MATERIALS AND METHODS

Experiment No. I.

Effect of long and normal photocycles as well as presowing hardening treatments on resistance of barley varieties to wilting after 20 and 40 days of growth in terms of growth and yield as well as ascorbic acid, carbohydrate and sulfhydryl metabolism.

The basic aim of this complex type of experiment was to study the effect of long and normal photocycles as well as presowing hardening treatment on resistance to wilting after a certain period of growth in terms of growth and yield data as well as in terms of ascorbic acid, carbohydrate and sulfhydryl metabolism. For this, the following two cultivars of barley were used:

1. New Pusa - 21 (Indian - Early variety).

2. Spartan - 5027 (American - Late variety).
The seeds of both the varieties were 'pretreated' with distilled water and ascorbic acid. Untreated seeds served as a control series.

Moreover, the plants were exposed to the following two photoperiodic cycles:

1. Long-day of 24 hours (LD).
2. Normal-day of 11 hours (ND).

There were in all 360 pots for each variety (180 pots for each of the two photocycles). These 180 pots of each photocycle were divided into three lots of 60 pots (20 pots for each pretreatment) each for the following three wilting treatments:

1. Full watering (F)
2. Wilting after 20 days of growth in which the watering was stopped on 21st day of growth and was resumed after six days of wilting to bring the moisture level back to 15 to 20% in the soil.
3. Wilting after 40 days of growth in which the watering was stopped on 41st day of growth and was resumed after six days of wilting to bring the soil moisture 15 to 20%.

During the duration of wilting at two periods in the life cycle of the plants, three soil moisture determinations in triplicate were made on second, fourth and sixth day for each wilting treatment.

Moreover, determinations in duplicate of \(-SH,\) ascorbic acid, ascorbigen, net ascorbic acid bound, ascorbic acid utilization and carbohydrates in the upper most unfolded leaves of plants of untreated seeds of both the varieties were carried out for all the drought and full water series of both the photocycles during the periods of wilting and revival as well as during the pre-wilting period.
Experiment No. II.

Effect of long and normal photocycles as well as of presowing hardening and wilting treatments at shooting and anthesis stages on metabolism, growth and yield:

The aim of this experiment was to study the effect of long and normal photocycles as well as presowing hardening treatment of varieties to wilting at different growth and developmental stages in terms of growth and yield data as well as ascorbic acid, ascorbigen, ascorbic acid utilization, catalase activity, general peroxidase activity, AA-free radical peroxidase activity, invertase activity, carbohydrate, protease activity, protein, sulfhydryl content, RNase activity and nucleic acids.

The following two cultivars of barley were selected for this experiment:

1. New Pusa - 20 (Indian - Early variety).

2. Peatland (American - Medium Late variety).
The seeds of both the varieties were pretreated with distilled water and ascorbic acid.

The plants were subjected to the following two photoperiodic cycles:

1. Long-day of 24 hours (LD).
2. Normal-day of 11 hours (ND).

There were in all 360 pots for each variety (180 pots for each of the two photocycles). These 180 pots of each photocycle were divided into three lots of 60 pots (20 pots for each pretreatment) each for the following three wilting treatments:

1. Full watering (F).
2. Drought at shooting stage (S).
3. Drought at anthesis (A).

Drought of six days was given to each pretreatment of a variety at two critical stages such as shooting and anthesis.
During the duration of wilting periods, three soil moisture determinations in triplicate were made on second, fourth and sixth day.

Determinations in duplicate were made for ascorbic acid, ascorbigen, ascorbic acid utilization, catalase activity, general peroxidase activity, AA-free radical peroxidase activity, invertase activity, carbohydrate, protease activity, protein, sulphydryl content, RNase activity and nucleic acids.

