

# *Lhb* Mutant – A Novel Mutant of Mungbean (*Vigna radiata* (L.) Wilczek) Induced by Gamma Radiation

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## ABSTRACT

In the present investigation an attempt was made to induce mutations in mungbean (*Vigna radiata* (L.) Wilczek) var. 'Vaibhav' by employing gamma radiation with the objective of obtaining novel and desirable mutants. Mungbean seeds were irradiated with 30, 40 and 50 kR doses of gamma radiation and sown in the experimental fields to raise M<sub>1</sub>, M<sub>2</sub> and M<sub>3</sub> populations. Mutations were screened at M<sub>1</sub>, M<sub>2</sub> and M<sub>3</sub> generations. All three doses of gamma radiation effectively produced various morphological mutations. However, the 50 kR dose effectively produced a novel mutant that showed multiple morphological mutations such as large flowers with dark-yellow petals, dense and thick hairy pods and black seeds. In this study, SDS-PAGE analysis of seed proteins of large flower, hairy pods and black seeds revealed a difference in the banding pattern between the *Lhb* mutant, other mutants (tall mutant, dwarf mutant, high-yielding mutant and early maturing mutant) and control.

**Keywords:** gamma rays, morphological mutations, SDS- PAGE analysis

**Abbreviations:** kR, kilorad; *Lhb*, large flower - hairy pods - black seed coat; RBD, randomized block design; SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

## INTRODUCTION

Pulses occupy an important position in world agriculture because of their high protein content, several essential amino acids and their capacity for fixing atmospheric nitrogen. Mungbean (*Vigna radiata* (L.) Wilczek) is an important pulse crop of semi-arid zones and sub-tropics. The genetic variability in most pulses, including mungbean, has been greatly reduced over the years, because of the role of natural selection under a low level of management (Singh and Singh 2003).

In mungbean, a self-pollinated crop, natural existing genetic variability might not be sufficient to achieve the desired improvement. As a consequence, lack of required amount of variability, limits the scope for the selection of better genotypes. Frequency of natural mutations is very low and information on mutagenesis induced population in mungbean is scanty (Singh 2008). Hence, artificial mutations are induced and genetic variability is best enhanced with the application of mutagens. Artificially induced mutations are the best way to enlarge genetic variability, considerably within a short time (Patil *et al.* 2003; Singh and Singh 2003).

Mutation breeding has been proved to be one of the important techniques to develop and release new genotypes and high yielding cultivars in mungbean. Mutation breeding is a supplement to conventional breeding and is useful for creation of desirable variability in crop and could be a driving force for evolution besides selection in mungbean (Tah 2009). A closer examination of the type of mutagens used and the number of mutant cultivars released in India indicates that the largest number of mutant varieties (65%) have been induced by physical mutagens, gamma ( $\gamma$ ) rays (168 cultivars) being the most commonly used and successful (Kharkwal *et al.* 2004). According to the Food and Agricultural Organization/International Atomic Energy Agency (FAO/IAEA) Mutant Varieties Database (<http://www.mvd.iaea.org>) (December 2011), 3191 plant varieties

were released worldwide which were derived from RT (radiation and isotope techniques) and chemical mutagens during the past 75 years, increasing by approximately 500 since 2007 (Kang *et al.* 2007). To increase production and productivity, several approaches have been used to develop 43 high-yielding mungbean varieties during 1985-2006 (Reddy and Dhanasekar 2007). Mutation breeding resulted in the development of 15 high-yielding and disease-resistant varieties during 1979-2006. BARC contributed 7 varieties which are under popularization programme and the average yield is expected to increase (Reddy and Dhanasekar 2007).

Although traditional mutation breeding has lost its prominent position, induced mutations continue to be in great demand for various biotechnological applications (Chopra 2005). New molecular approaches have greatly simplified forward genetic approach with conventionally derived mutants. Saturated molecular maps are now being used to delimit mutant locus with molecular markers and the gene is cloned through positional cloning or chromosome walking (Brown *et al.* 2003). In addition stable mutants can be used as source material for tagging of specific loci with DNA markers and for molecular analysis for gene function. Induced mutagenesis, which later was to become the most important tool in locating genes on chromosomes, studying gene structure, expression and regulation, and for exploring genomes. The sequencing of some plant genomes are completed but assigning functions to many of the DNA sequences would not be possible, but for the mutants induced with gamma rays, fast neutrons or chemical mutagens, the identification and analysis of mutants using molecular techniques of DNA fingerprinting and mapping with PCR based markers such as RAPD, AFLP and STMS and mutant tagging could bring a new dimension in gene technology (Ahloowalia and Maluszynski 2001).

Induced mutations contributed significantly to plant improvement programs, even though most of the induced mutations are recessive and deleterious from a breeding point of view (Maluszynski *et al.* 1995). Significant in-



Fig. 1 Large flower, hairy pods and black seeds of *Lhb* mutant. (A) Large flower; (B) Hairy pods; (C) Black seeds of control.

duced variability among various mungbean genotypes for various traits have been reported by Patra (1997), Khan *et al.* (2004) and Lal and Mishra (2006). Mutation breeding has become increasingly popular in recent times as an effective tool for crop improvement (Acharya *et al.* 2007). The present investigation was undertaken with the objective of inducing mutations in locally adapted mungbean variety 'Vaibhav' employing  $\gamma$  radiation to obtain novel mutants with desirable and economically important characters.

## MATERIALS AND METHODS

Healthy seeds of mungbean (*Vigna radiata* (L.), Wilczek) var. 'Vaibhav' (released by Mahatma Phule Agricultural University, Rahuri, India) selected for the present study was procured from Pulses Improvement Division of Mahatma Phule Krishi Vidya-peeth (MPKV), Rahuri (Ahmednagar District, Maharashtra State, India). Dry and uniform seeds were irradiated with  $\gamma$  radiation at 30, 40 and 50 kR.  $\gamma$ -Rays, an ionizing radiation from a  $^{60}\text{Co}$  (Cobalt-60) source, fixed in the Gamma Cell 200 installed at the Department of Biophysics, Government Institute of Science, Aurangabad, India, was used in the present work.

