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Scanning Electron Microscope Observations of Growth and Ochratoxin - A Production of *Aspergillus alutaceus* Variety *alutaceus* (Formerly *A. ochraceus*) on Gamma-Irradiated Barley

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SCANNING ELECTRON MICROSCOPE OBSERVATIONS OF GROWTH AND OCHRATOXIN-A
PRODUCTION OF ASPERGILLUS ALUTACEUS VARIETY ALUTACEUS (FORMERLY A.
OCHRACEUS) ON GAMMA-IRRADIATED BARLEY

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Abstract

Scanning electron microscope examination, colony counting and biochemical studies were conducted to describe the effect of gamma-irradiation on growth and ochratoxin A production by Aspergillus alutaceus. Irradiation at a dose of 1.0 or 2.0 kGy reduced the level of mold growth greatly relative to unirradiated controls. Growth in the irradiated samples after 7 to 12 day incubation was mainly in cracks in the hull, and less mycelium was seen on the grain surface. In unirradiated controls, mycelial growth was heavy and, although conidial heads were most abundant in cracks in the hull, they were seen over the whole surface. When the barley was inoculated before irradiation, the number of colony forming units (cfu) at 5 days after 1.0 or 2.0 kGy irradiation was lower than in the unirradiated controls; however, the number increased over the control by 30 days. A dose of 4.0 kGy eliminated viable fungi. Ochratoxin A production decreased from the control level of 17.6 µg/g with increased dose and was below the detection limit above 4.0 kGy. When barley was inoculated after irradiation the spore count and the ochratoxin A level were higher than the unirradiated control after 27 days. We conclude that the difference in growth and ochratoxin A production on irradiated and unirradiated barley is due to the effect of irradiation on the natural competitive microflora on the grain surface and the reduction of inoculum size of the A. alutaceus by radiation.

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Introduction

Insect disinfestation of imported grain by ionizing radiation is used on a commercial scale (400 000 tons of grain/a) in the Soviet Union (IAEA, 1986). Radiation processing of grain may become more widespread in the future (Chuaqui-Offermanns, 1987). In Canada, regulations allow grain to be irradiated to a maximum dose of 0.75 kGy for insect disinfestation (Health and Welfare Canada, 1988). Various other countries allow doses ranging from 0.3 to 1.0 kGy (IAEA, 1988). In all, 13 countries allow grain irradiation for insect disinfestation on a commercial or conditional basis (IAEA, 1988). The increasing interest is based, in part, on the extensive literature describing the usefulness of irradiation to disinfest grain (Erhart, 1990; Szlendak et al., 1990; Getoff, 1989; Ng et al., 1989; Zakladnoy et al., 1989).

Although it is clear that the radiation treatment kills insects within grain, some workers have suggested irradiation might increase mycotoxin production by surface molds. There are a number of conflicting reports, which suggest that the production of mycotoxins is either increased (Applegate and Chipley, 1976; Paster et al., 1985; Schindler et al., 1980; Priyadarshini and Tulpule, 1976, 1979), decreased (Odamtten et al., 1986; Farkas, 1989; Sharma et al., 1990) or unaffected (Paster and Bullerman, 1988) after irradiation of grain or spores under various laboratory conditions. It appears that the fungal strain, conditions of storage, humidity, inoculum size and irradiation dose affect mold growth and toxin production (Mitchell, 1988).

To resolve some of the conflicting results in the literature, Chelack et al. (1991a) have studied the growth and toxigenicity on barley of the ochratoxin-producing fungus, Aspergillus alutaceus var. alutaceus, after exposure to ionizing radiation. Ochratoxin A is of interest because it is one of the principle toxins produced in moist grain on the Canadian prairies and it is sometimes detected in commercial seeds and feeds (Abramson et al., 1990; Marquardt et al., 1990; Mills, 1990). Although the risk of mycotoxin development in western Canadian grains is considered low, there is a continuing need for research into safe storage of grain to reduce mycotoxin production, and into treatments, such

as irradiation, to assure that stored grain remains free of insect pests.

Questions have also been raised about the possibility of radiation-induced mutants of toxigenic fungi, which have an increased mycotoxin production capability, being formed in irradiated grain (Murray, 1990). In a controlled laboratory setting it is possible to generate variants with increased or decreased mycotoxin production. Three such variants have been studied and characterized (Chelack et al., 1991b). The scanning electron microscope (SEM) was used to confirm by spore morphology the taxonomy of variants produced after irradiation as suggested by the work of Kozakiewicz (1989). We observed the pattern of growth of these three variants as seen in the SEM to check whether the morphology or growth characteristics had been altered.

The principle objectives of this study were to use the SEM to elucidate the effect of irradiation on the morphology and growth characteristics of *A. Alutaceus* on barley grains and to ascertain the effect of irradiation on ochratoxin A production in barley during storage under various simulated conditions.

