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Bacterial consortium and microbial metabolites increase grain quality and soybean yield

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1 **Bacterial Consortium and Microbial Metabolites Increase Grain Quality and Soybean**
2 **Yield**

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50

51

52 **Abbreviations:**

53 PGPR, Plant-growth-promoting-rhizobacteria;
54 MSM, Microbial secondary metabolites;
55 BNF, Biological nitrogen fixation;
56 AEI, Agronomic efficiency index;
57 SEMIA, Section of Agricultural Microbiology;
58 USDA, United States Department of Agriculture;
59 CIAT, International Center for Tropical Agriculture;
60 CPAC, Embrapa Cerrados;
61 CNPSo, Embrapa Soybean;
62 LCOs, Lipo-chitooligosaccharides;
63 CEC, Cation exchange capacity;
64 MPN, Most probable number;
65 CFU, Colony forming units;
66 SI, Standard inoculation.

67

68 **Highlights**

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

76
77 (FIGURE 1)
78

79 Abstract

80 **Purpose:** The effects of *Bradyrhizobium* inoculation on soybean growth and productivity are
81 well known, but plant responses to consortia of other beneficial microbes and microbial
82 molecules have not yet been well explored. Therefore, the main aim of this study was to
83 evaluate the effect of different combinations of beneficial bacteria with and without microbial
84 secondary metabolites (MSM) on two soybean cultivars in three cropping seasons under
85 tropical field conditions. **Methods:** The bacterial consortia consisted of *Bradyrhizobium*
86 *japonicum* (strain SEMIA 5079) plus *Bradyrhizobium diazoefficiens* (strain SEMIA 5080)
87 inoculated with different combinations of *Bacillus subtilis* (strain QST 713), *Azospirillum*
88 *brasilense* (strains Ab-V5 and Ab-V6) and MSM (metabolites enriched in lipo-
89 chitooligosaccharides (LCOs) extracted from *B. diazoefficiens* (strain USDA 110) and from
90 *Rhizobium tropici* (strain CIAT 889)). **Results:** Standard inoculation of *Bradyrhizobium*
91 combined with *Azospirillum brasilense* and microbial secondary metabolites increased leaf
92 total N (7.1%), total P (11.1%) and N-ureide (16.5%); nodule number (NN, 26%) and dry
93 weight (NDW, 22%); root (RDW, 15.4%) and shoot dry weight (SDW, 6%); 100-seed weight
94 (3.7%); grain yield (up to 516 kg ha⁻¹); grain crude protein concentration (2.4%); and the
95 agronomic efficiency index (AEI) (11%). **Conclusions:** Inoculation with bacterial consortia
96 and metabolites increased grain yield and quality, representing a promising technology for
97 sustainable soybean cropping in tropical regions.

98
99 **Keywords:** *Azospirillum brasilense*; *Bacillus subtilis*; *Bradyrhizobium diazoefficiens*;
100 *Bradyrhizobium japonicum*; Microbial metabolites; *Rhizobium tropici*.

102 1 Introduction

103 The sustainable development of agricultural ecosystems requires improvements in crop
104 yield and quality. The adoption of sustainable production systems has increased considerably
105 in recent years, driven mainly by society's demand for high-quality food whose production
106 results in low environmental impacts (Santos et al. 2019). Legumes encompass important grain
107 crops, the economically most important being soybean (*Glycine max* [L.] Merrill), which, due
108 to its high protein concentration, is used for animal and human consumption (Sugiyama et al.
109 2014). In the 2019–2020 cropping season, it is expected that approximately 338 million Mg of
110 soybean grains will be produced, and 123 million ha will be cultivated worldwide (USDA
111 2020), among which Brazil has been moving towards becoming the largest global producer.
112 (Brasil 2019).

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

120 Strategies to improve *Bradyrhizobium*-soybean symbiosis and increase the effectiveness
121 of BNF have been extensively surveyed (Hungria et al. 2006; Chibeba et al. 2015; Moretti et
122 al. 2018; Vanlauwe et al. 2019). Among the strategies, the use of a bacterial consortium of
123 *Bradyrhizobium* with plant growth-promoting rhizobacteria (PGPR) has been identified as
124 beneficial for promoting BNF and improving crop performance, resulting in increases in grain
125 yield (Marks et al. 2013, 2015; Moretti et al. 2020). Bacteria belonging to the genus
126 *Azospirillum* are the best studied and most widely employed PGPR for agriculture worldwide
127 (Fukami et al. 2018a). In Brazil, *Azospirillum brasilense* strains Ab-V5 and Ab-V6 have been
128 broadly used in commercial inoculants for grain crops, including both legumes and nonlegumes
129 (Hungria et al. 2010, 2016; Fukami et al. 2018a; Santos et al. 2019).

130 A consortium of rhizobia and *Azospirillum* is feasible to improve grain yield (Hungria
131 et al. 2013; Hungria and Mendes 2015), the tolerance of biotic stresses, usually by improving
132 the plant intrinsic tolerance against pathogens (Bashan and de-Bashan 2010; Cerezini et al.
133 2016), and attenuate damages caused by abiotic stresses, such as salinity and drought (Fukami
134 et al. 2018a, b). Another important application of microorganisms is as biocontrol agents, which
135 are particularly important when resistance to fungicides has been developed (Leroux et al. 2002;
136 Standish et al. 2015). In this context, *Bacillus subtilis* has been used as a biological fungicide

137 that can induce systemic acquired resistance and release biocide molecules that will provide
138 biocontrol against several plant pathogens (Nicholson 2002; Araújo et al. 2005; Sansinenea and
139 Ortiz 2011).

