Mechanism of Biocontrol of *Aspergillus flavus* in Groundnut by Species of *Trichoderma*

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Abstract

Aflatoxin contamination of groundnut by *Aspergillus flavus* is a serious problem affecting the quality and commercial value of groundnut kernels. Biological management of *A. flavus* infection at pre- and post-harvest levels by using species of *Trichoderma* is a promising approach. *Trichoderma* isolates from the fields of Andhra Pradesh and Karnataka states of India were evaluated for their ability to control *A. flavus* (strain Af 11-4) in the laboratory. Out of 212 isolates of *Trichoderma*, 36 were potent antagonists and these were analyzed for the production of volatile and diffusible antibiotics. Twenty-one of the 36 isolates produced volatile antibiotics and 15 produced diffusible antibiotics. Biocontrol mechanism of their action was analyzed by estimating extracellular enzyme production viz, chitinase, protease and glucanase. Maximum chitinase activity was associated with the isolate *T. viride* (Tv 24), *T. pseudokoningii* (Tp 29) and *T. harzianum* (Th 42). *T. harzianum* showed a maximum protease activity while *T. koningii* (Tk 83) and *T. longibrachiatum* (Tl 102) produced glucanases in the presence of glucose.

Keywords: *Aspergillus flavus*, biocontrol, *Trichoderma* spp., chitinases, glucanases and proteases

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Groundnut (*Arachis hypogaea* L.) is the major oilseed crop in the rainfed farming system in the arid and semi-arid tropics. Groundnut is an important legume crop grown on 19.3 million ha in about 82 countries worldwide (Reddy *et al* 2003). Groundnut is rich in niacin and other B vitamins and also contains appreciable amounts of calcium, iron, phosphorus and potassium (McWatters et al 1995). Aflatoxin contamination of groundnut by the mold fungi *Aspergillus flavus* and *A. parasiticus,* both pre- and post-harvest, is a serious problem and has a tremendous impact on the global trade and groundnut industry, and pose serious public health risks (Will et al 1994; Gachomo et al 2004). *Aspergillus flavus* is a widely distributed fungus that survives in soil as conidia and microsclerotia to overcome extremely dry conditions, and is mostly associated with nuts, grain and seed crop products. Some *Aspergillus* species, predominantly *A. flavus* and *A. parasiticus* produce aflatoxins as secondary metabolites, which cause harmful effects in humans and animals. Effective control of groundnut infection by aflatoxigenic fungi is still a major challenge. No single method of control has been found to be highly effective (Gachomo et al 2008) and chemical control methods are not eco-friendly as they increase environmental pollution and health hazards. Biological control by the use of non-toxigenic strains of *A.flavus* to counteract toxin-producing strains in the environment by 'competitive exclusion' has 30been demonstrated on corn (Brown et al 1991), cottonseed (Cotty 1990) and groundnut (Dorner et al 1992, 1998). Use of microbial antagonists as biocontrol agents in the management of aflatoxicogenic fungi is a promising approach in preventing the pre- and post-harvest infection by *A. flavus* in groundnut (Anjaiah et al 2006). We have shown that some *Trichoderma* species present in groundnut-cultivated soils are antagonistic to *A. flavus* (Srilakshmi et al 2001; Kucuk and Kivanc 2003). *Trichoderma* are ubiquitous, soilborne, green-spored ascomycetes fungi with multiple attributes in biocontrol, enzyme production and novel antibiotics (Schuster and Schmoll 2010). Species of *Trichoderma* were proven biocontrol agents due to their antagonistic ability, mycoparasitism and resistance to fungicides, competition for nutrients and space and secretion of chitinolytic enzymes (Harman 2000). Antibiosis is one of the mechanisms of biocontrol in which antibiotics destroy the target fungi either by volatile or diffusible antibiotics (Schirmbock et al 1994). Enzyme induced lysis of fungal cell walls through extra cellular chitinases has also been implicated as one the chief mechanisms of biological control by microorganisms. Being an important biocontrol agent and producer of lytic enzymes, *Trichoderma* species have become a safe

and ecofriendly biological choice in the management of soil borne plant pathogens. The enzyme producing capability may vary from species to species and even strain to strain within a species. Verma et al (2007) reported the involvement of complex group of extracellular enzymes as a key factor in pathogen cell wall lysis during mycoparasitism. The nature of inhibition by *Trichoderma* isolates was estimated based on the production of antifungal compounds and extracellular enzymes so as to understand the mechanism of the biocontrol. Objectives of this study were to assess the nature of inhibition and characterization of *Trichoderma* isolates as per their enzyme production ability. This paper also addresses the question of whether the enzyme ability, volatile and non volatile antibiotic production are the same for isolates within the *Trichoderma* species that are isolated from diverse groundnut growing areas of Andhra Pradesh and Karnataka.

