APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1987, p. 230–234
0099-2240/87/020230-05$02.00/0

Vol. 53, No. 2

Simple Method for Screening Aflatoxin-Producing Molds by UV Photography

K. YABE,* Y. ANDO, M. ITO, AND N. TERAKADO

National Institute of Animal Health, Tsukuba-Science City, Ibaraki 305, Japan

Received 27 May 1986/Accepted 29 October 1986

UV absorption by aflatoxins was monitored in GY agar medium by UV photography. In the UV photographs, aflatoxin-producing molds were identified as gray or black colonies, whereas aflatoxin-nonproducing molds appeared as white colonies. By cellophane transplantation experiments and silica gel thin-layer chromatography, the products absorbing UV light substantially were found to be mainly aflatoxins B1 and G1 excreted from the mold mycelium into the agar medium. UV absorption did not occur when the agar medium contained aflatoxin-noninducible carbon sources instead of glucose. Various inhibitors of aflatoxin production, such as dichlorovos and dimethyl sulfoxide, also decreased the intensity of UV absorption. These results indicate that this technique can be used as a simple, safe, and rapid method of screening aflatoxin-producing molds.

Aflatoxins comprise a family of toxic secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *A. parasiticus*. These toxins have been reported to be acutely toxic, teratogenic, and potent carcinogenic and mutagenic agents (5). Although a number of screening methods have been reported for the detection of aflatoxin-producing molds, these can be mainly divided into three groups. The first group includes the standard methods, which are based on toxin production on solid or liquid substances, followed by some purification procedures and final detection by thin-layer chromatography (TLC) (3, 20) or high-performance liquid chromatography (21). The second group includes methods for the detection of fluorescence from aflatoxins in liquid culture (19) or on agar plates (6, 12, 18) under UV light. In the third group, a small agar plug from a pure mold culture is used directly to apply the sample to the TLC- plate (14).

In this paper, we present a new screening method based on the use of UV photography (16). Aflatoxin-producing molds could be detected by UV photography, because the absorbance spectra of aflatoxins produced by the molds show peaks of around 362-nm wavelength (9). This method is a simple system without tedious extraction and purification stages and is very safe, because throughout the study molds under test are fixed on an agar plate and the results can be obtained after a short incubation time. Therefore, this screening method is available for studies of the biochemical or genetic mechanisms of aflatoxin biosynthesis, which require a large number of analyses compared with the other screening methods cited above. In this report we also analyze the effects of variations in the experimental conditions.

MATERIALS AND METHODS

Microorganisms. The aflatoxin-producing strain *A. parasiticus* SYS-4 (NRRL 2999) and the aflatoxin-nonproducing strain *A. oryzae* SYS-2 (IFO 4251) were used throughout the study. Other strains used during the course of the investigations were the aflatoxin-nonproducing strains *A. oryzae* SYS-1 (IFO 4214), *A. oryzae* SYS-7 (IFO 4203), *A. oryzae* SYS-11 (IFO 5785), *A. oryzae* SYS-12 (IFO 30113), *A. sojae* SYS-13, and *A. sojae* SYS-14 and the aflatoxin-producing strains *A. flavus* SYS-3 (IFO 30180), *A. parasiticus* SYS-5 (NRRL 3145), and *A. parasiticus* SYS-6 (ATCC 15517). All strains were obtained from K. Tanaka and M. Manabe. Freshly prepared conidiospore suspensions were prepared by the method of Buxton et al. (8) by using 30-μm nylon mesh.

Media. GY agar medium (2% glucose, 0.5% yeast extract, 2% agar) (14) was used unless otherwise specified. Various carbon sources and inhibitors were separately sterilized from carbon source-free medium and then mixed. Dimethyl sulfoxide and dichlorovos (supplied by Kureha Chemical Co. Ltd.) were added aseptically after the medium had been autoclaved. Sodium benzoate and boric acid solutions were separately sterilized by filtration. The pH of the medium was not adjusted, because the intensity of UV absorption did not significantly change in the initial pH range of 4.0 to 11.0.

Culture technique. One microliter of spore suspension (approximately 400 conidia) was inoculated onto the solid agar medium. Unless otherwise specified, the aflatoxin-nonproducing strain *A. oryzae* SYS-2 was inoculated as a control together with the aflatoxin-producing strain *A. parasiticus* SYS-4 to measure only the specific changes of UV absorption induced by aflatoxin production. The plastic petri dishes were shielded with Silkylite tape (Tokyo Eizai Laboratory Co. Ltd.) and then placed upside down and incubated at 28°C in the dark for 3 days. All cultures were tested in duplicate.

