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Antimicrobial activity of secondary metabolites from *Bacillus* and *Trichoderma* against pathogens of red pitaya (*Hylocereus undatus*)

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Introduction

The exportation of pitaya has distinguished Ecuador as a leader in the exportation of the yellow variety and a prestigious producer of the red variety. However, the losses caused by phytopathogenic fungi in pitaya destined for exporting are significant to both the agricultural industry and the national economy. It is estimated that these losses can reach up to 50% in developing countries and 25% in developed countries (Andrés Cadena, 2020).

Various phytopathogenic fungi have been identified in pitaya crops, such as *Fusarium* sp., *Botrytis* spp., *Curvularia* sp., *Lasiodiplodia* sp., and *Neoscytalidium* sp., causing pathological deterioration. To address this problem, it is essential to implement post-harvest management strategies that minimize the risk of fungal infections. One of the alternatives for controlling phytopathogens is to use biological control, which involves employing organisms or their natural antagonistic by-products to reduce plant damage (Martínez, 2017).

The production of secondary metabolites through liquid fermentation using microorganisms such as *Trichoderma* sp. and *Bacillus* sp. has been studied due to their potential as biocontrol agents to combat pathogens in crops and preserve quality during storage or transportation (INIAP, 2020).

The combined use of *Trichoderma* sp. and beneficial bacteria such as *Bacillus* sp. and *Pseudomonas* has shown promising results in the development of synergistic microbial inoculants for sustainable agriculture (Daniel Eugui, 2022). *Bacillus* sp. and *Trichoderma* sp. are important due to their ability to synthesize antifungal metabolites and other bioactive substances that promote plant growth and control pathogens; *Bacillus* sp. produces antibiotics such as bacilysin, iturin, and fengycin, while *Trichoderma* sp. produces trichodermin, 6-pentyl-2H-pyran-2-one, and viridin, which have inhibitory properties against certain phytopathogenic fungi (Tingting Li, 2020).

Materials and Methods

Obtaining conidia from microorganisms

Isolates of *Bacillus* sp. previously isolated from the rhizosphere of rice plants were used. Additionally, two strains of rice phytopathogenic fungi, *Fusarium* spp. and *Curvularia cactivora*, were obtained from the fungal culture collection of the Laboratory of the Biotechnology Research Center of Ecuador (CIBE), within the Phytopathology Area of the Life Sciences Faculty at the Escuela Superior Politécnica del Litoral. Before conducting the antagonism study, the growth capacity of the bacterial strains on Potato Dextrose Agar (PDA) medium was evaluated for its use in dual culture.

For the subculturing process of *Trichoderma* sp. derived from the C9 strain (mother strain of *Trichoderma* sp.), the following method was used to obtain an aqueous conidial solution (Pedraza, 2022). Nine days after the initial subculture of the C9 strain, spores were washed using autoclaved water to produce a *Trichoderma* C9 solution. Subsequently, three progressive dilutions of this solution were made in 15 ml Falcon tubes, with concentrations of 1x10-1, 1x10-2 and 1x10-3, respectively, until reaching a final volume of 10 ml. Finally, the conidia were counted under a microscope using a Neubauer chamber. During this stage, a concentration of 3,17 x107 spores/ml and 1x109 spores/ml was obtained in the Neubauer chamber.

Liquid fermentation

Liquid fermentation (LF) is characterized by the use of dissolved nutrients, which provides extensive control over culture factors. This capability is essential as it allows us to adapt and improve the development conditions of the microorganisms (Arango, 2015).

For the preparation of the *Bacillus* sp. culture medium, a 1000 ml flask containing a commercial medium was used. During this process, 10 ml of the specific commercial medium for *Bacillus* sp. was extracted, to which the bacterial inoculum was added to reach a concentration of 1x108 on the McFarland scale. This resulted in a final volume of 990 ml in the flask. The remaining 10 ml of the medium along with the inoculum were reintroduced into the original 1000 ml flask containing a saline solution. This led to liquid fermentation for a period of 24 hours, with constant agitation and at an ambient temperature of 30 °C.

