APPLIED MICROBIOLOGY, June 1971, p. 1011-1016
Vol. 21, No. 6

Printed in U.S.A.

# Brine Shrimp (*Artemia salina* L.) Larvae as a Screening System for Fungal Toxins

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Received for publication 24 February 1971

Concentrations resulting in 50% mortality, determined with brine shrimp (*Artemia salina* L.) larvae exposed to known mycotoxins for 16 hr, were (μg/ml): aflatoxin $G_1$, 1.3; diacetoxyscirpenol, 0.47; gliotoxin, 3.5; ochratoxin A, 10.1; and sterigmatocystin, 0.54. 4-Acetamido-4-hydroxy-2-butenoic acid γ-lactone gave no mortality at 10 μg/ml. Used as a screening system involving discs saturated with solutions of known mycotoxins, the larvae were relatively sensitive to aflatoxin $B_1$, diacetoxyscirpenol, gliotoxin, kojic acid, ochratoxin A, rubratoxin B, sterigmatocystin, stemphone, and T-2 toxin. Quantities of 0.2 to 2 μg/disc caused detectable mortality. The larvae were only moderately sensitive to citrinin, patulin, penicillic acid, and zearalenone which were detectable at 10 to 20 μg/disc. They were relatively insensitive to griseofulvin, luteoskyrin, oxalic acid, and β-nitropropionic acid. The disc screening method indicated that 27 out of 70 fungal isolates from foods and feeds grown in liquid or solid media produced chloroform-extractable toxic material. Examination of toxic extracts by thin-layer chromatography for 17 known mycotoxins showed that the toxicity of eight isolates could be attributed to aflatoxin $B_1$ and $B_2$, kojic acid, zearalenone, T-2 toxin, or ochratoxin A. Nine out of 32 of these fungal isolates grown in four liquid media yielded toxic culture filtrates from at least one medium. Chemical tests for kojic, oxalic, and β-nitropropionic acids showed the presence of one or two of these compounds in filtrates of seven of these nine isolates.

In the search for hitherto unknown toxic fungal metabolites, evaluation of toxicity on the basis of mortality or pathological symptoms, such as kidney or liver damage in higher animals, is laborious and puts severe restrictions on the number of fungal isolates and culture conditions that can be tested. Besides, it is possible that the toxicity observed in animal experiments is due to the presence of one or more of the many known fungal metabolites, viz., known mycotoxins (3, 10) or commonly occurring compounds such as oxalic and kojic acids. Toxicity of feedstuffs inoculated with *Aspergillus* spp. was attributed in some instances to oxalic acid production (21), and many of the filtrates and extracts of fungi isolated from Japanese fermented foods contained kojic and β-nitropropionic acids (12). From a practical view point, it may therefore be advantageous to (i) test fungal isolates for toxicity with one or more easily manageable screening systems, (ii) screen the toxic extracts by chemical screening techniques for the presence of known toxins, and (iii) test those cultures, whose toxicity cannot be explained, against higher animals. It must be borne in mind, however, that the screening system used may not be sensitive to some fungal metabolites that are toxic to higher animals.

In this paper, we describe the use of brine shrimp larvae as a screening system for toxic fungi and investigate their sensitivity to some known mycotoxins. This system has the advantage that brine shrimp eggs are commercially available. Active larvae can be obtained within 1 to 2 days, and maintenance of live cultures is not required. The organism has been widely used, e.g., for quantitation of the potency of anesthetics (15), evaluation of toxicity of dichloro-diphenyl-trichloroethane (11) and other insecticides (13), of heavy metal salts (7) and antibiotics (8), and for testing the inhibitory effects of carcinogens on the hatching of the eggs (6). Brown et al. (5) described a bioassay involving brine shrimp larvae for aflatoxin $B_1$. Under the conditions of this test, 0.5 μg/ml in artificial seawater resulted in mortality of over 60%. The larvae were also relatively sensitive to ochratoxin A and highly sensitive to an acetone extract of *Fusarium tricinctum* (4). These data suggested that the

brine shrimp might be a suitable screening system for testing fungi for toxicity.