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

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

161

162 **2 Materials and Methods**

163 **2.1 Site Description**

164 The study consisted of three field experiments carried out under rainfed conditions
165 during the 2016–2017, 2017–2018 and 2018–2019 cropping seasons, at the Lageado
166 Experimental Farm of São Paulo State University in Botucatu, São Paulo State, Brazil
167 (48°26’W, 22°51’S, 786 m altitude) (Supplementary Figure 1). The soil is classified as clayey
168 textural class, kaolinitic, thermic Typic Haplorthox (Soil Survey Staff 2014). According to
169 Köppen’s classification (Alvares et al. 2013), the climate is Cwa, which corresponds to a humid
170 subtropical zone, with dry winter and hot summer. The long-term (1956–2020) average annual

171 temperatures are 26.1 °C maximum and 15.3 °C minimum, with a 20.7 °C average. The average
 172 annual rainfall is approximately 1,360 mm (CEPAGRI 2020). Climatological data during the
 173 experiments are presented in Figure 2.

174

175 (FIGURE 2)

176

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

186

187 (TABLE 1)

188

189 2.2 Experimental Design and Treatments

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

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

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

208

209 **2.3 Microbial Inoculants and Secondary Metabolites**

210 Liquid inoculants containing *B. japonicum* strain SEMIA 5079 (=CPAC 15, =CNPSO
211 07) and *B. diazoefficiens* strain SEMIA 5080 (=CPAC 7, =CNPSO 06) were prepared at a
212 concentration of 7×10^9 colony forming units (CFUs) mL⁻¹ and applied to provide 1.2×10^6
213 cells seed⁻¹. The MSM enriched in lipo-chitoooligosaccharides (LCOs) were extracted from *R.*
214 *tropici* strain CIAT 899 (= CNPSO 103, = SEMIA 4077) and *B. diazoefficiens* strain USDA 110
215 (=CNPSO 56) and produced as described before (Marks et al. 2013, 2015) by Embrapa Soybean.
216 Prior to sowing, lyophilized metabolites were resuspended in a mixture of acetonitrile and water
217 (20%) as previously described (Marks et al. 2015). The concentration was adjusted to 1.0 mL
218 L⁻¹, corresponding to approximately 10⁻⁸ M, and applied in a volume of 200 mL per 50 kg of
219 seeds.

220 For foliar spraying at the V₃ soybean phenological stage (Fehr and Caviness 1977), 3
221 L of inoculant containing *B. subtilis* strain QST 713 at a concentration of 1×10^9 CFUs mL⁻¹
222 was diluted in 200 L ha⁻¹ in water. For foliar spraying at the V₄ soybean phenological stage
223 (Fehr and Caviness 1977), 300 mL of inoculant containing *A. brasilense* strain Ab-V5 (=CNPSO
224 2083) and strain Ab-V6 (=CNPSO 2084) at a concentration of 2×10^8 CFUs mL⁻¹ was diluted
225 in a total volume of 150 L ha⁻¹ in water. Foliar sprays containing the two *A. brasilense* strains
226 and *B. subtilis* were always applied late in the afternoon (5:00 pm).

227

228 **2.4 Agronomic Practices and Measures**

229 In the three growing seasons, soybeans were sown after black oats (*Avena strigosa*
230 Schreb.) that had been cropped in the winter as a mulch crop in a no-till system, providing an
231 average of 4.5 Mg ha⁻¹ straw in a dry land area (without irrigation). The treatments were applied
232 to the same plots in all growing seasons. Each plot consisted of 10 rows that covered an area of
233 45 m² (10 m x 0.45 m). Plots were separated by 0.5-m-wide rows and 1.5-m-wide terraces to
234 avoid cross-contamination from surface runoff containing bacteria or fertilizers that may occur
235 as a consequence of heavy rainfall. In the three growing seasons, all plots were fertilized with
236 00-20-20 of N-P₂O₅-K₂O at 300 kg ha⁻¹. The seeds were treated with fungicides (carboxin +
237 thiram at 100 g + 100 g a.i. per 100 kg of seeds) prior to inoculation and sowing. Seed
238 inoculation was performed 1 h before sowing by evenly coating the seeds with the appropriate

239 amount of inoculant. Foliar-spray inoculations were performed by a tractor-mounted sprayer.
 240 In all treatments, foliar spray containing 20 g ha⁻¹ Mo (as Na₂MoO₄.2H₂O) and 2 g ha⁻¹ Co (as
 241 CoCl₂.6H₂O) was applied to plants at the V₄ stage (Fehr and Caviness 1977). Phytosanitary
 242 treatments were carried out according to the needs of and recommendations for the soybean
 243 crop (Embrapa 2013).

244 Plant nutritional status was evaluated at the R₂ phenological stage (Fehr and Caviness
 245 1977) by collecting the third fully developed leaf and its petiole from 30 plants in each plot,
 246 according to Ambrosano et al. (1997). The material was used to determine the N, phosphorus
 247 (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), copper (Cu), iron (Fe), zinc
 248 (Zn), manganese (Mn), and boron (B) concentrations according to the methodology described
 249 by Malavolta et al. (1997). Additionally, at the full flowering (R₂) stage, dry leaves with petioles
 250 were used for the determination of the N-ureide (allantoin and allantoic acids) concentration as
 251 described by Herridge and Giller (2016). Additionally, at the R₂ stage, five plants were
 252 harvested per plot and separated into shoots and roots according to the methodology described
 253 by Hungria et al. (2006). Roots were washed to remove substrate particles, and nodules were
 254 removed. Shoots, roots and nodules were oven dried at 65 °C for 72 h, and from this material,
 255 the nodule number (NN), nodule dry weight (NDW), root dry weight (RDW) and shoot dry
 256 weight (SDW) were determined.

