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Research Article

IN VITRO INHIBITION OF CELLULOLYTIC ENZYMES OF *FUSARIUM OXYSPORUM* BY *TRICHODERMA SPP* AND *PSEUDOMONAS FLUORESCENS* ON *ARACHIS HYPOGAEA.* L

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Abstract

In an attempt to develop biocontrol system for management of *Fusarium* wilt in groundnut, *Trichoderma viride, Trichoderma harzianum,* and *Pseudomonas fluorescens* were evaluated for their antagonistic activity against *Fusarium oxysporum in vitro. .Fusarium* wilt diseases caused by the fungus *Fusarium oxysporum* lead to significant yield losses of crops. Experiments were conducted on the effect of culture filtrates of *T.viride* (1%), *T. harzianum (*1.5%), and *P. fluorescens* (2%) on the *in vitro* inhibition of cellulolytic enzymes of *Fusarium oxysporum.* The activity of 1,4 endoglucanases, 1,4exoglucanase Cellobiase produced by *Fusariumoxysporum* was higher, when compared to control. Maximum inhibition of above Cellulolytic enzymes (1, 4 endoglucanases, 1,4exoglucanase, Cellobiase) was shown by *T. viride* treatment was followed by *T. harzianum* and *P. fluorescens*. Of all the treatments, *T. viride* treatment showed higher rate of inhibition of Cellulolytic enzymes of *Fusarium oxysporum* followed by that of *T. harzianum* and *P. fluorescens*.This present study indicates that culture filtrate of *T.viride*(1%) is the best biocontrol agent in the inhibition of *Fusarium oxysporum* causing *Fusarium* wilt of *Arachis hypogaea* .L

Key words: *Fusarium oxysporum;* 1,4 endoglucanases; 1,4 exoglucanase; Cellobiase; *Arachis hypogaea.*

Introduction

Arachis hypogaea L. is an important crop in many areas of the world and is found throughout the tropical, subtropical, and warm temperate regions between 40° north and 40° south latitudes. Groundnut is susceptible to many pathogens, with most damage being caused by fungi. Depending on severity of field infestation, yield losses due to such soil borne disease may be as high as 50 % (Melouk and Backman,1995).According to Busso *et al.*(2004), the increasing damage by soil borne fungi, reaching a disease incidence of 95% in some fields, are the main reason why producers are leaving groundnut production. Fusarium wilt caused by *Fusarium oxysporum* is one of the common diseases of groundnut. It has equally larger implications for groundnut yield. *Fusarium oxysporum*, the soil borne pathogen causes vascular wilt diseases in a wide variety of economically important crops (Beckman, 1987). It has been known as most distributed and important fungal disease on field crops for many years. Vascular wilt has been a major limiting factor in the production of many agricultural and horticultural crops. Robert Nyvall (1999) reported that Fusarium wilt is one of the prominent diseases of groundnut. Some structural defences are present in the plant even before the pathogen comes in contact with the plant. Agrios (1997) has elaborately explained these structural defences. The surface of a plant constitutes its first line of defence that pathogens must penetrate before it can cause infection. The structure of epidermal cell walls, further hinders the advance of the pathogen. Cell walls consist of celluloses, hemicelluloses, pectins, structural proteins and the middle lamella, which consist primarily of pectins. To gain entrance to plant cells, fungi generally secrete a mixture of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases .After penetration, the fungus often secretes toxins or plant hormone- like compounds that manipulate the plants' physiology to the benefit of the pathogen (Knogge, 1996). This is often achieved through the production of phytotoxins with varying degrees of specificity towards different plants (Walton, 1994).*Fusarium oxysporum* produces several enzymes that act upon the pectic and cellulose components of cell walls of host plant (Agrios, 1997). According to Husain and Dimond (1960) *Fusarium* produces both the Cx and C1 enzymes. Results of the investigation done by Fisher (1965) have shown that cellulolytic enzyme production is induced in surface rot and Fusarium wilt of sweet potato.

Trichoderma spp. have also been used in a wide range of commercial enzyme production, *viz.* cellulases, hemicellulases, proteases and β- 1,3-glucanase. Reino *et al.* (2008) reviewed the structural and biological studies of the

