Available online at www.elixirpublishers.com (Elixir International Journal)

Applied Botany

Elixir Appl. Botany 146 (2020) 54799-54803

ISSN: 2229-712X

Increase in Proteins and Pigments using Physical and Chemical Mutagenesis in Faba Bean

Shahnawaz Khursheed* and Samiullah Khan

Mutation Breeding Lab. Aligarh Muslim University Aligarh, India, 202 002.

ARTICLE INFO

Article history:

Received: 5 August 2020;

Received in revised form:

5 September 2020;

Accepted: 17 September 2020;

Keywords

Faba Bean,

Gamma Rays,

Ethyl Methnesulphonate,

SDS-PAGE.

ABSTRACT

Induced mutations through physical and chemical mutagens is one of the sophisticated and convenient approach to induce desirable variability in plants compared to the conventional methods. Induced mutagenesis has served an important source in producing high yielding mutants. The present experiment was conducted to induce, isolate and analyse the high yielding mutants of *Vicia faba* L. var. Vikrant and PRT-12 of M_3 generation using single and combination treatments of gamma rays and ethyl methanesulphonate (EMS). The leaves of plants are known to carry out the most important processes of plants relating to yield and productivity. The pigments like chlorophyll and carotenoids play key roles in the process of photosynthesis. Biochemical analysis like estimation of chlorophyll and carotenoid contents, nitrate reductase activity (NRA) and protein profiling of the leaves of high yielding mutants were carried out using different methods and approaches. High yielding mutant plants were selected from the lower doses/concentrations of both single and combined treatments of γ-rays and ethyl methanesulphonate (EMS). Increased levels of pigments and NR activity were observed in the variety Vikrant as compared to PRT-12, after treatment of the mutagens. Furthermore, SDS-PAGE analysis of leaf proteins of the mutants of the variety Vikrant showed higher polymorphism and number of protein bands as compared to variety PRT-12. Altogether, the increase in leaf protein content may enhance crop yield.

Introduction

Induced mutagenesis has gained a lot of popularity throughout the world because of its property to induce desirable variations in a very short period of time. The high content of proteins in pulses makes scientists focus on developing high yielding mutants of plants to overcome various worldly problems like hunger and malnutrition (Khursheed *et al*., 2015). The pigments and proteins present in leaf controlling various important processes in plants like photosynthesis are the major point of focus in developing high yielding varieties. Because of direct correlation between leaf proteins and pigments with food synthesizing machinery in plants, it very interesting to work on these proteins during the induction of mutation. *Vicia faba* L. is an important nutritional and hardy pulse crop. The continuously increasing demand of protein rich material for human and animal consumption has made the pulse crops as an interesting material because of high protein contents (Santalla *et al*., 2001).Low variability in most of the pulses due to autogamous nature makes scientists to think and develop some new and improving polygenic characters and yield (Khursheed *et al*., 2015). Mutation breeding is of great importance in crop improvement involving refined selection and detection innovative methods for inducing the variability in plants. Various approaches have been used these days to induce the desirable variability in plants of which mutation breeding forms an important part. Mutation breeding can be effectively utilised in method. The varietal description and assessment of different cultivars because of changes in the protein complement has become possible due to induced mutation (Khursheed and Khan, 2016).

The genetic makeup of plants can be best investigated by the technique known as SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Amjad *et al*., 2012). The protein profiling using SDS-PAGE has been considered as an important tool for germplasm characterization (Hameed *et al*., 2009). The diversity in different crop varieties, wild relatives and phylogenetic relationships of plants can be best analysed and characterised by protein profiling [32]. The genetic diversity based on protein profiling has also been reported in *Phaseolus vulgaris* and *Cicer arietinum* (Hameed *et al*., 2009). The different varieties exhibit a particular banding pattern of proteins on the basis of which the particular species can be identified through SDS-PAGE technique. Protein profiling through SDS-PAGE forms an important part of proteomics and is utilised in quantifying and identifying the proteins contained in biological samples. Protein profiling through SDS-PAGE has been used as an important tool to determine the taxonomic and evolutionary aspects in several crop plants. The identification of protein increase through SDS-PAGE in the high yielding mutants can prove a handy tool to the scientists by identifying the selected protein increase and target them in the future crop improvement programmes. Protein profiling is considered as an important tool for germplasm characterization because of unaltered effect of environment on storage proteins (Hameed *et al*., 2009).

