CHAPTER-III

MATERIALS AND METHODS

PLANT MATERIAL

Pan-mala

The studies on enzymes of mycoparasitism in Control, Infected by foot rot disease and *Trichodema* treated leaves of *Piper betle* plant were undertaken. The plant material for studies were collected from the selected betle leaf cultivars Mrs. Rajendra Rajunath Kanase, At/Po: Shanoli, Tal – Karad, Dist. Satara.

A selected pan mala is three kilometer from Rethare Sugar factory (Shivnagar), Tal – Karad, Dist – Satara. The area of selected pan mala is ½ acre (20 gunthas) , soil is alkaline and well irrigated field, grown mainly for commercial and economical purpose.

The studies were conducted on one and half year during 2006-2007. Mainly variety local Koppari grown in selected pan mala, which is a ruling local type at Karad taluka.

The soil type in selected pan mala for study is moderately alkaline (pH 7-44). The sample for study contain control leaves, Infected spot, *Trichoderma* treated and Foot – Rot (diseased yellow) leaves were collected from field at same day, date and time of the season. The experiments were carried out in thrice for definite conclusions.

Various standard methods were used for the analysis of organic, inorganic, contents and enzymes assays in control, infected ,

Trichoderma treated leaves of betel plant. The studies were further extended for pH of soil and soil temperature etc. of the locality.

***Trichoderma* Sample preparation and uses**

The betle vine plant is infected by Root-Rot disease leaves. Are selected for the treatment of *Trichoderma* is biological controlling agent.

The *Trichoderma* pure culture in solid (powder) is obtained from National Agricultural Research Station Kasabe Digraj, Dist – Sangli. In Research Station two main species i.e. contain *Trichoderma viride* and *Trichoderma harzani*um are cultured and used for commercial for sale. The *Trichoderma viride* variety is to find out the most effective one to the respective series against *Phytophthora parasitica* Var *piperina* which cause leaf spot and foot-rot of betel vine.

In this study we are selected *Trichoderma viride* Culture in Power Form.

Culture method and application

The easiest and most direct approach of application of *Trichoderma* to infected betel vine is (a) To drench the soil with a suspension of Trichoderma propagules (b) To spray on infected leaves.

There are other different treatments techniques are -

(a) To add in the soil colonized grain bran (Wells et al., 1972), wheat straw composed hard wood bark (Hoitink, 1980 ; Nelson and Hoitink, 1983).

(b) To dip the roots of the plants in the propagule suspension of *Trichonderma* spp. (Dutta 1981, Jordan and Tarr, 1978)

(c) Addition of *Trichoderma* spores to soil with a liquid fertilizers with seedling sets used for fluid drilling of small seeds (Fisher et al., 1983 ; Walker and Commick, 1983).

(d) Incorporation of *Trichoderma* through sprinkler irrigation (Lewis and Papavizas, 1983).

Drenching Treatment

The under cultivation of betle garden for studies has variety, Kapoori. The disease is soil borne and hence drenching was done in root zone of infected vine. *Trichoderma* Suspension prepared by mixing 10 gram *Trichoderma* viride formulation in (powder form) /1 litre of water. Every infected vine was drenched with half litre of *Trichoderma* suspension.

The *Trichoderma* formulation was so prepared or to have 10^8 spores/gram. The vines were irrigated next day drenching with

Trichoderma. The change in enzymic activity were recorded 30 days after treatment.

The biochemical analysis for various leaf components such as organic and inorganic also taken in to account. Several workers used *Trichoderma* to combat Plant diseases. It covers soil drenching, seed treatment (Harman et al; 1981). Some researchers viz. Tiwari and Meherotra;1973 and Vyas et al,1981) reported the usefulness of *Trichoderma* viride as a biocontrol agent in betelvine disease management. However, Tiwari and Mehrotra;1968; Mehrotra and Tiwari; 1976) showed earlierwork on stem rot of betle vine reduced by dipping of cuttings in a *Trichoderma* viride cell suspension.

Owing to fast growth rate and antagonistic nature, not just phytophthora but also several other species of soil born pathogens are checked by *Trichoderma* viride. For studies on enzymic activity affected. Due to treatment of *Trichoderma* on vine before after treatment (30 DAT) were collected from candidate vines which revealed positive reactions in treated vine.

