Full Length Research Paper

Effect of pH, temperature and water activity on the inhibition of *Botrytis cinerea* by *Bacillus amyloliquefaciens* isolates

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A total of 321 bacterial strains were isolated from the rhizosphere of healthy strawberry (*Fragaria* X *ananassa* Duch.) plants harvested from different places in Zlaoula area (Larache, Morocco). They were screened by dual testing for *in vitro* antagonism towards *Botrytis cinerea* (Bt7). Nine antagonistic strains were retained. Their effectiveness was evaluated according to temperature (5 to 30°C), pH (4 to 10) and water activity (aw, 0.80 to 1). Low values of water activity and acid pH were unfavourable for the growth inhibition. All the isolates were identified to belong to *Bacillus amyloliquefaciens*.

Key words: Antifungic activity, water activity, *Bacillus amyloliquefaciens, Botrytis cinerea*, pH, temperature.

INTRODUCTION

The strawberry plants undergo fungal attacks such as the gray mold due to *Botrytis cinerea* Pers.:Fr., which particularly touches the crop plants under greenhouse (Jarvis, 1989). *B. cinerea* infects the leaves, stems, flowers and fruits of the plants by direct penetration or through wounds (Hausbeck and Pennypacker, 1991; O' Neill et al., 1997). The infection is supported by high moisture and low temperatures (Eden et al., 1996; Morgan, 1985; O' Neill et al., 1997). The application of fungicides during flowering is the principal method of control of *B. cinerea* in the culture of the strawberry plant (Maas, 1984). However, this control is often difficult to realize (Sutton, 1990). Often chemical treatments are not allowed by regulatory agency policy unless applied well in advance of harvest, thus limiting their effectiveness for the control of disease during postharvest storage. Other treatments, such as surface sterilization and high-dose irradiation, can damage fruit tissue or leave residues that adversely alter taste or other quality factors.

The presence of chemical residues in the food chain and the development of resistant strains to fungicides aroused the interest of a development of more effective and respectful alternatives of the environment and health of the consumer (Wilson, 1993). Among those explored, biological control using antagonistic microbial agents showed a significant potential (Wilson, 1994). Biological control using antagonistic microbes alone, or as supplements to minimize the use of chemical pesticides in a system of integrated plant disease management, has become more important in recent years (Harman, 1991; Papavizas and Lewis, 1989).

The isolation and the selection of biological control agents (BCA) are the first two stages which condition the success of the later phases of the development of a biopesticide (Lepoivre, 2003). Several bacteria were isolated from the soil and the rhizosphere to increase the growth and the productivity of the plants or to control the pathogenic ones (Thakuria et al., 2004). The rhizosphere is a place of intense microbial activity with important exchanges between ground, root and microflora. The microbial density is high compared to that of a naked ground (Stengel, 1998). The studies of *in vitro*

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antagonism make it possible to select a great number of effective agents of biocontrol (Edwards and Seddon, 2001). Biological control of postharvest diseases of fruits and vegetables has proved feasible in numerous studies (Chalutz and Droby, 1998; Janisiewicz, 1998; Wisniewski and Wilson, 1992), and research has led to several commercial biological control products (Fravel and Larkin, 1996).

A good BCA must have a capacity of survival and adaptation to the various environmental conditions (Lepoivre, 2003). The environment affects the survival and the activity of the agents of biocontrol (Benbow and Sugar, 1999). Abiotic factors (temperature, pH, relative humidity) in which the air part of the plant develops act on the development of pathogenic and antagonist agents. Before biological control by any antagonistic agent can be practically implemented, it is essential to determine how biological control may be affected by changing environmental conditions. Overall, it is important to learn as much as possible regarding the ecology of these biocontrol organisms and their interactions with the pathogen, host plant and their surrounding environments (Cook, 1993; Handelsman and Stabb, 1996; Larkin et al., 1998).

The objective of this work was to select and identify some bacterial strains effective against *B. cinerea* and evaluate the *in vitro* effects of different environmental conditions including temperature, water activity, and pH on the efficacy of control of *B. cinerea* by these selected isolates of *Bacillus amyloliquefaciens*.

