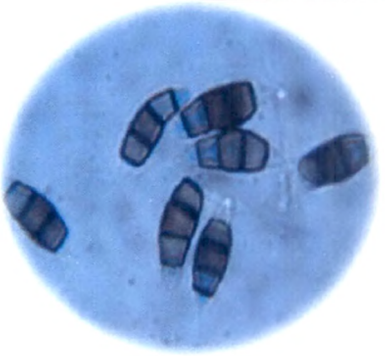
MATERIALS AND METHODS



METHODOLOGY

1. **SURVEY**

A survey was undertaken in Jambughoda WLS, Ratanmahal WLS, Shoolpaneshwar WLS, Pavagadh forest area during January 2007 – December 2009.

Diseases symptoms as leaf spot in forest trees was observed and samples were collected in clean polythene bags.

2. Isolation of Fungi

The infected leaves of plants were collected and washed thoroughly with running water. The infected portions were surface sterilized with 0.1% mercuric chloride for 1 min and after this treatment the tissue sections are transferred to dishes containing sterile distill water and wash thoroughly to free them from the chemicals. In some cases the central core of infected plant tissue, cut with a sterilized pair of scissors or a knife sterilized by momentary dipping in 90 percent alcohol and then flaming for a few seconds. The fungi were cultured and maintained on Potato Dextrose Agar Medium. PDA medium amended with 250µg Streptomycin sulphate per ml.

The organisms thus isolated from the diseased tissues was then be purified by single tip or single colony/ spore method. These plates were incubated at 25±1°C for 7days. Once fungal colonies were formed in the agar plates, each colony was transferred to a new agar slant to obtain a pure culture.

3. Identification of fungus based on morphological characters

During field survey the Materials were collected in clean polythene bags from different locations and brought to the laboratory. The identification of cultures was

done based on morphological characters of conidia/ spore and final confirmation was done from IARI, New Delhi and Agharkar Research Institute, Pune.

4. Diseased symptoms

Survey was conducted in and nearby forests of Vadodara to find out the diseased symptoms in leaves. The progress of Foliicolous spots was monitored and isolation was done from infect leaves. The artificial inoculation in healthy leaves confirmed the Koch's postulates. The physiological studies were undertaken to know the cultural behavior of some of the pathogens and Ecofriendly management was tried.

5. Pathogenicity test

Healthy leaves of plants were inoculated with fungal culture *in vivo* condition after surface sterilizing them with ethyl alcohol and then covering them with plastic bags tied along with wet adsorbent cotton tied near the leaf base. If the plants are susceptible to the pathogen, then the symptoms appeared after a few days. Plants were monitored for the development of disease symptoms and pathogen was reisolated from the leaf after seven days to confirm the pathogenicity according to Koch's Postulates.

6. Bio – control of foliicolous fungi by (Poisoned Food Technique)

The healthy leaves were collected and washed well and dried in oven at 60 ^{0}C for 48h. The dried leaves were powdered and stored in plastic bags. Twenty grams of leaf powder was extracted in a Soxhlet extractor with 200 ml methanol for 8 hours. The extract was concentrated then the residue was treated with 20 % of methanol. It was added to dry residue and water soluble compounds were filtered out.

Effect of Methanolic and aqueous leaf extracts were obtained by Soxhlet Extraction method of 24 plants. It was tested on 4 different foliicolous fungi. The leaf extracts were mixed with appropriate volume of medium (PDA) to obtain concentrations ranging from 2.0 to 10.0% in the final volume of 100 ml of medium. This 100 ml medium was dispensed into 90 mm petri plates with triplicates. (Nene and Thapliyal, 1979)

Fungal isolates of selected fungi were placed in the centre of each plate. Control sets were also prepared without plant extract. The plates were incubated at 25 °C ±2°C and growth of colony was measured after 7 days of inoculation. The radial growth of mycelium was measured at two points along the diameter of the plate and the mean of these two readings was taken as the diameter of the colony. The growth of the colony in control sets was compared with that of various treatments and the difference was converted into percent inhibition by following formula

$$\text{Percent inhibition} = \frac{\text{Diameter of control set - diameter of treated set}}{\text{Diameter of control set}} \times 100$$

CULTURAL STUDIES

Single spore cultures of the organisms were prepared by subculturing. The cultures were made bacteria free by the method described by Brown (1924). They were maintained on 2% agar slants of modified Asthana and Hawkers's medium 'A' (which was further selected for detailed nutritional studies with the constituents

D – glucose - 10 g
KNO_3 - 3.5 g
$KH_2 PO_4$ - 1.75 g
$MgSO_4. 7H_2O$ - 0.75 g
Distilled water – 1000ml

Borosil glassware and pure reagents supplied by Qualigens and SRL were used throughout the present investigation.

