Growth and Aflatoxin Production by Aspergillus parasiticus in the Presence of Sodium Chloride

FATHY E. EL-GAZZAR, GULAM RUSUL and ELMER H. MARTH*

Department of Food Science and The Food Research Institute, University of Wisconsin - Madison, Madison, Wisconsin 53706

(Received for publication November 7, 1985)

ABSTRACT

Twenty-five milliliters of glucose-yeast-salt medium containing 0, 2, 4, 6, 8, or 10% NaCl was inoculated to contain, approximately \(10^5\) or \(10^7\) conidia of Aspergillus parasiticus NRRL 2999 and then incubated at 13 or 28°C. Amounts of aflatoxin produced were determined using Reversed-Phase High Performance Liquid Chromatography (HPLC). Increasing the concentration of NaCl reduced accumulation of aflatoxin and also induced a lag in growth of the culture. At 13°C, the mold produced small amounts of aflatoxin after an extended lag phase, and NaCl was markedly more inhibitory at 13 than at 28°C.

Aflatoxin is a collective term for a group of heterocyclic, oxygen-containing mycotoxins that possess the biscyclopyrone ring system. These secondary metabolites are produced by certain strains of Aspergillus flavus and Aspergillus parasiticus. The ubiquity of the aflatoxin-producing fungi and the potent biological activity of their mycotoxins at very low concentrations have stimulated much research on different aspects of toxin production.

Two main types of substrates are used for aflatoxin production in the laboratory, e.g. chemically simple and chemically complex media. Depending on the medium, production of aflatoxin generally commences the second day of incubation and approaches a maximum at about the seventh day. Thereafter, degradation and interconversion of the aflatoxin take place, e.g. the amounts of aflatoxins \(B_1\) and \(G_1\) begin to diminish, whereas those of other aflatoxins such as \(B_2a\) and \(G_2a\), increase (7).

Sodium chloride is commonly used in foods as a preservative or flavoring agent. It is well recognized that growth and activity of some microorganisms at different temperatures is influenced by the concentration of sodium chloride in their media. Sodium chloride exerts its antimicrobial effects either by reducing water activity of the substrate or the sodium ion interferes with ion transport in the organism (8,13). Bullerman et al. (3) reported that toxigenic aspergilli produced small amounts of aflatoxin in salami containing 2.2% sodium chloride and stored at 20°C and in country hams which were aged at 30°C. They also observed that sodium chloride inhibited aflatoxin production in a glucose-ammonium nitrate broth. The salt content of many cheeses is between 2 and 7% (2). Many investigators (8,10,12,15) reported the presence of low levels of aflatoxin when either \(A.\) parasiticus or \(A.\) flavus grew on various types of cheese, but no explanation was given for this minimal production of toxin.

This study was conducted to determine the effect of various concentrations of sodium chloride on aflatoxin production by \(A.\) parasiticus NRRL 2999 in a glucose-yeast-salt medium at 13 and 28°C. This study was divided into three phases: (a) the pH of the medium was adjusted to 5.5 after addition of NaCl and the inoculum size was \(10^5\) conidia/25 ml of medium, (b) in the second study, the pH was 5.75, 5.67, 5.56, 5.46, 5.36 or 5.26 after addition of 0, 2, 4, 6, 8 or 10% NaCl and the inoculum size was \(10^6\) conidia/25 ml of medium, and (c) since some foods like cheese and cured meats are held below 15°C, it was thought appropriate to examine the effect of various concentrations of NaCl on aflatoxin production at 13°C; \(10^5\) conidia/25 ml of medium was the inoculum.

MATERIALS AND METHODS

Media

A glucose-yeast-salt medium (20) was used throughout these experiments. The medium contained the following per liter: glucose, 60 g; \((\text{NH}_4)_2\text{SO}_4, 4\) g; KH\(_2\)PO\(_4, 10\) g; MgSO\(_4\cdot7\)H\(_2\)O, 2 g; \(\text{Na}_2\text{B}_4\text{O}_7\cdot10\)H\(_2\)O, 0.7 mg; \((\text{NH}_4)_6\text{Mn}_7\text{O}_{24}\cdot4\)H\(_2\)O, 0.5 mg; Fe\(_2\)(SO\(_4\))\(_3\)·6H\(_2\)O, 10 mg; CuSO\(_4\cdot5\)H\(_2\)O, 0.3 mg; MnSO\(_4\cdot\)H\(_2\)O, 0.11 mg; ZnSO\(_4\cdot7\)H\(_2\)O, 17.6 mg; and 20 g of yeast extract.

