

In Vitro Evaluation of Efficacy of *Trichoderma* Isolates Against *Sclerotium Rolfsii*

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ABSTRACT

Sclerotium rolfsii is a plant pathogen causing collar rot diseases in several plants including Chickpea (*Cicer arietinum* L.). An *in-vitro* experiment was conducted to study the potential of different isolates of *Trichoderma spp* collected from different agro ecological regions of Nepal on inhibition of growth of *Sclerotium rolfsii* Sacc. in Plant pathology Laboratory of Agriculture and Forestry University, Nepal. The pathogenic isolate of *S. rolfsii* was isolated from symptomatic tomato and maintained in PDA. Several *Trichoderma spp* were isolated from soil of several locations using *Trichoderma* selective media (TSM) and maintained in PDA plates. Potential ability of *Trichoderma* isolates in controlling the pathogen was observed using dual culture and sclerotial parasitization techniques. Colony morphology and morphological features of sporulating structures of different isolates of *Trichoderma spp* were compared. Among all eight tested isolates *Trichoderma* isolate isolated from Palpa district of Nepal gave maximum inhibition percent of *S.rolfsii* on 2(90.500%), 3(94.666%), 4(97.746%) and 5(100%) days after inoculation in dual culture. The average growth of mycelium of *S. rolfsii* on PDA plates treated with liquid culture filtrate (LCF) of *Trichoderma* was maximum in isolate isolated from Tarahara and minimum in Palpa isolate. Among eight sclerotia of *S.rolfsii* inoculated in PDA plates treated with LCF of different isolates of *Trichoderma spp*, minimum germination of sclerotia was obtained in the plates treated with Palpa isolate at 3 (25%), 5 (29.16%), 7 (29.16%) and 9 (29.16%) days after inoculation. Morphological variation of *Trichoderma spp* was observed even within the isolates of same sample and same location. Rampur isolates (collected from rice and vegetable farm) showed slightly lighter green color whereas Palpa isolate gave darker green color than all other isolates.

Keywords: *Trichoderma*, *S. rolfsii*, Mycelium, Mycelium inhibition, Isolate

INTRODUCTION

Sclerotium rolfsii Sacc. is a necrotrophic soil-borne plant pathogen of worldwide occurrence that infects more than 500 plant species (Aycock, 1966 and Punja, 1985). The pathogen *S. rolfsii* is found causing various symptoms like crown rot, root rot, stem rot, rotting of pseudo bulbs, wilt and the gradual death of the plants etc (Agrios, 2005). The pathogen is very common in tropical, subtropical and warm temperate region in the world. Collar rot is prominent in one month old seedlings. Under field conditions, the pathogen has been reported to cause 30 to 60 % reduction in yield of chickpea. Because of prolific growth of *S. rolfsii* and ability to produce persistent sclerotia, it is contributing in high degree of economic losses. Under conducive conditions it can causes 55-95% mortality of the crop at seedling stage (Gurha and Dubey, 1982).

Since use of chemicals are hazardous to soil and users, use of biological control agents are the best alternative to the toxic chemicals. Among the bio control agents, *Gliocladium virens* and *Trichoderma viride* were found to be the most effective against the *S.rolfsii*. *Trichoderma spp.* have been found as an effective BCA against many soil borne pathogens (Eziashi et al., 2006). Dennis and Webster (1971) described the antagonistic properties of *Trichoderma* in terms of antibiotic production and hyphal interactions. The species of *Trichoderma* capable of hyper parasitizing pathogenic fungi, are highly efficient antagonists (Barnett et. al. 1973). Sanchez et al (2006) reported that *Trichoderma* species can inhibit the growth of plant pathogens especially fungi through competition for nutrients, enzymes, substrate, oxygen and space.

The dual culture technique can be used for rapid evaluation of the antagonistic capacity of *Trichoderma* stains against *S. rolfsii* of chickpea. Mukherjee et al. (1995) found that *G. virens* and *T. harzianum* were equally effective in parasitizing the hyphae of *R. solani*; *T. harzianum*, *Gliocladium virens* parasitized the hyphae of *S. rolfsii*, where as *T. harzianum* destroyed the sclerotia. The antagonistic potential of *T. harzianum* and *G. virens* against *S. rolfsii* based on antagonism *in-vitro* in dual culture as colony degradation tests, hyphal interaction antibiosis and parasitism of sclerotia and observed a positive response as evaluated by Jamduang and Sariah (1997).

