Role of the Competitive Microbial Flora in the Radiation-Induced Enhancement of Ochratoxin Production by *Aspergillus alutaceus* NRRL 3174

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The radiation sensitivity and the toxigenic potential of conidiospores of the fungus *Aspergillus alutaceus* var. *alutaceus* were determined after irradiation with 60Co gamma rays and high-energy electrons. Over the pH range of 3.6 to 8.8, the doses required for a 1 log10 reduction in viability based on the exponential portion of the survival curve ranged from 0.21 to 0.22 kGy, with extrapolation numbers (extrapolation of the exponential portion of the survival curve to zero dose) of 1.01 to 1.33, for electron irradiation, and from 0.24 to 0.27 kGy, with extrapolation numbers of 2.26 to 5.13, for gamma irradiation. Nonsterile barley that was inoculated with conidia of the fungus and then irradiated with either electrons or gamma rays and incubated for prolonged periods at 28°C and at a moisture content of 25% produced less ochratoxin A with increasing doses of radiation. Inoculation of barley following irradiation resulted in enhanced ochratoxin levels compared with unirradiated controls. In these experiments, inoculation with 102 spores per g produced greater radiation-induced enhancement than inoculation with 105 spores per g. There was no radiation-induced enhancement when the barley was surface sterilized by chemical means prior to irradiation. These results are consistent with the hypothesis that a reduction in the competing microbial flora by irradiation is responsible for the enhanced mycotoxin production observed when nonsterile barley is inoculated with the toxigenic fungus *A. alutaceus* var. *alutaceus* after irradiation.

There has been increasing interest in the use of ionizing radiation, particularly high-energy electron beams, for killing endogenous insects in stored cereal grains. Indeed, for several years grains imported into the USSR through the port of Odessa have been disinfested, when necessary, by irradiation with electrons. Since the work of Jemmali and Guibot (11) and several subsequent studies (1–3, 19–22), concerns about enhancement of production of mycotoxin resulting from gamma irradiation have been raised. In these studies, higher levels of mycotoxins, such as aflatoxins produced by toxigenic strains of *Aspergillus flavus* or *Aspergillus parasiticus* or ochratoxins produced by *Aspergillus ochraceus*, were found in irradiated grains or foods than in unirradiated controls. In other studies (4, 10, 17, 18), increased levels of mycotoxins were not observed in irradiated grains. In many of the studies (20–22) the workers used heat-sterilized substrates that were inoculated and then irradiated or that were inoculated with irradiated toxigenic cultures. These experiments often demonstrated enhanced mycotoxin production compared with unirradiated substrates or cultures. The interpretation of such results in relation to normal grain- or food-handling practices is difficult.

Grains in commercial trade undergo various handling and storage practices, depending on climatic conditions and market demands. Exposure of grains to high moisture levels is most likely to occur during harvest, but may occur at other stages between production and final consumption. Mycotoxin contamination of grain is difficult to predict because it depends on a complex interaction of factors, such as temperature, moisture, the kind of grain, endogenous fungal species, storage history, storage time, type of transit, and transit time. The moisture content and temperature are the most important variables in determining the rate of deterioration and the rate of mycotoxin production by fungi.

If irradiation for disinfestation were to be utilized, the most suitable location for irradiation would be at major terminals. Consequently, any experimentation on the effects of irradiation on mycotoxin production must examine these effects after both pre- and postirradiation inoculation of toxigenic fungi, since in actual practice contamination can occur at any time. Ideally, experimentation should be done under conditions that are representative of the conditions present during actual grain handling, but since fungi require certain minima of moisture and temperature for growth and toxin production, which generally are avoided in practice, such an approach would be extremely time consuming. Consequently, we examined the effects under conditions that are almost ideal for growth of the test organism, realizing that this is close to a worst-case scenario as far as grain-handling conditions are concerned. The test organism which we used was strain NRRL 3174 of *Aspergillus alutaceus* var. *alutaceus* Berkley et Curtis, which has been reported to yield enhanced ochratoxin production (3, 18) with irradiated substrates. This study was intended to determine whether radiation enhancement of ochratoxin production does occur and, if so, under what conditions it occurs. We also attempted to describe the mechanisms involved in this phenomenon.

