IMMUNOLOGICAL STUDIES ON BROWN BLIGHT OF TEA [*Camellia sinensis* (L.) O. KUNTZE]

THESIS SUBMITTED FOR THE DEGREE OF
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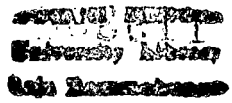

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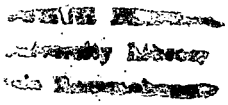

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This is to certify that Miss Ruma Das has carried out her research work under our joint supervision . Her thesis entitled " Immunological studies on brown blight of tea [*Camellia sinensis* (L.) O. Kuntze]" is based on her original work and is being 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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CONTENTS

Introduction

Tea is the most widely consumed hot beverage in the world, the habit of tea drinking being well established for more than half of the world's population. Tea is made from the young leaves and unopened leaf buds of the tea plant, *Camellia sinensis* (L.) O. Kuntze. The centre of origin of the tea plant is considered to be near the source of the Irrawady river and further north (Kato, 1989). In India, tea is one of the most important cultivated crops, its cultivation being spread over 3,96,000 hectres of land divided into 2 distinct regions - the North Indian tea belt located between 22-27° North and South Indian tea belt located at 7° North. North East India produces 75% of the total Indian tea in 3 different land scapes (Jain, 1991). Darjeeling produces the world's finest quality teas in the steep hill slopes of Eastern Himalayas upto an elevation of 2000 m. The extensive riverine flat plains at the base of Himalayas are the tea districts of the Terai and Dooars. Brahmaputra valley of Assam is located 100 m. above sea level and is the largest flood plains of the world growing tea which accounts for more than half of Indian production. Tea is also grown in the slopes of Nilgiris and Annamalai hills of Peninsular South India.

A number of 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 and Schrenk, causing brown blight disease (Agnihothrudu, 1995). 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 disease patches usually start on the margin of the leaves and spread in wards. When two or more patches occur side by side whole leaf may be affected. The edges of the patches are sharply defined and mostly marked with a delicate concentric zonation. The colour on the upper surface is yellowish to chocolate brown at first gradually changing to grey from centre outwards. Minute black, scattered dots which are the fructifications, appear on both sides of the diseased patch. The affected portion of the leaf finally turns over and shrivels up (Plate I, figs. A & B).

Plants respond to infection by a pathogen in a number of ways which are triggered by the initial recognition phenomenon. The initial recognition triggers the activation of immune system of plants, which though different from that of animals, is functionally similar. The immune system in plants involves several mechanisms, some of which are known to us but

many of them are still unknown. The complexities of the interactions that affect the selection of parasites and allow their establishment and survival among host cells is manifest in the frequency and variability of cell surface antigens. Some intriguing research work suggests that antigenic similarities between host and pathogen may be a prerequisite for compatible reaction or in other words successful establishment of the pathogen in its host depends on some kind of molecular similarities between the two partners (DeVay and Adler, 1976; Chakraborty and Purkayastha, 1983; Heide and Swedegaard - Peterson, 1985 ; Alba and DeVay, 1985 ; Chakraborty, 1988 ; Purkayastha, 1989 ; Protsenko and Lady Zhenskaya, 1989 ; Chakraborty and Saha, 1994 b, Chakraborty *et.al.*, 1995).

An important area of immunological studies of diseases involves the use of pathogen antiserum for detection of infection in the host beginning from the very early stages of host pathogen interaction. Disease detection by immunological means though of common use in viral diseases is only gaining ground in case of fungal diseases (Mohan, 1988; Sundaram et.al., 1991; Lyons and White, 1992; Linfeld, 1993; Holtz *et.al.*, 1994; Beckman *et.al.* 1994; Chakraborty *et. al.*, 1996, Wakeham and White, 1996). The recent diagnostic techniques for pathogen detection include enzyme linked immunosorbent assay, dot immunobinding assay, radioimmunosorbent assay, dip-stick immuno-assay, immunofluorescence and immunoenzymatic staining. Though significant advances have been made in the development of rapid, sensitive assays for fungi in recent years, commercially available techniques are limited to a few pathogens and diseases (Hansen and Wick, 1993). Such detection techniques makes it possible to detect the microquantities of the pathogen within a few hours of infection; this is definitely much more advantageous than the conventional techniques involving pathogen isolation, visible symptoms and microscopy. This has tremendous potential for plant disease control measures since detection of a pathogen at the initial stages of infection can lead to formulation of control measures before much harm has been done.

Since such immunological studies on brown blight disease of tea caused by *G.cingulata* are lacking, and, considering the importance of such studies, this study has been undertaken. The main objectives of this study are as follows: (a) to screen all available tea varieties (Tocklai, Darjeeling and UPASI) for resistance to *Glomerella cingulata* ; (b) to extract antigens from mycelial and cell wall preparations of *G.cingulata* ; healthy, naturally infected and artificially inoculated tea leaves ; non host species and non pathogen; (c) to raise antisera against antigen preparations from tea leaves, mycelia

and cell wall of *G.cingulata* as well as *F.oxysporum*, a non pathogen ; (d) to detect serological cross reactivity between isolates of *G.cingulata* and tea varieties following agar gel double diffusion, immunoelectrophoresis and enzyme linked immunosorbent assay; (e) optimization of the sensitivity of ELISA using antisera raised against both mycelial and cell wall preparations of *G.cingulata* ; (f) to detect infection in tea leaves of different varieties by ELISA ; (g) to determine the earliest period after inoculation with *G.cingulata* when detection can be done with ELISA and to quantify the fungal biomass in the tissues; (h) to purify the antigenic protein from the crude mycelial extract of *G.cingulata*; (i) to evaluate the antiserum raised against partially purified antigen of *G.cingulata*; (j) to characterize the chemical nature of cell wall of *G.cingulata* ; (k) to determine the cellular location of cross reactive antigens in mycelia and conidia of *G.cingulata* and tea leaf tissues.

Before going into details of the present work a brief review in conformity with this study has been presented in the following pages.

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

Literature Review

In nature a large number of interactionsbetween microorganisms and plants take place but most of them do not result in the entry of the pathogen into the host. Succesful host pathogen interaction generally depends on the recognition of the pathogen by the host. It is generally accepted that the cells recognise one another through pairs of complimentary structures on their surfaces; a structure on one cell carries encoded biological information that the strucrture on the other cell can decipher. Thus disease develops in individual plants by a series of sequential steps begining with the arrival of the inoculum at the plant surface and ending with the terminal stages of pathogenesis (Cowling and Horsfall; 1978). These stages in between the first and the last are unique to each host and pathogen and also depends to some extent on the prevailing environmental conditions. Serological relationship between host and pathogen is one of the first determinants in the establishment of pathogen within the host. Consdering the importance of serological relationships for host pathogen interaction a short selective review along these lines pertaining to the present study has been presented in the following pages. The review has been divided into three parts: (a) serological cross selectivity between host and parasite (b) detection of plant pathogenic fungi and (c) serological differentiation amongst and fungal species.

Serological cross reactivity between host and parasite

It is now well established that an immune system functionally similar to that of animal exist in plants. The serological cross reactivity between host and pathogen has been a subject of considerable interest to a number of workers and a number of review pertaining to this area have been published previously (DeVay and Adler, 1976; Damien, 1964; Clark, 1981; Chakraborty, 1988; Purkayastha, 1989; Purkayastha *et.al* 1991 and Purkayastha, 1994).

Serological methods for the differentiation between resistant and susceptible varieties of cotton infected with *Fusarium oxysporum* and *Citrus* sp. with *Phytophthora citrophthora* have been described by Abd-El-Rehim and Hashem (1970) and Abd El Rehim *et al.,* (1971 a). Serological and immunoelectrophoretical investigation on water melon varieties, resistant and susceptible to *Fusarium semitectum* also revealed that the cultivars could be differentiated by the titre or the time after which reaction occurred between antisera specific to the pathogens and seed globulins. It was noted that a,b globulin was present only in the resistant varieties. (Abd-El-Rehim *et. al.*, 1971 b).

Wimalajeewa and DeVay (1971) detected common antigenic relationship between *Zea mays* and *Ustilago maydis*. A pair of compatible haploid lines and two diploid solopathogenic lines of *U. maydis* were used in serological studies. *Avena sativa* var.'Victory' and *Hordeum vulgare* var. 'California Mariout' were taken as resistant hosts.Certain antigens were found common between Corn and *U. maydis*. A strong antigenic relationship existed between the solopathogenic lines 132 and 3 day oat seedlings. Barley did not have any antigen in common with any of the *U.maydis* did not indicate any qualitative significant serological difference among them.

Charudattan and DeVay (1972) compared antigenic preparation from four varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species for the presence of common antigen. At least one antigenic substance was common between the varieties of cotton and isolates of *Fusarium oxysporum* f. sp. *vasifectum*, *F. solani* f.sp. *phaseoli*, *Verticillium alboatrum*, and *V. nigrescens*. Cotton varieties which were resistant or susceptible to *Fusarium* wilt as well as pathogenic and nonpathogenic isolates of *F. oxysporum* f,sp. *vasinfectum* shared the common antigen. The common antigen was not shared between *F. moniliformae* (nonpathogenic) and cotton. In gel diffusion tests five to eight precipitation bands were observed in the homologous reactions; of these, only one or two bands were common in heterologous reactions between the fungal and cotton preparations. The common antigenic determinants shared by cotton and the fungal isolates did not appear to be related to the severity of wilt symptoms , but possibly affected host pathogen compatibility during the process of root infection. Cross reactive antigens were also found between eight legume hosts and three *Rhizobium* species (Charudattan and Hubbell, 1973). These common antigens among hosts and bacteria were not related to the specificity of compatible *Rhizobium* - legume association. The cross-reactive antigens were absent between rhizobia and eight non-legume plants tested, but present between five out of eleven gram-negative phytopathogenic bacteria and legumes.

Abbott (1973) determined the antigenic affinity among the saline soluble proteins of *Triticum aestivum* and *Avena sativa* and soil borne fungus *Ophiobolus graminis*. Single precipitin band in immunodiffusion test was formed when antisera of the wheat and oat roots were allowed to diffuse with the antigens of O.graminis.

Antisera raised against soluble extracts of (Race-4) and tubers of 'Arranbanner' and "Golden wonder" potato cultivars showing field susceptibility and resistance respectively

to late blight were used to test for the presence of common antigens between extracts of the fungus and various host and non host plants (Palmerley and Callow, 1978). Cross reactive antigen was detected between *P. infestans* (Race-4) and potato tubers of both the field susceptible and field resistant cultivars and also between the fungus and leaves of tomato and tobacco.Common antigens were not detected between *P.infestans* (Race-4) and leaves of non host (mungbean, pea, radish, cucumber and maize), or between potatoes and the alternative pathogen, (*Fusarium solani* var. *acruleum*) and two non pathogens (*Ustilago maydis* and *Phytophthora cinnamoni*)

Kalyanasundaram *et.al* (1978) studied the antigenic relationship between host and parasite in *Fusarium* wilt of cotton. Common antigen was shared by both avirulent and virulent isolates of *Fusarium oxysporum* f. sp. *vasinfectum* with disease resistant and susceptible line of cotton. In all cases, the fungal isolates invaded and parasitized cortical tissues of cotton roots, but only those fungal isolates that caused disease became established in the vascular system.

