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PRODUCTION AND PURIFICATION OF CHITINASE BY *TRICHODERMA HARZIANUM* FOR CONTROL OF *SCLEROTIUM ROLFSII*

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ABSTRACT

Trichoderma species are potent biological control agents against several plant pathogens. In the present study twenty isolates of *Trichoderma* were isolated and screened for their antagonistic activity against *Sclerotium rolfsii.* All the isolates were able to inhibit the *S.rolfsii* in vitro, but T_7, T_9, T_{10}, T_{13} and T_{18} were superior antagonists compared to other isolates of *Trichoderma*. T_{10} isolate is used for the production and purification of chitinase, due to their high enzymatic activity when screened in the medium containing chitin as sole a carbon source. T10 isolate produced the chitinases with molecular mass of 74 kDa (N-acetylglucosaminidase) and 32 kDa (endochitinase).

KEYWORDS: Antagonism, Chitin, Chitinases, *Sclerotium rolfsii*, Stem Rot, *Trichoderma*

INTRODUCTION

The fungi from the genus *Trichoderma* as biocontrol agents is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanism. Different mechanisms have been suggested as being responsible for their biocontrol which include mycoparasitism, antibiosis, competition for nutrients and space. It plays an important role in antagonism by secretion of different hydrolytic enzymes like glucanases, chitinases, cellulases and proteases (2, 7, 19).

Chitin, a homopolymer of β-(1, 4)-N-acetylglucosamine (GlcNAc) is the second most abundant source of nutrients and energy after cellulose (25, 3, 26, 18). It is widely distributed in the nature as the integuments of insects and crustaceans and as a component of fungi and algae (1, 9). Chitinases are a group of enzymes that decompose chitin into a variety of products that include the deacylated oligomer chitosan, the disaccharide chitobiose and the monomer N-acetyl glucosamine (3, 23, 10).The present study is aimed for screening of different *Trichoderma* species to select potent isolate, production and purification of chitinase followed by determination of its molecular weight.

MATERIALS AND METHODS

Isolation of Pathogen

The culture of *Sclerotium rolfsii* was isolated from the plants showing stem rot or southern blight symptoms of groundnut from the area of Sadhanavaripalem, Chittoor District in Kharif season and grown on potato dextrose agar (PDA) medium. The pure culture of the fungus was obtained and maintained on PDA for further study. The stock culture was maintained on PDA slants in a refrigerator and subcultured every two months.

Isolation of *Trichoderma* Species

Twenty *Trichoderma* isolates were isolated from the rhizospheric soil of groundnut fields from different areas of Chittoor District by serial dilution plate technique using modified *Trichoderma* specific medium. The isolates were maintained on PDA slants at 4^0C for further use.

Screening of *Trichoderma* Species for their Antagonistic Activity against *S.rolfsii*

The antagonistic activity against the *S. rolfsii* was tested by dual culture method on PDA medium (5). A 3 mm disc of actively growing test pathogen was placed at one end of the petri plate over the PDA medium. Similarly antagonist disc was also placed just opposite to the test pathogen i.e., at an angle of 180^0. The test pathogen without an antagonist serves as a control. For each treatment three replicates were maintained along with the control. All the plates were inoculated at room temperature for 5 days. Plates were observed at 24 hr intervals for the antagonism of the antagonists.

The per cent of reduction in serial growth and sclerotial production of the test pathogen was calculated using the following formula. The effective antagonists were selected and used for further studies.

$$I = \frac{C - T}{C} \times 100$$

Where

I = per cent reduction in growth of *S.rolfsii*

C = Radial growth (mm) in control

T = Radial growth (mm) in treatment

The effect of *Trichoderma* species on sclerotial number, sclerotial size and germination of sclerotia were also studied in dual culture plates.