In addition, since the use of electron beam radiation for disinfestation purposes is relatively new, we also examined the efficiency of this method for inactivating conidiospores of our test fungus compared with the efficiency of 60Co gamma radiation.

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MATERIALS AND METHODS

Media and chemicals. The dehydrated potato dextrose agar (PDA) and yeast extract which we used were products of Difco Laboratories, Detroit, Mich. Tween 80 was obtained from Koch-Light Laboratories, Ltd., Colmbrook Bucks, England. Sodium chloride, mercuric chloride, sodium acetate, sodium sulfide, sodium thiosulfate, and chloroform (certified American Chemical Society grades) were obtained from Fisher Scientific Co., Fair Lawn, N.J. High-pressure liquid chromatography grade solvents were used. Tris preset pH crystals were purchased from Sigma Chemical Co., St. Louis, Mo.

Toxicogenic strain. Strain NRRL 3174 of A. ochraceus Wilhelm was obtained from the American Type Culture Collection, Rockville, Md. The name A. alternata var. alutaceus Berkely et Curtis was assigned to this organism for reasons given previously (5). The culture was maintained by regular transfer onto slants of PDA containing 0.1% yeast extract and 2% sodium chloride.

Radiation sensitivity determination. Spores from 7-day-old cultures grown at 23°C on slants of PDA containing 0.1% yeast extract and 2% sodium chloride were harvested in sterile 0.1% Tween 80, filtered through four layers of sterile cheesecloth, pelleted by centrifugation at 12,000 × g for 5 min at 5°C, and then resuspended and washed in fresh 0.1% Tween 80. The pellets were resuspended in fresh Tween 80, and the spores were counted with a hemacytometer. The spore suspension was dispensed in appropriate volumes into 0.05 M acetate buffer (pH 3.6 and 5.4) and 0.05 M Tris buffer (pH 7.0, 8.0, and 8.8) to give final concentrations of conidia of 10^5 conidia per ml. These preparations were irradiated in air with a 60Co radiation unit (Gammacell model 220 apparatus; Atomic Energy of Canada, Ltd.) by using doses of 0, 0.2, 0.4, 0.8, and 1.2 kGy and a dose rate of 200 GY/min. Similarly, irradiation with 10-MeV electrons was accomplished by using a model I-10/1 electron accelerator (Atomic Energy of Canada, Ltd.) at an average dose rate of 4.5 kGy/s (the instantaneous dose rate within each pulse was 3.8 × 10^4 kGy/s). Dosimetry was performed by using radiocromic film and previously described methods (15). The survival levels of the spore suspensions were determined by serial dilutions of the preparations and plating duplicate samples onto PDA plates acidified to pH 3.5 with tartaric acid. The plates were incubated at 28°C for 4 days, after which colonies were counted and survival curves were constructed.

Radiation effects on ochratoxin production. Samples (200 g) of barley (Hordeum vulgare cv. Bedford) were irradiated with various doses ranging from 0 to 4 kGy by using either 60Co gamma rays or 10-MeV electrons. The barley samples were inoculated with spores (10^7, 10^8, or 10^9 spores per g) of A. alternata var. alutaceus either before or after irradiation. Prior to irradiation the barley was preconditioned for 24 h at 4°C to a moisture content of 25% (dry weight basis). Following irradiation the samples were maintained at 28°C in a humidified incubator for 50, 51, or 113 days. The ochratoxin A levels and numbers of fungal colony-forming units either were determined at the end of the incubation period or were followed serially by aseptically withdrawing samples (approximately 30 g) at various times during the incubation period. These samples were thoroughly mixed, and duplicate subsamples (1 g) were shaken in 10 ml of sterile water with approximately 5 g of sterile sand. Decimal dilutions of the supernatant were plated onto acidified PDA. The plates were incubated for 4 days at 28°C, the colonies were counted, and the data were expressed as the number of colony-forming units per gram.

For ochratoxin A determinations, 25-g samples of barley were air-dried and ground with a Cyclotec sample mill equipped with a 1-mm screen (Tecator AB, Höganäs, Sweden). Mycotoxin extraction and a high-pressure liquid chromatography analysis were carried out as described previously (9).

