CHAPTER II

Materials and Methods

Root initiation and root elongation
Nucleus/Cytoplasm ratio, mitotic index,
induced mitotic and meiotic chromosomal anomalies
Concentration range
Experimental layout (A to C)
Exposure to specific treatments
Recovery periods
Statistical test
Expected research output.
scales of *Allium sativum* L. and flower buds of *Allium cepa* L. were taken as experimental materials. For determination of various cytological and mitotic parameters roots of *A. sativum* were used. Sharma and Sharma (1965) described the advantages of using *Allium* sps. as experimental materials. These are rather inexpensive and give sufficient root crop in tap water within a short period (43-72 hours). *A. sativum* is a plant with comparatively low heterochromatin content and shows more resistance to cytological changes than the other species, *Allium cepa*. *A. sativum* scales are small, handy and make experimental replication quite convenient. The plant, therefore, represents an innately stable system and can be used in the studies evaluating the induced cytological and chromosomal changes. *A. cepa* flower buds were used for meiotic studies.

Allium tests as described by Sharma and Sharma (1965) were followed. *A. sativum* scales were subjected to a pregermination period of 72 hours in tap water in order to obtain fresh crop of roots, and were then exposed to various fungicidal treatments. Fixations and orcein staining were done according to the methods described by Sharma and Sharma (1965). During root initiation and root elongation tests, *A. sativum* scales were exposed directly
to various fungicidal suspensions without pregermination.

**Root Initiation And Root Elongation**

Root initiation and root elongation in *Allium sativum* scales were scored at different concentrations of fungicidal compounds. The scales were directly brought in contact with various concentrations of fungicides and direct counting of the emerging roots and measurements of their length in mm were recorded for five consecutive days at interval of 24 hours. For each concentration 8 scales were studied and all the emerged roots were counted and measured, and data so obtained were analysed for standard deviation, error and significant test.

**Nucleus/Cytoplasm (N/C) Ratio, Mitotic Index (MI), Induced Mitotic And Meiotic Chromosomal Anomalies**

Root tip squashing and PMC smears were the basic cytological techniques adopted in this work. Root tips from pregerminated scales (72 hrs) of *A. sativum* L. were treated with different concentrations of test fungicides and were fixed in acetic alcohol (1:3 v/v) for 24 hours and were then preserved in 70% alcohol for further studies.

For mitotic and chromosomal studies, root tips were stained in 9:1 mixture of aceto-orcein and 1 N HCl and then mounted in 45% acetic acid according to Tijo and Levan (1950) and Sharma and Sharma (1968). Extreme caution was taken to avoid the
overheating (not more than 10 sec.) of Orcein - HCl mixture. This was further checked and confirmed by the absence of chromosome abnormalities in the control treatments.

PMCS smears were prepared in 1% aceto-orcein without heating.

N/C Ratio :

For this, the areas of nuclei and cytoplasm in Camera lucida sketches, were measured with the help of a mm square grid, prepared on a transparent paper and was used to determine the nuclear (N) and cytoplasmic (C) areas. From these values the nucleus - cytoplasm area ratios were calculated by dividing the area of the nucleus by the area of the cytoplasm (N/C).

Mitotic Index :

Mitotic indices were computed by working out the total number of cells and total number of the dividing cells. The formula followed for determination of mitotic index is given below:

\[ MI = \frac{\text{Total number of cells in division}}{\text{Total number of cells observed}} \times 100 \]

Induced Mitotic And Meiotic Chromosomal Anomalies :

Different concentrations of each of the fungicides were
tested for the induced chromosomal anomalies. Total number of cells showing different types of chromosomal anomalies were noted down and per cent anomalies were calculated by the following formula:

\[
\text{Per cent anomalies} = \frac{\text{Total number of cells showing chromosomal anomalies}}{\text{Total number of dividing cells}} \times 100
\]

**Concentration Range:**

TMTD is toxic if consumed orally. It has also been reported to be irritating to nose, throat and skin (Nene, 1971). It is very effective as seed protectant and in general, the rate of application for dry seed dressing is about 0.25% as aqueous suspension. It is also used as soil treatment at the rate inbetween 15-25 kg per hectare.

Chloranil is toxic to mammals if consumed orally. It is mainly used as seed protectant at the rate of 4-8 oz/lbs of dry seeds. In a few cases it has also been used in soil treatments (Crandall, 1950).

PCNB is a soil fungitoxicant. It is used variously from 30 to 50 lbs. per acre for different diseases. It may cause skin irritation if contacts are frequent.

Aretan-6 is variously used for seed-borne smuts, seed and seedling diseases. The usual rate of its application may vary
from 0.2 to 6% as aqueous suspension (Nene, 1971).

Vitavax is found to be highly toxic to Basidiomycetes (Edgington and Arnon, 1967). Its usual application dosages for different plant diseases or seed treatments may vary from 0.25 to 1.0%. For better soil treatment 30-35 kg/ha of vitavax has been recommended (Nene, 1971).

In a vast country like India, possessing a wide variety of climate and soil conditions and differing agricultural practices, the distribution pattern of plant protection-chemicals varies considerably. States like Tamil Nadu, Andhra Pradesh and Gujrat use pesticides upto about 2 kg/ha, whereas Madhya Pradesh hardly about 100 gm/ha (Rangaswami, 1981). Also, of the total tonnes of pesticides, about 8 to 10 per cent is used for plantation crops like rubber, coffee, tea, cardamom, citrus, apple etc. grown in higher elevations. This works out to an average of about 4 to 5 kg/ha. Each state, in the country has conducted investigations on the utilization of various plant-protection-chemicals on a regional basis for a very long period of time and the state Agricultural Departments are therefore, good information centres. For the present investigation, various concentrations of test fungitoxicants were finally selected on the basis of personal talks with agricultural officers, various reports on recommended dosages, and pilot experiments by using eleven concentrations (0.001 to 30 mg/ml) of each fungitoxicant.
The used concentrations are as follows:

For TMTD, Chloranil, PCNB and Vitavax:

Root initiation and root elongation studies:
0.1, 1.0, 5.0, 10.0, 15.0 and 20.0 mg/ml.