MATERIALS AND METHODS

Fungal pathogen strain

The Bt7 isolate was the isolate of *B. cinerea* used in this study. It was isolated from naturally infected strawberry fruits presenting gray mold symptoms. It was selected for its aggressiveness among several isolates found in different strawberry cultivars. *B. cinerea* (Bt7), originated from fields of strawberry plants of Loukkous area (Larache, Morrocco), developed well in Potato Dextrose Agar (PDA, Biokar Diagnostics) and were incubated seven to ten days in 25°C before use. The identification was carried out by macroscopic and microscopic observations of the isolates using keys of determination (Samson et al., 1984; Botton et al., 1990). The isolate was maintained at 4°C in the PDA medium tilted in tubes (200 × 20 mm).

Isolation of antagonistic bacterial strains

The 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 Region of Loukkous (Larache, Morrocco). A mother suspension was prepared (5 g of soil in 120 ml of sterile peptone water 0.1% w/v). Then, a decimal dilutions series were prepared and 0.1 ml of each dilution was plated in Petri dishes containing medium Plate Count Agar (PCA, Biokar Diagnostics). Roots were shaken to remove excess soil and rinsed with 0.1% (m/v) sterile peptone water. The rinsing solutions was then serially diluted and plated on PCA. The rinsed roots were dried and crushed with pestle and mortar. 2 g from the crushed roots were transferred to 50 ml sterile peptone water [0.1% (w/v)] and serial decimal dilutions were prepared. 0.1 ml of each dilution was plated in Petri dishes containing medium PCA.

After 24 to 48 h incubation at 28°C, the colonies observed were transferred on others Petri dishes of PCA to purify the bacterial strains. The antagonistic bacteria were maintained at -20°C in brain heart infusion (BHI) with glycerol 20%.

Selection of the antagonistic bacteria of *B. cinerea in vitro*

The activity of the isolated bacteria was tested on the Bt7 isolate by the method of the dual culture on Petri dishes containing PDA medium. A mycelial disk (5 mm diameter) of a 7 day old culture of *B. cinerea* was placed on one side of a Petri dish containing PDA. Each bacterium strain, cultivated beforehand on PCA medium during 24 h, was placed 2 cm away from the *B. cinerea* disk. The dishes were incubated at 25°C in the dark. After 7 to 10 day incubation when inhibition zones appeared, the results were noted and observed visually. An equivalent zone of the medium on which *B. cinerea* had grown alone was used as the control.

Effect of environmental factors

Among the bacterial strains isolated, those having expressed a strong antifungal capacity against *B. cinerea* (Bt7) were selected and retained. Their inhibition was evaluated by calculating the percentage inhibition of radial growth (PIRG) (Ezziyyani, 2004), according to various parameters (temperature, water activity and pH).

Temperature

The effect of temperature on the inhibition of mycelial growth of *B. cinerea* by the antagonistic bacteria was evaluated. For this, a disc of mycelium cut from the edge of a growing colony of the pathogen was placed on one side of a Petri dish containing PDA. A bacterial spot was placed on the other side of the Petri dish making them equidistant from the centre. An equivalent zone of the medium on which *B. cinerea* had grown alone was used as the control. The dishes were incubated in the dark at 5, 15, 20, 25 and 30°C. After seven day incubation, the PIRG was calculated.

$$PIRG\ (\%) = [(R1-R2)/R1] \times 100$$

Where, R1 is the ray of the colony of *B. cinerea* in the absence of the antagonist (control) and R2 is the ray of the colony of *B. cinerea* in the presence of the antagonist.

Water activity (aw)

The aw)represents the water vapor pressure of a wet product to the water vapor pressure of pure water at the same temperature. It represents the availability of water for the biochemical reactions for the development of the micro-organisms. Various values of aw were tested (1, 0.95, 0.90, 0.85 and 0.80) by glycerol addition in PDA medium (Maouni, 2002), which will fix part of the water and will make it unusable to the micro-organisms. The same technique of confrontation used in temperature tests was followed. After seven dayS incubation in darkness at 25°C, the PIRG was calculated.

pH

With the same technique of confrontation in Petri dishes, the

Table 1. pH values and buffers added in PDA medium.

