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In vitro and *In vivo* Antifungal Activity of Culture Filtrates and Organic Extracts of *Penicillium* sp. and *Gliocladium* spp. against *Botrytis cinerea*

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Abstract

Eight isolates of *Penicillium* sp. and two isolates of *Gliocladium* spp. were tested *in vitro* and *in vivo* for their inhibitory effects against *Botrytis cinerea*, causal agent of tomato fruit grey mold. The biocontrol essays conducted *in vitro* revealed that the culture filtrates of the isolates tested have significantly reduced the mycelial growth of the pathogen. The filtrates of the isolate CH6 of *Penicillium* sp. applied at different concentrations (10, 15 and 20% v/v), was most effective in reducing *B. cinerea* colony diameter. The ethyl acetate and chloroform extracts of the isolates CH6 of *Penicillium* sp., Gc1 of *G. catenulatum* and Gv1 of *G. virens* have shown an inhibitory effect of the pathogen radial growth, at the concentrations used (1, 2.5 and 5% v/v). In addition to the reduction of the mycelial growth of *B. cinerea*, these antagonistic agents have induced important morphological alterations to the mycelium of the pathogen. These antagonists were applied to tomato fruits 2 hours before their inoculation with the pathogen. Tested as culture filtrates, the most effective isolates CH11 and MC1 of *Penicillium* sp. and Gv1 of *G. virens* had significantly reduced the severity of the disease compared to the inoculated and untreated control fruits. Similar effects were recorded using the ethyl acetate and chloroform extracts of the tested antagonists; those of CH6 and CH5 of *Penicillium* sp. and Gc1 of *G. catenulatum* were found to be the most effective in reducing severity grey mold. Thus, this study showed the presence of bioactive molecules in the culture filtrates of the antagonistic agents used and also allowed the selection of effective isolates for grey mold disease control.

Keywords: Biological control; Culture filtrates; *Gliocladium* spp.; Organic extracts; *Penicillium* sp.; Tomato rot

Introduction

Tomato (*Solanum lycopersicum* L.) is the second most consumed and most economically important vegetable crop worldwide after potato and constitutes a major agricultural industry [1]. Its adaptability to different climatic conditions [2] as well as different latitudes makes it the most cultivated crop on nearly a third acreage of global vegetable crop. Tomatoes are consumed either fresh or as economically important processed products [3] and have significant nutritional value; they are a good source of vitamin C, vitamin A and antioxidants.

In Tunisian agriculture, tomato crop occupied a strategic position. It is cultivable over 24,231 ha with a production of more than 1,040,100 T/year [4]. However, this crop is subjected to several viral, bacterial, and fungal attacks, especially in pre-and post-harvest stages [5]. Among common tomato fruit rot diseases, grey mold caused by *Botrytis cinerea* Pers. is of particular concern [6,7]. This pathogen has an exceptionally wide host range and is widespread in almost all tomato production areas and is responsible for considerable economic losses of up to 50% in some African countries [5]. *B. cinerea* can infect the plants either by direct penetration or through wounds caused by cultivation practices and during transport, storage, and marketing [8]. High humidity, free moisture on the plant surface, and low temperatures are conducive to fungal development [9].

Management of *B. cinerea* has heavily relied on the use of chemical fungicides which have sometimes yielded satisfactory results. However, the misuse of several chemical fungicides like benzimidazoles and dicarboximides led to the development of resistant strains making more difficult the control of this pathogen [10]. New safer, environmentally friendly alternative strategies have been developed such as, biological control [11]. Several species of biocontrol agents have been isolated and are becoming increasingly interesting in controlling plant pathogens on various crops, such as *Gliocladium virens*, *G. catenulatum* [12] and *Penicillium* sp. [13].

As grey mold is still causing severe losses for tomato production in Tunisia, the current study focused on the use of biocontrol agents to manage *B. cinerea* rot on tomato fruits. Isolates of *Penicillium* sp. and *Gliocladium* spp. have been chosen as they have shown satisfactory degree of protection against several pathogens attacking tomato in Tunisia, i.e, *Verticillium dahliae*, *Colletotrichum coccodes* and *Alternaria solani*.

Thus, the aim of the present study is to evaluate the *in vitro* antifungal activity of the culture filtrates and the organic extracts of the antagonistic isolates of *Penicillium* sp. and *Gliocladium* spp. against *B. cinerea* and to elucidate their effect on the severity of grey mold on tomato fruits.

Materials and Methods

Pathogen

The isolate of *B. cinerea* used in this study was gratefully provided by the laboratory of Phytopathology of the Regional Center of Research on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia. It was originally recovered from tomato cv. Rio grande fruit, showing typical symptoms of grey mold. Pathogen cultures were grown on Potato Dextrose Agar (PDA) amended with streptomycin sulphate* (300 mg/L) (Pharmadrug Production Gmbh, Hamburg, Germany) and incubated at 25°C in the dark for 7 days before use [14].

