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Research Article
Antagonistic Interactions Between the Foliar Pathogen *Botrytis fabae* Sard. and *Trichoderma harzianum* Rifai

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Abstract

Background: In Egypt, chocolate spot disease caused by *Botrytis fabae* Sard. and *Botrytis sinerea* Pers. is the most serious disease affecting bean. *Trichoderma harzianum* Rifai is one of the most potent bioagents used for the control of many plant pathogens. This biocontrol agent has not harmful effects on humans, wild life and other beneficial organisms, safe and effective biocontrol agent in both natural and controlled environments that doesn't accumulate in the food chain. **Materials and Methods:** The antagonistic effect of *T. harzianum* against *B. fabae* was investigated on Potato Dextrose Agar (PDA) medium using dual culture technique. Also the antifungal activity of *T. harzianum* metabolites was also tested on the linear growth of *B. fabae* using cellophane method. Scanning electron microscopy was also used to investigate the mycoparasitic nature of *T. harzianum* on *B. fabae*. **Results:** An overgrowth of *T. harzianum* on *B. fabae* was observed, indicating the antagonistic behavior of *T. harzianum* against *B. fabae*. A complete reduction in the linear growth of *B. fabae* was observed indicating the antifungal activity of *T. harzianum* metabolites. By using the slide culture method, light microscopy observations showed an evidence about the mycoparasitic nature of the tested isolate of *T. harzianum* on *B. fabae*. Scanning electron microscopic observations confirmed the mycoparasitic nature of *T. harzianum* on *B. fabae*. **Conclusion:** The above results confirmed the mycoparasitic and aggressive nature of *T. harzianum* on *B. fabae*.

Key words: *Trichoderma harzianum*, *Botrytis fabae*, mycoparasitism, antagonistic interactions

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Egypt, chocolate spot disease caused by *Botrytis fabae* Sard and *Botrytis sinerea* Pers. is the most serious disease affecting bean[1]. It is the major disease affecting the crop causing losses ranging from minor to complete failure of crop. This disease is a limiting factor for faba bean production in the north and middle Egypt[2].

Trichoderma has considerable antagonistic activity against many phytopathogenic fungi[3-7]. In all cases *Trichoderma* over grow the phytopathogens and subsequently developed a conidial lawn over the surface, the inhibition of growth appears directly related to its ability to hydrolyze the cell walls of the tested microorganisms rather than through the inhibitory action of antibiotics or toxins[8].

The most common biological control agents of the *Trichoderma* genus are strains of *Trichoderma virens*, *T. viride* and above all *T. harzianum* which is a soil living fungus and has the ability to control diseases in the phyllosphere. *Trichoderma harzianum* is one of the most potent bioagents used for the control of many plant pathogens[9]. This biocontrol agent has not harmful effects on humans, wild life and other beneficial organisms, safe and effective biocontrol agent in both natural and controlled environments that doesn't accumulate in the food chain and to which it has not been described resistance[10].

Morris and Lane[11] found that growth of *Botrytis fabae* was inhibited by antibiotics produced by culture filtrates of *Trichoderma*. The present study aimed at studding the antagonistic activity and the mechanism of antagonism of *T. harzianum* against *B. fabae* in an attempt to use it as a biocontrol agent for chocolate spot disease of faba bean.

MATERIALS AND METHODS

Isolation, purification and identification of the pathogen: The diseased leaves of faba bean plants were collected from different sites of Mansoura districts in Dakahlia governorate, Egypt, namely Sinbellawen, Aga, Talkha, Temay El-Amded, Mansoura and Bany Ebeed by sterile water. The leaves were cutted into small pieces (1-2 cm^2), disinfected in 0.01% $HgCl_2$ for 1-3 min for surface sterilization. Surface sterilized pieces were then rinsed in sterilized water several times to remove the remaining disinfectant solution, dried on sterilized filter papers. Using sterilized forceps, the dried pieces were then transferred into petri-dishes containing Potato Dextrose Agar (PDA) medium supplemented with 200 ppm streptomycin sulphate to inhibit bacterial contamination. The dishes were then incubated at 24-26°C for 7-10 days and checked for microbial growth two days after inoculation. Purification of the resulting isolates was done by using single spore technique to obtain them in pure cultures[12].

