Materials and Methods
CHAPTER - II
MATERIALS AND METHODS

In the present work the effect of physical mutagen (γ rays), chemical mutagens (SA and aniline) and their combinations (γ + SA and γ + caffeine) have been studied on the cytomorphological characters on *Vicia faba* L.

The certified, healthy and dry seeds of *V. faba* L. were used for all the experiments. In each concentration or dose of the mutagen 50 seeds were treated in three replicates and sown in randomized block design. The schedule of the treatment is given below -

2.1 TREATMENT SCHEDULE

<table>
<thead>
<tr>
<th>Mutagens</th>
<th>Doses (KR)/Concentrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gamma rays</td>
<td>5, 10, 20, 30, 40KR</td>
</tr>
<tr>
<td>gamma rays + SA</td>
<td>5+0.25, 5+1.00, 5+2.00</td>
</tr>
<tr>
<td>and</td>
<td>10+0.25, 10+1.00, 10+2.00</td>
</tr>
<tr>
<td>gamma rays + caffeine</td>
<td>20+0.25, 20+1.00, 20+2.00</td>
</tr>
<tr>
<td>(KR+ % Concentrations)</td>
<td>30+0.25, 30+1.00, 30+2.00</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>40+0.25, 40+1.00, 40+2.00</td>
</tr>
<tr>
<td>and Aniline</td>
<td>0.50, 0.75, 1.00, 1.25, 1.50, 1.75</td>
</tr>
<tr>
<td></td>
<td>and 2.00%</td>
</tr>
</tbody>
</table>

The source of gamma rays was Co$^{60}$ at National Botanical Research
Institute (NBRI), Lucknow, India. Three sets of 50 seeds each were soaked simply in distilled water as control.

2.2 EXPERIMENTAL PROCEDURE:

2.2.1: Preparation of Mutagenic Solution:

2% stock solution of all the chemical mutagens were prepared. Different concentrations were made from the stock solution, by using the formula:

\[ S_1 V_1 = S_2 V_2 \]

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

2.2.2: Pre-treatment and treatment of seeds with mutagens:

Seeds of uniform size were used for different treatments. Prior to chemical mutagenic treatment the seeds were presoaked in distilled water for 6 hrs. at room temperature, in order to activate the embryo. Thereafter, the seeds were treated in chemical mutagens for 24 hours.

2.2.3: Sowing of the treated seeds:

After the treatment with different concentrations of mutagens for 24 hrs., the seeds were thoroughly washed in running water in order to remove the mutagen from the seed surface. Three replications of 50 seeds each were sown
for each treatment. The cytomorphological observations were recorded from treated populations as well as controls.

2.3 OBSERVATIONS RECORDED IN M₁ GENERATION:

A detailed study of the effects of different mutagenic treatments was done using the following parameters:

2.3.1 Seed Germination:

In control and lower doses of mutagens the seed germination started 10th day after sowing. Germination data were recorded every alternate day upto 30 days till the maximum germination was attained.

The germination percentage, based on the number of seeds sown and germinated, is calculated by the following formula:

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

The delaying effect of mutagens on germination was recorded on the basis of the extra days taken for germination in the treated populations as compared to control.

2.3.2 Seedling Injury:

Seedling injury or percentage inhibition in germination was calculated by using following formula:
Seedling Injury (%) =
\[
\frac{\text{No. of seeds germinated in control} - \text{No. of seeds germinated in treated population}}{\text{No. of seeds germinated in control}} \times 100
\]

2.3.3: Seedling Morphology:

The parameters of morphological variations were the size and shape of cotyledonary and vegetative leaves and habit of seedlings.

2.3.4: Frequency of Morphological Variations:

The morphological variations were scored on the basis of visible physical characters and deviations from the normal behaviour of the plants as compared to control. Following method was adopted to calculate the frequency of variations.

Variation frequency (%) =
\[
\frac{\text{No. of varied seedlings/Total No. of germinated seedlings}}{\text{X 100}}
\]

2.3.5: Plant Height:

The height of plants was recorded from the point above the ground to the tip of the main axis, at different intervals i.e. at seedling stage and at maturity.

2.3.6: Number of leaves per plant:

\textit{V. faba} plant bears compound leaves. Each rachis, considered as single leaf, bears the number of leaflets. Leaves were counted in all the branches of the plant.
2.3.7 : Leaflet size ratio :

Leaflet size ratio is calculated by using the following formula :

\[ \text{Leaflet size ratio} = \frac{L}{B} \]

where, \( L = \text{Length of leaflet} \)

\( B = \text{Breadth of leaflet} \)

2.3.8 : Number of Branches :

Branches were counted emerging out of main stem in all the treatments along with the control.