For each irradiation dose, 700 seeds were used. 100 seeds from each treatment were used for germination studies. The remaining lot of treated seeds from each dose was sown in an experimental field in a randomized block design (RBD) with three replications together with non-treated seeds (control) for raising the  $M_1$  generation. The spacing between rows and plants was  $30 \times 10$  cm, respectively. All the surviving  $M_1$  plants were individually tagged. Seeds of single plants from each treatment were harvested and kept separately. The  $M_1$  plants were harvested individually and the seeds obtained were used to raise the  $M_2$  generation as plant-to-row progenies. The  $M_2$  population was carefully screened for different morphological mutations. Different types of mutants like tall mutant, dwarf mutant, high yielding mutant, early maturing mutant and *Lhb* mutant has been recorded. *Lhb* mutant showed poor performance in all agronomic characters over all obtained mutants and control plant. Present paper gives comparison of *Lhb* mutant with only control plant (Table 2).

The mutant plants which showed multiple mutagenic effects in the form of a large flower, hairy pod, black seed coat, leaf modification and semi-dwarf habit were selected. The name of the mutant was assigned on the basis of flower size, presence of thick hairs on the pod and black colour seed coat. *Lhb* (large flower - hairy pods - black seed coat) mutant plants (25) were selected and from each plant 25 seeds were sown in the field together with the control to raise the  $M_3$  generation in order to multiply the mutant and confirm its stability.

Seeds of the *Lhb* mutant were analysed for protein, albumin and globulin content (Lowry *et al.* 1951). Albumin and globulin were isolated by using dialysis bags. The protein profile of the *Lhb* mutant was analysed by SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) following the method of Dadlani and Varier (1993).

## Statistical analysis

Morphological and agronomical data obtained from 20 mutant plants was analysed for range, mean, standard deviation, coefficient of variation and one-way analysis of variance to assess quantitative differences in the various characters of the mutant and the

control using SPSS 9.0 software (SPSS Inc., Chicago, Illinois, USA). Significant differences between mutant and control means were determined using least significant difference (LSD) values at  $P = 0.05, 0.01$  and  $0.001$ .

## RESULTS AND DISCUSSION

In the present investigation, mungbean variety 'Vaibhav' proved to be very sensitive to all employed doses of the  $\gamma$  radiations (30, 40 and 50 kR). The mutagen also exerted inhibitory effects on the percent germination, seedling survival and increased the rate of sterility in  $M_1$  generation. It was also observed that these effects were dose dependent. The dose levels of 40 to 50 kR were critical for inducing mutations (Sharma and Haque 1997). The  $M_1$  population showed various morphological variations included different leaf, flower, pod and seed mutations. In  $M_2$  generation three types of chlorophyll mutations namely *striata*, *xantha* and *chlorina* was noted. Maximum number of *chlorina* and *xantha* types was observed. Average chlorophyll mutation frequency for  $\gamma$  radiation is 2.02%. In this variety as dose of the  $\gamma$  radiation increased, the effectiveness was decreased. 30 kR dose of  $\gamma$  rays was found to be more effective in inducing chlorophyll mutations and 50 kR  $\gamma$  dose induces prominent mutations.

Along with simple viable mutations, multiple mutagenic effects on two or more characters were also found in all the mutagenic treatments. However their frequencies differed with dose of mutagen and variety. In the present work 50 kR dose of  $\gamma$  radiation induced a novel mutant that showed multiple morphological mutations like large flowers with dark yellow petals, dense thick hairy pods and black coloured seeds (Fig. 1). It is named as large flower, hairy pod and black seed mutant (*Lhb* mutant). Such a mutant has not been reported earlier in mungbean. These *Lhb* mutants were also recovered in  $M_3$  populations of mungbean. *Lhb* mutant is homozygous in nature and whether it is recessive or dominant is yet to be established.

These mutants also showed multiple mutagenic effects on various other traits. Important among them are semi-dwarf habit, late flowering, large-sized flowers, dark-yellow petals, late maturing, broad and short pods, thick dense hairs on pods.

The large flower, hairy pod and black seed mutant (*Lhb* mutant) raised through  $\gamma$  radiation induced mutagenesis, seems to be promising at least for two characters i.e. semi-dwarf habit and presence of thick dense hairs on the pods. The semi-dwarf habit of the plant enables it as lodging resistant. The thick dense hairs on the pod act as a mechanical barrier and helps in protecting them from insects and caterpillar predators and seems to be promising economical important character (Pfeiffer *et al.* 2003). It can be used or incorporated in breeding programmes that are aimed at genetic improvement of mungbean. Large flowers minimize the consequent difficulties of emasculation during the crossing practices. Large flower mutant can be used for crossing to exploit heterosis (Jones 1945). The mutant heterosis has been described in many plant species (Maluszynski *et al.* 1989).

A comparative account of morphological characters of control and large flower, hairy pod, black seed mutant (*Lhb*

**Table 1** Comparative account of control and *Lhb* mutant on the morphological characters.

Characters	Control Plant – ‘Vaibhav’	Large flower, hairy pod and black seed color ( <i>Lhb</i> ) mutant
Habit	Herbaceous, erect	Herbaceous, erect
Root	Tap system provided with nodules	Tap system provided with nodules
Stem	Green with pigments	Green with pigments
Leaf	Trifoliate, thick leaves; no. of leaves: 6.8; leaf area index: 5.82; leaf area: 165.5 cm <sup>2</sup>	Trifoliate, thick leaves; no. of leaves: 6.9; leaf area index: 5.10; leaf area: 155 cm <sup>2</sup>
Flower	Bisexual, zygomorphic, Faint yellow, medium sized flower	Bisexual, zygomorphic, dark yellow color, large sized flower.
Pod	Green pod with prominent constriction, pod length medium, produced green seeds.	Small pod with dense thick hairs on the pod, produced black seeds.

