Chapter 3
MATERIALS & METHODS
3. MATERIALS AND METHODS

The experimental plant material used in the present investigation was mung (commonly known as mungbean) [Vigna radiata (L.) Wilczek, Family, Papilionaceae]. Two local varieties of this plant namely Vaibhav and Kopargaon-1 (Plate 3.1) were selected for induction of genetic variability employing physical and chemical mutagens and to carry out mutational studies. Germplasms of these two local varieties were procured from the Pulses Improvement Division of Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, (District Ahmednagar, Maharashtra state, India). The salient features of these two varieties of mungbean are shown in table 3.1. Experiments were carried out in the experimental fields of Padmashri Vikhe Patil College, Pravaranagar during the three years viz. Kharif 2002, 2003 and 2004.

3.1. Mutagens used

Two chemical mutagens viz. Ethyl methane sulphonate (EMS) and sodium azide (SA) and one physical mutagen, gamma radiation were used in the present investigation to induce mutations in the selected plant materials.

3.1.1. Chemical Mutagens

Ethyl methane Sulphonate and SA were procured from Sigma chemical company Ltd. U.S.A. were used in the present study.

3.1.2. Preparation of working solutions of chemical mutagens

The Fresh aqueous stock solution of ethyl methane sulphonate (1.0 Molar concentration) was prepared in phosphate buffer just before the use. double distilled water prior to treatments. Stock solution of sodium azide (1.0 molar concentration) was prepared in phosphate buffer (pH. 3.5). From these stocks, working solutions of 0.01, 0.02, 0.03, and 0.04 Molar concentrations each of EMS and SA were prepared.

3.1.3. Method of administration of chemical mutagens

Healthy dry seeds of the two local varieties of mungbean (About 7200 each) were selected for their uniformity in size. They were surface sterilized with 0.1% mercuric chloride solution for about one minute, washed thoroughly and soaked in
Table 3.1: Taxonomic and morphological features of mungbean \([Vigna\ radiata\ (L.)\ Wilczek]\) cvs. Vaibhav and Kopargaon-1

<table>
<thead>
<tr>
<th></th>
<th><strong>Vaibhav</strong></th>
<th><strong>Kopargaon-1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parentage</strong></td>
<td>KDM-1 x TARM-18</td>
<td>Local selection</td>
</tr>
<tr>
<td><strong>Year of release</strong></td>
<td>2001</td>
<td>1956</td>
</tr>
<tr>
<td><strong>Evolved by</strong></td>
<td>MPKV, Rahuri</td>
<td>Nagpur</td>
</tr>
<tr>
<td><strong>Area of adaptation</strong></td>
<td>Maharashtra (India)</td>
<td>Maharashtra (India)</td>
</tr>
<tr>
<td><strong>Growth habit</strong></td>
<td>Erect, 45-120 cm</td>
<td>Erect, 45-120 cm</td>
</tr>
<tr>
<td><strong>Root system</strong></td>
<td>Tap system provided with nodules</td>
<td>Tap system provided with nodules</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td>Green with light pigmentation, sparsely hairy</td>
<td>Green with pigments</td>
</tr>
<tr>
<td><strong>Leaves</strong></td>
<td>Trifoliate, broad green leaves with brown midrib, pigmented petiole</td>
<td>Trifoliate, large, dark green with greenish midrib, Leaf pubescence sparse</td>
</tr>
<tr>
<td><strong>Flower</strong></td>
<td>Bisexual, zygomorphic, light yellow flower, 5 petals, 5 sepals, 10 stamens diadelphous condition (1+9) and single carpelled ovary with hairy style.</td>
<td>Bisexual, zygomorphic, Faint greenish, 5 sepals, 10 stamens diadelphous condition carpelled ovary with hairy style</td>
</tr>
<tr>
<td><strong>Days for flowering</strong></td>
<td>38-40</td>
<td>32-36</td>
</tr>
<tr>
<td><strong>Pod</strong></td>
<td>Long sparsely hairy pod, unripe pods faint green, green marking at midrib, pod length medium, mature pod constrictions high. Medium pod curvature.</td>
<td>Long pod, greenish, at maturity turns black, suture of pod is green colour, unripe pods dense mature pod pubescence with medium</td>
</tr>
<tr>
<td><strong>Maturity (Days)</strong></td>
<td>70-75</td>
<td>60-65</td>
</tr>
<tr>
<td><strong>Seed size and colour</strong></td>
<td>Bold, shiny, oval, green, hilum size medium,</td>
<td>Bold, shiny, oval, green, hilum size medium,</td>
</tr>
<tr>
<td><strong>100 seed weight (g)</strong></td>
<td>4.6-4.8</td>
<td>4.6-4.8</td>
</tr>
<tr>
<td><strong>Yield (q/ha)</strong></td>
<td>14-15</td>
<td>8-10</td>
</tr>
<tr>
<td><strong>Special features</strong></td>
<td>Bold seeds, resistant to powdery mildew, high yield potential and suitable for Kharif and summer season.</td>
<td>Suitable for Kharif</td>
</tr>
<tr>
<td><strong>Area for cultivation</strong></td>
<td>Maharashtra</td>
<td>Vidharbha region (Maharashtra)</td>
</tr>
</tbody>
</table>
Plate 3.1

