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Biocontrol Potential of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR against Strawberry Anthracnose under Laboratory and Field Conditions

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Abstract

The increasingly strict regulation of the use of phytosanitary products and the will of the populations to move towards sustainable development allow bio-sourced products and more particularly for biocontrol in the field of pesticides to progress. The study carried out here concerns the evaluation of the potency of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* to fight against strawberry cultures infected with anthracnose (*Fragaria* × *ananassa* Duch.). The studies were carried out in the laboratory and in the field. The results indicate that *B. amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR are effective for the biological control of anthracnose, gray mold and powdery mildew on strawberries grown in field conditions. The increase in plant size and the number of fruits produced with these control agents has also been observed.

Keywords

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

1. Introduction

Agriculture is heavily dependent on the use of chemical fertilizers and pesticides to get better returns. This dependence is associated with problems such as environmental pollution and health risks. The use of biological control agents, as an alternative, has been the subject of much research over the past 30 years. The concept of biological control has been gradually integrated into the development of alternative methods of postharvest disease management. Since then, biocontrol industry deals with biologics development to include them in a professional company [1] [2].

Screening of novel microorganisms for commercial purposes under biocontrol against plant pathogens is a complex process. Besides the effectiveness of antagonists, there are different categories of criteria, from ecological characteristics necessary for good performance on the field toxicological profiles, development of bioreactors for mass production, a successful formulation and rights protection intellectual property and marketing [1] [3] [4].

Like all living organisms, plants have to cope with infections and diseases following the attacks of a mass of plant pathogens. These diseases may be causing only minor reduction in plant growth or capacity can cause much more serious damage leading to plant death in the worst case [5]. To prevent or control these diseases, growers have become increasingly dependent on agrochemicals, especially in recent decades, as agricultural production intensified. However, despite the high efficiency and ease of use of these products, their use or misuse has caused many problems.

Many microorganisms producing molecules surround the root zone, they contain some volatile compounds that can affect the growth via different mechanisms, such as biochemical signals causing local defense reactions or systemic resistance [6] [7]. The use of these organisms for biological control against plant pathogens has received attention because of the prevalence of pathogens resistant to pesticides and their prohibitions. Benefits include a lower environmental impact, higher specificity, lower costs and identifying new mechanisms for the elimination of new diseases. The genus Bacillus encompasses a wide genetic biodiversity forming endospores shaped Gram positive rod [8]. It is known to produce several antibiotics and is often found in soil and associated plants [9]. Trichoderma (teleomorph Hypocrea) is a fungal genus present in many ecosystems. Some strains have the ability to reduce the severity of plant diseases by inhibiting plant pathogens, mainly in the soil or on plant roots, thanks to their strong potential antagonist and mycoparasitic [10] [11].

Among the various biological approaches, the use of antagonistic microorganisms has become popular. Such natural interference between beneficial microorganisms and plant pathogens contributes to the storing of natural buffers culture systems, thereby preventing or limiting the development of plant pathology [1]. The mechanism by which microbial antagonists suppress postharvest diseases includes primarily the competition for nutrients and space, the pro-

duction of antibiotics, direct parasitism and possibly induced resistance [12] [13].

Many microorganisms with good potential are not developed for practical use because of uneven performance associated with adverse environmental conditions. In addition, the concentration of the antagonist used, the timing and method of application are also crucial in determining the success of an antagonist in the competition with other microorganisms [14]. Therefore, the implementation and optimization of biocontrol agents before harvest require considerable understanding of the culture.

The aim of this study is the evaluation of power of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* to fight against anthracnose cultivated strawberry (*Fragaria* × *ananassa* Duch.) under laboratory and field conditions.