Mold

\(A.\) parasiticus NRRL 2999 was obtained from the Northern Regional Research Center, U.S.D.A., Peoria, IL. The mold was grown on Mycological agar slants that were incubated at 28°C. After 7 d, spores were harvested using sterile distilled water with glass beads; the glass beads helped to dislodge spores from the mycelium when shaken. The spore suspension was pooled in a sterile 125-ml Erlenmeyer flask. The number of spores present per milliliter was determined by plate count.

Three hundred milliliters of the medium was dispensed into each of a series 500-ml Erlenmeyer flasks and appropriate amounts of NaCl (Columbus Chemical Industries, Inc., Colum-
bus, WI) were added to obtain the following concentrations of NaCl: 0, 2, 4, 6, 8 and 10%. The initial pH of each flask was determined using a pH meter (model 601A/digital, Ionanalyzer, Orion Research, Cambridge, MA). The pH was adjusted to 5.5 using 0.1 N KOH. Twenty-five milliliters of the medium was dispensed into each of a series of 125-ml Erlenmeyer flasks. The medium was autoclaved at 121°C for 15 min, cooled, and inoculated with 1 ml of the appropriate spore suspension. Inoculated flasks were incubated at 28°C and the contents were analyzed after 3, 7 and 10 d of incubation.

Analysis

Contents of flasks, with and without (which served as the control) NaCl, were analyzed in triplicate for pH, dry weight of mycelium and aflatoxin.

Dry weight of mycelium. The medium was filtered through a modified Büchner funnel (20) fitted with preweighed Whatman No. 1 filter paper (12.5 cm in diameter) and attached to a 125-ml separatory funnel. Vacuum was applied to the funnel using an aspirator. The mycelium was washed with 10 ml of distilled water followed by 10 ml of chloroform (AR, Mallinckrodt). The water was transferred to the modified Büchner funnel and the chloroform was transferred directly into the separatory funnel. We observed that when the chloroform wash was added to the Büchner funnel, it could not pass through because an emulsion was formed. The filter paper with the mycelium was dried at 50°C for 48 h, cooled in a desiccator, and weighed.

Extraction of aflatoxin. Extraction of aflatoxin was done as described by Yousef and Marth (21), but with some modifications. As soon as the filter paper with mycelium was removed from the Büchner funnel, 40 ml of chloroform was added to the separatory funnel and the contents were shaken for 2 min. The liquid phases were allowed to separate and the chloroform layer was washed through 10 g of anhydrous granular Na₂SO₄ in a funnel and collected in a 250-ml round-bottom flask. Extraction was repeated once more with 50 ml of chloroform and the chloroform phase was combined with the earlier phase in the round-bottom flask. Chloroform in the round-bottom flask was dried using a rotary evaporator (Rotavapor, Büchi, Brinkmann, Westbury, NY); the temperature of the waterbath was between 50 and 55°C. The resulting dried films were redisolved in known volumes of methanol (LC grade, Baker, Phillipsburg, NJ).

Analysis of aflatoxin. Aflatoxins B₁ and G₁ were determined using a reversed phase HPLC system. The column was a reverse-phase 5 μC₁₈ (Nova Pak No. 86344, Waters, Milford, MA), the detector was of the fluorescence type (Waters, Model No. 420) with an aflatoxin lamp (Waters, No. 78409), 365-nm excitation filter (Waters, No. 78225) and 425-nm emission filter (Waters, No. 78155); and the injector (Model 7120, Rheodyne) was equipped with a 10 μl sampling loop. The pump was Waters, Model 7120 and the recorder was Model 252A, Linear.

Aflatoxin B₁ and G₁ standards were obtained from Sigma (St. Louis, MO), and dissolved in a known volume of methanol to give the desired concentrations. The mobile phase used was 1% acetic acid and acetonitrile (LC grade, Baker), 65:35, as described by Waters (1). The mobile phase before use was degassed for 3 min using vacuum and a sonicator.

Aflatoxins were assayed by injecting 50 μl of the methanol extract into the HPLC system and concentrations were determined by comparing peak heights to those of the standard. Bioassay analysis of samples, aflatoxin B₁ and G₁ standards were injected until uniform peak heights were obtained. Each sample was injected twice.

RESULTS AND DISCUSSION

pH of media and mycelial growth

Figures 1-3 describe the changes in pH of the medium during mold growth under different experimental conditions. Figures 3-6 illustrate the rate of mold growth under the same conditions. Initially the pH of the medium decreased, but as growth progressed there was an increase in pH. The extent of this initial decrease in pH was influenced by the initial pH of the medium and concentration of NaCl in the medium. When the initial pH was unadjusted, with an initial inoculum of 10⁷ conidia/25 ml of medium (Fig. 1), the decrease in pH with increasing concentration of NaCl was less than when the initial pH was 5.5 and the initial inoculum was 10² conidia/25 ml of medium (Fig. 2). After 7 d of incubation, the pH of the medium increased and the rate of increase was influenced by amounts of mycelium and NaCl. After 10 d of incubation, there was only a slight additional change in pH from that at 7 d, indicating the end of the growth phase. At 13°C, the rate of pH change was slow in the medium containing 6, 8 or 10% NaCl when compared to that in the medium with 0, 2 or 4% NaCl (Fig. 3). At 30 d, the pH of the culture without NaCl began to increase, whereas this was not true for any cultures containing NaCl.

