MATERIALS AND METHODS

The experimentation in this investigation related to the physiological, biochemical and anatomical studies on the initiation and development of roots on hypocotyl cuttings of *Impatiens balsamina*.

PLANT MATERIAL

*Impatiens balsamina* L. (family: Balsaminaceae) was selected because of the ability of hypocotyls to root easily (Arnold and Alstron, 1961; Bala, 1965) and also on account of the uniformity of material because of delayed development of epicotylar leaves.

SOWING

Healthy and uniform seeds presoaked in water for 24 hr were placed equidistant on thin moist cotton pads in enamel trays and were allowed to germinate at 28±2°C either in continuous light (3200 lux) or in continuous dark till the
seedlings were about 3.5 cm in length and the cotyledons had fully unfolded.

**PREPARATION OF STOCK SOLUTIONS**

The stock solution of IAA (100 mg/l) was prepared by dissolving 10 mg in a few drops of ethanol and making the volume to 100 ml by adding distilled water.

Ten mg each of the water soluble antimetabolites, namely cycloheximide (cyc), 5-fluorouracil (FU), actinomycin-D (act-D) and 5-fluorodeoxyuridine (FudR) was dissolved in water and made to 100 ml. The stock solutions were stored in a refrigerator and diluted suitably with distilled water to prepare solutions of desired concentration whenever required. All the test solutions dealing with the use of Glucose contained chloramphenicol (30 uM) to prevent microbial growth and were changed on alternate days.

**PLANTING OF CUTTINGS**

Healthy seedlings of uniform size and thickness were selected. The root system was excised and the cotyledons and the apex were removed or left intact according to the design of the experiment. The 3.0 cm long hypocotyl cuttings, thus obtained, were planted in test solutions in holes on polythene sheets stretched over Petri-dishes (10 cm dia.) and held in position by rubber bands. The experiments were run at 28±2°C (except when mentioned otherwise) either in continuous light (3200 lux) from fluorescent tubes or in continuous dark.
Morphological observations on the number of rooted cuttings and roots were maintained at periodic intervals. Visual observations on callous formation, incipient primordia that became visible on fixing the material in formalin acetic acid (FAA) and the length of roots were also recorded.

ANATOMICAL STUDIES

Anatomical studies were made according to Johnson (1950) with minor modifications. The hypocotylar parts of the cuttings were fixed and kept in FAA (45 ml rectified + 45 ml distilled water + 5 ml formaldehyde + 5 ml glacial acetic acid) for one day. These were then transferred to 70% alcohol till sectioned.

Dehydration: The samples were dehydrated by passing them through tertiary butyl alcohol (TBA) grades. The concentration of TBA along with the other components used and the duration for which the cuttings were kept in each grade are given below:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Water</th>
<th>Rectified</th>
<th>TBA</th>
<th>Absolute Alcohol</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>40</td>
<td>10</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>50</td>
<td>20</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>50</td>
<td>35</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>45</td>
<td>55</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>
Penetration: A few drops of molten paraffin wax (m.p. 60-62°C) were added to the tube containing the sample in 100% TBA. After 15 min, half the mixture of the tube was poured off and molten wax was added. This procedure was repeated 6-7 times each at an interval of 30 min, till the smell of TBA disappeared.

Blocks were made in overnight ripe wax. The sections were cut at 18-20 μ. The ribbons were mounted on slides with uniformly spread adhesive and stretched, using 3% formalin water. After drying them overnight, dewaxing and staining was done.

Dewaxing and staining: The slides were kept in xylol for 4 hr till the wax was completely removed. These were then passed through xylol and alcohol grades in the following order:-

- Pure xylol
- 75% xylol (in absolute alcohol)
- 50% xylol
- 25% xylol
- Absolute alcohol
- 90% alcohol
- 75% alcohol
- 50% alcohol
- Saffranine in 50% alcohol (16 hr)
- Water
- 25% alcohol
- 50% alcohol
- 75% alcohol
- 95% alcohol
- Absolute alcohol
- 50% clove oil (in absolute alcohol)
- Pure clove oil
- Fast green
clove oil
- Xylol and finally mounted in Canada balsam.

Photomicrographs of the important stages were taken.

