Bacillus subtilis as growth-promoting rhizobacteria co-inoculated on *Bradyrhizobium*-treated soybean seeds in the planting furrow

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) can ensure the sustainability of agricultural growth. The objectives of this study were to characterize and determine the effects of *Bacillus subtilis*, isolate IMA Bs/170005, applied as co-inoculant in the soybean planting furrow. In all treatments, the seeds had been pre-inoculated with *Bradyrhizobium japonicum*. The experiments were carried out in a greenhouse, and in the field. Different doses of formulated (8.10^8 spores mL^{-1}) product with *B. subtilis* (0; 0.2; 0.4; 0.8; 1.2; 1.6 and 2.0 L ha^{-1}) were tested. The isolate proved efficient for *in vitro* auxin production. Under greenhouse conditions, the response to *B. subtilis* co-inoculation consisted of an increase of up to 26% in length of the root system. In the field, co-inoculation in the furrow proved beneficial for crop growth and yield and can be recommended. The best response rate was 0.4 L ha^{-1}. At this dose, averaged over 20 and 40 days after sowing and compared to the control with *Bradyrhizobium* inoculation alone, increases of 5.3% were observed for plant height, 14.8% for shoot fresh weight, 14.1% for shoot dry weight, 8.5% for root dry weight and 6.5% for soybean yield, demonstrating the efficiency of this *B. subtilis* isolate as a PGPR.

Keywords: inoculant; Radilix SC®; bioproduct; bacteria.

INTRODUCTION

Brazil is the world's largest soybean producer, where 138 million tons of soybean grain were harvested on 39.1 million hectares in the 2020/21 growing season (Conab, 2022). A major part of this area lies in the Cerrado biome. Some headway has been made in soybean cultivation since it has expanded into the Brazilian Cerrado, where the geological formations of the tropical soils is mostly ancient, acidity is high and fertility low (Hungria & Vargas, 2000). Some of these technological advances are soil acidity correction, breeding and intensive agricultural mechanization, which contributed to raise yields and optimize land use efficiency. In addition, a major milestone in the technological evolution of soybean cultivation was the discovery and use of nitrogen-fixing bacteria adapted to tropical conditions – the rhizobia. The practice of inoculation with these microorganisms has resulted in savings in nitrogen fertilization and contributed fundamentally to the sustainability and success of soybean cultivation in the Cerrado region (Hungria & Vargas, 2000).

Regardless of the improvements in recent years, efforts are continuously being made to increase the mean soybean yield in the Mato Grosso state, currently at 3,485 kg ha^{-1} (Conab, 2022). According to the Brazilian Soybean Strategic Committee (CESB), the yield potential could exceed 5,000 kg ha^{-1} in certain regions of the country (Battisti *et al.*, 2018). Technologies that minimize losses caused by biotic

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and abiotic stresses, especially in tropical regions, have been a key focus of research. In this sense, the combined use of different PGPR and the adoption of agricultural practices that favor rhizobacteria interaction in soybean appear promising to increase yield sustainably.

PGPR are a group of naturally-occurring soil bacteria found in the rhizosphere, i.e., in soil under the influence of plant roots and their exudates. The bacteria populations in the surroundings of plant roots are generally 10 - 100 x higher than in the bulk soil. Directly or indirectly, PGPR influence plant growth and root development by secreting regulatory substances or enzymes in the rhizosphere vicinity (Vejan *et al.,* 2016).

The inoculation of PGPR associated with rhizobia, i.e., co-inoculation with two or more microorganisms that contribute to different microbial processes that optimize plant growth to produce synergistic effects. The 2020, on the market of agricultural inoculum, 20% consisted of associations of *Bradyrhizobium* with other microorganism, of which 15% improved soil phosphorus solubilization and 5% promoted plant growth (Borsani & Vieira, 2022). Strains of *Azospirillum* spp. and *Bacillus subtilis* are considered promising for co-inoculation (Zeffa *et al.*, 2020)

The PGPR can promote plant growth through direct mechanisms such as phytohormone production (auxins, cytokinins, gibberellins), phosphorus solubilization and siderophore production (Olanrewaju *et al.*, 2017); as well as by indirect mechanisms such as the production of phytopathogen-antagonistic substances and resistance induction in plants (Lanna Filho *et al.*, 2010).

Microorganisms capable of producing phytohormones have been recommended for agricultural use in view of the great importance of these molecules in the regulation of all plant physiological processes. Auxins, of which the most important is indole-3-acetic acid (IAA), are key phytohormones in the regulation of root system architecture, for controlling primary root elongation and lateral root formation (Poveda & González-Andrés, 2021). The PGPR can synthesize auxins, particularly IAA, which is similar to that of plants. In bacteria, most auxin/IAA is synthesized from the amino acid tryptophan present in plant root exudates at varying low concentrations, according to the plant genotype (Olanrewaju *et al.*, 2017; Olenska *et al.*, 2020).

