MATERIAL AND METHODS

Experimental procedure:

During early stages of germination, a study of the changes in enzymic activities as well as on ascorbic acid, nucleic acids, protein and carbohydrate metabolism in wheat seedling was undertaken. The same study was also extended during the growth and development of wheat plant.

In order to elucidate the mechanism of action of temperature pretreatment of seeds in relation to its growth and development as well as differentiation and maturation, the entire work was divided in the following three experiments:

Experiment 1: Effect of temperature pretreatment on some biochemical changes during germination.

Experiment 2: Comparative studies on the effect of temperature treatments on biochemical (70°C and 70°C) changes during various phases of growth and reproductive differentiation, seed development and
maturation of plants.

**Experiment 3**: Histochemical localization of AA, nucleic acids (RNA and DNA), histone and AA-FR-peroxidase enzyme during the differentiation of shoot apex of plants raised from 70°C temperature treated seeds and untreated seeds.

Graded and selected seeds or grains of wheat (*Triticum aestivum* L.) Cv. N.P. 718 were subjected in an oven to two temperature treatments.

1. **70°C gradually raised temperature treatment** (70°C) in which seeds were placed in an oven and temperature was gradually raised to 70°C by increasing 6°C per 24 hours.

2. **70°C direct temperature treatment** (70°D). In this treatment, seeds were kept directly at 70°C in an oven for 24 hours.

**Experiment 1**: Effect of temperature pretreatment on some biochemical changes during germination.

After giving the temperature pretreatments, the seeds of control, 70°C and 70°D wheat (*Triticum aestivum* L.) Cv. N.P. 718 were germinated at room temperature
(28-30°C) under normal day light in sterilized petridishes (12.5 cm diameter) lined with sterilized filter paper (Whatman No. 1) and using glass distilled water as the germinating medium. The medium-distilled water was changed twice a day, the embryo axis and endosperm were analysed in duplicate for the following estimations at 24 hours interval up to 96 hours of germination. Fresh weight, dry weight, percentage moisture content of embryo axis and endosperm were also separately recorded. Methods used for following biochemical estimations are given at the end of this chapter.

1. Percent moisture content,
2. Dry weight as g./10 embryo axis or endosperm,
3. Ascorbic acid (AA), ascorptigen (ASG), ascorbic acid utilization (AAU) and AA-macromolecule complexing (AA-MM-complex),
4. DNA content as mg./g.fr.wt.
5. RNA content as mg./g.fr.wt.
6. Protein content as mg./g.fr.wt.
7. Histone as O.D./g.fr.wt.
8. -SH content as mg./g.fr.wt.
9. Total sugar content as mg./g.fr.wt.
10. Reducing sugar content as mg./g.fr.wt.
11. Starch content as mg./g.fr.wt.
12. Peroxidase activity as O.D./min./g.fr.wt.
13. AA-FR-peroxidase activity as O.D./20 min./g.fr.wt.
14. Catalase activity as ml C<sub>2</sub> evolved./min./g.fr.wt.
15. Amylase activity as mg starch digested/30 min./g.fr.wt.
16. Invertase activity as mg glucose released/
    30 min./g.fr.wt.
17. RNase activity as mg ribose released/30 min./
    g.fr.wt.
18. Protease activity as mg. tyrosine liberated/
    hr./g.fr.wt.

Experiment 2: Comparative studies on the effect of
temperature treatment (70°C and 70°C)
on biochemical changes during various
phases of growth and reproductive
differentiation, seed development and
maturation of the plant.

Untreated and temperature treated seeds of wheat
(Triticum aestivum L.) Cv. N.P. 718 were sown in earthen-
ware pots 9" in diameter, filled with loamy soil and farm
yard manure in 3:1 proportion. In each pot 10 seeds were
sown. These pots were kept under natural day light
condition. Pots were watered daily depending upon the
environmental conditions. Manuring was done once a week
at the rate of one gram per pot and the manure consisted
of a mixture of ammonium sulphate and superphosphate in 2:1.

This experiment was divided into two parts:

I  Growth and developmental studies: Various growth characters namely height of the plant, leaf number, tiller number and tiller leaf number were recorded at weekly interval from the plants raised from untreated and 70°C and 70¹⁰D temperature pretreatment of the seeds. Ten plants were selected by the method of random sampling.

Fresh weight and dry weight in triplicate of root, stem, leaves, tiller leaves, main spike and tiller spikes were also recorded.

Relative growth rate (RGR), Net assimilation rate (NAR) and leaf weight ration (LWR) were worked out for root, stem, leaf and of whole plant.

Rates of ear emergence, flowering (anthesis) as well as ripening were determined by keeping a daily record of total number of plants, that reached the stage of ear emergence, anthesis and ripening respectively. Rate of spike emergence was calculated as number of spikes emerged/100 plants. Similarly, flowering data was calculated as the number of spikes in anthesis/100 spikes. Likewise, the rate of ripening was determined as number of spikes
ripened/100 spikes.

At the end of season, ten plants were harvested and the following yield characters were studied: Height of the plant, length of main spike, dry weight of main spike, dry weight of total spikes, total number of grains, total grain weight and 1000 kernel weight were also recorded.

Metabolic and enzymic studies:

Throughout the growth and development, the following biochemical estimations were done in two replicates from various stages of shoot apex (vegetative, transforming, inflorescence, anther, carpel) and from different stages of grain development and maturation as well as from the topmost fully expanded subtending leaf at these stages as cited in experiments - 1).

