Scientific article

Bacillus thuringiensis RZ2MS9, a tropical plant growth-promoting rhizobacterium, improves maize and soybean growth under field conditions

Bacillus thuringiensis RZ2MS9, una rizobacteria tropical promotora del crecimiento vegetal, mejora el crecimiento del maíz y la soja en condiciones de campo

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Abstract

The insecticidal activities of *Bacillus thuringiensis* (*Bt*) are well known; however, *Bt* can also enhance plant development, a trait that has been less explored. The *Bt* RZ2MS9 has previously been shown to increase root dry weight of maize (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] by more than 200% under greenhouse conditions. The objective of this study was to test *Bt* effect on these crops under field conditions. We also compared *Bt* performance with that of commercial inoculants developed for these crops: *Azotobacter sp.* (*Az*) AZTBR19 for maize, and *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080 for soybean. Microbial inoculation was associated with different levels of nitrogen (N) fertilization in maize. *Bt* RZ2MS9 increased maize ear length, plant height, and ear insertion height. It also increased maize yield by 8%, while reducing N use by 30%. The inoculation with *Bt* RZ2MS9 increased soybean height more than *Bj* SEMIA 5080, and both strains increased plant mass by 29% and 39%, respectively. Under these experimental circumstances, our results confirmed the potential of using *Bt* RZ2MS9 as a microbial inoculant for maize and soybean cultivation in field conditions.

Keywords: *Azotobacter*; Biological product; *Bradyrhizobium*; Grain yield; Plant growth-promoting rhizobacteria.

Resumen

El efecto insecticida de *Bacillus thuringiensis* (*Bt*) es bien conocido; sin embargo, *Bt* también puede mejorar el desarrollo de la planta, un rasgo que está menos explorado. Se ha demostrado previamente que *Bt* RZ2MS9 aumenta el peso seco de la raíz en maíz (*Zea mays* L.) y brotes de soja [*Glycine max* (L.) Merr.] en más del 200 % en condiciones de invernadero. Por lo tanto, el objetivo de este estudio fue probar el efecto de *Bt* en estos cultivos en condiciones de campo. También comparamos el desempeño de *Bt* con el de inoculantes comerciales desarrollados para estos cultivos: *Azotobacter sp.* (*Az*) AZTBR19 en maíz y *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080 en soja. En el ensayo con maíz la inoculación microbiana se asoció con diferentes niveles de fertilización con nitrógeno (N). *Bt* RZ2MS9 aumentó la longitud de la mazorca de maíz, la altura de la planta y la altura de inserción de la mazorca. También aumentó el rendimiento del maíz en un 8 % y redujo el uso de N en un 30 %. La inoculación con *Bt* RZ2MS9 incrementó más la altura de la soja en comparación con *Bj* SEMIA 5080 y ambas cepas incrementaron la masa vegetal en 29 % y 39 %, respectivamente. Bajo estas circunstancias experimentales, nuestros resultados confirmaron el potencial del uso de *Bt* RZ2MS9 como inoculante microbiano para el cultivo de maíz y soja en condiciones de campo.

Palabras clave: *Azotobacter*; Bioinsumo; *Bradyrhizobium*; Producción de grano; Rizobacterias promotoras del crecimiento vegetal.

Introduction

The production of primary crops, such as maize (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] needs to increase significantly during the next decades to satisfy the demand of a growing world population (Aydinoglu *et al.*, 2020; FAO, 2020). Pesticides and fertilizers largely contribute to increasing crop production; however, the prolonged use of these agrochemicals can lead to soil degradation, and water pollution, among many other negative effects (Mitter *et al.*, 2021).

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Therefore, crop production needs to grow sustainably to protect the environment and to preserve soil quality (FAO, 2020; Mitter *et al.*, 2021). One of the main strategies to promote sustainable practices in traditional crop systems is the use of microbial inoculants formulated with plant growth-promoting bacteria (PGPB) (Hu *et al.*, 2017; Florio *et al.*, 2019; Aydinoglu *et al.*, 2020; Ullah *et al.*, 2020).

During the past decade, different species of bacteria have been studied in inoculant formulations in a variety of crops (Marks *et al.*, 2013; 2015; Hu *et al.*, 2017; Maqsood *et al.*, 2021). Among them, PGPB representatives of the genera *Azotobacter*, *Bacillus*, and *Bradyrhizobium* are reportedly notable plant-growth promoters (Gupta *et al.*, 2015; Gouda *et al.*, 2018; Pascale *et al.*, 2020). *Bacillus thuringiensis* (*Bt*) is a gram-positive, aerobic, spore-forming soil bacterium, well-known for its entomopathogenic ability (Mishra *et al.*, 2009; Qi *et al.*, 2016; Jouzani *et al.*, 2017; Azizoglu, 2019). *Bt*-derived products have been marketed as biopesticides for the control of important plant pests, including caterpillars, beetles, mosquito larvae, and black flies (Chattopadhyay *et al.*, 2017).