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

$$264 \quad \text{Agronomic efficiency index, \%} = \frac{Y2 \times 100}{Y1}$$

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

$$271 \quad \text{Crude Protein, \%} = \% \text{ Kjeldahl N} \times 6.25$$

272

273 **2.5 Statistical Analysis**

274 The data were initially analyzed using the Shapiro-Wilk's test (Shapiro and Wilk 1965)
275 for normality and Levene's homoscedasticity test (Levene 1960), both at 0.05 probability ($p <$
276 0.05), based on the UNIVARIATE procedure of SAS version 9.4 (SAS Institute 2015). The
277 data were also tested for sphericity by the Bartlett's test (Tobias and Carlson 1969) using
278 FACTOR procedure of SAS version 9.4 (SAS Institute 2015). The results indicated that all data
279 were distributed normally ($W \geq 0.90$) and no sphericity. All data were then analyzed using the
280 Linear Mixed Effect Model by PROC MIXED procedure of SAS and Satterthwaite
281 approximation to determine the denominator degrees of freedom for the tests of fixed effects.
282 Blocks and block interactions were considered random effects. Inoculations, cultivars, cropping
283 seasons, and their interactions were considered fixed effects. The results are reported as the
284 least square means and were separated using the probability of differences option (PDIF). The
285 means were compared using the LSD test. The main factor and interactive effects were
286 considered statistically significant at $p \leq 0.05$.

287

288 **3 Results**

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

298

(TABLE 2)

299

300

(TABLE 3)

301

302

303 At physiological maturity, positive effects on plant height were observed in plants
304 coinoculated with *Bradyrhizobium* and *A. brasilense* and in the number of pods of plants that
305 were coinoculated and received rhizobial metabolites (SI+ MSM + *A. brasilense*). Overall,

306 significant effects were not observed in the final population of plants (mean = 286,500 plant ha⁻¹)
307 ¹), the position of insertion of the first pod (mean = 13 cm), the number of branches per plant
308 (mean = 3), or the number of grains per pod (mean = 2.2) (data not shown).

309 Again, positive effects on the 100-grain weight and grain yield were observed in plants
310 inoculated with SI+ MSM + *A. brasilense* strains. In addition, there was an increase of up to
311 3.7% for the 100-seed weight and 2.4% for the crude protein concentrations, promoting an
312 increase of up to 516 kg ha⁻¹ of grain and 11% of AEI when compared to plants inoculated only
313 with *Bradyrhizobium* (Figure 3). However, no treatment differences were found for the ether
314 extract concentrations in seeds.

315

316 (FIGURE 3)

317

318 It is worth mentioning that in the 2017–2018 cropping season, higher averages were
319 obtained for most parameters, possibly due to the more favorable climatic conditions during
320 this season (Figure 3); nevertheless, no interaction with the factors (cultivar or inoculation) was
321 observed. Additionally, it is important to emphasize that plants inoculated with *B. subtilis* may
322 not have demonstrated their full potential because during the three cropping seasons; the
323 soybean crops had no suppression in relation to a high infestation of pests and disease inoculum.

324

325 **4 Discussion**

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

330 Although it has been reported that the response to bacterial inoculants may vary with
331 plant genotype (Wani et al. 1985; Penot et al. 1992), one reason for the variation is that the
332 growth habit may influence the source-sink relationship of the plants, mainly because of the
333 different hormonal balances and permanence of the alteration in the activity regime of the
334 photoassimilate source (Taiz et al. 2017). In addition, changes in plant C allocation in
335 association with BNF have been reported in several studies (Santachiara et al. 2017; Tamagno
336 et al. 2018), where the soybean growth types influence not only phenology but also growth and
337 allocation of biomass and N. However, in our study, the tested cultivars, one with a determinate
338 growth habit and nontransgenic and another that was indeterminate and transgenic, showed
339 similar responses to the treatments used. Kaschuk et al. (2016) also did not detect differences

340 in symbiotic performance and grain yield of soybean with different growth habits, while
341 Hungria et al. (2014) observed in a comparison of several parental and nearly isogenic
342 transgenic soybean tolerant to glyphosate that although the transgenic trait negatively affected
343 some BNF variables, over a three-year period, these effects had no significant impact on
344 soybean grain yield.

345 The results obtained revealed important increases in leaf total N, total P and N-ureide
346 concentrations in plants inoculated with SI + MSM + *A. brasilense*. The higher P uptake is
347 possibly due to the greater development of the soybean root system after inoculation. In a
348 previous study, Moretti et al. (2020) reported the capacity of the *Azospirillum* strains Ab-V5
349 and Ab-V6 to promote greater uptake capacity of soybean plants due to the greater development
350 of the root system, such as length, volume, surface area, and smaller diameter of roots (0.01–
351 0.5 mm). D'Angioli et al. (2017) reported positive correlations between the P supply and the
352 exudation of carboxylate in a corn root system stimulated by *A. brasilense* strains Ab-V5 and
353 Ab-V6, which was correlated with greater length and root area. This indicates positive feedback
354 in which the inoculation of *A. brasilense* stimulates root carboxylate exudation, influencing the
355 microbial community of the rhizosphere.

356 The bacterial consortium of *Bradyrhizobium*, *Azospirillum* and MSM improved leaf
357 total N by up to 7.1% and N-ureide concentrations by 16.5%. In legumes such as soybean, the
358 majority of N from BNF is transported in the xylem sap as N-ureides that will accumulate in
359 different organ tissues and in different concentrations (Baral et al. 2016), such that their
360 concentration has become a feasible method for the quantification of the contribution of BNF
361 (Herridge and Giller 2016). Since N is a constituent of plant cell components, and responsible
362 for proteins, amino acids, and nucleic acids synthesis, its deficiency limits grain yield,
363 nevertheless, the BNF can provide the N required by legume plants (Taiz et al. 2017, Oliveira
364 et al. 2019, Acuña et al. 2020).