2. Material and Methods

2.1. Control of Symptoms Caused by *C. acutatum* on the Leaflets of Strawberry Plants *In Vitro* and *In Vivo* by *B. amyloliquefaciens* and *T. harzianum*

2.1.1. Fungal Material

a) Pathogenic

The conidial suspension of the plant pathogen *Colletotrichum acutatum* (Ca6) was obtained by adding 10 ml of sterile distilled water in Petri dishes containing cultures of *C. acutatum* on older PDA (Dextrose Agar) 10 to 15 days, by gently rubbing the colonies using a sterile Pasteur pipette. To release the spores, the propagules suspension was filtered through a sterile nylon gauze having pores of 102 µm in diameter. The concentration was determined using a Malassez cell and adjusted to 10^4 spores/ml.

b) Antagonist

Each Erlenmeyer flask containing 15 ml of PDB (Potato Dextrose Broth) was inoculated with a disc 5 mm in diameter from the culture of *Trichoderma harzianum* and incubated for 12 days at 25˚C ± 2˚C with stirring at 200 rpm. After this period, the suspension was filtered to remove the mycelium. The suspension was adjusted to 10^5 spores/ml using a Malassez cell.

2.1.2. Bacterial Material

Antagonist isolate was grown on the PCA (Plate Count Agar) culture medium for 24 h at 37˚C. After, and using a sterile loop, a bacterial culture was prepared by inoculating 50 ml of the LBB (Luria Bertani Broth), by a colony of the isolate studied, in an Erlenmeyer flask of 100 ml. Cultures were incubated at room temperature with stirring at 125 rpm for two days. After incubation, a volume of 5 ml of bacterial suspension was centrifuged at 3000 rpm for 15 min. Cells were washed twice in the same volume of sterile physiological water, using the centrifuge under the same conditions cited above instructions. The bacterial cells were then suspended in 5 ml of sterile physiological saline and the suspension was adjusted to 3×10^5 CFU/ml depending on the scale of Mac Farland.

2.1.3. Plant Material

10 strawberry seedlings Sabrina variety have been reported in Ouled hamou region in Larache (Region Loukous), Morocco, aged five months at the Plant Biotechnology Laboratory of Faculty of Sciences Tetouan in pots containing black soil sand from the original field. The plants were watered daily with tap water.

a) Inoculation of the leaflets *in vitro*

Healthy, young leaflets were harvested, washed extensively with distilled water, and then disinfected with a sodium hypochlorite 2% solution for five minutes followed by three washes of 15 minutes with sterile distilled water. The sheets were dried under a vertical laminar flow hood airflow. They were placed in Petri dishes of 150 mm diameter sterile glass containing a double layer of sterile filter paper previously soaked in sterile distilled water in each leaflet, a central lesion was performed through an incision using a sterile scalpel [15].

Simultaneously depositing on each lesion a volume of 15 µl of a suspension of *Colletotrichum acutatum* mixed with 15 µl of sterile distilled water (control) or cell suspension of the studied antagonists.

Pathogenicity was rated on the following scale:

- 0 = no signs of visible disease,
- 1 = less than 15% of the surface of the leaflet is infected,
- 2 = 15% - 35% of the surface of the leaflet is infected,
- 3 = 36% - 49% of the surface is infected,
- 4 = 50% - 74% of the surface of the record is infected,
- 5 = more than 75% of the surface of the record is infected.

The percentage of occurrence of the symptoms of the disease (*PSM*) was calculated using the formula below:

$$PSM\,\% = \frac{S}{NSM \times Nmax}$$

With:

S: Sum of all notes

NSM: Rating corresponding to the infection percentage of the leaflet in presence of antagonist or distilled water (control)

Nmax: Maximum Rating

b) Inoculation *in vivo*

Inoculation of plants was made by spraying a volume of 50 µl of a suspension of *Colletotrichum acutatum* mixed with 50 µl of sterile distilled water (control) or cell suspension of antagonist (1 pot/isolate). The plants were then placed for 48 hours in the dark covered with black plastic bags, sprayed with sterile water to maintain high relative humidity required for germination and direct penetration of conidia (without injury). The pots were subsequently transferred to growth chamber and incubated for 15 days at 25˚C ± 2˚C in photoperiod.