Determinations of drought intensity: During the duration of wilting treatments, soil moisture determinations were made at definite intervals as mentioned above and intensity of drought was calculated from the following formula:

\[ I = \frac{t}{K \frac{m}{m}} \]  
(Chinoy, 1960)
where $I =$ Drought intensity

$t =$ Mean temperature during the wilting period

$m =$ Mean per cent moisture of the soil during the wilting period

$K =$ Constant (number of days in the wilting period)

The effect of wilting at different growth stages was determined by noting the percentage of plants surviving and making full recovery after removal of water stress. This is described as 'survival value'.

Method of pretreatment of seeds: This method is very simple. The point to be taken into consideration is that there should be good aeration and there should not be complete soaking. The seeds of barley were pretreated in the following manner:

25 gm. aliquots were taken in 8 oz. wide mouthed bottles and to these were added 10 ml. of -

1. Distilled water and

2. 25 mg./litre of ascorbic acid solution.
The seeds were allowed to stand in the wet condition for six hours at room temperature. During this soaking period, the bottles were shaken from time to time to ensure an even distribution of moisture in the seeds. Also at intervals the bottles were kept open for 1 or 2 minutes to prevent anaerobic respiration.

All the liquid was absorbed by the seeds in each bottle and after six hours the seeds were removed from the bottles and were spread out in a thin layer on sheets of blotting paper to dry in the shade in laboratory for 2 - 3 days. To ensure even drying the seeds were stirred on the paper from time to time. Every day the blotting paper was replaced by a fresh one. During this period the seeds got dried almost to their original weight. At this stage the seeds were again treated as before by soaking them in distilled water and ascorbic acid solution for six hours and then spreading them on blotting paper for drying for the second time. After ensuring that the water content of twice pretreated seeds was not much above the original, the
pretreatment was repeated for the third time and then the seeds were dried in the usual manner till the water content was almost the same as before the treatment. The soaking and drying periods were kept constant every time. These pretreated seeds were stored in bottles for further use.

**Statistical analysis:** The data of both the experiments were subjected to statistical analysis using Fisher's method (1954). Analysis of Variance was carried on data for various characters and their interactions have been worked out. Linear regressions of soil moisture on temperature and of survival value on drought intensity were also worked out. In all the statistical analysis 'S' denotes significance at 1% Point, whereas 'S*' denotes significance at 5% Point only.
Meteorological data: Records of maximum and minimum temperatures and per cent humidity during the growth periods of plants in both the experiments were recorded.

Total light hours: Growth period (days) and total light hours of both the varieties in each experiment as well as under both the photocycles were calculated. The lights were put off after anthesis in Long day treatment so as to avoid the deleterious effect of LD on grain filling processes.

Growth data: Methods of growth analysis originally formulated by Gregory (1921, 1926) and subsequently employed by Chinoy (1960, 1961, 1961a, 1962, 1962a) in his work were used here:

a) Height: Height of twenty plants selected by the method of random numbers from each row of pots were 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.

b) **Tiller production:** Tiller counts were made simultaneously with height measurements of the same plants. Main shoot was included in the count.

c) **Leaf number:** The number of unfolded leaves the whole plant also were noted.

d) **Drought coefficient and mean values:** Of growth characters were also calculated. For each character this was done by dividing the character mean value of growth period of the plants of drought series with the full water series and multiplying it with 100. The mean value of various treatment combinations also were worked out separately for control, distilled water and ascorbic acid pretreated plants with respect to the factors namely: wilting series, photocycles, wilting treatments, and varieties as follows:
i) Wilting series: This includes 20 replicates of each variety with 3 wilting treatments under two photo cycles and so the total value is divided by 240 \((20 \times 2 \times 3 \times 2 = 240)\) for control. The same is followed for DW and AA treated plants.

ii) Photocycles: This includes 20 replicates of each variety with 3 wilting treatments under two wilting series and so the total value is divided by 240 \((20 \times 2 \times 2 \times 2 = 240)\) for control. The same is followed for DW and AA treated plants.

iii) Wilting treatments: This includes 20 replicates of each variety of two wilting series and two photocycles and so the total value is divided by 160 \((20 \times 2 \times 2 \times 2 = 160)\) for control. The same is followed for DW and AA treated plants.
iv) **Varieties:** This includes 20 replicates of two wilting series and 3 wilting treatments under two photocycles and so the total value is divided by 240 \((20 \times 2 \times 3 \times 2 = 240)\) for control. The same is followed for DJ and AA treated plants.

