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ARTICLE

Application of Simple Biological Analyses to Screen Irradiated Brown Rice, Soybean and Sesame Seeds

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Abstract The efficacy of biological screening assays such as germination test and direct epiuorescent lter technique (DEFT) and aerobic plate count (APC) was evaluated to detect the irradiation status of different seeds. DEFT/APC help to calculate the difference between dead and living microorganisms in a sample after a possible irradiation treatment. Likewise, the irradiation can significantly affect the physiological and biochemical processes in germinating seeds, which provides the basis for the germination test. In the present study, three different seeds (brown rice, soybean, and sesame) of Korean and Chinese origins were subjected to gamma-irradiation (0.5, 1, 2, and 4 kGy) and the effects on the germination characteristics were evaluated. The results revealed that the growth rate and shoot length decreased with increasing irradiation doses. Particularly, 4 kGy of irradiation had a pronounced effect on all the germination characteristics in all seed samples. The DEFT counts did not change, which were independent of the irradiation dose, whereas the APC counts gradually decreased with dose increment. The results showed the potential of the germination test and DEFT/APC as useful screening methods for irradiated seeds.

Keywords brown rice · direct epifluorescent filter technique · germination test · irradiation · sesame seed · soybean seed

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Introduction

Irradiation processing has been considered a safe and effective technology and endorsed by the Food and Agriculture Organization, the World Health Organization, the International Atomic Energy Agency, and the Codex Alimentarius Commission. To date, more than 55 countries have approved the application of the irradiation process in more than 100 food items (Chauhan et al., 2009; Shahbaz et al., 2013). The process is useful in solving various agricultural problems: reduction of post-harvest losses through the suppression of sprouting and contamination, eradication or control of insect pests, reduction of food-borne diseases, extension of shelf life, and breeding of high-performance well-adapted and disease-resistant agricultural crop varieties (Andress et al., 1994; Delincée, 2002; Shahbaz et al., 2013).

Rice (*Oryza sativa*), soybean (*Glycine max*), and sesame (*Sesamum indicum*) are important agricultural crops in the world, providing nutrition and several health-promoting properties. Salmonella outbreaks linked with sesame seed-based products have been reported several times (Unicomb et al., 2005). Likewise, Agarwal et al. (2006) reported the detection of 21 pathogens associated with soybean seeds imported into India during 1978 to 2004. Similarly, mycotoxin-producing moulds (*Aspergillus* sp., F*usarium* sp., and *Penicillium* sp.) could contaminate rice grains and produce mycotoxins such as aflatoxin B1 (AFB1), fumonisin B1, and ochratoxin A, among others (Reddy et al., 2010). Irradiation is applied in different cereals and pulses for the purpose of destroying insects and harmful parasites associated with grains after harvesting (Crawford and Ruff, 1996). The recommended dose is up to 1 kGy for cereals and pulses mainly to eliminate insects and parasites and up to 8 kGy for seeds intended for sprout production to destroy food-borne pathogens (Code of Federal Regulations, 2000; Ding et al., 2013).

Regulatory authorities around the world have emphasized the implementation of various national and international regulations to promote trade and to ensure consumers' free choice of consuming irradiated food products (Akram et al., 2012). Several

methods have been developed so far to detect irradiated foods, and many methods have been approved as standard methods in the Codex Alimentarius Commission in the Codex General Standard. Until now, the European Committee for Standardization has approved about ten methods for the screening and identification of irradiated foods. However, most of these methods are time-consuming and require expensive equipment for analysis. Therefore, cost-effective and time-efficient screening methods are valuable for the screening of large food lots with minimal sample preparation (Delincée, 2002; Chauhan et al., 2009; Shahbaz et al., 2013). In present study, the applicability of the germination test, and the direct epiuorescent filter technique (DEFT)/ aerobic plate count (APC) method were for the first time evaluated to detect the irradiation status of different seeds.

Materials and Methods

Sample procurement and gamma-irradiation. About 5 kg each of brown rice, soybean, and sesame seeds, from Korean and Chinese origins, were purchased from a local grain market in Korea. The seed samples were separately packed in sterile polyethylene bags (pre-irradiated at 50 kGy) and labeled with the specific irradiation dose. Irradiation was undertaken at doses of 0, 0.5, 1, 2, and 4 kGy using a Cobalt-60 γ-irradiator (Sailor 100 kCi, IR-79, Nordion International Ltd., Canada) at the Korea Atomic Energy Research Institute. The irradiation process was done at room temperature with a dose rate of 1.5 kGy/h. The absorbed doses (±5.6%) were calibrated by alanine dosimeters with a 5-mm diameter (Bruker Instruments, Germany), in which the free-radical signals were determined with a Bruker EMS 104 EPR analyzer (Bruker Instruments).

