CHAPTER - 3

Materials and Methods
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Varieties used

The following two commercial varieties of broad bean (*Vicia faba* L.) were used in the present study.

i) *Vicia faba* L. var. minor

ii) *Vicia faba* L. var. major

A brief description of the two varieties is given below:

Table 3: Salient features of *Vicia faba* L. varieties used in the study.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Salient features</th>
<th>Var. minor</th>
<th>Var. major</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Habit</td>
<td>Erect</td>
<td>Erect</td>
</tr>
<tr>
<td>2.</td>
<td>Leaves</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>3.</td>
<td>Pod character</td>
<td>5–6cm (length, 4–5cm (girth))</td>
<td>6–7cm (length), 4.50–5.50cm (girth)</td>
</tr>
<tr>
<td>4.</td>
<td>Seed character</td>
<td>Round, grey in colour</td>
<td>Round, grey in colour</td>
</tr>
<tr>
<td>5.</td>
<td>Flowering</td>
<td>87–97 days</td>
<td>86–95 days</td>
</tr>
<tr>
<td>6.</td>
<td>Maturity</td>
<td>165–175 days</td>
<td>160–170 days</td>
</tr>
<tr>
<td>7.</td>
<td>Adaptability</td>
<td>All over India</td>
<td>All over India</td>
</tr>
<tr>
<td>8.</td>
<td>Height</td>
<td>56–62cm</td>
<td>47–54cm</td>
</tr>
<tr>
<td>9.</td>
<td>Chromosomes number</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

The certified, healthy and dry seeds of the above mentioned two varieties of broad bean were procured from Genetics Division, Indian Agricultural Research Institute (IARI), New Delhi. Both these varieties are
well adapted to the agro-climatic conditions of Uttar Pradesh, particularly the experimental site (University's Agricultural Farm, Aligarh).

3.1.2 Mutagens used

The following three types of mutagens were used separately as well as in combinations. The dose of each mutagen used in the present study is given in the Table-4.

3.1.2.1 Gamma rays

Air dried (8–10% moisture content) seeds of the two broad bean varieties were exposed to different doses of gamma rays from $^{60}$Co source at the Nuclear Research Division, Indian Agricultural Research Institute (IARI), New Delhi.

3.1.2.2 Ethyl Methane Sulphonate (EMS), (CH$_3$OSO$_2$CH$_2$H$_5$)

A set of unirradiated as well as irradiated seeds were subjected to treatment with different concentrations of EMS (E. Merck India Ltd., grade-AR) for separate and combination treatments respectively.

3.1.2.3 Methyl Methane Sulphonate (MMS), (C$_2$H$_6$O$_3$S)

A set of unirradiated as well as irradiated seeds were subjected to treatment with different concentrations of MMS (SISCO Research Laboratories Pvt. Ltd. Bombay) for separate and combination treatments respectively.

3.1.2.4 Mechanism of action of gamma rays and EMS

i) Gamma rays

Gamma rays are effective and efficient physical mutagen. They have shorter wave length and therefore, posses more energy per photon than x-rays. The unit of radiations is Roentgen (R-units) and are produced by the heavy isotopes of elements such as cobalt$^{60}$, p$^{32}$ etc. $^{60}$Co has a half life of 5.3 years with energy = 1.33 Mev ($\mu_1$) or 1.17 Mev ($\mu_2$). When radiation passes through a matter it collides with its atoms in tissues/cells and cause the release of
electrons from the atom leaving there positively charged free radicals or ions. These ions in turn collide with other molecules causing release of further electrons. The net result is that a core of ions is formed along the track of each radiation as it passes through matter or living tissue. Since this process gives rise to ions or free radicals, it is called ionization and hence the name ionizing radiations. The increased reactivity of atoms present in DNA molecules is the basis of mutagenic effects of ionizing radiations.

ii) Ethyl Methane Sulphonate (CH$_3$OSO$_2$C$_2$H$_5$)

