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Use of beneficial microorganisms as an alternative to resistance of *Botrytis spp*. to fungicides controlling gray mold on strawberry (*Fragaria* x *ananassa*)

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Abstract

The effect of four fungicides frequently used against gray mold in strawberry was evaluated on mycelial growth of two *Botrytis cinerea* isolates (Bt.MB and Bt.L) collected from two Moroccan regions. The results obtained showed that *B. cinerea* isolates were resistant and less sensitive to the active ingredient of each treatment at different degrees. At the pH 5.6, The chlorothalonil-carbendazim combination was very effective on Bt.L isolate (IC_{50}<¼ HD et CI90<HD) while the mancozeb was very effective on Bt.MB isolate (IC50<¼ HD) .On the other hand, the azoxystrobin treatment has no effect on the Bt.L isolate (IC_{50} and IC_{90}>2HD) unlike mancozeb that inhibits totaly the vegetative growth to Bt.L isolate ($IC_{50}$and IC_{90}<HD) whereas, the propanocarb HCL was moderately effective on Bt.MB isolate (IC_{50}<HD and IC_{90}>HD). At pH 7.5, the chlorothalonil-carbendazim inhibits completely the growth of *Botrytis cinerea* isolates (IC_{50} and IC_{90}< ¼ HD) while the other three fungicides are moderately effective on Bt.L isolate (IC_{50}< HD and IC_{90}> HD). Antagonists of the pathogenic of strawberry plants, *B. cinerea* (Bt.L and Bt.MB), were isolated, selecting two bacterial isolates belonging to the genus *Bacillus* (*Bacillus sp*. B1 and *Bacillus sp*. B2). During *in vitro* confrontations, the isolate produced inhibition zone by producing antibiotics. The radial growth of the pathogen was reduced. After 7 days the antagonist had completely invaded the pathogenic colony fragmenting it and sporulating throughout it. *Bacillus sp*. B1 gives a great inhibition for the two pathogenic isolate than *Bacillus sp*. B2.

1. Introduction

The phytopathogenic fungus *B. cinerea* is a ubiquitous and polyphagous microorganism that causes enormous economic losses on many plant products [1, 2]. Strawberry plants are among the most important crops that are attacked by grey mould. The fungus affects the quantity and the quality of the fruit due to the high abundance of conidia, the high humidity in the greenhouses and the storage facilities that provide ideal conditions for grey mould infections and disease initiation [3]. Protection against grey mould usually demands weekly fungicide sprayings during flowering time and often requires repeated fungicide application to protect the crop and to decrease the disease incidence which exerts strong selection on *Botrytis*, and has led to increasing resistance problems in recent years [4]. In Morocco, a wide range of fungicides were used, against grey mould remains ineffective because of their intensive use in the fields and also, because the rules of application and the homologated dose are often not followed by farmers when applying the treatment. Several studies have reported cases of resistance of *B. cinerea* to fungicides, resistance to dicarboxamides and benzimidazole (MBC) fungicides is common. There are reported cases of isolates resistant to thiophanate-methyl, pyraclostrobin, cyprodinil, fenhexamid, procymidone, azoxystrobin, iprodione, fludioxonil and boscalid fungicides, simultaneously [5-9].

Increased interest to find alternatives for chemical protection against grey mold have led to the search for efficient and economically viable alternatives, such as biological control using antagonistic microbial agents

[10]. Various bacterial strains (including *Bacillus sp*.) isolated from the rhizosphere have been reported to be effective in the control of *B. cinerea* in post-harvest treatment. *Bacillus sp*. strains have the ability to produce many antibiotics, and they are easy to manipulate under laboratory condition [11-13]. In addition, they offer an advantage over other bacteria due to their ability to form endospores that are resistant to changing environmental conditions, as well as for the product formulation [14-16]. In this study, we investigate the effect of four fungicides against two *B. cinerea* isolates and in the meanwhile, we isolated two *Bacillus sp*. from the soil of strawberry plant in order to evaluate their abilities for biocontrol by plate confrontation method.

