PRELIMINARY SURVEY ON THE EFFECTS OF IONIZING RADIATIONS ON ASPERGILLUS SPP. AND ON AFLATOXIN B1 CONTAMINATING MAIZE GRAINS

PRELIMINARNA ISTRAŽIVANJA UTICAJA JONIZUJUĆEG ZRAČENJA NA ASPERGILLUS SPP. I AFLATOXIN B1 ZARAŽENO ZRNO KUKURUZA

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ABSTRACT

The aim of this research project was to evaluate the effects of ionizing radiations on Aspergillus spp. and on aflatoxin B1 levels contaminating maize grains. Eight samples of maize were irradiated by ionizing radiations respectively at doses of 3, 5, 7 and 10 kGy. The control samples showed a mean total viable mycetes count of 10^6 CFU/g and the mean levels of aflatoxin B1, detected by HPLC/FL, were 1212.8 median 1319 ppb. The effects of γ radiations on Aspergillus spp. produced a dose-dependent decrease in total moulds count and the appearance of some mutants. The AFB1 contamination was evaluated in samples irradiated at the 10 kGy dose, the maximum overall average absorbed radiation dose allowed by the European Community Directive. The results showed an increased AFB1, with mean level of 1975.8 ppb and median 1924.5 ppb.

Key words: HPLC, Aspergillus spp., AFB1, γ rays, maize.

REZIME

Cilj ovog istraživanja bio je ocenjivanje učinka jonizujućeg zračenja na Aspergillus spp. i na aflatoxin B1 na nivoima kontaminacije kukuruznog zrna. Osim uzoraka kukuruza ozračeni su jonizujućim zračenjem odnosno dozama od 3, 5, 7 i 10 kGy. Kontrolni uzorci pokazali su ukupan broj održivih miceta od 10^6 CFU/g a srednji nivo aflatoxina B1, detektiran pomoću HPLC/FL, bio je 1212.8, medijana 1319 ppb. Učinak γ zračenja na Aspergillus spp. izaziva zavisno od dose zračenja smanjenje u ukupnom broju plśni i izgled nekih mutanata. AFB1 kontaminacija ocenjena je u uzorcima ozračenim sa 10 kGy, maksimalna ukupna prosečna apsorbovana doza zračenja koju dopušta Direktiva Evropske Zajednice. Rezultati su pokazali povećanje AFB1 sa srednjim nivoom od 1975,8 ppb i medijan 1924,5 ppb.

Ključne reči: HPLC, Aspergillus spp., AFB1, γ zraci, kukuruz.

INTRODUCTION

Mycotoxins are secondary metabolites produced primarily in the idiostage of fungal growth. Many of the foodborne filamentous fungi are capable of producing mycotoxins, and approximately 300 different mycotoxins have been identified. However, only 20 mycotoxins, produced by various species, are relevant to human and animal health (Geissen, 1998).

Aflatoxins, produced by Aspergillus spp., are reported to be among the most potent mycotoxins and maize is particularly susceptible to colonization and infection after silk emergence (Färber et al., 1997). Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated as causative agents in human hepatic and carcinogenesis (Rustom, 1997). The aflatoxins, as a group (AFB1, AFB2, AFG1, AFG2 and AFM1), are classified as group 1 carcinogens (IARC, 2002). Human exposure to aflatoxins can result directly from ingestion of contaminated foods, or indirectly from consumption of foods from animals previously exposed to aflatoxins in feeds (Rustom, 1997). Aflatoxins are highly resistant to various physical and chemical treatments (Patel et al., 1989); in many countries, both fumigation with ethylene oxide and heat sterilization have been tried with varying degrees of success. However, for sterilising cereal grains these methods have several disadvantages, such as toxic residues and altered organoleptic properties. For these reasons, reduction of pathogenic microflora by alternative techniques is highly desirable, and irradiation may be a method of choice, particularly because, unlike heat decontamination, it does not destroy nutrients (Campbell et al., 1986). For decades, radiation has been employed as an excellent tool in food sterilization, food preservation and different food engineering processes, which ultimately benefit human society (Dus’an, 2004; Hyun-Pa et al., 2006; Sameh et al., 2006; Maity et al., 2008).

