MATERIALS AND METHODS

Materials for the present histochemical studies were collected from plants of *Cyperus rotundus* L., growing in the Botanical Garden of University School of Sciences. Care had been taken to collect the material from one and the same spot so as to eliminate as far as possible the differences in growth due to differences in the environmental conditions.

Shoot apices from vegetative to reproductive stages, young spikes, florets and other reproductive organs were fixed in respective fixatives for ascorbic acid, DNA, RNA, basic proteins and protein bound -SH groups. The techniques used for the study of above mentioned biochemicals are as given under.

Histochemical localization of Ascorbic acid.

Taking into account the difficulties and possible errors involved in the previous histochemical techniques for the localization of ascorbic acid a new technique has been evolved in this laboratory. Difficulties in the localization of ascorbic acid have been highlighted by Chayen (1953), Jensen and Kavaljian (1956) and Pearse (1961). The previous methods (Glick, 1949; Barnett and Bourne, 1941; Bourne, 1936; Giroud and Leblond 1936, 1937)
are highly laborious as they involve treatment of sectioned tissues with \( \text{H}_2\text{S} \) to reduce dehydro-ascorbic acid before subjected to silver nitrate staining. This method may allow errors involving movement of ascorbic acid from the seat of reaction. However, Chayen (1953) has already confirmed the specificity of \( \text{Ag NO}_3 \) reaction. The problem of AA localization arises not from the final reaction but from the solubility of AA in aqueous solution prior to localization (Jensen and Kavaljian, 1956).

Keeping all the above points in mind a new technique has been evolved in our laboratories as reported by Dave et al., (1968) and applied by Madhavan Unni and Shah (1968), and Chinoy, N.J. (1969, 1969a). The new technique eliminated the above mentioned errors as the tissues are stained prior to sectioning.

The silver nitrate reagent is prepared as follows:–

5g. of analar silver nitrate crystals are dissolved in 34 ml. of glass distilled water. To this 66 ml. of absolute alcohol is added. The solution is acidified by adding 5 ml of glacial acetic acid and the solution is stored in dark bottle in refrigerator under 3-5°C.

Fresh materials were directly put in the above solution and kept in dark under 0-3°C for a week. After that the material was washed in 70% alcoholic ammonia (95 ml. of 70% ethanol + 5 ml. liquor ammonia) three
times in 15 minutes period, dehydrated in T.B.A. Series, para-
ffin embedded and microtomed at 10μ. After deparaffinization
sections were mounted in canadabalsam. Control sections were
made by keeping the material in 5% copper sulphate solution
overnight before treating with silver nitrate solution. Copper
sulphate oxidises ascorbic acid into dehydroascorbic acid which
will not reduce silver nitrate.

**Histochemical localization of DNA.**

Feulgen method was used for localizing DNA. The tissues
were fixed in P.A.A. (70% ethyl alcohol 90 ml. + 5 ml. formalin +
5 ml. acetic acid) section were cut at 10μ, deparaffinised and
brought to water. The sections were then hydrolyzed in a mixture
of 3 N HCl and ethanol in the proportion of 1:2 for about 15
minutes at 60°C. Sections were washed in tap water and stained
in standard Schiff's reagent for one hour. Standard Schiff's
reagent was made in the following way (de Tomasi, 1936) as given
by Pearse (1961). Dissolved 1 gm. of basic fuchsin in 200 ml.
of boiling distilled water. Shook for 5 minutes and cooled to
exactly 50°C. After filtering 20 ml. of N HCl was added to the
filtrate. Cooled to 20°C and 1 gm. of potassium metabisulphite
was added to it. The solution was kept in dark for 14 to 24 hr.
Added 2 gms. of activated charcoal, filtered and the colourless
solution was stored in the dark at 0-4°C. Sections were drained,
and rinsed three times with 10% potassium metabisulphite,
washed in water, dehydrated and mounted in canadabalsam.
Histochemical localization of DNA & RNA together.

