

# *In Vitro* Antagonistic Activity of *Trichoderma harzianum* and *Bacillus amyloliquefaciens* against *Colletotrichum acutatum*

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## Abstract

Biological control of plant diseases makes extensive use of the antagonists. The effectiveness of the control depends on the choice of an effective antagonist strains from criteria of implying a good knowledge of biological peculiarities of the material used. In this study, the antagonistic activities of nine isolates of *Bacillus amyloliquefaciens* and one isolate of *Trichoderma harzianum* were tested *in vitro* against seven strains of *Colletotrichum acutatum*, the causal agent of strawberry anthracnose. It was found that *T. harzianum* and *B. amyloliquefaciens* strains showed the ability to inhibit the mycelial growth of the pathogen by dual culture technique by more than 50 percent. The nonvolatile substances produced by the antagonists showed high inhibition percentages that are more than 99 percent, meanwhile their volatile compounds inhibited the mycelial growth of *C. acutatum* strains with low inhibition percentages starting from 30 percent except the substances produced by *B. amyloliquefaciens* Bc2 that showed a high inhibition percentage of more than 70 percent.

## Keywords

Biocontrol, Antagonism, *Bacillus amyloliquefaciens*, *Trichoderma harzianum*, Anthracnose, *Colletotrichum acutatum*

## 1. Introduction

Dissemination of phytopathogens has been detected from nurseries to plantations through asymptomatic plants. Consumers require less chemical residues on products and many fungi have developed resistance to commonly used fungicides [1]. In addition, the use of chemical pesticides is becoming increasingly restricted due to environmental and health concerns [2] [3].

The antagonistic microorganisms such bacteria and fungi provide an alternative source of control of these pathogens. Biological control is an alternative to the use of chemical pesticides with the benefits of greater consumer acceptance and reduced environmental impact.

In addition, microbial diversity in soil is considered important for maintaining the sustainability of agricultural production systems. The quantity and activity of microorganisms is a determining factor for the productivity of all types of soil [4].

The selection of antagonists for biological control of plant diseases usually involves collecting and examining a large number of microbial isolates to increase the likelihood of finding a highly effective strain. Natural antagonists on host surfaces are a promising component of organic crop protection [5]. An environmentally friendly biological control using antagonistic bacteria has been successful in controlling many post-harvest pathogens.

Natural compounds produced by bacteria and fungi antagonists are an important source of biopesticides and special conditions are required for the extraction and optimization of secondary metabolites that have an antibiotic effect [6].

In Morocco, the control of anthracnose in cultivated strawberry uses chemical pesticides in spite of their harmful effects. Many pathogenic microorganisms have developed resistance against chemical fungicides. This seriously hampers the management of phytopathologies. Considering the deleterious effects of chemical phytosanitary products on the maintenance of life systems, it is urgent to find an alternative for the management of pathogenic microorganisms. The present work aims to evaluate the antagonistic potential of *Bacillus amyloliquefaciens* and *Trichoderma harzianum* against *Colletotrichum acutatum* isolates.

## 2. Material and Methods

### 2.1. Pathogenic Material

Seven isolates of *Colletotrichum acutatum* (Ca1, Ca2, Ca3, Ca4, Ca5, Ca6 and Ca7) from naturally infected strawberries with anthracnose symptoms of strawberry field from Loukkos region (Larache, Morocco) were isolated and purified at the laboratory of plant biotechnology in Faculty of Science of Tetouan. They were grown on PDA (Potato Dextrose Agar) medium 7 to 10 days at 25°C in the dark. Successive subcultures were carried out until complete purification. Their identification was performed by macroscopic and microscopic observations of the isolates using determination keys [7] [8].

## 2.2. Antagonist Material

### 2.2.1. Bacterial Antagonists

Nine bacterial strains were isolated by serial dilution from the soil of rhizosphere and strawberry plant roots of the Loukkos region (Larache, Morocco), and identified by [9]. The molecular identification revealed that the nine bacterial isolates are *Bacillus amyloliquefaciens*. Strains were initially noted by an arbitrary notation I1, I2, I3, I18, B3, B12, RA9 and RA12 (Table 1).

### 2.2.2. Fungal Antagonist

The strain of *Trichoderma harzianum* (TR) was isolated from the soil in Petri dishes containing PDA using a spreading plate technique. The litter materials were grown in PDA culture medium for isolation. The TR strain was isolated in pure culture on PDA.

## 2.3. Effect on Mycelial Growth by the Direct Confrontation (Dual Culture)

### 2.3.1. Trichoderma Harzianum

*Trichoderma* strain was evaluated against the seven isolates of *Colletotrichum* by the technique of dual culture [10] [11]. Mycelial discs 5 mm indiameter were excised from the edge of the actively growing antagonist and each pathogen was cultured at the opposite end of a Petri dish at the same distance from the periphery. A completely random experimental setup was used with three replicates for each isolate. The inoculated Petri dishes were incubated at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 5 days. After the incubation period, the radial growth of pathogens was measured and the percent inhibition of mean radial growth was calculated relative to the control as follows:

$$L = [(C - T)/C] \times 100$$

*L*: Percent inhibition of radial mycelial growth,

*C*: Radial growth of the control pathogen,

*T*: Radial growth of the pathogen in the presence of *Trichoderma* [12].

