CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL MATERIAL

3.1 Collection of seeds

The dry and dormant seeds of the Pearl millet (*Pennisetum typhoides* (Burn.F.)Stapf.and C.E. Hubb.)

Variety- CO (cu)-9 were collected from Tamilnadu Agricultural University, Coimbatore, India.

3.2 The salient features of the genotype are given below.

<table>
<thead>
<tr>
<th>Features</th>
<th>CO(cu)-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parentage</td>
<td>Selection from ICMV 93752</td>
</tr>
<tr>
<td>Season- irrigated/ rainfed</td>
<td>Both</td>
</tr>
<tr>
<td>Duration</td>
<td>80-85 days</td>
</tr>
<tr>
<td>Plant height</td>
<td>186-222 cm</td>
</tr>
<tr>
<td>Shape of earhead</td>
<td>Candle to cylindrical</td>
</tr>
<tr>
<td>50% flowering</td>
<td>50-55 days</td>
</tr>
<tr>
<td>1000 grains weight</td>
<td>9-11 (gm)</td>
</tr>
<tr>
<td>Grain colour</td>
<td>Grey seed with yellow base</td>
</tr>
<tr>
<td>Rainfed</td>
<td>2354 (Kg/ha)</td>
</tr>
<tr>
<td>Irrigation</td>
<td>2865 (Kg/ha)</td>
</tr>
</tbody>
</table>
3.3 Mutagens employed

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

3.4 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.

3.5 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.

Treatments

3.6 Gamma rays

The dry seeds of Pearl millet variety CO(cu)9 were taken for induced mutation of physical mutagen (gamma rays). Six sets of five thousand seeds were packed in paper cover for irradiation and treated with 10, 20, 30, 40, 50 and 60 kR of 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. LD$_{50}$ value was determined based on the lethality of the seedling ($7^{th}$ day) with 10 replicates.

3.7 Ethyl methane sulphonate

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 $M_1$ generation.

Untreated dry seeds of *Pennisetum typhoides* variety CO(cu)-9 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 two replications.

3.8 Raising of $M_1$ generation

Field sowing was done on 21$^{st}$ February 2014 with spacing of 30 cm between rows and 10 cm between seeds in a plot size of 20 m x 15 m and all recommended package of practices was followed.
OBSERVATION IN M₁ GENERATION

3.9 Seed germination Percentage

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

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

3.10 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.

3.11 Seedling survival (%)

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

\[
\text{Seedling survival} (%) = \frac{\text{Number of seedlings survived}}{\text{Number of seeds germinated}} \times 100
\]

Survival was expressed as the percentage of plant survival.

3.12 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.

Field observations (M₁–M₄ generations)

3.13 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.

3.14 Plant height (cm)

Plant height was measured in cm using meter rule from the base of the plant to the top of the panicle.

3.15 Number of leaves

Number of leaves was taken by counting the number of leaves from the base to the flag leaf.

3.16 Number of nodes

The number of nodes was taken by counting the numbers from base to top.
3.17 Length of earhead (cm)

The length from the base of the panicle to the tip measured in cm using a meter rule.

3.18 Breadth of earhead (cm)

The breadth from the central part of the panicle one edge to the other edge measured in cm using a meter rule.

3.19 Thousand grains weight (g)

Thousand grains weight was worked out by using digital electronic balance and expressed in grams.

3.20 Yield per plant (g)

Seed yield was recorded in grams after weighing the seeds collected from each plant.

BIOCHEMICAL ANALYSIS

3.21 Estimation of chlorophyll content (Arnon, 1949)

0.5 mg of fresh leaf from the same plant of various conc. of M₂, M₃ and M₄ generation ground in a pestle and mortar with 20ml of 80 percent acetone. The homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was saved. The pellet was restricted with 5 ml of 80 percent acetone each time, until it become colourless. All the supernatants were pooled and utilized for the determination of chlorophyll. Absorbance was measured at 645 and
663nm in spectrophotometer. The chlorophyll content was
determined using acetone as standard and by using the following
formulae:

Chlorophyll ‘a’ (mg/g fr.wt.) = (0.0127) × (OD663) – (0.00269) × (OD645)
Chlorophyll ‘b’ (mg/g fr.wt.) = (0.0229) × (OD645) – (0.00468) × (OD663)
Total chlorophyll (mg/g fr.wt.) = (0.0202) × (OD645) + (0.00802) × (OD663)

