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ISOLATION AND IDENTIFICATION OF AN AFLATOXIN-PRODUCTING STRAIN OF *ASPERGILLUS FLAVUS* GROUP FROM STORED RICE[1,2]

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Abstract

Four *Aspergillus flavus* strains were isolated from local stored rice and one of which, *Aspergillus flavus* 13, was able to produce aflatoxin B_1 and B_2 compounds. The aflatoxin producing strain possessed the following significant characters: persistent green colony, biseriate, yellowish green and prominently echinulate conidia, black exudate, and rapid growth on Czapek solution agar.

Introduction

Numerous workers have reported that aflatoxins were mainly produced by *Aspergillus flavus* (Lancaster *et al.*, 1961; Carnaghan *et al.*, 1964; Ling *et al.*, 1967). According to Raper and Fennel (1965), the fungus *A. flavus* is a group of species that includes 11 species or subspecies. The name *A. flavus* is used to refer this group and also indicates that most of the individual species within this group are not sharply delimited from one another but have integrating forms. Among them, *A. parasiticus* Speare is evidently a more potent aflatoxin-producing strain than *A. flavus* Link. Ling *et al.* (1967) have made a thorough survey of local produced rice and the presence of aflatoxin in rice with the occurrence of *A. flavus*. Murakami *et al.* (1965) have made a study of the morphological characters of aflatoxin producing strains. Recently, Boller and Schroeder (1974) have investigated the growth conditions and the aflatoxin producing ability of *A. flavus* and *A. parasiticus* in rice. This study was dealing with the morphological characters and the aflatoxin-producing ability of *A. flavus* group which was obtained from rice samples as compared with

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the reference fungi in our laboratory.

Materials and Methods

Isolation and identification of ***Aspergillus flavus*** *group*

Rice samples were obtained from a store house nearby Taipei, which was kept at 28°C and relative humidity of 85–90%. The rice samples were stored for over 6 months. Five grams of rice grains were first polished and mixed with 10 ml of steriled water. They were shaken by a rotary shaker for 10 min. Few drops of the suspension were then added to melted Czapek solution agar plates. The plates were swirled to allow even distribution of the suspension in agar and then incubated at 28°C for 10 days. Twenty five ppm of Rose Bengal and 50 ppm of Streptomycin was added to Czapek agar prior to plating. After 10 days, the *Aspergillus*-like fungi were picked and examined under light microscope. The identification of the fungi were mainly based on morphological characters such as conidial heads, conidiophore, vesicles, sterigmata and color of colonies (Raper and Fennel, 1965). For measurement of the fruiting structures, due to great variability, at least ten measurements were taken for each fruiting structures, and the figures were taken from their averages. *A. flavus* Link, *A. flavus* 041, *A. parasiticus*, *A. tamari* Kita, and *A. oryzae* Ahlburg (Cohn) were used as reference fungi. All of the fungi were kindly obtained from Provincial Industrial Research Institute (Taiwan, Republic of China) except *A. flavus* 041 from Professor Kou-Huang Ling, Institute of Biochemistry, College of Medicine, National Taiwan University.

Extraction of aflatoxin compounds

The identified *A. flavus* strains were inoculated onto modified Czapek liguid rice medium (Ling *et al*, 1967) which consisted of 2g of NH_4NO_3; 0.5g KCl; 0.5g $MgSO_4 \cdot 7H_2O$; 0.01g $FeSO_4 \cdot 7H$ O; 0.01g $ZnSO_4$; 1g KH_2PO_4; 35g rice powder and 50g glucose in 1 liter of distilled water. An 100 ml flask contained 150 ml of the medium was inoculated and incubated at 28°C for 12 days. The mycelia sheet from each of the 150 ml medium was suspended in 60 ml of *n*-hexane and homogenizer with waring blendor (Virtis 45) for 15 min at medium speed. The homogenate was filtered by suction filtration and the residue was air-dried. Ten grams of the residue were mixed with 30 ml of *n*-hexane and 50 ml of 55% methanol in water. The mixture was then shaken by a rotary shaker for 30 min. After shaking, the filtrate was obtained by using a suction filtration. The aqueous methanol layer was separated by a separatory funnel and the hexane layer was removed. The aqueous methanol layer was washed with chloroform three times and then washed once with distilled water, and the final chloroform extract was concentrated to 10 ml.

