Chapter-II

Material & Methods

A) Material

Ratnagiri is situated at 17° 0' North and 73° 3' East (Fig.2). It is the nearest area from Kolhapur for mangrove collection and it has rich sea shore mangrove vegetation. This particular locality has served as study site of the Shivaji University research group for last several years. Hence for ecophysiological studies of Derris species this locality was chosen. The mangrove swamps at different places at Ratnagiri were served in order to find distribution of Derris species. Finally a study site at Takala along Bhatye creek was selected, because there was frequent occurrence of D. trifoliata in the mangrove swamps and presence of D. scandens in the border line vegetation. Since both the species show profuse nodulation during the late monsoon month August and September, the analysis of habitat and the plant material with respect to various ecophysiological parameters was done from this site in these two months. The plants produce pods and seeds in the month of April and May. Hence these two organs were analysed in these two months.

B) Methods

1. Habitat analysis :

a) Soil analysis :

The soil samples were collected from root zone of D. trifoliata and D. scandens in the month of September, 1988 Five soil samples collected from different sites in the mangrove

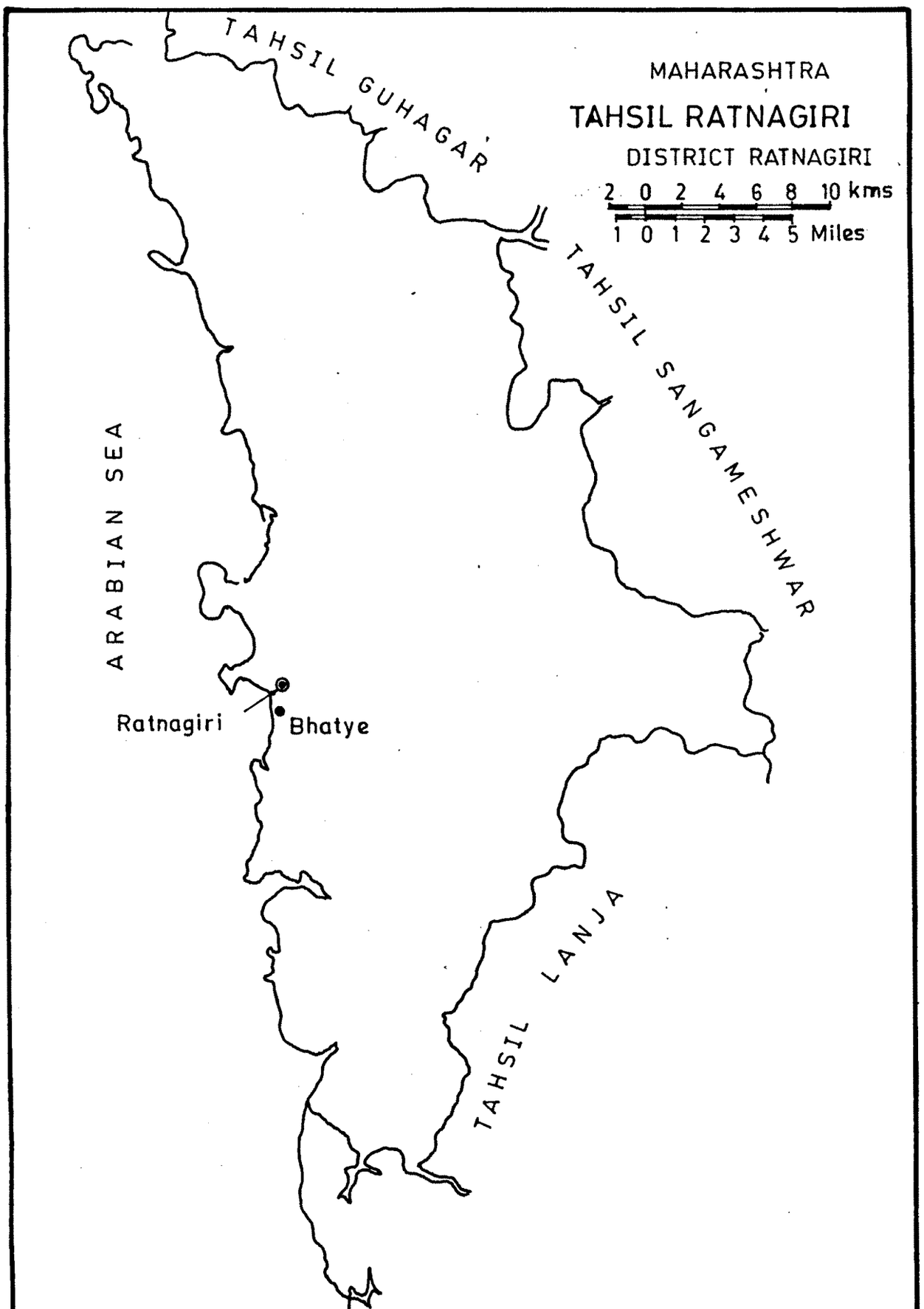

FIG. 2 – MAP OF RATNAGIRI TAHSIL SHOWING STUDY SITE.
(TAKEN FROM CENSUS OF INDIA – 1981 PUBL. 1986)

swamps were pooled to obtain as site soil sample. The samples were transferred to plastic bags and brought to laboratory for analysis. The fresh soil samples were used for determination of pH. For ion content determinations the samples were air dried.

Soil extract was prepared for mineral element determinations according to the method given below. Soil samples were leached for exchangeable cations and anions by placing 5g of dry powdered soil sample on a filter paper which was flushed with four 25 ml volumes of normal neutral ammonium acetate. Each volume was allowed to drain before the next was applied. The normal neutral ammonium acetate was prepared by adding 57.5 ml of glacial acetic acid to 800 ml of distilled water and then adding 65 ml of concentrated NH_4OH. The pH was adjusted to 7.0 with NH_4OH. The soil leachate was evaporated to dryness and the dried residue was dissolved in dilute HCl. It was then filtered into a 100 ml volumetric flask and made up to volume. This acid digest was used as a source to estimation of Na^+, K^+ and Ca^{2+} which were estimated flame photometrically. The elements Mg^{+2}, Fe^{+3}, Mn^{+2}, Co^{+2} and Zn^{+2} from this extract were estimated with the help of atomic absorption spectrophotometer, while P^{+5} was estimated colorimetrically, following the method of Sekine et al.