metabolites isolated from *Trichoderma* species. The study had especially focused the role of these metabolites in biological control mechanisms. The strains of *Trichoderma* spp. produce many types of secondary metabolites (Sivasithamparam and Ghisalberti, 1998) including antibiotics (Howel, 1998) and cell wall degrading enzymes (Kubicek and Penttila, 1998; Lorito, 1998), the role of which has been clearly established in biocontrol activity (Vinale *et al.*, 2008). *Trichoderma* is a genus which include species of free-living soil fungi, opportunistic, avirulent plant symbionts (Harman *et al*., 2004), asymptomatic endophytes (Wilberforce et *al*., 2003) and parasites of other fungi (Harman, 2006). *Trichoderma harzianum* and *Pseudomonas fluorescens* are important rhizosphere microorganisms and are efficient in suppressing root and foliar diseases of several plant species.There have been studies on the application of antagonistc microbes, such as *Pseudomonas* spp., for control of *Fusaruium* wilt (Duijfer *et al*., 1999 and Leonardo *et al*., 2006). Meena *et al.* (2001) studied the biological control of root rot of groundnut with antagonistic *Pseudomonas fluorescens*. Thus, the present study was carried out to determine the *in vitro* inhibition of Celluolytic enzymes of *F. oxysporum* with the application of *Trichoderma spp* and *Pseudomonas fluorescens* on *Arachis hypogaea*.L

Materials and Methods

Trichoderma viride, *Trichoderma harzianum* & *Pseudomonas fluorescens* were obtained from Institute of Microbial Technology (IMTECH),Chandigarh and were used for the present study. The pathogen *Fusarium oxysporum* was obtained from the infected leaves of *Arachis hypogaea* and was obtained from the infected leaves of *Arachis hypogaea* and was purified by single conidium isolation method. The purified culture was stored in the slants of PSA.

Fusarium oxysporum was grown on PSA for 30 days and further grown in Czapek's medium for 7 days and filtrate was taken. *Trichoderma viride* & *Trichoderma harzianum* were grown on Malt Extract agar and *Pseudomonas fluorescens on* ABM Medium and further grown on Czapek's medium in conical flask. It was further centrifuged and culture filtrate was taken.

For cellulolytic enzyme production the pathogens were grown in Czapek's broth, supplemented with as microcrystalline cellulose and carboxy methyl cellulose source replacing sucrose.To 50ml sterilized Czapek's liquid media in a 250ml Erlenmeyer conical flask, the culture filtrate of *T. viride*, *T. harzianum* and *P. fluorescens* in their OIC were amended to the media separately. The two discs of 9 mm were cut from the growing tip of the 7 days old culture of *F. oxysporum* with the help of a cork borer. They were inoculated in each flask and incubated in the BOD incubator at 28°± 0.2C for 7 days. The control and treated flasks were all maintained in triplicates. After incubation, the fungal mat and the liquid media were separated by double layered Whatman No. 1 filter paper placed in Buchner funnel under suction by vacuum pump. The filtrates were further centrifuged in a high speed, cooling centrifuge at 5000 rpm for 10 min and the supernatant was used as the enzyme source.

Estimation of Cellulolytic Enzymes

F.oxysporum produces 1,4-β-Exo-glucanase and 1,4-β-Endo-glucanase when grown in Czapek's broth.

Measurement of 1, 4-β-Exo-Glucanase (C1)

The activity of C_1 produced by *F.oxysporum* was assayed by measuring the reducing sugars released from microcrystalline cellulose and the activity was expressed in SAUs. Exo- β -1,4-glucanase activity was measured by estimating the reducing sugars released by the breakdown of avicel with anthrone reagent (Mahadevan and Sridhar, 1986).

To 1 ml of enzyme source, 1 ml of buffer and 0.5 ml of substrate were added in a test tube and incubated at room temperature for 2 h. The reaction mixture was mixed well with vortex mixer at regular interval of 30 minutes. At the end of the reaction, the volume of the reaction mixture was adjusted to 5 ml with distilled water. The tubes were centrifuged for 15 min at 2000 g to deposit the residual avicel cellulose. Soluble sugar in the supernatant was measured with the orcinol reagent. Two ml of the above supernatant, 3 ml of orcinol reagent was taken in the test tubes and 10 ml of anthrone reagent was added on ice. The tubes were mixed well with the help of vortex mixture and heated in a water bath at 80°C exactly for 20 minutes and immediately cooled under running tap water. The colour developed was read at 485 nm in Systronics Spectrophotometer. A blank was prepared with 2% H_2S0_4 instead of orcinol. Control was maintained with boiled enzyme reaction mixture and with zero time reaction mixture.

Measurement of 1, 4-β-Endo-Glucanase (C_x)

C_x, cleaves carboxyl methylcellulose randomly (endo-C_x) and terminally (exo-C_x). The activity of endo-C_x was assayed by the viscosity loss caused by enzyme in the substrate CMC.

Endo-β-l,4-glucanase (C_X **)** activity was determined by measuring the viscosity loss in reaction mixture (Mahadevan and Sridhar, 1986) and by estimating the reducing sugars released by the enzyme sources in the same reaction mixture (Wang *et al.*, 1997).

Substrate preparation

0.5 g of CMC (carboxyl methyl cellulose) was dissolved in 100 ml of sodium acetate-acetic acid buffer with pH 5.2 and kept in water bath at 50-60°C for 5-10 min. then the mixture is blended with the help of polytron homogenizer. The substrate was filtered in two layered cheese cloth and this was stored at 4°C with a layer of toluene.

