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ORIGINAL ARTICLE

Effect of Irradiation on Aflatoxin Content and Seed Viability of *Arachis hypogea* L.

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Abstract

Aflatoxins are toxic metabolites of fungi mainly *Aspergillus flavus* and *A. parasiticus*, which cause immune system suppression, liver cancer or even death. In present study different doses of gamma irradiation were used to reduce aflatoxin content in peanut, and the effect of irradiation on shelf life and seed viability was evaluated. The irradiation dose for total reduction of aflatoxins was optimized. Results obtained in this study indicate that a dose of 3 and 5 kGy reduced total background counts of fungi from $9.5\pm1.2x10^5$cfu/g to 20.0±5.0 and 4.0±1.0 cfu/g respectively and increased the shelf life of peanut by 2 months. Germination percentage and root and shoot length were measured to determine the effects of various irradiation doses on the peanut seed germination. There was marked reduction in germination percentage at 1.0 kGy, whereas at 3 kGy dose the peanut seeds completely lost their viability. Root and shoot lengths were found more sensitive to the gamma irradiation than the germination percentages.

Keywords: *Aspergillus flavus*, Gamma radiation, Peanut.

1. Introduction

Aflatoxins are a family of related bisfuranocoumarin compounds produced by fungi *A. flavus* and *A. parasiticus*. It has been reported that out of the known strains of *A. flavus* and *A. parasiticus*, only about one-half produce toxins. There are 14 known aflatoxins but most of these are metabolites formed endogenously in animals administered by one major toxin, i.e., aflatoxin B1, B2, G1 and G2. *A. flavus* produces B1 and B2 whilst *A. parasiticus* produces the four major toxins (Lillehoj, 1986). Aflatoxins G1 and G2 are formed only by *A. parasiticus* (Klich and Pitt, 1988). These toxins are usually found together with various foods and feeds in various proportions (Patil *et al.*, 2014; Katole *et al.*, 2013; Patial *et al.*, 2013); however, aflatoxin B1 is usually predominant and is the most potent naturally formed carcinogen (Squire, 1981).

Aflatoxin development in many stored cereal grains has constantly hampered the availability of good quality grains in Asian countries. The fungi *A. flavus* Link ex Fries and *A. parasiticus* Speare have been identified as the quality deterrent producing aflatoxin contaminated grain when stored. The most important group of toxigenic Aspergilli are the Aflatoxigenic molds, *A. flavus*, *A. parasiticus* and the lately described but much less common species *A. nomius* all of which are classified in *Aspergillus* section Flavi (Gams *et al.*, 1985). Although these three species are closely related and share many similarities a number of characteristics may be used in their differentiation. *A. flavus* is widely distributed in nature but *A. parasiticus* is less wide spread, the actual extent of its occurrence being complicated by the tendency for both species to be reported indiscriminately as *A. flavus*. In the last decade, aflatoxin levels were found to exceed an acceptable level limit of 20 µg/kg stipulated in most export specifications. Aflatoxin contamination has affected maize, peanuts and cottonseeds in Thailand, India and coconut in the Philippines, Sri Lanka and other Pacific countries. Aflatoxicosis both in humans and animals has been more prevalent in areas where maize and groundnut constitute a major part of the diet. Many private and government organizations have embarked on aflatoxin research. However, many problems are associated with the actual research activities concerning aflatoxin. Prevention of Food Adulteration Act (1954), amended in 1986, U.S. Food and Drug Administration (FDA) and the Codex Alimentarius Commission (1989) have recommended a permissible limit of aflatoxin of 30 µg/kg, 20 µg/ kg and 5 µg/ kg respectively. Few studies have been

undertaken in past sampling the food stuffs, available in market for consumption, for presence of fungi.

