MATERIALS AND METHODS

Wheat seeds cv. Loa-1 (Triticum vulgare L.) and cv. Arnej (Triticum durum L.) were used to incorporate gibberellic acid (GA $10^{-6}$ M), cycocel (CCC $10^{-5}$ M) and their combinations (GA $10^{-6}$ M + CCC $10^{-5}$ M) using presoaking technique of alternate soaking and drying for 6 hours (100 g of seeds in 60 ml solution). Three cycles were given and seeds were collected after each cycle. Seeds soaked under similar conditions in distilled water (H₂O) served as control.

Pretreated seeds of both varieties were germinated in sterilized petri dishes lined with filter paper (Bomol-X) under laboratory conditions ($25 - 26^\circ$C) and following studies were under taken:

I 1. Effect of soaking with different percent moistures and number of cycles on (a) seedling length and (b) fresh and dry weights of shoot, root and endosperm.

2. Physiological and biochemical changes during pretreatment and due to pretreatment.

II 1. Studies on cell number and cell volume as influenced by pretreatments.

2. Histochemical parameters at 24 hours of germination as affected by pretreatments.

3. Studies on the effect of pretreatments and number of cycles on the leaching behaviour of metabolites.
III 1. Studies on growth and development of pretreated and control seeds raised under field conditions.

2. Studies on growth indices, chlorophyll content, carotenoid content and mineral (K, Na, P) content during vegetative reproductive and senescence stages.

3. Studies on the effect of foliar spray with GA (10^-6 M), CCC (10^-5 M), CCC (10^-5 M) + GA (10^-6 M) and Dm at anthesis for comparative studies with pretreatments.

4. Studies on final yield attributes.

Experiment - I

1. Effect of soaking with different percent moistures and number of cycles on (a) seedling length and (b) fresh and dry weights of shoot, root and endosperm.

   a. Seedling length was measured after 96 hours of germination.

   b. Fresh and dry weights: Samples were weighed and dried in an oven at 60°C for 48 hours till constant weight was reached.

2. Physiological and biochemical changes during pretreatments and due to pretreatments.

   Embryo axis and endosperm were separated at 0, 3 and 6 hours during pretreatment at 12 and 18 hours (early
germination) and at 24 hourly intervals up to 72 hours for the following estimations.

1. **Fresh and dry weight (mg/organ)**
2. **Seeding length (after 24 hours germination)**
3. **Peroxidase (OD/min/mg protein)**
4. **Polyphenol oxidase (OD/3 min/mg protein)**
5. **Total amylase (mg starch hydrolysed/5 min/mg protein)**
6. **Invertase (mg reducing sugar released/30 min/mg protein)**
7. **Reducing and non-reducing sugars (mg/g dry wt)**
8. **FNA (mg/g dry wt)**
9. **DNA (mg/g dry wt)**
10. **Protein (mg/g dry wt)**

1. **Fresh and dry weight**: Samples were weighed and dried in an oven at 60°C for 48 hours till constant dry weight was reached.

2. **Seeding length**: Seeding length was measured after 72 hours of germination.

3. **Peroxidase activity** (Guaiacol method) was determined following in principle, the method of George (1953). 0.1 ml aliquot was mixed with 2 ml phosphate buffer (pH 5.8) + 0.02 µl of H₂O₂ (20 vol) and C.D. was recorded at 420 nm after 1 minute incubated at room temperature. Similarly blank had 0.1 ml enzyme extract, 2 ml phosphate buffer and 2 ml
guaiacol. O.D. was noted in spectronic-20-colorimeter against blank. Peroxidase activity was calculated and expressed as O.D./min/mg protein.

4. Polyphenol oxidase activity (Kar and Misra, 1976): For this 0.5 ml enzyme extract was mixed with 2 ml phosphate buffer (pH 7.0) + 2 ml pyrogallol (50 μl - 12.6 mg in 100 ml Dm) and incubated at room temperature for 3 minutes. Similarly blank had 0.5 ml enzyme extract + 4 ml phosphate buffer. O.D. was noted on spectronic-20-colorimeter at 420 nm. polyphenol oxidase activity was calculated and expressed as O.D./3 min/mg protein.