## MATERIALS AND METHODS

**Culture.** Most of the fungi tested had been isolated from foodstuffs (20). Some cultures which were implicated in death and illness of animals had been isolated by J. Forgacs, Good Samaritan Hospital, Suffern, N.Y. Others were isolated from naturally moldy wheat containing ochratoxin A (18) and from other cereals. Cultures were reisolated from sterile soil in which they had been stored and grown on fresh potato-dextrose agar for about 2 weeks; spores were then suspended in 0.05% aqueous polyoxyethylene sorbitan monooleate. The majority of the cultures were tested for toxicity after growth in liquid media. One milliliter of a suspension containing $10^6$ spores was added to 24 ml of yeast extract (2%)-sucrose (15%) medium (YES), Czapek-Dox broth (CD), CD supplemented with 0.5% yeast extract (CD + YE), and Sabouraud's maltose broth (SM) in 250-ml Erlenmeyer flasks. Cultures were incubated for 6 to 9 days at 25 C. Some isolates of *Aspergillus candidus* and *A. glaucus* from moldy wheat were tested for toxicity after 2 weeks of growth on 6 g of moistened and autoclaved wheat at 37 and 30 C, respectively. *Fusarium* and *Trichothecium* isolates were cultured on 3 g of moistened, crushed corn in addition to YES under conditions described previously for *Fusarium* isolates (17).

**Filtrates and extracts.** Mycelial mats were separated from the culture liquids and frozen in liquid nitrogen. Culture liquids were filtered with 0.45-$\mu$m Nalgene filter units (Nalge, Rochester, N.Y.). Mycelial mats and culture filtrates were stored at $-10$ C. Just before testing, culture filtrates were adjusted to *p*H 6.5 with 5 N NaOH. Mycelial mats and fungal cultures that had been grown on grains, and also some of those grown on liquid media, were extracted by immersion in boiling chloroform for 10 min; the chloroform fraction was removed with a pipette and the cultures were further extracted by dispersion in another portion of chloroform with a high-speed mixer (Silverson Machines Ltd., London, England). The two extracts of each culture were combined and reduced to 1 ml under nitrogen. The toxicity of each filtrate and extract was evaluated by the disc screening method described below.

**Larvae.** Methods involving the use of brine shrimp larvae for testing fungi for the production of toxic secondary metabolites (Higgens et al., Eighth Intersci. Conf. Antimicrob. Ag. Chemother., Abstr., 1968, p. 18) were followed with minor modifications. Brine shrimp medium (BSM) was composed of 3.0 g of dipotassium glycerophosphate; 30.0 g of NaCl; 0.3 g of $CaDl_2 \cdot 2H_2O$; 0.5 g of $MgSO_4 \cdot 7H_2O$; 1.5 g of $MgCl_2 \cdot 6H_2O$; 0.8 g of KCl; 0.1 g of $MgBr_2 \cdot 6H_2O$; 6.0 g of glycine; and deionized water to make 1,000 ml of BSM (C. E. Higgens, *personal communication*). The *p*H of the medium was adjusted to 6.5 prior to autoclaving for 15 min at 121 C. For each experiment, 100 to 200 mg of brine shrimp eggs (Connecticut Valley Biological Supply Co., Southampton, Mass.) was placed in 100 ml of BSM contained in a 750-ml Erlenmeyer flask and shaken at 140 rev/min on a rotary shaker for about 30 hr at 30 C. Larvae were separated from egg cases and eggs by means of a medicine dropper after they had started to cluster. The concentration of larval suspensions was adjusted to about 200 larvae/ml. Eggs were stored over $CaSO_4$ at room temperature. The percentage of larvae emerging from eggs thus stored remained generally satisfactory over a period of several months; in some batches, however, hatchability was low for unknown reasons.

