Chapter 3

Material and Methods

Comprehensive Work Plan of The Thesis
THE MATERIAL AND METHODS

Lentils (*Lens culinaris* Medik.)

Lentils are one of the oldest pulse crops and are grown in India Egypt Greece, Italy countries in the Mediterranean basin and Switzerland. In India the pulse is popularly called massur dal. The lentil dal is considered inferior to black gram but as good as red gram or pigeon pea. The young pod is also used as vegetable and the bhusa a fodder for cattle. For experimentation, certified seeds of Lentils were obtained from IARI, New Delhi, I I P R Kanpur, Chendra shaker azad Agriculture university Kanpur. The variety selected for the present work was – MORDEN.

ORIGIN AND HISTORY

Lentils are a native of Egyptians. Its wild relatives and other Lentils species are distributed widely across the plains of Canada from north to south. Archaeological evidence seems to indicate that the crop was domesticated in the central part of USA. In the 1500s, early Spanish explorers took Lentils back to Europe, where they were used as and pules, plant. It has been the
main source of the edible vegetable in the former Soviet Union and eastern European countries for decades. The food type Lentils was brought back to North America by immigrants and cultivated in the northern plains states.

WORLD PRODUCTION AND DISTRIBUTION

The leading commercial producers of Lentils seeds include the Egypt (1 million tonnes), Ukraine (2.1 million tonnes), Argentina (1.2 million tonnes), European Union (2.5-3.5 million tonnes), USA (1.2 million tonnes) and China (1 million tonnes). Central Europe is the major exporter of Lentils seeds. Lentils, in India were introduced in seventies. The major producers in India are Karnataka (33%), Andhra Pradesh (34%), Maharashtra (15%), Punjab (5%) and Haryana (4%), M P(4). The cultivation of Lentils in traditional areas of India increased after the introduction of hybrids utilizing CMS source and hybrid vigour. Availability of Cytoplasmic Genetic Male Sterile system (CMS system) coupled with high cross pollination nature of the crop makes the commercial hybrid production easier.

BOTANICAL DESCRIPTION

Herbaceous annual low growing bushy not exceeding twenty cm in height plant light green in colour stem oval leaves pinnately compound stipules linear leaflets five to seven pairs ovate mucronate, the end leaflets sometimes forming tendrils flowers carried on axillary slender peduncles two or four together small white tinged with blue or violet or pink ovary is short with one
or two ovules style curved and hairy on its inner surface fruit a legume or pod, short flattened one to one and a half cm long with a curved beak an persistent calyx seeds generally two per pod often light brown in colour lens shaped cotyledons flesh coloured.

ANTHESIS AND POLLINATION

The dehiscence of anthers takes place in the bud itself sometime before the opening of the flower in the next morning. The crop is generally self fertilized.

ROOTS: It has a root system comprising a central main tap root with extensive lateral roots. The tap root of Lentils is very short, often limited to the top 10cm and the lateral roots are extensive restricted to top layers. Numerous and strong lateral roots originating from the enlarged tap root in the top 10cm of the soil spread widely from 40-50 cm and concentrate in the upper 5 cm of the soil. Due to this high concentration soil surface, moisture exhausts quickly and demands for higher frequency of Irrigation.

STEM: Cultivated Lentils stems are typically unbranched. The stem is hairy and becomes very fibrous as the plant matures. Stem length is determined by the number of internodes. Stem diameter varies from 10mm lateral branching system is present in Lentils, in recent years due to rapid expansion of crop in wide range of agro-climatic conditions, the branching is frequented. Nitrogen triggers branching in Lentils and inorganic sources have greater
influence than organic sources (Sudhakara Babu, 1971 & 2001). Generally accepted ideotype for height of cultivated Lentils is a medium plant height of 40 cm.

**LEAF:** Number, size and shape of leaves varies with the plant type and the environment. Number of leaves on the stem or branch depends upon its growth patterns. The number of leaves on single stemmed plants may vary from many. The leaves are always opposite some alternate. The leaves are usually petiolate, vary in shape from linear to ovate and are usually entire or serrated. The colour intensity could vary from light to dark green.

