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Assessment of the Effect of Environmental Factors on the Antagonism of *Bacillus amyloliquefaciens* and *Trichoderma harzianum* to *Colletotrichum acutatum*

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Abstract

The effect of temperature (18˚C - 30˚C), water activity (0.85 - 1) and pH (4 - 9) was studied by dual culture technique on the antagonism of *Bacillus amyloliquefaciens* and *Trichoderma harzianum* to *Colletotrichum acutatum*, responsible of strawberry (*Fragaria x ananassa* (Weston) Duchesne ex Rozier) anthracnose. The antagonistic bacteria's strains behave significantly and differently according to the parameters studied. These results reveal useful information about the applicability of their biocontrol in agricultural culture with the change of environmental factors.

Keywords

Antagonism, Anthracnose, Biocontrol, Environmental Factors

1. Introduction

Strawberry (*Fragaria x ananassa* (Weston) Duchesne ex Rozier) is an important fruit crop, grown in Morocco in the areas of Souss, Gharb and Loukkos. A major constraint to the culture of strawberry is the low tolerance of this species to fungal diseases [1]. The number of phytopathogenic fungi attacking this culture is vast, more than 50 genera [2], resulting in severe economic losses. Fungal diseases can affect all parts of strawberry, but there are those who produce the crown rot, resulting in death of the plant, as anthracnose caused by *Colletotri-*

chum spp. [3] [4] [5], especially *C. acutatum*, considered among the more devastating phytopathogens. The control of fungal diseases attacking strawberry plants is mainly done by treatment of the soil and the plants using chemical pesticides. The intensive use of fungicides leads to the accumulation of toxic compounds potentially dangerous for humans and the environment, as well as in the induction of the resistance of phytopathogenic agents [6] [7] [8]. Biological control has received great attention as one of the non-hazardous pest management techniques against diseases caused by phytopathogenic fungi, including anthracnose [9]. The selection of the antagonists planned for the biological control of plant diseases usually involves examining a large number of microbial isolates to increase the probability of discovering a strain highly effective. The natural antagonists on the surfaces of the host are promising components of protection of organic crops [10]. Antagonistic bacteria such as *Bacillus subtilis* [2] [11], *B. amyloliquefaciens* [9] and *Pseudomonas fluorescens* [12] or fungi such as *Trichoderma harzianum* [13] [14] or yeast such *as Saccharomyces cerevisiae* [12] [15] have been found effective for the control of the anthracnose disease under controlled research conditions.

Some environmental factors can strongly influence the biological effectiveness of the antagonist against plant pathogens. Climate change and water conditions are among the crucial factors influencing microbial activity in natural systems [16]. Therefore it is reasonable to study the influence of temperature, pH and water activity on the *in vitro* antagonism of *Bacillus amyloliquefaciens* and *Trichoderma harzianum*. However, there is a lack of comparative information on the effects of these factors on potential biocontrol of *B. amyloliquefaciens* and *T. harzianum* against plant diseases.

The objective of this study is to evaluate the effect of temperature, pH and water activity on the antagonism of *Bacillus amyloliquefaciens* and *Trichoderma harzianum* to *Colletotrichum acutatum*.

2. Materials and Methods

2.1. Fungal Pathogen Strain

Strain Ca6 of *Colletotrichum acutatum* was isolated from naturally infected strawberry fruits presenting anthracnose symptoms. It was selected for its aggressiveness among several isolates found in different strawberry cultivars. *C. acutatum* Ca6 originated from fields of strawberry plants of Loukkos (Larache, Morocco), developed well in Potato Dextrose Agar and was incubated ten days in 25˚C ± 2˚C before use. The identification was carried out by macroscopic and microscopic observations of the isolates using determination keys [17] [18].