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Isolates	Name	Origin
CH5	*Penicillium* sp.	Compost
CH6	*Penicillium* sp.	Compost
CH7	*Penicillium* sp.	Compost
CH9	*Penicillium* sp.	Compost
CH11	*Penicillium* sp.	Compost
MC1	*Penicillium* sp.	Compost
MC3	*Penicillium* sp.	Compost
MC4	*Penicillium* sp.	Compost
Gv1	*Gliocladium virens*	Soil
Gc1	*Gliocladium Catenulatum*	Soil

Table 1: Antagonistic agents tested against *Botrytis cinerea*.

Biocontrol agents

Height isolates of *Penicillium* sp., one isolate of *Gliocladium virens* and one isolate of *G. catenulatum* were used in the present study (Table 1). They were isolated from compost and soil [15-17]. They were cultured on PDA and incubated at 25°C in the dark for 7 days before use [18].

Fruit material

Tomato fruits (cv. Rio Grande) used for the *in vivo* bioassays were selected according to their maturity, firmness, consistency and especially the absence of visible symptoms of rot on their surface. Before treatment, fruits were surface disinfected with 10% sodium hypochlorite for 5 min, rinsed with sterile distilled water (SDW) and dried at ambient temperature.

Assessment of the *in vitro* antifungal activity of *Penicillium* sp. and *Gliocladium* spp. culture filtrates against *Botrytis cinerea*

Liquid culture of each antagonistic agent tested was prepared on Potato Dextrose Broth (PDB) for 28 days at room temperature under continuous stirring at 150 rpm/min [19]. A non-inoculated PDB medium served as control filtrate [20]. A volume of 20 mL was collected from each liquid culture and centrifuged thrice for 10 min at 10000 rpm. The obtained supernatant was then sterilized by filtration through 0.22 µm millipore size filter [20,21]. The control was the PDB medium filtrate. Each antagonist filtrate was aseptically added to Petri dishes containing molten PDA medium supplemented with Streptomycin sulfate (300 mg/L) at the concentration of 10% (v/v). After solidification, three 6 mm diameter-agar plugs removed from 7 days-old pathogen cultures on PDA were placed equidistantly in each Petri plate [22,23]. Fungal cultures were incubated at 25°C [23]. The mean colony diameter of the pathogen (treated and untreated control) was measured after 5 days of incubation. The mycelial growth inhibition rate was calculated using the following formula:

$$I\% = [(C2-C1)/C2] \times 100$$

with C2: Mean diameter of the control colony and C1: Mean pathogen colony diameter in the presence of the antagonist filtrate.

The most active culture filtrates of the tested isolates in reducing *B. cinerea* mycelial growth were selected and tested at three concentrations (10%, 15% and 20%, v/v) as described above.

Assessment of the *in vitro* antifungal activity of *Penicillium* sp. and *Gliocladium* spp. organic extracts against *Botrytis cinerea*

Two organic solvents, chloroform and ethyl acetate, were used to extract the antifungal metabolites produced by the tested antagonists. Only antagonist filtrates showing antifungal activity were used for this assay. Ten mL-aliquots from each antagonist filtrate, prepared as described above, were poured into a separating funnel before adding 10 mL of each extraction solvent, chloroform, or ethyl acetate [24-27]. The funnel containing this mixture was then inverted several times, by degassing from time to time, and left to settle (open cap) to quickly reach the partition equilibrium between the two phases. The organic phase (the lower phase for extraction with chloroform and the upper one with ethyl acetate) were collected. The aqueous phase was replaced in the funnel and the extraction was repeated three times. The solvent was evaporated using a rotary evaporator at 90°C with a slight rotation at 150 rpm [28-30].

In order to test their antifungal potential against *B. cinerea*, 1 mg of each obtained extract was dissolved in 2 mL of methanol [25,31]. These extracts were added aseptically to Petri dishes containing the culture medium (PDA) supplemented with Streptomycin sulfate (300 mg/mL) and tested at three concentrations, 1%, 2.5% and 5% (v/v). The mixture was gently stirred for a better distribution of the extract in the medium [26]. After solidification, three 6 mm-pathogen plugs obtained from 7 days-old pathogen cultures on PDA, were equidistantly placed in the Petri plate. The colony diameter of *B. cinerea* was measured after 5 days of incubation at 25°C.

Effect of *Penicillium* sp. and *Gliocladium* spp. culture filtrates and organic extracts on fruit grey mold severity

Disinfected tomato fruits were wounded (4 mm deep and 6 mm in diameter) at the equator using a sterile cork borer and 100 µL of each culture filtrate (of all antagonists tested) or organic extract (of the most effective culture filtrate), or SDW for the untreated control, were injected into each wound. Approximately two hours after treatment, an agar plug (6 mm in diameter) colonized by *B. cinerea* was placed within each wound. Treated or untreated (control) and inoculated tomato fruits were placed on moist filter papers in plastic boxes to maintain a high relative humidity and incubated at 25°C for 5 days [11]. The lesion diameter of the occasioned rot developed from inoculation sites was measured.