**ANTHESIS AND FERTILIZATION/REPRODUCTIVE BIOLOGY**

The dehiscence of anthers takes place in the bud itself sometime before the opening of the flower in the next morning. The crop is generally self fertilized. They are high self-incompatible. Pollen is rather heavy, spiny and adapted to be transported by insects.

**BREEDING, SEED PRODUCTION AND AGRONOMIC PRACTICES**

Early breeding was by mass selection. Later on, Pustovoit (1964) in USSR in 1920s developed a much more successful technique called the method of reserves. Inbreeding as a method for improving The dehiscence of anthers takes place in the bud itself sometime before the opening of the flower in the next morning. The crop is generally self fertilized was used as early as 1922 (Cardon). The hybrids were produced by using a highly self incompatible...
female and crossing it with a highly self compatible male line. The resulting hybrid seed provided a considerable yield advantage. It was the discovery of cytoplasmic male sterility by Leclercq (1969) in France; followed by identification of fertility restorer genes that heterosis could be fully exploited.

Some of the promising varieties for commercial cultivation are –

**CLIMATE**

The temperature requirements ranges from 8 – 25 °C. However, optimum temperature is between 20 – 25 °C. Lentils requires cooler growing period and little warmer maturity period.

Planting time should be as such that flowering period not coinciding with continuous drizzling and fresh weather that causes pollen indehiscence.

**SOIL, SEEDBED PREPARATION, SOWING, HARVESTING**

Lentils grows best on loam, silty loam and silty clay loam soils with good drainage. Lentils have a low tolerance of saline conditions, and therefore soils with moderate to high levels of salinity should be avoided. Lentils grow well on neutral to moderately alkaline soils, with a pH range of 6.5 to 8.0 but can't tolerate acidic conditions. Seedlings are relatively frost tolerant upto the four leaf stage.

Lentils require a well prepared deep, friable seedbed for better germination, establishment and growth. The soil should be thoroughly tilled to a depth of
25 – 30 cm. Fertility nutrients required by Lentils are nitrogen, phosphorus and occasionally potassium. Lentils planting depth is typically from 2 cm. To plant in rows, space between seeds should be about 2 inches apart in a shallow trench between 1 & 2 inches deep. Watering should be done until seed sprout in 7 – 10 days. Lentils mature and develop seeds in 90 to 120 days.

Recently, it is performing best in the spring or zaid (November – March) season in northern India.

Lentils crop matures in about 90 – 120 days. Plant is harvested when the seed moisture content reduces to 10 %. Physiological maturity can be determined visibly by checking flower heads for sign of maturity.

COMMON PESTS AND DISEASES / WEEDS

They are sometimes infected with fungal diseases such as mildews and rusts. Various insects attack the crop.

Besides, these pests and diseases there are weeds which compete with the Lentils in the fields for moisture, light, nutrients etc. First four weeks after Lentils emergence are the most critical in determining damage from weed competition. The dominant weed species observed in Lentils crop in India include Cynodon, Digitaria, Commelina, Achyranthus, Phyllanthus,
*Convolvulus, Portulaca, Parthenium, Celosia* etc. Infestation with parasitic weed, *Orobanchec* is reported in European countries.

**METHODOLOGY**

The present investigation has been carried out to exploit mutation breeding in wild variety of *Lentil* for creation of some novel genotypes and also to evaluate the cyto-morphological response of *Lentil* against various mutagens. A comparative analysis of all the mutagens was also made to assess the relative efficiency, spectrum of mutagenicity of mutagens.

The Mutational breeding has been selected for present analysis.

**MUTATIONAL BREEDING**

**MUTAGENESIS:**

Mutation can be induced by exposure of organisms to physical mutagens which cause changes in DNA (or RNA in some viruses).

**PHYSICAL MUTAGENS:** These include various kinds of radiations which can be classified into two kinds.

a. **Ionizing radiations:** Particulate radiations: - such as X-rays, fast and thermal neutrons.

   Non-particulate radiations: - such as Gamma-rays, X-rays and cosmic rays.

b. **Non-ionizing radiations:** It includes UV-rays.
Ionizing radiations are of high energy and are useful for mutagenesis because of their high penetrance in living tissues. Gamma rays and X-rays were selected to induce mutations for the present work. These radiations have precise dosimetry showing wide spectrum of mutations even at lower doses. Radiation (X-rays) was the first mutagenic agent known, and its effects on genes were first reported in 1920s by Muller.