Arachis hypogea L. (Groundnuts or peanuts) provides an excellent source of protein and is a valuable cash crop for millions of small-scale farmers in the semi-arid tropics. As a groundnut-based fodder it is consumed by animals in India and elsewhere. Contamination of the groundnut by aflatoxin poses a serious threat to human and animal health not only from eating the diseased groundnuts themselves but also indirectly through drinking milk from cows that have eaten infected material. The fungus *Aspergillus* that produces aflatoxin enters the plant usually through the wounds left by an insect pest (Lee *et al.,* 1977; Marsh *et al.,* 1969).

Despite the availability of many food processing technologies developing countries still have high post harvest losses of food. According to the U.S. Food and Drug Administration (FDA), the consumption of pathogen-contaminated food can be a potential health hazard. To combat this problem, cereals, pulses and other stored products are preserved by chemical fumigation but use of chemicals has created problems relating to health and environment. In many countries, both fumigation with ethylene oxide and heat sterilization have been tried with varying degrees of success; however, these methods have several disadvantages for application to the sterilization of grains, such as toxic residues are left and organoleptic properties are changed. For these reasons, reduction of pathogenic micro-flora by alternative means is highly desirable. In 2000, the irradiation process was approved for use on sprout seeds for doses upto 8 kGy. Thus irradiation may be a method of choice, particularly because, unlike heat decontamination, it does not destroy nutrients (Campbell *et al.,* 1986). Radiation treatment is widely recognized as a suitable method for decontaminating food products. Commercial-scale use of radiation processing for food and feed commodities has been successful in several countries (Ley 1983; Giddings 1984; Grecz *et al.,* 1986; El-Zawahry *et al.,* 1991; Gharib and Aziz, 1995). Rajkoswki and Thayer (2001) also reported that gamma radiation could be used to reduce bacterial growth on sprouts.

Seed viability is another important aspect for quality maintenance of seeds as the same seed lot can be used for cropping; if the seed has no viability then the process of decontamination with these physical processes has no use for the farmers. Therefore, it is worthwhile to determine if the detoxification treatments have any effect on seed viability.

The present study was therefore conducted to determine the efficacy of gamma radiation on (1) aflatoxin level, (2) fungal count, (3) seed viability and (4) shelf life of peanut seeds.

2. Materials and Methods

2.1 Irradiation of Peanut

Peanut samples packed in polythene bag were irradiated with doses of 0.1, 0.2, 0.5, 1.0, 3.0, 5.0, 10.0 and 15.0 kGy in a Co-60 gamma irradiator (SARC, Delhi). Dose rate was 0.452 kGy/h. Variation in the dose absorbed by the experimental samples was minimized by moveable cycle within a uniform area of the radiation field. The actual dose was verified by dosimeter. In the studies of the storage (shelf life) after irradiation, the peanuts were stored at room temperature and the samples were withdrawn 15, 30 and 90 days after storage for determination of total fungal counts.

2.2 Determination of Fungal Micro-flora in Peanut Seeds

The peanut samples were triturated and 10g aliquots of each sample were transferred to Sterilized Erlenmeyer flasks containing 90 ml of 0.85% Normal saline. The mixture was then homogenized by shaking for 30 min and their 1 ml portion were used for serial dilutions in sterile test tubes. Two Petriplate (90mm) containing Rose Bengal Chloramphenicol Agar (RBCA) was used for each dilution. 0.1 ml aliquots of each sample was then transferred to Petriplate and evenly distributed over dried surface of media. The plates were incubated in incubator at 28^{o}C for 5 days. The number of colony forming unit per gram (CFU/g) were determined thereafter (Pitt *et al*., 1983)