Materials and Methods

Trichoderma species inhabiting the geocarposphere soils of groundnut was isolated by soil dilution method (Srilakshmi et al 2001; Siameto et al 2010) and were identified based on standard manual species identification keys (Rifai 1969; Bisset 1991a, b; Samuels 1996). The biocontrol ability of 212 isolates of *Trichoderma* spp. was assessed against the pathogen *A. flavus*, using dual culture technique (Dennis and Webster 1971c). Mycelial discs (5 mm dia.) of *Trichoderma* spp. from a four-day-old culture on 2% agar were placed on PDA plates on one side, 1 cm away from the edge of the plate. The disc of the *A. flavus* (Af11-4) was placed on the opposite side 6 cm apart. The plates were incubated at 24 ± 2 C for one week. The plates were observed for antagonism at regular intervals.

Production of volatile antibiotics. Volatile antibiotic study was done following the method of (Dennis and Webster, 1971b). Isolates of different species groups of *Trichoderma* were grown on 2% malt extract agar in Petri dishes and incubated at 28 C for 48 h. After a given time, the lid of each dish was replaced by a bottom dish containing 2% malt extract agar inoculated with *A. flavus* (Af11-4). The two dishes were fixed together with adhesive tape. The lids of control plates, which had not been inoculated with a *Trichoderma* strain, were also replaced in the same way. Test plates and control plates were set up in triplicate. The assembly was opened after 72 h and colony diameter of Af 11-4 was measured in each plate.

Production of diffusible antibiotics. Antagonism due to diffusible antibiotics was assessed according to (Dennis and Webster, 1971a). The antagonist was grown on cellophane membrane laid over 2% malt extract agar in Petri plates for four days and the metabolites produced were allowed to diffuse through the cellophane into the agar. Antibiotic activity was then assessed by growing *A. flavus* (Af 11-4) as the test fungus on the medium after removal of the antagonist along with the cellophane membrane. The plates were incubated for 48 h at 28 C and compared the colony diameter and sporulation with control. All treatments were maintained in triplicate.

Production of extracellular enzymes by *Trichoderma* species. *Trichoderma* species being known producers of extra cellular enzymes, 48 antagonistic isolates were screened preliminarily for chitinases by growing on a synthetic medium(SM) containing colloidal chitin as a carbon source and incubated at 28 C for 7 days and SM without any carbon source is the control.

Among 48 antagonistic isolates seven isolates belonging to ten different species of *Trichoderma* viz., *T. harzianum*, *T. fertile*, *T. fasiculatum*, *T. koningii*, *T. viride*, *T. atroviride*, *T. inhamatum*, *T. aureoviride*, *T. pseudokoningii* and *T. longibrachiatum* were grown in liquid medium containing colloidal chitin as a sole source of carbon. Amount of crude protein produced was calculated for all ten species (Table 3) and specific activity of chitinase enzyme (EC 3.2.1.14) was determined by doing chitinase assay. Chitinase - β (1-4) - Poly N - acetyl D-glucosaminidase (EC 3.2.165) assay was carried out following minor modifications (Reissing et al 1959). Sterilized potato dextrose broth flasks were inoculated with 1ml spore suspension of *Trichoderma* isolates separately in triplicate and incubated in shaker at 30 C and 120 rpm for 7 days. After 7 days the contents were filtered using Buchner funnel with 2 layers of what man filter paper under cold conditions (4 C). The filtrate was measured and ammonium sulfate @ 54.1 g/100 ml was added on a magnetic stirrer under cold conditions. Ammonium sulfate was added slowly with continuous stirring. The filtrate was centrifuged under cold conditions at 12000 rpm for 30 min and the pellet was dissolved in ice-cold distilled water and dialysis was done for 48 h with change of water at every 4 h interval. The water was tested in the beaker with barium chloride for the presence of any precipitation. Two to three drops of barium chloride (50 mg/50 ml) was added to 10 ml of water in the beaker, dialysis was stopped, as there was no precipitate in the water. The sample was lyophilized overnight after dialysis to get crude chitinase enzyme. One ml of crude chitinase enzyme (1mg of enzyme was dissolved in 1ml of borate buffer) was added to 2 mg of pure chitin in an eppendorf and incubated for 24 h at 37 C. The solution was centrifuged at 12000 rpm for 10 min under cold conditions. Supernatant was taken in a test tube (166 µl) and 33 µl of borate buffer was added and boiled exactly

