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ORIGINAL ARTICLE

Bacillus strains with potential for growth promotion and control of white mold in soybean

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are known to stimulate the biocontrol of phytopathogenic bacteria and fungi, induce plant growth, and increase the yield of important economic crops. In this study, we evaluated in vivo, the ability of *Bacillus* strains to control soybean white mold disease and promote the growth of soybean plants under greenhouse conditions. Initially, 27 *Bacillus* strains were analyzed by PCR for the presence of genes encoding antimicrobial molecules, followed by in vitro tests of the positive strains against four phytopathogenic fungi, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Colletotrichum truncatum*. The bacterial strains that returned positive results for antimicrobial genes were evaluated in vivo for their growth promoting capacity in soybean cultivars Potencia RR and M6210 IPRO, and white mold control in soybean plants. The results showed that eight strains presented the genes *bamD*, *ituD*, and *fenF*, while the *bacAB* gene was observed in 16 of all tested *Bacillus* strains. The greenhouse experiment showed that the inoculation of the strain VBN02 was the best treatment for increasing fresh shoot biomass of the soybean M6210 IPRO and Potencia RR cultivar in the single inoculation in relation to the control (111.9% and 103.57%, respectively). Co-inoculation of soybean inoculant and VBE01 was the superior treatment for increasing fresh shoot and root mass in both the cultivars. Two other strains, VBE05 and VBE01, reduced the disease progression of white mold by 39.1% and 37.5%, respectively. In conclusion, our results showed that the *Bacillus* strains have potential for biocontrol of white mold and for promoting the growth of soybean plants.

Keywords Antibiosis · Biocontrol · Inoculation · Microbiolization · Rhizobacteria

Abbreviations

PGPR	Plant growth-promoting rhizobacteria
TSA	Tryptic soy agar
h	Hours
°C	Degree celsius
PCR	Polymerase chain reaction

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nm	Nanometer
µL	Micro litre
ng	Nanogram
dNTPs	Deoxynucleotide triphosphates
mM	Millimolar
U	Unit
MgCl2	Magnesium chloride
min	Minute
s	Second
w/v	Weight by volume
mm	Millimeter
PDA	Potato dextrose agar
BOD	Biological Oxygen Demand
IAA	Indole-3-acetic acid
rpm	Revolutions per minute
TSB	Tryptic soy broth
NBRIP	National Botanical Research Institute`s Phosphate
$Ca_3(PO_4)_2$	Calcium phosphate
mL	Milliliter
YMA	Yeast mannitol agar
K_2HPO_4	Dipotassium phosphate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
NaCl	Sodium chloride
CFU	Colony forming units
kg	Kilogram
L	Liter
FB	Fertbio Soja
DAS	Days after sowing
µg	Microgram
AUDPC	Area under the disease progress curve

Introduction

Diseases are among the major factors that limit soybean productivity, and Brazil produces more of this cash crop than any other country globally. Approximately 40 soybean diseases caused by fungi, bacteria, nematodes, and viruses have been identified in Brazil (Hartman et al. 2015; Bandara et al. 2020). Among the main fungal diseases affecting soybean shoots is white mold caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, anthracnose from *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, soybean damping-off from *Rhizoctonia solani* Kühn, and charcoal rot from *Macrophomina phaseolina* (Tassi) Goid (Ajayi-Oyetunde and Bradley 2017; Smolińska and Kowalska 2018; Boufleur et al. 2021; Pandey and Basandrai 2021). Yield losses in soybeans due to diseases can vary depending on the incidence, causing up to 100% yield losses (Boufleur et al. 2021). For example, the incidence of *S. sclerotiorum* has been increasing in Brazil. In 2006, white mold disease caused losses of 200.000 metric tons in Brazil (Wrather et al. 2010). According to Pannullo et al. (2019), without proper management to control the disease, epidemiological modeling estimates that losses caused by white mold in the country can reach more than US$ 1 billion per year.

The control of these pathogens can be carried out through several methods, such as crop rotation with non-host species of fungi and chemical treatment with fungicides (Selim 2019; Willbur et al. 2019). However, all of them have resistance structures and/or produce substances to protect the spores, which makes the management of these diseases difficult. Biological control methods using bacteria opens an opportunity to reduce these phytopathogens, while minimizing the negative effects caused by the intensive use of agricultural defensives in the environment and in the feed (Thakkar and Saraf 2015; Carmona-Hernandez et al. 2019).

Plant growth-promoting rhizobacteria (PGPR) are beneficial microbes that can induce plant development through several mechanisms, including the production of various antimicrobial agents, prevention of disease, and provision of growth promoters, which help to improve plant health and consequently increase crop yields (Backer et al. 2018). PGPR-mediated biological control may be facilitated by several different types of "bacterial × pathogen" interactions, including antibiosis, space and nutrient competition, parasitism, and systemic resistance induction (Köhl et al. 2011; Kumar et al. 2012). Furthermore, these microbes can synthesize molecules that act as plant growth regulators, such as indole-3-acetic acid (IAA) (Teale et al. 2006), increase the availability of nutrients for plants (e.g., phosphorus) (Alori et al. 2017), and express enzymes that increase soil fertility by cycling nutrients (Hassan 2017).

