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# Effects of Temperature, Water Activity, and Incubation Time on Production of Aflatoxins and Cyclopiazonic Acid by an Isolate of *Aspergillus flavus* in Surface Agar Culture

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**An experiment with a full factorial design was used to study the effects of and interactions among temperature, water activity ($a_w$), incubation period, and substrate on coproduction of aflatoxins (AF) and cyclopiazonic acid (CPA) by an isolate of *Aspergillus flavus*. Analysis of variance showed that there was a complex interaction among all of these factors and that this influenced the relative concentrations of the mycotoxins produced. The optimum temperatures for the production of AF and CPA were 30°C and 25°C, respectively. Both mycotoxins were maximally produced (0.306 to 0.330 μg of AF · ml of medium$^{-1}$, 4.040 to 6.256 μg of CPA · ml of medium$^{-1}$) at an $a_w$ of 0.996 and after 15 days of incubation. No AF were produced in either yeast extract agar or Czapek yeast autolysate agar medium at an $a_w$ of 0.90 at 20 or 37°C after 15 days (minimum conditions), while 0.077 to 0.439 μg of CPA · ml of medium$^{-1}$ was produced under the same conditions. Yeast extract agar favored maximum AF production, and Czapek yeast autolysate agar favored maximum CPA production.**

Mycotoxins are secondary metabolites produced by specific filamentous fungi that cause a toxic response when introduced by a natural route in low concentrations to higher vertebrates and other animals (45). While some mycotoxins are produced by only a limited number of fungal species, others may be produced by a relatively large range of species from several genera (15, 39). It is increasingly apparent that most toxigenic fungi have the potential to produce more than one mycotoxin (7, 16).

The interaction between water activity ($a_w$) and temperature is a most critical determinant for fungal growth (2) and for mycotoxin production (25). Conditions for mycotoxin production are generally more restrictive than those for growth and can differ between different mycotoxins produced by the same species and between fungi producing the same mycotoxin (16). Bacon et al. (3) showed that a strain of *Aspergillus ochraceus* Wilhelm, producing both ochratoxin A and penicillic acid, produced the most ochratoxin A at an $a_w$ of 0.98 and 30°C and the most penicillic acid at an $a_w$ of 0.90 and 22°C.

*A. flavus* Link and *A. parasiticus* Speare are prominent in certain foods, animal feeds, and storage mycology because of their ability to produce aflatoxins (AF). Isolates of *A. flavus*, in particular, have been reported to coproduce AF, cyclopiazonic acid (CPA), and other mycotoxins in differing amounts (17, 22, 41, 47, 54). The coproduction of these mycotoxins may result in additive or synergistic effects on consumers (10, 37) and may thus increase the toxigenic potential of *A. flavus* (4, 7).

The effects of temperature and $a_w$ on the production of AF by *A. flavus* have been widely studied, but there is no information on how these factors affect CPA production. Reported optimum temperatures for the production of $AFB_1$ range from 24 to 35°C (9, 32, 35, 42). AF production declines with decreasing temperature and has been reported to cease between 10 and 13°C (22, 42). Reported minimal $a_w$ values for AF production by *A. flavus* range from only 0.81 to 0.82 (24, 31) to 0.83 to 0.87 (16).

A range of statistical designs have been used for biological experiments, some over several decades (5, 40). Many of these designs are valuable for the study of changes in more than one variable (43). Approaches in which one variable is varied at a time, although they have been useful and have invariably contributed to our knowledge of fungal growth and toxin production, not only are tedious but could lead to misinterpretation of the results since interactions between different factors could be overlooked. The usefulness of statistically designed experiments in the study of fungal growth and mycotoxin production has been well demonstrated. The designs applied include multifactorial designs analyzed by principal-component analysis (1, 44) and full factorial design (11). These statistical designs have been used as tools to enable workers to make intelligible interpretations of laboratory observations rather than as laboratory methods in their own right.

Most published studies on mycotoxin formation have been concerned with single mycotoxins. Few have examined how environmental factors can affect simultaneous production of two or more mycotoxins by a single isolate. This report describes the interaction of temperature, $a_w$, and time in determining the production of AF and CPA by a coproducing isolate of *A. flavus* on two agar media in an experiment with a full factorial design. Agar media were used to minimize other sources of variation and to identify clearly the effects of temperature and $a_w$.

