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REGULAR ARTICLE

IN VITRO ANTAGONISM OF *TRICHODERMA* SPECIES AGAINST *PYTHIUM APHANIDERMATUM*

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SUMMARY

Ten strains of *Trichoderma* species were screened against *Pythium aphanidermatum* by dual culture method. Efficacy of culture filtrates of the strains was also determined. Since mycoparasitism plays important role in antagonism mechanism of *Trichoderma* species, extracellular enzymatic activity of the strains was assayed. Among the strains tested, *T. viride* 1433 was found most effective against *P. aphanidermatum*.

Key words: *Trichoderma* species, *Pythium aphanidermatum*, Dual culture, Culture filtrate, Enzymatic activity

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1. Introduction

Pythium spp. are worldwide in distribution (Hendrix and Campbell, 1973) that attack cuttings, seeds, seedlings and all stages of the various crops causing significant losses to them. Almost all greenhouse crops are susceptible to one or more species of *Pythium* (Miller and Sauve, 1975; Stephens and Powell, 1982). Of the different species of *Pythium, P. aphanidermatum* (Edson) Fitz. is reported from a large number of hosts (Van der Plouts-Niterink, 1981). The most common means to check the disease caused by *P. aphanidermatum* in plants is by using fungicides. Frequent use of these 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 (Papavizas, 1985; Elad and Kapat, 1999; Howell, 2002). The antagonistic action of *Trichoderma* species against phytopathogenic fungi might be due to either by the secretion of extracellular hydrolytic enzymes (Chet, 1987; Di Pietro *et al*., 1993; Schirmbock *et al*., 1994) or by the production of antibiotics (Dennis and Webster, 1971a; Dennis and Webster, 1971b; Claydon *et al*., 1987; Howell, 1998).

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

Fungal isolates

The pure culture of the antagonists were obtained from the culture collection of Institute of Microbial Technology (Chandigarh), National Botanical Research Institute (Lucknow), Indian Agricultural Research Institute (New Delhi), Indian Institute of Vegetable Research (IIVR, Varanasi). A virulent strain of *Pythium aphanidermatum* was obtained from the Department of Mycology and Plant Pathology, Institute of Agriculture Science, Banaras Hindu University (BHU), Varanasi. The pathogenic and antagonistic strains was maintained on Potato-Dextrose Agar medium (PDA; Merck) at 25±□2°C by regular subculturings.

Colony growth inhibition assay

In vitro antagonistic activity of *Trichoderma* species against *P. aphanidermatum* was studied in dual culture by following the method described by Upadhyay and Rai (1987). The colony interactions were assayed as per cent

inhibition of the radial growth by the following formula (Fokkema, 1976): R1 – R2/ R1 ×□100, where, R1 denotes diameter of the radial growth of the pathogen towards opposite side and R2 denotes the radial growth of the pathogen towards the opponent antagonist.

Inhibitory effect of the culture filtrates

Trichoderma species were inoculated into 100 ml sterilized potato dextrose broth and incubated at 25°C for 10 days, after which the culture filtrates were filtered through Whatman filter paper no. 44 and again refiltered through Seitz filter (G4) by vacuum pressure to obtain cell-free culture filtrates.

Four ml of culture filtrates of each *Trichoderma* species were placed in sterilized petri dish which was immediately followed by pouring 16 ml of PDA, so as to make the final concentration of culture filtrates 20 %. After the agar solidified, mycelial discs of the *P. aphanidermatum* (5 mm in diameter) obtained from actively growing colonies were placed in the centre of the agar plates. The petri dishes were incubated at 25°C for 4 days.

The percent inhibition in the radial colony growth was calculated by the following formula:

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where, C = Radial growth in control set; T = Radial growth in treated set.

Assay of enzyme activity

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) contained the following components (in grams per liter): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4.7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source for cellulase, β- 1, 3 glucanase and protease assay. The pH was set to 6.3 with 50 mM phosphate buffer and autoclaved at 15 psi for 20 min. The medium was inoculated with a spore suspension to give a final concentration of ~5 $\times 10^6$ conidia per milliliter and placed on a rotary shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at fourth day of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4°C to get cell-free culture filtrate which were then used as enzyme source.

