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Antifungal properties of native *Trichoderma* isolates against *Sclerotium rolfsii* and *Pythium aphanidermatum* infecting tobacco

K.L.N. Mallikharjuna Rao[1], K. Siva Raju[2]* and H. Ravisankar[2]

[1]AKRG College of Pharmacy, Nallajarla, West Godavari-534 112, India
[2]AKMU, Central Tobacco Research Institute, Rajahmundry-533 105, India
*Corresponding Author E-mail: rajuks2002@yahoo.co.in

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Abstract

Isolates of *Trichoderma* native to tobacco rhizosphere, were evaluated for their antifungal properties against *Sclerotium rolfsii* and *Pythium aphanidermatum*, fungal pathogens causing collar rot and damping off diseases in tobacco. Isolate TvJt1 showed maximum HCN production (0.14 OD) followed by ThHt1 and minimum by isolates ThJnt and ThRt1 (0.08 and 0.09 OD). Indole acetic acid (IAA) production among the isolates varied from 6.48 to 14. 82 µg ml^{-1}. Isolate ThJO1 produced maximum siderophore (13.48 m mol ml^{-1}), whereas isolate TvJt1 produced minimum content (6.24 m mol ml^{-1}). Isolate ThJt1 showed maximum and significantly higher chitinase activity (62.12 p kat ml^{-1}) as compared to other isolates, followed by isolate TvHt2. Isolate TvHt2 showed maximum and significantly higher β-1, 3-glucanase activity (9.94 n kat ml^{-1}) when compared to all other isolates. Isolate ThJt1 showed second highest activity of β-1, 3-glucanase (6.75 n kat/ml). In dual culture, inhibition of mycelial growth of *Pythium* by isolate TvHt2 was maximum (72.45%) followed by ThJt1 whereas inhibition of mycelial growth of *S. rolfsii* by these two isolates was at a par (78.36% and 76.17%) and significantly higher than some isolates. Isolates TvHt2 and ThJt1 showed maximum and significantly higher inhibition of *Pythium* and *Sclerotium* growth (80.24% and 76%) by production of non-volatiles. Inhibition of *Pythium* and *Sclerotium* growth by production of volatile compounds by *Trichoderma* isolates ranged from 54.36 to 72.6% and 66.24 to 84.24% respectively. Hence, further investigation with potential isolates ThJt1 and TvHt2 in tobacco nursery may help to explore these bioagents for control of collar rot and damping off diseases in tobacco.

Key words

Antifungal properties, Fungal pathogens, Tobacco rhizosphere, *Trichoderma* isolates

Introduction

Biological suppression of plant diseases has been promoted as a means to achieve improved and sustainable crop production systems that are less reliant on chemical inputs (Ozbay *et al.,* 2004). The presence of fungal diseases on tobacco and its economical consequences require many fungicides. *Sclerotium rolfsii* and *Pythium aphanidermatum*, causing collar rot and damping off diseases in tobacco nursery, are wide spread in all tobacco areas causing huge loss to farmers. There is no resistance to *S. rolfsii* and *P. aphanidermatum* in the available cultivars. Modern production of tobacco is striving to involve biocontrol of diseases and limited chemical usage. For long, *Trichoderma* species have been known as biological agents for control of plant diseases. Biological control offers an environmental friendly approach for managing plant disease and can be incorporated into cultural and physical control and limited chemical use for effective integrated disease management (Biljana and Jugoslav, 2012).

Successful biological control systems commonly employ naturally occurring, antagonistic microorganisms that can effectively reduce the activities of plant pathogens. Microorganisms isolated from root or rhizospheree of specific crop may be better adapted to that crop, and may provide better control of diseases than organisms originally isolated from other plant species (Bunker and Mathur, 2001). *Trichoderma* spp. are well known biocontrol agents against a wide range of soil borne pathogens and some have plant growth promotion capacity (Ozbay *et al.,* 2004). Screening of diverse population of biocontrol

agents is an important requirement for developing efficient biocontrol agents.

