COMBINATORIAL EFFICACY OF *Trichoderma* spp. AND *Pseudomonas fluorescens* TO ENHANCE SUPPRESSION OF CELL WALL DEGRADING ENZYMES PRODUCED BY *Fusarium* WILT OF *Arachis hypogaea.* L

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Abstract

Fusarium oxysporum, the soil borne pathogen causes vascular wilt, on majority of crop plants. It has been demonstrated that two different species of *Trichoderma* and *Pseudomonas fluorescens* suppress disease by different mechanisms. Therefore, application of a mixture of these biocontrol agents, and thus of several suppressive mechanisms, may represent a viable control strategy. A necessity for biocontrol by combinations of biocontrol agents can be the compatibility of the co-inoculated micro-organisms. Hence, compatibility between *Trichoderma* spp. and *Pseudomonas fluorescens* that have the ability to suppress *Fusarium oxysporum in vitro* on the activity of pectinolytic enzymes of *Fusarium oxysporum.* The activity of pectinolytic enzymes, i.e. pectin methyl esterase, endo and exo polymethylgalacturonases and exo and endo pectin trans eliminases produced by *Fusarium oxysporum* (Control) was higher. Maximum inhibition of pectin methylesterase, exo and endo polymethylgalacturonase and exo and endopectin trans eliminase 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, pathogenecity suppression of *Fusarium oxysporum,* a causative of *Arachis hypogaea.* L by the compatible combination of *Trichodema viride + Pseudomonas fluorescens* (1+2%) was significantly better as compared to the single bio-agent. This indicates that specific interactions between biocontrol agents influence suppression of pathogenicity factors directly by combinations of these compatible bio-agents.

Keywords: *In vitro* interactions, Biological control, *Fusarium oxysporum*, *Arachis hypogaea*

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Introduction

Fusarium oxysporum is considered as an important fungus because of its ability to cause wilt disease on wide range of plants. *Arachis hypogaea* L. (Groundnut) is an important oil seed crop (Brown, 1999) and among diseases of groundnut, *Fusarium* wilt disease caused by *Fusarium oxysporum* (Schlecht. Emend. Snyder & Hansen) is a disease of considerable economic importance in groundnut production. *Fusarium oxysporum* causing wilt produces several enzymes that act upon the pectic and components of cell wall of host plant. These enzymes are involved in degradation of plant cell components and lignin. Pectic enzymes have been frequently associated with wilt disease on various crops (Cooper and Wood, 1980). Pectic enzymes consist primarily of pectin methyl esterase, polygalacturonase and pectate lyase (Kawano *et al.*, 1999; Verlent *et al.*, 2004). Pectin methyl esterase (PME) catalyzes the removal of methyl groups at C-6 from esterified anhydrogalacturonosyl units in HG and methanol is released and enzyme is active on methyl esters, where there is a free adjacent carboxyl group (Pedrolli *et al.*, 2009). Polygalacturonases catalyze the hydrolysis of α- (1→4)-glycosidic bonds between adjacent non- esterified D-GalpA units in HG, which leads to substrate depolymerization and viscosity reduction. Mechanism of depolymerisation could be differentiated into two enzymes: endo-PG and exo-PG. Endopolygalacturonase (endo-PG) with a random action and exopolygalacturonase (exo-PG) with a successive action have been reported by earlier studies. Fungal endo-PGs bind to 4 till 5 adjacent α-D-GalpA units from the main chain and hydrolyze between the last two residues at the reducing end (Prade *et al.*, 1999). Exo-PGs attack at the non-reducing end and monomers or dimers are cleaved (Benen and Visser, 2003). Transeliminases (Pectate lyase) catalyze the non-hydrolytic depolymerization of HG chains by α-

