CHAPTER II

METHODS
Microtomy

Buds were excised from corms together with a portion of surrounding tissue by means of a sharp scalpel, and were fixed in alcohol - acetic acid (3:1) for 48 hours. After fixation, the buds were washed with distilled water and transferred to 70% alcohol. Infiltration and embedding in paraffin wax was carried out by passing through usual alcohol, alcohol-xylene, and then xylene series. The dehydrated material was infiltrated with paraffin wax and finally embedded in paraffin wax (E. Merck, 56°C to 58°C). Longitudinal sections, 10 μ thick were cut with a Spencer AO microtome.
After dewaxing, the sections were double stained with safranin and light green and finally mounted in Canada balsam.

**Carbohydrate and nitrogen fractions**

For the estimation of sugars, starch, alcohol insoluble nitrogen and alcohol soluble nitrogen, fresh plant material was fixed in boiling 90% ethanol and macerated in a glass pestle and mortar. The alcohol soluble and insoluble fractions were separated by filtration through Whatman No. 1 filter paper in Büchner funnel under suction. The alcohol soluble portion and the subsequent washings from the residue were pooled and made to volume from which aliquots were then drawn for the determination of sugars and alcohol soluble nitrogen. Alcohol insoluble nitrogen, and starch were estimated in a suitable sample of alcohol insoluble material after drying it in an oven at 70°C for 48 hours.

For the determination of alcohol soluble solids an aliquot of ethanolic extract representing 1 gm fresh weight was partially dried in a current of air in pre-weighed petri dishes. The syrupy material was then vacuum dried over P₂O₅ overnight and the weight of alcohol soluble residue obtained by weighing. The dry residue was redissolved in a small volume of 10% isopropanol and the
solution utilized for chromatographic separation of free sugars.

**Sugars**: Aliquots for sugar estimation were made alcohol free over a boiling water bath and then made to volume. Reducing sugars were estimated by the method of Nelson (1944). Total sugars were estimated after hydrolysis of non-reducing sugars overnight by 0.01% invertase (BDH). Values for non-reducing sugars were obtained as the difference between total and reducing sugars.

A suitable amount of alcohol soluble fraction in 10% isopropanol was spotted on Whatman No. 1 chromatographic paper. A mixture of known sugars was spotted alongside a set of alcoholic extracts. Paper chromatograms were irrigated with butanol: ethanol: water (4:1:2.2). The solvent was allowed to overrun by fixing a pad of filter paper to the front edge according to Harborne (1973). After drying, the chromatograms were sprayed with either aniline-hydrgen phthalate or ammonical silver nitrate and heated at 105°C for 15 minutes to develop the spots, and the spot area determined by a planimeter. Samples were analysed in triplicate on separate chromatograms.

**Starch**: Estimation of starch was carried out by a modification of the method described in Official

A suitable quantity of alcohol insoluble fraction was taken in hard glass test tube and starch gelatinized in glass distilled water over a boiling water bath. After cooling to 50°C, 1 ml of 0.01% solution of freshly prepared Takka-diastase obtained from Parke Davis was added and temperature maintained at 50°C for one hour. Solution was again heated to boiling, recooled to 50°C and another 1 ml of 0.01% Takka-diastase solution added and maintained for one hour at 50°C. Solution was again boiled, cooled and 2 ml concentrated HCl added. The solution was refluxed with air cooled condensers over a boiling water bath for three hours. Refluxed solution was cooled, first nearly neutralized with 10% NaOH and neutralization completed with Na₂CO₃ solution using phenolphthalein as the indicator. Solution was filtered and made to volume. Reducing sugars were determined in a suitable aliquot of the digest by the method of Nelson (1944). Values for starch were obtained by multiplying the value of dextrose with a factor of 0.9.

**Total non-structural carbohydrates**: Values for total non-structural carbohydrates were computed by adding the values obtained for starch and total sugars.
Nitrogen: Nitrogen was estimated by the semi-micro-kjeldhal method. For the estimation of alcohol soluble nitrogen, alcohol was removed from the aliquots by heating over a heating mantle. The sample was then digested by the method of Chibnall et al (1943). 2 ml of N₂-free H₂SO₄ and 0.05 gm of copper-selenium catalyst were added and the digestion carried out in digestion flasks over a digestion rack, till digests were colourless. For alcohol insoluble nitrogen, dried alcohol insoluble material was digested as described above. Digests were made to volume and estimated for ammonia, which was distilled in a Markham's apparatus into a boric acid buffer and estimated by titration against 0.014 N H₂SO₄ containing phenolred-Bromocresol green indicator according to Conway and O'Malley (1942).