The *Bt* strain RZ2MS9 was originally isolated from guarana (*Paullinia cupana*) rhizosphere, a typical tropical plant from the Brazilian Amazon rainforest. The strain remarkably promoted maize and soybean growth under controlled conditions (Batista *et al.*, 2018; 2021). *Bt* RZ2MS9 inoculation increased maize and soybean root dry weight by more than 200% and 140%, respectively, as compared to the non-inoculated control, 60 days after seeding (Batista *et al.*, 2018). However, the performance of *Bt* RZ2MS9, as well as of other plant-growth promoting *Bts*, has not been evaluated under field conditions.

As most of the *Bt*-related studies focused on its entomopathogenic properties (Argôlo-Filho and Loguercio, 2014; Schünemann *et al.*, 2014; Parnell *et al.*, 2016; Qi *et al.*, 2016; Romeis *et al.*, 2019), our knowledge of the interaction between *Bt* and plants, as well as of its biofertilizer properties, is limited (Jouzani *et al.*, 2017; Azizoglu, 2019). In addition, as far as we know, there is not a commercially available *Bt*-based PGPR formulation in the biofertilizer market.

To help fill these knowledge and market gaps, our study evaluated the effect of *Bt* RZ2MS9 on the growth of maize and soybean under field conditions. We also compared the performance of *Bt* RZ2MS9 with that of commercial inoculants developed for these crops: *Azotobacter sp.* (*Az*) AZTBR19 for maize, and *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080 for soybean. In the maize trial, the effect of microbial inoculation was also associated with different levels of N fertilization to evaluate the possibility of reducing these inputs in the cropping system.

Materials and methods

Biological material

The bacterial strains used in this study were *Bacillus thuringiensis* (*Bt*) RZ2MS9, *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080, and *Azotobacter sp.* (*Az*) AZTBR19. The *Bt* RZ2MS9 belongs to the Laboratory of Genetics of Microorganisms "Prof. João Lucio de Azevedo" of the University of São Paulo, and it is kept at the Brazilian Collection of Microorganisms "Coleção Brasileira de Micro-organismos de Ambiente e Indústria – CBMAI", registered with number 823, and strain access number CBMAI1824. *Bt* RZ2MS9 is routinely grown in lysogeny broth (LB) medium (Giuseppe, 2004) at 28 °C. The *Bj* SEMIA 5080 was kindly provided by Dr. Mariangela Hungria from the "Culture Collection of Diazotrophic and Plant Growth Promoting Bacteria of Embrapa Soybean". *Bj* SEMIA 5080 is routinely grown in dextrose yeast glucose sucrose (DYGS) medium at 28 °C (Rodrigues Neto *et al.*, 1986). *Az* AZTBR19 strain, accession number MH538902 (Estrada-Bonilla *et al.*, 2021), was kindly provided by Barauna Biological Solutions (https://barauna.agr.br/). *Az* AZTBR19 is routinely grown in LB medium at 28 °C. We used the semi-flint maize hybrid 30A37PW® seeds (Morgan, Longping High-Tech, Hunan, China) and Intacta RR2-Pro® soybean seeds (Bayer, Leverkusen, Germany) in the field trials.

Experimental settings

Bacterial inocula were prepared by growing each bacterial strain in their respective culture medium at 28 °C and 150 rpm for 24 h. Initial cell density was adjusted to $1 \cdot 10^8$ CFU ml^{-1} in 50 ml of liquid medium. The inocula were kept at room temperature prior to seed inoculation. The seeds were washed with sterile distilled water and rinsed in a sterile 10% (w/v) sucrose solution for 10 min. Then, the seeds were drained and dried at room temperature. After drying, they were submerged into the bacterial treatments and incubated by shaking at 45 rpm for 30 min using an electronic shaker, at room temperature. After treatment with the bacterial inoculants, seeds were immediately sown (in both maize and soybean trials). The control treatment involved washing the seeds as previously described, but no microbial inoculant was applied.

Maize field experiment and agronomic measurements

Field experiment was carried out in the farm "Chapadão Chão Quente" in Taquarituba-SP, Brazil (23°31'59"S, 49°14'40"W, 669 m altitude, Köpen-Geiger climate type Cfa). The soil in the area is a dystrophic Red Latosol (Rhodic Hapludox) (Soil Survey Staff, 2010). Before sowing, soil fertility was corrected by adding 80 kg/ha of P_2O_5 and 60 kg/ha of K_2O, following routine farm management practices. The experiment was conducted in a split-plot design with three replicates. The treatments were: (i) single inoculation with *Bt* RZ2MS9, (ii) single inoculation with *Az* AZTBR19, and (iii) non-inoculated control, under four levels of N fertilization (0, 60, 70, and 100 kg/ha) each (assigned to sub-plots). The dose of 100 kg/ha of N represents the standard application adopted on the farm.

Maize seeds were mechanically sown in the 3 m × 20 m blocks, which were composed of 7 rows that were 45 cm away from each other; each row in a block corresponded to a plot. The application of different doses of N fertilizer (ammonium nitrate) was made manually at the V2 development stage. We also reapplied the bio-inoculants to the soil and close to the plants when they were at the V2 development stage, using a back-pack sprayer. Crop management followed standard farm practices and was kept under rainfed irrigation. Mean air temperature and precipitation in the region during maize experiment is shown in Figure 1.