2.2. Isolation of Antagonistic Bacterial Strains

Nine bacterial strains were isolated by the method of serial dilutions from rhizosphere soil and roots of strawberry plants taken from various agricultural zones of the Loukkos region (Larache, Morocco), and identified by Hamdache *et al.*

Table 1. Identification of antagonistic strains of *Bacillus amyloliquefaciens* [19].

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

[19]. A molecular identification revealed that the nine antagonistic bacterial isolates belong to the species *Bacillus amyloliquefaciens*. Strains at the beginning were noted by an arbitrary notation I1, I2, I3, I18, B3, B12, RA9 and RA12 (**Table 1**).

2.3. Fungal Antagonist Strain

Trichoderma harzianum (TR) strain was isolated from soil into PDA (Potato Dextrose Agar) plates using spread plate technique. Litter materials were cultured in PDA plates for the isolation. The TR strain was isolated into pure culture on PDA. The identification was carried out by macroscopic and microscopic observations [20].

2.4. Effect of Environmental Factors

The potential of biological control of the nine strains of *Bacillus amyloliquefaciens* and the strain of *Trichoderma harzianum* was assessed. The inhibition of *Colletotrichum acutatum* Ca6 according to the variation of some factors (temperature, pH, activity of the water) was evaluated by calculating the percentage of inhibition of mycelial growth on Petri dishes by dual culture technique on PDA. The antagonist and the phytopathogen were put on the opposite sides of Petri dish at the same distance from the periphery. A completely randomized experimental device was used with three replicates for each antagonist.

2.4.1. Effect of Temperature

The inoculated dishes were incubated in the dark at 18˚C; 23˚C; 25˚C; 27˚C and 30˚C for 7 days.

2.4.2. Effect of Water Activity

The water activity (aw) represents the availability in open water for the biochemical reactions for the development of microorganisms. Different values of activity of the water have been tested (1; 0.95; 0.90 and 0.85) by the addition of glycerol in PDA [19] [21], which will attach a part of the water and make it unusable to microorganisms. The same technique of dual culture has been fol-

Table 2. pH values and buffers used [19].

pH	4	4.5	5	5.5	6	6.5	7	7.5	8	9
Buffer	Trizma		MES		Pipes		Mops		Bicine	

lowed. After seven days of incubation in the dark at 25˚C, the PIGR (P*ercentage* of Radial Growth *Inhibition*) has been calculated.

2.4.3. Effect of pH

To assess the effect of pH on the inhibition of mycelial growth by antagonistic bacteria, the following pH, acid, neutral, and basic, have been tested: 4; 4.5; 5; 5.5; 6; 6.5; 7; 7.5; 8 and 9 (Table 2). The medium PDA has been stamped, according to the desired pH, by different buffers. Using a pH meter, the pH has been adjusted by addition of HCl to the pH acids and NaOH to the basic pH. The boxes were incubated at 25˚C. The PICR is calculated after seven days of incubation.

2.4.4. Percentage of Inhibition of Radial Growth

After the incubation period, the radial growth of the pathogens was measured and the percent inhibition (1) of mean radial growth was calculated relative to the control as follows:

$$PIGR = (T - C)/T \times 100 \quad (1)$$

PIRG: Percent Inhibition of Radial Growth of pathogen's mycelium;

T: Radial growth of control agent;

C: Radial growth of the pathogen in the presence of the antagonist.

2.4.5. Statistical Analysis

All analyses were conducted in triplicates. The percent of inhibition of mycelial growth of the phytopathogenic agent by the antagonists have been subjected to an analysis of variance (ANOVA) using the software STATISTICA for Windows v.6. The statistical significance of the results was determined by performing a test of Duncan's multiple range ($p < 0.05$). Results were expressed as mean ± standard deviation.

3. Results

3.1. Dual Culture Technique

Bacillus amyloliquefaciens strains *and Trichoderma harzianum* provide inhibitory effects on the mycelial growth of the phytopathogenic strain (Figure 1). The inhibition of development of *Colletotrichum acutatum* isolate varies within *B. amyloliquefaciens* strains.