**Mycotoxin solutions.** Aflatoxins $B_1$ and $G_1$, rubratoxin B, and diacetoxyscirpenol were purchased from Calbiochem, Los Angeles, Calif., oxalic acid was from Anachemia Chemicals Ltd., and $\beta$-nitropropionic acid and kojic acid were from Aldrich Chemical Co., Inc., Milwaukee, Wis. The latter compound was recrystallized from ethyl acetate with use of decolorizing charcoal. Ochratoxin A was obtained from *Penicillium viridicatum* (19) and stemphone was from *Stemphylium sarcinaeforme* (16). Citrinin, gliotoxin, griseofulvin, luteoskyrin, patulin, penicillic acid, T-2 toxin, zearalenone, sterigmatocystin, and 4-acetamido-4-hydroxy-2-butenoic acid $\gamma$-lactone were received as gifts (1, 17).

The toxicity of these mycotoxins was evaluated by means of the disc screening method or assay method, when possible in two independent experiments with different batches of brine shrimp eggs. Most toxins were dissolved in methanol to obtain concentrations of 1,000, 500, 100, and 10 $\mu$g/ml. Sterigmatocystin was dissolved in acetone; oxalic and $\beta$-nitropropionic acids were dissolved in water, and the *p*H was adjusted to 6.5 with 1 N NaOH. In the assay method, mycotoxin solutions were diluted 100-fold with BSM to obtain final concentrations of 10, 5, 1, and 0.1 $\mu$g/ml.

**Disc screening method.** Blank paper discs (7 mm diameter; BBL) were saturated with a solution of toxin or fungal extract in organic solvent (about 20 $\mu$liters/disc), the solvent was allowed to evaporate, and each disc was placed in a 0.5-ml well of a FB-48 Linbro Disposo Tray (Winley-Morris, Montreal). When aqueous solutions of toxins and culture filtrates (about 30 $\mu$liters/disc) were tested, discs were placed directly in the well. Toxicity of each solution was evaluated in triplicate. Two drops (about 0.1 ml) of a suspension of larvae (containing 20 to 40 larvae) were added to each well. Trays were incubated at 30 C for about 16 hr. Mortality was determined by counting the immobile (dead) larvae under a stereoscopic microscope, killing the living larvae with heat or Formalin, and then counting the total number. Mortality in controls was determined simultaneously with each screening test. Natural mortality associated with discs saturated in noninoculated media or water averaged 3 and 1%, respectively; that of discs soaked in chloroform or methanol, which was allowed to evaporate, averaged 2%. Chloroform extracts of quantities of corn meal and wheat, equal to that used for culturing some of the fungi examined, gave a mortality of 6 and 0.3%, respectively. Toxicity of filtrates and extracts was rated as follows: 0 to 9%

mortality, nontoxic (NT); 10 to 49% mortality, slightly toxic (ST); 50 to 89% mortality, toxic (T); 90 to 100% mortality, very toxic (VT).

**Assay method.** Of each mycotoxin solution to be tested, 4.8 ml was pipetted into a 10-ml beaker, and 4 drops (0.2 ml) of a larval suspension containing about 30 larvae was added to each beaker. Each concentration was tested in quadruplicate. Larvae were incubated at 30 C for about 16 hr. Mortality was determined on the basis of mobility, as viewed under a Quebec colony counter. Simultaneously run controls consisted of BSM containing 1% (v/v) of solvent. Natural mortality in 1% methanol solutions averaged 5% and ranged from 0 to 17%; in 1% acetone the mortality averaged 1%. Concentrations giving rise to 50% mortality ($LC_{50}$ values) were estimated from the weighted regression lines of the probit mortality on the logarithm of the concentration, as fitted by the maximum likelihood solution (9).