Cellulase assay

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Co.) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrates of different *Trichoderma* strains. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity is defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

β- 1, 3 Glucanase assay

β-1,3-glucanase was assayed similarly by incubating 1 ml 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH = 4.8) with 1ml enzyme solution at 50 °C for 1 h and by determining the reducing sugars with DNS (Nelson, 1944). The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1, 3 glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of glucose equivalents per min.

Protease assay

Protease activity was determined by a modified Anson's Method (Yang and Huang, 1994). The substrate used (1% casein in 50mM phosphate buffer, pH 7.0) was denatured at 100°C for 15 min in a water bath and cooled at room temperature. The reaction mixture containing 1 ml of substrate and 1 ml of enzyme solution were incubated at 37°C for 20 min with shaking and was stopped with 3 ml of 10% trichloroacetic acid (TCA). The absorbance of the liberated tyrosine in the filtrate was measured at 280

nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280 nm equivalent to 1 µmol of tyrosine in one minute under the assay condition.

3. Results

In vitro screening

The results of the colony interactions clearly demonstrate that *Trichoderma* species exhibited inhibition of the radial growth of *P. aphanidermatum* (Table 1). 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%). The least inhibition of *P. aphanidermatum* was recorded in case of *T. harzianum*-4 (38.5%) and *T. pseudokoningii*-2048(39.3%).

Table 1. Effect of strains of *Trichoderma* species on the per cent inhibition of radial colony growth of *P. aphanidermatum*

Trichoderma species	Percent inhibition
T. harzianum-4532	60.3±0.3e
T. harzianum-4572	69.8±0.3g
T. harzianum-801	54.1±0.5c
T. viride-1763	52.2±0.5b
T. viride-1433	72.0±0.3h
T. viride-793	62.1±0.3f
T. viride-2109	50.4±0.4a
T. koningii-2385	56.4±0.2d
T. virens-2023	53.5±0.6c
T. virens-2194	59.6±0.6e

Values are average of three replicates ± SEM
Values in the column followed by same letter are not significantly different (P< 0.05)

Table 2. Effect of culture filtrate of strains of *Trichoderma* species on the per cent inhibition of radial growth of *P. aphanidermatum*

Trichoderma species	Percent inhibition
T. harzianum-4532	27.4±0.3d
T. harzianum-4572	34.2±0.4g
T. harzianum-801	23.1±0.3c
T. viride-1763	21.7±0.5b
T. viride-1433	38.6±0.6h
T. viride-793	30.2±0.5f
T. viride-2109	18.6±0.3a
T. koningii-2385	24.2±0.4c
T. virens-2023	26.4±0.3d
T. virens-2194	28.8±0.5e

Values are average of three replicates ± SEM
Values in the column followed by same letter are not significantly different (P< 0.05)

Inhibitory effect of the culture filtrates

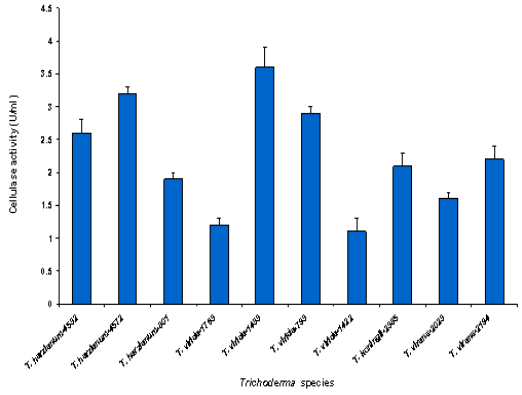
The effect of culture filtrates of selected strains of *Trichoderma* species on the per cent inhibition of radial growth of *P. aphanidermatum* has been presented in Figure 1. Out of ten strains of *Trichoderma* species, the maximum inhibition of the radial growth of *P. aphanidermatum* was observed by the culture filtrate of *T. viride*-1433 (38.6%), which was followed by *T. harzianum*-4572

(34.2%), and *T. viride*-793 (30.2%). The least inhibition was observed in case of *T. viride*-2109 (18.6%).

