CHAPTER III
MATERIALS AND METHODS

3.1. Materials

3.2. Variety used

In the present investigation, the variety of chick pea (Cicer arietinum L.) namely CO – 4 (Desi) were used and was obtained from Department of Pulses, Tamil Nadu Agriculture University, Coimbatore, Tamilnadu. The present study was conducted at Department of Botany, Annamalai University, Annamalai Nagar, India during 2012 – 2015. A brief description of CO – 4 variety is given below.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Variety CO -4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parentage</td>
<td>Cross derivative of ICC42 X ICC12237</td>
</tr>
<tr>
<td>Year of Release</td>
<td>1998</td>
</tr>
<tr>
<td>Source</td>
<td>TNAU, Coimbatore</td>
</tr>
<tr>
<td>50% flowering</td>
<td>40 days</td>
</tr>
<tr>
<td>Plant Height</td>
<td>35 – 40 cm</td>
</tr>
<tr>
<td>Branches</td>
<td>3 – 5</td>
</tr>
<tr>
<td>Flower colour</td>
<td>Light pink and Veined</td>
</tr>
<tr>
<td>Colour of grain</td>
<td>Brown</td>
</tr>
<tr>
<td>100 seed weight (g)</td>
<td>30-32</td>
</tr>
</tbody>
</table>
3.3. Experimental Procedure

3.4. Mutagens Used

Two types of mutagens were employed for the present study. Gamma rays are used as physical mutagen and EMS as a chemical mutagen.

3.5. Physical Mutagens: Gamma rays

Gamma rays were chosen as a physical mutagen. The dry seeds of chick pea with 12% moisture content were irradiated with 20, 30, 40, 50 and 60 kR of gamma rays with radioisotope $^{60}$Co (Cobalt – 60), source from Indira Gandhi Centre of Atomic Research, Kalpakkam, Tamilnadu, India.

3.6. Chemical Mutagen: Ethyl Methane Sulphonate ($C_3H_8O_3S$)

Ethyl Methane Sulphonate (EMS) is a nonfunctional alkylating agent, having molecular weight 124.16 with density of 1.20 and a half life period of 30 hours. The chemical was purchased from Hi-Media Laboratories, Mumbai, India. For chemical treatment, healthy seeds of uniform size were presoaked for six hours in distilled water and treated with different concentrations (10, 20, 30, 40 and 50 mM) of EMS for six hours with intermittent shaking at room temperature. The EMS solution was prepared in the phosphate buffer at pH 7. After the treatment, seeds were washed thoroughly under tap water to eradicate the excess of EMS.
Chapter III: Materials and Methods

Control

Three sets of 50 dry, well – matured, healthy and uniform size of non – dormancy seeds were soaked in the distilled water for six hours. The seeds were again soaked in phosphate buffer solution for six hours at room temperature (28±2°C) and they were sown along with treated seeds.

3.7 Sample size

A set of 50 seeds with three replications were used for each treatment and control in M₁ generation.

3.8 Handling and selection of the treated material in different generations

3.9 M₁ generation

Three replications of 50 seeds each, were sown for each treatment and control in a CRBD method at Botanical Garden, Department of Botany, Annamalai University, Annamalai Nagar. The spacing was maintained between 30cm x 15cm (within the row and between the rows) in the field. Another set of seeds were sown in pot condition and was used to determine the percentage of seed germination and seedling height. All the recommended practices were applied during field preparation, seed sowing and subsequent management for chick pea population.
3.10. Seed germination

Seeds were treated with various mutagens and placed in petriplates with moisture germinating paper to find out the germination percentage and LD$_{50}$ value. Seeds were counted on the day of emergence of cotyledonary leaf and were taken as the indication of germination. Germination percentage was worked out for the treatment in each concentration separately, and lethality was found out. The germination percentage is calculated as follows,

\[
\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}} \times 100
\]

3.11. Seedling height

The seedling height was recorded on the 15$^{th}$ day by measuring root and shoot length.

3.12. Seedling injury

Seedling injury was measured with respect to the reduction of seedling height on 30$^{th}$ day.

3.13. Lethality

The lethality was measured based on the number of seedlings died with respect to control.

