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Antagonistic Activity of *Trichoderma Flavofuscum* Against Peanut Pathogenic Mycoflora: Way to Formulate the Biocontrol System and Aflatoxin Bioremediation

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Abstract

The antagonistic metabolites released from *Trichoderma flavofuscum* were tested against different species of Aspergilli along with aflatoxigenic*A. flavus* and *A. parasiticus* associated with peanuts rhizosphere. Test pathogens (*A. flavus* and *A. parasiticus*) were further screened for the ability to produce aflatoxin using coconut agar medium, on the basis of blue green fluorescence shown by pathogens under UV radiation at 365nm along with yellow orange color pigmentation especially on the reverse side of the plate and also blue-yellow fluorescence on Czapek's Dox plate medium with $NaNO_3$ as nitrogen source. It was found out that the growth inhibition of pathogenic Aspergilli and aflatoxin bioremediation depends uponthe various factors likeas antagonistic fungalspecies and the metabolites produced along with the culture techniques. The growth of plant pathogen found to be inhibited up to 56.09 – 75.38% in diffusible (dual culture), from 66.97 – 78.98% in pathogen at center and from 38.33 - 63.07% in pathogen at periphery, where as volatile metabolite showed 57.50 to 76.18% growth inhibition. The test biocontrol agent *T. flavofuscum* showed highest inhibition at volatile metabolite. It was confirmed that the used antagonist is able to control the growth of harmful *Aspergillus* species along with prominently bioremediate aflatoxin.

The efficacy to control biosynthesis of aflatoxin in coculture system ranges from 62.5% (B_1) against *A. flavus*, 86.66% (B_1) and 83.33% (G_1) against *A. parasiticus.* Present results suggest that the *Trichoderma* – metabolomic in particular culture technique can be further analyzed and used as better biocontrol agent against growth of peanut mycoflora followed by reduction in aflatoxin production.

Keywords:*Trichoderma* species, Biocontrol agent, culture techniques, *Aspergillus* species, Aflatoxin B_1 and G_1, *Trichoderma* – metabolomic

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I. Introduction

Peanuts (*Arachis hypogaea*L.) are a major oil seed crop grown in tropical and subtropical areas (1) go by various names such as groundnut, earthnut, goobers etc. They are high energy food containing high amount of healthful fat, proteins and dietary fibers. Peanutsis a good source of niacin, other B vitamins and plenty of calcium, iron, phosphorous, potassium (2).Plays important role in human diet and health by supporting heart health, maintain healthy weight and also manage blood sugar level (3).As peanuts pods develop beneath the soil, which is the main source of toxigenic pathogen especially *A. flavus* which produce high level of toxins mainly aflatoxin (4)where fungus can easily colonize and contaminated peanut plant with mycotoxin at

different stages such as pre-post harvest, during drying, storage and transportation (5,6).

Aflatoxins are colorless, crystalline substances, small molecular weight fungal toxin having both acute and chronic toxic propertiesare well known to produce acute liver damage, liver cirrhosis, tumor induction and teratogenesis (7). However, a key challenge is the production and marketing of peanuts with acceptable level of aflatoxin contamination and the management of *Aspergillus* infection, which is becoming difficult due to lack of host resistance (8).

Contamination of mycotoxin especially aflatoxins in agricultural commodities now becomes the matter of concern (9), as they are highly stable and do not decompose quickly (10). Preventing the growth of aflatoxin producer fungal species and its bioremediation followed by improvement in crop yield by using biological control agents seems to be environmentally safe and sustainable alternative over the chemical control with the advantages of greater public acceptance, reduced environmental impact, nontoxic to human being, and is host specific and once colonized can last for years (11,12).

*Trichoderma*are well known biocontrol agent (13), soil borne, saprophyticaly living fungi. Several *Trichoderma* species have developed mechanisms of antagonistic activity towards broad range of plant pathogenic fungi via antibiosis, mycoparasitism, induced resistance of host cells, competition for nutrients and space and have been investigated as potential biocontrol agent against aflatoxigenic strains (14, 15). The parasitism of *Trichoderma* species on other fungi and the secretion of a lethal "principle" with antibiotic properties are known since 75 years (13,16).