A. Experimental material - cv. Vaibhav.

B. Experimental material - cv. Kopargaon-1.

C. Mungbean field - cv. Vaibhav.
distilled water for 8 hours. They were later divided into nine lots. Each lot was taken into 100 ml glass beakers. The first glass beaker was labeled as control and only distilled water was added to the beaker. Remaining lots of seeds were treated with various test concentrations of EMS and SA for 12 hours at room temperature.

To begin with, a pilot experiment was conducted to determine the lethal dose (LD50) and suitable concentrations of the mutagens and duration of treatment for both the varieties of mungbean. From such experiments it was finally established that concentrations 0.01, 0.02, 0.03 and 0.04M of both EMS and SA for duration of 12 hours are best suitable for mutational studies for both the varieties of mungbean. Hence remaining lots of seeds in the beakers were treated with these concentrations of chemical mutagens. Seeds soaked in distilled water for 12 hours served as control.

3.1.4. Post-treatment handling

Immediately after the completion of pre-treatment, the seeds were washed thoroughly under running tap water for 30 minutes to wash out the chemical residues. A batch of 150 seeds from each treatment were blotted between folds of filter paper and kept for germination in dark at room temperature (25 ± 2°C) in Petri plates lined with moist filter papers. These were used for studying various parameters of seed germination and seedling growth. The remaining lot of 450 seeds from each treatment was sown in the experimental field following randomized block design (RBD) with three replications along with control as the M₁ generation. The field experiments were carried out at the experimental fields of Padmashri Vikhe Patil College, Pravaranagar during the Kharif season of the years 2002, 2003 and 2004.

Different generations of treated plants were denoted and abbreviated as follows:

M₁ – It is the immediate generation following the mutagenic treatment.
M₂ – It is the progeny raised from the seeds of M₁ generation.
M₃ – It is the progeny raised from the seeds of M₂ generation.
3.1.5. Gamma radiation

The source of gamma radiation used in the investigation was $^{60}$Co. The facility available at the Department of Bio-physics, Government Institute of Science, Aurangabad (M.S. India) was availed. The doses employed were 30, 40 and 50 kR. Dry, uniform seeds of Vaibhav and Kopargaon-1 varieties (About 1800 each) of mungbean were exposed to different doses of gamma radiation (30, 40 and 50 kR). Seeds not exposed to gamma radiation were used as control. Treated and control seeds were germinated in petriplates and swon in the experimental as mentioned above.

3.2. Parameters employed in $M_1$ generation

Following parameters were studied from $M_1$ generation.

i. Percent seed germination

Percent of seed germination was calculated after eighth day of seed germination. Seeds, which have given rise to both plumule and radicle, were considered as germinated. The percent seed germination was calculated as follows.

$$\text{Percent seed germination} = \frac{\text{Number of seeds germinated}}{\text{total number of seeds kept for germination}} \times 100$$

ii. Seedling Survival

Survived seedlings from each treatment and their respective controls were recorded after 8 days of seed germination. Rate of seedling survival was expressed in percentages.

iii. Seedling injury

Data on seedling injury was recorded by the length of shoot and root of 8 days old seedlings.

iv. Pollen sterility

Pollen sterility was determined from 10 randomly selected plants belonging to each treatment. Aceto-carmine test was used to determine the pollen sterility. The pollen grains from freshly dehisced anthers were stained with 1% aceto-
carmine. Pollen grains that stained fully were considered as fertile, while the empty, partially stained and shriveled ones were considered as sterile.