**Table 1.** Identification of antagonist strains of *Bacillus amyloliquefaciens* [9].

Strain's name	Code of strain after identification	Similarity Percent %	Reference of strain
I1	<i>B. amyloliquefaciens</i> Bc1	99.8% (1014/1016 pb)	LMG 22478
I2	<i>B. amyloliquefaciens</i> Bc2	99.8% (1033/1035 pb)	CR-502
I3	<i>B. amyloliquefaciens</i> Bc3	100% (1030/1030 pb)	CR-502
I18	<i>B. amyloliquefaciens</i> Bc4	100% (1035/1035 pb)	CR-502
B3	<i>B. amyloliquefaciens</i> Bc5	99.9% (1020/1022 pb)	LMG 22478
B12	<i>B. amyloliquefaciens</i> Bc6	99.9% (1021/1022 pb)	LMG 22478
B24	<i>B. amyloliquefaciens</i> Bc7	99.9% (1019/1020 pb)	LMG 22478
RA9	<i>B. amyloliquefaciens</i> Bc8	99.9% (778/779 pb)	LMG 22478
RA12	<i>B. amyloliquefaciens</i> Bc9	99.9% (1035/1036 pb)	CR-502

### 2.3.2. *Bacillus Amyloliquefaciens*

The dual culture was performed using a mixed culture: a 5-mm diameter mycelial disc from culture of *Colletotrichum acutatum* was placed at one side of a Petri dish containing PDA medium and a bacterial isolate was seeded at 3.5 cm from the fungal strain in the opposite way. The dishes were incubated for 7 days at  $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Each treatment was repeated three times. The percent inhibition of radial growth (ICRP) was measured according to the following formula:

$$\text{PICR} = \frac{\text{Fungal growth control} - \text{Fungal growth near the antagonist}}{\text{Control fungal growth}} \times 100$$

## 2.4. Effect of Volatile Substances Produced by Antagonists by Remote Confrontation

This method consists in inoculating the antagonist and the pathogenic, in two plates separated thereafter, an assembly is carried out by the superposition of two plates, antagonist in bottom and the pathogenic one in top, the junction between the two plates is ensured by layers of Parafilm in order to avoid all loss of volatile substances [13].

### 2.4.1. *Trichoderma Harzianum*

To study the effect of the volatile substances of *Trichoderma harzianum* on the growth of pathogenic fungi on PDA, two disks (8 mm in diameter), one of the antagonist agent (*Trichoderma harzianum*) and the other of the pathogen, were placed in the center of two Petri dishes containing the PDA medium. The lids were removed aseptically, and then the bottom of the plate containing *Trichoderma* was placed below the one containing the phytopathogen, and the two juxtaposed plates were unsured by layers of Parafilm. The cultures were incubated for 4 days at  $25^{\circ}\text{C}$ . The test was repeated three times [14]. The percent inhibition of radial growth was measured according to the formula used in dual culture method.

### 2.4.2. *Bacillus Amyloliquefaciens*

The test of volatile antifungal compounds was carried out according to the method used by [14] [15]. Petri dishes containing LB medium were inoculated with 50  $\mu\text{l}$  of bacterial culture and incubated for 24 hours at  $28^{\circ}\text{C}$ . A 5 mm diameter mycelial disk of the phytopathogenic fungus was placed in the center of another PDA-containing Petri dish. The lids were removed and the box containing the bacillus was turned face to face on the one with the mycelial disc. The dishes were sealed with Parafilm and incubated at  $26^{\circ}\text{C}$  for 7 days. Each test was repeated three times. The percent inhibition of radial growth was measured according to the formula used in dual culture method.

## 2.5. Effect of Non-Volatile Compounds Produced by the Antagonists on the Growth of *Colletotrichum acutatum*

### 2.5.1. Suspension Preparation of *Trichoderma harzianum*

Each Erlenmeyer flask containing 15 ml PDB (Potato Dextrose Broth) was in-

oculated with a 5 mm diameter disc from the *Trichoderma harzianum* culture and incubated for 12 days at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  with shaking at 200 rpm. After this time, the suspension was filtered to remove the mycelium and spores then adjust concentration to  $10^8$  conidia/ml by using Malassez cell.

### 2.5.2. Preparation Suspension of *Bacillus amyloliquefaciens*

The bacterial strain was grown on Luria Bertani Agar (LBA) 24 h at  $28^{\circ}\text{C}$ . Then, using a sterile loop, a bacterial culture is prepared by inoculating 50 ml of the liquid medium Luria Bertani Broth with a colony of the strain studied, in a 250 ml Erlenmeyer flask. The cultures were incubated at room temperature with 125 rpm shaking for 2 days. After incubation, a volume of 5 ml of bacterial suspension is centrifuged at 4000 rpm for 10 min. The cells were washed, in the same volume of 5 ml of sterile distilled water, twice, using the centrifuge at the same conditions mentioned above [16].