This method is followed for calculating mean values for tiller number and leaf number also.

e) **Dry matter production:** Two plants were uprooted every week from each row of pots. The plants were thoroughly washed in slow running water to remove the soil and then gently pressed between blotting sheets to remove any moisture on their surface, and after taking fresh weights of different plant parts, separately the samples were dried in an oven at \(80^\circ\text{C}\) for a period of one week for complete and uniform drying. The dry weights were then recorded.
f) Relative growth rate (RGR): Relative growth rates were determined from the differences between the Naperian logarithms of dry weights of successive weekly samples as shown by Blackman (1919). The formula of relative growth rate is as follows:

\[ RGR = \frac{\log_e W_1 - \log_e W_0}{t} \]

g) 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):

\[ \frac{W_1 - W_0}{\log_e L_1 - \log_e L_0} \]

where, \( W_0 \) and \( W_1 \) represent the successive dry weights of the whole plant; \( L_0 \) and \( L_1 \) represent the successive dry weights 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 the leaf weight ratio:

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

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 row of pots of fully watered and wilted series and observations were recorded for height, dry weight of the plant, number of tillers with and without ears, length of main spike, spikelet number, grain number and grain weight of main and extra spikes, total grain weight and 1000 kernel weight.
Drought coefficients: Drought coefficients for various yield characters were calculated as follows: Values for each wilting treatment were divided by the corresponding values of the fully watered series and the value thus obtained was multiplied by 100 (Chinoy, 1961a). Thus for example, drought coefficient for height is obtained by dividing the mean values of the height of 10 plants of drought series (drought given at shooting or anthesis) with the mean values of the height of 10 plants of the full water series.

Mean values for various metabolic activities were calculated as follows:

1. Determinations: For the mean value of content or enzyme activity 'before drought', total value was calculated by 2 replicates x 2 wilting series x 2 photocycles x 2 wilting treatments and it was divided by 16. The same was followed for 3 days drought, 6 days drought, revival - 1 and revival - 2 for both the varieties.
2. Wilting series: For the mean value of wilting series, total value was calculated by 2 replicates x 5 determinations x 2 photocycles x 2 wilting treatments and it was divided by 40 for each variety.

3. Photocycles: For the mean value of photocycles, total value was calculated by 2 replicates x 5 determinations x 2 wilting series x 2 wilting treatments and it was divided by 40 for each variety.

4. Wilting treatments: For the mean value, total value was calculated by 2 replicates x 5 determinations x 2 wilting series x 2 photocycles and it was divided by 40 for each variety.

5. Variety: For the mean value, total value was calculated by 2 replicates x 5 determinations x 2 wilting series x 2 photocycles x 2 wilting treatments and was divided by 80 for each variety.
Metabolic activities:

The various metabolic activities were studied in the upper - most fully expanded leaf of the plant of fully watered and wilted series. The estimations were carried out only in the leaves of untreated (control) plants. Estimations in duplicate were carried out in each case. One estimation of each metabolic activity was made just before the commencement of the wilting treatment. Two estimations were done during the wilting period and lastly two estimations were carried out after 7 and 14 days after revival of the plants.

a) Ascorbic acid, ascorbigen, ascorbic acid utilization and net ascorbic acid bound:

A simplified titration method now modified to a photocolorimetric method in this laboratory (Chinoy et al., 1969) employing the specific dye 2,6-dichlorophenolindophenol for the determination of ascorbic acid has been used for the estimation of ascorbigen (ASG), AA utilization (AAU) as well as net ascorbic bound (NAB) over and above free ascorbic acid (AA).
The following solutions were used for the analysis:

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

2. i) Buffer solution - A (10.55 g. of citric acid dissolved in 100 ml. of 1N NaOH).
   ii) Buffer solution - B (31.65 g. of citric acid dissolved in 100 ml. of 3N NaOH).

