CHAPTER III :

MATERIALS & METHODS

A. MATERIALS :

It is very interesting to study the biochemistry and physiology of ferns. Hence the ferns were collected from wild area as well as from the garden for these studies.

The two ferns growing in different climatic conditions were selected for the studies. One is Gymnopteris contaminans Bedd. growing luxuriantly in the forest of Castle Rock and the other is Nephrolepis exaltata Schott. the most common garden fern. The identification of these ferns was done with the help of 'The Ferns of Bombay' written by E. Blatter and d' Almeida.

Physiology of the various parts viz. root, stolon, rhizome, rachis and leaflet was studied in the vegetative as well as in the reproductive stage of Nephrolepis exaltata Schott.

Gymnopteris contaminans Bedd. shows dimorphism of leaves and hence physiology of various parts viz. root, rhizome, rachis and the leaflet was studied in the vegetative stage as well as in the reproductive stage.

All the parts were washed first with tap water and then with distilled water. The plant parts were then separated for analysis.

For the present studies the plant species had been collected and phytochemistry is studied taking the parameters as Chlorophylls (photosynthetic pigments), TAN, Carbohydrates, Reducing sugars, Total sugars etc. Mineral nutrition, Nitrogen content, Organic and Inorganic constituents were studied separately from the vegetative as well as reproductive stages.

All the parts of the plant viz. roots, stolon, rhizome, rachis, leaflet were separated and blotted to dry. Then the fresh material was used for Chlorophyll, TAN, Nitrite and Nitrate reductase estimation and the remaining material was kept in the oven for drying. This dried material was used for analysis.

B. METHODS :

a. PHOTOSYNTHETIC PIGMENTS (CHLOROPHYLL) :

The chlorophylls from the leaves and rachis were estimated by following method of Arnon (1949). For this vegetative (before spore formation) and reproductive (after spore formation) leaflet and rachis were washed first with tap water and then with distilled water and then were blotted to dry and cut into small pieces. This material was homogenised in 80%

acetone and filtered through Whatman No.1 filter paper under suction by using Buchner's funnel. The residue was washed thoroughly 2-3 times with 80% acetone, collecting all the washings in the same containers. Final volume was made to 100 ml with 80% acetone.

The preparation of plant extract for chlorophylls was doe at 0 to 4°C in dark. The absorbance was taken at 663 nm and 645 nm for chlorophyll 'a' and Chlorophyll 'b' and total chlorophylls were calculated by the formula suggested by Arnon (1949).

Chlorophyll a = 12.7 x A663 - 2.69 x A645 = X

Chlorophyll b = 22.9 x A645 - 4.65 x A663 = Y

Total Chlorophyll (a+b)

= 8.02 x A663 + 20.2 x A645 = Z

$$\text{Chlorophyll a/b or Total (mg/100 gm)} = \frac{\text{X/Y/Z x Volume of extract x 100}}{\text{1000 x wt. of plant material (gm)}}$$

b. TITRATABLE ACID NUMBER (TAN) :

The TAN (Titratable Acid Number) of fresh plant material was determined by the method of Thomas and Beevers (1949).

The fresh tissue was washed and rinsed with distilled water and blotted to dry. It was accurately weighed (0.5 gm), The plant material was taken in 150

ml beaker and 50 ml distilled water was added to it and boiled for half an hour. After boiling it was filtered through muslin cloth and final volume of the extract was recorded. Then 10 ml of this filtrate was titrated against standardised N/40 NaOH (dissolve 1 gm of NaOH in distilled water and adjust the final volume to 1000 ml) using phenolphthalein as an indicator till permanent pink colour is obtained and then the readings were recorded.

Standardization of NaOH was done by N/40 oxalic acid (dissolve 1.75 gm of oxalic acid in distilled water, adjust the final volume to 1000 ml) using phenolphthalein as an indicator.

From the above readings TAN was calculated by using formula,

$$TAN = \frac{\text{Vol. of oxalic acid taken for titration ml.}}{\text{Titration reading ml.}} \times \frac{\text{Total vol. of extract ml.}}{\text{wt. of plant material gm.}} \times \frac{\text{Plant extract reading (ml)}}{\text{Vol. of extract taken for titration ml.}} \times \frac{100}{4}$$

c. CARBOHYDRATES :

Carbohydrates (total sugars, reducing sugars, non reducing sugars and starch) were estimated spectrophotometrically by the method of Nelson (1944).

Some known quantity of plant material or plant parts were homogenised in mortor with pestle and extracted with 80% ethanol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used for estimation of soluble sugars while the residue used for starch estimation.

The filtrate thus obtained was condensed on a boiling water bath till the volume was reduced to about 3 to 5 ml and treated with lead acetate and potassium oxalate (1gm:1gm) to decolourise it. To this 20 ml distilled water was added and filtered. It was again washed with distilled water two to three times, collecting the washing in the same filtrate. This filtrate was used for estimation of reducing sugars.