To know the changes taking place in diseased vines in respect to enzymic activities governed by *Trichoderma* in comparision with control, Healthy vine.

METHODS :

In the present investigation attempts have been made to study change in enzyme activity during mycoparasitism of *Trichoderma* spp. During control of Root-Rot disease of *Piper betel* leaves infected by *Phytophthora parasitica* Var. piperina Control (Healthy), Infected and , *Trichoderma* traeted leaves of *Piper betle* plant were collected from the selected pan mala near area of Rethare Bk., Tal – Karad, Dist – Satara.

The control, infected leaves were collected and *Trichoderma* treated and drenching treatment were collected from the same field and some day , date and time of the season.

Generally leaves from control (Healthy) leaves, Infected leaves of similar age are collected and used in the eperiments. Experiments were carried out in three sets to draw a definite conclusions. Various standard methods were used for the analysis of organic, inorganic and change in enzyme activity of the control (Healthy), Infected and *Trichoderma* treated leaves of *Piper betel* plant.

(A) Moisture Percentage

Control, infected and *Trichoderma* treated leaves of *Piper betel* are taken and cleaned well, with distilled water. Surface 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 :

$$\text{Moisture percentage} = \frac{\text{Fresh wt.} - \text{Dry. Wt.}}{\text{Fresh Wt. In grams}} \times 100$$

(B) Soil pH

pH of soil in selected pan mala of *Piper betle* is measured by the method of pH meter. Take 20 gms. Oven dried soil sample in 100 ml. distilled water. Stirr the solution for about an one hour at regular intervals. Allow to settle the solids. Filter the Supernant solution. Measure the pH of supernant solution using pH meter.

Express the results directly in pH units specifying the dilution of soil suspension = e.g. (pH of 1:5 soil suspension).

(C) Soil Temperature :

Soil Temperature is measured by soil thermometer. According to Z.S. Garvitch & M.C. Probine (1956) it is inserted into the ground, soil temperature at depth of 1 ft. or greater. Temperature is measured of the end of the probe. This allows to easily monitor the soil conditions while planting, growing or so this will make a great addition to any home. Temperature of Garden is also measured with addition of the soil thermometer. It is established practice in many part of the world to use a specially modified which is hung in a tube and withdrawn for reading. So that its indication will not change appreciably during the process of reading, the bulb of thermometer surrounded by a mass of paraffin wax to give it a high lag coefficient.

(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 reportedly with 80% acetone, collecting the washings in the same filtrate. The volume of filtrate was made to 100 ml with 80% acetone. Extraction 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.

Chlorophylls `a' = 12. 7 x A 663 – 2.69 x A 645 = X

Chlorophylls `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

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

(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 Krik and Allen (1965). Total carotenoids were estimated using the following formula of Liaaon – Jenson and Jenson (1971).

$$\text{Carotenoids mg /100 g} = \frac{\text{A 480 x volume of extract x 10}}{\text{2500 x Wt. Of the plant material in mg .}} \times 100$$

(iii) Total Polyphenols

Plyphenols were estimated by the method of Folin and Denis (1915). Polyphenols from control, Infected, *Trichoderma* treated leaves of betel vine (*Piper betel* L.) were extracted in 80% acetone and filtered through Whatman filter paper No.1 using Buchners funnel under section. 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 Nesselars 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 condenser. The volume was adjusted to 1 liter 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 standard curve of polyphenol obtained by tannic acid (0.1 ng/ml) from standard curve the unknown amount of polyphenols in the extract was determined to calculate the amount of polyphenols in % by using the following formula.

$$\text{Total polyphenols in (\%)} = \frac{\text{Absornace 660 x Std. T.A. x. Vol. Of Extract}}{\text{Std. Abs. X Volm. Taken for assay x Wt.of plant mat. (in gm.)}}$$

(iv) Titratable Acid Number (TAN)

TAN was estimated by method of Thomas and Beevers (1949). The control, Infected & *Trichoderma* sprayed and Root-rot leaves of *Piper betel* L. variety Kapoori were washed with distilled water. They were blotted to dry and cut in to small pieces. 1 gm of leaf material was accurately weighed and transferred tom 150 ml beaker containing 100 ml of 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 transfer to a clean conical flask and filtrated against N/40 NaOH using phenolphthalein as an indicator.

