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BIOCONTROL POTENTIAL OF BACTERIAL ISOLATES FROM THE CAATINGA BIOME AGAINST CHARCOAL ROT OF COWPEA CAUSED BY *MACROPHOMINA PHASEOLINA*

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ABSTRACT

Charcoal rot, caused by *Macrophomina phaseolina*, is one of the main difficult-to-control diseases that affect cowpea. Biocontrol has been gaining prominence for being an economically viable alternative with low risk to the environment. This study aimed to identify and evaluate bacterial isolates from the Caatinga biome for managing charcoal rot in cowpea. For this purpose, *in vitro* tests were performed, where the bacterial inhibition capacity under the growth of *M. phaseolina* strains was evaluated, and the production of non-volatile metabolites with an inhibitory effect on the mycelial growth of the fungus. *In vivo* tests, cowpea seeds were bacterized and sown in sterilized and natural soil with inoculum of *M. phaseolina* strains Fe 01 and Fe 02, for evaluation of Disease Severity Index (ISD%) and other growth variables and seedling development. Of the six isolates, four reduced more than 60% of fungal growth as well as their non-volatile metabolites. *In vivo*, the bacterium *B. licheniformis* FO5.7 applied to the seeds reduced the ISD% caused by *M. phaseolina* Fe 01 by more than 60% both in sterilized soil and in natural soil, and provided an increase in shoot and root mass, as well as the length of both. For *M. phaseolina* Fe 02, *Bacillus* sp. T 6.2 and M 7.1 reduced the severity in sterilized soil and positively influenced the growth and development of seedlings, however, they were not efficient in controlling the disease in natural soil.

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INTRODUCTION

Belonging to the *Botryosphaeriaceae* family, the fungus *Macrophomina phaseolina* (Tassi) Goid., is classified as a polyphagous and cosmopolitan phytopathogen, capable of infecting more than 500 plant species, among them, the cowpea [*Vigna unguiculata* (L.) Walp].

causal agent of charcoal rot, *M. phaseolina* causes considerable losses in cowpea production areas, causing deep and irregular necrotic lesions on the stem of adult plants with the presence of pycnidia, which extend towards the hypocotyls and roots, resulting in the wilting, rotting and death of the plant. (Kaur *et al*., 2012). In the case of infected seeds, the germination process may be interrupted, and pre-emergence and post-emergence damping-off may occur (Basandrai *et al*., 2021).

The control of charcoal rot is basically done through cultural practices and fungicide application. Cultural practices combine preventive control strategies, aiming to prevent the entry of the pathogen in the area, and

reduction of inoculum density in the soil through techniques such as moisture management, solarization, etc. (Marquez *et al*., 2021; Basandrai *et al*., 2021).

For chemical control, there are only three products registered in Brazil for bean culture, they are Anchor SC, Spectro$_o$ and Maxim. All for application in seed treatment, since this is one of the main sources of disease spread Anchor SC®, Spectro® e Maxim®. All for application in seed treatment, since this is one of the main sources of disease spread , they are Anchor SC, Spectro$_o$ and Maxim. All for application in seed treatment, since this is one of the main sources of disease spread (AGROFIT, 2022). However, the use of agrochemicals inappropriately in addition to bringing risks to the environment can also influence the development of resistant strains of the pathogen, something undesirable for producers, making the search for sustainable as the use of biological agents, essential for combating this disease in the field.

Despite advancements in biological control, few microbial-based products are commercially available for controlling *M. phaseolina*, necessitating the exploration of novel antagonists. Some studies prove the efficiency of microbial agents as potential antagonists of *M. phaseolina*, as demonstrated by Rangel-Montoya *et al*. (2022), where isolates of *B. amyloliquefaciens* reduced mycelial growth and the production of microsclerotia of *M. phaseolina* from strawberry plants.

Other species of the genus *Bacillus*, such as *B. subitilis* and *B. velezensis*, were able to delay the growth of *M. phaseolina* and reduce the incidence of the disease in tubers, through the production of secondary metabolites and mycolytic enzymes (Dhanabalan *et al*., 2024).

The microorganisms used in the biocontrol of pests and phytopathogens are obtained from various environments, whether on surfaces or interiors of biotic or abiotic media, such as plants and soil. Recently, the Caatinga biome has drawn the attention of researchers with regard to its biotechnological potential, both for the production of medicines and for the prospecting of microorganisms with the ability to promote growth and mitigate the effects of stresses on plants (Kavamura *et al*., 2013; Dias *et al*., 2023). As observed in the study by Santos *et al*. (2020), where *Bacillus* isolates obtained from the rhizosphere of *Caatinga cactaceae* were efficient in promoting corn growth under irrigated and water stress conditions.

For biological control purposes, studies related to the management of plant diseases using bacteria isolated from the Caatinga are still scarce. However, the characteristics of the biome, such as low rainfall, high temperatures and high evapotranspiration rates, can result in microorganisms with an aptitude for extreme conditions of survival and, consequently, more competitive, making them focus of study with high potential in phytosanitary management. (Lira-Cadete *et al*., 2011; Celestino, 2019).

Thus, considering the microbiota of the Caatinga biome as an important and little explored source of microorganisms and the growing need for new sources of biologically active molecules this study aimed to evaluate the antifungal potential of Caatinga bacterial isolates in the management of charcoal rot caused by *Macrophomina phaseolina* in cowpea.

MATERIAL AND METHODS

The assays were conducted at the Microbial Biotechnology and Phytopathology Laboratories of the State University of Bahia, *campus* III in Juazeiro-BA. Six bacterial isolates that showed promising results in the control of other similar fungal pathogens in previous studies were used: *Bacillus licheniformes* (S3.5), *Bacillus licheniformis* (S3.6), *Bacillus licheniformis* (FO5.7) and *Bacillus* sp. (P6.2), and three other strains not yet identified (T6.2, T7.2 and M7.1) from the collection of microorganisms isolated from the Caatinga of the Microbial Biotechnology Laboratory of UNEB (Table 1).

The fungal strains of *M. phaseolina* were isolated from soybean (GF 01), melon (Me 248, Me 249 and Me 250) and cowpea (Fe 01 and Fe 02) plants kindly provided by the Laboratory of Plant Pathology and Microbiology of the Federal Rural University of the Semiarid (UFERSA). And the seeds of cowpea cv. BRS Acauã were kindly provided by the Laboratory of Soil Microbiology of UNEB *campus* III in Juazeiro - BA.

Direct Antagonism Test

To evaluate the antagonistic potential of the bacterial isolates S3.5, S3.6, T6.2, T7.2, P6.2, M7.1 and FO5.7 against the fungal strains of *M. phaseolina*, the culture pairing method was used, where the bacterial isolates grown in Nutrient Agar medium for 24 hours were scratched at four opposite and equidistant ends in Petri plates of 9 cm in diameter with PDA (Potato Dextrose Agar), and fungal mycelium discs with 7 mm in diameter were deposited in the center of the plates.