3. Buffered HPO₃ - two volumes of 3% HPO₃ mixed with one volume of buffer solution - A (pH = 3.6).

4. Ascorbic acid solution prepared in boiled, cooled, CO₂ - saturated glass distilled water (freshly prepared) 10 mg. per 100 ml. for AAU.

5. Standard dye solution: 10 mg. dye (2, 6-dichlorophenolindophenol) was dissolved in 200 ml. distilled water at 90°C, cooled and adjusted to 390 reading of Klett Summerson
Colorimeter. In all the solutions glass distilled water was used.

**Extraction:** Plant material was weighed and extract was made with cooled \( \text{CO}_2 \) saturated glass distilled water by crushing it in a mortar with the addition of a pinch of purified silica sand. The mortar and pestle were washed with glass distilled water two to three times and the whole homogenate was made up to the required volume with cold \( \text{CO}_2 \) saturated distilled water in a pyrex test tube.

**Photocolorimetric readings:** The Klett Summerson Photoelectric Colorimeter was 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 were taken and the dye was adjusted for the value 330. The regression was worked out by standard concentrations (0.09 mg. to 0.01 mg./ml.) of \( A^3 \) solution prepared from a stock solution. 1 ml. ... of each standard
AA solution was mixed with 1 ml. of buffered HPO₄ and after adding 5 ml. standard dye the colour was read immediately in the Photoelectric Colorimeter using green filter. Sixteen readings of each concentration were taken and converted into O.D. by multiplying with 0.009. The data were statistically analysed to get the following regression equation:

\[ Y = 0.1103 - 0.14X \]

where \( Y \) = Concentration of AA in mg, and \( X \) = Optical density

Preparation No. 1: For the estimation of ascorbic acid: Immediately 3 ml. of the homogenate was pipetted out in a pyrex test tube and 3 ml. of cooled buffered HPO₄ (solution No.3) was added to it and kept in an ice bath. It was shaken well and 2 ml. aliquot was taken for adjusting the '0' for turbidity after addition of 5 ml. of distilled water and another 3 ml. aliquot was used for colorimetric reading after the addition of 5 ml. of the dye (solution No.5).
Calculations: Colorimetric reading is converted into optical density (O.D.) by multiplying with 0.002 (in case of Klett Summerson Photoelectric Colorimeter). Using the regressing formula: \( Y = 0.1103 - 0.14X; \) (where \( Y \) = Concentration of AA in mg. and \( X \) = Optical density), mg. of AA in 1 ml. of original extract is calculated.

After the addition of buffered HPO₃ 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 g. dry wt. of the material (AA) can be calculated as follows:

\[
AA = \frac{A\cdot V}{W} \times 1000
\]

where \( A \) = mg. AA/1 ml. of the original extract

\( V \) = total volume of the original extract (ml.)

and \( W \) = dry weight of the plant sample (mg.) taken for analysis.
Preparation No. 2: For the estimation of ascorbigen: 4 ml. of the homogenate was taken in another test tube to which 2 ml. of 15% HPO₃ was added and kept in a hot water bath maintained at 75°C for 15 minutes. After cooling the system was buffered at pH 3.6 using 2 ml. citric - NaOH buffer - B. 2 ml. aliquot was 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 was taken for the Colorimetric reading after the addition of 5 ml. of standard dye solution.

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 AA per g. dry wt. of the material can be calculated as follows:

\[ \text{ASG} = \frac{(T - A) \cdot V}{W} \cdot 1000 \]
where \( T = \) total ascorbic acid in mg./l. ml. of the original extract after hydrolysis with \( 15\% \text{ HPO}_4\).