2. Material and Methods

2.1. Fungal and Bacterial culture

The *Botrytis cinerea* strains used in this study were isolated from strawberry fruits with symptoms of gray mold collected from two Moroccan regions (Larache and Moulay Bousselham) and were identified with macroscopic and microscopic characteristics using the identification keys [17,18]. The both pathogenic fungi (Bt.L and Bt. MB) were cultured routinely in PDA at 24°C until total purification. The bacterial strains (B1 and B2) were isolated from the rhizosphere of strawberry plants and were identified according to Bergey's Manual of Determinative Bacteriology. In agar medium, the strain (B1) formed medium-sized whitish colonies that were smooth and opaque. In the same media, the strain (B2) formed medium-sized colonies. These colonies were creamy white with undulating borders, a convex profile and a rough surface. Cells of both strains were Gram positive and formed oval, regularly shaped spores that were subterminal in the strain (B1) but central in the strain (B2). The API 20E tests showed that the two strains belong to the genus *Bacillus*. *Bacillus* sp. B1 probably belongs to the *subtilis* species and *Bacillus* sp. B2 probably to the *lichinoformis* species.

2.2. Fungicides tested against B. cinerea

In this experiment, four fungicides frequently used in the strawberry field against gray mold were tested in vitro on the mycelial growth of *B. cinerea* (table 1).

Table 1: Fungicides used against *B. cinerea*

Fungicides Trade Names	Active Ingredients	Quantity (g/l)	Fungicides Class	Doses Homologated (cc/hl)	Doses Tested (µl/100 ml)
Dithane M 45	Mancozeb	80	Dithiocarbamate	200	150 **200** 250
Ortiva 25 sc	Azoxystrobin	250	Strobilurins	50	25 **50** 75
Previcur N	Propamocarb HCL	722	Carbamate	150	100 **150** 200
Banko plus	Chlorothalonil Carbendazim	500 100	Chloronitriles Benzimidazole	200	100 **200** 300

2.3. Effect on the growth of B. cinerea at pH 5.6 and pH 7.5

The effect on mycelia growth of *B. cinerea* was evaluated using the method described by Hamdache et al [19] with some modifications. Each fungicide was solubilised in sterile destillate water and mixed with a molten PDA medium at 60°C to obtain the desired dose then the pH values (5.6 and 7.5) were adjusted. Plugs of PDA media containing actively growing *B. cinerea* were individually placed in the centre of each Petri dish containing 15 mL of PDA+fungicide at different concentrations and incubated at room temperature for 3-6 days in the dark. The experiment was performed twice with three replicate and three Petri dishes for each concentration of fungicide [6].

2.4. The fungicides efficiency

The fungicides efficiency was evaluated by the average of two perpendicular diameters and the inhibition rate of each treatment was calculated using the following formula: (growth estimated in the absence of a fungicide - growth estimated in the presence of a fungicide)/growth estimated in the absence of a fungicide x 100% [6].

2.5. Inhibitory Concentrations of Fungicides

The determination of Inhibitory Concentrations of Fungicides IC_{50} and IC_{90} against *B. cinerea* was determined as described by Hamdache et al. [6].

2.6. In vitro assay of antagonistic activity

To detect strains with antagonistic abilities, the two bacteria of *Bacillus sp*. were tested against the two isolates of *Botrytis cinerea* on the PDA medium by the plate confrontation method. The mycelial plug was removed from a 10-day colony of *B. cinerea* and then placed on the PDA media 1 cm from the edge of the plate. Then, a plug from 24-hours bacterial colony was placed on the opposite side and the inhibition diameter was calculated. Negative control plates had no bacteria. Inhibition of radial growth percentage was calculated every 48 hours using the formula according to Ezziyyani et al. [16].

2.7. Statistical analysis

The statistical analysis of data was performed using the SPSS software, version 21(SPSS, Inc., Chicago, IL., USA). The statistical significance of the results was determined using "Duncan's multiple range" test ($P < 0.05$).