Several studies have demonstrated the positive effect of *B. subtilis* as growth-promoting inoculant in soybean (Araújo & Hungria, 1999; Bai *et al.*, 2002; Lanna Filho *et al.*, 2010; Costa *et al.*, 2014; Chagas *et al.*, 2017; Braga Junior *et al.*, 2018; Costa *et al.,* 2019; Tavanti *et al.*, 2019; Bavaresco *et al.*, 2020). The most commonly used form of bacterium co-inoculation is by treating seeds (Borsari & Vieira, 2022). However, depending on the interactions of the formulation, isolate, doses, soil and plant characteristics, results of other inoculation methods may be better (Lopes *et al.*, 2021).

Some studies show the effect of applying inoculants in the planting furrow of soybean (Vieira Neto *et al.*, 2008; Braga Junior *et al.*, 2018). By this inoculation method, higher doses per hectare can be applied if necessary, the product can be applied immediately at sowing, the time of direct exposure of the bacteria to chemical treatment on the seeds is shortened and the handling of seeds can be minimized, resulting in less mechanical damage (Possenti & Meneghello, 2022).

The objectives of this study were to characterize the *B. subtilis* isolate - IMA Bs/170005 and to evaluate how co-inoculating it in the soybean planting furrow with *Bradyrhizobium*-treated seed affects plant growth and yield under tropical climate conditions.

MATERIAL AND METHODS

Characterization of the B. subtilis isolate

The isolate IMA Bs/170005 of *B. subtilis* used in this study was extracted from a soil sample in the district of Campo Verde-Mato Grosso (S 15°29’55” W 55°12’18”), Brazil, in May 2017. In the agricultural area of the traditional production of cotton, soybean and corn. Soil type is red-yellow latosol with 38.6% clay. The bacterium was isolated from 1g of soil suspended in 10 mL sterile water, subjected to heat shock in a water bath (70 °C for 12 min), and seeded on nutrient agar culture medium. After incubation for 48 hours at 30 °C, bacterial growth was evaluated by optical phase contrast microscopy and the isolate was purified to obtain an axenic culture. The strain was preserved in the Mato Grosso Cotton Institute (IMAmt) collection for microorganism located in Primavera do Leste-MT, Brazil.

For the extraction of genomic DNA, the isolate *Bacillus subtilis* IMA Bs/170005 was grown in Luria-Bertani culture medium at 30 °C and 150 rpm for 14 h. Still in the exponential growth phase, the cell culture was centrifuged and genomic DNA extracted from the pellet using the NucleoSpin Microbial DNA Mini kit for microbial DNA (Macherey-Nagel, REF. 740235.50). Subsequently, DNA libraries for WGS sequencing were prepared using

the Illumina Nextera DNA Flex Library Preparation Kit. Sequencing was performed on an Illumina MiSeq platform with 2x250bp paired-end reads. The IMA Bs/170005 genome was deposited at the genomic database DDBJ /ENA/ GenBank (accession number JACTAU000000000.1).

To evaluate the production of IAA *in vitro* (Gordon & Weber, 1951), the bacterium was grown with and without tryptophan (1000 ug mL^{-1}) at 30 ºC and 200 rpm, until an optical density of 1 at 600 nm (OD600). The cell culture was centrifuged at 10000 rpm for 15 min, and 1 mL of the supernatant was mixed with 1 mL of Salkowski solution, followed by incubation at room temperature protected from light for 30 min. The samples were read in a spectrophotometer at 530 nm, and the IAA concentration was determined from a standard curve prepared with the synthesized hormone (Sigma – 99% purity).

To form a concentrated *B. subtilis* suspension for the experiments, strain IMA Bs/170005 was multiplied in a culture medium in a bioreactor under controlled pH, temperature and oxygen flow conditions. The multiplication process ended when 90% of the cells had free endospores at a final concentration of 8.10^8 viable spores mL^{-1}. Thereafter, the commercial product Radilix SC® (register No. MT 000245-3.000001, Brazilian Ministry of Agriculture-MAPA), was formulated with suitable stabilizers, emulsifiers and preservatives to ensure cell viability.

Rhizotron root trials

The effect of *B. subtilis* (formulated product with isolate IMA Bs/170005, 8.10^8 spores mL^{-1}) inoculation on soybean root length was evaluated in rhizotrons. Two identical trials were conducted in a greenhouse, one installed in March and the other in October 2021 (at 28 ± 2°C; coordinates S 15°32'08.98"/W 54°11'47.14 "). Each trial consisted of eight replications in a randomized complete block design and the plots of glass rhizotrons (120, 60.2 cm length width, thickness, respectively, and glass thickness 0.4 cm) (Galbieri *et al.*., 2018). The rhizotrons were filled with Vivatto slim® commercial substrate and sand (final composition: 15% clay, 5% silt and 80% sand with pH 6.0) at a ratio of 1:1 (v:v). The substrate was sterilized (autoclaved at 120 °C for 10 min) 25 days before planting.