Germination test. At least 25 seeds in a set were chosen to conduct the germination test, and each set was repeated four times. The seeds were imbibed in distilled water for about 10 h. Then, the seeds were placed on a distilled-water-moistened absorbent cotton layer of 9 cm in a Petri dish. The seeds were cultured under controlled condition of 25±1°C in a plant growth chamber (Seed & Grain Tech. Inc., USA). Germination percentage and shoot growth in terms of length for the un-irradiated and irradiated samples were measured using a Vernier caliper on Days 2, 3, 4, 5, and 6 after the start of experiment. Each measurement was carried out three times, and the mean length was recorded. The samples once used for measurement were discarded. The reported germination/shoot length data are the average of 100 seeds pooled for each set. For determination of germination percentage and shooting length, sprouts that were more than 0.2 cm in length were used (Chaudhuri, 2002).

DEFT/APC procedure and calculations. The microbiological screening method EN13783 (2001) is based on the comparison of the APC with the count obtained using the DEFT. The APC provides the number of viable microorganisms in a sample after irradiation treatment, whereas the DEFT count presents the total number of microorganisms, including nonviable cells, present in the sample. Microorganisms are captured by a membrane filtration process and stained with a acridine orange. The membrane is then rinsed and mounted on a microscope slide, which can be easily visualized and counted with an epiuorescent microscope. The process can be completed within 30 min (EN13783, 2001; Araúj et al., 2009).

The non-irradiated and irradiated seed samples were tested immediately after irradiation treatment according to EN13783 (2001) standard protocol. Briefly, 5 g of each the sample was added to 45 mL of peptone saline diluent (pH 7.2, 8.5 g sodium chloride and 1.0 g peptone/1,000 mL), diluted 10 times and vigously stirred. The saline solution was diluted with peptone saline diluent with a logarithmic dilution series (10^1–10^3 times) and then filtered on a manifold tower containing 10-μm polypropylene filter above a 0.6-μm polycarbonate filter under <20 mmHg. After the membrane filtration process, staining of microorganisms was performed with a acridine orange. The membranes were immediately rinsed with 2.5 mL of acetate buffer, pH 3.0. Finally, the membranes were rinsed by rapid filtration with 2.5 mL of isopropanol. Each membrane was mounted on a slide with a cover slip on which few droplets of non-fluorescing immersion oil were added and examined under an Epifluorescence microscope. The DEFT count (X) per gram was determined with the mean number of DEFT units per microscope filed (N/n), the dilution factor (DF) of the sample, and the microscope factor (MF) as follows: X=DEFT count/g=(N×MF×DF)/n.

Total viable count (APC) was determined after filtration through a fast filter paper (Whatman No. 4). Aliquots (0.2 mL) of suitable dilutions were spread onto plate count agar. The plates were incubated upside down at 30±1°C for 72 h and then counted (Wirtanen et al., 1993; Ahn et al., 2013). The difference between the DEFT count and APC count was then obtained by subtracting the APC count (logarithmic value) from the DEFT count (logarithmic value). All experiments were conducted in triplicate.

Results and Discussion

Effect of gamma-irradiation on the sprouting rate of shoot length. Although germination test can be easily performed, it is economical but too slow for routine analysis. This seedling assay is mainly confined to vegetable seeds. The basic principle of the germination test relies on the fact that irradiated seeds germinate at a considerably slower rate than control (non-irradiated) seeds (Stevenson and Stewart, 1995; Chauhan et al., 2009; Shahbaz et al., 2013).

