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Postharvest Production of Ochratoxin A by *Aspergillus ochraceus* and *Penicillium viridicatum* in Barley with Different Protein Levels

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The production of ochratoxin A (OA) in barley by *Aspergillus ochraceus* and *Penicillium viridicatum* was measured at 12 and 25°C. The grain had been fertilized with various amounts of nitrogen fertilizer (0, 90, or 240 kg/ha) and contained (at crop maturity) 9.1, 10.4, or 12.0% protein, respectively. The production of OA by both fungi increased as the protein concentration increased. Glutamic acid and proline were enriched relative to other amino acids as the protein concentration increased. The differences in OA production could not be explained by a differential effect of protein or amino acids on fungal growth in barley. However, glutamic acid and proline enhanced OA production in liquid cultures of both *A. ochraceus* and *P. viridicatum*.

Ochratoxins are a group of structurally related compounds produced by *Aspergillus* and *Penicillium* species (9). The most extensively studied is ochratoxin A (OA) which is toxic to many animals (4). Although OA has been found to be a natural contaminant in a number of agricultural commodities in different parts of the world, most studies have been done in Scandinavia and some Balkan countries, where OA has been found in animal feeds, mainly barley (10). The main pathological changes in farm animals include damage to and necrosis of the liver and kidneys. OA is also considered to be teratogenic (3).

Concerning the initiation of mycotoxin production, much of our present knowledge is based on mycotoxin production by fungi cultivated in liquid cultures. Several factors are known to influence OA production in liquid cultures, one of which is the source of nitrogen (5). The addition of glutamic acid and proline to the medium was shown to enhance the production of OA (6). Interestingly, proline was also found to stimulate the production of aflatoxins (13). This promotion of OA production by glutamic acid and proline was inhibited by several other amino acids as well as by analogs and antagonists of glutamic acid and proline. Our present knowledge concerning mycotoxin production on more complex substrates, such as grains, is still very limited, although many of the environmental factors that facilitate ochratoxin contamination of agricultural commodities have recently been summarized by Lillehoj and Elling (11). A relationship between OA production and environmental factors might be mediated by alterations in protein concentrations and amino acid composition; for example, a plant under water stress increases its proline content (16), and this might be expected to support the increased production of OA.

In the present investigation, we studied the effect of different amino acids on OA production by *Aspergillus ochraceus* and *Penicillium viridicatum* in liquid cultures. We also studied whether a solid substrate (barley) with different protein concentrations (or different proportions of amino acids) would influence the yield of OA.

MATERIALS AND METHODS

Ochratoxigenic organisms. Conidia from *A. ochraceus* Wilhelm (strain M4) or *P. viridicatum* Westling (strain M93) were used as inocula (cultures were gifts from K. Åkerstrand of the Swedish National Food Administration, Uppsala, Sweden).

OA production in liquid cultures. The basal medium of Ferreira (5) was supplemented with NH_4NO_3 (2 g/liter) and used as a control medium. L-Arginine (0.027 M) (Vitrum AB, Stockholm, Sweden), L-phenylalanine (0.075 M) (Mann Fine Chemicals, New York, N.Y.), L-glycine (0.075 M) (Schering AG, Berlin, Federal Republic of Germany), L-aspartic acid (0.075 M) (Sigma Chemical Co., St. Louis, Mo.), L-proline (0.075 M) (E. Merck AG, Darmstadt, Federal Republic of Germany), L-glutamic acid (0.075 M) (Calbiochem-Behring, La Jolla, Calif.), and L-asparagine (0.037 M) (E. Merck AG) were added separately to the liquid cultures to test the effect of amino acids on OA production.

The pH of the medium was adjusted to 6.0 with 1.0 M KOH, and the medium was sterilized at 121°C for 10 min. Conidial suspensions (1.6×10^6 conidia per ml) from 7-day-old cultures of *A. ochraceus* or *P. viridicatum* were added to Erlenmeyer flasks (200 ml) containing 50 ml of medium and incubated at 25°C in darkness for 9 days without agitation. At harvest, the pH was measured and the flask contents (medium and mycelium) were acidified (pH 1.5) and extracted with 50 ml of chloroform in a blender for 3 min. After filtration, the chloroform layer of the filtrate was separated and saved for OA analysis. The mycelium retained on the filter was dried at 70°C for 24 h and weighed.

