Bioagro 37(1): 53-66. 2025 doi: http://www.doi.org/10.51372/bioagro371.5

DETECTION OF ENZYMATIC COMPOUNDS AND ANTAGONISM OF BACTERIA SPECIES ON DEVELOPMENT OF *Macrophomina phaseolina*

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ABSTRACT

Macrophomina phaseolina (Tassi) Goid is a pathogen that causes damping off, stem rot and root rot disease in a broad host range. The method of biological control using bacteria of the genus *Bacillus* stands out as viable and effective in combating plant diseases. The aim of the present study was detecting the presence of enzymatic compounds produced by the *Bacillus* isolates and evaluating the *in vitro* antagonistic activity of the bacterial isolates against *M. phaseolina*, using the methods of paired culture, pathogen culture on antagonist culture, thermostable and volatile metabolites. The experiments were carried out at the Phytopathology Laboratory, Department of Technology and Social Science, *Campus* III – UNEB, in Juazeiro-BA. Of the nine *Bacillus* isolates provided by the LBM, all were molecularly identified, except for isolate B7. The fungus *M. phaseolina* was acquired from the Dept. of Micologia at Federal University of Pernambuco. The experiment was in a completely randomized design for the enzymatic test using three replicates, and for the antagonism studies a 4x5 factorial arrangement was used (four isolates and four methods of antagonistic activity and the control treatment) with five replicates. Data were subjected to analysis of variance and Scott-Knott test at 5% probability. The enzymes showed the rates: proteases (100 %), cellulases (88%), pectinases (77 %), and amylases (66 %), suggesting that the isolates could be promising in the biological control of pathogens. In the selection of isolates with greater antagonistic activity, the method of culturing the pathogen on the culture, proved to be the most efficient in inhibiting the growth of *M. phaseolina*.
Additional keywords: Biological control, hydrolytic enzymes, soil fungi

RESUMEN

Detección de compuestos enzimáticos y antagonismo de especies bacterianas en el desarrollo de *Macrophomina Phaseolina*
Macrophomina phaseolina (Tassi) Goid es un patógeno que causa enfermedades como el marchitamiento, la pudrición del tallo y de la raíz, en una amplia gama de huéspedes. El método de control biológico utilizando bacterias del género *Bacillus* se destaca como eficaz para combatir enfermedades en cultivos vegetales. El objetivo del estudio fue detectar la presencia de compuestos enzimáticos producidos por los aislados de *Bacillus* y evaluar la actividad antagonista *in vitro* contra *M. phaseolina*, utilizando los métodos de cultivo pareado de patógenos sobre cultivo antagonista, termoestable y metabolitos volátiles. Los experimentos fueron realizados en el Laboratorio de Fitopatología, Departamento Tecnología y Ciencias Sociales, UNEB, en Juazeiro-BA. De los nueve aislados de *Bacillus*, todos fueron identificados molecularmente, excepto el B7. El hongo *M. phaseolina* fue adquirido en el Departamento de Micología de la Universidad Federal de Pernambuco. El experimento fue un diseño aleatorio para la prueba enzimática, utilizando tres repeticiones y para los estudios de antagonismo se utilizó un arreglo factorial 4x5, cuatro aislados y cuatro métodos de actividad antagonista y el tratamiento control, con cinco repeticiones. Los datos se sometieron a análisis de varianza y prueba de Scott-Knott al 5% de probabilidad. Las enzimas mostraron las tasas: proteasas (100 %), celulasas (88 %), pectinasas (77 %) y amilasas (66 %), lo que sugirió que los aislados podrían ser prometedores para el control biológico de patógenos. En la selección de aislados con mayor actividad antagonista, el método de cultivo del patógeno sobre el cultivo, resultó ser el más eficaz para inhibir el crecimiento de *M. phaseolina*.
Palabras clave adicionales: Control biológico, enzimas hidrolíticas, hongos del suelo

Associated Editor: Prof. María Elena Sanabria Chópite

INTRODUCTION

Macrophominaphaseolina (Tassi) Goidanichis an Ascomycota fungus from the Botryosphaeriaceae family (Mycobank, 2020). It is an important pathogen that inhabits the soil,

Received: May 27, 2024 Accepted: November 8, 2024
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distinguished by being polyphagous, cosmopolitan, with high pathogenic variability and capacity for survival in the soil under adverse conditions. The fungus causes diseases such as stem and root rot, charcoal rot and seedling rust, and its growth is favored by high temperatures and low soil moisture, being spread by infected seeds (Gomes et al., 2008).

The control strategy for *M. phaseolina* is complex because it is a pathogen with great adaptability to a wide range of hosts and environmental conditions, and because it survives in the soil and in plant residues for up to 15 years through microsclerotia, which are the resistance structures of the fungus (Marquez et al., 2021).

