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Morphological and toxigenic study of *Aspergillus flavus* from indoor air

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Abstract

Aim : In the current investigation thirty four *A. flavus* cultures were isolated from indoor air samples of some farmers' house in coastal region of Odisha. Production of aflatoxin B1 and the pattern of formation of sclerotia by the isolates were studied *in vitro*.

Methodology : Morphological study and production of sclerotia were carried out by observing fungal colonies on culture media. Conidiophores were identified under Phase contrast microscope. Detection of aflatoxin B1 was done by Thin Layer Chromatography technique.

Results : The isolates exhibited variation in their colony characteristics and pattern of sclerotia formation. Overall 44% *A. flavus* isolates produced stable aflatoxin B1 and 41% were producers of sclerotia. About 32% *A. flavus* isolates produced both sclerotia and aflatoxin B1. Most of the non toxigenic isolates failed to form thick mycelia mat on β -CD-PDA media. Aflatoxin and sclerotia producing isolates were found more in coastal districts than inter-coastal regions. No significant variation in the pattern of sclerotia production was observed among isolates in less aerobic (sealed plates) and aerobic (unsealed plates) condition, however isolates had slightly more preference towards less aerobic environment for sclerotia production. The size of sclerotia was found to be inversely related to number of sclerotia.

Aspergillus flavus isolated from indoor air of farmers' house in coastal region of Odisha

44% were aflatoxigenic, 41% were producers of sclerotia and 32% produced both

Non toxigenic isolates failed to form thick mycelial mat on β -CD-PDA media.
Isolates preferred towards less aerobic environment for sclerotia production

The size of sclerotia was
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Presence of diverse *A. flavus* strains (toxigenic and sclerotic) in the farmers' indoor air was detected in the current study. Need for suitable management strategies has been put forward

Interpretation : The presence of diverse *A. flavus* strains in farmers' indoor air, having the ability to produce either aflatoxin or sclerotia or both, may lead to serious health implications. This issue need to be addressed and a suitable way for proper management of indoor environment of farmers' need to be designed.

Key words: *Aspergillus flavus*, Aflatoxin, β -Cyclodextrine, Indoor air

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Introduction

Aspergillus flavus (*A. flavus*) is ubiquitous fungus commonly occurring in soil, water, decaying vegetation and in indoor air (Ghosh *et al.*, 1997; Gorny *et al.*, 2002; Nielsen, 2003; Adhikari *et al.*, 2004; Khan and Karuppaiyil, 2012; Lai *et al.*, 2015). Inhalation of conidia and ingestion of *A. flavus* contaminated food is thought to be the cause of acquiring invasive aspergillosis (Balasubramanian *et al.*, 2012; Fakruddin *et al.*, 2015). This fungus also infects and produces aflatoxins in a wide variety of agricultural commodities such as maize, corn, vegetables, spices, chilies, sorghum, groundnut and other oil seeds and rice (Reddy *et al.*, 2009; Nayak *et al.*, 2014; Oyewale, 2015; Bala *et al.*, 2016). Since the infamous episode of Turkey-X disease in the United Kingdom in 1960, aflatoxins have been reported to be carcinogenic, mutagenic and immunosuppressive agent. Among the mycotoxins, aflatoxin B1 has been classified as group IA carcinogen by International Agency for Research on Cancer (IARC, 2002; Amaike and Keller, 2011).

Though, *A. flavus* is strictly a mitotic fungus, besides producing conidia asexually it also produces specialized resistant structures known as sclerotia. Sclerotia are pigmented, compact aggregates of specialized hyphae which are vestige of cleistothecia incapable of ascospore production. These can resist unfavorable environmental conditions and are capable of remaining dormant for long periods (Cotty, 1988; Horn *et al.*, 2016). Sclerotia are important survival structures in the life cycle of many fungi, stored with reserve food materials and sometimes detached and remain dormant until favorable conditions occur (McAlpin, 2001). *A. flavus* isolates that produce sclerotia can be divided into two types of strains, L and S on the basis of morphological, physiological and genetic criteria (Bayman and Cotty, 1991; Cotty, 1989). 'S' strain isolates produce numerous small sclerotia (<400µm in diameter) and fewer conidia than 'L' strain isolates. Typical isolates (L) of *A. flavus* may produce only B aflatoxins or no aflatoxins at all and all S strain isolates (atypical isolates by Saito *et al.*, 1986) reported to date produce large quantities of aflatoxins. Some strains produce only B aflatoxins and others produce both B and G aflatoxins. Formation of sclerotia by *A. flavus* has been linked positively, as well negatively with aflatoxin production.