(1→4)-glycosidic bonds breakage between adjacent α-D-GalpA monomers, and as a result a double unsaturated Δ-(4→5) bond is formed at the non- reducing end. Production of cell wall degrading enzymes (CWDEs) polygalacturonase (PG), pectate lyase (PL), and xylanase was studied in chickpea wilt and the result have implications for the role played by CWDEs in the early and later stages of pathogenesis in chickpea *fusarium* wilt (Jorge *et al.,* 2006). Akrami *et al.* (2011) demonstrated the ability of *Trichoderma harzianum* and *Trichoderma asperellum* isolates and their combination reduced fusarium rot disease and increased dry weight in lentil plants. Pushpavathi *et al.* (2015) observed that sucker treatment before planting with biocontrol agents *Trichodema viride* and *Pseudomonas fluorescens* and soil drenching with the same biocontrol agents twice as booster reduced the *Fusarium* wilt disease in banana and incidence thereby increasing the yield. Hoda *et al.* (2016) reported that combinations of *Pseudomonas fluorescens* (Pf2), *Bacillus subtilis* (Bs3) and *Rahnella aquatilis* (Ra39) is beneficial in controlling black leg rot of potato caused by *Pectobacterium atrosepticum*. There seems to be no report available on the control of *Fusarium* wilt with the combinations of biocontrol agents on *Arachis hypogaea* L. The present study was taken whether specific interactions between *Trichoderma* spp. and *Pseudomonas fluorescens* influence pathogenicity suppression by combinations of these two phylogeneically unrelated biocontrol agents on *Arachis hypogaea* L. as compared to single biocontrol agent.

Materials and Methods

Trichoderma viride, *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Fusarium oxysporum* were obtained from Institute of Microbial Technology (IMTECH, Chandigarh, MTCC Nos 2047,3112,664.2087, respectively) *Fusarium oxysporum* was grown on Potato Sucrose Agar for 30 days and further grown in Czapek s medium for 7 days and filtrate was taken. *Trichoderma viride* and *Trichoderma harzianum* were grown on Malt Extract agar and *Pseudomonas fluorescens* on Antartic Biotic Medium and grown on Czapek-Dox medium in conical flask. It was further centrifuged and culture filtrate was taken.

In vitro study

Conidial germination studies were carried out incavity slides by incubating in a moist chamber at room temperature. Conidial suspension of *Fusarium oxysporum* 8000-12000/ml was prepared, in sterile distilled water with the help of haemocytometer. 1 ml of conidial suspension was added in the cavity slides with each of six concentrations of culture filtrates of three biocontrol agents. For control, conidial suspension was maintained in the sterile distilled water. Slides in triplicates were maintained for each concentration. The slides were incubated in moist chamber at 30°C and conidial germination was observed after 24 h. The percentage of inhibition over control was calculated by the formula of Vincent (1927).

$$I = C-T \div C \times 100$$
Where,
I = Inhibition over control
C = % germination in control
T = % of germination in treated

Poisoned Plate Technique

The radial growth of the mycelium of *F. oxysporum* was measured by poisoned plate technique. After the sterilization of petriplates (9cm), PSA medium, corkborers and other glass wares in an autoclave at 121.5° C for 15 min with 15 1b/inch² pressure, the prepared culture filtrates of the biocontrol agents (7th day old culture) in six concentrations were added through a Seitz filter to the warm PSA medium separately. The plates were inoculated by placing 9 mm discs cut from the growing tip of 7 days old culture plates of *F. oxysporum*. All this was done under laminar flow chamber. PSA plates without any biocontrol agent served as control. The control and treated plates were maintained in triplicates. The inoculated plates were sealed with para film and incubated in BOD incubator at 28°± O.2C. The radial growth of the pathogen was measured in cm along the radial line of the mycelial growth in the petri plates after 7 days of treatment. The optimum inhibitory concentration of different biocontrol agents was determined based on the results of conidial germination and mycelial growth.