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

372 We hypothesize that the beneficial relationships between strains of *Bradyrhizobium* +
373 strains of *Azospirillum* and MSM observed in this study in the nodulation of soybean plants are

374 promoted by the following facts: (i) *Azospirillum* strains Ab-V5 and Ab-V6 carry *nif* and *fix*
375 genes demonstrated in draft genome sequences by Hungria et al. (2018) and produce high
376 amounts of phytohormones, with an emphasis on indole acetic acid (IAA) (Fukami et al.
377 2018c); (ii) The LCOs can affect various physiological processes of the host plant, inducing,
378 for example, root hair deformation, expression of host *nod* genes essential for infection,
379 infection thread formation and cell division in some root cortical cells (Schalaman et al. 1997);
380 (iii) As reported by Massoud et al. (2009), a bacterial consortium may promote greater
381 nitrogenase activity and increase the availability of macronutrients, in addition to plant growth,
382 resulting in greater productivity compared to single inoculation.

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

391 Previous reports have also shown that the application of *Azospirillum* influences the
392 crude protein concentration in several crops, such as rice (*Oryza sativa*) (Omar et al. 1993),
393 wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) (Ozturk et al. 2003), maize (*Zea mays*)
394 (Nadeem et al. 2007), sunflower (*Helianthus annuus*) (Stefan et al. 2013), and safflower
395 (*Carthamus tinctorius*) (Nosheen et al. 2016). However, studies evaluating the effect of a
396 bacterial consortium with PGPR and bacterial metabolites on soybean grain quality are still
397 needed.

398 In our study, the bacterial consortium of *Bradyrhizobium* strains + *A. brasilense* strains
399 with MSM increased crude protein concentration by up to 2.4%. According to Fukami et al.
400 (2018a, c), one main mechanism by which *Azospirillum* promotes plant growth is the synthesis
401 of phytohormones such as auxin, cytokines and gibberellin, which are closely linked to N
402 signaling. Lone et al. (2005) reported that phytohormones are the main drivers of protein
403 changes and can improve not only yield but also the quality of oilseed crops. Increases in the
404 synthesis of phytohormones may stimulate the biosynthesis of amino acids and the
405 accumulation of protein in grains (Greef 1994). Amino acid synthesis is an important feature
406 of PGPR, and amino acids synthesized by PGPR include glutamic acid, lysine, valine, serine,

407 isoleucine and leucine (Babalola 2010), which are essential components in the human and
408 animal food base (Karr-Lilienthal et al. 2004).

409

410 **5 Conclusion**

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

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

419

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431

432 **7 References**

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

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

677 ^aMeans ± SE (standard error); ^bTOC, Total organic carbon; ^cBS, Base saturation; ^dCEC, Cation
678 exchange capacity; ^eMPN, Most probable number; ^fCFU, colony forming units.

679 **Table 2.** Nutrients and N-ureide (U) concentrations in leaves with petioles of two soybean cultivars that received different bacterial consortia
 680 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 + <i>B. subtilis</i> (Bs)	42.2 ± 0.7 b	2.7 ± 0.1 b	24 ± 0.2	11 ± 0.2	3.5 ± 0.1	2.6 ± 0.3	8.9 ± 0.3	136 ± 1.7	26 ± 1.2	52 ± 1.3	59 ± 1.5	10.8 ± 0.4 b
SI + MSM + <i>A. brasilense</i> (Ab)	45.0 ± 0.3 a	3.0 ± 0.1 a	23 ± 0.2	10 ± 0.4	3.7 ± 0.2	3.0 ± 0.1	8.8 ± 0.2	136 ± 2.8	26 ± 1.1	54 ± 1.2	58 ± 1.6	11.9 ± 0.4 a
SI + MSM + Bs + Ab	45.0 ± 0.4 a	3.0 ± 0.1 a	23 ± 0.3	10 ± 0.4	3.5 ± 0.2	3.0 ± 0.1	9.1 ± 0.1	134 ± 1.9	26 ± 1.2	54 ± 1.2	60 ± 1.4	12.0 ± 0.3 a
SI + Bs	42.4 ± 0.8 b	2.7 ± 0.1 b	23 ± 0.2	10 ± 0.5	3.6 ± 0.2	2.8 ± 0.2	8.8 ± 0.3	136 ± 1.7	26 ± 1.1	53 ± 1.3	59 ± 1.7	10.4 ± 0.3 b
SI + Ab	44.8 ± 0.6 a	3.0 ± 0.1 a	23 ± 0.3	11 ± 0.2	3.7 ± 0.2	2.7 ± 0.2	8.8 ± 0.3	135 ± 2.8	26 ± 1.0	54 ± 1.4	59 ± 1.5	11.8 ± 0.3 a
SI + Bs + Ab	44.9 ± 0.7 a	3.0 ± 0.1 a	23 ± 0.2	11 ± 0.2	3.7 ± 0.2	2.7 ± 0.2	9.2 ± 0.1	133 ± 2.6	26 ± 1.2	53 ± 1.3	58 ± 1.6	11.8 ± 0.4 a
Cultivar (Cv)												
BRS 317	43.3 ± 0.6	3.0 ± 0.1	23 ± 0.3	11 ± 0.3	3.8 ± 0.1	2.7 ± 0.2	8.4 ± 0.2	135 ± 2.7	26 ± 1.2	55 ± 1.3	58 ± 1.4	10.6 ± 0.4
TMG 1264 RR	43.5 ± 0.3	2.9 ± 0.1	24 ± 0.3	10 ± 0.4	3.9 ± 0.2	2.8 ± 0.3	8.8 ± 0.3	135 ± 2.6	26 ± 1.1	53 ± 1.2	59 ± 1.3	10.8 ± 0.3
Cropping Season (CS)												
2016–2017	43.4 ± 0.3	2.8 ± 0.1	24 ± 0.3	11 ± 0.2	3.7 ± 0.2	2.8 ± 0.2	8.5 ± 0.2	136 ± 2.7	26 ± 1.2	53 ± 1.3	58 ± 1.4	10.4 ± 0.3
2017–2018	43.4 ± 0.5	3.0 ± 0.1	23 ± 0.4	11 ± 0.3	3.8 ± 0.1	2.7 ± 0.3	8.6 ± 0.3	134 ± 2.6	26 ± 1.2	52 ± 1.3	59 ± 1.3	10.6 ± 0.3
2018–2019	43.3 ± 0.3	2.9 ± 0.1	24 ± 0.3	11 ± 0.3	3.6 ± 0.3	2.8 ± 0.2	8.8 ± 0.2	135 ± 2.5	26 ± 1.1	54 ± 1.2	58 ± 1.4	10.2 ± 0.4
ANOVA (F probability)												
In	**	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
Cv	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
In 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

