MATERIALS AND METHODS

Plant Material

Experiments were carried out on barley (Hordeum vulgare var. Ratna) and maize (Zea mays var. Kissan). Seeds were obtained from the Indian Agricultural Research Institute, New Delhi. Plants were grown in the field or in earthen pots, placed in the open. Barley was grown during October-March and maize during the months of April to September. Irrigation and fertilisers were provided whenever necessary.

At the time of experiments, the age of barley varied from 20 to 25 days while that of maize varied from 15 to 20 days. In both the materials, the second leaf, which was the youngest fully expanded leaf, was sampled for the estimation of different parameters.

Parameter of Leaf Age

Leaf Area - The leaf age in the two months old barley plants was characterized by the average leaf area. Leaf area was measured for the maximum width and length and an average was obtained by measurement from at least eight plants.

Weight of ear head - To characterise the relative age of the flag leaf, the ear head weight was found to be a more
reliable parameter than the leaf area itself. The average weight of ear heads from six to eight plants was determined.

Source of Chemicals

L(-) Proline was obtained from Merck Company, England. Other chemicals were obtained from BDH, India.

Study of Moisture Stress

Methods of Study - Moisture stress was studied under laboratory conditions as well as under field conditions.

Osmotic stress - In the laboratory, water stress was simulated by treating the plants with a solution of polyethylene glycol 6000 (PEG). The concentration of PEG could be adjusted to create osmotic potentials of different strengths, at different temperatures. The PEG solution was prepared in 0.1 L KNO₃ in all the experiments on barley while for maize seedlings, it was prepared in 0.01 L KNO₃. The PEG treatment was given either to intact plants by immersing their roots in PEG, or to preweighed discs (8 cm diameter) or segments, by floating them in PEG solution in 5 cm diameter petri dishes. The treatments were given for 2 to 4 hours in a BOD incubator. The treatments were given under a light intensity of 5700 lux, unless mentioned otherwise. The temperature was usually maintained at 25 C for barley and at 30 C for maize, unless otherwise specified.
In mature plants of brinjal, Xanthium and mung bean, where it was inconvenient to give the PEG treatment via the roots, leaves were excised at the base of the petiole and the cut end dipped in PEG solution. Similarly, in barley plants in the reproductive phase of growth, stems were excised at the first node from the top and the excised stem containing the flag leaf was dipped in the PEG solution.

The pretreatment of the intact plants with amino acids or hormones was carried out by immersing the roots in the respective solutions, prepared in 0.1 M KNO$_3$ or 0.01 M KNO$_3$. Simultaneously, part of the plants (referred to as untreated plants) were incubated in a solution of KNO$_3$ alone. After two hours of pretreatment, the plants were removed from the amino acid or hormone solution (or the nitrate solution) and the roots rinsed with water and nitrate solution successively. Half of the (treated as well as the untreated) plants were transferred to PEG solution while the other half (of the treated and the untreated plants) were transferred to KNO$_3$ solution. The latter constituted the control (unstressed) for each PEG treatment. The estimates of NR activity, RWC (relative water content), proline content etc. were usually made after 2 to 4 hours of the PEG treatment.

In the studies with leaf discs, discs were vacuum infiltrated with the solution of the amino acid to be
tested and then incubated in it. After incubation for 30 minutes, the discs were washed and blotted lightly. Half of the discs from each set were transferred to PEG solution while the remaining half were transferred to 0.1 M KNO₃, which served as the control. The NR activity was estimated after 4 hours of PEG treatment.

**Drought stress** - Water stress, as experienced by the plants in the field or pots, was studied by withholding irrigation. The stress was released by re-irrigating the plants.

A study was carried out to evaluate the response of maize plants to different levels of water stress and also to study their subsequent recovery after the release of stress. Maize was grown in a total of 64 pots which were arranged in four rows. The first row of pots contained the control plants, i.e., those which were irrigated every day. In the second row, plants were given moisture stress for 1 day. In the third row, the stress was given for 2 days and in the fourth row, the stress was given for 3 days.

On the first day of experiment, the initial values of the physiological parameters like leaf RWC, nitrato reductase activity, peroxidase activity, proline synthesis, proline content, protein and free amino acid content,
Fresh and dry weight were measured, using samples from the pots in the first row. After the sampling, only the remaining pots of the first row was irrigated, leaving the other rows unirrigated. On the second day, samples were taken from the pots in the second row. The remaining pots in the second row, along with those in the first row, were irrigated. The rest of the rows were left unirrigated. Samples from the re-irrigated pots in the second row were next taken on the third day, fifth day and eighth day to study the pattern of recovery of 1-day-stressed plants. On the third day, samples were collected from the third row, after which the remaining pots of the third row, along with those in the first and the second rows, were irrigated. The fourth row was left unirrigated. Samples from the reirrigated pots of the third row were also collected 1 day, 2 days and 5 days later in order to study the recovery after 2-day-stress. On the fourth day, samples were collected from the pots in the fourth row and the remaining pots in this row irrigated, along with those in the first, second and the third rows. Samples were collected from the reirrigated pots of the fourth row, 1 day, 4 days and 5 days, after the 3-day-stress period. Samples from the first row, which served as the control, were taken on all the days.
Parameters of Study - The response to moisture stress under different conditions was largely assessed in terms of relative water content, nitrate reductase activity and proline content.

