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Effect of preventive and simultaneous inoculations of *Bacillus amyloliquefaciens* (Fukumoto) strains on conidial germination of *Botrytis cinerea* Pers.:Fr.

Ahlem Hamdache[1], Mohammed Ezziyyani[2] & Ahmed Lamarti[1]

1 Department of Biology, Faculty of Sciences, Abdelmalek Essaâdi University, 2121, Tetuan, Morocco.
2 Department of Life Sciences, Polydisciplinary Faculty of Larache, Abdelmalek Essaâdi University, Morocco.

Correspondence
A. Hamdache
E-mail: hamdach_ahlem@yahoo.fr
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Resumen

Efecto de inoculaciones preventivas y simultáneas de cepas de Bacillus amyloliquefaciens *(Fukumoto) sobre la germinacion de conidios de* Botrytis cinerea *Pers.:Fr.*

Botrytis cinerea es un patógeno fúngico necrotrófico de varias plantas distribuido en todo el mundo. En el presente estudio se analizó el efecto de nueve aislados bacterianos sobre la germinación de conidios de *B. cinerea*. Los aislados se identificaron como *Bacillus amyloliquefaciens*. Para evaluar la eficacia de los aislados, se realizaron confrontaciones duales tanto preventivas como simultáneas con los conidios de *B. cinerea*. Los resultados mostraron que cinco aislados fueron más eficaces cuando se co-inocularon simultáneamente con el patógeno. Cuatro aislados fueron más eficaces cuando se inocularon previamente cuatro horas antes el patógeno. La cepa denominada B24, mostró una inhibición de (84.04%) en concentraciones muy bajas ($3x10^3$ UFC/ml) y la cepa RA9 fue la cepa menos eficaz.

Palabras clave: Bacterias antagónicas, inhibición, concentración.

Abstract

Botrytis cinerea is a necrotrophic fungal plant pathogen distributed worldwide. In the current study, the effect of nine bacterial isolates, on germination of *B. cinerea* conidia were studied. The nine isolates were identified as *Bacillus amyloliquefaciens*. The efficacy of isolates was tested, at different concentrations, in preventive and simultaneous inoculations with *B. cinerea* conidia. Results showed that five *Bacillus* isolates were more effective when co-inoculated simultaneously with the pathogen. Four isolates showed more efficacies when inoculated previously four hours before the pathogen. The isolate denominated B24 was the only that showed an important percent inhibition (84.04 %) at the lower concentration tested ($3x10^3$ CFU/ml) and the isolate RA9 was the less effective strain.

Key words: Bacterial antagonists, inhibition, concentration.

Introduction

Strawberry gray mold, caused by *Botrytis cinerea* (de Bary) Whetzel, is one of the most serious diseases that affect fruits, leaves, petioles, stems, and flowers in cold and wet weathers (Agrios 2005). In addition, conidial spores contaminated during harvest can cause serious storage rot, especially when fruits are wet (Braun & Sutton 1987). In field, new infections usually occur by wind-dispersed conidia, which germinate on the plant surface and invade the tissue either through wounds or by direct penetration of cuticles and cell walls of epidermal cells (Holz *et al.* 2004). The chemical control and use of fungicides are the most effective way of preventing the occurrence of Botrytis disease (Rabosto *et al.* 2006). However, the use of chemicals is considered undesirable because of concerns over residues, their potential adverse effects on human health and the environment (Zhang *et al.* 2015) and the fast development of resistance to novel fungicides by fungi (Leroux 2004, Walker *et al.* 2013, Hahn 2014, Romanazzi & Feliziani 2014). Therefore, the difficulty in controlling *B. cinerea* has led to researchers to find alternative methods, which include biological control (Sutton 1995). Biological control has been shown to be successful in many other crops (Saligkarias *et al.* 2002) and as effective as chemical control (Dik & Elad 1999). However, the efficacy of biological control is occasionally inadequate and variability may be high. The mechanisms involved in biological control include, among others, induced resistance, competition for nutrients, and secretion of inhibitory compounds. *Bacillus* spp. have shown promise for controlling a wide range of fungi that cause decay, operating as an antagonist to plant pathogen growth through their production of antibiotics (iturin, surfactin, fengycin), enzymes that degrade fungal structural polymers (chitinase, β-1,3 glucanase), and antifungal volatiles (Leelasuphakul *et al.* 2006). *Bacillus amyloliquefaciens* has an antibacterial activity against *Xanthomonas oryzae* (Ishiyama 1922) Swings *et al.* (Wu *et al.* 2015) and antifungic activity against *Penicillium digitatum* (Pers.) Sacc., *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (=*Pyricularia grisea* Sacc.) and *Sclerotinia sclerotiorum* (Lib.) de Bary (Ji *et al.* 2013), *Fusarium verticillioides* (Sacc.) Nirenberg and *Aspergillus flavus* Link (Etcheverry *et al.* 2009) and also against *Pythium aphanidermatum* (Edson) Fitzp. (Zouari *et al.* 2016). In the present study, the inhibition of conidial germination of *B. cinerea* was investigated to characterize potential bacterial biocontrol agents isolated from rhizospheric soil of strawberry plants. The effect of nine isolates of *Bacillus amyloliquefaciens* Priest *et al.*, at different concentrations, was compared at two times of inoculations (T = 0h, as a simultaneous inoculation and T = 4h as a preventive inoculation).