The M1 generation was studied for various morphological characteristics like plant height, number of leaves, number of pods, length of pod, grains per pod and 100 seeds weight. The various leaf abnormalities and chlorophyll chimeras and other morphological mutations were recorded. The frequency of M1 plants carrying chlorophyll chimeras of different types (viz. chlorina, xantha etc.) was calculated treatment and variety wise separately.

3.2.1. Harvesting of seeds from M1 plants

All the surviving M1 plants were individually tagged. Seeds of single plants from each treatment were harvested and kept separately.

3.2.2. Raising of M2 and M3 generations

The individual M1 plant progenies seeds were sown in the experimental field of P.V.P. College campus, in separate rows. Each treatment consisted of 20-25 plants. M3 generation was raised from M2 single plant progeny along with controls. Only the elite progenies from M2 generation were carried forward in M3 generation. The spacing between rows and plants were 30 x 10 cm respectively. Agronomic practices followed in M1 generation, were followed to raise M2 generation also. The population was screened keenly for different types of chlorophyll and viable mutations.

3.3. Parameters employed to study the M2 and M3 generations

3.3.1. Chlorophyll mutations

The treated and the control population were carefully screened for chlorophyll mutations during the first 3-4 weeks after germination. The obtained spectrum of chlorophyll mutants was classified according to the terminology of Gustafsson (1940) and Blixt (1961). The frequency of chlorophyll mutations was calculated according to Gaul (1957) i.e., number of mutants/100 M2 seedlings.
3.3.1.1. Estimation of mutagenic effectiveness and efficiency

Mutagenic effectiveness and efficiency of different mutagens were calculated according to the formulae suggested by Konzak et al., (1965). Mutagenic effectiveness is a measure of the frequencies of the mutations induced by a unit dose of mutagens (kR or time x concentration). Mutagenic efficiency gives an idea of the biological damage such as lethality, seedling injury, pollen sterility and chromosomal aberrations.

Mutagenic effectiveness = Factor mutation (MF) / Time X concentration

Mutagenic efficiency = Factor mutation (MF) / Biological damage

= MF/L, MF/I, MF/S

Where, MF = % of chlorophyll mutation in M2 generation, L = % of lethality in M1 generation, I = % of seedling injury in M1 generation and S = % of pollen sterility in M1 generation.

3.3.1.2. Mutation rate

The mutation rate was calculated by the following formulae:

Mutation rate = \[
\frac{\text{Sum of values of effectiveness or efficiency of a particular mutagen}}{\text{Number of treatments of that particular mutagen}}
\]

This gives an idea of mutagens induced by a particular mutagen irrespective of dose.

3.3.2. Analyses of Quantitative traits

From each treatment 25 plants were randomly selected for recording data on different quantitative characters in both the M2 and M3 generations. Similarly 25 plants were picked up from the control for comparative assessment.

Data on the following nine quantitative characters were recorded.

i. Plant height: Plant height was measured at the time of harvesting in centimeters from the base of the plant to apex.
ii. **Number of Branching:** Number of branches was recorded at the maturated stage during 50% flowering for all the treatments and control.

iii. **Number of leaves and leaf area:** The observations on total number of leaves per plant and leaf area were recorded at the maturity stage during 50% flowering for all the treatments and control. The leaf area was measured with leaf area meter (Model NO. LI - 3100). The leaf area index (LAI) was calculated using the following formula. Leaf area index was calculated only for M₃ generation.

\[
\text{Leaf area index (LAI)} = \frac{\text{Leaf area (cm}^2\text{)}}{\text{Ground area (cm}^2\text{)}}
\]

iv. **Days to flowering:** The number of days has taken by the plant from the date of sowing to the opening of the first flower bud on the plant.

v. **Number of pods per plant:** Pods were counted for each plant separately and the average was recorded.

vi. **Days to maturity:** The number of days taken by the plant for the development of the first fully matured pod on the plant was recorded.

vii. **Pod length:** The length of pods per plant was measured. Mean was calculated for each plant and then for each treatment.

viii. **Number of seeds per pod:** Ten pods per plant were opened and the number of healthy seeds was counted. The mean was calculated for each plant and then for each treatment.

ix. **Yield per plant:** The total seed yield per plant was recorded.