**Table 2** Comparative account of control and *Lhb* mutant on their agronomic and biochemical characters.

	Control					Lhb				
	Min.	Max.	Mean	SD	CV%	Min.	Max.	Mean	SD	CV%
<b>Agronomic traits</b>										
Plant height (cm)	45.5	51.9	48.2	1.9	4.0	36.1	42.1	38.8###	1.7	4.2
Days to flowering	32.0	35.0	33.4	1.2	3.5	40.0	45.0	42.3***	1.3	3.0
Days to first pod bearing	42.0	46.0	44.4	1.3	2.9	48.0	54.0	51.1***	1.8	3.6
Days to maturity	74.0	78.0	74.9	1.2	1.6	80.0	84.0	81.9***	1.4	1.7
Pods/plant	18.0	28.0	21.5	3.1	14.2	13.0	18.0	16.2###	1.5	9.5
Pod length (cm)	5.7	7.2	6.4	0.4	7.0	4.2	5.2	4.6###	0.3	6.6
Grains/pod	4.0	6.0	5.0	0.7	14.8	4.0	5.0	4.3##	0.5	10.6
100 seed weight (g)	3.2	3.9	3.5	0.2	5.2	2.4	2.9	2.6###	0.2	5.9
Yield/plant (g)	4.5	4.7	4.6	0.1	1.7	2.5	3.5	3.0###	0.3	9.4
Harvest index	18.4	20.5	19.7	0.6	3.2	14.2	23.0	17.9#	2.2	12.1
Number of nodules	21.0	24.0	22.0	1.1	5.2	14.0	18.0	16.1###	1.2	7.2
<b>Biochemical traits</b>										
Total protein	21.8	22.2	20.0	0.2	1.0	17.6	18.9	18.6###	0.7	3.8
Globulin	12.2	12.9	12.6	0.4	2.9	9.8	10.5	10.1***	0.4	3.8
Albumin	9.9	10.2	10.1	0.2	1.5	6.4	6.5	6.4***	0.1	0.9

Lhb: Large flower, hairy pods and bold seeds; Min: minimum; Max: maximum; SD: standard deviation; CV: coefficient of variation

Values given are mean of 10 independent samples; \*\*\* Significantly higher than control at  $P < 0.001$ . #, ##, ### Significantly lower than control at  $P < 0.05$ , 0.01 and 0.001, respectively

mutant) is shown in **Table 1**. Obtained result showed that plant height, pod per plant, harvest index, and number of nodules were significantly lower than control at probabilities of 0.05, 0.01 and 0.001 respectively. While days to flowering and days to first pod formation were significantly higher than the control at probabilities at 0.01. *Lhb* mutant is stable for most of the agronomic traits in  $M_3$  generation. This mutant exhibit maximum coefficient of variation for harvest index (12.1%) and yield/plant (9.4%) over its control (**Table 2**). Therefore, these traits have high selection value and can be exploited for the improvement of mungbean.

Several scientists have reported viable mutants in leguminous plants such as *Cicer arietinum* (Kharkwal 2000; Atta *et al.* 2003; Badigannavar and Murty 2007; Barshile and Apparao 2009; Shah *et al.* 2010, 2011; Wani 2011), *Vigna radiata* (Sangsiri *et al.* 2005; Priya Tah 2006; Reddy and Dhanasekar 2007; Anand Kumar 2009; Reddy 2009). *Glycine max* (Ahire 2008; Tambe and Apparao 2009; Manjaya 2009; Diana *et al.* 2010), *Vigna unguiculata* (Kumar *et al.* 2007, 2009), *Arachis hypogaea* (Karthika and Subha Lakshmi 2006; Manjaya and Nandanwar 2007; Nadaf *et al.* 2009), *Phaseolus vulgaris* (Patil 2009; Borkar and More 2010; Mahamune and Kothekar 2010) and in *Vigna mungo* (L.) Hepper (Goyal and Khan 2010; Bhosale and Hallale 2011).

According to Patil (2009), although the genetic material of the cell is certainly sensitive to radiation damage, both the primary and secondary physiological effects may be proving responsible for various morphological changes.

*Lhb* mutant exhibited semi-dwarf habit. Dwarf and semi-dwarf mutants were also reported earlier in (*Arachis hypogaea* (Badigannavar and Murty 2007), dwarf and semi-dwarf mutant in *Cicer arietinum* (Kharkwal 2000; Barshile 2005), *Glycine max* (Ahire *et al.* 2005; Karthika and Subha Lakshmi 2006; Manjaya and Nandanwar 2007, Manjaya 2009; Tambe 2009) and *Vigna unguiculata* (Kumar *et al.* 2009). The mutants affecting the plant height in many crops was found to be due to changes in the internodal length and sometimes because of alterations in number of nodes

(Weber and Gottschalk 1973). Such mutants have been found to accumulate very little bioactive gibberellic acid ( $GA_3$ ) and are defective in  $GA$  biosynthesis but dwarf mutants in maize such as D8-1 is  $GA$  insensitive, and cannot be rescued by  $GA$  (Swain and Olszewski 1996). According to Patil (2010), the reduction in plant height is due to destruction of auxin affecting growth of seedling. The dwarf habit of the plant save it from lodging, it also helps plants for improvement.

In  $M_2$  and  $M_3$  population of mungbean, flower mutation was also observed. In the present work 50 kR irradiation has induced variations in size and colour of the flower. Barshile and Apparao (2009) reported white and open flower mutation in cv. ‘Vijay’ of *Cicer arietinum* L. by the treatment with three different concentration of ethyl methane-sulphonate (EMS) (8, 12 and 16 mM), SA (2, 3 and 4 mM) and gamma rays (400, 500 and 600 Gy). Pod mutations (small, roundish, gigas and narrow elongated. Flower colour mutations were recorded by Manjaya (2009) in soybean variety ‘VLS-2’ by the use of 250 Gy gamma rays, Nadaf *et al.* (2009). Macoumba *et al.* (2010) recorded flower mutation in sesame cultivar ‘32-15’ and ‘38-1-7’ and Turkish cultivar ‘Birkan’ by the irradiation with 300-400 Gy dose of gamma radiation. Flower mutant showed split corolla. This character is useful for open pollination and constitutes another mechanism for hybrid seed production. High sterility was noted in the mutant of var. ‘Birkan’, due to rudimentary floral parts. Wani (2011) reported flower mutants in two varieties of *Cicer arietinum* L. from EMS, gamma rays and combination (EMS + gamma rays) treatment. Mutants isolated in this category included double flower, white-flower and non-flowering vegetative mutants. Pollen sterility ranged from 40 to 50% and very few seeds were produced. The frequency of these mutants was 0.06% (var. ‘Pusa-212’) and 0.02% (var. ‘Pusa-372’). One true breeding mutant was isolated from 0.3% EMS in ‘Pusa-372’. This mutant was characterized by violet flowers which gradually turned white instead of pink flowers in the parent variety. Seed set in this mutant was normal. In addition to the above, some non-flowering mutants with no

seed set (0.11% in var. 'Pusa-212' and 0.18% in var. 'Pusa-372') were isolated from higher dose treatments of EMS, gamma rays.