NaOH was standardized 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 acid present in 100 g of fresh tissue. It was estimated by using following formula.

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

(V) Carbohydrates

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

The soluble carbohydrates were extracted from 1 gram 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 minutes. 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 bachners 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 plug and autoclaved for 30 minutes under 15 lbs pressure. After cooling to room temperature the content was neutralized with unhydrous Na_2CO_3. Filter again. The volume of filter was recorded. This was used for the estimation of total sugar (reducing + non reducing sugar).

(i)Starch

For estimation of starch the insoluble residue obtained at the beginning after filtering the alcoholic extract was transferred to a 100 ml capacity conical flask with 15 ml distilled water and 5 ml. Concentrated HCl. It was hydrolyzed at 15 lbs pressure for half an hour, and then cooled to room temperature. The contents were neutralized with unhydrous 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. The 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 along with different concentration of standard glucose solution (0.1 mg/1 ml) in other test tubes to the requisite amount of distilled water was added to make final volume 10 ml. In distilled water was added to begin with the reaction 1 ml of somogyis alkaline copper tartarate reagent (40 g

$CuSO_4$, $5H_2O$, 24 g unhydrous Na_2CO_3, 16 g Na-k tartarate and 180 g unhydrous Na_2SO_4 were dissolved in 1 ml filter distilled water) was added in each test tube and all these reaction mixture were transferred to boiling water bath for 10 minutes after cooling to room temperature 1 ml. Arsenomolybdate reagent, (25 g ammonium molybdate were dissolved in 450 ml distilled water to which 21 ml concentrated H_2SO_4 $7H_2O$,) 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 m10 ml with distilled water after 10 minutes 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 / 100 g of tissue.

(VI) Soluble Protein-

The soluble proteins in the enzyme extract were determinant according to the method of Lowry et al., (1951). 0.1 ml enzyme extract was taken in test tube and diluted to 1 ml with distilled water. To this 5 ml of reagent C solution)50 ml of A containing 2% sodium carbonate in 0.1 N aqueous NaOH was mixed with 1 ml of B containing 0.5%

copper sulphate in 1 %, Na - k tartarate) was added, mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes 5 ml Folin Cio-caltaue phenol reagent was added readily with immediate mixing. This was allowed to stand for 30 minutes in dark and intensity of developed blue colour was measured at 660 nm on spectronic 20.The Protein content was calculated by comparing with standard curve of different concentrations of Bovine Serum Albumin (0.1 mg/ml) prepare in a similar manner. The values were expressed as mg g^{-1} fresh tissue.

E) Inorganic Constituents

(i) Preparation of plant extracts (Acid digest)

Healthy and infected plant material of different vaieties of *Piper betle* 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 Acid digestion method of Toth et al., (1948).

0.5 g 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 followed to slow heating to dissolved solid particles completely.

(iii) Phosphorus

For the estimation of phosphorus, method of Sekin et al., (1965) was followed. Phosphorus react with `molybdate vanadate' reagent to give yellow colour complex. By estimating calorimetrically the intensity of colour developed and by comparing it with the colour intensity of known standards, phosphorus content was estimated.

4 ml of acid digest were taken in test tube and to two ml of 2N HNO_3 and 1 ml of `molybdate vanadate' reagent is added (A – 25 g ammonium molybdate in 500 ml of distilled water, B-1.25 g ammonium vanadate in 500 ml 1 N HNO_3, A and B were mixed at the time of experiment) were added. After 20 minutes, colour intensity was measured at 420 nm using a reaction blank containing no phosphorus.

Calibration curve of standard phosphorus was prepared from standard phosphorus solution containing mg per ml (0.110 g KH_2PO_4 per liter = 0.025 mg P^5ml^{-1}) with the help of standard curve. The amount of phosphorus in the plant material was calculated and it was expressed on dry weight basis.

After cooling to room temperature 10 ml of 60% perchloric acid were added and mixed thoroughly. It was heated strongly and vigorously until a dim 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 constituents.