The present study was undertaken in an attempt to isolate *Trichoderma* spp. from the various agro ecological regions of Nepal and also to study the morphological characteristics of isolates of *Trichoderma* spp in order to evaluate the most potential *Trichoderma* isolates to control *S.rolfsii*. Apart from that it was hypothesized that most of *Trichoderma* isolates can inhibit the growth of *S.rolfsii* according to their parallel evolution in the same location.

MATERIAL AND METHOD

Study Setting:

The study was carried out at the laboratory of Department of Plant Pathology, Agriculture and Forestry University (AFU), Rampur, Chitwan. Soil samples for isolation of *Trichoderma* spp were collected from Chitwan and other isolates from different agro ecological regions viz; Nepalgunj, Salyan, Illam, Taplejung, Palpa, Jumla and Sunsari were used which were available in lab.

Isolation of *Trichoderma* spp from soil

Trichoderma selective media (TSM) (Harman et al. 1998) was used for isolation of *Trichoderma* spp from the collected soils and the *Trichoderma* was isolated by using serial soil dilution technique.

Preparation of *Trichoderma* selective media (TSM)

Trichoderma selective medium was prepared as follows:

Following chemicals with their

respective amounts were dissolved in 1 litre distilled water and autoclaved for 15 minutes at 121°C.

Table 1. Chemicals used for preparation of *Trichoderma* selective medium

SN	Chemicals	Amount
1	Magnesium sulphate heptahydrate	0.20g
2	Dipotassium Hydrophosphate	0.90g
3	Potassium Chloride	0.15g
4	Ammonium Chloride	1.00g
5	Glucose	3.00g
6	Agar	20.00g
7	Water	1000ml

The autoclaved medium was left for cooling down to 30 minutes and 0.5g each of Antibiotic (Streptomycin and Tetracycline), Metalaxyl, Captan and Vitavax and 0.1g of Rose Bengal were thoroughly mixed and poured in Petridishes.

Serial dilution

Each soil samples were weighed to one gram and were dissolved in 10ml of distilled water in a test tube. The solution was shaken vigorously with hand until soil got dissolved uniformly into the distilled water remaining no residue at the bottom of the test tube. One ml of this suspension was then poured into 9ml of distilled water in second test tube and similarly 3rd, 4th and 5th dilutions were prepared.

Inoculation of the diluted soil suspension

The soil suspension of different dilutions were taken 0.1ml with sterilized micropipette and inoculated to TSM in petri plates. Inoculation was done at the center of the media in sterilized condition. It was then spread with sterilized glass rod spatula. Inoculated plates were then incubated at 25°C for about a week.

Fungal growth on TSM showing green or greenish color were picked up and transferred to the potato dextrose agar medium for their growth in pure form. The confirmation

test of the *Trichoderma* was done through microscopic observation and development of green color or fungal growth in the petri plate. Thus obtained two days old culture of *Trichoderma* spp was used as an inoculum. The inoculation of *Trichoderma* spp was done by cutting the young colonies into small disc by cork borer and transferring to the PDA medium.

Isolation of *Sclerotium rolfsii*

Sclerotium rolfsii was isolated from diseased plants collected from Chitwan district. The diseased plant was showing collar rot and wilting symptoms. The infected plant part was cut with sterilized blade and surface sterilized in 0.1% sodium hypochlorite solution followed by 2 to 3 times surface wash with distilled water, wiped with tissue paper and incubated at 24°C in PDA plates. Two to three days after the incubation, colonies of the pathogen showed white, silky mycelial growth on PDA radiating in all direction in petri plates. Pure culture was made by transferring mycelia from petri plates in to PDA slants and some of the plates were kept for sclerotia formation in plates.

