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EVALUATION OF VIABILITY OF *ASPERGILLUS FLAVUS* AND AFLATOXINS DEGRADATION IN IRRADIATED SAMPLES OF MAIZE

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ABSTRACT

One of the currently most important fungi in stored grains is *Aspergillus flavus*, which produce aflatoxins. This fungus can grow on diverse substrates and represents a serious public health and animal nutritional problem. Therefore, the study of techniques that can be applied to the control of aflatoxins is of great importance. The objective of the present study was to determine the effects of gamma radiation on the growth of *Aspergillus flavus* Link and on degradation of aflatoxin B_1 and B_2 (AFB_1 and AFB_2) at a relative humidity of 97 – 99% and a water activity (A_w) of 0.88-0.94. Samples of corn grains were irradiated using a cobalt 60 source emitting gamma rays at doses of 2, 5 and 10 kGy. Irradiation was found to be effective in reducing the number colony-forming units of *A. flavus*, per gram, in the corn samples analyzed. In addition, the fluorescent viability test (fluorescein diacetate and ethidium bromide) revealed a decrease in the number of viable cells with increasing irradiation doses and three different fluorescence patterns. Furthermore, irradiation induced a partial reduction in AFB_1 and AFB_2 levels at the doses of 2 and 5 kGy, whereas complete degradation of aflatoxins was observed in the assay employing 10 kGy.

Key words: gamma radiation, *Aspergillus flavus*, fluorescent viability test, water activity, maize

INTRODUCTION

Aspergillus flavus and *A. parasiticus* produce aflatoxins, which are among the most carcinogenic compounds known and cause serious problems worldwide in agricultural commodities such as maize, peanuts, tree nuts and cotton seed (10). Aflatoxins were reported to be among the most potent mycotoxins (9). Maize is particularly susceptible to colonization and infection after silk emergence. The tropical weather conditions which prevail during the greater part of the year in Brazil, favor fungal growth and mycotoxin production (5) and according to the Brazilian legislation, foods and feeds are not permitted to contain above 20 μg/kg of total aflatoxins (1).

The ability of ionizing radiation to kill microorganisms has been investigated since the late 19th century and as demonstrated by Aziz and Abd El-Aal (4) the complete elimination of toxigenic moulds in coffee beans and food commodities was achievable with doses from 5 to 10 kGy. The sensitivity of fungi to gamma-radiation has been established by Aziz *et al*. (3) who recorded that the dose required for complete inhibition of fungi in different food and feed products ranged from 4 to 6 kGy. There are a number of reports which suggest that moulds are very sensitive to gamma-radiation and in addition their mycotoxin production decrease after irradiation (16,21).

There are few methods used to determine the viability of cells. The cytotoxic test was improved by combining fluorescente

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diacetate (FDA) and ethidium bromide (EB) that showed a strong contrast between living and dead cells (12). The aim of this study was to evaluate the effects of irradiation on viablility of *Aspergillus flavus* by viability test (FDA-EB) and aflatoxins degradation in maize at a dose 2, 5 and 10 kGy.

MATERIALS AND METHODS

Irradiation

Twenty samples of maize grains (hybrid 3041 from Pioneer) grown in Pirassununga/SP, with 200 g each, were individually packaged and irradiated with 20 kGy, using a ^{60}Co gamma ray facility (Gammacell 220, A.E.C.L., dose rate: 4.74 kGy/h) to eliminate the natural microbial contamination. Thereafter, the samples were put in sterile dishes and the water activity was adjusted to *Aw* = 0.91 – 0.94 and RH to 97.5% (20) by sprinkling the samples with distilled water. To keep the equilibrium the dishes were placed in opened vials with 200 mL of 30% saturated salt solution (K_2SO_4). Then, the corn samples were inoculated with *A. flavus* IMI 190, which was previously shown to be an aflatoxin-producer, obtained from the International Mycological Institute, by spraying 2 mL of the fungal suspension with 10^6 spores/mL onto the corn samples. The samples were kept in a plastic container and incubated for 15 days at 25ºC with a RH of 97 – 98%. Thereafter, individual samples were irradiated at ambient temperature, with doses of 2; 5 and 10 kGy (five samples per dose). All samples, including the control (five samples), were used for A_w determination, plate counting, fluorescent viability test and aflatoxin analysis.

