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Research Communication

GAMMA RAYS INDUCED PROTEIN VARIATIONS IN M_4 GENERATION OF BLACK GRAM (*VIGNA MUNGO* L.)

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Abstract: Effects of various doses (45 krad, 55 krad and 65 krad) of gamma rays on proteins of black gram (*Vigna mungo* L.) in M_4 generation were investigated using Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) analysis. Higher doses of gamma rays resulted in reduced seed germination and affected further plant growth. SDS-PAGE results indicated differences in the protein banding patterns in 45 krad, 55 krad and 65 krad irradiated black gram samples. Absence of protein bands and appearance of new protein bands in M_4 generation black gram indicate the gamma rays-induced genomic lesions corresponding to these missing protein bands which could not be repaired even after M_3 generation and was quite stably inherited in M_4 generation. This study provides the useful information for the use of these black gram mutant lines in future breeding programme and further agronomic and nutritional study is needed for the analysis of the mutants.

Key words: Black gram; gamma irradiation; SDS-PAGE; *Vigna mungo*.

Note : Coloured Figures available on Journal Website in "Archives" Section

Introduction

The genus *Vigna* comprises of ~150 species, of which *Vigna mungo* or black gram is one of the most important food legume species having high nutritive value. It is reported to be originated in India and extensively grown in South-east Asia (Pratap and Kumar, 2011). India is the largest producer of this pulse crop in the world. It is a diploid (2n=2x=22) annual having a small genome size of ~515 Mb (Parida *et al.*, 1990). It contains about 26% proteins which are almost 3 times as that of cereals with a maximum contribution by globulins (63%), albumins and glutelins contributing 12% and 21% respectively.

A lot of research is going on for its improvement in terms of yield and nutrition quality. Mutation breeding as one of the conventional breeding methods has become an established tool to improve variability in terms of yield and quality of the crops (Acharya *et al.*, 2007; Ramya *et al.*, 2014). Among physical mutagens gamma rays are the ionizing radiation having the smallest wavelengths ($<10^{-12}$ m) with frequency ranges between 10^{-20} -10^{-24} Hz and therefore have energies above 100 keV. Its direct action is by increasing the frequency of G:C to A:T transitions in DNA and indirect action is to produce the free radicals which can damage the DNA, RNA,

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and proteins within the cell. Gamma rays induce shorter deletions and insertions and also frequent base substitutions in genome (Yatagai, 2004; Yoshihara *et al.,* 2013). Thus, after its treatment, it can enable the plant breeders to introduce new traits into a treated population and to select new genotype to obtain improved varieties (Shirley *et al.,* 1992; Kim *et al.,* 2006; Acharya *et al.,* 2007). Although, breaks produced in DNA molecules by gamma rays can be repaired but only within some limits. Further, this repair process works much slower in the case of a low dose exposure as compared to the higher dose exposure (Rothkamm and Löbrich, 2003). The mutagens cause genetic changes in an organism, break the linkages and produce many new promising traits to assist crop improvement (Shah *et al.,* 2011). According to the FAO/WHO, irradiation of any food commodity up to 1000 krad or higher doses has no toxicological hazard and it will be safe and nutritionally adequate (WHO, 1999).

In order to characterize the DNA and protein of gamma-irradiated plant samples, RAPD (Random Amplified Polymorphic DNA) and SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis) techniques have been generally employed by most of the researchers. SDS-PAGE and RAPD reflect the diversity of protein banding patterns and DNA banding pattern respectively, which can be used for cultivar identification also. Based on these two techniques, a considerable amount of genetic diversity has been analyzed in Pakistani black gram (Shafique *et al.,* 2011). In addition, earlier, the prostrate mutants, dwarf mutants, variegated mutants and bold mutants of gamma irradiated *Vigna mungo* were screened out (Pandey and Pandey, 2011). Further, morphological and biochemical effects of physical and chemical mutagens have been analyzed in the M_5 generation of black gram by treating its seeds with different concentration to induce the desirable mutation for the polygenic traits (Gandhi *et al.,* 2012). Recently, a new wheat germplasm has been developed with high anther culture ability by using a combination of ã-ray irradiation and anther culture (Zhao *et al.,* 2015). Recently, phenotypically and agronomically stable some novel mutants have been isolated at M_6 generation among three cultivated varieties of cowpea and are recommended for large scale production in Namibia (Horn *et al.,* 2016).

Keeping all the above views and facts in mind, hereby we characterized the gamma-irradiated (45 krad, 55 krad, and 65 krad treated) *Vigna mungo* (L.) seeds grown leaf protein samples in M_4 generation using SDS-PAGE analysis.