3.2. Effect of Temperature

The percent inhibition of mycelial growth by *Bacillus amyloliquefaciens* differs according the isolates and the temperatures studied. Isolate Bc2 is the most effective and presents the highest percent inhibition (Figure 2), whereas Bc5 has the lowest percent inhibition at all tested temperatures. *Trichoderma harzianum*

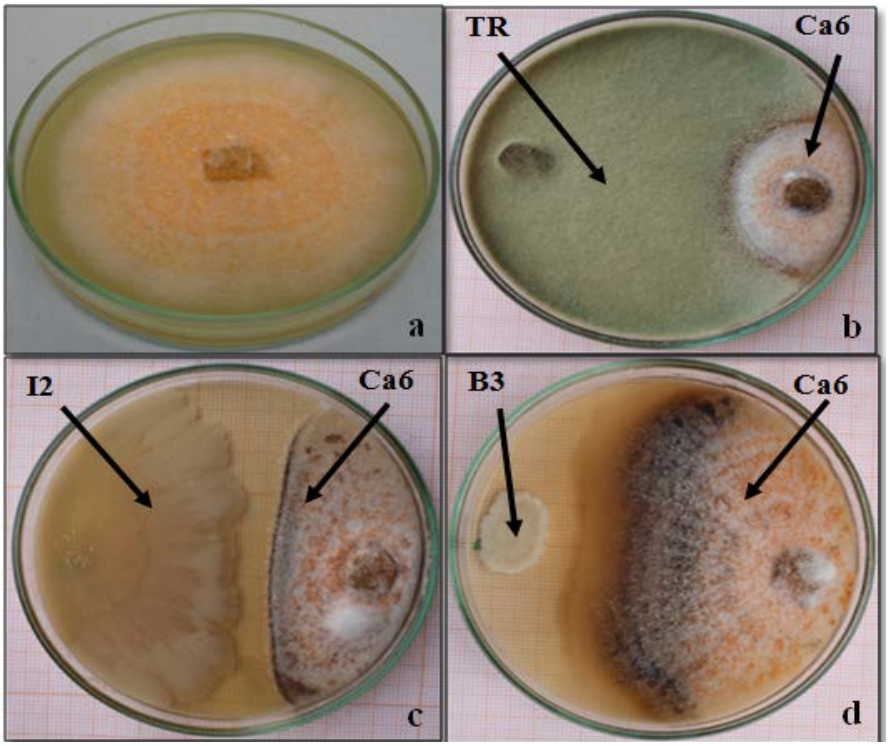

Figure 1. *In vitro* mycelial growth of *Colletotrichum acutatum* in PDA (a); dual culture between the antagonists and the phytopathogen in PDA (b, c and d); Inhibition of growth of *C. acutatum* by *Trichoderma harzianum* TR (b) and *Bacillus amyloliquefaciens* (isolate I2 (Bc2) (c) and isolate B3 (Bc4) (d)).

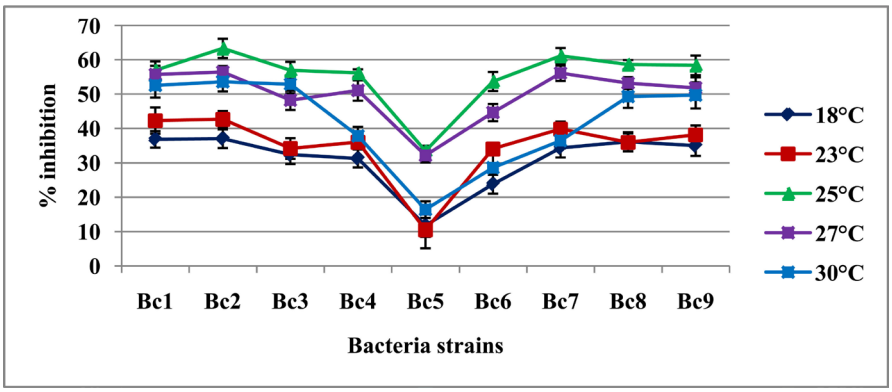

Figure 2. Effect of temperature on growth inhibition of *Colletotrichum acutatum* Ca6 by nine *Bacillus amyloliquefaciens* strains.

presents a very high efficiency on the inhibition of radial growth of *Colletotrichum acutatum* with an inhibitory effect exceeding 70% at all tested temperatures (**Figure 3**).

