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Biocontrol of Grey Mold on Strawberry Fruit by *Bacillus* spp. and Study of the Mechanisms Involved

Ilham Barakat[1*], Noureddine Chtaina[1], Kaoutar Caidi[1] and Fatiha Bentata[2]

[1]Laboratory of Phytopathology, Department of Production, Protection and Biotechnology, Agronomic and Veterinary Institute Hassan II, Rabat, Morocco; [2]Laboratory of Phytopathology, Regional Center for Agronomic Research, Rabat, Morocco

[*]*Corresponding author:* barakat.iavcha@gmail.com

Abstract

Grey mold, caused by the fungus *Botrytis cinerea*, is one of the most prevalent diseases affecting strawberry plants (*Fragaria ananassa*). The objective of this study was to assess the antagonistic effect of 5 bacterial strains belonging to the genus *Bacillus* spp. (BA1, BF2, BB3, BI3, and BO4) against *B. cinerea*, tested both *in vitro* and *in vivo* on strawberry fruits. The strains exhibited antifungal activity against *B. cinerea* under *in vitro* conditions, both through direct confrontations and antibiosis tests, as well as through the effect of organic compounds. Strain BO4 could inhibit mycelial growth by 62.92% through direct confrontation and 64.58% through the secretion of volatile organic compounds. Additionally, the strain BF2 demonstrated a high antibiosis effect (74.64%) compared to the control at a concentration of 25%. Treating fruits with the bacterial suspension and culture filtrate of the 5 studied strains controlled grey mold growth *in vivo*, as indicated by low severity indices in strawberries treated with strains BI3, BF2, and BA1, marked by percentages of 24.44%, 24.44%, and 37.78%, respectively, for preventive treatment. The difference in the effectiveness of various strains depended on the treatment mode; preventive treatment proved to be more effective compared to curative treatment.

Keywords: antagonism; *Bacillus* spp.; biological control; *Botrytis cinerea*; postharvest diseases

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INTRODUCTION

Grey mold, caused by *Botrytis cinerea*, is responsible for significant post-harvest losses in a wide range of crops. Primary infection begins in the field before harvest, and symptoms develop during storage as the disease spreads from fruit to fruit. The pathogen also takes advantage of fruit injuries during harvesting, transport, and packaging operations to establish itself and infect fruit tissue. *B. cinerea* can develop even at 0 °C and spread among fruit by mycelial growth and conidia. To minimize post-harvest grey mold, control programs mainly focus on chemical control using fungicides (Romanazzi et al., 2016). However, growing concerns among consumers and regulatory authorities about the risks associated with chemical residues in food have prompted strict regulations, prohibitions on using certain chemical groups, and consumer preference to avoid chemically treated crops. This has prompted researchers to develop biological controls, such as biological control agents, plant extracts, minerals, and organic compounds (Nicot et al., 2011). In this context, the present study set out to develop a biological control method for post-harvest grey mold using new antagonistic bacterial strains belonging to the *Bacillus* genus and to demonstrate the mechanisms of action they employ against *B. cinerea*.

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Species of the bacterial genus *Bacillus* spp. have been extensively studied and utilized as biocontrol agents against phytopathogenic fungi in agriculture, owing to the diversity of their secondary metabolism and their ability to produce a wide variety of structurally different antagonistic substances (Fira et al., 2018). As a result, high inhibition rates have been achieved through both the secretion of metabolites and the action of volatile organic compounds released by certain *Bacillus* species. This is attributed to compounds such as lipopeptides (iturin, bacillomycin, fengycin, and surfactin), whose effectiveness has been determined both *in vitro* and *in vivo* by *Bacillus velezensis* (Toral et al., 2018; Nifakos et al., 2021), *Bacillus amyloliquefaciens* (Ilham et al., 2020; Maung et al., 2021), and *Bacillus subtilis* (Kilani-Feki et al., 2016). Additionally, *B. velezensis*, *B. subtilis*, and *B. amyloliquefaciens* have been described as species capable of producing and releasing antifungal volatile organic compounds (VOCs) against *B. cinerea*, thereby inhibiting the germination of its spores and the growth of hyphae (Gao et al., 2017; Gao et al., 2018; Luo et al., 2022).

