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Research Article

Study of Host (*Michelia champaca*) and Pathogen (*Phomopsis micheliae*) interaction

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ABSTRACT

The purpose of this project is to identify and characterize the phyllosphere pathogenic organism, *Phomopsis micheliae* infecting *Michelia champaca*. The results were compared to that of a healthy, uninfected leaf. The physical characterization showed presence of acidic pH of 5.5 and electrical conductivity of 39.5. A high percentage of infection was seen in the host. Further, the stomatal efficiency was found to be significantly reduced with 89.8% decrease in stomatal frequency per mm^2 and a 75% decrease in stomatal diameter. Chlorophyll bioassay by Arnon's method (1949) showed a significant decrease in content of both chlorophyll a and b and hence, of total chlorophyll in the infected leaves with respect to the uninfected. Biochemical characterization included polyphenol oxidase (PPO), phenylalanine lyase (PAL), glucosidase and amylase assay. Increased PPO activity in the infected leaf suggested increased polyphenol content and thus, verified the low phenol content result in the same. An increased PAL activity suggested that *Phomopsis micheliae* is causing a biochemical change in host, *Michelia champaca*, leaves allowing them to produce phytoalexins, polyphenols and phenolic compounds etc. as a part of defensive strategy against the pathogen. Folin-Ciocalteau assay too showed an increased phenol and polyphenols content in the infected leaf. Thus, we may say that an increased PAL activity points out an increased production of flavonoids, phytoalexins, polyphenols and other proteins in the infected leaf and an increased PPO and PAL activity suggests ability of the host plant to activate defense mechanisms against the pathogen. Bradford Protein assay showed an increased protein content in the infected leaf. Glucosidase assay points out a possibility of fostering the glucosidase activity of the leaves by the fungus. It could be suggested that the fungus is utilizing starch in its own way and puts forward a greater demand for the same. Amylase activity of 0.15148mg/ml/minute as shown by the fungus might be helping in the development of the host. The pathogenic fungus was found to satisfy all of the Koch's postulates. The insecticide Folidol (Methyl Parathion) and the plant growth regulator Miraculan (Tricontanol) are found to inhibit the growth of the pathogen to some extent and folidol was found to be more effective than miraculan. Presence of 0.2% sugar solution further augments the inhibition. 6.4 mg/ml concentration of folidol was found to be the minimum lethal concentration of the pesticide at which it can completely kill the fungus. Miraculan, too, shows a progressive increase in inhibition of the fungal pathogen with gradual increase in concentration.

Key Words: Arnon's chlorophyll bioassay, polyphenol oxidase, phenylalanine lyase, β-glucosidase, α- amylase, phytoalexin and polyphenols, Methyl Parathion, Tricontanol, minimum lethal concentration, Koch postulate.

INTRODUCTION

Host:

Magnolia champaca, commonly called yellow jade orchid tree, is a large evergreen tree, native to the Indomalaya ecozone (consisting of South Asia, South East and some parts of China). It is best known for its strongly fragrant yellow or white flowers. It is, however, primarily cultivated for its timber, and is also used in urban landscaping. Its aril-covered seeds are highly attractive to birds.The glorious medicinal plant is a reservoir of numerous active principles and secondary metabolites and is extremely rich in its chemistry and is often widely used traditionally for indolent swellings, fevers and in nervousness. Parthenolide from leaves and root bark, michampanolide, 8-acetoxyparthenolide magnograndiolide, costunolide, dihydroparthenolide and micheliolide from root bark and β-sitosterol, liriodenine, ushinsunine, magnoflorine from stem

bark are some of the important chemical moieties reported from this plant. Michelia is a timber producing & fine grain wood is used for the construction of buildings, furniture, toys & carvings. It is a very tall tree that grows up to 30 m tall. *Magnolia champaca* is cultivated and used as an ornamental tree in temperate climate gardens, such as in coastal California. The leaves, flowers, and form of *Michelia* resemble *Magnolia*, but the blossoms of *Michelia* generally form clusters among the leaves, rather than singly at the branch ends as *Magnolia* does. Michelia champaca L. (family: Magnoliaceae), commonly known as Champa [Hindi], is traditionally used for fertility regulation by the women of Chhattisgarh state in India. No scientific evidence regarding the antifertility effect of this plant is available till date.

Fungus (Pathogen):

Phomopsis fungi attacks a wide range of plants, usually causing leaf spot disease. One of the species of this genus, *Phomopsis viticola*, cause a plant disease called phomopsis or *dead-arm*. Usually, infections begin during early growth stages in spring. This affects leaves, fruit, rachises, and shoots of a plant. This disease causes the formation of lesions on shoots, leaves, and rachises, but also can cause fruit rot. It causes significant economic damage to grape vines. Another *Phomopsis* species, *P. juniperovora*, infects junipers, and is a particularly important pest of seedlings and juvenile plants in the nursery industry. *Phomopsis amygdali* is the causal agent of twig canker and blight of almonds. In a recent survey of dieback of almonds in Portugal, the most frequent fungi detected were *Diaporthe*/*Phomopsis* species. Isolates from almond and other *Prunus* species were characterised and grouped according to their microsatellite-primed PCR (MSP-PCR) profiles and representatives of the different groups were selected for a phylogenetic study based on the ITS rDNA region (ITS1–5.8S–ITS2). Combining morphological, cultural, molecular and pathogenicity data, three species were distinguished. *Phomopsis amygdali* was shown to be the main pathogen on almond and is epitypified in the present work. *Diaporthe neotheicola* is reported for the first time on this host. A third species represented by a single isolate could not be unequivocally identified.