Tele:

E-mail address: shahnawazkhursheed95@gmail.com

Protein profiling has proven an excellent method to differentiate the varieties of same genus. Sometimes no clear differentiation is observed for origin and agronomic characters due to low inter-specific genetic diversity as has been already reported (Ghafoor *et al*., 2002). Protein profiling has proved reliable and economical technique for distinguishing different cultivars (Irfan, 2011). However, many other authors have reported contradictory to the above showing that cultivar differentiation from this method is not possible (Ahmad and Slinkard, 1992).

The present experiment was conducted to induce, isolate and analyse the high yielding mutants in M_3 generation of *Vicia faba* L. using single and combination treatments of gamma rays and EMS. Two varieties viz., Vikrant and PRT-12 were used in this experiment. Mutants for protein profiling were isolated from both the varieties.

Materials and Methods

Two varieties of *Vicia faba* L. viz., Vikrant and PRT-12 were used in this experiment. Thirteen sets of seeds of both the varieties were used in M_1 generation. First four sets of seeds were treated with individual doses of gamma rays (100, 200, 300 and 400 Gy). Irradiation of seeds was done through radioisotope ^{60}CO source (Gamma chamber Model-900 supplied by Bhabha Atomic Research Centre, Mumbai, India) at the National Botanical Research Institute, Lucknow, Uttar Pradesh, India. Another four sets of seeds were treated with individual concentrations of EMS (0.01, 0.02, 0.03 and 0.04 % EMS). Ethyl methanesulphonate (EMS) ($CH_3SO_3C_2H_5$), a monofunctional alkylating agent is manufactured by Sissco Research Laboratories Pvt. Ltd., Mumbai, India. For EMS treatments, healthy seeds of uniform size of each variety were presoaked for 9 hrs in distilled water and treated with 0.01, 0.02, 0.03 and 0.04 % of EMS for 6 hrs with intermittent shaking at room temperature of 22±1°C. The solution of EMS was prepared in the phosphate buffer of pH 7. Only freshly prepared solutions were used for all the treatments. The pH of the solution was maintained by using buffer tablets manufactured by MERCK manufactures, Mumbai, India. After treatment, the seeds were thoroughly washed in running tap water to remove the excess of mutagen. The last four sets of seeds were treated with combination treatments of gamma rays and EMS (100 Gy gamma rays + 0.01 % EMS, 200 Gy gamma rays+ 0.02 % EMS, 300 Gy gamma rays + 0.03 % EMS and 400 Gy gamma rays + 0.04 % EMS). The last one set of seeds was taken as control. The seeds were sown in the Agricultural Farm, Aligarh Muslim University, India to raise the M_1 generation. Seeds of M_0 generation, which are true breeding, were used in M_1 generation. Seeds were collected from the normal looking plants of M_1 generation and were sown to raise M_2 generation. For raising M_2 generation, seeds were taken only from the normal looking M_2 plants. For raising M_3 generation, only those treatments were selected which gave the maximum seed yield in M_2 generation. For each of these treatments, 10 M_2 progenies were selected which showed significant deviations in mean values in the positive direction from the mean values of control, particularly for the yield components under study in M_2 generation. Mutant plants which were much superior to others with regard to seed yield per plant were selected in M_3 generation. Mutants for analysis of pigments, NRA and protein profiling of leaf soluble proteins were isolated in M_3 generation. Leaves of both control and mutants selected from both Vikrant and PRT-12 were used for protein profiling.

