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MODE OF PARASITISM OF THE ANTAGONIST TRICHODERMA VIRIDE TOWARDS THE PHYTOPATHOGEN ALTERNARIA SOLANI

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ABSTRACT

In a previous work, the optimized crude chitinase of *Trichoderma viride* exhibited strong inhibitory activity against food spoilage moulds (phytopathogens) *Alternaria solani*, *Botrytis cinerea*, *Penicillium oxalicum* and *P. italicum*, with the first species being the most affected. Currently, on testing the antagonistic activity of *T. viride* against the previous phytopathogens, the antagonist exhibited a strong activity particularly towards A. *solani*. The antagonist displayed more than one mechanism of parasitism on *A. solani* including penetration of the pathogen hyphae, abnormal pathogen hyphae swelling, lack of conidia formation, conidia malformation as well as degradation of the pathogen cell wall chitin through secretion of chitinase enzyme which is a major cause of antagonism. Fortunately, the present chitinase secreted by *Trichoderma viride* is found to be a constitutive enzyme. The previously optimized enzyme showed also a powerful insecticidal activity against home insects cockroach, spider and ant where the exoskeleton was softened and hydrolyzed, followed by rapid killing of the insects within 12 minutes.

Contribution/ Originality: This study is one of very few studies which have investigated the in vitro mode of parasitism of Trichoderma viride on the food spoilage mold A. solani which exhibited more than one mechanism including hyphae penetration, abnormal hyphae swelling, conidia malformation and lysis of the cell wall chitin.

1. INTRODUCTION

A wide range of microrganisms including bacteria, fungi, actinomycetes and yeasts can produce chitinase enzyme [1-4]. Chitinases are hydrolytic enzymes that cause degradation of β-1-4-glucoside bond of chitin to *N*-acetyl D-glycosamine [5]. Chitin biodegradation is involved in fungal cell differentiation, nematode egg hatching, arthropods morphogenesis and in controlling pests containing chitin [6] like insects [7] and phytopathogenic fungi [8].

Trichoderma species secrete a number of hydrolytic enzymes, including chitinases, which degrade the chitin containing cell walls of other fungi [9] rendering this fungus to be used extensively as a biocontrol agent and effective antagonist for a wide range of plant pathogenic fungi [10, 11].

The antagonistic activity and the mode of parasitism of the antagonist towards the pathogen includes more than one mechanism comprising adhering then penetration to the host hyphae, competition for nutrients , secretion of lytic enzymes and others [12, 13].

This study was focused on the antagonistic and inhibitory activity of *Trichoderma viride* against some phytopathogenic fungi and some insects, respectively. The work was extended to elucidate the mode of parasitism of the antagonist on the most affected pathogen.

2. MATERIALS AND METHODS

2.1. The Antagonist and the Phytopathogens

In a previous work, the most abundant and the highly chitinase producer (among eight species) *Trichoderma viride* was isolated from soil sample on agar plates containing crude shrimp shell chitin. The previously optimized crude chitinase secreted by the antagonist *T. viride* exerted a strong inhibitory activity against the pathogens *Alternaria solani*, *Botrytis cinerea*, *Penicillium oxalicum* and *P. italicum* (isolated from some spoiled vegetables) using cup plate method, where the first species being the most affected. The optimized production medium (pH 4) contained (g%) 1.5 colloidal chitin, 0.25 peptone, KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05, KCl, 0.05 and 100 µg/ml $FeSO_4$ [14].

2.2. Antagonistic Activity of *Trichoderma Viride* against the Tested Phytopathogens

A disc (10 mm diameter) of the antagonistic *T. viride* obtained from the edge of 6 days old optimized culture were located at one side of plates (triplicate) containing Czapek-Dox's agar. The plates were incubated at 28 °C for 24 hrs. After that, a disc (10 mm diameter) of the phytopathogenic *Alternaria solani* (10 days old), *Botrytis cinerea*, *Pencillium oxalicum* and *P. italicum* (6 days old) were placed separately on the previous agar plates and at the opposite side of *T. viride* disc. The plates were incubated at 28° C for 6 days. At the end of the incubation period, the growth of the phytopathogens compared to the antagonist growth was visually observed and the plates were photographed using canon camera (CP780 IXUS). Since *A. solani* was the most sensitive to *T. viride*, it was chosen for the next experiments.