Materials and Methods

Parent strain

Aspergillus ochraceus Wilhelm, NRRL-3174 was obtained from the American Type Culture Collection, Rockville, Maryland. The culture was maintained by regular transfer onto slants of potato dextrose agar containing 0.1% yeast extract and 2% sodium chloride (PDA-YE-NaCl). In our laboratory this parental strain was given the accession number 001. The name, *Aspergillus alutaceus* var. *alutaceus* Berkeley et Curtis, was assigned to this organism because it is an earlier name suggested by Subramanian (1971) that was later endorsed by Samson and Gams (1986).

Irradiation conditions and treatment of barley

Five hundred grams of barley (variety Bedford) was adjusted to have a final moisture content of 25% after it was inoculated with spores. The moisture content was determined (dry weight basis, d.b.) by standard methods (Brook and Foster, 1981). The grain was inoculated with the appropriate concentration of spores in sterile 0.1% Tween 80 in water (10^2 to 10^6 spores/g), stored overnight at 4°C, and then irradiated in a ^{60}Co irradiator (Gamma Cell 220; AECL) at a dose rate of 198 Gy/min. The irradiated grain, as well as a comparable unirradiated but inoculated control, was incubated in loosely capped Nalgene bottles at 28°C and 98% relative humidity in a humidified incubator.

Variant strain production

Spores from 7-day cultures of the parent strain grown at 28°C on PDA-YE-NaCl slants were harvested in sterile 0.1% Tween 80, filtered through four layers of sterile cheesecloth and washed in fresh Tween 80 (0.1%). The spores were counted in a hemocytometer and added to barley at a rate of 10^6 spores/g. The inoculated samples were then irradiated at a dose of 1 kGy and incubated at 28°C. After 10 days of incubation, 25 seeds (~1 g) of each lot were shaken with 10 ml of sterile distilled water containing approximately 4 g of sterile sand in a 18-ml tube.

Serial dilutions of the supernatant were plated in PDA-YE-NaCl medium that had been acidified with 1.6 ml of sterile 10% tartaric acid per 100 ml of medium. After incubation, the colonies were examined for any characteristics that differed from those of the parental strain. Variant strains called 005, 006 and 007, with different ochratoxin A production than the parent, were isolated and cultured for further study. They are described in Chelack et al. (1991b).

Scanning electron microscopy

Conidia grown on agar medium or barley were prepared for SEM using the simplified method of Kozakiewicz (1989). Conidia were applied to standard aluminum stubs, coated with gold (Gold Sputter unit, Balzer Union), and then examined directly in the microscope (ISI Model DS130). Photographs were taken using Ilford 120 film. Barley kernels with surface mold were fixed in OsO_4 vapour for 1 h at room temperature before being mounted on aluminum stubs with conducting paint and then gold-coated.

Colony counting

Colony forming units (cfu) on the grain surface were quantified by serial dilutions and plating of duplicate samples on potato dextrose agar plates acidified to pH 3.5 with tartaric acid. The plates were incubated at 28°C for four days to allow for colony formation. More details are found in Chelack et al. (1991a).

Ochratoxin A measurement

Ochratoxin A was measured by the improved procedure of Frohlich et al. (1988). Briefly, two 5-g grain samples were extracted with acidified H_3PO_4 in chloroform (2.5 ml of 0.1 mol/l H_3PO_4 plus 30 ml of CHCl_3) and the extracts were subjected to reverse phase thin-layer chromatography in hexane to remove lipids followed by a second development in 70% methanol. The separated spots were scraped and collected. Ochratoxin A was eluted from the adsorbent and analysed quantitatively by either liquid chromatography with fluorescence detection or by direct spectrofluorometry.

Results

Scanning electron microscopy observations

Figure 1 shows mold growth on an unirradiated, autoclaved barley kernel that was innoculated with *A. alutaceus* and incubated for 12 days at 28°C and 98% humidity. The conidial heads were more frequently seen at crevices in the barley husk than on the surface. In addition, surface mycelium was seen over the husk along with newly forming conidial heads. When the barley kernels were inoculated without steam sterilization, as was the case in all the following figures, other forms of mold could also grow on the surface in addition to the *A. alutaceus*, which was the inoculant. In unsterilized samples that were incubated for approximately 30 or more days a number of variously coloured fungi were seen in low-magnification light microscopy on the control and irradiated barley kernels. The fungi were not identified.