Statistical analysis

Statistical analysis of all measured parameters was conducted through the software SPSS 16.0 (Statistical Package for the Social Sciences) using the procedures of general linear models (GLM). The *in vitro* test of culture filtrates of *Penicillium* sp. and *Gliocladium* spp. was analyzed according to a completely randomized design while for the essays of culture filtrates at different concentrations and the organic extracts a completely randomized design with two factors (culture filtrates or organic extracts tested, and concentrations used) was used. Three replications were used for each elementary treatment and the whole experiment was repeated once. The *in vivo* tests were conducted according to a completely randomized design where the culture filtrates or organic extracts tested are the only factor studied. Each treatment was replicated five times (five fruits). Experiments were conducted two times.

All these experiments were analyzed using a standard analysis of variance (ANOVA) with interactions. Means comparisons were conducted according to the Student-Newman-Keuls (SNK) or LSD tests at $P \leq 0.05$.

Results and Discussion

Effect of *Penicillium* sp. and *Gliocladium* spp. culture filtrates on the *in vitro* growth of *Botrytis cinerea*

Results shown in Figure 1 revealed that the culture filtrates of the antagonists tested exhibited significant (at P ≤ 0.05) inhibitory effect against *B. cinerea*, after 5 days of incubation at 25°C. The highest significant reduction of the pathogen mycelial growth, about 27%, was induced by the culture filtrate of the isolate CH6 of *Penicillium* sp. (Figure 2) followed by non-significant reductions, by 16%, 14%, 12% and 10% noted with filtrates of CH11 and CH5 of *Penicillium* sp., Gv1 of *G. virens* and Gc1 of *G. catenulatum*, respectively, as compared to the untreated control. Similar results have been reported by Di Pietro et al. [32] showing that the culture filtrate of *G. virens* contains metabolites such as endochitinase and gliotoxin with inhibitory effect on growth and spore germination of *B. cinerea.* In the same way, the culture filtrate of *T. harzianum* inhibited the growth of *B. cinerea* through chitinolytic glucanolytic, cellulolytic and xylanolytic enzymes [33]. Brunner et al. [34] also found active compounds in the culture filtrates of *T. atroviride*, such as glucose oxidase, able to inhibit *B. cinerea* spore germination. In the same sense, Chatterton [35] showed that chitinase and glucanase contained in the culture filtrate of *G. catenulatum* can inhibit by 50% *F. oxysporum* and *Pythium* spp. mycelial growth.

Effect of three concentrations of the of the most effective culture filtrates *in vitro* on mycelial growth of *Botrytis cinerea*

Analysis of variance showed that the mean diameter of *B. cinerea* colonies was significantly affected by the culture filtrates of the selected antagonists and the concentrations tested, as compared to the untreated control. As shown in Figure 3, the culture filtrates of the isolates CH5 and CH6 of *Penicillium* sp., Gv1 of *G. virens* and Gc1 of *G. catenulatum* have significantly limited pathogen mycelial growth at the concentrations used (10%, 15% and 20% v/v) (Figure 3). The highest inhibitory effect was induced by the culture filtrate of CH6 of *Penicillium* sp. applied at 10%, 15% and 20% (v/v), where pathogen radial growth was reduced by 27%, 37% and 34%, respectively. Used at 15% and 20% (v/v), the culture filtrate of CH5 significantly limited pathogen growth by 4% and 17%, respectively; whereas filtrate of Gc1 of *G. catenulatum* reduced by 18% *B. cinerea* when applied at 20% (Figure 4).

Our findings are similar to those obtained by Zhang et al. [36] who reported that the inhibitory effect of the culture filtrates of the biocontrol

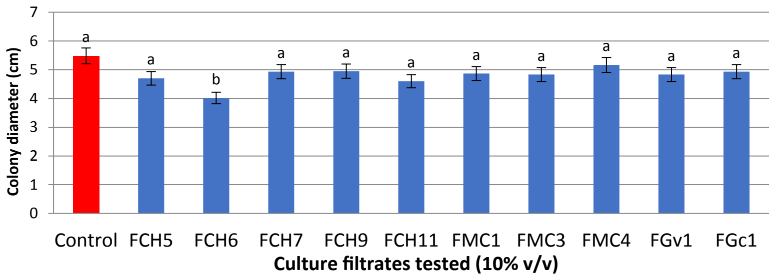

Figure 1: Effect of culture filtrates of ten isolates of *Penicillium* sp. and *Gliocladium* spp. on *Botrytis cinerea* mycelial growth recorded after 5 days of incubation at 25°C, compared to the untreated control. FCH5, FCH6, FCH7, FCH9, FCH11, FMC1, FMC3 et FMC4: Culture filtrates of isolates CH5, CH6, CH7, CH9, CH11, MC1, MC3 et MC4 of *Penicillium* sp.; FGv1: Culture filtrate of isolate Gv1 of *Gliocladium virens*; FGc1: Culture filtrate of isolate Gc1 of *G. catenulatum*. Bars assigned by the same letter are not significantly different according to the Student-Newman-Keuls test at P ≤ 0.05.