Fig. 1 shows the effect of gamma-irradiation on the sprouting rate of non-irradiated and irradiated seeds of rice, sesame, and soybeans over a 6-day germination period. It is obvious from the results that seed samples exhibited varied responses to different irradiation doses. The germination percentage gradually decreased

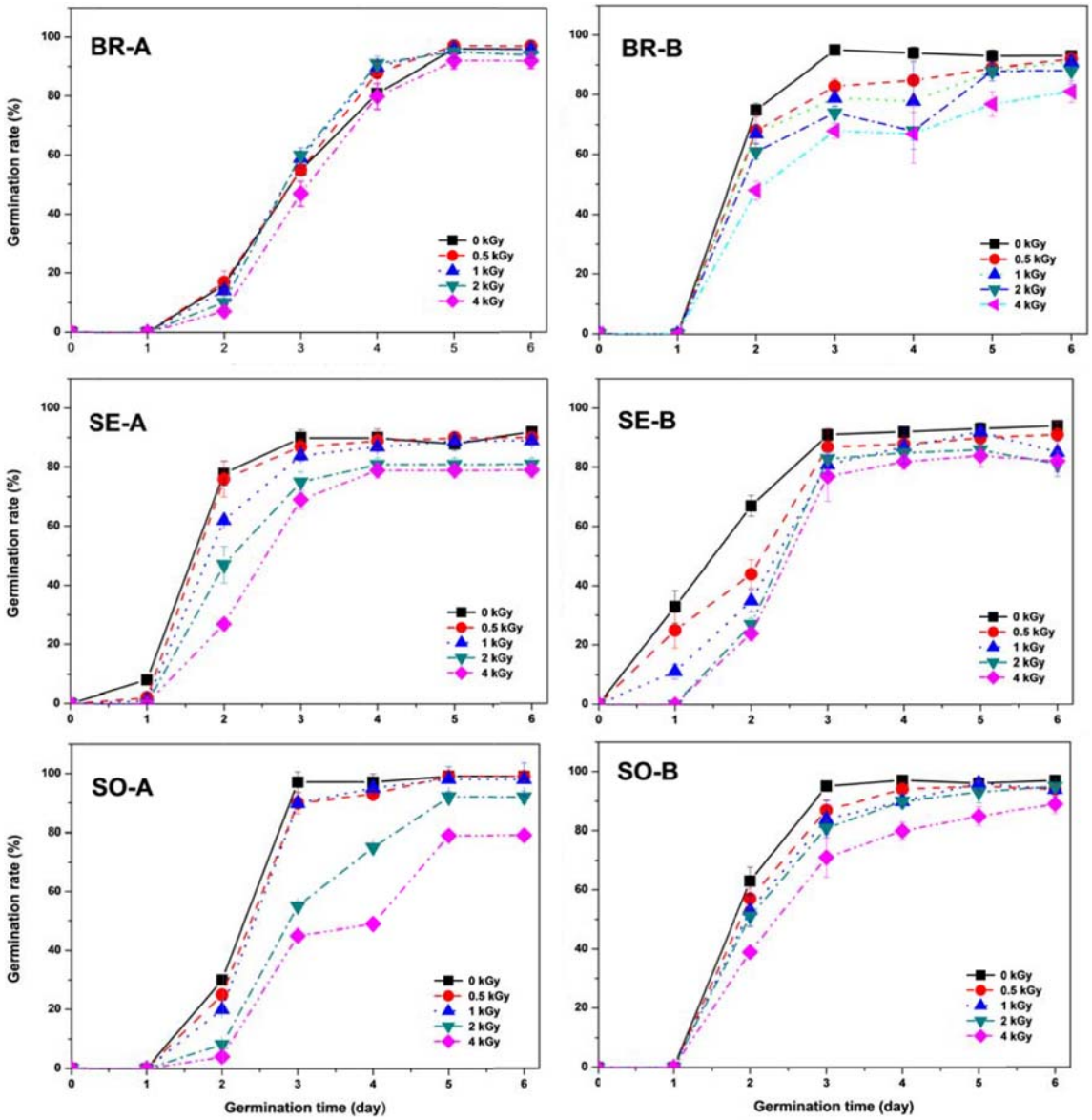

Fig. 1 Effect of gamma-irradiation on the sprouting rate of different seeds. (BR-A, brown rice grown in Korea, BR-B, brown rice grown in China; SE-A, sesame seeds grown in Korea, SE-B, sesame seeds grown in China; SO-A, soybean seeds grown in Korea, SO-B, soybean seeds grown in China).

with dose increments ranging from 0.5 to 4 kGy over the germination period. The effect of the irradiation treatment on germination was minor at a dose of 0.5 kGy but highly pronounced at 4 kGy. Moreover, the brown rice seeds from Chinese origin behaved slightly different to irradiation treatment in terms of germination. A slight increase in germination rate was recorded in brown rice seeds from Chinese origin up to 2 kGy irradiation than the rest of the samples (Fig. 1). The initial germination time was dependent upon the seed crop but independent of the origin.