As it is a soil fungus, the appropriate control measure is the combination of more than one technique, of which the most used are solarization, crop rotation, biological control, chemical control, and the use of more resistant cultivars (Bedendo, 2018; Nascimento et al., 2018).

Aiming to reduce the negative effects of chemical pesticides on food, the environment and pathogen resistance, efforts are being made to replace them with more sustainable methods to combat soil pathogens (Viteri and Linares, 2017). Biological control for this type of soil fungus is a method considered safe, as it does not leave residues for humans and the environment, and efficient, as it presents satisfactory results in controlling the pathogen (Kannojia et al., 2019).

In biological control, bacteria of the genus *Bacillus* stand out due to their aggressiveness in colonizing the cellular environment. This characteristic gives them a range of mechanisms to antagonize pathogens, mainly antibiosis, competition, production of hydrocyanic acid, siderophores, fluorescent pigments and antifungal compounds (Gouda et al., 2018). The successful colonization of biological control agents, combined with antagonistic mechanisms of action in plants, is essential for the effectiveness of biocontrol (Hakim et al., 2021).

In biological control, *in vitro* antagonism studies can select bacteria of great economic interest, with fungicidal or insecticidal action, which present antagonistic and efficient bioprotective properties. Therefore, research has focused on identifying new secondary metabolites produced by bacteria with the purpose of isolating bioactive substances capable of controlling phytopathogens (Khan et al., 2021). Likewise, volatiles are secondary compounds of interest in biological control studies, as they have the ability to diffuse into the environment and act in locations other than the site of infection. In addition to antibiotics, enzymes produced by bacteria are also powerful tools of biotechnological interest that may be promising in controlling fungal diseases (Arrarte et al., 2017).

Ground in more sustainable agriculture to meet increasingly demanding consumers in the search for products that have been obtained with low use of chemical pesticides and in the preservation of the environment. Therefore, the study aimed to evaluate bacterial strains that have an antagonistic effect on pathogenic fungi, as well as chemical compounds released by them that cause this effect.

MATERIALS AND METHODS

The experiments were conducted at the phytopathology laboratory, in partnership with the Microbial Biotechnology Laboratory (LBM), Department of Technology and Social Sciences – DTCS of Campus III – UNEB, in Juazeiro-BA. Of the nine *Bacillus* isolates provided by LBM, all were molecularly identified, except isolate B7. The fungus *M. phaseolina* was purchased from the Dept. of Mycology at UFPE, and its viability was proven by pathogenicity tests on melon trees using the pathogen infestation method on commercial substrate (Table 1).

Enzymatic compounds produced by bacterial isolates. All processing to evaluate enzymes and modes of antagonism took place in the laminar flow chamber of the Phytopathology Laboratory. In the first part of the bioassay, the enzymatic index of nine bacterial isolates with the ability to synthesize protease, cellulase, pectinase and amylase was evaluated.

A specific culture medium was used for each enzyme studied. In the production of amylase, the minimal medium of Pontecorvo (MM) (Pontecorvo et al.,1953) supplemented as soluble starch ($g \cdot L^{-1}$: 10.0 peptone, 5.0 yeast extract, 5.0 NaCl and 2.0 starch, 15.0 agar and then adjusted to pH 7.0). Bacterial colonies were grown overnight in the center of the plate and incubated at 30 °C for 2 days. Then, drops of 1 % Lugol's solution were added to the circumference of the plates, and the solution remained at rest to act for

30 minutes, and then the excess solution was discarded (King, 1965). The formation of a translucent halo indicated the presence of amylase production.

Two bioassays were carried out to control *M. phaseolina in vitro*. The first was the evaluation of the enzymatic compounds released by the bacterial strains, and the second was about the means of action of the antagonistic activity of *Bacillus* against the fungus.

Table1. Isolates obtained from the microbial biotechnology laboratory.

Isolates	Plant organ	Fragment (pb)	Species (BLAST-NCBI)	Acess number	Identity%	Reference
FO4.2	Leaf	1418	*Bacillus* sp.	KR094750	100	*Prestiamegaterium* NBRC 15308 ATCC
FO7.4	Leaf	1418	*Bacillus* sp.	KR094751	100	*P. Megaterium NBRC 15308 ATCC*
FO2.6	Leaf	1384	*Bacillus* sp.	KR094735	100	*B. pumilus* DMKUB39
B7	Not identified	_	_	_	_	_
FO5.5	Leaf	1405	*Bacillus* sp.	KR094734	99	*B. subtilis* 3DP2-2
S3.5	Soil	1405	*Bacillus* sp.	Kr094742	99	*B. licheniformis* TAD17
B1.9	Buzz	1418	*Bacillus* sp.	KR094745	100	*P. megaterium* NBRC 15308
FE3.7	Stalk	1406	*Bacillus* sp.	KR094736	99	*B. licheniformis* TAD17
B4.5	Buzz	1406	*Bacillus*sp.	KR094761	99	*B. licheniformis*

