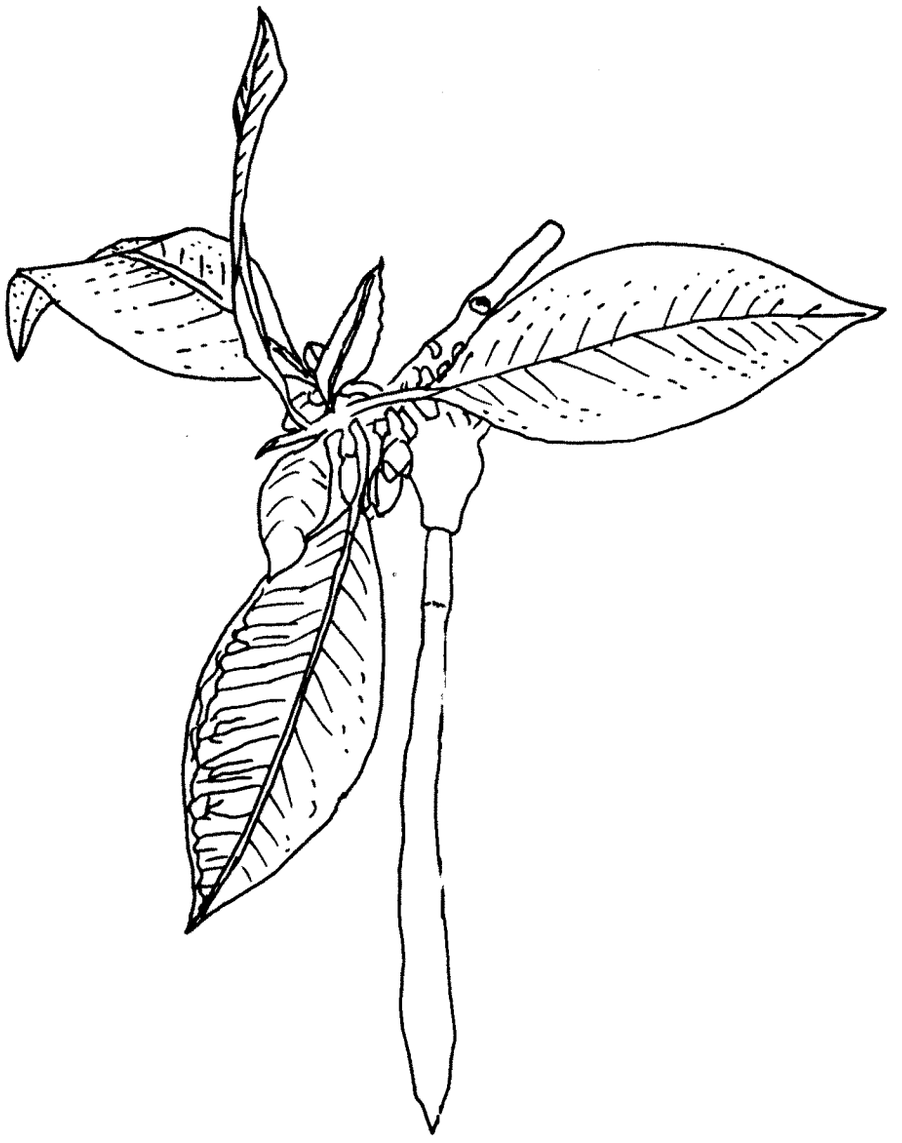

Material and Methods

The present study covers two mangrove species, Rhizophora apiculata and Kandelia candel. The aut ecological study covers the investigations with individual species regarding the morphology, systematic position, ecological adaptations along with soil conditions. The physiological study includes inorganic and organic constituents from the plants as well as the inorganic constituents from soil and water. The methods of collection of material and analysis thereafter, are described below.

A. Material :

The plant material was collected from Bhatya estuary situated to the south of Ratnagiri city (17^{o} $0^{'}$ N and 73^{o} $3^{'}$ E). Mature leaves of both of the species were collected in the polythene bags along with the seedling. The soil samples were collected from the root zone of each species in the layer 0-15 cms. At the same time the water sample was collected from the estuary in a plastic container, the material was brought to the laboratory and subjected to different analysis. The seedlings were excised in three parts, leaves, stems and roots. From mature plants, any leaves were used.

B. Methods :

i) Soil and Water analysis :

The collected samples of soil were dried in shade and all the estimations were carried out from air-dried soils. The water samples were filtered and used for analysis.

100 g soil was sieved through the sieve of different mesh and textural components were determined.

The soil solution was prepared by placing weighed amount of soil in distilled water (1:5) and then filtering the supernatent. This solution was used to record pH, electrical conductivity and major inorganic elements (water soluble). The water sample was also subjected to analysis of the different parameters, viz. pH, EC, sodium, potassium, calcium and chlorides. Na, K, Ca were determined flamephotometrically whereas chlorides were estimated by titrating the sample against 0.1 N $AgNO_3$.

ii) Plant analysis :

The floral buds were dissected to study and confirm the characteristic features in both the species. The percentage distribution of both the species was found out considering an area of 100 m x 200 m. Further, the plant material brought to the laboratory was subjected to analysis with respect to inorganic and organic constituents.