3.14. Pollen fertility

The pollen fertility was determined in 30 randomly selected plants (Ten plants from each replication) from each treatment and control at the time of flowering. The pollen grains were stained with
1% solution of acetocarmine in a clean glass slide and covered with cover slides. The pollen grains with regular outline and took the stain was considered as fertile. The unstained shrunken pollen grains were considered as sterile.

3.15. Plant survival

The plant survivability was observed on 30th day-after seed sowing. The number of surviving plants was counted and the survival was computed as percentage of seeds germinated.

3.16. Cytological observation – Mitotic studies

Mitotic studies were conducted on ten randomly selected plants from both treatment and control. For mitotic studies, the root tips were collected between 8.30 AM to 9.30 AM and fixed in acetic acid:ethyl alcohol (1:3) for 24 hours. The root tips were squashed by Iron alum - Haematoxylin squash technique according to Marimuthu and Subramanian (1960). After 24 hours of fixation, the root tips were washed thoroughly in distilled water for three times and kept in 40% iron alum for 3 minutes. Then the root tips were washed with distilled water and kept in haematoxylin stain for three hours. After staining, the root tips were washed thoroughly with distilled water and treated with 45% acetic acid for 1 minute to soften the tissue. Acetic acid is a destaining agent. One or two root tips were placed on a slide and squashed with coverslip and
examined under microscope for their behavior at various phases of mitosis. The photographs were taken from temporary preparations. Photographs were taken by phase contrast microscope.

3.17. Quantitative traits

Observations were made on 30 – 45 normal-looking plants for each progeny in each treatment along with control. The following ten quantitative traits were studied in all the four generations.

1. **Days to first flowering**: Days to first flowering was noted down as the number of days taken to opening first flower bud from the date of sowing.

2. **Days to 50% flowering**: Days to 50% flowering was noted for the number of days taken for 50% flowering.

3. **Plant height (cm)**: The height of the plant was measured from base to top of the plant at maturity and expressed in centimeter.

4. **Number of primary branches per plant**: The total number of primary branches arising from the main stem were counted and recorded at maturity.

5. **Number of secondary branches per plants**: Number of branches borne on the primary branches were recorded at harvest and expressed in number.
6. **Number of pods per plants**: The total number of pods were counted at ripeness and noted as the number of pods bearded on plant.

7. **Seeds per pods**: The best pods were thrushed and number of seeds per pod obtained from the plant were counted, recorded and averaged.

8. **Seed yield per plant (g)**: The total weight of the seeds harvested from the same plant used for recording seed yield per plant was noted in grams and averaged.

9. **Hundred seeds weight (g)**: It was a weight of random sample of 100 seeds from each plant.

10. **Protein content (%)**: Protein content was estimated and expressed in percentage for each concentrations and control plant.

**3.18. M₂ generation**

100 healthy seeds from M₁ plants were planted in plant progeny rows with three replications for each treatment along with control to raise M₂ generation.

**3.19. Chlorophyll mutation**

The Chlorophyll mutations were observed in 10–20 days old seedlings. Five types of chlorophyll mutants were identified (Gustafsson, 1940). The mutation frequency was estimated on M₂ seedling’s basis.
3.20. Morphological mutations

The M₁ – M₂ populations were screened for viable mutants for different morphological characters throughout the growth period of crop. The frequency of morphological mutations was calculated based on the M₂ population.

3.21. Mutagenic effectiveness and Efficiency

Mutagenic effectiveness of a mutagen is the measure of frequency of mutation induced by unit mutagen, whereas mutagenic efficiency gives an indication of the proportion of mutation in relation to undesirable changes like lethality and injury. The mutagenic effectiveness and efficiency of the both mutagens; gamma rays and EMS were worked out by using the formula suggested by Konzak et al., (1965).

\[ \text{Mutagenic effectiveness (\%)} = \frac{\text{MF}}{\text{kR/C t}} \times 100 \]

Mutagenic efficiency,

\[ \text{Lethality (L)} = \frac{\text{MF}}{L} \]
\[ \text{Seedling Injury} = \frac{\text{MF}}{I} \]

3.22. M₃ and M₄ generations

Seeds harvested from individual M₂ plant were used to raise M₃ generations in the field during winter season. For raising M₄ generation, the M₃ progenies were selected, which showed significant deviation in mean values from the mean values of the untreated plant (control). All the recommended cultural practices were carried out during the cultivation of crop.
3.23. **Statistical analysis**

The mean values for different characters in each treatment were calculated and expressed as percentage of increase and decrease over control. Analysis of variance methods was adopted for the statistical analysis of the data to find out the significant difference between the treatment and control.