## MATERIALS AND METHODS

**Experimental design.** The factors and levels of each factor used in this study included $a_w$ (0.996, 0.95, 0.9, and 0.85), temperature (37, 30, 25, and 20°C), and incubation time (20, 15, 10, and 5 days). Two media, yeast extract sucrose agar (YES) (27) and Czapek yeast autolysate agar (CYA) (38), were used. To deter-

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TABLE 1. ANOVA for CPA production by *A. flavus* in a full factorial experiment

| Source | df[a] | Mean square | $F^b$ | *P* value |
|---|---|---|---|---|
| Days | 3 | 43,798.800 | 70.45 | <0.001 |
| Medium | 1 | 625.974 | 1.01 | 0.317 (NS)[c] |
| Temp | 3 | 32,268.636 | 51.91 | <0.001 |
| $a_w$ | 3 | 16,6419.520 | 267.70 | <0.001 |
| Days-medium | 3 | 2,388.161 | 3.84 | 0.010 |
| Days-temp | 9 | 3,587.506 | 5.77 | <0.001 |
| Days-$a_w$ | 9 | 7,344.397 | 11.81 | <0.001 |
| Medium-temp | 3 | 5,407.812 | 8.70 | <0.001 |
| Medium-$a_w$ | 3 | 1,416.942 | 2.28 | 0.080 (NS) |
| Temp-$a_w$ | 9 | 9,388.483 | 15.10 | <0.001 |
| Days-medium-temperature | 9 | 1,422.274 | 2.29 | 0.017 |
| Days-medium-$a_w$ | 9 | 1,061.087 | 1.71 | 0.088 (NS) |
| Days-temp-$a_w$ | 27 | 1,858.047 | 2.99 | <0.001 |
| Medium-temp-$a_w$ | 9 | 4,187.771 | 6.74 | <0.001 |
| Days-medium-temp-$a_w$ | 27 | 1,796.132 | 2.89 | <0.001 |
| Error | 256 | 621.661 | | |
| Total | 383 | | | |

[a] df, degrees of freedom.
[b] *F*, variance ratio.
[c] NS, not significant ($P > 0.05$).

mine the effect of each factor on AF and CPA production and the interactions among the factors, a full factorial experimental design was used (12, 13). Measurements for each combination of factors were carried out in triplicate.

**Fungus, media, and culture conditions.** *A. flavus* F2R4FP1-5, a known coproducer of AF and CPA, was kindly supplied by R. J. Cole (U.S. Department of Agriculture National Peanut Research Laboratory, Dawson, Ga.). Cultures were maintained on Oxoid malt extract agar at 30°C for 7 to 10 days until conidiation occurred. Conidia were harvested by using a sterile glycerol-water solution containing 0.1% Tween, with the $a_w$ adjusted to match that of the growth medium by the methods of Gervais et al. (18) and Gonzalez et al. (20). The $a_w$ of representative samples of glycerol-water solutions and agar media was measured with a dew point meter (Protimeter Ltd.).

Petri dishes (90-mm diameter), each containing agar medium (ca. 20 ml) with the $a_w$ adjusted to 0.996, 0.95, 0.9, and 0.85 with glycerol as outlined by Dallyn and Fox (8), were inoculated with 1 ml of a conidial suspension containing $10^6$ conidia which was spread uniformly over the agar surface. The concentration of conidia had been adjusted with a Neubauer hemacytometer. Inoculated plates with the same $a_w$ were sealed in sterile polyethylene containers, in which the $a_w$ was controlled by a glycerol-water solution with the same $a_w$ prepared by the method of Gervais et al. (18) and which acted as humidity chambers (29), and were incubated at 20, 25, 30, and 37°C. Plates were withdrawn and analyzed for $AFB_1$, $AFB_2$, and CPA after 5, 10, 15, and 20 days of incubation.

