STUDIES ON THE BROWN BLIGHT DISEASE OF TEA [*Camellia sinensis* L. (O.) KUNTZE] CAUSED BY *Glomerella cingulata* (STONEMAN) SPAULD. & SCHRENK. AND ITS INTERACTION WITH PHYLLOPLANE MICROORGANISMS

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This is to certify that Miss Gouri Das, M.Sc. has carried out her reasearch work under my supervision. Her thesis entitled " Studies on the brown blight disease of tea (Camellia sinensis) L. (O.) Kuntze) caused by Glomerella cingulata (Stoneman) Spauld. & Schrenk. and its interaction with phylloplane microorganisms" is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

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INTRODUCTION

Tea, Camellia sinensis (L) O. Kuntze is one of the most important crops of India, its cultivation being spread over 3,96,000 hectares of land, divided into two distinct regions - the North Indian tea belt located between 22-27° N and the South Indian tea belt, located at 7° N. North-East India produces 75% of the total Indian tea in three different landscapes (Jain, 1991). Darjeeling produces the world's finest quality teas on the steep hill slopes of Eastern Himalayas upto an elevation of 2000 m. The extensive riverine flat plains at the base of Himalayan ranges are the tea districts of Dooars and Terai. Brahmaputra valley of Assam is located at 100 m above sea level and is the largest flat plains of the world growing tea which accounts for more than half of Indian production.

Tea is made from the young leaves and unopened leaf buds of the tea plant. A number fungal pathogens cause foliar diseases of tea, which assume extreme importance economically, as even slight damages to the leaves reduces the quality and quantity of tea production. A commonly occurring pathogen on tea leaves in all tea growing areas is Glomerella cingulata (Stoneman) Spauld. & Schrenk ., causing brown blight disease. The fungus generally gains entrance through a wound or into tissues that in some other way have been weakened (Baxter, 1974; Bertus, 1974; Dickens and Cook, 1989). The blight first appears on the upper surface of the leaf as a yellowish patch with an expanding border which is the actual seat of the growing disease. This expands to the edge of the leaf, causes a constriction there, and the affected portion of the leaf turns over and shrivels up (Plate I).

A thorough understanding of the physiology and biochemistry of host-pathogen interaction has made it clear, in recent years, that very effective mechanisms of defense enable plants to ward off potential attacks by an enormous number of pathogens. Resistance of a plant against pathogen depends chiefly on the metabolic processes of its cells preceding or following infection or other stresses (Harborne and Ingham, 1978; Ward, 1986). There is now enough evidence to indicate

Plate I- Tea plants (TV-18) grown in the Phytopathological Experimental Garden, naturally infected with *Glomerella cingulata*

PLATE-I

that some products of biotic or abiotic origin are capable of activating the hosts' defense reactionsby accumulating secondary metabolites or "stress" metabolites in infected or treated plants (Albersheim and Anderson-Prouty, 1975; Cruickshank, 1980; Keen and Legrand, 1980; Tepper et al., 1989). These metabolites are produced in appreciably greater amounts in resistant/incompatible reactions than in susceptible/compatible ones (Bhattacharyya and Ward, 1986; Rouxel et al., 1989; Nemestothy and Guest, 1990).

It is a well recognised phenomenon that a wide range of interactions take place between plants and microorganisms both around leaves and around roots. Therefore, under natural conditions, the activities of the pathogen on the host, are also modified to a great extent by its interaction with the vast array of microorganisms associated with the plant surface, mostly as saprophytic residents. The microorganisms are in a dynamic state of interaction among themselves as also with the host. The final outcome of any host-pathogen interaction is, therefore, determined not only by mechanisms governing the hosts compatibility/incompatibility, but also by factors influencing the interaction of the pathogen with other microorganisms.

Indigenous fungi and bacteria are recognised as common contributors to disease suppression and mediated effects of many cultural practices in reducing pathogen populations and limiting disease severity (Windels and Lindow, 1985). Biological control of foliar diseases of tea has become of paramount importance in the present day context as the application of fungicides and pesticides on the leaves is being restricted, on the basis of regulations. The minimum permissible level of residual pesticides and fungicides on tea leaves is very low, and growers are finding it more and more difficult to control diseases with the application of low doses of chemicals. Keeping this in mind, as well as the fact that studies on the brown blight disease of tea caused by G. cingulata are lacking, the study was undertaken. The main objectives of this study are as follows :

(i) to select varieties susceptible and resistant to G. cingulata, by pathogenicity test;

(ii) to make detailed studies on the growth, germination and appressoria formation of this pathogen;

(iii) to determine the levels of phenolics in healthy and infected, resistant and susceptible varieties;

(iv) to study the protein changes in tea leaves following infection and the protein profile of the pathogen;

(v) to isolate elicitors from the pathogen and determine their chemical nature;

(vi) to determine the nature of disease reaction and other biochemical changes elicited by the elicitor in the absence of the pathogen;

(vii) to isolate microorganisms from tea phyllosphere and study their interaction with G. cingulata;

(viii) to select potential antagonists from the isolated microorganisms;

(ix) to partially purify the antifungal compound, if any, from the antagonistic microorganisms;

(x) and to test the isolated compound in vivo and determine its efficacy.

REVIEW OF LITERATURE

Disease develops in individual plants by a series of sequential steps beginning with the arrival of inoculum at the plant surface and ending with the terminal stages of pathogenesis (Cowling and Horsfall, 1978). The stages in between the first and the last are unique to each host, pathogen and depends, to some extent on the prevailing environmental conditions. With the first penetration of the host by the pathogen, changes in host metabolism begin, and in most cases, dynamic defense responses are elicited. One of the most important and well documented host responses is the altered phenol metabolism following or preceding infection. Closely linked to the altered host metabolism is the plant-microbe interactions on the plant surface, which are also responsible for modifying the final outcome of the host-pathogen interaction. Several reviews have appeared on the microbial interactions on the phyllosphere (Dickinson and Preece, 1976; Blakeman, 1981; Cullen and Andrews, 1984; Fokkema and Heuvel, 1986; Andrews, 1990; Andrews and Hirano, 1991; Cook, 1993).

In the following pages, a short, selective review pertaining to the present study has been presented. The review has been divided into three parts : (a) phenolics in plant tissue in relation to disease development, (b) screening of phyllosphere microflora and (c) biological control of foliar diseases.

Phenolics in plant tissues in relation to disease development

Seasonal dynamics of the content of free, ester and glycoside phenolic acids in an ethanol-insoluble fraction were studied in the leaves of 1 year old shoots of the Antonovka apple - tree variety growing for 15 years. Nine different phenolic acids were discovered in them. The ethanol soluble fraction contained about 86-90% phenolic acids; among them 19-25% were free acids, 39-53% were ester-bonded acids, and 12-28% were glycoside-bonded acids. The ethanol insoluble

fraction contained 7-12% of the ester-bonded acids and 3-4% of the glycoside-bonded acids. The content of hydroxycinnamic acids increased towards autumn while that of hydroxybenzoic acids decreased. Hydroxycinnamic acids accumulated in the leaves during intensive growth in summer, regulating the elongation of the cells, whereas hydroxybenzoic acids accumulated in the leaves during autumn, suppressing metabolism (Miidla and Savisaar, 1977).

The dynamics of phenol compounds, stem lignification activity of phenylalanine and tyrosine - ammonia lyases, and the effect of chlorocholine chloride on these processes were studied in winter wheat varieties differing in lodging resistance. An inverse relationship was found between the intensity of the growth processes, activity of phenylalanine and tyrosine - ammonia lyases, content of phenol compounds, and stem lignification and formation of lodging resistance (Lyaskovskii and Kalinin, 1977). Role of phenols in resistance of tobacco to blue mold was established by Pashchenko (1978). From a study of the effects of pyrocatechol, pyrogallol and hydroquinone (0.33 mg/ml concentrations) and aqueous extracts from tobacco [Nicotiana didebta] tissues and leaf washing on conidial growth, no direct relationship was found between their quantity and the resistance of mature, non-damaged tobacco plants to Peronospora tabacina. Pyrocatechol and hydroquinone showed extremely high fungitoxicity in relation to P. tabacina. Pyrogallol somewhat stimulated conidia growth. Spore growth was more strongly inhibited by extracts from damaged plants of resistant tobacco plants than by extracts from tissues of receptive cultivars. Study of polyphenoloxidase of resistant cultivars demonstrated great sensitivity to products isolated from P. tabacina and was highly activated during infection. Lea (1978) reported that Ciders (apple) contain a series of procyanidins upto at least a 7-fold degree of polymerization. A procyanidin tetramer based on epicatechin was isolated by counter-current distribution between ethyl acetate and water. Further details were furnished by Lea and Arnold (1978) regarding the bitterness and

astringency of defined procyanidine fractions from bittersweet English [apple] Cider. They showed that while no. 1 procyanidin could be uniquely identified with bitterness or astringency, bitterness was associated with oligomeric procyanidins reaching a maximum with the epicatechin tetramer, whereas astringency is characteristics of higher molecular weight procyanidins. A method of phenol determination in Salvia occidentalis leaves was developed by Engelsma (1979). The development of the phenol pattern was studied in each separate leaf of a S. occidentalis plant grown in short and in long day. During the light period, the phenol content (mainly chlorogenic acid and isochlorogenic acids) increased in proportion to the length of the period; during the subsequent dark period the phenol content decreased. The decrease did not continue during the 2nd part of a dark period if that period was interrupted by a light break with red light. Instead, a small increase was observed. This effect of red light was reversed with far red light. He further suggested the continuous presence of certain O-dihydroxy phenols in the cytoplasm of leaf cells which inhibited the synthesis or the transport of a flowering hormone, in the case of flower induction in this short day plant.

Isolation and identification of phenolic acids from tobacco leaf was done by Snook et al. (1981). Compounds identified included : cis- and trans-isomers of p-coumaric, ferulic, caffeic and sinapic acids; O-, m- and p-hydroxybenzoic acids; O-hydroxyphenylacetic acid; 2,5- and 3,4-dihydroxybenzoic acids, and 2,3- and 2,5- and 3,4-dihydroxybenzaldehydes. Caffeic acid was the major compound, probably arising from the degradation of the chlorogenic acid of the leaf. Aliphatic acids having 2 or more carboxyl groups were also isolated and included the following : malonic, succinic, fumaric, malic and citric acids. Popravko et al. (1982) reported two new phenolic triglycerides from alcoholic extracts of propilis, Populus tremula and Triticum sativum. They were identified as : 1,3-diferuloyl-2-acetylglycerin and 1-feruloyl-3-p-cunaroyl-2-acelylglycerin. A chemosystematic study of the phenolics of Sonchus sp. was carried out by Mansour et al. (1983).

Thirty-three Sonchus, 1 Embergeria, 1 Babcockia and 5 Teackholmia spp. wre surveyed for their phenolic constituents. The coumarins, scopoletin and aesculetin were found as major constituent of Embergeria, Babcockia and Teackholmia spp. and in lesser amount in some Sonchus spp.

Phenolic acids were extracted from the nutmeats and/or testae of pine nut, almond, fibert, American chestnut, a hybrid American chestnut, Chinese chestnut, black walnut, butternut and Shagbark hickery. Qualitative and quantitative differences were observed among samples in the acids present, with gallic acid being predominant except in pine nut, almond and filbert. Caffeic was the predominant acid in pine nut; protocatechuic acid was predominant in almond and filbert. Phenolic acids isolated and identified were - p-hydroxybenzoic, p-hydroxyphenylacetic, vanillic, protocatechuic, syringic, gallic, caffeic and ferulic acids (Senter et al., 1983). A multistage continuous culture of Nicotiana tabacum cells showed that cells in the 2nd stage produced much higher levels of phenolics per unit weight of cells than cells in either the 1st stage or single stage unit (Sahai and Shuler, 1984). The steady-state was reproducted, when a glucose side stream was fed to the second stage, an increase in apparent cell division was observed with a simultaneous decrease in phenolics productivity. When the toxic precursor phenylalanine was pulsed into the reactor, the quantity of biomass decreased temporarily while phenolic productivity increased.

Hedin et al. (1984) reported that the terpenoid aldehyde content of cotton roots of a root-knot nematode-resistant strain (A 623) was higher initially and increased faster after inoculation than that of a susceptible strain (M-8). The presence of gossypol and 5 other terpenoid aldehydes in root exudates was confirmed. These terpenoid aldehydes were also found in the soil surrounding roots where cotton plants had grown; highest concentrations occurred in soils around the resistant inoculated strain. The terpenoid aldehyde concentrations of

roots of 17 cotton strains were significantly negatively correlated with fusarium (Fusarium oxysporum f. vasinfectum (AtK.) Snyd. et Hans) wilt incidence.

Wheat lines and cultivars known to vary phenologically were investigated in a serial sowing at two locations by Crofts et al. (1984). The wheat lines were classified into 5 categories : Winter wheats, groups I and II; semi-winter wheats; photoperiod - sensitive spring wheats; and relatively insensitive wheats. Only the winter wheats group I, and photoperiod-sensitive spring wheats were able to fully utilize the growing season available in the high rainfall areas of southwestern Victoria.

Resistance of maize to Helminthosporium carbonum and subsequent changes in host phenolics and their antifungal activity was studied by Werder and Kern (1985). Maize inbreds Pr 1 and Pr, resistant and susceptible respectively, to Helminthosporium carbonum race 1 were inoculated and phenolic material was extracted from maize leaf tissue. The components were then analyzed and resistance was studied with respect to phenol metabolism and accumulation of fungitoxic compounds. Host responses could be differentiated by changes in content of phenolic compounds. The pattern of changes of total phenolic content (hydrolyzed and unhydrolyzed ethyl acetate - soluble phenols) of resistant and susceptible inbreds did not differ much between 0 and 96h after inoculation. however, phenolics content in the resistant inbred increased between 96 and 120h after inoculation to a level two to three times higher than that of susceptible and non-infected control inbreds. They isolated four antifungal compounds, A, B, C and D from hydrolyzed maize leaf extracts. All four compounds were fungitoxic to H. carbonum in spore germination and chromatographic bioassays. Compounds. A and B were inhibitory to H. carbonum only in high concentrations. The investigators suggested a role of the phenol metabolism in the resistance of maize to H. carbonum based on the different content of

total phenolics in resistant and susceptible inbreds. The compounds C and D were supposed to play a role in the resistance mechanism as fungitoxic components.

Saxena et al. (1986) evaluated the changes in phenolics in response to brown rust - Puccinia recondita both at the vegetative and reproductive stages of the leaves of two resistant wheat varieties HD 2009 and HD 2285 and two susceptible varieties WG 377 and WG 357. They found that resistant varieties exhibited higher concentration of phenolics than the susceptible one.

Biochemical analysis of pea varieties resistant and susceptible to powdery mildew (Erisyphe polygoni) showed that the quantity of total phenol and orthodihydroxyphenol was high in stem and leaves of resistant varieties as compared to susceptible ones both at 60 and 90 days of plant growth (Parashar and Sindhan, 1987). After infection the quantity of total phenols and orthodihydroxyphenols increased in both stem and leaves of susceptible and resistant varieties. Further, total phenols and orthodihydroxyphenols decreased as the age of plant increased in all the varieties. In case of sorghum rust Naik and Anahosur (1987) showed that there was accumulation of sugars in the lower 3 leaves of susceptible genotype whereas in the resistant genotypes the quantity of sugars got reduced while the level of phenolics increased. In the topmost leaf both sugar and phenols were maximum. Dmitriev et al. (1988) studied the nature of protective response of the onion (Allium cepa) to pathogenic and non-pathogenic fungi (Botrytis allii, B. cinerea and Fusarium solani). Fungal infection was found to stimulate active protective reaction, thickening of cell walls and the formation of phenolic compound, containing cytoplasmic granules. The protective response in the onion developed immediately after the fungus penetrated the cuticle, which stimulated the accumulation of phenolic compound most probably in the endoplasmic reticulum. But they did not determine the degree of fungitoxic activity of the phenol compound. Changes in phenol contents was determined in

young, matured, healthy and Cassicola corynespora and Colletotrichum nicotianae infected leaves of tobacco. After infection the quantity of total phenols and orthodihydroxyphenol increased in both stem and leaves of susceptible and resistant varieties (Oke, 1988). Prasada et al. (1988) also reported that after infection total phenol increased in green and ripe tomato fruits in course of rotting due to Sclerotium rolfsii.

Polyphenol content in sweet cherry bark was reported by Bayer (1989) to be drastically changed after infection by Pseudomonas syringae and Cercospora personii close to the infected tissue. Biosynthesis of polyphenols already present in healthy bark increased after infection. Infected tissue and closely neighbouring areas were characterised by the appearance of phenolic aglycons which inhibited growth of both the pathogen. Mechanically wounded bark tissue showed different phenolic patterns than infected ones. Changes in phenolic and nitrogen metabolism throughout the infection - colonization period[(2, 4, 6, 8, 12 and 15 days after inoculation with Thielaviopsis basicola (Bark. & Br) Ferr.] were investigated in healthy and infected tobacco roots and leaves (Nicotiana tabacum L. - Bright cv. BC 60). Four days after inoculation the pathogen was isolated from infected roots and significant differences in root fresh weights were observed beginning on the 6th day after inoculation. The chlorogenic acid content increased in infected root and leaves compared with the control beginning on the 8th day after inoculation (Torre and Tosi, 1989). Quantitative changes in phenolic compound on barley varieties (Cebada capa, Cl 1243 and Rikal carrying Pa 7, Pa 9 and Pa 2 resistant genes respectively) inoculated by Puccinia hordei was studied by Etenbarian (1989) at different times after inoculation. When inoculated plants were incubated at 5°C there were no significant difference in total phenol content between infected leaves of barley cultivars and their healthy control two days after inoculation. But nine days after inoculation the phenol content of leaves of Cl 1243 and Rikal were

higher than those of the control. When inoculated plants were incubated at 5°C there was no significant difference between total phenol content of infected leaf of Cl 1243 and its healthy control, but the phenol content of infected Cebada capa and Rikal was significantly higher than those of the control thirty-eight days after inoculation.

Electron opaque (Eo) structures were observed in the xylem parenchyma cell of both incompatible and compatible Apium graveolens var. rapaceum and compatible Apium graveolens var. dulce with Fusarium oxysporum (Jordon et al., 1989). They found that Eo bodies were observed in the non-inoculated host. Histological, cytochemical and chemical tests indicated the presence of phenolic substance in Eo material. These Eo structure increased both in number and size as infection progressed. The incompatible produced 3-5 times more of the Eo materials than the compatible. The amount of Eo material and host compatibility were related to the absence and presence of fungal hyphae in the vascular system.

Baker et al. (1989) examined specific race interaction with cloves of resistant and suceptible genotypes. It was found that in resistant reaction accumulation of phenolic compounds were higher than susceptible reaction. They suggested that accumulation of phenolics including phytoalexins may play a role in natural and induced resistance interaction involving Colletotrichum trifolii and Medicago sativa. In his studies, Luthra (1989) determined levels of total phenol in sorghum leaves resistant and susceptible to Ramulispora sorghicola at 15 days interval after 25 days of sowing. Resistant variety exhibited high phenol content in comparison to susceptible ones at all stages of growth.

Host pathogen interactions between potato tubers and soft rot bacteria Erwinia carotovora subsp. atroseptica and dry rot fungi Fusarium spp. were investigated by a special descending paper

chromatographic technique for the elution of soluble compounds from the infected tissue region (Röber, 1989). The continuously formed sugars, phenolics and phytoalexins were determined by thin layer or gas chromatography after diffusing into the eluate. Both freshly harvested and stored potato tubers produced phenolics and phytoalexins after infection. The uninfected controls produced only low levels of these compounds.

Minimum inhibitory concentration (MIC) of polyphenolic components of tea against sixteen well known strains of food borne pathogenic bacteria was determined by Hara and Ishigami (1989a). As tea polyphenols, crude catechins from green tea extract and its four components, and crude theaflavins from black tea extract and its four components were used. The results showed that these tea polyphenols had antibacterial activity against Vibrio parahaemolyticus, V. fluvialis, V. metschnikovii, Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Plesiomonas shigelloides and Aeromonus sobria. But they hardly showed any such activity against A. hydrophila subsp. hydrophila, Salmonella enteritidis, S. typhimurium, enteropathogenic Escherichia coli, enteroinvasive E. coli, Yersinia enterocolitica, Campylobacter coli and C. jejuni. Further studies on antibacterial activity of tea polyphenols against Clostridium botulinum was undertaken by Hara and Watanabe (1989). They determined the minimum inhibitory concentration of tea polyphenols against spores and vegetative cells of C. botulinum. As tea polyphenols, crude catechins, and crude theaflavins were separated. These crude substances were further purified into each four catechins and theaflavins. Catechins and theaflavins were mixed at graded concentrations with TGC medium to make the test medium. Spores (5×10^2) were mixed with above test media anaerobically in pouches. Vegetative cells were preincubated and streaked on the solidified test medium. All these samples were incubated anaerobically at 30°C for 3 days to find out the growth inhibitory concentrations. Several hundred ppm concentrations of crude catechins, gallated catechins and all theaflavin fractions inhibited

the growth of both spores and vegetative cells of C. botulinum.

Anti-bacterial and anti-hemolysin activities of tea catechins and their structural relatives was also studied by Toda et al. (1990). Among catechins tested, (-) epigallocatechin (EGC), (-) epicatechin gallate (ECg) and (-) epigallocatechin gallate (EGC_g) inhibited the growth of Staphylococcus aureus, Vibrio cholerae. S. aureus was more sensitive than V. cholerae to these compounds. EGC_g also showed a bactericidal activity against V. cholerea. Pyrogallol showed a stronger anti-bacterial activity against S. aureus and V. cholerae than tannic acid and gallic acid. Rutin or Caffein had no effect on them. EC_g and EGC_g showed the most potent anti-hemolysin activity against S. aureus α-toxin, Vibrio parahaemolyticus thermostable direct hemolysin (Up-TDH) and Cholera hemolysin. Among catechin relatives, only tannic acid had a potent anti-hemolysin activity against α-toxin. These results showed that the catechol and pyrogallol groups were responsible for the anti-bacterial and bactericidal activities, while the conformation of catechins might play an important role in the anti-hemolysin activity.

Kumar et al. (1990) analysed certain biochemical changes in the pearl millet shoots infected with downy mildew pathogen (Sclerospora graminicola). The estimation revealed that the total phenol and free amino acids content were found to be low both in diseased shoot and roots of pearl millet (Pennisetum americanum). A marked accumulation of two caffeic acid esters after inoculation of maize with Glomerella graminicola or Cochliobolus heterostrophus was reported in both compatible and incompatible combinations (Lyons, 1990). Seasonal changes in the phenolics constituents of jack pine seedlings (Pinus banksiana) in relation to the purpling phenomenon was studied by Nozzolillo et al. (1990). A proanthocyanidin reaction was always present during the summer at variable levels but increased markedly in late fall. Total phenolics showed a gradual increase over the season.

Analysis of phenolics of bud exudate of Populus sieboldii by GC - MS was conducted by Greenaway et al. (1991). Analysis by GC-MS separated 50 components of bud exudate of which 36 were phenolics. The bulk of the bud exudate (66.7%) was composed of the flavonone naringenin and its methyl ethers sakuranetin and isosakuranetin, together with their corresponding chalcones. These flavonones were minor components (<1%) in bud exudate of poplars from other sections.

Screening of phyllosphere microflora

Interaction between a number of phyllosphere fungi of wheat and Puccinia graminis tritícii was studied by Mishra and Tewari (1976). They observed that several saprophytes, particularly Penicillium notatum, Myrothecium roridum, Cladosporium herbarum and Nigrospora sphaerica caused marked reduction in uredospore germination in vitro and the number of uredosori on leaves in glass house. Verma and Singh (1976) also isolated phyllosphere microorganisms and studied their interaction with Xanthomonas malvacearum, the causal agent of bacterial blight of cotton.

It was reported by Austin et al. (1977) that a number of phylloplane bacteria, particularly Pseudomonas fluorescens, isolated from leaves of Lolium perenne were antagonistic to the pathogen Drechslera dictyoides. The antagonists reduced spore germination and germtube growth and caused lysis of hyphae which led to a reduction in lesion development. Studies on blast, brown spot and bacterial leaf blight of rice was carried out by Jagadeesh et al. (1978). They observed that phylloplane bacteria of rice reduced the disease intensity in all the above cases. Verma et al. (1978) observed that avirulent strains, heat killed cells and phylloplane bacteria such as Flavobacterium sp. and Aeromonas sp. could reduce the bacterial disease of cotton caused by Xanthomonas malvacearum if inoculated 8 to 48h before infection. However, epiphytic bacteria caused an increase in the

pathogenicity of the anthracnose fungus by competing for nutrients and creating starvation situation in which appressoria were rapidly produced on short germtubes giving rise to infection pegs (Blakeman, 1978). Young (1978) studied the survival of epiphytic bacterial species of Pseudomonas, Xanthomonas and Erwinia groups on leaves of Prunus which usually exist in well protected sites in the leaves out of reach of common fungicidal sprays. More than 30 genera of fungi were isolated from leaves of two varieties of rapeseed (Tsuneda and Shoropad, 1978). These fungi were classified into three groups depending on their occurrence - those which developed with onset of senescence, or occurred rarely, or were present throughout the growing season. A survey of leaf inhabiting fungi of Quercus robur showed that the population varied between trees, increased towards the end of the season and occurred more profusely on the upper surface than on the lower (Cox and Hall, 1978).

The role of leaf surface yeasts and bacteria in reducing disease caused by plant pathogens has been emphasized by Fokkema (1978). According to him "phyllosphere fungi" behave as scavangers on leaves with enhanced nutrient conditions (e.g. by pollen, or honey dew) diminishing the nutrient availability to pathogens.

The yeast flora of field grown wheat was manipulated by spraying cell suspensions of common yeasts - Cryptococcus aurentii var. flavescens and Sporobolomyces roseus, along with nutrients, which caused an approximately ten-fold increase in yeast populations within a week. Inoculation of plants having enhanced yeast flora with Septoria nodorum and Cochliobolus sativus showed a 50% reduction of disease incidence from that on control leaves having normal flora (Fokkema et al., 1979).

An interesting study of phylloplane fungi in relation to Xanthomonas malvacearum infections of cotton by Wadje and Deshpande (1979) revealed that increased infection always caused an increase in

the number of fungi on the leaf surface. The dominant fungi were found to be Cladosporium, Phoma and Alternaria and the first two fungi along with Curvularia and Aspergillus inhibited the growth of the pathogenic bacterium. Grover et al. (1979) investigated the fungal population of leaf surfaces of Brassica campestris and isolated 29 spp. of fungi from leaves at various developmental stages. the maximum population was recorded at the flowering stage. While working on the pathogen Erwinia amylovora, Beer et al. (1980) observed that the inhibition of bacterial pathogens can result from bacteriocin production by closely related nonpathogenic epiphytes. They suggested that application of antagonistic phylloplane bacteria to leaves may probably be more successful for control of plant pathogenic bacteria rather than fungi, probably because conditions which favour multiplication of the saprophytes will be very similar to those for the pathogen. Hegde et al. (1980) used Bacillus subtilis to control foot rot of wheat caused by Sclerotium rolfsii.

Some authors (Reinecke, 1981; Spurr, 1981) studied phylloplane microorganisms mainly in relation with their possible use for controlling the incidence and development of leaf diseases through antagonism and competition. Spurr Jr. (1981) considered the role of microbial antagonists in control of foliar plant pathogens. Most examples included phylloplane fungi and bacteria antagonistic to pathogenic Alternaria sp., mainly A. alternata on tobacco leaves.

While investigating healthy and infected leaves of Trapaeolum majus, Saxena and Saxena (1982) found that leaves infected with Laveiliula taurica contained a distinct type of mycoflora which was absent in the healthy leaves.

It has been reported that the age of the cultivar, sowing time and soil amendment by fertilisers affect the phyllosphere microflora of rice plants (Sarkar and Samaddar, 1982). In general, 90 day old plants

had high population of microorganisms irrespective of sowing time. The leaf surface bacterial populations increased with increase in the doses of organic and inorganic fertilisers.

Effect of spraying aqueous suspension of eight phyllosphere fungi on the occurrence and development of powdery mildew disease of pea was studied by Srivastava and Bisht (1983). Seasonal incidence of phyllosphere microflora of guava (Psidium guayava) was studied from bud stage to senescent stage. The number of compositai phylloplane fungi was maximum in rainy and minimum in summer season. Pestalotia psidii, Fusarium oxysporum and Colletotrichum gloeosporioides were isolated from the foliage in different seasons with different stages of dominance. The number of phylloplane mycoflora increased with leaf age (Pandey and Dwivedi, 1984).

Ercolani (1985) analysed the factors influencing populations of Pseudomonas syringae pv. savastanoi on the phylloplane of olive. Forty-nine species of twenty fungal genera were collected from the wheat phyllosphere and phylloplane on 1% glucose and 50% Czapek's agar at 28°C. The total counts displayed seasonal periodicity. In the case of phyllosphere the most frequent species were Aspergillus niger, Penicillium corylophilum and Alternaria alternata; in the phylloplane Aspergillus niger and A. flavus (Mazen et al., 1985). An interesting observation from Egyptian tomato phylloplane was made by Zaher et al. (1985). The total number of epiphytic fungi on uninfected and artificially infected Pritchard cv. were low at the beginning of the Nili season and increased with the plant age. Isolates from healthy leaves were identified as Aspergillus flavus, A. niger, A. ochraceus, Penicillium sp. and pink yeast. A. ochraceus appeared only on Pritchard leaves artificially infected with Ulocladium botrytis.

Zaher and his colleagues (1985a) also reported that the total count of epiphytic bacteria were low and progressively increased through winter season but they were higher in the resistant cultivars

of Lycopersicum esculentum. Actinomycetes were only detected after 45 days from transplanting, disappeared after that, and were more abundant on resistant cultivars than on susceptible cultivars. More actinomycetes were also detected on uninfected Pritchard when compared to the artificially infected one.

Leaves of Spinacea oleracea infected by Cercospora beticola harboured more fungal population/cm^2 leaf surface and more fungal species than non-infected leaves. There was a characteristic mycoflora on the leaf surface of Spinacea oleracea. Some fungi were restricted either to infected or non-infected leaves. Disease severity was greatly influenced by atmospheric temperature, humidity and age of the plant (Singh et al., 1986).

The phylloplane microflora of the medicinal desert plant Aloe vera L. was studied by Mahmoud et al. (1986). They noted that the microflora varied widely from summer to winter and on the upper leaf surface to the lower one. Salem et al. (1986), on the basis of their studies on the phyllosphere of grape and orange trees, reported that the highest population of yeast in the phyllosphere was recorded in spring period reaching 2.9 x 10^2/g leaves in grape and 2.2 x 10^2/g leaves in orange. The most common genera of the two hosts were Debaryomyces, Saccharomyces, Cryptococcus, Rhodotorula, Torulopsis and Candida. Yeasts belonging to the genus Rhodotorula were found in comparatively high number compared to those of Cryptococcus in the summer period. Candida and Torulopsis were less dominant in phyllosphere of both the trees. There were no significant variations in the frequencies of ascosporogenous genera Debaryomyces and Saccharomyces with season.

Different field grown plants in Egypt were screened for different phyllosphere yeasts which ranged between 1.4 x 10 to 4.3 x 10^3 cells/g. Their highest count were found in the phyllosphere of

grape as well as Egyptian clover and wheat while their lowest counts were in the phyllosphere of cabbage and cowpea. Rhodotorula glutinis, Cryptococcus albidus, Cryptococcus diffluens, Torulopsis famata, T. aeria, Candida curvata, C. humicola and Debaryomyces kleockii were the yeast species found in the phyllosphere. The pigmented yeast R. glutinis was the common yeast in the phyllosphere where it constituted 50% of the total isolates, while Cryptococcus albidus did not exceed 20% of the total isolates. Spore forming yeast were less dominant in the phyllosphere (Bard-El-Din et al., 1986).

Khanna (1986) carried out a comparative study of the phylloplane and phyllosphere microflora of Croton bonplandianum, Musa sapientum and Tectona grandis growing at air polluted and non-polluted sites. The phyllosphere of plants growing at the polluted site harboured a higher population of fungi and actinomycetes; on the other hand, a reduction in the population of bacteria was recorded at this site. At the non-polluted site, Curvularia lunata, Acremonium sp. and Pestalotia sp. were the only fungi present.

Using a direct observation technique, Thomas and Shattack (1987) observed that filamentous phylloplane fungi on the senescent, damaged and diseased portions of leaves of Lolium perenne had 40 spp. at a single locality. The most common genera were Cladosporium, Drechslera, Phoma, Epicoccum, Alternaria and Leptosphaeria. Drechslera andersenii and D. siccans were found to be the most common pathogens.

A seasonal variation of quantity in the population of soft rot bacteria on the surface of the white cabbage leaves was studied by Matveena and Odintsova (1987). They showed that the level of epiphytic population of the pathogen increased by the end of vegetative season and depended on the variant resistance. Quantitative and qualitative estimation of the microflora on the surface of Eichhornia leaves during a period of one year was undertaken. Both bacteria as well as fungal

population in the phyllosphere and phylloplane was found to vary with the seasons. The quality and relative proportion of the fungal forms including a severe pathogen, Curvularia lunata varied with the changes in the climatic conditions. The dominance of the pathogen in the mycoflora from August to October was shared by Aspergillus, Cladosporium and Fusarium (Kaur and Chandra, 1987).

Selected fungi recovered from the phylloplane of lettuce were assayed by Mercier and Reeleder (1987), using in vitro and in vivo techniques, to determine their effectiveness as antagonists of ascospores of the lettuce drop pathogen, Sclerotinia sclerotium. On leaf discs, Trichoderma viride, Alternaria alternata and Epicoccum purpurascens significantly reduced ascospore germtube elongation by 34.2, 60.6 and 38.3% respectively. On whole plants these three fungi significantly reduced infection by 40-90.3%. Isolates of Cladosporium cladosporioides and a red yeast inhibited ascospore elongation significantly in vitro leaf disc assay. Hyphae of E. purpurascens did not come into direct contact with ascospore and inhibition may have been due to production of antifungal compound. Phyllosphere actinomycetes showed inhibition zones against the growth of Alternaria, Helminthosporium oryzae and Pyricularia oryzae (Kausar and John, 1987).

Babu et al. (1988) screened the phyllosphere mycoflora of Citrus sinensis Osbek. The fungi Acrophialophora fusispora, Acremonium terricola, Alternaria alternata, Curvularia pallescens, C. prasadii, C. lunata var. aeria, Drechslera hawaiiensis, D. rostrata, Fusarium moniliforme, Myrothecium roridum, Penicillium oxalicum, Phoma tracheiphila, Pyrenochaeta fallax and Sordamia fimicola were present only on healthy plants, while C. tuberculata, Trichoderma viride and Trichothecium roseum were only associated with the leaves of diseased plants. The phyllosphere mycoflora of healthy plants was qualitatively rich. Reducing sugars, free amino acids and phenols were more in healthy plants. The reverse was true with the proteins.

Extensive studies on phyllosphere and phylloplane were carried out by Alhubaishi and Abdel-Kader (1991). Fungi of 86 species belonging to 31 genera were collected from the qat phyllosphere (30 genera and 78 species) and phylloplane (20 genera and 64 species) of 24 varieties tested. In the phyllosphere the most frequent genera were Cladosporium, Aspergillus and Alternaria followed by Penicillium, Drechslera, Fusarium, Curvularia, Phoma and Chaetomium. From the preceding genera the most prevalent species were C. herbarum, C. macrocarpum, A. niger, A. flavus, A. alternata, P. citrinum, P. notatum, D. specifera, D. hawaiiensis, F. oxysporum, F. moniliforme, C. lunata, P. humicola, P. herbarum and C. globosum. In the phylloplane the order of genera frequency was more or less the same while the most common species were C. herbarum, C. sphaerospermum, A. niger, A. flavus, A. alternata and A. tenuissima.

Similar type of observation was made by Roy et al. (1991) from potato phylloplane, who isolated 20 fungi, viz. Pestalotiopsis mangifera, P. neglecta, P. versicolor, Penicillium purpurogenum, P. oxalicum, P. aurantiogriseum, Myrothecium varrucaria, Fusarium equisetti, F. moniliformae, F. pallidoroseum, Alternaria solani, A. alternata, Aspergillus terreus, A. niger, Trichoderma harzianum, T. koningii, Epicoccum nigrum, Chaetomium globosum, Cladosporium cladosporioides, C. herbarum. Out of these, nine fungi viz. A. terreus, F. moniliformae, P. mangiferae, P. neglecta, P. purpurogenum, P. oxalicum, P. aurantiogriseum, M. varrucaria and T. harzianum showed inhibitory effect on lesion size/sporulation against Phytophthora infestans.

Habber et al. (1991) isolated effective antagonists of several pathogen of sunflower from the leaves and other parts. The antagonistic bacteria recovered from leaves were mainly actinomycetes and Gram-positive bacteria. Candidate antagonists were specifically selected for broad-spectrum inhibitory activity in vitro.

It was reported by Smith and Couche (1991) that novel variants of Bacillus thuringiensis were present in the phylloplane of deciduous and conifer trees as well as other plants, so they considered B. thuringiensis as a part of the common leaf microflora of many plants.

Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time was studied by Ercolani (1991). He isolated mesophilic, heterotrophic, aerobic or facultatively anaerobic bacteria from the surface of olive leaves of 3 or 4 different ages. From the identity of the representative strains, the frequency of occurrence among the phylloplane bacteria over the 6-year period was estimated at 51% for Pseudomonas syringae followed by Xanthomonas campestris (6.7%), Erwinia herbicola (6%), Acetobacter aceti (4.7%), Gluconobacter oxydans (4.3%), Pseudomonas fluorescens (3.9%), Bacillus megaterium (3.8%), Leuconostoc mesenteroides subsp. dextranicum (3.1%), Lactobacillus plantarum (2.8%), Curtobacterium plantarum (2.2%), Micrococcus luteus (2.2%), Arthrobacter globiformis (1.4%), Klebsiella planticola (1.2%), Streptococcus faecium (1.2%), Clavibacter sp. (0.98%), Micrococcus sp. (0.82%), Serratia marcescens (0.81%), Bacillus subtilis (0.57%), Cellulomonus flavigina (0.4%), Erwinia sp. (0.37%), Zygomonus mobilis (0.3%), Bacillus sp. (0.29%), Alcaligenes faecalis (0.27%), Erwinia carotovora (0.08%) and Pseudomonas aeruginosa (0.04%). Communities on the leaves of a given age at a given time of a year were invariably dominated by one or another of only 9 taxa, which accounted for 22 to 98.5% of the isolates from those leaves.

Biological control of foliar diseases

Phylloplane of corn was screened for antagonists to reduce Bipolaris maydis infection. It was reported that Pseudomonas cepacia inhibited B. maydis by producing antibiotics which produce inhibition zone against the fungus in culture (Sleesman et al., 1976). Similar type of investigation was carried out by Vidaver (1976) who isolated specific protein containing antibiotic substances, known as

bacteriocins from leaf-inhabiting bacteria such as Erwinia, Corynebacterium and Pseudomonas.

Outstanding success in greenhouse biocontrol were those of cucumber powdery mildew by antagonists in the fungal genera Ampelomyces, Tilletiopsis and Stephanoascus. Ampelomyces quisqualis, a parasite of many mildew pathogens including Sphaerotheca fuliginea and Erysiphe cichoracea, when applied interspersed with water sprays, improved disease control indicating need of moisture for spore dispersal and infection (Jarvis and Slingsby, 1977). Swinburne (1978) reported that canker caused by Nectria galligena could be controlled using Bacillus mycoides, B. subtilis, B. thuringiensis and Pseudomonas cepacia. Similar type of observation was also made by Spurr (1981). Spray with those organisms successfully controlled Alternaria leaf spot of tobacco and peanut Cercospora leaf spot in field condition.

Alternaria blight of chilli was partially controlled by both Trichoderma viride (83.5% reduction) and Streptomyces sp. (79.5% reduction) isolated from phylloplane (Tyagi and Chouhan, 1981).

Bacillus megaterium was found to be effective in controlling anthracnose of jute caused by Colletotrichum corchori. This bacterium reduced the germination of conidia of C. corchori, thus reducing its pathogenicity. Moreover, the bacterium also secreted a toxic metabolite into the surrounding medium which was inhibitory to the growth of the fungus (Bhattacharyya and Purkayastha, 1982). This bacterium was originally isolated from the phyllosphere of jute along with 26 fungi and 11 other bacteria. In paired cultures Aspergillus nidulans, Penicillium oxalicum and two strains of Bacillus megaterium were highly antagonistic to the pathogen. An ethyl-acetate soluble, partially termolabile antifungal substance was detected in culture filtrate of B. megaterium (B-23) which significantly reduced spore germination and germtube growth of C. corchori. Foliar spray of bacterial suspension

and culture filtrate 24h prior to inoculation markedly reduced the production and spread of lesions on jute leaves by C. corchori (Purkayastha and Bhattacharyya, 1982).

It was reported by Baker et al. (1985) that autoclaved culture filtrates of Bacillus subtilis applied three times per week controlled bean rust in field significantly better than the fungicide mancozeb applied once per week. Bacillus megaterium, one of the predominant components of microflora on the phylloplane of rice plants was a potent antagonist against phytopathogens like Drechslera oryzae, Alternaria alternata and Fusarium roseum (Islam and Nandi, 1985). Singh (1985) emphasized on the use of Epicoccum purpurascens as antagonist against Macrophomina phaseolina and Colletotrichum capsici. The antagonistic compound was identified as Chrysin and parahydroxybenzoic acid. E. purpurascens was also antagonistic to Gloeosporium psidii and 7 other pathogens causing fruit rot of cucurbits. Bacillus subtilis isolated from soybean was tested for antagonism against 26 fungi, commonly associated with soybean in dual culture. On Potato Dextrose Agar (PDA) medium the bacteria were fungicidal to Penicillium species and fungistatic to all others. Autoclaved filtrates of B. subtilis culture inhibited growth and stroma formation of Phomopsis species. A chloroform soluble component from autoclaved B. subtilis culture filtrate was active against 7 soybean pathogen on PDA. Suspension of B. subtilis applied as a soybean seed treatment reduced stem infection caused by Phomopsis species in a growth chamber but not in the field (Cubeta et al., 1985).