Pure cultures of the isolated fungus were identified according to the cultural characteristics, morphological and microscopic features (mycelial development and spore formation) described by Ellis[13]. Stock cultures were stored on PDA medium under sterile mineral oil at 4°C.

Pathogenicity test: The different *Botrytis fabae* isolates were grown on PDA medium and incubated at 20°C for 12 days[14] under light regime of UV and normal fluorescent (12 h/12 h) to enhance sporulation. Conidia were harvested and transferred to 10 mL of sterilized water for each plate. Conidial concentration was estimated using a haemocytometer and adjusted to about 5×10^4 spores mL^{-1}.

Giza 40 seeds of faba bean were sown in plastic pots (35 cm in diameter) containing 12-13 kg clay: sand (3:1, v/v) soil. Five seeds were sown in each pot, plants were left to grow and watered when necessary. After 45 days, the intact plants were sprayed with the spore suspension of the pathogen and covered with polyethylene bags to maintain enough humidity around the plants. Control plants were sprayed with sterilized water. All pots were kept in a glass house under natural conditions. Disease assessment was recorded after 10 days of microbial spraying.

The Disease Severity (DS) was recorded according to the disease index which based on the standard scale of Gondran[15] as follows: (1) Healthy plants, (2) Small spots, (3) Increasing spots number and spreading, (4) Coalesce spots together and about ¼ of leaf surface is necrotic, (5) Half of the leaf surface is necrotic and (6) Leaves die and fall.

Antagonistic effect of *Trichoderma harzianum* against *Botrytis fabae* (dual culture test): The isolate of *Trichoderma harzianum* which is used through the present study was obtained from the Department of Biological Control, Institute of Plant Pathology, Giza, Egypt.

The antagonistic effect of *Trichoderma harzianum* was investigated using dual culture technique[16]. Six millimeter diameter discs taken from the growing edge of 5 days old culture of *B. fabae* were placed 10 mm from the edge of each 90 mm PDA plate. Six millimeter diameter discs taken from the growing edge of 4 days old culture of *T. harzianum* were also placed 10 mm from the edge of the plate oppositely to *B. fabae*. A control PDA plates inoculated with 6 mm diameter discs of *B. fabae* or *T. harzianum* were used. All plates were incubated at 26±2°C under alternating

luminosity (12 h light/12 h darkness) for 8 days. The inward linear growth (distance between the center of the disc and the edge of the colony) was measured after 2, 4, 6 and 8 days and the interaction between the two fungi was recorded either in the form of inhibition zone or overgrowth of *T. harzianum* on *B. fabae*[17].

Antifungal activity of non-volatile metabolites of *Trichoderma harzianum* against *B. fabae*: The effect of non-volatile metabolites from *Trichoderma* sp., against *B. fabae* was tested by the method described by Kucuk and Kivanc[18]. Initially, mycelial agar plugs (6 mm diameter) taken from the edge of a young culture of *T. harzianum* were transferred to the center of petri-dishes (90 mm diameter) containing 20 mL PDA sterilized medium covered by a sterilized cellophane membrane. The plates were incubated at 25°C for 72 h. Then the cellophane membrane covered with *Trichoderma* mycelial growth was removed before the growth reaches the edges of the plates. On the same media a disc of 6 mm diameter taken from the growing edge of *B. fabae* culture 5 days old was placed. Then the plates were incubated at 22°C for a further 6 days. Control plates of *B. fabae* growing on PDA medium were used for comparison. Five replicates (plates) were used for each treatment. The mean diameter of *B. fabae* was measured and the percentage of growth inhibition due to the non-volatile metabolites from *Trichoderma* was calculated.

