CHAPTER TWO

MATERIALS AND METHODS

(A) Material:

C.cajan and P.tetragonolobus seeds collected from local farmers were sown in the plots of 100 m^2 area in the Botanical Garden, Willingdon College, Sangli. The plants were watered regularly every alternate day. Every care was taken to protect the plants from pests and fungal diseases. Healthy pods at their different developmental stages from anthesis were selected and harvested for analysis. Pods were harvested at 10, 15, 20, 25, 30, 35 and 40 days after anthesis. Every care was taken to keep uniformity in sampling by selecting the pods of uniform size and age, at every developmental stage in each plant.

(B) Methods:

(a) Pod Development:

To study pod development in C.cajan and P.tetragonolobus, pods at different developmental stages were selected. For each stage 100 pods were taken. Length of the pods in each developmental stage was measured from the point of attachment of the pedicel to the base of the style. Breadth of the pods was measured at the middle of the pods from edge of wing parallel to true axis of fruit and pod covers.

(b) Moisture Content:

The pods were first washed with tap water and then with

distilled water and blotted to dry. Seeds and pod-covers were weighed accurately for their weight and kept in the oven for drying at 60°C. After 7-8 days, the dried seeds and pod-covers, when showed constant weights, were used for further analysis. For the fresh and dry weight measurements, 100 pods at every developmental stage were used.

Moisture percentages of seeds, pod covers in seed and pod cover was calculated as follows:

$$\text{Moisture percentage } \frac{\text{Fresh wt.-dry wt.}}{\text{Fresh wt.}} \times 100$$

(c) Organic Constituents:

(i) Total Nitrogen:

Total nitrogen of seed and pod-cover was determined by the method of Hawk et al. (1948). 0.5 gm oven dried plant material was digested in a Kjeldahl flask with sulphuric acid (1:1 dilution) and a pinch of microsalt (mixture of anhydrous copper sulphate and potassium sulphate in the proportion of 1:40) till a colourless liquid was obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to the volumetric flask and the volume was made to 100 ml with distilled water. It was kept overnight and was filtered through the filter paper. The filtrate was used for the estimation of total nitrogen.

Two ml of this filtrate was taken in Nesslor's tube (35 and

50 ml marked). In other such tubes different concentration of standard ammonium sulphate (0.05 mg nitrogen ml^{-1}) were taken. One tube was kept as a blank without ammonium sulphate. To these tubes was added a drop of 8 per cent potassium bisulphate and 1 ml H_2SO_4 (1:1 whenever needed). The volume of all the tubes was adjusted to 35 ml with distilled water. Fifteen ml of Nesselor's reagent was then added in each tube. (Nesselor's reagent is a mixture of reagent A (7 g KI and 10 g HgI_2 dissolved in 40 ml distilled water) and B (10 g NaOH dissolved in 50 ml of distilled water) in the proportion of 4:5). The colour intensity of the orange-brown product ($NH_4Hg_2I_3$) produced by the reaction between NH_3 liberated from the sample and the reagent was measured at 520 nm in Specol-Carlzeiss Jena Hergestelt in der DDR. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

(ii) Enzymes of Nitrogen Metabolism

Nitrate Reductase and Nitrite Reductase:

Nitrate reductase (in vivo) was studied by Evans (1982) method 0.5 g of seed and pod-cover at different developmental stages were incubated separately in 5 ml mixture of 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO_3, and 5 per cent propanol taken in vials kept at 25°C in the dark. Incubation was continued for 1 hour. After this period nitrite produced in the enzymatic reaction was determined by mixing 0.5 ml reaction mixture with 0.5 ml each of 1 per cent sulphanilamide in 3 M HCl and 0.02 per cent N-1-napthyl-ethylene amide hydrocloride and kept for 20 minutes. After

appropriate dilution (3 ml with distilled water) the absorbance was read at 540 nm. Activity of the enzyme was expressed as g nitrite liberated hr^{-1} g^{-1} fresh weight.

(iii) Proteins:

Proteins of seeds and pod-covers were obtained by multiplying the total nitrogen content by a factor 6.25.

