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In vitro evaluation of *Trichoderma* spp. against *Pythium myriotylum* and *Pythium aphanidermatum*

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Abstract
The genus *Trichoderma* belongs to ascomycetic fungi that are present in nearly all soils and other diverse habitats. In soils they frequently are the most prevalent culturable fungi. These fungi have been widely studied for their biocontrol activities *viz*., micoparasitism, antibiosis, competition for nutrient and space, niche exclusion, stress tolerance, induced resistance in plants as well as inactivation of the pathogen's enzymes by producing various antimicrobial compounds thus it reduces the pathogen populations, enhances the vegetative growth and protect the plants under numerous agricultural conditions. Hence in the present study was conducted to assess the biocontrol activities of *Trichoderma* spp. *viz.*, *T. asperillum, T. virens* and *T. aureoviridae* was carried out against most important soil borne plant pathogens like *Pythium myriotylum* causing ginger rhizome rot and *Pythium aphanidermatum* causing damping off in tomato using dual plate method, invert plate technique and culture filtrate technique method. Among three species of *Trichoderma, T. virens* showed 71.62 and 71.23 percent inhibition of *Pythium myriotylum* and *Pythium aphanidermatum* followed by *T. asperillum* (55.13 and 60.86%) and *T. aureoviridae* (50.65 and 57.16%) respectively in dual plate technique. Moderate inhibition was observed in culture filtrate technique (51.42 & 45.25%) and least inhibition was observed in invert plate technique (43.49 & 29.11%).

Keywords: *Trichoderma* spp. *Pythium myriotylum, Pythium aphanidermatum* and evaluation

1. Introduction
At present situation the substantial increase in food grain production over the years has helped to meet the food security needs of the country, but the number of biotic and abiotic stress resulting in the severe yield reduction in the biotic constraints include fungi, bacteria, virus, nematodes, weeds, and insects which causes yield loss up to 31 to 42 percent. To manage these, farmers also using exhaustive amount of pesticide increases year by year. These results in environment pollution as well as negative effect on non-target organisms. Continuous and tremendous uses of chemical pesticides create high selection pressure on pathogens and force them to undergo mutation and develop pesticide resistance races.

The use of biofertilizers and biopesticides is an alternative management practice for sustaining high production with low ecological impact. The most commonly used microbial biopesticides are living organisms, which are parasites for the pest of interest. These include biofungicides, bioherbicides, and bioinsecticides (Gupta and Dikshit, 2010) [10].

Trichoderma spp. is ubiquitous and often predominant components of the mycoflora in soil, litter, organic matter and rhizospheric ecosystem of all climatic zones as saprophytes. Strains of *Trichoderma* spp. are endophytes establish robust and ability to sense, invade, and destroy other fungi has been the major driving force behind their commercial success as biopesticides. *Trichoderma* defend the plants by their direct and indirect effect on plant-pathogen-soil environment interaction. These fungi not only protect plants by killing the pathogens mainly other fungi and nematodes but also induce resistance in the hosts against plant pathogens, impart abiotic stress tolerance, improve plant growth and vigor, nutrients uptake and bioremediation of heavy metals and environmental pollutants.

In India, *Trichoderma* was isolated for the first time by Thakur and Norris in 1928 from Madras. The potential significance of the genus *Trichoderma* as biocontrol agent was first reported by Weindling in 1936 [35] (Pandya *et al*., 2012) [21].

Bio-control agents, manage pathogens either by producing many toxic metabolites specific to the pest, preventing establishment of other microorganisms through their modes of action. Many species of *Pythium* considered to be economically important in agriculture as they

causes disease in different crop plants severe economic yield loss in agriculture, as they cause rots, damping off, blight and death of the plants. These pathogens have developed resistance to fungicides hence to manage these diseases in another way biocontrol agents are necessary. The success of biological control of plant diseases depends on the availability of effective biocontrol agent, antagonism, parasitic nature and compatibility with other beneficial micro-organisms.

With respect to above circumstances, the present investigation was undertaken to study the "*In vitro* evaluation of *Trichoderma* spp. against *Pythium myriotylum* and *Pythium aphanidermatum*."

2. Materials and Method

2.1 Isolation of *Pythium* spp.

Disease samples of ginger soft rot and tomato damping off caused by *Pythium myriotylum* and *Pythium aphanidermatum* were collected from the market and nursery then brought to the laboratory. Infected samples were washed thoroughly using clean water and diseased portion was cut into small bits. These bits were surface sterilized with one percent sodium hypo chloride solution for one minute. Such bits were washed thoroughly in sterilized distilled water thrice to remove traces of sodium hypochlorite solution, if any and then kept for sporulation on carrot slices in a petriplate by maintaining temperature at 12-18 ºC. After 24-48 hours sporulated bits are aseptically transferred to sterilized petri plates containing carrot agar.

