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Effect of gamma radiation on *Aspergillus flavus* and *Aspergillus ochraceus* ultrastructure and mycotoxin production

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ABSTRACT

The aim of this work was to study the effect of gamma radiation (2 kGy) on *Aspergillus flavus* and *Aspergillus ochraceus* ultrastructure. Moreover, the influence on aflatoxin B_1 and ochratoxin A production was also observed. Irradiated *A. flavus* strain showed a dull orangish colony while control strain showed the typical green color. Minor differences were observed on stipes, metulae and conidia size between control and irradiated *A. flavus* and *A. ochraceus* strains. Irradiated fungi showed ultrastructural changes on cell wall, plasmalema and cytoplasm levels. The levels of mycotoxins produced by irradiated strains were two times greater than those produced by control strains. Successive transferences of irradiated strains on malt extract agar allowed the fungus to recuperate morphological characteristics. Although minor changes in the fungal morphology were observed, ultrastructural changes at cell wall level and the increase of mycotoxin production ability were observed. Inappropriate storage of irradiated food and feed would allow the development of potentially more toxicogenic fungal propagules.

1. Introduction

The Brazilian poultry production chain has undergone an efficient modernization process aimed at reducing costs and increasing productivity. Considered one of the most organized in the world, it has contributed significantly to the country's economy. Brazil is the third largest producer and world leader in exports of chicken meat (IBGE, 2001/2006). Such achievement has been accomplished due to appropriate infrastructure and sanitary conditions. The poultry production is a dynamic activity depending on the agribusiness of grains, especially corn and soybeans, which provides food to feed exclusively. Microbial activity can cause undesirable effects in grains including discoloration, heating and losses in dry matter. Microorganisms can utilize carbohydrates as energy sources, degrade lipids and proteins, alter digestibility, produce volatile metabolites associated to off-odors, cause loss of germination and baking and malting quality; harming their use as animal feed or as seed. Fungi can cause damage to animal health through infectious processes and intoxication called mycotoxicoses, which might be acute, sub-acute or chronic (most common). *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp., considered the main mycotoxin producers, are the fungal genera mostly frequently isolated from food and feed (Fraga et al., 2007; Rosa et al., 2006). The use of gamma radiation in Brazil has been estimated to reduce or eliminate mycobiota from different substrates, such as maize; guarana used as a stimulant (Aquino et al., 2007) and peanuts (Prado et al., 2006). Exposure to ionizing radiation (irradiation) may cause direct damages to DNA through ionization leading to mutations in some cases, in others, killing the cell, depending on several factors. It has also an indirect effect as a result of radiolysis of cellular water and formation of active oxygen species, free radicals and peroxides causing single and double strand DNA breakages (McNamara et al., 2003). The sources of ionizing radiation employed in the treatment of foods are the radioisotopes cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs), emitters of gamma radiation, and fast electrons (Hernandes et al., 2003).

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The Food and Drug Administration (FDA) in the United States has primary regulatory responsibility for ensuring the safe use of irradiation on all foods. Irradiation has been approved for use of uncooked meat and poultry and fresh shell eggs, as well as a variety of other foods including spices and fresh fruits and vegetables. Levels from 1.5 to 3.0 kGy have been suggested for bacterial pathogen reduction in poultry meat (FDA, 2007). Scarce studies have shown the effects of this physical treatment on survival of fungal pathogens (Aziz et al., 1997; Ferreira-Castro et al., 2007). Moreover, no studies have reported on the influence of gamma irradiation on the ultrastructure and mycotoxin production by prevalent mycotoxigenic fungi in poultry feed such as *Aspergillus* spp. The aim of this work was to study the effect of gamma radiation on ultrastructure of *A. flavus* and *A. ochraceus* strains. Therefore, the influence on aflatoxin B_1 and ochratoxin A production was also studied.

2. Experimental

2.1. Strains

A. flavus (NRRL 5520) and *A. ochraceus* (ATCC 3174) strains were provided by the Department of Biology of the Oswaldo Cruz Foundation, originally obtained from the American Type Culture Collection (ATCC), Marassas, USA and the Agriculture Research Service Culture Collection (Peoria, USA), former National Centre for Agricultural Utilization Research (NRRL).

