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Relationship of mould count, ergosterol and ochratoxin A production ☆

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Abstract

The relationship between viable mould count, ergosterol content and ochratoxin A (OA) formation was studied at different inoculum concentrations of *Aspergillus ochraceus* NRRL 3174 and *Penicillium verrucosum* NRRL 3260 grown on sterile long-grain enriched white rice as the substrate. Ergosterol was determined by extraction, saponification and quantification using high performance thin layer chromatography (HPTLC) with UV detection. Ergosterol and ochratoxin A were detected after 3 days of incubation and reached their maximum at 7–10 days of incubation. After that, a decline in the concentrations in both ergosterol and ochratoxin was observed. Ergosterol measurement by HPTLC appeared to be a useful method to detect fungal activity, which corresponded to ochratoxin production. Thus, the ergosterol assay may have a use as an early indicator of potential mycotoxin production.

Keywords: Mould count; Ergosterol; Ochratoxin A

1. Introduction

Conventional plate count methods have long been used to detect and measure mould populations in foods and commodities. Recently, chemical and biochemical methods to measure fungal growth have been developed, which involve measuring specific components of fungi such as ergosterol (Ride and Drydale, 1972; Seitz et al., 1977, 1979; Naewbanij et al., 1984; Lin and Cousin, 1985; Matcham et al., 1985; Patel and Williams, 1985; Jarvis and Williams, 1987). Ergosterol is an important constituent of cell walls of most fungi and is either absent or a minor constituent in most higher plants and insects (Nes, 1977; Weete, 1980); yet, the ergosterol assay has not been widely used. Seitz et al. (1979) reported the ergosterol assay to be more sensitive, rapid and convenient than other methods. Ergosterol, used as an indicator of fungal growth, may also signal the possible synthesis of mycotoxins (Seitz et al., 1979).

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Zill et al. (1988) found that the formation of zearalenone started when the ergosterol level reached 50 mg kg^{-1} and increased rapidly in the stationary phase of growth, which was characterized by decreasing rates of ergosterol formation.

Ochratoxin A (OA) is a mycotoxin produced by *Aspergillus ochraceus*, some strains of *A. niger*, and *Penicillium verrucosum* (Pitt and Hocking, 1997). Ochratoxin A is a nephrotoxin, which has been found as a contaminant of barley and wheat and has been associated with porcine nephropathy. It has also been considered as possible cause (but never proven) of a human disease known as Balkan Endemic nephropathy. OA has also been shown to be a teratogenic, potentially carcinogenic and immunosuppressant substance, and is considered to be a potential foodborne hazard in certain parts of the world, particularly Europe (Krogh, 1987; Pitt and Hocking, 1997).

The objective of this study was to compare viable mould counts, ergosterol formation and ochratoxin A (OA) concentrations at different inoculum levels and incubation periods to determine if correlations might exist between these parameters.

2. Materials and methods

2.1. Fungi

A. ochraceus NRRL 3174 and *P. verrucosum* NRRL 3260 (received as *P. viridicatum*), obtained from the National Center for Utilization Research, US Department of Agriculture, Peoria, IL) were used for this study. Cultures of these fungi were maintained on potato dextrose agar (PDA) slants (Difco Laboratories, Detroit, MI). After the cultures had spored well, spore suspensions were made by adding sterilized phosphate buffer solution containing Tween 80 (0.05%) to the slant cultures. The spores were dislodged with a sterile loop and filtered through four layers of sterile cheesecloth. Total spore concentrations were determined using a Petroff–Hausser counting chamber, and viable spore counts were determined by plate counts on PDA plates. The original spore suspensions were diluted to contain the different inoculum levels in 1.0 ml of buffer.

2.2. Experimental design

Long-grain enriched white rice prepared as described by Park and Bullerman (1983) was used as a substrate in this study. Autoclaved rice (65 g dry weight), in Mason jars with perforated lids lined with filter paper, were inoculated at 10^1, 10^3 and 10^6 spores per jar and incubated at 25 °C for 0, 1, 3, 7, 10 and 15 days. The cultures were incubated in triplicate, and analyzed at various times. At the appropriate time, the mouldy rice was aseptically blended, and samples of 10 g of rice (wet weight) for ergosterol, 50 g for ochratoxin A, and 1 g for viable mould count analyses were taken. The control consisted of uninoculated sterile rice. The experiment was repeated twice.