Irradiation at a dose of 1.0 or 2.0 kGy after inoculation with 10^6 spores/g reduced the level of mold growth on the kernels, but did not eliminate it entirely. The irradiated kernels were

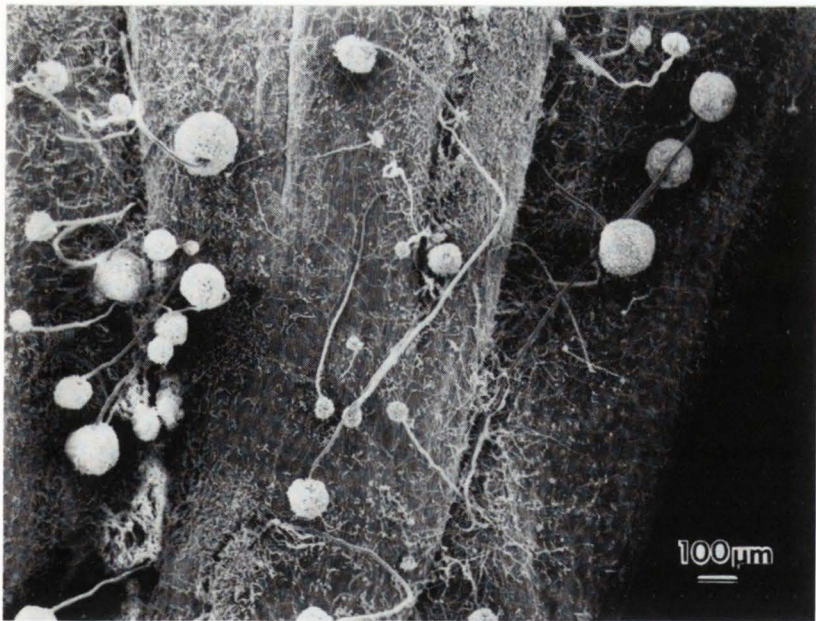


Figure 1. A SEM micrograph of an autoclaved barley kernel infected with *A. alutaceus*, after 12-day incubation at 28°C, showing mycelium on the surface and conidial heads, whose origins are concentrated at cracks in the hull.

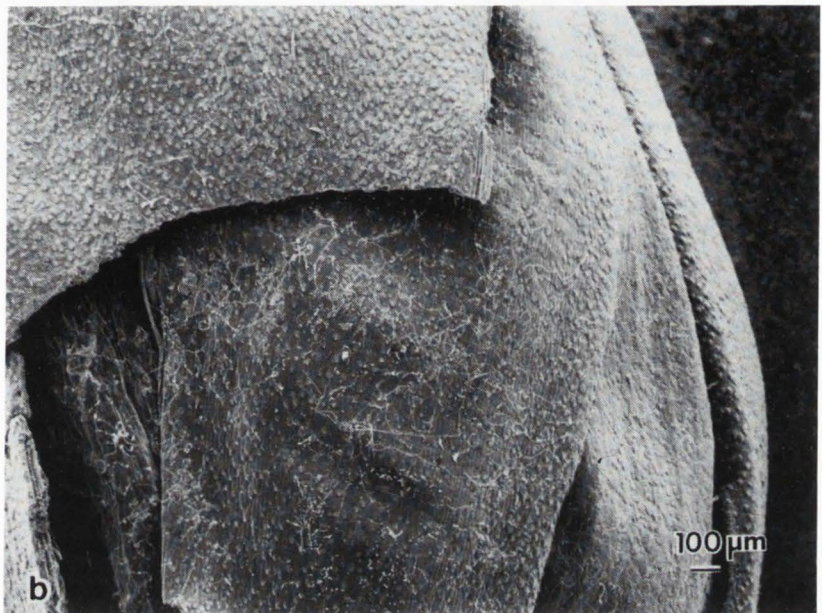
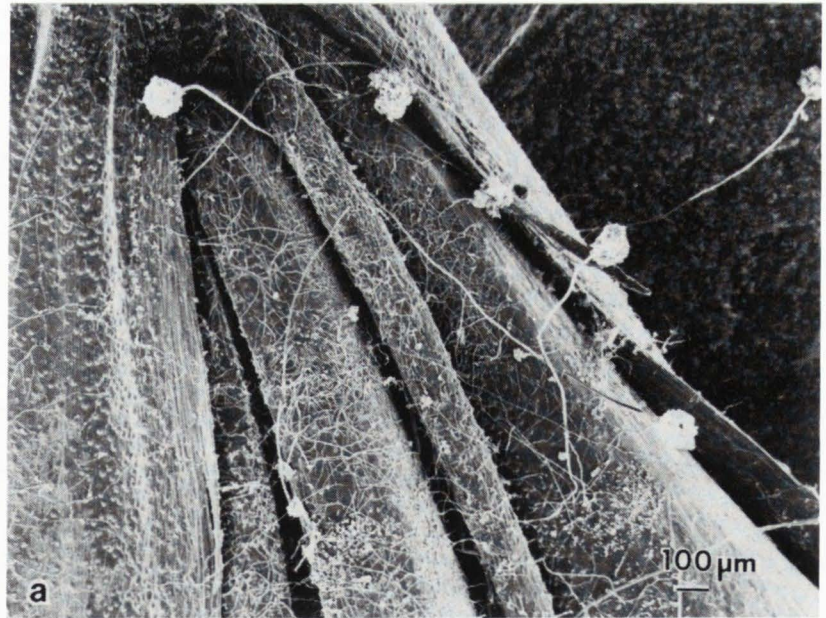


