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Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests

Alka Gupta* and Murali Gopal
Central Plantation Crops Research Institute, Regional Station, Kayangulam, Krishnapuram 690533, Kerala, India
**Author for correspondence: Tel.: +91-479-442160/442104, Fax: +91-479-445733,*
E-mails: alkagupta70@rediffmail.com, cpcrikgm@vsnl.com

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Summary

Aspergillus flavus isolated from naturally infected leaf-eating caterpillar (*Opisina arenosella* W.), lace bug (*Stephanitis typica* D.) and plant hopper (*Proutista moesta* Westwood), insect pests of the coconut palm, were tested for aflatoxin (AT) production by employing various media formulations. These *A. flavus* isolates were earlier found to be entomopathogenic in laboratory bioassays. A laboratory contaminant and four standard aflatoxigenic *A. flavus* isolates were also included in this study as reference strains. All *A. flavus* isolates were tested on seven AT detection media: coconut extract agar, coconut extract-sodium desoxycholate agar, coconut extract-ascorbic acid agar, coconut extract-Czapek Dox agar, coconut extract-milk powder agar, 10% commercial coconut milk powder agar (CCMPA) and 20% CCMPA. Only two isolates of *A. flavus*, originally isolated from *O. arenosella* and *P. moesta*, produced ATs. AT production was detected within 48 h of incubation and was detected continually up to 1 month. These AT-producing *A. flavus* isolates also produced bright yellow pigmentation in the medium. Of all the seven media used for AT detection, CCMPA (10%) was found to be the best one, followed by 20% CCMPA, for direct and rapid AT detection. AT production was not necessary for pathogenicity in the insects.

Introduction

Aspergillus flavus Link ex Fries is a ubiquitous saprophytic mould which produces a diverse array of secondary metabolites. Among these, the most notable are mycotoxins; and aflatoxins (ATs), which are polyketide secondary metabolites, are the most thoroughly studied mycotoxins. ATs are produced by a complex pathway involving over 16 steps after the synthesis of the first stable intermediate, norsolorinic acid (Bhatnagar *et al.* 1992). The production of these compounds is highly regulated and involves a commitment of cellular energy and metabolites. However, unlike other secondary metabolites, ATs do not play any role in the growth of the producing organisms, but presumably are involved in their ecology.

These ATs are highly toxic and carcinogenic, and AT B_1 is the best studied and ranks as the most potent naturally occurring toxin known (Squira 1989). Because of the production of ATs, the contamination of agricultural products (Golumbic & Kulik 1969), most notably corn and peanuts (Diener & Davis 1966, 1983), by *A. flavus* is a cause for concern for the sake of human and animal health. These ATs are known to cause both acute and chronic toxicity to mammals and can induce mutagenic and carcinogenic effects in a wide range of organisms (Heathcote 1984), including chronic aflatoxicosis in animals (Miller & Wilson 1994).

This fungus is also reported to attack a number of insect pests (Table 1). ATs have also been documented to have significant insecticidal, larvicidal and chemosterilizing properties against several insect and mite species (Moore *et al.* 1978; Gaston & Llewellyn 1980). Of the ATs, AT B_1 is mostly associated with insecticidal activity, apart from AT G_1 (Al-Adil *et al.* 1972).

The isolates of *A. flavus* from naturally infected *Opisina arenosella*, *Stephanitis typica* and *Proutista moesta*, insect pests of coconut, were screened for the production of AT, by either modifying the earlier media used or formulating new and improved detection media.

Materials and methods

Fungal isolates and cultures

Two isolates from *S. typica*, three from *O. arenosella* and one from *P. moesta* were used in this study. These were isolated from naturally infected insects collected from various coconut-growing regions of Kerala, India. All these isolates were identified as *A. flavus* using the taxonomic criteria described by Klich & Pitt (1988) and

Table 1. Record of *A. flavus* from some important insect pests.