1. Ascorbic acid (AA), ascorbigen (ASG), AA-macromolecule complexing (AA-MM-complex) as mg./g.fr.wt. and ascorbic acid utilization as per cent AA utilized within 3 hours.
2. DNA content as mg./g.fr.wt.
3. RNA content as mg./g.fr.wt.
4. Protein content as mg./g.fr.wt.
5. Histone as O.D./g.fr.wt.
6. -SH content as mg./g.fr.wt.
7. Total sugar content as mg./g.fr.wt.
8. Reducing sugar content as mg./g.fr.wt.
9. Starch content as mg./g.fr.wt.
10. Peroxidase activity as O.D./min./g.fr.wt.
11. AA-FR-peroxidase activity as O.D./20 min./g.fr.wt.
12. Catalase activity as ml O₂ evolved/min./g.fr.wt.
13. Amylase activity as mg starch digested/30 min./g.fr.wt.
14. Invertase activity as mg glucose released/30 min./g.fr.wt.
15. RNase activity as mg ribose released/30 min./g.fr.wt.
16. Protease activity as mg tyrosine liberated/hr./g.fr.wt.

Experiment III: Study of histochemical localization of AA, RNA, DNA, histone and AA-FR-peroxidase enzyme in the vegetative shoot apex, transforming apex, anther and carpel of untreated (control), and 70°G plants.

Seeds of wheat (Triticum aestivum L.) Cv. N.P. 718 were sown in earthenware pots (9" in diameter), each pot contained loamy soil and farmyard manure in 3:1 proportion.
Pots were watered daily, manuring was done once a week at the rate of 1 gram per pot and the manure consisted of a mixture of ammonium sulphate and superphosphate 2:1.

Sampling was done for the histochemical studies at the various stages of growth and reproductive differentiation. The samples of shoot apex were fixed in different fixatives and the qualitative as well as quantitative procedure was followed as described in detail under experimental methods. After staining the microtomed sections, the readings were taken on a cytophotoelectrometer. With the help of a filar micrometer, micromeasurements of cell area ($\mu^2$), nuclear volume ($\mu^3$), nucleolar volume ($\mu^3$) were taken during different phases of the life cycle.

The extinction value was calculated by subtracting it from the transmission values and this value was then either multiplied or divided by cell area to obtain the total content per cell or the content of the metabolite per unit area of the cell.

As ascorbic acid is found to be the most important regulatory substance an attempt is being made to find out the regression trend on the basis of regression equations. Linear regressions of DNA, RNA, basic proteins (histone) on ascorbic acid were determined.
Experimental methods:

Fresh weight, dry weight and % moisture determinations:

Plant materials of embryo axis and endosperm were weighed immediately after dissection and transferred to an oven at 80°C for 48 hours when a constant dry weight was recorded. The difference between fresh weight and dry weight was taken as moisture content and percentage moisture content was calculated on fresh weight basis. The dry weight mean of 3 replicates is expressed in all the cases in terms of mg. per embryo or endosperm.

Ascorbic acid, Ascorbigen, Ascorbic acid utilization and Ascorbic acid macromolecule complexing:

Photocolorimetric method was developed in this laboratory (Chinoy et al., 1969) employing the specific dye 2,6-dichlorophenol-indophenol (EDH) for the estimation of ascorbic acid (AA), ascorbigen (ASG), ascorbic acid utilization (AAU), as well as AA macromolecule-complexing (AA-MM-complex).

The following solutions are used in the analysis:

1. Metaphosphoric acid - i) 3% solution
   ii) 15% solution

2. Buffer solution A: (10.55 gm. of citric acid dissolved in 100 ml of 1 N NaOH).
Buffer solution B: (31.65 gm. of citric acid dissolved in 100 ml. of 3 N NaOH).

3. Buffered $\text{HPO}_4$ : Two volumes of 3% $\text{HPO}_4$ are mixed with one volume of buffer solution A pH (3.6).

4. Ascorbic acid solution prepared in boiled, cooled, CO$_2$ - saturated glass distilled water (freshly prepared), 10 mg. per 100 ml. for AAU.

5. Standard dye solution: 10 mg. dye (2,6-dichlorophenol-indophenol) was dissolved in 200 ml. distilled water at 80°C, cooled and adjusted to 390 reading of Klett Summerson photoelectric colorimeter. In all the solutions glass distilled water was used.

Photocolorimetric readings:

The Klett Summerson photoelectric colorimeter is kept at '0' by adjusting the knob with glass distilled water to serve as a blank.

2 ml. glass distilled water and 5 ml. dye solution are taken and the dye is adjusted for the value 390. This standardization of the dye solution is done every time just before starting estimation. The regression is worked out by using standard concentrations (0.09 mg. to 0.01 mg/ml) of AA solution prepared from a stock solution of
0.1 mg/ml. 1 ml of each standard AA solution is mixed with 1 ml of buffered HPO₃ and after adding 5 ml standard dye, the color is read immediately in the photoelectric colorimeter using green filter (520 ). 10 readings of each concentrations are taken and converted into O.D. by multiplying with 0.002. The data are statistically analysed to get the following regression equation

\[ X = 0.1103 - 0.14 y \]

where, \( X \) = concentration of AA in mg.

\( y \) = optical density

Extraction:

Plant material is weighed and extract is made with cooled CO₂ saturated glass distilled water by crushing it in a mortar with addition of a pinch of purified silica sand. The mortar and pestle are washed with CO₂ saturated distilled water two to three times and the whole homogenate is made upto 11 ml. with cold CO₂ saturated water in a pyrex test tube.

The homogenate is divided into 3 parts:

i. 3 ml. for the estimation of ascorbic acid.

ii. 4 ml for the estimation of ascorbigen.

iii. 4 ml for the estimation of AAU and NAB.
Determination of ascorbic acid:

Immediately 3 ml. of the homogenate is pipetted out in a pyrex test tube and 3 ml. of cooled buffered HPO$_3$ (solution no. 3) is added to it and kept in an ice bath. It is shaken well and 2 ml. aliquot is taken for adjusting the '0' for turbidity after addition of 5 ml. of distilled water and another 2 ml. aliquot is used for colorimetric reading after the addition of 5 ml. of the standard dye (solution no. 5).

Calculation:

Colorimetric reading is converted into the optical density O.D. by multiplying with 0.002 (in case of Klett Summerson photoelectric colorimeter). Using the regression formula:

$$X = 0.1103 - 0.14Y$$

where, $X =$ concentration of AA in mg.

