PHYTOPATHOLOGICAL INVESTIGATION AND BIOMETRIC STUDIES OF FUNGAL DISEASE IN FRENCH BEAN

(Phaseolus vulgaris L.)

By

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SUPERVISOR's CERTIFICATE

I certify that the thesis entitled: **"Phytopathological Investigation and Biometric Studies of Fungal Disease in French Bean (Phaseolus vulgaris L.)"** *submitted by Gopal Kumar Niroula Chhetry for the Degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. Degree. This work has not been submitted for any degree of any other University.*

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Date: **December 1989**

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GENERAL INTRODUCTION

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Beans (Phaseolus vulgaris L.) are widely grown legumes all over the world. This crop is extensively grown in Latin America particularly in Brazil and Mexico. The second most important areas of production is Eastern and Central Africa. In both these regions, a substantial proportion of the crop is grown in association with maize on small farms in predominantly subsistence system in which there are few added inputs (Allen and Russell, 1986). Southern Mexico and Central America are considered to be the primary centre of origin, while Peruvian-Equadorian-Bolivan area to be the secondary centres of origin of French bean (Bose and Som, 1986).

French bean cultivars are broadly classified as Dwarf or Bush type and Climbing pole types. Most of the local types native to North-Eastern region of India are pole types (Parthasarathy, 1986).

French bean can be grown on all types of soils except extreme acidic and alkaline, medium textured silt loam or clay loam are best for obtaining high yields (Parthasarathy, 1986).

French beans are grown as winter crop in plains, while in hills it can be grown throughout the year except winter season. In North-Eastern Region, particularly in Meghalaya, pole beans are grown from March to December. French bean is sown during September to November in South Indian plains, during February in north Indian plains, while it is grown from April to October in the hills (Parthasarathy, 1986).

Foliar diseases of P. vulgaris caused by Colletotrichum lindemuthianum and Phaeoisariopsis griseola (Sacc.) Ferraris are distributed in worldwide in many tropical and temperate regions (Inglis et al., 1988). Important reviews on the pathology of Phaseolus vulgaris (Zaumeyer and Thomas, 1957; Schwartz and Gálvez, 1980) have referred principally to American conditions. The nature and prevalence of bean diseases varies considerably with environment. In Latin Amerca, beans produced at intermediate to high altitude (1700-2800m) are affected by virus (BCMV), anthracnose (Colletotrichum lindemuthianum), aschochyta blight (Aschochyta Phaseolorum), angular leaf spot (Phaeoisariopsis griseola), rust (Uromyces appendiculatus), powdery mildew (Erysiphae polygoni), white leaf spot (Pseudocercosporella albida) and grey leaf spot (Cercospora vanderysti). Beans produced at lower altitudes and under hotter conditions are liable to infection by rust, angular leaf spot, web blight (Rhizoctonia solani) and various root rots.

This economically important vegetable crop (French bean) though thrives best in North Eastern Hill region in general and Meghalaya in particular with their abundant rainfall, high humidity and highly drained soil, is prone to many fungal diseases, important being the leaf spot resulting heavy losses in vegetable economy. More attention needs to be given to the various aspects of the disease problem of this vegetable crop in the field.

Most of the major diseases in French bean are seed-borne in nature. Impact of fungi on seeds is to reduce yields of seeds both qualitatively and quantitatively. Saprophytic and very week parasites may lower the quality of seeds by causing discoloration. Relatively very less information related to fungi associated with the French bean seed is available (Lokhande et al., 1986, Santosh et al., 1986. Muniz and Muchovez, 1987) and therefore, investigations were undertaken to study the seed borne mycoflora associated with the French bean seeds and their seasonal variations.

The microbiology of aerial plant surface has received increased attention (Sinha, 1965; Preece and Dickinson, 1971). It is now well established that a large number of micro-organisms inhabit the phylloplane of crop plants (Leben, 1965; Preece and Dickinson, 1971).

The term "phyllophere" has been introduced independently by Last (1955a) and Ruinen (1961) and subsequently "Phylloplane" by Dickinson (1965) for phyllosphere of Last. Since then the study of phyllosphere microflora has attracted the attention of different workers (Lamb and Brown, 1970; Sinha 1971; Dickinson, 1971; 1976; Mishra and Srivastava 1971b, 1974; Mishra and Tiwari 1976b).

Qualitative and quantitative composition of micro-organisms on the leaf surface as well as their activities exhibit an interrelation between plants and micro-organisms. Physico-chemical characteristics of leaf surface environment is also important for better understanding of the population dynamics of phylloplane mycoflora (Mishra and Tiwari 1976b). The interaction of the phyllosphere saprophytic micro-organisms with folior pathogen of the crops and consequent changes in disease potential have been studied by few workers (Leben, 1965; Fokkema, 1976; Skidmore and

Dickinson, 1976).

Leachates on the leaf surface attract micro-organisms. The different microbial population in such an environment interact with one another for space and nutrients. The role of phylloplane saprophytic fungi in biological control in certain epidemic diseases may be important and if properly exploited it may offer an alternative to synthetic fungicides (Srivastava et al., 1981; Purkayastha and Bhattacharya, 1982).

Relatively few studies dealt with the spatial distribution of disease (Strandberg, 1973; Rouse et al., 1981; Campbell and Pennypacker, 1980; Boivin and Sauriol, 1984). Since the number of sample necessary to obtain an estimate of the density of the diseased plants with a known precision varies with the spatial distribution of the disease in a field (Southwood, 1978), therefore, dispersion statistics are required to establish sampling procedures.

Boivin and Sauriol (1984) used the Iwao's regression parameters to establish the sequential sampling plan. The advantage of Iwao's procedure is that it does not require a theoretical mathematical model approaching the spatial distribution of the pathogen (Boivin and Vincent, 1987). Since the evaluation of the diseased density before and after fungicide treatment involves an important part of the human and economic resources in disease monitoring, efficient sampling is quite essential. Sequential sampling is one such sampling procedure which allows the observer to estimate the population density of a disease after each sample with respect to the economic threshold. Sampling methods specially the statistical ones are greatly affected by spatial distribution. Spatial distribution can also provide useful information on the etiology of diseases or their method of spread through a field or area (Strandberg, 1973). Sequential sampling can reduce time and labor costs associated with disease monitoring by deciding whether or not control is needed, while staying within a pre-determined error level, instead of giving an exact estimate of the disease level (Boivin and Sauriol, 1984).

Keeping these points in view, the spatial distribution of leaf lesions caused by Colletotrichum lindemuthianum of three locally cultivated French bean was studied and the dispersion statistics obtained was used in the establishment of sequential sampling plan to determine disease level in the field. This sampling plan may also be useful for the estimation of disease level in the field before and after the application of fungicide. The count data of leaf lesions were also fitted using the negative binomial distribution. Thus, spatial distribution was studied by using mean crowding relation and statistical distribution model.

The count data were also subjected for the analysis of variance to test the significant difference of varieties with regard to their susceptibility of the leaf lesions.

In certain areas of biological research the parametric assumptions of the shape of the distribution is only partly fulfilled. However, in many instances in which the underlying distribution is not known, or even if known they are found to be some other distribution than normal. In such cases non-parametric methods are very useful.

In the present investigation the distribution of Colletotrichum leaf spots was found to follow negative binomial distribution, analysis of variance with the assumption of normal distribution is erroneous and therefore, a non-parametric test such as the Friedman two way analysis of variance and Kruskal-Wallis one way **ANOVA** test by ranks were followed for the analysis of leaf spot data from the Randomized Complete block design besides other applicable non-parametric tests.

The present investigation has been classified into the following five chapters:-

i) Survey of leaf spot fungal diseases associated with the French bean (*Phaseolus vulgaris* L.).

ii) Screening of seeds for fungal association.

iii) Assessment and analysis of phyllosphere fungal population.

iv) Interaction studies between leaf surface fungi and the leaf pathogen.

v) Spatial distribution, sampling plan and analysis of variance of leaf lesions.

REVIEW OF LITERATURE

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HISTORY AND NOMENCLATURE

Bean Anthracnose (Pod spot, Leaf spot or Canker):

The term anthracnose literally means 'like coal'. Bean anthracnose - a disease caused by seed-borne fungus has been named as Gloeosporium lindemuthianum sacc. and Magn., (1878); Colletotrichum lindemuthianum (sacc. and Magn.), Scribner (1888); C. lindemuthianum (sacc. and Magn.) Br. and Cav. (1889). Pathogenic variability of C. lindemuthianum was first observed by Barrus (1911). Later he used 69 cultivars of bean to characterize 15 isolates of the fungus and found two distinct races which he designated as α and β, and this was the first time that pathogenic races had been demonstrated in non-obligate parasite (Barrus, 1918). Wheeler (1969) reported that the leaf lesions on beans caused by C. lindemuthianum are typically leaf spots.

Angular leaf spot:

The angular leaf spot disease of French bean (Phaseolus vulgaris L.) caused by Phaeoisariopsis griseola (sacc.) Ferraris was for the first time reported in Spain in 1944 (Benlloch, 1950). In India, this disease was for the first time reported from the Nilgiris plateau (Srinivasan, 1953).

The causal organisms of the disease has also been named as Isariopsis griseola (sacc.) (Cardona and Walker, 1956) and Cercospora culumnaris (Chupp and Sherf, 1960). Hocking (1967) described a new virulent form of P. griseola that caused large circular and symmetrical leaf lesions.

DISTRIBUTION AND HOST RANGE

Colletotrichum lindemuthianum (sacc. and Magn.) Br. and Cav.:

Leaf spot disease of bean caused by C. lindemuthianum (sacc. and Magn.) Br. and Cav. has been reported from different parts of the world including northern and southern India (Zaumeyer and Thomas, 1957; Walker, 1969, Wheeler, 1969). While the disease has been most severe on Phaseolus vulgaris L., it also affects P. lunata L., P. multiflorus wild, P. acutifolius, P. aureous Raxb. Vigna sinensis Savi, Dolichos biflorus L, and broad bean (Vicia faba L.) (Walker, 1969). A recent review (Allen and Russell, 1986) covered the extensive literature on the distribution and pathology of this disease. The disease has been reported to be most severe in highly rained subtropical to temperate climate than in tropical one (Bose and Som, 1986).

Phaeoisariopsis griseola (Sacc.) **Ferraris:**

Angular leaf spot of bean incited by P. griseola (sacc.) Ferraris has been reported by Cardona and Walker

(1956) as a common disease in tropical and subtropical region. Lima bean (*P. lunata*) has been reported as the only other host of *P. griseola* (Cardona and Walker, 1956). According to Barros *et al* (1958) angular leaf spot occurred in all parts of the world wherever the crops were grown. In India, angular leaf spot of French bean has been reported from the Northern and Southern hill regions (Bose and Sindhan, 1972; Sohi and Sharma, 1974).

DISEASE INCIDENCE, SEVERITY, EPIDEMICS AND LOSSES:

C. lindemuthianum (Sacc. and Magn.) Br. and Cav.:

According to Wheeler (1969) the leaf spot disease of French bean incited by *C. lindemuthianum* caused a considerable damage to *Phaseolus* beans in the U.S.A. and Britain. He stated that the severity of the disease has been influenced by three factors such as age of the host tissue, temperature and moisture. Moisture was very essential for spore formation and germination. Spore dispersal was largely by rain splash and the disease becomes most severe when heavy rains caused partial watersoaking of the young tissue. Robert and Bothyroyd (1972) mentioned that vast numbers of Conidia were produced during wet weather. Production and dissemination of inoculum depends upon rainfall during the growing season which may result in an epiphytotic of this disease.

Olarje et al (1981) explained the mechanism of primary infection by C. lindemuthianum in bean. They showed that infection was directly proportional to the spore concentration in the soil and infected plant litter functioned as an important source of primary infection.

Spread of the C. lindemuthianum from an infection focus was demonstrated by Tu (1981, 1982, 1983). Sindhan (1983) found 18° to 27°C optimum range of temperature and 91.2 to 100 per cent relative humidity for development of disease. Shyam and Chakraborty (1985) showed a range of 24 to 59 percentage of bean pods infection due to C. lindemuthianum in four cultivars.

Frederica and Teri (1985) reported the heavy yield losses in Tanzania due to anthracnose in Phaseolus vulgaris L. Arya et al (1986) studied the incidence of anthracnose in bean and suggested that the incidence of the disease and its seed transmission were higher during rainy season. They found a significant positive correlation between percentage infected pods and transmission of C. lindemuthianum through the seed but no correlation was observed between seed transmission of anthracnose and its incidence on leaves.

Dhingra et al (1986) reported that inspite of high disease prevalence on leaves of bean during rainy season, the disease prevalence on pods and proportion of seeds transmitting the pathogens was low and thereby established a lack of relationship between field incidence of bean anthracnose and production of seeds, transmitting *C. lindemuthianum*. Fernandez *et al* (1987) used extensive statistical tools to investigate the influence of primary inoculum on bean anthracnose prevalence at two locations during rainy and dry seasons. The multiple regression analysis with dummy variables for season and locality showed a positive and linear relationship. The influence of anthracnose on leaf spot was much higher during the rainy than during the non-rainy season. They also reported the low correlation coefficient (r=0.26) between leaf and pod anthracnose.

Phaeoisariopsis griseola (Sacc.) Ferraris:

Brock (1951) reported that the losses due to *P. griseola* were the greatest in seed crops and regarded as the most important disease affecting seed crops on the south coast, Queensland.

Srinivasan (1953) surveyed leaf spot disease in the various parts of Nilgiri plateau, India and reported that the *P. griseola*, was widely prevalent on French bean

(*Phaseolus vulgaris* L.) during and after rainy season. He proved the pathogenicity on three local varieties of *P. vulgaris* after 12 days of inoculation with the conidial suspension. Cardona and Walker (1956) studied the host range and physiology of *P. griseola*, its relation to the host tissue and various aspect of the disease cycle and concluded that only Lima beans were infected. Barros *et al* (1958) made an extensive survey of the disease in Cauca valley (Columbia) and inferred that cultural practices played an important part in the development of epiphytotic of angular leaf spot disease.

Bose and Sindhan (1972) discussed in length the etiology of the disease caused by *P. griseola* in the hill districts of Uttar Pradesh. They observed that the fungus generally appeared during the latter half of August, causing premature defoliation and seed deformation to a great extent. Singh and Sharma (1975) also reported that *P. griseola* caused considerable reduction in yield by causing premature defoliation.

Investigation on perpetuation and survival of *P. griseola* studied by Sindhan and Bose (1979) revealed that the survival of mycelium in the seed, plant debris and in the soil during the off season may provide a potential source of primary infection of this disease. Correa and Saettler (1987) observed numerous outbreaks of angular leaf spot caused by *P. griseola* in Michigan fields of red kidney beans and reported that the pathogen survived at least two winters under Michigan condition both in burried tissue and in infected standing plants.

EFFECT OF FUNGICIDES

Bernat and Raicu (1974) reported the control of *C. lindemuthianum* in French bean using 0.2% Thiram, 0.2% captafol, 0.2% Mancozeb and Captan fungicides. However, Rolim *et al* (1981) found that chlorothalonil and captafol gave the best result followed by thiabendezole and chlorothalonil + copper oxychloride in controlling natural infection of bean by *C. lindemuthianum*. Subsequently they have evaluated the fungicide on the basis of percentage of infected pods and damage seeds.

Tu (1983) found that seed treatment with benomyl formulation (0.3kg a.i./45 kg of seeds) was not successful as the recurrence of *C. lindemuthianum* leaf lesions in grower's field comes mainly from two sources and therefore, suggested disking down of infected plant debris and avoiding reintroduction of infected seeds might check the spread of the disease. Issae (1985) made a comparative study of fungicides and reported from his field trials that C. lindemuthianum was best controlled by captafol, benomyl and mancozeb used singly, alternately or in mixture. Fernandez (1987) emphasised that information of the influence of primary inoculum on bean anthracnose prevalence might be taken for formulation of fungicide spray schedules on the basis of primary inoculum and the rain distribution pattern.

SEED MYCOFLORA

Kaul (1973) studied the comparative effect of long storage period on the viability and microflora of P. vulgaris seeds and reported that fungicide treated seeds effectively controlled the seed mycoflora and did not affect the germinability of seeds even after 4 years of storage.

According to Neergaard (1977) seeds play a vital role as a carrier of economically important pathogen and several fungal propagules and with this understanding he made a critical analysis of the significance of seed transmission of various economically important diseases. Similar view was shared by Sinha and Khare (1977). They reported as many as 25 species of seed-borne fungi from 40 samples of cow pea (Vigna sinensis savi.) seeds with higher percentage incidence of Macrophomina phaseolina and Fusarium equisiti.

Kore and Solanke (1982) concluded that fungicides treatment of P. vulgaris seeds reduced the mycoflora significantly. Nitsche and Cafati (1985) isolated few pathogenic fungi like Rhizopus stolonifer, Rhizoctonia solani, Botrytis cinera and Fusarium oxysporum from seeds of P. vulgaris Similarly, species like F. solani, F. phaseoli and Penicillium sp. were frequently isolated from P. vulgaris seeds (Khare, 1985). From the fungicide treated seeds they found that Benlate was the most effective followed by Captan and Copper oxychloride in reducing incidence of fungi and increasing germination in vitro.

Fungi associated with common bean (Phaseolus vulgaris L.) were extensively studied by Lokhande et al (1986). They found 5 species of pathogenic and rest saprophytic out of 14 species belonging to 10 genera and the correlation of external disease symptoms on the seeds and the fungi associated with them were determined. Santosh et al (1986) reported high percentage of Fusarium sp. followed by Aspergillus, Rhizopus, Penicillium, Verticillium, Trichoderma sp. and *Colletotrichum lindemuthianum* in French bean seeds. Muniz and Muchovez (1987) found *Aspergillus glaucus*, *Curvularia intermedia*, *C. lunata*, *Fusarium* sp., *M. phaseolina* and *Sphaeriopsis* sp. associated with dry beans (*P. vulgaris*).

Sharif *et al* (1987) isolated *Aspergillus flavus*, *A. niger*, *Fusarium solani*, *Penicillium pinophilum* and *Drechslera spicifera* from broad bean. Tu (1988) observed the association of *Sclerotina sclerotiorum* with *P. vulgaris* seed testa and cotyledons.

PHYLLOSPHERE

DeBary noticed the presence of fungi on the plant surface as early as 1866 and the term "Phyllosphere" was proposed by Last (1955a) and Ruinen (1956) to denote the mileu on the leaf surface. Saprophytic activity of micro-organism on leaf surfaces had long been described by Last (1955a), Kerling (1964), Leben (1964, 1965), Last and Deighton (1965), Sinha (1965), Dickinson (1967), and Sharma and Sinha (1972). The phyllosphere has been an inspiring and unifying theme since 1970 when the first symposium on this subject was organised by T.F. Preece and C.H. Dickinson.

Leaf Surface Mycoflora:

According to Hudson (1962) the micro-organism which exist on the leaf surface in active state are known as colonizers and classified into the common primary saprophytes and restricted primary saprophytes. Lamb and Brown (1970) categorised the leaf surface mycoflora into two groups - the resident group consisted of actively growing saprophytic forms and the transient groups that consisted of inactive forms and were deposited on the surface of the leaves as wind-borne propagules.

The phyllosphere microflora of P. vulgaris was studied by Oblisami and Balagopal (1973). Mishra and Dickinson (1981) investigated the phyllosphere and litter fungi of Ilex aquifolium using leaf impression peels, scanning microscopy and cultural techniques. They reported a marked seasonal fluctuations of microbial population in older green leaves than on newly formed leaves. Phyllosphere and phylloplane microflora of a healthy and diseased (Phytophthora infestans) leaves of Solanum khasianum were studied by Sharma and Tiwari (1981) and reported maximum fungal species on diseased leaves whereas yeasts and bacteria were recorded maximum on healthy leaves.

Daniel (1985) investigated the saprophytic fungal communities of the phyllosphere of Eucalyptus viminalis by serial washing, superficial sterilization and direct observation. According to him the fungi form two well differentiated groups - phylloplane species and endophytes on leaving leaves. The phylloplane community was similar to that found on other plants but endophyte exhibited host specificity. Both groups showed variation in frequency but phylloplane species showed a distinct seasonal pattern with maximum in autumn-winter and minimum in summer with positive and negative correlation with humidity and temperature.

Leaf Surface Mycoflora in relation to Environmental Factors:

Abiotic factor (micro and macro environment) and biotic factors (interactions between micro-organism and between host and micro-organism) may affect the microbial colonization on the leaf surface. Host specificity was found to be important in the microbial pattern of phyllosphere as the former controlled the physical and chemical nature of leaf (Kerling, 1964). Plant height was also found to be an important factor for microbial colonization on the phylloplane (Mishra and Tiwari, 1969). Kumar and Gupta (1974) recorded as many as sixty micro-organisms from the phyllosphere of three potato varieties in two successive Rabi seasons with significant variation with respect to host, maturity of leaf, age of the plant and climatic conditions.

Kehri and Chandra (1987) observed the variation of both bacterial as well as fungal population of phyllosphere and phylloplane with season and climatic conditions in *Eichhornia* spp. having *Aspergillus*, *Cladosporium* and *Fusarium* spp. as the dominant form. Vardavakis (1988) studied the phyllosphere mycoflora of *Cistus incanus*, *Arbutus unedo* and *Quercus coccifera* by leaf impression, culture method and reported seasonal variation of dominant and resident fungal species. He also observed variation in abundance of each fungal species.

Scanning Electron Microscopy (SEM) of Leaf Surface Micro-Organism:

The first SEM views of aerial plant surfaces were provided by Amelunzen *et al* (1967) in Germany where much information on aerial plant surface morphology has since been gathered. Barnes and Neve (1968) first suggested that SEM could extend knowledge of micro-organism in relation to plant surfaces. They published micrographs of *Erysiphe polygoni* on clove leaves.

Lisker *et al* (1976) observed many details of the infection of *P. vulgaris* by *Rhizoctonia solani* through SEM.

Bashi and Fokkema (1976) found that SEM was of advantage for observation of the mucillage and the wax layer which were difficult to distinguish with conventional light microscope in Sporobolomyces roseus on wheat leaves.

Mishra and Dickinson (1981) reported that SEM was very useful for recording the occurrence of conidiophores, sporulating fungi on the leaf surface and distinguishing Yeast. They highlighted the role of SEM for providing valuable information on the area occupied by various microbial structure, on their distribution in relation to leaf topography and on the occurrence of fungal conidiophore.

King and Brown (1983) described the techniques for preserving aerial fungal structures for use in SEM. Similarly, Jones et al (1984) described a technique for the examination of fungi in SEM using frozen hydrated specimens.

Scanning electron microscopic studies on Phyllosphere microflora of Sambucus nigra, Ribes nigrum, Crataegus monagyna and Bosquiera phobiros by Dickinson (1986) showed a mucillage sheathing that enabled them to attach on the leaves.

INTERACTIONS

Extensive literature on interactions, biological control principles, practices, underlying mechanism and various strategies for improving the efficacy of biological control agents have been extensively reviewed (Baker and Cook, 1974; Fokkema, 1976; Blackman and Fokkema, 1982; Niwas and Sharma, 1988).

Interaction Between Leaf Surface Micro-Organism and Pathogens:

It is well established that large number of saprophytic and parasitic micro-organisms inhabit the phylloplane of crop plants (Leben 1965). Several reviews on interactions between leaf saprophytes and pathogen and their selective possibility of using as biocontrol agent of some plant pathogens had been focussed (Sinha, 1965; Heuvel, 1970, 1971; Dennis and Webster, 1971b; Preece and Dickinson, 1971; Dickinson and Preece, 1976; Skidmore, 1976; Fokkema, 1976; Mishra and Tiwari, 1976b).

Hadar *et al* (1979) observed that *Trichoderma harzianum* directly attacked the mycelium of *Rhizoctonia solani* when the two fungi were grown together on a glucose plus minerals medium but they could not detect the antibiotic activity of *T. harzianum* towards the pathogen.

They concluded that members of the genus Trichoderma were active both as hyperparasites and as antibiotic producers.

Purkayastha and Bhattacharya (1982) studied the interaction between phyllosphere fungi and the jute pathogen - Colletotrichum corchori and found a well-marked space of aversion between the pathogen and Aspergillus nidulans and Penicillium oxalicum. Further test on the culture filtrate of these organism revealed that the inhibition of the growth of C. corchori was due to a marked change in pH of those culture filtrates.

Spore germination and effect of culture filtrate of saprophytes towards pathogens:

Chaudhury (1937) reported that the minimum requirement of atmospheric humidity for germination of C. lindemuthianum was 95%. The literature relating to spore germination in fungi was first reviewed by Gottlieb (1950). Martinez et al (1957) reported 90% spore germination of C. lindemuthianum after 24 hours at optimum temperature (28°C).

Mercer et al (1971) observed that orange extract and glucose casmino acid mixture stimulated germination of conidia of C. lindemuthianum. These two additive completely suppressed the lesion formation when added to spore suspension placed on hypocotyle. Landes and Hoffman (1979) reported that *in vitro* germination of conidia was stimulated by complex nutrient media but not by individual sugars, vitamins or nuclic acid.

Bera and Samajpati (1984) reported that the culture filtrates of *Aspergillus flavus*, *A. oryzae*, *A. terreus* and *Streptomyces* species caused inhibition of conidial germination and mycelial growth of *Phytophthora parasitica* and *Colletotrichum capsici*. They also observed the zone of inhibition on PDA that ranged from 4mm to 10mm. Sarkar and Samaddar (1984) studied the interactions of phylloplane saprophytic microflora of rice, mustard and lentil with their respective pathogens and reported that culture filtrate of *A. terreus* inhibit the germination of conidia and germ tube growth of *Helminthosporium oryzae*. Their data also suggested the production of fungitoxic metabolites by *A. terreus*.

Kono (1985) reported the effect of culture filtrate of oat leaf surface fungi on germination and invasion of crown rust urediniospore. He observed that metabolites produced by the phylloplane fungi reduced the rate of spore germination and formation of appresoria. Similarly, Pandey (1985) observed the retarded growth of *Alternaria alternata*, *Drechslera* sp., *Fusarium oxysporum* and *Rhizoctonia bataticola* by the culture filtrates of *Aspergillus* spp. and *Trichoderma viride*. Srivastava and Suman (1986) observed the suppression of conidial germination of powdery mildew by the phyllosphere fungus *Penicillium fillutanum in vitro*.

Elad *et al* (1986) reported that four isolates of *Trichoderma harzianum* inhibited linear growth and microsclerotia production in *Macrophomina phaseolina in vitro*. Niwas and Sharma (1988) studied the inhibition of *Alternaria solani in vitro* through antagonistic phylloplane microfungi and reported that biological inhibition of *A. solani* differed according to species of antagonists in dual culture. *A. solani* were mutually inhibited by *Aspergillus clavatus* *A. niger*, *Cladosporium cladosporoides*, *Epicoccum purpurascens* and *Penicillium citrinum*.

SPATIAL DISTRIBUTION

Bliss and Fisher (1953) while fitting the negative binomial distribution to biological data concluded from the analysis of wide variety of biological counts that negative binomial distribution was the most widely applicable in describing data involving aggregation or clustering of organisms. They have estimated the parameters mean ($\bar{X}$) and exponent(r) of the negative binomial distribution from the frequency distribution of sample and described three efficient methods for the estimation of r: (a) based upon the first and second moments, (b) based upon the ratio of the total number of units in the sample (N) to the number of units without organism (f_o), and (c) based upon the method of maximum likelihood.

McGuire, et al (1957) reported the results of fitting three biologically significant statistical model such as negative binomial, Neyman's type A and the Poison binomial to entomological field data. He used Fishers' method of maximum likelihood in fitting the negative binomial while the method of moments was used for the other two distributions mentioned.

Morisita (1959) proposed I_δ-index for measuring aggregation tendency in plants and animals. The parameter r or 1/r of the negative binomial distribution also was widely used as good measure of aggregation if the data could be fitted to the negative binomial distribution model (Waters, 1959).

Taylor (1961) concluded from the study of biological population that (a) the variance (S^2) was equal to the mean ($\bar{X}$) in populations where the individuals were distributed at random, (b) the variance (S^2) was greater than the mean ($\bar{X}$) in natural populations where individuals were not independent of each other but aggregated and (c) the variance (S^2) less than the mean ($\bar{X}$) in populations where there was repulsion among individuals that ultimately led to regularity. However, he pointed out that the concept of aggregation (variance greater than the mean) was inappropriate at low densities and suggested that sample consisting mainly of O's and 1's may need special statistical treatment.

The study of spatial pattern has long been an important part of ecology and there were many proposed indices for analysing and measuring the dispersion pattern of plants and animal populations without any prior assumptions of the type of distributions (Grieg-Smith, 1964).

Llyod (1967) proposed a new parameter "mean crowding" which is defined as the mean number per individual of other individuals in the same quadrate and showed that the ratio of mean crowding to mean density ("Patchiness" in his term) was essentially identical with I_δ. He mentioned that the mean crowding or patchiness ($\overset{*}{X}/\bar{X}$) would be meaningful only when the population was loosely aggregated and quadrate size was not too large as compared with the size of "ambit" for individuals of the species concerned.

Lloyd's mean crowding was given by

$$\overset{*}{X} = \frac{\sum_{i=1}^{N} x_i(x_i-1)}{\sum_{i=1}^{N} xi}$$

where x_i was the number of individual in the quadrate and N the total number of quadrates contained in the population. It was related to the mean density (X) and variance (S^2) as

$$\overset{*}{x} = \bar{x} + \left(\frac{S^2}{\bar{x}} - 1\right)$$

Iwao (1968) pointed out that the concept of mean crowding could be applied to any kind of organisms and for any size of quadrat unit and the mathematical relationship between mean density ($\bar{x}$) and mean crowding ($\overset{*}{x}$) describes certain characteristics of the spatial distribution that are inherent to each species in a habitat. He found that in a wide variety of situations including both theoretical and biological distributions the mean crowding was linearly related with the mean density over a range of density.

The relation was shown by $\overset{*}{x} = b_o + b_1\bar{x}$. Therefore, the regression of mean crowding on mean density could be obtained as a means of detecting the aggregation pattern proposed by Iwao (1968).

Studies on the spatial frequency distribution of black rot in cabbage field by Strandberg (1973) showed that the patterns of distribution of both the lesions and infected plants were best described by the negative binomial distribution. Southwood (1978) was of the opinion that a certain number of samples is necessary to obtain an estimate of the density of disease plants with a known precision varies with the spatial distribution of the disease in a field. He pointed out that negative binomial distribution is a model which could be used to describe a contagious distribution.

Campbell and Pennypacker (1980) studied the spatial distribution of hypocotyl rot caused by Rhizoctonia solani in snap bean and concluded that the infected plant was randomly distributed whereas the fungal lesions were clustered as indicated by variance to mean ratio greater than unity and goodness of fit of the negative binomial distribution. The spatial frequency distribution of disease incidence of powdery mildew on wheat leaves was reported to follow the negative binomial distribution (Rouse *et al*, 1981). Waggoner and Rich (1981) reported that the lesions of few of the 112 observed cases of plant disease caused by virus and fungi were mostly fitted by the negative binomial function with exponent $r \approx 1$.

Boivin and Sauriol (1984) studied the spatial distribution of *Botrytis squamosa* on onion using Iwao's patchiness regression procedure and found that the aggregated leaf lesions were contagiously distributed which did not alter due to fungicidal treatment. Similar technique was applied to determine the spatial distribution of *Meloidogyne hapla* in Muckgrown - carrots by Be'lair and Boivin (1988) and represented the results in terms of regression of mean crowding ($\overset{*}{x}$) over mean ($\bar{x}$) as $\overset{*}{x}$ = $0.7286 + 0.7219\bar{x}$. which indicated that aggregates were the basic component of the nematode population and that those aggregates were randomly distributed in carrot fields.

SEQUENTIAL SAMPLING

According to Wald (1945) the concept of sequential sampling was brought to fruition in 1943 under wartime military contact for use in quality control. The savings realized from sampling sequentially were so significant (often exceeding 50%) and the potential for other uses was so obvious that the information was released to the public in 1945. A decade later, Waters (1955) observed that application of sequential sampling to biological problems had been "somewhat limited" and gave an excellent discussion of the disadvantages, applications, basic requirements and essential features of sequential sampling plans in relation to entomological problems. Subsequently Southwood (1966) had given more emphasis of sequential sampling for use in ecological and pest management and also in the study of plant disease.

Strandberg (1973) used a modified sequential sampling method based on the spatial distribution of lesions and infected plants to obtain the average infection rate for the black rot disease. Iwao (1975) proposed a method of sequential sampling for grading population level in relation to a critical density. This method was based on the $\overset{*}{x}-\bar{x}$ relationship which could be used without restriction on the distribution pattern. Jerome (1976) designed sequential sampling plan simply to choose between two alternatives (i.e. the density higher or lower than a critical level). He pointed out that the apparent mathematical complexity inhibited its use by the biologists.

Rouse *et al* (1981) discussed the sequential sampling procedures based on their result of the study of distribution of wheat powdery mildews in relation to disease incidence and severity. Samarjit *et al* (1982) developed a sequential sampling plan on the basis of negative binomial distribution which was very useful in the proper intervention of control operations particularly at very low and very high level of infestation.

Boivin and Sauriol (1984) developed sequential sampling plan based on Iwao's patchiness regression parameters for *Botrytis* leaf blight lesions on onions along with two additional elements; economic threshold of the disease as one half lesion per leaf and an error level of 0.1 to calculate the stoplines of the sequential sampling plan. Vincelli and Lorbeer (1987) established a sequential sampling plan that optimizes sampling intensity for determining disease levels of *Botrytis* leaf blight in onion fields. They used one lesion per leaf as an action threshold, for the initiation of fungicide spray programme.

According to Boivin and Vincent (1987) the economic threshold has been defined as the function of crop value and pest control, which should be established for each cultivar as the susceptibility of plant varies from one cultivar to another for better precision. It has been reported by them that a sequential sampling plan could be implemented on the basis of preliminary threshold called the action level if the economic threshold of the species concerned was unknown. Similarly, they have illustrated the use of provisional or action level economic threshold in establishing the sequential sampling plan for early nymph of Lygocoris communis using Iwao's regression technique as well as Wald's procedure.

ANALYSIS OF VARIANCE

Friedman (1937) illustrated the use of ranks to the data of Randomized Complete Block Design to avoid the assumption of normality implicit in the analysis of variance and emphasized to use non-parametric test when there were enough reason to believe that population distribution was not normal. Kruskal and Wallis (1952) described the non-parametric test for data obtained from the completely randomized Design and gave more emphasis to the use of ranks in one criterion variance analysis.

Siegel (1956) suggested the use of χ^2 test for K independent samples to avoid restrictive assumptions of F-test in the analysis of variance to determine the significance of the differences among K independent samples.

He pointed out that the hypothesis of no difference among K independent samples may be tested by applying formula:

$$\chi^2 = \sum_{i=1} \sum_{j=1} \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

where

O_{ij} = observed number of cases categorized in i^{th} row of j^{th} column

E_{ij} = number of cases expected under H_o to be categorised in the i^{th} row of j^{th} column.

$\sum_{i=1}^{r} \sum_{j=1}^{K}$ = Sum over all Cells.

CHAPTER - I

SURVEY OF LEAF SPOT FUNGAL DISEASES ASSOCIATED WITH THE FRENCH BEAN (Phaseolus vulgaris L.)

INTRODUCTION

French beans are an important food legume crop and provide an essential part of the daily diet for millions of people especially in Latin America, Central and Eastern Africa. This crop is prone to many fungal diseases and pose serious problems in its cultivation and production despite the introduction of high yielding, disease resistance varieties and use of fertilizers.

Anthracnose and angular leaf spots are among the most widespread and economically important fungal diseases of French beans in Latin America and many other regions of the world. The anthracnose also known as leaf spot, pod spot or canker (Wheeler, 1969; Robert and Bothyroyd, 1972) is most destructive and common disease of bean in India and abroad as well. The causal agent of anthracnose (Colletotrichum leaf spot), Colletotrichum lindemuthianum (Sacc. and Magn.) Br. and Cav. may induce upto 100% yield loss when a susceptible variety is grown in a region with cool to moderate temperature (13-26°C) and abundant moisture (Zaumeyer and Thomas, 1957). On the other hand, angular leaf spot, caused by Phaeoisariopsis griseola (Sacc.) Ferraris may induce upto 80% yield loss when a susceptible variety is grown in a region with moderate temperature

(20-26°C) and abundant and fluctuating moisture conditions; (Schwartz et al, 1982).

Various control strategies have commonly been advocated in attempts to reduce losses caused by Colletotrichum leaf spot and angular leaf spot. Planting pathogen free seeds, field sanitation, crop rotation, shifting planting dates, application of fungicides and plant resistance are some of the recommended practices (Schwartz et al, 1982; Zaumeyer and Thomas, 1957; Fernandez, 1987).

Besides these diseases, few other fungal diseases of minor importance that attack French bean include Alternaria leaf spot (Alternaria alternata), Aschochyta blight (Aschochyta phaseolorum sacc.), Phyllosticta leaf spot (Phyllosticta phaseolina), Chaetoseptoria leaf spot (Chaeto-septoria wellenanii stev.), Grey leaf spot (Cercospora vanderysti P. Henn.), Cercospora leaf spot (Cercospora canescens Eu. and Mart.) and white leaf spot caused by Pseudocercosporella albida (Matta and Bell.) Yoshii and Aamodt (Allen and Russel, 1986).

In India there is a paucity of literature available on the follior diseases of French bean. Srinivasan (1953) reported the angular leaf spot of French bean in his survey work from Nilgiri Hills and the disease was also reported from Himachal Pradesh by Sohi and Sharma (1969). Extensive studies on the perpetuation, control measures and survival of *P. griseola* was made by Sindhan and Bose (1979). The host range and physiology of *P. griseola*, its relation to the host tissue, various aspects of the disease cycles, the symptoms and the environmental factors that favour the development of the angular leaf spot disease have been described by Cardona and Walker (1956). Sindhan (1983) reported that temperature and humidity play major role in the development of anthracnose disease of French bean in Haryana. Similarly, the effect of temperature on disease incidence and severity of anthracnose on white bean was observed by Tu (1982). Recent survey work on fungal disease by various workers yielded new records from India. Few of these diseases were powdery leaf spot caused by *Ramularia phaseolina* from Uttar Pradesh hills (Tripathy, 1985), leaf spot caused by *Alternaria brassicicola* from Kalimpong (W.B.) (Berra, 1986) and Farinose leaf spot caused by *Vellosiella phaseoli* in Sikkim (Srivastava and Gupta, 1989).

It is thus evident, that not much work has been done on the diseases of this crop as far as the systematic survey and epidemiological aspect are concerned in India in general and in Meghalaya, Shillong in particular. Therefore, the present investigations were carried out in order to conduct a systematic survey of fungal diseases and their few epidemiological aspect of five locally cultivated French bean cultivars of Meghalaya, Shillong.

MATERIALS AND METHODS

Selection of study site

The present investigation was conducted at Shillong, the Capital of Meghalaya. It is located at 24°34'N latitude and 91°56'E longitude. The exact study site was selected at the botanical garden in the University Campus located at an altitude of 1540m. Physiographically it is hilly surrounded by pine forest. The soil is of laterite type usually acidic in reaction. The seasonal wind such as the south-west monsoons and the north-east wind mostly controls the climate of Shillong.

Selection of varieties

Survey of fungal diseases in French bean was conducted in the University garden for two years. Five locally

PLATE 1.1

Showing the field view of the experimental site.