Regarding cellulase detection, Pontecorvo minimal medium (MM) supplemented with 1 % carboxymethylcellulase (CMC) was used (1 L: 1 g of glucose, 2.5 g of yeast extract, 1 % CMC and 15 g of agar). For this, the process was similar to that used in the method da amilase, differing only from the solution dripped into the culture medium. To detect cellulase production, the plates were dripped with 1 % Congo red solution for 30 minutes. After draining the solution, the plates received 1 M NaCl for 60 minutes (King, 1965). The appearance of a translucent halo around the bacterial colony indicated the presence of cellulase synthesis.

Regarding the detection of pectinase, agar medium supplemented with pectin (PSAM) was used (1 L: 1 g of $NaNO_3$, 1 g of KCl, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of extract of yeast, 10 g of pectin, 15 g of agar and then the medium was adjusted to pH 7.0), and as well as the amylase assessment, 1 % Lugol was dripped (King, 1965). Bacteria that tested positive for pectinase production displayed a clear halo around the colony.

For the protease, nutritional agar medium added with 1 % skimmed milk powder (skimmed milk agar) was used. To prepare the medium, both solutions, i.e., nutrient agar and skimmed milk, were prepared and autoclaved separately. These two were mixed before pouring into the plates. Unlike the other methods, this one did not use any solution to detect the enzyme. The development of a clear halo zone around the bacterial colonies indicated the presence of the protease.

The clear halo zones produced by bacteria for hydrolytic enzymes were measured using the method of Hankin and Anagnostakis (1975), through the relationship between the average diameter of the degradation halo and the average diameter of the colony, expressed as enzymatic index (IE). IE = colony diameter (cm) + halo zone diameter (cm)/colony diameter (cm). All variables were subjected to analysis of variance (ANOVA) using the software Sisvar (version 5.6) assumptions, such as normality of residuals and homoscedasticity of variance, using the Shapiro-

Wilk and Bartlett tests, respectively. The means were contrasted using Tukey tests. A significance level of $p \leq 0.05$ was adopted to detect differences between treatments.

Antagonism of *Bacillus* spp. against *M. phaseolina*. The second bioassay evaluated the best method for controlling *M. phaseolina in vitro* using paired culture techniques, pathogen culture on antagonist culture, thermostable metabolites and volatile metabolites. In the paired culture technique, a disc of Potato Dextrose Agar (PDA), measuring 7 mm in diameter, colonized by the fungus, was placed 1.5 cm away from the left edge of a Petri dish measuring 9 cm in diameter, containing 20 ml of PDA medium and, with the aid of a platinum loop, a streak of *B. subtilis* was made on the right side, 1.5 cm away from the edge of the plate. A second method consisted of cultivating the pathogen on the antagonist culture. For this, a 7 mm disc of PDA, colonized by the fungus, was placed in the center of the Petri dish, previously streaked with the bacteria. In a third method, the inhibition of the growth of *M. phaseolina* was evaluated through the production of thermostable metabolites by *Bacillus* spp. To this end, Petri dishes, containing 20 ml of PDA, were streaked with *Bacillus* spp. and incubated in a germination chamber for 48 hours. After this period, the plates were autoclaved at 121 ºC for 15 minutes at 1 atm and then placed in a laminar flow chamber to solidify the medium in an aseptic environment. After the medium solidified, PDA discs colonized by *M. phaseolina* were placed in the center of the plates for subsequent growth assessment.

In the fourth method, antagonistic activity was assessed through the production of volatile metabolites. This method consisted of superimposing bases of Petri dishes, containing PDA medium, inoculated with discs of BDA medium, measuring 7 mm in diameter, colonized by *M. phaseolina*, on bases of Petri dishes with *Bacillus*spp in another base of plates containing half NYDA (NutrientYeast Glucose Agar). As a control treatment, discs of BDA medium, colonized by *M. phaseolina*isolates, were added to the center of Petri dishes containing BDA medium.

