MATERIALS AND METHODS
### MATERIALS AND METHODS

#### 3.1. Materials

The Soybean (*Glycine max* (L.) *Merill*) varities JS 335, Punjab-1 and their hybrid (JS335X Punjab-1), were utilized for the investigation. The genotypes used, their pedigree, source and distinguishing features are given in the following table.

<table>
<thead>
<tr>
<th>Character</th>
<th>Varieties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JS 335</td>
</tr>
<tr>
<td><strong>Pedigree</strong></td>
<td>JS 78-77 X JS 71-05</td>
</tr>
<tr>
<td><strong>Age in days</strong></td>
<td>85-90</td>
</tr>
<tr>
<td><strong>Height of plant</strong></td>
<td>Short</td>
</tr>
<tr>
<td><strong>Leaf Colour</strong></td>
<td>Dark Bluish Green</td>
</tr>
<tr>
<td><strong>Flower Colour</strong></td>
<td>Violet</td>
</tr>
<tr>
<td><strong>Yield in Kg/Hectare</strong></td>
<td>800-1000</td>
</tr>
</tbody>
</table>
3.2. Crossing Techniques

Two different crossing blocks were raised at intervals of 15 days. Crossing was effective using JS 335 as female parent and Punjab-1 as male parent. Conventional crossing method as well as rapid method of hand pollination techniques, as adopted by Rachie et al (1975), were followed.

3.3. Mutagen

The physical mutagen, gamma rays were used in the present investigation. Gamma irradiation was done in gamma chamber-900 installed at sugar cane by exposing the dry seeds to a Co-60 gamma source. Pre-examination of gamma rays from the cell at the time of treatment was at the rate of 0.532486 million roentgen units per hour.

The following four treatments including the control were formulated for the investigation.

1) Control
2) 30 Kilo rad
3) 40 Kilo rad and
4) 50 Kilo rad

3.4. Methods

3.5. Study of M1 generation

After mutagenic treatments, under laboratory studies 25 seeds of each treatment were sown in a tray filled with sterilized sand and each treatment was
replicated twice under laboratory conditions (28± 1 c). Observation were made of shoot and root length.

Under field condition 250 seeds of each treatment were sown in a factorial randomised block justify with 5 replication at the post graduated and Research Department of Botany, Jamul Mohamed College, Tricky. The seeds were sown in a single row of 4 m length, adopting a spacing of 45 cm rows and 20 cm between plants with a single seeds per hole. Recommended fertilizers, plant protection methods and general cultural practices were uniformly followed for all the treatments. The following observations were made M1 and method adopted for recording them are given below. The observations were recorded on 25 plants affected at random in each treatment, in each replication.

3.5.1. Germination

The seed germination counts were taken daily from 3rd to 15th day after sowing. The emergence of cotyledotery leaves was taken as the variation of germination.

3.5.2. Survival

On 30th day of sowing, the survival of seedling were recorded by counting the number of survived plants percentage of total seeds germinated.
3.5.3  **plant growth**

3.5.3 a.  **Shoot length**

The height of the plant was measured from the ground level to the tip of the plant on 30\textsuperscript{th} day.

3.5.3 b.  **Root length**

The root length was measured from ground level to the tip of the root on, 20\textsuperscript{th} day. The average were recorded and expressed and percentage of the root length.

3.5.4.  **Number of primary branches / plant**

The total number of branches arising from the main stem was recorded at the time of maturity.

3.5.5.  **Length of branches**

The length of branches were measured in centimeter and tabulated for each and every branch at a various periods.

3.5.6.  **Number of pods per plant**

The total number of productive pods per plant was count and the mean value was arrived at the time of maturity.

3.5.7.  **Pod length**

A sample of 100 pods were taken for recording the length of the pod.

3.5.8.  **Number of seeds per pods**

For recording the number of seeds per pod, a sample of 100 pods were taken.
3.5.9. Seed yield per plant

After thrashing, the grains were weighted and the yield of each plant was recorded in grams.