Preparation of specimen for Scanning Electron Microscopy (SEM): The PDA plate was inoculated at a constant distance from the edge of the petri dish with a mycelial disc (5 mm) cut from the leading edge of both colonies of *T. harzianum* and *B. fabae*. Both of the two fungi grew toward each other and their hyphae intermingled. After 6 days of incubation, the plate cultures were observed under a light microscope to verify the early stage of interaction. The interaction sites were marked and agar blocks of 1 cm^2 were removed for SEM preparation. Sample preparation was performed using the tissue processor model Lynexel, Leica. Where the mycelial samples from the interaction region were fixed with osmium oxide and then dehydrated using serial dilution of ethyl alcohol then finally acetone. The processed samples were then dried using a critical point drier (EMS 850), coated with gold using sputter coater (EMS 550) then the samples were examined using a SEM (JEOL100CX-ASID-4D)[19].

RESULTS

Pathogenicity test: The results illustrated in Table 1 indicated that all *B. fabae* isolates have the potency to cause faba bean chocolate spot disease, where disease incidence ranged from 52-70% and disease severity ranged from 2-5 but *B. fabae* isolated from Sinbellawen (B1) recorded the highest values of both disease incidence (70%) and disease severity (5).

Antagonistic effect of *Trichoderma harzianum* against *B. fabae* on solid medium (dual culture test): Data in Table 2 showed that in single culture plates, the linear growth of both *B. fabae* and *T. harzianum* increased by the time after inoculation. It was noticed that, the linear growth of *T. harzianum* was more rapid than that of *B. fabae*. In dual culture plates, a great inhibition to the growth of *B. fabae* was occurred after 6 days in comparison with control. Six days after inoculation, it was observed that *T. harzianum* overgrow *B. fabae* (Fig. 1) indicating the antagonistic behavior of *T. harzianum* against *B. fabae*.

Antifungal activity of the non-volatile metabolites of *Trichoderma harzianum* against *B. fabae:* A complete inhibition of the mycelial growth of *B. fabae* (except the growth on the disc itself) was observed compared with the control (Fig. 2), which indicated the antifungal activity of *T. harzianum* metabolites.

Table 1: Pathogenicity test for the different isolates of *Botrytis fabae*

Isolates	Location	Disease incidence (%)	Disease severity**
B1	Sinbellawen	70*	5
B2	Aga	66	4
B3	Temay El-Amded	69	4
B4	Mansoura	56	2
B5	Talkha	53	2
B6	Bany Ebeed	52	3

*Each value represents the mean of 3 replicates, **2: Small spots, 3: Increase in spots number and its spread, 4: Coalesce of spot together and 1/4 of leaf surface is necrotic and 5: 1/2 of leaf surface is necrotic

Table 2: Antagonistic effect of *Trichoderma harzianum* against *Botrytis fabae* (dual culture test)

	Inward linear growth* (cm) (Days)			
Treatments	2	4	6	8
Botrytis fabae	2.70**	4.10	4.50	4.90
Trichoderma harzianum	3.15	7.10	7.50	7.85
Botrytis fabae +	2.30	2.25	2.45	2.60
Trichoderma harzianum	3.10	4.35	5.10	7.55

*Distance between the disc's center and the margin of the colony, **Each value represents the mean of 5 replicates