The toxicity of some chloroform extracts was evaluated with the assay method on a lipid weight basis. The chloroform was removed under nitrogen from the remaining portions of the extracts. Residues were dissolved in 1 ml of methanol. A 0.2-ml portion of this solution was diluted 100-fold with BSM and tested as described above. The weight of the lipid residue from another 0.2-ml portion was determined after removal of the methanol.

**Thin-layer chromatography.** Thin layers (0.25 mm) of silica gel (Adsorbosil 5, Applied Science Laboratories, Inc., State College, Pa.) were activated at 100 C for 2 hr. Five $\mu$liters of T or VT filtrate, or T or VT chloroform extract (concentrated to 0.5 ml), were spotted on the thin-layer chromatography (TLC) plate together with several known mycotoxins (17). Plates were developed 15 cm in the solvent systems toluene-ethyl acetate-(90%) formic acid (6:3:1) and benzene-methanol-acetic acid (24:2:1). Chromatograms of T and VT extracts were examined under ultraviolet light and after spraying and heating with an acidic methanolic solution of *p*-anisaldehyde (17). This method was used for examination of extracts for aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$, aspertoxin, citrinin, diacetoxyscirpenol, gliotoxin, luteoskyrin, nivalenol, nivalenol acetate, ochratoxin A, patulin, penicillic acid, sterigmatocystin, T-2 toxin, and zearalenone. Kojic acid and $\beta$-nitropropionic acid were detected by spraying with 0.1% of diazo blue B salt in methanol-water (1:1) followed by brief exposure to ammonia fumes; they formed mauve and orange spots, respectively, at average $R_F$ values of 0.15 and 0.51 in the first solvent system. Amounts were estimated by visual comparison with standards (10 mg of kojic acid/ml and 2 mg of $\beta$-nitropropionic acid/ml). Kojic acid was confirmed by spraying with ferric chloride solution after repeat TLC; all filtrates were also tested with this reagent in test tubes.

**Oxalic acid.** T and VT filtrates suspected to contain oxalate were treated with Moir's calcium acetate reagent (14). The precipitate was purified and checked for reduction of 0.02 N potassium permanganate on heating in 2 N sulfuric acid (14).

## RESULTS

**Sensitivity of brine shrimp larvae to known mycotoxins.** The data in Tables 1 and 2 show that the larvae are sensitive to aflatoxin $B_1$, aflatoxin $G_1$, diacetoxyscirpenol, gliotoxin, kojic acid, ochratoxin A, rubratoxin B, sterigmatocystin, stemphone, and T-2 toxin. Toxicity from these toxins would have been detected if they had been present in the chloroform extract of fungal cultures at a concentration of 10 to 100 $\mu$g/ml or more. The larvae were less sensitive to citrinin, patulin, penicillic acid, and zearalenone. These would have been detectable only if present in the chloroform extracts at a concentration of about 500 $\mu$g/ml or more. Griseofulvin and luteoskyrin had no toxicity at the concentrations tested. The

TABLE 1. *Toxicity of mycotoxins to brine shrimp larvae (disc screening method)*

| Toxin | Amt of toxin ($\mu$g/disc) | Mortality[a] (%) |
|---|---|---|
| Aflatoxin $B_1$ | 0.2 | 21 (0) |
| Citrinin | 10 | 40 (1) |
| Diacetoxyscirpenol | 0.2 | 96 (0) |
| Gliotoxin | 0.2 | 74 (0) |
| Griseofulvin | 20 | 3 (3) |
| Kojic acid | 2 | 92 (2) |
| Luteoskyrin | 10 | 3 (5) |
| $\beta$-Nitropropionic acid | 90 | 35 (1) |
| Ochratoxin A | 0.2 | 17 (0) |
| Oxalic acid | 150 | 11 (1) |
| Patulin | 10 | 20 (3) |
| Penicillic acid | 20 | 26 (3) |
| Rubratoxin B | 2 | 91 (1) |
| Stemphone | 2 | 83 (6) |
| T-2 toxin | 2 | 100 (0) |
| Zearalenone | 10 | 18 (2) |

[a] Most values are means of two independent experiments; the toxicity of aflatoxin $B_1$, T-2 toxin, and stemphone was evaluated in one experiment only. Control mortality in brackets.