This chemical has been known to react particularly with the base guanine (Bautz and Freese, 1960). Krieg (1963) has shown that the reactivity of this compound with three of the four bases decreases in the order of guanine, adenine and cytosine. Like any other alkylating agents, ethyl methane sulphonate can react with either phosphate group of nucleic acids or the purine bases especially the guanine of DNA molecule (Freese, 1963). The action on guanine base i.e. ethylation of N$_7$ is inferred to be important pathway in mutations and chromosome breakage. The alkylation of purine in 7$^\text{th}$ position gives to quaternary nitrogens which are unstable. Either the alkyl group itself hydrolyses away from the purine or else the alkylated purine separates from the deoxyribose leaving it depurinated. Liberation of ethylated and methylated purines from DNA base has been observed (Bautz and Freese, 1960; Lett et al., 1962). The gap might interfere with DNA duplication or cause the incorporation of a wrong base. Bautz and Freese (1960) from their experimental results and theoretical considerations strongly suggested the removal of guanine to be the main cause of mutations induced by ethyl methane sulphonate.

iii) Methyl Methane Sulphonate (C$_2$H$_6$O$_3$S)

Among the numerous radiomimetic chemicals now known, the alkylating agents have been found to be the most potent in a wide array of organisms. Within the alkylating groups, MMS has been found to be a very
effective chemical mutagen; like other alkylating agents, MMS react with DNA by alkylating the phosphate group as well as purine and pyrimidine bases and create a gap in the DNA molecule causing mutation. It is a colourless liquid with a molecular weight of 110.13.

3.2 PREPARATION OF MUTAGENIC SOLUTIONS

One percent stock solution of EMS and MMS were prepared and from this stock solution different concentrations of EMS and MMS were prepared by using the formula \( S_1 V_1 = S_2 V_2 \), where,

- \( S_1 \) = Strength of stock solution
- \( V_1 \) = Volume of stock solution
- \( S_2 \) = Strength of desired solution
- \( V_2 \) = Volume of desired solution

The specificity of action of chemical mutagen depends upon the particular conditions of treatment, the more important of which are temperature and hydrogen ion concentration. In the course of present study, EMS solutions were prepared by dissolving appropriate quantity of this chemical in Sorensen's phosphate buffer having a pH of 7.0 and the final pH adjusted to 7.0 by adding few drops of normal NaOH with the help of Backman's pH meter.

3.2.1 Method of treatment with chemical mutagens

Prior to the chemical mutagenic treatment, the seeds were presoaked in distilled water for 12 hours at room temperature (25±1°C). After the presoaking period is over the seeds were kept on blotting paper so as to remove small droplets of water adhering to the surface of seeds. Thereafter, the seeds were treated with different concentrations of chemical mutagens for 6 hours. One set of seeds in each variety was kept untreated to act as control for comparison. Thus the control seeds, although not treated with the chemical mutagen; were exposed to the similar physiological conditions before sowing as that of the treated seeds.
During chemical mutagenic treatments, intermittent shaking was given throughout the treatment period to facilitate sufficient aeration. For uniform absorption, large quantities of mutagenic solution, approximately three times the volume of seeds (Konzak et al., 1965) were used. After the treatment period the treated seeds were thoroughly washed in running tap water for 1 hour to remove the residual effect of the mutagen sticking to the seed coat before they were sown in the nursery beds.

3.2.2 Combination treatments

In case of combination treatments the gamma ray doses viz. 10kR and 20kR were combined with four concentrations of the monofunctional alkylating agents, i.e. ethyl methane sulphonate (EMS) 0.1, 0.2, 0.3, and 0.4% and methyl methane sulphonate (MMS) 0.01, 0.02, 0.03 and 0.04%.

3.2.3 Sample size

In each variety a set of 650 seeds were used for each dose/treatment including the control. Out of these 650 seeds, 500 seeds in each treatment/dose were sown in the field for morphological and cytological studies, whereas the remaining set of 50 seeds was grown on moist cotton in petriplates for measuring root-shoot length. The petriplates were kept in B.O.D. incubator at 25±1°C temperature with relative humidity at 95%.