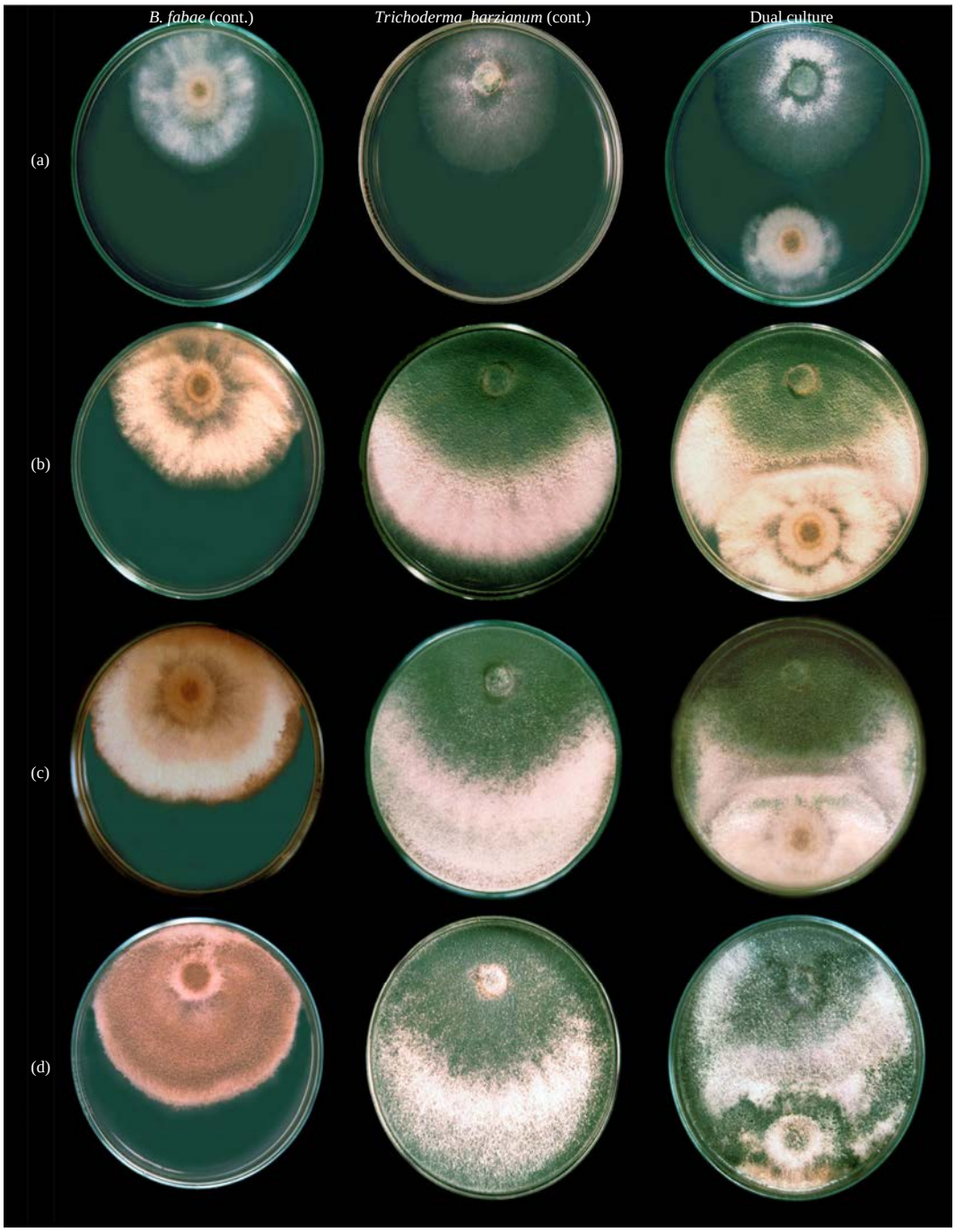

Fig. 1(a-d): Antagonistic effect of *Trichoderma harzianum* against *B. fabae* on solid medium after (a) 2 days (b) 4 days (c), 6 days and (d) 8 days