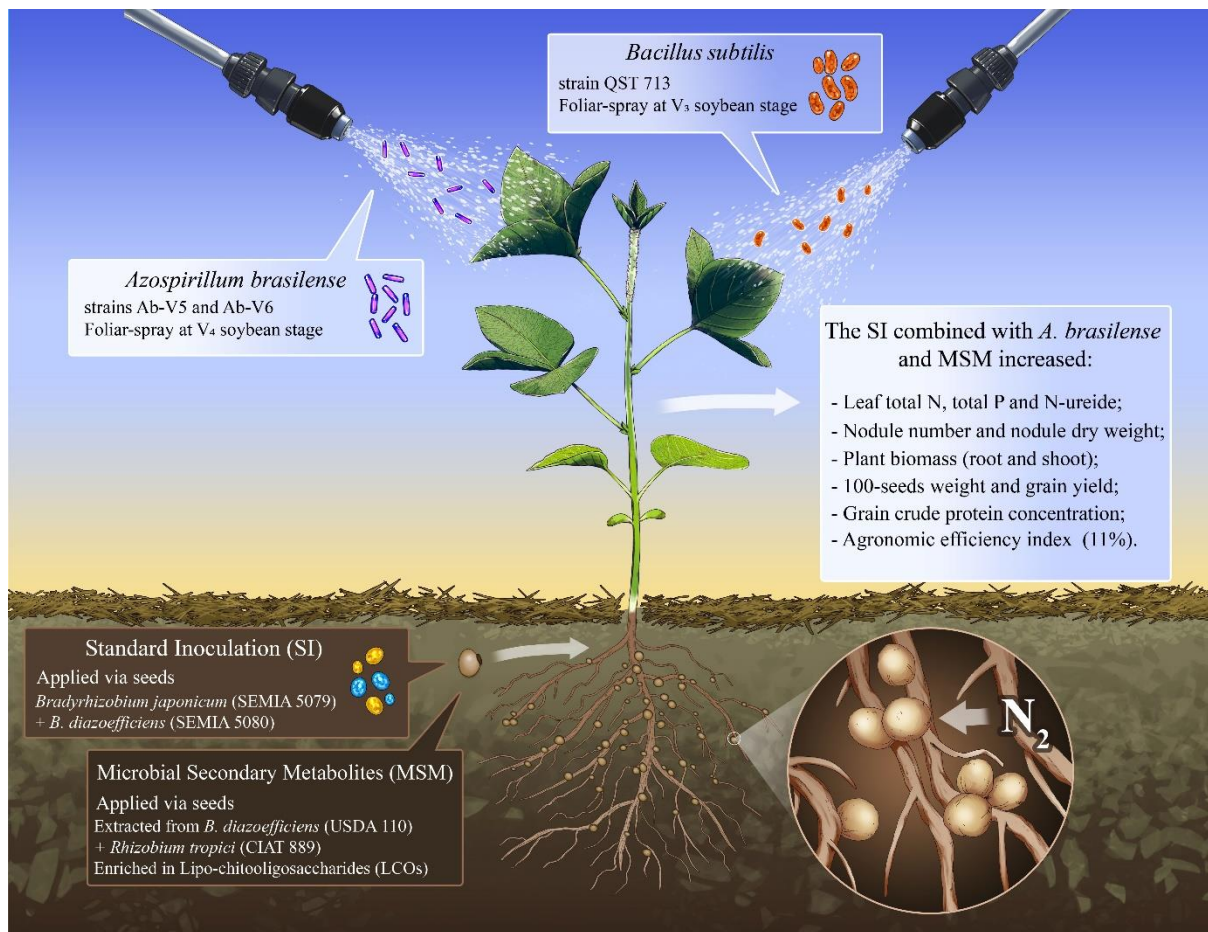
681 ^aInoculation treatments: SI = standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 + *B. diazoefficiens* strain SEMIA 5080 inoculated together on seeds;
 682 MSM = application of microbial secondary metabolites extracted from *B. diazoefficiens* strain USDA 110 and *Rhizobium tropici* strain CIAT 889 on seeds; *B. subtilis* = foliar-
 683 spray inoculation with *Bacillus subtilis* strain QST 713 at V₃ stage; *A. brasilense* = foliar-spray inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 at V₄ stage.
 684 ^bThe statistical model used was the linear mixed effect (LME). Means ± SE (standard error) followed by the same letter do not differ (ns) by the LSD test (Fisher's least
 685 significant difference) at **p* ≤ 0.05 and ***p* ≤ 0.01 probability.