Sangsiri *et al.* (2005) reported flower and pod mutations in mungbean varieties 'KPS 2' and 'VC 6468-11-1B' with  $\gamma$  rays (Cs-137 source) at a dose of 50 kR. The flower mutant was cock's comb raceme while the pod mutant was a lobed one, with fewer seeds per pod. This trait may associate with partial sterility, causing constriction at the point where there was undeveloped seed.

Barshile (2006) has recorded white coloured flower mutant, seed coat colour and seed size mutations in chickpea variety 'Vijay' induced by 60 kR dose of  $\gamma$  radiation. Many of these were associated with various leaf and plant type mutations. Atta *et al.* (2003) reported that white flower mutants in chickpea showed an increase in yield as compared to control. Barshile (2006) does not observe any increase in yield with white flower mutations. White flower mutants were reported by Diana *et al.* (2010) and Manjaya and Nandawar (2007) in *Glycine max* by the use of 150 to 200Gy dose of gamma radiation. Flower colour mutation can be exploited as genetic markers in breeding experiments (Datta and Sengupta 2002; Atta *et al.* 2003). Patil (2009) and Borkar and More (2010) has recorded different colour flower mutations in *Phaseolus vulgaris* by the treatment of EMS and irradiated with gamma radiation.

In soybean the mutants with off-colour flower (Ahire *et al.* 2005; Manjaya 2009) and pink flower (Smutkupt 1996; Ahire *et al.* 2005; Manjaya and Nandanwar 2007; Barshile and Apparao 2009; Manjaya 2009; Tambe and Apparao 2009) were obtained in the M<sub>2</sub> population. Different flower colours in soybean were used in germplasm characterization, evolution studies, and cultivar identification (Buzzell *et al.* 1977). Pigmentation in the flowers is controlled by a number of Mendelian loci in the anthocyanin pathway. Many of these loci act pleiotropically (Fasoula *et al.* 1995). According to Datta and Banerji (1995), chromosomal aberrations, changes in chromosome number, gene mutations, rearrangement of different histogenic layers and changes in biochemical pathways leading to pigment formation, may be prime cause for flower colour mutations. Flower color mutation appeared or produced due to qualitative and quantitative changes in pigments during pigment biosynthetic pathways due to  $\gamma$  radiation (Datta 1994).

In the present investigation pod colour, pod size, pod with dense pubescence and pod shape mutants were obtained in the M<sub>2</sub> generation. Pod mutants were obtained from  $\gamma$  rays treatments in *Cicer arietinum* (Barshile 2006; Barshile and Apparao 2009), *Vigna unguiculata* (Kumar *et al.* 2007), *Glycine max* (Ahire 2008) and *Vigna radiata* (Anand Kumar 2009) and *Cyamopsis tetragonoloba* (More and Shinde 2010).

Pods of the *Lhb* mutant showed dense hairs on its surface. Pubescence density can be an important factor in controlling *Soybean mosaic virus* (SMV) infection because increased pubescence density can bring a delay in time of infection by acting as a mechanical barrier to aphid probing (Pfeiffer *et al.* 2003). Bhosale and Hallale (2011) have recorded lobed and hairy pod mutation in *Vigna mungo* (L.) Hepper. Lobed pod mutations contained fewer seeds per pod, this trait may associated with partial sterility, causing constriction at the point where there was undeveloped seeds. Diouf *et al.* (2010) reported 8 mutants in Sesame. Two mutants from a "Birkan" background had dense hairs both on capsules and stems whereas 6 from '32-15' and '38-1-7' had hairs only on the capsules. Cultivar '38-1-7' had the highest frequency ( $5.9 \times 10^{-4}$ ) while the lowest frequency was observed in '32-15' ( $1.6 \times 10^{-4}$ ). Hairiness may be related to drought tolerance. Goyal and Khan (2010) has recorded pod mutations in *Vigna unguiculata*. Pod mutations e.g. small, roundish, gigas and narrow elongated has reported by Barshile and Apparao (2009) in *Cicer arietinum* by irradiating the seeds with gamma radiation and EMS. Manjaya (2009) in Soybean variety 'VLS-2' and Tambe and Apparao (2009) in 'MACS-450' cultivar of by the use of

250Gy gamma rays, and 10, 20, 30 and 40 kR dose of  $\gamma$  radiation. Kumar *et al.* (2009) have reported non-hairy mutant and short pod mutant in *Vigna mungo* cv. 'Pant URD-19'. A non-hairy mutant line was isolated from the combination treatment of EMS (2%) and  $\gamma$  rays (30 kR). Short pod mutant was isolated from 10 kR.

Pundir and Reddy (1989) obtained a glabrous mutant from ethyl methanesulphonate (EMS)-treated chickpea seeds. The character is governed by a single recessive gene. This mutant can be useful in certain pathological studies. Glabrous plants are more damaged by insects; on the other hand there is a cost for the plant to develop these structures (Karkkainen and Agren 2002). Production of trichomes is a variable character and the inheritance acts in a simple Mendelian fashion, where glabrousness is recessive to trichome production (Karkkainen *et al.* 2004).

Hanna Engstrom (2006) studied molecular and morphological analysis of genetic polymorphisms causing glabrousness in wild populations of *Arabidopsis lyrata*. Trichome mutants were first used as convenient genetic markers (Marks 1997). The *glabrous1* (*gll*) mutant, which lacks trichomes on most surfaces, was used in early gene mapping studies (McKelvie 1965). Trichome mutants were used to calculate mutation frequencies generated using several different mutagens (Koornneef *et al.* 1982). Hulskamp *et al.* (1994) used the *Arabidopsis* mutants to define steps in a pathway to trichome development. Trichomes in *Arabidopsis* are single-celled hairs that develop from epidermal cells regularly distributed on leaves, sepals and stems. Trichome formation in *Arabidopsis* is emerging as a genetic and molecular model system for the analysis of the spatial regulation of cell type specification in plants (Uhrig and Hulskamp 2010). Interaction between mutant alleles of two genes *gl3-sst* and *sim*, which causes trichome development. The mutation in the *gl3-sst* protein modifies its ability to form a complex with the GL1 protein (a MYB transcription factor required for trichome formation), leading to changes in gene expression compared with wild type during *gl3-sst* mutant trichome development (Mark *et al.* 2007; Uhrig and Hulskamp 2010).

