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Antagonism of Three *Trichoderma* Species Against *Botrytis fabae* and *B. cinerea*, the Causal Agents of Chocolate Spot of Faba Bean (*Vicia faba* L.) In Algeria

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Abstract: Three species of *Trichoderma* (*T. harzianum*, *T. viride* and *T. longibrachiatum*) were compared *in vitro* against two isolates of *Botrytis fabae* and *Botrytis cinerea* isolated from faba bean leaves showing symptoms of chocolate spot disease grown at western Algeria. The ability of each strain of *Trichoderma* to reduce the growth and sporulation of *B. fabae* and *B. cinerea* was measured. Two techniques were used for this purpose : the direct confrontation of culture medium and confrontation remote action of volatile substances. Direct confrontation of the colonies of *T. viride* with those of the pathogen results in an inhibition and growth arrest at a distance of the parasite. Sporulation of *Botrytis* sp colonies along the zone of inhibition is greatly reduced. In the case of *T. harzianum* and *T. longibrachiatum*, no inhibition zone was observed and their mycelium quickly invade the colony of the tested pathogen.

Key words: Botrytis fabae · Botrytis cinerea · Trichoderma spp · Antagonism · Growth inhibition

INTRODUCTION

The economic importance of bean (*Vicia faba* var. Major) cultivation in the world can be explained by its high nutritional value due to its richness in vitamins and protein. In addition, beans helps improve the soil fertility through to fix nitrogen. Therefore, improving the production of this crop is one of the objectives in agriculture in many countries. Chocolate spot disease, caused by *Botrytis fabae* Sard. and *Botrytis cinerea* Pers. is the most serious disease affecting bean in Algeria and in several countries in all the countries of North Africa [1].

It occurs on all top growth of the plant. The leaves are usually the first infected organs are first chocolate circular spots 1 to 3 mm in diameter. When the temperature conditions are favorable, these spots enlarge, reaching from 10 to 15 mm are zoned merge and thus covering a large part of the leaf surface, causing defoliation of plants. Mode and the development of infection have been described by Mansfield and Deverall [2].

The use of chemical control against this pathogen has sometimes given good results. However, improper use of fungicides leads mostly to the phenomena of resistance in *Botrytis fabae* and *Botrytis cinerea* [3,4], that in addition to the adverse effects on the development of the antagonistic microflora.

To overcome the difficulties previously stated, it is clear that an alternative strategy gainst the disease of the beanis needed. Biological control has in recent years many advances in the fight against many pathogens of crops. *Trichoderma* showed an interesting potential control against various pathogens [5]. In this context, we consider it appropriate to make our contribution through the study *in vitro* the power antagonist *Trichoderma harzianum*, *T. viride* and *T. longibrachiatum* against *Botrytis fabae* and *B. cinerea*, disease-causing agents 'chocolate spot' in bean.

MATERIALS AND METHODS

Fungal Material: Four isolates of *Botrytis* spp were used throughout this study. They were isolated from samples with fresh bean chocolate spot disease.

Isolates of *B. cinerea* were collected from the region of Ain Temouchent (C1) and Mascara (C2). While those of *B. fabae* (F2 and F3) are from the region of Relizane. Strains of *Trichoderma harzianum* (TH), *T. longibrachaitum* (TL 10202) and *T. viride* (TV 1001) were used as antagonists come from laboratory of Mycology in University of Chlef, Algeria.

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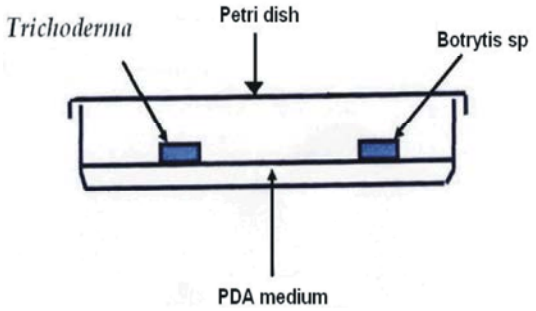

Fig. 1: Confrontation between Botrytis and Trichoderma strain by direct contact in PDA medium.