Materials and methods

Pathogenic fungi

B. cinerea pers.; Fr. the causal agent of gray mold disease on strawberry fruits was kindly isolated from diseased strawberry fruits (Fig. 1A) from a field of Lokkous (Larache, north of Morocco). The isolate was purified, identified by macroscopic and microscopic observations using keys of determination (Samson *et al.* 1984, Botton *et al.* 1990), noted Bt7 and maintained on PDA (Potato Dextrose Agar) medium (Fig. 1B).

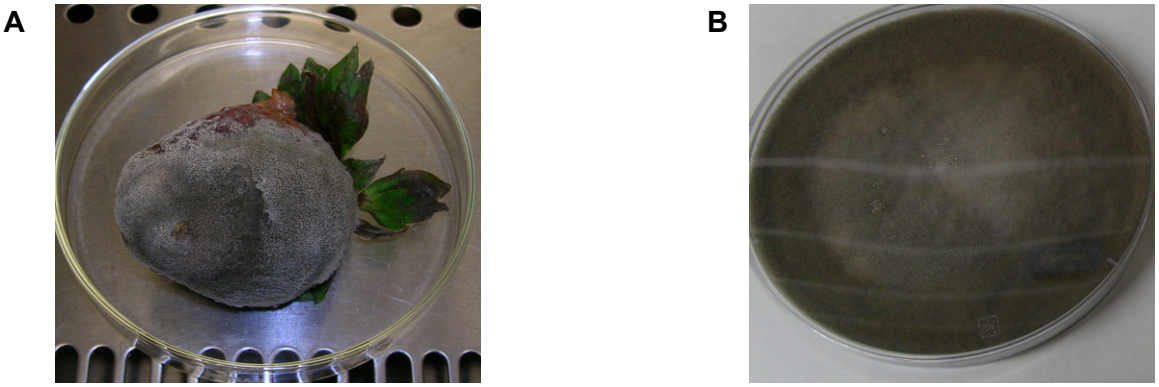

Figura 1. Podredumbre de la fresa causada por el aislado *Botrys cinerea* (Bt7) (**A**) y aspecto macroscópico del hongo en medio PDA (**B**).
Figure 1. *Botrys cinerea* (Bt7) on diseased strawberry fruit (**A**) and on PDA medium (**B**).

Conidia were harvested from 10- to 14-day-old cultures by agitating small pieces of agar bearing mycelia and conidia in a glass tube containing 4 ml of sterile distilled water. The suspension was filtered through cheesecloth, and the spore concentration was calibrated with a Malassez chamber and adjusted to $1x10^6$ spores per ml.