Several species of bacteria can be classified as PGPR, including some members of the genus *Bacillus* and *Pseudomonas* (Hashem et al. 2019). However, the genus *Bacillus* has been commonly studied worldwide because of its capacity to induce resistance, directly antagonize the production of antimicrobial compounds (Deketelaere et al. 2017; Shafi et al. 2017), and produce IAA (Barnawal et al. 2017) and siderophores (Radhakrishnan et al. 2017). *Bacillus* also possesses abilities of an inoculant due to the capacity of endospore formation that enables the survival to drought, and has as an ease of manipulation (Kavamura et al. 2013; Martins et al. 2018).

Thus, the aim of this study was to evaluate, in vitro, the potential of *Bacillus* strains to control fungal phytopathogens and to characterize the beneficial growth related traits in plants, and analyze, in vivo, the biocontrol activity of soybean white mold disease and the capacity for growth promotion when co-inoculated with the commercial inoculant content of two strains of *Bradyrhizobium*.

Materials and methods

Origin of bacterial strains

Previously, the 27 bacterial isolates used for this study were taxonomically and molecularly classified as *Bacillus* spp. (Viana et al. 2020). The strains were previously isolated and classified by Viana et al. (2020) and obtained from the Culture Collection of the Microbiology Laboratory of the Federal University of Mato Grosso do Sul, Campus Pantanal, Brazil. The bacteria were grown in tryptic soy agar (TSA) medium for 24 h at 30 °C to verify the purity before DNA extraction and the other assays.

PCR amplification of genes encoding antimicrobial substances

Bacterial DNA was extracted using the Wizard® Genomic DNA Purification Kit, following the manufacturer's instructions. The DNA was quantified using a spectrophotometer at 260 nm and stored at -20 °C. The detection of genes involved in the biosynthesis of antimicrobial substances was conducted by PCR amplification using specific primers (Table 1), as previously described by Chung et al. (2008). The 25 µL PCR reaction consisted of 30 ng of DNA, 1.6 pM of each initiator, 2.5 µL of PCR buffer, 0.5 µL of dNTP at 0.2 mM, 1.5 U of Taq DNA polymerase (Invitrogen, USA), and 3.0 mM of $MgCl_2$. PCR was conducted in a Veriti 96-Well Thermal Cycler (Applied Biosystems, CA, USA) according to Chung et al. (2008), with modifications, under the following conditions: denaturation at 95 °C for 1 min, followed by 40 cycles of 30 s at 95 °C, 45 s at 55 °C or 58 °C of annealing, and 45 s at 72 °C for 45 s, and a final elongation step of 5 min at 72 °C. The PCR products were stained with SYBR Safe DNA gel stain (Invitrogen, USA) and analyzed by electrophoresis on a 1.5% (w/v) TBE agarose gel. The amplified products were visualized using a Safe Imager 2.0 Blue-Light Transilluminator (Invitrogen). The strains that presented genomic amplified products were used in the subsequent experiments.

In vitro antagonism assays

The antifungal activity of the strains was tested against four pathogenic fungi, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Colletotrichum truncatum*, and *Macrophomina phaseolina*, provided by the Culture Collection of the Phytopathology Laboratory of University Anhanguera—Uniderp, Campo Grande, MS, Brazil. The fungal phytopathogens were cultured on a potato dextrose agar (PDA) medium at 22 °C for 7 days. For the assay, the bacteria were streaked on Petri plates containing PDA for pairing with mycelium disks (5.0 mm) from each fungus. Plates containing only the mycelial disks of phytopathogenic fungi were used as controls. The assay plates were incubated in a BOD (Biochemical Oxygen Demand) incubator at 22 °C with a photoperiod of 12 h. Mycelial growth was evaluated daily until total coverage of the plate surface of the control treatments. The assay evaluations were qualitative (presence of halos of inhibition of the phytopathogenic fungi growth) and quantitative (mycelial growth), with measurements performed with the aid of a ruler. The experiment was set up in a completely randomized design with three replicates and a control for each bacterial strain. The experiment was repeated twice. The inhibition of fungal growth was measured by recording the diameter of mycelial growth (mm). The percentage of inhibition was calculated using the following formula proposed by Shakeel et al. (2015):

$$\text{Inhibition rate (\%)} : \frac{C-T}{C} \times 100,$$

where C represents the mycelium diameter of pathogens growing on control plates, and T is that of the fungi growing in the presence of strains.

Table 1 Primers for amplification of antibiotic biosynthesis genes*

Antibiotic	Gene	Primers	Sequences (5′-3′)	Amplicon size
Bacillomycin D	*bamD*	*ITUD*-F	TTGAAYGTCAGYGCSCCTTT	482
	ituD	*ITUD*-R	TGCGMAAATAATGGSGTCGT	
	fenF			
Bacilysin *bacD*	*bacAB*	BACAB-F	CTTCTCCAAGGGGTGAACAG	815
		BACAB-R	TGTAGGTTTCACCGGCTTTC	
Mersacidin	*mrsA*	MRSA-F	GGGTATATGCGGTATAAACTTATG	597
		MRSA-R	GTTTCCCCAATGATTTACCCTC	
Surfactin	*sfp*	*sfp*-f	ATGAAGATTTACGGAATTTA	675
		sfp-r	TTATAAAAGCTCTTCGTACG	

*Reference:Chung et al. (2008)

In vitro characterization of bacterial strains for PGP traits

The isolates VBN02, VBE05, VBE57, VBE01, and VBE17 were evaluated for the presence of traits that improve plant growth and development. Previously, Galeano et al. (2021) screened the isolates of the culture collection, including VBE03, VBE19, and VBE23, and evaluated indole-3-acetic acid (IAA) production, phosphate solubilization activity, ammonia production, and protease, cellulase, and amylase enzyme presence. The results are presented in the same table as the evaluations of the strains used in this study.

