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Evaluation Of *Trichoderma Harzianum* As A Biocontrol Agent On *Fusarium* Wilt Of Tomato Grown In Eastern Nepal

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Abstract: *Fusarium wilt acts as a limiting factor for the yield of tomato for which Trichoderma spp. has been evidently used as a biological control agent. The most dominant species and causative agent of Fusarium wilt was identified as Fusarium oxysporum. The main purpose of this study was to investigate the activity of Trichoderma harzianum isolates towards to control Fusarium wilt on controlled tomato plant . Investigation of T. harzianum was performed under in vitro and in vivo conditions against the pathogen (Fusarium oxysporum). Three native Trichoderma antagonists were isolated from fifteen soil samples of different geographical regions of Eastern Nepal. Under in vitro conditions, the results revealed that Trichoderma harzianum, isolate Th-TJ, was found to inhibit effectively the radial mycelial growth of the pathogen by (57%). Under greenhouse conditions, the application of T. harzianum, Th-TJ exhibited the least disease incidence.Also, tomato plants treated with T. harzianum, Th-TJ isolate showed a significant stimulatory effect on plant height by (78.33cm) and the dry weight by (3.33g) of tomato plants, in comparison to untreated control (1.4g) which was statistically significant ($p<0.05$).Therefore, the antagonist T. harzianum, Th-TJ is chosen to be the most promising bio-control agent for F. oxysporum f.sp. lycopersici. On the base of this study,the biocontrol agents of plant diseases mightbe exploited for sustainable disease management programs to save environmental risk.*

Keywords:*Biological control,Fungiantagonist,Fusarium wilt,Trichoderma harzianum , F. oxysporum*

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I. Introduction

Tomato is known as productive as well as protective food considered crucial in large production of processed food products [1]. Tomato is also known as the poor man's apple in Nepal with an average national consumption of 11.97kg/person/year [2]. However tomato cultivation has been challenged by the fungal diseases one in many of them is *Fusarium* wilt [3]. *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* is a devastating disease in major tomato growing regions worldwide and has been reported in at least 32 countries [4].

Trichoderma species is one of such fungus showing inhibition of plant pathogen that has gained immense importance due to its biological control ability against several deadly plant pathogens by production of secondary metabolites and enzymes[5]. *T harzianum* stimulates the growth of plants by producing metabolites that promote developmental processes, which allow greater root development and absorbent hairs, which favors the mobilization of nutrients from the soil and also accelerates the decomposition of organic matter and minerals [6].The co-inoculation of tomato plants with *T. harzianum* provides a basis for the comparison of various agronomic parameters such as number of flowers, fruits number, leaves number, aerial part length, root length, fresh weight of the aerial part and fresh weight of root part [7].

Utilization of modern pesticides and chemical compounds has been done by farmers to control such plant pathogen. However, these chemicals do not degrade completely leaving behind toxic residue in soil [8].Chemical treatments against soil-borne root pathogens are very dangerous thus limits of chemical control and the high concern for the preservation of the environment are necessary [9].*Trichoderma* strains are appealing alternatives to hazardous fumigants and fungicides[10]. Therefore, the objectives of the present study were to assess the ability of *Trichoderma* spp. in decreasing the disease severity of *Fusarium* spp. in tomato under *in vitro* and *in vivo* conditions.

II. Materials And Methods

2.1 Study Area

The study was conducted from December 2018 to May 2019. This study was a laboratory based cross-sectional study. All the work concerning this research was carried out in microbiology laboratory of Central Campus of Technology, Dharan and at its garden. In this study, 50gm soil sample was taken from each 15 region of different ecological habitat (forest area and agricultural area) of Eastern Nepal for the isolation of *Trichodermaharzianum*. The samples were collected from top 2-5cm depth of rhizospheric soil. The soil sampleswere collected from a field in the polythene bag labeled separately. The samples were stored at 4^oC in the laboratory.

2.2 Isolation and identification of *Trichoderma harzianum*

For the isolation of *Trichoderma harzianum* ,1 g of soil sample was taken and added to 1ml of sterilized distilled water to make dilution of 10^{-1}. Six-fold serial dilution of each soil samples were prepared in sterilized distilled water and 0.5ml of each diluent from dilutions 10^{-4} and 10^{-6} dilutions were poured onto *Trichoderma harzianum* Selective Medium(THSM) (HiMedia,India) contained in petriplates and spread uniformly by adopting spread plate method. The petriplates were incubated at 25±3°C for 168hrs. [11]. After the isolation of all isolates, growths observed on those plates were taken for studying colony characteristics, morphology and microscopic examination of each *Trichoderma* isolates. Identified isolates were purified in the Potato Dextrose Agar (PDA) and were preserved at 4°C until use.

