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RESEARCH PAPER

Beneficial effects of the bacterial co-inoculation of *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus pumilus* on the promotion of growth in soybean and maize crops

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Abstract

Despite the great diversity of plant growth-promoting bacteria (PGPB) with potential to somewhat substitute the use of N fertilizers in agriculture, the use of *Bacillus* ssp as new eco-friendly bacterial consortium inoculants are a promising strategy to increase plant growth and crop yield by improving nutrient accessibility in the sustainable agricultural era. Therefore, the aim of this work was to evaluate agronomic efficiency and the viability of using a bacterial consortium of *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus pumilus* as growth promoters in soybean and maize crops, two of the most important grain and cereal crops, respectively. They both have high economic and strategic relevance, due to their nutritional value, and are therefore widely used in human and animal nutrition and as raw materials in various industrial sectors. Experiments were carried out in different locations and treatments. The application of the bacterial consortium of Bacillus was compared to the Absolut and Nitrogen controls, as well as to with a registered commercial product. The inoculant containing Bacillus consortium, associated with inoculation of Bradyrhizobium, provided an increase in soybean grain productivity, being statistically superior to the standard inoculation treatment and equivalent to co-inoculation with *Azospirillum brasilense* (AzoTotal). For maize crop, the co-inoculation with the bacterial consortium resulted in a 25% reduction in the amount of nitrogen used, in addition to promoting the growth of the maize crop, with productivity equivalent to the full dose of N and the application of a microorganism already registered for this purpose (*Pseudomonas fluorescens*). Thus, the results attest to the agronomic efficiency of the aforementioned inoculant in the field, as well as the viability of its use, and can be recommended as a growth-promoting microorganism for soybean and maize crops.

Keywords: ***Bacillus subtilis***; ***Bacillus amyloliquefaciens***; ***Bacillus pumilus***; ***Zea mays; Glycine max***; **plant growth-promoting bacteria.**

Introduction

Soybean (*Glycine max* L. Merrill) is one of the main plant species cultivated in the world, and its production is also mainly intended for animal feed and biofuel production. Food industry and pharmaceutical application are also relevant examples of the widespread use of this vegetable (Hil et al., 2006; Ibáñez et al., 2020; Kim et al., 2021). In addition to soy, maize (*Zea mays* L.) is one of the main cereal crops and a source of food for humans and animals due to the nutritional value of its grains. It is also in high demand across various industrial sectors, including the food industry, biofuel production, and the pharmaceutical industry (Miner et al., 2018; Chavez-Arias et al., 2021; Li et al., 2023).

The growing global demand for food and derivatives has made maize and soy cultivation the target of several studies aiming to increase the agricultural productivity (Ray et al., 2013; Malhi et al., 2021). Soybean is the fourth most cultivated grain globally and the biggest highlight of

Brazilian agribusiness. In the 2021/2022 season, Brazil estimates a production record of 142.009 million tons of soybeans. This value was obtained from the increase of 3.4% of the cultivated area compared to the 2020/2021 season and 4.3% of increased productivity per hectare (EPAGRI/CEPA, 2020; Kist, 2021). This virtuous cycle of expansion of soybeans and other grains in Brazil is closely linked to the success of inoculation technology with nitrogen-fixing bacteria (Barros-Carvalho et al., 2019; Cordeiro and Echer, 2019; Garcia et al., 2021).

Co-inoculation could be defined as the assemblage of at least two microorganisms that contribute to diverse microbial processes, improving plant growth and development (Fukami et al., 2016; Redondo-Gómez et al. 2021; Mortinho et al., 2022). The combined inoculation of two or more PGPB species has become an emerging agriculture technology recently, showing high reproducibility and efficiency under field conditions (Du Jardin, 2015; Mesa-Marín et al., 2019). Recent reports highlighted the potential for increased agricultural productivity through the use of microbial co-inoculants that promote enhanced growth and contribute to more sustainable agriculture. Compared to the single inoculation, co-inoculation with two or more bacterial species was positively correlated with the growth and nitrogen accumulation in soy and corn crops, as well as other important cultivation such as rice and wheat (Vargas-Díaz et al., 2019, Nascimento et al., 2021).

The pioneering practice of inoculating soybean seeds with standard strains of *Bradyrhizobium* made it possible to cultivate this oilseed without any use of nitrogen fertilizers (Hungria et al., 2005; Hungria et al., 2007). Subsequently, the practice of co-inoculation using *Bradyrhizobium spp.* and *Azospirillum brasilense* proved to be a practice with significant effects on productivity, generating increases of up to 16% (Bulegon et al., 2016; Hungria et al., 2013). Other groups of plant growth-promoting bacteria (PGPB) have been receiving attention from researchers and comprise the genera *Pseudomonas*, *Rhizobium* and *Bacillus* (Goswami and Deka, 2020). They can influence plant growth by increasing nutrient availability, producing phytohormones and suppressing harmful microorganisms in the rhizosphere. A meta-analysis study, comprising 42 published on the interaction between PGPB and soybeans from 1987 to 2018 across different regions of the world, revealed that co-inoculation with these bacteria promotes increases of 11% in the number of nodules, 6% in nodule mass, 13% in root mass and 6 % in aerial part mass, highlighting the importance of PGPB in promoting improvements in soybean crop (Zeffa et al., 2020).

Species of the *Bacillus* genus are widely explored for their potential of biological control in soybeans (Zhang et al., 2011) and maize crops (Fessia et al., 2022). Furthermore, studies focusing on promoting growth have been growing, both in Brazil and around the world, and bring promising results. In this aspect, the usage of *Bacillus* as new eco-friendly microbial consortium inoculants is a promising approach to rise plant growth and crop yield by improving nutrient accessibility in sustainable agricultural systems. *Bacillus subtilis* is the most studied species, research reveals positive results in nodulation, nodule mass, and plant height (Braga-Júnior et al., 2018; Marinković et al., 2018; Costa et al., 2019), as well as productivity increases of up to 25% (Braga-Júnior et al., 2018; Tavanti et al., 2020). Studies involving *Bacillus amyloliquefaciens* and *Bacillus pumilus* also highlight the potential use of these species in soybean inoculation, resulting in increased growth, nodulation, leaf area, and protein content in grains (Stefan et al., 2010; Masciarelli et al., 2014; Kim et al., 2017).