Production of Chitinases

Preparation of Colloidal Chitin

The colloidal chitin was prepared by following the method of Kang *et al* (22). Chitin (20 g) from crab shell (Sigma Co, Practical Grade) was dissolved in 200 ml of concentrated HCl with stirring for 3 min at 40^0C. The chitin was precipitated as a colloidal suspension by slowly adding water (2 L) adjusted to 5^0C. Colloidal suspensions were collected by filtering through coarse filter paper, and then the filtered colloidal suspension was washed with tap water until the pH of the suspension was about 4.0.

Screening for Chitinase Production

The potent *Trichoderma* isolates (T_7, T_9, T_{10}, T_{13} & T18) selected by dual culture method were screened for the production of chitinase enzyme. The composition of the chitinase detection medium ($g/^{-1}$) is as follows: chitin, 4.5; $MgSO_4$. $7H_2O$, 0.3; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 2.0; Citric acid, 1.0; Bromo cresol purple, 0.15; Agar, 15.0; Tween 80, 200µl; pH 4.7. The 3 day old culture of above 5 isolates inoculated at the centre of the medium individually, incubated at room temperature and observed for purple color zone around the colony.

Production of Chitinase

For enzyme production, 3 mm mycelial disc of T_{10} isolate was inoculated in 250 ml of liquid culture medium (0.1% bactopeptone; 0.03% urea; 0.2% KH_2SO_4; 0.14% $(NH_4)_2SO_4$; 0.03% $MgSO_4.7H_2O$; 0.03% $CaCl_2.6H_2O$; 1 ml of trace elements solution ($Fe^{2+,}$ Mn^{2+}, $Zn^{2+,}$ and Co^{2+}), 0.02% glucose, pH 5.5 containing colloidal chitin (0.5%) and incubated for 72h at 28^0C with agitation speed of 110 rpm. The culture supernatants were separated from the mycelium by filtration through filter paper, and used for enzyme assay and /or enzyme purification as described in the section below.

Chitinase Assay

Chitinase activity was assayed using the colorimetric method described by Molano *et al* (15) with minor modifications (22). The assay mixture contained 1 ml of 0.5% regenerated chitin (suspended in 50 mM acetate buffer pH 5.2) and 1 ml of enzyme solution. The reaction mixture was incubated for a minimum of 6 h at 37^0C with shaking, and was stopped by centrifugation (5,000 rpm^{-1}) for 10 min and the addition of 1 ml of dinitrosalicylate reagent ([14). The amount of reducing sugar produced was estimated using a reference curve constructed with N-Acetylglucosamine as the standard. One unit of enzyme activity (U) corresponds to the amount of protein necessary to release one µ mol of GlcNAc equivalent in 1 h at 37^0C.

Purification of Chitinases

To the supernatant of T_{10} isolate obtained as described above, $(NH_4)_2SO_4$ was added to a concentration of 40%. The mixtures were kept for 15 min at 4^0C under gentle agitation. Suspensions were centrifuged at 28,800 g for 30 min, and the pellets were discarded. Ammonium sulphate was then added to supernatants for a final concentration of 60%. After 15 min at 4^0C under gentle agitation, the supernatants were centrifuged as above, and the resulting pellets is resuspended in a small volume of 50 mM sodium acetate pH 5.0 which acts as crude enzyme preparation. Samples of crude enzyme were then injected into a DEAE-Sepharose chromatography column, equilibrated and eluted at a flow rate of 63 ml h^{-1} with 50 mM sodium phosphate buffer, pH 6.5, and 150 ml of the same buffer containing 1.2M NaCl. Fractions containing chitinase activity were pooled, dialyzed, concentrated by lyophilization and stored at -20^0C.

SDS-PAGE Analysis

Chitinase samples were subjected to SDS-PAGE analysis according to the method of Laemmli (12) in 12% acrylamide gels. The proteins were stained with Coomassie R-250 brilliant blue.