Scanning electron microscopy observations on the mycoparasitic nature of *Trichoderma harzianum* on *B. fabae*: Mycelial samples from the interaction region of dual culture of *B. fabae* and *T. harzianum* were observed in a scanning electron microscope, the events of mycoparasitism mechanism are shown in the scanning electron micrographs (Fig. 3). Hyphae of *T. harzianum* frequently grew parallel to the hyphae of the host (*B. fabae*) and sticks itself onto it's surface (Fig. 3a), this process followed by rapid and excessive coiling and formation of appressoria-like structures on the

Fig. 2: Antifungal activity of the non-volatile metabolites of *Trichoderma* against *B. fabae*

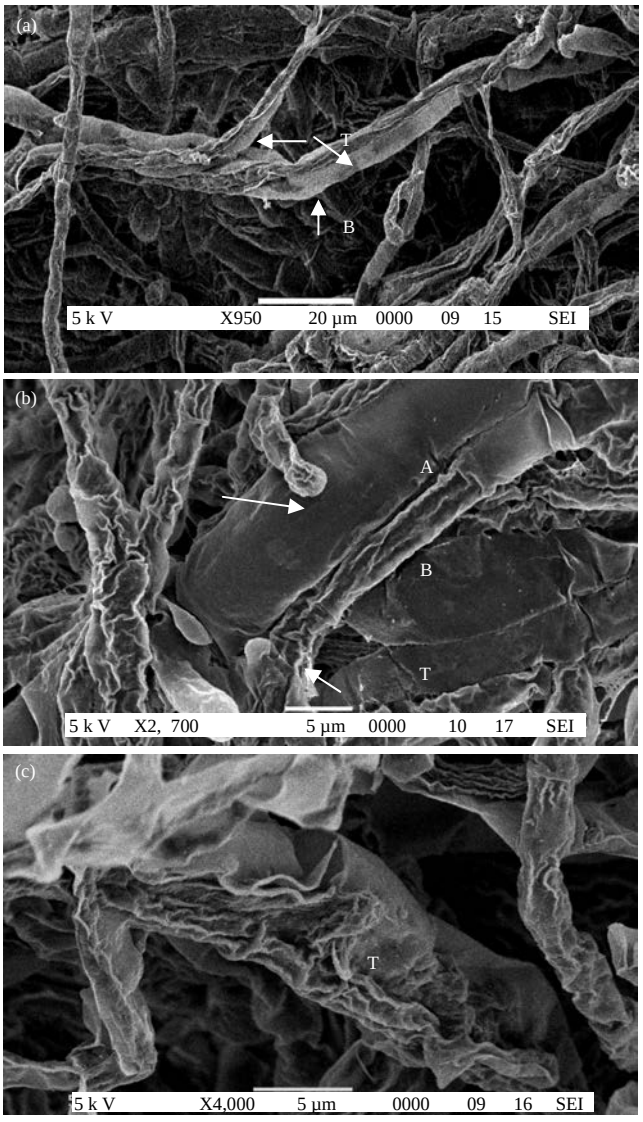

Fig. 3(a-c): Scanning electron micrograph showing hyphae of *B. fabae* 'B' hyphae of *Trichoderma harzianum* 'T' and appressoria like structures, 'A'. (a) *Trichoderma harzianum* attach to the host and coil around it, (b) Form appressoria on the host surface and (c) Finally the host cell wall lysed

host surface (Fig. 3b) and finally lysis of host cell walls was also observed (Fig. 3c), these observations indicated the mycoparasitic nature of *T. harzianum* on *B. fabae.*

DISCUSSION

Since the chemical pesticides are the most commonly known methods for the management of fungal diseases in fields and greenhouses, some considerable problems threaten to limit the continued use of them. Firstly, some fungi develop resistance to a number of chemicals; secondly, some synthetic fungicides are not readily biodegradable and tend to persist for many years in the environment. The toxic effects of the fungicides on the non-target organisms may also have undesirable changes in the environment[20]. Because of these associated problems, scientists are trying to test environmentally safe alternative means for the disease control, including the biological control methods[21].