The agronomic variables were evaluated at maturity stage in 6 plants from the three inner rows of each plot along five linear meters (useful area). Plant height (PH) was measured from the base of the stalk to the apex of the plant. Ear insertion height (EIH) was measured from the base of the stalk to the insertion point of the lowest ear. In each plot, 10 ears were randomly selected, stripped, and ear length (EL) was measured with a millimeter ruler. All maize ears were hand-stripped, and the grains were removed by a mechanical thresher. Grain mass (GM) per plot was measured using a digital scale. The values obtained from the useful area were adjusted to 16% of moisture and extrapolated to kg/ha to indicate maize grain yield per hectare. From each plot, 1000 grains were randomly collected for mass measurement (1000GM).

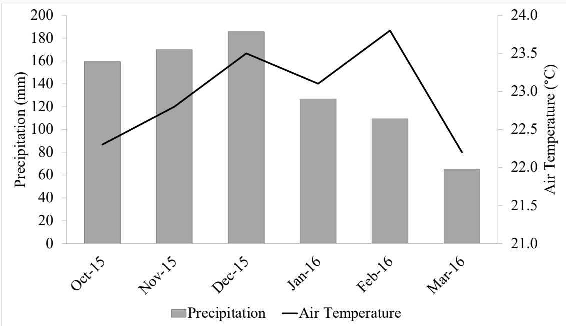

Figure 1: Mean air temperature (°C) and precipitation (mm) in the maize experimental area (Taquarituba – SP, Brazil) in the period of the experiment: October (2015) to March (2016) (Source: NASA Power, 2017 – Air temperature; Integrated Center for Agrometeorological Information - Centro Integrado de Informações Agrometeorológicas - CIIAGRO, 2017 – precipitation).

Soybean field experiment and agronomic measurements

Field experiment was carried out in the "Luiz de Queiroz" College of Agriculture in Piracicaba-SP, Brazil (22°42'23"S, 47°38'14"W, 535 m altitude, Köpen-Geiger climate type Cfa). The soil in the area is a dystrophic Red Latosol (Rhodic Hapludox) (Soil Survey Staff, 2010). Before sowing, soil fertility was corrected by adding 500 kg/ha of 4-14-8 NPK fertilizer following routine field management practices. The experiment was conducted in a randomized block design with three treatments and five replicates. The treatments were: (i) single inoculation with *Bj* SEMIA 5080, (ii) single inoculation with *Bt* RZ2MS9, and (iii) non-inoculated control.

Soybean seeds were mechanically sown in 3 m × 20 m blocks, which were composed of 7 rows that were spaced 45 cm apart; each row in a block corresponded to a plot. Crop management varied according to the needs of the culture, thus applying: (i) 5 L/ha of glyphosate Roundup® (Bayer, Leverkusen, Germany) at the V4 stage; (ii) a sub-dose of 1 L/ha of glyphosate at V5 stage; (iii) a preventive application of 0.6 L/ha of Opera® (BASF, Ludwigshafen, Germany) fungicide at R1 development stage; (iv) 2 L/ha of liquid fertilizer Sett® CaB (Stoller, Texas, USA) at R3 stage; and (v) 0.30 L/ha of Orkestra® (BASF, Ludwigshafen, Germany) fungicide at R4 growth stage. The crop was kept under rainfed irrigation. The mean air temperature and precipitation in the region during soybean trial is shown in Figure 2.

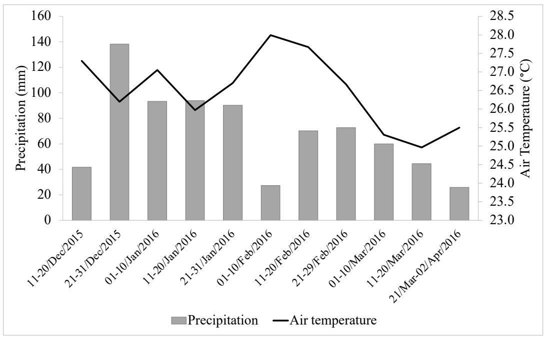

Figure 2: Mean air temperature (°C) and precipitation (mm) in the soybean experimental area (Piracicaba – SP, Brazil) in the period of the experiment: from December (2015) to April (2016) (Source: Climatological Data Series - Série de Dados Climatológicos - ESALQ, 2017).

The agronomic variables were evaluated at flowering (R1 stage), and harvest occurred after full maturity, at R8 stage. Twenty plants per treatment and per block were collected for measurements of plant height (PH), which was recorded as the length of the main stem from the soil surface to the terminal node; stem diameter (SD), which was measured at the height of the first node using a digital pachymeter; plant mass (PM); pod number (PN) and grain mass (GM). From each plot, 1000 grains were randomly collected for mass measurement (1000GM). The values of the agronomic variables obtained in the useful area were adjusted to 13% of moisture and extrapolated to kg/ha to indicate grain yield per hectare at the R8 stage.