for 3 min at 100 C and cooled immediately under tap water one ml of DMAB reagent (diluted 10 times in acetic acid) was added and incubated for 20 min at 37 C in a shaking water bath. OD values were taken at 580 nm. Blank was maintained. Specific activity of chitinase was expressed as µ moles of Glc NAc released /mg of protein/h. 200 µl reaction mixture containing 1% chitin, 20 µg of protein (crude enzyme) in 0.1 M acetate buffer (p^H 5.0) is incubated for the desired time in water bath shaker (100 rpm) at 37 C. The reaction is stopped by boiling at 100 C and then centrifuged at 10,000 rpm for 10 min; the supernatant is used for assay.

Glucanase (3.2.1.6) activity of *Trichoderma*-culture filtrates. Glucanase was assayed by estimating the glucose released from laminarin (Miller et al 1960). The reaction mixture contained 20 µg of lytic enzyme in 400 µg of 0.1 M acetate buffer pH 5 and 100 µl of 1% laminarin (10 mg/1ml). Total volume of reaction mixture was 1500 µl. The reaction was carried out at 37 C in a shaking water bath for 30 min (100 rpm). After incubation, 3 ml of DNS reagent was added to reaction mixture and boiled for 5 min in a boiling water bath, followed by cooling in running tap water adjusted to ambient temperature. Reading of absorbance was finally made in a spectrophotometer (Beckman model DV-20) at a wavelength of 640 nm. Specific activity of β-1-3 glucanase was expressed as glucose (µ. moles) released mg/ protein/ h.

Protease (EC 3.4.23.18) activity of *Trichoderma*-culture filtrates. This enzyme was assessed according to the method of Prestige et al (1971). Three mg of Azocaesein was taken in 0.1M Tris HCl (p^H 8) and incubated for 1h at 30 C in a total volume of 1ml. The reaction was terminated by adding 2 ml of ice-cold 7% potassium perchlorate. The protein was allowed to precipitate for 15 min at room temperature and centrifuged at 4,000 rpm for 10 min. To the supernatant 0.3 ml of 10N NaOH was added and intensity of colour was determined at 440 nm. Specific activity of β -1-3 glucanase was expressed as µmol of glucose released/mg/protein/h. Quantification of all three enzymes was done by running the samples on SDS-PAGE gels (Laemmli UK 1970) using prestained protein Molecular Weight Marker (118kDA, Fermentas).

Results

Out of 48 antagonist isolates, only 21 belonging to different species of *Trichoderma* exhibited antagonism through production of volatiles while 15 produced diffusible antibiotics that inhibited the growth of *A. flavus* (strain Af 11-4). Among the 21 isolates, *T. longibrachiatum* (Tl 102) was highly effective antagonist in producing volatiles and controlling the growth of Af 11-4 besides 8 isolates of *T. koningii* and *T. viride* (Table 1). Diffusible antifungal compounds played a major role in case of *T. koningii* isolates Tk 83 and Tk 12 along with other species *T. harzianum* (Th 42), *T. pseudokoningii* (Tp 29) and *T. viride* (Tv 60) (Table 2).

Chitinase production was evident in 7 isolates belonging to six *Trichoderma* species and among them *T. koningii* (Tk 83) and *T. longibrachiatum* (Tl 102) produced maximum amount of crude protein on colloidal chitin as a sole source of carbon in the medium (Table 4). Maximum chitinase activity was associated with *T. viride* (Tv 24) more than the other isolate of *T. viride* (Tv 179). Isolates *T. longibrachiatum* and *T. koningii* also showed appreciable enzyme activity on colloidal chitin when supplied as a sole carbon source. Glucanase activity was restricted to few species among the seven isolates tested on colloidal chitin as a sole carbon source (Table 3). Only *T. koningii* and *T. longibrachiatum* produced glucanase in the presence of glucose while all others failed to induce any enzyme activity. On the other hand, all the isolates were capable of producing proteases in liquid medium containing colloidal chitin (Table 3).