3.3. Effect of Water Activity

The inhibition of mycelial growth of *Colletotrichum acutatum* is important in the presence of the isolate Bc2 and low in the presence of Bc5 (**Figure 4**). *Trichoderma harzianum* has a high potential of radial growth inhibition of the

phytopathogenic agent that increases with the water activity (Figure 5).

3.4. Effect pH

The pH presents a large effect on the antagonistic potential of *Trichoderma harzianum* (Figure 6) and *Bacillus amyloliquefaciens* (Figure 7). Among the bacterial isolates, Bc2 shows a large inhibitory effect on the growth of *Colletotrichum acutatum*; contrariwise, Bc5 has the lowest inhibitory effect (Figure 7).

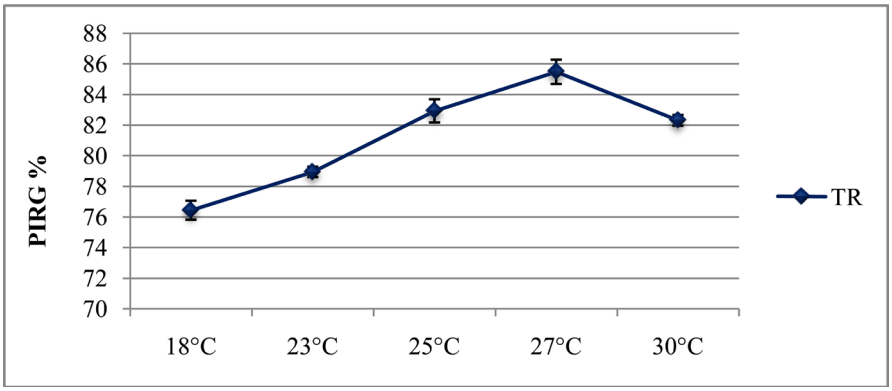

Figure 3. Effect of temperature on growth inhibition of *Colletotrichum acutatum* Ca6 by *Trichoderma harzianum*.

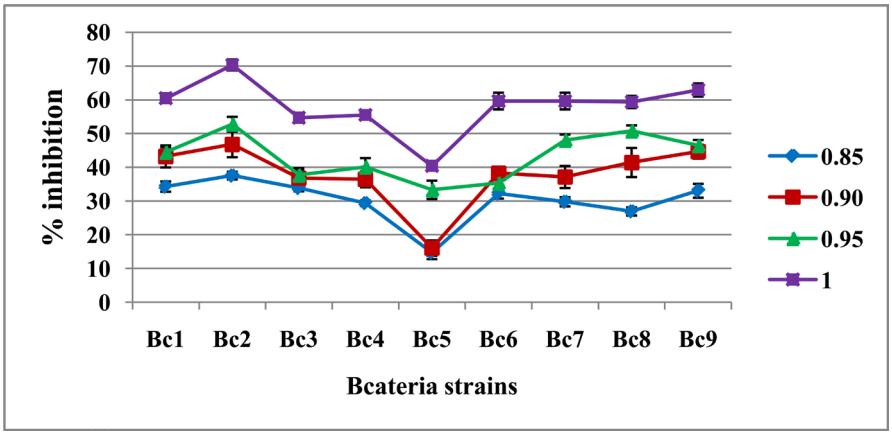

Figure 4. Effect of water activity on growth inhibition of *Colletotrichum acutatum* Ca6 by nine *Bacillus amyloliquefaciens* strains.