Alteration of endogenous microbial flora. Two types of treatment (other than irradiation) were used to inactivate the endogenous microbial flora on the barley. In the first method 600 g of barley (H. vulgare cv. Bedford) was treated with 1 liter of 0.1% mercuric chloride for 10 min and drained, the residual mercuric chloride was inactivated with 2% sodium sulfide, and the barley was washed five times in sterile distilled water. The grain was then covered with nonabsorbent cotton and air dried at 45°C to its original weight. Lots containing 75 g of the surface-sterilized barley and control barley were conditioned to a moisture content of 25% (dry weight basis) and irradiated with gamma rays by using doses of 0.3, 0.75, 1.0, 2.0, 5.0, and 10.0 kGy. The 10-kGy dose was chosen because it was a sterilizing dose for this barley lot and should have allowed us to determine the effect of complete elimination of the endogenous microflora. Each lot was divided into three 25-g replicates, which were inoculated with conidia of the toxigenic fungus (10^5 conidia per g of barley), incubated at 28°C for 50 days, air dried, ground, and analyzed for ochratoxin A as described above.

The second type of surface sterilization involved treatment with 2% sodium hypochlorite for 10 min, draining, and neutralizing with 2% sodium thiosulfate. This was followed by five washes in sterile distilled water. The subsequent experimental procedure for determining the effect of irradiation on ochratoxin A production in the inoculated grain samples was the same as that described above.

To determine the numbers of bacteria, yeasts, and fungi in the barley samples, 10 g of barley and approximately 25 g of sterile sand were shaken for 3 min with 100 ml of sterile distilled water. Decimal dilutions of this extract were plated onto nutrient agar to determine the total plate counts and onto acidified PDA to determine the fungal and yeast counts. Aliquots (0.5 ml) of the extract were also aseptically spread onto the surfaces of several plates containing these agar media to recover microorganisms to be used as a source for reconstitution of the competing flora in irradiated and/or surface-sterilized barley. After incubation for 4 days at 28°C, the bacterial cells and fungal conidia were collected separately from the plates in sterile distilled water, centrifuged at 12,000 × g, washed by centrifugation, and suspended in sterile distilled water. The bacteria and fungal conidia in the suspensions were counted with a hemacytometer. The reconstitution experiment was performed by adding the appropriate volumes of bacterial and fungal suspensions to the barley based on the total plate counts and the fungal and yeast plate counts determined for the unirradiated control sample.

RESULTS AND DISCUSSION

Radiation sensitivity of A. alternata var. alutaceus. The study of the effects of gamma irradiation and electron beam irradiation on the toxigenicity of A. alternata var. alutaceus required us to determine the sensitivity of this organism to these types of ionizing radiation. During mold growth non-sterilized grain can undergo wide changes in pH; therefore, the radiation sensitivity of the conidia was determined in buffers ranging in pH from 3.6 to 8.8. As Table 1 shows, the doses required for a 1 log_10 reduction in viability based on
TABLE 1. Electron and gamma radiation sensitivity of A. alutaceus var. alutaceus conidia irradiated in air at 23°C when suspended in acetate buffer (pH 3.6 and 5.4) and Tris buffer (pH 7.0, 8.0, and 8.8).

<table>
<thead>
<tr>
<th>pH</th>
<th>D10 (kGy) Electron radiation</th>
<th>Extrapolation no. Correlation coefficient</th>
<th>D10 (kGy) Gamma radiation</th>
<th>Extrapolation no. Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>0.21</td>
<td>1.07</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>0.21</td>
<td>1.20</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>0.21</td>
<td>1.01</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>0.22</td>
<td>1.16</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td>8.8</td>
<td>0.21</td>
<td>1.33</td>
<td>0.996</td>
<td></td>
</tr>
</tbody>
</table>

The exponential portion of the survival curve (D10 values) were constant over this pH range, with the values for gamma irradiation slightly higher than those for electron beam treatment. Similarly, the extrapolation numbers (extrapolation of the exponential portion of the survival curve to zero dose) were larger for gamma radiation. These results may indicate that damage from ionizing energy at very high dose rates, such as in the electron beam treatment, is less readily repairable. The D10 values obtained in this study (average, 0.25 kGy) after gamma irradiation were somewhat higher than the value of 0.17 kGy found by Choi et al. (6) for a Korean strain of the same organism irradiated at pH 6.5. These authors also observed a reduction in the D10 value with increased dose rate. There is no information available on the radiation sensitivity of A. alutaceus var. alutaceus to high-energy electrons, but our results indicate that this organism is at least as sensitive to electrons as it is to gamma radiation.

