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Use of PCR for Detecting *Aspergillus flavus* in Maize Treated by Gamma Radiation Process

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The aim of this study was to verify the effects of gamma radiation process on the fungal DNA and the application of PCR in the detection of *Aspergillus flavus* in irradiated maize grains. The samples were inoculated with a toxigenic strain and incubated under controlled conditions of relative humidity, water activity, and temperature for 15 days. After incubation, the samples were treated with gamma radiation with doses of 5 and 10 kGy and individually analyzed. The use of PCR technique showed the presence of DNA bands of *Aspergillus flavus* in all irradiated samples that showed no fungal growth in agar medium.

Key Words: gamma radiation; Aspergillus flavus; aflatoxin; maize; PCR; DNA

INTRODUCTION

Aflatoxins are secondary metabolites produced by filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus.* There are four naturally occurring aflatoxins, designated B_1, B_2, G_1, and G_2, with aflatoxin B_1 the most common and toxic. The limit for aflatoxins in food and feeds according to Brazilian regulation is 20 μ g/ kg aflatoxins $B_1+B_2+G_1+G_2$ (Brasil, 2002). These substances cause carcinogenic (especially in liver tissue), mutagenic, teratogenic, and

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immuno-suppressive effects on human and animals (Diehl, 1995; Diener et al., 1987; IARC, 1993). The genus *Aspergillus* is widespread in nature and found in the air, soil, and as a contamination of plants and grow on commodities including spices, cereals, nuts, herb teas, medicinal plants and corn (Ellis et al., 1991; Martins et al., 2001).

Corn is the main cereal crop grown in Brazil, which is the third major corn producer in the world after China and the United States. The tropical weather conditions that prevail during the year in Brazil favor fungal growth and mycotoxin production, mainly when the freshly harvested product is kept at a high moisture content (Castro, 2003).

Ellis et al. (1991) analyzed the influence of water activity (*Aw*), pH, the availability of nutrients, host resistance, interactions with other fungi, temperature, and atmospheric composition on aflatoxin production. Diener and Davis (1966) indicated that below of certain level of humidity the *A. flavus* has its growth retarded. According to Hunter (1969), the value of 0.87 was the minimal limit of *Aw* for aflatoxin production in maize grains. Spores of *A. flavus* grew on ground Indonesian nutmeg and peanut, in equilibrium with RH of 91, 95, or 97% but did not grow under RH of 85% or less (Hilmy et al., 1995).

Conventional methods for isolating and identifying fungi are time consuming and require admirably dedicated taxonomists (Paterson, 2004a). The PCR has been used for the detection of *A. flavus* with a specific target sequences for the aflatoxigenic fungi and nonviable cells and to reduce the detection time from several days. The detection of unculturable fungi is a large advantage of PCR (Färber et al., 1997).

A positive PCR is an indicator that the sample potentially contains mycotoxins and should be analyzed further (Paterson, 2006; Geisen, 1998). Shapira et al. (1996) used PCR to select aflatoxigenic fungi from other fungi and organisms commonly found on grains. The genes involved were identified and one of them is the *nor*-1 gene, which is associated with the conversion of norsolorinic acid to averantin (Shapira et al., 1996; Mayer et al., 2003).

As a phytosanitary treatment irradiation is adopted in many countries, since radiation was employed for food disinfestations. Food irradiation uses radiant energy electron beams, gamma rays, or x-rays to rid food of insects and to retard spoilage. Irradiation kills microorganisms and makes them incapable of reproduction and decreases post-harvest food losses (IAEA, 1988; ICGFI, 1995).

When ionizing radiation is absorbed in biological material, there is a possibility that it will act directly on the DNA in the cell, causing biological damage (direct effect of radiation). The second way of damage of ionizing radiation occurs in the interaction with water to produce free radicals, which can diffuse and damage the DNA as an indirect effect (Diehl, 1995). The same author reported the possibility of treatment with ionizing radiation to change properties of pathogenic microorganisms to such an extent they could no longer be correctly identified.

Until now there is little information about the effect of irradiation alterations in the DNA of the toxigenic fungi. To ensure that future mistakes are minimized, the effects of gamma radiation on detection of toxigenic *A. flavus* in maize were verified in this present study, by using PCR technique at doses of 5 and 10 kGy, under controlled conditions of *Aw*, relative humidity (RH) and temperature. A control group (0 kGy) inoculated with *A. flavus* was analysed in parallel, with irradiated samples.