Statistical analysis

Maize trial

The response variables PH, EIH, EL, GM, and 1000GM of maize plants were analyzed via a split-plot analysis of variance (split-plot ANOVA) using R software v. 3.5.2 and ExpDes package (Ferreira *et al*., 2014). The factors evaluated were the inoculants and the N doses. So, for each response variable, the interaction of these factors was tested, followed by a Tukey's test at 5% significance level to determine differences between means for each phenotypic attribute or the adjustment of regression models at the 5% significance level to analyze the influence of N fertilization levels.

Soybean trial

The response variables PH, SD, PM, PN, GM, and 1000GM of soybean plants were analyzed via a one-way analysis of variance (one-way ANOVA) using R software v. 3.5.2 and ExpDes package (Ferreira *et al*., 2014), followed by a Scott-Knott test at 5% significance level to determine differences between means for each phenotypic attribute. In this case, only one factor was evaluated: The inoculants. To verify the assumptions of normality of the residuals and homogeneity of variance, the Shapiro-Wilk's and O'Neill & Mathews' tests were performed at a 5% significance level.

Results

Effects of Bt RZ2MS9 inoculation and N dose on maize growth

In the maize trial, the inoculation with *Bt* RZ2MS9 and *Az* AZTBR19 did not influence maize growth. However, the interaction between the N doses applied and the inoculants tested (Table 1) had a significant influence on the response variables EL ($P < 0.001$), EIH ($P < 0.01$), and PH ($P < 0.05$). With the highest N dose (100 N), *Az* AZTBR19 significantly ($P < 0.05$) increased EL by 9% with respect to the non-inoculated control. On the other hand, *Bt* had no effect on EL (Table 2).

PH and EIH variables were significantly influenced by the interaction between N doses and inoculation (Table 1), with the adjustment of a quadratic polynomial regression model for *Bt* RZ2MS9 as a function of the different N doses (Figure 3). The adjustment of an increasing linear model for *Az* AZTBR19 and the non-inoculated control

Table 1. Analysis of variance of ear length (EL), plant height (PH), ear insertion height (EIH), grain mass (GM) and 1000-grain mass (1000GM) for maize plants inoculated with *Bacillus thuringiensis* (*Bt* RZ2MS9) and *Azotobacter sp.* (*Az* AZTBR19) and fertilized with four levels of nitrogen.

Factors	EL		PH		EIH		GM		1000GM	
	DF [a]	F	DF [a]	F	DF [a]	F	DF [a]	F	DF [a]	F
Inoculants (I)	2	2.2189	2	0.3279	2	0.5539	2	0.8903	2	1.6160
Residual 1	6	-	6	-	6	-	6	-	6	-
N dose (N)	3	9.3651 ***	3	23.7814 ***	3	8.4634 ***	3	4.6663 *	3	4.3804 *
I x N	6	5.3425 ***	6	2.2511 *	6	2.9769 **	6	0.9034	6	0.3987
Residual 2	342	-	198	-	198	-	18	-	18	-
CV [b] (%)		9.67		3.89		6.63		10.53		5.28

[a]Degrees of freedom.
[b]Coefficient of variation.
Significance codes, 0.001 '***' 0.01 '**' 0.05 '*'

Table 2. Tukey's test of ear length for maize plants inoculated with *Bacillus thuringiensis* (*Bt* RZ2MS9) and *Azotobacter sp.* (*Az* AZTBR19) and fertilized with four levels of nitrogen.

Nitrogen dose (kg/ha)	Treatments[a]		
	Bt RZ2MS9	*Az* AZTBR19	Control [b]
0	15.77±0.35 aA	14.10 ± 0.21 bA	15.57 ± 0.31 aA
60	15.87±0.24 aA	15.43 ± 0.28 aA	16.13 ± 0.29 aA
70	16.57±0.26 aA	16.07 ± 0.28 aA	16.07 ± 0.27 aA
100	16.30±0.29 abA	16.77 ± 0.28 aA	15.37 ± 0.30 bA

[a]Means followed by different lowercase letters in the same rows, and different capital letters under the same columns differ significantly (P < 0.05). Values are means of 30 replicates.

[b]Non-inoculated control (control).

as a function of the different N doses was observed (Figure 3). By modeling, the highest value of PH (2.197 m) was obtained when inoculating *Bt* RZ2MS9 and applying 67 kg/ha of N. For EIH, the highest value (1.217 m) was obtained when inoculating *Bt* RZ2MS9 and applying 53 kg/ha of N. To achieve the same maximum PH and EIH values with *Az* AZTBR19 inoculation, we would need to apply 73 and 122 kg/ha of N. As for the control, 87 and 66 kg/ha of N, respectively, would be necessary to obtain the same maximum values of PH and EIH (Figure 3).

The inoculation did not affect the GM and 1000GM variables (Table 1) and, therefore, nor did it affect grain yield. These variables were only influenced by N dose, with a linear increase in their values as N fertilization levels rose (Figure 4). The application of *Bt* RZ2MS9, along with a 70 N dose (a reduction of 30% in standard N fertilization), yielded a final grain yield of 12,785.19 kg/ha, which was 977.78 kg/ha (or 8%) higher than the yield obtained with the non-inoculated control (Table 3). However, this difference was not statistically significant according to the Tukey's test.

