MATERIALS AND METHODS

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Source of seeds

Certified seeds of Vigna radiata (L.) wilczek var.Guj.2 were obtained from the State Department of Agriculture, Gujarat, India.

Rhizobium inoculum (cowpea rhizobium-GAU) was obtained from the plant pathology division of Gujarat Agricultural University (GAU), Anand, Gujarat.

Method of cultivation

Plants were raised in pots containing 7.0 kg garden soil (three volumes land soil and one volume compost) under wire house conditions. Rhizobium inoculated seeds of mungbean, 10 per pot were sown at a depth of 1 cm and were allowed to germinate. When seedlings were a week old they are thinned to 3 per pot. Pots were divided in to four groups for different treatments. On day 14, plants in the first, second and third groups of pots were sprayed with 500, 1000 and 1500 ppm solution of (2-chloroethyl) trimethyl ammonium chloride (CCC) in .02 % (v/v) Tween 20 respectively to the point of run off. The fourth group (control plants) received only sprays of distilled water containing 0.02% (v/v) Tween 20. After the treatments were given, the pots were arranged at random. On day 32nd (the day of flowering) half of the treated plants

were again sprayed with the same concentration of CCC and the other half along with the controls received only Tween-20 spray. 20 replicates were earmarked for recording the data on yield parameters. All pots were received about one litre water every three days. The maximum and minimum temperature during the growth period were 31 ± 3°C and 21 ± 2°C respectively.

Growth measurements

Plants were harvested at every 14 days, washed with water, blotted and shoots and roots were separated. Immediately after separating, the length and fresh weights of shoot and root system, and number and fresh weight of nodules were recorded. The separated shoots, roots and nodules were dried at 80°C for 72 h and dry weights were determined. Length of internodes and circumference of the stem at the 5th internode were measured at the time of final harvest.

Leaf area measurements

Leaf area measurements were made planimetrically. Total leaf area per plant is tabulated and expressed as cm^2.

Leaf Area Index (LAI)

Leaf area index was calculated as $\frac{LA}{GA}$ and expressed as cm^2/cm^2.

Net Primary Productivity (N.P.P.)

N.P.P. was calculated by using the formula $\frac{W_2-W}{t_2-t_1}$ and expressed as g. plant^{-1} day^{-1} (Leith 1965).

Net Assimilation Rate (NAR)

Net assimilation rate (mg cm^2 day^{-1}) was worked out as $\frac{W_2-W_1}{A_2-A_1} \times \frac{\ln A_2 - \ln A_1}{t_2 - t_1}$ (Briggs, Kidd and West 1920).

Where

W_1 = Initial dry weight
W_2 = Final dry weight
A_1 = Initial leaf area
A_2 = Final leaf area
LA = Total leaf area
GA = Ground area
T_1 = Time of initial observation
T_2 = Time of final observation

Leaf thickness

The leaf samples (middle leaf let of the 5th trifoliate leaf) were collected from plants and fixed on the spot in Craf III (Berlyn and Miksche 1976). Later each sample was trimmed to the required size and aspirated for 5-10 min and then left in the fresh fixative for 24-48 h. Dehydration of the samples was carried out in a TBA series and infiltrated with paraffin (56-58°C).(Berlyn and Miksche,1976). Transverse sections of 8-10 µm thickness were cut on weswox rotary microtome, and stained with aqueous Toluidine blue O (pH 4.4) (Sakai 1973).

The photographs were taken on orwo black and white film using Leitz dialux 22 photomicroscope. Leaf thickness was measured with the help of a micrometer.

Extraction and estimation of reducing sugars, total soluble sugars and starch.

The method used for the extraction of starch and sugar is essentially the one described by McCready et al. (1950).

50 mg powdered plant material (Oven dried plant materials were powdered using a mortar and pestle) was taken in centrifuge tubes. Few drops of cold 80% ethanol and 1 ml of distilled water were added to each tube. 10 ml of boiling 80 % ethanol was then added and the tubes were allowed to cool and were spinned at 10,000 g for 10 min. The supernatant was collected and the residue was repeatedly (4 times) extracted with hot ethanol and all the supernatants were pooled together. The supernatant was evaporated to dryness at 40°C. The residue was dissolved in a little warm water and after appropriate dilutions, the contents of total soluble and reducing sugars present in this sample were estimated according to the procedure of Yem and Willis (1954) and Somogyi (1952) respectively. To the residue remained after ehtanolic extraction 2.5 ml of distilled water was added. The tubes were then placed in ice water and 3.25 ml of diluted perchloric acid (52%) was added to each tube. 10 ml of water was added after 15 minutes and the residue was sedimented by

centrifugation at 10,000 g for 10 min. The supernatant was collected and the residue was extracted again with perchloric acid and the supernatants were pooled together. The final volume of the supernatant was dry filtered through Whatman filter paper No.1 and the residue was discarded. An aliquot of this extract was used for the estimation of its glucose content by the procedure mentioned above (Yemm and Willis, 1954). The estimated value of glucose was multiplied by 0.9 to convert it to starch.

Extraction of total nitrogen

50 mg of powdered plant material was digested with 2 N H_2SO_4 containing copper selenite (20 mg/100 ml) in a micro-kjeldahl's flask till the solution became colourless. The volume of the colourless solution was made up to 25 ml. The nitrogen content in an aliquot of digested sample was estimated using double iodide reagent (Umbreit et al.1959).

Extraction and estimation of total protein

50 to 100 mg powdered plant sample was suspended in 10 ml of 0.3 N KOH and incubated for 18 h at 37°C. The supernatant was collected by centrifugation of the suspension at 10,000 g for 15 min and the residue was washed four times with KOH. The supernatants were pooled and the final volume was made up to 25 ml with distilled water and an aliquot of this extract was

used for the estimation of protein content following a modified Lawry's method (Hartee,1972) using bovine serum albumin as standard.