(ii) Estimation of Sodium , Potassium, Calcium, Magnesium, Iron, Copper

Sodium potassium, calcium Magnesium Iron and copper were estimated using atomic absorption pectrophotometer.For standardization various concentrations of sodium (20 – 100 ppm) and potassium (20-100 ppm) and calcium (20 – 100 ppm) from NaCl, KCl and $CaCl_2$ respectively were prepare using standard solutions. Standard curve for these elements were prepared. The plant extract (acid digest) was analyzed in the similar manner. In case needed appropriate dilution of plant extract were made with distilled water.

(F) Enzyme Studies -

(a) Phenol Oxidase (E.C. 1. 10. 3. 2)

Activity of enzyme phenoloxidase from leaves of control (Healthy), Infected, *Trichoderma* treated was studied following the method of Mahadevan and Sridhar (1982).

One gram of fresh leaves (control), Infected and *Trichoderma* treated of *Piper betle* were crushed in 15 ml of 0.1 M phosphate buffer (pH-6.1).The resultant homogenate was filtered through four layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 4 ml 0.1 M phosphate buffer (pH 6.1) 1 ml 0.01 M cotechol prepared in 0.1 M phosphate buffer (pH 6.1), p.5 ml enzyme and mixed well. The increase in OD at 30 seconds interval up to 180 seonds at 495 nm was recorded. The enzyme activity was expressed as ΔOD min^{-1} mg^{-1} of protein.

(b)peroxidase (EC 1. 11 .1.7)

To study peroxidase activity the method of Machly (1951) was followd one gram of leaves of *Piper betle* were homogenized in 15 ml ice cold (1/15 M) phosphate buffer (pH – 6.8) & filtered through four

layers of musclin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 5 ml of 1/15 M Acetate buffer (pH – 5), 0.5 ml of 0.1 % guaiacol, 1 ml enzyme extract, 2 ml D.W. p.5 ml p.08 % H_2O_2. The reaction mixture was incubated at 30°C. After 15 minutes of incubation, 1 ml $1NH_2SO_4$ was added to stop the reaction, followed by measurement of absorbance at 470 nm. The enzyme activity was expressed as units h^{-1} mg^{-1} protein.

(c) Cellulase (E.C. 3. 2.1.4)

Cellulase activity was studies by the method of Dinitrosalicyclic Acid method. Reference Densen, DA and Koehn, RD (1977) Mycologia Lx I x 592.

Accurately weighed one gram of fresh leaves of *Piper betle* were homogenized in 0.1 M sodium citrate buffer (pH 5.0) in a mortar with pestle, pipette out 0.45 ml 1 % carboxymethyl cellulose solution (CMC) at a temperature 55°C and 0.05 ml of enzyme extract. The mixture is incubated at 55°C for 15 minutes.

Immediately after removing the enzyme substrate mixture from the boiling water bath, 0.5 ml DNS reagent. Heat a mixture in a boiling water bath for 5 minutes. While the tubes are warm, add 1.0

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Shri.Yadav Sanjay.	At / Po.- Lavan Machi. Tal.-Walwa, Dist- Sangli	Half Acre.
Shri.Kanse Raghunath Appa	At / Po- Shenoli. Tal. -Karad, Dist-Satara.	Half Acre.
Smt. Mulla Lalbi Aabu.	At/Po.- Killemachandragad. Tal.-Walwa, Dist.- Sangli.	Half Acre.

ml potassium sodium tartarate solution.The reaction mixture allow to cool at room temperature. Add water to make volume 5 ml. The absorbance was measured on spectronic 20 at 540 nm.

A standard graph with glucose is prepared in the concentration range 50 mg to 1000 mg/ml. The enzyme activity is expressed as the mg glucose released per minute per mg protein.

G) Users of *Trichoderma spp.*

Piper betle is on large scale in Maharashtra , especially in the districts like Sangli ,Satara , Kolhapur,Nasik,Ahmadnagar etc. The cultivers in Sangli and Satardistrict now adapted the biological method of *Trichoderma spp.*treatement for controlling various diseases of Betle vine.Some of the farmers in our neighbouring area enlisted as fallows.