2.3.9 : Number of Pods per plant :

Number of pods borne on the whole plant were counted at maturity.

2.3.10 : Plant yield :

Plant yield was the weight of total number of seeds harvested per plant in grains.

2.3.11 : 100 Seeds weight (gms) :

It was the average weight of random samples of 100 seeds from each treatment.

2.4 CYTOLOGICAL STUDIES :

2.4.1 (i) Fixation of flower buds : For study of meiosis, the flower buds of proper size were fixed, between 8.00-10.30 a.m., in Carnoy’s fluid (6 parts absolute alcohol : 3 parts chloroform : 1 part glacial acetic acid)
for 40-45 minutes or until complete dissolution of chlorophyll. Buds were then transferred to propionic acid (saturated with ferric acetate) for 24 hrs. and then material was washed with 70% alcohol and stored in it.

(ii) Preparation of slide: Anthers were squashed in 0.5% propionocarmine stain (Swaminathan et al., 1954). These slides were dehydrated in NBA series (Bhaduri & Ghosh, 1954) mounted in Canada balsam and dried in incubator at 45°C for 3-5 days. Cytological observations and microphotographs were taken from temporary as well as permanent slides.

2.4.2: Chiasma Frequency:

The number of chiasmata per cell and per bivalent was estimated in treated as well as control plants by scoring 200 PMCs at random, at diakinesis and metaphase I stages.

2.4.3: Meiotic Abnormalities:

Randomly selected 200 PMCs were analysed from diakinesis to telophase II stages for the study of meiotic anomalies, such as multivalents, stickiness of chromosomes, unequal division, bridges with or without fragments, laggards, precocious separation, disoriented chromosomes, micronuclei and multinucleate PMCs etc.

2.4.4: Pollen Fertility:

Anthers of the randomly selected control and treated plants were
squashed in 0.5% acetocarmine. Fully stained, full size pollen grains with smooth and regular outline were counted as fertile while unstained, empty, shrunken and deshaped pollen grains were counted as sterile. The percentage of pollen fertility in each concentration was calculated by using the formula:

\[
Pollen \ fertility \ (\%) = \frac{\text{No. of fertile pollen grains}}{\text{Total No. of pollen grains}} \times 100
\]

2.4.5: Percentage Reduction in Pollen Fertility:

The following formula was used to calculate the percentage inhibition or injury:

\[
\text{Percentage Inhibition or Injury in pollen fertility} = \frac{\text{Pollen fertility in control plants} - \text{Pollen fertility in treated plants}}{\text{Pollen fertility in control plants}} \times 100
\]

**M₂ GENERATION**

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.

2.5 OBSERVATIONS RECORDED IN M₂ GENERATION:

2.5.1/i) Chlorophyll mutation frequency: Chlorophyll mutations were scored and classified when seedlings were 8-15 days old. The frequency of chlorophyll mutations was calculated by the following formula:
Chlorophyll mutation frequency (%) =

\[
\frac{\text{No. of mutant seedlings}}{\text{Total No. of } M_1 \text{ Seedlings}} \times 100
\]

The chlorophyll mutation frequency was calculated on progeny as well as on plant basis.

2.5.2 ii) Mutagenic Effectiveness and Efficiency:

Mutagenic effectiveness is a measure of the frequency of mutations induced by unit dose of a mutagen (time x concentration) while mutagenic efficiency represents the proportions of mutations in relation to biological damage.

The formula suggested by Konzak et al. (1965) were used to evaluate mutagenic effectiveness and efficiency of the mutagens.

a) (i) Mutagenic effectiveness (in case of physical mutagen)

\[
= \frac{\text{Percentage of mutated plant progenies (Mp)}}{\text{KR}}
\]

(ii) Mutagenic effectiveness (incase of chemical mutagen)

\[
= \frac{\text{Percentage of mutated plant progenies (Mp)}}{\text{Conc. of mutagen x duration of treatment in hrs.}}
\]

(iii) Mutagenic effectiveness (in combined treatment)

\[
= \frac{\text{Percentage of mutated plant progenies (Mp)}}{\text{KR x time x concentration}}
\]

b) Mutagenic efficiency = % of Mutated plant progenies (Mp)/*Biological damage in M₁ Generation
*Biological damage: For measuring the biological damage two different criteria were used.

(i) Injury: Percentage seedling germination reduction (Mp/I)

(ii) Sterility: Percentage reduction in pollen fertility (Mp/S).

2.5.3: Studies on different morphological traits:

For morphological studies the parameters were similar to M₁ Generation. These include height, No. of leaves, leaflet size L/B ratio, number of branches, No. of pods per plant, No. of seeds per plant, wt. of seeds per plant and weight of 100 seeds (gms.)