**Preparation No. 3:** For the estimation of AAU and NAB. The 5 ml. of extract was taken in a test tube and 5 ml. of ascorbic acid solution (10 mg./100 ml.) prepared in \( \text{CO}_2\) saturated glass distilled water was added. This mixture was kept for 3 hours with thorough shaking after every 15 minutes at a temperature of 30°C ± 2°C.

1) **Ascorbic acid utilization (AAU):** After 3 hrs., 3 ml. was taken and 3 ml. of buffered \( \text{HPO}_4\) was added. 2 ml. were used to adjust the Colorimeter to '0' for turbidity factor and another 2 ml. was taken for Colorimetric reading after adding 5 ml. of standard dye solution. In Experiment No. I AAU was also recorded after one hour and the preparation as well as calculations are same as 3rd hour utilization.
ii) **Net ascorbic acid bound (NAB):** 4 ml. of the solution was taken from preparation No. 3 and to it 2 ml. of 15% HPO₄ was added and was kept for hydrolysis in the water bath at 37°C for 15 minutes. After cooling the system was buffered at pH = 3.6 using 2 ml. citric - NaOH buffer - B.

2 ml. aliquot was taken to adjust the colorimeter to '0' for turbidity factor and was another 2 ml. aliquot taken for Photoelectric Colorimeter reading ... after the addition of 5 ml. of standard dye solution.

**Calculation: Ascorbic acid utilization**

(Preparation No. 3): In this 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 was converted into O.D. and the amount of AA remaining unutilized after 3 hours was calculated out using the above mentioned regression formula. This amount was
deducted from the total AA which was originally present in 1 ml. of extract plus that contained in 1 ml. of solution No. 4 which was added to it. In this 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

- $E = \text{mg. AA/1 ml. of the original extract plus 0.1 mg. AA which was added exogenously per ml. of the original extract.}$
- $U = \text{mg. AA in 1 ml. of the original extract which remained unutilized after 3 hours of incubation.}$

**Calculation:** Net ascorbic acid bound:

After the utilization period of 3 hours half of the test solution was subjected to hydrolysis.
Thus, the bound ascorbic acid was released as free AA. Therefore, the reading obtained from this set consists of the free form of AA which remained unutilized as well as the AA which was released from the bound form. In this case also the Colorimetric reading was converted into O.D. by multiplying it with 0.002, and the amount of ascorbic acid (in mg.) present in the aliquot which was hydrolyzed with 15% HPO₄ after incubating it for 3 hours was determined from the regression equation given earlier. The quantity of ascorbic acid so determined is multiplied by 2 as the aliquot taken for colorimetric estimation contains only 0.5 ml. of the original extract.

The calculation of NAB as mg. of AA/g. dry wt. is, therefore, as follows:

\[
NAB = \frac{(H - B) \times V}{W} \times 1000
\]

where \( H \) = mg. of AA in 2 ml. of (Preparation No. 3) hydrolyzed with 15% HPO₄ after 3 hours of incubation.
b) Catalase activity: Catalase activity was determined by the method of manometric technique of Chance and Maehly (1955). The tissue was thoroughly ground in a mortar with 1 gm. of CaCO₃, until it was reduced to a fine pulp. A few drops of distilled water were added during the grinding. This crushed material was made up to 20 ml. in a measuring cylinder by adding to it the required amount of distilled water. This 20 ml. of crushed tissue was placed in a bottle. 10 vol. H₂O₂ with an excess of CaCO₃ was kept ready. A small tube containing 5 ml. of this 10 vol. H₂O₂ and 2 ml. phosphate buffer (pH = 7.0) was carefully placed in the bottle containing plant material avoiding any mixing of the two solutions. After placing the small tube of H₂O₂ into the bottle, it was closed with rubber
cork. This rubber cork had an outlet for the escape of oxygen through a pressure rubber tubing which was connected to a manometer to measure the volume of oxygen liberated. The level of water in the manometer was adjusted initially at zero. The two liquids in the bottle were then mixed by tilting it to one side. The time taken for the evolution of 2 ml. of oxygen was recorded.