Figure 2. SEM micrographs of nonsterile barley kernels that were irradiated to 1 kGy after infection with *A. alutaceus*. (a) after 12 day incubation occasionally a kernel is seen with conidial heads; (b) most kernels show only mycelium growth.

easily identified in the SEM by the reduced amounts of mycelium and the small number of conidial heads compared to the controls. Although some kernels had conidial heads visible, especially at cracks in the surface (Figure 2a), most kernels seen in the micrographs of the 1.0-kGy-irradiated barley contained areas without visible mold growth (Figure 2b). Even the kernels with growth showed less mycelium on the surface and fewer conidial heads in the surface cracks than were seen in the parallel unirradiated control samples. A radiation dose of 2.0 kGy reduced the amount of growth on the surface, but, in samples inoculated with high titers of spores (10^6 spores/g), a dose of 2 kGy was not sufficient to completely eliminate the growth of *A. alutaceus* in all kernels (Figure 3a). Most kernels, however, did not have conidial heads developed on the kernel (Figure 3b). The type and level of *A. alutaceus* growth in unirradiated samples were similar on both autoclaved and nonsterile barley at the observation times we selected.

Scanning electron microscope observations of variants

The growth pattern of the variants 005, 006 and 007 was similar to that of the parent strain. The conidia of variant strains 005 and 006 were more variable in size and produced conidial heads that were rougher looking than those of the parent or 007 (Figures 4 and 5). This was also seen in the spore morphology. Although conidia of the variants and the parent strain had the same shape and decoration, those of 005 and 006 were slightly larger and more variable in size (Figure 6). Size measurements of the conidia of variants have been reported earlier in Chelack et al. (1991b) where it was shown that a majority of the conidia were mononucleated and the strains exhibiting variable conidial size were associated with larger nuclei.

Fungal population and ochratoxin A measurements

After irradiation the fungal population, as measured by the number of cfu found on the barley samples, was reduced by five log cycles at doses greater than 1.0 kGy (see Table I); however, the numbers increased above the unirradiated control after 30 days in those irradiated to doses between 0.5 and 2.0 kGy. A dose of 4.0 kGy was required to reduce the population by six log cycles after 30 days. The levels of ochratoxin A production on day 51 were also measured in the samples shown in Table I. As shown in Figure 7, radiation exposure reduced the ochratoxin A from 17.6 to 14.4 $\mu\text{g/g}$ after a 0.5-kGy exposure and eliminated it at a dose of 4.0 kGy. A sharp drop in ochratoxin A level occurs by 3.0 kGy, the same dose at which a drop in the number of cfu at 30 days occurs.

In a second experiment, samples of moist barley (25% dry basis) were inoculated with 10^5