Fig. 2 shows the effect different gamma-irradiation doses have on the shoot length of the seed samples over a 6-day growth time. Similar to the sprout rate behavior, shoot lengths were also inhibited in the seed samples with increasing irradiation doses. The higher irradiation dose of 4 kGy clearly depressed the shoot lengths. However, the sesame and soybean seeds proved to be more sensitive, and the shoot length significantly decreased with increasing irradiation doses.

Toker et al. (2005) indicated the effect of 0.2, 0.3, and 0.4 kGy of gamma-irradiation on the shoot and root length in germinated seedlings from two types of *Cicer* species (*C. reticulatum* Ladiz. *and C. bijugum* K.H. Rech.). The lower dose caused slight difference in the shoot length compared to the control, whereas the upper dose resulted in a significant decrease in the seeds

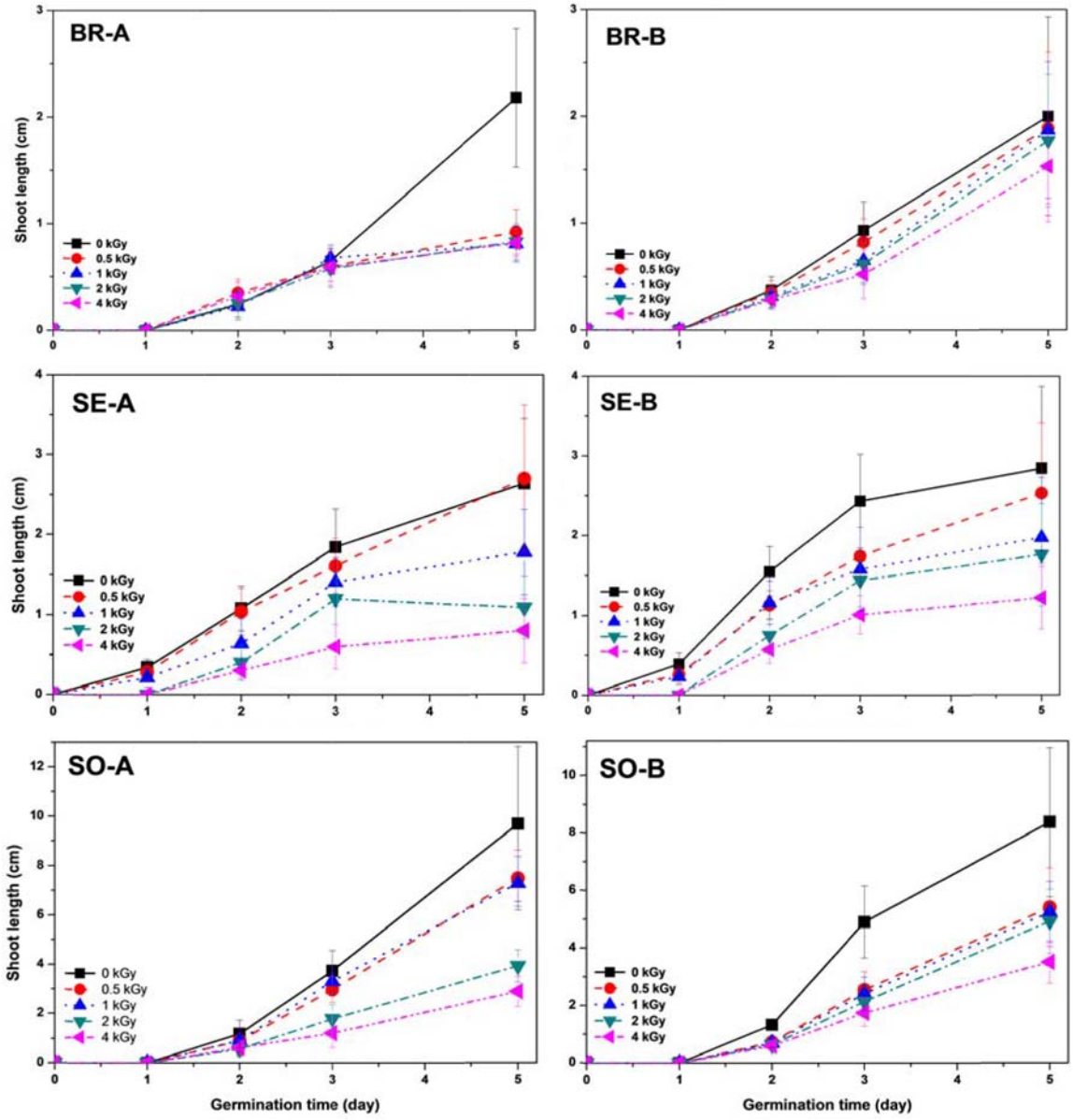

Fig. 2 Effect of gamma-irradiation on the shoot length of different seeds. (BR-A, brown rice grown in Korea, BR-B, brown rice grown in China; SE-A, sesame seeds grown in Korea, SE-B, sesame seeds grown in China; SO-A, soybean seeds grown in Korea, SO-B, soybean seeds grown in China).

belonging to different species. The critical dose that prevented shoot and root elongations varied among species and also changed from genotype to genotype within species. Maity et al. (2004) reported that germination in Bengal gram seeds was reduced upon exposure to gamma-irradiation of 6 kGy with a germination time of 96 h. Hameed et al. (2008) reported that the germination percentage and sprout growth rates were inversely related to irradiation by gamma-rays (100 to 1000 Gy) in two cultivars of chickpea seeds. Cutrubinis et al. (2004) showed the potential of the germination test as a detection method for garlic treated with irradiation up to 25 kGy in the dormancy period.