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

Factor	NN	NDW	RDW	SDW	PH	P	W100	CP	EE
Inoculation (In^a)	<i>n^o plant⁻¹</i>	<i>mg plant⁻¹</i>	<i>g plant⁻¹</i>		<i>cm</i>	<i>n^o plant⁻¹</i>	<i>g</i>	<i>g kg⁻¹</i>	
Standard Inoculation (SI)	50 ± 4 b ^b	205 ± 6 b	5.2 ± 0.4 b	18.2 ± 0.4 b	82 ± 3 b	46 ± 3 b	16.4 ± 0.2 b	420 ± 3 b	208 ± 3
SI + MSM	54 ± 2 b	216 ± 7 b	5.2 ± 0.3 b	18.3 ± 0.3 b	82 ± 2 b	46 ± 2 b	16.6 ± 0.1 b	423 ± 2 b	211 ± 2
SI + MSM + <i>B. subtilis</i> (Bs)	54 ± 3 b	215 ± 6 b	5.3 ± 0.3 b	18.4 ± 0.3 b	82 ± 2 b	47 ± 2 b	16.7 ± 0.1 b	424 ± 2 b	212 ± 3
SI + MSM + <i>A. brasilense</i> (Ab)	61 ± 2 a	246 ± 5 a	5.9 ± 0.2 a	19.0 ± 0.2 a	87 ± 1 a	52 ± 2 a	17.0 ± 0.1 a	430 ± 2 a	210 ± 3
SI + MSM + Bs + Ab	63 ± 3 a	250 ± 4 a	6.0 ± 0.2 a	19.3 ± 0.2 a	88 ± 2 a	54 ± 3 a	17.0 ± 0.1 a	430 ± 2 a	210 ± 2
SI + Bs	52 ± 5 b	212 ± 6 b	5.3 ± 0.3 b	18.4 ± 0.2 b	83 ± 2 b	46 ± 2 b	16.4 ± 0.2 b	424 ± 2 b	209 ± 3
SI + Ab	60 ± 2 a	240 ± 6 a	5.8 ± 0.1 a	18.9 ± 0.1 a	87 ± 1 a	47 ± 2 b	16.6 ± 0.1 b	424 ± 2 b	209 ± 3
SI + Bs + Ab	60 ± 2 a	242 ± 5 a	5.8 ± 0.1 a	19.0 ± 0.2 a	87 ± 1 a	47 ± 2 b	16.5 ± 0.1 b	425 ± 2 b	210 ± 2
Cultivar (Cv)									
BRS 317	57 ± 2	230 ± 4	5.5 ± 0.3	18.5 ± 0.3	84 ± 3	49 ± 3	16.6 ± 0.2	423 ± 4	209 ± 4
TMG 1264 RR	55 ± 3	225 ± 5	5.4 ± 0.2	18.9 ± 0.4	86 ± 2	48 ± 3	16.8 ± 0.3	424 ± 3	210 ± 3
Cropping Season (CS)									
2016–2017	55 ± 3	227 ± 4	5.6 ± 0.3	18.3 ± 0.4 b	83 ± 2 b	47 ± 2 b	16.3 ± 0.3 b	427 ± 3	210 ± 3
2017–2018	58 ± 3	231 ± 5	5.5 ± 0.3	18.9 ± 0.1 a	87 ± 1 a	52 ± 2 a	16.9 ± 0.1 a	429 ± 4	211 ± 3
2018–2019	56 ± 2	224 ± 5	5.6 ± 0.2	18.4 ± 0.3 b	84 ± 1 b	46 ± 2 b	16.5 ± 0.2 b	423 ± 5	210 ± 4
ANOVA (<i>F</i> 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

689 ^aInoculation treatments: SI = standard inoculation with *Bradyrhizobium japonicum* strain SEMIA 5079 + *Bradyrhizobium diazoefficiens* strain SEMIA 5080 inoculated together
 690 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*
 691 = foliar-spray inoculation with *Bacillus subtilis* strain QST 713 at V₃ stage; *Azospirillum brasilense* = foliar-spray inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 at

692 V₄ stage. ^bThe statistical model used was the linear mixed effect (LME). Means ± SE (standard error) followed by the same letter do not differ (*ns*) by the LSD test (Fisher's
693 least significant difference) at * $p \leq 0.05$ and ** $p \leq 0.01$ probability.



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695 **Figure 1.** Graphic abstract

