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GAMMA IRRADIATION EFFECTS ON SEED GERMINATION AND GROWTH, PROTEIN CONTENT, PEROXIDASE AND PROTEASE ACTIVITY, LIPID PEROXIDATION IN DESI AND KABULI CHICKPEA

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Abstract

Desi and kabuli chickpea seeds irradiated with 100 to 1000 Gy gamma rays (with an interval of hundred) were grown in incubator for 8 days at 25±°C. Germination, growth (seedling fresh weight, root shoot length and ratio), lipid peroxidation, protease and peroxidase activity were measured in leaves. Results showed that percent germination of the seeds and the rates of growth of sprouts were inversely related to the irradiation doses. In kabuli chickpea, peroxidase and protease activities (two folds) and MDA contents were higher as compared to desi chickpea while *vice versa* for protein contents, revealing inherent differences between two types. Data for protein contents, peroxidase and protease activities therefore suggested that irradiation dose should not exceed 600Gy in kabuli chickpea and 500Gy in desi chickpea. In kabuli chickpea 500Gy irradiation dose non-significantly affected the protein contents and peroxidase activity and lowered MDA contents and protease activity. In desi chickpea 400Gy irradiation dose increased the peroxidase activity, lowered the MDA contents and did not affect the protein content and protease activity. It was concluded that protein contents, protease, peroxidase and lipid peroxidation may be used in early assessment of effectiveness and superiority of radiation dose to induce mutations.

Introduction

Physiological and biochemical processes in plants are significantly affected by gamma-irradiation stress. The irradiation of seeds with high doses of gamma rays disturbs the synthesis of protein (Xiuzher, 1994), hormone balance (Rabie *et al.*, 1996), leaf gas-exchange (Stoeva & Bineva, 2001), water exchange and enzyme activity (Stoeva *et al.*, 2001). The morphological, structural and the functional changes depends on the strength and the duration of the gamma-irradiation stress. In the case of moderate stress, the adaptability capacity of the plants is preserved and the observed changes are reversible.

Antioxidants and peroxidase are involved in the compensatory mechanisms for the inhibition of free radicals formed upon UV irradiation of seeds (Rogozhin *et al.*, 2000). Correlation between growth and antioxidant enzyme activity of seedlings after gamma and neutron irradiation of pea seeds has been reported. Depending on the gamma radiation dose between 15 and 300 Gy the height of pea seedlings was found shorter and parallel with this peroxidase activities were higher than in the unirradiated controls (Bagi *et al.*, 1988). Similarly an increased level of the glutathione peroxidase activity (Marchenko *et al.*, 1996) after low doses of gamma irradiation has also been reported in corn (*Zea mays* L.).

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Protein breakdown and recycling, which depend on the levels of proteolytic enzymes, are an essential part of the plant response to environmental stress (Hieng *et al.*, 2004). In response to environmental abiotic and biotic factors cellular proteins should be rebuilt. Degradation of damaged, misfolded and potentially harmful proteins provides free amino acids required for the synthesis of new proteins (Schaller, 2004; Grudkowska and Zagdanska, 2004). After gamma and neutron irradiation of pea seeds, an inverse correlation between growth and degrading enzyme activity has also been reported (Bagi *et al.*, 1988).

A dose-dependent decrease in the triacylglycerol content and a concomitant increase in free fatty acids were observed after gamma-irradiation of nutmeg (*Myristica fragrans* Houtt.) (Niyas *et al.*, 2003). Similarly a prolonged irradiation of seeds with UV light (for 1-6 h) led to an increase in the level of lipid peroxidation in wheat sprouts (Rogozhin *et al.*, 2000). This suggested a breakdown of acylglycerols during radiation processing, resulting in the release of free fatty acids (Niyas *et al.*, 2003).

A number of radiobiological parameters are commonly used in early assessment of effectiveness of irradiation to induce mutations. Seed germination, seedling survival and pollen and ovule sterility have been used extensively. Fluorescence and light absorption spectra of chlorophyll attributed to different doses treatments of corn grains have been used to find out the superior irradiation doses in stimulating corn plants (Al-Salhi *et al.*, 2004). Previously on the basis of increase in free fatty acids it have also been suggested that radiation processing of nutmeg should be limited to a dose of 5 kGy (Niyas *et al.*, 2003). However protein contents, peroxidase, protease and lipid peroxidation, which have an important role in oxidative stress and are indicators of cellular damage should have been taken, as an important criterion, in the chickpea mutation breeding studies.

