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Exploration and Interaction of *Trichoderma* species and their Metabolites by Confrontation assay against *Pythium aphanidermatum*

Anuradha Singh[1], Mukesh Srivastava[2], Vipul Kumar[3], Antima Sharma[4], Sonika Pandey[5], Mohd. Shahid[6]

[1]Biocontrol Laboratory, Department of Plant Pathology, C.S.A. University of Agriculture & Technology, Kanpur-208002, Uttar Pradesh, India

Abstract: ***Four species of Trichoderma when screened against Pythium aphanidermatum by dual culture technique had varied antagonistic effects against the pathogen. The efficacy of Trichoderma culture filtrates was also determined. Since mycoparasitism plays an important role in antagonistic mechanism of Trichoderma species, extracellular enzymatic activity of the strains was assayed. Damping off of Chilli caused by Pythium aphanidermatum is a major disease in vegetables. In vitro experiments evaluated the effect of four isolates of Trichoderma species viz. Trichoderma harzianum (Th Azad), Trichoderma viride (01PP), Trichoderma asperellum (T_{asp}/CSAU) and Trichoderma longibrachiatum (21PP) were tested against Pythium aphanidermatum (both grown on PDA). T. harzianum (Th Azad) recorded maximum growth inhibition (60.38%) against P. aphanidermatum and produced more amounts of volatile and non-volatile metabolites. The culture filtrate of Trichoderma harzianum recorded complete inhibition on the mycelial growth of pathogen. The experimental results of different seed treatments in chilli revealed significant responses against all the seven seed quality attributes viz. germination, shoot length, root length, seedling length, seedling dry weight, vigour index I and vigour index II. T_1 treatment (T. harzianum (Th Azad) was found to be significantly superior and effective in increasing 8.39 per cent more germination of chilli from control.***

Keywords: *Trichoderma* species, *Pythium aphanidermatum,* interaction, metabolites, germination

1. Introduction

Management of *Pythium* is very difficult due to its wide host range, soil-borne nature and prolonged survival of propagules in the soil. Traditionally, this disease is controlled by the application of synthetic fungicides. But the indiscriminate use of fungicides resulted in the accumulation of residual toxicity, environmental pollution and altered the biological balance in the soil by over killing the non-targeted microorganisms. Besides development of resistance to fungicides in the pathogen [1] *Pythium* spp. are worldwide in distribution [2] that attack cuttings, seeds, seedlings and all stages of the various crops causing significant losses to them. *Pythium* root rot is a familiar crop disease caused by a genus of organisms called *Pythium,* The most common means to check the disease caused by *P. aphanidermatum* in plants is by using fungicides. Frequent use of chemicals leads to environmental pollution. The increasing awareness of fungicide-related hazards has emphasized the need of adopting biological methods as an alternative disease control method. Species of the genus *Trichoderma* are well documented fungal biocontrol agents [3]. The antagonistic action of *Trichoderma* species against phytopathogenic fungi might be due to either by the secretion of extracellular hydrolytic enzymes [4] or by the production of antibiotics [5]. It is therefore essential to develop an effective, cheap and environmentally safe non-chemical method for the management of damping-off disease. Hence, biological control has been developed as an alternative to synthetic fungicides and considerable success has been achieved by utilizing antagonistic microorganisms for controlling soil-borne pathogens. The need for alternative control strategies, particularly those involving biological control, has increased greatly in the past two decades. Growth inhibition of *Pythium* species by the *Trichoderma* metabolites has been well researched [6]. The successful application of *Trichoderma* species for the management of damping-off caused by *Pythium* species in chilli and tomato has been previously reported [7]. In view of the above, the present study was carried out to investigate the effective strain of *Trichoderma* species against *P. aphanidermatum*.

