Exploration of microbial antagonists in chili to control anthracnose disease *in vitro*

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Abstract. Anthracnose disease causes deterioration in chili production by 50–100%. Farmers often use synthetic fungicides to control this disease. However, these synthetics are unsafe for humans, ecosystems, and the environment. Microbial antagonists are expected to take the place of these synthetics in the control of this disease. This study aimed to assess several microbes from chilli plants for their potential as antagonists against anthracnose disease in chili. The research was conducted at the Plant Disease Laboratory of the Faculty of Agriculture, Universitas Sumatera Utara, from January to December 2023 through microbial isolation and characterization. Microbes producing inhibition zones were re-inoculated using the streak plate method in triplicates to obtain pure cultures. These bacterial and fungal pure cultures were labelled for further assessment. *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* were used for pathogenicity and antagonistic tests in duplicates. From the assessments, seven bacterial isolates (11, 34, 58, 90, 111, 188, and 215) and three fungal isolates (*Trichoderma* spp. 1, *Trichoderma* spp. 2, and *Rhizopus* spp.) were found to exhibit antagonism (> 60%), synergism, and the ability to produce siderophores.

1 Introduction

Anthracnose is a devastating disease caused by *Colletotrichum* spp. fungi in chili in tropical and subtropical regions. Fungal infections can occur both in the field and the post-harvest stage, causing significant losses in yield by 50–100%. The pathogens infect all parts of the host plants, including stems and leaves, damaging the apical shoots and resulting in top rot. Severe outbreaks resulting from heavy rainfall, high soil moisture, and excessive irrigation

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can lead to total crop failures, putting farmers and consumers at a disadvantage, as horticultural commodities play an important role in the food industry [1].

Farmers often use high doses of synthetic fungicides to rapidly control anthracnose. However, excessive application of these synthetics negatively impacts the environment, ecosystems, and human health. Additionally, it can compromise beneficial microbial populations, increase synthetic residues in harvested commodities, and increase pathogen resistance [2]. Biocontrol application, including the use of microbial antagonists, is expected to replace the intensive use of synthetics. Microbilal antagonists have been shown to increase plant growth, protect plants from pathogens, and increase plant tolerance to abiotic and biotic stresses. Exploration and application of microbial antagonists from various plant parts, such as roots, leaves, and fruits, have reduced the reliance on synthetic fungicides in agriculture [3].

Microbial antagonists, particularly bacteria and fungi, are crucial for plant growth and development, providing nutrients, growth hormones, and protection from soil-borne and seed-borne pathogens through various substances that inhibit pathogen growth and spread. These substances, including antibiotics and enzymes, directly target pathogens or interfere with their ability to colonize and damage plant tissues. *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., and *Trichoderma* spp. are some of the microbes known to produce antibiotics.

Despite previous studies on microbial antagonists' activity against *C. acutatum and C. gloeosporioides*, two *Colletotrichum* species known to be responsible for anthracnose, such as those assessing *B. tequilensis* and *B. velezensis* [4, 5], investigations into this subject have been rare. Therefore, this study was conducted to contribute to the understanding on microbial antagonists' role in controlling anthracnose disease by identifying microbial isolates capable of suppressing the growth of these microbes by more than 60%.

2 Research methods

2.1 Sample collection

This study used chili plant parts (roots, stems, and leaves) obtained from chili plantation centers in four different locations in North Sumatra—Deli Serdang, Simalungun, Langkat, and Karo Regencies—at three different altitudes—low (0–200 m asl), moderate (400–600 m asl), and high (> 1,200 m asl)—where the plantations experienced anthracnose with disease incidence rates >50%. The diagonal systematic sampling method was employed to collect the samples. Root samples (infected and uninfected) were excavated by digging a soil layer to a depth of 30 cm. Stem samples were also taken from selected plants (infected and uninfected). Collection of leaf samples focused on plants facing east, where direct sunlight was most prevalent, including young leaves (second leaves) or medium leaves (third leaves). Sample collection was conducted using sterilized equipment. Each plant part was inserted into a separate labelled plastic bag, brought to the laboratory, and then stored in a cooled box at 4°C for further use.

