CHAPTER II

MATERIAL AND METHODS.

I. MATERIAL

For the present investigation attempts have been made to study pathophysiological changes in healthy and infected leaves of plant varieties of turmeric (Curcuma longa L.) such as

1) Salem
2) Rajapuri
3) Krishna

Healthy and infected leaves of above varieties of turmeric (Curcuma longa L.) were collected from the farms of the turmeric research scheme K. Digraj, Dist : Sangli. The healthy and infected leaves were collected from the same field and same day, date and time of the season. Generally Healthy and Infected leaves of similar age were collected. Experiments were carried out in triplicate to support definite conclusions, various standard methods were used for the analysis of organic, inorganic and enzyme contents of the healthy and infected leaves.

II. METHODS

(A) Physical parameters - (Morphological features)

The morphological features of leaves were studied by the method of Linacre (1954). The healthy and infected leaves of different varieties of turmeric such as Salem, Rajapuri and Krishna were taken for the investigations. The leaves were cleaned to remove dust and dirt. Then the Average

leaf area, Leaf thickness and leaf weight were measured from these average volume and densities were calculated by using following formula :

Leaf volume = Average leaf area x Average leaf thickness

$$\text{Density} = \frac{\text{Average leaf weight}}{\text{Average leaf area} \times \text{Average leaf thickness}}$$

(B) Moisture Percentage

Healthy and Infected leaves of turmeric varieties Salem, Rajapuri and Krishna were taken and cleaned well with distilled water. Surfaces were blotted to dry and weighed accurately. Then the leaves were dried at 80°C in an oven till constant weight obtained. The moisture percentage can calculated by using following formula :

$$\text{Moisture Percentage} = \frac{\text{fresh wt. - Dry wt.}}{\text{Fresh wt.}} \times 100$$

(C) R.W.C. (Relative Water Content)

The clean washed and surface dried healthy and infected leaves of turmeric varieties such as Salem, Rajapuri and Krishna were taken. The leaf discs were prepared by punching leaf lamina and weighed accurately. Then the same leaf discs were kept for 4 hours in distilled water and weighed. Later on these discs were dried at 80°C in an ovan till constant weight obtained. The RWC can be calculated by using the following formula.

$$RWC = \frac{\text{Fresh Wt - Dry Wt.}}{\text{Turgid Wt - Dry Wt.}} \times 100$$

(D) Organic Constituents

(i) Chlorophylls :

The chlorophylls were estimated by the method of Arnon (1949). Chlorophylls were extracted in 80% Acetone from 0.5 g of leaves. The extract was filtered through Buchner's funnel using whatman filter paper No.1. 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. Extration was carried out in dark and ice cold conditions. The absorbance of the filtrate was read at 663 nm and 645 nm. Chlorophylls in mg 100^{-1} g. Fresh leaves were calculated by using following formula :

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= 8.02 x A 663 + 20.2 x A 645 = Z

$$\text{Chlorophyll a, or b or Total Chlorophylls (mg } 100^{-1} \text{ g)} = \frac{X/Y/Z \times \text{volume of extract} \times 100}{1000 \times \text{Wt. of the plant material in g.}}$$

(ii) Carotenoids :

Carotenoids were extracted by crushing 0.5 g of fresh leaves in 80% acetone. Procedure is similar to that of

chlorophylls described earlier and carotenoids were estimated spectrophotometrically at 480 nm by following the method of Kirk and Allen (1965). Total carotenoids were estimated using the following formula of Liaaon-Jenson and Jenson (1971) :

$$\text{Carotenoids mg/100g} = \frac{A\ 480 \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{Wt of plant-material}}$$

(iii) Total Polyphenols :

Polyphenols were estimated by the method of folin and Denis (1915). Polyphenols from healthy and infected leaves of turmeric (Curcuma longa L.) were extracted in 80% acetone and filtered through Whatman Filter Paper No.1 using Buchners funnel under Suction. Polyphenols were extracted repeatedly from the residue. The volume of filtrate was made to 50 ml.

Then 2 ml of filtrate and 10 ml of 20% Na_2CO_3 was taken in 50 ml marked Nesselors tube. The volume was adjusted to 35 ml with distilled water. Then add 2 ml of folin-Dennis reagent. (100 g Sodium tungstate and 20 g phosphomolybdic acid were dissolved in 200 ml distilled water. 50 ml 25% phosphoric acid were added and it was refluxed for 2-3 hours using water condensor. The volume was adjusted to 1 litre with distilled water). Final volume of reaction mixture adjusted to 50 ml with distilled water. Absorbance was measured after half an hour at 660 nm using a blank which was prepared without polyphenols.