Enzyme production

Czapek-Dox broth supplemented with pectin as carbon source replacing sucrose was used. To 50 ml sterilized Czapek -Dox broth, the culture filtrate of *Tv+Pf* (1+2%), *Th+Pf* (1.5+2%) *Tv+Th* (1+1.5 %) in their OIC (Optimum Inhibitory Concentration) were amended to the media separately. The disc of 9 mm was cut and were inoculated in each flask and incubated in the BOD incubator at 28± 2°C for 7 days. The control (with pathogen) 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 double layered Whatman No. 1 filter paper. The filtrates were further centrifuged in a high speed, at 5,000 rpm for 10 min and the supernatant was used as the enzyme source.

Assay of pectin methyl esterase (PME) activity

The enzyme activity was done according to the method of Muse *et al.* (1972).

Assay of endo polygalacturonase (endoPG) activity

The activity of endo (PG) was assayed as per the method described by Mahadevan and Sridhar (1986) and the enzyme activity was calculated as

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

Where,

V = percent loss in viscosity.

T_o = 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 exo polygalacturonase (exoPG) activity

The activity of exo-PMG was estimated as per the method described by Mahadevan and Sridhar (1986) and was assayed by measuring the monomeric galacturonic acids released by the enzyme by catalysing the pectin degradation. The results were expressed as specific activity units (SAU). From the three-hour incubated reaction mixture, 2.0 ml aliquots were taken and 2 ml of DNS reagent was added and then heated in boiling water bath for 10 min. Then cooled and diluted with 10 ml of distilled water. The orange red colour was read at 575 nm. Control was maintained with boiled enzyme reaction mixture.

Assay of endo and exo pectin trans eliminase (endo and exo PTE) activity

The activity of the endo (PTE) was estimated by the viscometric method as described by (Mahadevan and Sridhar, 1986) and exo by determining the production of TBA reacting substances.

Statistical analysis

The statistical analysis of the experimental data was carried out according to ANOVA and significance within the column with Tukey HSD multiple range test (TMRT) at 5% level of significance (n=3).

Results

The highest PME activity was recorded in enzyme source of control by specific activity unit (89.25 SAU). The lowest rate of enzyme activity was observed in *Tv+Pf* treated enzyme source (17.16 SAU) followed by those treated *Th+ Pf* (21.97 SAU) and *Tv+Th* (22.52 SAU). Maximum inhibition of enzyme activity was recorded in *Tv+Pf* treated culture (80.77%) followed by *Th+Pf* (75.38%) and *Tv+Th* (74.76 %) (Fig. 1).
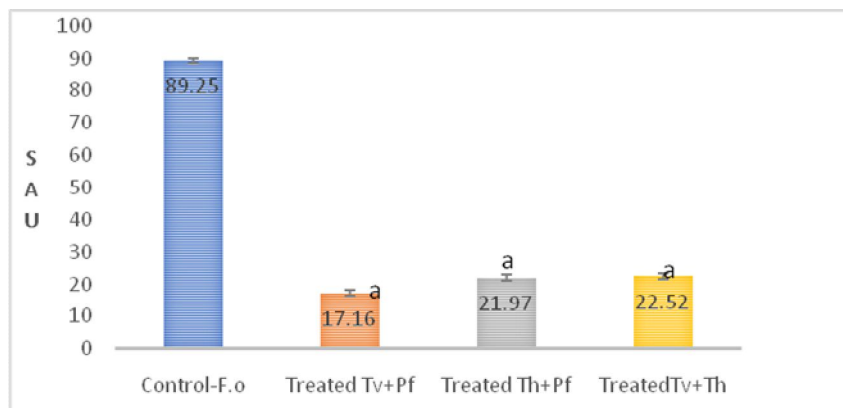

Fig.1 Effect of culture filtrates of *Trichoderma viride + Pseudomonas fluorescens* (*Tv + Pf*), *Trichoderma harzianum+ Pseudomonas fluorescens* (*Th + Pf*), *Trichoderma viride+ Trichoderma harzianum (Tv+ Th*) on the activity of polymethyl esterase of *Fusarium oxysporum* in *vitro*.