S.No.	Pest	Common name	Crop	Country	Effect on pest	Reference
1.	*Cimex lectularius*	Bed bug	–	England	Pathogenic	Cockbain & Hastie (1961)
2.	*Musca domestica*	Housefly	–	India	100% Mortality in 21 days	Amonkar & Nair (1965)
3.	*Atta texana*	Fire ant	–	USA	Pathogenic	Steinhaus & Marsh (1967)
4.	*O. arenosella*	Leaf eating caterpillar	Coconut	India	90% Mortality	Oblisamy *et al.* (1969); Muthukrishnan & Rangarajan (1974)
5.	*Orthaga euadrusalis*	Leaf webber	Mango	India	100% Mortality	Srivastava & Tandon (1980)
6.	*Calliptamus italicus*	Locust	Various crops	USSR	>60% Mortality	Nurzhanov & Lachininskii (1987)
7.	*Malacosoma indica*	Tent caterpillar	Fruit trees	India	Pathogenic	Joshi & Agarwal (1987)
8.	*Scirpophaga excerptalis*	Pyralid insect pest	Sugarcane	China	Pathogenic	Fang & Tan (1986)
9.	*Cossus cadambae*	Carpenter worm	Teak	India	57% Mortality	Mathew & Mohamed Ali (1987)
10.	*C. hemipterus*	Maize insect	Maize	USA	Toxic	Wicklow *et al.* (1988)
11.	*Bombyx mori*	Silkworm	Mulberry	India	Pathogenic	Patil (1989)
12.	*Dysdercus cingulatus*	Cotton stainer	Cotton	India	100% Mortality	Kshemkalyani *et al.* (1989)
13.	*Epilachna vigintioctopunctata*	Epilachna beetle	Potato	India	Toxic	Rajagopal & Trivedi (1989)
14.	*Heliothis zea (Helicoverpa zea)*	Corn earworm	Maize	USA	Toxic	Wicklow & Dowd (1989)
15.	*Spodoptera frugiperda*	Fall armyworm	Maize	USA	Toxic	Wicklow & Dowd (1989)
16.	*S. sacchari*	Mealybug	Sugarcane	Cuba	Toxic	Martinez & Bravo (1989)
17.	*Planococcus citri*	Mealybug	Sugarcane	Cuba	Pathogenic	Martinez & Bravo (1989)
18.	*Pogonomyrmex rugosus*	Queen harvester ant	–	Arizona	Pathogenic	Gilliam *et al.* (1990)
19.	*Helopeltis antonii*	Tea mosquito bug	Cashew	India	22.5–47.5% Mortality	Sathiamma & Saraswathy (1990)
20.	*Latoia viridissima*	Nettle caterpillar	Oil palm	Nigeria	60–80% Mortality	Igbinosa (1992)
21.	*Noorda blitealis*	Leaf caterpillar	Drum stick	India	70–80% Mortality	Kalia *et al.* (1996)
22.	*Oxya nitidula*	Grasshopper	Rice	India	23–94% Mortality	Ambethgar & Kumaran (1998)
23.	*P. moesta*	Plant hopper	Coconut	India	62.5% Mortality	Ponnamma *et al.* (2000)

were found to be entomopathogenic in laboratory bioassays (Gopal *et al.* 2000 a, b). Apart from these, a laboratory contaminant of *A. flavus* was also included in the study. Four standard aflatoxigenic *A. flavus* isolates obtained from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India were used as reference strains (details given in Table 2).

The *A. flavus* isolates were maintained as single spore cultures on Sabouraud's dextrose agar (SDA) kept at 4 °C (stock cultures) and 28 °C (working cultures). All the other isolates were also maintained on SDA in a similar manner.

Preparation of media

Seven different media were employed for detecting AT production by entomopathogenic *A. flavus* isolates, either by modifying the media used by earlier workers or formulating new improved ones. The composition of these media is given in Table 3.

Table 2. A. flavus isolates used in the study.

Culture designation	Source from which isolated	Location from where collected[a]	Specific place where isolated[a]
AF 1 (ITCC 5004)	*S. typica*[b]	Quilon, Kerala	CPCRI, Kayangulam, Kerala
AF 1a	*S. typica*[b]	Quilon, Kerala	CPCRI, Kayangulam, Kerala
AF 2 (ITCC 5005)	*O. arenosella*[b]	Quilon, Kerala	CPCRI, Kayangulam, Kerala
AF 2a (ITCC 5003)	*O. arenosella*[b]	Quilon, Kerala	CPCRI, Kayangulam, Kerala
AF 2b	*O. arenosella*[b]	Quilon, Kerala	CPCRI, Kayangulam, Kerala
PTM 1 (ITCC 5006)	*P. moesta*[b]	Trivandrum, Kerala	NRCOP, Palode, Kerala
LAC 1[c]	–	–	CPCRI, Kayangulam, Kerala
ITCC 3959[d]	Soil	New Delhi	CBT, New Delhi
ITCC 1717[d]	*Arachis hypogaea*	Hyderabad, Andhra Pradesh	RRL, Hyderabad
ITCC 1655[d,e]	*A. hypogaea*	Hyderabad, Andhra Pradesh	RRL, Hyderabad
ITCC 1652[d,e]	*A. hypogaea*	Hyderabad, Andhra Pradesh	RRL, Hyderabad

CPCRI – Central Plantation Crops Research Institute; NRCOP – National Research Centre For Oil Palm; CBT – Centre For Biochemical Technology; RRL – Regional Research Laboratory.

[a] All places mentioned are in India.

[b] Naturally infected by the fungus.

[c] Laboratory contaminant.

[d] Confirmed AT producers.

[e] Produced B_1 and B_2 ATs.

Table 3. Composition of different media[a] used for detection of AT production by entomopathogenic *A. flavus* isolates.