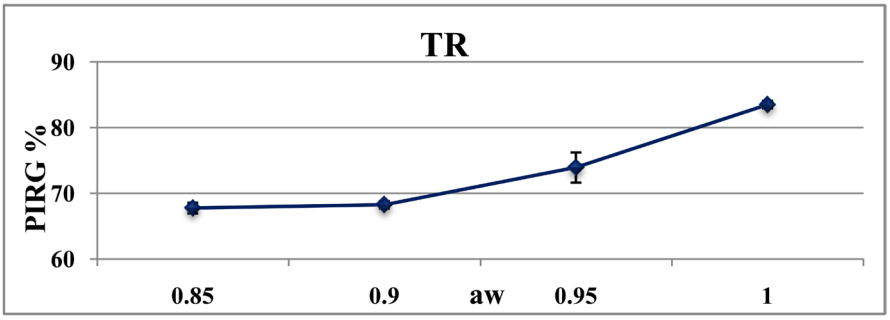

Figure 5. Effect of water activity on growth inhibition of *Colletotrichum acutatum* Ca6 by *Trichoderma harzianum*.

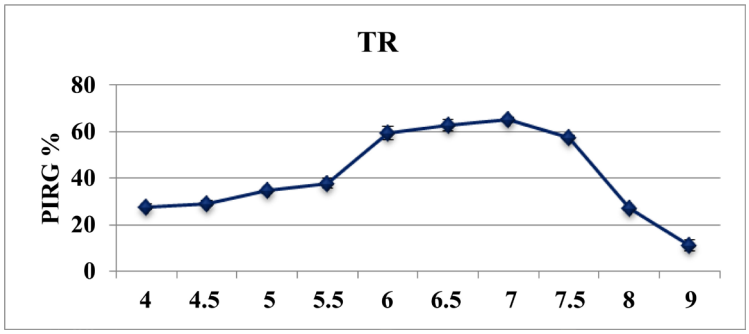

Figure 6. Effect of pH on growth inhibition of *Colletotrichum acutatum* Ca6 by *Trichoderma harzianum*.

