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Production of Ochratoxin A in Barley by *Aspergillus ochraceus* and *Penicillium viridicatum*: Effect of Fungal Growth, Time, Temperature, and Inoculum Size

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Moistened barley was inoculated with 1.4×10^3 and 1.4×10^5 spores, respectively, from ochratoxin A-producing strains of *Aspergillus ochraceus* and *Penicillium viridicatum*. To estimate fungal tissue in the barley, the amount of glucosamine was followed for 28 days at 10 and 25°C. Ochratoxin A was also followed during the same period and under the same conditions. The data show that ochratoxin A could be detected 4 to 6 days after inoculation at 25°C, and the maximal accumulation of ochratoxin A was observed 28 days after inoculation. After 28 days at 25°C, the quantities of ochratoxin A were between 7 and 46 μg/g of grain. At 10°C only *P. viridicatum* produced ochratoxin A. The results indicated that production of ochratoxin A is not associated with rapid increase of glucosamine in the barley.

Ochratoxin A is a mycotoxin produced by *Aspergillus* and *Penicillium* spp. (3, 4). The mycotoxin has been isolated from a number of agricultural commodities in various geographical regions of the world, but so far the principal interest in studying ochratoxin A has been in Scandinavia and certain Balkan countries (3, 4, 8). In Scandinavia the mycotoxin has been isolated from animal feeds, mainly barley (8). Ochratoxin A has been associated with naturally occurring porcine nephropathy in Denmark and Sweden (4).

Much of our knowledge concerning production of secondary metabolites by fungi such as mycotoxins is based on work with submerged liquid cultures. The knowledge we have obtained from these studies is that secondary metabolites often start to be produced after a decrease in growth rate (5). We do not presently know if this principle also holds true for in situ production of secondary metabolites such as ochratoxin A on grains or animal feeds. The reason for this lack of information might be due to difficulties in measuring fungal growth on solid substrates. The plate dilution method has often been used, but this method only reflects the number of live fungal elements and not the accumulated level of fungal mycelium that may well have contributed to toxin production earlier during the incubation.

Harwig and Chen (1), using nonsterile ground wheat as substrate, found that the water activity was important for ochratoxin A production by *Penicillium viridicatum*. Incubation at 12 and 25°C at a moisture content of 22% gave high concentrations of ochratoxin A after 4 weeks. The levels of ochratoxin A were related to the number of live fungal elements in the nonsterile wheat. A correlation between fungal growth and toxin production in situ could not be obtained from the above-mentioned work.

With sterile barley grain as substrate, the present study was made to investigate the relationship between fungal growth (measured as glucosamine equivalents) and ochratoxin A production at different inoculum sizes, temperatures, and incubation times.

Conidial suspensions of *Aspergillus ochraceus* W CBS589.68, *A. ochraceus* SLV, and *P. viridicatum* M93 were used. *A. ochraceus* SLV and *P. viridicatum* M93 were gifts from K. Åkerstrand of Swedish National Food Administration, Uppsala. All isolates were found to produce ochratoxin A when cultivated in liquid media.

Conidia were washed off 10-day-old cultures grown on malt-agar plates at 20°C with sterile aqueous 0.05% Tween 80 and counted on a hemacytometer. Two spore concentrations were used, 1.4×10^5 and 1.4×10^3 spores per flask. A 25-g amount of autoclaved barley grain (121°C, 25 min) was arbitrarily added to 125-ml Erlenmeyer flasks to a depth of 1 cm. An initial moisture content of 22% (wet-weight basis) (7) was used. The same moisture content was used by Harwig and Chen (1) and supported high ochratoxin A production. Noninoculated flasks

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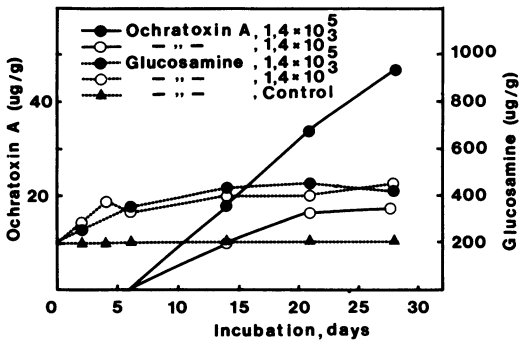

FIG. 1. Ochratoxin A and glucosamine content of barley with 22% moisture incubated with different spore concentrations of *A. ochraceus* 589.68 at 25°C for different periods.

with the same moisture content served as controls.