3. Results and discussion

The results of the fungicide's efficiency showed that the combination chlorothalonil-carbendazim was highly and totally effective against Bt.MB in both pHs values (Fig. 2a, Fig. 4a) and allows a very significant inhibition of Bt.L growth with an inhibition rate of 71.16% and 91 % in the homologated dose in the pH 5.6 and 7.5 respectively (Fig. 1a, Fig. 3a). The mancozeb was similar to the combination chlorothalonil-carbendazim in the inhibition of vegetative growth and colonial development of Bt.BM isolate in both pH values (Fig Fig.2a and Fig.4b). Furthermore, the inhibitory action of Mancozeb on mycelial growth of Bt.L at pH 5.6 was higher than the inhibitory action of chlorothalonil-carbendazim (Fig. 1a, Fig. 1b) but less effective when comparing its action on the vegetative growth of the same isolate in the pH 7.5. (Fig.3b).

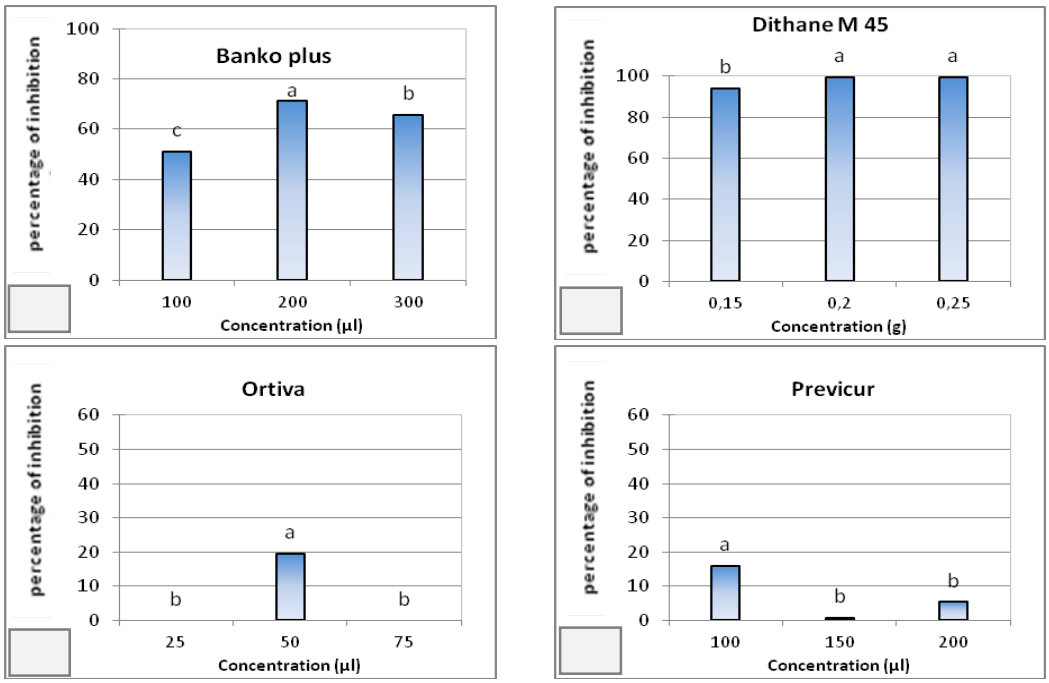


Figure 1: Effect of four fungicides on mycelial growth of *B. cinerea* (Bt.L) isolate at pH 5.6

The propanocarb HCL showed a lower efficacy than mancozeb. It showed that his action inhibits the growth of B.MB with 52% in the homologated dose (Fig. 2d) but in high dose it showed an increase in the resistance with 37.27% (Fig.4d), while for the Bt.L the inhibition rate was 15.81% in low dose and the inhibition effect was more efficient compared to the homologated dose at pH 5.6 (Fig.1d). However, the inhibition rate increased at the pH 7.5 for the Bt. L isolate (Fig. 3d), toward 30% in high dose and 38% in low dose. The azoxystrobin weakly inhibits the vegetative growth of the two isolates of *Botrytis cinerea* (Bt.BM and Bt.L). At the pH 5.6, the inhibition rate of Bt.MB was 44% in high dose while for B.L was19.53% at the homologated dose. At the