X. **100-seed weight:** A composite sample was drawn from the seed yield of the plants and hundred seeds were counted, weighed and the weight was recorded on electronic balance in gram.

xi. **Fresh weight of the plant:** Fresh weight of the 25 plants was recorded and mean values were calculated. Fresh weight was recorded only for M₃ generation.

xii. **Dry weight of the plant:** A known quantity of individual plant material from each treatment was dried at a constant temperature in an oven at 60°C till
the constant dry weight is obtained. It was expressed as percent dry weight. Fresh weight was recorded only for M₃ generation.

xiii. Number of nodules per plant: Number of nodules per plant was counted immediately after harvesting and mean values were calculated.

3.3.3. Scoring of variants in M₁ and Mutants in M₂ and M₃ generations

i. In M₂ and M₃ generations

Mutations, which can be scored visibly and affect the morphological characters of the plant were considered as macromutants. They were scored during the entire life cycle of the plant in M₂ and M₃ generations. All-important details regarding morphological characters of such viable mutants were recorded and the frequency of plants carrying all such mutations was calculated.

ii. Micromutations

These are the mutations that cannot be detected visually but need biochemical analysis for their detection. Such plants were subjected to biometrical analysis and screened accordingly.

3.3.4. Statistical Analysis

The experiments were conducted in three replicates and results were expressed as mean of three determinations. The data of various quantitative characters, macromolecular analysis and physiological parameters were processed statistically, using the method for randomized block design (RBD) described by Panse and Sukhatme (1976).

\[
\text{Mean} = \frac{\Sigma X}{n}
\]

Critical difference (C.V) = S.E. (d) X t e. d. f (error degree of freedom)

Where SE (d) = S.E. difference = S.E. (mean) X \sqrt{2}

S.E (M) = S.E (Mean) = \sqrt{\frac{\text{Mean error sum of square}}{r}}

r = Number of replications

Refer to Chapter 3, Materials and Methods.
3.4. Biochemical and macromolecular analyses

3.4.1. Estimation of chlorophyll

Total chlorophyll was extracted and estimated following the method of Arnon (1949). The leaves were cut into small pieces and 1 gram of sample was homogenized in chilled 80% acetone in mortar with a pestle. The acetone extract was filtered through Whatman No.1 filter paper. The final volume of the extract was made to 100 ml with 80% acetone. The absorbance of acetone extract was read at 645 and 663 nm using UV-visible spectrophotometer (Shimadzu-1601) using 80% acetone as a reference.

3.4.2. Estimation of total carbohydrates

Total carbohydrates were estimated following the method of anthrone as described by Sadasivam and Manickam (1996). The seeds were crushed into powder and 1 gram tissue was hydrolyzed with 10 ml of 2.5 N HCl in a boiling water bath for 3 hours. The acid digested sample was cooled to room temperature and neutralized by adding sodium carbonate. About 5 ml distilled water was added in the neutralized sample and again centrifuged at 5000 rpm for 15 min. The final volume of supernatant was adjusted to 100 ml with distilled water and used for estimation of total carbohydrates. For estimation of carbohydrates 0.1 ml extract was used. The green colour developed in reaction mixture was read at 630 nm UV-visible spectrophotometer (Shimadzu-1601). D-glucose at the concentration of 100 ug ml\(^{-1}\) was used to prepare the standard curve.

3.4.3. Estimation of total free amino acids

The amino acids are colourless ionic compounds that form the basic building blocks of proteins. Apart from being bound as proteins, amino acids also exist in free form in many tissues and are known as free amino acids. Very often due to mutagenesis or other changes, the free amino acid composition exhibits a change and hence, the measurement of the free amino acids give the
physiological and health status of the plants. Free amino acids were estimated following the method suggested by Sadasivam and Manickam (1996).

500 grams of defatted powder of the seeds were dissolved in 5 to 10 ml of 80% ethanol. The homogenate was centrifuged and the supernatant was saved. The volume of the supernatant was reduced by evaporation and the extract was used for the estimation of free amino acids. The reaction gives intensely coloured bluish purple product, which is colorimetrically measured at 570 nm on a UV-visible spectrophotometer (Shimadzu-1601). Lucine at the concentration of 10 \( \mu g \) to 100 \( \mu g \) ml\(^{-1}\) was used to prepare the standard curve.