5. Amylase activity (Daleg, 1960): weighed material (100 mg) was crushed in 10 ml CaCl₂ + 2 M NaCl solution and volume was made to 10 ml and centrifuged. 0.5 ml aliquot was taken and mixed with 1 ml of citrate buffer (0.25 M, pH 5.0) + 2 ml 0.1% starch and incubated for 5 minutes at room temperature and then to this 1 ml of I₂KI (10.2 g of I₂ + 2 g of KI in 500 ml 0.1 M HCl) was added and again incubated for 5 minutes at room temperature and final volume made to 25 ml. The difference in O.D. between reaction and blank set was worked out using inactivated (by heating) enzyme (Malik and Singh, 1980). mg starch hydrolysed was calculated by referring to the standard curve.

6. Invertase activity was determined by the method of Hatch
STANDARD CURVE-GLUCOSE

OD

0.70
0.56
0.42
0.28
0.14

FIG 1

GLUCOSE (µg)

40
120
200
and Glassiou (1963). 1 ml aliquot + 1 ml of 0.25% sucrose + 1 ml of acetate buffer (0.2 M) pH 4.8 were mixed and reaction was carried out at 30°C for 30 minutes. The enzyme activity was inactivated by adding 2 ml of 5% perchloric acid and volume was made (10 ml). 1 ml of the mixture was taken to develop color by Nelson Somogyi method (Wharton and McCarty, 1972). The color was read at 540 nm by referring to standard curve of glucose (Fig. 2) invertase activity was calculated and expressed as mg glucose released/30 min/mg protein.

Enzyme protein: (Lowry et al, 1951): 0.5 ml aliquot was mixed with 5 ml reagent C (4 parts of 12.5% Na₂CO₃ + 1 part of 0.1% CuSO₄) and was incubated for 10 minutes at room temperature and then 0.5 ml of 1 N folin phenol reagent was added and after 30 minutes O.D. was taken at 600 nm in spectronic-20-colorimeter. By referring to standard curve prepared with casein (20 μg/ml) the amount of protein was calculated and expressed as mg protein/g fr wt.

7. Reducing and nonreducing sugars: Weighed material (100 mg) was crushed in 80% alcohol and boiled for 2-3 minutes in capped test tubes at 100°C and then it was centrifuged. Residue was extracted with 5 ml of 80% alcohol twice. The supernatant volume was made to 20 ml with D₂O. From this 10 ml was taken for reducing sugars and remaining 10 ml was used for total sugars. 3 ml of 1 N HCl (0.75 ml of conc.
HCl + γ1.25 ml of D₅ was added to hydrolyse nonreducing sugars and it was kept in boiling water bath for 20 minutes. It was neutralized by 1 N NaOH; 1 ml of 25% lead acetate and 1 ml of 25% sodium carbonate were added. Finally in both the sets final volume was made 20 ml and filtered. 1 ml aliquot was taken for sugar estimation by the method of Nelson Somogyi (Wharton and McCarty, 1972). 1 ml aliquot + 1 ml of Somogyi reagent (Nelson reagent 'A' - 12.5 g sodium carbonate + 12.5 g Na-K tartarate + 10 g sodium bicarbonate + 100 g sodium sulphate in 500 ml D₅; Nelson reagent 'B' - 15 g copper sulphate + 90 ml of D₅ + 1 or 2 drops of conc. H₂SO₄ in volume made to 100 ml) Nelson reagent' was prepared by mixing 50 parts of 'A' with 2 parts of 'B'. Tubes were capped and heated at 100°C for exactly 20 minutes and cooled rapidly. 1 ml of arsenomolybdate reagents (25 g ammonium molybdate dissolved in D₅ and volume made to 450 ml + 21 ml conc. H₂SO₄ + 3 g sodium arsenate in 25 ml D₅ was added) added and shaken for 5 minutes thoroughly to dissolve red ppt. Final volume was made to 25 ml. O.D. was taken at 540 nm on spectronic-20-colorimeter. By referring to the standard curve of glucose (Fig. 2) amounts of reducing and total sugars were calculated and expressed as mg/g dry wt.