**Extraction and analysis of mycotoxins.** On each sampling occasion, the contents of each test plate were macerated in a Waring blender with 100 ml of dichloromethane-methanol (80:20). The slurry was twice filtered through a Büchner funnel with Whatman no. 1 filter paper and then concentrated to dryness with a rotary evaporator. The residue was extracted by the method outlined by Gorst-Allman and Steyn (21) for multimycotoxin analysis, by using dichloromethane instead of chloroform. The residue was partitioned between 200 ml of dichloromethane-distilled $H_2O$ (1:1), and the dichloromethane layer was extracted three times with a saturated $NaHCO_3$ solution (100 ml). The dichloromethane layer, containing AF, was rotary evaporated and concentrated under a gentle stream of nitrogen. The aqueous layer, containing CPA, was acidified to pH 2.0 with 0.5 M HCl and extracted three times with dichloromethane (100 ml). The extract was evaporated and concentrated as for AF. The mycotoxins were stored in colored vials at 4°C until required.

The qualitative presence of AF and CPA was determined by thin-layer chromatography (TLC) separation on silica gel G60 plates (20 by 20 cm; Merck). The plates were first dipped in a 10% (wt/wt) solution of oxalic acid in methanol for 2 min, and after being heated at 110°C for 2 min and cooled, the plates were spotted with 50 μl of the respective extract (dissolved in 1 ml of methanol) and developed in the solvent mixture toluene-ethyl acetate-dichloromethane-formic acid (70:50:50:20) (19).

The developed plates were viewed under longwave UV light (366 nm). Any $AFB_1$ and $AFB_2$ present fluoresced blue under these conditions. CPA was

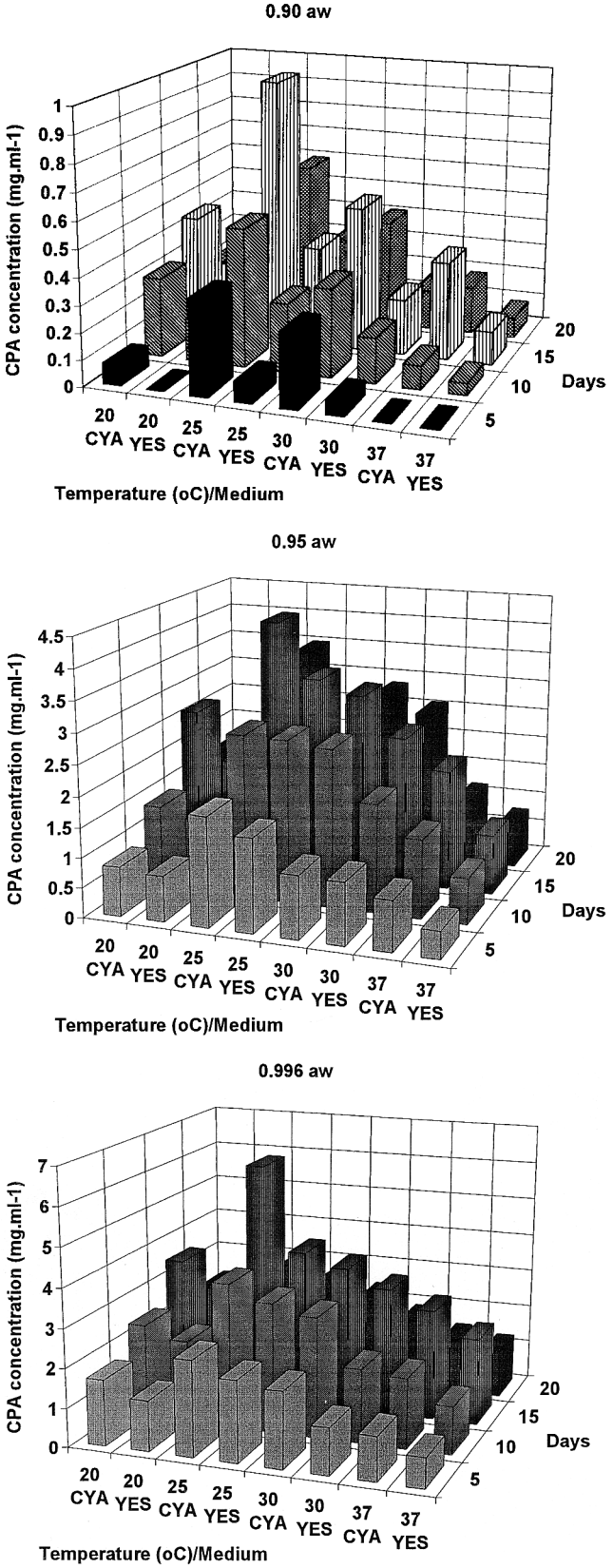


FIG. 1. Interaction between water activity and temperature and their effect on CPA production by *A. flavus* on YES or CYA over a 20-day period.