UV photography, conventional natural light photography, and fluorescence photography. Plastic petri dishes were placed upside down on black velvet. Plates were irradiated with long-wave UV light (365 nm) from a UV lamp (type SL-800F; Funakoshi Chemical Co. Ltd.) and photographed with a Nikon F3 camera, UV-Nikkor 105-mm F4.5S lens, and Kodak no. 18A filter on Kodak technical pan film 2415. When specified, the Schott UV-IL interference filter (360.9 nm; bandwidth, 6.3 nm; Schott Glaswerke) was used instead of the Kodak no. 18A filter. Development was performed by using a Kodak developer D-96 at 20°C for 6 min. To fix the conditions of contrast grade and quality of the prints, three

* Corresponding author.

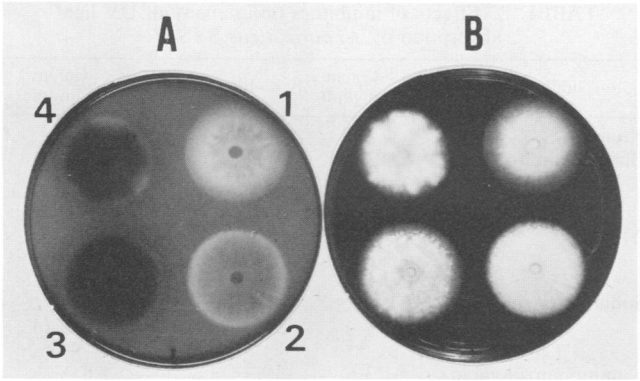

FIG. 1. UV light and natural light photographs of *Aspergillus* molds. The colonies of four kinds of molds, which had been cultured at 28°C for 3 days, were photographed by UV (A) and conventional natural light (B) photography. 1, *A. oryzae* SYS-2 (AF^-); 2, *A. flavus* SYS-3 (AF^+); 3, *A. parasiticus* SYS-4 (AF^+); 4, *A. parasiticus* SYS-6 (AF^+). AF^+, Aflatoxin-producing mold; AF^-, aflatoxin-nonproducing mold.

kinds of UV gray markers were photographed together with the petri dishes. While all photographs used in this manuscript were taken with a UV lens as described above, the photograph analogous to the UV photograph could be taken without a UV lens by using the Hasselblad 500 EL/M S-Planer C5,6/120 camera and Kodak no. 18A filter or Schott UV-IL interference filter (360.9 nm) on Polaroid film (type 667).

Conventional natural light photographs were taken with technical pan film 2415 and tungsten light.

Fluorescence photographs were taken by using Funa-UV-light type SL-800F, a Kodak no. 3 filter, and technical pan film 2415.

Cellophane transplantation of mold colonies. Conidiospores were inoculated and cultured on sterile cellophane paper (PS-1; Futamura Chemical Co. Ltd.) covering the surface of the GY agar medium. After incubation for 3 days at 28°C, the colonies were transplanted to another new GY agar medium by transferring the cellophane sheet. Both the remaining agar and the new transplanted agar plates were photographed from the bottom side by UV photography.

Extraction and analysis of aflatoxins. The agar medium immediately under the 3-day-old colonies was cut out after removing the 3-day-old mold colonies by taking off the cellophane sheet. The agar was disrupted by discharging it through a needle (0.70 by 32 mm; Terumo) and disposable plastic syringe. The slurry was blended twice for 30 s with a Vortex mixer with 2 ml of distilled water and 3 ml of chloroform per g of slurry. The mixture was centrifuged, and the chloroform layer was removed and retained. Chloroform extraction of the aqueous layer was repeated. The two chloroform fractions were combined and concentrated to dryness under nitrogen. Residues were solubilized in 0.5 ml of a benzene-acetonitrile (98:2) solution for TLC.

Aflatoxin analyses were performed by TLC, using silica gel plates (silica gel 60; E. Merck AG) developed with chloroform–ethylacetate–90% formic acid (6:3:1, vol/vol/vol) (22). Aflatoxins were quantified by using a Shimadzu TLC densitometer CS-930 with fluorometry attachment (Shimadzu Scientific Instruments, Inc.) by comparison with an aflatoxin (B1, B2, G1, G2) quantitative standard kit (Makor Chemicals Ltd.). The experiments were performed in duplicate and the results were reported as averages. Confirmatory tests for aflatoxins B1 and G1 were performed by treatment with trifluoroacetic acid (3).