Zaher et al.(1985a) isolated a number of phylloplane bacteria which caused inhibition zone and reduced radial growth of Ulocladium botrytis, the causal agent of tomato leaf spot. Two of these isolates were more antagonistic and were identified as Bacillus cereus and Erwinia herbicola. In using healthy cultivar of tomato (Pritchard), B. cereus and E. herbicola decreased U. botrytis infection by 74.7% and 77.7% respectively. Simultaneous spraying of these two antagonists gave

highly significant result on whole plant. They confirmed that pink yeast and Penicillium species isolated from Extra Marmande tomato CV were more inhibitive than those taken from Marmande and Pritchard. Aspergillus niger isolated from Marmande were more inhibitive against U. botrytis. In vivo study showed that A. niger, A. ochraceus, Penicillium species and pink yeast decreased infection by 45.7%, 61.5%, 73.7% and 78.6% respectively (Zaher et al., 1985b).

Sinha (1986) observed that among a large number of isolates of phyllosphere bacteria of rice tested for prevention of bacterial leaf blight and leaf streak disease, Erwinia herbicola completely suppressed the disease symptoms. Aqueous suspension of phyllosphere fungi Penicillium fellutanum, Drechslera spicifera, Aspergillus fumigatus and Fusarium oxysporum effectively reduced powdery mildew (Erysiphe cichoracearum DC.) on Cucurbita maxima (Srivastava and Bist, 1986). Melgarejo et al. (1986) reported antagonistic effect of 4 fungi (Aspergillus flavus, Epicoccum nigrum, Penicillium frequentans and Penicillium purpurogenum) against Monilinia laxa on peach twigs in spring and autumn. In spring, E. nigrum, P. purpurogenum and P. frequentans significantly reduced the success of infection when introduced before inoculation with the pathogen but A. flavus caused only a transient delay in the development of symptoms. However, when inoculation preceded antagonist introduction, only the treatments with P. frequentans resulted in a lasting reduction of symptom development. In autumn, only the treatments with E. nigrum resulted in a reduction of M. laxa infection.

Antifungal substances antagonistic to Monilinia fructicola were isolated from Bacillus subtilis (Mckeen et al., 1986). The active fraction was extracted from the cell-free medium after precipitation by acidification to less than pH 2.5. Finally, the precipitate was extracted with 80% ethanol; this extract contained antibiotics that were soluble in ethanol, methanol, isopropanol, and water above pH 7.5, but not soluble in ethylacetate, acetone, ether or methylenechloride. Thin layer chromatographic separation of the extract indicated four

biologically active bands. The extract was fungistatic, not fungicidal against spore of Monilinia fructicola. When tested for activity against M. fructicola on peach fruit the extract showed almost complete suppression of brown rot at 1 mg/ml.

Several yellow pigmented, rod-shaped bacterial strains with antagonistic activity against Erwinia amylovora were isolated from blighted ornamental shrubs by Isenbeck and Schulz (1986). In contrast to the non-inhibitory isolates of the same host origin the active forms obviously produced a substantial principle. This was active only under acid condition, was heat stable, dialysable and soluble in water and methanol. In disease control experiments using culture filtrates of the antagonistic bacterial isolates, fire blight was reduced to a limited extent following shoot tip inoculation of Cotoneaster bullatus under controlled conditions.

Culture filtrate of three phylloplane fungi (Fusarium sp., Helminthosporium sp. and Penicillium sp.) from sunflower inhibited the spore germination of Alternaria helianthi significantly. These fungi when sprayed on to the sunflower leaves three days prior to inoculation with A. helianthi, reduced the lesion production significantly (Somasundara and Kumar, 1986).

The amount and role of Hendersonia acicola Tub. in the epidemic caused by Lophodermella sulcigena (Rostr.) V. Hohn. was followed on Scots pine, Pinus sylvestris L. (Jal-Kanen and Laakso, 1986). From year to year H. acicola increased on needles primarily infected by L. sulcigena upto the end of the Lophodermella epidemic. Hendersonia was first abundant in the lower crown, while the upper parts were nearly free of it. The increase of H. acicola prevented L. sulcigena from producing ascocarps and led to the recovery of the trees and stands. L. sulcigena could fruit only in those needles which were free of H. acicola in the autumn. The autumnal presence of H. acicola in the needles led to a natural control of the pathogens. Mitchell et al.

(1987) studied the mycoparasitic activity of Dicyma pulvinata against Cercosporidium personatum. At 26°C, conidia of D. pulvinata close to both hyphae and conidia of C. personatum germinated within 11-17h. Visible signs of colonization of lesion of C. personatum by D. pulvinata appeared within 58-65h (21-31.5 h leaf wetness). D. pulvinata was an effective protectant when plants were exposed to continuous leaf wetness at 26°C for 5 days. In field microplot studies, lesions of C. personatum were visibly colonized by both mutant and wild type isolates of D. pulvinata within 4 days after applying their conidial suspensions. Environmental conditions during this 4 day period were 40h leaf wetness, 60h of 23-28°C (optimal temperature for growth of the wild type isolates) and 17.31 cm rainfall.

Seedlings in the cotyledon-to-first-leaf-stage of sickle-pod (Cassia obtusifolia) were inoculated with conidia or hyphal suspension of the biocontrol fungus Alternaria cassiae. Seedlings were examined for conidial germination, germtube growth, appressorial formation and extent of fungal host interaction with light and scanning electron microscopy. It was observed that conidia germinated 2-3h after incoulation, germtube growth reached 50-300 μm and growth usually terminated in appressoria (Van Dyke et al., 1987).

Biological control of Rhizopus rot of peach was investigated by Wilson et al. (1987). They observed that Enterobacter cloacae (isolate D-3) delayed the onset and reduced the development of rot in artificially wounded peaches inoculated with Rhizopus stolonifer. Rhizopus infection was completely inhibited in 70% of fruit upto 5 days after inoculation. The effectiveness of E. cloacae was related to relative inocula concentrations of the pathogen and antagonist. Fruit firmness affected Rhizopus infection, but not E. cloacae effectiveness when cells were applied with the culture medium in which they grew. However, no compounds toxic to Rhizopus were detected in culture filtrates of E. cloacae.

Blue mold, a major post harvest disease of apples caused by Penicillium expansum was controlled with an antagonistic bacterium and a yeast (Janisiewicz, 1987). Potential antagonists were isolated from apple plants throughout the year and from stored fruit. Lesion development was related to pathogen spore concentration and the concentration of the antagonist. The highest spore concentration of the pathogen (1×10^7 spores per milliliter) was totally controlled by higher concentrations of the antagonist. This protection was on going because subsequent reinoculation of wounds did not result in lesion development.

Isolates AF 1 and BACT 1 (obtained from Canada) of Bacillus subtilis was reported by Podile and Dube (1987) to cause wider inhibition zones against Phytophthora drechsleri f. sp. cajani. The pathogen failed to grow in 10 fold concentrated cell free culture filtrate of AF1 and in 5 fold concentrated extract the inhibitory effect on radial growth was proportional to concentration. Increasing concentration of cell free culture filtrate of AF1 in Richard's solution decreased the dry weight of the fungus. Two isolates of Eubacteria, twelve isolates of three actinomycetes, namely, Streptomyces albidoflavus, S. diastaticus, S. rochei and 4 fungal isolates from phylloplane - Botrytis cinerea, Penicillium chrysogenum, P. rubrum and Myrothecium roridum showed antagonistic potentialities against Phytophthora colocasiae in vitro (Narula and Mehrotra, 1987). In vivo bacterial isolates reduced the disease incidence upto 37-43%. Maximum reduction in percentage infection over control was caused by Streptomyces albidoflavus (90-93%), S. diastaticus reduced the percentage infection by 76% and S. rochei isolates resulted in 30-75% disease control over checks on fresh detached leaves of Calocasia esculanta. Among the fungi maximum control was obtained with Botrytis cinerea (33%).

Ordentlich et al. (1988) determined the role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsii. Serratia marcescens was grown on cell wall of Sclerotium rolfsii and its components, chitin and laminarin as a sole carbon source. A culture filtrate, possessing chitinolytic activity was obtained and incubated with different substrates including S. rolfsii cell wall, dry mycelium, washed mycelium. A release of N-acetyl-D-glucosamine at rates of 1-5.2 chitinase units indicated substrate degradation. Degradation of hyphae of the plant pathogen was observed by light and scanning electron microscopy. Crude chitinase caused very swift swelling at the hyphal tips of the fungus. Sixty three percent of the cells of the hyphal tips lysed.

Lysis of zoospores of Phytophthora in vitro was reported with culture filtrates of Bacillus cereus Franklin and Franklin strain UW 85 (Handelsman et al., 1988). Jindal et al. (1988) studied the antagonistic effect of six phylloplane fungi against Phytophthora infestans in the laboratory and green house condition. Maximum inhibition in vitro was caused by Penicillium aurantiogriseum followed by Fusarium equiseti, Mucor hiemalis, Trichoderma koningii, Epicoccum purpurascens and Stachybotrys atra. Application of spores of all the test fungi individually on potato plants significantly reduced the intensity of late blight and a maximum disease control of 93% was achieved with spores of P. aurantiogriseum. Culture filtrates of all the test organisms when applied on leaves simultaneously with the pathogen and 12h prior to inoculation also significantly reduced the severity of the disease.

Biological control of papaya fruit rot was achieved with Bacillus subtilis. Culture filtrate of B. subtilis caused radial growth inhibition of the fungus in poisoned food technique. In liquid culture, dry weight of the fungus was less in culture filtrate supplemented medium. In both the cases, inhibitory effect was proportional to

concentration of the culture filtrate. Test pathogen failed to form sporangia and the mycelium was malformed in presence of 40% culture filtrate of B. subtilis. Pretreatment with B. subtilis or its culture filtrate protected papaya fruits from Rhizopus nigricans infection (Podile and Dube, 1988).

Boland and Hunter (1988) considered protection of bean flowers as an important strategy for controlling white mould caused by Sclerotinia sclerotiorum. They found that several antagonists from flowers suppressed white mould under controlled condition, especially Alternaria alternata and Cladosporium cladosporioides.

Phyllosphere of geranium cultivars was screened for antagonists to geranium-rust by Rytter et al. (1989). Twelve strains of Bacillus were isolated and tested for their effect on spore germination of Puccinia pelargonii-zonalis, the causal agent of geranium rust. Of these, strain 3 of B. subtilis, inhibited spore germination and reduced the incidence of rust pustules on inoculated leaves in the green house. The culture filtrate was most inhibitory in decreasing the amount of pustules per leaf area, followed by the washed bacterial cell treatment. The investigators further reported that cells cultured and applied to leaves in nutrient broth were more effective in reducing rust development. Eugon broth enhanced the antagonistic effect in comparison to nutrient broth when bacteria were applied for different periods before inoculation with rust spores, the antagonistic effect persisted for at least 4 days after application. Kapoor and Kar (1989) studied the method of antagonism of Bacillus sp. to Fusarium oxyspor um f. sp. lycopersii, the causal organism of tomato with culture broth as well as cell-free filtrates of four potent bacterial isolates (Bacillus) showed inhibitory effect. Complete inhibition of the radial growth was observed with Bacillus sp.

Fungitoxic substance was isolated from culture filtrate of

Bacillus megaterium (B-23) as described by Bhattacharyya and Purkayastha (1989). Age of culture, and pH of medium influenced the fungitoxicity of its culture filtrate. Partially purified toxin was thermolabile, non-dialysable, ethyl acetate soluble, vanillin-sulphuric acid positive and effective within a rane of pH 5-9. It exhibited maximum UV absorption at 224 nm. Its melting point was 242°C. The efficacy of this compound was tested on 4 jute parasites namely, Colletotrichum corchori, C. gloeosporioides, Myrothecium roridum and Alternaria citri of which M. roridum and C. corchori were least and most sensitive to the toxin respectively.

Interaction between Debaryomyces hansenii and Penicillium digitatum were studied in culture and on fruit to better characterize the observed biological control of green mould on grape fruit by the yeast. The antagonist did not produce antibiotic substances in culture and was ineffective in protecting against the disease when killed by heat or chemicals. Incidence of green mould was dependent upon the concentration of both the pathogen spores and antagonist yeast cells. Control of green mould was most effective at 10^9 cfu/ml of D. hansenii. Significant inhibition of spore germination and hyphal growth of P. digitatum in culture was achieved by the addition of the yeast cells to a minimal synthetic growth medium. Inhibition of P. digitatum by the antagonist in culture and on the fruit peel could be overcome by the addition of exogenous nutrient. Results indicated that competition for nutrients may play a role in the biocontrol of P. digitatum by D. hansenii on grape fruit (Droby et al., 1989).

The ectomycorrhizal fungus Pisolithus tinctorius secreted a metabolite that lysed hypha and conidia and inhibited the germination of conidia in a range of phytopathogenic fungi (Kope and Fortin, 1990). The culture filtrate containing the metabolite had an optimum incubation period of 42-56 days, gradual dilution of culture filtrate with fresh medium caused a gradient of inhibitory effects. For hyphal lysis the minimum ratio of culture filtrate to medium for complete

growth inhibition was 5:1 for Rhizoctonia praticola and Truncatella hartigii and 3:1 for Sphaerosporella brunnea. At higher dilutions, the hyphae that were formed were short celled and highly branched; many hyphae lysed. Conidial germination was completely inhibited at a ratio of 5:1 for both Fusarium solani and T. hartigii, 3:1 for a North American isolate of Brunchorstia pinea (NA), 1:1 for a European isolate of B. pinea (EU), and 1:3 for two strains of Cochliobolus sativus (0910, 0912). Conidial lysis was seen for T. hartigii at a ratio of 5:1 and at a ratio of 1:1 for B. pinea (EU) and C. sativus (0910). Characterization of the cell free culture filtrate through the separate additions of D-glucose and an adsorbant, activated charcoal, showed that hyphal lysis and conidia germination inhibition did not result from a depletion of carbohydrates from the growth medium but for the presence of some substance adsorbed by charcoal.

Roitman et al. (1990) described a new chlorinated phenylpyrrole antibiotic produced by the antifungal bacterium Pseudomonas cepacia, collected from apple leaves during a screening programme designed to detect agents for biological control of fruit spoilage fungi. They for the first time reported 2,3-dichloro-4-(2-amino-3-chlorophenyl) pyrrol. In vitro testing showed that all four of the phenylpyrrole showed fungal inhibitory effects on Golden Delicious apples inoculated with conidia of pathogenic organisms.

Biocontrol agents active against gray mould of apple were reported by Roberts (1990). These included isolates of the bacterium Pseudomonas cepacia, the mycelial fungus Acremonium brevae and the yeasts Cryptococcus laurentii, Kloeckera apiculata and Candida guilliermondii, from the surface of leaves or fruits of apple or lemon, all of which were active against B. cinerea. Wilson et al. (1990) isolated Erwinia herbicola from flowers and leaves of hawthorn (Crataegus monogyana). The isolates were tested against fire blight disease caused by Erwinia amylovora. Two isolates of E. herbicola WL 9

and WL 40, reduced both blossom- and shoot-blight. WL 9 provided over 80% control of blossom blight, equivalent to that provided by chemical agents and also gave total control of shoot blight when applied at a WL 9 : pathogen ratio of 10 : 1.

Biological control of foliar diseases of alfalfa was undertaken by Douville et al. (1990). They studied the pathosystem that included five diseases - spring black stem (Phoma medicaginis), leaf spot (Leptosphaerulina trifolii), common leaf spot (Pseudopeziza medicaginis), stemphylium leaf spot (Stemphylium botryosum) and anthracnose (Colletotrichum sp.). During the seed pathological studies of seed borne fungi on agar plate a change isolate from sorghum grain, Bacillus licheniformis was found to secrete inhibitory substances only against the species of Aspergillus. Primary investigation suggested that a dialysable relatively heat stable substance which was able to inhibit the infection of Aspergillus niger strongly, was secreted by B. licheniformis (Pingale and Kshirsagar, 1990).

Cultures and cell-free culture filtrates of the biocontrol agent Bacillus cereus UW 85 was found to lyse zoospores of Phytophthora cactorum in vitro (Gilbert et al., 1990). Changes in the ionic composition of the growth medium caused by growth of UW 85 account for the lytic activity. UW 85 raised the pH, excreted ammonia, and removed calcium from the medium during growth and sporulation. Zoospores lysed when pCa^{2+} : pNH_3 was greater than 0.8. The lytic activity was produced in uninoculated growth medium by adding ammonium chloride and base to create a pCa^{2+} : pNH_3 ratio similar to that of UW 85 culture filtrate.

Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties by Bacillus subtilis was studied by Sandrin et al. (1990). Of the 13 strains of Bacillus subtilis tested, only 1 produced both lipopeptides with a high yield. The cultures were made in synthetic medium. Several L-amino acids and various carbon sources were good substrates for the lipopeptide

production. The maximum yield of surfactin was about 110 mg/l and that of iturin A about 39 mg/l. Production of mycosubtilins B and C - the minor antibiotics from mycosubtilin producer Bacillus subtilis was similarly reported by Besson et al. (1990). Mycosubtilins B and C were isolated from the culture medium of Bacillus subtilis. The acid hydrolysates of these new antifungal antibiotics, like mycosubtilin, contained α-amino acids (Asp_3, Glu_1, Pro_1, Ser_1, and Tyr_1) and a mixture of iso-C_{16}, n-C_{16}, iso-C_{17}, and antiiso-C_{17} amino acids. Mycosubtilins B and C differed by the presence of a carboxyl group and a carboxymethyl group, respectively.

The culture supernatant of Bacillus sp. showed growth inhibition against several kinds of pathogenic fungi. The strain remarkably inhibited the growth of Fusarium sp. The supernatants of about 30-fold dilution and 20-fold dilution retained the antifungal activities against Pyricularia oryzae and Helminthosporium oryzae, respectively (Tsuji et al., 1990a). Further detailed study on isolation and purification of antifungal substance produced by Bacillus spp. revealed that this water soluble antifungal compound was acidic polypeptide. Moreover, it was thermostable and purified powder showed a strong antifungal activity to the test organisms Helminthosporium oryzae and Pyricularia oryzae (Tsuji et al., 1990b).

Suitable bioassay techniques to evaluate the antagonists against pearl millet downey mildew was outlined by Shishupala (1991). The isolated antagonists spores were smeared on the infected leaf bits and incubated in moist chambers to allow S. graminicola to sporulate asexually. Two species of Trichoderma prevented asexual sporulation of the pathogen, Sclerospora graminicola completely. The spores of the antagonists and sporangia of S. graminicola taken in distilled water and incubated revealed that both Trichoderma spp. and Bacillus subtilis were inhibitory to the sporangial germination of the pathogen. Efficacy of antagonists culture filtrates were tested against asexual

sporulation of the pathogen by its volatile metabolites. All the culture filtrates were found inhibitory for the sporangia/zoospore germination. These bioassay methods were found to be accurate, rapid and reliable to evaluate large number of antagonists against S. graminicola. Late blight of potato was controlled by different antagonist mycoflora from the phylloplane (Roy et al., 1991). Inocula used were zoospore/sporangial suspensions with detached leaf method used throughout the studies. The fungi were studied alone and in combinations for their inhibitory effect. Maximum inhibition (lesion size) in sporangial inoculation was observed in Aspergillus terreus and Penicillium purpurogenum (64.3%). There was marginal difference amongst the antagonists in zoospore inoculation. Amongst the combination of antagonists, P. oxalicum + P. aurantiogriseum, A. terreus + P. aurantiogriseum and A. terreus + P. purpurogenum showed better inhibition over other combinations.

Lazano and Laberry (1991) working on cassava screened a wide range of microorganisms from cassava for inhibitory activity against a wide range of microorganisms. Strains of fluorescent pseudomonads were found with promising biocontrol activity against cassava leaf blight caused by Xanthomonas campestris pv. monihotis when sprayed on the leaves.

Biocontrol of Claviceps fusiformis, the incitant of ergot of pearl millet was achieved by Mahadevamurthy et al. (1991). Effect of solvent (ethyl acetate) extracts of culture filtrate and mycelial mat of Aspergillus niger, Epicoccum andropogonis, Fusarium chlamydosporum, F. heterosporum, Trichoderma harzianum, T. viride and Bacillus subtilis (extracted with chloroform) on sclerotial germination of C. fusiformis was studied. As the concentration of the extracts increased, the percentage inhibition of sclerotial germination also increased. The mycelial mat extracts of A. niger, F. chladosporum and F. heterosporum were more effective in reducing the sclerotial germination compared to the culture filtrate and mycelial mat of different antagonists tested.

Douville and Boland (1992) observed the influence and mechanism of action of Bacillus subtilis on Colletotrichum trifolii, a causal agent of anthracnose of alfalfa (Medicago sativa) in vivo and in vitro. In growth room conditions, a cell-free culture filtrate of B. subtilis significantly reduced disease incidence and severity on alfalfa seedlings from 56% to 16% and from 2.0 to 1.2, respectively. Treatment of seedlings with washed cell suspensions of B. subtilis had no influence on disease. Application of crude filtrate on alfalfa leaflets inoculated with C. trifolii were associated with reduced germination and lysis of conidia, as well as reduced formation of appressoria. Under in vitro conditions, crude filtrate reduced germination of inflated germ tubes on germinating conidia. An antibiotic of the iturin family, iturin D, was tentatively identified as the active compound responsible for the suppressive effect of B. subtilis on C. trifolii. Leaf blight disease of Mentha arvensis L. subsp. haplocalyx Briquet var. piperascens Holms, caused by Corynespora cassicola, was controlled by Alternaria alternata. The radial growth inhibition of C. cassicola was 72% in relation to control. Active gradient from culture filtrate also exhibited antifungal activity. Partially purified fraction was subjected to liquid partitioning. The aqueous fraction recorded 83% inhibitory activity while organic fraction depicted 65.3%. A detached twig of M. arvensis sprayed with highly pathogenic isolate of C. cassicola followed by the treatment of aqueous fraction of antifungal products of A. alternata responded effectively in controlling the disease (Kumar et al., 1992).

Role of Saccharomyces cerevisiae in protecting maize plants against Colletotrichum graminicola, the causal agent of anthracnose, was studied by Dasilva and Pascholati (1992). The effect of previous or simultaneous application of suspension of filtrates of S. cerevisiae on condium germination and appressorium formation by C. graminicola as well as lesioned area, number of lesions and sporulation capacity of the fungus on the leaves was determined. Suspensions from washed or non-washed S. cerevisiae cells and filtrates of these suspension

reduced the development of C. graminicola as well as the expression of anthracnose on leaves when they were previously or concomitantly treated with these preparations. In the same way, when cells of S. cerevisiae were isolated from commercial baker's yeast and grown in potato dextrose-agar medium the cell suspension and their filtrates were also able to reduce the development of C. graminicola and the expression of the disease on the leaves. The different yeast preparations and their filtrates were shown to be thermolabile. The reduction of the development of C. graminicola and disease expression on the leaves were, therefore, due to the presence of a thermolabile substance or a complex of substances released from the cells into the filtrates. In vitro experiments exhibited a possible antagonistic activity of S. cerevisiae cells against C. graminicola due to antibiosis.

The investigations of Mostafa et al. (1992) showed the antagonistic action of fungal and bacterial microorganisms on the two major biological sequences of Drechslera teres. The antagonists that were the most efficient on mycelial growth belonged to the genus Trichoderma. The inhibition of mycelial morphogenesis was temperature and pH dependent. The outstanding efficiency of antagonistic actinomycetes as well as of Myrothecium verrucaria on conidial germination was related to the release of toxic antibiotic substances.

Antagonistic fungi from cauliflower petals against Sclerotinia rot of cauliflower were collected from each of four developmental stages. Petals from per developmental stages were surface sterilized and incubated on PDA for isolating associated mycoflora. The influence of selected phylloplane fungi were studied on mycelial growth, ascospores germination and on lesion development. The percentage of petals colonized ranged from 12.3% to 69.1% in different stages. Nine genera were isolated by Alternaria and Cladosporium predominated. Penicillium and Phoma were isolated from bleached flowers only.

Botrytis, Epicoccum and Gliocladium were not recovered from freshly opened flower. Sclerotinia and Verticillium sp. and Gliocladium sp. completely prevented lesion development under inoculated conditions. The naturally occurring isolates were effective enough to control Sclerotinia sclerotiorum (Kapoor, 1993).

Clinton et al. (1993) evaluated several species of Streptomyces for their ability to control Sclerotinia homoeocarpa (dollar spot) and Bipolaris sorokiniana (leaf spot) on the phylloplane of Poa pratensis (Kentucky bluegrass). Species evaluated included S. diastaticus (S32), S. galbus (S35) and S. hygroscopicus (isolates S13, S26). All evaluations were conducted on the upper epidermis of intact attached leaves of P. pratensis and antagonism was measured as the ability of Streptomyces isolates to prevent chlorophyll loss from leaves inoculated with B. sorokiniana or S. homoeocarpa during pathogenesis. Only S. hygroscopicus (S13) effectively prevented infection and subsequent chlorophyll loss from leaves inoculated with B. sorokiniana or S. homoeocarpa. Isolate S23 of S. hygroscopicus showed erratic antagonism of both pathogens depending upon how the isolate was prepared for use. Streptomyces diastaticus and S. glabus were antagonistic to S. homoeocarpa only in whole culture form.

Potential microbial antagonists of Colletotrichum gloeosporioides were isolated from blossom leaves and fruit of mango and screened using a series of assay techniques (Koomen and Jeffries, 1993). In total 648 microorganisms including bacteria, yeasts and filamentous fungi were isolated and tested for their inhibition of growth of C. gloeosporioides on malt extract agar. In vitro 121 organisms inhibited the fungus and were tested further for their ability to affect conidial germination. These were inoculated onto mangoes artificially infected with C. gloeosporioides and assessed for their potential to reduce the development of anthracnose lesions. Seven isolates were chosen for use in a trial using freshly harvested fruit.

This final screening procedure yielded two potential candidates for further trials, isolate 204 (identified as Bacillus cereus) and isolate 558 (identified as Pseudomonas fluorescens).

The antagonistic behaviour of a number of dominant leaf inhabitants isolated from leaf surfaces of different plants were studied on agar plates by Behera and Nayak (1993). Penicillium granulatum was found to be quite effective against Curvularia lunata exhibiting 80% inhibition in radial growth and upto 90% inhibition against Alternaria alternata. Antagonism between Cladosporium cladosporioides and Aspergillus awamori was seen with 75% and 45% inhibition respectively. Aspergillus awamori considerably checked the growth of C. cladosporioides, Aspergillus terreus and C. lunata. Out of the 30 fungi isolated 12 showed antagonistic effect of one against the other. Among them A. awamori, P. granulatum and A. niger showed better result over other fungi tested.

An interesting observation was made by Saikia and Chowdhury (1993). They evaluated different cell concentrations of phylloplane microflora for the control of bacterial leaf blight of rice caused by Xanthomonas oryzae pv. oryzae (Xoo). All the microorganisms including the heat killed and avirulent cells of the pathogen showed antagonistic effect towards Xoo and the control was better with their higher levels. However, Erwinia herbicola controlled the pathogen most effectively even at lowest ratio (1:1) and registered more than 90 per cent reduction in disease development at 50:1. Bacillus subtilis also performed better than other antagonists in reducing the build up of the bacterial leaf blight of rice. Tip clipping method of inoculation exhibited significantly more protection than swab and infiltration methods. The antagonistic protective effect of the phylloplane microflora while inoculated in combination got reduced against Xoo.

An antibiotic producing strain of Bacillus subtilis was shown to produce potent antifungal volatiles (AFV) as described by Fiddaman and

Rossall (1994). These volatiles were active against a range of fungal species and were produced on a range of growth media and in loam based compost. In vitro antifungal volatile activity on nutrient agar was enhanced with the addition of D-glucose, complex carbohydrates and peptones. The addition of L-glucose showed significantly less AFV activity than comparable levels of D-glucose. Growth studies in liquid culture revealed that B. subtilis failed to grow in response to L-glucose. Further growth studies on solid media showed no clear correlation between enhanced bacterial growth and increased in vitro AFV activity in response to supply of substrates. Low level AFV activity was also detected from oilseed rape roots inoculated with B. subtilis. Gas chromatography, mass spectrometry head space analysis of B. subtilis cultures grown on various substrates revealed common similarities between substrates promoting AFV activity, although it was not possible to isolate individual antifungal compounds.

Stangarlin and Pascholati (1994) reported that maize seedlings susceptible to Exserohilum turcicum (popcorn cultivars "South American Mushroom" and "Pirapoca Amarela"), were subjected to previous or concomitant treatments to the inoculation of Saccharomyces cerevisiae obtained from commercial baker's yeast. The results showed that S. cerevisiae cells, depending on the concentration of time of application were highly effective in protecting maize leaves against the necrotrophic pathogen E. turcicum. In vitro experiments showed that S. cerevisiae cells and filtrates of these suspensions were able to inhibit condium germination. In vivo experiments also showed that yeast cells inhibited conidium germination and penetration of the host by the fungus, although appressorium formation was stimulated. It was concluded that S. cerevisiae potects maize leaves against E. turcicum possibly through antibiosis (inhibition of conidium germination) and/or activation of resistance mechanisms in the host.

Antagonism of Erwinia herbicola towards Leptosphaeria maculans causing blackleg disease of Brassica napus was studied by Chakraborty

et al. (1994). Phyllosphere microorganisms of Brassica napus were isolated and their antagonism against the pathogen was tested in vitro. In paired culture, Erwinia herbicola was found to be highly antagonistic to L. maculans. Bioassay of the culture filtrate of the bacterium against the test fungus revealed that E. herbicola secretes an antifungal substance into the culture medium. This substance was partially thermolabile and markedly reduced the germination and germtube length of L. maculans. Aqueous bacterial suspensions and cold sterilized culture filtrates, when applied to the seedlings prior to inoculation, significantly reduced the severity of blackleg disease.

MATERIALS AND METHODS

3.1 Plant Material

3.1.1 Selection

Ten different tea varieties were selected for plantation in the Phytopathological Experimental Garden of Department of Botany, North Bengal University, based on their growing stability as observed under field conditions over the years by Bezbaruah and Singh (1988). The varieties used in the present investigation were TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, Teen Ali-17/1/54 and CP-1.

3.1.2 Collection

Tea plants (18 month old) of nine clonal varieties - TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28 and Teen Ali 17/1/54 were obtained from the clone house of Mohurgong and Gulma Tea Estate, Sukna, Darjeeling; one seed variety (CP-1) was collected from the nursery of Dey's Tea Plants, Khaprail, Darjeeling.

3.1.3 Plantation

Before planting programme pre-conditioning was done. Attention was paid towards decision on spacing, planting time, land preparation, weed suppression etc. Excavation of soil for pit preparation was carried out in 3 stages :

i) 15 cm deep x 45 cm across - top soil removed and placed separately.

ii) 30 cm deep x 45 cm across - sub-soil cut and again kept separately.

iii) 30 cm deep x 15 cm across - soil loosened to 75 cm minimum depth of worked soil; to this soil, Thimate was added after which soil conditioning was done as follows :

to the top soil 30-40g Super phosphate was mixed and sub-soil was mixed with 4 kg dry cattle manure.

Following soil conditioning plants were inspected, selected and brought to the exprimental garden and planted in the prepared pits. Pits were refilled with conditioned soil.

Tea plants of all the varieties were also grown in earthen pots (one plant/pot 30 cm dia.) each containing 5 kg soil mixture (soil : planting mixture - 1:1).

Mulching was done immediately after planting and a thin continuous layer of material (monocot leaves) was spread over the ground. As soon as convenient, a 5-7 cm thick layer was built up.

To control weed Simazine was applied @ 75 gms/20 litres water as pre-emergent and Glyphosate @ 1:200 against thatch type of grasses, as described by Barbora (1988).

3.1.4 Growth and maintenance

A soluble mixture of NPK consisting of 10 kg Urea - 46% N, 20 kg ammonium phosphate - 11% P_2O_5, 8 kg muriate of potash, 60% K_2O was prepared; 8 kg of this mixture in 200 litre water made a suitable spray fluid.

As soon as 1 or 2 leaves appeared, the fluid was applied to the under side of all leaves with a hand operated sprayer. Greenol (Triacontanol) was also sprayed at regular intervals for good growth of bush. The main stem and side shoots were trimmed 12 weeks prior to inoculation in order to obtain new shoots.

3.2. Fungal Culture

3.2.1. Source of culture

A virulent strain of Glomerella cingulata (Stoneman) Spauld. & Schrenk. was originally isolated from naturally infected leaves of tea growing in the experimental garden. The strain was identified by Dr. P.F. Cannon, Commonwealth Mycological Institute, Kew, Surrey, U.K. (IMI No. 356806). This strain was used in all studies after completion of Koch's postulate.

3.2.2 Completion of Koch's postulates

Fresh, young tea leaves were collected from experimental garden and inoculated with conidial suspension of the isolated Glomerella cingulata following detached leaf inoculation technique. After 72h of inoculation, the infected tea leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 min, washed several times with sterile distilled water and transferred aseptically into Potato-Dextrose-Agar (PDA) slants. These isolates were examined after two weeks of incubation at 28°C and the identity of the organism was confirmed by comparing with the stock culture.

3.2.3 Maintenance of stock culture

The fungus thus obtained was sub-cultured on PDA slants. After two weeks the culture was stored under three different conditions (5°C, 20°C and at room temperature 30 ± 2°C). Apart from weekly transfer for experimental work, the culture of Glomerella cingulata was also examined at regular intervals to test its pathogenicity.

3.2.4 Assessment of mycelial growth in liquid medium

To assess mycelial growth of G. cingulata in a liquid culture, the fungus was first grown in Petridishes, each containing 20 ml of PDA

medium and incubated for 4 days at 30°C. From the advancing zone of the mycelial mat, agar block (4 mm dia.) containing the mycelia, was cut with a sterilized cork borer and transferred to each Ehrlenmeyar flask (250 ml) containing 50 ml of sterilized medium for the desired period at 30°C. Finally the mycelia were strained through muslin, collected in aluminium foil cup of known weight, dried at 60°C for 96h, cooled in a desiccator and weighed.

3.3 Composition of media

A number of culture media were used in the course of the study. The names and composition of these media are given below :

Potato Dextrose Agar (PDA)

Potatoes (Peeled and sliced)	..	200.0 g
Dextrose	..	20.0 g
Agar	..	20.0 g
Distilled water	..	1 L

Richard's medium (RMA)

KNO_3	..	10.0 g
K_2HPO_4	..	5.0 g
$MgSO_4.7H_2O$	..	2.5 g
KCl	..	0.5 g
$FeSO_4.7H_2O$	..	0.01g
Sucrose	..	30.0 g
Agar	..	20.0 g
Distilled water	..	1 L
pH	..	7.3 + 0.2

Oat meal agar (OMA)

Oat meal	..	30.0 g
Agar	..	20.0 g
Distilled water	..	1 L

[Oat meal boiled with stirring for 1h. Squeezed through a muslin bag volume made upto 1 L, added agar, boiled till dissolved].

Bean Juice Agar (BJA)

Bean	..	100.0 g

[Crushed with distilled water, strained and final volume made upto 1 L].

Agar	..	20.0 g

Carrot Juice Agar (CJA)

Grated Carrot	..	20.0 g
Agar	..	20.0 g
Distilled water	..	1 L
pH	..	5.1 - 5.5

[Grated carrot boiled, strained through muslin bag, volume made upto 1 L, added agar, boiled till dissolved].

Green Tea Agar (GTA)

Green tea leaves	..	100.0 g

[Crushed with distilled water, strained and final volume made upto 1 L).

Agar	..	20.0 g

Boiled Tea Agar (BTA)

Tea leaves	..	100.0 g
(Cut, boiled and final volume made upto 1 L).		
Agar	..	20.0 g

Hoagland and Knop's solution

KNO_3	..	0.61 g
$Ca(NO_3)_2,4H_2O$	..	0.95 g
$MgSO_4,7H_2O$	..	0.49 g
$(NH_4)_2H_2PO_4$	..	0.12 g
$MnSO_4,4H_2O$	..	3.0 g
$ZnSO_4,7H_2O$	..	0.5 mg
H_3PO_3	..	0.5 ml
$CuSO_4,5H_2O$	..	0.025 mg
$Na_2MoO_4,2H_2O$	..	0.025 mg
H_2SO_4	..	0.05 L
$F_6C_6O_5H_7,5H_2O$	..	0.2 g
Distilled water	..	1 L

Nutrient Agar (NA)

Peptone	..	5.0 g
Beef extract	..	3.0 g
NaCl	..	3.0 g
Agar	..	20.0 g
Distilled water	..	1 L
pH	..	7.2-7.4

Esculine hydrolysis test medium
(Facklam and Wilkinson, 1981)

Esculine	..	1.0 g
$FeCl_3.6H_2O$	..	0.5 g
Peptone	..	5.0 g
Yeast extract	..	1.0 g
Agar	..	15.0 g
Distilled water	..	1 L
pH	..	7.2

Gelatine Agar (Sneath and Collins, 1974)

Beef extract	..	3.0 g
Peptone	..	5.0 g
Gelatine	..	10.0 g
Agar	..	20.0 g
Distilled water	..	1 L
pH	..	7.2

Milk Agar (Gordon et al. 1973)

Skim milk powder	..	5.0 g in 50 ml distilled water
Agar	..	1.0 g in 50 ml distilled water

[Autoclaved separately for 20 min., cooled at 45°C and mixed together].

Modified Davis and Mingioli's broth

K_2HPO_4	..	7.0 g
KH_2PO_4	..	3.0 g
Sodium Citrate, $3H_2O$	..	0.5 g
$MgSO_4,7H_2O$	..	0.1 g
$(NH_4)_2SO_4$	..	1.0 g
Yeast extract	..	0.2 g
Distilled water	..	0.2 g
pH	..	7.2

Glucose-yeast extract peptone agar (Kregar-van-Rij, 1984)

Glucose	..	20.0 g
Peptone	..	10.0 g
Yeast extract	..	5.0 g
Agar	..	20.0 g
Distilled water	..	1 L

Starch agar (Pronina, 1962)

Soluble starch	..	20.0 g
K_2HPO_4	..	1.0 g
$CaCl_2.2H_2O$	..	0.1 g
$MgSO_4.7H_2O$	..	0.6 g
NaCl	..	0.1 g
$FeCl_3.6H_2O$	..	0.01g
$NaNO_3$	..	2.5 g
Agar	..	15.0 g
Distilled water	..	1 L
pH	..	7.2

Tributyrin agar (Stolp and Gadkari, 1981)

Peptone	..	5.0 g
Yeast extract	..	3.0 g
Tributyrin	..	10.0 g
Agar	..	15.0 g
Distilled water	..	1 L
pH	..	7.4

[Agar was added after blending the mixture to rest of the ingredients at 60°C].

Voges-Proskauer broth (Norris et al., 1981)

Peptone	..	7.0 g
Glucose	..	5.0 g
NaCl	..	5.0 g
Distilled water	..	1 L
pH	..	6.5

Peptone liquid medium (Irschik et al., 1983)

Tryptone	..	6.0 g
Yeast extract	..	0.5 g
$MgSO_4.7H_2O$	..	2.0 g
$CaCl_2.2H_2O$	..	0.4 g
Distilled water	..	1 L
pH	..	7.2

Nitrate broth

K_2HPO_4	..	7.0 g
KH_2PO_4	..	3.0 g
Sodium citrate	..	0.5 g
KNO_3	..	2.0 g
Yeast extract	..	0.2 g
Glucose (autoclaved separately)	..	5.0 g
Distilled water	..	1 L
pH	..	7.0

Tryptophan broth

K_2HPO_4	..	7.0 g
KH_2PO_4	..	3.0 g
Sodium citrate	..	0.5 g
Yeast extract	..	0.2 g
Glucose (autoclaved separately)	..	2.0 g
L-Tryptophan	..	1.0 g
Distilled water	..	1 L
pH	..	6.8

Urea medium (Christensen, 1946)

Part A

Peptone	..	1.0 g
Glucose	..	1.0 g
NaCl	..	5.0 g
KH_2PO_4	..	2.0 g
Phenol red	..	0.012 g
Agar	..	20.0 g
Distilled water	..	1 L
pH	..	6.8

Part B

Urea	..	40.0 g
Distilled water	..	1 L

[Sterilized by filtration].

After sterilization of Part A in test tubes (5 ml/tubes), 0.25 ml of sterile Part B was added.

Antibiotic Assay Medium No. 1
(Grove and Randall, 1955)

Peptone	..	6.0 g
Pancreatic digest of casein	..	4.0 g
Yeast extract	..	3.0 g
Beef extract	..	1.5 g
Dextrose	..	1.0 g
Agar	..	15.0 g
pH	..	6.6

SIM Agar

Peptone	..	30.0 g
Beef extract	..	3.0 g
Ferrous ammonium sulphate	..	0.2 g
Sodium thiosulfate	..	0.025 g
Agar	..	15.0 g
pH	..	7.0

3.4 Inoculation techniques

3.4.1 Detached leaf

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded tea leaves were detached from plants and placed in plastic trays lined with moist blotting paper. Wounds were made on the adaxial surface of each leaf and inoculated either with spore suspension or mycelial block of the fungus. Mycelial block inoculation was done by placing plug (2 mm dia.) of mycelia bearing conidia from 10-day old culture aseptically on the inoculation site. Sterile Richard's medium blocks or sterile distilled water were used as control.

For inoculation with spore suspension, 20 µl droplets of conidial suspension (1.2 x 10^6 conidia/ml) of the fungus (prepared from 10-day old culture) were placed (2-4 drops/leaf) on the adaxial surface of each leaf with a hypodermic syringe after making appropriate needle scratches. In control sets, drops of sterile distilled water were placed on the leaves.

Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize the drying of drops during inoculation.

3.4.2 Cut shoot

Cut shoot inoculation technique was followed as described by Yanase and Takeda (1989). Twigs (with 3-4 leaves) of tea plant grown in the exprimental garden were cut carefully and immediately introduced into glass vials containing sterile tap water and taken to the laboratory. Leaves were inoculated by making 2 mm light scratch with a sharp sterilized needle on the adaxial surface of the leaf and placing mycelial plugs on the scratches. Mycelial plug inoculated cut shoots were placed into the holes of styrofoam board which was floated on modified Hoagland and Knop's solution and kept in a glass chamber (45 cm x 30 cm) for one week with aeration.