Sl. No.	Medium	Components
1.	CEA	20% (w/v) Coconut extract[b] 2.2% Bacto-agar[c] pH 6.9–7.0[d]
2.	CE-SDA	20% (w/v) Coconut extract[b] 0.8% Sodium desoxycholate[e] 2.2% Bacto-agar[c] pH 6.9–7.0[d]
3.	CE-AAA	20% (w/v) Coconut extract[b] 0.08% Ascorbic acid[f] 2.2% Bacto-agar[c] pH 6.9–7.0[d]
4.	CE-CDA	16% (w/v) Coconut extract[b] 0.2% Sodium nitrate 0.25% $MgSO_4$ 0.25% KCl 0.005% $FeSO_4$ 0.1% K_2HPO_4 2.2% Bacto-agar[c] pH 6.9–7.0[d]
5.	CE-MPA	16% (w/v) Coconut extract[b] 1% Skim milk powder 0.01% Peptone 0.6% Sodium nitrate 0.01% Triton ×-100/0.001% sodium dodecyl sulphate 2.2% Bacto-agar[c] pH 6.9–7.0[d]
6.	CCMPA – 10%	10% CCMP[g] 2.2% Bacto-agar[c] pH 6.9–7.0[d]
7.	CCMPA – 20%	20% CCMP[g] 2.2% Bacto-agar[c] pH 6.9–7.0[d]

[a] All media were autoclaved at 15 lb/in^2 for 20 min. except CE-MPA, 10 and 20% CCMPA, which were autoclaved at 15 lb/in^2 for 15 min.

[b] Hundred gram of coconut meat was shredded into pieces and homogenized with 500 ml distilled water for 5 min. It was filtered through four layers of muslin cloth. The clean filtrate was used as coconut-extract and prepared fresh every time.

[c] Bacto-agar (DIFCO Laboratories, USA) was used uniformly in all media; 1.8 and 2% agar were found to be insufficient for proper solidification of the medium.

[d] *p*H of coconut-extract was adjusted to 6.9–7.0 with 2 N NaOH.

[e] Sodium desoxycholate was used as a growth retardant.

[f] Ascorbic acid was used as a preservative in the medium (concentrations ranging from 0.05 to 0.1% were tried).

[g] CCMP (trade mark 'Palmo', preservative-free; Shriram Coconut Products Ltd., India) contained spray dried natural coconut milk, modified starch and milk protein and was first dissolved in small volume of lukewarm distilled water and then filtered through muslin cloth for media preparation.

Preparation of inoculum

Conidia from a test/reference culture were transferred to a plate containing *Aspergillus*-differential medium (ADM) (Bothast & Fennell 1974) and incubated at 28 ± 1 °C for 4 days. Mycelial plugs (6 mm in diameter) from the ADM plate served as inocula. Fungi of *A. flavus* group do not sporulate on ADM; thus, secondary colonies do not develop from conidia disseminated during handling. In addition, *A. flavus* – group fungi produce characteristic bright, yellow-orange pigment in this medium which aids their identification (Bothast & Fennell 1974).

Cultivation and detection of AT

A mycelial plug from ADM was placed in the centre of each plate as well as slant containing the test medium and incubated at 28 ± 1 °C. Each plate contained a single, large, central colony, the reverse side of which was examined daily visually for colony growth and then with a u.v. lamp in a dark room for fluorescence. The colony diameter was measured in mm and the diffusible zone of AT was detected as blue fluorescent zone under long wave (366 nm) u.v. light (portable u.v. lamp; 4w/366 nm, 13203 Mikrobiologie, Merck KGaA, D-64271 Darmstadt). In case of slants, the reverse side of the slant was observed for blue fluorescence. Intensity of fluorescence expressed by number of '+' signs was estimated subjectively. Uninoculated plates and slants served as controls. Five plates and five slants for each test medium were inoculated and observed.

Laboratory bioassay

The *A. flavus* isolates were tested for pathogenicity against the coconut insect pests by spray as well as crawl method. In the spray method, spore suspension (10^6 spores ml^{-1}) was sprayed on fresh surface sterilized coconut leaflets and the insects were allowed to feed on them. In the crawl method, the insects were made to crawl over the dense sporulating cultures of *A. flavus* for 10 min and then transferred to fresh coconut leaflets (Gopal *et al.* 2000a).

Results and discussion

The *A. flavus* isolates were obtained from naturally colonized cadavers of *S. typica* (nymphs and adults), *O. arenosella* (larval stages) and *P. moesta* (adults). The laboratory bioassay conducted with these fungi on the respective hosts yielded 80% mortality of *S. typica* nymphs within 3 days; 80–90% *O. arenosella* larvae were mycosed within 3–4 days (Gopal *et al.* 2000a) and 62.5% *P. moesta* insects died within 4 days (Ponnamma *et al.* 2000) under laboratory conditions. The mortality was cent percent when the experiment was continued for 2–3 days more. The ideal spore concentration for achieving 100% mortality in short period (5–6 days) was 10^6 spores ml^{-1}. In response to *A. flavus* infection, within 24 h, the insects became lethargic, refused food indicating loss of appetite and did not respond to external stimuli. After death due to fungal parasitism, their body became mummified and brittle. These *A. flavus* isolates also showed cross-infectivity (Gopal *et al.* 2000b).