Indole-3-acetic acid (IAA) production

IAA synthesis using the isolates was determined according to the method described by Gordon and Weber (1951) with modifications. Isolates were inoculated in test tubes containing tryptic soy broth (TSB) medium with or without tryptophan (0.1% w/v) and incubated at 28 °C for 24 and 48 h at 120 rpm. The cultures were centrifuged (10 000 rpm for 5 min), and the supernatant was recovered for metabolite quantification using Salkowski's reagent (1:1). The samples were incubated for 30 min in the dark, and the absorbance of the samples was measured at 530 nm. IAA concentration was determined using a standard curve of pure IAA (Sigma-Aldrich, USA). The assay was performed in triplicate.

Phosphate solubilization

Phosphate solubilization was determined in Petri plates containing solid NBRIP medium (Nautiyal 1999) with 0.5% (w/v) $Ca_3(PO_4)_2$ as an inorganic source of phosphate. The isolates were inoculated in triplicate, and the plates were incubated at 28 °C for 7 days. The formation of a halo around the colonies indicated the ability of the isolates to solubilize phosphate.

Ammonia production

Ammonia production was tested as described by Cappuccino and Sherman (1992). Strains were grown in peptone water (1% peptone, 0.5% NaCl) and incubated at 28 °C for 24 h at 120 rpm. Cultures were centrifuged, and the supernatant (1 mL) was homogenized with Nessler's reagent (50 µL). A brownish color indicated positive results for the test.

Amylase, cellulase, and protease production

Amylase production was detected in a yeast mannitol agar (YMA) medium containing starch as the substrate. Cellulase activity was determined according to the method described by Kasana et al. (2008). Protease production was observed in the culture medium as proposed by Saran et al. (2007). The formation of a halo around the colonies indicated a positive (+) result for the production of each enzyme.

In vitro test for compatibility of isolates and inoculant containing *Bradyrhizobium japonicum*

The compatibility of the isolates with the commercial liquid-formulated inoculant Fertbio Soja® (containing the strains SEMIA 7079 and SEMIA 5080 of *Bradyrhizobium japonicum*) was assayed following method described by Stockwell and Johnson (1996) with modifications. The inoculant bacteria were inoculated in YMA medium (1% mannitol, 0.06% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% yeast extract, and 1.5% agar), and grown at 28 °C for 48 h in an incubator. The isolates, previously grown in TSA medium for 24 h at 28 °C, were confronted separately with *B. japonicum* strains. The assay was carried out in duplicate and the plates were incubated at 28 °C for 24 h. After the incubation time, inhibition or not of the growth of microorganisms was evaluated.

Experiment 1: Plant growth-promoting bacteria as inoculants and co-inoculants in soybean varieties

For the in vivo experiment under greenhouse, the soybean seeds from the cultivars Potencia RR and M6210 IPRO were microbiolized with a suspension of each of the bacterial strains at 1.5×10^9 colony forming units (CFU) mL^{-1} according to the MacFarland scale, previously cultured in Dygs medium for 24 h at 28 °C. The microbiolization consisted of soaking the seeds for 30 min in a 3.0 mL suspension of each treatment containing only *Bacillus* spp. strains or 5.0 mL of the commercial liquid-formulated inoculant Fertbio Soja®, at 5.0×10^9 CFU mL^{-1}, followed the manufacturer's recommendations (100 mL for every 50 kg of seeds). Subsequently, seeds were sown in 5 L pots (five seeds per pot) containing Aortic Quartzarenic Neosol soil, previously fertilized. The corrective fertilization was made based on chemical soil analysis, deploying 0.4 g pot^{-1} of urea for each standard control. In the other treatments, we added 0.4 g pot^{-1} KCl and 0.8 g pot^{-1} of phosphate fertilizer.

There were 16 treatments: non-inoculated control, strains VBE19, VBE03, VBN02, VBE05, VBE57, VBE01, and VBE17, Fertbio Soja (FB) inoculated treatment, FB + VBE19, FB + VBE03, FB + VBN02, FB + VBE05, FB + VBE57, FB + VBE01, and FB + VBE17. The control consisted of only the *B. japonicum* strains with the commercial inoculant, while the treatments without inoculation received only saline solution (NaCl 0.85% w/v). In the case of co-inoculated treatments, the seeds were microbiolized with a mixture of 5.0 mL of commercial inoculant and

3.0 mL of each bacterial strain. The experiment was distributed in a randomized block design with five replications. The plants were harvested at 40 days after sowing (DAS), and the root length, and root and shoot fresh and dry weights were determined. The experiment was conducted twice.

Experiment 2: White mold control in soybean plants following seed microbiolization with various Bacillus spp. strains

Bacillus spp. strains were prepared as previously described and used to treat soybean seeds 'Potencia RR'. Potencia RR cultivar seeds were microbiolized as described above in experiment 1. The experimental design and randomization were performed as previously described under greenhouse. After plant emergence, thinning was performed, leaving only one plant per pot. There were nine treatments: non-infested plant (control) and strains VBE23, VBE19, VBE05, VBE01, VBE57, VBE03, VBE17, and VBN02.

