### III. MATERIALS AND METHODS

#### 3.1. EXPERIMENTAL MATERIAL

**Description of the Experimental Material**

The dry and dormant seeds of the green gram variety Vamban (VBN2) were obtained from National Pulses Research Centre, Vamban, Pudukkottai, Tamilnadu, India. The salient features of the genotype are given below.

<table>
<thead>
<tr>
<th>Features</th>
<th>VBN2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parentage</td>
<td>Cross derivatives of VGG4× MH309</td>
</tr>
<tr>
<td>Year of release</td>
<td>2001</td>
</tr>
<tr>
<td>Season</td>
<td>Sep – Oct; Dec- Jan</td>
</tr>
<tr>
<td>Duration</td>
<td>65 – 70 days</td>
</tr>
<tr>
<td>Plant height</td>
<td>60 - 75cm</td>
</tr>
<tr>
<td>Leaves</td>
<td>Trifoliate with lobes</td>
</tr>
<tr>
<td>50% flowering</td>
<td>30-35 days</td>
</tr>
<tr>
<td>Colour of pod</td>
<td>Sparsely hairy pods</td>
</tr>
<tr>
<td>Colour of grain</td>
<td>Shiny green</td>
</tr>
<tr>
<td>Rainfed</td>
<td>750 (Kg/ha)</td>
</tr>
<tr>
<td>Irrigation</td>
<td>820 (Kg/ha)</td>
</tr>
</tbody>
</table>

#### 3.2. Mutagens employed

The two mutagens *viz.*, physical gamma rays ($^{60}$Co) and chemical (ethyl methane sulphonate) were used in the study.
Gamma rays

Gamma rays one of the electromagnetic radiations, having the low wave length with high penetrable power. The source of gamma rays is $^{60}$Co, one of the labeled metals, which emit the rays. The radiation was accomplished in Sugarcane Breeding Institute, Coimbatore, India.

Ethyl methane sulphonate (EMS) ($\text{CH}_3\text{SO}_3\text{C}_2\text{H}_5$)

The chemical was obtained from HI-MEDIA Laboratories, Mumbai having a half-life period of 30 hours with a molecular weight of 124.16 and density of 1.20.

3.3. METHODS

Gamma rays treatment

The dry seeds of green gram variety VBN2 were taken for induced mutation of physical mutagen (gamma rays). Five sets of hundred seeds were packed in paper cover for irradiation and treated with 10, 20, 30, 40 and 50 kR gamma rays to fix and determine the 50% lethal dose ($\text{LD}_{50}$) value. The irradiated seeds were repacked separately with wet paper and all the seeds in different doses were immediately placed on moist paper in the petriplates (9×3 cm size) separately in the laboratory condition. For each treatment 10 replicates were studied and the untreated seeds were germinated as control lines. $\text{LD}_{50}$ value was determined based on the lethality of the seedling ($7^{th}$ day) with 10 replicates.
EMS treatment

Hundred seeds of each treatment were presoaked for 4 hrs in distilled water, blotted dry, and treated with 10, 20, 30, 40 and 50 mM of freshly prepared solutions of ethyl methane sulphonate for 4 hrs with intermittent shaking at room temperature (28±2°C). All seeds were uniformly exposed to EMS solution by stirring with a glass rod. After treatment, seeds were thoroughly washed in running water for 8 to 10 times to leach out the residual of chemical. After the treatment, the seeds were sown in the field following randomized block design method with three replications in each to rise M₁ generation.

Control

Untreated dry seeds of green gram variety VBN2 pre-soaked in distilled water for 4 hours were used as control. After pre-soaking, the seeds were sown in the field following randomized block design method with three replications.

3.4. RAISING OF M₁ GENERATION

Field sowing was done on 3rd January 2012 with spacing of 30 cm between rows and 10 cm between seeds in a plot size of 11 m x 4 m and all recommended package of practices was followed.
OBSERVATION IN M₁ GENERATION

Seed germination (%)

Number of seeds germination was counted on 7th day and germination percentage was calculated by using following formula.

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

Seedling height (cm/ seedlings)

The seedling height was measured from the cotyledonary node to the tip of the root for 10 randomly selected seedlings (10 replications) on the 7th day due to the effect of physical and chemical mutagens along with control.

Seedling survival (%)

Seedlings survived on 30th day after sowing were counted. Survival percentage was calculated by using the following formula.

\[
\text{No. of seedlings survived} \\
\text{Seedling survival (\%)} = \frac{\text{No. of seedlings survived}}{\text{No. of seeds germinated}} \times 100
\]

Survival was expressed as the percentage of plant survival.

