CHAPTER - II

-:- MATERIALS AND METHODS -:-

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MATERIALS AND METHODS :

The materials for these studies were collected at various stages of development from Satara and localities in vicinity of Satara (Maharashtra) and Castle Rock (Goa). The infected leaves were collected, pressed, dried and preserved for taxonomical studies from which semipermanent slides were prepared.

For the cytological and developmental studies the infected leaves were fixed on the spot and in our laboratory in F.A.A. fixative. Other fixative like Carnoy's fluid, Navashin's fixative and Chrom Acetic fixative were also used after pre-treatments. The material after fixation was passed through various grades of alcohol to paraffin microtome sections of the thickness of 5 to 10-12 u were cut and stained in various stains and stain combinations and were fixed, mounted either in Euperol or D.P.X.

Fixation :

Following fixatives with the following formulae were tried.

(1) Formol-Acetic-Alcohol (F.A.A.)

Formoldehyde 40%	...	5 ml
Glacial acetic acid	...	5 ml
Ethyl alcohol 50% or 70%	...	100 ml

(2) Navashin's Fixative

Solution - A

Chromic acid 2%	...	100 ml
Acetic acid 20%	...	100 ml

Solution - B

Formoldehyde 40%	...	80 ml
Distilled water	...	20 ml

Solution A and B were mixed in equal parts just befor fixation.

(3) Chrom-Acetic Fixative

Aqueous chromic acid 10%	...	1 ml
Aqueous acetic acid 10%	...	10 ml
Distilled water	...	100 ml

(4) Carnoy's Fluid

Ethyl Alcohol (Absolute)	...	60 ml
Chloroform	...	30 ml
Glacial acetic acid	...	10 ml

(5) Modified Carnoy's Fluid

Ethyle Alcohol (Absolute)	...	60 ml
Glacial acetic acid	...	10 ml
Lactic acid	...	10 ml

These two fixatives were satisfactory for squash and smear techniques. Navashin's fixative proved to be the best

for the ascocarps while for the nuclear details, F.A.A. gave satisfactory results. Carnoy's fixative was tried for the rapid fixation of the material.

Pretreatments :

The pretreatment of the material was also found to be useful. Following pretreatments were tried.

(1) Material was kept in Methyle Alcohol in the refrigerator for 15-20 minutes which helped to get the proper divisional stages of the nucleus and the chromosome structures.

(2) Keeping the material in ice cold water for one to three minutes before the fixing, avoided contraction and shrinkage of the cytoplasm and the walls of the asci.

(3) Material pretreatment with aqueous solution of Paradi-chlorobenzen helped the counting of chromosomes with accuracy.

(4) During squash technique hydrolysis of intact perithecia in decinormal hydrochloric acid helped the spreading of the asci in proper plane.

Staining :

Following stains and stain combinations were tried.

(1) Cotton blue in Lactophenol :-

Anilin blue	...	1 gm
Lactophenol	...	100 mls

(Lactophenol -

Phenol crystals	...	20 gms
Lactic acid	...	20 mls
Glycerine	...	40 mls
Distilled Water	...	20 mls)

Semipermanent preparations were made by mounting the sections either in pure lactophenol or in cotton blue in Lactophenol.

(2) Heidenhain's Haematoxylin :-

It has the following composition

Haematoxylin	...	0.5 gm
Ethyle Alcohol (Absolute)	...	5.0 ml
Distilled water	...	100 ml

The haematoxylin powder was first dissolved in absolute alcohol to which distilled water was added. It was then ripened either by exposing to the atmosphere for few days or by passing air in it for few hours.

(3) Heidenhain's Haematoxylin in cellosolve with following composition was also tried.

Haematoxylin	...	0.5 gm
Absolute alcohol	...	5 ml
Methyl cellosolve	...	100 ml
Distilled water	...	50 ml
Tap water	...	50 ml

These two with or without Orange G were used frequently on the material fixed in Alcoholic Fixatives.

(4) Newton's Gentian Violet-Indine :-

This was used especially on material fixed in any of the chromic acid fixatives.

(5) Acetocarmine :-

A saturated solution of carmine in 45% glacial acetic acid was made. This was boiled under Leibig condensor for several hours, cooled and filtered. This stain was used in smears and squashes.

(6) Aceto-Orcein :-

It was prepared by dissolving 1 gm of Orcein in 45 ml of hot glacial acetic acid which was then cooled and to which 55 ml of distilled water was added. This stain was used in smears and squashes.

Of various stains and stain combinations tried, Heidenhain's Haematoxylin followed by counter staining in Orange G. proved to be the best and gave satisfactory results with sharp pictures of the various structures as well as of nuclei and their divisional stages.

For cultural studies :- The infected living leaves showing white to dark brown dried patches towards the margins or near the apices were collected. Dead fallen leaves with minute spots

of fruiting bodies were also collected. These were pressed, dried and stored in the paper envelopes and later used for macro-phetographs, microscopic observations and for cultural and cytological, taxonomical and developmental and cultural studies.