Table 1. Identification of bacterial isolates from the Caatinga used in the research.

ISOLATES	PLANT	ORGAN/SOIL
FO5.7	*Agave sisalana*	Endophytic/leaves
S3.5	*Agave sisalana*	Rhizospheric soil
S3.6	*Agave sisalana*	Rhizospheric soil
P6.2	*Pilosocereus catingivola* subsp. *salvadorensis*	Rhizospheric soil
M7.1	*Melocactus zehntneri*	Rhizospheric soil
T6.2	*Tacinga werneri*	Rhizospheric soil
T7.2	*Tacinga werneri*	Rhizospheric soil

The control treatment consisted of plates with fungi, without the presence of bacterial isolates. The plates were stored in BOD at 28 ºC, and the evaluations consisted of measuring the opposite diameters of the growth of the fungal strains on the 4th and 8th day after the test. The mycelial growth inhibition of the fungi was estimated, using the mathematical calculation according to Nascimento *et al*. (2013), using the following formula: PIC = (witness diameter - treatment diameter) / (witness diameter) x 100.

Non-volatile Bacterial Metabolites with Antifungal Action

The isolates that promoted greater inhibition of mycelial growth were selected to determine the inhibitory effect of their non-volatile metabolites. The test was performed according to the methodology described by Marroni and Germani (2011), with adaptations. Bacterial isolates were subcultured in Petri plates containing culture medium Trypic Soy Agar (TSA). After 24 hours, the isolates were removed from the plates using an inoculating loop and inoculated into 250 mL Erlenmeyers containing nutrient broth (0.2 g yeast extract, 0.5 g sodium peptone, and 100 mL distilled water) sterile. The Erlenmeyers containing the isolates were placed in a Shaker incubator at 28 ºC and shaken at 120 rpm for a period of 72 hours. Then, the suspension was filtered through Millipore Sartorius Stedium® Minisart filters, with 0.20 µm. The extracted metabolites were stored in a freezer at -14°C until the test was set up. Fifteen ml of Dextrose Agar Potato (BDA) were poured into 9 cm diameter Petri plates, and after solidification, 5 mm diameter wells were made 2 cm away from the edge of each plate, where aliquots of 200 µl of the metabolites were deposited. At the opposite end, 7 mm discs of mycelia from fungal strains (Me 248, Me 249, Me 250, Fe 01, Fe 02 and GF 01) grown on BDA medium for 7 days were also placed 2 cm away from the edges of the plates. The control treatment consisted of the deposition of sterile distilled water in the wells. The plates were stored in BOD at 28 ºC, and the evaluations consisted of measuring the opposite diameters of the growth of the fungal strains on the 4th and 8th day after the test. The inhibition of mycelial growth of fungi was estimated using the mathematical calculation according to Nascimento *et al*. (2013), using the following formula: PIC = (control diameter - treatment diameter) / (control diameter) x 100.

Pathogenicity Test

Fungal isolates Fe 01 and Fe 02 (isolated from cowpea) were selected for the other tests with the aim of representing cowpea infection more closely and the possible reduction of infection by bacteria.

The inoculum was prepared according to the methodology described by Abawi and Pastor - Corrales (1990), where 100 g of rice moistened for 10 minutes in 100 mL of distilled water were autoclaved in Erlenmeyers with 250 mL capacity and subsequently inoculated with 10 discs of fungal mycelium of each strain grown in BDA type culture medium, for 7 days. The Erlenmeyers with inoculated rice were kept for 15 days in B.O.D. at 25 ºC with 12h photoperiod.

The soil used was previously sterilized in an autoclave (120 ºC for one hour) twice with an interval of 24 hours. After the soil had rested for 7 days and the fungus incubation time, nine grains of rice inoculated with the fungus were deposited in each planting pit in plastic containers with a capacity of 500 mL containing sterilized soil. The soil was moistened, and the containers were covered with plastic bags to promote a humid chamber in order to favor fungal growth. After 24 h of soil inoculation, seeds of cowpea cv. BRS Acauã were disinfected with 70% alcohol (30s), 1.5% hypochlorite (30s) and sterilized distilled water (1 min and 30s) and then dried at room temperature. When dried, the seeds

were sown in the pits with the presence of the inoculum. Two seeds per plastic container and three replicates per treatment were used, considering one plant per container and one experimental unit. The witness consisted in the deposition of disinfected seeds in pits without the inoculum.

The plants were kept in B.O.D. at 25 2ºC with 12h of photo period for seven days. The evaluation consisted of normal seedling count and incidence of symptoms of charcoal rot.

Protection of Cowpea Seeds with Bacterial Isolates *In Vivo*

For the experiment in greenhouse were selected the two fungal strains that were isolated from bean plants, Fe 01 and Fe 02. The inoculum was prepared and introduced into the soil according to the methodology described in the pathogenicity test.

Previously disinfected cowpea seeds (BRS Acauã) were immersed for 30 minutes in suspensions of bacterial isolates (M 7.1, T 6.2, T 7.2 and FO5.7) prepared in xanthan gum (0.1%), for better adherence of bacteria to seeds, and adjusted to optical densities of 0.3 and 0.5 (OD_{600}) and concentrations of 10^7 UFC/mL. The controls consisted of: a) seeds treated with xanthan gum in infested soil (control) and, b) seeds treated with fludioxonil based chemical, commercial product Maxim ®.

The severity of the disease in seedlings was evaluated according to the scale of notes adapted from Abawi and Pastor-Corrales (1990), according to the following notes: 0 = no visible symptoms; 3= from mild discoloration and absence of necrotic lesions up to a maximum of 10% of hypocotyl and root tissues with lesions; 5= approximately 25% of hypocotyl and root tissues have lesions, but tissues remain firm. There is little deterioration or damage to the root system; 7= approximately 50% of the hypocotyl and root tissues present lesions. The root system undergoes considerable decay and reduction. Fungal structures are visible; 9 = approximately 75% or more of the hypocotyl and root tissues show lesions. The root system undergoes advanced stages of decomposition and considerable reduction. There is extensive fungal growth; 10 = pre-emergence damping-of . With the data obtained, the Disease Severity Index (ISD) was calculated per cup/pot, by the adapted formula: ISD = (V/N*X)*100, where V = level of the observed scale, N = total number of plants evaluated and X = maximum level of the scale (McKinney, 1923).

In addition, the parameters of growth and development of seedlings were evaluated through the weight of Fresh and Dry Matter and, Length of Aerial Part and Root. For Fresh Matter analysis, the seedlings were weighed on precision scales and for Dry Matter, the seedlings were placed in Kraft paper bags and stored in an oven at 60ºC for 48h, for weighing on precision scales. The length of the seedlings was measured with the aid of a millimeter ruler.

The experiment was conducted in a period of 20 days from sowing. The experimental unit was represented by one plant per plastic container. In all, there were 25 treatments with 4 replications.

The isolates and their concentrations that showed better results were selected for evaluation simulating the field conditions, in pots of 1 L, with non-sterile soil and in open environment.