TABLE 2. ANOVA for AF production by *A. flavus* in a full factorial experiment

| Source | df[a] | Mean square | $F^b$ | *P* value |
|---|---|---|---|---|
| Days | 3 | 31.215 | 22.49 | <0.001 |
| Medium | 1 | 23.359 | 16.83 | <0.001 |
| Temp | 3 | 42.168 | 30.39 | <0.001 |
| $a_w$ | 3 | 461.583 | 332.63 | <0.001 |
| Days-medium | 3 | 9.310 | 6.71 | <0.001 |
| Days-temp | 9 | 6.046 | 4.36 | <0.001 |
| Days-$a_w$ | 9 | 12.359 | 8.91 | <0.001 |
| Medium-temp | 3 | 13.978 | 10.07 | <0.001 |
| Medium-$a_w$ | 3 | 12.847 | 9.26 | <0.001 |
| Temp-$a_w$ | 9 | 22.654 | 16.32 | <0.001 |
| Days-medium-temp | 9 | 2.919 | 2.10 | 0.030 |
| Days-medium-$a_w$ | 9 | 3.842 | 2.77 | 0.004 |
| Days-temp-$a_w$ | 27 | 8.277 | 5.96 | <0.001 |
| Medium-temp-$a_w$ | 9 | 1.7360 | 12.51 | <0.001 |
| Days-medium-temp-$a_w$ | 27 | 2.984 | 2.15 | <0.001 |
| Error | 256 | 1.388 | | |
| Total | 383 | | | |

[a] df, degrees of freedom.
[b] *F*, variance ratio.

viewed after spraying with Ehrlich's reagent (2.0 g of *p*-dimethylaminobenzaldehyde in 100 ml of HCl) with subsequent development of a purple color.

Quantitative determination of AF was achieved by scraping the fluorescent AF spots on TLC plates (not dipped in oxalic acid as described above) and dissolving them in 5 ml of methanol. The mixture was filtered through a sintered glass filter (Sintaglass no. 4) and a Büchner funnel under a low vacuum. This clear methanol mixture was used to determine total AF by solution fluorometry with bromine (46) by using a Sequoia-Turner 450 Digital Fluorometer (excitation, 360 nm; emission, 450 nm) after reaction of a 1-ml sample with 1 ml of a bromine solution (diluted $10^6$ times with distilled $H_2O$). This method had a detection limit of 0.001 μg of total AF · ml of sample$^{-1}$.

Small quantities of CPA (<0.05 μg · ml of sample$^{-1}$) were quantified by comparing the intensity of spots from samples, developed on TLC as described above, with that from a standard range of concentrations of CPA (0.01, 0.02, 0.03, and 0.04 μg · ml$^{-1}$) and by spectrophotometric measurement of CPA sample spots in methanol solution (log ε = 4.31 at 284 nm; molecular weight, 336) (26). The detection limit of this method was 0.02 μg of CPA · ml of sample$^{-1}$. Higher concentrations of CPA (>0.05 μg · ml$^{-1}$) were determined by the spectrophotometric method of Chang-Yen and Bidasee (6). This method had a detection limit of 0.08 μg of CPA · ml of sample$^{-1}$.

**Statistical analyses.** The data were analyzed by using the analysis of variance (ANOVA) and GLM commands in the statistical software package Minitab, version 9.2.

## RESULTS

**Interactive effects of $a_w$, temperature, incubation time, and type of medium on CPA production by *A. flavus*.** The ANOVA for CPA production (Table 1) showed that the most significant single factors ($P < 0.001$) affecting CPA production by this isolate of *A. flavus* were $a_w$, incubation time, and temperature. Alone, composition of the agar media had no significant effect ($P > 0.05$). However, all two-, three-, and four-factor interactions were highly significant ($P < 0.001$), except incubation time-medium and incubation time-medium-temperature, which were significant, respectively, at $P \leq 0.01$ and $P < 0.05$. The $a_w$-medium and $a_w$-incubation time-medium interactions were not significant ($P > 0.05$).

