Production of Penicilllic Acid and Ochratoxin A on Poultry Feed by *Aspergillus ochraceus*: Temperature and Moisture Requirements

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A strain of *Aspergillus ochraceus* Wilhelm, isolated from poultry feed, produced both penicilllic acid and ochratoxin A. Studies demonstrating the ability of this fungus to colonize poultry feed and produce these two mycotoxins under various temperatures and moistures indicated that the interaction was complex. The optimal temperature for conidial development did not vary with moisture, but accumulation of both toxins did. A combination of low temperature, 15 or 22 °C, and low moisture favored the production of penicilllic acid, whereas high temperature, 30 °C, and high moisture favored the production of ochratoxin A.

The development of fungi proceeds only in an aqueous system whether the substrate is liquid or solid; thus, moisture is of major significance in determining the extent of fungal colonization. In addition, development is influenced by other factors such as temperature, light quality, and the chemical nature and availability of substrata. Studies of these factors suggest that temperature and moisture relations primarily determine the relative abundance of fungi on stored products (2, 4, 27, 31). Knowledge of the manner in which these factors interact to influence the nature of secondary metabolites of fungi is needed, especially since many of these metabolites are toxic. Furthermore, quantitative data on the effects of these factors on the ecology of fungi on stored feed products are essential if we are to control their growth or predict their biochemical activities.

While investigating the occurrence and distribution of possible toxigenic mycoflora in poultry houses and poultry feed, two strains of *Aspergillus ochraceus* were isolated, and both were found capable of producing penicilllic acid and ochratoxin A. Penicilllic acid, a carcinogenic lactone, first was isolated from *Penicillium puberulum* Bainier (1) but has since been reported from several species of *Aspergillus* and *Penicillium* (15). Ochratoxin A, a nephrotoxic dihydrosoconuamarin, first was isolated from *A. ochraceus* Wilhelm (32) and is now known to be produced by other members of the ochraceus group (12, 15) as well as by *P. viridicatum* Westling (33). The mean lethal dose (LD₅₀) (subcutaneous) of penicilllic acid for mice is 100 mg/kg, although a dose as low as 0.1 mg is carcinogenic (5); the LD₅₀ (oral) of ochratoxin A is 100 to 200 μg/day-old cockerel and 20 mg/kg in rats (28). The toxicology of these two mycotoxins has been reviewed by Lillehoj et al. (15). Poultry feed is subjected to varying degrees of moisture during transportation and storage and while in the feed trough due to differences in relative humidity and the feeding habits of the birds. Consequently, a study was initiated to determine whether these two toxins could be produced on poultry feed at different moisture contents and under constant light as practiced in broiler houses. Such information would be significant because many investigations have indicated that poultry are susceptible to mycotoxins (9, 11, 16, 26). Recently, the work of Choudhury et al. (3) demonstrated that graded levels of ochratoxin A caused effects ranging from delayed sexual maturity and reduced egg production at low levels (1 ppm) to mortality at high levels (4 ppm).

In measuring the availability of water to microorganisms, the quantity of water activity (a_w) or the equilibrium relative humidity often is used (25). In this paper a_w will be used to describe the availability of water in feed under equilibrium conditions and is to be differentiated from some experiments in which moisture content was adjusted as a percentage weight of the feed.
MATERIALS AND METHODS

Source of culture. The A. ochraceus strains (103 and 107) used in this investigation were isolated from feed and litter samples obtained in local broiler houses. On the semisynthetic medium (7) strain 107 produced higher yields of both penicillic acid and ochratoxin A and was, therefore, used in this study. The strain was maintained at 5°C on Czapek agar. For the preparation of spore suspensions used for inoculations, subcultures were made on Czapek agar supplemented with 20% sucrose (20).

Moisture content. Flasks (125 ml) containing 50 g of poultry feed were stoppered with stainless-steel closures and autoclaved for 20 min at 121°C. After autoclaving, the sterilized feed samples were brought to the required moisture content by gradually adding sterile distilled water. Each gradual addition was accompanied by thoroughly shaking the sample to assure that the water content was as uniform as possible. Each sample was finally mixed for 15 min on a wrist action shaker. As a check for each of the required moisture contents, a 10-g sample was removed and the moisture was determined. All samples had an initial moisture content of 11.5% after autoclaving. Moisture contents were adjusted to the following final percentages: 14.0, 18.0, 23.0, 32.0, 42.0, 52.0, and 62.0. The feed was inoculated with spores from 10-day-old cultures and incubated for 12 days at ambient temperatures under constant light supplied by 40-W fluorescent lamps.