Cultural Medium: The choice of a suitable culture medium is essential for the proper development of the pathogen and the antagonist. The PDA medium (Potatos Dextrose Agar) provides good growing conditions for both *Botrytis* and *Trichoderma* [6,7].

The antagonistic activity *in vitro* of *Trichoderma* spp against *B. fabae* and *B. cinerea* was addressed in two ways : direct and indirect confrontation test.

Direct Confrontation Test: Confrontations are performed *in vitro* using the method of Patel and Brown (1969) [8]. In Petri dishes 90 mm in diameter, containing 15 ml of PDA medium, two agar pellets (6 mm in diameter), one bearing the strain of *Trichoderma* to test both the pathogen are placed following a diametrical axis to 5 cm a part and distant from the center of the box (Fig. 1). Incubation is carried out under alternating 12 hours light and 12 hours dark at 26 ± 2°C for 7 days. The indicator consists of an explant of the pathogen on the edge of the box. Each treatment is replicated 4 times.

Evaluation of Mycelial Growth: Mycelial growth of *Botrytis* was evaluated every 24 hours all by measuring the diameter of the Petri dish, the radius of the parasite on the side of the antagonist. After 7 days of incubation, measurements were made on the width of the zone of inhibition observed between the two colonies.

Evaluation of Sporulation: Assessment of sporulation is performed according to the principle of the method used by Maslouhy (1989) [9]. For every box sporulation is evaluated on the 12th day. Using a pastry cutter disinfected, we collected 10 slices (6 mm in diameter) on the periphery of the colonies of *Botrytis* side of the antagonist. These slices are then placed in 10 ml of an acetic acid solution N/10. Heating (45-50°C) for several minutes freeing the conidia. The concentration expressed in number of spores per ml was estimated using the

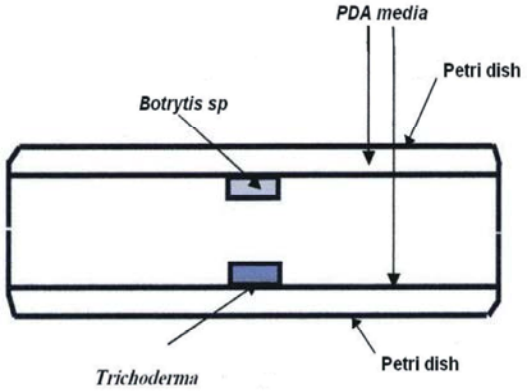

Fig. 2: Test of volatile substances influence emitted by Trichoderma sp. on mycelial growth and sporulation of Botrytis sp.

Malassez cell. If witnesses are estimates made ??in the same way by taking pucks to the periphery of the colonies of *Botrytis* inoculated alone.

Indirect Confrontation Test: The effect of volatile substances has been demonstrated by the technique of Shirmbock *et al.* (1994) [10]. A mycelial disc of 6 mm diameter of each pathogen and antagonist is at the center of Petri dishes containing PDA medium.

The lids are removed aseptically and the bottom of each box containing the antagonist tested is placed below the one containing the pathogen. Both funds are closed by three juxtaposed layers of Parafilm to prevent loss of volatile substances. For the witness box with a bottom of the middle one is placed below the bottom of a box containing the pathogen. After five days of incubation at room temperature laboratory (25 ± 2°C) under alternating 12 hours light and 12 hours of darkness, the inhibition of mycelial growth is estimated. All combinations between the four isolates of Botrytis and three strains of *Trichoderma* are performed and repeated 4 times.

Evaluation of Mycelial Growth of *Botrytis* Isolates: Scoring the average diameter of colonies treated is conducted every day for five days. Evaluation of the inhibition exerted by *Trichoderma* sp is estimated by calculating the percentage inhibition of mycelial growth using the following formula [11]:

$$I (\%) = (1 - C_n / C_o) \times 100$$

C_n : Average diameter of colonies in the presence of the antagonist

C_o : Average diameter of the control colonies.

Evaluation of Sporulation: Sporulation is estimated from the cultures of *Botrytis* aged 12 days. The technique used is to wash with 10 ml of sterile distilled water, the entire Petri dish containing the fungus, to release all the spores. The spore suspension is then poured into a beaker filled with sterile distilled water to 50 ml [12]. By using the Malassez cell under an optical microscope, counting the number of conidia for each sample.