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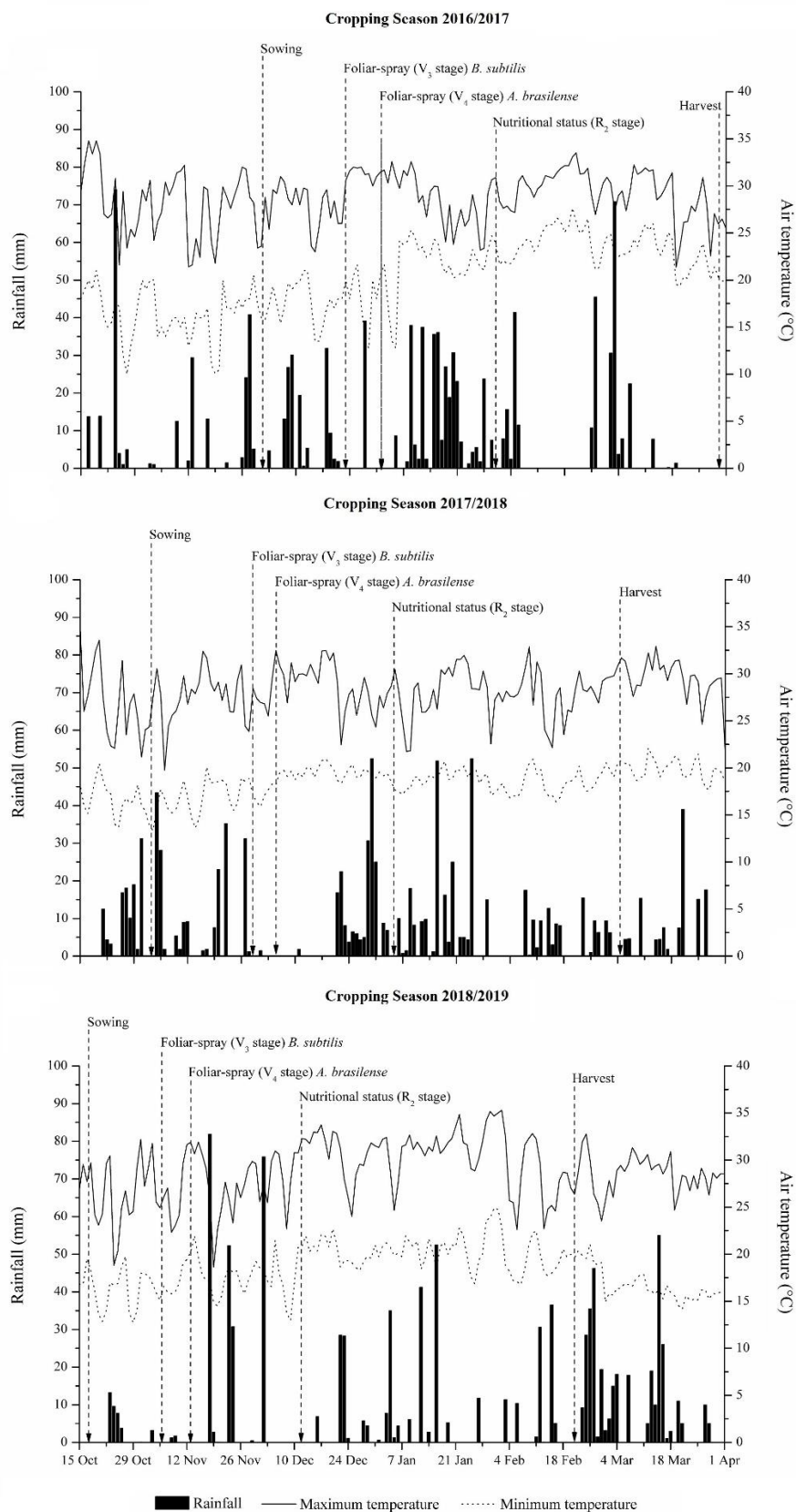
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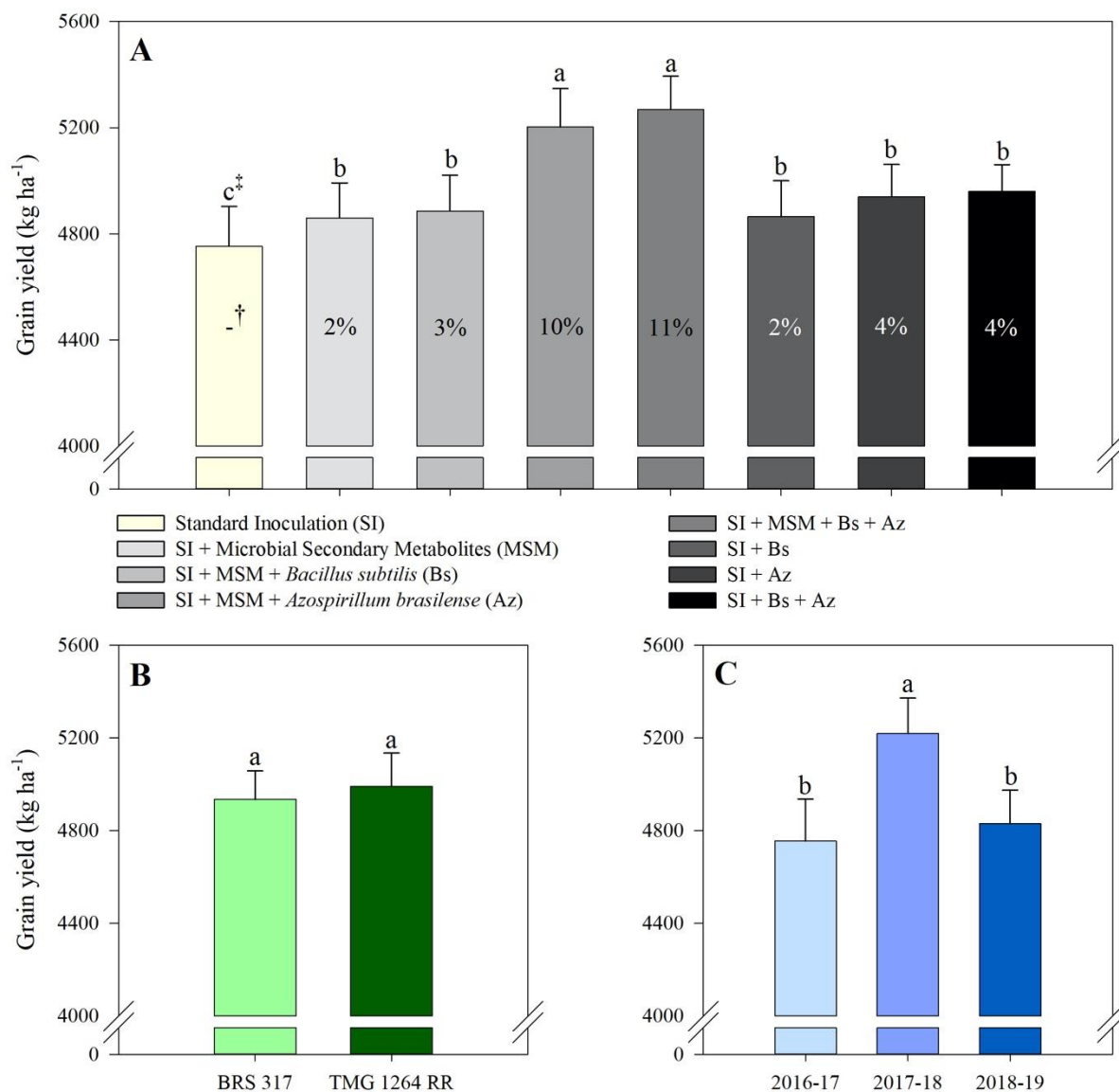


