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Research Article

Antagonistic efficacy of *Trichoderma* isolates against soil-borne plant pathogens, *Pythium aphanidermatum* and *Rhizoctonia solani*

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ABSTRACT: *Trichoderma* spp. are long been recognized as efficient fungal biocontrol agents for the control of plant disease and for their ability to increase plant growth and development. Management of soil borne diseases has become very much important since it causes high crop yield losses. The present study was carried out to isolate *Trichoderma* spp. from soil samples collected from different locations of Kerala and to test their in vitro efficacy against soil borne pathogens viz*., Pythium aphanidermatum* and *Rhizoctonia solani*. The *Trichoderma* spp. was isolated on Trichoderma Selective Medium (TSM) and observed that the isolates differed in radial growth and colony characters such as colony colour, texture and sporulation. In vitro studies revealed the potential of Trichoderma isolates against soil borne pathogens. Isolates TRPN3 and TRPN7 exhibited no sporulation and white mycelial colour. Isolates which completed their growth at four days after inoculation include TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18. Biocontrol activities against different pathogens resulted in inhibition of pathogens. Maximum inhibition percentage was observed by the isolates TRPN7, TRPN15 and TRKR2 against both the pathogens. The maximum inhibition exhibited against both the pathogens is due to the antagonistic property displayed by the isolates.

KEY WORDS: Antagonistic activity, inhibition, soil borne pathogens, *Trichoderma* spp.

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INTRODUCTION

The genus *Trichoderma* was described by Persoon in 1794 but captured the attention of agriculturists only after the discovery of one species of the genus could kill other fungi and control plant diseases (Weindling, 1932). *Trichoderma* spp. is soil borne, green-spored ascomycetes with broad spectrum biocontrol activities against different soil and air borne plant pathogenic fungi. *Trichoderma* species are widely used in agriculture and industry as biopesticides and sources of enzymes, respectively. The mechanisms of antagonism of *Trichoderma* spp. include mycoparasitism, antibiosis and competition for space and nutrients. Apart from these, production of antifungal enzymes including chitinases, β1,3 glucanases and induced systemic or localized resistance also contributed to pathogen suppression (Harman, 2006). Genus *Trichoderma* had been known for their ability to act as biocontrol agents against plant pathogens such as *Rhizoctonia*, *Fusarium*, *Alternaria*, *Colletotrichum* and *Helminthosporium*, which caused detrimental effects on crops of economic importance (Amin et al., 2010). Several species of *Trichoderma* like *T. viride* and *T. harzianum* have been successfully used in the management of plant diseases. These include *T. harzianum* and *T. viride* against rice sheath blight (Bhat et al., 2009), *T. harzianum* against rotting of ginger (Gupta et al., 2010) and collar rot of cowpea (Pan and Das, 2011), *T. flavofuscum and T. viride* used against *Pythium* damping off in tomato (Patil et al., 2012) and *T. harzianum* against *Phytophthora infestans* in potato and tomato (Fatima et al., 2015). In the present study the efficacy of *Trichoderma* isolates against soil borne pathogens were evaluated.

MATERIALS AND METHODS

Collection and determination of pH of collected soil samples

Soil samples were collected from virgin forest soils of different locations of Trivandrum district of Kerala. The pH of collected soil samples was determined by potentiometric method using a pH meter. Soil samples were shade dried

separately and sieved through a mesh size of 2 mm. The dried soil samples each with 10 g were taken in separate glass beakers and added 25 ml distilled water to make a soil water suspension of 1:2.5. This suspension was stirred continuously for 15 – 30 min and pH was recorded using a pH meter.

Trichoderma enumeration

Trichoderma spp. was isolated from virgin forest soils collected from different places of Kerala. The collected soil samples were pooled separately, shade dried and the total microbial population of *Trichoderma* spp. was estimated by serial dilution plate technique (Johnson and Curl, 1972). Microbial count of *Trichoderma* spp. was estimated at 10^{-3} and 10^{-4} dilutions on Trichoderma Selective Medium (TSM). The composition of TSM included $MgSo_4.7H_2O$: 0.2 g, K_2HPO_4: 0.9 g, KCl: 0.15 g, NH_4NO_3: 1.0 g, Glucose: 3.0 g, Chloramphenicol: 0.25 g, Rose Bengal: 0.15 g, Agar: 20 g/l.