This study aimed to assess the antagonistic potential of 5 bacterial strains belonging to the genus *Bacillus* spp.: BA1, BB3, BF2, BI3, and BO4 in the biocontrol of *B. cinerea* through both *in vitro* and *in vivo* trials on strawberry fruits. To achieve this objective, the kinetics of mycelial growth was monitored and the incidence of the pathogen and the antagonistic potential of the studied strains was assessed through the implementation of *in vitro* confrontations using 3 methods: direct confrontations, tests of antifungal activity of volatile organic compounds, and antibiosis tests between the 5 bacterial strains and *B. cinerea*. Furthermore, the efficacy of these antagonistic bacteria *in vivo* on mature and immature strawberries of the SABRINA variety was evaluated by examining the preventive and curative action of each strain.

MATERIALS AND METHOD

The biological material

The visit to the organic strawberry production farms in the Gharb region of Morocco identified that the SABRINA variety is highly susceptible to grey mold. This is the reason why researchers chose this variety for *in vivo* trials. The fruit were collected from the SABEMA farms using a random sampling technique. In the laboratory, the fruits with no symptoms of grey mold and of the same caliber were selected.

The *B. cinerea* strain used in this study was isolated from infected fruits of the SABRINA variety. Therefore, a mycelium fragment was taken under sterile conditions, placed on potato dextrose agar (PDA) medium acidified and incubated in darkness at 25 °C. Subsequently, the strain was purified through monosporic culture and maintained in culture at 25 °C in darkness.

The antagonistic bacterial strains were selected from the collection of the Laboratory of Phytopathology at the Hassan II Institute of Agronomy and Veterinary Medicine in Morocco (IAV Hassan II) based on their antifungal potential against several pathogens. The strains were stored in glycerol at -80 °C and then recultivated on non-acidified PDA medium in 9 cm diameter petri dishes and incubated in darkness at 25 °C (Table 1).

In vitro evaluation of the antagonistic potential of bacterial strains against *B. cinerea*

Direct confrontations

In the following, the modified method of Patel and Brown (1969), is described. A 5 mm explant was taken from the *B. cinerea* culture in PDA for 7 days and placed on a petri dish containing 20 ml of the same medium. The bacterial strain was inoculated diametrically opposite to the fungus and at a distance of 5 cm. This experiment was carried out with 3 replicates. The control consisted of replanting the fungus alone in the petri dish. Then, the petri dishes were incubated in darkness at 25 °C for 6 days.

Confrontations through volatile organic compounds

To highlight the effect of volatile substances secreted by bacteria during the inhibition of the mycelial growth of the pathogen, the modified method of Dennis and Webster (1971) was used. In the petri dishes containing PDA, the bacterium was replanted as a square in the center of the plate. The entire setup was incubated for 48 hours

Table 1. List of the tested bacterial strains for their antifungal activity

Strain code	Strain	Concentration (CFU ml^{-1})
BA1	*B. amyloliquefaciens*	10^8
BF2	*B. subtilis*	10^8
BB3	*B. subtilis*	10^8
BI3	*B. amyloliquefaciens*	10^8
BO4	*B. velezensis*	10^8

at a temperature of 27 °C. After incubation, the lid was replaced with the bottom of another petri dish containing PDA medium, where the pathogen was placed in the center as a 5 mm explant. The 2 dishes were sealed with parafilm, and the entire setup was incubated for 7 days in darkness at 27 °C. This experiment was carried out with 3 replicates. The control consisted of 2 dish bottoms containing PDA medium and the pathogen in a single compartment without the addition of the antagonist.

Tests of antibiosis

To highlight the action of culture filtrates on the inhibition of the mycelial growth of the pathogen, the modified technique of Sedra and Maslouhy (1995) was applied. The tested bacteria were separately cultured in sterile flasks containing 300 ml of Lysogeny Broth (LB) or Luria-Bertani medium and agitated in darkness for one week at 28 °C at a speed of 75 rpm. The cultures were first filtered through filter paper and then subjected to centrifugation at 6,000 rpm for 60 minutes. The supernatant was collected and filtered on a sterile millipore membrane (0.45 µm in diameter) using a sterile syringe. The obtained culture filtrate was stored cold at 4 °C in sealed sterile bottles.