2.3. Viable counts

For viable mould counts, 1.0 g mouldy rice was placed into a stomacher bag, 99 ml sterilized Butterfield's phosphate buffer solution was added, and the sample was mixed in the stomacher for 1 min (Acuff, 1992; Jarvis and Williams, 1987; Messer et al., 1992). Further serial dilutions were prepared in Butterfield's phosphate buffer and samples were spread on the surfaces of potato dextrose agar (PDA) plates containing 110 $\mu g\ l^{-1}$ tetracycline. The plates were incubated at 25 °C for 3 days. Colonies were counted using a dark field Quebec colony counter.

2.4. Ergosterol determination

The method of Seitz and Mohr (1992) was used for ergosterol determination. Briefly, 40 ml of chloroform and 10 ml of hexane were added to 10 g of ground sample. The jars were tightly closed and shaken (30 min) in a Gallenkamp orbital shaker (180 rpm). The mixture was allowed to settle, and the extract (30 ml) was transferred to dilution bottles capped with Teflon lined screw caps. Potassium hydroxide (3 g) was added, the bottles were incubated in a hot water bath (55–60 °C) for 20 min, then cooling water (5 ml) was added and mixed by agitation. After separation of layers, the hexane layer was transferred to a 4-dram vial (ca. 10 ml) using a Pasteur pipette. The extraction was repeated twice with hexane (5 ml). The extracts were combined and

evaporated to dryness on a hot plate under a stream of nitrogen. The dry extracts were dissolved in methanol and spotted on precoated high performance thin layer chromatography (HPTLC) plates along with ergosterol standards (Sigma, St. Louis, MO) and developed in toluene/acetone (99:1). The developed plates were exposed to iodine vapor in a developing tank for 45 s before observation under UV light for visual estimation of ergosterol concentrations (Rao et al., 1989).

2.5. Ochratoxin A determination

OA was determined according to AOAC (1984). Briefly, mouldy rice (50 g) was extracted with 0.1 M H_3PO_4 (25 ml) and chloroform (250 ml) in an Erlenmeyer flask (500 ml). The mixture was shaken for 80 min on a Gallenkamp orbital shaker and filtered through glass fiber paper covered with diatomaceous earth (10 g) on a Büchner funnel (9 cm). To remove acids, a purified cotton plug was placed in the bottom of a 700×17-mm chromatographic tube, and diatomaceous earth (2 g) mixed with 1.25% sodium bicarbonate solution (1 ml) was added and tamped firmly. Then, the sample extract (50 ml) mixed with hexane (40 ml) was added to the column and eluted at maximum flow rate, followed by elution with chloroform (75 ml). OA was eluted with 75 ml freshly prepared acetic acid/chloroform (1:99) and collected in an Erlenmeyer flask (250 ml). The extract was immediately evaporated to dryness on a steam bath, and the residue was transferred to a vial with chloroform and evaporated to dryness on a hot plate under a gentle stream of nitrogen. The residue was dissolved in acetic acid/benzene (1:99) and spotted on precoated HPTLC plates along with OA standards (Sigma), and then developed in toluene/ethyl acetate/formic acid (6:3:1). The plates were observed under long-wave UV light for visual estimation of OA concentrations.

3. Results and discussion

The relationships of ergosterol, total viable mould count and ochratoxin A production of *A. ochraceus* and *P. verrucosum* are summarized in Figs. 1 and 2. The amounts of ergosterol and OA were very low

