water Content

Prior to the establishment of the experiment, the soil water-holding capacity was determined according to the tension table and the Richards extractant chamber methods (Cassel and Nielsen, 1986), which allowed the determination of the soil water potential (ψ_w). The climatological water balance was monitored and calculated via electronic spreadsheets according to the methods proposed by Rolim et al. (1998) following the procedure of Thornthwaite and Mather (1955) to determine the real evapotranspiration (ETr). The climatological water balance of the two experimental growing seasons is shown in Figure 1.

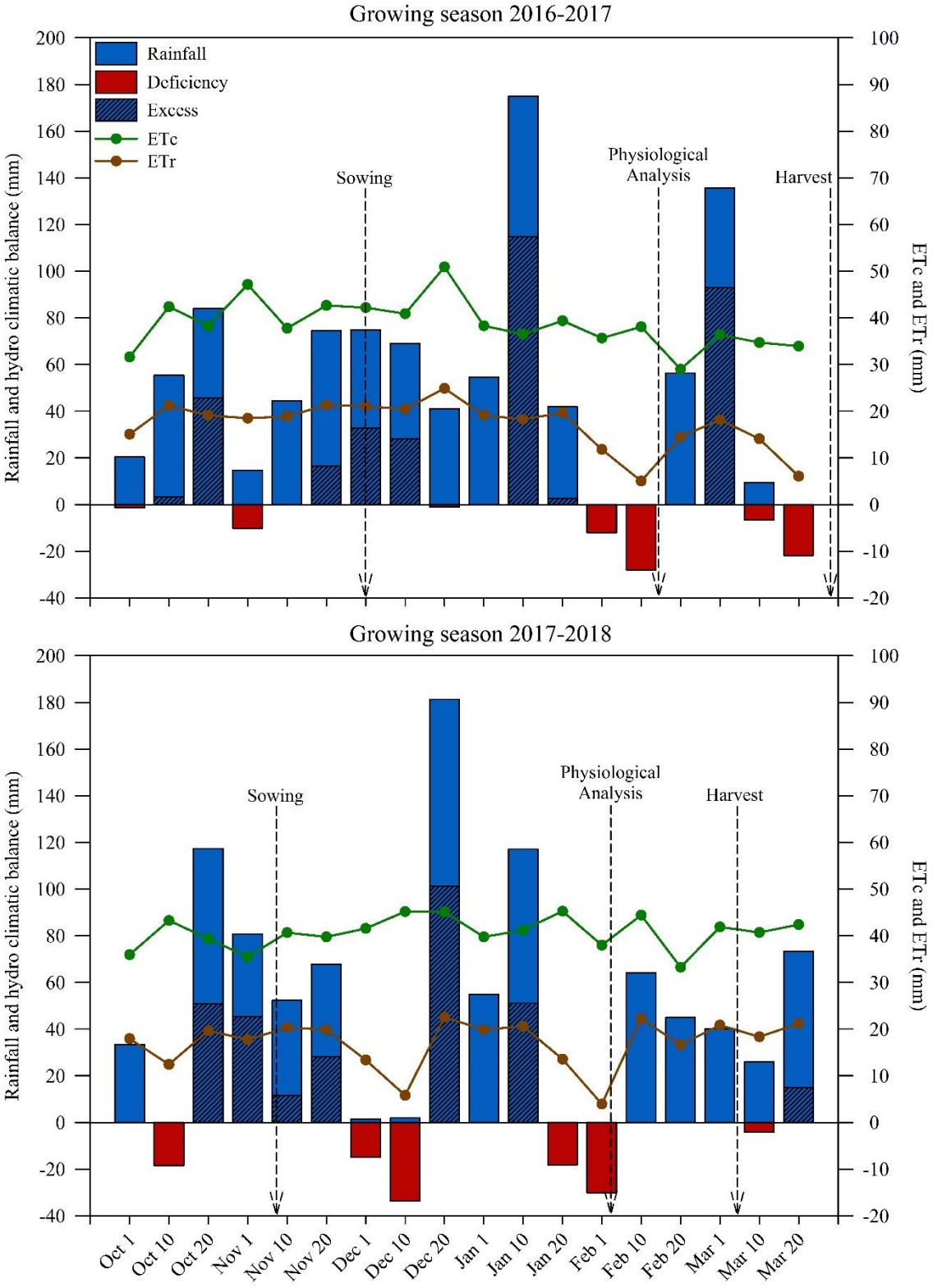


Figure 1. Climatological water balance at the experimental site during the 2016-2017 and 2017-2018 growing seasons.

ETc, crop evapotranspiration; ETr, real evapotranspiration; R, rainfall. The arrows indicate the key time points in the study.

In both growing seasons, at the R_5 growth stage (beginning of seed development) (Fehr and Caviness, 1977), which corresponds to seeds with a length of approximately 3.0 mm in the pod at one of the four uppermost nodes on the main stem, photosynthetic pigments, gas exchange, oxidative stress, antioxidant metabolism, and proline content were measured in the third fully expanded leaves of plants under moderate drought from short dry spells.

Photosynthetic Pigments

Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), total chlorophyll (total chl) and total carotenoid (total car) contents were determined in the third fully expanded leaves from five discs cut between the edge and the central rib of the soybean leaves using a paper punch (0.5 cm in diameter) at the R_5 growth stage. The discs were incubated for 24 h in capped glass vials containing 2 mL of N, N-dimethylformamide (DMF) and wrapped in aluminum foil according to the methodology proposed by Lichtenthaler (1987). Pigment concentrations were quantified using the spectrophotometric method at wavelengths of 664, 647, and 480 nm for chlorophyll *a*, *b* and carotenoids, respectively. The pigment concentrations were determined as proposed by Wellburn (1994).

Gas Exchange

Gas exchange was evaluated via nondestructive analysis with a portable gas exchange device (LI-6400 infrared gas analyzer (IRGA), LI-COR Biosciences Inc., Lincoln, NE, USA). Samples were taken from the central leaflet of the third fully expanded leaves and intact trifoliate leaf from the plant apex of the main stem of 10 plants per plot. The parameters of the instrument were as follows: 380–400 mol mol^{-1} atmospheric CO_2, 1100 µmol quanta m^{-2} s^{-1} of photosynthetically active radiation (PAR) supplied by LED lamps, 25–27°C leaf chamber temperature, and 60–70% relative humidity. The minimum equilibration time for each set of measurements was 3 min. The measurements were performed between 10:00 and 12:00 am. The following parameters were determined: net photosynthesis rate (*A*; µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_S; mol H_2O m^{-2} s^{-1}), internal CO_2 concentration in the substomatal cavity (*Ci*; µmol mol^{-1}), and transpiration (*E*; mmol H_2O m^{-2} s^{-1}).

Oxidative Stress and Antioxidant Metabolism

To evaluate the contents of H_2O_2 and malondialdehyde (MDA) as well as the activities of SOD (EC:1.15.1.1), CAT (EC:1.11.1.6) and APX (EC:1.11.1.11), sampling was performed on the same leaflets taken at the R_5 growth stage to assess the gas exchange parameters. The sampled tissue was then flash-frozen in liquid N and stored at −80 °C.

Lipid peroxidation was evaluated according to the method of Heath and Packer (1968). To calculate MDA content, a molar extraction coefficient of 155 mM^{-1} cm^{-1} was used, and the results were expressed in nanomoles of MDA per gram of fresh weight (FW). The H_2O_2 content was determined according to the method of Alexieva et al. (2001). The content was calculated based on a calibration curve and expressed in micromoles of H_2O_2 per gram of FW. Extractions for enzyme analysis were performed according to the methodology described by Silva et al. (2020). SOD activity was evaluated as described by Giannopolitis and Ries (1977), and the results were expressed in units (U) of SOD per milligram of protein. CAT activity was evaluated as described by Azevedo et al. (1998), and the results were expressed in micromoles per minute per milligram of protein. APX activity was evaluated according to Gratão et al. (2008), and the results were expressed in nanomoles per minute per milligram of protein.

Proline Content

Proline content was determined according to Torello and Rice (1986) in the same leaflets collected at the R_5 growth stage used for the other analyses. The absorbance at wavelengths of 647 and 664 nm was determined via a spectrophotometer, and the results were expressed per gram of FW, as described by Mauad et al. (2016).

Crop Yield

Additionally, at the R_5 stage, five plants per plot were harvested 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, plants were harvested from a 15-m^2 area from the central part of each plot and estimated the final population of plants, plant height, position of insertion of the first pod, numbers of branches and pods per plant, and number of grains per pod. Grain yield (kg ha^{-1}) and thousand-grain weight (W1000) were converted to values on a dry weight basis by correcting for 13% moisture. The moisture was determined with an automatic measuring device (Gehaka G650i, Brazil).

Statistical Analyses

All data were initially analyzed via the Shapiro-Wilk test (Shapiro and Wilk, 1965) for normality and the Levene test for homoscedasticity (Levene, 1960), both at $p < 0.05$; the UNIVARIATE procedure of SAS version 9.4 was used for the analysis (SAS Institute, 2015). The data were also tested for sphericity by the Bartlett test (Bartlett, 1937) via the FACTOR procedure of SAS version 9.4 (SAS Institute, 2015). The results indicated that all data were distributed normally ($W \geq 0.90$) and exhibited no sphericity. The data were then analyzed with the linear mixed-effect model by the PROC MIXED procedure of SAS and with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed-effects tests. The blocks and interactions between blocks were considered as random effects, whereas bacterial consortium, microbial metabolites, growing season, and their interactions were considered as fixed effects. The results were reported as the least square means and were separated by the probability of differences option (PDIFF). The means were compared using the least significant difference (LSD) test. The main factors and interactive effects were considered statistically significant at $p \leq 0.05$. The heatmap was built calculating the Pearson correlation coefficients ($p \leq 0.05$) and only the significant correlations were showed.

3.3 RESULTS

Photosynthetic Pigments

The photosynthetic pigments of soybean plants did not differ between those that received SI plus inoculation with *B. subtilis* and those that received SI (*Bradyrhizobium* spp. only) (Table S2). However, plants inoculated with *A. brasilense* strains exhibited higher concentrations of these pigments compared to plants that were not inoculated with *A. brasilense*, and application of MSM further increased these levels. A significant interaction between bacterial consortium and MSM application was observed ($p \leq 0.01$; Table S2) for both chlorophyll and carotenoid contents in soybean leaves at the R_5 reproductive stage in both growing seasons (Figure 2). Plants treated with SI plus *A. brasilense* and MSM exhibited increases in chl *a*, chl *b*, total chl, and total car contents of up to 14.5%, 30.8%, 17.2%, and 27.3%, respectively, compared with the plants receiving SI only. The results for the two growing seasons were similar.