Conidia of the *A. flavus* isolate germinated but failed to develop further during incubation at an $a_w$ of 0.85. At all other $a_w$-temperature combinations, the conidia germinated and grew well, although the rate was slow at an $a_w$ of 0.90. The amount of CPA produced was determined by the complex interaction of $a_w$, incubation time, temperature, and medium, and their effects on CPA production by the *A. flavus* isolate are shown in Fig. 1. The most CPA was produced after 10 to 20 days of incubation in both media. The highest levels of CPA (6.256 and 4.04 μg · ml$^{-1}$) were produced at an $a_w$ of 0.996, the highest $a_w$ tested when using media with no glycerol, after 15 days of incubation at 25°C. Very little or no CPA was produced in either medium at an $a_w$ of 0.90 and 37°C after 5 days. At an $a_w$ of 0.95 and 30°C, production of CPA was approximately 50 to 60% of that obtained at an $a_w$ of 0.996 and 25°C.

As the $a_w$ of the media was decreased, the concentration of CPA produced also decreased. At temperatures of 20 to 37°C at all $a_w$ values, the CPA concentration was the least at 37°C, followed by 20 and 30°C, and the most was produced at 25°C. No CPA was produced at an $a_w$ of 0.90 and 20 or 37°C or after 5 days of incubation in YES; when up to 0.078 μg · ml$^{-1}$ was produced in CYA. At an $a_w$ of 0.996 and 25°C, 4.040 μg of CPA · ml$^{-1}$ was produced in YES after 15 days incubation, compared to 6.256 μg · ml$^{-1}$ in CYA.

**Interactive effects of $a_w$, temperature, incubation time, and type of medium on AF production by *A. flavus*.** The ANOVA for AF production (Table 2) showed that all single factors and two-, three-, and four-factor interactions were highly significant at $P < 0.001$, affecting AF production by this isolate of *A. flavus*, with the exception of incubation time-medium-temperature and incubation time-medium-$a_w$, which were significant at $P < 0.01$ and $P < 0.05$, respectively. Among the single factors tested, $a_w$ had the greatest effect ($F = 332.63$).

The combined effects of $a_w$, incubation time, medium, and temperature on the production of AF on the two agar media are shown in Fig. 2. The concentrations of AF produced in both media by this isolate were generally small, although YES supported about 30% greater production than did CYA. No AF were produced at an $a_w$ of 0.90 and 20 or 37°C throughout the incubation period, and production was the least at this $a_w$ and other temperatures. The highest concentrations of AF produced at an $a_w$ of 0.90 (0.096 μg · ml$^{-1}$ at 30°C in YES and 0.076 μg · ml$^{-1}$ at 30°C in CYA) were produced after 15 days.

At an $a_w$ of 0.95, the highest concentrations of AF, produced at 30°C after 15 days of incubation, were 0.226 μg · ml$^{-1}$ on YES and 0.183 μg · ml$^{-1}$ on CYA. The lowest measured concentrations, produced after 5 days at 20°C, were 0.038 μg · ml$^{-1}$ on YES and 0.028 μg · ml$^{-1}$ on CYA.

At an $a_w$ of 0.996, the highest AF concentrations (0.330 μg · ml$^{-1}$ on YES and 0.306 μg · ml$^{-1}$ on CYA) were found at 30°C after 15 days of incubation. At all temperatures, AF were produced most rapidly from 5 to 15 days after inoculation, after which there was a decrease in the concentration. For each medium, the smallest measured AF concentrations (0.106 μg · ml$^{-1}$ on YES and 0.070 μg · ml$^{-1}$ on CYA) were produced after 5 days of incubation at 20°C.

In general, the greatest production of both mycotoxins was determined by a complex interaction among $a_w$, temperature, incubation time (days), and medium. However, conditions for maximum production of the mycotoxin were different. Interactions involving YES favored the production of greater concentrations of AF, while those involving CYA favored CPA. The optimum temperatures for AF and CPA production were, respectively, 30 and 25°C. The greatest amounts of both mycotoxins were produced at an $a_w$ of 0.996 after 15 days of incubation.