The percentage of the degree of infection in the petioles (*PIP*) and the leaflets (*PFF*) was calculated as follows:

$$PIP\% = \frac{\text{Nomber of infected petioles}}{\text{Total number of petioles}}$$

$$PFF\% = \frac{\text{Nomber of infected petioles}}{\text{Total number of leaflets}}$$

2.2. Biological Control under Field Conditions

2.2.1. Preparation Antagonists

Bacillus amyloliquefaciens Bc2 and a strain of *Trichoderma harzianum* TR were cultured in Petri dishes respectively containing PCA and PDA culture media, autoclaved at 121˚C for 15 minutes. The incubation of the Petri dishes was done in 24 h at 37˚C in the dark for Bc2 and ten days at 25˚C ± 2˚C in the dark for TR.

For each antagonist, the peat was used as substrate. The latter was placed in autoclavable bags (3 kg per bag) and sterilized three times by autoclaving for one hour at 121˚C. The contents of the bags were transferred to plastic food containers with lids (18.2 cm high, 35 in length and 21.5 in width) previously cleaned (as well as their lid) by the bleach, disinfected by ethanol 92% and placed under UV lamp (Philips Ultra-UV, 30W/G30T8, 254 nm) in a horizontal laminar flow hood for 20 min. These plates were inoculated with PCA and PDA antagonist cultures and then closed and incubated five days in the dark at 27˚C for both antagonists. After incubation, an amount of from 1 g of each mixture (substrate + antagonist) was introduced into two test tubes containing a 5 ml of distilled water and another 5 ml of physiological water for TR and Bc2 respectively. The suspensions were then stirred using a rotary shaker at 125 rpm for 10 min. And then a series of decimal dilution has been carried to the 10 - 4 dilution.

1 ml of the suspension and the first three dilutions were plated in Petri dishes containing PCA, the plates were incubated subsequently at 37˚C for 24 hours. The petri dishes containing more than 300 colonies were counted and the number (N) present in the sample was calculated (until 3×10^5 CFU/ml) according to the formula:

$$N = \frac{\sum C}{V} \times \left[n1 + \left(0.1 \times n2 \right) \right] \times d$$

With:

C = total colonies counted on selected dishes of two successive dilutions.

d = dilution ratio corresponding to the first dilution counted.

$n1$ = number of boxes retained in the first dilution.

$n2$ = number of boxes selected at the second dilution.

V = volume of the inoculum applied to each box in ml.

The results were reported in number of CFU per gram of peat.

TR spores of the suspension were obtained by adding a volume of 10 ml of sterile distilled water in petri dishes containing cultures of *T. harzianum* on PDA, aged 10 days, by gently rubbing the colonies to using a sterile Pasteur pipette. The propagule suspension was filtered through a sterile nylon gauze hav-

ing pores of 102 µm in diameter. The concentration was calculated using the Malassez cell until 10^5 spores/g of peat.

2.2.2. Performance of the Test on Field

a) Strawberry Field: location and planting conditions

The field experiment was conducted for one year in a field of strawberry cultivar Fortuna from his plantation in November 2018 in the region of Loukkos near Larache, Morocco (20 m, 35 N 06 813 and W 006 08. 152). Planting conditions were shown in Table 1.

b) Processing mode and application time

The treatments were carried out only one time during the planting strawberry explants. Six grams of substrate (peat/antagonist) were mixed with the soil at the roots of the explants at a rate 500 plantlets per antagonist (a ridge/antagonist) (Figure 1).

The control in our study was a billon treated by pesticides used on strawberry in Loukkos region. The treatment was performed once per 15 days in alternating products listed in Table 2.

c) Meteorological data

In experiments conducted in 2018/2019 we recorded the temperature every day in December 2018 and we recorded the minimum and maximum values for each month (Figure 2).

d) Observation and disease recording

The recordings on the initial attacks of fungal diseases including anthracnose on strawberry seedlings were conducted every month after planting the 2018/2019 year. On this occasion, all the logs were evaluated. All plants were carefully observed to search for signs of disease.