It is a well known fact that *A. flavus* is deleterious to human and animal health, besides infesting many

agricultural commodities, because of its ability to elaborate ATs. Because of this fungal character, some researchers are of the view that it could not be recommended for use as a biological control agent (Steinhaus & Marsh 1967; Martinez & Bravo 1989). In contrast to this, other workers emphasize that *A. flavus* is a ubiquitous mould and that not all strains produce ATs; thus, proper strain selection, formulation and method of application could conceivably nullify potential hazards and objections to its use (Gilliam *et al.* 1990). In this line, Wicklow *et al.* (1988) showed that non-aflatoxigenic strains of *A. flavus* are toxic to the maize insect *Carpophilus hemipterus*, owing to their sclerotial metabolites. About 20% of non-aflatoxigenic *A. flavus* isolates were reported from naturally infected sugarcane mealybugs (Drummond & Pinnock 1990). Studies by Diener & Davis (1966) found similar proportion of non-aflatoxigenic *A. flavus* agricultural isolates. In agricultural fields prior to or after harvest, *A. flavus* strains that do not produce ATs have been used to competitively exclude toxigenic *A. flavus* strains to prevent AT contamination of peanuts (Dorner *et al.* 1992) and cottonseed, which is primarily used as dairy feed (Cotty 1994). Since not all isolates of *A. flavus* can produce ATs (Detroy *et al.* 1971), determination of AT-producing ability of a particular isolate is important so that the entomopathogenic, non-AT producing *A. flavus* strains could be used for field release against crop pests. In this background, we tested the toxigenic nature of *A. flavus* isolates pathogenic to *S. typica*, *O. arenosella* and *P. moesta* for their possible use as biological control agents against some of the insect pests of coconut.

Seven isolates of *A. flavus*, two isolated from *S. typica* (lace bug), three isolated from *O. arenosella* (leaf eating caterpillar), one from *P. moesta* (plant hopper), one laboratory contaminant along with four reference strains (Table 2) were screened for AT production in solid state culture in seven different media over a 30-day period (Table 3). Out of seven *A. flavus* isolates, only two isolates (AF 2a and PTM 1) characteristically produced ATs detectable as blue fluorescence upon visual examination under long wave (366 nm) u.v. light (Tables 4 and 5). In both the isolates, fluorescence began to appear within 48 h after transfer to the media, indicating them to be strong AT producers, with the exception of Coconut Extract-Czapek Dox Agar (CE-CDA) medium. The intensity of AT production (fluorescence) increased with prolonged incubation. An incubation time of 4 days was sufficient for the detection of even weak AT production, which was detected continually upto day 30. However, in CE-CDA medium, AT production was observed only by day 7 and only in two *A. flavus* isolates which were AT-positive in other media; the colour of the medium further obscuring the fluorescence. The remaining five isolates (AF 1, AF 1a, AF 2, AF 2b, LAC 1) did not produce blue fluorescence on any of the media upto 30 days of incubation. The four confirmed aflatoxigenic *A. flavus* strains, used as reference cultures, produced blue fluorescence on all the media tested (Table 5). As discussed by Lin & Dianese (1976), a bright yellow pigmentation was found to be consistently associated with the production of fluorescence (AT-producing ability) by *A. flavus* isolates (including reference strains) on all the media with the exception of CE-CDA medium. None of the AT-negative isolates produced this pigmentation. The pigmentation was seen on the reverse side of the growth, at

Table 4. Comparative formation of fluorescence by selected *A. flavus* isolates[a] on different media (after 4 days of incubation at 28 ± 1 °C).

Medium	Bright yellow pigmentation on reverse side of growth[b]	Blue fluorescence visible at 366 nm[b]	Zone diameter[c] (mm)
A. flavus AF 2a			
CEA	+	+ + +	14.0
CE-SDA	+	+ + +	14.5
CE-AAA	+	+ +	10.5
CE-CDA[d]	–	–	–
CE-MPA	+ +	+ +	12.0
10% CCMPA	+ +	+ + + + +	21.0
20% CCMPA	+ +	+ + + + +	19.5
A. flavus PTM 1			
CEA	+ +	+ + +	10.0
CE-SDA	+	+ + +	8.2
CE-AAA	+	+ + +	8.0
CE-CDA[d]	–	–	–
CE-MPA	+ +	+ + +	9.8
10% CCMPA	+ + +	+ + + +	16.0
20% CCMPA	+ + +	+ + + +	15.0

–: Negative.
[a] Only those isolates which were AT-positive have been included; remaining isolates (AF 1, AF 1a, AF 2, AF 2b and LAC 1) did not produce AT on any of the media even after 30 days of incubation at 28 ± 1 °C. Standard aflatoxigenic *A. flavus* strains were inoculated in all the media as checks.
[b] Pigmentation and fluorescence intensities were estimated after 48 h of transferring the cultures to media. Number of '+' signs indicate the intensity of pigmentation and fluorescence.
[c] Average of five replications.
[d] On CE-CDA medium, pigmentation and fluorescence were detectable only by 7th day of incubation.

Table 5. Detection of blue fluorescence under long wave u.v. light (366 nm) by *A. flavus* isolates (test tube screening).