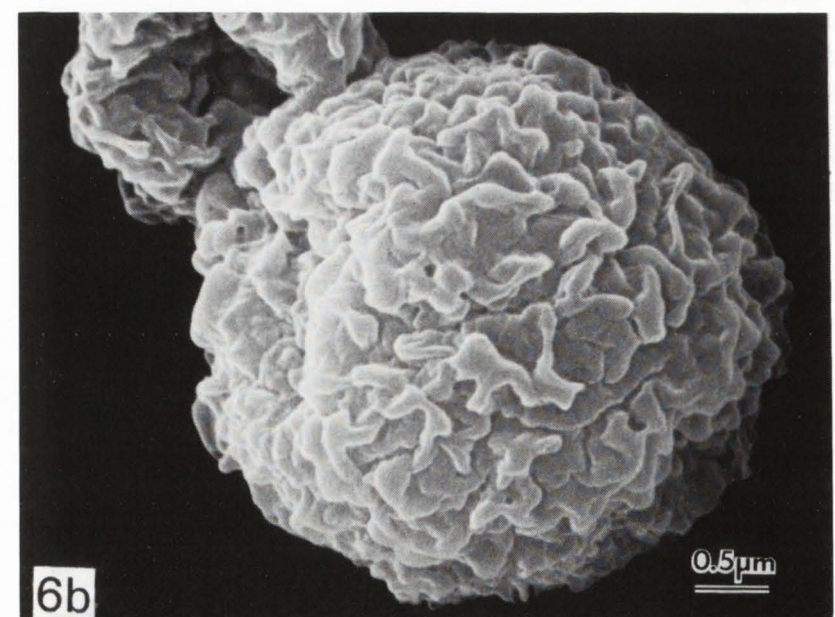
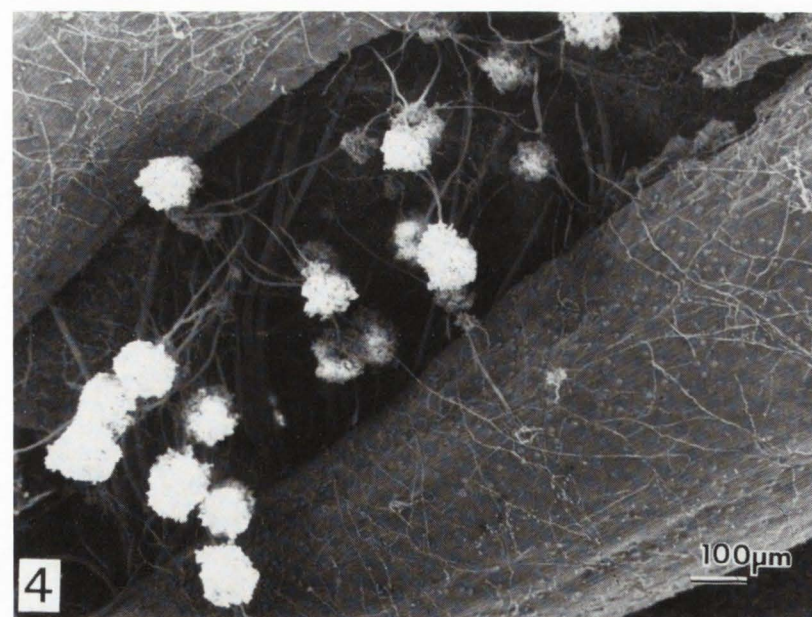
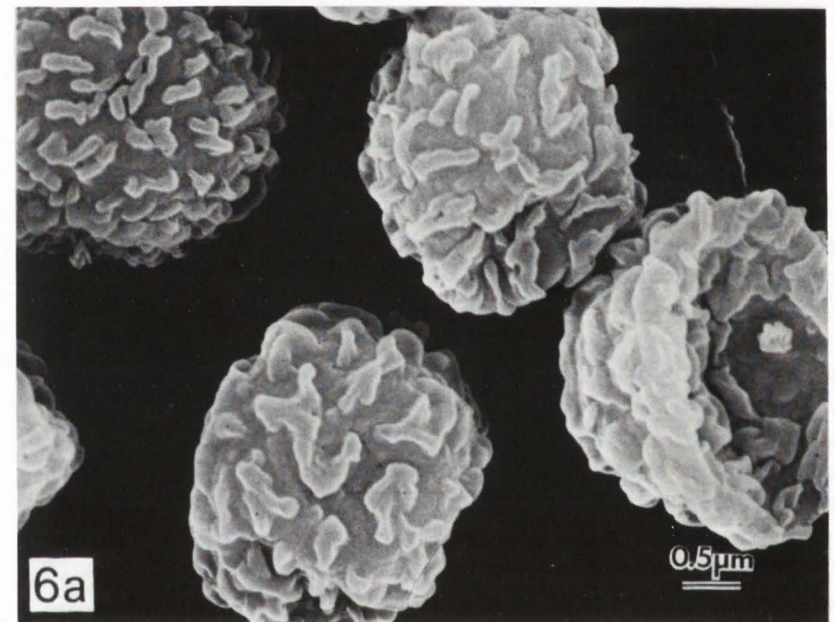
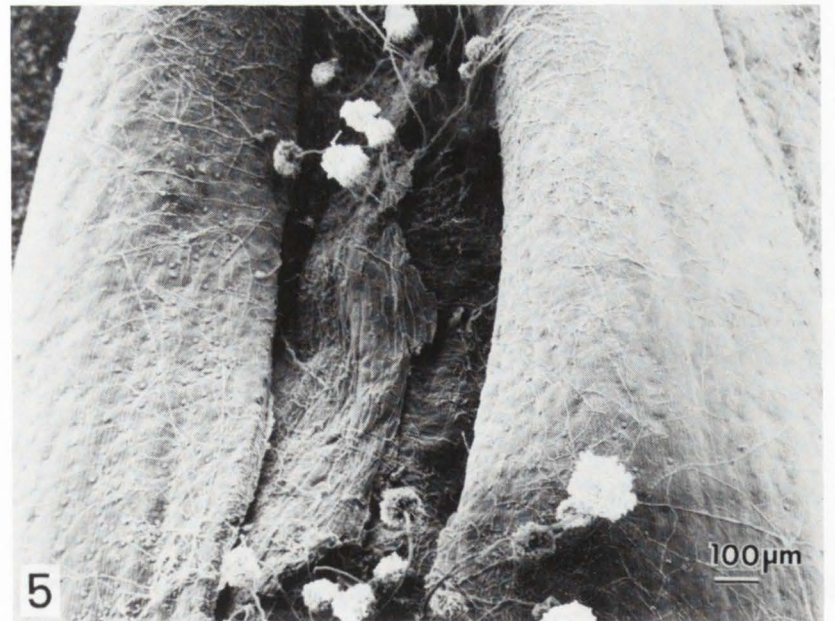
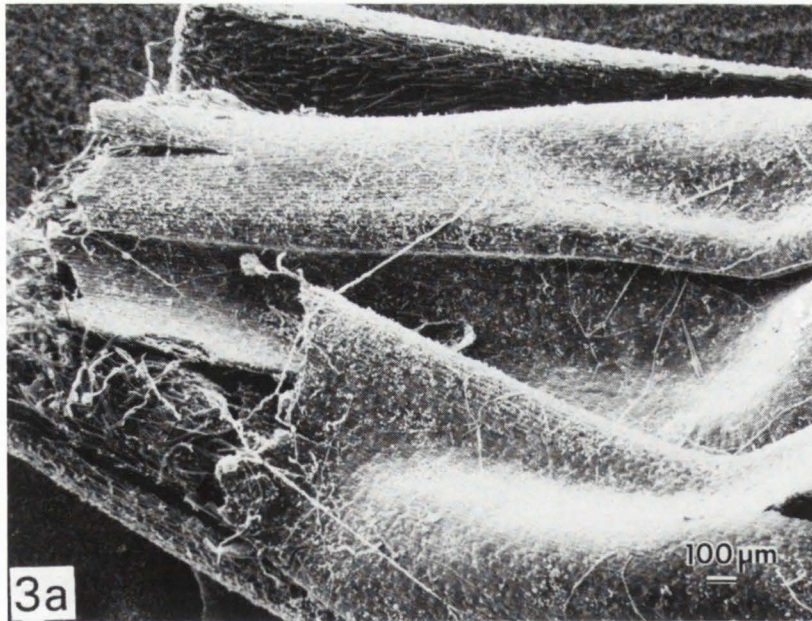