Ten grams of shade dried soil was transferred to 90 ml of sterilized water in 250 ml conical flask and shaken well for 10 min. in a shaker to get 10^{-1} dilution and again 1 ml from 10^{-1} was transferred to 9 ml sterile distilled water to make 10^{-2} dilution. The serial dilutions were continued up to 10^{-4} dilution. One ml each from 10^{-3} and 10^{-4} dilutions were transferred separately to sterilized Petri dishes, TSM was poured on the transferred dilutions and spreaded uniformly with a gentle swirl. Petri dishes were incubated at room temperature and next day onwards, the observations on total number of microbial colonies of *Trichoderma* spp. were recorded. The colonies showing the cultural characters of *Trichoderma* spp. were transferred to Potato Dextrose Agar (PDA) slants. These isolates were purified and sub cultured and maintained as pure culture for further work.

Isolation of different pathogens

The soil borne pathogens viz., *Pythium aphanidermatum* and *Rhizoctonia solani* were isolated from damping-off infected tomato seedlings and from collar rot infected cowpea plants respectively. The infected tissue was cut into small bit (2-4 mm) with a margin of healthy tissue around it, surface sterilized with 0.1% mercuric chloride solution for one min and then rewashed in two to three changes of sterile distilled water. These bits were placed on PDA medium in sterilized Petri dishes. The dishes were incubated at room temperature (28 ± 2 C). When the growth of the fungus was visible, mycelial bits were transferred aseptically to PDA slants. The slants were incubated at room temperature.

Radial growth of *Trichoderma* isolates

The *Trichoderma* isolates were transferred into Petri dish with PDA medium using cork borer. The growth of colonies was observed at 24 h interval. Radial growth (cm) was measured at third, fifth and seventh Day of Inoculation (DAI).

Colony characters of *Trichoderma* isolates

The cultural characters of 12 *Trichoderma* isolates were studied on PDA medium. From the actively growing edge of a fresh colony a cut was made using sterile cork borer and placed on the petriplates containing PDA medium. Three replications of each isolate were maintained. Petri dishes were incubated at room temperature and the colonies were examined at 24 h intervals for the colour of mycelium, texture of colony and growth pattern.

In vitro screening of *Trichoderma* isolates against fungal pathogens

Isolates were tested for their antagonistic efficacy against *P. aphanidermatum* and *R. solani*. The antagonistic index was calculated using parameters like overgrowth, lysis and antibiosis.

Dual culture assay of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani*

The isolates of *Trichoderma* spp. were preliminary screened for their antagonistic efficacy against two important soil-borne fungal pathogens, viz., *P. aphanidermatum* and *R. solani* by dual culture method outlined by Skidmore and Dickinson (1976) under in vitro condition on PDA medium. From actively grown culture of pathogens, 8 mm mycelial disc was cut off with the help of cork borer and placed at 2 cm away from edge of PDA medium in Petri dish. Mycelial disc of 8 mm diameter of each isolate of *Trichoderma* spp. was transferred to the same plate and was placed at 2 cm away from the opposite side. Three replications were maintained for each treatment. The pathogen grown as monoculture on one side of the Petri dish served as control. All the plates were incubated at room temperature and were examined for the antagonistic activity. The measurements on the radial growth of pathogen and the antagonists were taken daily till the control plate showed full growth. The native isolates of *Trichoderma* spp. showing antagonistic properties were selected for further studies.

Percent Inhibition (PI) on the growth of pathogen over control was calculated by the formula suggested by Vincent (1927).

$$PI = [C\text{-}T/C] \times 100$$

Where,

C = Radial growth of pathogen (cm) in control

T= Radial growth of pathogen (cm) in treatment

Assessment of zone of lysis, antibiosis and overgrowth

The modes of antagonism of like lysis, antibiosis and

overgrowth of isolates were tested against phytopathogens. A similar method as mentioned above. Observations were recorded when the control plate exhibited full growth. Overgrowth of isolates of *Trichoderma* spp. against phytopathogens after 72 h was recorded. The lysis between pathogen and *Trichoderma* spp. was observed as the region where pathogen was wiped out leading to formation of clear zone during the pathogen-antagonist interaction. Antibiosis could be observed at the point of interaction between the pathogen and antagonist which was observed as pigmented region.