2.2 Identification and isolation of causal agents of anthracnose from infected chili plants

A series of assays to identify and isolate causal agents of anthracnose from the samples was carried out at the Plant Disease Laboratory of the Faculty of Agriculture, Universitas Sumatra Utara. Before the assessments, the samples were sterilized using 70% alcohol, rinsed with distilled water, and air-dried. Nine pieces (5 mm × 5 mm) were cut from the infected skin tissue and fruit flesh samples each and placed on plates of a combination of PDA medium and chloramfenycol (100 mg L^{-1}), three pieces per plate. The plates were then incubated at room temperature (28 ± 2°C) for five days. Each colony detected as *Colletotrichum* spp. was cultured according to [6]. The pure cultures obtained from this process were used for identification according to their morphology under a microscope with 100–400× magnification.

2.3 Identification and isolation of microbial antagonists obtained from chili plants

Chili samples were sterilized using running water to avoid contamination with other fungi. The samples were then soaked in 50 mL of 70% alcohol for one minute and in 2% natrium hypochlorite for another minute, resoaked in alcohol for 30 seconds, and rinsed three times with distilled water. The samples were cut into pieces (1 cm x 1 cm) for assessments. Microbial isolation was conducted using two methods: spread plate method and inoculation method.

2.3.1 Spread plate method

In the spread plate method, samples (5 gr) were ground using mortar and added with 0.5 mL of distilled water. The suspension was diluted five times (final inoculum of 10^{-5}). Afterward, 1 µL of the suspension was pippeted into PDA- and NA-mediated Petri dishes and evenly spread over the surface of the medium using L-shaped cell spreaders.

2.3.2 Inoculation/streaking method

Some samples were introduced to NA medium and incubated at 28°C for 24 hours, and some others were introduced to PDA medium and incubated at the same temperature for 48 hours. The isolates producing clear zones were replaced to NA medium, where quadrant streaking was applied three times to acquire pure cultures.

Fungal isolates obtained from PDA medium were recultured using the dot method, where each isolate was placed into other PDA medium using a sterelized toothpick. The dishes were labelled and incubated for 5–7 days at room temperature. The fungal pure cultures were then divided based on their macroscopic and microscopic characteristics. The selected isolates were assessed for their antagonistic activity against *C. acutatum* and *C. gloeosporioides.* From this assesment, the isolates producing clear zones greater than 60% were used for haemolysis in blood agar and pathogenicity tests in cabbage plants.

2.4 Antagonistic activity of microbial antagonists (bacteria and fungi) against *C. acutatum* and *C. gloeosporioides*

Antagonistic tests were carried out using the dual culture method in a Completely Randomized Design (CRD) and replicated three times. In the dual culture method, bacterial isolates were introduced to PDA media, and pathogens were streaked 30 mm from them (Fig 1). Plates which contained only fungal pathogens served as controls. Similar treatments were implemented on fungal candidates as well. The radial growth of the pathogens in this method was measured after 18 days and 15 days after incubation for bacteria and fungi, respectively.

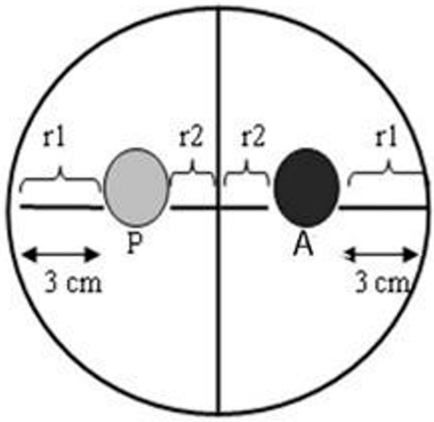

Fig 1. Antagonistic assessment in plates

Microbial growth was observed every three days. The clear zones produced by the microbes proved that the microbes (bacteria and fungi) are pathogenic. Inhibition zones greater than 60% that were produced by the isolates suggest the isolates' potential as antagonists. To investigate the mechanism of action of fungi a macroscopic observation was performed according to [7] with a slight modification.