In the present investigation large number of seed coat colour (brown, dark green, yellowish green and black) seed size (small, bold) seed shape (round, wrinkled and elongated) mutants were obtained from different mutagenic treatments in the M<sub>2</sub> generation. The various seed-coat colour and seed size mutants by using  $\gamma$  rays have been reported by The various seed-coat colour mutants by using gamma rays have been reported by Auti and Apparao (2009) in *Vigna radaita*, Kharkwal (2000) and Bashile and Apparao (2009) in *Cicer arietinum*, Patil (2009) and Mahamune and Kothekar (2010) in *Phaseolus vulgaris* and Kumar *et al.* (2007) in *Vigna unguiculata*, Manjaya (2009) and Tambe and Apparao (2009) in *Glycine max* and Satpute and Suradkar (2011) in *Arachis hypogaea*.

Bold and small seeded mutants in soybean were induced by  $\gamma$  ray and EMS treatments individually (Karthika and Lakshmi 2006). Increased seed size is generally attributed to increased cotyledonary cell volume, retaining similar cell number within unit area (Joshua and Bhatia 1983). Bashile and Apparao (2009) have reported green, dark brown, rough seed coat mutant in *Cicer arietinum* cv. 'Vijay' by employing EMS and gamma rays. Jambhulkar and Shitre (2009) induced white, brown and black seed coat colour mutations in sunflower (*Helianthus annuus* L.) cv. 'Surya'. Manjaya (2009) and Tambe and Apparao (2009) have obtained seed coat colour mutations in soybean variety 'VLS-2' by irradiation with 250 Gy  $\gamma$  rays. Mahamune and Kothekar (2010) has recorded seed coat colour mutant in *Phaseolus vulgaris* L. by the treatment of 0.05, 0.10 and 0.15% concentrations of EMS. Highest mutation frequency was induced by 0.10% EMS. Diouf *et al.* (2010) recorded seed coat colour mutations in sesame (*Sesamum indicum* L.) - the seed coat color of all these mutants was also different from that of the source cultivars of sesame cv. 'Birkan'. Eight of them had brown seed coat while the source parents

were whitish. The other one had whitish seed coat while the parent source ('Birkan') seed coat was yellowish light brown (data not shown). This trait is related with day length sensitivity and 'Birkan' is an insensitive genetic background in comparison to the African cultivars of this study, which are very sensitive to photoperiod and causes no capsule set in the first half of the plant. Consequently, it should be considered a useful drought escape mechanism. Satpute and Suradkar (2011) have reported different colour in TAG-24 variety of *Arachis hypogaea* by irradiating seeds with different doses of  $\gamma$  radiation. The frequency of plants carrying different seed coat colour was highest in 15 kR dose of  $\gamma$ -rays treated seeds. Seeds are sometimes big or small, long, round or oval, colour can also vary, some are yellow, others are green but can also be brown and some are even black, buff or with spots. Although some of these differences are determined by growing environments, others are genetic and stable and therefore can be used for cultivar identification (Liu 1997). According to Moh (1972) seed coat colour in beans is controlled by genetic factors especially modifying factors which do not produce colours, but influence the colour produced by other factors.

Chandlee and Vodkin (1998) reported the *r-m* (ring-molted) allele of soybean that produces a mottled or variegated seed coat colour such as spots and/or concentric rings of black pigmentation superimposed on an otherwise brown seed coat. The *r-m* allele is the mutable allele reported in soybean contains an autonomous transposable element. There are 2 other mutable alleles reported in soybean are the *Y18-m* allele and the *wp-m* allele (Xu and Palmer 2005). The mutable line with the *Y18-m* allele displays variegated green/yellow leaves and the mutable line with the *wp-m* allele produces variegated flower color.

The seed coat is a maternally derived tissue and should reflect the genotype of the parent plant. Todd and Vodkin (1996) reported that the dominant *I* gene inhibits accumulation of anthocyanin pigments in the epidermal layer of soybean seed coats. It was suspected that a transposon was responsible for the *k2* (tan-saddle seed coat) mutant phenotypes (Palmer *et al.* 1989; Xu and Palmer 2005).

Hilum color is also a major factor in cultivar identification. Hilum color as well as other physical appearances has become important factors in determining the type of food application for a particular soybean cultivar (Liu 1997). However, yellow hilum cultivars are more susceptible to low temperatures, resulting in reduced seed yield and poor seed quality compared with brown hilum cultivars (Takahashi 1997).

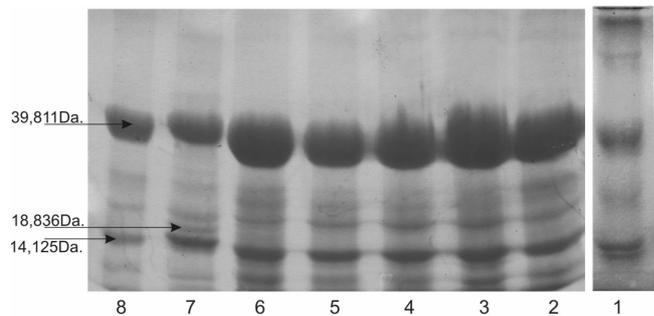
In the present investigation the 50 kR dose of gamma rays might be affecting the genes, which are causing changes in metabolism of mutant characters or causes gene mutations or various chromosomal aberrations may be the reason for semi-dwarf habit, flower mutation and seed mutations in mungbean. Rearrangement of different histogenic layers and alternations occurring in biochemical pathways leading to pigmentation formation, may be the reason for flower mutations (Datta and Banerjee 1995).