There has been increasing interest in the use of ionizing radiation for killing endogenous microorganisms in stored grains (Erhart, 1990). In fact, there are a number of reports which suggest that moulds are very sensitive to γ radiations but there are also conflicting reports of increased as well as decreased aflatoxin production following γ irradiation of spores of Aspergillus spp. (Sharma et al., 1990). Fungi have been successfully deactivated from different materials, such as paper, wood and soil with irradiation doses ranging from 6 to 15 kGy (De Silva et al., 2006). Moreover, the sensitivity of fungi to γ radiation has been established by Aziz et al. (1997) who recorded that the dose required for complete inhibition of fungi in different food and feed products ranged from 4 to 6 kGy. The Joint FAO/IAEA/WHO Expert Committee, on the Wholesomeness of Irradiated Food (JECFI) concluded in 1980 that the irradiation of any food commodity up to an overall average dose of 10 kGy, presented no toxicological hazard and no special nutritional or microbiological problems (Ingram and Farkas, 1977; WHO, 1981, 1991). The Study Group concluded that food irradiated to any dose appropriate to achieve the intended technological objective is both safe to consume and nutritionally adequate (WHO, 1999). But in order to achieve the wholesomeness of treated foodstuffs, European Community rules authorize 10 kGy as the maximum overall average absorbed radiation dose (European Community Dir. 2, 1999).

The aim of this study was to evaluate the effects of different doses of γ radiations (3,5,7 and 10 kGy) on Aspergillus spp., naturally affecting maize, and also to examine aflatoxin B1
(AFB₁) contamination at 10 kGy, the maximum dose permitted by the European rules.

**MATERIALS AND METHODS**

**Maize Samples**

Eight samples (20 kg each) of fresh harvest maize grains were collected from the storage centre before drying treatment and kept at room temperature for two months. Five subsamples (240 g) of maize grains were obtained from each sample, packaged in polyethylene bags and stored at 5±3°C before irradiation. One of these was used as the control sample, while the others were irradiated at 3, 5, 7 and 10 kGy of γ rays respectively. After treatment with ionizing radiations, all irradiated subsamples and the unirradiated control were analyzed to detect the total viable mycetes. The subsamples irradiated at 10 kGy were also checked to detect the AFB₁ contamination with HPLC-FL.

**Irradiation**

Irradiation treatment with ionizing radiations was performed at the CALLIOPE γ ray facility at the ENEA Research Centre Cassino in Rome (Italy). The subsamples were exposed to a 60Co γ source with a dose rate of 5143 Gy/h at room temperature. In order to obtain the 3 kGy dose, subsamples were irradiated for 35’, while the 5, 7 and 10 kGy doses were achieved in 58’ and 20’, 1 hr, 21’ and 40’ and in 1 hr, 56’ and 40’, respectively. The absorbed dose was monitored by an ESR (Electron Spin Resonance) dosimeter. The subsamples were re-stored at 5±3°C until analysis.

**Chemicals and Standards**

Peptone, yeast extract and agar were purchased from DIFCO (Michigan, USA). Sodium chloride, dextrose, chloramphenicol and citric acid were from Sigma-Aldrich (Steinheim, Germany). Water, acetonitrile, dichloromethane, isopropyl alcohol, acetic acid [HPLC grade], were from Mallinckrodt Baker Italia (Milan, Italy). Trifluoroacetic acid was from Fluka. AFB₁ standard was from Sigma-Aldrich (Steinheim, Germany).

**Total Mould Count**

The count of viable moulds in the irradiated and control samples was performed by means of a colony count technique at 25°C, according to ISO 7954:1987 and Dragoni et al. (1997) method. 40 g of each sample were added (1:10 ratio) to peptone saline solution (1 g peptone, 8.5 g NaCl, 1000 ml deionized water) and homogenized by a stomacher (PBI int., Milan, Italy) for 2 minutes. Four further dilutions (10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵) were obtained for each sample. For each dilution, 1 ml of suspension was transferred, in double, into sterile Petri dishes. About 15 ml of yeast extract-dextrose-chloramphenicol–agar medium (yeast extract 5 g, dextrose 20 g, chloramphenicol 0.1 g, agar 12 g, water 1000 ml) maintained at 45°C in a water bath was put in each Petri dish. The inoculum was carefully mixed and the mixture allowed to solidify by leaving the Petri dishes to cool on ice (10 min.). The suspension was filtered through a Büchner funnel with filter paper and was drawn down into a conical flask under vacuum. The final filtered volume was corrected with dichloromethane, used to rinse the filtering flask, to achieve 40 ml. Only 15 ml was centrifuged (3,500 rpm, 15 min) and 8 ml of the lower organic layer was transferred into a conical tube and evaporated under vacuum (Unipvano Martinsried/Munich, Germany). The dry residue was dissolved in 20 μl trifluoroacetic acid for derivatization. After ten minutes, 1,980 ml of water:acetonitrile mixture (90:10 v:v) was added, each sample was mixed by vortex (1 min) and maintained in the dark for 20 minutes before injection into the HPLC instrument. The HPLC system consisted of a 126 Solvent Delivery System, and a 507 autosampler fitted with a 20 μl loop (Beckman, San Ramon, CA, USA), coupled to a fluorometric detector (Jasco 821 FP, USA) set at 365 nm excitation and 418 nm emission wavelengths. The HPLC separation was performed using a Merck Chromolith Performance RP-18e column, 100 x 4.6 mm (MERCK, Darmstadt, Germany) and the mobile phase (flow rate of 0.8 ml/min) was an 86% of mixed solvents water:isopropyl alcohol:acetonitrile:acetic acid 1% (91:1:1:7 v:v) and a 14% solution of the same mixture (43:25:25:7 v:v).

