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November 30, 2000

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Dockets Management Branch (HFA-305)  
Food and Drug Administration  
5630 Fishers Lane, Room 1061  
Rockville, MD 20852

RE: Docket No. 99F-2673

To whom it may concern:

This is a second letter submitted for this docket on behalf of the Organic Consumers Association. OCA is a nonprofit, grassroots national organization that promotes food safety, organic farming and sustainable agricultural practices in the U.S. and internationally.

In our letter dated November 22, 2000, we objected to the FDA approval of ionizing radiation on seeds for sprouting, and asked that the FDA reevaluate its decision. The reasons given were: 1) the sprouts were likely to be nutritionally affected; 2) sprouts would contain radiolytic products similar to other plant foods; 3) the petitioner did not provide any justification for the 8 kGray level approved by the FDA.

In this letter, we submit several studies and abstracts with the goal of showing that sprouts grown from irradiated seeds are not substantially the same as sprouts grown from nonirradiated seeds. For this reason, irradiation should not be permitted on seeds for sprouting.

**Attachment 1 (Sitton et al., Electron beam irradiation effects on wheat quality, seed vigor, and viability and pathogenicity of teliospores of *Tilletia controversa* and *T. tritici*, Plant Disease 1995; 79:586-589)**

This study specifically addresses the issue at hand. The authors compared irradiated fungus spores and wheat at doses of 0.0, 1.2, 2.6, 4.6, 6.7 and 10.2 kGray for spore viability, wheat usability in baking, and germination. Germination was tested by storing the seeds on germination paper in the dark for 7 days at 20°C, then planting them in soil in early spring. Table 3 ("germination test," the 'easiest' test) shows that *no* normal germination occurred at any irradiation dose. The percentage of seeds that did not germinate increased from 0.5% (control group) to 6.0% (10.2 kGray). *The remaining seeds germinated abnormally.* To reiterate, *all* the seeds that germinated from wheat irradiated at 1.2 kGray and above produced abnormal sprouts. (The lead author, in conversation November 29, 2000, said that he has unpublished data that confirms that the minimum irradiation dose studied (1.2 kGray) prevents normal germination of wheat seeds.)

The relevance for this docket is as follows: 1) The conditions under which the wheat was germinated (dark, temperature) were similar to those used by commercial sprouters. 2) Wheat has already been approved for irradiation, and there is very little literature on other seeds that are commonly sprouted which have not been approved. 3) Wheat and barley sprouts are sold by sprouting companies.

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Organic Consumers Association, November 30, 2000

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This study implies that sprouts grown from any irradiated seed at any dose will be morphologically abnormal due to genetic disruption, which will also affect enzyme activity (see attachment 3).

**Attachment 2 (Choi and Hwang, Detection of hydrocarbons in irradiated and roasted sesame seeds, JAOCs 1997; 64:469-472)**

One objective of this study was to determine if the presence of gamma-irradiation-created hydrocarbons could be used to determine if sesame seed had been irradiated. They irradiated unroasted sesame seeds at 0, .05, .1, .5, 1, 5 and 10 kGray and found that the radiolytic hydrocarbons 16:2, 16:3, 17:1, and 17:2 can be used as markers of irradiation at 0.5 kGray or higher. Table 1 shows this graphically, where the levels of radiolytic hydrocarbons in seeds irradiated at 1 kGray and above are approximately six to eight times higher, overall, than other hydrocarbons.

This study is relevant for the present docket, because oil-rich seeds like sunflower are often sprouted and sold commercially. Sesame seeds and sunflower seeds have 48.6% and 47.3% fat by weight respectively.

This study shows that gamma irradiation creates radiolytic hydrocarbons in food oilseeds. Sprouts from irradiated sesame (or sunflower) seeds are substantially different from sprouts from nonirradiated seeds.

**Attachment 3 (Patel et al, Behaviour of lipase activity of the gamma-irradiated groundnut during germination, Journal of the American Oil Chemists Society, 1965; 42:617-619)**

This study investigated the effect of gamma irradiation on lipase activity of the groundnut (peanut) during germination. The dose levels were 10, 30, 50, 70, 90 and 120 Kilo-roentgens, which convert to .1, .3, .5, .7, .9 and 1.2 kGrays. These doses range from one-eightieth to one-sixth the maximum dose requested in the subject docket. The suppression of lipase activity (Figure 1) is for almost all doses dose-dependent.

Figure 1 shows that a difference in the level and rate of lipase activity between control and irradiated seeds begins at day 6 and continues through the germination period. The authors state "irradiation of 50 kr [.5 kGray] and above induced damage to the active centers [of lipase production]." "In general the growth of irradiated seeds was poor (only epicotyls are developed) and seeds irradiated to higher dosage levels, i.e., 70 kr [.7 kGray] and above did not grow at all," although lipase increased without germination in these seeds. The authors attribute the abnormal timing of lipase production and failure to germinate to irradiation damage to the nucleus and mitochondria.

It is reasonable to conclude that sprouts grown from fat-rich seeds (peanuts have 40-50% fat, sunflower seeds have 47.3% fat) would be nutritionally affected by irradiation prior to germination, especially at FDA-approved doses six times the maximum dose in this study. This study suggests that sprouts from irradiated seeds may be different nutritionally from sprouts from nonirradiated seeds. Studies of the nutrition of irradiated food generally look only at vitamins, minerals, protein and macronutrients rather than enzymes, even though the presence of enzymes is the marker of raw food, and the human body needs these enzymes to thrive. The evidence of abnormal and impaired enzyme activity in this study suggests that further enzyme production during sprout growth may be impaired.

**Attachment 4 (Andrianarison et al, Alterations in polyunsaturated fatty acid composition of *Voandzeia subterranea* seeds upon gamma irradiation, J Agric Food Chem 1992; 40:1663-1665)**

The authors studied the effect of irradiation for disinfestation of a tropical legume, the bambara groundnut, on fatty acids. They irradiated green seeds, flour and lipid extract at 0, 2, 4, 6, 8 and 10 kGray. They found that ionizing irradiation induces peroxidation of fatty acids. Table II shows that, compared to the control group, polyunsaturated fatty acids in green seeds decreased by 15% at 4 kGray, 30% at 6kGray, and 52% at 8 kGray.

At the end of their Discussion, they say "The high concentration of hydroperoxy fatty acids in the diet may cause several abnormalities in the human body. In addition, linolenic acid, one of the unsaturated essential fatty acids, was reduced upon ionizing radiation treatment of *V. subterranea*, suggesting that the quality of seeds is rendered poorer upon ionizing radiation."

We recognize that seeds for sprouting do not constitute an important source of fatty acids in the American diet. However, this study shows that the chemical composition of the seeds are seriously altered by irradiation.

This study is relevant to the docket because alfalfa and clover are legumes, and their seeds may be affected in a similar fashion, perhaps proportionately to the amount of fat present in the seed.

## **Conclusions**

### **Irradiation of seeds impairs the nutritional quality of the sprout**

Wheat sprouts grown from seeds irradiated at 1.2 kGray and above are *always* visibly abnormal. This study was done because China's 'no tolerance' policy for wheat fungus made this an urgent and fundable study. Alfalfa seeds or clover seeds (the reason the FDA is considering irradiation of seeds for sprouting), which do not otherwise produce crops for human consumption and studies of irradiation of these seeds have no commercial benefit. Therefore, we have to look at studies of irradiation of other seeds.

In the docket, the FDA bases its approval of seed irradiation solely on the assumption that radiolysis products in the sprout will not be significant. The wheat study shows that disruption of cell division has occurred. The groundnut study shows that the rate of enzyme activity has been altered and the amount of enzyme activity suppressed, correlating to increasing doses of radiation. It is reasonable to assume that enzyme activity in the sprout is also affected. The food is substantially different, as in the well-known equation of irradiated food to cooked food.

### **Sprouts from irradiated seeds are nutritionally different from sprouts of nonirradiated seeds, and the consumer should be informed**

- Wheat sprouts grown from seeds irradiated at 1.2 kGray and above are *always* visibly abnormal, and probably have irradiation-induced enzyme damage.
- Irradiated oilseeds like sunflower have detectable and proportionately increased levels of radiolytic hydrocarbons.
- Legume sprouts from irradiated seeds will show enzyme abnormalities, when they germinate at all.
- The fatty acids of legumes are impaired, causing growth abnormalities in the sprouts.

**Seeds that are sprouted for food are eaten along with the sprout, therefore the sprouts should be labeled**

The sprouts of sunflower seeds, lentils, chickpeas, mung beans and other large seeds are not detached from the seed when eaten; the seed is visibly part of the sprout. The sprouts of small seeds like alfalfa, clover and fenugreek 'use up' the seed (the seed coat remains behind). In both cases—large and small (used up) seed—all the nutrition of the seed is ultimately transferred to the consumer. We direct you to expert opinion from the sprout industry for confirmation. To distinguish between seed and sprout, and claim that "the irradiated article is not what is generally eaten" (Docket, section III.B) is disingenuous.

**Irradiation of seeds for sprouting is not necessary**

The FDA's own document "Guidance for industry: Reducing Microbial Food Safety Hazards for sprouted seeds (October 27, 1999)" states that microbial testing for pathogens "can be done with irrigation water as early as 48 hours into what is generally a 3 to 10 day growing period."

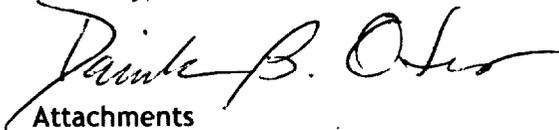
**Request**

The petition and the FDA's ruling is very broad, covering "seeds" in general, even though the public health concerns have centered on only two types of seeds, alfalfa and clover. The ruling perforce includes a variety of seeds for which there is no public health justification for irradiation, lacking any significant history of causing foodborne illness.

We would like to point out that there will be no tracking or monitoring of the use of irradiated seeds. Despite labeling requirements, these seeds could conceivably be planted, or sold to farmers or gardeners here or overseas, thus degrading the food supply. They could also be sold to consumers for home sprouting, without labels. Oilseeds could be used for oil.

We ask that the FDA reverse its present stance and disallow this petition. However, if it chooses to accept the petition despite the public interest, the FDA should use its discretionary power to acknowledge that the irradiated seeds of sprouts are an integral part of the food product sold to the consumer, and that therefore sprouts from irradiated seeds must be labeled like other whole fruits and vegetables.