### Total protein profile of *Lhb* mutant

In the present study the SDS-PAGE analysis of seed proteins of *tall mutant*, *dwarf mutant*, *high yielding mutant*, *early maturing mutant* and *Lhb* mutant and their controls showed interesting results. *Lhb* mutants did differ with other mutants and controls in their protein-banding pattern (Fig. 2).

No variation could be observed in the number, position and molecular weight of polypeptides of control and *tall mutant*, *dwarf mutant* and *high yielding mutant* except *Lhb* and Early maturing mutants. *Lhb* mutants differ from other mutants and controls in their protein-banding pattern.

The *Lhb* mutant had 7 polypeptide bands. These bands differed from those of control in position and molecular weight. Molecular weight of these polypeptides ranged from 94,406 to 11,885 Da. The molecular weight of the nine



**Fig. 2** Electrophoregram of total protein of *Lhb* mutant in mungbean. Lane (1) Marker, (2) Kopargaon-1 (control), (3) Vaibhav (control), (4) *Tall* mutant, (5) *Dwarf* mutant, (6) *High yielding* mutant, (7) *Early maturity* mutant, (8) *Lhb* mutant.

bands was 39,811, 28,184, 25,119, 22,387, 17,783, 14,125 and 11,885 Da. *Lhb* mutant was characterized by the presence of dense, narrow and light bands. Band number 7 and 3 in *Lhb* mutant was narrow and light as compared to 7<sup>th</sup> band of other mutants. Band number 3 was dark and broad in all the mutants and controls.

Mutation breeding is an important method used for the improvement of crops through the induction of mutations at loci that control economically important traits and/or by eliminating undesirable genes from elite breeding lines (Patil 2009). In common with other plant breeders, the legume breeder's prime objective has been to develop genotype capable of producing optimal yield of satisfactory quality. Improvement in either single or few polygenically controlled economic traits and quality attributes is not normally achieved by hybridization within shortest possible time. Furthermore, although selection for an economically useful spontaneous mutations still takes place with considerable success, purposeful induction of a specifically desired mutations at specific time and place and in selected genotype can be a much more attractive option (Harten 1998). One of the chief advantages of traditional mutagenesis is that it can give rise to many different mutant alleles with different degree of trait modification. This variation in expression is very useful in many basic studies, such as identification of amino acid residues critical for enzyme activity (Chopra 2005).

Mutation techniques have been successfully used in generating vast genetic variations and breeding thousands of new crop varieties (Harten 1998; Ahloowalia *et al.* 2004; Shu and Lagoda 2007). Recent advances in genomics have further stimulated great interest in induced mutations for functional genomic studies in plants (Waugh *et al.* 2006). In recent years, a couple of mutant germplasm panels have been developed for forward and reverse genetic studies (Wu *et al.* 2005; Jia *et al.* 2006).

### CONCLUSION

The *Lhb* mutant raised through  $\gamma$  radiation induced mutagenesis, in the present investigation, seems to be promising at least for two characters i.e., semi-dwarf habit and presence of thick dense hairs on the pods. The semi-dwarf habit of the plant enables it as lodging resistant. The thick dense hairs on the pod helps in protecting them from insects and caterpillar predators and seems to be promising economical important character. It can be used or incorporated in breeding programmes that are aimed at genetic improvement of mungbean.