3.2.4 Sowing of seeds in the field

The treated as well as untreated (control) seeds were sown in three replicates (200 seeds in each replicate) in a complete randomized block design (CRBD) in the rabi season of 2003 at the University Agriculture Farm, A.M.U., Aligarh. The distance between the seeds along a row was kept 60 cm whereas row to row distance was maintained at 100 cm in each experimental plot in a replication. Recommended agronomic practices were employed for the preparation of field, sowing and subsequent management of populations to raise a good crop.
Table 4: Details of mutagenic treatments given to broad bean varieties.

<table>
<thead>
<tr>
<th>Mutagen used</th>
<th>Dose/Conc.</th>
<th>Duration of presoaking (h)</th>
<th>Duration of treatment</th>
<th>pH</th>
<th>No. of seeds treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>12.0</td>
<td></td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td>EMS</td>
<td>0.1% (L)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.2% (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.3% (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.4% (H)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td>Gamma rays</td>
<td>10kR (L)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>20kR (I)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>30kR (I)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>40kR (H)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>650</td>
</tr>
<tr>
<td>MMS</td>
<td>0.01% (L)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.02% (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.03% (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>0.04% (H)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td>Gamma rays + EMS</td>
<td>10kR+0.1% EMS (L)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>10kR+0.2% EMS (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>20kR+0.3% EMS (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>20kR+0.4% EMS (H)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td>Gamma rays + MMS</td>
<td>10kR+0.01% MMS (L)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>10kR+0.02% MMS (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>20kR+0.03% MMS (I)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>20kR+0.04% MMS (H)</td>
<td>12.0</td>
<td>6.0</td>
<td>7.0</td>
<td>650</td>
</tr>
</tbody>
</table>

L = lower dose/concentration; I = Intermediate dose/concentration; H = Higher dose/concentration
3.3 EVALUATION OF M₁ GENERATION

A detailed study of the effect of different mutagenic treatments in the two varieties was undertaken using the following parameters.

3.3.1 Seed germination

Seed germination was recorded right from the emergence of first shoot in each treatment as well as control in the field on alternate days. After recording the germination counts, the percentage of seed germination and percent inhibition were calculated by using the formula.

\[
\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100
\]

\[
\text{Inhibition (\%)} = \frac{\text{Control - Treated}}{\text{Control}} \times 100
\]

3.3.2 Seedling height (cm)

Seedling height was estimated on 7th day of germination by measuring root and shoot lengths of 30 randomly selected seedlings from each treatment as well as control. Seedling injury as measured by the reduction in the root and shoot length was calculated in terms of percentage of root and shoot injury.

\[
\text{Percent Injury} = \frac{\text{Control - Treated}}{\text{Control}} \times 100
\]

3.3.3 Plant survival

The surviving plants in different treatments were counted at the time of maturity and the survival percentage and percent lethality were calculated by the following formula:

\[
\text{Survival (\%)} = \frac{\text{Number of plants at maturity}}{\text{Number of seeds germinated}} \times 100
\]

\[
\text{Lethality (\%)} = \frac{\text{Control - Treated}}{\text{Control}} \times 100
\]
3.3.4 Pollen fertility

Fresh and young flowers from 25-30 randomly selected plants were taken from each treatment and the control. Pollen fertility was determined by staining the pollen grains with 2% acetocarmine solution. Pollen grains which took the stain and had a regular outline were considered as fertile, while the shrunken, empty and unstained ones as sterile. The following formula were used to calculate percent fertility and percent reduction (sterility):

\[ \text{Pollen fertility} \% = \frac{\text{Number of fertile pollen}}{\text{Total number of pollen}} \times 100 \]

\[ \text{Percent reduction (Sterility)} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100 \]

3.3.5 Coefficient of interaction (k)

To evaluate the effects of combined treatments on various biological parameters as well as mutation frequency the data were analysed by using the following formula:

\[ K = \frac{(a + b)}{(a) + (b)} \times 100 \quad (\text{Sharma and Swaminathan, 1969}) \]

Where ‘a’ and ‘b’ stand for two mutagens and k is a hypothetical interaction coefficient. The value of k should be one, if the interaction is additive. Any deviation from this value would show synergistic or less than additive effects.

3.3.6 Cytological studies

Cytological studies were carried out on pollen mother cells by fixing young flower buds from each treatment as well as control. The purpose of fixation is to kill the tissue without causing any distortion of the components to be studied. It should not only increase visibility of the chromosome structure but should also clarify the details of chromosome morphology such as the
euchromatic or heterochromatic regions and the primary and secondary constrictions.