3.4.3 Whole plant

Well established and branched tea plants grown in pots were inoculated with fungal isolate following the method of Dickens and Cook (1989). Inoculation was done by spraying the plants with conidial suspension (1.2 x 10^6 conidia/ml) prepared from 10-day old culture of fungus grown on Richard's medium. In control sets the plants were sprayed with sterile distilled water. After spraying the plants were kept for 48h in transparent polythene chamber to maintain a high level of humidity. Subsequently, the plants were removed from the chamber and grown with soil surface watering. Twenty plants were inoculated in each treatment.

3.5 Assessment of disease intensity

3.5.1 Detached leaf

Percent drops or mycelial plugs that resulted in lesion production was calculated after 48, 72 and 98h of inoculation. Diameter of the lesions were noted. Observations were based on 50 inoculated leaves for each treatment and average of three separate experiments.

3.5.2 Cut shoot

In laboratory experiment, the actual number of lesions that developed on the artificially inoculated shoots were counted after 48, 72 and 98h. Diameter of the individual lesions were measured. They were graded into four groups and a value was assigned to each group. Very small restricted lesions, 1-2 mm dia. = 0.1; 2-4 mm dia. with sharply defined margin = 0.25; lesions with slow spread beyond 4 mm = 0.5 and spreading lesions variable in size with diffused margin = 1.0. Number of lesion in each group was multiplied by the value assigned to it and the sum total of such values were noted and disease index were computed as the mean of observation of 50 cut shoot per treatment.

3.5.3 Whole plant

Symptoms on leaves were assessed after 4, 8, 12 and 16 days of inoculation as described by Sinha and Das (1972), taking into consideration both number and size of the lesions. On the basis of visual observation the lesions were graded into four sized groups, such as very small, small, medium and large with respective values of 0.1, 0.25, 0.5, 1.0 assigned to give an appropriate idea of their relative size. Number of lesions in each size groups was multiplied by the values assigned. The sum total of such values for all the leaves gave the disease index for a plant. Results were always computed as the mean of observation of 20 plants (50 young leaves randomly picked off from each plant) per treatment.

3.6 Leaf clearing technique

Tea leaves were washed well, dried and on them drops of conidial suspension of G. cingulata were put with a hypodermic syringe. The leaves were then placed on moist filter papers in petridishes and incubated for 10h. Pieces were cut from the leaves with a cork borer, taking care that the pieces were cut from the places where the drops were put. These pieces were then kept on petridishes containing moist filter paper and the whole set was placed in a bell-jar. For leaf clearing concentrated HCl was added to $KMnO_4$ in a flask which was connected to another flask containing water where the excess HCl was absorbed. The Cl_2 was then passed to the bell-jar containing the leaf discs. When chlorine was passed for 1-1½ h the leaf discs became completely white. They were then taken out and washed with ammonia. The discs were then stained with 0.05% Lactophenol cotton blue, mounted and observed under the microscope.

3.7 Collection of diffusible compounds from tea leaves

Leaf diffusates were obtained following drop diffusate technique of Muller (1958) with modifications. First, young leaves were collected from tea plants, washed in sterile distilled water and blotted dry with blotting paper. Fifty leaves were placed on moist blotting paper in each plastic tray (30 cm x 30 cm). Twenty µl droplets (2-4 per leaf) of sterile distilled water or conidial suspension of G. cingulata (1.2×10^6 conidia/ml) prepared from 10-day old cultures with sterile distilled water were placed on the adaxial surface of each leaf. Each tray was covered with a glass lid and sealed with a smear of white petroleum jelly to maintain necessary humidity and incubated for 24-72h at 28°C. Drops of spore suspension were collected from leaf surfaces, combined and centrifuged ; this supernatant was treated as the diffusate. Similarly, the water drops were also collected from the leaf surfaces, combined and centrifuged and the supernatant obtained was the exudate. Finally, the diffusates and exudates were passed through

sintered glass filter and then immediately assayed for their effect on spore germination of the pathogen.

3.8 Extraction of total phenol and orthodihydroxy-phenol from healthy and infected tea leaves

Following the method of Mahadevan and Sridhar (1982) total phenol and orthodihydroxy-phenol were extracted from tea leaves. Fresh, young leaves (first, second and third) were collected from experimental garden and kept in plastic trays. Leaves were mounted with drops of conidial suspension (1.2×10^6 spores/ml) of the pathogen, G. cingulata. Control sets were prepared by mounting the leaves with drops of sterile distilled water. Healthy and infected (48h after inoculation) leaves (10g) were taken out and placed separately in boiling 80% ethanol (4 ml ethanol per gram fresh weight of leaf tissue) in Ehrlenmeyer flask (250 ml) for 30 min., cooled and crushed thoroughly in Waring blendor for 5-10 min. passed through two layers of cheese cloth and filtered through filter paper. The volume was raised with 80% ethanol to represent 1g tissue in 5 ml alcohol.

3.9 Estimation of total phenol and orthodihydroxy-phenol content

Total phenol

The total phenol was estimated by Folin-Ciocalteau's method as described by Bray and Thorpe (1954). One ml alcohol extract was taken in a 25 ml test tube; 1 ml of Folin-Ciocalteau's reagent was added to it, followed by 2 ml of 20% Na_2CO_3 solution. The tube was shaken and heated on a boiling water both for 1 min. The tube was cooled under tap water and volume raised to 25 ml. A blank was prepared with all reagents except the sample, to adjust the zero reading. Quantity of total phenol was estimated with caffeic acid standard in a Systronic Photoelectric colorimeter Model 101 at 515 nm wavelength.

Orthodihydroxyphenol

Quantitative estimation of Orthodihydroxyphenols was done by Arnow's method (1933). Initially 1 ml of the alcohol extract was taken in a colorimetric tube and the following reagents were added - 2 ml of 0.05 N.HCl, 1 ml of Arnow's reagent ($NaNO_3$ - 10g; Na_2MoO_4 - 10g; distilled water 100 ml) and 2 ml of 1N NaOH. The volume was raised to 25 ml and absorbance was estimated using a Photoelectric colorimeter Model 101 at 515 nm wavelength. A blank containing all the reagents except Arnow's was used to adjust the absorbance to zero. Quantity of phenol was estimated following the standard curve of caffeic acid.

3.10 Extraction of proteins

3.10.1 Extraction of leaf proteins

Soluble proteins were extracted from healthy and G. cingulata infected leaves of the varieties following the method of Alba and Devay (1985) with modification. Fresh, young leaves (first, second and third) were collected from the experimental garden and kept in plastic trays as described in detached leaf inoculation technique. Leaves were mounted with drops of conidial suspension (1.2×10^6 spores/ml) of G. cingulata. Control sets were prepared by mounting the leaves with drops of sterile distilled water. Healthy or infected (48h after inoculation) leaves (30g) were harvested and crushed with 30g of insoluble polyvinylpyrrolydone (Sigma) in mortar in cold (4°C) and stored at -20°C for 1h and homogenized with 30 ml of 0.05 M Tris-HCl buffer, pH 7.4. The crushed leaves were then strained through cheese cloth and the filtrate was centrifuged (12,100g) for 60 min. at 4°C. The supernatants were collected and were used as crude protein extract for further experimental purposes.

3.10.2 Extraction of mycelial protein

To extract soluble mycelial protein, G. cingulata was grown in sterilized liquid Richard's medium for 10 days at 30° ± 1°C. Mycelia (50g fresh weight) were harvested, washed with 0.2% NaCl solution, rewashed with sterile distilled water and crushed in cold (4°C), kept at -20°C for 2h and homogenized with 30 ml of 0.05 M Tris-HCl buffer, pH 7.4 and sea sand (10g). The mycelial mass was strained through cheese cloth, followed by centrifugation (12,100g) for 60 min. at 4°C; the supernatant was separated and known quantity of ammonium sulfate $[(NH_4)_2SO_4]$ was added to it gradually for 100% precipitation, kept at 4°C for 12h and centrifuged (10,000g) for 15 min. at 4°C. The precipitate was dissolved in the same extraction buffer (pH 7.4) and dialysed against 0.005 M Tris-HCl buffer for 24h at 4°C. during dialysis ten changes were given ; the dialysate (i.e. soluble protein) was used for gel electrophoretic study.

3.11 Estimation of protein contents

The soluble proteins were estimated following the method of Lowry et al. (1951). Initially an alkaline mixture was prepared by adding 0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% N - K - tartarate to 50 ml of 20% Na_2CO_3 in 0.1 N NaOH. Finally, reaction mixture was prepared by mixing 0.1 ml of the protein sample, 0.9 ml distilled water and 5 ml of the alkaline mixture and incubated for 10 minutes. Subsequently 0.5 ml of Folin-phenol solution (Folin-phenol reagent : water :: 1:1) was added and again incubated for 15 min. In case of blank, distilled water was added instead of protein sample. At the end of incubation period, optical density value of each sample was determined by Systronic Photoelectric colorimeter Model 101 at 710 nm wavelength. Quantity of protein was estimated using Bovine Serum Albumin (BSA) as standard.

3.12 Preparation of mycelial wall extract

3.12.1 Isolation of cell wall

Isolation of cell wall was done following the method of Keen and Legrand (1980) with modification. Mycelium of 8-day old log phase fungus cultures was collected on filter paper in a Büchner funnel, and 20g of fresh packed cells were ground for 15-30 sec. in a high speed blendor with 80 ml of water. The slurry was centrifuged for 1 min. at 1500g, the supernatant fluids discarded, and the sedimented cell walls washed with 200 ml water and pelleted by centrifugation repeatedly until the supernatant fluids were visually clear. The pellet after these washings gave the isolated cell walls.

3.12.2 Extraction of isolated cell walls

Isolated cell walls (1.5g) were suspended in 60 ml of ice-cold 0.1 N NaOH by blending in a chilled mixer-cup at full speed for 20 sec. Then the suspension was slowly stirred in an ice bath for 15h. Following centrifugation for 10 min. at 8000g, the residue was washed with 40 ml of ice-cold water and the pooled supernatants were carefully neutralized to pH 7 with 1 N HCl at 0°C. The supernatants were finally dialysed against distilled water and concentrated. The final dialysates were used as the mycelial wall extracts for further experiments.

3.13 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed following the method of Laemmli (1970) with modifications.

Stock Solutions : Following tock solutions were made for SDS-PAGE:

A. **Acrylamide/bis solution** (29.9%) (0.9%) - 75g Acrylamide
2.6g N,N'Methylenebis-acrylamide
Dissolved in distilled water and made up to 250 ml. Stored at 4°C.

B. **Stacking gel buffer,** 1 mol/L Tris-HCl, pH 6.8
- 30.3g Tris
Dissolved in distilled water and made up to 200 ml. Adjusted to pH 6.8 with HCl. Made up to 250 ml with distilled water. Stored at 4°C.

C. **Resolving gel buffer** (1.5 mol/L Tris, pH 8.8)
- 45.4g Tris
Dissolved in 200 mL distilled water and adjusted to pH 8.8 with HCl. Made up to 250 mL with distilled water. Stored at 4°C.

D. **Sodium dodecylsulfate** 10%
- 50g SDS
Made up to 500 mL with distilled water. Stored at room temperature.

E. **Ammonium persulfate** 10%
- 1.0g Ammonium persulfate
Dissolved in 10 mL distilled water, always made fresh, before use.

F. **TEMED** 10%
- 1.0 ml TEMED
Added to 9.0 mL distilled water.

G. **Electrode buffer Stock Solution**
0.25 mol/L Tris, 2 mol/L glycine, 1% SDS, pH 8.3
- 30g Tris
144g Glycine
100 mL 10% SDS (D)
Added to 1 litre distilled water. Adjusted to pH 8.3 if necessary. Diluted 1:10 with distilled water before use.

H. **Stacking gel stock solution**
- 17 mL Acrylamide/bis solution (A)
12 mL Stacking gel buffer (B)
1 mL 10% SDS (D)
Mixed together and made up to 100 mL with distilled water. Stored at room temperature.

I. **Stacking gel solution** T = 3%
- Deaerated 20 mL stacking gel stock solution (H) and then 10 µL Ammonium persulfate (E) and 10 µL TEMED (F) was added immediately before use.

J. **Resolving gel solution** T = 10%
- 10 mL Acrylamide/bis solution (A)
7.5 mL Resolving gel buffer (C)
0.3 mL 10% SDS (D)
12.2 mL Distilled water Deaerated and 10 µL Ammonium persulfate (E) and 10 µL TEMED (F) was added immediately before use.

K. **Sample buffer** 0.08 mol/L Tris-HCl pH 6.8
- 0.3g β-mercaptoethanol
4 mL 10% SDS (D)
1.6 mL Stacking gel buffer (B)
2.5g 87% Glycerol
0.5g Bromophenol Blue
Mixed together and made up to 20 mL with distilled water. Stored at -20°C in 1 mL aliquots.

L. **Fixing solution**
- 57.0g Trichloroacetic acid
17.0g Sulphosalicylic acid
150 ml Methanol
Made up to 500 ml with distilled water and stirred until clear.

M. **Staining solution**
- 1.25g Coomassie Blue R 250
230 mL Methanol
230 mL Distilled water
40 mL Acetic acid
Coomassie Blue dissolved in methanol and distilled water, and then acetic acid was added.

N. **Destaining solution**
- 500 mL Acetic acid
1500 mL Ethanol
Made up to 5 litres with distilled water.

O. **Preserving solution**
- 50 mL Glycerol
450 mL Destaining solution (N)
Solution was mixed thoroughly.

Clean gel tubes (12 cm long, 4 mm dia.) were kept vertically on the tube stand. End of each tube was closed with rubber cap. Resolving

gel solution (without TEMED and APS) was degassed for 20 min. TEMED and APS (Ammonium persulfate) was then added and the gel was poured slowly into the tubes upto a height of about 9 cm, a drop of distilled water was placed on the top of each gel tube to get a plain surface instead of concave one. The gels were allowed to polymerize. After polymerization, water drops from the surface were soaked with filter paper and the lower gel overlayed with the upper gel (stacking gel) solution. After polymerization of the upper gel, the rubber caps were removed and the gel tubes with gel columns were fitted on to the upper reservoir.

3.13.1 Mycelial protein of G. cingulata

Samples were added to sample buffer and taken in small tubes, boiled in boiling water bath for 2-3 min for denaturing the proteins. Samples were then loaded on the gel columns (50 µl - 100 µl/tube). Along with the sample, protein marker (consisting of a mixture of six proteins ranging in molecular weight from 30 to 200 KD [Carbonic anhydrase - 29,000, Albumin (egg) - 45,000, Albumin (bovine) - 66,000, Phosphorylase b - 97,400, β-Galactosidase - 116,000 and Myosin - 205,000] was also taken in a separate tube, boiled and loaded as above. Tris glycine buffer i.e. the electrode buffer was poured into both the upper and lower buffer reservoirs.

3.13.2 Cell wall of G. cingulata

Samples were dissolved in a solution of 1% SDS and 1% mercaptoethanol and heated at 50°C for 2h. Then they were diluted by the addition of 1 volume of 4 M urea containing 5% sucrose and 50-100 µl of sample were applied per gel tube.

Electrophoresis

Electrophoresis was performed at 1.5 mA per gel until the samples penetrated the upper gel and then at 2.5 mA per gel for 3-4

hours until the dye front reaches the bottom of the gel column.

Fixing

After removal of the gels from the tubes, gels were soaked in fixer solution (F) for atleast 1½-2h for tubes containing mycelial protein sample.

In case of cell wall samples, each gel was fixed by shaking 20h in 50 ml of 25% isopropanol in 10% acetic acid for protein staining and in 40% ethanol in aqueous 5% acetic acid prior to periodate - Schiff's staining.

Staining

For protein, Coomassie Blue R 250 (Sigma) was used (as formulated in solution M) and gels were stained for about 2h and destained with solution N until the bands were clear. Molecular weights were calculated by comparison with molecular weight markers. But for carbohydrate, gels were stained with the periodic acid - Schiff's (PAS) reagent as devised by Segrest and Jackson (1972) with modifications. Details of PAS staining solutions and the procedure was as follows:

Schiff Reagent (Segrest and Jackson, 1972)

Solutions

1.5g basic fuchsin dissolved in 500 ml boiling distilled water. Filtered at 55°C then cooled to 40°C. 25 ml 2 N HCl added and finally 3.75g $Na_2S_2O_5$ (Sodium metabisulphite) was added. Agitated rapidly, stoppered and kept in freeze for 6h, 1.2g charcoal mixed vigorously for 50 sec. and filtered rapidly, stored with stopper in freeze.

(b)	Periodic acid	..	1.0 g
	Anhydrous sodium acetate	..	0.82 g
	Distilled water	..	100 ml

(c)	Glacial acetic acid	..	0.54 ml
	Anhydrous sodium acetate	..	0.89 g
	Hydroxyamine hydrochloride	..	10.0 g
	Distilled water	..	90 ml
(d)	This solution was prepared before use :		
	10% (W/V) $Na_2S_2O_5$	..	5 ml
	2N HCl	..	5 ml
	Distilled water	..	90 ml
(e)	Glycerin	..	20 ml
	Solution (d)	..	100 ml

Method of Carbohydrate staining

Gels were soaked in solution (c) for 15 mins., then washed in running tap water. Next, the gels were soaked in solution (b) for 15 mins., washed in running water for 10 mins., and transferred to solution (a) for 3 mins.; this step was repeated thrice before washing in solution (d) for 2 mins. and finally were washed three times, 1h for each time in solution (e).

3.14 Fluorescence binding technique

Mycelium or isolated cell walls were incubated for 20 min. in 0.85% NaCl in 0.01 M potassium phosphate, pH 7.4, containing 1 mg/ml fluorescein isothiocyanate (FITC) labelled concanavalin A (Con A, Sigma). The fungus or walls were then washed with the saline solution only (3X) by repeated low speed centrifugation and re-suspension. Both the preparations were viewed under Leica microscope equipped with fluorescence optics (BP 450-490 exciting filter, RKP 520 Beam splitting mirror, 515 suppression filter).

3.15 Isolation of phyllosphere microorganisms

To isolate tea-phyllosphere microorganisms, leaf washing

technique as described by Dickinson (1971) was followed. Leaves (10g) were randomly collected from the gardens ; placed in 100 ml sterile distilled water and shaken for 1h on a mechanical shaker (200 cycles/min). Leaves were removed and the remaining suspension was centrifuged at 15,000 rpm for 10 min. The pellet was resuspended in 10 ml of sterile distilled water and dilution series (10^{-1} to 10^{-6}) were made. Aliquots of 1 ml each of these dilutions were added to 20 ml of either Potato-Dextrose Agar (PDA) or Nutrient Agar (NA) medium in 90 mm dia. Petridishes (4 replications/dilution) and incubated at 30±1°C. Potato-Dextrose Agar (PDA) supplemented with 100 µg/ml chlorotetracycline and Nutrient Agar (NA) supplemented with 20 µg/ml nystatin were used for isolation of fungi and bacteria respectively. The fungal and bacterial colonies arising on the agar plates were transferred to PDA or NA slants for preservation and identification.

3.16 Characterization and identification of the isolated microorganisms

Fungi

The isolated fungi were allowed to grow in Petridishes (90 mm dia.) containing sterile PDA medium for 7 days. Nature of mycelial growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were prepared from individual culture. Drops of spore suspensions were placed on clean, grease-free glass-slides, mounted with lactophenol-cottonblue, covered with cover-slip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

Bacteria

For bacterial characterization, morphology of the cells were studied and specific physiological and biochemical tests were performed.

Morphology of cells

Shape and size of cells

To examine shape and size of the cells a drop of cell-suspension of the test organisms were placed on a clean grease-free slide, air dried and stained with safranine [2.5% W/V Safranin in 95% ethanol 10 ml; distilled water 100 ml., Norris et al., 1981] and observed under the Phase-contrast microscope (Model : Leica WILD MPS 28). Diameter of cells were measured with a standardized ocular micrometer.

Endospore staining

The smears of test organisms were fixed by passing the slide about 20 times over the flame. The slides were placed on the edge of a Petridish with the film side upward and covered with Malachite green (saturated aqueous solution). The slides were warmed gently over the flame till fuming and removed and allowed to react for 10 min. The slides were then rinsed with tap water for 10 sec., counter stained with safranin for 1 min., rinsed with running tap water till no colour came out and excess water blotted off. The slides were air dried and examined under the same Phase-contrast microscope.

Negative staining for slime-capsule

A drop of Nigrosine blue (saturated aqueous solution) was added to two loopfuls of bacteria on a clean, grease-free slide for each of the organism. The solution was mixed thoroughly with inoculating needle and the thickness of the stain was graduated from a relatively thick to a fairly thin smear. Heat-fixation was done after proper air drying and then the slides were examined under oil immersion objective of the microscope.

Motility

To test motility a drop of 24h-old bacterial suspension in

nutrient broth was used. The slides were observed under oil immersion objective of Phase-contrast microscope.

Physiological and biochemical tests

Gram staining

Smears of test organisms prepared from 24h-old culture on Nutrient Agar slant with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, heat-fixed and flooded with crystal violet [Crystal violet 2.0g, 95% alcohol 20 ml, ammonium oxalate 1% W/V aqueous solution 80 ml] stain for 1 min, washed for 3 sec. with tap water, flooded with Burke's iodine solution [Iodine 1.0g, KI 2.0g, Distilled water 100 ml] and allowed to react for 1 min. Slides were washed for 5 sec. in 95% ethanol which was poured drop by drop by holding the slides in slanting position against a white background till no colour came out from the lower edge of the slides. After washing with tap water the smears were stained with safranin for 1 min and rinsed with water. The slides were air dried and observed under oil immersion objective (Bartholomew, 1962).

Production of Catalase

Bacterial cultures (24h-old) were flooded with 0.5 ml of 10% H_2O_2 solution and gas bubble production indicated the positive reaction (Norris et al., 1981).

Production of Oxidase

For this test freshly prepared 1% aqueous dimethyl-p-phenylenediaminohydrochloride solution was poured over 24h-old Nutrient Agar slant of bacterial cultures. The solution was poured off immediately; reddish purple colour in 3-5 min. and intense jet black colour in 30 min. indicated the presence of oxidase.

Hydrolysis of Starch

Streaks of test organisms were made on sterile starch agar plates and incubated for 2 days at 37°C. Plates were flooded with Lugol's iodine (Iodine 1.0g, KI 2.0g, distilled water 300 ml.) and after 15 min, observed for a clear zone underneath (after the zone was scrapped off) and around the zone indicating the presence of amylase (Gordon et al., 1973).

Hydrolysis of Casein

Dried milk-agar plates were streaked with 24h-old bacterial cultures and incubated for 96h. The plates were observed for any clear zone around and underneath the growth indicating hydrolysis of casein (Gordon et al., 1973).

Hydrolysis of Esculine

Slants containing esculine hydrolysis test medium were inoculated with 24h-old test bacterial cultures and incubated at 30°C for 7 days. Blackening of the medium indicated hydrolysis of esculine (Facklam and Wilkinson, 1981).

Hydrolysis of Fat

To test fat hydrolysis, tributyrin agar plates were surface-dried, streaked with 24h to 48h-old bacterial cultures and incubated at 37°C for 3 days. Formation of clear zones around the growth indicated the lipolytic activity of the isolates (Stolp and Gadkari, 1981).

Hydrolysis of Protein

Gelatine agar plates were streaked with 24h-old cultures and incubated at 37°C for 1-3 days. The plates were flooded with protein precipitating reagent [1 N Sulphuric acid saturated with ammonium

sulphate]. Hydrolysis was indicated by clear zone around and underneath the growth, in contrast to the opaque precipitate of unchanged gelatine.

Hydrolysis of Arginine

The stabs with arginine medium were inoculated with the test organisms and incubated at 37°C for 2-3 days. The change in colour from yellow to red was an indication of arginine hydrolysis (Thornley, 1960).

Growth in Anaerobic agar

Anaerobic agar medium tubes (depth of the medium 7.5 cm) were stabbed with the test organisms upto the bottom of the column, incubated at 30°C for 7 days and observed for the growth of the test organisms along the length of the stab (anaerobic) and surface of the agar (aerobic) (Claus and Barkeley, 1986).

Reduction of Nitrate to Nitrite

Sterile nitrate broth (5 ml in each case) was inoculated with test organisms and incubated at 37°C till the medium became turbid; three drops of reagent of nitrate reduction test [Solution A : Sulphuric acid 0.8g, 5N Acetic acid (Glacial acetic acid : water, 1:2.5) 100 ml; Solution B : α-Naphthylamine 0.5g, 5N Acetic acid 100 ml. The solutions (A and B) were mixed in equal volume just before use.] was mixed with 1 ml of the culture and observed for the development of a red or yellow colour indicating the presence of nitrate (Norris et al., 1981).

Production of Indole

Ten ml of Davis Mingioli's broth supplemented with 0.1% of tryptophan were inoculated with the isolates and incubated anaerobically at 37°C for 7 days. The cultures were then layered

carefully with 2 ml of Ehrlich-Böhme reagent [p-Dimethylaminobenzaldehyde 10.0g, HCl concentrated 100 ml] (1-2 ml) on the surface, allowed to stand for a few minutes and observed for the formation a ring at the medium - reagent interface indicating the production of indole (Iswaran, 1980).

Production of Urease

Streaks were made on slants containing Urea-medium and incubated at 37°C for 3-7 days. The change in colour of the medium indicated the presence of urease (Christensen, 1946).

Production of H_2S

Slants containing SIM agar were inoculated with the test bacteria and incubated at 37°C for 48h. Darkening along the line of the slants indicated the production of H_2S by the organisms.

Voges-Proskauer reaction

Culture tubes each containing 10 ml sterile VP broth were inoculated with isolates and incubated at 37°C for 3 days. To the cultures 0.6 ml of 5% w/v ethanolic α-napthol and 0.2 ml of 40% w/v aqueous potassium hydroxide were added and kept at room temperature for 1h for production of any pink colour indicating positive reaction.

Acid and gas production from carbohydrates

Tubes with 10 ml modified Davis and Mingioli's broth supplemented with 0.01% w/v bromothymol blue, 1% w/v different carbohydrates and inverted Durham tubes were inoculated with 24h bacterial isolates and incubated at 37°C for 1-2 weeks. Change in colour of the medium to yellow and production of gas in Durham tubes indicates positive reaction for acid and gas production respectively.

3.17 *In vitro* interactions

3.17.1 Solid medium

Antagonism of microorganisms was tested on Potato-Dextrose-Agar (PDA) following the method of Skidmore and Dickinson (1976). Agar blocks (4 mm dia.) containing 7-day old mycelia of the fungi were placed 3.5 cm apart on the medium in a Petridish and incubated at 30±1°C. Bacteria (24h-old) were streaked on to the medium in Petridish 4 cm away from the test fungus inoculum. Five replicates were taken for each treatment. The paired cultures were examined after regular intervals upto three weeks and the nature of reactions noted.

3.17.2 Liquid medium

To assess the possible mutualistic antagonism between the fungus and bacteria in mixed culture, methods outlines by Chakraborty and Chakraborty (1989) was followed. Nutrient broth medium was selected because it allowed vigorous growth of both the fungus and the bacteria. One agar block (4 mm dia.) containing 7-day old mycelia of the fungus and 0.5 ml of bacterial suspension (1 x 10^6 bacteria/ml) were used as inocula for each flask (50 ml nutrient broth/250 ml flask). The cultures were incubated at 30°C and after 14 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and harvested by straining through muslin and mycelial dry weights were determined. The suspension was centrifuged at 3000 rpm, which removed the mycelial fragments but not the bacteria. The bacterial growth was determined by measuring the optical density in a Systronics Photoelectric Colorimeter Model 101. The polysaccharides in the medium could be discounted because the O.D. values were taken using the medium as a blank.

3.18 Preparation of cell-free culture filtrates from bacteria

To prepare bacterial culture filtrate loopful of bacteria from a

24h old Nutrient Agar slant were inoculated into nutrient broth (50 ml/250 ml flash) and incubated at 37°C with vigorous shaking on a platform shaker at 175 rpm for 96h. Centrifugation was done at 15,000 rpm for 20 min. and the supernatant was sterilized by cold and hot sterilization. Cold-sterilization was done by passing through a sterilized microbial filter (G-5). This was plated on Nutrient Agar plates to test for the presence of viable bacteria before use. Heat-killed culture filtrate was prepared by autoclaving the supernatant at 15 p.s.i. for 15 min.

3.19 *In vivo* test

Four-day old culture of bacteria on Nutrient Agar slants were scraped off and suspended in sterile distilled water to give a concentration of 1 x 10^6 bacteria/ml . This was the aqueous cell suspension. Culture filtrate was prepared as described earlier. The pellets obtained after centrifuging the bacterial culture was washed with sterile distilled water to remove the trace of medium and resuspended in sterile distilled water and considered as the washed cell treatment. All treatments and other test solutions (active solvent - fraction, semi-purified antifungal compound) were sprayed on to the upper and lower leaf surface of 18 month old plants until run off.

3.19.1 Detached leaf

Following two sprays of each treatment/test solutions at 72h intervals the leaves were removed (after 24h of the second spray) and brought to the laboratory and inoculated with *G. cingulata* as described earlier.

3.19.2 Cut shoot

For cut shoot inoculation the sprayed shoots (two and a bud) were removed (after 24h of the second spray), brought to the laboratory and inoculated following the method as described earlier.

3.20 Bioassays

3.20.1 Spore germination bioassay

Spore germination bioassay was performed following the method of Trivedi and Sinha (1976) with modifications. One drop (10 µl) of the test solution was placed on a clean grease-free slide and it was allowed to dry, where necessary. A drop of the fungal spore suspension was placed on top of the test solution and incubated in moist Petridishes for 24h at 25±1°C. Finally one drop of Lactophenol-cottonblue was added to each spot to fix the germinated spores. Slides were observed under the microscope and percentage germination and appressoria formation were determined.

3.20.2 Radial growth bioassay

The inhibitory activity of a test solution was assessed by radial growth inhibition bioassay (Fiddaman and Rossall, 1994). Richard's medium (10 ml) was mixed with 0.5 ml of test compound solution and plated in Petridishes (50 mm dia.) at 45°C, cooled and mycelial block (2 mm dia.) of the pathogen (4-day old) was placed in the centre of each Petridish. The plates were incubated at 30±1°C and radial growth of mycelia was determined after every 24h.

3.20.3 Agar cup bioassay

The medium (PDA, 20 ml/Petridish) was seeded with 1 ml of spore suspension of the test pathogen. After solidifcation the plates were chilled for 30 min, then bored with sterile cork-borer (8 mm dia.). Test solution (0.1 ml) was added to each cup and incubated at 30°C for desired period. Diameter of inhibition zones were recorded.

3.21 Solvent extraction of the cell-free culture filtrates

In order to isolate the active principle from the culture filtrates, the bacteria were grown in Nutrient broth for 96h at 37°C in

shaking condition (175 rpm). Centrifugation was done at 15,000 rpm for 20 min. and supernatants were pooled and were used as the culture filtrates. Cell-free cold sterilized culture filtrate (1000 ml) was extracted separately three times with equal volume of acetone, benzene, chloroform, ethylacetate or diethylether. The organic fractions and corresponding aqueous fractions were evaporated to complete dryness in a rotary evaporator at room temperature and residue in each case was redissolved in 2.5 ml of 80% ethanol and stored in a capped bottle at 4°C.

3.22 Thin layer chromatography

The crude active solvent fraction was spotted onto 20 cm x 20 cm Silica gel plates (Silica Gel, E . Merck). The plates were developed in benzene : ethanol (6:1) and the spots were visualised with iodine vapour as described by McKeen et al. (1986). All detected spots and areas between spots were scraped off from the plates. These were then eluted with spectral methanol (2 ml/1 cm silica gel). Each part was bioassayed for anti-fungal activity .

For further purification of the parts showing antifungal activity, TLC eluates were again spotted onto Silica gel plates as described earlier and were developed using the same solvent system. Detected spots and areas in between were similarly scraped off and finally were taken to spectral methanol. To test antifungal activity 100 µl eluates from each area was bioassayed following spore germination bioassay.

3.23 UV-Spectrophotometry

The eluates from TLC plates showing antifungal activity was analysed for UV-Spectrophotometry using Shimadzu 160 UV-Spectrophotometer.

E X P E R I M E N T A L

4.1 Varietal resistance test of tea against G. cingulata

Varietal resistance of ten varieties of tea (TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, Teen Ali 17/1/54 and CP-1) released by Tocklai Experimental Station, Jorhat were tested against G. cingulata. Inoculation techniques (whole plant, cut shoot and detached leaf) and disease assessment procedures have been described in detail under Materials and Methods. Results are presented in Tables 1-3 and Fig. 1.

4.1.1 Whole plant

Ten varieties of tea plants grown in the experimental garden were inoculated with conidial suspension of G. cingulata under identical conditions. Disease assessment was done after 4, 8, 12 and 16 days of inoculation as described in the Materials and Methods.

Results (Table 1) revealed that among all the tested varieties TV-18 was most susceptible followed by TV-23 while TV-26 and CP-1 exhibited resistance. The other varieties are moderately susceptible or resistant.

4.1.2 Cut shoot

Pathogenicity of G. cingulata on the selected varieties were also tested by cut shoot inoculation technique as described in Materials and Methods. Disease was assessed on the basis of mean number of lesions per shoot from which mean disease index per shoot was calculated. Disease symptoms appeared as early as 24h after inoculation. Results (Table 2) confirmed the trend shown in the field test. G. cingulata was most susceptible on TV-18 and least on TV-26 after 72h of inoculation (Plate II, fig. 3).

Table 1 : Varietal resistance test of different varieties of pot grown tea plants against *G. cingulata*

Variety	Days after inoculation							
	4		8		12		16	
	Mean No.of lesion/ shoot	Mean disease index/ shoot	Mean No.of lesion/ shoot	Mean disease index/ shoot	Mean No.of lesion/ shoot	Mean disease index/ shoot	Mean No.of lesion/ shoot	Mean disease index/ shoot
TV-18	40.1	11.2	42.7	14.7	48.0	19.0	51.9	23.1
TV-20	25.0	6.3	27.1	8.0	28.9	10.3	30.4	12.7
TV-22	12.9	3.7	15.7	4.9	20.2	7.7	25.3	9.6
TV-23	37.8	8.7	39.0	10.1	40.8	15.9	42.3	17.2
TV-25	31.9	6.1	34.7	9.2	35.5	11.0	39.1	0.7
TV-26	1.7	0.3	2.0	0.4	2.3	0.6	3.1	0.7
TV-27	29.7	6.0	31.0	9.5	32.4	14.7	36.0	14.9
TV-28	24.5	6.1	26.9	8.3	29.7	10.6	31.2	11.0
Teen Ali 17/1/54	11.5	5.9	19.1	6.4	21.0	6.7	24.2	7.8
CP-1	2.9	1.8	3.7	2.1	4.5	3.0	5.0	4.3

Average of 20 plants/variety.

Table 2 : Varietal resistance test of different tea varieties against *G. cingulata* (cut shoot inoculation)

Variety	24 h		48 h		72 h	
	Mean No.of lesion/shoot	Mean disease index/shoot	Mean No. of lesion/shoot	Mean disease index/shoot	Mean No. of lesion/shoot	Mean disease index/shoot
TV-18	9.0	3.03	11.0	4.70	12.0	6.03
TV-20	4.5	1.20	6.9	2.00	7.3	3.70
TV-22	3.9	1.11	4.2	2.70	6.1	3.15
TV-23	6.0	3.77	8.0	4.02	9.0	4.35
TV-25	5.0	2.42	6.0	2.58	8.0	3.00
TV-26	2.0	0.30	3.0	0.48	5.0	0.63
TV-27	4.0	1.18	7.0	2.27	8.0	3.25
TV-28	3.1	2.20	4.1	2.80	5.7	2.90
Teen Ali 17/1/54	2.0	0.42	4.0	0.80	5.0	0.88
CP-1	1.0	0.17	4.0	0.73	5.0	0.83

Average of 50 shoots/variety

4.1.3 Detached leaf

Artificial inoculation of detached leaves of the ten varieties was carried out as described previously. Assessment of disease was done after 48, 72 and 96h of inoculation on the basis of percentage lesion production. Lesions appeared only after 48h of inoculation and started coalescing after 96h (Plate II, fig. 1).

Results (Table 3; Fig. 1) showed TV-18 to be most susceptible followed by TV-23 and TV-26 to be most resistant followed by CP-1.

Results from all the three inoculation and disease assessment techniques conclusively proved that TV-18 and TV-23 were highly susceptible to G. cingulata while TV-26 and CP-1 were resistant. The degree of susceptibility or resistance in the other varieties was moderate.

4.2 Cultural characteristics of G. cingulata

Growth and sporulation of G. cingulata varies depending on the media. The fungus grows well in different media including Richard's agar (RMA), Czapek's (Dox) agar (CDA), Oat meal agar (OMA), Carrot juice agar (CJA), Potato dextrose agar (PDA), Bean juice agar (BJA), Green tea agar (GTA) and Boiled tea agar (BTA). Colonies usually grow with a daily radial increment of 5-7 mm at 28°C reaching approximately 60.0 mm diameter in four days at their optimum temperature and medium. Maximum growth occurred in RMA (62.0 mm), followed by CJA (51.5 mm). Boiled tea agar (BTA) supported least growth (32.0 mm). Mycelial growth is fluffy and white in colour in RM, showing deep orange on the reverse side of the Petridish. In both GTA and BTA, deep brown concentric rings are seen on the reverse side; however, the upper side showed mycelial growth with brown hyphae intermingled with whitish grey hyphae. In PDA and CDA, grey-white mycelia were observed on the upper side but clear,

Table 3 : Varietal resistance test of different tea varieties against *G. cingulata* (detached leaf inoculation)

Variety	% Lesion production after		
	48 h	72 h	96 h
TV-18	83.0 ± 3.9	91.5 ± 3.5	95.2 ± 2.3
TV-20	41.1 ± 2.7	47.1 ± 2.1	54.7 ± 1.8
TV-22	41.2 ± 2.0	49.4 ± 3.6	51.2 ± 1.7
TV-23	66.8 ± 2.3	78.8 ± 2.8	82.3 ± 2.4
TV-25	64.4 ± 3.6	73.0 ± 2.6	76.2 ± 2.3
TV-26	25.0 ± 1.2	27.4 ± 1.5	28.0 ± 1.7
TV-27	61.2 ± 2.3	72.5 ± 1.9	74.2 ± 2.1
TV-28	29.3 ± 1.7	37.1 ± 2.7	39.5 ± 2.1
Teen Ali 17/1/54	31.7 ± 1.6	33.2 ± 2.7	35.1 ± 1.3
CP-1	24.2 ± 2.3	31.2 ± 1.8	32.2 ± 1.9

Average of 3 experimental sets .
Average of 50 leaves inoculated per variety.

Plate II (figs. 1-3)- Healthy and artificially inoculated leaves and twigs of TV-18 (72h after inoculation); (1) inoculation with G. cingulata on detached leaves; (2) healthy twigs; (3) inoculated cut shoots

PLATE - II

PATHOGENICITY TEST OF G. cingulata ON DETACHED LEAVES OF DIFFERENT VARIETIES OF TEA (96h after Inoculation)

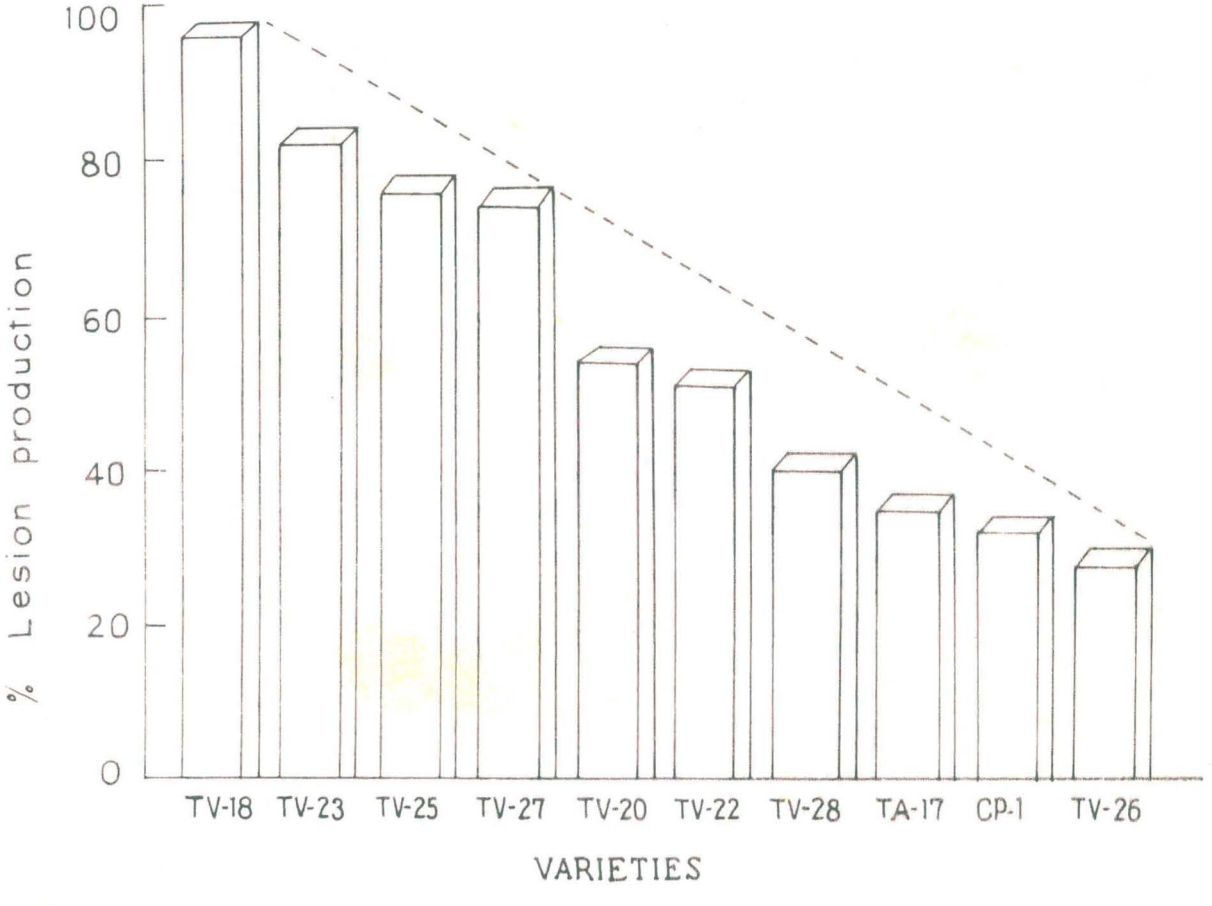


Fig. 1.

black concentric rings with orange acervuli is prominent on reverse side of the Petridish. Sporulation is very abundant in RM, OMA and CJA, moderate in PDA and CDA while in case of GTA, BTA or BJA sporulation is rare.