We counted diseased plants for each studied log and assessed the severity of each disease individually for all plants using the following scale:

Table 1. Terms of planting seedlings of strawberry in the field studied.

Area	2.5 ha total field, with 1.5 ha planted by strawberry
Soil Treatment before planting	No
The Plastic Mulching	Black opaque plastic film from 25 to 35 µm thick and 1m to 1.20 m
Planting (Figure 1)	- Twin ridges on a bank 60 cm wide - The plants are arranged in staggered rows 20 cm away - Each ridge contains 500 plants - Planting date: November 21, 2018
Irrigation	Localized irrigation Drip, alternate days from 13/12/2018
Coverage of small tunnels Nantes	The tunnel cover film is transparent, a thickness of 100 to 120 microns and a width of 1.5 m
Fertilizing	Twice, 26/01/2019 and 29/03/2019 50 kg/ha nitrogen; 100 kg/ha phosphate; 100 kg/ha of potassium

Table 2. Phytosanitary products used in billon witness during the experiment.

Products	Category	Active ingredient	Content	Use against	Dose
ALMECTIN™	Insecticide-miticide	abamectin	18/l	mites	50 cc/hl
				noctuelles	60 cc/hl
Benevia	Insecticide	cyantraniliprole	100 g/l	aphids	60 cc/hl
				thrips	75 cc/hl
KAISER 10 SC	Fungicide	cyflufenamid	100 g/l	powdery mildew	10 cc/hl
Teldor 50 WG	Fungicide	fenhexamid	500 g/kg	gray mold	150 g/hl
THIRAMCHIM 80	Fungicide	thiram	80%	anthracnose	250 g/hl

Figure 1. Strawberry field studied by specifying the 3 ridges covered: (A) *T. harzianum*; (B) Bc2; (C) chemical pesticides.

- 0% - 24%: no symptoms,
- 1% - 49%: symptoms one to two leaves/petioles or fruit per plant,
- 50% - 74%: more than two leaves/petioles or fruits attacked by plant,
- 75% - 100%: symptoms observed throughout the plant.

3. Results

3.1. Control of Symptoms Caused by *C. acutatum* on the Leaflets of Strawberry Plants *In Vitro* and *In Vivo* by Selected Antagonistic

Bc2 has been selected for its effectiveness to inhibit growth of *C. acutatum* at low concentrations against other isolates, in this assay we used suspensions and *Trichoderma harzianum* isolate Bacillus Bc2 to 10^5 spores/ml and 3×10^5 CFU/ml, respectively.

The results showed that these antagonists perfectly control the appearance of the disease suppression of symptoms caused by Ca6 reaches 100%, corresponding to 0% of incident disease (Figure 3).

3.2. Biological Control under Field Conditions

After confirmation of the concentration of antagonist in the peat at a rate 3×10^5