c) General peroxidase activity: Peroxidase activity was determined by the method of Maehly (1954) and George (1953). The sample was weighed, homogenized, made to a volume and centrifuged. The supernatant served as a source of the enzyme. The reaction mixture was prepared by adding 2 ml. phosphate buffer (pH 7.0), 1 ml. of 20 mM aqueous guaiacol (0.22 ml. of guaiacol in 100 ml. distilled water) and 1 ml. enzyme extract. Blank was run by preparing the reaction mixture
in the above manner except that the enzyme was excluded and replaced with 1 ml. distilled water. The scale of Klett Summerson Colorimeter was zeroed with blank mixture and the optical density of the reaction was read using blue filter (470 mλ). Then the scale of colorimeter was taken 26 divisions ahead from the point of optical density noted for reaction mixture. At this stage the photoelectric colorimeter switch was put off and 0.02 ml. of 10 nM. H₂O₂ (0.4 ml. of 20 vol. H₂O₂ + 9.6 ml. of distilled water) was added by dipping a thin rod in the cuvette containing the reaction mixture and stop watch was started simultaneously. The Colorimeter was put on. The time taken in seconds to reach the adjusted optical density was noted. The results are calculated and enzyme activity is expressed in terms of optical density of colour developed / min. / g. dry wt.
d) **Free radical peroxidase activity.** Free radical peroxidase activity was assayed by the method of Gurevich (1963).

The plant material was weighed, crushed in ice cold distilled water and centrifuged. The supernatant was used for the enzyme assay. To 2 ml. of the supernatant liquid, 1 ml. of ascorbic acid (5 mg./ml.) and 1 ml. of orthodinitrobenzene (100 mg. dissolved in 12 ml. distilled water by boiling in water bath for 15 minutes) and 0.1 ml. of 20 vol. H₂O₂ were added. Blank contained all the above except 0.1 ml. H₂O₂, which was replaced with distilled water. The solutions were allowed to stand for 20 minutes to develop colour at room temperature (25°C ± 1°C).

The optical density of the yellow colour (formed due to the reduction of ortho-dinitrobenzene to ortho-nitrophenyl hydroxylamine by the free radical of ascorbic acid) developed was recorded using the blue
filter in Klett Summerson Colorimeter.
The activity is expressed as optical density of the colour developed per 20 minutes per gram dry weight of the material.

e) **Invertase activity:** The method of Hatch and Glasziou (1963) was used for invertase assay. A weighed amount of material was crushed in a mortar using cold distilled water with sterilized sand and made upto a volume of 10 ml. Out of this, only 1 ml. aliquot was added to a reaction mixture containing 1 ml. of 0.2% sucrose and 1 ml. citrate buffer (pH = 5.5). The reaction was carried out at 30°C for 30 minutes. The enzyme activity was inactivated by adding 2 ml. of 5% perchloric acid. The solution was made upto a constant volume (10 ml.) 1 ml. of this final solution was taken to develop colour by anthrone method (please see McCready et al., 1950 - reducing sugars). The colour was read on a photoelectric
colorimeter. The amount was calculated by using regression formula and it is expressed as mg. glucose produced / 30 min. / g. dry wt.

f) Carbohydrates: The method of estimation of sugars is that of McCready et al., (1950). Plant tissue was dropped in boiling 80% alcohol and crushed thoroughly and left overnight to extract sugars and centrifuged. For the determination of reducing and nonreducing sugars separately, part of the aliquot was hydrolysed for the total reducing sugars and the unhydrolysed remaining aliquot gave reducing sugar content. Deduction of the latter from the former gave the value for nonreducing sugars.