The bacterial cells were then suspended in 5 ml of sterile physiological saline, and the suspension was adjusted to  $3 \times 10^8$  cell/ml according to the Mac Farland scale [17].

### 2.5.3. Mixture of Suspension of Antagonist with Pathogen Inoculum

The phytopathogenic fungi (*C. acutatum*) were inoculated ( $10^6$  conidia) and cultured in 50 ml of PDB medium for one week at  $28^{\circ}\text{C}$  at 200 rpm with presence (1% (v/v)) of the antagonist suspensions (*B. amyloliquefaciens* and TR) already prepared previously. For the control, distilled water was used instead of the antagonists.

The biomass was separated, dried 24 h at  $100^{\circ}\text{C}$  and weighed to obtain the dry weight. Values were expressed as a percentage:

$$\% \text{inhibition} = 1 - \frac{\text{Dry matter in the presence of the antagonist}}{\text{Dry matter of control}} \times 100$$

Three repetitions were performed.

## 3. Results

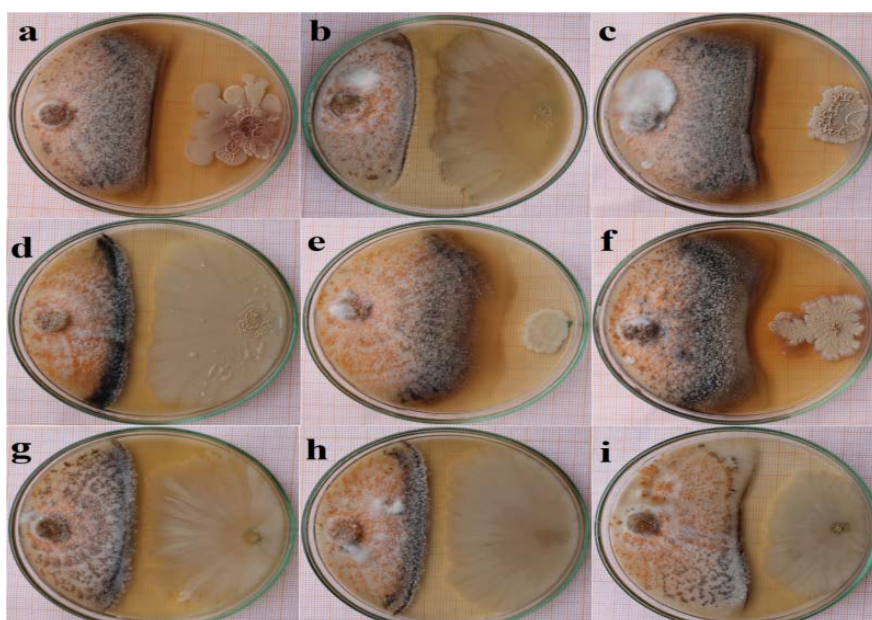
### 3.1. Effect on Mycelial Growth by Direct Confrontation (Dual Culture)

*In vitro* comparison showed that *Trichoderma harzianum* (Figure 1) and *Bacillus amyloliquefaciens* strains inhibit the growth of *Colletotrichum acutatum*-strains with varying efficiencies (Figure 1, Table 2).

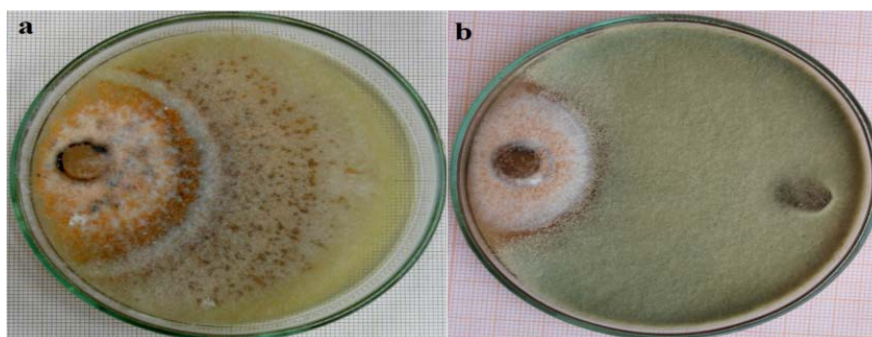
Direct challenge of *Trichoderma* with *C. acutatum* isolates resulted in up to 86.64% inhibition of the Ca1 isolate (Figure 2, Figure 3). The Bc2 isolate was effective against *C. acutatum* with percent inhibition ranging from 79.80% growth of the Ca7 isolate to 92.33% growth of the Ca5 isolate.