2.1. Estimation of chlorophyll and carotenoid contents

The chlorophyll and carotenoid contents of leaves were estimated by the method of MacKinney (1941).

One gram of finely cut fresh leaves was ground to a fine pulp using a mortar and pestle after pouring 20 ml of 80 % acetone. The mixture was centrifuged at 5,000 rpm for 5 minutes. The supernatant was collected in 100 ml volumetric flask. The residue was washed three times, using 80 % acetone. Each washing was collected in the same volumetric flask and volume was made up to the mark (100 ml) using 80 % acetone. The absorbance in terms of optical density was measured at 645 and 663 nm for chlorophyll and 480 and 510 nm for carotenoid against the acetone (80 %) as blank on the spectrophotometer (Spectronic 20D, Milton Roy, USA [4].

Total chlorophyll (mg g^{-1}leaf fresh mass) =

$$(20.2(OD645) + 8.02(0D633)) \times \frac{V}{1000 \times W}$$

Total chlorophyll (mg g^{-1}leaf fresh mass) =

$$\frac{7.6\,(OD480) - 1.49\,(OD510)}{d \times 1000 \times W} \times 100$$

Where,

OD_{645}, OD_{663}, OD_{480}, OD_{510} = Optical densities at 645, 663, 480 and 510 nm respectively

V = Volume of an extract

W = Mass of leaf tissues

d = Length of light path (d= 1.4 cm)

2.2. Assay for nitrate reductase activity (NRA)

The activity of nitrate reductase (EC 1.7.7.2) was measured in fresh leaf samples (Jaworski, 1971). The leaves were cut into small pieces avoiding the mid veins. 200 mg of these chopped leaves were weighed and transferred to plastic vials. To each vial, 2.5 ml of 0.1M phosphate buffer pH 7.4 and 0.5 ml of 0.2M potassium nitrate solution was added followed by the addition of 2.5 ml of 5% isopropanol. These vials were incubated in B.O.D. incubator for 2 hours at 30±2 ºC in dark. 0.4 ml of incubated mixture was taken in a test tube (Borosil) to which 0.3 ml each of 0.1% sulphanilamide solution and 0.02 % NED-HCL (N-1-nehthyl-ethylenediamine dihydro chloride) were added. The test tube was left for 30 minutes, for maximum colour development. The mixture was diluted to 5 ml with double distilled water (DDW). The absorbance in terms of optical density was measured at 540 nm on the spectrophotometer (Spectronic 20D, Milton Roy, USA). A blank consisting of 4.4 ml DDW and 0.3 ml each of sulphanilamide and NED-HCL were used simultaneously for comparison with each sample. A standard graded concentration of sodium nitrite ($NaNO_2$) from a standard aqueous solution of the salt were used. The absorbance of each sample was compared with that of the calibration curve and NRA (nmol $g^{-1}h^{-1}$ FW) was computed on a fresh mass basis.

2.3. Preparation of gel for protein profiling

2.3.1. PREPERATION OF SAMPLE

Extraction of protein for gel electrophoresis was made from a fresh leaf of the plant. Leaf tissues were homogenized using a cold mortar and pestle in extraction buffer (potassium phosphate–KOH buffer 0.2 M, pH 8.0) containing:5 mM EDTA, 5mM DTT, 0.2 mM PMSF and 50 % (w/w) PVP with a small amount of quartz sand. Extracts were centrifuged at 27,000×g for 10 min at 4 °C, and the resulting supernatant was desalted through sephadex PD10 columns Amersham Biosciences equilibrated with the extraction buffer (50 mM, pH 7.5). All procedures took place at 4°C. The desalted

extracts were stored at −20 ˚C and used as the source of total soluble protein for all subsequent analyses**.**

2.3.2. Preperation of Gel for SDS-PAGE

SDS-PAGE was essentially performed by the Tris-glycine system using slab gel electrophoresis apparatus (Laemmli, 1970).. Concentrated stock solution of 30 % acrylamide containing 0.8 % N, N' methylene bis-acrylamide and 1.5 M Tris, pH 8.8 were mixed in appropriate proportions to give desired percentage of gel. Protein samples were prepared in solution containing 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.001 % (w/v) bromophenol blue. The samples were incubated at 100 ^{o}C for 5 min. Electrophoresis was performed at 100 V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCl (pH 6.8).