3.5.10 Related water content of the plants

It was the dried weight of the plants, determined at 15\textsuperscript{th} day.

\[ \text{R.W.C} = \frac{\text{WEIGHT OF FRESH PLANT} - \text{DRIED PLANT}}{\text{WEIGHT OF FRESH PLANT}} \times 100 \]

3.5.11 Determination of moisture content in seeds

5 gm of seeds were collected, powdered and weighed accurately into a silica crucible kept in a Hot air oven at 100 degree centigrade for two hours. An alternated consecutive weights were taken at a frequent time interval. The procedure was continued until there was a constant weights were obtained. The moisture content of seeds were recorded and tabulated.

\[ \text{Weight of seeds} - \text{constant weight} = \frac{\text{WEIGHT OF SEEDS} - \text{WEIGHT OF SEEDS}}{\text{WEIGHT OF SEEDS}} \times 100 \]

3.5.12. Determination of ash and minerals

After ash content was completed, the silica crucibles were placed into a Muffle furnace and maintained the temperature of about 700 degree for six hours. Now crucibles were transferred into a desiccators. Using 5\% hydrochloric acid, the ashes were dissolved into a litre of the standard flask. This was transferred into a plastic bottle and analysed for the estimation of minerals in an
atomic absorption analyzer. The ash and mineral contents were determined and recorded.

3.5.13 Estimation of chlorophyll (Arnon's 1949)

Chlorophyll estimation was done according to Arnon's (1949). Fresh leaves were washed thoroughly in distilled water and blotted dry using Whatmann filter paper. They were cut into small pieces and 500 mg fresh leaf tissue was centrifuged at 5000 rpm for 5 minutes and the supernatent was decanted and stored. The residue was again washed with 5.0 ml of 80% acetone and centrifuged and the procedure was repeated until the pellet become colourless. The supernatants were pooled and the total volume measured. The absorbance was measured using spectronic 20 at 663 nm and 645 nm and the contents of chlorophyll 'a' and 'b' and total chlorophyll were calculated using the following formula.

\[
\text{Total chlorophyll (mg/g)} = \frac{20.2 (\text{OD 645} \times 8.02 (\text{OD 665}))}{1000 \times W} \\
\text{Chlorophyll 'a' (mg/g)} = \frac{12.7 (\text{OD 665}) - 2.69 (\text{OD 645})}{1000 \times W} \\
\text{Chlorophyll 'b' (mg/g)} = \frac{22.9 (\text{OD 645}) - 4.68 (\text{OD 665})}{1000 \times W}
\]
3.5.14. Estimation of carbohydrate (Anthrone method)

The basic units of carbohydrates are monosaccharides which cannot be split by hydrolysis into more simple sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant mono saccharides.

PRINCIPLE

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

Materials

- 2.5 N - HCl
- Anthrone reagent
- Stock and standard glucose

Procedure

- 100 mg of powdered seed was weighed and transferred into a boiling tube
• It was kept in a boiling water bath for three hours with 5ml of 2.5 N – Hcl and cooled in room temperature.

• It was neutralized with solid sodium carbonate until no effervescence and made upto 100 ml and centrifuged.

• The supernatent was collected and 0.5 and 1.0 ml taken for the analysis.

• The standards were prepared by 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml. The contents were made upto 1.0 ml using distilled water. And 4.0 ml of Anthrone reagent was also added and kept in a boiling water bath for eight minutes and cooled in a room temperature.

• The green to dark green colour was read a 630 nm.