### 3.2. Effect of Volatile Substances Produced by Antagonists by Remote Confrontation

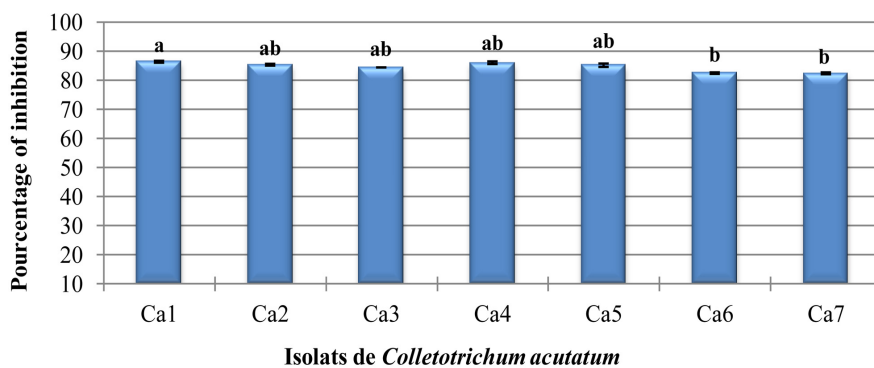
All bacterial isolates produce volatile substances inhibiting mycelial growth of *C.*



**Figure 1.** Growth of the Ca6 isolate in the presence of *Bacillus amyloliquefaciens* isolates: (a) Bc1, (b) Bc2, (c) Bc3, (d) Bc4, (e) Bc5, (f) Bc6, (g) Bc7, (h) Bc8 and (i) Bc9.



**Figure 2.** Growth of Ca6 isolate (a) without antagonist (b) and in the presence of *Trichoderma harzianum*.



**Figure 3.** Inhibition percent of mycelial growth of each isolate of *Colletotrichum acutatum* by *Trichoderma harzianum* by direct confrontation.

*acutatum* isolates. The percentage of inhibition did not exceed 42% for the Bc1, Bc3, Bc4, Bc5, Bc6, Bc7, Bc8 and Bc9 isolates, but in the case of the Bc2 isolate,



the inhibition of the Ca5 isolate reached up to at 72% (Table 3, Figure 4).

The volatile substances of *Trichoderma harzianum* have a variable inhibitory effect depending on the degree of resistance of the isolates of *C. acutatum*, the percentage inhibition of Ca3, Ca4 and Ca6 exceeding 46% whereas for the other isolates the inhibition is less than 44% (Figure 4 and Figure 5).

### 3.3. Effect of Non-Volatile Compounds Produced by Antagonists on the Growth of *Colletotrichum acutatum*

*Bacillus amyloliquefaciens* filtrates have a strong influence on the growth of *Colletotrichum acutatum* isolates in liquid medium and the percentage inhibition varies from one bacterial isolate to another insignificantly (Table 4). The same is true for the *Trichoderma harzianum* filtrate (Figure 6).

## 4. Discussion and Conclusion

In this study, *Bacillus amyloliquefaciens* and *Trichoderma harzianum* were found to have inhibitory effects against the pathogen *Colletotrichum acutatum*

**Table 2.** Inhibition percent of mycelial growth of *Colletotrichum acutatum* isolates in the presence of nine *Bacillus amyloliquefaciens* isolates by direct comparison.