Effects of Bt RZ2MS9 inoculation on soybean growth

The inoculation of *Bt* RZ2MS9 increased soybean PH and SD by 4% and 8%, respectively, in comparison with the non-inoculated control (Figure 5A and B). Moreover, the inoculation of *Bt* RZ2MS9 and *Bj* SEMIA 5080 significantly (P < 0.05) increased total PM by 29% and 39%, respectively, in comparison with the non-inoculated control (Figure 5C). The application of *Bt* RZ2MS9 and *Bj* SEMIA 5080 increased the average PN of soybean (Figure 6A). Considering GM (Figure 6B), the treatments were not

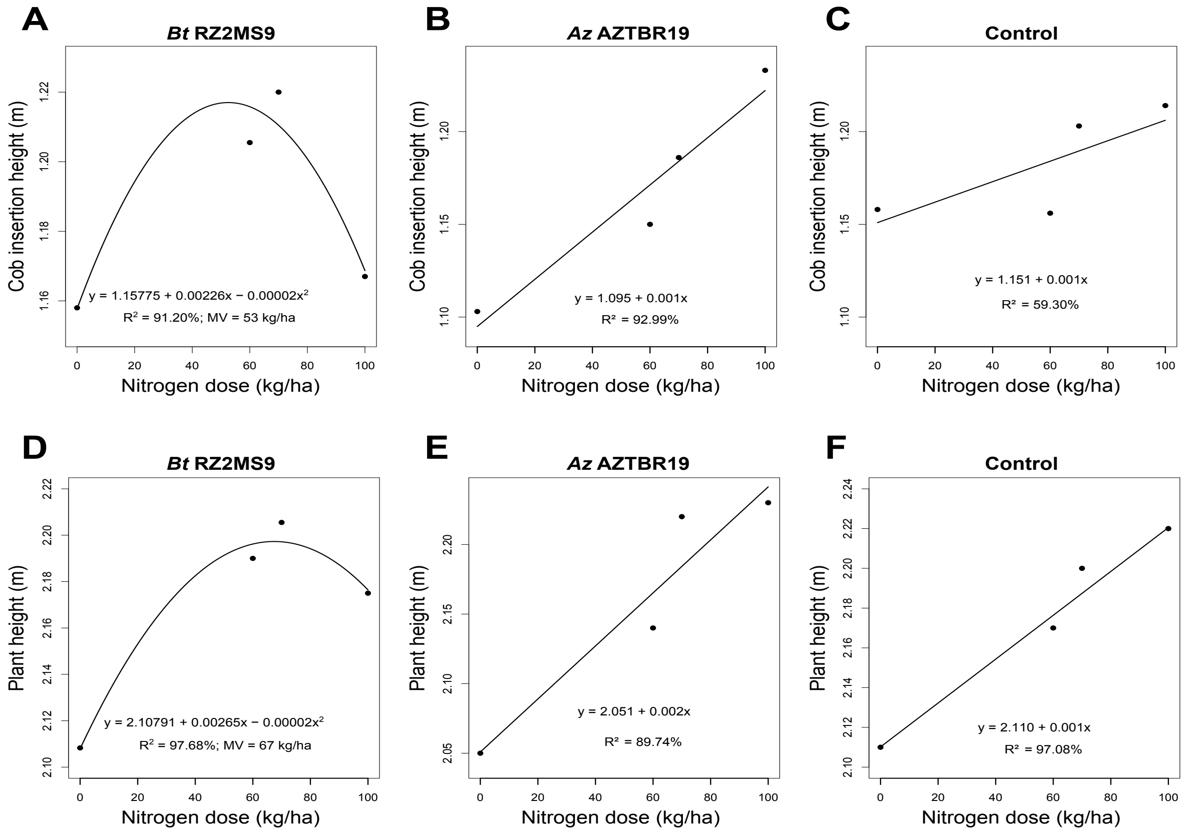

Figure 3. Ear insertion height and plant height of maize plants according to treatments: *Bacillus thuringiensis* (*Bt*) RZ2MS9 (A, D); *Azotobacter sp.* (*Az*) AZTBR19 (B, E); and non-inoculated control (C, F) associated with different levels of nitrogen fertilization (0, 60, 70 and 100 kg/ha). MV = maximum value.