PLATE 1.1

cultivated French bean varieties viz., Shillong local selection (V_1); Manipur variety (V_3); Premier variety (V_4); Local variety (V_5) and Masterpiece (V_6) were sown for the first year of preliminary survey. In the second year only three comparatively more susceptible varieties viz: Shillong local selection (V_1); Manipur variety (V_3) and Premier variety (V_4) were sown based on the preliminary survey. Manure of any sort was not applied and therefore, the survey work was carried out in a natural soil.

Design of experiment

The experimental site was well prepared by digging three times before showing the seeds. A 50cm wide shallow channel was made around the whole experimental site. The net experimental area measuring (4x37)m=148m^2 was divided into blocks. Each block was divided into plots of size 1mx0.6m. The experimental materials (French bean seeds) were sown in each plot of randomized block design in double rows. Plant to plant distance and row to row distance was maintained at 25cm and 30cm respectively. A part of the experimental lay out is presented in Fig.1.10 and Fig.1.11.

Isolation of pathogen in pure culture

Samples of diseased French bean leaves were collected asceptically in a polythene bag from the experimental site into the laboratory and the disease portion of the leaf along with some healthy tissue was cut into small pieces of appropriate size (1^2mm). These cut pieces were surface sterilized using 0.1% mercuric chloride solution for two minutes and subsequently they were removed and passed through the four changes of sterile distilled water. After removing from the sterile water the pieces were kept on the sterile filter paper (Watman No.1) for some time and finally placed on potato dextrose agar (PDA) slants separately with the help of sterilized inoculating needle under asceptic condition and plugged tightly with the help of non-absorbant cotton plug. These slants were labelled properly. Inoculated slants were incubated at 25±1°C for 5-6 days to obtain the growth of the pathogens. The hyphal tip of the young mycelium was transferred to another freshly prepared PDA slants to obtain pure culture.

For P. griseola-a pathogen causing angular to circular leaf spot of bean, the fungus was not readily isolated from the host tissue by the usual tissue plating technique, and therefore, a modification of the method developed was used (Cardona and Walker, 1956). When typical lesion were observed in the field, diseased leaf samples were collected and a section of diseased leaf tissue along with healthy portion was placed in 1:1000 mercuric chloride for 1 to 2 minutes, washed in sterile distilled water 4 to 5 times, and transferred to sterile Petridishes containing moistened sterile filter paper. After 6 to 8 days at 25±1°C spores were transferred from the coremial tips to PDA . slants. When colonies formed, the hyphal tip of the developing mycelium was transferred to another newly prepared slants of PDA containing one per cent bean leaf extract (PDAB).

Sporulation and stock culture

The isolated Pathogen did not sporulate well on PDA, and therefore, various media were tested at different occasions as detailed below along with their composition:

1. Potato-Dextrose-Agar Medium: Peeled and sliced potato - 250.g, Dextrose - 20.0g; Agar agar - 20.0g; Distilled water - 1000.0 ml.

2. Czapek's Dox Agar (Raper and Thom, 1949): Agar - 15.0g; $NaNo_3$ - 3.0g; KH_2PO_4 - 1.0g; $MgSO_4 7 H_2O$ - 0.5g; KCl - 0.5g; $FeSO_4 7 H_2O$ - 10.0mg; Sucrose - 30.0g; Distilled water - 1000.0 ml.

3. Richard's medium: KNO_3 - 10.0g; KH_2PO_4 - 5.0g; $MgsO_4,7H_2O$-

2.5g; $FeCl_3$ - 0.02g; Sucrose - 50.0g; Distilled water - 1000.0 ml.

4. <u>Mathur's Agar (MA)</u>: Glucose - 2.8g; $MgSO_4$ - 1.23g; KH_2PO_4 - 2.72g; Bacto peptone - 1.0g; Yeast extract - Trace; Agar - 20.0g; Distilled water - 1000.0ml.

5. <u>Potato Dextrose Agar containing 1 per cent bean leaf extract (PDAB)</u>: Peeled and sliced potato - 250.0g; Dextrose - 20.0g; Agar - 20.0g; Host extract - 10.0ml; Distilled water - 990.00ml

6. <u>Asthana and Hawker medium (Asthana and Hawker, 1936)</u>: Glucose - 5.0g; KNO_3 - 3.5g; KH_2PO_4 - 1.75g; $MgSO_4$ - 0.75g; Agar - 15.0g; Distilled water - 1000.0ml.

7. *<u>Host extract agar medium</u>: Agar - 20.0g; Host extract - 1000.0ml.

8. <u>Water agar</u>: Agar agar - 15.0g; Distilled water - 1000.0ml.

*Host extract from bean leaves was made by steaming the leaves in a pressure cooker for 20 minutes, crushing in a mortar and filtering through the cheese cloth. The one per cent bean leaf extract was made by steaming 10.0g of leaves in a manner stated above and adding 10.0ml filtrate to the PDA.

The media mentioned above were prepared according to the standard formula prescribed and pH of all media tested were adjusted in between 5 and 6.

Determination of disease incidence

Daily observations were made to note the appearance of the disease on the leaves just after germination. Regular observations were made at weekly interval after the first appearance of the disease to note the disease incidence by counting the diseased plants, healthy plants and total plants.

Disease rating

Disease rating was made on 10 randomly selected leaves from each plot. Disease ratings were based on the extent of number of leaf lesions and area of leaf damaged by the pathogen involved and over all rating pattern was adopted within the 9 point scale divided into 5 categories as used in International Institute for disease scoring (table 1.1) with certain modification (Mayee and Datu, 1987).

Determination of disease severity index

The per cent disease index (PDI) was determined

Table 1.1 Showing scale used for rating the disease.

Disease rating	% of leaf area destroyed	Description	Interpretation
1	0	no lesion/spot	Resistant
3	1 - 6 or less	spot present on a few plants upon careful examination	Moderately resistant
5	7 - 15 or less	Spots/lesions visible on many plants	Tolerant
7	16 - 50 or less	Spots present on almost all plants	Moderately susceptible
9	51 - 100	Spots commonly found on all plants	Susceptible

at weekly interval on 10 randomly selected leaves mentioned above. The general formula followed for the analysis of PDI using disease severity rating was as follows:

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves observed x maximum rating scale (i.e.9)}} \times 100$$

In actual calculation certain modification was done on this general formula. The disease incidence and PDI values obtained were converted into degree by angular transformation and with the transformed values the statistical analysis was done (Sharma, 1986).

Pathogenicity

The pathogenicity test experiments were conducted in the net house with V_4 and V_5 (susceptible) cultivars taking five replications separately for each isolate tested. Buckets containing steam sterilized soil were used. Three surface sterilized seeds with 1:1000 mercuric chloride were seeded per bucket. Leaves of these varieties were inoculated with standard spore suspension prepared from 10 days old culture using atomizer after 15 days of germination. The inoculated plants were covered with polythene bags moistened inside previously with sterile distilled water to maintain high humidity around the inoculated plants. Sufficient control sets were maintained in a separate net house. Observation on the extent and development of symptoms upto a period of 15 days was made.

Analysis of variance

The analysis of variance is a simple arithmatical process of sorting out the components of variation in a given data. It is a procedure by which the variation embodied in the data of the samples may be resolved into component variation due to independent factors. Each of the component yields an estimate of the population variance and these estimates are tested for homogenity by means of F-test.

Regression and correlation analysis

Regression analysis describes the effect of one or more variables (independent variables (X)) on a single variable (dependent variable (Y)) by expressing the latter as a function of the former. Coefficient of regression is the rate of change in dependent variable for unit change in the independent variable.

The formula for calculating the same is

$$Y = a + bx$$

where a and b are calculated as follows:

$$a = Y - bx$$

$$b = \frac{\sum_{i=1} xiyi - n\bar{x}\bar{y}}{\sum_{i=1}^{n} xi^2 - n\bar{x}^2}$$

Correlation analysis on the other hand, provides a measure of the degree of association between the variables. The formula for calculating the same is given below:

$$\text{Correlation Coefficient(r)} = \frac{\sum_{i=1}^{n} x_i y_i - n\bar{x}\bar{y}}{\sqrt{\sum_{i=1}^{n} xi^2 - n\bar{x}^2}\ \sqrt{\sum_{i=1}^{n} y_i^2 - n\bar{y}^2}}$$

Multiple regression

Regression analysis involving more than one independent variable is called multiple regression analysis. This method is commonly used for developing predictive models which may be highly useful under certain set of conditions. The analysis was done manually following two techniques - linear and quadratic (second degree polynomial). The quadratic regression equation has wide application because most of the non-linear relationship found in agricultural research can be linearized through creation of new variables. The coefficient of determination (R^2) was calculated for each technique. The size of R^2 gives the proportion of variance of character measured (Y) attributable to the joint action of the independent variables x_1 and x_2.

The following multiple regression equation and quadratic (second-degree polynomial) were tested for determining the R^2 in disease incidence and severity.

(1) Linear $Y = a + b_1 x_1 + b_2 X_2$

(2) $Y = a + b_1 x + b_2 X^2$

where

Y = the expected dependable variable

a = a constant

b_1 = regression coefficient for X_1 variable

b_2 = regression coefficient for X_2 variable.

Collection of meterological data

The meteorological data such as temperature, relative humidity and rainfall were obtained from the field by using ordinary thermometer (0-100°C), Huger Hygrometer (85mm model : 3123) and rain guage (fixed in the field) on weekly interval coinciding the sampling date of observation for two cropping seasons (1987 and 1988). The data so obtained were utilized to correlate with the disease incidence and severity of the disease.

RESULTS

During the two years of survey, two fungal diseases were found to attack the plants of French bean (*Phaseolus vulgaris* L.) (table 1.2). The *Colletotrichum* leaf spot caused by *Colletotrichum lindemuthianum* (sacc. and Magn.) Br. and Cav. and *Phaeoisariopsis* leaf spot disease was found to cause by *Phaeoisariopsis griseola* (sacc.) Ferraris.

C. lindemuthianum (sacc. and Magn.) Br. and Cav. was found to attack the all above ground parts of the host plant (*Phaseolus vulgaris* L.) right from the primary shoot and cotyledons of the seedlings to the leaves, stem and pods whereas the *Phaeoisariopsis* leaf spot disease was mostly confined to the matured leaves near the ground surface.

Isolation and identification

The pathogen *C. lindemuthianum* (sacc. and Magn.) Br and Cav. was readily isolated initially in PDA whereas *P. griseola* (sacc.) Ferraris was isolated in PDA with little modified technique. In both the cases the pathogens were isolated in pure culture and identification was done strictly on the morphological characters and with the help of pre-existing literature.

Table 1.2 Showing the causal organisms of disease on different bean varieties (Phaseolus vulgaris L.)

Diseases	Causal organisms	Varieties				
		V_1	V_3	V_4	V_5	V_6
Colletotrichum leaf spot	Colletotrichum lindemuthianum (sacc. and Magn.) Br. and Cav.	+	+	+	-	+
Phaeoisariopsis leaf spot	Phaeoisariopsis griseola (Sacc.) Ferraris.	-	+	-	+	-

+ = Presence of disease

- = Absence of disease.

Sporulation and pure culture

C. lindemuthianum (sacc. and Magn.) Br. and Cav. was found to sporulate well on Mathur's agar (MA) whereas the pathogen P. griseola sacc. was found to sporulate on 1% bean leaf extract Potato dextrose agar (PDAB). On PDA sporulation was almost nil in both the cases. Asthana media could produce little spores of C. lindemuthianum but not in P. griseola (sacc.) Ferraris. Since the best media of sporulation was found in MA and PDAB, pure culture made on this media were used for further investigation.

Pathogenicity

Isolate of C. lindemuthianum and P. griseola were used for pathogenicity test on variety (V_4) and (V_5) respectively. They were pathogenic to the test cultivar and symptoms were typical of C. lindemuthianum and P. griseola The tests satisfied the Koch's postulation in both the isolates of the pathogen. Typical lesions developed on the leaves after 8 days of inoculation in case of P. griseola whereas in C. lindemuthianum they appeared just after 9 days.

Symtomatology

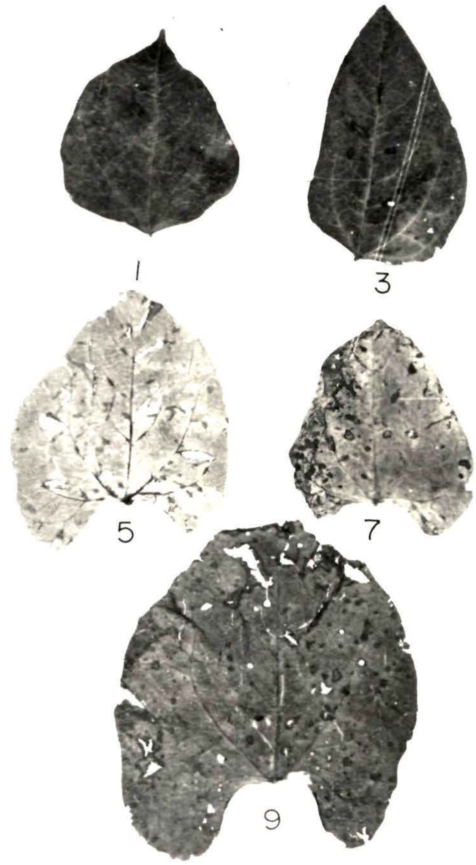
Colletotrichum leaf spot: Leaf spots caused by Colletotrichum lindemuthianum (sacc. and Magn.) Br. and Cav. were observed

PLATE 1.2

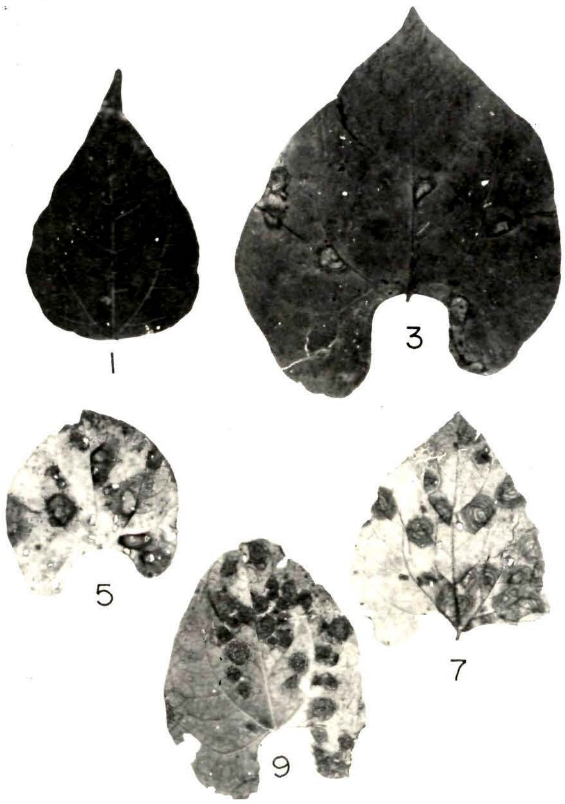
1. Showing leaf spot symptoms of Colletotrichum lindemuthianum and rating scale used for rating the severity of the disease.

2. Showing leaf spot symptoms of Phaeoisariopsis griseola and rating scale used for rating the severity of the disease.

PLATE 1.2



1 *Colletotrichum lindemuthianum*



2 *Phaeoisariopsis griseola*

PLATE 1.3

Transverse Section of a diseased leaf caused by Phaeoisariopsis griseola showing Coremia with conidia observed under scanning electron microscope.

PLATE 1.3

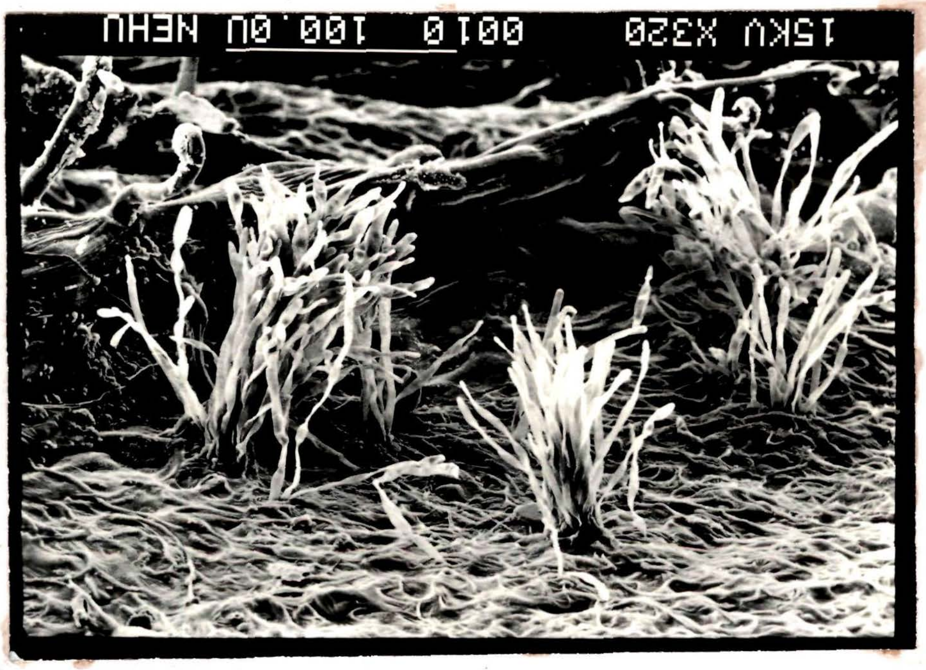


PLATE 1.4

1. Pathogenicity test of *Colletotrichum lindemuthianum* in variety - V_4

   A = control, B = Treated.

2. Pathogenicity test of *Phaeoisariopsis griseola* in variety - V_5.

   A = Control; B = Treated.

PLATE 1.4

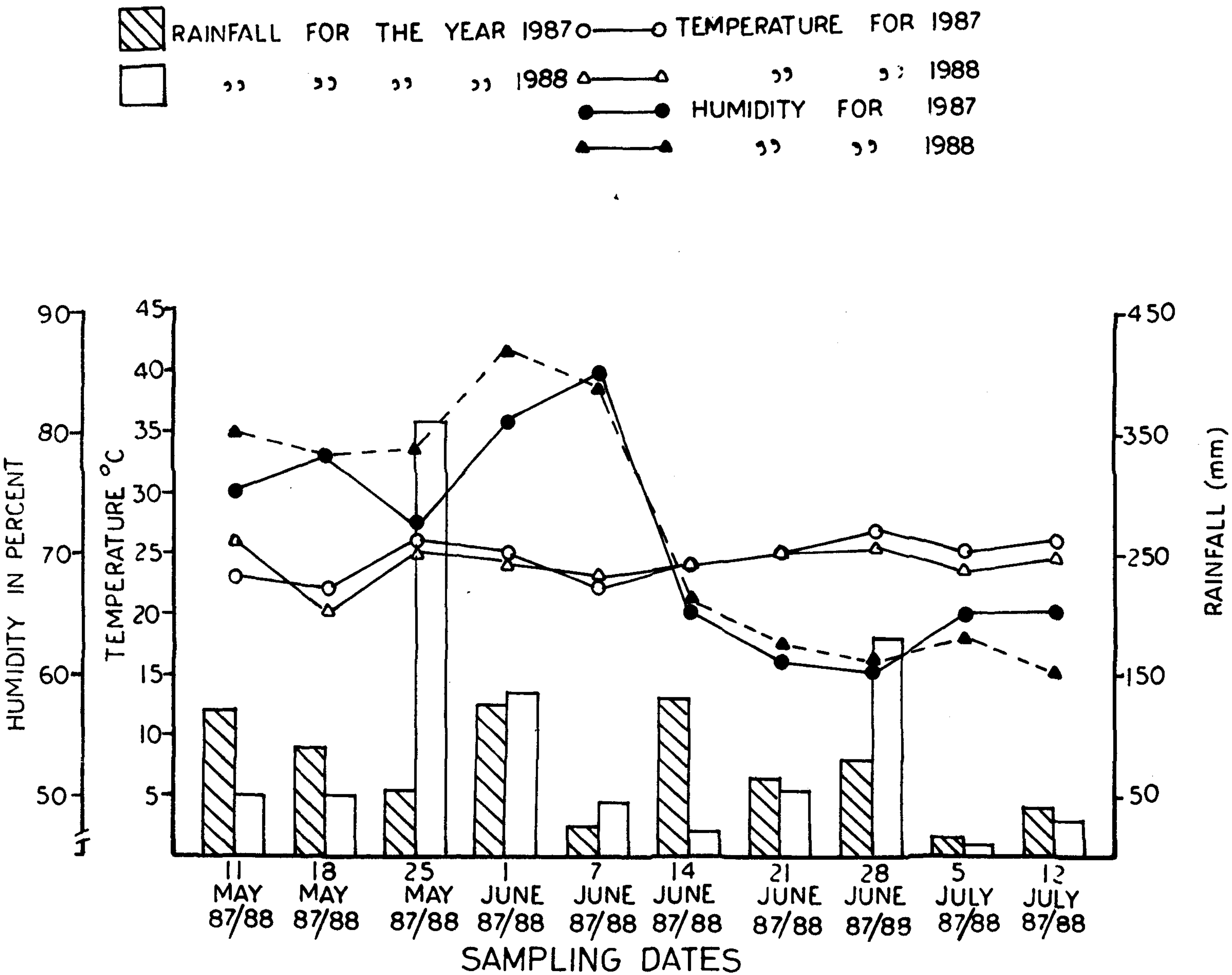
first on the lower side of the leaf as elongated, more or less angular to round spreading slightly into the surrounding tissue and finally appear on the opposite side as greyish to black, little sunken with slightly raised margins and thereby giving the appearance of leaf spots. In more severe form, prominent greyish to dark spots are produced all over the leaf. Prominent and most characteristic symptoms are frequently produced on the pod and on the stem. Spots on the leaf coalesced to form larger necrotic area in severe form.

Phaeoisariopsis leaf spots: Greyish leaf spots, which become light brown with age. Initially the pathogen causes small, light brown angular spots. Finally, the spots became brown circular, regular and symmetrical. On the lower surface of leaves brown to dark synnemata occur scattered on the affected parts. First symptom occured on the old leaves near the ground and then advances to other leaves. Premature defoliation was found common in the field.

Correlation and regression analysis

The meteorological data such as rainfall, temperature and relative humidity for two years sampling has been presented (fig. 1.1). The correlation coefficient between

Fig.1.1 Meteorological data for temperature (in °C), relative humidity (in percentage) and rainfall (in mm) of two years (1987 and 1982) of the study site at Shillong.



RAINFALL FOR THE YEAR 1987
" " " " 1988
TEMPERATURE FOR 1987
" " 1988
HUMIDITY FOR 1987
" " 1988
HUMIDITY IN PERCENT
TEMPERATURE °C
RAINFALL (mm)
90
80
70
60
50
45
40
35
30
25
20
15
10
5
450
350
250
150
50
11 MAY 87/88
18 MAY 87/88
25 MAY 87/88
1 JUNE 87/88
7 JUNE 87/88
14 JUNE 87/88
21 JUNE 87/88
28 JUNE 87/88
5 JULY 87/88
12 JULY 87/88
SAMPLING DATES

FIG. 1·1

rainfall, relative humidity and temperature with disease incidence of C. lindemuthianum was highly significant but not with severity. The simple linear regression equation for disease severity as well as disease incidence showed insignificant regression coefficient (table 1.4). However, simple linear in correlation and regression coefficients were insignificant for disease incidence and severity caused by P. griseola (table 1.3).

Out of the two methods tried, the coefficient of determination (R^2) of multiple linear regression and quadratic equations for disease incidence of C. lindemuthianum in variety (V_1) varied from 22.94% to 33.02% for linear equation and from 5.80% to 56.05% for quadratic equations. Similarly, the R^2 for severity varied from 5.61% to 8.77% in linear and 38.18% to 52.72% in quadratic (table 1.5).

The correlation and regression coefficient between disease severity (Y) and disease incidence (X) were highly significant at 1% probability level for C. lindemuthianum in V_1 (Y=7.85+0.31x; r=0.92) showing the R^2 value 88.28%. However, correlation and regression coefficients were insignificant for P. griseola.

The progress of the disease incidence and severity showed that initially disease progress slowly and reached

Table 1.3 Correlation coefficient and regression equation between disease characters and environmental factors for the leaf spot disease caused by <u>Phaeoisariopsis griseola</u> (Sacc.) Frerraris on French bean variety (V_5).

Disease characters (dependent variables)	Environmental factors (independent variables)	Correlation* coefficient (r)	Regression Equations*
Disease incidence(I)	Temperature(T)	0.09	Y = 19.45 + 0.84T
Disease severity(S)	Temperature(T)	-0.49	Y = 82.03 - 2.35T
Disease incidence(I)	Rainfall(R)	0.68	Y = 35.71 + 0.12R
Disease severity(S)	Rainfall(R)	0.68	Y = 29.76 - 0.07R
Disease incidence(I)	Relative humidity(RH)	-0.55	Y = 57.31 - 0.68RH
Disease severity(S)	Relative humidity(RH)	0.41	Y = 19.53 + 0.05RH

I = Incidence in degrees; S = Severity in degrees; T = Temperature(°C); R = Rainfall(mm); RH = Relative humidity(%).

* Correlation coefficient and regression coefficient not significant.

Table 1.4 Correlation coefficient and regression equation between disease characters and environmental factors for the leaf spot disease caused by <u>Colletotrichum lindemuthianum</u> on French bean variety (V_1).

Disease characters (dependent variables)	Environmental factors (independent variables)	Correlation coefficient (r)	Regression Equations
Disease incidence(I)	Rainfall(R)	-0.421**	$Y = 45.469 - 0.029R^{ns}$
Disease severity(S)	Rainfall(R)	-0.122^{ns}	$Y = 20.661 - 0.008R^{ns}$
Disease incidence(I)	Relative humidity(RH)	-0.445**	$Y = 57.260 - 0.510RH^{ns}$
Disease severity(S)	Relative humidity(RH)	-0.210^{ns}	$Y = 26.591 - 0.081RH^{ns}$
Disease incidence(I)	Temperature(T)	0.517**	$Y = (-)15.790 + 2.41T^{ns}$
Disease severity(S)	Temperature(T)	0.155^{ns}	$Y = 12.821 + 0.330T^{ns}$

I = Incidence in degrees; S = Severity in degrees; T = Temperature(°C); R = Rainfall(mm); RH = Relative humidity(%).

** = Significant at 1%
ns = not significant

Table 1.5 Showing the predicting equations of the progress of *Colletotrichum lindemuthianum* by environmental factors.

Methods		Estimated predicting equations	Coefficient of determination (R^2)
	I	$Y = 80.9007 + 0.042R + 0.516H$	0.2294
	S	$Y = 25.1601 - 0.011R - 0.052H$	0.0561
Multiple linear	I	$Y = -50.9310 - 0.079R + 4.032T$	0.3302
	S	$Y = 4.8630 - 0.013R + 0.688T$	0.0877
	I	$Y = 19.2801 + 2.036T - 0.397H$	0.2845
	S	$Y = 6.5910 + 0.515T + 0.020H$	0.0569
	I	$Y = 93.7991 + 9.0091T - 0.1391T^2$	0.2923
	S	$Y = 276.2640 + 4.0690T - 0.0790T^2$	0.5272
Quadratic (Second-degree Polynomial)	I	$Y = 645.2151 - 16.5231H + 0.1110H^2$	0.5605
	S	$Y = 217.7450 - 5.5301H + 0.0381H^2$	0.3818
	I	$Y = 26.875 + 0.1098R - 0.0007R^2$	0.058
	S	$Y = 39.795 - 0.2374R + 0.00008R^2$	0.4335

I = Incidence (in degrees)

S = Severity (in degrees)

Y = Expected severity and incidence

T = Temperature (°C)

R = Rainfall (mm)

RH = Relative humidity (%)

their peak stage when the average temperature (°C), rainfall (mm) and humidity (%) recorded were 24°C, 80mm and 65% respectively for C. lindemuthianum. Similar trend was observed for P. griseola. The disease progress curves more or less appeared sigmoid shaped (fig. 1.2-1.9).

DISCUSSION

During the survey of leaf spot fungal diseases in French bean for two years only two pathogens viz. C. lindemuthianum and P. griseola causing leaf lesions were observed although as many as 52 diseases for the temperate zone and 253-280 for the tropical were reported (Echandi, 1975). This could possibly be due to the varietal differences in their susceptibility to different diseases. C. lindemuthianum is distributed all over the world and in more severe form (Zaumeyer and Thomas, 1957; Walker, 1969) including India (Butler, 1973) whereas the P. griseola is a common angular leaf spot disease in tropical and subtropical region where the crop is grown and it occurs in less severe form, only sometimes it occurs spasmodically in epidemic propotion (Cardona and Walker, 1956; Barros et al, 1958). In India it has been reported from Nilgiri Hills (Srinivasan, 1953), Almora (Bose and Sindhan, 1972) and Himachal Pradesh

Fig.1.2 Showing disease incidence and disease severity caused by Colletotrichum lindemuthianum in variety - V_1.

Fig.1.3 Showing disease incidence and disease severity caused by Colletotrichum lindemuthianum (1) and Phaeoisariopsis griseola(2) in variety - V_3.

Fig.1.4 Showing disease incidence and severity caused by Colletotrichum lindemuthianum(1) in variety - V_4.

Fig.1.5 Showing disease incidence and disease severity caused by Phaeoisariopsis griseola (2) in variety - V_5.

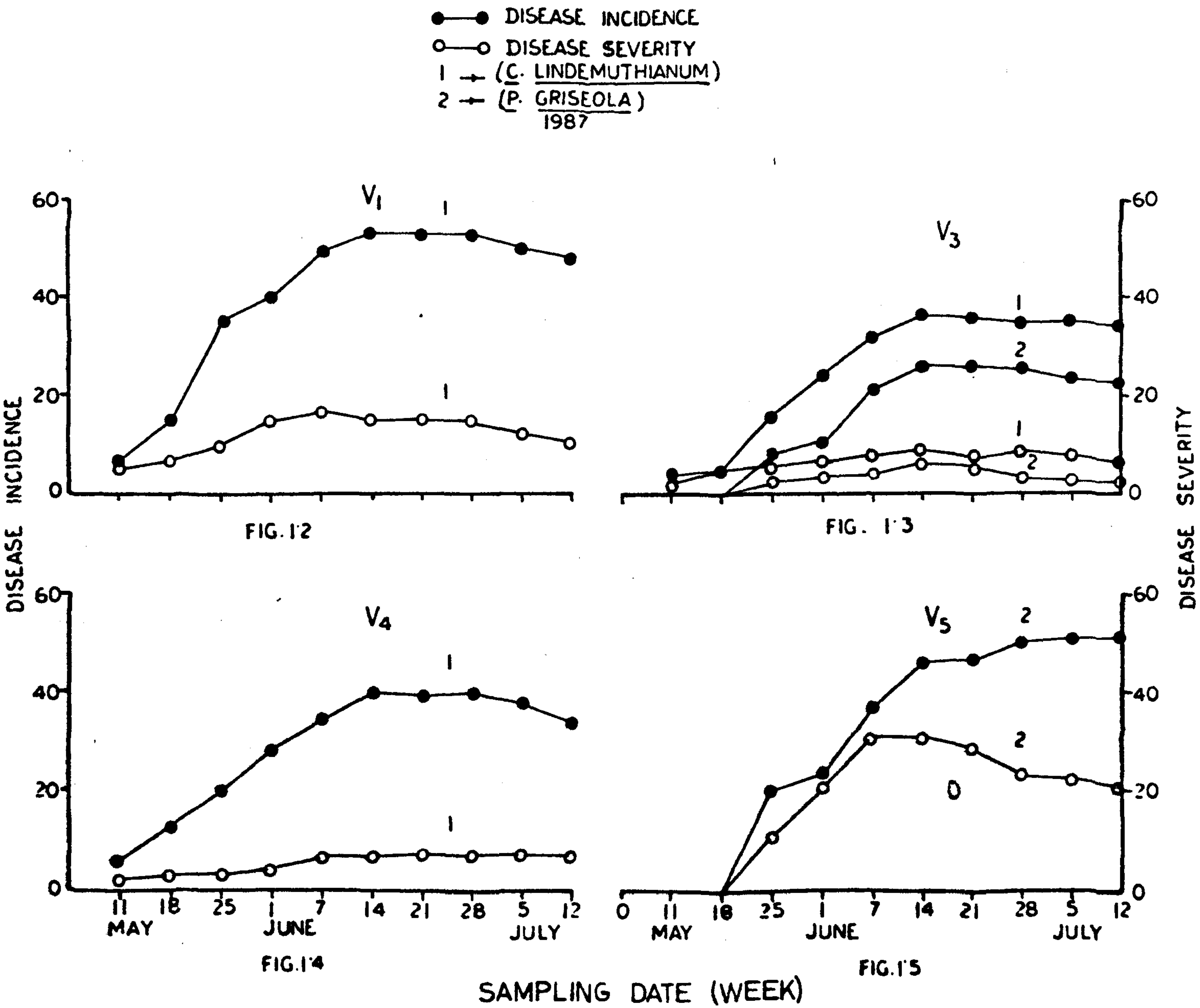
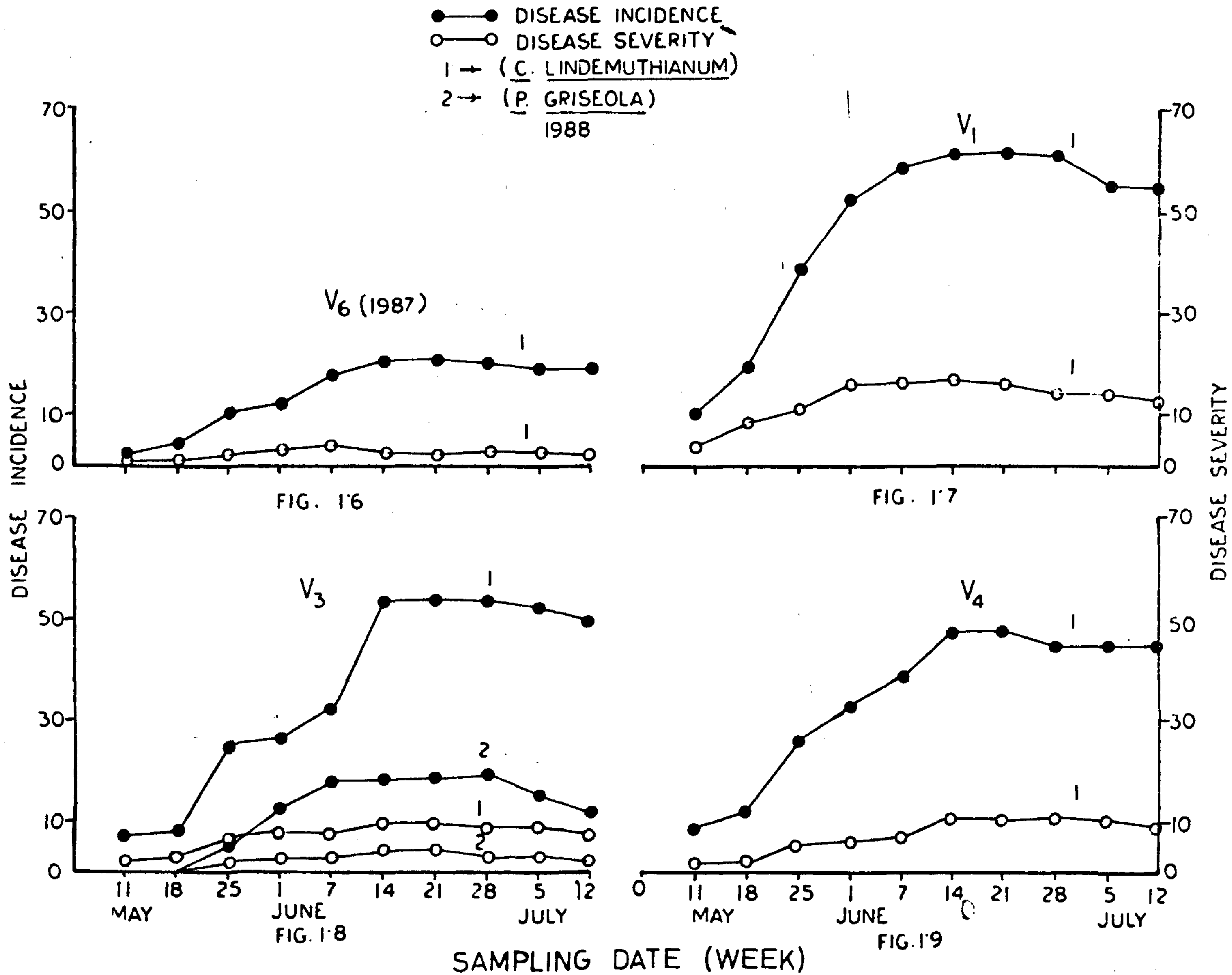

DISEASE INCIDENCE
DISEASE SEVERITY
1 → (C. LINDEMUTHIANUM)
2 → (P. GRISEOLA)
1987
V1
V3
V4
V5
FIG. 1·2
FIG. 1·3
FIG. 1·4
FIG. 1·5
DISEASE INCIDENCE
DISEASE SEVERITY
MAY
JUNE
JULY
SAMPLING DATE (WEEK)

Fig.1.6 Showing disease incidence and disease severity caused by C. lindemuthianum(1) in variety - V_6 for the year 1987.

Fig.1.7 Showing disease incidence and disease severity caused by Colletotrichum lindemuthianum(1) in variety - V_1.

Fig.1.8 Showing disease incidence and disease severity caused by Colletotrichum lindemuthianum(1) and Phaeoisariopsis griseola(2) in variety - V_3

Fig.1.9 Showing disease incidence and disease severity caused by Colletotrichum lindemuthianum(1) in variety - V_4.