RAISING OF M₂, M₃ and M₄ GENERATIONS: M₂ generation seeds were raised from M₁ generation; the seeds were collected from different individual
mutagenic treatments. Seeds harvested from individual M₂ plants and developed as M₃ generation in the field during kharif season. For rising M₄ generation, the M₃ progenies were selected which showed significant deviation in mean values in the negative directions from the mean values of the parental varieties (control) from days to flowering to days to maturity. All the recommended cultural practices namely, irrigation, weeding and plant protection measures were carried out during the growth period of the crop.

3.5. FIELD OBSERVATIONS (M₁–M₄ generations)

Plant height (cm)

The plant height was measured from base of the plant to the tip of fully opened leaf on the main shoot and the plant height was expressed in centimetre.

Number of branches per plant

The number of branches arising on the main stem in the five randomly selected and tagged plants were recorded at different growth stages. The mean of branches per plant was worked out and expressed in number.

Number of leaves per plant

The number of leaves arising on the stem in the five randomly selected plants were recorded at maturity period. The mean number of leaves per plant was worked out and expressed in number.
Days to first flowering

Daily observations were made on the five randomly selected and tagged plants for flowering. The number of days taken from the date of sowing to flowering was recorded and expressed in number as days to first flowering.

Number of pod cluster per plant

The number of pod cluster obtained from the plant were counted and recorded.

Number of pods per plant

The number of pods obtained from the plant were counted and calculated.

Number of seeds per pod

The number of seeds per pod obtained from the plant were counted and recorded.

Hundred seed weight (g)

Hundred seed weight was worked out by using digital electronic balance and expressed in grams.

Seed yield per plant (g): Seed yield was recorded in grams after weighing the seeds collected from each plant.
CHLOROPHYLL MUTANTS (M₁ and M₂ GENERATIONS)

The chlorophyll mutants were scored on 20th day after sowing. Four types of chlorophyll mutants were scored (Veena, 1997).

The M₁ and M₂ seedlings were screened from 15 to 30th day respectively to record the various chlorophyll mutants periodically. The mutation frequency was estimated on M₂ seedlings basis.

MORPHOLOGICAL MUTATIONS (M₁-M₄ Generations)

The M₁ to M₄ populations were screened for viable mutants for different morphological characters throughout the growth period of crop. The mutation frequency was estimated on M₂ seedling basis.

MUTAGENIC EFFECTIVENESS AND EFFICIENCY

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

\[
\text{Mutagenic effectiveness (Gamma rays) } = \frac{\text{Mutationrate}}{kR \times \text{time in h}} \times 100
\]
Mutagenic effectiveness (EMS) = \[ \frac{\text{Mutation rate}}{\text{concentration of EMS in mM} \times \text{time in h}} \times 100 \]

\[
\text{Mutation efficiency} = \frac{\text{Mutation rate}}{\text{Percentage of lethality or biological injury in } M_1}
\]

\[ M \] - Mutation frequency for 100 \( M_2 \) plants

\[ T \] - Period of treatment with chemical mutagen in hours

\[ \text{kR} \] - Kilo radiation

\[ C \] - Concentration of chemical mutagens in mM

\[ L \] - Reduction in height of seedling on 30\(^{th}\) day

\[ I \] - Lethality percentage or survival reduction of seedling

**STATISTICAL ANALYSIS**

**\( M_2, M_3 \) and \( M_4 \) GENERATION DATA ANALYSIS**

The mean values for different characters in each treatment were calculated and expressed as percentage of increase or 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 (H²), genetic advance (GA) and genetic advance as per cent of mean (GA%) were computed by ANOVA for RBD developed by NPRC-STAT, National Pulses Research Centre, Vamban, Pudukkottai, Tamilnadu, India.

**Range**

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

**Mean**

The mean value for each character was worked out by using the following formula

\[
\bar{y} = \frac{1}{n} \left( \sum y_i \right)
\]

\[
\sum x_i = \text{Sum total of the character}
\]

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

**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.
3.6. VARIABILITY ANALYSIS

Analysis of variance

The variations observed among the replication were exclusively non-heritable and hence treated as environmental variance. The variance of $M_2$ population was partitioned 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>$\sigma^2 + \sigma g^2$</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 ($\sigma g^2$) = $\frac{M_1 - M_2}{r}$

Phenotypic variance ($\sigma ph^2$) = $\sigma g^2 + M_2$

Where,
R = number of replications

G = number of genotypes

$(\sigma e^2) = \text{environmental variance}$

$(\sigma g^2) = \text{genotypic variance}$

$(\sigma ph^2) = \text{phenotypic variance}$

$M_1$ and $M_2 = \text{Mean sum of squares for genotype and error column}$

**Co-efficient of variation**

Phenotypic co-efficient of variation (PCV) and genotypic co-efficient of variation (GCV) were computed using the following formulae adopted by Burton (1952).