Bacterial antagonists

Nine bacterial isolates were selected, for their inhibitory effect against *B. cinerea in vitro*, from a mass selection (321 isolates) made by Hamdache (2012). These nine selected isolates were obtained from rhizospheric soil of healthy strawberry plants from the Loukkous zone in the north of Morocco. These included isolates I1, I2, I3, I18, B3, B24, B12, RA9 and RA12. Isolates were identified by amplification of the 16S rRNA gene by PCR as Bacillus sp. and showed 100% similarity with sequences of the 16S rRNA gene of strains of *B. amyloliquefaciens* (Hamdache *et al.* 2012) contained in data bases (Table 1). For the preparation of the bacteria suspensions, isolates were streaked onto Petri dishes containing Luria Bertani medium and maintained at 28 ºC. After 24h they were transferred to 50 mL fresh Luria Bertani liquid medium in a 250 mL Erlenmeyer flasks and maintained for 48 h under constant agitation (125 rpm). For each antagonistic strain, and after 48 h cultivation, 5 ml of medium was taken and cells were harvested by centrifugation at 4000 rpm for 10 min. The cells collected by centrifugation were washed twice with sterile distilled water by repeating the two centrifugation steps. Finally, cells were re-suspended in 5 ml of sterile distilled water and adjusted to $3x10^8$ colony-forming units (CFU)/ml, according to the scale of Mac Farland. Then, five decreasing concentrations were prepared: $3x10^7$, $3x10^6$, $3x10^5$, $3x10^4$ and $3x10^3$ CFU/ml.

The strains are deposited and available at the Faculty of Sciences-Tetuan, Abdelmalek Essaâdi University, Tetuan, Morocco.

Effect of bacterial antagonists on conidial germination of *B. cinerea*

To evaluate the effect of bacterial isolates on the conidia germination of *B. cinerea*, a volume of a 200 µl *B. cinerea* conidia suspension adjusted to 106 conidia/ml was added to 200 µl of bacterial suspensions of each of different concentrations ($3x10^7$, $3x10^6$, $3x10^5$, $3x10^4$ and $3x10^3$ CFU/ml). The two suspensions were mixed in tubes containing 5 ml of sterile liquid medium PDB (Potato Dextrose Broth). The tubes were incubated 24 hours at 25°C. Two applications time were compared; the first was simultaneous inoculation or co-inoculation (T=0h) of the two microorganisms (antagonist-pathogen) and the second was a pre-inoculation (T=4h) of the antagonist four hours before the addition of the pathogen. Each experiment using a single *B. amyloliquefaciens* isolate was run in triplicate. Results are expressed as the means of the percentage of inhibition of conidial germination of *B. cinerea* isolate in the presence of any of the strain of antagonistic bacteria. Percent inhibition was calculated using the following formula:

% inhibition = [1 - (Conidial germination / Control conidial germination)] x 100

The data were statistically analyzed 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.

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**	100,0 % (1030/1030 pb)	CR-502
I18	*B. amyloliquefaciens* **Bc4**	100,0 % (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

Tabla 1. Identificación de cepas antagonistas de *Bacillus amyloliquefaciens* (Hamdache *et al.* 2012)
Table 1. Identification of antagonistic strains of *Bacillus amyloliquefaciens* (Hamdache *et al.* 2012)

Results and discussion

In order to evaluate antagonistic activity of *Bacillus* strains against spore germination of *B. cinerea*, *in vitro* assays were performed. The effectiveness of the *Bacillus* isolates on the conidial germination of *B. cinerea*, in two different times of application of the bacterial suspension, (T = 0 hours and T = 4 hours) was compared. In the first case (T = 0h), the two microorganisms, pathogen and antagonist, were co-inoculated at the same time. In the second case (T = 4h), the antagonist was pre-inoculated four hours before introducing the pathogen. The results showed that an important inhibition of conidial germination of *B. cinerea* was observed with all of isolates (Fig. 2). The bacterial antagonists I1, I2 and I3 were more efficient when co-inoculated with the pathogen. However, strains I18 and B24 showed more efficacy when inoculated four hours before the pathogen. B3 was very effective in both cases of inoculation at high concentration, but this efficacy decreased at low dose when it was co-inoculated with the pathogen. For the two isolates B12 and RA9, no difference was found between the two times of application (T=0h and T=4h) at low concentrations ($3x10^4$ and $3x10^3$ CFU/ml), but at high concentration simultaneous application was more effective. For all of the isolates and in the two cases of application of the antagonist the inhibition of conidial germination increased proportionally with the inoculum concentrations. The results showed that the highest percentage of inhibition (100 %) was observed at high concentration tested ($3x10^7$ CFU/ml) and the lowest (0.04%) (I2, T=4h) was at low concentration ($3x10^3$ CFU/ml). At $3x10^5$ CFU/ml, the best inhibition percent of conidial germination by I18, I1, I2, I3, B24 and B3 isolates were 63.16, 77.79, 85.26, 89.93, 98.62 and 100 % respectively. B24 and B3 showed the best inhibition and were more efficient than other antagonists when they were pre-inoculated, at low concentration with no statistically differences between the different concentrations tested $3x10^7$, $3x10^6$, $3x10^5$ and $3x10^4$ showing inhibition percentages of 100, 98.95, 98.62 and 98.09% respectively for B24 and 100, 100, 100 and 96.33% respectively for B3. The B24 was the only isolate that showed an important inhibition percent (84.04 %) at the low concentration tested $3x10^3$ CFU/ml. RA9 was the less effective, with percentage of inhibition lower than 40% at high concentration.