Filtration sterilization

The cold syringe filtration technique is used to separate solid particles or impurities from a liquid. Filters composed of two layers are used: an overlay of polypropylene prefilters with pores of 10 µm and 5 µm, followed by a membrane for efficient separation. To simplify and avoid rapid filter saturation, the samples are centrifuged beforehand. The supernatants are then filtered with a 5 ml syringe and 0,22 µm filters under sterile conditions. 20 ml of each supernatant are stored at 6 °C, labeled as "sterile supernatant of *Trichoderma* sp." and "sterile supernatant of *Bacillus* sp."

Mixture of sterile supernatants for the preparation of treatments and respective controls, with these antimicrobial tests being evaluated.

A mixture called "M" was created by combining equal parts of the sterile supernatant of *Trichoderma* sp. in PDB and the sterile supernatant of *Bacillus* sp., in a 50/50 ratio. This mixture will be used in one of our treatments.

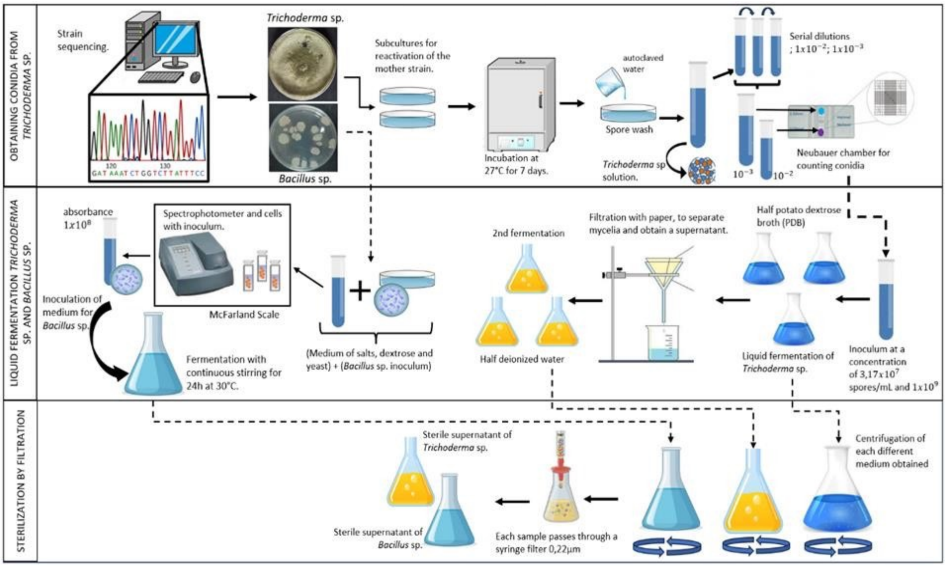
Petri dishes with PDA were prepared for each of the strains isolated from the symptoms of pitaya fruits, totaling 99 Petri dishes (11 strains, 4 treatments, and 4 controls). Each Petri dish was divided into four sectors for each treatment and control.

Finally, *in vitro* tests were evaluated using the modified agar well diffusion method on PDA medium, where each well contained 90 µl of solution. Themain objective of this study was to analyze the efficacy of various treatments in *in vitro* models. These qualitative methods are easy to standardize and are suitable for studying microorganisms, as they do not require very specific conditions and allow rapid reproduction of results.

The purpose was to determine the compatibility and growth of the evaluated microorganisms (Peña, 2017). After a period of 5 days, the antimicrobial capacities of each treatment and control were qualitatively evaluated by measuring the distance in millimeters between the inhibition halo of the microorganism and the wells containing different solutions: sterile supernatant of *Trichoderma* sp. in PDB, sterile supernatant of *Trichoderma* sp. in deionized water, sterile supernatant of *Bacillus* sp., and a mixture of sterile supernatant of *Trichoderma* sp. in PDB and sterile supernatant of *Bacillus* sp. each test was replicated four times per strain.

Figure. 1

Methodological description of the research process



Note. The diagram illustrates the systematic steps involved in obtaining sterile supernatants of the different microorganisms used in this project.

Isolation and sequencing of microorganisms with the molecular identification of strains isolated from the symptoms of red pitaya fruit.

Finch TV software was used to edit the sequences, selecting the optimal parts of the chain. Consensus sequences were then generated for each isolation sample by aligning the ITS1 and ITS4 sequences in Geneious Prime. These consensus sequences were used to identify the isolated strains using the BLAST (Basic Local Alignment Search Tool) algorithm, successfully identifying the strains associated with the symptoms of red pitaya.