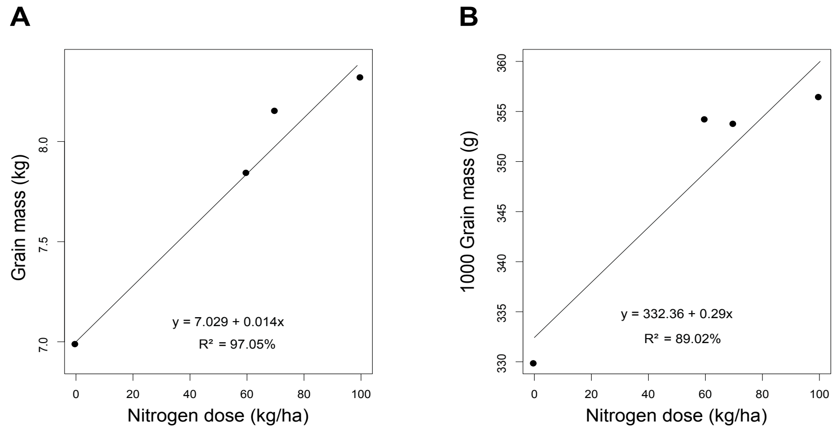

Figure 4. Grain mass (A) and 1000-grain mass (B) of maize associated with different levels of nitrogen fertilization (0, 60, 70 and 100 kg/ha).

statistically different from the non-inoculated control. The same was true for 1000GM (Figure 6C). Inoculating soybean with *Bj* SEMIA 5080 and *Bt* RZ2MS9 increased grain yield by 2,110.7 (36%) and 655.5 kg/ha (11%), respectively, as compared to the non-inoculated control. However, these increments were not significantly different from those shown by the control (Table 4).

Discussion

Bt RZ2MS9 has previously displayed four important plant growth-promoting (PGP) traits in vitro: indole-3-acetic acid (IAA) production, siderophore production, biological N fixation activity, and phosphate solubilization (Batista *et al.*, 2018). Therefore, we hypothesize that *Bt*

RZ2MS9 improves the growth and development of maize and soybean by employing at least one of those PGP mechanisms. As previously mentioned, the inoculation with *Bt* RZ2MS9 and *Az* AZTBR19 did not influence maize growth in field conditions. However, maize response to *Bt* RZ2MS9 inoculation was N-dependent.

Other studies have demonstrated the benefits of combining chemical fertilizers and microbial inoculants for agricultural production (Naher *et al*., 2019; Reddy *et al*., 2020). Our results are consistent with the findings of a previous study in which *Bacillus pumilus* inoculation in combination with N fertilization increased tomato growth and leaf transpiration in greenhouse conditions (Masood *et al*., 2020). Another study suggested that the co-inoculation of two strains of *Azotobacter chroococcum* (AC1 and AC10) allowed reducing N fertilization levels by up to 50% in cotton grown under greenhouse conditions (Romero-Perdomo *et al*., 2017).

From an agronomic perspective, morphological characteristics such as PH and EIH can help predict maize yield (Koca and Erekul, 2016; Martins *et al*., 2017). Furthermore, PH and EIH have a direct and positive correlation with the production of plant biomass and dry matter (Martins *et al*., 2017), highly desirable characteristics in the production of whole-plant corn silage (Borreani *et al*., 2018). The increase of maize PH and EIH with the application of *Bt* RZ2MS9 may be associated with the bacterial production of IAA (Batista *et al*., 2018; 2021). This phytohormone is responsible for the cell division and expansion processes leading to shoot elongation (Wu *et al*., 2021), and could thus have affected final plant height.

Recently, Batista *et al*. (2021) demonstrated that *Bt* RZ2MS9 harbors in its genome the complete set of genes required for IAA production through the indole-3-pyruvate (IPA) and tryptamine (TPM) pathways. *Bt* RZ2MS9 inoculation increased shoot dry weight of micro-tom tomato by 24% and increased its average lateral root length by 26%, as compared to the non-inoculated control. Likewise, Gomes *et al*. (2003) reported that an IAA-producing *Bt* strain C25 isolated from cabbage (*Brassica oleracea*) significantly enhanced the growth of *Lactuca sativa* in a greenhouse trial. Similarly, working with pea and lentil plants, Mishra *et al.* (2009) concluded that the co-inoculation of *Rhizobium leguminosarum* PR1 with the IAA-producing *Bt* strain KR1 significantly enhanced plant growth in comparison to single inoculation with *R. leguminosarum* PR1.

It is worth mentioning that other studies have reported using Bt strains as consortia (Bai *et al*., 2003; Mishra *et al*., 2009; Armada *et al*., 2016; Khan *et al*., 2020), and not in single inoculation, as we did in the present study. There are reports of bacterial strains that show little or no effects as single inoculants, but exhibit plant growth promotion effects when used in a consortium (Bai *et al*., 2003; Mishra *et al*., 2009; Armada *et al*., 2016; Khan *et al*., 2020; Zhikang *et al*., 2021). Future studies are needed to assess the performance of *Bt* RZ2MS9 in consortia.

As previously mentioned, GM, 1000GM and grain yield were influenced only by the nitrogen dose applied. Nitrogen is the most important mineral nutrient for cereal crops, since cereal seeds contain storage protein reserves with about 6% N (Zuluaga and Sonnante, 2019). Therefore, these crops highly depend on an adequate N supply. Haarhoff and Swanepoel (2018) investigated the correlation between N fertilizer inputs and plant population, which affects maize grain yield (Van

Table 3. Grain yield (kg/ha) of maize plants inoculated with *Bacillus thuringiensis* (*Bt* RZ2MS9) and *Azotobacter sp.* (*Az* AZTBR19) and fertilized with four levels of nitrogen.