Conidia of *F. oxysporum* f. sp. *vasinfectum* was reported to contain an antigen that cross-reacted with antiserum to cotton root tissue antigens (Charudattan and DeVay, 1981). In agar gel double diffusion tests, one precipitin band was formed when antiserum to cotton antigens was reacted with crude fungal antigens, or when antiserum to crude fungal antigens was reacted with cotton antigens. The cross reactive antigen from fungal conidia (CRA) was isolated, purified and partially characterized. The CRA migrated as a single band in polyacrylamide or agar gel electrophoresis, and sedimented as a single band during analytical ultracentrifugation. It was antigenic in rabbits, and was a protein carbohydrate complex. The major cross-reactive antigenic substance (CRA), common to cotton (*Gossypium hirsutum*) and certain fungal parasites of cotton roots, was further purified to homogeneity from conidial cultures of *F. oxysporum* f.sp. *vasinfectum* (DeVay *et al.*, 1981). Agar gel double diffusion tests indicated the presence of CRA not only in *F. oxysporum* f. sp. *vasinfectum* and in cotton roots and seed but also in *Thielaviopsis basicola*. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross-sections of roots, cut near or just below the root hair zone, the CRA was concentrated mainly around xylem elements, the endodermis and epidermis cells and was present throughout the cortex tissue. Protoplasts prepared from cross-sections of young cotton roots also contained the CRA which was concentrated in the region of the plasmalemma. Treatment of conidia and mycelia of *F. oxysporum*

f. sp. *vasinfectum* with antiserum to cotton and using indirect staining with FITC indicated that the CRA was mainly present in hyphal tips and in patch-like areas on conidia.

Chakraborty and Purkayastha (1983) detected cross reactive antigen shared between soybean cultivars and *Macrophomina phaseolina* causing charcoal rot disease. Rabbit antisera were raised against root antigens of soybean cultivars (Soymax and UPSM-19) and *M.phaseolina* isolate (M.P. 1) and tested against homologus and heterologous antigens following immunodiffusion test. When antiserum of *M. phaseolina* was reacted against its own antigens and antigens of susceptible soybean cultivars (Soymax, R-184) strong precipitation reactions were observed. In case of resistant cultivars (UPSM-19 and DS-73-16) no such reactions were observed. Reciprocal cross reactions between antiserum of the resistant cultivars and antigens of three isolates of *M. phaseolina* also failed to develop even weak precipitation bands. Four antigenic substances were found to be common between the susceptible soybean cultivars and isolates of *M. phaseolina* in immunoelectrophoretic tests, but no common antigens were detected between resistant cultivars and the fungus. Purkayastha and Chakraborty (1983) further detected that in susceptible soybean plants (cvs. Soymax and R-184) a close relationship exists between lower production of glyceollin and presence of common antigens. The production of glyceollin was much higher in resistant soybean cultivars (cvs. UPSM-19 and DS-73-16) where common antigens were absent. Alba *et al.*, (1983) also detected common antigens in extracts of *Hemileia vastatrix* urediniospores and of *Coffea arabica* leaves and roots.

Alba and DeVay (1985) detected cross-reactive antigens in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* Race-4 and Race-1, 2,3,4,7 with antisera for potatoes cv. King Edward and cv. Pentland Dell by using an indirect ELISA technique. It was suggested that the fungal mycelia do not easily release cross-reactive antigens into synthetic media where they grow; that most of *P. infestans* cross reactive antigens were thermolabile and that they could be concentrated by precipitation in the presence of 40% saturated ammonium sulphate (SAS). An antigenic disparity was observed when 40% SAS from *P. infestans* Race-4, mycelial preparation was assayed with antisera for King Edward and Pentland Dell.

Antigenic determinants responsible for a precipitation line specific for *Mycena galopus* were partially purified using salt fraction (with ammonium sulphate) and

ion exchangechromatography (Chard *et al,* 1985). Fractions were assessed by immunodiffusion and immunoelectrophoresis for the presence of the specific line. Proteins with associated lipid and carbohydrate moieties were detected in the antigenic fraction, which was used for further antiserum production. The new (F-) antiserum was tested for its specificity to *M. galopus* by immunodiffusion. Antigens extracted from four near isogenic barley lines when cross reacted with the antisera of *Erysiphe graminis* f.sp. *hordei*) were shown to share immunologically identical antigens (Heide and Swedegard Peterson, 1985).

Immunodiffusion, immunoelectrophotetic and crossed immunoelectrophoretic analyses of rice antigens in relation to sheath rot disease revealed a serological relationship between susceptible rice cultivars and isolates of the causal organism of sheath rot, *Acrocylindrium oryzae*. One precipitation band was observed when the antigen preparation of *A. oryzae* was cross-reacted with its own antiserum or against the antisera of four susceptible rice cultivars. No precipitin band was detected between the antiserum of the resistant cv. Mahsuri and antigen preparations from three isolates of *A. oryzae* or between the antigens of the resistant cultivars Mahsuri and Rupsail and the antiserum of *A. oryzae*. Crossed immunoelectrophoretic tests confirmed that there was a common antigen between Mahsuri and Jaya, and between Mahsuri and CR-126-42-1. The precipitin band between the antigen preparation of Jaya and *A. oryzae* was found to be similar (Purkayastha and Ghosal, 1985). The common antigenic relationship between soybean cultivars and *Colletotrichum dematium* var. *truncata* was also ascertained by Purkayastha and Banerjee (1986) following immunodiffusion, immunoelectrophoretic and crossed immunoelectrophoretic tests. At least one antigenwas found to be common between host cultivar and the pathogen. No antigenic relationship was observed either between soybean cultivars and the non pathogen (*C.corchori*) or avirulent pathogen (*C.dematium*).

Purkayastha and Ghosal (1987) also compared the antigenic preparations from two isolates of *Macrophomina phaseolina* , a pathogen of groundnut, four non-pathogens of groundnut (viz., *Corticium sasakii, Colletotrichum lindemuthianum, C. corchori,* and *Botrytis allii*) and five cultivars of *Arachis hypogea* by immunoserological techniques. Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M.phaseolina,* but not between nonpathogens and groundnut cultivars. No antigenic similarity was found between nonpathogens and *M.phaseolina* isolates. Cross-immunoelectrophoretic tests confirmed that at least one antigen was common between cv. J-11 and cv. TMV-2, cv. Kadiri 71-

1 and cv. TMV-2 and cv.Kadiri 71-1 and isolates of *M.phaseolina.*

Chakraborty and Purkayastha (1987) reported the changes in antigenic patterns after chemical induction of resistance in susceptible soybean cultivar (Soymax) to *Macrophomina phaseolina.* Sodium azide (100 μg/ml) altered antigenic patterns in susceptible cultivar (Soymax) and reduced charcoal rot disease. Similar results were also obtained by Ghosal and Purkayastha (1987) in susceptible rice cultivar (Jaya) and *Sarocladium oryzae* after altering disease reaction (sheath rot) by the application of gibberellic acid and sodium azide (100 μg/ml).

Antigens prepared from two resistant cultivars (UPSM-19 and DS 73-16) and two susceptible cultivars (DS-74-24-2 and PK-327) of soybean and three strains of *Myrothecium roridum* (M-1, ITCC-1143 and ITCC-1409), a causal organism of leaf spot disease were tested against antisera of pathogen. Immunodiffusion tests revealed that common antigens were present only between the virulent strain and susceptible host cultivars. But no such cross reactive antigen was detected in case of resistant cusltivar (UPSM-19). Immunoelectrophoretic analysis showed that one common antigen was shared by susceptible host and the virulent strain (M-1) which was further confirmed by both crossed and rocket immunoelectrophoresis (Ghosh and Purkayastha, 1990). Cross reactive antigens (CRA) were also detected between susceptible soybean cultivars and the virulent strain of *Colletotrichum dematium* var. *truncata* but CRA could not be detected between soybean cultivars and an avirulent strain of *C.dematium* or non-pathogen (*C.corchori*). Results of immunodiffusion and immunoelectrophoresis showed absence of common antigen between resistant cv. 'UPSM-19' and the pathogen, while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. Alteration in antigenic patterns of soybean leaves after induction of resistance by the treatment of cloxacillin(100 μ g/ml) were further detected by Purkayastha and Banerjee(1990).

Purkayastha and Pradhan (1994) observed that three strains of *Sclerotium rolfsii* were serologically different and their pathogenecities also differ markedly with host cultivars. Virulent strains 266 and 23 showed common antigenic relationship with their respective susceptible cost cultivars but not resistant cultivars. Antigenic change in a susceptible cv. PK-12-24 after treatment with a systemic fungicide kitazin was also evident. They suggested that the resistance could be induced in susceptible plants of specific antigens were

eliminated by suitable treatment.

Antigens obtained from six Tocklai varieties of tea, four isolates of *Bipolaris carbonum* and non-pathogens of tea (*Bipolaris tetramera* and *Bipolaris setarae*), were compared by immudiffusion, immunoelectrophoresis and indirect ELISA in order to detect cross-reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties (TV- 9, 17 and 18) and isolates of *B.carbonum* (BC 1,2,3, and 4). Such antigens were not detected between isolates of *B.carbonum* and resistant varieties (TV-16, 25 and 26), non-pathogens and tea varieties, as well as non pathogens and isolates of *B.carbonum*. Indirect straining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and conidia of *B.carbonum* with antisera to leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia (Chakraborty and Saha, 1994 b).

Among the 12 varieties of tea tested against three isolates of *Pestalotiopsis theae*, causal agent of grey blight disease, Teen Ali-17/1/54 and TV-23 were found to be highly susceptible while CP-1 and TV-26 were resistant under identical conditions. Leaf antigens were prepared from all the tea varieties, three isolates of *P.theae* and a non-pathogen of tea (*Bipolaris tetramera*). Polyclonal antisera were raised against mycelial suspensions of *P.theae* (isolate Pt-2) and leaf antigens of Teen Ali-17/1/54 and CP-1. These were compared by an immudiffusion test and enzyme-linked immunosorbent assay to detect cross-reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties and isolates of *P.theae* (Pt-1,2 and 3). Such antigens were not detected between isolates of *P.theae*.. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves the CRA was concentrated in the epidermal cells and mesophyll tissues. CRA was present in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P.theae* (Chakraborty, *et.al.*, 1995).