The inoculation of *S. sclerotiorum* was performed when the plants reached the V4 stage. A cut was made on the stem at 2.5 cm from the fourth petiole of the trifolium, and a fungal mycelium disk with 36 h of growth was inserted using sterilized tips (Petzoldt and Dickson 1996). The severity of the disease was evaluated at 3, 5, 7, 9, and 11 days post inoculation using the Petzoldt and Dickson (1996) scale adapted by Terán et al. (2006) (Table 2). The mean values for the severity of each treatment were used to calculate the area under the disease progress curve (AUDPC) (Campbell and Madden 1990). The AUDPC was calculated using the following formula:

$$AUDPC = \Sigma[(I_i + I_i + 1)/2.(T_{i+1} - T_i)],$$

where I_i is the incidence at the time of evaluation, and T_i is the time of evaluation. The experimental design was completely randomized with five replicates and was conducted twice.

Table 2 Descriptions of the note scale used to evaluate white mold infections, based on disease progression in infected plants*

Note	Severity
1	Plants without symptoms
2	Invasion of the fungus beyond the inoculation site
3	Invasion of the fungus near the first node
4	Invasion of the fungus to the first node
5	Invasion of the fungus beyond the first node
6	Invasion of the fungus near the second node
7	Invasion of the fungus to the second node
8	Invasion of the fungus beyond the second node
9	Death of the plant

*Reference: Petzoldt and Dickson (1996), adapted by Terán et al. (2006)

Statistical analysis

The data obtained in the plant growth-promoting and white mold control experiments in soybean plants were subjected to analysis of variance (ANOVA). In experiment 1, the means were compared using the Scott-Knott mean range test ($p < 0.05$) with Sisvar software version 5.6 (Ferreira 2011). The values of the white mold control experiment were compared using the Tukey test ($p < 0.05$) with the statistical software Sisvar v.5.6.

Results

PCR amplification of genes encoding antimicrobial substances

Only two primer pairs used for PCR amplification of genes coding for antimicrobial substances were positive among the strains used in this study. Positive amplification was observed with the primer pair *ituD* (simultaneously detecting genes *bamD*, *ituD*, and *fenF*) in eight strains. The *bacAB* primer pairs presented 16 bacterial strains with positive amplification of the *bac*AB gene (Table 3). There were no PCR products for other target genes. The 16 isolates that showed amplification were selected for evaluation of antagonism against four phytopathogenic fungi.

In vitro antagonism assays

Although no significant statistical difference was verified in the antagonism assay with paired cultures, a few bacterial strains presented inhibitory potential for some of the phytopathogenic fungi evaluated. The VBE03 and VBE57 strains inhibited the mycelial growth of *S. sclerotiorum* by 27% and 40.8%, respectively (Fig. 1). In the case of *M. phaseolina,* treatment with VBE19 showed the greatest inhibition of mycelial growth of 24% compared to the control (Fig. 1). Lesser inhibition of the fungi *R. solani* was observed; however, the strain VBE05 promoted an inhibition of 8.5% in the mycelial growth as compared to the control (Fig. 1). The strains VBE23 and VBE19 inhibited *C. truncatum* by 8.5% and 9.8%, respectively (Fig. 1).

In vitro characterization of bacterial strains for PGP traits

In the current study, five strains were able to synthesize IAA in the presence of tryptophan (Table 4). The highest concentration of IAA in the supernatant was observed for isolate VBE17 (33.27 ± 0.46 µg mL^{-1}), followed by VBE01 (26.1 ± 0.92 µg mL^{-1}) and VBN02 (16.08 ± 0.52 µg mL^{-1})

Table 3 Detection of PCR products for the genes of bacillomycin D (*bamD*, *ituD,* and *fenF*), bacilysin *bac*D (*bacAB*), mersacidin (*mrsA*) and surfactin (*sfp*) in 27 *Bacillus* spp. used in this study

Strain	Genus	Antimicrobial compound			
		Bacillomycin D	Bacilysin *bac*D	Mersacidin	Surfactin
VBE01	*Bacillus* sp.	+	+	-	-
VBE02	*Bacillus* sp.	-	+	-	-
VBE03	*Bacillus* sp.	+	+	-	-
VBE05	*Bacillus* sp.	+	+	-	-
VBE06	*Bacillus* sp.	-	-	-	-
VBE08	*Bacillus* sp.	-	-	-	-
VBE11	*Bacillus* sp.	-	-	-	-
VBE12	*Bacillus* sp.	-	-	-	-
VBE16	*Bacillus* sp.	-	-	-	-
VBE17	*Bacillus* sp.	+	+	-	-
VBE19	*Bacillus* sp.	+	+	-	-
VBE21	*Bacillus* sp.	-	+	-	-
VBE23	*Bacillus* sp.	+	+	-	-
VBE22	*Bacillus* sp.	-	+	-	-
VBE29	*Bacillus* sp.	-	+	-	-
VBE41	*Bacillus* sp.	-	+	-	-
VBE54	*Bacillus* sp.	-	-	-	-
VBN01	*Bacillus* sp.	-	+	-	-
VBE57	*Bacillus* sp.	+	+	-	-
VBN02	*Bacillus* sp.	+	+	-	-
VBN03	*Bacillus* sp.	-	-	-	-
VBN04	*Bacillus* sp.	-	-	-	-
VBN05	*Bacillus* sp.	-	+	-	-
VBN06	*Bacillus* sp.	-	-	-	-
VBN11	*Bacillus* sp.	-	+	-	-
VBN13	*Bacillus* sp.	-	-	-	-
VBN39	*Bacillus* sp.	-	-	-	-

Antimicrobial gene: + = presence; - = absence

(Table 4). Six isolates were able to solubilize phosphate in the NBRIP solid medium, and seven produced ammonia (Table 4). Six isolates showed enzymatic activity of cellulase, protease, and amylase in the culture medium with their respective substrates (Table 4).