Biological control involving *Trichoderma* spp. operates by way of mycoparasitism, antibiosis and competition. Antagonistic abilities of *Trichoderma* spp. are combination of several mechanisms including direct mycoparasitism which involves production of cell-wall degrading enzymes (CWDE) (Lorito *et al*., 1993; Qualhato *et al*., 2013). *Trichoderma* spp. secrete cell wall degrading enzymes like chitinases and volatile and non- volatile compounds which enter cell in the form of signal and triggers secondary messengers and altering the metabolic pathway of the pathogen (Howell, 2003). Most *Trichoderma* strains produce volatile (Vey *et al*., 2001) and non-volatile toxic secondary metabolites, among these production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vinale *et al*., 2009). They are frequently associated with biocontrol activity and promotion of plant and root growth (Saba *et al*., 2012). There is no solution, therefore biocontrol system must be developed for each crop. Before development and application of a biocontrol agent, it is necessary to study the various aspects of biocontrol agents. The first and the quickest way is to screen native *Trichoderma* isolates (*i.e,.* isolates from tobacco rhizosphere) for their biocontrol efficacy and cultural conditions for biomass production (Harman, 2006). In view of the above, the objective of the present study was to screen native *Trichoderma* isolates from tobacco rhizosphere for their antifungal properties against *S. rolfsii and P. aphanidermatum*.

Materials and Methods

Isolates of *Trichoderma* were isolated from different tobacco areas. The isolates of *T. harzianum* included ThRt1-FCV tobacco, ThRt2-FCV tobacco nursery and ThC-commercial isolate, Rajahmundry; ThJt1-FCV tobacco, ThJnt-*Natu* tobacco, ThJO1-oil palm, Jeelugumilli; ThDt1-*Motihari* tobacco, ThDt2-*Jati* tobacco, Dinhata; ThHt1-FCV tobacco, ThHt3-FCV tobacco, Hunsur, while isolates of *T. viride* included TvJt1-FCV tobacco, Jeelugumilli and TvHt2-FCV tobacco, Hunsur. *Trichoderma* spp. were isolated using dilution plate and soil plate techniques on PDA. Among various isolates, 12 isolates of *Trichoderma* were selected. *Trichoderma* colonies were identified according to the identification key, primarily based on growth characters of the medium and branching of conidiophores, shape of phialides, emergence of phialides and spore characters (Gams and Bisset, 1998). Isolates of *Trichoderma* were tested against *S. rolfsii* and *P. aphanidermatum in vitro* by dual culture technique. Periodical observations on growth of *Trichoderma* isolates and their ability to inhibit growth of *S. rolfsii* and *P. aphanidermatum* were recorded. Diffusible antibiotic production of *Trichoderma* isolates was tested by the method of Dennis and Webster (1971a). The plates were incubated for 72 hr and diameter of pathogen colony was compared with that of control. Production and inhibitory effect of volatile compounds of *Trichoderma* isolates were assessed against the pathogen by the method of Dennis and Webster (1971b). Production of volatile inhibitor compound, HCN was estimated by the method of Wei *et al*. (1991), with slight modification. Strips of filter paper soaked in picric acid solution were exposed to growing *Trichoderma* isolates in 150 ml conical flasks containing 25 ml liquid medium which turned brown. They were then soaked in 40 ml water for 10 min for complete leaching of brown colour from the filter papers and OD of colour solution was read at 515 nm in spectronic 20. The values of OD were recorded after subtracting the blank value. Indole acetic acid production was quantitatively estimated by the method of El-Khawas and Adachi (1999). Siderophore production was measured as per Lone Gram (1996). For all the experiments, test plates and control plates were set up in triplicate.