Protection of Cowpea Seeds with Bacterial Isolates *In Vivo* Simulating Field Conditions

Based on the results obtained in the previous experiment, bacterial isolates were selected at certain concentrations that showed greater potential to reduce the severity of charcoal rot, and that promoted better growth and development of seedlings for evaluation in conditions analogous to in field cultivation.

For this, cowpea seeds cv. BRS Acauã, previously disinfected, were immersed for 30 minutes in bacterial suspensions based on xanthan gum (0.1%) of the isolates selected and adjusted to OD_{600}.

The inoculum of the fungal strains Fe 01 and Fe 02 was prepared and introduced into the soil according to the methodology described in the pathogenicity test. However, for this trial, 1 L pots with natural (unsterilized) soil were used and placed in an open environment to simulate field cultivation.

The controls consisted of: a) seeds treated with xanthan gum in infested soil (control) and, b) seeds treated with a chemical product based on fludioxonil, commercial product Maxim ®.

Again, the severity of the disease in seedlings was evaluated according to the scale of notes adapted from Abawi and Pastor-Corrales (1990), as the following notes: 0 = no visible symptoms, 3= from mild discoloration and absence of necrotic lesions to a maximum of 10% of hypocotyl and root tissues with lesions, 5= approximately 25% of the tissues of the hypocotyl and the root have lesions, but the tissues remain firm.

There is little deterioration or damage to the root system, 7 = approximately 50% of the hypocotyl and root tissues present lesions. The root system undergoes considerable

decay and reduction. Fungal structures are visible, 9 = approximately 75% or more of the hypocotyl and root tissues have lesions. The root system undergoes advanced stages of decomposition and considerable reduction. There is extensive fungal growth, 10 = pre-emergence damping-off. With the data obtained, the Disease Severity Index (ISD) per plant was calculated by the adapted formula: ISD = (V/N*X)*100, where V = level of the observed scale, N = total number of plants evaluated and X = maximum level of the scale (McKinney, 1923).

The experiment was conducted in a period of 20 days from sowing, and the experimental unit was represented by one plant per cup.

Statistical Analysis

The in vitro experiments were conducted in a completely randomized design with three replicates each, with each replicate represented by a Petri dish.

The in vivo experiments consisted of four replicates, with each replicate consisting of one plant per pot in a completely randomized design.

The data collected from the experiments were analyzed using the ANOVA test. Post-analysis, Scott-Knott test was used to compare the means of the treatments with the help of the Sisvar program (version 5.6).

RESULTS

Direct Antagonism Test

The culture pairing experiment of the bacterial isolates and the six fungal strains of *M. phaseolina* showed that all isolates were able to inhibit between 36% and 100% the mycelial growth of all strains in the first four days of incubation (Figure 2). The most sensitive strain was Fe 01, which had its mycelial growth inhibited at least 92% by isolate *B. licheniformis* S3.5 and at most 100% by isolate *B. licheniformis* FO5.7 and the other isolates P6.2, T 7.2 and M 7.1 in the first four days of incubation (Figure 2A).

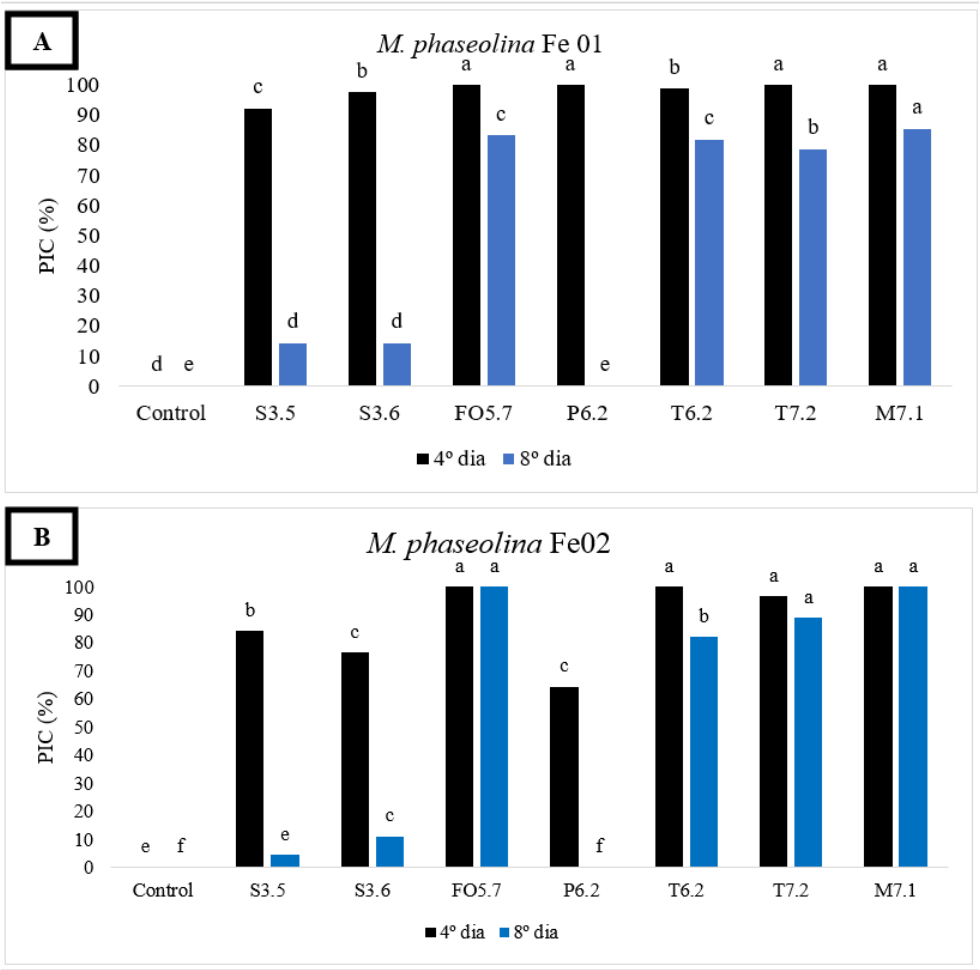

Figure 2 (A and B). Inhibition of *M. phaseolina* (Fe 01 and Fe 02) mycelial growth by bacterial isolates in dual culture assay. 1,2 Averages followed by the same letter did not differ according to the Scott-Knott test.

The results obtained at eight days of incubation showed that the bacterial isolate *Bacillus* sp. P6.2 did not provide inhibition of any of the fungal strains, and did not differ from the control. Similarly, *B. licheniformis* isolates S3.5 and S3.6 lost their efficiency in inhibiting the mycelial growth of. Me 248 and GF 01 strains after 8 days of incubation (Figure 2C and 2F). In contrast, the *B. licheniformis* isolate FO5.7 and the other isolates, M7.1, T6.2, and T7.2 were able to maintain inhibition of all strains at a minimum of 67% and a maximum of 91%, proving that they have the ability to control different strains of *M. phaseolina* for a longer period of time, and consequently have the potential for longer crop protection (Figure 2).