The data for each character in all the treatments were analyzed separately by an appropriate analysis of variance. The statistical parameters like Range, Mean, Standard Error, Phenotypic Co-efficient of Variation (PCV), Genotypic Co-efficient of Variation (GCV), Heritability ($H^2$), Genetic Advance (GA) and Genetic Advance as per cent of mean (GA%) were computed by ANOVA for CRBD developed by NPRC-STAT, National Pulses Research Centre, Vamban, Pudukkottai, Tamilnadu, India.

3.24. **Range**

The lowest and highest values for each character were recorded as the range.

3.25. **Estimation of standard error (SE)**

To compute standard error of estimates with respect to different character studied, standard deviation value and number of individual plant observation recorded were considered.

3.26. **Variability analysis**

3.27. **Analysis of variance**

The variations observed among the replication were exclusively non-heritable, and hence it considered as environmental variance.
In \( M_2 \) population onwards, the variance was separated into heritable and non-heritable components as indicated below (Mather and Jinks, 1971).

**ANALYSIS OF VARIANCE**

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Df</th>
<th>MSS</th>
<th>Expectation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>( r-1 )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Genotypes</td>
<td>( g-1 )</td>
<td>( M_1 )</td>
<td>( \sigma^2 + \rho \sigma^2 )</td>
</tr>
<tr>
<td>Total</td>
<td>( (rg-1) )</td>
<td>( M_2 )</td>
<td>-</td>
</tr>
</tbody>
</table>

Experimental variance \( (\sigma^2) \) = \( M_2 \)

Genotypic variance \( (\rho \sigma^2) \) = \( \frac{M_1 - M_2}{r} \)

Phenotypic variance \( (\sigma^h) \) = \( \rho \sigma^2 + M_2 \)

Where,

\( R \) = number of replications
\( G \) = number of genotypes
\( (\sigma^2) \) = experimental variance
\( (\rho \sigma^2) \) = genotypic variance
\( (\sigma^h) \) = phenotypic variance
\( M_1 \) and \( M_2 \) = Mean sum of squares for genotype and error column.

**3.28. Co-efficient of variation**

Phenotypic co-efficient of variation (PCV) and genotypic co-efficient of variation (GCV) were computed based on the formula suggested by Burton (1952).

\[
PCV = \frac{(\text{Phenotypic variance})^{1/2}}{\text{General mean}} \times 100
\]

\[
GCV = \frac{(\text{Genotypic variance})^{1/2}}{\text{General mean}} \times 100
\]
Categorization of the array of variation was carried out by the method as suggested by Sivasubramanian and Madhavamenon (1978).

Above 20 per cent  -  High
10-20 per cent   -  Moderate
Below 10 per cent  -  Low

3.29. Heritability

Heritability in broad sense was computed as the ratio of genotypic and phenotypic variance and expressed in percentage (Lush, 1940).

\[
\text{Heritability (} h^2 \text{)} = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times 100
\]

Heritability was classified as follows (Robinson, 1966).

Above 30 per cent  -  High
10-30 per cent   -  Moderate
Below 10 per cent  -  Low

3.30. Genetic advance

Genetic Advance (GA) for a particular trait was computed by following the method recommended by Johnson et al., (1955).

\[
\text{GA} = h^2 \times \sigma_{ph} \times K
\]

Where,

- \( h^2 \) = heritability
- \( \sigma_{ph} \) = phenotypic standard deviation
- \( K \) = selection differential 2.06 at 5 per cent level.
3.31. Genetic advance as per cent of mean (GA %)

\[
\text{GA as } \% \text{ of mean} = \frac{\text{GA}}{\text{General mean}} \times 100
\]

Genetic advance as per cent of mean classified as follows (Robinson 1966).