2.3 Quantification of Total Aflatoxin Content

2.3.1 Test Procedure for Total Aflatoxin

According to Ridascreen Total Aflatoxin (Art No. R4701) test kit manual (R-Biopharm, GmbH), 50 µl aflatoxin standard solutions and 50 µl prepared test samples were added into separate wells of micro-titer plate, in duplicate. Then, 50 µl of the diluted enzyme conjugate was added to each well, mixed gently and incubated for 2 h at room temperature (20-25^{o}C) in the dark. The liquid was then removed completely from the wells and then each well was washed with 250 µl washing buffer (PBS–Tween Buffer, pH 7.2). The washing procedure was repeated for three times. After the washing step, 50 µl enzyme substrate (urea peroxide) and 50 µl chromogen (tetramethyl-benzidine) were added to each well and incubated for 30 min at room temperature in the dark. Finally, 100 µl of the stop solution (1 N H_2SO_4) were added to each well and

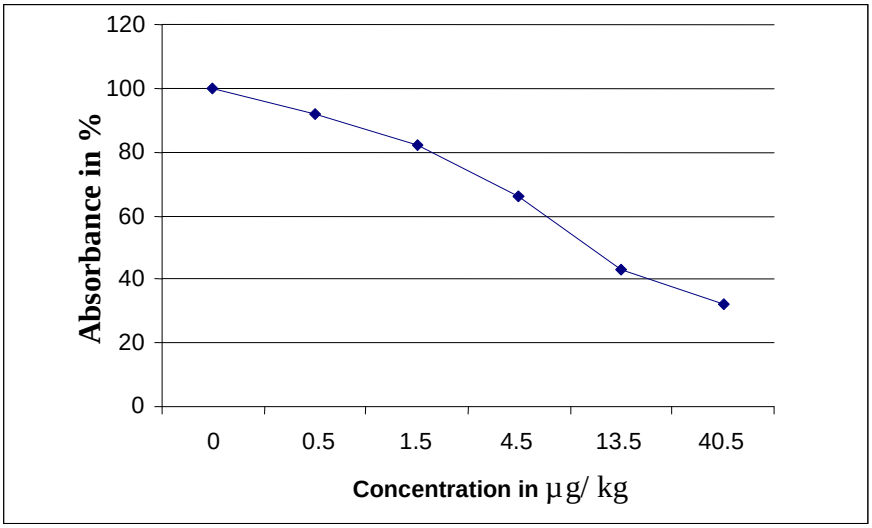

Fig 1: Calibration curve for total aflatoxin using different concentrations of total aflatoxin standards

the absorbance was measured at 450 nm in ELISA plate reader (Bio-Rad Model 680). Aflatoxin standard 0, 0.5, 1.5, 4.5, 13.5 and 40.5 µg/kg was used for the preparation of calibration curve (Fig 1).

2.3.2 Irradiation Effects on Germination of Peanut Seeds

Germination potential of the peanut seeds (irradiated and control) was estimated in accordance with the International Rules for Seed Testing (ISTA 1985). Germination percentages, using 3 replicates of 50 seeds, were determined by placing the seed samples in 100 mm Petridishes on filter paper (Whatman No.1) moistened with distilled water. Seeds were distributed evenly within each dish. Petri dishes were covered with their lid and then placed in an incubator at 25^{o}C. Observations were made after 7 days of planting. Radicle emergence (at least 2 mm) was taken as the index of germination.

2.3.3 Plant Growth Test

Gamma irradiated as well as control seeds were tested for plant growth studies to check the effect of different energy doses of irradiation. For this purpose, earthen pots were taken and filled with a mixture of sand and soil in ratio of 50:50. Twenty seeds from each sample were sown in single pots. Seeds were sown at a depth of 1 inch. After sowing pots were kept covered with straw till the emergence of seedling. Growth parameters such as plant height and root length were measured after one month of planting.

3. Results and Discussion

3.1 Effect of Gamma Radiation on Fungal Count and Aflatoxin Content

Table 1 shows the effect of gamma radiation on total fungal count and total aflatoxin content of seeds.

Table 1: Effect of Gamma irradiation on fungal microflora of Peanut samples.