A. flavus isolates	Blue fluorescence	Toxigenic potential
AF 1	–	Non-toxigenic
AF 1a	–	Non-toxigenic
AF 2	–	Non-toxigenic
AF 2a	+	Toxigenic
AF 2b	–	Non-toxigenic
PTM 1	+	Toxigenic
LAC 1[a]	–	Non-toxigenic
ITCC 3959[b]	+	Toxigenic
ITCC 1717[b]	+	Toxigenic
ITCC 1655[b]	+	Toxigenic
ITCC 1652[b]	+	Toxigenic

–: Negative for blue fluorescence; +: positive for blue fluorescence.
[a] Showed bright green fluorescence.
[b] Standard aflatoxigenic strains used as checks.

the same time as blue fluorescence became visible, initially appearing along the periphery of the growth and later on covering whole of the growth on the medium (Table 4). However, contrary to the work of Lin & Dianese (1976), the degree of pigmentation was not found to be proportional to the intensity of fluorescence consistently in all the media (Table 4). Thus, though the production of yellow pigmentation was associated with AT production by toxic strains (Wiseman *et al.* 1967), the intensity of pigmentation was not a reliable indicator of AT-producing ability (Davis *et al.* 1987) which could neither be judged sooner nor without the use of a u.v. lamp. Arseculeratne *et al.* (1969) also reported abundant production of yellow pigments and AT on freshly grated coconut inoculated with *A. flavus*. A similar yellow pigment produced by *A. parasiticus* was identified as averufin (Donkersloot *et al.* 1972; Lin *et al.* 1973).

Previously different groups of workers formulated many types of media for screening *A. flavus* isolates from agricultural commodities, animal feeds and raw and processed foods for contamination with AT. These include a groundnut-based complex agar medium (De Vogel *et al.* 1965), corn steep liquor-based medium (Hara *et al.* 1974) and coconut-based media (Lin & Dianese 1976; Davis *et al.* 1987; Lemke *et al.* 1989; Pallavi *et al.* 1997). For our requirement of testing entomopathogenic *A. flavus* isolates, we formulated or modified the previously reported procedures. In this study, all the seven media which were used were coconut-based, since coconut-based media are known to be efficient for growth and AT production by *A. flavus* and also by *A. parasiticus* (Arseculeratne *et al.* 1969; Davis *et al.* 1987; Lemke *et al.* 1988; Lin & Dianese 1976) and specifically support only the production of AT-related blue fluorescence (Lin & Dianese 1976). Furthermore, the agar plates/slants prepared with coconut have a white background which enhances visualization of blue fluorescence in the agar, so much so that the plating method with the coconut-based media for detection of AT production was described as technically analogous to examining coated thin-layer chromatography plates under u.v. light for fluorescent AT spots (Lemke *et al.* 1989).

In the present investigation, fresh coconut milk was essentially used for media preparation except for commercial coconut milk powder agar (CCMPA) medium where commercial coconut milk powder was used (Table 3). The media used by earlier workers employed preserved shredded coconut for preparing coconut extract (Davis *et al.* 1987; Lin & Dianese 1976). Coconut extract agar (CEA) medium used by us is a modification of the medium used by Lin & Dianese (1976); coconut extract-sodium desoxycholate agar (CE-SDA) medium is a further modification of CEA, where sodium desoxycholate is used as a growth inhibitor so that *A. flavus* colonies do not grow much radially in the petri plates but sporulate efficiently and produce ATs, to make the task of measuring the fluorescence zone diameter easy. Similarly, CEA was modified to include ascorbic acid (a preservative) which was reported to support intense AT-related fluorescence (Lin & Dianese 1976). All three media namely, CEA, CE-SDA and coconut extract-ascorbic acid agar (CE-AAA) showed only moderate production of ATs by *A. flavus* isolates as determined by the fluorescent zone measurement (Table 4). Corn steep liquor-based APA (AT-producing-ability) medium used by Hara *et al.* (1974) inspired the formulation of CE-CDA medium. However, the medium was found to allow very slow development of the AT production (after 7 days of incubation) by *A. flavus* isolates; the dirty-grey colour of the medium further obscuring the blue fluorescence. The precipitation of the medium was also found to be quite high after autoclaving (Table 6). The colour and precipitation characters of coconut extract-milk powder agar (CE-MPA) medium were satisfactory and supported moderate aflatoxin production (Tables 4 and 6). The newly designed CCMPA medium was found to be the best in all aspects *viz.*, colour, precipitation and AT production. Both 10 and 20% CCMPA supported good growth and intense AT production in a very short incubation period. The presence of either modified starch or milk protein in the medium might have contributed to such a result. However, 10% CCMPA was found to have advantage over 20% CCMPA as precipitation was minimal in that medium (Table 6).

The CCMPA medium was found to be definitely advantageous as a plating medium in the context of the present investigation as: (i) the medium surface was highly absorptive for u.v. light and, as such, provided an effective background for detecting zones of fluorescent AT surrounding the fungal colonies, (ii) the commercial coconut milk powder (CCMP) used as a substrate in the medium formed much less precipitate after autoclaving than the freshly prepared coconut extract, used in other media. Thus, the medium could be distributed evenly in the petri plates and did not obscure the direct visual detection of the blue fluorescence, (iii) with this medium, the isolates can also be cultured on slants, besides petri plates, making it easier to handle large number of isolates at a time, and (iv) the method described is specific, rapid and simple with only minimal equipment

Table 6. Colour and precipitation characteristics of AT-detection media[a] used.