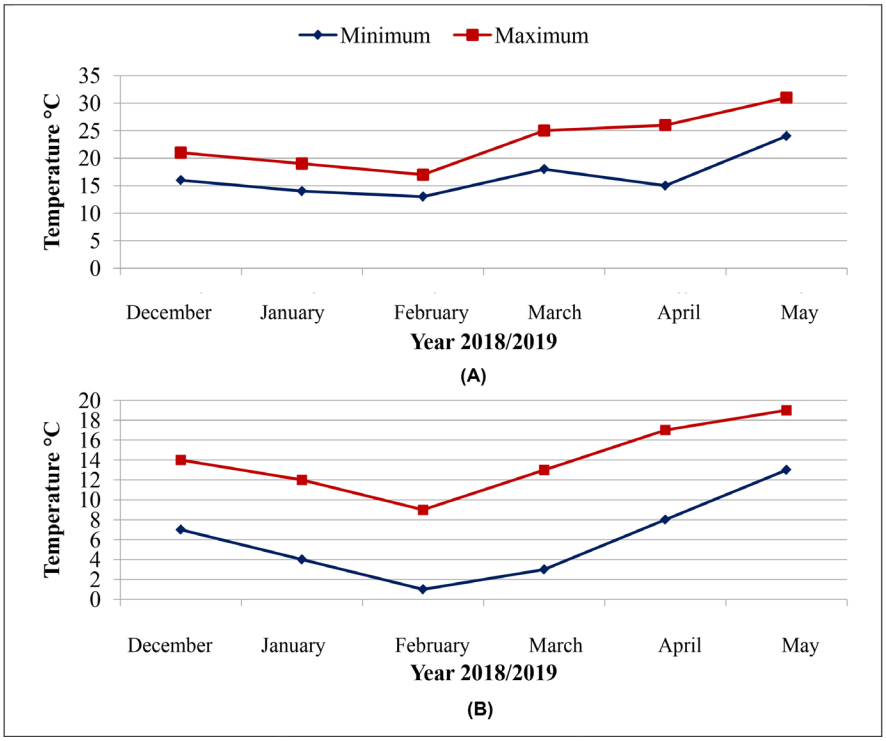

Figure 2. The maximum and minimum temperatures recorded during (A) the day and (B) night during the experimental period (2018/2019).

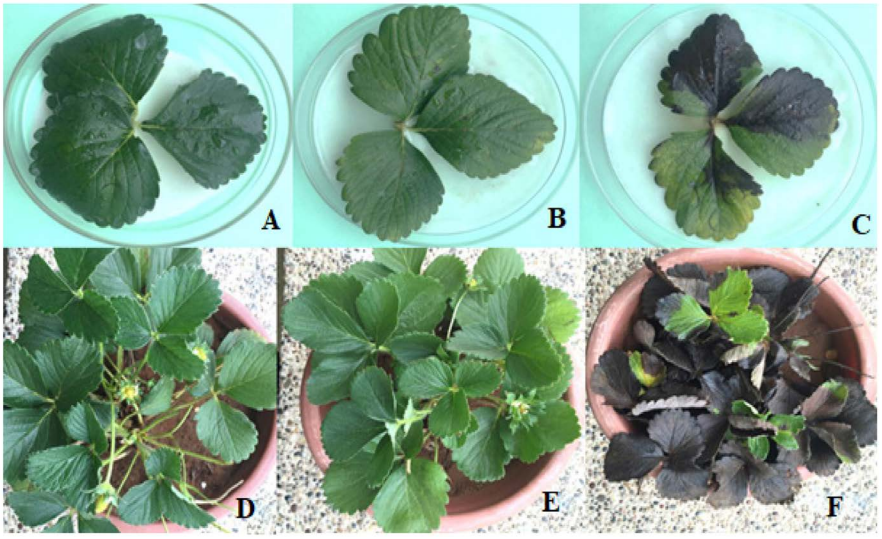

Figure 3. Inhibition test *in vitro* on strawberry leaves: (A) inoculation of Ca6 and antagonistic strain Bc2. (B) Inoculation of *C. acutatum* Ca6 and antagonist *T. harzianum*. (C) Inoculation of Ca6 and distilled water (control). *In vivo* on Strawberries: (D) Sprayed by Ca6 and antagonistic strain Bc2. (E) Sprayed by *C. acutatum* Ca6 and antagonist *T. harzianum*. (F) Sprayed by Ca6 and distilled water (control).

CFU/g and 10^5 spores/g of substrate for Bc2 and TR respectively. We have started the treatment on grounds of seedlings strawberry.

The results obtained allowed us to note that during the year of strawberry cul-

tivation, the number of diseased plants in the ridge treated control chemical pesticides began to increase at the end of December 2018 (Figure 4 and Figure 5). And we have seen the emergence of gray mold and powdery mildew (Figure 6) during the months of January, February and March, and anthracnose (Figure 6) during the month of February; March and April and after the plants of this ridge have started to dry and die in about late May (Figure 4 and Figure 5).