The present study was designed with following objectives: (1) to observe the effects of different doses of gamma rays on seed germination, seedling growth, protease, peroxidase and lipid peroxidation in chickpea. (2) Investigating the feasibility of gamma rays irradiation of seeds using lipid peroxidation, peroxidase and protease as an index of mutation frequency and to determine the possible role of these biochemical parameters in determination of appropriate radiation dose for inducing mutation in chickpea. (3) Based on radiobiological effects on lipid peroxidation, protease and peroxidase activity an attempt was made to study differential radiosensitivity of desi and kabuli chickpea varieties.

Material and Methods

Seeds of desi (97086) and kabuli (90395) chickpea (*Cicer arietinum* L) genotypes were treated with 10 doses of gamma rays ranging from 100 to 1000Gy with an interval of 100Gy by a ^{60}Co source. After irradiation, thirty seeds were sown per port filled with autoclaved sand along with untreated controls in three replicates with completely randomized design. The pots were placed in an incubator at 25^{o}C. Number of germinated seeds was recorded after 1, 2, 3, 4, 5 and 6 days. Different parameters like final percent germination (FPG), mean germination time (MGT) and time to 50% germination (T_{50}) were calculated from resulting data.

One week after sowing, root shoot lengths (cm), root/shoot ratio and seedling fresh weight were recorded. For different biochemical estimations leaves were grounded with a mortar and pestle under chilled condition in 50mM Potassium phosphate buffer. The homogenate was centrifuged at 14000rpm for 10 min at 0°C. The supernatant was separated and used for assay of enzyme activities and the level of lipid peroxidation.

Proteases activity: Protease activity was determined by the casein digestion assay described by Drapeau *et al.,* (1974). Briefly by this method one unit is that amount of enzyme, which releases acid soluble fragments equivalent to 0.001 A280 per minute at 37°C and pH 7.8.

Peroxidase activity: Peroxidase (POD) activity was determined as described by Liu & Huang (2000). The POD reaction solution (3ml) contained 50mM potassium phosphate buffer (pH 7.8), 20mM guaiacol, 40mM H_2O_2, and 100µl enzyme extract. Changes in absorbance of the reaction solution at 470nm were determined every 20 sec. One unit of peroxidase activity was defined as an absorbance change of 0.01 units per min.

MDA contents: The lipid peroxidation level was determined in terms of malondialdehyde (MDA) content by the method of Dhindsa *et al.,* (1981) and Zhang & Kirkham (1994). A 2ml aliquot of enzyme solution was added to a tube containing 1ml 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The mixture was heated in a water bath at 95^{o}C for 30min., cooled to room temperature and then centrifuged at 14,000rmp for 10 min. The absorbance of supernatant at 532nm was determined and nonspecific absorbance at 600nm was subtracted from it. The MDA content was calculated by using extinction coefficient of 155mM^{-1} cm^{-1} (Heath & Packer, 1968).

Protein contents: Total soluble protein contents were measured using Bradford's method (Bradford, 1976).

Statistical analysis: All experiments were repeated three times (90 and 30 seedlings per replication for germination and biochemical studies respectively). The descriptive statistics were applied to analyze and organize the resulting data. The F-test was applied to find differences in variance among samples. The significance of differences between means (irradiated and non-irradiated) for different parameters was measured using Student's t-Test (two tailed) at 0.01 and where applicable at 0.05 significance level. All the statistical calculations were performed using computer software Microsoft Excel 2000.

Results

Germination and growth: Seed germination test after gamma irradiation of seeds (100-1000Gy) revealed that mean germination time was increased with increasing irradiation dose for both desi and kabuli chickpea. The delay in germination was more pronounced in case of kabuli chickpea as compared to desi chickpea (Fig. 1).

Final germination percentage was non-significantly affected in desi chickpea with all irradiation doses. However in kabuli chickpea, final germination percentage was decreased significantly after higher irradiation doses ranging from 800 to 1000Gy. Maximum decrease in germination percentage was observed after 800Gy dose.

Shoot length was decreased in both desi and kabuli chickpea after all doses of gamma irradiation of seed. Generally shoot length of seedling was decreased gradually with increasing dose. Maximum decrease in shoot length was observed in both chickpea types after irradiation dose of 800Gy.

