Chapter-3

MATERIALS AND METHODS
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2. MATERIALS:

*Capsicum annuum* L. has been selected for the present experiment. The effect of chemical mutagens (MMS and DES) has been studied on cytomorphological characters of *Capsicum annuum* L.

2.1 Varieties Used:

Two commercial varieties of chilli (*Capsicum annuum* L.) viz. Pusa jwala and G4 were used for the study. A brief description of these two varieties is given below.

Table 3. Brief Description of the two varieties viz. Pusa jwala & G4 of *Capsicum annuum* L.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Procured from</th>
<th>Salient Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pusa jwala</td>
<td>IARI New Delhi</td>
<td>Plants 50-60 cm tall erect, fruits thin, red; 2-4 fruits of early flush are erect to semi-erect and subsequent are pendent on the same plant. Adaptable throughout India, mature up to 115-125 days, highly pungent, high yielding under irrigated conditions, most popular virus resistant variety. Chromosome number 24.</td>
</tr>
<tr>
<td>G4</td>
<td>IARI New Delhi</td>
<td>Plants average height 53 cm. Fruits olive green turning red, thin pendent pointed tip, mature upto 175-185 days. Adaptable throughout India, less pungent than Pusa jwala. Resistant to bacterial leaf spot but susceptible to root knot nematode. Chromosome number 24.</td>
</tr>
</tbody>
</table>
2.2. Mutagens Used:

The following two mutagens were used separately. The concentrations of each mutagen used in the present study are given below (Table 4).

2.2.1 Methyl Methane Sulphonate (MMS), \( [(C_2H_5O_4S) \] \).

The alkylating agents have been found to be the most potent in a wide array of organisms. Within the alkylating groups, MMS has been found to be a very effective chemical mutagen. Like other alkylating agents, MMS reacts with DNA by alkylating the phosphate groups as well as purine and pyrimidine bases and create a gap between DNA molecule causing mutation. It is a colourless liquid with a molecular weight of 110.3.

2.2.2 Diethyl sulphate (DES), \( [(C_2H_5)_2SO_4] \).

It is also an alkylating agent. Rapoport (1947) studied the mutagenic effect of DES in Drosophila and postulated that ethylation is a mutation inducing process. Thereafter DES has been largely used as a plant mutagen. It reacts with DNA bases, predominantly with the N-7 of guanine but also with the N-1, N-3 and N-7 of adenine and the N-1 and N-3 of cytosine (Singer and Fraenkel-Conrat 1975). Besides having two alkylating groups, it acts as monofunctional agents, since each group alkylates separately. It is highly toxic and suspected carcinogenic agent with a molecular weight of 154.20.

2.2.3 Preparation of Mutagenic Solutions:

One percent stock solutions of methyl methane sulphonate (MMS), dimethyl sulphate (DMS) and diethyl sulphate (DES) were prepared and then different concentrations were prepared by using the following formula:

\[
V_1 = \frac{S_1 V_2}{S_2}
\]

Where:
- \( S_1 \) = Strength of stock solution
- \( V_1 \) = Volume of stock solution
- \( S_2 \) = Strength of desired solution
- \( V_2 \) = Volume of desired solution

The specificity of the action of chemical mutagen depends upon particular conditions of treatment, the more important of which are temperature and hydrogen ion concentration of mutagenic solution. During the course of present study, solutions
of MMS, DMS and DES were prepared by dissolving appropriate quantities of these chemicals in phosphate buffer having a pH 7.0 and the final pH adjusted to 7.0 by adding few drops of normal NaOH/HCl with the help of Backman’s pH meter.

Table 4. Details of MMS and DES treatments given to chilli seeds of var. Pusa jwala & G4.