MATERIALS AND METHODS

Inoculation with *A. flavus* and Adjustment of Water Activity (*Aw*)

Thirty samples (10 for each dose assay containing 200 grams) of the hybrid 3041 Pioneer maize purchased from Pirassununga city were analyzed for fungal counts and identification. The maize was previously gamma-irradiated sterilized in sterile dishes with 20 kGy and re-humidified up to the desired level of *Aw* to 0.94, by adding sterilized distilled water and RH to 94.0–97.5% by sprinkling the samples with distilled water. The whole corn samples were inoculated by spraying of the fungal suspension (1×10^6 spores/ ml) onto the corn samples with *A. flavus* strain IMI 190 (obtained from the International Mycological Institute). This strain was previously shown to be an aflatoxin-producer and thereafter they were incubated for 15 days and than irradiated at ambient temperature with a dose of 5 and 10 kGy. To keep the equilibrium the dishes were placed in opened vials with 200 ml of saturated salt solution of potassium sulphate 30% (Winston and Bates, 1960) kept inside in a plastic container.

Processing by Gamma-Radiation of Samples

The samples were treated with ionizing radiation using ^{60}Co gamma rays facility (Gammacell 220, A.E.C.L., dose rate: 4.74 kGy /h). The maize substrate was irradiated with a dose of 20 kGy at 29 °C to eliminate the natural microorganisms, according to Shapira et al. (1996), who used gamma radiation to sterilize ground corn to use as substrate to test the ability of the PCR to detect inoculated aflatoxigenic fungi. After inoculation with *Aspergillus flavus* aflatoxin producer and incubated during 15 days at 25°C, the samples were submitted at dose of 5 and 10 kGy. Ten sterilized samples were used as control, which were not irradiated after incubation.

Water Activity Determination

Water activity (*Aw*) of the samples was determined in AQUALAB CX-2 equipment from DECAGON Devices Inc. Moisture content determination was carried out with a digital thermo-hygrometer.

Number of Colony Forming Units of Fungi (CFU/g)

Grounded samples of about 10 g of were thoroughly mixed with 90 ml sterilized distilled water. Spore counting was performed by plate count technique on *Aspergillus flavus* and *parasiticus* agar (AFPA) medium after incubation for 7 days at 25°C using each suspension in a serial dilution from 10^{-1} up to 10^{-6} (Pitt et al., 1983) in three replicates for each essay.

Aflatoxin Analysis

Twenty-five grams (25 g) of each sample were extracted with methanol and potassium chloride 4% (9:1). The extracts were clarified with 30% ammonium sulfate solution and then the aflatoxins were extracted by adding chloroform. Identification and quantification was conducted via thin layer chromatography by comparison with standards (Soares and Rodrigues-Amaya, 1989).

Isolation of Fungal DNA from Inoculated Maize

Samples of 100 g of grounded maize were frozen in liquid nitrogen and grounded to powder in a mortar and for the isolation of DNA for PCR was used with the Dneasy kit Qiagen as described by the manufacturer.

Polymerase Chain Reaction

The PCR reaction was performed as described by Färber et al. (1997). The PCR reaction mixture contained: 5 µl DNA solution, 2.5 µl *Taq* polymerase buffer (with magnesium), 11.3 µl bi-distilled water, 4 µl DNTP, 1 µl primer and 0.2 µl *Taq* polymerase. Thirty PCR reaction cycles were performed with the protocol: 95°C - 1 min; 62°C - 2 min; and 72°C - 4 min. The target sequence used was enclosing a fragment of 400 bp of gene *nor 1* mRNA and the primers used were:

Primer nor-1. 5' ACCGCTACGCCGGCACTCTCGGCAC -3'

Primer nor-2. 5'-GTTGGCCGCCAGCTTCGACACTCCG -3'.

RESULTS

In this present study it was observed that the *Aw* average of control maize samples before irradiation with the dose of 20 kGy was 0.76; after irradiation the average decreased to 0.61. After the gamma radiation treatment, the average of *Aw* level was adjusted up to 0.94, as shown in Table 1.

The irradiation of the inoculated maize samples with a dose of 5 kGy resulted in a reduction of 4 to 6 log cycles of colony forming units per gram (cfu/g) of *A. flavus*, compared to the control group (3.0×10^6 to 8.0×10^7), while irradiated samples (5 and 9) with 10 kGy decreased 5 log cycles (Table 2).

Table 1: The values of *Aw* of maize before and after irradiation (20 kGy) and after the adjustment of *Aw (*before inoculation).

