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Combination of *Trichoderma viride* and *Pseudomonas fluorescens* for the enhanced control of *Fusarium wilt* disease caused by *Fusarium oxysporum* infecting *Arachis hypogaea* L.

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Abstract

Fusarium wilt caused by *Fusarium oxysporum* is a devastating disease of peanut. The fungus causes severe yield loss in groundnut. Combinations of biocontrol agents that are compatible with each other is a viable approach to control the plant disease. The study was conducted to determine the beneficiary aspects of combining different species of *Trichoderma* and *Pseudomonas fluorescens* i.e *Trichoderma viride*+*Pseudomonas fluorescens* (Tv+Pf), *Trichoderma harzianum*+*Pseudomonas fluorescens* (Th+Pf) and *Trichoderma viride* +*Trichoderma harzianum* (Tv+Th) to control the *Fusarium wilt* in biochemical parameters such as DNA, RNA, Amino nitrogen, phenols, dihydroxy and proline contents of *Arachis hypogaea* L. Among the three combinations tested, *Trichoderma viride* + *Pseudomonas fluorescens* (1+2%) sprayed leaves provided greater suppression of *Fusarium oxysporum* by increasing the levels of DNA, RNA, Amino nitrogen contents resulting in the suppression of *Fusarium wilt* disease of *Arachis hypogaea* L. Maximum reduction of DNA, RNA, Amino nitrogen was observed in the infected *Fusarium oxysporum* leaves Phenol, Dihydroxy phenols and proline contents increase sharply in the treated plants treated with (Tv+Pf) as compared to the control plants. At the same time the other two combinations resulted in enhanced control in comparison with individual ones. This present study indicates that specific combination of *Trichoderma viride* and *Pseudomonas fluorescens* could have the greater efficacy in the inhibition of pathogen in the biocontrol of *Fusarium wilt* disease as compared with individual strains.

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

How to Cite

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is the most versatile legume and important oil seed crop grown worldwide. Grover (1981) documented that more than 55 pathogens were causing damage to ground nut crop. The crop improves soil fertility through the fixation of atmospheric dinitrogen into the soil and reducing to synthetic nitrogen fertilizers required. (Dakora *et al.*, 1987; Mokgehle *et al.*, 2014). Ground nut is the fourth largest edible oil crop in the world. (Shilman *et al.*, 2011) *Fusarium oxysporum* is the causal agent of vascular wilt disease that affects large variety of economically important plants (Beckman 1987). *Fusarium wilt* disease caused by *Fusarium oxysporum* (Schlecht. Emend.Snyder and Hansen) is a damaging disease which causes considerable yield loss in groundnut. The fungus invades through roots and causes complete loss in yield, when occurs at vegetative and reproductive stages of crop. (Navas *et al.*, 2000).

Infection by pathogen interferes with host nucleic

acids and protein metabolism especially enzymes. Padma Singh (2000) reported that disruption of cell structure coupled with enhanced proteolytic enzyme activity results in enhanced disease and decreased nitrogen content in the *Alternaria sp.* - onion infected leaves. Duthie *et al* (2003); Fraga *et al* (2010) studied that phenolic compounds are produced by plants as secondary metabolites participating in growth, lignification, pigmentation, pollination, and resistance against pathogens, predators, and environmental stresses They also participate in oxidation-reduction reactions, in the inhibition of production of cell wall degrading enzymes by the pathogen and involved in stimulation of auxin activity. (Mandavia *et al.*, 2003). Proline is a proteinogenic amino acid and is essential for primary metabolism (Szabados and Savoure 2009; Verslues and Sharma 2010). Dar *et al* (2016) reported that Proline accumulation occurs under various kinds of environmental stresses in many plant species. Earlier studies demonstrated successfully that *Trichoderma* spp. and *Pseudomonas fluorescens* have been con-