Conidial morphology was studied under bright field, phase contrast and scanning electron microscope (Plate III, figs. 1-4 & Plate IV, figs. 1-4). Conidia appearing scantily on solitary phialides but normally in orange sporodochia; a basal stromatic cushion is covered with dense layer of cylindrical, slightly tapering phialides upto 20 µm long. Sometimes these are interspersed with dark brown, tapering, blunt, septate setae. Conidia cylindrical with a rounded apex and slightly truncated base, hyaline filled with granular cytoplasm, 12-18 x 3-5 µm; forming orange-red slimy masses, germinating by irregularly rounded brown appressoria.

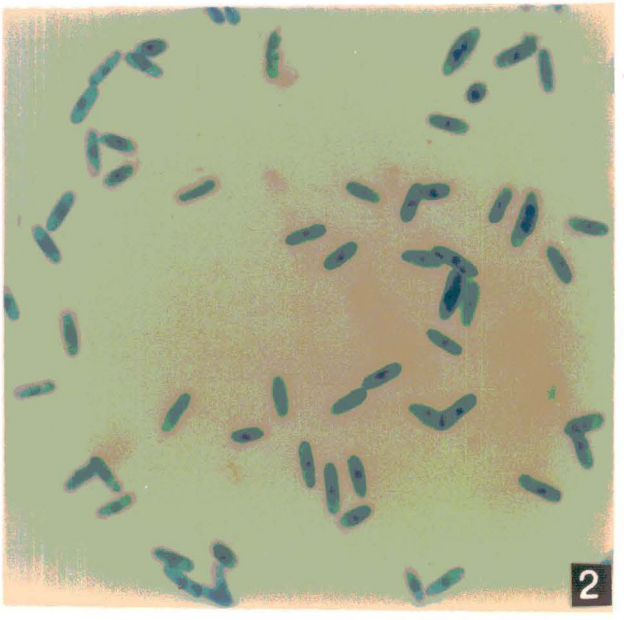
4.2.1 Factors influencing mycelial growth of G. cingulata

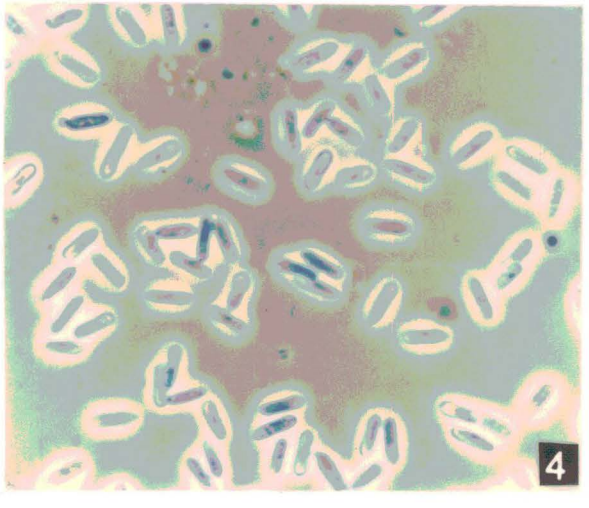
The growth of fungi both in vitro and in natural conditions is greatly influenced by different factors like temperature, pH of the surrounding medium and available nutrients. To determine the effect of such factors on mycelial growth of G. cingulata in vitro, the following experiments were undertaken. Effects of incubation period, pH of the medium, different carbon sources and nitrogen sources on the mycelial growth of G. cingulata were studied.

4.2.1.1 Incubation time

G. cingulata was grown in Richard's medium (RM) for a period of 30 days. Mycelial growth was recorded after 5, 10, 15, 20, 25 and 30 days of growth. Maximum growth (570 mg) of G. cingulata was recorded after 10 days of incubation (Table 4). There was a steady decline in growth after 10 days, growth rate decreasing by 56% from 10 days to 30

Plate III (figs. 1-4)- Mycelia and conidia of G.cingulata; (1) G.cingulata grown on RM showing orange acervuli; (2) conidia under bright field, low power microscope (X250) and (3) under high power (X750); (4) conidia under phase contrast microscope





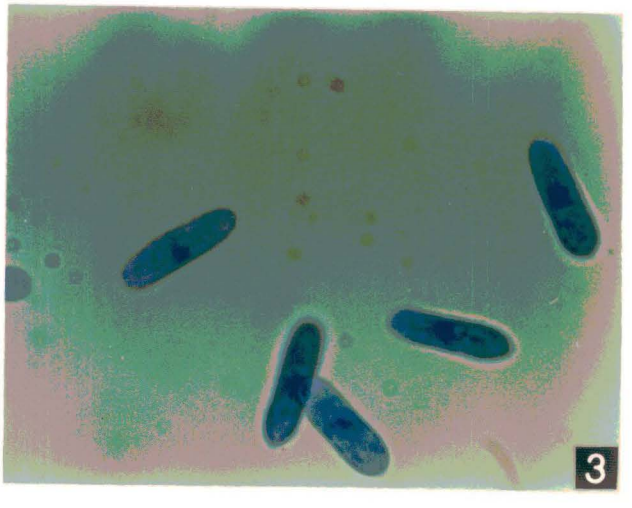


PLATE - III

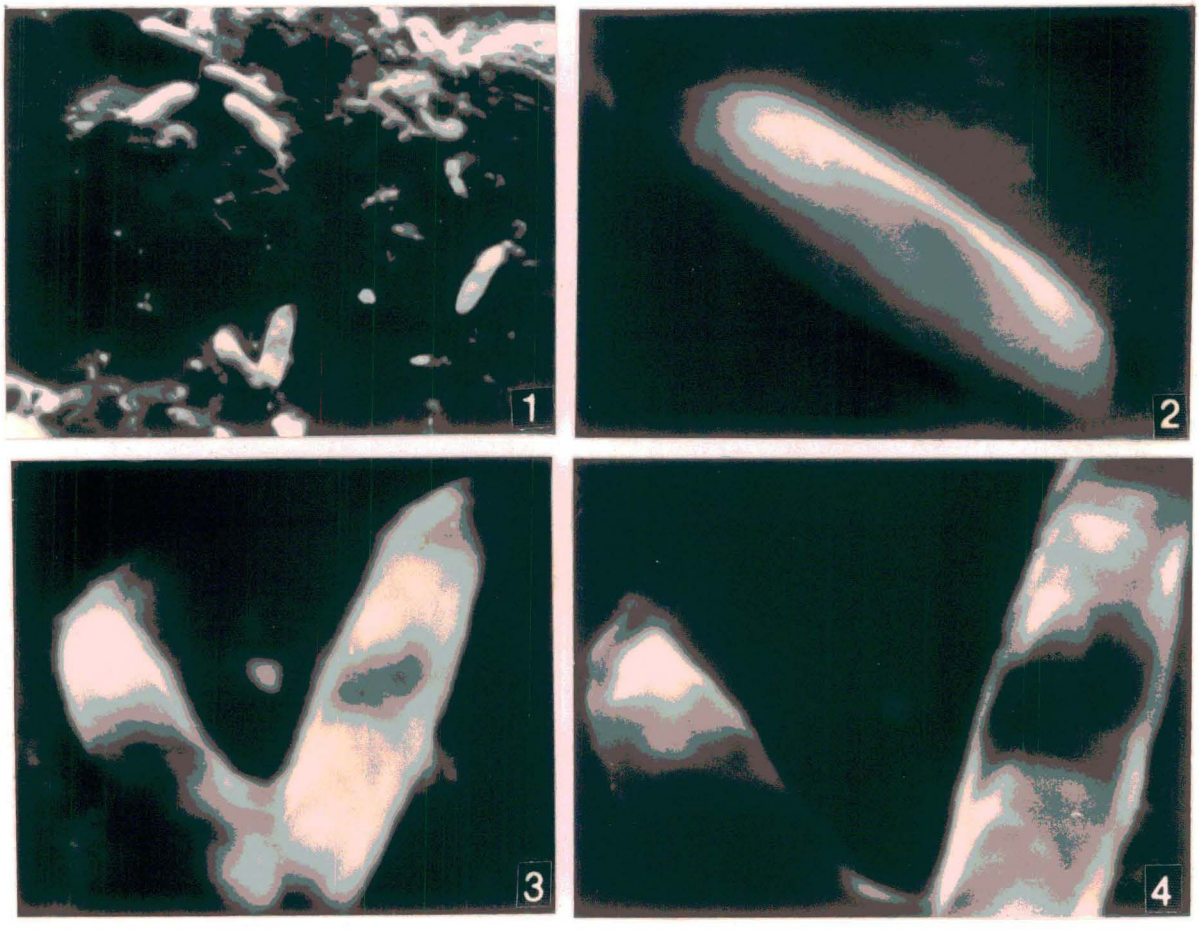


PLATE - IV

Plate IV (figs. 1-4)- Scanning Electron Micrograph of G. cingulata conidia; (1) group of conidia (X500); (2) single conidium (X3500); (3) two conidia (X2500) and (4) enlarged conidia (X5000)

Table 4 : Effect of incubation period on mycelial growth of G. cingulata

Incubation period (in days)	Mean mycelial dry wt.[a] (mg)
5	50.0 ± 3.8
10	570.0 ± 2.9
15	380.0 ± 3.3
20	320.0 ± 4.1
25	280.0 ± 3.8
30	250.0 ± 3.6

[a] Average of 5 replicates; Temperature 28+2°C; pH of medium 5.4.

days. After 5 days of incubation there was a growth of only 50 mg. Therefore, the mycelial growth at 10 days was more than 11 times that of 5 days. Sporulation was abundant at 10 days' growth. Spores were also observed at 5 days' growth.

4.2.1.2 pH

It is well known that the pH of the medium usually plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH. In the present investigation buffer solutions with pH values ranging from 4.0 to 8.0 (4.0, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. The medium and the buffer was sterilized separately by autoclaving for 15 min. at 15 lb. p.s.i. pressure. Equal parts of the buffer solution and the medium (RM) were mixed before use. Each flask containing 50 ml of the medium was then inoculated with mycelial block of G. cingulata and incubated for 10 days at 28 ± 1°C. Results (Table 5) revealed that G. cingulata grew to a lesser or greater extent in all the pH tested.

Table 5 : Effect of pH on mycelial growth of G. cingulata

pH of medium	Mean mycelial dry weight (mg)[a]
4.0	298.0 ± 4.2
5.0	351.0 ± 3.6
5.5	372.0 ± 3.7
6.0	410.0 ± 2.7
6.5	452.0 ± 4.2
7.0	365.0 ± 3.7
8.0	339.5 ± 2.8

[a] Average of 3 replicates
Temperature 28 ± 2°C.
pH of medium (RM) 5.4

Maximum growth was recorded at pH 6.5, while minimum growth occurred at pH 4.0. Sporulation was very less in high pH 8.0 ; pH 6.0 and 6.5 supported good sporulation.

4.2.1.3 Carbon sources

The ability of fungi to grow in different media depends on their capacity to utilize the available nutrients, of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In this investigation, 9 different carbon sources (Fructose, Sorbose, Glucose, Galactose, Mannose, Mannitol, Sucrose, Starch and Maltose) were tested for their effect on the growth of G. cingulata. The equivalent amount of carbon present in 1 percent glucose was used as standard and added separately to the basal medium (0.2% asparagine, 0.1% KH_2PO_4, 0.05% $MgSO_4,7H_2O$, 2 ppm Zn^{++}, Mn^{++} and Fe^{+++}). Data were

recorded after 3, 6 and 9 days of incubation. A control set without any carbohydrate was also set up. Both mycelial weight and sporulation were determined. Results given in Table 6 revealed maximum growth and sporulation of G. cingulata using maltose as the carbon source. Sucrose and glucose also supported comparatively good growth. Good sporulation was observed in case of maltose, sucrose, glucose and mannose. Among the tested carbohydrates least growth occurred in mannitol. There was little growth in absence of any carbohydrate.

Table 6 : Effect of different carbon sources on mycelial growth of G. cingulata

Carbon source	Mycelial dry weight (mg)[a]		
	3 days	6 days	9 days
D-Fructose	32.8 ± 3.9	153.0 ± 4.1	313.0 ± 3.1
L-Sorbose	35.4 ± 4.1	115.5 ± 3.3	265.7 ± 2.8
D-Glucose	40.3 ± 2.3	147.8 ± 2.4	374.9 ± 3.7
Galactose	33.8 ± 3.3	211.0 ± 2.1	348.3 ± 2.9
D-Mannose	31.0 ± 1.9	219.6 ± 2.7	338.7 ± 4.1
Mannitol	28.5 ± 3.7	91.7 ± 2.2	141.0 ± 3.1
Sucrose	32.3 ± 3.2	261.3 ± 2.9	382.9 ± 3.2
Starch	21.1 ± 2.8	189.2 ± 3.9	228.0 ± 3.2
Maltose	39.9 ± 4.1	297.0 ± 4.1	410.6 ± 3.4
Control (without carbon)	10.9 ± 2.2	18.5 ± 2.1	21.8 ± 2.6

[a] Average of 3 replicates.

4.2.1.4 Nitrogen sources

Nitrogen is undoubtedly the most important single nutrient necessary for the growth of any organism. The availability of nitrogen

depends to a great degree on the form in which it is supplied. Hence, the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effect of inorganic nitrogen sources (Calcium nitrate, Sodium nitrate, Ammonium sulphate, Ammonium nitrate and Potassium nitrate) as well as complex organic sources (Peptone, Urea, Yeast extract and Beef extract) on the mycelial growth of G. cingulata was tested. A basal medium without any nitrogen source was considered as control. Data, recorded after 3, 6 and 9 days of growth showed potassium nitrate to be optimum for growth of G. cingulata, followed by sodium nitrate, among all tested sources both inorganic and organic (Table 7). Among the organic sources beef extract supported

Table 7 : Effect of different nitrogen sources on mycelial growth of G. cingulata

Nitrogen source	Mycelial dry weight (mg)[a]		
	3 days	6 days	9 days
Inorganic			
Calcium nitrate	21.3 ± 1.2	256.0 ± 1.7	367.1 ± 2.9
Sodium nitrate	20.0 ± 2.9	303.5 ± 2.7	427.0 ± 2.9
Ammonium sulphate	15.5 ± 3.1	185.7 ± 3.0	235.0 ± 3.3
Ammonium nitrate	18.0 ± 4.1	281.0 ± 3.8	333.5 ± 3.9
Potassium nitrate	23.7 ± 3.1	319.5 ± 3.7	433.0 ± 4.1
Organic			
Peptone	12.5 ± 2.9	193.0 ± 3.1	265.0 ± 3.8
Urea	9.0 ± 3.7	153.4 ± 3.6	211.7 ± 3.2
Yeast extract	14.0 ± 2.9	171.5 ± 3.1	202.7 ± 4.0
Beef extract	11.8 ± 4.1	189.9 ± 4.4	290.0 ± 4.2
Control (without nitrogen)	8.7 ± 3.1	10.0 ± 3.7	13.5 ± 3.5

[a] Average of 3 replicates.

maximum growth. Only insignificant growth was noted without nitrogen. Sporulation was also maximum in potassium nitrate followed by sodium nitrate, calcium nitrate and beef extract.

4.2.2 Factors influencing spore germination and appressoria formation of G. cingulata

Spore germination and appressoria formation is the first step in the establishment of disease by any pathogen and factors affecting this process are of vital importance in deciding the fate of the pathogen in the initial stages. these factors include both biotic and abiotic ones. In view of the importance of spore germination and appressoria formation in the establishment of disease, effect of several factors including incubation period, pH, temperature, light period, concentration of spores and age of culture on the above process have been studied in this investigation.

4.2.2.1 Incubation period

Spores of G. cingulata were suspended in sterile distilled water and allowed to germinate for different time periods ranging from 4h to

Table 8 : Effect of different incubation hours on spore germination and appressoria formation of G. cingulata

Incubation hours	% spore germination[a]	% appressoria formed [b]
4	19.0 ± 3.7	6.2 ± 1.2
6	24.1 ± 2.1	14.2 ± 2.9
8	36.1 ± 3.0	23.7 ± 3.7
10	57.5 ± 2.4	37.7 ± 2.7
12	60.0 ± 1.8	37.2 ± 2.0
14	61.0 ± 3.2	43.1 ± 3.4
16	67.9 ± 2.6	66.9 ± 2.9

(Contd....)

Table 8 (Contd.)

Incubation hours	% spore germination [a]	% appressoria formed [b]
18	70.4 ± 1.8	67.5 ± 1.6
20	70.7 ± 3.8	68.4 ± 3.1
22	75.1 ± 2.7	68.3 ± 2.2
24	87.8 ± 3.4	82.4 ± 2.9

[a] Average of 200 spores
[b] Average of 50 germlings
Temperature 28 ± 2°C.

24h. Both spore germination and appressoria formation progressively increased and reached the maximum at 24h (87.8% spore germination and 82.5% appressoria formation). Even at 4h incubation 19% spore germination and 6.3% appressoria formation was noted (Table 8; Fig. 2).

4.2.2.2 pH

Results of the effect of different pH (4.0-9.0, 0.001 M Sodium phosphate buffer) on spore germination and appressoria formation

Table 9 : Effect of different pH on spore germination and appressoria formation of G. cingulata

pH	% spore germination [a]	% appressoria formation [b]
4.0	76.6 ± 3.1	81.5 ± 3.2
5.0	89.2 ± 2.4	80.6 ± 2.2
6.0	58.8 ± 3.2	28.4 ± 2.8
6.5	24.7 ± 3.1	25.5 ± 1.7
7.0	15.0 ± 1.9	13.7 ± 3.1
8.0	14.0 ± 2.8	3.5 ± 2.6
9.0	12.1 ± 1.9	-

[a] Average of 200 spores. [b] Average of 50 germlings.
Temperature 28±2°C.

EFFECT OF DIFFERENT INCUBATION PERIOD ON SPORE GERMINATION AND APPRESSORIA FORMATION OF G.cingulata .

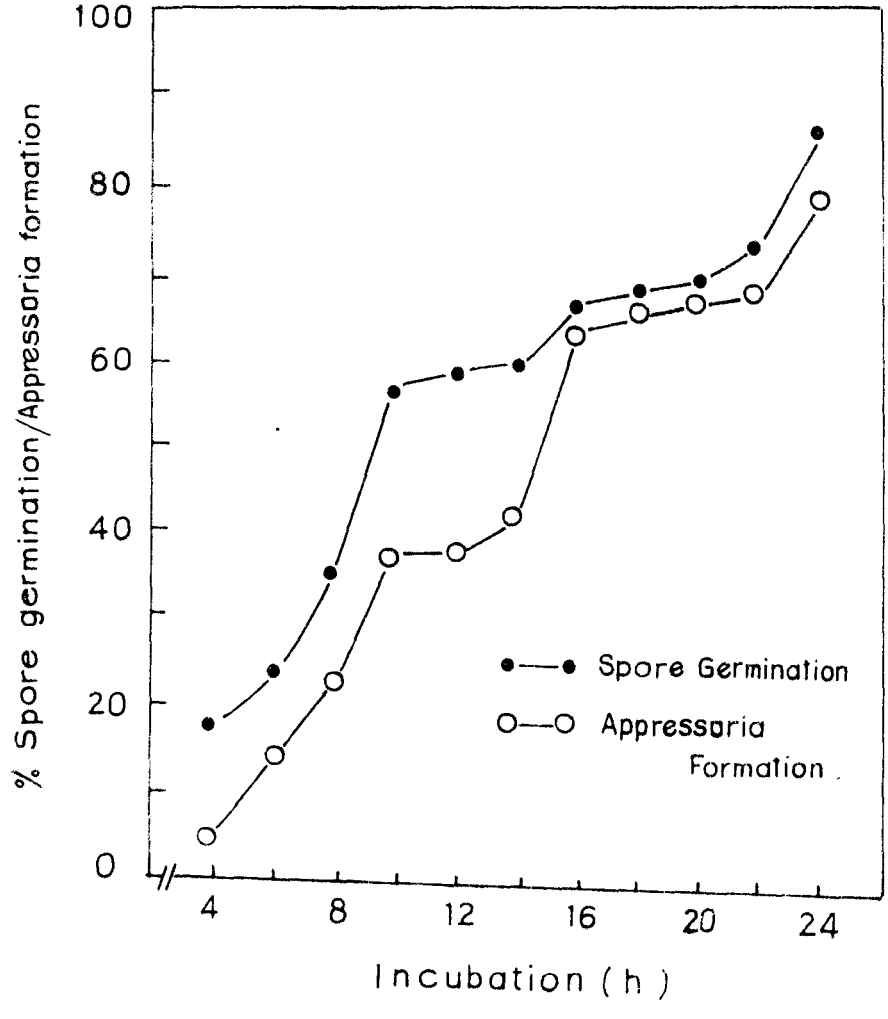


Fig. 2.

revealed that the optimum pH in both cases was 5.0 (Table 9; Fig. 3). Percentage of germination and appressoria formation was quite high at pH 4.0 (76.7% and 81.6% respectively), while at the higher pH germination and appressoria formation reduced very significantly. No appressoria formation was noted at pH 9.

4.2.2.3 Temperature

Spores were allowed to germinate at different temperatures ranging from 10°C to 40°C. Results (Table 10; Fig. 4) revealed that

Table 10 : Effect of different temperature on spore germination and appressoria formation of G. cingulata

Temperature	% spore germination[a]	% appressoria formation[b]
10	65.2 ± 2.6	8.7 ± 2.9
15	67.3 ± 3.1	22.6 ± 3.7
20	70.0 ± 2.2	17.9 ± 2.9
25	88.0 ± 2.9	82.9 ± 3.3
30	72.3 ± 1.4	44.5 ± 2.7
35	47.7 ± 2.3	30.4 ± 3.1
40	50.0 ± 3.8	21.5 ± 3.6

[a] Average of 200 spores
[b] Average of 50 germlings

at 25°C spore germination and appressoria formation were maximum, being 88.0% and 83.0% respectively. At 10°C, a germination of 65.3% was evident whereas approximately 50% spores germinated at 40°C. Appressoria formation at 10°C was only 8.7%, while it was 22% at 40°C.

EFFECT OF DIFFERENT pH ON SPORE GERMINATION AND APPRESSORIA FORMATION OF G. cingulata .

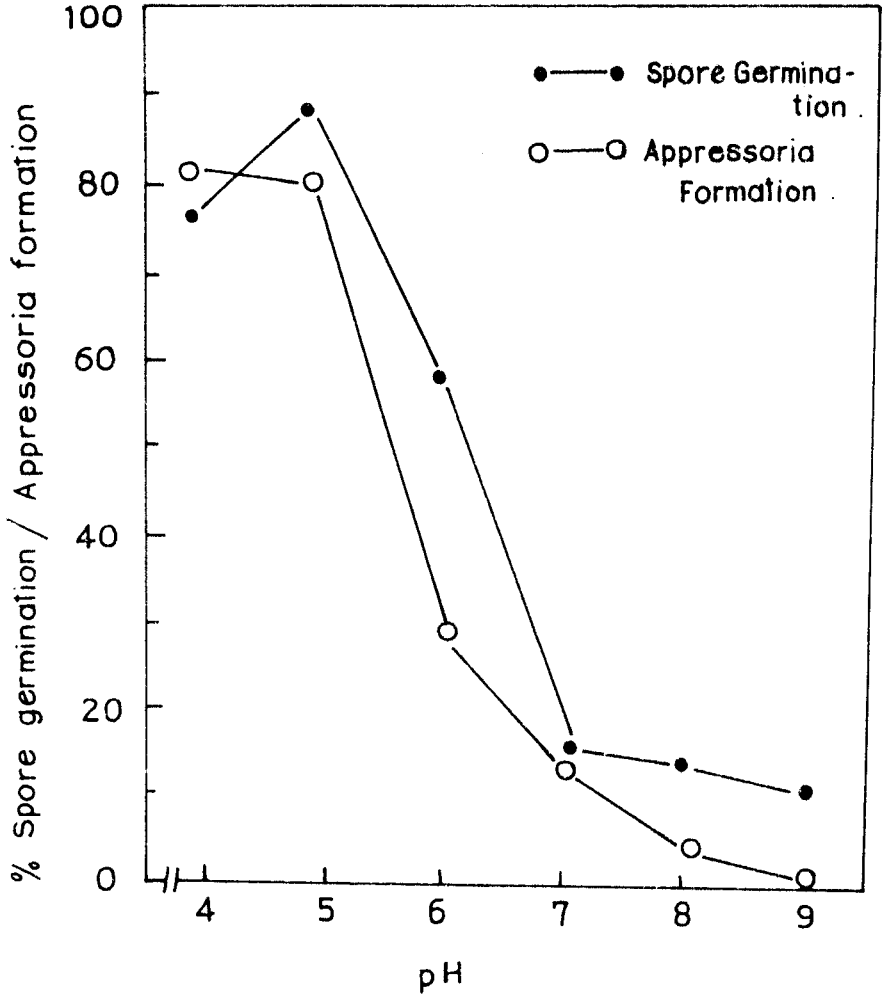


Fig. 3 .

EFFECT OF DIFFERENT TEMPERATURE ON SPORE GERMINATION AND APPRESSORIA FORMATION OF G. Cingulata

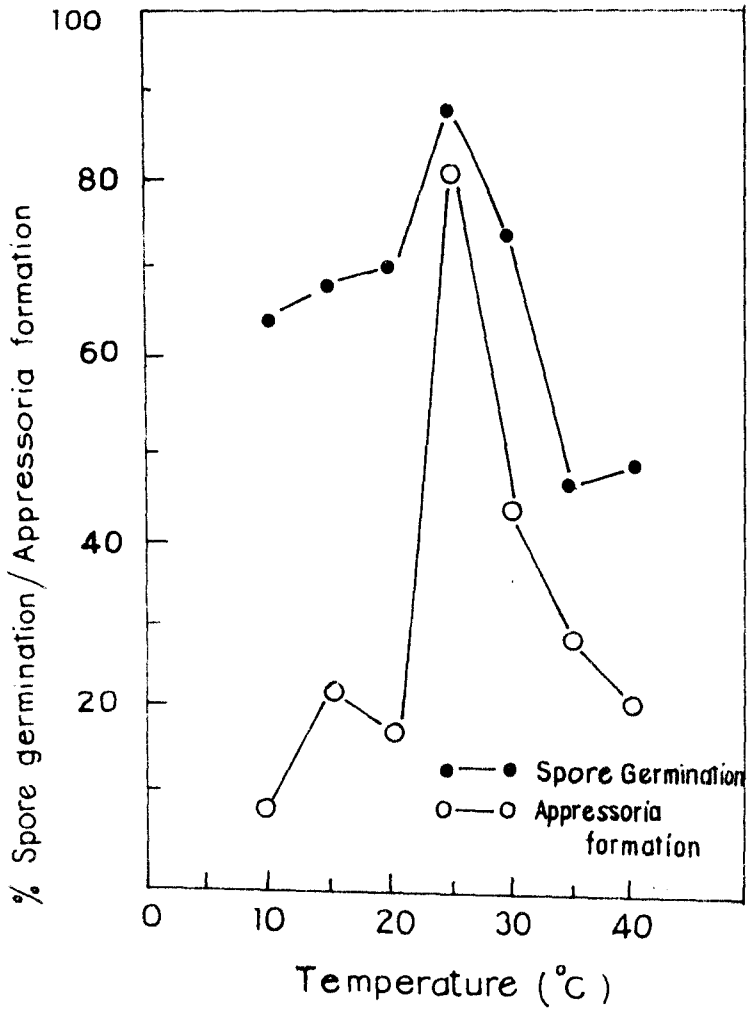


Fig. 4 .

4.2.2.4 Light period

Results of germination tests carried out under different light periods showed that 8h light period was optimum for spore germination (81.9%), while 6h light period was optimum for appressoria formation (Table 11; Fig. 5). At 12h light and dark periods 60.0% spore germination and appressoria formation were noted.

Table 11 : Effect of different light period on spore germination and appressoria formation of G. cingulata

Light period (hours)	% spore germination [a]	% appressoria formation[b]
2	37.5 ± 2.9	42.4 ± 3.1
4	46.7 ± 1.6	48.8 ± 2.2
6	72.7 ± 2.2	90.4 ± 3.3
8	81.9 ± 2.3	75.4 ± 2.6
10	55.3 ± 1.9	63.2 ± 2.6
12	60.3 ± 3.1	59.5 ± 2.9

[a] Average of 200 spores.

[b] Average of 50 germlings

4.2.2.5 Concentration of spores

Different concentrations of spores (1.2×10^4 - 1.2×10^8 spores/ml) were tested for their effect on spore germination and appressoria formation of G. cingulata. A concentration of 1.2×10^6 spores/ml was optimum for both spore germination and appressoria formation (95.1% and 89.7%, respectively) as given in Table 12. Spore germination reduced by 33.4% in the high concentration (1.2×10^4 spores/ml) with respect to the optimum concentration. However, there were no significant differences in three of the tested concentrations (1.2×10^6, 1.2×10^7 and 1.2×10^8 spores/ml).

EFFECT OF DIFFERENT LIGHT HOURS ON SPORE GERMINATION AND APPRESSORIA FORMATION OF G. cingulata

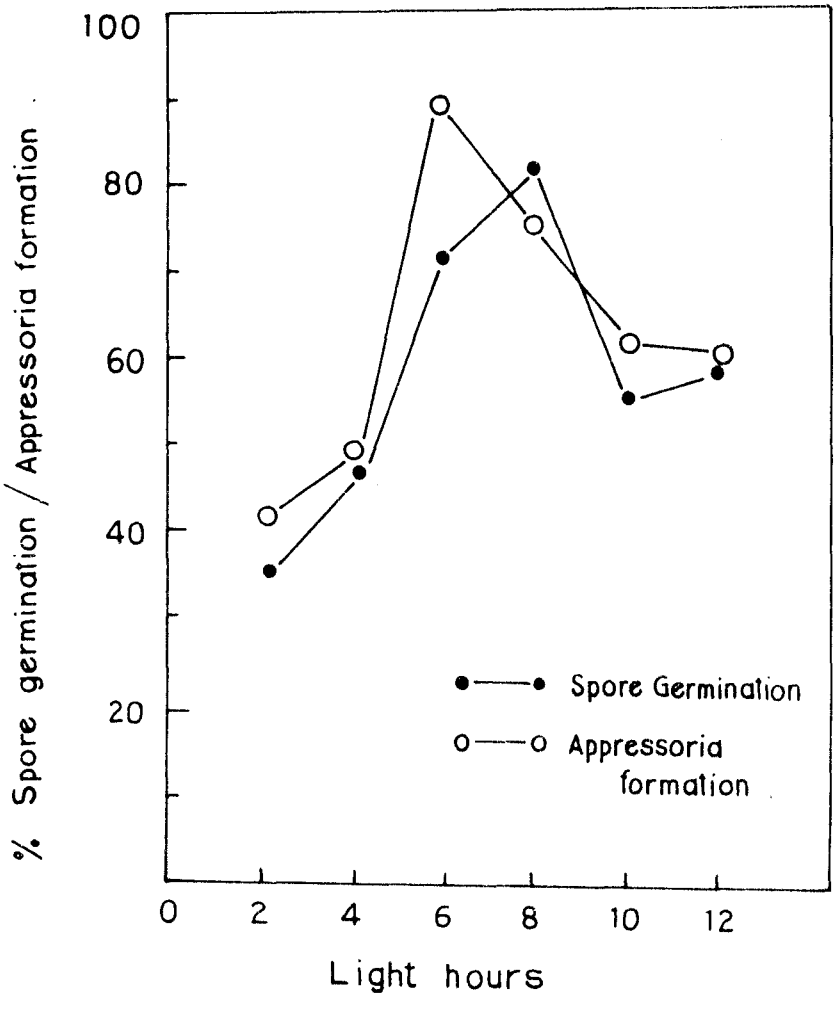


Fig. 5.

Table 12 : Effect of different spore concentrations on the *in vitro* germination and appressoria formation of *G. cingulata*

Spore concentration (Conidia/ml)	% spore germination[a]	% appressoria formation[b]
1.2×10^8	94.2 ± 3.6	74.5 ± 3.8
1.2×10^7	95.1 ± 2.9	81.6 ± 3.9
1.2×10^6	97.5 ± 3.7	89.7 ± 2.2
1.2×10^5	67.6 ± 1.2	73.9 ± 3.2
1.2×10^4	60.0 ± 2.6	66.6 ± 1.9

[a] Average of 200 spores.
[b] Average of 50 germlings.

4.2.2.6 Age of culture

Spores from 5 to 30 day old cultures germinated with appressoria formation to a greater or lesser degree, the optimum being 10-day old culture (Table 13). Spore germination increased by 25.0% from 5-day old culture to 10-day old culture and then decreased gradually.

Table 13 : Effect of age of culture on the *in vitro* germination and appressoria formation of *G. cingulata*

Age of culture (days)	% spore germination [a]	% appressoria formation [b]
5	73.7 ± 2.3	68.2 ± 3.4
10	92.5 ± 3.8	89.0 ± 2.6
15	86.2 ± 2.7	61.1 ± 3.1
20	82.0 ± 2.6	52.1 ± 2.9
25	81.3 ± 3.3	47.0 ± 1.4
30	79.9 ± 1.8	39.2 ± 2.0

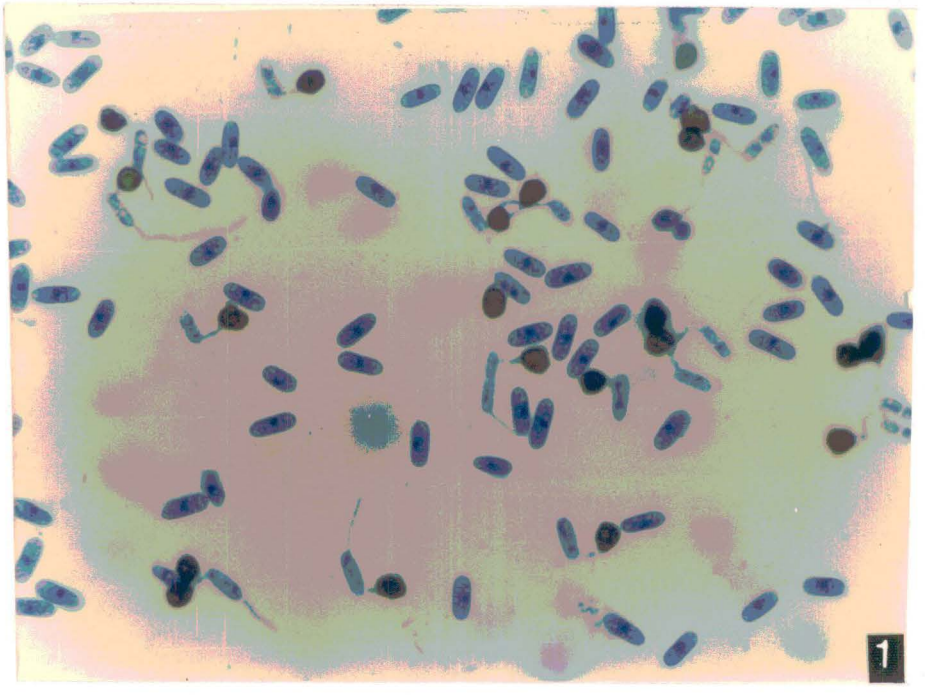
[a] Average of 200 spores.
[b] Average of 50 germlings.

4.2.3 Conidial germination of G. cingulata on glass and leaf surfaces and influence of light period and spore concentration on its infective capacity

Other than the abiotic factors, host factors greatly influence spore germination and appressoria formation and the ultimate establishment of disease. These include the stimulants or inhibitors of fungal development in infection droplets, nature of cuticle, nature of substrates and other such factors. It was therefore considered worthwhile to study the effect of glass surface and leaf surfaces on germination and appressoria formation of G. cingulata. Drops of spore suspension (1.2×10^6 spores/ml) were placed on glass slides, and on leaves of susceptible (TV-18) and resistant (TV-26) varieties of tea. Leaves of three categories were selected -- young leaves, middle leaves and mature leaves. Spores were allowed to germinate for 10h in all cases. For microscopic observation of spore germination on the leaf surfaces, leaves were cleared, fixed and stained as described in Materials and Methods. Maximum spore germination and appressoria formation were observed on the glass surface (Plate V, figs. 1 & 2). Germination on the resistant leaf surface was significantly lower than that on the susceptible leaves. Differences were also apparent depending on the age of the leaves (Table 14; Plate VI, figs. 1 & 2). Spore germination on the resistant surface of young leaves was inhibited by 65.0% in comparison to similar leaves of susceptible variety. Appressoria formation was inhibited by 80% in the resistant variety.

As the factors which affect spore germination and appressoria formation finally influence the disease production, two of the factors studied in vitro (light and spore concentration) were considered here for their effect on the infective capacity of G. cingulata.

Plate V (figs. 1&2)- Germination of G. cingulata conidia on glass surface, showing appressoria; (1) under low power (X250) and (2) under high power (X750) of microscope



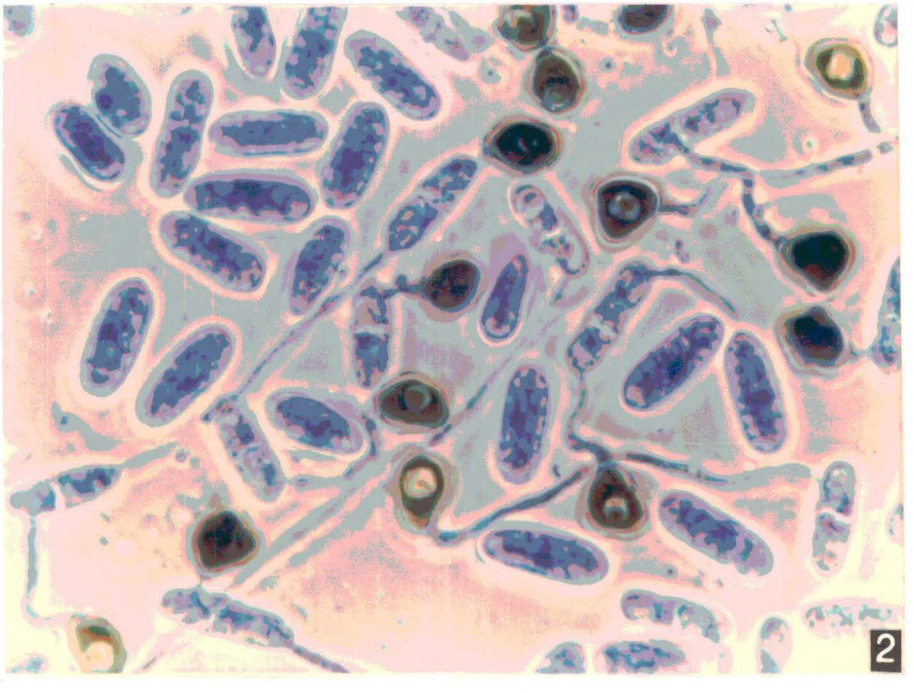
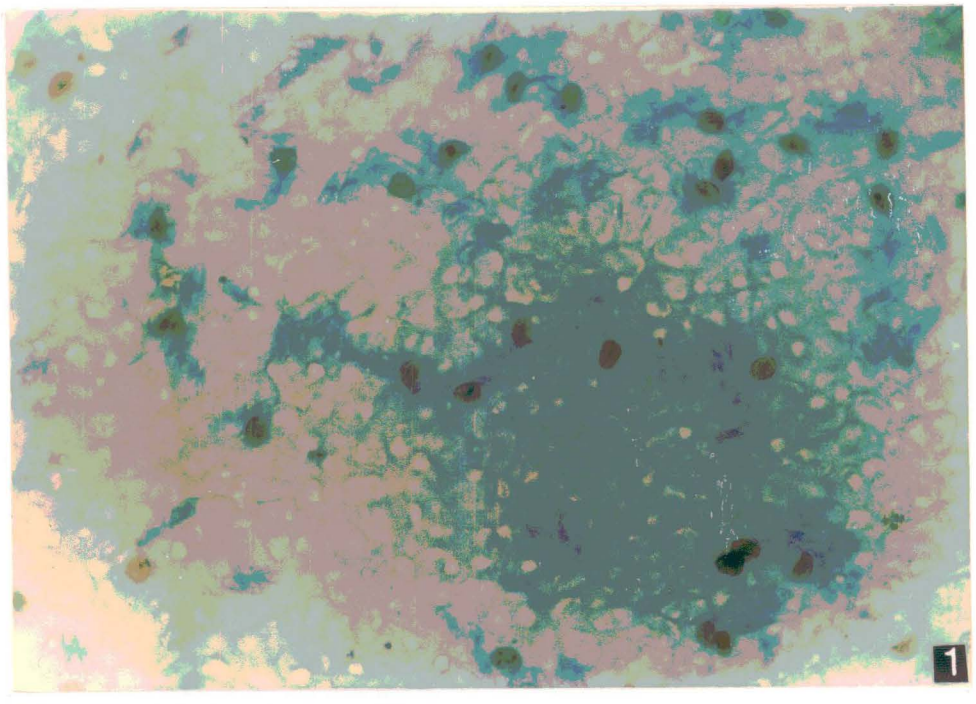


PLATE - V

Plate VI (figs. 1&2)- Germination of *G. cingulata* conidia on tea leaf surface; (1) under low power (X250) and (2) under high power (X750) of microscope



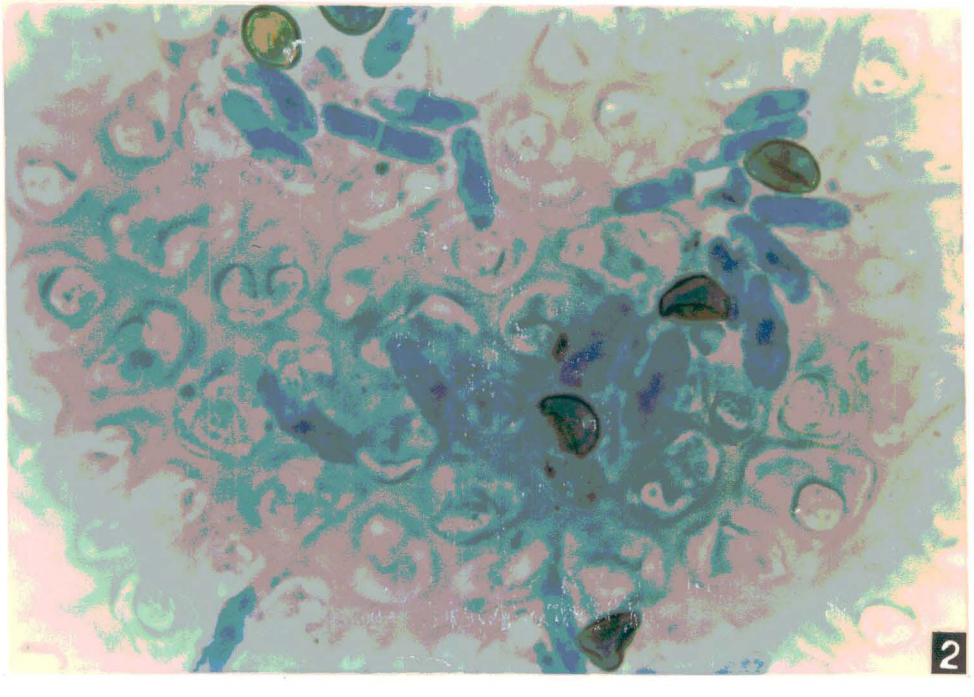


PLATE - VI

Table 14 : Spore germination of G. cingulata on leaf surface and glass surface

Variety	Nature of leaves	% spore germination [d]	% appressoria formation[e]
TV-18 (Susceptible)	a	77.2 ± 3.0	91.7 ± 2.8
	b	61.8 ± 2.9	83.0 ± 3.7
	c	52.2 ± 3.3	79.9 ± 4.0
TV-26 (resistant)	a	27.1 ± 3.7	17.7 ± 4.3
	b	23.7 ± 4.1	15.1 ± 4.0
	c	20.1 ± 3.9	11.1 ± 3.9
Glass surface		72.7 ± 4.2	94.5 ± 3.9

[a] Young leaves, [b] Middle leaves, [c] Matured leaves,
[d] Average of 200 spores, [e] Average of 50 germlings.