Thus, we analyzed the effects of co-inoculation with *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus* on the vegetative development, nutrition and productivity of soybean and maize under field conditions in Brazil.

Materials and methods

Site of studies with soybean

Two experiments were carried out in locations with different edaphoclimatic conditions, namely: Santa Maria do Oeste (PR, Brazil) and Lapa (PR, Brazil). A third experiment was conducted in Frei Rogério (SC, Brazil). All tests were conducted in the 2018/2019 harvest. Information related to each location is provided in Table 1.

Table 1: Description of the locations and management carried out in the tests with soybean.

Description	Location		
	Santa Maria do Oeste	Lapa	Frei Rogério
Previous Crop Summer	Perennial Pasture	Perennial Pasture	Perennial Pasture
Previous Crop Winter	Perennial Pasture	Perennial Pasture	Perennial Pasture
Hybrid	TMG 7062 IPRO	TMG 7062 IPRO	NA 5909 RG
Latitude	25° 50' 52.3" S	24° 54' 4.91" S	27°11' 55" S
Longitude	49° 39' 8.4" W	51° 51' 57.02" W	50° 43' 40" W
Altitude Meters	855	1051	815
Seeding	11/29/2018	11/07/2018	11/09/2017
V3-V5	12/28/2018	12/22/2018	12/18/2017
Harvest	04/08/2019	03/29/2019	04/06/2018
Soil Texture			
Clay (g/kg)	220	550	ND
Silt (g/kg)	310	300	ND
Sand (g/kg)	470	150	ND
Texture Class	Average	Clayey	Clayey
Soil Chemical Analysis (0 to 20 cm deep)			
pH (CaCl)	5.42	5.63	5.7
O.M. (g/dm^3)	21.75	3.50	4.0
P - Mehlich (mg/dm^3)	4.89	44.75	6.8
K (cmol/dm^3)	0.12	0.34	0.2
Ca (cmol/dm^3)	4.06	8.29	6.16
Mg (cmol/dm^3)	1.35	2.82	4.37
Al (cmol/dm^3)	0	0.05	0.3
H+Al (cmol/dm^3)	3.06	5.49	4.4
SB (cmol/dm^3)	5.63	11.46	6.62
CTC- pH 7.0 (cmol/dm^3)	8.69	16.94	13.0

ND: Not defined

Site of studies with maize

Three experiments were conducted in locations with different edaphoclimatic conditions, namely: Serranópolis do Iguaçu (PR, Brazil), São Miguel do Iguaçu (PR, Brazil) and Laguna Carapã (MS, Brazil). All tests were conducted in the 2019 growing. Information related to each location is provided in Table 2.

Table 2: Description of the locations and management carried out in the tests with corn.

Description	Location		
	Laguna Carapã	Serranópolis do Iguaçu	São Miguel do Iguaçu
Previous Crop Summer	Soy	Soy	Soy
Previous Crop Winter	Brachiaria	Corn	Corn
Hybrid	MG580 PowerCore	MG580 PowerCore	MG580 PowerCore
Latitude	22° 38' 09" S	25° 23' 08.4" S	25° 22' 27.9" S
Longitude	55º 02' 35" W	53° 58' 38.1" W	54° 12' 55.4" W
Altitude Meters	495	325	321
Seeding	02/15/2019	02/18/2019	02/16/2019
V5	03/08/2019	03/15/2019	03/14/2019
Harvest	07/05/2019	07/10/2019	07/09/2019
Soil Texture			
Clay (g/kg)	300	710	690
Silt (g/kg)	200	190	150
Sand (g/kg)	500	100	160
Texture Class	Average	Clayey	Clayey
Soil Chemical Analysis (0 to 20 cm deep)			
pH (CaCl)	4.39	5.29	656
O.M. (g/dm^3)	5.2	6.1	6.8
P - Mehlich (mg/dm^3)	29.53	45.62	40.29
K ($cmol/dm^3$)	1.16	4.42	2.50
Ca ($cmol/dm^3$)	0.46	0.61	0.46
Mg ($cmol/dm^3$)	2.61	5.01	3.44
Al ($cmol/dm^3$)	1.89	1.96	1.81
H+Al ($cmol/dm^3$)	0.36	0.01	0.11
SB ($cmol/dm^3$)	4.46	4.58	5.30
CTC- pH 7.0 ($cmol/dm^3$)	4.96	7.58	5.70

Commercial Inoculants

In the studies with a soybean liquid inoculant AzoTotal (Total Biotechnology, Curitiba, Brazil), with a guaranteed minimum concentration of 2 x 10^8 CFU/mL on the expiration date, was used. The inoculant contains two strains of *Azospirillum brasilense*: A. *brasilense*-YS (CNPSo 2083) and *A. brasilense*-V6 (CNPSo 2084). Furthermore, a commercial soybeans liquid inoculant Total Nitro Full was also used, composed of *Bradyrhizobium japonicum* SEMIA 5079 and 5080 strains, guaranteeing 7 x 10^9 CFU/mL. In the studies with the maize liquid inoculant produced by Total Biotechnology (Curitiba, Brazil) guaranteeing a minimum concentration of 1 x 10^8 UFC/mL of *Pseudomonas fluorescens* (strain CCTB03) from the expiration date.