Keeping this in view, the present study was conducted to screen the potential of antagonistic secondary metabolites and its mechanism produced by especially *T. flavofuscum* isolated from soil sample against various pathogenic *Aspergillus* species associated with peanut rhizosphere. The inhibitory potential of non-volatile, direct diffusible and volatile metabolites were screened in variable culture techniques which was an attempt to characterize the *Trichoderma*–metabolomic activity and suggest formulation of particular biocontrol system effectively against pathogenic *Aspergillus* species.

II. Materials and methods

A. *Isolation of rhizosphere associated Aspergilli (test pathogen) by soil dilution Method*

Serial dilutions of the experimental soil sample were carried out by using up to 10^4dilutions using sterile distilled water. 1ml of each dilution were removed and introduced onto the surface of the agar medium containing bacteriostatic agent and Rose Bengal (30mg)(17). Plates wereincubated for 72 hours at 27°C for fungal growth. Morphologically distinct colonies were purified and maintained by single spore isolation method. *Aspergillus* species isolates were again screened for their ability to produce aflatoxins based on the detection of UV-induced fluorescence and specific orange-yellow pigmentation on coconut agar medium (18), blue-yellow fluorescence on Czapek's Dox plate medium with $NaNO_3$ as nitrogen source (19,20).

B. *Isolation and identification of potential antagonist*

Biocontrol agent was isolated from the agriculture field where the pathogen(s) were known to exist but the disease occurrence is low. The soil samples from different agroclimatic ecosystems of peanut rhizosphere were collected at a 15 cm depth in the upper profile (21). The soil-tube method for assaying soil for isolation of antagonistic *Trichoderma* species was used (22). The mixed populations of isolated antagonist were further grown on selective *Trichoderma* medium. The colonies were successively purified and maintained on Rose Bengal Agar plates for further use.

For the identification of required antagonist, cultural features and microscopic characters such as colony growth, color, and texture of colony along with conidiophore, vesicles, phialides and conidia of isolated fungal isolates were assessed. Further confirmation of genus and species level was done by of ITCC, Indian Agriculture Research Institute, New Delhi (India).

C. *Antagonistic effect of T. flavofuscum against test pathogen*

To evaluate the antagonistic activity, a coculture of the isolated pathogenic fungi and the antagonist was established on Rose Bengal Agar in three different culture techniques viz; dual culture technique, pathogen at center and pathogen at periphery to evaluate the effect of diffusible metabolites. For volatile metabolites, two sealed base plate technique was used. Each experiment was carried out with three replication, five plates were used for each replication.

1. Effect of diffusible metabolites

a)Dual culture technique: Mycelial disc ofbiocontrol agent and test pathogen (5mm each) were inoculated in each half of the plate opposite to each other 2cm away from the edge and incubated at 25ºC. The plate without *Trichoderma* served as a control. After six days of inoculation, growth of pathogens along with their control was recorded in the form of colony diameter. Reduction in colony size was analyzed and expressed in percentage in relation with the control as a referencein relation with control (23, 24).

b) Pathogen at center:In this type of culture technique, disc of pathogen was placed aseptically in the center of the plate surrounded by four disc of biocontrol agent with the help of 5 mm cork borer at the distance 35mm away from pathogen disc. Inoculated plates were then incubated at 25º C for 6 days and percentages of growth inhibition were calculated in relation to the growth of control (25)

c) Pathogen at periphery: In this antagonistic method, 5mm disc of antagonistic agent was placed aseptically at the center of agar plate. Four similar size disc of pathogen were placed at the periphery of antagonistic agent. Inoculated plates were then placed for incubation at 25°C for 6 days. Mycelial growth of fungal pathogen was measured and reduction in growth was expressed in percentage. (26).

2. Effect of volatile metabolites

In vitro antifungal activity of volatile metabolites produced by BCA was carried out using two sealed base plate assay (27, 28). The method involves culturing and growth of pathogen in the trapped atmosphere of biocontrol agent to allow only volatile metabolites interaction.

