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ORIGINAL ARTICLE

Application of DNA comet assay for detection of radiation treatment of grams and pulses

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Abstract Several types of whole pulses (green lentils, red lentils, yellow lentils, chickpeas, green peas, cowpeas and yellow peas) and grams (black grams, red grams and white grams) have been investigated for the identification of radiation treatment using microgel electrophoresis of single cells (DNA comet assay). Pulses and grams were exposed to the radiation doses of 0.5, 1.0 and 5 kGy covering the legalized commercial dose range for protection from insect/pest infestations. All irradiated samples showed comet like stretching of fragmented DNA toward anode, which is expected for irradiated samples. Unirradiated samples showed many intact cells/nuclei in form of round stains or with short faint tails, which is typical for unirradiated food samples. The study shows that DNA comet assay can be used as a rapid, inexpensive and highly effective screening test for the detection of radiation treatment of foods, like pulses and grams.

Keywords Food irradiation · Pulses · Grams · Irradiation identification · DNA comet assay

Introduction

Radiation processing of foods is gaining increased attention as a result of improved understanding of the process and its benefits (Diehl 1995; Loaharanu and Thomas 2001; Molins 2001). The outcome of a Joint FAO/IAEA/WHO Study Group concluded that food irradiation is a safe and an effective treatment process and the products so treated are nutritionally adequate when produced under Good Manufacturing Practices (WHO 1999).

The pulses and grams are an important class of foods in South Asia as well as in other countries throughout the world. In usual practice, these food items are stored for longer periods before consumption, therefore infestation of such kinds of foods by moulds, insects, pests and egg larvae during storage are some of the major concerns. Infestation causes a serious problem in international distribution and supply of such kinds of foods due to gross losses during the storage. At the same time, the phase out of the traditional fumigant, such as methyl bromide on basis of environmental concerns and increasing resistance of many pests to phosphine fumigant is posing difficulties for disinfestations. So, irradiation of these food items as an alternate process, can contribute significantly to alleviate the post-production losses in staple grain crops and other such agriculture commodities (Anon 1999). Irradiation of pulses and grams with low doses of radiation (1 kGy or below 1 kGy) can be used for disinfestations and consequently prolongation of storage time can be achieved (Anon 1991).

A number of analytical methods have been investigated for the detection of radiation treatment of foods. In 1996, the European Committee for Standardization (CEN) adapted several methods based on Electron Spin Resonance (ESR) spectroscopy, Gas Chromatography (GC) and Thermoluminescence (TL) (Delincée 1998). Since all these techniques for the detection of irradiated foods are expensive, time consuming and need special expertise, therefore, there is a need to find out rapid and simple tests, which should be versatile and require inexpensive instrumentation.

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The majority of the foodstuffs, including pulses and grams, contain DNA. The large molecule of DNA is particularly a sensitive target to ionizing radiation, leading to its fragmentation, either by single- or double-strand breaks. In addition, denaturations of the DNA helix, cross-linking and base modifications also occur in the irradiated foods. These radiation-induced changes in DNA could be a basis for detection of irradiation in a number of foods (Delincée 1996; Cerda et al. 1997). A sensitive technique to detect DNA fragmentation is the microgel electrophoresis of single cells, commonly called 'Comet Assay' (Mckelvey-Martin et al. 1993; Cerda et al. 1997; Rojas et al. 1999).

Pulses and grams are commonly used in subcontinent and other countries. These foods with high starch contents are important because of a number of beneficial health properties (Yadav et al. 2010). Although use of processed foods has increased, there is concern to increase the dietary fibre level in these foods and meat products to promote healthy lifestyle (Verma and Banerjee 2010). A significant amount of these pulses and grams are imported and it is important to check if these have been irradiated. A simple screening method for detection of irradiation treatment of pulses and grams will also protect consumer interests and discourage exporters the practice of over irradiation beyond permissible limits for long storage life. The present work was carried out to find out suitable working conditions for DNA comet assay to identify irradiated pulses and grams as a screening test and extension of our work on DNA comet assay for detection of different types of foods (Khan and Khan 2008; Khan et al. 2003, 2005).

Materials and methods

Irradiation of food samples

Pulses and grams were purchased from the local markets of Karlsruhe and Leipzig (Germany) and were stored at room temperature. About 300 g of each food in form of single layer was packed in small transparent polyethylene bags for radiation treatment. A 10 MeV electron beam (Circe III linear accelerator, Thomson-CSF Linac, St. Aubin, France) having a dose rate in the pulse of 10^5 kGy/s was used for irradiation. GAF DM-1260 (radiochromic films, International Specialty Products, Wayne, USA) was used for the measurement of radiation doses (McLaughlin et al. 1991) by evaluating them at 405 nm for change in optical density with the help of a filter photometer (Ciba Corning Halstead, Essex, UK). The radiation doses applied to pulses and grams corresponded to the guidelines of the International Consultative Group on Food Irradiation (ICGFI 1994). Besides a control sample, the foods were irradiated to radiation doses of 0.5 to 5 kGy, covering the commercial dose range of radiation treatment for the purpose of insect/pest disinfestations. The samples after irradiation were immediately processed for cell suspension preparation.