Light

Percentage of lesion production on detached leaves of two varieties (TV-18 and TV-23) were noted under varying light/dark

Table 15 : Effect of different light conditions on lesion production by G. cingulata on detached tea leaves

Variety	Light/Dark (h/day)	% lesion production		
		48h	72h	96h
TV-18	24/0	31.1 ± 3.4	41.7 ± 2.8	48.2 ± 2.3
	12/12	47.3 ± 1.7	58.0 ± 1.1	62.2 ± 2.1
	0/24	35.6 ± 1.9	46.1 ± 3.0	49.1 ± 1.6
TV-23	24/0	24.1 ± 2.9	32.0 ± 2.1	39.5 ± 1.1
	12/12	38.4 ± 3.4	43.5 ± 1.3	56.6 ± 1.9
	0/24	23.1 ± 2.7	29.0 ± 0.09	53.3 ± 1.1

Average of 3 experimental sets.
Average of 50 leaves inoculated per treatment.

periods. Results were recorded after 48, 72 and 96h of inoculation. Maximum lesion production was obtained in both the varieties at 12h light and dark periods (Table 15). there was no difference in lesion production either in complete darkness or in complete light.

Concentration of spores

Detached leaves of TV-18 and TV-23 were inoculated with spore suspension ranging in concentration from 1.2×10^4 to 1.2×10^8 spores/ml and lesion production noted after 48, 72 and 96h. Results (Table 16) revealed that maximum lesion production occurred at a concentration of 1.2×10^6 spores/ml.

Table 16 : Effect of different spore concentrations of *G. cingulata* on lesion production on detached tea leaves

Spore concentration of *G. cingulata* [a]	Tea varieties [b]	% lesion production after		
		48h	72h	96h
1.2×10^7	TV-18	64.9 ± 2.9	69.1 ± 3.5	81.1 ± 1.9
	TV-23	59.2 ± 2.1	61.1 ± 2.0	75.2 ± 1.6
1.2×10^7	TV-18	68.2 ± 1.6	77.1 ± 3.5	82.1 ± 1.9
	TV-23	63.3 ± 1.8	64.0 ± 3.1	83.0 ± 1.5
1.2×10^6	TV-18	79.3 ± 2.8	85.1 ± 2.3	91.9 ± 3.2
	TV-23	71.0 ± 2.3	79.1 ± 1.6	88.3 ± 2.7
1.2×10^5	TV-18	72.1 ± 1.7	79.0 ± 3.7	80.1 ± 2.7
	TV-23	52.3 ± 2.1	65.5 ± 1.9	85.0 ± 1.3
1.2×10^4	TV-18	61.2 ± 1.5	71.9 ± 3.5	76.1 ± 1.8
	TV-23	47.2 ± 2.0	51.9 ± 1.7	69.0 ± 1.4

[a] Spore suspension prepared from 10-day old culture of *G. cingulata*

[b] Average of 50 leaves inoculated per treatment.

4.3 Studies on the biological activities of exudates and diffusates of tea leaves

One of the factors responsible for the successful defense mechanism of a host is its ability to produce antifungal compounds which inhibit the growth of the pathogen in its tissues. Many of these occur as preformed compounds while others are formed in response to infection by the pathogen. As results of pathogenicity test revealed that the different varieties of tea showed differential resistance to G. cingulata, the role of preformed and post-infectionally formed compounds, if any, in the differential resistance was investigated. Leaf exudates and diffusates were collected from resistant and susceptible varieties by drop diffusate method as described in Materials and Methods. Biological activities of exudates (preformed compounds) and diffusates (post-infectionally formed compounds) were tested by spore germination bio-assay. Results (Table 17 and Fig. 6)

Table 17 : Effect of leaf exudates of different tea varieties on spore germination and appressoria formation of G. cingulata

Exudates collected from	% spore germination*	% inhibition in spore germination +	% appressoria formation	% inhibition in appressoria formation +
TV-23	36.8 ± 1.4[a]	53.78	13.0 ± 1.2[a]	81.66
TV-25	19.7 ± 0.5[a]	75.26	10.0 ± 0.87[a]	85.89
TV-26	33.3 ± 1.2[a]	58.21	16.9 ± 1.3[a]	76.05
CP-1	35.7 ± 2.3[a]	35.17	6.2 ± 1.2[a]	91.21
Teen Ali 17/1/54	61.1 ± 3.4	23.35	2.4 ± 0.9[a]	96.54
TV-18	62.6 ± 3.6	21.44	31.3 ± 2.1[a]	55.83
Control	79.7 ± 2.8	-	70.8 ± 2.5[a]	-

* Average of 200 spores.
\+ Inhibition in relation to control
a Difference between control significant at P = 0.05 & 0.01.

EFFECT OF LEAF DIFFUSATES AND EXUDATES ON SPORE GERMINATION OF G. cingulata

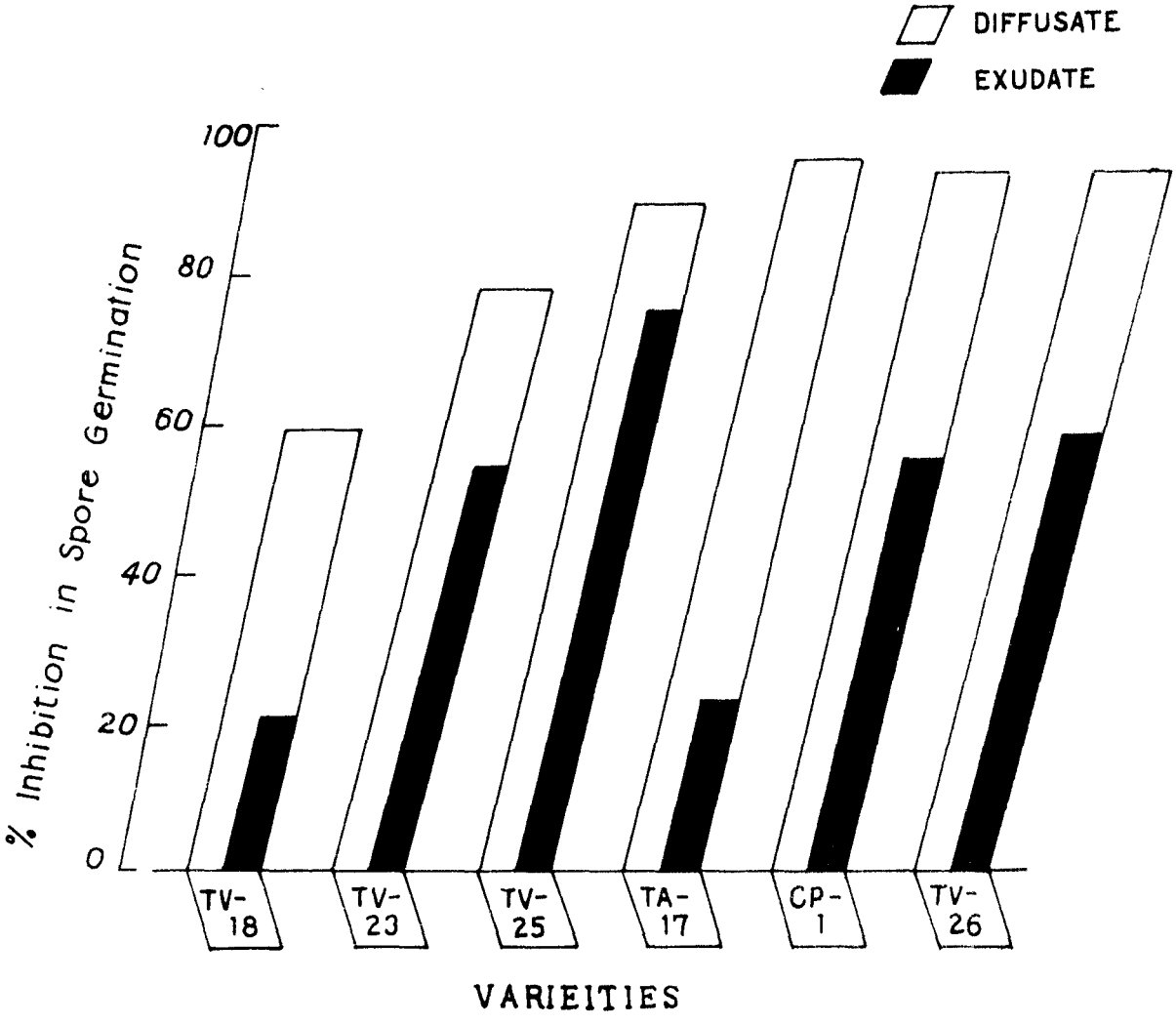


Fig. 6.

revealed that spore germination was inhibited by the exudates from all varieties. Inhibition percentage ranged from 21.4% to 75.0%. However, no clear correlation between the inhibition of spore germination and resistance could be drawn. Significant inhibition in appressoria formation was also observed. Diffusates from all varieties were highly fungitoxic but the activity of the diffusates collected from the resistant varieties (TV-26, CP-1 and Teen Ali 17/1/54) were at least four to five times higher than those from the susceptible ones (TV-18, TV-25 and TV-23). The results have been presented in Table 18 and Fig. 6.

Table 18 : Effect of leaf diffusates of different tea varieties on spore germination and appressoria formation of *G. cingulata*

Duffusates collected from	% spore germination*	% inhibition in spore germination+	% appressoria formation *	% inhibition in appresoria formation+
TV-23	24.1 ± 1.3[a]	78.39	13.6 ± 1.3[a]	71.79
TV-25	16.5 ± 1.1[a]	89.24	9.0 ± 0.9[a]	87.30
TV-26	5.8 ± 2.3[a]	92.62	0	100.00
CP-1	5.3 ± 1.1[a]	93.29	3.4 ± 0.89[a]	95.18
Teen Ali 17/1/54	3.8 ± 0.9[a]	95.19	0	100.00
TV-18	32.3 ± 1.6[a]	59.48	26.4 ± 1.5[a]	62.73
Control	79.7 ± 1.8	-	70.4 ± 2.5	-

* Average of 200 spores
\+ Inhibition in relation to control
a Difference between control significant at P = 0.05 & 0.01.

4.4 Determination of levels of phenolics in healthy and *G. cingulata* infected leaves of *C. sinensis*

Phenolic compounds are known to accumulate in numerous plant species following infection with plant pathogen and in many cases there

is a greater increase in phenolic biosynthesis in resistant host species than susceptible ones (Mahadevan, 1991; Borkar and Verma, 1991). As polyphenols are the major constituents of tea leaves it was decided to compare quantitative changes in the phenolics of resistant and susceptible varieties. At the onset, the simple phenolics present in the healthy leaves were characterized following which quantitative estimation was done.

4.4.1 Characterization of simple phenolics in healthy leaves

The phenolics of the healthy tea leaves were extracted and characterized by thin layer chromatography (TLC). The phenol extracts

Table 19 : Rf values and colour reaction of phenol extracts from healthy tea lea leaves

Phenolics	Rf[a] (x 100)		Colour reactions with Folin-Ciocalteau's reagent spray	Corresponded with authentic phenol & phenolic acid
	Solvent I Acetic acid: Chloroform (1:9)	Solvent II Ethylacetate: Benzene (9:11)		
1	03	16	Blue	-
2	06	32	Blue	-
3	24	40	Blue	-
4	19	44	Blue	Protocatechnic acid
5	03	47	Blue	Gallic acid
6	19	72	Blue	-
7	16	80	Blue	-
8	32	62	Blue	Catechol
9	51	80	Blue[b]	Caffeic acid
10	58	74	Blue[b]	p-Coumaric acid
11	74	65	Blue[b]	-
12	92	97	Blue	-

a On thin-layer chromatograms of silica gel.
b Blue colour after ammonia fuming.

were chromatographed two dimensionally on silica gel plates with acetic acid - chloroform (1:9) as first solvent and ethylacetate - benzene (1:1) as second solvent. The plates were sprayed with Folin-Ciocalteau's reagent and the Rf values of the blue spots which appeared were determined. Fuming with ammonia vapour after the spray revealed the presence of some more blue to grey spots. Rf values of the separated compounds (Table 19) were compared with those of some authentic phenols known to be present in tea leaves (gallic acid, catechol, caffeic acid, p-coumaric acid, protocatechuic acid) which were simultaneously run on the TLC plates. All the five phenolics mentioned above were detected on the TLC plates. Besides, a number of other spots were also present.

4.4.2 Levels of total phenols and orthodihydroxyphenols in healthy and infected leaf tissues

Total and orthodihydroxyphenols from healthy and G. cingulata inoculated tea leaves of TV-18, TV-23 and TV-25 (susceptible) and TV-26, CP-1 and Teen Ali 17/1/54 (resistant) were extracted after 48h of inoculation and estimated. Results depicted in Table 20 and Fig. 7,

Table 20 : Level of phenolics in healthy and G. cingulata infected tea leaves of resistant and susceptible varieties

Variety	Phenol contents (mg/g fresh wt. leaf tissue)			
	Total Phenol		Orthodihydroxy Phenol	
	Healthy	Infected	Healthy	Infected
Susceptible				
TV-18	35.2	29.4	2.0	1.7
TV-23	30.9	28.9	1.8	1.3
TV-25	31.9	28.2	1.9	1.5
Resistant				
TV-26	30.4	38.0	2.0	2.3
CP-1	25.7	34.1	1.5	2.1
Teen Ali 17/1/54	27.1	32.0	1.8	2.0

Average of 5 replicates/treatment.

LEVEL OF TOTAL PHENOLS IN HEALTHY AND
G. cingulata INFECTED TEA LEAVES.

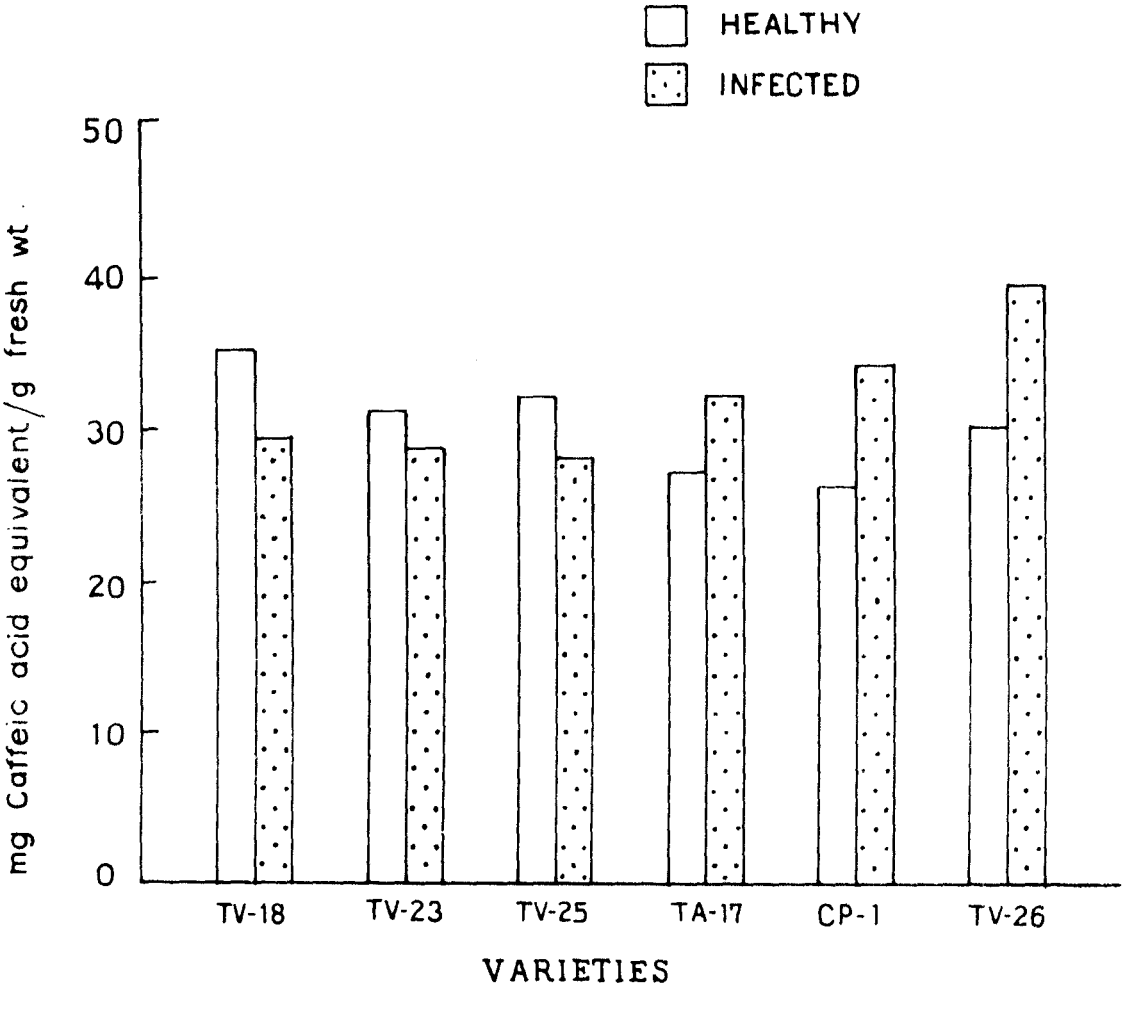


Fig. 7.

revealed that both total and orthodihydroxyphenol contents decreased following inoculation with G. cingulata in the susceptible varieties. In the resistant varieties, however, there was an increase in the phenol contents. TV-26, CP-1 and Teen Ali 17/1/54 showed 24.9%, 24.4% and 15.0% increases in total phenol content respectively over their healthy controls. Percentage of phenolic content decrease in the susceptible varieties ranged from 6.50 to 16.30. Similar trend was also noted in case of orthodihydroxyphenols.

4.5 Studies on host and parasite proteins

Besides changes in phenol metabolism, protein changes are also commonly correlated with disease susceptibility or resistance (Jooster and Dewit, 1977). The resistance of plants to a pathogen may depend to some extent on the speed and amount of protein synthesis induced in the host by the pathogen. In this study, the changes of protein contents of tea leaves after infection with G. cingulata have been determined and the mycelial protein of the pathogen has been analysed.

4.5.1 Comparison of protein content of healthy and G. cingulata infected leaves of C. sinensis

Leaf proteins from three susceptible and three resistance varieties were extracted and estimated as described earlier. Extractions were done after 48h of inoculation with G. cingulata. Results (Table 21 and Fig. 8) revealed that the protein content of all varieties increased significantly in the infected leaves in comparison to the healthy ones. No significant differences were noted between the different varieties of either healthy or infected leaves.

4.5.2 Mycelial protein content of G. cingulata

As protein content increased in all varieties following inoculation, it was considered worthwhile to estimate the protein

Table 21 : Level of proteins in healthy and G. cingulata infected tea leaves of resistant and susceptible varieties

Variety	Protein content (mg/g fresh wt.)	
	Healthy	Infected
Susceptible		
TV-18	8.6	17.1
TV-23	7.8	20.0
TV-25	8.0	17.2
Resistant		
TV-26	7.1	17.2
Teen Ali 17/1/54	6.8	14.6
CP-1	8.1	18.4

Average of 5 replicates/treatment.

content of the pathogen because protein preparations from the infected leaves might contain proteins of the pathogen. Mycelial protein of G. cingulata was prepared from 10-day old mycelia and estimated following the method described earlier. Results revealed that G. cingulata contain 37.5 mg/g fresh weight protein.

4.5.3 SDS-PAGE analysis of mycelial protein

Mycelial proteins of G. cingulata were analysed by SDS-PAGE. Seventeen protein bands ranging in molecular weight from 27 to 210 KD were detected. Four proteins of molecular weights 54, 69, 115 and 200 KD were very prominent (Fig. 9). The molecular weights were determined by comparison with the molecular weight markers as described previously.

TOTAL PROTEIN CONTENT OF HEALTHY AND G. cingulata INFECTED TEA LEAVES.

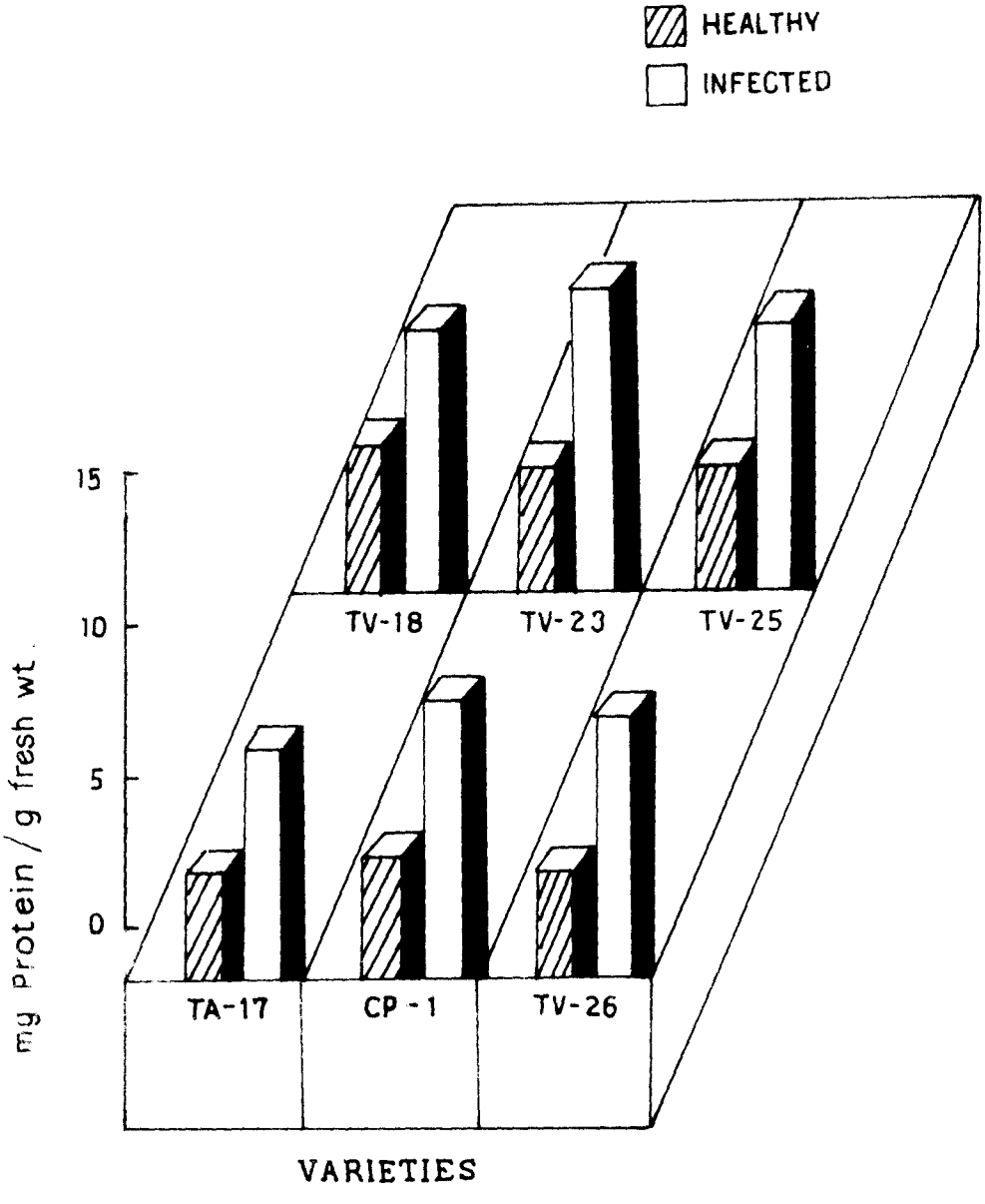
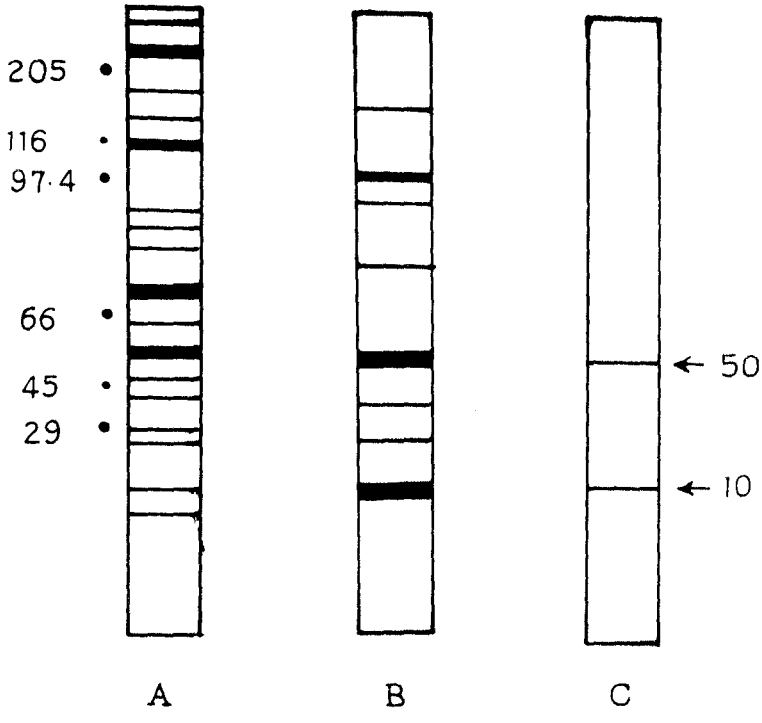


Fig. 8.

SDS- PAGE ANALYSIS OF MYCELIA AND CELL WALL EXTRACT OF G. cingulata



A : Mycelial Protein

B : Cell Wall Extract (Protein)

C : Cell Wall Extract (Carbohydrate)

Fig. 9

4.6 Studies on elicitors of G. cingulata

It is known that many plants accumulate antifungal compounds or phytoalexins as a part of the inducible defense mechanism in response to pathogen invasion or treatment with biotic or abiotic elicitors (Ransom et al., 1992). It has also been reported that many of the elicitor molecules are glycoproteins and fungi are known to possess them on their cell walls and plasma membranes (Ballou, 1976; Nakajima et al., 1977). Since results of this study showed enhanced accumulation of antifungal compounds in the resistant varieties of tea following infection by G. cingulata, it was decided to study the role of elicitors of the pathogen in this mechanism. For this, mycelial wall extracts were prepared, their chemical nature determined and their role in elicitation of antifungal compounds studied.

4.6.1 Determination of chemical nature of mycelial wall extract

Cell walls were isolated from G. cingulata and the isolated cell walls further extracted with NaOH as described earlier. The chemical nature of the cell walls were determined by SDS-polyacrylamide gel electrophoresis and by binding with fluorescein labelled concanavalin A.

4.6.1.1 SDS-Polyacrylamide gel electrophoresis

The mycelial wall extracts were analysed by SDS-PAGE and stained for both protein and carbohydrate to determine the presence of glycoproteins in the wall extracts. Staining of gels with Coomassie blue R250 exhibited eight protein bands.

Carbohydrate staining on the other hand showed two bands at molecular weights 10 and 50 KD. These two bands coincided with two of the bands of proteins. The coincidence of Coomassie blue and PAS

staining indicated the presence of two glycoproteins of molecular weights of 10 and 50 KD in the mycelial wall extract of G. cingulata Fig . 9).

4.6.1.2 Con A - FITC binding

To further confirm the glycoprotein nature of the cell walls, mycelia or isolated cell walls of G. cingulata were treated with FITC-labelled Con A and observed under the microscope as described in Materials and Method section. Strong fluorescence was observed under the microscope in both mycelial as well as cell wall preparations (Plate VII, figs. 1 & 2). The occurrence of Con A binding substance in the cell walls further confirmed the glycoprotein nature of mycelial wall.

4.6.2 Determination of nature of disease reaction elicited by the elicitor

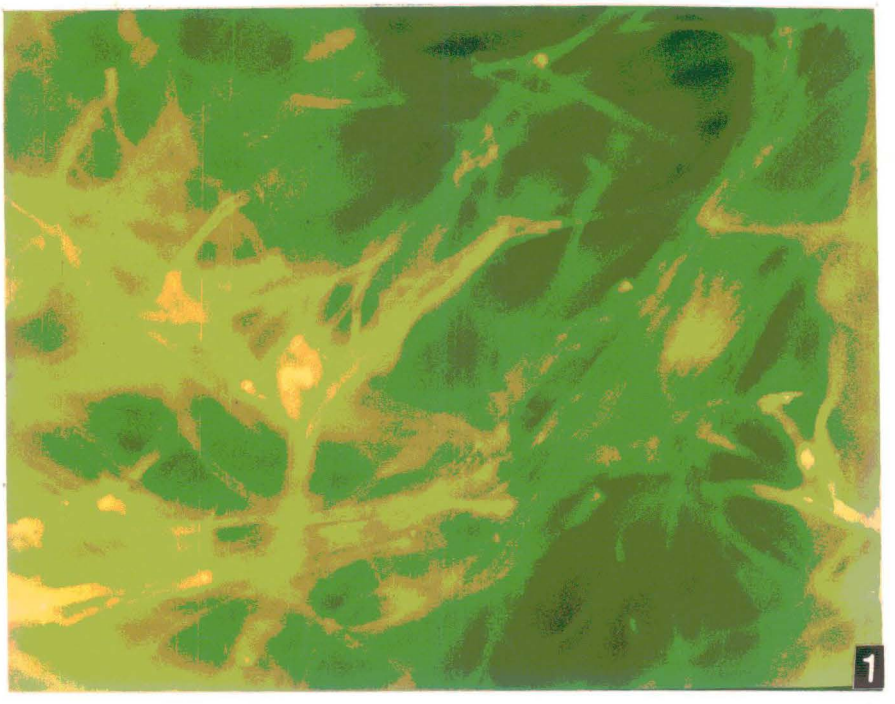
To determine the nature of disease reaction elicited by the elicitor, detached tea leaves (TV-26) were inoculated with spore suspension of G. cingulata, mycelial wall extract (MWE), spore suspension + mycelial wall extract or sterile distilled water. Percentage lesion production was calculated after 48, 72 and 96h of inoculation. Results (Table 22) showed that the disease reaction elicited by the mycelial wall extract was similar to that shown by the spore suspension.

Table 22 : Comparison of lesion production by mycelial wall extract and spores of G. cingulata on detached leaves of tea (TV-26)

Treatment	% lesion production		
	48h	72h	96h
Spore suspension	26.5 ± 2.6	28.1 ± 2.9	29.6 ± 3.3
Mycelial wall extract (MWE)	26.2 ± 3.1	29.0 ± 3.0	32.7 ± 2.1
Spore suspension + mycelial wall extract (MWE)	27.1 ± 2.8	30.2 ± 3.6	32.1 ± 2.7
Distilled water	0	0	0

Average of 3 experimental sets.
Average of 50 leaves/treatment.

Plate VII (figs. 1&2)- Fluorescence of living hyphae (1) and isolated cell walls (2) of G.cingulata after staining with fluorescein isothyacanate concanavalin A (FITC-CON A)



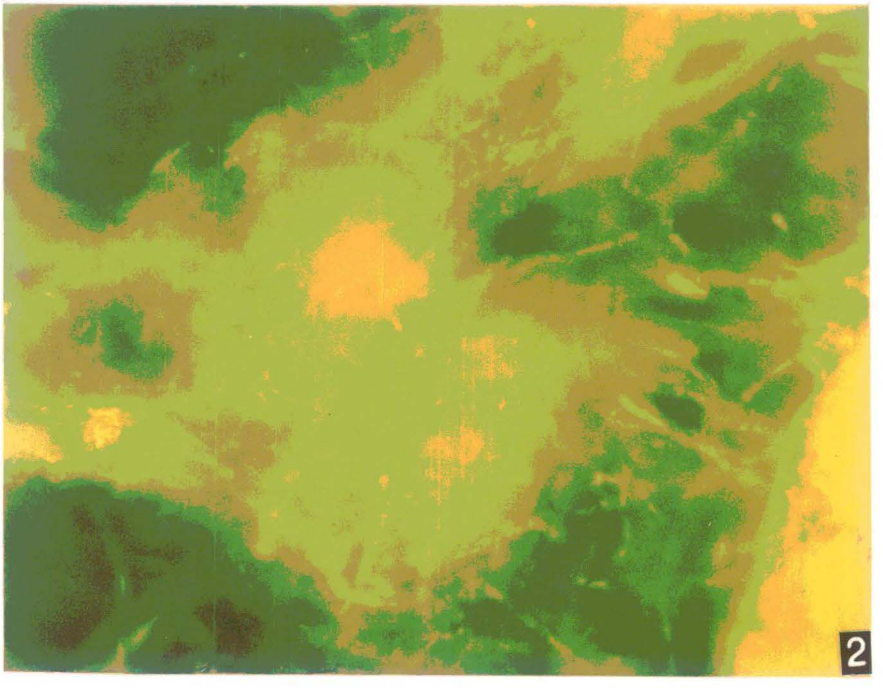


PLATE - VII

4.6.3 Bioassay of diffusible compounds elicited by the elicitor

Since the mycelial wall extract could elicit the resistant reaction in tea leaves it was bioassayed to determine whether it elicited any antifungal compound. For this, the following four treatments were applied separately on the adaxial surface of the leaves of resistant variety (TV-26) : distilled water, distilled water with mycelial wall extract, spore suspension and spore suspension with mycelial wall extract. After 48h of incubation the drops of each treatment were collected, centrifuged and bioassayed by spore germination method. It was found that fungitoxic compound was present in all cases. The mycelial wall extract was almost as effective as the spore suspension in eliciting the antifungal compound (Table 23). When

Table 23 : Spore germination bioassay of diffusible compounds elicited by the mycelial wall extract of *G. cingulata*

Treatment	% spore germination [a]	% appressoria formation [b]
Leaf exudate (Distilled water)	35.5 ± 3.9	14.2 ± 3.9
Leaf diffusate (Spore suspension)	9.0 ± 3.7	1.5 ± 1.1
Leaf exudate + Mycelial wall extract (MWE)	24.1 ± 3.3	11.4 ± 2.3
Leaf diffusate + Mycelial wall extract (MWE)	9.7 ± 2.2	1.6 ± 1.9
Mycelial wall extract (MWE)	87.5 ± 3.1	83.2 ± 3.9
Control	89.2 ± 3.7	94.4 ± 4.1

[a] Based on 200 spores

[b] Average of 50 germlings.

the spores were suspended in mycelial wall extract and allowed to germinate on glass slide, germination similar to the control was observed. The mycelial wall extract as such was therefore not fungitoxic but it induced the formation of antifungal compound in the host.

4.7 Studies on phylloplane microorganisms of tea and their interaction with G. cingulata

In the previous chapters results on studies conducted on the pathogen and host-pathogen interactions have been presented. Some of the metabolic changes occurring in tea leaves following infection with the brown blight pathogen, G. cingulata have been dealt with. However, in nature the pathogen does not occur in an isolated manner on the leaf surface but rather as part of a whole complex of microorganisms forming the phyllosphere. Hence, it was felt that the indepth study of the host-pathogen interaction would only be complete if the influence of phyllosphere microorganisms on the pathogen, G. cingulata and their role in disease development was studied. Keeping this in mind, such studies were undertaken and in the following chapters results will be presented on the work done pertaining to the phyllosphere microorganisms and their interaction with G. cingulata.

4.7.1 Microorganisms isolated from the phyllosphere and their identification

A large number of microorganisms were isolated from the phyllosphere of tea grown in the tea estates of Terai and hill districts of Jalpaiguri and Darjeeling, West Bengal, India (Table 24, Plate VIII). Detailed procedure of isolation has been outlined earlier. For identification of the fungi, microscopic and morphological characters were considered. List of characters on the basis of which the different fungi were identified have been tabulated in Table 25.

Table 24 : A list of microorganisms isolated from tea phyllosphere

Microorganisms	Name of the microorganisms
Bacteria[a]	*Bacillus cereus*
	B. pumilus,
	B. sphaericus
	Bacillus sp.
	Micrococcus luteus
	Micrococcus sp.
	Stenotrophomonas maltophilia
	A member of Coryneform group
Fungi[b]	*Acremonium fusidioides*
	Alternaria alternata
	A. solani
	Aspergillus flavus
	A. fumigatus
	A. nidulans
	A. niger
	A. terreus
	A. versicolor
	Bipolaris carbonum
	Cochliobolus sativus
	Colletotrichum gloeosporioides
	Curvularia lunata
	C. geniculatus
	Fusarium oxysporum
	F. solani
	Penicillium alicum
	P. frequentans
	P. simplicissium
	Pestalotiopsis theae
	Phoma exigua
	Rhizopus stolonifer

a Isolated using NA medium b Isolated using PDA medium.

