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Ochratoxin A Production by *Aspergillus ochraceus* Wilhelm Grown Under Controlled Atmospheres†

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When *Aspergillus ochraceus* NRRL 3174 was grown under controlled atmospheres with 1 and 5% O_2 and without CO_2, the amount of ochratoxin produced was the same as that produced by the control colonies. Increasing the O_2 level up to 40% reduced ochratoxin production by 75%, whereas at 60% O_2, ochratoxin production was enhanced. In atmospheres enriched with 10 or 20% CO_2, ochratoxin production was reduced when O_2 concentrations were below 20% and enhanced when the O_2 concentration was 40 or 60% O_2. Ochratoxin production was completely inhibited at 30% CO_2 and above, regardless of the O_2 level. Colony growth was partially inhibited at 60% CO_2, and no growth occurred at 80% CO_2 or above. However, when colonies inhibited by 60% CO_2 or above were subsequently exposed to air, radial growth, number of sclerotia formed, and the amount of ochratoxin produced were the same as in the control colonies. The results indicate that *A. ochraceus* is tolerant to CO_2 concentrations higher than those required to control storage insects.

Ochratoxin is a mycotoxin produced by many strains of *Aspergillus ochraceus*, and its presence in corn, barley, and wheat has been demonstrated. In animals, it causes tubular necrosis of the kidney and degeneration of the liver (8). In humans, a kidney disease (Balkan endemic nephropathy) has been correlated to the presence of ochratoxin (4).

The common way to prevent mycotoxin, including ochratoxin, production is to avoid mold growth on foodstuffs by either fungistatic agents or physical means (cooling, aeration, drying, modified atmospheres, etc.). Since the use of modified atmospheres has shown promising results in controlling insects in stored products (1, 3), the effects of such atmospheres on the growth of storage fungi and the subsequent production of mycotoxins were studied (2, 5, 6, 15).

Recently, Paster and Chet (11) studied the effects of modified atmospheres on sclerotia formation in *A. ochraceus* and found that high levels of CO_2 (20% and above) completely inhibited this morphogenetic process. However, the effect of these atmospheres on ochratoxin was not investigated. The present study constitutes an attempt to provide basic information on the effect of modified atmospheres on ochratoxin production.

A. ochraceus Wilhelm NRRL 3174 was used throughout this study. The fungus was grown in 100-ml Erlenmeyer flasks containing 20 ml of solid synthetic media (10). Agar disks (0.3 cm in diameter) covered with the mycelium of a 3-day-old colony were used as inoculum. A single disk was placed in the center of each Erlenmeyer flask, and a batch of six Erlenmeyer flasks was then connected to the system described by Navarro and Donahaye (9), which enables the formation of modified atmospheres. The system consisted basically of compressed CO_2, O_2, and N_2, a container in which the mixtures were prepared, and a series of valves that regulated the pressure and the air flow in the test chambers.

Experiments were carried out for 14 days at 16 ± 1°C. Erlenmeyer flasks in which the fungus was grown under normal air served as controls. Each experiment was conducted at least twice, and analysis of data was carried out with Duncan's multiple-range test at the 5% level (7).

A modification of the method of Takeda et al. (13) was used for ochratoxin determination. The contents of three inoculated Erlenmeyer flasks were blended together twice for 1 min in a mixture of 200 ml of acetonitrile–4% KCl–20% H_2SO_4 (89:10:1, vol/vol/vol). After filtration through a filter paper (Whatman no. 1), 50 ml of

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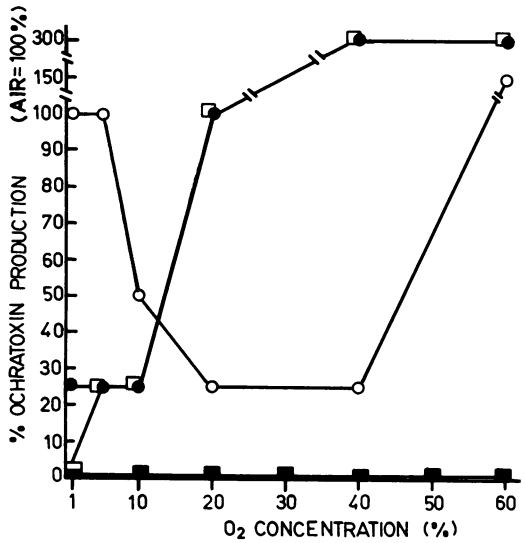