Water activity determination

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

Plate counting

Ten grams (10 g) samples were grounded and thoroughly mixed with 90 mL of sterile distilled water. Spore counting was performed by plate count technique on a selective medium for *A. flavus* and *A. parasiticus* (AFPA medium) after incubation for 7 days at 25ºC using each suspension in a serial dilution from 10^{-1} up to 10^{-6}. The fungus appeared as isolated yellow or orange colonies on the plates (15).

Aflatoxin analysis

Twenty-five grams (25 g) of each sample were extracted with methanol / 4% KCL (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 (18).

Fluorescent viability test

For viability testing, 1 g of each sample was suspended in 1 mL of distilled water. Thereafter 0.1 mL of a 1:1 mixture of a fluorescein diacetate solution (2 µg/mL in PBS buffer pH 7.4) and ethidium bromide (50 µg/mL in PBS buffer pH 7.4) was added to 0.1 mL of the suspension. This mixture was incubated at 25ºC for 30 minutes.

RESULTS AND DISCUSSION

It was shown, that irradiation effectively reduces the number of colony forming units in the maize samples under investigation at all doses used (Table 1), but the effect was more dominant at higher irradiation doses. This result is in accordance to the data shown by Rustom (17) that reported the reduction of fungal growth increased with increasing irradiation doses, but even using a dose of only 3 kGy resulted in a more than 99.9% reduction in the numbers of colony forming units of *A. flavus*. Hilmy *et al*. (11) reported no growth of *A. flavus* under RH of 85% or less in ground nutmeg and peanut and under 91-97%, growth of mycelium and toxin production of the mould were inhibited by irradiation, although, the effectiveness of irradiation varied with different RH and media during post-irradiation incubation.

The presence of water has an important role in the destruction of aflatoxin by gamma energy, since radiolysis of water leads to the formation of highly reactive free radicals. These radicals can readily attack AFB_1 at the terminal furan ring, giving products of lower biological activity. The mutagenic activity of AFB_1 in an aqueous solution (5 g μL^{-1} water) was reduced by 34%, 44%, 74% and 100% after exposure to gamma rays at 2.5; 5; 10 and 20 kGy, respectively (19). Addition of 1 mL

Table 1. Number of cfu of *Aspergillus flavus* after 15 days of incubation at 25ºC, RH of 97.5%, inoculated with 10^6 spores/mL and irradiated with 2; 5 and 10 kGy and control samples (0 kGy).

Samples	Control	2 kGy	5 kGy	10 kGy
1	500×10^5	0.5×10^5	0.001×10^5	0
2	40×10^5	0.05×10^5	0.001×10^5	0.0005×10^5
3	600×10^5	1.5×10^5	0	0.002×10^5
4	600×10^5	0.2×10^5	0	0
5	650×10^5	0.03×10^5	0.001×10^5	0.001×10^5
Average	478×10^5	0.456×10^5	0.0006×10^5	0.00070×10^5
S. D.	250.8×10^5	0.613×10^5	0.0005×10^5	0.00084×10^5

- cfu = colony forming units;
- S. D. = Standard Deviation;
- The control is different comparing with the all irradiated samples ($p<0.01$), which are not different each other ($p>0.05$) by the *Student* and *Tukey* test.

of 5% hydrogen peroxide to an aqueous AFB_1 solution (50 µg/mL) resulted in 37-100% degradation of the toxin at a dose of only 2 kGy (17). In this present work, irradiation resulted in a reduction of the aflatoxin content of the maize samples under investigation. Using 2 and 5 kGy, the reduction in AFB_2 (97.6% at 2 kGy, 94% at 5 kGy) was more efficient than the reduction in AFB_1 (68.9% at 2 kGy, 46% at 5 kGy). A radiation dose of 10 kGy resulted in a complete reduction in AFB_1 and AFB_2 (Table 2, Table 3).

The higher sensitivity of AFB_1 and AFB_2, respectively, to irradiation with 2 kGy compared to 5 kGy may be explained by higher water activity at 2 kGy (A_w 0.91) compared to 5 kGy (A_w 0.88) (Table 4) and a concomitantly higher gamma energy which may result in an increased formation of highly reactive free radicals (the radiolytic products of radiolysis of water). There are a number of conflicting reports that show different results in the increase, decrease or even unaffected the production of mycotoxins after irradiation of fungi under various laboratory conditions (2,14).

Table 2. Results of extraction of Aflatoxin B_1 (µg/kg) of control samples and irradiated samples.