Toxin production as a function of pre- and postirradiation inoculation. When barley was inoculated with conidia of the toxigenic fungus (10^6 conidia per g), irradiated with specified doses of either gamma or electron radiation, and incubated as described above, there was less ochratoxin A production at all doses after 51 days than in the unirradiated control (Table 2). Mycotoxin production was not detected after 3.03 kGy of electron radiation and 4.9 kGy of gamma radiation. These inactivation doses that resulted in complete elimination of the inoculum spores (assuming that lack of mycotoxin was due to a loss of viability of all spores) were somewhat higher than the values expected on the basis of the determined D10 values and the inoculum size, but this may have been due to differences in hydration state. Kume et al. (14) reported that D10 values for Aspergillus sp. increased when conidia were irradiated in the dry state. The D10 values that we determined were for conidia in suspension, while in the toxigenicity experiments the conidia were at the moisture content of the grain (25%), which would have made them more radiation resistant.

Figure 1 shows toxin production in the inoculated grain samples as a function of incubation time following inoculation into damp grain, both before and after treatment of the grain with 2 kGy of gamma irradiation. The changes in the mycoflora are shown in Fig. 1A through C. When we used preirradiation inoculation with 10^6 conidia per g of grain, gamma irradiation reduced ochratoxin A concentrations to undetectable levels for the first 51 days of incubation, and after 113 days of incubation the concentration was much lower than the concentration in the corresponding unirradiated control (Fig. 1D). Unirradiated and uninoculated control barley produced no ochratoxin A throughout the entire

![Graphs](http://aem.asm.org/)

**Table 2.** Ochratoxin A production in irradiated barley (moisture content, 25%) that was inoculated with A. alutaceus var. alutaceus (10^6 conidia per g) and incubated at 28°C for 51 days.

<table>
<thead>
<tr>
<th>Radiation type</th>
<th>Dose (kGy)</th>
<th>Ochratoxin A concn (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unirradiated)</td>
<td>0</td>
<td>17.60 ± 1.94</td>
</tr>
<tr>
<td>Electrons 0.49</td>
<td>12.43 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>16.76 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>2.21</td>
<td>8.30 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>2.10</td>
<td>5.89 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>3.03</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Gamma radiation 0.5</td>
<td>14.40 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>14.75 ± 3.04</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>12.85 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>3.96 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
incubation period. As shown in Fig. 1A, the mycoflora were reduced to levels of less than 10 CFU/g by the 2-kGy radiation dose, as determined immediately after irradiation, and following regrowth the maximum level was not reached until after 37 days of incubation. The other fungi were mostly Penicillium and Aspergillus species, but after 18 days of incubation there was an increasing proportion of A. alutaceus var. alutaceus, indicating that some conidia of this toxigenic fungus survived the radiation treatment and that the numbers of this organism increased with increasing incubation time to become the major fraction of the colony-forming propagules at later times in the incubation period. However, the toxin production was lower than that in the unirradiated control. In the unirradiated but inoculated control sample the major proportion of fungus propagules were A. alutaceus var. alutaceus propagules throughout the entire incubation period.

As Fig. 1 also shows, postirradiation inoculation of the barley resulted in enhancement of ochratoxin A production, with greater enhancement if the barley was inoculated with \(10^5\) conidia per g (Fig. 1F) than if it was inoculated with \(10^7\) conidia per g (Fig. 1E). These results are consistent with those of other workers (12, 17) who have shown similar effects with aflatoxin-producing fungi. The unirradiated and inoculated control produced only low levels of ochratoxin A (Fig. 1F).