This study allowed us to determine the minimal inhibitory concentration of each isolate of *B. amyloliquefaciens* (Table 2) to inhibit conidial germination of *B. cinerea*. In biological control studies, a good biocontrol agent (BCA) must be effective at low concentration (Wisniewski & Wilson 1992). Hence, we suggest that *B. amyloliquefaciens* B3 (Bc5) and *B. amyloliquefaciens* B24 (Bc7) can be considered as the best biological control agent with optimal inhibition of conidial germination of *B. cinerea* at low concentration.

	Time of application	
Antagonistic isolates	**Simultaneous (T = 0h)**	**Preventive (T = 4h)**
B3	$3x10^5$	$3x10^4$
B24	$3x10^7$	$3x10^4$
I2	$3x10^7$	$3x10^7$
I3	$3x10^6$	$3x10^7$
I18	$3x10^6$	$3x10^6$
I1	$3x10^6$	$3x10^7$
RA12	$> 3x10^7$	$> 3x10^7$
B12	$> 3x10^7$	$> 3x10^7$
RA9	$> 3x10^7$	$> 3x10^7$

Tabla 2. Determinación de la concentración mínima inhibitoria de cepas de *B. amyloliquefaciens* sobre la germinación de conidios de *B. cinérea*.

Table 2. Minimal inhibitory concentration of *B. amyloliquefaciens* strains on conidial germination of *B. cinerea*.

B. amyloliquefaciens isolates used in this study were capable of reducing the germination of the pathogen *B. cinerea*.

These isolates differed by their mode of action and by their effectiveness according to the concentration and the time of application to the pathogen. In the dual confrontations of the antagonist against the pathogen in PDA medium (Fig. 3), the inhibition zone produced by *B. amyloliquefaciens* increased with time, an increase that was accompanied by the necrosis of the fungal mycelium that had developed so far (Hamdache *et al.* 2012). The first thing to be noticed was suppressed growth of *B. cinerea* with the suppression zone increasing as the growth of *B. amyloliquefaciens* increased. This was followed by a marked antibiosis and competition for space and nutrients, effect that was manifested by reduced pathogen mycelial growth. After seven days, *B. amyloliquefaciens* stopped the growth of *B. cinerea* colonies.

Bacillus species are well known for their ability to control plant diseases through various me-