3.3. Disease Severity

From the results obtained above we concluded that *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* were perfectly inhibited the emergence of fungal diseases namely anthracnose, powdery mildew and Gray mold. Here we evaluated the severity of these diseases on plants in the control ridge and we calculated the average percentages of the disease severity of all diseased plants of each fungal plant pathology in the ridge.

During the months of November and December 2018, we have observed no disease in the month of January 2019 gray mold and powdery mildew appeared with equal incidence of disease at 75% and 63% respectively (Figure 7), against anthracnose has not developed on seedlings of strawberries. The degree of seedling infection anthracnose begins to rise in the month in February by 25.67% and it is maximum in the month of March, April and More by 57.33%; 34.95% and 26.67% respectively (Figure 7), with rising temperatures in these months (Figure 2). So we can see that climate change may influence the onset and degree of infection of plant pathology under field conditions.

4. Discussion

Fungal diseases that mate more often on the cultivation of strawberries in the control ridge are powdery mildew, gray mold and anthracnose; these plant diseases are considered principals diseases attacking cultivated strawberry [15]-[25].

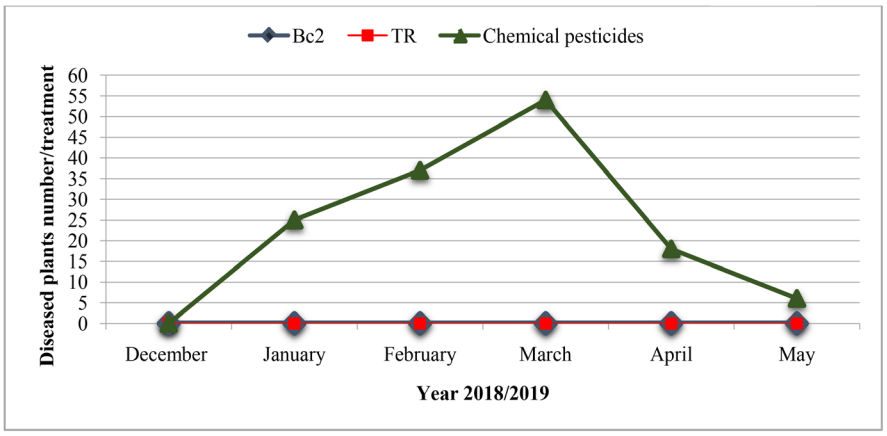

Figure 4. Number of diseased plants per treatment, processed Bc2, TR and chemical pesticides.

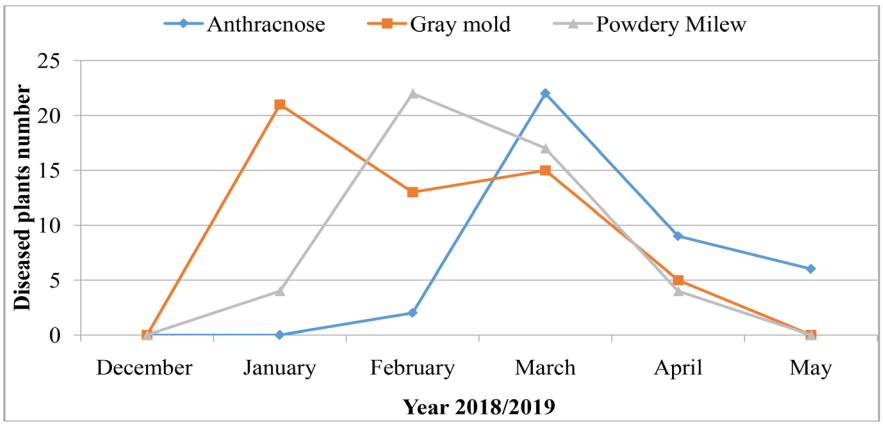

Figure 5. Number of diseased plants with gray mold, powdery mildew and anthracnose symptoms.

Figure 6. Apparition s of: (A) Oïdium; (B) Anthracnose; (C) Gray mold and (D) whiteflies in plants treated by chemical pesticides.