2.3. Mode of Parasitism of *Trichoderma viride* on *Alternaria Solani*

A disc (10 mm diameter) of *T. viride* (6 days old) and a disc of *A. solani* (10 days old) were placed simultaneously in flasks containing 50 ml sterilized basal mineral salts medium (g %: $NaNO_3$, 0.2; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05 and KCl, 0.05) without any carbon source then incubated at 28°C for 7 days. The mode of parasitism of *T.viride* and conidia formation by *A. solani* were observed using a light microscope (Leica DMLS) provided with a camera. *A. solani* grown in Czapek-Dox's medium was used as control.

2.4. Lytic Activity of the Antagonistic *Trichoderma viride* on *Alternaria solani* Mycelia

One gram of *A. solani* fresh mycelia (grown on liquid Czapek-Dox's medium for 10 days at 28°C) and a disc (10 mm diameter) of *T. viride* (grown on Czapek-Dox's medium for 6 days at 28°C) were placed aseptically together into flasks containing 50 ml sterile basal mineral salts medium. The flasks were incubated under shaking (120 rpm) at 28 °C for 6 days and the medium was filtered. The lytic activity of the antagonist towards the pathogen cell wall chitin was measured in terms of the activity of the produced chitinase using clear zone technique. 100 µl of the filtrate was introduced into wells made on plates (triplicate) holding assay medium for chitinase. The medium contained 0.2 % colloidal chitin and 1.5% agar in phosphate buffer (0.05 M, pH 5.2). The plates were incubated at 28 °C for 48 hrs. The produced clear zone was measured (mm) and taken as a criterion for lytic activity of chitinase. Culture filtrate of *T. viride* grown for 6 days at 28 °C on the optimized production medium (containing colloidal chitin) or on Czapek-Dox's medium containing sucrose (free of any chitin) were used for comparison.

2.5. Insecticidal Activity of *Trichoderma Viride* Crude Chitinase on Some House Insects

One ml of the previously prepared optimized crude chitinase [14] with 50 µl Tween 80 was sprayed on the surface of some house insects (cockroach, ant and spider). The insects were left at room temperature in beakers covered with a piece of perforated cloth. The lytic activity of chitinase on the insect exoskeleton was observed. The insects were photographed using canon camera (CP780 IXUS). Control insects were treated with distilled water and Tween 80 replacing the enzyme.

3. RESULTS AND DISCUSSION

Biological control of plant diseases can occur through different mechanisms, which are generally classified as antibiosis, competition, suppression, direct parasitism, induced resistance and others. The antagonistic activity has often been associated with production of secondary metabolites or the use of beneficial microorganisms, such as specialized fungi or yeast or bacteria to attack and control the plant pathogens [15]. commercially produced *Trichoderma harzianum* is an efficient biocontrol agent that prevent development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds [16].

The present results clearly showed prominent growth suppression of the phytopathogens *Alternaria solani*, *Botrytis cinerea*, *Pencillium oxalicum* and *P. italicum* in presence of the antagonist *T. viride* (Figure1$_{a-d}$). The antagonist *T. viride* grows quickly in all Petri plates leaving a small space for growth of the pathogens. The least colonized pathogens were *A. solani* followed by *Botrytis cinerea* and *P. italicum* but *P. oxalicum* was slightly affected.

