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Production of Antifungal Compounds and Hydrolytic Enzymes by *Bacillus*spp. As Mechanisms of Action against*Phyllostictacitricarpa*

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Abstract: *Citrus black spot, which is caused by the fungus Phyllostictacitricarpa, affects almost all varieties of citrus and causes major problems in the commercialization of fruits in natura. To minimize the use of chemicals, biological control strategies have become increasingly attractive, and many Bacillus species have been reported to act as biocontrol agents. Therefore, this study aimed to elucidate the different mechanisms of action of bacteria against P. citricarpa. The production of antifungal substances (volatile, thermostable and cell-free molecules) and hydrolytic enzymes (β-1,3-glucanase and chitinase) were studied. The most promising bacterial isolates were identified by molecular techniques. We found that all 70 isolates of Bacillus spp. tested produced antifungal substances that tolerated high temperatures (120 ºC) and resulted in up to 98% inhibition of pathogen colonies. With the exception of ACB-66, all other isolates produced cell-free metabolites in sufficient quantities to inhibit up to 100% of the P. citricarpa development. The four most promising isolates (ACB-08, ACB-12, ACB-63 and ACB-69) produced β-1,3-glucanase and were identified as Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus methylotrophicus and Bacillus amyloliquefaciens, respectively. This study demonstrated the potential use of Bacillus spp. to control P. citricarpa.*

Keywords:*Antifungal compounds, Cell-free, Hydrolytic enzymes, Thermostable, Volatile.*

I. Introduction

Citrus black spot (CBS), which is caused by the fungus *Phyllostictacitricarpa* (McAlpine) Petrak (teleomorph: *Guignardiacitricarpa*Kiely), affects almost every species of citrus, causing an esthetic depreciation of the fruit and consequent financial loss in its *in natura* commercialization. Thus, CBS is an economically important disease [1]. Marketing is primarily affected to the foreign market, due to phytosanitary barriers imposed by countries of the European community, where the pathogen is absent and the suitability of the weather conditions to complete its life cycle is unfavorable [2,3].

The current incidence and severity of the disease in São Paulo's citrus orchards have shown that the fungus is highly adapted to its conditions, and the chosen control procedures have only contributed to minimize the potential loss.The use of fungicides constitutes the major control measure for CBS, despite having a limited efficiency, high cost and problems with the appearance of fungal strains resistant to these products [4].

With the reduction in fungicide use due concerns about their impact on the environment and human health, farmers and researchers have considered alternative strategies, such as the use of biological control.

In this context, the use of antagonistic species of *Bacillus* has been extensively researched due to the fact that these bacteria produce several types of antimicrobial compounds, including antibiotics [5, 6, 7] and hydrolytic enzymes [8, 9, 10], such as chitinases and β-1,3-glucanases, which degrade fungal cell walls. These bacteria produce spores that are resistant to desiccation, UV radiation and organic solvents [11] and are potential antagonists of various pathogens found pre- and post-harvest [5, 7, 8, 9, 10].

Given the above, this study aimed to assess the capacity of different isolates of *Bacillus* spp. in controlling the development of the fungus *Phyllostictacitricarpa*, under laboratory conditions, through different mechanisms of action and identify the most effective biocontrol isolates.

II. Materials and methods

2.1. Microorganisms

The fungus *Phyllostictacitricarpa*and seventy *Bacillus* spp. isolates were obtained from the collection of the Phytopathology and Biological Control Laboratory, in Centro APTA CitrosSylvio Moreira/IAC, Cordeirópolis/SP, Brazil.

2.2. Study of the mechanisms of action

2.2.1.* Effect of *Bacillus* isolates on mycelial growth of*Phyllostictacitricarpa

The dual culture technique in Petri dishes containing potato-dextrose-agar (PDA) [12] has been used to study the antagonistic activity of *Bacillus* spp. cultures on *P. citricarpa*mycelial growth.The 5 mm diameter mycelium discs, gathered from the active colonies of *P. citricarpa*with ten days of growth, were placed in Petri dishes containing PDA at 3 cm distance from the colony of each *Bacillus* spp. isolate. The control group was represented by the pathogen without antagonists.To verify the effect of *P. citricarpa* on the size of the *Bacillus* spp. colony, there was a parallel test using the same methodology described above, and the control treatments were represented by bacterial isolates without the pathogen.

***2.2.2.* Production of volatile antifungal compounds**

To verify the production of volatile compounds by *Bacillus*, split plates were which prevented nonvolatile compounds produced by the bacteria from reaching the plant pathogen. A bacterium culture disk (5 mm in diameter) was placed on one side of the plate, and on the other side, a mycelial disc (5 mm) from the pathogen was added, following the methodology described [13].

***2.2.3.* Production of thermostable antifungal compounds**

The *Bacillus* spp. isolates were transferred to 250 mL Erlenmeyer flasks containing 50 mL of potato dextrose. Then, the cultures were incubated in laboratory conditions, under 150 rpm agitation, over a seventy-two hour period. After the given time, a 10 mL aliquot was collected from each flask and transferred to other 250 mL Erlenmeyer flasks containing 90 mL of PDA. The media were autoclaved for twenty minutes, at 120ºC and one atm pressure, and poured into Petri dishes. After solidification, a 5 mm diameter disc obtained from active *P. citricarpa* colonies was transferred to the center of each dish, which contained medium and metabolites produced by the bacteria [14].