Statistical Analysis: The variances (σ^2), averages and standard deviation (SD) of various repetitions were calculated and analyzed by the software of statistics (STAT BOX 6.0.4. GRIMMERSOFT) and the device used are the unifactorielle total randomization (one studied factor) by the test of Newman and Keuls ($P_{0.05}$ and $P_{0.01}$) [13].

RESULTS AND DISCUSSION

Direct Confrontation: Mycelial growth effect: Highly significant effect ($P < 0.01$) was observed in the study of mycelial growth of *Botrytis* colonies faced with *Trichoderma* strains whitch showed reduction in growth compared to the control (Table 1). Transplanting simultaneous TH or TL with one of *Botrytis* isolates showed a faster growth of *Trichoderma* isolates as *Botrytis*. After 2 days of incubation, the box is almost completely invaded by TH, whereas the colonies of C1 and C3, respectively, that occupy 12.75 and 13.75 mm radius. At the same time isolates C1 and C3 face 13 and reached TL 13.75 mm radius.

The growth of colonies of C1 and C3 is stopped on the second day of confrontation, that of F2 and F3 is stopped during the third day when one of *Botrytis* came into direct contact with either the TH or with colony colony TL. After 7 days of confrontation, the colonies of TH and TL are completely overlapped and covered the colonies of the parasite (*B. fabae* and *B. cinerea*).

Trichoderma is a fungus known for its mycoparasitism. Elad *et al.* (1983) [14] described the action of parasitic and *T. harzianum* and *T. hamatum* on *Rhizoctonia solani* and *Sclerotium rolfsii*. *Trichoderma* attacks its host by winding the mycelium around the host hyphae. Subsequently, the mycoparasite penetrates the host cells and uses the cytoplasmic contents.

TV by the strain has against mycelial growth very slow. It showed a different antagonist power, which is the ability to remotely stop parasite development with formation of an inhibition zone between colonies face whose varies with the *B/trytis* isolates (Fig. 3).

Table 1: ANOVA of *Trichoderma* species effect on mycelial growth of *Botrytis* isolates.

Confronted isolates	Mycelial growth (mm) (Mean ± SD)	Test F
Controled	39.81[a]	5093.2**
TV	26.93[b]	
TH	12.25[c]	
TL	11.87[c]	

Table 2: ANOVA of *Trichoderma viride* effect on sporulation of *Botrytis* isolates.

Confronted isolates	Mycelial growth (condia/cm^2) (Mean ± SD)	Test F
C1	28548[a]	5093.2**
C3	25572[b]	
F3	8248.75[c]	
F2	4353.5[d]	

Table 3: ANOVA of *Trichoderma* species effect by volatiles substances on mycelial growth of *Botrytis* isolates.

Confronted isolates	Mycelial growth (mm) (Mean ± SD)	Test F
Controled	71.46[a]	4644.33**
TV	51.90[b]	
TH	38.68[c]	
TL	32.90[d]	

** Highly significant effect at $P < 0.01$, SD : Standard deviation, TV : *Trichoderma viride*, TH : *T. harzianum*, TL : *T. longibachiatum*, a, b and c : Homogenate groups.

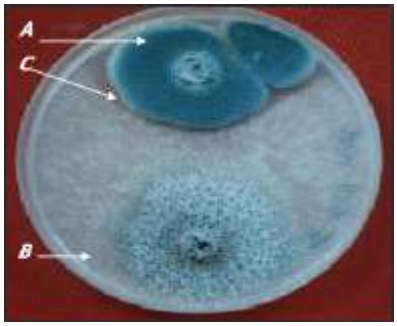

Fig. 3: Botrytis fabae confronted with T. viride: Zone of inhibition. A, Botrytis fabae ; B, T. viride; C, Zone of inhibition.

At the seventh day, the average radius of colonies of the parasite (C1, C3, F2, F3) faces the strain of TV is significantly lower than control colonies. About the behavior of *Trichoderma* strains, data showed that the average growth 39.81 mm is significatly different (5% level) facing lots of other TV, TL and TH. TH and TL strains which showed no significant difference between them.