2.6 M₃ GENERATION:

M₃ generation was raised from the seeds obtained from treated populations of M₂ generation. Different mutants were also selected in M₃ generation and studied separately. The characters studied in M₃ generation were same as in M₂ generation.

2.7 STATISTICAL ANALYSIS:

The data recorded on different characters relating to each of the different treatments have been subjected to statistical analysis with a view to find the individual and comparative effects of different mutagens.

2.7.1: Mean ($\bar{X}$):

The mean was computed by taking the sum of a number of observations and dividing it by the total number of observations recorded. Therefore,
\[ \bar{X} = \frac{(X_1 + X_2 + \cdots + X_n)}{N} \]
\[ \text{or } \bar{X} = \frac{\sum X}{N} \]

where \( X_1, X_2 \ldots X_n \) = observations

\( N \) = Total number of observations recorded.

**2.7.2: Standard Deviation (SD, } \sigma \text{):}**

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:

\[ \sigma = \sqrt{\frac{(X_1 - \bar{X})^2 + (X_2 - \bar{X})^2 + \cdots + (X_n - \bar{X})^2}{N}} \]

\( \bar{X} \) = Mean of observations involved

\( X_1 \ldots X_n \) = observations.

\( N \) = No. of observations.

**2.7.3: Coefficient of Variations (CV):**

It measures the relative magnitude of variations present in observations relative to the magnitude of their arithmetic mean. It is defined as the “Rate of standard deviation to arithmetic mean expressed as a percentage”.

\[ CV = \frac{SD}{\bar{X}} \times 100 \]
2.7.4: Critical Difference (C.D.):

The critical difference was applied and computed as follows:

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

The data were compiled in such a way that each treatment occupies a row & their replicates were arranged in columns. e.g. taken from γ rays.

<table>
<thead>
<tr>
<th>Rows (Treatments)</th>
<th>Column (Replicates)</th>
<th>Total of Rows (Treatments)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R₁</td>
<td>R₂</td>
</tr>
<tr>
<td>T₁</td>
<td>A₁</td>
<td>A₂</td>
</tr>
<tr>
<td>T₂</td>
<td>B₁</td>
<td>B₂</td>
</tr>
<tr>
<td>T₃</td>
<td>C₁</td>
<td>C₂</td>
</tr>
<tr>
<td>T₄</td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>T₅</td>
<td>E₁</td>
<td>E₂</td>
</tr>
<tr>
<td>T₆</td>
<td>F₁</td>
<td>F₂</td>
</tr>
</tbody>
</table>

Total of A₁ + ... + F₁ = XC₁; A₂ + ... + F₂ = XC₂; A₃ + ... + F₃ = XC₃ = Grand total (G)

Step 2: Correction Factor (C.F.)

\[
C.F. = \frac{(\text{Grand total})^2}{T \times R}
\]

where, T = Number of treatments
R = Number of Replicates

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Step 3: Total sum of squares (SSQT): This is the sum of squares of all the observations minus correction factor.

\[ SSQT = [(A_1)^2 + (B_1)^2 + \ldots + (F_3)^2] - CF \]

Step 4: Sum of squares of Replicates (SSQR):

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

where \( T = \) Number of treatments

Step 5: Sum of squares of treatments (SSQt)

\[ SSQt = (X_{r1})^2 + (X_{r2})^2 + \ldots + (X_{re})^2/R - CF \]

where, \( R = \) Number of replicates

Step 6: Sum of squares of Error (SSQE):

\[ SSQE = SSQT - (SSQR + SSQt) \]

Step 7: Construction of ANOVA table

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom (n-1)</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>C-1</td>
<td>SSQR</td>
<td>SSQR/C-1 = A</td>
<td>A/C</td>
</tr>
<tr>
<td>Treatment</td>
<td>r-1</td>
<td>SSQt</td>
<td>SSQt/r-1 = B</td>
<td>B/C</td>
</tr>
<tr>
<td>Error</td>
<td>(c-1)(r-1)</td>
<td>SSQE</td>
<td>SSQE/(C-1)(r-1) = C</td>
<td>C/C</td>
</tr>
</tbody>
</table>

Step 8: Estimated variance of error (MSE)

\[ MSE = \frac{SSQE}{(C-1)(r-1)} \]
Step 9: Critical difference based on ordinary t test

CD at 5% level = $\sqrt{2 \text{MSE}/R} \times (t \text{ value at 5% level})$

CD at 1% level = $\sqrt{2 \text{MSE}/R} \times (t \text{ value at 1% level})$

If the difference between any two sample means exceeds the CD values obtained at 5% and/or 1% level, the difference between the two means is said to be significant at 5% and/or 1% level respectively.