Water activity and temperature. The required a,w, in the feed was achieved and controlled by bringing the feed into water vapor equilibrium with solutions of NaCl (21). To determine the effects of a,w and temperature on toxin production, feed was ground in a Wiley mill (40-mesh screen) and air dried for 24 h. Samples (5 g) were placed in wide-mouth sampling jars (40 mm), capped, and sterilized for 15 min. The samples were then uncapped and transferred aseptically to desiccators. Each desiccator contained 250 ml of NaCl solution of known water activity (Table 1) and each was evacuated and maintained at 15, 22, and 30°C for 2 weeks to permit rapid equilibration of the feed with the corresponding relative humidity. A subsequent experiment determined that the feed in the evacuated desiccator reached equilibrium after 8 days at each of the temperatures. The water sorption isotherm of the poultry feed is given in Table 1.

| NaCl (m) | a,w | Water content (% dry wt) |
|---------|-----|------------------------|
|         |     | 15°C | 22°C | 30°C |
| 6.0     | 0.7598 | 10.22 | 14.03 | 14.02 |
| 5.0     | 0.8068 | 15.41 | 15.04 | 15.51 |
| 4.0     | 0.8515 | 21.26 | 22.47 | 22.20 |
| 2.8     | 0.9011 | 26.34 | 26.93 | 27.15 |
| 1.4     | 0.9532 | 38.25 | 38.49 | 39.34 |
| 0.1     | 0.9966 | 52.43 | 53.08 | 53.73 |

After equilibration, spores of A. ochraceus were dusted onto the feed, and the sampling jars were suspended over 200 ml of the various salt solutions contained in 950-ml wide-mouth Mason jars. The jars were sealed and immersed in pans of water which were placed in constant-temperature rooms under constant light. The water in the pans was adjusted to one of the three temperatures prior to introduction of the Mason jars. The feed was analyzed after 2 weeks of incubation. At each a,w the feed was examined under a binocular dissecting microscope and a compound microscope for growth and development of conidial heads.

Toxin analysis. Penicillic acid and ochratoxin A were extracted from the moldy feed with chloroform and methanol (1:1, vol/vol) in a Waring blender for 3 to 5 min. The suspension was filtered and the resulting chloroform-methanol extract was worked up by the sodium bicarbonate procedure of Steyn et al. (29). The resulting extract was evaporated under reduced pressure and the volume was adjusted to 4 ml with chloroform.

Penicillic acid was purified by preparative thin-layer chromatography on a Silica Gel G-254 by using chloroform as the moving phase. Crystallization from benzene gave a product (mp 86-87°C) which was identical to an authentic sample as determined by thin-layer chromatography, mass spectral data, and ultraviolet and infrared spectra. Penicillic acid was quantitatively determined in the final chloroform extracts as its trimethylsilyl derivative by using a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector. The column was 6 ft by ½ inch (182.88 by 0.318 cm) stainless steel and packed with 3% SE-30 Chromosorb W. Nitrogen flow rate was 45 ml/min. At a program rate of 8 degrees per min from 60 to 250°C, penicillic acid had a retention time of 11.5 min. Peak areas and retention times were measured with an Infotronics CRS-101 digital integrator. This procedure is similar to that described by Pero et al. (19), who demonstrated linearity of penicillic acid concentration with detector response.

Ochratoxin A was initially identified by chromatographing a sample on Silica Gel G (24). The identity of ochratoxin A was confirmed by co-chromatography with an authentic sample on Silica Gel G thin-layer plates with several solvent systems (24, 29, 30) and by comparison of ultraviolet spectra in acid and base. The concentration of ochratoxin A was determined spectrophotometrically at 333 nm after elution of the toxin from the plates with methanol (30). All experiments were done in triplicate and yields were reported as averages.