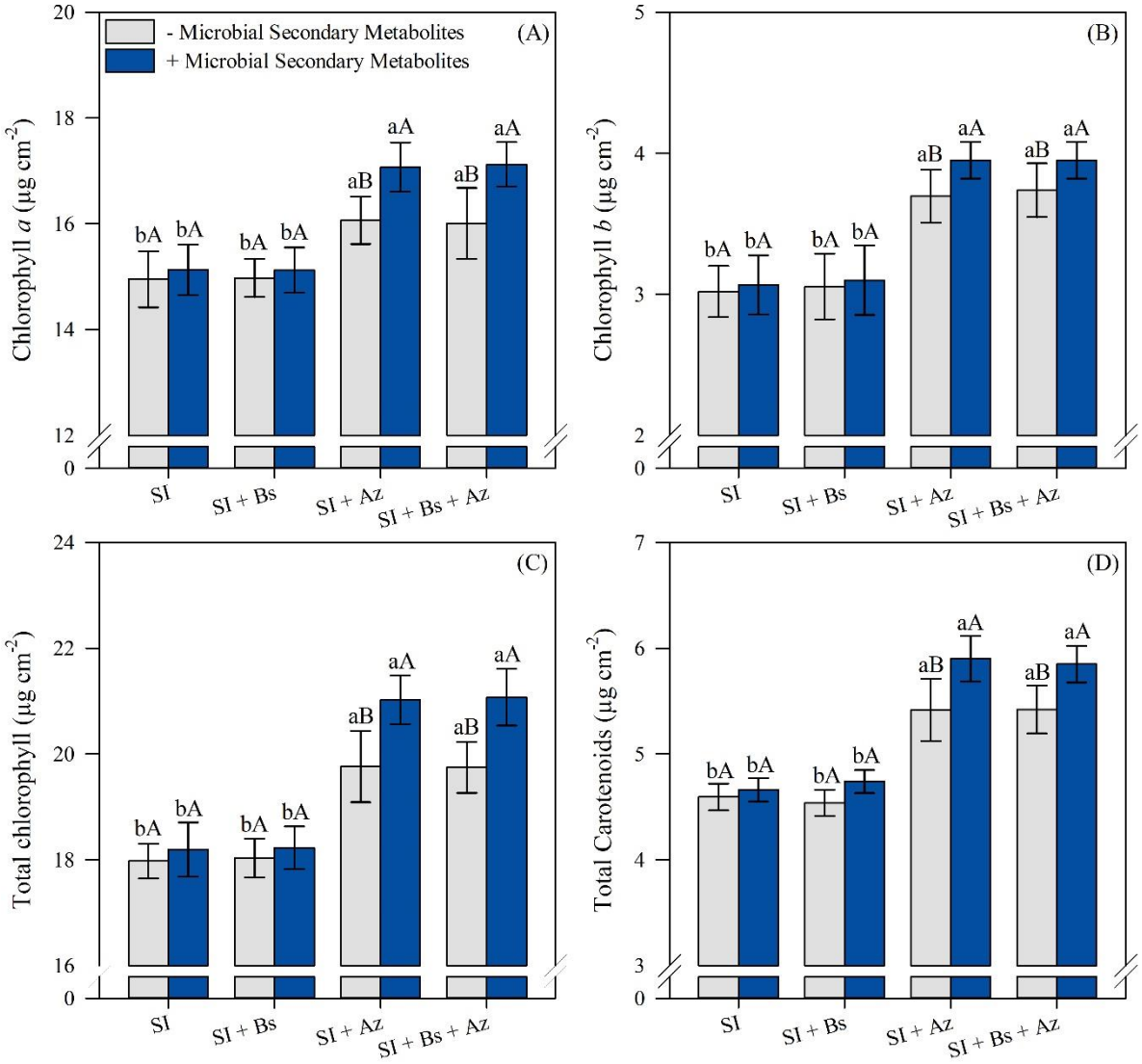


Figure 2. Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (total chl), and total carotenoids (total car) in soybean leaves at the R_5 reproductive stage as a function of bacterial consortium and microbial metabolites under field conditions.

Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher's least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

Gas Exchange

The effects of the different treatments on gas exchange parameters were similar to those on photosynthetic pigments, highlighting the benefits of foliar spraying of *A. brasilense* and MSM application (Table S3). Interaction effects of bacterial consortium and MSM inoculation on the net photosynthesis rate (*A*) ($p \leq 0.01$), stomatal conductance (*gs*) ($p \leq 0.01$), internal CO_2 concentration in the substomatal cavity (*Ci*) ($p \leq 0.01$) and transpiration (*E*) ($p \leq 0.01$) were detected (Table S3). Additionally, *A*, *gs*, *Ci*, and *E* increased significantly by up to 17.7%, 56.5%, 8.3%, and 44.0%, respectively, in all plants receiving SI plus foliar spraying of *A. brasilense* strains and MSM application (Figure 3A-D) compared to plants receiving SI alone.

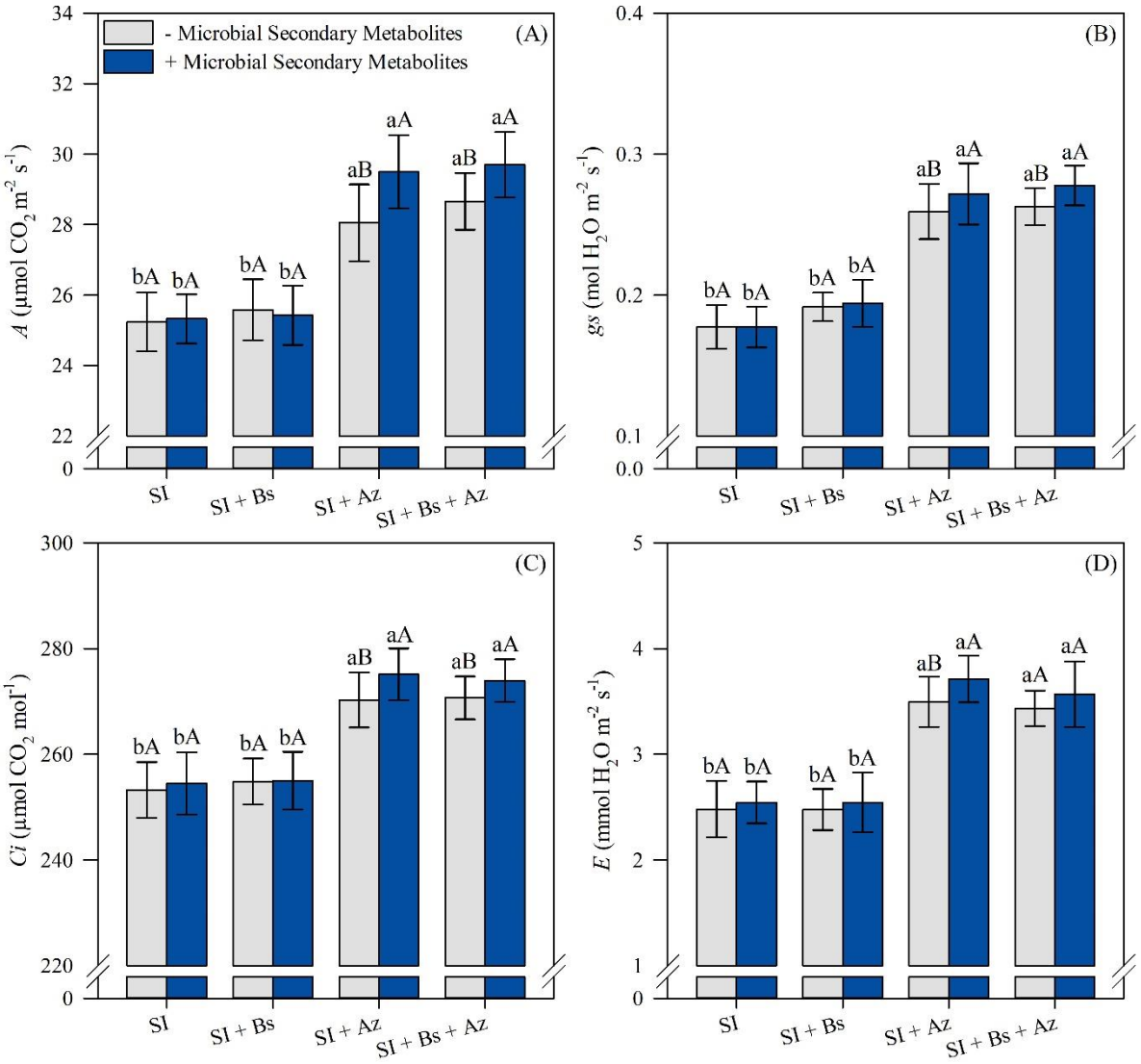


Figure 3. Net photosynthetic rate (*A*), stomatal conductance (g_S), internal CO_2 concentration in the substomatal chamber (*Ci*), and transpiration (*E*) of soybean leaves at the R_5 reproductive stage as a function of bacterial consortium and microbial metabolites under field conditions.
Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher's least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

Oxidative Stress, Antioxidant Metabolism and Proline Content

Interaction effects of bacterial consortium and MSM inoculation on H_2O_2 content ($p \leq 0.01$), lipid peroxidation (assayed via the MDA content) ($p \leq 0.01$), SOD ($p \leq 0.01$), CAT ($p \leq 0.01$), and APX ($p \leq 0.01$) activities, and proline content ($p \leq 0.01$) in soybean leaf tissue were detected (Table S4). In addition, H_2O_2 content, lipid peroxidation rates, and proline content were highest in plants receiving SI only (Figure 4A-B-F). By contrast, plants receiving SI plus foliar spraying with *A. brasilense* strains and MSM application exhibited 18.8% lower H_2O_2 content, 29.4% lower proline content, and 15.9% lower lipid peroxidation in leaf cell membranes. Moreover, this treatment resulted in significant decreases in SOD (-18.2%), CAT (-21.2%), and APX (-19.1%) activities compared to SI treatment alone (Figure 4C-D-E).