**GAMMA RAYS AND X-RAYS**

**Nature:** These are electromagnetic radiations with higher energy, higher penetrance power and lower wavelength ranging between 0.001-10 \( \mu \text{m} \). Gamma rays and X-rays are energetic enough that they produce reactive ions (i.e. charged atoms or molecules) when they react with biological molecules. Gamma rays are produced by radioactive decay of some elements like \(^{60}\text{Co}\), \(^{238}\text{U}\), \(^{131}\text{I}\) while X-rays are emitted when very fast moving electrons strike a high melting point element like Tungsten in X-ray tube.

**Mode of action:** These ionizing rays easily pass through the plasma membrane into the cell where they are absorbed by intracellular molecules such as water. These high energy rays collide with atoms and cause the release of electrons, leaving positively charged free radicals or ions. The ions, in turn collide with other molecules and cause the release of additional electrons. The free radicals react energetically with anything nearby from enzymes to RNA to DNA. Molecules bearing atoms in their ionic form/excited
states are chemically more reactive than those of stable states. The increased reactivity of atoms of DNA molecules is responsible for the mutagenicity of ionizing radiations.

**Genetic effects:** Ionizing radiations produce a wide range of effects on DNA either through free radical effects or direct action on DNA. It causes breaks in sugar phosphate backbone of one or both strands of DNA. Consequently, leading to the rearrangements through tautomerization, deletions, chromosome loss etc. Mutations are also caused by damage or loss of bases. Sometimes the effect may result in cross linking of DNA to itself or proteins, breaking of H- bonds of bases, blockage of cell division, organelle failure or cell death.

**Measurement of radiation:** Ionizing radiations are quantitated in Roentgen (r) units. It is a measure of the number of ionizations per unit volume under a standard set of conditions. Specifically, one Roentgen unit is a quantity of ionizing radiation that produces $2.083 \times 10^9$ ion pairs in one cubic centimeter of air at $0^\circ$C and a pressure of 760 mm of mercury. Similar dosage could be obtained by increasing exposure time and decreasing intensity or vice versa. Another measurement of radiation is in terms of energy released/absorbed by the surrounding tissues which is expressed as rad. 1 rad (radiation absorbed dose) = 100 ergs/gm.

One method for the measurement of radiation is to count the number of nuclear transformations or explosions which occur in a given unit of
radioactive substance per second. This measure is usually standardized to radium. 1 gm radium undergoes $3.7 \times 10^{10}$ nuclear transformations or disintegrations per second. Activity of 1 gm of radium is also referred as 1 Curie (Ci). A new unit of radiation dose is called Gray (Gy) where, $1 \text{Gy} = 100$ rad of the irradiated object. Measurement of the number of ionizations which radiation causes per unit distance as it traverses the living cell or tissue is called the Linear Energy Transfer of the radiation (LET). It involves lateral damage along the path in contrast to path length or penetration capability. X-rays and Gamma-rays are low LET and are called sparsely ionizing radiations while alpha particles have high LET so known as densely ionizing radiations.

**METHODOLOGY**

Seed packets were made for the exposure to different doses of radiations. For Gamma-rays treatment, seed packets were exposed to Gamma-rays irradiated from the controlled decay of a $^{60}\text{Co}$ source (at the rate of 1.6 KR/min) in Gamma irradiation chamber at NBRI, Lucknow.

**GENERAL EXPERIMENTATION**

1. **CONTROL**: Dried and healthy seeds of Lentil were presoaked in distilled water for 5h. These seeds were then and sown alongwith other treated seeds under similar conditions but without the treatment for comparison with mutagen treated population.

2. **DOSE SELECTION**: Dose selection is a parameter that is higly dependent on a number of experimental variables, such as germination,
survivability etc. Dose ranges should be selected on the basis of LD_{50} which includes concentrations that range from little effective dose level upto 90% cell lethality. LD_{50} (half lethal dose) is the dose which depicted 50% germination at particular dose.

3. RAISING OF M₁ GENERATION: After the treatment, these treated seeds were sown in pots. These pots contained equal amount of soil manured with dried and decomposed cowdung. The sowing was performed in 5 replicates for each type of mutagenic treatment. Alongwith all the treated sets, control seeds were also sown in each replicate corresponding to each mutagenic treatment for the comparative analysis.