Leaf Spot Disease:

A few spots on the leaves do little harm to a tree and are far more unsightly than they are injurious. However, leaf spot infections that start early in the growing season can lead to premature defoliation. If it occurs over two or more successive years, it can seriously weaken a tree, reduce its growth, and increase its susceptibility to bark borers, winter injury, and other diseases. Leaf spots commonly increase in number and size in late summer and early autumn as the leaves begin to senesce. The occurrence of a leaf spot disease late in the growing season generally does not seriously affect the health of a tree. Certain leaf spots have special names, such as anthracnose, black spot, downy spot or white mold, ink spot, spot anthracnose, leaf blister or curl, scab, shot-hole, sooty blotch, and tar spot. Many leaf spot diseases are caused by fungi that overwinter infallen leaves. Other fungi overwinter in infected buds, fruits,twigs, and branch cankers. In most cases a fungus that causes a certain leaf spot attacks only one species of tree; a few mayattack several species. Most leaf spot diseases develop as small, scattered, circular to oval dead areas in the leaves; usually tan,dark brown, yellow, gray, purple, or black. Some spots are raised, shiny, and coal black, others may dropout leaving ragged holes; some are marked with light and dark concentric zones. Numerous spots developyellow, purple, red, or reddish brown to black margins; and later, in damp weather, increase in size andnumber and merge into large, angular to irregular dead areas. Dark areas and speck-sized, fungus-fruitingbodies (known as pycnidia, acervuli, and perithecia) commonly form in the dead tissues of many olderspots. Heavily infected leaves may turn yellow to brown, wither, and drop early, weakening the tree. Occasionally, some leaf spotting fungi deform or kill flowers, buds, fruits, twigs, or even small branches.

Host-pathogen interaction:

There are several molecular and cellular approaches that are likely to supply insights into the host-fungus interaction. Fungi present intra- and/or extracellular host-parasite interfaces, the parasitism phenomenon being dependent on complementary surface molecules. The entry of the pathogen into the host cell is initiated by the fungus adhering to the cell surface, which generates an uptake signal that may induce its cytoplasmatic internalization. Furthermore, microbial pathogens use a variety of their surface molecules to bind to host extracellular matrix (ECM) components to establish an effective infection. On the other hand, integrins mediate the tight adhesion of cells to the ECM at sites referred to as focal adhesions and also play a role in cell signaling. The phosphorylation process is an important mechanism of cell signaling and regulation; it has been implicated recently in defense strategies against a variety of pathogens that alter host-signaling pathways in order to facilitate their invasion and survival within host cells. The study of signal

transduction pathways in virulent fungi is especially important in view of their putative role in the regulation of pathogenicity. This review discusses fungal adherence, changes in cytoskeletal organization and signal transduction in relation to host-fungus interaction.

MATERIALS AND METHODS

Fresh uninfected *Michelia champaca* leaf, Fungus infected *Michelia champaca* leaf, pH meter, Spectrophotometer, Mortar pestle, 80% Acetone, PDA, NA, Glasswares,0.1% $HgCl_2$, Tris buffer (pH = 7.5 -8), Catechol (10mM), Centrifuge machine, Phenylalanine (50mM), Acetate Buffer (pH=6) (Preparation - 52.2 ml of 0.1(M) Acetic acid and 947.8 ml of 0.1(M) Sodium Acetate mixed), 1(N) Sodium Hydroxide (NaOH) (Preparation – 0.4 gm of NaOH was dissolved in 100ml of distilled water.), Glucose Asparagine media,5 Dinitrosalicylic acid (DNS) (1% dinitrosalicylic acid+1% NaOH+0.05% sodium sulphite), 40% Sodium Potassium tartarate, Glucose standard (1 mg/ml), Gallic acid 0.1ml, Sodium carbonate (Na_2CO_3) (2%), Folin–Ciocalteu (F–C) reagent, Miraculan., Folidol, 2% sugar solution.

2.1 Study of the infected leaf: The leaf was taken in a petri-dish and the physical characters including diameter of the lesion, were noted down. Further, the leaf was washed with water and this water was collected to check the pH using a pH meter. Dry weight of the leaf was calculated.

2.2 Determination of Stomatal diameter and frequency: The method employed includes use of clear glue to make an impression of the epidermis. Such impressions of both, healthy and infected leaf, were prepared by coating the epidermal surface of the leaves with stationary glue. The dried layers of the glue were peeled off and they were stick onto separate grease-free slides. The impressions were then mounted in water on the slides and placed under the microscope to obtain stomatal diameter and frequency for both the leaves.

2.3 Chlorophyll Bioassay: by Arnon's Method (1949) - 1 gm fresh weight of both infected and non-infected was washed, crushed separately in 80% acetone and extracted in mortar-pestle. The extracted sample was centrifuged at 1000 rpm for 15 minutes
Supernatant was removed while the residues were collected.
The residues were further crushed and homogenized with the same solution and centrifuged at 1000 rpm for 15-20 minutes repeated. Final residues were discarded and the supernatants were collected. Absorbance was measured at 645 nm and 663 nm.

2.4 Isolation and Characterization of the pathogen: PDA and NA slants were prepared. Infected portion of the leaf were carefully cut off from the leaf & kept separately. They were dipped in 0.1% mercuric chloride ($HgCl_2$) for 20 sec for surface sterilization. Then they were poured into distilled water for few seconds. They were inoculated into the PDA and NA slant and kept in incubator at 37˚C.Growth that has come was taken on a slide & stained with lactophenol cotton blue. (Slides& cover slips should be made grease free. Cover slips is placed on the slide and the slide was subjected to microscopic study.

2.5 Polyphenol Oxidase Assay: 200mg of fresh weight of both infected and non-infected is washed and weighed and crushed in a motor pestlewith silica dust and 10 ml of Tris buffer into a fine paste. Pastes were centrifuged at 10,000 rpm for 15mins.The supernatants were collected for the two samples and the pellet was discarded.Then 1ml of 10mM Catechol was added to both of the supernatants. It works as a substrate for the enzyme PPO.The two samples were incubated at 37˚C for 15minutes.1 ml of 1(N) NaOH was added to stop the reaction in both the samples. Absorbance was measured at 475 nm.

2.6 Phenylalanine Lyase Assay: 200mg of fresh weight of both infected and non-infected was washed and weighed and crushed in a motor pestlewith silica dust and 10 ml of Tris buffer into a fine paste.The pastes were centrifuged at 10,000 rpm for 15mins. The supernatants were collected for two samples and the pellet was discarded carefully.Then 1ml of 50mM Phenylalanine was added to both of the supernatants. It will work as a substrate for the enzyme PAL. The two samples were incubated at 37 degree C for 15minutes. 1 ml of 1(N) NaOH was added to stop the reactions in both the samples Absorbance was measured at 290 nm.