A std. curve of polyphenol obtained by tannic acid (0.1 mg/ml) from std. curve the unknown amount of polyphenols in the extract was determined to calculate the amount of polyphenols in % by using the following formula.

$$\text{Polyphenols \%} = \frac{\text{Value from curve}}{\text{2 ml extract}} \times \frac{\text{50 ml}}{\text{0.5 g.wt.}} \times 100$$

(iv) Titratable Acid Number (TAN) :

TAN was estimated by the method of Thomas and Beevers (1949). The healthy and infected leaves of Curcuma longa L. Varieties were washed with distilled water. They were blotted to dry and cut into small pieces. 1 gm of leaf material was accurately weighed and transferred to 150 ml beaker containing 100 ml distilled water. Then boiled for half an hour and cooled to room temperature. The extract was filtered through muslin cloth and volume of filtrate was measured. 5 ml of filtrate was transfered to a clean conical flask and titrated against N/40 NaOH using phenolpthalein as an indicator.

NaOH was standardised against N/40 oxalic acid using the same indicator. Titratable acid number (TAN) represents the number of ml of decinormal NaOH required to neutralize the acids present in 100g of fresh tissue. It was estimated by using following formula :

$$TAN = \frac{\text{Volume of oxalic acid taken for titration}}{\text{Titration reading of NaOH}} \times \frac{\text{Total volume of extract}}{\text{Wt of plant material in g}} \times \frac{\text{Extract titration reading}}{\text{Volume of extract taken for titration}} \times \frac{100}{4}$$

(v) Curcumin :

Estimation of curcumin from plant Rhizome powder of different varieties of Curcuma longa L. was determine by "ASTA" official analytical method of the American spice trade association.

0.5 g of finely ground turmeric powder was extracted by refluxing a water cooled condenser with 40 ml of distilled alcohol for 2.5 hours. The extract was transferred to a 100 ml volumetric flask and made to volume with alcohol. It is then filtered and an aliquot of 5 ml is transfered to a 100 ml volumetric flask and made to volume. It is mixed well and the absorbance of this solution is measured at 425 nm against alcohol blank using the absorbance value of a standard solution of curcumin (0.00025 g/100 ml gives an absorbance of 0.42) the curcumin percentage is calculated by following formula

$$\text{Curcumin} = \frac{0.00025 \times \text{absorbance of sample} \times 100 \times 100}{\text{absorbance of std.} \times \text{wt. of sample} \times 5}$$

(Note - The alcohol used for the extraction should be in the pH range below 6.5)

(vi) Carbohydrates :

The sugars were estimated by following the method of Nelson (1944).

The soluble carbohydrates were extracted from 1 g oven dried plant material with 80% alcohol. The extract was filtered through buchners funnel whatman filter paper No.1. The filtrate thus obtained was condensed on water bath to about 5 ml. To this 2 g of lead acetate and potassium oxalate (1:1) were added with constant stirring the contents and 20 ml distilled water was added and filtered through buchners funnel using whatman No. 1 filter paper. The volume of filtrate is served as extract "A" for estimation of reducing sugar. A known volume of this filtrate was transferred to a conical flask and closed with a cotton pluge and autoclaved for 30 min. under 15 lbs pressure. After cooling to room temperature the content was neutralized with unhydrous Na_2CO_3. Filter again the volume of filtrate was recorded. This filtrate was used for the estimation of total sugar (reducing + non reducing sugar).

Starch

For estimation of starch the insoluble residue obtained at the begining after filtering the alcoholic extract was transferred to a 100 ml capacity conical flask with 15 ml distilled water and 5 ml Conc. HCl. It was hydrolysed at 15 lbs pressure for half an hour, and then cooled to room temperature.

The contents were neutralised with unhycrous Na_2CO_3 and filtered. The volume of filtrate was measured. This filtrate was used for estimation of starch.

The filtrate contains reducing sugars (glucose) formed as a result of hydrolysis of starch. These sugars represent the starch content in this residue.