The evaluation consisted of measuring, with the aid of a caliper, the average diameter of *M. phaseolina* colonies, eight days after inoculations, a period in which one of the isolates, in the control treatment, reached the edges of the plates. Soon after, the percentage of inhibition (% I) of *M. phaseolina* from the treatments in relation to the control was calculated, using the formula: $\%I = (C-T)/C \times 100$. Where %I: percentage of inhibition, C: diameter average mycelial growth of the control, and T: average diameter of mycelial growth of the treatment. The experimental design used was completely randomized (DIC), in a 4x5 factorial arrangement (four isolates and four methods of antagonistic activity and the control treatment) with five replications. All variables were subjected to the assumptions of analysis of variance (ANOVA). However, the parameters of inhibition percentage, mycelial growth, cellulase and pectinase did not meet the ANOVA assumptions so they were analyzed by the Generalized Linear Model (GLM) with quasipoisson model and probit link. The means were contrasted using Tukey tests. A significance level of $p \leq 0.05$ was adopted to detect differences between treatments.

RESULTS AND DISCUSSION

Enzymatic compounds produced by bacterial isolates. Most of the bacteria evaluated showed the presence of a solubilization halo, thus indicating that lytic enzymes were produced.

The most frequent enzymes produced by the isolates were proteases (100 %), cellulases (88 %), pectinases (77 %), and amylases (66 %). After calculating the solubilization halo, it was possible to observe that the majority of bacteria produced the enzymes studied.

Almost all isolates presented positive results for the four enzymes studied. Except for isolates FO7.4, which presented negative results for the production of pectinase and amylase, FO4.2, which showed a lack of production of the enzyme cellulase and amylase and FO2.6, which did not produce pectinase and amylase, while all isolates were positive for the production of protease (Table 2).

All bacteria showed positive results for the presence of cellulase production, except F04.2. The production of cellulase capacity in antagonistic bacteria is essential for entry into plant tissues, as the antagonistic bacteria can colonize the plant and prevent the pathogen from advancing into the host (Li et al., 2018).

Table 2. Production of cellulase, pectinase, amylase and protease enzymes by isolates of *Bacillus* spp.

Isolates	Index of celulase production[2]	Index of pectinase production [2]	Index of amylase production [1]	Index of protease production [1]
FO7.4	3.1 ± 0.1a	0.0 ± 0.0d	0.0 ± 0.0d	4.3 ± 0.5a
FE3.7	4.1 ± 0.6a	5.2 ± 0.0ab	3.2 ± 0.0bc	3.1 ± 0.2b
FO4.2	0.0 ± 0.0b	3.7 ± 0.2c	0.0 ± 0.0d	3.9 ± 0.1ab
B7	3.3 ± 0.1a	4.9 ± 0.0abc	3.3 ± 0.0bc	4.2 ± 0.1a
FO5.5	3.3 ± 0.1a	6.3 ± 0.6a	3.1 ± 0.1c	3.9 ± 0.2ab
B4.5	4.2 ± 0.2a	4.7 ± 0.0bc	3.1 ± 0.0c	4.4 ± 0.0a
B1.9	3.9 ± 0.3a	4.4± 0.5bc	4.2 ± 0.2a	4.4 ± 4.4a
FO2.6	3.1 ± 0.1a	0.0 ± 0.0d	0.0 ± 0.0d	3.7 0.1ab
S3.5	3.8 ± 0.0a	4.9 ± 0.0abc	3.7 ± 0.1ab	4.3 ± 0.0a
CV (%)	14.90	13.81	8.59	9.56

Means followed by different letters in the column differ from each other using the Tukey test ($p \leq 0.05$). [1]ANOVA; [2]GLM quasi poisson

Several factors can affect the production of the cellulase enzyme. Studies carried out by Selvankumar et al. (2011), with *B. pseudomycoides*, showed that the presence of cellulase reached its highest production peak at 72 h after the incubation period, similar to what was found by Tiwari et al. (2017), which used *Streptococcus* and *Bacillus* sp. Acharya and Chaudhary (2012) observed that *B. licheniformis* and *Bacillus* sp. obtained the highest cellulase production at pH 6.5 and 7.0.

The bacteria FO5.5 presented the highest level of pectinase production in relation to the other isolates. Only the bacteria FO7.4 and FO2.6 showed negative results for the enzyme. Alqahtani et al. (2022), when evaluating 20 bacterial isolates, they found that 9 of these isolates showed a positive result for the production of pectinase and of these 9 bacteria, the one that showed the highest production were the *B. subtilis* isolates. Kashyap et al. (2000), observed that the period of 36 h at 37 °C were the optimal conditions for the best production of pectinase by *Bacillus* sp.

For amylase, all isolates, except F07.4, FO4.2 and FO2.6, indicated production of the enzyme, with greater emphasis on isolate B1.9. Carvalho (2008), observed a better resourcefulness in the production of amylase, when the bacteria *Bacillus* sp. was conditioned in culture supplemented with soluble starch (5 g·L-¹), peptone (2·g.L-¹) and whey proteins (0.5 g·L-¹). According to the author, the period of amylase production was relatively quick, starting 4 hours after the test and ending 12 hours later, and from this period onwards, it was noticed that production entered a stationary stage.