BIOCHEMICAL ANALYSIS

DRY WEIGHT

The cotyledons and hypocotyls were separated and weighed on an electric balance. These were over dried at 80°C for 12 hr and weighed again and the percentage dry weight (wt) calculated.
AUXIN CONTENT

Auxin content was determined by the method of Brunner and Antoni (1971):

Extraction and estimation: 2.5 gm of the basal part of the cuttings was weighed in duplicate. The sample A was extracted with 5 ml of 35% PCA and sample B with 5 ml of Gordon Weber (GW) reagent in the dark for one hr. The solutions were centrifuged at 15000 g for 15 min and poured into tubes for colorimetric readings. Solution A was zeroed with 35% PCA while solution B was zeroed with undiluted GW reagent.

Calculation: The relative auxin content was determined by subtracting the extinction of adjusted A from the extinction of adjusted B measured at 530 nm. The free auxin content was calculated from a calibration curve using indole acetic acid (IAA) as standard and expressed as ug/gm fresh wt.

CHLOROPHYLL CONTENT

The chlorophyll content was measured by the method of Moore and Lovell (1970): One gm of fresh cotyledons/hypocotyls were treated with 80% acetone till white and the optical density of the green supernatent measured at 660 nm after adjusting the volume. The chlorophyll content is expressed as O.D. units per gm fresh wt.

CARBOHYDRATES

Carbohydrates were estimated by the methods of Morris (1948) and Loewus . (1952) by anthrone reagent and concentrated sulphuric acid as follows:
Extraction: The plant sample was taken and weighed. The cotyledons were then separated from the hypocotylar part and each weighed separately. The fresh cotyledons and the hypocotyle were separately boiled in 10 ml distilled water for 30 min over a water bath, cooled and the volume of the supernatant equalled to 10 ml by adding more distilled water to replace the loss by evaporation. This was the water soluble fraction.

The residue was boiled with 10 ml of 2.5 N HCl for 15 min over a water bath, cooled and the volume of the supernatent then equalled to 10 ml by adding more acid. This was the acid soluble fraction.

Estimation: Two ml anthrone reagent (0.2% anthrone in ethyl acetate) and 7 ml of concentrated sulphuric acid (AR) were added to 1 ml of the extract in a test tube. This was suitably diluted with sulphuric acid and the optical density of the blue-green solution measured with the help of a Bausch and Lomb spectronic 20 photocolorimeter at 610 nm after 15 min.

Calculations: The contents of the water and acid soluble carbohydrates were expressed as mg/gm fresh wt using glucose as standard.

Each sample of the plant material taken for the estimation of DNA, RNA, proteins and amino acids was repeatedly washed with acetone till free of pigments.

DNA and RNA

The RNA and DNA were extracted according to the method of Bonner and Zeevart (1962).
RNA

Extraction: Two ml of 3N perchloric acid (PCA) previously cooled at 4°C, was added to 1 gm pigment-free fresh material and kept for 30 min. The material was centrifuged at 2000 g for 10 min and the supernatant collected. The sediment was once more extracted with PCA and the pooled supernatant used for the estimation of RNA, while the residue stored for the extraction of DNA. The RNA content was estimated by the method of Mezbaum (1939):

Estimation: Two ml orcinol (1% orcinol and 0.5% FeCl₃ in conc. HCl) was added to 1 ml of the supernatant and the tubes boiled for 10 min on a water bath. The optical density of the green solution was noted in a photo colorimeter at 610 nm. The RNA content was calculated using ribose as standard and expressed as ug/gm fresh wt.

DNA

Extraction: To the residue of RNA, was added 2 ml of 3N PCA and kept in a water bath at 60°C for 15 min and in this way extracted twice. The material was centrifuged at 2000 g for 10 min and the supernatant collected. The DNA content was estimated by the method of Burton (1956):

Estimation: Two ml of DNA reagent (1 gm diphenylamine + 1.5 ml conc. sulphuric acid (AR) + 0.5 ml of CH₃CHO diluted 50 times and the total made to 100 ml with glacial CH₃COOH) was added to 2 ml of the supernatant and the tubes boiled for 30 min on a water bath. The optical density of the
bluish-green solution was noted at 610 nm and the DNA content calculated using deoxyribose as standard and expressed as ug/gm fresh wt.