Detection of aflatoxin compounds

Fifty microliters of the chloroform extract was applied on a thin-layer chromatoplate (Wako gel B5, activated at 150°C), and the plate was developed with a solvent of 2% methanolic chlorform. After 1 hr, when the desired distance (ca. 13 cm had been travelled by the solvent front, the plate was removed from the developing chamber, and air dried before observation under an Ultra-violet light (2537 Å). R_f values of blue and green fluroscent spots were determined. Aflatoxins (B_1, B_2, and G_1) which purchased from Calbiochem (San Diego, Calif. 92112, U.S.A.) were used as standard compounds.

Results

Although a number of *Aspergillus*-like fungi were isolated from the samples tested, only four *A. flavus* strains were classified. They are *A. flavus* 11, *A. flavus* 13, *A. flavus* 18L, and *A. flavus* 18D. Generally, the colonies of the species belonging to *A. flavus* group are green color with yellow shades. The texture are usually compact and velvety with submerged mycelia; occassionally, the colonies are furrowed. Most species shift to brown color in old age. Under microscope observation, conidial heads are radiate to columnar; conidiophore is usually colorless, its wall roughened and pitted; vesicles are globose and subglobose; sterigmata uniseriate, biseriate or both; conidia in most species are globose when matured, with conspicuous echinulation. The morphological characters of each identified strain were described in the following paragraphs:

1. *Aspergillus flavus* 11 (Figs A and B)

Rate of growth of colonies on Czapek solution agar rapid, loose textured and velvety, greenish brown shifting to dark brown in age; mycelia submerged, light yellow and medium remaining colorless; conidial heads dark green and loosely radiating, attaining a diameter of 400 μ; long conidiophore, usually 800 μ in width; pitted at anterior end and colorless; vesicle subglobose, 20 to 33 μ in diameter and light green in color; biseriate, both primary and secondary sterigmata present, 9×4 μ in primary and 5×7 μ in secondary sterigmata, both green in color; conidia prominently roughened and greenish brown in color, 6 μ and 7 μ in diameter and mostly globose when matured.

2. *Aspergillus flavus* 13 (Figs C and D)

Rate of growth of colonies on Czapek solution agar rapid; colony velvety, loose textured, greenish color with yellow shades, color remaining so even in old age; submerged mycelia and white in color; conidial heads yellowish green, columnar and reaching up to 500 μ long and 60 μ in diameter; conidiophore long, measuring 520 μ, diameter measuring 7 to 13 μ, and wall roughened and pitted; vesicle globose, 42 μ in diameter and yellowish green in color; biseriate

and both primay and secondary sterigmata present, primary sterigmata measuring 12×4 μ, and secondary sterigmata measuring 9×3 μ; conidial yellowish green in color, size variable from 3 to 6 μ in diameter, roughen and echinulate, mostly globose, though pyriform also found in early stage of maturation; sclerotia present, red brown and hard globose body, shifting to black coloration. Occassional production of dark brown gummy exudate one or two weeks after culture.

3. *Aspergillus flavus* 18L (Figs E and F)

Rate of growth of colonies on Czapek solution agar rapid, velvety, loose textured, green, shifting to greenish brown in old age; mycelia submerged, white in color, on reverse, substratum turning to yellowish red; abundant loose conidial structures with columnar conidial heads, measuring 500 μ in length and 30 μ in diameter; conidiophore 500 to 800 μ diameter 6 to 13 μ from base, with pitted wall colorless or light green; vesicle subglobose or globose, great variations in size, 30 μ, 40 μ, and 160 μ in diameter, and green in color; usually uniseriate, primary sterigmata 6×2 μ in size, not fertile throughout, only at anterior portion of vesicle, usually light green in color; bright greenish yellow conidia, measuring 3 μ, 4 μ, 5 μ, and 6 μ, not prominently roughened and mostly subglobose.

4. *Aspergillus flavus* 18D (Figs G and H)

Rate of growth of colonies on Czapek solution agar rapid, the colonies velvety, loose textured, greenish brown shifting to dark brown in old age; abundant conidial structures, with large conidial heads; the mycelia submerged, white above, reverse light yellow in color; the conidial heads attaining a diameter of 500 to 700 μ, loosely radiating and columar, mostly greenish brown; conidiophore long, meauring 1 to 1.5 mm, with increasing thickness from the base, wall pitted and colorless; vesicles variable in size, globose when matured and greenish brown in color, usually uniseriate, sterigmata measuring 7×3 μ;

Explanation of Figure 1

Fig. 1. *Aspergillus flavus* group cultured on Czapek solution agar, 10 days' incubation at 28°C, magnification of conidial structures (×300)

A. Single conidial head of *A. flavus* 11, showing uniseriate sterigmata and large conidia.
B. Loose, velvety colonies of *A. flavus* 11.
C. Conidal head of *A. flavus* 13, biseriate and dense sterigmata, and wall of conidiophore roughened.
D. Colonies of *A. flavus* 13.
E. Conidial structures of *A. flavus* 18L, showing pitted and septate conidiophore.
F. Colonies of *A. flavus* 18 L, of lighter coloration.
G. Conidial head of *A. flavus* 18D, showing clear and typical uniseriate sterigmata and echinulate conidia.
H. Dark, dense velvety colonies of *A. flavus* 18D.