Figure 4: SEM micrograph of an autoclaved barley kernel that was inoculated with variant strain 005 and incubated for 12 days.

Figure 6: SEM micrographs of conidia from (a) the parent, (b) the variant strain identified as 006.

Figure 3: Micrographs of nonsterile barley kernels that were irradiated to 2 kGy after infection with *A. alutaceus*. (a) a kernel showing some surviving fungi visible; (b) a kernel showing no apparent fungal growth.

Figure 5: SEM micrograph of an autoclaved barley kernel that was inoculated with variant strain 006 and incubated for 12 days.

Table I: Fungal population (\log_{10} cfu/g) obtained from barley irradiated after inoculation with 10^6 spores/g of *A. alutaceus* and stored at 28°C for the indicated periods.

dose (kGy)	Population (\log_{10} cfu/g)		
	Initial	5 day	30 day
0.0	5.04	5.70	7.28
0.5	3.84	4.76	7.38
1.0	<1.0	4.62	7.69
2.0	<1.0	<1.0	7.45
3.0	<1.0	<1.0	6.57
4.0	<1.0	<1.0	<1.0

Table II: Fungal population (\log_{10} cfu/g) obtained from unirradiated and 2.0-kGy irradiated barley after incubation at 28°C for the indicated periods.

Treatment	Inoculum Size (spores/g)	Population (\log_{10} cfu/g)		
		Init.	27 day	51 day
unirr. control pre-irr.	10^5	4.85	7.30	7.41
	10^5	<1.0	6.08	7.45
unirr. control post-irr.	10^5	4.85	7.26	7.26
	10^5	4.84	7.48	7.54
unirr. control post-irr.	10^2	2.45	7.36	7.41
	10^2	2.46	7.45	7.53

spores/g before (pre-irradiation) or after (post-irradiation) the barley received a 2.0-kGy radiation exposure. The cfu measurements found in this experiment at days 0, 27 and 51 are shown in Table II. In the sample that was irradiated after inoculation, the cfu are lowered immediately on day zero, but they re-grew so that by day 51 they were slightly higher than the unirradiated control. When the barley was irradiated before inoculation with 10^5 spores/g, the cfu were similar to the unirradiated control on day zero; however, the number of cfu in the irradiated samples increased quickly and by days 27 and 51 the irradiated barley had higher levels than the corresponding control. Barley inoculated with 10^2 spores/g, as expected, had a lower number of cfu at day zero, but by day 27 the values were larger than the unirradiated control