- Above 20 per cent - High
- 10-20 per cent - Moderate
- Below 10 per cent - Low

3.32. Biochemical analysis

**Complex-forming reagent:** The solution was prepared immediately before use.

- **Reagent A**: 2 % (W/V) sodium carbonate (NaCO₃) in distilled water
- **Reagent B**: 1% (W/V) copper sulphate (CuSO₄) in distilled water
- **Reagent C**: Alkaline copper sulphate solution obtained by adding 50ml of Reagent A with 1 ml of Reagent B.
- **Reagent D**: 2% (W/v) sodium potassium tartarate in distilled water
- **1N NaOH**: Commercially available

**Folin-Ciocalteu’s Reagent** (Commercially available): Folin-ciocalteu’s reagent was diluted with equal quantity of double distilled water so as to make it 1N and stored in a refrigerator under complete darkness.
3.33. **Estimation of seed protein content (%)**

0.5 g seed was dehulled and ground with 50mM of phosphate buffer (pH 7.8) in a pestle and mortar. These extracts were defatted by washing with cold acetone for 4 to 6 hours. The acetone was removed by filtration from the extract and kept in room temperature for air drying. Then the protein was precipitated by adding 10% of trichloro acetic acid and centrifuged for 30 minutes at 4000 rpm. The protein content was estimated by the method suggested by Lowry *et al.*, (1951) using bovine serum albumin as a standard.

3.34. **Seed protein estimation**

The percentage of seed protein was determined by the method proposed by Lowry *et al.*, (1951). For the extraction of both soluble and insoluble protein, the seed powder was kept overnight in an oven at 80°C. Then it was cooled. 50mg of sample was ground with 5ml of double distilled water in a pestle and mortar. Then the ground material was collected and centrifuged for 10 minutes at 4000 rpm. The supernatant was transferred to 25ml volumetric flask and was made up to the mark by adding double distilled water. This solution was used for the soluble protein estimation. The remaining residue was used for insoluble protein estimation.

3.35. **Insoluble protein estimation**

5ml of 5% trichloro acetic acid was added to the residue. The solution was shaken thoroughly and kept for 30 minutes in room temperature and centrifuged at 4000 rpm for 30 minutes and
discarded the supernatant and 5ml of 1N sodium hydroxide was added and kept for 30 minutes. And after that the residue was kept in water bath at 80°C for 30 minutes. Afterwards, it was cooled at room temperature and again centrifuged at 4000 rpm for 5 minutes. The supernatant was transferred into a 25ml volumetric flask and volume was made up to the mark with 1N sodium hydroxide.

For estimating seed insoluble protein, 1ml of extract was taken in 10ml test tube along with 5ml of reagent D and was kept for 10 minutes. 0. 5ml of Folin – ciocalteu’s reagent was added rapidly and it mixed well. After 30 minutes, the solution becomes blue. A blank was run for each sample, and the optical density (OD) value of the solution was read at 660nm by using a spectrophotometer (U – 2001, HITACHI).

3.36. Soluble protein estimation

For estimating seed soluble protein, 1ml of supernatant was taken in a test tube and added 5ml of reagent C. The solution was mixed well and kept in room temperature for 10 minutes. 0. 5ml of Folin's phenol reagent was rapidly added with instant mixing and left for 30 minutes. After 30 minutes, the solution turned to blue colour, was taken in calorimetric tube and the intensity was observed by optical density at 660nm on a spectrophotometer. A blank was run simultaneously.
3.37. **Standard for protein**

50 mg of Bovine Serum Albumin was transferred in a 100ml volumetric flask along with 1-2 ml of 0.1N NaOH. The volumetric flask was carefully rotated and placed on a water-bath for 5-10 minutes for heating. When the albumin became solubilized, and was made up to the mark by using double distilled water. From this prepared standard solution, a range of 10 volumes 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml was pipetted out into 10 ml test tubes. And the solution was diluted into 1 ml by adding volumes of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.0 ml double distilled water in that order.

In all test tubes, 5 ml of reagent C was added and kept at room temperature for 10 minutes. Then 0.5 ml of Folin’s phenol reagent was added to the test tube with immediate mixing. The value of optical density was read at 660nm in a spectrophotometer. A blank was also run at the same time and the calibration curve was plotted.

The content of soluble protein was finding out by comparing the optical density value of each sample, and the curve was plotted.