For meiotic studies, young flower buds from 40-50 randomly selected plants were fixed in freshly prepared Carnoy’s fluid (alcohol: chloroform: acetic acid in a 6:3:1 ratio), supplemented with crystals of ferric chloride for 24 hours. The material was then washed and preserved in 70% alcohol at 4°C. The anthers were squashed in 2% acetocarmine, dehydrated in NBA series (50% acetic acid + 50% normal butyl alcohol), mounted in canada balsam, and dried at 45°C (Bhaduri and Ghose, 1954). More than 500 dividing PMCs from each treatment, as well as control populations were studied and analysed at metaphase I/II, anaphase I/II and telophase I/II stages. The photomicrographs were taken from temporary as well as permanent slides with the aid of “Nicon” photomicrographic unit using 10 X eye piece x 100 X objective lens.

3.3.6.1 Chiasma frequency

The number of chiasmata per cell and per bivalent were estimated in treated as well as control plants by scoring 100 PMCs at random, at diakinesis and metaphase-I stages.

3.4 EVALUATION OF M2 GENERATION

Seeds from each M1 plant were harvested separately in treated as well as control populations. For raising M2 generation, 100 M1 plants and 50 seeds from each plant were selected in each treatment and control and were sown in the plant progeny rows in three replicates following complete randomized block design (CRBD). Thus, M2 population of each treatment consisted of 100 M1 plant progenies and a total of 5000 M2 plants. The seed to seed distance was maintained at 60cm and row to row spacing at 100cm.
3.4.1 Observations recorded in M₂ generation

3.4.1.1 Chlorophyll and viable mutations

The treated as well as control populations were carefully screened for chlorophyll mutations from the emergence till the age of four weeks after germination, whereas viable morphological mutations were scored throughout the growth period of plants in the field. The identification and classification procedure of Gustaffson (1940, 1947) was followed with suitable modifications.

Mutation frequency was calculated by the following methods:

1. Mutation frequency (%) = \[ \frac{\text{Number of mutated progenies}}{\text{Total number of progenies}} \times 100 \]

2. Mutation frequency (%) = \[ \frac{\text{Number of mutated plants}}{\text{Total number of plants}} \times 100 \]

3.4.1.2 Mutagenic effectiveness and efficiency

Mutagenic effectiveness is a measure of the frequency of mutations induced by unit mutagen dose, whereas, mutagenic efficiency is the measure of proportion of mutations in relation to undesirable changes like injury, lethality, sterility and meiotic aberrations etc. Mutagenic effectiveness and efficiency was calculated on the basis of formula suggested by Konzak et al. (1965).

3.4.1.2.1 Mutagenic effectiveness

a) Effectiveness (Physical mutagen) = \[ \frac{\text{Mutation rate (Mf)}}{\text{Dose in kiloroentgens (kR)}} \]

b) Effectiveness (Chemical mutagen) = \[ \frac{\text{Mutation rate (Mf)}}{(\text{Conc. of mutagen}) \times (\text{time of treatment in h})} \]

c) Effectiveness (combination) = \[ \frac{\text{Mutation rate (Mf)}}{\text{Dose of physical mutagen (kR)} \times \text{concentration of chemical mutagen} \times \text{time (h)}} \]
3.4.1.2.2 Mutagen efficiency

Mutation rate on the basis of $M_1$ plant progenies (MP) or $M_2$ population (Mf)

a) Efficiency = \[
\frac{\% \text{ injury (I)} \text{ or } \% \text{ lethality (L)} \text{ or } \% \text{ sterility (S)} \text{ or } \% \text{ meiotic aberrations (M)}}{\text{MP or Mf}}\]

3.4.2 Sensitivity studies on economic traits

Observations were recorded on 10-15 normal looking plants of each progeny from treated as well as control populations. The progenies segregating for macromutations were not used for such analysis. The following twelve quantitative characters were thoroughly studied in different generations.

1. **Days to flowering**

   Days taken to flowering were noted from the date of sowing till first flower appeared in 50% plants in a treatment.