The climate of Odisha is characterized by higher rainfall, temperature and humidity which form a favourable condition for growth of storage fungi. The poor farmers in this region usually keep harvested paddy inside their house due to lack of separate storage facility. Paddy associated fungi like *A. flavus* and its fragments may contaminate the indoor air in this situation. Continuous exposure of the inhabitants to these conditions make them prone to inhale fungal mycelial fragments, conidia, sclerotia, fungal metabolites, mycotoxins like aflatoxins etc. which act as indoor air biocontaminants (Gorny *et al.*, 2002), a condition similar to 'sick building syndrome'. Numerous studies have shown that exposure to fungi may be associated with acute toxic effects, allergies and asthma (Khan and Karuppaiyil, 2012).

There seems to be no reports available on the presence of *Aspergillus flavus* in the indoor air of farmers' house at coastal region of Odisha, hence the present study was undertaken to isolate *Aspergillus flavus* strains from air samples of farmers' indoor environment of coastal region of Odisha and to study the pattern of sclerotia and aflatoxin B1 production.

Materials and Methods

Isolation, identification and study of culture characters of *A. flavus* isolates: Thirty four *A. flavus* isolates (ID A-1 to A-34) were taken for analysis in the current study. The *A. flavus* cultures were isolated by passive air sampling method with settle culture plates. Potato Dextrose Agar (PDA) plates were exposed for 1 min to the open respirable indoor air of farmer's house from five coastal districts of the state of Odisha, India such as Balasore, Dhenkanal, Puri, Nayagarh and Khurda. Plates were then incubated at 28°C for 3 days and the fungal colonies were isolated on PDA plates. *A. flavus* cultures were identified by observing the colour of mycelia on culture plate having typical lime green or yellow green colour of mycelia as described by Fakruddin *et al.* (2015). Identification of *A. flavus* isolates was further confirmed by observing the typical structural arrangement of conidiophores under microscope (Olympus) as described by Koh and Tseng (1975). All fungal isolates were preserved at 4°C on PDA slants in the Plant Pathology Division of National Rice Research institute, Cuttack, India. The culture characters of isolates (colour of colony front and back), formation of sclerotia and production aflatoxin were further observed by growing isolates on Czapekdox Agar media (CZA) and β-Cyclodextrine Potato Dextrose Agar (β-CD-PDA) medium. The composition of CZA was constituted of 30 g Sucrose, 3 g NaNO₃, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 1g K₂HPO₄, 15 g Agar and 1000 ml distilled water (final pH 6.2 ± 0.2). Colour of isolates was observed by comparing with RHS colour chart (The Royal Horticultural Society, London: Flower Council of Holland, Leiden).

Detection of aflatoxin B1 by Thin Layer Chromatography: To detect the production of aflatoxin B1, isolates were grown in PD and CZ broth for seven days. The mycelial mat thus formed was harvested and blot dried and sclerotia (if any) were separated by using a small sterilized paint brush. Mycelia was ground with extraction buffer (60% Methanol with 0.5% KCl) and then centrifuged at 12000rpm for 10 min at 40C. The supernatant was used for detection of aflatoxin B1 by Thin Layer Chromatography method. Silica gel G (Hi-media) (20g) mixed with 40 ml of distilled water and applied manually on a clean glass plate (22×21cm) to make a thin and uniform gel. After air drying properly it was kept inside oven at 121°C for one hour and then cooled at room temperature. A 20µl each of standard aflatoxin B1 and sample was spotted at 4cm from the bottom of the plate. The plate was developed with 100ml of developing solvent containing chloroform to acetone at 90:10 proportions. After development, the plate was dried and spots were visualised and documented under 360nm on an UV Trans Illuminator (WEALTEC).

Study of vegetative growth and sclerotia production: To study the sclerotia characteristics, isolates were cultured on Czapekdox Agar media. After inoculation, isolates were grown in three types of culture environments. One set of culture plates were sealed tightly with paraffin film strips making a less aerobic or anaerobic like environment and another set was kept as such, thereby growing in aerobic environment. One set of cultures were also grown in CZA slants with cotton plugging. Periodical observations were taken for colony diameter, sclerotia number, colour, texture, shape and microscopic size. Variations in vegetative growth and sclerotia production pattern were studied from data of Balanced Analysis of Variance (ANOVA) using CROPSTAT Version 3.0 software. Correlations between different parameters were determined using DATA ANALYSIS option from Microsoft Excel sheet.

