II MATERIALS AND METHODS

1. **Survey :**

Survey of host plants for the occurrence of *Cephaeluros* in Kolhapur and adjoining areas was made in the month of September 1999 by visiting the different localities.

2. **Disease Intensity :**

Infected leaves were randomly collected from the hosts to study the disease intensity by calculating total area of the leaves and the area covered by the algal colonies using graph paper.

3. **Moisture Content :**

Freshly harvested and randomly sampled healthy and infected plant leaves were sun-dried and then oven-dried at 60°C till we get constant dry weight. This data was used to express the moisture percentage / dry weight percentage in the plant sample.

4. **Stomatal Regulation :**

Stomatal regulation in healthy and infected plant was studied in-situ by using Steady State Porometer (LI-1600, LiCOR, USA).

5. **Leaf Anatomy :**

Leaf anatomical study was performed by taking fine hand cut sections with sharp razor blade from cleanly washed alga infected plant tissue in order to know the host-parasite relationship.

6. Organic Constituents

Organic constituents such as chlorophyll, carotenoids, nitrogen, polyphenols and sugars were analysed from healthy and alga infected leaf tissue.

a) Chlorophylls :

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

0.5 g randomly sampled fresh plant material was crushed in a mortar with pestle and extracted in 80% chilled acetone containing 4 ml liquor ammonia per litre in a dark and cold room. A pinch of magnesium carbonate ($MgCO_3$) was added during crushing. This extract was filtered through Buchner's funnel using Whatman No.1 filter paper and the volume of filtrate was measured. In order to avoid destruction of chlorophylls by light, the flasks containing chlorophyll extracts were covered with black paper and stored at low temperature. The absorbance was measured at 645 nm and 663 nm on double beam spectrophotometer (Shimadzu).

Chlorophylls (mg 100^{-1} g fresh weight) were calculated using the formulae given below –

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

Chlorophyll '(a+b) = 8.02 x A 663 + 20.2 x A 645 = Z

$$\text{Total chlorophylls (mg } 100^{-1}\text{g fresh tissue)} = \frac{\text{X / Y / Z x volume of extract x 100}}{\text{Weight of plant material (g)} \times 1000}$$

b) **Carotenoids :**

Total carotenoids were estimated by using the method and formula suggested by Jensen (1978).

The acetone extract prepared for chlorophyll estimation was used for measuring the absorbance at 480 nm for carotenoid determination.

$$\text{Total carotenoids} = \frac{D \times V \times F \times 10}{2500}$$

Where,

D = Absorbance at 480 nm
V = Volume of extract
F = Dilution factor
2500 = Average extinction

$$\text{Dilution factor} = \frac{\text{Total volume of extract}}{\text{Moisture content in plant material used.}}$$

c) **Nitrogen :**

Nitrogen was estimated by the method of Hawk *et.al.* (1948). 0.5 g dried plant material was taken in Kjeldahl's flask containing 10 ml 1:1 H_2SO_4 and water. A pinch of microsalt and few glass beads were added to the flask. This was digested on low flame till colourless solution was obtained. Then it was cooled and transferred quantitatively to volumetric flask and volume was made 100 ml with distilled water and filtered through Whatman No.1 filter paper. From the filtrate 2 ml of extract was taken in Nesslle's tube to which a drop of 8% $KHSO_4$ was added and volume was

made to 35 ml with distilled water. Then 15 ml Nessler's reagent (freshly prepared) was added to it.

After 10-15 min. the absorbance was recorded at 520 nm on spectrophotometer (Shimadzu). The blank contained all the ingradients except nitrogen source.

Standard curve was obtained by using different concentrations of ammonium sulphate (0.1, 0.2, 0.3, 0.4 ml.) by using same procedure of nitrogen estimation. The values are expressed in g 100^{-1} g dry weight.

i) Preparation of microsalt :

Microsalt was prepared by grinding anhydrous copper sulphate and potassium sulphate in the proportion of 1:40 i.e. 0.1 g $CuSO_4$ + 4.0 g K_2SO_4.

ii) Preparation of Nessler's reagent :

a) 7 g KI + 1 g HgI_2 dissolved in 40 ml distilled water.

b) 10 g NaOH dissolved in 50 ml distilled water. Both a and b were mixed immediately before use.

iii) Preparation of standard ammonium sulphate solution :

$(NH_4)_2 SO_4$ was kept in an oven for 10 h and 0.266 g it was dissolved in water. A few drops of conc. H_2SO_4 were added to it and the volume was made to 1 litre. This contains 0.05 mg of nitrogen / ml.

d) Polyphenols :