For cultural studies Petri dishes of (90mm internal diameter, containing 20 ml agar) were inoculated with a piece of mycelium at the edge kept in diffused daylight at room temperature (20-25°C) and examined at 7 days intervals. Separate slides were prepared for fungi and mounted in lactic acid with cotton blue.

LACTOPHENOL – COTTON BLUE STAIN

Phenol : 20ml
Lactic acid : 20 ml
Glycerol : 40 ml

The above mentioned chemicals were mixed and heated at 70 ^{0}C and then 5 ml of 1 % aqueous cotton blue was added.

For Physiological studies 25 ml of the liquid basal medium was taken in 150 ml Erlenmeyer conical flasks. Unless other wise stated the culture media were autoclaved at 15 lbs psi for 30 min whenever the medium contained complex substances liable to decomposition or denaturilazation, fractional sterilization was done which involved

exposure to steam for 30 min on three successive days. With the help of agar disc method (Garrett 1936) 10 days old culture were used for inoculating the flasks containing different media. Inoculated flasks where incubated at 25 + 2 ^{0}C for 15 days. At the end of incubation period change in pH of the medium and degree of sporulation of the organisms was recorded.

In order to access the growth of organisms their fungal mats were harvested at the end of incubation period on previously dried and weighed Whatman filter paper no: 1. The filter paper were again dried in a electric oven at 60 $^\circ$ C for 72 h and then they were cooled in a dessicator atleast for 48h and finally weighed. The difference between the final an initial weight of the filter paper indicated the dry weight of the fungal mats. Dry weights of the mycelial mat and degree of sporulation were considered as measure of response of the organisms to different treatment. Each set of treatment run in triplicates and only the average dry weight was always taken as a standard value for comparison of growth. The dry weight results were statistically analyzed and standard error (S.E) was calculated by the formula:

$$S.E. = \sqrt{\frac{\text{Mean square of the error}}{\text{No of replicates}}}$$

And Critical difference (C.D.) by the formula:

$$C.D. = S.E. \times t \times \sqrt{2}$$ where t represented probability at 5% level

Dry weights of mycelial mats were graded into Good, moderate and poor. The general mean (G.M.) of the experiment + C.D. at 5 % level has been considered moderate. The dry weights higher or lower than the moderate have been designated as good or poor respectively.

Inoculated flasks were incubated at 25± 2°C for 5, 10 and 15 days. At the end of incubation period, change in pH of the medium was determined. In order to assess the

growth of organisms, their fungal mats were harvested at the end of incubation period on previously dried and weighed Whatman filter paper No. 1. the filter papers were again dried in an electric oven at 60°C for 48h and then they were cooled in a desiccators at least for 2 h and finally weighed to calculate the growth of foliicolous fungi by the following formula

$$\% \text{ Growth} = \frac{\text{Initial Dry weight} - \text{final dry weight}}{\text{Initial dry weight}} \times 100$$

The difference between the initial and final weights of the filter paper indicated the dry weight of fungal mat. Dry weights of the mycelia mat were considered as measure of response of the organisms to different treatments. Each set of the treatment was run in triplicates and only the average dry weight was always taken as standard value for comparison of growth. The dry weight results were statistically analyzed by using the MS office Excel software and the significant values were taken for study.

Dry weight of mycelial mat was graded into good, moderated and poor. The general mean (G.M) of the experiment ± SD with Annova has been considered moderate. The dry weights higher or lower than the moderate have been designated as good or poor respectively.

(A) Selection of suitable culture media

The following culture media were employed:

(a) Natural Medium

1. **Host – decoction medium**: 200g of the host tissue was cut into small pieces and boiled for an hour in a steamer. It was then filtered through a cloth and total volume was raised to 1000 ml.