RESULTS

Observation of colonies by UV photography. After culture at 28°C for 3 days, the plates were placed upside down and photographed by UV and conventional natural light photography. In the UV photographs, aflatoxin-producing strains (SYS-3, SYS-4, and SYS-6) were observed as gray or black colonies, indicating that these colonies absorbed radiated UV light (Fig. 1). In contrast, the aflatoxin-nonproducing strain (SYS-2) formed white colonies, indicating that most of the UV light was reflected by the mold mycelium. Similar results were obtained in other aflatoxin-producing and -nonproducing molds (SYS-5, SYS-1, SYS-7, SYS-11, SYS-12, SYS-13, and SYS-14). Even by using a Schott UV-IL interference filter (360.9 nm), almost identical pictures could be taken (data not shown). These differences in absorption between aflatoxin-producing and -nonproducing molds could be determined after culturing for 1.5 days in GY agar medium (colonies about 8 mm in diameter).

On the other hand, in the conventional photographs, there was no marked difference in absorption among the aflatoxin-producing and -nonproducing strains, and all of them appeared as white colonies in the pictures.

To identify the products absorbing UV light, the cellophane transplantation method was used with *A. parasiticus* SYS-4. Most UV absorption of the colonies remained in the first agar medium (Fig. 2). Thereafter, the agar immediately under the colonies of both SYS-4 and the aflatoxin-nonproducing mold SYS-2 was extracted by chloroform and analyzed by TLC (Fig. 3). The fluorescence of aflatoxins B1 and G1 was detected in the extract of SYS-4, in which 1 g of agar medium contained 16 μg of aflatoxin B1 and 54 μg of aflatoxin G1, whereas no aflatoxins were detected in SYS-2. The agar immediately under the SYS-3 colonies, which appeared pale gray in the UV photograph, contained 0.06 μg of aflatoxin B1 and 0.04 μg of aflatoxin G1 per g of agar medium. With both SYS-4 and SYS-3, the amounts of aflatoxins B2 and G2 were negligible compared with the

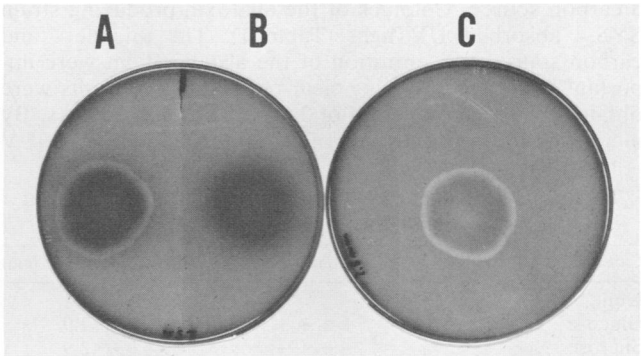

FIG. 2. Excretion of UV light-absorbing substances from mold mycelium into GY agar medium. One of the two colonies of *A. parasiticus* SYS-4 (aflatoxin producing), which had been cultured for 3 days on the cellophane sheet covering GY agar medium, was transplanted by transferring half of the cellophane sheet to a new agar medium. The resultant two plates were photographed from the bottom side by UV photography. (A) Half of the agar medium covered with cellophane sheet, on which the mold colonies had been cultured; (B) remaining half of the agar medium after the colonies together with the cellophane sheet were removed; (C) new agar medium onto which the mold colonies were transplanted.