Mycelial growth was influenced by concentration of NaCl, size of the initial inoculum and temperature. The rate of mold growth at 13 and 28°C generally decreased as the concentration of NaCl was increased; this was more evident at 13 than at 28°C (Fig. 4-6). Mycelial growth was more abundant when the inoculum was 10⁷ conidia/25 ml of medium and the initial pH of the medium was not adjusted than when the inoculum was 10² conidia/25 ml of medium and the pH of the medium was 5.5. Sharma et al. (16) made a similar observation.
and attributed it to an increase in lag phase when a small inoculum was used. Similar results were also reported by Yousef and Marth (20). Shih and Marth (17) reported that growth of *A. flavus* and *A. parasiticus* was reduced by 6% NaCl in a liquid medium also containing 2% yeast extract and 20% sucrose; growth was completely inhibited when the medium contained 14% NaCl. The dry weight of the mycelium in the medium with or without NaCl after 30 d at 13°C was similar to that achieved after 3 d at 28°C regardless of the pH of the medium (Fig. 4-6). Growth of *A. parasiticus* and *A. flavus* at and below 13°C has been reported (13,14,15,18,19). Maximum mycelial growth occurred after 7 d at 28°C in the absence of and in the presence of 2, 4 or 6% NaCl, whereas in the presence of 8 or 10% NaCl, the mold was still growing after 10 d. At the end of 10 d, there

Figure 2. Changes in pH caused by *A. parasiticus* in cultures containing sodium chloride. Incubation was at 28°C, the initial pH of cultures was adjusted to 5.5, and the inoculum was $10^5$ conidia/25 ml of medium.

Figure 3. Changes in pH caused by *A. parasiticus* in cultures containing sodium chloride. Incubation was at 13°C, the initial pH of cultures was adjusted to 5.5, and the inoculum was $10^5$ conidia/25 ml of medium.

Figure 4. Dry weight of mycelium produced by *A. parasiticus* in cultures containing sodium chloride. Incubation was at 28°C, initial pH of cultures was not adjusted and the inoculum was $10^7$ conidia/25 ml of medium.

Figure 5. Dry weight of mycelium produced by *A. parasiticus* in cultures containing sodium chloride. Incubation was at 28°C, initial pH of cultures was 5.5 and the inoculum was $10^5$ conidia/25 ml of medium.

Figure 6. Dry weight of mycelium produced by *A. parasiticus* in cultures containing sodium chloride. Incubation was at 13°C, initial pH of cultures was 5.5, and the inoculum was $10^5$ conidia/25 ml of medium.
was a decrease in weight of mycelium in cultures containing 0, 2, 4 or 6% NaCl. This loss in weight could have resulted from loss of soluble intracellular solutes which were released during hydrolysis of the mycelium during filtration.

**Production of aflatoxin**

Results shown in Fig. 7-12 indicate that in these experiments production and accumulation of aflatoxin by *A. parasiticus* was influenced most by concentration of NaCl in the medium and temperature of incubation. Increasing the concentration of NaCl decreased the amount of aflatoxin produced by the mold. Presence of 6, 8 or 10% NaCl in the medium had a pronounced effect on aflatoxin production in the beginning of the incubation period, as reflected both by slow mold growth and low accumulation of aflatoxin. Maximum production and accumulation of

![Figure 7](image1.png)  
**Figure 7.** Production of aflatoxin B1 by *A. parasiticus* in the presence of sodium chloride. Incubation was at 28°C, initial pH of cultures was not adjusted and the inoculum was 10^7 conidia/25 ml of medium.

![Figure 8](image2.png)  
**Figure 8.** Production of aflatoxin G1 by *A. parasiticus* in the presence of sodium chloride. Incubation was at 28°C, initial pH of cultures was not adjusted and the inoculum was 10^7 conidia/25 ml of medium.

![Figure 9](image3.png)  
**Figure 9.** Production of aflatoxin B1 by *A. parasiticus* in the presence of sodium chloride. Incubation was at 28°C, initial pH of cultures was 5.5, and the inoculum was 10^5 conidia/25 ml of medium.

