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Short Communication

A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor

Michihiko Saito and Sachiko Machida

National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Tsukuba, Ibaraki 305–8642, Japan

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The colony reverse of aflatoxin (AF)-producing strains of *Aspergillus flavus* and *A. parasiticus* turned pink when their cultures were exposed to ammonia vapor. The color change was visible for colonies grown on media suitable for AF production such as potato dextrose, coconut, and yeast extract sucrose agars after 2 d incubation at 25°C. Of the 120 strains of *A. flavus*, *A. parasiticus*, and two related species in *A. flavus* group: *A. oryzae* and *A. sojae* tested in this study, only the AF-producing strains of *A. flavus* and *A. parasiticus* showed the pink pigmentation. The color change occurred immediately after the colony was contacted with ammonia vapor. This method was useful for rapid screening the AF-producing strains of *A. flavus* and *A. parasiticus*.

Key Words——aflatoxin-producing strains; ammonia vapor; *Aspergillus flavus*; screening method.

Aflatoxins (AF) are a group of carcinogenic mycotoxins produced by *Aspergillus flavus* Link and *A. parasiticus* Speare (Diener and Davis, 1969). These fungi can infest a number of foods and feeds such as peanuts and maize and cause AF contamination (Diener and Davis, 1969; Heathcote and Hibbert, 1978). Diener and Davis (1969) summarized data of investigations from six countries and reported that 40% of *A. flavus* strains collected throughout the world did not produce AF. Bilgrami and Choudhary (1993) reported that the frequency of non-toxigenic strains of *A. flavus* to be comparatively higher (ratio= 1.07) than toxigenic strains and the percentage incidence of the toxigenic strains of *A. flavus* was highest (73.3%; ratio=0.36:1) in those from the soil samples. Since not all strains of *A. flavus* can produce AF and the ratio of non-toxigenic and toxigenic strains of *A. flavus* varied with sources and location of their isolations, several simple or rapid methods for identification (detection) of AF-producing strains have been reported: e.g., ultraviolet (UV) detection of AF diffused into agar medium using a modified Czapek's solution containing corn steep liquor (Hara et al., 1974) or coconut agar (Lin and Dianese, 1976; Davis et al., 1987; Cotty, 1988; Lemke et al., 1989). Yabe et al. (1987) reported another simple method using UV photography. Bothast and Fennell (1974) described *Aspergillus* Differential Medium (ADM) on which *A. flavus* and *A. parasiticus* produced an orange yellow colony reverse. The essential ingredient in this medium was reported to be ferric citrate (Bothast and Fennell, 1974; Assante et al., 1981). The medium was modified for the rapid detection of *A. flavus* and *A. parasiticus* known as *Aspergillus flavus* and *parasiticus* agar (AFPA) (Pitt et al., 1983; Beuchat, 1984). However, on these media the AF-producing ability was not always confirmed. In this paper, we present a rapid identification method for AF-producing strains of *Aspergillus* species depending on the color change with ammonia vapor.

Two strains of *A. flavus* (87-76 and IG34-1), each one strain of *A. parasiticus* (NRRL2999), *A. oryzae* (Ahlburg) Cohn (IFO30102) and *A. sojae* Sakaguchi & Yamada (IFO4244) were used. *Aspergillus flavus* 87-76 and *A. parasiticus* NRRL2999 were AF-producing strains. *Aspergillus flavus* IG34-1, *A. oryzae* IFO30102 and *A. sojae* IFO 4244 were non AF-producers.

Potato dextrose agar (PDA) was used throughout the study. To determine the effect of media on color change, coconut (COA), yeast extract-sucrose (YES), Czapek solution (CZA), glucose-mineral salts (GMS), and peptone-mineral mix (PMS) agars were also used. COA was prepared according to Davis et al. (1987). A commercial coconut powder was used instead of shredded coconut: 100 g commercial coconut powder was homo blended with 300 ml hot water and filtered through 4 folded cheese cloth. Filtrate was made up to 300 ml with water and agar was added (1.5%) and autoclaved. YES consisted of yeast extract (Difco), 20 g; sucrose, 200 g; agar, 20 g and water, 1,000 ml (Davis et al., 1966). CZA consisted of $NaNO_3$, 3 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; sucrose, 30 g; agar, 15 g and distilled water, 1,000 ml. GMS (Buchanan and Lewis, 1984) consisted of glucose, 60 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; KH_2PO_4, 10 g; minerals, 1.0 ml; and water, 1,000 ml. The miner-

