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# AN *IN VITRO* ANTAGONISTIC EFFECT OF Trichoderma spp. AGAINST *Fusarium oxysporum* f.sp. *lycopersici*

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**ABSTRACT**
The experiment was conducted in the Department of Plant Pathology, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, India. Diseased tomato stems and roots showing symptoms of Fusarium wilt were collected from the Research and Training plots of the Department. The pathogen isolated was identified as *Fusarium oxysporum* f.sp. *lycopersici.* Nine native *Trichoderma* strains were isolated from healthy tomato rhizosphere soils from three different locations of Allahabad; viz Naini, Sangam and Prayagraj. Six isolates were found to be strains of *Trichoderma harzianum* (SP-4, Th-6, Th-3, Th-5, RTM-16 and EPI-5) while the three were of *Trichoderma viride* (SP-1, KI-9, SP-3). They were examined in the laboratory for the control of pathogen *Fusarium oxysporum*. The results revealed that all Trichoderma spp. isolates were found to effectively inhibit the mycelial growth of the pathogen compared to control. However, significant differences between the species and strains were found ($P$<0.005). The isolates SP-1, SP-3 and Th-6 showed significant inhibition of mycelial growth of the pathogen. In this study, while both Trichoderma strains had a considerable antagonistic effect on the tested pathogen, SP-3 and SP-1 were respectively found to be more successful having the higher percent inhibition of 97.68% and 92.83% at 7 days after incubation. It shows that the strong repressive effect of *T. viride* isolates (SP-3 and SP-1) can be applied in biological control of *Fusarium oxsporum.*

**Keywords:** Antagonistic, Fusarium, Tomato and Trichoderma

## INTRODUCTION

Tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the prevalent diseases of tomato crop and can cause losses ranging from 30 to 80%, especially in local varieties and under favorable climatic conditions for the pathogen development (Buitrago *et al*., 2006; FENALCE, 2007). The disease symptoms starts with an ascending chlorosis, followed by plant stunting, in the outside of roots, a brown coloration is seen and in the inner part, a longitudinal lesion of reddish tonality. The fungus elicits a premature death on the host plant which is associated to redness and blockage of vascular bundles (Buitrago *et al*., 2006; FENALCE, 2007). As Fusarium wilt is soil-borne, application of fungicides to control this disease is found to be difficult. Besides, chemicals pose serious health hazards to an applicator as well as to a consumer of the treated material (Hayes and Laws, 1991). In addition to target organism, pesticides induce various hazards to beneficial organisms. Their toxic forms persist in soil and contaminate the whole environment (Hayes and Laws, 1991). Prospects of biological control of soil-borne plant pathogens using most promising biocontrol agent, the genus *Trichoderma* has been described (Morsy *et al.* 2009 ; Sabalpara *et al*. 2009).

*Trichoderma* is a common saprophytic filamentous fungus which inhibits rhizospheric soil. It acts as a biocontrol agent against various plant pathogens that causes several diseases in mono and dicotyledonous crop plants (Galarza *et al*., 2015; Singh *et al.*, 2014; Padmaja *et al.*, 2015). *Trichoderma* spp. are equally commercialized as biopesticides, biofertilizers, and soil enhancers. They are nonpathogenic microorganisms that provide protection against fungal diseases caused by Phytophthora, Rhizoctonia, Sclerotium, Pythium, and Fusarium genera (Peteira *et al.*, 2001) additionally, they promote high yields in crops. These traits are derived from their ability to produce antifungal metabolites, release hydrolytic enzymes, and their mycoparasitic behavior, as well as the production of other substances that enhance plant growth (Suárez-Mesa *et al*., 2008). Therefore the objectives of the present study were to assess the ability of nine isolates of both *Trichoderma viride* and *Trichoderma harzianum* in suppressing the populations of *Fusarium oxysporum* f. sp. *lycopersici* in tomato under *in vitro* condition and to identify isolates with the highest capacity for pathogen inhibition.

## MATERIALS AND METHODS

### Study Area

The experiment was carried out in the Plant Pathology Laboratory, Department of Plant Pathology and Entomology, Sam Higginbottom University of Agriculture, Allahabad (25º27'N 81º51'E), India. Tomato plant samples were collected from the research and training plots of the department. Soils from Naini, Sangam and Prayagraj regions of Allahabad were used for isolation of the antagonists.

