ABRATES

Journal of Seed Science

ISSN 2317-1545

www.abrates.org.br/revista

ARTICLE

Journal of Seed Science, v.44, e202244040, 2022

http://dx.doi.org/10.1590/2317-1545v44263329

Methods to quantify *Bacillus simplex*-based inoculant and its effect as a seed treatment on field-grown corn and soybean in Brazil

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ABSTRACT: Growth-promoting bacteria in agriculture have become an important tool to improve crop performance and productivity in the face of climate change and deteriorating soil conditions. *Bacillus simplex* is a recently developed active ingredient for the growth promotion of corn and soybean in Brazil. This study compared three methods to quantify *B. simplex* colony-forming units in the inoculant product and evaluated the treatment effects of four different concentrations of a *B. simplex*-based inoculant on corn and soybean root and shoot dry weight, the Normalized Difference Vegetation Index (NDVI), and yield. Field trials were performed at four different locations for each crop, in Mato Grosso do Sul and Paraná for corn, and in Mato Grosso do Sul, Minas Gerais, and Paraná for soybean. The performance of *B. simplex* was compared to an *Azospirillum brasilense*-based inoculant, a polymer seed treatment, and untreated controls. The results showed that the official MAPA method for quantifying microbes in inoculants recovered the highest number of *B. simplex* colonies. However, all three evaluated quantification methods recovered over 100 million colony-forming units per mL (10^8 CFU.mL^{-1}). The field results showed that the *B. simplex* inoculant generally increased corn and soybean yields as much or more as the *A. brasilense* product and that the polymer seed treatment had no impact on yield. The treatment effect on root and shoot weight, and NDVI, was inconsistent. This research shows that *B. simplex* is quantifiable with three different methods and that it can improve yield in corn and soy. The *Bacillus simplex*-based inoculant has the potential to become widely used in Brazil.

Index terms: bioinputs, biological product, inoculant, plant growth-promoting, seed treatment.

RESUMO: As bactérias promotoras de crescimento na agricultura tornaram-se uma ferramenta importante para melhorar o desempenho e a produtividade das culturas em face das mudanças climáticas e da deterioração das condições do solo. *Bacillus simplex* é um ingrediente ativo recentemente desenvolvido para a promoção do crescimento de milho e soja no Brasil. Este estudo comparou três protocolos para quantificar unidades formadoras de colônias de *B. simplex* no produto inoculante e também os efeitos do tratamento de sementes com quatro concentrações do inoculante à base de *B. simplex* sobre o peso seco da raiz e da parte aérea, o Índice de Vegetação por Diferença Normalizada (NDVI) e a produtividade de plantas de milho e soja. Ensaios de campo foram realizados em quatro locais diferentes para cada cultura, em Mato Grosso do Sul e Paraná para milho, e em Mato Grosso do Sul, Minas Gerais e Paraná para soja. O desempenho das quatro concentrações de *B. simplex* foi comparado a um inoculante à base de *Azospirillum brasilense*, a um tratamento de sementes com polímero e também ao controle não tratado. Os resultados mostraram que o método oficial MAPA para quantificação de microrganismos em inoculantes recuperou o maior número de colônias de *B. simplex*. No entanto, todos os três métodos de quantificação avaliados recuperaram mais de 100 milhões de unidades formadoras de colônias por mL (10^8 UFC.mL^{-1}). Os resultados de campo mostraram que o inoculante de *B. simplex*, em geral, aumentou a produtividade de milho e soja tanto ou mais quanto o produto de *A. brasilense* e que o tratamento de sementes com polímero não teve impacto na produtividade. O efeito do tratamento sobre o peso da raiz e da parte aérea, e NDVI, foi inconsistente. Esta pesquisa mostrou que *B. simplex* é quantificável pelos três métodos avaliados e que pode aumentar a produtividade de milho e soja. O inoculante à base de *Bacillus simplex* tem potencial para se tornar amplamente utilizado no Brasil.

Termos para indexação: bioinsumo, produto biológico, inoculante, promotor de crescimento de plantas, tratamento de sementes.

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Received: 04/22/2022.
Accepted: 08/28/2022.