RESULTS

Toxin production at added moisture. Growth was not observed in feed samples at an initial moisture content of 11.5%. Microscope examination revealed that spores at this moisture level had not germinated. Growth and conidial development were observed at 14% moisture and higher. Although both mycotoxins

TABLE 1. Water activities and water sorption isotherms of poultry feed

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| 6.0     | 0.7598 | 10.22 | 14.03 | 14.02 |
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| 1.4     | 0.9532 | 38.25 | 38.49 | 39.34 |
| 0.1     | 0.9966 | 52.43 | 53.08 | 53.73 |
were produced under constant light, their patterns of accumulation differed (Fig. 1). Maximum production of penicillic acid was reached at 23% moisture, whereas maximum production of ochratoxin A was reached at 42% moisture. Furthermore, near maximum production of penicillic acid occurred at 18% moisture, a level at which ochratoxin A production was still extremely low. The results also showed an interesting correlation between higher moisture content and toxin production. There was a marked decrease in the yields of penicillic acid at 42% moisture until, at 62%, the yield was about one-half the maximum. There was a similar pattern for ochratoxin A production, but the decrease began at a higher moisture level, i.e., 52%.

**Water activity and temperature.** Table 2 illustrates the effect of temperature on the tolerance of low $a_w$ by *A. ochraceus*. At 15°C the fungus developed conidial heads only at the highest $a_w$ activity, but at 30°C it did so readily at all levels of $a_w$. Generally, the optimal temperature for development of conidia as determined in this study was 30°C regardless of the $a_w$. Although no accurate measure was used to quantitate the extent of growth and conidial development, samples with water activities of 0.90 and higher had more conidial heads than samples at lower $a_w$. Furthermore, sclerotia were produced only at the lowest temperature (15°C) until moisture became a limiting factor. At an $a_w$ of 0.80 no growth occurred at 15°C, and sclerotia were produced at 22°C.

These observations on the presence of mycelia, sclerotia, and conidial heads do not permit correlations with the quantity of toxins produced. The currently used methods for measuring fungal growth are not suitable for use on such a complex solid substrate as poultry feed.

Generally, maximum production of both mycotoxins was temperature and moisture dependent (Table 2). The accumulation pattern for each toxin, however, was different. Penicillic acid began to accumulate at an $a_w$ of 0.80 at 22°C and 30°C, whereas ochratoxin A began to accumulate at an $a_w$ of 0.85 at 30°C. The optimal conditions for the production of each toxin were different. Maximum production of penicillic acid occurred at 22°C and an $a_w$ of 0.90 (Fig. 2),

![FIG. 1. Production of penicillic acid and ochratoxin A on moistened poultry feed by *A. ochraceus* after 12 days of growth. Symbols: ▲, penicillic acid; O, ochratoxin A.](image)

**Table 2. Growth of *A. ochraceus* and production of mycotoxins at various $a_w$ and temperatures**

| $a_w$   | Penicillic acid$^a$ | Ochratoxin A |
|---------|---------------------|--------------|
|         | 15°C | 22°C | 30°C | 15°C | 22°C | 30°C |
| 0.7598  | 0    | 0    | 0    | 0    | 0    | 0    |
| 0.8068  | 0    | +    | ++   | +    | ++   | ++   |
| 0.8515  | -    | ++S  | ++   | +    | ++S  | +    |
| 0.9011  | 15   | 164  | 115  | 0    | 0    | 0    |
| 0.9532  | 183  | 498  | 281  | 0    | +S   | 46   |
| 0.9966  | 284  | 324  | 106  | +S   | 201  | 302  |
| 1.0000  | 360  | 297  | 35   | +S   | ++   | ++   |

$^a$ Both mycotoxins expressed as micrograms of toxin per gram of feed (dry wt).

Symbols: -, no spore germination; +, mycelial growth only; ++, both mycelial growth and the development of conidial heads; S, the presence of sclerotia.
the production of both penicillic acid and ochratoxin A. Although moisture and temperature studies on fungal growth are available, very little research has been done on the effect of controlled moisture and temperature on the production of secondary metabolites. The only studies that are sufficiently parallel deal with either the ability of mycotoxic organisms to colonize stored grains or the ability of the organisms to produce toxins at various temperatures on various agricultural products or nutrient media. In the ensuing discussion, some comparison will be made with these independent studies; any conclusions may or may not be comparable.