3.4.4. **Study of peroxidase isozyme profiles**

Peroxidase isozyme profiles were analysed on polyacrylamide gels of 7.5% pore size, employing horizontal submerged polyacrylamide gel electrophoresis. Peroxidase isozyme profiles were carried out using seeds of selected mutants.

i. **Extraction of peroxidase isozymes from the plant material**

Peroxidase isozymes were extracted from control and mutagen administrated seeds. One gram dry seeds were homogenized in 2 ml of cold Tris HCl buffer (1.5 M, pH 8.8). Homogenates were centrifuged at 5,000 rpm, for 10 to 20 minutes at 4°C until the supernatant was clear. The supernatants were decanted and stored at -20°C until subjected to electrophoresis. 500 \( \mu l \) of supernatant was then mixed with equal quantity of glycerol (50% V/V) containing bromophenol blue (0.05 mg/ml). 50\( \mu l \) sample was used for loading per well.

ii **Casting of polyacrylamide gels**

Horizontal gel mould, used in the submerged horizontal slab gel electrophoresis, was cleaned thoroughly and its both the open ends were closed with a cellophane tape. A comb of desired size was affixed approximately at one third of its length. Separating gel mixture of 7.5% pore size was poured in the gel mould taking care that the thickness of the gel does not exceed 1.5 mm. No stacking gel was used in this method. Polymerization took about 15 to 20 minutes.

The following solutions are required in ready stock for preparation of polyacrylamide gel slabs of 7.5 pore size.

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Chapter 3. Materials and Methods
1. Stock Acrylamide: - (30 % acrylamide + 0.8 bis-acrylamide). Dissolve 30 gm acrylamide and 0.8 gm N,N, Methylene bis-acrylamide in 80 ml water and then make up the final volume to 100 ml. Filter and store in dark bottle preferably in a refrigerator.

2. Separating gel buffer: - (1.5 M Tris-HCL buffer, pH 8.8) Dissolved 18.17 gm. Tris- (Hydroxy methyl, methyl amine (2 amino, 2-(hydroxy methyl)propane-1-3-diol) in 80 ml water and adjust the pH to 8.8 with concentrated HCL make final volume to 100 ml.

3. Electrode buffer: - (0.05 M Tris+0.38 M glycine pH 8.3) dissolved 6.0 gm Tris and 28.8 gm glycine in 980 ml water and make the final volume to 1,000 ml. The pH of the buffer will be between 8.2 – 8.4.

4. Ammonium persulphate (10%): - dissolved 1.0 gm ammonium persulphate in 7.0 ml D.W. and make the final volume to 10.0 ml. This should be freshly prepared every time, just before use.

5. TEMED (N,N,N,N,tetramethylethylenediamine): Fresh from refrigerator.

The above solutions are mixed in the following ratio to obtain separating gel of 7.5% pore size.

1) Stock acrylamide 2.5 ml.
2) Separating gel buffer 3.4 ml.
3) Distilled water 4.0 ml
4) Ammonium persulphate 50 µl.
5) TEMED 50 µl.

iii. Electrophoresis

After the gels were completely polymerized, the mould containing the polymerized gel was placed in the migration chamber in such a way that the wells face towards the cathode. The migration chamber was filled with electrode buffer (Tris-Glycine, pH 8.2) until the polymerized gel is completely immersed in the electrode buffer. At this stage the comb was removed carefully, without disturbing the wells. 30µl sample (plant extract of both control and mutagen administered plants) was loaded in to each well, using micropipettes. The gel
was run at 25 to 30 mA at room temperature until the marker dye migrated to the edges of gel.

iv. Activity staining of peroxidase isozymes

The peroxidase isozymes present in the polyacrylamide gel slabs were detected using activity staining method of Shrauwen (1966). The staining was done using benzidine solution in presence of hydrogen peroxide. The preparation of incubation mixture for the activity staining of peroxidase is as follows. Incubation medium was prepared by mixing the following solutions.

1. Saturated benzidine

Take 200ml distilled water and boil it for few minutes. Add 4 grams of benzidine to the boiling water and boil it for another 4-6 minutes. Cool it and filter. Store it in an amber coloured bottle preferably in a refrigerator, until its use.