sidered as the most effective antagonistic microbes in biocontrol of Fusarium wilt (Sharavanan *et al.* 2003). Kemerait Jr. (2000) evaluated the pathogenicity of soil borne fungi associated with groundnut. The strains of *Trichoderma* spp. produces cell wall degrading enzymes, antibiotics and also many types of secondary metabolites the role of which has been established in biocontrol activity. Woo and Lorito (2007) demonstrated that a strain of *T. harzianum* produced different secondary metabolites that could further focus on the inhibiting the pathogen invasion infecting that plant. Alabouvette *et al.* (2009) reported that the success of biological control depends not only on the mode of action of biocontrol agents but also on protective effects of BCA'S in targeting the pathogen to improve the production, formulation and application processes. Talaviya and Jadega (2015) reported that combined application of *T. viride*+ *T. harzianum*+ *Pseudomonas fluorescens* was significantly effective in controlling cumint wilt disease and improves higher yield. Rajeswari and Kapoor (2017) reported that the combination of *Trichoderma viride* and *Pseudomonas fluorescens* is effective in managing the *Fusarium* wilt disease in *Arachis hypogaea* L as compared to single strains. Zhang *et al.* (2008) has established combined application of *Basillus subtilis* SQR-5 and *Paenibacillus polymyxa* SQR-21 is beneficial in control of *Fusarium* wilt disease in cucumber plants. Wu *et al.* (2009) studied that combinations of antagonistic microorganisms (*Paenibacillus polymyxa* and *Trichoderma harzianum*) had a greater efficacy to suppress *Fusarium* wilt of watermelon. Somasekhara *et al.* (1996) reported that different species of *Trichoderma* i.e *T. viride*, *T.harzianum* and *T.hamatum* are effective in controlling *F.oxysporum* *F.sp.udum*. in pigeon pea wilt. Nikam *et al.* (2007) established that *T. viride* showed maximum growth inhibition of *F. oxysporum* f.sp.ciceri. Najar *et al.* (2011) evaluated the efficacy of *Trichoderma viride*, *Trichoderma harzianum* and *Pseudomonas fluorescens* in the inhibition of *Fusarium solani* f.sp. *melongenae* causing wilt of Brinjal. The present work was taken to determine that whether specific interactions of *Trichoderma* species and *Pseudomonas fluorescens* influence the suppression of *Fusarium* wilt disease by combination of these biocontrol agents on *Arachis hypogaea* L. as compared to individual strain.

MATERIALS AND METHODS

Microbial cultures: *Trichoderma viride*, *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Fusarium oxysporum* cultures used in this study were obtained from Institute of Microbial Technology (IMTECH, Chandigarh. MTCC Nos 2047, 3112, 664. 2087, respectively). *Fusarium oxysporum* was cultured on Potato Sucrose Agar

(PSA) for 30 days. *Trichoderma viride* and *Trichoderma harzianum* were grown on Malt Extract Agar (MA) .*Pseudomonas fluorescens* was cultured on Antarctic biotic Medium (ABM) for 30 days. The above mentioned cultures further grown on Czapek's medium separately for 7 days at 28° ±0.2C. After the centrifugation culture filtrates were taken.

Plant material: The plants of *Arachis hypogaea* (JLR –variety) were grown up to 75 DAS in twenty earthen pots (25cm diameter)and grouped into three sets- control, infected and infected treated. The first set of four pots as control plants was sprayed with distilled water (sample 1). The second set of four pots as infected plants were sprayed with culture of pathogen, *Fusarium oxysporum* on 30 DAS and left without any treatment (sample 2). The third set of twelve pots marked as infected treated was sprayed with pathogen on 30 DAS and these infected plants were sprayed with OIC of culture filtrates of combinations of biocontrol agents, *Trichoderma* spp. and *Pseudomonas fluorescens*: *Trichoderma viride* + *Pseudomonas fluorescens*(Tv + Pf)(1+2%) as sample 3, *Trichoderma harzianum* + *Pseudomonas fluorescens* (Th +Pf)(1.5+2%)as sample 4, and *Trichoderma viride* + *Trichoderma harzianum* (Tv+ Th)(1+1.5%) as sample 5 on 40 DAS. The leaves of control, infected and infected treated plants were collected on 50 DAS for estimating the biochemical parameters.