The soybean cultivar used in both trials was IMA 731 IPRO. The seeds were previously inoculated with Brasilec® (*Bradyrhizobium japonicum,* 5.10^9 CFU mL^{-1}) at a dose of 2 mL kg^{-1} of seed, by hand, in a plastic bag. Sowing in the rhizotrons was carried out by opening a planting furrow and placing two seeds in each rhizotron. Before closing the planting furrow, the following treatments were applied: **T1**: control (no co-inoculation with *B. subtilis*); **T2**: *B. subtilis* at a dose of 0.15 L ha^{-1}; **T3**: *B. subtilis* at a dose of 0.4 L ha^{-1} and **T4**: *B. subtilis* at a dose of 1.2 L ha^{-1}. All inoculation was carried out in the planting furrow on the seeds at an approximate volume of 100 L ha^{-1} or 2.7 mL per rhizotron (60 cm wide). After seedling emergence, thinning was performed, leaving one plant per rhizotron. Automatic drip irrigation provided near-ideal growing conditions.

Thirty days after sowing, the rhizotrons were opened and each root system was carefully rinsed in running water over a sieve, so that small root debris could be recovered. Roots from each rhizotron were chopped into approximately 1.5 cm-long pieces and placed on a 30x40x2cm acrylic tray with 700mL of water. The tray was scanned by an Epson® Expression 10,000 XL model scanner and a digital image was saved. Root length data for each root system were obtained using software WinRHIZO® (Regent Instruments Canada Inc.). Data of total root system length were subjected to analysis of variance and the means separated by Duncan's test ($P \leq 0.05$).

Field trials

To test the agronomic efficiency of co-inoculation with a *B. subtilis*-based formulation in soybean, seven trials were carried out across five production regions of the crop in the State of Mato Grosso, Brazil, in two agricultural growing seasons (2018 and 2019) (Table 1). All experimental areas lie in the Cerrado biome where the climate is predominantly Aw (Tropical Savanna), according to the Köppen-Geiger classification, with two well-defined seasons (rainy, from October to April and dry, from May to September). The soil in the areas is typically classified as red-yellow latosol. The means (variation intervals between the seven tests) of the soil parameters in the experimental areas were: 45.5 (60-29)% sand, 8.2 (15.6-5.5)% silt, 46.3 (55.4-34)% clay, with 3.2 (3.75-2.31)% organic matter, pH 5.9 (6.1-5.3) and base saturation of 53.4 (58.8-42.0)%.

Sowing was carried out under no-tillage system, with fertilization of 400 kg ha^{-1} of single superphosphate, broadcast before planting, 150 kg ha^{-1} of KCl, broadcast 20 days after emergence and foliar fertilization, consisting of 2 L ha^{-1} of 10% manganese. The soybean cultivars used were selected according to their adaptation in the planting regions (Table 1). The experiments were installed from October 1 to 15 in each year.

Table 1: Description of test locations in the State of Mato Grosso, Brazil

Mato Grosso (MT) district	Growing season	Region (MT)	Coordinates		Altitude	Soybean cultivar
			Latitude	Longitude		
1. Campo Verde	2018	central	15°30'06.83"	55°2'26.94"	690	NS 7901 RR
2. Lucas do Rio Verde	2018	medium-northern	13°00'27.05"	55°58'06.67"	387	M 8372 IPRO
3. Primavera do Leste	2018	central-eastern	15°31'50.68"	54°12'00.90"	621	IMA 84114 RR
4. Sorriso	2019	northern	12°45'45.37"	55°50'38.60"	394	IMA 731 IPRO
5. Campo Verde	2019	central	15º30'07.95"	55º02'20.18"	682	IMA 731 IPRO
6. Primavera do Leste	2019	central-eastern	15º31'41.89"	54º12'05.19"	617	IMA 731 IPRO
7. Rondonópolis	2019	southern	16º33'40.37"	54º37'49.42"	309	IMA 731 IPRO

The seeds had previously been treated with thiamethoxam and fludioxonil at doses of 105.0 and 3.75 g a.i. per 100 kg^{-1} of seeds, respectively, and with the micronutrients cobalt sulfate at 20 g 100 kg^{-1} of seeds and sodium molybdate at 10 g 100 kg^{-1} of seeds. In all treatments, the seeds were inoculated with *Bradyrhizobium* which is a well-established technology, applied in almost all soybean production areas in Brazil. Inoculation based on *Bradyrhizobium japonicum* (Brasilec®, 5.10^9 CFU mL^{-1}) was performed before planting, at 2 mL kg^{-1} of seeds. Thus, the purpose of this study was to evaluate the effect of *Bacillus subtilis* as co-inoculant, applied in the planting furrow.

The experiment was arranged in randomized blocks with six replications. Each experimental plot consisted of eight 6-m long rows, spaced 0.45 m apart, i.e., a total plot area of 21.6 m^2 and treatment area of 129.6 m^2. The treatments used were: **T1** (control without co-inoculation of *B. subtilis*); **T2**: co-inoculation with a commercial product based on *B. subtilis* - UFPEDA 764, at a concentration of 3 x 10^9 CFU ml^{-1} and dose of 0.2 L ha^{-1}; T3 to T7 co-inoculation with formulated product based on *B. subtilis* - isolate IMA Bs/170005 - at different doses. **T3**: 0.4; **T4**: 0.8; **T5**: 1.2; **T6**: 1.6 and **T7**: 2 L ha^{-1}.