OA production in barley. Barley grown at different levels of nitrogen fertilization (0, 90, or 240 kg/ha) was collected near Uppsala, Sweden. Three batches of barley (designated low-protein barley, medium-protein barley, and high-protein barley) contained 9.1, 10.4, and 12.0% protein, respectively. Protein content was measured by the method of Lowry et al. (12). Amino acid concentrations in ground barley hydrolyzed with phenol (1 mg/ml) for 24 h at 100°C were determined on a Durrum model D-500 amino acid analyzer with norleucine as an internal standard. The initial moisture content of the grain ranged from 6.3 to 7.5% (wet-weight basis) (15). The grain was sterilized by gamma irradiation before inoculation (120,000 Ci, batch-type ^{60}Co source; Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada; dose rate, 1.18 kGy/h). After the grain moisture was elevated to 22% (7) by the addition of sterile distilled water, the grain was inoculated with a conidial suspension of *A. ochraceus* or *P. viridicatum* (1.4×10^5 conidia per flask, 25 g of grain) obtained by washing 10-day-old cultures on malt agar with

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TABLE 1. Effect of certain amino acids on the growth of and the production of OA by *A. ochraceus* and *P. viridicatum* after 9 days of incubation at 25°C[a]

Amino acid	*A. ochraceus*		*P. viridicatum*	
	Growth (mg [dry wt])	OA (μg/liter)	Growth (mg [dry wt])	OA (μg/liter)
Arginine	721.6 ± 23.9	252 ± 13	767.0 ± 12.6	576 ± 12
Phenylalanine	187.6 ± 11.9	248 ± 17	684.4 ± 10.3	807 ± 42
Glycine	766.0 ± 40.7	912 ± 16	733.7 ± 16.4	1,121 ± 35
Leucine	356.9 ± 7.5	195 ± 8	734.7 ± 26.2	547 ± 15
Aspartic acid	265.8 ± 15.2	1,632 ± 31	745.2 ± 30.1	588 ± 37
Proline	657.1 ± 20.4	5,491 ± 27	901.7 ± 28.7	3,196 ± 39
Glutamic acid	523.0 ± 28.8	4,880 ± 42	798.9 ± 22.6	2,657 ± 25
Asparagine	305.4 ± 22.6	2,240 ± 11	719.2 ± 48.8	1,358 ± 35
Control	98.2 ± 15.9	124 ± 9	350.9 ± 19.3	142 ± 8

[a] Data represent the mean ± standard error of the mean of three replicates.

Tween 80 (0.05%). Noninoculated grain with the same moisture content served as the control. The flasks were covered with aluminum foil, sealed with Parafilm, shaken thoroughly, and incubated at 12 or 25°C in darkness. Samples were harvested after 3, 6, 9, 12, 20, and 28 days. The flask contents were dried overnight at 105°C, ground to pass through a 0.4-mm screen, and kept in a refrigerator until OA analysis. Chitin, a cell wall component of many fungi, was measured in cultures as an indicator of fungal growth (14).

Analysis of OA. The analysis of OA was done by a modification of the method of Howell and Taylor (8). Grain samples (20 g) were extracted with a combination of 0.1 M H_3PO_4 (10 ml) and $CHCl_3$ (100 ml) for 30 min on a wrist-action shaker and filtered through folded Munktell V150 filter paper. The filtrate (40 ml) was evaporated to dryness under a vacuum at 50°C, and the residue was dissolved in hexane (10 ml) before being passed through a Sep-Pak silica cartridge (Waters Associates, Inc., Milford, Mass.) prewet with hexane (10 ml). The silica was then washed with 10 ml of hexane-chloroform (9:1) followed by 10 ml of methanol-chloroform (3:97) before elution with 10 ml of toluene-acetic acid (9:1). The eluate was evaporated to dryness under N_2 and dissolved in 200 μl of benzene-acetic acid (9:1) before being spotted on thin-layer chromatography plates (DC Alufolien, Kiselgel 60; E. Merck AG) and developed with benzene-acetic acid (9:1). Chloroform extracts of liquid cultures were evaporated to dryness at 50°C and analyzed similarly.

TABLE 2. Chitin content in barley inoculated with *A. ochraceus* and *P. viridicatum*

Inoculum	Temp (°C)	Chitin content (μg/g of barley)[a] in:		
		Low-protein barley	Medium-protein barley	High-protein barley
A. ochraceus	12	120 ± 2 (100)	127 ± 1 (95)	142 ± 2 (89)
	25	222 ± 4 (270)	230 ± 3 (254)	248 ± 5 (230)
P. viridicatum	12	120 ± 4 (100)	135 ± 3 (108)	148 ± 6 (97)
	25	233 ± 2 (288)	242 ± 4 (272)	255 ± 5 (240)
None (control)		60 ± 3	65 ± 3	75 ± 3

[a] Data represent the mean ± standard error of the mean of three replicates. Numbers in parentheses represent the percent increase in the chitin content in barley over that in the control.