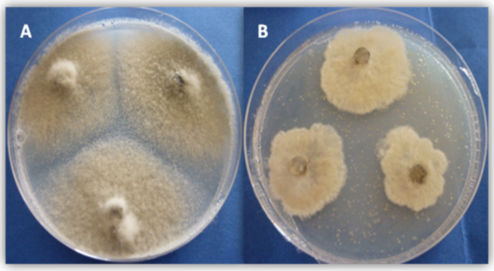

Figure 2: Inhibition of the mycelial growth of *Botrytis cinerea* (B) and morphological alteration of its colony induced by the culture filtrate of the isolate CH6 of *Penicillium* sp. as compared to the untreated control (A).

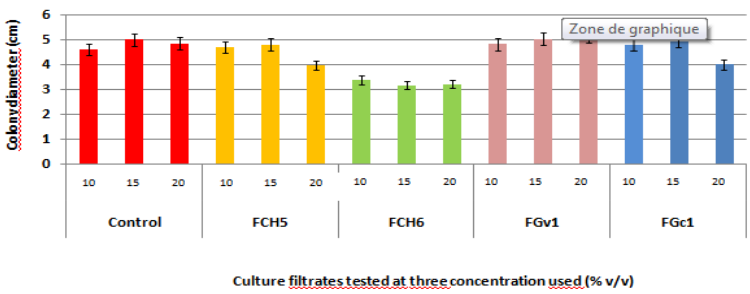

Figure 3: Effect of three concentrations of culture filtrates of *Penicillium* sp. and *Gliocladium* spp. on *Botrytis cinerea* mycelial growth recorded after 5 days incubation at 25°C as compared to the untreated control. FCH5, FCH6: Culture filtrates of isolates CH5, CH6 of *Penicillium* sp.; FGv1: Culture filtrate of isolate Gv1 of *Gliocladium virens*; FGc1: Culture filtrate of isolate Gc1 of *G. catenulatum*. LSD (Culture filtrates tested × Concentrations used)=0.48 cm at P ≤ 0.05.

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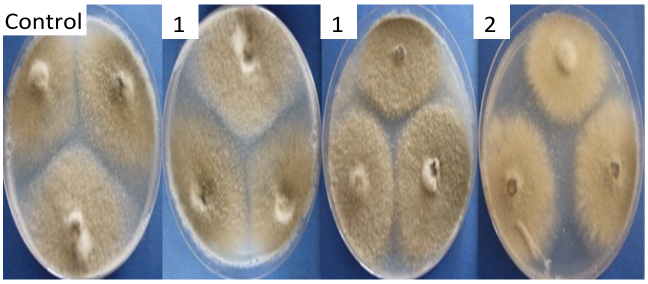

Figure 4: Inhibition of *Botrytis cinerea in vitro* growth induced by Gc1 of *Gliocladium catenulatum* depending on the concentrations tested (% v/v) as compared to the untreated control.

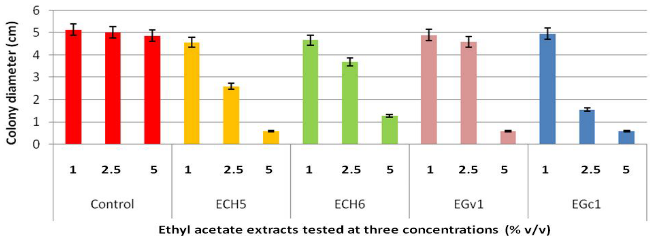

Figure 5: Effect of three concentrations ethyl acetate extracts from *Penicillium* sp. and *Gliocladium* spp. *Botrytis cinerea* mycelial growth recorded after 5 days incubation at 25°C. ECH5 and ECH6: Ethyl acetate extracts of the isolates of *Penicillium* sp. CH5 et CH6; EGv1: Ethyl acetate extracts of the isolates Gv1 of *Gliocladium virens*; EGc1: Ethyl acetate extracts of the isolates Gc1of *G. catenulatum.* LSD (Extracts tested × Concentrations used)=0.29 cm at P ≤ 0.05.