### Isolation and Purification of *Fusarium oxysporum*

Infected vascular tissues from stem and root regions of tomato cultivar showing wilt symptoms were collected separately from farmer's field. Tissue bits were surface sterilized with 2 per cent sodium hypochlorite for 5- 10 minutes and subsequently three washings with sterile distilled water. Then, they were placed on potato dextrose agar (PDA) medium separately and incubated at the laboratory conditions at 25 ± 2º C for five days. The fungus was purified separately by transferring the tip of the mycelia into PDA slants and maintained as stock cultures.

### Source of Trichoderma Species Isolates

*T. viride* isolates (SP-1, KI-9, SP-3) and *T. harzianum* isolates (SP-4, Th-6, Th-3, Th-5, RTM-16 and EPI-5) were obtained from Microbial Culture Collection of the Department of Plant Pathology and Entomology, Sam Higginbottom University of Agriculture, Allahabad, India. The isolates were maintained on a potato dextrose agar (PDA) medium and stored at 4°C for further use.

### Evaluation of *in vitro* Antagonistic Effect of Trichoderma spp. against *F. oxysporum*

Each of the nine strains of Trichoderma species was studied with *F. oxysporum* strain in a dual culture assay using sterile PDA as the growth medium. 90 mm Petri plates were used as described by (Altinok 2009). In each plate, a 5mm disc of culture medium containing mycelium and conidia of *F. oxysporum* was transferred to one side of the Petri plate containing the PDA. Another disc of culture medium containing mycelium and conidia of the Trichoderma isolate was transferred to the opposite position of the disc with *F. oxysporum* microstructures. The plates were incubated at 25 ± 2 °C and after seven days, the diameter of colonies of the pathogen were measured with the aid of a digital caliper and the antagonism degree was evaluated according to mycoparasitism of Trichoderma strains on the pathogen. The mycoparasitism was determined based on the ability of the Trichoderma strain to inhibit the growth of the Fusarium in each plate (Bell *et al.*, 1982). Similar antagonism test was performed among the three best isolates and Mycelial growth of *F.oxysporum* was measured on the 3rd to 9th days after inoculation (DAI). This dual culture assay was carried out in a completely randomized experimental design and replicated four times.

### Statistical Analysis

Data were analyzed using statistical package SPSS version 22.0 and means were separated using DMRT at ($p<0.05$).

## RESULTS

### Identification of Trichoderma Species

Fungal hyphae of *Trichoderma* species were found to be septate, hyaline and smooth-walled that produces highly branched numerous conidiophores. Normally, the branches were formed at or near 90° with respect to the main branch. Conidia were one-celled, and either ellipsoidal (3-5 × 2-4µm) or globose. They were typically light to dark green or sometimes white to whitish green with typically smooth or rough surfaces. Some strains produced chlamydospores which play important role in survival. The chlamydospores are either formed within hyphae or at the hyphal tips (Bisset 1984; 1991).

**Table 1: Identification of Trichoderma Species**

| SI. No. | Trichoderma specie | Designation of Trichoderma Isolates | Colony Colour |
|---|---|---|---|
| 1 | *Trichoderma viride* | SP-1 | Dark green |
| 2 | *Trichoderma viride* | KI-9 | Green |
| 3 | *Trichoderma viride* | SP-3 | Whitish dark-green |
| 4 | *Trichoderma harzianum* | SP-4 | Pale green |
| 5 | *Trichoderma harzianum* | Th-6 | Whitish pale-green |
| 6 | *Trichoderma harzianum* | Th-3 | Whitish green |
| 7 | *Trichoderma harzianum* | Th-5 | White |
| 8 | *Trichoderma harzianum* | RTM-16 | Dark green |
| 9 | *Trichoderma harzianum* | EPI-5 | Dark green |

**Evaluation of Antagonistic Activity of Trichoderma Species Isolates**

During initial screening of the Trichoderma isolates a variety of reactions were recorded as a result of antagonism. The results revealed that after 7 days of incubation a significant inhibition of Fusarium growth was produced by Trichoderma spp. Contact between Fusarium and Trichoderma isolates occurred but the ability to overgrow and to parasitise the mycelia of the fungus was highly dependent on the antagonistic potential of each Trichoderma isolate and the resistance of the challenged fungus to antagonism (Table 2 & 3). The isolates SP-1, SP-3 (from *Trichoderma viride*) and Th-6 (from *Trichoderma harzianum*) showed the strongest antagonistic potential with a statistically similar performance ($P<0.05$). RTM-16 and Th-3, however, had respectively the weakest effect (30.40 and 32.14%).