Viscosity Measurement

Ostwald viscometer 150 size was used to determine the viscosity loss of cellulose substrate.

4 ml of carboxyl methyl cellulose, 1 ml of the buffer and 2 rnl of enzyme was pipetted out into the viscometer. The contents were mixed by drawing air gently through the large arm of the viscometer. Suction was applied to the small aim and the efflux time of the mixture was determined at every 30 min interval for 3 h incubation .The percentage loss in viscosity was calculated by employing the formula of the viscosity assay of Endo-PMG.

Estimation of Cellobiase

The amount of reducing sugars released from cellobiose by cellobiase was used to assay the enzyme.

1.5 ml of the buffer, 2.5 ml of 5 mM cellobiose and 1 ml of the enzyme was taken in a test tube and incubated at 30°C for **2** h. The reaction was terminated by placing the test tube in a boiling water bath for 10 min. 'The amount of glucose liberated by the enzyme using DNS (dinitro salicyclic acid) reagent was measured at 575 nm in Systronic Spectrophotometer. Glucose was used as standard.

Results

The activity of endo-C_x was assayed by the viscosity loss caused by the enzyme in the CMC substrate and the activity of exo-$C_1$was obtained by measuring the amount of reducing sugar liberated by the breakdown of CMC substrate and the activity was expressed in SAU. The endo- C_x of control reduced the viscosity of the substrate to 70% at 180min. The least 1, 4- β endoglucanase activity was observed in the enzyme source obtained from the culture treated with *T.viride* (10% viscosity loss at180 min), followed by those of *T. harzianum* (13.04%) and *P. fluorescens* (14.28%) at 180 min (Fig.1).

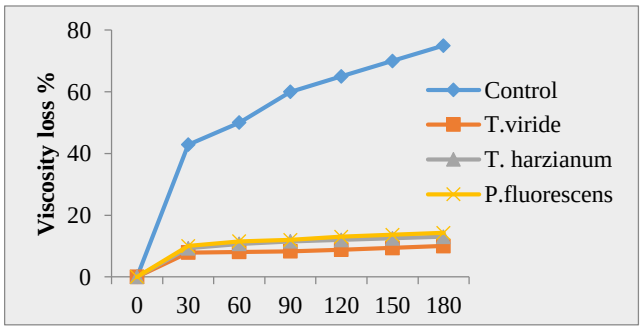

Fig. 1: Effect of culture filtrates of *T.viride*, *T. harzianum* *P. fluorescens* on the activity of 1, 4-β-endo-glucanase of *Fusarium oxysporum* *in vitro* condition.

The activity of exo-C_1 was expressed in specific activity units (SAU). All the treatments inhibited the activity of exo-C_1 at varying degree. Higher exo-C_x activity was observed in the case of enzyme source obtained from the control (27.55 SAU), followed by those of *P. fluorescens* (10.55 SAU) *T. harzianum* (9.73 SAU) Among the treatments the least amount of sugar was liberated in the case of enzyme source obtained from *T. viride* treatment (9.11SAU).(Fig.2)

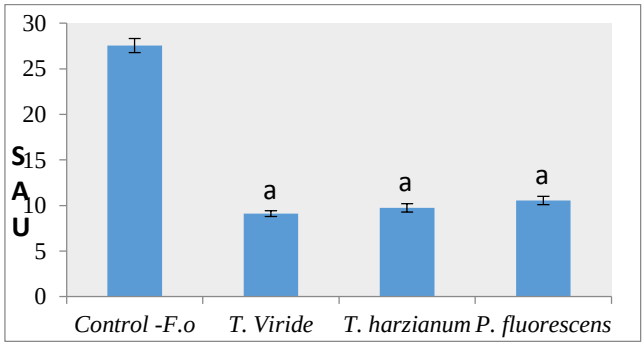

Fig. 2: Effect of culture filtrates of *T.viride*, *T. harzianum* and *P. fluorescens* on the activity of 1, 4- β -exo-glucanase of *Fusarium oxysporum in vitro* condition.

p< 0.001 as compared to control
SAU= micro g of maltose equivalent liberated /h
The values within a column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3)

The highest cellobiase activity was observed in case of control (40.88SAU) followed by the enzyme sources obtained from *P. fluorescens* (16.38SAU) and *T.harzianum*(14.76SAU) The lowest rate of enzyme activity was observed in *T. viride*-treated enzyme source (13.54SAU) (Fig.3).