![Figure 10](image4.png)  
**Figure 10.** Production of aflatoxin G1 by *A. parasiticus* in the presence of sodium chloride. Incubation was at 28°C, initial pH of cultures was 5.5, and the inoculum was 10^5 conidia/25 ml of medium.

![Figure 11](image5.png)  
**Figure 11.** Production of aflatoxin B1 by *A. parasiticus* in the presence of sodium chloride. Incubation was at 13°C, initial pH of cultures was 5.5, and the inoculum was 10^5 conidia/25 ml of medium.
aflatoxin at 28°C occurred after 7 d in the control (0% NaCl) and in the presence of 2, 4 or 6% NaCl (Fig. 7-10). Generally A. parasiticus produced more aflatoxin G₁ than B₁ in the medium with or without NaCl (Fig. 7-10). This was true after 3, 7 and 10 d at 28°C and after 14, 21 and 30 d at 13°C. Similar results were obtained by Bullerman et al. (2).

Sporulation occurred except in cultures containing 8 or 10% NaCl. This was followed by a decrease in accumulation of aflatoxin in the same cultures at the end of 10 d, and an increase in degree of sporulation. Sporulation indicated that the mold ceased to grow and began to autolyse. Yousef and Marth (20) speculated that maximum accumulation of aflatoxin is associated with autolysis of the mycelium and subsequent release of mycelial aflatoxin into the medium (glucose-yeast-salt). They postulated further that the decrease in aflatoxin resulted from degradation by intracellular fungal enzymes that are liberated following autolysis. Doyle and Marth (5) reported that 9-d-old mycelia degraded the maximum amount of aflatoxin at a pH in the range of 5 to 6.5 and at 28°C. The mycelium was unable to degrade aflatoxin at or below pH 4 or at 7 or 45°C. The amount of aflatoxin degraded was dependent upon its initial concentration, and aflatoxin G₁ was degraded more rapidly than B₁.

At 8 and 10% NaCl, there was no decrease at 10 d in amounts of aflatoxin or mycelial weight, which meant that the mold was still growing and producing aflatoxin at a very slow rate. Shih and Marth (17) observed that a concentration of NaCl above 4% in broth (2% yeast extract and 20% sucrose) markedly reduced, and 14%, completely inhibited production of aflatoxin by A. parasiticus and A. flavus when incubated at 28°C. Bullerman et al. (2,3) reported that Italian-type salamis contaminated with A. flavus were more likely to develop aflatoxin during aging than were contaminated smoked Hungarian-type salamis when both were held under the same conditions. Holding salamis at a temperature below 15°C and a relative humidity of less than 75% prevented development of aflatoxin in the sausages. These investigators also found that high concentrations of salt prevented growth and aflatoxin production by A. parasiticus during aging of country-cured hams. Kulik and Hanlin (10) indicated that 15% NaCl in malt agar was needed to inhibit growth of A. flavus, and they postulated that high NaCl concentrations adversely affected the water activity required for growth and toxin production.

A larger amount of aflatoxin was produced when the initial inoculum was 10^7 (pH not adjusted) rather than 10^5 conidia/25 ml of medium (pH 5.5). This effect was most pronounced in the control (0% NaCl) and in the medium containing 2% NaCl. Similar results were reported by Yousef and Marth (20).

Temperature also influenced production of aflatoxin in the presence of NaCl. At 13°C, production of aflatoxin was limited (Fig. 11, 12) because of slow mold growth. Production of aflatoxin was greatly inhibited by NaCl at concentrations above 4%. Small amounts of aflatoxin were produced when the medium contained 6% and negligible amounts when 8 or 10% NaCl were present and incubation was for 30 d. Schindler (14) reported that neither A. flavus nor A. parasiticus produced any aflatoxin when incubated at temperatures below 7.5 or above 40°C. Northolt et al. (13) noted that aflatoxin was produced by A. parasiticus NRRL-2999 at 13 and at 32°C. Schroeder and Hein (15), using four isolates of A. flavus, found aflatoxin production occurred at temperatures ranging from 10 to 40°C after an incubation period of 10 d. Wallbeek et al. (19) reported that five isolates of A. flavus produced aflatoxin at 7.5 and 10°C. None of these investigators studied the effect of different concentrations of NaCl at the temperatures mentioned.

Results of this study indicate that NaCl alone, in concentrations normally used in foods, will not prevent growth and toxin production by A. parasiticus. Thus, when storing foods containing NaCl, other means must be used to control mold growth and thus to safely preserve the foods.

ACKNOWLEDGMENTS

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by the American-Mideast Educational and Training Services through a Peace Fellowship to Fathy E. El-Gazzar and by the University Pertanian Malaysia through support of Gulam Rusul.

REFERENCES