Experimental Design

The experiment was conducted in a Randomized Block Design (RBD) with six replications. In the soybean crop, the following treatments were used in the study:

Treatment a1 - Absolute Control: the seeds were neither inoculated nor treated with nitrogen fertilizers.
Treatment a2 - Nitrogen Control: no seed inoculation. Nitrogen fertilizer was applied at a dose of 200kg N/ha (Super N, Koch Agronomic). Fifty percent of this dose was applied at sowing, and the remaining 50% was applied 31 days after emergence.
Treatment a3 - Standard Inoculation: Liquid inoculant TotalNitro Full (Biomagna, Rosario, Argentina) was

applied at a rate of 100 mL/50 kg of seed. The seeds were mixed and dried in the shade before sowing.
Treatment a4 – Co-inoculation Control: AzoTotal (100 mL/50kg of seed) + Total Nitro Full (100 mL/50kg).
Treatment a5 – Bacillus co-inoculation *[Bacillus subtilis* (BS), *Bacillus amyloliquefaciens* (BA) and *Bacillus pumilus* (BP)] at 50 mL/ha + Total Nitro Full (100 mL/50kg). For furrow inoculation, a mixture of the inoculant containing the three Bacillus species (50 mL/ha) was prepared with the inoculant additive Protege Max (100 mL/ha). Both were mixed and added to water so that the total volume corresponded to the application of 150L of syrup per hectare. Then, the mixture was sprayed into the sowing furrow.

For corn:
Treatment b1 - Absolute Control: the seeds were neither inoculated nor treated with nitrogen fertilizers.
Treatment b2 - Nitrogen Control: no seed inoculation and 100% of the recommended dose of N (200 kg/ha of N) was applied.
Treatment b3 – *P. fluorescens* + application of 75% of the recommended dose of N (200 kg/ha of N).
Treatment b4 - Bacillus Co-inoculation [*Bacillus subtilis* (BS), *Bacillus amyloliquefaciens* (BA), and *Bacillus pumilus (*BP)] + 75% of the recommended dose of N (200 kg/ha of N) was applied.

Seed treatment

Bacteria treatments were applied by seed treatment (ST). ST was conducted in a shaded place, shortly before sowing. In addition to the bacteria, the seeds were treated with Standak Top (Basf) at a dose of 80 mL of the product for 60,000 seeds for pest management.

Bacillus spp.: concentration and purity analysis

The concentration and purity analysis of the inoculant containing *B. subtilis* (CCTB04), *B. amyloliquefaciens* (CCTB09) and *B. pumilus* (CCTB05) was carried out by the laboratory of Total Biotechnology (Curitiba, Brazil), using the scattering method in trypticase soy agar (TSA) medium and observation under a microscope. The results of the analysis of the inoculant used in the experiments are provided in Table 3 for soybean and Table 4 for corn.

Soy, maize and Sowing

The soy hybrid was purchased from TMG 7062 IPRO (Tropical Melhoramento & Genética, Rondonópolis, MT, Brazil), and sowed a population of 240,000 plants per hectare. For maize, the hybrid used was the MG 580 Power Core (Morgan, Floresta/PR, Brazil), with a population of 60,000 plants per hectare for maize crop. Each experimental unit consisted of 10 rows with a spacing of 0.4 m between rows and a length of 6.0 m, totaling an area of 24m². Sowing dates, as well as other management information, are provided in Table 1 for soybean crop and Table 2 for maize crop. Sowing was carried out in a no-tillage system. The experimental area of each location was desiccated with glyphosate (720 g/ha) 15 days before sowing. In the background fertilization, 28 kg/ha of N, 105 kg/ha of phosphorus (P_2O_5) and 70 kg/ha of potassium (K_2O) were used, respectively, for all locations. During the crop cycle, agrochemicals were applied to manage pests, diseases and weeds following standard practices. Top dressing nitrogen fertilization was applied to plots at the V5 growth stage.

Table 3: Total microbial count, in colony forming units (CFU), in the inoculant used in the experiments with soybean, containing *B. subtilis*, *B. amyloliquefaciens* and *B. pumilus*, as well as presence or absence of contaminants in the 10^5 dilutions, as provided in Brazilian legislation.

Inoculant	Guarantee (CFU/mL)	Concentration CFU/mL	Contaminant (10^{-5})
Total microbial count	1.8 x 10^8	3.4 x 10^8	Absent

Table 4: Total microbial count, in colony forming units (CFU), in the inoculant used in the experiments with corn, containing *B. subtilis, B. amyloliquefaciens and B. pumilus*, as well as presence or absence of contaminants in the 10^5 dilutions, as in the Brazilian legislation.

Inoculant	Guarantee (CFU/mL)	Concentration CFU/mL	Contaminant (10^{-5})
Total microbial count	1 x 10^8	3.6 x 10^8	Absent

Variables Studied:

Productivity (kg/ha), mass of a thousand grains (TMG); number of productive branches per plant (plant branches); height of insertion of the first productive branch (cm); final plant height (cm); number of pods per plant; number of grains per pod; number of grains per plant; number of nodules; nodule mass; production of dry matter (DM) in the aerial part; production of dry matter in the root; nitrogen content in aerial part and grains.

From the 10 collected plants, we directly counted the number of pods and the number of grains per pod. By multiplying the values obtained for each variable, we calculated the number of grains per plant. For these evaluations, the measurement basis was the soil level up to the place where the first pod is inserted and up to the top of the plant, to determine, respectively, the insertion height of the first pod and plant height. Still from the 10 plants collected, through direct counting, the number of pods and the number of grains per pod were obtained. By multiplying the value obtained for each variable, the number of grains per plant was obtained.

To evaluate the number of nodules, three plants were collected per plot at the R1/R2 stage. The plants were cut at ground level, and with a cylinder with a volume of 942 cm^3 (cylinder with 10 cm in diameter and 12 cm in height) the roots of these plants were collected and subsequently washed, after washing the roots, proceeded the removal and counting of nodules. Subsequently, the nodules were placed in a forced-air ventilation oven at 65°C for 72 hours, and then their dry mass was determined.