Two observations in this study indicate that the interaction of moisture and temperature on the production of both mycotoxins is complex. First, the production of penicillic acid is favored by low temperatures and moisture near saturation. Second, at higher temperatures there is a decrease in the production of both mycotoxins as the moisture content increases.

Production of penicillic acid by several species of Penicillium on various agricultural commodities demonstrates that maximum production occurs at low temperatures, between 1 and 10 C (6). Another study (14) reported that P. martensii produced this toxin on high-moisture corn (25%) between 1 and 15 C; at higher temperatures production gradually decreases. A similar conclusion may be drawn from this study with A. ochraceus on poultry feed. No information is available on the influence of

whereas maximum production of ochratoxin A was at 30 C and an a_w of 0.95 (Fig. 3). The linear increase in penicillic acid production at 15 C suggests that cooler temperatures favor the production of this compound, and this hypothesis is strengthened by the fact that more of this toxin was produced at 22 C than at 30 C. The reverse is suggested by the data on ochratoxin A production.

The water sorption isotherm data (Table 1) show that at an a_w of 0.99 the percentage of water is 53.08 and 53.73% at 22 and 30 C, respectively. At these high water contents, there is a decrease in the production of both mycotoxins at 22 and 30 C. This correlates well with the data in Fig. 1, which show that production of both mycotoxins decreased as the moisture level increased.

**DISCUSSION**

The data obtained in this investigation show the interaction of moisture and temperature on
temperature on ochratoxin production. However, high yields of ochratoxin A have been reported on synthetic and semisynthetic media at 25 C (7, 8).

Apparently, low substrate moisture is optimum for the production of penicillic acid. Accumulation of the compound at low moisture and temperature may simply indicate that penicillic acid is synthesized more rapidly than ochratoxin A since, in shaken and stationary liquid culture, penicillic acid appears in the medium several hours before ochratoxin A (unpublished data). Apparently, the biosynthetic pathways for these two mycotoxins are controlled by different limiting factors. The accumulation of both mycotoxins decreased at 22 and 30 C as the moisture level increased to near saturation. Perhaps near saturating conditions on this substrate are least favorable for maximum growth, and thus these data reflect nothing more than a growth phenomenon. Reduced growth near saturation has been reported for all members of the A. glaucus group (2). The production of these mycotoxins then would be a consequence of suboptimal growing conditions; this possibility appears especially strong in the case of penicillic acid. Linear production of this toxin parallels the development of sclerotia, the presence of which is considered to represent suboptimal growing conditions (23). The favored production of other mycotoxins at suboptimal growth conditions supports the generality of this hypothesis (6, 13, 14, 18, 22).

Since the volume of air available to the fungus in the jars was only about 600 ml, the differential production of the mycotoxins may simply reflect an aeration effect to which the two pathways have different affinities. However, results were parallel from the moisture experiment in which air was not limiting. Furthermore, similar experiments present evidence that oxygen is not a limiting factor under these conditions (10, 17). Although the total amount of oxygen may not be limiting, the amount available in the feed under near saturating conditions may be. As the moisture increases, the air-filled porosity (texture) of the feed may change, creating a waterlogged condition. Under such a condition reduced growth, O2, and high levels of CO2 may affect the production of these two toxins.

The fundamental effects of temperature and moisture on the production of these two toxins have not been explained entirely. Perhaps accumulation of these mycotoxins is not controlled by a simple temperature-humidity relationship. Generally, the controls and regulations of secondary metabolites are not well understood and therefore warrant investigations. This study demonstrates that, although growth of fungi may be barely visible on relatively dry poultry feed and during a relatively short incubation period, quantities of penicillic acid may be produced which are capable of inducing tumors in mice. The data further suggest that after long incubation and a cool temperature (15 C), lethal quantities of the toxin may be produced. It appears that ochratoxin A may become a problem in poultry feed under more obvious conditions of fungal decay where lethal levels of this compound may occur. Thus, A. ochraceus appears to produce ochratoxin A as near optimal conditions for conidial development are approached and to produce penicillic acid under suboptimal growth conditions. Studies of the possible interaction of other environmental factors which may be responsible for the accumulation patterns of these toxins are underway.

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