Medium	Colour	Precipitation[b]
CEA	White-buff	+ + +
CE-SDA	White-buff	+ + +
CE-AAA	Milky white	+
CE-CDA	Dirty grey	+ + + + +
CE-MPA	White	+
10% CCMPA	Milky white	–[c]
20% CCMPA	Milky white	+

[a] After autoclaving and cooling down.
[b] Extent of precipitation is expressed as number of '+' signs.
[c] Minimal precipitation.

Table 7. AT production by *A. flavus* isolates (petri plate screening) and pathogenicity[a] against their respective hosts (after 4 days).

A. flavus isolates	Zone diameter[b] (mm)	Degree of toxigenicity[c]	Pathogenicity (% mortality)
AF 1	–	–	80.0[d]
AF 1a	–	–	62.0[d]
AF 2	–	–	86.0[e]
AF 2a	21	+ + + +	76.0[e]
AF 2b	–	–	80.0[e]
PTM 1	16	+ +	62.5
LAC 1	–	–	ND

–: Negative; ND: not done.
[a] Fungal inoculation dose of 1×10^6 spores/ml was used uniformly.
[b] Maximum value obtained among all the screened media after 4 days of incubation.
[c] Number of '+' signs indicate the intensity of AT-related fluorescence and were estimated after 4 days of incubation at 28 ± 1 °C.
[d] Average of five replications for a total of 50 *S. typica* nymphs.
[e] Average of five replications for a total of 25 *O. arenosella* larvae.

requirements and thus, can find wide application for rapid and economical screening of the toxigenic potential of entomopathogenic *A. flavus* isolates.

Although all the *A. flavus* isolates included in the study (except LAC 1) inflicted mortality ranging from 62 to 86% on their respective hosts, only two isolates out of these produced ATs. It is interesting to note that *A. flavus* isolates AF 1 and AF 2 which were found to be more pathogenic to *S. typica* and *O. arenosella* (80 and 86% mortality) respectively, did not elaborate AT into the media (Table 7). In other words, entomopathogenicity by *A. flavus* isolates was not always associated with AT production. Probably the sclerotial metabolites were involved in the pathogenicity of non-aflatoxigenic *A. flavus* isolates, as was found in maize insect *C. hemipterus* (Wicklow *et al.* 1988). Further, it was demonstrated in the mealy bug *Saccharicoccus sacchari* that loss of AT production ability of an otherwise highly aflatoxigenic and also highly pathogenic *Aspergillus* isolate WF5BC did not affect its pathogenicity against *S. sacchari* (Drummond & Pinnock 1990), which led the authors to conclude that the ability of *Aspergillus* isolates to produce ATs was not essential to cause mortality to the host *S. sacchari.*

Considering the fact that four of the five *A. flavus* isolates from naturally infected *O. arenosella* (leaf-eating caterpillar) and *S. typica* (lace bug) were not aflatoxigenic and still showed high level of pathogenicity in laboratory bioassays, it is possible that the conclusion drawn by Drummond & Pinnock (1990) may also be true in the case of above mentioned pests of coconut also.

Conclusion

Two (designated as AF 2a and PTM 1) out of seven entomopathogenic *A. flavus* isolates showed a distinct AT-producing ability in that they produced AT-related blue fluorescence throughout the 30-day culture period and the characteristic bright yellow pigmentation was associated with AT-positive *A. flavus* isolates. The CCMPA medium reported in this paper permits one to easily and rapidly detect AT synthesis by direct observation of blue fluorescent zones of AT surrounding the fungal colonies. The ability of *A. flavus* isolates to produce AT was not necessary for the entomopathogenic activity of these fungi against the coconut pests – *O. arenosella* and *S. typica.*

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References

Al-Adil, K.M., Kilgore, W.W. & Painter, R.R. 1972 Toxicity and sterilization effectiveness of aflatoxin B_1 and G_1 and distribution of aflatoxin B_1-C^{14} in houseflies. *Journal of Economic Entomology* **65**, 375–378.

Ambethgar, V. & Kumaran, K. 1998 Biological suppression of grasshoppers. *Science Express Supplement, The Indian Express*, Tuesday, November 17, 4 pp.

Amonkar, S.V. & Nair, K.K. 1965 Pathogenicity of *Aspergillus flavus* Link to *Musca domestica nebulo* Fabricius. *Journal of Invertebrate Pathology* **7**, 513–514.

Arseculeratne, S.N., De Silva, L.M., Wijesundera, S. & Bandunatha, C.H.S.R. 1969 Coconut as a medium for the experimental production of aflatoxin. *Applied Microbiology* **18**, 88–94.

Bhatnagar, D., Ehrlich, K.C. & Cleveland, T.E. 1992 Oxidation – reduction reactions in biosynthesis of secondary metabolites. In *Handbook of Applied Mycology vol. 5 Mycotoxins in Ecological Systems*, eds. Bhatnagar, D., Lillehoj, E.B. & Arora, D.K. pp. 255–286. New York: Marcel Dekker. ISBN 0-82478551-7.

Bothast, R.J. & Fennell, D.I. 1974 A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. *Mycologia* **66**, 365–369.

Cockbain, A.J. & Hastie, A.C. 1961 Susceptibility of the bed bug, *Cimex lectularius* Linnaeus, to *Aspergillus flavus* Link. *Journal of Insect Pathology* **3**, 93–102.