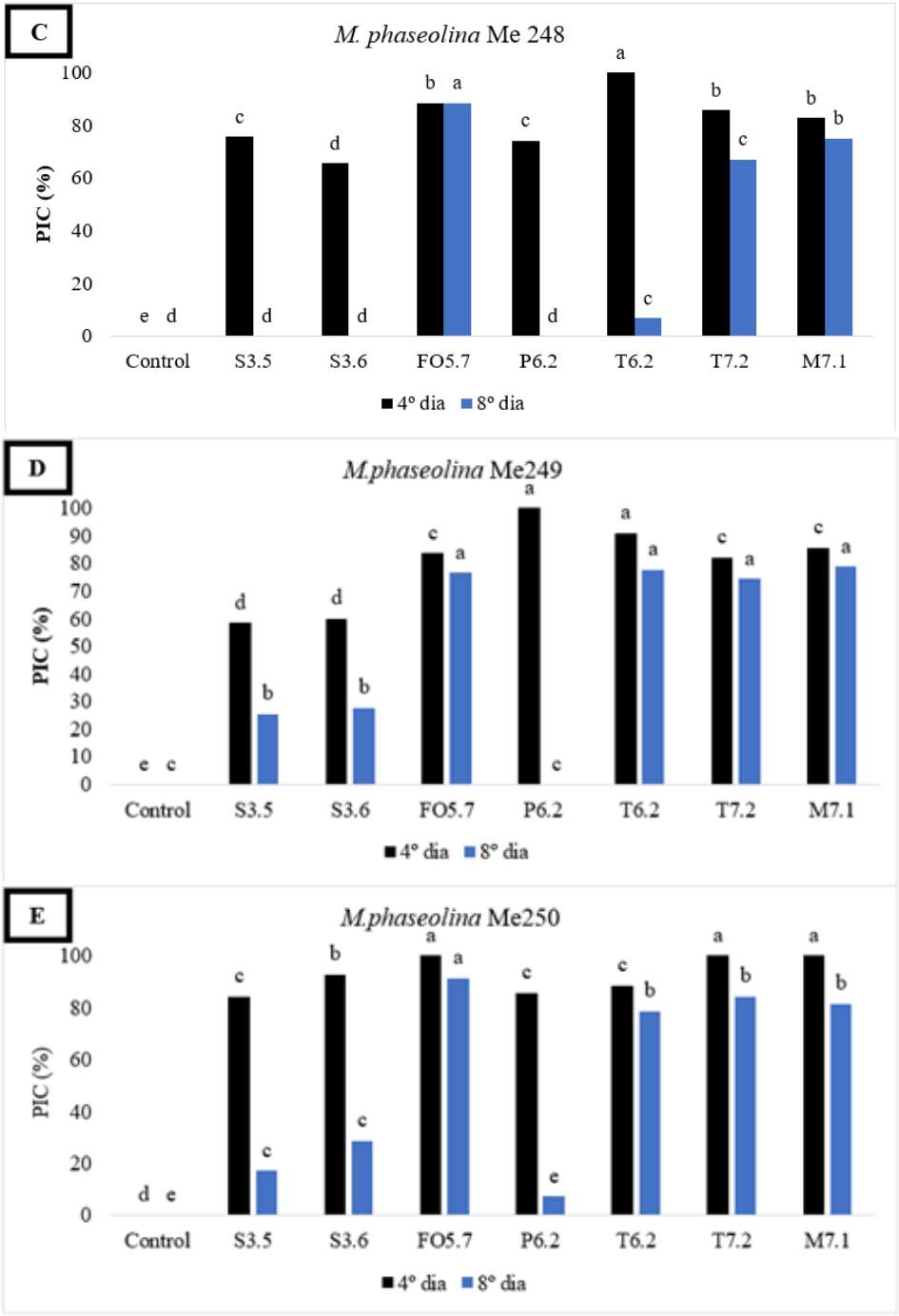

Figure 2 (C, D and E). Inhibition of *M. phaseolina* (Me 248, Me 249 and Me 250) mycelial growth by bacterial isolates in dual culture assay. 1,2 Averages followed by the same letter did not differ according to the Scott-Knott test.

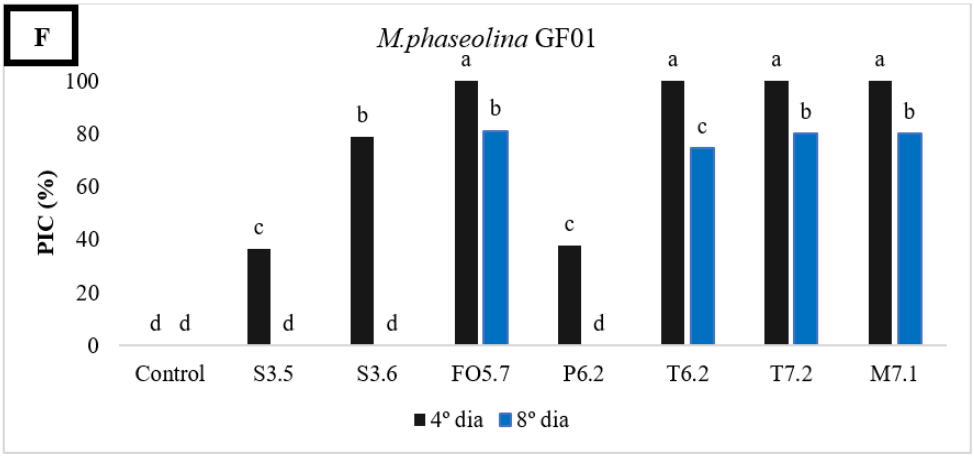

Figure 2 (F). Inhibition of *M. phaseolina* (GF01) mycelial growth by bacterial isolates in dual culture assay. 1,2 Averages followed by the same letter did not differ according to the Scott-Knott test.

Non-Volatile Bacterial Metabolites

Based on the results of the previous experiment, four isolates were selected for analysis of the inhibitory capacity of the non-volatile metabolites produced by them, they were *B. licheniformis* FO5.7 and isolates M7.1, T6.2, T7.2. In the first four days of incubation, all metabolites of bacterial isolates inhibited 27 to 50% of mycelial growth of fungi (Tables 2, 3 and 4). And, at eight days of incubation, the metabolites promoted an inhibition of 31 to 48% of the fungal growth of the strains, except the metabolite produced by the isolate *B. licheniformis* FO5.7, which did not maintain the growth inhibition of the strain Me 248 in this period, not differing from the control treatment (Table 3).

Pathogenicity Test

The pathogenicity test was positive, showing that the fungal strains (Fe 01 and Fe 02) have the ability to promote the symptoms of charcoal rot in cowpea when the inoculum is in the soil.

Protection of Cowpea Seeds with Bacterial Isolates *In Vivo*

The results presented in Table 5 show that bacterial isolates applied to seeds were able to reduce the severity of charcoal rot caused by both strains (Fe 01 and Fe 02) in cowpea seedlings.