(1965) 1 ml of leaf extract 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 were dissolved in 500 ml N HNO_3. B - 25 g of ammonium vanadate in 500 ml distilled water. The 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 colorimetrically at 420 nm using reagent blank. Compared with the colour density of known standards of phosphorus (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. This solution contained 25 ppm phosphorus) and the amount of phosphorus in the plant material was calculated. Chloride from the same soil sample was determined from the extract prepared in distilled water by titrating against $AgNO_3$ (USDA Book No.60).

2. Plant analysis :

a) Introduction :

The identification of the two Derris species i.e. D. trifoliata and D. scandens was confirmed with the help of key adopted by Thothathri (1982) and the taxonomist (Dr.S.R.Yadav) of Botany Department, Shivaji University, Kolhapur. The study site was visited at different times during the years 1988-89 to observe the phenological behaviour

of the Derris species with respect to associations, nodulation, leaf senescence, flowering, pod development, seed production and seedling emergence.

b) Anatomical studies :

For anatomical studies fresh, mature and healthy leaves and nodules from the identical positions on the plants of both the species of Derris were fixed in FAA (Formaline 5 ml + 5 ml acetic acid + 50% alcohol 90 ml). The material was preserved in same solution in specimen bottles at laboratory conditions. The selected leaf pieces and nodules were passed through usual grades of dehydration, alcohol-xylol grades and infiltrated with paraffin wax (Melting point 52 - 54°C). The sections were cut at 8 to 20 µm thickness on Rotary microtome. The egg albumin was used as adhesive. After dewaxing and dehydration sections were stained in alcoholic safranin for 30 minutes and alcoholic light green for few seconds. The slides were passed through usual grades of alcohol and xylol series and made permanent using DPX as a mounting medium. Photomicrographs were taken by using MFAK's system of Jenaval Carlzeiss microscope.

For the stomatal studies the method described by Stoddard (1965) was followed. The impressions of upper and lower epidermis of leaf of Derris trifoliata and Derris scandens were obtained with nail polish. Stomatal frequency

was calculated by counting the number of stomata in one square milimeter. Stomatal index was calculated by the formula

$$SI = \frac{S}{(S + E)} \times 100$$

where

SI = stomatal index

S = number of stomata/mm^2 (stomatal frequency)

E = number of epidermal cells/mm^2

Stomatal frequency and stomatal index were calculated as described above.

c) Analysis of Inorganic Constituents :

The different plant parts viz. roots, nodules, stem, petiole, young, mature and senescent leaves were separated from the plant material collected during September visit and immediately brought to the laboratory. These plant parts were cut into small pieces and were kept in oven at 65°C till the constant dry weight was obtained.