2. Materials and Methods

An experiment was conducted in October, 2013 at Department of Plant Pathology, Biocontrol Laboratory of CSAUA&T, Kanpur, to evaluate the efficacy of *Trichoderma* species against *P. aphanidermatum* that were collected from rhizospheric soils of chickpea, pigeonpea and lentil crops from different places of Uttar Pradesh, India.

2.1 Isolation of *Trichoderma* sp.

Table 1 lists the different isolates and their details that have been used in this study. All the isolates were isolated on PDA medium with serial dilution plate technique described by Johnson and Curl [8] . Isolates were identified up to species level based on phenotypic characters like colony colour and growth; size and shape of conidiophore, phialides and conidia. The cultures were identified using the available literature and also confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12.

Table 1: Identification of potential strains of *Trichoderma sp*

Strain No.	Name of Bioagent	ITCC Acc. No	GenBank Accession No.	Strain code	Source	GPS Location
T1	*T. harzianum*	6796	KC800922	*Th* Azad	CSA Kanpur Nagar	Latitude: 25° 8′ 34.821″ Longitude: 81° 59′ 2.979″
T2	*T. viride*	8315	JX119211	01PP	Hardoi	Latitude: 27° 23′ 40.729″ Longitude: 80° 7′ 47.751″
T3	*T. asperellum*	8940	KC800921	T_{asp}/ CSA U	CSA Kanpur Nagar	Latitude: 25° 8′ 34.821″ Longitude: 81° 59′ 2.979″
T4	*T. longibrachiatum*	7437	JX978542	21 PP	Kaushambi	Latitude: 26° 34′ 27.61″ Longitude: 79° 18′ 24.623″

2.2 Isolation and Characterization of Pathogen

Pythium aphanidermatum was isolated from the soil sample of CSAUA&T Kanpur, vegetable farm by using root trapping method with some modifications on potato dextrose agar (PDA) according to Patil *et al*. [1]. Further species-level identification and characterization were carried out by using multiple identification key such as oogonia wall, antheridia position, swollen hyphae, sporangia size, etc.

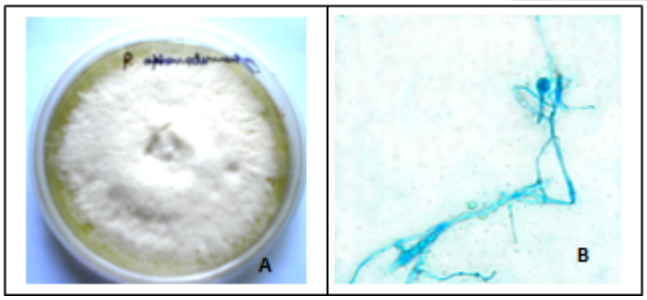

Figure 1 : *P. aphanidermatum* (A) Growth on PDA medium (B) Microscopic observation at 40x

2.2 Confrontation assays *in vitro*

2.2.1 Dual culture technique: About 7-day old culture, mycelial disc (5mm) from a *Trichoderma sp.* and test pathogens were placed on the plate opposite to each other equidistant from the periphery and were incubated at 27°C. After 7 days of the incubation period, radial growth of pathogen was recorded and percentage inhibition calculated in relation with control [9] by following formula:

$$L= (C-T)/C*100$$

2.2.2 Effect of non-volatile metabolites: The activity of non volatile metabolites of *Trichoderma* species were studied using the antagonist culture filtrate [10]. Mycelial discs of 5 mm size from 7 days old culture of *Trichoderma* species were grown in conical flask containing 150 ml potato dextrose broth (PDB) and incubated at 27˚C for 7 days. The culture medium was filtered using Whatman No.1 filter paper after removing the mycelial mats. The filtrate was centrifuged at 9000 rpm for 10 min. The supernatant was filter sterilized using Millipore membrane filter paper (0.22µm). The filtrate at a concentration of 10% was added to potato dextrose agar (PDA) before pouring in to petri dishes. Mycelial discs of the pathogen were inoculated at the centre of the petri plates. The plates were incubated at 27°C for five days. The pathogen alone inoculated on to PDA was kept as control. The radial growth was recorded and the percentage of inhibition was calculated as above.