als were prepared by dissolving $Na_2B_4O_7 \cdot 10H_2O$, 700 mg; $NaMoO_4 \cdot 2H_2O$, 6,080 mg; $FeSO_4 \cdot 7H_2O$, 10 g; $CuSO_4 \cdot 5H_2O$, 300 mg; $ZnSO_4 \cdot 7H_2O$, 1.76 g and $MnSO_4 \cdot H_2O$, 110 mg in 100 ml of distilled water. PMS was identical, except that glucose was replaced by peptone (Buchanan and Lewis, 1984). GMS and PMS were developed for the study of AF biosynthesis by Abdollahi and Buchanan (1981a, b). They reported that *A. parasiticus* was not capable of synthesizing AF when cultured on PMS but was induced to produce the toxins if transferred to GMS. Each medium was autoclaved at 121°C for 15 min.

Each strain was inoculated at the center of solidified agar medium in 9-cm glass Petri dishes and incubated at 25°C. To observe the color change of colony reverse after incubation, dishes were placed upside down and a drop (0.2 ml) of 25% ammonia solution was put into the lid of the Petri dish. To determine the effect of incubation temperature, plates were incubated at 20, 25, 30, 35 and 40°C on PDA for 4 d. The effect of incubation time was measured by incubating plates for 2, 3, 4, 5, 6 and 7 d at 25°C on PDA.

Including the 5 strains mentioned above, a total of 83 strains of *A. flavus*, 13 of *A. parasiticus*, 19 of *A. oryzae* and 5 of *A. sojae* were examined the relation of AF-producing ability and the color change by ammonia vapor method. Among the *A. flavus* strains, 55 and 28 were AF-producing and non-producing strains, respectively. All of the *A. parasiticus* were AF-producing strains. *Aspergillus oryzae* and *A. sojae* did not produce any AF. Most of the *A. flavus* and *A. parasiticus* strains were collected from field soils in Thailand (Saito et al., 1986, 1989) and Okinawa Islands in Japan (Tsuruta, 1990). AF productivity of the strains was determined quantitatively by HPLC according to the method of Minamisawa et al. (1980) and by the UV irradiation method.

Immediately after the ammonia solution was put into Petri dish, the colony reverse of AF-producing strains of *A. flavus* 87–76 and *A. parasiticus* NRRL2999 turned to pink (Fig. 1). No change of color was observed with plates of AF-non-producing strains of *A. flavus* IG34-1 as well as *A. oryzae* IFO30102 and *A. sojae* IFO4244. The color change was restricted to the colony reverse. The surrounding agar did not show any color change.

To confirm that the color change was associated with AF productivity, several media including those supporting or enhancing AF-production: PDA, YES, COA and GMS, and media reported unsuitable for AF-production: PMS and CZA were tested (Fig. 2, Table 1). The pink color change was observed with plates incubated with *A. flavus* 87–76 and *A. parasiticus* NRRL2999 on PDA, YES, COA and GMS. The color intensity was strongest for YES and COA, less intense for PDA and much less for GMS than others. No color change occurred on PMS and CZA. No color change was observed on any media for AF-negative strains of *A. flavus* IG34-1 and *A. oryzae* IFO30102 and *A. sojae* IFO4244.

The effect of temperature on the color intensity was tested using *A. flavus* 87–76 grown on PDA at 20, 25, 30, 35 and 40°C. There was a slight reduction in color intensity at 20°C. At 25, 30, and 35°C, no significant differences in the intensity of color change were observed, while at 40°C the color change was obscure. The color change was checked daily with plates incubated for 1 wk (25°C). The color change was detected after 2 d incubation and increased with time, the maximum intensity being observed at 3 d, the color remaining almost the same with 4, 5, 6, and 7 d incubation.

Fig. 1. The color change of the colony reverse of AF-producing strain of *Aspergillus flavus* induced by ammonia vapor. Left: An AF-producing strain of *A. flavus* before (top) and after (bottom) exposed to ammonia vapor. Right: An AF-non-producing strain of *A. flavus* before (top) and after (bottom) exposure to ammonia vapor.