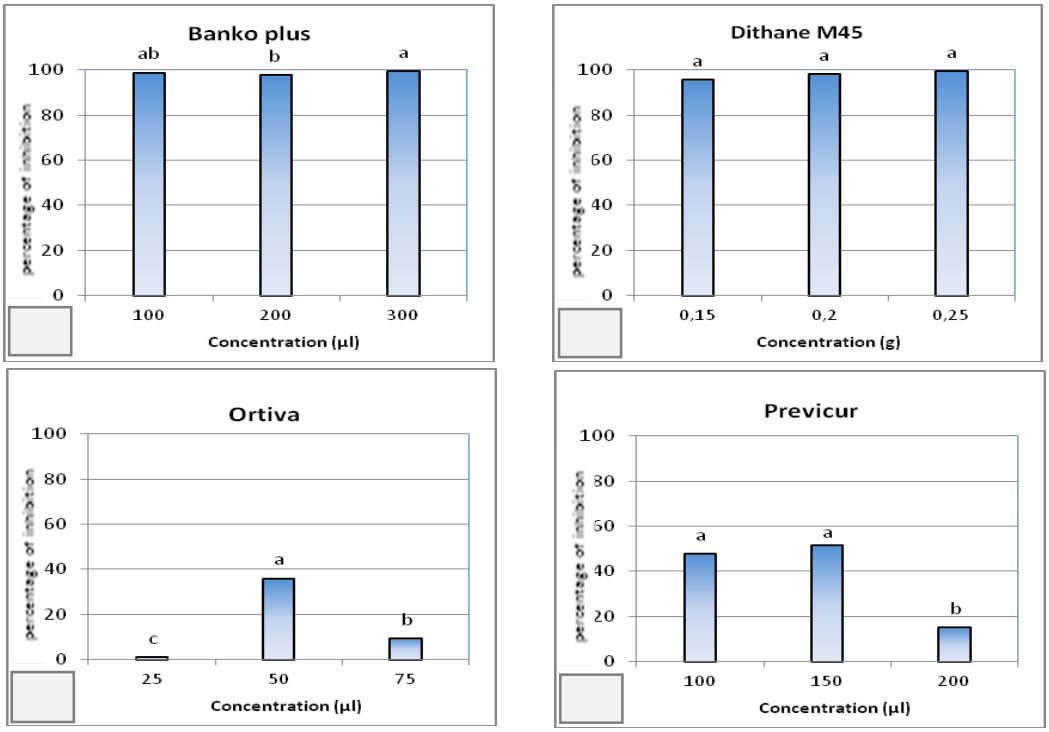
pH 7.5, Bt.MB showed a resistance to azoxystrobin with an inhibition rate of 36% at the homologated dose. The results of the inhibitory concentrations of fungicides in the homologated dose are represented in the Table 2.

In both pHs tested, the mancozeb was very effective on the isolate of Bt.MB (IC_{50} <¼ HD). Likewise, the combination of chlorothalonil-carbendazim inhibits completely the growth of the two isolates of *Botrytis cinerea* (IC_{50} <¼ HD) 2). At pH 5.6, the azoxystrobin had no effect on the Bt.L (IC_{50} and IC_{90}> 2 HD), whereas the chlorothalonil-carbendazim combination was very effective against this isolate (IC50 <¼ HD and CI90 < HD)[a]. The propanocarb HCL is moderately effective for Bt.MB (IC_{50} < HD and IC_{90}> HD) in contrast to mancozeb that shows a high inhibitory effects against Bt.L (IC50 and IC_{90} < HD). At pH 7.5, chlorothalonil-carbendazim completely inhibits the growth of the two isolates of *Botrytis cinerea* (IC_{50} and IC_{90} <¼ HD) while the other three fungicides are moderately effective against Bt.L (IC_{50} < HD and IC_{90}> HD).

Table 2: Inhibitory concentration of the four fungicides evaluated on the growth of *B. cinerea* isolates.