2.2. Fungal inoculation of substrate

Erlenmeyer flask bottles containing 25 g of corn grains were autoclaved for 20 min at 120 °C to eliminate the initial mycobiota, in order to guarantee sterilization prior to inoculation. Corn grains were hydrated with distilled water and stored for 24 h at 4 °C with periodic shaking to allow absorption and equilibrium (a_W 0.995). Level of a_W was then measured by using an Aqualab Series 3 (Labcell Ltd., Basingstoke, Hants, UK).

Corn grains were inoculated with 5 μl of spore suspensions (1×10^6 spores ml^{-1}) of *A. flavus* or *A. ochraceus* strains from 7-day-old cultures on 2% malt extract agar (MEA), separately.

2.3. Irradiation

The shielded-cavity-type irradiator used in this experiment has the movement of its source and door controlled by a pneumatic system. The maximum dose rate generated by its cesium-137 source inside its two irradiating chambers having a total effective volume of 80 l each is 1.8 kGy h^{-1}. Two (2) kGy gamma radiation dose was used based on the range suggested by the FDA (2007) for poultry meat. The treatment was done by triplicate. Control Erlenmeyers containing sterilized and inoculated corn grains without irradiation and Erlenmeyers containing sterilized and non-inoculated corn grains were incubated for 7 days at 25 °C.

The exposure time was calculated by using a program that takes into account the natural decay of the source and the position, geometry and density of the samples.

2.4. Fungal strains isolation

The last day of incubation, *Aspergillus* strains developed in each treatment were isolated using the surface spread method by blending 10 g portion of each Erlenmeyer with 90 ml of 0.1% peptone water solution for 30 min. Serial dilutions were made and 0.1 ml aliquots were inoculated in triplicates onto solid media Dichloran Rose Bengal Chloranphenicol agar (DRBC) (Pitt and Hocking, 1997). The plates were incubated in darkness for 7–10 days at 25 °C. The last day of incubation, *Aspergillus* colonies were transferred on MEA, developed under the above conditions and transferred to MEA and Czapeck Yeast Agar (CYA) media according to the inoculation scheme proposed by Klich (2002).

2.5. Morphological and microscopic assessment

Morphological characteristics of irradiated fungal strains isolated from corn grains were compared with control strains. Color, texture and reverse of colony, pigment production, conidia size and aspect, were observed macroscopically and by light microscopy according to Klich (2002).

2.6. Transmission electron microscopy

Fungal strains from treatments were obtained from 7-day-old cultures grown on MEA and processed for Transmission Electron Microscopy (TEM) (Bozzola and Russel, 1999). All samples were pre-fixed in 3% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffers, pH 7.2 for 3 h at room temperature followed by thorough washing with phosphate buffer. Fixed materials were then post-fixed in 1% aqueous osmium tetroxid for 3 h at room temperature after infiltrating with 2% molten agar. Dehydration of samples was achieved by transferring to vials containing graded water–acetone series (10% steps for 30–90% each of 60 min, 100% for 180 min and finally 100% overnight). Dehydrated specimens were embedded with Epon 812 and then polymerized in spur's resin (Epon 812 with 1.5% hardening agent, DMP-30) at 45 °C for 24 h and 65 °C for 72 h. Thin sections (80 nm thickness) were prepared using an electron microscope (Elmiskop 101-Siemens) staining with uranyl acetate for 20 min and with lead citrate for 5 min.

2.7. Mycotoxins production

2.7.1. Aflatoxin B_1 production ability

Aflatoxin B_1 production ability was determined following the methodology described by Geisen (1996). The strains were grown at 25 °C for 7 days in MEA in darkness. Mycelium and conidia were collected from the agar surface with a sterile brush and transferred to an Eppendorf tube. Aflatoxin B_1 was extracted with 500 μl chloroform and centrifuged at 4000 rpm for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and re-dissolved in chloroform. The extract was spotted together with standards and screened for AFB_1 by using the Thin Layer Chromatography (TLC) method on silica gel plates without a fluorescent indicator (0.25 mm, G60 Merck) and using chloroform: acetone (9:1, v/v) as developing solvent. Aflatoxin B_1 concentration was determined by visual comparison to the standards. Chromatograms were dried and observed under 365 nm UV light. The detection limit of the technique was 0.1 μg g^{-1}.