$Y =$ optical density

Thus, mg. of AA in 1 ml. of original extract is calculated.

After the addition of buffered HPO$_3$, when 2 ml. of the solution is taken for colorimetric determination, it contains only 1 ml. of the original extract. Therefore, the ascorbic acid content per gm.fr.wt. of the material can be calculated as follows:
\[
AA = \frac{A \cdot V}{W} \cdot 1000
\]

where, 
\( A = \text{mg. AA/1 ml. of the original extract} \)
\( V = \text{total volume of the original extract} \)
\( W = \text{weight of the plant sample in mg. taken for analysis.} \)

Determination of ascorbigen (ASG):

For the estimation of ascorbigen (ASG), 4 ml. of the homogenate is taken in another test tube to which 2 ml. of 15% \( HPO_3 \) solution no. (ii) is added and kept in a hot water bath maintained at 70°C for 15 minutes. After cooling the system is buffered at pH 3.6 using 2 ml. citric-NaOH buffer - B solution no. 2(ii). 2 ml. aliquot and 5 ml. of distilled water is taken for adjusting the colorimetric reading to '0' for turbidity factor. Another 2 ml. aliquot to which 5 ml. of standard dye solution added is taken for the colorimetric reading.

Calculation:

The total AA obtained from the ASG estimation includes both free form of AA as well as the bound form. Therefore, from the reading obtained, the free form is subtracted and ascorbigen (ASG) as mg. of ASG per gm./fr.wt. of the material can be calculated as follows:
where, $T = total\ ascorbic\ acid\ in\ \text{mg./l}\ of\ the\ original\ extract\ after\ hydrolysis\ with\ 15\%\ \text{HPO}_3$

$A, V\ and\ W$\ are\ same\ as\ in\ case\ of\ free\ AA\ calculation.

Ascorbic acid utilization (AAU):

4.0 ml. of ascorbic acid solution for AAU (10 mg./100 ml.) are added to 4.0 ml. of the original homogenate and the mixture is incubated at 30±2°C with thorough shaking after every 10 minutes.

After 3 hours, 4 ml. is taken and 4 ml. of buffered \text{HPO}_3 is added, 2 ml. are used to adjust the colorimeter to '0' for turbidity factor and another 2 ml. are taken for colorimetric reading after adding 5 ml. of the standard dye solution.

Calculation:

In the preparation, over and above the endogenous ascorbic acid, each ml. of the original extract is provided with 0.1 mg./ml. of ascorbic acid exogenously. The colorimetric reading is converted into O.D. and the amount
of AA remaining utilized after 2 or 3 hours was calculated out using the above mentioned regression formula. This amount is deducted from the total AA which is originally present in 1 ml. of extract plus that contained in 1 ml. of solution no. 4 which is added to it. In the case, the test aliquot contained only 0.5 ml. of the original extract.

Therefore, the calculation for per cent ascorbic acid utilization AAU in 3 hours of incubation will be as follows:

\[ AAU = \frac{(E-U)}{E} \times 100 \]

where, \( E = \) mg. AA/1 ml. of the original extract plus
0.1 mg. AA which was added exogenously per ml. of the original extract.

\( U = \) mg. AA in 1 ml. of the original extract which remained unutilized after 3 hours of incubation.

Determination of AA-MM-complex:

AA-macromolecule complexing:

After 3 hours, 4 ml. is taken and to which 2 ml. of 15% \( \text{HPO}_4 \) is added and kept in a hot water bath maintained at 70°C for 15 minutes. After cooling, it is buffered at pH 3.6 using 2 ml. Buffer-B. 2 ml. aliquot is taken for adjusting the colorimetric reading to '0' for
turbidity factor after the addition of 5 ml. of distilled water and another 2 ml. aliquot is taken for the colorimeter reading after the addition of 5 ml. of standard dye solution.

**Calculation:**

Colorimetric reading is converted into O.D. and the AA content in the aliquot is calculated from the regression equation. Then, it is multiplied by 2, because 2.0 ml. aliquot taken for colorimetric reading contains only 0.5 ml. of the original homogenate. Subtracting the value of AA of 1.0 ml. of the original homogenate utilized before hydrolysis from the above value gives the amount of AA released by hydrolysis of AA-MM-complex (in mg./ml.). The amount of AA complexing with macromolecule per g.fr.wt. can be calculated for the aliquot taken immediately after incubation period of 3 hours as follows:

$$\text{AA-MM-complex NAB} = \frac{(D - U)V}{W} \times 1000$$

where, $D = \text{mg. of AA in 1 ml. of the original extract}$ which released from the complex by the hydrolysis after 2 or 3 hours incubation.

$U = \text{mg. AA in 1 ml. of the original extract which remained unutilised after 3 hours incubation.}$

$V$ and $W$ are same as in case of free AA calculation.
Colorimetric estimation of nucleic acids:

The nucleic acids were extracted employing a slightly modified method of Bonner and Zeevaart (1962). The method is as follows:

The weighed material is dropped in 80% boiling ethanol for 1-2 minutes. The material is then crushed thoroughly in 80% ethanol and left overnight in a refrigerator for extraction. It is extracted repeatedly with 80% ethanol until a pigment free powder is obtained. This powder is extracted with ice-cold 5% HClO₄ three times to remove all the acid soluble components. Next, perchloric acid and phospholipids are removed with two changes of ethanol; chloroform: solvent ether (2:1:1) (V/V). During first change, the residue is boiled in the above mentioned mixture at 70°C for 1 to 2 minutes. After these 2 changes, residue is dissolved in 0.3N KOH and incubated at 37°C for 20 hours.