20 ml of this extract was hydrolysed with HCl (2m) in a pressure cooker at 15 lbs pressure for half an hour. The contents were cooled, neutralised with Na_2CO_3 and filtered through filter paper. The filtrate was used for the estimation of total sugars.

The residue obtained in the first filtration was transferred to a conical flask with 50 ml of distilled water and 5 ml of 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 sugars so available were estimated to determine the starch present in the tissue.

The requisite quantity (preferably 0.1 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, 0.3, 0.4 and 0.5 ml) of standard glucose solution (0.1 mg/ml) were taken. 1 ml of Somogyis alkaline copper tartarate solution (4 gm $CuSO_4$ $5H_2O$, 24 gm anhydrous Na_2CO_3, 16 gm Na-K tartarate, Rochelle salts and 180 gm anhydrous Na_2SO_4 dissolved in 1000 ml distilled water) was added to each test tube. All the reaction mixtures were then subjected to boiling water bath for about 10 minutes. After cooling to room temperature 1 ml of arsenomolybdate reagent (25 gm ammonium molybdate in 450 ml distilled water, to which 21 ml concentrated H_2SO_4 were added. To this 3 gm sodium arsenate, Na_2HASO_4 $7H_2O$ dissolved in 25 ml distilled water. All ingredients were mixed well and the solution was placed in an incubator at 37°C for 48 hours before use) was added to each reaction mixture.

The contents of each test tube were then diluted with distilled water to a volume of 50 ml. A blank was prepared in the same way but without sugar solution. After 10 minutes the absorbance of each reaction mixture was read at 560 nm on spectrophotometer.

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

d. PROLINE :

Proline content of root, stolon, rhizome, rachis and leaflet of Nephrolepis exaltata Schott. in both vegetative and reproductive stages while in Gymnopteris contaminans Bedd. proline content in root, rhizome, rachis, leaflet in vegetative stage as well as reproductive stage has been determined following the method of Bates et al. (1973)

For this 0.5 gm oven dried plant material was homogenised in 10 ml sulphosalicylic acid (3 %) and the extract was filtered through whatman No. 1 filter paper. For assay known quantity of filtrate was mixed with 2 ml of acid Ninhydrin reagent. (Mixture of 1.25 gm Ninhydrin, 30 ml glacial acetic acid and 20 ml 6m orthophosphoric acid, heated for few minutes till ninhydrin was completely dissolved and kept in freeze at $0^{o}c$). The contents were boiled for 1 hour on boiling water bath and then cooled rapidly in freeze ice bath. 4 ml of toluene was added to each test tube and vigorously shaken for few seconds. The absorbance of toluene chromophere was recorded at 520 nm against toluene as a blank. Standard curve of proline (0.1 mg ml^{-1}) was prepared taking different concentartions of proline. From this standard curve the proline content of plant material was calculated.

e. POLYPHENOLS :

Polyphenols were estimated from the various parts using the oven dried powdered material by Folin-Denis method (1915).

Polyphenols from dried plant material were extracted in 80% acetone (30 ml). The 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 this filtrate was taken in 50 ml marked Nesseler's tube. In other such tubes different concentrations (0.5, 1.0, 2.0 and 4.0 ml) of standard polyphenol solution (tannic acid, 0.1 mg/ml) were taken, 10 ml of 2% Na_2CO_3 were then added to each testtube to make the medium alkaline. 2 ml of Folin Denis reagent (100 gm of sodium tungstate and 20 gm of phosphomolybdate acid, dissolved in 200 ml of distilled water were mixed with 25% phosphoric acid. It was then refluxed for two and half hour, cooled to room temperature and diluted to 1 liter with distilled water) were then added to each tube and finally the volume was made to 50 ml with distilled water. A blank was prepared without polyphenol solution. The ingredients were allowed to mix thoroughly. After 10

minutes the optical density of each mixture was read at 660 nm on spectrophotometer.

f. NITROGEN METABOLISM :

i. TOTAL NITROGEN AND CRUDE PROTEINS :