Detection of Plant Pathogenic Fungi

Detection of plant pathogenic fungi within host tissues by serological means is a relatively recent development in the field of plant pathology. The merit of this method of detection lies in its ability to detect even very small amounts of pathogen in tissues which is generally not detected by conventional techniques. A number of recent reviews have been published by workers along this line (Hansen and Wick, 1993 and Werres and Steffens, 1994)

Detection of *Ophiobolus graminis* on the roots of cereals and its rhizosphere by the use of direct and indirect fluorescent antibody staining was reported. *Aspergillus* and *Penicillium* spp. were the predominant fungi isolated in culture from 5 samples of stored grain. Using an immunofluorescence method, the presence of these fungi in most grains was confirmed but the amounts of mycelium involved were shown to be small. *Alternaria* spp. were not isolated from 3 of the samples but small amounts of *Alternaria* mycelium were detected in grains of all the samples (Warnock, 1973).

Casper and Mendgen (1979) used an ELISA format to estimate *Verticillium lecanii* at different stages of infection in leaves of wheat infected with yellow rust pathogen (*Puccinia striiformis*). Following immunofluorescence technique, *V.lacanii* could also be detected in pustules of rust pathogen (*Uromyces phaseoli*) of bean

The red stele pathogen, *Phytophthora fragariae* could be detected by a "root -tip-bait" test in disease free strawberry plants. Susceptible plants were grown in a mixture of compost and root tip cut from runner plants. When root tip samples were prepared from a combination of 1:99 infected and uninfected runner plants, the test gave positive results in all 10 trials (Duncan, 1980).

Antibodies raised to the ribosomes of *Fusarium moniliforme* and corn (*Zea mays.*) were shown to be specific by the Ouchterlony double diffusion test and sucrose density gradient analysis of the antigen-antibody reaction. Extracts of total ribosomes from pith of infected corn could be separated into host, pathogen and other organism(s) fractions by the use of antibodies and sucrose density gradient centrifugation (Michael and James, 1981).

Johnson *et al.* (1982) used antiserum prepared to homogenates of washed *Epichloe typhina* mycelium grown in a liquid medium in an enzyme-linked immunosorbent assay (ELISA) to detect antigens of the fungus in tall fescue (*Festuca arundinacea*) tissue samples. Very low concentrations (100 µg/ml) of freeze-dried *E.typhina* mycelium could be detected and the pathogen could also be detected in individual seeds of tall fescue. Of 14 fungal genera tested, including *Acremonium, Claviceps, Helminthosporium, Pythium, Rhizoctonia* and *Sclerotium* all showed reactivities less than 0.1% that of *E. typhina.*

Antisera were made to both a whole cell and cell wall preparation of *Eutypa armeniacae*. Rhodamine isothiocynate (RITC) conjugated antisera were tested for reactivity with various fungi on glass slides. Both antisera showed low specificity, but specificity was

improved by cross-adsorption of the RITC-conjugated cell wall antiserum with *Phomopsis viticola*. Woody cross sections from concord grapevines inoculated with *E.armeniacae* and also inhabited by various other fungi were stained directly with the conjugated anti-*Autypa* rabbit serum. In an indirect staining procedure, sections were treated with anti-*Eutypa* rabbit γ globulin. Both procedures specifically stained hyphae in wood sections. Hyphae stained indirectly in woody vine sections showed a much brighter fluorescence than analogous hyphae stained by the direct method. Fungi of some species that reacted strongly with the direct method on glass slides did not always react when stained indirectly in woody vine sections in which they were growing (Gendloff *et al.*, 1983).

Indirect immunofluorescence, performed by using antisera to culture filtrate molecules of *Phaseolus schweinitzii* has been used to demonstrate the presence of mycelium, and on occasions chlamydospores, in naturally and artificially infested soil samples. The authors could thus identify the kind of propagule most likely to be the source of field isolates of the organisms; this information, which could not be obtained by using selective media, strongly suggested that the pathogen could survive saprophytically in the soil. In contrast, isolated mycelial cell wall preparations did not prove to be a suitable source of immunogenic material for these studies (Dewey *et al*, 1984).

Aguelon and Dunez (1984) used double antibody sandwich ELISA and indirect ELISA technique for the detection of *Phoma exigua* in inoculated tubers and sprouts and in stems grown from these tubers. The fungus was detected in these different tissues with var. *foveata* being more aggressive, demonstrating the applicability and sensitivity of the techniques. The antibodies produced to the two varieties of the fungus were not specific to their own varieties. They also reacted with *Phoma tracheiphila* but did not react with several other common potato pathogens. Preliminary results obtained with antibodies from mouse ascite liquid suggest the possibility of producing specific monoclonal antibodies.

Reddy and Ananthanarayanan (1984) investigated the presence of *Ganoderma lucidum* in betelnut by the fluorescent antibody technique. Presence of *Sclerotinia sclerotiorum* in sunflower was also detected by Walcz *et al*, (1989) by enzyme-linked immunosorbent assay (ELISA).

Sections of leaves of *Nicotiana tabecum* infected with *Peronospora hyoscyami* f.sp. *tabacina* and of *Erythronium americanum* infected with *Ustilago heufleri* treated

with an antiserum directed against the fimbriae of *U. violacea* Fuckel and other fungi were then treated with protein-A-gold complexes to detect the presence and location of fimbrial antigens following transmission or scanning electron microscopy. The infected leaf sections were heavily labelled with gold particles indicating the presence of fimbrial antigens, whereas the control preparations showed only a low background level of labelling. Gold particles were detected on the sections of hyphae, on haustoria and on the nearby plant cells. The intensity of labelling was much higher for *P. hyoscyami* f.sp. *tabacina* than for *U. heufleri* and was particularly high in the walls of the former species. Relatively high levels of labelling occurred over the cells of infected host tissues, but little or low occurred over the cells of uninfected host tissues or of the infected host tissues treated with a range of serological controls. This level of labelling was not associated with specific host structures in *P. hyoscyami* , but was frequently associated with the chloroplast in *U. heufleri*. The antigens detected inside the host plant cells appeared to indicate that fungal fimbrial protein, either as polymerised fibrils or as isolated sub-units, could penetrate the host plasma membrane and therefore entered the host cytoplasm.(Day *et al.*, 1986).

Hyphae of *Verticillium dahliae* were detected in cotton root tissue with an ELISA. A soluble protein extract of *V.dahliae* was used to prepare a specific rabbit antiserum. The reaction of this rabbit antibody to the hyphae of *V. dahliae* was detected with an alkaline phosphatase antirabbit IgG conjugate that hydrolyzed the substrate, napthol-AS-phosphate, to a product that reacted with a diazonium salt, yielding a colored precipitate outlining the fungal hyphae. The hyphae were readily observed on and in the root cortex of the host using a dissecting microscope (Gerik *et al*, 1987).

Gerik and Huisman (1988) further observed that colony density of *V.dahliae* increased with distance from the root tip, with the maximal density occurring more than 1 cm from the root apex. Colony densities at distances more than 1 cm from the tip were relatively constant. The mean colony length of *V.dahliae* was 7.3 mm, and increased colony length was correlated with distance from root apices. Hyphae of *V. dahliae* were present through the entire depth of the cortex, and were greatest in the interior of the root cortex at the surface of the vascular cylinder. The colony appearance was consistent with growth of hyphae from the root surface toward the stele. Hyphae of *V.dahliae* also were found within numerous cortical cells. Colonies of *Fusarium oxysporum* similarly stained, were found to be mostly confined to the root surface and the outer cortex.

In enzyme-linked immunosorbent assay, antiserum raised against pooled mycelial suspensions from five isolates (designated pf 1, pf 2, pf 3, pf 10 and pf 11) of *Phytophthora fragariae* detected homologous soluble antigens at protein concentrations as low as 2 ng/ml (Mohan ; 1988) Fungal antigens could also be detected in extracts of strawberry plants infected with *P. fragariae.* Root extracts prepared from the alpine strawberry *Fragaria vesca* and *F. ananassa* cv. Cambridge Favourite infected with any of the five isolates studied produced strong reactions in ELISA. In *F. vesca*, ELISA positive material was detectable 6-8 days after inoculation before macroscopic symptoms appeared. The cultivar Red Gauntlet, resistant to pf 1, 2 and 3 but susceptible to pf 10 and 11, reflected this differential response in ELISA : the absorbance produced by extracts of plants infected with virulent isolates was significantly higher than that obtained with the corresponding extracts of plants inoculated with avirulent isolates.

An agri-diagnostic *Phytophthora* multiwell ELISA kit, developed for detection of *Phytophthora* in plant tissue, also readily detected *Phytophthora* in soil where soybeans were damaged by *P. megasperma* f. sp. *glycinea* (Pmg) (Schmitthenner, 1988). Only low level of *Phytophthora* were detected in soil stored at 3°C. Following cold storage high levels of *Phytophthora* could be detected directly from soil, after *Phytophthora* damping off of soybean seedlings was induced. But *Phytophthora* detection was obtained from soybean leaf discs floated on water over infested soil for 24 hrs. Pmg was the only *Phytophthora* isolated from such leaf discs using selective media. It was concluded that *Phytophthora* was detected best with an ELISA test of soil with actively rotting roots or from leaf disc baits with actively growing mycelium.

Amouzou-Alladaye *et al.* (1988) reported that the antiserum obtained by injecting rabbits with mycelial protein extracts of one strain of *Phytophthora fragariae* had a dilution end point of 1:64 in double diffusion and 1/512,000 in indirect ELISA. This serum could detect 11 different strains of *P. fragariae* in pure culture and the pathogen in naturally infected or inoculated roots. Although the sensitivities of direct DAS and indirect ELISA were comparable, the direct DAS-ELISA was more specific for the detection of *P.fragariae* in strawberry roots. The antiserum failed to react with 18 fungal species isolated from underground parts of strawberry but reacted with some strains of *P. cactorum* , which parasitized only rhizomes but not roots, and *Pythium middletonii*, which was isolated sometimes in association with *P. fragariae* from strawberry roots. In inoculated strawberry

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roots *P. fragariae* was detected reliably by ELISA several days before oospores were found and before symptoms developed. Thus, direct DAS-ELISA may be useful for early detection of infection and for the detection of latent infections of strawberry plants by *P. fragariae.*

Arie *et al.* (1988) detected resting spores of *Plasmodiophora brassicae* from soil and root by indirect fluorescent antibody technique. Infested soil and roots were stained by fluorescent antibody technique with the IgG and FITC conjugated antirabbit IgG-sheep IgG. Resting spores were effectively detected and also clearly differentiated from small particles of soil and tissues of plant in the reflected light fluorescence microscope. An indirect ELISA for quantitative detection of *P. herpotrichoides* infections in wheat was developed by Unger and Wolf (1988) . All tested isolates of the virulent varieties *P. herpotrichoides* var. *herpotrichoides*, *P.herpotrichoides* var. *acuformis* or the W-and R- type reacted on a high level in the test, while the less virulent *P. angnioides* was assessed only with 40% and the avirulent *P. aestiva* with 20% of the homologous reaction. No cross reactions occurred with extracts of 11 other species of *in vitro* cultivated fungi nor with plant material infected with other pathogens. The infection profile throughout the leaf sheaths was clearly reflected by ELISA. The examination of 24 stem base samples from the field showed that the values assessed by ELISA correlated well also with the disease indices of naturally infected plant material.