[a]*p< 0.001 as compared to control SAU= µ ml of 0.02 N NaOH required to maintain pH 7/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 endo- PMG of control reduced the viscosity of the substrate to 80% at 180 min. The least endo-PMG activity was observed in the enzyme source obtained from the culture treated with *Tv+Pf* (10.52% viscosity loss at 180 min) followed by those of *Th+Pf* (19.04%) and *Tv+Th* 26.66%). (Fig. 2).
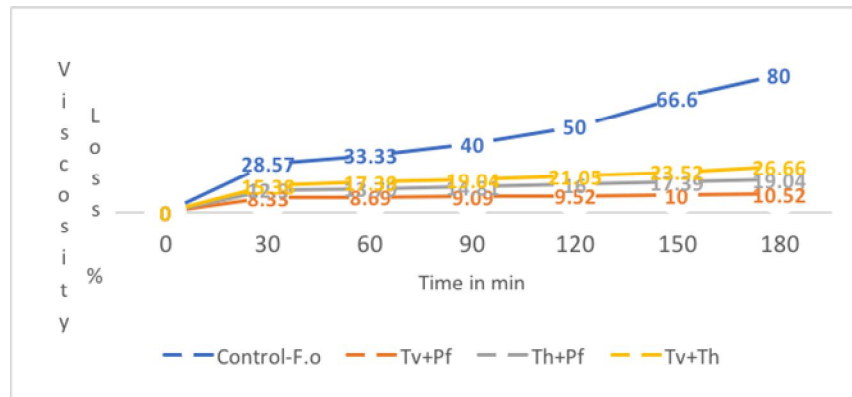
Fig.2. Effect of culture filtrates of *Trichoderma viride + Pseudomonas fluorescens* (*Tv + Pf*), *Trichoderma harzianum+ Pseudomonas fluorescens* (*Th + Pf*), *Trichoderma viride+ Trichoderma harzianum (Tv+ Th*) on the activity of Endo polymethylgalacturonase of *Fusarium oxysporum* in *vitro*.

Higher amount of monogalacturonic units was released in the case of enzyme source obtained from the control (507.33 SAU), followed by those of *Tv+Th* (226.54 SAU) and *Th+Pf* (170.55 SAU). Among the treatments the least amount of sugar was liberated in the case of enzyme source obtained from treatment *Tv+Pf* (91.00 SAU). (Fig. 3).
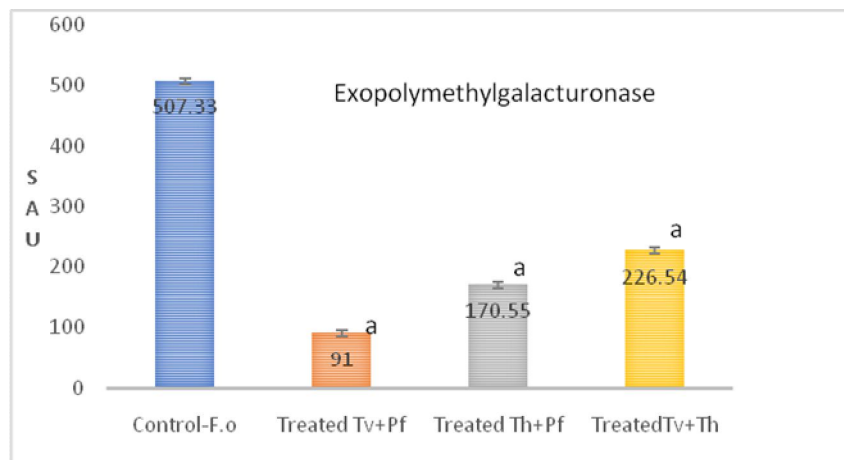