Results and Discussion

The presence of allergenic *Aspergillus flavus* in indoor air of many areas of India including rice grain handling areas have been reported (Adhikari *et al.*, 2004; Ghosh *et al.*, 1997). Evancho *et al.* (2001) discussed about the sampling of indoor air in food processing areas. Hence, in the current investigation thirty four *A. flavus* cultures A-1 to A-34 were isolated from the indoor air of farmers' house from five coastal districts of Odisha. Production of aflatoxin and sclerotia by these *A. flavus* cultures (A-1 to A-34) was investigated. The isolated *A. flavus* cultures had typical yellow green or lime green colony with typical conidiophores structures. The culture colour 'on surface' of all the isolates were recorded to have RHS no. 146A, 147A & 152A on CZA and 146A, 148A & 152A on β -CD-PDA indicating various shades of yellow-green colour. However, reasonable variations were observed in the colour of colony reverse (observed at petridish bottom) in both the culture media, which was actually the colour of mycelia mat formed by the isolates. The reverse of colony colour in most of the isolates varied between shades of yellow orange and grayed orange group on CZA. Mycelia mat was not formed by 50% of the isolates on β -CD-PDA media, hence the reverse back was noted as 'colourless'.

Variation in the yellow colouration of mycelia mat (back of the colony) might be due to large number of secondary metabolites produced by *A. flavus* cultures. Lin and Dianese (1976) first linked this yellow pigmentation with the production of aflatoxins which are also secondary metabolites. These bright yellow pigments have been known as anthraquinone pigments which are associated with the aflatoxin biosynthetic pathway in *Aspergillus* spp. (Shier *et al.*, 2005). Abbas (2011) also found many indoor toxigenic *A. flavus* strains producing yellow pigments. However, some reports have delimited the linkage of yellow pigmentation with the production of aflatoxins where some media were identified in which semi quantitative differences were observed (Davis *et al.*, 1987; Gupta and Gopal, 2002; Pitt and Hocking, 2009). In the current study no such correlation could be firmly established, but generally it could be observed that most of the non-toxicogenic isolates failed to form mycelia mat on β -CD-PDA media.

Sclerotia formation was observed in 41% of the isolates on CZA media and in 21% on β -CD-PDA media. Production of sclerotia in the strain A28 isolated from Balasore district was much higher than other isolates on β -CD-PDA (Table 1). Nearly half of the isolates showed the ability to produce sclerotia in either of the media but CZA media proved to be containing better substrates for this purpose. This might be due to the optimal balance of carbon and nitrogen in CZA along with sodium nitrate as an excellent source of nitrogen (Nesci *et al.*, 2006; McAlpin, 2004).

As far as toxigenic potential is concerned, fifty percent of the *A. flavus* isolates were found to have the ability to produce aflatoxin B1 when both media were considered. Only two isolates, A11 and A28 (5%) were detected to produce aflatoxin B1 on CZ broth by TLC method whereas 41% isolates produced aflatoxin B1 on β -CD-PD broth (Table 1). Most of the isolates which were unable to form mycelia mat on β -CD-PDA medium were also unable to produce sclerotia and aflatoxin B1.

Production of aflatoxins by *A. flavus* isolates could be detected better in β -CD-PD broth than CZ broth. The β -cyclo dextrines present in this medium have excellent cavities for exciting the fluorescence responses of aflatoxins B1 and G1 through the formation of inclusion complexes. Addition of this complex to a suitable agar medium enhances detection of aflatoxin production by *A. flavus* and *A. parasiticus* by blue fluorescence under 365-nm light (Fente *et al.*, 2001; Galaverna *et al.*, 2008). Indoor air of farmers' house of Dhenkanal district was devoid of sclerotia as well as aflatoxin B1 producing *A. flavus* and less numbers of such isolates were also obtained from Puri district. However, *A. flavus* isolates from indoor air of farmers' house in Balasore district were prolific producers of both sclerotia and aflatoxin B1, with A28 being the most aggressive one. Dhenkanal district is almost 100km away from sea coast, hence seems to be an inter-coastal region. On the other hand, Balasore is a coastal district with high humidity, temperature and receive long periods of rain, making it a congenial environment for fungal growth and production of secondary metabolites like aflatoxins.

Overall, 44% *A. flavus* isolates were found to be aflatoxigenic and 41% were producers of sclerotia when both media are considered. Among the isolates which produced aflatoxin B1, 73% of isolates produced sclerotia whereas 78% of sclerotia producers also produced aflatoxin B1. Out of the total number (34) of *A. flavus* isolates, 32% produced both sclerotia and aflatoxin. All the sclerotia producing isolates were found to be L-type strains *i.e.*, the size of the sclerotia were more than 400 μ m (Table 3).