So that, this study was planned to replace the undesirable and unsafe chemical control by another effective, inexpensive and safe options for faba bean chocolate spot disease control. *Trichoderma* spp., specially *T. harzianum* were suggested to be used as a biocontrol agents for plant pathogens of faba bean plants[18,22].

The antagonistic effect of *T. harzianum* against *B. fabae* on solid medium was investigated using dual culture technique and it was observed that, the linear grow th of *T. harzianum* was more rapid than that of *B. fabae*. A great inhibition to the growth of *B. fabae* was clearly observed. An overgrowth of *T. harzianum* on *B. fabae* was observed 6 days after inoculation and dots of conidia are seen on the surface of the phytopathogen. The antagonistic mode of action of the fungus and the ability of conidia to germinate on phytopathogen's surface has been proposed for the production of antibiotics[9] and the fact that, they are already possess a set of cell wall degrading enzymes such as chitinases, glucanases and proteases[23].

The antifungal activity of *T. harzianum* metabolites was also tested against the linear growth of *B. fabae* using the cellophane method. A complete inhibition of the growth of *B. fabae* was observed indicating the antifungal activity of *T. harzianum* metabolites, these results were in agreement with the findings of Bennet and Lane[24] and Doi and Mori[25]. This inhibition can be explained according to the generally accepted definition of antibiosis "The mechanism mediated by specific metabolites such as antibiotics, enzymes and non-volatile compounds[26]". Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonized microorganisms among these metabolites, the production of harzianic acid, tricholin, peptaibols, viridian, heptelidic acid and antibiotics[27]. The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanisms alone[28].

In the present study the mycoparasitic nature of *T. harzianum* on *B. fabae* was also investigated by the light microscope using slide culture method. It was observed that the two fungi grew toward each other and their hyphae intermingled with slight mycoparasitic behavior. Light microscopic observations revealed that there was evidence about the mycoparasitic nature of the tested isolate of *T. harzianum* on *B. fabae.* In this connection, the scanning electron microscopy observations confirmed that mycoparasitism appeared to contribute to the aggressive nature of the tested isolate of *T. harzianum* against *B. fabae*. These results are in good agreement with Monte and Liobell[29] reported that most isolates of the genus *Trichoderma* were found to act as mycoparasites of many economically important aerial and soil borne plant pathogens. Also, these observations were in agreement with that of Labudova and Gogorova[8] and Harman[9], in these studies the lytic action of the pathogen was clearly appearant and the inhibition of the growth appears directly related to the ability of *T. harzianum* to hydrolyze the cell walls of the tested microorganisms rather than through inhibitory action of the antibiotics or toxins, their results revealed that, the nature of *T. harzianum* antagonism is based on mycoparasitism (lysis) and appeared to optimize with contact between the mycelia.

In contrast, Elad *et al.*[23] found that, the mycoparasite *T. harzianum* was unable to degrade the cell wall of *Fusarium oxysporum* and that these hydrolases of *Trichoderma* were actively involved in the microbiological control. Also, Rashad[30] showed that there was no evidence about the mycoparasitic nature of *T. harzianum* on *Bipolaris orayzae*, this mean that the mycoparasitism phenomenon may be host specific. In all experiments dealing with *Trichoderma* spp., specificity of antagonist for a range of host fungi has been reported.

CONCLUSION

The above results indicated the antagonistic action of *T. harzianum* against *B. fabae*. Moreover, the antifungal activity of *T. harzianum* metabolites was also indicated. Light microscopic observations revealed that there was evidence

about the mycoparasitic nature of the tested isolate of *T. harzianum* on *B. fabae.* In this connection, the scanning electron microscopy observations confirmed that mycoparasitism appeared to contribute to the aggressive nature of the tested isolate of *T. harzianum* against *B. fabae*. These results strongly recommend using the save biological control agent *T. harzianum* as an alternative to the harmful chemical fungisides to manage the phytopathogen *B. fabae.*

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