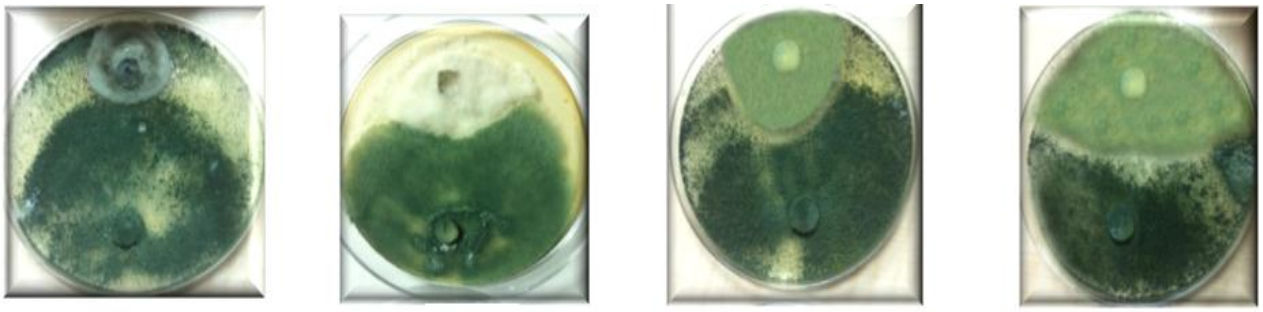


Figure-1.a-d. Antagonistic activity of *Trichoderma viride twards* some phytopathogens (a):*Alternaria solani.* (b): Botrytis cinerea. (c): *Penicillium italicum* (d): *Penicillium oxalicum.*
Source: a live photo was taken for the plates using canon camera.

In this connection, the rapid growth of the antagonist *T. viride* in Petri plates might be due to its heavy sporulation ability and/or to competition for nutrients which together give a chance to occupy more growth area leaving little for the pathogens [17]. Such observations is an indicator for a biocontrol agent having a strong antagonistic activity.

Recently, Narasimha, et al. [18] tested the antagonistic activity of *T. koningii*, *T. flavofuscum*, *T. harzianum*, *T. asperellum and T. viride* against virulent strains of *Ralstonia solanacearum* where the highest activity was observed by *T. asperellum.* More recently, Schöneberg, et al. [10] found that 10 Trichoderma strains significantly antagonized and reduced the colony area of *Fusarium graminearum* and *F. crookwellense* by 45-93%.

In our study, the mode of parasitism of the antagonist *T. viride* towards the phytopathogen *A. solani* involved more than one mechanism. One of these mechanisms is that the antagonist became contacted, adhered then finally penetrated the host hyphae (Figure 2$_{a, b}$). Penetration seems to occur mechanically and/or through secretion of cell wall degrading chitinase [12].

The second mechanism is induction of abnormal hyphae swelling of the pathogen (*A. solani)* under antagonist stress (Figure 2b). Similar observation was detected by Sandhya, et al. [19] when studied antagonism between *T. harzianum* and the pathogen *Colletotrichum gloeosporioides.*