Total nitrogen was estimated from roots, stolon, rhizome, rachis and leaflet of *Nephrolepis exaltata* Schott. in vegetative as well as reproductive stage separately while in *Gymnopteris contaminans* Bedd. it was estimated from root, rhizome, rachis, leaflet, sterile rachis, sterile leaflet, fertile rachis, fertile leaflet by following the method of Hawk *et al.* (1948). 0.5 gm oven dried plant material was digested in a Kjeldhal's flask with sulphuric acid (1:1) and a pinch of microsalt (mixture of anhydrous copper sulphate and potassium sulphate in 1:40 proportion) till the colourless liquid is 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 it was filtered through the filter paper. The filtrate was used for the estimation of nitrogen. 2 ml of this filtrate was taken in Nesseler's tube (35 and 50 ml marked) in other such tubes different concentrations (0.5, 1.0, 2.0 and 4.0 ml) of standard ammonium sulphate (0.05 mg nitrogen/ml) were taken. One tube is kept as blank without ammonium

sulphate. To these test tubes a drop of 8% potassium bisulphate and 1 ml of H_2SO_4 (1:1) were added. The volume of all these tubes was adjusted to 35 ml with distilled water. 15 ml of Nesseler's reagent was then added to each tube. Nesseler's reagent is a mixture of A(7 gm KI and 10 gm HgI_2 dissolved in 40 ml distilled water) and B (10 gm NaOH, dissolved in 50 ml distilled water) in the proportion of 4:5. The colour density of the orange brown product produced by the reaction between NH_3 liberated from the sample and the reagent was measured at 520 nm on spectrophotometer. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

Protein content was calculated by multiplying the total nitrogen with the factor 5.7.

ii. NITRATE REDUCTASE (EC 1-6.6-1) :

Activity of this enzyme in vivo was determined by following the method described by Jaworski (1971). The leaf tissue was incubated in the medium containing 1 ml 1M KNo_3, 2 ml 5% n-propanol, 5 ml 0.2 M phosphate buffer pH 7.5 and 2 ml 0.5% titron-X-100 for 1 hour in dark under anaerobic conditions. After 1 hour, 1 ml of reaction mixture was taken out and mixed with 1 ml 1% sulfanilamide in 1 ml HCl and 1 ml 0.02% NEEDA (N-1 Naphthylethylene diamine dihydrochloride). The absorbance was read at 540 nm on spectrophotometer (Elico).

Standard curve was prepared with 0.03 mM KNO_2 (0.0026 mg KNO_2/ml distilled water) while mixture of 1 ml incubation medium, 1 ml sulphanilamide and 1 ml NEEDA served as a blank.

Enzyme activity is expressed as nm NO_2 liberated per gm fresh tissue per hour.

iii. NITRITE REDUCTASE (EC 1.6.64) :

The activity of enzyme nitrite reductase was determined following the same method described already for nitrate reductase except that KNO_3 was replaced by 0.3 mM KNO_2 in the incubation medium and the incubation was done in the light. Changes in KNO_2 present in the incubation medium was determined by reading the optical density of the reaction mixture containing 1ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA. The difference between the two readings one at zero minute and other after the enzymatic reaction gives an amount of KNO_2 utilized by the enzyme (reduced).

g. INORGANIC CONSTITUENTS :

i. 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).

For acid digestion, 0.5 gm plant material was taken in 150 ml beaker. Then 20 ml concentrated HNO_3

was added to it and allowed to stand for 30 minutes till initial reaction subsides. Then the mixture was heated until plant material was dissolved, cooled to room temperature and added to it 10 ml perchloric acid and heated it gradually. The mixture becomes clear. Then volume was reduced to 2-3 ml by heating, then cooled to room temperature and adjusted the volume to 100 ml with distilled water and allowed it to stand overnight. On next day it was filtered through Whatman No.1 filter paper. The solution was used for estimation of some elements viz. Sodium, Potassium, Calcium, Magnesium, Iron, Manganese, Copper and Zinc. These elements were estimated by using Atomic Absorption Spectrophotometer (Perkin Elmer).

ii. CHLORIDES (Cl^-) :

Chlorides were extracted according to the method described by Imamul Huq and Larher (1983) with slight modifications and estimated according to the method of Chapman and Batt (1961). The chlorides were extracted in distilled water at 45^0C for 1 hour and hot distilled water was added to prevent drying. After cooling the extract was filtered through the layer of cheese cloth. The filtrate was collected in 50 ml volumetric flask and the volume was made with distilled water. From this 10 ml extract was taken for titration against standardised $AgNO_3$. Few drops of acetic acid (20%) solution (dilute 200 ml concentrated acetic acid

with 800 ml distilled water) were added to a filtrate until the pH of the solution was 6 to 7. The five drops of potassium chromate solution (1%) were added and titrated with standardized 0.05 N silver nitrate (dissolve 8.5 gm AR grade $AgNO_3$ in distilled water. Transfer it to 1 liter volumetric flask and make-up the volume with distilled water to 1 lit) until the first permanent reddish brown colour appears.

STANDARDIZATION - Put 10 ml 0.1 N sodium chloride standard into Erlenmeyer flask and add 50 ml distilled water. Titrate with prepared silver nitrate solution.

1 ml 0.05 N $AgNO_3$ = 1.77 mg chlorides.

for 1 gm of sample % of chloride =

ml 0.05 N $AgNO_3$ x 0.177.