The formula below was applied to calculate the percentage of clear zone produced by an antagonistic agent:

$$\text{PIRG} = (r_1 - r_2)/r_1 \text{ x } 100\%$$

where PIRG is the percent inhibition of radial growth, r_1 is the radial growth of the pathogen colony (P) in the control plate, and r_2 is the radius of the pathogen colony in the direction toward the antagonist colony (A) [8]. The data collected were subjected to statistical analysis using SPSS Univariate. Significant differences between means were separated using Duncan's Multiple Range Test (DMRT) at the 0.05 probability level.

2.5 Microbial synergism

The assessment for microbial synergism was conducted using the zigzag streak method in NA-mediated plates. A sterile inoculation loop was used to streak the first microorganism in a straight line across the center of an agar plate. Then, the second microorganism was streaked across the first streak (Figure 2). Further, the plate was incubated, and the growth patterns were observed. Synergism occurred when the two microorganisms Exhibit éd increased growth compared to their individual growth patterns with no clear zones of inhibition between the streaks. Antagonism might be observed as clear zones or reduced growth at the intersection, indicating that one microorganism was inhibiting the other.

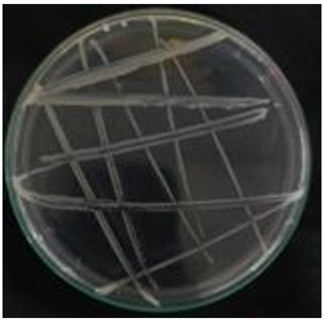

Fig 2. Zigzag streak method for the microbial synergism test

2.6 Siderophore-producing microbes

Arnow's assay was employed to determine siderophore production by bacteria. One-day bacterial isolates were inoculated on sterilized Nutrient Broth (NB) media. The selected isolates were aseptically inoculated into Fe-CAS media using the streak plate method and incubated for 24 hours. The bacteria producing siderophores were yellow or orange in color, which was in contrast with the blue color of the agar media. The experiments were carried out in UV light. The isolates were further used for metal bio-removal assays. An Fe-resistant assay was to be applied if all isolates were confirmed to be producing siderophores.

3 Results and discussion

3.1 Antagonistic activity of microbial isolates (bacteria and fungi) against *C. acutatum* and *C. gloeosporioides*

3.1.1 Antagonistic activity of bacterial isolates against C. acutatum and C. gloeosporioides

There were 218 bacterial isolates obtained from chili plantation centers in the four study areas in North Sumatra. After haemolysis and pathogenicity tests, we found 42 potential candidates for antagonistic tests against *C. acutatum* and *C. gloeosporioides* (Tables 1 & 2). We obtained 40 fungal isolates from chili plants and 10 fungal candidates for antagonistic tests against the two pathogens (Tables 3 & 4).

Table 1. Antagonistic activity of selected bacteria against *C. acutatum*

Isolates	Bacterial antagonistic activity			
	Day 9	**Day 12**	**Day15**	**Day 18**
Control	.0000 a	.0000 a	.0000 a	.0000 a
11	43.6985 fg	57.8630 ef	64.7148 l	71.4065 p
29	31.7645 cdefg	34.8851 bcdef	37.8026 bcd	39.7792 bc
30	28.4994bcdefg	32.8965 bcde	42.1800 bcdefg	44.4483 bcdefg
33	17.1220 b	25.9533 bcd	35.3187 bc	37.8823 b
34	44.0151 fg	44.3934 bcdefg	63.9143 l	65.4590 mnop
36	27.1155 cdefg	24.0426 b	39.0717 bcde	53.0837 ghijk
42	41.0965 efg	40.1714bcdefg	54.5220 hijkl	61.6483 lmnop
48	40.7151 efg	49.6374 efg	51.9100 ghijk	61.3657 lmnop

Table 1. Antagonistic activity of selected bacteria against *C. acutatum* (continue)

