Materials and Methods
MATERIALS AND METHODS

GLASSWARE AND CHEMICALS

In all the experiments, acid washed (0.4 N HCl) pyrex glassware rinsed with double distilled water was used. For the preparation of culture media and other chemical reagents analytical grade chemicals (E Merck GR/BDH, Analar R) were used.

STERILIZATION

All the glassware used in the experiments were sterilized in hot air oven at 160°C for 4 hours.

The media used in the experiments were sterilized in an autoclave at 15 lbs per square inch for 15 minutes.

SOURCE AND RAISING OF TEAK SEEDLINGS

One year old teak seedlings were obtained from Divyaramam nursery, Tirupati, Andhra Pradesh, India.

The seedlings were raised in 20 earthen-ware pots of 30 cm diameter. The pots were filled with soil and farmyard manure in 4:1 ratio. One seedling per pot was planted and watered daily. These seedlings were used to study the effect of phylloplane fungal spore suspension and biochemical changes of diseased (powdery mildew) plants in the present investigation (Plate-1).
PLATE-1

A. One year old teak seedlings
SAMPLE COLLECTION

The leaf samples of both healthy and diseased plants were collected in separate sterilized polythene bags from different areas of teak plantations located at Rangampet, Chandragiri, Tirupati and Palamner in Chittoor district, A.P., India.

COMPOSITION OF THE MEDIA EMPLOYED

For the isolation and the study of phylloplane fungi Czapek-Dox agar, Martin’s rose-bengal agar, Potato dextrose agar medium and Czapek-Dox liquid medium for nutritional studies of mycoparasites were used in the present work.

The composition of each medium was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit.</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>
All the ingredients except phosphate was dissolved in half litre of the water and then sucrose was added. Phosphate was dissolved separately, added to the rest and made up to one liter.

**b. Martin's Rose-Bengal agar medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit.</td>
</tr>
<tr>
<td>Rose Bengal</td>
<td>0.035 g</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

Streptomycin was added to the medium after autoclaving and cooled at 42-45°C.

**c. Potato dextrose agar medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato (peeled)</td>
<td>200 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit.</td>
</tr>
<tr>
<td>pH</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Peeled off potatoes were cut into small bits and boiled in 500 ml of water, filtered through cheese cloth and dextrose was added to the filtrate. Agar was dissolved in hot water added to the above mixture and made up to one liter.
Czapek-Dox liquid medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit.</td>
</tr>
</tbody>
</table>

MOUNTING LIQUIDS

Lactophenol (equal quantities of phenol, glycerol, lactic acid and distilled water) and lactophenol-cotton blue mixture were used as mounting media and stains for semi-permanent microscopic preparations and then the slides were sealed with canada balsam.

Details of the materials and methods used in the investigation are presented under three sections.

1. Incidence of powdery mildew disease on teak plantations and identification of the pathogen. Studies on pathogen conidial germination and physiological changes in the infected host leaves.

2. Isolation, identification and screening of mycoparasites and their nutritional studies.

3. Disease management by chemicals, fungicides and plant extracts.
SECTION - I

IDENTIFICATION OF THE PATHOGEN

The collected leaf samples from different areas were observed for type, colour and position of leaf spots. The samples were brought to the laboratory and examined under microscope to detect the presence of anamorphs and teleomorphs of the pathogen.

Microslides were prepared by scraping the infected area with needle moistened in lactophenol and mounted in lactophenol-cotton blue mixture. Slides of free hand sections were also made to observe the structure of mycelium and origin of conidiophore. All the prepared slides were examined under the microscope to study the morphological characters of the pathogen. The microphotographs were also taken. The measurements were taken with an ocular micrometer and the scale with a stage micrometer.

1. INCIDENCE OF POWDERY MILDEW DISEASE

Randomly selected teak plants around Chittoor district, Andhra Pradesh were surveyed for two consecutive years (1998-2000) during September-March and progress of the disease was recorded. The percentage of affected plants and older leaves was calculated.

In four locations of Chittoor district viz., Rangampet, Chandragiri, Tirupati and Palamaner (Plate-2), fifty plants in each location about 3-5 years old were selected and about 300 older leaves per location were tagged for the observation of disease incidence. The observations were recorded at 10 days interval.
PLATE-2

Teak Plantations

A. Rangampet location
B. Chandragiri location
C. Tirupati location
D. Palamaner location
3.2. EFFECT OF TEMPERATURE AND RELATIVE HUMIDITY ON CONIDIAL GERMINATION UNDER IN VITRO CONDITIONS

Conidia from fresh mildew affected teak leaves were gently dusted on clean glass slides and kept over U-shaped glass rod placed in petri plates containing moistened cotton wool. The petriplates were sealed with parafilm and incubated at 0, 5, 10, 15, 20, 25, 30 and 35° C.