Nitrogen doses	Treatments [a]		
	***Bt* RZ2MS9**	***Az* AZTBR19**	**Control [b]**
0	10651.85 ± 924.80	9377.78 ± 143.35	11022.22 ± 592.33
60	12074.07 ± 465.95	11674.07 ± 361.83	11214.81 ± 1266.51
70	12785.19 ± 782.28	11629.63 ± 361.83	11807.41 ± 291.01
100	12562.96 ± 1159.40	11933.33 ± 513.84	11511.11 ± 538.41
Mean [c]	12018.52 ± 553.28	11153.75 ± 687.91	11388.89 ± 198.56

[a]Values are means of three replicates.

[b]Non-inoculated control (control).

[c]This value is the mean of means.

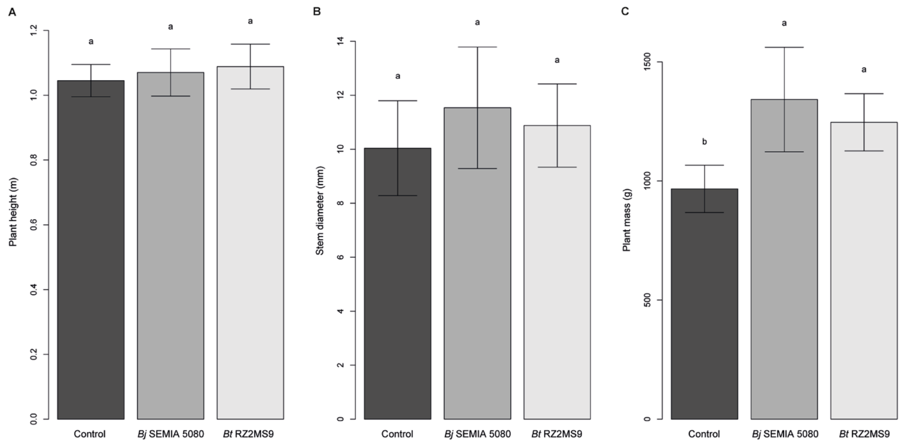

Figure 5. Effects of inoculation on plant height **(A)**, stem diameters **(B)**, and plant mass **(C)** of soybean plants according to treatments: non-inoculated control, *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080 and *Bacillus thuringiensis* (*Bt*) RZ2MS9. Means followed by different letters differ according to Scott-Knott test (P < 0.05).

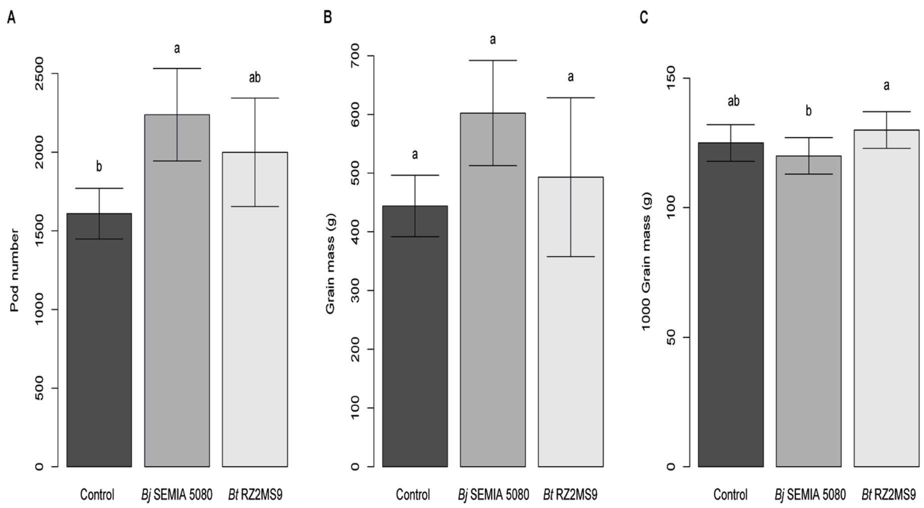

Figure 6. Effects of inoculation on pod number **(A)**, grain mass **(B)**, and 1000-grain mass **(C)** of soybean plants according to treatments: non-inoculated control, *Bradyrhizobium japonicum* (*Bj*) SEMIA 5080, and *Bacillus thuringiensis* (*Bt*) RZ2MS9. Means followed by different letters differ according to Scott-Knott test (P < 0.05).

Roekel and Coulter, 2011). The authors found that in high-N-input (R2 = 0.19, P < 0.001, N = 2,018) production systems, the response of plant population to applied N is weaker than in medium-N-input (R2 = 0.49, P < 0.001, N = 680) systems (Haarhoff and Swanepoel, 2018). Therefore, the use of *Bt* RZ2MS9 in maize crop systems demonstrates a high potential as a contribution to boosting maize grain yield and reducing N fertilization.