DISEASE INCIDENCE
DISEASE SEVERITY
1 → (C. LINDEMUTHIANUM)
2 → (P. GRISEOLA)
1988
V6 (1987)
V1
V3
V4
FIG. 1·6
FIG. 1·7
DISEASE INCIDENCE
DISEASE SEVERITY
11 18 25 1 7 14 21 28 5 12
MAY
JUNE
JULY
FIG. 1·8
FIG. 1·9
SAMPLING DATE (WEEK)

LAY OUT PLAN (A PART) FOR 1987

DESIGN: R.B.D

TREATMENTS: BEAN VARIETIES (V_1 V_3 V_4 V_5 V_6)

REPLICATION: 20

PLOT SIZE: 1m × 0·6m

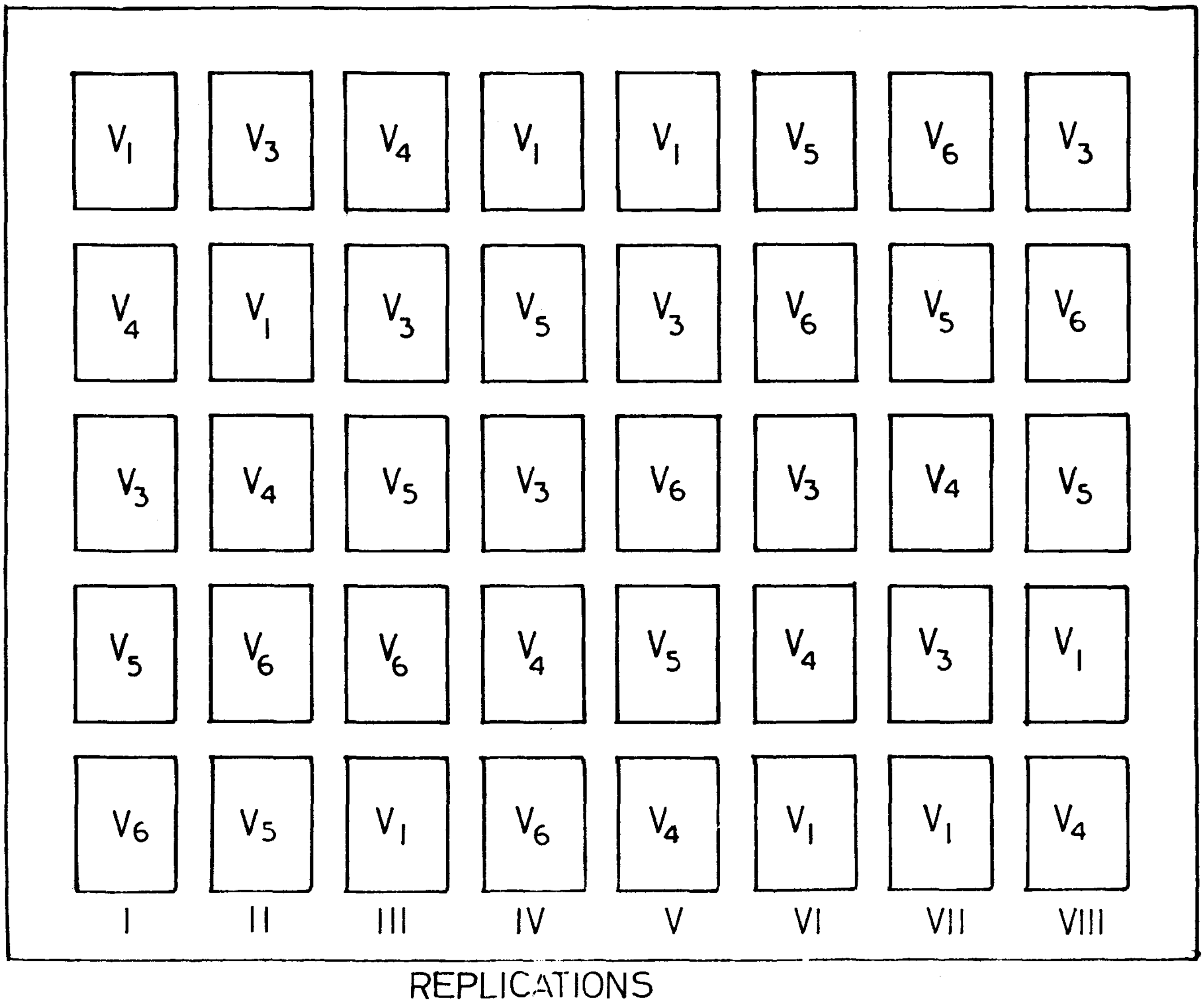


FIG. 1·10

LAY OUT PLAN (A PART) FOR 1988

DESIGN: R.B.D

TREATMENTS: BEAN VARIETIES (V_1 V_3 V_4)

REPLICATION: 40

PLOT SIZE: 1m x 0·6m

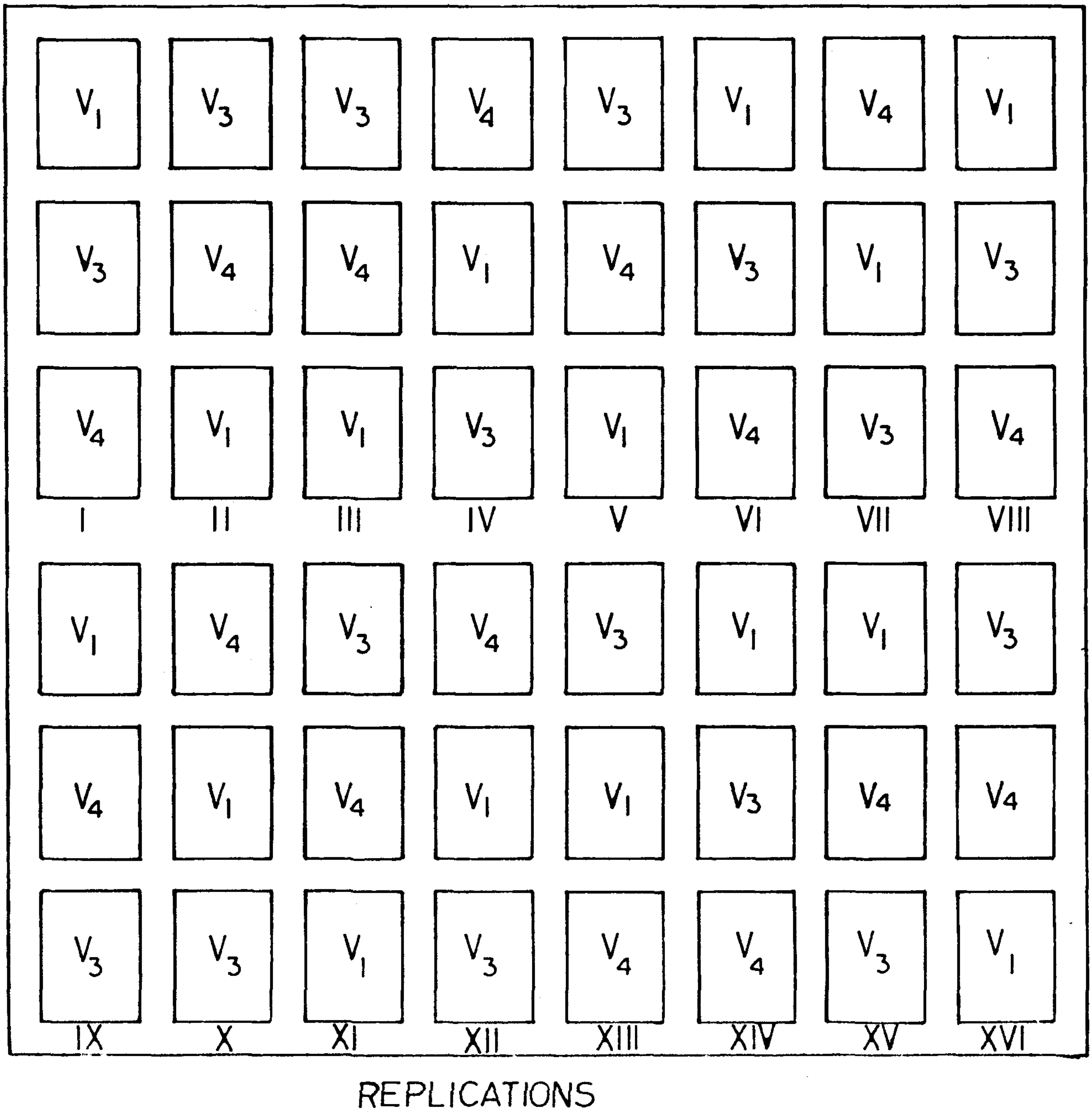


FIG. 1·11

(Sohi and Sharma, 1974). However, variation of leaf spot symptom of the disease could be due to the outbreak of new and highly virulant form of P. griseola. Similar observation of circular and symmetrical leaf spot caused by virulent form of P. griseola was reported by Hocking (1967) from Tanzania.

C. lindemuthianum appeared soon after the cotyledonary stage. This could be due to its seed borne nature, as well as the remains of infected plant debris (Walker, 1969). Occurrence of P. griseola bit late in the season might be due to the survival of the pathogen in winter in plant debris as dormant mycelium that becomes active in the favourable season causing primary infection on the leaves near the ground surface (Cardona and Walker, 1956). Survival of mycelium and spores of P. griseola in seed, plant debris and in the soil during the off season also could be the potential source of primary infection of angular leaf spot (Sindhan and Bose, 1979).

The micrometeorological data and disease incidence of P. griseola was in broad agreement with the findings of Cardona and Walker (1956) who have reported that the most favourable climatic conditions for epidemic development of the disease include, moderate temperature, high humidity alternating with periods of low humidity and wind action.

In case of C. lindemuthianum, the lesions appearing on the cotyledons serve as the source of secondary inoculum when the secondary cycle of the disease starts due to the splash conidia from the affected parts as a result of which disease developed rapidly and reached epiphytotic stage. Gregory et al (1959) observed that the local dispersal of C. lindemuthianum conidia from the inoculum source depends on rain splashes. Our micrometeorological data and disease development of C. lindemuthianum is also in broad agreement with the observations of Sindhan (1983). The slight variation in the present investigation could be due to the topographical and altitudinal variation as well as the varietal differences in their susceptibility to the pathogen. Van Arsdel (1965) also observed the changes in disease incidence on plants due to the microclimatic variations of a place.

The more or less sigmoid shaped disease progress curves observed in the present investigation was in agreement with that of Vander plank (1963) who defined epidemic as an increase of disease with time and advocated that sigmoid shaped disease progress curve could result when graphical method is used to plot the amount of disease against time on an arithmatical scale.

The rapid progress of the disease incidence and severity of C. lindemuthianum and P. griseola could be due to the fulfilment of all four essential factors for disease development (Epiphytotics) i.e. susceptible host, virulent pathogen, congenial environmental factors and date of sowing (a human factor). This was further supported by the significant correlation between disease incidence and environmental factors in the most susceptible host of C. lindemuthianum that indicate the existence of relationship between disease incidence and environmental factors. However, multiple linear regression and quadratic regression analysis showed that the variation in disease incidence and severity of C. lindemuthianum in French bean due to environmental factors could not be adequately described by the multiple linear and quadratic equations. Similar use of regression model for predicting certain Maydis blight epidemic was used by Zhou et al (1981). However, no such effort was made in French bean by previous workers but the effect of the percentage of introduced primary inoculum on the disease progress in two season and two locality using multiple regression analysis with dummy variable by Fernandez et al (1987) showed that epidemic of anthracnose initiated from introduced primary inoculum provided by infected seed largely depended on the subsequent rain pattern.

The larger coefficient of determination (R^2) value for quadratic equation indicates a better fit compared to the linear method. Though R^2 was statistically insignificant in both the methods used the size of the R^2 determines how useful the regression equation is. Kwanchai and Gomez (1984) made remark on the size of R^2 and pointed out that significance and insignificance of R^2 does not make any difference as long as the size of the R^2 value is larger. Hence, if the value of R^2 is low and even if the F-test on R^2 is significant the estimated regression equation may not be meaningful. Obviously the larger the R^2 value is, the more important the regression equation is in characterizing the dependent variable. Therefore, the computed R^2 values of quadratic regression equation indicated that 5.80% to 56.05% and 38.18% to 52.72% of total variation in the mean disease incidence and severity due to the variation of temperature, relative humidity and rainfall could be explained by the quadratic regression equation.

Significant correlation between disease incidence (X) and severity (Y) and their high R^2 indicated that disease severity (Y) could be fitted as linear function of the disease incidence (X) in C. lindemuthianum. James and Shih (1973) observed the relationship between disease incidence and severity of powdery mildew and fitted a simple linear regression which could adequately estimate the severity for incidence value of 65% or below. However, insignificant correlation coefficient and low R^2 in P. griseola could be due to its more virulent nature and freak outbreak of the disease (Hocking, 1967).

CHAPTER - II

SCREENING OF SEEDS FOR FUNGAL ASSOCIATION

INTRODUCTION

In India, French bean (Phaseolus vulgaris L.) is an important vegetable as well as pulse crop. The crop suffers due to a number of diseases few of which are seed borne. Seeds play a vital role for the healthy production of a crop. They are known to carry several pathogenic as well as saprophytic fungi which cause considerable damage either directly to the seeds or to the crops.

Colletotrichum lindemuthianum (sacc. and Magn.) Br. and Cav., an important seed-borne pathogen of French bean, serves as a limiting factor in vegetable cultivation. Considerable progress was made in Western countries with regard to the research on the seed-borne diseases of vegetable crop but in India, little attention has been paid to the diseases of vegetable seeds, particularly to the micro-organisms carried with the seeds and their significance in causing plant diseases (Suryanarayan and Bhombe, 1961). Good quality of seed is one of the basic and most important input for a successful vegetable seed industry which in India is not well organized and thoroughly exploited. Only recently more attention is being directed towards improvement of vegetable seeds in the country with a view to increase production and build up seed industry. The potentially adopted method for vegetable production is obviously the use of improved seeds. This may be achieved by building up of stocks of superior quality of seeds which in turn becomes possible if the seeds are free from disease and pests. Thus for laying down the health standard against seed-borne diseases of vegetable seeds, considerable background is very much essential with regard to the pathogenic as well as saprophytic fungi associated with seeds, their role if any, on the disease outbreaks and the nature and extent of damage they cause. Recently Lokhande *et al* (1986) have isolated various fungi including the seed-borne pathogen *C. lindemuthianum* from French bean. Suryanarayana and Bhombe (1961) also isolated *C. lindemuthianum* from the seed. There is a paucity of information on this aspect in general and in French bean in particular and therefore, the present investigation was carried out in order to conduct a survey of seed-borne microflora of five locally cultivated French bean varieties.

MATERIALS AND METHODS

Experimental materials

Five varieties of locally cultivated French bean

(Phaseolus vulgaris L.) seeds viz; Shillong local selection (V_1), Manipur variety (V_3), Premier variety (V_4), Local variety (V_5) and Masterpiece (V_6) were selected for the survey of seed mycoflora.

Collection of samples

All samples were collected from the local market and got identified at the vegetable research station, Shillong. Sufficient seed samples were collected at a time in bulk for the whole period of investigation. While drawing samples from the bulk storage, International Rules for Seed Testing Association (ISTA, 1966), were followed. The composite samples drawn from the top, middle, bottom and side of bulk storage were collected in sterile polythene bags and brought to the laboratory. Care was taken to avoid contamination during sampling by hands and other instruments which were sterilized with 90% alcohol.

Nutrient media used for isolation of fungi

The following nutrient media were used for the whole period of investigation:

(1) Potato dextrose Agar (PDA): Peeled and sliced potato - 250.0g; Dextrose - 20.0g; Agar agar - 20.0g; Distilled water - 1000.0ml.

(2) Tap Water Agar: Agar - 15.0g; Distilled water - 1000.0ml.

Sterilisation of the media

The culture tubes and flasks containing PDA and water agar media were sterilized in autoclave for 20 minutes at 15 Psi pressure. The tubes were slated in slanting board to make slants. After solidification the slants and the flasks were stored in refrigerator till they were used.

Survey of seed borne mycoflora

Survey of seed borne mycoflora on five varieties of French bean (Phaseolus vulgaris L.) was conducted seasonally for two consecutive years 1987 and 1988 using the following two methods:

(1) Standard Blotter Method: Standard blotter method as recommended by Internation Seed Testing Association (ISTA, 1966) with certain modification was used for detection of fungi. Three pieces of sterile moistened filter papers in sterile petridish (9.50cm dia.) containing 10 equispaced seeds in each petridish were incubated at 25±1°C for seven days. One hundred randomly selected seeds were taken from each variety. Plating of medium was carried out in a Laminar Flow Chamber.

(2) Agar Plate Method: The agar plate method as proposed by the International Seed Testing Association (1966) was followed. Ten out of hundred randomly selected seeds of each variety were equispaced using sterile forcep in a petriplate (9.50cm dia.) containing potato dextrose agar (20ml) and incubated at 25±1°C for five days.

Identification and Purification of fungal isolates

Fungi isolated from seeds were grown in pure culture on PDA slants. Identification was done in pure culture following strictly the keys and manuals provided by different workers (Subramaniam, 1971; Barnett and Hunter, 1972).

why don't tap water agar?

Interpretation of result

The data collected during the experiment were expressed as an average of isolation for two years of survey. The percentage frequency of occurrence of individual fungus was calculated using the formula:

$$\text{Percentage of frequency occurrence} = \frac{\text{Total number of individual species of fungus}}{\text{Total number of all the species of the fungi}} \times 100$$

RESULTS

Comparison between isolation techniques

Forty four species of fungi belonging to 33 genera were isolated from five varieties of bean seeds using two techniques. More number of fungi was detected in blotter test than the agar plate method (fig.2.1). Blotter test could detect 37 spp. belonging to 32 genera whereas agar plate method detected only 24 genera comprising of 31 spp. (Table 2.1). However, statistical test showed no significance difference between the two techniques in the isolation of total fungi.

Seasonal and varietal variation of seed-borne mycoflora

Seasonal as well as varietal variation of seed-borne mycoflora was observed in both the techniques. Table 2.2 showed that 33 spp. belonging to 24 genera in summer, 29 spp. of 24 genera in rainy and 21 spp. belonging to 15 genera were recorded in winter season by the two techniques from all the varieties (fig.2.2). Similarly seasonal variation was also observed between the two techniques. Maximum number of fungi was detected in summer followed by rainy and least in winter season. Blotter method was superior over agar plate method in all the seasons (fig.2.3). Isolation of total number of fungal species showed a decreasing trend from summer to winter season (Fig.2.2 and 2.3).

'F'-test of the analysis of variance showed that the total number of fungal species isolated by blotter

Table 2.1 Showing the comparison of two techniques used for isolatior of seed mycoflora of French bean.

Fungi	Agar Plate	Blotter Method
Absidia sp.	+	+
Acremonium cerealis	-	+
Alternaria alternata	+	-
A. tenuis	+	+
Aspergillus alutaceus	+	+
A. fumigatus	+	+
A. flavus	+	+
A. niger	+	-
Botryosporium sp.	-	+
Candida albicans	+	+
Chaetomella atra	-	+
Colletotrichum lindemuthianum	+	+
Coniothyrium sp.	-	+
Cunninghamella elegans	+	+
Curvularia lunata	+	-
Dendrophion nonum	+	-
Fusarium oxysporum	+	+
F. semitectum	-	+
F. verticilloides	-	+
Gliocladium sp.	+	+
Heterocephalum sp.	+	-

Table 2.1 contd..

Fungi	Agar Plate	Blotter Method
Humicola fuscoatra	+	+
Leptosphaeria maculans	+	+
Mucor racemosus	+	+
Myrothecium roridum	-	+
Oedocephalum lineatum	+	+
Penicillium brefeldianum	+	+
P. expansum	+	+
P. lanosum	+	-
P. nigricans	+	+
Paecelomyces variotii	+	+
Periconia sp.	-	+
Pestalotia sp.	+	+
Phomopsis vexans	-	+
Pseudostemophillum sp.	+	+
Rhizopus oryzae	+	+
Sclerotium sp.	+	-
Scopuloriopsis brymptii	+	+
Sphaeceloma sp.	-	+
Sterile mycelia (brown)	-	+
Sterile mycelia (white)	+	+
Trichochladium asperm	+	+
Verticillium albo-atrum	-	+
V. chlamydosporum	-	+

+ = Presence of fungi; - = Absence of fungi.

Table 2.2 Showing the seasonal incidence of fungi on five varieties of French bean seeds isolated by two techniques (Average of two years observation)

Fungi	Summer										Rainy										Winter									
	V_1		V_3		V_4		V_5		V_6		V_1		V_3		V_4		V_5		V_6		V_1		V_3		V_4		V_5		V_6	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Absidia sp								+		+																				
Acremonium cerealis										+																				
Alternaria alternata					+											+														
A. tenuis	+	+				+					+	+					+				+	+						+	+	
Aspergillus alutaccus	+	+		+			+			+	+	+	+	+	+	+			+	+	+		+		+					
A. flavus	+	+	+	+	+	+	+	+	+	+		+	+	+				+		+	+	+		+			+		+	
A. fumigatus		+		+			+																				+			
A. niger	+				+			+	+	+		+	+		+	+		+		+						+		+	+	
Botryosporium sp														+																
Candida albicans															+							+						+		+
Chaetomela atra												+																		
Colletotrichum lindemuthianum	+	+			+		+	+	+						+		+	+	+								+			
Caniothyrium sp														+																
Cunninghamella elegans			+	+			+	+	+					+		+			+			+							+	
Curvularia lunata							+		+																					
Dendrophion nonum			+																											
Fusarium oxysporum			+	+		+			+			+							+									+	+	
F. semitectum						+																								
F. verticilloides						+																								
Gliocladium sp																+	+													
Heterocephalum auriaurum											+																			
Humicola fuscoatra							+	+																						
Leptosphaeria maculans	+	+				+																								
Mucor racemosus			+	+			+						+		+		+		+											
Myrothecium roridum		+				+												+		+										
Oedocephalum lineatum									+				+									+								
Paecelomyces variotii					+			+	+	+																				
Penicillium brefeldianum						+			+	+	+						+								+					
P. expansum							+	+		+		+			+		+		+		+	+	+	+				+		+
P. lanosum							+						+	+	+	+		+	+	+		+		+		+	+		+	+
P. nigricans	+	+			+		+		+			+									+							+		
Periconia sp								+							+	+	+													
Pestalotia sp		+				+																								+
Phomopsis vexans						+								+																+
Pseudostemophillum sp					+			+														+		+						+
Rhizopus orysae																+														
Sclerotium sp												+								+					+	+				
Scopulariopsis brymartii	+							+											+		+		+							
Sphaceloma sp																														
Sterile mycelia (brown)																	+			+										
Sterile mycelia (white)																														
Trichochladium asperm											+				+						+		+			+		+		
Verticillium albo-atrum			+				+			+																				+
Verticillium chlamydosporum			+							+																				

A = Agar plate, B = Blotter method, + = Presence of fungi test was significantly different between seasons but not between varieties (Table 2.4). Critical difference (CD at 5% = 2.583) showed that the significant difference was between summer and winter but not between summer-rainy and rainy-water season. However, no significant difference was observed between season as well as between varieties for total number of fungal species isolated by agar plate method. Thus, the various fungal species detected in different seasons by two techniques may be categorised into the following two categories:

(i) Category 'A' - Fungi occurring in all the seasons

(ii) Category 'B' - Fungi detected only in a particular season.

Maximum number of fungi belong to the first category (A) which consists of 14 spp. belonging to 10 genera. Dominant species were Penicillium sp., Aspergillus sp., and Fusarium sp. Fungi detected either in one or two seasons were Acremonium cerealis, F. semitectum, F. verticilloides, Verticillium albo-atrum and V. chlamydosporum.

Trichocladium asperm, V. albo-atrum, V. chlamydosporum, Leptosphaeria maculans, Humicola fuscoatra and Dendrophion nonum were recorded only in summer season. Sphaeceloma sp., Gliocladium sp., and Heterocephalum aurianrum were restricted to rainy season whereas fungi detected in winter season were common to other two seasons.

Variation of total number of fungal species among five varieties showed that maximum number of fungi was isolated from V_6 followed by V_1, V_5, V_4 and least in V_3 by both the techniques when all the seasons were considered (Fig.2.4).

Varietal variation was also exhibited by each of the two methods of isolation in three different seasons. Mostly, fungi were detected more in all varieties during summer season and least in winter season by both the methods. This variation among varieties was more clearly depicted together with season and technique (fig. 2.5 and 2.6).

Based on their frequency of occurrence on different varieties, the fungal species may be classified into the following three groups:

(i) fungi occurring commonly in all varieties
(ii) fungi restricted to only one variety
(iii) fungi that occurred sporadically in various varieties.

Aspergillus sp., Penicillium sp., sterile mycelia, Cunninghamella elegans, Fusarium oxysporum, Phomopsis vexans were commonly detected in all the five varieties. Chaetomella atra and H. aurianrum were found only in V_1 during rainy season. Scopuloriopsis brymptii was detected only in V_1 and V_5. Humicola fuscoatra and Curvularia lunata were observed in V_5 only. Similarly, Alternaria alternata, F. semitectum, F. verticilloides were found to occur in V_4 whereas Botryosporium sp. was recorded only in V_3.

Fungal species such as A. alternata, A. tenuis, Aspergillus fumigatus, Candida albicans, H. fuscoatra, Myrothecium roridum, Oedocephalum lineatum, Penicillium spp., Periconia sp., Pestalotia sp., S. brymptii, Sphaeceloma sp., Trichocladium asperm, V. albo-atrum and V. chlamydosporum were quite infrequent and sporadic in their occurrence on different varieties under different seasons. Colletotrichum lindemuthianum was detected in all varieties except in V_3.

Table-2.3 showed the percentage frequency occurrence of different fungi on various varieties during the three seasons. Frequency occurrence of fungi was maximum in summer followed by rainy and least in winter.

Aspergillus alutaceous, A. flavus, Penicillium expansum, P. lanosum were detected with higher frequency whereas Absidia sp., A. cerealis, C. atra, F. semitectum, T. asperm, C. lunata occurred with low frequency. The frequency of C. lindemuthianum was more during summer

Table 2.3 Showing the seasonal percentage frequency occurrence of different fungi on five varieties of French bean seeds isolated by Agar Plate and Blotter technique (Average of two years observations)

Fungi	Summer										Rainy										Winter									
	V_1		V_3		V_4		V_5		V_6		V_1		V_3		V_4		V_5		V_6		V_1		V_3		V_4		V_5		V_6	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Absidia sp.								5.66		11.11					14.28															
Acremonium cerealis										9.25																				
Alternaria alternata					14.28											6.94														
A. tenuis	8.69	13.36				8.82					12.90	6.66					11.90				17.24	10.59						9.09	18.79	
Aspergillus alutaceus	13.04	6.81		17.14			14.14			11.11	22.58	13.33	17.14	8.57	14.28	25.00			13.33	18.91	17.24		20.00		40.00					
A. fumigatus		13.63		8.57			9.42																				26.66			
A. flavus	21.73	15.90	35.71	14.28	20.00	20.58	10.99	9.43	12.19	9.25		13.33	20.00	20.00				21.21		18.91	17.24	7.89		33.33			20.00		18.79	
A. niger	13.04				20.00			5.66	9.75	12.96		13.33	14.28		6.12	20.83		15.15		13.51						13.25		9.09	11.27	
B. cyosporium sp.														8.57																
Candida albicans															12.24							7.89						18.18		16.08
Chaetomella atra												8.88																		
Colletotrichum lindemuthianum	13.04	13.36			14.28		4.72	13.32	4.87						6.12		14.28	9.09	6.06								13.33			
Coniothyrium sp.														20.00																
Cunninghamella elegans			7.14	14.28			4.72	5.66	14.63					8.57		6.94			20.00			15.78							11.27	
Curvularia lunata							7.85		7.31																					
Dendryphion nanum			14.28																											
Fusarium oxysporum			21.48	8.57		8.82			7.31			15.55							13.33									15.15	18.79	
F. semitectum						8.82																								
F. verticillioides						14.70																								
Gliocladium sp.																11.11	11.90													
Helrocephalum aurianrum											19.35																			
Humicola fuscoatra							7.85	9.43																						
Leptosphaeria maculans	4.34	6.81				11.76																								
Mucor racemosus			21.42	14.28			4.72						14.28		10.20		11.90		11.11											
Myrothecium roridum		13.36				5.88												15.15		13.51		18.42								
Oedocephalum lineatum									9.75				20.00																	
Penicillium brefeldianum						8.82			7.31	12.96	29.03						19.04								30.00					
P. auranum							9.42	9.43		11.11		11.11			10.20		14.28		11.11		10.34	18.42	20.00	22.22				21.21		16.09
P. lanosum							10.99						14.28	20.00	14.28	16.66		21.21	13.13	16.21		13.15		27.77		18.75	40.00		9.77	16.59
P. africans	21.73	13.36			14.28		7.85		7.31			15.55									17.24							18.18		
Paecilomyces varioti					8.57			9.43	7.31	9.25																				
Periconia sp.								13.32								5.55	16.16													10.13
Pestalotia sp.		13.36				2.94			12.19																					10.13
Phomopsis vexans						8.82								14.28								7.89		16.66						10.13
Pseudostemphylium sp.					8.57			9.43								6.94									30.00	31.25				
Rhizopus oryzae												2.22								10.81	10.34		40.00							
Sclerotium sp.																			11.11											
Scopulariopsis brumptii	4.34							13.20																						
Sphaceloma sp.																		18.18		8.10										
Sterile mycelia (brown)																														
Sterile mycelia (white)											16.12				12.24											18.75		9.09		
Trichocladium asperum							7.85			5.55											10.34		20.00							
Verticillium albo-atrum				14.28																										20.27
V. chlamydosporium				8.57						7.40																				

A = Agar plate method

B = Blotter method

Table 2.4 Two way analysis of variance for total number of fungal species isolated by Blotter method from different varieties at different seasons.

Sources of variation	Degrees of Freedom (d.f.)	Sum of squares (SS)	Mean sum of Square (MS)	Computed F-value
Between seasons	2	32.934	16.467	8.745**
Between Varieties	4	9.334	2.333	1.238^{ns}
Error	8	15.066	1.883	
Total	14			

C.D. at 5% = 2.583

** = Significant at 1% probability level.

ns = not significant

and rainy seasons than the winter.

Average percentage frequency occurrence of fungi, such as A. tenuis, A. flavus, C. lindemuthianum, C. elegans, F. oxysporum and P. lanosum showed that A. flavus occurred with higher percentage frequency in all the varieties during summer whereas the other fungi ennumerated above showed fluctuation in their dominance in a particular season. P. lanosum and A. flavus appeared with high average percentage frequency in winter on variety (V_5) and in rainy season on variety (V_3) respectively (Fig.2.7).

DISCUSSION

Of the two methods employed, blotter test was found to be the most reliable than the agar plate method which was in agreement with the findings of many other workers (Agarwal et al., 1972; Singh et al., 1973). While comparing four laboratory methods for detection of carrot seed mycoflora, de Tempe (1964) obtained more reliable result from the blotter test experiment and consequently recommended the blotter test as being reliable, simple and cheap. However, Gill et al (1983) observed that PDA Plate method was better than the blotter test. In the present investigation Aspergillus sp., Penicillium sp.,

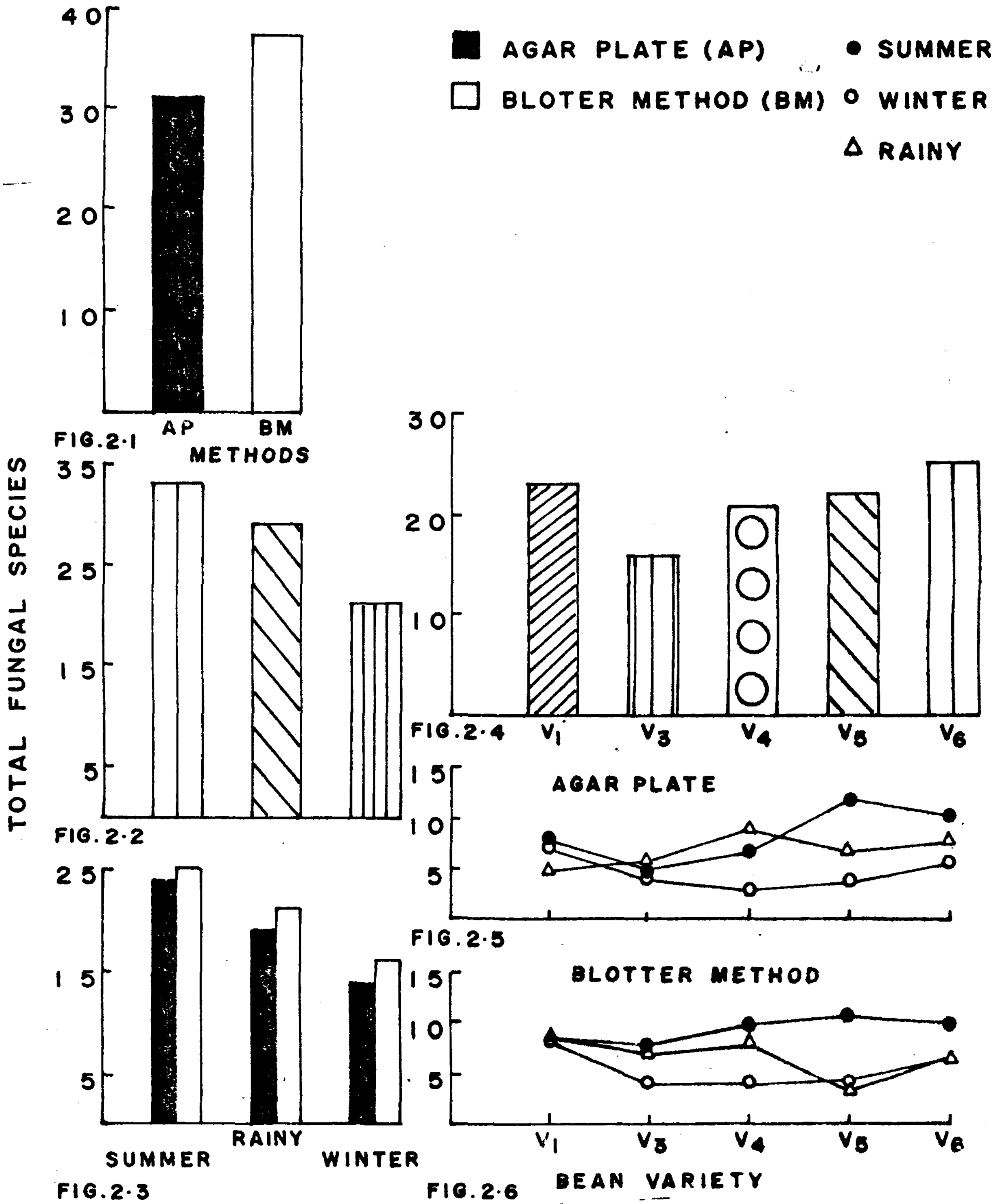


FIG.2·1

FIG.2·2

FIG.2·3

FIG.2·4

FIG.2·5

FIG.2·6

Fig.2.7 Showing average percentage frequency occurrence of the following fungal species isolated from five different varieties of French bean seeds during different seasons.

(1) *Alternaria tenuis*

(2) *Aspergillus flavus*

(3) *Colletotrichum lindemuthianum*

(4) *Cunninghamella elegans*

(5) *Fusarium oxysporum*

(6) *Penicillium lanosum*

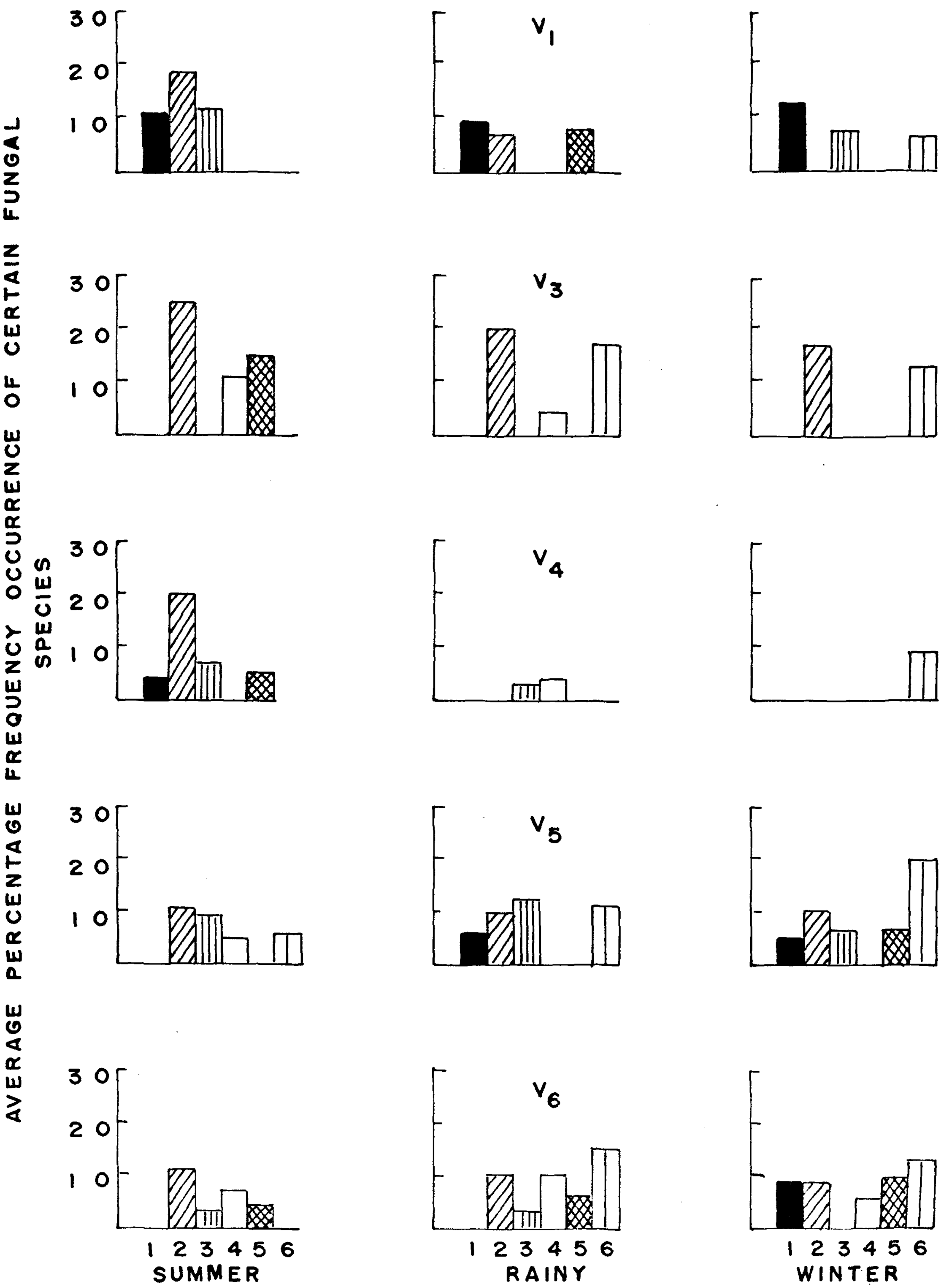

AVERAGE PERCENTAGE FREQUENCY OCCURRENCE OF CERTAIN FUNGAL SPECIES
V1
V3
V4
V5
V6
30
20
10
1 2 3 4 5 6
SUMMER
RAINY
WINTER

FIG. 2·7

Fusarium sp., Mucor sp., and Rhizopus sp., were frequently observed in agar plate which could be due to their fast growing nature resulting in suppression of other slow growing ones. Aulakh et al (1976) concluded that agar test gives erratic observations because of the chance of contamination and over growth by fast growing fungi. While studying the comparative method of seed health testing, Neeragard and Saad (1962) commented that the blotter and agar plate methods are both equally valuable and supplementary to each other. Further, both agar plate and blotter methods have been selected as standard procedures for seed health testing by International Seed Testing Association (ISTA, 1966).

The commonly observed fungi such as Absidia sp., Alternaria sp., Penicillium sp., Aspergillus sp., Fusarium sp., Paecelomyces sp., and C. lindemuthianum were in accordance with the findings of Lokhande et al (1986) on French bean. They have reported similar composition of saprophytic and pathogenic fungi on seeds of this pulse crop. Similarly, Suryanarayana and Bhombe (1961) reported the isolation of Colletotrichum sp., from French bean seeds on PDA plate.

In the present investigation, seasonal variation of seed-borne mycoflora was observed during summer, rainy and winter season. Seasonal changes were statistically significant. A gradual decline of fungi both in taxonomic composition as well as in numerical strength was observed as the storage period increased from summer to winter. Succession of mycoflora could be due to their different role in deteriorating the quality of seeds favoured by different seasons as the storage length increased. Bhikane et al (1982) observed variation in species content and intensity of fungal attack due to the influence of temperature and other conditions. Gradual decline of fungi as observed in the present investigation could possibly be due to storage of seed bulk sample in the laboratory condition. Similarly, Neergard and Saad (1962) pointed out that storage for a few months under laboratory conditions may change the composition of seed-borne fungal flora considerably. As evident from the tabulated result, few species of fungi were restricted to a particular season of the year which might be because of their greater sensitivity and adaptibility to storage condition of a particular season. The other category of fungi occur in all seasons independently. This could possibly be because of their being versatile in their food requirements and tolerance capacity to varying environmental conditions of the year. Fungi of rare occurrence reflect their more demanding nature for nutrients and environmental conditions as suggested by Bilgrami et al (1976) whereas the frequently occurring species or absence of some other species of fungi might be due to unfavourable storage conditions or lack of competing capacity with other fungi (Campbell, 1962).