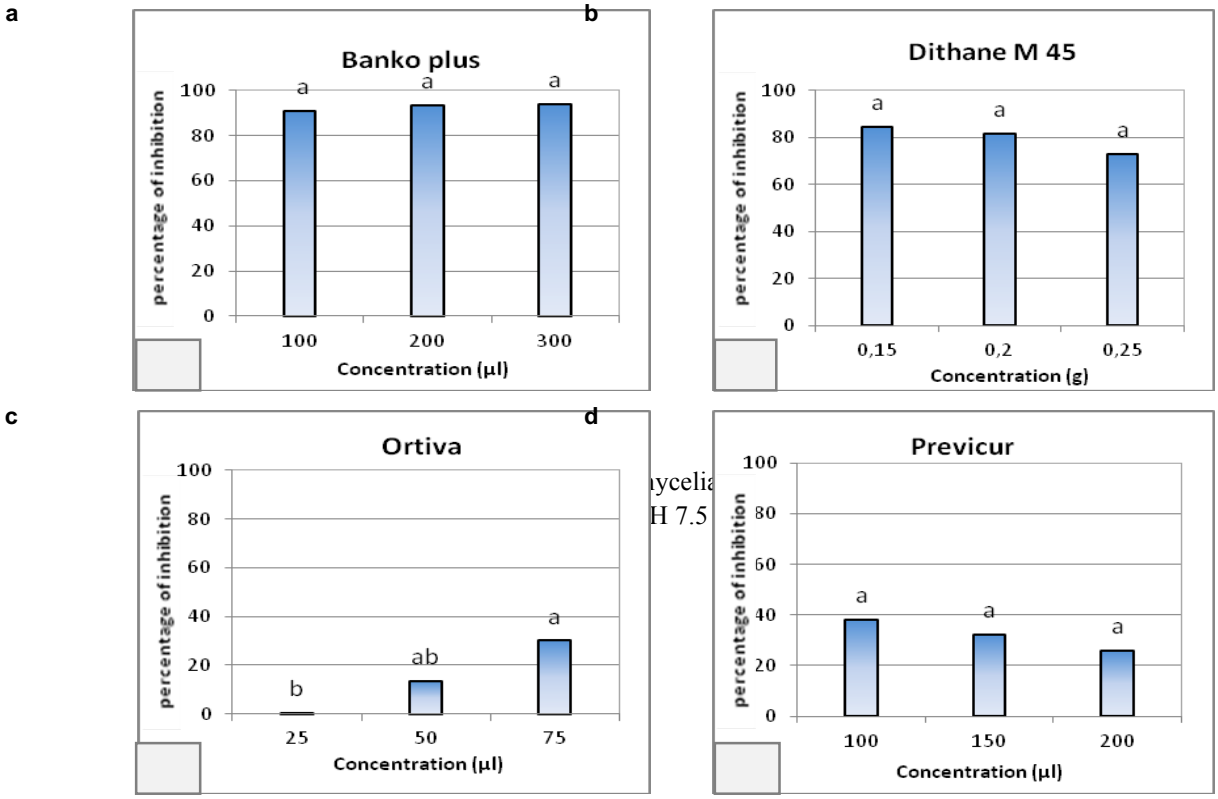
	pH 5.6				pH 7.5			
	Bt.L		Bt.MB		Bt.L		Bt.MB	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Chlorothalonil-Carbendazim	< ¼ HD	< HD	< ¼ HD	< ¼ HD	< ¼ HD	< ¼ HD	< ¼ HD	< ¼ HD
Mancozeb	< HD	< HD	< ¼ HD	< ¼ HD	< HD	> HD	< ¼ HD	< ¼ HD
Propanocarb HCL	< HD	>2 HD	< HD	> HD	< HD	> HD	> HD	> HD
Azoxystrobin	>2 HD	> 2 HD	< HD	>2 HD	< HD	> HD	> HD	> HD

