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Original Research Article

In vitro Inhibitory Activity of *Bacillus amyloliquefaciens* Isolates against *Botrytis cinerea* Mycelial Growth and Determination of the Minimal Inhibitory Concentration

Ahlem Hamdache*, Anass Ben Moula, Ahmed Bagayou, Mohamed Mchita, Mohammed Ezziyyani

Department of Life Sciences, Plant Microbial Marine Biotechnologies and Precision Agriculture, Polydisciplinary Faculty of Larache, 92004, Abdelmalek Essaâdi University, Tetouan, Morocco.

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ABSTRACT

Gray mold disease, caused by *Botrytis cinerea* is a major postharvest disease impacting fruits such as strawberries. Bacillus species are promising agent for the biological control of postharvest diseases. Biological control agents at suitable population, should be able to effectively interact with pathogens to produce satisfactory disease control. Knowledge on the relationships between biocontrol agent and pathogen inoculum concentration can determine the population levels of the biocontrol agent required to achieve adequate disease control. This study aimed to determine the inhibitory effect of nine *Bacillus amyloliquefaciens* isolates from rhizospheric soil and roots of strawberries plants against *Botrytis cinerea* mycelial growth *in vitro*. Nine bacterial isolates (I1, I2, I3, I18, B3, B24, B12, RA9, and RA12) were selected from rhizospheric soil and roots of healthy strawberry plants. The bacterial isolates at different concentrations ($3x10^1$ to $3x10^7$ cfu/mL) were tested for their inhibitory activity against mycelial growth of *Botrytis cinerea* using the plate confrontation assay in a potato dextrose agar. The results showed that all the isolates inhibited *Botrytis cinerea* mycelial growth in a concentration-dependent manner, with isolates B3 and B24 exhibiting the most effective activity showing 50.68% inhibition at $3x10^3$ cfu/mL (B3), and 31.91% inhibition at $3x10^1$ cfu/mL (B24). The minimum inhibitory concentration (MIC) for both B3 and B24 was $3x10^5$ cfu/mL. The MIC for isolates I1, I2 and I18 was $3x10^6$ cfu/mL, while the other isolates had MIC $\geq$.$3x10^7$ cfu/mL. These findings suggest that the two isolates B3 and B24 could serve as biocontrol agents for Gray mold.

Keywords: *Botrytis cinerea*, *Bacillus amyloliquefaciens*, **S**trawberry, Mycelial growth.

Introduction

Strawberry (*Fragaria* x *ananassa* Duch.) is an important and highly valued plant that is grown worldwide.[1,2] Strawberries are of economic importance due to their unique fruit flavour and nutritional benefits.[3] Strawberry fruits are rich in sugar, vitamins, dietary fiber, and amino acids. They can be processed into various products with high economic value.[4] However, the strawberry plants are prone to damage caused by environmental factors such as dehydration of the fruit or plant diseases. It can also be infected by different pathogens such as nematodes, viruses, bacteria and, various fungi, causing enormous economic losses to strawberry cultivators. Strawberries are more susceptible to microbial infections during pre- and post-harvest periods, particularly fungal infection which has the highest impact on the economic benefits of these plants.[5] Gray mold caused by the phytopathogenic fungus *Botrytis cinerea* is one of the most disturbing diseases of strawberries plants, generally causing a 10 to 25% yield reduction and serious economic losses of more than 50%.[6,4] Generally, Botrytis species are serious plant pathogens, which are implicated in many diseases of flowers, fruits, and vegetables.