The culture filtrate obtained from each strain was incorporated into the PDA medium at 2 concentrations, 25% and 50% of the crude filtrate diluted with PDA. Then, 5 mm explants were taken from *B. cinerea* cultures and placed in the center of each petri dish containing the PDA and culture filtrate mixture at the mentioned concentrations. For each bacterial strain, the experiment was conducted with 3 technical and biological repetitions. The control consisted of replanting the pathogen in the center of a petri dish containing a mixture of PDA and sterile LB medium at 25% and 50% concentrations.

Methods of evaluation

For all 3 types of confrontations, the mycelial growth of the pathogen was assessed every 24 hours by measuring the diameter of the pathogen's growth. The test concluded when the control fully covered the petri plate.

The assessment of antifungal activity was estimated by the percentage of inhibition calculated using Equation 1.

$$I\,(\%) = \frac{(D_C - D_B)}{D_C} \qquad (1)$$

Where D_C = diameter of the *B. cinerea* colony in the control petri dishes (in cm), D_B = diameter of the *B. cinerea* colony in the petri dishes with bacteria or its filtrate (in cm).

To account for the kinetics of *B. cinerea* pathogen growth for each strain, the average speed of pathogen growth was calculated using Equation 2.

$$V\,(\text{cm day}^{-1}) = \frac{\Delta D}{\Delta t} \qquad (2)$$

Where D = diameter of pathogen, t = the number of days of incubation for each confrontation method.

In vivo evaluation of the antagonistic potential of bacterial strains against *B. cinerea* on fruits

Disinfection and preparation of the fruits

The selected fruits are mature and uniform in size and color. The harvested fruits were randomly divided into batches of 3 fruits each. To test the pathogenicity of the *B. cinerea* isolate, the strawberry fruits were firstly surface-disinfected by immersion in a 2% sodium hypochlorite solution for 3 minutes, then rinsed twice in sterile distilled water and finally dried under a laminar flow hood (Biobase BBS-H1300). After preparation, each fruit was wounded (1 mm wide and deep) using a sterile pipette tip (10 µl) (Kahramanoğlu et al., 2022). The trials were conducted to examine the preventive and curative activity of cultures of antagonistic bacterial strains and their sterile filtrates. The time interval between treatment and inoculation by the pathogen was 8 hours. The experiments were carried out in a completely randomized design with 3 repetitions per treatment and each repetition contained 3 fruits.

Bioassay of direct confrontation

The direct confrontation assay was conducted following the modified method of Gao et al. (2018). The fruits were treated by injection with the bacterial suspension, previously adjusted at a concentration of 10^8 CFU ml^{-1}, of each bacterial strain used in each treatment mode (preventive or curative). The pathogen was used at 5×10^4 CFU ml^{-1} concentration. For the control, the fruits were inoculated with *B. cinerea* and injected with sterile distilled water instead of antagonist suspensions. After treatment, the strawberries were placed in a sterile plastic container closed to maintain high relative humidity (RH = 95%), and incubated at 25 °C in darkness for 24 hours

1: Infection less than 20% | 2: Infection between 20.1% and 40% | 3: Infection between 40.1% and 60% | 4: Infection between 60.1% and 80% | 5: Infection more than 80.1%

Figure 1. The disease severity scale (Laboratory of Phytopathology, IAV Hassan II)

to initiate infection. They were then kept at a temperature of 10 °C for 10 days to monitor the development of symptoms.

Bioassay of antibiosis

The *in vivo* antibiosis test was conducted identically to the direct confrontation assay on fruits (2.2.3), by injecting the fruits with the sterile filtrate obtained after filtering each bacterial suspension. The control treatment involved injecting the fruits with nutrient broth (LB) only. The incubation conditions were the same as those in the direct confrontation assay (2.3.2).

