Chapter-3

MATERIALS AND METHODS

3.1 Materials

3.1.1. Varieties used

Two varieties of lentil (*Lens culinaris* Medik) namely, K-75 and L-4076 were used in the present study. Seeds of both the varieties K-75 and L-4076 were obtained from Genetic Division of Indian Agriculture Research Institute, New Delhi. Both the varieties are popular for cultivation in this region. A brief description of both the varieties is given below:

3.1.1.1. Variety K-75

It is a *microsperma* variety. This variety matures in 135-140 days, foliage green, semi-spread medium plant height (40-45 cm), flower violet, pod green in early stage and brown at maturity, seed bold and mottled gray (2.7 g/100 seeds), average yield is 10-16 q/ha.

3.1.1.2. Variety L-4076

It is a *macrosperma* variety. This variety matures in 135-140 days, plants are dwarf, semi-spreading type, grains are bold (3.0 g/100 seeds) and pinkish in colour. This variety is resistant to wilt disease. It is suitable for growing under irrigated as well as un-irrigated conditions, average yield is 14-16 q/ha.
3.1.2. **Mutagens used**

3.1.2.1. **Methylmethane sulphonate (MMS)**, CH$_3$OSO$_2$CH$_3$, monofunctional alkylating agent, is manufactured by Sissco Research Laboratories Pvt. Ltd., Mumbai.

3.1.2.2. **Hydrazine hydrate (HZ)**, NH$_2$-NH$_2$-H$_2$O, a base analogue, is manufactured by Sigma Chemical Company, USA.

3.2. **Experimental procedures**

3.2.1. **Preparation of mutagenic solutions**

All solutions of chemical mutagens were prepared in phosphate buffer of pH-7. Only freshly prepared solutions used for all the treatments.

3.2.2. **Pretreatment**

Healthy seeds of uniform size of each variety were used in the present experiments. The seeds were soaked in distilled water for 9 hours prior to the treatment with mutagens.

3.2.3. **Mutagen Administration**

**Concentrations used:** Four different concentrations viz., 0.01, 0.02, 0.03 and 0.04% of MMS and HZ were used for treating the presoaked seeds.

**Treatment time:** The treatments were given at temperature of 25±1°C for 6 hours.

**Sample size:** 273 seeds were used for each treatment.

**Controls:** For each variety 273 pre-soaked seeds were again soaked in phosphate buffer for 6 hours to serve as controls.
3.3. **M₁ generation**

Three replications of seventy-five seeds each, were sown for every treatment in each variety in the field. The remaining lot of forty-eight seeds of each treatment with their respective controls of both the varieties were spread over moist cotton in petriplates, in order to determine percentage of seed germination and seedling height i.e. root and shoot length. The petriplates were kept in the B.O.D. incubator at 25±1°C temperature.

3.3.1. **Observation recorded in M₁ generation**

Following parameters were studied in M₁ generation:

3.3.1.1. **Seed germination:** After recording germination counts, the percentage of seed germination was calculated on the basis of total number of seeds sown in petriplates. Seeds which gave rise to both radical and plumule were considered as germinated.

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

3.3.1.2. **Seedling height**

On the tenth day, the seedling height was estimated in centimeters by measuring the root and shoot length of fifteen randomly selected seedlings for each treatment. Seedling injury, as measured by the reduction in the root and shoot length, was calculated in terms of percentage of root and shoot injury.
3.3.1.3. Plant survival

The surviving plants in different treatments were counted at the time of maturity and the survival was computed as percentage of the germinated seeds.

3.3.1.4. Pollen fertility

Pollen fertility was estimated from fresh pollen samples. From mature anthers, some amount of pollen was dusted on a slide containing a drop of 1% acetocarmine solution. Pollen grains, which took stain and had a regular outline were considered as fertile, while empty and unstained ones as sterile.