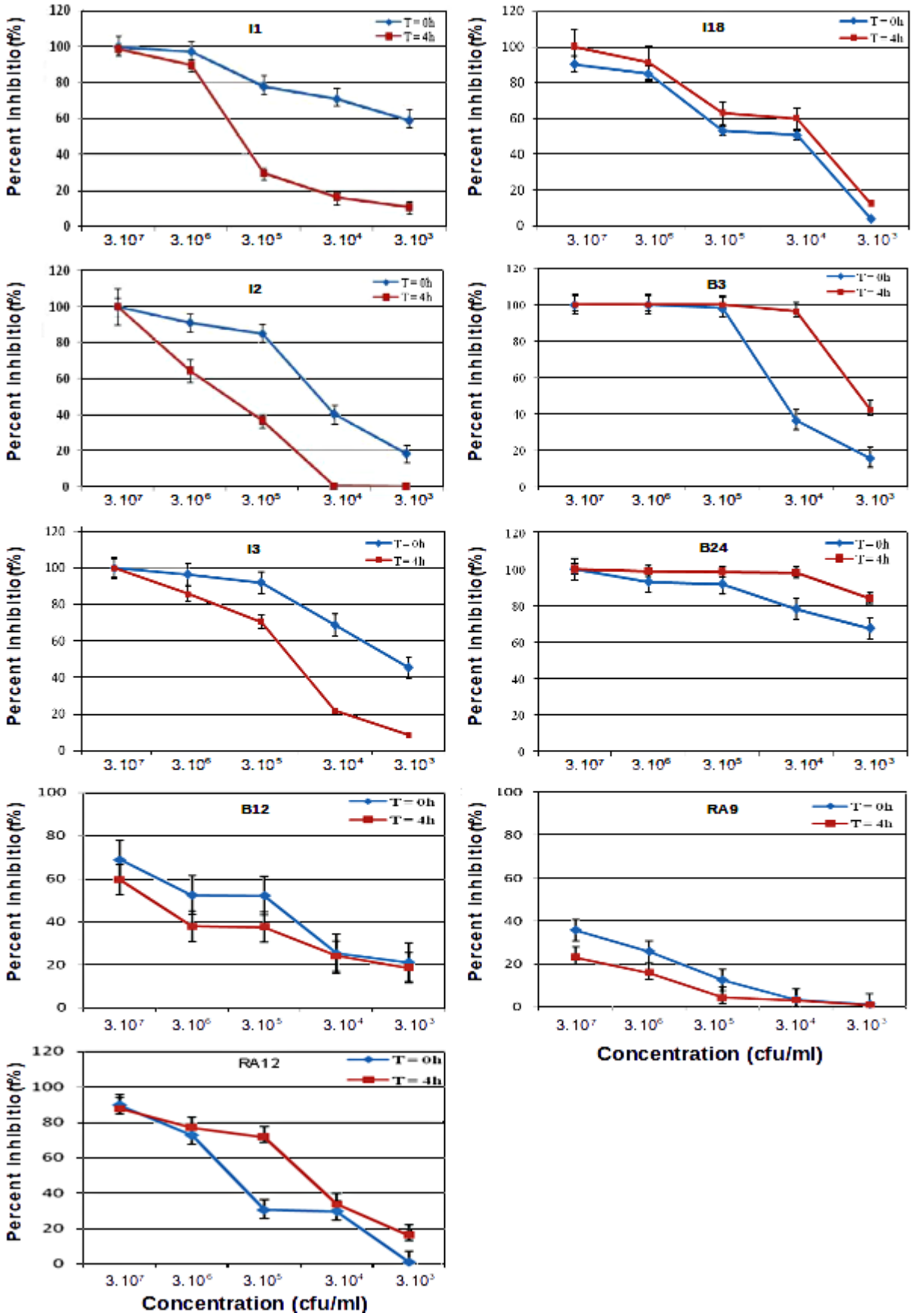

Figura 2. Efecto de la concentración y tiempo de inoculación de los aislados de *B. amyloliquefaciens* (I1, I2, I3, I18, B3, B24, B12, RA12 y RA9) sobre la germinación de conidios de *B. cinerea*.

Figure 2. Effect of concentration and time of inoculation of *B. amyloliquefaciens* isolates (I1, I2, I3, I18, B3, B24, B12, RA12 and RA9) on conidial germination of *B. cinerea*.

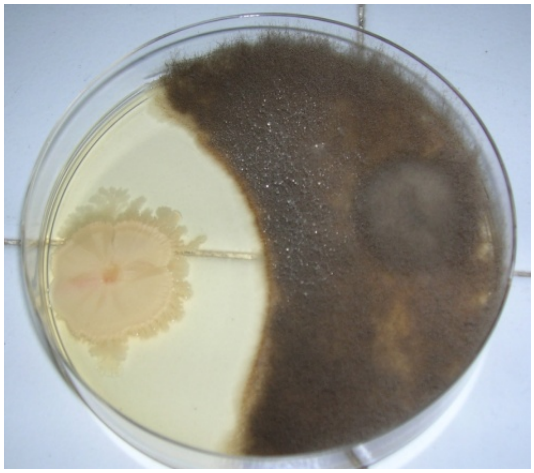

Figura 3: Inhibición del crecimiento vegetativo de *B. cinérea* por *B. amyloliquefaciens* B3 en el medio PDA.
Figure 3: Inhibition of the vegetative growth of *B. cinerea* by *B. amyloliquefaciens* B3 on PDA medium.