Statistical analysis

The data obtained was subjected to the analysis of variance using package OPSTAT. Wasp 2.0 was used to compare the values of significant treatment at 5% level of significance.

RESULTS

Collection and pH of collected soil samples

The pH of soil samples collected from different locations was estimated and pH of each location is listed in Table 1. The pH range between 6 and 7 was observed. The pH of soil samples T1PND and T2KTR were recorded 6.50 and 6.80 respectively.

***Trichoderma* enumeration**

The isolation of *Trichoderma* spp. from collected soil samples was done by serial dilution technique on Trichoderma Selective Medium (TSM) at 10^3 and 10^4 dilutions. The microbial colonies were observed from two Days after Inoculation (DAI). A total of 12 isolates were obtained and named as TRPN3, TRPN7, TRPN9, TRPN10, TRPN11, TRPN14, TRPN15, TRPN 17, TRPN18, TRKR1, TRKR2 and TRKR3.

Radial growth of *Trichoderma* isolates

Trichoderma isolates were inoculated on PDA media and the radial growth of isolates was recorded. Most of the isolates were fast growers. The radial growth of isolates showed in Table 2. Isolates TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18 completed growth by 4 DAI.

Colony characters of *Trichoderma* isolates

The cultural characters of isolates were evaluated by subculturing the isolates on PDA medium. The mycelial growth started from 2 DAI. The cultural characters of the isolates are shown in Table 3. Growth pattern of isolates was observed after completion of mycelial growth on the petriplate.

Varying degree of growth pattern from no sporulation to heavy circular green sporulation was observed. Sporulation patterns with thick dense, sporulation at centre or circular, spreading sporulation were seen. Most of the isolates produce green sporulation except isolates like TRPN3 and TRPN7. Yellow sporulation was observed in isolate TRPN10. Texture of isolates showed variations like smooth, fluffy and spreading type. Majority of the isolates showed fluffy type. Colour of culture differed with different isolates of *Trichoderma* spp. They exhibited wide ranges of green colour ranging from light green to dark green. Some isolates showed white colour (TRPN3, TRPN7 and TRPN10).

Dual culture assay of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani*

The in vitro evaluation of *Trichoderma* isolates was carried out by dual culture method against fungal pathogens *P. aphanidermatum* and *R. solani*. The percent inhibition of pathogen by the antagonist isolates is presented in Table 4. Isolates which showed complete inhibition of *P. aphanidermatum* are TRPN7, TRPN11, TRPN15 and TRKR2. Majority of the isolates exhibited more than 90 per cent inhibition (Figure 1).

Table 1. pH of soil samples collected from different locations

Soil sample	Location	pH of the soil
T1PND	Trivandrum	6.50
T2KTR	Trivandrum	6.80

Table 2. Radial growth of different isolates of *Trichoderma* spp.

Isolate	Radial growth on PDA medium		
	3 rd day (cm)	5th day (cm)	7th day (cm)
TRPN3	6.90 ± 0.05 ab	9.00 b	9.00
TRPN7	6.16 ± 0.06 c	9.00 c	9.00
TRPN9	4.60 ± 0.05 c	8.56 ± 0.08 c	9.00
TRPN10	6.36 ± 0.08 c	9.00 c	9.00
TRPN11	3.56 ± 0.08 c	7.36 ± 0.08 c	9.00
TRPN14	4.50 ± 0.05 c	6.90 ± 0.03 c	9.00
TRPN15	5.00 ± 0.05 c	8.73 ± 0.03 c	9.00
TRPN17	3.76 ± 0.08 c	6.90 ± 0.05 c	9.00
TRPN18	6.70 ± 0.11 c	9.00 c	9.00
TRKR1	5.93 ± 0.12 ab	9.00 a	9.00
TRKR2	6.00 ± 0.01 a	8.96 ± 0.03 a	9.00
TRKR3	5.13 ± 0.08 b	8.73 ± 0.03 b	9.00
CD (0.05)	1.05	0.73	
SE (m)	0.08	0.06	