Kawamura et al. (1992) found that the germination test is helpful for discriminating the γ-irradiated and non-irradiated rice even after long storage periods. Chaudhuri (2002) reported on the efficiency and reliability of the germination method to detect γ-irradiated lentil seeds even after a 1-year storage period. The effect of irradiation varied greatly on shoot length compared to the sprout rate. The treatment and cultivar effect proved to be statistically significant for different seed samples of domestic and imported origins.

Gamma-irradiation significantly affects the physiological and biochemical processes in plants, which provide the basis for the

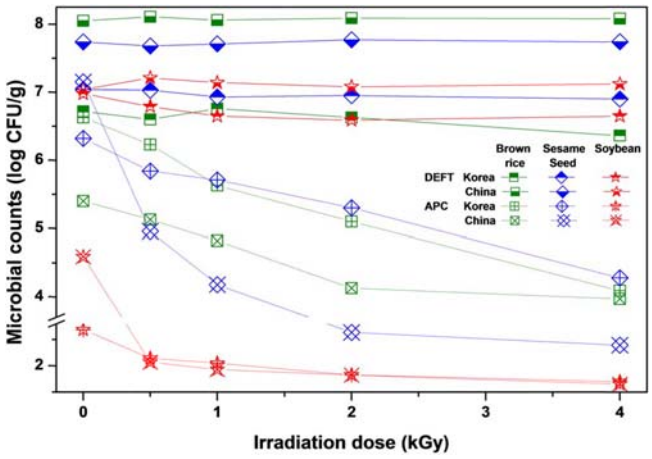

Fig. 3 The log DEFT and log APC values for the gamma-irradiated seeds.

germination test. Furthermore, the morphological, structural, and functional changes depend on the strength and duration of the gamma-irradiation stress (Hameed et al., 2008).

Effect of gamma-irradiation on DEFT and APC. Fig. 3 shows the influence of different irradiation doses on log DEFT and log APC counts. The DEFT counts remained quite stable at all dose levels, whereas the APC counts significantly decreased with the increasing irradiation dose. The control samples investigated in the present study had a total number of microorganisms in the range of 10^7–10^8 CFU/g at the initial stage. The viable microorganisms were reduced to a level of 10^1–10^4 CFU/g at the maximum applied treatment of 4 kGy. In general, irradiation treatment caused almost one log cycle reduction of aerobic microorganisms in most of the samples after each successively applied dose. Furthermore, the effect of origin was also prominent, because non-irradiated sesame and soybean seeds showed remarkably higher APC values. Analyzing previous studies, if the APC count is found to be considerably lower than the obtained DEFT, indicating that the sample could have been irradiated (Chauhan et al., 2009).