FIG. 1. Ochratoxin A production by *A. ochraceus* grown under modified atmospheres. Air (atmospheric conditions): ochratoxin production under these conditions was 2.0 μg/g. The CO_2 concentrations used were 0% (○), 10% (●), 20% (□), and 30% (■).

distilled water was added to 100 ml of the filtrate in a separatory funnel. The whole mixture was then fractionated twice with chloroform (50 and 30 ml, respectively). Both chloroform fractions were combined and fractionated twice with 50 ml of 0.5 N sodium bicarbonate. Both sodium bicarbonate phases were combined and carefully acidified to pH 2.5 with 1.2 N HCl; the acidified fraction was further fractionated, first with 75 ml and later with 50 ml of chloroform. The chloroform phases were combined and evaporated first to near dryness under reduced pressure on a steam bath and then under a N_2 stream until dryness. The residual preparation was resuspended in 0.5 ml of benzene-acetonitrile (98:2, vol/vol) and loaded on precoated Silica Gel 60 thin-layer chromatography plates without a fluorescent indicator (catalogue no. 5721; E. Merck AG, Darmstadt, Germany). Plates were developed in toluene–ethyl acetate–90% formic acid (50:40:5, vol/vol/vol), as this mixture gave the best separation between the toxin and accompanying pigments. Ochratoxin was estimated under a long-wave UV light (366 nm), and the fluorescence of the spots was compared with that of the standards. Ochratoxin A was purchased from Makor Chemicals Ltd., Jerusalem, Israel.

Mycelial dry weight was determined as follows. Erlenmeyer flasks were heated in a water bath until complete melting was attained. Mycelium was then removed from each flask, washed with distilled water to remove the remaining agar, and dried over filter paper (Whatman no. 1) at room temperature, followed by overnight drying at 80°C. Sclerotia were collected as described by Paster and Chet (10).

When *A. ochraceus* was grown under 1 or 5% O_2 without CO_2, the amount of ochratoxin produced was the same as that produced by the control (Fig. 1). Increases in O_2 concentrations of up to 40% reduced ochratoxin production by 75%. However, at 60% O_2 without CO_2, ochratoxin production was enhanced. Under 1, 5, or 10% O_2 in combination with 10 or 20% CO_2, the amount of ochratoxin produced was only 25% that produced by the control colonies (except for the combination 1% O_2–20% CO_2, under which ochratoxin was not produced).

The addition of 20% O_2 to atmospheres enriched separately with 10 or 20% CO_2 did not cause a reduction in the amount of ochratoxin produced (Fig. 1), whereas further increases of O_2 levels of up to 40 or 60% enhanced toxin production over the control. Ochratoxin production was completely inhibited at 30% CO_2 and above (40 and 60% CO_2), regardless of the O_2 levels given in combination with these levels.

In all the above combinations (except for 60% CO_2), dry weight of mycelia and diameters of exposed colonies were similar to those of the control at the end of the experiments.

Colonies grown for 14 days under 60% CO_2 were partially inhibited, and radial growth of these colonies was 63.6% that of the controls (Table 1). A reduction in the dry weight of mycelia was also noticed (to 60% of the control). After disconnection of test chambers from the modified atmosphere and exposure for the following 14 days to atmospheric conditions, colony diameter, number of sclerotia produced, and dry weight of the pretreated mycelia were the same as those of the control colonies. However, the amount of ochratoxin produced by the colonies after disconnection from the modified atmospheres was about three times higher than that formed by the control (55 versus 15 μg/g).