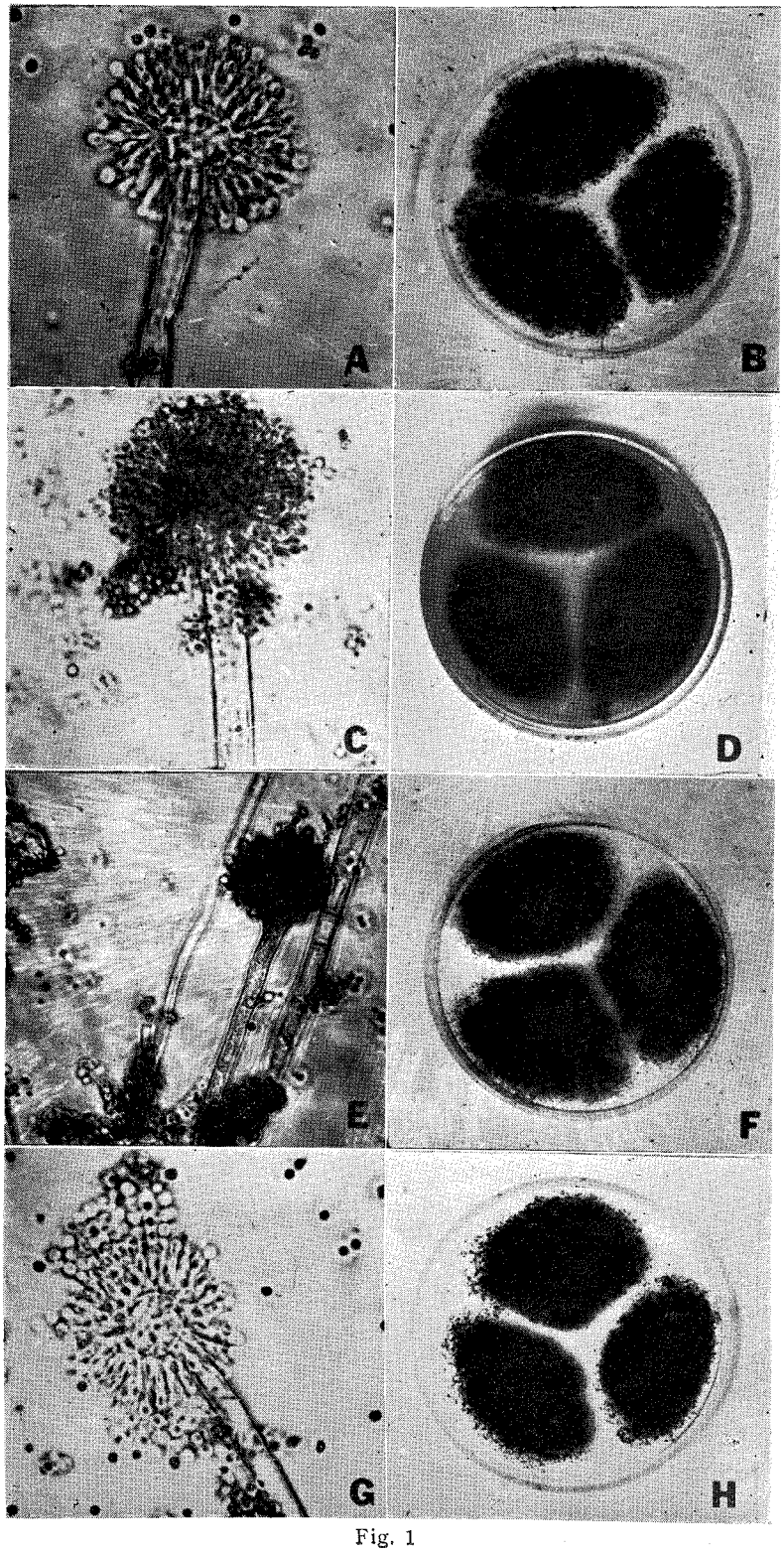

Fig. 1

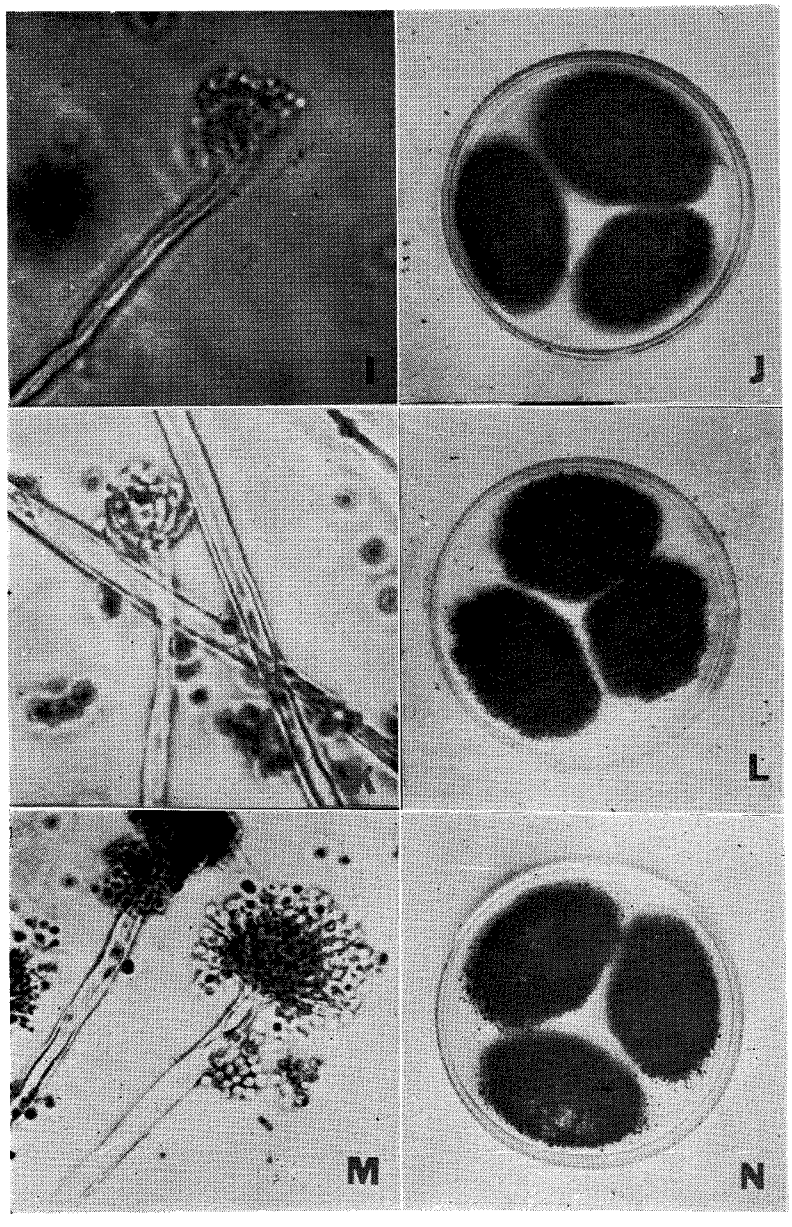

Fig. 2

condia greenish brown in color, diameter 6 to 7 μ, very prominenty roughened and mostly globose.

5. *Aspergillus flavus* Link (Figs I and J)

The growth rate of colonies on Czapek solution agar rapid, usually close textured, wrinkled and radially furrowed, with abundant conidial structures directly from the substrate mycelia; the color of the colonies green with yellow shade, shifting to deep grape green in old age; the mycelia usually submerged, reverse white or occasionally pink; conidial heads green in color, measuring 300 μ to 400×50 μ and columnar shaped; length of conidiophore 500 μ with diameter 4 to 10 μ, thick walled, coarsely roughened; vesicle subglobose, measuring 23 to 65 μ, and light green in color. Usually large vesicle, biseriate; primary sterigmata measuring 6–10×3–4 μ, and secondary sterigmata measuring 6–10×3–5 μ; conidia mostly globose, rough and lightly echinulate, 3.5 to 4.5 μ in diameter.