***2.2.4.* Production of cell-free antifungal compounds**

After cultivating each bacterial isolate in potato dextrose for seventy-two hours, as described above, a 10 mL aliquot was collected, filtered with a Millipore® membrane (0.22 µm) and transferred to 250 mL Erlenmeyer flasks containing 90 mL of PDA medium. After pouring and solidifying the medium in a Petri dish, a 5 mm diameter disc obtained from the active pathogen colony was transferred to its center.

2.2.5. Production of hydrolytic enzymes

Four isolates of *Bacillus* spp. (ACB-08, ACB-12, ACB-63 and ACB-69) were analyzed for the production and release of hydrolytic enzymes [15]. These four isolates were selected based on their performance in the interaction tests between microorganisms (pathogen/antagonist).A loopful of each isolate was transferred to 2.5 mL of potato dextrose broth (BD) and incubated at 32 °C for 12 hours. After this period, 1 mL of the culture was transferred into Erlenmeyer flasks (125 ml) containing 50 mL of BD or 50 mL of modified BD (cell wall of the fungus to 1% (w/v), replacing glucose). The cultivation was performed in triplicate at 32 °C with stirring at 90 rpm for 24 hours. After this period, a 2 mL aliquot was removed and centrifuged at 10,000 g for 10 minutes at 5 °C. The supernatant was recovered and used for analysis of chitinase activity and β-1,3-glucanase activity (Miller, 1959). For preparation of the cell wall of the pathogen, four mycelial discs from the fungus *P. citricarpa* were transferred to 250 mL Erlenmeyer flasks containing 100 mL of BD. The fungus cultivation was performed with shaking at 150 rpm in room temperature and light for 10 days. The resulting mycelium was collected by vacuum filtration, washed several times with distilled water, homogenized in 100 mL of distilled water and centrifuged at 5,000 g for 10 minutes. The supernatant was discarded, and mycelial tissue maceration was carried out in a mortar in the presence of liquid nitrogen. Subsequently, 100 mL of distilled water was added to the material obtained, and this was subjected to 3 cycles of centrifugation and resuspension. Then, the pellet was subjected to drying at 55 °C for 12 hours, forming the prepared cell wall [16].

2.2.5.1. Quantification of reducing sugars

Analysis of the reducing sugars released during the enzyme assays (chitinase and β-1,3-glucanase) was performed according to the method described by Miller (1959), using the acid reactant 3,5-dinitrosalicylic acid (DNS).

***2.2.5.2.* Quantification of β-1,3-glucanase activity**

The production of β-1,3-glucanase was assessed using colorimetric quantification of glucose liberated from laminarin substrates by measuring the reducing sugars. The reaction mixture was incubated at 37 ºC for 1 hour and contained 200 µL McIlvaine buffer (pH 6.0), 100 µL sample of culture and 100 µL of laminarin (4 mg/mL). After this period, the reaction was stopped by adding 200 µL of the reagent DNS to the reaction mixture, thus defining thecontent of reducing sugars released. The absorbance readings at 540 nm were subtracted from the

absorbance of the reaction mixture in the presence of a buffer instead of culture medium. Furthermore, the absorbance of the negative control (buffer solution instead of the substrate) was subtracted from each experimental reading. The absorbance values were compared to a standard curve of glucose, and the enzyme activity was expressed as U/L, where one unit of activity (U) was defined as 1.0 g of reducing sugar (glucose) liberated from laminarin under the test conditions used.

2.2.5.3. Quantification of chitinase activity

The chitinase activity was determined by colorimetric quantification of N-acetyl glucosamine released from a glycol chitin substrate. For this assay, 100 µL of the culture sample was mixed with 200 µL of McIlvaine buffer, pH 6.0 and 100 µL of chitin glycol 0.01% (w/v) in this same buffer. After incubation for 60 minutes at 50 °C, the reaction was stopped by addition of 200 µL of the reagent DNS to the reaction mixture, thus defining the reducing sugars released, as previously described. The reference cuvette (white) had buffer to replace the culture medium. This reading was subtracted from the value obtained for the negative control, which had the cap to replace the substrate (glycol chitin). The enzymatic activity was expressed as U/L, where one unit of activity (U) was defined as 1.0 g of reducing sugar (N-acetylglucosamine) released from glycol chitin under the test conditions used.

2.2.6. Evaluation and statistical delimitation

The analysis of all experiments was carried out after 10 days of incubation of the greenhouse crops to BOD chamber at 26 °C and a photoperiod of 12 h by measuring the mycelial growth of colonies of *P. citricarpa* and/or *Bacillus* spp. in two perpendicular directions. Altogether, there were seventy isolates of *Bacillus* spp. divided into four trials.

A completely randomized design with four replicates was used to evaluate the paired cultivation and the effect of the antifungal compounds produced by bacteria on the mycelial growth of *P. citricarpa*. Data were assessed by analysis of variance (ANOVA), and the comparison of average values was performed using Tukey's test, at 5% probability.