Root length also decreased after all doses of irradiation as compared to non-irradiated control in both desi and kabuli chickpea. Maximum decrease in root length was observed after 1000Gy dose in desi while after 600Gy dose in kabuli chickpea. Root/shoot ratio was increased at 800Gy dose in desi while at 900Gy dose in kabuli chickpea.

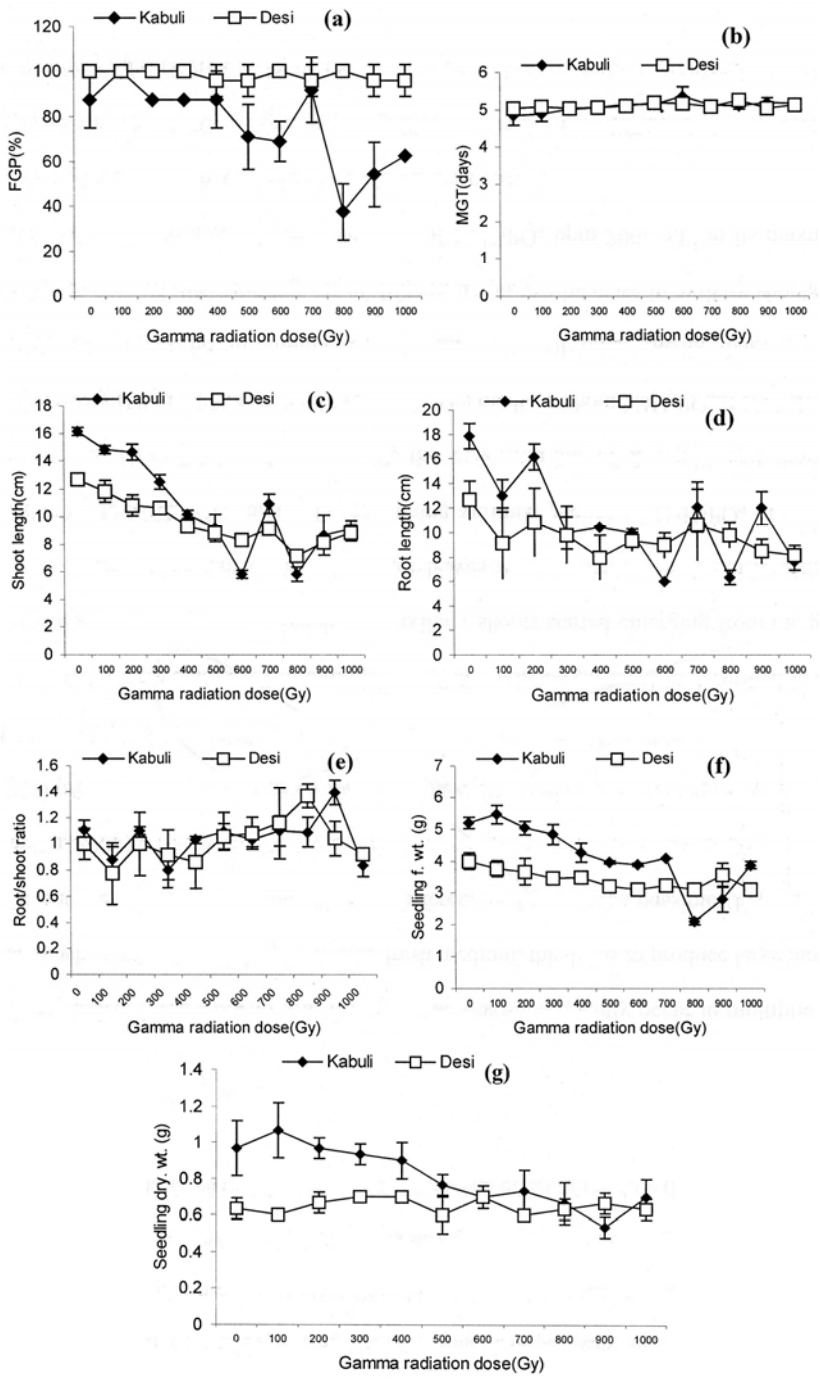

Fig. 1. Germination (a, b) and seedling growth (c, d, e, f, g) in desi and kabuli chickpea genotypes after gamma irradiation of seeds.