Fig.3 Effect of culture filtrates of *Trichoderma viride + Pseudomonas fluorescens* (*Tv + Pf*), *Trichoderma harzianum+ Pseudomonas fluorescens* (*Th + Pf*), *Trichoderma viride+ Trichoderma harzianum (Tv+ Th*) on the activity of Exo polymethylgalacturonase of *Fusarium oxysporum* in *vitro* [a]p< 0.001 as compared to control

The endo- PTE of control reduced the viscosity of the substrate to 66.66% at 180 min. The least endo- PTE activity was observed in the enzyme source obtained from the culture treated with *Tv+Pf* (14.28 %viscosity loss at180 min) followed by those of *Th+Pf* (17.39%) and *Tv+Th* (23.52%) at 180 min. (Fig. 4)
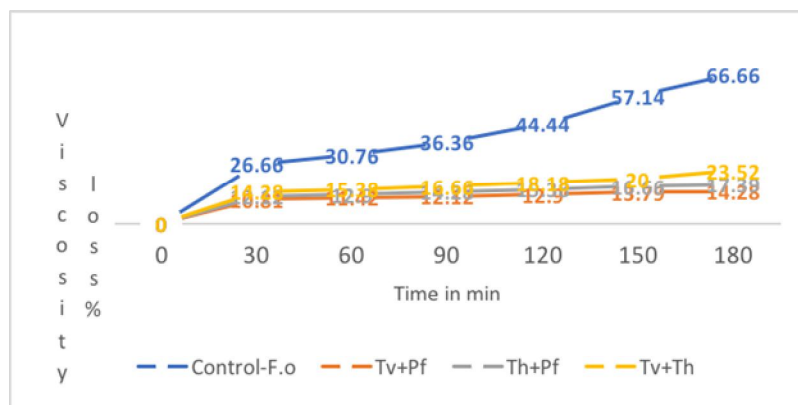
Fig. 4. Effect of culture filtrates *of T. viride + P. fluorescens* (*Tv + Pf*), *T. harzianum+ P. fluorescens* (*Th + Pf*), *T. viride+ T. harzianum (Tv+ Th*) on the activity of endopectintranseliminases of *Fusarium oxysporum in vitro*

The activity of exo-PTE was expressed in specific activity units (SAU). Higher amount of monogalacturonic units was released in the case of enzyme source obtained from the control (47.71 SAU). Maximum inhibition of enzyme activity was recorded in *T. viride+ P. fluorescens* (9.96 SAU) followed by those of *T. harzianum+ P. fluorescens* (17.22 SAU) and *T. viride + T. harzianum* (29.04 SAU). (Fig. 5)
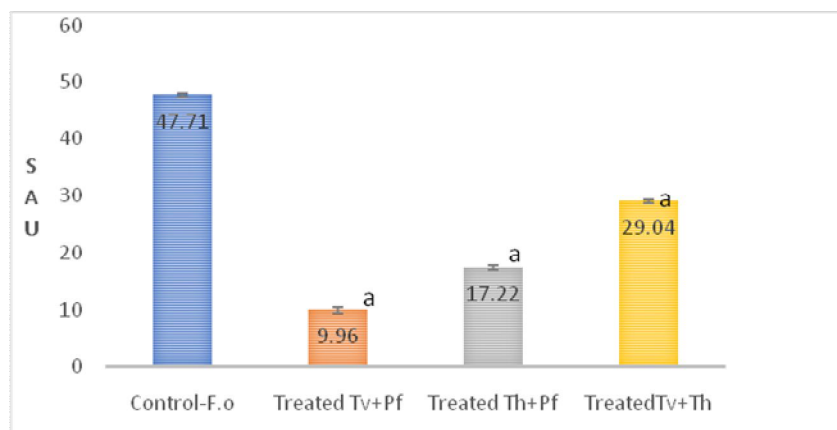