2.3 Isolation and purification of plant pathogenic fungi

Infected vascular tissues from root andleaf regions of tomato showing wilt symptoms were collected separately from field. Tissue bits were surface sterilized with 10% sodium hypochlorite for 5-10min. and subsequently three washings with sterile distilledwater. Then, they were placed on potato dextrose agar (PDA)(HiMedia, India) medium separately and incubated at the laboratory conditions at 25±3°C for five days.The fungi were purified separately by transferring the tipof the mycelia into PDA slants and maintained as stock cultures forfurther studies. Isolated fungus was identified according to their morphological characters [12].Cultures were stored at 4^0C.

2.4 In vitro effect of *Trichoderma* antagonists against *Fusarium* pathogen

2.4.1 Slide culture preparation

Pertiplatesand glass slides were sterilized for slide culture. A thin layer of Corn Meal Agar (CMA)(HiMedia,India) of 1×1cm was placed on glass slide. From actively growing plate cultures of each *Trichoderma* isolates and *Fusarium oxysporum* thin mycelium was taken with the help of sterile inoculating loops and inoculated at the edge of CMA on opposite sides; a cover slip was then placed on the slide cultures; and incubated for 5days at 25°C. Microscopic observations were performed by transferring the cover slip to another microscope slide, Lacto Phenol Cotton Blue was used to stain the fungi [13].

2.4.2 Dual plate culture

Dual plate culture technique was followed to determine the antagonistic activity of *Trichoderma harzianum* isolates against plant pathogen *Fusarium oxysporum.* 4mm disc of fifteen days old antagonistic fungi and pathogen cultures were placed on PDA medium one cm away from the edge of the plate, separately. Five replicated plates for each treatment was maintained and incubated at 25±3°C.Control plates were inoculated with phytopathogen only. Growth of each phytopathogen isolates in dual culture and in control (without antagonist) was measured after different intervals from the 5^{th}day i.e., 120hrs., 144hrs., 168hrs., 192hrs.and 216hrs. [14] . Percent inhibition overcontrol was calculated as per the formulae:

$$\mathrm{PI} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100\%$$

Where, PI = Percent inhibition over control
C = Growth of test pathogen with absence of *Trichoderma harzianum* (cm)
T = Growth of test pathogen with *Trichoderma harzianum* (antagonist) (cm)

2.5 Development of *Trichoderma harzianum*

Trichoderma harzianum in the form of wheat bran culture as soil treatment was effectively known to control the wilting of tomato caused by *Fusarium oxysporum*. Wheat bran: water in1:1 (w\v) was autoclaved in 500ml flask for one and half hour at 121°C at 1.5kg\cm^2 pressure. 5mm bit of *Trichoderma harzianum* was inoculated after cooling of the medium and incubated at 25±3°Cfor three weeks until all substrates were covered with *T. harzianum* mycelium[15].

2.6 Development of phytopathogen

For this purpose, *Fusarium oxysporum* was grown on PDA for 15 days. Inoculum of *F. oxysporum* was multiplied by transferring the 5cm diameter culture to Erlenmeyer flasks containing Maize meal sand medium (100gm sand, 5gm maize meal and 20ml of sterile distilled water). Then, the inoculated substrates were incubated at 25±3°C for three weeks until all the substrates were covered by pathogen mycelium [16].

2.7 *In vivo* experiment of *F. oxysporum* and *Trichoderma harzianum* on tomato

A pot culture study was conducted to test the antagonistic potential of selected antagonists (*Trichoderma harzianum*-DN, *Trichoderma harzianum*-PN and *Trichoderma harzianum*-TJ) against *F. oxysporum*-TL. The soil was sterilized by autoclaving it for 1 hr. for twoconsecutive days and filled in plastic pots (25cm diameter) of 5 kg capacity.Tomato (F1 hybrid) seeds were sown in autoclaved soil inplastic pot. After 25 days, the seedlings were transplanted in another pots at the rate of three seedlings per pot. Both multiplied inoculums of the pathogen and antagonists were incorporated into the soil of pots at 5% (w/w) .*F. oxysporum* was inoculated one day before transplanting and *Trichoderma* isolates were applied just the day of seeding. The observation on the percent disease incidence was recorded at the time of harvest [17].

$$\mathrm{DI} = \frac{\text{Total No. of infected plant}}{\text{Total No. of plant assessed}} \times 100\%$$

Where, DI = Disease Incidence (%)

Each treatment was replicated thrice inCompletely Randomized Block Design (CRD). Treatments were:

T0:Uninoculated control (healthy) , T1: Inoculated control with *F. oxysporum*-TL

T2*: F. oxysporum*-TL + *T. harzianum*-PN ,T3: *F. oxysporum*-TL + *T. harzianum*-TJ

T4: *F. oxysporum*-TL + *T. harzianum*-DN

Plants were maintained in normal conditions at garden by watering daily and equal moisture was maintained in each pot. In all the treatments, plant height and number of leaves were measured at 10, 20, 30, 40 and 45 days after transplantation (DAT). While the fresh root and dry roots were measured after 45 DAT.The measured parameters were:

a) The height of the plant (cm), (b) Number of leaves (c) The fresh weight of the roots (gm) (d) The dry weight of the roots (gm).