Extraction and bioassay of endogenous growth regulators

Auxins:

Extraction and bioassays of endogenous auxins was carried out by the method of Hemberg (1958) with some modifications.

Extraction: 200 gm of corms from which fibrous sheaths had been removed were surface washed with distilled water and homogenized in a Braun homogenizer.
was extracted in cold 90% ethanol. Ethanolic extract was filtered through Whatman No. 1 filter paper and the filtrate partitioned 4 to 5 times with peroxide free diethyl ether. Peroxide free ether was prepared by the method described by Vogel (1968). Ether extract was dried in vacuo and the extract redissolved in 2 ml of ether.

**Chromatography:** 0.5 ml aliquot representing 50 gm fresh weight was subjected to chromatography employing both paper and thin layer chromatography.

1) **Paper chromatography:** The ether extract was streaked across strips of Whatman No. 1 chromatographic paper and the chromatograms were run in isopropanol : ammonia : water (100 : 14 : 6) as used by Hemberg (1953c). The solvent was allowed to run upto front distance of 40 cm and then dried. Chromatograms were divided into 10 equal parts from the line of start to the solvent front. Paper chromatogram strips were directly placed in 4 cm diameter petri dishes, 5 ml glass distilled water added and left overnight for elutions. A 4 cm strip below the starting line was used to serve as control.

11) **Thin layer chromatography:** A layer of silica gel - G (Merck) of 0.50 mm thickness was uniformly spread over 5 X 20 cm glass plates with a spreader.
After drying, the plates were activated at 105°C for 15 minutes. Ether extract was streaked across the plates and plates run up to a 15 cm front in methyl acetate: ammonia : isopropanol (45 : 20 : 35) as recommended by Hemberg and Orvelid (1967). After irrigation the plates were dried and then divided into 10 equal parts. Each part was scrapped and the scrappings quantitatively transferred into centrifuge tubes. Scrappings were thrice eluted with 5 ml portions of peroxide free ether, centrifuged and supernatants pooled in 4 cm diameter petri dishes. Ether was evaporated and residues taken up in 5 ml of glass distilled water.

**Bioassay:** Avena coleoptile straight growth test as recommended by Hemberg (1953b) was employed for assaying auxin activity. Grains of oat (*Avena sativa* Linn. var. Kentucky white) were germinated in dark at 30°C in a polypropylene tray over moist tissue paper. Coleoptiles were collected when about 2 cm long. A 3 mm segment from the tip of the coleoptile was excised and discarded. From the remaining portion a 5 mm long segment was excised with the help of a locally fabricated coleoptile cutter. The excised part of first leaf from within the coleoptile was carefully pulled out. Five coleoptile segments were floated on each test solution, and their lengths measured.
after incubation in the dark at 28° C for 24 hours.

The method was standardized with recrystalized \( \beta \)-indolyl-acetic acid (BDH) in the range of 0 to 10 \( \mu g/ml \) to obtain a standard calibration curve (Fig. 2.1).

Auxin activity due to IAA was located by chromatographing 50 \( \mu g \) IAA on thin layer chromatographic plates and subjecting eluates from different zones of the chromatogram to bioassay.

**Gibberellins:**

**Extraction:** 500 gm of corms from which sheaths had been removed were surface washed with distilled water and then frozen by placing in the ice chest of a refrigerator for 48 hours. Chilled corms were then extracted for gibberellin like substances by the method of Aung and De Hertogh (1968) with modifications.

Chilled corms were macerated in 250 ml of cold methanol in a Braun homogenizer; the homogenate stored in a refrigerator at about 5° C for 48 - 72 hours. Homogenates were then filtered under suction through Whatman No. 1 filter paper. Methanol from filtered extracts was evaporated by placing them in a strong current of air under a fan and then drying in a vacuum desiccator. The residues were taken up in 250 ml of water and the aqueous extract was partitioned 3 - 4 times with petroleum ether. Petroleum ether phase was washed with
50 ml of 0.33 M KH$_2$PO$_4$ (pH 3.00) buffer and then discarded. Aqueous extract was now partitioned 3-4 times with ethyl acetate and then acidified with concentrated HCl to pH 3.00. Acidification was followed by repartitioning of the extract with ethyl acetate 3-4 times. All ethyl acetate fractions were pooled and traces of water were frozen out by chilling. Ethyl acetate fraction was then dried at room temperature, and the residue taken in 1 ml of methanol. This represented free-acidic fraction of the extractable gibberellin-like substances.