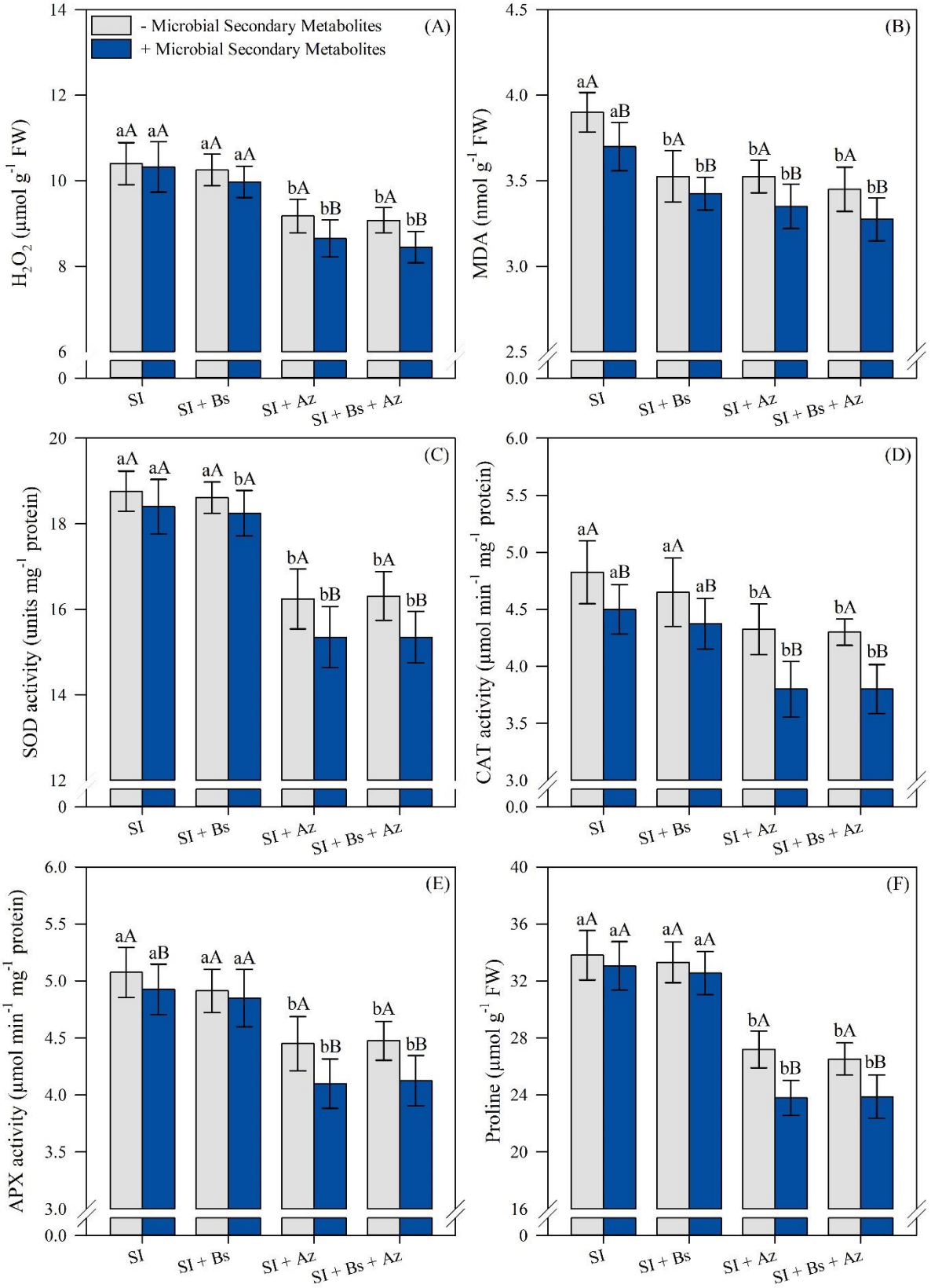


Figure 4. Hydrogen peroxide (H_2O_2) content, malondialdehyde (MDA) content, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities, and proline content in soybean leaves at the R_5 reproductive stage as a

function of bacterial consortium and microbial metabolites under field conditions. Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher's least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

Crop Yield

Positive interactions effects of bacterial consortium (*Bradyrhizobium* plus *A. brasilense* strains) and rhizobial metabolites application were observed on soybean nodule number (NN, 19%), nodule dry weight (NDW, 21%), root dry weight (RDW, 17.2%) and shoot dry weight (SDW, 20.6%) when compared to exclusively SI ($p \leq 0.01$, Table S5) (Figure 5A-D). At physiological maturity, significant interactions effects of bacterial consortium and MSM application on soybean plant height (PH), number of pods, thousand-grain weight (W1000) and grain yield (GY) were detected ($p \leq 0.01$, Table S5). Plant height, pods, W1000 and GY increased considerably when plants received SI plus foliar application of *A. brasilense* strains and MSM application, by up to 7.6% (PH), 25.3% (pods), 5.0% (W1000) and 12.2% (GY), respectively, compared to SI alone (Figure 6A-D). No benefits of foliar application of *B. subtilis* were observed. Overall, significant effects were not observed in the final population of plants (mean = 288,886 plant ha^{-1}), position of insertion of the first pod (mean = 12 cm), number of branches per plant (mean = 3), nor in the number of grains per pod (mean = 2.2) (data not shown).

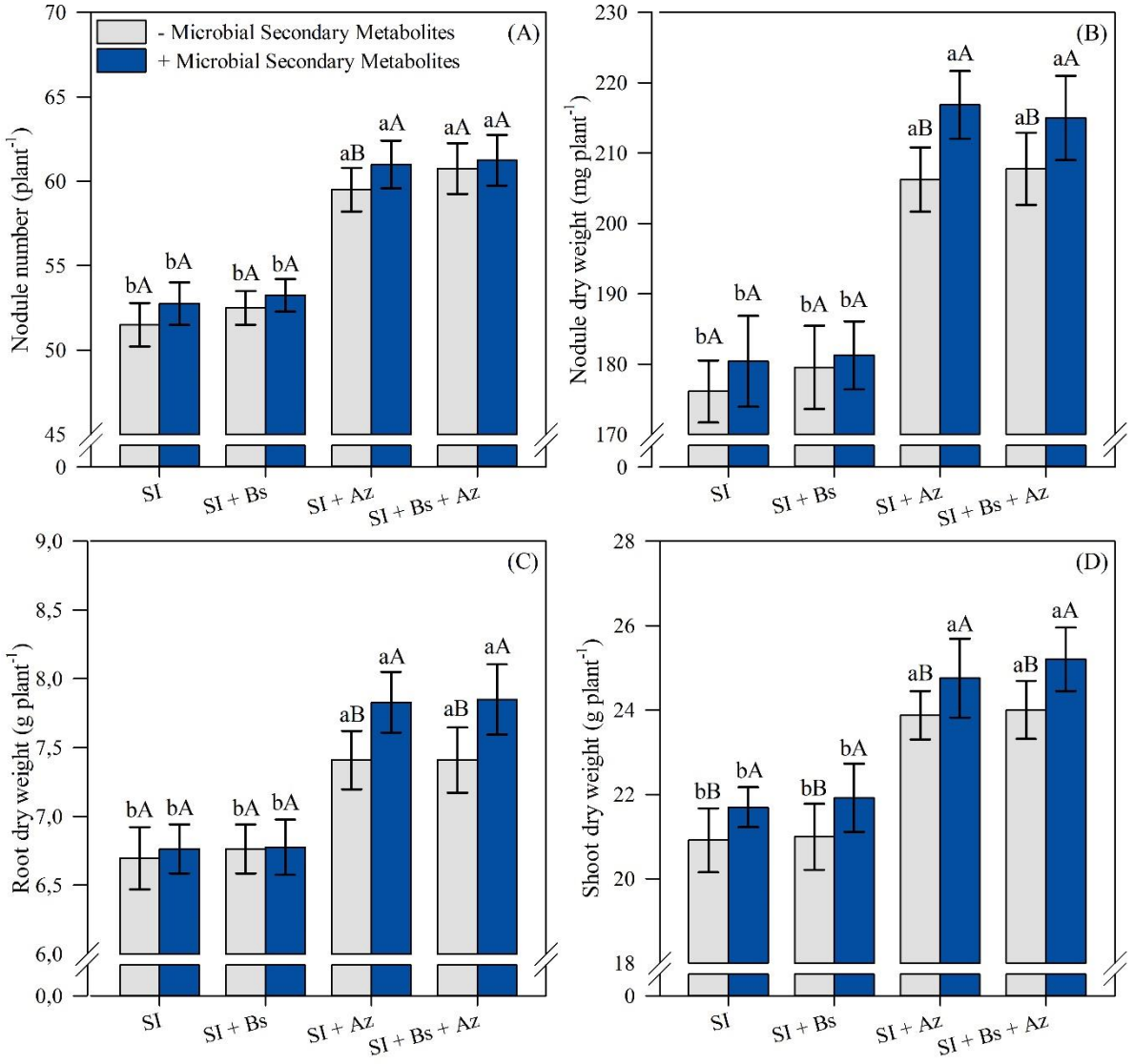


Figure 5. Nodule number, nodule dry weight, root dry weight, shoot dry weight in soybean at the R_5 reproductive stage as a function of bacterial consortium and microbial metabolites under field conditions.

Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher's least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

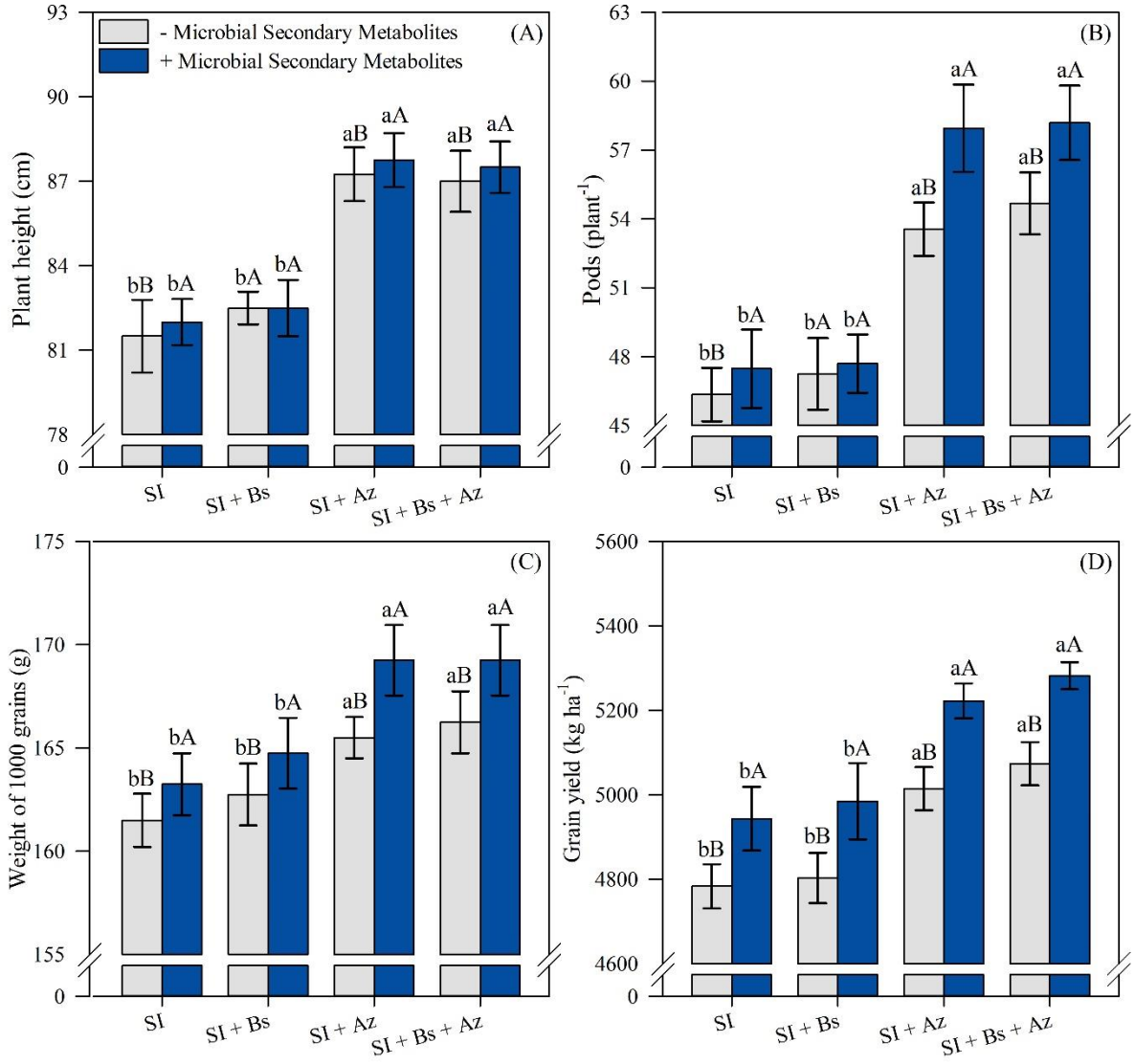


Figure 6. Plant height, pods, soybean 1000-grain weight (W1000) and grain yield (GY) as a function of bacterial consortium and microbial metabolites under field conditions. Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher's least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

Correlation analysis between plant measurements

Changes in pigments concentrations correlated positively with all photosynthetic parameters from soybean gas exchange measurements, and both correlated with

soybean plant responses, including root nodulation and dry weight, as well as production of shoot, pods and grains (Figure 7). On the other hand, oxidative stress and antioxidant metabolism correlated negatively with all other parameters studied and positively with themselves.