Polyphenols from healthy and alga infected leaf tissue were estimated following the method of Folin and Denis (1915). 1 g dried powder was crushed in a mortar with pestle using 80% acetone. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue on

the filter paper was washed several time with 80% acetone and final volume of extract was adjusted to 50 ml using 80% acetone. 2 ml of plant extract was taken in Nessler's tube along with the series of standards (Std. Tannic acid having concentration 0.1 mg ml^{-1}) to which 10 ml 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water. Then 2 ml of Folin Denis reagent was added in each test tube and the final volume was adjusted to 50 ml with distilled water. After about 20-30 min. absorbance was measured at 660 nm using reaction blank. Polyphenols were calculated from standard curve of tannic acid and the values are expressed in g 100^{-1} g dry tissue.

i) Preparation of Folin-Denis reagent :

100 g. sodium tungstate and 20 g phosphomolybdic basic acid were dissolved in 800 ml distilled water. To that 50 ml 80% phosphoric acid was added. The entire mixture was refluxed to 2 h on water bath using water condenser. After cooling to room temperature, the final volume of the mixture was made to 1 litre and stored in an amber coloured bottle at low temperature.

Standard tannic acid was prepared by dissolving 25 mg tannic acid in 250 ml distilled water so as to get the final concentration, in the range of 0.1 mg ml^{-1}.

e) Sugars :

The sugars were estimated following the method suggested by Nelson (1944). Known quantity of oven dried plant material (1 g) was extracted with 80% ethanol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper and the residue was saved for starch analysis.

The filtrate was then condensed on waterbath to about 2 to 3 ml. To this a mixture of lead acetate and potassium oxalate (1:1) was added with constant stirring and mixed with 30 ml distilled water and filtered through ordinary filter paper. The volume of extract was adjusted to 50 ml with distilled water. From this extract, reducing sugars were determined.

For the estimation of total sugar, 20 ml extract from the above was taken in a conical flask to which, 5 ml concentrated HCl was added and the same was hydrolysed at 15 lbs pressure for half an hour and cooled to room temperature. It was neutralized with anhydrous Na_2CO_3 and filtered. The filtrate was used to estimate total sugars.

The residue saved for starch analysis was transferred to 150 ml. conical flask along with filter paper. To this, 50 ml distilled water and 5 ml concentrated HCl were added. The flasks were hydrolysed at 15 lbs pressure for half an hour. They were cooled to room temperature, neutralized with Na_2CO_3 and filtered. The volume of filtrate was measured. This filtrate contained reducing sugars (mostly glucose) formed as a result of hydrolysis of starch.

The sugars from all the three filtrates were estimated using arseno-molybdate reagent (Nelson, 1944).

Aliquot of plant extract was taken in a test tube, along with the standard series of glucose (standard glucose 0.1 mg/ml) in other test tubes. In case of blank, standard glucose and plant extract was not added. 1 ml of alkaline copper tartarate reagent was added to each test tube and all these test tubes were kept in boiling waterbath for 10 min. After cooling to room temperature, 1 ml arseno-molybdate reagent was added to each test tube and the volume of each reaction mixture was adjusted to 10 ml

with distilled water. After 10 min. the absorbance was read at 560 nm on spectrophotometer (Shimadzu).

Using calibration curve of standard glucose, the sugar content in each fraction was calculated.

7. Inorganic Constituents

a) Acid digestioin and mineral analysis

Mineral constituents were analysed from acid digested plant extract as per the method of Toth *et al.* (1948). 0.5 g dried plant sample was acid digested firstly with 20 ml concentrated nitric acid on hot plate till the solid particles get dissolved and then with 15 ml perchloric acid till the solution becomes colourless. After cooling, the volume was made to 100 ml with distilled water and kept overnight. It was then filtered and the filtrate was used for analyzing different mineral element *viz.* Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+} and P^{5+} on Atomic Absorption Spectrophotometer (PERKIN ELMER-3030).

b) Phosphorus

The phosphorus content was determined from the acid digested extract spectrophotometrically by the method of Sekine *et al.* (1965).

8. Chromatography of Polyphenols

a) Preparation of extract

The polyphenols were extracted by the method suggested by Glass and Bohm (1969). 1 g fresh healthy and algal infected leaf was crushed separately in 80% ethanol. The extract was refluxed for 2 h on waterbath using water condenser and filtered hot through a sintered glass funnel. The

solvent was removed under reduced pressure. The residue was triturated in 50 ml hot water. The combined extract was reduced to 3 ml by condensation, centrifuged at 5000 g for 5 min. and supernatant was used for chromatography.

b) Unidimensional paper chromatography of polyphenols

Unidimensional paper chromatography was performed using whatman No.1 filter paper with slight modifications and chromatograms were spotted with 20 µl healthy and algal infected leaf extract using microsyringe with frequent drying. The solvent system used was n-butanol, acetic acid and water in the proportion of 80:20:44 (v/v). The position of individual phenolic compounds on chromatogram was determined by marking fluorescent area under UV light as well as under UV light in presence of ammonia fumes. Phenolic compounds and flavonoids that could not be located under UV and UV + NH_3 were detected by dipping the chromatogram in a mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$ in equal proportion.