For this assay, the mycelial agar disc (5mm diameter) of BCA as well as test pathogen were centrally placed on separate plates andallowed to incubated at 25ºC for 4 days. After the completion of incubation period, lid of all plates were replaced with each other's bottom and sealed withparafilm so as test pathogen were directly exposed to antagonistic environment created by *Trichoderma* species. The inhibition rate of fungal growth was calculated after 4 days of incubation relation to control.

D. *In vitro Aflatoxin production in coculture system*

The spore suspension ($1x10^{6}$spores/ml) of aflatoxigenic *A. flavus* and *A. parasiticus* were co-cultivated with *T. flavofuscum* in Richard broth medium (pH 5.5) for 8 days at 25ºC.

E. . *Qualitative assay of aflatoxin by TLC*

On 9^{th} day mycelial mat were separated from the medium through Whatman filter paper No. 1. The 5 ml of cell free culture filtrate was extracted thrice with 10 ml of chloroform in a separating funnel. The pooled chloroform extract mixed and were passed through a bed of anhydrous sodium sulphate, which were then evaporated to dryness on water bath (60ºC). The obtained residue was again redissolved in the used solvent.

The extract was then passed through silica gel column for aflatoxin purification (29). The column was washed with hexane for defatting and with ethyl ether for pigment extraction and pure aflatoxin was then eluted with 15 ml of chloroform: methanol (3: 1) (30). The extra water content was removed out by passing the extract through bed of anhydrous sodium sulphate, further evaporated to dryness. The residues were then reconstituted in 100 µl of toluene: acetonitrile (9:1), about 5 µl of sample was spotted on TLC plates along with standard aflatoxin (B_1 and G_1, 10µg/ml, HiMedia). The plates were then developed in chloroform: acetone (9:1) solvent system, air dried and observed under UV transilluminator for the presence of aflatoxins.

F. Spectrophotometeric quantitative assay of aflatoxin

The detected bands of aflatoxins on the basis of color fluorescence from the silica gel coated plates were marked under UV light. The required band containing the toxin was scrapped and collected separately in centrifuge tube. About 3 ml of toulene: acetonritrile (9:1) was added to silica gel portion and centrifuged at 1000 rpm for 10 min. The diluted samples were read (OD) in spectrophotometer (Shimadzu) at 350 nm wavelength for aflatoxin B_1 and G_1 determination (31). The readings were extrapolated to standard curve of pure aflatoxin B_1 and G_1 and the sample concentration was determined.

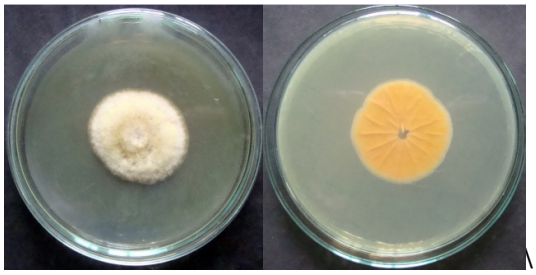
III. Results and Discussion

In the present study, potential antagonist *T. flavofuscum* found to be attack or interfere with peanut rhizosphere pathogens in variable culture mechanisms. Used antagonistic agent control the pathogenic as well as aflatoxigenic Aspergilli in varied range of percent inhibition along with decreased production of aflatoxin.

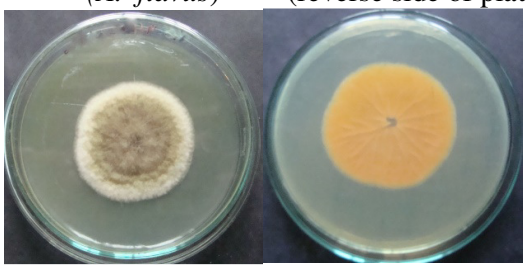
For fungal identification conventional methods were used which involves morphological and microscopic characters such as colony features including diameter after 7 days, color of conidia, mycelial color, colony reverse, colony texture and shape with conidial head; conidia shape, roughness and vesicle serration were taken into consideration.