## DISCUSSION

Semisynthetic agars have been used extensively to investigate mycotoxin production by *Aspergillus* species at different $a_w$

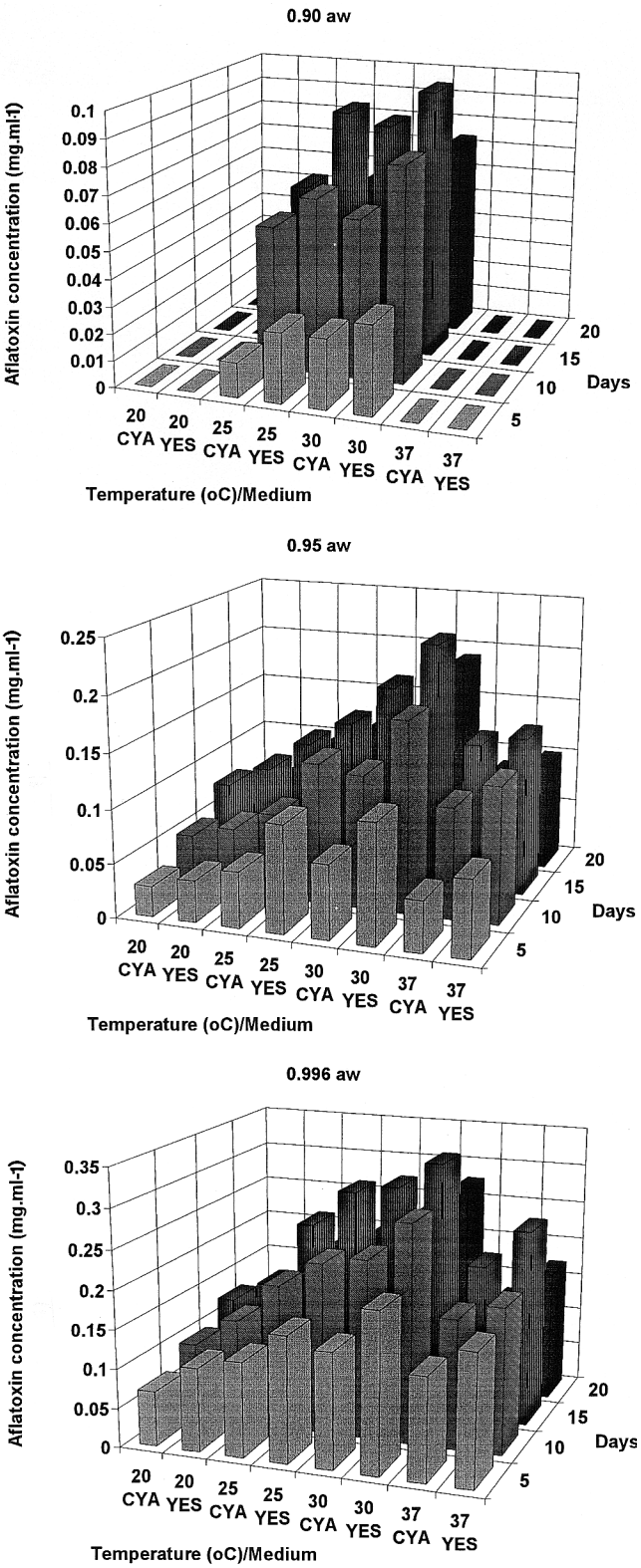


FIG. 2. Interaction between $a_w$ and temperature and their effect on AF production by *A. flavus* on YES and CYA over a 20-day period.

values and temperatures (32–35). All of these studies involved single mycotoxins from individual *Aspergillus* isolates. Northolt et al. (32) demonstrated that *A. flavus* isolates can differ in their optimum temperatures for AF production. Similar to the present studies, theirs also showed that the optimum $a_w$ for AF production was determined not only by temperature and fungal isolate but also by substrate.

Le Bars (26) reported optimum CPA production on Czapek agar at 25°C, while Kheiralla et al. (23) showed that several *A. flavus* isolates produced the most AF at 30°C. Frisvad (14) and Frisvad and Filtenborg (15) showed that YES was useful for mycotoxin production by filamentous fungi. Park and Bullerman (36) showed that YES was particularly suitable for AF production.

