

Combined Application of Different Species of *Trichoderma* and *Pseudomonas fluorescens* on the Cellulolytic Enzymes of *Fusarium oxysporum* for the Control of *Fusarium wilt* Disease in *Arachis hypogaea*. L

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Fusarium oxysporum causes *Fusarium wilt* of crop plants leads to considerable yield loss. The study was conducted to determine the beneficial effects of combining *Trichoderma* species and *Pseudomonas fluorescens* i.e *Trichoderma viride*+ *Pseudomonas fluorescens* (Tv+Pf) (1+2%), *Trichoderma harzianum*+*Pseudomonas fluorescens* (Th+Pf) (1.5+2%), *Trichoderma viride* +*Trichoderma harzianum* (Tv+Th) (1+1.5%) on the activity of cellulolytic enzymes of *Fusarium oxysporum* to control *Fusarium wilt* of *Arachis hypogaea*. L wilt in vitro. The activity of 1,4- β -Endoglucanase, 1,4- β -Exoglucanase, Cellobiases produced by *Fusarium oxysporum* (Control) was higher. Maximum inhibition of Cellulolytic enzymes was shown by culture filtrate of *Trichoderma viride* + *Pseudomonas fluorescens* (Tv+Pf) (1+2%), followed by *Trichoderma harzianum* + *Pseudomonas fluorescens*, (Th +Pf) (1.5+2%) and *Trichoderma viride* + *Trichoderma harzianum* (Tv+Th) (1+1.5%). However, disease suppression of *Fusarium wilt* of *Arachis hypogaea*. L by the compatible combination of *Trichoderma viride* + *Pseudomonas fluorescens* (1+2%) was considerably better as compared to other two strains. At the same time the other two combinations resulted in enhanced disease suppression as compared to single strains. This indicates that the potential benefits of using combination treatments to suppress *Fusarium wilt*. The study suggests the significance of interactive effects of *Trichoderma* and *Pseudomonas* in biocontrol of wilt disease.

Keywords: *In vitro* interactions, *Trichoderma viride*, *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Fusarium wilt*, *Arachis hypogaea*.L.

Fusarium oxysporum was considered as the most important phytopathogenic and toxigenic genus of filamentous fungi worldwide (O'Donnell, 1996; Langseth *et al.*, 1999; Eskola *et al.*, 2001; Kosiak *et al.*, 2003; Zhang *et al.*, 2007; Suga *et al.*, 2008). *Fusarium wilt* caused by *Fusarium*

oxysporum (Schlecht. Emend.Snyder& Hansen) leads to significant loss to crop yield and quality of nuts in groundnut plants. Cellulose is the main component of the plant cell wall. It is synthesized by plasma membrane protein complexes. It is deposited directly on the wall in a directional way (Taylor N.G 2008). It is a linear homopolymer composed of β (1 \rightarrow 4) linked D-glucose units. Cellobiose is a glucose dimer linked by β (1 \rightarrow 4) and found as repeating unit of cellulose. It is found that plant pathogens produce the exocellular enzymes