An enzyme immunosorbent assay (ELISA) for isomarticin, a napthazarin toxin produced by *Fusarium solani* was developed by Phelps *et al*, (1989). A carbodimide procedure was used to couple the hapten isomarticin to BSA for the immunogen and to alkaline phosphatase for enzyme linked tracer. The resulting assay had a detection limit of 2 ng/ml for isomerticin, other napthazarin toxins were detectable at less than 10 ng per well in ELISA plates. The assay was specific for napthazarine. The cross reactivity with a number of phenolic compounds including the closely related napthoquinones was 3 order of magnitude less sensitive.

Gleason *et al* (1989) used freeze dried powdered mycelium of *Phomopsis longicola*, seed decay pathogen, in an indirect ELISA and an immunoblot assay for detecting seedborne infection. Antigen of *P. longicola* was detected by indirect ELISA in as little as 250 ng of dried mycelium/ml of extract. The antiserum reacted with mycelium preparation of *Diaporthe*

phaseolarum var. *sojae* showed comparatively little or no reaction when tested against 7 other seed borne fungi. *P.longicola* could be detected in individual symptomless infected seeds. A single infected seed coat could not be readily detected by indirect ELISA. Results of immunoblot assay for incidence of *P.longicola* and *D. phaseolarum* var. *sojae* in halved seeds from 10 seed lots correlated ($P< 0.001$) with agar plate bioassays of the corresponding seed halves but not with incidence of symptomatic seeds. Indirect ELISA absorbance values for bulked samples of seed-coat halves from the same 10 seed lots correlated weakly with agar plate bioassay but strongly ($P< 0.01$) with incidence of symptomatic seeds. Because immunoblot assays detected only viable *P. longicola* and ELISA did not discriminate between live and dead fungus, the authors concluded that former would be a better indicator of pathogen activity on seeds after planting. Werres and Casper (1987) detected *Phytophthora fragariae* in roots of strawberry cultivar 'Teniva' by ELISA, even before symptoms were detected microscopically.

Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reactions by scanning electron microscopy was also done (Large, 1989). A semipurified suspension of spores of *P. brassicae* was used as antigens, obtained by filtration and Percoll gradient centrifugation of infected roots. Crude root suspension samples were bound to a nitrocellulose paper and tested by dot immunobinding assay. The surface of the resting spores of *P. brassicae,* race-7, appeared smooth, while the dot immunobinding processed spores had a heavy, irregular coating, which was demonstrated to originate from incubation in the primary antiserum. Noraml serum did not give rise to a coating on the resting spores. The antiserum against *P.brassicae* did not react with surface antigens of resting spores of *Polymyxa.* Further no cross reaction with other root pathogens was observed.

A double antibody ELISA to detect *Pythium* blight in turfgrass, with a monoclonal antibody directed against *Pythium* sp. has been developed. The monoclonal antibody was the product of hybridoma cell line PAS 111F11, produced using *P.aphanidermatum* as immunogen. The antibody bound *P.aphanidermatum, P.graminicola, P. myristylum* and *P. ultimum* all of which were involved with *Pythium* blight. *Pythium* spp. were detected in inoculated and naturally infected turfgrass samples, and the color intensity of the final reaction product of the immunoassay was directly related to the level of disease present. No cross reactivity was observed with other common turf grass pathogens (Miller *et al.,* 1989). Antiserum (anti-PfM) raised against mycelial suspensions of *Phytophthora fragariae* isolates

reacted strongly with antigen from several *Phytophthora* species. Some cross-reactions with antigens from *Pythium* species were decreased by fractionating on an affinity column of sepharose 4B bound to extracts of *Fragaria vesca* roots infected with *P.fragariae.* The affinity purified anti-PfM retained its high cross-reactivity with the various *Phytophthora* species tested. It also detected infection of raspberry and strawberry roots by some *Phytophthora* species (Mohan, 1989). He however, reported that anti-PfM could not be made specific for *P.fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However, immunoblotting with the affinity purified anti-PfM produced distinct patterns for *P.fragariae, P.erythroseptica* and *P.cactorum* : three serotypes were identified for the latter species. It was concluded that this antiserum might prove useful in classifying *Phytophthora* species.

Watabe (1990) reported on the usefulness of immunofluorescent antibody technique for detection of *Phytophthora* in soil. Autofluorescence and the nonspecific staining of soil particles interfered with the detection of the fungi in soil. Pretreatment of the samples with gelatin-rhodamine conjugate prevented the samples from the autofluorescence and the nonspecific staining and therefore permitted the immunofluorescent antibody staining in soil. Stained *Phytophthora* was easily detected on the yellow orange background.

A polyclonal antiserum, prepared in a rabbit immunized with a mycelium extract of *Phytophthora infestans* reacted in an enzyme-linked immunosorbent assay (ELISA) with mycelial extracts of two *Phytophthora* species but not with those of 10 other micro-organisms found on potato. *P.infestans* mycelium in leaf tissue was readily detected by ELISA using either the plate-trapped antigen or $F(ab')_2$ antibody fragment techniques. The amount of mycelium in leaf extracts was estimated by comparing the values obtained in ELISA with those for known concentrations of *P.infestans* mycelium (Harrison *et al.*, 1990).

Ricker et al, (1991) detected water soluble antigens produced by *Botrytis cinerea* in picked and naturally infected grape juice by using an enzyme immunoassay with an indirect format of antibody HRPO conjugated bound to polyclonal rabbit antibodies directed against *B.cinerea* (anti Bc IgG). Protein A purified gamma globulin from an early bled antiserum (803.7), which reacted primarily with low molecular weight compounds present only in extracts of *B.cinerea*, was used to specifically detect *B.cinerea* and quantify levels of infection in juice from infected grape berries. Late bled higher titre antiserum (803-19), which cross reacted with proteins and carbohydrates present in extracts from species of *Botrytis, Aspergillus, Penicillium* and *Uncinula*, was used to quantify the levels of rot caused

by presence of multiple fungi. Minimum detectable levels of infections, based on mixture of clean and infected juice, were .25-.5% with (803-7)/IgG, and 0.2% with 803-19 IgG. Cross reactivity of anti Bc. IgG with extracted antigens (water soluble) from sterile and reproductive strains of several fungi was negligible in early bled antiserum and increased in subsequent collections. The increase in cross reactivity in late bled antiserum corresponded with an increase in the overall serum titres for anti Bc IgG to antigens from *B.cinerea.*

Polyclonal antisera prepared against purified mycelium proteins from *Verticillium dahliae* , the predominant fungus species in the potato early drying complex was tested against crude mycelial preparations of *Verticillium* spp. using indirect ELISA (Sundaram, 1991). It reacted positively with 11 of 12 *V. dahliae* isolates from potato, cotton and soil but negatively with one isolate from tomato. The antisera did not react with mycelial proteins from *Fusarium* spp. from potato and cotton, with a *Colletotrichum* sp. from potato or with on isolate of *Rhizoctonia solani* from sugarbeet. Double antibody sandwich ELISA, using polyclonal antisera, detected *V.dahliae* and *V.allo-atrum* in infected roots and stems of potato.

Two commercial serological assay kits were compared to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from infected azaleas by Benson (1991). Both the multiwell E kit and the rapid assay F kit detected *P. cinnamoni* on azalea roots beginning 1 week after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P.cinnamomi* was not consistent beginning 3-5 week after inoculation. Root symptoms, but not foliar symptoms, of *Phytophthora* root rot were evident during this period. There was a positive correlation between root rot severity in green-house trials and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immunoassay results. The multiwell kit detected *P.cinnamomi* in root samples containing as little as 1.0% infected root tissue. In a commercial nursery survey, 5 and 15% of the azalea root samples of two nurseries had positive ELISA values that were unconfirmed by culture plate. The rapid assay kit detected *P.cinnamomi* , was easy to use, and gave results in a short time.

Methods for sampling turf grass tissue were compared for their effectiveness in monitoring *Pythium* blight epidermis with enzyme-linked immunosorbent assay (ELISA). Samples consisted of whole plants picked by hand and assayed as whole plants; whole plants sectioned into lower, middle and upper strata components; leaf clippings collected with a reel mower set at a 1.2 cm cutting height. ELISA readings for mowed samples generally

matched those for whole plucked samples. Fluctuations in detectable *Pythium* antigens were most pronounced on the uppermost stratum compared with moderate to very little change in ELISA readings for the two lower strata. Several episodes of *Pythium* antigen increase were detected by ELISA assays of mowed samples, although signs and symptoms of *Pythium* blight were not evident. However, increase in ELISA readings for *Pythium* blight were not evident . However, increase in ELISA readings for *Pythium* coincided with, but did not generally precede, the onset of blight symptoms with a 2 to 3 day sampling interval, Shane (1991) concluded that antibody aided detection was useful for verification of diagnosis and determination of general *Pythium* population fluctuations, but was not satisfactory for advanced detection of blight epidemics.

Bossi and Dewey (1992) developed a monoclonal antibody - based immunodetection assay for *Botrytis cinerea* using three hybridoma cell lines secreting antibodies that specifically recognize *Botrytis cinerea* and *B. fabae* but not *B. allii,* in strawberries. Supernatants from the three specific cell lines recognized mycelial fragments, saline extracts of mycelia and germinating conidia by both ELISA and immunofluorescence. Recognition of non-germinating spores was poor. Supernatants from the specific cell lines did not recognize other fungi normally involved in post-harvest spoilage of fruits and vegetables. Supernatants from KH4 gave the lowest background values with healthy tissue. Indirect evidence from heat, protease and periodate treatment of the antigens indicated that antibodies from all three specific cell lines recognized carbohydrate epitopes on a glycoprotein.

Results of conventional isolation techniques for *Pythium violae* were compared with the assay of cavity spot lesions using polyclonal antibodies raised to *P. violae* or *P. sulcatum*, in competition ELISA (Lyons and White, 1992). Where lesions were artificially induced the test confirmed which pathogen was causal. With cavities developed on the field grown carrots *P. violae* again predominated and the ELISA confirmed this. In one sample *P. sulcatum* was also isolated from a small number of lesions and was not detected in ELISA. The competition ELISA did not indicate presence of either *Pythium* in a range of non-cavity spot lesions from which attempts at isolation were also negative.

A double antibody sandwich ELISA test was developed for the detection of *Pseudocercosporella herpotrichoides* using a highly specific monoclonal antibody pH-10 as the capture antibody and genus specific polyclonal rabbit antisera as test antibody. The assay recognized extracts from plants both artificially and naturally infected with *P. herpotrichoides*, at least three fold higher absorbance values with extracts *P. herpotrichoides*

infected tissue than with extracts from healthy tissues. The assay tested positiuively with all isolates of *P.herpotrichoides* including W type and R type isolates. The assay could be used to detect the pathogen in presymptomatic infected seedlings. The immunogen used was a mycelial extract from high molecular weight proteins and glycoproteins were removed by SAS. The high mol. wt. fraction was shown to contain cross reactive antigens: it induced antiserum in mice that cross reacted with the other stem base fungi even at high dilution (Priestley and Dewey, 1993).