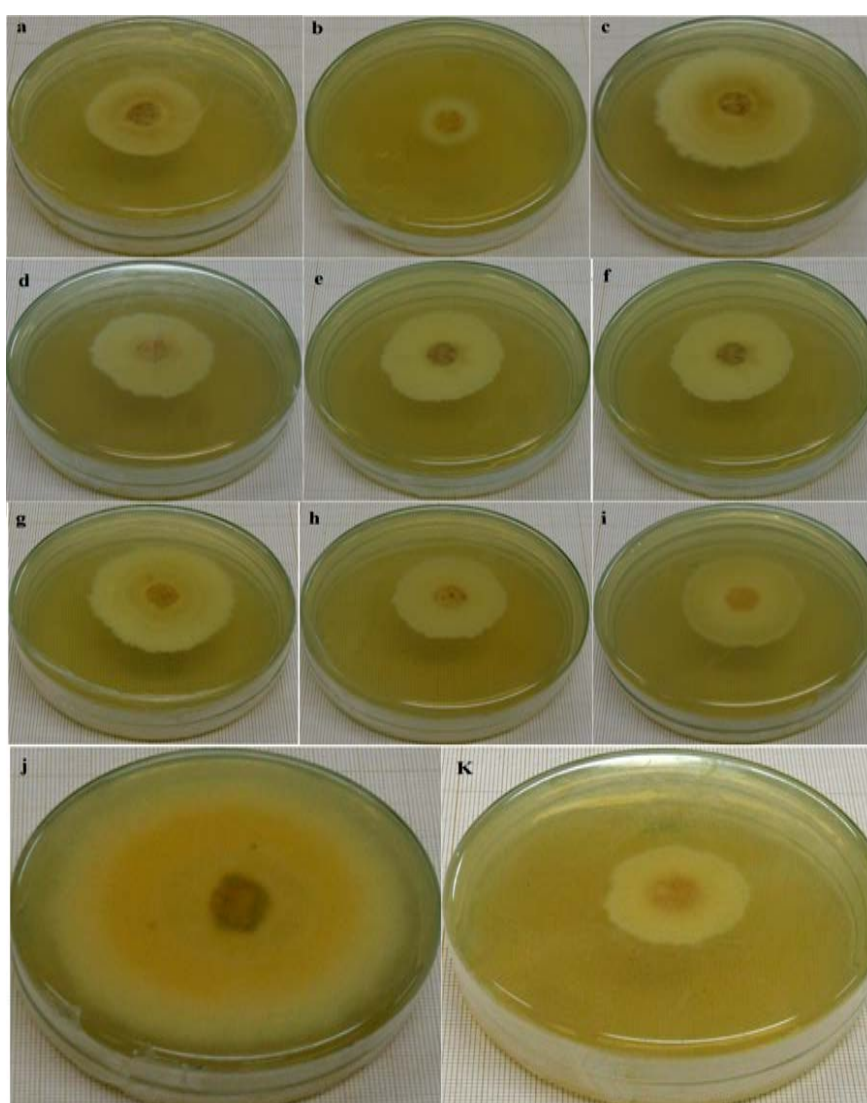
	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7
<b>Bc1</b>	76.21 ± 2.53 <sup>b</sup>	77.00 ± 1.14 <sup>c</sup>	76.86 ± 0.92 <sup>b</sup>	77.49 ± 0.30 <sup>bc</sup>	76.29 ± 2.61 <sup>c</sup>	76.97 ± 0.60 <sup>bc</sup>	70.38 ± 0.38 <sup>b</sup>
<b>Bc2</b>	88.40 ± 0.68 <sup>a</sup>	88.23 ± 0.29 <sup>a</sup>	82.31 ± 1.03 <sup>a</sup>	83.63 ± 0.59 <sup>a</sup>	92.33 ± 0.66 <sup>a</sup>	81.73 ± 0.10 <sup>a</sup>	79.80 ± 0.21 <sup>a</sup>
<b>Bc3</b>	64.07 ± 0.85 <sup>c</sup>	64.20 ± 1.33 <sup>f</sup>	61.26 ± 0.15 <sup>e</sup>	64.62 ± 1.46 <sup>d</sup>	82.07 ± 0.40 <sup>b</sup>	59.25 ± 0.75 <sup>f</sup>	55.23 ± 1.23 <sup>d</sup>
<b>Bc4</b>	84.62 ± 2.16 <sup>a</sup>	71.14 ± 0.45 <sup>e</sup>	77.76 ± 1.83 <sup>b</sup>	75.77 ± 0.33 <sup>bc</sup>	82.02 ± 1.32 <sup>b</sup>	71.85 ± 0.94 <sup>de</sup>	65.34 ± 1.34 <sup>c</sup>
<b>Bc5</b>	58.94 ± 1.05 <sup>c</sup>	64.81 ± 0.71 <sup>f</sup>	64.01 ± 1.05 <sup>e</sup>	65.93 ± 1.02 <sup>d</sup>	75.31 ± 1.63 <sup>cd</sup>	72.23 ± 1.32 <sup>de</sup>	65.93 ± 2.07 <sup>c</sup>
<b>Bc6</b>	75.65 ± 0.21 <sup>b</sup>	73.68 ± 0.46 <sup>de</sup>	76.27 ± 0.34 <sup>b</sup>	74.56 ± 0.88 <sup>c</sup>	73.47 ± 1.54 <sup>cd</sup>	75.71 ± 1.16 <sup>bcd</sup>	80.82 ± 0.82 <sup>a</sup>
<b>Bc7</b>	72.46 ± 0.53 <sup>b</sup>	84.04 ± 0.44 <sup>b</sup>	72.17 ± 0.06 <sup>c</sup>	80.12 ± 1.17 <sup>ab</sup>	71.53 ± 1.35 <sup>d</sup>	73.78 ± 1.05 <sup>cd</sup>	67.70 ± 1.70 <sup>bc</sup>
<b>Bc8</b>	84.05 ± 1.59 <sup>a</sup>	74.46 ± 2.05 <sup>cd</sup>	68.18 ± 0.34 <sup>d</sup>	66.38 ± 3.22 <sup>d</sup>	81.19 ± 0.49 <sup>b</sup>	77.98 ± 1.62 <sup>b</sup>	71.72 ± 0.28 <sup>b</sup>
<b>Bc9</b>	75.35 ± 0.10 <sup>b</sup>	77.00 ± 0.56 <sup>cd</sup>	74.77 ± 0.70 <sup>bc</sup>	67.25 ± 2.34 <sup>d</sup>	72.08 ± 0.14 <sup>cd</sup>	69.13 ± 1.79 <sup>e</sup>	68.67 ± 1.33 <sup>bc</sup>

**Table 3.** Percentage of inhibition of mycelial growth of *Colletotrichum acutatum* isolates by volatile substances produced by *Bacillus amyloliquefaciens*.