Media :

Culture was done on Potato Dextrose Agar (P.D.A.) and Asthana and Hawker's medium (Basal Medium). Composition of media used were as described by Kamat (1971) and Tandon (1961) and have been cited at the end.

A variation of Corn Meal Agar (C.M.A.) was also used along P.D.A. line. The formulae are cited at the end.

Sterilization :

Conical flasks were filled to the three fourth of their capacities by a medium and plugged by non-absorbant cotton.

Basal medium was sterilized in an autoclave under pressure of 15 pounds for 15 minutes while 12 pounds pressure for 15 minutes was used for P.D.A. and C.M.D.A. media.

The sterilization was done for three successive days as per Koch's method (Purvis et al.,1964). Autoclaved flasks were taken out from the autoclave when these were cooled to room temperature and stored in the refrigerator.

Inoculation :

Inoculation table was sterilized by wiping it with 95% alcohol. Petriplates were sterilized in the oven at 160°C for one hour and cooled down to room temperature.

The stored conical flasks with enclosed media were brought to room temperature. These were later heated in waterbath to liquefy the medium. The edges of the flasks and petriplates were sterilized with mercuric chloride (1 ppm) followed by rectified sprit. Lequefied medium was poured to occupy the one third volume of the petriplates of size 3" diam. by slightly opening from lid from one side only. Petriplates were allowed to cool in the slanting position.

The needles, forceps etc. were sterilized in the boiling water for 10 minutes. These were kept in cotton pads soaked in sprit, and when cooled, these were used for lifting up and transferring the inoculum to the culture medium, now settled at room temperature.

Inoculation was done by keeping petriplates in vertical position to avoid chance air-borne contamination (Collins and Patrica, 1970).

Inoculum to be cultured was separated from the host tissue with the help of two sterilized needles after surface sterilization by 1 : 1000 mercuric chloride. Then, it was washed 2-3 times by sterilized water. Inoculum was now freed

from other contaminations. Single perithecium was transfered to the culture media.

Incubation :

Inoculated petriplates were incubated at 30°C. The moist chamber was used to prevent early drying of the media during the hot seasons.

For the development of the fungus, inoculated petriplates were kept on a wooden block covered by blotting paper whose margins were allowed to dip in a petriplate - containing water. Belljar was covered over this arrangement (Miller and Blaydes, 1962).

Well developed petriplates with cultures were stored in low petriplate lids of size 20 cms in diam. Moistened saw-dust was kept in the lower lid covered by a blotting paper (Miller and Blayde,1962).

Mounting :

For the microscopic studies the fungus material was separated, cleaned and mounted in the lactophenol and was covered with a coverglass after staining with cotton blue when necessary. Natural colour nail-polish was used to seal the slides and semipermanent slides were prepared.

Instruments :

The observations were made with the Oskar (J2 Model) microscope, Ernst Leitz Gmbh Wetzlar (Germany) research microscope

was used for observations and measurements were made under oil immersion. Macrophotographs were taken on Asahi Pentax Spotomatic 35 mm Camera. This camera with microscope attachment and Momya single lens reflex camera were used for photographs.

Culture Media :

1) Potato Dextrose Agar (P.D.A.) :

Pealed potato slices	...	200 gms
Dextrose	...	20 gms
Agar-agar	...	20 gms
Distilled water	...	1000 mls

2) Asthana and Hawker's Medium :

Glucose	...	5.00 gms
KNO_3	...	3.50 gms
KH_2PO_4	...	1.75 gms
$MgSO_4,7H_2O$	...	0.75 gms
Agar-agar	...	20.00 gms
Distilled water	...	1000 mls

3) Conn Meal Dextrose Agar (C.M.D.A.) :

Corn grains (crushed)	...	200 gms
Dextrose	...	20 gms
Agar-agar	...	20 gms
Distilled water	...	1000 mls

4) Czapek-Dox-Agar :

Sucrose	...	30.00 gms
Sodium nitrate	...	2.00 gms
(K_2HPO_4) Potassium Phosphate	...	1.00 gms
Magnesium sulphate	...	0.50 gms
Potassium Chloride	...	0.50 gms
Ferrous sulphate	...	0.01 gms
Agar	...	15.00 gms
Water	...	1000 mls

For cytological and developmental studies of cultured material :

The cultured material from media were fixed in the fixatives like F.A.A. and Carnoy's at different times and later was passed through various grades of alcohol to paraffin. Microtome sections of the thickness 5 to 8 μ were cut and stained in various stains and stain combinations and were mounted either in Euperol or D.P.X.

In the text, the references on fungi which are directly related to the species under study, are only cited. Other references which are used for identification of developmental pattern, nuclear division stages are interpretation of nuclear behaviour are listed at the end.

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