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708 **Figure 2.** Rainfall and maximum and minimum air temperatures during the experimental period

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(2016–2019).

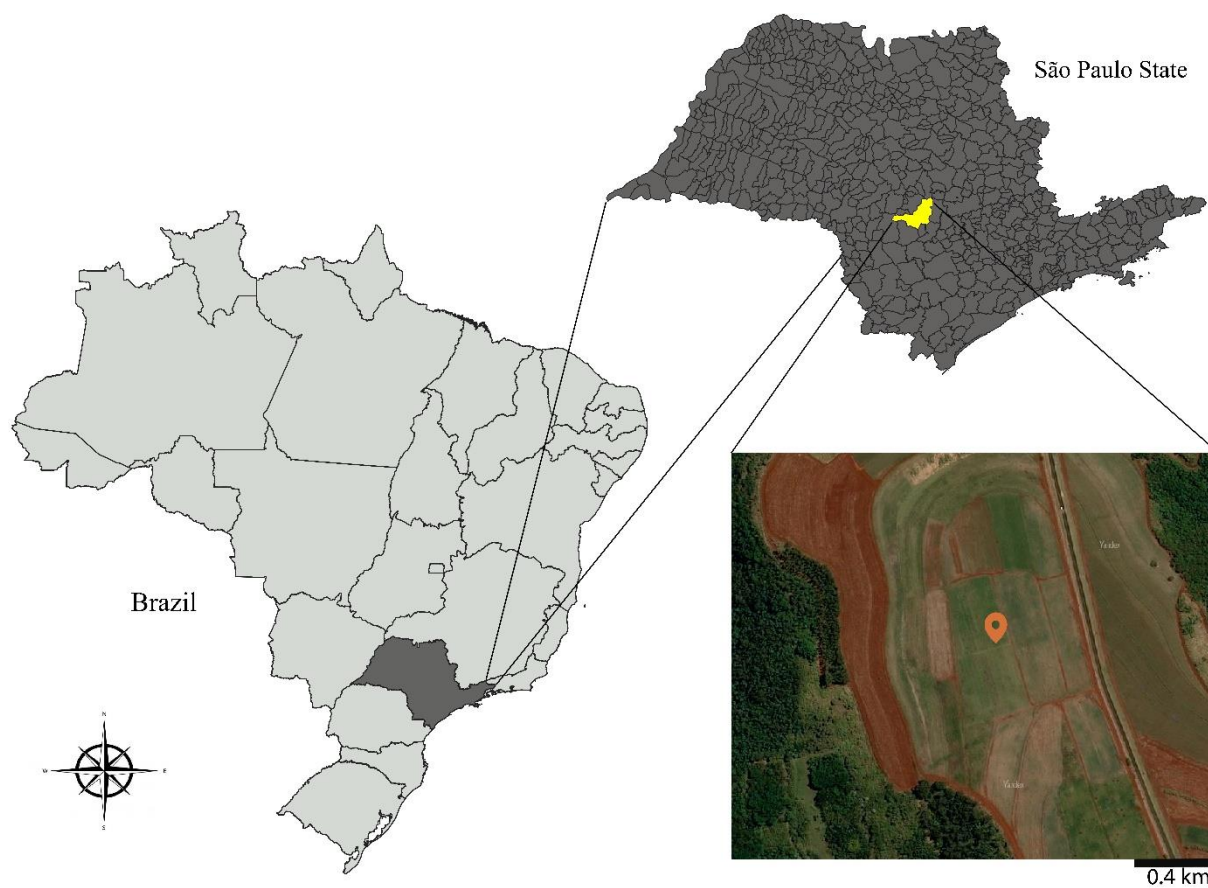


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 711 **Figure 3.** Average soybean grain yield as a function bacterial consortia (A), in two soybean
 712 cultivars (B), in the field during three cropping seasons (C). †Agronomic efficiency index
 713 (AEI). ‡The statistical model used was the linear mixed effect (LME). Means ± SE (standard
 714 error) followed by different letters differ from each other by LSD test (Fisher's least significant
 715 difference) at $p \leq 0.05$. There was no statistical interaction between bacterial consortia, cultivar
 716 or cropping season.

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720 **Supplementary Figure**

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723 **Supplementary Figure 1.** Lageado Experimental Farm – São Paulo State University (UNESP),

724 Botucatu-SP, Brazil. 📍 The Field Experiment.