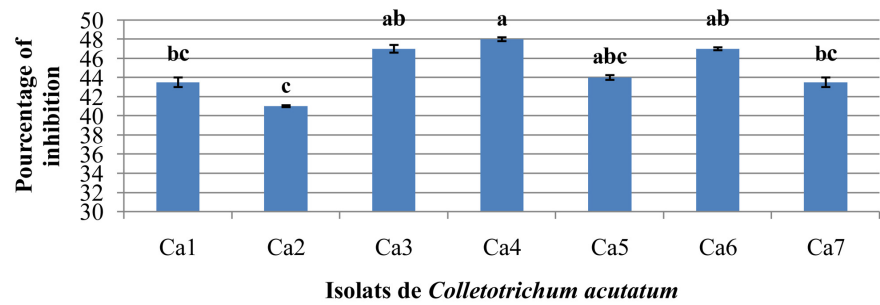
	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7
<b>Bc1</b>	37.50 ± 2.50 <sup>b</sup>	36.50 ± 0.50 <sup>b</sup>	35.00 ± 0.00 <sup>c</sup>	35.00 ± 2.00 <sup>b</sup>	40.00 ± 0.00 <sup>b</sup>	40.00 ± 0.00 <sup>b</sup>	41.00 ± 4.00 <sup>b</sup>
<b>Bc2</b>	71.00 ± 1.00 <sup>a</sup>	70.00 ± 1.00 <sup>a</sup>	71.00 ± 2.00 <sup>a</sup>	71.50 ± 1.50 <sup>a</sup>	72.00 ± 2.00 <sup>a</sup>	70.00 ± 1.00 <sup>a</sup>	70.50 ± 0.50 <sup>a</sup>
<b>Bc3</b>	37.00 ± 1.00 <sup>b</sup>	36.50 ± 0.50 <sup>b</sup>	36.00 ± 1.00 <sup>bc</sup>	37.50 ± 2.50 <sup>b</sup>	36.00 ± 2.00 <sup>b</sup>	36.00 ± 4.00 <sup>b</sup>	38.00 ± 2.00 <sup>b</sup>
<b>Bc4</b>	38.00 ± 1.00 <sup>b</sup>	37.50 ± 2.50 <sup>b</sup>	37.00 ± 0.00 <sup>bc</sup>	37.50 ± 0.50 <sup>b</sup>	36.00 ± 0.00 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>
<b>Bc5</b>	37.50 ± 2.50 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>	38.50 ± 1.50 <sup>bc</sup>	37.50 ± 1.50 <sup>b</sup>	36.50 ± 1.50 <sup>b</sup>	38.50 ± 2.50 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>
<b>Bc6</b>	33.50 ± 1.50 <sup>b</sup>	39.00 ± 1.00 <sup>b</sup>	39.50 ± 0.50 <sup>b</sup>	39.00 ± 1.00 <sup>b</sup>	38.00 ± 2.00 <sup>b</sup>	38.00 ± 3.00 <sup>b</sup>	39.00 ± 2.00 <sup>b</sup>
<b>Bc7</b>	37.00 ± 2.00 <sup>b</sup>	38.00 ± 1.00 <sup>b</sup>	39.00 ± 1.00 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>	36.00 ± 1.00 <sup>b</sup>	35.00 ± 0.00 <sup>b</sup>	36.50 ± 1.50 <sup>b</sup>
<b>Bc8</b>	37.50 ± 2.50 <sup>b</sup>	38.50 ± 1.50 <sup>b</sup>	38.50 ± 1.50 <sup>bc</sup>	36.00 ± 1.00 <sup>b</sup>	37.00 ± 2.00 <sup>b</sup>	36.00 ± 1.00 <sup>b</sup>	36.50 ± 0.50 <sup>b</sup>
<b>Bc9</b>	41.00 ± 5.00 <sup>b</sup>	38.00 ± 1.00 <sup>b</sup>	36.00 ± 1.00 <sup>bc</sup>	36.00 ± 1.00 <sup>b</sup>	35.50 ± 1.50 <sup>b</sup>	38.50 ± 3.50 <sup>b</sup>	39.50 ± 2.50 <sup>b</sup>

**Table 4.** Percentage of inhibition of mycelial growth of *Colletotrichum acutatum* isolates in the presence of nine *Bacillus amyloliquefaciens* filtrates on PDB.