To evaluate the development of *Bacillus* spp. colonies in the presence or absence of the pathogen, a factorial design with two factors and three replications was used. Data were assessed by analysis of variance (ANOVA), and the comparison of average values was performed using the Scott-Knott test at 5% probability.

2.3. Identification of *Bacillus* isolates by amplification of the 16S rRNA region

Using molecular biology techniques, the same four strains of *Bacillus* (ACB-08, ACB-12, ACB-63 and ACB-69) used in enzyme assays were identified based on their genetic material. DNA was extracted from the bacteria using the Wizard Genomic DNA Purification Kit from Promega®. The amount and the quality/purity of the extracted material were determined by optical density using a spectrophotometer (NanoDrop 2000c). The amplification of the 16S rRNA region was performed by PCR using two universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCTTACGACTT- 3') [17] . The PCR cycling conditions consisted of an initial denaturation step at 95 °C for 3 min; 27 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 sec and extension at 72 °C for 1 min and 40 sec and a final extension at 72 °C for 7 min. The PCR product was purified using a Quick Gel Extraction PureLink™ and Combo PCR Purification Kit (Invitrogen), which is commercially available [18]. The sequences were aligned and compared with the NCBI database using the BLAST tool (Basic Local Alignment Search Tool) [19].

III. Results

3.1.* Effect of *Bacillus* isolates on mycelial growth of *Phyllostictacitricarpa

All *Bacillus* spp. isolates were tested for their antagonistic capacity towards *P. citricarpa*, and the results are shown in Tables 1, 2, 3 and 4. The results revealed that among the seventy isolates, sixty-seven significantly inhibited the pathogenic fungus, with more than 25% inhibition of growth; ACB-12 and ACB-13, which showed the best results, resulted in approximately 90% inhibition (Fig. 1A).

The effect of *P. citricarpa* on the *Bacillus* spp. colonies was assessed, and 52 isolates were not significantly different compared with the control treatments (cultivation of *Bacillus* spp. alone) (Table 5 and Fig. 1C). However, 18 isolates showed altered growth, and, among these, 17 isolates (ACB-25, ACB-26, ACB-28, ACB-47, ACB-48, ACB-53, ACB-56, ACB-58, ACB-59, ACB-64, ACB-65, ACB-67 ACB-69, ACB-76, ACB-83, ACB-85 and ACB-91) showed inhibitions of their colonies ranging from 23.28% to 82.60%. Moreover, the ACB-08 *Bacillus* strain showed increased growth in the presence of the pathogen (Fig. 1B).

3.2. Production of volatile antifungal compounds

The results revealed that among the seventy bacteria isolates that were tested, twenty-seven produced volatile compounds that significantly inhibited fungal growth. In the first assay, the best results were obtained by the isolates ACB-19, ACB-15, ACB-22, ACB-16, ACB-20 and ACB-18, in ascending order of efficiency, showing inhibition of 72.79% to 42.33% (Table 1 and Fig. 1D). In the second assay, the isolates that had the greatest effect were ACB-47, ACB-41, ACB-52, ACB-24 and ACB-46, with a variation of 42.29% to 36.76% (Table 2). In the third assay, all *Bacillus* spp. isolates produced volatile compounds that significantly inhibited the fungal growth, with inhibition values that ranged from 35.46% (ACB-59) to 20.50% (ACB-63) (Table 3). In the fourth assay, only ACB-81 was significantly different from the control, with 37.18% inhibition of the phytopathogen growth (Table 4).

3.3. Production of thermostable antifungal compounds

Regarding the *Bacillus* spp. production of antifungal compounds that were stable at high temperatures, the results showed that all seventy tested isolates produced thermostable metabolites that affected pathogen colony growth (Tables 1, 2, 3 and 4), with inhibition values that ranged from 41.37% (ACB-73) to 98.15% (ACB-57).

3.4. Production of cell-free antifungal compounds

With the exception of ACB-66, which resulted in only 10.84% fungal growth inhibition, the bacteria isolates showed significant control of the phytopathogen (Tables 1, 2, 3 and 4). These isolates produced cell-free metabolites that affected the pathogen colony growth, with fungal colony inhibition values that ranged from 26.55% (ACB-43) to 100% (ACB-63, ACB-69).

3.5. Production of hydrolytic enzymes

All the *Bacillus* spp. tested exhibited β-1,3-glucanase activity, and none of the isolates tested had chitinase activity under the conditions shown (Table 6).

3.6. Identification of *Bacillus* isolates by amplification of the 16S rRNA region

The most effective bacterial isolates in the control of *Phyllostictacitricarpa* were identified as *Bacillus amyloliquefaciens* (ACB-08), *Bacillus subtilis* (ACB-12), *Bacillus methylotrophicus* (ACB-63) and *Bacillus amyloliquefaciens* (ACB-69).

IV. Discussion

Due to the economic loss caused by *Phyllostictacitricarpa* and to the growing desire to develop control methods that are harmless to the environment, farmers and researchers have assessed the use of biological control to contain diseases. By using antagonistic microorganisms, it was possible to observe different levels of disease control [20].