For relative humidity studies, similarly slides were incubated at 25, 40, 50, 55, 75, 80, 90 and 100 percent relative humidity at room temperature (27° C). These appropriate humidity levels were maintained by using different proportions of concentrated H₂SO₄ and distilled water (Solomon, 1951). The treatments were repeated for three times. After 24 hrs incubation, observations on percent germination, length of germ tube were recorded on 50-100 conidia per treatment.

3.3. BIOCHEMICAL STUDIES

For biochemical studies, two sets of one year old plants were raised in pots. In one set the plants were inoculated with spore suspension of 4000-6000 spores/ml and incubated in moist chamber for 48 hrs, then transferred in the open. The second set was grown without inoculation. After 10, 20 and 30 days of incubation, the leaves were collected and studied with healthy leaves as control (Plate-3).
PLATE-3

A. 10 days of inoculation
B. 20 days of inoculation
C. 30 days of inoculation
3.3.a. **ESTIMATION OF CHLOROPHYLL**

Total chlorophyll content of the leaves was determined according to the method of Arnon (1949). One gram of leaf sample was cut into small pieces and ground with the pestle in a mortar using 80% acetone. The extract was centrifuged at 3000 rpm for 15 min and the absorbance of the supernatant was measured in a Schimadzu spectrophotometer at 645 nm and 663 nm.

The following formulae were used for the estimation of total chlorophyll, chlorophyll ‘a’ and chlorophyll ‘b’ (Arnon, 1949).

- Total chlorophyll (µg/ml): \((20.2 \times OD_{645}) + (8.02 + OD_{663})\)
- Chlorophyll ‘a’ (µg/ml): \((12.7 \times OD_{663}) - (2.69 + OD_{645})\)
- Chlorophyll ‘b’ (µg/ml): \((22.9 \times OD_{645}) - (4.68 + OD_{663})\)

The chlorophyll content was expressed as mg chlorophyll per gram fresh weight of the leaf.

3.3.b. **ESTIMATION OF CARBOHYDRATE FRACTIONS**

The method of extraction was similar to that described by Mahadevan et al., (1965). The plant material was chopped into small pieces and one gram of the material was transferred to about 10 ml of boiling 80% ethanol for five minutes on a hot water bath by refluxing and cooled. The material was homogenized in a mortar with pestle and squeezed through a wet cheese cloth.
The material was transferred back to a small quantity of fresh boiling 80 per cent ethanol and re-extracted for five minutes. Both the extracts were pooled and centrifuged. The supernatant was concentrated on a boiling water bath to 5 ml.

3.3.c. ESTIMATION OF REDUCING SUGARS

Reducing sugars in the ethanol extract were determined by Nelson Somogy’s Method (Nelson, 1944).

Estimation

To one ml of ethanol extract in a test tube, one ml of a mixture of reagent ‘A’ and reagent ‘B’ prepared by mixing 25 parts of reagent ‘A’ with one part of reagent ‘B’ was added. The mixture was heated for 20 min in a boiling water bath and cooled in tap water. One ml of the arsena-molybdate colour reagent was then added. The solution was thoroughly mixed and diluted to 25 ml with distilled water. The resulting blue colour was read in Schimadzu spectrophotometer at 497 nm. Reducing sugar content was calculated using a standard graph with glucose.

Preparation of Copper Reagent ‘A’

In 800 ml of distilled water, 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartarate (Rochelle salt), 20 g of sodium bicarbonate and 200 mg of anhydrous sodium sulphate were dissolved. The resulting solution was diluted to one litre with distilled water.
Preparation of Copper Reagent 'B'

Fifteen grams of copper sulphate was dissolved in 100 ml of distilled water and to this 1 or 2 drops of conc. H₂SO₄ was added.

Preparation of Arseno-Molybdate Colour Reagent

To 450 ml distilled water, 25 g of ammonium molybdate, 21 ml of conc. H₂SO₄ and 3 g of sodium arsenate in 25 ml of distilled water were added and the mixture kept in an incubator at 37°C for 48 hrs. The reagent was stored in a glass stoppered brown bottle.

