SCREENING OF *Trichoderma* SPECIES FOR BIOLOGICAL CONTROL ACTIVITY ON *Aspergillus flavus*

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ABSTRACT

Sixteen species of *Trichoderma* isolated from soil were screened according to the dual culture essays, antibiosis inhibiting test, and aflatoxin inhibition test on peanuts. TISTR3167 and C1-1 were selected for their better inhibition of aflatoxins and growth of *Aspergillus flavus*.

Keywords: *Trichoderma*, *Aspergillus flavus*, aflatoxin

1. INTRODUCTION

Aspergillus flavus and the closely related subspecies *parasiticus* have a world-wide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter [1]. These fungi readily colonize several important crops such as corn, cottonseed, peanuts, and tree nuts. *Aspergillus flavus*, *A. parasiticus, A. nomius, A. tamarii* and *A. bombycis* [2, 3] can produce polypeptide-derived secondary metabolites known as aflatoxins, which are highly toxic, mutagenic, and carcinogenic to animals. They may also be involved to some degree in primary liver cancer in humans [4]. Possible cases of aflatoxicoses in humans have been reported in many countries in Southeast Asia and Africa [5]. Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis in malnourished children, and kwashiorkor [6, 7]. Concern for human and animal health has led to regulatory limitations on the quantity of aflatoxins permitted in foods and feeds throughout most of the world. WHO and FAO regulate the content of aflatoxin B (AFB) should be less than 0.5μg/kg in milk in 23th Congress of CAC (Codex Alimentarius Commission) in1999 [8].

Aflatoxin contamination of crops can be minimized by early harvest, prevention of insect damage, and proper storage [9, 10]. However, even under careful management, unacceptable aflatoxin levels may occur from unpreventable insect damage to the developing crop or from exposure of the mature crop to moisture either prior to harvest, or during storage in modules, handling, transportation, or even use [11].

For many diseases, traditional chemical control methods are not always economical nor are they effective, and fumigation as well as other chemical control methods may have unwanted health, safety, and environmental risks. The antifungal abilities of some beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then. However, they are only now beginning to be used commercially.

The filamentous fungus *Trichoderma* spp. has been investigated for more than twenty years for its role as a mycoparasite of plant pathogens and has been accepted as one of the most potent biological control agents for certain fungal plant diseases [12, 13]. Its mycoparasitism involves the complementary action of antibiosis, nutrient competition and cell wall degrading enzymes such as β-1,3-glucanases, proteases and chitinases. Since chitin is the major component of most fungal cell walls, a primary role has been attributed to chitinases in the biocontrol activity of *Trichoderma* [14, 15].

In the present study our objective was to screen promising *Trichoderma* spp. that could inhibit aflatoxin production and growth of *Aspergillus flavus*.

2. MATERIALS AND METHODS

2.1 Fungal species

Aspergillus flavus IMI242684 and 16 species of *Trichoderma* spp. were obtained from Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Thailand. The fungi were grown on potato dextrose agar (PDA) slants for 7-10 days at 30 °C and kept in the refrigerator at 5 °C.

2.2 Screening for growth inhibition

2.2.1 Direct opposition test of antagonism between *Trichoderma* spp. and *A. flavus*

Trichoderma spp. were tested for their antagonistic property against *A. flavus*. The promising antagonistic ones were put aside for further studies. Mycelial disc (5 mm) of *A. flavus* was transferred onto PDA medium at one side of agar plate and mycelial disc (5 mm) of *Trichoderma* spp. was placed 6 cm apart on the other side of the plate. Observation was carried out seven days later at 30 °C. The inhibition of mycelial growth of *A. flavus* by the antagonist was measured by the formula of Fokkema [16]. The presence of inhibition zone that might develop between the two colonies was also observed.

2.2.2 Antibosis Test

Cellophane membrane (8.5 cm diameter) was autoclaved and overlaid on sterile PDA. Mycelial disc (5 mm diameter) of *Trichoderma* spp. was transferred onto the center of the cellophane membrane and incubated for 36 h before both cellophane membrane and mycelial disc were removed. Mycelial disc of *A. flavus* (5mm diameter) was then placed on PDA medium at the same location as *Trichoderma* spp. and incubated at 30 °C for 4 days. Mycelial disc of *A. flavus* on PDA medium served as control. The diameters of *A. flavus* on both control and test plates were then measured. Three replicates were used for this experiment. Percentage of inhibition was calculated according to the formula:

$$\text{Percentage of inhibition} = [(D_1 - D_2)/ D_1] \times 100\%$$

And D_1 = the diameter of *A. flavus* on control plate

D_2 = the diameter of *A. flavus* on test plate

2.3 Assay for inhibition of aflatoxin production and growth of *A. flavus* by *Trichoderma* spp.

2.3.1 Samples

Fifty grams of peanut kernels purchased from the local markets were put into the 250 ml flask plugged with cotton and then autoclaved for 30 minutes. The sterilized peanuts were dried for 48 hrs at 70 °C in a hot air oven before inoculation.