2. Ammonium chloride (30%)

Dissolve 30 grams of Ammonium chloride in 50 ml distilled water and then make it up to a final volume of 100 ml.

3. Hydrogen peroxide (0.2%)

Add 0.2 ml of Hydrogen peroxide to 99.8 ml distilled water. This should be prepared fresh just before use. Mix 1, 2 and 3 in 100:15:20 ratio. Soak the gel after electrophoresis for peroxidase isozymes in this incubation medium for 10-15min. Sites (bands) indicating the presence of peroxidase appears reddish brown. Peroxidase isozymes present in the acrylamide gel react with the hydrogen peroxide present in the incubation medium and produces nascent oxygen at the site of peroxidase. The nascent oxygen reacts immediately with Benzidine in the presence of ammonium chloride and oxidizes the Benzidine into a brown coloured end product appeared on the acrylamide gels was as bands. The gel was fixed in 7% acetic acid to stop the activity of staining and later it preserved in 2% acetic acid if necessary. The gel was photographed immediately to make a permanent record of the bands. They were also traced on a tracing paper to facilitate the calculation of the relative mobility (Rm values). The relative mobility of each isozyme was calculated using following formula.

*Chapter 3. Materials and Methods*
3.4.5. Nitrate reductase - In vivo anaerobic assay

The in vivo anaerobic nitrate reductase assay was carried out following to the method of Sawhney et al., (1978). For this about 200 mg leaves were weighed and cut into small pieces. They were transferred to test tubes containing 5 ml of assay mixture (2.5 ml of 0.1 molar phosphate buffer-pH 7.2, 0.2 ml n-propanol and 2.3 ml distilled water). The tubes were vacuum infiltrated for 45 minutes in dark at 30°C. The reaction was terminated by keeping the tubes in boiling water bath for 10 minutes. The assay mixture was cooled and 1.0 ml of this was used for developing colour with sulphanilamide and napthyl ethylenediamine reagent for 10 minutes. The optical density was measured at 540 nm against the blank on UV-visible spectrophotometer (Shimadzu - 1601). The amount of NO₂ produced was calculated from the standard curve and the nitrate reductase activity was expressed in terms of μ moles of NO₂ produced g⁻¹ fresh weight hr⁻¹. Sodium nitrite (NaNO₂) was used at the concentration of 200 μg ml⁻¹ as a standard nitrite to prepare the standard curve.

3.4.6. Analysis of Seed Storage Proteins

3.4.6.1. Extraction and Estimation of total seed proteins

i. Extraction of proteins

Healthy, mature seeds of control and isolated mutant plants were used for extraction of proteins. The seeds were ground in mortar and pestle to make a fine powder. This fine seed powder was defatted with acetone. After defatting the fine seed powder was air dried and the powder was dissolved in 2.5 ml of 0.1 molar phosphate buffer (pH 7.0). The extract was centrifuged at 5000 rpm for 15 minutes at 4°C. The clear supernatant was collected and used as the source of protein.

ii. Estimation of Proteins

Total proteins were estimated following the method of Lowry et al., (1951). 0.2ml extracted protein sample was used to prepare the reaction mixture. To this 0.8
ml distilled water, 3 ml of alkaline mix and 1ml folin phenol reagent was added. The whole mixture was shaken well and kept at room temperature. The blue colour developed was read at 660 nm on UV-visible spectrophotometer (Shimadzu-1601). The BSA (Bovine serum albumin fraction V) was used at the concentration of 1mg–1 as standard protein, to prepare standard curve.

iii. SDS-PAGE Analysis of proteins.

The methodology adapted for SDS-PAGE analysis of total proteins is seeentially similar to that mentioned under SDS-PAGE analysis of albumins and globulins.