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INTRODUCTION

The intense agricultural practices in many parts of the world have resulted in the unsustainable degradation of soils involving loss of organic matter, the release of greenhouse gases, erosion, and the excessive application of fertilizers (Kopittke et al., 2019). In addition, climate change has contributed to the frequency, increase, and severity of many biotic and abiotic stresses, mainly due to high temperatures and droughts, which drastically reduce productivity (Teixeira et al., 2013). Among the sustainable approaches to mitigating adverse impacts of soil degradation and climate change in agriculture is using plant growth-promoting microorganisms (Etesami and Maheshwari, 2018). Different microbes present plant growth-promoting properties (Pandey et al., 2019), among which *Bacillus* species are among the most-studied bacteria (Tiwari et al., 2019). Beneficial *Bacillus* and relatives are associated with plants as root endophytes (Santoyo et al., 2016), rhizoplane (Cavaglieri et al., 2009), and rhizosphere inhabitants (Lugtenberg and Kamilova, 2009). These microorganisms exhibit a variety of mechanisms involved in the plant growth-promotion (Ibanhes-Neto et al., 2021; Tiwari et al., 2019), including nitrogen fixation and phosphorus solubilization (Elkoca et al., 2007), production of siderophores for iron acquisition (Kushwaha et al., 2020), 1-amynocyclopropane-1-carboxylate deaminase (ACC deaminase) involved in the alleviation of drought stress (Gowtham et al., 2020), phytohormones promoting plant growth (Martínez-Viveros et al., 2010), and antibiotics, catabolic enzymes and other strategies to control plant pathogens (Choudhary and Johri, 2009; Schönbichler et al., 2020). Plant growth promotion may manifest in increased yield, root and shoot growth, overall plant health as measured by the normalized difference vegetation index (NDVI), and by other phenotypic changes (Tiwari et al., 2019; Witkowicz et al., 2021).

In Brazil, products of a certain degree of purity containing living organisms capable of providing benefits to plant growth are classified as inoculants. *Bacillus* species such as *B. licheniformis* (Akhtar et al., 2020), *B. subtilis* (Lee et al., 2014; Hashem et al., 2019), *B. amyloliquefaciens* (Kim et al., 2017; Ngalimat et al., 2021) and *B. simplex* (Barneix et al., 2005; Schwartz et al., 2013) are among well-known growth promoters that add value to crops and have been used in the country. Brazil stands out as a global leader in using *Bradyrhizobium*, which supplies almost the entire nitrogen demand for soybean cultivation in Brazilian agriculture (Campo et al., 2009; Hungria et al., 2015). Another commonly used nitrogen-fixing inoculant for non-leguminous crops in Brazil contains *Azospirillum brasilense* as the active ingredient (Santos et al., 2021).

Seed inoculation is one of the ways biological inoculants can be deployed in the field (Santos et al., 2021). Microbes added to the seeds rapidly colonize the rhizosphere upon germination and confer benefits to the plants from the beginning of crop establishment (McQuilken et al., 1998). *Bacillus* species are Gram-positive, spore-forming bacteria that can survive in dehydrated conditions, including on seed, longer than other bacteria (Schisler et al., 2004). This translates to extended shelf life and added flexibility in the planting process.

Current Brazilian regulations for registering microbial inoculant products require a demonstration of the product's efficacy under field conditions. Still, they do not specify any reference methods to quantify the viable cell count of *Bacillus* species in the product (MAPA, 2010). However, such methods are essential for the assessment of product quality.

Modern seed-coating technology uniformly applies a wide range of active ingredients onto crop seeds at desired dosages to facilitate sowing and enhance crop performance (Afzal et al., 2020). Polymers are an essential component of seed-coating, assure adherence to the seed and improve plantability.

The aimes of this study were: (i) to compare the efficacy of three methods to quantify *Bacillus simplex* strain SYM00260; (ii) to assess the field performance of a *B. simplex* strain SYM00260 inoculant as a seed treatment in corn and soybean on yield, root and shoot weight, and NDVI; and (iii), to compare the *B. simplex* strain SYM00260 field performance to a commercial *Azospirillum brasilense* seed inoculant and a polymer seed treatment.

MATERIALS AND METHODS

The *Bacillus simplex* strain SYM00260, registered as an inoculant by Indigo Brazil Agriculture Ltda in Brazil (registration number: SP004627-2.000001), was used in this study.

Comparison of quantification methods for Bacillus simplex in formulated products

Method 1: This method was based on an EMBRAPA protocol for quantifying *Bacillus subtilis* and *Bacillus licheniformis* from formulated products (Embrapa, 2012) with modification of point 7.2, where the product was measured by volume. The concentration of viable *B. simplex* cells was assessed by performing a series of 10-fold sequential dilutions in 10 mL solution volumes and a total dilution factor of 10^{-7}. Peptone water (Merck) was used as the diluent. After each transfer, dilutions were homogenized on a shaker three times for 1 minute, sonicated without heating for 5 minutes, homogenized on a shaker for 1 minute, incubated in a double boiler at 80 ± 2 °C for 12 minutes, and cooled in ice water for 10 seconds. From the final dilution, 0.1 mL aliquots were transferred to five Petri plates containing nutrient agar (Merck) culture medium, and spread with a Drigalski loop. The plates were inverted after two minutes and incubated at 37 ± 2 °C for 17-20 h. Serial dilutions were repeated a total of three times.

Method 2: This method is based on a MAPA quantification protocol for *Bradyrhizobium*-containing formulated products (MAPA, 2010), and is the officially recognized method for quantification of microbial organisms from inoculants. Serial dilutions involved 10-fold sequential dilutions in 10 mL solution volumes and a total dilution factor of at least 10^{-5}, such that colony forming unit (CFU) counts from plating of 0.1 mL aliquots onto a standard-sized Petri dish reached 30 to 300 CFU. The diluent was sterile saline solution (NaCl 0.85%), and the growth medium for CFU counting was yeast mannitol agar (YMA) + Congo Red. A Drigalski loop was used for spreading, and inoculated plates were incubated at 29 ± 1 °C for 5 days. Two replication series, Series A and B, were prepared for each dilution, and CFU counts were averaged between corresponding series A and B plates. Serial dilutions were repeated a total of three times.

