CHAPTER - II

MATERIAL AND METHODS

1. Plant material :

Nicotiana tabacum L. and Solanum melongena L. plants, healthy and infected with Orobanche, a total root parasite, were collected from Lingnoor (Tal. Kagal, Dist. Kolhapur). Plant material was collected fresh from time to time for analysis. To study the anatomical changes in the roots of these plants, roots of healthy and infected plant with its parasite were used. A random sampling of mature leaves of healthy and infected plants and the parasite was done. Plant material was washed thoroughly with tap water first and then with distilled water to remove debris and surface dust if any. The plant material was blotted to dryness and cut into small pieces (approx. cm^2). This fresh material was used for the estimation of chlorophylls, polyphenols, carbohydrates and chromatographic separation of organic and amino acids and sugars. For determination of total nitrogen, various nitrogen fractions, inorganic constituents and nicotine oven-dried plant material was used.

2. Methods :

A) Pathological Anatomy -

Anatomy of healthy and infected root with its parasite was studied by microtomy procedure. Material was washed with

tap water first and then with distilled water to remove debris and surface dust if any. Plant material was cut into small pieces (approx. 2 cm). Such material was fixed in 10% formalin for 48 hours. After fixation material was washed thoroughly for three hours under running tap water to remove formalin completely.

This material was dehydrated in the following series of alcoholic grades (30%, 50%, 70%, 90% and absolute alcohol two changes). After complete dehydration material was transferred to xylene : alcohol grades (3:1, 1:1, 1:3). In the pure xylene (100%) 2-3 changes were given. For infiltration paraffin wax of melting point 56-58°C was used. This infiltrated material was embeded in paraffin wax and blocks were made.

By using Rotary microtome (Johansen, 1940) sections of 8-12 μ thickness were cut. Slides were passed through series of xylene : alcohol grades.

Xylene : Alcohol ----> Xylene : Alcohol ----> Xylene : Alcohol
3 : 1 — 10 min
1 : 1 — 10 min
1 : 3 — 10 min

---> 100% Alcohol ---> 90% Alcohol ----> 70% Alcohol
10 min — 10 min

---> Safranin ----> 70% Alcohol ----> 70% Alcohol
24 hr — 5 min — 5 min

----> 90% Alcohol ----> 100% Alcohol ----> Light green
5 min 5 min few seconds

----> Clove oil ----> Xylene ----> Mount in DPX.
5 min 5 min

After complete drying slides were observed under microscope. Microphotographs were taken.

Preparation of Safranin :

4 g Safranin dye in 200 cc methyl cellosolve + 100 cc of 95% alcohol and distilled water + 4 g sodium acetate + 8 cc formalin (1%).

Preparation of Light green :

0.5 g light green dye in 100 ml clove oil (0.5%).

B) Physiological studies :

a) Organic constituents -

i) Chlorophylls :

Chlorophylls were estimated by the method of Arnon (1949). Chlorophylls were extracted in 80% acetone from 0.5 g fresh plant material. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue on filter paper was repeatedly washed with 80% acetone, collecting all the washings in the same filtrate. The flask containing the filtrate was wrapped with black paper to protect the

pigments from photooxidation. Volume of the filtrate was made to 50 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophyll 'a' and 'b' respectively, using double beam Spectrophotometer (Shimadzu, UV-190).

Chlorophylls (mg 100^{-1} g fresh tissue) were calculated using the following formulae.

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

Chlorophyll 'b' = 22.9 x A 645 - 4.68 x A 663 = Y

Total Chlorophylls (a+b) = 8.02 x A 663 + 22.2 x A 645 = Z

$$\text{Chlorophyll 'a'/'b'/total (mg } 100^{-1} \text{ g fresh tissue)} = \frac{X/Y/Z \times \text{Vol. of extract} \times 100}{1000 \times \text{wt. of plant material (g)}}$$

ii) Polyphenols :

Polyphenols were estimated by the method of Folin and Denis (1915). Polyphenols were extracted by homogenizing 0.5 g fresh plant material in morter with pestle in 80% acetone. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. The residue on filter paper was repeatedly washed with 80% acetone, collecting the washings in the same filtrate. The volume of the filtrate was made to 50 ml with 80% acetone. This filtrate was used for estimation of polyphenols.