4. RAISING OF M₂ GENERATION: Seeds from randomly selected plants of M₁ generation were sown in the next season. Seeds were again sown in 5 replicates in pots to raise M₂ generation.

MORPHOLOGICAL INVESTIGATIONS

Analysis of all the morphological parameters was done and recorded. This was studied both in M₁ & M₂ generations with reference to efficiency of mutagen on growth parameters, fertility parameters and the yield parameters. Data from randomly chosen plants from each treatment dose was undertaken.

(I) Efficiency of mutagens: This was estimated by analyzing the percentage of seeds germination and plant survived from the population of sown seeds. Germination percentage was observed and recorded after 10 days
of sowing, taking into account that up to this time all the potential seeds must have germinated. Survivability was the next parameter which was studied. This was observed after 25 days of sowing. Survived plants were determined in form of survival percentage.

(II) Stem: Height of the plant, stem diameter, no. of nodes and internodes were recorded.

(III) Leaf: Length & Breadth of leaves was observed. Different types of leaves regarding shapes and serrations were observed and recorded.

(IV) Flower: Days to flowering was recorded when 50% of the plants were in flowering stage.

(VI) Fertility parameters: Fertility parameters were studied by counting total number of filled seeds in each flower. Pollen sterility/fertility was estimated by Acetocarmine-glycerine stainability test. For this, anthers from unopened buds were squashed in Acetocarmine-glycerine stain. Deeply stained and filled larger pollen grains were considered as fertile while, undersized and unstained pollen grains were taken as sterile.

(VII) Yield parameters: For this, total number of seeds per pods was counted and recorded. Average weight of 50 seeds was also recorded to assess yield.
(VIII) Scoring of mutants/variants: Different types of mutants/variants regarding plant size, leaf size & shape, seed type and shape, content etc. were observed and recorded from M₁ generation and M₂ generations. Chlorophyll mutants of different types were scored from the M₂ generations.

CYTOLOGICAL INVESTIGATION

Treated plants were cytologically analyzed for various cytological parameters. For the meiotic studies, following preparation was made.

(I) Fixation: For the fixation, commonly used fixatives are Carnoy's fixative (6:3:1, ethanol: chloroform: glacial acetic acid) and Farmer's fixative (1:3 ethanol glacial acetic acid). At the time of flowering, young floral buds of 20 randomly selected plants from each treated set were fixed in Farmer's fixative.

(II) Storage: Fixed material was stored in the fixative for 24h and then preserved in 70% alcohol at 4°C.

(III) Staining and squash preparation: 1% Acetocarmine was used for the staining of chromosomes for the cytological studies. The staining property of Carmine is due to the Carminic acid (C₂₂H₂₈O₁₃) which belongs to anthraquinone group. The Carmine dye is obtained from the dried up bodies of female Coccus cacti (an American bug). This dye imparts a crimson red colour. The 2% acetocarmine stain is prepared by dissolving 2g Carmine
powder in 100 ml of 45% Glacial acetic acid. This mixture was stirred well
and kept in a condenser for gentle heating for 6-8h.

For slide preparation, 1-2 anthers from the preserved materials were taken
on a slide and one drop of 2% acetocarmine was placed over it. This was
covered with a coverslip over it. After 2-3 mins, slide was slightly warmed on
a burner. This slide was then kept in between a pile of filter papers and the
stained material was squashed/tapped by thumb pressure or by using a glass
rod. The slides were waxed around the coverslip to avoid entry of air bubbles.

(IV) Permanent preparation: Slides with good preparations can be made
permanent. This procedure was suggested by Celarier (1956) and Darlington
and La Cour (1963). Temporary waxed slides were firstly dipped in 1:1
tertiary butyl alcohol and glacial acetic acid solution till the coverslip was
detached from the slide. The slide and the coverslip both were then passed
through a series of solution mixture viz. 3:1 Glacial acetic acid (GAA) and
Tertiary Butyl Alcohol (TBA), 1:1 GAA : TBA, 3:1 TBA : GAA and were
finally transferred to absolute TBA. The slide and the coverslip was kept in
each solution for 10 min. Both slide and coverslip were air dried and then
they were mounted separately using DPX, Euparol or Canada balsam. This
preparation gives a better resolution as it bears refractive index very near to
that of glass.