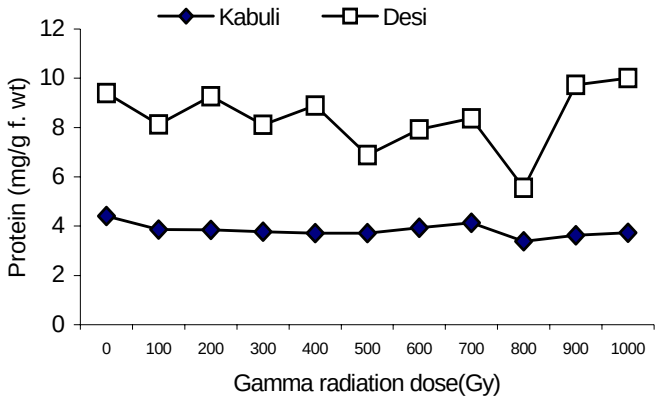

Fig. 2. Comparison of total soluble protein contents in kabuli and desi chickpea.

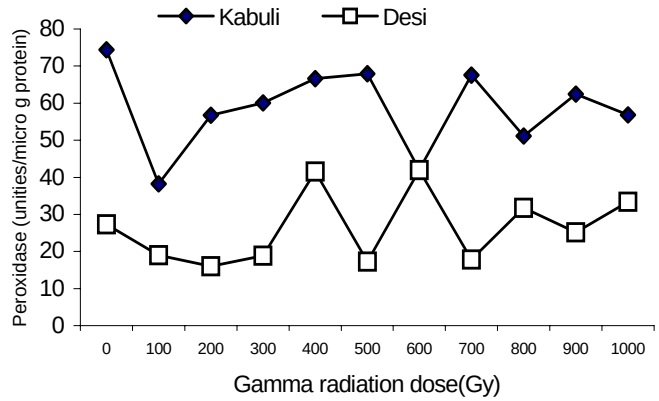

Fig. 3. Comparison of peroxidase activity in kabuli and desi chickpea.

Seedling fresh weight was decreased in desi as well as kabuli chickpea as compared with non-irradiated control after almost all irradiation doses. Minimum seedling fresh weight was observed after 800Gy dose in kabuli while 1000Gy dose in desi chickpea. Seedling dry weight was decreased in kabuli chickpea after all irradiation doses as compared to non-irradiated control. However seedling dry weight was non-significantly affected by seed irradiation and it was slightly increased after some irradiation doses as compared with non-irradiated control (Fig. 1).

Total soluble protein contents: Leaf protein contents were estimated in kabuli and desi chickpea after different doses of gamma irradiation of seeds (Fig. 2). In kabuli chickpea genotype, leaf protein contents were slightly decreased after different levels of gamma irradiation of seeds as compared with non-irradiated control. However in desi chickpea genotype, protein contents were lower after 100 to 800Gy dose as compared with non-irradiated control. However higher radiation doses (900 and 1000Gy) caused an increase in leaf protein contents in desi chickpea as compared with non-irradiated control. Maximum decrease in protein contents as compared to control was observed after 800Gy dose in desi as well as kabuli chickpea genotypes, however difference was highly significant in former one (Fig. 2).

Peroxidase activity: Leaf peroxidase activity was also affected after gamma irradiation of seeds (Fig. 3). In desi chickpea change in leaf peroxidase activity was dose dependent. Leaf peroxidase activity in desi chickpea was higher after 400, 600, 800 and 1000Gy dose while lower after all other doses as compared with non-irradiated control. Initially leaf peroxidase activity showed a decreasing trend up to 200Gy followed by an increase in activity up to 400Gy and then a cyclic decrease and increase after every 100Gy dose interval till 1000Gy dose.

Leaf peroxidase activity was generally decreased in kabuli chickpea after seed irradiation as compared with non-irradiated control. In kabuli chickpea, initially leaf peroxidase activity was decreased after 100Gy dose followed by a gradual increase in activity up to 500Gy dose and then a cyclic decrease and increase after every 100Gy dose interval till 1000Gy dose. Maximum decrease in activity was observed after 100Gy dose (Fig. 3).

Protease activity: Leaf protease activity was also affected by gamma irradiations of seeds in both desi and kabuli chickpea (Fig. 4). In desi chickpea change in leaf peroxidase activity was dose dependent. Leaf protease activity in desi chickpea was higher after 100, 400 and 800 Gy dose while lower after 700 and 900 Gy doses as compared with non-irradiated control. The difference was significant as compared to non-irradiated control after 100Gy (higher) and 900Gy (lower) dose only.