Qualitative variation of fungi was noticed in different varieties during different seasons of the year. Such variation in commonly grown French bean cultivars was also noticed by Lokhande et al (1986) in different French bean graded seeds. They found 14 spp., belonging to 10 genera by blotter and agar plate method. In general, fungi were detected more frequently in variety (V_6) and variety (V_1) than the other three varieties. Qualitative and quantitative differences of fungi between varieties could possibly be attributed to their physico-chemical nature of the seeds, agricultural operation, storage, climatological conditions of the crop growing site, during harvesting and sampling occasions as pointed out by Neergard (1977).

The pathogen C. lindemuthianum of French bean was detected in all the varieties except variety V_3 by both agar plate and blotter test in summer and rainy seasons. Possibly this could also be due to Physico-chemical nature of the seed and varietal differences in their susceptibility to the seed-borne pathogen. Frequent detection of *C. lindemuthianum* by the two methods mentioned above might be because of the survival of dormant mycelium in the infected seed testa and cotyledons (Robert and Bothyroyd, 1972).

CHAPTER - III

ASSESSMENT AND ANALYSIS OF PHYLLOSPHERE FUNGAL POPULATION

INTRODUCTION

Plant surfaces are colonized by a heterogeneous population of micro-organisms. Definite microbial community which harbour leaf surface has been termed as phylloplane/ phyllosphere analogous to rhizosphere/rhizoplane of roots (Kerling, 1964).

Some of the surface colonizers are responsible for disease development and few others are saprophytic (Last, 1955a; Kerling 1964; Sinha, 1965; Dickinson, 1971). A considerable interest has been developed in investigating the ecology of leaf surface micro-organisms. Some factors such as host species, leaf maturity and weather may play an important role in the distribution of fungi on leaf surface both qualitatively and quantitatively (Rao and Monoharchari, 1981). However, only few workers (Sharma and Sinha, 1972; Sharma and Gupta, 1979, 1984, 1985; Kumar and Gupta, 1976) have studied the changes in the phyllosphere microflora of crop plants influenced by varietal characters.

The fungi on the leaf surface have been investigated by a variety of techniques. The merits and demerits of cultural techniques commonly used in phyllosphere studies have been reviewed by Dickinson (1971) who emphasized the use of suitable combination of various cultural methods along with the direct or indirect microscopic examination of leaf surface.

Techniques such as leaf impression peels, scanning electron microscopy and cultural techniques have been used to investigate the fungi inhabiting leaves (Mishra and Dickinson, 1981). Relatively little work has been done to understand the ecology of micro-organisms of annual crops within their plant surface using suitable combination of various techniques. Therefore, the present investigation was taken up to understand the ecology, nature, composition and the extent of variation of leaf surface microflora both in quality and quantity among five french bean varieties influenced by the growth stage of the plant using different techniques including scanning electron microscope.

MATERIALS AND METHODS

Experimental materials

Five local varieties of French bean viz. Shillong Local Selection (V_1), Manipur variety (V_3), Premier variety (V_4), Local variety (V_5) and Masterpiece (V_6) were sown in the experimental garden plot of the Botany Department of the University for two years 1987 and 1988. These varieties were used as the experimental material throughout the course of the present investigation. Variety V_1, V_3 and V_5 were pole type or climbers whereas V_4 and V_6 were dwarf/ bushy in habit.

Field sampling

Composite samples of old as well as young leaves were collected randomly in sterile polythene bags with the help of sterilized scissor and forcep from the field at fortnightly interval just after 15th day of germination (table 3.1).

Isolation of fungi

The following techniques were used to examine the leaf surface mycoflora:

(1) Moist Chamber (Keyworth, 1951): Three leaves of each variety were kept in each moist chamber prepared by moisting 3 to 4 sterile filter papers kept in a petriplate by sterile distilled water. The prepared plates were incubated at 25±1°C and observation taken at the interval of 5 days till one month. Five replicates were maintained for each variety.

(2) Impression Plate (Potter, 1910): In this technique

Table 3.1 Showing the sampling dates and ages of the plants: sowing date - 20 April 1987/88; Germination date - 30 April 1987/88.

Sampling dates	Age of the plant	Growth stages of the plant
15 May 1987/88	15 days	Seedling stage
30 May 1987/88	30 days	Young stage
14 June 1987/88	45 days	Pre-flowering stage
29 June, 1987/88	60 days	Flowering stage
14 July 1987/88	75 days	Podding stage
29 July 1987/88	90 days	Senescent stage

leaves of different varieties were gently pressed asceptically using sterilized forcep in separate petriplates containing Czapek's Dox Agar and leaves were subsequently removed leaving the impression. Observations were taken by counting the number of colonies of each fungus after 5 days of incubation at 25±1°C. Five replicates were maintained for each variety.

(3) Washing leaf disk: In this technique, 50 leaf disks each of 5mm diameter of different varieties were cut from the leaves sampled with a sterilized cork borer and taken in 100.0ml of sterilized distilled water separately. 1.0ml of this aliquote was plated on Czapek's Dox Agar medium after shaking vigorously for 15 minutes for each variety separately. Observation on the incubated petriplates at 25±1°C were taken after five days of incubation by counting the number of colonies of different fungi in each plate. Five replicates were maintained for each variety.

(4) SEM observation of leaf disk (Mishra and Dickinson,1981): Leaf material was prepared for the scanning electron microscope (SEM) by cutting 1cm diam. disk from leaves, avoiding the mid rib and leaf margin. Preparations were made with either adaxial or abaxial surface of leaves upper most. The leaf pieces were stuck on the SEM specimen stubs with

Durofix adhesive, gold coated and they were examined at low magnification.

Nutrient media used for isolation of fungi

Following media were used at different occasions to achieve isolation of maximum number of fungi:

1. Potato Dextrose Agar: Peeled and sliced potato - 250.0g; Dextrose - 20.0g; Agar - 20.0g; Distilled water - 1000.0ml.

2. Czapek's Dox Agar (Raper and Thom, 1949): Agar - 15.0g; $NaNO_3$ - 3.0g; KH_2PO_4 - 1.0g; $MgSO_4$ $7H_2O$ - 0.5g; KCl - 0.5g; $FeSO_4$ $7H_2O$ - 10.0mg; Sucrose - 30.0g; Distilled water - 1000.0ml.

Isolation and identification of fungi

The incubated plates were regularly examined and the slow growing fungi were transferred to fresh nutrient plates to avoid over running by fast growing ones. Identification of fungi was done mostly in pure culture raised in slants containing PDA or Czapek's Dox Agar following the keys provided by different workers (Subramaniam, 1971; Barnett and Hunter, 1972).

1. Qualitative estimation

The qualitative estimations were expressed as percentage frequency of occurrence of each fungus (isolated by Leaf Impression Technique) which was calculated using the following formula:

$$\text{Percentage frequency of occurrence} = \frac{\text{Total number of individual species of fungus}}{\text{Total number of all the species of fungi}} \times 100$$

2. Quantitative estimation:

For quantitative estimations, the total number of fungal population as well as the population of different fungal components were computed in terms of population per square cm of the leaf surface. It is calculated by the formula (Sharma and Tiwari, 1981) with certain modification as given below:

$$\text{Total number of fungi per cm}^2 = \frac{\text{Total number of fungi in 100 ml}}{\text{Total area of leaf disks (=50xarea of leaf diskx2)}}$$

Physical analysis of sampled leaves

PH: 10g of fresh leaves were crushed in 25ml of double distilled water and filtrate was used for determining pH using electric pH meter.

Moisture: Moisture content of leaves was determined by drying 10g of fresh leaves in hot air oven at 105°C for 24 hours, cooled at room temperature and weighed to get dry weight expressed in terms of percentage dry weight.

Statistical analysis: The experimental data collected during the present investigation were statistically analysed using student's 't'-test and 'F'-test with critical differences (CD). The correlation coefficient (r) was worked out for fungal population of different varieties with pH and moisture content of leaves at the different growth stages of the plant.

RESULTS

Total 67 fungal species belonging to 52 genera were isolated by three techniques viz. Leaf impression (LI), Moist chamber (MC) and Washing leaf disk (WLD) from the five varieties of French bean (V_1, V_3, V_4, V_5 and V_6) at different growth stages of the plant for two cropping seasons (Table 3.2).

Scanning electron microscope (SEM) provided information mostly on the conidiophores and spores population at low magnification (Plate 3.1;1,2). Detailed surface structure at high magnification as an aid of identification becomes difficult because of the fact that actual structure more or less get shrivelled during the SEM coating process. In SEM observation the spores or conidial population of *Spiromyces* sp. and *Sporothrix* sp. were more in spite of

Table 3.2 Showing the comparison of three techniques used for isolation of Phyllosphere mycoflora of French bean.

Fungi	Techniques		
	LI	WLD	MC
Absidia sp.	-	-	+
Acremonium cerealis	+	-	+
Alternaria alternata	+	+	+
A. brassicicola	+	+	+
A. tenuis	+	+	-
Aspergillus flavus	+	+	+
A. niger	+	+	-
A. versicolor	-	+	+
Botrytis cinera	-	+	+
Candida albicans	+	+	+
Cephalosporium sp.	+	+	-
Chaetomium globosum	-	-	+
Chaetophoma confluens	-	+	-
Cladosporium cladosporoides	+	+	+
C. herbarum	-	-	+
Colletotrichum lindemuthianum	+	+	+
Curvularia lunata	+	+	+
Epicoccum nigrum	-	-	+
Endosporostible sp.	-	+	+
Erysiphae graminis	-	+	-
Fusarium moniliforme	+	+	+
F. oxysporum	+	+	+

Table 3.2 contd..

Fungi	Techniques		
	LI	WLD	MC
F. semitectum	+	+	+
Geotrichum sp.	+	+	-
Gliocladium deliquescens	+	+	-
G. penicilloides	+	+	+
Gliomastix muororum	-	+	-
Gloeosporium fructigenum	+	+	+
Haplosporangium parvum	+	+	+
Humicola grisea	-	+	+
Isariopsis griseola	-	-	+
Leptosphaeria maculans	+	+	-
Macrophomina phaseolina	-	-	+
Monilia fructigena	-	+	-
Mucor haemalis	+	+	+
Myrothecium roridum	+	+	+
Oedocephalum coprophilum	-	+	+
Oedodendron griseum	-	+	+
Paecelomyces variotii	+	-	+
Penicillium chrysogenum	+	+	+
P. funiculosum	+	+	+
P. granulatum	+	+	+
P. wortmanii	+	+	+
Periconia palludosa	+	+	+

Table 3.2 contd..

	Techniques		
	LI	WLD	MC
Pestalotia sp.	-	-	+
Phaeoseptoria sp.	+	-	-
Phoma glomerata	-	+	+
Phylosticta minima	-	+	-
Pseudostemophylium consortiale	+	+	-
Pyrenochaeta decipiens	-	-	+
Rhizoctonia solani	-	-	+
Rhizopus nigricans	+	+	+
Septocylindrium leucum	+	-	+
Sphacelia segetum	+	+	-
Spiromyces sp.	-	+	-
Sporobolomyces salmonicolor	+	+	-
Sporothrix sp.	+	+	+
Stemphyllium sp.	-	+	-
Sterile mycelia (brown)	+	+	+
Sterile mycelia (white)	+	+	+
Tieghemiomyces sp.	+	+	+
Torula herbarum	-	-	+
Trichothecium roseum	-	-	+
Verticillium albo-atrum	+	+	+
V. dahlae	+	+	-
Verticicladium trifidium	+	+	-
Xenosporium sp.	+	-	-

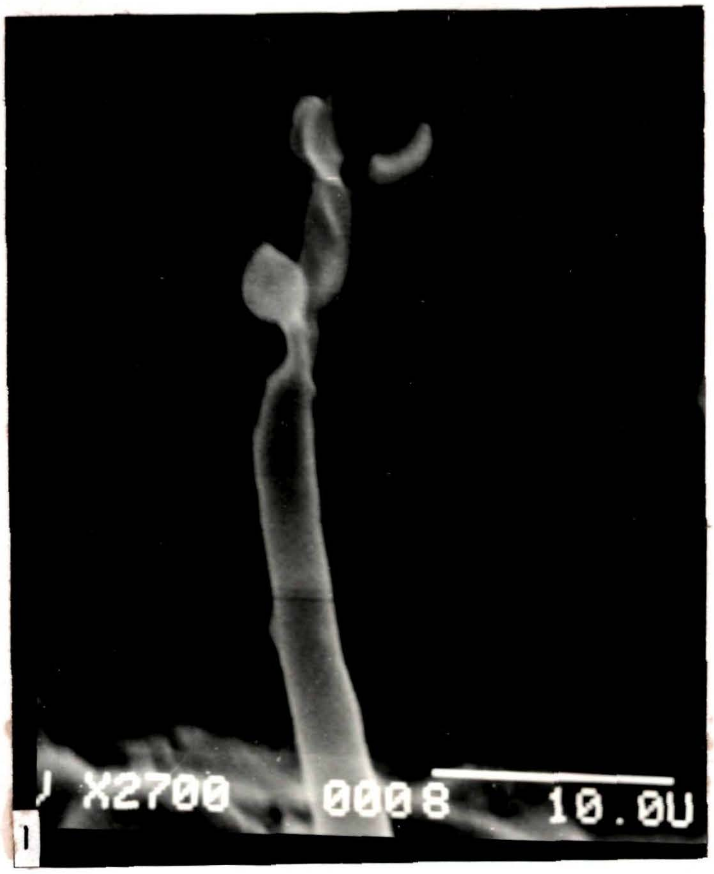
LI = Leaf impression; WLD = Washing leaf disk; MC = Moist chamber.

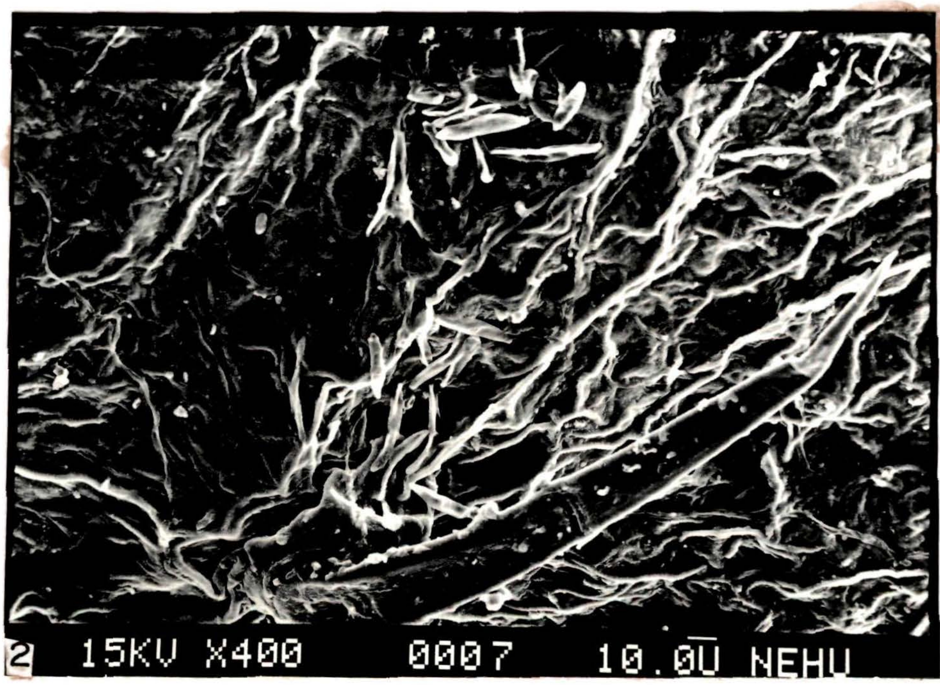
PLATE 3.1

1. A scanning electron micrograph showing conidiophore of Botrytis sp. with attached conidia X 2700.

2. A scanning electron micrograph showing conidia of Phaeoisariopsis griseola accumulated at the base of leaf hair x 400.

PLATE 3.1





their low presence in the cultural studies (Plate 3.2; 1, 2.). At the senescent stage, the spore population of Uromyces appendiculatus was more. (Plate 3.3; 1, 2.).

A comparative result of different fungal species recorded by three different techniques are presented (Table 3.2). It showed that maximum number of fungi (51 species) was recorded by washing leaf disk followed by moist chamber (47 species) and least (42 species) by leaf impression technique (Fig.3.1). Of these 26 species belonging to 19 genera were common to all the three techniques. They mostly belonged to Hyphomycetes and their frequency occurrence was maximum followed by Ascomycetes.

Table 3.2 to 3.7 showed that fungal colonisers on the leaf surface of five varieties of French bean belonged to Phycomycetes (Mucorales), Ascomycetes, and Hyphomycetes which show maximum frequency occurrence of species under the order Moniliales. Few Melanconiales such as Pestalotia sp. and sterilia mycelia were also recorded.

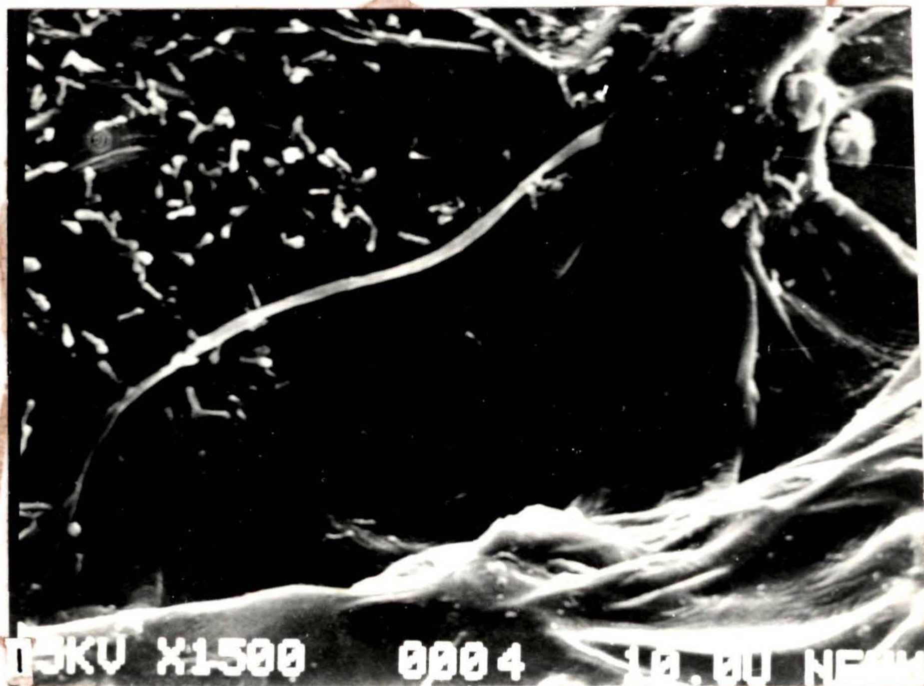
Statistical analysis showed that the total number of fungal species recorded in all the growth stages were statistically significant at 1% probability level between techniques but not between varieties (Table 3.12).

PLATE 3.2

1. A scanning electron micrograph showing spores population of *Spiromyces* sp. with a conidiophore x 1500.

2. A scanning electron micrograph showing spores population of *Sporothrix* sp. x 1300.

PLATE 3.2



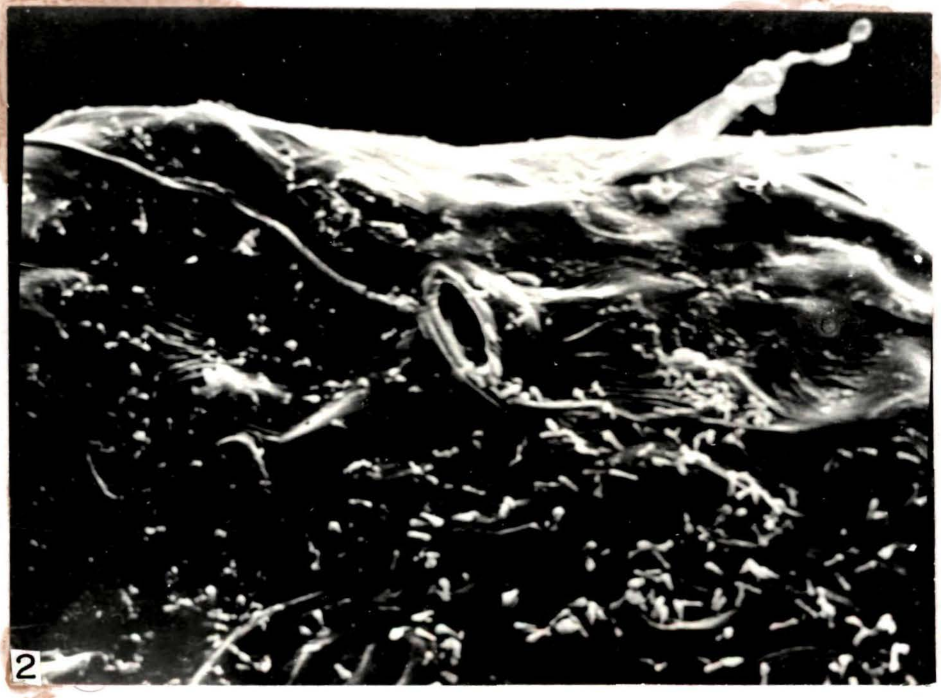
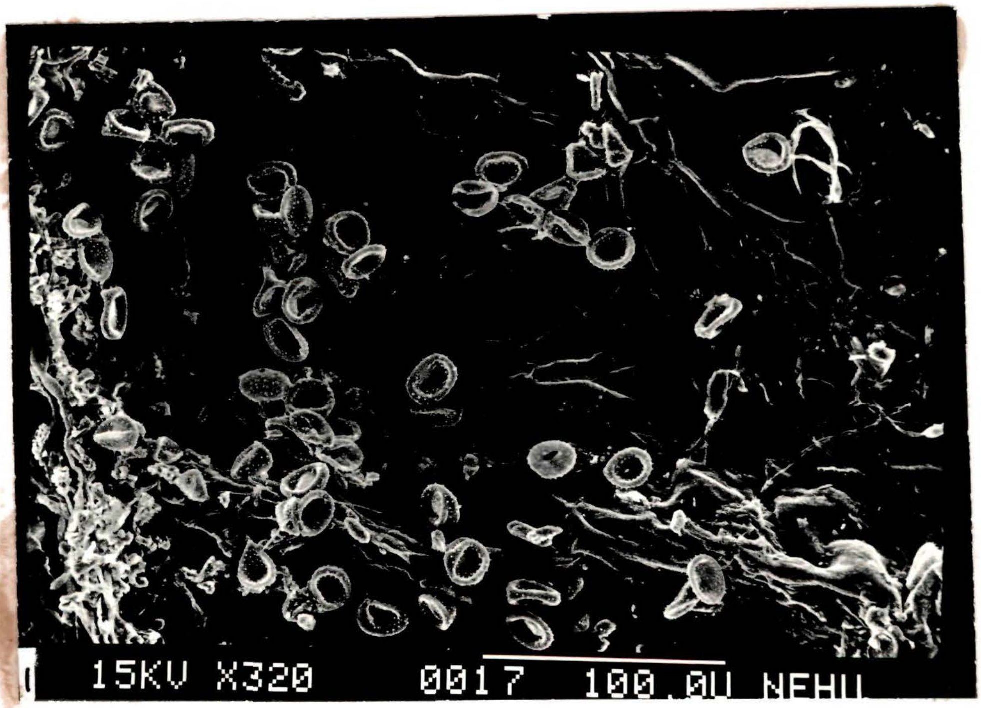


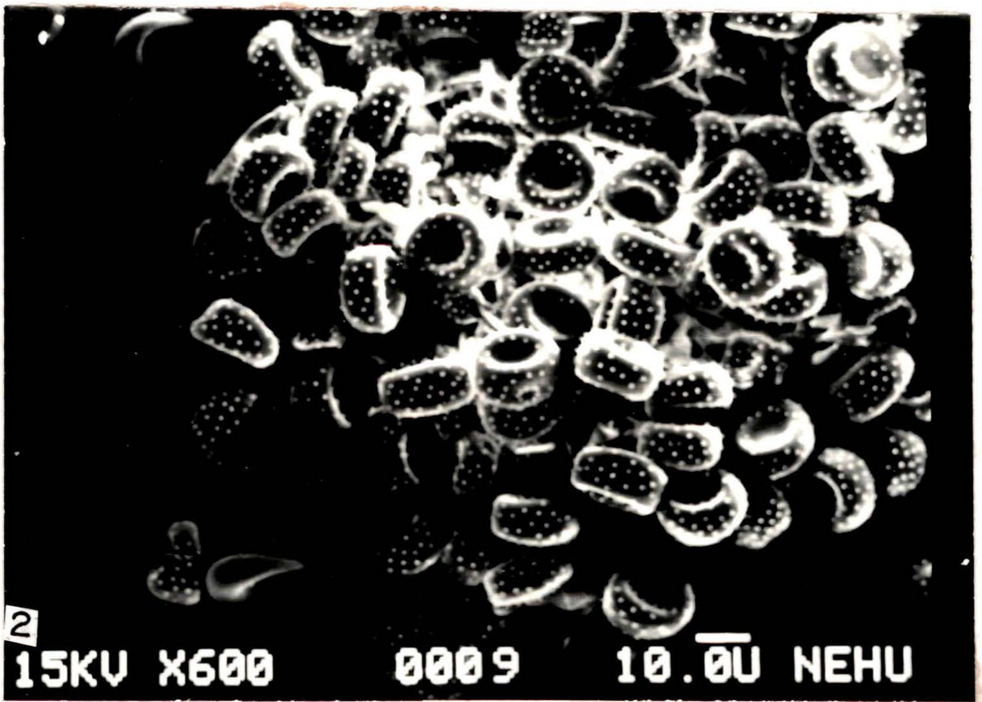
PLATE 3.3

1. Uredospores population of Uromyces appendiculatus observed under scanning electron microscope at low magnification x 320

2. Uredospores population of Uromyces appendiculatus observed under scanning electron microscope at high magnification x 600.

PLATE 3.3





Occurrence of different fungal species varied in different varieties at their various growth stages (Table 3.6 and 3.7). Variation in total fungal species among five bean varieties isolated by three different techniques is depicted (Fig.3.2). Among individual varieties, isolation of total number of fungi initially showed an increasing trend till the age of 30 days but suddenly decreased at the age of 45 days. They showed a second peak of increase at the flowering stage of the crop and at the latter stage they generally showed downward fluctuations with the exception in variety V_4. Significant varietal differences were observed as regard the occurrence of total number of fungal species at the senescent stage (Fig.3.3 to 3.7).

The qualitative distribution of different fungal species in different varieties at different growth stages of the plants has been presented (Table 3.3 to 3.5). The maximum total frequency occurrence of fungi was recorded in V_1(55) followed by V_3(47), V_6(44) and V_4(41) using leaf impression technique. Similarly, washing leaf disk recorded maximum number of fungi in V_1 (44) followed by V_5(43), V_3(42), V_4(42) and V_1(31) whereas moist chamber recorded almost equal number of fungi in all the varieties.

Statistical analysis of the number of fungal species recorded in different varieties showed that the changes in the number of fungal species were significantly different at different growth stages of the plant in both the techniques employed. This is evident from Tables (3.9 and 3.10) which showed that F-values for various growth stages were significant with critical difference of 2.26 and 2.46 for leaf impression (LI) and washing leaf disk (WLD) respectively. The statistical significance of changes in the total number of fungal species were in between young stage and pre-flowering stage of the plant in both the techniques. Table 3.7 showed the percentage frequency occurrence of different fungi in five different varieties at different growth stages of the plant. Fusarium sp., Penicillium sp., Sporothrix sp., Rhizopus nigricans were encountered with more percentage frequency in all the varieties.

Table 3.2 also showed that few species belonging to Hyphomycetes such as Cladosporium herbarum, Epicoccum nigrum, Isariopsis griseola, Macrophomina phaseolina, Pestalotia sp., Pyrenochaeta dicipiens, Rhizoctonia solani, Trichothecium roseum, Torula herbarum etc. were restricted to moist chamber technique except Chaetomium globossum

(Ascomycetes) and Absidia sp. (phycomycetes) whereas fungi Gliomastix muororum, Erysiphae graminis, Monilia fructigena, Chaetophoma confluens and Phyllosticta sp. were recorded in washing leaf disk only and few other fungi such as Xenosporium sp. and Phaeoseptoria sp. were restricted to leaf impression technique.

Leaf impression plate method showed that fungi such as Penicillium sp., Alternaria sp., Aspergillus sp., Sporothrix sp., Rhizopus sp., and Fusarium sp. were more frequent at various growth stages of the plant compared to Xenosporium sp., Gliocladium sp., Septocylindrium leucum and few other Melanconiales (Table 3.5).

In moist chamber technique the pathogens Colletotrichum lindemuthianum and Isariopsis griseola were observed along with common colonizers such as Penicillium sp., Aspergillus sp., Sporothrix sp. (Table 3.4).

A comparative glance at the fungi isolated from five French bean varieties using all the three techniques revealed that Fusarium spp., Sporothrix sp., sterile mycelia, Penicillium spp., Rhizopus nigricans, Aspergillus flavus were dominant in leaf impression and washing leaf disk technique whereas the pathogen C. lindemuthianum was dominant in moist chamber throughout the period of investigation.

Quantitative estimation of fungal propagules expressed as propagules/cm^2 of leaf surface showed the sequential changes of fungal population during the whole period of observation (Table 3.6). Maximum fungal population per square cm of leaf area was supported by V_1 and V_3 followed by V_4, V_6 and V_5 (Table 3.6). Total fungal population was found more in all varieties at 30 days old leaves but abruptly decreased at 45 days. Almost all varieties showed similar population at the 60 days showing another peak of increase at 75 days. At the senescent stage (90 days) variety V_1 and V_3 showed increased fungal population whereas the other varieties showed decreasing trend (Fig.3.8).

Compared to other species, the fungal population of <u>Sporothrix</u> sp., <u>Fusarium</u> sp., <u>Penicillium</u> <u>wortmanii</u>, sterile mycelia was high in all the varieties whereas the population of <u>Periconia</u> <u>palludosa</u>, <u>Oedodendron</u> <u>griseum</u>, <u>Verticillium</u> <u>trifidum</u> present in V_6 and <u>Spriomyces</u> sp., <u>Tieghemiomyces</u> found in V_5 was less. Respective population of different fungal species isolated by washing leaf disk has been shown in Table 3.6.

The 't'-test analysis showed that the changes in fungal population was significant at 15 to 45 days and 15 to 90 days of plant growth in V_3 whereas in V_5 it was found significant at 15 to 45 and 45 to 90 days of plant growth. Similarly, V_6 showed significant differences in 45 to 90 days and 15 to 90 days of plant growth (table 3.8). To determine the overall changes if any, in between the fungal population and growth stages as well as in between the varieties, two way analysis of variance showed that the changes in fungal population was statistically significant between the different ages of the plant whereas the varietal differences were not statistically significant. The critical difference (CD=60.00) showed that the significant changes in fungal population determined by analysis of variance was due to the significant changes of population in seedling stage (15 days), young stage (30 days) and pre-flowering stage (45 days) (Table 3.11).

Classification of mycoflora based on their occurrence at different growth stages of the plant

The fungi isolated by the three techniques from all the varieties were arbitrarily classified into the following three groups:

(i) Fungi found commonly in all the growth stages of the plant

(ii) Fungi occurred sporadically but not restricted to any growth stage of the plant

(iii) Fungi restricted to one particular growth stage only.

Fungi such as Aspergillus sp., Penicillium sp., Fusarium sp., Rhizopus nigricans, sterile mycelia and C. lindemuthianum, belonged to the first category of classification.

Diverse group of fungi belonged to the second group of classification. Worthy to mention were Sphacelia segetum, Aspergillus flavus, Phoma glomerata, Myrothecium roridum, Curvularia lunata, Verticillium sp., Mucor haemalis and Alternaria alternata.

Rare occurrence of fungi such as Verticillium trifidum, Tieghemiomyces sp., Spiromyces sp., Pseudostemophylium consortiale, Gliocladium penicilloides, Gliocladium deliquescens, Gloeosporium fructigenum, Endosporostible sp., Xenosporium sp., Phaeoseptoria sp., Leptosphaeria maculans, Acremonium cerealis, were restricted to one of the growth stages of the plant. Further, fungi like Monodictys nitens and Periconia palludosa were restricted to early growth stage of the plant whereas the other fungi such as Gliomastix muororum, Erysiphae graminis and Alternaria brassicicola were found mostly at the senescent stage of the plant (Table 3.6 and 3.7).

Relationship between pH, moisture content of leaves and fungal population

The total fungal population of all growth stages of each variety isolated by washing leaf disk were tried for correlation with the pH and moisture content of leaves. It was found that pH of the leaves was positively correlated whereas the moisture content of the leaves was negatively correlated. However, correlation coefficient(r) was not statistically significant between the variables except in V_6 where the fungal population showed significant negative correlation ($r=-0.81$) with the moisture content (Table 3.13). Figure 3.9 depicted the relationship between pH and moisture content of leaves with increasing age of the plant.

DISCUSSION

The results obtained regarding the isolation of different fungal species by the three techniques suggested that a combination of various techniques are needed to isolate the maximum number of leaf saprophytes. Dickinson (1971) and Sharma et al (1974) were of the opinion that simultaneous use of several techniques would result maximum

Table 3.3 Showing the presence of fungi on the leaf surface of five bean varieties isolated by washing leaf disk technique at different growth stages of the plants (Average of two years observations)

Fungi	Age of the plants in days																													
	15					30					45					60					75					90				
	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6
Alternaria alternata	+	+	+		+													+										+		
A. brassicola									+							+														+
A. tenuis																+													+	
Aspergillus flavus				+	+					+						+											+			
A. niger																+					+						+	+		
A. versicolor									+																					
Botrytus cinerea																	+									+				
Cephalosporium sp							+	+																						
Chaetophoma confluens																														+
Cladosporium cladosporoides											+								+											
Cladosporium herbarum													+																	
Colletotrichum lindemuthianum				+			+												+				+							
Curvularia lunata																	+													
Endosporostible sp.							+																							
Erysiphae graminis																										+				+
Fusarium moniliforme							+	+	+				+	+																+
F. oxysporum	+	+		+		+			+			+		+				+			+		+			+	+		+	
F. semitectum			+		+				+		+								+			+	+	+		+				
Geotrichum sp																								+						
Gliocladium deliquescens															+												+			
G. penicilloides																				+						+				
Gliomastix muororum																							+				+			
Gloeosporium fructigenum												+	+																	
Haplosporium parvum													+																	
Humicola grisea																							+							
Leptosphaeria maculans														+																
Monilia fructigena							+	+																		+				
Mucor haemalis																+			+				+	+		+				
Myrothieum roridum							+																						+	
Oedocephalum coprophilum																												+		
Oedodendron griseum																		+												+
Penicillium chrysogenum			+															+										+		
P. funiculosum					+	+	+			+																+				
P. granulatum	+	+		+					+		+	+	+	+	+		+		+	+			+	+		+				
P. wortmanii					+	+		+					+			+				+	+	+	+				+		+	
Periconia palludosa															+															
Phoma glomerata																												+	+	
Phyllosticta minima																													+	+
Pseudostemophylium conosortiale							+																							
Rhizopus nigricans				+	+			+	+		+	+		+	+							+								+
Sphacelia segetum	+	+	+			+																+								
Spiromyces sp																							+							
Sporobolomyces salmonicolor						+																								
Sporothrix sp	+	+	+	+		+		+	+	+	+	+				+	+	+	+	+			+			+				
Stemphylium sp							+						+															+		+
Sterilia mycelia (brown)	+	+					+										+		+				+			+	+			
Sterilia mycelia (white)			+	+		+		+	+		+					+	+	+	+	+	+	+		+	+	+	+			
Tieghemiomyces sp																												+		
Verticicladium trifidum																				+										
Verticillium albo-atrum							+		+	+																				
V. dahliae									+																	+				

+ = Presence of fungi

Table 3.4 Showing the presence of different fungi on the leaf surface of five bean varieties isolated by moist chamber technique at different growth stages of the plant (Average of two years observation)

Fungi	Age of the plants in days																													
	15					30					45					60					75					90				
	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6
Absidia sp.	+																													
Acremonium cerealis			+																											
Alternaria alternata				+					+					+																
A. brassicicola																										+				
Aspergillus flavus							+			+								+										+		+
A. versicolor																			+											
Botrytis cinera	+		+	+																										
Candida albicans			+													+	+													
Chaetomium globossum				+					+					+																
Cladosporium cladosporoides			+																		+									
C. herbarum				+																									+	
Colletotrichum lindemuthianum	+					+		+		+	+	+			+	+		+		+	+				+		+	+		+
Curvularia lunata			+																+					+						
Entosporostilbe sp.												+																		
Epicoccum nigrum			+																											
Fusarium moniliforme			+																											
F. oxysporum				+			+											+												
F. semitectum						+			+																		+			
Gliocladium penicilloides		+											+										+							
Gloeosporium fructigenum							+	+									+													
Haplosporangium parvum								+																				+		
Humicola grisea		+																												
Isariopsis griseola									+	+									+	+				+				+	+	
Macrophomina phaseolina																		+												
Mucor haemalis	+	+								+							+										+			
Myrothecium roridum											+											+	+	+					+	
Cedocephalum coprophilum												+																		
Oedodendron griseum																									+					
Paecilomyces variotii					+	+																								
Penicillium chrysogenum								+			+		+	+	+	+		+	+							+	+	+		+
P. funiculosum					+	+	+												+			+								
P. granulatum																							+	+	+					
P. wortmanii									+	+							+													
Periconia paludosa													+																	
Pestalotia sp.																				+										
Phoma glomerata		+							+		+																			
Pyrenochaeta decipiens	+																					+				+				
Rhizoctonia solani																													+	
Rhizopus nigricans																+										+				
Septocylindrium leucum							+															+								
Sporothrix sp.																						+			+					+
Sterile mycelia (brown)											+		+	+	+		+													
Sterile mycelia (white)				+		+	+		+					+							+									
Tieghemiomyces sp.												+							+				+			+				
Torula herbarum							+									+									+					
Trichothecium roseum							+				+				+															
Verticillium albo-atrum											+	+			+											+				

+ = Presence of fungi

Table 3.5 Showing the presence of different fungi on the leaf surface of five varieties of French bean isolated by leaf impression technique at different growth stages of the plants (Average of two years observations)