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

$$\text{GCV} = (\text{Genotypic variance})^{1/2}/\text{General mean} \times 100$$

Categorization of the range of variation was done as proposed by Sivasubramanian and Madhavamenon (1978).
Above 30 per cent - High

10-30 per cent - Moderate

Below 10 per cent - Low

**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

**Genetic advance**

Genetic Advance (GA) for a particular trait was estimated adopting the method suggested by Johnson *et al.* (1955).

\[ GA = h^2 \times \sigma_p h \times K \]
Where,

\[ h^2 = \text{heritability} \]

\[ \sigma_{ph} = \text{phenotypic standard deviation} \]

\[ K = \text{selection differential 2.06 at 5 per cent level} \]

**Genetic advance as per cent of mean (GA %)**

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

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

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

**3.7. CYTOLOGICAL ANALYSIS**

The root tips were collected from the control and treated seedlings and fixed in 1:3 acetic alcohol. The root tip squashes were made by using Iron alum Haematoxylin squash technique (Marimuthu and Subramanian, 1960). The root tips were hydrolyzed in 0.1N HCl for 5 to 10 minutes at 60°C and then they were thoroughly washed in distilled water and transferred to 4% iron alum for 3 minutes. The root tips were washed in distilled water and transferred to ripened dilute haematoxylin stain and kept for 3hrs. The root
tips were thoroughly washed in distilled water and then they were treated in 45% acetic acid for 1 minute to soften the tissues. One or two root tips were placed on the tissue clean slide and squashed by using a cover slip and the slide was sealed and mounted with DPX solution. The normal and abnormal mitotic stages were examined and photographed.

3.8. PROTEIN PROFILING USING SDS-PAGE

Reagents:

1. Complex-forming reagent: Prepare immediately before use by mixing the following 3 stock solutions A, B, and C in the proportion 100:1:1 respectively.

Solution A : 2% (W/v) sodium carbonate (Na$_2$CO$_3$) in distilled water
Solution B : 1% (W/v) copper sulphate (CuSO$_4$) in distilled water
Reagent C : 2% (W/v) sodium potassium tartrate in distilled water

2. 2N NaOH

3. Folin-Ciocalteu’s Reagent (Commercially available): Folin-ciocalteu’s reagent was diluted with equal quantity of distilled water so as to make it 1N and stored in refrigerator under complete darkness.

Extraction of Total protein

For extraction of soluble protein, seeds were ground in 50 mM phosphate buffer (pH 7.8) and centrifuged in micro-centrifuge machine (Eppendorf 5415c) for 10 min at 14,000rpm. The extraction buffer contains the following final concentrations (1M Tris (pH 7.5), 1M KCl, 5% glycerol, 0.5M EDTA, 0.1% Triton, 1M DTT, 60mM DMSO). The supernatant was
separated and used for protein profiling. Protein concentration of extracts was measured by (Lowry et al., 1951).

**Estimation methods**

0.5ml of extract was taken and added with 0.1ml of 2N NaOH and hydrolyzed at 100°C for 10 min in a heating block or a boiling water bath. Cool the hydrolyzed seed sample at room temperature and added 1ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 minutes. Add 0.1ml of Folin-ciocalteu’s Reagent, using a vortex mixer, and let the mixture stand at room temperature for 30-60minutes (do not exceed 60 minutes). Read the absorbance at 750nm if the protein concentration was below 500μg/ml or at 550nm if the protein concentration was between 100 and 200μg/ml. Plotted a standard curve of absorbance as a function of initial protein concentration and used to determine the unknown protein concentrations.

**Standards:** Use a stock solution of standard protein (e.g., Bovine serum albumin fraction V) containing 4mg/ml protein in distilled water stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

<table>
<thead>
<tr>
<th>Stock Solution (μl)</th>
<th>0</th>
<th>1.25</th>
<th>2.50</th>
<th>6.25</th>
<th>12.5</th>
<th>25.0</th>
<th>62.5</th>
<th>125</th>
<th>1.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water μl</td>
<td>500</td>
<td>499</td>
<td>498</td>
<td>494</td>
<td>488</td>
<td>475</td>
<td>438</td>
<td>375</td>
<td>250</td>
</tr>
<tr>
<td>Protein Conc. ml</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>500</td>
<td>1000</td>
<td>2000</td>
</tr>
</tbody>
</table>
Reagents