Samples	* *Aw* of samples before irradiation*	* *Aw* of samples after irradiation	* Adjusted *Aw* to inoculation after Irradiation
1	0.73	0.61	0.93
2	0.75	0.60	0.91
3	0.76	0.63	0.93
4	0.75	0.62	0.92
5	0.76	0.62	0.93
6	0.75	0.62	0.97
7	0.77	0.61	0.92
8	0.74	0.62	0.96
9	0.74	0.63	0.94
10	0.76	0.60	0.92
11	0.75	0.60	0.95
12	0.79	0.63	0.94
13	0.77	0.60	0.92
14	0.75	0.60	0.92
15	0.76	0.60	0.92
16	0.74	0.60	0.95
17	0.77	0.61	0.91
18	0.76	0.60	0.98
19	0.79	0.60	0.92
20	0.77	0.60	0.94
21	0.75	0.60	0.93
22	0.75	0.63	0.96
23	0.78	0.60	0.95
24	0.77	0.64	0.97
25	0.76	0.63	0.94
26	0.76	0.60	0.94
27	0.78	0.61	0.93
28	0.75	0.60	0.96
29	0.76	0.60	0.97
30	0.78	0.60	0.98
Average of three replicates	0.76	0.61	0.94

Table 2: Number of cfu/g of *A. flavus* in control samples and irradiated with 5 and 10 kGy.

Samples	0 kGy*	5 kGy*	10 kGy*
1	5.0×10^7	8.0×10^1	0
2	4.2×10^6	1.2×10^2	0
3	**5.3×10^7**	**0**	**0**
4	**6.0×10^7**	**0**	**0**
5	6.5×10^7	6.0×10^1	1.0×10^2
6	**5.5×10^7**	**0**	**0**
7	**8.0×10^7**	**0**	**0**
8	4.9×10^7	1.4×10^2	0
9	3.0×10^6	2.0×10^2	5.0×10^1
10	**5.2×10^7**	**0**	**0**

*Average of three replicates.

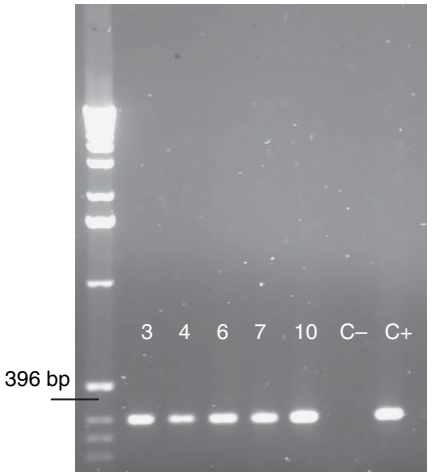

Figure 1: The agarose gel of control samples (3, 4, 6, 7, 10), negative (C-) and positive control (C+).

In 50% of irradiated samples with dose of 5 kGy, a complete decontamination was observed and 80% of samples irradiated with 10 kGy did not show the growth of *A. flavus* (cfu/g) in the AFPA medium, after 7 days of incubation. The samples 3, 4, 6, 7, and 10 showed no fungal growth in both treatments with gamma radiation (5 and 10 kGy) and were submitted to analysis by PCR technique (in three replicates) to demonstrate if the damage of fungal DNA could lead to interference in the primers performance during PCR reaction in the detection of toxigenic fungi in irradiated maize. The PCR technique showed the presence of DNA bands of *Aspergillus flavus* in all samples (3, 4, 6, 7, and 10), as demonstrated in Figures 1, 2, and 3.

DISCUSSION

According to the authors previously cited, the growth and aflatoxin production would be impossible, in an irradiated substrate with *Aw* around 0.61 after inoculation with a spore suspension. The decrease of *Aw* is explained by radiolysis of free water in maize grains during the radiation process with formation of free radicals as an indirect effect of radiation (Diehl, 1995).

The adjustment of *Aw* average to 0.94, the temperature to 25°C, and relative humidity (94.0–97.5%) were favorable to the growth and aflatoxin production of toxigenic *A. flavus*, in this study. The expression of the genes involved in the aflatoxin biosynthetic pathway was demonstrated with average production of aflatoxin B_1 of 2200 μg/kg.

The results showed that gamma radiation was effective in decreasing the load of viable *A. flavus* in maize by inactivation of the fungi. Onyenekwe et al.

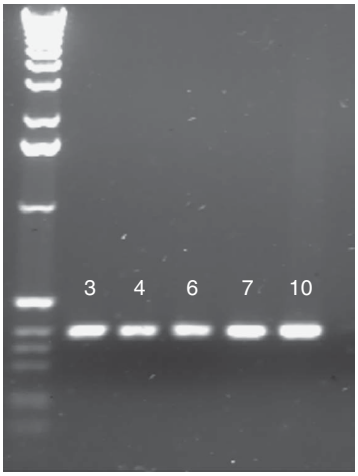

Figure 2: The agarose gel of irradiated samples with 5 kGy.