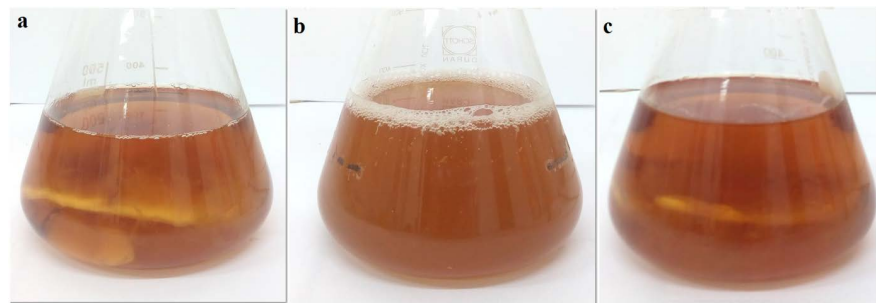
	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7
<b>Bc1</b>	99.16 ± 0.09 <sup>a</sup>	99.39 ± 0.02 <sup>a</sup>	99.35 ± 0.04 <sup>a</sup>	99.15 ± 0.08 <sup>a</sup>	99.20 ± 0.04 <sup>a</sup>	99.27 ± 0.06 <sup>a</sup>	99.19 ± 0.13 <sup>a</sup>
<b>Bc2</b>	99.44 ± 0.06 <sup>a</sup>	99.58 ± 0.02 <sup>a</sup>	99.66 ± 0.04 <sup>a</sup>	99.46 ± 0.03 <sup>a</sup>	99.64 ± 0.02 <sup>a</sup>	99.69 ± 0.02 <sup>a</sup>	99.61 ± 0.08 <sup>a</sup>
<b>Bc3</b>	99.22 ± 0.03 <sup>a</sup>	99.41 ± 0.00 <sup>a</sup>	99.35 ± 0.04 <sup>a</sup>	99.28 ± 0.05 <sup>a</sup>	99.18 ± 0.02 <sup>a</sup>	99.27 ± 0.06 <sup>a</sup>	99.09 ± 0.03 <sup>a</sup>
<b>Bc4</b>	99.16 ± 0.09 <sup>a</sup>	99.36 ± 0.05 <sup>a</sup>	99.29 ± 0.02 <sup>a</sup>	99.20 ± 0.03 <sup>a</sup>	99.14 ± 0.02 <sup>a</sup>	99.40 ± 0.06 <sup>a</sup>	99.17 ± 0.10 <sup>a</sup>
<b>Bc5</b>	99.29 ± 0.03 <sup>a</sup>	99.39 ± 0.02 <sup>a</sup>	99.27 ± 0.04 <sup>a</sup>	99.25 ± 0.03 <sup>a</sup>	99.22 ± 0.06 <sup>a</sup>	99.31 ± 0.02 <sup>a</sup>	99.04 ± 0.03 <sup>a</sup>
<b>Bc6</b>	99.16 ± 0.09 <sup>a</sup>	99.34 ± 0.07 <sup>a</sup>	99.33 ± 0.02 <sup>a</sup>	99.15 ± 0.08 <sup>a</sup>	99.11 ± 0.04 <sup>a</sup>	99.35 ± 0.02 <sup>a</sup>	99.14 ± 0.08 <sup>a</sup>
<b>Bc7</b>	99.10 ± 0.16 <sup>a</sup>	99.31 ± 0.10 <sup>a</sup>	99.27 ± 0.04 <sup>a</sup>	99.30 ± 0.08 <sup>a</sup>	99.24 ± 0.08 <sup>a</sup>	99.44 ± 0.10 <sup>a</sup>	99.22 ± 0.16 <sup>a</sup>
<b>Bc8</b>	99.13 ± 0.12 <sup>a</sup>	99.44 ± 0.02 <sup>a</sup>	99.38 ± 0.06 <sup>a</sup>	99.18 ± 0.05 <sup>a</sup>	99.18 ± 0.02 <sup>a</sup>	99.25 ± 0.08 <sup>a</sup>	99.01 ± 0.05 <sup>a</sup>
<b>Bc9</b>	99.19 ± 0.06 <sup>a</sup>	99.36 ± 0.05 <sup>a</sup>	99.38 ± 0.06 <sup>a</sup>	99.36 ± 0.13 <sup>a</sup>	99.26 ± 0.11 <sup>a</sup>	99.48 ± 0.15 <sup>a</sup>	99.11 ± 0.05 <sup>a</sup>

**Figure 4.** Growth of Ca6 isolate in the presence of the volatile substances of the bacterial isolates: (a) Bc1, (b) Bc2, (c) Bc3, (d) Bc4, (e) Bc5, (f) Bc6, (g) Bc7, (h) Bc8 and (i) Bc9, (j) in the absence of antagonist and in the presence of the volatile compounds of (k) *Trichoderma harzianum*.





**Figure 5.** Percentage of inhibition of mycelial growth of *Colletotrichum acutatum* isolates by the volatile substances produced by *Trichoderma harzianum*.



**Figure 6.** Growth of the Ca6 isolate in the PDB medium: (a) in the absence of antagonist in the presence of the filtrate of (b) I2, (c) *Trichoderma harzianum*.

causing anthracnose in cultivated strawberry (*Fragaria x ananassa* Duch.). Biological control is therefore an alternative to the use of chemical plant protection products.

The sensitivity of the isolates of *Colletotrichum acutatum* appears variable for both bacterial and fungal antagonists. All bacterial isolates and *Trichoderma harzianum* reduce mycelial growth of *C. acutatum* isolates. The growth of *Trichoderma harzianum* seems more marked than that of *Colletotrichum acutatum*.