The synthesis of α-amylase depends on the way the experiment was conducted and the type of *Bacillus* used. In the first condition, enzyme production grows exponentially as the colony expands, and decreases as the bacteria enters stationary phase (Bajpai, 1989; Stephenson et al., 1998).

According to studies carried out by Coca (2022), *B. amyloliquefaciens*VR002 showed better amylase production, at pH 6.5 and 7.0. Teodoro and Martins (2000), observed that at pH 5.5 there was no enzymatic activity, which could also explain the lack of enzymatic activity of the bacteria FO7.4, FO4.2 and FO2.6.

For the production of the protease, the bacteria FO7.4, B7, B4.5, B1.9 and S3.5 showed higher enzymatic activity than FE3.7. According to Silva (2007), the optimal temperature for protease production for *Bacillus*sp SMIA-2 is 70 °C. Bacteria are the group that synthesize the most proteases, with the *Bacillus* genus being the largest source of this enzyme, mainly used for industrial use, depending on the inoculated pathogen, the seedlings may present different patterns of protein expression (da Costa et al., 2023).

Baard (2023), evaluating the antagonistic effect of *B. subtilis* and *B. tequilensis*, observed that only one isolate (B1) did not present protease synthesis of the four isolates tested, while only B2 and B

showed the presence of chitinase. Ramyabharathi and Raguchander (2014), reported that the isolated *B. subtilis* EPCO16 inhibits the mycelial growth of *F. oxysporum in vitro*, through the synthesis of chitinase and protease.

Rangel et al. (2022), evaluated *Bacillus* strains, the BsA3MX and BsC11MX strains, and through chemical analyzes identified the presence of protease and amylase production, but negative for pectinase, therefore, it was deduced that the antagonistic effects of these strains were caused by antifungal, volatile or diffusible compounds, rather than the action of hydrolytic enzymes.

Antagonism of *Bacillus* spp. against *M. phaseolina.* Of the nine isolates used to evaluate enzyme production, the four isolates that presented the best results were selected to evaluate which would present the best control for the pathogen.

The GLM quasipoisson analysis showed a significant difference between the bacterial isolates (F= 48.44; df= 4; $p \leq 0.001$), the *M. phaseolina*control methods (F= 60.32; df= 3; $p \leq 0.001$) and in the interaction between the isolates and the methods (F= 28.55; df= 12; $p \leq 0.001$), for the average diameter of the isolates (Table 3).

Among the control methods, the most effective for B1.9 bacteria was CP, with the lowest mycelial growth (4.7 cm) compared to the other treatments. In contrast, the result that presented the least efficiency for B1.9 was TE, not differing from the other methods, PSA and V, in which mycelial growth was 8.2 cm, that is, the entire plate was covered by the pathogen, with no control. In the CP method, all bacteria exhibited greater inhibition of the mycelial growth of the fungus, which infers the effectiveness of the isolates evaluated by this method.

Rangel et al. (2022), with the aim of evaluating two isolates of *B. amyloliquefaciens*in the control of *M. phaseolina*, observed that the two isolates inhibited the fungal growth of *Macrophomina in vitro*. The isolate BsA3MX showed 66.8 % growth inhibition in 5 days of incubation in PDA, while BsC11MX showed 62.8 %.

For the bacteria FO5.5, the method that was most expressive was PSA and CP, in which a mycelial growth of 1.2 cm and 3.8 respectively was observed (Table 3) followed by lower efficiency of V and TE, which, despite presenting lower values, did not present divergent statistical values. Bacteria B7 was the one that showed the highest inhibition rate when subjected to the PSA method, followed by F04.2 and F05.5, and of the four, B1.9 was the one that had the least satisfactory result.

Table 3. Mycelial growth of *M. phaseolina* subjected to the antagonistic action of *Bacillus* spp.

Bacteria	Control methods*			
	CP	**PSA**	**V**	**TE**
B1.9	4.67 ± 1.0bB	7.08 ± 0.9aA	6.20 ± 0.6abA	8.20 ± 0.0aA
FO5.5	3.87 ± 0.3bB	1.20 ± 0.1 bB	6.88 ± 1.0aA	7.21 ± 0.7aA
FO4.2	2.97 ± 0.1bB	0.50 ± 0.2bB	7.51 ± 0.3aA	6.68 ± 0.5aA
B7	3.42 ± 0.3bB	0.00 ± 0.0bB	7.63 ± 0.3aA	3.08 ± 0.5bB
Control	8.20 ± 0.0aA	8.20 ± 0.0aA	8.20 ± 0.0aA	8.20 ± 0.0aA

* Means followed by different letters, lowercase in the column and uppercase in the line, differ from each other using the Tukey test ($p \leq 0.05$). (CP) paired culture; (PSA) pathogen over antagonist; (V) volatile; and (TE) thermostable.