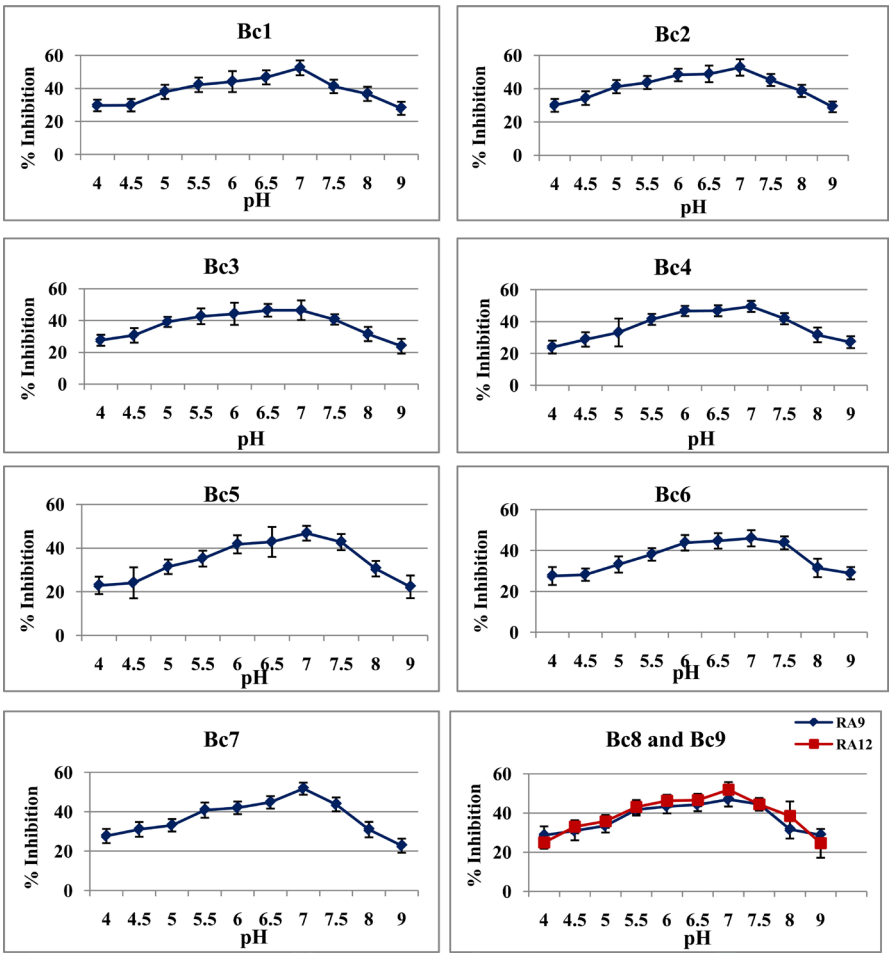

Figure 7. Effect of pH on the inhibition of radial growth of *Colletotricum acutatum* Ca6 by nine antagonistic bacterial strains.

The percent of growth inhibition of the phytopathogen by the fungal antagonist increase by the pH values up to neutral pH 7, and then start to decrease in alkaline pH (**Figure 6** and **Figure 7**).

4. Discussion

Biological control is an alternative to the use of phytochemicals in involving bi-

ological products in the control of plant diseases. In this study, we found that *Bacillus amyloliquefaciens* and *Trichoderma harzianum* provide inhibitory effects on the development of *Colletotrichum acutatum*, the phytopathogenic agent of the anthracnose of strawberry (*Fragaria x ananassa*). Biological control of Colletotrichum species has been demonstrated in other studies using Trichoderma species [13] and Bacillus species [9]. This study shows that *T. harzianum* grows faster than *C. acutatum* Ca6 strain. This rapid growth suggests a mycoparasitism, at least in the experimental conditions tested, and gives Trichoderma an important advantage in the competition for nutrients and space with phytopathogenic fungi [22]. Biological control is important in crop production disease control [23] [24]. *Bacillus subtilis* and *B. amyloliquefaciens* have been used in commercial biological control products due to their potential of biocontrol and high stability in harsh environmental conditions caused by spore forms [25].

The environmental factors play an important role, since they affect the biological life of the microbial species and the physiology/metabolism of pathogen antagonist and host plant [26] [27]. Several species antagonists of *Bacillus spp*. have shown efficiency in the fight against the anthracnose of multiple hosts [9] [28] [29] [30] [31].

The inhibition of mycelial growth of *Colletotrichum acutatum* by the *Bacillus amyloliquefaciens* strains tested and by changing the temperature, water activity and pH, shows that the potential of biological control varies from an isolate to the other. *Bacillus amyloliquefaciens* isolates have a different effect on mycelial growth of the phytopathogen. Bc2 shows a great effect compared to other isolates, Bc5 has a weak influence on radial growth. *Trichoderma harzianum* has a large antagonistic effect on the mycelial growth of the phytopathogenic agent. Hamdache *et al*. [19] have worked on the same bacterial strains to fight against *Botrytis cinerea* and have found that strain Bc7 has an efficiency of upper control against *Botrytis cinerea* compared to other strains to the different conditions tested, while strain Bc4 is the least effective. For the control against *Colletotrichum acutatum* we found that the isolate Bc2 is the more efficient compared to other isolates and Bc5 the less effective to the different conditions tested.

B. amyloliquefaciens show good antifungal activity on various plant pathogens, can be effectively used for controlling phytopathogens including *Colletotrichum acutatum* [32]. *Trichoderma harzianum* also has a great potential of biocontrol against anthracnose caused by *C. acutatum* [13] [33] [34].