Dry mass

To evaluate the dry mass of the aerial part, five plants were collected from the useful area of each plot at the R1 stage. The total fresh mass of the aerial part was weighed, and 200 grams were sampled; subsequently, it was placed in a forced air ventilation oven at 65ºC for 72 hours to determine the percentage of dry mass. After this evaluation, the dry mass per plant was estimated. The formula for this determination was as follows:

$$Dry\,\frac{mass}{Plant} = \frac{\left[fresh\ weight\ x\ \left(\frac{200g}{dry}\ mass\right)x\ 100\right]}{5}$$

For productivity, after maturation, the three central lines of the plot were harvested, and 0.50 m from each headland was discarded. The harvested material was then threshed, dried, and the grain yield at 14% humidity was determined Subsequently, from a subsample of this material, 300 grains were counted and weighed for each plot, and from these values, the mass of one thousand grains was calculated.

Nitrogen measurements

To analyze the N content in the corn, samples were taken from the grains at the time of harvest. The N analyzes in the plant and grains were carried out in an outsourced laboratory (Solum Brasilis, Guarapuava, PR, Brazil), according to the method described by Brazilian Agricultural Research Corporation (EMBRAPA, 2009). For soybean, the nitrogen content of the aerial part was measured according to the methodology described by Tedesco et al. (1995). The analysis consists of an adaptation of the Kjeldahl method.

Statistical Analyses

First, an outlier analysis was performed to identify outliers from the experiment. Outlier values were replaced by the average value of the other replications. Next, the data were analyzed for compliance with the assumptions necessary for Analysis of Variance. When the assumptions were met, the data were subjected to Analysis of Variance (using the F test) considering a level of 5%. When significant variations were detected, the treatment means were compared using the LSD Test or Duncan's test. The variables whose data did not meet the necessary assumptions for ANOVA were analyzed using a non-parametric test, Kruskal Wallis. When the p value was lower than 0.1, the means were compared with each other using the Kruskal-Wallis ranking test, also considering a 10% probability level. All statistical procedures were carried out using the Rbio software (Bhering, 2017).

Results

Soybean crop - site 1: Santa Maria do Oeste

The results of the first experiment with soybean, conducted in Santa Maria do Oeste (Paraná, Brazil) are shown in Tables 5 and 6. According to the analysis of variance (Table 5), a treatment effect was observed only for grain productivity and the number of nodules per plant. The results indicate that the Bacillus co-inoculation treatment had the highest productivity. This treatment was significantly superior to the treatments Absolute Control, Nitrogen Control and Inoculated Standard. It was also found out that the treatment with *Bacillus consortium* did not differ from the Standard Co-inoculated treatment with *A. brasilense*, a microorganism registered as a growth promoter for soybean crops. The use of *A. brasilense* for the inoculation of soybeans, together with *Bradyrhizobium*, has been disseminated by the Brazilian Agricultural Research Corporation (EMBRAPA) since 2013, and has provided significant increases in productivity for this crop. Consequently, co-inoculation with the *Bacillus Consortium* was equivalent in productivity to treatment with *A. brasilense*, and therefore demonstrates enormous potential as a growth promoter in this culture.

Regarding nodulation, it has been noticed that the Inoculated Control treatment did not differ from the Absolute Control treatment, which can be justified due to the presence of a native population of rhizobia in the soil, which allows the formation of nodules even in the absence of inoculants. The co-inoculation with A. *brasilense* and Bacillus Consortium treatments showed higher nodulation than the Absolute Control and Nitrogenated Control, demonstrating a positive impact on this parameter. The highest number of nodules was obtained by the *Bacillus Consortium* treatment (Table 6), which did not differ from the Control Inoculated and Co-inoculated treatments with *A. brasilense*. These results demonstrate that treatment with *Bacillus Consortium* does not harm culture nodulation and can be recommended in co-inoculation with *B. japonicum.*

Table 5: Summary of analysis of variance with mean squares of productivity, thousand grain mass (TGM), pod per plant, grains per pod and grains per plant, nitrogen in grains and plant, dry mass of shoots and roots, number and mass of nodules per plant, insertion height of the first pods and plants and number of productive soybean branches in Santa Maria do Oeste.

FV	DF	Mean Square Productivity (kg/ ha)	TGM (g)	Pod/Plant (Number)	Grain/Pod (Number)	Grain/plant (Number)	N Grain mg kg^{-1}	N Aerial Part mg kg^{-1}
Block	4	598901*	34.76 ns	70.3 ns	0.033 ns	355.33 ns	37.26 ns	31.28 ns
Treatment	5	149329	23.94	58.59	0.012	343.98	21.38	12.19
Error	20	141888	41.33	145.16	0.025	1038.45	21.34	23.46
Average		6443	3.54	47.03	2.57	120.76	54.10	55.69
CV (%)		5.85	181.57	25.62	6.19	26.69	8.54	8.70

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

FV	DF	DM/Plant (g)	DM/root (g)	Nodules/ Plant (Number)	Nodules/ Plant (Mass)	Insertion/ height (cm)	Plant height (cm)	Branches (Number)
Block	4	31.39 ns	0.346 ns	2261.91*	1727.45 ns	6.83 ns	43.30 ns	1.093 ns
Treatment	5	10.84	0.334	1348.37	29038.83	5.61	33.21	0.306
Error	20	23.45	0.404	483.26	13636.45	7.16	27.58	0.689
Average		23.32	2.38	38.33	276.37	21.68	92.94	2.03
CV (%)		20.7	18.8	57.35	42.25	12.34	5.65	40.83

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

Table 6: Effect of soil inoculation with different Inoculants in soybean crop in Santa Maria do Oeste.

Treatment	Productivity (kg/ha)	TGM (g)	Branches (number)	Insertion/height (cm)	Plant/height (cm)	N Grain mg kg^{-1}	N Aerial Part mg kg^{-1}
Absolut Control	6283 b c	181.20 ns	2.3 ns	21.6 ns	88.9 ns	52.54	54.22
Nitrogen Control	6041 c	178.60	2.00	21.8	95.7	51.94	53.69
Inoculant Control	6472 a b	180.10	1.9	21.4	94.1	56.65	57.77
AzoTotal	6528 a	183.50	1.5	23.3	94.5	56.98	58.66
Bacillus co-inoculation	6894 a	184.50	2.6	20.4	91.6	52.39	54.23
Average	6444	181.60	2.0	21.7	92.9	54.10	55.69

Means followed by the same letter (a, b or c) within columns do not differ from each other by Duncan's test at 5%. ns – not significant.