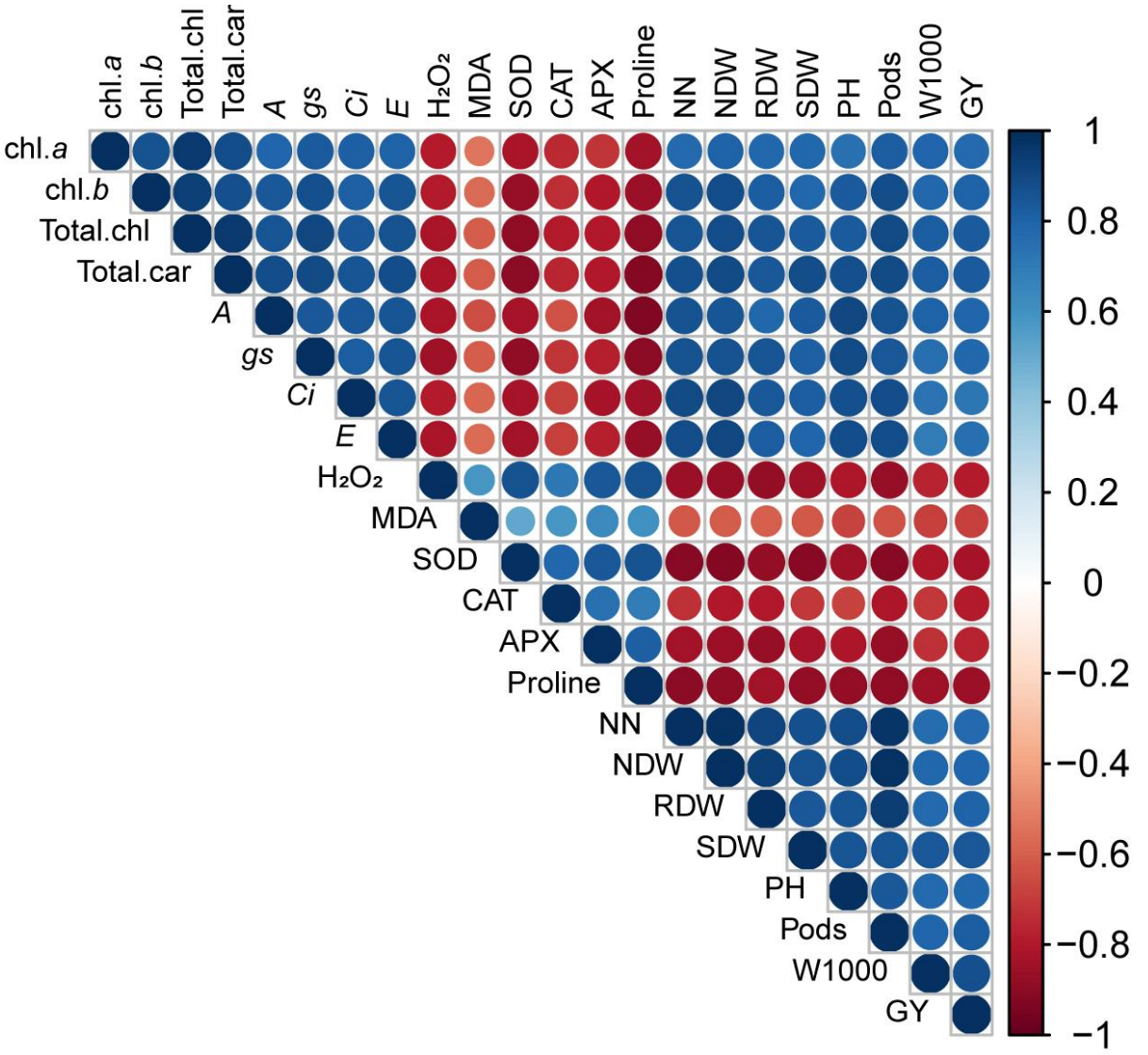


Figure 7. Heatmap of the Pearson correlation coefficients matrix between soybean physiological, biochemical and agronomic parameters.

Only significant correlations at $p \leq 0.05$ are shown. Chlorophyll *a* (Chl *a*), chlorophyll *b* (chl *b*), total chlorophyll (Total chl), total carotenoids (Total car), net photosynthesis rate (*A*), stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), transpiration (*E*), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), proline, nodule number (NN), nodule dry

weight (NDW), root dry weight (RDW), shoot dry weight (SDW), plant height (PH), pods, thousand-grain weight (W1000) and grain yield (GY).

3.4 DISCUSSION AND CONCLUSIONS

For successful yield potential, soybean cultivation in South America requires 450 to 800 mm of rainfall (Embrapa, 2020). The maximum crop requirement for water occurs between flowering and grain filling, with ETr of ~7–8 mm day^{-1} (Embrapa, 2020) and a crop coefficient (Kc) of ~1.5 (Farias et al., 2001). In the present study, total rainfall was 789 and 705 mm in the 2016–2017 and 2017–2018 growing seasons, respectively. However, there was an uneven distribution of rain during the crop cycle. Short dry spells with negative hydric balance lasting 17 and 14 days were observed in the first and second growing seasons, respectively, both prior to the R5.1 reproductive stage (Fig. 2) and during the critical stage of early grain filling. According to Flexas et al. (2004), the stress level can be quantified via stomatal conductance, where values ≥ 0.2 mol H_2O m^{-2} s^{-1} represent well-watered plants, values of 0.1–0.2 mol H_2O m^{-2} s^{-1} represent moderate drought-stressed plants, and values ≤ 0.1 mol H_2O m^{-2} s^{-1} represent severely drought-stressed plants. According to this classification, in both growing seasons the plants treated with SI only were under moderate drought stress at the R_5 stage.

Short dry periods during the summer cropping season are common in tropical regions (Silva et al. 2019) and, combined with the high temperature and evapotranspiration typical of this period of the year, greatly impact crop productivity, particularly during critical periods of plant development such as flowering and grain filling (Basu et al., 2016). In addition, water restriction during the reproductive stage (R_1–R_5; beginning of flowering until grain formation) leads to several physiological, morphological, and biochemical changes resulting in loss of leaf turgor, premature flower dropping, pod abortion and ultimately lower grain yields (Nelson et al., 2005).

Chlorophyll is the main photosynthetic pigment in the Calvin–Benson–Bassham (CBB) cycle (Croft et al. 2017; Busch 2020). Reduced chlorophyll content decreases the light energy uptake and light utilization capacity of plants, resulting in a decrease in photosynthesis (Ansari et al. 2019). Carotenoids are responsible for plastid adaptation to stress conditions such as light and energy dissipation and avoiding excessive ROS production (Rodriguez-Concepcion et al., 2018). One of the main

effects of drought in higher plants is reduction and/or inhibition of photosynthesis (Reddy et al., 2004), as reflected by decreased synthesis of chlorophyll and carotenoids. As schematized in Figure 8, our field experiments in this tropical region clearly demonstrated that the bacterial consortium represented by seed treatment with *Bradyrhizobium* spp. and rhizobial metabolites (MSM) and foliar application of *A. brasilense* at the V_4 stage increased photosynthetic pigments, namely, chl *a*, chl *b*, total chl, and total car. However, the increased growth of plants inoculated with MSM could be at least partially related to indirect effects of LCOs on photosynthesis and plant growth acceleration by stimulating mitotic activity in the meristematic tissue of leaves (Khan et al., 2020).

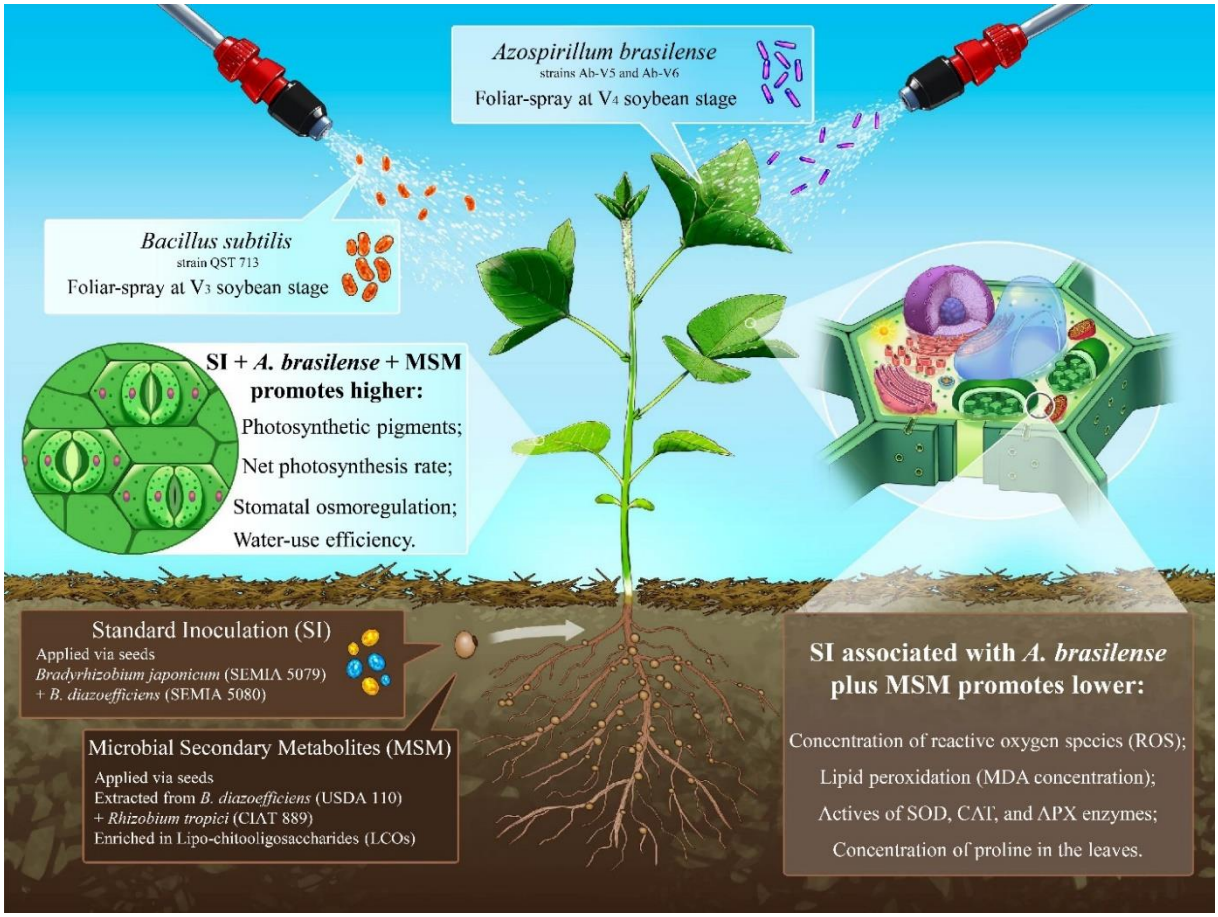


Figure 8. Schematic graphic representing the treatments and corresponding results in the field experiment.