2.7.2. Ochratoxin A analysis

Ochratoxin A was determined following the methodology described by Téren et al. (1996) with some modifications as follows: the strains were grown in stationary cultures in 25 ml quantities of Yeast Extract Sucrose (YES—2% yeast extract, 15% sucrose) medium for 10 days at 30 °C in the dark. After incubation, a portion of these culture media (1 ml) was mixed with 1 ml of chloroform and centrifuged at 4000 rpm for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and re-dissolved in 0.5 ml of methanol. Detection and

quantification of OTA were performed by using a High Performance Liquid Chromatography (HPLC) system with fluorescence analysis carried out in a Hewlett Packard Series 1100 chromatograph, following the methodology proposed by Scudamore and Macdonald (1998). The detection limit of the used technique was 1 ng g^{-1}.

Mycotoxin production results were expressed as the media $\pm$ standard deviation (SD) of AFB_1 of OTA levels ($\mu g\ ml^{-1}$) from triplicates.

2.8. Statistical analysis

Data analyses from mycotoxin levels were performed by analysis of variance (ANOVA). Fisher's protected LSD test was used for comparing means of mycotoxin production between control and irradiated strains (Quinn and Keough, 2002). The analyses were conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Morphological characteristics of control and irradiated *Aspergillus* strains

Tables 1 and 2 show the macro- and micro-scopic characteristics for control and irradiated strains of *A. flavus* and *A. ochraceus*, respectively. Few macroscopic differences among control and irradiated strains were observed on MEA medium. The main difference between *A. flavus* control and irradiated strain was the reproductive mycelia color. Irradiated strain was dull orangish while control strain showed the typical green color described in the literature. Minor differences were observed on stipes, metulae and conidia size.

Only minor differences were observed on stipes, metulae and conidia size between control and irradiated *A. ochraceus* strains.

3.2. Ultrastructural study

Results were similar for *A. flavus* and *A. ochraceus* strains. Fig. 1 shows a photomicrography by TEM of the control strain ($\times$ 16,700). The cell wall of control *Aspergillus* strains was uniform and thoroughly surrounded by an intact febrile layer. Plasma membrane was unfolded with a uniform shape in all parts. All the organelles, such as nuclei, mitochondria, endoplasmic reticulum, vacuoles, electron dense granules and septum appeared normal.

Fig. 2 shows a photomicrography by TEM of the irradiated strain. Ultrastructural changes of irradiated *Aspergillus* strains were noticed on cell wall, plasmalema and cytoplasm levels. The main pathologic changes were found to occur on endomembrane systems mainly affecting plasma membrane and membranous organelles specially nuclei and mitochondria. In this study, initial cell depression signs including abnormal shaped and swelled hyphae, increased vacuolation of cytoplasm accompanied with vacuole fusion, swelling of septum and early degradation of electron-dense granules were noticed. Also a complete autolysis and disorganization of hyphae cytoplasm characterized by disrupted membranes and accompanied of the destruction and breaking down of plasma membrane leading to massive formation of membrane-bounded vesicles was observed.

Obvious damages in the cell wall were detected. A marked depletion of the cytoplasmic hyphae content with rupture of membranes of organelles like nucleus, mitochondria and endoplasmic reticule was also observed, demonstrating that radiation not only crosses the cell wall but also the plasma membrane.

3.3. Mycotoxins production

Irradiated strains were found to display different toxigenic profiles under the same conditions. The control *A. flavus* NRRL

Table 1
Morphological characteristics of control and irradiated *A. flavus* strains.

***Aspergillus flavus* strains**	**Macroscopic characteristics**			**Microscopic characteristics (μm)**				
	Colony							
	Diameter (mm)	**Textures**	**Colors**	**Vesicles**	**Stipes**	**Metulaes**	**Phialides**	**Conidia**
Control (NRRL 5520)	40–30	Floccose, low reproductive mycelium and abundant vegetative mycelium	White, brown to green reproductive mycelium, uniform yellow reverse	Spherical to elongate, radiate to columnar, mostly uniseriate	150 × 4.8	5 × 2.4	7–12 × 3–4	Spherical, smooth 1.5 to 2 × 2.4 μm
Irradiated (NRRL 5520)	Covering almost the plate	Floccose, low reproductive mycelium and abundant vegetative mycelium	White, dull orangish on reproductive mycelium, yellowish brown to brown clear uniform reverse	Spherical to elongate, radiate to spathulate, uniseriate	100 × 2.4	3 × 2.4	7–12 × 3–4	Spherical occasionally roughened 3 × 2.4 μm

Table 2
Morphological characteristics of control and irradiated *A. ochraceus* strains.