The inorganic constituents were determined from oven dried plant parts. The oven dried plant parts were powdered and subjected to acid digestion, following the standard method of Black (1965). Accurately weighed (0.5 g or less) plant material was taken in a 100 ml clean Corning beaker and to it 10 ml of acid mixture was added (Acid mixture was prepared

by mixing 75 ml HNO_3 + 15 ml H_2SO_4 + 30 ml $HClO_4$). The beaker was kept in fuming hood for 1 hour till the primary reaction subsided. Then beaker was subjected to heating first on low flame and then vigorously till clear solution remained. It was cooled and transferred to 100 ml volumetric flask and volume was made to 100 ml with distilled water. Then it was filtered through a dry Whatman No.1 filter paper. The filtrate was used for the estimation of inorganic constituents such as Na^+, K^+, Ca^{+2}, Mg^{+2}, Mn^{+2}, Fe^{+3}, P^{+5}, Co^{+2}, Zn^{+2}. Of these Ca^{+2}, Mg^{+2}, Fe^{+3}, Mn^{+2}, Co^{+2}, Zn^{+2} were estimated on Atomic absorption spectrophotometer (Perkin Elmer 3030) at common facility centre, Shivaji University, Kolhapur. Na^+ and K^+ were estimated flame photometrically following the standard procedure. For flame photometric estimation standard solutions in parts per million (ppm) of K (K of KCl) and Na(Na of NaCl) were prepared. The standard curve was obtained by using various concentrations (2,4,6,8,10 ppm) of Na^+ and K^+ made with the help of NaCl and KCl. From these standard curves the concentrations of Na^+ and K^+ in the acid digest were calculated.

Phosphorus was estimated according to the method of Sekine et al. (1965). In clean labelled test tube 1 ml of acid digest was taken. To it 2 ml of 2N HNO_3 and 1 ml of molybdate vanadate reagent (A : 1.25 g of ammonium molybdate were dissolved in 500 ml N NHO_3. B : 25 g of ammonium vanadate were dissolved in 500 ml distilled water. Then A and B were

mixed in equal volumes) were added. The volume of reaction mixture was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes for full colour development. Absorbance was read at 420 nm using blank reaction mixture containing no phosphorus.

Standard curve for phosphorus was prepared by using various concentrations of phosphorus (0.025, 0.05, 0.1, 0.2, 0.4 mg phosphorus) from standard KH_2PO_4 solution containing 0.025 mg phosphorus/ml. By using the standard curve the amount of phosphorus in the plant material was calculated.

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 Pratt (1961). The chlorides were extracted in distilled water at 45°C for 1 hour with addition of hot distilled water to prevent drying. After cooling, the extract was filtered through a layer of cheese cloth. The filtrate was collected in 50 ml volumetric flask and volume was made with distilled water. For this 10 ml extract was taken for titration against standardized $AgNO_3$. Few drops of acetic acid solution (200 ml conc. acetic acid diluted with 800 ml of distilled water) were added to the filtrate until the pH of the solution was 6 to 7. Then five drops of potassium chromate solution (1%) was added and titrated with standardized 0.05 N silver nitrate

8.5 g AR grade $AgNO_3$ dissolved in distilled water. This was transferred to 1 litre volumetric flask and made up to volume with distilled water) until the first permanent reddish brown colour appears.

Standardization : 10 ml of 0.1 N Sodium chloride standard were put into an Erlenmeyer flask and 50 ml of distilled water were added. This was titrated with the prepared Silver nitrate solution.