Method of evaluation

The assessment of antifungal activity was estimated by the disease severity index (DSI), evaluated from the rating of each fruit on a scale of 1 to 5, as indicated in Figure 1 (Kahramanoğlu et al., 2022). Then, the DSI (%) was calculated using Equation 3.

$$\text{DSI}\,(\%) = \left[\frac{1*a+2*b+3*c+4*e+5*e}{N*5}\right] \times 100 \quad (3)$$

Where a, b, c, d, and e correspond to the number of fruits belonging to levels 1, 2, 3, 4, and 5, respectively, and N corresponds to the total number of fruits observed per treatment (Chiang et al., 2017).

Statistical analysis

Statistical analyses were performed using analysis of variance with SPSS software. Inhibition percentage and severity index data were tested for normal distribution using the Shapiro-Wilk test ($\alpha = 0.05$). To demonstrate differences between treatments, an analysis of variance was performed with a single classification factor at a significance level of 5% for the inhibition rate. Means were compared using Duncan's test at a 5% significance level.

For severity index data, a non-parametric Kruskal-Wallis ANOVA and multiple comparisons to detect differences between control and treatments were performed for non-normally distributed data.

RESULTS AND DISCUSSION

Inhibition of the mycelial growth of *B. cinerea* through direct confrontation between the pathogen and bacterial strains

Among the 5 bacterial strains (BA1, BF2, BB3, BI3, BO4), only BO4, BF2, and BA1 exhibit significant antagonistic activity against *B. cinerea* compared to the control. The antagonistic action of the bacterial strains is reflected in the limited mycelial growth of the pathogen over the 7-day incubation period and a significant decrease ($p < 0.001$) in the average growth rate of the fungus compared to the control. Indeed, the average growth rate of *B. cinerea* in control dishes is 1.07 cm day^{-1}, whereas in the presence of strains BO4, BF2, BA1, BB3, and BI3, the average growth rates are 0.34, 0.53, 0.56, 0.72, and 0.73 cm day^{-1}, respectively (Table 2). The assessment of antifungal activity was estimated by a percentage of inhibition ranging from 30.56 to 62.92% (Figure 2a).

In studies conducted by Jiang et al. (2018) to determine whether the antifungal activity of *B. velezensis* 5YN and DSN012 was due to the direct effect of bacteria or the secretion of antifungal substances. The metabolic results indicated that the secondary metabolites of strains 5YN8 and DSN012 could significantly inhibit the mycelial growth of *B. cinerea* by secreting effective antifungal substances. Furthermore, Nifakos et al. (2021) evaluated the inhibition activity of *B. velezensis* against a strain of *B. cinerea* through direct confrontation. In all repetitions of the experiment, a clear zone of more than 0.7 cm was formed between the fungus and the *Bacillus* inoculum, suppressing the radial growth of fungal mycelium by 70% compared to the control, correlating with the results of this study.

Inhibition of the mycelial growth of *B. cinerea* by the effect of volatile organic compounds from bacterial strains

The inhibition of mycelial growth of *B. cinerea* through the production of volatile organic compounds was observed for all 5 strains of antagonistic bacteria. The production of volatile antifungal compounds was observed in all strains of antagonistic bacteria with very high statistical significance ($p < 0.001$). The assessment of antagonistic activity using the method of confrontations with VOCs highlighted the presence of volatile organic compounds with inhibitory effects in strain BO4, supported by an inhibition percentage of 64.58%, significantly higher than that of the other tested strains which generated inhibition rates of approximately 58.61% for strain BI3, 57.92% for strain BF2, 57.50% for strain BA1, and 55.97% for strain BB3 (Figure 2b).

Furthermore, the VOCs secreted by antagonistic bacteria significantly reduced ($p < 0.001$) the average growth rate of *B. cinerea* compared to the control. The growth rate of *B. cinerea* in control dishes was 1.06 cm day^{-1}, whereas in the presence of strains BO4, BI3, BA1, BF2, and BB3, the growth rates were respectively 0.41, 0.48, 0.49, 0.53, and 0.53 cm day^{-1} (Table 2).