Values followed by similar superscripts are not significantly different at 5% level

Table 3. Colony characters of different isolates of *Trichoderma* spp. grown on PDA medium at 7 Days after inoculation (DAI)

Sl. No	Isolates	Colour of mycelium	Texture of colony	Sporulation/Growth Pattern
1	TRKR1	Dark green	Spreading smooth	Thick circular sporulation
2	TRKR2	Dark green	Spreading	Circular sporulation
3	TRKR3	Dark green	Smooth	Sporulation as ring
4	TRPN3	White	Fluffy raised	No sporulation
5	TRPN7	White	Fluffy raised	No sporulation
6	TRPN9	Dark green	Spreading smooth	Sporulation at centre
7	TRPN10	White	Fluffy	Yellow sporulation at centre
8	TRPN11	Green	Spreading	Circular sporulation
9	TRPN14	Green	Fluffy	Less sporulation
10	TRPN15	Green	Spreading	Circular sporulation
11	TRPN17	Green	Fluffy raised	Sporulation at centre
12	TRPN18	Dark green	Fluffy raised	Less sporulation

Against *R. solani,* isolates TRPN7, TRPN9, TRPN14, TRPN15, TRPN17, TRPN18 and TRKR2 exhibited complete inhibition. Except TRKR1 isolate all other isolates exhibited more than 90 per cent inhibition. All the isolates were fast growing and overgrow above the pathogen inhibiting the growth of mycelia and sclerotial production of pathogen (Figures 2 and 3).

Assessment of zone of lysis, antibiosis and overgrowth

Antagonist and pathogen interaction during dual culture shows mechanisms like antibiosis, lysis and overgrowth. It was found that except TRKR1, TRKR2 and TRKR3 all other isolates produced antibiotic against *P. aphanidermatum.* Lysis of pathogen was observed in TRKR1, TRKR2, TRKR3, TRPN14, TRPN15 and TRPN18. All the isolates exhibited overgrowth over *P. aphanidermatum.* Interaction of the *Trichoderma* isolates from Southern zone against *R. solani* resulted in lysis of pathogen and overgrowth by all the antagonists. Except TRKR1, TRKR2, TRKR3, TRPN7, TRPN9 and TRPN18 all other isolates showed antibiosis against *R. solani*. Isolates TRPN14 and TRPN15 displayed all the antagonistic properties against both the soil-borne pathogens.

DISCUSSION

Trichoderma spp. are widely used in management of soil borne pathogens. pH has influence on the occurrence of *Trichoderma* spp with enzyme production which involved in degradation of fungi (Kredics et al., 2003). *Trichoderma* isolates also obtained based on the sclerotial viability showed acidic pH of 8.08 (Khattabi et al., 2004). Delgado et al. (2000) has reported maximum stability of β1,6 glucanase produced by *T. harzianum* at a pH of seven. Native Trichoderma strains found in acidic soils were reported by Garcia-Nunez et al. (2012) and Hima (2017).