2.8 Data Analysis

The data recorded from dual culture and pot culture were documented and tabulated. The data were statistically analyzed using SPSS version 16. One way ANOVA test was used to determine the association of plant growth parameters with different treatments. The test was statistically significant if p-value was less than and equal to 0.05 with 95% confidence interval.

III. ResultsAnd Discussion

3.1 Distribution of *Trichoderma harzianum*in soil

Trichoderma harzianum was isolated from different soil of Eastern Nepal. Out of fifteen samples, three samples (20%) gave the positive result for isolation of *Trichoderma harzianum* .One isolate was isolated from Dharan (Th-DN), one fromPanwari (Th-PN) and one from Tahrara Jungle (Th-TJ).

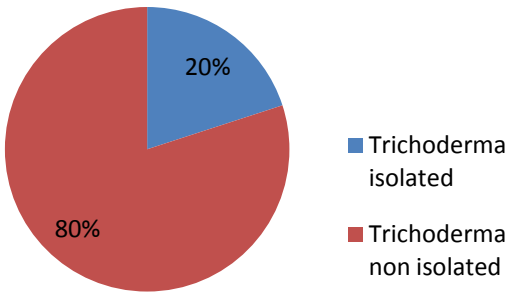

Figure -1: Distribution of *Trichoderma harzianum* in soil sample

3.2 Effect on dual plate culture and on slide culture method

Among the 3 *Trichoderma* isolates collected from Eastern Nepal, Dharan (Th-DN), Panwari (Th-PN) and Tahrara Jungle (Th-TJ), Th-PN showed the highest inhibition (41%) at day 5 in comparison to Th-DN (2% only) which was statistically significant ($p<0.05$). However, the data recorded day 6 to day 9 showed increased inhibitions for Th-TJ from 47% to 69%. This outcome is similar to earlier experiments that have demonstrated that *Trichoderma spp*. mycoparasitize the hyphae and resting structures of plant pathogens in vitro and also in natural soil [18]. Growth inhibition ofthe pathogen by all the *Trichoderma harzianum* isolates was evident from the fifth day of incubation.*Trichoderma harzianum*-TJ showed significantly higher inhibition of the mycelial growth of the pathogen (56%) than *Trichoderma harzianum*-PN (55%) and *Trichoderma harzianum*-DN (36%) which was statistically significant ($p<0.05$).On slide culture method the *Fusariumoxysporum* was enclosed by *Trichoderma* isolate with remarkable interaction.The result of slide culture was similar with the dual culture method.Hence, most *Trichoderma* strains were more efficient for control of some pathogens than others and may be largely ineffective against some fungi [19].

Picture -1: Dual culture, *Trichoderma*vs*Fusarium* at 8 DAI (Days after Inoculation)

Table-1. Effect of *Trichoderma harzianum* isolates on mycelium Percent (%) inhibition on growth of *Fusarium oxysporum*

	% Inhibition on growth of *Fusarium oxysporum*					
Treatments	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Average**
Th–PN	41%	45%	59%	*62%*	68%	55%
Th–DN	2%	21%	45%	*53%*	59%	36%
Th–TJ	39%	47%	57%	*65%*	69%	56%

3.3 Pot assay experiment

In this study the inoculated plant showed wilting symptoms and percentage of diseases incidence was (100%). The result is supported by the findings which observed that *F. oxysporum* f.sp. *lycopersici*was able to produce wilting symptoms in tomato plants [20].It was revealed from the results, *Trichoderma* isolates varied in their effect on tomato plants and ability to reduce the effect of *F. oxysporum* when subsequently applied in the pot experiment. Reduction of *Fusarium* wilt disease was observed by 100% and 67% in comparison to the control. Best disease control was achieved in treatment (T3) demonstrating only 33% of disease incidence followed by treatment (T2) and treatment (T4) with 67%.In one study, two *Trichoderma* isolates significantly ($p<0.05$) reduced tomato *Fusarium* wilt incidence, as shown by 69% fewer plants with vascular discoloration[21]. In several studies, *T. harzianum* has also been reported to be effective against the *Fusarium* wilt pathogen and improving the growth and yield of tomato [22] [23] [24]

Figure-2: *In-vivo* trial (pot culture)

Table-2: Efficacy of *Trichoderma harzianum* on *Fusarium* wilt control in tomato at 45 DAT

Treatments	Fungal Native Antagonist	FO-TL	Total no. of Plant examined	Infected plants with Wilt	% Disease Incidence
T0	Healthy Control	-	3	2	67%
T1	Inoculated Control	+	3	3	100%
T2	FO-TL + Th-PN	+	3	2	67%
T3	FO-TL + Th-TJ	+	3	1	33%
T4	FO-TL + Th - DN	+	3	2	67%

FO-TL= *Fusarium Oxysporum* isolated from tomato plant. There was significant differences ($p<0.05$) ,DAT- Days after transplantation.