2.7 Glucosidase Assay: 200mg of fresh weight of both infected and non-infected was washed and weighed.The leaf fragments of both were then crushed separately in Acetate buffer and volume made up to 10 ml by the same buffer. Extraction was done in mortar-pestle. Centrifugation of extracted sample at 1000 rpm for 10 minutes. Supernatant was removed while the residues are collected. 1ml of supernatant take in a test tube and 1ml of PNPG was added to it.The mixture was allowed an incubation period of 15 mins.1ml of 1(N) NaOH solution was

added to stop the reaction. Absorbance measured at 410 nm.

2.8 Amylase activity assay of the Fungal Mycelia: 100 ml of glucose asparagine broth was inoculated with the fungal culture for mycelia growth and incubated at room temperature.After growth of adequate mycelia, filtration was done and the filtrate was centrifuged at 8000 rpm for 15 minutes. The pellet was discarded and the supernatant was kept.1ml of this supernatant was taken in a test tube. To this 1 ml of DNS reagent was added. Now this was incubated in water bath at 90° C for 15 minutes.After the completion of the incubation time a notable colour change was present in the test tube and the further reaction was stopped by adding 1 ml of sodium potassium tartarate. In another test tube 1 ml of distilled water and 1 ml of DNS reagent was added and this was considered as control. Standard Glucose curve was prepared

2.9Estimation of total phenolic content of infected and uninfected *Michelia champaca* leaf: 200mg of fresh weight of both infected and non-infected was washed and weighed.
and crushed in a motor pestlewith silica dust and 10 ml of Tris buffer into a fine paste. The two fine pastes were centrifuged at 10,000 rpm for 15mins. The supernatants were collected for two samples and the pellet was discarded carefully. Then added 4.5 ml of water and 0.1 ml of gallic acid. It will work as a standard solution in spectrophotometric count. In the supernatant 1ml of F-C reagent was added and incubated for 3minutes. After that 2% sodium carbonate solution was added and incubated for 2 hours in room temperature. Absorbance was measured at 725nm.

2.10 Total Protein content: by Bradford methodology- The Bradford reagent was diluted (1:4) and stored at 4˚C. The bottle was covered with aluminum foil to avoid interaction with light. 200mg of fresh weight of both infected and non-infected was washed and weighed.
And crushed in a motor pestlewith silica dust and 10 ml of Tris buffer into a fine paste. The paste were centrifuged at 1000rpm for 10 minutes.10ml of the soup taken in a test tube covered with aluminum foil and 250µl of reagent was added.The mixture was incubated in dark for 15 minutes at room temperature.Absorbance was measured at 595nm. Standard curve was prepared.

2.11 Verification of Koch's Postulates:
Day 1 - Infected portion from the infected leaf was taken & inoculated in PDA slant.
Day 3 - Incubated for 48 hours at 37^0C and observation was taken.
Day 6 -After 5 days of incubation spores were observed. Lactophenol cotton blue staining was performed and observation taken was noted down.
Sterile water in added in to the fungal culture tube and rubbed slowly with the loop to make a spore suspension. That suspension was then filtered in another test tube through a cheese cloth to make the final spore suspension.
Portion of the healthy leaf is taken into a petri plate containing a filter paper made moist with sterile water. 3 to 4 drops (10µl) of the spore suspension was given on each side of the mid-rib of that leaf. Incubated at 37^0C for 24 hours in a wet filter paper in petri plate.
Day 7 – Infection was seen at the site of application of spore suspensions. The infection obtained was allowed to grow for next 24 hours.
Day 8 – Observations of newly infected leaf taken.
Day 9 - Infected portion from that leaf is inoculated with a scalpel into the PDA slant and incubated at 37^0C for 48 hours.
Day 11- Observations of the growth in PDA slants noted down after lactophenol cotton blue staining.
Day 13 - Spores were observed. Lactophenol cotton blue staining was performed

2.12 Fungicide and Insecticide Bioassay: Spore suspension was prepared. The fungicides were prepared according to the following concentrations:

a) 0.40mg/ml - 4 mg folidol was dissolved in 100ml sterile water.
b) 0.20gm/ml - 2ml of miraculan was added to 100 ml sterile.

The control and the test slides were prepared as:-

- Positive control : 10µl of spore suspension was added to10µl sterile water on a slide
- Negative control : 10µl of spore suspension was added to 10µl of 0.2% sugar solution

Test Slides:-

- 10µl of folidol and 10µl of spore suspension
- 10µl of folidol, 10µl of sugar solution and 10µl of spore suspension.
- 10µl of miraculan and 10µl of spore suspension
- 10µl of miraculan, 10µl of sugar solution and 10µl of spore suspension.

All the samples on the slide were covered with cover slips and placed in petri dishes with filter paper wet with sterile distilled water. The petri plates were kept for overnight incubation.

2.13 Minimum Lethal Concentration: Spore suspension was prepared. The fungicides were prepared according to the following concentrations:

Folidol:-

a. 0.80mg/ml - 8 mg folidol was dissolved in 100ml sterile water. (F2)
b. 1.60mg/ml - 16 mg folidol was dissolved in 100ml sterile water. (F3)
c. 3.20mg/ml - 32 mg folidol was dissolved in 100ml sterile water. (F4)
d. 6.40mg/ml – 64 mg folidol was dissolved in 100ml sterile water. (F5)

Miraculan

a. 0.40gm/ml - 4ml of miraculan was added to 100 ml sterile water. (M2)
b. 0.80 gm/ml – 8ml of miraculan was added to 100ml of sterile water. (M3)
c. 1.60 g/ml – 16ml of miraculan was added to 100 ml of sterile water. (M4)

The control and the test slides were prepared as :-

- Positive control : 10µl of spore suspension was added to10µl sterile water on a slide
- Negative control : 10µl of spore suspension was added to 10µl of 0.2% sugar solution

Test Slides:-

- 10µl of folidol and 10µl of spore suspension
- 10µl of folidol, 10µl of sugar solution and 10µl of spore suspension.
- 10µl of miraculan and 10µl of spore suspension
- 10µl of miraculan, 10µl of sugar solution and 10µl of spore suspension.

4) All the samples on the slide were covered with cover slips and placed in petri dishes with filter paper wet with sterile distilled water.

5) The petri plates were kept for overnight incubation.