Assay of enzyme activities : β-1, 3-glucanase activity was estimated according to the method of Elad *et al*. (1993). The reaction mixture contained 0.5 ml of culture filtrate, 1 ml citrate buffer (pH 4.5, 0.1 M) and 0.5 ml laminarin. Test tubes containing the reaction mixture were incubated at 40ºC for 1 hr and kept in boiling water bath for 5 min to stop the reaction. 2 ml of dinitrosaliccylic acid reagent (1% solution of dinitrosalycilic acid in 0.7 M NaOH) was added to the reaction mixture and kept in boiling water bath for 15 min. After cooling to room temperature, the absorbance of the reaction mixture was measured at 575 nm and the amount of glucose released was estimated from the standard curve prepared with glucose. Enzyme activity was expressed as nkat ml^{-1} (nmol sec^{-1}). Chitinase activity was estimated (El-Katatny *et al*. 2000) by incubating the reaction mixture containing 0.5 ml colloidal chitin, 1 ml Mcllavains buffer (pH 4) and 0.5 ml culture filtrate at 37 ºC for 2 hr. At the end of incubation, 3 ml of potassium ferricynide reagent (0.05% potassium ferricynide in 0.05% sodium carbonate) was added and incubated in boiling water bath for 15 min. The amount of N-acetyl glucosamine released was estimated by measuring the absorbance at 420 nm, and comparing with the standard curve prepared with N- acaetyl glucosamine. Enzyme activity was expressed as pkat ml^{-1} (pmol sec^{-1}). Data if all the experiments were analyzed using SAS 9.3 and expressed as mean of three replicates ± SD. Different letters in each column represented significant differences ($p<0.05$).

Results and Discussion

All isolates showed HCN production ranging from 0.08 to 0.14 OD. Isolate TvJt1 showed maximum HCN production followed by ThHt1 and minimum by isolates ThJnt and ThRt1. Isolate TvJt1 produced maximum content of HCN, but was at a par with isolate ThHt1 and ThJt1 (Table 1).

Indole acitic acid (IAA) production among the isolates varied from 6.48 to 14.82 µg ml^{-1} (Table 1). Isolate ThJnt produced

maximum IAA followed by ThHt3 and were found to be significantly higher than other isolates. Isolate TvJt1 and TvHt2 were at par in IAA production and lower than all other isolates, except for isolate ThDt1. Isolates ThJt1, ThRt2 and ThJO1 were at par in the production of IAA. To study siderophore production, isolates were grown in Czepack-Dox liquid medium supplemented with tryptophane. Isolate ThJO1 produced maximum siderophore, whereas isolate TvJt1 produced minimum. Isolate ThJO1 and ThHt3 were at par in the production of siderophore and were significantly higher than other isolates (Table 1). Isolates ThRt1, ThRt2, ThDt2 and ThC were at par, while isolates ThJt1, ThJnt, ThDt1 and ThHt1 were at par in the production of siderophore. Isolate TvHt2 produced significantly higher content of siderophore when compared to the isolate TvJt1.

Isolate ThJt1 showed maximum and significantly higher chitinase activity as compared to other isolates (Table 1). Isolate TvHt2 was second highest producer of chitinase and produced significantly higher than other isolates. Isolates ThDt1 and ThDt2, collected from Dinhata, were at par in chitinase activity.

Isolate TvHt2 showed maximum and significantly higher β-1,3-glucanase activity when compared to all other isolates (Table 1). Isolate ThJt1 showed second highest activity of β-1,3-glucanase. All other isolates showed β-1,3- glucanase activity but they were all at par. Isolate TvHt2 showed nearly 61.66% higher β-1,3-glucanase activity when compared to isolate TvJt1. Isolates ThDt1 and ThDt2 Dinhata showed similar activity of β-1,3-glucanase.

Antifungal activity of *Trichoderma* isolates was tested against the pathogenic fungi *S. rolfsii* infecting tobacco in dual culture technique. All the isolates inhibited mycelial growth of *S. rolfsii* ranging from 58.56 to 78.36% (Table 2). Inhibition of mycelial growth of *S. rolfsii* by ThJt1 and TvHt2 was at par and significantly higher than other isolates. Isolate TvHt2 showed significantly higher inhibition when compared to isolate TvJt1, whereas isolate ThDt2 showed maximum inhibition as compared to isolates ThDt1. Isolates ThJnt and ThHt1 were at par, and showed significantly higher inhibition when compared to isolates ThRt1, ThDt1, ThJO1 and ThC, respectively.