1. Extraction buffer consisting of 0.25M Tris buffer (pH 8.0)
2. Acrylamide mix (30%)
3. Separating gel buffer consisting of 1.5M Tris (pH 8.8).
4. Stacking gel buffer consisting of 1.0M Tris (pH 6.8).
5. Polymerizing agents:
   a. Ammonium per sulphate (10%)
   b. TEMED
6. Electrode buffer (10X)
7. Sample buffer (5X conc.)
8. SDS: 10% solution.
9. Stainer
10. Destainer
11. Standard marker proteins

Protein profiling of samples was performed using Sodium Dodecyl Sulphate-Poly acrylamide gel electrophoresis as described by Laemmli (1970). Equal quantities of proteins (150 micro grams) from each sample along with protein molecular weight marker (SM0441, Fermentas) loaded into 10% gels. Electrophoresis was performed at constant voltage (100 volts). At end of the electrophoresis, gels were fixed in solution containing 10% Acetic acid and 40% ethanol for 15 min, with constant agitation on a shaker. After fixing, gel was washed with distilled water for 15 min, with change of water every
5 min. Gels were then stained with Coomassie blue G-250 dye and kept in water overnight for destaining.

**Preparation of polyacrylamide gel**

<table>
<thead>
<tr>
<th>Separating gel 10% (6ml to 8ml)</th>
<th>Stacking gel 10% (4ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>H₂O</td>
</tr>
<tr>
<td>2.8ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>30% Acrylamide</td>
</tr>
<tr>
<td>2.31ml</td>
<td>0.8ml</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.5M Tris (pH 6.8)</td>
</tr>
<tr>
<td>1.75ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% SDS</td>
</tr>
<tr>
<td>70µl</td>
<td>0.04µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>10% APS</td>
</tr>
<tr>
<td>70µl</td>
<td>0.04µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>TEMED</td>
</tr>
<tr>
<td>10µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

**Gel documentation and analysis**

Finally gels were photographed using UVI proplatinum gel documentation system (UVItec UK). Computerized gel analysis was performed using UVI proplatinum 1.1 Version 12.9 for window.

**3.9. Random amplified polymorphic DNA assay (Williams *et al.*, 1990)**

Among the physical and chemical mutagenic treatments, some of the macro mutants (morphological) were isolated in M₂ generation and these mutants used to raise M₃ and M₄ generations. Seed samples were germinated in plastic cups until seedlings were grown up to two to three leaf stage. Five gram leaves of seedlings were taken from each mutant for DNA assay. The
different mutants recruited to the study were tall, dwarf, bushy, early maturity and bold seed mutants along with control. Mutants observed in M₄ generation were analyzed for their morphological and biochemical content. In order to analyze the molecular basis, RAPD technique was used to detect the polymorphisms in the base pairs of DNA among the mutants.

**DNA isolation**

**Solutions required**

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

- phenol-chloroform-isoamylalcohol (25:24:1 [v/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 by adopting the CTAB method outlined by Doyle and Doyle (1990) with some modifications as follows.

1. The young seedling leaf samples (two grams) were ground into fine powder in liquid nitrogen using sterilized pestle and mortar. Pre-warmed extraction buffer in the amount of 800μL was added to the
samples and ground again in buffer. Samples were taken in 1.5 ml eppendorf tube with a small spatula or glass rod after the contents were incubated at 55°C for 45mts.

2. After following the incubation periods, samples were cooled at room temperature then centrifuged at 10,000rpm for 10mts and supernatant was transferred to a fresh tube.

3. Samples were extracted with equal volume of Chloroform: Isoamyl alcohol (25:1) gently mixed by tilting over 40-50 times. At this stage, it was observed that incubating the samples for at least 15 min on ice (4°C) increased the efficiency.

4. After centrifugation, the supernatant was transferred to a fresh tube having 7.5M ammonium acetate and isopropyl alcohol and then the samples were incubated on ice for 15mts. The step is not mandatory, but it can be prolonged for several hours.

5. The samples were centrifuged for 10mts at 10,000 rpm and 70% ethanol (500μl) was added to the pellet and centrifuged again for 10mts at 10,000rpm.

6. Then the supernatant was discarded and the pellet saved air dried for 10mts and pellet dissolved in an appropriate amount of TE Buffer (30-60 μl). The samples were treated with ribonuclease (RNase -10mg/ml) and stored at -20°C.

7. DNA was quantified via spectrophotometric measurement of UV absorption at 260 nm (Shimadzu UV-260). DNA was also quantified
by means of agarose gel electrophoresis with ethidium bromide fluorescence and a 100-bp DNA ladder (Fermentas) as the DNA size marker.