To develop a commercial bioproduct, it is necessary to characterize an antagonistic agent with regard to effectiveness, the nature of its action, survivability, colonization and toxicity to non-target species [21, 22]. The *Bacillus* genus is among the most commonly used, as it can form endospores, allowing the development of more stable and viable products [11], has a rapid growth rate in liquid medium and is not toxic to most species [23].

In the present study, all *Bacillus* spp. isolates strongly inhibited pathogen growth by producing antifungal compounds. The results can be compared to previous studies, which have shown that filtered amounts of *Bacillus* species inhibit the growth of many pathogenic fungi [24, 25, 26].

Studies *in vitro* interaction between *P. citricarpa* and endophytic bacteria found that some *Bacillus* spp. were inhibited and others were stimulated by the supernatant from fungal cultures [27]. Performing antibacterial activity tests with thirty isolates of *Phyllosticta*, researchers showed that twenty-four isolates inhibited the development of colonies of *B. subtilis*[28]. These results are consistent with those obtained in this study, where seventeen isolates of *Bacillus* spp. showed inhibited growth and one (ACB-08) had increased growth in the presence of the pathogen in paired cultivation. The data presented in this study suggest that although some bacterial isolates were affected by the fungus, they still maintained their antagonistic activity, as demonstrated by the production of antifungal substances.

Analyses of thermostable metabolites of all the bacterial isolates showed that they significantly inhibited the development of the fungus *P. citricarpa*, revealing that the metabolites produced by *Bacillus* spp. maintained their antagonistic activities even after exposure to high temperature.

Regarding the production of free antifungal substances of bacteria cells, with the exception of the ACB-66, all other strains produced compounds that affected the development of the pathogen, while this isolate produced antifungal substances only when subjected to autoclaving. We hypothesized that solubilization of substances present in the environment occurred, making them available to inhibit *P. citricarpa*. A second hypothesis is that components present in the microorganism with activity against the pathogen were released to the medium after exposure to high temperatures [29].

The results obtained in their study showed that while all isolates except ACB-66 produced cell-free metabolites in sufficient amounts, the greatest inhibition of pathogen colonies occurred by thermostable

metabolites. In contrast, researchers [30] studied the strain NSRS 89-24 of *B. subtilis* in controlling *Pyriculariagrisea* and *Rhizoctoniasolani* and showed that the sterilized culture filtrates or cell-free bacteria had the strongest inhibition of growth of pathogenic fungi compared to bacterial metabolites produced and subjected to autoclaving.

In this study, twenty-seven isolates of *Bacillus* spp. produced volatile compounds in concentrations sufficient to inhibit the development of the fungus *P. citricarpa*, which was significantly different from the control. The ACB-19 isolate produced volatile metabolites that inhibited 73% of the *Phyllosticta* colonies, while others showed an average of 35% of the control. Similar results were found by other authors [31] who worked with strains of *Bacillus amyloliquefaciens* to control five pathogens and showed that the isolates were effective in the production of volatile organic compounds with fungistatic effects *in vitro*. Additionally, the volatile compounds promoted growth in *Arabidopsis thaliana*.

Researchers studied the biological control of the fungi *P. citricarpa*, by the yeast *Saccharomyces cerevisiae* and noted plant pathogen control up to 87.2% by production of volatile compounds; this was attributed to the production of eight substances, mostly alcohols [4].

Interestingly, ACB-74 and ACB-76 did not produce antifungal volatile metabolites but produced a volatile compound that favored the growth of the pathogen. Moreover, these microorganisms produced cell-free and thermostable metabolites that inhibited more than 80% of the fungal colonies.

Analysis of 4 isolates of *Bacillus* spp. (ACB-08, ACB-12, ACB-63 and ACB-69) showed the production of β-1,3-glucanase when the bacterial isolates were grown in medium supplemented with cell wall fragments of the fungus. However, no chitinase production was observed in any of the bacterial isolates studied, suggesting that β-1,3-glucanase may be more important than chitinase at degradation of the wall of *P. citricarpa*.

Studies have shown that the extracellular lytic enzymes and antibiotics of *B. subtilis* are important for biocontrol of phytopathogens, and these substances may act alone or synergistically to degrade the cell walls of fungi [8, 9, 10, 30]. By studying the NSRS 89-24 isolate of *B. subtilis*, scholars showed that it produced β-1,3-glucanase and an antibiotic extracts (cell-free metabolites) that acted synergistically in controlling *P. grisea* and *R. solani*[30]. According to the authors, the antifungal compounds directly attacked the cell wall of fungi, which contained chitin, β-1,3-glucans and other oligosaccharides.

Isolates that showed enzymatic activity in this study, ACB-08, ACB-12, ACB-63 and ACB-69, also produced cell-free metabolites that yielded 86.68%, 68.48%, 100% and 100% inhibition of the mycelial growth of *P. citricarpa*, respectively. These results indicate the possible synergistic activity of these metabolites with the hydrolytic enzymes in the pathogen biocontrol, as shown by the *in vitro* results. Therefore, the bioactive compounds produced by *Bacillus* spp. can function in vivo as biofungicides to control fungal diseases.