Aspergillus ochraceus strains	Macroscopic characteristics			Microscopic characteristics (μm)				
	Colony							
	Diameter (mm)	Textures	Colors	Vesicles	Stipes	Metulaes	Phialides	Conidia
Control (ATCC 3174)	28–28	Low, abundant reproductive mycelium. Scarce vegetative mycelium (4 mm). Central elevation (6 mm) covered with conidia	Ochraceus central reproductive mycelium and cream at the periphery. Short and abundant ochraceus conidiophores. Dark brown reverse in the center and clearer in the periphery	Radiate 10×2.4 μm	140×4.8	8×2.4	5×2.4	Spherical, smooth 1×2.4 μm
Irradiated (ATCC 3174)	25–25	Low, abundant reproductive mycelium. Scarce vegetative mycelium (4 mm). Central elevation (6 mm) covered with conidia	Ochraceus central reproductive mycelium and cream at the periphery. Short and abundant ochraceus conidiophores. Dark brown reverse in the center and clearer in the periphery	Radiate 10×2.4 μm	$70–80 \times 4.8$	4×2.4	3×2.4	Spherical, smooth 1.5×2.4 μm

Fig. 1. Photomicrography by TEM of *A. ochraceus* control ($\times 16{,}700$). CW: Woronin, GE: electron dense granules, N: nuclei, NO: nucleoli, M: mitochondria, MP: plasma membrane, P: cell wall, S: septum, V: vacuole.

5520 produced 8.58 ± 5.25 μg g^{-1} of AFB_1 whereas the same irradiated strain produced 20.06 ± 1.99 μg g^{-1} of AFB_1. On the other hand, the control *A. ochraceus* NRRL3174 produced 76.29 ± 17.81 μg ml^{-1} of OTA and the same irradiated strain produced 150.36 ± 23.16 μg ml^{-1}.

4. Discussion

The effect of gamma radiation on ultrastructure and AFB_1 and OTA production by *A. flavus* and *A. ochraceus* strains, respectively, was studied.

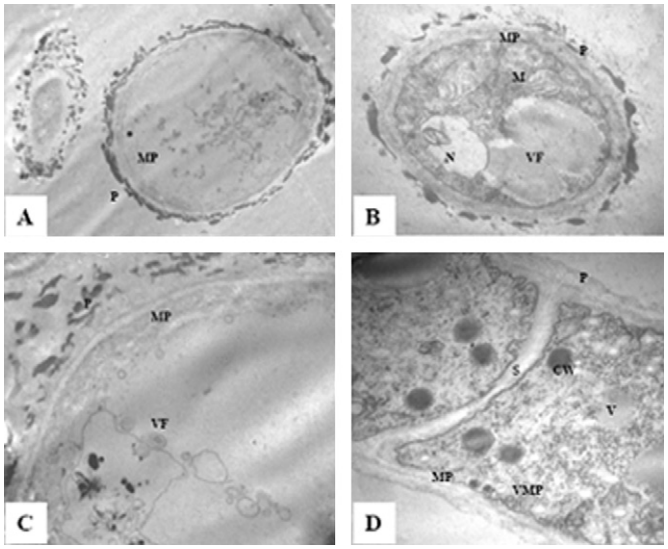

Fig. 2. Photomicrography by TEM of irradiated *A. ochraceus*. A (×5800); B (20,700); C (13,100); D (34,600). CW: Woronin, N: nuclei, NO: nucleoli, M: mitochondria, MP: plasmatic membrane, P: cell wall, S: septum, V: vacuole; VF: vacuole fusion; VMP: vesicle.

Minor macroscopic and optical microscopic differences among control and irradiated strains were not observed in this study. However, when strains were subject to successive transferences on MEA, they showed similar morphological characteristics compared to the reference strain. In contrast with our results, Chelack et al. (1991) observed that some *A. ochraceus* irradiated strains at 1 kGy level, exhibited light-yellow spores and produced a diffused red pigment while the reference strain showed the characteristic ocher spores. Gamma irradiation has been proposed as a suitable method of sterilization to study the digestion of cereal grains (such as corn grains) by a mixed population of ruminal microorganisms. McAllister et al. (1991) used a dose of 1500 krads (15 kGy) and differences between unsterilized and sterilized corn grains were determined. Moreover, they studied the effect of radiation (among other sterilization methods) on dry matter (DM), protein (P) and starch disappearance. Results showed that gamma irradiation did not affect ($P > 0.05$) the disappearance of DM, CP or starch. They also stated that sterilization by irradiation did not affect ($P > 0.05$) the rate of corn digestion by microorganisms. Based on these comments, the dose of 2 kGy used in our study did not have significant effect on starch granules degradation. Therefore, the slight alteration in color seen in the mycelia was not an effect of starch granules degradation but probably a result of the 2 kGy dose used.

