CHAPTER II

MATERIALS AND METHODS

Chemicals used.

Chemicals for experimental purposes were obtained from various sources which are presented on the facing page with the formulae (cf. Chart).

Preparation of Solutions.

Carbohydrate (sucrose, maltose, glucose, fructose and arabinose) solutions (10%) were prepared fresh for daily use by dissolving the measured quantity in sterile distilled water in volumetric flasks. Similarly, mineral salts, coumarin and gibberellic acid were dissolved in sterile distilled water. Vitamins and amino-acid were prepared fresh for use in the same manner and filtered through Whatman's bacteriological filter paper for sterilization. IAA solution (100 p.p.m.) was prepared by dissolving 10 mg. of the crystals in about 60 c.c. of sterile distilled water in a 100 c.c. volumetric flask. The solution was prepared by heating the volumetric flask in a boiling water bath. The volume was made up before autoclaving and the solution was stored in refrigerator for use in the next day. In preparing the stock solutions
of skatole (200 p.p.m.) and NMSA (500 p.p.m.), the measured quantity of the compounds were added to sterile distilled water in volumetric flasks which were then heated in boiling water bath. Few drops of N/10 KOH were needed to dissolve the compounds. The solutions were stored in refrigerator for 24 hours. Next-day the pH was adjusted to a neutral point (i.e. pH 7) and the volume was made up. Kinetin was prepared (100 p.p.m.) after several hours of boiling in water bath. B-(2-Furyl)-Acrylic acid solution (200 p.p.m.) was prepared by adding few drops of 50% alcohol to dissolve the compound. The volume was then made up by adding sterile distilled water.

Plant Material.

The healthy seeds of Phaseolus vulgaris L.cv. Sutton's Premier obtained from Messrs. Sutton & Sons, Pvt.Ltd., Calcutta, were selected for experimental purpose. The seeds were first surface-sterilized followed by thorough washings with sterile distilled water.

Surface Sterilization of Seeds.

Surface sterilization of the seeds prior to sowing was considered necessary, as the micro-organisms that lodge on the surface are likely to infect the young leaves to the detriment of their normal growth. Further, if they are carried to the culture solution with the
leaf tissues, they are likely to interfere the growth of the disks cultured therein.

Das (1965) working in this laboratory on the growth of excised roots of *Pisum* observed luxuriant growth of fungi and bacteria over the surface of the non-sterilized seeds. He observed that fungal and bacterial growth could be avoided by sterilizing the surface of the seeds with Br-water or HgCl$_2$ solutions and aureomycin. He succeeded in controlling the fungal growth by using 1:1000 HgCl$_2$ as surface-sterilizer.

This success prompted the present author to use (1:1000) HgCl$_2$ solution as a surface-sterilizer.

**Sterilization of Glasswares, Cutter, Solutions etc.**

The glasswares used in the experiments were first rinsed in water to remove the foreign materials. Then, they were kept immersed in absolute alcohol for about 24 hours and washed thoroughly with soap water. The pipettes and volumetric flasks were rinsed with dilute acid and washed several times with water. Then all the petri-dishes, pipettes, volumetric flasks, horse-shoe shaped glass rods were washed with distilled water prior to their insertion in hot air-oven. They were then sterilized in a hot air-oven at 200°C for one hour. Filter papers used were also sterilized in hot air-oven.
The cork borer and section lifters were sterilized before use in each experiment. They were first dipped in spirit and burnt over the flame of a spirit lamp. When cooled, these were ready for use.

Distilled water was sterilized by autoclaving at 15 lb pressure for 15 minutes. Sterile distilled water was used in preparation of solutions and in dilutions from the stock solutions. Stock solutions were sterilized by autoclaving at 15 lb pressure for 15 minutes. Thus every possible care was taken to avoid fungal and bacterial contamination which might adversely affect the growth of the leaf disks in culture.

**Culture of Seedlings.**

The seeds were washed with (1:1000) \( \text{HgCl}_2 \) solution for half a minute and then washed thrice with sterile distilled water. Wheeler (1965) observed a deleterious effect on presoaking the seeds in water. He reported that the longer the seeds of dwarf bean were soaked in water before sowing, the smaller were the seedlings they produced. Soaking resulted in the loss of detectable amount of gibberellins and a betaine. Therefore, all these operations before sowing were completed within two minutes. The seeds were immediately sown on earthen-pots containing sands. Sands used were sieved and washed.
thoroughly and placed in flat bottom bored earthen pots. Pots were then sterilized by autoclaving at 20 lb pressure for half an hour. After sterilization, the pots were allowed to dry and were placed on aluminium trays. Dried sands were then moistened with sterile distilled water and surface-sterilized seeds, ten in each pot at an equal distance from each other were sown. The pots were then kept in a dark room. Sterile distilled water was sprinkled time to time to keep the sands moist and the temperature of the room was maintained at 22.5±1°C. The importance of temperature on germinating seeds and young seedlings was noted by Dale (1964) who reported that the bean leaves are well-developed in the embryo and at 22.5°C exhibited an exponential increase in fresh weight, dry weight and leaf area until about 8 days from planting.