Efficacy of *Trichoderma* isolates against *Sclerotium rolfsii*

Antagonism between *Trichoderma* isolates and *Sclerotium rolfsii*

Culture discs (5 mm) of *Trichoderma* and *Sclerotium rolfsii* were taken from the margin of the actively growing cultures and placed diagonally approximately at seven cm apart on to 90mm Petri plates containing 20ml PDA. For each treatment minimum four replications were maintained and controls were maintained by placing the disc of only *Sclerotium rolfsii* (Singh et al., 2004). Petri plates werethen incubated at 28 ± 2°C. The growth of the *Sclerotium rolfsii* andthe ability of the *Trichoderma* to inhibit the pathogen was recorded by periodical observations. The percent growth reduction (P_i) of the test pathogen was calculated when the growth of the *Sclerotium rolfsii* was full in control plates by using the following formula (Vincent, 1927):

$$P_i = \frac{C - T}{C} \times 100$$

Where,

- P_i = Percent growth reduction of test pathogen
- C = Radial growth of test pathogen in control (mm)
- T = Radial growth of test pathogen in treatment (mm)

Parasitization test of pathogen

Antagonistic properties of the isolated *Trichoderma* were evaluated on the basis of sclerotial parasitization of the pathogen and inhibition of germination. *Trichoderma* spore suspension of the eight different isolates was prepared by mixing 2mm *Trichoderma* isolates spore containing PDA block into 10ml distilled water. The mixture was then shaken thoroughly. The suspension was then poured into PDA plates and spread uniformly throughout the plates. Eight freshly formed sclerotia per plate were kept maintaining equidistance in plate. The plates were then incubated at 25°C. Control was maintained without antagonist. The experiment with eight treatments was replicated three times in completely randomized design. Observations on sclerotial parasitization and germination of sclerotia were recorded. The treatment combinations were as mentioned below.

Treatments <i>Trichoderma</i> spp	
I	Palpa Isolate
II	Rampur Rice field isolate
III	Rampur Vegetable field isolate
IV	Illam isolate
V	Jumla isolate
VI	Nepalgunj, Banke isolate
VII	Kapurkot, Salyan isolate
VIII	Tarahara, Sunsari isolate
IX	Control (sclerotia without antagonist)

Result and Discussion

Morphological characteristics of *Sclerotium rolfsii*

Colonies were silky white radiating in all direction on PDA plates at two days after incubation at 24°C. Mycelium was hyaline, thin walled, sparsely septate hyphae. Light brown to dark brown mustard seed like formation bodies within a week were observed in-vitro conditions. Size of sclerotia varies from 1.2-1.6mm in diameter. Similar morphological characters of sclerotia of the pathogen were shown by several others (Swart et al., 2003; Jeeva et al., 2005; Singh 1998).

Isolation of *Trichoderma spp* from soil

All the soil samples collected from different agro ecological regions yielded colonies of *Trichoderma spp* growing after 7 days of incubation on *Trichoderma* selective medium (TSM). *Trichoderma* isolates from different soil samples varied in their morphological characteristics. Variation was observed even within the isolates of same sample and same location. Rampur isolates (collected from vegetable and rice farm) showed slightly lighter green color whereas Palpa isolate gave darker green color than all other isolates. Ranganath and Sheeba (2002)

reported that identification and classification of *Trichoderma spp* based on morphological data like cultural characteristics, structure of conidiophores and conidia etc is erroneous.

Antagonism of *Trichoderma* isolates against *Sclerotium rolfsii*.

All the isolates of *Trichoderma* isolated from different agro ecological regions were found effective against germination of sclerotia of *S. rolfsii*. Sclerotia in control germinate earlier (2 days after inoculation) than with antagonist. Jha (2008) reported similar result. Isolates isolated from Palpa district of Nepal gave maximum inhibition percent against *S. rolfsii* in 2(90.500%), 3(94.666%), 4(97.746%) and 5(100%) days after inoculation followed by isolates of Tarahara in 2(54.570%) and 3(58.720) days after inoculation and isolates of vegetable farm of Rampur in 4(74.200%) and 5(74.843%) days after inoculation (Table 3). The present results were similar to those obtained by Rajput (2016) who stated that *T. harzianum* caused 80 % inhibition of mycelial growth after 72 h of incubation; and it also caused 35.5 % inhibition of sclerotial formation after 10 days of incubation.