Treatment	Pod number	Grain/pod number	Grain/ Plant number	Plant mass	Root mass	Nodules number	Nodules mg
Absolut Control	44.7 ns	2.5 ns	111 ns	21.95 ns	3.09 ns	19 b	251
Nitrogen Control	47.9	2.55	123	22.32	3.35	16 b	273
Inoculant Control	45.0	2.58	116	22.27	3.40	48 a b	287
AzoTotal	44.9	2.69	122	22.69	3.31	53 a	295
Bacillus co-inoculation	52.7	2.53	132	27.39	3.75	56 a	276
Average	47.0	2.57	121	23.32	3.38	38	276

Means followed by the same letter (a, b or c) within columns do not differ from each other by Duncan's test at 5%. ns – not significant.

Soybean crop - site 2: Lapa

The results from the second site of experiment with soybean, conducted in Lapa (Paraná, Brazil), are shown in Tables 7 and 8. By analysis of variance (Table 7), a treatment effect was found only for grain productivity. For this parameter, all treatments that received nitrogen fertilization (Nitrogenated Control) or inoculation with *Bradyrhizobium* (Nitrogen Control, Inoculant Control, AzoTotal, and *Bacillus Consortium*) performed better than the control without nitrogen (Absolute Control). The treatment that obtained the highest productivity was the *Bacillus Consortium* (Table 8), which achieved values statistically equal to the Nitrogen Control and the Co-

Inoculated Standard with *A. brasilense* (AzoTotal). The *Bacillus Consortium* treatment was also superior to the inoculated control, demonstrating that the addition of *Bacillus* increases productivity over inoculation with *Bradyrhizobium*.

Table 7: Summary of analysis of variance with mean squares of productivity, thousand grain mass (TGM), pod per plant, grains per pod and grains per plant, nitrogen in grains and plant, dry mass of shoots and roots, number and mass of nodules per plant, insertion height of the first pods and plants and number of productive soybean branches in Lapa.

FV	DF	Mean Square						
		Productivity (kg/ha)	TGM (g)	Pod/Plant (number)	Grain/Pod (number)	Grain/plant (number)	N Grain mg kg^{-1}	N Aerial Part mg kg^{-1}
Block	4	345379*	6.38 ns	120.69 ns	0.064	142.47 ns	9.84 ns	9.74
Treatment	5	41523	54.23	71.76	0.099	786.10	13.25	13.32
Error	20	67686	36.45	92.48	0.077	861.76	14.25	14.22
Average		4896	179.06	59.75	2.46	147.28	53.33	57.34
CV (%)		5.31	3.37	16.10	11.30	19.93	7.08	6.57

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

FV	DF	DM/Plant (g)	DM/root (g)	Nodules/ Plant (Number)	Nodules/ Plant (Mass)	Insertion/ height (cm)	Plant/ height (cm)	Branches (number)
Block	4	8.31 ns	0.092 ns	477.5 ns	16906.83 ns	7.38 ns	33.76 ns	1.104 ns
Treatment	5	24.18	0.123	349.5	6062.00	4.40	25.83	1.087
Error	20	35.2	0.150	297.7	18091.63	7.81	32.54	0.566
Average		23.32	2.38	38.33	276.37	21.68	92.94	2.03
CV (%)		20.7	18.8	57.35	42.25	12.34	5.65	40.83

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

Table 8: Effect of soil inoculation with different Inoculants in soybean crop in Lapa.

Treatment	Productivity (kg/ha)	TGM (g)	Branches (number)	Insertion/ height (cm)	Plant/height (cm)	N Grain mg kg^{-1}	N Aerial Part mg kg^{-1}
Absolut Control	4524 c	177.7 ns	2.1 ns	17.2 ns	81.7 ns	51.80	55.86
Nitrogen Control	5008 a b	180.2	2.7	17.7	81.3	55.25	59.31
Inoculant Control	4815 b	178.4	2.1	17.0	77.9	53.01	57.07
AzoTotal	4984 a b	180.0	2.9	15.1	76.0	52.83	56.89
Bacillus co-inoculation	5151 a	179.0	3.0	15.6	79.3	53.74	57.71
Average	4896	179.1	2.6	16.5	79.2	53.33	57.37

Means followed by the same letter (a, b or c) within columns do not differ from each other by Duncan's test at 5%. ns – not significant.

Treatment	Pod number	Grain/pod number	Grain/ Plant number	Plant DM	Root DM	Nodules number	Nodules mg
Absolut Control	55.0 ns	2.47 ns	135 ns	23.69 ns	2.41 ns	47 ns	324 ns
Nitrogen Control	65.0	2.47	162	20.90	2.60	65	286
Inoculant Control	55.2	2.33	127	21.19	2.62	70	420
AzoTotal	61.1	2.61	160	21.73	2.35	64	391
Bacillus co-inoculation	62.4	2.42	151	22.89	2.59	56	364
Average	59.7	2.46	147	22.08	2.51	61	357

Means followed by the same letter (a, b or c) within columns do not differ from each other by Duncan's test at 5%. ns – not significant

Regarding nodulation, differences were observed in the number of nodules among the different treatments. The highest nodulation averages were promoted by standard inoculation and co-inoculation with *A. brasilense* (Table 8).

The mass of dry nodules was also influenced by the treatments studied. As for the number of nodules, the highest values were observed in treatments with standard inoculation and co-inoculation with registered standard (AzoTotal).

For the dry mass of the aerial part, differences were observed among treatments. The mean separation test indicates that the lowest value was observed in the Absolut Control treatment. The other treatments, both inoculation and nitrogen fertilization, presented equivalent mass averages. The evaluated treatments did not affect the nitrogen content in either the aerial parts or grains.