Linfield (1993) elicited polyclonal antiserum against a strain of *Fusarium oxysporum* f.sp. *narcissi* (GCRI 80/26) and a specific and sensitive enzyme-linked immunosorbent assay was developed. Antiserum raised to cell-wall fractions gave better recognition than that to cytoplasmic fractions. Recognition was equally good in artificially and naturally infected bulbs. Little cross reactivity in bulb tissue was shown by three other bulb-rotting fungi. Nine isolates of *F.oxysporum* f.sp. *narcissi* from a wide geographic area gave similar results in an indirect ELISA of mycelial extracts, although some cross reactivity was observed with two other *Fusarium* spp. Four *Fusarium* spp. and four other fungi showed little cross reactivity. Ten days after inoculation the pathogen was readily detected in the base plate area of three *Narcissus* cultivars and points remote from the inoculation site in the most susceptible cultivar. A direct correlation was observed between positive results in the enzyme-linked immunosorbent assay and recovery of pathogen on selective medium.

New monoclonal antibody (MAb) raised against haustorial complexes (UB10) isolated from pea leaves infected with the powdery mildew fungus *Erysiphe pisi* recognised a 45KDa N-linked glycoprotein which was specially located in the haustorial plasma membrane. This glycoprotein was clearly distinct from a previously characterised 62 KDa plasma membrane (identified with MAb UB8) which was also specially located in the haustorial plasma membrane. These antibodies were used, along with MAb UB7 which binds to a major 62 KDa glycoprotein in the cell wall and plasma membrane of both haustoria and surface hyphae, to label haustoria within epidermal strips from infected pea leaves using indirect immunofluorescence. Results showed that all three glycoproteins recognized by MAbs expressed early in haustorial development (Mackie *et al.* 1993). Molecular differentiation in the extra haustorial membrane of pea powdery mildew haustoria of early and late stage development was subsequently focussed.

Lherminer *et al* (1994) used an indirect ELISA to monitor the distribution of a mycoplasma like organism (MLO), causal agent of grapevine yellow disease in the experimental host plant *Vicia faba*, during the course of infection after inoculation by leafhopper *Euscelidius variegatus*. Post-embedding collodial gold indirect labelling was developed to identify, the various forms of MLO in the different parts of the plant by TEM. Silver enhancement of the gold probe gave accurate histological and cellular location of MLOs in tissue sections by light microscopy. Both ELISA and immunolocalization first detected MLO in roots 17 days after inoculation. ELISA indicated the occurrence of a multiplication phase in the roots 17-24 days after inoculation. MLO then reached the collar and systemically colonized the basal axillary shoot. They preferentially multiplied in this apical area. MLO bodies were located in mature seive tubes and in non-functional phloem cells on the periphery of the vascular bundles, and necrotic cells were observed in the phloem tissue. Electron dense and distorted MLOs were identified in these collapsed cells.

Polyclonal mouse ascites antibodies were raised against soluble protein extracts of chlamydospores and mycelium. The IgG fraction was purified and biotin labelled to device a fungal capture sandwich ELISA. ELISA detected both brown and grey cultural types of *T.basicola* and had negligible cross reactivity with other soil borne fungi. The minimum detection limit of ELISA was between 1 and 20 ng of *T.basicola* protein depending on the assay. *T.basicola* could be detected in cotton roots two days after inoculation. At this time, initial symptoms were apparent. The antibody also was used to observe *T.basicola* on cotton roots with immunofluorescence microscopy (Holtz *et. al.*, 1994).

Polyclonal antibodies produced against culture filtrate and mycelial extracts immunogen preparations from the soybean (*Glycine* max) fungal pathogen *Phomopsis longicolla* were purified to the immunoglobulin fraction and tested in indirect ELISA and in direct DAS ELISA (Brill *et.al.*, 1994). The PABs raised to culture filtrate were more specific but less active in binding to members of the *Diaparthe-Phomopsis* complex than were those to the mycelial extract immunogen preparation. DAS ELISA was more specific and 100 fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PABs was lower in DAS ELISA compared with indirect ELISA. Immunization of one rabit with culture filtrate over an extended time resulted in maximum anti *P.longicolla* activity after three immunization, and the activity became constant against most members of the complex at the same time. Reactivity to some culture of *P.longicolla* was undetectable following the fourth and fifth immunizations, whereas reactivity to all of other cultures of the complex remained high.

A serological test was developed by Jamaux and Spire (1994) that allows the early etection of infection ofyoung petals by *Sclerotinia sclerotiorum,* an important pathogen of rapeseed. Soluble mycelial extracts of *S.sclerotiorum* were used to produce the first generation antiserum. This was not specific for *S.sclerotiorum* in double-DAS ELISA and allowed the screening of cross reacting fungal species such as *Botrytis cinerea,* a pathogen commonly present on rapeseed petals. Using a polyclonal anti-*B.cinerea* serum enabled the adsorption by serial cycles, of *S.sclerotiorum* antigens commonly to *B.cinerea.* Residual antigens were used as immunogens for the production of two second generation antisera (S1 and S2) which were then tested by DAS-ELISA. Cross reactions with *B.cinerea* decreased with purification cycles of the immunogen whereas cross-reactions with some unrelated fungi slightly increased. *S.sclerotiorum* and *B.cinerea* were distinguishable using antiserum S2.

An immunoassay for the detection of phomopsin was used by Williamson *et al,* (1995) to detect levels of the mycotoxin in epidermal peels from resistant and susceptible lupin cultivars asymptomatically infected with *Diaporthe toxica.* Quantifiable levels (>6,25μg/kg) of phomopsin were detected in susceptible lupin cultivars, but not in very resistant breeding lines or in the control. These differences reflects the differences in resistance observed in microscopical assays and mature plants in the field.

Wakeham and White (1996) raised polyclonal antisera against whole (coded : 16/2), and sonicated (coded : 15/2) resting spores of *Plasmodiophora brassicae* as well as soluble components prepared by filtration and ultracentrifugation (coded : SF/2), Cross-reactivity of all three antisera with a range of soil fungi, including *Spongospora subterranea* was low. Test formats including Western blotting, dip-stick, dot blot, indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence were assessed for their potential to detect resting spores of *P.brassicae* in soil. Dot-blot was least sensitive, with a limit of detection level of 1×10^7 resting spores g^{-1} in soil. With Western blotting the lower limit of detection with antiserum 15/2 was 1×10^5. This antiserum showed the greatest sensitivity in a dip-stick assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of 1×10^2. The indirect ELISA was successful only after the substitution of alkaline phosphatase by protein. A conjugated horseradish peroxidase. Of the assays tested, indirect immunofluorescence appears to be the most rapid and amenable assay for the detection in soil of low levels of resting spores of *P.brassicae.*

Polyclonal antiserum was raised against mycelial extract of *Pestalotiopsis theae* and immunoglobulin fractions were purified by ammonium sulfate fractionation and

chromatography on DEAE sephadex. In ELISA, antiserum dilution upto 1:16000 detected homologous antigen at 5 mg/L concentration and at 1:125 antiserum dilution fungal antigens could be detected at concentration as low as 25 μg/L. In 15 varieties of tea tested, from Darjeeling, UPASI and Tocklai Research Stations, absorbance values of infected leaf extracts were significantly higher than those of healthy extracts at 40 mg/L concentration in indirect ELISA. ELISA positive material was detected in tea leaves as early as 12 h after inoculation with *P.theae.* At serum dilution upto 1:125, pathogen could be detected in infected leaf extracts upto 2 mg/L concentration. The results demonstrated that ELISA can be used for early detection of *P.theae* in leaf tissues even at very low level of infection (Chakraborty et al, 1996).

Serological differentiation among fungal species

It is possible to differentiate fungal species on the basis of serological relationship. Serological cross reactivity within species is found to occur in several cases and this is an area of research which is also receiving much attention.

Three species of the genus *Phytophthora* were studied serologically by means of gel diffusion and immunofluorescence. The type and concentration of the nitrogen source, the amount of inoculum, and the age of mycelium greatly affected the antigenic efficacy of the preparations. The antigens were found to be localized in the young growing tips of the hyphae. Species specific sera were obtained and proved useful and efficient for the identification of *P.cactorum, P.cinnamoni* and *P.erythroseptica* (Burrell *et al*, 1966).

Amos and Burrell (1967) reported serological differentiation in the genus *Ceratocystis* by using the serological techniques of agglutination, gel diffusion and immunofluorescence. Eight species within the genus *Ceratocystis* were identified. All three serological techniques proved to be applicable in the identification of three fungi. It was found necessary to absorb selectively cross-reacting antibodies from the sera to make them species specific. The immunofluorescence techniques proved to be most useful in differentiating among these species. Although all of the species were shown to have serological differences, no differences could be seen in the A and B compatibility types of *C.fagacearum.*

Immunodiffusion tests were used to study the taxonomic relations of six heterothallic species of *Phytophthora. P.cinnamomi* was serologically distinct, whereas two serological groups were evident among the five closely related species. When tested with antiserum to *P.arecae, P.citrophtoora,* and one isolate of *P.palmivora* comprised one group, and *P.meadii, P.mexicana* and one isolate of *P.palmivora* the second group. The five species were serologically identifical when tested with antisera to either *P.meadii* or *P.palmivora.* On the basis of their results Merz *et al.* (1969) suggested that the five species might best be incorporated into one, *P.citrophthora.*

The immunological responses of *Verticillium alboatrum* and *V.nigrescens* pathogenic to cotton were compared by Wyllie and DeVay (1970). On the basis of antigenic pattern *Verticillium* sp. were definitely differentiated from one another. Defoliating strain of *V.alboatrum* (T9) was shown to differ antigenically from the non defoliating strain (SS4). It appeared to be more closely related serologically to the midly virulent *V.nigrescens* isolates than was the defoliating T9 isolates.

The serological cross-reactivity of *Sporothrix schenckii* with various unrelated fungi was investigated. A strain specific antiserum prepared against the mature hyphae of *Fusarium culmorum* was used by Hornok and Jagicza (1973) for direct and indirect methods of the fluorescent antibody staining technique. There was a significant difference in the intensity of fluorescence between the mycelia of the homologous *F.culmorum* strain and those of the heterologous *F.graminearum* and *F.culmorum* strains. All the other *Fusarium* species tested showed no detectable fluorescence. The heterologous strains of *F.culmorum* and *F.graminearum* could not be distinguished. No significant difference was found between the 'culmorum' strains from different host plants. The serum specific for *F.culmorum* and *F.graminearum* was suitable to separate these species from other fungi.

Soluble proteins from the mycelia of 30 isolates of *Phytophthora cinnamoni* collected by Gill and Zent (1977) from 17 different hosts and from widely separated geographic locations and of five isolates of *P.cactorum,* when fractionated by disc electrophoresis, yielded 22 and 26 bands with different densities. The two species differed markedly and each exhibited its distinct, characteristic protein pattern making it possible to

identify them. With one exception, there was little or no variation in the protein patterns within the isolates of *P.cinnamoni*. Also, identical or nearly identical protein patterns of each species were obtained regardless of date of isolation, host on geographic locality. No differences in the sprotein patterns were seen between the mating types of *P.cinnamoni*.