3.3.6. ESTIMATION OF NON-REDUCING SUGARS

Hydrolysis of the extract

Non-reducing sugars present in the ethanol extract were first hydrolysed to reducing sugars as described by Inman (1965). One ml of ethanol extract was taken in a test tube and evaporated to dryness on a water bath. To the residue, 1 ml of distilled water and 1 ml of 1N H₂SO₄ were added. The mixture was hydrolysed by heating at 49°C for 30 minutes. The resulting solution was neutralized with 1 N NaOH using methyl red indicator.
Estimation

Total sugars in the hydrolysed sample were estimated by Nelson Somogy's method (Nelson, 1944). Reagent blanks were prepared with one ml of distilled water instead of ethanol extract. The quantity of non-reducing sugars was calculated by subtracting the reducing sugar content from total sugars and expressed as glucose equivalents.

3.3.e ESTIMATION OF STARCH

The residue left behind after ethanolic extraction of the original material was used for extraction of starch and estimated according to the method of McCready et al. (1950). Starch was solubilized with 52% perchloric acid for 30 minutes, filtered and made the volume upto 100 ml with distilled water. One ml of this extract was diluted to 5 ml with distilled water in a boiling test tube and 10 ml of fresh anthrone reagent (prepared by dissolving 200 mg of anthrone in 100 ml of cold 95% H₂SO₄) was added. The tube with its contents was heated for 7.5 minutes at 100°C in a boiling water bath. The tube was cooled rapidly to room temperature and the colour intensity measured at 630 nm on Schimadzu spectrophotometer. The starch content was calculated by reading the value on a standard curve for glucose and multiplying the glucose equivalent present in the sample with 0.9.
TOTAL PROTEINS

Protein content was estimated by method of Lowry et al., (1951).

Preparation of Reagents

A 2% sodium bicarbonate in 0.1 N NaOH
B 0.5% copper sulphate in 1% sodium tartarate
C Mixture of 50 ml of reagent ‘A’ with 1 ml of reagent ‘B’
D Folin phenol reagent

All the above reagents were freshly prepared for the protein estimation.

1 gm of tissue was taken for protein extraction and ground in a mortar using 20 ml of 20% TCA. The contents were centrifuged and the supernatant was discarded. The protein precipitate was washed twice with cold TCA, centrifuged again and the protein precipitate was dissolved in 0.1 N sodium hydroxide and made upto 2 ml, out of this 0.1 ml was taken in a test tube, 5 ml of reagent C was added, mixed thoroughly and the solution allowed to stand for 10 min at room temperature. Then 0.5 ml of reagent D was added with instantaneous and vigorous shaking. After 30 min, the colour intensity was read at 660 nm on Shimadzu spectrophotometer. A blank was prepared by using the same volume of glass distilled water in place of protein extract. A reference curve was prepared with the known concentration of bovine serum albumin.

ESTIMATION OF TOTAL PHENOLS

Total phenols were estimated by employing Folin ciocalteau reagent (Bray and Thorpe, 1954).
**Preparation of Folin-ciocalteu reagent**

To 700 ml of distilled water, 100 g of sodium tungsten, 25 g of sodium molybdate, 50 ml of 80% ortho-phosphoric acid and 100 ml of conc. HCl were added. The mixture was refluxed for 10 hrs and cooled. To it, 180 g of lithium sulphate, 50 ml of water and a few drops of bromine were added. The mixture was boiled for 15 minutes, to remove the excess bromine, cooled and diluted to one litre with distilled water. The reagent was stored in a dark glass bottle and diluted with an equal volume of distilled water, just before use.

**Estimation**

To one ml of ethanol extract in a test tube one ml of Folin-Ciocalteu reagent and 2 ml of per cent sodium carbonate were added. The mixture was heated on a boiling water bath, for exactly one minute and cooled, resulting in development of blue colour. It was diluted to 25 ml with distilled water and absorbance determined in Schimadzu spectrophotometer at 725 nm. A reagent blank was maintained with one ml distilled water in the place of ethanol extract. Total phenols were calculated from the standard curve plotted for catechol.

3.3.k. **PEROXIDASE**

Peroxidase enzyme activity was estimated according to the method of Manoranjankar and Dinabandu (1976). The leaf material was homogenized with 0.1 M phosphate buffer pH 7.0 in a pre-chilled mortar and pestle and the homogenate was centrifuged at 15,000 rpm at 4°C for 30 min and an aliquot was used as the source of the enzyme.