Plate VIII (figs. 1-20)- Fungi isolated from the phylloplane of tea (1)Aspergillus niger; (2) Aspergillus tereus; (3) Fusarium solani; (4) Rhizopus stolonifer; (5) Phoma exigua; (6) Alternaria alternata; (7) Aspergillus flavus; (8)Colletotrichum gloeosporioides; (9) Acremonium fusidioides (10) Aspergillus fumigatus; (11) Bipolaris carbonum (12) Penicillium oxalicum; (13) Alternaria solani; (14) Rhizopus sp. (15) Pestalotiopsis theae; (16) Fusarium sp. (17) Curvularia geniculatus; (18) Penicillium frequentans; (19) Penicillium simplicissimum; (20) Fusarium oxysporum

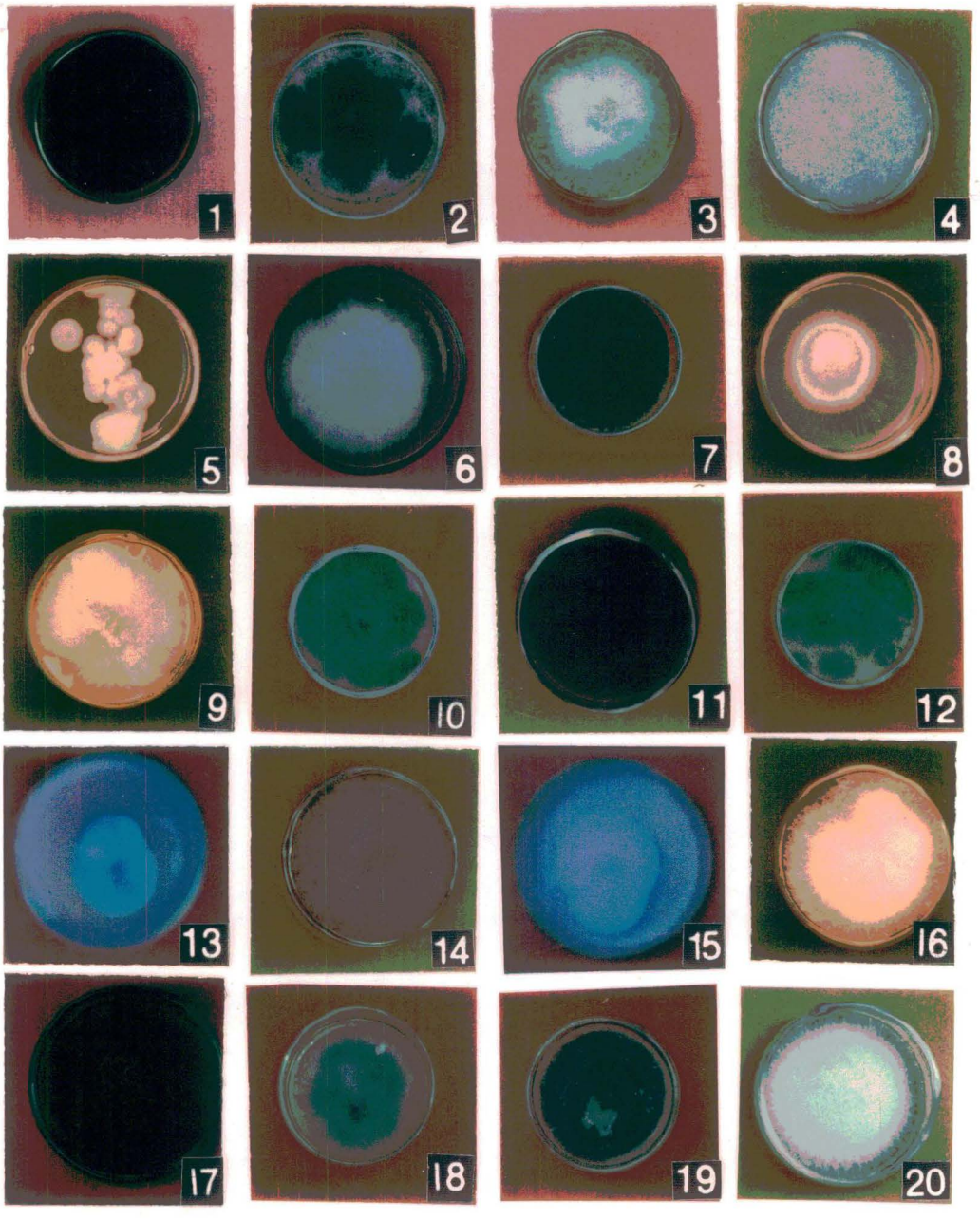


PLATE - VIII

Table 25 : Characterization and identification of fungi from tea phyllosphere

Mycelial characteristics	Conidiophore	Nature and size of conidia	Name of organism
In PDA, colonies reach 6-9 mm in 7 days at 20°C, ochraceous brown		Catenulate, of two kinds (a) predominantly slightly pigmented, fusiform with truncate ends, hyaline, 4.9-6.4 x 1.2-2.1 µm in dia. (b) Globose, hyaline, slightly warty 3.4-4.7 µm.	Acremonium fusidioides
Colonies reach 6 cm dia. in 7 days in PDA at 22-30°C, light to dark brown	Simple, straight or curved, 1-3 septate, 50 um long, 3-6 µm wide, golden brown	Long branching chains, ovoid, obclavate, obpyriform with a short apical beak, smooth walled with 3-8 septa, each portion of lower part with 1-2 longitudinal septa, 18-63 x 7-18 µm.	Alternaria alternata
Colonies fast growing on PDA at 25°C, hyphae septate, branched, light-brown becoming darker with age.	Simple, become geniculated by sympodial elongation.	Single, muriform, beaked, dark, 5-10 transverse and a few longitudinal septa, 120-296 x 12-20 µm.	Alternaria solani
Colonies reach 2-6 cm dia. in 7 days on PDA, at 28°C, yellow-green	Hyaline, 0.4-1.0 µm long and rough walled, on large conidiophores a layer of melulae supports the phialides.	Conidial head radiating, globose to subglobose, finely roughened to echinulate, 1-3 nucleate 3.5-4.5 µm.	Aspergillus flavus
Colonies reach 6-7 cm dia. in 7 days at 24-26°C on PDA, spread broadly, thin, bluish green.	Pigmented conidiophores with clavate vesicles arise from clearly differentiated thick walled foot cells.	Strictly columner conidial heads, conidia globose to subglobose, echinulate, 2.5-3.0 µm.	Aspergillus fumigatus

(Contd....)

Mycelial characters	Conidiophore	Nature and size of conidia	Name of organism
In PDA colonies reach 2.5-3 cm in 7 days, at 24-27°C, powdery, black	Arise from long broad thick walled, mostly brownish, sometimes branched foot cells about 1.5-3.0 mm.	Large radiating heads, mostly globose, irregularly roughened, 4.0-5.5 µm.	Aspergillus niger
Rapidly growing colonies in PDA reaching 3-3.5 cm dia. in 7 days, cinnamon to orange-brown or brown	Conidiophores normally 100-250 µm long, smooth walled, hyaline, with hemispherical vesicles, metulea present, conidial heads strictly columner	Conidia globose to slightly ellipsoidal, smooth-walled, uninucleate, 1.8-2.4 µm.	Aspergillus terreus
Colonies reach 2-3 cm dia. in 7 days, variable in colour, light yellowish to yellowish green	Conidiophores colourless or yellowish, smooth walled, 500-700 µm long, vesicles elongate with metulae and Phialides covering most of the surface, conidial heads radiating.	Conidia globose, echinulate, uninucleate, 2-3 µm.	Aspergillus versicolour
Mycelia usually hyaline to subhyaline, hyphae septate, branched, white becoming blackish-grey with age.	Brown, 150-550 µm long, 4-8 µm broad, unbranched or sparingly branched, producing conidia through apical pore	Straight or curved with 3-9 septa, septa always transverse, fusioid, 12-100 x 6-20 µm.	Bipolaris carbonum
Fast growing colonies, reach 5.0 cm in 7 days at 20°C in PDA, olivaceous black.	Erect, straight, olivaceous brown but later becoming black.	Ellipsoidal, gradually tapering towards the ends, straight or slightly curved, dark brown, smooth-walled, glossy, 6-9 distoseptate, 60-100 x 18-23 µm.	Cochliobolus sativus

(Contd....)

Mycelial characters	Conidiophore	Nature and size of conidia	Name of organism
Colonies with a daily radial increment of 5-7 mm, reaching approximately 5 cm.dia. in 7 days at 26°C in PDA, variable colonies white in colour.	Conidia appearing scantily on solitary phialides but normally in orange sporodochia, basal stromatic cushion covered with dense layer of cylindrical, slightly tappering phialides upto 20 µm long.	Cylindrical with a rounded apex, slightly truncated base, hyaline, filled with granular cytoplasm forming orange-red shiny masses, 9-24 x 3-4.5 µm.	Colletotrichum gloeosporioides
Colonies fast growing; form compact, black stromata on PDA at 25°C, brown in colour	Erect, pigmented, geniculated from sympodial elongations	Olive brown, curved, ellipsoidal, 3-septate, rounded at the apex, slightly accuminate at the base, the middle septum below the centre and the third cell strongly curved, 20-30 x 9-15 µm.	Curvularia lunata
Fast growing colonies on PDA at 25°C, often forming stromata, black	Pigmented, erect conidiophores	Conidia predominantly 4-septate, the central cell distinctly geniculate, tapering gradually towards each end, 18-37 x 8-14 µm.	Curvularia geniculatus
Colonies fast growing reaching 6.5 cm dia. in 7 days at 25°C on PDA, aerial mycelium sparse to abundant, then floccose, delicate, white with purple or violet tinge.	Simple, lateral phialides short, sparsely branched	Microconidia never in chain, mostly 0-septate, ellipsoidal to cylindrical, 5-12 x 2.3-3.5 µm. Macroconidia fusiform, moderately curved, pointed at both ends basal cells pedicellate, 27-46 x 3.0-4.5 µm.	Fusarium oxysporum
Fast growing colonies, 6-8 cm dia. in 7 days at 25°C aerial mycelium but abundant cream to puff conidial slime formed in sporodochia.	Short, branched conidiophores 8-16 x 2-4 µm.	Microconidia abundant, chlamydospores bornesingly, sometimes in pairs, in terminal, lateral, hyaline, smooth walled, 6-10 µm.	Fusarium solani

(Contd....)

Mycelial characteristics	Conidiophore	Nature and size of conidia	Name of organism
Colonies reaching 4-7 cm dia. in 7 days at 25°C on PDA, velvety, dark green, reverse variable in colour, typically yellow-brown.	Conidiophores 100-200 µm long in compact columns.	Globose to subglobose, smooth walled, sometimes finely roughened, 3.0-3.5 µm.	Penicillium frequentans
Colonies spreading, reaching 3.0-4.0 cm dia. in 7 days at 24°C on PDA, dull green, reverse uncoloured, sometimes pink.	Conidiophores typically two-stage branched, phialides cydlindrical, tip distinctly tapering	Conidia form deep crusts which appear silky, strongly ellipsoidal, smooth-walled, 4.5-6.5 x 3-4 µm.	Penicillium oxalicum
Very fast growing colonies reaching 3.2-4.1 cm in 7 days at 25°C on PDA, uncoloured to yellow.	Very dense ramification of the conidiophores, conspicuously roughened stipe.	Subglobose, finely echinate, long 2.5-3.0 µm.	Penicillium simplicissimum
On PDA, mycelia hyaline, with whitish aerial mycelium sparse on the periphery but dense in the centre, acervuli develop form small yellowish hyphal aggregations, form conspicuous greenish-black spore mass	Conidiophores annellidic formed from upper cells of the acervulus, hyaline, cylindrical or obovoid to obpyriform, 10-15 µm long with 1-5 successive proliferations.	Conidial mass black, diffuse, spread on maturity, conidia long fusiform, straight, rarely curved 5-celled, slightly constricted at septa, basal situlae hyaline, straight 4-10 µm long. Size of conidia ranging from 23-25 x 5.5-8 µm.	Pestalopsis theae
Colonies reaching 4.0-6.0 cm dia. on PDA at 20°C, irregularly lobed, whitish to olivaceous grey .		Oblong, sometimes two-celled, 4.0-8.5 x 2.0-3.0 um.	Phoma exigua
Very fast growing colonies on PDA, often over 2 cm high. Stolons hyaline to brown, 13-20 um wide, abundantly branched rhizoids (300-350 um long), whorls of sporangiophores produced terminally, mycelia reddish grey brown in colour.	Sporangiophores pale to dark brown, straight, mostly 1.5-3 mm tall, 20-25 µm wide.	Sporangiospores subglobose, biconical to oval, ridged, mostly 4 nucleate, 7-12 x 6-8.5 µm.	Rhizopus stolonifer

Bacterial identification was performed on the basis of morphological, physiological and biochemical tests (Table 26). The identity of the bacteria were also confirmed from the Mycological Institute, Kew, Surrey, England.

4.7.2 *In vitro* interaction studies on solid medium

At the onset, the interaction of *G. cingulata* with the isolated phylloplane microorganisms were studied *in vitro* on solid medium. *G. cingulata* was paired separately with the different microorganisms and incubated for upto three weeks. On solid media four types of reactions were noted, when *G. cingulata* was paired with the microorganisms on PDA or NA media, (a) homogenous - free intermingling between pairing organisms; (b) overgrowth - *G. cingulata* overgrown by the test organism, (c) cessation of growth at line of contact and (d) aversion - a clear zone of inhibition. The names of microorganisms exhibiting the different types of reactions are given in Table 27. Results indicate that among the tested organisms *Aspergillus flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, *Bacillus cereus*, *B. pumilus*, *Bacillus* spp., Coryneform bacteria, *Micrococcus luteus*, *Micrococcus* sp., *Penicillium oxalicum* and *Pnicillium simplicissium*, *Penicillium frequentans* showed antagonistic reaction with *G. cingulata* (Plates IX; figs. 1 & 2; Plate X, figs. 1-4). The antagonistic bacteria were considered for further studies on interaction with *G. cingulata* since they exhibited more significant antagonism.

4.7.3 *In vitro* interaction studies in liquid medium

All the bacteria which showed antagonistic reaction in solid medium were grown separately with *G. cingulata* in nutrient broth. In each case mycelial dry weight of *G. cingulata* growing alone as against growing in dual culture were noted and percentage inhibition in mycelial growth determined. Results depicted in Table 28 and Fig. 10

Table 26 : Characterization and identification of some of the bacterial isolates from tea phyllosphere

Characteristics	*M. luteus* (359392)	*Bacillus* sp. (359388)	*Micrococcus* sp. (359384)	*Bacillus pumilus* (359391)	Coryneform bacterium (359393)	*Bacillus cereus* (359386)
Morphological						
Shape	Spheres Tetrads often clusters	Rods	Spheres Irregular cell cluster	Rods	Very short rods	Rods
Size (μm)	0.9-1.8	2-3 x 0.7-0.8	0.5-2.0	2-3 x 0.6-0.7	1.0-1.7	3-5 x 1.0-1.2
Pigment	Y	W	Y	W	O	W
Spore	-	+	-	+	-	+
Spore shape	-	E	-	E	-	E
Capsule/Slime layer	-	-	-	-	-	-
Motility	-	+	-	+	+	+
Physiological and Biochemical						
Gram reaction	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+
Nitrate reduction	-	+	-	-	-	+
Indole production	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	+	+
NH_3 from Arginine	-	+	-	+	+	+
Esculine hydrolysis	-	+	-	+	$\pm$	+
Growth at 37°C	+	+	+	+	+	+
Growth in anaerobic agar	-	-	-	-	+	+

(Contd..)

Table 26 (Contd.)

Characteristics	M. luteus (359392)	Bacillus sp. (359388)	Micrococcus sp. (359384)	Bacillus pumilus (359391)	Coryneform bacterium (359393)	Bacillus cereus (359386)
Gelatine liquefaction	-	+	-	+	-	+
VP reaction	-	-	-	+	+	+
Urease production	-	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-
Fat hydrolysis	-	+	-	+	ND	+
Utilization/gas production from						
Glucose	+/-	+/-	+/-	+/-	+/-	
Sucrose	+/-	+/-	+/-	+/-	+/-	
Lactose	+/-	+/-	+/-	+/-	+/-	
Glycerol	+/-	+/-	+/-	+/-	+/-	

+ Positive reaction, ± Weak reaction, - Negative reaction, ND Not detected,

Y = Yellow, W = White, O = Orange

Based on Bergey's Manual of Systemic Bacteriology

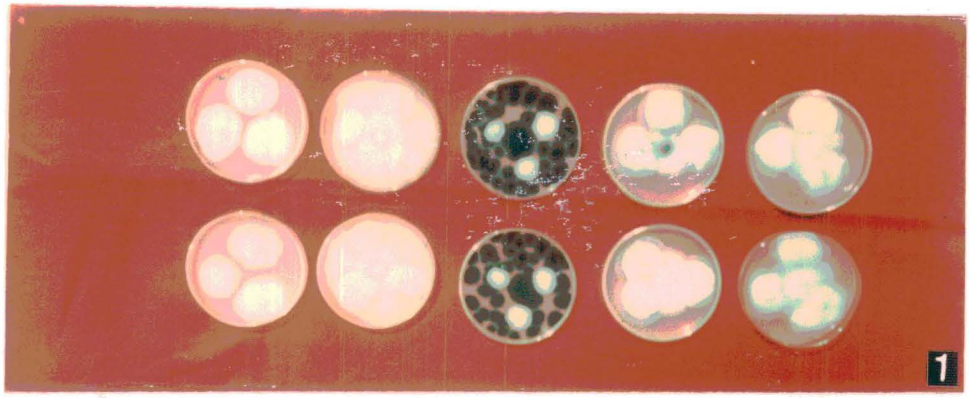
IMI numbers within the parenthesis indicate further confirmation of the characters of the isolates.

Table 27 : Interaction of phylloplane microorganisms with G. cingulata on solid medium

Type of reaction	G. cingulata
Homogenous[a]	Acremonium fusioidis
	Alternaria alternata
	A. solani
	Bipolaris carbonum
	Cochliobolus sativus
	Colletotrichum gloeosporioides
	Curvularia lunata
	Fusarium oxysporum
	F. solani
	Pestalotiopsis theae
Overgrowth[b]	Rhizopus stolonifer
Cessation of growth at line of contact[c]	Curvularia geniculatus
	Phoma exigua
Aversion[d]	Aspergillus flavus
	A. fumigatus
	A. nidulans
	A. niger
	A. terreus
	Bacillus cereus
	B. pumilus
	Bacillus sp.
	Coryneform bacterium
	Micrococcus luteus
	Micrococcus sp.
	Penicillium frequentans
	P. oxalicum
	P. simplicissium

[a] Free intermingling between paired microorganisms.
[b] G. cingulata overgrown by the test organism.
[c] Two organisms grew towards each other but growth stopped at common margin.
[d] A clear zone of inhibition was observed.

Plate IX (figs. 1-3)- Pairing of G.cingulata with phyllosphere microorganisms on solid medium (NA); (1) G.cingulata paired with fungi and (2) with Bacillus cereus; (3) G. cingulata growing alone (control)



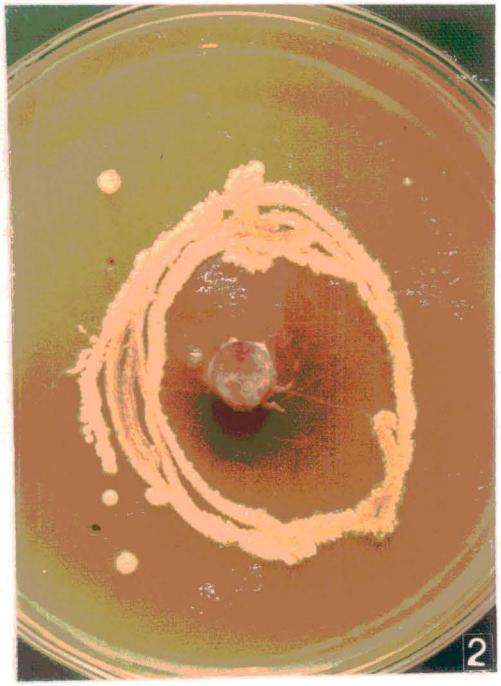


PLATE - IX

Plate X (figs. 1-4)- Pairing of *G.cingulata* with bacteria isolated from phyllosphere of tea; *G. cingulata* growing alone (1); *G. cingulata* paired with *Micrococcus luteus* (2); *Micrococcus* sp.(3); and with coryneform bacterium (4)

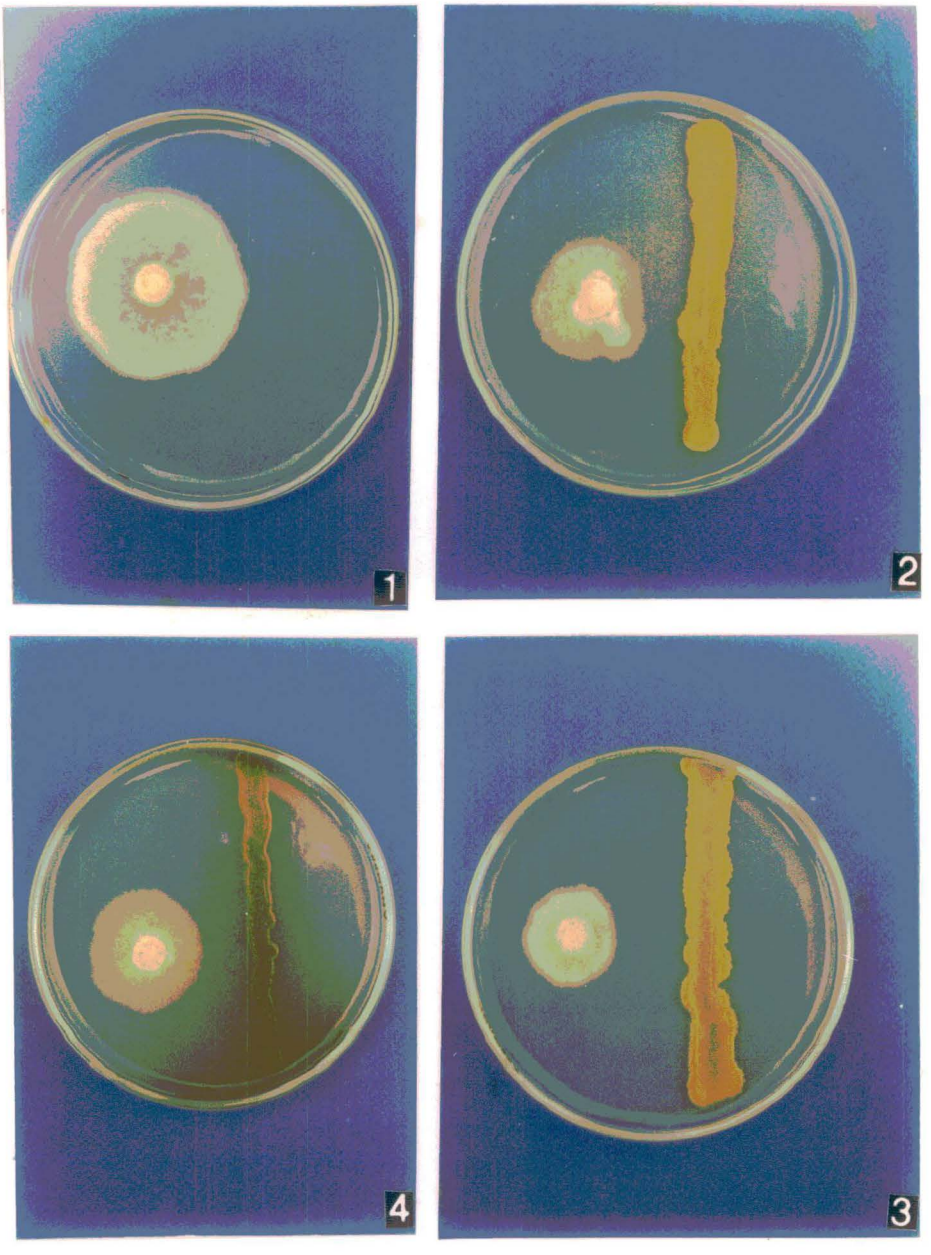


PLATE - X

Table 28 : Mycelial growth of *G. cingulata* in dual culture

Interacting microorganisms	Mean mycelial dry wt. (mg)[a]	% inhibition in mycelial growth	Change in pH of the medium
G. cingulata	197.5 ± 2.4	55.26	5.5
M. luteus + *G. cingulata*	88.3[c] ± 1.6		
G. cingulata	189.7 ± 2.8	36.74	7.1
B. pumilus + *G. cingulata*	120.0[c] ± 4.4		
G. cingulata	183.2 ± 2.9	62.45	4.7
B. cereus + *G. cingulata*	68.8[c] ± 3.4		
G. cingulata	197.3 ± 1.4	54.81	5.0
Micrococcus sp. + *G. cingulata*	89.1[c] ± 3.9		
G. cingulata	201.0 ± 2.4	53.94	
Bacillus so. + *G. cingulata*	92.5[c] ± 4.1		
G. cingulata	175.9 ± 2.2	53.28	6.6
Coryneform bacterium + *G. cingulata*	82.2[c] ± 4.0		

pH of the medium (NB) 7.2

a Average of 3 replicates/treatment

b Inhibition in relation to growth of *G. cingulata* alone.

c Difference between growth of *G. cingulata* alone significant at LSD 1%.

EFFECT OF ANTAGONISTIC BACTERIA ON MYCELIAL GROWTH OF *G. cingulata* IN MIXED CULTURE .

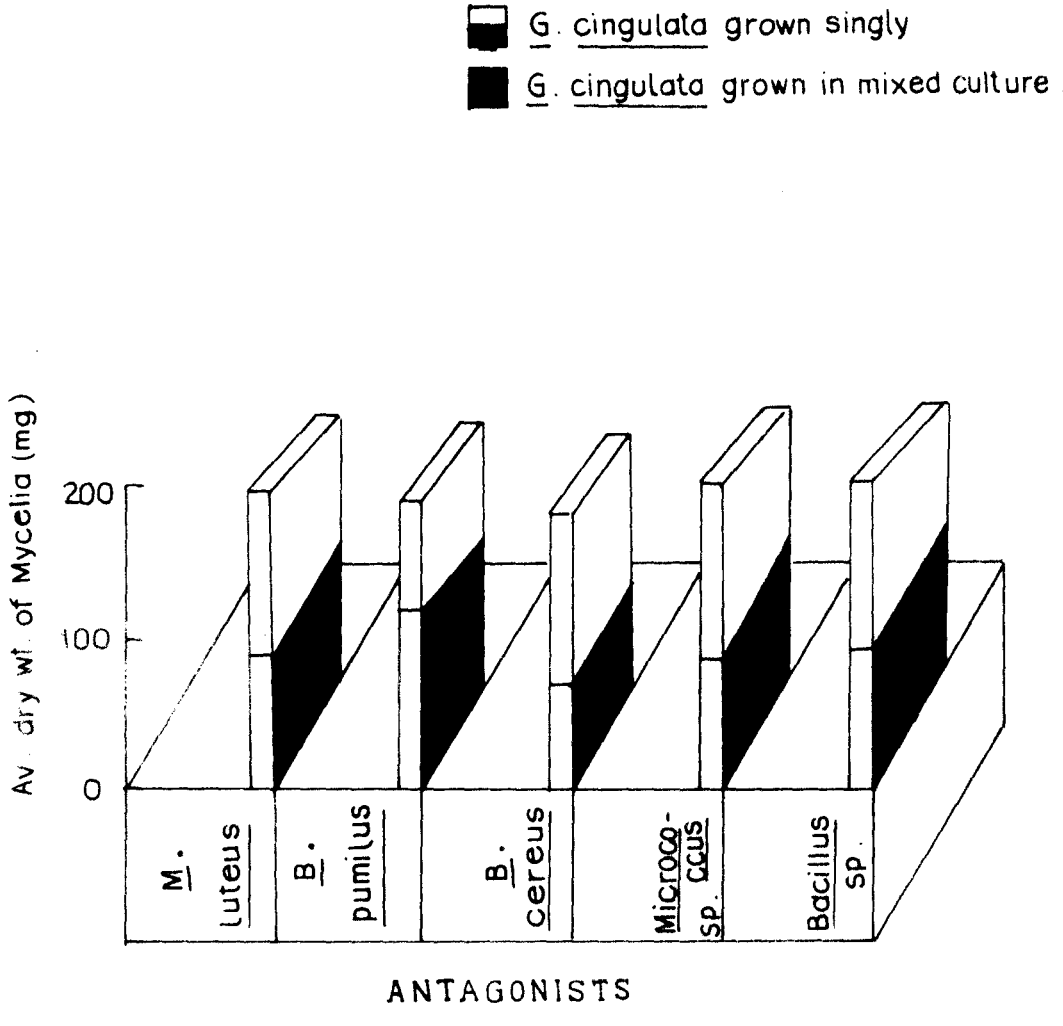


Fig. 10 .

revealed that all the tested bacteria inhibited mycelial growth to a greater or lesser degree. The most effective were Bacillus cereus and M. luteus, where 62.45% and 55.26% inhibition in growth were noted respectively in relation to the growth of G. cingulata alone. The change in pH of the medium was also noted.

Inhibition of growth of G. cingulata observed in mixed culture may be either due to competition for nutrients between the two interacting microorganisms or fungitoxic metabolites may be released by the bacteria into the culture medium. Experiments have been conducted later to investigate the actual mechanism.

4.8 Tests with selected antagonistic microorganisms

On the basis of in vitro tests on solid and in liquid media, four bacteria, Bacillus cereus, Micrococcus luteus, Bacillus sp. and one of the member of Coryneform bacteria was selected for further studies both in vivo and in vitro. In vivo studies included pretreatment of tea leaves with either aqueous cell suspension, cell-free culture filtrate or cell wash of the antagonistic microorganisms followed by inoculation with G. cingulata. Besides, the proportion of antagonists and G. cingulata on the leaf surface affecting disease development has also been studied.

4.8.1 Effect of aqueous cell suspension, cell wash and cell free culture filtrate on disease development in vivo

Aqueous cell suspension, cell-free culture filtrate and cell wash from the different bacteria were prepared as described in Materials and Methods and these were sprayed on leaves of susceptible variety (TV-18). Spraying was done on the leaves of whole plants in the field. Two sets of controls were maintained by spraying either sterile distilled water or nutrient broth. After 24h of second spray, the leaves were inoculated with G. cingulata by detached leaf inoculation

Table 29 : Effect of pre-treatment with *Micrococcus luteus* on disease development by *G. cingulata* on tea leaves (TV-18)

Treatment	% lesion production[a]			Mean of trials
	Trial 1[b]	Trial 2[b]	Trial 3[b]	
Aqueous cell suspension	20.0±1.7	23.9±1.2	20.1±2.7	21.3[c]±1.9
Washed cell	33.2±2.0	35.0±2.9	30.7±2.1	32.0[c]±1.9
Cell-free culture filtrate	10.0±2.4	8.2±1.9	8.7±2.5	9.0[c]±0.46
Control : Sterile distilled water	73.9±1.9	75.2±2.3	78.0±2.1	75.7 ±0.75
Nutrient broth	65.2±2.4	62.5±1.7	64.7±3.1	64.1 ±1.70

[a] After 48h of inoculation with *G. cingulata* on detached leaf.
[b] Average of three experimental sets.
[c] Difference with control significant at LSD 1%

Table 30 : Effect of pre-treatment with *Bacillus cereus* on disease development by *G. cingulata* on tea leaves (TV-18)

Treatment	% lesion production[a]			Mean of trials
	Trial 1[b]	Trial 2[b]	Trial 3[b]	
Aqueous cell suspension	21.0±1.5	24.0±2.2	20.5±1.9	21.8[c]±1.2
Washed cell	28.0±2.0	29.1±1.7	26.7±2.1	27.9[c]±0.48
Cell free culture filtrate	8.5±1.7	7.0±1.0	7.5±1.2	7.6[c]±0.61
Control : Sterile distilled water	73.2±2.3	71.7±1.8	75.2±1.1	73.4 ±0.59
Nutrient broth	65.0±2.0	62.7±2.1	67.5±1.9	65.1 ±0.92

[a] After 48h of inoculation with *G. cingulata* on detached leaf.
[b] Average of three experimental sets.
[c] Difference with control significant at LSD 1%

Table 31 : Effect of pre-treatment with *Bacillus* sp. on disease development by *G. cingulata* on tea leaves (TV-18)

Treatment	% lesion production[a]			Mean of trials
	Trial 1[b]	Trial 2[b]	Trial 3[b]	
Aqueous cell suspension	22.8±1.5	24.7±1.0	21.5±1.7	23.0[c]±0.80
Washed cell	31,2±2.2	29.5±1.8	30.7±1.3	30.4[c]±0.68
Cell-free culture filtrate	10.1±1.2	7.5±0.8	11.0±1.0	9.5[c]±1.0
Control :				
Sterile distilled water	65.0±2.1	67.1±3.1	69.1±1.4	67.3±0.4
Nutrient broth	61.0±1.9	64.4±2.1	66.9±1.7	64.1±1.4

[a] After 48h of inoculation with *G. cingulata* on detached leaf.
[b] Average of three experimental sets.
[c] Difference with control significant at LSD 1%

Table 32 : Effect of pre-treatment with Coryneform bacterium disease development by *G. cingulata* on tea leaves (TV-18)

Treatment	% lesion production[a]			Mean of trials
	Trial 1[b]	Trial 2[b]	Trial 3[b]	
Aqueous cell suspension	24.9±1.0	22.5±1.6	19.9±1.6	22.4[c]±0.9
Washed cell	33.4±1.5	37.0±2.3	32.7±1.9	34.4[c]±0.4
Cell-free culture filtrate	17.7±1.7	10.1±2.3	15.4±2.1	14.4[c]±3.1
Control :				
Sterile distilled water	70.9±1.1	75.0±2.0	73.7±1.3	73.2 ±1.6
Nutrient broth	69.0±1.4	68.1±3.1	62.0±1.9	66.4 ±0.3

[a] After 48h of inoculation with *G. cingulata* on detached leaf.
[b] Average of three experimental sets.
[c] Difference with control significant at LSD 1%.

technique. Disease intensity was assessed after 48h of inoculation. In each case average of three experimental sets was considered. Results (Tables 29-32) revealed that disease intensity was reduced significantly by all the four tested microorganisms. Cell-free culture filtrates of the organisms were most effective followed by aqueous suspension and washed cells. Percentage inhibition of lesion production by culture filtrates of *B. cereus*, *M. luteus*, *Bacillus* sp. and of the bacterium of Coryneform group were 88.2, 85.9, 85.0 and 78.3 respectively.

4.8.2 Effect of different ratios of antagonists and *G. cingulata* on disease development

In natural conditions the actual ratio of antagonists to the pathogen necessary for disease reduction is important. As the antagonistic bacteria decreased disease severity, it was decided to test the effect of different ratios of antagonists and *G. cingulata* on disease development. Six ratios of antagonists to *G. cingulata* were prepared -- 50:1, 25:1, 10:1, 5:1, 1:1 and 0:1. The concentration of *G. cingulata* was kept constant in all cases while that of the different antagonists were decreased serially. The mixed suspension were inoculated onto detached tea leaves and percentage lesion production determined after 48, 72 and 96h of incubation. Percentage lesion production increased with decreasing proportion of the antagonists (Table 33 and Fig. 11). There was no lesion production at 50:1 ratio indicating that at this ratio the antagonists could totally inhibit the growth of *G. cingulata*. At 25:1 ratio, *M. luteus* and *B. cereus* inhibited lesion production by 85.3% and 84.2% respectively after 48h of inoculation which decreased to 25% and 33.6% inhibition respectively at 1:1 ratio. Similar trend was noticed in all the other cases also. After 96h of inoculation, percentage lesion production in the absence of the antagonists ranged from 80-90%; at 25:1 ratio it ranged from 22-28%.

Table 33 : Effect of different ratios of antagonists and *G. cingulata* on lesion production in leaves of tea (TV-18)

Ratio of antagonists and *G. cingulata*	Selected antagonists	% lesion production[a]		
		48h	72h	96h
50:1	*M. luteus*	0	0	0
	B. cereus	0	0	0
	Bacillus sp.	0	0	0
	Coryneform bacterium	0	2.2±2.1	4.3±3.1
25:1	*M. luteus*	11.5±3.9	19.6±2.8	21.9±3.3
	B. cereus	12.9±3.1	14.7±3.6	17.2±2.7
	Bacillus sp.	14.7±2.1	17.3±1.6	22.5±4.0
	Coryneform bacterium	18.1±2.0	21.1±2.0	28.9±2.0
10:1	*M. luteus*	21.1±1.5	27.9±2.6	34.1±2.7
	B. cereus	24.1±2.1	28.3±3.0	32.1±3.4
	Bacillus sp.	32.2±3.4	38.2±4.0	41.2±3.3
	Coryneform bacterium	27.7±2.5	29.5±3.2	28.9±1.9
5:1	*M. luteus*	49.9±2.8	52.6±2.3	58.2±3.0
	B. cereus	40.5±2.9	43.1±2.8	49.0±2.7
	Bacillus sp.	42.2±3.1	49.1±2.7	50.2±2.9
	Coryneform bacterium	52.1±3.1	57.7±1.7	61.1±3.2
1:1	*M. luteus*	58.0±3.0	59.7±3.6	60.7±2.3
	B. cereus	54.2±3.3	58.5±3.4	60.9±2.1
	Bacillus sp.	52.2±2.8	57.9±3.2	58.1±3.9
	Coryneform bacterium	58.7±1.7	61.2±1.8	65.7±3.9
0:1	*M. luteus*	78.2±2.7	80.0±3.8	83.2±4.1
	B. cereus	81.7±2.9	83.2±1.1	88.5±3.2
	Bacillus sp.	80.2±3.1	85.7±3.4	87.7±2.4
	Coryneform bacterium	84.7±2.9	89.4±2.7	93.3±2.1

[a] Lesion produced after 48, 72 and 96h
[b] Average of three experimental sets.

EFFECT OF DIFFERENT RATIOS OF ANTAGONISTIC BACTERIA (M. luteus AND Bacillus Sp.) AND G. cingulata ON LESION PRODUCTION ON TEA LEAVES (TV-18) .

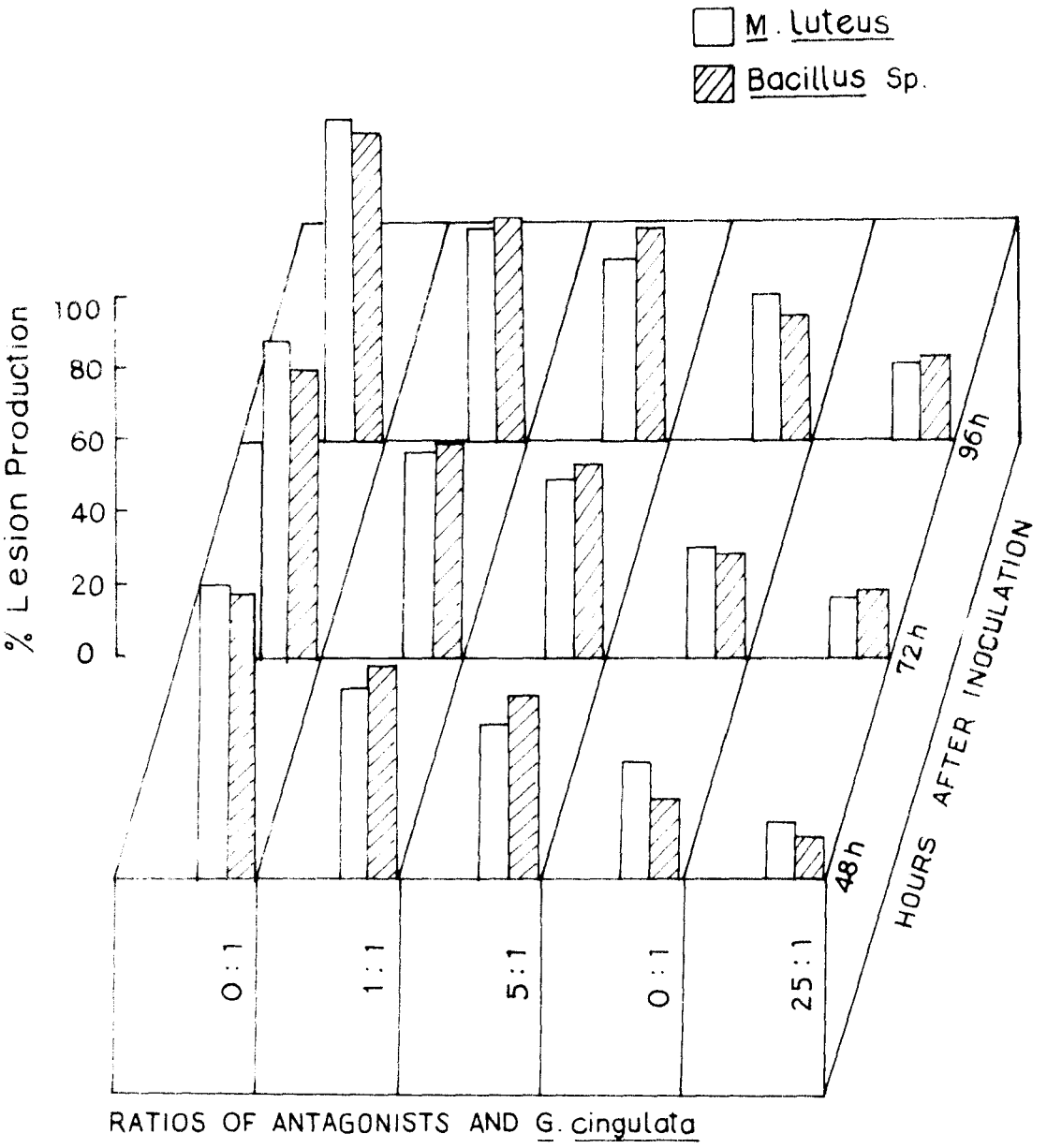


Fig. 11.

4.8.3 Bioassay of cell free culture filtrates

Since the cell free culture filtrates were most effective in vivo, their activity were further tested in vitro by spore germination bioassay. Cell-free culture filtrates of the four bacteria were prepared, and sterilized either by autoclaving or by passing through sterilized G-5 sintered glass filter. These were then separately bioassayed against G. cingulata. Percentage germination of spores and appressoria formation were determined in each case and compared with that of control. Cold sterilized culture filtrates of all the four bacteria inhibited spore germination of G. cingulata completely while the heat-killed culture filtrates inhibited spore germination upto a certain extent (Table 34). Results, therefore, indicate the presence of antifungal compounds in culture filtrates and these are partly thermolabile.

Table 34 : Effect of culture filtrates of antagonistic bacteria on the spore germination of G. cingulata

Treatment	% spore germination[a]	% appressoria formation[b]
Sterile distilled water	93.3 ± 3.2	97.5 ± 2.8
Medium	94.2 ± 2.4	81.6 ± 3.1
Culture filtrates of *Bacillus cereus*		
Cold sterilized	0	0
Heat-killed	26.3 ± 1.2	28.4 ± 1.9
Micrococcus luteus		
Cold sterilized	0	0
Heat-killed	21.7 ± 2.7	27.1 ± 2.4
Bacillus sp.		
Cold sterilized	0	0
Heat-killed	21.1 ± 3.1	17.1 ± 2.1
Coryneform bacterium		
Cold sterilized	0	0
Heat-killed	17.7 ± 3.9	10.8 ± 2.8

[a]Based on 200 spores [b]Average of 50 germlings

4.8.4 Effect of replacement of culture medium on the growth of G. cingulata

To confirm that inhibition in growth of G. cingulata in vitro and in vivo was not due to competition for nutrients, an experiment was performed. G. cingulata was grown in nutrient broth medium for 7 days, initial weight of mycelial was noted, washed with sterile distilled water and culture medium was replaced by sterile distilled water, nutrient broth medium or cold sterilized culture filtrates of the antagonistic bacteria (50 ml/flask) and incubated for another 3 days at 30°C. At the end of the experimental period mycelia were collected, dried, weighed and results are presented in Table 35.

Table 35 : Effect of replacement of medium with culture filtrate of antagonistic bacteria on the growth of G. cingulata

Treatment	Average dry weight of mycelia (mg)[a]		Difference in growth[d]
	Initial weight[b] (mg)	Final weight[c] (mg)	
Medium[e]	202.5	374.0 ± 3.1	172.4
Sterile distilled water	202.5	331.0 ± 3.7	128.5
Cold sterilized cell-free culture filtrate of			
M. luteus	198.2	214.5 ± 4.0	16.2
B. cereus	207.1	227.5 ± 3.8	20.4
Bacillus sp.	203.5	226.0 ± 3.7	22.5
Coryneform bacterium	199.0	218.3 ± 2.9	19.3

[a] Average of three replicates/treatment;
[b] After 7 days in the original medium;
[c] After 4 days in the replaced medium;
[d] Difference in mycelial growth owing to replacement of medium;
[e] Nutrient broth.

The culture filtrate of all tested bacteria significantly inhibited the growth of G. cingulata. Maximum growth occurred in the control medium. It was interesting to note that the fungus grew better in distilled water rather than in bacterial culture filtrate although it contained residual nutrients. The results suggest that minimum growth in culture filtrate may be due to inhibitory action of toxic metabolite released by the bacteria and not due to lack of nutrition. These two tests (spore germination bioassay of culture filtrate and replacement of medium with culture filtrate) conclusively proved that the antagonistic bacteria secrete antifungal metabolite(s) into the culture filtrate.