2.1.1 *In vitro* evaluation of *Trichoderma* spp. against *Pythium* spp.

2.1.1.1 Dual plate technique

Antagonistic potential of three species of *Trichoderma viz.*, *Trichoderma asperillum*, *Trichoderma virens*, and *Trichoderma aureoviridae* were assessed against *Pythium* spp. by dual plate inoculation technique (Upadyay and Rai, 1987) [30].

Actively growing mycelium disc of 5 mm diameter was cut from the margin of 7 days old culture of test pathogen and antagonistic agent respectively and placed opposite to each other on PDA in 3 petriplates having diameter of 90 mm. The discs were placed 20 mm away from periphery. The petriplate inoculated with disc of *Pythium* spp. alone served as control.

The inoculated plates were incubated in inverted position at 27±1 ºC in BOD for 7 days. The radial growth of *Pythium* spp. was measured to assess the antagonistic potential of *Trichoderma* spp. against pathogens. The percent growth inhibition of pathogen was calculated based on the formula suggested by Vincent (1947) [32].

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

Where,

C = Growth in control plate

T = Growth in treatment plate

I = Percent inhibition

2.2 Invert plate technique

Similar sized two petriplates were surface sterilized followed by transfer of twenty ml of sterilized and cooled potato dextrose agar into two seperate sterile Petri plates under aseptic condition and allowed to solidify. On one petriplate *Trichoderma* spp. was placed at the centre, wrapped and incubated for 24 hours. In other plate mycelial disc (5 mm) of test fungus, *Pythium* was placed at the centre and incubated for 24 hours.

After 24 hours, petriplates were unwrapped and kept one other above is such a fashion that, *Trichoderma* isolate containing plate was at lower side and test pathogen contained petriplate is at the top in inverted manner. Both the plates were wrapped and incubated at 27 ± 1 ºC in BOD for 7 days. The radial growth of *Pythium* was measured to assess the volatile effect of *Trichoderma* spp. against the test pathogen. The percent growth inhibition of pathogen was calculated as per formula suggested by Vincent (1947) [32].

2.3 Culture filtrate technique

Trichoderma species were inoculated into flasks containing sterilized potato dextrose broth and incubated for 15 days. After that the contents were filtered by passing through membrane filter twice. Further filtrate will be autoclaved and added at different concentrations into sterilized and liquified potato dextrose agar. This media was poured into the sterilized petriplate and pathogen, *Pythium* spp. was placed at the centre and incubated. Percent inhibition was calculated using the formula given by Vincent, 1947 [32].

3. Results

During dual plate technique all the species of *Trichoderma* significantly inhibited the growth of the both the species of *Pythium* ten days after inoculation compare to control the results revealed that, Among different biocontrol agents tested *T. virens* showed maximum inhibition with 75.88 and 71.76 percent of *P. aphanidermatum* and *P. myriotylum* respectively. Followed by *T. asperillum* with 70.00 and 71.96 percent. Whereas *T. aureoviridae* inhibits about and 65.55 and 54.90 percent of *P. aphanidermatum* and *P. myriotylum* respectively. Where they were on par with each other (Table 1).

Due to effect of volatile metabolites secreted by *Trichoderma* species against test pathogen, the range of inhibition was observed from 29-48.94% in *in vitro* condition. The highest inhibition of *Pythium aphanidermatum* and *Pythium myriotylum* was observed in *Trichoderma virens* about 53.16 and 43.69 percent respectively followed by *T. asperillum* (48.94 & 46.34%) and least was observed with *Pythium aphanidermatum* about 29.11 percent from *T. aureoviridae* in comparison with the control (Table 2).

Among the three species tested that culture filtrate of *Trichoderma virens* showed highest inhibition of *Pythium aphanidermatum* to an extent of 74.74, 45.25, 38.07 and 31.66 percent at 50, 25, 10 and 5 percent concentration of culture filtrate respectively. Followed by culture filtrate of *Trichoderma asperillum* (63.71, 44.28, 26.92 and 23.07% at 50, 25, 10 and 5%, respectively) and *Trichoderma aureoviridae* (61.53, 38.01, 29.01 and 16.66 at 50, 25 10 and 5%). Where they were on par with each other in comparison with the control (Table 3).