The tests conducted to determine the potential of inhibiting trough concentrations Bc2 and TR previously recognized to inhibit the proliferation of *Colletotrichum acutatum* in the leaflets *in vitro* and in seedlings strawberry (*Fragaria* × *ananassa* Duch.) enabled us to deduce that these antagonists have potential important biological control, and allowed us to think about testing the ground to confirm their effectiveness.

Anthracnose mainly attacks the flowers and fruits, causing yield losses may also exceed 50%, even in well-managed crops [26] [27]. *Colletotrichum* spp.

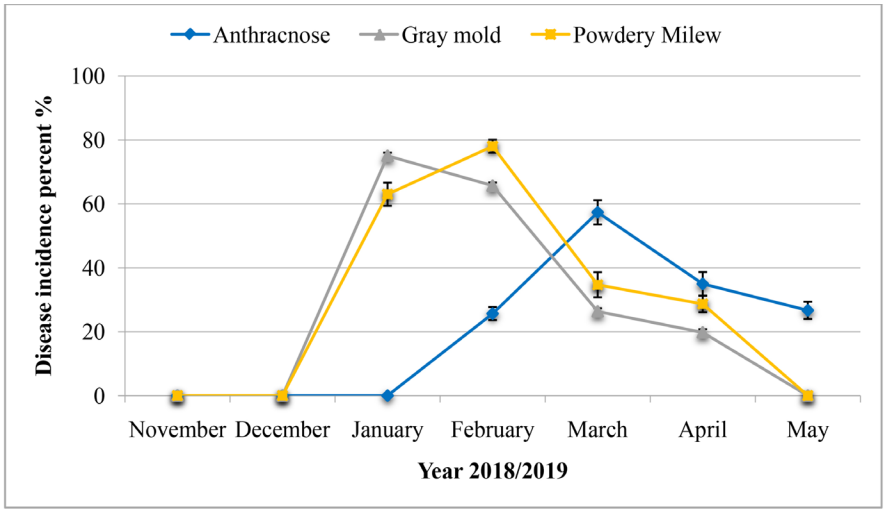

Figure 7. Percentage severity of disease caused by each plant pathology on strawberries control ridge.

require relatively warm temperatures and are mainly [28] [29], and we also found that the favorable temperature for optimal development of isolates of *Colletotrichum acutatum* is 25˚C ± 2˚C [30], and in this study the onset of symptoms of the disease begins in the month in February but only 2/500 plants were sick in this month. Gray mold can cause losses that can be severe, reaching 25% in the main crop and 37% in the second. However, the main losses occur during the post-harvest stage, with a maximum incidence of 89% during the second crop [31]. During periods when weather conditions are conducive to the development of gray mold with temperatures between 15˚C and 25˚C [32] [33], we observed the appearance of the disease in the month of January with temperatures ranging from 14˚C - 19˚C during the day and 4 to 15 overnight with relative humidity of 9% - 16%, and the disease has continued to grow during the month of May. Powdery mildew development on strawberries is promoted by high humidity and an optimum temperature of between 20˚C and 22˚C [34] [35]. The disease appeared on the plant strawberries in January and disappeared in the month of May as botrytis.

The appearance of diseases in ridge treated by chemical pesticids and mainly by fungicides based on cyflufenamide, fenhexamide and thiram used respectively to fight against powdery mildew, gray mold rot and anthracnose of strawberries cultivated in Morocco, especially in the Lokkous region, is proof of the resistance of these phytopathogenic fungi to the treatments carried out. And we found that isolates of *Colletotrichum acutatum* grow even applying large doses of fungicides Thiram and copper [36]. The Thiram is ineffective to control anthracnose strawberry [37] [38] [39]. The cyflufenamids remain effective against powdery mildew of cucurbits [40] [41] [42]. The fenhexamides were found ineffective for treating gray rot [43] [44] [45].