Conidia were added to flasks, which were then sealed with aluminum foil and shaken frequently to ensure a uniform distribution of conidia and moisture. The samples were incubated in plastic containers at 10 and 25°C for 2, 4, 6, 14, 21, and 28 days. The final moisture content of the samples incubated at 25°C was 21%, and that at 10°C was 21.5%. The flask contents were dried at 104°C for 24 h, then ground to pass through a 40-mesh sieve, and kept frozen until used. The enzymatic method of Hult and Gatenbeck (2), using carboxypeptidase A, was followed for the extraction and quantitation of ochratoxin A, except that in the final extraction of ochratoxin A two 2.5-ml portions of Tris-hydrochloride were used instead of two 5-ml portions. Chitin was estimated by the method of Ride and Drysdale (6). Figures 1 to 3 represent typical levels of ochratoxin A and glucosamine in respective samples. Each experiment was carried out twice with at least two replicates.

A linear relationship was found between dry weights of molds (1 to 10 mg) and glucosamine content when the molds were cultivated on malt-agar plates. Growth of *A. ochraceus* 589.68 (Fig. 1) grown on barley at 25°C, expressed as glucosamine equivalents, approximately doubled during the time of incubation without any differences between the inoculum sizes. For *A. ochraceus* SLV (Fig. 2) the glucosamine levels for both inoculum sizes increased about three times during incubation for 28 days at 25°C.

For *P. viridicatum* (Fig. 3) the glucosamine content increased 3 to 3.5 times during the incubation period without any significant difference between inoculum sizes.

Incubation of *A. ochraceus* 589.68 at 10°C for 28 days (Table 1) on barley showed that the glucosamine content was approximately the same as after incubation at 25°C. *A. ochraceus* SLV and *P. viridicatum*, however, gave lower yields of glucosamine at 10°C compared with incubation at 25°C.

The molds tested produced considerable amounts of ochratoxin A on barley after 28 days at 25°C (Fig. 1 to 3). For *A. ochraceus* 589.68 (Fig. 1) the higher spore concentration in the inoculum gave a level of about 46 μg of ochratoxin A per g of barley, whereas the lower inoculum size gave 17 μg/g. For *A. ochraceus* SLV (Fig. 2) there was a linear increase in the yields of ochratoxin A with time, the lower inoculum size yielding slightly higher levels. With *P. viridicatum* (Fig. 3), the size of inoculum did not affect the yields of ochratoxin A within 28 days. Ochratoxin A started to be produced by *A. ochraceus* 589.68 (Fig. 1) and *P. viridicatum* after 6 days of incubation and by *A. ochraceus* SLV after 4 days. At 10°C only *P. viridicatum* produced ochratoxin A (Table 1) when 1.4×10^5 spores were used as inoculum, whereas 1.4×10^3 spores per flask yielded no ochratoxin A.

In control experiments, gamma-irradiated barley was used as substrate to reduce any adverse effects of autoclaving. Essentially the same levels of glucosamine and ochratoxin A were obtained as with autoclaved barley for all incubation times (data not shown).

Autoclaved or gamma-irradiated barley with an initial moisture content of 22% incubated at 25°C was shown to be a good substrate for ochratoxin A production by *A. ochraceus* and *P. viridicatum* (Fig. 1 to 3). Fungal growth, measured as glucosamine equivalents, was slow for *A. ochraceus* 589.68 (Fig. 1), for which the levels reached 400 to 450 μg of glucosamine per g of grain after 28 days. For *A. ochraceus* SLV the levels went up to 550 to 650 μg/g (Fig. 2) after 28 days. For both *A. ochraceus* SLV and *P. viridicatum* the yields of ochratoxin A were

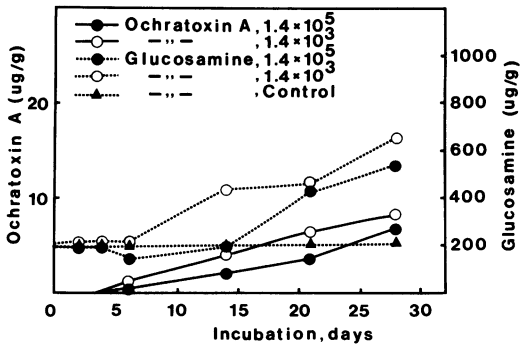

FIG. 2. Ochratoxin A and glucosamine content of barley with 22% moisture incubated with different spore concentrations of *A. ochraceus* SLV at 25°C for different periods.