<table>
<thead>
<tr>
<th>Mutagen Used</th>
<th>Conc.</th>
<th>Duration of Presoaking (h)</th>
<th>Duration of Treatment (h)</th>
<th>pH</th>
<th>No. of treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.01%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.02%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td>MMS</td>
<td>0.03%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.04%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.05%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.01%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.02%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td>DES</td>
<td>0.03%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.04%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.05%</td>
<td>12 h</td>
<td>6 h</td>
<td>7.0</td>
<td>300</td>
</tr>
</tbody>
</table>

2.2.4 Method of Treatment with Chemical Mutagens:
Prior to the mutagenic treatment the seeds were presoaked in distilled water for 12 hours at room temperature (25±1). After the completion of presoaking period the seeds were kept on blotting paper so as to remove small droplets of water adhering to the surface of seeds. Thereafter the seeds were treated in different concentrations of chemical mutagens for 24 hours.

The control seeds were also soaked in distilled water but kept untreated for simultaneous physiological activities, as that of treated seeds.

During chemical mutagenic treatments the intermittent shaking was given throughout the treatment period to facilitate sufficient aeration and maintenance of uniform concentration of mutagen around the seeds. After the treatment period, the
treated seeds were thoroughly washed in running tap water before they were sown in earthen pots.

2.3 Sample Size:

In each variety a set of 200 seeds was used for each dose including the control. Out of these seeds, 150 seeds in each treatment were sown in earthen pots and then transplanted to field at 4 to 5 leaves stage for morphological and cytological studies, whereas the remaining set of 50 seeds was also sown in separate earthen pots for measuring root-shoot length (Seedling Height).

2.4 Sowing of Seeds:

The treated as well as untreated seeds were sown in 30 cm diameter earthen pots (50 seeds in each pot) for raising the seedlings. The seedlings at 4 to 5 leave stages were transplanted to well prepared experimental field in a complete randomized block designs (CRBD) in three replicates. Recommended agronomical practices were employed for the preparation of field, sowing and subsequent management of populations to raise a nice crop.

2.5 EVALUATION OF M1 GENERATION:

A detailed study of the effect of different mutagenic treatments in the two varieties was undertaken using the following parameters.

2.5.1 Seed Germination:

Germination data were recorded every alternate day upto 30 days after sowing, till the maximum germination was attained. The germination percentage based on number of seeds sown and germinated, was calculated by the following formula

\[
\text{Germination percentage (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100
\]

\[
\text{Inhibition (\%)} = \frac{\text{Control – treated}}{\text{Control}} \times 100
\]

2.5.2 Seedling Height:

Seedling height was calculated on 12\textsuperscript{th} day of germination of seeds in pots by measuring root and shoot length of randomly selected seedlings from each treatment as well as control. Seedling injury was estimated by reduction in the root and shoot lengths, calculated in terms of percentage.

\[
\text{Percent Injury (\%)} = \frac{\text{Control – treated}}{\text{Control}} \times 100
\]
2.5.3 Plant survival (%)

The surviving plants in different treatments were counted at the time of maturity and following formula was used to calculate percent survival and percent lethality.

\[
\text{Survival} \% = \frac{\text{Number of plants at maturity}}{\text{Number of seeds germinated}} \times 100
\]

\[
\text{Lethality} \% = \frac{\text{Control - treated}}{\text{Control}} \times 100
\]

2.6 CYTOLOGICAL STUDIES

2.6.1 Meiotic abnormalities:

Cytological studies were carried out on pollen mother cells (PMCs) by fixing young flower buds from each treatment as well as control. The purpose of fixation is to kill the tissue without causing any distortion of the components to be studied. For meiotic studies, young flower buds from 40-45 randomly selected plants were fixed in freshly prepared Carnoy’s fluid (alcohol : chloroform : acetic acid in 6:3:1 ratio) supplemented with crystals of ferric chloride for 24 hours. The material was then washed and preserved in 70% alcohol at 4°C. Meiosis was studied by squashing the anthers in 2% acetocarmine. The slides were made permanent by dehydrated them in n-butyl alcohol series (NBA series) (Bhaduri and Ghose, 1954) followed by mounting in canada balsam and then slides were kept in incubator at 45°C temperature till drying. After drying, the extra amount of canada balsam remained outside the cover slip was cleaned with xylene. Analysis of various stages of meiosis was done from each treatment as well as control at metaphase I/II, anaphase I/II and telophasce I/II by studying more than 200 dividing PMCs. The abnormalities were recorded on the basis of variations in structure and behaviour of chromosomes as compared to control. The photomicrographs were taken from temporary as well as permanent slides with the aid of “Nicon”, photomicrographic unit at the magnification of 1000 X (10 x eye piece X 100 x objective lens).