**RAPD-PCR amplification**

**Requirements**

- **Random primers:** Commercial kits were obtained from Progen Biotech, Chennai and operon technologies Inc., Alamedas, USA. The total of eight random decamer DNA primers had been used for RAPD-PCR assay.

- **Template DNA:** Purified genomic DNA extracts from the selected mutants of green gram have been used as template DNA.

- **dNTPs:** The four individual dNTPs viz., dATP, dGTP, dCTP and dTTP were obtained from M/S Bangalore Genei Pvt. Ltd., Bangalore and were used at a concentration of 2.5mM each.

- **Taq DNA polymerase:** Taq DNA polymerase (3U/μl) and 10 X Taq assay buffer were obtained from Bangalore Genei Pvt. Ltd., Bangalore.

- **Chemicals:** Analytical grade chemicals were obtained locally.

- **Deionised distilled water and thermal cycler.**

**Stock solutions**

- **a. 100 μM random primer**

- **b. 25ng μl-1 template DNA**

- **c. 3.0 U μl-1 DNA polymerase**
List of primers used in the study

A total of eight random primers used for the study are as follows:

<table>
<thead>
<tr>
<th>S. No</th>
<th>Primer Code</th>
<th>GC content</th>
<th>Primer sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>OPS11</td>
<td>70%</td>
<td>5'-CAGGTGTTGC-3</td>
</tr>
<tr>
<td>2.</td>
<td>OPS17</td>
<td>70%</td>
<td>5'-CTGGACAGAC-3</td>
</tr>
<tr>
<td>3.</td>
<td>OPC11</td>
<td>70%</td>
<td>5'-CGTCTAGGTC-3</td>
</tr>
<tr>
<td>4.</td>
<td>OPC12</td>
<td>60%</td>
<td>5'-GCAGGCTAAC-3</td>
</tr>
<tr>
<td>5.</td>
<td>PG07</td>
<td>60%</td>
<td>5'-GCTGCAGTAG-3</td>
</tr>
<tr>
<td>6.</td>
<td>PGF04</td>
<td>60%</td>
<td>5'-GCATCGGCC-3</td>
</tr>
<tr>
<td>7.</td>
<td>OPC03</td>
<td>60%</td>
<td>5'-GGGTTCTTT-3</td>
</tr>
<tr>
<td>8.</td>
<td>OPA13</td>
<td>60%</td>
<td>5'-AATCTGCGG-3</td>
</tr>
</tbody>
</table>

Master Mix for PCR (25 μl tube-1)

<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>2.5 μl</td>
</tr>
<tr>
<td>2</td>
<td>MgCl₂ (25mM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>3</td>
<td>dNTPs (2.5 mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>4</td>
<td>Primer (5 mM/μl)</td>
<td>4.5 μl</td>
</tr>
<tr>
<td>5</td>
<td>Template DNA</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>6</td>
<td>Taq DNA polymerase (3.00 U/μl)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>7</td>
<td>De-ionised distilled water</td>
<td>11.0 μl</td>
</tr>
</tbody>
</table>

Total reaction volume 25.0 μl
**Thermal cycling**

- Sterile microfuge tubes were numbered from 1 to 10.
- 1.0 μl of template DNA from individual genotypes was added to each tube.
- 24 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, Germany). 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</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Primer annealing</td>
<td>38</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Primer extension</td>
<td>72</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Repeat cycle</td>
<td></td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>6</td>
<td>Final extension</td>
<td>72</td>
<td>4</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 40°C until the gel electrophoresis was done.
Separation of amplification products by agarose gel electrophoresis

Requirements

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

Procedure

- 1.8 g of agarose was weighed and added to a conical flask containing 100ml of 1X TAE buffer.
- The agarose was melted by heating the solution on an electric heater and the solution was stirred to ensure even mixing till 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) was added.
- The solution was poured into the pre-levelled gel casting platform after inserting the comb in the trough. 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 TAE) so as to cover the well completely.
The amplified products (25μl) to be analyzed were carefully loaded along with the marker (λDNA EcoRI and Hind III double digest, Bangalore Genei, Bangalore) 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 50-55 volts, 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.

**Gel scoring and data analysis**

RAPD-PCR analysis was repeated at least 3 times and only the primers producing strong and reproducible bands were considered for further analysis. The presence (1) or absence (0) of the RAPD fragment was considered as a single trait and the binary data were used to generate Jaccard’s similarity coefficients for RAPD bands (Jaccard, 1908).

The matrix of similarity coefficients was subjected to unweighed pair-group method using arithmetical averages (UPGMA) to generate a dendrogram using linkage procedure. 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. Per cent of polymorphism was calculated by using the following formula.

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