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# Action of Phosphine on Production of Aflatoxins by Various *Aspergillus* Strains Isolated from Foodstuffs

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**Phosphine is a food fumigant, used until now as an insecticide and rodenticide. The present work researches the action of phosphine treatment on growth and aflatoxin production of 23 *Aspergillus* strains. Production of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ decreased in almost all cases by a ratio of 10 to 100. Phosphine treatment therefore seems favorable to prevent growth of various *Aspergillus* strains, in the context of keeping food safe.**

Use of phosphine to fumigate stored foodstuffs is likely to become more widespread owing to both its effectiveness and its rapid desorption (13). Phosphine is a gas which is released from aluminum phosphide on contact with water vapor in the air. It is a strong insecticide (1, 5, 6, 14, 17, 21) and has antimicrobial activity (3, 18). Its target appears to be the respiratory chain (9, 15, 19). Since it diffuses rapidly, it is well adapted for use as a food preservative in sealed containers such as silos. It is possible to adjust the dose and duration of fumigation in relation to the pests and microorganisms present. Fumigated stocks are ventilated after use, and this use leaves very little residue. Maximum permitted levels are now set at 0.01 mg/kg (20, 26) when it is used as an insecticide.

This study was designed to evaluate the action of a nonmicrobicidal dose of phosphine on aflatoxin production. We investigated the toxicity of a nonlethal dose of phosphine on various mold strains isolated from foodstuffs, and we then measured the levels of various aflatoxins produced. It was conceivable that a preservative dose could halt mold growth but nevertheless stimulate toxin synthesis (4).

## MATERIALS AND METHODS

Mold strains were cultured on synthetic medium in the presence or absence of phosphine. After extraction and preliminary purification, the aflatoxins produced were analyzed by thin-layer and high-pressure liquid chromatography (HPLC).

**Organisms.** The different strains which were kindly given by the Department of Microbiology, Institut National de la Santé et de la Recherche Médicale, Le Vesinet, namely, *A. flavus*, *A. parasiticus*, *A. chevalieri*, *A. repens*, *A. ruber*, and *A. niger*, were isolated from various foodstuffs (see Table 2). These strains produce various amounts of aflatoxins.

The purity of each strain and its identity were verified by the morphological criteria of Raper and Fennel (23).

**Culture medium.** The medium for the fungi contained 2% yeast extract (Difco Laboratories, Detroit, Mich.), $MgSO_4$ (1%), $FeSO_4$ (0.05%), and 20% sucrose. Demineralized water was used throughout the study.

**Aflatoxins.** Standard aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ (Sigma Chemical Co., St. Louis, Mo.) were dissolved in benzene-acetonitrile (90:10) at a concentration of 0.1 μg/ml.

**Fumigation procedure.** Fumigation was carried out in a closed chamber which was constructed from transparent polyvinyl chloride and which had a volume of 1/14 $m^3$. It was equipped with a sealed door and a tap with no metallic parts accessible to the gas.

Phosphine is normally liberated over a period of 48 h by the reaction of aluminum phosphide tablets in contact with atmospheric water vapor. However, this was too long a period to allow accurate evaluation of the effects of this agent on the mold cultures. The speed of release was thus increased by placing the aluminum phosphide tablets in a petri dish containing an ice cube and a few drops of Teepol. The petri dish was placed inside the chamber containing the cultures as described in the next section. As the ice melted, the liberated water mixed with the detergent and then came into contact with the aluminum phosphide. Ninety-five percent of the phosphine was thus liberated over a 5- to 6-h period. Very little gas was liberated before the chamber was sealed.

A 3-g aluminum phosphide tablet released 1 g of phosphine, and to obtain a level of $3 \times 10^{-4}$ g of phosphine per liter of air in the tank, 0.0634 g of aluminum phosphide was required.

Mold cultures were exposed to the phosphine for 21 days at ambient temperature.

At the end of the experiment, the tank was opened in a well-ventilated room. Face masks were worn for protection. The flasks were aerated to eliminate phosphine residues.