2.3.2 Inoculation and incubation

Moisture content of samples was adjusted to 22% by adding sterile distilled water spore suspension (0.1 ml 1×10^8 cfu/ml) of *Trichoderma* spp. was inoculated into the sample and incubated at room temperature (30±2 °C). Three days later, 0.1 ml 1×10^8 cfu/ml spore suspension of *A. flavus* was added to the sample. The sample without *Trichderma* spp. was kept as a control. Three replicates were made for each treatment. Seven days later the growth of mold was measured and aflatoxin was analyzed by HPLC.

2.3.3 Aflatoxin analysis

Aflatoxin was extracted by modified methods of Seitz and Morh [17] and Lichrolut Vacuum Manifold and analyzed by HPLC.

2.3.4 Fungal growth assessment

Fungal growth was visually assessed using a semi quantitative scale, viz. (1) very little growth; (2) 25% of the peanuts covered; (3) 50% of the peanuts covered; (4) 75% of the peanuts covered; (5) all of the peanuts covered.

2.4 Statistical analysis

The experimental data were analyzed statistically using SAS (Statistic Analysis Software).

3. RESULTS AND DISCUSSION

3.1 Antagonism between *Trichoderma* spp. and *A. flavus*

The success of an organism in colonizing organic substrates in competition with other organisms can be determined by physiological characteristics such as rapid growth rate, abundant sporulation and rapid germination of spores, necessary enzyme systems for breaking down substrate tissues, production of antibiotic substances, and tolerance to antibiotics by the other microorganisms [18, 19].

In this study, most of the *Trichoderma* spp. significantly inhibited the growth of *A. flavus* (P=0.05), but the percentage of inhibition varied considerably (from 7.04% to 92.67) (Table 1). Even if *A. flavus* was inoculated three days before *Trichoderma* spp., the inhibition of *A. flavus* could be observed. Wheeler and Hocking [20] classified the interactions between two fungal colonies into six types. Three types were observed in this study. *Trichoderma* spp. strains TISTR3167, TISTR3331, C1-1, R1-1, S38-2, S73-1 S84-1 overgrew the colony of *A. flavus*. For the other *Trichoderma* spp., an inhibition zone was observed between the pathogen and antagonist. In addition, the color of inhibiting band was different. Some *Trichoderma* spp. might produce colorful metabolite inhibiting the growth of *A. flavus*. *Trichoderma* spp. used in this study grew rapidly on PDA medium, and could cover the whole Petri dish in 72 h; while *A. flavus* needed 6-7 days. So competing for nutrients and space might be one mechanism for inhibiting the growth of *A. flavus* on the plate.

3.2 Antibiosis assay

The results of antibiosis test (Table 2) showed that growth of *A. flavus* was not strongly inhibited by *Trichoderma* spp. used in the study (no significant difference at P=0.05) on PDA medium, which meant *Trichoderma* spp. did not produce antibiotics that could inhibit the growth of *A. flavus* on PDA medium. However, whether they could produce some other metabolites to inhibit the aflatoxin production need to be further investigated.

3.3 Screening assays for inhibition of AFB_1 and growth of *A. flavus* by *Trichoderma* spp. in peanuts

The ability of *Trichoderma* spp. in inhibiting the growth and aflatoxin production of *A. flavus* ranged from 24% to 99.7% in peanuts. Strains S11-1, Ku-1, TISTR3331 promoted the production of aflatoxin. R1-1 and R18-1 could inhibit aflatoxin, but didn't suppress growth of *A. flavus* (Table 3). They might produce some metabolites to hinder the formation of aflatoxin. Some *Trichoderma* spp. like S38-2, TISTR3167, C1-1 significantly (P = 0.01) inhibited both colony growth and aflatoxin production.

Fungal and bacterial species could affect the growth and/ or toxigenicity of mycotoxin producing fungi. In some cases, they reduce mycotoxin level, while in other enhance toxin production [21].