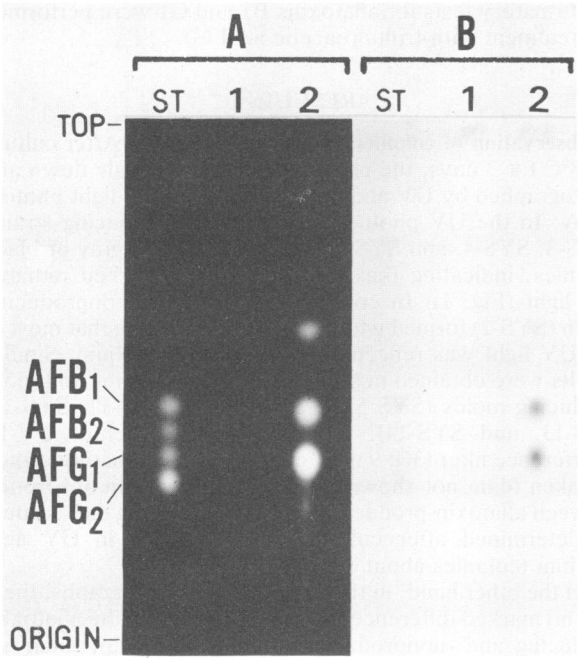

FIG. 3. Analysis of chloroform extract from agar medium by TLC. The chloroform extract was prepared from the agar immediately under the mold colonies as described in Materials and Methods. The chloroform extract equivalent to 20 mg of agar medium was developed by TLC and photographed by fluorescence (A) and UV (B) photography. ST, Aflatoxin standard; 1, *A. oryzae* SYS-2 (aflatoxin nonproducing); 2, *A. parasiticus* SYS-4 (aflatoxin producing). AFB_1, Aflatoxin B1.

amounts of B1 and G1. These results indicate that UV absorption of the colonies in the UV photographs corresponded to the aflatoxins, mainly B1 and G1, produced by the molds.

Effects of carbon sources on UV absorption. Glucose, sucrose, raffinose, glycerol, and sorbose are known to be able to induce aflatoxin production (1, 2, 11). Each of them was incorporated into the GY medium instead of glucose as a carbon source. Colonies of the aflatoxin-producing strain SYS-4 absorbed UV light (Table 1). The thickness and carbon source concentration of the agar medium were important factors in obtaining clear results. Good results were obtained with agar medium of 2.0- to 2.5-mm thickness. By increasing the concentration of glucose, the intensity of UV absorption of SYS-4 increased and reached a constant value of >2% glucose. Therefore, the concentration of the carbon sources was fixed at 2%. On the other hand, when cultured on agar medium containing citrate, fumarate, pyruvate, or peptone, which cannot induce aflatoxin production (1, 2, 11), SYS-4 did not show specific UV light absorption. In the case of the aflatoxin-nonproducing strain SYS-2, absorption of UV light in the medium was not observed regardless of carbon source.

TABLE 1. Effects of carbon sources on intensity of UV light absorption by *A. parasiticus* SYS-4[a]

Carbon source, 2%	Intensity[b]	Colony diam (cm)
None	−	2.1
Glucose	+ + + +	2.9
Sucrose	+ + + +	2.9
Raffinose	+ + + +	2.7
Glycerol	+ + +	2.8
Sorbose	+ +	2.3
Citrate, Na^+	−	2.6
Fumarate, Na^+	−	1.5
Pyruvate, Na^+	−	2.4
Peptone	−	1.9

[a] 28°C, 3 days.
[b] The intensity of UV absorption of mold colonies in the print was read by using the Kodak Gray Scale (Q-13), which was divided into six grades (− to + + + + +).

TABLE 2. Effects of inhibitors on intensity of UV light absorption by *A. parasiticus* SYS-4[a]

Inhibitor	Concn (mg/ml)	Intensity[b]	Colony diam (cm)
None		+ + + +	2.9
Dimethyl sulfoxide	33	−	2.1
	66	−	0.7
K_2SO_2	4.5	+ + +	3.1 (1.8)[c]
	10	+ +	3.2 (1.7)[c]
Sodium benzoate	2	+ + +	2.4
	8	+	2.2 (1.6)[c]
p-aminobenzoic acid	3.6	−[d]	2.0
	12	−[d]	0.9
$CuSO_4$	0.2	−[d]	2.4
	0.4	−[d]	1.7
Boric acid	0.3	+ + +	2.8
	1.3	+ + +	2.4
Caffeine	0.5	+ + +	1.7
	1.0	−	0.8
	2.0		No growth
Dichlorovos	0.014	+ +	2.8
	0.14	+	2.6
	1.4	−	1.0

[a] 28°C, 3 days.
[b] The intensity of UV absorption was read as described in footnote *b*, Table 1.
[c] The number in parentheses is the diameter of the region which absorbs UV light.
[d] The difference in absorption intensity between SYS-4 and the control (SYS-2) was measured in this case.