The sugars from all these filtrates were estimated by determining the reducing power by employing arsenomolybdate reagent introduced by Nelson (1944) for the calorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent. For this 0.5 ml filtrate (aliquots) (Reducing sugar and Total sugars) and 0.1 ml aliquot for starch were taken in test tubes alongwith different concentration of stardard glucose solution (0.1 mg/1 ml) in other test tubes to the requisit amount of distilled water was added to make final volume 10 ml. In case of blank instated of filtrate standard glucose distilled was added to begin with the reaction. 1 ml of somogyis alkaline copper tartarate reagent (40g $CuSO_4$, 5 H_2O, 24g unhydrous Na_2CO_3, 16g Na-K tartarate and 180g unhydrous Na_2SO_4 were dissolved in 1 litre distilled water) was added in each test tube and all these reaction mixture were transferred to boiling water bath for 10 min. after cooling to room temperature 1 ml. Arsenomolybdate reagent, (25g ammonium molybdate were dissolved in 450 ml distilled water to which 21 ml concentrated H_2SO_4 were added. 3g of Sodium arsenate

(Na_2HASO_4, 7 H_2O) were dissolved in 25 ml distilled water. The solutions were mixed well and kept in an incubator at 37°C for 48 hours before use) was added to each reaction mixture which were further diluted to 10 ml with distilled water after 10 min absorbance was read at 560 nm on spectrophotometer.

Using calibration curve of standard glucose, the sugar percentage in the above these fractions were calculated. Value of soluble sugars are expressed as g/100g of tissue.

Protein content :

Protein was estimated by Biuret method described by Gornall et al., (1949).

1 g of fresh plant material (Healthy and infected leaves of Curcuma longa L.) was homogenized in 0.14 Mol cold saline (NaCl). The extract was filtered and centrifused for 15 min. The supernente was used as a source of protein.

1 ml of plant extract was mixed with 8 ml Biuret reagent and it was incubated at 37°C for 30 min. The absorbance of violet colour developed was measured at 540 nm. Simultaneously a set of reaction mixture containing different concentration of Std. curve of protein. The curve was used to determine the amount of proteins.

(E) Enzyme activity

(i) Nitrate reductase :

Activity of nitrate reductase was determined by following the invitro technique developed by Jaworski (1975).

2 g of plant leaf material (Healthy and Infected leaves of Curcuma longa L.) incubated in the medium containing 1 ml 1 Mol KNO_3, 2 ml 5% n-propanol, 5 ml 0.2 M phosphate Buffer pH 7.5) and 2 ml 0.5% Triton in 100 ml sealed tube for one hour in dark condition.

1 ml reaction mixture was taken out and mixed with 1 ml 1% sulphanilamide in 1 M HCl and 1 ml 0.02% NEEDA (N-1 Napthyalene amide dihydrochloride). The absorbance was read at 540 nm on Spectrophotometer (Spectronic 20).

A standard curve of Nitrate was prepared with 0.03 KNO_3 (0.0026 mg KNO_3).

(ii) Acid phosphatase : (E.C. 1:1:3:2)

Activity of enzyme acid phosphatase was studied by method of Malachlam (1980). Accurately weighed •5 g of plant leaf material (Healthy and Infected leave of Curcuma longa L.) was homogenized separately in 10 ml cold acetate buffer (0.1 M· pH 5) in a chilled mortar with pastle. The resultant extract was filtered through four layered muslin cloth and filtrate was centrifuged at 6000 rpm. for 10 minutes. The supernent was used as a enzyme source.

The assay mixture contained 3 ml of p-nitrophenol phosphate (0.1 mg/ml of acetate buffer pH-5) 2 ml of acetate buffer (0.1 M pH-5) and 1 ml enzyme. The reaction was allowed

to proceed for 60 min. and then terminated by adding 1.5 ml. of NaoH (1.68 N). the reaction was terminated immediately to detain enzyme activity at 0.min, optical density of the yellow colour developed was read at 420 nm. using a substrate blank. The enzyme activity expressed as $OD\text{-}h^{-1}$ fresh weight.

(iii) Amylase : (E.C-3:1:1)

A modified method of Kalsumi and Fukuhani (1969) was used to study the amylase activity 0.5 g of fresh leaves from healthy and infected plants were collected, washed with distilled water and homogenised in pre-chilled mortor and pestle in 10 ml cold acetate buffer (0.1 M PH-5.00). The extract was filtered through four layered muslin cloth and centrifuged at 6000 rpm for 10 minutes. The supernent was stored on an ice bath and used for enzyme assay. All the operations were carried out at 0.4°C .