2 ml of each of the extracts prepared from healthy and infected leaves and from parasite was taken in three separate

50 ml marked Nessler's tubes. In other such tubes different concentrations (1.0, 2.0 and 4.0 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml of 20% Na_2CO_3 were added to each tube to make the medium alkaline. 2 ml of Folin Denis reagent (a mixture of sodium tungstate, phosphomolybdic acid and Orthophosphoric acid) were then added to each tube and finally the volume was made to 50 ml with distilled water. A blank was prepared in the same way with all reagents except the standard tannic acid. The ingredients were allowed to mix thoroughly. After some time, the absorbance of each reaction mixture was read at 660 nm on spectrophotometer (Shimadzu, UV-190). Polyphenols were calculated from the calibration curve of standard tannic acid.

iii) Carbohydrates :

Carbohydrates were estimated following the method by Nelson (1944). Fresh plant material (healthy, and infected leaves and parasite) was extracted in 80% ethanol. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel. The residue on filter paper was used for starch estimation. The filtrate thus obtained was used for estimation of reducing and total sugars.

Reducing sugars :

The filtrate thus obtained was condensed on boiling water bath till the volume was about 5 ml. This extract was

then treated with lead acetate and potassium oxalate (1 g each) to decolorize it. The aliquots were filtered through Whatman No.1 filter paper using Buchner's funnel. Volume of the filtrate was recorded and used for estimation of reducing sugars.

Total sugars :

20 ml of the extract prepared for estimation of reducing sugars was taken in 150 ml capacity conical flask. To this 2 ml of concentrated HCl was added and hydrolysed at 15 lbs pressure for half an hour in autoclave. The contents were cooled, neutralized with sodium carbonate and filtered. The volume of the filtrate was recorded. This filtrate was used for estimation of total sugars (reducing + non-reducing sugars).

Starch :

The residue obtained at the beginning while filtering the alcoholic extract, was transferred quantitatively to a conical flask along with filter paper. 50 ml distilled water and 5 ml of concentrated HCl were added to the flask. The residue was hydrolysed at 15 lbs pressure in pressure cooker for half an hour and cooled to room temperature, neutralized by sodium carbonate and filtered. Volume of the filtrate was noted. This filtrate was used for estimation of reducing sugars.

In 10 ml marked test tubes different concentrations of the above extracts (0.05 ml for starch and total sugars, 0.1 ml for reducing sugars) were taken separately. In other such test tubes, different concentrations (0.1, 0.2, 0.4 and 0.8 ml) of standard glucose solution (0.1 mg ml^{-1}) were taken. 1 ml of Somogyi's alkaline copper tartarate reagent (4 g $CuSO_4$, 5 H_2O, 24 g unhydrous Na_2CO_3, 16 g Na-K tartarate, Rochelle salt and 180 g unhydrous 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 min. After cooling to room temperature, 1 ml of arsenomolybdate reagent (25 g ammonium molybdate in 450 ml water to which were added 21 ml of concentrated H_2SO_4. To this was then added 3 g sodium arsenate, Na_2HASO_4, 7 H_2O dissolved in 25 ml water. All ingredients were mixed well and incubated at 37°C for 48 hours before use) was added to each reaction mixture. The contents of each tube were then diluted to 10 ml with distilled water. A blank was prepared in the same way but without sugar solution. After 10 min the absorbance of each reaction mixture was read at 560 nm on Spectrophotometer (Shimadzu, UV-190). From the calibration curve of standard glucose, the sugar percentage of the above three fractions were calculated.

iv) Nitrogen :

For estimation of various fractions of nitrogen viz. NO_3-N, NO_2-N, Protein-N and insoluble nitrogen from healthy and infected leaves and the parasite, 0.5 g of each oven-dried

powdered material was used. The material was extracted in 20 ml 80% ethanol and filtered through Whatman No.1 filter paper using Buchner's funnel. The filtrate was used to estimate the soluble fractions, while from the residue insoluble nitrogen was estimated.

The filtrate was condensed on boiling water bath upto 1-2 ml. 10 ml of distilled water was then added to it and mixed well. It was filtered through Whatman No.1 filter paper using Buchner's funnel with frequent washing the residue on the filter paper. All the washings and the filtrates were collected together and diluted to 100 ml with distilled water. From this filtrate NO_3-N, NO_2-N (Jaworski, 1971) and protein-N (Lowry et al., 1951) were determined.

Nitrate (NO_3^-) nitrogen :

The principle underlying the estimation of NO_3 is that, the diphenylamine H_2SO_4 reagent forms blue coloured complex with nitrate (Kolhaff and Noponen, 1933). The blue coloured complex has the absorption maxima at 590 nm. The reaction mixture contained 1 ml plant extract, 1 ml distilled water and 1.8 ml diphenylamine H_2SO_4 reagent (1 g diphenylamine 100^{-1} ml H_2SO_4). After vigorous shaking of the reaction mixture, the colour was allowed to develop for 10 min and the absorbance was read at 590 nm on spectrophotometer (Shimadzu, UV-190). Instead of plant extract distilled water was used to prepare

blank. The standard curve for NO_3^- was prepared with KNO_3 and with the help of this nitrate content was calculated.