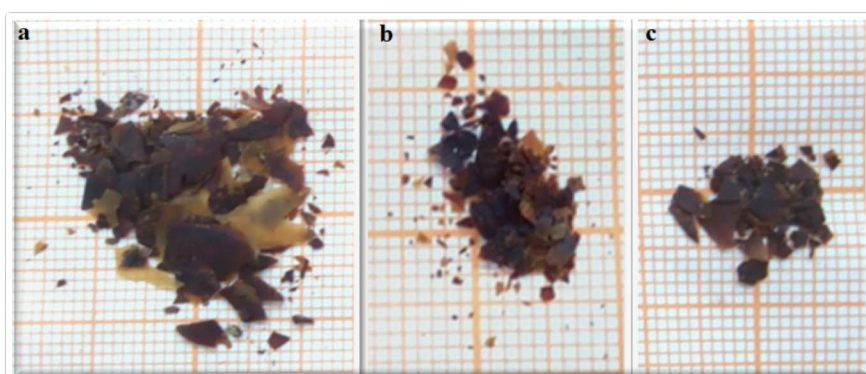
The Bc2 isolate has superior antagonistic activity: the Bc2 isolate inhibits the growth of Strain *Colletotrichum acutatum* from 79.8% to 92.33% in direct confrontation and its volatile substances by 70% to 72% the mycelial growth. The nonvolatile substances of all isolates indiscriminately inhibit the development of *Colletotrichum acutatum* isolates.

Several studies have been made to evaluate the *in vitro* antagonism of *Bacillus amyloliquefaciens* with respect to the development of different phytopathogenic agents: [18] found that all *Bacillus* spp. in their study as antagonists resulted in a significant reduction in mycelial growth of anthracnose isolates by direct confrontation. Similar results have been reported by [19]. [20] reported that *Bacillus* were promising bacterial antagonists for the control of plant diseases, because of their simple nutritional requirements, which allow them to colonize on dry surfaces for long periods of time, the rapid use of many available nutrients and endospores resistant to withstand many environmental risks. *Bacillus* species, in-

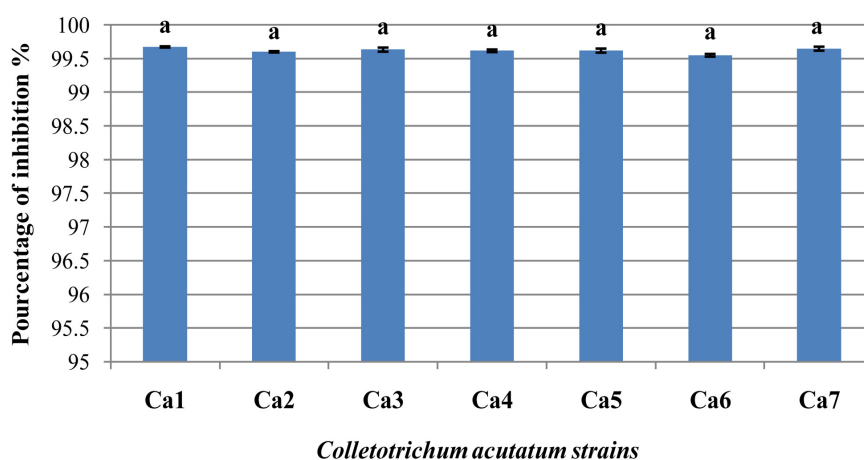
cluding *B. amyloliquefaciens* [21] [22] [23], significantly reduce the severity of plant pathologies on various hosts under greenhouse or field conditions [24]. *B. subtilis* and *B. amyloliquefaciens* have been used in commercial biological control products because of their excellent antagonistic effects and high stability under harsh environmental conditions [25].

The results of this study revealed that *Trichoderma harzianum* has a high inhibitory effect against different isolates of *C. acutatum* at three life stages. Its growth during the confrontation is faster than that of the pathogen (Figure 2(b)), hence a significant advantage in the competition for space and nutrients, even before deploying its arsenal of mycotoxins [26]. Pathogen development is inhibited by volatile (Figure 4) and non-volatile compounds produced by the antagonists (Figures 6-8). The decrease in mycelial growth of *Colletotrichum* isolates with *T. harzianum* is similar to that of *Phytophthora cinnamomi* with 24 manure fungi including *T. harzianum* [27].

The use of products based on *Trichoderma harzianum* and *Bacillus amyloliquefaciens* in the fight against phytopathogenic fungi is safe for farmers and consumers and good for the environment. However, much remains to be done



**Figure 7.** Dry matter of the mycelium of the Ca6 isolate: (a) in the absence of antagonist and in the presence of the filtrate of: (b) I2 and (c) *Trichoderma harzianum*.



**Figure 8.** Percentage of inhibition of mycelial growth of seven isolates of *Colletotrichum acutatum* by *Trichoderma harzianum* on PDB.

to develop a stable, cost-effective formulation that is easy to produce and apply.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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