3.4.6.2. Estimation and SDS-PAGE analysis of Albumins and Globulins

In the present investigation albumin and globulin fractions of seed storage proteins of seeds of isolated mutant, were analysed on Polyacrylamide slab gels. For this purpose control and mutagen treated seeds were first ground to flour and then it was defatted to remove oily substances. From such defatted seed meal albumins and globulins were extracted by using dialysis bags. They were later used for quantitative estimation and qualitative analysis of protein profiles on SDS – PAGE. The procedures followed are as follows:

i. Defatting of seed meal

Seed sample (3 gram) was weighed and ground to a fine powder in mortar with a pestle. The seed powder (flour) was sieved through a fine mesh to get fine powder. This powder was transferred to a glass column packed with glass wool and sodium sulphate. The powder was first defatted with sufficient acetone (100 ml) and then with a mixture of acetone and ether in equal proportions and finally with pure ether. The defatted meal was then baked at 60°C in an oven for 1-2 hours to evaporate and remove the excess ether. The dry defatted seed meal was stored at 4°C until further use for extraction and purification of albumins and globulins.

ii. Extraction and purification of albumins and globulins

One gram defatted seed meal was extracted in 10 ml extraction buffer for 1 hour at 4°C on magnetic stirrer. The extract was then centrifuged at 10,000 rpm for 15 minutes. The supernatant was saved as source of albumins and globulins. For purification of albumins and globulins supernatant was taken in dialysis bags and
dialyzed against sodium acetate buffer (pH 4.75) at 4°C overnight. Globulins usually get precipitated because of decrease in ionic strength of the extract and alteration in pH by sodium acetate buffer. The precipitate of globulin was removed by centrifuging the contents of dialysis bag at 10000 rpm for 10 minutes. The supernatant contained albumins, as albumins are soluble in water as well as in salt solution with low ionic strength. The globulin fraction was dissolved in 5 ml of Tris-HCl buffer. The quantity of albumins and globulins were estimated employing biochemical methods. These albumin and globulin fractions were also used for the SDS-PAGE analysis of protein profiles.

iii. Biochemical Estimation of albumins and globulins

Albumins and globulins thus extracted were estimated using Lowry et al., (1951) method. 0.2 ml sample was taken in a test tube and diluted to a final volume of 1.0 ml. To this 3.0 ml of alkaline mix and 1.0 ml of folin phenol reagent was added. The contents were shaken thoroughly and kept undisturbed for 30 minutes at room temperature. The blue colour developed was read at 660 nm on UV-visible spectrophotometer (Shimadzu-1601). The BSA (Bovine serum albumin, fraction V) was used at the concentration of 1mg/ml as standard protein, to prepare the standard curve.

iv. Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) of albumins and globulins.

Electrophoresis is widely used to separate and characterize proteins by applying electric current. Electrophoretic procedure is rapid and relatively sensitive requiring only micro-weight of proteins. Electrophoresis in the polyacrylamide gel is more convenient than in any other medium such as paper and starch gel. Separation of proteins in SDS-free polyacrylamide gels relies on both the charge and size of the proteins, whereas it depends only upon the size in the SDS-gels. Analysis and comparison of proteins in a large number of samples is easily made on polyacrylamide gel slabs.

SDS is an anionic detergent, which binds strongly to, and denatures proteins. The number of SDS molecules bound to polypeptide chain is approximately half the number of amino acid residues in that chain. The protein-SDS complex carries net negative charges, hence moves towards the anode.
and separation is based on the size of the protein. SDS-PAGE was carried out by the method of Davis (1964), Laemmli (1970) and Dadlani and Varier (1993).

a. Preparation of solutions

The following solutions are required to prepare the polyacrylamide gels.

1. Stock Acrylamide: (30 % acrylamide + 0.8 bis-acrylamide). Dissolve 30 gm. acrylamide and 0.8 gm N, N, Methylene bis-acrylamide were dissolved in 80 ml water and then made to a final volume of 100 ml. The solution was filtered and store in a dark bottle in a refrigerator.

2. Separating gel buffer: (1.5 M Tris- HCl buffer, pH 8.8). 18.17 gm. Tris-(Hydroxy methyl, methyl amine (2 amino, 2-(hydroxy methyl) propane-1-3-diol) was dissolved in 80 ml water and the pH was adjusted to 8.8 with concentrated HCl. The final volume was made to 100 ml by adding distilled water.

3. Stacking gel buffer: (pH 6.8). 7.26 gm of Tris (Hydroxy methyl, methyl amine (2 amino, 2-(hydroxy methyl) propane-1 3-diol) was dissolved in 100 ml water and the pH was adjusted to 6.8 with concentrated HCl. The volume was made to 100 ml by adding distilled water.