Ramyabharathi et al. (2016) evaluated the antagonistic effect of *B. subtilis* EPCO16 using the pathogen method on antagonistic culture and noticed that there was an antagonistic effect of 44.44 % on the mycelial growth of *F. oxysporum* sp. *lycopersici*. Kejela et al. (2017) evaluated 40 isolates of *Pseudomonas* sp. and found that the PT11 isolate showed 70 % inhibition of mycelial growth of *C. gloeosporioides* and 72 % of control of *F. oxysporum* in the paired culture assay.

Through molecular analyses, it was found that the main antagonistic compounds produced by *B. amyloliquefaciens* were lipopeptides such as bacillomycin D, macrolactin, iturin A, surfactin

and phenycin showed high antagonistic activity against *M. phaseolina* (Torres et al., 2016), *F. oxysporum*, *R. solanacearum* (Yuan et al., 2012), *B. cinerea* and *C. orbiculare* (Arrebola et al., 2010).

Rangel et al. (2022) using *Bacillus* strains, BsA3MX and BsC11MX realized that in addition to the inhibition of *M. phaseolina*, it was also possible to clearly see that there was an induction of morphological changes in *M. phaseolina*, from which irregular growth was noticed, with color brown in the center, creamy-white edges and reduced production of microsclerotia. As seen in the present work, from the fourth day onwards, the pathogen showed morphological changes, and some plates had brown edges while others had a woolly appearance.

In studies focused on the FO4.2 bacterium, it was observed that the most efficient method was PSA, in which there was little mycelial growth of the fungus, without differing from CP, and the V and TE methods showed less expressive results.

In the analysis of variance, both the individual factors and the interactions between them showed highly significant effects ($p \leq 0.001$) on the variables mycelial growth and percentage of inhibition (Table 4).

The intersection of the *Bacillus* genus with the physical-chemical properties of the soil and environmental factors interfere with the behavior of bacteria, and consequently also their volatile emission (Effmert et al., 2012; Burns et al., 2015)

Rezende et al. (2015), when evaluating the antagonistic effect of *B. subtilis* through the production of volatile compounds, proved that 3-methyl-1-butanol was the compound causing the antagonistic effect on *C. goeosporioides* and *C. acutatum*, causing an inhibition of 100 % mycelial growth. Zhang et al. (2019), evaluating *B. pumilus*, found that methyl isobutyl ketone is the volatile compound with an antagonistic effect synthesized by it and that in a study it obtained an inhibition of 95% to 100% in the mycelial growth of *Penicillium italicum*, after 5 days of experiment..

Table 4. Significance of ANOVA (*p*-value) of micelial growth and percentage of inhibition of *M. phaseolina* in the presence of different bacteria and control methods during seven days of evaluation.

Source of variation	Df	Micelial growth	Percentage of inhibition
Bacteria (BA)	4	<0.001*	<0.001*
Control Methods (CM)	3	<0.001*	<0.001*
Day (DA)	6	<0.001*	-
BA x CM	12	<0.001*	<0.001*
BA x DA	24	<0.001*	-
CM x DA	18	<0.001*	-
BA x CM x DA	72	<0.001*	-
CV (%)		32.88	19.3

* significant

Biological control against *M. laxa*, *M. fructicola* and *B. cinerea* was more significant when *B. amyloliquefaciens* CPA-8 was cultivated in TSA medium instead of NYDA or NAglu20 medium. Fiddaman and Rossall (1994) noticed that there is a variation in the production of volatile compounds depending on the carbohydrate used as a carbon source. In *B. subtilis* they noticed that when cultivated in agar with basic nutrients there was not as expressive a production as when it was cultivated in medium with the addition of D-glucose or complex substrates such as cellulose gum or carboxymethylcellulose to the medium, which obtained an extremely higher.

Through the seven days in which the experiment was analyzed, it was possible to see the interaction between the factors days, bacteria and control methods, which were significant (Table 5).

Table 5. Interaction of the antagonistic effect of different bacterial isolates and different control methods on the mycelial growth of *M. phaseolina* during the evaluation days.