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

For 1 g sample

% Cl = ml of 0.05 N $AgNO_3$ X 0.177.

d) Organic Constituents :

i) Moisture percentage :

Moisture percentage in various plant parts was determined from the difference between fresh weight and dry weight (weight taken after drying the plant material in oven at 80°C till a constant weight was obtained).

ii) Carbohydrates :

Carbohydrates were estimated according to method of Nelson (1944). Fresh leaves (0.3 g) were extracted with 80% ethanol. Then extract was, filtered through Buchnner's funnel by using filter paper (Whatman No.1). The filtrate was collected and used for the estimation of sugars while the residue was used for the estimation of starch. The filtrate

Thus collected was condensed to 3-5 ml on water bath in porcelain dish. Then decolorizing agents lead acetate and potassium oxalate (1 g : 1 g) were added and thoroughly mixed with condensed filtrate. Then about 15 ml of distilled water was added to it and filtered through filter paper (Whatman No.1). The residue was washed for three times with distilled water. The final volume of aliquot was measured and noted down. 20 ml of this extract (aliquot) was hydrolysed with 2 ml conc. HCl in 150 ml conical flask with a bored cork by autoclaving at 15 lbs pressure for half an hour. The contents were cooled, neutralized with sodium carbonate and filtered. This filtrate was used for the estimation of total sugars.

The insoluble residue of starch along with filter paper was transferred to 150 ml conical flask. Then 50 ml distilled water and 5 ml conc. HCl were added. The conical flask was corked with bored cork and contents were hydrolysed by autoclaving at 15 lbs. pressure for half an hour. The contents were cooled, neutralized with sodium carbonate and filtered through filter paper (Whatman No.1). The final volume was measured and noted down. This filtrate contains reducing sugars produced as a result of hydrolysis of starch. These sugars were estimated.

The appropriate quantity (0.5 ml) of extract prepared for estimation of sugars and starch was taken separately in 10 ml marked test tubes. In other such test tubes different

concentrations (0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml) of standard glucose solutions were taken (standard glucose solution contains 0.1 mg glucose ml^{-1} distilled water) 1 ml of somogyi's alkaline copper tartarate reagent (4 g of $CuSO_4$, $5H_2O$; 24 g of anhydrous Na_2CO_3 ; 16 g of Na-K-tartarate (Rochelle salt) and 180 g of anhydrous Na_2SO_4 dissolved in 1000 ml distilled water) was added to each tube. All test tubes containing mixtures were kept in water bath for about 10 minutes. Afterwards they were cooled to room temperature and 1 ml of arsenomolybdate reagent (25 g of 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_2HASO_4, $7H_2O$; dissolved in 25 ml distilled water. All ingradients were mixed well and 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 minutes the absorbance of each reaction mixture was read at 560 nm.

With the help of glucose standard curve, the amounts of these carbohydrate fractions were determined.

iii) Study of sugar composition of leaves

2 gm of plant material (of each species) was homogenised in 80% ethanol and the homogenate was filtered

through Buchner funnel. The filtrate was condensed in evaporating dish on water bath to 2 ml. 3 ml of distilled water was added to each evaporating dish and this was centrifuged at 5000 rpm for 10 minutes. The volume of supernatant was made equal in all the samples and the supernatant was used as an extract of sugars. This was employed for qualitative analysis of sugars which was done with the help of descending paper chromatography. An aliquot (50 λ; Means 0.05 ml) was loaded on Whatman chromatographic paper with the help of micropipette with frequent drying of the paper with hair dry. The aliquots of leaf extracts of both species were loaded in one line in order to get a comparative picture of sugar composition. The chromatogram was developed in a descending manner using a solvent system. Butanol - Acetic Acid - Distilled water (80 : 20 : 44) When the chromatogram was sufficiently developed, it was removed and dried. The sugars were located with the help of para-anisidine.

The chromatogram was dried in the oven and the individual sugar bands were marked with the help of pencil. The RF value of each band was determined according to formula -

$$\frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The sugars were identified by comparing their RF values with those of authentic standards.

iv) Total nitrogen :

Total nitrogen was estimated colorimetrically by the method of Hawk et al. (1948). 0.5 g of oven dried material was digested in a Kjeldahl flask with sulphuric acid (1:1) and a pinch of microsalt (mixture of anhydrous copper sulphate and potassium sulphate in the proportion of 1:40) till a colourless liquid is obtained. It was then cooled and diluted to 100 ml with distilled water. It was filtered next day through dry Whatman No.1 filter paper and the filtrate was used for estimation of nitrogen.

Reagents : Nessler's reagent - prepared fresh by mixing Nessler's A(7 g of KI and 10 g of Hg I in 40 ml d.w.) and Nessler's B (10 g of NaOH in 50 ml d.w.) in the proportion of 4:5 at the time of expt.; 8 % $KHSO_4$ and std. ammonium sulphate - dissolve 0.236 g of ammonium sulphate (ovendried at 60°C for 12-18h) in d.w., add a few drops of H_2SO_4 and adjust the volume to one litre with distilled water.