Mycelia growth of the pathogen was measured using a scale rule in mm while percent inhibition over control was calculated using the equation (Vincent, 1927).

$PI\% = (C-T)/C \times 100$

Where,

PI = Per cent inhibition over control

C = Growth of test pathogen with absence of antagonist (mm)

T = Growth of test pathogen with antagonist (mm)( Hajieghrari, 2008).

**Table 2: Antagonistic Effect of Trichoderma spp. on Mycelial Growth of *Fusarium oxysporum* f.sp. *lycopersici***

| Trt. No. | Trichoderma Isolates | Mycelial Growth (mm) | Percent Inhibition Over Control |
|---|---|---|---|
| T1 | SP-1 | 17.22 ± 5.21 | 80.03[a] |
| T2 | KI-9 | 45.50 ± 5.51 | 47.22[b] |
| T3 | SP-3 | 27.37± 2.10 | 69.28[ab] |
| T4 | SP-4 | 37.50 ±3.30 | 56.50[b] |
| T5 | Th-6 | 24.11 ±6.13 | 72.83[ab] |
| T6 | Th-3 | 58.50 | 32.14[d] |
| T7 | Th-5 | 41.37 ±4.00 | 52.01[b] |
| T8 | RTM-16 | 60.00 ±6.12 | 30.40[d] |
| T9 | EPI-5 | 49.99 ±7.35 | 42.01[c] |
| T10 | Control | 86.21 ±4.98 | 0.00 |

Values represent mean ± SD of four replicates. Measurements of radial growth were taken 5 days after inoculation. Means within columns followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

**Effect of Trichoderma spp. on Fusarium *oxysporum* Days after Inoculation**

The results of dual culture indicated that Trichoderma spp. significantly ($P<0.05$) inhibited the growth of *F. oxysporum* at varying degrees across duration of incubation (Table 3). The findings revealed that the inhibitory activity averagely started at 72 hours after inoculation. Later, the activity kept rising till reaches maximum, at 144 hours after which diminishes significantly up to 216 hours after inoculation. This shows that all the treatments inhibited the pathogen best from 168 hours after inoculation, with *T. viride* isolate, SP-3, having the highest percent inhibition (97.68%). This was followed by SP-1 (92.83%) which were found to be statistically similar ($P<0.05$). Among the promising antagonists, *T. harzianum* isolate (Th-6), performed least with 70.24% as percent inhibition against the fungus.

**Table 3: Promising antagonists based on Incubation Period and Inhibition of the Pathogen**

| Incubation Period | Zone of Inhibition(mm) | | | Percent Inhibition | | |
|---|---|---|---|---|---|---|
| | SP-1 | SP-3 | Th-6 | SP-1 | SP-3 | Th-6 |
| 72 hours | 79.10±8.60 | 84.12±8.10 | 61.22±8.12 | 8.25[d] | 2.42[c] | 28.99[d] |
| 96 hours | 62.11±1.22 | 79.80±11.11 | 48.00 | 27.95[c] | 7.44[c] | 44.32[c] |
| 120 hours | 32.10±7.10 | 29.22±2.22 | 31.00±2.00 | 62.77[b] | 66.11[b] | 64.04[b] |
| 144 hours | 14.10±1.61 | 8.99±3.30 | 28.18±6.60 | 83.64[ab] | 89.58[a] | 67.31[b] |
| 168 hours | 6.18±0.91 | 2.00±0.61 | 25.66±7.11 | 92.83[a] | 97.68[a] | 70.24[a] |
| 192 hours | 6.00±1.23 | 2.11±1.10 | 25.12±6.66 | 93.04[a] | 97.55[a] | 70.86[a] |
| 216 hours | 6.81±0.99 | 2.10±0.88 | 24.99±6.54 | 92.10[a] | 97.56[a] | 71.01[a] |

Values represent mean ± SD of four replicates. Measurements of radial growth were taken 3 to 9 days after inoculation. Means within columns followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).