Isolates	Bacterial antagonistic activity			
	Day 9	**Day 12**	**Day15**	**Day 18**
Control	.0000 a	.0000 a	.0000 a	.0000 a
49	37.7785 cdefg	48.8167 defg	51.3733 fghijk	53.9097 hijkl
58	40.0460 defg	58.3775 f	63.0347 l	70.2373 p
60	30.6138 bcdefg	31.1043 bcde	38.1557 bcd	53.8340 hijkl
73	30.4000bcdefg	36.4658bcdefg	38.8367 bcde	44.6400 bcdefg
82	30.8889bcdefg	34.3649 bcde	41.3835 bcdefg	51.5831 fghijk
90	46.0006 g	46.5242bcdefg	55.7973 ijkl	65.9211 nop
101	28.4239bcdefg	31.0409 bcde	36.1595 bc	44.5267 bcdefg
111	40.5020 defg	49.6720 efg	61.6436 kl	65.4440 mnop
116	35.8291bcdefg	36.5838 bcdefg	41.7271 bcdefg	49.9867 efghij
117	23.2533 bcdef	25.2833 bc	31.6846 b	49.9557 fghijk
118	29.4683bcdefg	30.6200 bcde	32.0447 b	41.0772 bcd
122	38.3527 cdefg	46.8351 bcdefg	51.2181 fghijk	56.0897 ijkl
129	29.7577bcdefg	31.3900 bcde	39.8563 bcdef	48.768923w
131	28.7305bcdefg	33.0069 bcde	37.7333 bcd	43.7633 bcdef
133	25.7291bcdefg	33.0069 bcde	34.9708 bc	51.4576 fghijk
136	25.7935bcdefg	34.7646 bcdef	34.8471 bc	51.3289 fghijk
137	41.8032 efg	48.4279 cdefg	50.3841 efghijk	56.9683ijkl
138	40.2255 defg	49.1594 defg	50.1297efghij	56.0052ijkl
139	37.5279bcdefg	38.3804 bcdefg	43.5758bcdefgh	44.6529 bcdefg
157	24.4075 bcdef	25.8800 bcd	33.9061 bc	43.8885 bcdef
161	21.2651 bcdef	23.4600 b	35.2490 bc	51.7468 fghijk
179	40.6892 efg	43.8256bcdefg	50.9569 fghijk	57.8270 jklm
184	26.9110bcdefg	40.4582bcdefg	43.3100bcdefgh	51.5231efghijk
187	33.0591bcdefg	37.5606bcdefg	40.1067 bcdef	52.3045 fghijk
188	44.0033 fg	48.8115 defg	60.5967 jkl	67.3488nop
190	29.7967bcdefg	32.2030 bcde	33.8719 bc	47.0905cdefgh
202	19.1400 bc	27.2035 bcde	32.7364 bc	44.6602bcdefg
207	19.7159 bcd	28.7031 bcde	32.0911 b	42.3996 bcde
214	21.5546 bcde	35.7657bcdefg	41.3267 cdefg	43.9867 bcdef
215	39.4227 cdefg	50.0072 efg	61.0474 jkl	69.6825 op
216	25.3036bcdefg	39.1989bcdefg	44.2999 defgh	50.5091 efghij
217	30.0297bcdefg	36.7549bcdefg	42.6142 cdefg	44.6467bcdefg
218	27.5066bcdefg	37.1877bcdefg	48.0580 defghi	50.2983efghij

According to Table 1, the 42 bacterial isolates tested had different levels of inhibitory activity against *C. acutatum* and different inhibition zone percentages. On day 18, isolate 11 produced the largest inhibition zone (71.40%), followed by isolate 58 (70.24%), isolate 215 (69.68%), isolate 188 (67.34%), isolate 34 (65.45%), isolate 90 (65.92%), isolate 42 (61.64%), and isolate 48 (61.36%). Inhibition zone percentages above 60% show that the bacterial isolates had strong potential for antagonistic activity against the anthracnose-causing pathogen. This was due to the production of antimicrobial compounds such as hydrolytic enzymes (chitinase, protease, and β-1,3-glucanase), as well as secondary metabolites such siderophores, surfactants, and antibiotics, by the bacterial isolates. Research [9] has confirmed that siderophores, produced by microbes such as *Pseudomonas* sp., can suppress pathogen growth by competing for iron availability. In addition, the antagonistic activity of these isolates is related to secretion of several enzymes such as protease, amylase, and chitinase. The consistently large inhibition zones observed around several isolates in this study further support their potential development as microbe-based biofungicides. The lowest antagonistic effect was recorded for isolate 33 (37.88%).