Preparation of cell suspension

The specific amount of pulses and grams (see Table 1) from each sample for each commodity were crushed finely and transferred to about 3–5 mL cold PBS solution. The suspension for each food was stirred at a rate of 500 rpm for about 5 min. The stirring was achieved in presence of ice surrounding all the beakers containing suspensions. Filtration was carried out through 200 and 100 μm nylon sieves and then each suspension was allowed to sediment for specific time periods (see Table 1) for different samples of food. After the required sedimentation period, the supernatant was used as cell suspension (Khan and Delincée 1998; Khan and Khan 2008; Khan et al. 2002a).

DNA comet assay

In principle, the DNA comet assay was carried as described previously (Cerda et al. 1997; Khan and Delincée 1998; 1999; Khan et al. 2002a, b; Delincée et al. 2003). For each radiation dose, a pair of microscope slides was used for the purpose of duplicate analysis. About 100 μL of the supernatant as cell suspension was mixed thoroughly with 1000 μL of warm 0.8% agarose gel solution. 100 μL of this mixture was spread on a microscope slide (76×26 mm). The casted slides from each food sample were immersed in lysis buffer (45 mM Tris-borate, 1 mM EDTA, pH~8.4 containing 2.5% SDS) for different lysis times (see Table 1 for optimised conditions for each food). Electrophoresis was carried out using the same TBE buffer, but devoid of SDS at a potential of 2 V/cm for about 2 min. Silver

Table 1 Optimum conditions for DNA comet assay of lentils, peas and grams

Name of grams or pulses	Amount mg/mL PBS	Sedimentation time (min)	Lysis time (min)	Ag-staining time (min)
Green lentils	350/3	15	25	80
Red lentils	300/3	15	25	80
Yellow lentils	300/3	15	25	80
Chick peas	300/3	15	25	60
Cow peas	600/4	30	35	100
Green peas	800/5	25	40	80
Yellow peas	300/3	15	25	60
Black gram	350/3	15	35	120
Red grams	500/5	25	40	110
White grams	500/5	20	22	80

staining was employed to visualize DNA material on the microscope slides. Standard transmission microscope was used for the evaluation of unirradiated and irradiated samples of grams and pulses (Khan and Delincée 1998, 1999; Khan et al. 2002a, b; Delincée et al. 2003).

Results and discussion

Among the pulses and grams, which were investigated for the qualitative analysis leading to identification of radiation treatment, the major categories included lentils, peas and grams.

For DNA comet assay, the extracted cells or nuclei of the food samples were embedded in agarose on microscope slides. The cell membranes were made permeable by a detergent and then electrophoressed at selected voltage. DNA fragments were stretched or migrated out of the cells forming a tail (comet) towards the anode. The shape, length and intensity of comets indicate the degree of DNA damage, which in turn is an indication of the radiation treatment applied to the food samples. On the other hand, non-irradiated cells appear round or conical or as a nucleus with a thinly dispersed DNA nearby, indicating the absence of radiation treatment.

The results obtained for each category of food have been discussed below.

Green, red and yellow lentils

The unirradiated samples of green lentils showed regular stained nuclei. The sizes and the shapes of these intact cells were similar. However, irradiated samples showed the DNA staining in form of comets and no intact cells were observed in these samples. The size and shape of comets were dose dependent and gave some idea about dose delivered. Photographs for DNA comet assay of unirradiated and irradiated samples of green lentils are given in Fig. 1a.

In case of yellow and red lentils, the unirradiated samples showed fairly round intact cells. In both these lentils, the DNA material was varied and some time bigger round stains of DNA were observed. All the unirradiated samples showed the intact cells as a major population and only few comets were present along with these intact cells. In the irradiated samples, homogeneous migration patterns of comets were observed in their respective doses of radiation. The sizes of comets for different doses were different and a rough dose estimate was also possible.

Among these three kinds of the lentils, red and yellow lentils showed the signs of polyploidy, which was observed in both the irradiated and unirradiated samples. Such behaviour is common in plant commodities and has been reported in some other kinds of plants also, where the nuclei were varied considerably in size and susceptibility against radiation were quite large (e.g. sunflower nuclei), whereas DNA comet assay in sesame showed much smaller size comets (Delincée 1996).