*Corresponding author. E mail: a.hamdache@uae.ac.ma
Tel: +212 663850721

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In particular, *B. cinerea* attacks economically important crops such as lettuces, carrots, tobacco, grapes and strawberries.[7,8] In fact, *B. cinerea* is the main cause of agricultural losses during the postharvest period due to its unspecific host and the variety of organs it infects.[9] The control of postharvest pathogens relies mainly on the use of synthetic fungicides, and the application of these fungicides has for many years been an efficient way of controling these pathogens. However, fungicides have negative environmental consequences coupled with development of fungicide-resistant pathogens. This challenge has elicited public debate on the need to reduce the use of synthetic pesticide and explore alternative control strategies such as new and improved biological control agents to combat these pathogens and pests.[10]
Among the Bacillus species, *B. amyloliquefaciens* are known for their ability to combat a wide range of plant-associated diseases, and they are considered promising biocontrol agents.[11,12] Several studies have reported that *B. amyloliquefaciens* could control diseases caused by a variety of pathogens.[13,14] Furthermore, *B. amyloliquefaciens* has demonstrated the ability to promote plant growth.[15,16] The degree of disease control obtained depends on the concentration of the biocontrol agent, the concentration of the pathogen, the efficiency of the biocontrol agent in suppressing the pathogen, and the proportion of the pathogen population that is potentially affected by the agent.[17,18] A good biocontrol agent must be effective at low concentrations.[19] The aim of this study was to investigate the inhibitory effect of different concentrations of nine *Bacillus amyloliquefaciens* islates, isolated from roots and rhizospheric soil of strawberries plants, on mycelial growth of *Botrytis cinerea*, and to determine the minimal inhibitory concentration of the nine bacterial isolates to select the most potent inhibitor.

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Materials and Methods

Pathogenic fungi
Botrytis cinerea Pers.: Fr., was isolated from infected strawberry fruits harvested from a field at Loukkous, Laâouamra (Larache, north of Morocco). The isolate was purified, and identified by macroscopic and microscopic observations.[20,21] The bacterial usolate identified as *Botrytis cinerea* Bt7 was maintained on Potato Dextrose Agar medium (PDA) (Difco,USA), at 25°C. 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 isolates
Nine bacterial isolates were selected, for their inhibitory effect against *B. cinerea in vitro*, from a mass selection of 321 isolates according to the procedure described by Hamdache *et al* (2012).[22] The nine selected isolates were obtained from rhizospheric soil and roots of healthy strawberry plants from the Loukkous zone in the north of Morocco. The selected isolates include isolates I1, I2, I3, I18, B3, B24, and B12, isolated from rhizospheric soil of strawberries plants, and isolates RA9 and RA12 isolated from strawberries roots. The 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*.[22]
Each of the nine selected bacterial isolates was cultured for 24 hours on Luria Bertani (LB) nutrient medium in the dark at 28°C. Liquid cultures of the antagonistic bacterial strains were grown in 250 mL Erlenmeyer flasks containing 50 mL of Luria Bertani liquid medium which had been inoculated with a loop of the culture. The flasks were incubated at 25°C on a rotary shaker at 125 rpm for two days. Following incubation, cells were centrifuged at 4000g for 10 min, to separate the colonies (pellet) from culture filtrates (supernatant). Thereafter, the cell pellets were washed twice with sterile distilled water in order to remove the growth medium. Cell pellets were re-suspended in sterile distilled water and adjusted to an initial concentration of $3x10^8$ cfu/mL according to the Mac Farland scale.[23,24] Then, seven decreasing concentrations ($3x10^7$, $3x10^6$, $3x10^5$, $3x10^4$, $3x10^3$, $3x10^2$ and $3x10^1$ cfu/mL) were prepared and tested against the mycelial growth of *Botrytis cinerea* (isolate Bt7).

Effect of antagonistic bacterial concentrations on mycelial growth of B. cinerea
The effect of the different concentrations of the antagonist bacterial isolates on the fungal pathogen was evaluated using the plate confrontation assay in a potato dextrose agar (PDA) medium. A 20 µL quantity of $1x10^6$ spores per mL of a suspension of *Botrytis cinerea* was added to 20 µL of sterile distilled water (control) or a 20 µL quantity of washed bacterial cell suspension of *B. amyloliquefaciens* strains that was adjusted to these different concentrations; $3x10^7$, $3x10^6$, $3x10^5$, $3x10^4$, $3x10^3$, $3x10^2$ and $3x10^1$ cfu/mL, and inoculated into each PDA plates. The plates were incubated for five days in the dark at 25°C. The inhibition of radial growth of *Botrytis cinerea* was evaluated as a percentage using the following formula:

$$Inhibition\ (\%) = \frac{(Dt - Di)}{Dt}\ x\ 100$$

Where;
Dt = Diameter of the mycelial colony of *Botrytis cinerea* in the absence of the antagonist (control).
Di = Diameter of the mycelial colony of *Botrytis cinerea* in the presence of the antagonist.
All interactions consisted of three replicates, and experiments were repeated three times.