Relative water content (RWC) - 2.5 cm long leaf segments were used to determine the RWC according to the method described by Weatherly (1950). The excised segments were weighed and floated in distilled water for three to four hours. The turgid weight was determined after carefully pressing the wet segment between filter papers to remove the adhering water. The segments were dried in an oven at 50°C till they attained a constant dry weight. Percent relative water content was calculated using the formula,

\[
\text{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100
\]

The values were taken from at least three leaves for a particular set.

Nitrate reductase activity - Nitrate reductase catalyses the conversion of nitrate to nitrite and the amount of nitrite released gives a measure of the nitrate reductase activity. The activity of the enzyme was determined by the in vivo method of Klepper et al. (1971). Leaf material was chopped into small segments with a blade. Segments (100 mg) were weighed and placed in 50 ml
Börnemeyer flasks containing 5 ml of 0.1 M KBrO₃. Three replicates were taken for each sample. The flasks were evacuated for 30 seconds and the vacuum released. During the infiltration process, the tissue in all cases was visibly wetted and sank below the surface of the solution. The flasks were then incubated at 30°C in dark, in a BOD incubator for 30 minutes. One ml of the sample from each flask was pipetted into a test tube. To this was added 1 ml of sulfanilamide (1 per cent in 1 N HCl) followed by 1 ml of 0.01 per cent N-(1-naphthyl) ethylene-diamine hydrochloride solution. The pink colour developed was read at 540 nm in spectronic 20. Nitrate reductase activity was computed using a standard curve of nitrite employing the above colour reaction. One unit of enzyme activity was defined as nanomoles nitrite released per hour of incubation at 30°C in dark. The activity was expressed as units per gram fresh weight (gFW) or as units per gram dry weight.

Proline content - This was estimated according to the method of Bates et al. (1973). Samples of 100 to 200 mg of leaf material were weighed and homogenized in 5 ml of sulphocalicylec acid. After homogenisation and centrifugation, the clear extract was made up to 5 ml. To 2 ml of 5 ml aliquot of the extract, an equal volume of acetic acid was added, followed by an equal volume of ninhydrin reagent
(2.5 per cent, prepared in a mixture containing 30 volumes of glacial acetic acid and 20 volumes of 6N phosphoric acid). This was placed in a boiling water bath for one hour, cooled immediately and extracted into 5 ml or 10 ml of toluene after shaking. The red chromophore was extracted into the upper layer and the absorbance was read at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

\[
\text{micrograms proline per gram fresh weight} = \frac{\text{mc proline per ml (from std. curve)} \times \text{volume of toluene}}{\text{gram sample}} \times \frac{\text{volume of total extract}}{\text{volume of aliquot}}
\]

From the above, proline could also be expressed as milligram per gram dry weight of the sample. All the samples were estimated in triplicate.

**Other physiological parameters of study** - The effect of drought stress was also evaluated in terms of the total protein and free amino acid content, as well as the rate of protein synthesis. In addition to nitrate reductase, the responses of two other enzyme activities, i.e., peroxidase and PEG carboxylase, were studied.

**Protein content** - Leaf material (0.1 gm) was homogenized in 5 ml of 10 per cent TCA. The homogenate
was centrifuged and the pellet was washed once with 5 per cent TCA. The supernatant and the washing were pooled, made upto 5 ml, and kept aside for estimation of amino acids. The pellet was digested in 5 ml of 1 N NaOH at 37 C. The insoluble material was removed by centrifugation and the amount of protein in the supernatant was determined according to the procedure of Lowry et al. (1951).

Free amino acid content - This estimation was carried out according to the method of Roberts and Smith (1971). The supernatant, obtained after homogenization of the plant material in 10 per cent TCA, as mentioned in the previous paragraph, was used for free amino acid estimation.

Reagents

Solution A - Prepared by dissolving 0.6 gm of stannous chloride in 500 ml of citrate buffer (0.2 M, pH 5).

Solution B - 20 gm of ninhydrin dissolved in 500 ml of ethylene glycol monomethyl ether.

Solution C - Prepared by mixing equal parts of solutions A & B.

Apparatus - To 0.1 ml of the amino acid extract in a boiling tube, was added 0.4 ml distilled water, 0.1 ml of 0.1 N NaOH and 5 ml of solution C. Tubes were covered with aluminium foil and kept in a boiling water bath for twenty minutes. They were then cooled and the absorbance read at 570 nm.
**Peroxidase extraction** - Leaf material (0.2 gm) was ground in 4 ml of 0.1 M phosphate buffer, pH 7.2 with 50 mg insoluble polyvinyl pyrrolidine, in a chilled mortar and pestle. The homogenate was centrifuged at 12,000 x g at 0°C for 20 minutes. The supernatant was used for enzyme assay.