The analysis of three kinds of lentils during the present investigation showed that DNA comet assay could help to identify the irradiated and unirradiated samples just at a glance under the microscope. The results were good because there was no sign of contaminating debris and a clear background was observed behind stained DNA material. Therefore a shorter sedimentation time and shorter lysis time of 15–25 min could be used to achieve a rapid analysis in case of these foods. Even low doses like 0.5 kGy could be identified just by visual inspection. So development of a frequency histogram was not needed as it was carried out during an earlier study in our laboratory for the detection of radiation treatment of red lentils, where the identification of samples irradiated to 1 kGy was made possible using frequency histogram (Khan and Delincée 1998).

Chickpeas, green peas, cowpeas and yellow peas

The unirradiated samples of chickpeas showed round intact stains of the cells/nuclei. The stains were varied in size; some being large and others were smaller in size. The major population of intact cells was smaller in size. There were high densities of DNA contents in round stain thus making them dark black. All the irradiated samples showed only the comets and not even a single intact cell was visible thus providing an evidence of radiation treatment. The density of DNA in the tails of the comet increases from lower to higher radiation dose showing linear relation with fragmentation of DNA. From this experience, samples irradiated to different doses of radiation could also be differentiated; giving rough dose estimate. The majority of comets were same in size with respect to applied dose.

The same kind of behaviour was noted in case of the unirradiated and irradiated samples of yellow peas. The analyses were quite good and the samples could be discerned as unirradiated and irradiated just by a quick visual inspection.

The analyses for samples of green peas were positive and were achieved without any difficulty using DNA comet assay and quick detection of radiation treatment was possible. Almost round intact cells/nuclei were observed in case of unirradiated samples, showing no or a few comets. All the intact cells were of same size and shape. The intact cells were well populated showing the ease of extraction of DNA material under the employed conditions. The other merit of this analysis was total absence of any contaminating material for both unirradiated and irradiated

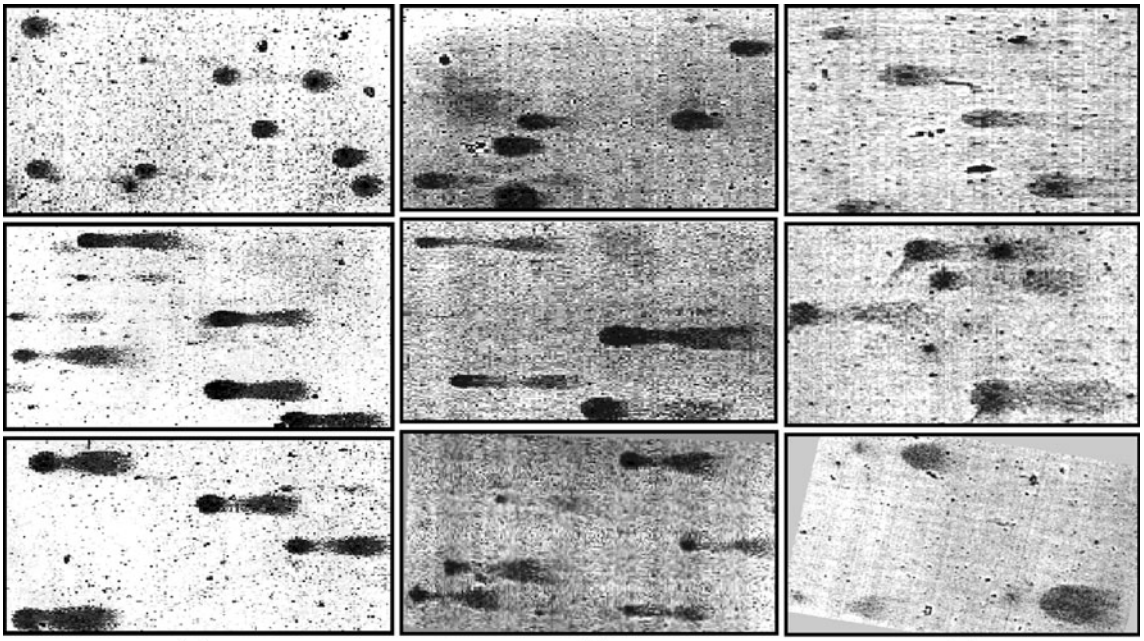

Fig. 1 Typical pictures for DNA Comet Assay of (**a**) green lentils (**b**) green peas and (**c**) black grams (from top to bottom: 0, 0.5 and 5 kGy)

samples and thus background of stained DNA was clean. However, in case of irradiated samples (0.5 and 5 kGy), regular comets were observed. The sizes of almost all the comets were same for a given applied radiation dose. Fig. 1b shows the photographs of unirradiated and irradiated samples of green peas. Shapes of the comets were dose dependent and were consistent with the applied radiation doses.