RESULTS AND DISCUSSION

3.1.a **Percentage of Infection:** (Lesion dimension/leaf dimension) ×100 =53.2%

3.1.b **Lesion Texture:** Brown, colored lesion, having a pale yellow outline

3.1.c **Dry weight of the leaf sample:** 0.14 g

3.1.d **pH** of the collected water = 5.5

3.1.e **Electrical conductivity** shown by the water = 39.50

3.1.f OD for **turbidity** measurement = 2

3.2.a **The stomatal counts:**

For healthy leaf impression- 27

For infected leaf impression – 3

Percent of reduction in frequency {(435-48/435} X 100 = 89%

3.2.b **Stomatal Diameter:**

Percentage reduction in stomatal diameter = 75%

3.3 **Chlorophyll Bioassay**:

The amount of chlorophyll a (C_a), b (C_b) and total chlorophyll is calculated according to:-

Equation I :- O.D. at 663 = 82.04 C_a + 9.27 C_b

Equation II:- O.D. at 645 = 16.75 C_a + 45.6 C_b

Total Chlorophyll = C_a + C_b

The optical density of the prepared leaf extracts for both infected and uninfected when measured at 645 nm and 663 nm , gave results as:-

Uninfected
O.D. at 645nm = 0.939
O.D. at 633nm = 0.988
Infected
O.D. at 645nm = 0.461
O.D. at 663nm = 0.568
Uninfected –
C_a = 10.137 g/lt
C_b = 16.868 g/lt
Total Chlorophyll = 27.01 g/lt
Infected –
C_a = 6.03 g/lt
C_b = 7.91 g/lt
Total Chlorophyll = 13.94 g/lt

3.4. **Isolation of the pathogen:**

a. After 24 hours incubation no growth was observed. Incubating the tubes for another 24 hours showed growth in the PDA tubes.

On PDA : Cottony growth are observed.

Characteristic : a) cottony growth.
b)Blackish zonation
c) Marzine are slightly raised.
d) Reddish brown ting

No growth was observed in case of NA slants. Fungal culture is then examined under microscope. Reproductive structures – Conidia, chlamydospore& mycelia was seen.

Conidia- appeared round & hyaline in nature.

Mycelia- appeared forced to penetrate in nature.

b MEASUREMENT OF THE REPRODUCTIVE STRUCTURES:

Chlamydospore = 0.066mm

Mycelia = 0.132mm

- After incubation for 4 days spores were observed.

c. Result of staining : Huge spore are observed.

Characteristic of the spore :

Color : Black .

Nucleus : Mono-nucleated and bi-nucleated.

Diameter : Mono-nucleated spore = 0.04 mm
Bi-nucleated spore =0.07mm.

β spores were mostly seen.

3.5 **Polyphenol Oxidase activity:**

OD measures as:-

Healthy Leaf extract – 0.77

Infected Leaf extract – 0.59

By lambert-beer's law-

in case of infected leaf, c= 4.538 X 10^{-4}nmole

in case of healthy leaf = 5.923 X 10^{-4}nmole

From Bradford test –

The total protein content of infected leaf sample 0.4 ug

The total protein content of healthy leaf sample 0.2 ug

Specific activity of Healthy Leaf = {3.025 X 10^{-5}}/0.2 = 1.51 X 10^{-4}nmole/min/ ug of total protein

Specific activity of infected leaf= {3.95X 10^{-5})}/0.4 = 9.875 X 10^{-4}nmole/min/ug of total protein.

3.6 **PAL activity:**

OD_{290} :-

Healthy leaf extract – 1.695

Infected leaf extract – 2.377

Specific activity of the enzyme in the infected leaf extract = 123.5 nmole/min/ug of total protein

Specific acyivity of the enzme in the healthy leaf extract = 174.6 nmole/min/ug of total protein

3.7 **Glucosidase activity:**

Uninfected - Test - OD_{410} = 0.427

Blank- OD_{410} = 0.408

Infected - Test - OD_{410} = 2.456

Blank - OD_{410} = 1.7

Activity for Infected leaf = 0.37 /min

Activity for Uninfected leaf = 0.01 /min

3.8 **Amylase activity:**

OD_{575} = 0.968

From the standard glucose graph,

Absorbance of 0.426 corresponds to the concentration of glucose = 1.0 mg/ml

So,the absorbance of 1.542 corresponds to the concentration of glucose =1.0/0.426×0.968

=2.2723 mg/ml

Hence the amylase enzyme activity =dc/dt

=concentration of product/incubation time

= 2.2723/15

= 0.15148 mg/ml/minute

3.9 **Total Flavonoid and Polyphenol content:**

OD_{725} measured as:-

Healthy Leaf extract – 0.756

Infected Leaf extract – 0.995

3.10 **Total Protein content:**

OD_{595} measured as :-

Healthy leaf extract – 0.2

Infected leaf extract – 0.4

Total protein concentration in infected leaf = 630 µg/ml.

3.11 **Verification of Koch's Postulate:**

The infection started to develop after 18 hours of incubation. After 3days of incubation well developed infection was established.

Brownish-yellow colored lesions were observed as it was observed in the original infected leaf.

When this infected part is inoculated & incubated in the PDA slant, growth occurred after 7days.

Further staining & microscopic study of the fungal culture revealed the reproductive structures like mycelia, conidia &chlamydospore as seen in the original infected leaf.

β-spores were seen with some mono & bi-nucleated spores.

3.12 **Fungicide and Insecticide Bioassay:**

Refer to Table VI for the detailed study results

1ul contains 7.75 spores. So, 1 ml will contain 77500 spores

Dilution Factor = 1:10

Thus, concentration of spores in the suspension = 77500 * 10 = 7.75 * 10^5 spores/ml

3.13 **Minimum Lethal Concentration:**

Refer to Table 8, 9 and 10 for the MLC in different cases

DISCUSSION

This entire work is based on the infection of the leaves of *Michelia champaca* by some pathogen . The physical characters and chemical properties on that leaf were then studied to observe the massiveness of the infection. The leaf texture pointed towards this massiveness by bearing brownish-yellow lesions. The leaf texture indicated that the presence of pathogen is very much pronounced, maybe it is a fungal or bacterial or viral pathogen. Low pH (5.5) of the sample indicated the presence of slight acidic compounds liberated by the pathogen. The electric conductivity value indicated the presence of small amount of metal ions which can be acquired from the environment. Lastly the turbidity indicated the presence of organisms or other contaminants.