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like cellulolytic, hemi cellulolytic, pectinolytic and proteolytic enzymes which degrade the major components of Cellwall (Wheeler, 1975; Riou, 1991). Most pathogens capable of producing more cellulolytic than pectolytic enzymes (Sadik *et al.*, 1983). The chemistry of cellwall affects by enzymes produced by pathogens and further leads to cellwall degradation (Albersheim and Jones 1969; Miettinen- Oinonen and Suominen 2002). The production of cellulolytic enzymes and degradation of cellulose by several fungi has been reported by many workers, (Basu and Ghose 1960; Bateman 1964; Sohaila *et al.*, 2011). The studies reported that most of the plant pathogenic organisms are capable of degrading cellulose by producing a cellulose complex which involves the synergistic action of three main enzymatic complexes. (1) endo- β -1,4-glucanases (endo- β -1,4-d-glucan 4-glucanohydrolase, (2) exo- β -1,4-glucanase or cellobiohydrolase (exo- β -1,4-d-glucan 4-cellobiohydrolase, and (3) β -glucosidase or cellobiase. These components act synergistically to hydrolyze cellulose to glucose. Endo-glucanase hydrolyzes internal β -1,4 linkages of cellulose chains and creates new reducing and non-reducing ends. Exoglucanase cleaves disaccharide cellobiose from the non-reducing end (cellobiohydrolase). In some cases, it cleaves from the reducing end (cellobiosidase) of the cellulose chain. These cellobiose units and short-chain cello dextrins are hydrolyzed by β -glycosidase into individual monomeric units of glucose (Nelson 1994; Moreria 2005; Beguin and Aubert 1994). Cellulases is associated with pathogenicity of number of microorganisms (Jan and Chen 2003). There are many studies on biological control of soil borne diseases caused especially by *Fusarium oxysporum* (Marois *et al.*, 1981; Sivan and Chet, 1986). It was successfully demonstrated that biological control of *Fusarium* wilt by *Trichoderma* spp and *Pseudomonas* spp Saravanan *et al.*, 2003; Tu and Chang 1983; Duijff *et al.*, 1999). Combined application of different biocontrol agents has improved disease protection (Jetiyanon and Kloepper 2002). Studies on banana demonstrated that biocontrol agents in different combinations have improved plant growth parameters than individual treatments by reducing the *Fusarium oxysporum* f.sp. cubense infection in field conditions. (Sukhada

et al., 2010). Abeysinghe (2009) suggested that combination of *B. subtilis* with *Pseudomonas* strains can lead to greater plant protection against *R. solani* and *S. rolfsii* than individual application. Combining antagonists plays more effective biological control treatments of infection rather than individual antagonists (Janiesiewicz 1996). Weller (1994) found that combinations of several fluorescent *Pseudomonads* have greater biocontrol activity against take all of wheat as compared to application of single strains. Wahid (2006) reported that combination of biocontrol agents gave better results than using them singly. Talaviya and Jadega (2015) established that combined application of *T.viride*+*T.harzianum*+ *Pseudomonas fluorescens* was found most effective in controlling cumin wilt disease and also had highest seed yield. There seems to be no report on the control of *Fusarium* wilt with the combinations of biocontrol agents on *Arachis hypogaea*.L. The present study was taken to determine the significant interactive effects of *Trichoderma* spp and *Pseudomonas fluorescens* in biocontrol of *Fusarium* wilt of *Arachis hypogaea* L. in comparison with application of single strains.

MATERIALS AND METHODS

Microbial Cultures

The bacterial and fungal cultures used in the study were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. *Fusarium oxysporum* was cultured on Potato Sucrose Agar (PSA) for 30 days. *Trichoderma viride* and *Trichoderma harzianum* were cultured on Malt Extract Agar (MA) and *Pseudomonas fluorescens* on Antarctic biotic Medium (ABM) for 30 days. All these cultures further grown on Czapek's medium separately for 7 days at $28^{\circ}\pm 0.2^{\circ}\text{C}$. The culture filtrates were taken after centrifuged.

Enzyme Production

For the assay of 1,4 β – endoglucanases, Czapek's broth was supplemented with carboxy methyl Cellulose and for 1,4- β exo glucanases microcrystalline cellulose was used. Culture filtrates of Tv+Pf (1+2%), Th+Pf (1.5+2%) Tv+Th (1+1.5%) Tv+Th (1+1.5 %) in their OIC (Optimum Inhibitory Concentration) were amended to 50ml Czapek's liquid media separately. The two discs of 9 mm of *F. oxysporum* was inoculated in each flask and incubated in the BOD incubator at $28^{\circ}\pm 0.2^{\circ}\text{C}$

for 7 days. The control (with *Fusarium oxysporum*) and treated flasks (Tv+Pf, Th+Pf, Tv+Th) were all maintained in triplicates. After incubation, the fungal mat and the liquid media were separated by Whatman No.1 filter paper. The filtrates were further centrifuged at 5000 rpm for 10 min and the supernatant was used as the enzyme source.