(b) Semi Synthetic Media

2. Potato Dextrose medium: 200g of potato was peeled and sliced into small pieces. It was boiled in an autoclave for 40 min in 500 ml of distilled water and then filtered through a cloth. Twenty grams of dextrose was added and total volume was raised to 1000ml.

(c) Synthetic Media

3. Asthana and Hawker's Medium 'A'

D – glucose	- 5 g
KNO_3	– 3.5g
KH_2PO_4	– 1.75g
Mg SO_4 $7H_2O$	– 0.75g
Distilled water	– 1000ml

4. Modified Asthana and Hawker's Medium 'A'

D – glucose	-10g
KNO_3	– 3.5g
KH_2PO_4	– 1.75g
Mg SO_4 $7H_2O$	– 0.75g
Distilled water	– 1000ml

5. Czapek Dox's Medium

Sucrose	– 30g
Na NO_3	– 2g

K_2HPO_4	– 1g
Mg SO_4 $7H_2O$	– 0.5g
KCl	– 0.5g
$FeSO_4$ $7H_2O$	– 0.01g
Distilled water	– 1000ml

6. Coon's Medium

Maltose	- 3.5 g
L – aspargine	- 0.25 g
KH_2PO_4	- 1.25 g
$MgSO_4.7H_2O$	- 0.50 g
Distilled water	- 1000 ml

7. Richard's medium

Sucrose	- 50 g
KNO_3	- 10 g
KH_2PO_4	- 5 g
$MgSO_4.7H_2O$	- 2.5 g
$FeCl_3$	- 0.02 g
Distilled water	- 1000 ml

8. Czapek's Medium

Sucrose	- 30 g
$NaNO_3$	- 2 g
KH_2PO_4	- 1 g
KCl	- 0.5 g
$FeSO_4$ .7 H_2O	- 0.01 g
$MgSO_4.7H_2O$	- 2.5 g
Distilled water	- 1000 ml

9. Elliot's Medium

D-glucose	- 5 g
L-aspargine	- 1.0 g
KH_2PO_4	- 1.36 g
$Na_2 CO_3$	- 1.06 g
$MgSO_4.7H_2O$	- 0.50 g
Distilled water	- 1000 ml

(B) Effect of suitable Hydrogen ion concentration

To select suitable hydrogen ion concentrations for better growth of foliicolous fungi, the following initial hydrogen ion concentrations of 2, 4, 6, 8 and 10 were adjusted. The pH of the Modified Asthana and Hawker's medium was adjusted by using 1N HCl or 1N NaOH solutions. After adjustment of suitable hydrogen ion concentration 25 ml of the medium was transferred to 150 ml Conical flasks (Borosil grade). These flasks were autoclaved and inoculated with previously grown culture of decay fungi. After inoculation the flasks were incubated for 15 days. After completion of incubation period, each test fungus was filtered by using Whatman filter paper no 1. The filtrate was used to determine final pH. The filter papers were dried in oven for 48h at 60°C and weighed to calculate the growth of foliicolous fungi. The pH of the filtrate was determined with the help of pH meter.

(C) Selection of suitable temperature

To select a suitable temperature for the growth of foliicolous fungi, the temperatures like 0,5,10,15,20,25,30,35 and 40°C were used. The same procedure was followed as above mentioned in suitable growth and hydrogen ion concentration tests except that flasks were incubated at different temperatures in BOD incubator.

(D) Effect of carbon sources on foliicolous fungi

To study the effect of carbon sources on growth of foliicolous fungi amount of individual substance in the basal medium was calculated, and a quantity equivalent to that was singly substituted in the basal medium by replacing the original corresponding substance. The amount of polysaccharides was similar to the amount of glucose present in the basal medium. The medium devoid of glucose was served as control for carbon. Ten different carbon sources *i.e.* Sucrose, Raffinose, D - Arabinose, L – Arabinose, Xylose, Fructose, Maltose, Mannitol, D- Galactose, Starch, Rhamnose were studied. After completion of incubation period the fungi were filtered with Whatman filter paper No.1 and dried for 48 h at 60 °C in oven. The dried filter papers were weighed to calculate the growth of each test fungi. The filtrate was used to determine the final pH.