Total inhibition of mycelial growth occurred when the fungus was exposed for 14 days to atmospheres enriched with 80 or 100% CO_2. However, after disconnection from the system, followed by 14 days of exposure to air, no significant differences in dry weight of mycelia, radial growth, number of sclerotia formed, or amount of ochratoxin produced were observed between these colonies and the controls (Table 1).

The findings of the present work show that ochratoxin production was totally inhibited at high levels of CO_2 (30% and above), whereas at lower levels of CO_2, the inhibitory effect was pronounced only when the concentrations of O_2 were lower than 20%. Nevertheless, the inhibi-

TABLE 1. Effect of modified atmospheres on ochratoxin production, growth, and sclerotia formation by *Aspergillus ochraceus*[a]

CO_2, O_2, N_2 (%)	Expt I			Expt II			
	Dry wt (mg)[b]	Colony diam (mm)[b]	Sclerotia formation[c]	Dry wt (mg)	Colony diam (mm)	Ochratoxin A (μg/g)[d]	No. of sclerotia[e]
60, 20, 20	120^{B}	35^{B}	−	200^{A}	55	5.5^{A}	$1{,}720^{A}$
80, 20, 0	NG			190^{A}	55	1.5^{B}	$1{,}468^{A.B}$
100, 0, 0	NG			190^{A}	55	1.5^{B}	$1{,}814^{A}$
Control[f]	200^{A}	55^{A}	+	210^{A}	55	1.5^{B}	$1{,}878^{A}$
Control[g]				210^{A}	55	1.5^{B}	$1{,}751^{A}$

[a] Expt I, After 14 days of growth under controlled atmospheres; Expt II, after disconnection from the controlled atmosphere and 14 days of exposure to normal air.

[b] Average of six replicates; means with a common letter are not significantly different at $P < 0.05$. NG, No growth.

[c] −, No sclerotia; +, sclerotia appearance.

[d] Average of two replicates; means with a common letter are not significantly different at $P < 0.05$.

[e] Average of three replicates; means with a common letter are not significantly different at $P < 0.05$.

[f] Control (atmospheric conditions) from the beginning of the experiments.

[g] Control (atmospheric conditions) added after CO_2-treated mycelia were disconnected from the controlled atmosphere.

tory effect of CO_2 cannot be due to a direct effect on mycelial growth, as growth was retarded only at 60% CO_2 and above. Landers et al. (5) reported that aflatoxin production in peanut kernels was strongly inhibited by 80% CO_2 or above. Working with *Penicillium martensii*, Lillehoj et al. (6) found a general reduction in penicillic acid accumulation at 40% CO_2 or above. Therefore, it seems that toxin production by some species of *Penicillium* and *Aspergillus* can be controlled by elevated CO_2 levels; however, the inhibitory concentrations may differ among different species.

Many fungi can grow at low levels of O_2 or high levels of CO_2 (12, 14, 15). In the present study, *A. ochraceus* was found to be highly tolerant to high levels of CO_2, and only 80% CO_2 and above caused total inhibition of fungal growth. Moreover, these inhibitory concentrations were not lethal to the fungus, and after removing the colonies from these atmospheres, normal growth, along with ochratoxin production and sclerotia formation, occurred. A correlation between ochratoxin production and sclerotia formation was found by us (unpublished data) in studying the effect of methionine and closely related compounds on these processes. We found that in most cases when sclerotia formation was inhibited, toxin production was also reduced. Recently, Paster and Chet (11) found that high levels of CO_2 (20% and above) inhibited the formation of sclerotia in *A. ochraceus*. The results of the present study confirm their findings. The possibility arises that both processes are partially controlled by some common metabolic pathways which are affected by high levels of CO_2 or some chemicals, resulting in the inhibition of both ochratoxin production and sclerotia formation.

The use of modified atmospheres to control insects in stored products was found to be promising. However, the levels of CO_2 needed to control these insects did not exceed 40% (1, 3). The data of our study, along with those of other reports (2, 15), indicate that CO_2 concentrations needed for insect control are far below those required for the control of storage fungi.

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