Assay mixture for peroxidase contained 2 ml of 0.1 M phosphate buffer pH 7.0, 1 ml 0.01 M pyrogallol, 1 ml 0.005 M H₂O₂ and 1 ml of diluted enzyme extract.
3.4. **ISOLATION OF PHYLLOPLANE MYCOFLORA**

The phylloplane fungal populations were enumerated by standard methods (Aneja, 2001).

The collected leaf samples of both healthy and infected plants were cut into small pieces and weighed about 1 gm. The leaf bits were transferred into 9ml of sterile water blanks to get $10^1$ dilutions. From this, serial dilutions up to $10^5$ were prepared. One ml aliquots were transferred to labeled sterile petriplates. Triplicates were maintained for each dilution. The molten and cooled PDA medium was poured into the petriplates and rotated gently for proper distribution of the inoculum suspension with the medium. After solidification, the plates were incubated at $25 \pm 1^\circ C$ for one week. After incubation, the colonies were qualitatively enumerated, sub-cultures were maintained and identified.

**Maintenance of cultures**

The identified colonies were sub-cultured on potato dextrose agar slants and preserved in cold room. Sub-culturing was maintained at three months interval.
3.5. IDENTIFICATION OF PHYLLOPLANE FUNGI

Micro slides of each culture were prepared in lactophenol-cotton blue, examined under the microscope, observed their morphological characters and identified with the help of the keys provided by Ellis (1971, 76), Rifai (1969), Raper and Fennell (1965), Raper and Thom (1949), Booth (1971) and Subramanian (1971) in their representative manuals besides consulting relevant published literature. The identification of phylloplane saprophytes was done at Mycology and Plant Pathology Research Laboratory under the direction of Bagyanarayana, G., Department of Botany, Osmania University, Hyderabad. The measurements of the spores and vegetative structures were taken with the help of an ocular micro meter. The microphotographs of selected fungi were also taken wherever it was found necessary.

3.6. EFFECT OF PHYLLOPLANE SAPROPHYTES ON POWDERY MILDEW FUNGUS

The effect of phylloplane saprophytes against the pathogen was studied by spore germination method. A loopful of spores of each phylloplane fungus was kept in 2 ml of sterile distilled water for 48 hrs. It was centrifuged for 45 minutes at 300 rpm at 20°C and the supernatant liquid was used as suspension.

A drop of each phylloplane fungal spore suspension was placed on a microslide. The powdery mildew fungal spores were dusted over it and incubated in moist chambers for 24 hrs. Control was maintained in distilled water. After incubation, the germination percentage of pathogen spores (50-100 per microscopic field) was recorded from triplicates.
Alternatively, the effect of these saprophyte suspensions was studied on one-year-old seedlings by spraying each suspension followed by inoculation of spore suspension ($5 \times 10^5$ ml$^{-1}$) of powdery mildew fungus. The plants were incubated for two weeks in a glasshouse to maintain relative humidity which avoids evaporation of suspensions. Seedlings sprayed with distilled water served as control. After two weeks of incubation, the average number and size of the lesions from three leaves were recorded.

3.7. MYCOPARASITISM

The naturally infected powdery mildew leaves were collected and observed for mycoparasites. The infected leaves were scraped with sterile needle on to clean glass slides. The slides were stained with lactophenol-cotton blue and observed under microscope.

For light microscopy, 0.5 ml of the spore suspension of each phylloplane saprophyte was spread evenly on a cellophane membrane disk about 8 cm in diameter. These were placed on 2% water agar in a petri dish. The mature spores of *Uncinula tectonae* were dusted on to the membranes and kept for incubation at $25^\circ$C. After 2nd or 4th day of incubation, the cellophane membranes were removed and cut into strips of 1x1 cm size, mounted in lactophenol-cotton blue mixture and observed under the microscope for mycoparasitism.
One year old seedlings of teak were inoculated with spore suspension of *U. tectonae* and plants were kept in a growth chamber at 25°C. When *U. tectonae* colonies were about 10 days old and sporulating profusely, they were sprayed with conidial suspension of the mycoparasite *Cladosporium oxysporum* and incubated at 25°C. Such leaves were scanned under an electron microscope at different intervals of 24, 48 and 72 hrs.

**Scanning electron microscopy**

Powdery mildew infected leaf segments (3 x 4 mm) with mycoparasite were fixed overnight with 2% glutaraldehyde in 0.1M cacodylate buffer pH 7.4, rinsed and post fixed in 2% osmium tetraoxide in the same buffer. After several rinses in the buffer, the samples were dehydrated in a graded series of ethanol and critical point dried. The dried samples were mounted on specimen holders and sputter coated with gold. The specimens were examined in a Philips XL 30E Scanning Electron Microscope operating at an accelerating voltage of 10 KV.