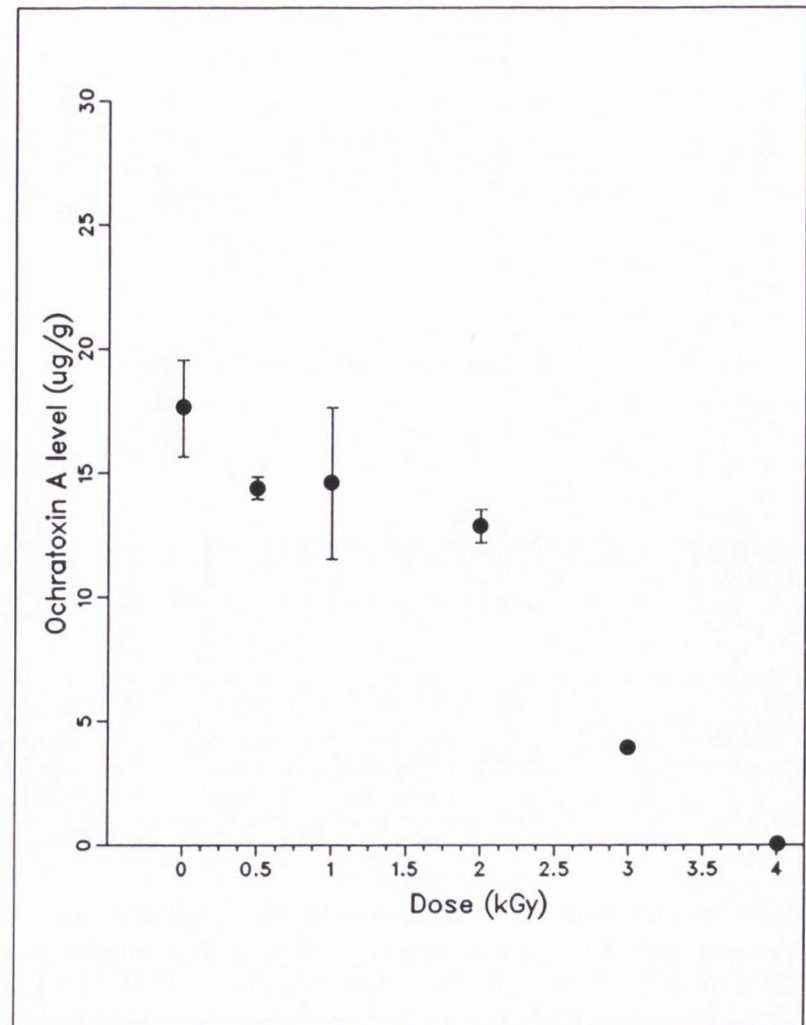


Figure 7: Ochratoxin A level in γ -irradiated barley samples after a 51-day incubation at 28°C and 98% relative humidity. The total length of each error bar is two standard deviations of the mean taken from multiple measurements in the same experiment.

and similar in magnitude to those measured in the samples inoculated at 10^5 spores/g. The ochratoxin A levels showed a pattern that was generally similar to the cfu count (see Table III). An exception, however, was the barley that had been inoculated with 10^5 spores/g before irradiation. In this case, the amount of ochratoxin A was negligible at 0-, 27- and 51-day incubation. The difference appears because the cfu represent *A. alutaceus* and any other fungi that will form colonies on the test plates, whereas ochratoxin A is only produced by *A. alutaceus*. The samples inoculated with 10^2 spores/g after irradiation rapidly reached the same level of ochratoxin A and cfu as those inoculated with 10^5 spores/g. Both inoculum levels produced higher fungal population values than the unirradiated controls after 27 and 51 days of incubation.

Discussion

The micrographs and Tables I and II show that at doses of 1.0 and 2.0 kGy the growth of *A. alutaceus* was decreased, but not eliminated. The most dramatic change seen by SEM at the surface of the barley was the decrease in development of mycelium and conidial heads in the irradiated samples. Growth was seen mainly in the cracks in the hull. The difference between

Table III. Ochratoxin A levels in unirradiated (control) and gamma-irradiated (2 kGy) barley given the indicated treatments.