After isolation of the pathogen, growth on the PDA slants showed that it was a fungal pathogen. Microscopic study pointed towards the presence of reproductive structures like chlamydospores, conidia & mycelia. The nature of spores after four days of incubation and mycelia led us to identify the fungus as *Phomopsis micheliae*, a common fungus infecting *Michelia champaca*. No bacterial growth was found

on the nutrient agar and thus, we may say that there was no bacteria infecting the leaves.

While verifying the koch's postulate the fungus was found in abundance in all infected *Michelia champaca* leaves but absent in the healthy ones, verifying the first postulate. Pure culture of the fungus, *Phomopsis micheliae*, was isolated in PDA tubes and after 48 hours, cottony growth with blackish zonation and reddish brown tinge and the slightly raised margins was seen in the tubes. After 72 hours incubation, on staining a pinch of the sample with lactophenol-cotton blue and observing it under microscope, reproductive structures- conidia (round and hyaline in nature), β and α spores and penetrating mycelia – were seen and thus verifying the second postulate. After inoculation was the healthy *Michelia champaca* leaves with the above spore suspension and incubation at 30˚C, infection developed in the healthy leaves in form of brownish-yellow colored lesions verified the third postulate. Hence, we can state that this fungus is an opportunistic pathogen. Thus same infection was observed in that healthy leaf infected by the original fungal culture Re-inoculation of the infected area into PDA tubes, isolation of the fungus from the newly infected leaf and presence of same characters and reproductive structures, especially the large number of β-spores and few alpha-spores clearly reveals the fact the fungal pathogen infecting the healthy *Michelia champaca* leaf is *Phomopsis micheliae*. Thus, the fourth postulate was also verified.

The impression of the epidermis when viewed under microscope showed a significant decrease in the frequency of the stomata per mm^2 by 89.8% (TABLE 2 & 3) and study of each stoma showed a decrease in the diameter, on an average, by 75%(TABLE 4 & 5). Such a reduction may be linked to the reduction in transpiration rate of the infected leaf leading to wilting. Infection is bringing about damage to the stomatal apertures. Indah Anita-Sari et al (2013) in a paper showed a decrease in stomatal aperture and number in cocoa plant due to fungal infection and our results are in line with it [3].

Chlorophyll bioassay by Arnon's method (1949) showed that the chlorophyll content with respect to chlorophyll a, chlorophyll b and total chlorophyll have reduced in infected leaf compared to the uninfected, fresh leaf. The fungi being non – photosynthetic, it did not help in augmenting the chlorophyll content rather decreased it, thus, may be reducing the photosynthetic ability of *Michelia champaca*. Thus, the fungus is having an attrition effect with respect to photosynthesis. The total chlorophyll content is found to decrease by almost 7.2% in the infected leaf with respect to the healthy leaf. These results are in line with the results given by Scott KJ et al (2006) and H.S. Aldesuquy et al (1991) who studied the infection of barley leaves by powdery mildew[35] and *Senecioglaucus*infected by *Puccinialagenophorae* respectively .

A major focus of research in PPO has been its potential role in defense mechanism in plants. The common approach now is to examine the expression of specific gene coding for PPO during injury, herbivore or pathogen attack or during exposure to external stresses. Polyphenol oxidases or tyrosinases (PPO) are members of oxidoreductases that catalyses the *o*-hydroxylation of monophenols (phenol molecules in which the benzene ring contains a single hydroxyl substituent) to *o*-diphenols (phenol molecules containing two hydroxyl substituent). They can also further catalyse the oxidation of *o*-diphenols to produce *o*-quinones. It is the rapid polymerization of *o*-quinones to produce black, brown or red pigments (polyphenols). There is a correlation between levels of PPO activity and environmental factors, attacks by pathogens. Increased PPO activity in the infected leaf as compared to the healthy, uninfected leaf suggested increased polyphenol content and thus, verified the low phenol content result in the same. So we may say that enhancement of the infection by the pathogen caused a mechanical stress inside the leaf tissue causing it to activate certain defence mechanisms. Thus, an increased PPO activity will result in decreased phenol content in the plant tissue because the phenol is being broken down into quinone products and an increased content of phenolic acids and polyphenols due to the oxidation-reduction reaction. So PPO has emphasized potential roles in defense. Thipyapong et al (2004) introduced antisense PPO cDNA into tomato plants and examined the resistance of the plants to the pathogen *Pseudomonas syringae* and received similar results[37]. PPO overexpression in tomato plants as studied by Li and Steffens et al (2002) resulted in enhanced resistance to the pathogen [21]. Though the mechanism behind such a phenomena is not clear Thipyapong et al (2004) puts forward some possible explanations, such as, generation of ROS[37].

The brownish coloration of the lesions may be ascribed to increased PPO activity. Spagna G. et al (2005) related PPO activity with color changes associated with browning and lycopene degradation, because lycopene is an antioxidant agent that reconstitutes the polyphenols oxidized by the action of PPO in tomato plant.Coetzer et al (2001) too showed that reduced PPO activity shows reduced browning[9].

Phenylalanine ammonia lyase (PAL) is an enzyme that catalyzes a reaction converting L-phenylalanine

to ammonia and trans-cinnamic acid and is the first and committed step in the phenyl propanoid pathway and is therefore involved in the biosynthesis of the polyphenol compounds such as flavonoids, phenylpropanoids, and lignin in plants. An increased PAL activity suggests that *Phomopsis micheliae* is causing a biochemical change in host, *Michelia champaca*, leaves allowing them to produce phytoalexins, polyphenols and phenolic compounds etc. as a part of defensive strategy against the pathogen. Folin-Ciocalteau assay too showed an increased polyphenols content in the infected leaf. Thus, we can say that an increased PAL activity suggests an increased production of flavonoids, phytoalexins,polyphenols and other proteins in the infected leaf. We may say that the infected leaf has sensitized itself to defend them against pathogen attack by triggering various defence mechanisms including production of phytoalexins, synthesis of phenolics, and accumulation of pathogenesis-related proteins. So this particular fungal infection, phomopsis is able to cause a biochemical change within the leaf tissue. Jogaiah S et al (2013) showed that the susceptible tomato cultivar is triggered synthesis of PAL, POX, and GLU, which activate defence resistance against bacterial wilt disease, thereby contributing to plant health improvement[19].Presence of a probable relationship between PAL and disease resistance of rice against *Rhizoctoniasolani* causing sheath blight has been explained. An increase in glucosidase activity is seen in case of infected leaf (0.37/min) with respect to the uninfected, fresh leaf(0.01/min). Thus, it might be said that the fungus infecting the life fosters the glucosidase activity causing more and more breakdown of starch. It may be proposed that the fungus is itself utilising the starch in its own way and thus, demanding more breakdown of starch. Arthur F.Olah et al (1972) too showed an increase in beta-glucosidase activity on infection of *Medicago sativa* leaves with *Ascochyta imperfect*[28].