3.22 Determination of Total Carbohydrate by Anthrone Method
(Hedge and Hofreiter, 1962)

1. Weigh 100 mg of the sample into a boiling tube.

2. Hydrolyse by keeping it in a boiling water bath for three hours
with 5 mL of 2.5 N HCl and cool to room temperature.

3. Neutralise it with solid sodium carbonate until the effervescence
ceases.

4. Make up the volume to 100 mL and centrifuge.

5. Collect the supernatant and take 0.5 and 1 mL aliquots for
analysis.

6. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL
of the working standard.'0' serves as blank.

7. Make up the volume to 1 mL in all the tubes including the
sample tubes by adding distilled water.

8. Then add 4 mL of anthrone reagent.
9. Heat for eight minutes in a boiling water bath.

10. Cool rapidly and read the green to dark green colour at 630 nm.

**CALCULATION**

Amount of carbohydrate present in 100 mg of the sample = \( \frac{\text{mg of glucose}}{\text{volume of test sample}} \) \times 100

**3.23 Chlorophyll mutants (M₁ and M₂ Generations)**

The chlorophyll mutants were recorded on 20\(^{th}\) day after sowing.

Four types of chlorophyll mutants were scored (Veena, 1997).

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

**3.24 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.

**3.25 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).
Chapter III: Materials and Methods

Mutagenic effectiveness (Gamma rays) = \( \frac{\text{Mutation Rate}}{\text{kR} \times \text{time in h}} \)

Mutagenic effectiveness (EMS) = \( \frac{\text{Mutation Rate}}{\text{Concentration of EMS in mM} \times \text{time in h}} \)

Mutagenic effectiveness = \( \frac{\text{Mutation Rate}}{\text{Percentage of lethality or biological injury in } M_1} \)

M - Mutation frequency for 100 M2 plants
T - Period of treatment with chemical mutagen in hours
kR - Kilo radiation
C - Concentration of chemical mutagens in mM
L - Reduction in height of seedling on 30th day
I - Lethality percentage or survival reduction of seedlings

3.26 Pollen sterility

Pollen sterility was determined from 20 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% acetocarmine. Pollen grains that stained fully were considered as fertile, while the empty, partially stained shriveled ones were considered as sterile.

STATISTICAL ANALYSIS

3.27 M2, M3 and M4 Generation

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 ($H^2$), genetic advance (GA) and genetic advance as per cent of mean (GA%) were computed by ANOVA for RBD developed by NPRC-STAT, Tamilnadu Agricultural University, Coimbatore, 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

$$ n = \frac{1}{n} \left( \sum y_i \right)$$

$$ i=1$$

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

$n = \text{Number of observations}$

**3.28 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.
VARIABILITY ANALYSIS

3.29 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>$\sigma e^2 + \sigma g^2$</td>
</tr>
<tr>
<td>Total</td>
<td>$(rg-1)$</td>
<td>$M_2$</td>
<td>-</td>
</tr>
</tbody>
</table>

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

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

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

Where,

$R$ = number of replications
$G$ = number of genotypes
$(\sigma e^2)$ = environmental variance
$(\sigma g^2)$ = genotypic variance
$(\sigma p^2)$ = phenotypic variance
$M_1$ and $M_2$ = Mean sum of squares for genotype and error column
3.30 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).

\[
PCV = \frac{\text{Phenotypic variance}}{\text{General mean}} \times 100
\]

\[
GCV = \frac{\text{Genotypic variance}}{\text{General mean}} \times 100
\]

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

3.31 Heritability

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

\[
\text{Heritability (h2)} = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times 100
\]

Heritability was classified as follows (Robinson, 1966).

- Above 30 per cent - High
- 10-30 per cent - Moderate
- Below 10 per cent - Low
3.32 Genetic advance

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

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

Where,

\[ h^2 \] = heritability

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

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

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

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

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

3.34 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.

3.35 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 are used to raise M₃ and M₄ generations. Seed samples were germinated in small pots until seedlings were grown up to two to three leaf stage. One gram leaves of seedlings were taken from each mutant for DNA assay. The different mutants recruited to the study were tall, dwarf, long panicle, flower mutant, early maturity, tillers 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.