2.6.2 Pollen fertility (%)

Fresh anthers of randomly selected control and treated plants were squashed in 2% acetocarmine. The pollen grains which took the stain and had a regular outline were considered as fertile, while the empty ones without stain and having irregular
shape were considered as sterile. The percent fertility and percent reduction was calculated as follows:

\[
Pollen\ fertility\ (\%) = \frac{\text{Number of fertile pollen grains}}{\text{Total number of pollen grains}} \times 100
\]

\[
\text{Percent reduction (sterility)}\ (\%) = \frac{\text{Control–treated}}{\text{Control}} \times 100
\]

2.6.3 Chiasma frequency:

The number of chiasmata per cell and per bivalent were estimated in treated as well as control plants by scoring 100 PMC at random at metaphase-I stages.

2.7 Frequency of Morphological Variations/Mutations:

The morphological variations/mutations were scored on the basis of characters in control plants and their deviations in the treated populations at older stage (90 days old). Following formula was adopted to calculate the frequency of variations/mutations.

\[
\text{Frequency of variations (\%)} = \frac{\text{Number of varied plants at older stage (M}_1\text{generation)}}{\text{Total no. of germinated seedlings}} \times 100
\]

\[
\text{Mutation Frequency (\%)} = \frac{\text{Number of mutated plants at older stage (M}_2\text{ & M}_3\text{generation)}}{\text{Total no. of germinated seedlings}} \times 100
\]

2.8 Quantitative characters:

Following seven quantitative characters were statistically analyzed to assess the extent of induced variability in M1 generation.

Days to flowering: Days to flowering were noted as the number of days taken by the plant from the date of sowing till the first flower appeared in the plant.

Plant height (cm): Plant height (cm) was measured at maturity from the base up to the apex of plant.

Days to maturity: Days to maturity were noted as the number of days taken by the plant from the date of sowing to the date of harvesting of the plant.
Number of fruits per plant: The average number of fruit per plant was determined at maturity.

Fruit length (cm): Twenty fruits were selected randomly from each selected plant and their length was measured and mean length of fruits was determined in each treated populations as well as control.

Fruit girth (cm): The girth at the mid position of the twenty randomly selected fruits from each selected plant was measured and the mean value of girth from each selected plant was calculated.

Total yield per plant (g): Total yield per plant was the weight of total number of fruits harvested from each plant and the yield of each plant was recorded in grams. Data related to these characters were taken from 30 randomly selected plants in control and treated populations and divided by the number of plants or observations to obtain the average in their respective units.

Selfing:

The selected variants were selfed in M₁ generation to induce homozygosity and for screening of the mutants in M₂ generation.

2.9 EVALUATION OF M₂ GENERATION:

The collected seeds in M₁ generation were harvested separately in treated as well as control populations. A set of control seeds and all those obtained from treated populations of M₁ generation were sown for study in M₂ generation. Three replicates were maintained in each treatment.

2.9.1 Mutagenic Effectiveness and Efficiency:

Mutagenic effectiveness is a measure of the frequency of mutations induced by unit dose of mutagens, whereas, mutagenic efficiency is the measure of proportion of mutations in relation to undesirable changes like injury, lethality, sterility and meiotic aberrations etc. Mutagenic effectiveness and efficiency was calculated on the basis of formula suggested by Konzak et al (1965).

\[
\text{Mutagenic Effectiveness} = \frac{\text{Mutation rate (M₂ family or population basis) MP}}{\text{Concentration of mutagen (C) × Duration of treatment in hours (T)}}
\]

Percentage of mutated plant progenies (mutation rate in
Mutagenic efficiency = \[
\frac{M_2 \text{ MP}}{\% \text{ injury (I) or } \% \text{ lethality (L) or } \% \text{ sterility(S) or } \% \text{ meiotic aberrations (M)}}
\]

2.9.2 Studies on Different Morphological Traits:

For morphological studies the parameters were similar to those in M1 generation. These include: Days to flowering, Days to maturity, plant height, No. of fruits, fruit length, fruit girth, and yield per plant.