Extracellular enzymatic activity

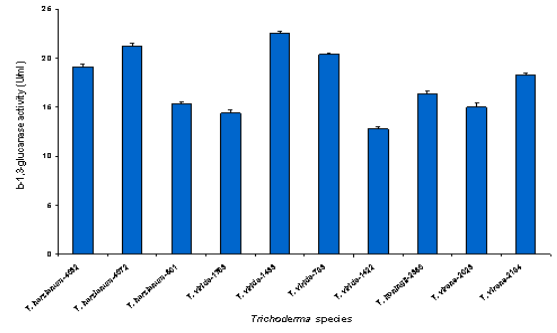
It is evident from the data in Figure 3 that the highest cellulase activity was observed in case of *T. viride*-1433 (3.6 U/ml), which was followed by *T. harzianum*-4572 (3.2 U/ml). The cellulase activity of *T. viride*-793 (2.9 U/ml) showed no significant difference ($P < 0.05$) with *T. harzianum*-4532 (2.6 U/ml). The least cellulase activity was produced by *T. viride*-2109 (1.1 U/ml) and T. viride-1763 (1.2 U/ml).

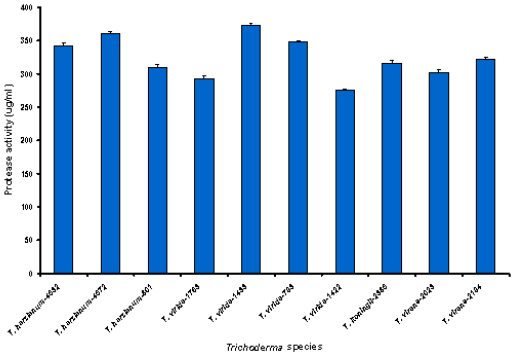
Figure 3. Cellulase activity of the selected strains of *Trichoderma* species



The β-1, 3-glucanase activity of the strains varied from 12.8 to 22.5 U/ml (Figure 4). The highest activity was recorded in case of *T. viride*-1433 (22.5 U/ml). The β-1, 3-glucanase activity of *T. harzianum*-4572 (21.2 U/ml) and *T. viride*-793 (20.4 U/ml) was not significantly different ($P < 0.05$). *T. viride*-2109 significantly produced minimum activity of β-1, 3-glucanase (12.8 U/ml).

Figure 4. β-1,3 glucanase activity of the selected strains of *Trichoderma* species





The data in Figure 5 showed that *T. viride*-1433 significantly produced maximum protease activity (373 µg/ml), which was followed by *T. harzianum*-4572 (360 µg/ml). Among the tested strains, the least protease activity was recorded in case of *T. viride*-2109 (275 µg/ml).

4. Discussion

Antagonism of *Trichoderma* species against several pathogens has been reported (Chet and Baker, 1980; Bell *et al*., 1982; Papavizas, 1985; Elad, 2000; El-Katatny *et al*., 2001; Howell, 2002). As dual culture method is widely used in antagonistic studies (Huang, 1978; Pachenari and Dix, 1980; Bell *et al*., 1982), the present investigation, was carried out to screen ten strains of *Trichoderma* species against *P. aphanidermatum* by this method. The degree of inhibition varied from one strain to another. Similarly, isolates of different *Trichoderma* species to control *Sclerotium rolfsii* have been reported to differ in their effectiveness (Elad *et al*., 1980; Maity and Sen, 1984). Maximum inhibition of the pathogen (72.0%) was observed with *T. viride*-1433. Formation of inhibition zone at the contact between *Trichoderma* and *P. aphanidermatum* in dual cultures could be explained on the basis of production of volatile and non-volatile metabolites as well as the production of extracellular hydrolytic enzymes by *Trichoderma* (El-Katatny, 2001).