Isolate ThJt1 showed maximum inhibition of *S. rolfsii* growth by production of non-volatiles, whereas isolate ThDt1 showed minimum inhibition (Table 2). Inhibition of *S. rolfsii* by isolate TvJt1 was at par with isolate TvHt2. All the *Trichoderma* isolates inhibited mycelial growth of *S. rolfsii* by producting of volatile compounds. Inhibition of *S. rolfsii* growth by production of volatile compounds by ThJt1, TvJt1, ThDt1 and ThHt1 isolates was at par and significantly higher than other isolates (Table 3). Isolate ThDt1 from Dinhata showed significantly higher inhibition of *S. rolfsii* growth when compared to ThDt2 isolate from the same region.

All the isolates of *Trichoderma* inhibited mycelial growth of *P. aphanidermatum* ranging from 48.55 to 72.45% in dual culture (Table 3). Inhibition of mycelial growth of *P. aphanidermatum* by isolate TvHt2 was maximum followed by ThJt1, and both were significantly higher than other isolates. Isolate ThDt2 from Dinhata showed significantly higher inhibition when compared to isolate ThDt1. Inhibition of *Pythium* growth by isolates ThRt2, TvJt1, ThDt2, ThHt3 and ThC was at par. Inhibition of *P. aphanidermatum* by production of non-volatiles varied from 48.50 to 78.77% (Table 3). TvHt2 isolate showed maximum and significantly higher inhibition of *P. aphanidermatum* by production of non-volatiles, followed by ThJt1. TvJt1, ThDt1, ThDt2, ThHt3 and ThJO1 isolates were at par in inhibiting *P. aphanidermatum* growth. Commercial isolate ThC showed lower level of inhibition when compared to other isolates, except for ThRt1. Inhibition of *P. aphanidermatum* growth by production of volatile compounds by *Trichoderma*

Table 1 : Activity of extracellular enzymes produced by *Trichoderma* isolates

Isolates	HCN (OD)	IAA ($\mu g\ ml^{-1}$)	Siderophore ($m\ mol\ ml^{-1}$)	Chitinase ($p\ kat\ ml^{-1}$)	β-1,3-glucanase ($n\ kat\ ml^{-1}$)
ThRt1	0.09±0.005^{ef}	10.46±0.44^{d}	8.9±0.39^{c}	40.26±1.96^{f}	4.26±0.19^{c}
ThRt2	0.12±0.017^{bc}	12.88±0.46^{bc}	9.64±0.17^{c}	42.82±1.49^{ef}	5.22±0.10^{c}
ThJt1	0.126±0.003^{ab}	13.04±0.6^{b}	8.14±0.2^{d}	62.12±3.15^{a}	6.75±0.14^{b}
ThJnt	0.08±0.006^{f}	14.82±0.29^{a}	8.46±0.26^{e}	40.12±1.61^{f}	4.06±0.13^{c}
TvJt1	0.14 ±0.004^{a}	8.24±0.38^{f}	6.24±0.30^{d}	52.12±2.42^{bc}	3.86±0.17^{c}
ThDt1	0.106±0.004^{cde}	6.48±0.2^{g}	8.24±0.29^{cd}	48.24±1.61^{cd}	4.26±0.07^{c}
ThDt2	0.098±0.003^{de}	12.08±0.17^{c}	9.80±0.22^{d}	46.14±1.22^{de}	4.34 ±0.27^{c}
ThHt1	0.128±0.004^{ab}	11.08±0.28^{d}	8.48±0.22^{bc}	41.34±1.50^{f}	4.86±0.25^{c}
TvHt2	0.096±0.007^{de}	8.98±0.38^{ef}	10.48±0.30^{bc}	56.24±0.59^{b}	9.94±0.19^{a}
ThHt3	0.11±0.006^{d}	14.12±0.2^{a}	12.03±0.24^{ab}	48.26±1.48^{cd}	5.08±0.11^{c}
ThJO1	0.102±0.002^{de}	12.66±0.26^{bc}	13.48 ±0.21^{a}	46.12±1.41^{de}	4.75±0.21^{c}
Th C	0.094±0.005^{ef}	9.48±0.4^{e}	10.26±0.20^{c}	38.26±1.14^{f}	4.48±0.30^{c}