For the molecular identification of the strains isolated from the symptoms of red pitaya fruit, DNA was extracted from fungal mycelium obtained from pure cultures on PDA medium, following the Cenis protocol. Amplification of the ITS1, 5.8S, and ITS2 regions was performed by PCR using universal primers ITS-1. The PCR reaction volume and amplification conditions were detailed according to the protocol of Suárez (2021), and the products were visualized on 2% agarose gel.

For this study, experiments were conducted under controlled conditions to analyze the antimicrobial activity of filtered supernatants; eleven culture plates were used, each replicated four times to evaluate the *in vitro* interaction between bacteria and fungi. Four different treatments were applied: two sterile filtered supernatants of *Trichoderma* sp., one of *Bacillus* sp., and a mixture of the sterile supernatant of *Trichoderma* sp. in PDB medium with *Bacillus* sp., to test against 11 pathogens, with four control groups corresponding to each treatment. The objective was to obtain data to statistically evaluate the results and observe the efficiency of each treatment.

Results

Molecular identification of isolated strains

Table. 1
Molecular identification of the sequencing of the ITS1, 5.8S, ITS2 region of strains isolated from red pitaya fruit using the BLAST database

Code	Strain	Identification number in Gen Bank	Identify percentage (%)
PIT1-H2	*Fusarium dimerum*	EU 926267.1	98,87
PIT2-6	*Fusarium verticillioides*	MK790046.1	92,90
PIT2A-6	*Fusarium verticillioides*	MK790046.1	92,90
PIT2-H6	*Fusarium falciforme*	MG189935.1	99,81
PIT2-H9	*Fusarium verticillioides*	MK790046.1	92,90
PIT5-21	*Fusarium dimerum*	EU926267.1	98,57
PIT2-8	*Curvularia cactivora*	KJ909775.1	95,56
PIT3-H10	*Curvularia cactivora*	MN688803.1	99,81
PIT3-H12	*Curvularia cactivora*	MN688803.1	99,81
PIT4-H19	*Curvularia cactivora*	MN688803.1	99,81
PIT5-H25	*Curvularia cactivora*	MN688803.1	99,81

According to Crespo (2022),regarding morphological identification, two parameters must be considered: the e-value, which should approach a value of 0,0 and genetic similarity, which should range between 90% and 100%.

Based on this, it can be confirmed that all isolates show genetic similarity above 90% and an E-value of 0,0 in the ITS region.

Antimicrobial activity

Bacillus *sp. and* Trichoderma *sp. vs.* Fusarium dimerum

The measurement of the inhibition halo diameter of the replicates indicates that the halo of the fungus *Fusarium dimerum* was found above the wells with sterile supernatant containing the secondary metabolites produced by *Bacillus* sp. (B) and the mixture of sterile supernatants of *Trichoderma* sp. and *Bacillus* sp. (M). This suggests that the secondary metabolites of *Trichoderma* sp. and *Bacillus* sp. were not effective in inhibiting the growth of the fungus *Fusarium dimerum*.

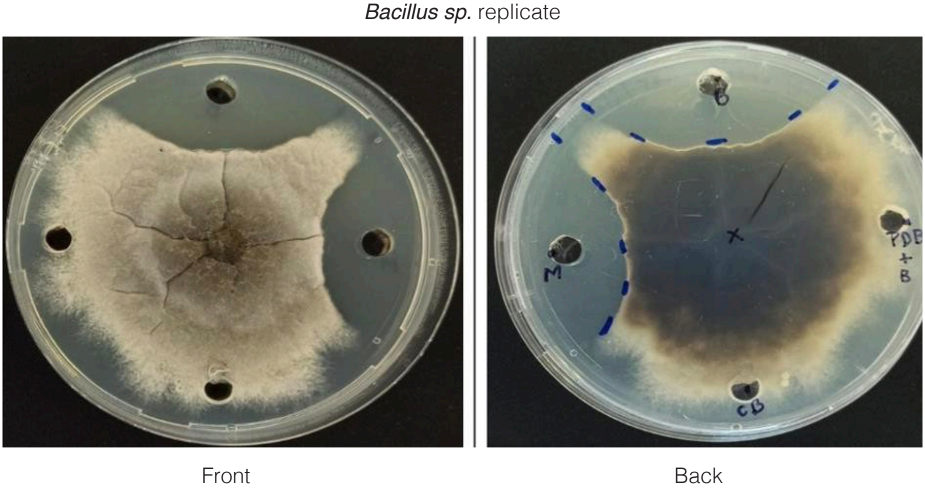
Bacillus *sp. and* Trichoderma *sp. vs.* Fusarium verticillioides