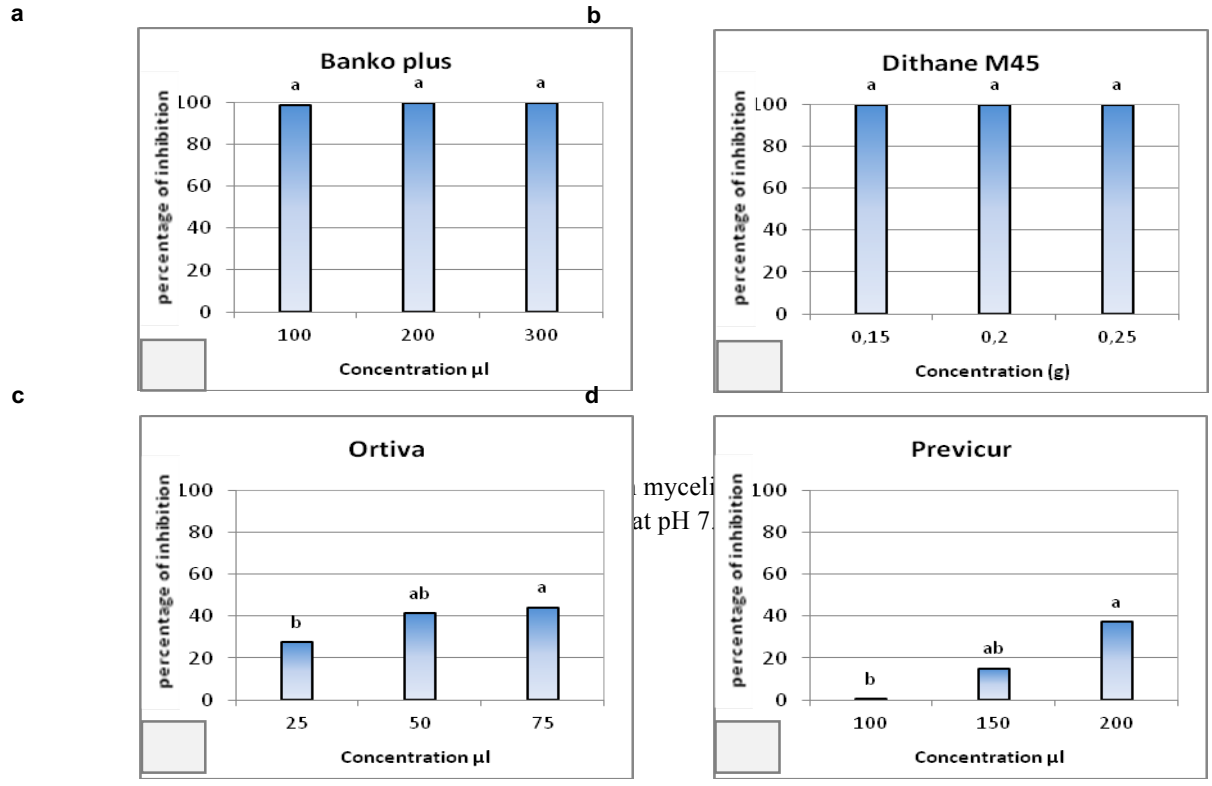
Figure 2. Effect of four fungicides on mycelial growth of *B. cinerea* (Bt.MB) isolate at pH 5.6



The interactions between *Bacillus sp.* B1 and *Bacillus sp.* B2 and the pathogen was observed in the antagonistic activity test, the *Bacillus sp*, strains showed an inhibition of the mycelial growth of the two pathogens (Bt.MB and Bt.L) by antibiosis and a very clear necrosis zone at the border of the mycelial colony which indicated the cell death of the pathogen by disorganization and disintegration followed by a mycelial hypertrophy (Figures 5, 6). The results suggest the potential of these strains to control the disease.

The different inhibition percentages evaluated using the diameter of inhibition zone (fig. 7) shows *Bacillus* sp. B1 gives a great inhibition for the two pathogenic isolate than *Bacillus sp*. B2. After six days of incubation, *Bacillus sp.* B1 shows an inhibition of the vegetative growth of *B. cinerea* Bt.L with 75.83% while for Bt.MB the inhibition was 82.22%. The confrontation of *B. cinerea* Bt.L with *Bacillus sp*. B2, shows an inhibition of 63.33% and 74.44% for *B. cinerea* Bt.MB