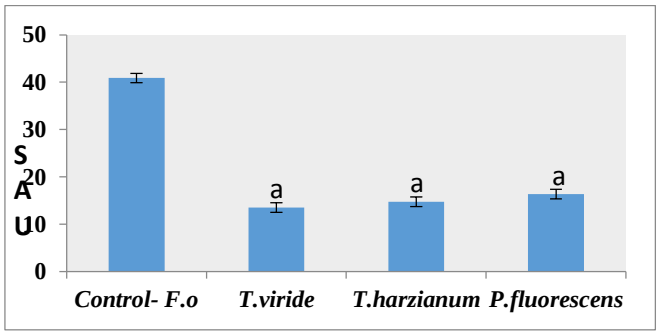

Fig. 3: Effect of culture filtrates of *T. viride*, *T. harzianum* and *P. fluorescens* on the activity of cellobiase of *Fusarium oxysporum in vitro* condition.

[a]p< 0.001 as compared to control
SAU= micro g of maltose equivalent liberated /h
The values within a column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3)

Discussion

Diverse molecules have been described and their role in the suppression of several plant pathogens has been documented (Fravel,1988; Weller and Thomashow, 1993).They include not only antibiotics *sensu stricto*, but also bacteriocins, enzymes such as cell wall degrading enzymes (CWDEs) and votatile compounds with an antifungal activity.

The fungal plant pathogens produce different types of cellulose lytic enzymes, which act as the main agents for disease development. The cellulolytic enzyme activity and

the cellobiase enzyme activity of pathogen *in vitro* were higher. Aktar and Dimond (1960) brought out the fact that the cellulolytic enzymes secreted by *Fusarium* sp. attack the primary and secondary cell wall of the host (tomato) and disintegrate them. The degraded products may get into the transpirational stream, which block the vessels leading to wilt symptoms formation.

Activity of cellulolytic enzymes was inhibited significantly by the addition of culture filtrate of *T.viride* (1%) in the medium followed by that of *T. harzianum* and *P. fluorescens* Cellobiase activity was inhibited significantly by amending the medium with the culture filtrate of *T. viride* 1% followed by that of *T. harzianum* and *P. fluorescens* . The inhibition of cell wall degrading enzymes of the pathogen by the antagonistic microorganisms was due to the production of enzymes or antifungal compounds which can degrade or inactivate the enzymes produced by the pathogen (Tokimoto, 1982; Ramamoorthy and Samiyappan, 2001) .

Trichoderma spp. have also been used in a wide range of commercial enzyme production, *viz.* cellulases, hemicellulases, proteases and β- 1,3-glucanase. Reino *et al.* (2008) reviewed the structural and biological studies of the metabolites isolated from *Trichoderma* species. The study had especially focused the role of these metabolites in biological control mechanisms. The treatment might inhibit the activity of lytic enzymes of the pathogen either by antibiotic action or ISR in the host cell such as thickening cell wall or it induces the host cell to produce lytic enzymes which are able to inactivate or inhibit the pathogen's lytic enzymes. The accumulation of phenolic compounds in the treated leaves may be toxic to the enzyme activity of the pathogen. The present result substantiates the observation made by Borowitz *et al. (*1992) who reported that *P. fluorescens* treatment degraded the lytic fungal pathogen's cellulase, pectinase and xylanase mainly by the action of different antibiotics.

Frindlender *et al. (*1991) observed the activity of chitinase and β-1, 3-glucanase of *Pseudomonas* participating in the inhibition of lytic enzymes of different fungal pathogens. Similar reports are available on F. *solani* (Lim *et al.,* 1991), *F. oxysporum* in tomato (M' Piga *et al*., 1997) and *Pythium* sp. in cucumber (Chen *et al.,* 2000).

Trichoderma spp. are reported to produce many antifungal lytic enzymes such as chitinases, proteases, glucanases, lipases. laminarinase and xylanase, which are responsible for the degradation of lytic enzymes of other fungal species. This was observed in *Trichoderma* spp.-treated bean plant against *Pythium* sp. (Cotes *et al.,* 1996). *Trichoderma* spp. as bio-control agents induced the accumulation of some enzymes chitinase, peroxidase and polyphenol oxidase which played an important role in plant defense mechanisms against pathogen infection and significantly reduced the disease. (Rajeswari, 2014). *P. fluorescens* treatment degraded the lytic fungal pathogen's cellulase, pectinase and xylanase mainly by the action of different antibiotics. In this study all the Celluolytic enzymes ((1,4 endoglucanases, 1,4exoglucanase Cellobiase)of *Fusarium oxysporum* was inhibited by *T. viride* followed by *T. harzianum* and *P. fluorescens.* Of all the treatments, *T. viride* treatment showed higher rate of inhibition of cellulolytic enzymes of *Fusarium oxysporum* followed by that of *T. harzianum* and *P. fluorescens*. The present study concludes that among the three biocontrol agents the culture filtrate of *T. viride* (1%) is the best biocontrol agent in the inhibition of *Fusarium oxysporum* causing *Fusarium* wilt of *Arachis hypogaea* .L

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