Statistical analysis
Data were presented as mean ± standard deviation of triplicate measurements. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (SPSS Statistics V21.0). Comparison between mean values was done using Duncan's multiple range test. Significant difference was established at P-value < 0.05.

Results and Discussion

The percentage inhibition of *B. cinerea* mycelial growth by the different bacterial concentrations is shown in Figure 1. The result shows that all the antagonistic bacterial isolates exhibited a concentration-dependent decrease in mycelial growth; the percentage inhibition of mycelial growth decreased as the bacterial concentration decreased. At $3x10^7$ cfu/mL, the selected bacterial isolates completely inhibited the growth of *Botrytis cinerea*, with 100% inhibition except for isolates B12 and RA12 which showed a percentage inhibition of 90.44 and 94.41%, respectively. At $3x10^6$ cfu/mL, mycelial growth was also completely inhibited by isolates B3, B24, I1, I2 and I18, while isolates I3, B12 and RA12 resulted in 74.76, 66.18 and 52.94% inhibition, respectively. Isolate RA9 showed no activity against mycelial growth from $3x10^6$ cfu/mL. At $3x10^5$ cfu/mL, only isolates B3 and B24 showed 100% inhibition, followed by bacterial isolates I1 and I2 with 53.19 and 51.87% inhibition, respectively, while the other isolates demonstrated an inhibition of less than 40%. At bacterial concentration of $3x10^4$ cfu/mL, the isolates became less effective, with percentage inhibition of less than 20%, except for isolates I2, B3 and B24 which demonstrated 51.87, 77.54 and 90.92% inhibition, respectively. Finally, at the lowest concentration of $3x10^1$ cfu/mL, all the bacterial isolates showed minimal inhibition which varied from 0 to 6.62%, however, isolate B24 showed significantly higher inhibition than the others, with percentage inhibition of 31.91% (Table 1).

Table 1: Comparison of *Botrytis cinerea* mycelial growth inhibition by *Bacillus amyloliquefaciens* isolates at seven different concentrations

Concentration	Percentage inhibition								
(cfu/mL)	I1	I2	I3	I18	B3	B12	B24	RA9	RA12
$3x10^7$	**100.00 a**	**100.00 a**	**100.00 a**	**100.00 a**	**100.00 a**	90.44 a	**100.00 a**	**100.00 a**	94.41 a
$3x10^6$	**100.00 a**	**100.00 a**	74.76 b	**100.00 a**	**100.00 a**	66.18 b	**100.00 a**	0.00 b	52.94 b
$3x10^5$	53.19 b	51.87 b	38.75 c	23.62 b	**100.00 a**	37.94 c	**100.00 a**	0.00 b	34.70 bc
$3x10^4$	11.09 c	51.87 b	18.75 cd	4.27 c	77.54 b	2.38 d	90.92 b	0.00 b	16.47 cd
$3x10^3$	7.12 c	51.65 b	5.06 d	3.80 c	50.68 c	1.39 d	44.56 b	0.00 b	2.20 d
$3x10^2$	7.07 c	28.95 bc	2.14 d	3.80 c	0.00 d	0.00 d	41.11 b	0.00 b	0.73 d
$3x10^1$	6.62 c	3.56 c	1.94 d	2.48 c	0.00 d	0.00 d	31.91 b	0.00 b	0.00 d

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

B. cinerea mycelial growth was completely inhibited (100%) by two of the selected bacterial isolates; B24 and B3 at moderate concentration of $3x10^5$ cfu/mL (Table 1). These two antagonists demonstrated an inhibitory effect much greater than all the other bacterial isolates tested.
The minimum inhibitory concentrations varied significantly between assays and between antagonistic bacterial isolates within the same assay. Thus, the comparison of the percentage inhibition of mycelial growth according concentration, and the minimum inhibitory concentrations among the selected Bacillus isolates are presented in Figure 2 and Table 2, respectively. Based on the analysis of the results obtained, the isolates can be categorized into four groups;

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1. B24 and B3, with maximum inhibition of *B. cinerea* mycelial growth at a minimum inhibitory concentration (MIC) of $3x10^5$ cfu/mL (Figure 1A).
2. I1, I2 and I18, with maximum inhibition of *B. cinerea* mycelial growth at a minimum inhibitory concentration (MIC) of $3x10^6$ cfu/mL (Figure 1B).
3. I3 and RA9, with maximum inhibition of *B. cinerea* mycelial growth at a minimum inhibitory concentration (MIC) of $3x10^7$ cfu/mL (Figure 1C).
4. B12 and RA12, with maximum inhibition of *B. cinerea* mycelial growth at a minimum inhibitory concentration (MIC) greater than $3x10^7$ cfu/mL (Figure 1D).