Fungi	Age of the plants in days																													
	15					30					45					60					75					90				
	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6
Acremonium cerealis			+									+																		
Alternaria alternata	+	+	+																							+				
A. brassicicola																						+				+				
A. tenuis																+				+								+		+
Aspergillus flavus		+			+				+							+					+		+	+	+	+		+		
A. niger					+		+									+	+		+	+								+		
Candida albicans																+	+	+	+	+										
Cephalosporium sp.								+	+			+														+				
Cladosporium cladosporoides		+																								+			+	+
Colletotrichum lindemuthianum						+		+																						
Curvularia lunata																					+									
Fusarium moniliforme						+	+	+	+						+														+	
F. oxysporum	+	+	+	+	+	+	+	+	+	+	+	+	+			+			+	+			+		+					+
F. semitectum	+	+		+	+	+				+							+		+		+	+		+	+				+	+
Geotrichum sp.																					+	+				+				
Gliocladium diliquescens									+																					
Gliocladium penicilloides																										+				
Gloeosporium fructigenum																								+		+				
Haplosporangium parvum						+	+	+															+						+	+
Leptosphaeria maculans																							+							
Monodictys nitens		+	+	+												+														
Mucor haemalis														+				+	+			+	+	+	+					
Myrothecium roridum	+												+		+												+			+
Paecelomyces variotii															+		+													
Penicillium chrysogenum											+		+	+	+						+	+				+	+		+	
P. funiculosum		+				+	+				+					+	+							+	+					
P. granulatum	+	+		+	+	+		+		+												+	+			+			+	
P. wortmanii	+		+		+		+	+	+									+	+	+										
Periconia palludosa							+	+		+																				
Phaeoseptoria sp.																														+
Pseudostemophylium consortiale										+																				
Rhizopus nigricans	+		+	+			+	+		+	+	+	+	+						+		+		+		+	+	+	+	+
Septocylindrium leucum																											+			
Sphacelia segetum	+																													
Sporobolomyces salmonicolor																				+										
Sporothrix sp.	+	+		+	+	+	+	+	+	+				+	+	+	+	+		+	+	+	+		+	+				
Sterile mycelia (brown)	+	+													+									+						
Sterile mycelia (white)	+		+	+		+					+						+	+	+	+	+		+	+	+					+
Tieghemiomyces sp.													+			+														
Verticillium albo-atrum												+					+	+	+					+						+
Verticillium dahliae						+	+					+	+							+							+			
Xenosporium sp.																+														

+ = Presence of fungi

Table 3.6 Population of fungi in the phyllosphere of five French bean varieties at different growth stages of the plant (Population : no. of propagules/cm^2 leaf surface)(Average of two years observations)

Fungi	Age of the plant in days																													
	15					30					45					60					75					90				
	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V,	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6
Aspergillus flavus				5.09	12.72					7.63						10.18		10.20		10.18			10.19		10.19		10.19			
A. niger																7.63					7.63						22.90	22.90		
A. versicolor									5.09												5.09									
Alternaria alternata	15.28	5.09	10.19		5.09													15.26					15.27			12.73				
A. tenuis					7.63																							10.19	10.19	10.19
A. brassicicola									5.09							20.36														20.36
Botrytis cinera																	[illegible]									10.19				
Colletotrichum lindemuthianum					7.63		5.09												7.63					5.09				7.63		
Cephalosporium sp.						10.19		12.73																						
Cladosporium cladosporoides											15.27		10.19						5.09					5.09						
Curvularia lunata																	15.27					10.19								
Chaetophoma confluense																														17.81
Endosporostilbe sp.							12.72																							
Erysiphae graminis																										10.19				20.36
Fusarium moniliforme						35.88	25.45	31.81	25.45				10.19	20.36																20.36
F. oxysporum	12.72	12.72		15.27		20.20				7.63		25.45		17.81			20.36	20.36	17.81		10.19	10.19	15.27	10.19	17.82	17.81	10.18		10.19	
F. semitectum			17.81		10.19				25.45		10.19				12.73							22.90		15.27	20.36	10.19				
Geotrichum sp.																								7.63						
Gloeosporium fructigenum												15.27	12.73																	
Gliocladium deliquescens															5.09												15.27			
G. penicilloides																				7.63						15.27				
Gliomastix murorum																											22.90	5.09		
Haplosporangium parvum													17.81																	
Humicola grisea																							12.73	15.27						
Leptosphaeria maculans														7.63																
Monilia fructigena							5.09	25.45																		10.19				
Mucor hiemalis																			5.09					22.90	22.90	10.19				
Myrothecium roridum												15.27																	5.09	
Oedocephalum cyprophilum																		7.63					5.09					12.73		
Oedodendron griseum																														
Penicillium chrysogenum		10.19	25.45							38.17																				12.73
P. funiculosum					20.36	61.08	50.9			7.63														10.19				12.73	12.73	
P. granulatum	5.08			15.27							50.9	50.9			12.73		20.36													
P. wortmanii					20.36	50.9	76.35	45.53					17.81			20.45				25.45	5.09			25.45		22.90	22.90			
Periconia pullulosa															5.09						25.45	76.35	5.09	40.72		25.45	50.9	33.08	17.81	
Phoma glomerata																														
Phyllosticta minima																												25.45	10.19	
Pseudostemphylium consortiale							17.82																							10.19
Rhizopus nigricans				5.09	5.09			15.27	10.19		10.19	17.81		17.81	15.27														10.19	20.36
Sphacelia segetum	7.64	10.19				10.19																15.27							15.27	
Spiromyces sp																							20.36							
Sporobolomyces salmonicolor						12.72																		5.09						
Sporothrix sp	22.90	17.82	7.63	10.19		30.54	50.9	38.17	25.45	22.50	38.11	50.9		20.36		27.79	22.90													
Stemphylium sp							15.27						10.19							25.45	61.08			17.81	25.45	61.08	25.45			
Sterile mycelia (brown)	22.91	10.19		12.73		12.72		10.19	15.27	7.63						20.45	10.19											20.36		
Sterile mycelia (white)	20.38						12.72				12.73						10.19			33.08	10.19	20.36	55.99	15.27	17.81	20.36	25.45		12.73	
Tieghemiomyces sp																								20.36	50.9	25.45	10.19			
Verticillium albo-atrum							5.09		12.73															5.09						
V. dahliae									15.27																	20.36				
Verticicladium trifidum															15.27												10.19			

Table 3.7 Showing the percentage frequency occurrence of different fungi in the phyllosphere of five bean varieties isolated by leaf impression technique (Average of two years observation)

Fungi	Age of the plant in days																													
	15					30					45					60					75					90				
	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6	V_1	V_3	V_4	V_5	V_6
Acremonium cerealis												3.03																		
Alternaria alternata	34.96	4.44	23.43	13.24																						11.61				
A. brassicicola																						15.26				29.41				11.62
A. tenuis																3.31		10.71		4.25								17.17		26.64
Aspergillus flavus		4.44			4.25				14.08							8.33					2.81		1.06	3.5	6.57		12.51	5.26		
A. niger					8.51		15.38									13.74	6.75		2.89	10.12								6.57		
Candida albicans									11.81							17.08	16.21	12.77	7.24	4.25										
Cephalosporium sp								7.93	2.81			15.15														9.80		32.89		
Cladosporium cladosporioides	16.57																									9.65		6.57	32.6	
Colletotrichum lindemuthianum						8.08		5.61	1.40																					
Curvularia lunata																					2.86									
Fusarium moniliforme						3.03	16.2	16.05	19.26					18.05														6.57		
F. oxysporum	20.48	15.55	15.62	19.04	18.79	15.15	16.48	16.05	2.81	21.77	21.11	26.68	45.45		10.51	3.33			36.23	21.27			19.45		19.73					28.79
F. semitectum	16.25	11.11		9.52	17.81	19.19				9.43							9.45		19.24		21.12	24.59		15.09	39.07					
Geotrichum sp.																					13.93	8.19					18.80			
Gliocladium deliquescens									11.81																					
G. penicillioides									17.15																		10.00			
Gloeosporium fructigenum																								3.50						
Haplosporangium parvum						11.11	16.20	13.48															19.73			3.92			10.86	11.62
Leptosphaeria esculens																							1.06							
Monodictys nitens		11.11	6.25	2.38																										
Mucor haemalis														23.61		9.99		26.53	11.37			17.71	2.12	16.91	14.07				4.34	
Myrothecium roridum	2.63												11.36														10.60	2.63		18.31
Paecilomyces variotii															20.36		27.67													
Penicillium chrysogenum											30.71		18.18	10.66	37.03						14.08	21.36			5.26	26.24			21.73	
P. funiculosum	3.94	26.74			36.78	15.15	13.18		14.08		12.12					16.66	17.62				29.95		10.63	31.82	6.57					
P. granulatum	1.31	11.11		19.04	21.27	10.10		28.34		18.86																	22.91		19.71	
P. martensii	13.15		32.81	32.81	8.51		16.48	14.78	7.04	18.86						3.33		30.76	14.49	20.36						13.49				11.62
Periconia pellucida								19.47	7.04	3.77																				
Phaeoseptoria sp																														4.65
Pseudostemphylium consortiale								1.12		21.38																				
Rhizopus nigricans	2.63		18.75	11.90			17.03	1.12	2.81	20.04	27.4	26.80	22.72	15.27						12.12	2.86				2.63	3.92	17.36	6.57	16.14	13.95
Septocylindrium leucum																											10.00			
Sphacelia segetum	2.63																													
Sporobolomyces salmonicolor																				10.69										
Sporothrix sp	21.05	23.41	12.5	9.62	10.63	20.20	16.48	8.96	14.08	18.86				33.33	51.84	41.66	22.97	28.15			54.15	31.14	28.58		12.73	7.84				
Sterile mycelia (brown)	2.63	8.88				10.10				3.77														8.77	8.77			13.15		
Sterile mycelia (white)	3.94		40.62	14.64		27.77					13.98						14.77	7.69	7.39	25.95	64.08		11.47	26.31	13.15			14.70	8.69	
Tieghemiomyces sp.																1.66														
Verticillium albo-atrum												25.29	27.72				12.21	3.07	13.22						15.02					
V. dahliae						2.02	2.19					12.12	18.05			1.66			7.24	4.25							14.58			
Xenosporium sp							5.09																							

Table 3.8 Showing changes in fungal counts of three growth stages of plants using student's 't'-test.

Variety	Seedling — Pre-flowering stage (15-45 days) 't'-value	Pre-flowering — senescent stage (45-90 days) 't'-value	Seedling - senescent stage (15-90 days) 't'-value
V_1	1.84^{ns}	0.81^{ns}	1.60^{ns}
V_3	2.78*	1.61^{ns}	2.47*
V_4	0.65^{ns}	0.02^{ns}	0.54^{ns}
V_5	2.28*	2.45*	0.50^{ns}
V_6	0.40^{ns}	2.33*	2.69*

* = significant at 5% probability level.

ns = not significant.

Table 3.9 Showing two way analysis of variance for fungal species recorded by leaf impression technique between different varieties and stages.

Sources of variation	Degree of Freedom (d.f.)	Sum of squares (SS)	Mean sum of squares (MS)	Computed F-value	Tabular F-value	
					5%	1%
Growth stages	5	46.167	9.233	3.129*	2.71	4.10
Varieties	4	16.20	4.05	1.37^{ns}	2.87	4.43
Error	20	59	2.95			

CD at 5% = 2.26

* = significant at 5%.
ns = not significant.

Table 3.10 Showing two way analysis of variance for fungal species recorded by washing leaf disks technique between varieties and growth stages.

Sources of variation	Degree of Freedom (d.f.)	Sum of squares (SS)	Mean sum of squares (MS)	Computed F-value	Tabular F-value	
					5%	1%
Growth stages	5	51.767	10.353	2.97*	2.71	4.10
Varieties	4	19.867	4.966	1.42^{ns}	2.87	4.43
Error	20	69.733	3.486			

CD at 5% = 2.46

* = significant at 5%.
ns = not significant.

Table 3.11 Showing two way analysis of variance of fungal population (no. of propagules/cm^2 leaf surface) between varieties and growth stages of the plant.

Sources of variation	Degree of Freedom (d.f.)	Sum of squares (SS)	Mean sum of squares (MS)	Computed F-value	Tabular F-value	
					5%	1%
Growth stages	5	41823.61	8364.72	4.04*	2.71	4.10
Varieties	4	13754.43	3438.60	1.66^{ns}	2.87	4.43
Error	20	41370.65	2068.53			

CD at 5% = 60.003

* = Significant at 5%
ns = not significant.

Table 3.12 Two way analysis of variance for total number of fungal species isolated from five varieties of French bean leaves using three techniques in all the growth stages of the plant.

Sources of variations	Degree of Freedom (d.f.)	Sum of Squares (SS)	Mean sum of squares (MS)	Computed F-value
Techniques	2	706.535	353.267	24.336**
Varieties	4	166.266	41.566	2.863^{ns}
Error	8	116.134	14.516	
Total	14			

CD at 5% = 9.288.

** = Significant at 1% probability level

ns = not significant.

Table 3.13 Correlation between total fungal population of French bean varieties and physical factors (pH and moisture content) of leaves at various growth stages of the plant.

Variety	pH (r - value)	Moisture content (r-value)
V_1	0.205^{ns}	-0.384^{ns}
V_3	0.188^{ns}	-0.057^{ns}
V_4	0.25^{ns}	-0.078^{ns}
V_5	0.45^{ns}	-0.409^{ns}
V_6	0.29^{ns}	-0.811*

* = significant at 5%.

ns = not significant

r = correlation coefficient.

Fig.3.1 Comparison of three techniques used (Leaf Impresion, Washing Leaf Disk and Moist Chamber) in the isolation of total number of fungal species from the phyllosphere of five different varieties of French bean.

Fig.3.2 Showing total number of fungal species isolated from each of the five different varieties of French bean leaves by three different techniques.

Fig.3.3-3.7 Showing total number of fungal species isolated from the Phyllosphere of various varieties of French bean (V_1, V_3, V_4, V_6 and V_5) by three different techniques at various ages of the plants (15, 30, 45, 60, 75 and 90 days).

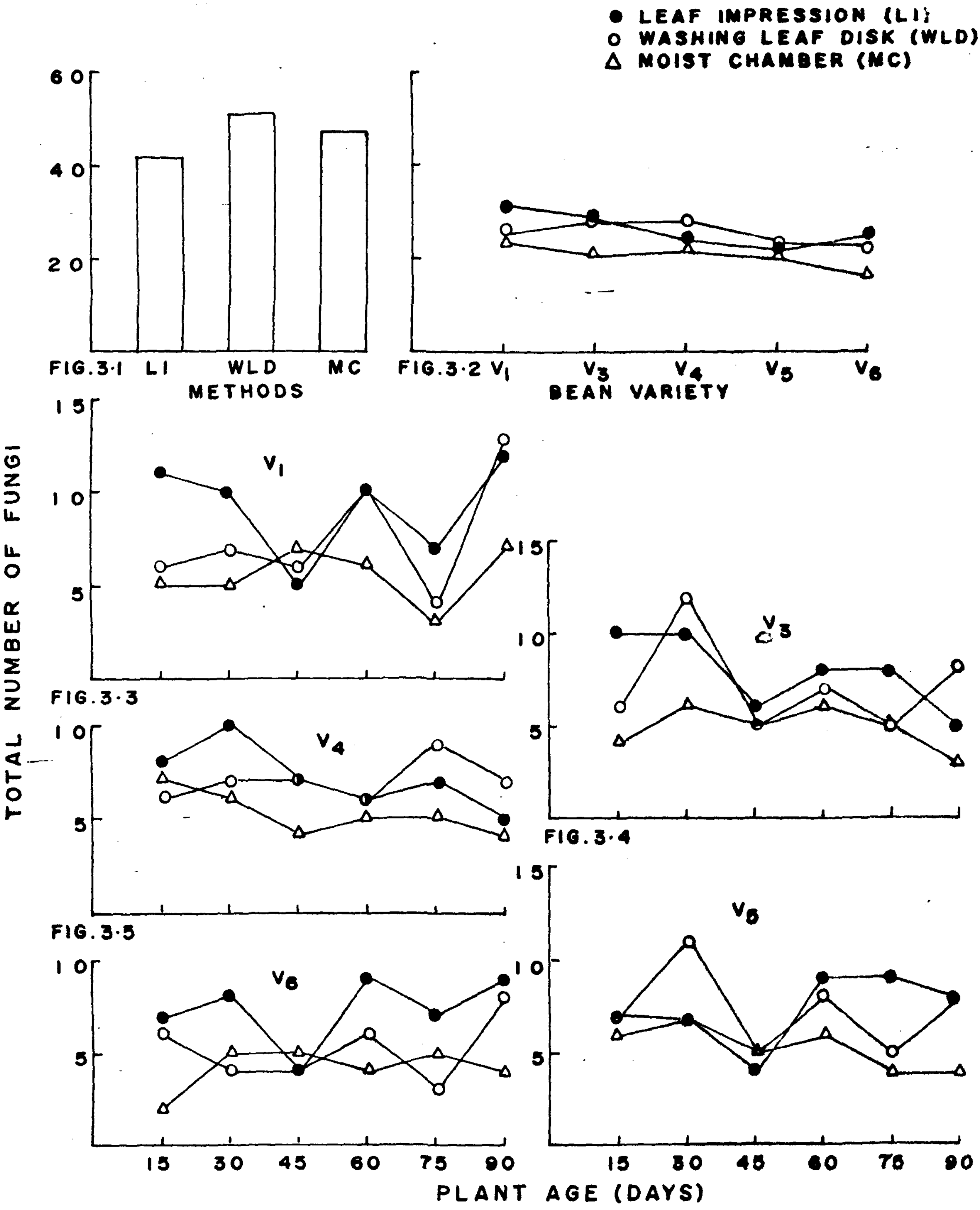


FIG.3·1

FIG.3·2

FIG.3·3

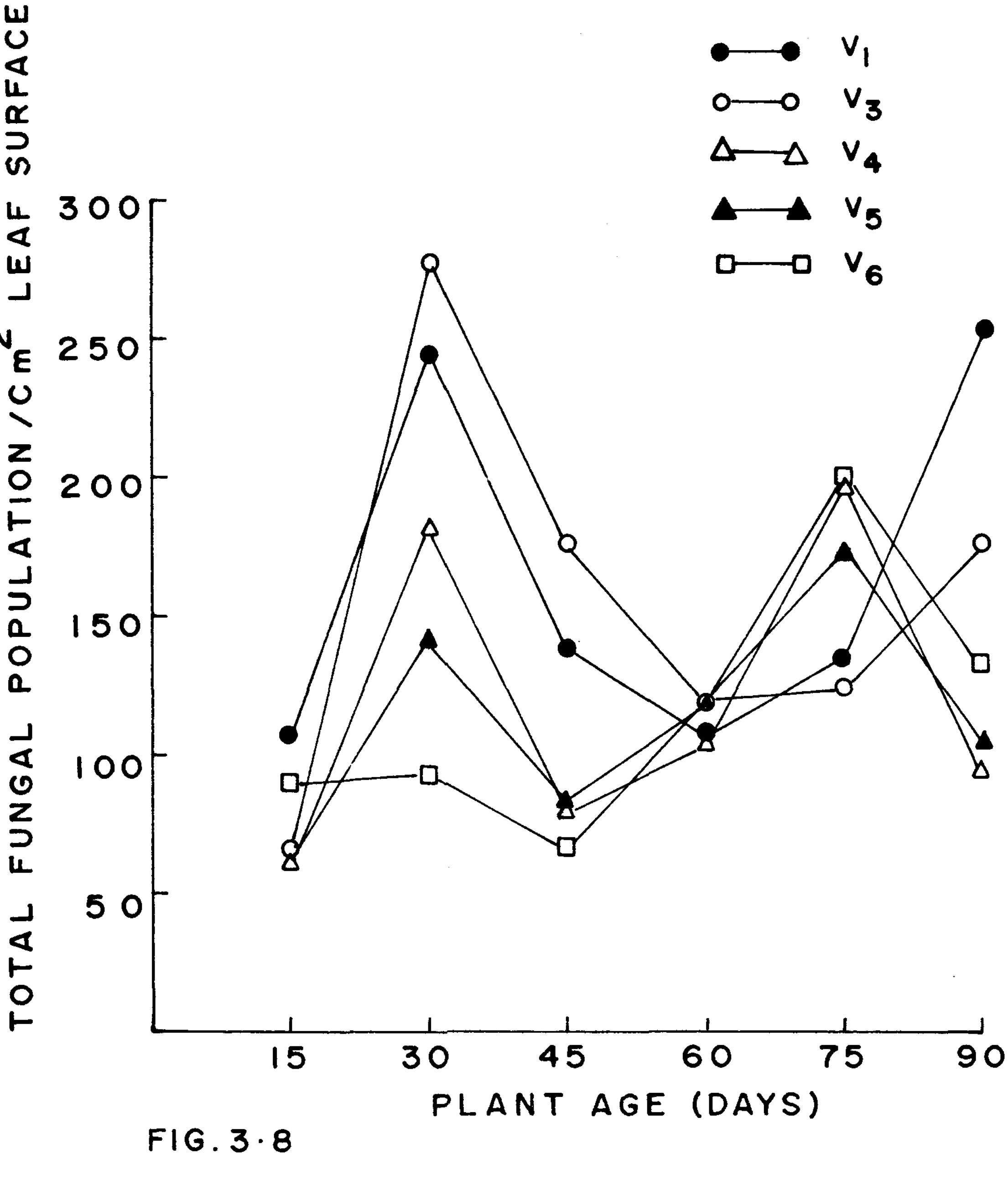
FIG.3·4

FIG.3·5

FIG.3·6

FIG.3·7

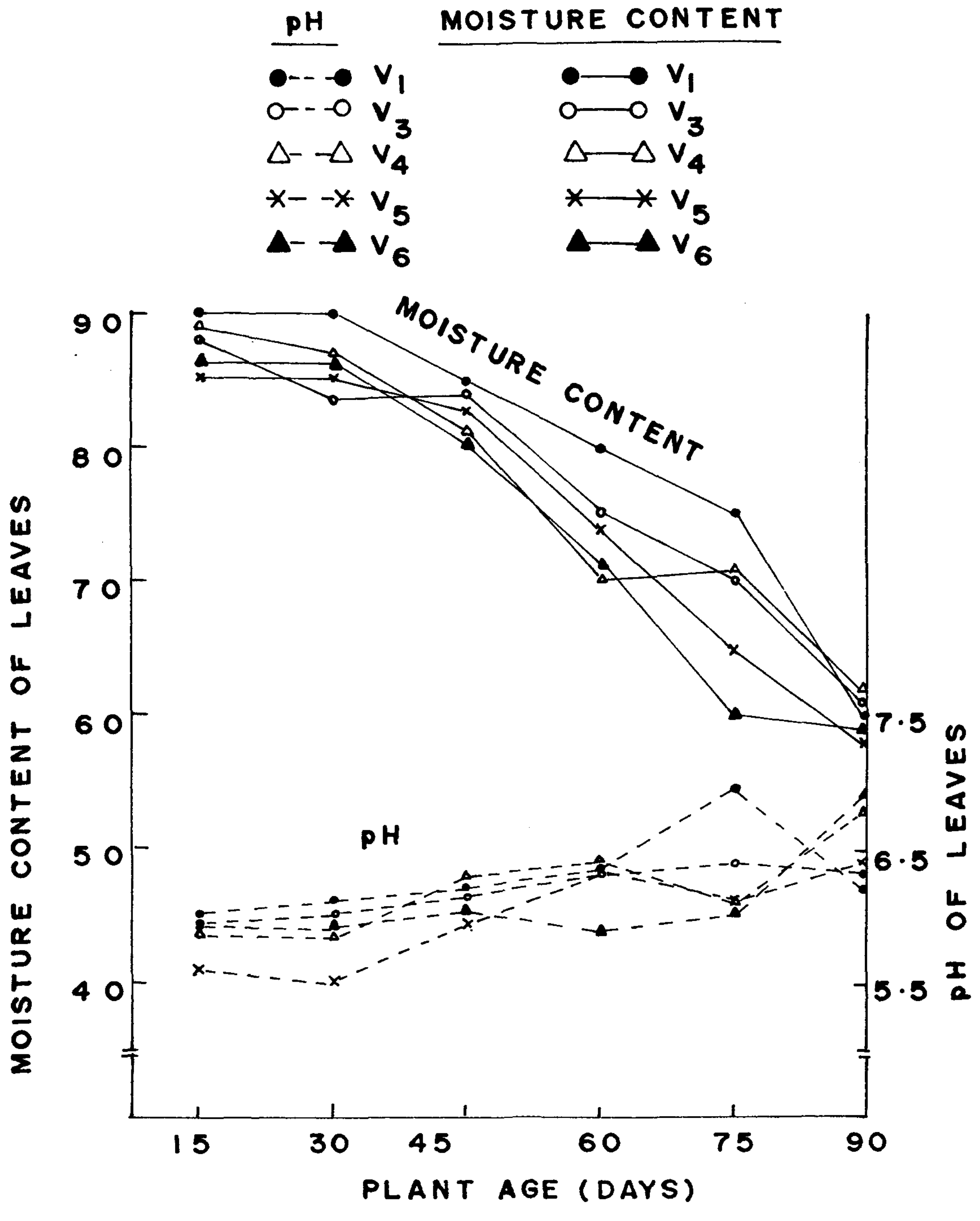
Fig.3.8 Showing the total fungal population/cm^2 of leaf surfaces of five different varieties of French bean isolated by washing leaf disk technique at different ages of the plants.



TOTAL FUNGAL POPULATION / Cm² LEAF SURFACE
300
250
200
150
100
50
15
30
45
60
75
90
PLANT AGE (DAYS)
V1
V3
V4
V5
V6

FIG. 3·8

Fig.3.9 Comparison of two factors viz. pH and moisture content in (percentage) of leaves of five varieties of French bean (V_1, V_3, V_4, V_5 and V_6) at different ages of the plant.



pH
MOISTURE CONTENT
V1
V3
V4
V5
V6
V1
V3
V4
V5
V6
MOISTURE CONTENT
pH
90
80
70
60
50
40
MOISTURE CONTENT OF LEAVES
7·5
6·5
5·5
pH OF LEAVES
15
30
45
60
75
90
PLANT AGE (DAYS)

FIG.3·9

isolation of phyllosphere mycoflora both qualitatively and quantitatively.

In the present investigation moist chamber technique was found to be the most suitable for isolating saprophytic as well as the parasitic fungi that may be responsible for the decomposition of leaves. This could be the probable reason for frequent isolation of leaf pathogens (C. lindemuthianum and I. griseola) by moist chamber. Sharma et al (1974) also reported that moist chamber technique was the best for isolation of decomposers from healthy and senescent leaves.

Scanning electron microscope (SEM) tgechnique provided information on the conidiophores and conidial population within their plant surface habitat at low magnification. Mishra and Dickinson (1981) also reported the usefulness of SEM in recording the occurrence of cnidiphores and Conidia. However, the rare detection of these reproductive and vegetative structures on the green leaves in situ could probably be due to frequent washing by rain splashes and blowing away by wind action as they might not have fully adhered on the leaf surface or their rare occurrence on the green leaves.

Washing leaf disk technique possibly recorded the loosely attached propagules of highly sporulating fungi which were mostly surface colonisers and therefore, the nutrient medium used might have played important role in their isolation. In cultural techniques only few fungi such as Fusarium sp., Sporothrix sp., Rhizopus nigricans, Aspergillus flavus were found to occur with maximum frequency. Possibly this could be due to the overgrowth by fast growing fungi such as Rhizopus nigricans and Mucor sp. in Czapek's Dox Agar medium. Eicker (1976) has also reported that dilution plate technique was less effective and yielded only nine species due to overgrowth by fast growing fungi such as Mucor sp.

Information obtained from different techniques gave an idea about the colonization and successional pattern of different fungi on the leaf surface since till today there is no single method which can selectively remove the fungal components directly from the leaf surface for cultural purpose which can be taken as a measure of fungal activity in vivo.

Aspergillus flavus, Fusarium oxysporum, Penicillium sp., Rhizopus nigricans, Sporothrix sp. and sterile mycelia contributed maximum percentage frequency of occurrence.

The other fungi ennumerated were found to occur with varying percentage frequency of occurrence (Table 3.7).

The percentage frequency occurrence of fungi revealed that the fungal population fluctuate with the varying age of the plants. This could be due to the fluctuation of environmental factors and the nutritional status of the leaves because there is often fluctuation in the nutrient containing leachates from the aerial plant parts (Sinha, 1971). The varietal differences in the frequency occurrence of different fungi could possibly be due to the growth habit of the varieties since variety V_6 and V_4 were dwarf type whereas V_1, V_5 and V_3 were pole type. Dwarf varieties get senesced earlier than the pole type and therefore variation in frequency occurrence of fungi might have resulted. The senescent leaves were colonized by Aspergillus sp. Alternaria spp., Phaeoseptoria sp., Cladosporium sp., whereas most of the early surface colonizers were Fusarium spp., Penicillium spp., Sporothrix sp. and sterile mycelia. The important filamentous yeast Candida albicans was exclusively found at the flowering stage of the plant (Table 3.7).

The dominant fungi such as Aspergillus spp., and Penicillium spp. occurred at high frequency reflecting their higher relative abundance in the atmosphere. An abrupt fall in the population of these fungi in the senescent stage and low frequency occurrence in moist chamber suggest that their role is negligible in the decomposition process (Dickinson, 1976) (Table 3.7).

Qualitative assessment of the fungi such as Cladosporium sp., Alternaria sp., Epicoccum nigrum, Aspergillus flavus and some sterile mycelia may be grouped under 'residents' and other species like Aspergillus niger, Phoma sp., Macrophomina Phaseolina and many other infrequently occurring forms may be treated as 'Casuals' or 'transients' (Lamb and Brown, 1970).

The specificity of the occurrence of different fungi in a particular growth stage could be due to the influence of weather conditions prevailing at the different growth stages of the plant. Possibly the different environmental factors might have affected the trapping of spores on leaf surface (Gregory, 1971; Sinha 1971; Narula and Mehrotra, 1981). The rare occurrence of few fungal species mentioned in result might be because of their colonization behaviour which depends more on the habitat condition and growth stage of the plant than on the availability of the inoculum density.

In the quantitative estimation, the sequential changes of fungal population showing marked increase at 30 and 75 days of the plant growth might have been possibly affected by the nutrient release from the leaf surface. Further, the varietal differences in supporting the fungal population indicated that the susceptibility of variety might have affected the amount of fungal population. It is evident from the higher fungal population in V_1 which was more susceptible to disease than any other variety (Table 3.6). This is in accordance with the findings of many other workers (Sharma and Sinha, 1972; Kumar and Gupta, 1974), who have observed higher fungal population in susceptible varieties than the resistant one. However, the overall varietal differences in supporting the fungal population was not statistically significant but the changes in the total fungal population with increasing age was statistically significant indicating that the growth stages of the plant had a highly significant effect on the total fungal population. This is in agreement with the findings of Bainbridge and Dickinson (1972); Sharma and Mukherjee (1974).

Moisture content of leaves decreased with the increase in plant age whereas the pH changed from acidic to alkaline which could be due to succulent leaves containing more moisture at the initial stage than the matured leaves at the senescent stage (Rajeevalochana 1983). The result indicated that the pH and moisture content of leaves may have some effect on the variation of fungal population. However, the effect was not significant despite the report that pH of the leaves might play an important role in the distribution of fungi both qualitatively and quantitatively (Dickinson, 1967).

CHAPTER - IV

INTERACTION STUDIES BETWEEN LEAF SURFACE FUNGI AND THE LEAF PATHOGEN

INTRODUCTION

Plant surface is a natural habitat which represents a heterogeneous population of micro-organisms comprising of both pathogens and saprophytes (Dickinson, 1967). Review of literature indicates that considerable amount of work has been done to understand the biology of the pathogen in relation to the leaf surface saprophytes. In nature, interactions are known to take place between pathogenic and saprophytic microbes as well as within the pathogen themselves and the net effect of the phylloplane saprophytes is to reduce the effective inoculum dose of pathogens (Mukerji, 1983).

On the plant surface antagonistic interactions might be going on and probably because of this the plant does not get attacked by a virulent pathogen. The practical use of antagonist in disease control as a form of biological control provides a great challenge for plant pathologists and phytomicrobiologists (Mukerji, 1983).

The non-pathogenic micro-organisms may have significant effect on the folliar pathogen and this has attracted many workers to study the interaction of saprophytes and pathogen to explore the possibility of biological control of folliar plant pathogen. According to Hudson (1978) "The first colonizers of leaf are usually parasites but their development may be affected by the saprophytic microbial population in the phyllosphere. These population increase in numbers as leaves mature and senesce and may influence disease incidence directly by competing with the parasites for space or nutrients by stimulating or inhibiting spore germination, by exerting some form of antagonism or even by altering the surface of leaf." Mukerji (1983) found that the presence of Alternaria tenuissima, a saprophyte on leaves of Phaseolus inhibited Alternaria zinniae which is a pathogen. According to him this inhibition was due to the production of some inhibitory substances by A. tenuissima.

Skidmore and Dickinson (1976) described the possibility of different types of interaction between paired fungi on agar media. Porter (1924) made a detailed study of various types of inhibition shown by more than 100 fungal species on agar media and he finally selected one isolate for testing in vivo. This is still one of the common methods of selecting antagonists in vitro and the production of antibiotics by the antagonists was far long considered as the only relevant mechanisms (Fokkema, 1976).

He, however, pointed out the serious problem concerning the decision to disregard potential antagonists on the basis of their failure to cause inhibition on agar. According to him, Cladosporium sp., Sporobolomyces sp., and Aureobasidium sp. differed greatly in their induction of inhibition zones with Cochliobolus sativus on agar medium but found equally effective in reducing infection by C. sativus on rye. Bhatt and Bahuguna (1963) showed that Penicillium, Aureobasidium and Cladosporium inhibited Botrytis cinera on agar showing distinct inhibition zone.

Literature on the antagonistic abilities of common phylloplane fungi showed that a lot of research in control environment will be needed to obtain antagonistic mycoflora so that enhanced biological control may become practically possible. Prior to this, emphasis should be given on finding which organisms were present and on elucidating successions which occur during growing season and stage of the plant since these backgrounds are essential for the exploitation of biological control (Fokkema, 1976).

Knowledge of interaction studies in vitro particularly the dual culture may be useful in determining the pattern of colonization of various saprophytic and pathogenic fungi on the natural leaf surface habitat. Biocontrol of several disease with phylloplane micro-organisms has been investigated and shown to have some promise .(Blakeman and Fokkema, 1982). However, there is paucity of references on the antagonists of Colletotrichum lindemuthianum - the causal agent of the leaf spot disease of french bean. Therefore, the purpose of this study is to see the antagonistic activities of certain saprophytic fungi isolated from five different cultivars of french bean towards C. lindemuthianum in vitro in relation to colony interaction, spore germination and the saprophytes antibiotic activities in spore germination and mycelial growth.

MATERIALS AND METHODS

Colletotrichum lindemuthianum (sacc. and Magn.) Br. and Cav., the causal organism of leaf spot disease, isolated from the leaf of French bean (Phaseolus vulgaris L.) mentioned in chapter I was taken as pathogen and the following fungi isolated from the leaf surface of the host plants mentioned in chapter III, maintained in pure culture were used as saprophytes for the interaction studies: Aspergillus flavus, Fusarium semitectum, Torula herbarum, Paecelomyces variotii, Alternaria tenuis, Curvularia lunata, Pestalotia macrotricha, Cunninghamella elegans, Acremonium cerealis, and Penicillium wortmanii.

The pure culture of pathogen and the epiphytic fungi was obtained in Czapek's Dox Agar. All test fungi and pathogen incubated at 25±1°C were 7 days and 15 days old respectively.

Spore germination studies

(i) Three colony discs of the test fungi (5mm in diameter) were punched using sterile cork borer from the periphery of the colonies cultured for 7 days at 25±1°C in petridishes (9.50cm dia.). The discs were placed invertedly in a small empty petriplates and seeded with 0.025ml of washed and centrifuged (1200rpm for 10 min.) C. lindemuthianum spores. The spore suspension of the pathogen was prepared by adding 15ml sterile distilled water to a two week old colony of the fungus grown on a Mathur's Agar plate and surface of the colony was scrapped to dislodge the spores. The suspension was adjusted to $1x10^7$ spores per ml of water. As a control, three agar discs from uninoculated plates kept at 25±1°C for 7 days were also seeded with the C. lindemuthianum spores in the same manner. Three replicates were maintained for all the test fungi.

Observation of 100 C. lindemuthianum spores chosen at random on disc with and without the test fungi after 24 hours of incubation at 25±1°C was taken and the percentage inhibition of spore germination was calculated by formula given below:

$$G_i = \frac{G_c - G_t}{G_c} \times 100$$

where,

G_i = inhibition of spore germination (%)

G_c = spore germination in control discs(%)

G_t = spore germination on discs with test fungi(%)

(ii) The spore suspension (1×10^7 spore per ml in sterile distilled water) of the saprophytes (s) and pathogen (P) was mixed in different proportion (S:P = 3:1, 3:2, 2:3, 1:3) and the percentage germination of the pathogen was determined by germinating the spores in hanging drop slides incubated at 25±1°C for 24 hours. 10 replicates with control set in sterile distilled water were maintained.

In another set of experiment equal volume of the spore suspension of the pathogen and saprophytes was mixed and hanging drop slide was prepared with 5 replicates to examine the percentage of spore germination and germ tube length of the pathogens at different incubation time

(12 hours, 24 hours and 48 hours) with triplicate control set in sterile distilled water.

Mycelial growth

To see the effect on mycelial growth of the pathogen, colony discs (5mm dia.) punched using sterile cork borer from the periphery of the test fungi and the pathogen grown in Czapek's Dox Agar were placed invertedly side by side (3.5cm apart) in a freshly prepared petriplate containing the same media with 6 replications.

Observations on the radial growth of the pathogen were made along two axes from colony centre upto the remote periphery and adjoining side of the test fungus disc. The former represents the "normal" and the latter "influenced" growth. Five replicates were maintained for each test fungus. Percentage inhibition of the mycelial growth of C. lindemuthianum was calculated using the formula given by Heuvel (1970) as follows:-

$$M_i = \frac{M_c - M_t}{M_c} \times 100$$

where,

M_i = inhibition of mycelial growth(%)
M_c = 'normal' mycelial growth(mm) and
M_t = 'influenced' mycelial growth(mm)

Assessment of antibiotic activity of the saprophytes

(i) A 5mm inoculum disc, each 7 days old of the test fungus grown on Czapek's Dox Agar at 25±1°C was centrally inoculated over the asceptically placed single sterilized 50µm thick cellophane in the prepared petriplates containing 15ml Czapek's Dox Agar, kept overnight to let excess of moisture to evaporate and incubated under a bank of light at the same temperature for two days letting metabolites (antibiotics) to diffuse through the cellophane into the medium. Subsequently the cellophane along with adhering inoculum disc was removed. A 5mm inoculum disc of the pathogen was then placed in the same position occupied by the antagonist in the aforesaid petriplates. Five replicates were maintained. In control, sterilized cellophane sheet was incubated for 2 days and inoculated with the pathogen after removing the cellophane. Observations were taken after 7 days of incubation at 25±1°C by measuring the radial growth of the treated and control sets.

(ii) To determine the antibiotic activity, saprophytes were cultured in liquid Czapek's medium for 30 days and filtrate through the microfilter was used for germinating pathogen spores ($1x10^7$ per ml) in hanging drop slides. The percentage germination and germ tube length of the pathogen spores were measured in culture filtrates of different test fungi after 24 hours of incubation at 25±1°C Two control sets - one 30 days old uninoculated Czapek's liquid medium and the other sterile distilled water were maintained for germinating pathogen spores.

(iii) Autoclaved and unautoclaved 10, 20 and 30 days old culture filtrate of different test fungi were used for germinating the pathogen spores. Observations on the percentage germination were taken after 24 hours. Two controls were maintained, one in sterile distilled water and the other in Czapek's liquid medium.