0.5 ml of the digested filtrate was taken in Nessler's marked (35 and 50 ml) tube. To this was added a drop of 8% potassium sulphate. The volume was adjusted to 35 ml with distilled water. 15 ml of Nessler's reagent (fresh) were added in each tube and the intensity of colour (orange brown, product ($NH_4Hg_2I_3$) produced formed was measured immediately at 520 nm on spectrophotometer. The amount of nitrogen in

the sample was calculated from standard curve of ammonium sulphate. A blank was run without adding ammonium sulphate.

v) Free proline :

Free proline was estimated from various plant parts (viz. root, stem and leaf) according to method of bates et al., (1973). 0.5 g fresh plant material was homogenized in 10 ml of 3% sulfosalicy-lic acid. After complete homogeniza-tion it was filtered through Buchnner's funnel by using Whatman No.1 filter paper. Then filter paper washed repeatedly with small amount of 3 % sulfosalicylic acid. The filtrate was collected and volume was made 20 ml with 3% sulfosalicylic acid. 0.5 ml extract was taken in clean dry test tube and 2 ml of acid ninhydrin reagent was added to it (Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid with agitation cooled and stored at 4°C). Then 2 ml of glacial acetic acid was added to it. The reaction was allowed to continue for about 1 hour by keeping test tubes in boiling water bath at about 100°C. At the same time reaction mixtures for standard proline curve were prepared by taking different concentrations of proline (0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml). Standard proline solution contains 0.1 mg proline ml^{-1} of 3% sulfosalicylic acid. After 1 hour the reactions in all test tubes were terminated by keeping them in ice bath. Then 4 ml of toluene were added to each test tube with vigorous shaking for 15 to

to 20 seconds. The reaction mixtures were brought to room temperature and absorbance of tolune chromophore layer was read at 520 nm using tolune blank. Tolune chromophore layer was pipetted out with the help of vaccupipette. The standard curve of proline was used for the determination of proline in various samples. The values are expressed as mg 100^{-1} g dry tissue.

vi) Study of amino acid composition of leaves

2 gm of plant material (of each species) was homogenised in 80% ethanol and the homogenate was filtered through Buchner funnel. The filtrate was condensed in evaporating dish on water bath to 2 ml. 3 ml of distilled water was added to each evaporating dish and this was centrifuged at 5000 rpm for 10 minutes. The volume of supernatant was made equal in all the samples and the supernatant was used as an extract of amino acids. This was employed for qualitative analysis of amino acids which was done with the help of descending paper chromatography. An aliquote (50λ) was loaded on Whatman chromatographic paper with the help of micro-pipette with frequent drying of the paper with hair dry. The aliquote of leaf extracts of both the species were loaded in one line in order to get a comparative picture of amino acid composition. The chromatogram was developed in a descending manner using a solvent system. Butanol-Acetic Acid - Distilled water (80:20:44). When the chromatogram was sufficiently developed it was removed and dried. The amino acids were

located with the help of ninhydrine.

The chromatogram was dried in the oven and the individual amino acid bands were marked with the help of pencil. The RF value of each band was determined according to formula -

$$\frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The amino acids were identified by comparing their RF values of authentic standards.

vii) Organic Acids (TAN)

Titratable Acid Number (TAN) values was estimated according to the method of Thomas and Beevers (1949). The plant material (root, stem, leaf, podwall and seed) was washed with distilled water and blotted to dryness. Then it was cut into small pieces. 1 g plant material was accurately weighed and put in boiling distilled water in a 150 ml beaker and boiled for half an hour. Then it was allowed to cool to room temperature and filtered through cheese cloth. The volume of extract was made to 25 ml with distilled water. 10 ml. of extract was titrated against N/40 NaOH using phenolphthalein as an indicator. Before this titration NaOH was standardized against N/40 oxalic acid using the same indicator. Titratable acid number is helpful in determining the acidity of the plant material. It represents the number of ml of decinormal

alkali (NaOH) required to neutralize acids present in 100 g of the plant material.