Temperature has a great influence on the development of microorganisms as well as their biological activity. All bacterial isolates represent a large inhibitory effect of radial growth at 25˚C. Studies have been made on the influence of environmental parameters on the development of *Colletotrichum acutatum* and have found that the optimal values of the temperature is 25˚C ± 2˚C [35] [36] [37], Bc2 is the most effective among the other isolates and Bc5 the least effective. The fungal antagonist has a great inhibitory effect at all temperatures studied, and rises by increasing the temperature. *Trichoderma harzianum* has high

efficacy at 33˚C or lower against *Sclerotium rolfii*, another phytopathogenic agent, and produces secondary metabolites and mycotoxins at high temperatures that will help control plant pathologies [38]. Antagonistic activity *in vitro* of *T. harzianum* to *B. cinerea* is more effectively at a near-optimal 25˚C is consistent with a more rapidly increasing conidial respiratory rate at the higher temperature [39] [40]. The optimum temperature for radial growth of *Trichoderma spp.* is between 25˚C and 30˚C [41].

The water activity has a remarkable way on the mycelial growth, which is optimal between 0.95 and 1. The activity of water has also been favorable to inhibit the growth of *Verticillium dahliae* and *Rhizoctonia solani* by *Trichoderma harzianum* [42]. At 37˚C the optimal growth of B. amyloliquefaciens was at aw 0.960 [43] [44], this antagonist has inhibited the growth of *A. flavus* and *F. verticillioides* at aw = 0.99; 0.97; 0.95 and 0.93 [45] and inhibited the growth and aflatoxin B1 production by *Aspergillus* section Flavi at aw = 0.982 [46]. Maximal growth rates of *T. harzianum* were observed at aw 0.997 [16] [47], the optimal aw values for mycelial growth and *in vitro* enzyme activities were similar [16]. The *in vitro* enzyme activities of *T. harzianum* were also affected by aw, but significant enzyme activities were measured for most of the enzymes even at aw values less than the limit of mycelial growth [48].

Bacillus amyloliquefaciens and *Trichoderma harzianum* were influenced by pH, which was low at acid pH, and increased by increasing pH values to 7 (neutral pH) and then began to decrease in alkaline pH. *Colletotrichum musae*, agent to anthracnose of the banana tree, develops at an optimal pH equal to 4.5 [49]. The tolerance of *B. amyloliquefaciens* to grow under different pH-temperature was studied by Gotor-Vila *et al.* [43]; they have found that the optimum growth was observed at 37˚C and pH 5-7 [50]. *B. amyloliquefaciens* has exhibited significantly low activities of starch-degrading enzymes and high resistance to low pH [51]. The crude lipopeptides of *B. amyloliquefaciens* were insensitive to pH variation. The activity was not affected at pH 2 to 11, and was reduced at pH 12 [52] [53] which means variation of pH affect the antifungal activity of this antagonist. *T. harzianum* were able to grow on a wide range of pH from 2 to 6, and the optimal growth was observed at pH 4, the mycelial growth ceased at pH 8 and 7, also pH had an effect on the *in vitro* of enzymes activities of *T. harzianum* [16]. Jackson *et al.* [54] have found that optimum biomass production *Trichoderma harzianum* occurred at pH 4.6 - 6.8.

5. Conclusion

The antagonists behave differently depending on the environmental parameters. We found that isolate Bc2 show more efficiency compared to other bacterial strains, and that *Trichoderma harzianum* always inhibits the development of the pathogen despite the change in the factors studied. However, other parameters could be considered to develop and improve the control efficiency by these antagonists in order to acquire a viable biological control system against the brown

spot disease caused by *Colletotrichum acutatum*. Adaptation of biocontrol potential of the antagonists studied to environments with different temperature; aw and pH characteristics seems to be an important mechanism of evolution enabling the effective competition for nutrients under a wider range of these environmental parameters.

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