Savage and Sall (1981) used radioimmunosorbent assay (RISA) for somatic antigens of the pathogen *Botrytis cinerea* to detect the presence of *Botrytis* antigens in homogenized samples. As little as 100 ng of original fungal dry weight could be detected and the sensitivity curve was log-log linear in response upto 10 mg. The assay was highly specific for *B.cinerea* although some reaction was obtained with other members of Sclerotiniaceae. *B.allii* showed 48% reactivity relative to *B.cinerea,* and species of *Sclerotinia* showed 10-24% reactivity. All other fungi tested showed less than 0.1% reactivity. The usefulness of the assay for detection of the fungus within host tissue was demonstrated by the high correlation of the assay results with an estimation of rot weight from field-infected lots.

Ishizaki *et al* (1981) by use of immunodiffusion test. A rabbit anti-*S.schenckii* sera was obtained which reacted with *Cladosporium werneckii, C.carrionii, C.bantianum, Coccidioides immitis, Phialophora geanselmei, P. gougerotii, P.dermatitidis, Fonsecaea pedrosoi, Aspergillus fumigatus, Histoplasma capsulatum* and *Trichophyton mentegrophytes,* but not with *Saccharomyces cerevisiae* antigens. The serological determination responsible for the cross reactions were suggested to be galactosyl residues.

Four formae speciales of *Fusarium oxysporum (dianthi, melonis, pisi, lycopersici*) and three physiological races (1,2,3) of *F.oxysporum* f.sp. *melonis* have also been differentiated using specific antisera and the techniques of double diffusion and radial double diffusion in agar (Iannelli *et. al.* 1982). Three isolates of *F.oxysporum* f.sp. *dianthi* race-2 and 10 of f.sp. *lycopersici* race-1 tested against the appropriate antisera reacted the same within each race.

Immunologic analyses of teliospore surfaces by Banowetz *et al* (1984) using polyclonal antisera and monoclonal antibodies indicated that *Tilletia controversa* and *Tilletia caries* were very similar. Although two polysaccharide antigens were present in teliospore extracts, these components appeared to be immunologically identical in both species and no protein antigens were demonstrated by either electrophoretic or immunologic means. Spectrophotometric analyses of surface extracts also indicated no extractable protein. None of seven fluorescein-labelled lectins bound these teliospores, even after the spores were treated

with 8M Urea to enhance exposure of potential lectin-binding sites. An antibody 'double sandwich' enzyme immuno-assay demonstrated quantitative differences in the numbers of certain monoclonal antibody binding sites of the two fungi although these differences did not provide a basis of the unambiguous detection of either bunt species in contaminated wheat shipments. No quantitative distinction of the two species was found by any of the methods used in this study.

Immunological comparison between isolates of *Botrytis cinerea* by crossed immunoelectrophoresis has been done by Ala-El-Dein and El-Kady (1985). CIE techniques showed that the tested isolates were serologically different; some antigens were specific for each isolate, *Botrytis cinerea* isolate no.1 had four specific antigens; these antigens were absent in the other isolates. At least sixteen antigens were common in the isolates tested. Some isolates were serologically similar when tested by double gel diffusion test, while they were distinguishable when CIE techniques were used. Numbers of precitptin peaks obtained with CIE techaniques were more than double the number of precipitin lines detected with double gel diffusion test. Results revealed that CIE techniques could be used as valuable analytical tools in resolving the spectrum of antigens present in *Botrytis cinerea* isolates. They also compared the antigenic structures of *Botrytis cinerea, B.tulipae, B.paeoniae* and *B.allii* isolates by using CIE techniques. Antisera against antigens of these isolates gave 24, 15, 20 and 15 precipitin peaks, respectively, when analyzed in homologous reactions, CIE with an intermediate gel and CIE with antibody absorption *in situ* reacted that each isolate was serologically different from the other and had species-specific antigens. Eight antigens distinguished *Botrytis cinerea* from the other species of *Botrytis*, these were present only in the former species. *Botrytis allii* had less common antigens than the other species. These results showed that CIE techniques were more successful and sensitive tools for these comparative studies than the double gel diffusion test.

Immunofluorescence tests, involving 34 species of fungi, were carried out on an anti-*Mycena galopus* serum raised against a partially purified antigenic fraction (Chard *et al,* 1985). Cross reacting fluorescence was produced primarily by *Mycena,* deuteromycetes and ascomycete species, non-*Mycena* basidimycetes generally showing less fluorescence. Absorption of the antiserum with mycelium from cross reacting fungi resulted in a reduction in fluorescence of cross-reacting species, mostly to an acceptable control level.

Cross-reactivity between antisera produced against fimbriae of either *Ustilago violacea* (AU) or of *Rhodotorula rubra (*AR) and cell surface proteins of two ascomycete fungi,

Ascocalyx abietina and *Ophiostoma ulmi* was revealed by means of dot-immunobinding and immunocytochemical methods. Following treatment with antiserum AR, the walls, septa, and plasma membrane of *A.abietina* and *O.ulmi* cells were appreciably labeled by gold particles, but the labelling intensity was always found to be greater over the plasma membrane. The fibrillar sheath surrounding cells of *A.abietina* reacted with antiserum AU while all other structures did not. No significant labelling with this antiserum occurred over cells of *O.ulmi* indicating that they either lacked these antigens or that these were more easily removed during the fixation process (Benhamou *et al,* 1986).

The agglutinating effect of 5 lectins having different sugar binding specificities on the conidia of a variety of strains from 8 *Fusarium* sp. were examined by Christenzio *et.al.* (1988). Conidia of all the strains of *F.solani* and *F.oxysporum* were strongly agglutinated by concanavalin A and *Helix pomatia* agglutinin; D-mannose (and D-glucose) and N-acetyl D-galactose amine inhibited the agglutination induced by concanavalin A and *Helix pomatia* agglutinin, respectively. Conidia of the other species of *Fusarium* examined (*F.culmorum, F.sambucinum, F.graminearum, F.avenaceium, F.moniliforme* and *F.xylarioides*) were not agglutinated by either lectin. No conidia from the *Fusarium* sp. were agglutinated by wheat germ agglutinin, potato lectin, *Ulex uropeas* agglutinin-1. These results have provided some insight into the chemical nature of the outermost layer of the fusaria conidia and this information may be of taxonomic significance.

The potential of polyclonal antisera and monoclonal antibodies to differentiate the EAN and NAN aggressive subgroups of *Ophiostoma ulmi* was explored by Dewey *et al,* (1989). Polyclonal antisera, when tested by ELISA, cross reacted widely with unrelated species and failed to distinguish between the two aggressive subgroups but small quantitative differences were found, particularly between antigens secreted overnight by EAN and NAN germlings. Out of 33 cell lines that secreted monoclonal antibodies specific to *O.ulmi*, one third were non-specific, 11 were specific either to species or subspecies. Two cell lines differentiated mycelial antigens of the aggressive isolates of *O.ulmi* from those of the non-aggressive subgroup, but not antigens from surface washings. Only quantitative differences were detected between the EAN and NAN aggressive subgroups. Almost all the monoclonal antibodies and antiserum recognized antigens present in surface washings of cultures on solid medium, in cell free extracts of mycelial homogenates, in cell free culture fluids, and in substances secreted overnight by germinating spores. Most of the monoclonal antibodies appeared to have potential diagnostic value, since they gave readings two-fold to ten-fold higher with extracts from diseased than from healthy tissue.

Soluble proteins from 34 isolates representing 16 species of the genus *Phytophthora* from different geographic locations and host plants, when analysed by two dimensional electrophoresis, were resolved into 100 to 200 protein spots with different densities (Masago *et al*, 1989). Protein patterns differed markedly among different species and each species exhibited a distinctive and characteristic pattern. Although slight differences were occasionally observed in patterns of faintly-stained minor protein spots, those of densely stained major protein spots were highly similar, if not identical, among isolates of the same species regardless of their geographic origin, original host plant, mating type, or virulence race. Authors have suggested that the two dimensional electrophoretic patterns, being species-specific may be a useful measure to differentiate and identify species of the genus *Phytophthora.*

Fuhrmann *et al.* (1989) tested antisera produced against *Penicillium verrucosum* var. *verrucosum* for their reactivity with 44 strains of moulds by immunofluorescence and by indirect enzyme linked immunosorbent assay. Antigenically *P. verrucosum* var. *verrucosum* (subgenus *Penicillium*) appeared to be similar to strains belonging to subgenus *Furcatum* but strongly different from *Penicillium frequentans* (subgenus *Aspergilloides*). Specific absorption of antibodies to antigens confirmed the existence of similar biochemical structures on *Penicillium frequentans, Aspergillus versicolor,* and *Aspergillus fumigatus.*

Kitagawa *et al* (1989) have developed competition types of 2 novel enzyme linked immunosorbent assays (ELISA) for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* as well as for general detection of 10 strains of common *Fusarium* sp. that show specific pathogenecities to different plants. Antiserum against a strain of *F.oxysporum* f.sp. *cucumerinum* (F 504) was elicited in rabbits and a highly specific, sensitive and accurate ELISA for the homologous strains was developed by using the antiserum with β-D galactosidase labelled anti rabbit IgG as the secondary antibody and cell fragments of the strain to amino dylark balls as the solid phase antigens. This assay was specific for strain F 504 and showed little cross reactivity with 9 other strains of *Fusarium* sp. including strains 501 of *F.oxysporum* f.sp. *cucumerinum.* Strain F 501 possessed pathogenicity against cucumber similar to that of strain F 504 although slight differences have been observed between these two strains regarding their spore formation and pigment production.

Antibodies of three isolates each of *Armillaria mellea, A.astoyae, A. tabescens and Lentinula edodas* were tested against the different isolates by an enzyme-linked immunosorbent assay (ELISA). The cross reactivity of the antibody preparations to a given

Armillaria species varied considerably when tested against isolates of other *Armillaria* species. Several antibody preparations were capable of distinguishing isolates of the homologous species from isolates of the heterologous species. The specificity of the antibodies present in eggs was dependent on time elapsed since immunization. Eggs laid 3 weeks after immunization with *Armillaria* species isolate possessed antibodies that were most specific for isolates of that species. The intergeneric cross-reactivity was found to be smallest with antibodies from eggs laid 5 weeks or more after immunization. (Burdsall, 1990).