DNA estimation: In DNA estimation, the method of Burton (1956) was used. To 1.5 ml of PCA extract, 3ml of diphenylamine reagent was added. The tubes were kept at 70°C in a boiling water bath for 20 min and then cooled. The colour development was read at 600 nm on a Spectrophotometer. A Standard calibration curve was prepared by using known concentration of calf thymus DNA. The DNA content was expressed in mg DNA/g fresh weight of the leaf tissue.

Estimation of RNA: RNA was estimated by using the method of Rawal *et al.* (1977). To the 0.5 ml nucleic acid fractions, 3 ml of orcinol reagent was added. The tubes containing the mixture were kept in water bath for 20 min at 90°C and then cooled. The colour that was produced was measured at 665 nm in Spectrophotometer. By taking the known concentration of purified RNA the standard curve was prepared.

Estimation of aminonitrogen: Amino nitrogen was estimated by using the method of Levine and Chargoff (1951).

The pH of the alcoholic extract was adjusted to 7.0 by adding 0.1 N NaOH/HCl. To 1 ml of the above extract 1 ml of ninhydrin reagent was added. The tubes was heated for 20 min and cooled. To the above mixture, 5 ml of methyl cellosolve was added and the absorbance was read at 570 nm in Systronics Spectrophotometer.

Estimation of total phenol: The method of Bray and Thorpe (1954) was used for Total phenol estimation. 1 ml of alcoholic extract was taken in the test tube. To this, 1 ml of Folin-Ciocalteau reagent and 2 ml of 20% sodium carbonate were added and shaken well. The above mixture was heated in a boiling water bath for 1 min and cooled under running tap water. The blue colour solution formed was diluted to 25 ml with distilled water and read at 650 nm in Systronics Spectrophotometer. Total phenols were calculated using a standard curve catechol as standard.

Estimation of ortho Di-hydroxy phenols: Ortho Di-hydroxy phenols was estimated by the method proposed by Johnson and Shoal (1952) was used. 1 ml of alcoholic extract was taken in the test tube. To this, 1 ml of 0.5 N HCl and 1 ml of Arnov's reagent was added. To the above mixture 2 ml of 1 N NaOH and 10 ml of distilled water were added. NaOH was added slowly. The pink colour appears and the colour intensity was reduced by diluting it to 25 ml with distilled water and the absorbance read at 515 nm. Standard curve was prepared using catechol as standard.

Estimation of proline: Proline extraction and estimation was done according to the method of Bates *et al* (1973). Fresh plant material was homogenized with 10 ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman No. 2 filter paper. The residue was re-extracted 3 to 4 times. The extracts were pooled

and made up to 20 ml with aqueous sulfosalicylic acid and used for the estimation.

2.0 ml of the above filtrate was taken in the test tube. To this, 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid was added. The mixture containing tubes were incubated for 1h at 100°C on a water bath. The tubes were transferred on ice to terminate the reaction After that 4.0 ml of toluene was added and shaken vigorously for 15-20 seconds. The chromophore containing toluene was aspirated from the aqueous phase It was allowed to reach room temperature and the absorbance measured at 575 nm. By taking the known concentrations of Proline standard curve was prepared.

RESULTS AND DISCUSSION

In this study among the three combinations of *Trichoderma* spp and *Pseudomonas fluorescens*, *Trichoderma viride* and *Pseudomonas fluorescens* (1+2%) was found significantly effective in increasing the DNA, RNA, and Amino nitrogen content of the leaves. This study confirms to the earlier findings of Rudresh *et al.* (2005) that the combined inoculation of *Rhizobium* spp with *Trichoderma* spp resulted in increased growth, nutrient uptake and yield of chickpea. John *et al.* (2010) found that soybean plants treated with *Trichoderma viride* showed higher total nitrogen, carbon and dry weight and established the enhancement

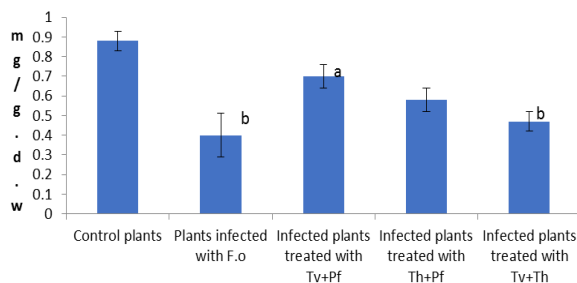


Fig. 1. Effect of culture filtrates of *T. viride* + *P. fluorescens*, *T. harzianum* + *P. fluorescens* and *T. viride* + *T. harzianum* on Amino nitrogen content of *Arachis hypogaea* leaves infected with *F. oxysporum*. ^a*p*< 0.001 as compared to control, ^b*p*<0.01 as compared to control; Values within column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3).