**PROTEINS**

The protein content was estimated by the method of Lowry et al. (1951):

**Reagents used**

(A) 2.0% sodium carbonate in 0.1N sodium hydroxide.
(B) 0.5% copper sulphate in 1.0% sodium citrate.
(C) 1.0 ml of reagent B + 50 ml of reagent A.
(D) Folin-ciocalteu reagent.

The Folin-ciocalteu reagent was prepared as follows:

- Hundred gm of sodium tungstate (AR) and 25 gm of sodium molybdate (AR) were dissolved in 700 ml of distilled water.
- To this were added 50 ml of orthophosphoric acid (sp. gr. 1.75) and 100 ml of conc. HCl. The reaction mixture was refluxed in a water bath for 10 hr using an air-condenser. 150 gm of Lithium sulphate, 50 ml of distilled water and a few drops of bromine water were added and the solution boiled for another 15 min, cooled and the volume made up to one litre in a volumetric flask. The reagent was stored in a coloured bottle at 15°C and diluted in a ratio 1:2 with distilled water before use.

**Estimation:** To 1 gm pigment-free material was added 5 ml of reagent C and after 10 min 0.2 ml of reagent D. After 30 min the volume was made with distilled water and the optical density of the blue solution measured at 660 nm. The protein
content was calculated using bovine albumin as standard and expressed as ug/gm fresh wt.

**AMINO ACIDS**

Amino acids were estimated by ninhydrin reaction according to the method of Moore and Stein (1948).

**Extraction:** One gm pigment-free material was treated with 80% alcohol for 1 hr. A known volume of the supernatant was evaporated to dryness.

**Estimation:** While the dried residue was still hot, 2 ml of ninhydrin reagent (0.1% in 70% ethyl alcohol) was added and the solution heated on a water bath for 10 min with constant shaking. After cooling, the solution was diluted to 5 ml by isopropyl alcohol and the optical density noted at 520 nm. The free amino acid content was calculated from the standard curve prepared by using glutamic acid and expressed as ug/gm fresh wt.

**ASSAY OF ENZYMES AND ISOCENZYMES**

**Extraction of crude enzyme:** The cotyledons and hypocotyls were separated and washed with distilled water. These were homogenised in the buffer in pre-chilled glass pestle-mortar at 4°C with a small amount of sterilized sand.

For the assay of peroxidase and IAA-oxidase, the samples were homogenised in 0.67M phosphate buffer (pH 7.0). These were centrifuged at 3000 g to remove cell debris and the supernatant re-centrifuged at 15,000 g for 3 min. The supernatant obtained was used as the crude extract for enzyme assay.
For the assay of amylases the samples were homogenised in 0.05M Tris-HCl buffer (pH 7.2) containing 0.5M sucrose, 6mM cysteine hydrochloride and 6mM ascorbic acid (Staples and Stahman, 1964) and the supernatant collected after centrifugation at 15000 g for 3 min.

**ENZYME ACTIVITY**

An aliquot of crude enzyme equivalent to 1 mg protein was taken in all the enzyme assays, the protein being determined by the method of Lowry et al. (1951).

**Peroxidase:** The extracts containing an equal amount of protein were incubated with 1.0 ml of a (1:1) mixture of 1% H$_2$O$_2$ and 1.5% Benzidine in 25% glacial acetic acid. The rise in optical density of the blue solution was recorded with a photocolorimeter at 620 nm and the peroxidase activity expressed as O.D. units per mg of enzyme protein.

**IAA-oxidase:** The extract was incubated with 1.0 ml of 0.01% IAA at 37°C in the dark for 30 min. The IAA left unoxidised was estimated according to modified Gordon-Meher method.

The Salkowski's reagent comprised of 50 ml of 35% conc. H$_2$SO$_4$ added to 1.0 ml of 0.5M FeCl$_3$ solution. 2.0 ml of the above reagent was added to the test solution and incubated at 37°C in dark for 30 min. The volume was made up to 10 ml with distilled water and the optical density of the pink solution measured at 530 nm.