Different letters in each column represent significant differences ($p<0.05$)

Table 2 : Inhibition of *S. rolfsii* growth (%) by *Trichoderma* isolates

Isolates	Dual culture	Non-volatiles	Volatiles
Th Rt1	62.64±2.79 ^{def}	72.88±1.69 ^{bc}	66.24±1.53 ^{e}
ThRt2	58.56±2.40 ^{f}	68.18±3.00 ^{d}	70.44±1.90 ^{cde}
ThJt1	78.36±2.00 ^{a}	80.24±1.88 ^{a}	80.44±2.64 ^{ab}
ThJnt	72.47±4.60 ^{bc}	76.24±2.26 ^{ab}	74.26±1.27 ^{bc}
TvJt1	64.23±2.43 ^{def}	72.46±1.63 ^{bcd}	80.84±2.49 ^{ab}
ThDt1	60.47±2.13 ^{ef}	64.07±1.87 ^{e}	79.42±3.33 ^{ab}
ThDt2	66.24±1.43 ^{d}	74.68±2.19 ^{ac}	70.66±1.98 ^{cde}
ThHt1	70.26±1.54 ^{b}	68.46±2.04 ^{de}	84.28±3.37 ^{a}
TvHt2	76.17±1.97 ^{a}	76.00±0.95 ^{ab}	75.24±2.99 ^{b}
ThHt3	68.44±1.59 ^{cd}	72.47±1.12 ^{bcd}	68.46±1.46 ^{de}
ThJO1	66.82±3.12 ^{cde}	75.46±2.18 ^{a}	74.26±2.08 ^{bcd}
Th C	62.46±1.01 ^{ef}	69.24±3.78 ^{cde}	74.56±1.08 ^{bcd}

Different letters in each column represent significant differences (p<0.05)

Table 3 : Inhibition of *P. aphanidermatum* growth (%) by *Trichoderma* isolates

Isolates	Dual culture	Non-volatiles	Volatiles
ThRt1	51.72±1.29 ^{d}	48.50±2.42 ^{e}	54.36±1.21 ^{c}
ThRt2	60.21±2.49 ^{b}	54.35±2.40 ^{de}	68.52±1.17 ^{ab}
ThJt1	69.22±1.52 ^{a}	70.19±1.97 ^{b}	70.47±2.06 ^{abc}
ThJnt	52.84±1.71 ^{c}	54.55±1.64 ^{de}	56.63±1.78 ^{d}
TvJt1	58.26±2.06 ^{b}	62.44±1.73 ^{c}	72.64±1.39 ^{a}
ThDt1	52.33±2.11 ^{d}	60.32±1.65 ^{c}	66.36±1.67 ^{abc}
ThDt2	58.33±1.94 ^{b}	62.30±2.46 ^{c}	64.33±2.93 ^{bc}
ThHt1	48.55±2.36 ^{d}	54.33±2.19 ^{de}	70.52±2.54 ^{ab}
TvHt2	72.45±1.50 ^{a}	78.77±1.52 ^{a}	66.44±3.51 ^{abc}
ThHt3	54.58±1.35 ^{bcd}	60.40±2.18 ^{cd}	69.36±2.21 ^{abc}
ThJO1	49.74±0.74 ^{d}	62.55±1.07 ^{c}	63.29±3.19 ^{cd}
ThC	58.42±2.93 ^{bc}	54.29±1.61 ^{de}	58.50±1.50 ^{de}

Different letters in each column represent significant differences (p<0.05)

isolates ranged from 54.36 to 72.6% (Table 3). TvJt1 Isolate showed maximum inhibition of *P. aphanidermatum* growth followed by ThHt1. ThRt2, ThJt1, TvJt1, ThDt1, ThHt1, TvHt2 and ThHt3 isolates were at par in inhibiting mycelial growth in *P. aphanidermatum*. ThRt1 isolate showed least inhibition of *P. aphanidermatum* growth by producing of volatile compounds.