Assay of 1,4 - β – Endoglucanase (Cx) activity

The activity of 1,4 endoglucanases was assayed as per the method described by Mahadevan and Sridhar (1986) and the enzyme activity was calculated as percent reduction in viscosity of the substrate. The reducing sugars released by the enzyme sources in the same reaction mixture was estimated according to Wang *et al.*, (1997).

Substrate preparation

Carboxyl Methyl Cellulose (0.5g) 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. The substrate was filtered and stored at 4°C with a layer of toluene.

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 ml of enzyme was pipetted out into the viscometer. The contents were mixed well and the efflux time of the mixture was determined at every 30-min interval for 3 h incubation and the enzyme activity was calculated from the following formula.

$$V = \frac{T_0 - T_1}{T_0 - T_W} \times 100$$

Where V = percent loss in viscosity T_0 = flow time of reaction mixture at 0 minute

T_1 = flow time of reaction mixture at a particular time interval T_W = flow time of distilled water

Assay of 1,4 - β – Exoglucanase activity

1,4- β -Exo glucanase activity was measured by estimating the reducing sugars released by the breakdown of avicel with anthrone reagent. 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 and centrifuged. To 2 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 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% H₂S₀₄ instead of orcinol. Control was maintained with boiled enzyme reaction mixture and with zero-time reaction mixture.

Estimation of Cellobiase

To estimate the cellobiase enzyme, the mixture of 1.5 ml of the buffer, 2.5 ml of 5 mM cellobiose and 1 ml of the enzyme was incubated at 30°C for 2 h. Then the reaction was terminated. By using DNS (dinitro salicylic acid) reagent, the amount of glucose liberated by the enzyme was measured at 575 nm in Systronics Spectrophotometer. Glucose was used as standard.

Statistical analysis. The data of experiment was statistically analyzed according to ANOVA and significance with in the column with Tukey HSD multiple range test (TMRT) at 5% level of significance (n=3)

RESULTS AND DISCUSSION

The 1,4 - β -Endoglucanase of control reduced the viscosity of the substrate to 80% at 180min. The lowest activity was observed in the enzyme source obtained from the culture treated with *Trichoderma viride* + *Pseudomonas fluorescens* (Tv+Pf) (13.3% viscosity loss at 180 min) followed by *Trichoderma harzianum* + *Pseudomonas fluorescens* (Th+Pf) (17.39%) and *Trichoderma viride* + *Trichoderma harzianum*(Tv+Th) (21.05%). (Fig.1)

The activity of 1,4 - β - Exoglucanase was expressed in specific activity units (SAU). All the treatments inhibited the activity of 1,4- β -Exoglucanase at varying degree. Higher amount of monogalacturonic units was released in the case of enzyme source obtained from the control (487.53 SAU), followed by Tv+Th (*Trichoderma viride*+ *Trichoderma harzianum*) (234.00 SAU) and Th+Pf (*Trichoderma harzianum* + *Pseudomonas fluorescens*) (165.05 SAU). Among the treatments the least amount of sugar was liberated in the case of enzyme source obtained from treatment Tv+Pf (*Trichoderma viride* + *Pseudomonas fluorescens*) (95.14 SAU). (Table 1)

The Cellobiase activity was observed in enzyme source of control (297.36 SAU). The lowest rate of enzyme activity was observed in Tv+Pf (*Trichoderma viride* + *Pseudomonas fluorescens*)-treated enzyme source (91.50 SAU) followed by *Trichoderma harzianum* + *Pseudomonas fluorescens* Th+ Pf (155.12 SAU) and *Trichoderma viride* + *Trichoderma harzianum* and (Tv+Th) (195.18 SAU). (Fig.2)

Maximum inhibition of 1,4 β-Endoglucanases, 1,4 Exoglucanases, and Cellobiase enzyme activity was recorded in Tv+Pf (*Trichoderma viride* + *Pseudomonas fluorescens*) treated culture. Our results are in agreement with the earlier studies recorded by Karthikeyan *et al.* (2006) that *T. viride*, *P. fluorescens*, neem cake,

T. viride + neem cake, *P. fluorescens* + neem cake that applied to soil has considerably reduced the stem rot incidence compared to control in groundnut plants 60 days after sowing. Khan *et al.* (2004) reported that combination of *Trichoderma* and *Pseudomonas* has better efficacies in control of *Fusarium* infection on chickpea than using them singly.