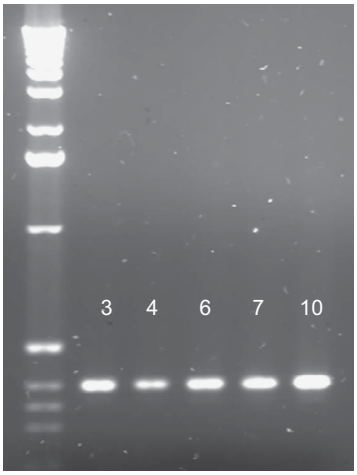

Figure 3: The agarose gel of irradiated sample with 10 kGy (samples 3, 4, 6, 7 and 10).

(1997) and Aziz et al. (2006) used the dose of 10 kGy for the total decontamination of spices and cereal grains. These studies are similar range to our results that showed the reduction of fungal growth using the dose of 10 kGy, and had 80% of decontamination.

DNA techniques have been developed to detect bacterial contamination of foods and molecular techniques could be used with food treated with a radiation dose high enough to leave no viable bacteria in the sample (Hill and Keasler, 1991). Once the PCR method is based on the molecular structure of DNA and according to Pollard (1966), an estimated dose of 0.1 kGy will damage 0.28% of the DNA in bacterial cells. This effect on DNA will be lethal to a large fraction

of the irradiated cells and is easily recognized with fewer colonies developing upon inoculation of a culture medium. All samples showed DNA amplification products from the samples 3, 4, 6, 7, and 10, respectively, as indicated in Figures 1, 2, and 3.

The identification of critical control points in food systems relevant to toxin-producing fungi is important part of a hazard analysis critical control point (HACCP) approach (FAO, 2003). This assumes there is a close link between fungus and toxin. Isolation and identification of structural and regulatory genes from the aflatoxin pathway provide a unique opportunity to study the regulation of production of these secondary metabolites in *Aspergillus* spp. (Trail et al., 1994). Potential mycotoxin production can be detected by PCR which may permit the establishment of HACCP and is a significant advantage for the detection of uncultivable fungi (Paterson, 2006).

The PCR is sensitive for detection if all the genes of a pathway can be expressed by incubating a sample and assessing whether concentrations of the mycotoxin increase. However, the gene may be present but is not expressed. Issues of inhibition and nuclei acid damage need to be carefully considered (Paterson, 2004b). Mayer et al. (2003) obtained data using real-time PCR correlating fungal growth, gene expression and aflatoxin production, showing that the produced AFB_1 could be detected 6 days after inoculation, 2 days later than the induction of the *nor-1* gene. Its concentration reached a maximum after 10 days and stayed constant. This indicated that no more aflatoxin is synthesized after degradation of the *nor-1* mRNA.

However, it is sometimes necessary to emphasize the potential pitfalls of PCR technique. The gene may be present but is not expressed. Issues of inhibition and nuclei acid damage need to be carefully considered (Paterson, 2004b). Changes in morphological, biochemical, or serological may appear after irradiation. Robern and Thatcher (1968) observed a change in requirements of amino acids for growth of *E. coli.* Similarly, Parisi and Antoine (1974) reported the same effect in *Bacillus pumilus*. The irradiation treatment apparently caused sufficient genetic damage that the enzymes critical in the synthesis of certain amino acids were either altered to the extent of being nonfunctional or lost (Diehl, 1995).

The argument often heard from opponents of food irradiation is that the process could be used to mask unhygienic conditions of food production and handling by eradicating detectable microorganisms. As demonstrated by Rowe and Towner (1994), gross bacterial contamination detected by hybridization techniques in irradiated food samples (not containing viable bacteria), showed reduced detection compared with otherwise identical untreated samples.

It was possible to detect the products of amplification of gene *nor-1*, by PCR in this present study, in all inoculated samples (control). This was shown in the maize samples irradiated with 5 and 10 kGy (Table 2), where the fungal growth in AFPA agar was not observed in samples 3, 4, 6, 7, and 10. The

products of amplification of all irradiated samples exhibited strong positive signals, compared to the DNA bands of control samples, as demonstrated in Figures 1, 2, and 3.

The PCR is a valuable tool for the qualitative microbiological detection in irradiated food, and the process of treatment by gamma radiation did not damage the fungal DNA at applied doses.

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