Trichoderma isolates produced IAA and siderophore to various extents. Siderophores are iron chelating agents which deprive the surrounding pathogens of iron and thus concentrated iron is made available to the plant. In addition to competition for limited carbon sources in the rhizosphere, antagonism can be mainly attributed to the production of antibiotics, siderophores and cyanides (Bae Hanhong, 2011). Production of IAA varied among the isolates. The role of microbial production of IAA and the response of host plant was reported by Vinale *et al.* (2008). With this system, microbe produced IAA may stimulate plant cell proliferation or elongation, and result in plant production of ACC (1- aminocyclopropane-1-carbomylate).

All the isolates of *Trichoderma* inhibited mycelial growth of *P. aphanedermatum* ranging from 48.55 to 72.45% in dual culture. Inhibition of mycelial growth of *P. aphanedermatum* by TvHt2 isolate was maximum followed by ThJt1. Inhibition of mycelial growth of *S. rolfsii* by ThJt1 and TvHt2 isolates was at a par and significantly higher than other isolates. Nagamani *et al.* (2013) evaluated 12 *Trichoderma* isolates against *S. rolfsii* by dual culture and reported maximum inhibition to an extent of 82.1% by isolate TRI6. Prasad *et al.* (2003) reported that *T. harzianum* was superior to *T. viride* in inhibiting growth of *S. rolfsii.* The mechanism of inhibition may be competition for food and space, production of antibiotics and mycoparasitism. Such identification of effective isolates to suit the purpose of locality is important (Harman, 2000).

Inhibition of *P. aphanedermatum* and *S. rolfsii* growth by production of non-volatiles varied from 48.56 to 78.77% and 64.07 to 80.24%, respectively. TvHt2 isolate showed maximum and significantly higher inhibition of *Pythium* growth by production of non-volatiles followed by ThJt1. ThJt1 isolate showed maximum inhibition of *S. rolfsii* growth by production of non-volatiles, whereas ThDt1 isolate showed minimum inhibition. Inhibition of *P. aphanedermatum* and *S. rolfsii* growth by production of volatile compounds by *Trichoderma* isolates ranged from 54.36 to 72.6% and 66.24 to 84.24% respectively. Siva Raju *et al*. (2009) reported inhibition of *Sclerotium* growth by production of non-volatile compounds varying from 62 to 92.33% among the 10 isolates of *Tichoderma*. Variation in antagonistic potential of different aggregates of *Trichoderma* against different pathogens has been reported (Pan and Bhagat, 2007). Production of non-volatile substances by *Trichoderma* spp. was considered more advantageous than volatile substances as they diffused through air filled pores in soil, and actual contact between pathogen and antagonists might not be necessary for inhibition of pathogen. Kumar and Dubey (2001) reported inhibition of growth of various soil borne pathogens by producing volatile and non-volatile compounds by *Trichoderma* spp. The present study revealed that different strains have different capacities, as biological weapons, in inhibiting the pathogen.

In the present study, all the isolates collected from the rhizosphere of tobacco from different regions and commercial isolate produced HCN, IAA and siderophore to different levels. ThJt1 and TvHt2 isolates produced maximum chitinase and β-1, 3-glcanase activity and also showed maximum inhibition of pathogens. It is clear from the present investigation that *Trichoderma* isolates native to tobacco ecosystem, showed good antifungal properties in suppressing the mycelial growth of *S. rolfsii and P. aphanedermatum* infecting tobacco.

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