TABLE 2. *$LC_{50}$ values of mycotoxins to brine shrimp larvae (assay method)*

| Toxin | $LC_{50}$ ($\mu$g/ml)[a] | |
|---|---|---|
| | Expt. 1 | Expt. 2 |
| Aflatoxin $G_1$ | 1.3 ± 0.4 | 1.3 ± 0.1 |
| Diacetoxyscirpenol | 0.47 ± 0.11 | |
| Gliotoxin | 3.2 ± 0.2 | 3.7 ± 0.4 |
| Ochratoxin A | 10.1 ± 1.6 | |
| Sterigmatocystin | 0.54 ± 0.11 | |

[a] Mean ± SE.

TABLE 3. *Toxicity to brine shrimp larvae of culture filtrates and chloroform extracts of 70 fungal isolates from feeds and foods (disc screening method)*

| Species | Isolate no. | Filtrate | | | Extract | | |
|---|---|---|---|---|---|---|---|
| | | Toxicity[a] | Medium[b] | Toxin found | Toxicity[a] | Medium[b] | Toxin found |
| *Alternaria tenuis* | 1 | NT | | | VT | YES | ND[c] |
| *Alternaria* sp. | 2 | E[d] | | | T | Corn meal | ND |
| *Aspergillus candidus* | 3–4 | NT | | | T | CD + YE | ND |
| | 5–14 | E | | | NT | | |
| *A. glaucus* | 15–16 | NT | | | NT | | |
| | 17–34 | E | | | NT | | |
| *A. flavus* | 35 | E | | | VT | YES | Aflatoxin $B_1$, $B_2$ |
| *A. niger* | 36–37 | NT | | | NT | | |
| | 38–39 | NT | | | ST | YES | |
| | 40 | NT | | | ST | SM | |
| | 41 | NT | | | ST | CD + YE | |
| | 42–44 | NT | | | T | YES | ND |
| | 45–46 | NT | | | VT | YES | ND |
| | 47 | T | CD + YE | Oxalate, 3.6 mg/ml | VT | YES | ND |
| | 48 | T | YES | Oxalate-positive | VT | YES | ND |
| | 49 | VT | SM | Oxalate, 6.1 mg/ml | T | YES | ND |
| *Aspergillus* spp. | 50 | T | YES | Kojic acid | VT | YES | Kojic acid |
| | 51 | VT | YES | Kojic acid | T | CD + YE | L[e] |
| | 52 | VT | YES | ND | VT | CD + YE | ND |
| | 53 | VT | YES | Kojic acid | VT | YES | Kojic acid |
| | 53 | T | SM | Kojic acid, β-nitropropionic acid | T | SM | Kojic acid |
| *Fusarium* spp. | 54–56 | E | | | VT | Corn meal | ND |
| | 54–56 | E | | | VT | YES | ND |
| | 57 | E | | | VT | Corn meal | Zearalenone |
| | 57 | E | | | T | YES | ND |
| | 58 | E | | | VT | Corn meal | T-2 toxin |
| | 58 | E | | | ST | YES | |
| | 59 | E | | | VT | Corn meal | ND |
| | 59 | E | | | VT | YES | T-2 toxin and related compound |
| *Geotrichum candidum* | 60 | NT | | | NT | | |
| *Mucor* sp. | 61 | NT | | | NT | | |
| *Penicillium viridicatum* | 62 | E | | | T | YES | Ochratoxin A |
| *Penicillium* spp. | 63–67 | NT | | | VT | YES | ND |
| | 68 | T | YES | ND | ST | YES | |
| | 69 | VT | YES | Kojic acid | VT | YES | Kojic acid |
| | 69 | T | SM | Kojic acid, β-nitropropionic acid | T | SM | Kojic acid |
| *Trichothecium roseum* | 70 | E | | | VT | Corn meal | ND |
| | 70 | E | | | VT | YES | ND |

[a] Toxicity expressed as NT, nontoxic, 0 to 9% mortality; ST, slightly toxic, 10 to 49% mortality; T, toxic, 50 to 89% mortality; VT, very toxic, 90 to 100% mortality.

