:: Chapter I ::

MATERIALS AND METHODS
The experimental techniques, procedures adopted in the present work and a technique making use of a microdensitometer for RNA, DNA, Protein and Ascorbic Acid comparisons, are briefly described.

Seeds of *Pisum sativum* and bulbs of *Allium sativum* were taken as experimental materials. These were chosen because they are (a) inexpensive, (b) easy to handle, and (c) yield sufficiently long roots in tap water within a short period of 2-3 days. Besides, in *Pisum* a single root tip represents one whole plant and it is, therefore, possible to study the cell population variability in comparatively larger number of individual plants with obvious statistical advantages.

Seeds of *Pisum sativum* were surface sterilized with 0.2% mercuric chloride solution for few minutes. After a thorough wash, sterilized seeds were transferred to sterilized, moist petridishes lined up with filter papers. As mentioned earlier the cell populations were studied in two types of root meristem systems — (i) Normal Cell System (NCS) and (ii) Aging Cell System (ACS). A normal cell system (NCS), in these studies, is represented by meristematic cells of intact and growing roots, while the aging cell system (ACS) is constituted by meristem cells of excised and aging roots.
I. Cytological Techniques:

The basic technique adopted in this work is that of root-tip squashing. However, microtome sections were also made to make morphological comparisons with squash preparations. (Plates 42, 43 & 44).

Squash:

Root-tip squashes were made in 2% aceto-orcein according to the schedule given by Sharma and Sharma (1965). From every single squashed root-tip, five readings were taken as counts, and 5 Camera Lucida drawings were made. Out of these five camera lucida drawings, two were in the form of detailed sketches and three were in the form of what may be called as Position Diagrams (Fig. 1) in which the respective positions of various cells together with other relevant particulars were noted. For each parameter, 10 root tips, in *Allium* and *Pisum* respectively, were scored in this manner. However, for Karyotype studies a 9:1 mixture of 2% aceto-orcein and IN HCl was used (Tijo and Levan, 1950; Sharma and Sharma, 1957), and for aging root tips instead of 9:1, a 9:3 mixture was used as it gave much better spreading.

Microtomy:

NCS root tips were fixed in 1:3 acetic alcohol after a pre-germination period of 72 hours. Microtome sectioning and crystal violet staining were done according to the schedules given by Johansen (1940). Longitudinal sections were cut at 10-12 μ and
transverse sections at 8-10 μ.

II. Cytological Parameters:

1. Mitotic Indices (MI):

Mitotic Indices were computed by working out the total number of cells and total number of dividing cells from camera lucida drawings of squash spreads as well as from direct counts. The formula (Mishra, 1973) used is given below:

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

2. Mitotic Stages:

Percentages of prophase, metaphase, anaphase and telophase cells in different cell populations were calculated on the basis of the formula given below:

\[ \text{Percentage of mitotic stage} = \frac{\text{Total number of cells showing a specific stage}}{\text{Total number of dividing cells}} \times 100 \]

3. Cell Population Percentage:

I, R, S, C and T population percentages were calculated with the help of the following formula:
Percentage of specific cell population = \( \frac{\text{Total number of population cells}}{\text{Total number of cells}} \times 100 \)

4. Nucleolar Indices (NI):

From camera lucida sketches, each cell population was scored in terms of mono-, bi-, tri- and tetranucleolate condition and nucleolar indices were computed on the basis of following formula:

\[ \text{NI} = \frac{\text{Total number of nucleoli}}{\text{Total number of interphase cells}} \times 100 \]

5. Cell Area:

Cell area measurements were made from camera lucida sketches with the help of a planimeter. The Vernier position on the tracer bar was adjusted to 33.39, thus making 100 cm\(^2\) of the scale equivalent to 100 cm\(^2\).

6. Nuclear-Cytoplasmic Ratio (N/C):

In this case too, the areas of cytoplasm and the nucleus were measured with the help of a planimeter. N/C ratio was calculated by dividing the area of the nucleus by the area of cytoplasm.

7. Karyotype Study:

This was done for NCS only. After a pre-germination period of 72 hrs root tips of Pisum and Allium were treated with 0.25%
colchicine for 3½ to 4 hrs. Squashes of colchicine pretreated root tips were prepared according to the schedule of Tijo and Levan (1950). Camera lucida sketches were drawn under a magnification of 100 x 15 in oil and photomicrographs were taken.

8. X-irradiation:

Bulbs of Allium and seedlings of Pisum were X-irradiated in polythene bags after a pre-germination period of 72 hrs (Plate 8). Care was taken to produce minimal moist environment inside the polythene bags as higher levels of moisture interfere with irradiation effects. Pilot experiments were performed to fix the exposure time in order to get a good number of chromosomal abnormalities. In Pisum, the decided exposure time was 40 min whereas in Allium, 60 min. Two sets were X-irradiated, out of which, one was fixed immediately after the exposure without any recovery, while the other was given a recovery period in the sterilized and modified White's nutrient medium (Rangaswami, 1961). Data were analysed for percentage of cells showing abnormalities, percentage of mitotic stages, percentage of cell populations and mitotic indices. Following formula was used for percentage abnormalities:

\[
\text{Percentage of cells with abnormalities} = \frac{\text{Number of cells with abnormalities}}{\text{Total number of cells}} \times 100
\]
III. Physiological and Biochemical Parameters:

Physiological (Indole Acetic Acid (IAA) Treatments):

These treatments were given in dark at 28°C after 72 hrs pre-germination of root tips. Pilot experiments were performed to decide the concentration and duration of treatments. On the basis of these experiments following concentrations and treatment schedules were finalised.