**Measurement of phosphine concentration.** The phosphine concentration was measured by using a calibrated bellows pump connected to the tap on the tank. A known volume was pumped through a Draeger tube containing the reagent for phosphine. The intensity of the color reaction was read directly on a graduated scale on the side of the tube.

**Cultures.** Mold strains were cultured in liquid medium. Flasks (300 or 500 ml) containing 50 ml of medium were stoppered with cotton plugs. One gram of cork powder washed with methanol in a Soxhlet was placed in each flask. The cork powder allowed better development of the molds on the liquid medium. The media were inoculated with 1 ml of spore suspension in water from 1- to 3-week-old cultures of *A. flavus*, *A. parasiticus*, *A. chevalieri*, *A. repens*, *A. ruber*, and *A. niger* and incubated for 21 days at 25°C as stationary cultures. Eight flasks were inoculated for each strain; four flasks were treated and four were not. In each lot, two flasks were used for determination of the dry weight of the mycelium and the other two were used for the aflatoxin assays.

**Determination of dry weight of the mycelium.** At the end of the experiment, two treated cultures and two control cul-

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TABLE 1. Effect of a 21-day phosphine treatment at ambient temperature on growth of various strains of *Aspergillus*

| Strain | Dry wt of mycelium (g) | | Ratio, control/ treated (%) |
|---|---|---|---|
| | Control | Phosphine treated | |
| *A. flavus* XLIIC | 2.77 | 1.58 | 56.89 |
| *A. flavus* 1326 | 3.24 | 0.44 | 13.67 |
| *A. parasiticus* 126-62 | 2.61 | 1.68 | 64.36 |
| *A. parasiticus* 2050 | 2.74 | 1.40 | 51.09 |
| *A. flavus* BIIB | 2.66 | 1.32 | 49.43 |
| *A. flavus* 2135 | 2.70 | 1.61 | 59.62 |
| *A. chevalieri* B119B | 2.57 | 1.49 | 57.97 |
| *A. chevalieri* 1415 | 3.42 | 1.71 | 50.05 |
| *A. repens* B588A | 2.76 | 1.32 | 47.75 |
| *A. ruber* B38C | 1.88 | 1.81 | 96.48 |
| *A. ruber* 2310 | 2.33 | 0.67 | 28.54 |
| *A. flavus* ITM9 | 3.39 | 1.81 | 53.51 |
| *A. flavus* B591A | 1.82 | 0.90 | 49.17 |
| *A. flavus* B641 | 2.03 | 0.68 | 33.25 |
| *A. flavus* AF15 | 2.07 | 1.00 | 48.42 |
| *A. flavus* AF70 | 2.31 | 0.91 | 39.50 |
| *A. flavus* J32 | 1.84 | 1.31 | 71.28 |
| *A. flavus* JA104A | 1.94 | 1.16 | 59.76 |
| *A. flavus* A36B | 1.85 | 1.33 | 71.54 |
| *A. flavus* 75032 | 2.03 | 1.20 | 59.05 |
| *A. flavus* 25A40 | 1.97 | 0.87 | 44.16 |
| *A. flavus* JJ24B | 1.81 | 0.69 | 37.86 |
| *A. niger* X | 1.76 | 1.21 | 68.78 |

tures were filtered in a Buchner, and the mycelial mats were weighed after drying for 12 to 14 h at 80°C (to constant weight).

**Extraction procedure.** Aflatoxins were extracted from the mixed cultures with chloroform-water (10:1) by published methods (2). The extracts were filtered through paper filters (Whatman no. 1) and purified on a silica gel column. The aflatoxins were eluted with chloroform-methanol (97:3), evaporated to near dryness, transferred to small vials in chloroform, and then evaporated to complete dryness under nitrogen.