Localisation of DNA & RNA together following the methyl green-pyronin method (Taft, 1951) as described by Jensen (1962) was done. The tissues were fixed in Carnoy's fluid (60 ml. ethanol + 30 ml. chloroform + 10 ml. acetic acid) dehydrated through TBA series, paraffin infiltrated and sectioned at 10 μ. The sections were deparaffinised, hydrated and placed in stain for 5 minutes. The methyl green-pyronin stain was prepared by dissolving 0.5g methyl green in 100 ml. of 0.1 M acetate buffer at pH 4.4. Extracted this solution with chloroform several times (as many as 8 times) to remove the residual methyl violet. Dissolved 0.2 g of pyronin Y in the solution of methyl green. After staining the sections were rinsed briefly and blotted. Before the tissue was completely dry the sections were immersed in differentiating solution of tertiary butyl alcohol + absolute ethanol (3:1) for 2 to 3 minutes. Cleared in xylene and mounted in canadabalsam. DNA registered greenish to blue colour while RNA registered red colour.

Histochemical localization of RNA alone was done by following the same method with modifications. Instead of a mixture of methyl green-pyronin solution, a solution of pyronin alone in acetate buffer at pH 4.4 was used.

Controls for DNA and RNA were made by perchloric acid extraction. The sections were brought to water and then placed in
1 N perchloric acid for 24 hrs. for removing RNA. Washed in running water and stained. Cytoplasm and nucleolus took no red colour.

The sections were washed in running water and were placed in 0.5N perchloric acid at 70°C for 40 minutes to remove DNA. Sections were then immersed in 1% sodium carbonate solution for 5 minutes and stained. No staining for DNA was observed.

**Histochemical localization of Basic protein.**

Alkaline fast green test (Alfert and Geshwind, 1953) was followed for the localization of histones. The tissues were fixed in neutral formalin (10% formalin adjusted to pH 7 by 1 N potassium hydroxide), paraffin infiltrated and sections were cut at 10μ. After deparaffinisation the sections were coated with celloidin and hydrated. The sections were placed in 15% trichloroacetic acid in boiling water bath for 15 minutes. After giving three changes of 70% ethyl alcohol sections were stained for 30 minutes in 0.10% aqueous solution of Fast green FCF at pH 8.0-8.1 adjusted by 1 N sodium hydroxide. Washed the sections in distilled water for 5 minutes. Placed the sections in 95% ethyl alcohol, dehydrated and mounted in canadabalsam. The basic proteins, histones were coloured green.
Histochemical localization of Sulfhydryl-disulfide.

Sulfhydryl-disulfide was localised by following Barnett and Seligman's (1952) DDD method. Neutral formalin fixed (4 hrs. of fixation) tissues were washed in running water overnight, dehydrated in TBA series, paraffin infiltrated, sectioned at 10\(\mu\)m and mounted. Removed the paraffin and celloidin coated the sections (0.5% celloidin in 1:1 ethanol ether mixture).

The sulfhydryl group which was converted to disulfide in the paraffin infiltrated tissues was reduced by the following step. The sections were placed in fresh aqueous 0.2 - 0.5 M solution of thioglycollic acid adjusted to pH 8.0 with 0.1 N NaOH for 1 - 2 hrs. at 50°C. Washed in several changes of distilled water. A stock solution of DDD (2,2'-dihydroxy-6,6'-dinephthyl disulfide) by dissolving 100 mg. of DDD in 60 ml. of absolute ethyl alcohol. Immediately before using added 15 ml. of this stock solution to 35 ml. of barbital buffer at pH 8.5. Incubated the sections in this solution for 1 hr. at 56°C and then brought it to room temperature. Rinsed the tissues with distilled water, washed the sections in distilled water adjusted to pH 4 with acetic acid. Dehydrated in alcohols, kept in absoluted ether for 5 minutes and returned to water through alcohol series. Placed the sections in a fresh solution containing 50 mg.
of diazo blue B in 50 ml. of phosphate buffer at pH 7.4 for 2 - 3 minutes at room temperature. Washed in running tap water dehydrated and mounted. The site of sulfhydryl group appeared blue.

The controls were made by treating the sections with 0.1 M N-ethyl maleimide solution in a phosphate buffer at pH 7.4 for 4 hr. at 37°C before incubating in DDD solution.

Measurement of stain intensities.

After histochemical processing the intensity of the stain reaction was measured by a simple cytophotometer devised in this laboratory as shown/Plate 1. The instrument is set up in a dark room. A powerful microscope lamp (A) produces a beam of light which passes through the condenser (B) and illuminates the preparation on the slide (D) kept on the stage (C). The light passes through the objective (E) and the prism (F) and comes out through the eye piece (G) which protrudes out of the dark chamber and is reflected by the mirror (H) to the platform (I) with a small aperture below which a photoelectric cell (J) (Photronic cell, Weston Model S.123) is fitted. The photocell is connected to a lamp and scale galvanometer. The reading is taken by bringing the image of the cell or the nucleus concerned in alignment with the aperture on the platform, and noting the deflection on the galvanometer scale (M). The difference
Plate 1. Figure of cytophotoelectrometer
between the transmission value of the stained cell and that of the corresponding control gives the extinction value \((e)\). At least ten such readings were taken for every tissue.