2.3.3. Coommassie blue staining

After electrophoresis the gels were stained with five times of a gel volume of coommassie brilliant blue R-250 in 50% methanol and 10% acetic acid for at least 4 hrs. For destaining, the gels were incubated with shaking in 5% methanol and 7.5 % glacial acetic acid at room temperature.

2.4. Significance test

Data was statistically analysed and the means were compared by Duncan's Multiple Range Test (DMRT) at p≤0.05 through *SPSS 17.0 software* (Chicago: *SPSS Inc*.).

Results

Mutant plants were selected from 100 Gy gamma rays and 0.01 % EMS in the variety Vikrant and in 100 Gy gamma rays and 100 Gy gamma rays+0.01 % EMS in the variety PRT-12(Table 1). Mutants selected from the variety Vikrant showed more positive response in increasing chlorophyll, carotenoid and NRA than the mutants selected from the variety PRT-12. Protein profiling of mutants selected from the variety Vikrant showed more polymorphism and expression of proteins than of the variety PRT-12.

Table 1. Selection of mutants in two varieties of *Vicia faba* L. and their respective dose/concentration of mutagen.

Treatments	Selection of mutants	
	Variety Vikrant	VarietyPRT-12
100 Gy	*	*
200 Gy	#	#
300 Gy	#	#
400 Gy	#	#
0.01% EMS	*	#
0.02% EMS	#	#
0.03% EMS	#	#
0.04% EMS	#	#
100 Gy + 0.01% EMS	#	*
200 Gy + 0.02% EMS	#	#
300 Gy + 0.03% EMS	#	#
400 Gy + 0.04% EMS	#	#

*= Mutants selected #= Mutants not selected

3.1. Chlorophyll, carotenoid and NRA of the mutants of both the varieties

Data showing the chlorophyll, carotenoid and NRA is presented in Table 2.

3.1.1. Chlorophyll content

In the variety Vikrant, the control plants showed the chlorophyll content of 2.71 mg.g^{-1}FW, while as the mutant plants selected from the variety Vikrant showed significant increase in chlorophyll content from 3.09-3.16 mg.g^{-1}FW. In the variety PRT-12, the control plants showed the chlorophyll content of 2.53 mg.g^{-1}FW, whereas the mutant plants selected from this variety also showed significant increase in chlorophyll content from 2.87-2.96 mg.g^{-1}FW.

Table 2. Variation in the photosynthetic pigments and nitrate reductase activity of high yielding mutants selected from two varieties of *Vicia faba* L. in M_3 generation.

Strain No.	Chlorophyll (mg.g^{-1}FW) $\bar{x}$ ± S.E	Carotenoid (mg.g^{-1}FW)$\bar{x}$ ± S.E	NRA (nmol. h^{-1}.g^{-1}FW)$\bar{x}$ ± S.E
Control (Vikrant)	2.71^{b}	0.25^{c}	772.35^{c}
Mutant-A	3.09^{a}	0.35^{b}	784.13^{b}
Mutant-B	3.16^{a}	0.46^{a}	788.40^{a}
Control (PRT-12)	2.53^{c}	0.23^{c}	771.43^{c}
Mutant-C	2.87^{b}	0.33^{a}	776.13^{a}
Mutant-D	2.96^{a}	0.30^{b}	774.56^{b}

Different letters show significant difference at p≤0.05(Dunkan's multiple range test). Strains- A, B, C and D are used for the names of mutants.