The activity of α amylase produce by fungi is 0.15148mg/ml/minute. Thus it can be stated that the fungus is able to produce amylase & this enzyme somewhat is responsible for this infection. Ming-Chang Wang et al in his paper showed an increased amylase activity resulting in decreased amount of starch and increased amount of reducing sugars in banana plant infected with *Gleosporiummusarum*.

The absorbance is found to be greater in the infected leaf sample compared to the healthy leaf. Thus, the results suggest an increased phenol and phenolic compounds' content in the infected leaf with respect to the healthy leaf. A Jamiokowska et al as well showed an increased flavonoid and phenolic acid content in sweet pepper leaves and rooton infection with *Alternaria*sp, *Fusarium*sp and a negative correlation between high flavonoid and phenolic acid content and concentration intensity of the fungus suggesting resistance by plant[18]. Arthur F.Olah et al (1972) too showed an accumulation of aglycones of certain flavonoids on infection of *Medicago sativa* leaves with *Ascochyta imperfect*[28].

In the healthy leaf protein content is 250µg/ml, while in the infected leaf protein content is 630µg/ml. This increase in the amount of the protein content in the infected leaf with respect to the non-infected one indicates that proteins are being produced due to the infection by the fungus. Md. AbulKashem Tang et al (2005) showed increase in protein content in mulberry leaves on infection with *Cercosporamoricola*[36].

Fungicide and Insecticide Bioassay of Miraculan(Tricontanol) and Folidol((dimethoxy-(4-nitrophenoxy)-sulfanylidene) or methyl parathion) showed that the fungicide as well as the insecticide act on the pathogen causing a reduction in germination percentage of the spores(TABLE 6, 8 & 9). .However, presence of 0.2% sugar solution is found to be more effective resulting in better germination percentage compared to their corresponding samples without the sugar solution (TABLE 10). The effect of the pesticide Folidol is seen to be quite significant compared to the fungicide Miraculan which has acted to a lesser extent. Calculation of the minimum lethal concentration of the corresponding fungicide and insecticide showed that folidol at concentration of 6.4mg/ml or 6407.31 ppm shows no spore and no occurrence of germ tube in both presence and absence of sugar. In cases of other concentrations of folidol (0.4-3.2 mg/ml), the presence of sugar is found to bring about a greater inhibitory effect on of the fungus compared to the absence of sugar i.n the same concentrations

While in case of Miraculan, at highest concentration tested i.e. 1.6gm/ml germination of spore is observed and there is a gradual decrease in percentage of spore germinating with increase in concentration of the fungicide. The MLC for miraculan was not achieved.

So,in case of folidol, MLC is 6.4 mg/ml and Miraculan at the highest concentration tested i.e. 1.6gm/100ml no such complete inhibition is observed.

N.V. RamarajeUrs et al (1967) too showed inhibitory effects of folidol on growth of fungi [32].

In summary we can say that *Phomopsis micheliae* is an opportunistic pathogen and infects *Michelia champaca* plant as verified by Koch postulates. The infection is detrimental to the plant as it reduces the chlorophyll content of the infected plant. Moreover, the infection causes a decrease in the frequency of the number of stomata per sqr. mm and a decrease in

aperture of the stomata too. Amylase activity of the fungus and increased glucosidase activity of the infected leaves could be linked to the fungal infection as a part of high demand for sugars. However, the plant has evolved defense mechanisms against the infection. An increased polyphenol oxidase and phenylalanine ammonia lyase activity proves so. Further, an increased amount of flavonoids and polyphenols that help in combating fungus too support the fact. The fungicide, Miraculan, and the insecticide, Folidol, are seen to have inhibitory effect on the fungus though the effect of folidol was more pronounced. Presence of 0.2% sugar solution enhanced the inhibitory effects.

CONCLUSION

Michelia champaca is a valuable plant native to Indomalaya ecozone. It finds its most important usage in perfume industry. *Phomopsis micheliae* infection is a very common fungal infection in this plant and has detrimental effects, such as, reduction in cholorophyll content, decrease in stomatal aperture and frequency per mm^2. As our experiments showed, folidol is a very good agent in controlling such detrimental effects of the fungus. We have characterized the fungus, *Phomopsis micheliae* and its infection on *Michelia champaca*. Our results also showed how the plant in various ways tries to combat this infection. Thus, induction and up-regulation of polyphenol oxidase and phenylalanine ammonia lyase activity of the plant can actually help the plant combat the *Phomopsis micheliae* infection as suggested by Thipyapong et al (2004) by introduction of antisense cDNA[37] .