2. **Number of flowers per plant**

   Number of flowers were noted as the number of flowers borne on a whole plant.

3. **Days to maturity**

   Days to maturity was noted as the number of days taken from the date of sowing to the date of harvesting of plants.

4. **Plant height (cm)**

   Plant height was measured at maturity in centimeters from the base up to the apex of plant.

5. **Number of fertile branches per plant**

   Fertile branches were counted at maturity as the number of branches which bore more than one pod.
6. **Number of pods per plant**
   
   Mean number of productive pods were counted at maturity and noted as the number of pods borne on the whole plant.

7. **Number of seeds per pod**
   
   Mean number of seeds per pod was first calculated from each individual plant and then the pooled mean was calculated from different mean values.

8. **Pod length (cm)**
   
   The pods were measured in centimeters and the mean for each selected plant was calculated for each plant.

9. **Pod girth (cm)**
   
   The pods were measured in centimeters and the mean for each selected plant was calculated for each plant.

10. **100-seed weight (g)**
    
    It was the weight of a random sample of seeds from each plant.

11. **Total seed yield per plant (g)**
    
    Seed yield per plant was the weight of total number of seeds harvested per plant and the yield of each plant was recorded in grams.

12. **Number of leaves per plant**
    
    *Vicia faba* L. plant bears compound leaves. Each rachis, considered a single leaf bears the number of leaflets. Leaves were counted in all the branches of the plant.

**3.5 STUDIES IN M$_3$ GENERATION**

On the basis of performance of yield and other desirable traits, the following treatments were selected in M$_2$ for raising M$_3$ generation.

a) 0.1% EMS

b) 0.2% EMS
c) 0.3% EMS  
d) 10 kR gamma rays  
e) 20 kR gamma rays  
f) 30 kR gamma rays  
g) 0.01% MMS  
h) 0.02% MMS  
i) 0.03% MMS  
j) 10 kR + 0.1% EMS  
k) 10 kR + 0.2% EMS  
l) 20 kR + 0.3% EMS  
m) 10 kR + 0.01% MMS  
n) 10 kR + 0.02% MMS  
o) 20 kR + 0.03% MMS  

From each treatment such M₂ progenies were selected which showed significant deviations in mean values in the positive direction particularly for the yield components under study in M₂ generation. Thereafter, seeds of each selected progeny were bulked by taking equal number of seeds from individual plants. Plants showing morphological, chlorophyll and other variations were discarded from each progeny for quantitative studies. However, seeds of such plants were collected separately and sown in individual rows in M₃ to study their mutant nature.

A random sample of each selected progeny of treatments as well as control were sown as M₂ progeny rows to raise the M₃ generation. The plant to plant and row to row distance was maintained same as in previous experiments. Observations were recorded on all 12 quantitative traits as in M₂ generation.
3.6 STATISTICAL ANALYSIS

The data of each quantitative trait were subjected to statistical analysis to assess the extent of induced variation in different generations. Mean, standard error, standard deviation and coefficient of variation were calculated as per the standard statistical procedures. Components of variance (genotypic and phenotypic) were estimated as per the formula suggested by Singh and Chaudhary (1977). The broad sense heritability (h²) was estimated by the formula suggested by Johnson et al. (1955), whereas, genetic advance (GA) expressed as percentage of mean was calculated as suggested by Allard (1960) and modified by Khan (1979a).

3.6.1 Mean (\(\bar{X}\))

The mean was computed by taking the sum of a number of observations and dividing it by total number of observations recorded, thus

\[
\bar{x} = \frac{X_1 + X_2 + \ldots + X_n}{N}
\]

\[
\bar{x} = \frac{\sum_{i=1}^{n} x_i}{N}
\]

where \(x_1, x_2, \ldots, x_n\) = observations

\(N\) = Total number of observations involved

3.6.2 Standard deviation (S.D.)

Standard deviation is the positive square root of the average of sum of squares of deviations of all observations from their means. It was computed on the basis of following formula.