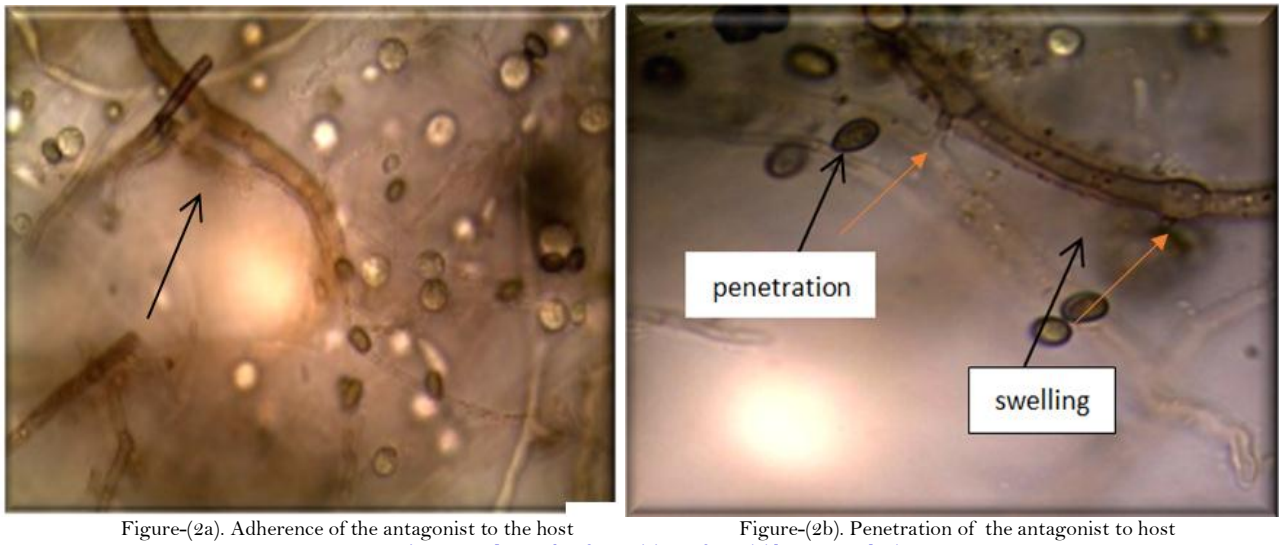


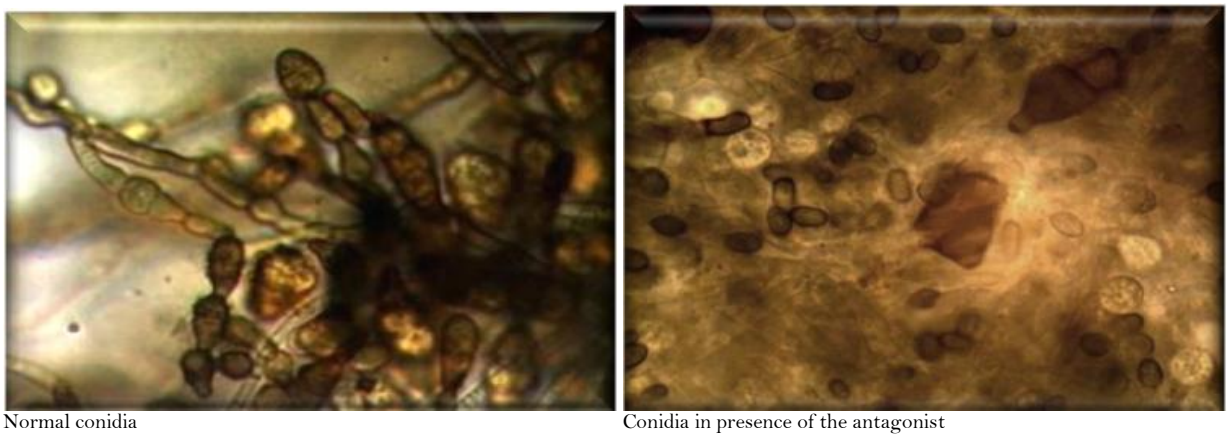
Figure-(2a). Adherence of the antagonist to the host

Figure-(2b). Penetration of the antagonist to host

Figure-2a-b. Mode of parasitism of *T. viride* on *A. solani.*

Source: A live photo was taken under microscope

The third mechanism is the lack of conidiogenesis and malformation of the formed conidia of *A. solani.* Conidia malformation appeared as absence of the transverse and longitudinal septa, reduction in conidia size and weakly attachment to the conidiophores as they were seen scattered not arranged in chains (Figure3). Amazingly, the lack in conidia formation and conidia malformation by *A. solani* might be due to the fact that the antagonist *T. viride* can compete and utilize the medium nutrients more rapidly than the pathogen (*A. solani*) which is reflected on inhibition of growth and conidial formation [12].