3.36 DNA isolation Solutions required

➢ Extraction buffer:

100 mMTris-HCl (pH 8.0)

50 mM EDTA

1.4 M NaCl

2% CTAB

1% PVP (addition of PVP is optional)

➢ Phenol-Chloroform-Isoamylalcohol (25:24:1 [v/v/v])

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

➢ Isopropyl alcohol, 7.5M Ammonium Acetate

➢ 70% ethanol

Procedure

Genomic DNA was extracted by adopting the CTAB method
outlined by Sharma et al., (2003) with some modifications as follows.

1. Weighed 5g of leaf material in 5ml of alcohol for 30 min and
allow alcohol to evaporate

2. Grind the tissue with a mortar and pestle.
3. Transfer the homogenized tissue to prewarmed 2X CTAB DNA extraction buffer (100mM Tris ) 20mM EDTA (pH 8), 1.4M NaCl, 2% CTAB, 2μl/ml β-mercaptoethanol).

4. Incubate for 1 hour in a 60°C water bath, occasionally mixing by gentle swirling.

5. Remove from water bath. Add 0.6 volume of phenol chloroform isoamylalcohol (25:24:1). Mix by inversion for 15 min.

6. Spin at 15,000 rpm (15,000g) for 10 min.

7. Transfer the aqueous phase to another tube.

8. Add twice the volume of isopropanol to precipitate the DNA.

9. Spool out or centrifuge briefly to pellet the DNA.

10. Wash with 70% ethanol. Invert the tubes and drain on a paper towel for approximately 1 hour. Dry overnight (Cover with parafilm with tiny holes).

11. Dissolve the dried DNA in TE buffer (pH 8).

12. Add 2.5μl of RNAse to 0.5ml of crude DNA (2.5μl of RNAse= 25μg of RNAse, thus treatment is at 50μg/ml of DNA preparation).

13. Mix thoroughly but gently and incubate at 37°C for 1 hour.
14. Add 0.3-0.4ml of phenol chloroform- isoamylalcohol (25:24:1). Mix thoroughly for 15 min.
15. Centrifuge for 15 min at 15,000 rpm (10,000).
16. Remove the supernatant (avoid the whitish interface layer).
17. Reprecipitate the DNA by using double the quantity of absolute alcohol.
18. Remove the DNA with a pasture pipette centrifuge the tube to pellet the DNA.
19. Wash the pellet with 70% alcohol. Dry overnight.
20. Redissolve the DNA in TE buffer.
21. Dilute the DNA 1000 times in TE buffer and quantify by taking the optical density (OD) at 260 to obtain the 260/280 ratio as an indicator of DNA purity.
22. Observe purified DNA on 0.8% agarose gel after staining with ethidium bromide to ascertain its integrity.

3.37 RAPD-PCR amplification

Requirements

➢ **Random primers:** Commercial kits were obtained from NEXT GEN BIO, Chennai, Tamil nadu, INDIA. The eleven random decamer DNA primers had been used for RAPD-PCR assay.
- **Template DNA**: Purified genomic DNA extracts from the selected mutants of Pearl millet have been used as template DNA.

- **dNTPs**: The four individual dNTPs viz., dATP, dGTP, dCTP and dTTP were obtained from NEXT GEN BIO, Chennai, Tamilnadu, INDIA. It is 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 NEXT GEN BIO, Chennai, Tamilnadu, INDIA.

- **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 Taq polymerase

d. **List of primers used in the study**

A total of eleven random primers used for the study are as follows:
### Master Mix for PCR Analysis

<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>
<tr>
<td></td>
<td><strong>Total reaction volume</strong></td>
<td><strong>25.0 µl</strong></td>
</tr>
</tbody>
</table>
**Thermal cycling**

- Sterile microfuge tubes were numbered from 1 to 10.

- 2.0 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 thermal 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°C</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Primer annealing</td>
<td>38°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Primer extension</td>
<td>72°C</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Hold temperature</td>
<td>4°C</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.
3.38 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 (λDNAEcoRI 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.

3.39 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 coefficient 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. Percent of polymorphism was calculated by using the following formula.

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