Salman *et al.* (2017) concluded that application of *Pseudomonas* to field crops such as watermelon and similar crops that are affected by *Fusarium* spp improve their productivity and yields of such crops. The mixtures of bioagents also acts a PGPR. The phytohormones viz. auxins, gibberellins, cytokinins and ethylene produced by bacteria play a major role in growth

Table 1. Effect of culture filtrates of *Trichoderma viride* + *Pseudomonas fluorescens* (Tv + Pf), *Trichoderma harzianum* + *Pseudomonas fluorescens* (Th + Pf), *Trichoderma viride*+ *Trichoderma harzianum* (Tv+ Th) on the activity 1,4 -² - Exoglucanases of *Fusarium oxysporum* *in vitro*

In Vitro	SAU (Specific Activity Units)
Control- <i>Fusarium oxysporum</i>	487.53±0.30
Treatment with Tv+Pf(<i>Trichoderma viride</i> + <i>Pseudomonas fluorescens</i>)	95.14±0.52 ^a
Treatment with Th+Pf(<i>Trichoderma harzianum</i> + <i>Pseudomonas fluorescens</i>)	165.05±0.95 ^a
Treatment with Tv+Th(<i>Trichoderma viride</i> + <i>Trichoderma harzianum</i>)	234±0.44 ^a

^ap< 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)

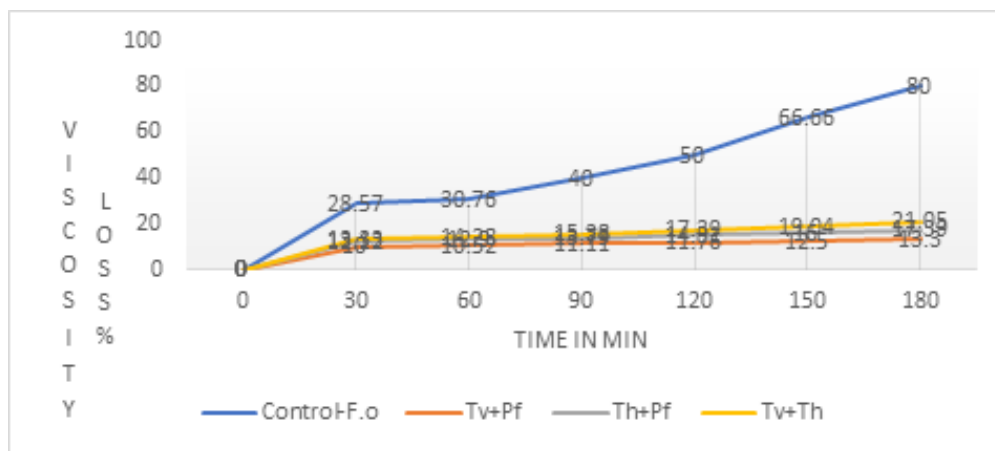


Fig. 1. Effect of culture filtrates of *Trichoderma viride* + *Pseudomonas fluorescens* (Tv + Pf), *Trichoderma harzianum* + *Pseudomonas fluorescens* (Th + Pf), *Trichoderma viride*+ *Trichoderma harzianum* (Tv+ Th) on the activity 1,4 -² - Endoglucanases of *Fusarium oxysporum* *in vitro*

promotion. The same is reported by Lehar *et al.* (2016) stating that administration of biological agents of *T. viride* combined with *P. fluorescens* and *Streptomyces* sp. produce growth hormone or PGPR which stimulates better plant growth and thereby increasing yield in potato and ability to control disease caused by *Phytophthora infenstan* and *Ralstonia solanacearu*.