chanisms, including the production of secondary metabolites. *Bacillus* species are both taxonomically and metabolically diverse, and they exhibit enormous metabolic capabilities and versatile biochemistry through the production of structurally diverse bioactive chemical structures. Although *Bacillus* species mainly synthesize peptides, antibiotics belonging to other chemical classes are also produced by these microorganisms, and the determination of their chemical structure and the mechanism of their biological action are of fundamental and practical interest (Arguelles-Arias *et al.* 2009, Hamdache *et al.* 2013, Chowdhury *et al.* 2015). Although the isolates I1, I2 and I3 provide an inhibition of conidial germination when co-inoculated simultaneously with the pathogen, the pre-inoculation of the B3 and B24 isolates gives a more important inhibition even at low concentration. The B24 was the only isolate that showed an importante inhibition percent 84.04 % at the low concentration tested ($3x10^3$ CFU/ml). Therefore, it could be selected as a good bacterial antagonist and used for further studies for controlling *B. cinerea*. B3 also gives a strong inhibition (100%) at $3x10^5$ CFU/ml. As stated before, a good biocontrol agent (BCA) must be effective at low concentration (Wisniewski & Wilson 1992). A combination of two compatible micro-organisms, *Trichoderma harzianum* Rifai and *Streptomyces rochei* Berger *et al.*, both antagonistic to the pathogen *Phytophthora capsicik* Leonian, was used to control root rot in pepper where the optimal dose of the antagonists in the compound formulation was $3.5x10^8$ spores/ml of *T. harzianum* and $1.0x10^9$ CFU/ml of *S. rochei* (Ezziyyani *et al.* 2007). The strong inhibition of conidial germination during the pre-inoculation can be explained as an important bacterial colonization in the culture medium before the onset of the pathogen *B. cinerea* and consequently a competition for nutrients or a high secretion of antifungal metabolites. Several mechanisms can be used for controlling the pathogens, either directly inside the plant, by antibiosis against the pathogen (Sturz *et al.* 1998) and by the competition for nutrients (Mari *et al.* 1996), or indirectly by inducing a resistance response in the plant (M'Piga *et al.* 1997). *B. cinerea* is highly susceptible to competition because external nutrients are required for conidial germination (Elad 1996), germ tube growth and the successful completion of infection (Elad & Stewart 2004). Nutrients are essential for the development of populations of epiphytic microorganisms, necrotroph pathogens, and nonpathogens alike. Competition for nutrients on plant surfaces is an important mechanism of biological control against pathogens that depend on external nutrition (Blakeman & Brodie 1977, Elad & Chet 1987, Morris & Rouse 1985, Roberts 1990). For example, isolates of *Rhodotorula glutinis* (Fresen.) F.C. Harrison and *Cryptococcus albidus* (Saito) C.E. Skinner compete for nutrients with germinating conidia of *B. cinerea* (Elad *et al.* 1994, Elad 1996). Similarly, several bacterial isolates compete for glucose and asparagine with germinating oospores of *P. aphanidermatum* in the rhizosphere of various crops (Elad & Chet 1987), and *Pseudomonas fluorescens* Migula suppresses *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker by competing with the pathogen for glucose (Mohamed & Caunter 1995). It is well known that some Bacillus species may synthesize numerous antimicrobial or, more generally, bioactive compounds with well-established activity *in vitro* (Stein 2005, Hamdache *et al.* 2011). A *Bacillus subtilis* (Ehrenberg) Cohn strain was shown to reduce post-harvest infection of apples caused by *B. cinerea*, the causative agent of grey mold disease (Toure *et al.* 2004). Also it was shown to possess biocontrol activity against *X. oryzae* strains by producing the antibiotic compounds difficidin and bacilysin (Wu *et al.* 2015). Germination of *B. cinerea* conidia is inhibited by antifungal metabolites of *Pseudomonas antimicrobica* Attafuah and Bradbury. It was also shown that germination was almost completely inhibited

when metabolites were added prior to germination (Walker *et al.* 2001). In this study we can conclude that B3 (Bc5) and B24 (Bc7) can be selected for their effectiveness at low concentration and might be applied preventively for best inhibition of conidial germination of *B. cinerea*.

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