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## REFERENCES

- Acharya SN, Thomas JE, Basu SK (2007) Improvement in the medicinal and nutritional properties of fenugreek (*Trigonella foenum-graecum* L.). In: Acharya SN, Thomas JE (Eds) *Advances in Medicinal Plant Research*, Research Signpost, Trivandrum, Kerala, India 554 pp
- Ahire DD (2008) Isolation and characterization of induced mutants for some morphological and agronomic traits and oil quality in soybean (*Glycine max* (L.) Merr.). PhD thesis, University of Pune, 252 pp
- Ahire DD, Thengane RJ, Manjaya JG, George M, Bhide SV (2005) Induced mutations in soybean [*Glycine max* (L.) Merr.] cultivar 'MACS 450' *Soybean Research* 3, 1-8
- Ahloowalia B, Maluszynski M, Nichterlein K (2004) Global impact of mutation derived varieties. *Euphytica* 135, 187-204
- Atta BM, Ahsan ul Haq M, Shah TM, Sadiq M, Hassan M, Syed H (2003) Induced flower colour mutations in chickpea. *International Chickpea and Pigeonpea Newsletter* 10, 6-7
- Auti SG, Apparao BJ (2009) Induced mutagenesis in mungbean (*Vigna radiata* (L.) Wilczek). In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 97-100
- Badigannavar AM, Murty GS (2007) Genetic enhancement of groundnut through gamma ray induced mutagenesis. *Plant Mutation Reports* 1 (3), 16-21
- Barshile JD (2006) Induction of genetic variability in *Cicer arietinum* employing sodium azide, ethyl methane sulphonate and gamma radiations. PhD thesis, University of Poona, 205 pp
- Barshile JD, Apparao BJ (2009) Genetic improvement of chickpea (*Cicer arietinum* L.) using induced mutations. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 91-94
- Borkar AT, More AD (2010) Induced flower colour mutations in *Phaseolus vulgaris* L. through physical and chemical mutagen. *Advances in Bioresearch* 1, 22-28
- Brown GG (2003) The radish *Rfo* restorer gene of ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. *Plant Journal* 35, 262-272
- Bhosale UP, Hallale BV (2011) Gamma radiation induced mutations in black gram (*Vigna mungo* (L.) Hepper) *Asian Journal of Plant Science and Research* 1 (2), 96-100
- Buzzell R, Butter B, Bernard R (1977) Inheritance and linkage of a magenta flower color gene in soybeans. *Canadian Journal of Genetic and Cytology* 19, 749-751
- Chandlee JM, Vodkin LO (1998) An unstable mutation affecting soybean seed coat colour. *Soybean Genetics Newsletter* 15, 177-179
- Chopra V (2005) Mutagenesis: Investigating the process and processing the outcome for crop improvement. *Current Science* 89, 353-359
- Dadlani M, Varier A (1993) Electrophoresis for variety identification. Technical Bulletin published by Division of Seed Science and Technology. IARI, New Delhi
- Datta AK, Sengupta K (2002) Induced viable macromutations in coriander (*Coriandrum sativum* L.). *Indian Journal of Genetics and Plant Breeding* 62 (3), 273-27
- Datta SK (1994) Radiation induced ornamental varieties developed at NBRI Lucknow. In: *DAE - BRNS Symposium on Nuclear Application in Agriculture, Animal Husbandry and Food Preservation*, 16- 18 March, New Delhi, pp 11-12
- Datta SK, Banerji BK (1995) Chromosome counting in original and gamma ray induced mutants of chrysanthemum. *Journal of Nuclear Agricultural Biology* 24 (1), 58-61
- Datta SK (1992) Radiosensitivity of garden *Chrysanthemum*. *Journal of Indian Botanical Society* 71, 283-284
- Datta SK, Chakrabarty D (2009) Management of chimera and in vitro mutagenesis for development of new flower colour/shape and chlorophyll variegated mutants in chrysanthemum. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 303-305
- Diana SH, Trikoese M, Sudirman Y, Desta W (2010) Induced mutations by gamma rays to Argomulyo soybean (*Glycine max*) variety. *Biosciences* 2 (3), 121-125
- Diouf M, Boureima S, Tahir D, Ihan M, Irgan ÇA (2010) Gamma rays-induced mutant spectrum and frequency in sesame. *Turkish Journal of Field Crops* 15 (1), 99-105
- Fasoula D, Todd J, Stephens P, Nickell C (1995) Linkage analysis with genes and molecular markers of the soybean anthocyanin pathway. Vodkin Plant Genome IV Conference Town & Country Conference Center, San Diego, CA
- Goyal S, Khan S (2010) Induced mutagenesis in urdbean (*Vigna mungo* L. Hepper): A review. *International Journal of Botany* 6, 194-206
- Hanna E (2006) Molecular and morphological analysis of genetic polymorphisms causing glabrousness in wild populations of *Arabidopsis lyrata*. MSc theses, Molecular Cell Biology, Södertörn University College
- Harten VAM (1998) *Mutation Breeding, Theory and Practical Applications*, Cambridge University Press, Cambridge, pp 1-353
- Hulskamp M, Misera S, Jurgens G (1994) Genetic dissection of trichome cell development in *Arabidopsis*. *Cell* 76, 555-556
- Jia X, Li S, Xiao X, Guo X, Zhang Q (2006) Molecular epidemiology of mtDNA mutations in 903 Chinese families suspected with Leber hereditary optic neuropathy. *Journal of Human Genetics* 51 (10), 851-856
- Jambhulkar SJ, Shitre AS (2009) Development and utilization of genetic variability through induced mutagenesis in sunflower (*Helianthus annuus* L.). In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 104-105
- Jones DF (1945) Heterosis resulting from degenerative changes. *Genetics* 30, 527-542
- Kang SY, Kim DS, Lee GJ (2007) Genetic improvements of crop plants by mutation techniques in Korea. *Plant Mutation Reports* 1 (3), 7-15
- Karkkainen K, Agren J (2002) Genetic basis of trichome production in *Arabidopsis lyrata*. *Hereditas* 136, 219-226
- Karkkainen K, Loe G, Agren J (2004) Population structure in *Arabidopsis lyrata*: Evidence for divergent selection on trichome production. *Evolution* 58, 2831-2836
- Karthika R, Subbalakshmi B (2006) Effect of gamma rays and EMS on two varieties of soybean. *Asian Journal of Plant Sciences* 5 (4), 721-724
- Khan S, Wani MR, Kouser P (2004) Induced genetic variability for quantitative traits in *Vigna radiata* (L.) Wilczek. *Pakistan Journal of Botany* 36 (4), 845-850
- Kharkwal MC (2004) Mutation breeding for crop improvement. In: Jain HK, Kharkwal MC (Eds) *Plant Breeding - Mendelian to Molecular Approaches*, Narosa Publishing House, New Delhi, India, pp 602-638
- Kharkwal MC (2000) Induced mutations in chickpea (*Cicer arietinum* L.) IV. Types of macro mutations induced. *Indian Journal of Genetics* 60, 305-320
- Koornneef M, Dellaert SWM, Veen JH (1982) EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L.) Heynh. *Mutation Research* 93, 109-123
- Kumar A, Mishra MN, Kharkwal MC (2007) Induced mutagenesis in blackgram (*Vigna mungo* L.) Hepper. *Indian Journal of Genetics* 67 (1), 41-46
- Kumar A, Parmhansh P, Prasad R (2009) Induced chlorophyll and morphological mutations in mungbean (*Vigna radiata* L. Wilczek). *Legume Research* 32 (1), 41-45
- Kumar V, Sharma AK, Singh VP, Kumar M (2009) Characterization of pre-breeding genetic stocks of urdbean (*Vigna mungo* L. Hepper) induced through mutagenesis. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 391-394
- Lal N, Mishra R (2006) Induced genetic variability and divergence in M<sub>2</sub> generation of mungbean. *Indian Journal of Pulses Research* 19 (1), 47-49
- Liu KS (Ed) (1997) Soybeans - chemistry, technology and utilization. In: *Agro-nomic Characteristics, Production and Marketing*, Chapman and Hall, New York, pp 1-24
- Lowry OH, Rosebroough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. *Biochemistry* 15, 529-536
- Mahamune SE, Kothekar VS (2010) Induced mutations in French bean (*Phaseolus vulgaris* L.) affecting seed coat colour. *Journal of Experimental Sciences* 3 (5), 1-4
- Maluszynski M, Fuglewicz A, Szarejko I, Micke A (1989) Barley mutant heterosis. In: Maluszynski M (Ed) *Doubled Haploids, Mutants and Heterosis*, Kluwer Academic Publisher, Dordrecht, The Netherlands, pp 129-146
- Maluszynski M, Van Zanten L, Ashir A, Brunner H, Ahloowalia B, Zapata FJ, Week E (1995) Mutation techniques in plant breeding. In: *IAEA/FAO Symposium on Induced Mutations and Molecular Techniques for Crop Improvement*, 19-23 June, Vienna, pp 489-504
- Manjaya JG, Nandanwar RS (2007) Genetic improvement of soybean variety JS-80-21 through induced mutations. *Plant Mutation Reports* 1 (3), 36-40
- Manjaya JG (2009) Genetic improvement of soybean variety VLS-2 through induced mutations. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 106-107
- Marks MD (1997) Molecular genetic analysis of trichome development in *Arabidopsis*. *Annual Review of Plant Physiology and Plant Molecular Biology* 48, 137-141
- Marks M, Gilding E, Wenger J (2007) Genetic interaction between glabra3-shapeshifter and *siamese* in *Arabidopsis thaliana* converts trichome precursors into cells with meristematic activity. *The Plant Journal* 52, 352-361
- McKelvie A (1965) Preliminary data on linkage groups in *Arabidopsis*. In: Robbelen G (Ed) *Arabidopsis Research*, Gerd Wasmund & Co., Gelsenkirchen, pp 79-84
- Moh CC (1971) Mutation breeding in seed coat colour of bean (*Phaseolus vulgaris* L.). *Euphytica* 20, 119-125
- Nadaf HL, Kaveri SB, Madhusudan K, Motagi BN (2009) Induced genetic variability for yield and yield components in peanut (*Arachis hypogaea* L.). In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 346-348
- Palmer RG, Hedges BR, Benavente RS, Groose RW (1989) w4-mutable line in soybean. *Developmental Genetics* 10, 542-51
- Patil MT (2009) Genetic improvement of cowpea for agronomic traits through mutation breeding. PhD thesis, University of Pune, 210 pp