Effect on sporulation: The effect of TH and TL on sporulation has not been studied because of the tangle of their colonies with colonies of the pathogen, making it difficult counting spores. As against the study of

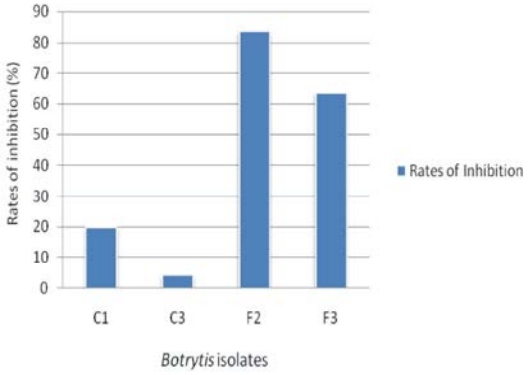

Fig. 4: Rates of sporulation inhibition of Botrytis isolates by Trichoderma species.

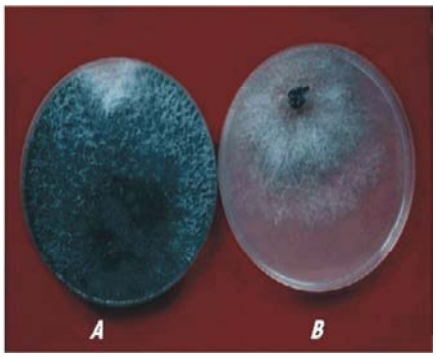

Fig. 5: Effect of T. harzianum on mycelial growth of isolate F2. (A: mycelium of T. harzianum colony covering the isolate F2, B: F2 (control)).

sporulation of *Botrytis* isolates on the edge of the zone of inhibition caused by TV showed a significant inhibition compared to control. The percentage of inhibition of sporulation in front of the mycelium ranged from 83.59, 63.61, 4.08 and 19.84% for the isolates F2, F3, C3, C1 (Fig. 4).

The reaction of *Botrytis* isolates revealed that each isolate reacts In significant differences of F2 which was the most likely because its average sporulation is only 4353.5 conidia / cm^2 and the isolate C1 being the most tolerant (28548 conidia / cm^2).

Indirect Confrontation

Mycelial Growth Effect: This technique allowed us to demonstrate the inhibitory effect even at a distance of three strains of *Trichoderma* into all isolates of *Botrytis*.

Indeed, after 5 days of incubation the average diameter of colonies of *Botrytis* in the presence of antagonists was significantly lower than the control (Table 3 ; Fig. 5).

The average diameter of colonies faced C1 TV, TH and TL respectively 64.5, 51.5 and 47.5 mm instead of 90 mm in the control, reflecting an inhibition of about 28, 47 and 43%.

Inhibition of growth of about 28, 51 and 43% for C3, 25, 60 and 52% for F2 and 26, 50 and 66% for F3, respectively facing TV, TH and TL.

The statistical analysis of the reaction of *Botrytis* isolates revealed a significant difference between the four isolates. C1 represents the isolate that is resistant to the best action of volatile substances (average diameter 63.37 mm), followed by C3 (62.59 mm), F2 (35.31 mm) and F3 (33.68 mm).

In the same vein, Camporota (1985) [8] showed the inhibitory effect of *T. viride* and *T. harzianum* against *Rhizoctonia solani* Kühn. This inhibition was more pronounced in the case of *T. viride*.

It appears that despite the absence of direct contact between *Trichoderma* spp and isolates of *Botrytis*, the first may have had an inhibitory activity on the development of colonies of *Botrytis fabae* and *B. cinerea*. This can be explained by the ability of *Trichoderma* to produce volatile substances that are able to limit and even stop the development of the pathogen.

Macroscopic observations made in the mycelium of the parasite in the presence of *Trichoderma* isolates revealed a significant transformation in strips of the mycelium of the pathogen.

These results confirm those obtained by Cherif and Benhamou (1990) [16] reported the presence of alterations due to the action of *T. harzianum*, despite the absence of direct contact between the two fungi.