The resultant slurry is cooled and separated. The supernatant containing RNA is adjusted to pH 3.0 with perchloric acid. A known volume (30 ml.) is made up. The residue are reextracted twice with 5% HClO₄ to remove residual ribonucleotides and HClO₄ in turn is removed by ethanol; chloroform:solvent ether (2:1:1) by giving two changes. Here also, during first change, the residue is
boiled in the above mentioned mixture at 70°C for 1 to 2 minutes. Then the residue is hydrolysed by dissolving it in 0.5N HClO₄ and is incubated at 90°C for 15 minutes. It is centrifuged to remove the KClO₄. The supernantant is adjusted to pH 7.0 with KOH and made up to a known volume (35 ml.).

RNA and DNA are estimated colorimetrically employing orcinol method (Markham, 1955) and modified diphenylamine reaction (Burton, 1956) respectively.

A: Or cinol method:

Reagent:  i. Or cinol 1% in distilled water  
ii. Concentrated HCl  
iii. 1.0 % FeCl₃, 6 H₂O  

The reagent is prepared freshly by mixing 10 volumes of 1% orcinol with 40 volumes of concentrated HCl and one volume of FeCl₃ 6H₂O (10%).

0.6 ml. of solution containing 5.50 ug of pentose sugar (ribose sugar) is mixed with 6 ml. reagent, stoppered and heated for 8 minutes, on a water bath at 100°C. It is then cooled. The solution is read for measurement of optical density in a photoelectric colorimeter using a red filter. The standard curve is obtained by determining O.D. of solutions containing known amount of RNA and working out
the regression of O.D. upon RNA concentration. The regression equation is as follows:

\[ X = 502.036 \, Y - 0.4275 \]

where, \( X \) = concentration in mg.

\( Y \) = optical density of the solution.

B: Diphenylamine reaction:

6 gm. of diphenylamine in 460 ml. acetic acid + 6 ml. concentrated \( H_2SO_4 \) is prepared freshly and immediately before use. 0.1 ml. acetaldehyde (16 mg/ml) is added to each 20 ml. of the reagent.

To 2 ml. of the unknown solution containing DNA, 4 ml. of diphenylamine reagent is added and the contents are incubated for 18 hours at 28-30°C, the color of the solution is read in a photoelectric colorimeter employing a red filter. In this case also, a calibration curve is worked out using purified DNA. The regression is as follows:

\[ X = 1.676 \, Y - 2.18848 \]

where, \( X \) = concentration in mg.

\( Y \) = optical density

Thus, from the known optical density, in both the cases, the amount of RNA and DNA is calculated per g.fr.wt.
Estimation of protein:

The method used for determination of protein is that of Lowry et al. (1951).

Principle:

The final colour is a result of (1) biuret reaction of the protein with copper ion in alkali and (2) reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophane present in the treated protein.

Reagents:

1. Reagent A: 2% Na₂CO₃ in 0.1 N NaOH
2. Reagent B: 0.5% CuSO₄·2H₂O in 1% Na or K tartarate
3. Reagent C: alkaline CuSO₄ solution i.e. 50 ml. of reagent A + 1 ml. of reagent B.
4. Reagent D: folin reagent.

Foline - CiO catechu reagent: A mixture consisting 100 g. of Na-tungstate (Na₂WO₄·2H₂O), 25 g. of Na-molybdate (Na₂MoO₄·2H₂O), 700 ml. of water, 50 ml. of 85% phosphoric acid and 100 ml. of concentrated HCl in a 1.5 litre flask is refluxed gently for 10 hours. 150 g. of lithium sulphate, 50 ml. of water and few drops of bromine water are added and the mixture is boiled for 15 minutes without condensor to remove excess of bromine, then it is cooled and diluted to
1 litre and filtered. The reagent should have no greenish tinge.

The acid concentration of the reagent is determined by titration with NaOH with phenolphthalein as an indicator.

Protein standard: 10 mg. of casein is dissolved in 100 ml. of 1 N NaOH 1 ml. of this solution contains 0.1 mg. of protein. Range: 5 mg. - 100 mg.

Procedure: Different aliquots of standard protein solution containing 10-100 mg. and 5 ml. of the reagent C are added in the test tube, both are mixed and allowed to stand for 10 minutes or so at room temperature. 0.5 ml. of 1 N of folin reagent D is added rapidly with immediate mixing. After 30 minutes or so, the optical density is read on photoelectric colorimeter using red filter (660 m\(\mu\)).

The plant tissues are fixed in 80% ethanol and boiled for 5 minutes. It is then homogenized in 80% ethanol and centrifuged. The residue is suspended in water and perchloric acid is added to remove sugars and soluble nitrogen fraction. The residue is now dissolved at room temperature in 1N NaOH by keeping for half an hour. It is then made to a volume 6 ml. and a known aliquot (0.1 ml.) is taken and the color is developed in the above manner. A standard curve is prepared with known amounts of casein (or egg albumen). The regression formula is as follows:
\[ X = 1263.15 Y + 0.464 \]

where, \( X \) = concentration of protein in \( \mu g \)
\( Y \) = optical density

Protein content is calculated as \( \text{mg./g.fr.wt.} \).

**Histones:**

Histone was extracted following the modified method of Bonner and Huang (1962) and was estimated by following the method of Lowry et. al. (1951).

**Procedure:**

The plant tissue is fixed in 80% ethanol and boiled for 5 minutes. It is then homogenized in 15% perchloric acid (10 ml.). The tubes are kept at 70°C for 20 minutes to remove nucleic acids (Smillie and Krotkov, 1960). Then it is centrifuged and supernatant is discarded. Residue is dissolved in 6 ml. of 0.2N hydrochloric acid and the tubes are kept in a boiling water-bath for 30 minutes (Bonner and Huang, 1962) to extract histones. After cooling, it is centrifuged and supernatant is collected for histone estimation.

A known aliquot of histone solution is taken in a test tube to make up the final volume to 2 ml. with distilled water. Then to it 5 ml. of reagent C is mixed immediately
and it is allowed to stand for 10 minutes at room temperature.

0.5 ml. of 1N folin reagent D is added rapidly with immediate mixing. After 30 minutes, the optical density is read on a photoelectric colorimeter using red filter (660 mu) (see: protein determination). The content is calculated and expressed as O.D. of the color developed/g.fr.wt.