(iv) Proline:

Proline was estimated by the method of Bates et al. (1973). The extract was prepared in 3 per cent sulfosalicylic acid and filtered through Whatman No. 2 filter paper. Requisite amount (2 ml) of the extract was mixed with 2 ml of freshly prepared acid ninhydrin reagent (prepared by warming 1.25 g. of ninhydrin in 30 ml glacial acetic acid and 20 ml phosphoric acid (6 M) with agitation until dissolved. It was then cooled and stored at 4°C) and the whole reaction mixture was boiled in water both for one hour. It was then cooled rapidly at 0°C and the colour formed was extracted in 4 ml of toluene by vigorous shaking. Known concentration of L-proline was used to obtain the standard curve. The toluene chromophore was sucked by vaccupate in cuvette and optical density was read at 520 nm.

(v) Chlorophylls:

Chlorophylls were estimated following the method of Arnon

(1949). Chlorophylls were extracted in 80 per cent acetone from 0.5 g of fresh plant material. A pinch of $MgCO_3$ was added during homogenization to protect and stabilize the chlorophyll nucleus. This extract was filtered through Whatman No. 1 filter paper using Buchner's funnel. Residue was washed repeatedly with 80 per cent acetone collecting the washings in the same filtrate. The volume of the filtrate was made to 100 ml with 80 per cent acetone. In this extract liquor ammonia was added in the concentration of 4 ml per litre. The absorbance was read at 663 and 645 nm for chlorophylls a and b respectively. Chlorophylls (mg 100 g^{-1} fresh tissue) were calculated using the following formula (Arnon, 1949):

Chlorophyll a = (12.7 x A 663) - (2.69 x A 645) = 'X'

Chlorophyll b = (22.7 x A 645) - (4.68 x A 663) = 'Y'

Total Chlorophylls = (8.02 x A 663) + (20.2 x A 645) = 'Z'

$$\left.\begin{array}{l}\text{Chlorophyll a or}\\ \text{Chlorophyll b or}\\ \text{Total Chlorophylls}\\ \text{(mg 100 } g^{-1} \text{ fresh tissue)}\end{array}\right] = \frac{\text{'X'/'Y'/'Z' x Vol. of Extract}}{1000 \times \text{wt of material (g)}} \times 100$$

(vi) Carbohydrate Metabolism:

(a) Carbohydrates:

Carbohydrates were estimated according to the method described by Nelson (1944). 1 g fresh plant material was homogenised in morter with pestle and extracted with 80 per cent alcohol. It was filtered through Buchner's funnel using Whatman No. 1 filter paper. The filterate was

used for starch determination. The filterate thus obtained was condensed on a water bath till the volume was about 3 ml and treated with lead acetate and potassium oxalate (1:1) to decolourize it. To this distilled water was added and filtered. It was again washed with distilled water 2-3 times collecting the washings in the same filterate. This filterate was used for estimation of reducing sugars (A). A known volume of this extract was hydrolysed with N/HCl in an autoclave at 15 lbs pressure for half an hour. The contents were cooled, neutralized with Na_2CO_3 and filtered. The filterate was used for the estimation of total (reducing + non-reducing) sugars (B).

The residue in the first filteration (ethanol extract) was transferred to a conical flask with 50 ml of water and 2.5 ml of concentrated HCl. This was hydrolysed, neutralized and filtered as stated above. This filterate contains reducing sugars produced as a result of hydrolysis of starch. The sugars so available were estimated to determine the starch present in the tissue (C).

The requisite quantity (preferably 0.1 ml) of the above filterates A,B and C was taken separately in 10 ml marked test tubes. In other such test tubes different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of standard glucose solution (0.1 mg ml^{-1} were taken. 1 ml of Somogyi's alkaline copper tartarate solution [4 grams $CuSO_4$, $5H_2O$, 24 g anhydrous Na_2CO_3, 16 g Na-K-tartrate (Rochelle salt), and 180 g anhydrous Na_2SO_4 dissolved in 1000 ml distilled water] was added to each tube. All the reaction mixtures were then subjected to

boiling water bath for about 10 min.. After cooling to room temperature 1 ml of areseno molybdate reagent (25 g ammonium molybdate in 450 ml distilled water, to which were added 21 ml concentrated H_2SO . To this was then added 3 g sodium arsenate, Na_2HASO_4 , $7H_2O$;dissolved in 25 ml distilled water. All ingredients were mixed well and the solution was placed in incubator at 370 °C for 48 h before use) was added to each reaction mixture. The contents of each test tube were then diluted with distilled water to a volume of 10 ml. A blank was prepared by the same way without sugar solution. After 10 minutes the absorbance of each reaction mixture was read at 560 nm on spectrophotometer (Electronic corporation of India).