Table 1. Antagonistic action between *Trichoderma* spp. and *A. flavus*

Fungi	Diameter of *Trichoderma*	Diameter of *A. flavus*	Inhibition of growth (%)	Types of interaction[a]	Colony of *A. flavus*[b]
S11-1	37.76	22.49	7.04	B	D
S27-1	40.47	22.03	31.00	B	E
S33-1	49.53	12.97	59.38	B	E
S36-1	44.73	17.77	44.35	B	E
S38-2	57.36	5.14	83.90	A	E
S40-1	38.87	23.63	25.99	B	E
S40-2	38.45	25.43	20.29	C	E
S63-1	39.21	23.29	27.06	B	E
S73-1	39.15	23.35	26.87	A	E
S84-1	56.45	6.05	81.05	A	D
R1-1	59.78	2.72	91.48	A	D
R18-1	45.83	16.67	47.79	B	E
TISTR3331-1	58.23	4.27	86.63	A	D
TISTR3167-1	60.16	2.34	92.67	A	D
Ku-1	40.56	21.94	31.29	B	D
C1-1	58.48	4.02	87.41	A	D
A. flavus					

[a]Types of interaction: A - *Trichoderma* spp. overgrowing *A. flavus*; B - mutal inhibition; C - *A. flavus* overgrowing *Trichoderma* spp.

[b]Colony of *A. flavus*: D - The colony of *A. flavus* is olive green, the same as control; E - the colony of *A. flavus* become yellow proximal to the *Trichoderma* colony, and the *Trichoderma* spp. produce yellow pigment around the edges of the boundary.

Table 2. The effects of antibiotics from *Trichoderma* spp. on the growth of *A. flavus*

Trichoderma species	Diameter of *A. flavus* colony (mm)	Percentage of inhibition	Comparison of significant difference
S11-1	39.76	29.69	b
S27-1	57.34	-9.68	a
S33-1	50.20	3.98	a
S36-1	44.08	15.68	a
S38-2	56.82	-8.68	a
S40-1	47.14	9.83	a
S40-2	42.06	19.55	b
S63-1	46.50	11.06	a
S73-1	51.72	1.07	a
S84-1	43.88	17.07	a
R1-1	43.08	17.60	a
R18-1	63.50	-21.46	a
TISTR3331-1	54.18	-3.63	a
TISTR3167-1	54.47	-4.21	a
Ku-1	44.39	15.11	a
C1-1	54.08	-3.44	a
Control	52.28	0	

*a - no significant difference at P = 0.05; b - significant difference at P = 0.05

Table 3. Inhibition of AFB_1 production and growth of *A. flavus* by *Trichoderma* spp. in peanuts

Fungi	Mean AFB_1 (µg/g)	Inhibition of growth (%)	Growth of *A. flavus***
A. flavus	0.749 a	0	5
Trichoderma S11-1	2.068 b	-176.10	5
Trichoderma S27-1	0.222 b	70.28	3
Trichoderma S33-1	0.616 a	17.43	4
Trichoderma S36-1	0.0778 b	89.57	2
Trichoderma S38-2	0.0770 b	89.72	1
Trichoderma S40-1	0.541b	27.77	3
Trichoderma S40-2	0.450 b	39.92	3
Trichoderma S63-1	0.883 a	17.89	4
Trichoderma S73-1	0.273 b	63.40	4
Trichoderma S84-1	0.0273 b	96.35	3
Trichoderma R1-1	0.149 b	80.03	5
Trichoderma R18-1	0.300 a	59.89	5
Trichoderma Ku-1	1.245 a	-42.45	4
Trichoderma TISTR3331	0.868 a	-15.89	5
Trichoderma TISTR3167	0.00566 b	99.24	1
Trichoderma C1-1	0.00358 b	99.52	1
Transformant strain of *Trichoderma*	0.0110 b	98.53	1

*a - no significant difference at P=0.05; b - significant difference at P = 0.05

** (1) very little growth; (2) 25%of the peanutscovered; (3) 50% of the peanuts covered; (4) 75% of the peanuts covered; (5) all of the peanuts covered.

4. CONCLUSION

Studies on the control of aflatoxin have normally taken two approaches, namely: 1) the control of the toxin-producing fungi and 2) detoxification of aflatoxin. Future research will target the prevention of *A. flavus* growth.

Among the 16 *Trichoderma* spp. studies, TISTR3167 and C1-1 were most active in inhibiting aflatoxin production and growth of *A. flavus*. They were selected for further research.

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