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

inhibition of the mycelial growth was studied on PDA medium, at various pH values (4, 4.5, 5, 5.5, 6.5, 7, 7.5, 8, 9 and 10). The medium was buffered, according to the pH desired, by different buffers (Table 1). The pH was adjusted by addition of HCl and NaOH using a pH-meter until the desired pH was reached. The PIRG was calculated after seven days incubation at 25°C.

Statistical analysis

Each treatment was replicated three times. The data were statistically analysed by applying a one-way ANOVA, for comparison of mean values, followed by Duncan's multiple range test at the 0.05 level of significance.

Identification of bacterial isolates

Bacterial strains with antifungal activity were identified, at first, on the basis of some morphological and physiological characteristics and Gram stain. Then, a molecular technique was used to confirm the identification. The bacterial isolates were identified at the Spanish Type Culture Collection (CECT "Colección Española de Cultivos Tipo"), Valencia-Spain, for confirmation of the *Bacillus* species.

The identification was made according to the following method: direct amplification by polymerase chain reaction (PCR) of the 16S rRNA gene, partial sequencing of the same gene (with reading on the two directions) and analysis of the sequences (Arahal et al., 2008). Later, the results of the BLAST analysis were indicated (Zhang et al., 2000) in front of the NCBI databases, collecting the fragment extension, the percentage of similarity and the number of microorganisms with a major grade of sequence identity.

RESULTS

Isolation and selection of the antagonistic bacteria of *B. cinerea*

Among the total strains isolated, 56% originated from soil rhizosphere, and 44% from roots. All of the bacterial

strains (180 originated from rhizosphere soil and 141 originated from roots) were confronted with *B. cinerea* Bt7. After seven to 10 days incubation, the ineffective strains were completely invaded by the mycelium of the pathogen. Seven isolates from soil rhizosphere (I1, I2, I3, I18, B3, B12 and B24) and two strains from roots (RA9 and RA12) were retained for their inhibiting effect on the growth. Inhibition appeared either with a direct contact between the bacterium and the pathogen, or with the appearance of a clear zone separating the mycelium from the bacterial colony (Figure 1).

Effect of the environmental factors

Temperature

The percentages of inhibition of the radial growth by the antagonistic bacteria differed according to the studied strains and the temperature (Table 2). At 5°C, after seven day incubation, no growth was observed for the pathogen as for the antagonist. At 25°C, all the antagonistic strains did not show any significant difference. Strain B24 proved to be most effective with the highest percentages at 15, 20 and 25°C, whereas strain I18 showed lower inhibition at all the temperatures tested.

Activity of water

The inhibition of the mycelial growth of *B. cinerea* was less important at low values of aw and better at high values (Figure 2). Below 0.90 (0.80 and 0.85), all the bacterial isolates presented an inhibition lower than 30%. At high aw values (0.90 to 1) the isolates reached or exceeded 50% of inhibition. Except the normal condition when aw = 1, the isolate I18 appeared clearly less effective than other isolates (8.97% at 0.80) and it had a clear difference between each condition.

pH

For all the antagonists, the acid pH gave an inhibition lower than 50% (Figure 3). Strains B24, RA9 and RA12 reached 50% of inhibition at pH 5.5, I2, I18 and B12 at pH 6.5, B3 at pH 7 and I1 and I3 at pH 7.5. The optimum was variable and it varied especially between pH 7.5 and 10. Thus, maximum inhibition was obtained at alkaline pH for I3 and neutral pH for I1 and B24. It reached 62.27% at pH 7.5 for B24.

Identification of antagonistic isolates

The effective biocontrol strains were selected for identification. The isolates were subjected to first stage diagnostic tests including Gram stain. All of them were Gram-positive bacilli. The molecular identification revealed that the nine antagonistic bacterial isolates belong to the species *B. amyloliquefaciens*. Strains at the beginning were noted by an arbitrary notation I1, I2, I3, I18, B3, B12, RA9 and RA12. Then, they were coded by order from Bc1 to Bc9 beside *B. amyloliquefaciens* after molecular identification (Table 3).