3.1.2. Carotenoid content

In the variety Vikrant, the control plants showed carotenoid content of 0.25 mg.g^{-1}FW, whereas the mutants showed the content ranging from 0.35-0.46 mg.g^{-1}FW. In the variety PRT-12, the control plants showed the carotenoid content of 0.23 mg.g^{-1}FW, whereas the mutant strains from this variety showed the content ranging between 0.30-0.33 mg.g^{-1}FW.

3.1.3. Nitrate reductase activity (NRA)

Nitrate reductase activity of control leaves of the variety Vikrant was observed as 772.35 nmol. h^{-1}.g^{-1}FW, whereas the mutants isolated from the variety showed NRA from 784.13-788.40 nmol. h^{-1}.g^{-1}FW. In variety PRT-12, the control plant leaves showed NRA as 771.43 nmol. h^{-1}.g^{-1}FW, whereas, the range of NRA of mutant plants from the variety PRT-12 showed range from 774.56-776.13 nmol. h^{-1}.g^{-1}FW.

3.2. Protein Profiling

Protein profiling of mutants (Fig. 1) isolated from both the varieties are shown in Fig. 2. Proteins were isolated in different ranges of their molecular weights ranging from 29-97 kD. Mutants selected from the variety Vikrant showed more polymorphism and expression of proteins than of the variety PRT-12. More increase and expression of proteins was observed in 29 kD proteins, 43 kD and 66 kD proteins in the mutants of the variety Vikrant than of the PRT-12.

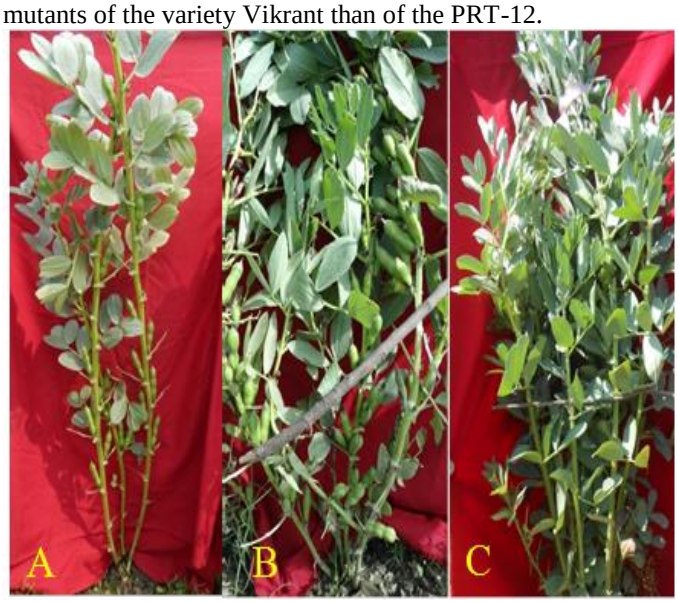

Fig 1. Control plant A: and Mutant plants B: Variety PRT-12 & C: Variety Vikrant.

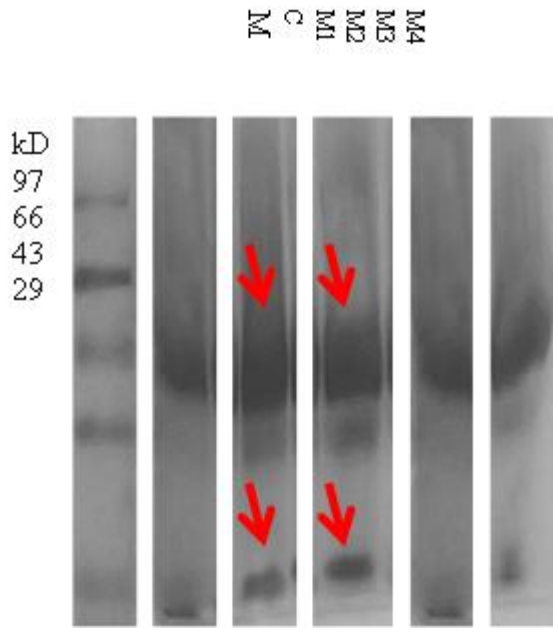

Fig 2. SDS-PAGE of leaf soluble proteins of high yielding mutants of faba bean.