**Calculations:** IAA-oxidase activity was calculated and expressed as ug of IAA oxidised per hr per mg enzyme protein.
Amylase: The enzyme extract was incubated with 1.0 ml of 0.5% soluble starch in 0.04M phosphate buffer pH 7.0 and 2.0 ml of phosphate buffer. After one hr the reaction was stopped by adding 1.0 ml of 1N HCl. The unhydrolysed starch was estimated by adding 0.2 ml of 0.6% iodine in 6% KI. The volume was made up to 10 ml with distilled water and the optical density of the blue colour measured with a photocolorimeter at 620 nm. The enzyme activity was expressed as mg starch hydrolysed per hr per gm fresh wt and was calculated from a calibration curve using starch as standard.

DETECTION OF ISOENZYMES

The isoenzymes were separated on 10% polyacrylamide gels (unless stated otherwise) by the methods of Ornstein (1964) and Davis (1964).

Preparation of gels

The following stock solutions were prepared in distilled water and stored in a refrigerator at 15°C till used:

**Solution A.**

- **IN HCl**: 48 ml
- **Tris**: 36.6 gm
- **N,N-dimethylamino propionitrile**: 0.23 ml
- **Water to make up**: 100 gm
- **pH**: 8.9

**Solution B.**

- **Acrylamide**: 28.0 ml
- **Bis-acrylamide**: 0.735 gm
- **Water to make up**: 100 ml
Solution C
Riboflavin 4.0 mg
Water to make up 100 ml

Solution D
Ammonium Persulphate 0.14 gm
Water to make up 100 ml

The above solutions were allowed to warm up to room temperature before use and mixed as follows:

| Solution A | 3 parts |
| Solution B | 9 parts |
| Solution C | 6 parts |
| Solution D | 6 parts |
| Water      | 3 parts |

The mixture was poured into glass tubes (0.5 cm x 9.0 cm) fitted with rubber stoppers in a perspex stand. The top portions of the tubes (2.0 cm) were layered gently with distilled water to eliminate any meniscus and to obtain a flat smooth gel surface. The tubes were placed before a fluorescent tube for 15 min to allow photopolymerisation. When the gels were set, these were removed from the stoppers and were stored in distilled water at 15°C till used.

**GEL ELECTROPHORESIS**

Electrophoresis was carried out on 10% polyacrylamide gels at 4°C by the methods of Ornstein (1964) and Davis (1964). 9.0 cm long tubes were used, having 7.0 cm gel and 2.0 cm space for loading the extract. Lithium hydroxide-borate buffer 0.01M (pH 8.9) was used in the compartments.
Crude extract containing a known amount of protein was loaded on the gel at the top and was overlayered by 60% urea solution or estimated sucrose solution. The buffer was then carefully pipetted into the chambers so as not to disturb the extract. The lower chamber was connected to +ve terminal and upper chamber to -ve terminal of a constant DC supply power unit.

The voltage was maintained at 250 V with a current strength not exceeding 5mA per tube. After completion of the electrophoresis the gels were taken out of the glass tubes with the help of a fine needle and stained for different enzymes. These were then washed thoroughly with distilled water and band positions recorded against a fluorescent tube.

**DETERMINATION OF RF-VALUE**

Gels were always run up to a known distance. The last band which itself is coloured dark yellow occupied the same position as the bromophenol blue dye (marker). Hence Rf values of the bands were determined by calculating from the last band taking it as Rf 1.0

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Rf = \frac{\text{Distance of a band from the top}}{\text{Distance of the last band}}
\]

**STAINING OF ISENVZO-BANDS ON GELS**

**Peroxidase:** Peroxidases were visualized by the method of Mitra et al. (1970). The incubation mixture comprised of 1.5% benzidine in 25% glacial CH₃COOH mixed with an equal volume of 1% H₂O₂. The gels were incubated at 30°C for one min, washed and the positions of bands noted immediately.
IAA-oxidase: The gels were stained by the method of Endo (1968). The staining mixture consisted of 0.8 mg potassium indole acetate, 0.06 mg trichlorophenol sodium salt and 2.0 mg fast blue BB salt per ml of 0.04M phosphate buffer at pH 6.0. The brown bands became distinct indicating zones of IAA-oxidase activity.

ELUTION OF ISOENZYME BANDS

After the completion of electrophoresis, the gels were taken out under ice-cold water and sliced horizontally with a sharp blade into 2 mm discs. These were placed separately in serially numbered test tubes, crushed in 1 ml cold distilled water with a glass rod and the elutes, thus, obtained were used for determining peroxidase and IAA-oxidase activities.