According to a study conducted by Zhang et al. (2017), *B. subtilis* can achieve an inhibitory effect against *B. cinerea* ranging from 71 to 80% in *in vitro* biological assays. The results of this study revealed inhibition rates of 60.39% for the tests of volatile organic compounds' activity for the BF2 strain and 58.56% for the BB3 strain. Thus, the results of this study align with the findings of previous research. Indeed, the results of the study by Gao et al. (2018) demonstrated that VOCs in the 24-hour fermentation liquid of *B. subtilis* CF-3 inhibited the mycelial growth of *B. cinerea* with an average inhibition rate of 59.97%. In another *in vitro* assay conducted by Gao et al. (2017), the VOCs produced by *B. velezensis* ZSY-1 showed significant antifungal activity against *B. cinerea* with an inhibition rate of 92%.

Inhibition of the mycelial growth of *B. cinerea* through antibiosis

Test with a concentration of 25% of bacterial culture filtrate

Secondary metabolites secreted in the culture medium by antagonistic bacteria had a significant ($p < 0.001$) effect on the growth kinetics of the fungus, leading to a substantial reduction. Specifically, due to the presence of 25% bacterial culture filtrates, the mycelial growth rates of *B. cinerea* were 0.29, 0.39, 0.48, 0.55, and 0.71 cm day^{-1} for BF2, BI3, BA1, BO4, and BB3, respectively. In contrast, the growth rate of the pathogen in the control group was 1.07 cm day^{-1} (Table 2).

The filtrate from strain BF2 exhibited a significantly high antibiosis effect on the *B. cinerea* fungus with an inhibition percentage of 74.64%. Nevertheless, the other strains showed antibiosis effects, resulting in the cessation of mycelial growth by the third day of incubation. The inhibition rates reached percentages of 69.80% for BI3, 61.70% for BA1, 55.56% for BO4, and 45.10% for BB3 (Figure 2c).

Test with a concentration of 50% of bacterial culture filtrate

The inhibitory effect was significantly correlated with the filtrate concentration ($p < 0.01$). Indeed, in the presence of 50% bacterial filtrate, the average growth rate of the pathogen reached 0.31 cm day^{-1} for BI3, 0.42 cm day^{-1} for BO4, 0.43 cm day^{-1} for BA1, 0.49 cm day^{-1} for BB3, and 0.51 cm day^{-1} for BF2, compared to 1.14 cm day^{-1} for the control (Table 2). However, no significant difference was noted between the inhibition rates of different strains. There was an antibiosis effect resulting

Table 2. Average growth rates of *B. cinerea* for the 3 confrontation methods

Treatment	The average rate of pathogen growth (cm jour^{-1})			
	Direct confrontation	Confrontation with COV	Antibiosis test	
			25% of filtrate	50% of filtrate
Control	1.07^{c}	1.06^{c}	1.07^{e}	1.14^{c}
BO4	0.34^{a}	0.41^{a}	0.55^{c}	0.42^{ab}
BI3	0.73^{b}	0.48^{ab}	0.39^{ab}	0.31^{a}
BF2	0.53^{ab}	0.53^{b}	0.29^{a}	0.51^{b}
BB3	0.72^{b}	0.53^{b}	0.71^{d}	0.49^{b}
BA1	0.56^{ab}	0.49^{ab}	0.48^{bc}	0.43^{ab}

Note: Averages with a common letter belong to the same group according to the Duncan test at a 5% significance level