Calculation

\[
\text{Amount of carbohydrate present in 100 g of the sample} = \frac{\text{Mg of glucose}}{\text{Volume of test sample}} \times 100
\]

3.5.15. Estimation of protein (Lowry's et al, 1951)

5gm of the seed was homogenized with 5ml of 80% ethanol and centrifuged at 10000rpm for 15 minutes. The supernatent was made upto 5ml. To one ml of this solution, 2 or 3ml of 10% ice cold TCA was added and incubated at four degree centigrade for an hour. The precepitated protein was
centrifuged and the pellet was collected and dissolved in 1ml of 0.2N NaOH. To 0.1 ml of the protein solution, 5ml of alkaline copper reagent was added. It was shaken well and allowed to stand at room temperature for 10 minutes. Then 0.5 ml of Folin reagent was rapidly added and the volume made up to 6 ml with distilled water. For the blank 0.1 ml of distilled water was taken all the reagents were added as above after 30 minutes, the optical density was read at 660 nm in spectronic 20. Protein content was calculated by referring to standard curve of BSA and the values were expressed as mg Protein/gram weight.

3.5.16. **Estimation of lipid in oil seeds**

2 gm of the seed was extracted with petroleum ether. It was then distilled off completely, dried, the oil weighed and the % oil was calculated with the help of Soxhlet apparatus.

\[
\text{\% of oil content} = \frac{\text{Weight of oil (g)}}{\text{Weight of sample (g)}} \times 100
\]

0.1N KOH till the pink colour persisted.

3.5.17. **Estimation of phenol** (Mahadevan and Sridhar, 1996)

5 gm of the powdered seed was ground in Mortar and Pestle using 80% ethanol. 10000 rpm centrifuged to collect the supernatent for the estimation purpose. The 1 ml of extract in a test tube, 1 ml of folin’s phenol reagent was added. To the content 2 ml of 20 % sodium carbonate was
added and mixed thoroughly and boiled for few minutes in a boiling water bath. It was cooled under the tap water and measured the OD at 650 nm.

The amount of total phenol present in the sample was measured in mg / g weight. linear phase of the reaction (20-60). Enzyme activity was expressed as units minutes per milligram per protein (Beers and Sizer, 1952). One unit of catalase was equal to one μ mole of H2O2 decomposed per min. at 25°C.

\[
\text{Acid value (mg KOH/g)} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample (g)}}
\]

3.5.18. Ortho dihydroxy phenol

500 mg of the sample was ground well with 80.5 ethanol and centrifuged the homogenate and the supernatent was used for the estimation of the ortho dihydroxy phenol. To 2 ml of the extract was made up to 6 ml with water. 1.0 ml of 10 % sodium nitrate, 20 % urea and 0.1 ml of 10% Acetic acids were added. After 4 min. 2.0 ml of 2N Sodium hydroxide was added and read at 620 nm in a spectrophotometer against the blank.
3.5.19. Assay of catalase

500 mg of seed was homogenized at 4°C by using Mortor and pestle with polyvinyl pyrrolidine (25 % weight) and four volumes of buffer consisting of 50 mM KH₂PO₄/ K₂HPO₄ and 0.1 mM EDTA at pH 7.0. the macerate was filtered through muslin cloth and then centrifuged at 8000 rpm using refrigerated centrifuge for 20 min. at 4°C to remove the cell debris. The supernatent was used as crude enzyme. Catalase was measured by a modified spectrophotometer procedure based on the rate of decomposition of H₂O₂ by following the decline in absorbance at 240 nm (Claiborne, 1985). The assay was performed in a 3.0 ml quartz cuvette containing 200 μl enzyme extract, 50 mM potassium phosphate buffer pH 7.0 and 37.5 mM H₂O₂. The rate of H₂O₂ decomposition was determined during the linear phase of the reaction (20-60). Enzyme activity was expressed as units minutes per milligram per protein (Beers and Sizer, 1952). One unit of catalase was equal to one μ mole of H₂O₂ decomposed per min. at 25°C.

\[
\text{Units mg per protein} = \frac{\text{Absorbance / min.} \times 1000}{43.6 \times \text{mg protein / ml reaction mixture}}
\]