Materials and Methods

For the present investigation, gamma irradiation 45 krad (0.45 kGy), 55 krad (0.55 kGy) and 65 krad (0.65 kGy) doses were applied to the seeds of *Vigna mungo* var. Mash 1008 in the Plant Breeding Department of Punjab Agricultural University, Ludhiana. The M_1 to M_3 generations of *Vigna mungo* were analyzed for any morphological variations and stability of irradiated germplasm. Further, M_4 generation seeds were used in the present study for protein characterization of the germplasm at School of Agricultural Biotechnology, PAU Ludhiana.

Plant materials- Gamma irradiated seed samples (M_4 generation) of *Vigna mungo* var. Mash 1008 were sown in pot soil in trays under greenhouse conditions.

Extraction of Protein and its quantification- For protein extraction, 200 mg of young leaf tissues from 45 days old plants (four-leaf stage) were taken and ground in 600 µl of protein extraction buffer contained 2.5 M Tris, pH 7.5, 0.1% β-mercaptoethanol and 0.5% NaCl in autoclaved double distilled water. The ground plant samples were centrifuged at 13,000 rpm for 15 min at room temperature and 400 µl of clear supernatant containing total protein was transferred to the fresh tube and stored at -20 °C until use.

For quantification of protein samples, a standard protocol of Lowry assay (Lowry *et al.,* 1951) was followed. Different reagents used for Lowry assay were prepared and Bovine serum albumin (BSA) was used as a standard solution for protein quantification. The absorbance was read at 520 nm in the spectrophotometer. The amount of protein in the samples was calculated from the standard curve prepared by taking different concentrations of BSA (20-100 µg) from using its 1 mg/ml of stock solution. The concentration of protein (µg/µl) in each of protein samples were calculated according to the standard formula.

SDS-PAGE analysis of protein samples- The extracted protein was analyzed through SDS-PAGE using 12% polyacrylamide gel measuring 16.5x17.5 cm. SDS-PAGE of total protein was carried out according to the methods of Laemmli and Favre (1973). The molecular weights of dissociated

polypeptides were determined using protein molecular weight marker (low) (14.3 kDa to 97.2 kDa) (Takara Bio Inc. Japan). The gels were stained with Coomassie Brilliant Blue R-250 and then destaining was done (Green and Sambrook, 2012). The gel photograph was taken using a gel documentation system (UV-P Ltd., UK).

Results and Discussion

M_1 to M_3 generations of gamma irradiated (45 krad, 55 krad and 65 krad) samples of *Vigna mungo* were analyzed consecutively for the presence of morphological variations related to the yield and disease resistance. After stabilization of desirable changes at genomic as well as protein levels during M_1 to M_3 generations under field conditions, M_4 generation seeds produced were used in the present study for analyzing the protein variation in seedlings using SDS-PAGE.

Germination and growth rate variation

Out of total 129 gamma irradiated seed samples, 23 samples of 45 krad, 27 samples of 55 krad and 34 samples of 65 krad samples of black gram were analyzed. We observed that the non-irradiated seeds of black gram which were kept as control showed the highest germination (96%). However, among irradiated samples, 91.9% germination percentage was recorded in 45 krad treated samples, followed by 88.5% in 55 krad and 77.1% in 65 krad treated samples. Morphological effects were observed among gamma radiation treated samples of black gram. Despite to the fact that higher dose of gamma irradiation can easily produce a high genetic variability but with higher damaging effects on seed tissues. But a low dose of gamma radiation did not adversely affect the seed germination and also growth rate of black gram M_4 plants as compared to that of higher doses (Fig. 1.). Although, Rady *et al.* (2002) reported no real effect of gamma rays on the moisture content of seeds. But low seed germination with a higher dose of gamma ray indicates its adverse effects on seed as reported earlier (Chaudhuri, 2002; Sengupta *et al.*, 2013). In whole experiment, 3 samples each of 45 krad (numbered as 16, 24 and 32) and 65 krad (97, 113 and 114), 8 samples of 55 krad (38, 44, 47, 50, 51, 56, 60 and 61) could not germinated because of some physiological reasons.

Protein variations

Total protein from each of the leaf samples was extracted with same volume of extraction buffer for grinding. The extracted protein from each of the samples was equally loaded into SDS-PAGE and that resulted in the appearance of new bands and also some missing bands were found (data not shown). Based on this protein variation, we selected some protein samples each from 45 krad, 55 krad and 65 krad irradiated samples and an equal protein (3.5 µg) was loaded into SDS-PAGE. There were several protein bands which were present in control sample but either absent or less expressed in other irradiated samples (Fig. 2A). Thus, there were differences in protein banding patterns in SDS-PAGE as reported earlier (Ghafoor *et al.*, 2002; Ghafoor and Ahmad, 2005). In total, 7 protein bands were recorded (Fig. 2A). Protein subunits with lower molecular weight (<22 kDa) were not considered here due to lack of their reproducibility. These protein variations in terms of SDS-PAGE bands appearance (+) and missing (-) in gamma irradiated black gram samples compared to the control samples were showed in Table 1. The protein variations could be detected only in higher sized (>26 kDa) polypeptides. Although, there was