Soybean crop - site 3: Frei Rogério

The results from the third site of experiment with soybean, conducted in Frei Rogério (Santa Catarina, Brazil), are shown in Tables 9. In these trials, promising results were observed in relation to nodulation. Differences were observed in the number of nodules among the different treatments. The highest nodulation averages were promoted by Standard Inoculation and Co-inoculation with *A. brasilense*. The mass of dry nodules was also influenced by the treatments studied. As for the number of nodules, the highest values were observed in treatments with Standard Inoculation and Co-inoculation with registered product.

Additionally, in relation to dry mass of the aerial part, differences were observed between treatments. The mean separation test indicates that the lowest value was observed in the Absolute Control treatment. The other treatments, both Inoculation and Nitrogen fertilization, exhibited similar average mass. The evaluated treatments did not affect nitrogen content in the aerial part of the plant. Importantly, the productivity values showed statistical differences among the treatments. Table 9 displays the data on productivity, and it indicates that the highest average productivity was achieved through co-inoculation with a consortium of three *Bacillus* species applied in the furrow, specifically in conjunction with *B. japonicum*. The mean of the Co-inoculation treatment with *Bacillus* consortium was statistically equal to the mean of the Co-inoculation treatment control, which corresponds to the co-inoculation with *B. japonicum* and *A. brasilense*. Furthermore, it was superior to the standard seed inoculation treatment with *B. japonicum* alone.

Table 9: Effect of soil inoculation with different Inoculants in soybean crop soybean crop in Frei Rogério.

Treatment	Productivity (kg/ha)	Plant DM	Nodules number	Nodules mg
Absolut Control	4312.8 c*	1.00 b*	12.85 a b*	21.92 b*
Nitrogen Control	4988.1 b	1.45 a	2.10 c	0.77 c
Inoculant Control	4314.3 b c	1.57 a	15.90 a	40.33 a
Azo Total	5108.3 a b c	1.50 a	15.10 a	38.68 a
Bacillus co-inoculation	5361.4 a	1.62 a	9.92 b	25.62 b
Average				

* Means followed by the same letter do not differ from each other using the LSD Test ($p<0.05$).

Maize crop - site 1: Serranópolis do Iguaçu

'The results of the first experiment with maize, conducted in Serranópolis do Iguaçu, are shown in Tables 10 and 11. By analysis of variance (Table 10), a treatment effect was verified only for grain productivity and dry mass at the R1 stage of the crop. For grain productivity and dry mass, it was found that the Nitrogen Control, *P. fluorescens* and *Bacillus Consortium* treatments performed better than the Absolute Control. There was no significant difference for the other variables.

Table 10: Summary of the analysis of variance with mean square values of the variables productivity of grains and dry matter (DM), thousand-grain mass (TGM), N content in the plant and N content in the grain, in the test carried out with corn in Serranópolis do Iguaçu.

		Mean Square				
FV	DF	Productivity Grains (kg ha^{-1})	Productivity DM (kg ha^{-1})	TGM (g)	N Aerial Part mg kg^{-1}	N Grain mg kg^{-1}
Block	5	2290410**	21203942**	37.57 ns	18.77 ns	2.16 ns
Treatment	3	278049	352934	48.35	20.48	2.68
Error	15	136766	265869	98.74	15.12	2.00
CV (%)		4.63	6.39	3.74	12.51	12.04

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

Table 11: Productivity, thousand-grain mass (TGM), dry matter (DM) production per hectare, N content in the plant and N content in the grain, in the test carried out with corn in Serranópolis do Iguaçu.

Treatment	Productivity kg ha^{-1}	TGM (g)	DM/ha Kg ha^{-1}	N Aerial Part mg kg^{-1}	N Grain mg kg^{-1}
Absolute Control (0% N)	7070 b	262.16 ns	5263	28.5 ns	10.9 ns
Nitrogen Control (100% N)	8434 a	267.89	9086 a	32.2	12.0
P. fluorescens	8231 a	266.34	9173 a	31.6	12.4
Bacillus co-inoculation	8202 a	266.74	8761 a	32.1	11.8
Average	7928	265.69	7855	31.2	11.8

Means followed by the same letter (a or b) within columns do not differ from each other by Duncan's test at 5%. ns – not significant

Maize crop - site 2: São Miguel do Iguaçu

The results of the second experiment, conducted in São Miguel do Iguaçu (Paraná, Brazil), are provided in Table 12 and 13. There was a treatment effect for grain productivity and dry mass at the R1 stage and thousand grain mass (TGM). For grain and dry mass productivity, it was found that the Nitrogen Control, *P. fluorescens* and *Bacillus* co-inoculation treatments (Table 13) performed better than the Absolute Control. Besides, in absolute numbers, the *Bacillus* co-inoculation presented the highest grain productivity (6516 kg/ha) and the second highest dry mass productivity (7369 kg/ha). These results demonstrate that, even with a 25% reduction in nitrogen, grain and dry mass productivity were similar to those obtained with a full dose of nitrogen (Nitrogen Control).

Regarding the weight of one thousand grains (TGM), it was found that the Nitrogenated Control, *P. fluorescens* and *Bacillus* co-inoculation treatments were statistically similar to each other. However, only the Nitrogen Control and *Bacillus* co-inoculation treatments were superior to the Absolute Control. Notably, in this variable, the Bacillus co-inoculation treatment achieved the highest absolute value, with 300.21 grams for every thousand grains.

Table 12: Summary of the analysis of variance with mean square values of the variables productivity of grains and dry matter (DM), thousand-grain mass (TGM), N content in the plant and N content in the grain, in the test carried out with corn in São Miguel do Iguaçu.

		Mean Square				
FV	DF	Productivity Grains	Productivity DM	TGM (g)	N Aerial Part mg kg^{-1}	N Grain mg kg^{-1}
Block	5	1571172**	16827295**	491.33*	20.355*	3.35*
Treatment	3	373209	242101	48.42	3.105	0.51
Error	15	141244	255980	89.12	0.130	1.30
CV (%)		6.09	7.98	3.23	7.69	7.68

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

Table 13: Productivity, thousand-grain mass (TGM), dry matter (DM) production per hectare, N content in the plant and N content in the grain, in the test carried out with corn in São Miguel do Iguaçu.

