CHAPTER II
MATERIALS AND METHODS

Chemicals used:

The chemicals used for experimental purposes were obtained from various sources which are presented below in their chemical structures.

1. Gibberellic acid (GA₃), (Merck and Co. Inc. Rahway, NJ)

2. Indole-3-acetic acid (IAA), (Sigma chemical company, St. Louis, MO, USA).

3. (2-Chloroethyl)-trimethyl-ammonium chloride (CCC), (American Cyanamid Co., Stamford, Conn., USA).

4. Abscisic acid (ABA), (Sigma Chemical Company, St. Louis, MO, USA).

Preparation of the Chemical Solutions:

1. Gibberellic acid: 200 mg. of Gibberellic acid were accurately weighed out and dissolved with a very small amount of absolute alcohol in a 100 c.c. volumetric flask making a volume of 100 c.c. in order to prepare a 2000 ppm stock solution. The required grades of solutions were prepared from this stock solution by adding distilled water.
2. **Indole-3-acetic acid**: Accurately weighed out 100 mg of Indole acetic acid were transferred to a 100 c.c. volumetric flask, and dissolved with a little alcohol, making a volume of 100 c.c. stock solution in order to prepare a 1000 ppm solution. Further dilutions were made by using distilled water.

3. **(2-Chloro-ethyl)-trimethyl-ammoniumchloride**: A stock solution of 5000 ppm was prepared by dissolving 500 mg of the chemicals in distilled water and the volume was made up to 100 c.c. The required grades were prepared from this stock solution and the remaining portion of the solution was preserved in the refrigerator for further experimental use.

4. **Abscisic acid**: 1 mg of abscisic acid accurately weighed out and dissolved in a small amount of absolute alcohol in a 100 c.c. volumetric flask and the volume was made up to prepare a 10 ppm solution. From this solution different concentrations were prepared by adding distilled water.

**Sterilization of glass wares solutions, etc.:**

The glassware used in the experiment was first rinsed in water and then immersed in absolute alcohol for about 24 hours before being washed thoroughly with soapy water. Pipettes and volumetric flasks were rinsed with dilute acid and washed several times with water. Then they were dried in a hot air-oven at a temperature of 160°C for 3 hours prior to use.

Distilled water was sterilized by autoclaving at 15 lb pressure for 15 minutes. Sterilized distilled water was used in the preparation of different chemical solutions.
Germination: Seeds of a local variety of Bean (*Phaseolus vulgaris*), Cucumber (*Cucumis sativus*) and Pea (*Pisum sativum*) were purchased from the Guwahati Nursery in Assam, India. The healthy seeds were sorted out carefully from the seed stock and washed with a 0.001% mercuric chloride solution and then with distilled water several times. The Cucumber and Pea seeds were soaked in the test solution for 10 hours. The Bean seeds were soaked for a comparatively shorter period, i.e., 4 hours, since Wheeler (1965) observed a deleterious effect of pre-soaking bean seeds for a longer period.

Sterile forceps were used to count the seeds and place them on the filler paper moistened with distilled water in a petri dish. The lids of each of the pairs of petri dishes were closed and the petri dishes were transferred to a dark chamber maintaining 25 ± 2°C.

The germinating seeds were counted at 24, 30, 36 and 42 hours for the Pea and Bean seeds, and at 36, 42, 48 and 54 hours for the Cucumber seeds. The seeds were considered to have fully germinated when the emerging radicle was first observed.

Culture of the seedlings: The germinated seeds were carefully transferred to sterilized earthen pots (12" x 10") containing sterilized sand. 5 germinated seeds were planted and after their emergence, the seedlings were thinned leaving 3 plants per pot. The pots were kept in a glass house which corresponded to the natural environmental condition usually encountered for growth of such crops at that time of year. Sand was moistened with half-strength "Hoagland Solution" to ensure normal growth for the seedlings.
Apical application of the seedlings: Healthy seeds were selected and soaked in distilled water for a definite period: Pea and Cucumber seeds for 10 hours and Bean seeds for 4 hours. The soaked seeds were then placed on the moist filter paper contained in the petri dishes. The fully germinated seeds were transferred to the earthen pots containing sterile sand moistened with half-strength "Hoagland Solution."

Three days after the transplantation, seedlings of uniform growth and appearance were selected, and 0.2 ml of the test solution was applied to the apex of each seedling with a micro pippette. The sand was protected from any spray and run-off with a covering of polythene paper. The paper was removed only when the plants had dried completely.

Growth measurement: The pots were removed to the laboratory to measure shoot and root growth at two different growth phases. The first growth phase was from the day of planting up to the seventh day, and the second growth phase was from the seventh day up to the 14th day. For each treatment 5 pots, i.e. 15 samples, were observed. For the whole experiment 50 pots containing 150 samples were observed.

The shoots were cut off at the soil level. The roots were carefully collected from the sand pots so that no root was damaged, and then washed several times with water to remove any sand adhering to the roots. The length of the shoots and the roots were measured with a m.m. ruler. The fresh weights were recorded immediately. The plant materials were then placed inside a dry-air-oven and were dried at 80°C till the weight became constant and the dry weights of the shoots and roots were thus determined.
Determination of the chlorophyll content: In the determination of the chlorophyll content of Peas (Pisum sativum), only the foliar portion of the plant was considered.

150 mg of the fresh leaf tissues were extracted with 80% acetone. During maceration CaCO₃ was added to prevent pheophytin conversion. Filtration was done through a sintered glass filter using suction. Extracts were made up to 20 c.c. by adding 80% acetone. The extracts were analyzed with a spectrophotometer (Pye-Unicam) by optical density measurement at 663 nm and at 645 nm in 1 cm cell; using the specific absorption co-efficients given by Maclachlan and Zalic (1963).

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\text{Chl-a mg/g} = \frac{12.3 D_{663} - 0.8 D_{645}}{d \times 1000 \times w} \times V
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\text{Chl-b mg/g} = \frac{19.30 D_{645} - 3.6 D_{663}}{d \times 1000 \times w} \times V
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Where, \(V\) = Volume of the extract, \(d\) = length of the light in cm, \(w\) = fresh weight in gm.

Total Chl = Chl-a + Chl-b

Analysis of the results: The germination was expressed as a percentage and the growth and the chlorophyll content were expressed as percentages of the control. The concentration response curves were drawn from the average of the corresponding replications.

The pooled data were analysed statistically using Fisher's method of analysis of variance. The L.S.D. was calculated using the following
The calculated L.S.D. was utilized in testing whether the difference between the two mean values was significant or not.