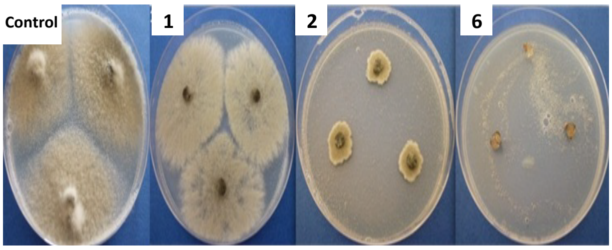

Figure 6: Inhibition of *Botrytis cinerea in vitro* growth induced by Gc1 (*Gliocladium catenulatum*) ethyl acetate extract depending on the concentrations tested (% v/v) as compared to the untreated control.

agents depends on the concentrations used. In fact, they demonstrated that increasing the concentration of the filtrate obtained from *Rhodotorula glutinis* led to a limited incidence of the grey mold in strawberries. In the same context, Pietro et al. [32] found that the reduction of *B. cinerea* spore germination is correlated with the concentrations of the secondary metabolites released in the culture filtrate of *G. virens*. Indeed, 25 µg/mL to 50 µg/mL of endochitinase and 0.5 µg/mL to 0.75 µg/mL of gliotoxin were reported to be responsible of 95% reduction of *B. cinerea* mycelial growth. In the same sense, Allen et al. [37] showed that the ability of the yeast species *R. glutinis* to inhibit gray mold pathogen is concentration-dependent. In fact, this antifungal activity was significantly greater at the concentration of 10^7 yeasts/mL compared to the lower concentrations. Moreover, Chen et al. [38] found that applying five concentrations (50, 150, 250, 350 and 450 mg/L) of *T. harzianum* filtrate led to 73.7% reduction of *F. oxysporum* f. sp. *cucumerinum* mycelial growth. An and Ma [39] showed that 2 mL of the culture filtrate of *Bacillus subtilis* diluted in five doses (1%, 10%, 20%, 50% and 100%) had inhibitory effects against the development of *B. cinerea* at 25°C.

Antagonistic effect of organic extracts on the mycelial growth of *Botrytis cinerea*

Ethyl acetate extracts: Analysis of variance showed that *B. cinerea* colony diameter varied significantly depending on the ethyl acetate extracts tested and the concentrations used (1%, 2.5% and 5% v/v) (Figure 5). A significant interaction was recorded between both fixed factors. Indeed, when applied at 1%, the ethyl acetate extracts of CH5 and CH6 of *Penicillium* sp. have significantly reduced pathogen growth by 11 and 9%, respectively. Used at 2.5%, the extracts of CH5 of *Penicillium* sp. and Gc1 of *G. catenulatum* limited by 48 and 69%, respectively, the growth of *B. cinerea*. The highest inhibition, by 74 to 88%, was recorded with CH6, CH5, Gv1 and Gc1 extracts tested at 5% (Figure 6).

The antifungal potential of organic fungal extracts was reported in numerous studies as for ethyl acetate and methanolic extracts of *G. virens* with ability to inhibit the germination of *P. ultimum* oospores [32]. Furthermore, Tapwal et al. [40] demonstrated the inhibitory effect of ethyl acetate extract of *Penicillium* sp. used in five concentrations (1, 12.5, 25, 50 and 100 mg/mL) against some phytopathogenic species of the genus *Aspergillus* (*A. flavus*, *A. versicolor* and *A. candidus*). They also found that *P. oxalicum* organic extracts exhibited a significant antifungal activity against *B. cinerea*. These authors showed also that the appropriate solvents for extraction of *Penicillium* sp. secondary metabolites were ethyl acetate and hexane. Chatterton [35] found that the enzymatic extracts of *G. catenulatum* were found to be effective at different concentrations in reducing the conidial germination of *F. oxysporum* and in inhibiting growth of *Pythium* species. Vio-Michaelis et al. [41] have recently reported the inhibitory effect of ethyl acetate, n-hexanoic, n-butanol, methanolic and ethanolic extracts of the *Ephedra breana* plants and *Nolana sedifolia* on mycelial growth of *B. cinerea* at the concentration 250 µg/mL. The acetone crude extracts of the fungi strain Basidiomycetes also inhibited *in vitro* the colony growth of *B. cinerea* [42].

Chloroform extracts: Analysis of variance revealed a significant effect ($P \leq 0.05$) of the chloroform extracts tested and the concentrations used as well as their interaction on *B. cinerea* radial growth. Tested at 1% (v/v), the chloroform fractions of CH5 of *Penicillium* sp. and Gc1 of *G. catenulatum* have significantly reduced pathogen mycelial growth by 16 and 9%, respectively. Applied at 2.5%, chloroform extracts obtained from Gc1 and Gv1 cultures were more active, showing respectively 88 and 81% inhibition of pathogen mycelial growth, than those from *Penicillium* sp. isolates, CH5 and CH6, reducing growth by 53 and 45%. Used at 5%, extracts of Gv1 (*G. virens*), Gc1 (*G. Catenulatum*) and CH6 (*Penicillium* sp.) have significantly limited *B. cinerea* colony diameter by 88, 84 and 81%, respectively (Figure 7) as compared to the untreated control.