Thus we identified *Aspergillus* species mainly *A. sydowii, A. nidulans, A. terreus, A. ochraceus, A. fumigatus, A. flavus* and *A. parasiticus* are predominantly associated with the peanut rhizosphere. The Aspergilli species were further characterized with respect to their ability to produce aflatoxin by growing on Coconut Agar media (CAM) and Fluorescence media. Only *A. flavus* and *A. parasiticus* show blue-green fluorescence when grown on CAM and CzA media when observed under UV light at 360nm. Moreover, colonies also show yellow-orange color on reverse side of the plate with orange pigmentation on CAM (Fig. 1).

Fig 1. Colonies of aflatoxin-positive (A)*A. flavus* and (B) *A. parasiticus* on coconut agar medium shows yellow-orange color on reverse side of the plate with orange pigmentation.



(A. flavus) (reverse side of plate)



(A. parasiticus) (reverse side of plate)

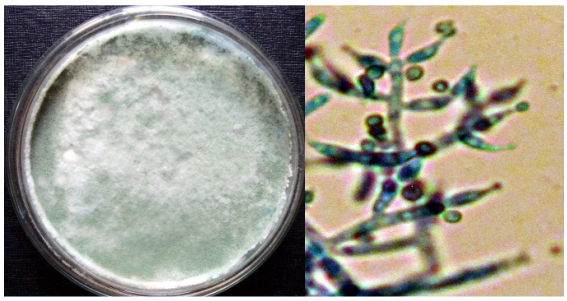
A.Isolation and characterization of potential Trichoderma antagonist

T.flavofuscum was isolated by dilution plate method (32), purified and species level identified based on the growth of colony on culture media, coniditation, exudates formed, branching of

conidiophore, septatation of mycelium, color, structure and size of conidia.

The colony of *T. flavofuscum* grew rapidly, 7 to 9.5 cm in diameter in 4 days at 25° C. Aerial mycelium appears to be floccus to wooly, white or grayish. The colony color changes, yellowish to olive green with completely branched conidiophores. Conidiation was abundant mostly effused and covering the plate forming fascicle occasionally or spreading, flat to cushion shape near the margin. Conidiophores arise from aerial mycelium, basal part is unbranched (Fig. 2).

Fig 2**.** Plate culture and microscopic view of *Trichoderma flavofuscum.*

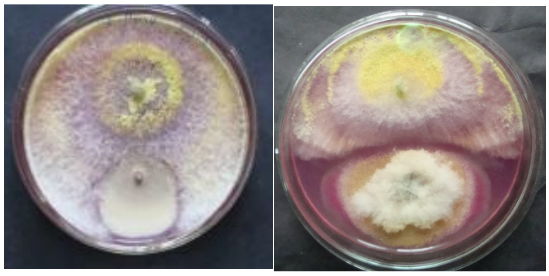


B.Antagonistic effect of T. flavofuscum in variable culture technique

The interactive results between the *T. flavofuscum* and the *Aspergillus* species associated with peanut in variable culture technique ranges from 56.09 - 75.38% in dual culture technique, 66.97 - 78.98% in pathogen at center, 38.33 - 63.07% in pathogen at periphery and 57.50 - 76.18 % in volatile metabolites (Table 1). It has been observed that, *T. flavofuscum* selectively overgrew on all Aspergilli, which results into highest growth suppression at pathogen at center techniques as compare to other culture technique, while the volatile metabolites emitted by *T. flavofuscum* gives highest inhibition with remarkable inhibition against *A. fumigatus* (78.98%) and *A. terreus* (76.66%) (Fig. 3,4,5).