		Control methods			
Day	**Bacteria**	**CP**	**PSA**	**TE**	**VL**
1	B1.9	0.00 Aa	0.00 Aa	0.00 Aa	0.00 Aa
	B7	0.00 Aa	0.00 Aa	0.00 Aa	0.00 Aa
	Control	0.00 Aa	0.00 Aa	0.00 Aa	0.00 Aa
	F04.2	0.00 Aa	0.00 Aa	0.00 Aa	0.00 Aa
	F05.5	0.00 Aa	0.00 Aa	0.00 Aa	0.00 Aa
2	B1.9	1.35 Aab	0.13 Ab	2.92 Aa	1.08 Ab
	B7	1.03 Aa	0.00 Aa	0.83Ba	1.05 Aa
	Control	1.63 Aa	1.63 Aa	1.63 ABa	1.63 Aa
	F04.2	1.32 Aab	0.00 Ab	2.07 Aba	0.67 Aab
	F05.5	1.88 Aa	0.00 Ab	2.28 ABa	1.27 Aab
3	B1.9	1.77 Ab	0.22 Bb	4.75 Aa	1.65 Ab
	B7	3.22 Aa	0.00 Bb	1.17 Cb	2.88 Aa
	Control	3.15 Aa	3.15 Aa	3.15 ABa	3.15 Aa
	F04.2	2.10 Ab	0.32 Bc	3.75 ABa	2.17 Aab
	F05.5	2.37Aab	0.97 Bc	2.50 BCab	2.88 Aa
4	B1.9	3.08 Bb	1.38 Bc	6.58 ABa	2.80 Cbc
	B7	1.03 Cbc	0.00 Bc	2.00 Db	4.73 Ba
	Control	8.00 Aa	8.00 Aa	8.00 Aa	8.00 Aa
	F04.2	2.78 Bb	0.50 Bc	5.83 BCa	3.53 BCb
	F05.5	2.83 Ba	0.93 Bb	4.22 Ca	4.20 BCa
5	B1.9	3.52 Bb	4.17 Bb	7.68 Aa	4.00 Cb
	B7	3.47 Bb	0.00 Cc	2.08 Cb	6.90 ABa
	Control	8.00 Aa	8.00 Aa	8.00 Aa	8.00 Aa
	F04.2	2.88 Bb	0.50 Cc	6.53 ABa	5.20 Ca
	F05.5	3.20 Bb	1.20 Cc	5.87 Ba	5.47 BCa
6	B1.9	4.25 Bb	5.08 Bb	7.88 Aa	5.32 Bb
	B7	3.47 Bb	0.00 Cc	2.67 Bb	7.70 Aa
	Control	8.00 Aa	8.00 Aa	8.00 Aa	8.00 Aa
	F04.2	2.97 Bb	0.50 Cc	6.73 Aa	6.63 ABa
	F05.5	3.42 Bb	1.20 Cc	6.53 Aa	6.35 ABa
7	B1.9	4.67 Bc	7.08 Aab	8.30 Aa	6.20 Cbc
	B7	3.42 BCb	0.00 Bc	3.08 Bb	7.92 Aa
	Control	8.00 Aa	8.00 Aa	8.00 Aa	8.00 Aa
	F04.2	2.97 Cb	0.50 Bc	7.02 Aa	7.52 ABa
	F05.5	3.87 BCb	1.20 Bc	7.42 Aa	6.88 ABa

Means followed by different capital letters in the column and lowercase letters in the row do not differ from each other by the Tukey test ($p \leq 0.05$). Capital letters: refer to the comparison of mycelial growth in the presence of different bacteria on each day and control method alone; Lowercase letters: refer to the comparison of mycelial growth in the different control methods in each bacteria and day alone.

Observing the interaction of the CP method during the 7 days of evaluation, a significant difference was recorded only from the 3rd day onwards, with emphasis on the lowest growth in the bacterial isolate B7. On the 7th and last day, the isolate FO4.2 differed from the other treatments and the control, presenting the lowest growth.

For the PSA method, the isolates B7 and FO.4 were the ones that differed from the control, with emphasis on B7, which did not present mycelial growth during the seven days of cultivation, indicating high antagonism of the isolate to *M. phaseolina*.

For the TE method, the lowest growth of M. phaseolina was again recorded for the isolate B7, with a statistical difference from the other bacterial isolates and the control.

For the VL method, differences were observed only from the 4th day onwards, but unlike what was recorded for the other methods, the isolate B1.9 was the one that stood out with the greatest antagonism.

Evaluating the days, no differences were observed for the 1st day in general, isolate B1.9 showed the highest mycelial growth of M. phaseolina when used with the TE method and the lowest growth (highest inhibition) in the CP method, during the 7 days evaluated.

For isolate B7, the highest mycelial growth of M. phaseolina was observed when using the VL method and the lowest growth (highest inhibition) in the PSA method, during the 7 days evaluated.

For isolates FO4.2 and FO.5, the highest mycelial growth of *M. phaseolina* was observed when using the VL and TL methods and the lowest growth (highest inhibition) in the PSA method, during the 7 days evaluated.