Fungal growth and mycotoxin production are determined by the $a_w$ of the medium and its temperature. However, studies have generally been limited to the production of single mycotoxins by different fungi growing on semisynthetic agars. The production of two or more mycotoxins by a single fungus has rarely been studied. However, Bacon et al. (3) studied ochratoxin and penicillic acid production by *A. ochraceus*, and Magan et al. (28) studied alternariol, altenuene, and alternariol monomethyl ether production by *Alternaria alternata.*

Nielsen et al. (30), when studying the effects of temperature, light, and $a_w$ on the fumitremorgin A and C production by *Neosartorya fischeri* on CYA, found that the organism also coproduced verruculogen. The optimum temperatures for production of the three mycotoxins were, respectively, 25, 30, and 37°C. Fumitremorgin production was retarded at 15°C. The greatest fumitremorgin production occurred at an $a_w$ of 0.980 when CYA was supplemented with glucose or fructose and at an $a_w$ of 0.990 on CYA supplemented with sucrose. Fumitremorgins were produced in glucose-supplemented CYA down to an $a_w$ of 0.925 but not below an $a_w$ of 0.97 on sucrose-supplemented CYA. Production of verruculogen was greatest on all of the test media, followed by that of fumitremorgin A and that of fumitremorgin C.

Statistical assessment of the significance of different treatments requires the application of valid experimental designs and sound statistical analysis. Factorial designs allow the analysis of interactions between a range of different factors applied at different dose levels simultaneously and are economical and save time. They have previously been used with principal-component analysis to determine the interactions among fungal colonization, insect and mite infestation, and environmental factors in cereal grains but have not been used before to determine the effects of environment on the production of different metabolites by a single fungus. However, Ellis et al. (11) used a factorial design to study the effects of inoculum concentration, modified atmosphere, $a_w$, and temperature on AF production by *A. flavus*.

In the present study, the use of a full factorial design demonstrated the complex factors controlling mycotoxin production by fungi and helped to explain their variable concentrations in natural substrates. None of the factors studied had an overriding effect on mycotoxin production, but the level and duration of each contributed to the final outcome. The two toxins were not necessarily affected in the same way, as they had different optimum temperatures and minimum $a_w$ values for production, although both were produced optimally at an $a_w$ close to 1.00. Similarly, relative amounts of the toxins produced by *Alternaria alternata* in cereal grains differ with environmental conditions (28).

The role of two or more toxins produced simultaneously by a fungus has yet to be fully explained. Coproduction of such toxins may have additive or synergistic effects on competitors

or consumers (10, 37), thus increasing the toxigenic potential of the producer fungus, in this example, *A. flavus*, allowing it to colonize more substrate and aiding its survival in a particular ecological niche. It may thus modify the competitive ability of competing fungi and other microorganisms and inhibit their invasion of already colonized substrate. It may also decrease consumption of the substrate by animals, insects, or mites by making the substrate distasteful or by decreasing the populations of such predators. The inhibition by aflatoxigenic *A. flavus* of colonization of maize grains by *Fusarium moniliforme* has been shown by Wicklow and Shotwell (52). Invertebrates, because they may consume large quantities of mycotoxin-containing material in stored grains, could be particular targets for inhibition by mycotoxins (49). Dowd (10) has shown synergism between the activities of AF and kojic acid against insects. The effects of CPA and AF on lethality, body weight change, and hepatic cell pathology in guinea pigs are also synergistic (37). Synergism between ochratoxin and penicillic acid has also been reported (48). The presence of mycotoxins in survival structures, e.g., sclerotia of *A. flavus*, could also be of particular importance, preventing their colonization by hyperparasites or predation by insects during the period between crops (50, 51, 53).

The production of two mycotoxins may be a consequence of coevolution of predators and host and reciprocal adaptation. A predator feeding on a host induces production of antipredator substances by the host. Such interactions may result in mutually dependent biochemical systems (46). Coevolution of predator and host is likely to be further forced by changes in agricultural practice or storage conditions. The production of several mycotoxins may aid survival of the fungus at the cost of making the substrate unusable for subsequent consumption by animals and humans.

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