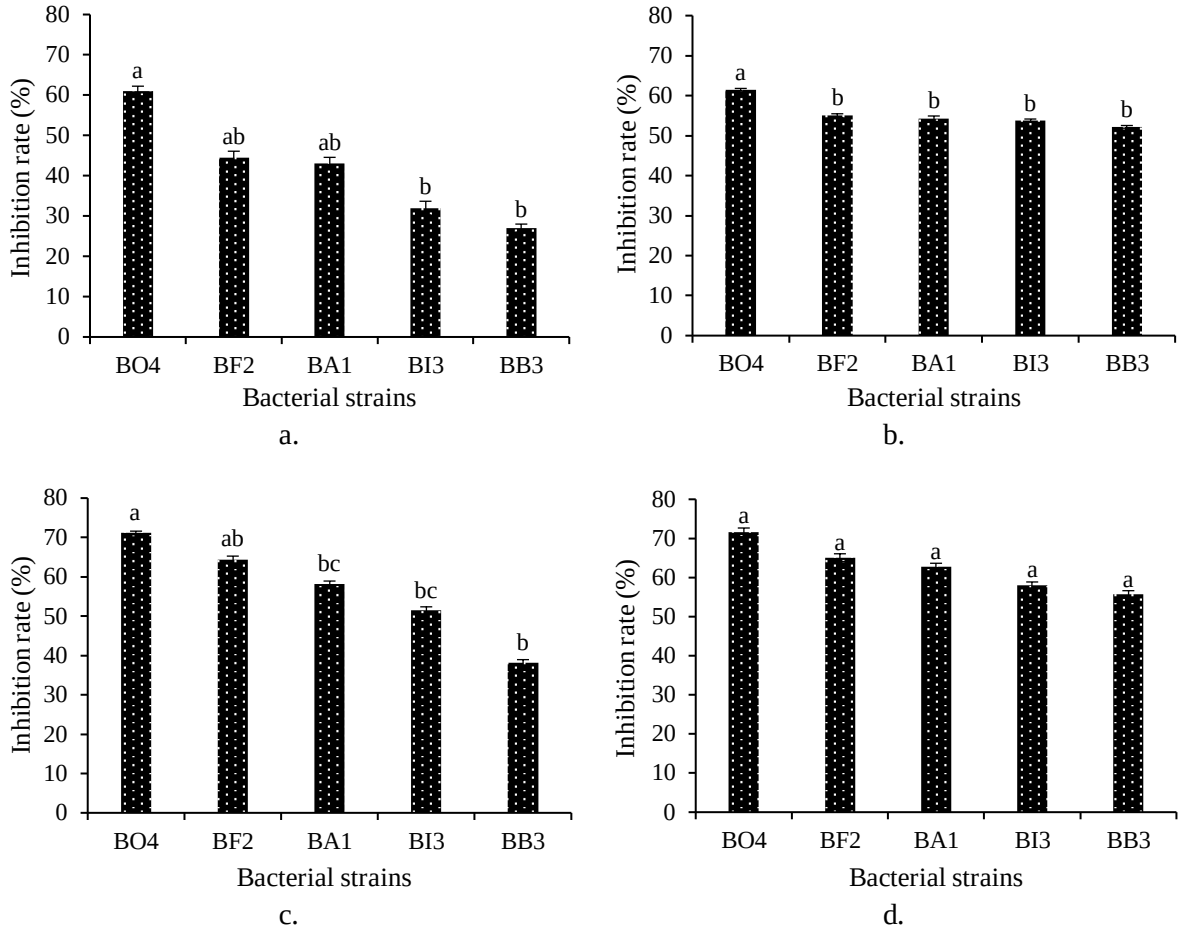

Figure 2. Inhibition rates (%) for the 5 studied bacterial strains after 6 days of incubation at 25 °C for direct confrontation method (a), confrontation with VOCs (b), and the 25% (c) and 50% (d) bacterial culture filtrate antibiosis test

Note: Bars indicate standard deviations of the means. Means with a common letter are not significantly different according to the Duncan test at a 5% significance level

In the inhibition of mycelial growth with percentages of 75.95% for BI3, 70.45% for BO4, 68.5% for BA1, 64.44% for BB3, and 62.48% for BF2 (Figure 2d).

Moreover, *B. amyloliquefaciens* has been proven to significantly inhibit the mycelial growth of *Alternaria panax*, *B. cinera*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea*, and *Sclerotinia sclerotiorum*, as well as the germination tube elongation of *B. cinerea* spores (Ji et al., 2013). On their part, the study by Kefi et al. (2015) demonstrated the antifungal activity of the BF11 strain filtrate of *B. amyloliquefaciens* and its ability to inhibit the mycelial growth of *B. cinerea* by 53% compared to the control. This is consistent with the results of the present study. Indeed, the BA1 strain of *B. amyloliquefaciens* inhibited mycelial growth by 52% compared to the control through antibiosis.

In vivo inhibitory effect of bacterial strains against grey mold disease on fruit

To test the antagonistic activity of strains BO4, BI3, BF2, BB3, and BA1 against *B. cinerea* on strawberries, the inhibitory effect of bacterial cultures with a bacterial density of 10^8 CFU ml^{-1} and their filtrate on strawberries either before (preventive treatment) or after (curative treatment) artificial inoculation with a conidial suspension of *B. cinerea* at a concentration of 5×10^4 CFU ml^{-1} were assessed.