It is important to note that at $3x10^5$ cfu/mL, only isolates B3 and B24 were able to produce significant effectiveness by completely inhibiting *Botrytis cinerea* mycelial growth.

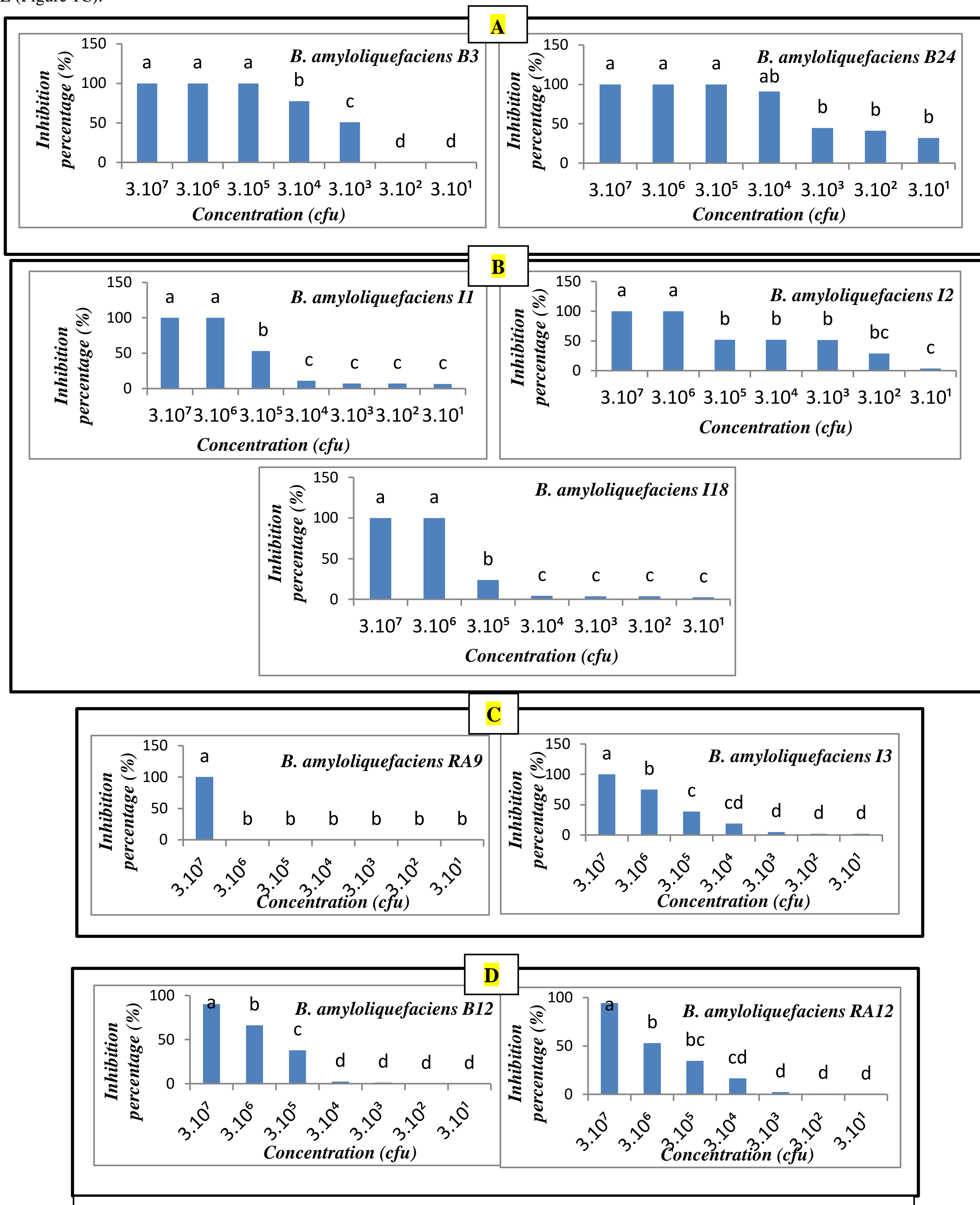

Figure 1: Percentage inhibition of *B. cinerea* (Bt7) mycelial growth at different concentrations of the antagonistic bacterial isolates.

