MATERIALS AND METHODS

The experimentation in this investigation mostly involved the culturing of etiolated stem segments or epiphyllous buds in liquid as well as in agar media of known ingredients under controlled light and temperature conditions.

Plant materials

Plant material used in this investigation was Populus nigra and Salix tetrasperma belonging to the family Salicaceae and Bryophyllum tubiflorum belonging to the family Crassulaceae.

Etiolated branches of Populus nigra and Salix
tetrasperma were obtained by planting stem cuttings taken from the trees growing in the University Campus, in earthen-ware pots in river sand. The pots were adequately watered and were kept in the dark. The axillary buds developed into branches that were pale and etiolated and had poorly developed thin leaves. These were relatively poor in food materials and other metabolites (see Fig. 1). Stem segments of requisite size were cut from these etiolated branches for experimentation. The experiments involved the culturing of 2.5 cm long etiolated stem segments from which the leaves and the apex had been excised, in test solutions except where otherwise mentioned in experiments that were conducted to study the effect of size of the segments or the role of the leaves and the apex when these were left intact. These details are described under experimentation separately in each experiment.

Epiphyllous buds of Bryophyllum tubiflorum, each with a basal undifferentiated disc and two pairs of opposite decussate leaves separated by 0.5 mm long internode, were taken from the plants grown under long day conditions (Fig. 2). These buds arise in the marginal notches of the leaves when the plants are grown under long day conditions. Five epiphyllous buds
were floated on 10 ml of each test solution in each Petri-dish (5 cm dia.) as described separately in each experiment.

Preparation of stock solutions

The stock solutions of auxins, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were prepared by dissolving 10.0 mg of each in a few drops of ethanol and making the volume to 100 ml by adding distilled water. Ten mg of each of the water soluble chemicals, namely, gibberelic acid (GA₃), cycloheximide (cyc.), actinomycin-D (Act. D), 5-fluorodeoxyuridine (5 FUDR), 5- fluorodeoxyuracil (5 FU) was dissolved in water and made to 100 ml. The stock solutions were stored in the refrigerator and were diluted suitably with distilled water to prepare solutions of desired concentrations, whenever required. All the test solutions in liquid culture dealing with the use of sugars contained chloramphenicol (30 UM) to prevent microbial growth and were changed at 2-day intervals for 20 days.

Light and temperature treatments

The cultures were exposed to varying light and temperature conditions as described separately under experimentation and results. For continuous light (CL), the cultures were exposed to artificial light with an
intensity of about 3200 lux obtained from fluorescent tubes with incandescent bulbs in between fitted on light banks. For continuous darkness (CD) the cultures were kept in dark cabinets in the same air-conditioned room. The temperature under both the light and dark conditions was maintained at 28 ± 2°C except where mentioned otherwise.

Scope and method of experimentation

In all 23 experiments were carried out. Most of the experiments involved the culturing of etiolated stem segments in liquid media. Stem segments in these experiments were planted vertically in holes on polythene sheets stretched over Petri-dishes (10 cm dia.) containing test solutions as described in each experiment (see Fig. 3). In experiment 7 the segments were cultured in agar-agar medium with the procedure as follows:

Sterilization of plant material

Stem segments were washed in running tap water and were sterilized by immersing them in 0.1% solution of mercuric chloride for 5 minutes. These were taken out and washed with 5 changes of sterile distilled water given inside a glass inoculation chamber that was previously sterilized by ultraviolet radiation.
The segments were left in sterile distilled water till they were inoculated in agar medium containing different test solutions in culture tubes.

Preparation and composition of medium

The basic medium consisted of 0.9% agar prepared by heating it in glass distilled water. The medium was supplemented with auxins and nutrition as described under experimentation. Fifteen ml of the medium was poured in each culture tube that was then plugged with a cotton plug and sterilized in an autoclave at 15 lbs pressure for 15 minutes. After autoclaving, the culture tubes were taken out and were kept vertically on wooden racks to form a gel in an air-conditioned room.

Inoculation of plant material

Sterilized material was inoculated with the help of a forceps over a flame to the medium in an inoculation chamber which was previously sterilized by ultraviolet radiation. The culture tubes were plugged immediately after inoculation to avoid contamination, were then taken out from the inoculation chamber and kept vertically in wooden racks in an air-conditioned room with controlled light and temperature conditions.

In experiment 9 estimates were made of starch
contents in relation to hydrolysing enzymes. The etiolated sterilized segments were tied loosely into bundles with cotton thread and were placed vertically in culture tubes containing different test solutions. The details of culture solutions are given under experimentation. As in experiment 7 all operations in this experiment were also carried out aseptically. For hydrolytic activity 0.2 ml 0.6% iodine in 6% aqueous potassium iodide solution was added to the extract and optical density measured with the help of a Spectronic 20 Bausch and Lomb photocolorimeter using a yellow filter. The amount of starch was determined from the standard curve previously prepared from the known concentration of starch sol. The percentage hydrolysis was calculated by subtracting the residual value of the starch from the initial value.

The determinations of the contents of carbohydrates, amino acids, total protein and nucleic acids in segments cultured in different test solutions in experiments 21 to 23 were made as follows. The samples for these determinations included only part of the segments on which roots were produced. The samples were dried in the oven at 90°C for 48 hrs and were powdered. The requisite amount of the fine dry powder was used in
each case and determinations were made on three replicates collected at random at periodic intervals.

**Sugars**

**Water and acid soluble**

Sugars were estimated by the following method used by Morris (1948), Villies and Silverman (1949) and Loewus (1952).