S. No.	Dose in kGy	Total fungal count (cfu/g)	TAF[a] (µg/Kg)
1	Control	9.5± 1.2 x10^5	123.0±22.0
2	0.1	7.0± 1.0 x10^4	112.0± 12.0
3	0.2	3.2±0.5 x10^4	93.3±10.2
4	0.5	4.5±0.2 x10^3	75.0± 3.5
5	1.0	2.3±0.2 x10^2	56.6± 6.0
6	3.0	20.0±5.0	11.0± 1.2
7	5.0	4.0±1.0	0.6± 0.1
8	10.0	Nil	ND[b]
9	15.0	Nil	ND

[a]*Total Aflatoxin,* [b]*Not Detected*

Fungal count of control i.e. non irradiated sample was 9.5 x 10^5 cfu/g and aflatoxin content was 123 µg Kg-1 that decreased significantly with increasing dose of gamma radiation. No aflatoxin and fungal count was observed above 5 kGy dose. The results indicate that the fungal flora in the peanut samples is sensitive to gamma-radiation, and were completely inhibited after 5.0 kGy radiation dose. Grains and cereals are treated with low doses of irradiation to eliminate fungi since these organisms can produce mycotoxin. Increasing dose up to 5 kGy totally kills the spores of many fungi that survive in lower dose**.** The sensitivity of fungi to gamma-radiation has been established by Saleh and Aziz (1996) and Abd El-

Aal and Aziz (1997) 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 mycotoxin production decreased after irradiation of foods (Refai *et al.,* 1996; Youseff *et al.,* 1999). Significant reduction in fungal load in walnut was reported by Al-Bachir (2004). Recently, Jalil *et al*. (2010) also reported that gamma rays are able to reduce aflatoxin level in black pepper. Malveille *et al*. (1979) found that aflatoxin after irradiation at 400 Krad showed complete loss of its toxicity in the Ames mutagenicity tests.

3.2 Effect of Gamma Radiation on Shelf life of Peanuts

Effect of gamma radiation on shelf life was evaluated by determining fungal count after different intervals of time i.e. 0, 15, 30 and 90 days subsequent to irradiation. Before irradiation fungal load of peanut was 9.5 x 10^5 cfu/g which decreased gradually with increasing dose of gamma radiation reaching almost negligible level at 5.0 kGy where only 4 cfu/g were recorded (Table 2). The fungal load remained unchanged in case of 3.0 and 5.0 kGy doses till 1 month but subsequently there was slight increase in fungal load recorded after 90 days. However, it remained minimum in seeds irradiated with 3.0 kGy and 5.0 kGy doses. There are a number of conflicting reports which suggest that the production of mycotoxin is increased (Paster *et al.,* 1985), decreased (Aziz *et al.,* 1990) or unaffected (Paster and Bullerman, 1988) after irradiation of fungi under various laboratory conditions. It appears that the fungal strain, condition of storage, humidity and irradiation dose affect mould growth and toxin production (Mitchell, 1988). The shelf life of various refrigerated fresh food may be extended to three weeks by applying low doses (less than 10 kGy) of ionizing radiation. The intention is to reduce the number of spoilage organisms and to alter their growth patterns (e.g., extension of lag phase) to enhance keeping quality (WHO, 1988). An irradiation dose of 1.5–3.0 kGy extended the storage life of refrigerated fresh strawberries by reducing the numbers of the principal spoilage fungi such as species of *Botrytis*, *Rhizopus* and *Mucor* (WHO, 1981). Navaiz *et al.* (1992) irradiated almonds at doses of 1.0, 1.5 and 2.0 kGy and stored the products at 5°C for a period of six months and reported that the initial mold and yeast load was reduced to acceptable values which were then maintained throughout the storage period. In the present study, the seeds irradiated with 5.0 kGy and 3.0 kGy doses of gamma radiation could contain the fungal load at the lowest level after 90 days of storage whereas in seeds with lower doses the fungal counts were high.