We found that different isolates of *Bacillus* spp. produce inhibitory substances of *P. citricarpa*, and the data showed that some substances are more effective than others. Therefore, it is important to elucidate the possible mechanisms of action that are involved in controlling the plant pathogen, which will allow for the possibility of controlling the disease using synthetic substances and not only the mass introduction of biocontrol agents.

In general, antagonistic bacteria, such as *B. subtilis*, *Pseudomonascepacia*, *Pseudomonas fluorescens* and others, act via antibiosis and occasionally by competition and parasitism [32]. Microorganisms that act via antibiosis generally have a broad spectrum of action, and the production of toxic substances is more effective at inhibiting fungi than any other mechanism of action. In our work, among the four isolates identified, one of them (ACB-12) belongs to the species *B. subtilis*; however, the others were classified as *Bacillus amyloliquefaciens* (ACB-08 and ACB-69) and *Bacillus methylotrophicus* (ACB-63), both of which are closely related to *B. subtilis* [33, 34]. Considerable interest has been shown in the development of strains of these species as biocontrol agents because they produce antifungal substances that are effective against various pathogens and also promote plant growth [10, 31, 35, 36, 37].

Therefore, rapid and efficient production of metabolites by microorganisms with antimicrobial activity, as observed in this study, suggests the feasibility of using them in biological control programs.

V. Conclusions

This study demonstrated the potential use of *Bacillus* spp. in inhibiting the development of the fungus *P. citricarpa* through various mechanisms, such as production of antifungal compounds and hydrolytic enzymes. However, more studies are needed to identify the bioactive compounds against *P. citricarpa* to develop a product that can be used commercially for the control of diseases under natural conditions.

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Table 1. Inhibition percentage of *Phyllostictacitricarpa* colony after paired cultivation with different isolates of *Bacillus* spp. or under the influence of antifungal compounds produced by the bacteria. Test 1.

Treatments	PairedCultivation (cm)	Inhibition (%)	Antifungal Compounds					
			Volatile (cm)	Inhibition (%)	Thermostable (cm)	Inhibition (%)	Cell-free (cm)	Inhibition (%)
Control	4,93 a(a)	0,00	5,79 a	0,00	4,60 a	0,00	4,60 a	0,00
ACB-01	1,86 bcde	62,18	4,88 ab	15,77	2,21 b	51,90	3,03 b	34,24
ACB-02	1,29 cde	73,86	4,76 ab	17,71	1,44 b	68,75	2,14 c	53,53
ACB-07	0,86 de	82,49	4,20 ab	27,43	1,73 b	62,50	1,84 cde	60,05
ACB-08	1,31 cde	73,35	4,56 ab	21,17	1,34 b	70,92	0,61 hi	86,68
ACB-09	1,08 de	78,17	4,06 ab	29,81	1,66 b	63,86	0,85 ghi	81,52
ACB-10	2,43 bc	50,76	4,40 ab	23,97	1,75 b	61,96	0,59 i	87,23
ACB-11	0,84 de	82,99	3,48 abc	39,96	1,54 b	66,58	1,34 efg	70,92
ACB-12	0,55 e	88,83	3,51 ab	39,31	1,48 b	67,93	1,45 defg	68,48
ACB-13	0,58 e	88,32	4,79 ab	17,28	1,66 b	63,86	1,25 cdef	72,83
ACB-15	0,65 e	86,80	2,64 bc	54,43	1,75 b	61,96	1,50 cdef	67,39
ACB-16	2,64 b	46,45	3,01 bc	47,95	2,01 b	56,25	0,58 i	87,50
ACB-17	0,89 de	81,98	3,66 abc	36,72	1,41 b	69,29	0,95 fghi	79,35
ACB-18	2,03 bcd	58,88	3,34 bc	42,33	1,90 b	58,70	1,35 efg	70,65
ACB-19	1,05 de	78,68	1,58 c	72,79	1,69 b	63,32	1,54 cdef	66,58
ACB-20	2,05 bcd	58,38	3,21 bc	44,49	2,11 b	54,08	1,53 cdef	66,85
ACB-21	0,94 de	80,96	4,64 ab	19,87	2,01 b	56,25	2,06 cd	55,16
ACB-22	1,16 cde	76,40	2,79 bc	51,84	1,79 b	61,14	1,24 efg	73,10
ACB-AP3	2,65 b	46,19	3,85 abc	33,48	2,20 b	52,17	1,74 cde	62,23

(a) Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 2. Inhibition percentage of *Phyllostictacitricarpa* colony after paired cultivation with different isolates of *Bacillus* spp. or under the influence of antifungal compounds produced by the bacteria. Test 2.