Sincerely,



**Attachments**

Attachment 1 (Sitton et al., Electron beam irradiation effects on wheat quality, seed vigor, and viability and pathogenicity of teliospores of *Tilletia controversa* and *T. tritici*, Plant Disease 1995; 79:586-589)

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## Electron Beam Irradiation Effects on Wheat Quality, Seed Vigor, and Viability and Pathogenicity of Teliospores of *Tilletia controversa* and *T. tritici*

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### ABSTRACT

Sitton, J. W., Borsa, J., Schultz, T. R., and Maguire, J. D. 1995. Electron beam irradiation effects on wheat quality, seed vigor, and viability and pathogenicity of teliospores of *Tilletia controversa* and *T. tritici*. Plant Dis. 79:586-589.

Wheat seed infested with sori or free teliospores of *Tilletia controversa* and *T. tritici*, respectively, were irradiated with high energy electrons at doses ranging from 0-10.2 kGy to determine the suitability of electron irradiation to disinfect wheat. The germination of spores was then assayed to determine the sensitivity of each smut species to irradiation. Data indicated that *T. controversa* teliospores were somewhat more resistant to electron beam radiation than were teliospores of *T. tritici*. For *T. tritici*, doses of 4.6 and 6.7 kGy completely eliminated germination of free teliospores and teliospores in intact sori. For *T. controversa*, 10.2 kGy was required to completely eliminate germination of both free teliospores and teliospores in sori. Substerilizing doses of radiation delayed germination of the teliospores of both species. There was no significant deleterious effect of irradiation on wheat quality measurements, except for reduced surface texture and water absorption. As expected, irradiation significantly reduced seed germination and seedling vigor. Irradiation dosages above 2.6 kGy on teliospores significantly eliminated *T. tritici* infection of wheat, while irradiation doses of 10.2 kGy on sori reduced infection from 75.5 to 0.08%. No *T. controversa* infection was observed in wheat seed inoculated with irradiated or nonirradiated teliospores.

Dwarf bunt of wheat (*Triticum aestivum* L.), caused by *Tilletia controversa* Kühn in Rabenh., sporadically infects winter wheat (11) in the Pacific Northwest of the United States. The Peoples Republic of China (PRC) has implemented a "zero tolerance" on importation of wheat infested with this fungus (11,23). Difenoconazole (Dividend 3FS, CIBA Corp., Greensboro, NC), the only effective chemical seed treatment (21), has been registered in the United States for control of dwarf bunt in winter wheat, but its ability to totally eliminate dwarf bunt is questionable. Common bunt of wheat, caused by *T. tritici* (Bjerk.) G. Wint. in Rabenh., can be controlled in the United States. There are resistant wheat varieties, effective chemical seed treatments, and crop rotations for control of this disease (11). The effectiveness of Dividend and dwarf bunt resistant wheat cultivars will not solve the export problem to the PRC. The need therefore exists for effective methods to treat grain infested with *T. controversa*, prior to loading into clean ships to satisfy the

demands of the PRC quarantine inspectors.

Chemical control methods that have been tried include use of sodium hypochlorite (8) and gaseous hydrogen peroxide (22), which kills teliospores of *T. controversa* (8). Sodium hypochlorite and hot water (70 C for 5 s) have been used effectively in an experimental treater to decontaminate grain infested with teliospores of *T. controversa* and *T. tritici* (R. P. Cavalieri, unpublished). The disadvantages of these methods are that liquid sodium hypochlorite and gaseous hydrogen peroxide are hazardous to worker health, the chemicals require special methods of disposal or decontamination, and there is an added cost of seed drying following treatment with these solutions.

Schultz and Maguire (19) used irradiation with cobalt-60 gamma rays, at 0-20 kGy, to effectively decontaminate wheat with *T. tritici* and *T. controversa* teliospores. There was no significant reduction of wheat quality with these treatments. Gamma irradiation has been successfully used throughout the world for disinfestation of many foods (7,9, 10,16,20). However, shippers in the Pacific Northwest of the United States are skeptical of using a radioactive source to irradiate *T. controversa*-contaminated grain (T. R. Schultz, unpublished).

Treatment with high-energy electrons from an electron accelerator can be used to decontaminate a variety of food products (4,5,13). This type of irradiator

does not contain any radioactive materials, but instead converts electricity to ionizing energy. An electron beam facility has several advantages over gamma facilities. It is more suitable for on-line treatment of grain, it can be shut off when there is no grain to be treated, it has no long-term storage requirement for a potentially hazardous material, it is more easily transported, there is no perceived waste disposal problem, and it is more acceptable to the public. In this preliminary evaluation, we examined the effectiveness of various doses of electron beam irradiation to decontaminate wheat in which both sori and teliospores of *T. controversa* and *T. tritici* were present, and determined the changes in wheat quality, seed vigor, and teliospore pathogenicity and viability.

### MATERIALS AND METHODS

Wheat heads infested with *T. controversa* were collected from a commercial wheat field in Cavendish, ID, in 1993, and heads infested with *T. tritici* were collected from an experimental plot at Pullman, WA, in 1990. The sori were removed from the wheat heads. Half of the sori of each pathogen were hand crushed by means of a mortar and pestle. Teliospores were sieved through a 150- $\mu$ m screen, prior to use, to eliminate most material other than individual spores. Experiments were carried out in which the spores were irradiated as clean preparations, either as sori or teliospores, and also as contaminants on wheat, to simulate the expected commercial condition.

The teliospores, sori, and wheat cv. Stephens were shipped to AECL Research in Pinawa, Manitoba, Canada, for irradiation. Aliquots (ca. 0.3 g) of teliospores and sori, respectively, were transferred to small, screw-topped glass vials. Samples (ca. 750 g) of contaminated wheat were packaged in plastic bags prior to irradiation. Duplicate samples of teliospores, sori, and wheat seed were exposed to graded doses of 0-10.2 kGy of electron beam radiation in two tests in an AECL 110/1 linear accelerator (Fig. 1) supplying approximately 1 kW of 10 Mev electrons. Irradiation was at an ambient temperature of 22 C throughout the treatment. Following irradiation, the samples were shipped to Washington State University (WSU) for testing wheat quality, seed vigor, and pathogenicity

This investigation was partially supported by a special grant from the WSU International Marketing Program for Agricultural Commodities and Trade (IMPACT) Center, CSS Paper 9409-27, College of Agriculture and Home Economics, Research Center, Washington State University, Pullman

Accepted for publication 12 January 1995.

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VOL

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and viability of *T. controversa* and *T. tritici* teliospores.

The viability of free teliospores and teliospores extracted from intact sori was assessed by suspending ca. 0.01 g of teliospores in 10 ml of sterile water. The spore suspensions were agitated on a vortex-type mixer prior to assay. For *T. controversa*, 0.5-ml aliquots of teliospore suspensions were spread on 2% soil extract agar and incubated in continuous light (95 lux) at 5 C for 1 mo (15). For *T. tritici*, the teliospores were spread on 2% water agar or a laboratory bench (301 lux) at ca. 23 C for 5 days. The percentage of spore germination was determined by examining 6-68 teliospores, for a total of nine microscope fields at 100X on three petri dishes.

Wheat quality was evaluated by analyzing triplicate samples at the USDA-ARS, Western Wheat Quality Laboratory, WSU, for test weight, flour yield, break flour yield, flour ash and protein, milling score, water absorption, surface texture, and cookie diameter (1). These tests were repeated twice.

Germination (3) and cold tests (2,17) were used to test the vigor of electron beam-treated seed. For standard germination, the seeds were placed on saturated germination papers (76 lb., 1,153 cm sq; Anchor Paper Co., St. Paul, MN), rolled into rag dolls, and incubated in the dark for 7 days at 20 C. In cold tests, the seeds were placed on saturated seed germination paper coated with Palouse Silt Loam soil, rolled into rag dolls, and incubated in the dark for 7 days at 5

C, then for 4 days at 20 C. Following incubation, the seeds were visually examined to determine percentage of seed germinated and germination abnormalities, if present. These tests were repeated twice.

Stephens wheat samples (ca. 750 g), sealed in plastic bags, were shipped (January 1994) to AECL Canada for electron beam irradiation (0 and 10.2 kGy) and to Battelle PNL (Richland, WA) for irradiation in a GB650 irradiator at doses of 0, 4, 6, 8, or 10 kGy. The electron beam treatments were repeated twice, while the gamma irradiation treatments were repeated once. On receipt at WSU, Pullman, WA, the seed was stored at ca. 23 C. The seed was planted at Pullman, WA, at 5 g/1.5-m-long row in randomized blocks, covered with 2.5 cm soil on 7 March 1994. There were 15 replications per treatment. Seedling emergence was assessed on 7 April 1994.

The pathogenicities of irradiated *T. controversa* and *T. tritici* were assessed on wheat by transferring eight sori or 0.1 g of free teliospores in three drops of a 0.5% methyl cellulose solution into a sterile 17-ml shell vial. The sori were crushed by means of a sterile glass rod to liberate the teliospores. An additional 27 drops of methyl cellulose solution were transferred to the spore solution, and the mixture was agitated for ca. 15 s on a vortex mixer. To eliminate possible contamination of seed with nonirradiated spores, seeds of wheat cvs. Orin and Lemhi were surface disinfested by

immersion in a 3:1,000 formalin/water solution for 15 min and air dried on absorbent paper in a smut-free greenhouse (14). Six drops of the spore-methyl cellulose suspension were transferred to a sterile vial along with 5 g of surface-disinfested wheat seed. An additional 27 drops of methyl cellulose were transferred to the wheat-teliospore mixture and agitated for ca. 20 s. The seeds (5 g) were planted in 1.5-m-long open furrows, covered with 2.5 cm of soil, 40 cm apart in randomized blocks at Observatory Hill (site 1) and Plant Pathology Farm (site 2) near Pullman, WA, on 27 October 1993 (run 1 on Orin at site 1), 14 November 1993 (irradiation run 2 on Orin at both sites), and 11 March 1994 (irradiation run 2 on Lemhi at both sites). Disease (percentage of heads with smut) was assessed on 9 August 1994 after the wheat was mature. Each replication (five to six) consisted of at least 250 heads per plot.

Teliospore germination, seed vigor tests, wheat quality assessments, and pathogenicity data from test plots were analyzed with SAS-GLM and Fisher's protected least significant difference (18).