Fig. 5. Effect of culture filtrates of *Trichoderma viride + Pseudomonas fluorescens* (*Tv + Pf*), *Trichoderma harzianum+ Pseudomonas fluorescens* (*Th + Pf*), *Trichoderma viride+ Trichoderma harzianum (Tv+ Th) on* the activity of Exo pectin transeliminases of *Fusarium oxysporum* in *vitro*

[a]*p< 0.001 as compared to control   SAU= change in the absorbance at 547nm of 0.001/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

In the present study, the results show the highest Poly methyl galacturonase, polygalacturonase, pectin transeliminase activity was observed in enzyme source of control. Waggoner *et al.* (1955) reported that increased level of PME in diseased plants is due to the enzyme produced by the pathogen and not increased production of PME by the host in response to pathogen. Shukla and Dwivedi (2012) demonstrated that polygalacturonase, pectinmethylesterase, and Cellulose enzymes play an important role in inducing pathogenesis and the enzyme activity was increased in *Fusarium* sp. causing wilt in pigeon pea which determines higher pathogenicity in comparison to *Fusarium oxysporum* f.sp. *ciceri* causing wilt in chickpea.

Maximum inhibition of Pectin methyl galacturonase, polygalacturonase and pectin transeliminase enzyme activity was recorded in *Tv+Pf* (*Trichoderma viride + Pseudomonas fluorescens*) treated culture. Our results are in agreement with the earlier studies reported by (Manjula *et al.,* 2004) that the combined application *Pseudomonas fluorescens* and *Trichoderma viride* has improved the biocontrol activity against stem rot in groundnut.

Combination of biocontrol agents with different mechanisms of disease control will have an additive effect and results in enhanced disease control compared to their individual application (Guetsky *et al.,* 2002). 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 infenstans* and *Ralstonia solanacearum.* Also, our results indicated that inhibition of PME, PG, PTE was recorded in *Th+Pf* (*Trichoderma harzianum+ Pseudomonas fluorescens*) treated culture. Our results are in confirmation with the reports of several workers (Lutz *et al.,* 2004) who reported that the use of bacteria and fungi singly or in combination is a promising approach to improve efficacy of biocontrol treatments. This could be attributed to the involvement of different mechanisms in disease suppression like mycoparasitism, antibiosis or competition for place and nutrients. Pyrones such as 6-pentyl pyrone (produced by *T. atroviride* and *T. harzianum*) have antifungal activity against various pathogens including *Fusarium oxysporum* and *Bipolaris* (Reithner *et al.,* 2005; Mathivanan *et al.,* 2008; Rubio *et al.,* 2009). Glick (2015) demonstrated that inhibition of fungal pathogens by *Pseudomonas* spp., could be exerted by several mechanisms including production of antibiotics, (2, 4 diacetylphloroglucinol, fengycin, herbicolin, pyoluteorin, Phenazine-1-carboxylic acid, amphisin) HCN, siderophores, volatile compounds and competition for nutrients. Results revealed that inhibition of PME, PG, PTE was recorded in *Trichoderma viride + Trichoderma harzianum* (*Tv+Th*) treated culture. Our results also support the earlier findings of Khan *et al.* (2004). *Trichoderma harzianum* and *Trichoderma viride* are active rhizosphere colonizers and fungi produce antibiotics such as gliotoxin, viridian, cell wall degrading enzymes and biologically active heat stable metabolites such as ethyl acetate which are involved in disease suppression and plant growth promotion.

Conclusion

Pathogenicity suppression of *Fusarium oxysporum* a causative of *Arachis hypogaea*. L by the compatible combination of *Trichodema viride + Pseudomonas fluorescens* (1+2%) was significantly better as compared to other two combinations. This enhanced cell wall degrading enzyme suppression by the combination of bio-agents could be the possible mechanisms *viz.,* Production of antibiotics, volatile compounds, and competition for nutrients. The present study concluded that specific interactions between biocontrol agents could influence reduction of pathogenic virulence, which may leads to disease suppression by combinations of these bioagents.

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