0.2 g. of anthrone was dissolved in 100 ml. of 96% sulphuric acid (A.R.) and cooled in refrigerator. Solution to be analysed and anthrone were 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 complex. The optical density of this coloured complex was read in the photoelectric Russian colorimeter using red filter (660 nm). The amount of reducing sugars is calculated using the following regression formula:

\[ Y = 0.03102X - 0.00035 \]

where \( Y \) = Sugar concentration in per cent and \( X \) = Optical density.

g) Protease: The sample was 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 was added to 3 ml. phosphate buffer (pH = 7.0) and 2 ml. of 0.5% casein solution (pH = 7.0) and the reaction mixture was incubated at 30°C for 1 hour. The enzymatic activity was stopped by adding 2 ml. of 15% TCA to the reaction.
mixture. After 20 minutes the contents were centrifuged and supernatant was used to
determine the amount of tyrosine liberated
from casein using folin reagent of
Lowry et al., (1951). 1 ml. reaction aliquot
was mixed with 1 ml. glass distilled water
and 4 ml. of 0.5N NaOH, to this was added
1.2 ml. of folin reagent and allowed to stand
for blue colour development. The optical
density of the colour was read using
photoelectric colorimeter and red filter against
blank. The blank contained all solutions
except 1 ml. reaction mixture which was
replaced with 1 ml. water. The activity was
calculated and it is expressed in terms of mg.
tyrosine liberated / hour / g. dry wt. using
the following regression formula. Casein was
used for standard curve preparation.

\[ Y = 142.4 \, X - 0.13 \]

where \( Y \) = amount of tyrosine in mg.
and \( X \) = Optical density.
h) **Protein:** The method described below is that of Lowry et al., (1951). **Principle:** The final colour is a result of - (i) biuret reaction of the protein with copper ion in alkali; and (ii) reduction of the phosphomolybdicphosphotungstic reagent, by the tyrosine and tryptophane present in the treated protein.

**Reagents:**

1. Reagent A : 2% Na₂CO₃ in 0.1N NaOH
2. Reagent B : 0.5% CuSO₄₅H₂O 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 1N.

**Folin-Ciocalteu reagent:** A mixture consisting 100 g. of sodium tungstate 25 g. of sodium molybdate 700 ml. of water, 50 ml. of 85% phosphoric acid and 100 ml. of
con. HCl in a 1.5 litre flask was 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 was boiled for 15 minutes without condenser to remove excess bromine. Then it was cooled and diluted to 1 litre and filtered. The reagent should have no greenish tinge. The acid concentration of the reagent was determined by titration with 1N NaOH with phenolphthalein as an indicator.

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

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

The plant tissue was fixed in 80% alcohol and boiled for 5 minutes. It was then homogenized in 80% ethanol and centrifuged. The residue was suspended in water and perchloric acid was added to remove sugars and soluble nitrogen fractions. The residue was dissolved at room temperature in 1N NaOH by keeping for half an hour. It was then made to a volume (6 ml) and a known aliquot (0.1 ml) was taken and the color was developed in the above manner.

The standard curve was prepared with known amounts of casein (or egg albumin). The regression formula is as follows:

\[ Y = 516.7 \times X - 1.19 \]

where \( Y \) = Concentration of protein in mg.
and $X = \text{Optical density}$.

1) **Sulphydryl content**: The -SH content was measured using the colorimetric method of Grunert and Phillips (1949, 1951).

**Reagents:**

1. Sodium nitroprusside = 2% solution

2. 0.067 NaCN in 1.5M Na$_2$CO$_3$ (15.9 g. in 100 ml.)

3. Saturated sodium chloride solution prepared at room temperature.

**Procedure**: 6 ml. of NaCl was taken and to it was added 2 ml. of plant extract, 1 ml. of sodium nitroprusside solution and 1 ml. of Na$_2$CO$_3$ - NaCN mixture. The optical density was measured at 520 \(\mu\) (using green filter) in a photoelectric colorimeter. Blanks were prepared in the same manner except that 2 ml. distilled water was used instead of 2 ml. extract.
(Blank I) and 1 ml. distilled water was used instead of 1 ml. of nitroprusside (Blank 2).