Sclerotia development and aflatoxin biosynthesis have been found to be closely related in previous studies. Increased production of the aflatoxin precursors is associated with progressive decrease in sclerotial size, alteration in sclerotial shape and weakening in the sclerotial structure (Chang *et al.*, 2002). Duran *et al.* (2007) reported that production of aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene also necessary for

Table 1: Culture characters, sclerotia and toxin producing ability on CZA and β -CD-PDA media of *A. flavus* cultures isolated from indoor air of farmers' house

Isolate ID	*Culture colour on CZA media		Culture physiology on CZ broth		*Culture colour on β -CD-PDA media		Culture physiology on β -CD-PD broth	
	On surface	Petri dish bottom (mycelia mat)	** Sclerotia	Afla toxin	On surface	Petridish bottom (mycelia mat)	*** Sclerotia	** Afla toxin
A1	146A	20A	-	-	148A	colorless	-	-
A2	146A	18A	-	-	148A	colourless	-	+
A3	146A	20A	-	-	146A	colourless	-	-
A4	146A	20A	-	-	148A	colourless	-	-
A5	146A	19A	-	-	146A	colourless	-	-
A6	146A	20A	-	-	148A	colourless	-	-
A7	146A	20A	-	-	146A	colourless	-	-
A8	146A	20A	-	-	146A	colourless	-	-
A9	146A	21B	-	-	146A	colourless	-	-
A10	147A	20A	-	-	146A	colourless	-	-
A11	147A	22A	-	+	146A	160B	-	-
A12	146A	20B	-	-	146A	colourless	-	-
A13	146A	18B	+	-	146A	colourless	-	-
A14	146A	21B	+	-	146A	17B	+	++
A15	146A	20A	+	-	146A	17A	-	++
A16	146A	18A	+	-	146A	17C	+	++
A17	146A	18A	+	-	146A	colourless	-	-
A18	152A	19A	-	-	146A	colourless	-	-
A19	146A	18A	-	-	146A	12C	-	-
A20	152A	19A	+	-	146A	17B	+	++
A21	152A	19A	+	-	146A	17A	+	++
A22	152A	163B	-	-	152A	12B	-	-
A23	152A	163B	+	-	146A	17A	+	++
A24	146A	20A	-	-	146A	12C	-	-
A25	146A	19A	+	-	146A	17B	+	+++
A26	146A	18A	+	-	146A	colourless	-	++
A27	146A	164C	+	-	146A	12C	-	-
A28	152A	18B	+	+	152A	164B	++++	++++
A29	146A	18B	-	-	146A	colourless	-	-
A30	146A	164	+	-	146A	12C	-	++
A31	146A	164C	+	-	146A	12C	-	++
A32	146A	164B	-	-	146A	colourless	-	++
A33	146A	164C	-	-	146A	12C	-	-
A34	146A	164C	-	-	146A	17C	-	+

Colourless: No mycelia mat formed by the isolate on petridish bottom. *Culture colour as per RHS colour chart as follows: 14 to 23 yellow orange group; 144 to 154 is yellow green group; 160 greyed yellow; 163 to 165 greyed orange group. A, B, C and D etc along with RHS no. indicate the various shades

** Sclerotia of L type

***Quantity of aflatoxin: (-) No toxin; (+) Less than $2\mu\text{g ml}^{-1}$; (++) $2\mu\text{g ml}^{-1}$ to $9\mu\text{g ml}^{-1}$; (+++) $10\mu\text{g ml}^{-1}$ to $25\mu\text{g ml}^{-1}$; (+++++) More than $25\mu\text{g ml}^{-1}$

sclerotial formation. However, not all isolates of *A. parasiticus* and *A. flavus* which produce aflatoxins have the ability to produce sclerotia. The results of many investigations suggested that sclerotia formation was poorly related to aflatoxin production (Razzaghi-Abyaneh *et al.*, 2006). Some of clinical *A. flavus* isolates could have aflatoxin and sclerotia producing ability, but not necessarily all aflatoxigenic *A. flavus* isolates were capable of producing sclerotia (Dehghan *et al.*, 2008). Singh *et al.* (2015) also observed no association between morphological-diversity

and toxigenic potential of *A. flavus* isolates from pea nut. Though it is generally agreed upon that aflatoxin biosynthesis and sclerotial development in *A. flavus* and *A. parasiticus* are related, the relationship between these processes is yet to be defined. In the present study such correlation was observed on a higher percentage in indoor *A. flavus* isolates. However, more vigorous study is required to establish a significant correlation, might be more than 90%. Even if all isolates in the current study were of L-strains, higher concentrations of aflatoxin could be