**A**: *T. harzianum* (Th-6) with *F. oxysporum* **B**: *T. viride* (SP-1) with *F. oxysporum* **C**: *T. viride* (SP-3) with *F. oxysporum*

***In vitro* antagonistic effect of *Trichoderma* spp. isolates on tomato wilt pathogen (*Fusarium oxysporium* f.sp. *lycopersici*) hours after inoculation**

## DISCUSSION

*T. harzianum* and *T. viride* isolates used in this work were able to reduced mycelial growth of *F. oxysporum* when grown in dual culture irrespective of the time of introduction of the antagonist. However, it is found that the highest inhibition is observed at seven days after inoculation. The study also showed that among the strains of *Trichoderma* spp. isolates tested against *F. oxysporum*. f.sp. *lycopersici*, the strains SP-1, SP-3 and Th-6 showed a significant inhibitory effect on the mycelial growth of pathogen compared to other strains and control. It was found that during parasitism *Trichoderma* spp. showed a target-directed growth towards the mycelia of its hosts and an increased formation of conidiophores, phialides and conidia. Formation of apressoria-like structures enabled the hyphae of *Trichoderma* spp. to attach firmly to the surface of its host mycelia. Penetration of the mycelia occurred with fie hyphae. The secretion of lytic enzymes and fungicidal substances lead to complete cell wall degradation and efflux of cytoplasm.

This study agreed with the work of Ekefan *et al*. (2009) who showed that *T. harzianum* isolates suppressed the growth of *Colletotrichum capsici* eventually overgrowing it within seven days. It is equally similar with the findings of Azza and Allam (2004) who demonstrated that the *Trichoderma* isolates have a strong antagonism against wilt diseases caused by Fusarium sp, *in vitro*, on potato dextrose agar medium, whereby the mycelia growths were decreased by 88%, 86% and 80% by *Trichoderma harzianum*, *T. hamatum* and *T. viride* respectively. Also, our results tally with Hibar *et al.* (2005) who demonstrated that *Trichoderma harzianum* inhibited the growth of pathogenic fungus, *Fusarium oxysporium*, *in vitro*, with a ratio more than 65%. Moreover, the volatile metabolic substances of the antagonist reduced the pathogenic fungus growth by 63% compared with controls. Our work is in harmony with the dual culture experiments of Hacer *et al.* (2015), who studied the *in vitro* effect of *T. harzianum* against *F. oxysporum* with the strains T16 and T23 showing a significant inhibitory effect on the mycelia growth of the pathogen when compared to the control. *T. harzianum* (T16) grew much faster on PDA than the tested pathogens under the same culture conditions. The maximum inhibition was recorded when the *T. harzianum strain* T16 was used (72.69%). The study concluded that T16 was more efficient than strain T23 in inhibiting colony growth of the pathogen *F. oxysporum* isolates.

Nevertheless, Calistru *et al.* (1997) and Elkatatny *et al*. (2006) discovered that microscopic studies showed no overgrowth, hyphal penetration, or hyphal coiling (hyperparasitism) of Fusarium around hyphae of pathogenic *F. oxysporum* strains, suggesting that mycoparasitism was not a major mechanism for the observed inhibitory effects. The pathogen and Trichoderma hyphae did not interfere with each other in dual cultures and this was explained by the production of extracellular mycolytic enzymes by *T. harzianum.* They suggested that the extracellular mycolytic enzymes secreted by *Trichoderma* might play an important role in antibiosis against pathogenic *F. oxysporum.* On the other hand, fungitoxic metabolite secretion by *Trichoderma* might not be the primary mechanism in biocontrol, instead it could be through the competition or parasitism of pathogen hyphae (Mukherjee and Raghu, 1997).

## CONCLUSION

The *in vitro* study showed that the two species of *Trichoderma* used have antagonistic potential against *Fusarium oxysporum* f. sp. *lycopersici*, causal organism of tomato wilt. Among the isolates, SP-1 and SP-3 from *Trichoderma viride* and Th-6 from *Trichoderma harzianum* performed significantly better. Following the screening of the promising antagonists, including their effectiveness with duration of incubation to select one effective strain, SP-3 having the highest percent inhibition of the pathogen can be included in the biological control of Fusarium wilt disease of tomato. However, positive results obtained from *in vitro* studies are only indicative, as experimental conditions do not take all ecological and endemic factors into account. For this reason field studies are essential to test the selected competitive biocontrol agents under field conditions.

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