MATERIALS AND METHODS

A) Plant Material :

Sesbania grandiflora Pers., Moringa pterygosperma Gaertn.Fruct., Emblica officinalis Gaertn.Fruct., Tamarindus indica Linn., Azadirachta indica A.Juss., Acacia arabica Willd., Polyalthia longifolia Benth.and Hook, and Leucaena leucocephala, Linn., growing in the University Campus conditions were used for the present study. The studies were carried out in the conditions where day light intensity was 0.64 Cal Cm^{-2} min^{-1} and with day night temperatures, 30° and 14°C respectively, particularly in winter season. As every tree species has its own and different life span, the sampling of leaves at different developmental stages was done with respect to their size and pigmentation. The developmental stages at which the leaves were selected are -

1. Young and vigorously growing,
2. Fully expanded green and mature, and
3. Senescent leaves.

In addition to leaves, stem, bark, fruits, and seeds of the above mentioned tree species were used for analysis.

The plant material was first washed with tap water and then with distilled water and blotted to dry and cut into small

pieces. It was weighed accurately and used for the physiological investigations.

B) Methods :

1. Organic constituents :

Organic constituents like moisture content, chlorophylls, carotenoids, carbohydrates, polyphenols, proteins and proline were determined from the fresh plant material. However, proline was determined from both oven dried as well as fresh plant material.

a) Moisture content : Moisture percentage was determined by subjecting accurately weighed fresh plant material to oven drying at 60°C for eight days till a constant dry wt was obtained and finding the loss of moisture.

b) Chlorophylls : Chlorophylls were estimated following the method of Arnon (1949). Chlorophylls were extracted in 80% acetone from 0.5 g of fresh plant material. A pinch of $MgCO_3$ was added during homogenization to protect and stabilise the chlorophyll nucleus. This extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue was washed repeatedly with 80% acetone collecting the washings in the same filtrate. The volume of the filtrate was made to 100 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophyll a and b respectively.

Chlorophylls (mg g^{-1} fresh tissue) were calculated using the following formulae (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

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

c) Carotenoids :

Carotenoids were estimated by reading the absorbance of the above acetone extract at 480 nm (Kirk and Allen, 1965). Total carotenoids were estimated using the formulae by Liaaen-Jenus and Jensen and Jensen (1971) :

$$C = D \times \frac{v.f.10}{2500}$$

$$\text{Total carotenoids (mg .100 } g^{-1}) = \frac{A\ 480 \times \text{Volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}}$$

Where C = Total carotenoids in mg

D = Optical density

V = Total volume in ml.

f = dilution factor and

2500 = Average extinction.

d) Carbohydrates :

Carbohydrates were estimated according to the method described by Nelson (1944). 2 g fresh plant material was homogenised in mortar with pestle and extracted with 80% ethanol. It was filtered through Whatman No.1 filter paper using Buchner's funnel. The filtrate was used for estimation of soluble sugars. While the residue on filter paper was used for starch determination. The filtrate so obtained was condensed on boiling water bath till the volume was reduced to about, 1-2 ml, cooled and treated with lead acetate and potassium oxalate (1:1) to decolourise it. To this about 40 ml distilled water was added and filtered. The residue on filter paper was washed with distilled water 2-3 times, collecting the washings in the same filtrate. The volume of the filtrate was recorded. A known volume of this extract was hydrolysed with 1N HCl in autoclave at 15 lbs pressure for half an hour. The contents were cooled, neutralised with Na_2CO_3 and filtered. The filtrate was used for estimation of soluble (total) sugars.

The residue obtained in the first filtration (ethanol extract) was transferred quantitatively to a conical flask with 50 ml distilled water and 5 ml concentrated HCl. This was hydrolysed, neutralised and filtered as stated above. This filtrate contains reducing sugars produced as a result of hydrolysis of starch.

The requisite quantity (soluble sugars, 0.1 ml and starch, 0.05 ml) of the above filtrates was taken separately in 10 ml marked test tubes. In other such test tubes different concentrations (0.1, 0.2 and 0.4 ml of standard glucose solution, 0.1 mg ml^{-1}) were taken. 1 ml of somogyi's alkaline copper tartarate solution (4 g $CuSO_4.5\ H_2O$; 24 g anhydrous Na_2CO_3; 16 g Na-K-tartarate, 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 arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water, to which were added 21 ml concentrated H_2SO_4. To this was then added 3 g sodium arsenate, $Na_2H\ AsO_4$, $7\ H_2O$, dissolved in 25 ml distilled water. All ingradients were mixed well and the solution was placed in an incubator at 37°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 (10 ml). A blank was prepared by the same way but without sugar solution. After 10 min the absorbance of each reaction mixture was read at 560 nm on spectrophotometer (UV-VIS, Shimadzoo Japan). The sugar percentage was calculated with the help of standard curve of glucose.

e) Polyphenols :

Polyphenols were estimated by the method of Folin and Denis (1915). 0.5g fresh plant material was homogenized in

mortar with pestle and extracted with 80% acetone (30 ml). Extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Polyphenols were extracted repeatedly from the residue. The volume of the filtrate was made to 50 ml. This filtrate was used for the estimation of polyphenols.