In vitro test for compatibility of isolates and inoculant containing *Bradyrhizobium japonicum*

Regarding the compatibility between the *Bacillus* strains and *B. japonicum*, none of the *Bacillus* strains interfered with the growth of *B. japonicum*, suggesting that both species could be used in the co-inoculation experiments.

Experiment 1: Plant growth-promoting bacteria as inoculants and co-inoculants in soybean varieties

The plant growth promotion experiments revealed no significant differences in shoot dry mass and root length of the cultivar Potencia RR inoculated with *Bacillus* strains in the presence or absence of commercial inoculant (Table 5). The treatments with the strains VBN02, VBE57, VBE05, VBE01, and FB + VBE01 resulted in higher values of fresh biomass of the aerial part. Moreover, the treatments with VBN02, VBE05, VBE01, VBE17, FB + VBE19, FB + VBE01, and FB + VBE05 showed the highest values of the root fresh biomass. For the root dry biomass parameter, the FB + strain VBE05 and FB + strain VBE01 treatments presented significantly larger root increments than the other treatments (Table 5). For cultivar M6210 IPRO, no substantial differences were found in the dry biomass of shoots (Table 6). The treatments with VBN02, FB + VBE57, and FB + VBE01 promoted increases in fresh biomass of shoots and roots, dry biomass of roots, and length of roots (Table 6).

Experiment 2: White mold control in soybean plants following seed microbiolization with various Bacillus spp. strains

In the in vivo assay for evaluation of the *Bacillus* strains on the control of the white mold, significant statistical differences were observed among the treatments (Table 7). Although there was an increase in disease severity in inoculated plants during the 11 days of evaluation, the progression stopped at day seven and remained stable up to day 11. The highest inhibition percentage values were observed for strains VBE19 (34.29%), VBE05 (39.1%), VBE01 (37.5%), and VBE17 (33.33%) (Table 7). Although the disease progress in the infected regions of the plants continued, treatment with these strains resulted in lower disease severity indices from the first day of evaluation.

Discussion

Bacillus spp. are endospore-forming bacteria and some species and strains can associate with plants, improving growth and development (Sansinenea 2019). The ability to promote plant growth is attributed to several mechanisms, including phytohormones production, increased nutrient availability, and suppression of phytopathogens by the production of antimicrobials and enzymes (Saxena et al. 2019). Although these abilities are well known, reports on *Bacillus* strains adapted to Brazilian regions are limited, and research with native strains to control diseases, such as white mold, opens up new possibilities for disease management in the agricultural production of crops such as soybeans.