The measurement of the inhibition halo diameter of the replicates indicates that the halo of the fungus *Fusarium verticillioides* extended to the edges of the wells containing the sterile supernatant of *Bacillus* (B) and the mixture of sterile supernatants of *Trichoderma* and *Bacillus* (M). This indicates that, although to a lesser extent, the antimicrobial activity of the produced secondary metabolites exerted an inhibitory action on the growth of the fungus *Fusarium verticillioides.*

Bacillus *sp. and* Trichoderma *sp. vs.* Fusarium falciforme

The measurement of the inhibition halo diameter of the replicates indicates that the halo of the fungus *Fusarium falciforme* was found above the wells with sterile supernatant containing the secondary metabolites produced by *Bacillus* sp. (B) and the mixture of sterile supernatants (M). However, despite the absence of manifested antimicrobial activity by the secondary metabolites, an effect impacting its growth or evolutionary process was observed.

Figure. 2
Pathogen inhibition test replicate 2

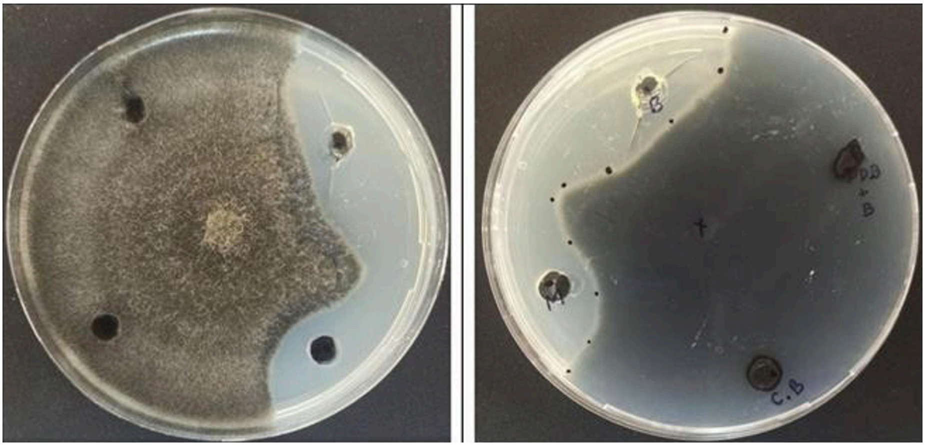


Note. Antimicrobial activity (modified agar well diffusion method) of the sterile supernatant of *Bacillus* sp. (B) and the mixture of sterile supernatants of *Trichoderma* sp. and *Bacillus* sp. (M) against *Curvularia cactivora* with sample code PIT2-8.

Figure. 3

Pathogen inhibition test replicate 1

Bacillus sp. replicate 1



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Note. Antimicrobial activity (modified agar well diffusion method) of the sterile supernatant of *Bacillus* sp. (B) and the mixture of sterile supernatants of *Trichoderma* sp. and *Bacillus* sp. (M) against *Curvularia cactivora* with sample code PIT5-H25.

The measurement of the inhibition halo diameter in each of the replicates indicates that the secondary metabolites produced by *Bacillus* sp. (B) and the combination of secondary metabolites from *Trichoderma* sp. and *Bacillus* sp. (M) caused a significantly high inhibition in the growth of the fungus *Curvularia cactivora*. This suggests that the secondary metabolites have a potent effect in suppressing the growth of this fungus.

The readings of the inhibition halos of the isolated strains are presented, where we can observe and interpret the effectiveness of treatments T3 and T4.

Table. 2

Results of the antimicrobial activity of treatments and controls with Bacillus sp. against the isolated strains

Isolated strains	Treatments and controls	
	T3	T4
Code	**Bacillus sp. (mm)**	**Mix (mm)**
PIT2-6	14,4166	12,6667
PIT2A-6	11,4375	6,5

Isolated strains	Treatments and controls	
	T3	T4
Code	**Bacillus sp. (mm)**	**Mix (mm)**
PIT2-H9	13,125	8,6667
PIT2-8	16,0833	11,9167
PIT3-H10	14,875	14,125
PIT3-H12	15,8125	11,3125
PIT4-H19	16,375	14,5625
PIT5-H25	18,8125	16,5

Note. The values detailed in this table correspond to the diameter of the inhibition zones observed in the two different treatments with bacillus and "mixture" against the pathogens.