2.2.3 Seed treatment: Chilli seeds were surface sterilized with 2% sodium hypochlorite for 30 seconds, rinsed in sterile distilled water and air dried overnight. 10 mL of biocontrol inoculum containing 2×10^8 CFU mL^{-1} was placed in a Petri plate. A small amount of Carboxymethyl Cellulose (100 mg) was added as an adhesive material. Fifty seeds were soaked in 10 mL of biocontrol suspension for 2 h and air dried overnight in a sterile Petri plate [11].

2.2.4 *In vitro* effect of Seed treatment: Seed treatments not only protect the seeds from soil borne diseases but also provide protection to the emerged seeding from sucking insect pests affecting crop emergence and its early growth. The seed treatment test included five treatments with one control viz., T_1 (*T. harzianum*), T_2 (*T. viride*), T_3 **(***T. asperellum)*, T_4 (*T. longibrachiatum*) and T_5 (control). Freshly harvested fifty chilli seeds were counted and weighed to apply the recommended dose of biocontrol agent into three replications. Seed treatment was carried out with the help of paper towel method [12]. The treated seeds were subjected to assess the germination and vigour as per the procedure recommended by ISTA at Biocontrol laboratory, Dept of Plant Pathology, CSAUA&T, Kanpur. For observing the dry weight of seedling, about10 seeds are randomly picked from all treatments and put in separate petri plates in hot air oven for eight hours at 30^0 C.

Two recommended methods *viz.,* germination percent x seedling length for vigour index I [13] and germination x dry weight for vigour index-II was adopted during the course of investigation.

3. Results and Discussion

3.1 Characterization of *Pythium*

The microscopic characterization and for species-level identification, slides of isolated cultures were prepared and stained with lactophenol cotton blue. The slides were observed under light microscope. The culture was found to be *Pythium aphanidermatum* by the following characteristics: colony color, sporangia consisting of a terminal complex of swollen hyphal branches of varying length; oogonia terminal, globus smooth with 24-29 µm diameter. Antheridia terminal and intercalary of broadly sac shaped [14].

3.2 Confrontation assays *in vitro:*

Dual culture technique was carried out while studying the interaction between *Trichoderma* species *viz. T. harzianum* (*Th* Azad), *T. viride* (01PP), *T. asperellum* (T_{asp}/CSAU), *T. longibrachiatum* (21PP) and *P. aphanidermatum.* Effect of

different species of *Trichoderma* with respect to suppression of mycelial growth of the test pathogen was recorded. It is evident from the data that *Trichoderma harzianum* (69.70%) suppressed the radial growth of *P. aphanidermatum* significantly followed by *Trichoderma viride* (67.74%) and *T. longibrachiatum* (62.94%). The least suppression was showed by *Trichoderma asperellum* (61.76%) (Fig 2).

Figure 2: Plate confrontation test of *Trichoderma* sp. against *Pythium aphanidermatum*

The main objective was to identify the most promising *Trichoderma* isolate for the management of root rot. The present study involved preliminary screening of the isolates by three methods i.e., dual plating, activity of non-volatile metabolites. The promising antagonistic activity of the isolates *T. harzianum* (*Th* Azad), *T. viride* (01PP), *T. asperellum* (T_{asp}/CSAU) and *T. longibrachiatum* (21PP), on dual culture may be due to mycoparasitism. The ability of *Trichoderma* to inhibit the growth of plant pathogens like *Pythium* sp. has been reported by several authors [15], [11], [1] and [16]. Hyphal parasitism of *Pythium* sp. by *Trichoderma* was also observed *in vitro* by many workers. Amongst them, Chet I [17] reported coiling and puncturing of the mycelium of *Pythium* by *Trichoderma* sp. during hyphal interactions. Since the nature of interaction between the antagonist and pathogen is important in biocontrol approach it is evident that mycoparasitism and competition are the two modes of biocontrol mechanism showed by these isolates.