Exposure:

(a) IAA 100 ppm
(b) IAA 1 ppm
(c) IAA $10^{-4}$ ppm
(d) IAA $10^{-7}$ ppm

For 24 hrs

In ACS of *Pisum*, root tips were treated after 3, 6 and 9 days respectively and in ACS of *Allium* after 3 days only, as roots in this case do not survive long. From the recorded observations of IAA treated root tips, the independent response of each cell population was studied in terms of percentage cell populations, mitotic indices, percentages of mitotic stages, nucleolar indices, N/C ratios and cell areas.

IV. Biochemical (RNA, DNA, Basic Protein *Histones*, and Ascorbic Acid Comparisons):

Inspite of specific histochemical staining of RNA, DNA,
Proteins and Ascorbic Acid, the follow-up techniques were found unsatisfactory, at least, in the present study. The commonly followed method of subjective comparisons of stain intensities in terms of some arbitrarily fixed stain intensity classes was rejected an account of its subjectivity as well as apparent crudeness. Histochemical specific staining of RNA, DNA, basic proteins and ascorbic acid was done in order to explore population homogeneity or heterogeneity in terms of these parameters and, therefore, it demanded many readings at the level of a single cell in its cytoplasm as well as the nucleus. This was not possible by visual stain intensity comparisons or even by normally available gadgets for cytophotometry.

The use of microdensitometer (Plate 9.) proved to be a reliable and elegant technique for scanning the stained cell and it was possible to take as many as 25 readings in a single cell. The technique so developed and adopted is described below.

After a pre-germination period of 3 days histochemical localizations of DNA, RNA, Basic Protein (Histones) and Ascorbic Acid were done in root tip squash preparations. Photomicrographs of different cell populations were then taken at a constant exposure of 20 seconds, keeping magnification also constant, at 250 volts kept constant with the help of a transformer. Microdensitometer readings were taken from the negatives so obtained. Sensitivity of the microdensitometer was adjusted to its maximum. Negatives were fixed in between two glass plates.
Densitometer aperture was adjusted and was kept constant. 25 readings per cell were taken, covering almost the total cell area. Microdensitometer gives a higher scale reading for a lighter spot and a lower scale reading for a darker spot. In a negative, darkly stained portions appear light, whereas non-stained and lightly stained portions appear darker. In this way there is a positive correlation between the contents of DNA, Basic Proteins and Ascorbic Acid and microdensitometer readings. Five readings were taken as blank in those areas where only the mountant was present. Average of these blank readings was then deducted from cell readings.

Histochemical Stain Techniques:

Deoxyribose Nucleic Acid:

Feulgen stain was prepared according to the modified Lacour's method of Feulgen preparation. Feulgen squash method was the one followed for staining DNA (Darlington and La Cour, 1960).

Ribose Nucleic Acid:

The technique followed in the present investigation, is a slight deviation from Bhaduri and Mukarjee's (1961) modified schedule for plant tissues. Staining was done with pyronin y, omitting the methyl green. Root tips were hydrolysed in IN HCl for 10-12 min, and stained with pyronin y for 20 min. After
washing the stained tips in distilled water, root tip squashes were prepared in the mountant glycerine.

**Basic Protein (Histones):**

Alkalize fast green test of Albert and Geschwind (1953) was slightly modified for root tip squashing.

1. Root-tips were fixed in 10% neutral formalin the pH of which was adjusted to 7 by IN potassium hydroxide.

2. Fixed root-tips were hydrolysed in IN HCl for 30 min.

3. Root-tips were washed in distilled water and transferred to 15% trichloroacetic acid which was kept in boiling water bath for 15 min. After giving three changes of 70% ethanol root-tips were stained for 30 min in 0.1% aqueous solution of fast green FGF at pH 8.0 to 8.1 adjusted by IN sodium hydroxide solution.

4. After washing for 5 min in distilled water regular root-tip squashes were made.

**Ascorbic Acid:**

The technique of Dave et al. (1968) for histochemical localization of Ascorbic Acid in plant tissues was slightly modified for squashing. After fixing, staining and washing according to the original technique, the root-tips were washed in distilled water, hydrolysed in IN HCl for 15 min to be followed
by another washing in distilled water and lastly mounted in glycerine.
Fig. 1: Position diagram sketched with the help of camera lucida.

Numbers recorded beside mitotic stages indicate the number of nucleoli.

Abbreviations:

R = rectangular;  S = square;
C = Circular  I = irregular
T = triangular  P = prophase
M = metaphase;  a = anaphase
T = telophase;  i = interphase