4. Electrode buffer: - (0.05 M Tris +0.38 M glycine pH 8.3). 6.0 gm Tris and 28.8 gm glycine were dissolved in 980 ml water and the final volume was made to 1,000 ml. To this 2 grams of SDS was added. The pH of the buffer was between 8.2 – 8.4.

5. Ammonium persulphate (10%): 1.0 gm ammonium persulphate was dissolved in 7.0 ml distilled water and the final volume was made up to 10.0 ml. This was prepared fresh every time, just before use.

6. TEMED (N,N,N,N, tetramethylethlenediamine): This was used Fresh from refrigerator.

The above solutions were mixed in the following ratios to obtain resolving (separating) gel of 12.0% pore size and stacking gel of 4% pore size.

**Preparation of resolving (separating) gel**

| Stock Acrylamide solution | 12 mL |

*Chapter 3. Materials and Methods*
Tris-HCL (pH 8.8) 7.5 mL
Water 12.05 mL
Degas on a water pump for 3-5 min and then add
10% SDS 30 µL
TEMED 20 µL
Ammonium persulphate solution 15 µL

**Preparation of stacking gel (4%) (Total volume 10 mL)**

Stock Acrylamide solution 1.35 mL
Tris-HCL (pH 6.8) 1.00 mL
Water 7.50 mL
Degas as above and add
Ammonium persulphate solution (5%) 50 µL
10% SDS 0.1 mL
TEMED 20 µL

**Sample Buffer (Laemmli buffer)- 5x concentration.**

Tris-HCL Buffer pH 6.8 1.0 mL
SDS 10 % 1.6 mL
Glycerol 0.8 mL
β Mercaptoethanol 0.4 mL
Water to 4.2 mL
Bromophenol Blue (0.5% W/V solution in water)

Sufficient Bromophenol blue solution was added to get appropriate colour to protein samples. Samples were stored frozen in small aliquots. They were dilute to 1x concentration at the time of use. Sodium dodecyl sulphate 10% solution—store at room temperature. Marker proteins were dissolved in single strength sample buffer (Laemmli buffer) at a concentration each of 1 mg per mL. The wells were loaded with 25-50 µL samples.

**b. Method**

The glass plates and spacers were thoroughly cleaned, and assembled them properly. They were held together firmly with bulldog clips. The clamps were placed an upright position. 3% agar (melted in a boiling water bath) was then
applied around the edges of the spacers to hold them in place and prevent any leakage between the glass plates. Holding the glass plates vertically, the separating gel mixture was poured into the chamber between the glass plates to ¾ of volume and allowed to polymerize.

After polymerization water was removed from the top of the gel and wash with little stacking gel solution. Stacking gel solution was poured quickly on top of the polymerized gel. A comb was placed to form the sample well and the gel was allowed to polymerize. After the stacking gel was polymerized, the comb was removed without distorting the shapes of the well. The electrophoresis unit was filled with electrode buffer and the glass plates with the gel were installed in the electrophoresis apparatus. 30ul protein samples were mixed with equal volume of sample buffer in 1:1(v/v) ratio and were heated in boiling water for 2-3 min. to ensure complete interaction between proteins and SDS. They were loaded in the sample wells. Electrophoresis was carried out at a constant current at 30 mA until the bromophenol blue reaches the bottom of the gel. After the completion of run, the gel was removed and was fixed in 10% TCA for 30 minutes. Molecular weights of these proteins, globulins and albumins of mutants and controls were determined employing the protein molecular weight marker (PMW-M of Genei, Bangalore, India Ltd.) were stained with coomassie brilliant blue R 250 overnight and destained in destaining solution till the background became colourless. The coomassie brilliant blue R 250 stain was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie brilliant blue R 250</td>
<td>0.1g</td>
</tr>
<tr>
<td>Methanol</td>
<td>40.0 mL</td>
</tr>
<tr>
<td>Glacial Acetic acid</td>
<td>10.0mL</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>50.0 mL</td>
</tr>
</tbody>
</table>

The destaining solution (destainer) was prepared as mentioned above but without including the dye (Coomassie brilliant blue R 250). The Relative Mobility Values (RM) were calculated using the formula:

\[
RM = \frac{\text{Distance traveled by the band}}{\text{Distance traveled by the tracking dye}}
\]