Table 2. Mycelial growth of *M. phaseolina* strains Fe 01 and Fe 02 are confronted with non-volatile metabolites of bacterial isolates and the percentage of growth inhibition.

Treatments	Strain Fe 01				Strain Fe 02			
	4 days of incubation		8 days of incubation		4 days of incubation		8 days of incubation	
	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø Colony (cm)	PIC (%)
Control	8,37 b[1]	-	9,00 b	-	8,02 b	-	9,00 b	-
M7.1	5,71 a	31,8	5,85 a	35,0	5,72 a	28,7	5,85 a	35,0
T6.2	5,57 a	33,5	5,70 a	35,0	5,80 a	27,7	5,90 a	34,4
T7.2	5,72 a	31,7	5,82 a	35,3	5,78 a	27,9	5,92 a	34,2
FO5.7	5,03 a	40,0	5,48 a	39,1	5,53 a	31,1	5,98 a	33,6
CV (%) =	8,94		4,67		3,58		1,27	

[1]Averages followed by the same letter do not differ, according to the Scott-Knott test. Ø = diameter of the fungal colony (cm). PIC (%) = Percentage of mycelial growth inhibition.

Table 3. Mycelial growth of *M. phaseolina* strains Me 248 and Me 249 confronted with non-volatile metabolites of bacterial isolates and the percentage of growth inhibition.

Treatments	Strain Me 248				Strain Me 249			
	4 days of incubation		8 days of incubation		4 days of incubation		8 days of incubation	
	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)
Control	8,50 b[1]	-	9,00 b	-	9,00 b	-	9,00 b	-
M7.1	5,58 a	34,4	5,90 a	34,4	5,43 a	39,7	5,53 a	38,6
T6.2	5,43 a	36,1	5,90 a	34,4	5,77 a	35,9	5,82 a	35,3
T7.2	5,80 a	31,8	5,80 a	35,6	5,83 a	35,2	5,95 a	33,9
FO5.7	6,07 a	28,6	9,00 b	0,00	5,82 a	35,3	6,13 a	31,9
CV (%) =	6,70		2,93		4,55		3,85	

[1]Averages followed by the same letter do not differ, according to the Scott-Knott test. Ø = diameter of the fungal colony (cm). PIC (%) = Percentage of mycelial growth inhibition.

Table 4. Mycelial growth of *M. phaseolina* strains Me 250 and GF 01 confronted with non-volatile metabolites of bacterial isolates and the percentage of growth inhibition.

Treatments	Strain Me 250				Strain GF 01			
	4 days of incubation		9 days of incubation		4 days of incubation		9 days of incubation	
	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)	Ø colony (cm)	PIC (%)
Control	9,00 b[1]	-	9,00 b	-	9,00 b	-	9,00 b	-
M7.1	5,82 a	35,3	5,82 a	35,3	5,17 a	42,6	5,67 a	37,0
T6.2	5,40 a	40,0	5,72 a	36,4	4,42 a	50,9	4,63 a	48,6
T7.2	4,92 a	45,3	5,10 a	43,3	5,87 a	34,8	6,00 a	33,3
FO5.7	4,30 a	52,2	6,00 a	33,3	4,42 a	50,9	5,87 a	34,8
CV (%) =	14,41		7,58		14,36		11,24	

[1]Averages followed by the same letter do not differ, according to the Scott-Knott test. Ø = diameter of the fungal colony (cm). PIC (%) = Percentage of mycelial growth inhibition.

Table 5. Severity charcoal rot of the stem in seedlings of cowpea cv. BRS Acauã resulting from seeds previously treated with bacteria isolated from the Caatinga.

Treatments	[1]ISD (%)	
	Strain Fe 01	Strain Fe 02
Control	100,00 b	100,00 c
T1 (fludioxonil)	47,40 a	33,21 a
T2	36,15 a	64,65 b
T3	36,15 a	36,15 a
T4	36,15 a	75,80 c
T5	33,21 a	100,00 c
T6	78,75 b	62,50 b
T7	36,15 a	100,00 c
T8	100,00 b	78,75 c
T9	33,21 a	100,00 c
CV (%) =	30,81	25,72

[1]Calculate by the Disease Severity Index (ISD) per plant according to the formula developed by McKinney (1923) with adaptations, considering a scale of grades from 0 to 10. [2]To meet normality assumptions, data were transformed to arcsine$\sqrt{x}/100$. [3]Mediun followed by the same letter in the column do not differ significantly by Scott-Knott test. T1 = Treatment with the fludioxonil-based fungicide; T2= Trat. with M 7.1 (0.3); T3= Trat. with T 6.2 (0.3); T4= Trat. with T 7. 2 (0.3); T5= Treat with FO5.7 (0.3); T6= Treat with M 7.1 (0.5); T7= Treat with T 6.2 (0.5); T8= Treat with T 7.2 (0.5); T9= Treat with FO5.7 (0.5).

In the D.O. suspension of 0.3, all isolates of *Bacillus* reduced the severity promoted by the strain Fe 01, not differing from the fungicide treatment (T1), where the fungicide registered for seed treatment was used, Maxim®. Likewise, for the suspension of D.O. of 0.5, isolates T6.2 and *B. licheniformis* FO5.7 also differed from the control, reducing by 63.85% and 66.79%, respectively, the symptoms caused by *M. phaseolina* in cowpea seedlings (Table 5).

For strain Fe 02, only the isolate *Bacillus* sp. T 6.2 in the DC of 0.3 reduced the severity index of the charcoal rot to a level statistically similar to that promoted by the fungicide treatment (T1). Severity rates below 65% were also observed in treatments with the isolate M 7.1 at optical densities of 0.3 and 0.5, differing from the control (Table 5).

Observing Tables 6 and 7, it is understood that the effect of treatment with bacteria under seedling development was also positive. For aerial fresh matter (MFPA), all bacterial isolates applied at 0.3 O.D. were statistically similar to the fungicide treatment, for strain Fe 01. However, only isolates of *Bacillus* sp. M 7.1 and T 6.2 in O.D. of 0.3 were significantly similar to the fungicide treatment in the evaluation of aerial part dry matter (ASM), proving a greater accumulation of leaf mass in seedlings in soil infested with Fe 01 strain (Table 6).

For fresh matter (MFR) and root dry matter (MSR), *B. licheniformis* FO5.7, in both optical densities, was superior to all treatments, which explains the best results obtained in reducing the severity promoted by the same isolate, proving that this was able to protect the root of the seedlings of cowpea to the point of preventing the fungus *M. phaseolina* strain Fe 01 from interfering with mass accumulation (Tables 5 and 6).

Table 6. Fresh (MFPA/MFR) and dry (MSPA/MSR) matter of shoots and roots of cowpea seedlings cv. BRS Acauã submitted to seed treatment with Caatinga bacteria and cultivated in soil infested with the fungus *M. phaseolina*, strain Fe 01.