Here, our treatments have slightly affected maize growth, with inferior increases to those reported by Batista *et al.* (2018) with inoculation of RZ2MS9 and RZ2MS16 in maize under controlled conditions. The authors reported gains of up to 47% in shoot dry weight of both maize and soybean crops. Although reports about the beneficial effects of PGPR inoculation on plant growth are widely available, there has been variability and inconsistency of results observed under laboratory, greenhouse, and field trials (Gouda *et al*., 2018; Saad *et al*., 2020). We hypothesize that the inconsistent performance of the PGPR across different trials may be due to the various uncontrolled biotic and abiotic factors that can affect the bacterial survival and performance of the applied microbes (Saad *et al*., 2020).

Bj SEMIA 5080, a symbiotic rhizobium that has high specificity with soybean, has been studied for decades and is marketed for soybean cropping

Table 4. Grain yield of soybean plants inoculated with *Bacillus thuringiensis* (*Bt* RZ2MS9) and *Bradyrhizobium japonicum* (*Bj* SEMIA 5080).

Treatments	Grain yield (Kg/ha)
***Bt* RZ2MS9**	6584.2 ± 808.9
***Bj* SEMIA 5080**	8039.4 ± 535.5
Control [a]	5928.7 ± 311.4

[a]Non-inoculated control (control). Values are means of five replicates. Means did not differ according to Scott-Knott test.

systems in Brazil and other South American countries (Hungria and Vargas, 2000; Souleimanov *et al*., 2002; Hungria *et al*., 2006; 2015; Marks *et al*., 2013). Considering that *Bt* RZ2MS9 had effects on soybean growth comparable to those of *Bj* SEMIA 5080, we can suggest that the strain could be developed as an additional microbial inoculant for soybean.

Soybean yield is determined by various agronomic traits, such as height, pod number, and effective branching number (Yang *et al*., 2015; Fan et al., 2018). Here, the application of *Bt* RZ2MS9 and *Bj* SEMIA 5080 increased average PN of soybean. The primary constituents of soybean seed yield are flower number (flower/plant) and pod number (pod/plant) (Raza *et al*., 2019). Pod number is directly related to flower number, and only a small number of flowers mature into pods with seeds (Fan *et al*., 2018; Raza *et al*., 2019).

Previous studies reported that co-inoculation of *Bj* SB1 with *Bt* KR1 promoted soybean growth and increased shoot weight, nodule number, root weight, root volume, and total biomass compared to rhizobial inoculation alone and the control (Mishra *et al*., 2009; Jouzani *et al*., 2017). Bai *et al.* (2003) reported that *Bt* strain NEB17 co-inoculated with *Bj* significantly improved soybean nodulation, growth, and yield. There has been growing evidence of the benefits of the combined effect of PGPR and N-fixing bacteria (Bai *et al*., 2003; Mishra *et al*., 2009; Hungria *et al*., 2015; Armada *et al*., 2016). For instance, the PGPR *Bacillus amyloliquefaciens* LL2012 enhanced the capacity of *Bj* to colonize soybean roots and increase the number of nodules (Masciarelli *et al*., 2014). This suggests that the combination of *Bt* RZ2MS9 with *Bj* SEMIA 5080 should be further investigated.

The high variability in plant response, which is also highly affected by the many uncontrolled external factors, might help explain the lack of statistical significance when comparing bacterial inoculation with the control in this study. It is worth mentioning that in our study the strain was not prepared as a bioformulation (with the microbial cells being combined with some carrier material). Bioformulations can increase microbial inoculant potential by protecting microbial cells (Chaudhary *et al*., 2020), which can greatly improve the performance of the strain in the field.

Another important fact to consider is the specificity of the association between the bacterium and the host plant. It is known that the performance of a microbial inoculant is influenced by plant genotype (Vidotti *et al*., 2019). To obtain more consistent results, future investigation should aim to assess the effect of the bacterium on different plant genotypes over time (Cregger *et al*., 2018; Compant *et al*., 2020). Moreover, although compatibility between the inoculant and pesticides used in our study was not tested most studies have raised issues when microorganisms are subjected to a long-term exposure (Santos *et al.*, 2021). Here, the inoculant was applied alone in the seeds. So, although some effect could be expected we did not believe the agrochemicals impaired the benefits of inoculation (Gomes *et al*., 2017). Finally, our findings suggest that the beneficial features of *Bt* RZ2MS9 should not be limited to its insecticidal properties. Indeed, this strain holds great potential for its development as a novel microbial inoculant for primary crops such as maize and soybean.

Conclusion

Bt RZ2MS9 increased maize EL, PH, and EIH, even under low N doses, with a performance comparable to that of *Az* AZTBR19, a commercial inoculant for maize. Besides that, *Bt* RZ2MS9 increased soybean PH with a performance superior to that of *Bj* SEMIA 5080 inoculation. In addition, *Bt* RZ2MS9, similarly to *Bj* SEMIA 5080, significantly improved soybean PM and PN in comparison with the non-inoculated control. To obtain more consistent results when using *Bt* RZ2MS9, future studies should consider assessing strain performance in bioformulations and under field conditions. Further analyses should also include applying *Bt* RZ2MS9 to different maize and soybean genotypes, in combination with other beneficial microbes, and even under stress conditions.

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