N/C ratio, MI and Mitotic chromosomal effects -
0.1, 1.0, 5.0 and 10.0 mg/ml.

Meiotic studies -
1.0 and 10.0 mg/ml.

For Aretan-6:

Root initiation and root elongation studies -
0.1, 1.0, 5.0, 10.0 and 20.0 mg/ml.

N/C ratio -
0.1, 1.0, 5.0 and 10.0 mg/ml.

MI -
0.001, 0.005 and 0.01 mg/ml

Mitotic chromosomal effects -
0.001, 0.005, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/ml.

Meiotic studies -
1.0 and 10.0 mg/ml.

Thus, on an overall basis, these treatments ranged between
0.001 to 20 mg/ml (0.0001 to 2.0%) as compared to 0.2 to 6.0%
or more agriculturally used concentrations of these chemicals.

Experimental design, specific layouts and the choice of statistical test:

Experimental layout: (A)

Parameters: Root initiation (number) and root elongation.
Treatment: X, Experimental material: *Allium sativum* L.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>B_1</th>
<th>B_2</th>
<th>B_3</th>
<th>B_4</th>
<th>B_5</th>
<th>B_6</th>
<th>B_7</th>
<th>B_8</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>duration</td>
<td>S_1</td>
<td>S_2</td>
<td>S_3</td>
<td>S_4</td>
<td>S_5</td>
<td>S_6</td>
<td>S_7</td>
<td>S_8</td>
<td>C_1</td>
</tr>
</tbody>
</table>

120 h 120 h

Total roots in every scale were scored after 120 h.

B_1 - B_8 = Bulb number (All the eight bulbs were approximately of the same size).

S_1 - S_8 = Scale number.

C = Control in distilled water.
Experimental layout: (B)

Parameters: N/C ratio, MI and chromosomal abnormalities (Mitotic).

Treatment: X. Experimental material: Allium sativum L.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>B₁</th>
<th>B₂</th>
<th>B₃</th>
<th>B₄</th>
<th>B₅</th>
<th>Total No. of readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>duration (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>12 + No recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
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<tr>
<td>+ 24 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
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<tr>
<td>+ 48 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 72 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
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<tr>
<td>24 + No recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
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<tr>
<td>+ 24 h recovery</td>
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<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
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<td>50</td>
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<tr>
<td>+ 48 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 72 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>48 + No recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 24 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 48 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 72 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>72 + No recovery</td>
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<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 24 h recovery</td>
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<td>10 Re</td>
<td>10 Re</td>
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<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 48 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
<tr>
<td>+ 72 h recovery</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
<td>50</td>
</tr>
</tbody>
</table>

MI values were computed for every root tip separately.

B₁ - B₅ = Bulb number (All the five bulbs were approximately of the same size).

S₁ - S₅ = Scale number; RT = Root tips as examined. Re = Number of readings taken. C = For each set of experiments separate control samples were used.
Experiment layout: (C)

Parameter: Meiotic abnormalities, Treatment X.

Experimental material: *Allium cepa* L. (flower buds).

<table>
<thead>
<tr>
<th>Treatment duration (hours)</th>
<th>1 $F_1$</th>
<th>$F_2$</th>
<th>No. of total readings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{B1}$</td>
<td>$F_{B2}$</td>
<td>$F_{B3}$</td>
</tr>
<tr>
<td>24</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
</tr>
<tr>
<td>48</td>
<td>10 Re</td>
<td>10 Re</td>
<td>10 Re</td>
</tr>
</tbody>
</table>

**IF** = Inflorescence.

$F_{B1}$ - $F_{B10}$ = Flower bud number

Re = Readings taken.
Exposure to Specific Treatments:

In all the experimental layouts (A) and (B), the treatment time has been started from 12 hours duration to 72 hours duration.

Recovery Periods:

Cells were scored at three recovery periods - (i) 24 hours (ii) 48 hours and (iii) 72 hours. Since in *A. sativum* one mitotic cycle is completed in 16-17 hours the extension of recovery periods upto 72 hours provides with the opportunity of testing the stability of fungicide effects upto 4-5 mitotic cycles.

Expected Research Output:

a. Whether the treatment accelerates or inhibits the number of roots?

b. Whether the treatment accelerates or inhibits the elongation of roots?

c. Whether the treatment alters Nucleus/Cytoplasm ratio?

d. Whether the treatment alters the size of the nucleus or the size of the cytoplasm?

e. Whether the treatment interferes with cell cycle and cell division synchrony as indicated by altered mitotic index?
f. Whether the treatment induces mitotic chromosomal anomalies and in what proportion?

g. What type of mitotic chromosomal anomalies are induced?

h. Whether the treatment induces meiotic abnormalities and in what proportion?

i. What type of meiotic abnormalities are induced?

Data analysis and computation were done with the help of DCM MOSCAL 1400 electronic calculator. t - significance tests were applied. The results are expressed as population mean ± standard error, 't' values and percentage values. The treatment effects were scored at the room temperature.