Treatments	Strain Fe 01			
	MFPA (g/plant)	MSPA (g/plant)	MFR (g/plant)	MSR (g/plant)
Witness	0,70 c	0,70 c	0,70 d	0,70 d
T1 (fludioxonil)	1,41 a	1,04 a	0,89 b	0,75 b
T2	1,45 a	1,08 a	0,88 b	0,74 c
T3	1,42 a	1,11 a	0,84 c	0,74 c
T4	1,40 a	0,99 b	0,87 b	0,71 d
T5	1,45 a	0,99 b	0,99 a	0,76 a
T6	0,70 c	0,70 c	0,70 d	0,70 d
T7	1,36 b	0,92 b	0,82 c	0,73 c
T8	0,70 c	0,70 c	0,70 d	0,70 d
T9	1,26 b	0,90 b	0,84 c	0,74 c
CV (%) =	5,09	7,21	3,52	0,90

[1]Averages followed by the same letter in the column do not differ significantly by Scott-Knott test. [2]For the purpose of best meeting the analysis of variance (ANOVA), data were transformed into $\sqrt{x}$+0.5. T1 = Treatment with the fludioxonil-based fungicide; T2= Trat. with M 7.1 (0.3); T3= Trat. with T 6.2 (0.3); T4= Trat. with T 7. 2 (0.3); T5= Treat. with FO5.7 (0.3); T6= Treat. with M 7.1 (0.5); T7= Treat. with T 6.2 (0.5); T8= Treat. with T 7.2 (0.5); T9= Treat. with FO5.7 (0.5).

For seedlings sown in soil inoculated with strain Fe 02, as well as in the analysis of disease severity, the treatments that differed statistically from the others and stood out, maintaining means significantly similar to the means obtained by the control and the fungicide treatment in the analyzes of MFPA, MSPA, MFR and MSR, were the isolates *Bacillus* sp. T 6.2 and M 7.1, at optical densities of 0.3 and 0.5, respectively (Table 7).

Significant differences were also observed in the analyses of the variables of aerial part length (AAC) and root length (RAC), where the bacteria M 7.1, T 7.2 and *B. licheniformis* FO5.7 at an optical density of 0.3, and T 6.2 and *B. licheniformis* FO5.7 at an optical density of 0.5, promoted statistically higher growth of the aerial part than the controls for the seedlings in soil infested with strain Fe 01 (Table 8). For the same strain, all isolates at

the optical density of 0.3 and again, bacteria T 6.2 and *B. licheniformis* FO5.7 at the optical density of 0.5, provided root growth similar to fungicide (T1) (Table 8).

For seedlings sown in soil infested with strain Fe 02, only isolate T 6.2 to D.O. of 0.3 (T3) was higher than fungicide in the shoot growth variable. Similarly, for root length, T3 did not differ from T1, standing out from other treatments, and promoting root growth similar to fungicide treatment (Table 8).

Table 7. Fresh (MFPA/MFR) and dry (MSPA/MSR) matter of aerial part and roots of cowpea seedlings cv. BRS Acauã submitted to seed treatment with Caatinga bacteria and cultivated in soil infested with the fungus *M. phaseolina*, strain Fe 02.

Treatments	Strain Fe 02			
	MFPA (g/plant)	MSPA (g/plan)	MFR (g/plant)	MSR (g/plant)
Control	0,70 d	0,70 c	0,70 d	0,70 c
T1 (fludioxonil)	1,49 a	0,91 a	0,99 a	0,76 a
T2	0,85 c	0,80 b	0,78 c	0,72 b
T3	1,51 a	0,94 a	0,96 a	0,76 a
T4	0,70 d	0,70 c	0,70 d	0,70 c
T5	0,70 d	0,70 c	0,70 d	0,70 c
T6	1,37 b	0,80 b	0,83 b	0,75 a
T7	0,70 d	0,70 c	0,70 d	0,70 c
T8	0,70 d	0,70 c	0,70 d	0,70 c
T9	0,70 d	0,70 c	0,70 d	0,70 c
CV (%) =	5,82	4,28	3,83	1,19

[1]Averages followed by the same letter in the column do not differ significantly by Scott-Knott test. [2]For the purpose of best meeting the analysis of variance (ANOVA), data were transformed into $\sqrt{x}$+0.5. T1 = Treatment with the fludioxonil-based fungicide; T2= Trat. with M 7.1 (0.3); T3= Trat. with T 6.2 (0.3); T4= Trat. with T 7. 2 (0.3); T5= Treat with FO5.7 (0.3); T6= Treat with M 7.1 (0.5); T7= Treat with T 6.2 (0.5); T8= Treat with T 7.2 (0.5); T9= Treat with FO5.7 (0.5).

Table 8. Aboveground length (AAC) and root length (RAC) of cowpea seedlings submitted to seed treatment with bacteria from Caatinga and grown in soil infested with different strains of *M. phaseolina*.

Treatments	Strain Fe 01		Strain Fe 02	
	AAC (cm)	RAC (cm)	AAC (cm)	RAC (cm)
Control	0,00 c	0,00 b	0,00 d	0,00 d
T1 (fludioxonil)	11,96 b	8,35 a	18,23 b	10,78 a
T2	18,10 a	8,91 a	6,50 c	3,50 c
T3	15,60 b	10,65 a	24,12 a	11,93 a
T4	17,25 a	9,65 a	0,00 d	0,00 d
T5	20,48 a	10,00 a	0,00 d	0,00 d
T6	0,00 c	0,00 b	14,73 b	7,20 b
T7	18,98 a	9,25 a	0,00 d	0,00 d
T8	0,00 c	0,00 b	0,00 d	0,00 d
T9	18,37 a	8,30 a	0,00 d	0,00 d
CV (%) =	28,75	26,92	33,17	33,45

[1]Averages followed by the same letter in the column do not differ significantly by Scott-Knott test. T1 = Treatment with the fludioxonil-based fungicide; T2= Trat. with M 7.1 (0.3); T3= Trat. with T 6.2 (0.3); T4= Trat. with T 7. 2 (0.3); T5= Treat with FO5.7 (0.3); T6= Treat with M 7.1 (0.5); T7= Treat with T 6.2 (0.5); T8= Treat with T 7.2 (0.5); T9= Treat with FO5.7 (0.5).

Protection of Cowpea Seeds with Bacterial Isolates *In Vivo* Simulating Field Conditions

Based on the results of the previous experiment, the isolates that showed greater efficiency in reducing the severity of charcoal rot and that promoted the growth and development of cowpea seedlings were selected, for the evaluation of their protective capacity under conditions similar to those of field situation. For seedlings grown in soil infested with strain Fe 01, the isolate of *B. licheniformis* FO5.7, in both optical densities: 0.3 (5.68 x 10^7 UFC/mL) and 0.5 (4.54 x 10^7 UFC/mL), significantly reduced the severity of the disease, with a difference of 66% and 63% respectively, in comparison to the witness. However, these also differed from seedlings from seeds treated with fungicide (Figure 3).