**Method validation**

Before the analysis of experimental samples, a complete validation of the analytical procedure for AFB₁ extraction and quantification was performed in accordance with the “Guidelines on validation of analytical procedures” suggested by the European Agency for the Evaluation of Medicinal Products (EMEA) (CVMP, 1998).

AFB₁ stock standard solution in benzene:acetonitrile (98:2 v:v) was prepared to achieve the final concentration of 1 mg/ml. From this stock, standard solution reference standards (1, 2, 5, 10, 20 ppb) in water:acetonitrile (90:10 v:v) were prepared to generate the reference curve. In order to prepare the calibration curve, aliquots of blank maize ground grains were spiked with AFB₁, reference standard solutions were then extracted and derivatized with the same protocol described for unknown samples. Calibration curve was analyzed using the least squares linear regression analysis and the determination coefficient (r²) was calculated. The mycotoxin concentration in unknown samples was calculated from its peak area using the slope and the intercept of the calibration curve. Specificity, related to the absence of interfering substances under the experimental conditions, was determined calculating the mean values (±standard deviation) of the retention times of AFB₁ present in calibration curves and in unknown samples. Mycotoxin recovery was established comparing the peak areas of the AFB₁ calibration solutions and the peak areas of AFB₁ reference standards. Percentage of recovery, accuracy and precision were obtained in the range 1-20 ppb. The limit of quantification (LOQ) was the lowest concentration of the calibration curves. The limit of detection (LOD) was established on the basis of a Signal-to-Noise ratio of three at the mycotoxin retention time. The study was performed according to ISO 9001:2000 requirements.

**RESULTS**

**Total Mould Count**

A mean contamination level of 10⁶ CFU/g was observed in unirradiated samples. The irradiated samples reported a significant dose-dependent CFU/g decrease compared to the control. At the radiation dose of 3 kGy, a mean fall of 98.3%
was observed; at 5 kGy the diminution was greater, and only 0.003% of colonies were born. At the 7 kGy and 10 kGy doses, the CFU/g decrease was nearly 100% (Graph 1). Identification of representative colonies was executed according to gross morphology of conidial heads. Mycological analysis showed that maize grains were contaminated with many yeasts and fungal genera such as *Mucor* spp., *Alternaria* spp., *Rhizopus* spp., but *Penicillium* spp. and *Aspergillus* spp. were the most abundant isolated genera of fungi.

Several modified conidiophores in colonies irradiated at 5 kGy indicated the presence of some mutants. In fact, it was observed that some hyphae projected from these conidiophores, instead of conidia (Figure 1A and 1B), that subsequently become as many conidiophores producing conidia (Figure 1C and 1D).

**AFB<sub>1</sub> Measurement**

The AFB<sub>1</sub> calibration curve was linear in the concentration range considered (1-20 ppb) with a determination coefficient (r²) of 0.999. The AFB<sub>1</sub> mean recovery percentage was 93.94±7.12%. The limit of quantification (LOQ) was 1 ppb, and the limit of detection (LOD) was 0.5 ppb. Under the experimental conditions, the specificity of the method was good, and no interfering substances were observed at the AFB<sub>1</sub> retention time (about 13 minutes) (Figure 2). The mean levels of AFB<sub>1</sub> contamination, detected in the control and in 10 kGy irradiated samples, were respectively 1212.8 median 1319 ppb and 1975.8 median 1924.5 ppb (Table 1).

**DISCUSSION**

According to Aziz and Mahrous (2004), the total count of viable mycetes showed a dose-dependent decrease of *Aspergillus* spp. The results of the present study confirm the sensitivity of fungi to γ radiation and demonstrate that a complete inhibition of fungi in different food and feed products can be observed even at doses of 4-6 kGy.

The increased AFB<sub>1</sub> concentration observed in samples irradiated at the 10 kGy dose confirms some previous studies (Applegate and Chipley, 1973; Bullerman and Hartung, 1974; Jemmali and Guilbot, 1969; Paster et al., 1985; Schindler et al., 1980) but contrasts with others. Several hypotheses could be put forward to explain these outcomes.