Treatment	Productivity kg ha^{-1}	TGM g	DM/ha kg ha^{-1}	N Aerial Part mg kg^{-1}	N Grain mg kg^{-1}
Absolute Control (0% N)	5011 b	279.13 b	3877 b	39.14 ns	13.9 ns
Nitrogen Control (100% N)	6439 a	295.07 a	7414 a	38.5	15.4
P. fluorescens	6304 a	293.83 ab	6703 a	38.7	15.5
Bacillus co-inoculation	6516 a	300.21 a	7369 a	36.41	14.5
Average	6184	292.55	6117	37.2	14.9

Means followed by the same letter (a or b) within columns do not differ from each other by Duncan's test at 5%. ns – not significant

Maize crop - site 3: Laguna Carapã

The results of the third experiment with corn, conducted in Laguna Carapã (MS, Brazil), are presented in Tables 14 and 15. A treatment effect was observed for grain productivity and dry mass at the R1 stage. Additionally, the number of grains per row, grains per row, and grains per ear yielded positive results. For grain productivity and dry mass, grains per row and grains per ear, it was found that the Nitrogen Control, *P. fluorescens* and *Bacillus* co-inoculation treatments (Table 15) performed better than the Absolute Control. It was also found that those treatments did not differ from each other. For number of rows, only *Bacillus* co-inoculation treatments was superior to the Absolut Control.

Table 14: Summary of the analysis of variance with mean square values of the variables productivity of grains and dry matter (DM), thousand-grain mass (TGM), N content in the plant and N content in the grain, in the test carried out with corn in Laguna Carapã.

FV	DF	Mean Square						
		Productivity Grains	Productivity DM	TGM (g)	N Aerial Part mg kg-1	N Grain mg kg-1	Grain/ row	Grain/ ear
Block	5	4521702**	9099145**	202.62 ns	31.28 ns	4.60 ns	33.25**	12060**
Treatment	3	477848	926103	77.43	14.07	2.88	44.6	4033
Error	15	474571	591745	198.13	25.96	3.12	3.29	1257
CV (%)		24.4	26.04	4.44	18.06	18.56	8.66	11.35

CV (%) – coefficient of variation; DF – degrees of freedom; ** and * - significant at 1 and 5%, respectively; ns – not significant

Table 15: Productivity, thousand-grain mass (TGM), dry matter (DM) production per hectare, N content in the plant, N content in the grain, as well as number of grains per row and ear, in the test carried out with corn in Laguna Carapã.

Treatment	Productivity kg ha^{-1}	TGM (g)	DM/ha kg ha^{-1}	N Aerial Part mg kg^{-1}	N Grain mg kg^{-1}	Grain/ row	Grain/ ear
Absolute Control (0% N)	1523 b	317.25 ns	1115 b	27.6 ns	9.2 ns	17.6 b	248 b
Nitrogen Control (100% N)	3296 a	322.03	3720 a	28.4	9.8	21.2 a	320 a
P. fluorescens	3278 a	308.80	3452 a	27.3	9.4	22. 1 a	330 a
Bacillus co-inoculation	3196 a	319.94	3532 a	28.1	9.6	22.9 a	352 a
Average	2847	316.90	2894	27.8	9.5	21.0	312

Means followed by the same letter (a or b) within columns do not differ from each other by Duncan's test at 5%. ns – not significant

Discussion

It is known that in natural environments, plant growth-promoting events are the result of the joint action of several species of bacteria, and not of a single bacterial isolate. Therefore, bacterial consortium that contains compatible and synergistic microorganisms can have better results than the application of a single microorganism (Hungria et al., 2013; Barbosa et al., 2022; Galindo et al., 2022; Ngosong et al., 2022), as verified in the present study. A study carried out by Bai et al. (2002) reveals that the association of *Bacillus* and *Bradyrhizobium* in soybean plants is a naturally occurring process. According to the authors, different species of *Bacillus* are associated with *Bradyrhizobium japonicum* inside soybean nodules, where they coexist harmoniously and act to promote plant growth (Bai et al., 2002).

According to the literature, the distinct species of the genus *Bacillus* have different mechanisms for promoting plant growth. *Bacillus subtilis*, for example, produces phytohormones that stimulate plant growth, including gibberellins, auxins, and organic acids (Islam et al., 2016). *Bacillus amyloliquefaciens*, in addition to producing some phytohormones, has the ability to promote plant growth through the production of phytases, which act on the phytate present in organic matter, making phosphate available to the plants (Idriss et al., 2002; Makarewicz et al., 2006). Besides, *Bacillus pumilus* stands out mainly for its high ability to colonize, associated with its ability to promote an increase in the activity of the enzymes peroxidase, chitinase and two isoenzymes 13-1,3-glucanase, and the salicylic acid metabolite, involved in the plant defense system (Dey et al., 2014; Zhang et al., 2002).

Taken together our data showed that the association of these *Bacillus* species in bacterial consortia enables the synergistic action of these mechanisms, promoting plant growth, which culminates in increases in productivity. For instance, the increase in productivity promoted by co-inoculation with three species of *Bacillus* was 24% in relation to that observed with standard inoculation (*B. japonicum), and was statistically equal to standard co-inoculation with *A. brasilense*. Data from other researchers corroborate the positive effect of co-inoculation with Bacillus on this parameter. A previous study showed the effects of co-inoculation of soybeans with *B. japonicum* and *Bacillus subtilis* in two areas in Tocantins (Brazil), in which significant increases of 8.9 and 14.9% in crop productivity was observed (Braga-Junior et al., 2018). Besides, Tavanti et al. (2020) tested the response of soybeans to two strains of *Bacillus subtills* in Mato Grosso do Sul. The increase in productivity was 18 and 25% for each strain, respectively, when they were used in co-inoculation with *Bradyrhizobium*. The increase in the production of soybeans by *Bacillus amyloliquefaciens* has also been reported by Sharma et al. (2013) observed a statistical difference in the number of grains per plant, which increased from 7.34 to 9.06 g.