(iv.) The pathogen was allowed to grow near the periphery of the petriplates containing Czapek's Dox Agar medium for 2 days. Four folds of sterilized filter paper discs, 8mm diameter tied together and socked in 20 and 30 days old culture filtrate of the test fungus (Autoclaved and unautoclaved) were placed near the opposite side of the pathogen in the petriplates. Observations were taken on the percentage inhibition in radial growth of the pathogen by comparing the petriplates containing filter paper disc soaked in culture filtrates of the antagonists with the control after a week of incubation at 25±1°C. Five replicates were maintained.

(v) The autoclaved and unautoclaved 15 and 30 days old culture filtrates of different concentrations (25, 50, 75, 100%) were prepared by incorporating required quantity of autoclaved and unautoclaved Czapek's Dox Agar medium and poured into the petriplates which were then inoculated with the pathogen. In control, uninoculated Czapek's liquid medium mixed with Czapek's Dox Agar medium was inoculated with the pathogen. Inhibition in radial growth of the pathogen was measured after a week of incubation at 25±1°C by comparing the control and the treated set.

RESULTS

1. Effect on spore germination of the pathogen

(a) Germination of Pathogen spores on the agar disc of test fungi:

The result of the percentage inhibition of pathogen spores germination exerted by 10 test fungi belonging to different genera on the agar disc is depicted in fig.4.1 which showed that the degree of inhibition ranged from 8 to 35%. Among the test fungi tested, Torula herbarum showed higher (35%) inhibition followed by Paecelomyces variotii (31%), Cunninghamella elagans (26%), Curvularia lunata (23%), Fusarium semitectum (20%), Pestalotia macrotricha

(18%), Alternaria tenuis (17%), Aspergillus flavus (15%), Acremonium cerealis (9%) and Penicillium wortmanii (8%).

(b) **Slide germination of Pathogen and saprophytes spore mixed in different proportions:**

The result on germination of spores of the pathogen when mixed with the spores of test fungi in different proportions has been presented in table 4.1. It showed that certain degree of inhibition was exerted by all the test fungi. Percentage germination of pathogen spores was considerably reduced when the test fungus spores and the pathogen spores were mixed in 3:1 ratio. However, the percentage germination of spores increased as the proportion of saprophytes spores decreased. The inhibitory effect was more in case of T. herbarum spore mixture at 3:1 proportion. However, the percentage germination increased as the proportion of the pathogen spores increases in all the test fungi and showed less percentage germination as compared to control even when the proportion of saprophyte(s) and pathogen(s) mixture was 1:3. Almost equal germination to the control was observed in case of Penicillium wortmanii at the proportional concentration of 1:3 between saprophytes and the pathogen. Rest of the test fungi showed certain degree of inhibition even at the 1:3 proportion

Table 4.1 Percentage germination* of Colletotrichum lindemuthianum after 24 hours when spores mixed in different proportion of test fungi.

Test Fungi	Proportions			
	I	II	III	IV
Aspergillus flavus	25.39	31.26	35.62	40.32
Fusarium semitectum	19.25	21.36	30.67	35.29
Torula herbarum	12.26	15.29	29.19	32.26
Paecelomyces variotii	15.23	21.34	29.36	32.46
Alternaria tenuis	29.26	32.05	33.97	43.02
Curvularia lunata	31.04	39.26	40.01	45.01
Pestalotia macrotricha	28.37	29.30	41.00	45.29
Cunninghamella elegans	17.02	29.16	35.03	39.05
Acremonium cerealis	20.01	25.03	39.08	42.39
Penicillium wortmanii	39.26	45.1	50.32	65.29
Control (sterile distilled water)	66.50			

*Germination based on 300-500 spores counted.

I S:P	II S:P	III S:P	IV S:P
3:1	3:2	2:3	1:3

of spore mixtures.

Table 4.2 showed the percentage germination and germ tube length of the pathogen when mixed in equal proportion with the spores of the 10 test fungi at three different incubation periods. It showed increasing trend in germination and germ tube extension as the incubation period increases from 12 hours to 48 hours. T. herbarum, and Paecelomyces variotii showed more inhibition at the 24 hours of incubation times. Besides these two fungi, other test fungi also showed general trend of inhibition in the spore germination and germ tube extension.

(2) **Effect on mycelial growth of the pathogen:**

The percentage inhibition on the radial growth of the pathogen and the types of colony interaction exerted by each test fungus has been depicted in fig.4.2. It showed that the type of colony interaction as well as the percentage inhibition differed with the different test fungi. Assessment of colony interaction is depicted in fig.4.3.

The nature of colony interaction of C. elegans and A. cerealis was of the mutual intermingling with no sign of interaction type (A). F. semitectum showed complete overgrowth type (C). P. varioti and T. herbarrum indicated

Table 4.2 Spore germination** and germ tube length* of Colletotrichum lindemuthianum when mixed with the spores of different test fungi in equal proportions at different period of incubations.

Test Fungi	Incubation Period (Hours)					
	12 hours		24 hours		48 hours	
	Germination (%)	Germ tube length(μ)	Germination (%)	Germ tube length (μ)	Germination (%)	Germ tube length (μ)
Aspergillus flavus	2.47	5.94	25.63	47.52	26.39	99.01
Fusarium semitectum	2.63	2.57	22.96	51.48	27.32	110.88
Torula herbarum	1.45	2.04	19.27	28.76	20.04	91.08
Paecelomyces variotii	1.81	2.94	21.05	37.72	24.14	95.04
Alternaria tenuis	3.27	5.94	26.83	59.43	29.30	110.88
Curvularia lunata	3.26	11.88	29.64	67.32	34.25	122.76
Pestalotia macrotricha	3.45	7.24	23.25	63.36	40.32	138.62
Cunninghamella elegans	3.61	3.92	37.27	65.34	39.34	134.64
Acremonium cerealis	2.25	5.24	37.49	47.52	41.12	128.79
Penicillium wortmanii	3.81	3.70	39.20	49.53	56.32	162.36

**Percentage germination based on 300-500 spores.

* Average germ tube length based on 100 spores in μ

a mutual inhibition at a distance and therefore assigned type (E). Most of the other fungi were of the type (D) showing mutual slight inhibition. However, overgrowth of pathogen by antagonist was not observed in any of the test fungi.

The percentage inhibition of mycelial growth by T. herbarum and P. variotii was found to be slightly higher as compared to other test fungi. F. semitectum apparently showed complete overgrowth but still the pathogen was found to grow underneath of the antagonist. Narrow inhibitory zone was observed in case of P. variotii and T. herbarum. Percentage inhibition by other test fungi was very low.

(3) Assessment of antibiotic activity towards spore germination and mycelial growth:

(a) Spore germination:

(i) The germ tube length and the spore germination of C. lindemuthianum in 30 days old culture filtrate of different test fungi showed that T. herbarum and P. variotii inhibited the germination and germ tube length of pathogen considerably (Table 4.3). The other test fungi too showed certain degree of inhibition.

(ii) When culture filtrate of different test fungi

Table 4.3 Germ tube length and germination of Colletotrichum lindemuthianum spores in 30 days old culture filtrate of different test fungi after 24 hours.

Test Fungi	Germination*(%)	Germ tube length**(μ)
Aspergillus flavus	38.25±1.68	52.37
Fusarium semitectum	40.25±1.96	65.28
Torula herbarum	29.30±2.01	37.12
Paecelomyces variotii	29.35±1.23	40.34
Alternaria tenuis	35.25±2.12	65.34
Curvularia lunata	34.19±1.79	72.37
Pestalotia macrotricha	32.56±2.18	72.13
Cunninghamella elegans	40.19±1.78	75.27
Acremonium cerealis	39.35±1.06	59.32
Penicillium wortmanii	59.39±2.16	65.32
Control (uninoculated 30 days old Czapek's Dox liquid medium)	69.25±1.98	92.37
Control (sterilized distilled water)	65.75±2.26	85.24

*Average germination based on 300-500 spores.

* Average germ tube length based on 100 spores.

collected at 10, 20 and 30 days interval were used for spore germination of the pathogen, it was found that germination was lowest in 30 days culture filtrate compared to 10 and 20 days interval culture filtrate both in the autoclaved and unautoclaved sets. However, the comparative result of autoclaved and unautoclaved sets indicates that germination was less in unautoclaved culture filtrate than the autoclaved. Similar results were obtained in 10 and 20 days culture filtrates (autoclaved and unautoclaved). The percentage germination was found comparatively less in *T. herbarum* and *P. variotii* culture filtrate. Other test fungi also exerted slight inhibition on the spore germination as shown in table 4.4.

(b) **Mycelial growth:**

(i) The inoculum disc of the test fungi over the cellophane membrane when replaced by the pathogen, it was found that 15% and 10% of the radial growth of the pathogen was inhibited by *T. herbarum* and *P. variotii* respectively. The other test fungi such as *A. flavus*, *P. macrotricha* and *C. elegans* exhibited quite a low percentage of inhibition whereas the other test fungi did not show any sign of inhibition (table 4.6).

(ii) Percentage inhibition in radial growth of

Table 4.4 Percentage germination* of Colletotrichum lindemuthianum spores after 24 hours in autoclaved and unautoclaved culture filtrate of different test fungi collected at different time intervals.

Test Fungi	Time (days) intervals of filtrate collection					
	10		20		30	
	A	UA	A	UA	A	UA
Aspergillus flavus	45.5	45.00	43.5	42.00	40.00	38.25
Fusarium semitectum	49.35	46.39	45.20	43.19	42.05	40.25
Torula herbarum	35.21	34.30	33.10	30.39	30.50	29.30
Paecelomyces variotii	38.25	32.19	32.35	29.37	30.15	29.35
Alternaria tenuis	40.32	39.42	40.15	39.40	37.34	35.25
Curvularia lunata	45.34	41.25	41.00	39.25	35.36	34.19
Pestalotia macrotricha	39.75	38.34	35.39	34.30	32.19	32.56
Cunninghamella elegans	51.35	50.45	45.32	45.19	43.45	40.19
Acremonium cerealis	47.42	46.35	45.34	42.39	40.35	39.35
Penicillium wortmanii	61.34	60.95	60.84	61.74	62.30	59.39
Control(Czapek's liquid medium)	69.25	69.25	69.25	69.25	69.25	69.25
Control(sterilized distilled water)	65.75	65.75	65.75	65.75	65.75	65.75

* Germination based on 300-500 spores

A = autoclaved

UA = Unautoclaved

the pathogen ranged from 10-20% when filter paper discs soaked in 20 and 30 days old culture filtrate (autoclaved and unautoclaved) of T. herbarum were kept near the periphery of the pathogen inoculated petriplates (table 4.5). Comparative result showed that percentage inhibition was less in autoclaved than in the unautoclaved culture filtrate.

(iii) When 25 to 100% of the culture filtrate of T. herbarum was incorporated into the medium and the pathogen was allowed to grow on it, it was found that percentage inhibition in radial growth of the pathogen though quite less showed increasing trend of inhibition as the concentration of the culture filtrate increases both in the autoclaved and unautoclaved as well as with the increase of incubation period of the culture filtrate from 15 to 30 days (Fig.4.4).

DISCUSSION

Almost all the test fungi exerted some degree of inhibition on agar discs as well as in hanging drop experiments. The general inhibitory effect shown in agar disk could possibly be due to the exposure of spores to substrate (agar disc) which might have developed an unfavourable pH or might have contained antifungal substances.

Table 4.5 Percentage inhibition of *Colletotrichum lindemuthianum* by *Torula herbarum* culture filtrate soaked filter paper discs.

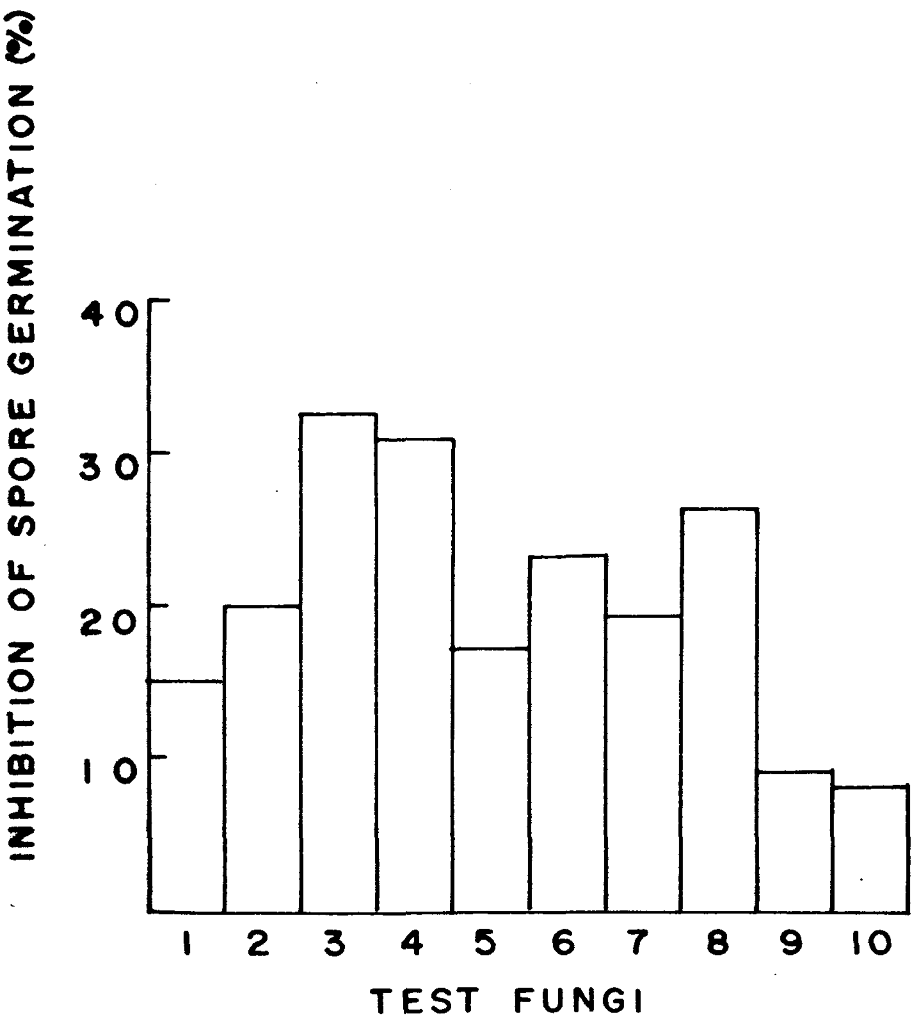
Filter paper discs soaked in:	% inhibition in radial growth of the pathogen
20 days old autoclaved culture filtrate of *Torula herbarum*	10
20 days old unautoclaved culture filtrate of *Torula herbarum*	15
30 days old autoclaved culture filtratrate of *Torula herbarum*	12
30 days old unautoclaved culture filtrate of *Torula herbarum*	20

Table 4.6 Percentage inhibition of Colletotrichum lindemuthianum when it replaces the test fungi grown on the cellophane.

Test Fungi	*% inhibition*
Aspergillus flavus	5
Fusarium semitectum	-
Torula herbarum	15
Paecelomyces variotii	10
Alternaria tenuis	-
Curvularia lunata	-
Pestalotia macrotricha	6
Cunninghamella elegans	5
Acremonium cerealis	-
Penicillium wortmanii	-

Fig.4.1 Showing the percentage inhibition of the germination of pathogen (Colletotrichum lindemuthianum) spores on agar exerted by the following test fungi:

(1) Aspergillus flavus
(2) Fusarium semitectum
(3) Torula herbarum
(4) Paecelomyces variotii
(5) Alternaria tenuis
(6) Curvularia lunata
(7) Pestalotia macrotricha
(8) Cunninghamella elegans
(9) Acremonium cerealis,
(10) Penicillium wortmanii



INHIBITION OF SPORE GERMINATION (%)
40
30
20
10
1
2
3
4
5
6
7
8
9
10
TEST FUNGI

FIG. 4·1

Fig.4.2 Showing percentage inhibition of mycelial growth of Colletotrichum lindemuthianum by the following test fungi:

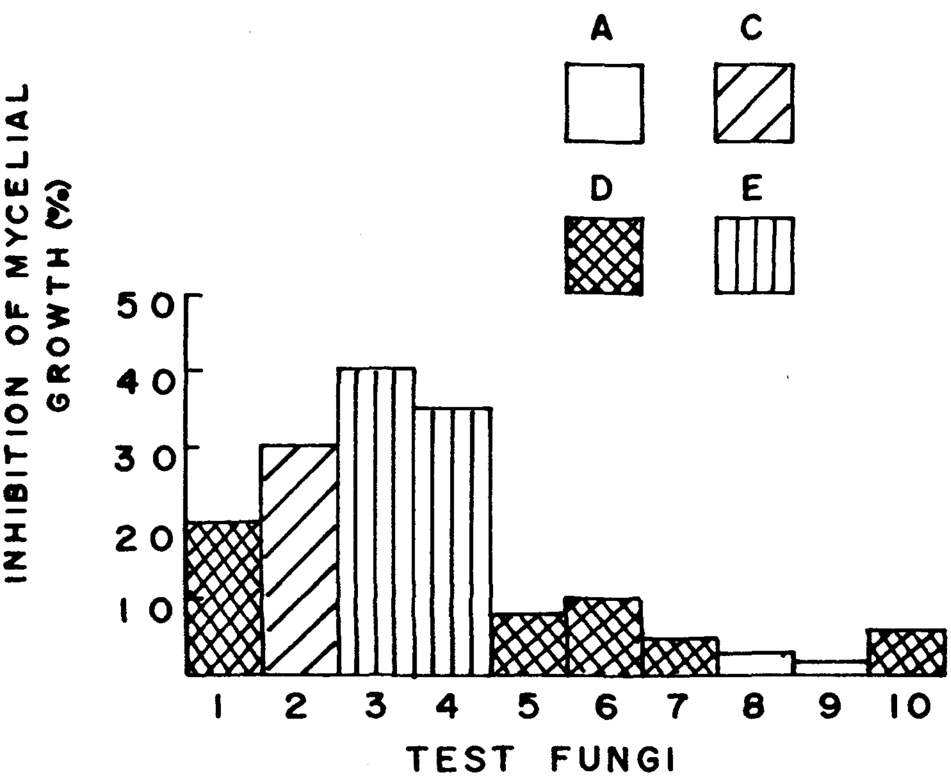
1. Aspergillus flavus
2. Fusarium semitectum
3. Torula herbarum
4. Paecelomyces variotii
5. Alternaria tenuis
6. Curvularia lunata
7. Pestalotia macrotricha
8. Cunninghamella elegans
9. Acremonium cerealis
10. Penicillium wortmanii

A = Mutual intermingling with no sign of interaction

C = Complete overgrowth by antagonist

D = Mutual slight inhibition when they are almost in contact.

E = Mutual inhibition at a distance showing clear zone of inhibition.



A
C
D
E
INHIBITION OF MYCELIAL GROWTH (%)
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
TEST FUNGI

FIG. 4·2

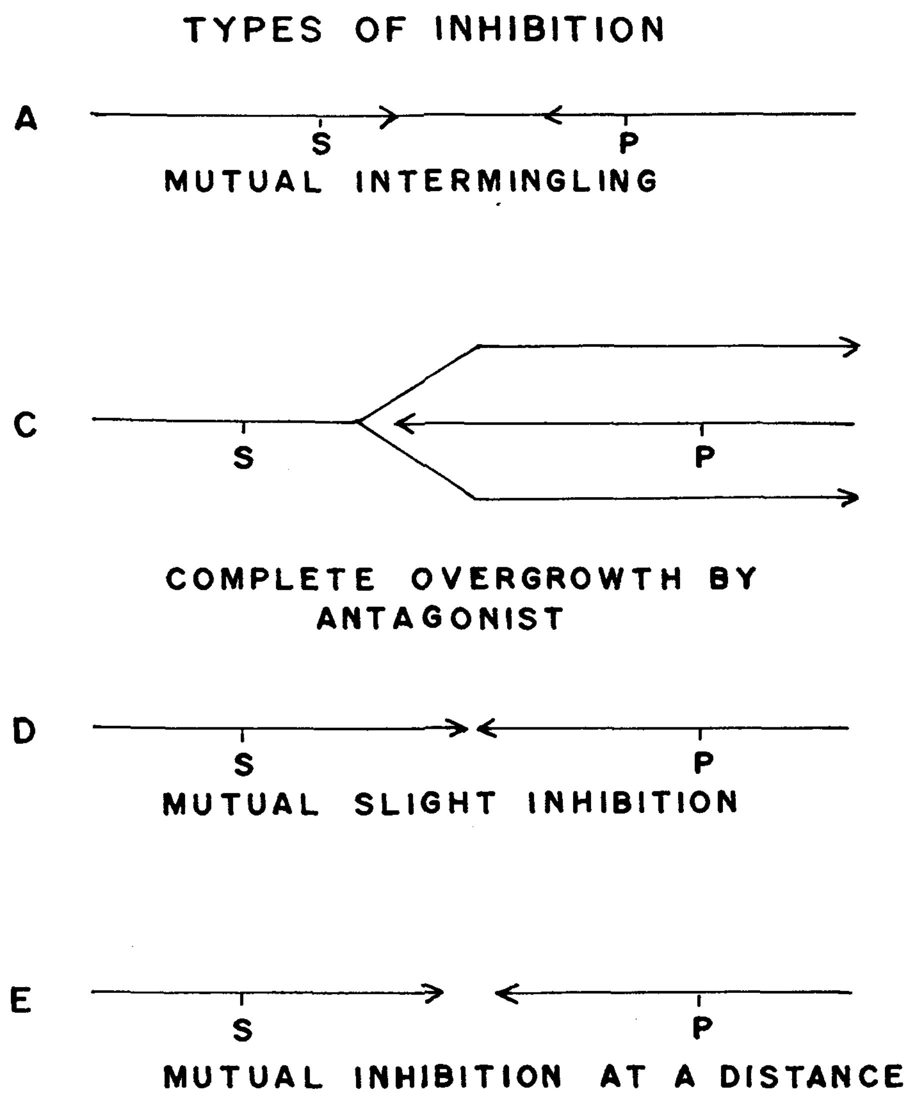

TYPES OF INHIBITION
A
S
P
MUTUAL INTERMINGLING
C
S
P
COMPLETE OVERGROWTH BY ANTAGONIST
D
S
P
MUTUAL SLIGHT INHIBITION
E
S
P
MUTUAL INHIBITION AT A DISTANCE

FIG. 4·3

Fig.4.4 Showing the effect of various concentrations of outoclaved and unautoclaved 15 day old and 30 day old culture filtrates of *Torula herbarum* on the percentage inhibition of radial growth of the pathogen - *Colletotrichum lindemuthianum*.

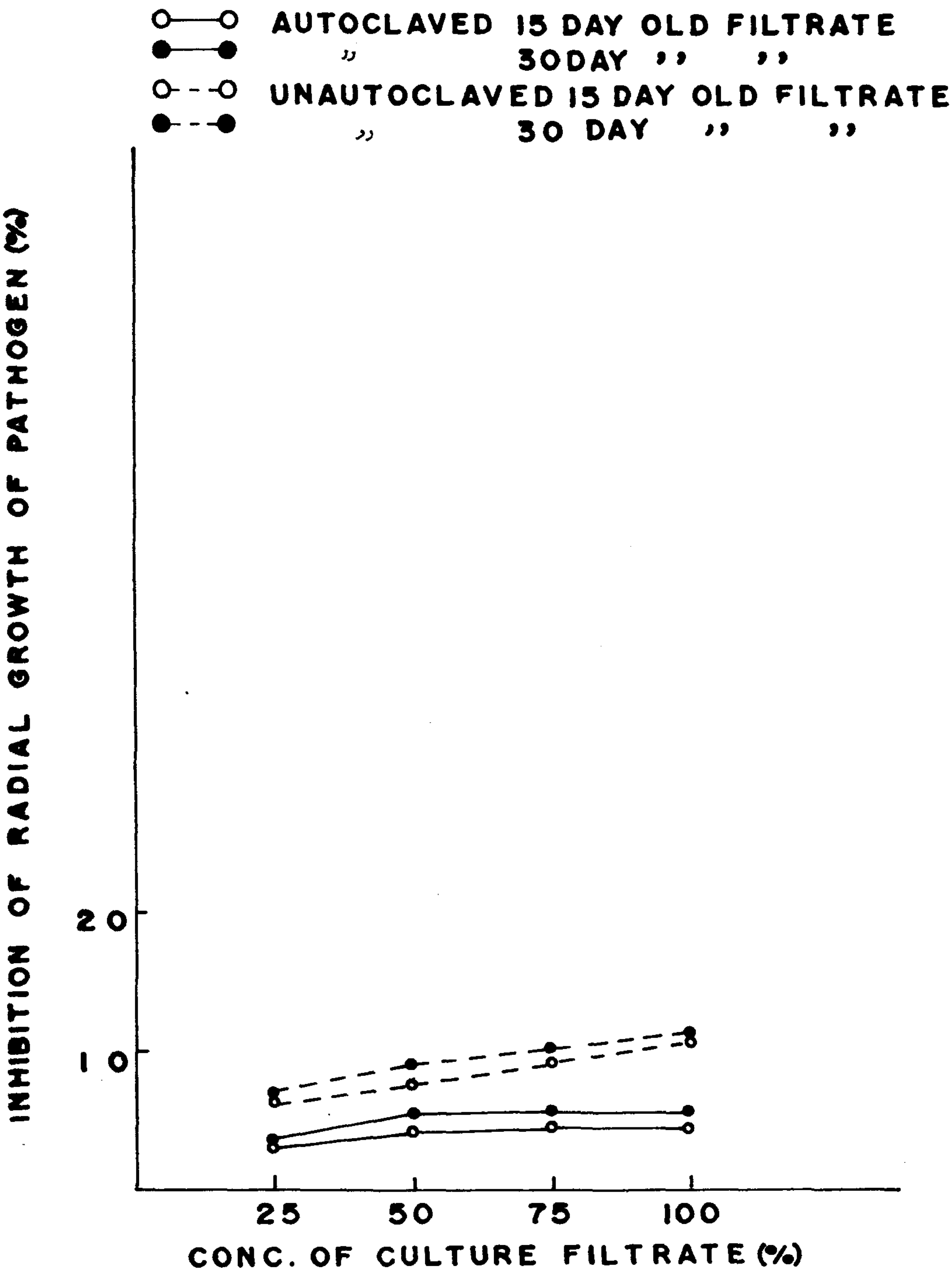

AUTOCLAVED 15 DAY OLD FILTRATE
" 30DAY " "
UNAUTOCLAVED 15 DAY OLD FILTRATE
" 30 DAY " "
INHIBITION OF RADIAL GROWTH OF PATHOGEN (%)
20
10
25
50
75
100
CONC. OF CULTURE FILTRATE (%)

FIG. 4·4

The inhibition of spore germination of pathogen when mixed with different proportions of spores of saprophytes could be due to the suppressive effect of fast germinating spores of saprophytes over the pathogen spores which germinate only after 9 to 12 hours (Martinez et al, 1957). Further the fast germinating spores might consume the available oxygen resulting less availability of oxygen to the later germinating pathogen spores. Gottlieb (1950) observed a sharp increase in oxygen consumption associated with the germination of spores. Similar explanation could be attributed to Torula herbarum and Paecelomyces variotii which showed slightly more inhibition compared to other test fungi when spores of both the saprophytes and pathogen were mixed in equal proportion. Upadhyay (1981) had observed the germinating spores of phylloplane microfungi and Pestalotiopsis funerea, leaf spot pathogen of Eucalyptus in vitro by mixing them in equal proportion, and found that Aspergillus flavus, Fusarium oxysporum and Penicillium oxalicum were most effective in causing more than 60% inhibition of spore germination of the pathogen.

In dual culture experiment four different kinds of interactions were observed which indicate that the antagonistic effect of leaf saprophytes on the pathogen differed between different species and different types of interactions are possible between paired fungi on agar media. Similar interactions have been shown by many workers (Heuvel, 1970; Skidmore, 1976; Purkayastha and Bhattacharya, 1982). P. variotii showed a marked aversion that might indicate an antagonistic reaction. Purkayastha and Bhattacharya (1982) attributed the well marked aversion of Aspergillus nidulans and Penicillium oxalicum against Colletotrichum corchori as the antagonistic reaction in paired culture.

When antibiotic activity was tested by different experiment it was found that no significant inhibition was exerted by the different test fungi towards pathogen. The general slight inhibition could be due to the changes in pH or due to the presence of certain antifungal substances in the culture filtrates of different test fungi. However, no effort was made to identify and isolate the tentative possibility of the presence of antifungal substances. Since it is a known fact that fungi produces certain volatile components inhibitory to the growth of the other fungi (Dennis and Webster, 1971b). Robinson and Garrett (1969) have also described the volatile sporostatic substances produced by Fusarium oxysporum, Rhizopus stolonifer, Penicillium expansum and Geotrichum candidum. Similarly, Heuvel (1971) reported the production of inhibitory substance by a Phaseolus leaf saprophyte Alternaria tenuissima which inhibit the leaf pathogen Alternaria zinniae. Allen (1955) demonstrated the production of toxic substances in spores of Uromyces phaseoli and Puccinia graminis tritici which inhibit the germination. However, Dorran (1922) and Lilly and Barnett (1951) were of the opinion that inhibition of spore germination was due to the competition of the limited supply of oxygen rather than the toxic substances produced by the germinating spores. In C. lindemuthianum also the general inhibition shown by different test fungi probably could be due to the secretion of inhibitory substances into the medium as well as the limited supply of oxygen in the culture filtrates. Vasudeva et al (1961) observed that aeration is a factor of considerable importance in the germination of Colletotrichum falcatum spores.

CHAPTER - V

SPATIAL DISTRIBUTION, SAMPLING PLAN AND ANALYSIS OF VARIANCE OF LEAF LESIONS

INTRODUCTION

The new concept in ecology and pest management in relation to entomological research may be useful in advancing the knowledge of quantitative aspect of plant disease but many of these new ideas are not applied in plant pathology (Strandberg, 1973). One such concept is spatial distribution of organisms, most of which are distributed in aggregation (Waters, 1959; Southwood, 1966). Such aggregated pattern of several type of organisms are better described by the negative binomial distribution (Bliss and Fisher, 1953). Distribution pattern has tremendous contribution in the estimation of population mean and formulation of sampling plan (Bliss, 1958; Waters, 1959). Information on distribution patterns and their change over time are essential for choice of an appropriate sampling technique and better disease assessment.

Spatial distribution of organisms are characterize by two methods such as theoretical distribution using χ^2-test and the other is using the relation between mean crowding index ($\overset{*}{X}$) (Lloyd, 1967) and Iwao's regression technique (Boivin and Vincent, 1987).

Sequential sampling was developed during the second world war (Wald, 1945 and 1947) and it is useful in determining whether the pest population is above or below a certain economic threshold (level of the population above which intervention become necessary) by reducing the number of sample required and thereby reducing time and labor cost associated with the disease monitoring while staying within a predetermined error level instead of giving an exact estimate of the disease level (Southwood, 1966; Kuno, 1969; Waters, 1955). When the economic threshold is unknown, sequential sampling plan can be formulated on the basis of preliminary action level threshold (Lincoln, 1978).

In the sequential sampling plan two statistical errors i.e., Type I and Type II errors are made. Most of the sequential sampling plan have error levels of about 0.05 to 0.1 (Boivin and Vincent, 1987).

Few quantitative studies of spatial distribution of disease in some economically important crops are available but not of Colletotrichum leaf lesions (Strandberg, 1973; Byrde et al 1973; Campbell and Pennypacker, 1980; Boivin and Sauriol, 1984).

The objective of the present investigation was to determine the spatial distribution of *Colletotrichum* leaf lesions in French bean leaves using both the methods mentioned above. Secondly we were interested to know the effect of different fungicides on the distribution pattern of the leaf lesions. The spatial distribution parameters obtained from Iwao's regression technique were used to prepare a sequential sampling plan for the disease and the feasibility for obtaining the estimate of disease level in the field has been discussed. The knowledge of the spatial distribution of leaf lesions has also been used in the analysis of variance of the mean number of leaf lesions on three varieties to determine the significant varietal differences with regard to the susceptibility of the leaf lesions caused by *C. lindemuthianum*.

MATERIALS AND METHODS

Spatial distribution and sampling plan

The present investigation was conducted in the University garden. A net experimental area measuring 148 m^2 (4 x 37m) was divided into 40 blocks each measuring 1.8 m^2 (3 m x 0.6m). Each block was further sub-divided into three plots of size 0.60 m^2 (100cm x 60cm) for growing the experimental material (French bean). After well preparation of the land, seeds of the three susceptible varieties of French bean viz. Shillong local selection(V_1), Manipur variety (V_3), and premier variety (V_4) were sown in the plots using random numbers. Plant to plant and row to row distance was maintained at 25cm and 30cm apart respectively.

Initially high seed rate was applied to ensure proper plant stand and after 7 days of germination thinning was done maintaining the distance of plant to plant and row to row as mentioned above. Sampling was done at weekly interval after 20 days of germination. At each sampling, 10 leaves were randomly selected from the population of each variety in a plot having 40 replications. Therefore, observations on 400 leaves were made at each pre-fungicide sampling for each variety. The sampling unit consisted of leaf (prematured and matured) and the datum recorded was the number of characteristic Colletotrichum leaf lesion in it. To determine the nature of leaf lesion distribution the count data i.e. the number of leaf lesions on the leaves (including non-occurrence of leaf lesion = 0) was recorded weekly and subjected to the various statistical tests. The spatial distribution of leaf lesions was studied using Iwao's patchiness regression technique and theoretical distribution model.

(A) **Iwao's patchiness regression technique:**

Iwao's patchiness regression parameters were calculated using the relation given by Lloyd (1967).

$$\overset{*}{X} = \bar{X} + (S^2/\bar{X}) - 1$$

where,

$\overset{*}{X}$ = the mean crowding of the sample

$\bar{X}$ = the mean number of lesion per leaf, and

S^2 = the variance of the number of lesion per leaf.

At each sampling the mean number of lesions per leaf and variance were determined and used to calculate the mean crowding of sample ($\overset{*}{X}$) as mentioned above. The relationship between mean density ($\bar{X}$) and mean crowding ($\overset{*}{X}$) can be described by a simple linear regression of $\overset{*}{X}$ on X ($\overset{*}{X} = b_o + b_1 \bar{X}$), which quantifies the aggregation of individuals (Iwao, 1968).

In the regression equation on $\overset{*}{X} = b_o + b_1 \bar{X}$, there are two parameters that describe the type of spatial distribution of the organisms : the Y-intercept of the regression line (b_o) which is the index of basic contagion, and the slope of the regression (b_1) which is the density contagiousness coefficient. The first of these parameters characterize the basic unit of the population, whereas the second characterizes how the basic units are distributed in space. A single individual is the basic component of the distribution if $b_o = 0$ and when b_o is > or < o, it indicates a positive or negative association between individuals. When $b_1 < 1$, the distribution is regular in space; when $b_1 = 1$ the basic components are randomly distributed in space and when $b_1 > 1$, the basic components are distributed contagiously in space (Boivin and Sauriol, 1984). The regression of $\overset{*}{X}$ on $\bar{X}$ was calculated for each variety for pre-fungicide and post fungicide and also the pooled regression of all varieties. The value of b_o and b_1 were tested for significance difference from 0 and 1 by means of t-test (Chatterjee and Price, 1977).

Sequential sampling plan:

The sequential sampling plan is to ascertain the maximum number of samples required for the determination of disease level whether it is below, or above or equal to the economic threshold necessary for intervention of chemical control operation was prepared following the Iwao's procedure. The observed pre-fungicide regression parameters ($b_o = 2.132$ and $b_1 = 2.921$) of the spatial distribution of C. lindemuthianum leaf spots on French bean leaves in terms of regression of mean crowding ($\overset{*}{X}$) over the mean ($\bar{X}$) together with a provisional or action level economic threshold (i.e., the level at which fungicide is to be applied) of one leaf spot per leaf and the error level α =0.1 were used in the sequential sampling procedures. The student's t-test for an error level of α =0.1 for two tailed test at an infinite number of degrees of freedom was chosen to calculate the maximum number of samples in the sequential sampling plan.

Curves of the upper and lower acceptance limits of sequential sampling plan:

Since dispersion statistics of Colletotrichum leaf spots was known and after checking the regression validity in terms of significant correlation coefficient, the dispersion statistics were used to calculate the upper acceptance and lower acceptance limit curves of sequential sampling plan following the equations of Iwao (1975) and Southwood (1978) as follows:

$$T_{upper} = nxA' + t\,[n(b_o+1)A'+(b_1-1)A'^2]^{\frac{1}{2}}$$

$$T_{lower} = nxA' - t[n(b_o+1)A'+(b_o-1)A'^2]^{\frac{1}{2}}$$

where T = total count of lesions, n = number of samples taken, A'=economic threshold (action level) and t = value of students t - test at chosen level of significance for a two-sided test at an infinite number of degrees of freedom. b_o=index of basic contagion (Y-intercept), b_1=density-contagiousness coefficient (slope).

Maximum number of samples:

The maximum number of samples to be taken was calculated by

$$N(max) = \frac{t^2}{d^2} [(b_o+1)A' + (b_1-1)A'^2]$$

where d = the confidence interval (d=0.5) allowed for the estimation of population density when the disease level (X) equals A' (action level). When this calculated N_{max} sample is observed before a sample stop line is crossed, the disease level may be X = A'±d.

(B) Theoretical distribution:

Before considering this distribution, the frequency data were subjected to the following preliminary tests for fitting the theoretical distribution:

(i) Variance - mean ratio test:

Here the mean ($\bar{X}$) and variance (S^2) of the count data was worked out and inferred if,

$S^2 = \bar{X}$, the distribution is poison or random,

$s^2 < \bar{X}$, the distribution is binomial or normal,

$s^2 > \bar{X}$, the distribution is negative binomial or contagious.

(ii) Third moment test:

It involves the calculation of the value of third moment (T) and comparing it with its standard error (SE(T)). These two quantities were calculated using the formula:

$$T = \left(\frac{\Sigma fx^3 - 3\bar{x}\Sigma fx^2 + 2\bar{x}^2\Sigma fx}{N}\right) - s^2 \left(\frac{2s^2}{\bar{x}} - 1\right)$$

$$SE(T) = \sqrt{2\bar{x}(r+1)\frac{\bar{x}^2}{r^2}(1+\frac{\bar{x}}{r})^2 \; [2(3+5\frac{\bar{x}}{r})+3r(1-\frac{\bar{x}}{r})]} \times (\sqrt{N})^{-1}$$

where,

X = the number of spots

f = the frequency

$\bar{X}$ = mean of the leaf spots

r = the clumping parameter or index of aggregation (as in negative binomial)

s^2 = the sample estimated variance

N = sample size

When SE(T) > (T), the leaf lesions are said to follow the negative binomial series.

Negative binimial distribution

For the negative binomial distribution, a random variable X is said to follow this distribution if its probability mass function is given by

$$f(x) = \begin{cases} \binom{r+x-1}{r-1} p^r q^x & \text{if } x = 0, 1, 2, \ldots \\ 0 & \text{Otherwise.} \end{cases}$$

That is, the probability P_x, that a frequency class X will contain 0,1,2 individual (i.e. leaf spots) is

$$P_x = \binom{r+x-1}{r-1} p^r, q^x$$

in which P = the probability of lesion occurrence ($0<p<1$), $q = 1-p$, r = relative index of aggregation (a positive integer) and x=frequency class r and P can be obtained from the relation of mean and variance of the distribution.

$$\text{Thus,} \quad \mu_1 = \frac{r.p}{p} \rightarrow \text{(a)}$$

$$\mu_2 = \frac{rq}{p^2} \rightarrow \text{(b)}$$

Solving (a) and (b) we get the value of P and q and hence $r = p/q \times \mu_1$.