## RESULTS AND DISCUSSION

Table 1 presents the effects of electron beam doses on teliospore germination. For *T. tritici*, the germination of teliospores was reduced to undetectable levels at 4.6 kGy for irradiated free spores, and at 6.7 kGy for irradiated sori. *Tilletia controversa* teliospores were resistant to radiation; 10.2 kGy were required to eliminate germination of either free-spores or spores in intact sori. With *T. controversa* teliospores irradiated to a dose of 6.7 kGy, only promycelia (non-infective for wheat) (11) were observed in such spores. Sporidial "H" structures (wheat infective) (11) were observed in samples irradiated at low doses (1.2 and 2.6 kGy) and in nontreated controls. Wheat is most susceptible to infection when it has 2-3 leaves, or 1-3 tillers, under snow cover (12,21). Since a dose of 6.7 kGy retards the rate of sporidial development, it is possible that a dose of 6.7 kGy would be just as effective as 10.2 kGy, because the delay in development would cause the spores to effectively miss the "window of infection" in the field.

There were no significant reductions in test weight, flour yield, break flour yield, flour ash, flour protein, milling score, or cookie diameter. There were significant differences in surface texture and water absorption (Table 2), which should not affect consumer acceptance of electron beam irradiated wheat.

Results of the seed germination and cold tests are presented in Table 3. Irradiation reduced the germination of seeds; even in the lowest dose tested (1.2 kGy) germination was near zero. In the cold test the effect was even more dramatic.

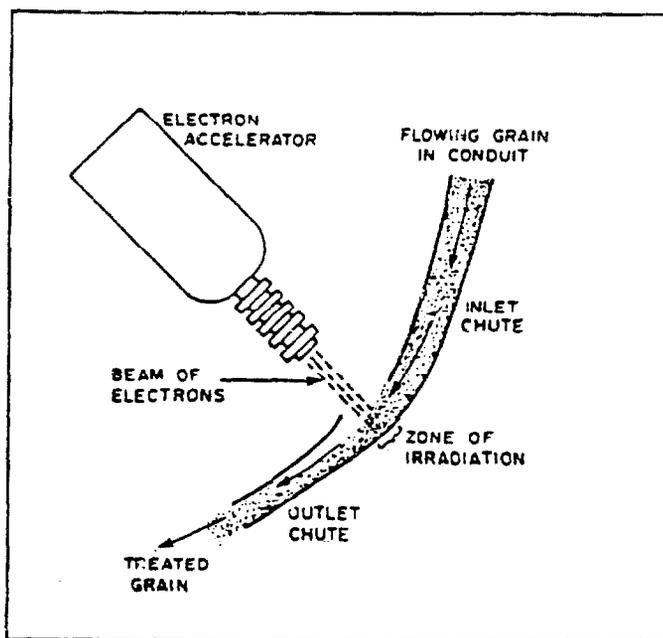


Fig. 1. Key elements of grain irradiation using an electron accelerator.

**Table 1.** Effect of electron beam doses on the germination of free teliospores or teliospores treated in sori of *Tilletia controversa* after 33 days at 5°C on 2% soil extract agar and of *T. tritici* after 5 days at 23°C on 2% water agar

Electron dose kGy <sup>a</sup>	Germination (%) <sup>a</sup>			
	<i>T. tritici</i>		<i>T. controversa</i>	
	Free spores	Spores from sori	Free spores	Spores from sori
0.0	89.5 <sup>c</sup>	97.7	92.8	96.0
1.2	87.5	75.8	88.9	95.0
2.6	12.7	7.3	19.4	16.2
4.6	0.0	7.1	5.4	2.4
6.7	6.0	0.0	1.3	0.8
10.2	0.0	0.0	0.0	0.0
LSD ( <i>P</i> = 0.05)	7.3	5.8	6.2	4.5

<sup>a</sup>Mean number of germinating spores with two irradiation replications in three microscope fields (6-68 teliospores per field).

<sup>b</sup>kiloGray (kGy) = Joules per cubic meter of material treated.

<sup>c</sup>Means within a column were compared with Fisher's protected least significant difference (LSD) (*P* = 0.05).

**Table 2.** Effect of electron beam irradiation on surface texture, water absorption, and cookie diameter

Electron dose kGy <sup>a</sup>	Wheat quality assessments <sup>a</sup>		
	Surface texture	Water absorption	Cookie diameter (cm)
0.0	7.0 <sup>c</sup>	54.1	9.2
1.2	7.0	52.3	9.0
2.6	7.0	52.5	9.2
4.6	7.0	51.3	9.0
6.7	5.7	51.4	8.9
10.2	5.7	51.5	9.0
LSD ( <i>P</i> = 0.05)	0.9	0.6	0.3

<sup>a</sup>Mean of three wheat samples of 200 g per sample.

<sup>b</sup>kiloGray (kGy) = Joules per cubic meter of material treated.

<sup>c</sup>Means within a column were compared with Fisher's protected least significant difference (LSD) (*P* = 0.05).

**Table 3.** Effects of electron beam treatments on germination (normal, weak or abnormal germination [Abn], and no germination [No Germ]) of cv. Stephens wheat<sup>a</sup>

Electron dose (kGy) <sup>a</sup>	Seed germination % <sup>b</sup>					
	Germination test			Cold test		
	Normal	Abn.	No Germ.	Normal	Abn.	No Germ.
0.0	94 <sup>c</sup>	5.3	0.5	88	11.0	1.3
1.2	0	99.0	0.8	0	95.8	4.3
2.6	0	98.0	2.0	0	70.5	27.0
4.6	0	98.8	1.3	0	42.3	57.8
10.2	0	94.0	6.0	0	1.8	98.3
LSD ( <i>P</i> = 0.05)	2	3.5	2.9	2	8.4	6.8

<sup>a</sup>In germination tests with rolled paper towels according to Association of Off. Seed Analyst (2) procedures at 20°C for 7 days and cold tests rolled paper coated with 28.3 g of saturated loam soil at 5°C for 7 days, then 20°C for 4 days.

<sup>b</sup>Mean percentage of germinating seeds representing 400 seeds (100 from each of four electron beam replications).

<sup>c</sup>kiloGray (kGy) = Joules per cubic meter of material treated.

<sup>d</sup>Means within a column were compared with Fisher's protected least significant difference (LSD) (*P* = 0.05).

In the standard germination test, the 10.2 kGy treatment showed 94% germination (all but weak or abnormal) and 6% with no germination. In the cold test, the same treatment had only 1.8% germination (all weak or abnormal) and 98.3% no germination.

No wheat irradiated with 4 kGy and above emerged in test plots at Pullman, WA (Table 4).

None of the plants from seed inoculated (0-10.2 kGy) with *T. controversa* teliospores (free-teliospores or sori) at two locations became infected with dwarf

**Table 4.** Emergence of cv. Stephens wheat seed irradiated with electron beams and gamma rays (Co60) planted in 1.5-m rows, 2.5 cm deep in Pullman, WA, on 7 March 1994

Dose (kGy) <sup>b</sup>	Emergence (no. coleoptile per row) <sup>a</sup>	
	Electron beams	Gamma rays
0	14.7 <sup>c</sup>	19.3
4	...	0
6	...	0
8	0	0
10	...	0
LSD ( <i>P</i> = 0.05)	3.0	4.1

<sup>a</sup>Mean number of coleoptiles emerging from 5 g of seed per row in each of 15 replications determined on 7 April 1994.

<sup>b</sup>kiloGray (kGy) = Joules per cubic meter of material treated.

<sup>c</sup>Means within a column were compared with Fisher's protected least significant difference (LSD) (*P* = 0.05).

bunt (Table 5). This agrees with previous work (11) that showed that dwarf bunt infection from seedborne teliospores is rare. Irradiated free-teliospores and sori of *T. tritici* lost pathogenicity in doses above 4.3 kGy, with the exception of one test in which a trace amount (0.08%) of common bunt occurred at 10.2 kGy. This trace amount of common smut may be due to natural, soilborne infection from inoculum from previous tests at this site. It is unlikely to represent trace survivors of the radiation process since there was no infection at 4.6 kGy. We believe that this minute level of infection is insignificant.

In summary, electron beam treatments of wheat seed infested with *T. controversa* and *T. tritici* were effective in eliminating the viability and pathogenicity of spores of both species. In addition, low doses (4 kGy) of electron beam irradiation prevented emergence, hence disease development, of wheat seed potentially infected with bunt fungi. At the required doses, there was only minimal reduction in wheat quality using a variety of indicators. The process itself is flexible, cost effective (6), and suitable for use in export terminals as a decontamination method for grain being loaded into ships. Based on these findings and considerations, we conclude that irradiation has the potential of solving the dwarf bunt problem in wheat designated for export to the PRC, and elsewhere where these microorganisms may be a problem.

#### ACKNOWLEDGMENTS

The authors would like to thank Lisa M. Lucht, AECL Whiteshell Laboratories, Pinawa, Manitoba, Canada, for technical assistance in treating the samples with electron beams; H. Jeffers and C. Morris of the USDA-ARS, WWQL, for assistance with the wheat quality assessments; J. T. Waldher for assistance in assessing common bunt severity in the test plots; and J. D. Maguire for financial support and use of the Seed Technology Laboratory facilities.

**Table 5.** Pathogenicity of free teliospores and teliospores in intact sori of *Tilletia tritici* treated with 0–10.2 kGy doses of electron beams<sup>a</sup>

Cultivar and dose (kGy) <sup>b</sup>	Infected heads (%) <sup>b</sup>			
	Site 1		Site 2	
	Free TS	TS in sori	Free TS	TS in sori
Lemhi				
0.0	98.4 <sup>d</sup>	94.4	95.0	90.5
4.3	0.0	0.3	0.0	0.0
7.5	0.0	0.0	0.0	0.0
9.6	0.0	0.0	0.0	0.0
10.2	0.0	0.0	0.0	0.0
LSD ( <i>P</i> = 0.05)	0.5	2.8	1.0	2.2
Orin				
0.0	80.8	75.5		
1.2	69.2	66.7		
2.6	3.2	0.6		
4.6	0.0	0.0		
6.7	0.0	0.1		
10.2	0.0	0.1		
LSD ( <i>P</i> = 0.05)	14.1	10.7		

<sup>a</sup>Cultivars Orin and Lemhi wheat (*Triticum aestivum*) were inoculated with treated teliospores and planted at two sites near Pullman, WA on 27 October 1993 (Orin) and 11 March 1994 (Lemhi). The severity of wheat infection (% infected heads) was determined on 8 August 1994.

<sup>b</sup>Mean percentage of heads with common bunt assessed on a minimum of 250 heads per plot in each of five replications at the Plant Pathology Farm and six replications at Observatory Hill.