Irradiation treatment reduced viable microorganisms effectively, and the log DEFT/APC ratio gradually increased with the dose increments in all seed samples (Fig. 4). The log DEFT/APC ratio was less than 2.0 for non-irradiated rice and soybean seeds from domestic and imported origins. The soybean seeds irradiated with 4 kGy had few viable counts, and the corresponding log DEFT/APC ratio was very high. The log DEFT/APC ratio varied according to the difference in counts of total and viable microorganisms. This is probably due to the different degree of initial contamination and the nature of the contaminating microorganisms, which are greatly variable depending on a number of factors. The results showed the potential of DEFT/APC as a screening method for irradiated seeds.

Oh et al. (2003) reported the applicability of the DEFT/APC method for the screening of irradiated spices produced in Korea.

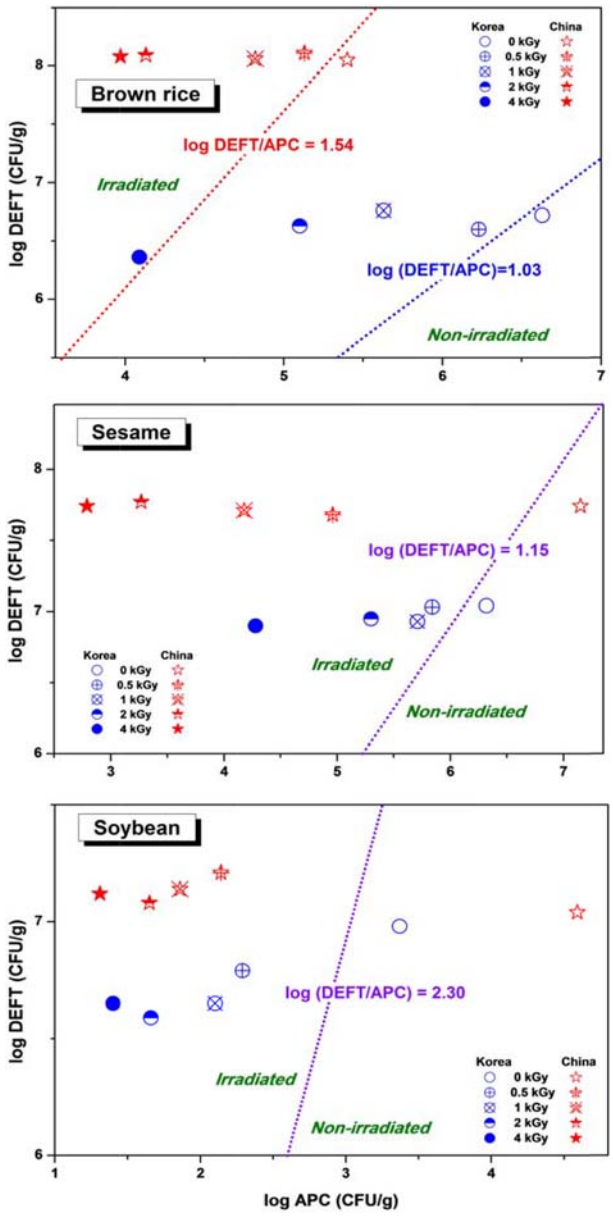

Fig. 4 The log (DEFT/APC) values for the gamma-irradiated seeds.

Irradiation doses of 3.0 kGy or over effectively destroyed viable microorganisms, and the log DEFT/APC ratio gradually increased with the dose increments in all spice samples. Similarly, Araúj et al. (2009) reported the efficiency of the DEFT/APC method for the screening of minimally processed vegetables treated with 0.5 and 1.0 kGy gamma-rays. More recently, Ahn et al. (2013) reported that the DEFT/APC technique was helpful for clear screening through the changes in microbial profiles in irradiated (0–10 kGy) paprika, red pepper and cinnamon samples.

Generally, the DEFT/APC technique can be applied for the screening of irradiated foods, where a log DEFT/APC difference of around 2.0 logs could give a preliminary indication of the

irradiation history of the samples (Araújo et al. 2009). On the basis of the present findings, it can be suggested that a DEFT/APC ratio of 1.03 to 2.30 logarithmic units could be a criterion for the screening of seed samples from Korean markets as non-irradiated or irradiated depending on the type of sample as well as the filtration step. In conclusion, biological screening methods are cost-effective and easy to perform. The results showed that DEFT/APC method can be applied as a screening assay for irradiated seeds. Similarly, the germination test verifies that irradiated seeds germinate at a considerably slower rate than control seeds. However, it is necessary to confirm positive results with more validated techniques such as thermoluminescence and electron spin resonance (Delincée, 2002; Shahbaz et al., 2013).

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