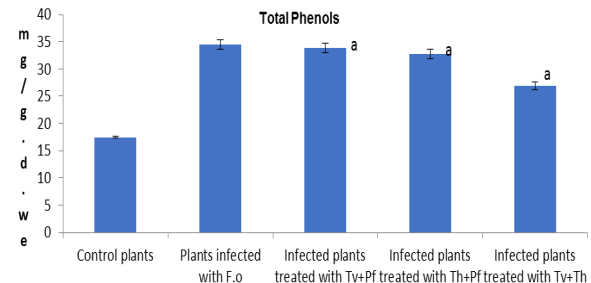


Fig. 2. Effect of culture filtrates of *T. viride*+*P. fluorescens*, *T. harzianum*+*P. fluorescens* and *T. viride* + *T. harzianum* on Total phenols content of *Arachis hypogaea* leaves infected with *F. oxysporum*. ^a*p*< 0.001 as compared to control; Values within column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3).

Table 1. Effect of culture filtrates of *T. viride*+*P. fluorescens*, *T. harzianum*+*P. fluorescens* and *T. viride* + *T. harzianum* on DNA and RNA content of *Arachis hypogaea* leaves infected with *F. oxysporum*. ^a*p*< 0.001 as compared to control; Values within column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3)

Treatments	DNA (mg/g.f.w)	RNA(mg/g.f.w)
Control plants	33.93± 0.34	48.39 ±0.34
Plants infected with <i>F. oxysporum</i>	6.42 ±0.0.34 ^a	12.24 ±0.35 ^a
Infected plants treated with <i>T. viride</i> + <i>P. fluorescens</i>	26.90 ±0.0.34 ^a	24.49 ±0.34 ^a
Infected plants treated with <i>T. harzianum</i> + <i>P. fluorescens</i>	18.47 ±0.34 ^a	17.06 ±0.34 ^a
Infected plants treated with <i>T. viride</i> + <i>T. harzianum</i>	17.20 ±0.34 ^a	16.66±0.34 ^a

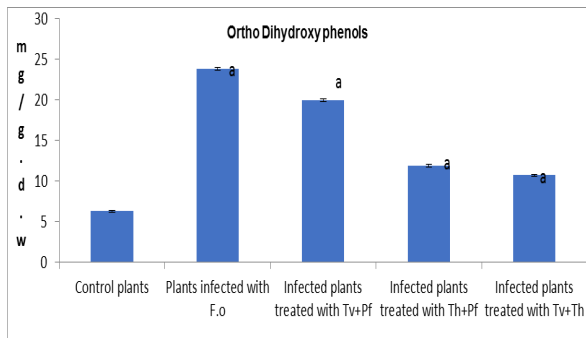


Fig. 3. Effect of culture filtrates of *T.viride*+*P.fluorescens*, *T.harzianum*+*P.fluorescens* and *T.viride* + *T. harzianum* on ortho dihydroxy content of *Arachis hypogaea* leaves infected with *F.oxysporum*. ^ap< 0.001 as compared to control; Values within column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3).