DISCUSSION

Several strains belonging to the genus *Bacillus* and particularly to *Bacillus subtilis* and the closely related *B.*
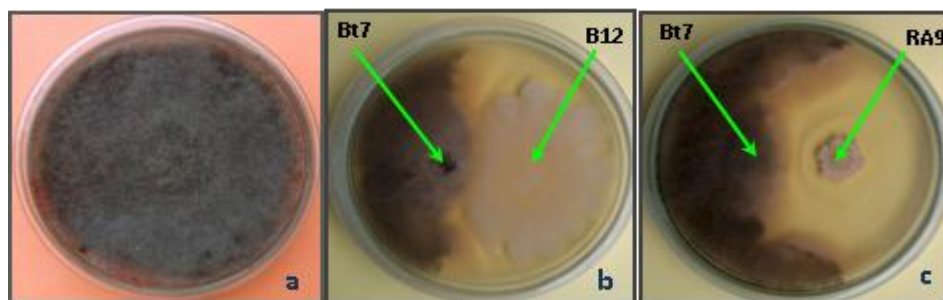
Figure 1. *In vitro* growth of the pathogen *Botrytis cinerea* in potato dextrose agar (PDA) (Control) (a). Dual confrontations of the antagonists against the pathogen Bt7 in PDA medium (b-c); Inhibition of growth of *Botrytis cinerea* by the B12 isolate originated from soil (b) and the RA9 isolate originated from root (c).

Table 2: Effect of the temperature on the inhibition of the mycelial growth of *Botrytis cinerea* by nine antagonistic bacteria. No growth of pathogenic as of the antagonists is observed with 5°C.

Origin of isolate	Isolates name	Temperature (°C)			
		15	20	25	30
Soil	I1	48.89 abc	47.62 bc	63.81 a	45.88 abcd
	I2	44.00 c	48.57 bc	58.57 a	38.82 bcd
	I3	47.11 bc	48.10 bc	59.52 a	47.06 abc
	I18	27.56 c	37.14 c	47.62 a	32.94 d
	B3	45.78 bc	43.33 bc	54.28 a	50.59 ab
Root	B12	49.33 abc	67.14 ab	50.47 a	57.65 a
	B24	73.33 a	73.33 a	64.28 a	48.24 ab
	RA9	70.22 ab	55.71 abc	53.33 a	34.12 cd
	RA12	34.22 c	47.14 bc	60.00 a	38.82 bcd

Means within a column, followed by the same letter, are not significantly different (P = 0.05) according to Duncan's multiple range test.
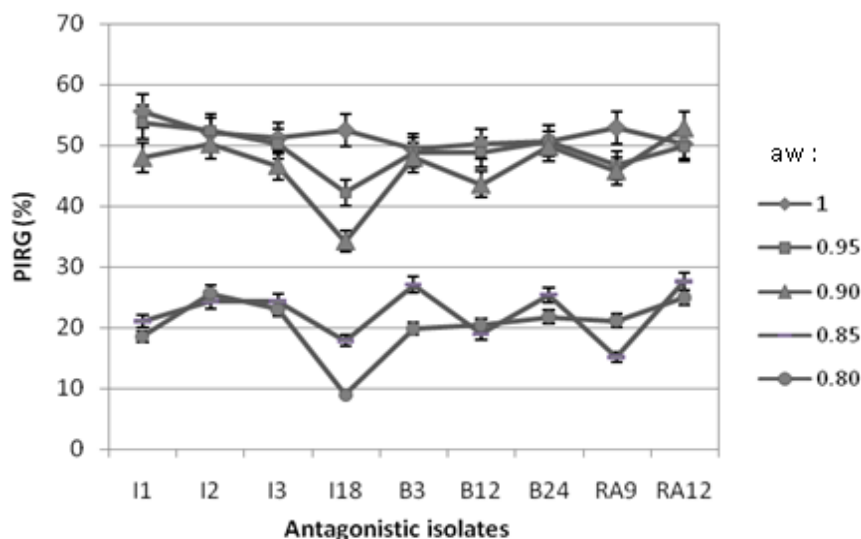