(V) MEIOTIC STUDIES

Cytological parameters like chiasma frequency, chromosomal abnormalities,
total abnormality percentage etc. were examined and results were recorded.
Chiasma frequency: Chiasma frequency was assessed by observing the number of chiasmata depicted by each bivalent. The chiasmata are the point of attachment between chromatids. This can be well demonstrated at diakinesis and metaphase – I stage. In general, two kinds of bivalents were encountered viz. ring & rod. Rod bivalents exhibited one chiasma while ring bivalents show 2 chiasmata points. There may be different configurations of bivalents which form multivalents with greater number of chiasmata or some may exhibit univalent behaviour with no chiasmata. Chiasma frequency was examined in terms of chiasmata/bivalent and chiasmata / PMC.

Chromosomal Abnormalities: Chromosomal abnormalities were assessed by studying 10 plants from each treated set. 5 slides per plant were prepared and 10 fields/slide were cytologically investigated. Chromosomal behaviour in terms of normal and abnormal structures at Diakinesis, Metaphase I & II & Anaphase I & II were scored and tabulated. Best results were photomicrographed.

STATISTICAL ANALYSIS OF THE DATA

The formulae used for the computation of the data are as follows:

I. Range: it represents the difference between the lowest and the highest value of the data.

II. Mean : \( X = \frac{\sum X}{N} \)

Where,
\( \bar{X} = \text{Mean} \)

\[ \sum X = \text{Sum of all the observations} \]

\( N = \text{Total number of observations} \)

III. Variance :

\[ \delta^2 = \frac{1}{N-1} \sum (x - \bar{x})^2 \]

Where,

\[ \delta^2 = \text{Variance} \]

\( \bar{x} = \text{Individual observations} \)

\( N = \text{Number of observations} \)

\( \sum = \text{Summation} \)

IV. Standard Deviation :

\[ SD = \sqrt{\text{Variance}} \]

V. Standard Error of Mean :

\[ SE = \delta / \sqrt{N} \]

Where,

\( SE = \text{Standard Error} \)

\( \delta = \text{Standard deviation} \)

\( N = \text{Sample size} \)

VI. Co-efficient of Variation (CV) :

\[ CV = SD / \text{Mean} \times 100 \]

VII. Skeleton of Analysis of variance (ANOVA):

Data recorded from M₂ and M₃ generations were subjected to analysis of variance.
<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Df</th>
<th>Sum of Squares</th>
<th>Mean Sum of squares</th>
<th>F. Cal</th>
<th>F tab (5%)</th>
<th>F tab (1%)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>r-1.0</td>
<td>SSr</td>
<td>$SSr/(r-1)=MSr$</td>
<td></td>
<td>MSt/MSr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>t-1.0</td>
<td>SST</td>
<td>$SSt/(t-1)=MSt$</td>
<td></td>
<td>MSt/MSe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>(r-1)</td>
<td>SSe</td>
<td>$SSe/(r-1)/(t-1)=MSe$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>rt-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Where,

Df = Degree of freedom

SS = Sum of squares

MSS = Mean Sum of squares

SSr = Replication Sum of squares

SSt = Treatment sum of squares

SSe = Error sum of squares

r = Number of replications

t = Number of treatments

F (Cal) = Calculated value of 'F'

F (tab 5%) = Table value of 'F' at 5% level of significance.

F (tab 1%) = Table value of 'F' at 1% level of significance.

> Significance was tested using F Table (Fisher & Yate's, 1963).

VIII. Standard Error due to Deviation: Values were calculated at 5% and 1%

of significance level where ratio of variance (F) test was found significant.
S.E_D for Treatment = \sqrt{2} \text{ EMSS}

Where,

\text{EMSS} = \text{Error mean sum of squares},
\text{r} = \text{No. of replication},

\textbf{IX. Critical Difference:}

\text{C. D for treatment (at 5\% level)} = S.E_D \times t.05 \text{ degree of freedom}
\text{C. D for treatment (at 1\% level)} = S.E_D \times t.01 \text{ degree of freedom}

Where,

\text{t} = \text{Tabulated value at 5 \% level of significance at the same d.f. of EMSS}.