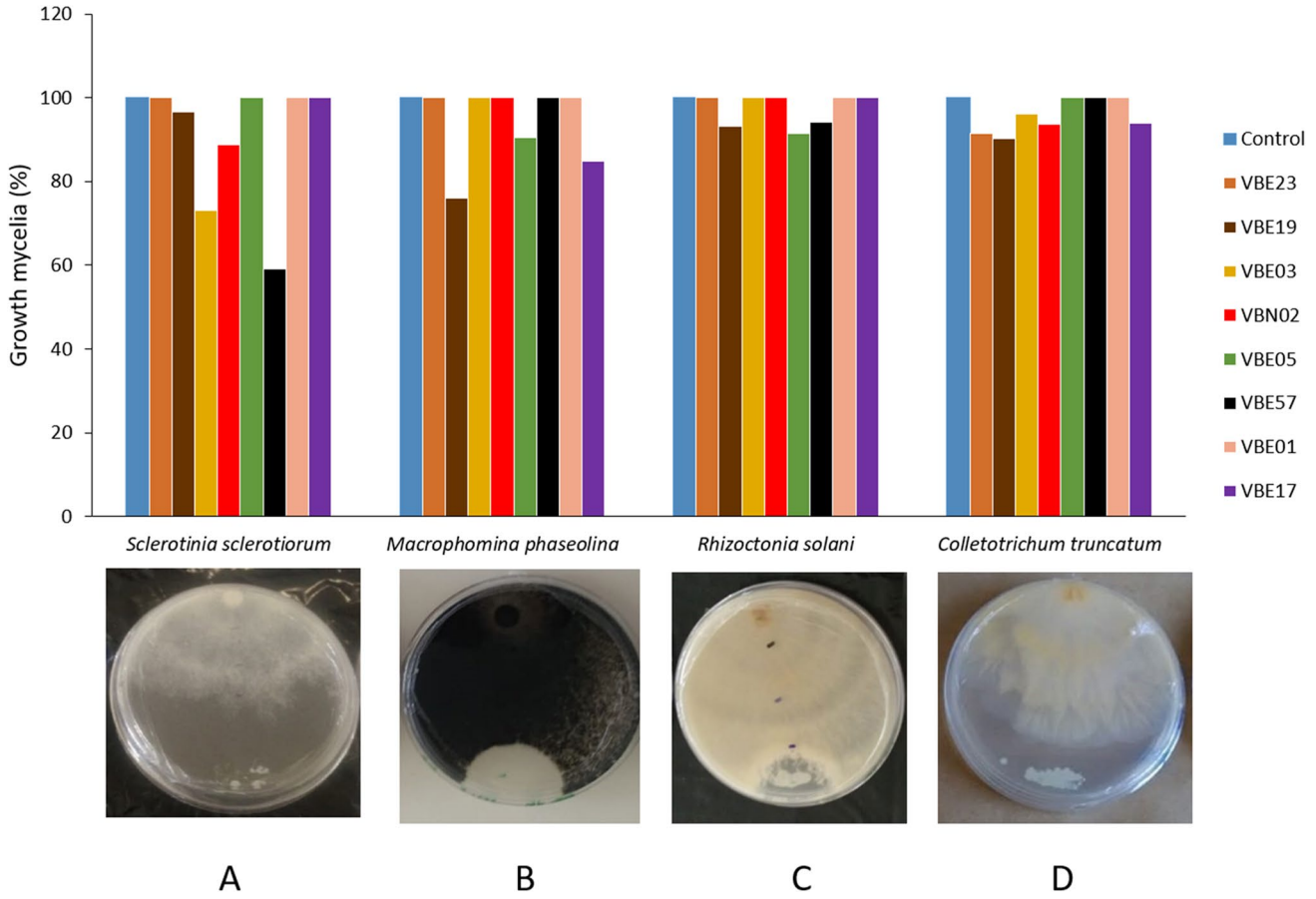

Fig. 1 Antagonist effect of different strains of *Bacillus* on growth of four phytopathogenic fungi evaluated in in vitro assays. (**a**) *Sclerotina sclerotiorum* against VBE57, (**b**) *Macrophomina phaseolina* against VBE19, (**c**) *Rhizoctonia solani* against VBE05, (**d**) *Colletotrichum truncatum* against VBE19

In this study, we observed amplification products for genes encoding antimicrobial substances with the expected sizes for the primers *ituD* and *bacAB*, but not for *sfp* and *mrsA*; these results have also been reported in other *Bacillus* studies with similar patterns of gene expression (Stanković et al. 2012; Carrer Filho et al. 2015). The results are in accordance with those obtained by Chung et al. (2008) and Ayed et al. (2014) regarding the existence of antibiotic and antifungal substances produced by *Bacillus* species. Bacteria of the genus *Bacillus* have been widely studied in biocontrol

Table 4 Indole-3-acetic acid (IAA) production, phosphate solubilization, ammonia and enzyme production (amylase, cellulase, and protease) by *Bacillus* spp. strains

Strain	IAA (μg mL^{-1})[a]	Phosphate[b]	Ammonia[b]	Cellulase[b]	Protease[b]	Amylase[b]	Reference
VBE19	5.41 ± 0.26	+	+	+	+	+	Galeano et al. (2021)
VBE03	–	–	+	+	+	+	Galeano et al. (2021)
VBN02	16.08 ± 0.52	+	+	+	+	+	This study
VBE05	13.08 ± 0.28	+	+	+	+	–	This study
VBE57	–	+	+	+	+	+	This study
VBE01	26.1 ± 0.92	–	–	+	+	–	This study
VBE17	33.27 ± 0.46	+	+	+	+	+	This study
VBE23	–	+	+	+	+	+	Galeano et al. (2021)

[a]Data are the mean values of three replicates

[b]Did not show any activity (–); Positive production (+)

Table 5 Seed microbiolization effect of *Bacillus* spp. strains on the fresh biomass of shoots (FBS), dry biomass of shoots (DBS), fresh biomass of roots (FBR), dry biomass of roots (DBR), and length of roots (RL) of the soybean Potencia RR cultivar grown in soil pots for 40 days. Numbers in parentheses represent the percentage of growth

Treatment	Potencia RR				
	FBS*	DBS†	FBR*	DBR*	RL†
Control (no inoculant)	1.12 d	0.65	3.02 c	0.19 d	53.50
VBE19	1.78 (58.93) b	0.88	4.18 (38.41) b	0.26 (36.84) c	65.37
VBE03	1.82 (62.5) b	0.93	4.85 (60.6) a	0.30 (57.83) c	72.62
VBN02	2.28 (103.57) a	1.10	4.45 (47.35) a	0.33 (73.68) b	65.00
VBE05	1.97 (75.83) a	1.01	4.92 (62.91) a	0.37 (94.73) b	70.12
VBE57	1.99 (77.68) a	0.99	4.08 (35.1) b	0.30 (57.89) c	61.37
VBE01	2.02 (80.34) a	1.27	4.47 (48.01) a	0.37 (94.74) b	67.00
VBE17	1.78 (58.93) b	0.96	5.01 (65.89) a	0.35 (84.21) b	72.62
FB soybean inoculant	1.54 (37.5) c	0.88	2.89 (-5.69) c	0.21 (10.52) d	55.87
FB + VBE19	1.81 (61.61) b	1.04	4.39 (45.36) a	0.30 (57.89) c	65.50
FB + VBE03	1.71 (52.68) b	1.10	4.01 (32.78) b	0.28 (47.37) c	62.62
FB + VBN02	1.60 (42.86) c	1.09	3.99 (32.11) b	0.29 (52.63) c	71.25
FB + VBE05	1.79 (59.82) b	1.11	5.08 (68.21) a	0.43 (126.31) a	60.00
FB + VBE57	1.81 (61.61) b	1.04	4.05 (34.1) b	0.32 (68.42) c	61.12
FB + VBE01	2.12 (89.28) a	1.05	5.26 (74.17) a	0.41 (115.79) a	70.50
FB + VBE17	1.42 (26.79) c	1.02	3.72 (23.17) b	0.36 (89.47) b	60.88
CV (%)‡	17.12	18.43	18.51	17.51	19.90

*Means followed by different letters in the column differ from each other according to the Scott-Knott test ($p > 0.05$)