Method 3: This method comprised 10-fold sequential dilutions, a total dilution factor of 10^{-7}, 1 mL solution volumes, 1x phosphate buffered saline (PBS) as the diluent and tryptone soy agar (TSA) culture medium for CFU counting. Product aliquots of 500 µL were transferred to 2 mL deep well microplates, incubated at 65 °C for 15 ± 2 minutes in a double bath, mixed by shaking, followed by serial dilution. Dilutions were homogenized by pipetting up and down five times. Aliquots of 100 µL from the final dilutions were spread onto TSA Petri dishes in three replications using a Drigalski's loop and allowed to soak into the medium. Petri dishes were inverted and incubated at 30 ± 2 °C for 17-24 hours. Serial dilutions were repeated three times.

Negative controls were included for all methods and consisted of two plates inoculated with the respective diluent. The positive control was *Bacillus subtilis* strain AGR 013.3-B acquired from the André Tosello Collection (code CCT 2576 - ATCC 6051), using the recommended dilution suggested by the supplier.

Counting of *B. simplex* colonies was performed after the incubation period described for each method. The descriptions from Rosenberg et al. (2016) and Parte et al. (2020) were used for the morphological characterization of the colonies.

The data were analyzed through a linear mixed-effects model conducted in the R 3.6.3 software package (R Core Team, 2021) and the lmerTest v3.1-1 package (Kuznetsova et al., 2017). Fisher's least significant difference (LSD) method was used to assess whether the mean colony-forming unit (CFU) counts recovered by each quantification method were equal.

Assessment of Bacillus simplex performance in the field

Corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merrill) seeds were sown at a total of 4 localities for each crop in January 2018. The sowing locations, hybrid/variety, geographic coordinates of each field trial, germination and purity

of the seed lots, soil type, and climate classification are described in Table 1. Hybrids/varieties were selected for each crop based on location and because they are commercially relevant for each region. The experiment was not designed to compare differences between hybrids/varieties. All experimental fields were located in experimental agronomic research areas. The soil chemical and granulometric characteristics of the field sites are shown in Table 2.

The experimental design was a randomized complete block design with seven treatments and four replications. Each plot had a total area of 24 m^2 (3.0 m x 8.0 m). Analysis and results presented here focus on comparing the performance of seeds treated with different microbial products versus untreated controls.

Soybean seed was base-treated with Cruiser 350 FS® (thiamethoxam 350 $g.L^{-1}$) at a rate of 100 $mL.100\ kg^{-1}$ of seeds, Maxim Advanced® (metalaxyl-m 20 $g.L^{-1}$ + thiabendazole 150 $g.L^{-1}$ + fludioxonil 25 $g.L^{-1}$) at a rate of 50 $mL.100\ kg^{-1}$ of seeds, and Fortenza 600 FS® (cyantraniliprole 600 $g.L^{-1}$) at a rate of 30 $mL.100\ kg^{-1}$ of seeds. Corn seed was base treated with Cruiser 350 FS® at 120 $mL.100\ kg^{-1}$ of seeds and Fortenza 600 FS® at 40 $mL.100\ kg^{-1}$ of seeds. After treatment with the base chemical products, all soybean seed was inoculated with the product Rizoliq TOP® (*Bradyrhizobium japonicum* 6 x 10^9 $CFU.mL^{-1}$) at a rate of 100 $mL.50\ kg^{-1}$ of seeds, and left to dry in the shade at room temperature. After the *Bradyrhizobium* treatment, part of the seeds was treated with Azototal® (*Azospirillum brasilense* 2x10^{11} CFU. mL^{-1}) at a rate of 100 $mL.50\ kg^{-1}$ for soybean, and 100 $mL.60\ kg^{-1}$ for corn. The remaining seeds were treated with the *Bacillus simplex* strain SYM00260 inoculant at the inoculum concentrations of 5x10^4, 5x10^5, 5x10^6, and 5x10^7 $CFU.mL^{-1}$. A BIOCROMA® polymer was subsequently applied at a rate of 1 $mL.kg^{-1}$ for soybean and corn, and the seeds were immediately sown. The amount of *B. simplex* inoculum used was 9 $mL.kg^{-1}$ for soybean and 6 $mL.kg^{-1}$ for corn.

Seed treatments were performed in plastic bags with 1 kg of seeds per bag, and mixed by hand using circular movements to simulate industrial seed treatments centrifuges according to the methodology proposed by Nunes (2005). Seed was treated separately at each field trial location. Non-inoculated controls without *B. simplex* and without BIOCROMA, and non-inoculated controls without *B. simplex* but with BIOCROMA, were used in each trial.