2.10 Cytological Studies:

Cytological studies in M2 progeny were carried out as in M1 generation.

Selfing:

The selected mutants were selfed in M2 generation also.

2.11 EVALUATION OF M3 GENERATION:

The plants in M3 generation were raised from the seeds obtained from the selected mutants of M2 generation and studied separately along with control population. The characters studied in M3 generation were same as in M2 generation.

2.12 STATISTICAL ANALYSIS

The data recorded on different characters due to mutagenic treatments have been subjected to statistical analysis with a view to find the individual and comparative effects of different mutagens. Mean, standard error, standard deviation and coefficient of variation were calculated as per the standard statistical procedures.

2.12.1 Mean (\( \bar{X} \)):

The mean value was computed by taking the sum of a number of observations and dividing it by the total number of observations recorded, thus

\[
\bar{X} = \frac{x_1 + x_2 + \ldots + x_n}{N}
\]

\( \bar{X} \) (Mean) = \( \frac{\sum x_n}{N} \)

Where, \( x_1, x_2, \ldots, x_n \) = observations

\( N = \) Total number of observations involved
2.12.2 Standard deviation (S.D.):

Standard deviation is the positive square root of the average of sum of squares of deviations of all observations from their means. It is calculated by the following formula.

\[
S.D. = \sqrt{\frac{(x-x_1)^2 + (\bar{x}-x_2)^2 + \ldots + (\bar{x}-x_n)^2}{n}}
\]

\[
S.D. = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}
\]

where,

\[\Sigma x = \text{sum of all individual observations}\]

\[\bar{x} = \text{Mean of all observations}\]

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

2.12.3 Standard Error (S.E.):

It is the measure of uncontrolled variation present in a sample. It is estimated by dividing the estimate of standard deviation by the square root of the total number of observations in the sample, Thus

\[S.E. = \frac{\text{S.D. of sample}}{\sqrt{N}}\]

Where,

\[\text{S.D.} = \text{Standard deviation}\]

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

2.12.4 Coefficient of variation (C.V.):

It measure the relative magnitude of variation present in the observations relative to magnitude of their arithmetic mean. It is defined as the ratio of standard deviation to arithmetic mean expressed as percentage and is a unit less number. It is computed by applying the following formula:

\[C.V. (\%) = \frac{S.D.}{\bar{X}} \times 100\]

Where,

\[\text{S.D.} = \text{Standard deviation}\]

\[\bar{X} = \text{Arithmetic mean}\]
2.12.5 Least Significant Difference (LSD):

The least difference was applied and computed as follows:

**Step 1: According to treatment given, construction of data table for treatment and 3 replicates.**

The data were compiled in such a way that each treatment occupies a row and their replicates were arranged in column.

<table>
<thead>
<tr>
<th>Column (Treatments)</th>
<th>Column (Replicates)</th>
<th>Total of Rows (Treatments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;+A&lt;sub&gt;2&lt;/sub&gt;+A&lt;sub&gt;3&lt;/sub&gt;=X&lt;sub&gt;t1&lt;/sub&gt;</td>
</tr>
<tr>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>B&lt;sub&gt;1&lt;/sub&gt;+B&lt;sub&gt;2&lt;/sub&gt;+B&lt;sub&gt;3&lt;/sub&gt;=X&lt;sub&gt;t2&lt;/sub&gt;</td>
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<tr>
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<tr>
<td>T&lt;sub&gt;6&lt;/sub&gt;</td>
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<td>F&lt;sub&gt;1&lt;/sub&gt;+F&lt;sub&gt;2&lt;/sub&gt;+F&lt;sub&gt;3&lt;/sub&gt;=X&lt;sub&gt;t6&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Total of A<sub>1</sub>+...F<sub>1</sub>=XC<sub>t1</sub>, A<sub>2</sub>+...F<sub>2</sub>=XC<sub>t2</sub>, A<sub>3</sub>+...F<sub>3</sub>=XC<sub>t3</sub>=Grant Total (G)