Remaining aqueous fraction was made 0.4 N with concentrated HCl and heated to 60° C in the water bath for one hour. After cooling to room temperature it was adjusted to pH 3.00 with 5 N KOH. Partitioning was done with ethyl acetate 4-5 times, and the pooled fractions were vacuum dried at room temperature after water from the fractions was first frozen out. The residue was taken up in 1 ml of methanol. This fraction represented the bound gibberellin-like substances.

**Chromatography:** For separation thin layer chromatography was employed throughout.

Glass plates 20 x 5 cm were coated with 0.7 mm thick silica gel-G layer (BDH), air dried and then
Fig. 2.1 Standard calibration curve for bioassay of auxin activity prepared with Indolyl-3 acetic acid (IAA) employing *Avena* Coleoptile straight growth test.

Fig. 2.2 Standard calibration curve for bioassay of gibberellin activity prepared with gibberellic acid (GA₃) employing rice mesocotyl – second leaf sheath elongation test.
FIG. 2.1

mm
coleoptile length

0.001 0.01 0.1 1.0 5 10
Indolyl acetic acid conc. μg/ml

FIG. 2.2

mm Length mesocotyl - 2nd. leaf sheath

0.001 0.01 0.1 1.0 10 100
Gibberellic acid conc. μg/ml
activated in an oven at 105° C for 15 minutes. 0.2 ml aliquots from the extracts in methanol representing 100 gm fresh tissue were streaked across one end of the plate and then run in an ascending direction in benzene : acetone : acetic acid ( 13 : 6 : 1 ) as employed by Takahashi et al ( 1970 ). Plates were irrigated to a distance of 15 cm and then air dried. Zones 1.5 cm wide were scrapped off, and transferred to 15 ml centrifuge tubes. The scrapped adsorbant was eluted with 5 ml portions of methanol 3 - 4 times, centrifuged and supernatants pooled in 5 cm diameter petri dishes. Methanol was evaporated and fractions taken up in 3 ml of sterile glass distilled water and a thin swab of sterilized cotton placed in each petri dish. Scrapping from a 1.5 cm zone below the starting line served as the control.

Bioassay: Bioassay of gibberellin activity was carried out by the rice seedling test (Murakami, 1959). Seeds of a locally bred semi dwarf variety of rice ( Oryza sativa Linn. var. K - 34 ) were used.

Rice seeds were washed thoroughly with distilled water, and then surface sterilized with 0.01% cetrimide ( Cetavelon ) for 20 - 30 minutes. After being thoroughly washed the seeds were soaked in sterile glass distilled water at 30° C in the dark till the coleoptiles just emerged. Uniformly germinated seedlings were then
transferred to petri dishes containing test solutions. Petri dishes were covered and left for incubation at 30° C in an illuminated cabinet under constant illumination of about 700 lux for 3 days. After 3 days the petridishes were uncovered, three ml of water added and placed into a chamber at 30° C under constant illumination and 95 - 100% relative humidity for six days. After 6 days, length of seedling from mesocotyl to the second leaf sheath was measured to the nearest millimeter.

A standard calibration diagram was prepared in the range of 0 - 100 μg/ml with pure gibberellic acid (GA₃) as obtained from M/S Abbot, U.S.A. (Fig. 2.2).

Two additional bioassays were employed at one stage for confirmation of gibberellin-like activity.

Cucumber hypocotyl elongation: Seeds of cucumber (Cucumis sativus Linn. var. Long green) were soaked for one hour and then washed with glass distilled water. Ten seeds were placed on filter papers soaked in 3 ml test solutions in each petri dish. Hypocotyl length was measured after 72 hours incubation at 30° C.

Lettuce hypocotyl elongation: Lettuce (Lactuca sativa var. Great lakes) seeds were pre-soaked in glass distilled water and ten seeds were placed on filter paper in petri dishes containing 3 ml
of test solution. Petri dishes were incubated at 25°C under constant illumination and hypocotyl lengths of seedlings measured to nearest mm after 96 hours.