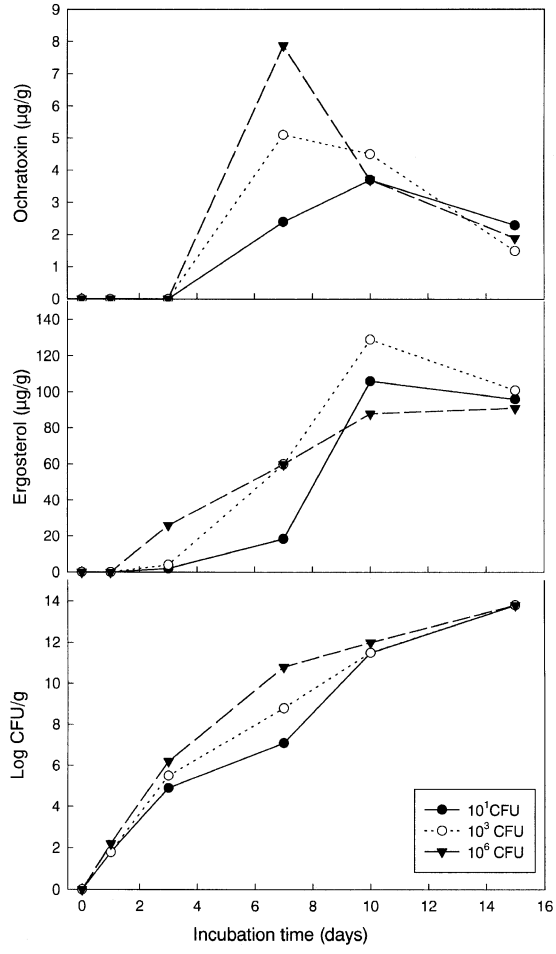

Fig. 1. Colony counts (log cfu/g), ergosterol (µg/g), and ochratoxin A (µg/g) production by *A. ochraceus* NRRL 3174 in sterilized long white grain.

during the first 3 days of incubation. No growth was visible on the rice just after inoculation (day 0) or at day 1 of incubation at all inoculum levels studied. Growth of *A. ochraceus* became visible after 3 days, and there was a sharp increase in total viable mould count, followed by a more gradual increase in counts thereafter. Growth of *P. verrucosum* became visible by day 1 and gradually increased thereafter, slowing after 10 days.

Ergosterol was not detected after 1 day of incubation at inoculum levels of 10^1 and 10^3 spores per jar with either fungus tested, but was detected in trace amounts at the 10^6 inoculum level of *A. ochraceus*

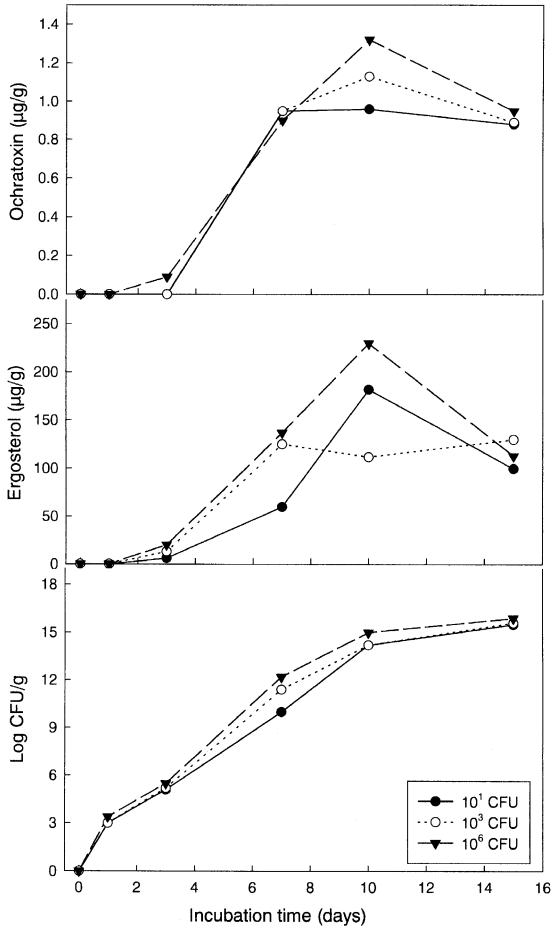

Fig. 2. Colony counts (log cfu/g), ergosterol (µg/g), and ochratoxin A (µg/g) production by *P. verrucosum* NRRL 3260 in sterilized long white grain.

(Fig. 1). Ergosterol production reached a maximum level after 10 days, and after that, was stationary or decreased slightly. With *A. ochraceus*, the increase in ergosterol content was very sharp at the 10^6 inoculum level during the first 3 days of incubation compared to more gradual increases at 10^1 and 10^3.