Now, to test the goodness of fit, we should consider the recurrence relation formula, which is given by

$$P\ (x+1;\ r,p) = \frac{x+r}{x+1} .\ q.\ P(x)$$

In all cases, the probability of a given X was multiplied by N, the total number of observations counted to obtain the expected frequency of class with X individuals. Goodness of fit of expected frequency values to observe values for each distribution function was evaluated by χ^2 analysis

$$\chi^2 = \sum_{i=1}^{n} \left(\frac{O_{fi}^{\,2}}{E_{fi}} \right) - n$$

which follows χ^2 distribution approximately with (n-1) d.f. where,

O_{fi} = the observed frequency

E_{fi} = the corresponding expected frequency of the ith class (i = 1,2,.....n) and

N = the total frequency.

Post-fungicide spatial distribution of leaf lesions

To determine the effect of fungicide on the distribution pattern of leaf lesions, three fungicides viz: Mancozeb, Benomyl and Captafol at the concentration of 0.13g (% a.i) were applied thrice at weekly interval after pre-fungicide sampling with eight replication for each treatment. Observations on the distribution pattern of leaf lesions after a week of each spray were taken following the same procedure as used in the pre-fungicide spatial distribution mentioned before. However, three observations were taken after the final spray.

Analysis of variance

The occurrence of number of leaf spots on the three varieties of French bean recorded for the spatial distribution analysis was also subjected for the analysis of variance to test the significance difference of mean occurrence of leaf spots among three varieties for drawing a general inference of their degree of susceptibility to the disease using both the parameter and the non-parametric methods.

For the parametric test, usual F-test (one way and two way analysis of variance) was used and for the non-parametric counterpart the Kruskal-Wallis (one way analysis of variance) and the Friedman (two way analysis of variance) by ranks were followed. Computational formula for:

(a) Kruskal-Wallis method (corrected for ties) is given by:

$$H = \frac{\frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{R_j^2}{n_j} - 3(N+1)}{\frac{\Sigma T}{N^3 - N}}$$

where

k = number of samples

n_j = number of cases in j^{th} sample

$N = \Sigma n_j$, the number of cases in all samples combined

R_j = sum of ranks in j^{th} sample (column)

$\sum_{j=1}^{k}$ = sum over the k samples (columns).

$T = t^3 - t$ (when t is the number of tied observations in a tied group of scores)

ΣT = sum overall group of ties.

(b) Friedman's method is given by:

$$\chi_r^2 \quad \frac{12}{Nk(k+1)} \sum_{j=1}^{k} (R_j)^2 - 3N(k+1)$$

where,

N = number of rows

k = number of columns

R_j = sum of ranks in j^{th} column

$\sum_{j=1}^{k}$ = sum the squares of the sums of ranks over all k.

Protected rank sum test

Protected rank-sum test for multiple comparison of the non-parametric test based on Kruskal Wallis H-test was used to determine which pairs of populations differed significantly in mean disease occurrence. The analysis was quite similar to the normal curve procedures:

$$Z = \frac{T_1 - T_E}{\sigma_T}$$

$$= (T_1 - T_E)\ [\{\sqrt{N_1\ N_2 (N+1)}\}/12]^{-1}$$

Instead of comparing a sample mean (X) to a null hypothesized population value (μ) and dividing by the standard error of the mean in the normal curve procedure, comparison was made between the sample rank sum (T_1) to the null hypothesized expected value (T_E) and divided by the standard error of T. By doing so the sampling distribution of T may become approximately normal and therefore, one may determine whether the observed T_1 departed significantly from T_E by using the customary Z - procedure. N_1 and N_2 are the total number of observations in group 1 and 2 (i.e. varieties compared).

Since data of present investigation constitute frequencies in discrete categories, χ^2-test was also used to determine the significance of the differences among three varieties with respect to their frequencies of leaf spot occurrence by applying formula:

$$\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{k} \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

where,

O_{ij} = observed number of cases categorized in i^{th} row of j^{th} column.

E_{ij} = number of cases expected under H_o (that the three samples do not differ among themselves) to be categorized in i^{th} row of j^{th} column.

$\sum_{i=1}^{r} \sum_{j=1}^{k}$ = sum overall (r) rows and all (k) columns, i.e. to sum overall cells.

RESULTS

(i) Spatial distribution

(a) Spatial distribution of leaf spots based on Iwao's regression technique and sequential sampling plan: Result of the spatial distribution showed that the regression of $\overset{*}{X}$ on $\bar{X}$ had highly significant correlation coefficient in all the three varieties (Table 5.1). The intercept (b_o) and slope (b_1) were significantly different from 0 and 1. When data were pooled from all the three varieties and analysed, it was found that regression of $\overset{*}{X}$ on $\bar{X}$ ($\overset{*}{X}$ = 2.132+2.921$\bar{X}$) showed very high significant correlation (r=0.90) and the intercept (b_o=2.132) and slope (b_1=2.921) were also significantly different from 0 and 1 (Tables 5.1, fig. 5.1).

The upper (T_{upper}) and lower (T_{lower}) acceptance limits of the sequential sampling plan were found to be

$$T_{upper} = nx1 + 1.64\sqrt{nx5.053}$$

$$T_{lower} = nx1 - 1.64\sqrt{nx5.053}$$

Table 5.1 Showing statistics of the mean crowding ($\overset{*}{X}$) on mean ($\bar{X}$) regression for each of the three varieties and for data pooled from all the varieties (with and without fungicide treatment).

Varieties	Regression Parameters		Correlation coefficient $(r)^a$	Number of sampling occasions(n)
	Intercept (b_0)	Slope (b_1)		
V_1	5.328	2.031	0.92*	5
V_3	2.267	3.081	0.94*	5
V_4	1.072	2.782	0.93*	5
Pooled data from all varieties	2.132	2.921	0.90**	15
Benomyl treated pooled data from all varieties	1.628	2.522	0.83**	15
Mancozeb treated pooled data from all varieties	1.815	2.272	0.82**	15
Captafol treated pooled data from all varieties	2.431	2.372	0.81**	15

[a]Statistically significant at α=0.05 (*) and at α = 0.01 (**)

The sequential sampling plan generated by these two limits (T_{upper} and T_{lower}) required a maximum number (N_{max}) of 54 samples to know the disease level for chemical intervention (Fig. 5.2).

(b) Variance to mean ratio test for leaf spots distribution: The observed mean number of leaf spots and the variance about the mean for each set of data showed that variance was significantly greater than the mean (Table 5.2).

(c) Third moment (T) test of leaf spot distribution: The analysis of the third moment test showed that T value was less than its standard error (SE(T)) for each set of data (TAble 5.2).

(d) χ^2-test for the goodness of fit of negative binomial distribution of leaf spots: The discrete lesion count data were found to fit the negative binomial distribution (Fig.5.3-5.8). The χ^2 analysis showed that the probability of fit ranged from 0.01 to 0.50. The index of aggregation(r) of negative binomial distribution for lesion count data was observed to ranged from 0.10 to 0.45 (Table 5.2). Counts such as 0 and 1 were encountered with maximum frequencies whereas other higher values represented a long, flat tail to the figure that constituted a very small

Table 5.2 Showing the distribution and dispersion statistics of Colletotrichum leaf spots on French bean leaves (two sets of data for each variety)

Varieties	Mean ($\bar{X}$)	Variance (S^2)	Variance-mean ratio ($S^2/\bar{X}$)	Third moment test (T)	[SE (T)]	Clumping parameter (r)	Probability (P)	Cal. χ^2-value	d.f.	Probability of fit	*Distribution fitted
V_1 (S-1)	1.56	12.16	7.79	-2743.22	43.11	0.23	0.13	18.00	7	0.25-0.50	NBD
V_1 (S-2)	3.98	44.68	11.23	-22062.13	139.07	0.45	0.10	17.59	13	0.10-0.25	NBD
V_3 (S-1)	0.52	2.76	5.30	-192.88	8.63	0.12	0.19	6.74	3	0.05-0.10	NBD
V_3 (S-2)	1.07	4.91	4.58	-1009.91	8.56	0.30	0.22	8.5	5	0.10-0.25	NBD
V_4 (S-1)	0.62	2.13	3.44	-50.87	2.83	0.25	0.29	8.99	3	0.10-0.05	NBD
V_4 (S-2)	1.28	6.73	5.26	-1951.26	10.18	0.35	0.21	9.04	5	0.10-0.25	NBD

*NBD = Negative Binomial Distribution.

frequencies (Fig.5.3-5.8).

(ii) Effect of fungicide on the distribution pattern of leaf spots:

Table 5.1 showed that the regression parameters (b_o and b_1) of post fungicide treated pooled data from all varieties were not significantly different from the prefungicide pooled regression parameters (Fig.5.1). The result showed that the pattern of the distribution was not affected. Further statistical analysis of the fungicide treated data based on different statistical test also showed that the basic pattern of the distribution was not changed due to the application of fungicide. (Table 5.3-5.5; Fig. 5.9 & 5.10). However, the comparative result of the pre and post fungicide showed slight variation of different statistics such as mean ($\bar{X}$), Variance (S^2) and index of aggregation (r) in post-fungicide treated data (Table 5.6). Coefficient of variation (Cv) of the same showed that post fungicide data revealed less coefficient of variation than the pre-fungicide in all the post-fungicide treated data. However, ratios of the coefficient of variations between pre-fungicide and post fungicide treated with benomyl showed comparatively less than the other two fungicides such as Mancozeb and Captafol

Table 5.3 Showing the distribution and dispersion statistics of Colletotrichum leaf spots on French bean leaves (Mancozeb-treated).

Varieties	Mean ($\bar{X}$)	Variance (S^2)	Variance-mean ratio ($S^2/\bar{X}$)	Third moment test (T)	[SE(T)]	Clumping parameter (r)	Probability (P)	Cal. χ^2-value	d.f.	Probability of fit	*Distribution fitted
V_1	0.82	1.25	1.52	-0.26	0.615	1.72	0.67	1.47	1	0.10-0.25	NBD
V_3	0.70	1.75	2.50	-0.88	2.06	0.61	0.44	1.11	1	0.25-0.50	NBD
V_4	1.27	3.25	2.55	-3.67	3.99	0.85	0.40	2.61	2	0.25-0.50	NBD

*NBD = Negative binomial distribution.

Table 5.4 Showing the distribution and dispersion statistics of <u>Colletotrichum</u> leaf spots on French bean leaves (Benomyl treated).

Varieties	Mean ($\bar{X}$)	Variance (S^2)	Variance-mean ratio ($S^2/\bar{X}$)	Third moment test (T)	[SE(T)]	Clumping parameter (r)	Probability (P)	Cal. χ^2-value	d.f.	Probability of fit	*Distribution fitted
V_1	0.77	2.07	2.68	0.47	3.28	0.48	0.38	0.40	1	0.75-0.50	NBD
V_3	0.86	2.22	2.58	-0.70	3.15	0.56	0.39	0.17	1	0.50-0.75	NBD
V_4	0.63	1.24	1.97	-0.48	1.14	0.66	0.51	0.06	1	0.75-0.90	NBD

*NBD = Negative binomial distribution.

Table 5.5 Showing the distribution and dispersion statistics of *Colletotrichum* leaf spots on French bean leaves (captafol treated).

Varieties	Mean ($\bar{X}$)	Variance (S^2)	Variance-mean ratio ($S^2/\bar{X}$)	Third moment test (T)	[SE(T)]	Clumping parameter (r)	Probability (P)	Cal. -value	d.f.	Probability of fit	*Distribution fitted
V_1	1.5	4.87	3.24	1.66	8.68	0.69	0.31	4.02	2	0.10-0.25	NBD
V_3	0.74	1.48	2.00	-0.77	1.34	0.77	0.51	0.40	1	0.50-0.75	NBD
V_4	1.23	2.35	1.91	-0.21	1.45	1.86	0.58	1.85	2	0.10-0.25	NBD

*NBD = Negative binomial distribution.

Table 5.6 Showing comparative statistics for Pre and Post fungicides distribution of Colletotrichum leaf spots on French bean leaves.

rieties	Pre-fungicide				Post-Fungicide											
					Mancozeb				Benomyl				Captafol			
	Mean ($\bar{X}$)	Variance (S^2)	Variance mean ratio ($S^2/\bar{X}$)	Clumping parameter (r)	Mean ($\bar{X}$)	Variance (S^2)	Variance-mean ratio ($S^2/\bar{X}$)	Clumping parameter (r)	Mean ($\bar{X}$)	Variance (S^2)	Variance mean ratio ($S^2/\bar{X}$)	Clumping parameter (r)	Mean ($\bar{X}$)	Variance (S^2)	Variance mean ratio ($S^2/\bar{X}$)	Clumping parameter (r)
V_1	1.56	12.16	7.79	0.23	0.82	1.25	1.52	1.72	0.77	2.07	2.68	0.48	1.5	4.87	3.24	0.69
V_3	1.07	4.91	4.58	0.30	0.70	1.75	2.50	0.61	0.86	2.22	2.58	0.56	0.74	1.48	2.00	0.77
V_4	1.28	6.73	5.26	0.35	1.27	3.25	2.55	0.85	0.63	1.24	1.97	0.66	1.23	2.35	1.91	1.86

except in V_3 where mancozet showed less CV value. (Table 5.7).

(iii) Parametric and non-parametric analysis of variance of leaf spots:

The usual parametric analysis of variance both one way and two way showed that varietal differences in the mean occurrence of leaf spots was highly significant at 1% probability level. Similar results were obtained by non-parametric one way (Kruskal-Wallis test) and two way (Friedman's test) analysis by ranks. χ^2-test also showed statistically significant varietal difference (Tables 5.8 and 5.9). Test of significance by Z-test showed that varietal combination V_1 and V_3 and V_1 and V_4 were significantly different in the mean occurrence of leaf lesions. However, V_3 and V_4 were not statistically significant (Table 5.10). Therefore, the result may be represented symbolically as $V_1 > V_3 > V_4$ in order of their susceptibility to the occurrence of leaf spots disease caused by *C. lindemuthianum*.

DISCUSSION

The significant regression of $\overset{*}{X}$ on $\bar{x}$ in terms of correlation coefficient indicated a relationship between $\overset{*}{X}$ and $\bar{x}$ for the occurrence of *Colletotrichum* leaf spot on all

Table 5.7 Showing coefficient of variations of pre and post fungicide distribution of _Colletotrichum_ leaf spots.

Varieties	Pre-fungicide	Post-Fungicide			Ratio		
		Mancozeb	Benomyl	Captafol			
	Coefficient of variation (CV_1)	Coefficient of variation (CV_2)	Coefficiant of variation (CV_3)	Coefficient of variation (CV_4)	CV_1/CV_2	CV_1/CV_3	CV_1/CV_4
V_1	223.53	136.34	186.85	147.12	1.63	1.19	1.51
V_3	207.08	188.98	173.25	164.39	1.09	1.19	1.25
V_4	202.67	141.95	176.75	124.63	1.42	1.14	1.62

Table 5.8 Showing Parametric and Non-Parametric analysis of variance and χ^2-test of leaf spots caused by Colletotrichum lindemuthianum on three varieties of French bean.

(Set-1)

Sources of variations	Degree of Freedom (d.f.)	Sum of Squares (SS)	Mean Squares (MS)	Computed F-value	Tabular F-Value 5%	Tabular F-Value 1%	Friedman Test by Ranks: Degree of Freedom (d.f.)	Friedman Test by Ranks: Computed χ_r^2-value	χ^2-test: Degree of freedom (d.f.)	χ^2-test: Computed χ^2-value
Replications	39	12548.08	321.745	1.300	1.54	1.84	2	30.91**	78	556.13**
Varieties	2	21340.83	10670.415	43.14**	3.11	4.88				
Error	78	19290.68	247.316							
Total	119									

(Set-2)

Sources of variations	Degree of Freedom (d.f.)	Sum of Squares (SS)	Mean Squares (MS)	Computed F-value	Tabular F-Value 5%	Tabular F-Value 1%	Friedman test by Ranks: Degree of Freedom (d.f.)	Friedman test by Ranks: Computed χ_r^2-Value	χ^2-test: Degree of Freedom (d.f.)	χ^2-test: Computed χ^2-value
Replications	39	18160.33	465.649	1.094	1.54	1.84	2	14.19**	78	952.817**
Varieties	2	14945.82	7472.910	17.561**	3.11	4.88				
Error	78	33191.85	425.536							
Total	119									

** = Statistically significant at 1%.

Table 5.9 Showing parametric and non-parametric analysis of variance (one way ANOVA) of leaf spots caused by Colletotrichum lindemuthianum on three varieties of French bean.

(Set-1)

Sources of variations	Parametric test: F-Test — Degree of freedom (d.f.)	Sum of Squares (SS)	Mean Squares (MS)	Computed F-Value	Tabular F-Value 5%	Tabular F-Value 1%	Non-parametric Test: Kruskal-Wallis Test by Ranks — Degree of Freedom (d.f.)	Computed H-Value
Varities	2	21053.02	10526.51	38.34**	3.07	4.78	2	41.26**
Error	117	32126.57	274.59					
Total	119							

(Set-2)

Sources of variations	Parametric test: F-Test — Degree of freedom (d.f.)	Sum of Squares (SS)	Mean Squares (MS)	Computed F-Value	Tabular F-Value 5%	Tabular F-Value 1%	Non-parametric Test: Kruskal-Wallis Test by Ranks — Degree of Freedom (d.f.)	Computed H-Value
Varieties	2	14945.82	7472.91	17.03**	3.07	4.78	2	13.06**
Error	117	51352.175	438.91					
Total	119							

** = Statistical significant at 1%.

Table 5.10 Showing Parametric and non-parametric test of significance of varietal differences in susceptibility to *Colletotrichum lindemuthianum*.

Parametric test		Non-parametric test			
Variety	Mean number of leaf spot	Varietal Combinations	Cal. Z-Values	Tab. Z-value (Two-tailed Test) 5%	Tab. Z-value (Two-tailed Test) 1%
V_1	39.801	V_1 and V_3	5.55**	1.96	2.58
V_4	12.825	V_1 and V_4	5.07**		
V_3	10.700	V_3 and V_4	1.28 ns		

LSD (P = 0.05) = 7.336
(P = 0.01) = 9.696

** = Significant at 1%

ns = not significant

Fig.5.1 Showing pre and post fungicide regression of mean crowding ($\overset{*}{X}$) on mean density (x) of Colletotrichum leaf spots for polled data (untreated and treated seperately with fungicides : Mancozeb, Captafol and Benomyl) from three varieties of French bean. leaves.

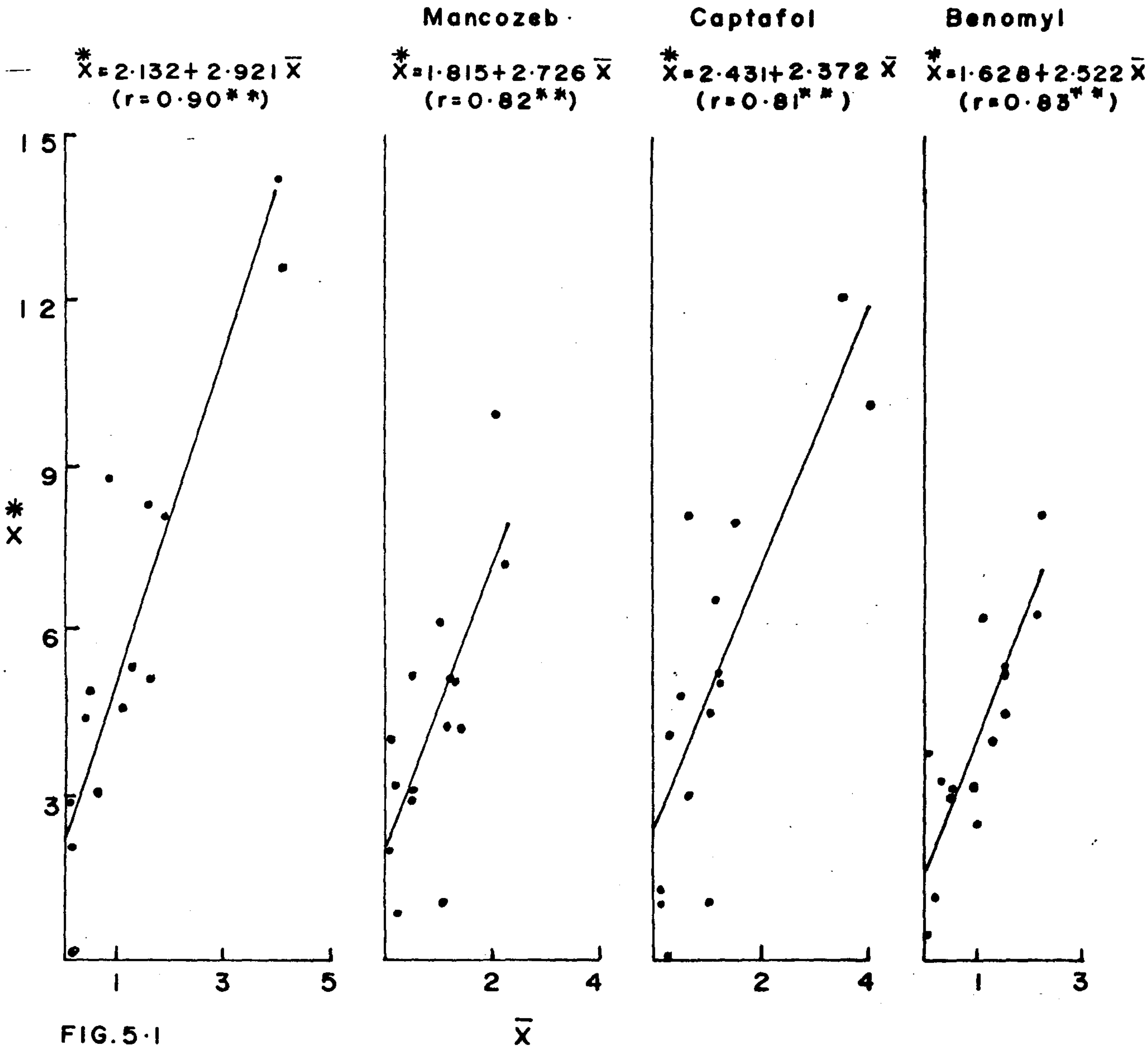
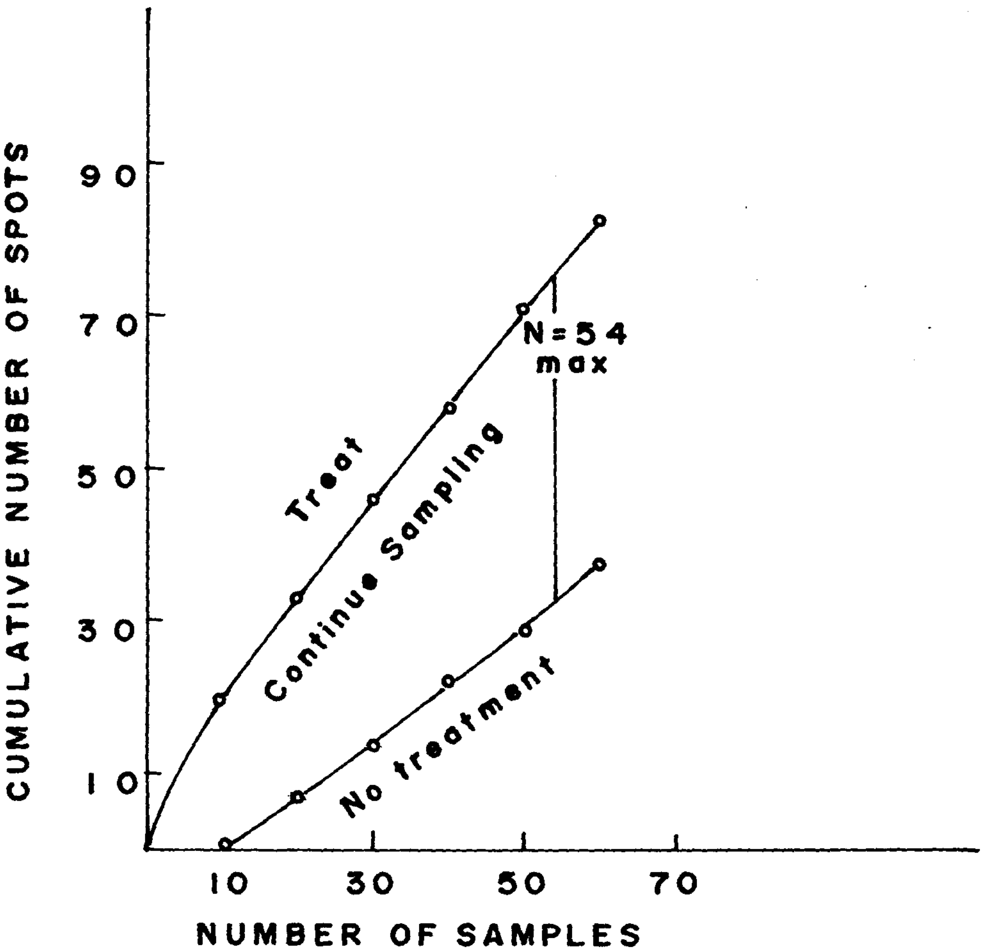

Mancozeb
Captafol
Benomyl
X* = 2·132 + 2·921 X̄
(r = 0·90**)
X* = 1·815 + 2·726 X̄
(r = 0·82**)
X* = 2·431 + 2·372 X̄
(r = 0·81**)
X* = 1·628 + 2·522 X̄
(r = 0·83**)
15
12
9
6
3
X*
1
3
5
2
4
2
4
1
3
X̄

FIG.5·1

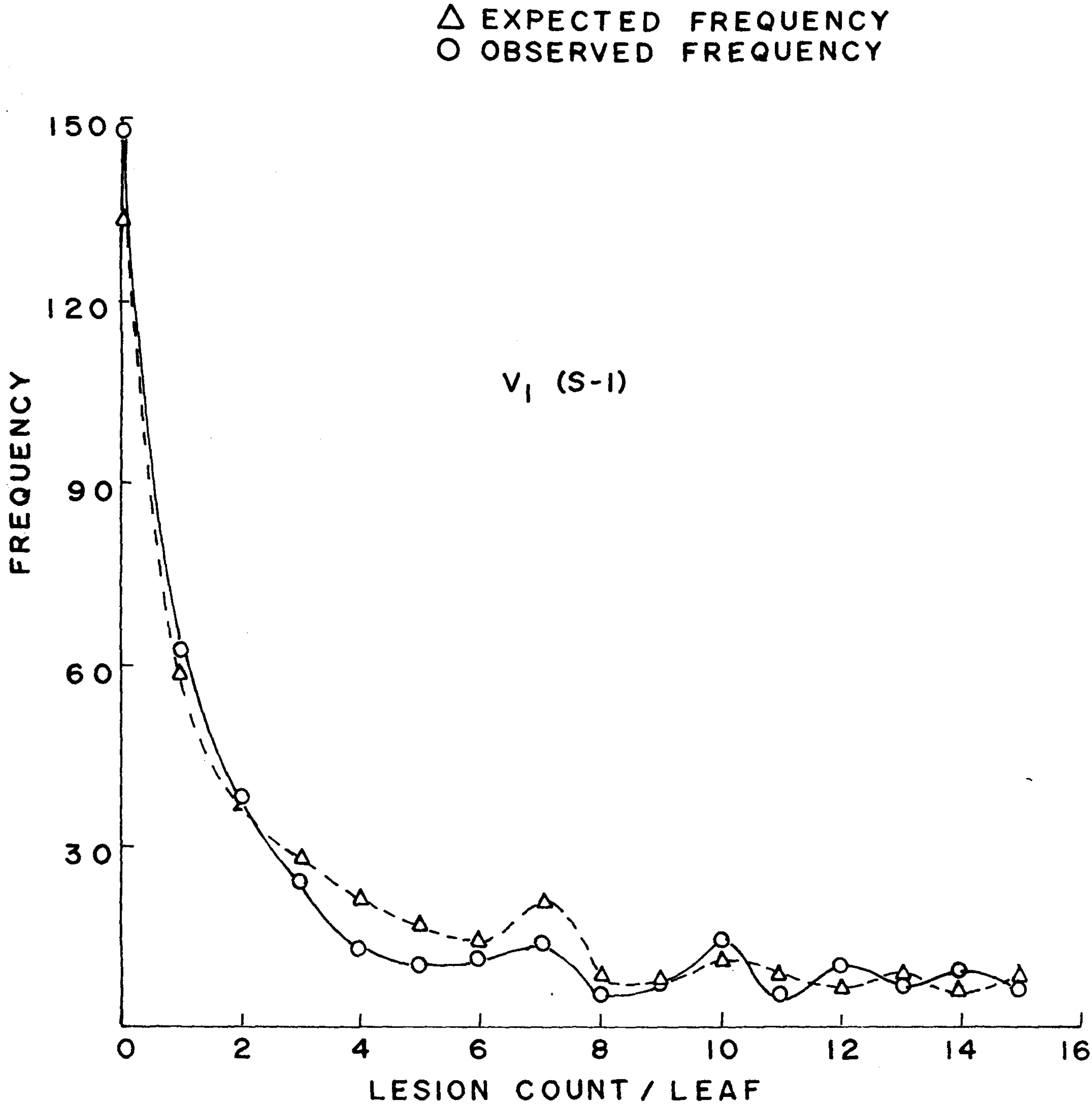
Fig.5.2 Sequential sampling plan for Colletotrichum leaf spots on French bean leaves.



CUMULATIVE NUMBER OF SPOTS
90
70
50
30
10
Treat
Continue Sampling
No treatment
N=54
max
10
30
50
70
NUMBER OF SAMPLES

FIG.5·2

Fig.5.3 The observed and expected (calculated) frequency distribution of Colletotrichum leaf lesions on leaves of French bean variety - V_1 for the first set of data (S-1).



△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
150
120
90
60
30
FREQUENCY
V_1 (S-1)
0
2
4
6
8
10
12
14
16
LESION COUNT / LEAF

FIG. 5·3

Fig.5.4 The observed and expected (calculated) frequency distribution of Colletotrichum leaf lesions on leaves of French bean variety V_1 for the second set of data (S-2).

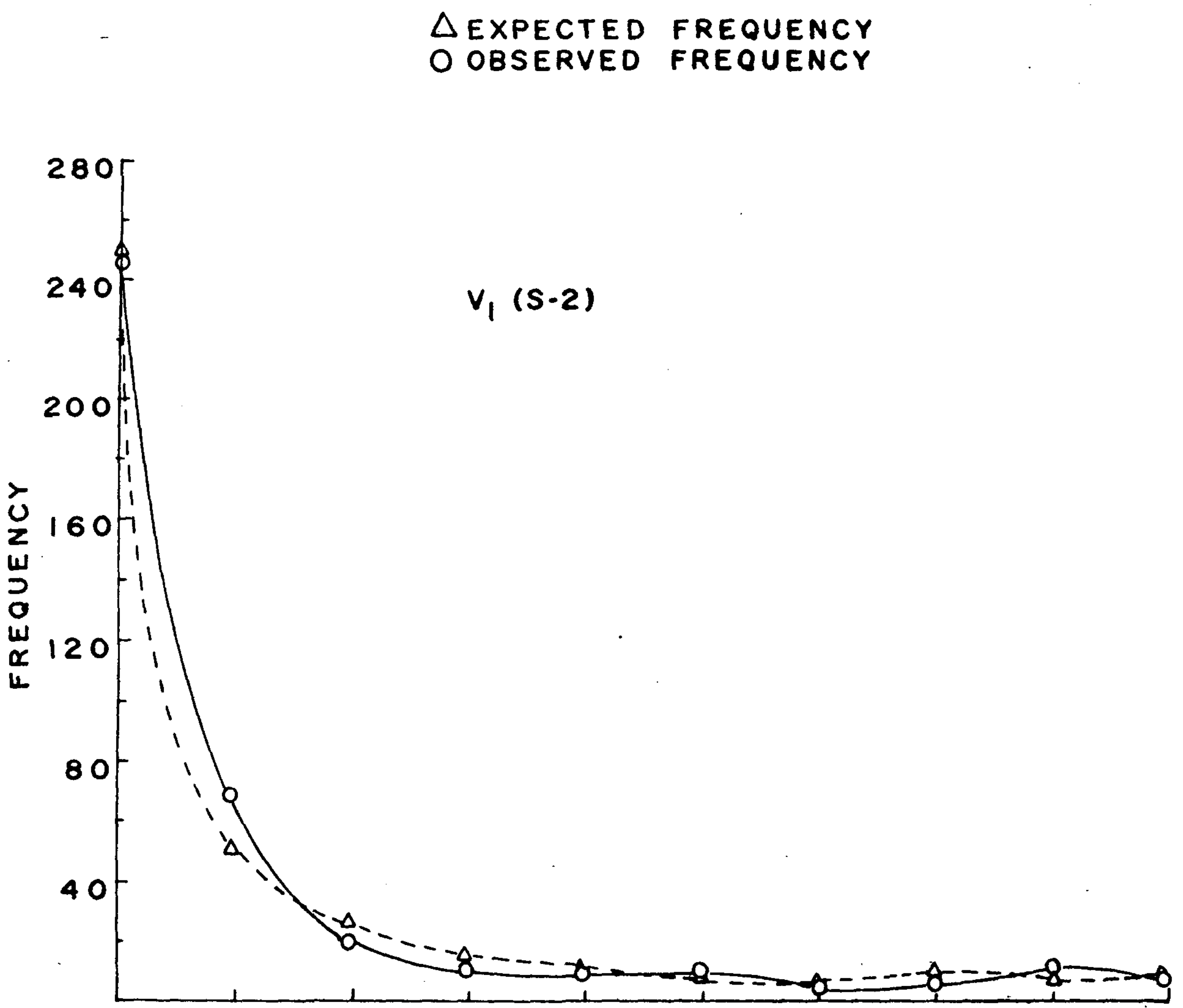
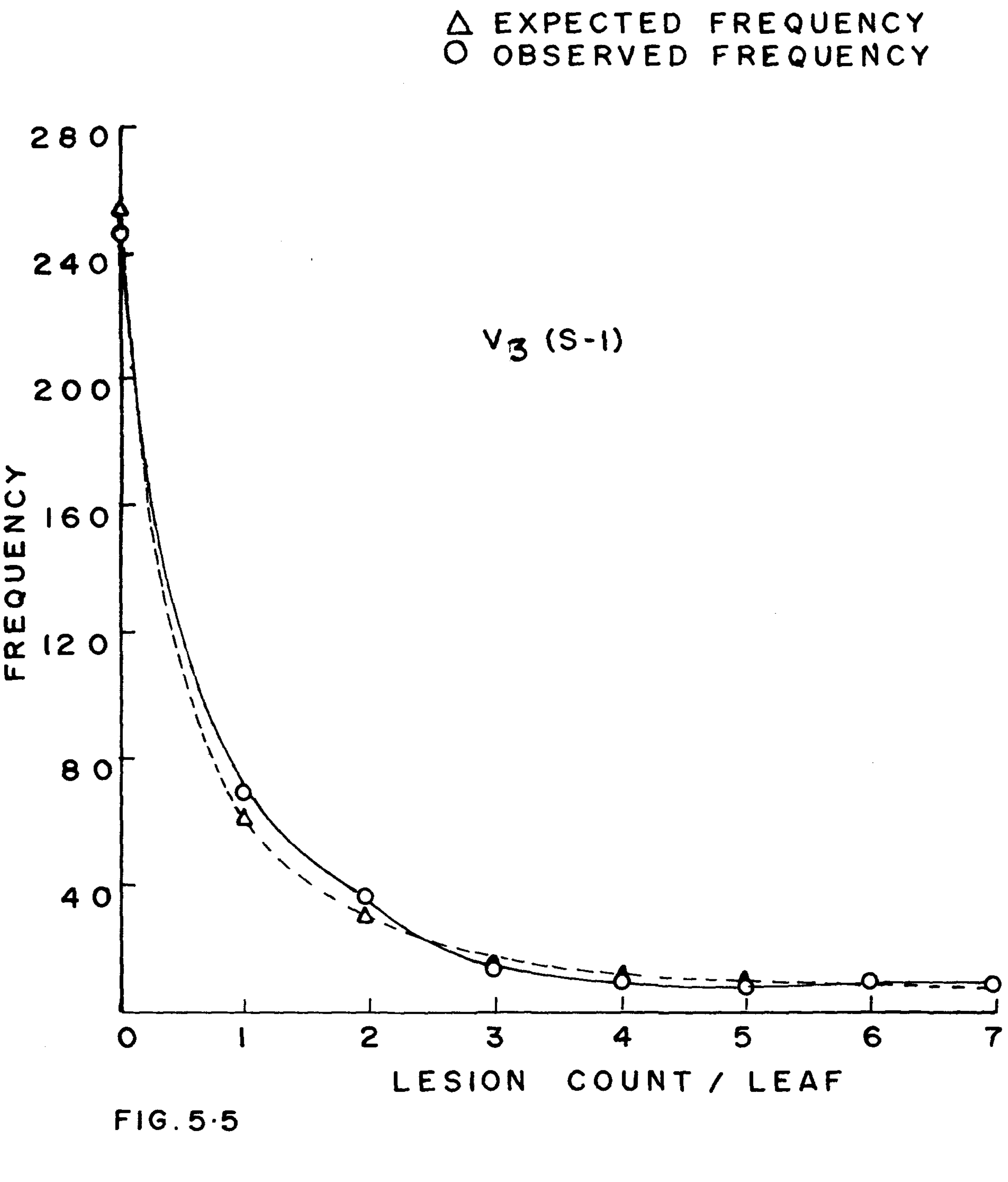

△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
280
240
200
160
120
80
40
FREQUENCY
V_1 (S-2)
0
1
2
3
4
5
6
7
8
9
LESION COUNT / LEAF

FIG. 5·4

Fig.5.5 Showing the observed and expected (calculated) frequency distribution of Colletotrichum leaf lesions on the leaves of French bean variety - V_3 for the first set of data (S-1).



△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
280
240
200
160
120
80
40
FREQUENCY
V3 (S-1)
0
1
2
3
4
5
6
7
LESION COUNT / LEAF

FIG. 5·5

Fig.5.6 Showing the observed and expected (calculated) frequency distribution of *Colletotrichum* leaf lesions on the leaves of French bean variety - V_3 for the second set of data (S-2).