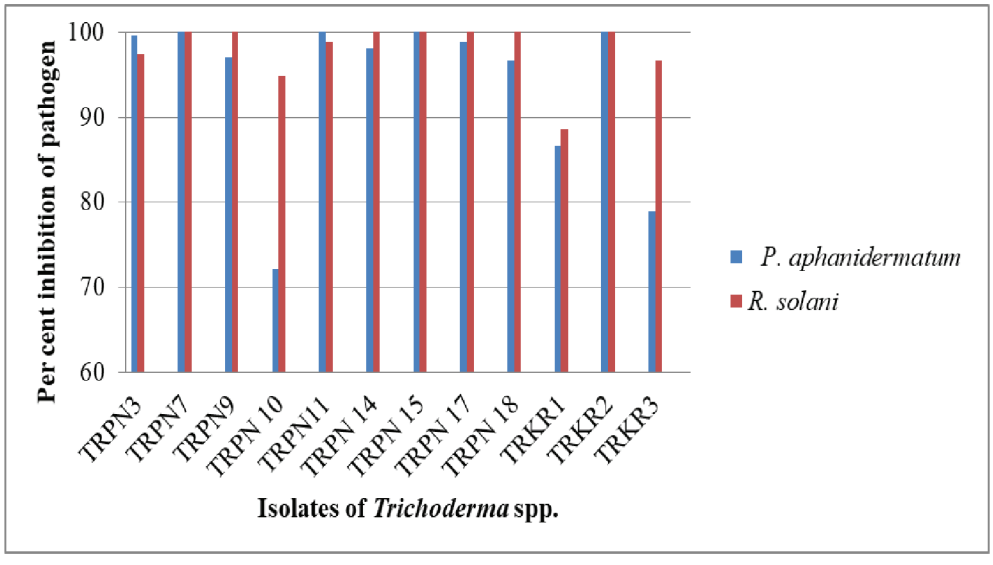

Fig. 1. Percent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolates

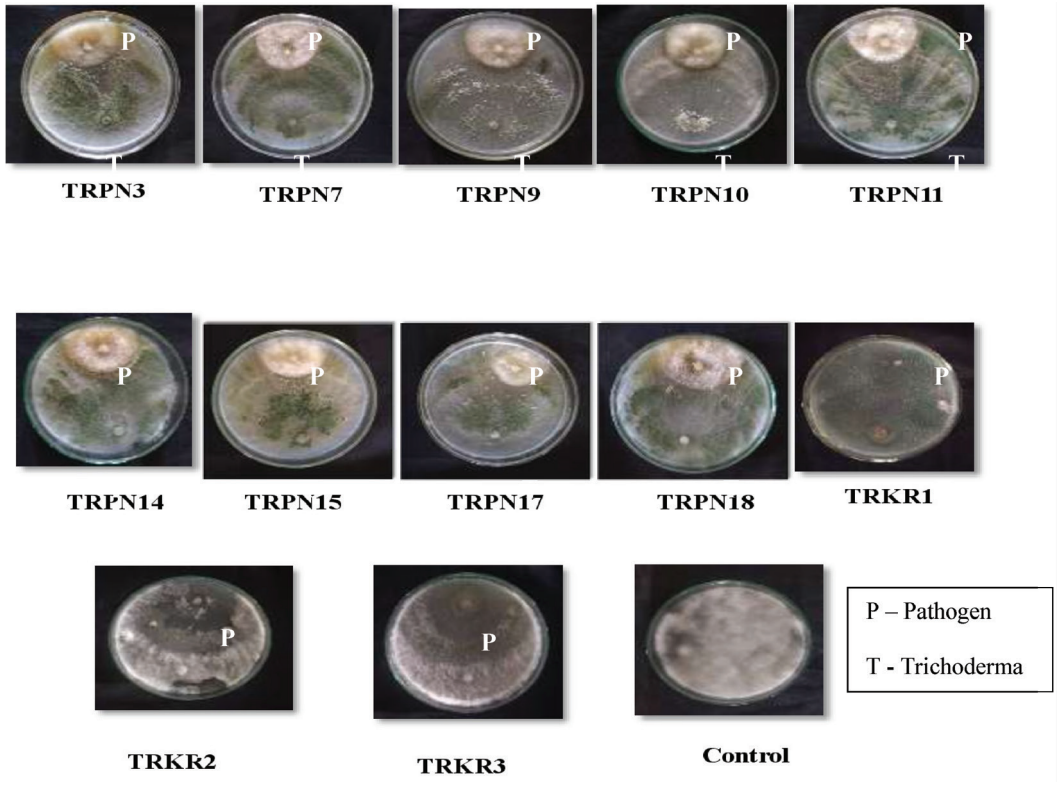

Fig. 2. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* isolates.