Table 2. Antagonistic activity of selected bacteria against *C. gloeosporioides*

Isolates	Bacterial antagonistic activity			
	Day 9	**Day 12**	**Day15**	**Day 18**
Control	.0000 a	.0000 a	.0000 a	.0000 a
11	44.3779 ij	54.6121 hijklm,	62.2640 hijk	69.9447 jkl
29	16.7781 abc	21.2380 bc	33.2101 b	52.6297 bc
30	14.5733 ab	27.3357 bcdef	33.3167 b	43.5333 b
33	17.1151 abc	27.9945 bcdefg	35.0697 bcd	57.9645 cdefgh
34	45.0577 j	52.7243 hijklm	68.1806 k	70.9311 kl
36	25.3821 bcdefghij	45.4950 fghijklm	47.3433 efg	50.5466 bc
42	44.8484 j	58.2993 jklm	65.2516 ijk	70.2448 jkl
48	41.2316 hij	57.7225 ijklm	64.2244 hijk	70.2827 jkl
49	38.4497 efghij	48.3753 ghijklm	60.0459 hijk	60.5028 hijkl
58	38.0896 defghij	57.0879 ijklm	68.2859 k	69.7054 jkl
60	31.4145 bcdefghij	37.5020 bcdefghi	46.8931 efg	58.6728 defghi
73	25.5867 bcdefghij	28.0700 bcdefg	32.9100 b	44.3400 b
82	43.4793 ij	46.1643 fghijklm	59.5374 hijk	59.6310 defghij
90	37.2507 cdefghij	59.3088 lm	64.2967 hijk	69.1049 ijkl
101	36.2333 cdefghij	45.1613 efghijklm	55.3789 ghi	59.8591 defghi
111	40.0716 ghij	61.8566 m	69.0306 k	70.1654 jkl
116	35.9129 cdefghij	38.6109cdefghijkl	48.5000 efg	56.1048 defg
117	28.8700 bcdefghij	44.1700 fghijklm	57.0267 ghij	60.6473 fghijkl
118	14.5756 ab	17.6890 b	31.7904 b	44.6433 bc
122	40.6172 ghij	56.6443 ijklm	67.4016 jk	69.7763 jkl
129	19.0673 ghij	38.9332cdefghijkl	39.6436 bcde	56.0127defg
131	17.7717 abcd	27.3460 bcdef	38.2333 cde	44.5267bc
133	17.4870 abc	44.8322efghijklm	59.0842 hijk	58.0319 defgh
136	36.3933 cdefghij	38.8988 defghijkl	42.5767 bcde	58.0541 defghi
137	40.1103 ghij	54.6444 hijklm	58.6167 hijk	60.8554 ijkl
138	40.0333 ghij	56.4175 ijklm	62.9746 hijk	69.6867 jkl
139	38.2169 defghij	42.0306 efghijklm	53.6592 fgh	55.2289 defg
157	18.3367 abcde	22.3400 bcd	39.1210 bcde	56.0116 efg
161	21.1838 bcdefgh	24.6199 bcde	34.9955 bcd	59.6359 efghi
179	39.7373 fghij	59.0745 klm	61.0881 hijk	61.1257 jkl
184	26.3784 bcdefghij	30.4841 bcdefg	36.1528 bcd	45.0393 dfghijk
187	33.2149 bcdefghij	35.4084 bcdefgh	35.5257 bcd	45.8798 fghijk
188	40.1142 ghij	59.5221 lm	60.2620 k	67.3172 k
190	24.1312 bcdefghi	27.0943 bcdef	33.6353 bc	58.1344 defgh
202	21.9467 bcdefgh	30.5533 bcdefg	32.4968 b	44.8533 ghij
207	19.3750 abcdef	20.8234 bc	31.8742 b	44.960 ghijk
214	20.4578 bcdefgh	29.8067 bcdefg	44.3600 def	44.8800 bc
215	40.4067 ghij	58.9656 klm	65.0455 ijk	70.1870 jkl
216	29.2997 bcdefghij	37.9163 bcdefgij	44.0689 cdef	54.5888 cdef
217	27.7624 bcdefghij	30.6400 bcdefg	31.8400 b	43.4914 b
218	28.5324 bcdefghij	42.4058 defghijklm	47.8067 efg	56.4066 defg