3.4Effect on shoot length of tomato

All the treatments of *Trichoderma* compared to the control and *Fusarium* resulted in better plant height of tomato. All the parameters related to the plant growth and yields were also maximum in *Trichoderma* treated soil; while most of them were the lowest in control and *Fusarium* treated soil. As seen in table below, the shoot length showed progressive growth with maximum length of 78.33cm in 45 DAT by T3 (*F. oxysporum*-TL + *T. harzianum*-TJ).

Table-3: Effect of *Trichoderma* and *Fusarium* on shoot length (cm) of tomato

Treatments	Fungal Native Antagonist	10 DAT	20 DAT	30 DAT	40 DAT	45 DAT
T0	Healthy Control	13.67	24	31.5	42.17	56.33
T1	Inoculated Control	8.67	19	27.5	34.5	42.5
T2	FO-TL + Th-PN	15.33	28	38.67	50.83	65.67
T3	FO-TL + Th-TJ	16.17	32.67	45.5	66.33	78.33
T4	FO-TL + Th - DN	13.5	30	40.67	55.67	67

There wassignificant differences ($p<0.05$) among the treatments according to Anova test, DAT- Days after transplantation.

3.5Effect on number of leaves of tomato

As seen in table below, the *Trichoderma* treated plant showed greater number of leaves as compared to the control and *Fusarium* inoculated plant ($p<0.05$). The highest number of leaves were found in tomato treated with T3 (*F. oxysporum*-TL + *T. harzianum*-TJ) at 90 leaves in 45 DAT. However, the *Fusarium* inoculated plant had only 33 leaves in same duration of time.

Table-4: Effect of *Trichoderma* and *Fusarium* on number of leaves of tomato

Treatments	Fungal Native Antagonist	10 DAT	20 DAT	30 DAT	40 DAT	45 DAT
T0	Healthy Control	34	44.33	48.67	48.33	51.67
T1	Inoculated Control	18	28.67	34.33	35	33
T2	FO-TL + Th-PN	39.33	51.33	60.33	64.67	74.67
T3	FO-TL + Th-TJ	45.33	61.33	71.67	82.67	90.67
T4	FO-TL + Th - DN	39	51.6	56.67	64.33	66.33

There wassignificant differences ($p<0.05$) among the treatments according to Anova test, DAT- Days after transplantation.

3.6Effect on fresh root weight of *Trichoderma* inoculated plants

It was evident from the root mean weight that the *Trichoderma* increase the overall biomass of root system due to promotion of root development ($p<0.05$). The maximum fresh and dry root mass was measured for treatment T3 and the minimum was for T1 (control).The results of *Trichoderma* treatments showed statistically significant effects of *Trichoderma* treatments on plant growth parameters root length, fresh weight and dry weight($p<0.05$). These results agree with study that reported that seedling treated with *T. harzianum* increases number of true leaves, fresh and dry weights of root of tomato plants [25]. *Trichoderma* spp., are known to be opportunistic plant colonizers that affect plant growth by promoting abundant and healthy plant roots, possibly via the production or control of plant hormones [26].

Table-5: Mean fresh root weight (gm) of control and *Trichoderma* treated plants

Isolates	**T0**	**T1**	**T2**	**T3**	**T4**
Mean Root fresh wt.(gm)	2.9	2.13	3.47	4.4	3.87
Mean Root dry wt.(gm)	2.13	1.4	2.6	3.33	2.95

IV. Conclusion

The results in this study are highly promising and they support the potential of *Trichoderma* as suppressor of pathogen growth in in-vitro condition and as growth promoter in in-vitro and in-vivo condition. *Trichoderma* sp. decreased disease incidence of *Fusarium oxysporum* f.sp.*lycopersici* by as less as 33%, and increased plant growth parameters such as shoot length, number of leaves and root weight in tomato. Thus, the finding of present investigation holds a good promise in tomato wilt management. However, further studies on the effect of these treatments in field conditions need to be undertaken so that *Trichoderma* could be recommended as a biocontrol and growth promoter agent.On the basis of this study, the biocontrol agents of plant diseases might be explored for sustainable disease management programs to save environmental risk.

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