Table 1. Crops, hybrids or varieties, germination and purity, locality, geographical coordinates, climate, and soil types of the experiments.

Crop	Hybrid/Varieties-Company	Germination/Purity (%)	Locality/State	Geographical coordinates/Climate (Köppen)	Type of soil
Corn	Status Viptera-Syngenta	99/100	Ponta Grossa - Paraná (PR)	25°10'11.61" S, 51°11'17.22" E/ Oceanic climate (Cfb)	Sandy clay loam
Corn	Supremo Viptera-Syngenta	97/100	Imbituva - Paraná (PR)	25°9'23.41" S, 50°32'57.68" E/ Oceanic climate (Cfb)	Sandy clay
Corn	Supremo Viptera-Syngenta	97/100	Lapa - Paraná (PR)	25°44'30.72" S, 49°48'16.91" E/ Oceanic climate (Cfb)	Sandy clay loam
Corn	Supremo Viptera-Syngenta	97/100	Dourados - Mato Grosso do Sul (MS)	22°05'23.08" S, 55°08'22.27" E/ Tropical monsoon climate (Am)	Clay
Soybean	Brasmax Garra IPRO-Brasmax	98/100	Imbituva - Paraná (PR)	25°9'20.13" S, 50°32'57.09" E/ Oceanic climate (Cfb)	Sandy clay
Soybean	Brasmax Garra IPRO-Brasmax	98/100	Uberlândia - Minas Gerais (MG)	19°2'24.21" S, 48°11'50.83" E/ Tropical savana climate (Aw)	Clay
Soybean	Monsoy 5947 IPRO-Bayer	95/100	Lapa - Paraná (PR)	25°44'33.62" S, 49°48'19.62" E/ Oceanic climate (Cfb)	Sandy clay loam
Soybean	Monsoy 5947 IPRO-Bayer	95/100	Dourados - Mato Grosso do Sul (MS)	22°05'24.69" S, 55°08'24.23" E/ Tropical monsoon climate (Am)	Clay

Soybean planting density was 300,000 seeds.ha^{-1}, and the spacing between rows was 0.50 m in Imbituva, Lapa and Uberlândia. In Dourados, the spacing between rows was 0.45 m. The average population density was 264,744 plants.ha^{-1}. A base fertilization of 130 kg.ha^{-1} of 04-30-10 N-P-K based on soil analyses (Table 2), was applied in-furrow at each trial immediately before sowing. Corn planting populations were 70,000 plants.ha^{-1} and the row spacing was 0.50 m, with an average population density of 69,761 plants.ha^{-1}. The base fertilization was 300 kg.ha^{-1} of 04-30-10 N-P-K with urea coverage of 350 kg.ha^{-1}.

For soybean, weed control was performed with Zapp Qi 620® (glyphosate potassium 620 g.L^{-1}). For pest control, the insecticides Engeo Pleno® (thiamethoxam 141 g.L^{-1}+ lambda-cyhalothrin 106 g.L^{-1}), Tiger 100 EC® (pyriproxifen 100 g.L^{-1} + xylene 800 g.L^{-1}), and Oberon® (spiromesifen 240 g.L^{-1}) were used. Disease control was carried out with the fungicides Unizeb Gold® (mancozeb 750 g.L^{-1}), Fox® (trifloxystrobin 150 g.L^{-1} + prothioconazole 175 g.L^{-1}), Previnil® (chlorothalonil 720 g.L^{-1}), Orkestra SC® (fluzapyroxad 167 g.L^{-1} + pyraclostrobin 333 g.L^{-1}), Ativum® (epoxiconazole 50 g.L^{-1} + fluxapyroxad 50 g.L^{-1} + pyraclostrobin 81 g.L^{-1}) and Sphere max® (trifloxystrobin 375 g.L^{-1} + cyproconazole 160 g.L^{-1}).

For corn, weed control was carried out with the application of Primoleo® (atrazine 400 g.L^{-1}) and Soberan® (tembotrione 420 g.L^{-1}). Pest control was carried out with the application of Engeo Pleno® (thiamethoxam 141 g.L^{-1} + lambda-cyhalothrin 106 g.L^{-1}) and Ampligo® (lambda-cyhalothrin 50 g.L^{-1} + chlorantraniliprole 100 g.L^{-1}). Disease control was performed with Opera Ultra® (pyraclostrobin 80 g.L^{-1} + metconazole 130 g.L^{-1}) and Fox® (trifloxystrobin 150 g.L^{-1} + prothioconazole 175 g.L^{-1}).

At the phenological stage V10 for soybean and corn, the Normalized Difference Vegetation Index (NDVI) was determined using a portable GreenSeeker® device on two central rows in each plot. Thirty-five to 50 days after sowing (DAS), five plants were collected randomly from each plot for root and shoot biomass evaluation. Plant materials were oven-dried at 65 °C for four days when the plants were weighed.