**Extraction:** Requisite amount of fine dry powder of the plant material was boiled in 10 ml distilled water in test tube in waterbath for 20 minutes, cooled, filtered through glass-wool and the volume made to 10 ml by adding distilled water. This was water soluble fraction.

The residue was boiled with 10 ml of 6 M HCl the solution centrifuged at 2000 rpm for 10 minutes. The supernatant obtained was acid soluble fraction.

**Estimation:** 2 ml anthrone reagent (0.2% anthrone in ethyl acetate) and 5 ml of conc. H$_2$SO$_4$ were added to 1 ml of the extract in a test tube and its optical density measured with the help of a photo-colorimeter at 610 nm after 10 minutes.

**Calculations:** The contents of water or acid
soluble sugars as the case may be, were expressed in mg/gm dry wt in terms of glucose equivalent values using glucose as the standard.

**Total sugar**

The content of total sugars (mg/gm dry wt) was calculated by adding up values of water and acid soluble sugars in each case.

**Free amino acids**

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

**Extraction:** 5 mg powdered material was boiled in 70% ethyl alcohol for 10 minutes in tubes (10 x 1 cm) on a water bath using air condensors. The material was centrifuged at 2000 rpm for 10 minutes and the supernatant collected and 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 was again boiled for 10 minutes with constant shaking. After cooling, the solution was diluted to 5 ml by iso-propyl alcohol and the optical density was noted with the help of a photo-colorimeter at 520 nm.
**Calculations:** The free amino acid content (mg/gm dry wt) was calculated from the standard curve prepared by using glutamic acid.

**RNA**

The RNA content was estimated by the method described by Mezbaum (1939).

**Extraction:** The RNA and DNA was extracted according to the method of Bonner and Zeevart (1962). The powdered sample was suspended in acetone till free from pigments. The residue was dehydrated by 1:1 mixture of acetone: ether and was air dried at room temperature for 24 hrs. 2 ml of 3 N perchloric acid (PCA) previously cooled at 4°C, was added to 5 mg of dry material in test tubes, which were then kept at 4°C for 30 minutes. The material was centrifuged at 2000 rpm for 10 minutes and the supernatant collected. The sediment was extracted once more with PCA in the similar way. Supernatant were pooled and used for the estimation of RNA and the residue was used for the extraction of DNA.

**Estimation:** 2 ml of orcinol (1% orcinol and 0.5% FeCl₃ in conc. HCl) was added to 1 ml of the supernatant and the tubes boiled for 10 minutes on a water bath. The optical density of the green solution was
noted with the help of a photo-colorimeter at 610 \textmu m.

**Calculations:** The RNA content (mg/gm dry wt) was calculated from the standard curve made by using ribose.

**DNA**

The DNA content was estimated by the method described by Burton (1956).

**Extraction:** To the residue of RNA, was added 2 ml of 3 N perchloric acid (PCA) and kept in a water bath at 60°C for 15 minutes and in this way was extracted twice. The material was centrifuged at 2000 rpm for 10 minutes and the supernatant collected and cooled.

**Estimation:** 2 ml of DNA reagent (1 gm diphenylamine + 1.5 ml of conc. H$_2$SO$_4$ (AR grade) + 0.5 ml of acetaldehyde diluted 50 times and the total volume made to 100 ml with glacial acetic acid) was added to 2 ml of the supernatant and the tubes were boiled for 30 minutes in a water bath. The optical density of the bluish green solution was noted with the help of a photo-colorimeter at 610 \textmu m.

**Calculations:** The DNA content (mg/gm dry wt)
was calculated from the standard curve made by using deoxy-ribose.

**Protein**

Protein was estimated by the method of Lowry et al. (1951).

**Reagents**

A) 2% sodium carbonate in 0.1 N sodium hydroxide

B) 0.5% copper sulphate in 1% sodium citrate

C) 1 ml of the reagent B is mixed with 50 ml of reagent A.

D) Folin- Ciocalteu reagent

**Method of preparation of Folin- Ciocalteu reagent**

A mixture consisting 100 gm of Sodium tungstate (AR), 25 gm of Sodium molybdate (AR), 700 ml of water, 50 ml of 85% orthophosphoric acid (sp. gravity 1.75) and 100 ml of conc. HCl in a 1.5 litre flask was refluxed gently for 10 hours. 150 gm of Lithium sulphate, 50 ml of distilled water and few drops of bromine water were added and the mixture was boiled for 15 minutes without condensor to remove excess bromine. Then it was cooled and diluted to 1 litre, filtered and stored in dark bottle. The stock solution was diluted with two volumes of distilled water before use.
**Estimation:** The requisite amount of fine dry powder of plant material was suspended in distilled water and 20% trichloroacetic acid (TCA) at 5°C for 24 hours to remove sugars, non-protein phenols and soluble nitrogen fractions, and centrifuged. To the residue 1 ml of the reagent-C was added and after 10 minutes, 0.1 ml of the reagent-D was added rapidly and mixed. The optical density of the blue solution was read with the help of a photo-colorimeter at 710 μm after an interval of 30 minutes.

**Calculations:** The protein content (mg/gm dry wt) was calculated from the standard curve made by using bovine albumin.

**Observations**

The number of segments that rooted in each Petri-dish and the number and length of roots were recorded at periodic intervals. In addition to these, observations were also recorded on the development of anthocyanin, the size, shape and characteristics of callus and the growth of secondary roots on adventitious roots. The length of the first internode of epiphyllous buds of *Bryophyllum tubiflorum* was also recorded in some experiments.
The details regarding the composition of the culture medium, the time and duration of treatment and the frequency and nature of observation recorded are given separately in each experiment.