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

\[
\text{Percentage inhibition} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100
\]

\[
\text{Percentage injury} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100
\]

3.3.2. Morphological variants

Some induced morphological variants affecting plant form, height and leaf were isolated in M1 generation. Frequency of each variant was calculated in both the varieties.
3.3.3. Quantitative traits

Observations were also made on 25-30 normal-looking plants in each treatment and control.

The following eight quantitative traits were studied in M₁ generation.

1. **Plant height**: Plant height was measured at maturity in centimeters from the base up to the apex of the plant.

2. **Days of flowering**: Days to flowering were noted as the number of days taken by the plant from the date of opening of the first flower bud.

3. **Days to maturity**: Number of days taken by the plant from the date of sowing up to the date of harvesting of the plant.

4. **Number of fertile branches**: Number of fertile branches were counted at maturity as the number for fertile branches which had more than one pods.

5. **Number of pods**: Number of pods were counted at maturity as the number of pods borne on the whole plant.

6. **Seeds per pod**: Twenty best pods were threshed and number of seeds per pod was counted. The mean was calculated for each plant.

7. **100 seed weight (g)**: It was the weight of a random sample of hundred seeds from each plant.

8. **Total plant yield**: Plant yield was the weight of total number of seeds harvested per plant and the yield of each plant was recorded in grams.
3.4. Cytological studies

For meiotic analysis, young flower buds from each treatment and controls in both the varieties were fixed in Cornoy’s fluid (1 part glacial acetic acid : 3 parts chloroform : 6 parts of ethyl alcohol) for 30 minutes. The material was then transferred to propionic alcohol saturated with ferric acetate for 24 hours. The flower buds were washed with and preserved in 70% alcohol. Anthers were smeared in 1% acetocarmine solution and pollen mother cells were examined for their behaviour at various stages of microsporogenesis. Photographs were taken from temporary preparations.

3.5. Statistical analysis

Assessment of variability

An insight into the magnitude of variability present in a crop species is of utmost importance, as it provides the basis of effective selection. The variability present in breeding population was assessed by using simple measures of variability. Data collected for eight quantitative traits in M₁ generation were subjected to statistical analysis to find out range, mean, standard error, standard deviation and coefficient of variability.

Range

It is the difference between the lowest and the highest values present in the observations included in a sample.
Mean ($\bar{X}$)

The mean is computed by taking sum of the number of values ($X_1, X_2, \ldots, X_n$) and dividing by the total number of values involved, thus

$$\bar{X} = \frac{(X_1 + X_2 + X_3 \ldots \ldots X_n)}{N}$$

or

$$= \frac{\sum X_n}{N}$$

Where, $X_1, X_2, X_3\ldots\ldots, X_n = $ Observations

$N = $ Total number of observations involved.

Standard error (S.E.)

It is the measures of the uncontrolled variation present in a sample. It is estimated by dividing the estimate of standard deviation by the square root of the sample and is denoted by S.E., thus

$$S.E = \frac{S.D. \text{ of the sample}}{\sqrt{N}}$$

Where, S.D. = Standard deviation

$N = $ Number of observations

Standard deviation (S.D.)

The standard deviation is calculated by the following formula for each parameter of study.
S.D. = \sqrt{\frac{(\bar{X} - X_1)^2 + (\bar{X} - X_2)^2 + \ldots (\bar{X} - X_n)^2}{N}}

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

\( X_1, X_2, \ldots, X_n \) = Observations

\( N \) = Total number of observations

**Coefficient of variability (C.V.)**

It measures the relative magnitude of variation present in observations relative to magnitude of their arithmetic mean. It is defined as the ratio of standard deviation to the arithmetic mean expressed as percentage and is a unitless number. The following formula is applied to compute coefficient of variability (C.V.).

\[
\text{C.V.} (\%) = \frac{\text{Standard deviation}}{\bar{X}} \times 100
\]

or

\[
= \frac{\text{S.D.}}{\bar{X}} \times 100
\]

Where, \( \text{S.D.} \) = Standard deviation of sample

\( \bar{X} \) = Arithmetic mean