Nucleic acids (Fujisawa, 1966): To the residue leftover after removing alcohol soluble fraction 5 ml of 5% ice cold HClO₄ was added and kept for 5 minutes to remove acid soluble
fraction. 2 changes of 5 ml each of alcohol, ether and chloroform (2:2:1) were given to the residue and kept for 10 minutes to extract lipids. Then to the residue 10 ml of 1 N HClO₄ was added and it was left in cold (in refrigerator) for 42 hours and finally centrifuged. Supernatant was collected as RNA fraction. 10 ml of 1 N HClO₄ was added to residue and the same was hydrolysed at 90°C for 10 minutes to extract DNA. It was cooled and centrifuged. Supernatant contained DNA.

8. **RNA determination by orcinol method (Modified method Marsham, 1955):** 1 ml of aliquot + 5 ml of orcinol (10 ml of 1% orcinol + 40 ml of conc. HCl + 1 ml of 10% FeCl₃) reagent were heated for 10 minutes and cooled. Control had 1 ml of HClO₄ instead of aliquot; O.D. was recorded at 600 nm on spectronic-20-colorimeter against control. RNA content was calculated from standard curve (Fig. 3), prepared with known PNA and expressed as mg/g dry wt.

9. **DNA determination using diphenylamine reagent (Barton, 1956):** 2 ml of aliquot + 4 ml of diphenylamine reagent (6 grams of diphenylamine in 400 ml of glacial acetic acid + 6 ml of conc. H₂SO₄) is prepared and immediately before use 0.1 ml acetaldehyde (10 ml/ml) is added to each 20 ml of reagent) is added and the contents were incubated for 18 hours at 28-30°C. O.D. was noted on spectronic-20-colorimeter at 600 nm against control. Control had 2 ml 1 N HClO₄ instead of
STANDARD CURVE-RNA

OD

FIG 3

RNA (µg)
STANDARD CURVE-DNA

FIG 4

DNA (μg)
aliquot. DNA content was calculated from standard curve (Fig. 4) and expressed as μg/g dry wt.

10. **Protein** (Lowry et al., 1955 - modified): The residue (after removal of nucleic acid fraction) was dissolved in 5 ml 1 N NaOH for 20-30 minutes and centrifuged. 0.5 ml of aliquot was taken and to this 4 ml of 12.5 % Na₂CO₃ and 1 ml of 0.1 % CuSO₄ was added and incubated for 10 minutes. 0.5 ml of 1 N folin phenol reagent was added and after 10 minutes O.D. was taken at 600 nm on spectronic-20-colorimeter. By referring to the standard curve of protein (Fig. 5) the amount of protein was calculated and expressed as mg/g dry wt.

**Experiment - II**

1. Studies on cell number and cell volume as influenced by pretreatments.

   Cell number and cell volume was determined following the method of Wright (1961). 10 embryos of 24 hours germinated seeds, weighed and macerated in 5 % acetic acid + 2 N HCl. The contents were incubated for overnight at 30°C. The solution was agitated with hypodermic syringe for proper maceration. Counts were made with the help of haemocytometer. Cell volume was calculated using the following formula.

\[
\text{Cell volume} = \frac{\text{Fresh weight of embryos}}{\text{Cell number}}
\]
2. **Histochemical parameters at 24 hours of germination as affected by pretreatments.**

**Histochemical studies:** Pretreated seeds along with controls were germinated in Petri dishes moistened with D.H. Shoot part was separated at 24 hours and fixed in Carnoy's (1 part of acetic acid + 3 parts of absolute alcohol) fixative. The tissue dehydration and paraffin infiltration processes were carried out as mentioned by Johansen (1940). Material containing paraffin block was fixed on block holder and after proper trimming sections were cut using microtome at 10 μ in series. Sections were affixed to slides by the use of an adhesive (egg albumin glycerine). Following parameters were studied.