Our results also demonstrated that, compared with plants inoculated exclusively with *Bradyrhizobium* spp., the combination of *Bradyrhizobium* spp. inoculation with *A. brasilense* and MSM treatment improved the efficiency of gas exchange, including

stomatal conductance and transpiration. Regulating stomatal conductance under conditions of low hydric availability is key to improving water-use efficiency (Gupta et al., 2020). Our results suggest that *A. brasilense* plus MSM positively influenced stomatal opening and closing, which directly affected the process of gas exchange and contributed to increased biomass accumulation. A previous study conducted on the same site also reported important roles of MSM in *Bradyrhizobium*-soybean symbiosis (Moretti et al., 2020b). This previous study suggested that a more developed root system increases soil exploration and, in turn, the distribution and activity of soybean roots, which might indirectly ensure plant metabolic activities and soybean establishment and favor tolerance to moderate water restriction.

The gradual loss of photosynthetic capability due to drought conditions results in accumulation of electrons in photosystems I and II, leading to increased ROS levels and lipid peroxidation (Croft et al., 2017). ROS can severely damage the photosynthetic apparatus, resulting in lipid peroxidation (Loix et al., 2018). To mitigate the effects of oxidative stress and reduce cell damage, a variety of enzymes in antioxidant metabolism are activated to inactivate ROS (Farooq et al., 2019). In the present study, the H_2O_2 concentration and lipid peroxidation rate in soybean leaves were greater in plants inoculated just with *Bradyrhizobium* spp. and with *B. subtilis* by foliar spraying at the V_3 stage. By contrast, the lower activities of SOD, CAT and APX in soybean leaves of plants treated with *Bradyrhizobium* spp. + MSM + *A. brasilense* indicate an increased ability to mitigate ROS compared with inoculation with *Bradyrhizobium* alone. Indeed, SOD, CAT and APX activities have been used as important indicators of PGPB-induced plant resistance to abiotic stresses, intracellular ROS removal, reduced membrane peroxidation, stabilization of membrane permeability and improved plant photosynthesis (Sandhya et al., 2010; Fukami et al., 2018b,c).

Fukami et al. (2017) reported that foliar spraying of maize (*Zea mays* L.) with *A. brasilense* resulted in regulation of oxidative stress genes in leaves (APX1, APX2, SOD2, SOD4); however, the up-regulation of these genes was always greatest when foliar spraying was combined with the application of rhizobial metabolites. This previous study corroborates our results and suggests that the persistence of oxidative stress is reduced when live cells (only *Azospirillum* spp.) are applied in combination with rhizobial metabolites.

Proline is an important stress marker molecule associated with osmoregulation that prevents dehydration under negative soil water potential (ψ_w) (Goswami et al. 2016). In our study, proline content was significantly lower in soybean leaves of plants treated with the consortium comprising *Bradyrhizobium* spp., MSM and *A. brasilense* strains compared with plants treated with SI alone, suggesting an important impact of the former consortium on avoiding oxidative damage and reducing the accumulation of proline in tissues under water stress. Similarly, Fukami et al. (2018b) reported that inoculation of maize with *Azospirillum* reduced proline synthesis, and the decrease in proline content was correlated with the maintenance of plant metabolism under abiotic stress.

Drought stress significantly affected the concentration of chlorophyll and carotenoids (Fig. 2), photosynthetic capacity and carbon assimilation of plants (Fig. 3), influencing root and shoot the dry weight (Fig. 5), seriously reflecting 1000-grain weight (Fig. 6). The photoassimilates produced in the source leaves, mainly sucrose, are the main components of C metabolism and amino acid biosynthesis for the development of the grains (Du et al., 2020).Water stress during the grains filling phase inhibited the synthesis and accumulation of carbohydrates, probably by blocking cell division (Awasthi et al., 2014; Sehgal et al., 2017), which reduced energy demand and promoted the early completion of the grain filling process, resulting in considerable losses in productivity (Fig. 6). In this study, plants treated only with SI presented significant decrease in nodulation, inhibition of root and shoot growth development, photosynthetic activity, and decrease in thousand-grain weight and yield. Taken together (Fig. 7), the results indicate that a beneficial bacterial consortium comprising *Bradyrhizobium* spp. and *A. brasilense* strains combined with MSM application help plants in the accumulation of osmolytes, antioxidants metabolism and upregulate stress-responsive gene for a better acquisition of drought tolerance, ensuring nodulation, root and shoot development, improving the photosynthetic activity of the plant and the production of photoassimilates and, finally, the filling of the grains.

Overall, the beneficial effects of the consortium of *Bradyrhizobium* spp., MSM and *A. brasilense* strains on alleviating soybean water stress under field conditions resulted in improvements in the 1000-grain weight and in grain yield by up to 574 kg ha^{-1} compared with inoculation with *Bradyrhizobium* spp. alone. Therefore, this consortium represents an effective strategy to guarantee and increase soybean grain yield by improving plant osmotic adjustment, maintenance of physiological processes

and biological nitrogen fixation during the short dry spells that occur frequently in tropical regions. This study illustrates the feasibility of identifying beneficial bacterial consortia that can promote plant growth and elicit plant tolerance to abiotic stress, which represents an important biotechnological strategy to achieve sustainable food production and security.

Author contribution statement

L.G.M., C.A.C.C. and M.H. worked on the research designing and conduction, data analysis, writing and formatting the manuscript. J.W.B., J.C.C., A.M., A.G., L.M., E.E.K. revised this draft by rewriting, discussing and commenting. All authors contributed significantly on the manuscript, confirm being contributor of this work and has approved it for publication. We confirm that none of the material in this manuscript has been published or is under consideration for publication elsewhere.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

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REFERENCES

Abbasi, S., Zahedi, H., Sadeghipour, O., Akbari, R., 2013. Effect of plant growth promoting rhizobacteria (PGPR) on physiological parameters and nitrogen content of soybean grown under different irrigation regimes. Res. Crop. 14, 798–803.

Alam, N.B., Ghosh, A., 2018. Comprehensive analysis and transcript profiling of Arabidopsis thaliana and Oryza sativa catalase gene family suggests their specific roles in development and stress responses. Plant Physiol. Biochem. 123, 54–64. https://doi.org/10.1016/j.plaphy.2017.11.018
Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell Environ. 24, 1337–1344. https://doi.org/10.1046/j.1365-3040.2001.00778.x

Alvares, C.A., Stape, J.L., Sentelhas, P.C., De Moraes Gonçalves, J.L., Sparovek, G., 2013. Köppen's climate classification map for Brazil. Meteorol. Zeitschrift 22, 711–728. https://doi.org/10.1127/0941-2948/2013/0507

Ansari, F.A., Ahmad, I., Pichtel, J., 2019. Growth stimulation and alleviation of salinity stress to wheat by the biofilm forming Bacillus pumilus strain FAB10. Appl. Soil Ecol. 143, 45–54. https://doi.org/10.1016/j.apsoil.2019.05.023

Assad, E.D., Sano, E.E., Masutomo, R., Castro, L.H.R. de, Silva, F.A.M. da, 1993. Veranicos na região dos cerrados brasileiros frequência e probabilidade de ocorrência. Pesqui. Agropecuária Bras. 28, 993–1003.

Awasthi, R., Kaushal, N., Vadez, V., Turner, N.C., Berger, J., Siddique, K.H.M., Nayyar, H., 2014. Individual and combined effects of transient drought and heat stress on carbon assimilation and seed filling in chickpea. Funct. Plant Biol. 41, 1148–1167. https://doi.org/10.1071/FP13340

Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J., 1998. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiol. Plant. 104, 280–292. https://doi.org/10.1034/j.1399-3054.1998.1040217.x

Bartlett, M.S., 1937. Properties of sufficiency and statistical tests. Proc. R. Soc. London. Ser. A - Math. Phys. Sci. 160, 268–282. https://doi.org/10.1098/rspa.1937.0109

Bashan, Y., de-Bashan, L.E., 2010. How the plant growth-promoting bacterium azospirillum promotes plant growth-a critical assessment. Adv. Agron. 108, 77–136. https://doi.org/10.1016/S0065-2113(10)08002-8

Basu, S., Ramegowda, V., Kumar, A., Pereira, A., 2016. Plant adaptation to drought stress. F1000Research 5, 1–10. https://doi.org/10.12688/F1000RESEARCH.7678.1

Battisti, R., Sentelhas, P.C., 2017. Improvement of soybean resilience to drought through deep root system in Brazil. Agron. J. 109, 1612–1622. https://doi.org/10.2134/agronj2017.01.0023

Battisti, R., Sentelhas, P.C., 2014. New agroclimatic approach for soybean sowing dates recommendation: A case study. Rev. Bras. Eng. Agric. e Ambient. 18, 1149–1156. https://doi.org/10.1590/1807-1929/agriambi.v18n11p1149-1156

Bottini, R., Fulchieri, M., Pearce, D., Pharis, R.P., 1989. Identification of gibberellins A1, A3, and Iso-A3 in cultures of azospirillum lipoferum. Plant Physiol. 90, 45–47. https://doi.org/10.1104/pp.90.1.45

Busch, F.A., 2020. Photorespiration in the context of Rubisco biochemistry, CO2 diffusion and metabolism. Plant J. 101, 919–939. https://doi.org/10.1111/tpj.14674

Cassel, D.K., Nielsen, D.R., 1986. Field Capacity and Available Water Capacity, in: Klute, A. (Ed.), Methods of Soil Analysis: Part 1 Physical and Mineralogical Method. American Society of Agronomy, Soil Sciense Society of America, Madison, Wisconsin USA, pp. 901–926. https://doi.org/10.2136/sssabookser5.1.2ed.c36

Cerezini, P., Kuwano, B.H., Neiverth, W., Grunvald, A.K., Pípolo, A.E., Hungria, M., Nogueira, M.A., 2019. Physiological and N2-fixation-related traits for tolerance to drought in soybean progenies. Pesqui. Agropecuária Bras. 54, e00839. https://doi.org/10.1590/s1678-3921.pab2019.v54.00839

Cerezini, P., Pípolo, A.E., Hungria, M., Nogueira, M.A., 2014. Gas Exchanges and Biological Nitrogen Fixation in Soybean under Water Restriction. Am. J. Plant Sci. 05, 4011–4017. https://doi.org/10.4236/ajps.2014.526419

Chen, G., Wiatrak, P., 2010. Soybean development and yield are influenced by planting date and environmental conditions in the southeastern coastal plain, United States. Agron. J. 102, 1731–1737. https://doi.org/10.2134/agronj2010.0219

Ciampitti, I.A., Salvagiotti, F., 2018. New insights into soybean biological nitrogen fixation. Agron. J. 110, 1185–1196. https://doi.org/10.2134/agronj2017.06.0348

Cohen, A.C., Bottini, R., Piccoli, P.N., 2008. Azospirillum brasilense Sp 245 produces ABA in chemically-defined culture medium and increases ABA content in arabidopsis plants. Plant Growth Regul. 54, 97–103. https://doi.org/10.1007/s10725-007-9232-9

Croft, H., Chen, J.M., Luo, X., Bartlett, P., Chen, B., Staebler, R.M., 2017. Leaf chlorophyll content as a proxy for leaf photosynthetic capacity. Glob. Chang. Biol. 23, 3513–3524. https://doi.org/10.1111/gcb.13599

Cunningham, C., 2020. Characterization of dry spells in Southeastern Brazil during the monsoon season. Int. J. Climatol. 40, 4609–4621. https://doi.org/10.1002/joc.6478

Donagema, G.K., Viana, J.H.M., Almeida, B.G., Ruiz, H.A., Klein, V.A., Dechen, S.C.F., Fernandes, R.B.A., 2017. Granulometric analysis, in: Teixeira, P.C., Donagema, G.K., Fontana, A., Teixeira, W.G. (Eds.), Soil Analysis Methods Manual. Embrapa Solos, Brasília, DF, pp. 95–116.