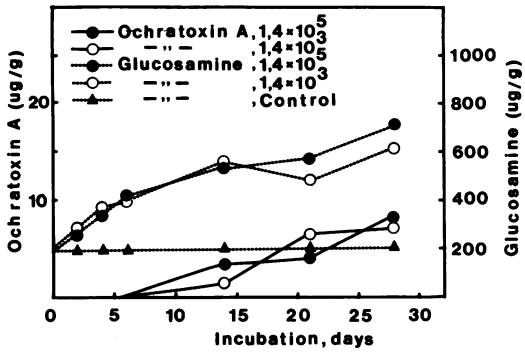

FIG. 3. Ochratoxin A and glucosamine content of barley with 22% moisture incubated with different spore concentrations of *P. viridicatum* SLV at 25°C for different periods.

considerably lower than for *A. ochraceus* 589.68. This indicates that production of ochratoxin A is not associated with rapid growth of the fungi. Rather, higher growth rates (Fig. 2 and 3) seem to restrict ochratoxin A production.

The decrease in inoculum size by 10^2 spores per flask had no significant effect on the levels of ochratoxin A after 28 days except for *A. ochraceus* 589.68 (Fig. 1), for which 60% less ochratoxin A was produced compared with the higher spore inoculum. The difference in ochratoxin A production by *A. ochraceus* 589.68 (Fig. 1) after 28 days for the two inoculum sizes is puzzling and cannot be explained from these data. These results, together with the results of Sharma et al. (10), may have practical implications because a small inoculum from ochratoxin A- or aflatoxin-producing fungi may yield large amounts of mycotoxins under suitable conditions.

The levels of glucosamine measured during the incubation indicate that all fungi had about the same growth irrespective of the initial inoculum size (Fig. 1 to 3). The reason for this is not clear but may be due to limited nutrients in the substrate which reduced growth or to otherwise nonoptimal conditions for growth.

At 10°C ochratoxin A production was inhibited in *A. ochraceus* but not in *P. viridicatum*, in which 2.5% of the amount of toxin produced at 25°C was formed after 28 days. This is in agreement with earlier reports in which *A. ochraceus*, in liquid culture, was unable to produce ochratoxin A at 10°C (9) and *P. viridicatum* produced ochratoxin A at 5 and 10°C after 4 weeks of incubation on ground wheat (1). In this work, *A. ochraceus* and *P. viridicatum* grew at 10°C at reduced growth rates compared with growth at 25°C (Table 1). No differences in levels of glucosamine could be detected after 28 days between the two strains of *A. ochraceus* or between *A. ochraceus* and *P. viridicatum*. These data may indicate that enzymes involved in ochratoxin A synthesis are more sensitive to low temperatures than the enzymes of primary metabolism.

TABLE 1. Effect of different temperatures and inoculum sizes on growth and ochratoxin A production by *A. ochraceus* and *P. viridicatum* on barley with 22% moisture after 28 days

Organism	Size of inoculum (no. of spores per flask)	Glucosamine (μg/g of barley)		Ochratoxin A (μg/g of barley)	
		10°C	25°C	10°C	25°C
A. ochraceus 589.68	1.4×10^3	400	450[a]	ND	17[a]
	1.4×10^5	395	420[a]	ND	48[a]
A. ochraceus SLV	1.4×10^3	375	650[b]	ND	8[b]
	1.4×10^5	375	570[b]	ND	6[b]
P. viridicatum M93	1.4×10^3	405	610[c]	ND	7[c]
	1.4×10^5	435	710[c]	0.2	8[c]

[a] From Fig. 1.
[b] From Fig. 2.
[c] From Fig. 3.

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