Similar results were obtained by Iftikhar and Alam [31] with the ethyl acetate and chloroform extracts of *T. harzianum* used against *F. oxysporum* f. sp. *ciceris*. Indeed, a significant inhibition of *B. cinerea* growth has been recorded with the two types of extracts due to the presence of antifungal metabolites in their culture filtrates. Other studies have highlighted the ability of the chloroform extracts of *P. janthillium* and *P. duclauxii* to inhibit spore germination and mycelial growth of *Grifola umbellate* due to the secondary metabolites present in their culture filtrates [30]. In the same way, according to Fu et al. [27], the chloroform extracts of *Phomopsis* sp. are able to significantly inhibit the growth of *B. cinerea in vitro* at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) (Figures 8 and 9).

Effect of the culture filtrates on grey mold severity

The culture filtrates of *Penicillium* sp. and *Gliocladium* spp. isolates were tested on tomato fruits before their inoculation with *B. cinerea*. Anova analysis showed a significant variation ($P \leq 0.05$) of the grey mold lesion diameter by the tested culture filtrates. Indeed, lesion diameter recorded after incubation at 25°C was reduced by 53, 52 and 50%, with the culture filtrates of MC1, CH11 of *Penicillium* sp. and Gv1 of *G. virens* (Figure 10), respectively, as compared to the untreated control. The culture filtrates of CH7, CH6 and CH5 of *Penicillium* sp. significantly limited disease severity by 35 to 37% compared to 23 and 26% noted with culture filtrates of MC3 of *Penicillium* sp. and Gc1 of *G. catenulatum*, respectively (Figure 11).

Similar effects were observed by Bridžiuvienė and Repečkienė [43] on grape bunches infected by *B. cinerea* and treated with the culture filtrates of *T. virens*. These authors also demonstrated the effectiveness of the culture filtrate of *Penicillium* sp. in the control of this pathogen. In the same sense, Burgess et al. [44] reported the protection of chickpea against *B. cinerea* using *G. roseum* culture filtrate. Similarly, filtrates of *T. harzianum* were shown to be effective in limiting by 52% the

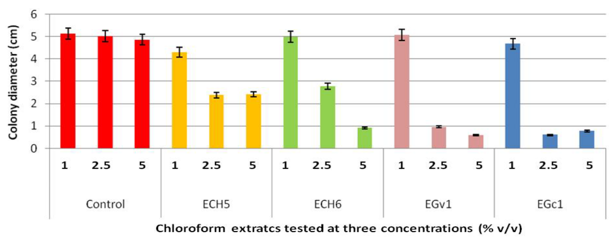

Figure 7: Effect of three concentrations of chloroform extracts from *Penicillium* sp. and *Gliocladium* spp. isolates tested at against *Botrytis cinerea* mycelial growth recorded after 5 days incubation at 25°C. ECH5 and ECH6: Chloroform extracts of the isolates of *Penicillium* sp. CH5 and CH6; EGv1: Chloroform extract of the isolate Gv1 of *Gliocladium virens*; EGc1: Chloroform extract of the isolate Gc1 of *G. catenulatum*. LSD (Extracts tested × Concentrations used)=0.29 cm at $P \leq 0.05$.

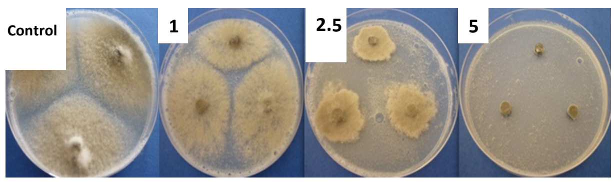

Figure 8: Inhibition of *Botrytis cinerea in vitro* growth induced by CH6 of *Penicillium* sp. chloroform extract depending on the concentrations tested (% v/v) as compared to the control.

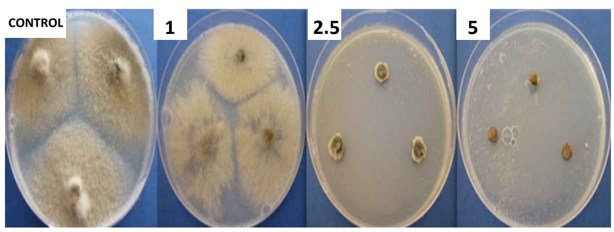

Figure 9: *In vitro* inhibition of *Botrytis cinerea* growth induced by chloroform extract from Gv1of *Gliocladium virens* depending on the concentrations tested (% v/v) as compared to the control.

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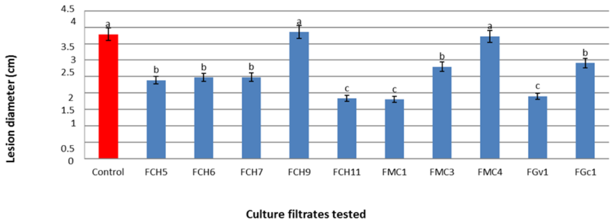

Figure 10: Effect of the culture filtrates of *Penicillium* sp. and *Gliocladium* spp. tested on grey mold severity on tomato fruits recorded after 5 days of incubation at 25°C compared to the inoculated and untreated control fruits. FCH5, FCH6, FCH7, FCH9, FCH11, FMC1, FMC3 et FMC4: Culture filtrate of isolates CH5, CH6, CH7, CH9, CH11, MC1, MC3 et MC4 of *Penicillium* sp.; FGv1: Culture filtrate of isolate Gv1 of *Gliocladium virens*; FGc1: Culture filtrate of the isolate Gc1 of *G. catenulatum*. Bars assigned with the same letter are not significantly different according to the test Student-Newman-Keuls at P ≤ 0.05.