Cotty, P.J. 1994 Method for the control or prevention of aflatoxin contamination using a non-toxigenic strain of *Aspergillus flavus. US Patent 5, 294, 442.* March 5.

Davis, N.D., Iyer, S.K. & Diener, U.L. 1987 Improved method of screening for aflatoxin with a coconut agar medium. *Applied and Environmental Microbiology* **53**, 1593–1595.

De Vogel, P., Van Rhee, R. & Blanche-koelensmid, W.A.A. 1965 A rapid screening test for aflatoxin-synthesizing aspergilli of the flavus-oryzae group. *Journal of Applied Bacteriology* **28**, 213–220.

Detroy, R.W., Lillehoj, E.B. & Ciegler, A. 1971 Aflatoxin and related compounds. pp. 3–178. In *Microbial Toxins*, vol. VI, eds. Ciegler, A., Kadis, S. & Ajl, S.J. 563. p. New York: Academic Press. ISBN 0-12-046506-X.

Diener, U.L. & Davis, N.D. 1966 Aflatoxin production by isolates of *Aspergillus flavus. Phytopathology* **56**, 1390–1393.

Diener, U.L. & Davis, N.D. 1983 Aflatoxins in corn. In *Xenobiotics in Foods and Feeds*, Symposium Series 234, eds. Finley, J.W. & Schwass, D.E. pp. 242–269. Washington DC: American Chemical Society. ISBN 0-84120809-3.

Donkersloot, J.A., Mateles, R.I. & Yang, S.S. 1972 Averufin production by a mutant of *Aspergillus parasiticus* impaired in aflatoxin

biosynthesis. *Biochemical and Biophysical Research Communication* **47**, 1051.

Dorner, J.W., Cole, R.J. & Blankenship, P.D. 1992 Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. *Journal of Food Protection* **55**, 888–892.

Drummond, J. & Pinnock, D.E. 1990 Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus*. *Journal of Invertebrate Pathology* **55**, 332–336.

Fang, H.M. & Tan, S.M. 1986 Pathogenic fungi of several insect pests on sugarcane. *Microbiology* **13**, 97–100.

Gaston, J.Q. & Llewellyn, G.C. 1980 Sex-determined differential mortality of milkweed bugs, *Oncopeltus fasciatus* (Hemiptera), to aflatoxins. *Journal of Invertebrate Pathology* **35**, 195–199.

Gilliam, M., Spangler, H.G., Prest, D.B. & Lorenz, B.J. 1990 Mycosis of a queen harvester ant, *Pogonomyrmex rugosus*. *Journal of Invertebrate Pathology* **55**, 437–438.

Golumbic, C. & Kulik, M. 1969 Fungal spoilage on stored crops and its control. In *Aflatoxin: Scientific Background, Control, and Implications*, ed. Goldblatt, L.A. pp. 307–327. New York: Academic Press. ISBN 0-12-288350-0.

Gopal, Murali, Gupta, Alka, Sathiamma, B., Mohan, Chandrika, Nair, K.R.C. & Soniya, V.P. 2000a A fungal pathogen of lace bug and leaf eating caterpillar, two insect pests of coconut palm. *Coconut Research and Development (CORD)* **16**, 49–59.

Gopal, Murali, Gupta, Alka, Nair, K.R.C., Sathiamma, B. & Nair, C.P.R. 2000b Studies on cross-infectivity with entomofungal isolates of *Aspergillus flavus* Link. against *Stephanitis typica* D. and *Opisina arenosella* W., two pests of coconut. *Indian Coconut Journal* **31**, 12–15.

Hara, S., Fennel, D.I. & Hesseltine, C.W. 1974 Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. *Applied Microbiology* **27**, 1118–1123.

Heathcote, J.G. 1984 Aflatoxins and related toxins. In *Mycotoxins – Production, Isolation, Separation and Purification. Developments in Food Science* 8, ed. Betina, V. pp. 90–130. The Netherlands: Elsevier. ISBN 0-44442289-7.

Igbinosa, I.B. 1992 Field and laboratory techniques for assessing infestations of the nettle caterpillar, *Latoia viridissima* Holland (Lepidoptera : Limacodidae). *Insect Science and its Application* **13**, 389–398.

Joshi, K.C. & Agarwal, S.B. 1987 Bionomics of the tent caterpillar, *Malacosoma indica* Wlk. *Bulletin of Entomology* **28**, 1–11.

Kalia, Shamila, Harsh, N.S.K. & Joshi, K.C. 1996 Pathogenic fungus of *Noorda blitealis* Walker (Lepidoptera – Pyralidae), a major pest of *Moringa oleifera* Tanss. *Indian Journal of Forestry* **19**, 94–96.

Klich, M.A. & Pitt, J.I. 1988 Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Transactions of the British Mycological Society* **91**, 99–108.

Kshemkalyani, S.B., Enliley, M.R., Hakim, S.S.Y. & Prabhakar, J.D. 1989 Pathogenic fungus of *Dysdercus cingulatus*. *Indian Journal of Entomology* **51**, 322–324.