The activity of amylase was assayed by incubating 1 ml enzyme and 1 ml amylose (0.2%) and acetate buffer - (0.1 M PH - 5.00). The reaction was killed after 0 to 30 minute with 2 ml acetic acid (0.5 N). The aliquote 1 ml from killed reaction mixture was then treated with 10 ml dilute I_2KI solution (o.25% I_2 prepared in 0.1% aqueous KI solution). The absorbance was measured on spectronic 20 at 700 nm using I_2KI solution as blank exzyme activity was calculated using the following formula.

$$DB = 2 \times \frac{d - D}{d} \times \frac{100}{10}$$

where d = O.D at 'O' minute.

D = O D at 30 minute

DB = is the unite enzyme activity expressed as Mg of hydrolysed amylose jodine complex at 700 nm was decreased 10% with 1 ml of enzyme in 30 minutes reaction at 40°C.

(F) Inorganic constituents

(a) Nitrogen

(i) Preparation of acid digest

Total nitrogen was estimated from healthy and infected leaves of Curcuma longa L. by the method of Hawk et al., (1948). .5g of oven dried powdered plant material was transferred to 300 ml Kjeldahls flask containing 10 ml of 1:1 H_2So_4 a pinch of microsalt and few glass bids (to avoid bumping). The flask was heated on a low flame till a colourless solution was obtained. Then it was cooled, dituted with distilled water and transferred quantitatively to a 100 ml volumetric flask. The volume was made with distilled water. It was then filtered through Whatman No.1 filter paper. The filtrate was used for nitrogen estimation.

(ii) Two ml of filtrate was taken in a Nessler's tube to which a drop of 8% $KHSO_4$ was added and volume was made to 35 ml with distilled watter. Then 15 ml of Nesslers reagent. (7 g of KI + 10g of H_gI_2 dissolved in 40 ml distilled water and 10 g of NaoH dissolved in 50 ml of distilled watter. The reagent was prepared fresh by mixing the both solution at the time of estimation) were added to it. After 10-15 minute the absorbance was recorded at 250 nm on spectronic 20. The blank contained all the ingradients except nitrogen source. Standard curve was obtained by using different concentration cf ammonium sulphate (0.05 mg/ml) in place of the filtrate.

(b) Minerals

(i) Preparation of acid digest (Extract)

Healthy and Infected plant material of different varieties of Curcuma long L. was taken, cleaned well with distilled water and dried at 80°C in an oven till constant weight obtained. This oven dried material was taken for the estimation of different inorganic elements by following the method of toth et al., (1948).

0.5g of oven dried powdered material was transferred to a 150 ml beaker, to which 20 ml cone HNO_3 were added. The beaker was covered with watch glass and kept till the primary reaction subside. It was then subjected to slow heating to dissolved solid particles completely. After cooling to room

temperature 10 ml of 60% perchloric acid were added and mixed throughly. It was then heated strongly and vigorously untill a deam and colourless solution reduced to about 2-3 ml. After heating the liquid was made to 100 ml with distilled water and kept over-night. Next day it was filtered through a dry whatman filter paper No. 44 (ashless) and the filtrate was used for the estimation of different inorganic constitutents.

(ii) Estimation of Phosphorus

For the estimation of phoshorus, method of Sekine et al., (1965) was followed. Here phosphorus react with "Molybdate Vanadate Reagent" to give yellow colour complex. By estimating calorimetrically the intensity of the colour developed and by comparing it with the colour intensity of known standards phosphorus content was estimated.

Take 2 ml of acid digest in a test tube add 2 ml of 2 N. HNO_3 and 1 ml of molybdate vanadate reagent (1.25 g of ammonium vanadate dissolved in 500 ml of 1 N HNO_3 and 25 g of ammonium molybdate dissolved in 500 ml distilled water. The reagent was prepared fresh by mixing the both solution in equal proportion at the time of estimation) and adjust the volume to 10 ml with distilled water. The ingradients were mixed well and where allowed to react for 20 minutes. After 20 minutes colour intensity was measured at 420 nm. using a reaction blank without phosphorus. Calibration curve of standard phosphorus

solution (0.110 g KH_2PO_4 per litre i.e. 0.0025 mg p^{+5}/ml taking different concentrations 0.025, 0.05, 0.1, 0.2 and 0.4 g of phosphorus. Other steps being essentially similar to the one described above, with the help of standard curve, amount of phosphorus in the plant material was calculated.

(iii) Estimation of Sodium, Potassium, Calcium, Magnesium manganese, Iron, Copper, Zinc.

The acid digest extract was used to estimate Na^{2+}, K^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} elements on automic absorption spetrophotometter (Perkin - Elmer model 3030) using acetylene air Flame. The light source employed was hollow cathod lamp.