CHAPTER LII

METHODS AND MATERIALS
CHAPTER III

METHODS AND MATERIALS

Plant materials:

Seeds of Tobacco (*Nicotiana rustica* L) were obtained from Tobacco Research Station, Dinhata (Cooch-Bihar). The seeds of tobacco (cv. Motihari) proved to be positively photoblastic in preliminary trials, and was selected, for experimentation. Only the healthy seeds were selected for experimental purpose.

Chemicals:

The different chemicals, including both growth promoters and growth inhibitors were used in the experiments: (1) Thiourea, (2) Potassium nitrate, (3) Gibberellic acid, (4) kinetin, (5) Coumarin, and (6) (2-chloroethyl) trimethyl ammonium chloride or chlorocholine-chloride.

Sterilization of Glassware:

The glasswares used in the experiments such as petri dishes, volumetric flask, pipettes, measuring cylinders, funnel, glass rod and test tubes were first dipped into 50% alcohol and then washed with soap water. These were then rinsed with distilled water, dried and then put into a hot-air-oven. The temperature was adjusted at 160°C. The oven was allowed to run for a period of two hours at this temperature.
Sterilization of the Distilled water:

Distilled water used in the experiment was sterilized in an autoclave at a pressure of 15 lb./sq.inch for about half an hour.

Sterilization of the Filter papers:

The filter papers ("Q. Sicerin and Biva, Grade No.1 01, size 11 cm.) used in the experiment were sterilized just before use. The process was same as in the case of glasswares, but the filter papers were kept inside the hot-air-oven only for few minutes.

Sterilization of the Forcep:

The steel forcep used for transfer of the seeds and to count the seeds is also sterilized before used by dipping the tip in alcohol and burning over a flame of a spirit lamp.

Preparation of the Chemical Solutions.

1) Thiourea: A stock solution of 3000 ppm. were prepared by dissolving 3 gm. of chemicals (thiourea crystals) in 1000 cc sterilized distilled water in a volumetric flask. This stock solution was kept in a refrigerator and the required graded solutions were prepared before use from this stock solution by adding sterile distilled water.

2) Potassium nitrate: 0.3 gm. (300mg.) of KNO₃ crystals were accurately weighted out and dissolved in 100 cc of sterile distilled water in a 100 cc volumetric
flask to prepare 3000 ppm of KNO₃ solution. This was used as the stock, solution and preserved in a refrigerator. The required graded solutions were prepared from this stock solution before use.

iii) Gibberellic acid: To prepare a stock solution of 1000 ppm Gibberellic acid, 100 mg of GA₃ (Gibberellic acid) power was accurately weighed out in an analytical balance and then was transferred to a 100 cc volumetric flask, where it was dissolved by adding sterilized distilled water up to the mark. This 1000 ppm GA₃ solution was kept overnight in a refrigerator and then used. The lower concentrations were prepared from this stock solution by adding sterile distilled water before use and the remaining portion of the stock solution was preserved in the refrigerator for further experimental work.

iv) Kinetin: To prepare 1000 ppm of kinetin solution, 5 mg of Kinetin powder was weighed out accurately and dropped into a 100 cc volumetric flask to which sterile distilled water was added. The flask was in a water bath until the powder completely dissolved. Then the volume was made up to the mark of 100 cc by the addition of sterile distilled water. The solution was kept overnight in a refrigerator and then used. Then required lower concentration were prepared from this stock solution by adding sterile distilled water.

v) Coumarin: To prepare a 500 ppm solution of coumarin, 500 mg of coumarin (crystals) were accurately
weighed out and dissolved in sterile distilled water in a hot water bath and then the volume was adjusted to 100 cc in a 100 cc volumetric flask. The required graded solutions were prepared from this stock solution of coumarin before use and the rest was kept in the refrigerator for further experimental use.

vi) Choro-choline-chloride (CCC): To obtain 1000 ppm of ccc solution 100 mg of crystals of the chemical was accurately weighed out and transferred to a 100 cc volumetric flask where it was dissolved by adding sterile distilled water upto the mark. From this stock solution required graded solutions were prepared before use and the rest was preserved in the refrigerator for further experimental purpose.