Nitrite (NO_2^-) nitrogen :

Nitrite content was determined following the method of Jaworski (1971). The reaction mixture contained 5 ml plant extract, 1 ml sulfanilamide (1% in 1 M HCl) and 1 ml NEEDA (0.02% N-1 Naphthylethylene diamine dihydrochloride) together in a test tube. After 15 min reaction the absorbance was read at 540 nm. The standard curve for NO_2^- was prepared by using 0.03 mM KNO_2. The blank was prepared with distilled water in place of any extract or nitrite.

Soluble protein nitrogen :

Soluble proteins were determined following the method of Lowry *et al.* (1951). In the assay mixture 0.5 ml of plant extract was diluted to 1 ml with distilled water and 5 ml of freshly prepared reagent C (50 ml 2% Na_2CO_3 in 0.1 N aqueous NaOH mixed with 1 ml 0.5% $CuSO_4.5\ H_2O$ in Na-K tartarate) were added. After 15 min, 0.5 ml Folin-Phenol reagent (100 g sodium tungstate mixed with 25 g sodium molybdate, in 700 ml distilled water, 50 ml 85% phosphoric acid and 100 ml concentrated HCl mixed together were refluxed gently for 10 h using water condenser. To this 150 g lithium sulphate, and 50 ml distilled water were added. The mixture was boiled for 15 min

without water condenser to remove excess bromine. The mixture was cooled and then adjusted to 1 N by titrating it against 1 N NaOH) was added. The colour was allowed to develop for 30 min and the absorbance was read at 660 nm. Albumin (egg albumin, crystalline powder, 0.1 mg ml^{-1}) was used for preparation of standard curve of protein. Blank was prepared with distilled water, reagent C and Folin-phenol reagent.

Insoluble Nitrogen :

From the residue, insoluble nitrogen (insoluble in alcohol and water) was estimated. For this purpose, the residue along with the filter paper was digested with 7 ml 1:1 H_2SO_4 in Kjeldehl's flask. A pinch of microsalt (200 g K_2SO_4 + 5 g dehydrated $CuSO_4$) was added to accelerate the digestion. Digestion was carried out till the clear and colourless solution was obtained. After cooling to room temperature it was transferred quantitatively to 100 ml capacity volumetric flask. Volume was made and stored overnight at room temperature. Next day, it was filtered through Whatman No.1 filter paper. From this filtrate nitrogen was estimated following the method of Hawk et al. (1948). 2 ml of extract was mixed with a drop of 8% $KHSO_4$, 15 ml Nessler's reagent (reagent A-7 g KI + 10 g HgI_2 in 40 ml distilled water. Reagent B-10 g NaOH in 50 ml distilled water. A and B were mixed in the proportion of 4:5 at the time of experiment only)

and diluted to 50 ml with distilled water. The absorbance was read at 520 nm on spectrophotometer (Shimadzu, UV-190) against reagent blank. A standard curve of ammonium sulphate (0.05 mg N ml^{-1}) was prepared with the help of which insoluble nitrogen content was calculated.

v) Organic composition (Chromatographic analysis) :

To study the composition of tissue from healthy and infected leaves and that of parasite with respect to amino acids, sugars and organic acids, the paper chromatography technique was followed. The compounds were separated from alcoholic extracts of the plant material on 46 x 46 cm sized Whatman No.1 paper chromatograms with appropriate solvent system. The solvent system used was n-butanol:acetic acid : water (80:22:50). The paper chromatograms were run in descending manner. For spotting the compounds on chromatograms, spray reagents, ninhydrin, aniline phthallate and bromophenol blue, were used respectively for colour reactions with amino acids, sugars and organic acids. The separated compounds on the chromatograms were identified with the help of their Rf values colour reactions and with the help of co-chromatography of authentic samples.

vi) Nicotine in Tobacco :