Table 3. Percent inhibition of *Sclerotium rolfsii* in dual culture with *Trichoderma* isolates

<i>Trichoderma</i> isolates	Inhibition% 2 days after inoculation	Inhibition% 3 days after inoculation	Inhibition% 4 days after inoculation	Inhibition% 5 days after inoculation
I	90.500 ^a	94.666 ^a	97.74683 ^a	100.00 ^a
II	17.726 ^f	27.063 ^e	52.780 ^d	60.090 ^d
III	32.603 ^d	47.250 ^c	74.200 ^b	74.843 ^b
IV	39.666 ^c	45.463 ^c	51.660 ^d	56.463 ^e
V	34.270 ^d	34.696 ^d	64.733 ^c	73.633 ^b
VI	22.186 ^e	36.363 ^d	53.890 ^d	66.176 ^c
VII	31.230 ^d	45.740 ^c	53.796 ^d	65.780 ^c
VIII	54.570 ^b	58.720 ^b	63.753 ^c	67.386 ^c
P- Value	4.47e-16 ***	6.75e-14 ***	2.2e-12 ***	3.44e-16 ***
LSD0.001(P=0.001)	0.186	0.228	0.216	0.142
C.V (%)	5.468	5.530	4.016	2.384

Browning area interception of mycelium of *Sclerotium rolfsii* and *Trichoderma spp*
 After 120 hours of inoculation Palpa isolate of *Trichoderma* had widest browning area

(1.033cm) (Table.4). Isolate from rice field of Rampur and Jumla district had lowest breadth of browning area (0.32cm and 0.400cm respectively).

Table 4. Browning area at the junction of *Trichoderma* and *Sclerotium rolfsii* in dual culture

<i>Trichoderma</i> Isolates	Width of the browning area at the region of interception in dual culture (cm)
I	1.075 ^a
II	0.4250 ^e
III	0.925 ^{ab}
IV	0.600 ^{de}
V	0.650 ^{cd}
VI	0.575 ^{de}
VII	0.800 ^{bc}
VIII	0.550 ^{de}
P- Value	1.55e-06***
S.Em±	0.0077
LSD0.001(P=0.001)	0.0091
C.V (%)	17.737

Effect of liquid culture filtrate (LCF) of *Trichoderma spp.* on mycelial growth of *Sclerotium rolfsii*

The average growth of mycelium of *S. rolfsii* on PDA plates treated with liquid culture filtrate (LCF) of *Trichoderma spp* was maximum in Tarahara isolate, 3 days after inoculation (0.775 cm) followed by Jumla

(0.675cm), Nepalgunj(0.625cm) and Salyan(0.625) isolates and lowest growth of mycelium of *S. rolfsii* was recorded in PDA plates treated with *Trichoderma* isolate of Palpa district (0.074 cm). Darvin *et al.* (2013) reported that *T. viride* isolate completely inhibited the mycelial growth of *S. rolfsii* through poisoned food technique.

Table 5. In-vitro analysis of bioefficacy of liquid culture filtrates of different isolates of *Trichoderma spp* against *Sclerotium rolfsii* (poisoned food technique)

Treatments <i>Trichoderma</i> Isolates	Mycelial growth (3 days after inoculation)	Mycelial growth (7 days after inoculation)
I	0.074 ^e	2.175 ^f
II	0.400 ^d	5.250 ^d
III	0.400 ^d	4.0252 ^e
IV	0.575 ^c	4.350 ^e
V	0.675 ^{bc}	5.500 ^{cd}
VI	0.625 ^{bc}	6.300 ^b
VII	0.625 ^{bc}	3.925 ^e
VIII	0.775 ^b	5.900 ^{bc}
IX	2.775 ^a	7.675 ^a
P- Value	<2e-16	6.48e-15
S.Em±	0.0063	0.1056
LSD0.001(P=0.001)	0.007646	0.0311
C.V (%)	13.5270	8.4577

CONCLUSION

This investigation sheds new light on the ways for the use of *Trichoderma* spp. in controlling the plant pathogen *S. rolfsii* which is one of the serious fungal pathogens which develops the collar rot in *Cicer arietinum*. The present result reveals that all the native isolates of *Trichoderma* spp isolated from different agro ecological regions of Nepal showed antagonistic effect against *Sclerotium rolfsii*. *Trichoderma* is potential antagonist for the bio control management of the disease if effective isolates or strains could be obtained as it has shown the inhibitory effect to the pathogen in laboratory conditions. The precise benefits and consequences of the present findings open several avenues for isolation of more isolates of *Trichoderma* spp and study their antagonistic property for more pathogens which will aid in future research in the field of biocontrol and biotechnology.

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