Figure 2. Effect of the water activity (aw) on the percentage of inhibition of the radial mycelial growth (PIRG %) of *Botrytis cinerea* by nine antagonistic bacteria.
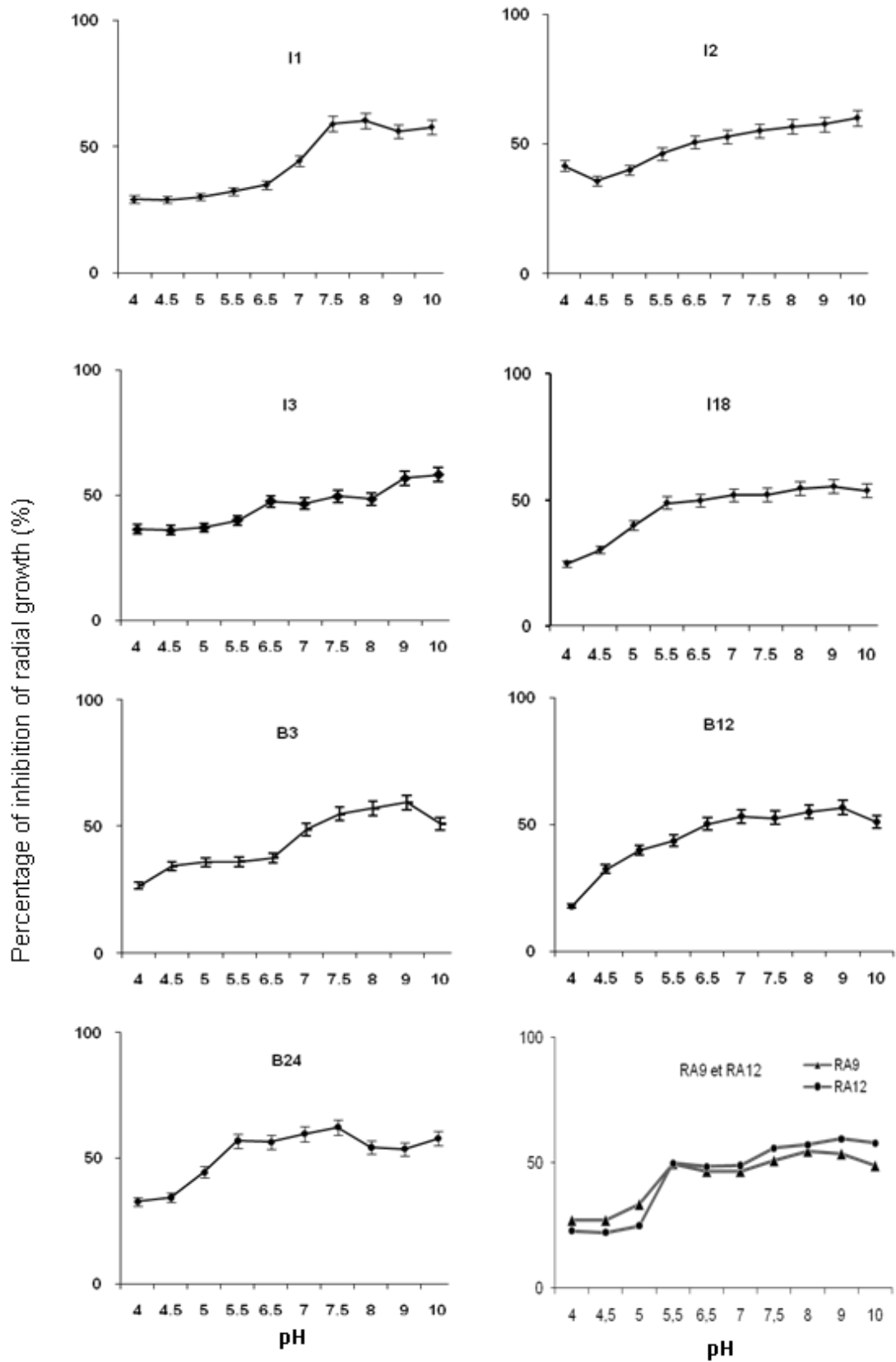
Figure 3. Effect of pH on the percentage of inhibition of radial growth of *Botrytis cinerea* (Bt7) by nine antagonistic bacterial strains.

Table 3: Identification of antagonistic strains.