4.9 Separation and bioassay of active principle from the cell-free culture filtrates

Results of previous experiments showed that all the four selected antagonistic bacteria secreted antifungal substances into the medium. For further characterization of these antifungal metabolites, the cell-free culture filtrates were extracted separately with five solvents - acetone, benzene, chloroform, diethylether or ethylacetate. The solvent extracts were dried in vacuum and were finally dissolved in ethanol as described earlier.

4.9.1 Bioassay of solvent extracts from M. luteus, B.cereus, Bacillus sp. and one member of Coryneform group

The different solvent extracts were bioassayed against G. cingulata to determine the fraction containing the active principle following two types of bioassay techniques.

(i) Spore germination bioassay

For spore germination bioassay a drop of each solvent extract was placed on clean, grease-free glass slide and allowed to evpaorate

following which drop of spore suspension of G. cingulata (1.2×10^6 spores/ml) was placed over it. Percentage spore germination and appressoria formation were determined after 24h. For control set drop of ethanol was used. Results (Table 36 and Fig. 12) showed that diethylether fractions of M. luteus and B. cereus, ethylacetate fraction of Bacillus sp. and chloroform fraction of the Coryneform

Table 36 : Effect of solvent extracts from culture filtrate of antagonistic bacteria on spore germination of G. cingulata

Solvent extracts of	% spore germination of G. cingulata						% appressoria formation[b]					
	Control	A	B	C	D	E	Control	A	B	C	D	E
M. luteus	89.1 ±4.1	40.0 ±3.9	47.0 ±3.1	24.2 ±3.3	0	56.5 ±2.9	91.7 ±4.2	0	0	0	0	0
B. cereus	90.0 ±2.1	62.0 ±3.7	45.0 ±3.1	34.2 ±3.3	0	55.0 ±1.9	88.2 ±2.1	10.2 ±3.1	0	0	0	40.0 ±1.9
Bacillus sp.	87.1 ±3.0	68.2 ±3.5	40.2 ±3.3	31.2 ±2.1	10.0 ±1.1	0	90.4 ±3.7	13.1 ±4.0	7.1 ±1.9	0	0	0
Coryne-form bacterium	90.7 ±3.1	70.9 ±3.2	51.6 ±1.7	0	8.0 ±2.7	25.8 ±1.1	94.2 ±3.9	23.0 ±3.3	2.0 ±1.8	0	0	0

A - Acetone extract; B - Benzene extract;
C - Chloroform extract; D - Diethylether extract;
E - Ethylacetate extract;

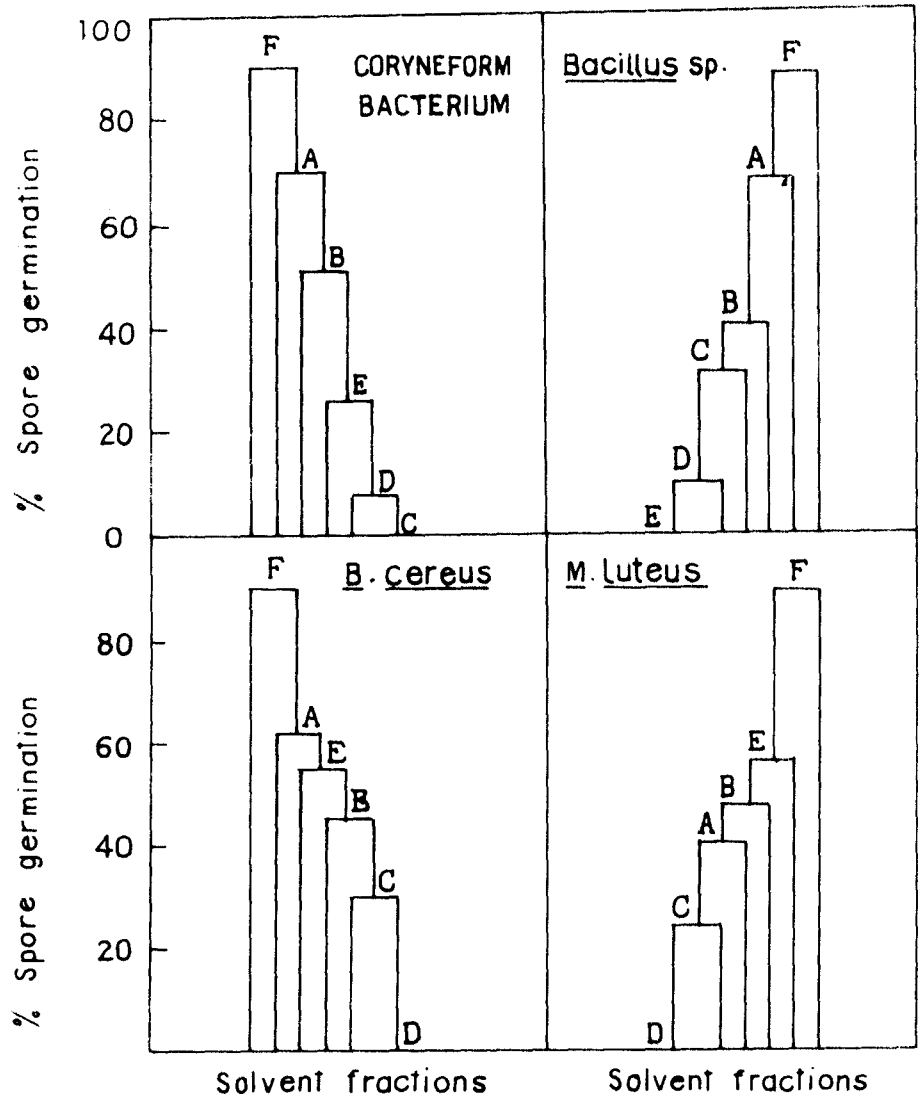
[a] Average of 200 spores/treatment,
[b] Average of 50 germlings/treatment.

bacterium totally inhibited spore germination and appressoria formation. Partial inhibition in spore germination was also observed in other fractions. Complete inhibition of appressoria formation was observed in most of the extracts tested.

(ii) Radial growth inhibition

In the second bioassay method, the effect of the different

EFFECT OF DIFFERENT SOLVENT EXTRACTS FROM ANTAGONISTIC BACTERIA ON SPORE GERMINATION OF G. cingulata .



A : ACETONE FRACTION B : BENZENE FRACTION C : CHLOROFORM FRACTION D : DIETHYLETHER FRACTION E : ETHYLACETATE FRACTION F : CONTROL .

Fig . 12 .

extracts on radial growth of G. cingulata was tested on solid medium. Procedure has been described in detail in Materials and Methods. After 4 days of growth in the control sets the mycelia completely covered the Petridishes (5 cm dia.) while varying degrees of inhibition in mycelial growth was noted in the different extracts (Table 37). In the diethylether fraction of M. luteus 92% inhibition in growth was recorded. Inhibition of 80%, 82.2% and 90.2% were observed in diethylether fraction of B. cereus, ethylacetate fraction of Bacillus sp. and chloroform fraction of the Coryneform bacterium respectively.

Table 37 : Effect of solvent extracts from culture filtrate of antagonistic bacteria on radial growth of G. cingulata

Solvent extracts of	Radial growth of G. cingulata (mm)[a]					
	Control	A	B	C	D	E
M. luteus	50.0±0.8	21.0±1.6	17.0±1.5	8.0±1.9	4.0±0.8	25.0±2.1
B. cereus	50.0±1.1	22.0±0.8	25.1±0.8	29.0±0.5	10.0±0.1	20.0±0.2
Bacillus sp.	50.0±0.9	26.5±2.0	28.0±1.7	19.0±1.9	13.9±1.0	8.9±0.9
Coryneform bacterium	50.0±1.0	23.0±1.9	21.5±1.1	4.9±0.7	7.9±1.9	20.0±1.0

A - Acetone extract; B - Benzene extract;
C - Chloroform extract; D - Diethylether extract;
E - Ethylacetate extract.

a Radial growth in RM supplemented with the respective solvent extracts.
Average of three replicates/treatment.
Radial growth measured after 4 days.
Size of mycelial block 2 mm.

4.9.2 Dose response of solvent extracts of *M. luteus*, *B. cereus*, *Bacillus* sp. and the Coryneform bacterium

The effect of serial dilutions of each of the active solvent fraction was tested on spore germination and appressoria formation of *G. cingulata*. In each case the final concentration of extracts obtained from the culture filtrates (5 ml extract/1000 ml culture filtrate) was considered as 'X'. From this, serial dilutions of X/2, X/4, X/8, X/16, X/32 and X/64 were prepared with ethanol and these were then bioassayed by spore germination method as before. It was observed that in all cases percentage spore germination increased concomitantly with the dilution of the extract (Table 38, Fig. 13). At X and X/2 concentrations total inhibition was observed. Inhibition percentage of X/64 concentration was 5.9% (Coryneform bacterium), 19.33% (*Bacillus* sp.), 30.10% (*M. luteus*) and 67.50% (*B. cereus*). Appressoria formation also increased considerably with the serial dilutions.

4.10 Further studies on production of active principle from *M. luteus*

Results of the previous experiments have established four bacteria as antagonistic to *G. cingulata* both *in vitro* and *in vivo*. Of these, *M. luteus* was selected for further studies on purification and to be used as biocontrol agent (Plate XI, figs. 1 & 2). It was the most effective on the basis of most of the tests performed. *B. cereus*, though very potent, could not be considered since it may be unsuitable as biocontrol agent due to the involvement of some strains of this bacterium in food toxicity (Koomen and Jeffries, 1993).

The production of antifungal metabolites by bacteria in culture is influenced by a number of factors including available carbon and nitrogen sources, pH of the medium, temperature, size of inoculum and time period. Maximum production of the antifungal principle will be at the optimum combinations. Considering the above, it was decided to

Table 38 : Response of *G. cingulata* to serial dilutions of active fraction from antagonistic bacteria

Concentration	% spore germination [a]				% appressoria formation [b]			
	M. luteus [c]	*B. cereus* [d]	*Bacillus* sp. [e]	Coryneform bacterium [f]	*M. luteus*	*B. cereus*	*Bacillus* sp.	Coryneform bacterium
X	0	0	0	0	0	0	0	0
X/2	0	0	0	4.2±1.9	0	0	0	0
X/4	5.7±0.07	1.1±1.0	5.4±2.9	20.0±1.9	0	0	4.8±0.2	6.7±0.8
X/8	16.7±0.3	6.8±1.3	27.8±2.7	46.9±1.1	1.7±0.8	0	21.0±0.7	14.9±1.4
X/16	23.1±0.5	20.8±1.7	45.2±1.9	67.4±1.9	4.9±1.0	2.4±1.0	29.9±1.9	20.8±2.5
X/32	45.6±0.5	25.0±2.0	66.5±2.0	85.9±1.7	7.1±1.1	6.1±1.1	37.7±3.1	32.1±2.9
X/64	67.3±1.1	31.5±1.2	78.0±1.0	91.7±1.3	12.3±1.1	9.6±0.5	42.5±2.0	52.7±2.9
Control	96.8±3.7				83.2±3.1			

[a] Average of 200 spore
[b] Average of 50 germlings
[c] Diethyl ether fraction from *M. luteus*
[d] Diethyl ether fraction from *B. cereus*
[e] Ethyl acetate fraction from *Bacillus* sp.
[f] Chloroform fraction from Coryneform bacterium.

RESPONSE OF G. cingulata TO SERIAL DILUTION OF ANTIFUNGAL COMPOUND (S) FROM ANTAGONISTIC BACTERIA

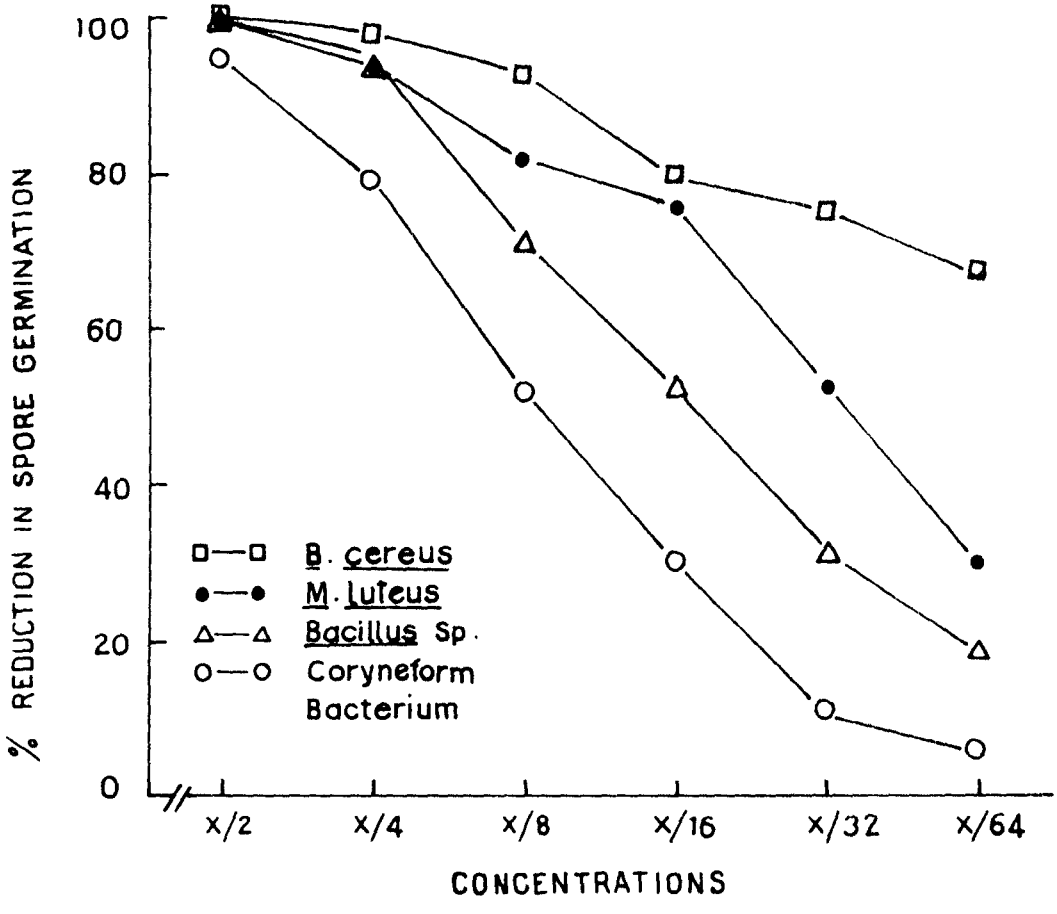
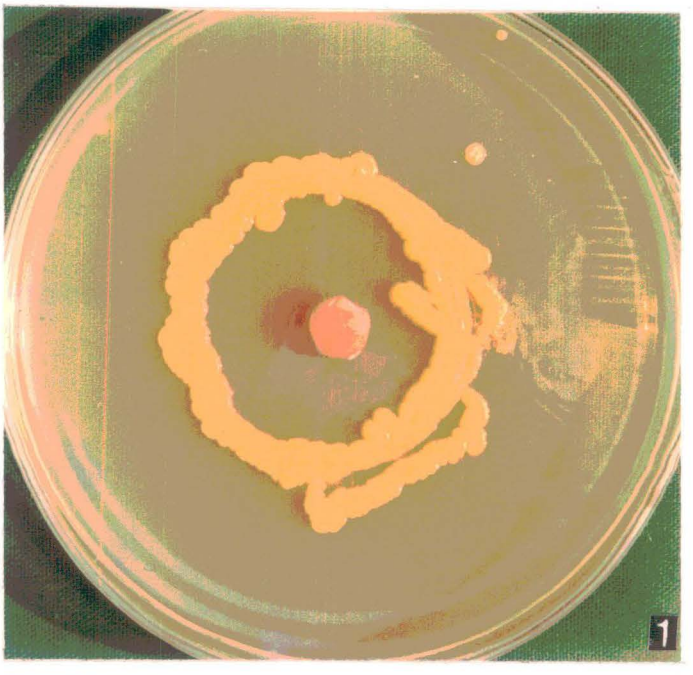


Fig. 13.

Plate XI (fig.1)- G. cingulata paired with M. luteus ; (fig 2) M. luteus cells after Gram staining (X2000)



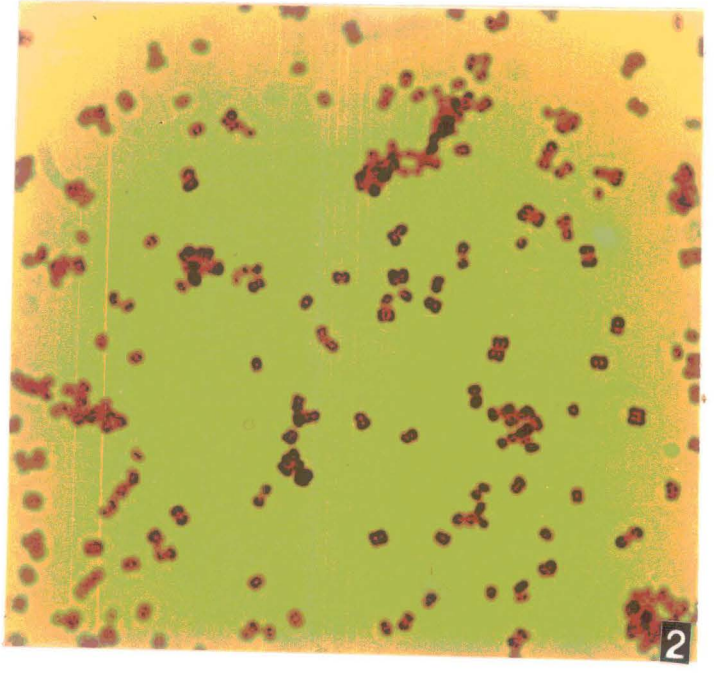


PLATE - XI

study the effect of the important factors on production of active principle by M. luteus. Incubation period, pH, different media and carbon sources were considered. The spectrum of fungitoxicity of the antifungal compound has also been tested.

4.10.1 Effect of incubation period

To determine the optimum incubation period for the production of antifungal compound by M. luteus, Nutrient broth (50 ml/250 ml flask) was inoculated with 0.5 ml inoculum and kept for 7 days at 37°C in shaking condition. The culture filtrate was collected at intervals of 24h upto 168h. The culture filtrates were extracted with diethylether as described earlier. The extracts were bioassayed by radial growth inhibition and agar cup methods. Results (Table 39, Fig. 14 and Plate XII, figs. 1 & 2) revealed that maximum production of antifungal compound was at 96h of incubation as evidenced by minimum radial growth

Table 39 : Effect of different incubation time on production of antifungal compound by M. luteus

Growth period of M. luteus [a] (h)	Radial growth of G. cingulata (mm)[b]	Inhibition zone in Agar cup assay (mm)[c]
0	50.0 ± 0.17	8.00
24	21.0 ± 1.1	17.2 ± 1.0
48	18.5 ± 1.4	21.7 ± 1.7
72	9.0 ± 0.7	23.5 ± 1.3
96	50.5 ± 0.6	27.0 ± 1.3
120	9.7 ± 1.4	17.3 ± 1.1
140	12.5 ± 0.8	16.7 ± 2.1
168	13.0 ± 1.0	14.4 ± 1.3

Average of three replicates/treatment.

[a] M. luteus grown in Nutrient broth

[b] Growth measured after 4 days.

[c] Seeding medium RM.
Cork borer size 8 mm.

EFFECT OF INCUBATION TIME ON THE PRODUCTION OF ANTIFUNGAL COMPOUND(S) BY M. Luteus ASSAYED BY RADIAL GROWTH OF G. cingulata.

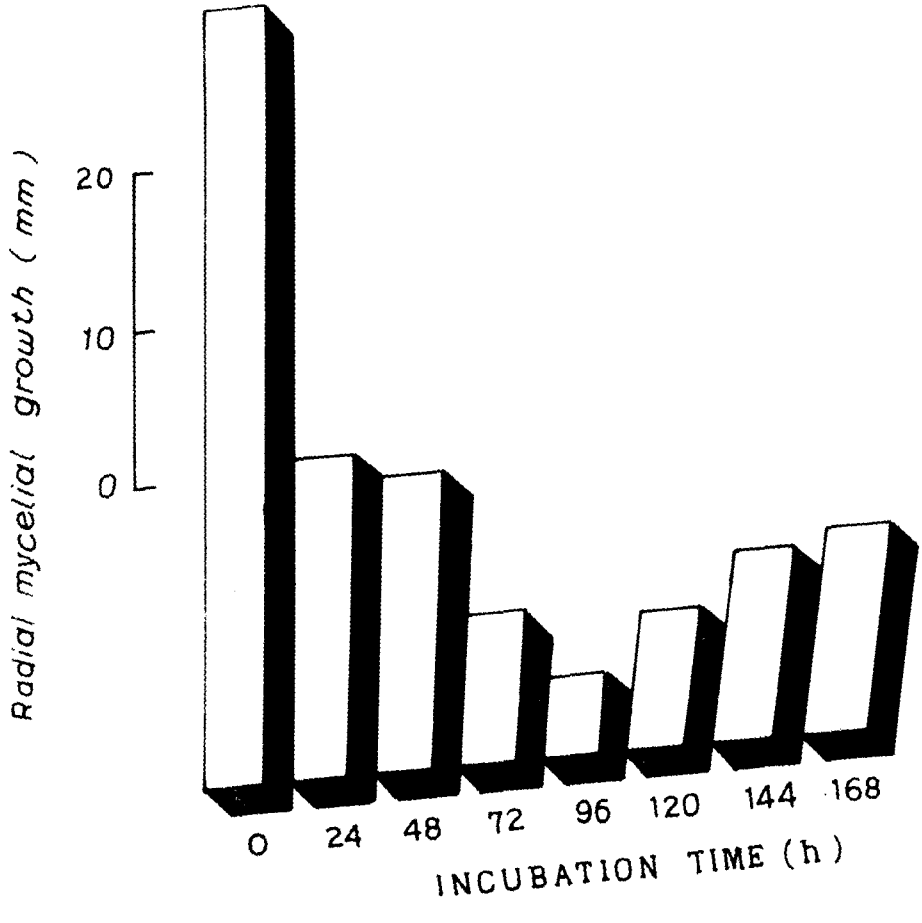
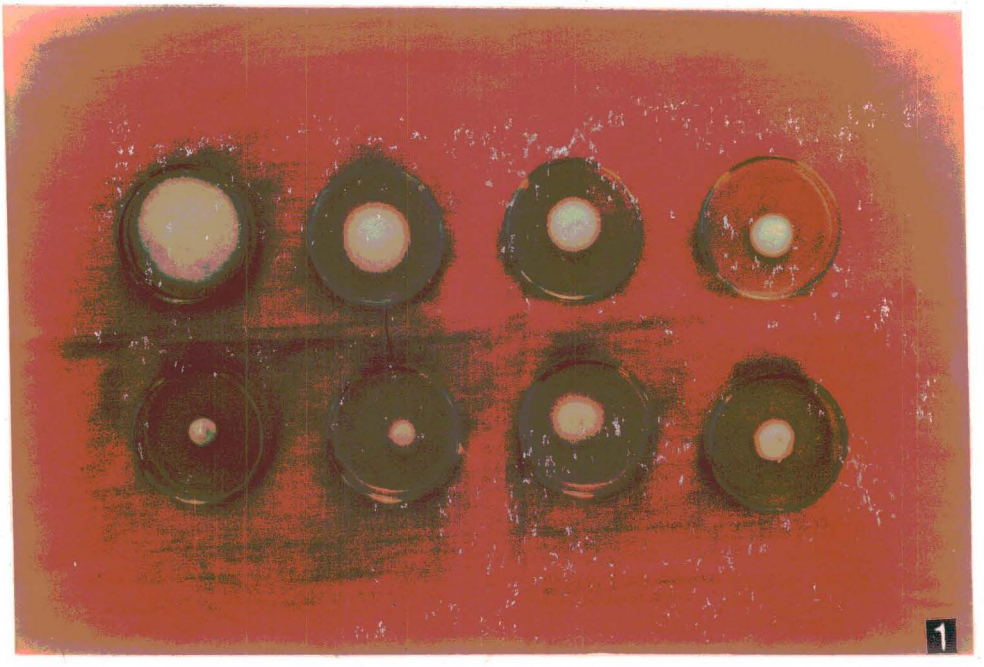


Fig. 14

Plate XII (fig.1)→ Radial growth bioassay of G.cingulata in diethyl ether extracts from M.luteus grown for 24, 48, 72, 96, 120, 144 & 168 h; (fig.2) enlarged view of bioassay after 96h incubation, along with control



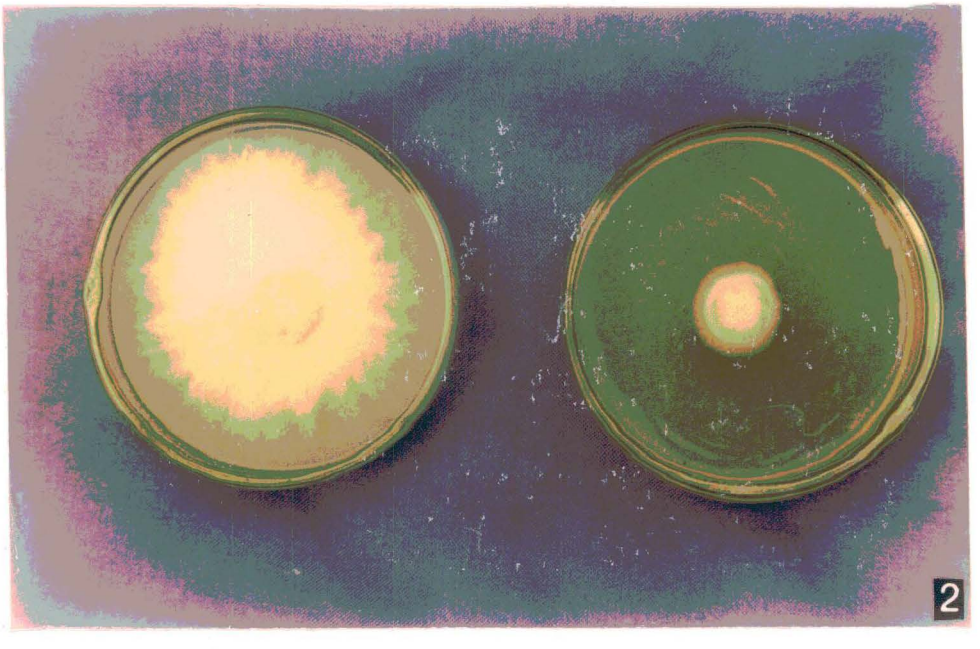


PLATE – XII

(5.5 mm) and maximum inhibition zone (27 mm dia). At 0h growth, there was no inhibition. Production of the metabolite increased from 24h to 96h and showed a decline. Results, therefore, indicate that 96h is the optimum incubation period for production of antifungal metabolite by M. luteus.

4.10.2 Effect of different media

Four different media (glucose yeast extract peptone, nutrient broth, antibiotic media no. 1 and peptone liquid) were considered to select the most suitable one for production of antifungal compound. After 96h of growth in the different media the bacterial culture filtrates were collected and extracted as before. Activity of the extracts were tested by radial growth inhibition and agar cup bioassays. It was observed that among the tested media, antibiotic media no. 1 was the most effective followed by nutrient broth (Table 40). In the extract from antibiotic media no. 1 only very little growth occurred followed by the extract from Nutrient broth.

Table 40 : Effect of different media on production of antifungal compound by M. luteus

Media	Radial growth of G. cingulata [a] (mm)	Inhibition zone in Agar cup assay (mm)[b]
Nutrient broth	6.0 ± 1.1	28.5 ± 1.8
Glucose yeast extract peptone broth	20.0 ± 1.1	22.5 ± 2.1
Peptone liquid medium	17.3 ± 1.9	25.5 ± 1.7
Antibiotic media no. 1	5.3 ± 0.8	32.5 ± 1.5

Average of three replicates/treatment.

[a] Growth measured after 4 days.

[b] Seeding medium RM.
Cork borer size 8 mm.

4.10.3 Effect of carbon sources

Of all four tested media, since antibiotic media no. 1 was most effective, it was selected as the basal medium for further studies. To determine the effect of carbon sources on production of antifungal compound, the original carbon source of the medium (dextrose) was replaced by fructose, maltose, sucrose, sorbose, starch, galactose, mannitol or mannose. In all cases the medium was supplemented with 0.1% w/v C-source to maintain the same concentration as in the original medium. Production of antifungal metabolites in the above cases were tested by radial growth inhibition and agar cup bioassay techniques. Both bioassays confirmed maximum production of antifungal compound in the medium containing maltose as carbon source, followed by dextrose (Table 41, Fig. 15).

Table 41 : Effect of different carbon sources on the production of antifungal compound by *M. luteus*

Carbon sources[a]	Radial growth of *G. cingulata* mm)[b]	Inhibition zone in Agar cum assay (mm)[c]
Dextrose	5.5 ± 1.7	20.0 ± 2.0
Fructose	24.9 ± 2.1	11.0 ± 1.1
Maltose	4.0 ± 0.08	31.0 ± 1.7
Sucrose	27.0 ± 1.9	14.0 ± 1.9
Sorbose	27.0 ± 1.2	12.5 ± 2.0
Starch	10.0 ± 1.6	19.0 ± 2.3
Galactose	26.0 ± 1.7	17.5 ± 2.0
Mannitol	8.5 ± 1.9	20.0 ± 2.1
Mannose	25.5 ± 2.1	12.0 ± 1.9

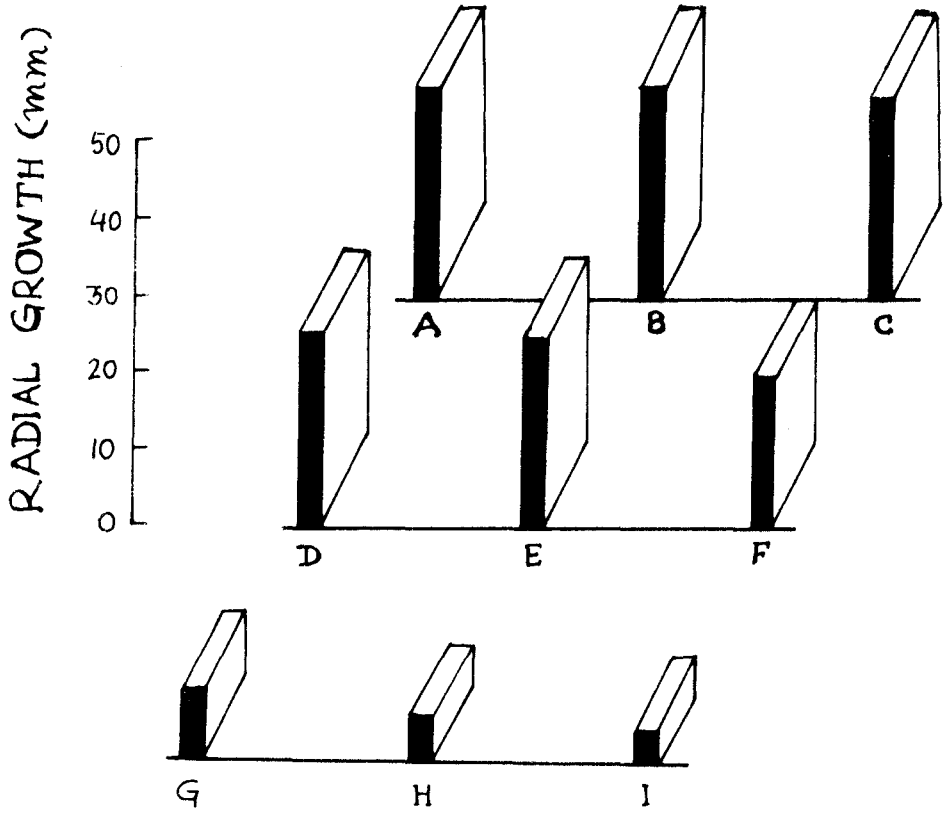
Average of three replicates/treatment.

[a] Basal medium - Antibiotic media no. 1
Concentration of carbohydrates 0.1% w/v.

[b] Growth measured after 4 days

[c] Seeding medium RM,
Cork borer size 8 mm.

EFFECT OF DIFFERENT CARBON SOURCES ON THE PRODUCTION OF ANTIFUNGAL COMPOUND(S) BY *M. luteus* ASSAYED BY RADIAL GROWTH OF *G. cingulata*



A : SORBOSE B : SUCROSE C : GALACTOSE

D : MANNOSE E : FRUCTOSE F : STARCH

G : MANNITOL H : DEXTROSE I : MALTOSE

Fig. 15

4.10.4 Effect of different pH

As pH of the medium is known to influence the production of metabolites by microorganisms, it was one of the factors considered here. For this the antibiotic media no. 1 was adjusted to different pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) with HCl or NaOH. Culture filtrates obtained from each pH was extracted separately with diethyl ether and tested for their activity. Results (Table 42) revealed that 6.5 was the optimum pH for production of antifungal compound from M. luteus. In pH 5.5, 6.0 and 7.0 also considerable amount of production was noted. Final pH in all cases tended to reach a maximum of 8.6-8.8.

Table 42 : Effect of different pH on the production of antifungal compound by M. luteus

Initial pH[a]	Radial growth of G. cingulata (mm)b	Inhibition zone in Agar cup assay (mm)c	Final pH
5.5	10.7 ± 1.8	21.5 ± 1.7	8.60
6.0	14.5 ± 1.3	21.0 ± 1.3	8.65
6.5	6.0 ± 1.7	29.0 ± 1.9	8.80
7.2	16.0 ± 1.9	17.0 ± 1.9	8.85
7.5	22.5 ± 1.7	15.0 ± 2.0	8.85
8.0	32.0 ± 2.1	12.0 ± 1.8	8.85

Average of three replicates/treatment.

[a] Basal medium - Antibiotic media no. 1

[b] Growth measured after 4 days.

[c] Seeding medium RM,
Cork borer size 8 mm.

4.10.5 Spectrum of fungitoxicity

The fungitoxicity of the compound extracted from the culture filtrate of M. luteus was also tested on fungi other than G. cingulata.

The diethylether extract was bioassayed against Bipolaris carbonum. Pestalotiopsis theae and Fusarium oxysporum by spore germination, radial growth inhibition and agar cup bioassays as described previously. Results (Table 43, Plate XIII, figs. 1-3) indicated that the compound was fungitoxic to all the tested fungi but the degree of fungitoxicity varied. It was most effective against B. carbonum followed by G. cingulata. The compound therefore exhibited a wide spectrum of fungitoxicity.

Table 43 : Spectrum of fungitoxicity of the antifungal compound produced by M. luteus

Tested against	Bioassay tests				
	Radial growth (mm)[a]		Spore germination[b]		Agar cup[c]
	Mycelial growth	% of inhibition[d]	Spore germination[b]	% of inhibition	Inhibition zone (mm)
G. cingulata	5.5±0.5	89.0	0	100	25.5±1.7
Pestalotiopsis theae	6.1±0.6	87.8	0	100	22.0±1.9
Bipolaris carbonum	4.5±0.8	91.0	0	100	27.5±1.3
Fusarium oxysporum	9.2±1.0	81.0	12.7±1.7	78.08	15.0±1.9

Average of three replicates/treatment.

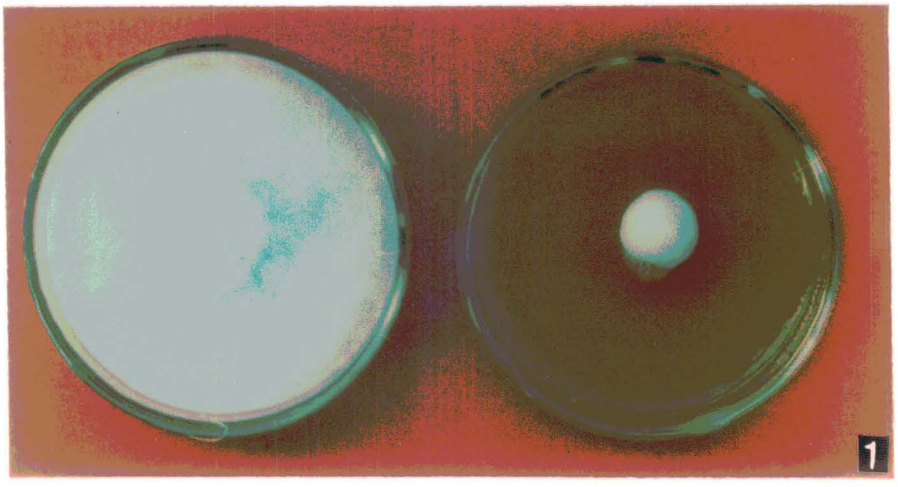
[a] Growth measured after 4 days

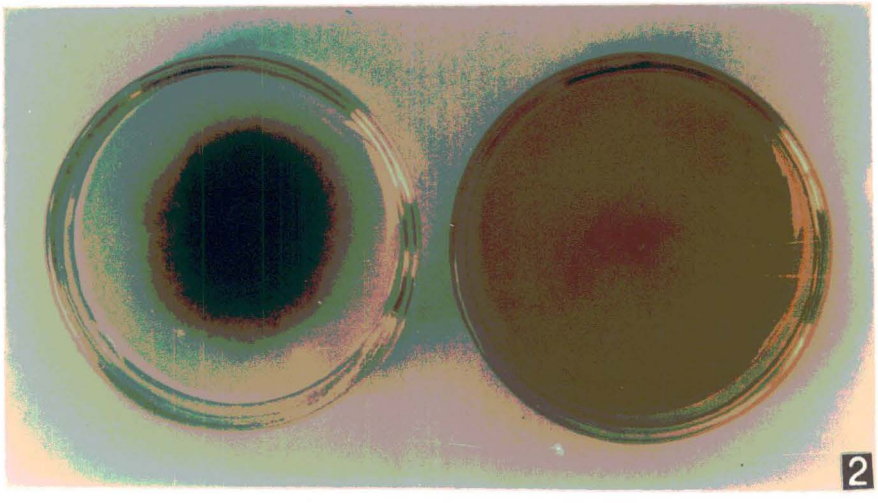
[b] Average of 200 spores

[c] Seeding medium RM,
Cork borer size 8 mm.

[d] % inhibition in relation to ethanol control.

Plate XIII (figs. 1-3)- Spectrum of fungitoxicity of the antifungal compound from M. luteus; (1) Glomerella cingulata ; (2) Bipolaris carbonum and (3) Pestalotiopsis theae, grown in RM supplemented with diethyl ether extract of M. luteus





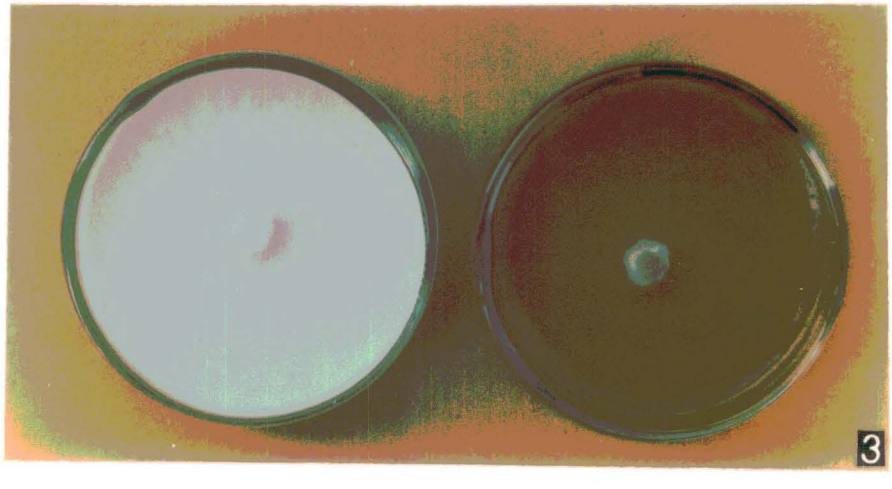


PLATE - XIII

4.11 Partial purification of active principle from M. luteus

Partial purification of active principle was carried out by Thin-Layer Chromatography followed by UV-Spectrophotometry.

4.11.1 Thin layer chromatography

The extracts were spotted on TLC plates and developed in the solvent system as described in Materials and Methods. The plates were exposed to iodine vapour. A number of brown spots appeared, of which the one with Rf 0.31 was very prominent. The silica plates were divided into nine zones and the silica gel from each zone was scraped off separately and eluted with spectrophotometric methanol. The eluates after centrifugation were bioassayed against G. cingulata by spore germination method. In fration 7 (Rf 0.31) no germination has observed while in fraction 2 (Rf 0.84) there was germination of only 1.2%. In the other fractions varying degrees of germination was observed (Table 44). No appressoria formation could be detected in any of the fractions.

Table 44 : Spore germination bioassay of eluates from Thin-Layer Chromatograms

Fractions	Rf*	% germination[a]	% appressoria formation[b]
Control[c]		72.1±3.7	83.5±2.7
1	0.96	41.0±1.7	0
2	0.84	1.2±0.04	0
3	0.76	27.6±1.3	0
4	0.64	33.1±2.3	0
5	0.54	24.7±2.1	0
6	0.45	12.1±1.6	0
7	0.31	0	0
8	0.19	52.5±1.2	0
9	0.05	43.0±1.0	0

* Rf in solvent system - Benzene : ethanol 6:1

[a] Based on 200 spores, [b] Average of 50 germlings,

[c] Control in spectral methanol.

4.11.2 UV-Spectrophotometry

Since fraction 7 was most fungitoxic it was further analysed by UV-Spectrophotometry along with the crude diethylether extract. Two peaks with absorption maxima at 220 nm and 292 nm appeared in both crude as well as the TLC eluate (Fig. 16). In the partially purified extract (TLC eluate at fraction 7) the peak at 292 nm was very sharp indicating the possibility of it being the absorption maximum of the antifungal compound.