\[
S.D. = \sqrt{\frac{(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 + (x_3 - \bar{x})^2 + \ldots + (x_n - \bar{x})^2}{n}}
\]

or \(S.D. = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}\)

where, S.D. = Standard deviation
\[ \sum x = \text{Sum of all individual observations} \]

i.e. \[ x_1 + x_2 + x_3 - \ldots - x_n \]

\[ \bar{X} = \text{Mean of all observations} \]

\[ n = \text{number of observations} \]

### 3.6.3 Standard error (S.E.)

\[ \text{S.E.} = \frac{\text{S.D. of sample}}{\sqrt{N-1}} \]

Where, S.D. = Standard deviation

\[ N = \text{Number of observations} \]

### 3.6.4 Coefficient of variation (C.V.)

It measures the relative magnitude of variation present in the observations relative to their magnitude of arithmetic mean. It is defined as the ratio of standard deviation to arithmetic mean expressed as percentage and is a unitless number. The following formula was applied to compute coefficient of variability (C.V.).

\[ \text{C.V.} = \frac{\text{Standard deviation}}{\text{Arithmetic mean}} \times 100 \]

Or \[ \text{C.V.} = \frac{\text{S.D.}}{\bar{X}} \times 100 \]

### 3.6.5 Analysis of variance (ANOVA)

Analysis of variance helps in sorting out the variance due to different sources and also provides a basis for test of significance. For computing the analysis of variance between and within families, ANOVA was prepared for testing the significance of variance.

#### 3.6.5.1 Components of variance

i) Genotypic variance \( (\sigma^2_g) \)

The estimate of genotypic variance was calculated by the following formula:
\[
\sigma^2_g = \frac{MSv - MSe}{N}
\]

Where, MSv and MSe = Mean sum of squares of between families/ within families and error, respectively,

\( \sigma^2_g \) = Genotypic variance

\( N \) = Number of replicates

Genotypic coefficient of variation (GCV) was calculated as

\[
GCV(\%) = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100
\]

**ii) Phenotypic variance (\( \sigma^2_p \))**

Phenotypic variance was estimated by summing up the estimates genotypic (\( \sigma^2_g \)) and the environmental variance (MSe or \( \sigma^2_e \))

\[
\sigma^2_p = \sigma^2_g + \sigma^2_e
\]

Phenotypic coefficient of variation (PCV) was calculated as follows:

\[
PCV(\%) = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100
\]

**3.6.6 Heritability (h^2)**

It is the ratio of genotypic variance to the total phenotypic variance. The broad sense heritability was calculated as

\[
h^2 (\%) = \frac{\sigma^2_g}{\sigma^2_p} \times 100
\]

where, \( \sigma^2_g \) = induced genotypic variance

\( \sigma^2_p \) = Phenotypic variance

**3.6.7 Genetic advance (GA)**

The genetic advance as percentage of mean at 1% selection intensity (k) was computed as follows
GA = k. σp.h²

Where,  

\( h^2 = \) broad sense heritability,  
\( \sigma p = \) phenotypic standard deviation of the mean performance of treated populations, and  
\( k = 2.64 \) constant for 1% selection intensity

\[
GA \left( \% \text{ of } \overline{x} \right) = \frac{GA}{\overline{x}} \times 100
\]

3.6.8 Test of significance

The significant difference between treated and control population means was obtained by using the least significant difference (L.S.D.) method (Snedecor and Cochran, 1968) with some suitable modifications. It was computed as follows.

**Step 1:** Construction of data table for 5 treatments and 3 replicates:

The data were computed such that each treatment occupied a column and their replicates were arranged in rows.