Culture filtrates have been used in the present study to demonstrate the possible presence and role of fungal metabolites in understanding the antagonistic behaviour of *Trichoderma* species. The culture filtrates of

the strains of *Trichoderma* species were found effective to inhibit the growth of pathogen in varied degrees. The maximum inhibition was due to culture filtrate of *T. viride*-1433 (44.0%). Growth inhibition of the pathogens by the *Trichoderma* metabolites has been reported by several workers (Dennis and Webester, 1971a, 1971b; Howell and Stipanovic, 1983; Sivan *et al*., 1984; Claydon *et al*., 1987; Ghisalberti and Sivasithamparam, 1991; Howell, 1998). The effect of culture filtrate of *Trichoderma* on the pathogen might be due to the production of antibiotics (Upadhyay and Rai, 1987). Fajola and Alasoadura (1975) found that culture filtrates of *T. harzianum* inhibit zoospore germination, germ tube elongation and mycelial growth of *P. aphanidermatum* causing the damping-off disease of tobacco. Metabolites produced from *T. viride* and *T. polysporum* reduced the growth of *Ceratocystis paradoxa*, the causal agent of black seed rot in oil palm (Eziashi *et al*., 2006). Huang *et al*. (1995) isolated peptaibols, named trichokonins from the culture broth of *T. koningii*. Calistru *et al*. (1997) reported that the culture filtrates of *T. viride* and *T. harzianum* were inhibitory to *Fusarium moniliforme*. Sivan *et al*. (1984) noted that culture filtrates of *T. harzianum* strongly inhibited growth of *P. aphanidermatum* in culture. Kapil and Kapoor (2005) reported that the culture filtrate of *T. viride* inhibited the mycelial growth of *Sclerotinia sclerotiorum* due to production of antibiotic like substance. Lee and Wu (1984) observed that *T. viride* produced metabolites that inhibited the mycelial growth of *Sclerotinia sclerotiorum*. The metabolites released in the culture filtrates by the *Trichoderma* species in the present investigation may be similar to the metabolites produced by other *Trichoderma* as mentioned above that may be toxic and/or fungistatic to *P. aphanidermatum*. This suggests that the antibiotics possibly play an important role in suppressing infection by the pathogen.

The extracellular enzymes produced by *Trichoderma* strains may be correlated with the antagonism. Elad *et al.* (1982) reported that the isolates of *T. harzianum*, which were found to differ in their ability to attack *Sclerotium rolfsii*, *Rhizoctonia solanii* and *P. aphanidermatum*, also differed in the levels of mycolytic enzymes produced by them. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1, 3-glucanases and proteases (Elad *et al*., 1982; Haran *et al*., 1996a). In the present study *T. viride*-1433 was observed to be the efficient producer of cellulases (3.6 U/ml), β-1, 3-glucanases (22.5 U/ml) and proteases (373 µg/ml). This might be one of the reasons for its biocontrol potentiality. As the cell wall of *Pythium* species are composed of cellulose and 1, 3-β-glucan (Bartinicki-Garcia, 1968), the enzymes, cellulase and β-1, 3-glucanase produced by *Trichoderma* might be involved in hydrolysis of *P. aphanidermatum* cell wall during antagonism (Thrane *et al*., 1997). Lorito *et al*. (1994) reported the involvement of glucanases in mycoparasitism. Jones *et al*. (1974) have shown that *T. viride* solubilized hyphae of *Sclerotinia sclerotiorum* by β-1, 3- glucanase activity. As fungal cell wall also contains lipids and proteins (Hunsley and Burnett, 1970), the antagonistic fungi synthesize proteases which might act on the host cell wall. Involvment of proteases in biocontrol processes has already been reported (Elad and Kapat, 1999; Pozo *et al*., 2004). De Marco and Felix (2002) observed that the biocontrol potential of an Indian *Trichoderma* isolate against *C. perniciosa* was due to protease activity.

In conclusion, the strain *T. viride* 1433 is found effective against *P. aphanidermatum*. If biocontrol potential of this strain is enhanced by generating mutants, it will become a promising biocontrol agent against *P. aphanidermatum*.

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