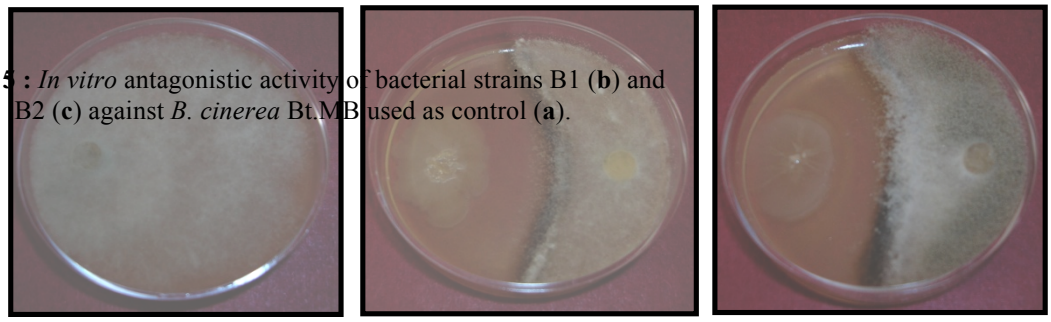


Figure 5 : *In vitro* antagonistic activity of bacterial strains B1 (**b**) and B2 (**c**) against *B. cinerea* Bt.MB used as control (**a**).

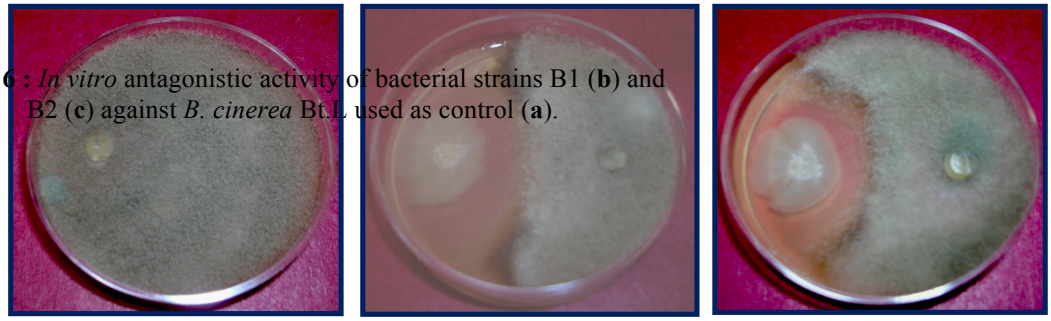


Figure 6 : *In vitro* antagonistic activity of bacterial strains B1 (**b**) and B2 (**c**) against *B. cinerea* Bt.L used as control (**a**).

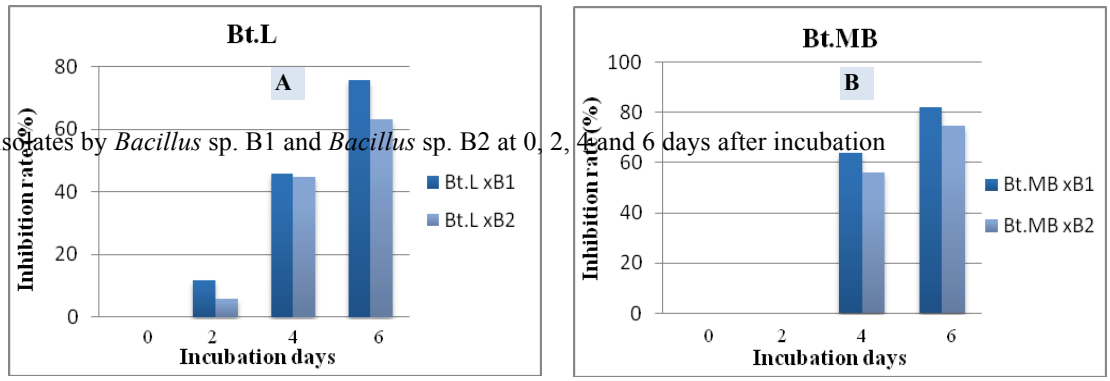


Figure 7 : Bt.MB isolates by *Bacillus* sp. B1 and *Bacillus* sp. B2 at 0, 2, 4 and 6 days after incubation