[b] Medium recorded for most isolates is that of highest toxicity; YES, yeast extract-sucrose medium; SM, Sabouraud's maltose agar; CD + YE, Czapek-Dox solution agar containing 0.5% yeast extract.

[c] Mycotoxins not detected.

[d] Whole culture extracted; see under "Extract" for toxicity.

[e] Sample lost.

TABLE 4. *Toxicity in relation to lipid residue from chloroform extracts of some fungal isolates grown in yeast extract-sucrose medium (assay method)*

| Isolate no. | Species | Lipid (mg/ml of BSM)[a] | Mortality[b] (%) |
|---|---|---|---|
| 54 | *Fusarium* | 0.02 | 98 |
| 56 | *Fusarium* | 0.06 | 100 |
| 59 | *Fusarium* | 0.04 | 94 |
| 70 | *Trichothecium* | 0.01 | 90 |

[a] Brine shrimp medium.
[b] Control mortality, 4%.

mycotoxin 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone caused no deaths when tested at 10 μg of BSM per ml with the assay method. β-Nitropropionate and oxalate were toxic at high concentrations only. Of the toxins tested, diacetoxyscirpenol and sterigmatocystin were more toxic to the larvae than aflatoxin $B_1$ and $G_1$. The $LC_{50}$ values obtained for aflatoxin $G_1$ and gliotoxin in two different experiments (Table 2) indicated good reproducibility with this method.

**Toxicity of culture filtrates and extracts.** Table 3 shows, for most isolates, only those media from which the most T or VT filtrates and extracts were obtained. Many isolates grew well in YES, SM, and CD + YE but only poorly in CD. Some isolates grew a thick mycelial mat on several media but proved T or VT in one medium only. In some instances, poorly developed mycelial mats yielded T or VT extracts. YES yielded T or VT filtrates and extracts more frequently than the other media. Nine out of 32 isolates produced, in at least one medium, culture filtrates that were either T or VT. Of the 70 isolates, 27 showed toxicity in chloroform extracts obtained from at least one medium. The data in Table 4 confirmed the high toxicity of the *Fusarium* and *Trichothecium* extracts observed with the disc screening method.

**Known mycotoxins detected.** Mycotoxins detected by TLC are recorded in Table 3. β-Nitropropionic acid was present in two T SM filtrates at a concentration of approximately 1 mg/ml (isolates no. 53 and 69); a lesser amount (about 0.25 mg/ml) was detected in a NT filtrate (isolate no. 50), out of several selected at random. No β-nitropropionic acid was detected in any of the T or VT mycelial extracts.

Concentrations of kojic acid, when present in the T or VT filtrates, ranged from approximately 3 to 10 mg/ml; about 0.2 to 0.5 mg/ml was present in several of the corresponding mycelium extracts. No kojic acid was detected in the ST or NT filtrates (ferric-chloride test).

The toxicity of four filtrates (isolates no. 50, 51, 53, and 69) can be explained by the presence of kojic acid. The presence of oxalate in culture filtrates from isolates no. 47 and 49, which contained 3.6 mg/ml and 6.1 mg/ml, respectively, may partially account for their toxicity. Isolate no. 48 produced little oxalate. In view of the low sensitivity of the larvae to oxalate, factors other than oxalate alone may have been responsible for the observed high mortality in this filtrate. No toxins were identified in the filtrates of no. 52 and 68. The toxicity of some chloroform extracts could be attributed to aflatoxin $B_1$ and $B_2$ (no. 35), kojic acid (no. 50, 53, and 69), zearalenone (no. 57), T-2 toxin (no. 58 and 59), and ochratoxin A (no. 62). In the remaining 26 extracts that were either T or VT no known mycotoxins were detected.