Our results indicated that the inhibition of 1,4 β Exoglucanases, 1,4 β Endoglucanases, and Cellobiases was recorded in Th+Pf (*Trichoderma harzianum* + *Pseudomonas fluorescens*) treated culture. Our results substantiate earlier reports that in management of plant diseases the use of biocontrol agents in combinations were more effective than individual agents (Thilagavathi *et al.*, 2007; Ganeshmoorthi *et al.*, 2008). The application of *T.harzianum*, captan and neem seed extract two days after pathogen inoculation significantly reduced damping off disease caused by *S. rolfsii* in greenhouse grown tomato plants (Okereke and Wokocha 2006). Thangavelu and Gopi (2015) found that the combination of bacterial antagonists could provide sustainable management of *Fusarium* wilt of banana under field conditions.

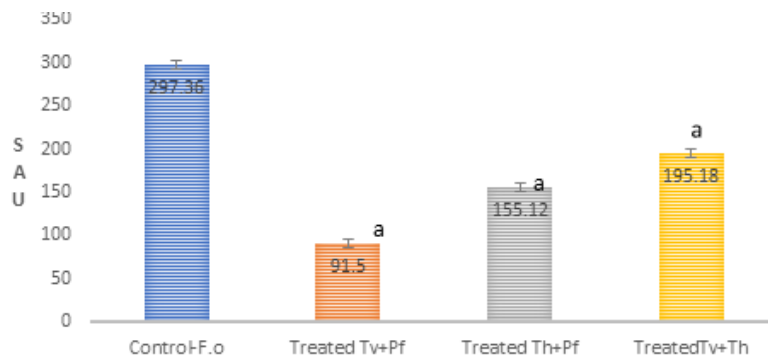
Our results also revealed that inhibition of 1,4 Exoglucanases 1,4 β Endoglucanases and Cellobiases was recorded in Tv+ Th (*Trichoderma viride* + *Trichoderma harzianum*) treated culture.

Similar observation were recorded by Rini and Sulochana (2007) that *T.viride* and *T. harzianum*, with *P. fluorescens* were found to be compatible and more effective in suppressing the seedling disease of chilli and tomato.

Bosah *et al.* (2010) reported that *Trichoderma* spp. proved to be the most effective biocontrol agent against *S. rolfsii* in inhibiting the growth of the pathogen by 80% under in vitro conditions. In greenhouse trial, inoculation of mint plants with either *T. harzianum* or *T. virens* significantly reduced the collar rot caused by *S. rolfsii* and was accompanied by significant increase in herb and oil yield (Singh and Singh 2004).

CONCLUSION

Results from the present study revealed that cellulolytic enzymes (Exo and endo Glucanases, and Cellobiases) produced by *Fusarium oxysporum* were inhibited by culture filtrate of *Trichoderma viride*+*Pseudomonas fluorescens* (1+2%) followed by *Trichoderma harzianum*+ *Pseudomonas fluorescens* (1.5+2%) and *Trichoderma viride* +*Trichoderma harzianum*(1+1.5%). The different disease suppression mechanisms together lead to enhanced disease suppression by the combined application of above strains. The reasons for the reduced wilt incidence and severity may attributed



^ap< 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)

Fig. 2. Effect of culture filtrates of *Trichoderma viride* + *Pseudomonas fluorescens* (Tv + Pf), *Trichoderma harzianum* + *Pseudomonas fluorescens* (Th + Pf), *Trichoderma viride*+ *Trichoderma harzianum* (Tv+ Th) on the activity of cellobiases - of *Fusarium oxysporum* in vitro

to various mechanisms of *Trichoderma* spp. viz. mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defence system. The *Pseudomonas* produced secondary metabolites like phenazine, 2,4-diacetylphloroglucinol, pyocyanine, pyrrolnitrin and pyrrolnitrin which were involved in enhancing plant growth by suppressing the disease caused by *Fusarium oxysporum*. In this study combinations of biocontrol agents showed significant reduction in *Fusarium* wilt disease. The application of fungal and bacterial combination, i.e. *T. viride* + *P. fluorescens* (1+2%) has proved to be more effective in managing the *Fusarium* wilt in *Arachis hypogaea* L as compared to other combinations and single strains.

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