Monoclonal antibody (Mab) LK50 was developed against *Leptosphaeria korrae* strain ATCC 56289 (Nameth et al, 1990). The antibody was capable of detecting *L.korrae* in cultures and in the naturally infected bluegrass samples from three states. In cross reactivity tests using indirect ELISA, MAb reacted positively to all 24 isolates of *L.korrae.* MAb reacted negatively with 38 of 42 isolates of related and non related fungi and negatively to apparently healthy grass. The limit of detection was less than 2 μg/ml of lyophilyzed mycelia

Daniel and Nilsson (1991) reported that polyclonal antiserum raised against mycelial extracts of the rot fungus *Phialophora mutabilis* reacted strongly with its homologous antigen and cross reacted strongly to moderately with six other *Phialophora* soft. rot spp. in ELISA. With the exception of *Ceretocystis* sp. the serum reacted weakly or not at all with 11 other mold, blue and rot fungi. The antiserum cross reacted strongly with antigens in extracellular filtrates from *P.mutabilis* cultures that contained about 40 ng/ml protein.. Ultrastructural and immunocytochemical studies on wood degraded by *P.mutabilis* showed specific localization of the antibody on the fungal cell wall and certain intracellular structures. The antiserum was assessed by ELISA for detecting the presence of the fungus and soft rot in untreated and preservative treated wood blocks of pine and birch degraded for periods of 1-12 months. *P.mutabilis* was detected in samples from all wood blocks degraded to low or high wt. loss. Highest ELISA readings were recorded for wood blocks with highest substrate losses and vice versa.

Extracts from *Fusarium oxysporum* (F.O) and *F.oxysporum* var. *redolens* (F.O.r.) isolates were compared by means of electrophoresis and cross immunoelectrophoresis (Rotej Guranowska and Walko, 1991). Both fungi appeared almost identical serologically. Relative amounts of their corresponding proteins differed but the quantitative patterns of the proteins were nearly the same. With the anti-*F.oxysporum* var. *redolens* serum, only one specific antigen was detected in the extracts from *F.oxysporum* var. *redolens* isolate. Although the

results obtained indicated a strong similarity between *F.oxysporum* and *F.oxysporum* var. *redolens.* The authors considered that they could not be separated into 2 species.

The sensitivity of a *Phytophthora* specific immunoassay kit was tested by Pscheidt (1992) on 17 species of *Phytophthora* collected throughout the world,. including 18 isolates each of *P.cinnamomi* and *P.cactorum;* kits were also used in the diagnosis of plant specimens with symptoms characteristic of *Phytophthora* infection. All *Phytophthora* isolates tested produced a positive reaction with the immunoassay kit. The lowest absorbances relative to other species were obtained from *P.cinnamomi* and *P.megasperma* (originally isolated from cherry). Variation in absorbance was high among isolates of *P.cinnamomi* but low among *P.cactorum.* Clinic samples with typical symptoms of *Phytophthora* infection produced a positive reaction with the immunoassay as did pure culture of *Phytophthora* sp. isolated from these samples. Cross-reactions occurred with several *Pythium* spp. isolated from clinic samples and with several specimens infected with *Peronospora* spp. Other samples without typical *Phytophthora* symptoms but associated with other pathogens did not produce a positive reaction with the immunoassay.

Two monoclonal antibodies and three polyclonal antisera were raised to cell wall/ membrane fractions of *Pythium violae* and *Pythium sulcatum.* When screened with a collection of 40 isolates of the genus *Pythium* including 20 species and the H-S group there was extensive cross reaction. However, when the binding of the antibodies was assessed in an enzyme-linked immunosorbent assay using cytoplasmic fraction antigens, the combined recognition patterns produced profiles unique to each species. Multivariate analysis methods were used to establish relationships between isolates on the basis of these profiles; isolates of the same species tended to group together. The affinity of these reagents for fungi other than members of the genus *Phytophthora* was low (White *et. al.*, 1994).

Materials and Methods

3.1. Plant material

3.1.1 Collection

Tea clones were collected mainly from three experimental stations from different geographical locations of India: (a) Tocklai Experimental Station, Jorhat, Assam (b) Darjeeling Tea Research Centre, Kurseong (c) United Planter's Association of South India(UPASI) Tea Research Station, Valparai, Tamilnadu.

Thirteen TV clones (TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-29, TV-30, TS-449 and Teenali 17/1/54) released by Tocklai Experimental Station were collected from the clone house of Mohurgaon and Gulma Tea Estate, Sukna, W.Bengal.

Fresh clonal cuttings of 15 Darjeeling vatrieties (BS/7A/76, B668, P312, B777, T-78, P-1258, B-157, TTV_1, CP-1, BT-15, AV-2, RR-17, HV39, K1/1 and T-135) generally grown in hilly regions were collected from Darjeeling Tea Research Centre, Kurseong, W.Bengal.

Cuttings of 6 clonal varieties (UPASI 2, UPASI 3, UPASI 8, UPASI 9, UPASI 17 and UPASI 26) and seeds of 3 varieties (BSS1, 2 and 3) were collected from UPASI Tea Research Station, Valparai, Tamilnadu.

3.1.2. Propagation

Tea plants are usually propagated either by cuttings or by seeds.

3.1.2.1 Cutting

The Sandy soil (sand 75% and soil 25% with a pH ranging from 4.5 to 4.8)was used for clonal propagation. Soil pH was adjusted by treating with 2% aluminium sulphate solution. It was followed by two waterings to remove excess aluminium sulphate. The heated soil was treated between 60°C to 80°C on a metal sheet with fire below to kill eelworms, if any, present in the soil.

Polythene sleeves (6") were filled up with the prepared soil and stacked in rows in a bed and then watered thoroughly. All cuttings were allowed for rooting in sleeves after dipping them in hormone obtained from UPASI Tea Research Centre mixture. These cuttings were kept in a polythene cloche and watered every 4th day, until new leaves were appeared. (Plate II, figs. A-D).

3.1.2.2 Seed germination

For the seed germination, pre treated (heat treated) seived sand was used to prepare beds of about 15 cm thickness. Sand in the bed was slightly compacted and watered thoroughly prior to putting out the seed. The seeds were treated with a 0.03% suspension of Dithane M45 and then sown in rows at a spacing of 5.0x5.0 cm triangle. Seeds were pressed down in the bed with the sear directed downwards; the dome of the seed was projected slightly above the level of the bed. The seed beds were then covered with a thick polythene sheet and watered when necessary.

At the "cracking stage" (between 25 and 35 days) the seeds were transplanted into the sleeves. Then all these sleeves were covered by polythene cloch and watered as and when necessary. At the four leaved stage the cloch was removed.(Plate III, figs. A-D)

3..1.3. Plantation

Before planting, simazine @ 75g/20 litre water and Glyphospate @ 1:200 were used in the experimental plots for the suppression of weeds (Borpujari & Banerjee; 1994). Then pits ($1^1/_2$' x $1^1/_2$' x $1^1/_2$') were dug at the intervals of 2 ' between plant and 3.5' between row to row. Planting mixture was prepared in the ratio of 4.5 kg well rotten dry cattle manure , 30 g rock phosphate, 30g super phosphate and 2.5 g phorate [0,0- diethyl S- (ethylthiomethyl) phosphoro dithioate]. At the botom of each pit, rock phosphate was placed following which half portion was covered with cattle manure soil mixture. Phorate was mixed with a portion of excavated soil and was applied approximately 5 cm below the ground level.

Following soil conditioning, plants were inspected, selected and brought to the experimental garden and planted in the prepared soil. 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). All these plants were maintained inside the Glass house under natural condition. (Plate IV, figs. A-D; Plate-V, fig. A-D)

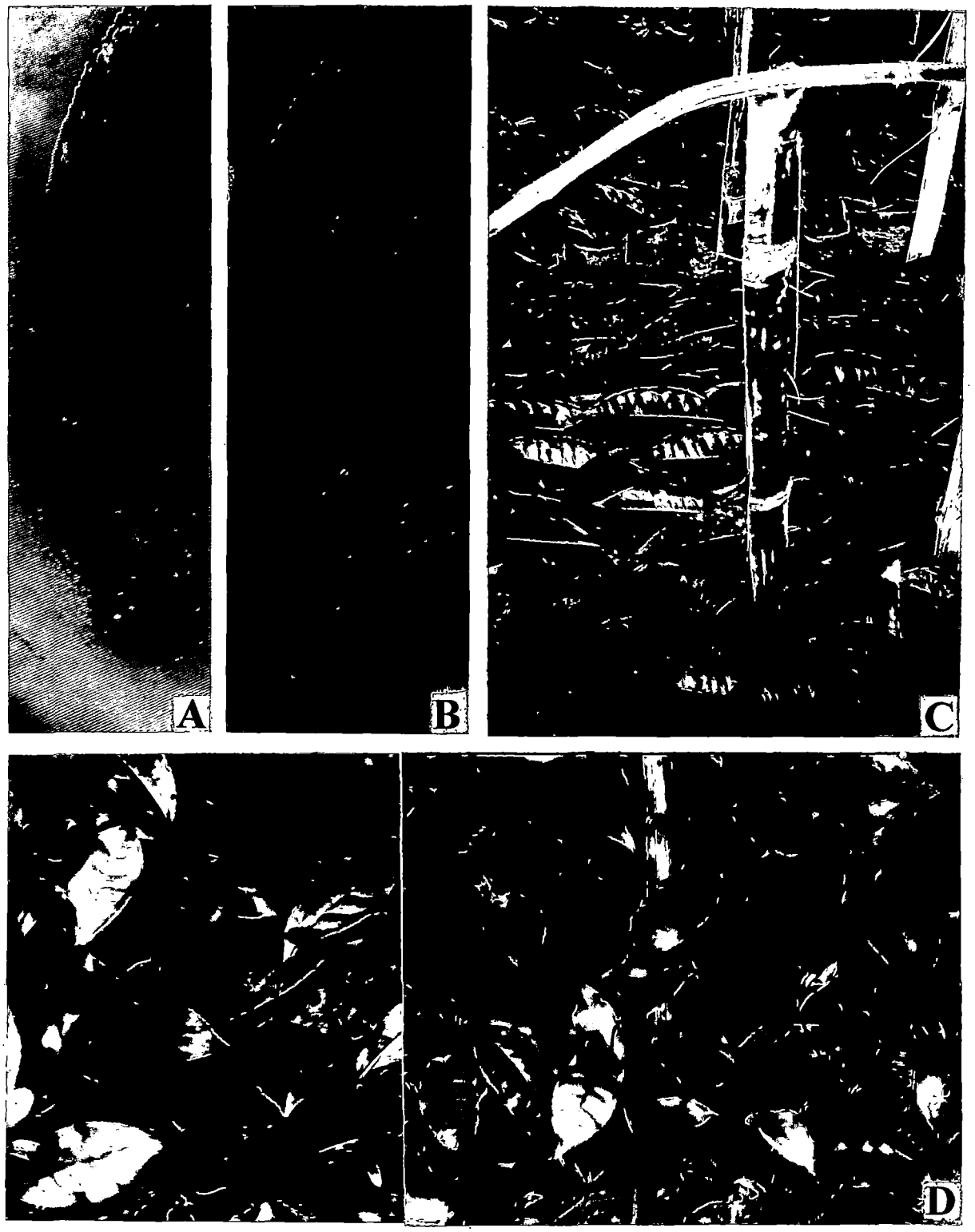


Plate II(figs. A-D) . Stages in propagation of tea (UPASI 9) by cuttings .
A- nodal cutting with excess callus ; **B**-nodal cutting after rooting ;
C- cuttings in plastic sleeves ; **D**-shoot development from cutting