Nodulation promoted by the three species of *Bacillus* consortium was lower than that observed with the other two inoculation treatments in our study in Frei Rogério. Even so, this reduction in nodulation did not impact the reduction in N content in the grains or in the aerial part of the plant, denoting the action of growth promotion mechanisms compensating for this effect. Furthermore, when analyzing the set of nodulation, mass and productivity, another interesting aspect can be noted: the treatment containing the three species of *Bacillus*, even resulting in lower nodulation, was able to maintain phytomass production similarly to other treatments. Furthermore, the consortium could also significantly increase the productivity. Therefore, it is believed that the evaluation carried out after 31 days did not express the real effect of the inoculant tested on nodulation. A second collection at a phenological stage closer to flowering could more adequately reflect the plant's nodulation. Data from Braga-Junior et al. (2018) support this hypothesis. The authors studied the co-inoculation of soybeans with Bradyrhizobium and *Bacillus subtilis* in early stages, the authors did not find significant differences in both number or mass of nodules. However, when a second evaluation was performed, positive statistical differences were observed. Plants inoculated with *Bradyrhizobium* alone showed 11 nodules, while those co-inoculated with *Bradyrhizobium* and *Bacillus* produced 18 nodules. The nodule mass values were 120 and 192 mg for the aforementioned treatments, respectively.

Still about the study with soybeans in Frei Rogério, differences in aerial part mass occurred only in relation to the control treatment. Other research groups also reported that co-inoculation of soybeans with *B. japonicum* and *Bacillus subtilis* resulted in no differences in the mass production of the aerial part (Bai et al., 2003; Araujo et al., 2008; Braga-Júnior et al., 2018; Costa et al., 2019). The use of *B. amyloliquefaciens*, isolated or in association with *B. japonicum*, also does not cause significant effects on the mass production of the aerial part when compared to standard inoculation. As mentioned, the nitrogen content in the shoots was not affected by inoculation treatments. Oliveira et al. (2019) evaluated the effects of co-inoculating soybeans with *B. japonicum* and the same three *Bacillus* species evaluated in the present report. Regardless of the method of application of the inoculants (via seed or post-emergence), this parameter was not affected by any species of *Bacillus* combined with *Bradyrhizobium.*

The results obtained with corn revealed that, in all experimental areas, there was promotion of plant growth in the corn culture inoculated with *Bacillus* co-inoculation, verified by the increase in mass in the R1 stage of the culture. The *Bacillus* species used in the bacterial consortium evaluated here belong to species widely described in the literature as PGPB. *Bacillus subtilis*, for example, is described as capable of producing phytohormones that stimulate plant growth, including gibberellins and auxins (Islam et al., 2016). *Bacillus amyloliquefaciens*, in addition to producing some phytohormones, has the ability to promote growth through the availability of organic phosphate present in the soil (Idriss et al., 2002; Makarewicz et al., 2006). Finally, *Bacillus pumilus* promotes an increase in the activity of key enzymes in plant metabolism, including those involved in the plant defense system against biotic stresses (Dey et al., 2014; Zhang et al., 2002). The action of these mechanisms may explain the promotion of plant growth observed in the present study. In lettuce (*Lactuca sativa),* increases in biomass with the use of *B. pumilus*, *B. subtilis* and *B. amyloliquefaciens*, in consortium or alone, were also verified, corroborating the results verified in the present study. The authors verified an increase in plant vigor and head weight by up to 49% compared to the control. According to the authors, the growth promotion observed may be related to the ability of Bacillus spp. produce and secrete phytohormones such as cytokinin and auxin, stimulate the uptake of nutrients via the root system, and produce ACC-deaminase, which allow the maintenance of growth and development under stress conditions, through inhibition of ethylene synthesis in plants. The results obtained in the present study indicate that these growth-promoting mechanisms mediated by *Bacillus* must also be acting on soybean crops, promoting the increase in grain production observed.

In addition to increasing plant biomass, experiments conducted in São Miguel do Iguaçu and Laguna Carapã demonstrated that inoculation with the *Bacillus consortium* also affected various productivity components, including the mass of one thousand grains, the number of grains per row, the number of grains per ear, and grain yield. This highlights the potential of using this consortium to enhance corn productivity. In fact, the grain productivity results obtained in the three experimental areas (Serranópolis do Iguaçu, São Miguel do Iguaçu and Laguna Carapã) demonstrate that inoculating with the *Bacillus consortium* led to increased productivity. This performance was superior to the Absolute Control and equivalent to the Control Nitrogenated and inoculated with *P. fluorescens*, reflecting the compensatory effect of the 25% reduction in nitrogen applied in this treatment. Equivalent results were obtained in corn cultivation with *P. fluorescens*, where inoculation by seed treatment resulted in increased biomass and grain productivity in standard and high-yielding maize (Sandini et al., 2019). Additionally, it is known that in natural environments, plant growth promotion is a result of the joint action of several species of bacteria, and not of a single species. Furthermore, bacterial consortia have a higher likelihood of success in heterogeneous environments, such as those with diverse soil and climate conditions, as encountered in this study.

Conclusion

The inoculant containing *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus*, associated with inoculation with *Bradyrhizobium japonicum*, provides an increase in soybean grain productivity. For maize crop, the results obtained in trials conducted in three locations, demonstrate that inoculation with the bacterial consortium promoted a 25% reduction in the amount of nitrogen used, as well as the growth of corn, with productivity equivalent to the full dose of N and the application of a microorganism already registered for this purpose (*Pseudomonas fluorescens*). Considering that one of the most relevant parameters in agronomic efficiency trials of an inoculant is productivity, these results confirm the viability of use and the recommendation of the *Bacillus* consortium as an inoculant that promotes plant growth in soybean and maize crops.

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