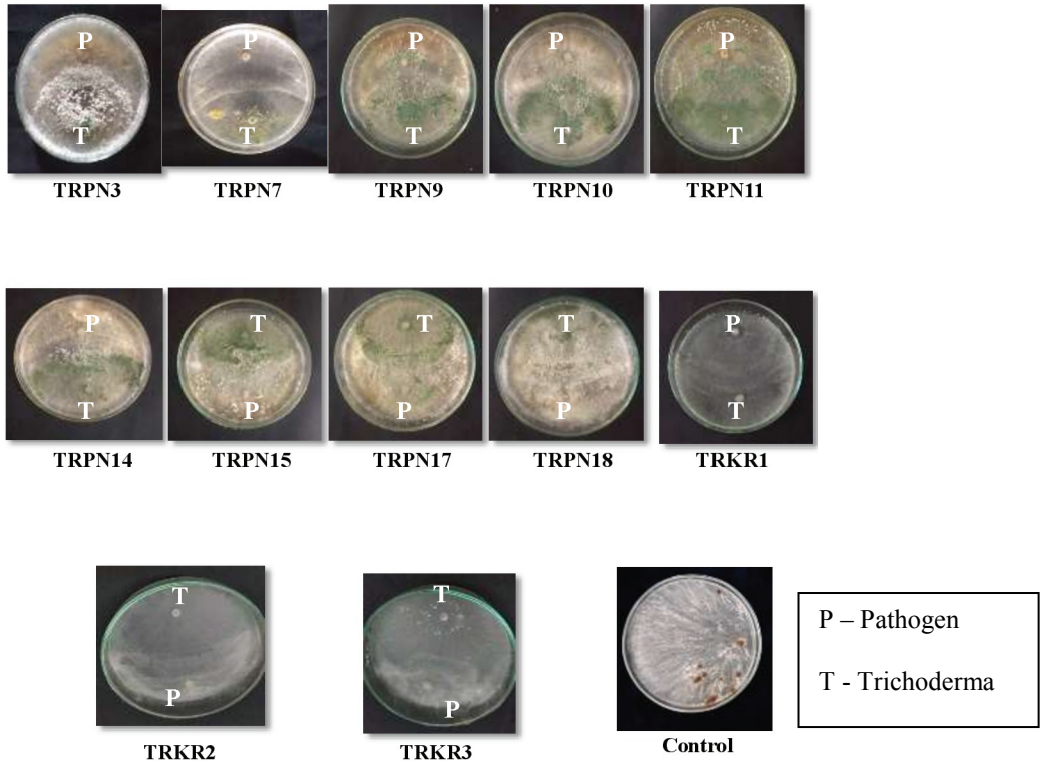

Fig. 3. Inhibition of mycelial growth of *R. solani* by *Trichoderma* isolates

Table 4. Percent inhibition of *P. aphanidermatum* and *R. solani* by different isolates of *Trichoderma* spp.

Isolates of *Trichoderma* spp.	*P. aphanidermatum*		*R. solani*	
	Radial growth (mm)*	Inhibition (%)**	Radial growth (mm)*	Inhibition (%)**
TRPN3	10.01	99.62^{ab} (87.71)	10.10	97.40^{bc} (82.20)
TRPN7	10.00	100^{a} (89.71)	10.00	100^{a} (89.71)
TRPN9	10.11	97.03^{de} (80)	10.00	100^{a} (89.71)
TRPN10	10.97	72.22^{h} (58.18)	10.20	94.81^{d} (76.83)
TRPN11	10.00	100^{a} (89.71)	10.04	98.88^{b} (84.90)
TRPN14	10.07	98.14^{cd} (83.4)	10.00	100^{a} (89.71)
TRPN15	10.00	100^{a} (89.71)	10.00	100^{a} (89.71)
TRPN17	10.04	98.88^{bc} (84.90)	10.00	100^{a} (89.71)
TRPN18	10.13	96.66^{e} (79.46)	10.00	100^{a} (89.71)
TRKR1	10.49	86.66^{f} (68.53)	10.43	88.51^{e} (70.18)
TRKR2	10.00	100^{a} (89.71)	10.00	100^{a} (89.71)
TRKR3	10.05	78.88^{g} 62.61)	10.13	96.66^{cd} (79.46)
Control	90		90	
CD (0.05)	0.28	3.70	0.27	4.37

*Log transformed; **Values in paranthesis are Arcsine transformed. Values followed by similar superscripts are not significantly different at 5% level

Enumeration of *Trichoderma* isolates was carried out using TSM. *Trichoderma* enumeration on TSM was reported by Elad et al. (1981). Isolation of *Trichoderma* isolates was also carried out in PDA medium which resulted in colonies after three days of incubation. (Shalini and Kotasthane, 2007). Enumeration of *Trichoderma* isolates was also performed in Rose Bengal Agar medium (Lunge and Patil, 2012). The use of antibiotics streptomycin and chloramphenicol favoured the growth of *Trichoderma* and reduced the amount of contamination in TSM compared to other culture media (Gil et al., 2009).