Using the above method readings for the intensity of stain reaction for ascorbic acid, DNA, RNA, basic proteins and \(-\text{SH}\) were found for the following tissues:

I. **Shoot apex.**
   1. Vegetative apex at tuber level
   2. Inflorescence primordium
   3. Inflorescence at spike initiation
   4. Young spike inside the leaf sheath
   5. Spike just emerged.

II. **Floret.**
   1. The primordium of floret
   2. The primordium of reproductive organs and the bract (glume) just differentiated
   3. Primordium of reproductive organs and the bract when the latter just overlaps the former
   4. Primordium of reproductive organs and subtending bract just before stamen and carpel are differentiated
5. Primordium of differentiated stamen and carpel and the subtending bract.

III. Anther.

1. Stamen primordium
2. Anther at sporogenous cells
3. Microspore mother cell and corresponding tapetum and the rest of the anther wall.
4. Meiocytes at first and second meiotic divisions and the corresponding tapetum and the rest of the anther wall.
5. Uninucleate microspores and corresponding tapetum and the rest of the anther wall

IV. Carpel.

1. Carpel primordium
2. Ovule primordium and ovary wall primordium
3. Megaspore mother cell corresponding nucellus, integument and ovary wall
4. Megaspore cell corresponding nucellus, integument and ovary wall
5. Egg and polar nuclei of unfertilized embryo sac and corresponding nucellus, integument and ovary wall
6. Zygote and primary endosperm nucleus of fertilized embryo sac and corresponding nucellus, integument and ovary wall.
V. Seed.

1. Globular embryo and embryo axis and cotyledon of developed embryo

2. Free nuclear endosperm, cellular uninucleate endosperm and cellular multinucleate endosperm.

Micromeasurements.

The cell width and length, the diameter of nuclei and nucleoli of the cells of the tissues studied were measured by using a Filar micrometer. The approximate sectional area of cell was calculated out. Approximate nuclear volume and nucleolar volume were calculated from the respective diameters and the ratios nucleolar/nuclear size were calculated. The approximate ratios of nuclear size and cell size also were calculated from nuclear area/cell area. The sectional area of cells, the nuclear volume nucleolar volume, the values for nuclear/cell size and nucleolar/nuclear size of the tissues studied are given in Tables 1-11. The area of cells with oval shape like pollen grains was obtained from the mean diameter. The nuclear/cell size in the case of multinucleate cells eg., 3 nucleate pollen and multinucleate endosperm was obtained by taking the total nuclear area in the cell. The area of those cells in which the cell walls are not visibly demarkated under the light microscope was arbitrarily determined by taking into consideration the dense cytoplasm.
surrounding the respective nucleus. Examples are egg, polar nuclei, zygote, primary endosperm nucleus, free nuclear endosperm etc. When the above values are expressed for parts composed of different kinds of cells the mean values are taken into consideration eg., anther wall composed of epidermis and hypodermis and ovary wall composed of epidermis, middle layers and endodermis.

The e. value for the AA, DNA, RNA, basic proteins and -SH group were multiplied by the corresponding cell areas to determine the total content of the metabolite in question per cell. The e. values were divided by the area of the cell to determine the content per unit area of the cell. The e. values, per cell content and content per unit area of the cell of the above mentioned metabolites are plotted as curves for the differentiation of (1) shoot apex (Plates 6 and 7) (2) Floret (Plates 6 and 7) (3) Anther development (Plates 12 and 13) (4) Ovule development (Plates 20 and 21) (5) Embryo, endosperm and ovary wall (Plates 25 and 26) and are presented. In order to further elucidate the relationship between the metabolites studied linear regressions of DNA on AA, RNA on AA, basic protein on AA, -SH on AA, RNA on DNA, basic protein on DNA, basic protein on RNA, DNA on -SH, RNA on -SH and basic protein on -SH were worked out for the data of cell content per unit area for all the tissues at different stages of
inflorescence differentiation, floret differentiation, anther development and ovule development. The trend lines were determined from the regression equation obtained in each case (Plate 27 - 34).