Soybean and corn yield measurements were made on a 5 m^2 area in the center of each plot. Plants were harvested manually with a sickle and processed using a threshing machine. Seeds were cleaned and weighed, and grain yield was estimated after correcting seed weights to 13% moisture.

Data were analyzed separately for each cultivar/hybrid at each location using analysis of variance (ANOVA). The Scott-Knott test was used to compare means in cases where the ANOVA F test detected statistical significance. Statistical analyses were performed using SASM-Agri® (Canteri et al., 2001).

Table 2. The soil chemical and granulometric analysis of the experimental localities in the top 0 -20 cm before installation of trials.

Localities	Chemical Analysis									Granulometry		
	P	MO	pH	H+Al	Al	K	Ca	Mg	CTC	Clay	Silt	Sand
	(mg.m^{-3})	(g.dm^{-3})	($CaCl_2$)	(mmolc.dm^{-3})						(g.kg^{-1})		
Ponta Grossa - PR	52	48	4.8	86	0.6	7.2	53	17	163	351	188	461
Imbituva - PR	43	37	6.2	13	N.D	1.8	115	21	151	390	66	544
Lapa - PR	17	36	4.6	65	3.7	4.1	18	8	95	230	156	614
Dourados - MS	59	26	5.0	38	N.D	7.4	34	13	92	584	125	291
Uberlândia - MG	31	23	5.0	30	N.D	3.8	21	8	63	499	157	344

RESULTS AND DISCUSSION

There were significant differences in colony counts between the three methods in this work (Figure 1). Method 2 recovered the highest number of *B. simplex* colonies with approximately $8x10^8$ CFU.mL^{-1}, followed by Methods 1 and 3 with about $2x10^8$ CFU.mL^{-1}. All three methods provided counts of over 100 million colony-forming units per mL (10^8 CFU.mL^{-1}).

The three methods compared in this work have been widely used to quantify different types of bacteria. None of the methods involve the use of any selective media. Both YMA culture media used in Method 2, and NA and TSA media used in Methods 1 and 3, respectively, provided nutrients necessary for growth of *B. simplex*. All methods were reproducible and consistent, although they differed from each other in some points described below.

Method 1 was developed to quantify *B. subtilis* and *B. licheniformis* - based products on the Brazilian market. The main disadvantage of this Method is that it requires a relatively high number of replications and dilutions, including two initial tubes per product, two serial dilutions from each tube, and five replications, for 20 dishes per sample. The results using Method 1 were similar to Method 3, which was less labor-intensive.

Method 2 is the official method used in Brazil for quantifying microbes classified as inoculants. By this method, a relatively rich medium is required for counting slow-growing bacteria such as *Bradyrhizobium*, which is not the case for the fast-growing bacteria like *Bacillus*. It is also relevant to point out that the use of the Congo Red dye in the medium to help distinguish rhizobia and contaminants is not convenient for *Bacillus.* This dye turns the *Bacillus* colonies slightly pink and less well-defined and may cause misinterpretation by the analyst. Another disadvantage of Method 2 is that it takes at least five days for the quantification of *Bradyrhizobium*. As *Bacillus* species grow faster, this relatively long incubation period for *Bradyrhizobium* may lead to quantification mistakes. Removal of the Congo Red dye should be investigated to adapt this method for counting *Bacillus*-based products.

Method 3 recovered a smaller number of colonies but the colonies were larger than the ones observed in the other two methods. Vieira and Nahas (2000) found similar results when evaluating *Bacillus* spp. isolates from soil samples. Method 3 has two significant advantages. First, it uses a TSA culture medium, which, due to its transparency, facilitates the distinction between *B. simplex* colonies and contaminants based on colony morphology. Second, it employs deep well plates for high-throughput analysis, which reduces costs and execution time. Method 3 is satisfactory for CFU determination of the *B. simplex* inoculant that has a minimum guaranteed CFU content of $9x10^7$.

Results showed that *B. simplex* strain SYM00260 significantly increased corn and soybean yield compared to the two non-inoculated controls, one with the BIOCROMA® polymer and the other without the BIOCROMA® polymer. The exception was soybean in Dourados (Tables 3-6), where a water deficit in the grain filling phase compromised productivity.

For corn, one to several *B. simplex* concentrations resulted in a significant yield increase at each location (Tables 1, 2). In Ponta Grossa, a yield increase of up to 26% was observed corresponding to an additional 35 bags hectare (60 kg.bag^{-1}) (Table 3). In Imbituva, yield increased by up to 23% or 23 bags.ha^{-1} (Table 3), and in Lapa by up to 24% or 24 bags.ha^{-1} (Table 4). In Dourados, only the highest *B. simplex* inoculation rate of $5x10^7$ CFU.mL^{-1} resulted in a significant yield increase of 24% or 29 bags.ha^{-1} (Table 4). For the remaining response variables, including root dry weight, shoot dry weight, and NDVI, significant differences between the *B. simplex* treatments and the non-inoculated controls were only observed at some locations: for root dry weight in Imbutiva (Table 3) and Dourados (Table 4); for shoot dry weight in Ponta Grossa, Imbituva (Table 3), and Dourados (Table 4); and for NDVI in Dourados (Table 4). Dourados was the only location where for all variables, at least one *B. simplex* concentration was significant (Table 4). For the *A. brasilense* treatment, yield increase was equal to the maximum *B. simplex* concentration as in Ponta Grossa, Imbituva, and Lapa, or significantly lower as in Dourados (Tables 3 and 4).