Plates were observed under long-wave (365-nm) UV light for the presence of OA. Positive samples were confirmed with BF_3 (14%) (1). OA was quantitated with a fluorodensitometer (Zeiss KM3 chromatogram spectrophotometer equipped with a mercury source). An exitation wavelength of 313 nm and a barrier filter (FL 43, <430 nm) were used. OA was calculated from the peak height with a standard curve. The recovery from spiked barley samples was 71%.

RESULTS

In liquid cultures, both the growth of and the production of OA by *A. ochraceus* and *P. viridicatum* were stimulated by all amino acids tested, although some specificities were observed (Table 1). In general, proline and glutamic acid were more efficient at stimulating growth and toxin production than were the other amino acids. It should be mentioned that sporulation of both fungi was observed after 6 days of incubation at 25°C and after 9 days of incubation at 12°C with all amino acids tested.

The production of OA in barley by both fungi was influenced by the protein content of the barley. At both 12 and 25°C, the amount of OA produced was greater in the high-protein barley than in the medium-protein barley, which supported more OA production than the low-protein barley (Fig. 1A through D). As expected, the onset of OA production was quicker at 25°C than at 12°C. *P. viridicatum* (Fig. 1C and D) produced more OA and did so more quickly than *A. ochraceus* (Fig. 1A and B) at both 12 and 25°C. Similar results were obtained with barley with protein concentrations of 8.5, 10.2 and 13.6% (data not shown). The growth of the two fungi in barley (measured as chitin content) was approximately equal under each condition (Table 2). The Chitin content was higher in barley incubated at 25°C than at 12°C. The chitin content was highest in the high-protein barley and lowest in the low-protein barley.

The possible role of amino acids in determining OA production in barley was investigated by determining the amino acid composition of barley with various protein concentrations. The amino acids present at the highest concentrations in all three batches of barley were glutamic acid and proline (Table 3). These amino acids were enriched

TABLE 3. Amino acids in barley with various protein concentrations

Amino acid	Amino acid concn (nmol/mg) in:		
	Low-protein barley	Medium-protein barley	High-protein barley
Aspartic acid	45.3	51.0	53.8
Threonine	29.1	33.8	36.8
Serine	43.0	48.2	54.1
Glutamic acid	133.5	164.2	196.2
Proline	77.5	103.0	117.4
Glycine	55.0	64.0	65.3
Alanine	47.9	53.0	56.1
Valine	41.7	47.3	51.5
Methionine	9.4	11.8	11.8
Isoleucine	25.4	30.7	33.5
Leucine	50.9	60.1	65.9
Tyrosine	12.6	14.3	16.5
Phenylalanine	27.1	36.1	37.7
Histidine	13.5	15.5	16.8
Lysine	26.3	27.1	30.2
Arginine	27.3	29.8	32.9