1.3. **EFFECT OF CULTURE FILTRATES OF MYCOPARASITES ON POWDERY MILDEW FUNGUS**

2 mm discs of each mycoparasite cultures of seven days old actively growing on PDA medium were inoculated into 200 ml Czapek-Dox liquid medium in 1 lit. Erlenmeyer flasks with the help of a sterilized cork borer and incubated for 15 days at 25 ± 1°C. The culture metabolites were collected by filtering through cotton wool. The solutions were centrifused for 20 minutes at 2000 rpm. Supernatant liquids were collected and examined under microscope
which revealed absence of spores. Solution of metabolites in different concentrations (10, 20 and 50%) were made with the help of sterilized distilled water. The effect of metabolites on conidial germination of *U. tectonae* was studied by using slide spore germination method (Anonymous, 1943). The slides were incubated in moist chambers for 24 hrs. at 20 ± 1°C. Conidial germination in distilled water served as control. Observations were recorded and inhibition percentage of spores was calculated by Bliss (1934) formula.

### NUTRITIONAL STUDIES

#### a. Effect of carbon and nitrogen sources on mycoparasites

The biomass production of mycoparasites was studied by using Czapek-Dox solution. To study the effect of carbon, sucrose, glucose, fructose, lactose, xylose, maltose, ribose, starch, galactose, manose and pectin at 30 g/l only, were used as carbon sources and sodium nitrate at 2g/l was used as nitrogen source.

Similarly for studying effect of nitrogen, sodium nitrate, potassium nitrate, ammonium chloride, casein hydrolysate, ammonium nitrate, sodium nitrite, ammonium sulphate, ammonium tartrate and ammonium oxalate at 2g/l were taken as nitrogen sources and sucrose at 30g/l as carbon source.

200 ml Czapek-Dox solution in 1 liter roux bottles were inoculated with 5mm discs of mycoparasite cultures of seven days old actively growing on PDA medium, with the help of a sterilized cork borer and incubated for 15 days at 25±1°C. The mycelial dry weight and sporulation were recorded.
Determination of mycelial dry weight and sporulation

Mycelial dry weight was determined by following the method of Monga (2001). After 15 days of inoculation of mycoparasites in different carbon and nitrogen sources provided with Czapek-Dox solution in roux bottles, the cultures were filtered separately on Whatman No.1 filter paper and the mycelial mats were dried at 60°C over night in a hot air oven. They were cooled, weighed and the dry weights were recorded. The extent of sporulation was determined by Haemocytometer counts (Aneja, 2001).
3.10. **DISEASE MANAGEMENT BY CHEMICALS, FUNGICIDES AND BIOLOGICAL METHODS**

a. **Effect of chemicals and fungicides on the pathogen**

Chemicals like copper sulphate, zinc sulphate, lead chloride, cobalt chloride, mercuric chloride, silver nitrate and manganous sulphate at three different concentrations of 200, 500 and 1000 ppm respectively were tested on the conidial germination of the powdery mildew fungus. Fungicides like Karathane, Capton, Dithane M-45, Blitox, Difolaton, Chlorothalonil and Ridomil MZ were also tested in the same above concentrations against conidial germination.

Spore suspension was prepared in 1 ml of sterile distilled water by transferring a loopful of spores from the surface of infected leaf spot.

A drop of each test chemical and fungicidal solutions of respective concentration was placed on clean cavity slides and allowed to dry. Soon after a drop of spore suspension having 30-50 spores per microscopic field (100 x) was placed over the dried chemical and fungicidal spots in each slide. The slides were incubated in moist chamber at 25 ± 1°C for 24 hrs. For each concentration triplicates were maintained with suitable control in sterile distilled water without chemicals and fungicides. After incubation, the percentages of germinated and
non-germinated spores were recorded. Inhibition percentage of spores was calculated by following formula (Bliss, 1934).

\[
\frac{T}{C} \times 100
\]

Where

- \(T\): percent inhibition of spores
- \(C\): percent spores germinated in control
- \(T\): percent spores germinated in treatment

b. Effect of plant extracts on conidial germination of pathogen

In the above manner, the effect of leaf extracts of some wild and medicinal plants on conidial germination of the pathogen was studied.


The percentage of conidial germination against each leaf extract was calculated using the Bliss (1934) formula.