First of all, the enhanced AFB<sub>1</sub> production could be attributed to the development of fungal mutants as observed in the present study, during γ irradiation at sublethal doses. Ionizing radiation can generate in biological organisms single-strand breaks (SSBs), double-strand breaks (DSBs), base damage, and DNA crosslinks that eukaryotic cells try to repair by specific mechanisms, potentially leading to large-scale genomic rearrangements. Such phenomenon may prove beneficial in evolving genetic variants that have growth advantages under genotoxic stress (Dadachova and Casadevall, 2008), but not necessarily induce an enhanced mycotoxin production.

In order to test the possibility that γ radiations on *A. parasiticus* spores lead to mutants with enhanced capabilities to produce aflatoxin, Sharma et al. (1990) studied the progeny of the survivors in a γ irradiated spores suspension in synthetic glucose salt medium and rice. The surviving colonies, however, did not produce more aflatoxin compared to control colonies. On the contrary, the greatest amount of aflatoxin was produced by the inoculum from the progeny of unirradiated spore suspension in both glucose salt medium and rice. Moreover, irradiated microorganisms usually exhibit reduced fitness and the survivors of sublethal radiation treatment do not compete well with unirradiated organisms (Diehl, 1995).

Another hypothesis is that a gradual enhancement in aflatoxin production could be observed when there is a decrease in the inoculum size (Odamten et al., 1987). This diminution resulted in a increased lag phase which is maximal when the number of spores are minimal. Irradiation reduces exponentially the spore population, acting as inoculum reduction, and thus the rate of aflatoxin production seems to be related to the growth rate, which in turn depends upon the inoculum size. This supposition could be valid if irradiated spores were kept at a temperature suitable for mycotoxin production, but in the present experiment maize samples were immediately stored after irradiation at 5±3°C, unlike other authors who stored samples at 25-30°C (Aziz and Mahrous, 2004; Bullerman and Hartung, 1974; Kume et al., 1989).

The increase of AFB<sub>1</sub> level after irradiation, cannot be due to an increased toxin production capability of surviving moulds but it could be related to structural modifications of aflatoxin molecules, leading to new products. This assumption is supported by Aquino et al. (2005) who suggest that new products, with lower biological activity, are created when highly reactive free radicals, formed during water radiolysis readily attack AFB<sub>1</sub> at the terminal furan ring, changing its molecular structure.

Moreover, the high radiosensitivity of AFB<sub>1</sub>, as shown by Albana and Miyaki (1970), is due to the double bond in the furan ring. Patel et al. (1989) also found that after ionizing irradiation, AFB<sub>1</sub> turns into a degradation product that shows a similar retention time but is more fluorescent. These findings are in agreement with Van Dyck et al. (1982) who reported a reduced mutagenic activity of AFB<sub>1</sub> when the mycotoxin was irradiated in an aqueous solution. They also asserted that the effect of irradiation on AFB<sub>1</sub>, when present as a precipitate, was markedly reduced (Kume et al., 1983; Van Dyck et al., 1982). Thus it is most probable that also the physicochemical state of the mycotoxin influences its sensitivity to ionizing radiation.

**CONCLUSIONS**

There are a number of conflicting reports about the increased, decreased or even unaffected production of mycotoxins after irradiation of fungi under various laboratory conditions (Aquino et al., 2005). These contrasting outcomes depend on several parameters such as inoculum size, activity water, incubation period, radiation dose and physicochemical state of the mycotoxin. Thus, as far mycotoxin contamination is concerned, ionizing radiation treatment would not yet seem to be effective for decontaminating foodstuff from AFB<sub>1</sub>. However, unlike other studies, it was not possible for our survivor moulds to create more mycotoxins after irradiation; therefore, the increased AFB<sub>1</sub> contamination after γ exposition could be due to a structural modification of the AFB<sub>1</sub> molecule with a consequent change in the fluorimetric response of that compound. It is possible that this molecular modification could lead to a degradation product which is more fluorescent than the unirradiated AFB<sub>1</sub> and thus can simulate an increased AFB<sub>1</sub> contamination. Nevertheless, further studies are required in order to investigate AFB<sub>1</sub> radio-sensitivity and its molecular change after irradiation, which has perhaps created a new product which is not distinguishable with HPLC-FL.

**ACKNOWLEDGEMENTS:** The authors would like to thank Prof. Tata and Dr. Adamo for their valuable help in making this research possible.
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Received: 14.02.2011. Accepted: 28.03.2011.