In soybean, *B. simplex* triggered a significant yield increase at most locations (Tables 5 and 6). In Imbituva, yield increased by up to 16% (7 bags.ha^{-1}) (Table 5); in Uberlândia by up to 16% (7 bags.ha^{-1}) (Table 5); in Lapa by up to

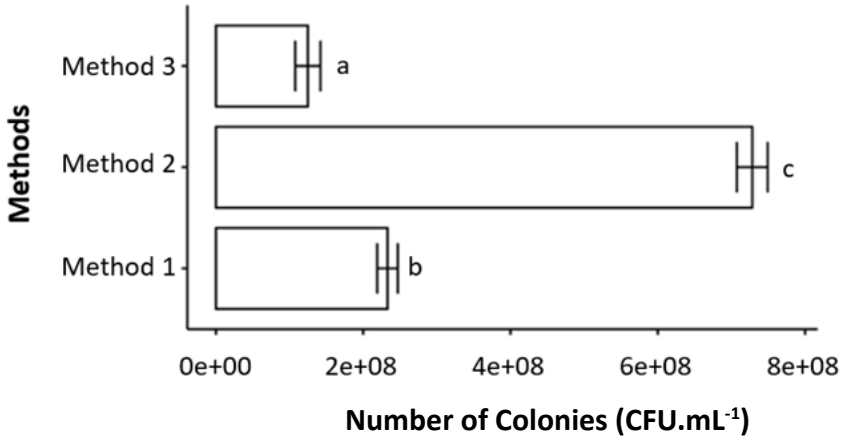

Figure 1. Mean colony-forming unit (CFU) counts of *Bacillus simplex* strain SYM00260 using three different quantification methods. Bars followed by different letters differ by the Scott-Knott Test at 5% probability. The data were analyzed using a linear mixed effects model, and LSD multiple comparisons of the number of colonies between the three methods described in the text.

Table 3. Mean values of variables root dry weight, shoot dry weight, NDVI, and yield used to assess the efficacy of corn seed treatment with *Bacilllus simplex* and *Azospirillum brasiliense* in field experiments with hybrids Status and Supremo in Ponta Grossa and Imbituva.

Treatment [a]	Status								Supremo							
	Ponta Grossa								Imbituva							
	Root dry weight		Shoot dry weight		NDVI		Yield		Root dry weight		Shoot dry weight		NDVI		Yield	
	(g)		(g)		(N)		(kg.ha^{-1})		(g)		(g)		(N)		(kg.ha^{-1})	
Non-inoculated	148	a	263	b	76	a	8028	b	2228	a	248	b	81	a	5865	b
Non-inoculated + Polymer	185	a	217	b	77	a	8230	b	188	b	202	b	82	a	5635	b
Ab 2x10^{8} CFU. mL^{-1}	231	a	285	b	80	a	9178	a	3149	a	350	a	81	a	6463	a
Bs 5x10^{4} CFU. mL^{-1}	200	a	287	b	77	a	7981	b	239	a	263	b	82	a	7224	a
Bs 5x10^{5} CFU. mL^{-1}	142	a	351	a	77	a	10136	a	145	b	251	b	82	a	6582	a
Bs 5x10^{6} CFU. mL^{-1}	161	a	314	a	77	a	9644	a	245	a	263	b	82	a	6927	a
Bs 5x10^{7} CFU. mL^{-1}	183	a	275	b	79	a	8978	a	245	a	290	b	83	a	7028	a
CV (%)	29		14		3		7		23		12		2		12	

[a]Means (n = 4) followed by different letters on the same column are significantly different ($p \leq 0.05$, Scott-Knott test).
Legend: Ab=*Azospirillum brasilense*, Bs=*Bacillus simplex,* CV=coefficient of variation.

Table 4. Mean values of root dry weight, shoot dry weight, NDVI, and yield used to assess the efficacy of corn seed treatment with *Bacilllus simplex* and *Azospirillum brasiliense* in field experiments with hybrid Supremo in Lapa and Dourados.