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3.1.c TABLE 1
DRY WEIGHT OF THE LEAF SAMPLE

At first the weight of the leaf	0.96g
After the collection of washed water	0.82g
Dry weight of the leaf	0.14g

3.2.a TABLE 2
STOMATA COUNT IN HEALTHY LEAF

No of stomata in a microscopic field	**count**
Field 1	20
Field 2	29
Field 3	33
Field 4	24
Field 5	30
Average	27

3.2.a TABLE 3
STOMATA COUNT IN INFECTED LEAF

No of stomata in a microscopic field	**count**
Field 1	1
Field 2	3
Field 3	5
Field 4	2
Field 5	1
Average	3

3.2.b. TABLE 4
STOMATA DIAMETER IN HEALTHY LEAF

No. of fields	Diameter
1	2
2	2
3	3
4	3
5	2
Average	2.4

3.2.b TABLE 5
STOMATA DIAMETER IN INFECTED LEAF

No. of fields	Diameter
1	1
2	1
3	0
4	0
5	1

3.12 TABLE 6
EFFECT OF FOLIDOL AND MIRACULAN ON GERMINATION AND GERM TUBE LENGTH OF *Phomopsis micheliae*

S. NO.	Treatment	No. of spore	No. of germ tube	% of germination	Length of germ tube.(mm)
1	control	85	20	23.52	0.0325
2	+ control	63	19	30.20	0.0195
3	F + spore suspension	57	11	19.30	0.013
4	F+ spore suspension+ sugar.	60	14	23.33	0.0134
5	M + spore suspension	43	8	18.60	0.013
6	M + spore suspension + sugar.	53	11	20.80	0.012

control = spore suspension(10ul) + sterile water(10ul)
+control = Spore suspension(10ul) + 0.2% sugar solution(10ul)
F = Folidol (0.4 mg/ml)
M = Miraculan (0.2gm/ml)

3.12 TABLE 7
HEMOCYTOMETER COUNT OF SPORE SUSPENSION

S. No.	Count per Large Square
1.	9
2.	8
3.	6
4.	8
AVERAGE	7.75

3.13 TABLE 8
EFFECT OF VARIED CONCENTRATIONS OF FOLIDOL AND MIRACULAN ON GERMINATIONS AND GERM TUBE LENGTH OF *Phomopsis micheliae*

Sl No	Treatment (without sugar)	Concentration of fungicide	No of spore	No of germ tube	% of germination	Length of germ tube (mm)	% reduction in germ tube length
1	Control	-	85	20	23.52	0.0325	-
2	-sugar	F1	57	11	19.3	0.013	60
3	-sugar	F2	52	8	15.4	0.01	69.23
4	-sugar	F3	50	3	6	0.0087	73.23
5	- sugar	F4	47	1	2.1	0.005	84.62
6	-sugar	F5	0	0	0	-	100
7	- sugar	M1	43	8	18.6	0.013	60
8	-sugar	M2	55	8	15	0.005	84.62
9	-sugar	M3	51	7	13.7	0.0037	88.62
10	-sugar	M4	53	5	9	0.003	90.8

3.13 TABLE 9
EFFECT OF VARIED CONCENTRATIONS OF FOLIDOL AND MIRACULAN ON GERMINATIONS AND GERM TUBE LENGTH OF *Phomopsis micheliae*IN PRESENCE OF SUGAR

SL. No	Treatment (with sugar)	Concentration of fungicide	No. of spore	No of germ tube	% of germination	Length of germ tube.	%reduction in germ tube length
1	Control(+ Sugar)	-	63	19	30.2	0.0195	-
2	+sugar	F1	60	14	23.33	0.013	33.33
3	+ sugar	F2	57	11	19.3	0.005	74
4	+ sugar	F3	54	9	16.67	0.005	74
5	+sugar	F4	60	12	6.6	0.0025	87.18
6	+ sugar	F5	0	0	0	-	100
7	+sugar	M1	53	11	20.80	0.012	38.46
8	+ sugar	M2	49	5	22	0.005	74
9	+ sugar	M3	49	3	15	0.003	84.62
10	+ sugar	M4	33	3	9	0.0025	87.18

3.13 TABLE 10
COMPARATIVE STUDY OF VARIED CONCENTRATIONS OF FOLIDOL AND MIRACULAN ON GERMINATION AND GERM TUBE LENGTH IN ABSENCE AND PRESENCE OF SUGAR

Sample	Percentage germination in absence of 0.2% sugar solution	Percentage germination in presence of 0.2% sugar solution	Germ tube length in absence of 0.2% sugar solution	Germ tube length in presence of 0.2% sugar solution
Control	24	30.2	0.0325	0.0195
F1	19.3	23.33	0.0130	0.0130
F2	15.4	19.3	0.0100	0.0050
F3	6	16.67	0.0087	0.0050
F4	2.1	6.6	0.0050	0.0025
F5	0	0	-	-
M1	18.6	20.80	0.0130	0.0120
M2	15	22	0.0050	0.0050
M3	13.7	15	0.0037	0.0030
M4	9	9	0.0030	0.0025