Average inhibition halo (mm)

Treatments 3 and 4, corresponding to the sterile supernatant of *Bacillus* sp. (B) and the mixture of sterile supernatants (M), showed antimicrobial activity against the isolated fungi from red pitaya. Meanwhile, treatments 1 and 2 with *Trichoderma* sp. did not show antimicrobial activity in any of the isolated strains from red pitaya.

The table presents the average inhibition halo (mm) found in various isolated strains symptomatic of pathogens from red pitaya fruit, highlighting the strain of the fungus *Curvularia cactivora* with code (PIT5-H25), which presented the highest average inhibition halo in treatments 3 and 4, being 18,8125mm and 16,5mm respectively. This indicates that treatments with *Bacillus* sp. are effective as antimicrobial agents in controlling the fungus *Curvularia cactivora*.

According to Heydrich (2012) in their study of the antagonism of *Bacillus* sp. against phytopathogenic fungi in rice cultivation, the inhibitory capacity of *Bacillus* sp. members against species of the genus *Curvularia* was demonstrated.

On the other hand, the strain of *Fusarium verticillioides* with code (PIT2A-6) presented the lowest inhibition averages in treatments 3 and 4.

These results differ from the claims of Ariza Yesid and Sánchez Ligia (2012), who affirm that *Bacillus* sp. is very effective as biological control against *Fusarium* sp. with inhibition rates ranging from 70 to 100%. Therefore, it is inferred that the performance of sterile supernatants of *Bacillus* sp. in our treatments could be linked to the concentration in each of them.

Statistical analysis

For the presentation and evaluation of the results, a statistical analysis of the data obtained was performed using the Infostat program version 2017, with a focus based on the analysis of variance (ANOVA). A Tukey test was applied, which shows the differences between the means and allows for a detailed observation of the results to analyze the inhibitory activity of the pathogens in the samples.

Table 3 presents the average values of the inhibition halos observed in the treatments of *Bacillus* sp. and in the mixture, used as biological control agents against the pathogens. A normality test was performed to determine the suitability of the statistical analysis, confirming the applicability of the one-way ANOVA.

Table. 3
Standard deviation of the inhibition percentage of Bacillus sp. and mixture

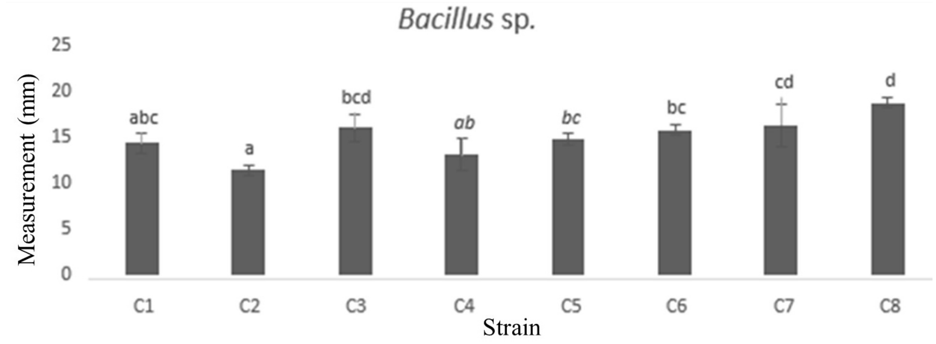
Strain	Bacillus	Desve	Mix	
C1	14,4166667	1,04748376	12,6666667	0,82495791
C2	11,4375	0,625	6,5	0,93541435
C3	16,0833333	1,47667043	11,9166667	0,82495791
C4	13,125	1,76186454	8,66666667	0,96465308
C5	14,875	0,66143783	14,125	1,88745861
C6	15,8125	0,55433895	11,3125	1,32876823
C7	16,375	2,25924029	14,5625	0,875
C8	18,8125	0,625	16,5	1,5411035

Note. Values are detailed that represent the inhibition percentage of the *bacillus* and the “mixture” sample against the pathogen. With this analysis, the probability value P = 0.001 which is less than 0,05 was obtained.