- Patil S, Nair B, Maheshwari JJ, Pillewan S** (2003) Variability studies in M<sub>2</sub> and M<sub>3</sub> generation of soybean mutants. *Advances in Plant Sciences* **16** (1), 295-299
- Pfeiffer TW, Peyyala R, Ren Q, Ghabrial SA** (2003) Increased soybean pubescence density: Yield and soybean mosaic virus resistance effects. *Crop Science* **43**, 2071-2076
- Pundir RPS, Reddy KN** (1989) Induction, genetics and possible use of glabrousness in chickpea. *Euphytica* **42**, 1-2
- Reddy KS, Dhanasekar P** (2007) Induced mutations for genetic improvement of mungbean, urdbean and cowpea pulse crops in India. In: Tomar BS (Ed) *LANCAS Bulletin 303* (Vol VI), Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Center, Trombay, pp 299-307
- Reddy KS** (2009) A new mutant for yellow mosaic virus resistance in mungbean (*Vigna radiata* (L.) Wilczek) variety SML-668 by recurrent gamma-ray irradiation. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 361-362
- Sahu BC, Patra G** (1997) Evaluation of quantitative characteristics in some mutant lines of green gram. *Indian Journal of Agricultural Sciences* **67**, 533-535
- Sangsiri C, Worawit S, Peerasak S** (2005) Gamma radiation induced mutations in mungbean. *Science Asia* **31**, 251-255
- Shah TM, Atta BM, Alam SS, Ali H, Haq MA, Hassan M** (2010) High yielding kabuli mutant chickpea (*Cicer arietinum* L.) variety "CM 2008". *Pakistani Journal of Botany* **42** (5), 3533-3545
- Sharma CM, Haque MF** (1983) Induced mutations in mungbean (*Vigna radiata* (L.) Wilczek). In: *XV<sup>th</sup> International Congress Genetics*, 12-21 December, 1983, New Delhi, India p 329 (Abstract)
- Shinde MS, More AD** (2010) Study of pollen sterility in *Cyamopsis tetragonoloba* through mutagenesis. *Asian Journal of Experimental Biological Sciences* **s1**, 31-34
- Shu QY, Lagoda PJJ** (2007) Mutation techniques for gene discovery and crop improvement. *Molecular Plant Breeding* **5**, 193-195
- Singh AK, Singh KP** (2003) Induced quantitative variation for yield and its components in okra (*Abelmoschus esculentus* (L.) Moench. *Advances in Plant Science* **16** (1), 519-525
- Singh A** (2009) Induced genetic variability in M<sub>3</sub> generation of mungbean. *Journal of Food Legumes* **22** (3), 166-170
- Smutkupt S** (1996) Observations of unstable genes of flower and seed coat colours in soybean derived from a 'Wakashima' mutant. *Soybean Genetics Newsletter* **23**, 120-122
- Swain SM, Olszewski NE** (1996) Genetic analysis of gibberellin signal transduction. *Plant Physiology* **112**, 11-17
- Tah P** (2006) Induced macromutation in mungbean [*Vigna radiata* (L.) Wilczek]. *International Journal of Botany* **2** (3), 219-228
- Takahashi R** (1997) Association of soybean genes I and T with low-temperature induced seed coat deterioration. *Crop Science* **37**, 1755-1759
- Tambe AB, Apparao BJ** (2009) Gamma-ray induced mutations in soybean (*Glycine max* (L.) Merrill) for yield contributing traits. In: Shu Q-Y (Ed) *Induced Plant Mutations in the Genomics Era*, Food and Agriculture Organization of the United Nations Rome, pp 95-96
- Todd JJ, Vodkin LO** (1996) Duplications that suppress and deletions that restore expression from a chalcone synthase multigene family. *Plant Cell* **8**, 687-699
- Uhrig JF, Hülskamp M** (2010) Trichome development in *Arabidopsis*. *Methods in Molecular Biology*, Springer, New York, pp 77-88
- Wani AA** (2011) Spectrum and frequency of macromutations induced in chickpea (*Cicer arietinum* L.). *Turkish Journal of Biology* **35**, 221-231
- Waugh R, Leader DJ, McCallum N, Caldwell D** (2006) Harvesting the potential of induced biological diversity. *Trends in Plant Science* **11**, 71-79
- Wu JL, Wu C, Lei C, Baraoidan M, Bordeos A, Madamba MRS, Ramos-Pamplona M, Mauleon R, Portugal A, Ulat VJ, Bruskiewich R, Wang G, Leach J, Khush G, Leung H** (2005) Chemical and irradiation induced mutants of indica rice IR64 for forward and reverse genetics. *Plant Molecular Biology* **59**, 85-97
- Xu M, Palmer RG** (2005) Genetic analysis and molecular mapping of a pale flower allele at the *W4* locus in soybean. *Genome* **48**, 334-340