Na, K, Ca were estimated by flamephotometrically from acid digest. Chlorides were determined following the method of Volhard (1956); TAN was estimated according to Thomas and Beevers (1949); chlorophylls according to Arnon (1949); polyphenols according to Horwitz and carbohydrates by the method of Nelson (1944).

The procedures of different estimations are given below.

i) Inorganic constituents :

The leaves were detached from the twigs and surface dust was washed with tap water. They were further rinsed with distilled water and blotted dry. The midrib was removed and the leaf blade was weighed and kept for drying in the oven at 60 C till a constant weight was acquired. The dry material obtained thus was used for the wet digestion by the method of Toth et al. (1948) as follows.

Reagents :

i) Nitric acid (conc).

ii) Perchloric acid 60 %.

Procedure :

To 1 g of plant ash in a pyrex beaker, was added 20 ml of reagent (i). The beaker was covered with watch glass and it was kept to subside the initial reactions. It was then heated till all particles disappeared. After cooling, 10 ml of reagent (ii) was added to this, it was then heated gently at first and more vigorously until a clear and colourless solution was obtained. Care was taken to see that it is not taken to dryness. It was then transferred to a 100 ml volumetric flask and made to volume and then allowed to stand overnight.

Next day it was filtered without washing, through a dry ashless filter paper. This acid digested extract was used for estimation of the cations employing the following methods.

i) Estimation of Na, K and Ca :

Na, K and Ca were estimated flamephotometrically following the standard procedure. Stock solution of known concentration in parts per million (ppm) of K (20 ppm) in KCl, Na (10 ppm) in NaCl and Ca (200 ppm) in $CaCl_2$ were prepared.

Various concentrations of Na in NaCl ranging from 1 to 10 ppm were prepared by diluting the stock solution of NaCl (10 ppm). The deflection in galvanometer for two (stock solutions and 1+9) different concentrations of Na, K and Ca using specific filters were noted. The amount of Na, K and Ca was calculated according to formula.

$$C = C_2 + \frac{C_1 - C_2}{a_1 - a_2} (a - a_2)$$

where C = concentration of unknown,

C_1 = concentration of stock solution,

C_2 = concentration of 1+9 standard solution,

a = corresponding galvanometer readings.

Estimation of Cl :

Reagents :

i) Silver nitrate 0.1 N

ii) Ammonium thiocyanate 0.1 N

iii) Ferric alum: 100 ml of saturated solution of ferric ammonium sulphate was prepared and to this 5 ml of conc. HNO_3 was added.

iv) Dilute HNO_3 (1+4)

v) Potassium chromate 5 %.

Procedure :

0.5 g of powdered oven dried plant material was mixed with 0.125 g CaO in water and made a paste in a crucible. This paste was dried on water bath. The plant material in the crucible was then subjected to the furnace at low temperatures at the beginning and then was ashed at about 500-600°C for 1 h; cooled to room temperature and was extracted in some quantity of hot water. The extract was filtered through Whatman No.44 filter paper and the residue on the paper was washed three times with hot water. The residue was returned again to crucible and was ashed once again as before for 1 h. Now it is extracted in dil. HNO_3 (1:4) and filtered through the same filter paper. The residue was washed thrice with dil. HNO_3 collecting the washing in the same filtrate. The filtrate was transferred to 250 ml volumetric flask and to it were added 20 ml 0.1 N $AgNO_3$. The

precipitate of AgCl was allowed to coagulate at the bottom. The solution in the flask was diluted to 250 ml with distilled water, filtered through Whatman No.44, and filtrate was collected. This filtrate was used for the determination of quantity of $AgNO_3$ remained unused.

100 ml of this filtrate was mixed with 5 ml of ferric alum (indicator) and 3 ml of dilute HNO_3 in 250 ml conical flask. Mixed well and titrated against standardized ammonium thiocyanate. Appearance of first reddish brown precipitate was considered to be the end point.

Ammonium thiocyanate and silver nitrate were standardized against silver nitrate and standard (0.1 N) potassium chloride solutions using ferric alum and potassium chromate indicators respectively.

1 ml of 0.1 N $AgNO_3$ = 3.55 mg Cl

Amount of Cl (%) present in the plant material was calculated using the formula -

$$\% \text{ chlorides} = \frac{A \times 3.55 \times 100}{\text{weight of the plant material (g)}}$$

where A = Amount of $AgNO_3$ (0.1 N) utilized in the 250 ml volumetric flask for precipitation of chlorides in the extract. (This amount is determined after considering corrected normalities of $AgNO_3$ and ammonium thiocyanate).

ii) Organic constituents :

The fresh leaves after cleaning were used for the determination of TAN, total polyphenols, chlorophylls and carbohydrates.

a) Titratable Acid Number (TAN) :

TAN was determined by the method of Thomas and Beevers (1949). Leaves were wiped with a moist cloth and after weighing were cut into small bits, placed in distilled water and boiled for half an hour. After making upto known volume, aliquots of 10 ml were taken and titrated with 0.1 N NaOH, using phenolphthalein as indicator. Number of ml required to neutralize aqueous extracts from 100 g of material was expressed as TAN.

b) Chlorophylls :

Chlorophylls were estimated by the method of Arnon (1949).