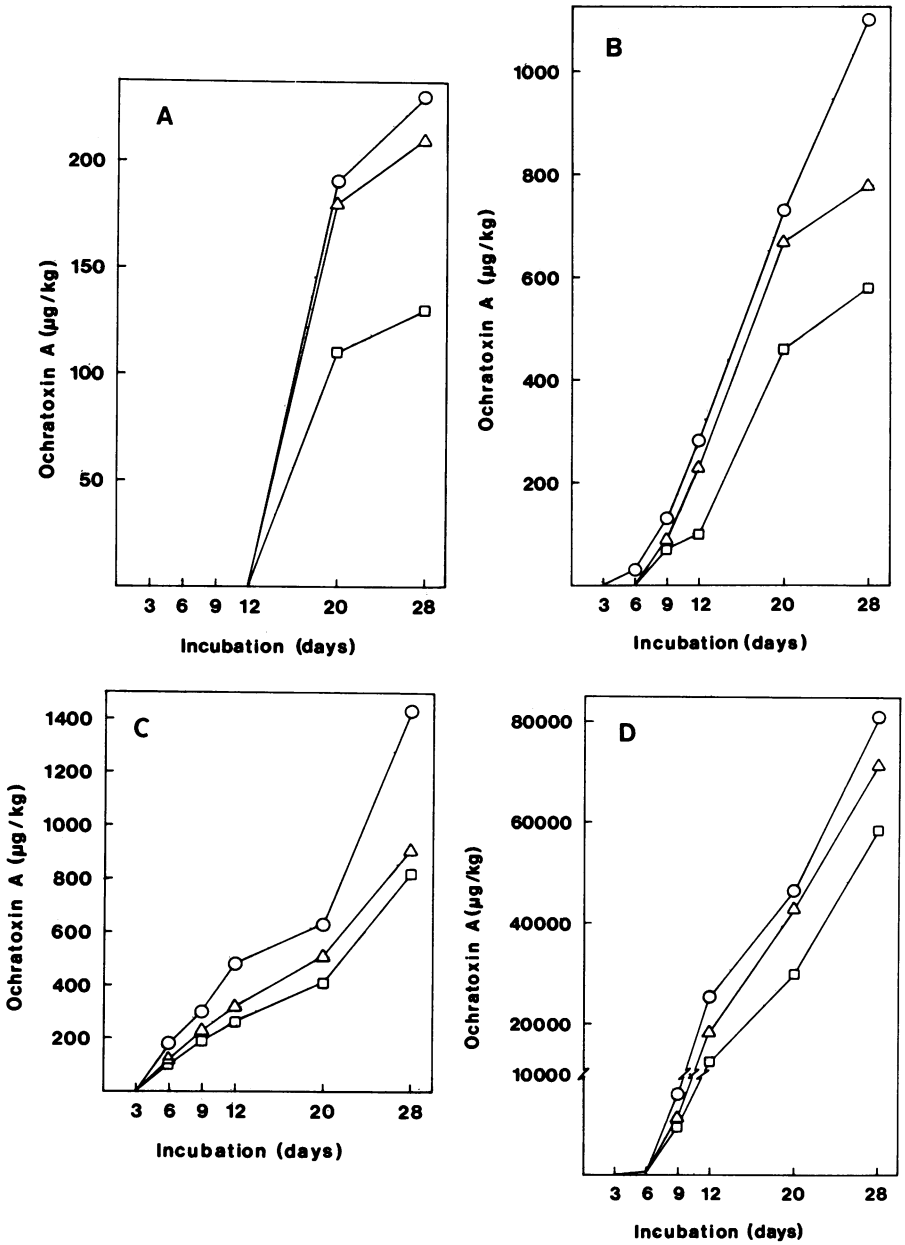

FIG. 1. Amounts of OA in barley (with a 22% moisture content) inoculated with *A. ochraceus* and incubated at 12°C (A) or 25°C (B) or inoculated with *P. viridicatum* and incubated at 12°C (C) or 25°C (D). Symbols: □, low-protein barley; △, medium-protein barley; ○, high-protein barley.

(a higher percentage of the total) as the protein concentration increased.

DISCUSSION

The stimulatory effect of amino acids, particularly glutamic acid and proline, on the production of OA in liquid cultures by *A. ochraceus* reported earlier (5) was confirmed by the present work. The present work also showed that *P. viridicatum* responded similarly. In barley, a positive correlation was found between the protein concentration of the barley and the production of OA by both *A. ochraceus* and *P. viridicatum* at 12 and 25°C.

The correlation between OA production and protein concentration could possibly be explained by differences in growth, but this reason was ruled out when we observed essentially identical growth at the three protein levels (Table 2). A more likely explanation rests in the finding in this study that the levels of glutamic acid and proline increased relative to other amino acid with increasing protein concentrations (Table 3). Such an increase was previously described for grain (17). These observations, combined with the finding that OA production in liquid cultures is increased by proline and glutamic acid, suggest that OA contamination of barley is a function of these two amino acids.

The stimulation of aflatoxin production described for proline (13) and glutamic acid (G. A. Payne, personal communication), particularly in stationary cultures of *Aspergillus flavus* and *Aspergillus parasiticus*, also makes it

essential to know the roles of these two amino acids. Glutamic acid is incorporated into both the phenylalanine and isocoumarin moieties of the OA molecule, indicating that the amino acid has no immediate effect on the amino acid metabolism of a fungus (2). To our knowledge, the incorporation of label from glutamic acid or proline has not been demonstrated for aflatoxins.

In contrast to aflatoxins, field contamination by OA has so far not been demonstrated. Therefore, it cannot be concluded that a high fertilization rate results in a product of lower acceptability. However, in a storage situation allowing the growth of *A. ochraceus* or *A. parasiticus*, higher levels of OA could be expected at higher protein concentrations in the grain resulting from high fertilization or any other factor, such as drought, that contributes to changes in the pool of free amino acids.

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