Determination of starch:

The material is weighed and transferred to a test tube containing about 15 ml. of 80% ethanol and placed in a boiling water bath to stop enzyme action and extract soluble sugars. The sample is homogenized in 80% ethanol using sterilized sand and centrifuged. The residue is dissolved in 20 ml. of 0.7% KOH and it is gelatinized in boiling water bath for 40 minutes. It is cooled, made to a volume and centrifuged. 0.5 ml. of 20% acetic acid is added to 1 ml. starch aliquot and to this 0.3 ml. of 0.1N KI and 1-2 drops of 3 volume H₂O₂ are added and the color is allowed to develop at 30°C for 30 minutes. It is made to a volume and 2-3 changes of 5 ml. chloroform are given until the last lot of chloroform is colorless as published earlier (Chinoy, 1939).

Standard solution of (0.2%) starch is prepared and the color is developed in 1 ml. of this solution as above. Readings are taken on a visual 'Kruss' colorimeter using blue
filter by keeping control (standard solution of starch in the cup) on left hand side and unknown starch solution on right hand side. Three readings are taken on left and right side scales of each sample. Mean reading was taken to calculate the amount of starch using the following formula

$$S = \frac{100 \cdot A \cdot L \cdot V}{g \cdot V' \cdot R}$$

where, $S$ = percent dry weight of starch content of the material.

$A$ = starch (in gm.) in the aliquot of the standard solution.

$V$ = total volume of the unknown solution.

$L$ = colorimetric reading of left hand side.

$g$ = fresh weight (g) of plant tissue.

$V'$ = ml. aliquot taken for color development.

$R$ = colorimetric reading of right hand side.

Sugars:

The method of estimation of sugars is that of Mc Cready et. al. (1950). Plant tissue is dropped in boiling 80% alcohol and crushed thoroughly and left overnight to extract sugars. After centrifuging, the supernatant is hydrolyzed with 1N HCl in a boiling water bath. The solution is neutralized, cooled, made upto a volume and analysed for total reducing sugars using anthrone reagent.
For the determination of reducing and nonreducing sugars separately, part of the aliquot is hydrolyzed for the total reducing sugars and the unhydrolyzed remaining aliquot gave reducing sugars content. Deduction of the latter from the former gives the value for nonreducing sugars.

0.2 g. of anthrone is dissolved in 100 ml. of 95% sulphuric acid (A.R.) and cooled in a refrigerator, solution to be analyzed and anthrone reagent are mixed in 1:2 proportion and heated over a water bath at 100°C for 7.5 minutes. The furfuryl groups of sugars combine with anthrone to form bluish green or green complex. The optical density of this color complex is read in the photoelectric colorimeter using red filter (660 m u). The amount of reducing sugars is calculated using the following regression formula:

\[ X = 317.00Y + 0.61993 \]

where, \( X \) = sugar concentration (in mg.)
\( Y \) = optical density

Sulphydryl content (-SH content):

The -SH content is measured using the colorimetric method of Grunert and Phillips (1949, 1951).
Reagents:
1. Sodium nitroprusside 2% solution.
2. 0.067M NaCN in 1.5M Na₂CO₃ (15.9 g. in 100 ml.).
3. Saturated sodium chloride solution - prepared at room temperature.

Procedure:
6 ml. of NaCl is taken and to it is added 2 ml. of plant extract, 1 ml. of sodium nitroprusside solution and 1 ml. of Na₂CO₃-NaCN mixture. The optical density is measured at 520 m u (using green filter) in a photoelectric colorimeter. Blanks are prepared in the same manner except that 1 ml. distilled water is used instead of 1 ml. sodium nitroprusside.

The amount of -SH content (mg./g.fr.wt.) is calculated by using the following regression formula

\[ X = 268.279 Y - 3.252 \]

where, \( X \) = concentration of -SH (in mg.)
\( Y \) = optical density.

AA-free radical peroxidase:
AA-FR-peroxidase is assayed by the method of Gurevich (1963). The sample is weighed, crushed in ice cold glass distilled water and centrifuged. The supernatant is used for
enzyme assay. To 2 ml. of the supernatant, 1 ml. of ascorbic acid (5 mg./ml) and 1 ml. of saturated solution of o-dinitrobenzene, (1 g. o-dinitrobenzene is suspended in approximately 100 ml. of glass distilled water, kept in boiling water bath for 15 minutes and filtered in cold. The volume is made up to 100 ml. this colourless solution is stored at 30°C) and 0.1 ml. of 20 vol. H₂O₂ are added. Blank contains all the above solutions except 0.1N H₂O₂, which is replaced with glass distilled water. The solution is allowed to stand at temperature 26±1°C for 20 minutes. The optical density of the yellow colour (formed due to the reduction of o-dinitrobenzene to o-nitrophenyl-hydroxylamine by the free radical of ascorbic acid developed was recorded using blue filter (λ20 μ) on Klett Summerson colorimeter against blank. The activity is calculated and expressed as O.D. of the colour developed per 20 min. per gram fresh weight of the material.

Peroxidase activity:

Peroxidase activity is determined by the method of Maehly (1954) and George (1953).

The sample is weighed, homogenized, made to a volume and centrifuged. The supernatant serves as a source of the enzyme. A reaction mixture is prepared by adding 2 ml. phosphate buffer (pH 7.0), 1 ml. of 20 m M aqueous guiacol
(0.22 ml. guiacol in 100 ml. distilled water) and 1 ml. enzyme extract. Blank is run by preparing the reaction mixture in the above manner except that the enzyme is excluded and replaced with 1 ml. distilled water. The scale of Klett Summerson photoelectric colorimeter is placed on zero with blank mixture and the O.D. of the reaction is read (blue filter, 420 m u). Then the scale of colorimeter is taken 25 divisions ahead from the point of O.D. noted for reaction mixture. At this stage the photoelectric colorimeter switch is put off and 0.02 ml. of 10 m M. H₂O₂ (0.4 ml. of 20 vol. H₂O₂ + 9.6 ml. of distilled water) is added by dipping a thin rod in the cuvette containing the reaction mixture and stope watch is started simultaneously. The time taken in seconds to reach the adjusted O.D. is noted. The results are calculated and enzyme activity is expressed in terms of O.D. of color developed per g.fr.wt. per 20 minutes.