3.3 Effect of Gamma Radiation on Germination and Plant Growth

Table 3 shows the effect of gamma radiation on the seed viability, moisture content of seeds and early seedling growth. As indicated above also, with increasing dose of gamma radiation a gradual decline in fungal load was recorded reaching near to complete inhibition at 5.0 kGy dose. However, irradiation of seeds also led to loss in seed moisture content and germination percentage. The control (non-irradiated) seeds had 14.0% moisture content and 98% germination both of which declined gradually with increasing dose of gamma radiation. Decline in seed germination was faster than moisture content as 0.1 kGy dose of radiation caused 12% reduction in moisture content and 54% reduction in germination. Seeds irradiated with 3.0 kGy gamma radiation contained 3.2% moisture content and failed to germinate. Thus irradiation of seeds with gamma radiation though effective in extinguishing the fungal load and in turn aflatoxin production, is of limited use in maintaining the viability of seeds.

Root length as well as shoot length also decreased after irradiation of seeds with gamma radiation as compared to the non irradiated control seeds (Table 3). Decline in early seedling growth was more than reduction in germination percentage. It indicated that the shoot and root growth were more sensitive than germination percentage to gamma radiation. Root/Shoot ratio decreased with increasing dose of gamma radiation indicating that irradiation had inhibitory effect more on root growth than on shoot growth. These results are in conformity with findings of previous workers (Bajaj *et al.,* 1970; Khanna, 1986; Al-Safadi and Sirnon, 1996) who recorded reduction in seedling growth with the increasing irradiation dose. Atsumi and Matano (1973) reported that the irradiation of wheat seeds reduces shoot and root lengths upon germination. Ramarathnamm *et al*. (1987) also reported that irradiation of rice seeds at level in excess of 5 kGy did reduce germination. Similar finding was also reported in case of Chickpea by Hameed *et al.* (2008).

Irradiation can be an effective alternative technology in postharvest pest control because of gamma rays ability to kill insects (Sirisoontaralak and Noomhorm, 2006) and inhibit mycotoxin biosynthesis during storage (Kabak *et al.,* 2006). As for control of mold growth, Chiou (1994) demonstrated that gamma irradiation at levels above 5 kGy was effective in reducing the mold population on the surface of peanut kernels but even at doses up to 15 kGy, complete elimination of the molds was not possible. However, -

Table 2: Effect of radiation on shelf life of peanut sample.

Sample	Initial Total fungal count (cfu/g)	Fungal count after 15 days (cfu/g)	Fungal count after 1 month (cfu/g)	Fungal count after 3 months (cfu/g)
Control	$9.5\pm1.2x10^5$	$5.0\pm2.1x10^5$	$5.4\pm0.4x10^7$	$4.2\pm0.8x10^8$
0.1 kGy	$7.0\pm1.0x10^4$	$3.5\pm0.7x10^4$	$4.1\pm0.6x10^4$	$3.2\pm1.2x10^6$
0.2 kGy	$3.2\pm0.5x10^4$	$3.3\pm0.5x10^4$	$2.0\pm1.1x10^4$	$1.9\pm0.0x10^5$
0.5 kGy	$4.5\pm0.2x10^3$	$6.2\pm0.2x10^3$	$5.1\pm1.3x10^3$	$7.8\pm2.0x10^5$
1.0 kGy	$2.3\pm0.2x10^2$	$2.9\pm0.4x10^2$	$4.6\pm2.0x10^2$	$4.9\pm0.1x10^4$
3.0 kGy	20.0±5.0	25.0±6.0	19.0±2.1	86.0±12.2
5.0 kGy	4.0±1.0	2.0±0.2	4.0±0.6	47.0±3.2

Table 3: Effect of Gamma radiation on seed germination and plant growth.