† Non-significant difference, ‡ variance coefficient

Table 6 Seed microbiolization effect of *Bacillus* spp. strains on the fresh biomass of shoots (FBS), dry biomass of shoots (DBS), fresh biomass of roots (FBR), dry biomass of roots (DBR), and length of roots (RL) of the soybean M6210 IPRO cultivar grown in soil pots for 40 days. Numbers in parentheses represent the percentage of growth increase relative to the control

Treatment	M6210 IPRO				
	FBS*	DBS†	FBR*	DBR*	RL*
Control (no inoculant)	2.10 c	0.95	2.62 b	0.16 b	60.50 b
VBE19	3.29 (56.66) b	1.09	3.51 (33.97) b	0.23 (43.75) b	83.37 (37.8) a
VBE03	3.46 (64.76) b	1.19	2.97 (13.36) b	0.27 (68.75) b	81.37 (34.5) a
VBN02	4.45 (111.9) a	1.26	4.70 (79.39) a	0.36 (125) a	88.50 (46.28) a
VBE05	3.94 (87.62) a	1.04	4.57 (74.42) a	0.26 (62.5) b	74.62 (23.34) b
VBE57	3.89 (81.24) a	0.90	3.83 (46.14) b	0.22 (37.5) b	84.25 (39.25) a
VBE01	4.04 (92.32) a	0.95	3.70 (41.22) b	0.24 (50) b	69.75 (15.29) b
VBE17	3.54 (68.57) b	1.43	4.94 (88.54) a	0.35 (118.75) a	94.00 (55.37) a
FB soybean inoculant	3.08 (46.66) b	0.87	3.76 (43.51) b	0.22 (37.5) b	67.12 (10.93) b
FB + VBE19	3.40 (61.9) b	0.89	4.47 (70.61) a	0.32 (100) a	77.25 (27.68) b
FB + VBE03	3.34 (59.04) b	0.90	3.78 (44.27) b	0.29 (81.25) a	66.87 (10.52) b
FB + VBN02	3.14 (49.52) b	1.08	4.48 (70.93) a	0.32 (100) a	82.25 (35.95) a
FB + VBE05	3.54 (68.57) b	1.04	3.71 (41.6) b	0.26 (62.5) b	72.81 (20.34) b
FB + VBE57	3.62 (72.32) a	1.25	5.36 (104.58) a	0.37 (131.25) a	92.50 (52.83) a
FB + VBE01	4.25 (102.38) a	1.81	4.24 (61.83) a	0.34 (112.5) a	89.50 (47.93) a
FB + VBE17	3.18 (51.42) b	1.08	4.81 (83.58) a	0.36 (125) a	62.11 (2.66) b
CV (%)‡	11.89	18.48	25.13	25.66	22.74

*Means followed by different letters in the column differ from each other according to the Scott-Knott test ($p > 0.05$)

† Non-significant difference, ‡ variance coefficient

Table 7 Effect of microbiolization of soybean seeds of cultivar Potencia RR with different *Bacillus* strains on the reduction of the severity of the white mold disease and area under the disease progress curve (AUDPC) of plants grown in a greenhouse

Treatment	Severity of disease						
	3 days*	5 days*	7 days†	9 days*	11 days*	AUDPC*	% reduction
Control	3.40 b	3.60 b	3.80	4.10 b	4.80 b	31.20 b	0
VBE23	2.30 a	2.90 ab	2.90	3.00 ab	3.20 ab	23.50 ab	24.68
VBE19	2.50 ab	2.50 ab	2.60	2.60 a	2.60 a	20.50 a	34.29
VBE05	1.90 a	2.20 a	2.50	2.50 a	2.50 a	19.00 a	39.10
VBE01	1.90 a	2.10 a	2.60	2.70 ab	2.80 a	19.50 a	37.50
VBE57	2.20 a	2.80 ab	3.00	3.10 ab	3.40 ab	23.40 ab	25.00
VBE03	2.00 a	2.60 ab	2.90	3.00 ab	3.10 ab	22.10 ab	29.20
VBE17	2.00 a	2.50 ab	2.70	2.70 ab	3.00 ab	20.80 a	33.33
VBN02	2.40 ab	2.60 ab	2.80	3.50 ab	4.00 ab	24.20 ab	22.44
CV (%)‡	22.32	22.72	21.67	23.74	29.09	21.24	

*Means followed by different letters in the column differ among themselves according to the Tukey test ($p > 0.05$)

† = non-significant, ‡ = Coefficient of variation

trials due to their facility in producing antimicrobial compounds (Caulier et al. 2019; Andrić et al. 2020).

Among the 27 *Bacillus* strains initially selected, eight presented both *ituD* and *bacAB* amplification products, and of these eight, the strains VBE01, VBE05, VBE17, VBE19, and VBE57 can be considered as potential candidates for the induction of phytopathogenic fungi resistance and growth promotion in soybean. The selection of strains that produce these antimicrobials is interesting since these molecules act in the disturbance of hyphal cells and/or permeabilization of spores of phytopathogenic fungi (Shahid et al. 2021). The strains promoted plant growth, decreased white mold disease severity, and induced metabolite production related to resistance induction to a certain degree.

The in vitro antagonism assay showed that phytopathogenic fungi had decreased mycelial growth in the presence of some strains tested. Cavalcanti et al. (2020) and Ribeiro et al. (2021) also reported biocontrol rates of *S. sclerotiorum* with strains of *Bacillus* spp. in an in vitro antagonism assay. Although the results with the other fungal species did not represent great antagonistic potential for *Bacillus*, the VBE19 strain presented the best performance against *M. phaseolina* and *C. truncatum* (24% and 8.5%, respectively), and VBE05 presented the superior results for *R. solani*.