The treatments were applied on the seeds in the planting furrow, before covering with soil, by means of a CO^2 spraying equipment, with a 80.02 flat fan nozzle and a spray volume of 100 L ha^{-1}. The plants were not irrigated but treated with herbicides and pesticides as needed.

Several plant growth parameters were evaluated at 20 and 40 days after sowing (DAS). *Plant height* was determined from ground level to plant apex, measured in four random plants of each experimental plot at the two timepoints described above and at the end of the crop cycle. *Shoot weight* was determined from 12 randomly collected plants from the third and eighth row of each experimental plot cut at ground level. *Shoot dry weight* was averaged from five representative plants of the sample used to determine the shoot weight. The shoots were placed in paper bags in a forced air circulation oven at 65 °C ± 2 °C for a minimum period of 48 h or until constant weight. *Root dry weight* was the mean of 12 randomly collected plants from the third and eighth row of each experimental plot cut at ground. The roots were placed in paper bags in a forced-air circulation oven at 65 °C ± 2 °C for a minimum period of 48 h or until constant weight. *Soybean yield* was calculated by harvesting and weighing the grains of the four central rows of the evaluated area of each plot and adjusted to 13% moisture.

The data analyses were subjected individually and together (seven trials) to analysis of variance by the F test ($P \leq 0.05$), and the means were compared by the Duncan test ($P \leq 0.05$) and, for the yield variable, also at ($P \leq 0.10$). All analyses were performed using the "ExpDes" v1.2.0 package of the R statistical system version 3.3.3.

RESULTS

Characterization of the B. subtilis isolate

The complete genome of *Bacillus subtilis* isolate IMA Bs/170005 was assembled in 23 contigs, a mean guanine-cytosine content of 43.5% and a total genome size of 4052094 bp. The genome contains 4331 genes, 4131 of which are protein-encoding. Among the 4131 genes annotated in the isolate genome, an indole-3-glycerol phosphate synthase (trpC) gene was identified, related to auxin biosynthesis.

In the presence of tryptophan, the IAA production capacity of isolate IMA Bs/170005 was 52.21 ug ml^{-1}, while in the absence of the precursor it produced 0.44 ug ml^{-1}.

Rhizotron root trials

Root length in each treatment was consistent between the two rhizotron trials (no treatment x trial interaction; (P = 0.2279), so a pooled data analysis was appropriate. The overall mean total root length was 2030 cm (2189.2 cm in trial 1 and 1870.3 cm in trial 2). All *B. subtilis* doses (T2, T3 and T4) induced a significant increase (P = 0.0133*) in total root length, with a mean of 15.1%. Root length increased most in response to the dose of 0.4 L ha^{-1}, with a 26.3% increase over the control (Figure 1).

Field trials

Regarding *plant height*, there was no significant interaction between treatments and trials in the three sampling times (P = 0.375, P = 0.83, P = 0.97), allowing combined data analysis and presentation of the seven trials. At 20 DAS, soybean plants were tallest when co-inoculated with *B. subtilis* at doses of 0.2 to 1.2 L ha^{-1}. At the highest doses of *B. subtilis* (1.6 and 2.0 L ha^{-1}), the plant height was intermediate, statistically equal to the control and the other doses (Table 2). At 40 DAS, all applied *B. subtilis* doses induced taller plant height than the control treatment. In the evaluation at harvest, there were no significant differences between the treatment means. There was no difference in plant height between IMA Bs/170005 and the commercial isolate of *B. subtilis* (UFPEDA 764).

Soybean biomass was measured 20 and 40 DAS. The variables obtained were shoot fresh and dry weight and root dry weight. The combined data of the seven tests are shown in Table 3. For fresh weight at 20 DAS, only three trials were considered in the combined analysis, considering the criterion of the ratio major to minor mean square residue to be less than seven (Cruz & Regazzi, 1997). For the other variables, there was no significant treatment x trial interaction, allowing combined data analysis.

The treatments significantly increased the fresh matter weight of soybean plants at 20 DAS. The treatments with highest shoot fresh weight were those treated with *B. subtilis* at 0.2 and 0.4 L ha^{-1}. Root dry matter data also increased in the *B. subtilis* treatment. As for shoot weight, dry matter was also highest in the treatment inoculated with *B. subtilis* at 0.4 L ha^{-1}, 17% higher than in the control treatment without *B. subtilis* and 3.83% higher than in response to *B. subtilis* at 0.2 L ha^{-1}. The mean for this variable was 0.385 g per plant. Root dry matter was significantly higher in all treatments co-inoculated with *B. subtilis*, with an increase of up to 11% compared to the control.