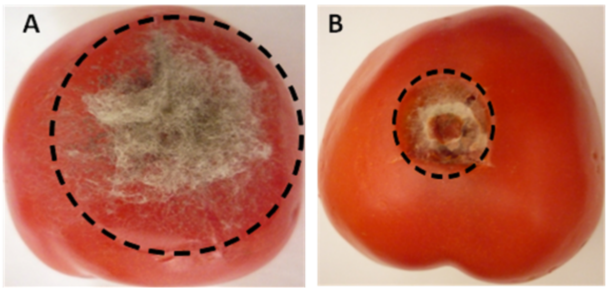

Figure 11: Effect of the culture filtrate of Gv1 of *Gliocladium virens* on grey mold severity on tomato cv. Rio grande fruits (B) compared with the untreated and inoculated control (A). N.B. The black dots are added to define the dimensions of the grey mold lesions on inoculated and treated fruits.

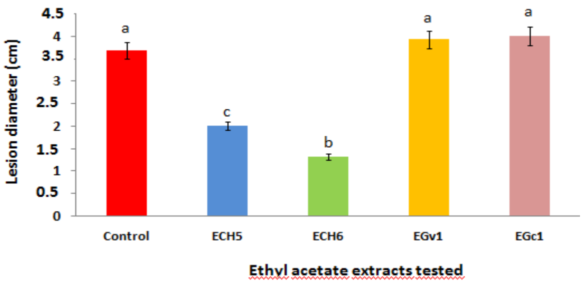

Figure 12: Effect of the ethyl acetate extract from *Penicillium* sp. and *Gliocladium* spp. tested on grey mold severity on tomato fruits recorded after 5 days of incubation at 25°C, compared to the inoculated and untreated control. ECH5 and ECH6: Ethyl acetate extracts of the isolates of *Penicillium* sp. CH5 et CH6; EGv1: Ethyl acetate extracts of the isolates Gv1 of *Gliocladium virens*; EGc1: Ethyl acetate extracts of the isolates Gc1of *G. catenulatum*.. Bars assigned the same letter are not significantly different according to the test Student-Newman-Keuls at P ≤ 0.05.

lesion diameter on *B. cinerea*-inoculated tomato fruits, five days after inoculation [33]. In the same way, Zhang et al. [36] demonstrated the antifungal activity of *Rhodotorula glutinis* towards *B. cinerea* infecting stored strawberries. Similar results were reported in the *in vitro* studies of Card [12] concerning the efficiency of *G. catenulatum* culture filtrate and its variable activity depending on the site of its application and the source of contamination with *B. cinerea*. Therefore, the greater distance of the application of *G. catenulatum* for increasing their protective effect was very low to the site of infection. The inhibitory effects were reported by Abou-Zeid et al. [45] where the filtrates of *G. deliquescens* and *G. virens* exhibited a strong suppressive potential of the severity of diseases caused by *A. alternata* and *F. oxysporum* on some medicinal plants as *Prunus peorsica* and *Pulicaria crispa*.

It should be also noted that all the culture filtrates tested could not completely inhibit the growth of *B. cinerea* on the site of inoculation but only decreased the diameter of the lesions on tomato fruits, compared to the control. Thus, pathogen sporulation depreciates the quality of the treated tomato fruits. The evaluation of the antifungal potential of the extracts from culture filtrates may give more interesting inhibitory effects.

Antagonistic effect of the organic extracts on grey mold severity

Suppressive effect of ethyl acetate extracts: Ethyl acetate extracts of *Penicillium* sp. and *Gliocladium* spp. were assessed for their potential to control *B. cinerea* infection on tomato fruits. Analysis of variance revealed that grey mold lesion diameter noted on inoculated and treated fruits varied significantly (P ≤ 0.05) depending on treatments tested. Ethyl acetate extracts of CH5 and CH6 of *Penicillium* sp. were found the most effective in reducing disease severity by 45% and 64%, respectively compared to inoculated and untreated control (Figure 12). Moreover, the tested extracts completely suppressed *B. cinerea* mycelial growth at the fruit inoculation site. Thus, these extracts were shown to possess promising antifungal active compounds for controlling *B. cinereal* (Figure 13).