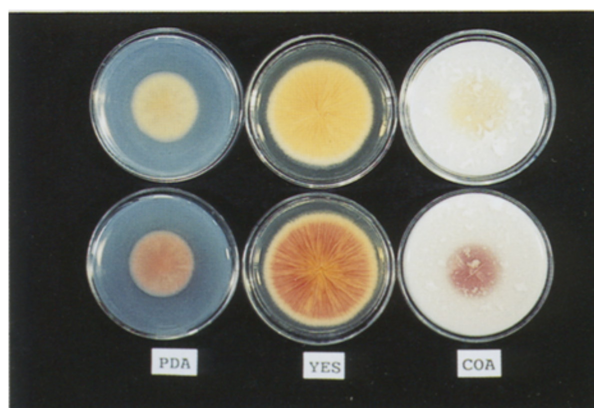

Fig. 2. The color of the colony reverse of AF-producing strain of *Aspergillus flavus* grown on PDA, YES agar and COA after 4 d incubation at 25°C.
Colnies at top and bottom shown before and after exposure to ammonia vapor, respectively.

To examine if this method was useful for identification of AF-productivity of *A. flavus* and *A. parasiticus*, a total of 120 strains including AF-non-producing *A. oryzae* and *A. sojae* were screened. As shown in Table 2, 53 of the 55 strains of AF-producing *A. flavus* and all of

the 13 AF-producing *A. parasiticus* showed apparently pink color change. The color change of other 2 strains of AF-producing *A. flavus* was obscured. The AF-production ranged from 1.9 ng/g to 40,200 ng/g of AF B_1 and that of the AF-producing strain whose color change was obscure was 8.0 ng/g. On the other hand, 25 of the 28 strains of AF-non-producing *A. flavus*, 19 of *A. oryzae* and 5 of *A. sojae* did not show any pink color change.

The present study shows that the color change of the colony reverse to pink by ammonia vapor is a useful indicator for identifying AF-producing strains of *A. flavus* and *A. parasiticus*. We need only a drop of ammonia solution for the test. The color change occurs immediately after the ammonia vapor contacted with colony. No special medium is not necessary for culturing fungi. Although the color change was visible after 2 d, 3–4 d was sufficient for detection of even very weak AF production. Incubation at 20°C slightly reduced the intensity of fluoresecnece, whereas no difference was observed at 25, 30, or 35°C. At 40°C the color change was obscure, because of the reduction of AF-production at this temperature. Diener and Davis (1967) reported the upper limiting temperature was slightly above 40°C and small amount of AF were formed at that temperature.

The fluorescence methods are based on UV detection of AF diffused into an agar medium (Hara et al., 1974; Lin and Dianese, 1976; Davis et al., 1987; Lemke et al., 1989). In the UV photography method developed by Yabe et al. (1987), the AF-producing strains were observed as gray or black colonies in UV photographs, indicating that these colonies absorbed radiated UV light. They indicated that UV absorption of the colonies in the UV photographs corresponded to the AF production. In the present method, the pigmentation was restricted to the area of the colony reverse and no change occurred in the surrounding agar. Lin and Dianese (1976) reported that the fluorescence-producing colonies always produced a conspicuous orange-yellow pigmentation in their study of COA and the pigmentation can be used to identify AF-positive strains without the use of UV light. On the other hand, Davis et al. (1987) found that the production of yellow pigment was not a reliable indicator of AF-producing ability. In the present study, we did not compare the intensity of the orange yellow pigmentation and that of the pink color. Among the yellow orange pigments produced by *A. flavus* and *A. parasiticus*, versicolorin, a precursor of AF B_1 (Lee et al., 1976), has been reported to give purple-red color in alkaline conditions (Hatsuda et al., 1955). We need further investigations to make clear the mechanism of the color change.

Table 1. Effect of media on the intensity of pink color induced by ammonia vapor (6-d incubation at 25°C).

Fungus	AF-productivity	Medium					
		PDA	YES	COA	GMS	PMS	CZA
Aspergillus flavus 87–76	High	+++[b]	++++[b]	++++	+[b]	−[b]	−
A. flavus IG34-1	ND[a]	−	−	−	−	−	−
A. parasiticus NRRL2999	High	+++	++++	++++	+	−	−
A. oryzae IFO30102	ND	−	−	−	−	−	−
A. sojae IFO4244	ND	−	−	−	−	−	−

a) ND=not detected.
b) Intensity of pink color: no change (−); weak (+) to strongest (++++).

Table 2. Result of screening for AF-producing strains by ammonia vapor method.

Fungus	No. of strains tested	No. of strains showing color change		
		apparent	obscure	no change
AF-producing				
Aspergillus flavus	55	53	2	0
A. parasiticus	13	13	0	0
AF-non-producing				
A. flavus	28	0	3	25
A. oryzae	19	0	0	19
A. sojae	5	0	0	5

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