In a opposite way, seedlings sown in soil containing the strain Fe 02 and seeds previously treated with isolates T 6.2 and M 7.1, at optical densities of 0.3 (5.92 x 10^7 UFC/mL) and 0.5 (6.12 x 10^7 UFC/mL), respectively, did not differ from the inoculated control and fungicide, with severity rates above 60% (Figure 4).

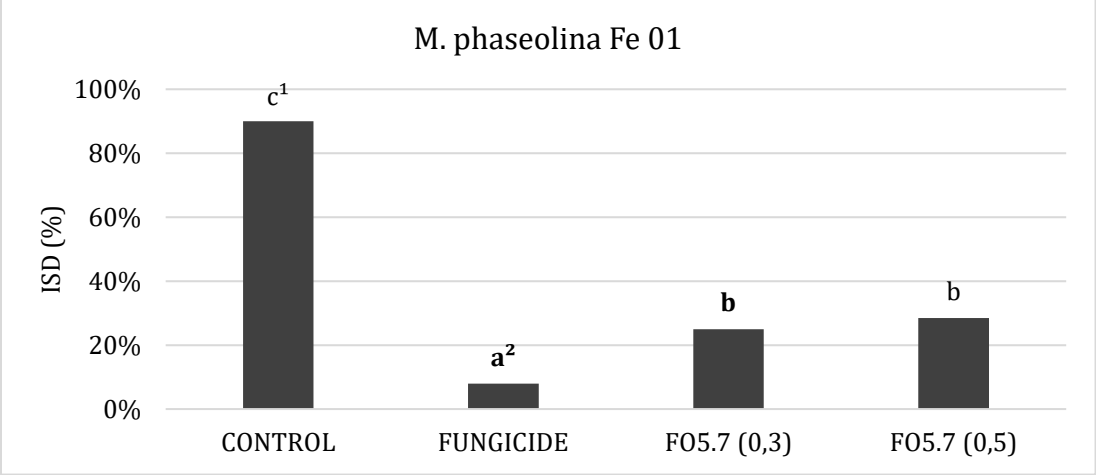

Figure 3. Severity index of the charcoal rot disease (strain Fe 01) in cowpea with seeds previously treated with *Bacillus licheniformis* isolate and sown in natural soil. [1]Average Severity index of the charcoal rot disease (strain Fe 01) in cowpea with seeds previously treated with *Bacillus licheniformis* isolate and sown in natural soil. [1]Mediums followed by the same letter in the column did not differ significantly by Scott-Knott test. [2]For the purpose of better meeting the analysis of variance (ANOVA), data were transformed into arcsine$\sqrt{x}$.

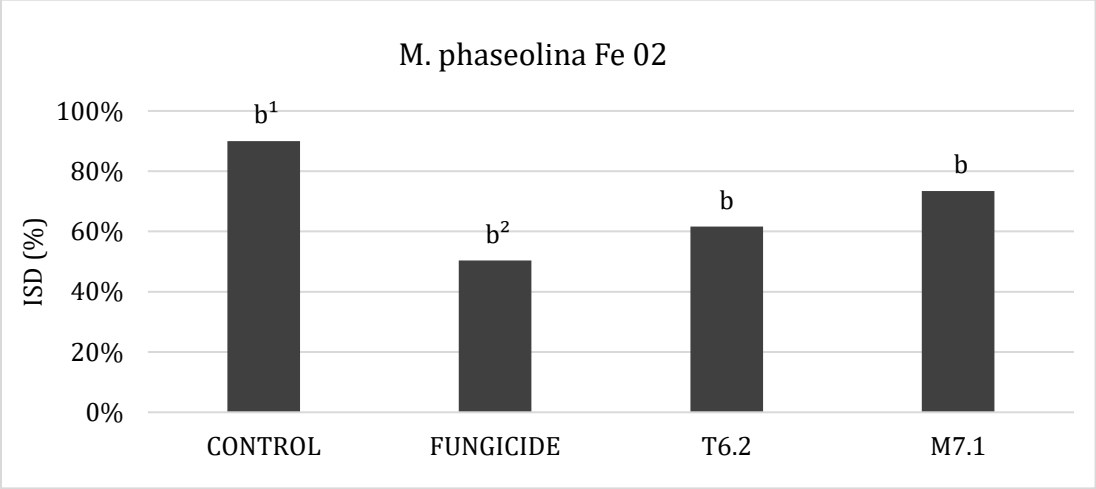

Figure 4. Severity index of cahrcoal rot disease (strain Fe 02) in cowpea beans with seeds previously treated with bacterial isolates from Caatinga and sown in natural soil. [1]Mediums followed by the same letter in the column did not differ significantly by Scott-Knott test. [2]For the purpose of better meeting the analysis of variance (ANOVA), data were transformed into arcsine$\sqrt{x}$.

DISCUSSION

Direct Antagonism Test

The effect of direct antagonism of bacteria of the genus *Bacillus* on different strains of *M. phaseolina* observed in the results can be attributed to several mechanisms of action such as the production of antimicrobial substances and competition for nutrients and space, which have been shown to inhibit the growth of several pathogens and consequently reduce the incidence and severity of diseases in plants (Chen *et al*., 2020).

Corroborating the present study, among 37 *Bacillus* spp. isolates, four were efficient in reducing the mycelial growth of *M. phaseolina* from common beans by 62.5%-85% (Bojórquez-Armenta *et al*., 2021).

Although the present study does not aim to investigate the modes of action of antagonistic bacteria, it can be stated that they are capable of reducing the mycelial growth of *M. phaseolina* (Figure 1). The antimicrobial capacity of the species *Bacillus licheniformis*, for example, may be associated with the potential for production and secretion of lytic enzymes, such as chitinase, proteases, amylases, pectinases and cellulases, which act in the degradation of the fungal cell wall (Nascimento *et al*., 2025; Nawaz *et al*., 2018).

Among the substances with antimicrobial activity produced by bacteria of the genus *Bacillus* and widely studied are bacteriocins, peptides synthesized ribosomally, and lipopeptides synthesized by enzymatic complexes and classified into three families: iturines, surfactants and phenergans (Fira *et al*., 2018; Lopes *et al*., 2018). The efficacy of iturin A and surfactin produced by *Bacillus subtilis* strain IAB/BS03 against the phytopathogenic fungi *Botrytis cinerea* (gray mold) in tomato and *Bremia lactucae* (mildew) in lettuce was proven in the work of Hinarejos *et al*. (2016).

The loss of efficiency of the isolates after the eight-day period can be attributed to low production of antimicrobial substances or competitive insufficiency. However, they may be efficient in inducing the expression of plant defense genes through the synthesis of molecules that act as elicitors of the systemic induced cognition process, another mechanism of action of bacteria of the genus *Bacillus* proven in several studies (Rashid *et al*., 2017), which can be evaluated by applying suspensions of the bacteria to plants and analyzed through the activity of pathogenesis-related proteins (Lorenzetti *et al*., 2018).