Reagent :

Acetone 80 %.

Procedure :

1 g of fresh material was weighed, crushed in 80 % acetone in mortar and pestle. The extract was filtered through Buchner funnel using Whatman No.1 filter paper. Residue was washed

repeatedly with 80 % acetone collecting the washings in the same filtrate.

The volume of filtrate was made to 100 ml with 80 % acetone. The absorbance was read at 645 and 663 nm.

Chlorophylls (mg/100 g fresh tissue) were calculated using the following formula :

$$\text{Chlorophyll } \underline{a} \text{ (mg/l)} = 12.7 \times A_{663} - 2.69 \times A_{645} = X$$

$$\text{Chlorophyll } \underline{b} \text{ (mg/l)} = 22.9 \times A_{645} - 4.68 \times A_{663} = Y$$

$$\text{Chlorophyll } \underline{a} \text{ or } \underline{b} \text{ (mg/100 g fresh tissue)} = \frac{X \text{ or } Y \text{ volume of extract} \times 100}{1000 \times \text{wt of the material (g)}}$$

c) Polyphenols :

Polyphenols were estimated by the method of Folin and Denis (1915).

Reagents :

i) Acetone 80 %

ii) Standard polyphenol solution : 1 mg of tannic acid is dissolved in 10 ml of distilled water.

iii) Sodium bicarbonate (Na_2CO_3) : 20 %

iv) Folin-Denis reagent : A mixture of sodium tungstate, phosphomolybdic acid and ortho-phosphoric acid.

Procedure :

Polyphenols were extracted in 80 % acetone from the well washed and blotted dry plant material (0.5 g). Extract was filtered through Buchner funnel under suction using Whatman No.1 filter paper. The residue was washed 2-3 times and volume was measured. And this was the source of polyphenols.

1 ml of filtrate was taken in 50 ml marked Nessler's tube. In others different concentrations (0.05, 0.1, 0.2, 0.3, 0.4 ml) of standard polyphenol solution were taken. 10 ml of 20 % Na_2CO_3 were then added to each tube to make medium alkaline. 2 ml of Folin-Denis reagent were then added to each test tube and finally the volume was made to 50 ml with water. A blank was prepared similarly, with distilled water. The ingradients were allowed to mix thoroughly. After some time the optical density of each mixture was read at 660 nm an spectrophotometer (ECIL). Polyphenols were calculated from the calibration curve of standard tannic acid.

1 ml standard = 0.1 mg polyphenols (tannic acid)

d) Carbohydrates :

Sugars were estimated by the method of Nelson (1944).

Reagents :

i) Somogyi's alkaline copper tartarate solution : 4 g of $CuSO_4$, $5H_2O$, 24 g of anhydrous Na_2CO_3, 16 g of Na-K-tartarate and 180 g of Na_2SO_4 were dissolved in 1000 ml of distilled water.

ii) Nelson's arsenomolybdate reagent : 25 g of ammonium molybdate were dissolved in 450 ml of distilled water. To this 21 ml of concentrated H_2SO_4 were added and mixed well. 3 g of sodium arsenate were dissolved in 25 ml of distilled water and mixed. The solution was placed in an incubator at 37 ^{o}C for 24 to 48 hours, which was stored in a brown bottle.

iii) Standard sugar solution : 10 mg of glucose were dissolved in 100 ml of distilled water.

Procedure :

1 g of green leaves were extracted with 80 % ethanol. It was filtered through Whatman No.1 filter paper. The residue was preserved and the filtrate was condensed to 2-5 ml. To this 2-3 g each of lead acetate and potassium oxalate were added and mixed thoroughly to decolourise it.

After decolourization, the aliquots were filtered and the volume was measured. This extract was used for estimation reducing sugars (A).

20 ml of extract (A) was taken in a conical flask and to it 2 ml of concentrated HCl were added and pluged with cotton. The sample was hydrolysed for 30 min under 15 lbs pressure. It was cooled to room temperature and Na_2CO_3 was added slowly, till the extract becomes neutral. It was filtered and the volume was noted down, which served as the sample for total sugars (B).

The preserved residue of alcoholic extract was taken in 150 ml capacity conical flask and to it 50 ml of distilled water and 5 ml of conc HCl were added. Then it was hydrolysed as above. It was cooled to room temperature and neutralized as already described. It was filtered and the volume was noted. This served as sample for starch estimation (C).

Estimation of sugars :

In 10 ml marked test tube 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml of standard glucose solution was added. To it 1 ml of Somogyi's alkaline copper reagent was added. All the tubes were placed in boiling water bath for 10 min. It was cooled to room temperature and to it 1 ml of arsenomolybdate reagent was added and the contents were mixed. The total volume was diluted to 10 ml and the absorbance was taken at 560 nm on spectrophotometer (ECIL). Similar procedure was followed for the sample.

By plotting the standard curve of glucose, the values for samples were obtained and the amount was calculated.