At 40 DAS, the highest weight was achieved in the treatment with *B. subtilis* at 0.4 L ha^{-1}, statistically higher than the un-inoculated control treatment. Shoot dry weight was highest in the treatments with *B. subtilis* co-inoculation at 0.2-0.8 and 1.6 L ha^{-1}, i.e., statistically higher than in the treatment with 2 L ha^{-1}. For root dry weight at 40 DAS, the treatment with the highest value was co-inoculation with *B. subtilis* IMA Bs/170005 at 0.2 L ha^{-1}, with a 7.8% higher root weight than the lowest values, i.e., in the control treatment and the treatment with co-inoculation at the lowest dose (0.2 L ha^{-1}).

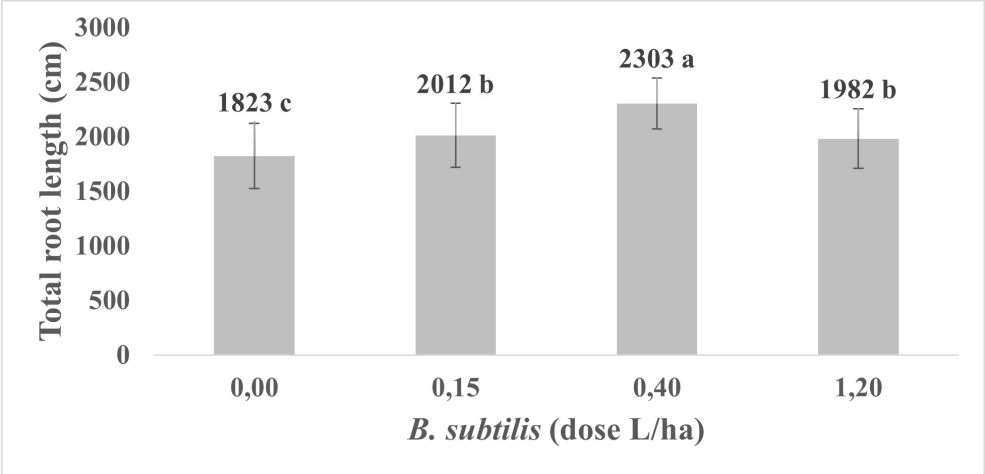

Figure 1: Total length of soybean root system 30 day after sowing (DAS) in rhizotrons in a greenhouse. *B. subtilis* (isolate IMABs/170005) applied in-furrow at planting. Bars with data followed by the same letter did not differ significantly by Duncan's test ($P \leq 0.05$). Results represent the pooled analysis of two trials.

Table 2: Height of soybean plants after co-inoculation with different doses of formulated product based on *B. subtilis* in the planting furrow under field conditions

Treatment (L/ha)	Plant height (cm pl^{-1})					
	20 DAS*		40 DAS*		Harvest	
	mean	sd†	mean	sd	mean	sd
0.0	13.1 b	2.2	39.8 b	9.4	72.5 a	10.6
0.2**	13.7 a	2.0	41.6 a	8.5	73.2 a	11.4
0.2	13.6 a	1.6	41.4 a	8.5	72.6 a	11.1
0.4	13.7 a	1.7	41.9 a	8.8	73.3 a	12.8
0.8	13.8 a	2.1	41.3 a	9.6	75.0 a	12.4
1.2	13.6 a	2.0	41.0 a	9.2	73.7 a	11.3
1.6	13.5 ab	2.0	41.1 a	9.2	74.4 a	13.1
2.0	13.5 ab	2.2	41.1 a	9.9	72.6 a	12.6
CV%	6.5		6.7		7.2	

Values in the same column followed by the same letter are not significantly different according to Duncan's test ($P \leq 0.05$). Results are the pooled analysis of seven trials.
*DAS: days after sowing; **Commercial product standard with *B. subtilis*
†Standard deviation

With regard to yield, there was no significant interaction between treatment x trial ($P = 0.857$) and combined data analysis was possible. According to Duncan's test ($P \leq 0.10$), there were significant differences between treatments. The treatments with the highest yields were those co-inoculated with *B. subtilis* at 0.2-0.4 and 1.2 L ha^{-1}. The greatest yield increase (245.0 kg ha^{-1}) in response to 0.4 L ha^{-1} (Figure 2).

DISCUSSION

Although IAA is a tryptophan-derived metabolite, it can be synthesized in bacteria by either dependent or independent pathways of this amino acid. In this study, the isolate was capable of producing IAA both in the presence and absence of tryptophan, although production was significantly higher in the presence of the precursor. Using tryptophan as a precursor for IAA biosynthesis is the most commonly used process of PGPR, since this amino acid is released by plant roots in the rhizosphere (Olenska *et al.*, 2020). In a literature review, Bokhari *et al.* (2019), showed that the ability to produce indole-3-acetic acid of PGPR is more frequently reported than that of phosphate-solubilization or zinc-solubilization.

