CHAPTER III

MATERIALS AND METHODS
ISOLATION, IDENTIFICATION AND DETERMINATION OF ECOLOGICAL RELATIONSHIPS

The observations for the present study were made on pulse crops, namely *Glycine max.* (L) Merr. and *Cajanus cajan* L. which are grown extensively in the Chattisgarh region. Samples were planted in the kitchen garden at sector-1 Bhilai Nagar, in a limited area which ensured uniform conditions regarding the climate and air spora.

31.1 ISOLATION AND IDENTIFICATION

Sampling fields were visited monthly for the two successive years 1986-87 and 1987-88. The seeds were sown in the month of early June and data were recorded from late July onwards. In each monthly visit 25 sampling plants of each host were surveyed. Young, matured, and old leaves from each plant at random were collected using clean sterilized forcep and scissor. These sample leaves were then mixed in a sterilized container and kept in the dark cloth bags. In the laboratory leaves were taken out from the container and small discs, each of 18 mm., diameter were cut with the help of sterilized cork borer and then transferred to 500 ml corning flasks containing
300 ml of sterilized water. These flasks were thoroughly shaken for 10 minutes. The process was repeated five times. In the last shaking 10% mercuric chloride was added for about a minute to sterilize the leaf surface. The fungal species loosely attached to the leaves, could be easily washed out by the former method while closely adhering ones (at the Phylloplane) were reserved for further studies.

The sample leaf pieces were plated on sterilized cooled nutrient plates containing modified Martin's media (Dextrose 10 gms, Peptone 5 gms, KH₂PO₄ 1 gms, MgSO₄.7H₂O 5 gms, Agar Agar 20 gms., in a litre of distilled water containing 1/30000 G'gms Rose Bengal). Each plate was then aseptically inoculated at random with 5 leaf pieces and then kept for incubation at 28°C±1°C for 5 days. After incubation the pieces of fungal mycelia were transferred to PDA-slants for obtaining pure cultures, and subsequently stored in a freeze carefully. The identification of fungal species were confirmed by the CMI Kew, London.

3.1.2 METEOROLOGICAL DATA

Monthly meteorological data, viz. temperature, relative humidity and rainfall were obtained from District Gazetteer for the concerned years to study their effect on the variations of phylloplane mycoflora populations. (Table 1; Figure 1)
3.1.3 ECOLOGICAL RELATIONSHIPS

The data in each case were processed for obtaining the frequency and abundance of the fungal species using following formulae:

\[ \text{No. of observations in which a species appeared.} \]

\[ \text{\% Frequency} = \frac{\text{No. of observations}}{\text{Total No. of observations}} \times 100 \]

\[ \text{Total No. of colonies of a species in all the observations.} \]

\[ \text{\% Abundance} = \frac{\text{Total No. of colonies}}{\text{Total No. of colonies}} \times 100 \]

3.1.2 PHYSIOLOGICAL STUDIES OF SOME SELECTED FUNGI

Preliminary experiments showed that Richard's medium adjusted to pH 5.5 was quite satisfactory (Table 4) for the growth and sporulation of most of the test and target organisms, thus this medium was selected as basal medium for all the physiological and other experiments. To study the effect of different carbon and nitrogen sources, they were singly substituted in a required concentration for sucrose and potassium nitrate of the basal medium respectively.

The following fungal species were selected for physiological and further studies. The selection was made on the
basis of their constant presence on the hosts, frequency, abundance and physiopathological or saprophytic nature.


TARGET FUNGI: - Epicoccum nigrum, Trichoderma harzianum, Trichoderma longibrachiatus.

3.2.1 EFFECT OF CARBON AND NITROGEN COMPOUNDS

The quantity of different compounds were so adjusted as to contain an amount of carbon equivalent to that present in the basal medium. The amount of polysaccharides (viz. starch, carboxymethyl cellulose, and pectin), and sugar alcohol used were similar to the amount of sucrose in per liter of basal medium as their molecular weights were not known.

In order to determine the effect of different inorganic and organic (amino acids) nitrogen sources the quantity of different nitrogen sources was so adjusted as to contain an amount of nitrogen equivalent to that in potassium nitrate present in the basal medium.
All glasswares were chemically cleaned with concentrated sulphuric acid saturated with potassium dichromate (Nolan 1983) and then rinsed with double distilled water. In the experiments using medium the components were mixed before autoclaving.

312.2 AUToclAVING AND INOCULATION

Aliquots of 25 ml media were dispensed in 150 ml capacity Erlenmeyer's flasks. The flasks were autoclaved at 15 lbs of pressure for 15 minutes; but in certain cases where there was a possibility of breaking of the compounds into sub units, they were fractionally sterilized by steaming for half an hour for three successive days. The pH of the media was adjusted at 5.5 before autoclaving and, if necessary after autoclaving, or just prior to inoculation by the addition of either sterile 0.5 N KOH or 0.5 N HCl.