**Preparative and analytical thin layer chromatography of active fraction**: Acidic ethyl acetate extract representing 200 gm of fresh weight was chromatographed on activated thin layer chromatographic plates coated with 1 mm thick layer of silica gel-G in benzene : acetone : acetic acid (13 : 6 : 1). After air drying, the zone between Rf 0.4 - 0.7 which contained the entire gibberellin like activity was eluted several times with methanol. The eluates were pooled and reduced to a small volume. The methanolic solution was then rechromatographed and a mixture containing GA3 and GA4+7 spotted alongside the extract to serve as markers on activated TLC plates coated with 0.3 mm layer of silica gel-G. Separate plates were run in the following solvent mixtures:

1) Benzene : acetone : acetic acid (13 : 6 : 1)

2) Chloroform : ethyl acetate : acetic acid (60 : 40 : 5)

3) Chloroform : butanol : acetic acid (70 : 25 : 5)

4) Chloroform : ethyl acetate : acetic acid (50 : 40 : 10)

5) Benzene : butanol : acetic acid (80 : 15 : 5)

6) Chloroform : methanol : acetic acid : water (45 : 15 : 3 : 2)
**Location of spots**: A plate each irrigated in different solvent systems was treated as follows:

a) 70% aqueous sulphuric acid was sprayed on the plate and then the plate heated for 5 minutes at 120° C.

b) 5% ethanolic sulphuric acid was sprayed on the plate and the plate heated for 20 minutes at 120° C.

Fluorescent spots were located under an ultraviolet lamp giving emission in the region of 280 - 350 nm. The boundaries of spots were marked with a sharp needle. Colour of the fluorescence emitted by each spot noted and \( R_f \) value of each spot was calculated.

**Inhibitors**

**Extraction**: In preliminary studies aimed at exploring the presence of inhibitor activity, crude extracts prepared from corm tissues were used. Corms collected from Pampore fields during May - June were freed of fibrous sheaths and washed in distilled water. Corms were then crushed in a pestle and mortar and the resulting slurry squeezed through muslin. The milky extract was centrifuged to sediment starch grains and other suspended material. The clear extract and several of its dilutions were tested for inhibitor activity.

For preparing ethanolic extracts 100 gm of
sliced tissue was ground in 100 ml cold 90\% ethanol in a glass pestle and mortar to obtain a thin slurry, which was filtered through Whatman No. 1 filter paper under suction in a Buchner funnel. The ethanolic extract was dried in vacuo and the residue redissolved in glass distilled water and several of its dilutions were assayed for inhibitor activity (crude ethanolic extract).

Partitioning of the inhibitor activity from ethanolic extracts into different solvents was attempted by phase separation and extraction in a separatory funnel. Aqueous solutions of ethanolic extracts were acidified to pH 3.5 with HCl and separately partitioned into petroleum ether, diethyl ether and ethyl acetate. Non aqueous phases were separated and dried in vacuo. Vacuum dried residues were redissolved in glass distilled water and assayed for inhibitor activity.

In subsequent experiments aimed at studying seasonal changes in inhibitor activity vacuum dried ethanolic extracts were dissolved in water and were first partitioned into petroleum ether. Petroleum ether phases were discarded. Aqueous phase was then brought to pH 3.5 with AR HCl and repartitioned twice into ethyl acetate. Ethyl acetate fractions were pooled and dried in vacuo.

**Chromatography:** In preliminary experiments chromatography of inhibitors was carried out using
Ethanolic extracts. Ethanolic extracts after vacuum drying were taken up in 10% isopropanol and streaked across a strip of Whatman No. 3 mm chromatographic paper and dried. Chromatograms were given an initial run in glass distilled water in descending order and dried. Some of these chromatograms were cut into 10 equal strips and assayed for inhibitor activity. Such assays showed that when irrigated with water the inhibitor activity was retained at the starting line. Subsequently, the chromatograms were given a second run in the same direction in isopropanol : ammonia : water (3 : 1 : 1) up to a 40 cm front, as used by Bennet-Clark and Kefford (1953). Chromatograms were air dried and cut into 10 equal strips of 4 cm each. These strips were lined into 10 cm diameter petri dishes and eluted with 10% isopropanol for 24 hours. Isopropanol was evaporated from these petri dishes and the eluate assayed for inhibitor activity.

In subsequent experiments acidic ethyl acetate fractions were streaked across Whatman chromatographic paper No. 3 mm. These chromatograms were run to a front line distance of 40 cm with isopropanol : ammonia : water (100 : 14 : 6) as solvent according to Hemberg (1953a,b). After drying, the chromatograms were cut lengthwise into ten equal strips, 4 cm each, and these strips were eluted with 10% isopropanol for 24 hours. The eluates were
evaporated to dryness and tested for inhibitor activity.