<table>
<thead>
<tr>
<th>Rows (replicates)</th>
<th>Column number (treatments)</th>
<th>Total of rows (replicates) ((\Sigma))</th>
<th>Square of total of rows (w^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_1 )</td>
<td>( A_1 ) ( B_1 ) ( C_1 ) ( D_1 ) ( E_1 )</td>
<td>( A_1 + \ldots + E_1 = \overline{x}_1 ) ((\overline{x}_1)^2)</td>
<td>(w = \overline{y}_1)</td>
</tr>
<tr>
<td>( R_2 )</td>
<td>( A_2 ) ( B_2 ) ( C_2 ) ( D_2 ) ( E_2 )</td>
<td>( A_2 + \ldots + E_2 = \overline{x}_2 ) ((\overline{x}_2)^2)</td>
<td>(w = \overline{y}_1)</td>
</tr>
<tr>
<td>( R_3 )</td>
<td>( A_3 ) ( B_3 ) ( C_3 ) ( D_3 ) ( E_3 )</td>
<td>( A_3 + \ldots + E_3 = \overline{x}_3 ) ((\overline{x}_3)^2)</td>
<td>(w = \overline{y}_1)</td>
</tr>
<tr>
<td>Total of column ((\Sigma))</td>
<td>( A_1 + \ldots + A_3 ) ( B_1 + \ldots + B_3 ) ( C_1 + \ldots + C_3 = \overline{y}_1 )</td>
<td>( D_1 + \ldots + D_3 = \overline{y}_1 ) ( E_1 + \ldots + E_3 = \overline{y}_3 )</td>
<td>(w = \overline{y}_1)</td>
</tr>
</tbody>
</table>

Squares of total of columns

\( (y_1)^2 \) \( (y_2)^2 \) \( (y_3)^2 \) \( (y_4)^2 \) \( (y_5)^2 \)

Sum of squares total of column \((\Sigma)^2\)

\( (A_1)^2 + \ldots + (B_1)^2 + \ldots + (C_1)^2 + \ldots + (D_1)^2 + \ldots + (E_1)^2 + \ldots + (A_3)^2 + Z_1 \) \( (B_3)^2 + \ldots + (C_3)^2 + Z_3 \) \( (D_3)^2 + Z_4 \) \( (E_3)^2 + Z_5 \)

\( Z_3 + \ldots + Z_5 = wz \) \( x_1 + \ldots + x_9 \)
Step 2: Correction factor (CF)

\[ CF = \frac{(\text{Grand Total})^2}{t \times r} \]

or \[ CF = \frac{(wx)^2}{t \times r} \]

where,

wx = Grand total

t = number of treatments

r = number of replicates

Step 3: Total sum of squares (SSQT)

This is the sum of squares of all the values in the table minus the correction factor

\[ SSQT = [(A_1)^2 + (B_1)^2 + \ldots \ldots \ldots \ldots (E_1)^2] - CF \]

Step 4: Sum of squares of treatments (SSQt)

\[ SSQt = \frac{(y_1)^2 + (y_2)^2 + \ldots \ldots \ldots (y_s)^2}{r} - CF \]

or \[ SSQt = \frac{wy}{r} - CF \]

Where, \( r \) number of replicates

Step 5: Sum of squares of replicates (SSQr)

\[ SSQr = \frac{(x_1)^2 + (x_2)^2 + \ldots \ldots \ldots (x_t)^2}{t} - CF \]

or \[ SSQr = \frac{wr}{t} - CF \]

Where, \( t \) = number of treatments

Step 6: Sum of squares of error (SSQE)

\[ SSQE = SSQT - (SSQt + SSQr) \]
Step 7: Estimated variance of error (MSE)

$$MSE = \frac{SSQE}{(t-1)(r-1)}$$

Step 8: Least significant difference based on ordinary t-test (L.S.D.)

L.S.D. at 5% level = $\sqrt{\frac{2MSE}{r}} \times t \text{ value at 5% level}$

L.S.D. at 1% level = $\sqrt{\frac{2MSE}{r}} \times t \text{ value at 1% level}$

If the difference between any two sample means exceeds the least significant difference (L.S.D.) value obtained at 5% or 1% level, the difference between the two means is said to be significant at 5% or 1% level, respectively.

### 3.7 PROTEIN ESTIMATION

Crude protein content in the seeds was estimated following the method of Lowry et al. (1951) with some laboratory modifications.

Besides the isolated mutant lines, protein estimation was done in all selected treatments as well as in control populations. At least 5 different samples of seeds were taken from each treatment as well as mutant lines. Each sample of seeds was crushed into powder with the help of mortar and pestle and 2-3 replicates were kept for all samples studied.