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(A): Isolates B3 and B24, **(B):** Isolates I1, I2 and I18, **(C):** Isolates I3 and RA9, **(D):** Isolates B12 and RA12
Values with the same letter are not significantly different at 5% probability level.

The results of the inhibition of mycelial growth (Table 1) clearly showed that isolate B3 is effective from $3x10^3$ cfu/mL (50.68% inhibition). On the other hand, isolate B24 still retained its inhibitory effect even at low concentration (31.91% inhibition at $3x10^1$ cfu/mL) (Figure 2 and Table 1). To illustrate the results, Figure 3 shows the radial growth of the pathogen *Botrytis cinerea* Bt7 and the pathogen-antagonist interactions. The Figure showed that the growth of *B. cinerea* was strongly inhibited (100%) when it was co-cultured with *B. amyloliquefaciens* at the MIC of the antagonistic bacteria (Figure 3b). On the other hand, there was no mycelial growth inhibition when *B. cinerea* was co-cultured with *B. amyloliquefaciens* at concentration less than the MIC of the antagonistic bacteria (Figure 3c) or when it was cultured alone (Figure 3a).

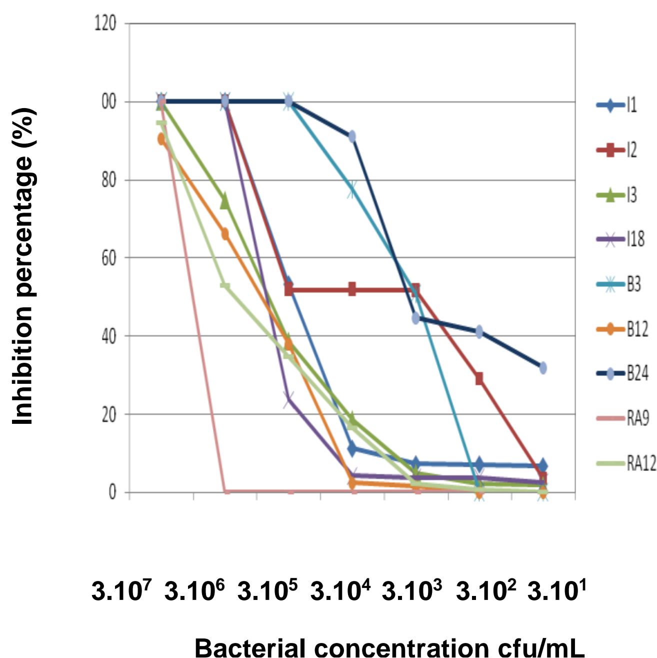

Figure 2: Concentration-dependent inhibition of *Botrytis cinerea* mycelial growth by Bacillus isolates.

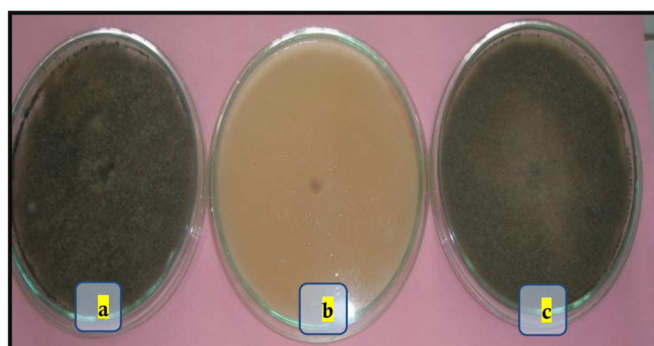

Figure 3: *Botrytis cinerea* Bt7 mycelial growth after ten days of incubation in the absence and presence of the antagonist
a: *Botrytis cinerea* pathogen alone (control), **b:** pathogen-antagonist interaction at minimum inhibitory concentration (100% inhibition), **c:** pathogen-antagonist interaction at less than minimum inhibitory concentration (0% inhibition).