1. Total protein content
2. Total nucleic acid content
3. Polysaccharides.

1. **Total protein content (Hazik et al., 1953):** Slides were deparaffinized and hydrated. Then these are kept in bromophenol (1 g of HgCl₂ and 50 mg of bromophenol in 100 ml of 2% aqueous acetic acid, solution for 30 minutes. After they were bleached in 0.5% acetic acid, washed and mounted. The absorption was measured at 590 - 620 nm. Control set was prepared by deaminating (keeping the sections in a mixture of 20 ml sodium nitrate and 10 ml of 1% acetic acid at room
temperature for 24 hours) and acetylation (placing the slides in 10% solution of acetic anhydride in pyridine at room temperature for 20 hours before staining (see: Jensen, 1962).

2. **Total nucleic acids** (Temer and Clark, 1960): Deparaffinized and hydrated slides were incubated for 10 minutes in water (pH 1.6). Then they were treated with gallocyanin (600 mg gallocyanin is dissolved in 200 ml of 5% chrome alum in a boiling water bath for 30 minutes, cooled pH adjusted to 1.6 with 1N HCl and volume made to 400 ml with D2O solution for 24 hours at 45°C. Again they were kept in water (pH 1.6), dehydrated and mounted. Control reaction were carried out by pretreating the sections with 5% boiling TCA for 15 minutes (Uabe, 1970). The absorption was measured at 500 - 570 nm.

3. **Polysaccharides** (Hotchkiss and McMannus, 1948): Slides were deparaffinized and treated with 0.5% periodic acid for 30 minutes. Then slides were washed and stained with Schiff’s reagent (0.5 g of fuchsin and 0.5 g of potassium metabisulphite is dissolved in 100 ml of 0.15 N HCl. Mixture was shaken at an interval of 2-3 minutes until dye is converted into fuchsin solution 0.3 g of fresh decoloring charcoal was added and shaken for 5 minutes, then filtered twice through filter paper. The reagent is stored in refrigerator in amber coloured bottle). Control - Periodic acid treatment is omitted.
Cytophotometry (Shah et al., 1975): The absorption of the exposed chromophore was measured by the help of cytophotometer. It records the transmittance of light passing through the histochemical preparation on an expanded scale of micrometer connected to a light dependent resistor. It can measure the light absorption of 10 μ in size. Sections were uniformly cut at 10 μ. Extinction or the absorption values were calculated by subtracting the log value of the stain reading from the log value of the control.

\[ \text{Extinction value} = \log_{10} \frac{I_0}{I_s} = \alpha K I \]

where
- \( I_0 \) = Background light coming through the control slide.
- \( I_s \) = Light transmitted through the histochemical preparation
- \( K \) = Extinction coefficient
- \( C \) = Concentration of chromophore
- \( l \) = Thickness of the section

\[ \text{content/cell or nucleus} = \text{Extinction value or nuclear value} \]

\[ \text{concentration/unit area} = \frac{\text{Extinction value/cell area or nuclear area}}{} \]

Content and concentration reflect the changes in the amount and concentration of the metabolite stained for respective dye. Cell area was measured with Camera lucida. The images
were drawn on the graph paper by projecting the section. The cell area was measured by counting the number of squares in the cell drawn. These divisions are again converted into micron squares (μ²) using stage micrometer. Camera lucida diagrams were drawn at 10 x 45 magnification for measuring cell area.

3. **Studies on the effect of pretreatments and number of cycles on the leaching behaviour of metabolites**

In order to know the amount and nature of the leachates given out by the pretreated seeds following experiment was undertaken. 1 g wheat seeds were soaked in 20 ml of sterilised distilled water in a test tube at room temperature (25-28°C). Leachates were collected in one set after 1, 6 and 24 hours while in the other set leachates were collected only after 24 hours and following parameters were recorded.

1. **Electroconductivity (μ mhos/cm² seed)**
2. **Sugars (mg/g initial seed wt.)**
3. **DNA (μg/g initial seed wt.)**
4. **Protein (mg/g initial seed wt.)**

1. **Electroconductivity**: Measurements of electroconductivity readings were done using Toshniwal conductivity bridge type Cl. 01/258 with cell constant 0.574 -1 cm. The cell is dipped in the total leachate (vol 20 ml) and the lids were connected to the terminal 'cell' with scale switch on "MHO"s position.
Balance is obtained by alternate adjustment of dial and phase control. Value of electroconductivity is the dial reading multiplied by suitable factor for that range switch. Electroconductivity readings are calculated by multiplying the conductance with cell constant (appropriate correction was done for temperature changes) and the results are expressed as umhos/cm/g initial seed wt.