Du, Y., Zhao, Q., Chen, L., Yao, X., Zhang, H., Wu, J., Xie, F., 2020. Effect of drought stress during soybean r2–r6 growth stages on sucrose metabolism in leaf and seed. Int. J. Mol. Sci. 21, 1–19. https://doi.org/10.3390/ijms21020618
Embrapa, 2020. Tecnologias de Produção de Soja, Sistemas de Produção 17. Embrapa Soja, Londrina, PR.

FAO, 2019. The State of Food Security and Nutrition in the World 2019, The State of Food Security and Nutrition in the World 2019. Food and Agriculture Organization of the United Nations. https://doi.org/10.4060/ca5162en
FAO, 2006. Guidelines for soil description.

Farias, J.R.B., Assad, E.D., Almeida, I.R., Evangelista, B.A., Lazzarotto, C., Neumaier, N., Nepomuceno, A.L., 2001. Caracterização de risco de déficit hídrico nas regiões produtoras de soja no Brasil. Rev. Bras. Agrometeorol. 9, 415–421.

Farooq, M.A., Niazi, A.K., Akhtar, J., Saifullah, Farooq, M., Souri, Z., Karimi, N., Rengel, Z., 2019. Acquiring control: The evolution of ROS-Induced oxidative stress and redox signaling pathways in plant stress responses. Plant Physiol. Biochem. 141, 353–369. https://doi.org/10.1016/j.plaphy.2019.04.039

Fehr, W.R., Caviness, C.E., 1977. Stages of soybean development.
Flexas, J., Bota, J., Loreto, F., Cornic, G., Sharkey, T.D., 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol. 6, 269–279. https://doi.org/10.1055/s-2004-820867

Fukami, J., Cerezini, P., Hungria, M., 2018a. Azospirillum: benefits that go far beyond biological nitrogen fixation. AMB Express 8, 73. https://doi.org/10.1186/s13568-018-0608-1

Fukami, J., De La Osa, C., Ollero, F.J., Megías, M., Hungria, M., 2018b. Co-inoculation of maize with Azospirillum brasilense and Rhizobium tropici as a strategy to mitigate salinity stress. Funct. Plant Biol. 45, 328–339. https://doi.org/10.1071/FP17167

Fukami, J., Ollero, F.J., de la Osa, C., Valderrama-Fernández, R., Nogueira, M.A., Megías, M., Hungria, M., 2018c. Antioxidant activity and induction of mechanisms of resistance to stresses related to the inoculation with Azospirillum brasilense. Arch. Microbiol. 200, 1191–1203. https://doi.org/10.1007/s00203-018-1535-x

Fukami, J., Ollero, F.J., Megías, M., Hungria, M., 2017. Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with Azospirillum brasilense cells and metabolites promote maize growth. AMB Express 7, 153. https://doi.org/10.1186/s13568-017-0453-7

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59, 309–14. https://doi.org/10.1104/pp.59.2.309

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Glick, B.R., 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica (Cairo). 1–15. https://doi.org/10.6064/2012/963401

Goswami, D., Thakker, J.N., Dhandhukia, P.C., 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food Agric. 2, 1127500. https://doi.org/10.1080/23311932.2015.1127500

Gratão, P.L., Monteiro, C.C., Antunes, A.M., Peres, L.E.P., Azevedo, R.A., 2008. Acquired tolerance of tomato (Lycopersicon esculentum cv. Micro-Tom) plants to cadmium-induced stress. Ann. Appl. Biol. 153, 321–333. https://doi.org/10.1111/j.1744-7348.2008.00299.x

Gupta, A., Rico-Medina, A., Caño-Delgado, A.I., 2020. The physiology of plant responses to drought. Science (80-.). 368, 266–269. https://doi.org/10.1126/science.aaz7614

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198. https://doi.org/10.1016/0003-9861(68)90654-1

Hu, M., Wiatrak, P., 2012. Effect of planting date on soybean growth, yield, and grain quality: Review. Agron. J. 104, 785–790. https://doi.org/10.2134/agronj2011.0382

Hungria, M., Franchini, J.C., Campo, R.J., Crispino, C.C., Moraes, J.Z., Sibaldelli, R.N.R., Mendes, I.C., Arihara, J., 2006. Nitrogen nutrition of soybean in Brazil: Contributions of biological N2 fixation and N fertilizer to grain yield. Can. J. Plant Sci. 86, 927–939. https://doi.org/10.4141/P05-098

Hungria, M., Mendes, I.C., 2015. Nitrogen Fixation with Soybean: The Perfect Symbiosis?, Biological Nitrogen Fixation. Hoboken, New Jersey. https://doi.org/10.1002/9781119053095.ch99

Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. F. Crop. Res. 65, 151–164. https://doi.org/10.1016/S0378-4290(99)00084-2

Juge, C., Prévost, D., Bertrand, A., Bipfubusa, M., Chalifour, F.P., 2012. Growth and biochemical responses of soybean to double and triple microbial associations with

Bradyrhizobium, Azospirillum and arbuscular mycorrhizae. Appl. Soil Ecol. 61, 147–157. https://doi.org/10.1016/j.apsoil.2012.05.006

Khan, N., Bano, A., Ali, S., Babar, M.A., 2020. Crosstalk amongst phytohormones from planta and PGPR under biotic and abiotic stresses. Plant Growth Regul. 90, 189–203. https://doi.org/10.1007/s10725-020-00571-x

Levene, H., 1960. Robust tests for equality of variances, in: Olkin, I., Ghurye, S.G., Hoeffding, W., Madow, W.G., Mann, H.B. (Eds.), Contributions to Probability and Statistics: Essays in …. Stanford University Press, pp. 278–292.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, in: Methods in Enzymology. Elsevier, pp. 350–382. https://doi.org/https://doi.org/10.1016/0076- 6879(87)48036-1.

Loix, C., Huybrechts, M., Vangronsveld, J., Gielen, M., Keunen, E., Cuypers, A., 2018. Reciprocal interactions between cadmium-induced cell wall responses and oxidative stress in plants. Front. Plant Sci. 9, 1–2. https://doi.org/10.3389/fpls.2018.00391
Marks, B.B., Megías, M., Nogueira, M.A., Hungria, M., 2013. Biotechnological potential of rhizobial metabolites to enhance the performance of Bradyrhizobium spp. and Azospirillum brasilense inoculants with soybean and maize. AMB Express 3, 21. https://doi.org/10.1186/2191-0855-3-21

Marks, B.B., Megías, M., Ollero, F.J., Nogueira, M.A., Araujo, R.S., Hungria, M., 2015. Maize growth promotion by inoculation with Azospirillum brasilense and metabolites of Rhizobium tropici enriched on lipo-chitooligosaccharides (LCOs). AMB Express 5, 1–11. https://doi.org/10.1186/s13568-015-0154-z

Mauad, M., Crusciol, C.A.C., Nascente, A.S., Filho, H.G., Lima, G.P.P., 2016. Effects of silicon and drought stress on biochemical characteristics of leaves of upland rice cultivars1. Rev. Cienc. Agron. 47, 532–539. https://doi.org/10.5935/1806-6690.20160064

Moretti, L.G., Crusciol, C.A.C., Bossolani, J.W., Momesso, L., Garcia, A., Kuramae, E.E., Hungria, M., 2020a. Bacterial Consortium and Microbial Metabolites Increase Grain Quality and Soybean Yield. J. Soil Sci. Plant Nutr. https://doi.org/10.1007/s42729-020-00263-5

Moretti, L.G., Crusciol, C.A.C., Kuramae, E.E., Bossolani, J.W., Moreira, A., Costa, N.R., Alves, C.J., Pascoaloto, I.M., Rondina, A.B.L., Hungria, M., 2020b. Effects of growth-promoting bacteria on soybean root activity, plant development, and yield. Agron. J. 112, 418–428. https://doi.org/10.1002/agj2.20010

Moretti, L.G., Lazarini, E., Bossolani, J.W., Parente, T.L., Caioni, S., Araujo, R.S., Hungria, M., 2018. Can additional inoculations increase soybean nodulation and grain yield? Agron. J. 110, 715–721. https://doi.org/10.2134/agronj2017.09.0540

Nelson, K.A., Motavalli, P.P., Nathan, M., 2005. Response of no-till soybean [Glycine max (L.) Merr.] to timing of preplant and foliar potassium applications in a claypan soil. Agron. J. 97, 832–838. https://doi.org/10.2134/agronj2004.0241

Nicholson, W.L., 2002. Roles of Bacillus endospores in the environment. Cell. Mol. Life Sci. 59, 410–416. https://doi.org/10.1007/s00018-002-8433-7

O'Hara, G.W., Hungria, M., Woomer, P., Howieson, J.G., 2016. Counting rhizobia, in: Howieson, J.G., Dilworth, M.J. (Eds.), Working with Rhizobia. Australian Centre for International Agricultural Research, Canberra, pp. 109–124.

Pozo, M.J., van Der Ent, S., van Loon, L.C., Pieterse, C.M.J., 2008. Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in Arabidopsis thaliana. New Phytol. 180, 511–523. https://doi.org/10.1111/j.1469-8137.2008.02578.x

Quaggio, J.A., van Raij, B., 1997. Correção da acidez do solo, in: van Raij, B., Cantarella, H., Quaggio, J.A., Furlani, A.M.C. (Eds.), Recomendações de Adubação e Calagem Para o Estado de São Paulo. Instituto Agronômico, Campinas, Brazil, pp. 14–19.