The amount of -SH content (mg.) was calculated by using the following regression formula:

\[ Y = 0.084X + 0.0019 \]

where \( Y \) = Concentration of -SH (mg.)
and \( X \) = Optical density.

j) RNase: The sample was weighed, homogenized in cold (temperature 5°C) and made to a volume and centrifuged. The supernatant was used for determination of RNase activity according to the modified method of McDonald (1955). 1 ml. enzyme aliquot was 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 was incubated at 37°C for 30 minutes. The enzyme activity was stopped by adding 2 ml. of uranyl acetate solution (0.25% uranyl acetate in 2.5% HClO₄)
and this was left overnight at a low temperature in a refrigerator. It was centrifuged next day and the supernatant was 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 was prepared with ribose sugar (H.B.C., Cleveland, Ohio, U.S.A.) and the RNase activity is expressed as mg. ribose released / 20 minutes / g. dry wt.

k) **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 was dropped in 80% boiling ethanol for 1 - 2 minutes. The material was then crushed thoroughly in 80% ethanol and left in overnight/a refrigerator for extraction. It was extracted repeatedly with 80% ethanol until a pigment free powder was obtained. The powder was extracted with ice - cold 8% HClO₄
(Perchloric acid) three times to remove all the acid-soluble components. Next perchloric acid and phospholipids were removed with 2 changes of ethanol : ethylether : chloroform (2:1:1) mixture. The residue was incubated with 0.3N KON at 37°C for 20 hours. The resultant slurry was cooled and separated. The supernatant containing RNA was adjusted to pH 3.0 with perchloric acid.

A known volume (50 ml.) was made up. The residue was re-extracted twice with distilled water. It was washed twice with 10% cold HClO₄ to remove residual ribonucleotides and HClO₄ in turn was removed by ethanol : ether : chloroform (2:1:1) by giving 2 changes. The residue was hydrolysed by dissolving it in 0.5N HClO₄ and was incubated at 90°C for 15 minutes. It was centrifuged to remove the HClO₄.

The supernatant was adjusted to pH 7.0 with KON and made up to a known volume (30 ml.).
The RNA and DNA were estimated colorimetrically employing orcinol method (Markham, 1955) and modified diphenylamine reaction (Burton, 1956) respectively.

Orcinol method: The reagent was prepared freshly by mixing 10 volume of 1% orcinol with 40 volume of conc. HCl and 1 volume of FeCl₃·6H₂O (10%).

0.2 ml. of solution containing pentose sugar was mixed with 2 ml. reagent, stoppered and heated for 8 minutes on a water bath at 100°C and then cooled. The solution was read for measurement of optical density in a photoelectric colorimeter using red filter. The standard curve was obtained by determining optical density of solutions containing known quantities of RNA and working at the regression of O.D. upon RNA concentration. The regression equation is as follows:

\[ Y = 0.845X - 0.004895 \]

where \( Y \) = Concentration of RNA (mg.)

and \( X \) = Optical density.
Diphenylamine reaction: 6 g. diphenylamine in 460 ml. acetic acid + 6 ml. conc. H₂SO₄ was prepared freshly and immediately before use. 0.1 ml. acetaldehyde (16 mg./ml.) was added to each 20 ml. of the reagent.

To 1 ml. of unknown solution containing DNA, 2 ml. diphenylamine reagent was added and the contents were incubated for 18 hours at 28 - 30°C. The colour of the solution was read in a photoelectric colorimeter using red filter. In this case also a calibration curve was worked out using purified DNA. The regression equation is as follows:

\[ Y = 1.48069 \times - 0.00119 \]

where \( Y \) = Concentration of DNA in mg. and \( X \) = Optical density.

The amounts of RNA and DNA are presented in mg. per gram dry weight.