PROCEDURE FOR THE EXPERIMENTAL SET UP AND MEASUREMENTS:

The seeds were first washed with sterile distilled water. From these only healthy seeds were selected for experimentation. Graded solutions were measured and poured into petridishes seeds after washings were directly placed in petridishes containing graded solutions of KNO₃, Thisurea GA₃, kinetin, ccc and coumarin seeds were counted into 2 lots of 20 and one lot was dispensed into each petri-dish. For interaction experiments two compounds were co-applied and were poured into test tubes and seeds were allowed to imbibe for 12 hours. The test tubes after insertion of seeds were kept in dark for 12 hours. Trials have proved this period of imbibition to be more responsive. The filter papers were
placed well inside the inner half of the petridishes. To keep the filter papers and the seeds well moistened, 5 cc of sterile distilled water was put into each pair. For each replication, for each treatment one set was maintained as control separately for light and dark. One set was exposed to different light (4 hours/24 hours) and the other set was placed in complete darkness. All the petridishes were placed in a BOD incubator and the temperature was maintained at 25°C. The transfer of the seeds to petridishes was carried out in a dark room under a dim green safe-light known to be photomorphogenetically inactive. Counting of germination for seeds placed in dark was also done under the same green-light.

The number of seeds germinated was counted after an equal interval of time of 24 hours, and germination was considered to have taken place just on the protrusion of the radicle. The germination was observed under a magnifying lens. The counting was continued up to 96 hours to obtain maximum germination.

All experiments were replicated thrice for convenience of statistical analysis.

Graphs were drawn to represent the action curves in light and dark for time and concentrations of every experiment done.

**Statistical Analysis:**

The data obtained from the experiments were subjected to statistical analysis for convenience of inter
pretation and for the study of the effect of the variable
factors on the germination of tobacco seeds. The results
were subjected to analysis of variance after the transpor-
tation to percentage of germinations.

The mean difference were compared with the
critical difference (C.D.) to test their significance.

**ANALYSIS OF VARIANCE:**

1) Determination of correction factor (C.F.)

ii) Determination of Sum of Squares (S.S.)

iii) Determination of the mean sum of squares (M.S.S.)

iv) Determination of variance ratio (F.value).

The formulae for determination of the above values
are given below:

1. C.F. = \((\text{Grand Total})^2\)
   Total units (i.e. 54 and 75)

2. S.S.

(a) Total S.S. = Summation of the squares of the experimental
   values ______________ C.F.

\[
\text{Concentration S.S.} = \frac{\text{Summation of the square of concentration total}}{\text{Total number of concentrations}} = \frac{\text{CF}}{
\text{Total number of concentrations (i.e. 9 and 15)}
}\]

(b) Time S.S. = \(\frac{\text{Summation of the squares of the time total}}{\text{Total number of time (i.e. 18 and 16)}}\) - CF

(d) Interaction S.S. = \(\frac{\text{Summation of the squares of treatment total}}{\text{CF - Concentration SS - Time SS}}\)
(e) Error S.S. = Total S.S. - Concentration S.S. - Time S.S
- Interaction S.S.

(ANALYSIS OF VARIANCE CONTD.)

DETERMINATION OF MEAN SUM OF SQUARES (M.S.S.)

Mean sum of squares (M.S.S.) are obtained by dividing the sum of squares (S.S.) by the respective degrees of freedom (D.F.)

\[
\text{Degrees of freedom (D.F.)} = \text{Number of cases} - 1 = (n-1).
\]

DETERMINATION OF VARIANCE RATIO (F.VALUE):

The mean sum of squares (M.S.S.) on concentrations, time and interactions are divided by the error M.S.S. to get calculated F.value for concentration, time and interaction respectively.

To test the significance of the calculated value of 'F' it is necessary to compare it with the value of 'F' given in the Fisher's F table, against the respective degree of freedom. If the calculated value is found to be greater than the table value at 5% and 1% probability, then the variation due to experiment, the main factors and their interactions as the case may be, are said to be significant and highly significant respectively. Otherwise i.e. if the calculated F.value is smaller than the table value of F. it is said to be insignificant. One star mark indicates the significant at 5% level of probability and the two star marks stand for significance at 1% level of probability and the latter is said to be highly significant.
CRITICAL DIFFERENCE (C.D.): The analysis of variance table gives only a broad indication of performance of the concentration and time as well as their interactions on the germination of tobacco seeds. But in order to get the clear appraisal of the specific phenomenon of different treatment combinations as well as the different levels of the main factors, the calculation of the critical difference (C.D.) is necessary.

C.D. is calculated as follows:

\[ \text{C.D. for concentration at 5\% or 1\% probability} = \sqrt{\frac{\text{error M.S.S.} \times \bar{x}^2}{n}} \times t \text{ value at 5\% or 1\% level of error degrees of freedom} \]

where:

- \( n \) = Total unit/individual unit
- \( \bar{x} \) = the actual number used for calibrating the means.

The calculated C.D. is utilized in testing the difference between the two mean value as significant or not. The same procedure was followed in the case of interaction experiments.