**Step 2: Correction Factor (CF).**

\[
CF = \frac{(G)^2}{T \times R}
\]

Where:

T = Number of treatments
R = Number of replications

**Step 3: Total Sum of Squares (TSS):** This is the sum of square of all the observations minus correction factor

\[
TSS = [(A<sub>1</sub>)^2 + (B<sub>1</sub>)^2 + ... + (F<sub>3</sub>)^2] - CF
\]

**Step 4: Replication Sum of Squares (RSS).**

\[
RSS = \frac{(XC_1)^2 + (XC_2)^2 + (XC_3)^2}{T} - CF
\]

Where:

T = No. of treatments
Step 5: Treatment Sum of Squares (TrSS).

\[
TrSS = \frac{(X_1) + (X_2) + (X_3) - CF}{R}
\]

Where: \( R \) = Number of replications

Step 6: Error Sum of Squares (ESS).

\[
ESS = TSS - (RSS - TrSS)
\]

Step 7: Construction of ANOVA table.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>SS</th>
<th>MS</th>
<th>F. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>R-1</td>
<td>RSS</td>
<td>RSS / R-1 = RMS</td>
<td>TrMS / EMS</td>
</tr>
<tr>
<td>Treatment</td>
<td>T-1</td>
<td>TrSS</td>
<td>TrSS / T-1 = TrMS</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>(R-1) (T-1)</td>
<td>ESS</td>
<td>ESS / (R-1) (T-1) = EMs</td>
<td></td>
</tr>
</tbody>
</table>

Step 8: Least significant difference based on ordinary test.

LSD at 5% level = \( \sqrt{\frac{2EMS}{R}} \times (t \text{ value at 5\% level}) \)

LSD at 1% level = \( \sqrt{\frac{2EMS}{R}} \times (t \text{ value at 1\% level}) \)

Where:

\( t \) = Tabulated value

If the difference between any two samples means exceeding the LSD value obtained at 5\% level and / or 1\% level, the difference between the two means is said to be significant at 5\% or 1\% level respectively.

2.13 CAPSAICIN ESTIMATION:

Capsaicin content of some of most promising isolated mutant lines in M_3 generation were analyzed. The selection of mutants from the isolated mutant lines in M_3 for capsaicin estimation was based on morphological/economic characters.

2.13.1 Preparation of Samples:

The harvested fruits were dried at 50°C in oven and grounded into fine powder and were kept in airtight containers at room temperature, prior to extraction. For
extraction of capsaicin, 1 gm finely grounded powdered sample of each mutant were dissolved in 10 ml of acetonitrile with two hr of shaking on a shaker. After that, flask was left overnight at room temperature. The supernatant was filtered with Whatman filter paper No.1, thereafter by 0.45µm filter paper and with a 10 ml disposable syringe into 1 ml glass vial. The volume of injection in HPLC was taken 10 µl. The standard solution of capsaicin (Sigma Chemicals Co. M2028) were prepared as 50 ppm stocks in absolute ethanol and used for retention time verification and instrument calibration.

2.13.2 Estimation of Capsaicin by High-Performance Liquid Chromatography (HPLC):

The samples were analysed using Watre’s Quaternary Gradient HPLC system equipped with Waters 717 autosampler, temperature controller, Water-966 photodiode array detector and Millennium$^2$ software for data processing. Reverse phase HPLC was carried out on a Spherisorb RP C-18 ODS-1 column (150mm×46mm), having particle size 5 µm. A pre-column guard cartridge Spherisorb RP C-18 were also used. The capsaicin was determined under uniform HPLC conditions: column temp, 30 °C, flow rate 1.5 ml/min. and run time was 20 minutes. The mobile phase was isocratic with solvent combination (Acetonitrile: water containing 1.00% acetic acid in 70:30) and the detection was at 280nm.