6. *Aspergillus parasiticus* Speare (Figs K and L)

Rapid growth in Czapek solution agar, colonies close textured, velvety, non-sporulating margin in early age, dull green with yellow shades; mycelia submerged and plane, white in color, attaining a diameter of 400 μ; conidiophore length varing from 260 μ to 800 μ and 7 to 10 μ in diameter, the wall roughened; vesicle globose or subglobose, 13 to 26 μ in diameter, and dark greenish yellow in color; sterigmata closely packed and fertile, and the measurement 3×7 μ; conidia dark yellowish green, attaining a diameter of 4 μ, 5 μ or 6 μ, the wall prominently and coarsely echinulate and its shape mostly globose.

7. *Aspergillus flavus* 041 (Figs M and N)

Rate of growth of colonies on Czapek solution agar rapid, velety compact textured and radially furrowed, deep yellowish green in color; mycelia submerged, white but light pink or tinted yellow on the reverse; conidial heads greenish yellow, radiating and columnar attaining a diameter of 500 μ, length of conidiophore varying from 190 to 240 μ, width 5 μ or 7 μ, roughened and

Explanation of Figure 2

Fig. 2. *Aspergillus flavus* group cultured on Czapek solution agar, 10 days' incubation at 28°C, magnification of conidial structures (×300)

I. Single conidial head of *A. flavus* Link, uniseriate sterigmata, and fertile at upper portion of vesicle only.
J. Colonies of *A. flavus* Link, showing close textured, furrowed, and abundant sporulation.
K. Conidial structures of *A. parasiticus* Speare, showing single condial head and thick conidiophore.
L. Colonies of *A. parasiticus* Speare.
M. Conidial head of *A. parasiticus* 041, uniseriate sterigmata, and echinulate conidia.
N. Colonies of *A. flavus* 041, showing white mycelia among dense sporulation, with large conidial heads.

pitted throughout the length; vesicle globose and green, measuring 20 μ in diameter; biseriate or uniseriate; primary sterigmata 7×3 μ, secondary sterigmata 8×3 μ, both green in color; conidia greenish yellow in color, echinulate and mostly globose, size varying between 4 μ and 6 μ.

Identification of ***Aspergillus flavus*** *group by Aspergillus Differential Medium*

Aspergillus differential medium (ADM) which consisted of 1.5% tryptone, 1% yeast extract, 0.05% ferric citrate, and 1.5% agar was used for identifying *A. flavus* group from other species of *Aspergillus* (Bothast and Fennel, 1974). It is based on the fact that the ability of pigment production in the medium was unique to members of *A. flavus* group but other species of *Aspergillus*. In this study, nine strains of *A. flavus* containing five reference strains and four isolated strains from rice were inoculated in ADM, and incubated at 28°C for six days before examination. Results show that *A. parasiticus*, *A. flavus* 041 and *A. flavus* 13 produced clear intense orange-yellow pigment, whereas the remaining strains show slight pigmentation except *A. flavus* Link and *A. tamari* Kita exhibit negative results (Table 1).

Table 1. *Ability of Aspergillus strains to produce orange-yellow pigment on Aspergillus Differential Medium*

The results were recorded after 6 days of incubation on the medium

Aspergillus strains	pigment (Orange-yellow)*
Aspergillus flavus Link	−
Aspergillus parasiticus Speare	+
Aspergillus flavus 041	+
Aspergillus oryzae Ahlburg (Cohn)	(+)
Aspergillus tamari Kita	−
Aspergillus flavus 11	(+)
Aspergillus flavus 13	+
Aspergillus flavus 18L	(+)
Aspergillus flavus 18D	(+)

*+: intense color; (+): slight color; −: absence

Detection of aflatoxin compounds by thin-layer chromatography

Aflatoxin compounds extracted from various *Aspergillus* strains were determined by thin-layer chromatoplate coated with Wako-gel B5 and developed in 2% methanolic chloroform solvent. It was revealed that *A. flavus* 041, *A. flavus* 13 and *A. parasiticus* were able to produce compounds emmiting either blue or green fluorescence or both under ultra-violet light illumination (Table 2). Non-detectable fluorescence spots, at least blue or green, was observed in *A. flavus* Link, *A. flavus* 11, *A. flavus* 18L and *A. flavus* 18D. The identity

of the green or blue spots was determined against standard aflatoxins. Only *A. flavs* 13 which isolated from stored rices was able to produce aflatoxin B_1 and B_2 compounds (Table 2). Reference fungi, such as *A. flavus* 041 was the producer of Aflatoxin B_1, B_2 and G_1, while *A. parasiticus* Speare was the producer of Aflatoxin B_1 and G_1. In th current study, no aflatoxins were produced by *A. flavus* Link although it was evidently a potent Aflatoxin-producing strain.