The curative treatment with bacterial suspensions showed a highly significant inhibition for the BI3 treatment compared to the control ($p = 0.005$) and a significant inhibition for the BA1 treatment ($p = 0.034$). Indeed, treatment with bacterial cultures of BI3 and BA1 after *B. cinerea* inoculation reduced disease severity from 51.11% (for the control) to 24.44% and 33.33%, respectively, on the tenth day of

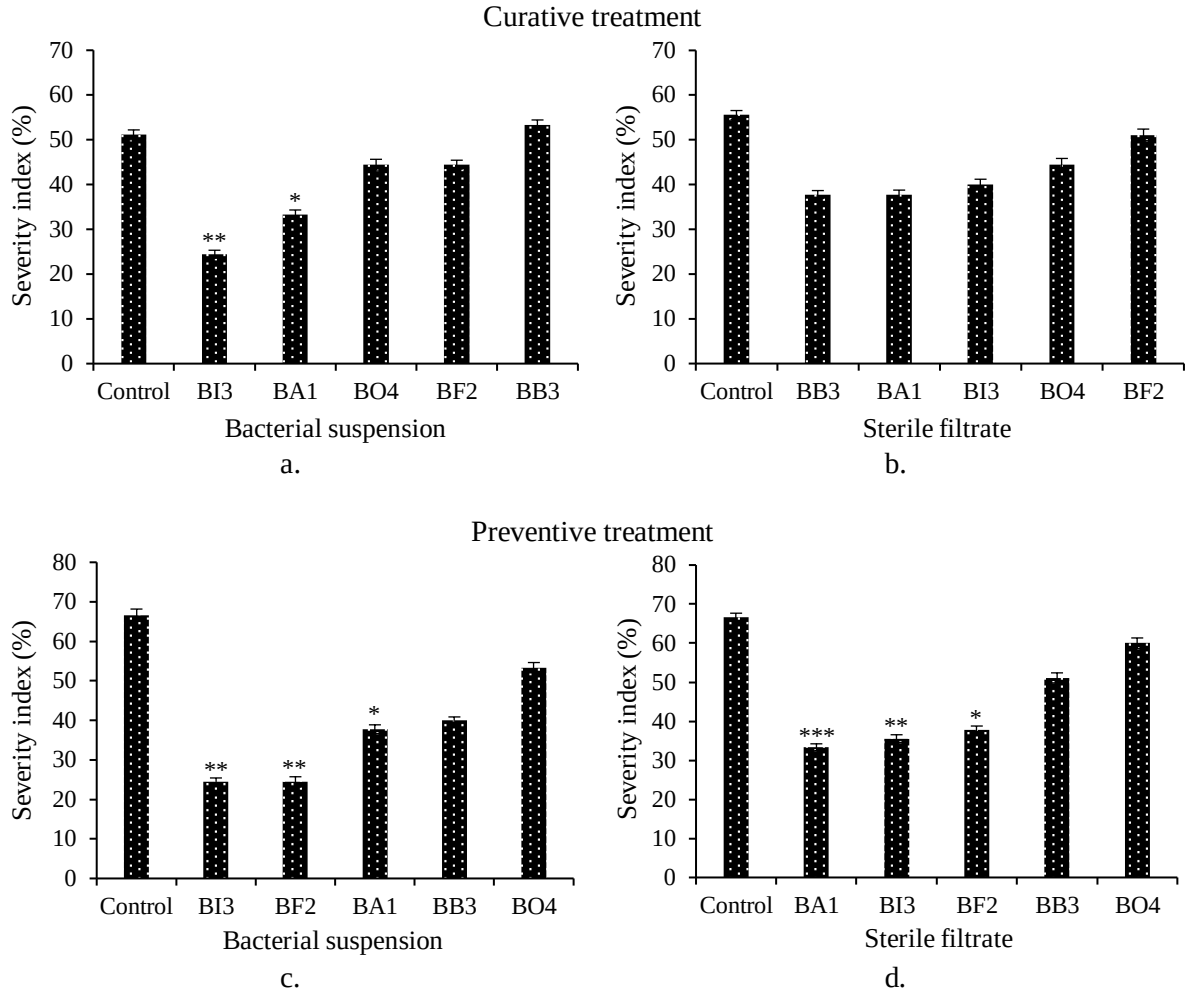

Figure 3. The effect of preventive and curative treatment with bacterial strains BO4, BI3, BF2, BB3, and BA1 against grey mold *in vivo* on strawberries after 10 days of incubation at 10 °C; (a) and (b) represent the severity index of curative bacterial treatment with bacterial cultures and culture filtrates, respectively, (c) and (d) represent the severity index of preventive treatment with bacterial suspensions and culture filtrates, respectively

Note: Data values represent the mean of 3 repetitions. Asterisks indicate statistically significant (*), highly significant (**), or very highly significant (***) differences of each treatment compared to the control

incubation (Figure 3a). However, for the curative treatments based on bacterial filtrate, no significant difference was observed between the treatments and the control (Figure 3b).

The preventive treatment proved to be more effective than the curative treatment, and disease severity was reduced compared to the control during the incubation period. Treatment with BI3 and BF2 cultures before artificial inoculation by *B. cinerea* significantly (p = 0.001) reduced grey mold severity 10 days after inoculation (Figure 3c). Disease severity on strawberry fruits was reduced to 24.44% for fruits treated with BI3 and BF2 compared to 66.67% for the control treatment.

Furthermore, preventive treatment with BA1, BI3, and BF2 filtrates also significantly reduced disease severity values after 10 days of incubation. As shown in Figure 3d, the disease severity of grey mold on treated fruits decreased compared to the control treatment with a percentage of 33.33% for strains BA1 and BI3, and 37.78% for strain BF2, suggesting the involvement of metabolites in their biocontrol activity. These results highlight that BI3, BF2, and BA1 were the most effective strains.