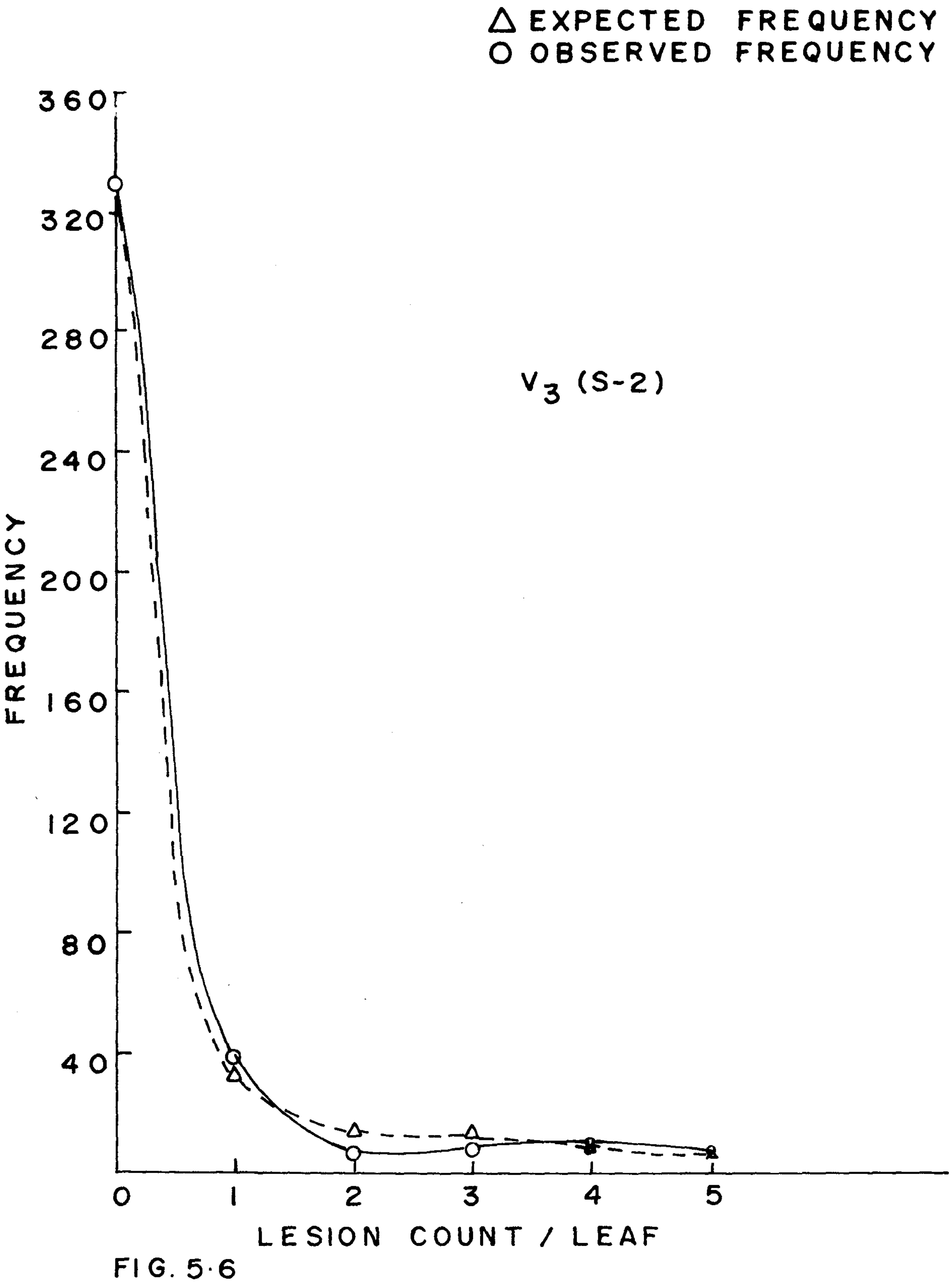

△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
360
320
280
240
200
160
120
80
40
FREQUENCY
V_3 (S-2)
0
1
2
3
4
5
LESION COUNT / LEAF
FIG. 5·6

Fig.5.7 Showing the observed and expected (calculated) frequency distribution of Colletotrichum leaf lesions on the leaves of French bean variety - V_4 for the first set of data (S-1).

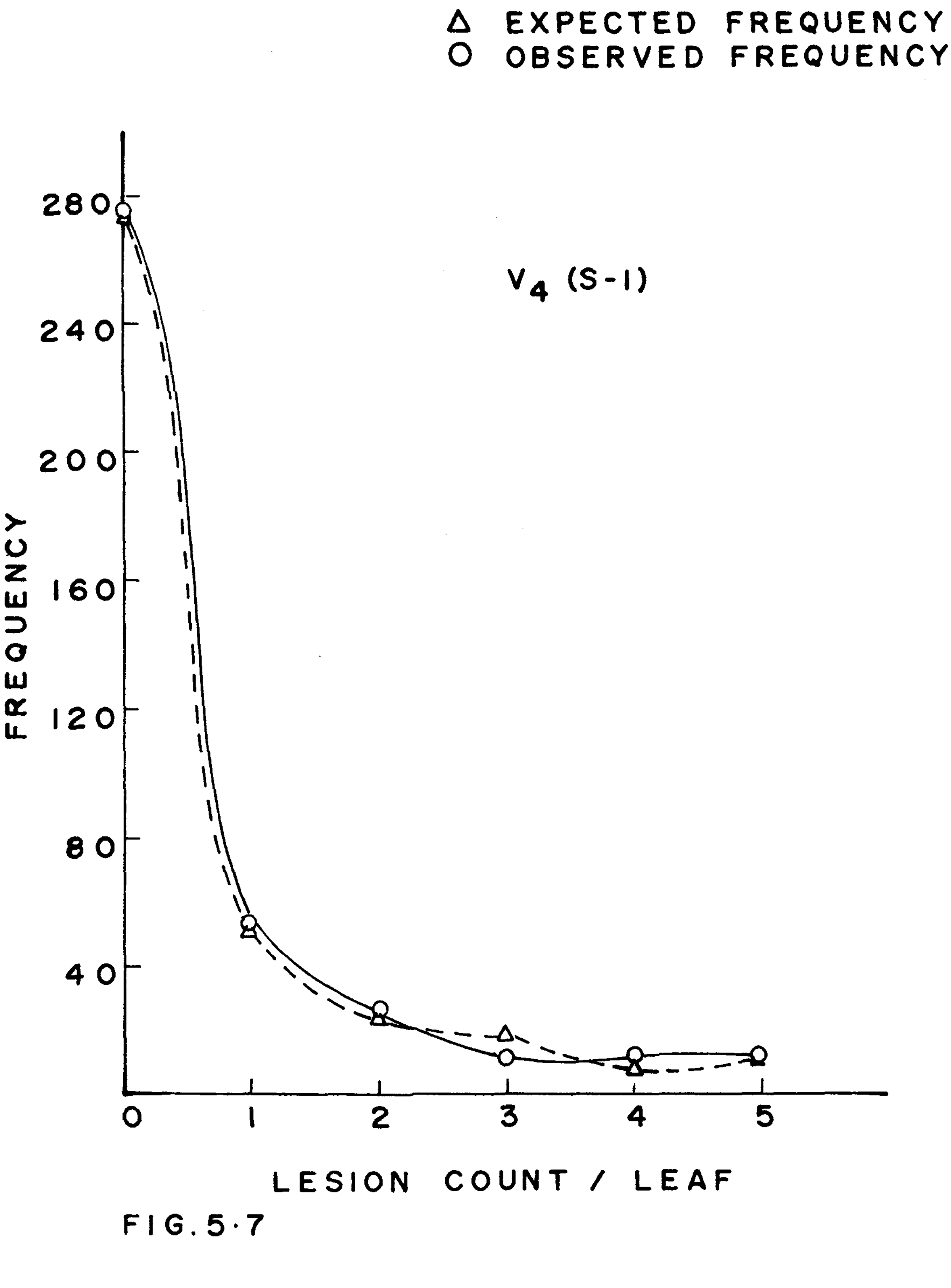

△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
V_4 (S-1)
FREQUENCY
280
240
200
160
120
80
40
0
1
2
3
4
5
LESION COUNT / LEAF
FIG. 5·7

Fig.5.8 Showing the observed and expected (calculated) frequency distribution of Colletotrichum leaf lesions on French bean variety - V_4 for the second set of data (S-2).

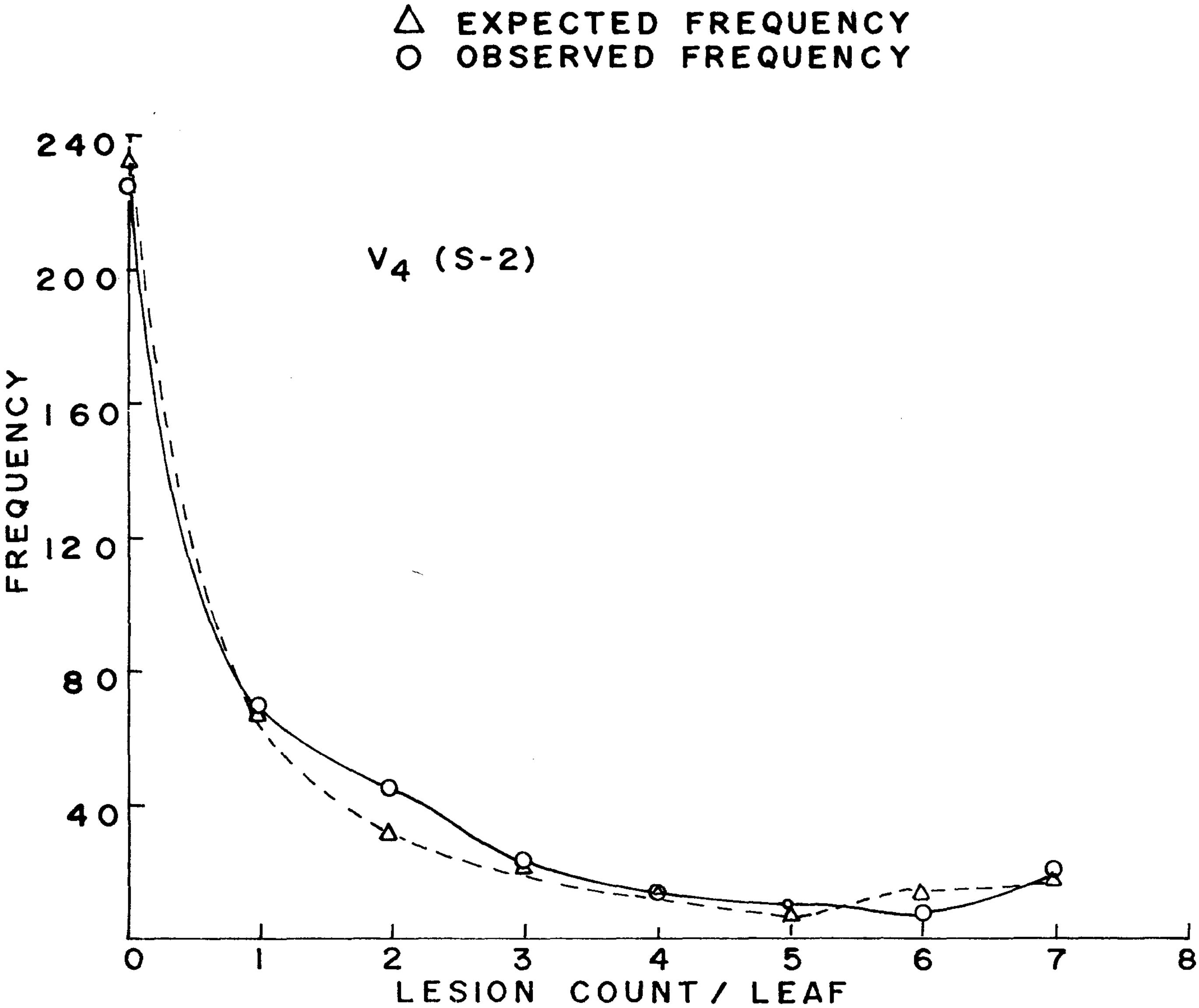

△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
V_4 (S-2)
FREQUENCY
240
200
160
120
80
40
0
1
2
3
4
5
6
7
8
LESION COUNT / LEAF

FIG.5·8

Fig.5.9 Showing observed and expected (calculated) frequency distribution of leaf lesions on three different varieties of French bean (V_1, V_3 and V_4) treated separately with fungicides: Mancozeb and Benomyl.

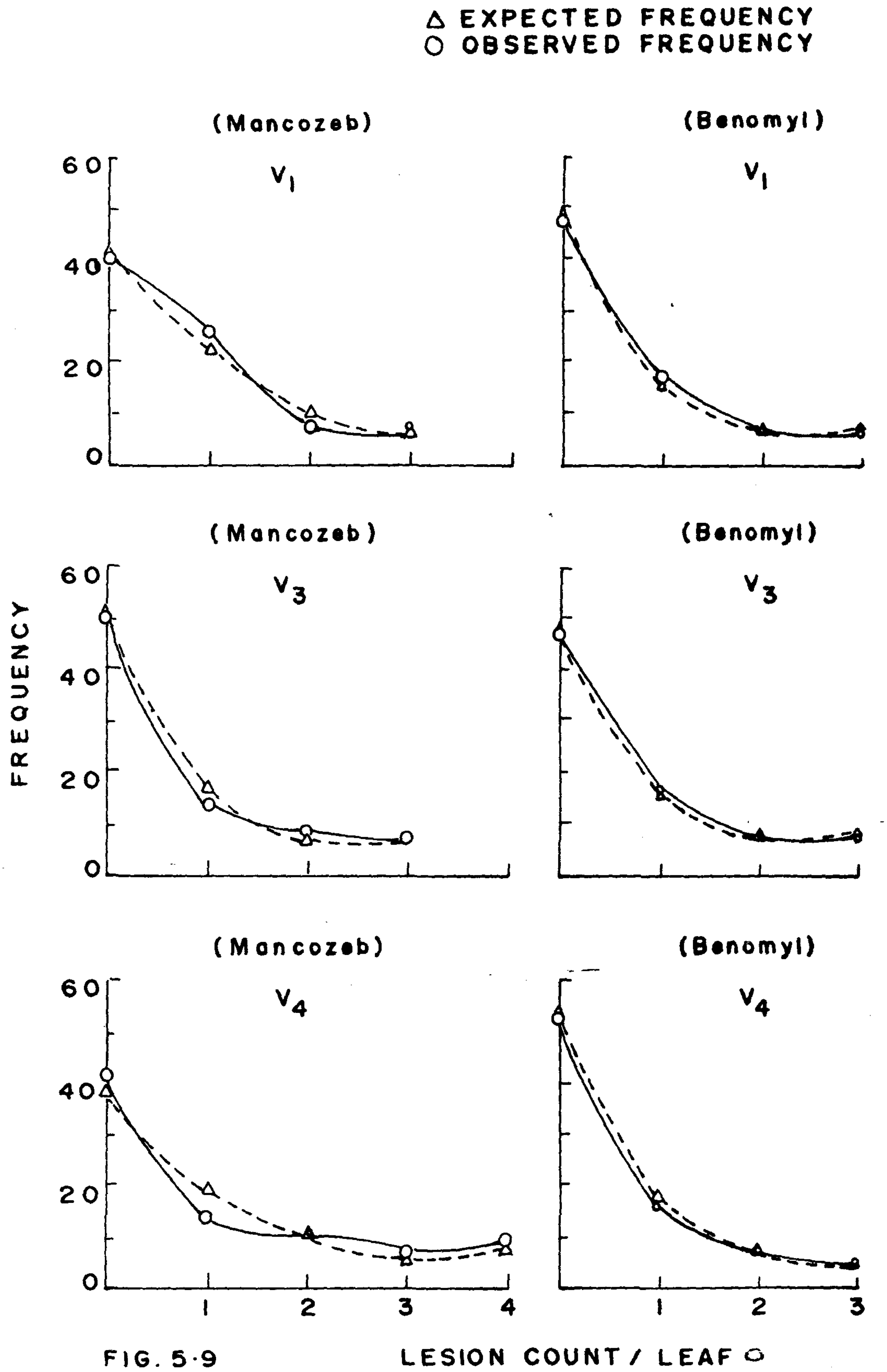
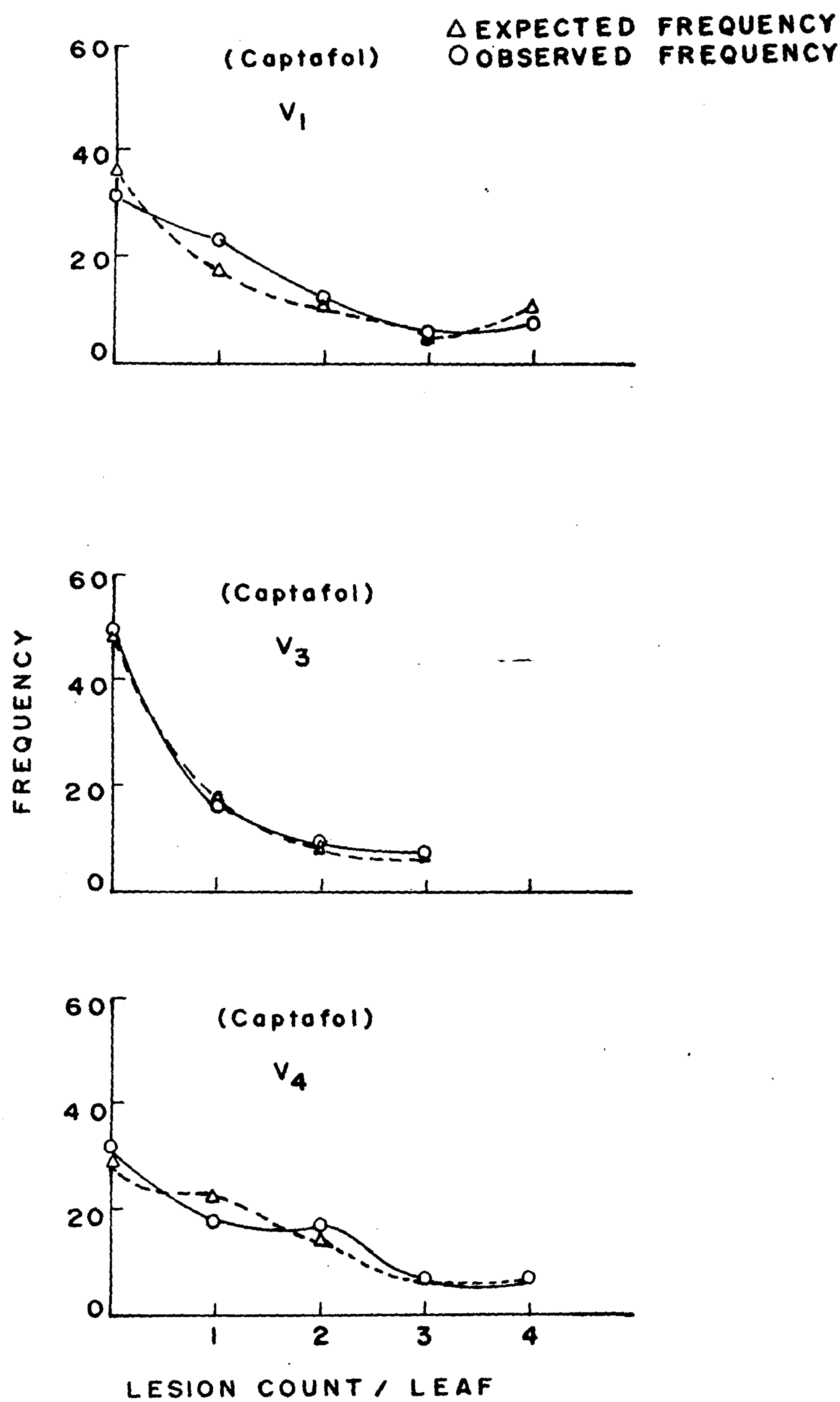

△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
(Mancozeb)
(Benomyl)
V1
(Mancozeb)
(Benomyl)
V3
(Mancozeb)
(Benomyl)
V4
FREQUENCY
60
40
20
0
1
2
3
4
LESION COUNT / LEAF

FIG. 5·9

Fig.5.10 Showing observed and expected (calculated) frequency distribution of leaf lesions on three different varieties of French bean (V_1, V_3 and V_4) treated with fungicide - Captafol.



△ EXPECTED FREQUENCY
○ OBSERVED FREQUENCY
(Captafol)
V1
(Captafol)
V3
(Captafol)
V4
FREQUENCY
60
40
20
0
1
2
3
4
LESION COUNT / LEAF

FIG. 5·10

varieties of French bean leaves. Iwao (1977) observed the existence of similar relation in a number of plants and animals species because of their specific way of utilizing the habitat as reflected by their basic component of the population distribution. Since the leaf spots (i.e. the basic component) distribution was considered here, they might have reflected their own way of spreading or utilizing the habitat i.e. on the leaves. The b_o and b_1 values of each variety were significantly different from 0 and 1 and that characterizes their (leaf spots) unique way of spreading on the leaves.

The significant pooled regression of $\overset{*}{X}$ on $\bar{X}$ of all varieties further confirmed the existence of relation between these two parameters. The significant difference of b_o and b_1 values from 0 and 1 indicates that aggregates were the basic component of the Colletotrichum leaf spots population when all the varieties were considered and that these basic components were contagiously distributed on the leaves of all french bean varieties in the field. Possibly this could be due to the infection of new site of leaf nearby the infected ones as a result of which more lesion might have formed resulting clustering of lesions on the leaves. Boivin and Sauriol (1984) observed similar aggregation of basic components and their contagious distribution of onion leaf lesions caused by Botrytis squamosa because of its polycyclic nature where contagious distribution is common in many polycyclic diseases of plants. Since Colletotrichum disease on Phaseolus vulgaris is also a polycyclic disease a contagious rather than random distribution is expected because of the spreading or infection of disease due to the pre-existence of inoculum source and their spread around the infection site. The possible mechanisms of spread of disease from an infection site and their possibility of the formation of aggregation of disease components on their habitat were fully discussed by Rotem (1978).

The sequential sampling plan was generated using the pre-fungicide pooled regression parameters of the spatial distribution which indicates that a maximum of 54 samples are needed to determine whether the disease level is above or below a certain economic threshold in the field when confidence interval of d=0.5 was taken (Fig.5.2). Here d=0.5 were choosen which means that when maximum number of samples (N_{max}) are observed, the population mean i.e., the disease level would be 1±0.5 leaf spot per leaf, with an error α of 0.1. Statistically this idea may be interpreted as follows: After maximum number of samples (N_{max}) are obtained, nine times out of ten the estimated mean falls within the 0.5-1.5 confidence interval.

To explain the use of this sampling plan, one has to count the number of Colletotrichum leaf lesions on the sampled French bean leaves collected randomly from the field and the cumulative number of leaf spots are plotted on the Y-axis of the sampling plan after each sample. In this process of collection, counting and plotting, if the upper acceptance limit of the sequential sampling curve is crossed upward, one may infer that the population density of the disease in question is significantly greater than one lesion per leaf and therefore the decision of the fungicide application is to be made. On the other hand if the lower acceptance limit of the sequential sampling curve is crossed downward, then one may easily conclude that the population density of the leaf lesions is significnatly lower than one lesion per leaf and decision of no fungicide application is necessary may be made. One has to continue the sampling till N_{max}=54 samples are observed if no two of the acceptance limits are crossed. At this point of N_{max}, sampling stops and the decision would be that population density of Colletotrichum leaf lesions is not significantly different from one lesion per leaf. Of course, one may start the fungicide application or more sampling could be done depending upon the weather forecast for the disease and phenological stage of the crop. Similar explanation was given for the sequential sampling plan for Botrytis leaf blight on onion caused by Botrytis squamosa by Boivin and Sauriol (1984).

Thus, sequential sampling plan is a useful tool for reducing time and labor cost associated in monitoring the disease especially while taking decision of whether or not control of the disease is needed. That is, the first fungicide application could be delayed untill the pre-determined economic threshold of the disease is reached (to be infered from the sequential sampling plan).

The variance significantly greater than the mean indicate that distribution of Colletotrichum leaf lesion may follow a negative binomial distribution. Possibly the leaf lesions are not independent to each other but dependent for the clumping of lesions around the infected site that resulted variance grater than the mean. Taylor (1961) pointed out that individuals in natural population are not, however, independent of each other but mutual attraction leads to aggregation that makes variance greater than the means ($S^2 > \bar{X}$). Similar indication of negative binomial distribution of leaf lesions was also observed in third moment test (T) which was in accordance with Samargit et al (1982) who also observed the standard error of the third moment (SE(T)) greater than the third moment (T) and inferred that the distribution of Earias vittella Fab. infestation on Okara fruit followed negative binomial series.

The fit between the observed and the expected frequency distribution quantified by χ^2 analysis and measure of clumping parameter 'r' of the negative binomial distribution indicates that the leaf lesions were clumped on the leaves rather than random. Since the relative degree of aggregation could be measured on parameter 'r', small values of r (<1) indicate extreme aggregation of the diseased units (leaf spots) and larger value of 'r' indicate smaller degree of aggregation (Rouse et al, 1981). The 'r' value in our study showed a trend towards extreme aggregation indicating a contagious distribution of leaf lesions due to the high probability of the formation of new lesions from the already infected leaf lesion. The infection of new sites on leaves might have favoured by the various mechanical transportation factors responsible for inoculum spread. Similar findings in many biological situations were reported by many authentic workers. Bliss and Fisher (1953), pointed out that negative binomial distribution often occurred in biology when the items being counted were likely to gather themselves in 'clumps' and commonly encountered in insect and bacterial population where a secondary spread of the populations arised from the randomly distributed primary foci. Rouse et al (1981) reported that negative binomial distribution was the result of wide variety of mechanism leading to aggregation of sampling units. Southwood (1978) pointed out the usefulness of negative binomial model in describing a contagious distribution.

From a plant pathological view point our negative binomially fitted leaf lesions data might be interpreted in terms of the primary infection of the Phaseolus vulgaris leaves by C. lindemuthianum as a result of seed-borne infection or as a result of wind-borne or water-borne conidia or by the presence of infected plant debris on the field where the crops were grown. Subsequently secondary spread of the disease would have occured, both from conidia of the primarily infected sites and from water and air-borne conidia as the disease in question sporulate quite frequently during rainy seasons producing a pinkish mass of conidia responsible for secondary infection of susceptible tissues on leaves. Cotyledonary leaves of seedlings, which were affected at the initiation might serve as the immediate secondary spread of the disease on the leaves which in turn spread subsequently following the favourable weather conditions leading to a pattern of multiple infection gradient and finally form aggregation of lesions on the leaves. Similar result with discussion in accordance with the multiple infection trend that fits the negative binomial distribution was reported by Byrde et al (1973) on apple trees cankers caused by Nectria galligena. Waggoner and Rich (1981) reported that most of the plant disease lesions were fitted by the negative binomial distribution. He observed that propagules of a contagious disease was not randomly distributed, instead the expected frequency distribution would be "contagious" or "over-dispersed" with variance larger than the mean and fitted by the negative binomial distribution since the presence of one lesions in an area or habitat increases the chance of other infection nearby that area of lesion occurrence.

The insignificant difference among the regression parameters of pre and post fungicide indicates that application of fungicide did not change the basic distribution pattern of leaf lesions.

The various statistical tests on fungicide treated data also indicate that application of fungicide did not affect the basic pattern of distribution. However, slight variation on dispersion statistics such as mean, variance, third moment (T) and clumping parameter 'r' could be due to the effect of fungicide that might have checked the further spread of the inoculum and subsequently the clustering tendency of lesions was reduced as indicated by the larger 'r' value. Boivin and Sauriol (1984) also observed that application of fungicide did not affect the distribution pattern of Botrytis leaf blight in onion field. McGuire (1957) further pointed out that negative binomial could be an excellent approximating distribution even at the low densities of the population that supports the present findings.

The coefficient of variation (Cv) of post fungicide data indicate that the variation in the spread of the disease was checked. The ratio of the Cv between pre-fungicide and post-fungicide treated data indicates that benomyl was more effective in controlling the variation of the spread of the disease than the other two fungicides.

Though the experiment was laid out in a randomized block design (RBD), analysis of variance (two way classification both for parametric and non-parametric) result indicates that varietal differences were statistically significant but not the replications indicating homogeneity of the experimental site and therefore, it was not meaningful to consider the block effect in variation. Hence one way analysis was found to be more suitable for both the parametric and non-parametric test and therefore further test of significance was carried out with the one way ANOVA results only.

Statistical significance of the analysis of leaf lesions computed by both the parametric and non-parametric test indicates that varieties differ with each other in their susceptibility to the occurrence of mean number of lesions caused by C. lindemuthianum. The significant LSD and Z values of both the tests further indicate that the statistical significance was between V_1 and V_3 and also between V_1 and V_4 varieties.

GENERAL DISCUSSION

Occurrence of two leaf spot disease in French bean caused by _Colletotrichum_ _lindemuthianum_ (sacc. and Magn.) Br. and Cav. and _Phaeoisariopsis_ _griseola_ (Sacc.) Ferraris were in accordance with the report of many other previous workers (Zaumeyer and Thomas, 1957; Allen and Russel, 1983; Walker, 1969; Roberts and Bothyroyd, 1972; Butler, 1973; Cardona and Walker, 1956 and Srinivasan, 1953). However, _C_. _lindemuthianum_ was found to attack four varieties of French bean whereas _P_. _griseola_ occurred in only two varieties. The variation in leaf spot diseases occurrence could be attributed due to the varietal differences in their susceptibility (Annual Report, ICAR for North-Eastern Region, 1977; 1978; 1979; 1980; Parthasarathy 1986).

The leaf spot disease caused by _P_. _griseola_ produced some variation in its symptoms. Instead of angular, irregular brown to grey spot lying in the angle between leaf veins (Cardona and Walker, 1956; Hocking, 1967), the regular and circular brown lesions on the leaves could possibly be due to an outbreak of highly virulent form of _P_. _griseola_. The virulent form of the fungus might have occurred due to environmental or mutational changes (Hocking, 1967).

Occurrence of elongated and necrotic leaf spot of C. lindemuthianum mostly around the leaf vein indicated that conidia of the pathogen preferentially settled around the veins to cause infection (Mercer et al, 1971).

Regression analysis of the data indicated that the quadratic regression equation was little useful than the multiple linear regression to describe the variation in disease incidence and severity due to environmental factors for the disease caused by C. lindemuthianum. Similar predictive model of the disease epidemic with the help of regression method has been suggested (Burleigh et al, 1972; Massie and Nelson, 1973; Zhou et al. 1981; Sharma, 1986).

C. lindemuthianum and P. griseola have been reported as seed borne pathogens of French bean (Walker, 1969, Sindhan and Bose, 1979). Survey of seed borne mycoflora of French bean indicated some similarities in species composition with the seed mycoflora of many other pulse crops reported by previous workers (Suryanarayana, 1961; Agarwal et al, 1972. Singh et al, 1973; Sinha and Khare, 1977; Sharif et al, 1987; Nitsche et al, 1985). Investigating into the seed mycoflora by two techniques suggested that a combination of both the techniques was necessary to study the saprophytic and pathogenic fungi. Blotter technique was comparatively better to give an idea of fungi associated with the seed than the agar plate method. The superiority of blotter test over agar plate could possibly be due to the development of fast growing fungi which hampered the growth of slow growing fungi in agar plate (Singh et al, 1973).

Seasonal variation in the quality of seed borne mycoflora could be due to the changes in environmental conditions and in the quality of seeds (Bhikane et al, 1982). Occurrence of few species of fungi throughout the year indicated their wide range of ecological amplitude (Bilgrami et al, 1976, Campbell, 1962). Varietal variation in seed mycoflora could be related to the physicochemical nature of the seeds (Lokhande, et al, 1986; Neergard, 1977).

Fungi on the leaves of French bean have been investigated by three techniques. Qualitative and quantitative variation in the isolation of both saprophytic and pathogenic fungi of phyllosphere could be due to the application of different techniques. Many earlier workers have suggested the use of different techniques for maximum isolation of phyllosphere fungi (Dickinson, 1967; 1971;

Mishra and Dickinson, 1981). Information gathered from the electron microscopic study indicated that few conidiophores with abundant conidia/spores of different fungal species were found often associated with leaf hair which could possibly be due to more fungal activity at the basal area of leaf hair (Pugh and Bukley, 1976). In general, species composition of phyllosphere mycoflora of French bean was in broad agreement both in quality and quantity with those in several annual herbaceous crops (Leben, 1965; Sinha 1965; Dickinson and Preece, 1976).

Variations in the isolation of various fungi with respect to the varieties and age of the plant could be attributed to the nutrient composition of leaf, their maturity and adaptability of micro-organisms to adhere themselves to the leaf surface. These factors affected the nutrients availability to the micro-organisms on the surface of leaf.

Fluctuation in the phyllosphere population of microbes could also be related to the varietal characters and climatic factors like temperature, humidity and rain (Kumar and Balasubramanian, 1981; Sinha 1971).

Interaction between leaf surface fungi and leaf pathogen in vitro condition suggested that the saprophytic fungi used in this study were not antagonistic against *C. lindemuthianum*. Therefore, these fungi may not be useful as biological controlling agent for leaf spot disease. However, it has been reported that failure of certain saprophytes to inhibit the pathogen *in vitro* could potentially function as antogonist *in vivo* (Fokkema, 1976).

Spatial distribution of *C. lindemuthianum* leaf spot population was not random under natural condition but contagiously distributed and best described by negative binomial distribution model. Similar spatial distributions have been observed in certain biological population (Bliss, 1958; Iwao, 1970; Kuno 1969). The regression equation fitted to the mean crowding ($\overset{*}{X}$) on mean density ($\bar{X}$) of the leaf spot disease provided a characteristic nature of the distribution pattern of *C. lindemuthianum* in terms of two parameters (b_o and b_1) of the regression equation ($X=b_o+b_1\bar{x}$) which was in accordance with the observation of previous workers (Boivin and Saurio, 1984; Iwao and Kuno, 1971; Kuno, 1969).

The contagious distribution based on the relation of mean crowding ($\overset{*}{X}$) and mean density ($\bar{X}$) of leaf spot disease was also adequately described by the negative binomial distribution model which could possibly be due to the aggregation of leaf lesions nearby the infected site. Bliss and Fisher (1953) have concluded that the negative binomial distribution was the best in describing the biological data involving aggregation or clustering.

The mean-variance ratio test and third moment test also indicated the contagious distribution of C. lindemuthianum leaf lesions. Similar conclusions were drawn from such test (Taylor, 1961; Samarjit et al, 1982). The spatial distribution of Colletotrichum leaf spot population suggested that new lesions are likely to form neaby the already existed lesion resulting in aggregation of leaf spot population and therefore a contagious rather than random distribution was followed. Similar observations have already been reported for polycyclic fungal disease that showed contagious or negative binomial distribution (Boivin and Sauriol, 1984; Campbell and Pannypecker, 1980).

The sequential sampling plan generated by incorporating the regression parameters (b_o and b_1) indicated that a maximum number of 54 samples were required to assess the disease level in the field when the fixed level of precision (d) and an error level are 0.5 and 0.1 respectively. This finding was in accordance with the observation of Boivin and Sauriol (1984) and Boivin and Vincent, (1987). However in the sequential sampling plan, one lesion per leaf was taken as action level economic threshold based on the crop phenology, severity of the disease and their control strategies (Lincoln, 1978). Most sequential sampling plan of a disease were also established with a different economic threshold (Shoemaker and Lorbeer, 1977; Boivin and Sauriol, 1984).

An error level of 0.1 was considered for the precision of sequential sampling plan since it is more serious to fail to recommend a necessary treatment (β-risk) than to recommend an unnecessary action (α-risk) because the cost of treatment would be lower than the potential losses. Moreover, most sequential sampling plan has an error level of about 0.05 to 0.1 (Boivin and Vincent, 1977).

Out of the two methods used in the analysis of variance, the non-parametric procedure was more valid which suggested that assumption of the population was not necessary. On the other hand parametric procedure is based on a pre-assumption that population is normally distributed. Since the distribution of Colletotrichum leaf lesion population followed negative binomial distribution and to avoid the normality assumption, different non-parametric tests were used in the analysis of varietal differences with respect to their susceptibility in terms of the mean occurrence of leaf spot disease caused by C. lindemuthianum. Similarly, Friedman (1937) has emphasised the use of non-parametric method than parametric one to avoid normality implicit in the analysis of variance to the biological problems.

SUMMARY

Leaf spot fungal disease survey was carried out on five varieties of French bean for the first year (1987) and on three varieties in the second year (1988) grown in a randomised block design in the Botanical garden of North-Eastern Hill University, Shillong. Two leaf pathogens viz., Colletotrichum lindemuthianum and Phaeoisariopsis griseola were found to attack the crops. Variety V_1, V_3, V_4 and V_6 were found susceptible to C. lindemuthianum whereas V_3 and V_5 were prone to P. griseola. Pathogenicity of these two pathogens were established in variety V_4 (for C. lindemuthianum) and variety V_5 (for P. griseola). The disease incidence, severity and meteorological data collected at weekly interval were used for regression and correlation analysis. The variation in incidence and severity caused by C. lindemuthianum on variety V_1 could not be adequately described by the multiple linear regression as well as quadratic regression equation.

Study of seed borne fungi on five varieties of French bean grown for disease survey as mentioned above revealed the presence of C. lindemuthianum - the causal agent of colletotrichum leaf spot. All together forty four species belonging to thirty-three genera were detected. Thirty-seven species belonging to thirty-two genera were found alone by blotter test and thirty-one species of twentyfour genera by agar plate method. The blotter test method proved superior over agar plate method. Variety - V_6 supported the growth of more fungal species than other varieties studied but varietal differences wére not statistically significant. Fungal population was found to be influenced by the season. Summer season yielded maximum number of fungi than the rainy and winter season and this seasonal variation was found to be statistically significant.

Qualitative and quantitative composition of leaf surface mycoflora of different varieties of French bean was done by three techniques (Moist chamber, Leaf impression and Washing leaf disk). Scanning electron microscope (SEM) was also used in recording the spore population of fungi at low magnification. A total of 67 species belonging to 52 genera were isolated, Maximum number of fungal species was found in washing leaf disk technique. Variation in fungal population was statistically significant with the increasing age of the host variety whereas varietal difference was not significant although variety V_1 was found to support the maximum population. Fusarium sp., Penicillium sp., Sporothrix sp., and Mycelia sterilia dominated the leaf surface of almost all varieties. A filamentous yeast *Candida albicans* was also detected both in the cultural method and SEM observation. The leaf pathogen *Colletotrichum lindemuthianum* was detected often by moist chamber technique.

Interaction study showed that saprophytes tested were not found to be potential antagonist to vegetative growth of *C. lindemuthianum* since no significant zone of inhibition was observed on PDA except *Paecelomyces variotii* and *Torula herbarum* which showed slight inhibition at a distance. However, culture filtrate of *Torula herbarum* and *P. variotii* were found to inhibit the spore germination.

Spatial distribution of leaf spot disease of three varieties of French bean (*Phaseolus vulgaris* L.) caused by *Colletotrichum lindemuthianum* was studied using the mean crowding ($\overset{*}{X}$) in relation with Iwao's regression technique. The population of *colletotrichum* leaf lesion was aggregates which were distributed contagiously in French bean leaves. The mean-variance ratio test and third moment test indicated that the leaf spot follows negative binomial series. The frequency distribution of leaf lesion was also adequately fitted by the negative binomial distribution that confirmed the contagious distribution of the *Colletotrichum* leaf lesion population in

French bean. Three fungicides viz. Mancozeb, Benomyl and Captafol were used as a control measure to observe the changes in distribution pattern of leaf spots but it was found that the application of fungicide did not significantly altered the basic pattern of the spatial distribution. This information was used to establish a sequential plan using action level economic threshold of one leaf spot per leaf. To detect the disease level at or near the provisional action level economic threshold, maximum number (N_{max}) of 54 leaves samples were needed as determined by the N_{max} relation of the sequential sampling plan.

Parametric as well as non-parametric procedures were used in the analysis of variance of leaf lesion population to determine the significant diference of varieties in terms of their susceptibility to the mean occurrence of Colletotrichum leaf lesion. The V_1 variety was founa to be the most susceptible compred to the other two varieties (V_3 and V_4) tested as per the least significant difference (LSD) and Z-value of the parametric and non-parametric test respectively.

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* Not seen in original.