Table 1. Effect of volatile antibiotics of *Trichoderma* on the growth of *A. flavus* (Af 11-4)

Isolate no.	*Trichoderma* spp.	*A. flavus* (*Af 11-4*) (cm)	Reduction (%)
Th 2	*T. harzianum*	4.0	33.3
Tl 6	*T. longibraciatum*	2.6	56.6
Th 10	*T. harzianum*	3.8	36.6
Tk 13	*T. koningii*	2.0	66.6
Tk 21	*T. koningii*	3.0	50.0
Tv 24	*T. viride*	2.8	53.3
Tl 34	*T. longibrachiatum*	3.0	50.0
Thm 47	*T. hamatum*	4.5	25.0
Tk 49	*T. koningii*	3.3	45.0
Tv 50	*T. viride*	4.5	25.0
Th 53	*T. harzianum*	4.0	33.3
Tl 56	*T. longibrachiatum*	4.0	33.3
Th 58	*T. harzianum*	3.2	46.6
Tk 70	*T. koningii*	3.0	50.0
Tl 102	*T. longibrachiatum*	1.0	83.3
Tl 110	*T. longibrachiatum*	4.0	33.3
Th 129	*T. harzianum*	3.3	45.0
Tk 143	*T. koningii*	3.0	50.0
Tk 161	*T. koningii*	4.5	25.0
Th 170	*T. harzianum*	3.2	46.6
Tv 179	*T. viride*	2.8	16.6
control		6.0	-
CD (*P=0.05*)		1.5	

Table 2. Effect of diffusible antibiotics of *Trichoderma* on the growth of *A. flavus* (Af 11-4)

Isolate	Specific activity (µM / mg protein/ h)			
	Protein (mg/ml)	Chiti-nase	Glu-canase	Protease
T. harzianum (Th 20)	06.0	166.60	0.00	0.86
T. pseudokoningii (Tp 29)	07.0	071.40	0.00	0.87
T. hamatum (Thm 47)	52.0	096.15	0.00	0.21
T. viride (Tv 24)	29.0	551.70	0.00	0.44
T. koningii (Tk 83)	75.0	346.60	0.16	0.26
T. longi-brachiatum (Tl102)	66.5	360.00	0.15	0.24
T. viride (Tv 179)	61.0	327.80	0.00	0.19

No enzyme activity was observed in control without any carbon source in the medium. Isolates *T. pseudokoningii* and *T. harzianum* showed maximum protease activity compared to others. Isolate T 179 was the poor producer of protease enzyme (Table 3).

Table 3. Total protein produced and specific activity of chitinase, glucanase and protease produced by *Trichoderma* spp. in liquid medium containing colloidal chitin as sole carbon source

Isolate	*Trichoderma* spp.	*A. flavus* (cm)	Reduction (%)
Th 11	*T. harzianum*	1.8	32.7
Tk 12	*T. koningii*	1.4	25.4
Th 20	*T. harzianum*	2.3	41.8
Tp 29	*T. pseudokoningii*	1.0	18.1
Tk 33	*T. koningii*	2.5	45.4
Tp 37	*T. pseudokoningii*	3.0	54.5
Th 42	*T. harzianum*	1.0	18.1
Tv 51	*T. viride*	3.0	54.5
Tv 60	*T. viride*	1.5	27.2
Tv 62	*T. viride*	5.0	90.9
Th 72	*T. harzianum*	2.2	40.0
Tk 83	*T. koningii*	1.0	18.1
T 142	*Trichoderma sp.*	3.0	54.5
Tv 205	*T. viride*	2.0	36.3
Tp 206	*T. pseudokoningii*	4.0	72.7
Control		5.5	
CD (*P=0.05*)		1.0	

Trichoderma koningii and *T. longibrachiatum* produced good amount of crude protein, chitinases and glucanases in liquid medium with colloidal chitin as sole carbon source. These two species, on the other hand were poor producers of proteases. *T. harzianum* and *T. psedokoningii* produced maximum proteases and minimum crude protein with low chitinase activity in liquid medium containing colloidal chitin. No glucanase activity was observed by these *Trichoderma* species. *T. viride* isolate Tv 24 showed high chitinase activity though the amount of crude protein produced was less than the other isolate Tv 179.