<sup>c</sup>kiloGray (kGy) = Joules per cubic meter of material treated.

<sup>d</sup>Means within a cultivar and column were compared with Fisher's protected least significant difference (LSD) (*P* = 0.05).

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2

## Detection of Hydrocarbons in Irradiated and Roasted Sesame Seeds

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**ABSTRACT:** Hydrocarbons produced by  $\gamma$ -radiation of sesame seed were analyzed to determine the relation of irradiation dose to the production of hydrocarbons and to eventually use them as markers for identifying post-irradiated sesame seed. Hydrocarbons in sesame seed were determined by a sequential procedure of lipid extraction by hexane, Florisil column chromatography, and gas chromatography. 16:2, 16:3, 17:1, and 17:2 were prominently detected in irradiated sesame seed. 17:2 was detected in seed irradiated at 0.1 kGy or higher, and the others were detected at 0.5 kGy or higher. These hydrocarbons were not detected in unirradiated sesame seeds that were raw, roasted, whole, ground, or stored. *J. Agric. Food Chem.* 45, 469-472 (1997).

**KEY WORDS:** Detection of post-irradiation, Florisil column chromatography, gas chromatography, hydrocarbon(s), irradiation, sesame oil, sesame seed.

Sesame seed is one of the most important condiments for Korean foods. In Korea, a large amount of sesame seed is imported and may become infested with insects during shipping. Sesame seed is not permitted to be irradiated in any country except Cuba, which permits sesame seed to be irradiated up to 2 kGy for disinfestation (1). Sesame seed belongs to the spices and seasonings, for which irradiation is permitted to control insects, with doses ranging from 1 to 5 kGy in several countries (1). Although irradiated foods must be properly labeled, there is the possibility of irradiating them without any notification on the shipment. It is, therefore, necessary to develop an appropriate method to detect irradiation of imported sesame seed.

Since Nawar's group (2-4) reported that some hydrocarbons are exclusively produced by  $\gamma$ -radiation of lipids and lipid-containing foods, hydrocarbons have been extensively studied as a marker to detect irradiation of foods (5-9). Two types of hydrocarbons are predominantly produced by irradiation of fatty acids in lipids: one has one carbon less than the parent fatty acid ( $n-1$ ), and the other has two carbons less and an additional double bond at position 1 ( $n-2$ , 1-ene) (10). Many analyses of hydrocarbons have concentrated on meats,

although other foods have recently been included (9,11). A variety of methods to detect the radiation-induced hydrocarbons has been developed, including separation of the lipid fraction from foods, separation of the hydrocarbons from lipids, and gas chromatographic analysis of the hydrocarbons in accordance with food types and lipid composition. The separation of hydrocarbons from lipids is considered to be the most critical step in detecting hydrocarbons. Hydrocarbons have been separated from lipid fractions by cold-finger distillation (6), column chromatography (5,7,8,10), or high-performance liquid chromatography (12-14). Schreiber's group compared high-vacuum cold-finger distillation and Florisil column chromatography and concluded that the latter seemed to be more practical for routine application to meats (10,15). They also applied the Florisil chromatography method to some fresh fruits and obtained satisfactory results (11,16).

The objective of the present study is to determine whether a sequential procedure of lipid extraction by hexane, Florisil column chromatography, and gas chromatography (GC) is suitable for (i) detecting the hydrocarbons that are exclusively produced by  $\gamma$ -radiation of sesame seeds, (ii) examining how dose relates to the production of hydrocarbons in sesame seeds, and (iii) whether determining the hydrocarbons can be linked to irradiation of sesame seeds.

### MATERIALS AND METHODS

**Materials and reagents.** Sesame seeds were purchased from a farmer in Kimje, Korea. Sodium sulfate was of analytical grade (Pure Chemicals Co., Ltd., Osaka, Japan). *n*-Hexane was from J.T. Baker Inc. (Phillipsburg, NJ). The hydrocarbon standards [*n*-octane (8:0), *n*-nonane (9:0), *n*-decane (10:0), *n*-dodecane (12:0), *n*-tridecane (13:0), *n*-tetradecane (14:0), *n*-pentadecane (15:0), *n*-hexadecane (16:0), *n*-heptadecane (17:0), *n*-octadecane (18:0), *n*-nonadecane (19:0), *n*-eicosane (20:0), *n*-heneicosane (21:0), and *n*-docosane (22:0)] were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Hexadecene (16:1) and 1-tetradecene (14:1) were also from Sigma Chemical Co. Oleic and linoleic acids were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

**Sample preparation and irradiation.** Sesame seeds were irradiated at 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 kGy with a com-

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mercial  $^{60}\text{Co}$  source (Greenpia Technology Inc., Yaju, Korea). Oleic and linoleic acids, which are the predominant fatty acids in sesame oil, were irradiated at 41 kGy. Some irradiated sesame seeds were roasted for 10 min in an electric frying pan (temperature setting: 400°F; Sunbeam Appliance Co., Oak Brook, IL). A part of the roasted seeds was ground in a mortar. Untreated sesame seeds were also included as the control. All samples were kept at  $-40^{\circ}\text{C}$  until the subsequent fat extraction.

**Fat extraction.** Fat extraction, separation of hydrocarbons, and GC analysis followed Schreiber's group's method (10,11) with minor modification. Three grams of sample were ground in a mortar with 3 g of anhydrous sodium sulfate (previously heated at  $650^{\circ}\text{C}$  for 5 h). After that, 100 mL *n*-hexane was added, and the content was homogenized thoroughly with a homogenizer (Nissei AM-3; Nihonseiki Kaisha, Ltd., Tokyo, Japan) for 2 min. The mixture was transferred to Teflon centrifuge tubes (Nalge Co., Rochester, NY) and centrifuged at 3400 rpm for 20 min in a VS 5500 centrifuge (Vision Scientific Co., Ltd., Seoul, Korea). The supernatant was collected in a round-bottomed flask. The solvent was evaporated under a nitrogen stream. The extracted fat was stored at  $4^{\circ}\text{C}$  until subsequent Florisil column chromatography.

**Separation of hydrocarbons by Florisil column chromatography.** Florisil (60–100 mesh, F100-3; Fisher Scientific, Fairlawn, NJ) was heated at  $550^{\circ}\text{C}$  overnight. Just before packing the column, it was heated again to  $150^{\circ}\text{C}$  for 5 h and cooled down at room temperature. It was then deactivated by the addition of 3% distilled water. A glass column (2.3 cm i.d.) with a Teflon stopcock was rinsed with hexane and filled with 20 g Florisil. One gram of fat sample, mixed with 1 mL of hexane that contained  $2\ \mu\text{g/mL}$  *n*-eicosane as internal standard, was applied to the column, followed by 60 mL hexane to elute at 3 mL/min. The eluate was concentrated to a volume of about 3 mL under a nitrogen stream. The concentrated sample was filtered through a Nylon membrane (13 mm, 0.2  $\mu\text{m}$ ; Whatman International Ltd., Maidstone, England), contained in a 13-mm syringe holder (Nucleopore Corp., Pleasanton, CA), which was connected to a 10-mL Luer-lock syringe (Popper & Sons, Inc., New Hyde Park, NY). The filtrate was concentrated to 0.6 mL under nitrogen into a GC vial. Hydrocarbons from the oleic and linoleic acids, unirradiated or irradiated, were also separated in the same way.

**GC analysis of hydrocarbons.** The isolated hydrocarbons were analyzed on a Hewlett-Packard 5890 series II gas chromatograph (Avondale, PA), equipped with a flame-ionization detector and a split injector. Helium was used as the carrier gas. The column was 0.25 mm i.d.  $\times$  30 m with 0.25  $\mu\text{m}$  film thickness (DB-5; J&W Scientific, Folsom, CA). The initial column temperature was  $50^{\circ}\text{C}$  for 2 min, then programmed at  $10^{\circ}\text{C}/\text{min}$  to  $130^{\circ}\text{C}$  and  $5^{\circ}\text{C}/\text{min}$  to  $200^{\circ}\text{C}$ , where it was held for 2 min, then  $25^{\circ}\text{C}/\text{min}$  to  $250^{\circ}\text{C}$  with a final hold for 5 min. The injector and detector temperatures were 200 and  $250^{\circ}\text{C}$ , respectively. One  $\mu\text{L}$  of sample was injected. All experiments were in duplicate unless otherwise stated.

## RESULTS AND DISCUSSION

**Irradiated oleic and linoleic acids.** Palmitic, stearic, and linoleic acids are the principal fatty acids in sesame oil. Oleic and linoleic acids comprise 33–47% and 33–48% of total fatty acids in sesame oil, respectively (17). The predominant radiation-induced hydrocarbons could therefore be predicted as 8-heptadecene (17:1) and 1,7-hexadecadiene from oleic acid and 6,9-heptadecadiene (17:2) and hexadecatriene (16:3) from linoleic acid. Oleic and linoleic acids were irradiated at a fairly high dose (41 kGy) in this study to induce large amounts of the expected hydrocarbons and to identify the GC retention times of the predominant hydrocarbons from sesame seeds because these irradiated hydrocarbon standards were not commercially available. The GC chromatogram of the hydrocarbon extracted from the irradiated oleic acid showed two large peaks; one prior to the standard hydrocarbon 16:1 and the other after 17:0, suggesting they should be 16:2 and 17:1, respectively. The GC chromatogram from the irradiated linoleic acid showed two large peaks; they should be 16:3 and 17:2 hydrocarbons from the irradiated fatty acids were also confirmed by GC/mass spectrometry at Korea Ginseng and Tobacco Research Institute (Daejeon, Korea).

**Irradiated sesame seed.** Sesame seed that was irradiated even at 10 kGy was hardly distinguishable from unirradiated seed by appearance or flavor. The sesame seed in this study contained 48.6% fat and 4.5% moisture; therefore, less sample was needed than that for meats or fruits. Subsequently, less sodium sulfate was used to remove moisture.