Normal conidia

Conidia in presence of the antagonist

Figure-3. Malformation *of A. solani* conidia in presence of the antagonist

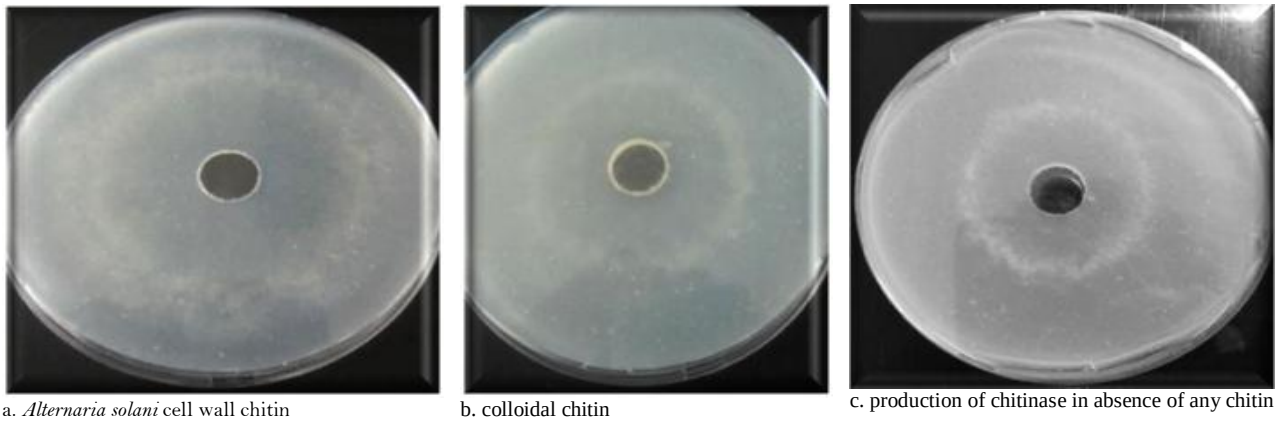
Source: A live photo was taken under microscope

The fourth mechanism of antagonism relied on the fact that the present *T. viride* could degraded *A. solani* mycelia through induction of chitinase enzyme (Figure 4a). The degradation power (clear zone diameter, 57 mm, data not presented) is much more than that achieved for the colloidal chitin (Figure 4b, clear zone diameter, 43 mm, data not presented)). Degradation of *Thielaviopsis paradoxa* hyphae by *T. longibrachiatum* [20] and that of *Sclerotium rolfsii* by *Trichoderma* sp. [9] was reported.

In this regard, chitinases produced by biocontrol agents are responsible for suppression of the fungal phytopathogens through degradation of cell wall chitin and in that way destroying cell identity [21]. This concept

is well observed with our potent *T. viride* which could produce chitinase even in absence of any chitin substrate i.e. constitutive enzyme (Figure3c) that supported its antagonistic activity and its ability to be a significant biocontrol agent. Similar findings were observed by Homthong, et al. [22].

In addition, [17] elucidated that *Trichoderma* achieves its biocontrol activity by means of competition, parasitism and production of enzymes. *Trichoderma* spp. are used as a strong fungal antagonist to control plant diseases [11].



a. *Alternaria solani* cell wall chitin b. colloidal chitin c. production of chitinase in absence of any chitin

Figure-4a-c. Production and degradation power of Trichoderma viride chitinase in presence of different chitin sources measured in terms of clear zone technique

Source: a live photo was taken for the plates using canon camera

In the current work, the observed dissolution of the exoskeleton followed by the amazing rapid killing of the tested home insects, cockroach, ant and spider, within 12 min. after spraying with our chitinase suggested its promising use as a powerful clean insecticide (Figure 5 a-f). Utilization of chitinolytic enzyme as bio pesticides agents for controlling insects and pests was reported [23].

In this Concern, the application of biological approaches as pesticides, insecticides included, is preferred since it is clean, non-pollutant and cheap replacing the traditional chemical ones which can be persistent in the environment with high concentrations, leading to major pollution problems and health risk [11, 24].

a. Cockroach (control). b. Cockroach treated with c. Ant (control). D .Ant treated with enzyme. e. Spider (control). f. Spider treated with enzyme.

Figure-5.a-f. Effect of crude chitinase on some insects

Source: live photo was taken for the insects using canon camera

4. CONCLUSION

The findings from the current study suggest the urged use of our ***Trichoderma viride*** to control phytopathogens and home insects. Such biological control is risk-free provides an eco-friendly safe, cheap and clean approach to overcome the pollution problems and hazards caused by traditional chemical ones.

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