For locating inhibitor activity due to abscisic acid a 30 μg sample of abscisic acid (Fluka) was chromatographed on separate strips in the same solvent and segments from the chromatogram were tested for inhibitor activity.

**Bioassay of inhibitor activity:** Inhibitor activity was tested employing cucumber seed germination test.

Cucumber seeds (Cucumis sativus L. var. long green) were used for bioassay. Cucumber seeds were first washed and then kept in water for about 30 minutes. Seeds which did not sink were discarded and seeds of uniform size from the ones which sank were put on filter paper discs in petri dishes containing test solutions. Petri dishes were incubated at 30°C in dark and periodical observations taken for percentage germination, radicle and hypocotyl length at 24, 48 and 72 hour intervals, together with water and solvent controls.

**Exploratory work on phenolic compounds:**
Separation and tentative identification of phenolic compounds was attempted by employing paper and thin layer chromatography.

1) **Paper chromatography**: Ethyl acetate
extracts were loaded on Whatman No. 1 filter paper and
cromatographed in ascending direction up to a front line
distance of 30 cm in following solvents:

a) Butanol : acetic acid 27% (1 : 1).

b) Benzene : methanol : acetic acid
(45 : 3 : 4)

c) Distilled water

ii) Thin layer chromatography: 5 X 20 cm
glass plates were coated with 0.5 mm thick layer of silica
gel - G (Merck), dried and activated for 15 minutes at
105°C. After cooling ethyl acetate extracts were spotted
on the plates, were run in ascending direction with benzene:
methanol : acetic acid (45 : 3 : 4) up to 15 cm front and
then dried.

Both, paper chromatogram and thin layer plates
were critically observed under visible and ultra violet
light. Chromatograms were also developed by spraying
acidic FeCl₃ (FeCl₃ in 0.5 N HCl), tetrazotised
benzidine and diazotized sulphanilic acid and then heated
at 105°C for 15 minutes. Colours of various spots
appearing on chromatograms and their Rf values were noted
and then compared with those given by Geissman (1955).

All work pertaining to endogenous growth
regulators was carried out with either AR grade solvents
and reagents or where AR grade solvents were not available
the solvents were purified by redistillation.

**Oxygen uptake**

Oxygen uptake of tissues was determined by conventional manometric method in Warburg's respirometer at a temperature of 30° C (Umbreit et al, 1959).

From freshly collected corms, 8 mm diameter tissue cylinders were bored out by means of a cork borer. These cylinders were then sliced into 1 mm thick discs. About 10 such discs were accurately weighed and transferred to the outer compartment of a Warburg flask containing 3 ml of glass distilled water, 0.1 ml of 10% KOH was cautiously put into the central well into which a filter paper wick was introduced. Flask was tightly sealed to the manometer with lanolin and the oxygen uptake followed for four hours. Time course of oxygen uptake during the four hour period was linear.

**Dry matter**

Dry matter of plants or plant parts was determined by drying fresh plant material in a forced draught oven at 70° C for 48 hours.

Computation of growth analysis parameters was carried out by the method of Evans (1972) using following formulae:
Leaf area ratio (LAR) = \( \frac{\text{Total leaf area per plant}}{\text{Total dry weight per plant}} \)

Net assimilation rate (NAR) = \( \frac{\text{Rate of increase in Dry weight per plant}}{\text{Total leaf area per plant}} \)

Relative growth rate (RGR) = LAR X NAR

Total leaf area: Ten 1 cm long leaf segments were taken and their breadth determined. They were dried in a forced draught oven at 70\(^\circ\) C and their dry weights determined. On the basis of this the total leaf area was computed from the total leaf dry weight of the plant by the following relationship:

\[ B = \frac{A}{x} \times y \]

where,

- \( B \) = Total leaf area of the plant
- \( A \) = Leaf area of the known sample of leaf segments
- \( x \) = Dry weight of the known sample of leaf segments
- \( y \) = Total leaf dry weight of the plant.

Statistical evaluation

As far as possible the data have been evaluated for variability. Each sample comprised 3 - 7 replicates. In tables the mean values are accompanied by standard deviations which have been calculated according to the formula of Arditti and Dunn (1969).
\[ \delta = \sqrt{\frac{\sum d^2}{n - 1}} \]

In experiments on endogenous growth regulators the data have been subjected to analysis of variance and the least significant difference (L.S.D.) calculated at \( P = 0.05 \).