4.12 *In vivo* test with the partially purified compound from *M. luteus*

After partial purification of the antifungal compound, it was decided to test its activity *in vivo* to determine whether it could reduce disease intensity. For the *in vivo* tests 2 ml of the extract was suspended in 10 ml of sterile distilled water for each spray. In case of control, spraying was done with sterile distilled water. After two sprays at 48h interval leaves or twigs of susceptible varieties (TV-18 and TV-23) were brought to the laboratory (after 24h of the second spray) and inoculated with *G. cingulata* by detached leaf or cut shoot inoculation methods. A set was also prepared by inoculating whole plant with spore suspension of *G. cingulata*, after spray with the test solution.

4.12.1 Cut shoot inoculation

In cut shoot inoculation technique disease intensity was assessed after 24, 48 and 72h of inoculation with *G. cingulata*. Disease intensity was significantly reduced by the semi-purified extract (Table 45). After 72h of inoculation mean disease index per shoot was 4.60 in control but only 0.23 in the treated leaves (TV-18).

UV-SPECTRA OF ANTIFUNGAL COMPOUND EXTRACTED FROM M. luteus

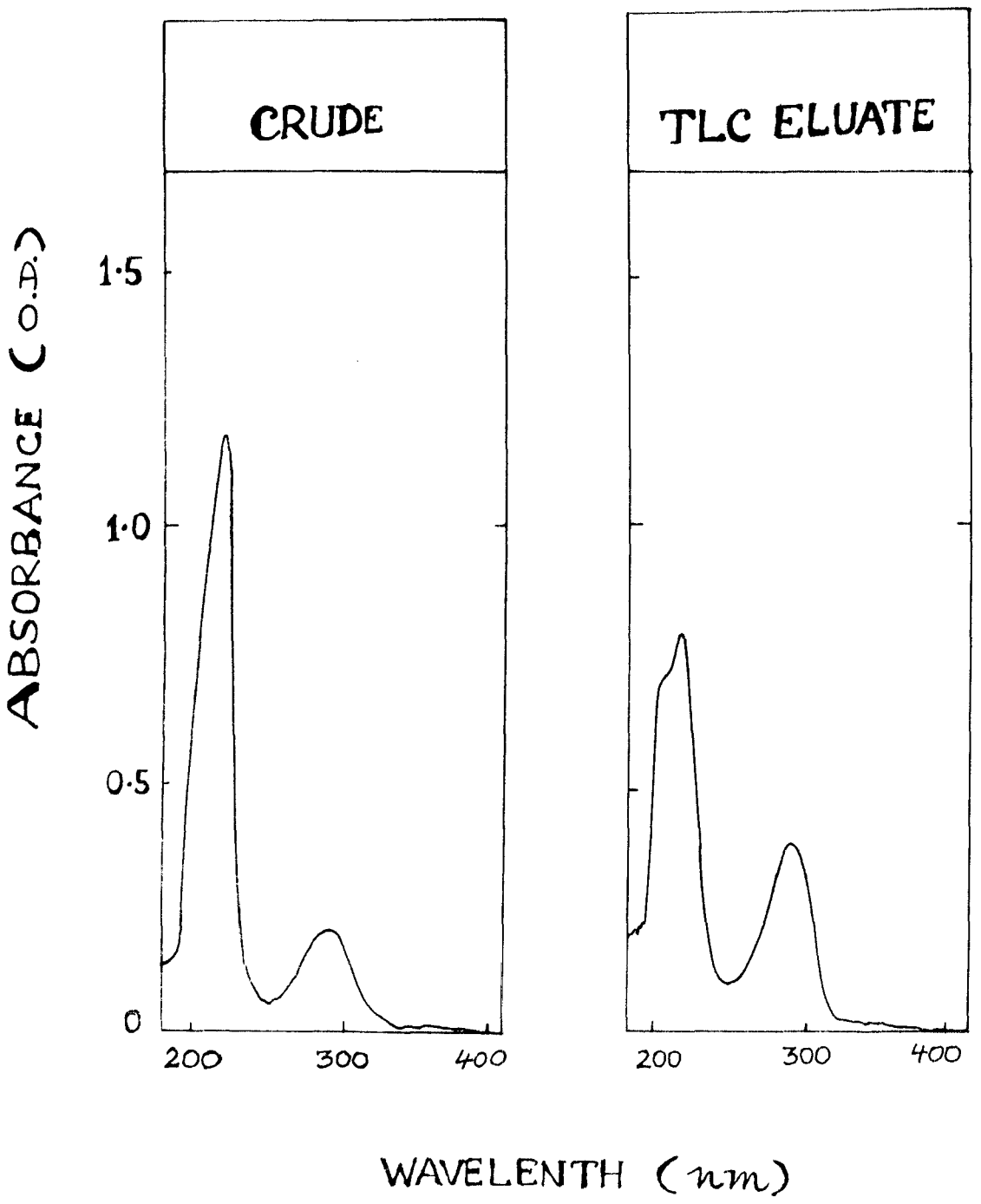


Fig. 16

Table 45 : **Effect of partially purified antifungal compound from M. luteus on disese development by G. cingulata (cut-shoot inoculation)**

Variety	Treatment	Mean number of lesion /shoot			Mean disease index/ shoot		
		24h	48h	72h	24h	48h	72h
TV-18	Control	7.00	10.00	14.00	1.50	2.80	4.50
	Treated[b]	0	2.00	4.00	0	0.06	0.23
TV-23	Control	5.00	7.00	9.00	1.00	1.66	2.33
	Treated[b]	0	2.00	3.00	0	0.16	0.20

[a] Average of 50 twigs per treatment

[b] Sprayed twice at 48h interval.

[c] Experiment set after 24h of second spray.

4.12.2 Detached leaf inoculation

Percentage lesion formed by G. cingulata on detached leaves after 48, 72 and 96h of inoculation was determined in both control and treated leaves. Spraying with the semipurified extract resulted in significant reduction in lesion production (Table 46). Percentage

Table 46 : **Effect of partially purified antifungal compound from M. luteus on disease development by G. cingulata (detached leaf inoculation)**

Variety	Treatment	% lesion production after[a]		
		48h	72h	96h
TV-18	Control	87.5±3.9	93.3±2.6	97.2±3.3
	Treated [b]	30.0±1.2	33.5±1.9	35.2±2.7
TV-23	Control	71.3±2.6	80.2±2.8	84.2±4.1
	Treated [b]	21.1±1.8	23.2±1.0	27.0±2.5

a Average of 50 leaves per treatment.
b Sprayed twice at 48h interval.
c Experiment set after 24h of second spray.

lesion production after 96h of inoculation was 35.22% in case of the treated leaves in comparison to 97.2% in control. These results clearly indicate the effectiveness of the partially purified antifungal compound in disease reduction.

4.12.3 Whole plant inoculation

Leaves sprayed with the semi-purified extract and inoculated later with G. cingulata, failed to develop any significant symptom, in comparison with the naturally infected plants (Plate XIV, figs. 1&2).

Plate XIV (fig.1)- Tea plants (TV-18) infected with G.cingulata; (fig.2) leaves sprayed with semipurified extract from M. luteus priorto inoculation with G. cingulata

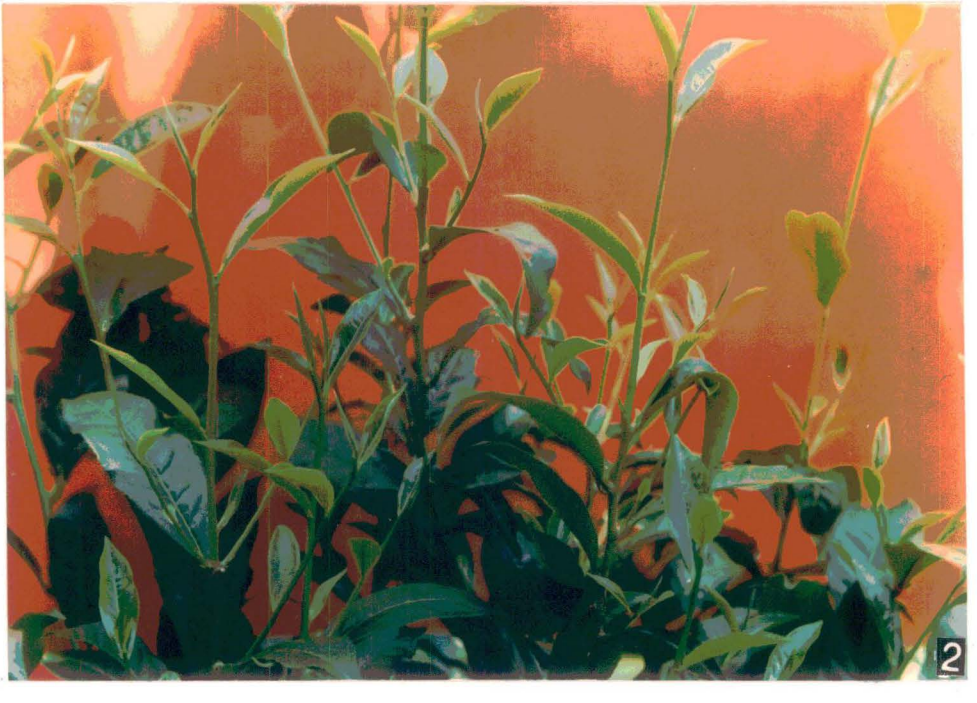


PLATE - XIV

D I S C U S S I O N

The invasion of host tissues by pathogen initiates a complex and variable developmental pattern between host and parasite which continues throughout their course of co-existence. Differences in the physiological responses and morphological structures of various host genotypes affect their susceptibility or resistance to invasion and its consequences, while similar variation in pathogens influence their growth rate and virulence (Loomis and Adams, 1983). In nature, a multitude of potential invaders are present in the plant environment. In spite of this, disease is still the exception rather than the rule, which indicates that plants are able to successfully ward off most of the invaders with the help of their well developed defense systems. Defense by the host generally begins at the perimeter i.e., the outer walls of plant surfaces including the cuticles, trichomes, the epidermis, the cells lining the stomatal and lenticel cavities, cellulose, hemicellulose, lignin and suberin of the peripheral walls (Cowling and Horsfall, 1980). More importantly, plants have effective internal defense mechanism against any form of stress - whether biotic or abiotic, which fall under two categories, "preformed" and "induced". The induced defense systems have been termed as 'alarms', which, according to Chessin and Zipf (1990) is a complex physiological phenomenon including a series of steps i.e., perturbation by particular biotic or abiotic stress, followed by production in the stressed tissue of a systematically transmitted signal (electrical, chemical or both) and finally induction of a new morphological or physiological state which protects such tissues from subsequent exposure to the same or other stresses.

In the present study, at the very beginning, pathogenicity test of the brown blight pathogen G. cingulata was carried out on ten different varieties of tea released by Tocklai Experimental Station. All the three inoculation techniques followed (whole plant, cut shoot and detached leaf) exhibited results which were in agreement with each other. TV-18 was the most susceptible to G. cingulata followed by TV-23, while TV-26 was the most resistant. The cut shoot method to detect

resistance of tea plants to grey blight disease caused by Pestalopsis longiseta was reported by Yanase and Takeda (1987). Studies on pathogenicity are extremely useful for several purpose, mainly in understanding more clearly the mechanism of disease development and the way and degree to which host plant performance is altered (Loomis and Adams, 1983).

Fungal plant pathogens invade host plant cells with variety of specialized infection structures of which, for most fungi, the appressorium is developmentally the first and most important infection structure formed in preparation of host colonization (Hoch and Staples, 1987). It must be positioned at an appropriate site on the host in the most advantageous way for subsequent infection to occur and it must be able to withstand adverse environmental conditions. For G. cingulata, which is able to infect only through wounds (Baxter, 1974; Bertus, 1974; Dickens and Cook, 1989), the positioning of an appressorium assumes great significance. Similarly in many rust fungi positioning of the appressorium is most critical because invasion of the host can occur only via the stomata (Littlefield and Heath, 1979; Staples and Macko, 1984). Ando and Hamaya (1986) reported that the anthracnose fungus Gloeosporium theae-sinensis could infect tea only through the trichomes of young leaves. In many cases this pathogen was inhibited from gaining entrance by a callosity which was produced by swelling of the trichome cell-wall inward in a way that enveloped and preceded the invading hypha. Formation of appressoria being the first step in establishing the disease, the factors affecting this process are of vital importance in deciding the fate of the pathogen in initial stages (Purkayastha and Menon, 1981). Aerial plant parts generally influence the behaviour of microorganism on their surface (Dickinson and Preece, 1976). In view of the importance of spore germination and appressoria formation in the establishment of disease, the effect of some important factors on spore germination, appressoria formation and disease development in tea leaves by G. cingulata has been investigated. A temperature of 25°C, pH 5.0, incubation period of 24h, 7-8h light

period and a concentration of 1.2 x 10^6 conidia/ml from 10-day old culture, were found to be optimum for both spore germination and appressoria formation. Maximum lesion production was also obtained at an inoculum concentration of 1.2 x 10^6 conidia/ml and in diffused light. There is evidence that number of appressoria of Puccinia helianthi decreased with increased light intensity but this was overcome by exposing it to alternate periods of light and dark (Sood and Sackston, 1972). In this investigation, optimum conditions necessary for growth of G. cingulata were also tested in vitro. Maximum growth occurred at an incubation period of 10 days, pH 6.5 and using maltose and potassium nitrate as carbon and nitrogen sources, respectively.

During host-parasite interaction, even when coincidence of location and time is provided and supplemented with optimum conditions for microbial development, most fungi just can not establish a parasitic relationship, or if they do so, it is only in plant tissue weakened by stress condition (Schlösser, 1980). The basis for this high level of resistance against microbial attack is a plant's chemical defense consisting of both preformed and post-infectional ones. Preformed defenses are often regarded as general or unspecific as compared to inducible defense systems which are highly specific. In the present investigation preformed as well as post-infectionally formed compounds (exudates and diffusates, respectively) from the susceptible and resistant varieties were tested against G. cingulata by spore germination assay. Both exudates and diffusates showed fungitoxicity, indicating that tea leaves contained preformed as well as post-infectionally formed antifungal compounds. The antifungal activity of the exudates was not specific with regard to resistance and susceptibility, but diffusates of the resistant varieties were much more effective than those of the susceptible ones. Schneider and Sinclair (1975) also found that young cowpea leaves which were resistant to Cercospora leaf spot, possessed toxic substances in leaf

diffusates. Suppression of appressoria formation of Colletotrichum gloeosporoides on resistant jute leaves has been reported by Purkayastha and Menon (1981). It was reported by Chakraborty and Saha (1989) that tea leaf diffusates from varieties susceptible to Bipolaris carbonum were more antifungal than those from the resistant variety. Nicholson et al. (1989) demonstrated that phenolic compounds inhibitory to germination of spores of Colletotrichum graminicola were leached out of the necrotic lesions of corn leaves by the fungus. The dynamic biochemical reactions activated in the host following penetration by the pathogen into its tissues are therefore, very significant in determining the success of the pathogen.

Among the many biochemical changes occurring in host tissues following fungal invasion, the one which is of most universal occurrence is the change in phenolics. Phenolic compounds accumulate in most plant species as a result of infection with plant pathogens (Friend, 1977; Bazzalo et al., 1985; Baker et al., 1989; Mahadevan, 1991). Many of the phenols in plants, on the other hand, occur constitutively and are thought to function as preformed inhibitors associated with non-specific resistance (Schonbeck and Schlosser, 1976; Stoessl, 1983; Mansfield, 1983). Heath (1980) has suggested that differentiation of the responses of the plants to pathogen is based on host and non-host interactions. In such relationship, it has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid cell death (Fernandez and Heath, 1989). Rapid accumulation of phenols at the infection site may result in the effective isolation of the pathogen or non-pathogen at the original site of ingress. In this investigation, the presence of both preformed and post-infectionally formed diffusible antifungal compounds (exudates and diffusates) has been demonstrated previously. Since polyphenols are major constituents of tea leaves, their involvement in the defense mechanism seemed highly probable. To explore this possibility, the levels of phenolics in leaves of

resistant and susceptible tea varieties were estimated before and after inoculation with G. cingulata. Host responses could be differentiated by changes in the content of phenolic compounds. In the susceptible varieties (TV-18, TV-23 and TV-25) both total and orthodihydroxyphenol contents decreased following inoculation with G. cingulata while there was an increase in the phenol content in the resistant varieties (TV-26, CP-1 and Teen Ali 17/1/54). These results are in conformity with the findings of previous workers who have also recorded similar results. Sridhar and Ou (1974) reported differences in total phenolics accumulation in the interaction of Pyricularia oryzae with rice. Accumulation of more phenol in the resistant rice cultivar as against the susceptible one following inoculation with Xanthomonas campestris pv. oryzae was reported by Purushothaman (1974). A clear difference between resistant and susceptible interactions of maize to Colletotrichum graminicola on the basis of phenol accumulation has been recorded (Hammerschmidt and Nicholson, 1977). Similarly, Borkar and Verma (1991) also reported that cotton cultivars accumulated greater amount of total and orthodihydroxyphenol in resistant interaction. On the other hand, there are also reports that phenolics accumulate after infection in both compatible and incompatible interaction. In maize, a marked accumulation of two caffeic acid esters after inoculation with C. graminicola or Cochliobolus heterostrophus in both compatible and incompatible combination has been reported (Lyons et al., 1990). Greater accumulation of phenolic compounds in resistant interaction of G. cingulata and tea varieties indicated that this may play a role in resistances of tea plants against this pathogen. It has been reported by Chakraborty et al. (1995) that among several phenolics detected in tea leaf extracts, catechin was antifungal in nature. They concluded, on the basis of further experimental evidences that catechin is broken down to simpler phenols leading to their accumulation following inoculation. Catechol which was also demonstrated to be antifungal, accumulated in a greater amount in the resistant varieties than in the susceptible ones after inoculation.

Besides phenolic compounds, proteins are also known to play a part in the host-pathogen interaction. Changes in protein content of resistant and susceptible tea varieties following inoculation with G. cingulata has been determined in this study. Protein content of all the varieties increased significantly in the infected leaves in comparison of healthy ones with no significant differences between resistant and susceptible varieties. Increased protein levels after infection of susceptible varieties by pathogens has been reported by several workers (Daly, 1972; Ouchi, 1974).

An important phenomenon in host-pathogen interaction is the fact that host cells "recognize" some common component of the microorganisms that they come into contact with and then respond to it in a predictable manner (Sequeira, 1980). Cell recognition has been defined as "the initial event of cell-cell communication which elicits a defined biochemical, physiological or morphological response" (Clarke and Knox, 1978). In the incompatible interaction between plant and microorganism the early recognition event generally leads to the activation of plant defense mechanisms. Surface molecules of eukaryote cells have been involved in cell-cell recognition and/or adhesion and as receptors for various effectors (Albersheim and Anderson-Prouty, 1975; Cunningham, 1977; Snary and Hudson, 1979). Many of these specificity imparting molecules are glycoproteins, and fungi are known to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980; Beissmann, 1992; Ransom et al., 1992). In this study, as G. cingulata was found to elicit diffusion of greater amount of antifungal compound in the resistant varieties, and previous reports indicate the involvement of cell wall glycoproteins as elicitors, it was considered worthwhile to isolate and extract cell walls from mycelia, determine their role in host response and elicitation of antifungal compound and, finally to determine the chemical nature of the mycelial wall extract. Characterization of mycelial wall extract by SDS-PAGE and ConA-FITC binding showed it to be glycoproteinaceous in

nature. Two glycoprotein bands of molecular weights 10 and 50 KD were detected in the extract. When these elicitors were spotted on the tea leaves of resistant variety (TV-26) they elicited a similar response to that shown by the spore suspension. Results of other experiments also revealed that the mycelial wall extract could elicit the formation of antifungal compound in tea leaves. Keen and Legrand (1980) isolated two surface glycoproteins of molecular weights 14 and 34 KD from the cell-wall of Phytophthora megasperma f. sp. glycinea. These glycoproteins were shown to function as phytoalexin elicitors. Ricci et al. (1992) demonstrated that P. parasitica isolates could be differentiated on the basis of their ability to produce a proteinaceous elicitor active on tobacco and of their pathogenicity to tobacco and that there is relationship between these two properties. Kumar et al. (1993) also isolated and characterized mycelial wall polysaccharide from Drechslera longistrata which could elicit the production of glycollins which were determinants of resistant reactions. Results of this investigation, along with those of other workers clearly demonstrate that the cell-walls of the fungi contain glycoproteins which have a role to play in the initial cell recognition leading to the activation of the defense mechanisms.

The host-pathogen interaction is influenced not only by the host and pathogen factors discussed above but also by the epiphytic microorganisms occurring on the leaf surfaces. A thorough understanding of the establishment of the disease by the pathogen would be complete only by a knowledge of the microbial communities occurring on the plant surfaces. Keeping this in mind, in the present investigation, further studies were conducted on the phylloplane microorganisms of tea and their interaction with G. cingulata. In an extensive survey of the tea estates of Dooars and Darjeeling region phylloplane microorganisms were isolated from tea leaves of over twenty estates. These microorganisms included fungi, bacteria and actinomycetes. The isolated microorganisms were identified. The most common of these were Acremonium fusidioides,

Alternaria alternata, A. solani, Bipolaris carbonum, Cochliobolus sativus, Colletotrichum gloeosporioides, Curvularia lunata, Fusarium oxysporum, F. solani, Pestalotiopsis theae, Rhyzopus stolonifer, Phoma exigua, Aspergillus flavus, A. fumigatus, A. nidulans, A. niger, A. terreus, Penicillium oxalicum, P. simplicissium, P. frequentans, Bacillus cereus, B. pumilus, Bacillus sp., Micrococcus sp., Micrococcus luteus and Coryneform bacterium.Bacteria, yeasts and filamentous fungi have been reported to form resident populations on leaves (Blakeman and Fokkema, 1982). Of the bacteria, most are Gram-negative, often chromogenic and include Erwinia, Pseudomonas, Xanthomonas and Flavobacterium, Gram-positive bacteria such as Lactobacillus, Bacillus and Corynebacterium are isolated less frequently. In addition to saprophytic bacteria, pathogenic bacteria such as P.syringae pv. syringae, P. syringae pv. morsprunorum, P. glycinea, E. amylovora and E. carotovora can live on a non-pathogenic epiphytic phase on foliar surface (Blakeman and Brodie, 1976) . Blakeman and Fokkema (1982) have suggested that bacteria often tend to be relatively more abundant on leaves early in the growing season. Spores of filamentous fungi such as Alternaria, Epicoccum and Cladosporium land on leaves throughout the growing season but they generally germinate to form colonies only towards end of the season when leaf senescence begins (Preece and Dickinson, 1971; Blakeman, 1981). The phyllosphere being a dynamic environment with cyclic and non-cyclic environmental variables, substantial variation can occur in time and space even on a scale appropriate to microorganisms (Andrews, 1992). The original sequence in colonization of the phylloplane is therefore a function of available inocula, the environment and host phenology. The colonization pattern on individual leaves is localized and heterogenous. Pattern of colonization may,however, be specific at least to class of microbes. When leaves of bean plants in a growth chamber were coinoculated with yeast and bacteria, a distinctive pattern emerged after 30h in which the yeasts colonized anticlinal walls mainly over the lamina, while the bacteria were concentrated on anticlinal walls of lamina along veins, stomatal pores and about glands (Blakeman, 1985).

The isolated microorganisms in this study were tested against G. cingulata for antagonistic interaction in vitro. Several fungi and bacteria were antagonistic to G. cingulata, out of which four were selected as the most antagonistic. These were Micrococcus luteus (359392), Bacillus cereus (359386), Bacillus sp. (359388), and one member of Coryneform group (359393). Similar antagonistic behaviour of different bacteria have been reported by several previous workers. Cubeta et al. (1985) isolated Bacillus subtilis from soybean and tested it for antagonism against 26 fungi commonly associated with soybean seeds in dual culture on potato-dextrose-agar. The bacteria were fungicidal to Penicillium sp.and fungistatic to all others. Two bacterial isolates, B. cereus and Erwinia herbicola were found to be antagonistic to Ulocladium botrytis, the causal agent of tomato leaf spot (Zaher et al., 1985a). Koomen and Jeffries (1993) also selected B. cereus and Pseudomonas flouroscences out of 121 organisms tested against Colletotrichum gloeosporioides. On the basis of antagonistic tests in vitro, Walker et al. (1994) reported that five Bacillus isolates showed distinct antagonism to two tested pathogens, Pythium mamillatum and Botrytis cinerea. E. herbicola was selected as a potential antagonist against Leptosphaeria maculans on the basis of in vitro reactions (Chakraborty et al., 1994), again supported antagonistic behaviour of phylloplane bacteria.

Following in vitro studies, selected microorganisms were tested in vivo for reduction of brown-blight disease. For in vivo tests, aqueous cell suspension, bacterial cell free culture filtrates and washed cells were used as foliar sprays. In all cases, the cell-free culture filtrates were the most effective in disease reduction followed by the aqueous cell suspensions. These results are substantiated by the works of several previous authors who have obtained inhibition in pathogen growth as well as disease reduction by the culture filtrates of antagonistic microorganisms. Roveratti (1989) showed that suspensions of Saccharomyces cerevisiae significantly reduced the germination of Hemileia vastatrix uredinospores on coffee leaves and under in vitro conditions although appressoria formation in vivo was

not affected. Application of crude filtrates on alfalfa leaflets inoculated with Colletotrichum trifolii was associated with reduced germination of conidia, lysis of conidia and reduced formation of appressoria. Fully sporulated cultures of B. cereus and sterilized filtrates of these cultures were effective in protecting alfalfa seedlings against damping off (Handelsman et al., 1990). A cell-free culture filtrate of Bacillus subtilis was reported to significantly reduce disease incidence and severity on alfalfa seedlings from 56% - 16% and from 2.0 - 1.2 respectively, although treatment of seedlings with washed cell suspension had no influence on disease (Douville and Boland, 1992). El-Abyad et al. (1993) reported that 80% concentration of culture filtrate of Streptomyces pulcher or S. canescens significantly inhibited spore germination, mycelial growth and sporulation of Fusarium oxysporum f. sp. lycopersici, Verticillium albo-atrum and Alternaria solani. Culture filtrates were effective in reducing disease severity in vivo. Suspensions from washed or non-washed Saccharomyces cerevisiae cells and filtrates of this suspension reduced the development of Colletotrichum graminicola as well as the expression of anthracnose of maize leaves when they were previously or concomitantly treated with these preparations (Dasilva and Pascholati, 1992). The authors attributed the reduction of the development of Colletotrichum graminicola and disease expression on the leaves by filtrates of cell suspension of S. cerevisiae to a thermolabile substance or complex of substances released from the cells into the filtrates.

Different ratios of the antagonists and G. cingulata on the tea leaf surface (TV-18) also influenced the activity of the antagonists. The inhibitory effect of all the four tested microorganisms increased with increase in their concentration. Minimum disease development occurred at the highest ratio of antagonists and pathogen tested 50:1 . Similar results have also been reported by previous authors. Sekhawat and Chakraborty (1977) reported 100% control of bacterial leaf spot of chilli when antagonistic bacteria were mixed with Xanthomonas

vesicatoria in the ratio 16:1, 32:1 and 64:1. It has also been reported that Erwinia herbicola controlled Xanthomonas oryzae pv. oryzae on rice most effectively even at lowest ratio 1:1 and registered more than 90% reduction in disease development at 50:1.

The antagonistic interaction between the phylloplane microflora and pathogen may be brought about by different mechanisms including parasitism, nutrient competition, antibiotic production or interaction with the host (Blakeman and Fokkema, 1982). Some of the saporphytes are hyperparasites and they may affect plant pathogens in two main ways - either by penetration of fungal tissues and production of metabolic substances which result in destruction by lysis of spores, sori or hyphae; or by displacement of tissues of the pathogens within pustules or by the formation of crusts of mycelium which overlay fruiting structures (Barnett, 1963). Ampelomyces telliopsis and Stephanoascus species are hyperparasites and have been reported to infect and kill many mildew pathogens including Sphaerotheca fulliginea and Erysiphe cichoracearum that attack cucumbers (Sutton and Peng, 1993). On the other hand, microorganisms may compete with each other for the available nutrients. It is well known that intraspecific and interspecific competition is one of the most important factors determining the density of population in nature (Schroth and Hancock, 1981). Water films on leaf surfaces often lead to low levels of available nutrients. Bacteria and yeasts, partly because of their more favourable surface to volume ratio are able to take up nutrients from the dilute solution of an infection droplet more rapidly and in greater quantity than the germtubes of fungal pathogens. The exogenous nutrients available to the pathogen are thus reduced and some of the endogenous nutrient reserves leaked from the germinating spore are also preferentially absorbed by the competing epiphytes surrounding the spores (Brodie and Blakeman, 1975). Under these conditions, spores of fungi such as Botrytis cinerea, Colletotrichum herbarum or Phoma betae either do not germinate or germinate poorly, failing to give rise to infections. The most important mechanism of antagonistic reaction is perhaps that of production of antibiotics by such microorganisms which

inhibit the growth of pathogens. Several saprophytic bacteria produce antibiotics capable of inhibiting the growth of pathogens (Douville and Boland, 1992; Koomen and Jeffries, 1993; Hodges *et al.*, 1993; Chakraborty *et al.*, 1994a). Kemp and Wolf (1989) attributed part of the *in vivo* activity of *Erwinia herbicola* against *Fusarium culmorum* to antibiosis. They suggested that competition between *F. culmorum* and *F. herbicola* may also play a role in the antagonistic interaction.

Finally, bacteria are also well known to induce protective responses in host tissues. Live cells of avirulent strains of the pathogen, heat killed cells or cell-free extract, as well as non-pathogen have been used to induce protective responses. Martins *et al.* (1986) reported that filtrates of *S. cerevisiae* extracts although not affecting uredinospore germination nor appressorium formation, caused a reduction in the expression of symptom by *Hemileia vastatrix* on coffee leaves. The possible activation of resistance mechanisms in coffee leaves have been suggested to be responsible for this reduction. *S. cerevisiae* extract has been shown to be a powerful elicitor of glyceollin accumulation in soybean tissues and it has been involved in disease resistance (Hahn and Albersheim, 1978).

In the present investigation, the possible mechanism of antagonistic interaction between the isolated bacteria and *G. cingulata* have been investigated. Mycoparasitism was ruled out in the very beginning as prominent inhibition zones had appeared in all *in vitro* tests on solid medium. To determine whether the bacteria secrete antifungal compounds into the culture medium, the cell free culture filtrates were bioassayed against *G. cingulata*. Inhibition in spore germination of *G. cingulata* by the culture filtrates indicated that these contained some toxic metabolites which are fungitoxic. These compounds were partly thermolabile as they lost some of their activity by heat sterilization. Results of replacement culture media test further confirmed that the observed reduction of growth of *G. cingulata*

in presence of the antagonistic bacteria was not due to competition for nutrients but due to the presence of antifungal compound(s) in the culture filtrates.

Having established that all the four antagonistic bacteria secrete antifungal compounds into culture, in the next step, attempts were made at partial purification of these compounds. The cell-free culture filtrates were accordingly extracted with different solvents - acetone, benzene, chloroform, diethylether or ehtylacetate and each extract was bioassayed separately against G. cingulata.

Results revealed that the compounds from different microorganisms had different solubilities - the one from M. luteus and B. cereus being most soluble in diethylether, the one from Bacillus species in ethylacetate and the compound from the Coryneform bacterium was most soluble in chloroform. In all cases, serial dilution of these extracts reduced their activity. Purkayastha and Bhattacharyya (1982) reported the presence of an ethylacetatesoluble partially thermolabile antifungal substance in the culture filtrate of Bacillus megaterium which was antagonistic to Colletotrichum corchori on jute. Mc.Keen et al (1986) have also reported that the culture filtrate extracts from B. subtilis contained antifungal compounds that were soluble in ethanol, methanol, isopropanol and water above pH 7.5, but not soluble in ethyl acetate, acetone, ether or methylene chloride.

Among the four antagonistic microorganisms tested so far, B. cereus and M. luteus seemed to be the most effective on the basis of all the in vitro and in vivo tests. Out of these two, M. luteus was selected for further studies on production of antibiotics and its probable use as a biocontrol agent against G. cingulata. There have been reports that some strains of B. cereus may be involved in food toxicity (Koomen and Jeffries, 1993). Use of this bacterium or its

products, as foliar sprays on tea leaves for bio-control was, therefore, considered unsuitable. Since the production of any metabolite by a microorganism is dependent on several factors, the optimum production would be obtained at specific combinations of these factors. Hence, experiments were conducted in this study to optimize factors for the maximum production of the antifungal compound. The factors considered here were different types of growth medium, pH of the medium, incubation period and different carbon sources. Maximum production was obtained after 96h of incubation of M. luteus, at pH 6.5, in antibiotic media no. 1 and using maltose as carbon source. Roitmann et al. (1990) reported that phenylpyrrole antibiotics produced by Pseudomonas cepacia were greatly affected by choice of culture medium and incubation time. Concentrations of the two nitrophenyl metabolites, pyrrolinitrin and 2-chloropyrrolnitrin rose throughout the 7-days incubation and were more than twenty times greater in Minimum salt medium than that in either King's B or Nutrient broth.

An antibiotics producing strain of B. subtilis was shown to produce antifungal volatiles (Fiddaman and Rossall, 1994). The ability of these compounds were enhanced with the addition of D-glucose, complex carbohydrates and peptones. The addition of L-glucose led to significantly less activity than comparable levels of D-glucose. They also reported that the antifungal volatiles produced by the strain of B. subtilis possessed activity against a wide range of fungi from different taxonomic groups. Some genera such as Pythium and Fusarium were relatively uninhibited. Moore-Landecker and Stotzky (1972) also found considerable variation in the inhibitory effect of the bacteria they tested. A strain of B. cereus produced fungistatic volatiles that were considerably more inhibitory to a strain of Trichoderma viride than a strain of Gelasinospora cerealis. Similarly, a strain of Aerobacter aerogens produced volatiles that were more active against Fusarium oxysporum f. sp. conglutinans. In the present investigation also, the antifungal compound from M. luteus exhibited different

degrees of inhibitory activity against the fungi tested. Besides G. cingulata it was effective on Bipolaris carbonum, the causal agent of leaf spot of tea, Pestalotiopsis theae, the causal agent of grey blight disease of tea and F. oxysporum.

For partial purification of the compound, Thin Layer Chromatography (TLC) was performed. Bioassay of the eluates from different zones revealed the antifungal compound to be present predominantly in fraction number 7 (Rf 0.31) and fraction number 2 (Rf 0.84). This was followed by UV-Spectrophotometric analysis of the crude diethylether extract as well as eluate from the TLC plate (fraction number 7). In studies on the production and partial characterization of antifungal substance antagonistic to Monilinia fructicola from B. subtilis Mc.Keen et al. (1986) found that when the crude extract was developed in TLC, it separated into four biologically active bands which inhibited M. fructicola. The bands with Rf values of 0.48 and 0.55 were ninhydrin positive while the ones at 0.60 and 0.67 were not.

In this study, it has been established that M. luteus is antagonistic to G. cingulata and the mechanism of antagonism has been shown to be by production of antifungal metabolite. This microorganism being a naturally occurring resident on the tea leaf surface has become adapted to survive and grow in this habitat. It is well known that if such organisms have been found to possess an effective antagonistic action against a pathogen, then their use for biological control purpose should be preferred to organisms from other habitats which may be equally antagonistic to the pathogen (Blakeman and Fokkema, 1982). The latter would be less likely to survive for as long in the phylloplane. In this respect M. luteus seems to be a good choice as bio-control agent. Besides, instances where antibiotics production has been identified as a main cause of antagonism by a saprophyte, a cell-free culture filtrate or semipurified antibiotics production may be used for biocontrol purpose as opposed to using a live inoculum. This

procedure may be more advantageous if the antagonists fail to effectively colonize host surfaces. Keeping this in mind, the semipurified extract from M. luteus was sprayed on tea leaves and inoculated later with G. cingulata. Development of disease was effectively controlled by the semipurified extract.

It can be generalized from the aforesaid considerations that the final appearance of disease on a host is dependent on a multitude of factors. Detailed studies on the brown blight disease and its causal agent, G. cingulata have shown that the pathogen is influenced by a number of environmental factors, biochemical responses of the host as well as on its interaction with the phylloplane microorganisms present on the leaf surface. As an outcome of this study, a phyllosphere antagonist has been selected which could be used as a potential biocontrol agent against tea pathogens. Biocontrol in the phyllosphere has not yet met with outstanding success so far, but the use of introduced antagonistic microorganism, for control of plant pathogens has attracted worldwide attention over the past few decades (Cook, 1993). The heightened scientific interest in biological control of plant pathogens is a response, in part, to growing public concerns over chemical pesticides. Biocontrol organisms present a unique challenge of the need for mass quantities of "competent" inoculum which is compatible with existing storage and application technology (Lisansky and Hall, 1983). the application of semipurified extracts where possible also needs considration. Possibly the biocontrol component of the phylloplane community could be selctively enhanced by recent techniques such as microencapsulation (Posillico, 1986) or bio-adhesives (Strauberg et al., 1989). Alternatively since host genotypes can influence epiphytic populations it may be possible to develop varieties of plants which support an antagonistic leaf epiflora or endoflora (Cullen and Andrews, 1986). However, the primary requisite for all these would be a thorough understanding of the host-pathogen interaction at all levels.

S U M M A R Y

(1) A review of literature pertaining to this investigation has been presented which deals mainly with phenolics in plant tissue in relation to disease development, screening of phyllosphere microflora and biological control of foliar diseases.

(2) Materials used in this investigation and experimental procedures followed have been discussed in detail.

(3) Pathogenicity of G. cingulata was tested on ten varieties of tea (TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, Teen Ali 17/1/54 and CP-1). TV-18 and TV-26 were found to be the most susceptible and resistant varieties, respectively.

(4) Maximum growth of G. cingulata occurred after 10 days of incubation and at pH of 6.5.

(5) Maltose was the most effective carbon source where optimum growth of G. cingulata was recorded. As a nitrogen source, potassium nitrate was the most effective.

(6) A temperature of 25°C, pH 5.0, incubation period of 24h, 7-8h light period and concentration of 1.2×10^6 conidia/ml from 10-day old culture were optimum for both spore germination and appressoria formation.

(7) Spore germination and appressoria formation on the glass-surface and leaf surface of susceptible variety (TV-18) were similar, whereas on the leaf surface of resistant variety (TV-26) it was significantly lower.

(8) Maximum lesion production was obtained at an inoculum concentration of 1.2×10^6 conidia/ml and in diffused light.

(9) Both leaf exudates and diffusates collected from resistant and susceptible varieties were fungitoxic. Diffusates from resistant varieties were four to five times more fungitoxic than the ones from susceptible varieties.

(10) Phenolics in healthy leaves were found to be protocatechuic acid, gallic acid, catechol, caffeic acid and p-coumaric acid. the levels of total and orthodihydroxyphenols in both healthy and G. cingulata inoculated tea leaves of resistant and susceptible varieties were determined.

(11) Both total and orthodihydroxyphenol contents of susceptible varieties decreased following inoculation with G. cingulata while there was increase in both cases in resistant varieties.

(12) Protein contents of all tested varieties (both susceptible and resistant) increased significantly in the G. cingulata infected leaves in comparison to the healthy ones.

(13) G. cingulata had a protein content of 37.5 mg/g fresh wt. SDS-PAGE analysis of mycelial protein revealed seventeen bands ranging in molecular weight from 27 to 210 KD.

(14) Cell walls from G. cingulata were isolated, extracted and characterized by SDS-PAGE. Two glycoproteins of molecular weights 10 and 50 KD were detected in the mycelial wall extract.

(15) ConA-FITC binding of the isolated cell walls showed strong fluorescence under the microscope which confirmed glycoprotein nature of mycelial wall extract.

(16) The resistant variety (TV-26) showed similar disease reaction with the mycelial wall extract as that of the spore suspension of G. cingulata.

(17) Mycelial wall extract elicited the production of antifungal compound in the tea leaves of resistant variety.

(18) A large number of microorganisms were isolated from the phyllosphere of tea from different tea estates of Dooars and Hill regions of Jalpaiguri and Darjeeling districts.

(19) The isolated bacteria were identified on the basis of morphological, physiological and biochemical tests while the fungi were identified on the basis of morphology and spore characteristics.

(20) All isolated microorganisms were paired with G. cingulata on solid medium. Aspergillus flavus, A. fumigatus, A. nidulans, A. terreus, Bacillus cereus, B. pumilus, Bacillus sp., Coryneform bacterium, Micrococcus luteus, Micrococcus sp., Penicillium frequentans, P. oxalicum, P. simplicissium showed antagonistic reaction against G. cingulata.

(21) Antagonistic bacteria were grown with G. cingulata in liquid medium. The growth of G. cingulata was inhibited by all the tested bacteria.

(22) Spraying of tea leaves of susceptible varieties with aqueous cell suspension, washed cell or cell-free culture filtrates of the selected antagonistic bacteria resulted in reduction in disease development. Cell-free culture filtrates were most effective in all cases.

(23) Reduction in disease development by the antagonistic bacteria were also dependent on the ratio of antagonists to pathogen on the leaf surface.

(24) Culture filtrates of the antagonistic bacteria inhibited spore germination and appressoria formation of G. cingulata in vitro.

(25) The fungitoxicity of the bacterial culture filtrates were due to the release of antifungal metabolites into culture.

(26) Antifungal compound from M. luteus and B. cereus were more soluble in diethylether, the one from Bacillus sp. in ethylacetate and the compound from the Coryneform bacterium in chloroform as determined by bioassays of different solvent extracts against G. cingulata.

(27) Serial dilution of the extracts reduced their activity.

(28) Micrococcus luteus was selected for further studies on the basis of all previous tests.

(29) Maximum production of antifungal compound by M. luteus occurred after 96h of incubation, at pH 6.5, in antibiotic media no. 1 and using maltose as carbon source.

(30) The compound was fungitoxic not only to G. cingulata but other fungi (Bipolaris carbonum, Fusarium oxysporum and Pestalotiopsis theae) also.

(31) The compound was partially purified by Thin Layer Chromatography and UV-Spectrophotometric analysis.

(32) The partially purified compound showed absorption maximum at 292 mm.

(33) In vivo tests with semipurified extract showed it to be very effective in disease reduction.

(34) Results of present investigation have confirmed that disease development is the net result of a number of factors - mainly the pathogen factor, the host factor and the surrounding network of interacting microorgansims.

(35) Implications of the results have been discussed.

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