Reddy, A.R., Chaitanya, K.V., Vivekanandan, M., 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 161, 1189–1202. https://doi.org/10.1016/j.jplph.2004.01.013

Reis, A.R. dos, de Queiroz Barcelos, J.P., de Souza Osório, C.R.W., Santos, E.F., Lisboa, L.A.M., Santini, J.M.K., dos Santos, M.J.D., Furlani Junior, E., Campos, M., de Figueiredo, P.A.M., Lavres, J., Gratão, P.L., 2017. A glimpse into the physiological, biochemical and nutritional status of soybean plants under Ni-stress conditions. Environ. Exp. Bot. 144, 76–87. https://doi.org/10.1016/j.envexpbot.2017.10.006

Riar, M.K., Cerezini, P., Manandhar, A., Sinclair, T.R., Li, Z., Carter, T.E., 2018. Expression of drought-tolerant N2 fixation in heterogeneous inbred families derived from PI471938 and hutcheson soybean. Crop Sci. 58, 364–369. https://doi.org/10.2135/cropsci2017.02.0089

Rodriguez-Concepcion, M., Avalos, J., Bonet, M.L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, D., Limon, M.C., Meléndez-Martínez, A.J., Olmedilla-Alonso, B., Palou, A., Ribot, J., Rodrigo, M.J., Zacarias, L., Zhu, C., 2018. A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. Prog. Lipid Res. 70, 62–93. https://doi.org/10.1016/j.plipres.2018.04.004

Rolim, G., Sentelhas, P., Barbiere, V., 1998. Planilhas no ambiente excel para os cálculos de balanços hídricos: normal, sequencial, de cultura e de produtividade real e potencial. Rev. Bras. Agrometeorol. 6, 133–137.

Sahoo, R.K., Ansari, M.W., Pradhan, M., Dangar, T.K., Mohanty, S., Tuteja, N., 2014. Phenotypic and molecular characterization of native Azospirillum strains from rice fields to improve crop productivity. Protoplasma 251, 943–953. https://doi.org/10.1007/s00709-013-0607-7

Sandhya, V., Ali, S.Z., Grover, M., Reddy, G., Venkateswarlu, B., 2010. Effect of plant growth promoting Pseudomonas spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. Plant Growth Regul. 62, 21–30. https://doi.org/10.1007/s10725-010-9479-4

Santos, H. G., Jacomine, P.T., Dos Anjos, L.H.C., de Oliveira, V.A., Lumbreras, J.F., Coelho, M.R., Almeida, J.A., Araujo Filho, J.C., Oliveira, J.B., Cunha, T.J.F., 2018. Brazilian Soil Classification System. - Portal Embrapa, 5th ed. re. ed. Embrapa Solos, Brasília, DF.

Santos, E.F., Santini, J.M.K., Paixão, A.P., Júnior, E.F., Lavres, J., Campos, M., Reis, A.R., 2017. Physiological highlights of manganese toxicity symptoms in soybean plants: Mn toxicity responses. Plant Physiol. Biochem. 113, 6–19. https://doi.org/10.1016/j.plaphy.2017.01.022

SAS Institute, 2015. Procedure guide for personal computers. Version 9.4.
Sehgal, A., Sita, K., Kumar, J., Kumar, S., Singh, S., Siddique, K.H.M., Nayyar, H., 2017. Effects of drought, heat and their interaction on the growth, yield and photosynthetic function of lentil (Lens culinaris medikus) genotypes varying in heat and drought sensitivity. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.01776

Shapiro, S.S., Wilk, M.B., 1965. An Analysis of Variance Test for Normality (Complete Samples). Biometrika 52, 591. https://doi.org/10.2307/2333709

Silva, F.A.M., Naudin, K., Corbeels, M., Scopel, E., Affholder, F., 2019. Impact of conservation agriculture on the agronomic and environmental performances of maize cropping under contrasting climatic conditions of the Brazilian Cerrado. F. Crop. Res. 230, 72–83. https://doi.org/10.1016/j.fcr.2018.10.009

Silva, V.M., Tavanti, R.F.R.F., Gratão, P.L., Alcock, T.D., Reis, A.R., 2020. Selenate and selenite affect photosynthetic pigments and ROS scavenging through distinct mechanisms in cowpea (Vigna unguiculata (L.) walp) plants. Ecotoxicol. Environ. Saf. 201, 110777. https://doi.org/10.1016/j.ecoenv.2020.110777

Soil Survey Staff, 2014. Keys to Soil Taxonomy. USDA - Natural Resources Conservation Service, Washington, DC, p. 360.

Spaepen, S., Vanderleyden, J., 2015. Auxin Signaling in Azospirillum brasilense : A Proteome Analysis, in: Biological Nitrogen Fixation. John Wiley & Sons, Inc, Hoboken, NJ, USA, pp. 937–940. https://doi.org/10.1002/9781119053095.ch91

Thornthwaite, C.W., Mather, J.R., 1995. The Water Balance Climatology. Laboratory of climatology, New Jersey.

Tien, T.M., Gaskins, M.H., Hubbell, D.H., 1979. Plant Growth Substances Produced by Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.). Appl. Environ. Microbiol. 37, 1016–1024. https://doi.org/10.1128/aem.37.5.1016-1024.1979

Torello, W.A., Rice, L.A., 1986. Effects of NaCl stress on proline and cation accumulation in salt sensitive and tolerant turfgrasses. Plant Soil 93, 241–247. https://doi.org/10.1007/BF02374226

Ueda, A., Shi, W., Sanmiya, K., Shono, M., Takabe, T., 2001. Functional analysis of salt-inducible Proline Transporter of barley roots. Plant Cell Physiol. 42, 1282–1289. https://doi.org/10.1093/pcp/pce166

Unicamp, 2020. Center of Meteorological and Climatic Research Applied to Agriculture. Botucatu: Municipalities climate of São Paulo State. [WWW Document]. URL www.cpa.unicamp.br/outras- informacoes/clima_muni_086.html (accessed 2.20.20).

van Raij, B., Andrade, J.C., Cantarella, H., Quaggio, J.A., 2001. Análise química para avaliação da fertilidade de solos tropicais.

Wellburn, A.R., 1994. The spectral determination of chlorophyll a and b, as well as carotenoids using various solvents with spectrophotometers of different resolution. J. Plant Physiol 144, 307–313.

Supplementary Tables

Table S1. Physicochemical and biological attributes (0.0–0.2-m depth) before sowing.

Soil properties		Unit	Value
Physical			
Clay		g kg^{-1}	602
Silt		g kg^{-1}	281
Sand		g kg^{-1}	117
Bulk density		g cm^{-3}	1.19
Chemical			
pH $_{(CaCl2)}$		–	5.10
Soil organic matter		g kg^{-1}	26.2
Phosphorus–available (P $_{resin}$)		mg kg^{-1}	59.0
Exchangeable	Calcium (Ca^{2+} $_{resin}$)	$mmol_c$ kg^{-1}	25.0
	Magnesium (Mg^{2+} $_{resin}$)	$mmol_c$ kg^{-1}	15.0
	Potassium (K^{+} $_{resin}$)	$mmol_c$ kg^{-1}	3.90
	Aluminum (Al^{3+} $_{KCl}$)	$mmol_c$ kg^{-1}	2.00
Potential acidity (H+Al)		$mmol_c$ kg^{-1}	42.0
S-Sulfate (S–SO_4^{2-} $_{Ca(H2PO4)2}$)		mg kg^{-1}	4.90
Boron (B $_{Hot\ water}$)		mg kg^{-1}	0.40
Copper (Cu $_{DTPA\text{-}TEA}$[a])		mg kg^{-1}	8.80
Iron (Fe $_{DTPA\text{-}TEA}$)		mg kg^{-1}	22.0
Manganese (Mn $_{DTPA\text{-}TEA}$)		mg kg^{-1}	26.2
Zinc (Zn $_{DTPA\text{-}TEA}$)		mg kg^{-1}	2.10
Base saturation (BS)		%	51.0
Cation exchange capacity (CEC $_{pH\ 7.0}$)		$mmol_c$ kg^{-1}	86.0
Biological			
Most probable number		CFU[b] g^{-1}	9.32×10^4

[a]DTPA-TEA, diethylenetriaminepentaacetic acid-triethanolamine;

[b] CFU, colony forming units.

Table S2. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Total Chl), and total carotenoids (Total car) in soybean leaves as a function of bacterial consortium and microbial metabolites under field conditions.

Factors	Chl a	Chl b	Total Chl	Total Car
Bacterial Consortium (BC)†			$\mu g \cdot cm^{-2}$	
Standard Inoculation (SI)	15.0 b§	3.0 b	18.1 b	4.6 b
SI + *B. subtilis* (Bs)	15.1 b	3.1 b	18.1 b	4.6 b
SI + *A. brasilense* (Ab)	16.6 a	3.8 a	20.4 a	5.7 a
SI + Bs + Ab	16.6 a	3.8 a	20.4 a	5.6 a
Microbial Secondary Metabolites (MSM)‡				
Absence	15.5 b	3.4 b	18.9 b	5.0 b
Presence	16.1 a	3.5 a	19.6 a	5.3 a
Growing Season (GS)				
2016–2017	15.8	3.5	19.2	5.1
2017–2018	15.6	3.4	19.3	5.0
ANOVA (*F probability*)				
BC	**	**	**	**
MSM	**	**	**	**
GS	*ns*	*ns*	*ns*	*ns*
BC × MSM	**	**	**	**
BC × GS	*ns*	*ns*	*ns*	*ns*
MSM × GS	*ns*	*ns*	*ns*	*ns*
BC × MSM × GS	*ns*	*ns*	*ns*	*ns*

†Bacterial Consortium: SI = standard inoculation with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) co-inoculated on seeds; SI + *B. subtilis* (Bs) = SI via seeds + foliar spray inoculation with *Bacillus subtilis* (strain QST 713) at the V_3 growth stage; SI + *A. brasilense* (Ab) = SI via seeds + foliar spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at the V_4 growth stage.

‡Microbial metabolites = Absence or presence of application of microbial metabolites extracted from *B. diazoefficiens* (strain USDA 110) and *Rhizobium tropici* (strain CIAT 889) on seeds.

§Means (n = 4) followed by the same letter do not differ (*ns*) by Fisher's least significant (LSD) test at *$p \leq 0.05$ and **$p \leq 0.01$

Table S3. Net photosynthetic rate (*A*), stomatal conductance (g_S), internal CO_2 concentration in the substomatal chamber (*Ci*), and transpiration (*E*) of soybean leaves as a function of bacterial consortium and microbial metabolites under field conditions.

Factors	*A*	*gs*	*Ci*	*E*
Bacterial Consortium (BC)†	μmol CO_2 m^{-2} s^{-1}	mol H_2O m^{-2} s^{-1})	μmol mol^{-1}	μmol CO_2 (mmol H_2O)$^{-1}$
Standard Inoculation (SI)	25.3 b§	0.177 b	254 b	2.5 b
SI + *B. subtilis* (Bs)	25.5 b	0.193 b	255 b	2.5 b
SI + *A. brasilense* (Ab)	28.8 a	0.266 a	273 a	3.6 a
SI + Bs + Ab	29.2 a	0.270 a	270 a	3.5 a
Microbial Secondary Metabolites (MSM)‡				
Absence	26.9 b	0.223 b	257 b	3.0 b
Presence	27.5 a	0.230 a	265 a	3.1 a
Growing Season (GS)				
2016–2017	27.6	0.321	70.6	9.1
2017–2018	27.0	0.331	71.9	9.3
ANOVA (*F probability*)				
BC	**	**	*	*
MSM	**	**	*	*
GS	*ns*	*ns*	*ns*	*
BC × MSM	**	**	*	*
BC × GS	*ns*	*ns*	*ns*	*ns*
MSM × GS	*ns*	*ns*	*ns*	*ns*
BC × MSM × GS	*ns*	*ns*	*ns*	*ns*

†Bacterial Consortium: SI = standard inoculation with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) co-inoculated on seeds; SI + *B. subtilis* (Bs) = SI via seeds + foliar spray inoculation with *Bacillus subtilis* (strain QST 713) at the V_3 growth stage; SI + *A. brasilense* (Ab) = SI via seeds + foliar spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at the V_4 growth stage.