Leaf protease activity was generally decreased in kabuli chickpea after seed irradiation except after 200Gy dose where activity was same as compared with non-irradiated control. Leaf protease activity was minimum after 1000Gy dose where it was many fold lower as compared to non-irradiated control (Fig. 4).

Lipid peroxidation (MDA contents): Leaf MDA contents were decreased significantly by all doses of gamma irradiation of seed in both desi and kabuli chickpea genotypes (Fig. 5). In desi chickpea MDA contents were many folds lower after 100Gy dose. A gradual increase in MDA contents was observed with increasing radiation dose. In kabuli chickpea leaf MDA contents were higher as compared with desi chickpea in non-irradiated control as well as after all irradiation doses except 1000Gy. Among irradiated seeds, maximum MDA contents were measured after 700Gy dose in kabuli while 1000Gy dose in desi chickpea.

Discussion

Knowing that water radiolysis, the predominant effect of ionizing radiation in organisms, induces reactive oxygen species (ROS) formation (De-Vita *et al.,* 1993), one can assume that plant, bacterial and animal enzymes that are involved in cell protection against oxidative stress will display similar responses under ionizing radiation stress as under other stress factors (Zaka *et al.,* 2002). Antioxidants and peroxidase are involved in the compensatory mechanisms of inhibition of free radicals formed upon irradiation of seeds (Rogozhin *et al.,* 2000).

Inverse correlation between growth and peroxidase enzyme activity of seedlings after gamma and neutron irradiation of pea seeds has been reported. Depending on the gamma dose between 15 and 300 Gy the height of pea seedlings was found shorter and parallel with this the peroxidase activities were higher than in the un-irradiated controls (Bagi *et al.,* 1988). Similarly in our case, leaf peroxidase activity in desi chickpea was higher after 400, 600, 800 and 1000Gy gamma irradiation doses while shoot length was decreased in both desi and kabuli chickpea after all doses of gamma irradiation of seed.

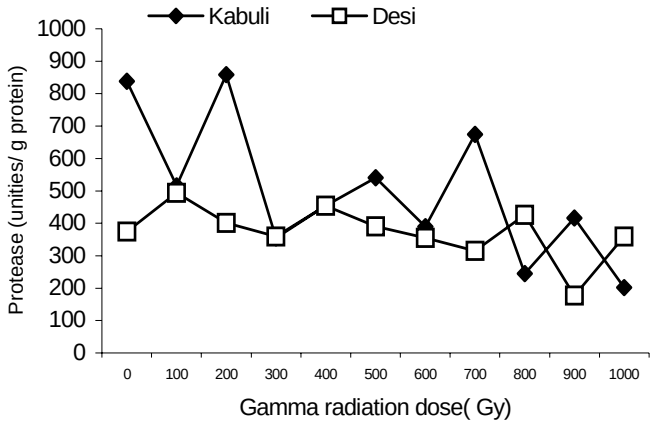

Fig. 4. Comparison of protease activity in kabuli and desi chickpea.

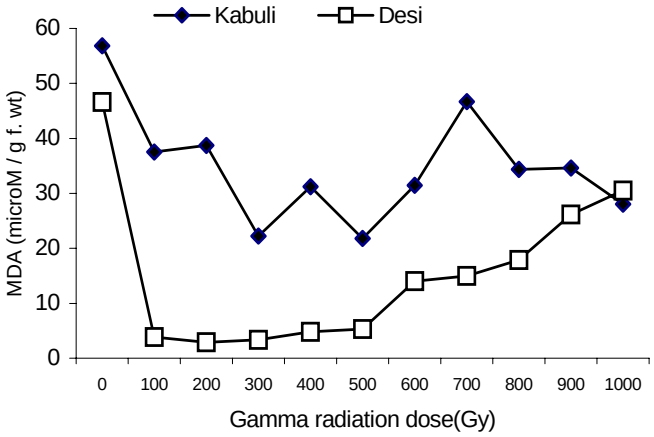

Fig. 5. Comparison of MDA contents in kabuli and desi chickpea.