Non-Volatile Bacterial Metabolites

Observing the results of the previous test and comparing them with those obtained in the experiment with the non-volatile metabolites is notorious that the levels of inhibition are higher when bacteria and not their metabolites are used, this can be explained by the fact that when alive and under favorable conditions, they continue to produce the metabolites. Low levels of inhibition can be attributed to the low concentration of antimicrobial compounds present in the filtrates of bacterial cultures, which can be elevated through different cultivation techniques that optimize the production of microbial metabolites (Kaspar *et al*., 2019).

The loss of efficiency of the metabolite of isolate *B. licheniformis* FO5.7 after 8 days and the stability of its inhibitory capacity in the previous test can be explained by the fact that this isolate does not produce antimicrobial compounds capable of inhibiting strain Me 248, but that can act in the competition for space and nutrients.

Some secondary metabolites produced by bacteria of the *Bacillus* genus can interfere with fungal growth by binding to enzymes responsible for fungal development, preventing the assembly of target proteins that determine the virulence of the pathogen (Dhanabalan *et al*., 2024).

The results of this experiment corroborate those of Marroni and Germani (2011), who used 19 isolates of *Bacillus* sp. obtained from the rhizosphere and castor bean roots (*Ricinus communis* L.) cultivated and wild to evaluate in vitro antagonism to the fungus *M. phaseolina*, and observed that six bacteria produced non-volatile metabolites that inhibited fungus growth by more than 15%, whereas in the present study, bacteria of the same genus were able to produce metabolites that largely inhibited more than 30% of the mycelial growth of six strains of *M. phaseolina*, representing twice the efficiency. Similarly, Vieira *et al*. (2017) tested the non-volatile metabolite of *B. subtilis* isolate BSV-05 by agar diffusion method against *M. phaseolina*, *Rhizoctonia solani* Kuhn, *Fusarium solani* f. sp. *phaseoli* and *Fusarium oxysporum* f. sp. *phaseoli*. In this work, the metabolite of BSV-05 isolate inhibited 100% mycelial growth of *R. solani*, 80.26% growth of *M. phaseolina*, 45.31% and 47.80% growth of *F. solani* f. sp. *phaseoli* and *F. oxysporum* f. sp. *phaseoli*, respectively.

Protection of Cowpea Seeds with Bacterial Isolates *In* Vivo

Observing the promising result of isolate *B. licheniformis*

FO5.7, which provided the lowest rates of disease severity, it is possible that this has produced the enzyme chitinase, responsible for degrading the cell wall of fungi, as demonstrated in a study conducted by Gomaa (2012), which using chitinases produced by isolates of *B. licheniformis* and *B. thuringiensis* promoted increased germination rates of soybean seeds infected with various storage fungi. In addition to chitinase, another enzyme produced by *B. licheniformis* is β-1,3-glucanase, also capable of promoting the lysis of fungal cells by degradation of β-1,3-glucan present in the cell walls of them, causing hyphae deformation and directly interfering with the growth and development of the fungus (Won *et al*., 2018).

Several studies prove the control potential exerted by bacteria of the genus *Bacillus*, corroborating with the results obtained in this study, as shown in the work of Hashem *et al*. (2017), where Mungo bean seeds [*Vigna radiata* (L.) Wilczek] treated with endophytic isolate of *B. subtilis* (3.6 x 10^9 CFU/100g of seeds) sown in soil previously infested with *M. phaseolina* significantly reduced the incidence and severity index of the disease, in addition to increasing the survival rate of plants by more than 80%, while inducing the production of plant hormones and maximizing the absorption of macro and micronutrients by plants. Similarly, isolated from *B. amyloliquefaciens* (B14) reduced the incidence by 62% and delayed the appearance of root rot symptoms in black bean seeds sown in inoculated soil (Sabaté *et al*., 2017).

The difference of the results between the strains (Fe 01 and Fe 02), where most of the bacterial isolates were efficient in reducing severity for the strain Fe 01, but only T2, T3 and T6 promoted a decrease in the severity index of the disease of the strain Fe 02, can be explained by genetic variability between strains, which may present morphological, metabolic and pathogenic differences (Almeida *et al*., 2014), directly or indirectly interfering with sensitivity to bacteria.

The significant increase in the growth of the aerial part and root promoted by the bacteria may be associated with auxin production, phosphate solubilization, and biofilm formation, as was attested to in the study by Rangel-Montoya *et al*. (2022), where two isolates of *B. amyloliquefaciens* promoted a 23.1% increase in the growth of bean roots.

Similar results were attested in work developed by Sukkasem *et al*. (2018), where *Arabidopsis* seedlings with roots inoculated with *B. licheniformis* CH102 showed increased root hairs and increment in seedling fresh matter.

Bacterial species used as biocontrol agents, especially those of the genus *Bacillus*, have the ability to produce indoleacetic acid (AIA), auxin responsible for promoting root growth and expressive increase in the number of root hairs (Fernandes *et al*., 2019).

Protection of Cowpea Seeds with Bacterial Isolates *In Vivo* Simulating Field Conditions

By comparing these results with those obtained in the previous trial, it is possible to attest to the biocontrol capacity that the isolate of *B. licheniformis* FO5.7 has against charcoal rot caused by strain Fe 01, since it reduced the severity of the disease in sterile and non-sterile soil.

The efficacy of the species *B. licheniformis* as a biocontrol agent of plant fungal diseases is already known, as was observed in the study developed by Nawaz *et al*. (2018) where the strain OE-04 of *B. licheniformis* produced biosurfactant that applied to cotton plants resulted in a reduction of approximately 50% of Anthracnose caused by *Colletotrichum gossypii*.

In addition to the high degree of virulence of this strain (Fe 02), it should be considered the fact that the natural soil (not sterilized) is habitat for several microorganisms, phytopathogenic or not, which can lead to competition for space and nutrients, therefore, biocontrol agents must have a high competitive capacity to establish themselves in the soil and exercise their control potential, factors that may have influenced the performance of isolates T 6.2 and M 7.1.

Regarding the performance of the bacteria being better in vitro than in vivo conditions, it can be inferred that factors such as temperature, wind speed, humidity and nutrient availability may have impacted the control capacity of these microorganisms. In this context, it would be ideal to conduct further studies on these isolates under different conditions, and to investigate which mechanisms they use are most efficient *against M. phaseolina*.

CONCLUSIONS

This study suggests that *Bacillus licheniformis* FO5.7 may be a potential biocontrol agent for field application against *M. phaseolina*, warranting further testing under different environmental conditions, with more applications at different stages of plant development and

at different concentrations, since it was observed that the number of bacterial cells applied may interfere with the effectiveness of the control.

The same should be considered for other *Bacillus* sp. strains, such as M 7.1 and T 6.2, since these were efficient under controlled conditions, indicating that they may have different mechanisms of action, but that they can serve as future biocontrol agents.

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