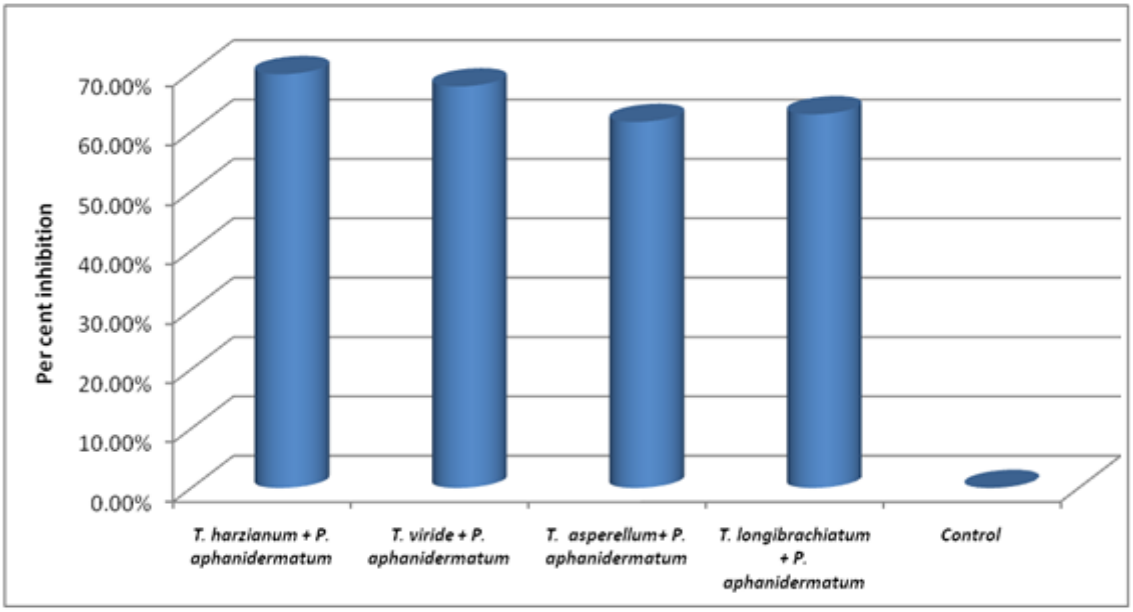

Figure 3: Antagonistic activity of *Trichoderma* sp. against *Pythium aphanidermatum*

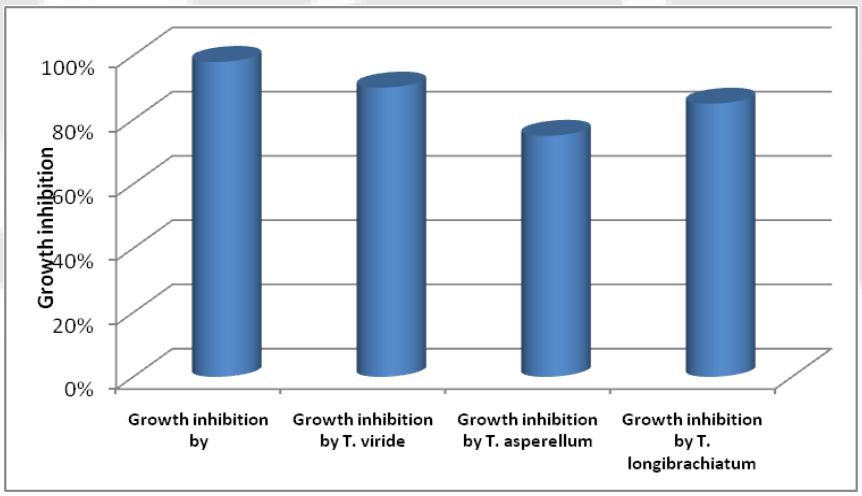

Figure 4: Effect of *Trichoderma* Metabolites on *Pythium aphanidermatum*

Pythium (pathogen) disc was placed at centre while four *Trichoderma* (biocontrol) disc were placed at periphery and more percentage inhibition was observed as compared to the results of dual culture technique. In the study of interaction