Table 1
Protein variations in terms of SDS-PAGE bands appearance (+) and missing (-) in gamma irradiated black gram samples

Protein bands in SDS-PAGE	*Control (untreated) sample*	*45 krad treated sample*	*55 krad treated sample*	*65 krad treated sample*
28 kDa	Present	Present in all except sample number 20	-	-
34 kDa	Present	Present	- (only in sample number 43)	Present
37 kDa	Present	-	-	- (only in sample number 79, 112)
95 kDa	Present	+	-	-

not much more protein variation found in all the irradiated samples but interestingly, a 34 kDa protein band was missing in case of 55 krad (sample number 43) sample while it was present in all 45 and 65 krad samples (Fig. 2A, B, C). Further, a 37 kDa protein band was altogether absent in all samples of 45 krad and 55 krad samples but absent only in 79 and 112 samples of 65 krad. In addition, a 28 kDa protein band was present in all samples of 45 krad samples except in sample 20 while it was absent in all 55 krad and 65 krad samples. A 95 kDa protein band was also present in all samples of 45 krad but it is altogether absent in 55 krad and 65 krad samples (Fig. 2A, B, C and Table 1). Seed protein content of *Vigna mungo* has been found gradually increased with increasing dose of gamma rays as compared to respective control (Arulbalachandran and Mullainathan, 2009). But in the present study, we did not find any such effect of gamma irradiation on *Vigna mungo*.

The gamma rays cause the changes in the protein patterns by inducing the appearance and/or disappearance of protein bands (Hegazi and Hamideldin, 2010). This is because it induces the shorter deletions and insertions of bases in DNA of irradiated plants (Yoshihara *et al.*, 2013). It is difficult to analyze the desired genomic regions with created lesions but modulation in the protein banding pattern as reported in our study also, which could be due to the altered genomic sequences in black gram. The missing of these protein bands proves that gamma rays induced genomic lesions corresponding to these missing protein bands which could not be repaired even after M_3 generation and was quite stably inherited in M_4 generation. Thus, SDS-PAGE analysis of 45 krad, 55 krad and 65 krad samples of black gram resulted in missing of some potential protein bands. Here, we could not able to correlate the presence/absence of these protein bands with any yield or quality attributes of black gram (data not shown) because the whole experiment was performed in a greenhouse on small scale basis. But we are in a way to cultivate these gamma irradiated black gram samples which showed the potentially clear presence of new or absence of some protein bands to evaluate their agronomic characters especially yield and disease resistance. More importantly, we have used the low doses of gamma irradiation which seems to produce more stable genomic changes in black gram. In addition to this, several protein bands, especially of 45 krad and 65 krad samples, were found with change in their intensity as compared to that of control even after equal loading which also shows the effect of gamma rays.

Conclusion

Creation of stable high genetic variability in the existing germplasm or cultivated variety of black gram is really needed in order to enhance its yield potential and disease resistance (Ramya *et al.*, 2014).

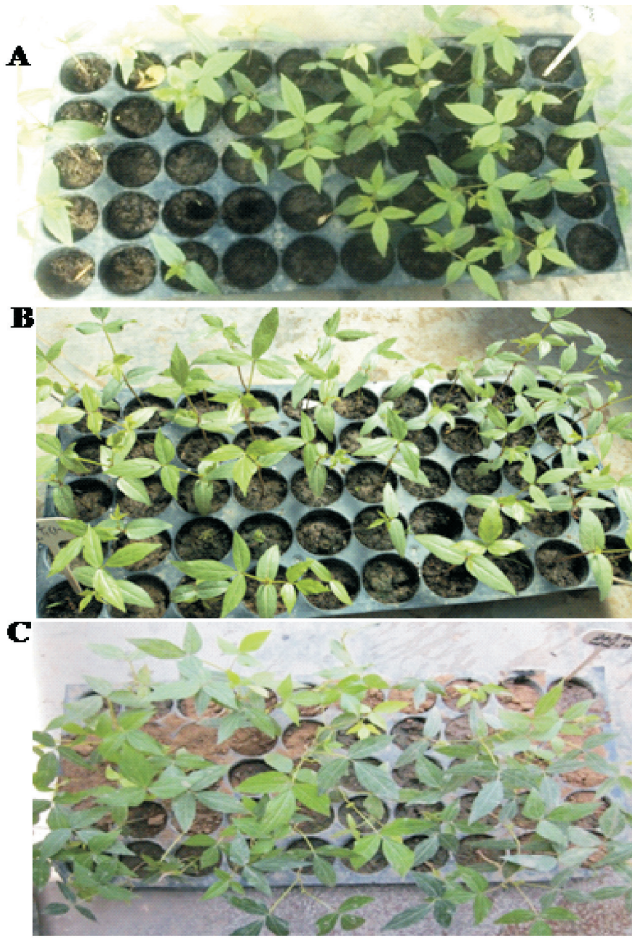
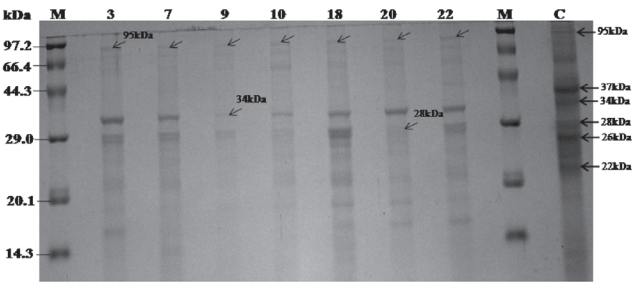