Trichoderma isolates exhibited faster growth rate ranging from six to nine cm at two days after inoculation on PDA media (Devi and Sinha, 2014). Sharma and Singh (2014) also reported the fast growth of *Trichoderma* isolates with eight to nine cm after 72 h at 28 ^{0}C and four to seven cm at 35 ^{0}C. Sekhar et al. (2017) had observed wide variations in colony colour like white, pale yellow, bluish green, and dull green in ten isolates of *Trichoderma* sp. isolated from groundnut rhizosphere. The colony colour of isolates of *T. harzianum, T. viride* and *T. aureoviride* exhibited variations from green to dark green (Shalini and Kotasthane, 2007). Devi and Sinha (2014) studied the cultural characteristics of *T. viride*, *T. harziaum* and *T. hamatum*. These isolates showed fluffy to spare growth, with different range of colony colour and different patterns of sporulations.

Isolates exhibited caused complete inhibition of *R. solani* due overgrowth of antagonist. Various *Trichoderma* spp. have been found to inhibit pathogens *in vitro* by overgrowth. According to Bastakoti et al. (2017) the colony growth of *S. rolfsii* on the fourth day incubation was found to be covered by the growth of *Trichoderma* sp. Due to overgrowth of *Trichoderma* species in the plate, the growth of test fungal pathogen was found to be highly inhibited. Manandhar et al. (2019) also reported that *Trichoderma* isolates showed more than 80 per cent inhibition of radial growth of *R. solani*. Some of the isolates completely overgrew the pathogen a week after inoculation.

Reduced sclerotia formation or overgrowth of the antagonist on sclerotia was found during the in vitro assay

of *Trichoderma* spp. against *R. solani*. Similarly, absence of *sclerotia* formation on *R. solani* by *Trichoderma* spp. was reported by Shalini and Kotasthane (2007). Mycoparasitic action including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm leads to parasitisation of sclerotia by heavy sporulation. Seema and Devaki (2012) also reported decreased sclerotia formation of *R. solani*. *T. harzianum* and *T. viride* completely overgrew the pathogen with percentage inhibition of 67 per cent and 70% respectively.

Mishra (2010) reported variations in inhibition percent and the maximum inhibition of *P. aphanidermatum* was by *T. viride*-1433 (72.0%), which was followed by *T. harzianum*-4572 (69.8%), *T. viride*-793 (62.1%), *T. harzianum*-4532 (60.3%) and *T. virens*-2194 (59.6%). Kamala and Indira (2011) studied the antifungal activity of *Trichoderma* spp. against *P. aphanidermatum* under *in vitro* and in vivo conditions. Maximum inhibition of *P. aphanidermatum* was observed in isolate T105.

Majority of the isolates exhibited lysis of pathogens during the antagonist-pathogen interaction. Lytic enzymes play a vital role in mycoparasitism and degrade the pathogen cell wall (Ridout et al*.,* 1986). Mycoparasitism is one of the most important mechanisms with antagonistic activity against soil-borne plant pathogenic fungi (Howell, 1998; Kubicek et al., 2011), in which hydrolytic enzymes, especially chitinases and β1,3-glucanases, play a crucial role. These enzymes were induced efficiently by pathogen cell walls.

Antibiosis also has an important role in antagonistic activity (Aluko and Hering, 1970). Production of antibiotics during the antagonist-pathogen interaction inhibits the growth of pathogens (Dennis and Webster, 1971). Antibiotics are microbial toxins that, at low concentration can poison or kill other microorganisms (Heydari and Pessarakli, 2010). Mendoza et al. (2015) also reported antibiosis of *Trichoderma* after 48 h with a colour change in medium due the production of secondary metabolite.

Thus, the present study revealed the antagonistic efficacy of *Trichoderma* isolates for controlling the soil borne pathogens. The antagonistic properties such as antibiosis, lyis and overgrowth resulted in maximum inhibition of pathogen under *in vitro* condition

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