Catalase activity:

Catalase activity is assayed by the manometric technique of Chance and Maehly (1955). The material is weighed and crushed in a mortar with a pinch of sterilized sand and calcium-carbonate.

The extract is made to 20 ml. and transferred to a reagent bottle. 5 ml. of H₂O₂ (10 volume) and 2 ml. phosphate
buffer (pH 7.0) are taken in a polythene tube 1"x1" and carefully transferred to the reaction bottle. The bottle is connected to the manometer at zero. The mixture is shaken for 2 minutes and the difference in reading which gives the volume of oxygen liberated is noted. The results are calculated per minute of time and per g.fr.wt. of the tissue which expresses the catalase activity.

RNase activity:

The sample is weighed, homogenized in cold (temperature 5°C) and made to a volume and centrifuged. The supernatant is used for determination of RNase activity according to the modified method of McDonald (1955). 1 ml. enzyme aliquot is added to a reaction mixture containing 1 ml. citrate buffer (pH 6.0) and 1 ml. RNA solution (pH 7.0 ; 0.05 %). The reaction mixture is incubated at 37°C for 30 minutes. The enzyme activity is stopped by adding 2 ml uranyl acetate in 2.5% HClO₄) and is left overnight at a low temperature in a refrigerator. It is centrifuged next day and the supernatant is used for the determination of release of ribose sugar by the method of Markham (1955) (please see Nucleic acids).

The amount of ribose released is calculated from a calibration curve which is prepared with ribose sugar (N.B.C.
Cleveland, Ohio, U.S.A.) and the RNase activity is expressed as mg. ribose released/30 min./g. fr.wt.

\[ X = 128.756 Y - 0.630 \]

where, \( X \) = concentration of ribose sugar in ug

\( Y \) = optical density.

Protease activity:

The sample is weighed homogenized in cold temperature (5°C), made to volume and centrifuged. The supernatant served as a source of enzymatic activity, which is assayed by the slightly modified method of Penner and Ashton (1967).

1 ml. enzyme aliquot is added to 3 ml. phosphate buffer (pH 7.0) and the reaction mixture is incubated at 30°C for 1 hour. The enzyme activity is stopped by adding 2 ml. of 15% TCA to 2 ml. of reaction mixture. After 20 minutes the content are centrifuged and supernatant is used to determine the amount of tyrosine released from casein using folin reagent of Lowry et. al. (1951). 1 ml. reaction aliquot is mixed with 1 ml. glass distilled water and 4 ml. of 0.5N NaOH, to this is added 1.2 ml. of folin-reagent and allowed to stand for blue colour development. The O.D. of the colour is read using a photoelectric colorimeter with red filter (660 m u) against blank. The blank contains
all solutions except 1 ml. reaction mixture which is replaced with 1 ml. distilled water.

The activity is calculated and it is expressed in terms of mg. tyrosine liberated/30 min./g.fr.wt. Using the following regression formula; casein is used for standard curve preparation

\[ X = 102.7Y + 0.080 \]

where, \( X \) = amount of tyrosine in ug and

\( Y \) = optical density.

Amylase activity:

The material is weighed, homogenized, made to a volume and centrifuged. The supernatant serves as the enzyme source. The reaction is carried out for 30 minutes at 30°C using 1-2 ml of 1-5% starch substrate as the case may be the control consists of enzyme inactivated by boiling before adding starch. The reaction is suspended by boiling in a water bath for 45 minutes.

In controls, as well as reaction sets 0.5 ml. of 10% acetic acid, 0.5 ml. of 0.1N KI and a drop of 3 vol. \( \text{H}_2\text{O}_2 \) are added and it is allowed to develop color for 30 minutes at 30°C. The excess of iodine is removed by giving repeated changes of chloroform. The intensity of the color is
compared using control set on left side and reaction set on right side of the Kruss colorimeter.

The amount of residual starch is determined by the method of Chinoy (1939) and thus amylase activity is calculated as g. starch hydrolyzed/30 min./g.fr.wt.

Invertase activity:

The method of Hatch and Glasziou (1963) is used for invertase assay. A weighed amount of material is crushed in a mortar using cold distilled water with sterilized sand and made to a volume 10 ml. Out of this, only 1 ml. aliquot is added to a reaction mixture containing 1 ml. of 0.25% sucrose and 1 ml. citrate buffer (pH 5.5). The reaction is inactivated by adding 2 ml. of 5% perchloric acid. The solution is made up to a constant volume (10 ml.) 1 ml. of this final solution is taken to develop color by anthrone method (Please see: McCready et.al., 1950 - sugars). The color is read on a photoelectric colorimeter. The enzyme activity is calculated using regression formula

\[ X = 317.0045 Y - 0.61993 \]

and expressed as mg. glucose produced/30 min./g.fr.wt.
Growth analysis:

Methods of growth analysis originally formulated by Gregory (1921, 1926) and subsequently employed by Chinoy (1961, 1961a, 1962, 1962a) in his work are used here.

Height:

Height of 10 plants selected by the method of random sampling from each row of pots was taken every week. The distance in cm. between the level of the soil and the base of the top-most unfolded leaf was taken as height before ear emergence and the distance between the level of the soil and the tip of the ear was taken as height after its emergence.

Tiller production: Tiller counts were made simultaneously with height measurements of the same plants.

Leaf number: Total number of leaves of the whole plant was noted. Besides this, the number of tiller leaves was also noted.