Effects of inhibitors on UV absorption. The effects of various inhibitors of aflatoxin production on the UV absorption are shown in Table 2. Dimethyl sulfoxide (4) decreased the intensity of UV absorption of SYS-4. At a higher concentration (66 mg/ml), growth inhibition occurred. Similar results were obtained with other inhibitors such as K_2SO_3 (10), sodium benzoate (24), *p*-aminobenzoic acid (10), $CuSO_4$ (23), boric acid (17), and caffeine (7).

The results obtained with dichlorovos (13, 15) are shown in Fig. 4. The UV absorption decreased when 0.14 mg of dichlorovos per ml was applied, without affecting mold growth. At a concentration of 1.4 mg/ml, growth was also inhibited. In the case of the aflatoxin-nonproducing strain SYS-2, no changes in UV absorption were observed irrespective of the presence of dichlorovos.

DISCUSSION

UV photography is a method enabling the recording of UV radiation as it is reflected by the subject (16). The present study shows that UV photography can be used as a rapid and

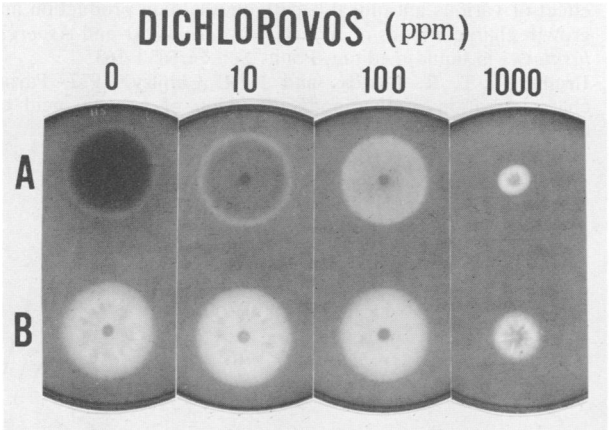

FIG. 4. Effect of dichlorovos on intensity of UV absorption. *A. parasiticus* SYS-4 (aflatoxin producing) (A) and *A. oryzae* SYS-2 (aflatoxin nonproducing) (B) were cultured on GY medium containing various concentrations of dichlorovos. The resultant colonies were observed by UV photography. Ten ppm = 0.014 mg/ml.

practical method for the identification of aflatoxin-producing molds. Moreover, by using UV photography, this screening method, is very safe, because a toxic mold can be identified without removing the lid of the plate. By this method, the aflatoxin-producing strains appeared as gray or black colonies in the UV photographs, whereas the aflatoxin-nonproducing strains appeared as white colonies. The substances absorbing UV light were mainly aflatoxins B1 and G1.

Hara et al. (18) previously proposed the fluorescence method, in which fluorescence of the aflatoxins diffusing into the agar medium from the mold mycelium was detected under UV light. With this method, however, it is difficult to examine a large number of colonies on agar in a petri dish, and it takes a long time (7 to 10 days) to detect aflatoxins diffusing into the agar medium. In contrast, the UV photography method described herein makes it possible to identify the production of aflatoxins even in the case of small colonies after culture for 1.5 days in GY agar, and many colonies can be examined on a single plate.

Filtenborg and Frisvad (14) also proposed a screening method for toxigenic molds in pure cultures. A small agar plug from a pure mold culture is spotted onto a TLC plate and analyzed by TLC. Although the method is simple, it seems to be dangerous because the spores are scattered during the operation.

The present study also shows that the aflatoxin-producing molds could be accurately identified by examining the effects of specific inducers or inhibitors of aflatoxin production on the intensity of UV absorption in the UV pictures. Furthermore, this method may be available for the screening of aflatoxin-producing molds among a number of heterogeneous molds in foods, feeds, and soil. Of the various inhibitors examined, dichlorovos showed the strongest inhibitory effect on the intensity of UV absorption without inhibition of mold growth at a concentration of 0.14 mg/ml. This chemical has been reported to be a specific inhibitor (15).

Finally, this screening method by UV photography should facilitate genetic studies of the aflatoxin-producing molds. For example, the use of this method in our laboratory demonstrated that isolation and characterization of aflatoxin-nonproducing mutant strains can be easily performed.

ACKNOWLEDGMENTS

We thank K. Tanaka and M. Manabe, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, for supplying all of the molds and for helpful discussions. We also thank H. Saginoya, University of Tsukuba Hospital Photo Studio, for supplying the UV gray markers.

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