One of the main benefits of IAA produced by PGPR is growth promotion of root and lateral root hair and, consequently, improved water and nutrient absorption and greater plant resistance to a number of environmental stresses (Poveda & González-Andrés, 2021). In addition, bacterial IAA loosens the plant cell walls and as a result facilitates intensified root exudation that provides additional nutrients (Glick, 2012). The expected effects depend, however, on the concentration of the plant-available phytohormone (Talboys *et al.*, 2014). The strain evaluated in our study produced a considerable IAA concentration (of 52.2 ug mL^{-1}) in the presence of tryptophan. Other studies on *Bacillus* isolates (PGPR) have also reported IAA production at levels from 6.9 to 15.0 ug mL^{-1} (Franco-Sierra *et al.*, 2020) and 8.56 to 31.33 ug mL^{-1}, with values above 19.40 ug mL^{-1} were considered highest production (Ferreira de Paula *et al.*, 2021).

The positive effect of promoting soybean root growth by co-inoculation of *B. subtilis* with *Bradyrhizobium* was demonstrated under controlled conditions in rhizotrons. Over the two trials, the total length of the root system averaged 2030 cm. In similar tests with soybean, Araújo *et al.* (2021) obtained values close to 3000 cm and Kanase & Guhey (2018) measured a total length of the root system close to 1800 cm, similar to the results of this study. Root length increased gradually from the *B. subtilis* dose of 0.15 to 0.4 L ha^{-1}, and at 0.4 L ha^{-1}, it was 26.3% longer than in the control without *B. subtilis*.

One of the advantages of inoculation in the planting furrow is to optimize the response rate per hectare, without taking into account the maximum volume of spray of a given product, as in the case of seed treatment. For example, the dose of 0.4 L ha^{-1} is a little higher than that normally applied in soybean seed treatments, however, in our study it resulted in more significant increases than the other doses. According to Possenti & Meneghello (2022), one must always consider the possibility of applying bio-

Table 3: Soybean shoot and roots weight in response to different doses of formulated product based on *B. subtilis* co-inoculated in the planting furrow in the field

Treatment (L/ha)	20 days after sowing (g pl^{-1})						40 days after sowing (g pl^{-1})					
	Shoot		Shoot dry		Root dry		Shoot		Shoot dry		Root dry	
	mean	sd†	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
0.0	2.14 b	1.10	0.35 c	0.14	0.126 b	0.06	29.12 c	15.27	5.30 bc	3.04	0.89 c	0.40
0.2**	2.58 a	1.04	0.39 ab	0.14	0.133 a	0.05	30.94 abc	15.48	5.81 ab	3.37	0.89 c	0.37
0.2	2.49 a	1.07	0.40 ab	0.14	0.139 a	0.06	31.40 ab	16.07	6.27 a	4.13	0.96 a	0.42
0.4	2.53 a	0.99	0.41 a	0.15	0.138 a	0.06	32.44 a	17.55	5.95 a	3.64	0.95 ab	0.42
0.8	2.24 b	1.07	0.38 ab	0.15	0.133 a	0.06	30.25 bc	16.24	5.92 a	3.66	0.90 bc	0.37
1.2	2.25 b	1.10	0.40 ab	0.16	0.140 a	0.06	30.13 bc	16.05	5.76 ab	3.48	0.92 abc	0.42
1.6	2.25 b	1.08	0.39 ab	0.14	0.140 a	0.05	30.89 abc	15.84	6.10 a	4.10	0.93 abc	0.41
2.0	2.15 b	1.17	0.37 b	0.17	0.134 a	0.06	30.50 bc	17.31	5.21 c	2.83	0.91 bc	0.41
CV%	11.1		14.5		11.0		13.10		21.6		12.0	

Values in the same column followed by the same letter are not significantly different by Duncan's test ($P \leq 0.05$). Results represent the pooled analysis of seven trials.
*Standard commercial product with *B. subtilis*
†Standard deviation

logical products in the planting furrow, which is the most recommended form of inoculant application. After several years of soybean cultivation, Vieira Neto *et al.* (2008) concluded that the best nodulation occurred in response to the application of liquid inoculant in the planting furrow.

At the highest inoculated dose (1.2 L ha^{-1}) there was a decline in root length increase (root length only 8.7% longer than in the control), demonstrating that the response to co-inoculation in promoting root growth in soybean plateaus at 0.4 L ha^{-1}. Studies carried out by Costa *et al.* (2019) with soybean co-inoculated with *B. subtilis* in seed treatments showed that higher doses (8 mL/kg of seed) impaired soybean development. It should be emphasized that the combinations of isolates must be carefully evaluated to establish a co-inoculation process. According to Costa *et al.* (2014), the combination of some bacterial strains had a negative effect on soybean plant growth.

In field trials, the results show consistent benefits of using co-inoculation with *B. subtilis* IMA Bs/170005 and *Bradyrhizobium*. There was no clear relationship between the *B. subtilis* doses applied with plant population per hectare (data not shown), with a mean of 260,032 plants ha^{-1} in the seven trials. This information differs from the data obtained by Braga Junior *et al.* (2018), Tavanti *et al.* (2019) and Chagas Junior *et al.* (2021), who showed improvements in plant population in response to *B. subtilis* inoculation.