**Isolation of "TLC-pure" unknown toxin from a Fusarium spp.** Most of the toxicity of the chloroform extract of a YES culture of isolate no. 59, after initial separation into seven fractions by preparative TLC, lay in the T-2 toxin fraction and a more polar fraction. The latter fraction contained a substance with an $R_F$ value half that of T-2 toxin in the toluene-ethyl acetate-formic acid solvent system. The material behaved the same as T-2 toxin toward the anisaldehyde spray (17) and was highly toxic to the brine shrimp larvae after further purification by TLC. Microacetylation with acetic anhydride and pyridine yielded a spot with the same $R_F$ value in two solvent systems as the spot obtained by acetylation of T-2 toxin. The unknown toxin is possibly a desacetyl T-2 toxin (2).

## DISCUSSION

Brine shrimp larvae were found to be convenient test organisms for toxic fungal metabolites. Larvae were readily obtained in large numbers, and their low natural mortality in controls involving media and chloroform made it possible to test crude fungal filtrates and extracts. As the natural mortality in 1% methanol and acetone solutions was low, these solvents can be used to help dissolve the less water-soluble components of lipid extracts. In most experiments in which the assay method was used, a straight-line relationship between probit mortality and logarithm of the concentration of mycotoxin could be demonstrated. Two failures (*not reported*) to establish this relationship may be attributed to variable natural mortality as observed in some control solutions. This may have resulted from toxicity associated with the glassware used and may be avoided by the use of disposable containers. Disposable containers were used in the disc screening method, and natural mortality was less variable. $LC_{50}$ values were determined by using

the maximum likelihood solution (9), which corrects for natural mortality. When the amount of toxin available is ample, the use of a greater number of concentrations and replicates would be preferable to reduce variation in $LC_{50}$ values.

As is probably the case with any one organism, brine shrimp larvae showed a wide range of sensitivity to different mycotoxins. The order of sensitivity of the brine shrimp larvae to many of the toxins tested was comparable to that of zebra fish larvae (1). Both systems were found highly sensitive to sterigmatocystin. The results indicate that most of the known mycotoxins tested (e.g., aflatoxins $B_1$ and $G_1$ and diacetoxyscirpenol) could have been discovered with the brine shrimp system, whereas a few of the toxins tested (e.g., luteoskyrin and 4-acetamido-4-hydroxy 2-butenoic acid $\gamma$-lactone) would have remained undetected. The use of additional biological screening systems may reduce the likelihood of this occurrence. Even with this one system, however, a number of filtrates and extracts were found to possess toxicity that could not be explained by the presence of the known mycotoxins looked for. It is unlikely that this finding is chiefly due to "false positives" resulting from sensitivity of the larvae to commonly occurring fungal constituents. Isolates that grew equally well on two different substrates proved T or VT in one substrate but NT in the other. This suggests that nonspecific toxicity from common fungal constituents is low. To evaluate the toxicity of a certain isolate, it may be advisable to determine the amount of lipid residue responsible for the mortality, as was done for three *Fusarium* and one *Trichothecium* isolate (Table 4). Since a large proportion of the lipid residue from these isolates probably consisted of nontoxic storage lipids and structural lipids, the toxicity of the actual toxin in these extracts is likely to be high. The high toxicity of isolate no. 59 (YES) could be attributed to the presence of two toxins in the lipid residue, T-2 toxin and a related compound.

ACKNOWLEDGMENTS

We are grateful to C. E. Higgens for providing unpublished information on the use of brine shrimp larvae. We thank J. W Lawrence, Doreen Ready, and Y-K. Chen for technical assistance.

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