The *in vivo* tests on fruits revealed that pretreatment with the filtrates of the BI3 and BA1 strains of *B. amyloliquefaciens*, as well as the BF2 strain of *B. subtilis*, resulted in significant inhibition of grey mold development on artificially inoculated fruits. Indeed, a study conducted by de Moura et al. (2021) showed that *B. subtilis* was the most effective in

controlling the mycelial growth of *B. cinerea* for the strawberry cultivar "Albion", which is highly susceptible to the disease. Furthermore, electrospray mass spectrometry analysis revealed that *Bacillus* strains produced a heterogeneous mixture of metabolites belonging to iturin and surfactin for the BR8 strain of *B. subtilis*; and to bacillomycin D, fengycin, and surfactin for the BF11 strain of *B. amyloliquefaciens*, explaining the inhibitory power of the sterile filtrates of these strains (Kefi et al., 2015).

According to Abbey et al. (2019), biofungicides are only preventive and cannot "cure" already infected crops. In this context, the study by Hang et al. (2005) detected that pretreatment with *B. subtilis* S1 before inoculation with *B. cinerea* was more effective in controlling grey mold in strawberries than post-application, concluding that the pre-colonization of antagonistic agents on host plants can be a critical factor in protecting the host against fungal pathogen infection. This confirms that in the case of the *Bacillus* genus, the preventive application of the biocontrol agent allows the colonization of bacterial tissues and the production of antifungal compounds that protect the fruit from infection by the pathogen, thereby achieving effective control of *Botrytis* infections (Hang et al., 2005; Arrebola et al., 2010; Calvo et al., 2017).

CONCLUSIONS

The *in vitro* and *in vivo* trials showed a statistically significant decrease in the average mycelial growth rate and the severity of *B. cinerea* compared to the control. This confirms the presence of an antagonistic action constraining the growth of the fungus. This occurs through competition between bacteria and the fungus for space and nutrients, the secretion of volatile organic compounds preventing the normal development of *B. cinerea* mycelium, and the secretion of metabolites with antifungal activity. The results obtained are highly encouraging and deserve to be continued in the field conditions, in addition to the identification of secondary metabolites and volatile compounds produced by these bacteria.

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