**Enzyme assay** - Peroxidase activity was determined by recording a change in absorbance per 15 sec, at 610 nm, after adding an aliquot of 20 ul or 50 ul of supernatant to 5.0 ml of assay mixture. 100 ml of assay mixture contained 4 ml of benzidine solution, 1 ml of hydrogen peroxide (3 x 10^-2 M) and 95 ml of distilled water. Benzidine solution was prepared by dissolving 1 gm benzidine in 9 ml warm glacial acetic acid and then adding 36 ml distilled water (Scandalios, 1964). An arbitrary unit of peroxidase activity was defined as the change in absorbance of 0.01 per 15 sec. The activity was expressed as units per gram dry weight.

**Phosphoenolpyruvate carboxykinase** - The enzyme was assayed by the modifications of the method of Haruyama and Daniellano (1962).

**Enzyme assay** - Leaf material (0.2 gm), obtained from the topmost fully expanded leaf, was chopped and extracted with 4 ml of extraction mixture using ice cold mortar and pestle. The homogenate was centrifuged at 12,000 x g for 10 minutes at -5°C. The final volume of the clear extract
was made up to 4 ml. Extraction mixture contained 0.04 N Tris HCl pH 8.0; 0.01 N MgCl₂; 0.925 µl EDTA and 5 ml cysteine.

The reaction mixture contained the following in micro moles: NaHCO₃, 2.5; Cysteine, 1.25; EDTA 0.1; MgCl₂, 2.5 and 10 of Tris HCl pH 8.1 ± 0.1. For the assay of PEP Case, 1 micro mole of PEP was added to the reaction mixture. In addition 0.5 microcurie of NaHCl¹⁴O₃ or Na₂ C¹⁴O₃ was present in the reaction mixture. In the control tubes, the reaction was carried out in the absence of added PEP. The counts obtained in the control tubes were subtracted to obtain the actual counts. The reaction was started by the addition of 0.2 ml enzyme extract. The reaction was stopped after 10 minutes by adding 0.5 ml of 0.3 per cent semicarbazide hydrochloride prepared in 1 N HCl. The reaction was carried out in the vials. After completion of the reaction, the vials were dried and counted as described. One unit was defined as micromoles of CO₂ fixed per hour.

**Ratio of protein synthesis** - 5 discs (8 mm diameter) were placed in 5 cm diameter petri dishes, having 2 ml of medium containing 0.5 µg chloramphenicol, 0.1 micro mole sucrose, 5 micro moles phosphate buffer pH 7.2, and 1 micro curie of C¹⁴ Chlorella hydrolysate (30 µC/millimole). The incubation was carried out for 2 hours at 30 C in light of intensity 5700 lux. Then, the discs were washed with
distilled water and homogenized in 2 ml of 10 per cent 
TCA. The residue, obtained after washing with additional 
TCA, was digested in 1 ml of 1 N NaOH. The insoluble 
material was removed by centrifugation and 0.5 ml of 
aliquots of the supernate were counted.

**Assay of radioactivity** - The vials containing the 
radioactive solutions were dried at 50 C. The radioactivity 
was determined in a Packard Liquid Scintillation Spectro-
meter using PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-2; 
,5-phenyloxazolylbenzene) in toluene medium. PPO, 2 gm and 
50 mg POPOP were dissolved in 500 ml of toluene. Corrections 
for quenching were made.

**Study of Heat Stress**

Studies on heat stress were carried out in maize 
only. Heat stress was applied to maize seedlings by 
subjecting them to a temperature of 50 C or 40 C. For 
reference, another set of plants was maintained at 30 C. 
In both cases, the plants were kept in a solution of 0.01 M 
KNO₃. The stress was given for two hours at a light 
intensity of 5700 lux, in a BOD incubator saturated with 
water vapor.

In experiments involving pretreatment with certain 
hormones and amino acids, plants were incubated for 2 hours 
in the required solution, which was prepared in 0.01 M KNO₃.
Simultaneously, one set of plants, i.e., untreated plants, were incubated in a solution of 0.01 M KNO₃ alone. Plants were subsequently washed and one set was transferred to a fresh solution of 0.01 M KNO₃, maintained at 50 °C while the other set to a similar solution maintained at 30 °C.

To study the recovery from heat stress, the plants incubated at 50 °C as well those incubated at 30 °C were transferred to a fresh solution of 0.01 M KNO₃, in the absence or presence of the hormones or amino acids to be tested. This solution was maintained at a temperature of 30 °C.

Parameter of the study - As in moisture stress studies, nitrate reductase activity was chosen as a parameter for the studies on heat stress. The enzyme was assayed as mentioned before.