RESULTS AND DISCUSSIONS

From the different disease suppressive rhizospheric soils 20 isolates were isolated, purified and stored for further use. The isolates were named as T_1, T_2, T_3……. T_{19} and T_{20}, respectively.

In vitro Screening of *Trichoderma* Species against *S.rolfsii*

In the present investigation twenty isolates of *Trichoderma* sp (T_1-T_{20}), were screened *in vitro* for their efficacy in the inhibition of growth of *S. rolfsii.* Varied range of inhibition was noticed among twenty isolates of *Trichoderma* sp. Initially all the antagonists and pathogen grew together without showing any zone of demarcation. After 3 days of inoculation, growth of *S.rolfsii* was found to be inhibited by the hyphae of fungal antagonists. It indicates that the pathogen stopped its growth in petriplates upon contact with the antagonists and hyphae began to lie back while the antagonists continued their growth over the test pathogen colony. Data in Figure 1 indicates that T_7, T_9, T_{10}, T_{13} & T_{18} were strong mycoparasites as they inhibited 71-100% colony of *S.rolfsii* after 6 days of inoculation. Similarly, the isolates T_6, T_8, T_{11}, T_{12}, T_{14}, T_{15}, T_{16}, T_{17} and T_{19} parasitized 51-70% colony growth and were moderate mycoparasites. The rest of the isolates T_1, T_2, T_3, T_4, T_5, and T_{20} showed 0-50% parasitism in dual culture and were categorized as poor mycoparasites.

The isolates of *Trichoderma* significantly inhibited the mycelial growth of the *S.rolfsii*. The antagonistic activity of the *Trichoderma* isolates against *S.rolfsii* has been reported by many workers (21, 17, 18, 24). *Trichoderma* isolates differ in their effectiveness to control *S.rolfsii* [6]. Depending on their growth rate, twenty isolates were categorized as very fast, fast and medium growing isolates. In the present study, the maximum growth inhibition by five isolates (T_7, T_9, T_{10}, T_{13} & T_{18}) of *Trichoderma* is due to rapid sporulation and fast growing nature of antagonists which is evident

from the Figure 1. Similar results were also reported by Suriachandraselvan *et al* (20). It is well known that there is sufficient selectivity of isolates of *Trichoderma* in their antagonistic efficiency towards a particular pathogen. Cook and Baker (4) have also reported variation in antagonistic efficiency of *Trichoderma* against *S.rolfsii.*

Elad *et al* (6) reported that in dual culture, *Trichoderma* controls the *S.rolfsii* through mycoparasitism by releasing active lytic enzymes that can digest the components of cell walls of *S.rolfsii*. Upon reaching host hyphae, the antagonistic fungus either coiled around the host

Figure 1: Effect of *Trichoderma* Isolates (T_1-T_{20}) on Growth and Sclerotial Production of *S.rolfsii* by Dual Culture Method $T_1 – T_{20}$ = *Trichoderma* Isolates; Sr= *Sclerotium rolfsii*

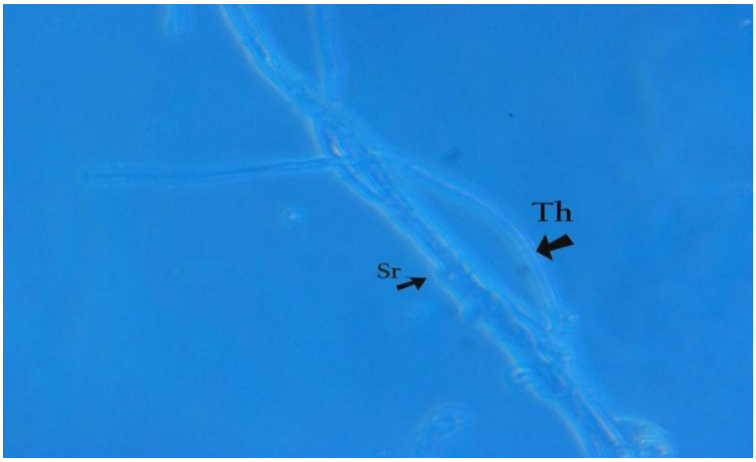

Figure 2: Coiling of *Trichoderma* Hyphae around the Plant Pathogen of *S.rolfsii*