3.38. **Total seed protein estimation**

The total seed protein content was obtained by adding the value of both soluble and insoluble protein content.
3.39. PROTEIN PROFILING USING SDS – PAGE

Reagents

A) Re suspension Buffer : Tris (pH 6.8) – 50mM; DDT-100mM; 2%
B) Resolving gel : DH₂O – 12. 3ml; 1. 5M Tris HCl (pH 8.8)
- 7.5ml; 20% SDS-0.15ml; 30% acrylamide
- 9.9ml; 10% APS-0.15ml; TEMED – 0. 015ml.
C) Stacking gel : DH₂O-3.075ml; 0.5M Tris HCl (pH 6.8)-
1.25ml 2% SDS-0.025ml; 30%Acrylamide solution-0.67 10% APS-0.025ml; TEMED
- 0.005ml. (APS and TEMED were added just before to pour the gel)

D) 5X Running buffer : Tris base – 15g; Glycine – 72g; SDS- 5g;
DH₂O – make up to 1 litre.

E) Staining
1. Washing solution: 1ml formaldehyde;40 ml methanol;60ml
DH₂O
2. Sodium thio sulphate: 200 mg in 1 litre water.
3. Silver nitrate solution: 0.1%
4. Na₂CO₃ (3g) in 80ml water; Sodiumthiosulphate solution
(1ml); formaldehyde (1ml) and make up to 100ml with distilled
water.
5. 1ml of Butanol
3.40. Extraction of total seed protein

Total seed storage protein from the seeds of isolated mutants and control of M₄ generations was extracted by the method described by Alsohaimy et al., (2007). Seeds of both the mutants and controls were grounded into a fine powder. 50mg of powder was collected in a fresh Eppendorf tube and the samples were dissolved with 600 µl of reverse osmosis water. The pH of the sample was adjusted to 11 with 0.1 N NaOH and made up the volume into 1ml with reverse osmosis water. The sample was incubated for 1 hour on a rocker at room temperature and centrifuged for 5 minutes at 12000 rpm. The supernatant was collected into a fresh Eppendorf tube, and the pH was brought down to 4.5 using 1M HCl. And the samples were again centrifuged for 2 minutes at 12000 rpm and discarded the supernatant. Finally dissolve the pellet in 200 µl of re suspension buffer, which is used as the total protein sample.

3.41. SDS-PAGE analysis

Protein profiling of samples was performed using sodium dodecyl sulphate – polyacrylamide gel electrophoresis as described by Sadashivam and Manickam (2008). The SDS apparatus was set up properly prior to the SDS – PAGE analysis. Then 10ml of resolving gel mixture was poured up to 2cm below from the upper edge of the plate. 1 ml of butanol solution was added in order to
ensure the even surfaced as well as to smooth the resolving gel mixture. Let them to settle down, it took about 30 – 40 minutes normally. After gel polymerization, it was washed to remove un polymerized acrylamide and then the comb was inserted leaving the space 1 cm between them. Then 2.5 ml of stacking gel mixture was added and kept for settling down. The comb was carefully removed once the stacking gel set without disturbing the wells. To remove un polymerized acrylamide, the wells were washed with reverse osmosis water. The glass plates were fixed in the running tray and filled with 1X running buffer. 15 μl from each mutant sample and control (2 μl protein; 8 μl RO water; 5 μl gel loading dye (Bromophenol blue dye) along with protein molecular marker (SM0441,Fermantas) were carefully injected into the wells. The established SDS-PAGE apparatus was connected to the power pack (45mA) and ran the gel until the dye reaches to the bottom of the glass plate. The glass plates were removed and carefully separated by using a spatula.

### 3.42. Staining

Once electrophoresis done, the stacking gel was removed. The resolving gel was washed in washing solution in a clean plastic container for 10 minutes with slow shaking. The washing solution was discarded, and gel was rinsed for 2 minutes with plenty of water. After that, gel was soaked in sodium thiosulphate solution
for 1-2 minutes. The gel was again washed twice by water and then stained for 10 minutes with the silver nitrate solutions with gentle shaking and poured developer into the plastic container. It was shaken until the yellow to dark-brown coloured bands was appeared. Once the band developed, the reaction was stopped up by adding acetic acid solution.

3.43. Gel documentation and analysis

Finally gels were photographed by using UV proplatinum gel documentation system (UVITEC, UK). The gel analysis was performed using UVI proplatinum 1.1 Version 12.9 for window.