Of the 42 bacterial isolates screened, 12 demonstrated antagonistic activity by greater than 60% against *C. gloeosporioides* at the end of the day of observation (11, 34, 42, 48, 58, 90, 111, 122, 138, 179, 188, and 125). Isolate 34 produced the largest zone (70.9311%) and isolate 30 produced the smallest zone (43.5333). Five isolates produced clear zones at more than 70% on day 18 (isolates 34, 42, 48, 111 and 215), exhibiting significant increases in antagonism against the pathogens consistently. The highest antagonisitic activity of the bacterial isolates rendered the production of antimicrobial compounds such hydrolytic enzymes (e.g., chitinase, protease, amylase, and β-1,3-glucanase), proving their potential as biocontrol agents. Research [10] has confirmed that antagonistic activity is related to the production of these enzymes by bacteria, as they are capable of damaging the cell walls of *Colletotrichum* spp.

3.1.2 Antagonistic activity of fungal isolates against C. acutatum and C. gloeosporioides

Tables 3 and 4 present fungal isolates that exhibited an antagonistic mechanism against *C. acutatum* and *C. gloeosporioides*.

Table 3. Antagonistic activity of selected fungi against *C. acutatum*

Fungi	Antagonistic activity of fungi				
	Day 3	Day 6	Day 9	Day 12	Day 15
Control	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
Rhizopus sp.	12.94 de	31.90 b	33.10 de	53.69 de	63.32 cd
Aspergillus spp. 1	7.65 bcd	28.31 b	29.56 bcd	51.87 cd	61.29 bc
Aspergillus sp. 2	7.58 bcd	27.67 b	28.97 bc	51.20 bcd	60.86 bc
Unidentified fungi	4.85 ab	26.40 b	28.48 bc	48.92 bc	59.32 b
Aspergillus sp. 3	7.73 bcd	27.21 b	27.54 bc	48.12 b	60.13 b
Aspergillus sp. 4	11.06 cde	26.28 b	30.09 cd	51.17 bcd	61.70 bc
Penicillium sp 1	5.32 abc	27.27 b	25.56 b	48.74 bc	61.08 bc
Penicillium sp 2	6.73 bc	28.06 b	28.56 bc	50.83 bcd	61.50 bc
Trichoderma spp 1	14.50 e	33.43 b	34.58 ef	55.58 e	65.00 d
Trichoderma spp 2	13.68 e	34.69 b	37.81 f	56.23 e	65.72 d

Table 4. Antagonistic activity of selected fungi against *C. gloeosporioides*

Fungi	Antagonistic activity of fungi				
	Day 3	Day 6	Day 9	Day 12	Day 15
Control	0.00a	0.00a	0.00a	0.00a	0.00a
Rhizopus sp.	13.31bcd	15.66bc	41.39b	54.94cd	64.45c
Aspergillus spp. 1	12.99bcd	13.81bc	37.51b	52.76bc	61.29c
Aspergillus sp. 2	12.73bcd	14.37bc	39.29b	52.84bc	60.86c
Unidentified fungi	10.80bc	12.16b	33.94b	49.97b	59.32bc
Aspergillus sp. 3	12.83bcd	14.43bc	43.60b	49.48b	53.37b
Aspergillus sp. *4*	15.34d	17.58c	36.28b	52.46bc	61.70c
Penicillium sp *1*	10.33b	11.21b	40.83b	50.68b	61.27c
Penicillium sp *2*	9.70bc	11.25b	41.13b	51.99bc	62.06c
Trichoderma spp 1	13.75bcd	15.53bc	52.95c	56.60d	65.15c
Trichoderma spp 2	14.11cd	16.59c	56.85c	57.27d	66.05c