‡Microbial metabolites = Absence or presence of application of microbial metabolites extracted from *B. diazoefficiens* (strain USDA 110) and *Rhizobium tropici* (strain CIAT 889) on seeds.

§Means (n = 4) followed by the same letter do not differ (*ns*) by Fisher's least significant (LSD) test at *$p \leq 0.05$ and **$p \leq 0.01$.

Table S4. Contents of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline; activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) as a function of bacterial consortium and microbial metabolites under field conditions.

Factors	H_2O_2	MDA	SOD	CAT	APX	Proline
Bacterial Consortium (BC)†	g^{-1} FW		mg protein^{-1}	μmoL min^{-1}		g^{-1} FW
Standard Inoculation (SI)	10.4 a§	3.8 a	18.6 a	4.7 a	5.0 a	33.4 a
SI + *B. subtilis* (Bs)	10.1 a	3.5 a	18.4 a	4.5 a	4.9 a	32.9 a
SI + *A. brasilense* (Ab)	8.9 b	3.4 b	15.8 b	4.1 b	4.3 b	25.6 b
SI + Bs + Ab	8.8 b	3.4 b	15.8 b	4.1 b	4.3 b	25.2 b
Microbial Secondary Metabolites (MSM)‡						
Absence	9.7 a	3.6 a	17.5 a	4.5 a	4.7 a	30.2 a
Presence	9.4 b	3.4 b	16.8 b	4.1 b	4.5 b	28.3 b
Growing Season (GS)						
2016–2017	9.5	3.5	17.2	4.3	4.6	29.3
2017–2018	9.7	3.4	17.0	4.1	4.7	29.6
ANOVA (*F probability*)						
BC	**	**	**	**	**	**
MSM	**	**	**	**	**	**
GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
BC × MSM	**	**	**	**	**	**
BC × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
MSM × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
BC × MSM × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

†Bacterial Consortium: SI = standard inoculation with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) co-inoculated together on seeds; SI + *B. subtilis* (Bs) = SI via seeds + foliar spray inoculation with *Bacillus subtilis* (strain QST 713) at the V_3 growth stage; SI + *A. brasilense* (Ab) = SI via seeds + foliar spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at the V_4 growth stage. ‡Microbial metabolites = Absence or presence of application of microbial metabolites extracted from *B. diazoefficiens* (strain USDA 110) and *Rhizobium tropici* (strain CIAT 889) on seeds. §Means (n = 4) followed by the same letter do not differ (*ns*) by Fisher's least significant (LSD) test at *$p \leq 0.05$ and **$p \leq 0.01$.

Table S5. Nodule number (NN), nodule dry weight (NDW), root dry weight (RDW), shoot dry weight (SDW), plant height (PH), pods, and soybean 1000-grain weight (W1000) and grain yield (GY) as a function of bacterial consortium and microbial metabolites under field conditions.

Factors	NN	NDW	RDW	SDW	PH	Pods	W1000	GY
Bacterial Consortium (BC)†	no. plant^{-1}	mg plant^{-1}	g plant^{-1}		cm	no. plant^{-1}	g	kg ha^{-1}
Standard Inoculation (SI)	52 b§	178 b	6.7 b	21.3 b	82 b	47 b	160 b	4775 b
SI + *B. subtilis* (Bs)	53 b	180 b	6.8 b	21.7 b	83 b	47 b	163 b	4890 b
SI + *A. brasilense* (Ab)	60 a	212 a	7.5 a	24.3 b	88 a	56 a	167 a	5124 a
SI + Bs + Ab	61 a	210 a	7.6 a	24.6 b	87 a	55 a	168 a	5178 a
Microbial Secondary Metabolites (MSM)‡								
Absence	53 b	192 b	7.0 b	22.1 b	85 b	50 b	162 b	4900 b
Presence	57 a	200 a	7.3 a	23.9 a	85 a	53 a	166 a	5173 a
Growing Season (GS)								
2016–2017	54	195	6.9	22.4	84	48	165	4895
2017–2018	55	199	7.0	22.6	85	50	166	5016
ANOVA (*F probability*)								
BC	**	**	**	**	**	**	**	**
MSM	**	**	**	**	**	**	**	**
GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
BC × MSM	**	**	**	**	**	**	**	**
BC × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
MSM × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
BC × MSM × GS	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

†Bacterial Consortium: SI = standard inoculation with *Bradyrhizobium japonicum* (strain SEMIA 5079) + *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) co-inoculated together on seeds; SI + *B. subtilis* (Bs) = SI via seeds + foliar spray inoculation with *Bacillus subtilis* (strain QST 713) at the V_3 growth stage; SI + *A. brasilense* (Ab) = SI via seeds + foliar spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at the V_4 growth stage. ‡Microbial metabolites = Absence or presence of application of microbial metabolites extracted from *B. diazoefficiens* (strain USDA 110) and *Rhizobium tropici* (strain CIAT 889) on seeds. §Means (n = 4) followed by the same letter do not differ (*ns*) by Fisher's least significant (LSD) test at *$p \leq 0.05$ and **$p \leq 0.0$

GENERAL CONCLUSIONS

The beneficial bacterial consortium comprising seed inoculation with N_2-fixing *Bradyrhizobium japonicum* (strain SEMIA 5079) and *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) combined with seed treatment with MSM (rhizobial metabolites enriched in LCOs) and foliar spraying in the V_4 soybean growth stage with plant growth-promoting *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) increased root system soil exploration and, in turn, the distribution and activity of soybean roots.

This beneficial bacterial consortium also increased nodulation, the production of N-ureides and, consequently, N nutrition. The combination of bacterial consortia and metabolites may represent an effective strategy to mitigate dry spell stress during soybean development by reducing the leaf contents of hydrogen peroxide and proline, lipid peroxidation, and the activities of the enzymes superoxide dismutase, catalase, and ascorbate peroxidase, resulting in greater soybean photosynthetic potential.
Ultimately, the combination of this bacterial consortium and MSM increased both grain yield (~11%) and crude protein concentration. This study illustrates the feasibility of identifying beneficial bacterial consortia that can promote plant growth and elicit plant tolerance to abiotic stress, which represents an important biotechnological strategy to achieve sustainable food production and security.

REFERENCES

CEREZINI, P.; KUWANO, B. H.; DOS SANTOS, M. B.; TERASSI, F.; HUNGRIA, M.; NOGUEIRA, M. A. Strategies to promote early nodulation in soybean under drought. **Field Crops Research**, *[s. l.]*, v. 196, p. 160-167, 2016. Disponível em: https://doi.org/10.1016/j.fcr.2016.06.017. Acesso em: 24 set. 2020.

FUKAMI, J.; CEREZINI, P.; HUNGRIA, M. Azospirillum: benefits that go far beyond biological nitrogen fixation. **AMB Express**, *[s. l.],* v. 8, n. 73, p. 1-12, 2018. Disponível em: https://doi.org/10.1186/s13568-018-0608-1. Acesso em: 13 out. 2020.

GOUGH, C.; JACQUET, C. Nod factor perception protein carries weight in biotic interactions. **Trends in Plant Science**, *[s. l.]*, v. 18, n. 10, p. 566-574, 2013. Disponível em: https://doi.org/10.1016/j.tplants.2013.06.001. Acesso em: 22 set. 2020.

HUNGRIA, M.; FRANCHINI, J. C.; CAMPO, R. J.; CRISPINO, C. C., MORAES, J. Z.; SIBALDELLI, R. N. R.; MENDES, I. C.; ARIHARA, J. Nitrogen nutrition of soybean in Brazil: Contributions of biological N2 fixation and N fertilizer to grain yield. **Canadian Journal of Plant Science**, *[s. l.]*, v. 86, n. 4, p. 927-939, 2006. Disponível em: https://doi.org/10.4141/P05-098. Acesso em: 1 out. 2020.

HUNGRIA, M.; NOGUEIRA, M. A.; ARAUJO, R. S. Alternative methods of soybean inoculation to overcome adverse conditions at sowing. **African Journal of Agriculture Research**, *[s. l.]*, v. 10, n. 23, p. 2329-2338, 2015. Disponível em: https://doi.org/10.5897/ajar2014.8687. Acesso em: 14 out. 2020.

KASCHUK, G.; HUNGRIA, M.; LEFFELAAR, P. A.; GILLER, K. E.; KUYPER, T. W. Differences in photosynthetic behaviour and leaf senescence of soybean (Glycine max [L.] Merrill) dependent on N2 fixation or nitrate supply. **Plant Biology**, *[s. l.]*, v. 12, n. 1, p. 60-69, 2010. Disponível em: https://doi.org/10.1111/j.1438-8677.2009.00211.x. Acesso em: 2 set. 2020.

MARKS, B. B.: MEGÍAS, M.; NOGUEIRA, M. A.: HUNGRIA, M. Biotechnological potential of rhizobial metabolites to enhance the performance of *Bradyrhizobium spp*. and *Azospirillum brasilense* inoculants with soybean and maize. **AMB Express**, *[s. l.]*, v. 3, n. 21, 2013. Disponível em: https://doi.org/10.1186/2191-0855-3-21. Acesso em: 9 set. 2020.

MARKS, B. B.; MEGÍAS, M.; OLLERO, F. J.; NOGUEIRA, M. A.; ARAUJO, R. S.; HUNGRIA, M. Maize growth promotion by inoculation with *Azospirillum brasilense* and metabolites of *Rhizobium tropici* enriched on lipo-chitooligosaccharides (LCOs). **AMB Express**, *[s. l.]*, v. 5, n. 71, p. 1-11, 2015. Disponível em: https://doi.org/10.1186/s13568-015-0154-z. Acesso em: 18 set. 2020.

MORETTI, L. G.; CRUSCIOL, C. A. C.; KURAMAE, E. E.; BOSSOLANI, J. W.; MOREIRA, A.; COSTA, N. R.; ALVES, C. J.; PASCOALOTO, I. M.; RONDINA, A. B. L.; HUNGRIA, M. Effects of growth-promoting bacteria on soybean root activity, plant development, and yield. **Agronomy Journal**, *[s. l.]*, v. 112, n. 1, p. 418-428, 2020. Disponível em: https://doi.org/10.1002/agj2.20010. Acesso em: 20 out. 2020.

MORETTI, L. G.; LAZARINI, E.; BOSSOLANI, J. W.; PARENTE, T. L.; CAIONI, S.; ARAUJO, R. S.; HUNGRIA, M. Can additional inoculations increase soybean nodulation and grain yield? **Agronomy Journal**, *[s. l.],* v. 110, n. 2, p. 715-721, 2018. Disponível em: https://doi.org/10.2134/agronj2017.09.0540. Acesso em: 23 set. 2020.