From the glucose standard curve, the sugar percentage in the above three fractions was determined.

(b) Enzyme:

α-Amylase:

Enzyme α-amylase was determined following the method of Katsumi and Fukuhara (1969).

0.5 g of thoroughly washed fresh plant material (seed and pod cover) was homogenised in a morter with pestle at about 0 to 4 °C temp. Enzyme was isolated in 20 ml cold acetate buffer (0.1 M, pH 5). The extract was filtered through 4 layered muslin cloth. The filterate was centrifuged at 10,000 X g and supernatant was saved and stored at 0 to 4 °C temp. This supernatant was

used as an enzyme source.

The assay mixture for α-amylase contained 1 ml 0.2 per cent amylase (pH 7), 1 ml acetate buffer (0.1 M, pH 5) and 1 ml enzyme preparation. The reaction was started by the addition of enzyme. The enzymatic reaction was terminated by the addition of 10 ml 0.5 N acetic acid, after 30 min.. In another such reaction mixture the enzymatic reaction was terminated at the beginning only (0 min. initial reaction). The amount of amylase unutilised during enzymatic reaction was determined by mixing 1 ml of the reaction mixture with 10 ml of Iodine solution (dil I_2 + KI). The optical density of blue, violet colour developed was read at 700 nm on spectrophotometer by using reagent blank.

The activity of enzyme amylase was calculated by the formula -

$$\text{D.B. Value} = 2 \times \frac{d-D}{d} \times \frac{100}{10}$$

where d = Optical density at zero min.

D = Optical density after 30 min.

Activity of enzymes is expressed as D.B. values.

(vii) Polyphenols:

Polyphenols were estimated by the method of Folin and Denis (1915). Polyphenols from fresh material were extracted in 80 per cent acetone (30 ml). Extract was filtered through Whatman

No. 1 filter paper using Buchner's funnel under suction. Polyphenol extracted was made to 50 ml.. This filterate was used for the estimation of polyphenols.

0.5 ml of the filterate was taken in a 50 ml marked Nesselor's tube. In other such tubes different concentrations (0.5, 1, 3, 4 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml of 20 per cent Na_2CO_3 were then added to each tube to make the medium alkaline. 2 ml of Folin Denis reagent (100 g of N-tungstate and 20 g of phosphomolybdic acid dissolved in 200 ml distilled water were mixed with phosphoric acid 25 per cent. It was refluxed for 2½ hours, cooled to room temperature and diluted to one litre with distilled water) were then added to each test tube and finally the volume was made to 50 ml with water. A blank was prepared without polyphenols, which helped to determine calibration curve. The ingredients were allowed to mix thoroughly. After some time the optical density of each reaction mixture was read at 660 nm on spectrophotometer. Polyphenols were calculated from the calibration curve of standard tannic acid.

(c) Inorganic Constituents:

Preparation of Acid digest for the estimation of different inorganic constituents (except chlorides), an acid digest was prepared following the method of Toth et al., (1948). 0.5 g of the oven dried, powdered material was transferred to 150 ml capacity beaker to which 20 ml concentrated HNO_3 were added. The beaker was covered

with watch glass and was kept till the primary reactions subsided. It was then heated slowly to dissolve solid particles. After cooling to room temperature, 10 ml of perchloric acid (60 per cent) was added to it and mixed thoroughly. It was then heated strongly until a clean and colourless solsution (about 2-3 ml) was obtained. While heating, the liquid was not allowed to dry. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day it was filtered through dry Whatman No. 44 (ashless) filter paper and the filterate was used as the source of different inorganic constituents.