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

amyloliquefaciens species were reported effective for the biocontrol of multiple plant diseases caused by soilborne (Asaka and Shoda, 1996; Chen and Wu, 1999; Harris and Adkins, 1999) or post-harvest pathogens (Ferreira et al., 1991; Sholberg et al., 1995; Mari et al., 1996a). The isolation from the rhizosphere soil and roots of cultivated strawberries plants allowed the obtaining of 321 bacterial strains. After a selection by dual culture, *in vitro* nine antagonistic strains showed an inhibiting effect of growth against *B. cinerea*. The morphological identification demonstrated that the strains were Gram-positive bacilli. The identification of the species was confirmed by the CECT, and all of the strains belong to *B. amyloliquefaciens*. Other isolates of this species also have a potential as biological control agents as evidenced in diverse studies. Recent works showed an important biological activity of this bacterium. *B. amyloliquefaciens* PPCB004 was selected as a potential antagonist to control *B. cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* on peach fruit (Arrebola et al., 2010). *B. amyloliquefaciens* strain RC-2 isolated from healthy mulberry leaves, showed an inhibition activity against anthracnose disease (Yoshida et al., 2001). This species is similar to *B. subtilis* (Sneath, 1986), and can be distinguished from *B. subtilis* by a slightly higher molecular percent G+C content of the DNA (Welker and Campbell, 1967). Although *B. amyloliquefaciens* shows properties similar to those of *B. subtilis*, it has not been as frequently reported as an antagonist of various plant diseases. Mari et al. (1996b) reported that *B. amyloliquefaciens* 2TOE reduced the severity of gray mold caused by *B. cinerea* in pears. They suggested that the antifungal activity of the bacterium was due to competition for nutrients. The antagonistic bacteria reduced the crown-rot caused by pathogens (*Lasiodiplodia theobromae*, *Thielaviopsis paradoxa*, *Colletotrichum musae* and *Fusarium verticillioides*) in banana (Alvindia and Natsuaki, 2009) and two *B. amyloliquefaciens* isolates were evaluated *in vitro* and *in vivo* as potential BCAs against *Sclerotinia sclerotiorum* (Abdullah et al., 2008); they inhibited the growth and

production of mycelia and sclerotia. Yu and Sinclair (1996) reported that strain B94 of the bacterium coated on soybean seeds readily colonized the seedling rhizosphere and controlled *Rhizoctonia solani*.

Biocontrol activity of antagonists may be influenced by the specific pathogen, host commodity and particularly by environmental conditions (Spotts et al., 1998; Tian et al., 2002). The *in vitro* inhibition of the mycelial growth of *B. cinerea* by antagonists depends on temperature, pH and activity of water. At 5°C, no growth of the micro-organisms was observed. It was shown that the maximum rate of growth of *B. cinerea* colonies decreases when the temperature of incubation is low (Lahlali et al., 2007). At 25°C, no significant difference was observed between the percentages of inhibition of the mycelial growth of the nine antagonistic isolates. At the other temperatures, the strains showed a variable effect. The isolate B24 proves to be most effective contrary to I18. The low values of water activity (0.80 and 0.85) were unfavourable for the development of the bacilli. The rate of growth of the colonies of *B. cinerea* decreases when the activity of water is low (Lahlali et al., 2007). The Gram-positive bacilli develop on high water activity, about 0.95 (Prescott et al., 2007). The high values are also favorable to the growth inhibition of *Verticillium dahliae* and *R. solani* by the antagonist *Trichoderma harzianum* (Santamarina and Roselló, 2006), with a maximum inhibition at 0.98 and 0.995. The inhibition of the mycelial growth of *B. cinerea* Bt7 was also influenced by the pH.

The neutral and/or alkaline pH gave a better inhibition than the acid one. Rosenzweig and Stotzky (1979), in a similar way, observed an antagonistic effect, in soil, of bacteria against fungus when the pH increases. In general, the bacteria prevail in the neutral or slightly alkaline soils (Davet, 1996). Their relative abundance in the acid soils is due only to the difficulty for majority of the bacteria to develop at pH lower than 6.5 (Davet, 1996). This study shows that the antagonistic bacteria behave differently according to environmental parameters. The B24 isolate proved to be the most effective one. Other parameters could be studied in order to define optimum

conditions for biological control of the gray mold caused by *B. cinerea*.

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