The other variables had a positive response to *B. subtilis* co-inoculation. The technique induced greater plant height up to 40 DAS, different from the data obtained by Costa *et al.* (2019), who observed no positive effect of *B. subtilis* inoculation on plant height, demonstrating the specific potential of the strain tested in this study. The response in plant height was greatest at 20 DAS compared to the doses between 0.2 and 1.2 L ha^{-1}. However, plant height measurements at the time of harvest showed no significant differences.

According to Tkacz *et al.* (2020), the community structure of rhizosphere microorganism become distinct after only one week of growth in soil. The community structure becomes more robustly established by the second week and remains stable for a minimum of three weeks thereafter. Between the third and sixth week after inoculation, Durham (2013) observed a decline in the root bacterial population, which reduces the effect of the bacteria at the end of the crop cycle. This reinforces, therefore, the need to re-inoculate the plants in subsequent crops to maintain the bacterial population at satisfactory levels. These statements can explain the results for biomass up to 40 DAS found in this study.

Soybean biomass (shoot and root) was highest 40 DAS with the use of co-inoculation with *B. subtilis* IMA Bs/170005, showing the effects of the bacterium in promoting plant growth. There was also a differentiated response to the applied doses, showing that at the higher doses, e.g., 2 L ha^{-1}, the benefits were reduced or even cancelled out, depending on the analyzed variable. This reinforces the data showing diminishing benefits of increasing dosage obtained in the rhizotron in this study and by Costa *et al.* (2019).

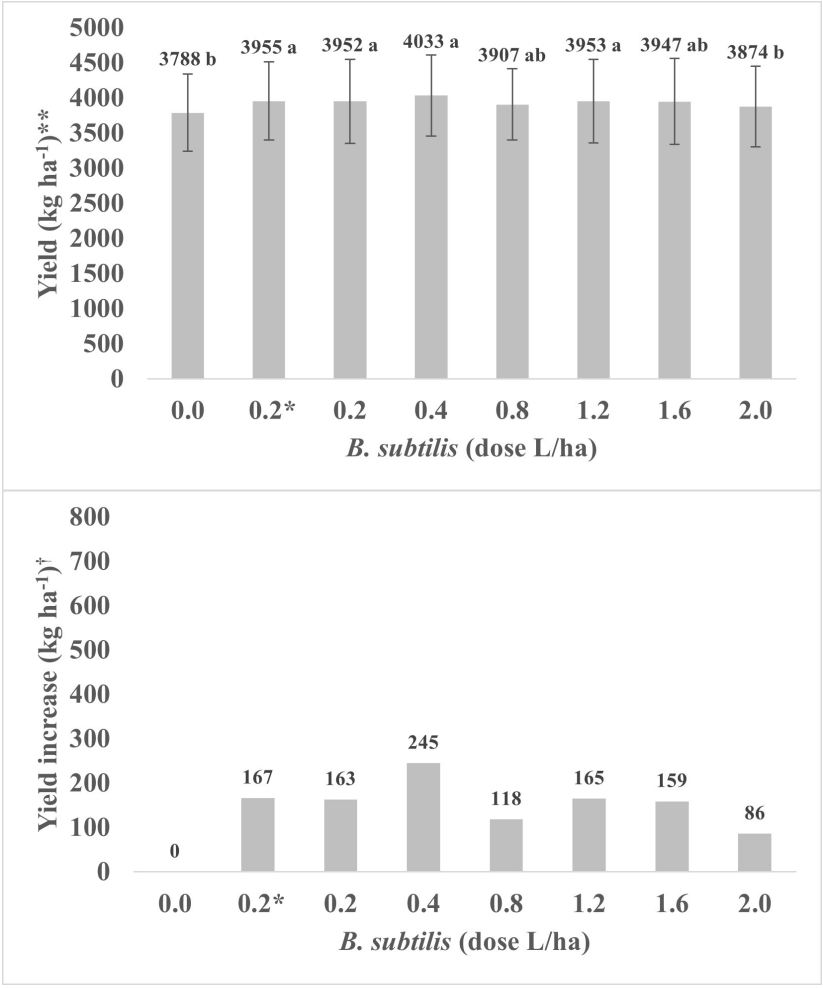

Figure 2: Soybean yield in response to different doses of formulated product based on *B. subtilis* co-inoculated in the planting furrow in the field.

**Bars with data followed by the same latter did not differ significantly by Duncan's test ($P \leq 0.10$). Results represent the pooled analysis of seven trials.

†Yield increase: yield increase compared to the control treatment without *B. subtilis*.