Table 2: Comparison of sclerotia production by *A. flavus* isolates in sealed and unsealed plates and slants

Isolate	Sclerotia formed Per cm ²			Mean sclerotia no. per isolate
	Sealed	Unsealed	Slant	
A13	0	22	15	12
A27	32	9	16	19
A17	25	18	20	21
A26	38	39	12	30
A31	45	40	12	32
A30	51	42	10	34
A16	76	45	17	46
A15	72	70	22	55
A14	77	65	30	57
A25	60	93	18	57
A21	91	73	11	58
A23	95	82	12	63
A20	102	106	11	73
A28	141	138	70	116
Mean sclerotia no in each environment	65	60	20	

LSD: Least Significance Difference. *Significant at $p < 0.05$; LSD for mean sclerotia no. per isolate: 14.18; LSD for mean sclerotia no. in each; environment : 7.09 and LSD for isolate and Sclerotia no. : 24.5

detected in culture broth and sclerotia. In general isolates showed little more preference to less aerobic environment over aerobic environment and least preference to slant environment to produce sclerotia though there was isolate specific preferences (Table 2). On an average isolates produced 65, 60 and 20 numbers of sclerotia cm⁻² in less aerobic, aerobic and slant environment respectively (LSD 7.09). When all three environments are taken into consideration, isolates showed variable ability to produce sclerotia

(LSD 14.18) and the number of sclerotia produced ranged from 12 numbers cm⁻² by A13 to 116 numbers cm⁻² by A28. A13 didn't produce any sclerotia in less aerobic environment. Overall, the sizes of the sclerotia were found to be inversely related to the number of sclerotia produced by isolates in all the three environments depicted by r value of -0.49 (Fig 1). Accordingly, the average sizes of sclerotia were 675.8 μ m, 744.8 μ m and 810.6 μ m in sealed, unsealed and slants, respectively, which

Fig 1: Overall comparison between sclerotia number and size produced by *A. flavus* isolates in sealed and unsealed plates and slants

Table 3: Comparison of sclerotia size (diameter in μm) produced by isolates of *A. flavus* in sealed and unsealed plates and slants

Isolate	Diameter of sclerotia (μm)			Mean sclerotia size. per isolate
	Sealed	Unsealed	Slant	
A28	566.66	577	429	524.2
A14	735.33	715	656	702.1
A31	681.6	685	750.6	705.7
A20	714.33	672.33	776.66	721.1
A16	713	782.66	749.66	748.4
A17	718.66	740.33	841	766.6
A15	750	721.33	843.66	771.6
A21	720.33	756.33	848.66	775.1
A25	778	759	811.66	782.9
A27	679.33	676.66	1011	789.0
A30	780	791	801.5	793.8
A26	867	882.66	685.66	811.7
A23	758	725.66	1109.67	864.4
A13	0	943.66	1025.33	984.95
Mean Sclerotia size in each environment	675.8	744.8	810.6	

LSD: Least Significance Difference. * Significant at $p < 0.05$; LSD for mean sclerotia size per isolate: 66.08; LSD for mean sclerotia size in each environment: 33.04 and LSD for isolate and Sclerotia size: 114.46

depicted significant variation with a LSD value of 33.04. Isolate A13 produced the largest size sclerotia ($984.95\mu\text{m}$) and A28 had the smallest sized sclerotia ($524.2\mu\text{m}$). The sclerotia size of rest of the isolates ranged between $702.1\mu\text{m}$ to $793.8\mu\text{m}$ (LSD at $p < 0.05$ was 66.08), except isolate A23 and A26 which had sclerotia size $811.7\mu\text{m}$ and $864.4\mu\text{m}$ respectively.

Reports suggested that sclerotia production may partly act as defense mechanism against oxidative stress (Chang, 2009). Physiologically sclerotia differentiation was mainly inhibited by the increase in intracellular H_2O_2 concentration (Grintzalis et al., 2014). Usually, it has been observed that lowering the oxygen concentration results in inhibition or suppression of sclerotia or cleistothecia formation in fungal species that naturally produce them during their life cycle (Rai et al., 1967; Barkai-Golan, 2001). Pathogenic species like *A. flavus* are capable of growing at low concentration of oxygen, but not completely in anaerobic environment. The geography, environment and climate of coastal region of Odisha is favorable for fungal growth and proliferation, including *A. flavus*. The presence of reasonable population of such isolates having the ability to produce both aflatoxin and sclerotia at the same time puts forward a serious issue to be addressed in this region to avoid the so called "Sick building", as observed in some part of the globe like in America and Europe. Designing of suitable management practice of this fungus by eco-friendly approach needs further research.

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