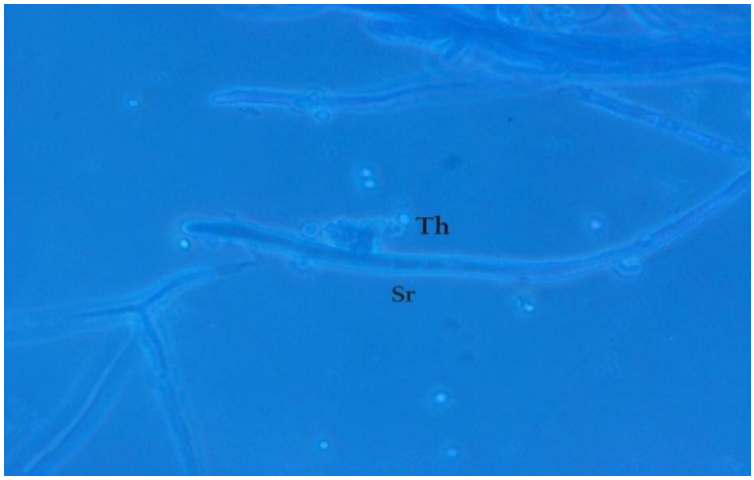

Figure 3: Attachment of Spores of *Trichoderma* to the Hyphae of Plant Pathogen *S. rolfs*ii

Or produced appressoria or hook-shaped contact branches. Removal of the coiling hyphae of *Trichoderma* by gentle shaking revealed "footprints" of partial lysis on the host hyphae. Moreover, holes that were observed in *S.rolfsii* hyphae apparently resulted from penetration by antagonist as observed in Figure 2 and Figure 3 of the present experiment.

Chitinase Production

Screening for Chitinase Production by *Trichoderma* Isolates

Five potent *Trichoderma* isolates (T_7, T_9, T_{10}, T_{13} & T_{18}) selected by dual culture method were screened for chitinolytic activity in the medium containing the chitin as a sole carbon source. Similarly, Kang *et al* (9); Gonzalez *et al.*, (8), also reported highest enzyme activity in medium containing chitin as the sole carbon source. Figure 4 indicates that the all the *Trichoderma* isolates had produced chitinase enzyme, but T_{10} isolate showed maximum activity followed by T_{18}, T_7, T_{13} and T_9, respectively. Therefore it has been selected for further studies on purification of chitinase. Chitinase enzymes produced by *Trichoderma* isolate T_{10} grown in liquid medium containing chitin as a carbon source, was purified in a single step using ion-exchange chromatography. The enzyme was has a specific activity of 190 U/ml of protein. Whereas Lime *et al*., (13) reported 264 U/ml of chitinase activity with 100 µl of enzyme solution of *Trichoderma* species.