#### 3.7.1 Materials used

1. Bovine serum albumin (Sisco India Ltd.)
2. Sodium carbonate (Na$_2$ CO$_3$ of Qualigen India, Ltd.)
3. Sodium hydroxide (NaOH of E. Merck India, Ltd.)
4. Sodium potassium tartarate (Qualigen India, Ltd.)
5. Copper sulphonate (CuSO$_4$ of BDH India, Ltd.)
6. Folins phenol reagent (Sisco India, Ltd.)
3.7.2 Preparation of reagents

1. Sodium dodicyl phosphate (SDS)

   1% SDS was prepared by dissolving 1 g SDS in 100 ml DDW. SDS is used to extract protein from the sample.

2. Lowery reagent

   A. Na₂CO₃ (Sodium carbonate) = 2%

      NaOH (Sodium hydroxide) = 0.4%

      Added 2 g of Na₂CO₃ and 0.4 g of NaOH to 100 ml DDW in a simple beaker.

   B. Sodium potassium tartarate (Na-K-tartarate)

      Added 2 g of Na-k-tartarate to 100 ml DDW to get 2% sodium potassium tartarate.

   C. Copper sulphate (CuSO₄) = 1%

      Added 1 g of CuSO₄ to 100 ml DDW to get 1% CUSO₄

   Lowery reagent = 100 ml A: 1ml B: 1 ml C

3. Folin phenol reagent

   It is available as 2 N in the market. 50% or 50ml of Folin phenol was diluted in 50 ml DDW to get 1 N Folin

3.7.3 Procedure for protein estimation

**Step 1:** Took 10 mg seed sample from each treatment in small test tubes.

**Step 2:** Added 1 ml of 1% SDS to each sample. Vertext or shook thoroughly and carefully. Left for 10-15 minutes.

**Step 3:** Took 0.2 ml aliquot from each tube and added 0.8 ml DDW to make 1 ml exactly. Vertext or shook well.
Step 4: Added 5 ml Lowery reagent to each tube and vertex. Left exactly for 10 minutes.

Step 5: Added 0.5 ml of 1 N Folin phenol to each sample and vertex. Left for 20 minutes. Read each sample at 660nm in DU-40 or Beckman's Spectrophotometer.

3.7.4 Test of significance

Data of protein estimation were subjected to T-test to study the significant shifts in treated population with respect to control.

\[
|t| = \frac{\bar{x} - \bar{y}}{\sqrt{SD^2 (1/n_1 + 1/n_2)}}
\]

\[
SD^2 = \frac{n_1SD_1^2 + n_2SD_2^2}{n_1 + n_2 - 2}
\]

where \( \bar{x} \) = mean of one group, e.g. control

\( \bar{y} \) = mean of 2\(^{nd} \) group, e.g. any treatment

\( n_1 \) = number of observations in 2\(^{nd} \) group

\( n_2 \) = number of observations in 2\(^{nd} \) group.

\( SD_1 \) = Standard deviation of one group

\( SD_2 \) = Standard deviation of 2\(^{nd} \) group

\( n_1 + n_2 - 2 \) = degree of freedom (df)

3.8 PROCEDURE FOR SDS-PAGE OF PROTEINS

For SDS-PAGE, five seeds from each treatment were ground in a pestle with mortar after removal of testa and 100 mg meal was extracted with 1 ml of TRIS-HCl buffer (pH 7.5, 50mM) by shaking in cold for 30 minutes. The extract was centrifuged and the protein in the supernatant was estimated by the method of Bradford (1976). The extract was subjected to SDS-PAGE by the method described by Laemmli (1970). Samples containing about equal amounts of protein were treated with TRIS buffer containing sodium dodecyl
sulfate (SDS), glycerol and bromophenol blue. For each treatment, 30 mg of protein was electrophoresed, on 2.5% stacking gel and 12.5% resolving gel. The gels were later stained with coomassic Brilliant Blue G-250. The variation in the position of the bands in any lane was analysed. The standard proteins used glumatic dehydrogenase (53 kDa Rf 0.66), transferage 76 (kDa, Rf 0.41), β-galactosidase (116 kDa, Rf 0.24), α-2-macroglobulin 170 (kDa, Rf 0.09) and myosin (220 kDa, Rf 0.02). SDS-PAGE was repeated at least three times to get uniform results.