Dry matter production:

Three replicates of plants were selected by the method of random numbers at weekly intervals. These plants were uprooted carefully to minimize damage to the roots and wrapped in wet cloth before bringing to the laboratory. The
plants were thoroughly washed by water to remove soil and then gently pressed between blotting papers to remove moisture on their surface. Plant parts viz. root, stem, leaf and spike separated and fresh weight of each organ is recorded. These are then placed in paper bags and transferred to an electrically operated oven at 80°C for a period of one week for complete and uniform drying. The dry weights were then recorded. From this RGR, NAR and LWR were calculated as follows:

Relative Growth Rate (RGR):

Relative growth rates were determined from the differences between the Naperian logarithms of dry weights of successive weekly samples as formulated by Blackman (1919). The formula of relative growth rate is as follows:

\[
R.G.R. = \frac{\log_e W_1 - \log_e W_0}{t}
\]

where, \(W_0\) = Initial dry weight of plant
\(W_1\) = Dry weight on the succeeding sampling dates
\(t\) = time interval between two succeeding sampling dates.
Net Assimilation Rate (NAR):

From the data of dry matter production of the whole plant and leaf, net assimilation rates were calculated using Gregory's formula (1926):

\[
\frac{W_1 - W_0}{\frac{L_1 - L_0}{\log e^{L_1} - \log e^{L_0}}}
\]

where, \(W_0\) and \(W_1\) represent the successive dry weight of the whole plant;

\(L_0\) and \(L_1\) represent the successive dry weight of leaves of the corresponding samples.

Net assimilation rates were determined on the basis of unit dry weight of leaf instead of unit leaf area.

Leaf Weight Ratio (LWR):

The following formula was used for determining leaf weight ratio:

\[
\frac{\frac{L_1 - L_0}{\log e^{L_1} - \log e^{L_0}}}{\frac{W_1 - W_0}{\log e^{W_1} - \log e^{W_0}}}
\]

Where, \(L_0\) and \(L_1\) represent the successive dry weights of the leaves and

\(W_0\) and \(W_1\) represent the successive dry weights of the whole plant.
Yield: At the end of the growth period, 10 plants were selected at random from each treatments and observations were recorded for height, total dry weight of the plants, number of tillers, length of the main spike, spikelet number, grain number and grain weight and 1000 kernel weight.

Histochemical localization of ascorbic acid:

A new technique has been evolved in our laboratory as reported by Dave et al., (1968), Chinoy, N.J. (1969, 1969a) and Unni Madhavan and Shah (1968).

The silver nitrate reagent was prepared as follows: 5 g. of silver nitrate crystals were dissolved in 34 ml. of glass distilled water. 66 ml. of absolute ethyl alcohol was added to this. The solution was acidified by adding 5 ml. of glacial acetic acid and the solution was stored in an amber coloured bottle and kept in the refrigerator under 3-5°C and 0-3°C.

Fresh materials (shoot apices) were directly put in the above solution and kept in the dark under 0-3°C and pH 2 to 2.5 for a week. At the end of the reaction period, the material was thoroughly washed two to three times in 50% alcoholic ammonia for 10 to 15 minutes. The material was dehydrated in tertiary butyl alcohol, embedded in paraffin and microtomed at 10 microns. After the ribbons were
were dry, they were deparaffinised in xylol and mounted in Canada balsam.

Histochemical localization of basic protein (histone):

Alkaline fast green test (Alfert and Geschwind, 1953) was employed for the detection of basic proteins. The tissues were fixed in neutral formalin (10% formalin adjusted to pH 7.0) by 1N potassium hydroxide) paraffin infiltrated and sections were coated with celloidin and hydrated. The sections were placed in 15% trichloroacetic acid in boiling water bath for 15 minutes. After giving three changes of 70% ethyl alcohol sections were stained for 30 minutes in 0.1% aqueous solution of fast green (FCF) at pH 8.0 - 8.1 adjusted by 1N sodium hydroxide. The sections were then washed in distilled water for 5 minutes, dehydrated through alcohol series and mounted in Canada balsam. The basic proteins viz. histones were coloured green.

Histochemical localization of RNA:

RNA was localized using the modified method of Taft (1951).

The tissues were fixed in a formalin acetic alcohol (70% ethyl alcohol, 90 ml. + 5 ml. of formalin + 5 ml. of acetic acid) the tissue were dehydrated, paraffin
infiltrated and the sections were taken. Paraffin was removed by keeping the slide in xylol and then hydrated the tissue by passing through the following series - absolute alcohol 90% alcohol 70% alcohol 50% alcohol and distilled water.

After that the slides were placed in stain for 10 minutes, the stain was prepared by dissolving 0.2 g. of pyronin B in 100 ml. of 0.1M acetate buffer (pH 4.4). Wash the slides in running water till no red colour in water is seen. Before the tissue was completely dry. Slides were immersed in solution of TBA:Alcohol (1:1, 2:1, 3:1) and cleaned in xylene and mounted canada balsam.

Histochemical localization of DNA:

Feulgen method was used for localizing DNA. The tissues were fixed in formalin-acetic-alcohol (70% ethyl alcohol 90 ml. + 5 ml. of formalin + 5 ml. of acetic acid) dehydrated through tertiary butyl alcohol series and sections were cut at 10 microns. Deparaffinized sections were then hydrolyzed in a mixture of 3N HCl and ethyl alcohol in the proportion of 1:2 for about 15 minutes at 60°C. After hydrolysis sections were washed in tap water and stained in standard Schiff's reagent prepared in the following way:
de Tomasi (1936) as given in Pearse (1961) 1 gm. of basic fuchsin was dissolved in 200 ml. of boiling distilled water thoroughly and cooled exactly to 50°C. After filtering 20 ml. of N HCl was added to the filtrate. 1 gm. of potassium metabisulphite was added after bringing it to the room temperature. The solution was kept in dark for 14 to 24 hours, and 2 gm. of activated charcoal was added to the solution.