As we came across several research papers regarding the study of volatile metabolites, it was found that *T. viride* and *T. harzianum* shows significant inhibitory activity, but no one has studied the activity of volatile metabolites of *T. flavofuscum*. It is considered that the production of volatile metabolites by *Trichoderma* spp. depends on strain characteristics along with growth conditions, stage of development, nature, quality and quantity of inhibitory metabolites secreted by antagonistic species. In the present investigation it was found that volatile metabolites of *T. flavofuscum* were as more efficient to restrict the growth of peanut mycoflora along with aflatoxigenic *Aspergillus* species. Raut et al, 2014[32]studied the maximum growth inhibition (100%) of plant pathogen *R. solani* by the volatile metabolites *Trichoderma* T36 isolates. Doi and Mori (1994)[33] deduced the volatile compounds of *Trichoderma* species control the mycelium growth of different fungal pathogens on agar plates. This finding also supports the research of Calistru et al. (1997)[34], they revealed that volatile metabolites and extracellular metabolites produced by *Trichoderma* species can significantly suppress the growth of *A. flavus* and *F. moniliforme* rather than mycoparasitism.

Fig. 3.Effect of diffusible metabolites of *T. flavofuscum* on plant pathogens (dual culture technique) against *A. flavus* and *A. parasiticus.*

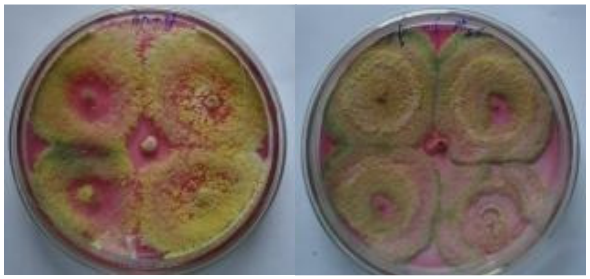


(*A. flavus*) **(*A. parasiticus*)**

Fig. 4. Effect of diffusible metabolites of *T. flavofuscum*on plant pathogens (pathogen at periphery) against *A. flavus* and *A. parasiticus*.

(*A. flavus*) (*A. parasiticus*)

Fig. 5. Effect of diffusible metabolites of *T. flavofuscum* on plant pathogens (pathogen at center) against *A. flavus* and *A. parasiticus.*



(*A. flavus*) (*A. parasiticus*)

Table. 1. Effect of *Trichoderma flavofuscum* on radial growth inhibition of peanut mycoflora by the production of metabolites on RBA medium in variable culture techniques

Sr. No.	Test pathogen	Inhibition index (%)[a]			
		Dual culture[a]	Pathogen at center[b]	Pathogen at periphery[b]	volatile metabolite[c]
1	*A. sydowii*	59.37%	68.75%	53.12%	76.18%
2	*A. nidulans*	61.11%	75.00%	38.33%	65.55%
3	*A. terreus*	60.00%	76.66%	39.16%	62.14%
4	*A. ochraceus*	56.09%	66.97%	51.21%	57.50%
5	*A. fumigatus*	72.46%	78.98%	59.78%	71.42%
6	*A. flavus*	75.38%	70.76%	63.07%	61.22%
7	*A. parasiticus*	64.00%	72.00%	40.00%	65.00%

[a] Radial growth inhibition caused by *T. flavofuscum* against *Aspergillus* species

[b] Within each column followed by same letter, readings were taken on 6th day at 25°C

[c] Growth reading were taken on 4th day at 25°C

Figures in parenthesis are percent inhibition values

C. Bioremediation of aflatoxin by Trichoderma1 species – A confirmatory test

Peanut and its extraction products have been recognized as high-risk commodities for aflatoxin contamination. The aflatoxin producing fungi *A. flavus* and *A. parasiticus* can invade peanut seed in the field before harvest, due to pod damage for various reasons including bores, during drying and curing after harvest, in storage and transit (35). Several fungal biocontrol agents have the capabilities to resist the production of aflatoxins as well as biodegradation of these compounds into several other metabolites that are either not or less toxic than the original aflatoxin (36).

In present study, the chromatographic qualitative assay (TLC) of aflatoxin production by peanut mycoflora revealed that *A. flavus* and *A. parasiticus* were the producers of aflatoxins, predominantly B_1 by both the species and G_1 specifically by *A. parasiticus.* Genus *Trichoderma*, due to varity of factor inhibits the growth of pathogenic test organisms, including a reduction in mycotoxins production when the mycopathogen grows together with *Trichoderma* species (37).