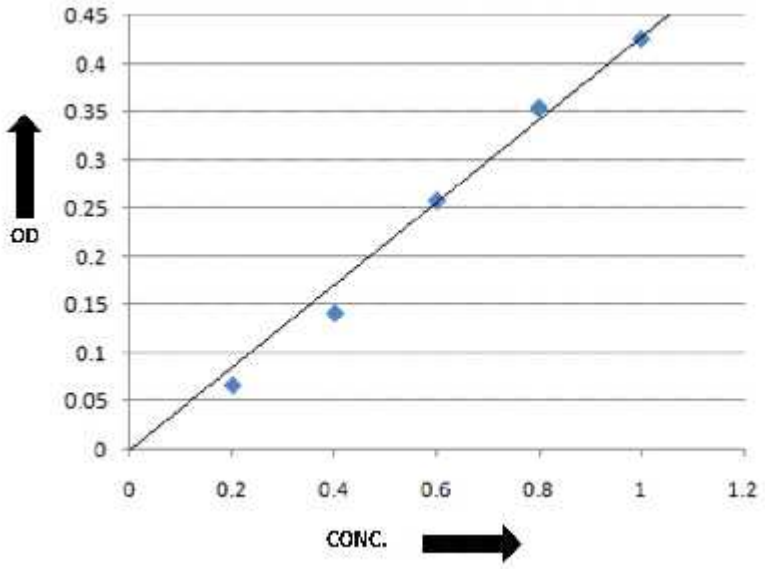

Fig I
Standard Glucose curve

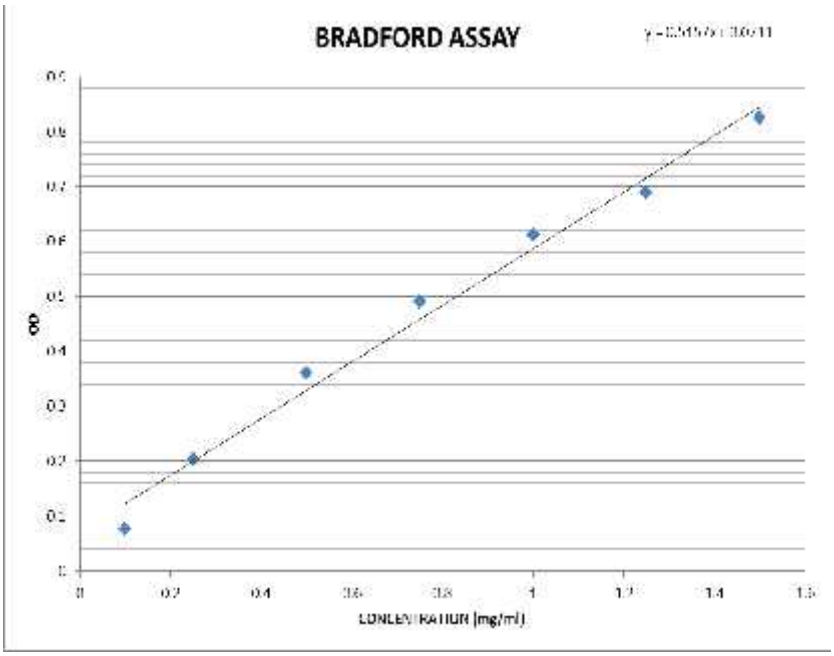

Fig II
Standard Gallic Acid Curve

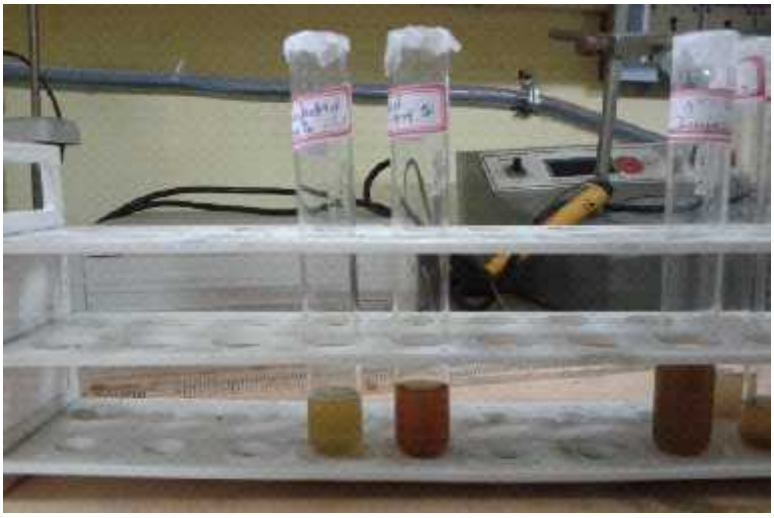

Figure III
Results of Chlorophyll Bioassay

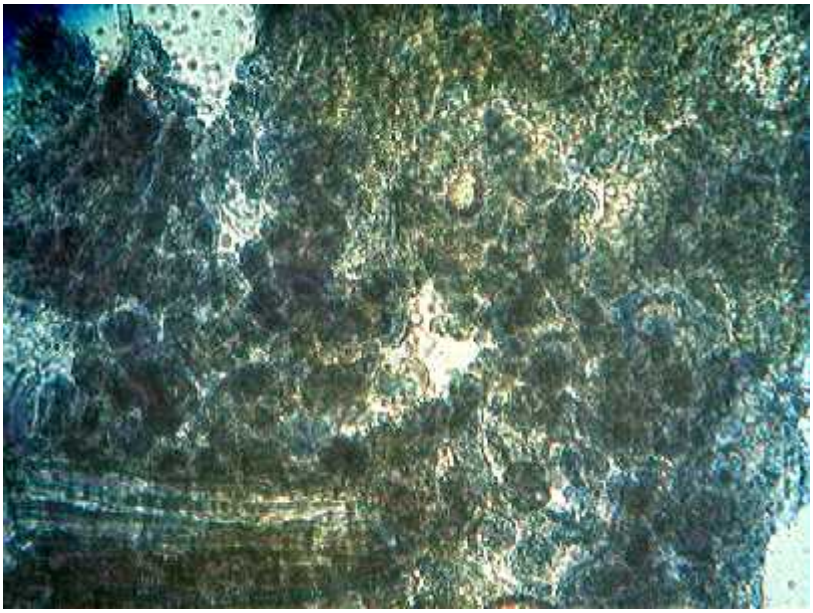

Figure IV
shows chlamydospore and penetrating mycelia when observed under 40X

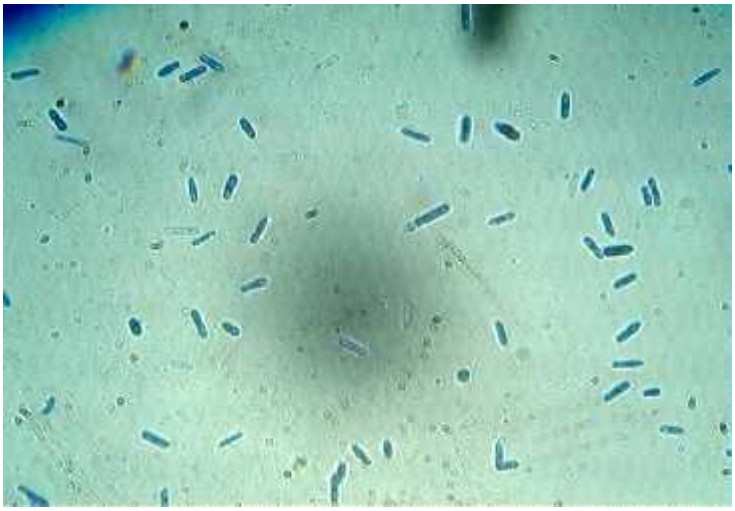

Figure V
shows β spores along with mono and bi-nucleated spores under 40X

Figure VI
shows results of PPO assay in infected leaf extract

Figure VII
shows results of PAL assay in (a) Healthy Leaf Extract (b) Infected Leaf Extract

Figure VIII
shows results of Glucosidase assay in (a) Infected Leaf Extract (b) Healthy Leaf Extract

(A)

(B)

Fig IX
shows (A) Inoculation of healthy leaf with spore suspension for verification of Koch Postulate
(B) Gradual development of infection after inoculation after 24 hours

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