No unsaturated hydrocarbons were detected in the hydrocarbons extracted from unirradiated sesame seed (Fig. 1). It has been reported that hydrocarbons 16:1, 16:2, 16:3, 17:1, and 17:2 were detected in unirradiated oils from peanut, sunflower, extra-virgin olive oils and that small amounts of 17:1 and alkenes were naturally present in avocado-peanut oils, which made quantitative analysis difficult (9). No predominant radiation-induced hydrocarbons were detected in sesame seed irradiated at 0.05 kGy (Table 1). Hydrocarbon 17:2 was first detected in the sample at 0.1 kGy. The major radiation-induced hydrocarbons, 16:2, 16:3, 17:1, and 17:2, were detected in the sample after 0.5 kGy. Hydrocarbons 16:2, 16:1 (from oleic acid), 16:3, 17:1, 17:2, and an unidentified peak (probably 17:3 from linolenic acid) were detected in the samples irradiated at 1.0 kGy or higher. The amount of hydrocarbons increased with the dose. The prominent hydrocarbons produced by  $\lambda$ -radiation of sesame seed were 16:2, 16:3, 17:1, and among which 17:2 was the most abundant (Fig. 1). The amount of these radiation-induced hydrocarbons increased most linearly with the irradiation dose; correlation coefficients were over 0.95 for the four hydrocarbons. The ratios of hydrocarbons *n*-2,1-ene (16:2 and 16:3) to *n*-1 (17:1) and in sesame seed irradiated at 5 and 10 kGy were about 0.5.

**Roasted and ground sesame seed.** The hydrocarbons detected in irradiated sesame seed should not be produced by other treatments if they are to be used as irradiation ma-

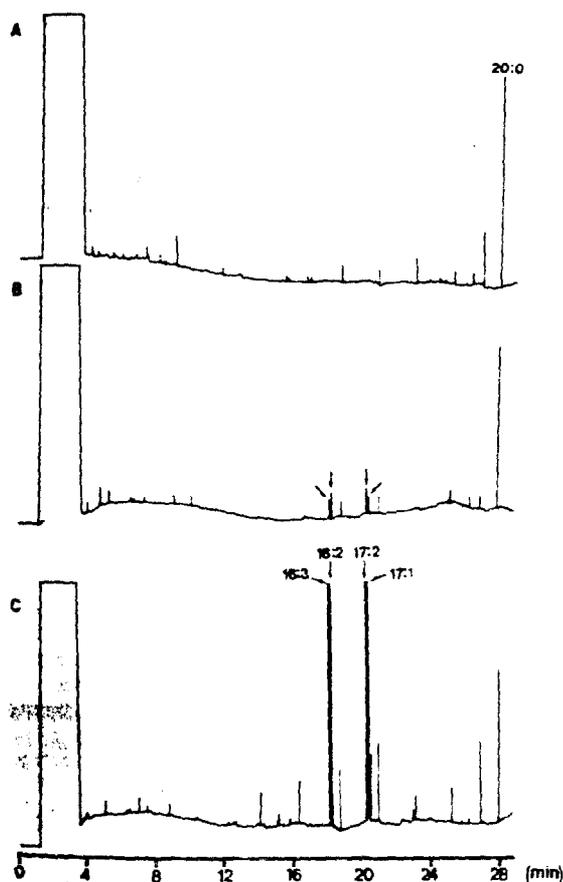


FIG. 1. Chromatograms of the hydrocarbons isolated from unirradiated and irradiated sesame seeds; (A) unirradiated; (B) 0.5 kGy; (C) 10 kGy.

Because sesame seed might be exposed to severe heat during storage, the hydrocarbons were analyzed in sesame seed after storage at 37°C for 3 mon. Some sesame seed was stored in an extreme condition, and part was ground and

stored for 3 mon in air. It was reported that peanut oil characteristically developed 13:1 and 14:0 by heating, as well as 14:2 exclusively by heating in the presence of oxygen (9). The untreated sample stored for 3 mon did not develop any hydrocarbons inducible by irradiation: some hydrocarbons, such as 14:0, 16:0, and 17:0, which were present in the initial sample, were not detected in the stored sample (Table 2). Roasted sesame seed showed few differences in the types of hydrocarbons from the control, with an increase in some saturated hydrocarbons. After storage of the roasted samples, the seeds lost some hydrocarbons. The saturated hydrocarbons in roasted and ground sesame seed increased significantly during the 3-mon storage. However, no unirradiated sesame seed, regardless of the treatment, contained any irradiation-inducible hydrocarbons.

In conclusion, detection of the prominent radiolytic hydrocarbons, such as 16:2, 16:3, 17:1, and 17:2, in irradiated sesame seed may make it possible to identify whether sesame seed was previously irradiated at 0.5 kGy or higher. Irradiation dose for sesame seed would typically range from 1 to 5 kGy because the purpose of irradiation is mainly to kill insects. Therefore, detection of hydrocarbons can be an applicable method to identify post-irradiation of sesame seed.

ACKNOWLEDGMENTS

This paper was supported by the Nondirected Research Fund of the Korea Research Foundation. We also thank Greenpia Technology Inc., Yozu, Korea, for generously allowing us to use the radiation source.

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Hydrocarbons in Irradiated Sesame Seed ( $\mu\text{g/g oil}$ )<sup>a</sup>

Hydrocarbon	Dose (kGy)						
	0	0.05	0.1	0.5	1.0	5.0	10.0
14:1	—	—	—	—	—	—	0.61 (0.12)
14:2	0.07 (0.12)	0.18 (0.10)	0.19 (0.15)	—	—	—	0.17 (0.10)
15:0	—	0.41 (0.31)	0.31 (0.11)	—	0.13 (0.02)	0.39 (0.00)	0.98 (0.39)
16:0	—	—	—	0.23 (0.07)	0.53 (0.01)	2.65 (0.00)	6.55 (0.01)
16:1	—	—	—	0.30 (0.08)	0.62 (0.01)	2.98 (0.02)	7.07 (0.46)
16:2	—	—	—	—	0.11 (0.00)	0.35 (0.00)	0.92 (0.29)
16:3	0.14 (0.04)	0.45 (0.14)	0.29 (0.04)	0.22(0.02)	0.15 (0.00)	0.17 (0.00)	0.29 (0.07)
17:0	—	—	—	—	0.14 (0.08)	0.53 (0.00)	1.41 (0.07)
17:1	—	—	0.16 (0.72)	0.38 (0.06)	0.55 (0.03)	3.67 (0.00)	8.25 (0.65)
17:2	—	—	—	0.26 (0.05)	0.42 (0.04)	3.17 (0.01)	7.59 (0.82)
18:0	0.09 (0.00)	0.26 (0.05)	0.36 (0.06)	0.23 (0.05)	0.31 (0.00)	0.44 (0.03)	1.27 (0.26)
18:1	0.18 (0.05)	0.31 (0.12)	0.60 (0.39)	0.24 (0.06)	0.13 (0.01)	0.27 (0.01)	0.28 (0.01)
18:2	0.12 (0.05)	0.22 (0.01)	0.22 (0.04)	0.53 (0.20)	0.16 (0.02)	0.31 (0.02)	0.44 (0.02)

<sup>a</sup>Standard deviation of duplicate samples.

**TABLE 2**  
Hydrocarbons in Raw, Roasted, and Ground Unirradiated Sesame Seed ( $\mu\text{g/g oil}$ )<sup>a</sup>

Hydrocarbons	Raw		Roasted		Roasted and ground	
	0 d	37°C; 3 mon	0 d	20°C; 3 mon	0 d	20°C; 3 mon
14:1	—	—	—	—	—	—
14:0	0.07 (0.12)	—	—	—	—	—
15:0	—	—	—	—	—	—
16:3	—	—	—	—	—	—
16:2	—	—	—	—	—	—
16:1	—	—	—	—	—	—
16:0	0.15 (0.00)	—	0.29 (0.07)	—	0.39 (0.00)	0.62 (0.14)
17:3 (?)	—	—	—	—	—	—
17:2	—	—	—	—	—	—
17:1	—	—	—	—	—	—
17:0	0.09 (0.00)	—	0.25 (0.07)	—	0.15 (0.04)	0.93 (0.00)
18:0	0.18 (0.05)	0.18 (0.15)	0.39 (0.05)	0.12 (0.02)	0.27 (0.14)	1.31 (0.29)
19:0	0.12 (0.05)	0.13 (0.32)	0.24 (0.04)	0.09 (0.03)	0.21 (0.01)	0.30 (0.05)

<sup>a</sup>Mean (standard deviation) of duplicate samples.

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[Received July 6, 1996; accepted December 12, 1996]

# Behaviour of Lipase Activity of the Gamma-Irradiated Groundnut During Germination

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## Abstract

Lipase activity of gamma-irradiated groundnut has been reported. The dosage levels: 10, 30, 50, 70, 90 and 120 Kilorontgen (kr) units have different effects on lipase. Radiation levels of 50 kr and above induced damage to the active centers. Lipase activity was found to decrease during the initial stages of germination, and to increase in later stages. After reaching maximum value, the activity decreased. The lipase of control seeds doubled during germination in light. Increase in irradiated seeds was about 1.5-fold. Maximum activity for seeds treated under different conditions was attained at different periods of germination. Plant growth and the behaviour of lipase has been explained on the basis of other metabolic factors such as: ascorbic acid oxidase, amino acids and free fatty acids liberated during germination, and their mutual effects.

## Introduction

LIPASE ACTIVITY plays a major role in liberation of fatty acids from the glycerides (1). It has been further shown that the lipase activity of oil-bearing seeds increases during the course of germination. Wetter (2) for example, has noted that rapeseed possessing very little activity in dormant condition, showed a remarkable increase (100-fold) in lipase activity after 3 days of germination. The activity was found to decrease later in the germination period. Similar observations have been made by Shabetai and Kamal (3), working with cotton seeds. They found that the enzyme activity increased gradually up to the fourth day of germination and, thereafter, it diminished gradually. Torzhinskaya (4) has observed that the lipase activity of cornseed increased during germination, with a corresponding decrease in percent of fat. The lipase activity of oats reached its maximum value within a short period (8 hr) of germination and then it suddenly decreased (5). In case of groundnut, Ramakrishnan (6) has pointed out that there was a quantitative increase in the lipase activity up to the third stage (appearance of 3 leaves) of germination, and then a decrease.

Thus it can be perceived that lipase activity increases gradually during germination in certain oil-bearing seeds. In some seeds it increases suddenly in the initial stages of germination then drops off rapidly. Little information is available on the effect of ionizing radiation on the lipase activity of oil-bearing seeds. The present investigation is part of a program for study of the changes in the glyceride composition of the fat, of the lipase, amylase, ascorbase activities, and of vitamin C, free amino acids and carbohydrate contents during germination of groundnut seeds which are irradiated to different dosage levels of  $\gamma$ -radiation.