2. **Sugar**: (Please see the method of Harten and McCarty, 1972).

3. **RNA**: (Please see the method of Harxham, 1955) and

4. **Protein**: (Please see the method of Lowry et al., 1955)

were determined in leachet and were expressed as mg metabolite/g initial seed wt.

**Experiment - III**

1. **Studies on growth and development of pretreated and control seeds raised under field conditions**.

   In order to test the effectiveness of the pretreated seeds and number of cycles (wheat seeds pretreated as discussed in the experiment no. 1) were sown in the field and standard agronomic practices regarding watering and manuring were followed. Untreated control were also raised for comparison. Following growth data of ten plants were recorded at 20 days interval up to 80 days.
1. Height of the plant (cm)
2. Dry weights of leaves, stem, root and spike (g)
3. Total plant dry weight (g)
4. Leaf area (cm;
5. Spike length (cm)
6. Spike number (No)
7. Total spike weight (g)

**Growth data:** Plant height (cm) was measured from ground to the tip of the spike of the main axis; then to the base of the spike of the main axis. Height was measured at an interval of 20 days of the plants which were fixed and tagged for final harvest data. Plant height of 10 plants were taken and mean values were taken out.

**Dry weight:** To record this data plants were watered previous day to take out the plants without loss of root system. The different parts were separated and washed. These parts (root, stem, leaf, spike) were kept in an oven at 60°C for 48 hours till constant dry weight was recorded. During flowering the length of spike, number of spikes, spike dry weight, grains/main spike were recorded. Leaf area was measured of the leaves on the main axis only of the plants which were fixed for stem height and final harvest data. Every 30 days, length and breadth of leaves were measured and leaf area calculated as \( L \times B \times 0.88 \) as per our preliminary findings and following, in principle, the method of Williams (1954) and Jain and Mishra (1968).
2. **Studies on growth indices, chlorophyll content, carotenoid content and mineral (K, Na, P) content during vegetative reproductive and senescence stages.**

1. **Relative growth rate (RGR)**
2. **Net assimilation rate (NAR)**
3. **Leaf weight rate (LWR)**
4. Chlorophyll 'a', chlorophyll 'b', a/b ratio, total chlorophyll (at vegetative, reproductive and senescence stages)
5. Carotenoid content (at vegetative, reproductive and senescence stages)
6. Mineral estimation (K, Na, P) at vegetative, reproductive and senescence stages)

1. **Relative growth rate (RGR)**: RGR is determined as difference between the Naperian logarithms of dry weights of dry weights of successive samples (20 days interval) as shown by Blackman (1919). The formula for RGR is as follows:

   $$ RGR = \frac{\log_{e}W_{1} - \log_{e}W_{0}}{t} $$

2. **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).

   $$ NAR = \frac{L_{1} - L_{0}}{\log_{e}L_{1} - \log_{e}L_{0}} $$
3. **Leaf weight ratio (LWR)**: The following formula is used for determing the leaf weight ratio.

\[ \text{LWR} = \frac{L_1 - L_0}{\log_{e} L_1 - \log_{e} L_0} \cdot \frac{w_1 - w_0}{\log_{e} w_1 - \log_{e} w_0} \]

where \( w_0 \) and \( w_1 \) represents the successive dry weights of the whole plant and \( L_0 \), \( L_1 \) represents the successive dry weights of the leaves.