Treatments	PairedCultivation (cm)	Inhibition (%)	Antifungal Compounds					
			Volatile (cm)	Inhibition (%)	Thermostable (cm)	Inhibition (%)	Cell-free (cm)	Inhibition (%)
Control	4,93 a(a)	0,00	3,16 a	0,00	3,63 a	0,00	3,63 a	0,00
ACB-23	1,11 fg	77,41	2,39 abc	24,51	0,68 cd	81,38	0,74 c	79,66
ACB-24	2,30 cde	53,3	2,00 bc	36,76	0,91 cd	74,83	1,03 c	71,72
ACB-25	1,06 fg	78,43	3,10 ab	1,98	0,86 cd	76,21	0,81 c	77,59
ACB-26	1,50 efg	69,54	2,79 abc	11,86	0,70 cd	80,69	0,65 c	82,07
ACB-27	1,31 efg	73,35	2,20 abc	30,43	0,66 cd	81,72	0,73 c	80,00
ACB-28	2,73 bcd	44,67	2,33 abc	26,48	1,05 bc	71,03	0,81 c	77,59
ACB-41	3,00 bc	39,09	1,95 c	38,34	0,75 cd	79,31	0,63 c	82,76
ACB-42	2,33 cde	52,79	2,88 abc	9,09	0,69 cd	81,03	0,60 c	83,45
ACB-43	3,66 b	25,63	2,44 abc	22,92	1,46 b	59,66	2,66 b	26,55
ACB-44	2,85 bcd	42,13	2,48 abc	21,74	0,75 cd	79,31	0,59 c	83,79
ACB-45	1,91 cdef	61,17	2,15 abc	32,02	0,71 cd	80,34	0,75 c	79,31
ACB-46	1,73 defg	64,97	2,00 bc	36,76	0,65 cd	82,07	0,55 c	84,83
ACB-47	1,09 fg	77,92	1,83 c	42,29	0,58 d	84,14	0,78 c	78,62
ACB-48	0,94 fg	80,96	2,15 abc	32,02	1,08 bc	70,34	0,69 c	81,03
ACB-51	1,10 fg	77,66	2,36 abc	25,30	0,69 cd	81,03	0,71 c	80,34
ACB-52	1,86 defg	62,18	1,98 c	37,55	0,80 cd	77,93	0,68 c	81,38
ACB-53	0,78 g	84,26	2,48 abc	21,74	0,80 cd	77,93	0,63 c	82,76

(a) Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 3. Inhibition percentage of *Phyllostictacitricarpa* colony after paired cultivation with different isolates of *Bacillus* spp. or under the influence of antifungal compounds produced by the bacteria. Test 3.

Treatments	PairedCultivation (cm)	Inhibition (%)	Antifungal Compounds					
			Volatile (cm)	Inhibition (%)	Thermostable (cm)	Inhibition (%)	Cell-free (cm)	Inhibition (%)
Control	3,08 a(1)	0,00	4,51 a	0,00	3,11 a	0,00	3,11 a	0,00
ACB-54	1,13 ab	63,41	3,01 b	33,24	0,50 efgh	83,94	0,71 bc	77,11
ACB-56	1,20 ab	60,98	3,15 b	30,19	1,13 cd	63,86	1,23 b	60,64
ACB-58	1,60 ab	47,97	3,19 b	29,36	1,18 c	62,25	0,70 bc	77,51
ACB-59	0,61 b	80,08	2,91 b	35,46	0,38 fgh	87,95	0,34 cde	89,16
ACB-60	1,84 ab	40,24	3,18 b	29,64	0,54 defgh	82,73	0,56 cde	81,93
ACB-63	0,55 b	82,11	3,59 b	20,50	0,63 cdefgh	79,92	0,00 e	100,00
ACB-64	1,91 ab	37,80	3,33 b	26,32	0,76 cdefg	75,50	0,05 de	98,39
ACB-65	0,88 ab	71,54	3,18 b	29,64	0,89 cdef	71,49	0,50 cde	83,94
ACB-66	1,24 ab	59,76	3,08 b	31,86	1,06 cde	65,86	2,78 a	10,84
ACB-67	2,19 ab	28,86	3,26 b	27,70	0,49 efgh	84,34	0,64 bcd	79,52
ACB-68	0,49 b	84,15	3,23 b	28,53	0,63 cdefgh	79,92	0,39 cde	87,35
ACB-69	0,96 ab	68,70	3,04 b	32,69	0,68 cdefgh	78,31	0,00 e	100,00
ACB-70	1,21 ab	60,57	3,06 b	32,13	0,20 gh	93,57	0,04 de	98,80
ACB-71	0,40 b	86,99	3,39 b	24,93	0,13 h	95,98	0,04 de	98,80
ACB73	0,88 ab	71,54	2,91 b	35,46	1,83 b	41,37	0,84 bc	73,09

(a) Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 4. Inhibition percentage of *Phyllostictacitricarpa* colony after paired cultivation with different isolates of *Bacillus* spp. or under the influence of antifungal compounds produced by the bacteria. Test 4.