The work carried out in this study was in laboratory conditions and showed the possibility of developing resistance to fungicides in *B. cinerea* isolates. The absence of any significant difference between the doses tested for growth confirms the resistance of *B.cinerea* to these fungicides. On the other hand, our work has shown that the inhibition of mycelial growth depends on the fungal isolate, the pH of the medium, the active substance or the fungicide used and its concentration. The present research showed that the treatments with azoxystrobin and propanocarb HCL, could promote the development of resistance in *B. Cinerea* isolates at pH 5.6, while the pH 7.5 could promote the inhibition of growth and mycelial development of pathogens. In the meanwhile, the combination of chlorothalonil-carbendazim and Mancozeb decreased the growth of *B. cinerea* isolates. Hmouni et al. [19] observed a high efficacy of mancozeb on mycelial growth. It inhibits a variety of enzymes, such as those of glycolysis [20, 21]. According to Hamdache, et al. [6] the chlorothalonil was effective when used in combination with chlorpyrifos, a higher concentration of chlorothalonil increases the inhibition of a population of soil microorganisms by acting on the respiratory processes [6]. Moreover, the chlorothalonil was effective when used in combination with other fungicides [6]. The mode of action and the resistance mechanisms in *B. cinerea* to this anti-*Botrytis* fungicides have been widely studied by Leroux et al. [22-24] and Walker et al. [25]. The chlorothalonil belong to Multisite inhibitors group, it has been used against

grey mold for a long time. Today, it plays a minor role compared to the more active site-specific compound. Due to their non-specific mode of action, the resistance risk is low [4]. The carbendazim belongs to benzimidazoles, which effectiveness in controlling *B. cinerea* has been reduced due to the development of high levels of resistance [26]. Shengming et al. showed that seven azoxystrobin-resistant isolates were also resistant to carbendazim [27]. A similar finding was gained by Jinhua et al [28], from 263 isolates of *B. cinerea* from diseased fruit and leaves of tomato plants 89% were resistant to carbendazim. The azoxystrobin below to the strobilurins group is highly active against a variety of fungi and oomycetes but considered to be less effective against *B. cinerea* which contains an alternative terminal oxidase that can bypass the inhibition of the respiratory chain [29, 4]. To overcome the problem of resistance related to the use of synthetic compounds, different biological control strategies have been studied. Biocontrol with beneficial bacteria has been shown to be an environmental and friendly solution to prevent pathogenic fungi. *Bacillus sp*. is present in the rhizosphere [30] and the most strains of *Bacillus sp*. that are antagonistic towards fungi have been isolated from the soil surface. *Bacillus sp*. have biocontrol capabilities such as cell wall degrading enzymes and antibiotics [31, 32]. The present study showed that gray mold can be controlled by *Bacillus sp.* because of its ability to inhibit mycelial growth of the fungus. Previous studies have also confirmed that this bacterium has a broad spectrum of antimicrobial activity against bacteria and phytopathogenic fungi [33-36]. It has been reported that the suppression of growth of *B. cinerea* and the formation of inhibition zones by selected isolates belonging to the genus *Bacillus* were probably due to the metabolites released by the bacteria in the culture medium [35]. Antibiosis is probably the most well-known and important mechanism used by *Bacillus* to limit invasion of the pathogen in the tissues of the host plant. It consists of a direct inhibition of the growth of the pathogen via the production of metabolites with antifungal and / or antibiotic properties. The strains of the genus *Bacillus*, produce a variety of antifungal metabolites, which may be either non-lipopeptidic molecule, such as polyketides [35], zwittermycin-A, kanosamine [35], or lipopeptides such as the families of surfactin, iturin and fengycin [36-38] and hydrolytic enzymes like the β-1,3- glucanase that can degrade fungal cell walls [39].

Conclusion

The experimental results reported in this paper revealed that the azoxystrobin and propanocarb HCL increased the resistance of *B. cinerea* isolate. The repeated use of this fungicide may be the reason of the occurrence of this resistance. The Chlorothalonil-Carbendazime and the mancozeb are effective in controlling gray mold in strawberries and consequently they can be used more in the field, even if it is preferable to turn toward less polluting treatment. Finally, it is now recognized that other factors could affect the development of resistances to fungicides in *B. cinerea*, in our case, the biodiversity of *B. cinerea* isolates, geographical distribution and the pH factor which proved to be a determining factor in overcoming the problem of the inefficiency of certain fungicides. In the future, the knowledge gained in this field could provide elements for the management of *B. cinerea*. Different strategies of biological control have been developed to replace the use of chemical treatment and several works have been done to confirm their efficiency. Nowadays, the antagonistic effect of two *Bacillus sp*. isolates (B1 and B2) against two isolate of *B. cinerea* (Bt.BM and Bt.L), has already been evaluated for further use as biocontrol agent in gray mold management.

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