Cultivation of *A. flavus* and *A. parasiticus* in mixed cultures showed the promising growth retardation and efficient reduction of B_1 and G_1 aflatoxins (Fig. 6). Aguero et al. (2008)[9] studied the effect of volatile metabolites on aflatoxin production in stored maize grown on malt extract agar and revealed the reduction of aflatoxin concentration to 12.62ng as compared to the control value of 24.33ng in relation with the

Biocontrol Agent / toxigenic strains	*A. flavus*		*A. parasiticus*		
	Mycelia weight (g/100ml)	Aflatoxin B_1 conc. µg/100ml	Mycelia weight (g/100ml	Aflatoxin B_1 conc. µg/100ml	Aflatoxin G_1 conc. µg/100ml
Control	2.96	16	1.92	30	18
T. flavofuscum	2.58	6	1.66	4	3

Table 2: Effect of *Trichoderma* species on growth inhibition and aflatoxin production by *A flavus*(B_1) and *A. parasiticus*(B_1 and G_1) in co-culture system

pathogen biomass. Gachomo and Kotchoni (2008)[37] found the significant reduction of aflatoxin production in peanut kernels when *T. harzianum* was inoculated before the test pathogen. *T. flavofuscum* were capable of considerable inhibition of *A. flavus* growth (70.10%), were as aflatoxin B_1(62.5%) reduced as compare to control, were as it showed inhibition of *A. parasiticus* growth (60.25%), B_1(86.66%) and G_1 (83.33%) reduction aflatoxin concentration.

In the present investigation, *T. flavofuscum* is able to significantly reduce the aflatoxin concentration when cocultured with aflatoxigenic test pathogen viz. *A. flavus* and *A. parasiticus* as they able to grow in the presence of aflatoxins in its growth medium. This aflatoxin reduction is thought to be an indirect result of the suppressed growth of aflatoxigenic pathogen by *Trichoderma* species, leading to the assumption that smaller colonies produced fewer toxins. The reduction in aflatoxin levels can, therefore, be attributed to one or a combination of the following factors: 1) physical competition for space and nutrition 2) *T. flavofuscum* may compete with aflatoxigenic *Aspergillus* species for a substrate required for toxin reduction but not growth 3) presence of *T. flavofuscum* might cause a change in the biochemical environment altering the metabolic pathway available to the toxigenic fungi and 4) degradation of aflatoxin following its formation.

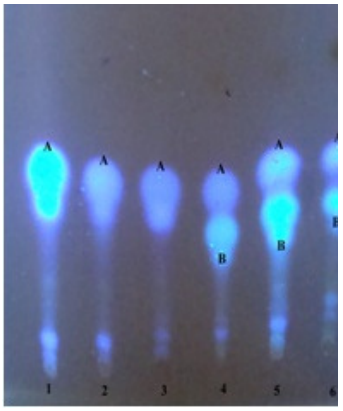


Fig. 6. Determination of aflatoxin content and reduction in concentration (fluorescence intensity of TLC bands) in *A. flavus*(lane 1: Aflatoxin B_1 (A) from *A. flavus* (Control), lane 2 & 3: with *T. flavofuscum*, and in *A. parasiticus* (lane 4 & 6: B_1 (A) and G_1(B) with *T. flavofuscum*, lane 5: B_1(A) and G_1 (B) from *A. parasiticus* (control).

IV. Conclusions

Selection of proper biological agents as well as understanding the mechanisms involved in the antagonistic effect of potential *T. flavofuscum* on peanut pathogen include aflatoxin production in the variable culture techniques are important in designing effective and safe biocontrol strategies. Therefore, one of the most interesting aspects of biocontrol study is the type of technique and mechanisms employed by individual *Trichoderma* species to affect the disease control.

It is very important to study these interaction in field conditions as well establishment of *Trichoderma* potential against pathogenic Aspergilli, as the environmental and biotic factors may had significant influence on biological control system.

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