## Experimental

### Irradiation and Germination of Groundnut (*Arachis Hypogaea* L.) Seeds

Seeds utilized for the present investigation were of AK-12/24 M.P. variety obtained from Junagarh Research Farm (Gujarat State, India). Gamma radiation was applied from a  $\text{Co}^{60}$ -source located at the Atomic Energy Establishment—Trombay, Bombay (India). Dosages of 10, 30, 50, 70, 90 and 120 kr units were used in the gamma radiation experiment. Control and irradiated seeds were weighed individually and planted in chemically purified and sterilized sand. Germination was carried out under laboratory environment at constant temp of  $25 \pm 1^\circ\text{C}$ . Distilled water was added daily in a measured quantity to the germinating seedlings. In order to study changes in lipase activity by light, control seeds were also planted in darkness. The periods of germination selected for the present work were: 0, 1, 3, 6, 12, 20 and 30 days. No nutrients were added during germination. At the end of each period, some of the germinating seedlings were removed from the sand, cleaned with cold distilled water and used for the extraction of the enzymic activity.

### Preparation of Enzyme

The cleaned seedlings were crushed with cold acetone in a mortar and pestle for about 10 min. The homogenates were filtered in the cold and washed with cold acetone until free of oil. The filtrate was utilized for the study of fat content and its glyceride composition. The residual, finely crushed plant materials were dried in a vacuum desiccator, weighed, and stored cold until ready for assay. Ungerminated groundnut cakes were prepared by the same method. It was observed that extraction with ether was unsuitable, as a prolonged period of extraction with ether diminishes the enzyme activity. Hence acetone, because of its dehydrating property, was used for extraction in the present work.

### Method

The method adopted for the estimation of the lipase activity in the present work is essentially the same as that described by Wetter (2).

Commercial groundnut oil was used as a source of glyceride. For assay purpose, 2 ml of 22%  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  followed by 10 ml of  $\text{NH}_4\text{Cl} \cdot \text{NH}_4\text{OH}$  buffer (pH = 8.7), were added to approx 0.5 g oil in a beaker (7,8). The contents of the beaker were stirred for about 5 min in order to have thorough mixing. Then 0.100 g of the test material was added with vigorous stirring and the time was recorded as zero. Hydrolysis was carried out at  $25^\circ\text{C}$  for 1 hr. The pH was maintained at 8.7 by the addition of 0.1 N NaOH from a microburette with mild stirring. Lipase activity has been expressed in terms of ml of 0.1 N NaOH con-

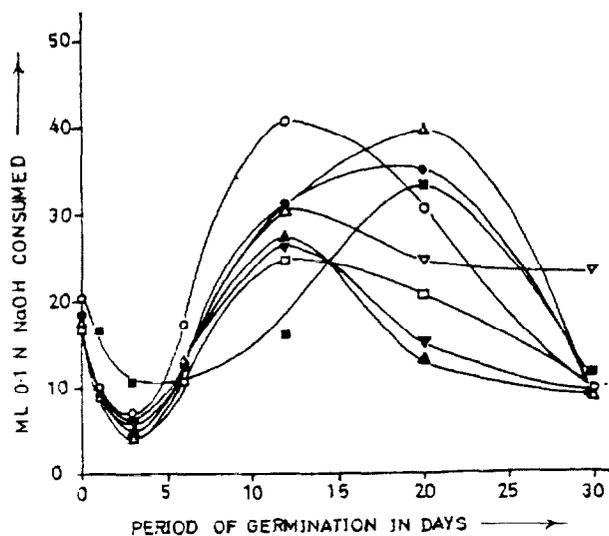


Fig. 1. The behaviour of lipase activity with the period of germination. Radiation doses: ■ dark; ○ control; ● 10 Kr.; △ 30 Kr.; ▽ 50 Kr.; ▲ 70 Kr.; ▼ 90 Kr.; □ 120 Kr.

sumed. From this and the weight of the total sample the activity per gram of the original seed was calculated.

#### Results

Seeds planted in dark grew faster than those grown in light. Irradiated seeds showed a comparatively poor rate of germination with increase in radiation dose as compared to control seeds grown in light. The increase in lipase activity of the seeds grown in the dark was poor in comparison to that of seeds grown in light. Results of the determinations of lipase activity are shown graphically in Figure 1 and Figure 2. Figure 1 indicates the change in activity with period of germination, while Figure 2 shows the variation of activity with different dosage level of  $\gamma$ -radiation. It should be noted (Fig. 1) that the activity present in both irradiated and nonirradiated seeds decreased in the initial stages of germination. This decrease in activity

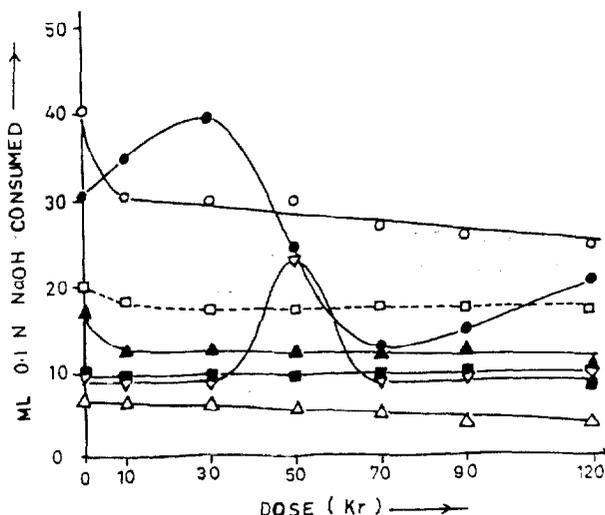


Fig. 2. The behaviour of lipase activity with various gamma doses: □ 0-day; ■ 1-day; △ 3-days; ▲ 6-days; ○ 12-days; ● 20-days; 30-days.

was observed up to the third day of germination, after there was an increase in lipase, with maximum being reached (Table I) at different periods of germination for the several treatments.

The activity of the control seeds attained maximum value on the 12th day of germination and was higher than that obtained for irradiated seeds germinated the same period. Further, the seeds irradiated to 30 units, showed maximum lipase content on 16th day of germination, but variation in activity between the 10 and 20-day periods is not great. Other irradiated seeds, except for 30 kr dosage level, attained maximum activity on the 12th day. Seeds grown in dark and irradiated at 30 kr units reach maximum lipase activity on 20th day of germination. The lipase activity maxima observed on 12-days germination has the following trend: control seeds > 10 kr seeds > 30 kr seeds > 70 kr seeds > 90 kr seeds > 120 kr seeds. Maximum activity of the seeds 20-day germination irradiated to 30 kr dosage level was greater than that of seeds grown in darkness.

From Figure 2 it may be noted that before germination the lipase activity of control seeds is slightly higher than that of irradiated seeds, and that it is greatly affected by the increase in dosage levels. This is also true for the 1-, 3- and 6-day periods. In case of 12-day germination, the activity of the control seeds is higher than that of irradiated seeds and there is a regular decrease in lipase with dosage level. It is noted that the lipase of all the seeds on 12th day of germination > that of zero day > that of sixth day > that of one day > that of third day of germination. This type of regularity in behaviour is not marked with the germination period of 20 and 30 days. In both these cases lipase attained maximum value for a definite dosage level (30 and 50 kr units, respectively) and thereafter there was a decrease in the activity.

#### Discussion

The present studies suggest that the lipase activity of dormant seeds is definitely affected by radiation. This might be ascribed to the fact that the radiation interferes with the functions of the mitochondria (9). The effect of radiation becomes more pronounced during the period of germination. This is visualized from the rate of production of lipase.

The activity in control as well as in irradiated seeds decreases to a certain level during the early stages of germination, in contradiction to the observation made by a previous investigator (6), who has shown that there was a gradual increase in the lipase on germination of groundnut. This is explained by the fact that the previous worker selected the stages of germination in terms of physiological aspects, i.e., the appearance of number of leaves. According to him the appearance of 2-leaves is the second stage of germination. In the present investigation the leaves appear after 3-day germination, and at that stage the activity is gradually found to increase. Thus the previous worker seems to have missed the observation on behaviour at intermediate stages. In cotton seeds similar decreases in activity have also been observed by other investigators (3). This decrease in activity in the initial stages of germination might be explained as follows: In the initial stages of germination cell division does not occur; hence the number of mitochondria may remain the same. Other chemical processes may lead to the increase in weight of the cake but not in activity. Hence, due to the increase in weight of the cake, the activity is found to decrease proportionately.

On the third day of germination the lipase once increases in all the cases. The rate of increase of lipase of seeds grown in dark is low in comparison with that of all other seeds. Of course the growth of the seeds planted in dark is faster than that of other seeds grown in light. This behaviour is in agreement with the observation made by Wetter (2). The faster production of lipase in seedlings grown in the light may result from the more active metabolic state of the plant grown in light. This suggests that growth might not be the only factor affecting the activity.

The rate of increase in activity for the seeds grown in light is different and is definitely affected by the irradiation. This is illustrated by the rates at which control and irradiated seeds attain maximum value. In case of control seeds, the maximum activity is attained on the 12th day of germination, in light. This is also the case with the dosage levels of 50, 70, 90 and 120 kr units. However, it may be noted that the activity of control seeds at maximum is higher than that of irradiated seeds under the same conditions. This may be interpreted by assuming that the active centers of control seeds, whatever number initially present, are undisturbed and can show a characteristic increase in the rate, while in case of  $\gamma$ -irradiated seeds there may have occurred some sort of radiation damage to the active centers. This view is supported by the work of Meisel (9), who observed that irradiation damaged the nucleus and interfered with the functions of the protoplasmic structure, including the mitochondria, which responded differently to irradiation.

Further, in case of 10 and 30 kr dosage levels, the lipase activity reaches its maximum value on 16th and 20th day of germination, respectively. The value of the lipase of control seeds and that of seeds irradiated to 30 kr unit, at their respective maxima, is practically the same. There is a little difference in the value of activity in case of seeds exposed to 10 kr unit. Here it may be assumed that the radiation-damage of the active centers was slight and that as germination proceeded the interfering effects of irradiation on mitochondrial activity became less pronounced. This could explain why there was a time lapse in attaining maximum value of lipase in the above-mentioned seeds. The activity after reaching maximum value diminished gradually in all the treatments. This is in agreement with the observations made by the previous workers (2,3,6) on different oil-bearing seeds.

The behaviour of the lipase with radiation for a given period of germination is indicated in Figure 2, which shows that the activity of control seeds germinated in light increases by 2-fold over that of dormant seeds. For irradiated seeds the increase is about 1.5-fold.