Samples	Control	2 kGy	5 kGy	10 kG
1	2597.2	398.3	1552.0	ND *
2	ND *	729.5	1120.5	ND *
3	1844.4	571.7	1121.1	ND *
4	2159.4	556.5	1096.0	ND *
5	2258.5	1187.4	1046.8	ND *
Average	2214.88	688.68	1187.28	ND *
S. D.	310.06	302.43	206.11	-

- S. D. = Standard Deviation;
- The control is higher than all samples. The irradiated samples with 5 kGy are superior comparing with the 2 kGy ($p<0.01$);
- ND = No detection of mycotoxins in the irradiated samples with 10 kGy. The detection limit was 2 µg/kg.

Table 3. Results of extraction of Aflatoxin B_2 (µg/kg) of control samples and irradiated samples.

Samples	Control	2 kGy	5 kGy	10 kG
1	778.2	11.3	97.9	ND *
2	ND *	27.2	20.5	ND *
3	485.0	5.7	17.1	ND *
4	481.1	ND *	19.8	ND *
5	540.1	9.7	15.1	ND *
Average	571.10	13.48	34.08	ND *
S. D.	140.67	9.45	35.74	-

- S. D. = Standard Deviation;
- The control is higher than all samples ($p<0.01$). The irradiated samples with doses of 2 and 5 kGy are equals ($p=0.14$);
- ND = No detection of mycotoxins in the irradiated samples with 10 kGy. The detection limit was 2 µg/kg.

Table 4. Levels of water activity (A_w) of maize samples after 15 days of incubation at 25ºC, under a relative humidity of 97.5%.

Samples	Control	2 kGy	5 kGy	10 kG
1	0.92	0.91	0.90	0.96
2	0.91	0.92	0.91	0.96
3	0.93	0.89	0.87	0.92
4	0.93	0.93	0.87	0.92
5	0.94	0.92	0.87	0.96
Average	0.926	0.914	0.884	0.944
S. D.	0.011	0.015	0.019	0.022

- S. D. = Standard Deviation;
- There are not significantly different between the irradiated samples 2; 10 kGy and the control values ($p>0.05$);
- The samples irradiated with doses of 2 kGy are inferior comparing with samples irradiated with 10 kGy ($p<0.02$) and 5 kGy are inferior comparing with all irradiated samples ($p<0.01$).

Mitchell (13) showed that the fungal strain, condition of sporage, humidity and irradiation dose affect mould growth and toxin production. The effect of irradiation on the aflatoxin content of food and feed was previously shown by Aziz and Moussa (2), who indicated that the fungal flora in the different fruit samples are sensitive to gamma-radiation, and were completely inhibited at 5 kGy radiation dose. The same study showed that the degradation of AFB_1, observed in plum stored at refrigeration and irradiated at 3.5 kGy, decreasing 380-500 µg/kg to 20 µg/kg and this result is in accordance with our results (Table 2).

The dead cells showed a bright red fluorescence due to ethidium bromide penetration and giving evidence of that esterases were inactived. Instead of, living cells (green fluorescence), staining by fluorescein diacetate (FDA), showed three different standards of fluorescence (SF), observed in Fig. 1:

SF1– Observed in fungi cells presents in the maize of control group, represented by stained cells visualized as a intensive green fluorescence;

SF2– Represented by fungi cells stained with a weak green fluorescence, in the irradiated samples of maize with doses of 2 and 5 kGy;

SF3– Represented by fungi cells stained with green fluorescence situated in the cellular wall, in a form of ring. The intracellular region, thereby, showed almost any fluorescence, in the irradiated samples of maize with doses of 5 and 10 kGy.

According to Corrêa *et al*., (7), the intensive areas stained by FDA, demonstrate that SF1 are points which have high hydrolyzing activity due to acetillesterases. The Standard SF2 (weak fluorescence intensity) is explained by a weak action enzimatic, problaby allied to age of cells (6,12), but in this case, due to loss of enzyme activity by effetcs of radiation, because the cells had the same age (seven days). Diehl (8) mentioned that a dose of 10 kGy caused 20% of loss of enzyme activity in a 1% solution and 60% loss in a 0.5% solution. The standard of green fluorescence 3 (SF3), showed the effect of radiolysis in the intracellular region of spores, where the concentration of water is higher than the cellular wall, which intesivity of fluorescence is preserved, giving the appearance of ring.

Table 5. Frequency of viable and dead cells of *Aspergillus flavus* using fluorescent method (FDA-BE) of control and irradiated samples with doses of 2, 5 and 10 kGy.