TAN was calculated by using following formula :

$$TAN = \frac{\text{Volume of oxalic acid taken for titration}}{\text{Titration reading (ml)}} \times \frac{\text{Total vol. of extract}}{\text{Wt. of plant material in g.}} \times \frac{\text{Extract reading (ml)}}{\text{Vol. of extract taken for titration}} \times \frac{100}{4}$$

viii) Chlorophylls :

Chlorophylls were estimated according to method of Arnon (1949). The fresh leaves were washed with distilled water and blotted to dryness. Exactly 0.25 g leaves were weighed. They were cut into small pieces and crushed in mortor with pestle in 80% chilled acetone in cold room. The extract was filtered through Buchnner's funnel using Whatman No.1 filter paper. The residue was washed with 80% acetone. Then filtrate was collected and the volume was made to 25 ml 80% acetone. The absorbance for chlorophylls was read at 663 nm and at 645 nm for chlorophylls a and b respectively.

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

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 A663) + (20.2 X A 645) ..... Z

Chlorophyll a or

Chlorophyll b or

$$\text{Total chlorophylls (mg g}^{-1}\text{ fresh tissue)} = \frac{X/Y/Z \times \text{volume of extract}}{1000 \times \text{Weight of material (g)}}$$

iv) Total polyphenols :

Polyphenols were estimated according to the method of Folin and Denis (1915). Polyphenols from fresh material were extracted in 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. 0.5 ml of filtrate was taken in a 50 ml marked Nesseler s tube. In other such tubes different concentrations (0.5, 1, 2, 3, 4 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml of 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%). This was refluxed for $2\frac{1}{2}$ 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. The ingradients were allowed to mix thoroughly. After sometime the optical density of each mixture was read at 660 nm. Polyphenols were calculated from the calibration curve of standard tannic acid.

X) Qualitative analysis of phenolic compounds

2 gm of leaf material (of each species) was homogenised in 80% ethanol and the homogenate was filtered through Buchner funnel. The filtrate was condensed in evaporating dish on water bath to 2 ml., 3 ml of distilled water was added to each evaporating dish and this was centfifuged at 5000 rpm for 10 minutes. The volume of supernatant was made equal in all the samples and the supernatant was used as an extract of phenolic compounds. This was employed for qualitative analysis of phenolic compounds which was done with the help of descending paper chromatography. An aliquot (50λ) was loaded on Whatman chromatographic paper with the help of micropipette with frequent drying of the paper with hair dryer. The aliquots of leaf extracts of both the species were loaded in one line in order to get a comparative picture of phenolic composition. The chromatogram was developed in a descending manner using a solvent system, Butanol - Acetic acid - Distilled water (80:20:14).

When the chromatogram was sufficiently developed it was removed and dried. The phenolic compounds were detected with the help of ultra violet lamp.

C) Pod and Seed analysis and Germination studies :

The pods of both Derri's species were collected in the months of April and May and the seeds and the podwall were separated. The various inorganic constituents from the oven dried podwall tissue and seed tissue were estimated according to the procedure described in earlier section. The organic constituents namely carbohydrates, crude proteins and polyphenols were estimated from the seed samples of both species. According to the procedure described earlier.

The crude lipid content was determined from ovendried seed material; Two clean corning beaker (150 ml) were taken and their weights(W_1) were recorded. Exactly 3 g seeds of each species were weighed and crushed thoroughly in mortar with pestle in petroleum ether. The extract was filtered through Buchnner's funnel using Whatman No.1 filter paper. Then filtrate was kept in preweighed beaker and condensed on water bath till all petroleum ether evaporated and weight of cooled beaker (W_2) was taken. Lipid content was calculated by using following formula :

$$\text{Lipids present in 100 g seed material} = \frac{W_2 - W_1}{\text{Weight of the seed material}} \times 100$$

where

W_1 = Weight of empty beaker

W_2 = Weight of beaker + extracted lipids.

For germination studies, healthy seeds of D. trifoliata and D. scandens were sorted out and surface sterilized with 0.1 % $HgCl_2$ solution for 5 minutes. After 5 minutes of soaking in $HgCl_2$ solution the seeds were washed thoroughly and dried. Ten seeds were kept in sterilized petridishes for germination over Whatman No.1 filter paper. The filter paper was moistened with 15 ml of distilled water. The experiment was carried out (in triplicate) at 28°C in germination chamber for 120 hours (5 days). The emergence of radicle from seed coat was acknowledged is a criterion for germination counts.

Note - The findings depicted in the part 'Results and Discussion' represent means of three independent determinations.