3.44. Random Amplified Polymorphic DNA assay

Among the different mutagenic treatments, some of the morphological mutants were identified and isolated in M₂ generation, and these seeds were used to raise the M₃ and M₄ generations. Seed samples of the mutants were germinated in plastic pots. The seedlings grown-up to two to three leafy stage were used for the isolation of DNA. The seedlings were taken from each mutant recruited to the study were control, early flowering mutant, bold pod mutant, bold seed mutant, high yielding mutant and high protein content mutant. Among the molecular techniques, RAPD technique was used to find out the polymorphisms in genetic level among the mutants with respect to their control.
3.45. DNA isolation

Solutions required

- Extraction buffer
  100 mM Tris-HCl (pH 8.0)
  50 mM EDTA
  1.4 M NaCl
  2% CTAB
  1% PVP (addition of PVP is optional)

- Chloroform-isoamylalcohol 24:1 [v/v]

- TE buffer: 10 mM Tris-HCl (pH 8), 0.1 mM EDTA

- 7.5M Ammonium acetate, Isopropyl alcohol

- 70% ethanol

Procedure

Genomic DNA was extracted based on the CTAB method described by Doyle and Doyle (1990) with slight modifications as follows.

1. One gram of the young seedling leaf samples was crushed in pestle and mortar and ground with 2ml of CTAB extraction buffer and 250 mg of Poly Vinyl Pyruvate. Then the extract solution was transferred into a centrifuge tube and kept in water bath at 65º C for 30 minutes. After the incubation period, the
sample was centrifuged at 12,000 rpm for 10 minutes and transferred the supernatant into a fresh tube.

2. The supernatant was extracted with equal volume of Chloroform: Isoamylalcohol (24:1) and centrifuged at 12,000 rpm for 10 minutes. To the collected supernatant equal volume of isopropanol was added and incubated for 1 hour.

3. Then centrifuged the samples for 10 minutes at 12,000 rpm. Discarded the supernatant. To the residue 1ml of 70 % ethanol was added and centrifuged again for 10 minutes at 12,000rpm.

4. After discarding the supernatant, pellet was collected and kept for air drying. The pellet was dissolved with 0.5ml of TE Buffer and 3μl of RNase and stored at -20°C for 30 minutes.

5. The residue was collected and added 50 μl of 3M Sodium acetate and the sample was centrifuged at 12,000 rpm for 10 minutes.

6. After centrifugation, the pellet was collected and washed with 0.5 ml of ice cold ethanol. The sample was centrifuged for 10 minutes at 12,000 rpm and evaporates the methanol. Finally dissolve the pellet in 500 μl of TE buffer for further analysis.

7. DNA quantification was done by Nanophotometer (IMPLEN) quantified at 260 nm. Quantification and quality of DNA was also done through run agarose gel electrophoresis.
3.46. RAPD-PCR amplification

Requirements

- **Random primers**: Commercial kits were obtained from Next Gen – Bio, Chennai. A total of 10 random decamer DNA primers have been used for RAPD-PCR assay.
- **Template DNA**: Purified genomic DNA extracts from the selected mutants of chick pea have been used as template DNA.
- **dNTPs**: The four individual dNTPs viz., dATP, dGTP, dCTP and dTTP were obtained from M/S Genei Pvt. Ltd., Bangalore and were used at a concentration of 5.0 μl each.
- **Taq DNA polymerase**: Taq DNA polymerase (5U/μl) and 10X Taq assay buffers were obtained from Genei Pvt. Ltd., Bangalore, India.
- **Chemicals**: Analytical grade chemicals were obtained locally.
- Deionised distilled water and thermal cycler.

Stock solutions

a. 100 μM random primer

b. 25 ng μl⁻¹ template DNA

c. 5.0 U μl⁻¹ Taq DNA polymerase

List of primers used in the study.