Treatment [a]	Supremo Lapa								Supremo Dourados							
	Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg.ha^{-1})		Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg.ha^{-1})	
Non-inoculated	230	a	253	a	81	b	5979	b	109	b	109	b	80	d	7312	b
Non-inoculated + Polymer	222	a	231	a	82	b	6214	b	110	b	111	b	80	d	6964	b
Ab 2x10^{8} CFU. mL^{-1}	226	a	258	a	84	a	6827	a	131	a	124	b	83	c	6906	b
Bs 5x10^{4} CFU. mL^{-1}	231	a	258	a	83	a	7399	a	112	b	109	b	84	b	6296	b
Bs 5x10^{5} CFU. mL^{-1}	257	a	268	a	83	a	6436	b	121	b	125	b	83	b	7556	b
Bs 5x10^{6} CFU. mL^{-1}	250	a	278	a	84	a	6792	a	145	a	121	b	85	a	7299	b
Bs 5x10^{7} CFU. mL^{-1}	276	a	287	a	84	a	7284	a	151	a	144	a	85	a	9043	a
CV (%)	15		11		1		7		10		9		1		10	

[a]Means (n = 4) followed by different letters on the same column are significantly different ($p \leq 0.05$, Scott-Knott test).
Legend: Ab=*Azospirillum brasilense*, Bs=*Bacillus simplex,* CV=coefficient of variation.

Table 5. Mean values of root dry weight, shoot dry weight, NDVI, and yield used to assess the efficacy of soybean seed treatment with *Bacilllus simplex* and *Azospirillum brasiliense* in field experiments with cultivar BMX in Imbituva and Uberlândia.

Treatment [a]	BMX Imbituva								BMX Uberlândia							
	Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg.ha^{-1})		Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg.ha^{-1})	
Non-inoculated	3	a	31	a	81	c	2562	b	4	a	30	a	83	a	2643	b
Non-inoculated + Polymer	3	a	31	a	79	d	2351	b	4	a	31	a	82	a	2717	b
Ab 2x10^{8} CFU.mL^{-1}	2	a	33	a	81	c	2581	b	3	a	26	a	82	a	2655	b
Bs 1x10^{4} CFU.mL^{-1}	3	a	36	a	79	d	2528	b	4	a	30	a	83	a	2946	a
Bs 1x10^{5} CFU.mL^{-1}	3	a	40	a	84	b	2859	a	4	a	30	a	84	a	3079	a
Bs 1x10^{6} CFU.mL^{-1}	3	a	37	a	86	a	2832	a	4	a	31	a	82	a	2933	a
Bs 1x10^{7} CFU.mL^{-1}	3	a	35	a	86	a	2985	a	4	a	31	a	83	a	2846	a
CV (%)	15		17		1		11		18		16		1		7	

[a]Means (n = 4) followed by different letters on the same column are significantly different ($p \leq 0.05$, Scott-Knott test).
Legend: Ab=*Azospirillum brasilense*, Bs=*Bacillus simplex,* CV=coefficient of variation.

Table 6. Mean values of root dry weight, shoot dry weight, NDVI, and yield used to assess the efficacy of soybean seed treatment with Bacillus *simplex* and *Azospirillum brasiliense* in field experiments with cultivar Monsoy in Lapa and Dourados.

Treatment [a]	Monsoy Lapa								Monsoy Dourados							
	Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg.ha^{-1})		Root dry weight (g)		Shoot dry weight (g)		NDVI (N)		Yield (kg ha^{-1})	
Non-inoculated	3	a	38	a	85	b	2317	b	4	a	5	b	72	d	1306	a
Non-inoculated + Polymer	3	a	29	a	85	b	2291	b	4	a	5	b	73	c	1315	a
Ab 2x10^{8} CFU.mL^{-1}	3	a	38	a	88	a	2677	a	4	a	6	b	74	c	1193	a
Bs 1x10^{4} CFU.mL^{-1}	3	a	31	a	87	a	2819	a	4	a	7	a	75	b	1373	a
Bs 1x10^{5} CFU.mL^{-1}	3	a	43	a	87	a	2660	a	4	a	7	a	75	b	1363	a
Bs 1x10^{6} CFU.mL^{-1}	3	a	33	a	87	a	2684	a	4	a	7	a	76	a	1419	a
Bs 1x10^{7} CFU.mL^{-1}	3	a	43	a	88	a	2665	a	5	a	8	a	78	a	1431	a
CV (%)	12		20		1		9		17		6		1		8	

[a]Means (n = 4) followed by different letters on the same column are significantly different ($p \leq 0.05$, Scott-Knott test).
Legend: Ab=*Azospirillum brasilense*, Bs=*Bacillus simplex,* CV=coefficient of variation.

22% (8 bags.ha^{-1}); and in Dourados there was no significant difference in terms of yield between any of the *B. simplex* concentrations and the non-inoculated controls (Table 6). For the remaining response variables, significant differences were only observed for some of the *B. simplex* concentrations for shoot dry weight in Dourados (Table 6) and for NDVI in Imbituva (Table 5) and Dourados (Table 6). For the *A. brasilense* treatment, yield increase was equal to *B. simplex* in Lapa (Table 6), or yield was significantly lower as in Imbituva and Uberlândia (Table 5). There was no difference between the *A. brasilense* treatment, the *B. simplex* treatments, and the non-inoculated controls in Dourados (Table 6).