As said before, plant growth might not be the only criteria for the increase of lipase, and vice versa. In general the growth of irradiated seeds was poor (only epicotyls are developed), and seeds irradiated to higher dosage levels, i.e., 70 kr unit and above did not grow at all. Even in that case lipase activity increased. This might be interpreted as follows: in the initial stages of germination certain amount of activity may be needed by the seedling for normal growth and other metabolic features. If this is not available the growth may be inhibited. Since lipase is affected by irradiation, as mentioned previously, the growth of irradiated seeds is poor. Further, with the increase in period of germination, other metabolic factors play a part in producing the lipase activity. Thus lipase activity of the irradiated seedlings might increase but not at the

TABLE I  
Lipase Activity of Groundnut\*

Kr units, irradiation.....	0		10		50		70		90		120	
	(Dark)		(Light)									
Period <sup>b</sup> .....	20	12	16	20	12	12	12	12	12	12	12	12

\* Except for "0" (Dark) irradiation treatment, all seeds were grown in optimum light condition.

<sup>b</sup> Period of germination in days for maximum activity.

usual time. Hence this increase in lipase at the later stages is ineffective for promoting the growth of seedling. Thus it may be that over and above plant growth, other metabolic factors such as: ascorbic acid (10), ascorbic acid oxidase (AAO), amylase, carbohydrates and free amino acids liberated during germination are also operative. They may also play a role in the production of lipase activity. Amino acids increase the stability of the enzyme (11). It has been observed that there was an increase in free amino acids during germination. Other factors also show corresponding increases (13) and reach maximum values after different periods of germination of these seeds. Thus it is assumed that these factors have their mutual effect on each other and are responsible for the observed increase in lipase activity of irradiated seeds.

The abnormal behaviour of lipase observed in case of seeds irradiated to 30 and 50 kr units, is explained on the following ground. The AAO and free fatty acids (ffa) of these irradiated seeds at their specific period of germination are found to attain maximum values. Further the increase in AAO is ultimately responsible for the corresponding increase in dehydroascorbic acid. It has been mentioned by Guida (12) that dehydroascorbic acid considerably raised the lipase activity. Thus the increase in AAO and ffa of the above-mentioned seeds may be responsible for abnormal increase in lipase activity.

Hence, in general, the assumption regarding the mutual effects of all the factors mentioned above is strengthened by the observations made in the present investigation. Further work for confirmation of the above views on the behaviour of lipase activity of other oil-bearing seeds having various degree of unsaturation is in progress.

#### ACKNOWLEDGMENTS

Prof. R. D. Patel provided facilities and useful suggestions; the authorities of the Atomic Energy Establishment-Trombay, irradiation. The data on irradiation were obtained at the AEET-Bombay, India, with the gamma-ray and sterilization unit presented by the U.S. Government. The Ministry of Scientific Research and Cultural Affairs, Govt. of India, provided G. M. Patel with a Research Scholarship.

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[Received May 12, 1964—Accepted March 25, 1965]

# Alterations in Polyunsaturated Fatty Acid Composition of *Voandzeia subterranea* Seeds upon $\gamma$ Irradiation

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Exposure of *V. subterranea* seeds, a herbaceous plant from Madagascar belonging to the family of legumes, to  $\gamma$  irradiation resulted in a polyunsaturated fatty acids decrease associated with the formation of UV-absorbing substances. The finding that products containing conjugated diene structure are formed during lipid extract irradiation indicates that hydroperoxy fatty acids may arise not only by enzymatic reactions but also by nonenzymatic oxygenation of polyunsaturated fatty acids promoted by ionizing radiation. Dehulled green seeds, flour made from dehulled green seeds, and lipid extract were studied for irradiation dose dependent changes in fatty acids compositions and hydroperoxydiene synthesis. The irradiation dose is more efficient in lipid extract than in dehulled green seeds or in flour made from these seeds, suggesting that the formation of UV-absorbing products is not a reliable clue for enzyme activity owing to the absence of protein in lipid extract. A homolytic pathway for the biogenesis of hydroperoxy fatty acids from polyunsaturated fatty acids is proposed. This involves an initiating radical which promotes a chain mechanism in which the  $O_2$  adsorbed is converted to hydroperoxide. Conclusively, preservation of fatty acid oxygenation should be a primary goal in the ionizing radiation processes of *V. subterranea* seeds and generally in the preservation of food of plant origin by ionizing radiation.

## INTRODUCTION

Recently, several investigations have reported the preservation of food of animal or plant origin by ionizing radiation (Dodd et al., 1985; Raffi et al., 1988). In animal cells, the metabolites of polyunsaturated fatty acids including hydroperoxy fatty acids represent an important class of biologic mediators which are released after ionizing radiation (Steel et al., 1988). It would be of great interest, therefore, to determine whether ionizing radiation may be involved in the synthesis of dioxygenated polyunsaturated fatty acids from plant cells and particularly in food of plant origin.

The polyunsaturated fatty acids are susceptible to oxidation by radical processes (Jore and Ferradini, 1988). On the other hand, it is well-known that free radicals are formed in food by ionizing radiation (Ehrenberg et al., 1980; Wills, 1980). However, little information exists about the mechanism of formation of hydroperoxy fatty acids resulting from exposure to ionizing radiation of fatty acids (Chipault and Mizuno, 1964; Hyde and Verdin, 1968). Therefore, efficient strategies for preventive lipid peroxidation in ionizing radiation processes of food of plant origin are also lacking. Chemical characteristics as well as fatty acid composition govern food quality. Studies conducted in our laboratory revealed alterations in fatty acid composition of *Voandzeia subterranea* seeds upon  $\gamma$ -irradiation treatment. The present investigation was undertaken to determine the effect of ionizing radiation on fatty acid composition of seeds, flour, and lipid extract made from these seeds.

## MATERIALS AND METHODS

**Plant Materials and Irradiation.** *V. subterranea* seeds were from Madagascar cultivated harvested in 1990. Samples (dehulled green seeds, flour, or lipid extract made from dehulled green seeds) were placed in plastic containers and irradiated in a  $\gamma$ -ray field using a IBL 460 ionizer. Total dose was 10 kGy, delivered at a dose rate of 331.967 Gy/h, carried out at room temperature.

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**Lipid Extraction.** *V. subterranea* samples were extracted by shaking (3000 rpm, at room temperature) for 15 min in 5 volumes of chloroform-methanol (2:1 v/v) (Folch et al., 1957) using an Ultra Turrax homogenizer. The extraction was repeated for 15 min with the same amount of chloroform-methanol. The extracts were combined and dried with anhydrous  $CaCl_2$ . After filtration, the organic solvent was removed under vacuum. Lipids were dissolved in 10 mL of hexane divided as 1-mL portions in test tubes and stored at  $-20^\circ C$  before analysis. The samples were used to determine fatty acid composition and hydroperoxide formation.

**Preparation and Analysis of Fatty Acid Methyl Esters.** Fatty acid methyl esters were prepared by saponification and methylation essentially as described by Suutari et al. (1990). The methyl esters were analyzed by gas-liquid chromatography. The major fatty acids were identified by comparing their retention times with those of standards (Sigma). The extractable fatty acids were determined by adding 30  $\mu g$  of heptadecanoic acid methyl ester (as internal standard) prior to saponification.

**Gas Chromatography.** A Packard-Becker Model 417 chromatograph equipped with a flame ionization detector, a capillary inlet system, and a OV-1 (25 m  $\times$  0.32 mm  $\times$  0.2  $\mu m$ ) column was used. The column temperature was programmed from 180 to 240  $^\circ C$  at the rate of 2  $^\circ C/min$ . The injector and detector were maintained at 300  $^\circ C$ . Peak areas were measured by using a Hewlett-Packard Model 3365 A integrator.

**Calculations.** The absolute amount of the individual fatty acids was calculated per 1 g of sample by comparison of the peak area to that of the methyl ester internal standard. The total amount of fatty acids was a sum of all fatty acids.

**UV Spectra Analysis.** Diene formation was monitored by taking UV spectra from 330 to 190 nm using a Perkin-Elmer Lambda 5 UV-vis spectrophotometer. Twenty-five microliters of lipid extract was used. Hexane was removed under  $N_2$  and replaced by 3 mL of ethanol. Formation of conjugated diene was visualized by the increase of absorbance at 234 nm as previously described (Andrianarison et al., 1990).

## RESULTS AND DISCUSSION

*V. subterranea* seeds, flour, or lipid extract from dehulled green seeds were studied for irradiation dose dependent changes in the fatty acids compositions and hydroperoxydiene synthesis. Details of dose measurements have been described previously (Snyder et al., 1986).

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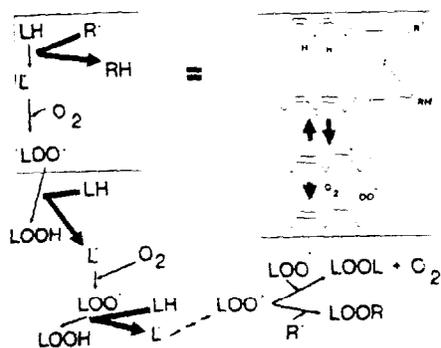


Figure 2. Proposed schematic mechanism for the formation of lipid hydroperoxide in samples from *V. subterranea* green seeds upon  $\gamma$  irradiation. LH, polyunsaturated fatty acid; R $\cdot$ , initiating radical; L $\cdot$ , pentadienyl radical; LOO $\cdot$ , peroxy radical.

initiating radical (R $\cdot$ ), produced during the radiation process, results in the formation of a new free pentadienyl radical (L $\cdot$ ) which would be oxidized rapidly by molecular oxygen. A peroxy radical would be expected (LOO $\cdot$ ). Such an equilibrium between peroxy radical and pentadienyl radical plus O $_2$  is already known from research of the free-radical chemistry of autoxidizing polyunsaturated lipids. The peroxy radical may promote a loss of hydrogen radical from another polyunsaturated fatty acid molecule followed by the formation of a hydroperoxy fatty acid (LOOH) and a free pentadienyl radical (L $\cdot$ ). This radical process may be ended by the reaction between two peroxy radicals (2LOO $\cdot$   $\rightarrow$  LOOL + O $_2$ ) or between the initiating radical and a peroxy radical (LOO $\cdot$  + R $\cdot$   $\rightarrow$  LOOR). Patterson and Redpath (1977), on the one hand, and Metwally and Moore (1987), on the other hand, have shown that the  $\gamma$  ray induced oxidation of unsaturated fatty acids in aqueous solution and the predominant chain-initiating radical was OH. The dienone chromophore, showing absorbance at 284 nm, may be the result of the reactions of water radiolysis species with hydroperoxy fatty acids (Greenstock and Wiebe, 1981).