Several fungal isolates demonstrated potential as antagonists with an ability to suppress the growth of the pathogens by > 60%. However, it is important to note that, of these isolates, *Penicillium* spp. did not pass the haemolysis test, indicating that it is unsafe for human health, rendering it unsuited to be an antagonist candidate. Only three fungal isolates investigated were found to be safe for both plants and humans, namely, *Trichoderma* spp. 1, *Trichoderma* spp. 2, and *Rhizopus* sp. *Trichoderma* spp. 2 exhibited the highest antagonistic activity against *C. acutatum* and *C. gloeosporioides* on day 15 (65.72% and 66.05%, respectively), followed by *Trichoderma* spp. 1 (65.0% and 65.15%, respectively) and *Rhizopus* sp. (63.32% and 64.45%, respectively).

These results highlight the ability of *Trichoderma* spp. in controlling the two causal agents of anthracnose. The rapid growth of *Trichoderma,* combined with the mycoparasitism effect, reduced the ability of pathogens to grow. These results align with [11], which evaluated the antifungal activity of five fungal isolates of *Trichoderma* spp. Against *C. gloeosporioides.* The results showed that all of these isolates had antifungal effect against *C. gloeosporioides*. In addition, [12] confirmed that *Trichoderma harzianum* IC-30 reduced fruit rot in oranges in storage by 80% after three weeks of incubation by breaking down the pathogen's cell walls, exhibiting high enzymatic activity (secretion of chitinase and glucanase).

3.2 Microbial synergism

3.2.1 Bacterial synergism

The 12 bacterial candidates (isolates 11, 34, 42, 48, 58, 90, 111, 122, 138, 179, 188, and 215) were tested for their synergism in achieving a combined effect, resulting in enhanced metabolic activity in suppressing the growth of *C. acutatum* dan *C. gloeosporioides* (Table 5).

Table 5. Bacterial synergism

Isolates	11	34	42	48	58	90	111	122	138	179	188	215
11	-	+	+	+	+	-	+	-	-	+	+	+
34	+	-	+	-	+	+ -	+	+	-	-	+	+
42	+	+	-	+	+	-	-	+	-	+	-	-
48	+	+	+	-	+	+	+	+	+	+	-	+
58	-	+	+	+	-	+	+	+	+	+	+	+
90	-	+	-	-	+	-	+	+	+	+	+	+
111	+	+	+	+	+	+	-	-	+	+	+	+
122	+	+	+	+	+	+	-	-	+	-	-	+
138	-	-	+	+	+	+	+	+	-	-	+	+
179	+	+	-	+	+	+	+	-	-	-	+	-
188	+	+	-	-	+	+	+	-	+	+	-	+
215	+	+	-	+	+	+	+	-	+	-	+	-

(+) = synergystic activity; (-) = no synergystic activity

Based on Table 5, of the total of 144 bacterial isolates tested, only 105 showed synergism. Higher levels of synergystic activity were demonstrated by nine isolates (11, 34, 48, 58, 90, 111, 122, 179, and 188), as presented in Figure 3. These nine isolates exhibited stronger efficacy in controlling the plant disease. According to [13], there are several factors affecting the success of synergistic activity between two antagonists: an absence of nutrient competition and an absence of toxins during the process of synergism. In addition, bioactive compound compatibility and carbon source complexity have been proven to be essential to the success of bacterial synergism.

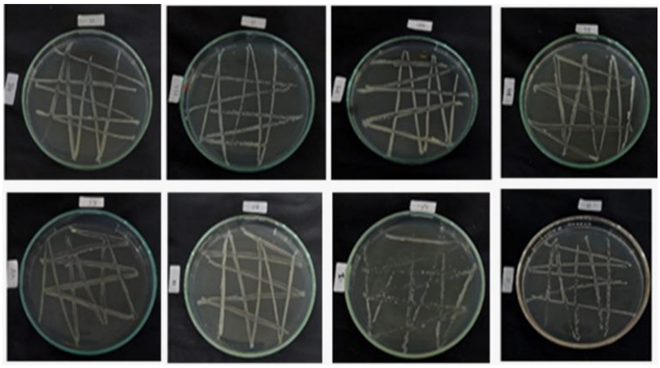

Fig 3. Synergysm tests on NA-mediated plates

3.2.2 Bacterial–fungal synergism

Table 6 shows the results of bacterial–fungal synergism identification between three selected fungal isolates (*Trichoderma* spp. 1, *Trichoderma* spp. 2, and *Rhizopus* spp.) and 12 bacterial isolates (isolates 11, 34, 42, 48, 58, 90, 111, 122, 138, 179, 188, and 215).