Yield increase was observed in both corn and soybean at *B. simplex* strain SYM00260 inoculum concentrations of 5x10^{4} CFU.mL^{-1} or above except in Dourados for soybean, demonstrating that *B. simplex* is stable and effective even at lower concentrations across a range of different environmental conditions. Higher *B. simplex* inoculum concentrations may be beneficial under high-stress situations such as those encountered in Dourados, where overall yield was low for soybean and corn crops, likely due to a combination of late planting and lower rainfall in the final grain filling phase. Even so, corn yield in Dourados for the *B. simplex* treatment was significantly increased by up to 24% or 1731 kg.ha^{-1}.

The impact of *Bacillus simplex* on corn and soybean root and shoot dry weight and NDVI was also investigated, but none of which was consistently impacted by the *B. simplex* treatments. Root and shoot dry weight, and NDVI, are commonly evaluated because they reflect plant vigor tied to higher photosynthetic activity, potentially translating into increased yield. The increase in the green area assessed by NDVI was significant for some *B. simplex* inoculum concentrations in five of the eight locations. Shoot dry weight was also significantly elevated at three of the eight locations for certain concentrations. Root dry weight increased significantly at the highest *B. simplex* inoculum concentration in Dourados. This shows that under certain situations, *Bacillus simplex*, in addition to yield, can also positively impact other key traits. The fact that simultaneous positive impact on all investigated traits was inconsistent, has been found with other inoculants (Hassen and Labuschagne, 2010).

Azospirillum in Brazil has been used as seed inoculant to increase nitrogen fixation in soybean together with *Bradyrhizobium* (Hungria et al*.,* 2015) or without *Bradyrhizobium* in corn. The yield increase of the *B. simplex* inoculant

was similar to the *A. brasilense* product, except for corn in Dourados, and soybean in Imbituva and Uberlândia, where only the *B. simplex* inoculant resulted in a significant yield increase.

The BIOCROMA polymer treatment did, in general, have no effect as compared to the untreated control. This was expected, as polymers are designed not to impact seed treatments or germination (Afzal et al., 2020).

Beneficial impacts on plants by *Bacillus* and related genera are well documented in the literature. In corn, Lima et al. (2011) observed a higher yield and overall improved plant development upon the use of *B. subtilis*. In soybean, Tavanti et al. (2019); and Araújo and Hungria (1999) reported increased yield using soybean seeds treated with *B. subtilis*. *Paenibacillus* strains isolated from the wheat rhizosphere and applied to soybean, maize, and wheat, showed significant increases in crop growth compared to the control (Akinrinlola et al., 2018). The application of *Bacillus*-based products has many benefits for plant growth and contributes to increasing crop yields and soil fertility (García-Fraile et al., 2015). *Bacillus* species can convert the complex form of essential nutrients, such as phosphorus and nitrogen in the soil, to available forms that can be taken up by plant roots (Kuan et al., 2016). Some species have the *nifH* gene and produce nitrogenase, which can fix atmospheric nitrogen and make it available to plants, thereby enhancing plant growth and yield (Ding et al., 2005; Szilagyi-Zecchin et al., 2014).

In this work, before *B. simplex* inoculation, standard chemical treatments were applied to corn and soybean seed, and a *Bradyrhizobium* inoculant to soybean seed, as is common in Brazil. It has been shown that the co-inoculation of compatible microorganisms can benefit *Bradyrhizobium* nodulation by increasing nitrogen fixation (Santos et al., 2019). *Bacillus* species are compatible with other beneficial microorganisms when applied as plant growth promoters to plants. Wu et al. (2005) reported improved growth and uptake of nitrogen, phosphorus, and potassium by corn plants when inoculated with *B. megaterium* and *B. mucilaginosus*. Schwartz et al. (2013) noticed a change in pea root architecture, nodule, and nodule size when co-inoculating *B. simplex* and *Rhizobium leguminosarum*. *Bacillus subtilis* and *Bradyrhizobium* co-inoculation also increased germination rate, plant height, dry root weight, and the number of nodules in soybean (Petkar et al., 2018). *Azospirillum* species in Brazil have been used as co-inoculants with *Bradyrhizobium* species in soybean (Hungria et al., 2015) or on their own in corn, aiming to increase nitrogen fixation.

The standard chemical base treatments applied to the seed did not appear to negatively affect *B. simplex*. This shows that the integration of *B. simplex* into the prevailing seed treatment regimen can be done easily for additional productivity gain.

CONCLUSIONS

The three methods are suitable for quantifying *Bacillus simplex* colony-forming units (CFU) in the inoculant product. In field trials, the *B. simplex* inoculant increased corn yield by up to 26% corresponding to an additional 2100 kg.ha^{-1}. Soybean yield was increased up to 22% or 500 kg.ha^{-1}. The *B. simplex* inoculant had no consistent effect on shoot and shoot weight, and NDVI. Yield increase of the *B. simplex* and *Azospirillum brasilense* inoculants was comparable. The polymer seed treatment had no impact on yield.

ACKNOWLEDGMENTS

We want to thank reviewers for valuable suggestions and Zhanshan Dong for help with statistical analyses of the *Bacillus simplex* quantification data.

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