No ochratoxin A was detected in cultures immediately after inoculation; however, at the 10^6 inoculum level, low amounts were found after 1 day, and at 10^3 after 3 days (Fig. 1). At all three inoculum levels, OA was not detected until after the 6th day of incubation of *A. ochraceus.* Production of OA reached maximum levels at 7 days for the 10^3 and 10^6 inoculum levels, but at the 10^1 level, did not reach maximum until 10 days. OA production subsequently declined sharply in all cultures. With *P. verrucosum*, OA was not detected at day 0 or 1 of incubation at any inoculum level and could be detected only at the 10^6 inoculum level at 3 days of incubation (Fig. 2). At 10^1 and 10^3, OA was detected after 6 days of incubation. Maximum ochratoxin production was observed at 10 days of incubation at all three inoculum levels. The data suggest that ergosterol and ochratoxin A followed similar patterns of production. However, total viable mould counts were detectable before either ergosterol or OA production at all inoculum levels with both *A. ochraceus* and *P. verrucosum.*

In this study, ergosterol was detected and quantified using HPTLC, which requires less sophisticated and expensive equipment than high performance liquid chromatography (HPLC), which makes the method more usable by laboratories that do not have access to HPLC equipment. The recovery of ergosterol by HPTLC from spiked rice was 86%. Seitz et al. (1977) reported 93–96 $\pm$ 5% recovery with HPLC. While this is somewhat higher, the difference is not so great that HPTLC cannot be used. Naewbanij et al. (1984) and Rao et al. (1989) have also advocated the use of TLC for routine testing of ergosterol. The minimum amount of ergosterol which could be detected in this study using HPTLC was 0.87 µg/g, while Seitz et al. (1977) have reported detection of as little as 0.05 µg/g of ergosterol by using HPLC.

Various workers have confirmed that ergosterol determination is a useful method to monitor fungal contamination of cereal grains (Seitz et al., 1977; Cahagnier et al., 1983; Seitz and Pomeranz, 1983). Matcham et al. (1985) tested three different methods —chitin, laccase and ergosterol—for the estimation of fungal biomass in a solid substrate, and found ergosterol to be a more sensitive indicator of low levels of mycelial growth than the other methods. In this study, viable plate counts detected mould growth sooner, but ergosterol can be determined in less time and can detect both viable and nonviable moulds. If ergosterol is detected by HPTLC instead of HPLC, the method may have wider application for detecting mould invasion.

With all inoculum levels, total viable mould counts reached 10^{12} cfu/g for *A. ochraceus* and 10^{15} cfu/g

for *P. verrucosum*, respectively (Figs. 1 and 2). Karunaratne and Bullerman (1990) found that mould growth reached maximum at 10^8 and 10^9 cfu/g at 28 and 35 °C irrespective of initial inoculant levels. Häggblom (1982) found a linear relationship between dry weights of moulds and glucosamine content when the moulds were cultivated on malt agar plates. Ergosterol production either started decreasing, or was constant after it reached the highest level in both moulds. Gourama and Bullerman (1995) reported similar results with *A. parasiticus.* After 7 days for *A. ochraceus* and 10 days for *P. verrucosum*, production of OA declined. Similar results were reported by Applegate and Chipley (1976) using the same strain of *A. ochraceus*.

Zill et al. (1988) compared the production of ergosterol to zearalenone formation by *Fusarium graminearum.* They found that zearalenone production started after 1 week of incubation when the ergosterol content of the fungal mycelium was estimated to be 0.05 μg/g of substrate. Gourama and Bullerman (1995) found ergosterol to be a sensitive early indicator of low levels of fungal activity and aflatoxin production by *A. parasiticus* in solid substrates. In that study, aflatoxin B_1 production followed the same pattern as ergosterol formation. At high inoculum levels, ergosterol and mould spore counts were detectable before aflatoxin B_1. Both aflatoxin B_1 and ergosterol remained constant after reaching maximum concentrations. However, there was no clear pattern between aflatoxin G_1 production and ergosterol content. In this study, ochratoxin and ergosterol commenced and reached maximum concentrations at the same times for *P. verrucosum.* For *A. ochraceus*, ergosterol was detected before ochratoxin, but maximum ochratoxin production occurred 3 days sooner than maximum ergosterol production. Thus, in this study, the ergosterol method did not detect mould activity at any earlier point than the viable plate count method; however, given the fact that the results can be obtained more quickly with the ergosterol method, with no need for the 3–5 day incubation period of the viable plate counts, detection of mould activity may still be quicker with ergosterol. And since ochratoxin and ergosterol production patterns were similar, ergosterol could be used as a more readily measurable indicator of potential ochratoxin production.

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