It has been reported that prolonged irradiation of wheat (*Triticum aestivum* L.) seeds with UV light (for 1-6 h) led to an increase in the level of lipid peroxidation in sprouts (Rogozhin *et al*., 2000). Moreover the effect of gamma-irradiation on the lipid constituents of nutmeg (*Myristica fragrans*) was examined at radiation doses between 2.5 and 100 kGy. A dose-dependent decrease in the triacylglycerol content and a concomitant increase in free fatty acids was observed (Niyas *et al.*, 2003). Similarly in present study a dose dependent increase in lipid peroxidation was also observed in desi chickpea. Based on lipid peroxidation data it is suggested that irradiation of desi chickpea should be limited to a dose of 500Gy while 600Gy for kabuli chickpea. Previously on the bases of increase in free fatty acids it has also been suggested that radiation processing of nutmeg should be limited to a dose of 5 kGy (Niyas *et al.*, 2003).

Recent results have shown the complexity of cellular regulation in plants by proteolysis. They are involved in protein maturation, degradation and protein rebuilt in response to different external stimuli and to remove abnormal, misfolded proteins (Grudkowska & Zagdanska, 2004). The rapidly growing amount of information indicates that proteases participate in turnover of proteins during response to abiotic stresses

(Grudkowska & Zagdanska, 2004). Protein breakdown and recycling, which depend on the levels of proteolytic enzymes, are an essential part of the plant response to environmental stress (Hieng *et al.*, 2004). Similarly in present study higher proteolytic activity in desi chickpea after 100 and 400Gy dose indicates protein degradation by proteases, for removal of abnormal, misfolded proteins and for rebuilt processes in response to gamma irradiation. Degradation of damaged, misfolded and potentially harmful proteins provides free amino acids required for the synthesis of new proteins.

Inherent differences in all biochemical parameters studied were observed in desi and kabuli chickpea. Desi chickpea has almost double protein content as compared to kabuli chickpea inheritably. On the other hand peroxidase and protease activities (two folds) and MDA contents were inheritably higher in kabuli chickpea as compared to desi chickpea. In growth parameters root shoot lengths and seedling fresh and dry weights were higher in kabuli chickpea as compared with desi chickpea. So it can be concluded that kabuli chickpea has higher antioxidant value (peroxidase activity) while desi chickpea has higher protein contents.

It was evident from all biochemical parameters included in the present study that desi and kabuli chickpea respond deferentially to gamma irradiation. Firstly, there was a substantial loss in protein contents after 500 and 800Gy gamma irradiation dose in desi chickpea, which was not seen in kabuli chickpea. Secondly peroxidase activity was enhanced by 400 and 600Gy gamma irradiation dose in desi chickpea while activity was suppressed after all irradiation doses in kabuli chickpea. Thirdly minimum MDA contents were observed after 500Gy dose in kabuli chickpea while after 200Gy dose in desi chickpea. Further MDA contents increased steadily from 200 to 1000Gy dose in desi chickpea while there was a decrease in MDA contents with increasing dose from 700 to 1000Gy in kabuli chickpea. Inherent differences in desi and kabuli chickpea may be the basis for their differential response to gamma irradiation.

A number of radiobiological parameters are commonly used in early assessment of effectiveness of irradiation to induce mutations. Seed germination, seedling survival and pollen and ovule sterility have been used extensively. Fluorescence and light absorption spectra of chlorophyll attributed to different doses treatments of corn grains has confirmed the superiority of 1.5 Gy irradiation dose in stimulating corn plants. (Al-Salhi *et al.*, 2004). Similarly in present study biochemical parameters like protein and MDA contents and protease and peroxidase activities has pointed towards the superiority of 500 Gy irradiation dose for kabuli chickpea (non-effected protein contents and peroxidase activity and lower MDA contents and protease activity) and 400Gy for desi chickpea (higher peroxidase activity, non-effected protein and protease activity and lower MDA contents).

Conclusion

Collective data for protein contents, peroxidase and protein activities therefore suggested that seed irradiation should be limited to a dose of 500Gy for desi chickpea while 600Gy for the kabuli chickpea. Peroxidase and protease activities were higher (two folds) in kabuli chickpea as compared with desi chickpea while *vice versa* for protein contents. It was concluded that peroxidase was involved in the compensatory mechanisms of inhibition of free radicals formed upon gamma irradiation of seeds. Biochemical parameters like protein contents, protease, peroxidase and lipid peroxidation may be helpful in early assessment of effectiveness and superiority of irradiation dose.

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