M: Marker
C: Control
M1: Mutant 1 (100 Gy)
M2: Mutant 2 (0.01% EMS)
M3: Mutant 3 (100 Gy)
M4: Mutant 4 (100 Gy gamma rays+0.01% EMS)

Discussion

Results show an increase in chlorophyll, carotenoid and NRA in mutant plants of both the varieties. But the increase in the variety Vikrant was more than the increase shown by variety PRT-12 (Table 2). Increase in chlorophyll, carotenoid and NRA in mutants may be due to the stimulatory effect of lower doses/concentrations of mutagens on the genes controlling the biosynthesis of the pigments and enzyme involved in nitrogen metabolism. Many authors have reported the increase of chlorophyll content compared to control after treatment with different doses of gamma rays as reported in wheat and rice (Shereen *et al*., 2009). This increase in photosynthetic pigments and NRA may be reason for the better increase in yield and other characters of plants.

The considerable effects of gamma rays on physiological and biochemical processes in plants has already been studied by many workers (Heidarieh *et al*., 2012). Effect of gamma rays on biological processes in cells occurs through the production and interaction of free radicals with the biological system [26]. Free radicals can modify and damage the plant cell components and many workers have reported the effect of gamma rays on biochemical, physiology and anatomy of plants (Ashraf, 2003). Enzyme activity, synthesis of proteins and chlorophyll are effected by gamma irradiation on seeds (Hameed *et al*., 2008). Change in the chlorophyll contents in response to different doses of gamma rays is an effective index to determine the mutagenic action (Verma *et al*, 2010). Effects on nitrate reductase activity by mutagens have been reported by many authors in *Cicer arietinum* and *Vigna* species (Kozgar *et al*., 2011).

Results also show the increase of different leaf soluble proteins during SDS-PAGE. But the increase of protein bands in the mutants selected from the variety Vikrant were observed more than the variety PRT-12. The selection of mutants was done in lower doses/concentrations of mutagens. The increase in protein bands might be due to the increase in RNA synthesis and subsequent protein synthesis by lower doses/concentrations of mutagens. The stimulatory action of low dosage has already been reported (Abdel-Hady *et al*., 2011).

Rubisco is the main photosynthetic enzyme consisting of eight small sub-units (14 kDa and eight large sub-units (56 kDa) [31]. As can be seen from the Fig. 2, the protein bands around 20 and 50 kDa proteins has increased considerably in mutant lanes. Different leaf soluble proteins involved in cellular processes like photosynthesis and other important processes can improve the cells and thus plants efficiency for making food and hence increasing of yield. The protein bands around 20, 60 and 200 kDa make up the sub units of nitrate reductase. The increment in the bands of this enhances the nitrogen fixing ability of plant and hence is directly related with the increasing yield of these high yielding mutants.

As results clearly indicate the increase of pigments, NRA and leaf proteins in the isolated mutants in M_3 generation. This indicates the fixation of character after maintaining this character in two generations. These mutants can be used in further experimental analysis. Similar increase of protein content in M_3 generation was observed in black gram (Rehman *et al*, 2001). By inducing mutation and selecting the mutants has proved efficient, rapid and simple method in increasing biochemical and yield characters after selecting the desired genotypes (Lee *et al*., 2003).

Conclusions

From the above experiment, it is clear that the chlorophyll, carotenoid and NRA of mutant strains of both varieties has increased but the increase in the variety Vikrant was more than the variety PRT-12. Furthermore, SDS-PAGE of leaf proteins of the variety Vikrant has shown more increase in different bands of proteins. Further experiments can be made on mutants in future to observe and analyse the different desired variations in plants.

References

Abdel-Hady MS, Okasha EM, Soliman SSA, Talaat M. Effect of Gamma Radiation and Gibberellic Acid on Germination and Alkaloid Production in Atropa belladonna L. Aust. j. basic appl. sci. 2008; 2: 401-405.