**Identification by thin-layer chromatography.** One-half milliliter of benzene-acetonitrile (90:10) was added to the dry residues in chromatography vials, which were then shaken in a Vortex shaker. Two 10-μl portions of the sample were spotted, and another portion was cospotted with 1 μl of the standards (10 μg of $B_1$ and $G_1$ per ml, 5 μg of $B_2$ and $G_2$ per ml) on thin-layer chromatographic plates (0.2 mm, 20 by 20 cm) consisting of aluminum sheets coated with Silica Gel G60 without fluorescence indicator (Merck). Plates were developed in the dark in chloroform-acetone-isopropanol (85:10:5) or in diethyl ether-methanol-water (96:3:1) (11, 25). After being dried, the plates were observed under UV light (365 and 254 nm) with a UV lamp (Ultra Violet Products, Inc., San Gabriel, Calif.). Aflatoxins appear as four spots in the order of mobility $B_1$, $B_2$ (blue), $G_1$, and $G_2$ (green) from $R_f$ 0.4 to 0.5. Sample spots were compared with the standards.

Identification was confirmed by spraying the plates with

TABLE 2. Levels of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ in several strains of *Aspergillus*

| Strain and origin | Aflatoxin production[a] (μg/g [dry wt] of mycelium) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $B_1$ | | $B_2$ | | $G_1$ | | $G_2$ | |
| | C | P | C | P | C | P | C | P |
| Pure medium | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* XLIIC (corn) | 700 | 16 | 120 | 0.95 | 2,000 | 15 | 100 | 1.04 |
| *A. flavus* 1326 (corn) | 500 | 58 | 70 | 6.6 | 1,600 | 78 | 100 | 5.2 |
| *A. parasiticus* 126-62 (line "Baarn," Holland) | 200 | 9.4 | 20 | 1.5 | 1,350 | 35 | 20 | 2.3 |
| *A. parasiticus* 2050 (line Laborat "Le Vesinet") | 100 | 35 | 20 | 8.1 | 320 | 52 | 1.1 | 2.8 |
| *A. flavus* BIIB (corn) | 2.6 | 0.56 | 0.01 | 0 | 1.2 | 0.23 | 0.001 | 0.014 |
| *A. flavus* 2135 (corn) | 0.085 | 0.042 | 0 | 0 | 0.076 | 0.053 | 0.014 | 0.0085 |
| *A. chevalieri* B119B (corn) | 1.2 | 0.29 | 3.4 | 0.18 | 2.0 | 0.058 | 0.32 | 0.13 |
| *A. chevalieri* 1415 (corn) | 0.03 | 0.017 | 0.06 | 0 | 0.014 | 0.016 | 0.0029 | 0 |
| *A. repens* B588A (pig food) | 2.0 | 0.85 | 3.4 | 0.6 | 0.091 | 0.028 | 1.9 | 0.074 |
| *A. ruber* B38C (jam) | 0.83 | 0.03 | 0.17 | 0.0015 | 1.3 | 0.03 | 0.04 | 0.0052 |
| *A. ruber* 2310 (bread) | 0.095 | 0.017 | 0.73 | 0 | 0.0044 | 0 | 0.00089 | 0.0077 |
| *A. flavus* ITM9 (line "Bari") | 0.84 | 0.28 | 0.01 | 0 | 0.42 | 0.0018 | 0.0083 | 0 |
| *A. flavus* B591A (groundnut oil cake) | 0.0057 | 0.24 | 0.087 | 0 | 0.2 | 0.066 | 0.032 | 0 |
| *A. flavus* B641 (Sudanese groundnuts) | 0.13 | 0.17 | 0.027 | 0 | 0.014 | 0 | 0 | 0 |
| *A. flavus* AF15 (groundnut oil cake) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* AF70 (Sudanese oil cake) | 0.32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* J32 (groundnut) | 110 | 0.21 | 0.05 | 0 | 0.097 | 0 | 0.0044 | 0 |
| *A. flavus* JA104A (groundnut) | 0.36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* A36B (groundnut) | 400 | 0.26 | 3.3 | 0 | 0 | 0.037 | 0.013 | 0 |
| *A. flavus* 75032 (pig food) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* 24A40 (pig food) | 0.91 | 0.11 | 0.02 | 0 | 0.44 | 0 | 0.022 | 0 |
| *A. flavus* JJ24B (groundnut) | 0.30 | 0.34 | 0.023 | 0.029 | 0.64 | 0.029 | 0.0074 | 0 |
| *A. niger* X (coffee) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] C, Control; P, phosphine treated. Limit of detection, $10^{-7}$ mg of aflatoxin per g (dry weight) of mycelium.