Calvo et al. (2020), evaluating the production of volatile compounds produced by different strains of *B. velezensis* species, observed that the BUZ-14 and I3 strains were the most efficient against brown and gray mold, respectively. It was possible to find at least 12 compounds for which *in vitro* tests highlighted diacetyl, benzaldehyde, 2-heptanone and isoamyl alcohol.

In relation to B7 bacteria, there was a significant difference between treatments, but it can be observed that PSA was the treatment that most inhibited the pathogen, with 100% control of the fungus, without differing from CP and TE, and lower efficiency in method V.

Sobrinho et al. (2018) evaluated the ability of *B. subtilis* isolates to control *F. solani*, with four antagonism methods, and noticed the highest percentages of inhibition of mycelial growth of *F. solani* isolates. Furthermore, they found that the method using thermostable metabolites was the one that presented the best results, inhibiting 81.7 and 78.2 % for the Anagé and Dom Basílio isolates. The metabolites produced by *B. subtilis* showed thermal stability at high temperatures, withstanding temperatures of up to 121 °C and yet continuing to maintain their ability to inhibit the development of pathogenic fungi (Pan et al., 2015; Amaro et al., 2018).

Petermann (2022) evaluated the control of *C .nymphaeae* (MdCn142) and *C. chrysophilum* (MdCc-110) based on thermostable products present in agricultural biological products and found that of the products used, the only one to have thermostable compounds that inhibited mycelial growth, was commercial product Serenade, a biological product based on *B. subitilis*.

It was also possible to observe in the work of Moreira et al. (2014), that *B. subtilis* from the product Serenade produced thermostable compounds and there was an antagonistic effect on the mycelial growth of *Colletotrichum* sp. Kupper et al. (2003), evaluating 53 isolates of *B. subtilis*, found that 52 of these isolates produced thermostable compounds and inhibited the mycelial growth of *C. acutatum*.

Regarding the percentage of inhibition, the GLM quasipoisson analysis showed a significant difference between the bacterial isolates and in the interaction between isolates and methods (Table 6).

The other variable evaluated was the percentage of inhibition that *M. phaseolina* suffered under the effect of the antagonistic bacteria to which they were subjected. In an interaction between methods and treatments, it was possible to observe that the method that presented the best results was CP, PSA and V, showing high levels of inhibition by the bacteria studied. The TE method was the second most effective in controlling the pathogen. In relation to bacteria, B7 was the one that presented the best result in terms of percentage of inhibition, followed by B1.9, FO5.5 and F04.2.

Table 6. Antagonistic effect of bacteria on *M. phaseolina*, with the percentage of inhibition as a variable.

Bacteria	Control methods			
	CP	PSA	V	TE
B1.9	43.06 ± 12.8 abA	13.58± 11.1bAB	24.36 ± 7.5 aAB	0.00 ± 0.0 bB
FO5.5	52.81 ± 4.6aA	85.31 ± 1.2aA	16.05 ± 12.3aB	11.21 ± 9.3 bB
FO4.2	63.78 ± 2.2aB	93.90 ± 2.7aA	8.31 ± 4.1 aC	16.98 ± 6.7 bC
B7	58.31 ± 3.6aB	100.00 ± 0,0aA	6.90 ± 4.3 aC	62.38 ± 6.9 aB
Control	0.00 ± 0.0 bA	8.20 ± 0.0bA	0.00 ± 0.0aA	0.00 ± 0.0bA

Means followed by different letters, lowercase in the column and uppercase in the line, differ from each other using the Tukey test ($p \leq 0.05$). (CP) paired culture; (PSA) pathogen over antagonist; (V) volatile; (TE) thermostable.

Camilo and Pietro (2023), when evaluating the antagonistic effects of *B. subtilis* RIZO-D and *B. amyloquefaciens* BRPJ on *M. phaseolina* and *A. alternaria*, noticed that the volatile compounds test and paired culture presented the best antagonistic effect, with a percentage index with more than 80 % inhibition.

Azevedo et al. (2020) tested different isolates of *Trichoderma* sp. as an antagonist for *Fusarium solani* and *F. oxysporume* and concluded that isolates of *Trichoderma* sp. Andobserved that of the methods used, the one that showed the highest percentage of inhibition was that of volatile organic compounds.

CONCLUSIONS

Of the nine bacteria evaluated to quantify the production of lytic enzymes, all demonstrated to be promising sources of enzymes of importance for biological control. Of the four methods, all showed an antagonistic effect on *M. phaseolina*, with emphasis on the pathogen method over antagonistic culture. The *Bacillus* B7 isolate demonstrated promise in controlling *M. phaseolina* using *in vitro* control methods. The *Bacillus* isolates evaluated exhibited an antagonistic effect in inhibiting the growth of the fungus depending on the method under study.

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