The samples after running on SDS PAGE molecular weight of enzyme proteins was compared with 118 kDa β - galactosidase prestained protein marker. Chitinase and protease enzyme production could only be quantified, as the bands were think and clear. Glucanase production was not quantified as the isolates produced very faint bands. Molecular wt. of chitinase was 28 kDa and that of protease 72 kDa.

Discussion

Trichoderma species successfully reduce the populations of their competitors, including pathogenic fungi from the ecological niches such as geocarposphere of groundnut. Success of these antagonists as biocontrol agents may be attributed to their ability to produce lytic enzymes and antifungal compounds. In this study, non-volatile antifungal compounds of *Trichoderma harzianum* (Th 42) and *T. pseudokoningii* (Tp 37) solely and effectively prevented the growth of *A. flavus* (Af 11-4). Two isolates of *T. viride* also inhibited *A. flavus* growth by diffusible antibiotics. Volatile and non-volatile compounds produced by *T. viride* was found to be most fungitoxic followed by *Gliocladium virens* (Pandey and Upadhyay 1997). Prasad and Rangeshwaran, 2001 tested nine *Trichoderma* isolates, comprising of *T. viride*, *T. harzianum* and *T. virens in vitro* against *Sclerotium rolfsii* and reported the 190inhibition of the mycelial growth by volatile and non-volatile antibiotics which were superior to fungicides.

Anjaiah et al 2006 isolated fluorescent *Pseudomonas*, *Bacillus* and *Trichoderma* spp. from geocarposphere that are potentially antagonistic to *A. flavus* and used successfully for the control of pre-harvest groundnut seed infection. Dennis and Webster (1971 a, b, c) differentiated the mechanism of biocontrol among isolates of *Trichoderma* into three categories, *viz*., volatile antibiotics, non volatile antibiotics and hyphal interactions. Doi and Mori, 1994 reported volatiles from *Trichoderma* species inhibiting the hyphal growth of different fungal pathogens.

In the present study *Trichoderma* species from groundnut growing areas of A. P. and Karnataka effectively restricted the growth of *A. flavus* Af 11-4 by producing volatiles or diffusing antifungal compounds into the medium. No isolate produced both types of compounds. Calistru et al 1997 attributed the suppression of *A. flavus* and *Fusarium moniliforme* by *Trichoderma* spp. to volatiles and extracellular enzymes rather than mycoparasitism. However, in the present investigation, isolates that produced volatiles or non-volatile compounds have shown the ability to produce chitinolytic enzymes on colloidal chitin. Specific activity of chitinases was at its maximum in *T. viride* (Tv 24) followed by *T. longibrachiatum* (Tl 102) which has also shown some glucanases activity along with *T. koningii* (Tk 83). Protease activity was evident in all the seven isolates. Early reports suggest *T. harzianum* as an effective producer of chitinases and also β – 1, 3 glucanase (Hadar et al 1979; and Harman et al 1989) that made it a potential biocontrol agent against many plant pathogens. Lypolytic, proteolytic, pectinolytic and cellulolytic enzymes and an unknown antibiotic was implicated in biological control of *A. flavus* and *F. moniliforme* (Calistru et al 1997). These results reaffirm the involvement of volatile or non volatile compounds in addition to extracellular enzymes in the inhibition of *A. flavus* Af 11-4 by isolates of different *Trichoderma* species. The volatile or non volatile trait was exclusive to different isolates where as lytic enzyme ability was a common characteristic among the antagonists.

The ability of indigenous strains to produce volatile or non-volatile substances as seen in this study may shift the balance in favour of antagonists in the geocarposphere for effective biocontrol of aflatoxigenic *A. flavus* (AF 11-4). This work also emphasis the cumulative effect of antibiotics and lytic enzymes in enforcing biological control by selected strains of *Trichoderma*. Isolation, identification and incorporation of genes responsible for secretion of chitinases and volatile and non-volatile antibiotics might be a potential source of strain improvement for effective biological control.

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