2 ml of the filtrate was taken in a 50 ml marked Nesseler's tube. In other such tubes different concentrations (0.5, 1, 2 and 4 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml 20% Na_2CO_3 were then added to each tube to make the medium alkaline. 2 ml of Folin Denis reagent (100 g of sodium tungstate and 20 g of phosphomolybdic acid dissolved in 200 ml distilled water were mixed with phosphoric acid, 25%). It was refluxed for 2.5 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 polyphenolics, which helped to determine calibration curve. After some time the optical density of each mixture was read at 660 nm on spectrophotometer. Polyphenols were calculated from the calibration curve of standard tannic acid.

Polyphenols from the leaves of E.officinalis, A.arabica, P.longifolia and L.leucocephala at different developmental stages such as young, mature and senescent were analysed qualitatively following the technique of two dimensional paper

chromatography.

2 g of plant material (leaves) well washed, blotted to dry and cut into small pieces, was homogenized in mortar with pestle. Polyphenols were extracted in about 50 ml of 80% alcohol. The extract was filtered through Whatman No.1 filter paper using Buckner's funnel under suction. The residue on filter paper was repeatedly washed with 10 ml aliquots of 80% alcohol. The filtrate so obtained was condensed to about 1 ml in evaporating dish under reduced pressure on boiling water bath. After cooling to room temperature, the contents in the dish were dissolved in about 1 ml distilled water. The solution was centrifuged at full speed for 10-15 min and the supernatent was carefully decanted, measured and stored at 0.4°C temperature. This extract was used to separate polyphenols.

In two dimensional paper chromatography the solvent system used were -

I Direction - 1. n-butanal:acetic acid:water (80 : 20 : 44 v/v/v),
II Direction - 2. 2% Acetic acid.

The phenolic compounds separated on chromatogram were spotted, identified and confirmed by co-chromatography of authentic samples following their colour reactions and Rf values. The position of individual phenolic compound on the chromatogram was determined by marking its flourescent area under UV light as well as in UV light in presence of ammonia. Phenolic compounds

that could not be located by these methods were compared with chromatograms developed with the mixture of 0.3% $FeCl_3$ and 0.3% $Fe_4(CN)_6$ in equal amounts for phenols.

f) Proteins :

Proteins were estimated according to the method described by Lowry et al. (1950). 0.5 g fresh plant material was homogenized in mortar with pestle and extracted with 80% ethanol (20 ml). Extract was condensed on a water bath till volume was about 2,3 ml. To this 20 ml distilled water was added and filtered through Whatman No.1 filter paper. The filtrate was used for estimation of soluble proteins.

0.1 ml of the filtrate was taken in a test tube. In other such test tubes different concentrations (0.1, 0.2, 0.3 and 0.4 ml) of standard protein solution (egg albumin, 0.1 mg ml^{-1}) were taken. 5 ml reagent C (50 ml of reagent a was mixed with 1 ml of reagent b. This reagent is stable for one day. Reagent a = 2% Na_2CO_3 in 0.1 N aquious NaOH, reagent b = 0.5 g $CuSO_4$, 5 H_2O in 1% sodium tartarate) was added to each reaction mixture. It was kept for 15 min at room temperature. Then 0.5 ml of Folin Phenol reagent. (100 g sodium tungstate, Na_2W_4, H_2O and 25 g sodium molybdate, Na_2MoO_4, 2 H_2O were added to 700 ml distilled water). To this 50 ml 85% phosphoric acid and 100 ml concentrated HCl were added. This mixture was then refluxed gently for 10 hours using water condenser. To

this mixture, 150 g Lithium sulphate, 50 ml distilled water and few drops of bromine water were added. It was boiled for about 15 minutes without water condenser to remove excess of bromine. Cooled to room temperature and adjusted to 1 N acidity by titrating it against 1 N NaOH), was added to each reaction mixture. After 30 min the absorbance was read at 660 nm on spectrophotometer.

g) Proline :

Proline contents were determined by the method of Bates et al. (1973). The extract was prepared in 3% sulfosalicylic acid and filtered through Whatman No.1 filter paper. Requisite amount (2 ml) of the extract was mixed with 2 ml of freshly prepared acid ninhydrin in 50 ml glacial acetic acid and 20 ml phosphoric acid, 6 M, with agitation until dissolved. It was then cooled and stored at 14°C and the whole reaction mixture was boiled in water bath for an hour. It was then cooled immediately at 0°C and the colour formed was extracted in 4 ml of toluence by vigorous shaking.known concentrations of L-proline were used to obtain the standard curve. The toluene chromophore was sucked by vaccupippete in cuvette and optical density was read at 520 nm on spectrophotometer using toluene blank.

2) Enzymes of nitrogen metabolism :

a) Nitrate reductase and Nitrite reductase :

Nitrate reductase (E.C. 1.6.6.1) was studied by in vivo method (Evans, 1982). Leaf disks, each of about cm^2 area, were placed in 10 ml of medium containing 0.1 M KNO_3, 5% (v/v) Triton X-100 and 0.2 M phosphate buffer at pH 7.5. The medium with leaf disks was vaccume infiltered and incubated for one hour in the dark at 28°C. After one hour 1 ml reaction mixture was assayed for NO_2 by adding 1 ml (N-(1-naphthyl) ethylenediamine dihydrochloride (0.1 g in 500 ml distilled water) and 1 ml sulphanylamide (1% sulphanylamide in 1 M HCl). The colour was allowed to develop for 15 min and the absorbance was read at 540 nm.

Nitrite reductase (E.C. 1.7.7.1) was measured by the same procedure except that 0.1 M KNO_3 was replaced by 0.3 mM KNO_2.