Table 6. Bacterial–fungal synergism

Bacterial isolates	***Trichoderma* spp. 1**	***Trichoderma* spp. 2**	***Rhizopus* spp.**
11	+	+	+
34	+	+	+
42	+	+	-
48	-	-	+
58	+	+	+
90	+	+	+
111	+	+	+
122	-	-	+
138	-	-	+
179	-	-	-
188	+	+	-
215	+	+	-

(+) = synergystic activity; (-) = no synergystic activity

We found that six bacterial isolates formed synergism with three fungal isolates (isolates 11, 34, 58, 90, and 111). The enzymes lipopeptide, cycloserine, and glucosidase, when present together, enhance a synergistic process in fungi, aiding their adaptation without causing toxicity. Lignolytic and cellulolytic enzymes play a crucial role in the degradation of lignocellulosic materials, and the formation of biofilms can enhance their activity and reduce competition. By forming a biofilm, they create a "cooperative" environment where enzymes are concentrated, protecting them from external factors and increasing their efficacy [14].

3.3 Detection of siderophore production by bacterial and fungal isolates

Table 7 shows that more than 50% of the bacterial isolates produced siderophores, indicated by an orange color that appeared in the media.

Table 7. Siderophore production by bacterial isolates

Bacterial isolates	The presence of siderophores
11	+
34	+
42	-
48	-
58	-
90	-
111	+
122	-
138	+
179	
188	+
215	+

(+) = siderophores produced ; (-) = no siderophores produced

There were six isolates producing siderophores, namely, isolates 11, 34, 111, 138, 188, and 215. These six isolates are considered potential as biocontrols in iron-deficient environments. In agriculture, siderophore-producing bacteria are essential. Bacterial siderophores help beneficial fungi by providing them important nutrients, particularly iron. Besides, they inhibit pathogen growth by rapidly lowering the number of ferric ions in the rhizosphere [15].

Table 8. Fungal isolates producing siderophores

Fungal isolates	The presence of siderophores
Rhizhopus sp.	+
Trichoderma spp. 1	+
Trichoderma spp. 2	+

(+) = siderophores produced ; (-) = no siderophores produced

Based on Table 8, all the three fungal isolates were found to have the ability to produce siderophores, as indicated by the presence of an orange color in the CAS media. This finding shows that *Trichoderma* spp. and *Rhizopus* sp. can inhibit pathogen growth, not only by producing enzymes to degrade the pathogens' cell walls, but also by producing iron to inhibit the infection, or by competing for iron with the pathogens in the soil.

4 Conclusion

Nine bacterial isolates (isolates 11, 34, 42, 48, 58, 90, 111, 188, and 215) demonstrated the best antagonistic mechanism against *C. acutatum*, while twelve bacterial isolates (isolates 11, 34, 42, 48, 58, 90, 111, 122, 138, 179, 188, and 215) showed antagonistic activity against *C. gloeosporioides* with inhibition greater than 60%. Eight bacterial isolates (isolates 11, 34, 48, 58, 90, 111, 188, and 215) exhibited synergism when cultured together, and among these, six isolates (isolates 11, 34, 58, 90, 111, and 188) were identified as siderophore producers. Furthermore, only seven bacterial isolates (isolates 11, 34, 58, 90, 111, 188, and 215) simultaneously exhibited antagonism (greater than 60%), synergism, and the ability to produce siderophores. In addition, three fungal isolates (*Trichoderma* spp. 1, *Trichoderma* spp. 2, and *Rhizopus* sp.) were found to simultaneously demonstrate antagonism, synergism, and siderophore production.

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