The probable identification of the compounds was made by calculating the Rf values, observing colour under UV and UV + NH_3 and by comparing with the Rf values of authentic standards obtained from Dr. P. Neuman, University of Texas, Austin.

9. Chromatography of Amino Acids and Organic Acids :

Amino acids and organic acids from healthy and algal infected leaves were studied by using the technique of ascending unidirectional paper chromatography (Block *et.al.* 1955).

a) Preparation of extract :

The leaves of healthy and algal infected plant (1 g) were crushed separately in 80% ethanol and filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was condensed under reduced pressure up to 2 ml and transferred into a centrifuge tube. The extract was then centrifuged at 5000 x g for 5 min. and the supernatant was collected in a small glass vial and stored at 4°C until use.

b) Unidimensional paper chromatography :

The amino acids and organic acids were separated by loading the aliquot of extract on chromatographic paper and by using the solvent system n-butanol : Acetic acid : water (4:1:5 v/v) for amino acid while n-butanol : formic acid : water (4:1:5 v/v) was used for organic acid separation.

0.5% ninhydrin prepared in 95% acetone was used as detecting reagent for amino acids. Identification of each amino acid was made by using the chromatography of authentic standards and comparing their colours and Rf values. The colour intensity of spots were compared and according to their concentrations they were graded as least (+), less (++), moderate (+++) and high (++++).

10. Polyphenol Oxidase (E.C. 1.10.3.2)

The activity of an oxidative enzyme polyphenol oxidase was studied spectrophotometrically by using the extraction and assay procedure suggested by Mahadevan and Sridhar (1982) with slight modification to suit our laboratory conditions.

a) Extraction :

0.5 g randomly sampled healthy and algal infected leaves were cleanly washed and cut into small pieces and extracted in 15 ml cold 0.1 M phosphate buffer (pH 6.1) in pre-chilled mortar with pestle.

The homogenate was filtered through 4 layers muslin cloth and centrifuged at 5000 g at 4°C. The supernatant was used for assaying the enzyme activity.

b) Assay :

In order to score the activity of polyphenol oxidase the oxidation of catechol was measured from the reaction mixture containing 2 ml phosphate buffer (pH 6.1), 0.5 ml enzyme extract and 1 ml 0.01 M catechol at 495 nm. The change in the absorbance between the first 30 sec. and 150 sec., of incubation at room temperature (27°C) was measured. The control reaction was maintained with heated enzyme.

11. Phenylalanine Ammonia Lyase (PAL) :

Activity of an enzyme phenylalanine ammonia lyase was assayed according to slightly modified method of Bopp and Murrach (1980) and Mahadevan and Sridhar (1982).

a) Extraction :

0.5 g cleanly washed fresh healthy and algal infected leaf material was homogenized separately in 0.025 M cold sodium borate buffer containing 0.01 M mercapto ethanol (pH 8.8) and filtered through 4 layered muslin cloth and the filtrate was centrifuged at 10,000 x g for 15 min. The supernatant was used as a source of an enzyme.

b) Assay :

The assay mixture contained 0.5 ml enzyme, 1 ml 0.025 M sodium Borate buffer (pH 8.8), 0.3 ml 0.01 M L-phenylalanine and 1.2 ml distilled water. The assay mixture was incubated at 30°C for 15 min to allow for an initial non enzymatic decrease in absorbance (which is usually detected in control reactions containing boiled enzyme or no substrate). The absorbance of the assay mixture was measured at 290 nm at an interval of 20 min for 1 h. A reaction mixture without either L-phenylalanine or enzyme extract was used as the control.

12. Algal Culture

The parasitic alga *Cephaleuros* was cultured on Bolds basal medium (Bischoff and Bold, 1963).

Stock solutions of the following were first prepared in distilled water (400 ml).

Salts		Quantity
$NaNO_3$	:	10.0 g
$CaCl_2.2H_2O$	:	1.0 g
$MgSO_4.7H_2O$	:	3.0 g
K_2HPO_4		3.0 g
KH_2PO_4	:	7.0 g
NaCl	:	1.0 g

10 ml of each was added to 940 ml distilled water and to this was added 1 ml each of following stock trace-element solutions prepared as follows.

1) 50 g Na_2EDTA / lit. distilled water
2) 4.98 g $FeSO_4$, 7 H_2O / lit. distilled water and acidified by adding 1 ml H_2SO_4
3) 11.42 g H_3BO_3 / lit distilled water.
4) Mixture of following in one lit. distilled water –

$ZnSO_4$, $7H_2O$	-	8.82 g
$MnCl_2$, $4H_2O$	-	1.44 g
MoO_3	-	0.71 g
$CuSO_4$, $5H_2O$	-	1.57 g
$CO(NO_3)_2$ $6H_2O$	-	0.49 g

The growth of *Cephaleuros* was also tried on sterilized leaf decoction of the host tissue as well as chopped host tissue. The suspension of algal growth was also studied by using algicide copper sulphate at 5%, 10% and 15% concentration.