Extraction, and estimation of total ureides

Finely ground plant material (50-150 mg) was suspended in 10 ml of extraction medium (equal volumes of 50 % ethanol and 0.1 M phosphate buffer pH 7.0) and heated in a water bath at 80°C for 5 min. The mixture was filtered through Whatman No.1 filter or centrifuged at low speed and decanted. Approximately 2 g (wet weight) of Dowex HCR-S resin (washed with 4 vol. water 2 vol. 0.5 M NaOH, enough water until the washings were neutral, 2 vol. 1 M HCl and enough water until the washings were neutral. The resin was drained, suspended in 50 % ethanol, well drained with vacuum filtration, and stored in a sealed bottle until use) was added to the vials containing the plant extract, and the vials were shaken in a reciprocating shaker for 30 min and the solutions were decanted from the resin. The content of the ureides in the extracts was estimated according to the procedure of Vogels and van der Drift (1970). 1 ml of sample was mixed with 2 ml of water and 1 ml of 0.5 N NaOH was added and the mixture was heated for atleast 4 min at 100°C. After cooling 1 ml 0.4 M phosphate buffer (pH 7) and 1 ml freshly prepared phenyl hydrazine solution (100 mg phenyl hydrazine HCl in 30 ml water) were added and the mixture was allowed to stand

for 5 min at room temperature. The tubes were cooled to 0°C in ice water bath and 5 ml concentrated HCl percooled to 0°C and 1 ml of freshly prepared ferricyanide solution (500 mg potassium ferricyanide in 30 ml water)were added. The tube were placed at room temperature and colour was read at 535 nm after 15 minutes.

Chlorophyll estimation

Fifty milligrams of fresh leaf tissue was homogenized in 80 % acetone and centrifuged at 3,000 g for 10 min. Chlorophyll levels in the supernatant, after appropriate dilutions, were determined spectrophotometrically according to the method described by Harborne (1984).

Preparation of enzyme extract

Fresh plant material was homogenized in cold 0.2 M borate buffer pH 8.6; 8 ml/g·fr wt) along with a pinch of glass powder, for 5 min using a chilled mortar and pestle. The homogenate was filtered through four layers of cheese cloth and centrifuged at 10,000 g for 10 min at 0°C. The protein present in the extract was precipitated by mixing it with chilled acetone at a ratio of 1:2 v/v at °C. The precipitated protein was sedimented by centrifugation (15,000 g) at 2°C for 15 min and was dissolved in 0.05 M acetate buffer pH 4.6 for the assay of amylase and acid invertase.

Total amylase assay

Total amylase activity was determined by the method of Bernfeld. The assay system (2.0 ml) consisted of 0.5 ml 0.1 M acetate buffer (pH 4.6), 0.5 ml 1 % soluble starch and 1.0 ml enzyme. The reaction was carried out at 30 ± 1°C for 60 min and an aliquot of 1.0 ml was estimated for the amount of maltose released using 3,5 dinitro salicylic acid. The enzyme unit is defined as the amount of enzyme required to liberate 1µmole of maltose per hour under the assay condition and the activity is expressed as units per mg protein.

Acid invertase assay

The activity of acid invertase was measured by estimating the reducing sugar produced in the assay system after an incubation period of one hour. The assay system (2.0 ml) contained 1.0 ml of 0.1 M acetate buffer (pH 4.6), 0.5 ml of 2 % sucrose and 0.5 ml enzyme extract. The reaction mixture was incubated at 30 ± 1°C for 60 min and the reaction was terminated by keeping it in boiling water bath for 10 min. An aliquot from the reaction mixture was used for the estimation of reducing sugars according to the procedure of Somogyi (1952). The enzyme unit is defined as the amount of enzyme required to produce 1 µg of glucose per hour under the assay condition and the enzyme activity is expressed as units per mg protein.

Nitrogenase assay

The nitrogenase was assayed by the acetylene reduction technique (Hardy et al.,1968). To measure acetylene reduction, plants were carefully uprooted and after removing the soil by washing in water, the root system possessing nodules were placed in 15 ml corning test tube and sealed by means of a serum cap. 2 ml of acetylene gas was injected into each tube and allowed to incubate for 1 h. After 1 h one ml of gas was withdrawn from the tube with an airtight syringe and injected in to a gas chromatograph (Chromatographic Instrument Company, India) fitted with chromosorb 104 column. Nitrogen was used as a carrier gas. Activity of nitrogenase was expressed as /u moles of C_2H_4 produced per g dry weight nodule per hour.

Pod growth

The first formed 2 or 3 flowers were tagged at the time of anthesis. The pods and the subtending leaves (source leaves) of the same age from all the treatments were collected after every 10 days and the fresh weights of the samples were recorded. The area of the subtending leaf was measured planimetrically. The samples were then dried at 80°C for 72 hours and dry weight were determined. Another batch of fresh samples from all the treatments were used for estimating the chlorophyll content and activity of amylase and invertase enzymes of the subtending leaf. The contents of starch, total

soluble sugars, reducing sugar, total protein and nitrogen of the ground dried samples were estimated as described earlier.

Yield

Yield parameters analysed included (a) total number of pods per plant (b) number of seeds per pod (c) total number of seeds per plant and (d) 1000 seed weight.

The values shown for extension growth, fresh weight and dry weight are the means of 15 measurements. In the case of total leaf area each value is the mean of 10 measurements and that of leaf thickness is the mean of 3 measurements. All the growth and yield data except that of leaf thickness have been subjected to statistical analyses. The values reported for various chemical and biochemical analyses are the average of 3 to 5 replicates $\pm$ SE.