4. **Chlorophyll estimation**: A fresh leaf was taken for chlorophyll estimation at vegetative (30 days), reproductive (60 days) and senescence (90 days) stages. Weighed material (200 mg) was crushed in 80% acetone and volume was made to 20 ml. O.D. was taken at 645 nm and 663 nm on Systronics colorimeter model 103. Chlorophyll 'a' and 'b' (mg/g fr wt) was calculated following the formulae of Arnon (1949).

\[
\text{Chl. a} = 12.7 \times \text{O.D. 663} - 2.69 \times \text{O.D. 645}
\]
\[
\text{Chl. b} = 22.9 \times \text{O.D. 645} - 4.68 \times \text{O.D. 663}
\]

5. **Estimation of carotenoids** (Jensen and Jensen, 1971): Leaf material was cut into small pieces and ground in 80% acetone. The extract was centrifuged and the residue was again extracted and centrifuged. The procedure was repeated until no more chlorophyll pigment could be extracted. The evaporated residue was dissolved in equal volume of petroleum ether and metholic OH (10%). The mixture was allowed to
stand at room temperature for 2 hours and then transferred
to a separating funnel. Separation of chlorophyll and
carotenoids was achieved by aqueous NaCl (5 %). The residue
was dissolved in acetone and reading was taken at 445 nm
in Systronics colorimeter. Carotenoids content was calculated
as follows:

\[ C = \text{D.V.F.} \times \frac{10}{2500} \]

where, \( C \) = Total carotenoid
\( V \) = Total volume
\( F \) = Dilution factor

6. **Estimation of mineral constituents**: Even dried ground
plant material was transferred to 50 ml kjeldahl flask. 10 ml
of nitric acid + 1 ml of perchloric acid + 1 ml of sulphuric
acid were mixed and heated until the fumes subsidised leaving
the digest colourless. The digest was made to desired
(50 ml) volume.

**Phosphorus** (Fiske and Subbarow, 1925): 1 ml of test solution
was mixed with 1 ml of acidic ammonium molybdate in 5 M \( \text{H}_2\text{SO}_4 \)
(100 ml - 15 ml conc. \( \text{H}_2\text{SO}_4 \) = 85 ml \( \text{D}_2 \)) and 0.5 ml reducing
agent (0.25 g 1, 2, 4 amino naphthol sulphonic acid, 1.5 g
\( \text{NaHSO}_3 \) and 1.25 g of \( \text{Na}_2\text{SO}_3 \) in 100 ml \( \text{D}_2 \)) was added. After
30 min 5 ml \( \text{D}_2 \) was added. O.D. was taken at 600 nm. By referring
to standard curve (Fig. 6) the amount of phosphorus present
was calculated. Sodium (Na) and potassium (K) were estimated
through systronics digital (Model-111) flame photometer.
STANDARD CURVE-PHOSPHORUS

OD

FIG6

PHOSPHATE (μg)

20

60

100
3. **Studies on the effect of foliar spray with GA (10^{-6} M), CCC (10^{-5} M), CCC (10^{-5} M) + GA (10^{-6} M) and De at anthesis stage for comparative studies with re-treatments.**

Untreated control of Arnej were raised in different plots and were given foliar spray (3 times with a 4 day interval) at the time of anthesis (60 days after sowing). Following parameters were recorded.

1. Plant height (cm)
2. Dry weights of stem, leaf, root and spike (g)
3. Total plant dry weight
4. Spike length (cm)
5. Spike number (No)
6. Total spike weight (g)
7. Main spike weight (g)
8. Number of grains/main spike (No)
9. Grain weight/main spike (g)
10. Tiller number (No)
11. 1000 grain weight (g).

4. **Studies on final yield attributes.**

Harvest data of the ten plants from each treatment were recorded for following parameters.

1. Plant height (cm)
2. Dry weights of stem, leaf, root and spike (g)
3. Total plant dry weight (g)
4. Spike length (cm)
5. Spike number (No)
6. Total spike weight (g)
7. Main spike weight (g)
8. Extra spike weight (g)
9. Number of grains/main spike (No)
10. Number of grains/extra spike (No)
11. Grain weight/main spike (g)
12. Grain weight/main spike (g)
13. Number of grains/extra spike (No)
14. Extra spike length (cm)
15. Tiller number (No)
16. 1000 grain weight (g).

Analysis of variance of harvest data and foliar spray was worked out and significance of the data at 1% and 5% level was expressed.