Treatment	Total fungal count (cfu/g)	Seed moisture Content (%)	Percent Germination (%)	Root length (in cm)	Shoot length (in cm)	Root/ Shoot ratio
Control	$9.5\pm1.2x10^5$	14.0±0.5	98	10.5±2.0	25.0±0.8	0.42
0.1 kGy	$7.0\pm1.0x10^4$	12.4±0.3	57	8.2±1.1	19.6±0.4	0.41
0.2 kGy	$3.2\pm0.5x10^4$	10.6±0.2	46	3.4±0.2	10.1±0.5	0.33
0.5 kGy	$4.5\pm0.2x10^3$	8.2±0.5	46	2.0±0.5	7.8±0.2	0.25
1.0 kGy	$2.3\pm0.2x10^2$	6.4±0.8	40	0.9±0.1	4.3±0.6	0.21
3.0 kGy	20.0±5.0	3.2±0.1	NG[c]	NG	NG	0.0
5.0 kGy	4.0±1.0	3.0±0.0	NG	NG	NG	0.0

[c]*No Growth*

Hilmy *et al*. (1995) reported that at irradiation doses of ≥3 kGy both mycelium growth and toxin production of *A. flavus* were found to be completely inhibited in ground nutmeg and peanuts. Likewise, Aziz and Mousa (2004) also reported inhibition of *A. alutaceus* and *A. flavus* growth after gamma radiation at a dose of 5 kGy while at a dose of 6 kGy detoxification of aflatoxin B1 (by 74.3-76.7%) and Ochratoxin A (by 51.3-96.2%) was achieved in chick-pea and groundnut seeds. The irradiation of food stuffs up to an overall dose of 10 kGy is permitted in numerous countries for commercial food processing (Lacroix and Quattara, 2000). In 1997, a Joint FAO/IAEA/WHO Study Group was convened to assess the safety and nutritional adequacy of food irradiated to doses above 10 kGy. It was concluded that food irradiated to any dose appropriate to achieve the intended technological objective is safe to consume and nutritionally adequate (WHO, 1999). The U.S. Food and Drug Administration (FDA) concluded that food irradiated at 50 kGy or less can be considered safe for human consumption (FDA, 1981) and therefore for animal consumption.

Nuts contain high levels of unsaturated fatty acids which are prone to lipid peroxidation when irradiated. On the other hand irradiation is known to produce free radicals interacting with both lipid and protein molecules. Such interactions lead to the formation of numerous oxidation products including aldehydes, esters, ketones, sulfur compounds etc. (Sajilata and Singhal, 2006). Such compounds in turn are responsible for flavor changes in irradiated foods (Diehl, 1981). Additionally, new trials for increasing biological activities of natural product by gamma irradiation showed advantage in increasing yields, improved the color and antioxidant activity (Byun *et al.*, 1999; Jo *et al*., 2003a-b; Kim *et al.*, 2006).

Thayer and Rajkowski (1999) concluded that ionizing radiation could penetrate the entire product to inactivate the pathogens, and it is a promising technology that could be used to improve the safety of ready-to-eat fruits and vegetables. WHO (1981) reported that gamma irradiation technology has positive effects in preventing decay of food products by eliminating microorganisms and by improving the safety and shelf-stability. Since major proportion of fungal and bacterial cell mass is water, it absorbs much of the radiation resulting in production of hydroxyl radicals and hydrated electrons that are important in irradiation-induced cell inactivation. Irradiation damage of DNA is considered as a major cause of cell inactivation. In addition to direct DNA damage, irradiation may induce functional changes in the cytoplasmic membrane resulting in cell death (Yatvin and Grummer, 1987). Ribeiro *et al.* (2011) observed the minor changes on cell wall, plasmalema, cytoplasm level, stipes, metulae, and conidia size of *A. flavus* after irradiation of gamma radiation

Overall results obtained in the present study indicate that peanuts irradiated with 5.0 kGy dose of gamma radiation reduced the fungal load and aflatoxin level to acceptable limits. However, it was of limited use for retaining the viability of seeds.

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