The use of legume inoculants containing multiple microbial strains, such as the combination of rhizobial and non-rhizobial strains, including Firmicutes, can promote synergistic or additive effects on legume growth and nodulation through several mechanisms (Rodrigues et al. 2012; Hungria et al. 2013, 2015; Ribeiro et al. 2022). The in vitro compatibility evaluation was based on the criteria used by Mafia et al. (2007) to select combinations of microorganisms for in vivo studies. This method was an efficient strategy here considering that none of the *Bacillus* strains affected *Bradyrhizobium japonicum* (Fertbio Soja®) growth. Our results showed a positive compatibility of the *Bacillus* strains with *B. japonicum* with a possible additive effect in co-inoculated treatments. Other researchers performed compatibility tests between different isolates and strains by the direct antagonism method, streaking bacteria in Petri dishes containing culture medium (Prasad and Babu 2017; Ribeiro et al. 2022).

Of the eight isolates evaluated, five strains (VBN02, VBE01, VBE05, VBE17, and VBE19) were positive for IAA production. This growth-regulating substance is one of the most physiologically active auxins, playing an important role in plant growth promotion by contributing to root growth and alleviating biotic and abiotic stresses (Shahzad et al. 2017; Galeano et al. 2021). The *Bacillus* strains studied here indicate the capacity of this microorganism in solubilize phosphate to increase soil fertility without causing environmental damage (Rawat et al. 2021). Ammonia produced by PGPR can indirectly improve plant growth through the control of phytopathogens (Hassan 2017). In the present study, seven strains tested ammonia positive in vitro. Amylase, cellulase, and protease are enzymes that play important roles in soil nutrient cycling. Furthermore, protease may participate in the suppression of pathogens by cell-wall degradation (Glick 2012).

Several studies with various crops have shown plant growth promotion by diverse *Bacillus* spp., such as with wheat (Ku et al. 2018), tomato (Kalam et al. 2020), and corn (Batista et al. 2018). In the Potencia RR soybean cultivar, inoculation with FB + strain VBE01 or FB + strain VBE05 increased the dry matter of roots by 115.78 and 126.31%, respectively, compared to the control, while in M6210 IPRO, inoculation with FB + VBE57, FB + VBE17, and strains VBN02 and VBE17, produced the better results, with an increase of 118.75 to 131.25% (Table 4). A study conducted by Pandey et al. (2018) found that co-inoculation

with *B. pumilus* and *B. subtilis* in *Amaranthus hypochondriacus* seeds provided a 32.2% increase in dry mass of plants, whereas inoculation with *B. subtilis* alone showed a 29.53% increase in the same variable. For soybean, Masciarelli et al. (2014) concluded that the co-inoculation of *B. amyloliquefaciens* strains with *B. japonicum* was an effective technique for use in commercial inoculant formulations following proper field evaluation. The success of co-inoculation in promoting plant growth was also reported for soybean with *Bacillus* and *B. japonicum*, with an increase in shoot dry weight (Tonelli et al. 2017). In this context, the *Bacillus* spp. used in this study can be classified as efficient for plant growth promotion in co-inoculation.

Soybean plants microbiolized with *Bacillus* strains showed lower disease severity and progression compared to the control as determined by the disease progress curve area. However, there was no relationship between the in vitro and in vivo assays, since the best strains in vitro of VBE57 and VBE03, showed no significant results in the in vivo assay. Although these strains showed the highest *S. sclerotiorum* inhibition values in the in vitro antagonism assay, disease suppression by antagonists depends on several factors, such as the ability of bacteria to establish in the plant-soil system, and inhibition by induced resistance, where the defense system of plants is activated to combat the pathogen (Latz et al. 2018). The results obtained for *Bacillus* strains VBE01 and VBE05 are encouraging because the disease was partially controlled in soybean (37.5 and 39.10%, respectively). Villareal-Delgado et al. (2021) and Ribeiro et al. (2021) also found that strains of *Bacillus* act as suppressors of white mold in plants. Sabaté et al. (2018) observed suppressive effects of *Bacillus* spp. on the mycelia of *S. sclerotiorum*. According to Lobo Junior (2013), the use of antagonistic microorganisms is a promising alternative for controlling the white mold because it helps to reduce the density of the pathogen inoculum in the soil and, consequently, lower the incidence and/or severity of the disease.

Thus, these results indicate the potential of VBE01, VBE05, VBE17, VBE19, and VBE57 for plant growth promotion and suppression of white mold, and as possible candidates for agricultural applications. Further experiments should be performed to understand the effects of microbiolization with these strains on the biocontrol of *S. sclerotiorum* in soybean cultivars.

Conclusions

In conclusion, eight strains showed genes encoding antimicrobial substances. The strains had PGP traits and promoted the growth of two soybean cultivars in a single inoculation or co-inoculation with a commercial inoculant containing *B. japonicum*. Five strains (VBE01, VBE05, VBE17, VBE19, and VBE57) decreased the severity and progression of white mold in soybean, suggesting their potential application for biocontrol of this disease.

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Authors' contributions LMMD and MSB conceived and designed the study. RMSG and TFCV contributed to the PCR experiment and interpretation of results. BOC and RM contributed to the plant growth promoting assay. CRG, GMP, BOC and MSB contributed to the analysis and interpretation of the results. LMMD and MSB wrote the paper. RMS, GMP and MSB reviewed and edited the manuscript. All the researchers read and approved the manuscript.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

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Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

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