*Standard commercial product with B. subtilis

In the two evaluations of 20 and 40 DAS, the soybean biomass of the shoot part was 30% and that of the roots 16% higher than in the control treatment, without taking into account the applied *B. subtilis* doses. In a study of Braga Junior *et al.* (2018), a mean increase of 22% (in two trials) of total dry matter (shoot and root) in soybean was recorded at 20 DAS compared to the control without *B. subtilis*. A 20% increase in shoot dry matter of soybean plants at 20 DAS using co-inoculated *B. subtilis* was also observed by Chagas *et al.* (2017). According to Bai *et al.* (2002), root dry weight in V3 soybean plants inoculated with *B. subtilis* increased close to 30%. In trials under controlled conditions, Costa *et al.* (2014) stated that the shoot dry matter of inoculated soybean plants increased between 25 and 56%. *Bacillus subtilis* also colonizes the plant rhizosphere and releases some volatile organic compounds (VOCs). According to Bavaresco *et al.* (2020), soybean root system biomass increased by 18% in response to exposure to volatile compounds only by *B. subtilis*.

The increase in dry root weight in co-inoculated treatments under field conditions corroborate the data obtained with *B. subtilis* co-inoculation under controlled conditions. The strong IAA production of isolate *B. subtilis* IMA Bs/170005 may explain these results, since auxin is a phytohormone responsible for root growth by cell division and elongation. In addition, there are also benefits of co-inoculation of *B. subtilis* with *Bradyrhizobium* in nodulation and nitrogen fixation. Several studies have suggested that

higher auxin levels in the host plant are necessary for nodule formation (Glick, 2012). With the promotion of root growth by *B. subtilis*, there is more volume for colonization and nodulation by *Bradyrhizobium*, clearly showing the importance of co-inoculation of two microorganisms. Studies have reported on the benefits of other bacteria co-inoculated with *Bradyrhizobium* (Zeffa *et al.*, 2020).

With a larger root system, plants become more tolerant to biotic and abiotic factors in the scenario of increasingly frequent climatic adversities (Lopes *et al.*, 2021). In tropical regions, soybean is under constant attack by soil and nematode diseases. In the latter case, the larger the root system, the lower the parasite load received by the plant and, consequently, the greater its tolerance (Galbieri *et al.*, 2018). A well-established root system, especially at the beginning of crop development, ensures good soil exploitation and greater water and nutrient uptake (Falk *et al.*, 2020).

In this study, the architectural structure of the root system and aspects such as topology, branching angles, and longevity were not addressed (Falk *et al.*, 2020). This is a topic for future research, since there may be relationships between *B. subtilis* inoculation and alteration of the root system architecture and soil exploitation, wich may influence soybean yield.

The final consequence of the co-inoculation interaction is an increase in soybean yield. In our study, all co-inoculated *B. subtilis* doses led to an increase in yield. The yields in response to the dose of 2 L ha^{-1} did not differ statistically from those in the control treatment, which demonstrates, similar to the other variables, that the co-inoculation response occurs plateaus, and that very high doses are not necessarily advantageous. The biggest increase in yield was 245.0 kg ha^{-1} at the dose of 0.4 L ha^{-1}. At 0.2 L ha^{-1}, the tested commercial product induced a mean increase of 166.6 kg ha^{-1}.

Different results have been reported regarding the effect of co-inoculation of *B. subtilis* on soybean yield in Brazil. In studies comparing *B. subtilis* co-inoculation with inoculation treatments of *Bradyrhizobium* alone, Araújo & Hungria (1999) recorded a mean yield increase of 57 kg ha^{-1}, Braga Junior *et al.* (2021) of 523.6 kg ha^{-1} and Chagas Junior *et al.* (2021) of 23 to 139.8 kg ha^{-1}. In two trials, Braga Junior *et al.* (2018) observed increases of 270 and 335 kg ha^{-1}, in a comparison of *B. subtilis* inoculation in the planting furrow with an uninoculated control. Abiotic factors such as soil (nutrients or heavy metal content and pH), water availability, light intensity and temperature influence the response of growth-promoting microbes (Lopes *et al.*, 2021), without however considering the quality of the applied product. In studies with microorganisms multiplied on a farm, "on farm" production, Bocatti *et al.* (2022) found a high level of contamination or even absence of activity, which influences the response intensity of plants to inoculation.

Finally, the best response rate of *B. subtilis* co-inoculation in this study was 0.4 L ha^{-1}, applied in the planting furrow under field conditions. The mean increases (20 and 40 DAS) in response to inoculation with *B. subtilis* IMA Bs/170005, compared to the control inoculated with *Bradyrhizobium* only, were 5.3% for plant height, 14.8% for shoot weight, 14.1% for shoot dry weight, 8.5% for root dry weight and 6.5% for soybean yield.

CONCLUSIONS

Co-inoculation of *B. subtilis* IMA Bs/170005 on soybean promoted an increase in total length of the root system, plant height and shoot and root biomass up to 40 DAS, and in soybean yield. Increments with *B. subtilis* occurred according to the dose applied in the planting furrow; results were best in response to 0.4 L ha^{-1}. Co-inoculation with *B. subtilis* in the planting furrow is an efficient technology for soybean cultivation under tropical climate conditions. *B. subtilis* associated with *Bradyrhizobium* proved agronomically advantageous compared to inoculation with *Bradyrhizobium* only.

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