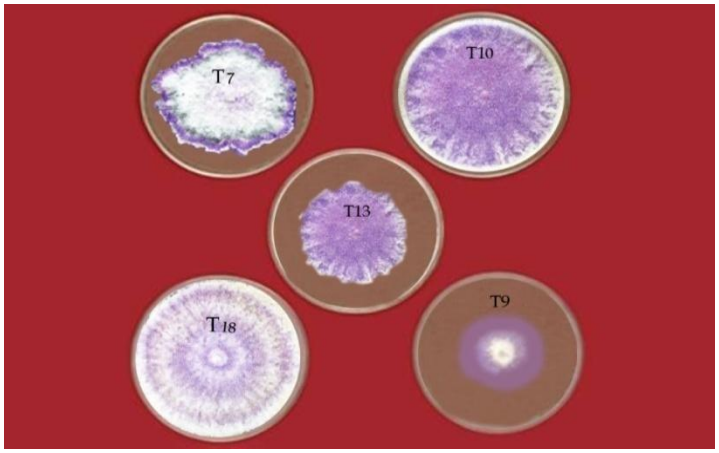

Figure 4: Screening of Potent *Trichoderma* Isolates for Chitinase Activity

SDS-PAGE analysis showed that the enzyme migrated as two bands with an estimated molecular mass of 76 kDa and 36 kDa (Figure 5). Coinciding with the present study, Ulhoa and Peberdy (22) also reported that chitinase from the culture fluid of *T. harzianum* had a molecular mass of 40kDa. Although chitinase producing microorganisms may be

effective in biological control and despite the possible role of chitinase on the antagonistic process, literature concerning purification, molecular and kinetic properties, physiological roles and molecular structures of extracellular chitinases from mycoparasitic fungi is scarce. Hence in the present study, chitinase enzyme has been purified by chromatography and SDS-PAGE.

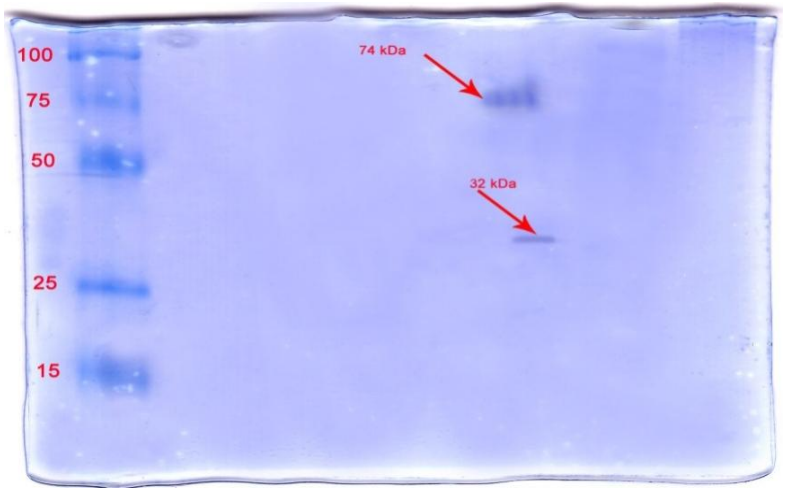

Figure 5: SDS-PAGE of *Trichoderma* sp. Isolates T_{10} for Chitinase Enzyme

All the isolates of *Trichoderma* showed the antagonistic activity against *S. rolfsii*, the causal agent of stem rot of groundnut. But, five isolates (T_7, T_9, T_{10}, T_{13} & T_{18}) were highly potent in controlling the growth of *S. rolfsii.* Probably, it may be due to the production of large quantities of antifungal metabolites by the fungal agent. *Trichoderma* spp. showed that highest chitinolytic activity when grown in the medium supplemented with chitin as a carbon source (9, 8). Several workers purified the chitinase enzyme from *Trichoderma* isolates and they reported that the molecular mass of chitinase was 46 kDa (13). In 1999, Kang *et al* (9) reported that *Metarhizium anisopliae* produced chitinase enzyme with molecular mass of 60 kDa. *Trichoderma harzianum* may produce seven individual chitinases two N-acetylglucosaminidases (102 and 73kDa), four endochitinases (52, 42, 33 and 31 kDa) and one chitobiosidase (40 kDa). They also reported that the chitinases of *T. harzianum* are substantially more active and effective against a wide range of fungi than chitinolytic enzymes from plants and other microorganisms.

CONCLUSIONS

Trichoderma are potential biocontrol agents which inhibit the growth of the phytopathogens by producing hydrolytic enzymes like glucanases and chitinases. In the present study T_{10} isolate produced two chitinases with molecular mass of 74kDa and 32kDa, respectively.

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