Treatments	PairedCultivation (cm)	Inhibition (%)	Antifungal Compounds					
			Volatile (cm)	Inhibition (%)	Thermostable (cm)	Inhibition (%)	Cell-free (cm)	Inhibition (%)
Control	6,76 a[1]	0,00	3,90 a	0,00	4,74 a	0,00	4,74 a	0,00
ACB-57	3,59 bcdef	46,95	3,29 ab	15,71	0,09 g	98,15	0,71 bcd	84,96
ACB-72	4,29 b	36,60	3,60 ab	7,69	1,33 b	67,81	0,70 bcd	85,22
ACB-74	4,05 bc	40,11	4,10 a	-5,13	0,78 cde	83,64	0,36 cdefgh	92,35
ACB-75	3,84 bcd	43,25	3,35 ab	14,10	0,81 cde	82,85	0,35 defgh	92,61
ACB-76	3,84 bcd	43,25	3,99 a	-2,24	0,79 cde	83,38	0,40 cdefgh	91,56
ACB-77	3,46 bcdef	48,80	3,74 a	4,17	0,63 def	86,81	0,51 bcdefg	89,18
ACB-78	3,11 def	53,97	3,61 a	7,37	0,64 def	86,54	0,65 bcde	86,28
ACB-79	3,41 bcdef	49,54	3,06 ab	21,47	0,89 cd	81,27	0,20 gh	95,78
ACB-80	3,31 cdef	51,02	3,70 a	5,13	0,81 cde	82,85	0,50 bcdefg	89,45
ACB-81	3,73 bcde	44,92	2,45 b	37,18	0,84 cde	82,32	0,36 cdefgh	92,35
ACB-82	3,15 cdef	53,42	3,25 ab	16,67	0,40 efg	91,56	0,50 bcdefg	89,45
ACB-83	2,80 f	58,60	3,70 a	5,13	0,78 cde	83,64	0,43 cdefgh	91,03
ACB-84	2,89 ef	57,30	3,89 a	0,32	0,70 de	85,22	0,85 b	82,06
ACB-85	3,41 bcdef	49,54	3,33 ab	14,74	0,21 fg	95,51	0,06 h	98,68
ACB-86	3,59 bcdef	46,95	3,54 ab	9,29	1,21 bc	74,41	0,73 bc	84,70
ACB -87	2,96 def	56,19	3,45 ab	11,54	0,20 fg	95,78	0,53 bcdefg	88,92
ACB-88	3,71 bcdef	45,10	3,59 ab	8,01	0,98 cd	79,42	0,16 gh	96,57
ACB-89	3,51 bcdef	48,06	3,44 ab	11,86	0,91 cd	80,74	0,30 efgh	93,67
ACB-90	3,45 bcdef	48,98	3,64 ab	6,73	0,89 cd	81,27	0,59 bcdef	87,60
ACB-91	3,16 cdef	53,23	3,50 ab	10,26	0,86 cd	81,79	0,28 fgh	94,20

(a) Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 5. Influence of *Phyllostictacitricarpa* on the size of *Bacillus* spp. colonies

***Bacillus* spp.**	**With Fungus (cm)**	**Without Fungus (cm)**	**Test F**
ACB-01	2,77 Ad[(a)]	1,83 Ad	1,17NS
ACB-02	1,50 Ad	1,42 Ad	0,01NS
ACB-07	1,77 Ad	3,07 Ac	2,27NS
ACB-08	5,32 Ac	1,73 Bd	17,27**
ACB-09	2,23 Ad	2,88 Ac	0,57NS
ACB-10	2,13 Ad	1,32 Ad	0,90NS
ACB-11	1,62 Ad	1,48 Ad	0,02NS
ACB-12	2,28 Ad	0,92 Ad	2,51NS
ACB-13	1,52 Ad	1,35 Ad	0,04NS
ACB-15	1,70 Ad	1,75 Ad	0,00NS
ACB-16	2,20 Ad	3,73 Ac	3,16NS
ACB-17	2,17 Ad	2,90 Ac	0,72NS
ACB-18	4,05 Ac	4,38 Ac	0,15NS
ACB-19	2,00 Ad	1,98 Ad	0,00NS
ACB-20	1,57 Ad	1,67 Ad	0,01NS
ACB-21	1,52 Ad	1,85 Ad	0,15NS
ACB-22	1,43 Ad	1,52 Ad	0,01NS
ACB-23	1,72 Ad	3,15 Ac	2,76NS
ACB-24	1,35 Ad	1,15 Ad	0,05NS
ACB-25	1,22 Bd	3,15 Ac	5,03*
ACB-26	0,87 Bd	3,08 Ac	6,61*
ACB-27	1,85 Ad	1,97 Ad	0,02NS
ACB-28	1,27 Bd	3,42 Ac	6,22*
ACB-41	1,30 Ad	1,75 Ad	0,27NS
ACB-42	2,28 Ad	1,78 Ad	0,34NS
ACB-43	1,52 Ad	1,58 Ad	0,01NS
ACB-44	1,23 Ad	1,52 Ad	0,11NS
ACB-45	1,20 Ad	1,38 Ad	0,05NS
ACB-46	1,95 Ad	2,58 Ad	0,54NS
ACB-47	2,20 Bd	4,00 Ac	4,36*
ACB-48	0,87 Bd	5,00 Ab	22,97**
ACB-51	1,68 Ad	2,03 Ad	0,16NS
ACB-52	1,20 Ad	2,22 Ad	1,39NS
ACB-53	1,18 Bd	4,43 Ac	14,20**
ACB-54	5,55 Ac	6,33 Ab	0,83NS
ACB-56	5,85 Bc	9,00 Aa	13,34**
ACB-57	2,13 Ad	2,33 Ad	0,05NS
ACB-58	5,78 Bc	7,83 Aa	5,65*
ACB-59	4,90 Bc	7,05 Aa	6,22*
ACB-60	5,17 Ac	4,78 Ac	0,20NS
ACB-63	8,45 Aa	8,42 Aa	0,00NS