The primary leaves of 8-day old seedlings were harvested for experimentation. Leaf disks of equal diameter were cut with the help of sharp cork-borer as described below.

**Cutting of Leaf disks.**

Primary leaves collected from 8-day old seedlings were placed in a petridish in sterile distilled water so as to prevent them from drying up. Leaf disks
were cut with the help of a sharp cork-borer. Cork borer had conveniently been used for cutting disks of equal diameter by Miller and Meyer (1950-51), Miller (1956), Powell and Griffith (1960), Scott and Liverman (1956) and subsequently by many investigators. Dale (1966) used a sharp punch to obtain such leaf disks. Disks 7 mm. in diameter were cut from the leaves with the help of a sterilized cork borer. Two pairs of disks were cut from each leaf carefully avoiding the mid-vein. A lateral main vein approximated the diameter of each disk. The disks were first rinsed in sterile distilled water just to remove the cell-sap extruded in cutting them. They were then transferred to a mercuric chloride solution (1:1000) for half a minute and then washed twice thoroughly in sterile distilled water. They were then put in a moist sterile filter paper kept in a petridish. The entire gamut of the operations were completed within a few minutes.

Culture of Disks.

Disks from a number of leaves were pooled in each experiment. The sections were surface dried with sterile filter paper and randomly chosen sets of 5 disks constituting a sample were transferred to one petridish containing 10 c.c. of culture solutions. In this, required number of petridishes were taken according to the plan of
the experiment. The disks were placed with lower epidermis up over a sterile filter paper which was inserted over a horse-shoe shaped glass rods. The Whatman No. 1 filter papers were so placed so as to touch the culture solutions in each petridish. The ends of the filter paper served as a wick for supplying the applied growth substances to the leaf disks. This constituted a device for aeration of the growing tissues. Flooding of the explants with solutions were reported to cause growth depression in root sections (Audus and Shipton, 1952). Parkin (1899) floated leaves on sucrose solution and observed that those which sank did not accumulate starch, probably through lack of air. Phillis and Mason (1937) performed experiments on the uptake of sucrose and starch formation by cotton leaf disks and arrived at a similar conclusion. On the otherhand, utilization of liquid medium was preferred over solid agar medium by Heller (1949). The difficulty arising from poor aeration was overcome by de Ropp (1946) who also favoured the utilization of liquid media. Caplin and Steward (1949) subsequently used an apparatus capable of placing the tissue colonies alternatively in the nutrient solution and in air. But, the technique was simplified by Heller (1949) who used ash-free filter paper support upon which the explant was main- tained at a convenient level.
The leaf disks were then covered with the lids of the petridishes and were incubated at 25±1°C and under saturated humidity.

**Growth Measurements.**

The expansion growth of the disks was measured perpendicular to the main vein under a horizontal microscope. The Vernier Constant of the microscope scale was worked out and the expansion growth was measured up to two decimal points. Each operation involved in measuring the expansion growth of individual disks. Growth measurements were made at regular time intervals of 0-24, 24-48 and 48-72 hours. Such observation at different time intervals was necessitated to understand the rate of growth with the progress of time. At the end of 72 hours slight contamination was observed but that too was never serious. Besides the growth rate of sections fell markedly leading virtually to cessation of growth. For this reason growth observation was abandoned after 72 hours.

All the manipulations involving the leaf disks, starting from cutting the disks, culturing them, and growth measurements were performed under dim green light. Green safe-lights were obtained following the technique of Bertsch and Hillman (1961) who wrapped green fluorescent tubes with 4 layers of dark green and 6 layers of amber cellophane.
Analysis of the Results.

After each interval of time the mean increase in expansion of 5 disks in each concentration was calculated. For all the experiments performed with carbohydrates control could not be maintained. Therefore the expansion growth was converted to per cent increase over the initial diameter after 24, 48 and 72 hours (cf. Tables in Appendix). The mean percentage increase of all the replications was calculated out which was used for constructing the action-curves. In other experiments control was maintained at 1% sucrose solution. The expansion growth was expressed as per cent of control (cf. Tables in Appendix). The mean of all the replications for each interval of time was used for drawing the concentration growth response curves or interaction curves.

The pooled data were analysed statistically using Fisher's method of analysis of variance. The L.S.D. was calculated using the formula as follows:

\[ L.S.D. = \sqrt{\frac{2 \times \text{Error M.S.S.}}{n}} \times t \text{ value at 5 or 1\% level for Error degrees of freedom,} \]

where \( n \) = total unit/individual unit

= the number of ultimate observations used in computing the means.
The calculated L.S.D. was utilized in testing the difference between two mean values as significant or not. The vertical lines drawn on the graphs represent least significant difference (L.S.D.) at 5% level of probability unless otherwise indicated below the respective lines and figures by the side of them represent the number of observations.