Table 2. *Detection of aflatoxin compounds produced by Aspergillus strains using thin-layer Chromatography*

Developing solvent system: 2% methanolic chloroform. The chromatography was applied after the developing chamber was saturated with the solvent for 1.5 hr. It was carried at 28°C in the dark for 1 hr. Each sample size was 50 microliters. Fluroscent spots were observed under an U.V. light (2537Å), and the R_f values were measured.

Chloroform extract of samples	R_f values		
	Blue fluorescence		Green fluorescence
Standard aflatoxin B1	0.86		
Standard aflatoxin B2	0.58		
Standard aflatoxin G1			0.44
Aspergillus flavus 041	0.86	0.58	0.45
Aspergillus parasiticus	0.58	—*	0.45
Aspergillus flavus 13	0.86	0.58	—
Aspergillus flavus 11	—	—	—
Aspergillus flavus 18L	—	—	—
Aspergillus flavus 18D	—	—	—
Aspergillus flavus Link	—	—	—

*—: not detectable

Discussion

From the observation of the color of colonies and fruiting structures and the measurements of the latter, it is found that *A. flavus* 13 and *A. flavus* 18L resembled closely the morphological characters of *A. flavus* Link; the fruiting structures were mostly greenish to yellowish green, and the measurements were consistent with that of *A. flavus* Link. For instance, the lenght of conidiophore of *A. flavus* Link was 500×4–10 μ, *A. flavus* 13 was 520×7–13 μ, beside these, their conidial heads were columnar. *A. flavus* 18D and *A. flavus* 11, on the other hand, possessed similar characters as *A. parasiticus;* they had close resemblance in features such as greenish brown fruiting structures, radiating conidial heads and consistent measurements of fruiting structures, additionally, their conidia were alike, *i.e.* very prominent echinulation and relatively larger size.

It was noted that the aflatoxin-producing strains such as *A. flavus* 041, *A. flavus* 13 and *A. parasiticus* Speare had common characters: (1) they are mostly biseriate, though uniseriate vesicles are also found in the same strains, (2) the conidia are yellowish green in color and prominently echinulate, (3) the colonies are persistently green in color even in old age, (4) the growth is rapid in Czapek solution agar, (5) they produce dark gummy exudate occassionally.

By using thin-layer chromatography, it is demonstrated that only *A. flavus* 13, *A. flavus* 041, and *A. parasiticus* Speare are aflatoxin-producing strains, while *A. flavus* Link used in this particular experiment does not give any aflatoxin; this might be interpreted as having emerged by processes of variation of mutation in our laboratory. Closer study of this strain has lead to the conclusion that it had become a variant, *A. flavus* Link var. columnaris, as described by Raper and Fennel (1965); it had abundant conidial heads predominantly columnar. There was no past evidence that this variant was capable of producing aflatoxin. The aflatoxin-producing potential varies much with culture media and growth conditions, for instance, the age of the culture was an important factor. If the culture was contaminated with other mycoflora, the aflatoxin-producing ability would be influenced (Boller and Schroeder, 1974). According to our results, not all strains belonging to *A. flavus* group was capable of producing aflatoxin and the production of the three closely related aflatoxin B_1, B_2, and G_1 by the fungi depends on the particular fungal strains, and on a particular growth conditions. We could not say with certainty, therefore, even the identity of one strain resembles very closely the identity of the aflatoxin-producing strain, that it is also an aflatoxin producer.

Bothast and Fennel (1974) indicated the ability of pigment production was unique to members of *A. flavus* group, and it could be used to distinguish from other species of *Aspergillus*. In the current study we found that those *A. flavus* strains and *A. parasiticus* producing the intense pigment in the ADM also produced aflatoxins (Tables 1 and 2). Thus, we suggest the ADM is a more useful medium for screening the aflatoxin-producing strains of *A. flavus* group as well as other species.

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儲藏稻米中一種黃麴毒素產生菌的分離和鑑定

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從臺北近郊取得的儲藏稻米中，分離出四種黃麴菌種，其中一種 *Aspergielus flavus* 13 已證明它會產生 B_1 和 B_2 型黃麴毒素。這種黃麴毒素產生菌在 Czapek 培養基下生長迅速而且具有下列特性：如菌落呈綠色雙層單子梗，黃綠色粗糙孢子和產生暗色粘膠似分泌物等。