ANOVA analysis revealed significant variability among the means of the samples, highlighting two representative values: one of 18,8125 for the inhibition halo of Bacillus sp. and another of 16,5 for the mixture in strain 8 indicating effective suppression of the phytopathogenic fungus.

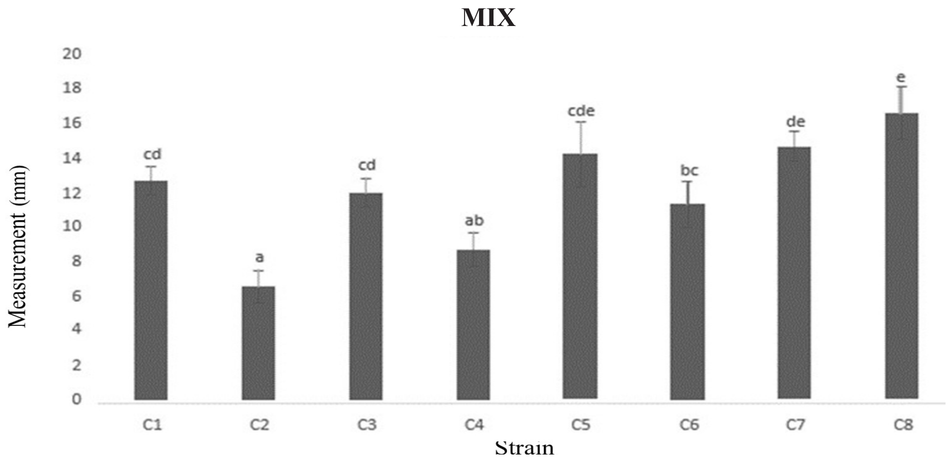
The Tukey test was applied to identify the most representative differences between the means of the samples in terms of the inhibition halo. Noticeable variations were observed among the strains, with strain 8 being the most effective in control, while strain 2 showed a lower variation. The Tukey test applied to the mixture also demonstrated its effectiveness as a method of biological control

Figure 4
Tukey histogram, Bacillus sp. assay



Note. As seen in the histogram, it details the highest peak of the clusters obtained, the most prominent being that of sample 8 in terms of the *bacillus* test.

Figure 5
Tukey histogram, mixture assay



Note. The variations in the means between the samples are evident, with one of the greatest variations observed in the mixture compared to strain 8. In comparison with the *bacillus* assay, the "mixture" strain represents a greater prominence in clusters values.

Conclusions

This study has conducted the molecular identification of strains isolated from red pitahaya, employing bioinformatics tools such as Geneious Primer, FinchTV, and NCBI. This analysis is crucial for a profound understanding of the diversity of pathogens affecting red pitahaya, as well as for the design and implementation of specific control strategies in this crop.

The focus of this research was on analyzing the inhibition halos, determining the capacity of secondary metabolites produced through liquid fermentation of *Trichoderma* sp. and *Bacillus* sp. to inhibit and/or control the proliferation of fungi isolated from red pitahaya (*Hylocereus undatus*).

It can be concluded that the secondary metabolites produced by *Bacillus* sp. exhibit a greater inhibitory action on pathogens, showing one of the most representative inhibition halos with 18,8125mm and 16,5mm respectively against strains of *Curvularia cactivora.*

The methods used in this study facilitated the production of a substantial number of metabolites, supporting the effectiveness of liquid fermentation as a viable strategy for generating natural antimicrobial agents for pathogen control in agriculture. Finally, this study yielded positive results regarding the capacity of secondary metabolites produced by *Bacillus* sp. to inhibit the growth of *Curvularia cactivora* and *Fusarium* spp. These findings suggest that these metabolites could play a significant role in controlling specific fungal pathogens affecting red pitahaya. On the other hand, further evaluation of the potential of secondary metabolites from *Trichoderma* sp. is suggested. Although this study did not observe effectiveness in inhibiting the growth of strains of *Curvularia cactivora* and *Fusarium dimerum*, it is important to consider that the efficacy of these metabolites could be influenced by specific environmental conditions or genetic characteristics of the strains. Therefore, additional research could provide insight into their true potential as antifungal agents.

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