ACB-64	5,03 Bc	8,00 Aa	11,83**
ACB-65	6,42 Bb	9,00 Aa	8,97**
ACB-66	5,50 Ac	6,42 Ab	1,13NS
ACB-67	6,23 Bb	8,12 Aa	4,77*
ACB-68	8,08 Aa	7,87 Aa	0,06NS
ACB-69	4,47 Bc	7,25 Aa	10,42**
ACB-70	7,20 Ab	8,58 Aa	2,57NS
ACB-71	6,13 Ab	6,58 Ab	0,27NS
ACB-72	2,35 Ad	2,97 Ac	0,51NS
ACB-73	5,62 Ac	5,62 Ab	0,00NS
ACB-74	2,37 Ad	3,00 Ac	0,54NS
ACB-75	2,88 Ad	3,67 Ac	0,83NS
ACB-76	2,47 Bd	4,57 Ac	5,93*
ACB-77	2,35 Ad	3,47 Ac	1,68NS
ACB-78	2,68 Ad	2,47 Ad	0,06NS
ACB-79	1,98 Ad	3,33 Ac	2,45NS
ACB-80	3,68 Ad	3,53 Ac	0,03NS
ACB-81	3,07 Ad	4,37 Ac	2,27NS
ACB-82	4,58 Ac	5,02 Ab	0,25NS
ACB-83	3,58 Bd	5,33 Ab	4,12*
ACB-84	4,32 Ac	5,25 Ab	1,17NS
ACB-85	3,37 Bd	6,28 Ab	11,44**
ACB-86	2,88 Ad	3,38 Ac	0,34NS
ACB-87	4,12 Ac	3,75 Ac	0,18NS
ACB-88	3,88 Ac	2,58 Ad	2,27NS
ACB-89	2,43 Ad	3,28 Ac	0,97NS
ACB-90	3,08 Ad	4,53 Ac	2,83NS
ACB-91	1,93 Bd	3,68 Ac	4,12*
ACB-AP3	4,23 Ac	3,93 Ac	0,12NS
Teste F	9,28**	13,42**	

(a) Means followed by the same capital letter in line and tiny column, do not differ by the Scott-Knott test at 5% probability.

Table 6. Production of chitinase and β-1,3-glucanase of *Bacillus* spp. evaluated by production of reducing sugar

Isolated	**ChitinaseRS(mg/mL)**[(a)]	**β-1,3-Glucanase RS (mg/mL)**
ACB-08	0,00	0,10
ACB-12	0,00	0,07
ACB-63	0,00	0,30
ACB-69	0,00	0,06

[(a)] RS – Reducing sugar

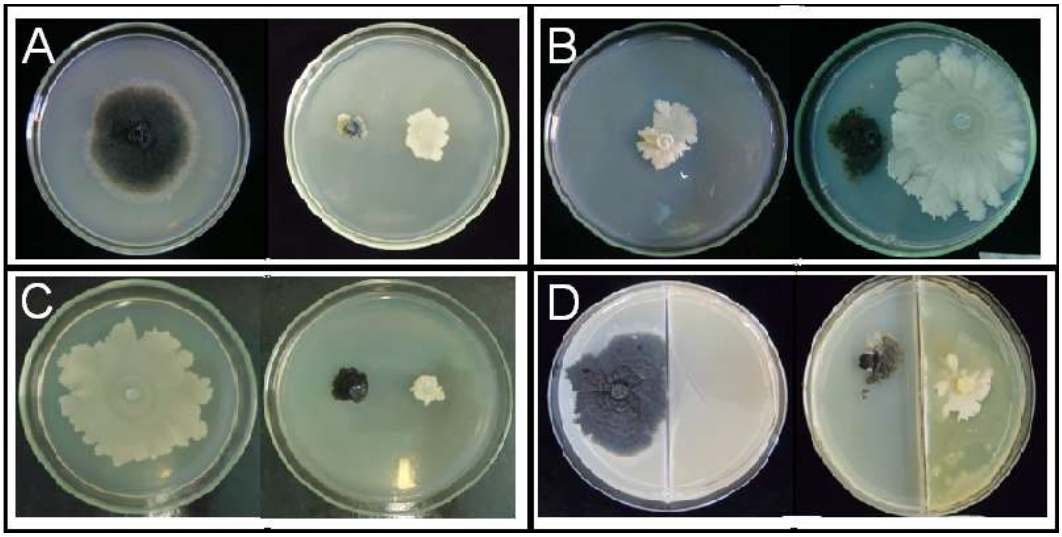

Figure 1. Microbial Interactions. A- Influence of *Bacillus* (ACB-12) on mycelial growth of *Phyllostictacitricarpa* in paired cultivation. B- *P.citricarpa* favors the growth of *Bacillus* (ACB-08). C- *P. citricarpa* inhibits the development of *Bacillus* colony (ACB-48). D- Influence of volatile metabolites of *Bacillus* (ACB-19) on mycelial growth of *P. citricarpa.*