(E) Effect of Nitrogen on growth of foliicolous fungi

The basal medium 'A' was used for studying the effect of different nitrogen sources on the mycelial growth and sporulation of the - fungi.

Modified Asthana and Hawker's medium

D – glucose	-10.0g
KNO_3	– 3.5g
KH_2PO_4	– 1.75g
Mg SO_4 $7H_2O$	– 0.75g

The quantity of various nitrogen sources was adjusted by replacing KNO_3 so as to give the same amount of nitrogen as furnished by 3.5g KNO_3 in the basal medium.

The basal medium supplemented with eight nitrogen sources was used for growth of test fungi. The basal medium supplemented with Potassium nitrate, Sodium nitrate, Ammonium acetate, Ammonium oxalate, Ammonium sulphate, Ammonium nitrate, Calcium nitrate, Peptone were used for growth of these fungi, which acted as nitrogen sources. Flasks containing 25 ml of basal medium were autoclaved at 121°C temperature for 20 min, inoculated with test fungi and incubated for 15days. After completion of incubation period, each test fungus was filtered by using Whatman filter paper no 1. The filtrate was used to determine final pH. The filter papers were dried in oven at 60 ^{0}C for 48 h and then kept in desiccator before weighing to calculate the growth of foliicolous fungi.

(F) Utilization of the sugars by foliicolous fungi

Utilization of different mono di and trisaccharides were studied. Paper chromatography was used to find out preferential utilization of Sucrose, Raffinose and Rhamnose for this purpose. The quantity of various sugars was similar to that used in experiment dealing with carbon requirements. Dry weights of mycelial mat and pH of the medium were recorded after incubation period of 5, 10 and 15 days and filtrates were analyzed daily to detect the presence of various sugars during their utilization. Drops of known volume (0.05 ml of sample was taken with the help of 1/100 ml pipette every second day and were placed on the chromatograms by micropipette at a position located for this purpose. The running solvent was n-butanol-acetic acid-water (4:1:5) v/v). In order to separate glucose and galactose the running solvent was n-butanol (100ml) aniline (0.91 ml), pthallic acid (1.6g), distilled water (10ml). (Daniel,) was used as spraying reagent for the detection of sugars. Chromatograms

were developed after drying at room temperature and by heating in an electric oven at 100°C for 90 sec. The Rf values were calculated by the following formula:

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

(G) Effect of vitamins by foliicolous fungi

Vitamins are organic molecules required in small amount and not used as a source of either energy or structure materials of protoplasm. Fungi like other organisms require minute amounts of these specific organic compounds for their normal development. It is believed that they are needed for metabolic reactions and functions as coenzymes or constituent parts of coenzymes.

Fungi in their ability to synthesize their vitamin requirements occupy a position in between the totally independent higher plants and completely dependent animals. Schopfer (1943) has distinguished two groups of fungi, i.e. (a) auxoautotrophs (b) auxoheterotrophs. The auxoautotrophs fungi are capable to synthesize all their vitamin requirements and the auxoheterotrohs include those fungi either completely or partially lack biosynthetic capacity for vitamin production. It has been suggested that different degree of vitamin deficiency in fungi operate through a complicated synthetic mechanism (Lilly and Barnett, 1948).

Our current knowledge of vitamin requirements of fungi indicates that they generally need only water soluble vitamins (Bilgrami and Verma, 1978). Although some of the organisms are reported to attain the same mycelial output even on a vitamin free medium yet usually an exogenous supply of vitamins accelerates the rate of growth of fungi. In the present investigation an attempt has been made to study the

effect of some vitamins on the growth and sporulation of the four pathogens under study.

Following four concentrations (in µg/l) of the vitamins were used in order to select the most suitable concentration for growth and sporulation:

Vitamins	**conc. of vitamins (µg/l)**			
Thiamine (vit. B_1)	50	100	150	200
Pyridoxine(vit. B_6)	50	100	150	200
Riboflavin (vit. B_2)	25	50	75	100
Ascorbic acid	25	50	75	100
Nicotinic acid	25	50	75	100
Folic acid	10	20	30	40
Biotin	5	10	15	20
Cyanocoblamin (vit.B_{12})	10	20	30	40