Nicotine in tobacco (Nicotiana tabacum L.) healthy and infected leaves, infected with Orobanche, a total root parasite

and that of the parasite was estimated following the Silicotungstic acid method by Horowitz (1965). 5 g of oven-dried plant material was taken in a 300 ml capacity Kjeldahl's flask. Few glass beads, about 10 g of NaCl and 10 ml of NaOH (30%) were added into the flask. The flask was stoppered well with bored cork having two holes (one for steam and another as an outlet for steam distillate). Steam was passed through the inlet which was just touching the bottom of the flask. A trap bulb, which was connected to the condenser, the lower end of which was dipped in 15 ml HCl (1+4) in a 500 ml capacity volumetric flask was fixed in the outlet hole. When the distillation was half way, the burner was lighted below the flask containing tobacco to reduce the volume in the flask. When about 475 ml of the distillate was collected, last few drops of the distillate were tested for nicotine with a drop of silicotungstic acid and hydrochloric acid (if no cloudy precipitate, the distillate is free from nicotine). The alkalinity of the residue in the distillation flask was confirmed with phenolphthalein indicator. The volume of the distillate was made upto the mark and thoroughly shaken. 50 ml of the distillate was taken in 100 ml beaker and 3 ml of HCl (1+4) and 10 ml of silicotungstic acid solution were added in the beaker, stirred and allowed to stand overnight at room temperature. A few drops of silicotungstic acid solution was added down the sides of the beaker to the clear supernatant solution

for testing complete precipitation. It was then filtered through previously weighed dry gooch crucible set up with asbestos matting and washed with HCl (1 + 1000) till it was free from silicotungstic acid. Crucible was dried at 105°C in oven for about four hours and then weighed.

Amount of nicotine in the distillate was calculated using the following formula :

$$\text{Amount of nicotine in 50 ml distillate} = \text{Weight of residue} \times 0.1012 \text{ g}$$

% nicotine in oven dried plant material =

$$= \text{Wt of residue} \times 0.1012 \times \frac{\text{Vol. of distillate}}{\text{ml of distillate taken}} \times \frac{100}{\text{wt of plant material (g)}} \times 100$$

b) Inorganic constituents :

i) Preparation of plant extract (Acid digestion) -

For the estimation of inorganic constituents an acid digest from the oven-dried plant material was used. The material was acid digested following the method of Toth et al. (1948). 0.5 g of oven-dried powdered material was transferred to 150 ml beaker to which 20 ml concentrated HNO_3 were added. The beaker was covered with watch glass and kept till the primary reactions subsided. It was then subjected to heating. It was heated first slowly till the solid particles were completely dissolved. After cooling to room temperature, 10 ml

of perchloric acid (60%) was added to it and mixed thoroughly. It was then heated strongly and vigorously until a clean and colourless solution remained. Heating was stopped when the volume of the extract was reduced approximately to 2-3 ml. It was then cooled and transferred quantitatively to a 100 ml capacity volumetric flask and the volume was made with distilled water. It was kept overnight and next day filtered through dry Whatman No.1 filter paper.

ii) Estimation of sodium and potassium :

Sodium and Potassium from the acid digest were estimated flame photometrically (ELICO CL-22-A), following the method standardized in our laboratory. The stock solutions of known concentrations in ppm of sodium (5,10,15,20 and 25 ppm) and potassium (10,20,40 and 80 ppm) were used as standards from which the sodium and potassium concentration in the plant material were calculated.

iii) Estimation of calcium, magnesium, iron, copper and zinc :

The filtrate was used for estimation of inorganic elements, Fe, Zn, Cu, Ca, Mg. Elemental constituents were analysed quantitatively in an Atomic Absorption Spectrophotometer (PERKIN-ELMER 3030).

iv) Estimation of Phosphorus :

P^{5+} was estimated following the method by Sekine et al. (1965). Phosphorus gives yellow colour reaction with Molybdate-

Vanadate reagent. The intensity of the colour developed is directly proportional to P content.

2 ml of the acid digest was pipetted in a test tube to which 2 ml of 2 N HNO_3 was added followed by 1 ml Molybdate-Vanadate reagent (A - 1.25 g ammonium vanadate (NH_4VO_3), dissolved in 500 ml 1 N HNO_3, B-25 g ammonium molybdate {$(NH_4)_6 MO_7O_{24}.4 H_2O$} in 500 ml distilled water. A and B are mixed in equal volumes at the time of experiment only). The volume was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 min. The yellow colour developed with Molybdate-Vanadate reagent was measured on spectrophotometer (Shimadzu, UV-190) at 420 nm with reagent blank. Simultaneously the reaction mixtures were prepared for standard phosphorus curve by pipetting various concentrations of phosphorus solution (Standard phosphorus solution was prepared by dissolving 0.110 g monobasic potassium phosphate (KH_2PO_4) in distilled water and making the volume to one litre. This solution contained 25 ppm phosphorus). The amount of phosphorus in the plant material was calculated by comparing the absorbance of the plant extract with that for standard phosphorus.