Lemke, P.A., Davis, N.D. & Creech, G.W. 1989 Direct visual detection of aflatoxin synthesis by minicolonies of *Aspergillus* species. *Applied and Environmental Microbiology* **55**, 1808–1810.

Lemke, P.A., Davis, N.D., Iyer, S.K., Creech, G.W. & Diener, U.L. 1988 Fluorometric analysis of iodinated aflatoxigenic minicultures of *Aspergillus flavus* and *Aspergillus parasiticus*. *Journal of Industrial Microbiology* **3**, 119–125.

Lin, M.T. & Dianese, J.C. 1976 A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology* **66**, 1466–1469.

Lin, M.T., Hsieh, D.P.H., Yao, R.C. & Donkersloot, J.A. 1973 Conversion of averufin into aflatoxin by *Aspergillus parasiticus*. *Biochemistry* **12**, 5167–5171.

Martinez, A.M. & Bravo, N. 1989 *Aspergillus flavus* Dirk., control natural de diferentes especies de chinches harinosas. *Revista de Proteccion Vegetal* **4**, 83–84.

Mathew, G. & Mohamed Ali, M.I. 1987 Microbial pathogens causing mortality in the carpenter worm, *Cossus cadambae* Moore (Lepidoptera, Cossidae), a pest of teak (*Tectona grandis* Lin. f.) in Kerala (India). *Journal of Tropical Forestry* **3**, 349–351.

Miller, D.M. & Wilson, D.M. 1994 Veterinary diseases related to aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance*, eds. Eaton, D.L. & Groopman, J.D. pp. 347–364. San Diego: Academic Press. 0-12-228255-8.

Moore, J.H., Hammond, A.M. & Llewellyn, G.C. 1978 Chemosterilant and insecticidal activity of mixed aflatoxins against *Anthonomus grandis* (Coleoptera). *Journal of Invertebrate Pathology* **31**, 365–367.

Muthukrishnan, P. & Rangarajan, M. 1974 Laboratory studies on the control of black headed caterpillar, *Nephantis serinopa* Meyr. by microorganisms. *Labdev Journal of Science and Technology* **B 12**, 106–108.

Nurzhanov, A.A. and Lachininskii, A.V. 1987. Entomopathogenic microorganisms of locusts in Uzbekistan. In *Saranchovye-ekologiya i mery bor'by*, ed. Nikitina, M.S. pp. 62–69. Leningrad: All-Union Scientific-Research Institute of Plant Protection.

Oblisamy, G., Ramamoorthi, K. & Rangaswamy, G. 1969 Studies on the pathology of some crop pests of South India. *Mysore Journal of Agricultural Sciences* **3**, 86–98.

Pallavi, R.M.V., Ramana, D. & Sashidhar, R.B. 1997 A simple test tube screening method for identifying aflatoxin-producing strains of *Aspergillus* sp. using coconut milk agar medium. *World Journal of Microbiology and Biotechnology* **13**, 713–714.

Patil, C.S. 1989 New record of fungal pathogen *Aspergillus flavus* Link on mulberry silkworm *Bombyx mori* L. from India. *Current Science* **58**, 683.

Ponnamma, K.N., Gopal, M. & Sharmila, S. 2000 Record of *Aspergillus flavus* (Link) on the plant hopper, *Proutista moesta* Westwood (Hemiptera: Derbidae). *Journal of Plantation Crops* **28**, 231–232.

Rajagopal, D. & Trivedi, T.P. 1989 Status, bio-ecology and management of epilachna beetle, *Epilachna vigintioctopunctata* (Fab.) (Coleoptera: Coccinellidae) on potato in India: a review. *Tropical Pest Management* **35**, 410–413.

Sathiamma, B. & Saraswathy, N. 1990 Mycosis on tea mosquito *Helopeltis antonii* S. *Indian Journal of Entomology* **52**, 516.

Squira, R.A. 1989 Ranking animal carcinogens: a proposed regulatory approach. *Science* **214**, 887–891.

Srivastava, R.P. & Tandon, P.L. 1980 Studies of insect pathogens on mango leaf webber, *Orthaga euadrusalis* Walker (Lepidoptera: Pyralidae). *Entomon* **5**, 219–221.

Steinhaus, E.A. & Marsh, G.A. 1967 Previously unreported accessions for diagnosis and new record. *Journal of Invertebrate Pathology* **9**, 436–438.

Wicklow, D.T. & Dowd, P.F. 1989 Entomotoxigenic potential of wild and domesticated yellow-green aspergilli: toxicity to corn earworm and fall armyworm larvae. *Mycologia* **81**, 561–566.

Wicklow, D.T., Dowd, P.F., Tepaske, M.R. & Glober, J.B. 1988 Sclerotial metabolites of *Aspergillus flavus* toxic to a detritivorous maize insect (*Carpophilus hemipterus*, Nitidulidae). *Transactions of the British Mycological Society* **91**, 433–438.

Wiseman, H.G., Jacobson, W.C. & Harmeyer, W.C. 1967 Note on removal of pigments from chloroform extracts of aflatoxic cultures with copper carbonate. *Journal of Association of Official Analytical Chemists* **50**, 982–983.