Treatment	inoculum size (spores/g)	ochratoxin A level ($\mu\text{g/g}$)		
		Initial	27 day	51 day
unirradiated control	10^5	neg	4.76 ± 0.02	16.86 ± 0.28
pre-irradiation	10^5	neg	neg	neg
unirradiated control	10^5	neg	4.90 ± 0.27	7.93 ± 0.25
post-irradiation	10^5	neg	6.42 ± 0.05	18.87 ± 0.20
unirradiated control	10^2	neg	neg	2.61 ± 0.43
post-irradiation	10^2	neg	7.48 ± 0.42	16.91 ± 0.66

neg = negligible. The level was below the detection level of the method.

the irradiated and unirradiated samples was in the extent of fungal infection. In the 12-day experiments shown here, the general results were the same for both steam-sterilized and nonsterile barley. In the nonsterile samples some other fungi, which were on the kernel as a natural contaminant, also grew during the experiment. This became important as the observations were continued for up to 51 days. The SEM micrographs for such late times are not shown here since the barley was too heavily overgrown for effective pictures to be obtained, but light microscope observations showed that nonsterile barley irradiated after inoculation ultimately became overgrown with survivors of the natural flora of fungi, which survived the irradiation and became competitors for the *A. alutaceus* inoculum. This was not unexpected since a number of microorganisms have been reported on stored grain by Abramson et al. (1990), Cuero et al. (1988), and Ramakrishna et al. (1991). They have shown that a number of species are present in pre-harvest and post-harvest wheat and barley, and their presence effects growth and mycotoxin production in storage. Cuero et al. (1987, 1988) have also shown in paired-growth experiments that aflatoxin production from *A. flavus* could be increased or decreased depending on the competing organism and water activity. In the colony counts shown in Tables I and II, the colonies were not identified and some may not be *A. alutaceus*.

These SEM results showed that the mode of growth of the *A. alutaceus* was similar when it was inoculated on both irradiated and unirradiated grain. This suggests that contrary to the results of Priyadarshini and Tulpule (1976, 1979), irradiation does not alter the substrate in such a way as to change the growth pattern of the mold. We have found that after 51-day incubation, irradiated grain, inoculated with 10^2 or 10^5 spores/g, had similar ochratoxin A and cfu levels. Chelack et al. (1991a) have shown that, after 100 days of incubation, samples inoculated

with 10^2 spores/g have higher levels of ochratoxin A. The effect of inoculum size, suggested to be the cause of increased mycotoxin production in a number of reports, plays a large part in determining the ultimate level of mycotoxin production, along with the water activity and the number and variety of competing natural molds on the grain surface (Sharma et al., 1980; Cuero et al., 1987, 1988; Odamtten et al., 1987; Karunaratne and Bullerman, 1990).

As we have reported, an irradiation dose of 1.0 kGy or 2.0 kGy was sufficient to kill most *A. alutaceus* spores on barley and delay the development of mature fungi for 7 to 12 days. Ultimately both *A. alutaceus* and natural occurring fungi re-grew, but the infected barley had a lower level of ochratoxin A than the unirradiated control. Barley that had been infected after irradiation, on the other hand, had higher ochratoxin A levels than the unirradiated controls after 27-day incubation, probably because the natural fungi, which compete with *A. alutaceus*, were decreased. Irradiated barley that had been inoculated with a lower titer (10^2 spores/g) of *A. alutaceus* reached the same ochratoxin A level as the 10^5 spores/g-inoculated barley after 27 days. Also, Chelack et al. (1991a) have reported increased ochratoxin A at 100-day incubation in autoclaved barley inoculated at 10^2 spores/g as compared to 10^5 spores/g. Thus irradiation, and presumably any chemical disinfection method that kills some of the toxin-producing fungi or delays fungal growth, is equivalent to inoculating the grain with a lower titer of spores; hence, the disinfected grain will have a higher than expected level of mycotoxin production in these artificial systems. This effect has also been reported with a number of disinfection procedures including irradiation (Vandegrift et al., 1973a, 1973b; Niles, 1978).

In actual practice, when grain is irradiated before storage or distribution, it is not stored in high-humidity and high-temperature conditions.

In addition, the high spore count used in laboratory experiments on radiation-pasteurized grain is not expected in normal grain handling. It must be emphasized that the findings we have reported are not representative of natural conditions, but are relevant to laboratory experiments reported in the literature. We used optimal conditions for mold growth to help understand the large disparity between results obtained by different workers. Grain stored under recommended storage conditions of about 13% moisture content does not allow the growth of storage fungi (Cuero et al., 1988; Brook and Foster, 1981), and irradiation does not encourage growth or mycotoxin formation.

Although high-ochratoxin-producing variants were isolated on autoclaved grain after radiation treatments in the laboratory (Chelack et al., 1991b), we have not found enhanced ochratoxin A levels on nonsterilized barley that was inoculated with *A. alutaceus* before irradiation (see Figure 7). This indicates that the highly toxigenic variants found in autoclaved grain are not good competitors against the normal microbiological flora that exists on the surface of nonsterile damp grain. When variants called 005 and 006 were inoculated on barley, they had the same growth characteristics as the parent strain except for a ragged appearance of the conidial head. This corresponded with the other observation that the conidia are slightly larger in these two variants.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.