In vitro confrontation is a important method because antibiosis is easily identified. Antagonistic activity *in vitro* depends on the antagonist strain. It varies depending on environmental parameters[22] and also depending on the concentration of bacterial cells.[27] The latter affect the morphophysiological growth of the pathogen, following the synthesis and release of secondary metabolites which are responsible for the partial or total inhibition of the pathogen.[25] A good biocontrol agent must be effective at low concentrations.[19] Determining the minimum inhibitory concentration (MIC) is mandatory because it allows for the specification of the actual concentration of the antagonist needed to exert its optimal effectiveness.[26]
Among the nine Bacillus isolates that produced zones of inhibition and necrosis in the vegetative growth of the *B. cinerea* colony,[22] it was observed that the two antagonistic strains B3 and B24 were the most effective. They completely suppressed the mycelial growth of the pathogen at $3x10^5$ cfu/mL (MIC).
According to a previous study, when the results of the effect of these bacterial antagonistic strains was compared at different concentration on conidial germination with the results of the effect on the mycelial growth, it was found that the nine bacterial antagonists (*B. amyloliquefaciens*) inhibited mycelial growth more than conidial germination.[27] The minimum inhibitory concentration capable of completely inhibiting the mycelial growth of *Botrytis cinerea* was low for the two isolates B3 and B24 ($3x10^5$ cfu/mL) and for the three isolates I1, I2 and I18 it was $3x10^6$ cfu/mL. With the exception of isolate B3 (MIC = $3x10^5$ cfu/mL), the other antagonists only inhibited conidial germination (100%) at high concentrations ($\geq 3.10^7$ cfu/mL).[27] Similarly, isolate B24 completely inhibits conidial germination at 3.10^7 cfu/mL (100% inhibition) and showed significant percentage inhibition at other concentrations (98.95, 98.62, and 98.09% at $3x10^6$, $3x10^5$, and $3x10^4$ cfu/mL, respectively).[27] Studies have shown that, the performances of antagonists vary according to the type of organisms involved. For example, it was observed that the bacteria *Aquaspirillum autotrophicum*, *Cellulomonas fimi*, and *Pseudomonas putida* produce antibiosis against mycelial growth without affecting the germination of the fungus *Helminthosporium solani* (causative agent of potato silver scab).[28] On the other hand, the germination of spores of this same fungus was strongly inhibited more than mycelial growth by the bacteria *Bacillus cereus*, *Kocuria rosea*, and *Pseudomonas fluorescens*. Similarly, it has been noted that the bacterial antagonist *Rahnella aquatilis* completely inhibits the germination of *B. cinerea* and *Penicillium expansum* spores *in vitro* at 10^6 cfu/mL.[29] In another study, *Bacillus amyloliquefaciens* inhibited both the mycelial growth and spore germination of *Bipolaris sorokiniana*.[30]
According to the findings from the present study, and reports by several authors, the application of the antagonist at doses higher than those required (MIC) does not increase the effectiveness in the biocontrol of the pathogen (*B. cinerea* Bt7).[25,31-34]

Table 2: Minimum Inhibitory Concentration (MIC) of antagonistic bacterial isolates against *Botrytis cinerea* mycelial growth

Antagonistic Bacterial isolates	Minimum Inhibitory Concentration (cfu/mL)
Bacillus B24	$3x10^5$
Bacillus B3	$3x10^5$
Bacillus I1	$3x10^6$
Bacillus I2	$3x10^6$
Bacillus I18	$3x10^6$
Bacillus I3	$3x10^7$
Bacillus RA9	$3x10^7$
Bacillus B12	$> 3x10^7$
Bacillus RA12	$> 3x10^7$

Conclusion

This work is complementary to a study which has previously been carried out on the same isolates against *B. cinerea* conidial

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germination. The results showed that the effect of bacterial antagonists on mycelial growth and on conidial germination depends largely on the concentration of bacterial cells. The effectiveness of the antagonistic isolates was greater on mycelial growth (elongation of the germ tube) than on conidial germination. The minimum inhibitory concentration of isolates B3 and B24 against *Botrytis cinerea* mycelial growth was $3x10^5$ cfu/mL, with 100% inhibition. Although the results of the *in vitro* confrontation experiments cannot be fully considered under greenhouse conditions, they can nevertheless give an approximate value of the minimum concentration necessary for the antagonist agent, depending on other abiotic factors, to be able to inhibit or limit the pathogen development. The findings from this study allowed for the selection of the two isolates B3 and B24 as potential biocontrol agents, due to their effectiveness at low concentrations, for use as biopesticides against Grey mold.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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