A total of ten random primers used for the study are as follows:
### S. No | Primer Code | GC content | Primer sequence 5′ to 3′
---|---|---|---
1 | OPA-01 | 70% | 5′- CAGGCCCTTC-3′
2 | OPA-02 | 70% | 5′- TGCCGAGCTG-3′
3 | OPA-03 | 60% | 5′- AGTCAGCCAC-3′
4 | OPA-04 | 60% | 5′- AATCGGGCTG-3′
5 | OPA-05 | 60% | 5′- AGGGGTCTTG-3′
6 | OPA-06 | 70% | 5′- GGTCCCTGAC-3′
7 | OPA-07 | 60% | 5′- GAAACGGGTC-3′
8 | OPA-08 | 60% | 5′- GATGGTCAC-3′
9 | OPA-09 | 70% | 5′- GGGTAACGCC-3′
10 | OPA-10 | 60% | 5′- GTGATCGCAG-3′

### Master Mix for PCR (30 µl)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Quantity (µl/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Taq assay buffer (10X)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2.</td>
<td>MgCl₂ (25mM)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>3.</td>
<td>dNTPs Mix (2 mM)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>4.</td>
<td>Primer (2 µM/µl)</td>
<td>8.0 µl</td>
</tr>
<tr>
<td>5.</td>
<td>Template DNA (50 ng)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>6.</td>
<td>Taq DNA polymerase (5U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>7.</td>
<td>De-ionised distilled water</td>
<td>19.5 µl</td>
</tr>
</tbody>
</table>

**Total reaction volume** | **50.0 µl**
**Thermal cycling**

- Sterile centrifuge tubes were numbered from 1 to 10.
- 2.0 µl of template DNA from individual genotypes was added to each tube.
- 48 µl of master mix was added to all the tubes and was given a short spin to mix the contents.
- The tubes were placed in the thermal cycler for amplification.

The PCR reaction was carried out using master cycler gradient (Eppendorf). This cycler was programmed as under

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Duration (Min)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial denaturation</td>
<td>94</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Denaturation</td>
<td>94</td>
<td>40 sec</td>
<td>34</td>
</tr>
<tr>
<td>3.</td>
<td>Primer annealing</td>
<td>36</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Primer extension</td>
<td>72</td>
<td>90 sec</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Repeat cycle</td>
<td></td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>6.</td>
<td>Final extension</td>
<td>72</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Hold temperature</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

After the completion of PCR, the products were stored at 4°C until the gel electrophoresis was done.
3.47. **Separation of amplification products by Agarose gel electrophoresis**

**Requirements**

- Electrophoretic unit: Gel casting tray, gel preparation comb, power pack, UV transilluminator
- Agarose (1.5 %)
- Bromophenol blue
- Ethidium bromide (0.5μg ml⁻¹)
- 1X TBE – pH-8.0
- Working solution (1X TAE)

**Procedure**

- 1.5 g of agarose was weighed and added to a conical flask containing 100ml of 1X TBE buffer.
- The agarose was melted by heating the solution on an electric heater, and the solution was stirred to ensure even mixing until dissolution of agarose was made complete.
- The solution was then cooled to about 40-45°C.
- Two to three drops of ethidium bromide (0.5 μg ml⁻¹) were added.
- The solution was poured into the pre leveled gel casting platform after inserting the comb in the casting system. While pouring, sufficient care was taken without allowing the air bubbles to trap in the gel.
The gel was allowed to solidify, and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1X TBE) to cover the well completely.

The amplified products (25μl) to be analyzed were carefully loaded along with the marker into the sample wells, after adding 2-3 μl of loading dye (Bromophenol blue) with the help of a micropipette.

Electrophoresis was carried out at 75V for 3 hrs, until the tracking dye migrated to the end of the gel.

Ethidium bromide stained DNA bands were viewed under UV transilluminator and photographed for documentation.

3.48. Gel scoring and data analysis

RAPD-PCR analysis was carried out a minimum of 3 times and only the primers which produce strong and reproducible bands were taken for further analysis. The data were scored in the presence (1) or absence (0) of the RAPD fragments were considered as a single trait and the binary matrix through Jaccard’s similarity coefficient (Jaccard, 1908).

The Jaccard’s similarity coefficients were generated by using an Unweighed Pair-Group Method with Arithmetical averages (UPGMA). Total number of bands, total number of polymorphic
bands, total number of monomorphic bands and percentage of polymorphism were calculated in control and mutant plants. Percentage of polymorphism was calculated by using the following formula.

\[
\text{Number of polymorphic bands} \times 100
\]

\[
\text{Per cent of polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of band}} \times 100
\]