Figure 1: **Enhancement of germination and growth rate of gamma irradiated *Vigna mungo*. A, B and C as 45 days old seedlings of 65 krad, 55 krad and 45 krad gamma irradiated *Vigna mungo*. Lower dose of gamma irradiation enhanced the seed germination and also further seedling growth rate as compared to higher dose**



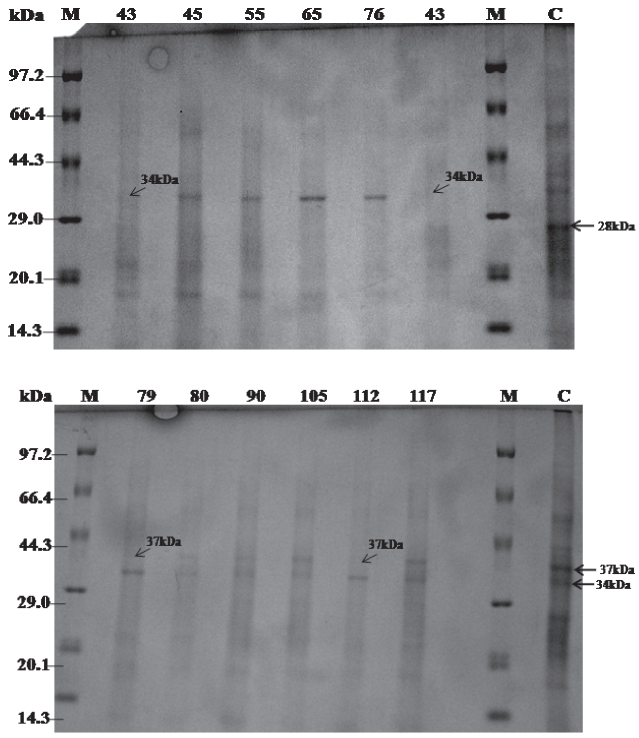


***Figure 2 (A)*: SDS-PAGE analysis of gamma irradiated samples (45 krad) of *Vigna mungo*. C as control where no irradiation while M as protein molecular weight marker (Takara) ranging from 14.3 KDa to 97.2 KDa. 3.5 µg protein from each of the 45 krad irradiated samples of 3, 7, 9, 10, 18, 20 and 22 was loaded for SDS-PAGE analysis.** *(B)*: **SDS-PAGE analysis of gamma irradiated samples (55 krad) of *Vigna mungo*. C as control where no irradiation while M as protein molecular weight marker (Takara) ranging from 14.3 KDa to 97.2 KDa. 3.5 µg protein from each of the 55 krad irradiated samples of 43, 45, 55, 65, 76, and 43 was loaded for SDS-PAGE analysis.** *(C)*: **SDS-PAGE analysis of gamma irradiated samples (65 krad) of *Vigna mungo*. C as control where no irradiation while M as protein molecular weight marker (Takara) ranging from 14.3 KDa to 97.2 KDa. 3.5 µg protein from each of the 65 krad irradiated samples of 79, 80, 90, 105, 112 and 117 was loaded for SDS-PAGE analysis.**

The gamma irradiation technology has been recognized as fast, reliable and safe means of producing genetic variability in various crops. From the present investigation, clear presence/absence of some protein bands evident due to gamma irradiation, proves the resolving efficacy of SDS-PAGE technique to visualize the potential variation created. Our results reflect the possibility of some genetic variation produced in the irradiated black gram samples or lines and further study is needed for the analysis of these mutants. Our results provide an important insight for evaluation of the mutational effects of gamma radiation on *Vigna mungo* plant system. This study can provide the useful information for the use of these black gram mutant lines for the future breeding program after their agronomic trait and nutritional analysis.

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Conflict of Interest

All authors have no conflict of interest to declare.

Abbreviations

RAPD, Random Amplified Polymorphic DNA; SDS-PAGE, Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis; rpm, revolution per minute; BSA, bovine serum albumin.

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