*V. subterranea* is a plant whose dry seeds are used as food in some countries of Africa and in Madagascar. The seeds of this plant are receiving nutritional interest because of their high-quality protein and good fatty acid composition. However, the development of food applications of *V. subterranea* is often hampered by conservation problem due to infection by seed parasite. One issue may be the use of ionizing radiation. Unfortunately, this process induces peroxidation of fatty acids.

The high concentration of hydroperoxy fatty acids in the diet may cause several abnormalities in the human body. In addition, linolenic acid, one of the unsaturated essential fatty acids (Achaya, 1987), was reduced upon ionizing radiation treatment of *V. subterranea*, suggesting that the quality of seeds is rendered poorer upon ionizing radiation. Then, preservation of fatty acid oxygenation should be a primary goal in the ionizing radiation processes of *V. subterranea* seeds and generally in the preservation of food of plant origin by ionizing radiation. Chipault and Mizuno (1966) showed that several antioxidants were also destroyed by high-energy radiations in the presence of oxygen and did not prevent the peroxidation of fats.

It is hoped that further studies utilizing free polyunsaturated fatty acids or polyunsaturated fatty acids esterified in phospholipids will aid in the investigation and understanding of the relevant effect of ionizing

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**Table I. Fatty Acid Composition (Percent) of *V. subterranea* Seeds**

fatty acid	dehulled green seeds <sup>a</sup>	dehulled dry seeds
16:0 $\omega$	13.73 $\pm$ 0.31	13.20 $\pm$ 0.20
18:0 $\omega$	8.93 $\pm$ 0.22	9.39 $\pm$ 0.11
18:1 $n$ -9	20.73 $\pm$ 0.51	24.64 $\pm$ 0.23
18:1 $n$ -7	1.72 $\pm$ 0.05	1.78 $\pm$ 0.10
18:2 $n$ -6,9	45.20 $\pm$ 0.70	43.29 $\pm$ 0.61
18:3 $n$ -6,9,12	4.96 $\pm$ 0.12	2.33 $\pm$ 0.15
20:0 $\omega$	2.66 $\pm$ 0.13	2.95 $\pm$ 0.12
22:6 $n$ -3,6,9,12,15,18	1.09 $\pm$ 0.10	0.87 $\pm$ 0.01
others <sup>b</sup>	0.98 $\pm$ 0.15	1.53 $\pm$ 0.05

<sup>a</sup> Mean values are from three replicated determinations. Mean  $\pm$  SD. <sup>b</sup> Includes 20:1( $n$ -9), 20:1( $n$ -7), and unidentified acids.

**Table II. Effect of Irradiation Dose (0–10 kGy) on Polyunsaturated Fatty Acid Composition in Samples<sup>a</sup> from *V. subterranea* Seeds**

sample	kGy	polyunsaturated fatty acids <sup>b</sup>		
		18:2 $n$ -6,9	18:3 $n$ -6,9,12	22:6 $n$ -3,6,9,12,15,18
A	0	45.21 $\pm$ 1.32	4.96 $\pm$ 0.06	1.09 $\pm$ 0.01
	2	45.49 $\pm$ 0.67	4.55 $\pm$ 0.14	0.90 $\pm$ 0.02
	4	44.68 $\pm$ 0.36	4.13 $\pm$ 0.21	0.68 $\pm$ 0.02
	6	43.03 $\pm$ 1.21	3.76 $\pm$ 0.33	0.49 $\pm$ 0.02
	8	42.11 $\pm$ 0.29	3.65 $\pm$ 0.22	0.44 $\pm$ 0.01
	10	40.43 $\pm$ 0.56	3.48 $\pm$ 0.25	0.23 $\pm$ 0.00
B	0	48.01 $\pm$ 2.19	5.96 $\pm$ 0.22	0.75 $\pm$ 0.06
	2	47.53 $\pm$ 1.65	5.35 $\pm$ 0.25	0.73 $\pm$ 0.04
	4	46.99 $\pm$ 1.38	5.07 $\pm$ 0.28	0.67 $\pm$ 0.02
	6	46.55 $\pm$ 1.56	4.91 $\pm$ 0.25	0.61 $\pm$ 0.02
	8	45.50 $\pm$ 1.47	4.34 $\pm$ 0.23	0.49 $\pm$ 0.01
	10	44.87 $\pm$ 1.06	4.72 $\pm$ 0.24	0.40 $\pm$ 0.02
C	0	46.74 $\pm$ 1.58	4.81 $\pm$ 0.23	0.96 $\pm$ 0.05
	2	45.77 $\pm$ 0.61	4.76 $\pm$ 0.22	0.94 $\pm$ 0.04
	4	45.73 $\pm$ 1.76	4.50 $\pm$ 0.21	0.82 $\pm$ 0.02
	6	43.75 $\pm$ 1.39	4.46 $\pm$ 0.22	0.86 $\pm$ 0.01
	8	42.71 $\pm$ 1.56	4.17 $\pm$ 0.10	0.46 $\pm$ 0.02
	10	41.68 $\pm$ 0.72	4.01 $\pm$ 0.25	0.39 $\pm$ 0.06

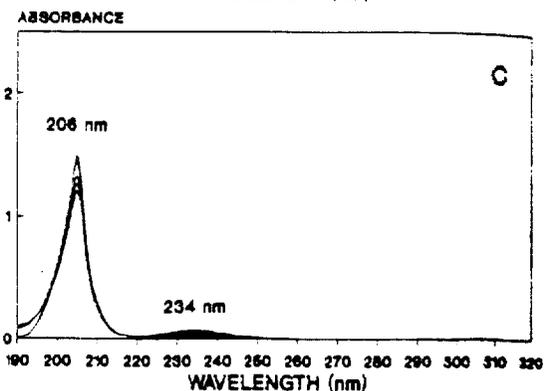
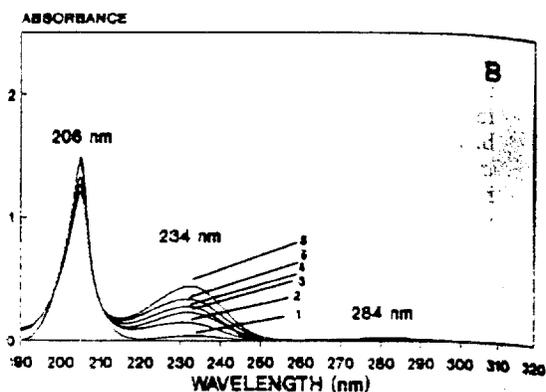
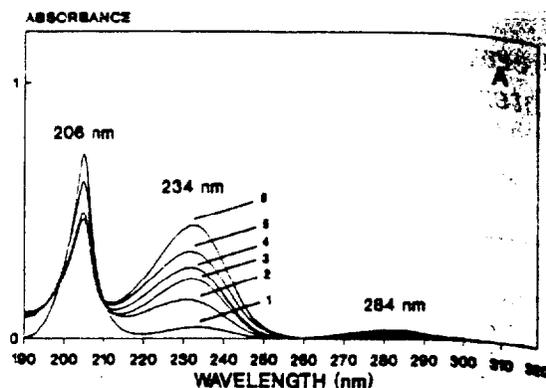
<sup>a</sup> A, lipid extract. B, flour from green seeds. C, green seeds. <sup>b</sup> Mean values were from three replicated determinations. Mean  $\pm$  SD.

**Fatty Acid Analysis.** Small differences were observed between fatty acid compositions of dehulled green or dry seeds of *V. subterranea* (Table I). In the two cases, the major fatty acids were linoleic, oleic, and palmitic acids. Arachidic and linolenic acids occurred in lesser amounts.

**Effect of  $\gamma$  Irradiation on Percentage Distribution of Polyunsaturated Fatty Acids.** The amount of polyunsaturated fatty acids decreased concomitantly with increase of irradiation dose (Table II), suggesting that the irradiation induced a selective loss of polyunsaturated fatty acids. Loss of fats was thought to be the result of radiolysis (Nawar, 1978), involving primary ionization followed by cleavage at preferential position near the carbonyl groups.

**Effect of Irradiation Dose on UV-Absorbing Product Formation.** When *V. subterranea* samples (lipid extract, dehulled green seeds, and flour made from those seeds) were subjected to ionizing radiation as described under Materials and Methods, a diene peak (234 nm) appeared concomitantly with increase of irradiation dose (Figure 1), while the peak of nonoxidized lipids (206 nm) decreased. The peak at 234 nm corresponds to those obtained by oxidation of polyunsaturated fatty acids by plant lipoxygenase (Andrianarison et al., 1991). Absorption near 234 nm was characteristic of lipid peroxidation, in vitro or in vivo studies (Kappus, 1984).

These results suggest that ionizing radiation may be of major contribution in the peroxidation of polyunsaturated fatty acids. This oxidation process is more effective in lipid extract than in dehulled green seeds or in flour made from these seeds (Figure 1).



**Figure 1.** UV spectra analysis. (A) UV spectra of irradiated lipids extract. (B) UV spectra of lipid extract from irradiated flour made from dehulled green seeds. (C) UV spectra of lipid extract from irradiated dehulled green seeds. The doses of irradiation were, from line 1 to line 6, 0, 2, 4, 6, 8, and 10 kGy.

During the exposure to ionizing radiation of lipid extract or flour, we found a second peak at 284 nm (Figure 1). Absorption near 280 nm was characteristic of a conjugated diene chromophore (Andrianarison et al., 1989).

This is evidence that the formation of conjugated diene and dienone chromophores is not a reliable clue to lipoxygenase activity owing to the absence of enzyme in lipid extract. It is well-known that radiation damage resulting from exposure to ionizing radiation is primarily an indirect effect initiated by free radicals produced during the radiation process (Ehrenberg et al., 1969).

The mechanism in the formation of the conjugated diene or dienone products was not investigated. However, we proposed a mechanism which can explain these syntheses (Figure 2). It seems likely that the conjugated diene products were formed from polyunsaturated fatty acids containing a pentadiene system, as, for example, linoleic or linolenic acid (the most abundant polyunsaturated fatty acids in *V. subterranea* seeds) (LH). Loss of a hydrogen radical ( $H^{\cdot}$ ), which is transferred into the

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