The ultrastructural study showed similar differences between *A. flavus* and *A. ochraceus* irradiated strains. The main pathologic changes were found at endomembrane systems mainly affecting plasma membrane and membranous organelles (nuclei and mitochondria). Razzaghi Abyaneh et al. (2006) obtained similar results to us when ultrastructural evidences were observed by TEM at cell wall level with the use of the biocide Akacid®plus on *Aspergillus parasiticus* strain.

Gamma irradiation has been proposed as an efficient process to eliminate toxigenic fungi before the initiation of mycotoxins production (Refai et al., 1996). However, stress situations may lead to a higher toxin production by the fungi. In this study, irradiated strains increased the mycotoxin production under irradiation conditions. The fact that irradiated foods can become more susceptible to recontamination by toxigenic fungi or fungi that can be stimulated to produce more toxins after exposure to gamma radiation was reported by Paster and Bullerman (1988). Chelack et al. (1991) observed that some *A. ochraceus* irradiated strains (1 kGy) increased its capacity to produce OTA, and remained constant during several months and continued to show the greatest ocratoxigenic potential. Other authors attributed the increase in the fumonisin production by *Fusarium verticillioides* in maize irradiated with 2 kGy in agreement with our results (Ferreira-Castro et al., 2007). However, they found a lower fumonisin production when the same strain was irradiated at 5 kGy.

The adaptative responses of toxigenic fungi to adverse environmental conditions have also been studied for fungicides, and it was assessed that the use of chemicals can stimulate the toxin production by resistant fungi (D'Mello et al., 1997). Barberis et al. (2009) demonstrated the in vitro control of growth and OTA production by butylated hydroxyanisole in *Aspergillus* section *Nigri* species. The stimulation of mycotoxin production in some treatments could be explained as response to fungal stress. Fungi produced amounts of mycotoxins in presence of low concentrations of antioxidants as a survival mechanism. The use of combined antioxidants could be an alternative to control ochratoxigenic fungi in peanut seeds in storage state and diminish the entry of OTA into the animal and human food chain.

Several works have shown that the use of additives in foods stimulates the production of toxins (Marin et al., 2002). Campos et al. (2008) observed that 80% of *A. flavus* strains isolated from pet foods were aflatoxin-producers. Ionizing radiation has been assessed by researchers aiming at the production of mutants, selected for the production of certain substances, such as yeast mutant for the production of higher amounts of carotenoids (Sun et al., 2004).

In Brazil, a range from 0.05 to 0.3 kGy of radiation doses allows disinfesting insect and pests. Applications below 1 kGy are said to involve low doses of radiation (Sharma, 2004). However, Hernandes et al. (2003) described that usually the smaller the organism, the greater is the dose of radiation needed to kill it. Likewise, mycobiota reduction is reached at doses over 1 kGy for beef, chicken and spices decontamination (Sharma, 2004). This study demonstrated that a dose of 2 kGy is not efficient to completely reduce the main toxigenic *Aspergillus* spp. Although minor changes in the fungal morphology were observed, ultrastructural changes at cell wall level and the increase of mycotoxin production ability at 2 kGy were observed. Inappropriate storage of irradiated food and feed would allow the development of potentially more toxicogenic fungal propagules.

It is important to point out that the use of irradiation is a complement of the good manufacturing practices and may constitute a strategy that could be applied together other methodologies to prevent and control the presence of toxigenic fungi. The determination of optimal application doses of the antifungal substances is very important. High levels could produce undesirable effect on the grain organoleptic properties and on its processed products, causing a negative economic impact. On the other hand, sub-inhibitory doses together with inadequate distribution could cause stimulation on fungal sporulation, growth and secondary metabolism, increasing mycotoxin production. Future studies should be conducted to determine the type of change induced at different irradiation doses on the toxigenic potential of fungi in contaminated cereals and feed.

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