Ahmad F, Slinkard AE. Genetic relationships in the genus *Cicer arietinum* L., as revealed by polyacrylamide gel electrophoresis of seed storage proteins. Theor. Appl. Genet. 1992; 84: 688-692.

Amjad H, Saddiqa A, Nadeem S, Shah TM. Genotypic variability and mutant identification in *cicer arietinum* l. by seed storage protein profiling. Pak. J. Bot. 2012; 44: 1303-1310.

Ashraf M. Relationships between leaf gas exchange characteristics and growth of differently adapted populations of Blue panicgrass (*Panicum antidotale* Retz.) under salinity or waterlogging. Plant Sci. 2003; 165: 69-75.

Ghafoor A, Ahmad Z, Qureshi AS, Bashir M. Genetic relationship in *Vigna mungo*(L.)Hepper and *V.radiata* (L.) R. Wilczek based on morphological traits and SDS-PAGE. Euphytica. 2002; 123: 367-378.

Hameed A, Mahmud shah T, Manzoor Atta B, Ahsanulhaq M, Syed H. Gamma radiation effects on seed germination and growth, protein content, peroxides and protease activity, lipid per oxidation in desi and Kabuli chickpea. Pak. J. Bot. 2008; 40: 1033-1041.

Hameed A, Shah TM, Atta, BM, Iqbal N, Haq MA, Ali H. Comparative seed storage protein profiling of Kabuli chickpea genotypes. Pak. J. Bot. 2009; 41: 703-710.

Heidarieh MA, Borzouei S, Rajabifar F., Ziaie SH, Shafiei. Effects of gamma irradiation on antioxidant activity of Ergosan. Iran. J. Radiat. Res. 2012; 9: 245-249.

Irfan E. Determination of genetic diversity in the *Vicia* L. (*section vicia*) by using SDS-PAGE. Pak. J. Bot. 2011; 43: 1429-1432.

Jaworski EG. Nitrate reductase assay in intact plant tissues. *Biochem. Biophys. Res*. *Commun*. 1971; 43: 1274-1279.

Khursheed S, Fatima S, Khan S. Differential genotypic response of two varieties of *Hordeum vulgare* L. in response to hydrazine hydrate alone and in combination with dimethyl sulfoxide. *J. Phytol*. 2015; 7: 19-25.

Khursheed S, Khan S. Genetic improvement of two cultivars of *Vicia faba* L. using gamma irradiation and ethyl methanesulphonate mutagenesis. *Legume Res*. 2016; DOI: 10.18805/lr.v0iOF.9614.

Kozgar MI, Khan S, Goyal S. EMS induced mutational variability in *Vigna radiata* and *Vigna mungo*. Res. J. Bot. 2011; 6: 31-37.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680-5.

Lee SY, Cheong TS, Kim J. Production of doubles haploids through anther culture of hybrid plants derived from mutagenized fertilized egg cells. Plant Cell Rep. 2003; 22: 218-223.

Mackinney G. Absorption of light by chlorophyll solution. *J. Biol. Chem*. 1941; 140: 315-322.

Rehman MV, Siddiqui BA, Din MV. Hydrazine hydrate induced dwarf bold seeded mutant in black gram cultivar Pv-19. Mutation Breeding Newsletter. 2001; 45: 1-56.

Santalla M, Amurrio JM, De Ron AM. Food and feed potential breeding value of green, dry and vegetable pea germplasm. Canadian Journal of Plant Science. 2001; 81: 601-610.

Shereen A, Ansari R, Mumtaz S, Bughio HR, Mujtaba SM, Shirazi MU, Khan MA. Impact of gamma irradiation induced changes on growth andphysiological responses of rice under saline conditions. Pak. J. Bot. 2009; 41: 2487-2495.

Verma AK, Banerji BK, Chakrabarty D, Datta SK. Studies on Makhana (*Euryale ferox* Salisbury). Curr. Sci. 2010; 99: 795-800.