(i) Potassium (K), Sodium (Na) and Calcium (Ca):

Potassium, sodium and calcium were estimated by flame photometry following the procedure standardized in our laboratory. Stock solutions of known concentrations in parts per million (ppm) of K in KCl (1 to 10 ppm), Na in NaCl (2 to 20 ppm) and Ca in $CaCl_2$ (20 to 200 ppm) were used for calibration curves. From these calibration curves the concentrations of K, Na and Ca respectively in the said digested samples were calculated.

(ii) Phosphorus (P):

Phosphorus was estimated following the method of Sekine et al.[1865]. One ml of acid digest was pipetted in a test tube to which 2 ml of 2N HNO_3 were added followed by 1 ml of molybdate vanadate reagent (A-1.25 g of ammonium molybdate dissolved in 500 ml N HNO_3. B-25 g of ammonium vanadate in 500 ml distilled

water. Then A and B were mixed in equal volumes). The volume was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes. The yellow colour developed with molybdate vanadate reagent was measured spectrophotometrically at 420 nm using reagent blank, compared with the colour density of known standards of phosphorus in KH_2PO_4 (0.025 mg ml^{-1}) and the amount of phosphorus in the plant material was calculated.

(Standard P solution was prepared by dissolving 0.11 g of monobasic potassium phosphate in distilled water and by adjusting the volume to one litre with one ml conc. HNO_3. This solution contained 25 ppm phosphorus).

(iii) Magnesium (Mg):

Estimation of Magnesium:

Mg^{2+} was estimated following the method described by Drosdoff and Nearpass (1948).

Five ml of acid digest was mixed with 1 ml of hydroxylamine hydrochloride (5 per cent W/v), 5 ml of 2 per cent starch compensating solution (Equal volumes of freshly prepared starch solution, 2 per cent and compensation solution of 3.7 g calcium chloride, 0.74 g of Aluminium sulphate, 0.36 g of Manganese chloride and 0.6 g of Trisodium phosphate dissolved in distilled water containing 10 ml concentrated HCl and then volume was made to one (litre) 1 ml.. Thiozole yellow (1 per cent) and 5 ml HaOH (2.5 N freshly prepared).

Final volume was made to 50 ml with distilled water. It was allowed to stand for 30 min and then the colour intensity was measured at 525 nm on UV double beam spectrophotometer (uv-VIS-190, Shimadzu) using reagent blank. Standard Mg^{2+} soluton used was 0.2 mg ml^{-1}

(iv) Iron (Fe):

Fe^{3+} was determined by the method described by Durie et al. (1965). In this method ferric ions are reduced to ferrous ions. The colour developed between the latter and O-phenenthroline is read at 510 nm.

Five ml of acid digest and 5 ml of standard iron solution (3 µg Fe_2O_3 ml^{-1}) were taken separately into 50 ml marked test tubes. One more 50 ml marked test tube was reserved for reagent blank containing no iron solution. To this 10 ml of hydroxylamine hydrochloride (10 per cent W/v) were added followed by small piece of congo red paper. It was mixed well. The acetate buffer solution (140 g sodium acetate in distilled water + 60 ml acetic acid, diluted to 1 litre with distilled water) was added dropwise until the indicator paper changed just from blue to red. Eight ml of O-phenenthroline (0.25 per cent W/v) were then added, volume was adjusted to 50 ml with distilled water and optical density was read at 510 nm against reagent blank.

By comparing the optical density of the test solution with that of standard Fe_2O_3 solutions, total amount of Fe in the plant

material was calculated.

(v) Manganese (Mn), Copper (Cu) and Zinc (Zn):

Mn^{2+}, Cu^{2+} and Zn^{2+} were estimated following the standard procedure on Atomic Absorption Spectrophotometer (Varien Techtron, Model 1100). For the estimation of these elements, plant samples were digested by the standard method (Black, 1965). One of dry materials was taken in a Kjeldahl flask and to this was added 10 ml of acid mixture 75 ml HNO_3 + 15 ml H_2SO_4 and 30 ml $HClO_4$). It was digested on low flame first, then vigorously till white clear mixture remained. It was coolod and filtered through acid washed Whatman filter paper No. 44. Filterate was made to volume (100 ml) and used for estimation.