between *Trichoderma* and pathogen (*Pythium aphanidermatum*) at centre technique, range of inhibition was observed between 75 and 98%. *T. harzianum* showed highest inhibition against *P. aphanidermatum* (98%); followed by *Trichoderma viride* (90%) and least by *T. longibrachiatum* (75%). While, *T. asperellum* (85%) showed less inhibition percentage than *T. viride* against *P. aphanidermatum* (Table 2).

Table 2: Effect of different seed treatments on quality of chilli seeds

T. No.	*Treatment*	*Germi-nation %*	*Root length (cm)*	*Shoot length (cm)*	*Seedling length (cm)*	*Dry weight (mg)*	*Vigour index -I*	*Vigour index II*
T1.	*T. harzianum* (*Th* Azad)	94%	5.52	5.25	10.77	0.35	506.19	16.45
T2.	*T. viride* (01PP)	90%	5.30	5.10	10.40	0.33	468.00	14.85
T3.	*T. asperellum* (T_{asp}(CSAU)	78%	5.35	5.28	10.63	0.32	414.75	12.48
T4.	*T. longibrachiatum* (21PP)	68%	4.80	4.75	9.55	0.31	324.70	10.54
T5.	*Control*	56%	4.92	3.85	8.77	0.28	245.56	07.84

The experimental results of different seed treatments in chilli revealed different significant responses against all the seven seed quality attributes viz. germination, shoot length, root length, seedling length, seedling dry weight, vigour index I and vigour index II (Table-2). T_1 treatment *(T. harzianum* (*Th* Azad) was found to be significantly superior and effective in increasing 10.39 per cent more germination of chilli from control followed by T_1 (94%), T_2 (90%), T_3 (78%) and T_4. Similarly, the beneficial impact of seed treatment was also recorded for root length, shoot length, seedling length and dry weight vigour index-I and vigour index-II in which T_1 treatment excelled overall significant superior performance by contributing 5.52 cm, 5.25 cm, 10.77 cm, 0.35 mg, 506.19 and 16.45, respectively followed by T_2 treatment (*T. viride*) for all these physiological attributes by contributing 5.30 cm, 5.10 cm, 10.40 cm, 0.33 mg, 468.00 and 14.85 as root length, shoot length seedling length, dry weight vigour index-I and vigour index-II respectively. Least performance was given by *Trichoderma asperellum* in all seven quality attributes. Cokkizgin & Cokkizgin [18] reported germination and vigour index in lentil. Singh *et al.* [19] also investigated to know the impact of pre-sowing seed treatment on germination, seedling establishment, seedling dry weight and vigour in lentil genotype (KLB 320). The various pre-sowing seed treatments showed different responses against all seven seed quality attributes and also supported by Shahid *et al.* [20] in case of chickpea seed treatment.

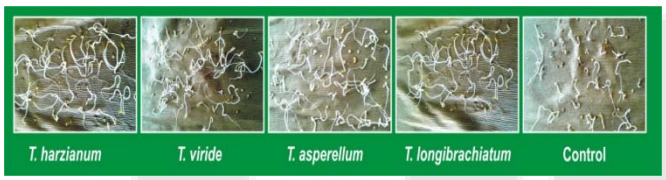

Figure 5: Effect of different seed treatments

4. Conclusion

The antagonist *Trichoderma* not only suppresses the growth of pathogen and controls the disease, but also has expresses growth promoting effects in plants. It can be concluded from this study that out of five treatments including control, T_1 treatment (*Th* Azad) is the best seed treatment to enhance the quality parameters of chilli seeds that can be helpful in increasing its yield even in adverse environmental conditions.

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