The evaluation of the biological power of *Bacillus amyloliquefaciens* and *Trichoderma harzianum* has enabled us to deduce that these antagonists can inhibit

the development of fungal plant diseases attacking cultivated strawberry, and we observed that the plants remain healthy and a limited number was dried and death against the witness billon, most plants were dead in May. This confirms our findings on the effect of environmental factors on the biological activity of *B. amyloliquefaciens* and *Trichoderma harzianum* against *Colletotrichum acutatum* of anthracnose agent cultivated strawberries, we found that the bacterial isolate Bc2 can inhibit the growth of *C. acutatum* by increasing the temperature, the same effect was observed for TR.

Trichoderma species are effective antagonists, able to fight against pathogens soil borne, parasitic plants of economic importance, and are present in abundance in almost all soil types [46] [47] [48]. Biocontrol antagonists have played an important role in plant disease management and parasitic microorganisms [49] [50]. Trichoderma attacked other plant pathogenic fungi and promote plant growth and roots. It uses various control mechanisms of plant pathogenic pathogens including antibiosis, mycoparasitism, induced resistance of the host cell and the competition for nutrients and space. Trichoderma species can monitor and thwart a wide range of plant pathogenic fungal pathogens and economically important plant pathogenic fungi after harvest, as well as bacteria and viruses [51] [52]. [53] found that *Trichoderma harzianum* T-39 is effective against Botrytis and anthracnose of cultivated strawberries. The same isolate T39 was used to fight against mildew and found that it is effective against this disease [54]. *T. harzianum* has inhibited the development of plant and even animals' pathogenic fungi such as *Botrytis cinerea*, *Mucor circinelloides*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Rhizoctonia solani* and *Candida albicans* [55]. This antagonistic fungus produces antifungal volatile compounds [56], their effectiveness was found against pathogens such as *Rhizoctonia solani*, Sclerotinia *sclerotiorum* and *F. oxysporum* [57] [58]. The 6-PP is a major biosynthesized composed by *T. harzianum* or *T. atroviride* [59] [60] [61]. It is able to promote plants growth and reduce the symptoms of their diseases.

Members of the genus Bacillus, such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* are among the most effective bacterial biocontrol agents [62]. The strong biocontrol activity of these bacteria is mainly due to their potential for producing multiple antimicrobial compounds whose inhibitory activity with respect to many plant pathogens [63] [64] [65] [66] [67]. A related study by [64] showed that the root colonization by *B. amyloliquefaciens* FZB42 induce an SRI and limit the reopening of stomata induced foliar pathogen of the oocyte *P. nicotianae* in *Nicotiana benthamiana*, thus blocking the penetration of the pathogen. FZB42 inhibited the growth of plant pathogenic fungi such as *Fusarium* spp. including *F. oxysporum*, *Gaeumannomyces graminis*, *Rhizoctonia solani, Alternaria alternatae*, *Botrytis cinerea* and *Pythium aphanidermatum* [68].

The semi-purified compound *B. amyloliquefaciens* CNU114001 significantly inhibited mycelial growth of pathogenic fungi (*Alternaria panax*, *Botrytis cinerea*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea and*

Sclerotinia sclerotiorum) at a concentration of 200 ppm [69]. Bacillus species are common soil bacteria, fermented foods and endophytic. Some Bacillus species have the ability to inhibit plant pathogens. *Bacillus amyloliquefaciens* has been studied to reduce green mold and blue mold of postharvest citrus [70], anthracnose of strawberry [71], and the melting of soybean plantings [7].

B. amyloliquefaciens Bc2 and *T. harzianum* TR completely inhibited the appearance of anthracnose on strawberry plants under laboratory and field conditions. The results of this study indicate that Bc2 and TR are effective for biocontrol against anthracnose, gray mold and powdery mildew on strawberries grown under field condition at concentrations of 3×10^5 CFU/g of substrate and 10^5 spores/g of substrate respectively.

In addition, the observation of the increasing of plant size and number of fruits produced in the ridges treated with these control agents, has allowed us to conduct a study on their effectiveness as plant growth promoters microorganisms.

Conflicts of Interest

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

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