In case of unirradiated samples of cowpeas, a few intact round nuclei were observed along with some debris. For irradiated samples, few comets could be observed for the radiation doses of 0.5 and 5 kGy along with some debris. The shapes of comets for different doses of radiation were different and were consistent with the applied doses. Although the unirradiated and irradiated samples could be discerned easily, two problems were encountered that made the DNA analysis difficult: first was the significant amount of contaminating crude debris that heavily interfered with the stained nuclei and the second problem was the low amount of extracted nuclei. This may be due to a shorter sedimentation time of 30 min, which lowered the amount of the nuclei/cells. Such problem has also been reported in case of soybeans where proper amount of cells were not available using the normal conditions of assay (Haine and Jones 1996). Hence, the conditions were modified in the way that isolated extracts of cells were not allowed to settle down, instead the mixing of extract was carried out with molten agarose prior to the settling of cells. Under these modified conditions, the cells could be obtained as round stains for unirradiated samples or like comets for irradiated samples. However, presence of larger amount of debris made the analysis difficult. Never the less, soybeans samples were correctly identified as irradiated or non-irradiated (Haine and Jones 1996). For analysis of cowpeas in the present study, when appropriate cell extractions were tried using zero sedimentation time, a large amount of debris contaminated the background. On the other hand, on prolonging the sedimentation time in order to cope with the problem of dirt, the number of extracted cells obtained was lowered. It is, therefore, suggested that more work should be done to find out the optimum conditions that could be helpful for irradiation detection of cowpeas.

Black grams, red grams and white grams

The analysis of unirradiated samples of the whole black grams showed numerous intact cells in form of round stains. The analysis of samples irradiated to different doses of radiation showed only the comets on microscope slide and no intact cells were visible. The lengths and shapes of the comets were consistent with the radiation doses applied to the samples and the tails of the comets were denser and wider. However, the DNA material in the necks of the comets was less making them thin and hence they were lightly stained. There was a small amount of contaminating crude debris making the evaluation easy just at a glance. However, interfering debris can be decreased by using more sedimentation time, in addition to removal of outer skins of the foods prior to extraction of cells. The present study showed that detection of irradiated black grams was

possible using the present conditions of comet assay (Table 1). Fig. 1c illustrates the DNA comet assay of black grams for unirradiated and irradiated samples.

The unirradiated samples of red grams showed a number of round cells with the majority of these nuclei having the same sizes. In case of irradiated samples, the comets of appropriate sizes were observed and no intact cells were found, even in the samples irradiated to low dose of 0.5 kGy. At the same time, there was no contamination by dirt and the background of stained cells was clear as compared to the samples of black grams as explained above.

In case of the unirradiated samples of the white grams (usually referred as grams), a large number of round intact cells were observed. The intact cells were smaller in size and cylindrical in shapes. The samples irradiated to 0.5 kGy showed comets on whole of the microscope slide and no intact cell was observed. The same was true for the samples irradiated to 1 and 5 kGy. The sizes of the comets increased with the applied doses of radiation. These analyses for grams revealed that the cell walls were sufficiently lysed for release of fragmented DNAs and there were no intact cells in irradiated samples that could falsify the results.

The present investigations for analysis for all kinds of grams were encouraging and successful as compared to some earlier studies on grams, where the stained images like intact cells were observed in the samples irradiated to 1 kGy (Khan and Delincée 1998). In case of present studies, the analyses of all these grams, even for a low dose of 0.5 kGy, was successful and difference between irradiated and unirradiated samples was fairly possible just by visual inspection under microscope. However, it should be recognised, that longer lysis times, used in the present study, were certainly helpful in the success of the comet assay test.

Conclusions

Among the ten types of pulses and grams investigated during the present study, nine types of foods showed very successful results with DNA comet assay. Typical round intact cells were present in majority of the unirradiated samples. In the irradiated samples, only comets were observed with no intact cells and the sizes and shapes of comets were consistent with the applied radiation doses. In case of cowpeas, some problems in analysis were encountered due to contaminating debris as well as the extraction of small amount of cells or nuclei. However, the detection of radiation treatment was also possible for this food. It can be concluded that DNA comet assay could be applied for identification of radiation treatment of all the types of pulses, grams and peas investigated in the present study. It should be recognized here that DNA comet assay is not radiation specific and any kind of heat treatment to these foods can also cause DNA fragmentations. Therefore, DNA comet assay is recommended as a screening test for the irradiated foods. In case of suspected samples, other validated methods for the detection of radiation treatment should be applied for confirmation.

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