The levels of enhancement that resulted from postirradiation inoculation could be explained by the following two possible mechanisms: (i) irradiation affected the grain substrate in a way that increased ochratoxin A production when preparations were inoculated with A. alutaceus var. alutaceus; and (ii) irradiation eliminated some of the competing flora so that inoculation with the fungus resulted in better growth and toxin production by the fungus. To better understand which mechanism was responsible, we performed radiation experiments after alteration of the endogenous flora.

**Mycoflora enhancement after radiation.** The two surface-sterilizing agents which we used, mercuric chloride (Fig. 2) and sodium hypochlorite (Fig. 3), had similar effects on ochratoxin A production on the substrate when the substrate was inoculated postirradiation with A. alutaceus var. alutaceus and incubated for 50 days. In both cases ochratoxin A levels were higher in the surface-sterilized grain than in the control grain at all radiation doses, including the unirradiated controls. The chemically untreated grain produced increasing levels of ochratoxin A as a function of radiation dose as described above (Fig. 1). These data are consistent with the hypothesis that elimination of the competitive flora is the major contributor to radiation enhancement of ochratoxin A production by A. alutaceus var. alutaceus when this organism is inoculated after irradiation into nonsterilized grain. If the effect had been due to a radiation effect on the grain substrate, there should have been increasing levels of ochratoxin A production in both surface-sterilized and nonsterilized grain as a function of radiation dose. The data clearly eliminated this possibility.

The endogenous flora in the barley lot used for these experiments, as determined by plate counting, was found to consist of \(4.1 \times 10^3\) bacteria per g of grain and \(5.3 \times 10^5\) fungi and yeasts per g of grain. When the competing flora isolated from this barley lot was reintroduced at the levels given above into irradiated, surface-sterilized barley or surface-sterilized, irradiated barley, the amount of ochratoxin A produced was reduced to approximately the level in the unirradiated control (Table 3). We realized that the isolation procedure may have enriched some microbial species relative to others present in the grain, but it was clear that the added organisms were derived from the grain and had a profound effect on ochratoxin A production by A. alutaceus var. alutaceus. This is additional evidence that the observed enhancement of ochratoxin production when irradiated grain was inoculated with the toxigenic fungus was caused by a reduction in the competing microbial flora by the radiation.

There have been several descriptions (7, 8, 13, 16) of various organisms, both fungal and bacterial, that affect the growth and/or toxigenicity of mycotoxin-producing fungi. In some cases, the effects were enhancement of toxin production, while in other cases the competing organisms resulted in reductions in mycotoxin levels. In the conflicting reports regarding the enhancement of mycotoxin production by irradiation, at least in those instances where experiments were done with nonsterilized grain, elimination of the competing flora could have caused the observed effects. The types and quantities of the competing microbial flora vary
from one grain lot to the next, resulting in lot-to-lot variability in the observed effects on toxin production. Any physical or chemical treatment that alters the quality and quantity of the competing microbial flora in the grain could have profound effects on the amounts of mycotoxins produced, if such treated grain is inadvertently contaminated with a toxigenic fungus and exposed to sufficient moisture to allow growth. In this context radiation and chemical fumigation should produce similar effects, since both agents can have microbicidal effects. There have been isolated reports (23, 24) of both decreases in and enhancements of mycotoxin levels after fumigation, but much more extensive work is needed. Fumigation has been used for several decades in the grain industry, and an estimate of the risk of mycotoxin enhancement arising from the elimination or reduction of the competing microbial flora can be made on the basis of this past work.

We have previously described (5) the isolation of A. alutaceus variants that exhibit both increased and decreased toxigenicity from grain that was inoculated and then irradiated. In our studies we have never encountered any enhancement of mycotoxin production in grain that was inoculated prior to irradiation. Consequently, any variants that have the potential for producing greatly increased amounts of ochratoxin A in sterilized grain must not be able to compete as effectively as the parent strain with the existing flora in nonsterilized grain. In this study we found that in the A. alutaceus var. alutaceus-ochratoxin A model, elimination of the competing flora may be the major contributor to enhancement of mycotoxin production by irradiation. However, the general applicability of this hypothesis to other mycotoxin systems will require further study.

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REFERENCES