Then a colourless solution was obtained by filtering and stored in the dark at 0-4°C. Sections were drained and rinsed three times after staining with 10% potassium metabisulphite, washed in water, dehydrated and mounted in canada balsam. DNA registered purple colour.

Histochemical localization of AA-FR-peroxidase activity:

Based on the biochemical method for the estimation of AA-FR-peroxidase activity, Chinoy, N.J. (1970) has developed a technique for its histochemical localization. It consists of treating o-dinitrophenyl-hydroxyl-amine formed by AA-FR-activity, with dilute ammonia to form a deep violet colour. The incubation medium is prepared as follows:

All reagents and media were prepared in double glass distilled water:
(a) Saturated aqueous o-dinitrobenzene (o-DNB) 5 ml
(b) ascorbic acid (Analar) 5 mg./ml: 5 ml.
(c) 3% H₂O₂ 1 ml.
(d) citrate buffer pH 6.4 - 6.6 - 2 ml.

Fresh tissues were rapidly frozen on a CO₂ freezing and sections were cut at 10 to 15 microns. After a brief wash in citrate buffer, the sections were immediately transferred to the incubation medium. The sections were incubated for 24 hours at room temperature. Post incubation, the sections were washed in citrate buffer and treated with dilute ammonium hydroxide for two minutes. Since the purple colour thus obtained is not stable, the sections were immediately mounted in glycerine jelly and photographed.

Measurement of stain intensities:

After histochemical processing the intensity of stain reactions were measured by a simple cytophotometer devised in this laboratory. As shown in plate 22 a powerful microscope lamp produces a beam of light which passes through the condensor and illuminates the preparation on the slide kept on the stage. The light passes through the eye piece which protrudes out of the dark chamber is reflected by the mirror to the platform with a small aperture
below which a photoelectric cell (J) (Photronic cell, Weston illumination model, S. 123) is fitted. The photocell is connected to a lamp and scale galvanometer. The reading is taken by bringing the image of the cell or the nucleus concerned in alignment with the apperture on the platform and noting the deflection on the galvanometer scale. The difference between the transmission value of the stained cell, nucleus or nucleolus and the corresponding control gives the extinction value. Atleast ten such readings are taken for every tissue.

Using the above method reading for the intensities of stain reaction for ascorbic acid DNA, RNA and basic proteins were found. Photomicrographs were taken on a Carl Zeiss Photomicroscope with planospochromatic objectives and Orwo N.P. 15 film with yellow filters.

Micromeasurements:

The length and width of the cell, the diameter of nuclei and nucleoli for the above tissues are also determined by using a Filar micrometer. The cross sectional area of the cell (in $\mu^2$) is calculated and is presented in tables, alongwith the diameter of the nuclei and nucleoli. Approximate nuclear volume and nucleolar volume are also calculated from the respective diameters. The extinction
values (Ex-value) are multiplied by the cross sectional area of the respective cells to obtain the total content/cell (total content per cell). Further, the extinction values are divided by cell area to obtain the content/unit area (total content per unit area of the cell). Using the values obtained by dividing the values with cell area, the correlations between AA - DNA, AA - RNA, AA - basic proteins, DNA - RNA, DNA - basic proteins, RNA - basic proteins are determined by calculating the regression formulae and trend lines are indicated.

Regression value:

To determine the correlation between DNA, RNA, basic protein with AA the regression values were obtained as follows:

Regression value established the important correlation between two types of data. The data collected on the basis of divided values (i.e. cell area/ext. value) were tabulated as in the following table:

\[
\frac{X}{AA} \quad dx \quad dx^2 \quad \frac{Y}{DNA} \quad dy \quad dy^2
\]

where, X and Y are two types of data

e.g. \( X = \) divided values of ascorbic acid;
\( Y = \) divided values of DNA.

By calculating totals and averages \( X, \bar{X} \) and \( Y, \bar{Y} \) were respectively obtained.
The value of 'X' and 'Y' were deducted from their average for getting dx and dy respectively.

With Barlows table $dx^2$ and $dy^2$ were found out and by multiplying dx with dy, dx-dy value were obtained. The regression coefficient "$(b_{xy})$" was calculated by the following formula:

$$b_{x y} = \frac{dx X dy}{dy^2}$$

For determining the relation between X and Y value the following formula was applied.

$$X = \bar{x} + b \times Y (Y - \bar{Y})$$

by simplifying the above formula and putting the necessary figures, the final relation between 'X' and 'Y' values was obtained. Selecting the value of 'Y' from the data and calculated the value of X for three readings this values were plotted on the graph paper and line was drawn passing through the above three points. The straight line indicated the correlation between two types of data.

Considering that there are changes in the content of metabolites and enzyme activities at various stages of germination, their increase or decrease can be calculated in percentage taking into consideration. The first reading
of untreated seeds at 24, 48, 72, 96 hours of germination and comparing them with the respective growth stages of treated seeds subsequently.

Percentage increase or decrease = \[
\frac{mr \times 100}{mv}
\]

where, \(mr\) = enzymic activity of treated seeds during the selected stage of germination
\(mv\) = enzymic activity of untreated seeds during the respective selected stage of germination.

Percentage increase or decrease in experiment 1 are calculated from the actual values presented (in table)

Statistical analysis:

It was carried out for the metabolites and enzymes in experiment I and II and for growth characters, dry weights, metabolites and enzymes by the method of analysis of variance (Fisher, 1948). In all the experiments, metabolites, enzymes, growth characters and dry weights are analyzed statistically for degrees of freedom, variance and F value. Analysis of variance, standard error of differences of the means and critical difference (.C.D) were worked out on the data of various estimations in experiment I and II. Mean value of each treatment and period was obtained by addition of all the determinations for a given period and
treatment and dividing total number of determinations.

Higher values than control (for treatment) and higher values than 24 hours (for period) are designated as + while lower ones as - and where differences are not significant, these figures are mentioned as such i.e. without + or - signs. The statistically analysed data for various determinations are given in an appendix (Table 1.2o § 2.6).