25% sulfuric acid or 5% nitric acid. Under long-wave UV light, the suspect spots and standards changed fluorescence color. Aflatoxin fluorescence is completely abolished by treatment with a 10% solution of hypochlorite.

Derivatization with trifluoroacetic acid was also employed to confirm the presence of aflatoxins $B_1$ and $G_1$ in the samples (22).

**Quantitation by HPLC.** The mobile phase consisted of toluene-ethyl acetate-formic acid-methanol (90:6:2:2) (12). The fluorescence detector (Jobin-Yvon JY3D) was set to excitation at 365 nm and emission at 425 nm. The flow rate of the pump (Chromatem M 380; Altex-Beckman) was stabilized at 1.5 ml/min. Aflatoxin standards were diluted with benzene-acetonitrile (90:10) to give a range of standard concentrations (0.001 to 10 μg/ml) for each aflatoxin. Each concentration of each aflatoxin was injected into a 100-μl loop through an HPLC column (Merck Lichrospher SI 100/II 5 μ). An integrator (L.D.C. Milton Roy CL 10B) was used to evaluate the heights or areas of the peaks. Limits were below 1 ng. Regression lines of peak heights and areas as a function of concentration were calculated with a microcomputer (Olivetti M24).

The amount of each aflatoxin was calculated by using the calibration equation [μg of aflatoxin = $(y - b)/a$], where $y$ is the peak height or peak area, $b$ is the intercept with the $y$ axis, and $a$ is the slope of the regression line.

Mold extracts evaporated to dryness under nitrogen were dissolved in 4.5 ml of benzene-acetonitrile (90:10), and 100-μl samples were injected.

## RESULTS

Table 1 shows the results for dry weights of mycelium of the treated and control cultures for each mold strain. This provided a measure of growth under the two conditions. The dose of phosphine used (150 ppm, gas/gas) did not halt growth, although the dry weights of the treated cultures were lower than those of controls. Table 2 shows the results of assays of the four aflatoxins for the different mold strains. Some strains (XLIIC, 1326, 126-62, and 2050) produced considerable amounts of aflatoxins, while others were much less productive, and strains such as AF15, 75032, and X produced none at all. Similarly, Kulik and Holaday (16) observed low production of aflatoxins by species such as *A. ruber*. After exposure to a treatment that was insufficient to halt growth completely, many strains of *Aspergillus* produced considerably less aflatoxin. With only a few exceptions, the quantities of the four aflatoxins were much reduced by treatment with phosphine. There was a 10-fold reduction for the weakly productive strains and a 100- to 1,000-fold reduction for the more productive strains.

## DISCUSSION

The results indicate that, as expected, phosphine not only slowed mold growth but also affected toxin production. Fortunately this effect was seen as an overall reduction in aflatoxin production. These results tend to confirm the value of phosphine as a preserving agent for various foodstuffs. Phosphine has an insecticidal action similar to that of carbamates, which both slow growth and reduce toxin production (10). However, phosphine poses less of a toxicity problem (7, 8) than the carbamates. Moreover, it has activity at levels which do not completely halt mold growth. This is of interest, since other agents, such as methyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate, benzoic acid, and sorbic acid, when tested at subinhibitory doses, have been shown to stimulate production of aflatoxin $B_1$ in *A. flavus* (4).

Treatment with doses of phosphine that were insufficient to completely halt mold growth did in fact also reduce overall production of aflatoxins by the remaining organisms in the treated material. This would indicate that improper treatment (suboptimal levels, uneven distribution, insufficient final ventilation, etc.) would probably not stimulate production of aflatoxins as might have been feared. Fumigation procedures could thus be expected to be fungistatic. It remains to be determined whether the mycelial population is capable of developing resistant or less sensitive strains during treatment with this agent. This would modify the microflora and lead to new and therefore less acceptable dangers. Further investigations are needed before this question can be answered satisfactorily.

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