In the similar way, *Pythium myriotylum* was inhibited significantly by *Trichoderma virens* about 70, 51.42, 50.00 and 46.71 percent at 50, 25, 10, 5 percent, respectively whereas *Trichoderma asperillum* inhibits with 67.57, 36.71, 31.00, 27.57 percent at 50, 25, 10 and 5 percent concentration and least was observed in *Trichoderma aureoviridae* with 62.00, 38.57, 24.48, 12.42 percent at 50, 25, 10 and 5 percent

concentration, respectively (Table 3).

In case of *Pythium aphanidermatum* and *Pythium myriotylum* the highest inhibition in mycilial growth was found at the same concentration of isolate *Trichoderma virens* followed by *Trichoderma asperillum* and *Trichoderma aureoviridae.*

Table 1: Antogonistc effect of *Trichoderma* spp. against *Pythium* spp. in dual plate technique

Treatments	Inhibition (%)	
	Pythium myriotylum	***Pythium aphanidermatum***
T. asperillum	71.96 (58.00)*	70.00 (56.76)
T. virens	71.76 (57.87)	75.88 (60.56)
T. aureoviridae	54.90 (47.79)	65.55 (54.03)
S.Em±	0.16	0.18
CD (1%)	0.86	0.96

*Figures in parenthesis are arc sine transformed values

Table 2: Antogonistc effect of *Trichoderma* spp. against *Pythium* spp. in invert plate technique

Treatments	Inhibition (%)	
	Pythium myriotylum	***Pythium aphanidermatum***
T. asperillum	46.34 (42.88)*	48.94 (44.37)
T. virens	43.69 (41.35)	53.16 (46.79)
T. aureoviridae	43.49 (41.24)	29.11 (35.63)
S.Em±	0.18	0.07
CD (1%)	0.96	0.37

Table 3: Antogonistc effect of *Trichoderma* spp. against *Pythium* spp. in culture filtrate technique

Treatment \ Concentration		Inhibition (%)			
		5%	**10%**	**25%**	**50%**
T. asperillum	*P. aphanidermatum*	23.07 (8.20)*	26.92 (10.57)	44.48 (22.54)	63.71 (37.30)
	P. myriotylum	27.57 (15.75)	31.00 (17.80)	36.71 (21.33)	67.57 (43.89)
T. virens	*P. aphanidermatum*	31.66 (13.88)	38.07 (17.80)	45.25 (22.79)	74.74 (47.76)
	P. myriotylum	46.71 (27.90)	50.00 (30.19)	51.42 (31.20)	70.00 (46.05)
T. aureoviridae	*P. aphanidermatum*	16.66 (4.37)	29.01 (11.94)	38.01 (17.80)	61.53 (35.42)
	P. myriotylum	12.42 (7.19)	24.48 (13.83)	38.57 (22.51)	62.00 (39.92)

S.Em+	0.35
CD at	1%
Factor A (*Trichoderma* spp.)	1.069
Factor B (*Pythium* spp.)	0.873
Factor C (*concentration*)	1.234
A×B	1.511
A×C	2.137
B×C	1.745
A×B×C	3.023

Discussion

The antagonistic potential of three *Trichoderrma* species *viz., Trichoderma asperillum, Trichoderma virens and Trichodeerma aureoviridae* were evaluated against *Pythium* spp. in dual culture, invert plate, and culture filtrate technique. The results revealed the efficacy of all the three species in reducing the growth of the pathogens in all the three form *viz.*, parasitism through direct parasitism, by producing volatile compounds as well as through secondary metabolites.

Primarily the pathogens suppression by *Trichoderma* is due to competition, antibiosis, inhibition and parasitism of mycelium. Mycoparasitism is accomplished by the production of several lytic enzymes like Cellulase, Chitinases, Glucanases, proteases and lipases which help in degrading the cells and utilizing the contents as nutrients. Production of antibiotics, cell wall degrading enzymes and competition for space and nutrients by *Trichoderma* spp. was solely responsible for the suppression of the pathogens.

These results were in agreement with earlier workers. (Dennis and Webster, 1971; Bell *et al.*, 1982; Howell, 1982; El-Katany *et al*., 2001; Rajan *et al*., 2002; Howell, 2002; Vinale *et al.*, 2006; Khare *et al*., 2010; Muthukumar *et al*., 2011; Anita *et al*., 2012; Biljana, 2013; Rajendraprasad *et al*., 2017) [7, 2, 13, 8, 22, 14, 31, 17, 20, 1, 3, 23] Who reported that *Trichoderma* spp. effectively inhibited the mycellial growth of *Pythium* spp. Invert plate technique, revealed the bioefficacy of *Trichoderma* species in the form of volatile compound against *Pythium* spp. by reducing the growth under *in vitro* condition The results showed the highest inhibition of *Pythium* spp. by *Trichoderma virens* followed by *T. asperillum* and least was observed with *T. aureoviridae*.