Suppressive effect of chloroform extracts: The chloroform extracts of the antagonists tested were also evaluated for their inhibitory effect on the development and severity of grey mold on tomato fruits. Analysis of variance showed that the external lesion diameter varied significantly (P ≤ 0.05) between treatments tested. As shown in Figure

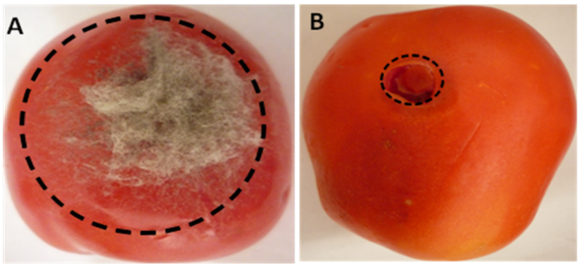

Figure 13: Effect of the ethyl acetate extract of the isolate CH6 of *Penicillium* sp. on grey mold severity (B) compared with the inoculated and untreated control (A). N.B. The black dots are added to define the dimensions of the grey mold lesions on inoculated and treated fruits.

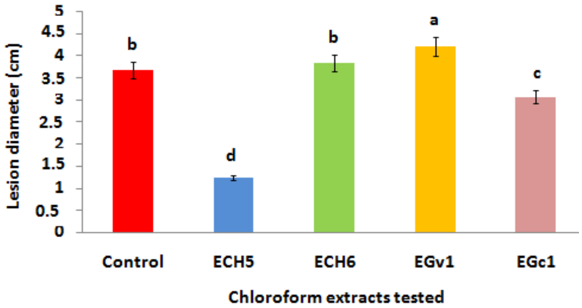

Figure 14: Effect of the chloroform extract from *Penicillium* sp. and *Gliocladium* spp. tested on grey mold severity on tomato fruits recorded after 5 days of incubation at 25°C compared to the inoculated and untreated control. ECH5 and ECH6: Chloroform extracts of the isolates of *Penicillium* sp. CH5 and CH6; EGv1: Chloroform extract of the isolate Gv1 of *Gliocladium virens*; EGc1: Chloroform extract of the isolate Gc1 of *G. Catenulatum*. Bars assigned with the same letter are not significantly different according to the test Student-Newman-Keuls at $P \leq 0.05$.

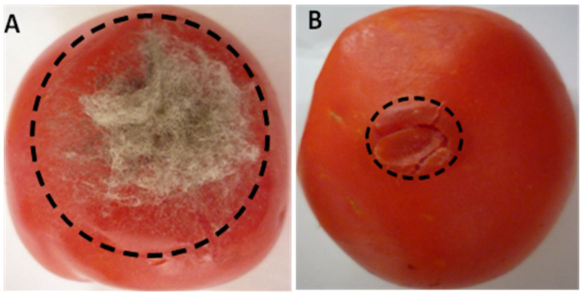

Figure 15: Effect of the chloroform extract of CH5 of *Penicillium* sp. on grey mold severity (B) compared with the inoculated and untreated control (A). N.B. The black dots are added to define the dimensions of the grey mold lesions on inoculated and treated fruits.

14, only chloroform extracts of CH5 of *Penicillium* sp. and Gc1 of *G. catenulatum* have significantly reduced the severity of the decay by 67% and 17%, respectively. The other extracts tested were founded to be ineffective in controlling the disease and behaved as the untreated control. Furthermore, these extracts, as already recorded with the ethyl acetate ones, completely inhibited *B. cinerea* mycelial growth and sporulation at the infection site. Similar inhibitory effects were also obtained with aqueous extracts of *Trichoderma* sp. found to be able to control *B. cinerea* on tomato plants [46] (Figure 15).

Conclusion

The present study investigates the suppressive effects of different culture filtrates and organic extracts of *Penicillium* sp. and *Gliocladium* spp. isolates on the *in vitro* growth of *B. cinerea* and toward the grey mold severity on tomato fruits.

The use of culture filtrates or organic extracts of the biocontrol agents *Penicillium* sp. and *Gliocladium* spp. against grey mold pathogen have shown encouraging results *in vitro* and led to a significant reduction of the mycelial growth of the pathogen, essentially with CH6 (*Penicillium* sp.) and Gc1 (*G. catenulatum*). Overall, the increase in the rate of inhibition of the pathogen was positively correlated with the increase of the concentration of the culture filtrate or extract used.

These results recorded *in vitro* were also evaluated *in vivo* using these biocontrol agents for the treatment of tomato fruits inoculated with *B. cinerea*. Culture filtrates of CH11, MC1 of *Penicillium* sp. and Gv1 of *G. virens* were found to be the most effective in reducing disease severity. Moreover, the use of ethyl acetate and chloroform extracts against *B. cinerea* showed that CH5 and CH6 of *Penicillium* sp. and Gc1 of *G. catenulatum* were effective in controlling of the disease.

Therefore, this application of antagonists is considered as a promising approach that can reduce the use of synthetic fungicides and their negative impact on the consumer and the environment. Furthermore, it would be interesting to study the efficiency of the bioactive metabolites contained in the aqueous phase of the antagonist tested, to elucidate the main compounds in the culture filtrates and their mechanisms of action and to promote these isolates as biological control agents against other tomato diseases.

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