The chemical nature of volatile substances is not yet determined. Dennis and Webster (1971) [17] presented a case involving acetaldehyde but they need 500 ppm of this compound for complete inhibition of their target organism. Hutchinson and Cowan (1972) [18] attribute the inhibition to the accumulation of carbon dioxyde and ethylen.

Effect on Sporulation: The action of volatile substances causes a significant inhibition of sporulation compared to untreated controls. TH and TL strains gave inhibition of sporulation close to 100%. This is normal since the inhibitory effect of these on the growing mycelium of the pathogen. As against the effect of TV on the inhibition of sporulation of C3 (56.18%) and C1 (66.02%), although it seems important, it is less relative to that of TH and TL (Fig. 6).

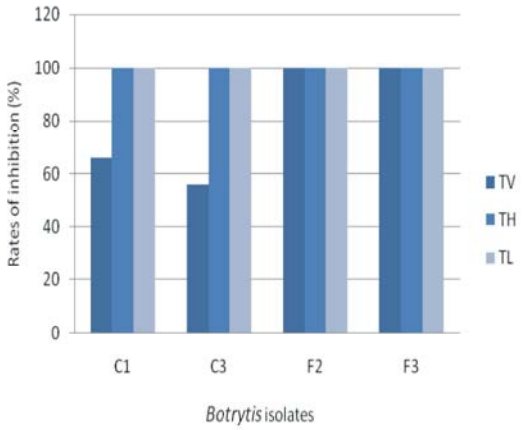

Fig. 6: Rates of sporulation inhibition of Botrytis isolates by volatiles substances of Trichoderma species.

The inhibitory action usually differs between TV and TV or between TH and TL, this difference could be interpreted by a difference in activity in the nature of inhibitory substances produced by these antagonists. On the other hand it is due to the quantity of substances produced by these microorganisms.

The obtained results in the present study showed that The three strains of *Trichoderma* inhibited effectively mycelial growth and sporulation of *Botrytis fabae* and *Botrytis cinerea*. The test of direct confrontation has highlighted the mycoparasm power TH and TL, as these have overlapped and covered the colonies of the parasite (*B. fabae* and *B. cinerea*) on which they sporulate. This is indicative of mycoparasitic power [19]. This phenomenon was described by Benhamou and Chet (1997) [20] during a test of confrontation between *T. harzianum* and soil fungus, *Pythium ultimum*. TV has a strain to different antagonist, which is the ability to remotely stop parasite development with formation of an inhibition zone between colonies face whose width varies with the species of *Botrytis*. These observations lead to think about the possibility of existence in a TV inhibitory action involving chemical substances.

Dennis and Webster (1971) [17] were able to isolate an antibiotic in *T. viride* (Trichodermin), but they did not rule out however the possibility of production in this species, due to other antibiotics.

Sporulation of *Botrytis* isolates on the edge of the zone of inhibition caused by TV showed a significant inhibition compared to control. This inhibitory action of sporulation is due to lysis of the mycelium and spores of the parasite. Indeed, during the confrontation experiments, a flattening of colonies of the parasite on the side of the antagonist. These observations suggested the possibility of secretion by the antagonistic substance which diffuses into the culture medium, which causes the lysis of the mycelium and spores of the pathogen. When testing the confrontation from a distance, *Trichoderma* strains have been even remotely effective in inhibiting the growth of two species of *Botrytis* [21, 22].

CONCLUSION

This study showed clearly the effect of the three antagonistic strains of *Trichoderma* on *Botrytis* isolates responsible for disease chocolate stain. Indeed, the confrontation test whether a direct way of culture medium or remotely showed inhibition of growth and sporulation of the pathogen. If There's contact between *T. harzianum* or *T. longibrachiatum* and the pathogen, *Trichoderma* colonies invade those of the parasite.

When the pathogen is confronted in a direct way of culture medium, *T. viride*, we noticed a remote breakdown of the mycelium and reduced sporulation on the edge of the zone of inhibition involving the secretion of antibiotic substances circulating in the culture medium.

The three strains of *Trichoderma* have therefore proved to be active against the pathogen through inhibiting its sporulation and mycelial growth.

Based on these results, it is of interest to use *Trichoderma* as a biocontrol agent against chocolate spot disease.

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