Control		2 kGy		5 kGy		10 kGy	
Dead Cells %	Viable Cells %	Dead Cells %	Viable Cells %	Dead Cells %	Viable Cells %	Dead Cells %	Viable Cells %
14	86	94	6	98	2	96	4
25	75	94	6	96	4	98	2
NC	NC	98	2	95	5	99	1
NC	NC	94	6	98	2	99	1
* X= 19.5 (S.D.) 7.8	* X= 81.5	X = 95 (S.D.) 2.0	X = 5	X = 96.8 (S.D.) 1.5	X = 3	X = 98 (S.D.) 1.4	X = 2

- S. D. = Standard Deviation;
- NC = Not Counted;
- *X = Average of three replicates.

CONCLUSIONS

The previously knowledge of humidity, free water and temperature of substrate and the combination with low doses of irradiation, to lead a good result in a fungal control. The irradiated samples of maize exposed to doses of irradiation of 2, 5 and 10 kGy, when submitted to the fluorescent test (FDA-BE), showed decrease of viable cells number of *A. flavus*, with the increasing of doses (Table 5). In this present investigation, the obtained results with the fluorescent method, in front of growth and plate count of colonies, showed consonance between these techniques, revealing a efficient effect of gamma radiation, observed in the decrease of viable cells number with the raise of irradiation doses. Meanwhile, comparing with plate counting, the fluorescent method demonstrated to be faster and an important indicator of damage or fungi cellular viability, when submitted to irradiation.

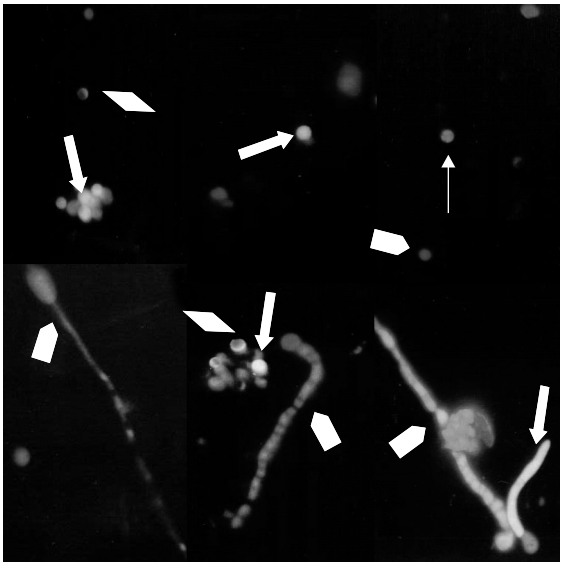

Figure 1. Viable and dead cells of *Aspergillus flavus* using the method of viability by fluorescence (FDA-EB).
⇨ SF1 - Viable cells with intensive green fluorescence;
→ SF2 – Viable cells with weak green fluorescence;
◇ SF3 – Viable cells with green fluorescence in the cellular wall (ring form);
⇨ DC – Dead cells with a bright red fluorescence.

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RESUMO

Avaliação da viabilidade de *Aspergillus flavus* e degradação de aflatoxinas em amostras de milho irradiadas

Um dos fungos mais importantes atualmente em grãos armazenados é o *Aspergillus flavus*, o qual produz aflatoxinas. Este fungo pode crescer em diversos substratos e representa uma séria preocupação em saúde pública e nutrição animal. Portanto, o estudo de técnicas que possam ser aplicadas no controle das aflatoxinas é de grande importância. Assim sendo, o objetivo do presente trabalho foi estudar os efeitos da radiação gama no crescimento de *Aspergillus flavus* Link e na degradação das aflatoxinas B_1 e B_2, (AFB_1 e AFB_2) em umidade relativa (UR) de 97-99% e atividade de água (Aa) de 0,88-0,94. Amostras de

grãos de milho foram irradiadas, utilizando-se uma fonte de Cobalto 60, emissora de raios gama, com as doses de 2; 5 e 10 kGy. A irradiação foi efetiva na redução do número de Unidades Formadoras de Colônias de *A. flavus*, por grama, nas amostras de milho analisadas. Adicionalmente, o teste de viabilidade fluorescente (solução de diacetato de fluoresceína e brometo de etídio) revelou diminuição no número de células viáveis com o aumento das doses de irradiação e três diferentes padrões de fluorescência. Além disso, a irradiação induziu a uma parcial redução dos níveis de AFB_1 e AFB_2, nas doses de 2 e 5 kGy, ao passo que uma completa degradação das aflatoxinas foi observada no ensaio empregado com 10 kGy.

Palavras-chave: radiação gama, *Aspergillus flavus*, teste de viabilidade fluorescente, atividade de água, milho

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