The flasks were inoculated by equal size (4 mm diam.) Agar discs of 8-10 days old stock culture (Garrett, 1936) and the organisms were allowed to grow for 12 days at 28°C ± 1°C. At the end of each incubation period (3, 6, 9 and 12 days) change in pH of the medium, and dry weight of the mycelial mat were determined. The average values of three replicates have been recorded.
312.3 DEGREE OF SPOREULATION

Degree of sporeulation was graded in four categories on the basis of visual observations viz. poor, moderate, good, and very good.

312.4 GROWTH RATE

Growth and Growth rate was measured in terms of the mycelial dry weight. The mycelia mats were harvested by filtering cultures on previously weighed Whatman filter papers No.42 at an interval of each incubation period. Mycelial mats were washed twice with distilled water to remove associated salts and colloidal particles. The filter papers containing harvested mycelium were dried at 70°C for 24 hours & finally allowed to cool in a desiccator and weighed in a digital balance. The mycelial dry weight was calculated by subtracting the initial weight from final weight. Only average values of the three replicates to the nearest milligrams have been recorded.

312.5 RATE OF SUGAR AND AMINO ACID DEPLETION

For investigating the rate of depletion of Sugar at each incubation period the culture filtrate was subjected to Benedict’s test. To test polysaccharides, such as Starch, CNC, and pectin, F1 reagent was used (Ghosh et al. 1965).
The rate of depletion of amino acid from the culture filtrate was investigated chromatographically by ascending method. Culture filtrate samples (5-20 μl) were plotted on the Whatman filter paper No.1 with the help of micropipette from each sample at each incubation interval. The chromatograms were run in n-butanol - acetic acid - water (4:1:5 V/V) for 24 hours and then dried at room temperature for two to three hours and subsequently sprayed with 0.1% ninhydrin (in acetone). After spraying the chromatograms were dried at room temperature for three to four hours and then placed in an electric oven at 70°C to get the spots discernible. The average Rf values were used for identification and confirmed with stahls (1965) standard values. The rate of depletion is presented here in histograms.*

**DETECTION OF BIOLOGICAL ANTAGONISM**

In the present study the test fungi exhibited high growth rate, and any kind of inhibition in growth could, therefore, be detected in a short period of time. Antagonism between phylloplane micro-organisms including antagonists and pathogens were studied by following methods: -

* Denotes that the chromatograms in support of the assimilation of amino acids have not been attached to help reduce the bulk of the thesis. Instead the graphs and histograms have been incorporated.
3.3.1 EFFECT OF CELL FREE CULTURE FILTERATES ON TEST FUNGI

The culture filterate of the target organisms - *Epichoccum nigrum, Trichoderma harzianum, and Trichoderma longibrachiatum* were taken for the study of their effect upon the mycelial growth, sporulation, and spore germination inhibition of which test organisms were tested *in vitro*.

3.3.1a Mycelial Growth and Sporulation

The target (antagonistic) fungi were isolated and were allowed to grow on liquid basal culture medium (Richard's medium) for fifteen days in dark. After 15 days the culture filterates were taken and filtered through Whatman paper No.42 followed by seitz filtering. Filterates were added in separate flasks with basal medium in different proportions, such as 4:1, 3:2, 2:3, 1:4 and 0:5 before sterilization. Each flask was inoculated with a uniform agar disc of test organism. Three replicates were taken for each treatment. The medium without culture filterate served as control. Inoculated flasks were incubated at 28°C ± 2°C for 8 days. After the end of 8th day fungal mats were harvested by filtering over previously dried and weighed Whatman paper No. 42, and dry weight of the mats was recorded. Sporulation was also registered based on visual observation.
3.3.1b **Spore Germination Inhibition**

Spore germination inhibition was evaluated by employing spore germination test in a covered cavity glass slide (Norse, 1972). Cell free culture filtrates were collected and centrifuged at 1500 rpm for 10 minutes and the supernatant were concentrated up to half of the original volume at 55°C in water bath. These culture filtrates were then used to study the spore germination. Percentage inhibition of spore germination was recorded after an incubation for 12 hours and results were compared with control. Spore germination on distilled water served as control.