Plate 1: *In vitro* evaluation of *Trichoderma* spp. against *Pythium* spp. in dual plate technique

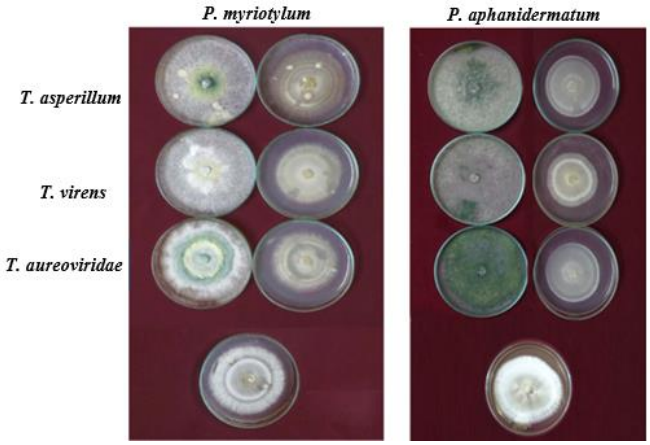

Plate 2: *In vitro* evaluation of *Trichoderma* spp. against *Pythium* spp. in invert plate technique

These results were in confirmatory with the results obtained by earlier workers (Claydon *et al.*, 1987; Howell, 1982; Rathore *et al.*, 1992; Harman *et al*., 2004; Khalid and Abdel 2017; Kuzmanovoska *et al*., 2018) [6, 13, 24, 12, 16, 18] who showed efficacy of both volatile and non-volatile antibiotics like trichodermin, trichodermol, harzianum A and harzianolide and acetaldehyde produced by *Trichoderma* which plays crucial role in inhibiting the growth of pathogen.

Culture filtrate study also revealed the antagonistic nature of *Trichoderma* species in the form of secondary metabolites which resulted in inhibition of mycelial growth of *Pythium* spp. at different concentration *in vitro*. *Trichoderma virens* inhibited the growth of *Pythium* spp. significantly followed by culture filtrate of *Trichoderma asperillum* and least mycelial growth observed with *Trichoderma aureoviridae.*

This inhibitory effect is due to production and release of Cell wall degrading enzymes (CWDEs), Glucan, Chitin synthases, chitinases, glucanases and antibiotics. Gliotoxin and gliovirin are the two most important *Trichoderma* secondary metabolites belonging to P and Q group strain, respectively a P group strain of *Trichoderma* (Gliocladium) *virens* is active against many plant pathogens. (Howell, 2002). *T. asperellum* strain produces two asperelines (A and E) and five trichotoxins (T5D2, T5E, T5F, T5G and 1717A) which can be associated with antibiosis inhibit the pathogens in culture filtrate effectively (Brito *et al.*, 2014) [4].

These results were similar with the work carried out by earlier workers (Sathpathi, 1998; Misra and Prasad 2003; Tondje *et al*., 2007; Guptan and Misra 2009; Singh *et al*., 2016; Rajendraprasad *et al*., 2017 and Jahangir *et al*., 2017) [25, 19, 28, 11, 27, 23, 15] who showed that the highest inhibition in culture filtrate of *Trichoderma* spp. was against *Pythium* spp.

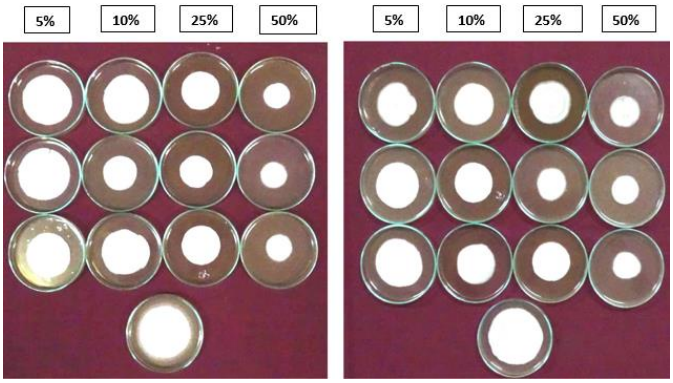

Plate 3: Inhibition of *Pythium* spp. by *Trichoderma* spp. in culture filtrate technique

Conclusion

Among the three species of *Trichoderma* tested, *Trichoderma virens* showed maximum antagonistic effect against *Pythium aphanidermatum* and *Pythium myriotylum*. Whereas *Trichoderma asperillum* and *Trichoderma aureoviride* showed moderate antagonistic effect. The antagonistic or inhibitory effect of *Trichoderma* species was observed more in dual plate and culture filtrate compare to invert plate technique under *in vitro*.

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