- = Molar absorbance index for hydrogen peroxide at 240 nm in a 1 cm cuvette
3.5.20. DETERMINATION OF AMINO ACID SEQUENCE IN PEPTIDE

The poly peptide of related molecular mass of 4400 was isolated from the Soybean (Glycine max) seeds by 60% ethanol in a mortar and pestle and followed by ion-exchange and reverse phase chromatography. The primary structure of complete sequence was determined by an Edman degradation method. The sequence was expressed in results. The C-terminal was a poly Aspartyl tail and N-terminal was the Serine. There are 43 amino acid with relative molecular mass of 44,000. The ethanol isolated extract was purified by ion exchange and reverse-phase chromatography with an elution of CM-cellulose. There are five peaks given the recorder. The 5th peak was largely due to Soybean molecular weight. It was further purified with reverse-phase chromatography. The yield of this peptide was calculated to be 3.5 mg per Kg of Soybean seeds.

The relative molecular mass was calculated by gel filtration in the presence of 6M guanidine Hydrochloride to be 44,000. Its biochemical and biological aspects were unknown.

3.6. Study of M2 generation

M2 Generation was raised in the field on individual M1 plant progeny basis in the randomised block design with four replications. The seeds were sown in the beds adopting a spacing of 45 cm bet rows and 20 cm bet plants with single seeds per hole. The following parameters were used to study the M2 generation.
3.6.1. Germination
3.6.2. Survival
3.6.3. Plant growth
3.6.4. Number of primary branches
3.6.5. Length of branches
3.6.6. Number of pods per plants
3.6.7. Pod length
3.6.8. Number of seeds per pod
3.6.9. Seed yield per plant
3.6.10. Related water content of the plant
3.6.11. Determination of moisture content of in seeds
3.6.12. Determination of ash content and minerals
3.6.13. Estimation of chlorophyll content
3.6.15. Estimation of proteins
3.6.16. Estimation of oil in seeds
3.6.17. Estimation of phenol
3.6.18. Estimation of ortho dihydroxy phenol
3.6.19. Assay of catalyse
3.6.20. Determination of amino acid sequence in peptide
3.7. M3 Generation

The M3 Generation was raised in the field on individual M2 plant progeny basis in the randomized block design. The seeds were sown in the beds adopting as spacing of 45 cm between rows and 20 cm between plants with single seeds per hole. 600 (6 x 4 x 25) families at the rate of 25 families per treatment per genotype, the significance was tested and regressions were also computed.

3.7.1 Germination

3.7.2 Survival

3.7.3 Plant Growth

3.7.4 Number of Primary Branches

3.7.5 Length of Branches

3.7.6 Number of pods per plant

3.7.7 Pod length

3.7.8 Number of Seeds per Pod

3.7.9 Seed yield per Plant

3.7.10 Related Water Content of the Plant

3.7.11 Determination of Moisture content in seeds

3.7.12 Determination of Ash content and Minerals

3.7.13 Estimation of Chlorophyll Content

3.7.14 Estimation of Carbohydrate
3.7.15 Estimation of Proteins

3.7.16 Estimation of Oil in Seeds

3.7.17 Estimation of Phenol

3.7.18 Estimation of Ortho Dihydroxy Phenol

3.7.19 Assay of catalase

3.7.20 Determination of Amino Acid Sequence in Peptide

3.8. Statistical Analysis

The M1, M2 and M3 generations, in respects of germination, survival were analysed. For complete statistical picture of the mutagenic effect in the generation, correlation, coefficients were calculated between dose of mutate and percentage reduction compared with controls for the genotypes. The significance was tested and regression were also computed (Panse and Sukhatme, 1967).

3.8.1. Abbreviations

K rad : Kilo rad

L D 50 : Dose causing 50 per cent reduction

M1 : First generation after mutagenic treatment

M2 : Second generation after mutagenic treatment

M3 : Third generation after mutagenic treatment