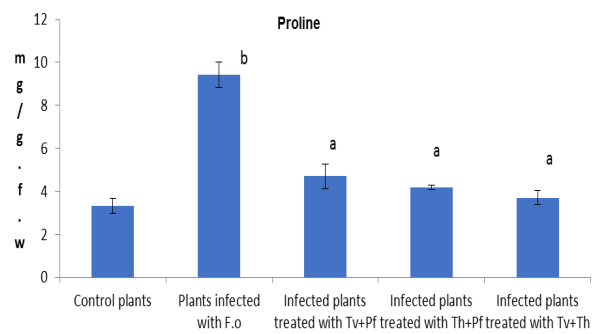


Fig. 4. Effect of culture filtrates of *T.viride*+*P.fluorescens*, *T.harzianum*+*P.fluorescens* and *T.viride* + *T. harzianum* on proline content of *Arachis hypogaea* leaves infected with *F.oxysporum*. ^ap< 0.001 as compared to control, ^bp<0.01 as compared to control; Values within column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3).

of plant growth compared with the plants infected with *Pythium arrhenomanes* and *Fusarium oxysporum f.sp. adzuki*.

Results revealed that 3-fold increase in phenol, dihydroxy and proline contents in *Fusarium oxysporum* infected leaf compared to control. (Fig 2,3,4). De Ascensao and Dubery (2003) studied the changes over time in specific phenolic compounds in banana in response to the pathogen *F. oxysporum f. sp. cubense*. They reported that induced phenolics included coumaric, ferulic, sinapic and vanillic acid. These are cell wall bound phenolics esterified to the cell wall and cross link to form lignin-like polymers reinforcing the cell wall in order to provide defense against the invading pathogen.

Panina *et al.* (2007) also observed the changes in phenolic compounds in leaves after exposing roots to *Fusarium oxysporum*. This indicates that the response is truly systemic and that the physiological state of the tomato plant has been altered. This is in agreement with reports of increase and decrease in tomato phenolics in response to other biotic and abiotic stressors (Tamietti *et al.*, 1993; Di'az *et al.*, 2005; Cavalcanti *et al.*, 2006). Ojha and Chatterjee (2012) observed that the phenol content was significantly higher in *F. oxysporum* -infected tomato plants. Induction of total phenol accumulation in the host plant treated with salicylic acid and *Trichoderma harzianum* could play a vital role in resistance and defense against *F. oxysporum*. Similar findings was made by (Alstrom 1995) that the higher phenol content due to *Pseudomonas* treatment in tomato plant infected with *P. syringe*. Beckman (2000) has pointed that the importance of phenolic compounds could play a

major role in host defence pathways and in signalling for host defences rather than their toxicity to the pathogen in reducing the wilt disease.

Combination of biocontrol agents with different mechanisms of disease control will have a beneficial effects and results in enhanced disease control compared to their individual ones (Guetsky *et al.*, 2002). Results also illustrated that leaves sprayed with combinations of *Trichoderma harzianum* and *Pseudomonas fluorescens* (*Th+Pf*) and *Trichoderma viride* and *Trichoderma harzianum* (*Tv+Th*) were significantly effective in increasing the amount of DNA, RNA, Amino nitrogen content when compared to individual strains.(Table1, Fig1) Phenols, Dihydroxy and proline contents were also significantly increased in the treated leaves. (Fig 2,3,4). Present study reveals that among the three combinations of *Trichoderma* spp and *Pseudomonas fluorescens*, a significant combination of *Trichoderma viride* and *Pseudomonas fluorescens* (*Tv +Pf*) (1+2%) was found to have greater efficacy than other two combinations in the control of *Fusarium* wilt in *Arachis hypogaea* L. The study substantiates the findings of Hoda *et al* (2016) who established that the combined application of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Rahnella aquatilis* have potential benefits in the enhanced suppression of black leg of potato. Similar findings was made by Manjula *et al.* (2004) *Pseudomonas fluorescens* combined with *Trichoderma viride* has improved the biocontrol activity against stem rot in groundnut.

Conclusion

Pathogenecity suppression of *Fusarium oxysporum* by the compatible combination of *Trichoderma viride* +*Pseudomonas fluorescens* (1+2%) was significantly better compared to the other two combinations. This enhanced control of

the disease by the combinations of biocontrol agents could be possible by different mechanisms i.e mycoparasitism, spacial and nutrient competition, production of antibiotics and volatile compounds compared to individual ones. The present study concluded that specific interactions of biocontrol agents could influence the suppression of *Fusarium* wilt disease by combination of these bioagents.

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