Percentage inhibition of spore germination was calculated using the formula:

\[
\% \text{Germination inhibition} = \left( \frac{100 \times \text{Germination in control} - \text{Germination in the treatment}}{\text{Germination in the treatment}} \right) \times 100
\]

3.3.2 **Colony Interaction in Dual Culture on Agar Plate**

Antagonism between the different antagonists and test organisms was measured in terms of "Zone inoculation method". Uniformly cut discs of the pure cultures of *Macrophoma phaeolina*, *Curvularia lunata*, *Fusarium equiseti*, and *Bipolaris hawaiiensis* were placed after 24 hours, separately
on each agar plate, where target organism was contained. The delayed planting of the above test organisms was done to avoid their over growth whereas all other test organisms were inoculated simultaneously with target organisms at the distance of two centimetres apart from each other. Paired cultures were then placed on a laboratory table at room temperature under fluorescent light in aseptic conditions. The degree of antagonism was assessed when the fungi achieved their growth equilibrium and no further alteration in their growth pattern could be detected. The degree of antagonisms was categorised into following six grades as suggested by Buxon (1960).

Category 0 : (-)  
Test organism grew without hinderance over the antagonists.

Category 1 : (+)  
Test organism grew well but not over the antagonist.

Category 2 : (+++)  
Growth of the test organism slightly affected, only a few hyphae of the test organisms grew towards the antagonist.

Category 3 : (++++)  
Growth of test organism hindered showing inhibition zone 2 to 3 mm wide.

Category 4 : (+++++)  
Mycelium of test organism checked leaving a well defined gap of about 5 mm from that of antagonist.
Pronounced inhibition of the test organism, mycelium stopping sharply about 1 cm away from that of antagonist.

**HOST FACTORS AFFECTING THE PHYLOPLANE FUNGI**

For both the host plants, namely *Glycine max* and *Cajanus cajan* leaf exudates and leaf extracts were taken for studying the effect of host factors against the selected organisms.

**PREPARATION OF SAMPLES FOR CHROMATOGRAPHIC ANALYSIS**

For obtaining leaf exudates, potted plants of *Glycine max* and *Cajanus cajan* were kept in moist chambers and sprayed with distilled water. At 4 hourly interval for twenty four hours droplets were collected from the young and old leaves which represented a solution of exudates. For analysis exudates were evaporated over a water bath and the residues were dissolved in a known amount of 20% Ethanol. Free Amino acids and sugars present in this solution were determined Chromatographically.

For obtaining leaf extract 25 gm of healthy and fresh leaves of each host plant randomly were taken and quinched in 100 ml. of 80% ethanol at boiling temperature, then ground in
a mortar with pestle. The juice was expressed through muslin cloth and then centrifuged at 3500 rpm repeatedly to yield a clear supernatant liquid. The pallet was discarded. The supernatant was concentrated by evaporation at room temperature and then filtered. The residue was made up to 100 ml. with sterile water. This served as aliquots for Chromatography and for other experiments.

3.4.2 CHROMATOGRAPHIC METHOD FOLLOWED

Descending unidirectional paper Chromatography method was employed for the separation and identification of free Amino acids and sugars for exudates and extracts both.

3.4.2a Detection of Free Amino Acids

To detect the presence of free amino acids the solvent used was n-butanol-glacial acetic acid—water (4:1:5 V/V). The Chromatograms were sprayed with 0.3% ninhydrine in acetone and heated at 100°C for 10 minutes.

3.4.2b Detection of Free Sugars

For detection of sugars plotted chromatographic paper was developed with solvent system n-butanol-glacial acetic acid—water (4:1:5 V/V) and allowed to run for 30 hrs. Dried Chromatograms were sprayed with p-anisidines (5 grams anicidin dissolved in 3 ml of artho phosphorac acid and 90 ml ethanol).
After spraying the chromatogram was heated at 100°C for 10 minutes. The Rf values of coloured spots were determined and confirmation of the sugar compounds was done as suggested by Stahl (1965).

3.4.3 SPORE GERMINATION/GERMINATION INHIBITION

For studying the effect of leaf exudates and extracts on spore germination and germination inhibition the exudates were collected and centrifuged at 3500 rpm for 10-15 minutes to separate the clear supernatant which was employed to test the percentage germination inhibition of spores. For getting leaf extracts the leaves were crushed and their filtered aqueous solutions (2:10 V/V) were employed.

Spores were collected by gently brushing the turf of a 10 days old colony with a sterile needle. The spores were then suspended in sterile water and stored in freeze for 48 hours before use as recommended by Biggs (1964). Spore density in the supernatant of extract/exudate was kept constant in successive experiments and inocula so standardized that its each loopful contained 40-50 spores.

For each organism one drop of extract/exudate with a loopful of spores suspension were poured in a cavity slide and covered with coverslip. Slides were allowed to incubate at 28±
2 °C for 12 hours. A spore suspension was prepared in a similar manner in distilled water which served as control. After incubation period at least 6 microscopic fields were examined to obtain percentage germination. Each treatment was replicated three times and the observations were analyzed randomly. Percentage spore germination-inhibition was calculated using the following formula for each fungus.

\[
\% \text{ of germination inhibition} = \frac{100 - \text{percentage germination in treatment}}{\text{percentage germination in control}} \times 100
\]