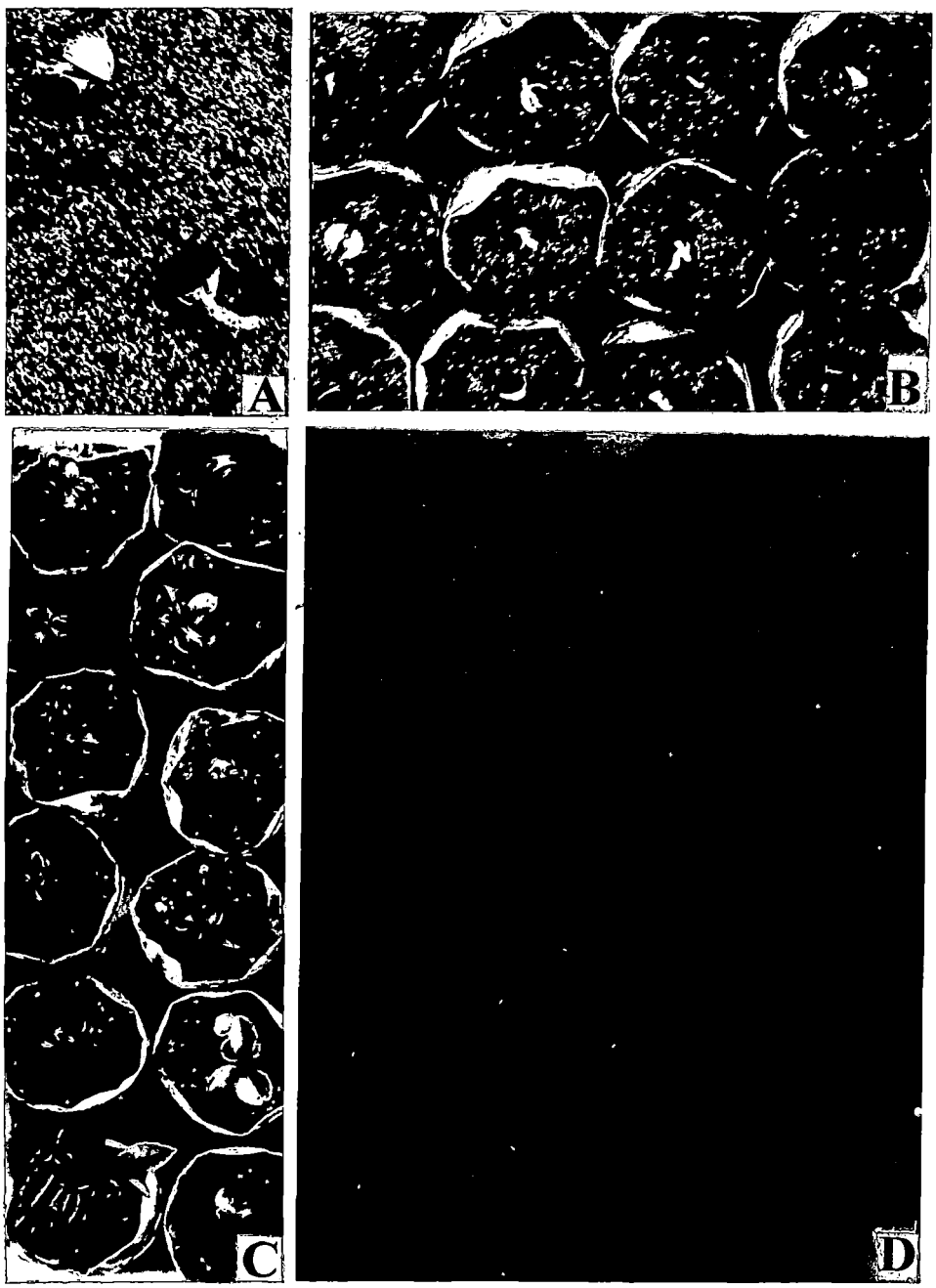


Plate III (figs. A-D). *Stages in propagation of tea by seed germination (BSS-3).* **A** - seed cracking in the bed ; **B** - seeds after cracking transferred to polythene sleeeves; **C**- seedlings (3- 4 leaved stage) ; **D**-seedling after being transferred to pot

PLATE IV (figs.A-D). Cuttings of tea in plastic sleeves **(A&B)** and after transfer to the field **(C&D)**.
A&C - UPASI 26 ; **B&D** - UPASI 9

PLATE V (figs.A-D). Potted plants of Tocklai varieties of tea maintained in glass house

3.1.4. Maintenance

In case of young plants manuring (Ammonium sulphate-8 parts by wt. Ammonium phosphate-sulphate(16:20)-35 parts by wt.; Potassium sulphate-15 parts by wt.; Magnesium sulphate-15 parts by wt. and Zinc sulphate-3 parts by wt.) was done after rooting following the method by Ranganathan and Natesan (1987) and continued upto 12 months once only in 15 days. The mixture was dissolved @ 30 g in 1 L of water and applied @ 50 ml/plant.

The mature plants (1 year & above) were maintained by applying a soluble mixture of N,P,K consisting of 10 kg Urea-46% N, 20 kg ammonium phosphate- 11% P_2O_5, 8 kg muriate of potash-60% K_2O in the soil. Grenol (Triacontanol) was sprayed at regular intervals for good growth of bush.

Only tipping was done once to promote lateral branching in young plants (3 years) but in case of mature plants 2-year deep pruning cycle was maintained.

Besides three other plants species such as *Glycine max* (cv. Soymax), *Cicer arietinum* (cv. JG-62) and *Camellia japonicum* were grown in earthen pots and maintained in the glass house. Seeds of *G.max* (cv. soymax) and *C.arietinum* (cv.JG-62) were collected from Pulses and Oil seed Research Station, Berhampore and International Crop Research Institure for Semi Arid Tropics (ICRISAT), Hyderabad respectively, while *C.japonicum* plants were obtained from the Botanical Garden, Darjeeling.

3.2 Fungal culture

3.2.1 Source of culture

Glomerella cingulata (Stoneman) Spauld & Schrenk isolated from naturally infected tea plants (TV-18) was identified at the International Mycological Institute, U.K., and was designated as GC-1 (IMI number-356806). Three more isolates of *G.cingulata* (GC-2,3 and 4) were collected from naturally infected leaves of TV-23, 27 and Teenali 17/1/54 respectively. Two other isolates (GC-5 and 6) were collected from naturally infected leaves of TV-9 of Chandmani Tea Estate. From Tirrihana Tea Estate, GC-7 was collected. GC-8 and GC-9 was collected from naturally infected leaves of UPASI-3.

Besides this a non-pathogen of tea, *Fusarium oxyporum* Schlecht (ITC number -2389) was obtained from Indian Type Culture Collection Centre, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. Five different species of *Colletotrichum* were also collected from IARI, New Delhi and used in different experiments.(Table-1)

Table 1: List of species of *Colletotrichum* and their isolates used.

Organism	ITCC number	Host
Colletotrichum gloesporioides	1726	*Musa* sp.
Colletotrichum gloesporioides	1809	*Capsicum annuum*
Colletotrichum papayae	1269	*Carica papaya*
Colletotrichum lindemuthianum	1119	*Phaseolus vulgaris*
Colletotrichum lindemuthianum	1764	*Phaseolus vulgaris*

A virulent strain of *Pestalotiopsis theae* Sawada (IMI No. 356807). and *Corticium invisum* Petch was collected from culture collection of Mycology and Plant Pathology Laboratory of the Department

3.2.2. Completion of Koch's postulate

Fresh, young tea leaves were collected from Phytopathological Experimental Garden and inoculated with conidial suspension of the isolated *Glomerella cingulata* following detached leaf inoculation technique. After 96h 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 Richard's medium (RM) slants. These isolates were examined after 15 days of inoculation at 30°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 RMA (Richard's Medium Agar) 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, all the isolates of *Glomerella cingulata* were also examined at regular intervals to test their pathogenicity.

3.2.4. Assessment of Mycelial Growth

3.2.4.1. Solid media

To assess mycelial growth of *G. cingulata* (GCI to GC 9) in solid media, the fungus was first grown in petridishes, each containing 20 ml of RM and incubated for 7 days at 30°C. Agar block (4mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. Following solid media were used for assessment of growth.

Potato dextrose agar (PDA)

Peeled Potato	-	40.00g
Dextrose	-	2.00g
Agar	-	2.00g
Distilled water	-	100ml

Richards medium (RM)

KNO_3	-	1.00g
K_2HPO_4	-	0.50g
$MgSO_4, 7H_2O$	-	0.25g
$FeCl_3$	-	0.002g
Sucrose	-	3.00g
Agar	-	2.00g
Distilled water	-	100 ml

Carrot Juice Agar (CJA)

Grated Carrot	-	20.00g
Agar	-	2.00g
Distilled water	-	100 ml

Czapek-dox agar (CDA)

$NaNO_3$	-	0.20g
K_2HPO_4	-	0.10g
$MgSO_4, 7H_2O$		0.05g
KCl	-	0.05g
$FeSO_4, 7H_2O$	-	0.05g
Sucrose	-	0.001g
Agar	-	3.00g
Distilled water	-	100 ml

Flentze's soil extract agar (FSEA)

Soil extract	-	1L
Sucrose	-	1.00g
KH_2PO_4	-	0.20g
Dried yeast	-	0.10g
Agar	-	25.00g

All these petridishes were then incubated at 30°C for the desired period. Finally the mycelia were strained through muslin cloth, collected in aluminium foil of known weight , dried at 60°C for 96h , cooled in a desiccator and weighed.

3.2.4.2. Liquid media

To assess the mycelial growth of *G. cingulata* (GC-1) in liquid medium, the fungus, was first allowed to grow in petridishes containing 20 ml of RMA and were incubated at 30°C for 7 days. From the advancing zone, the mycelial block (4mm-dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50 ml of sterilized Richards medium for the desired period at 30°C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60°C for 96h , cooled in a desiccator and weighed.

3.3. Inoculation techniques

3.3.1. 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 form plants were placed in plastic trays lined with moist blotting paper. Wounds were made on the adaxial surface of each leaf and inoculated with 20ml droplets of spores suspensions (1.2 x 10^6 conidia/ml) of the fungus (prepared from 10-day old culture). Spore suspensions were placed (2-4 drops/leaf) on the adaxial surface of each leaf with a hypodermic syringe on the wounds. 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.

3.3.2. Cut Shoot

Cut shoot inoculation technique was followed as described by Yanase and Takeda (1987). Twigs (with 3-4 leaves) of tea plant grown in the Experimental 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 (Cook, 1989) and placing mycelial plugs on the scratches. Mycelial plug inoculated cut shoots were inserted into the holes of styrofoam board which was floated on modified Hoagland and Knops solution and kept in a glass chamber (72cmx33 cmx 30 cm) for one week with aeration.

3.4. Assessment of disease intensity

3.4.1. Detached leaf

Percent drops that resulted in lesion production was calculated after 24, 48, 72 and 96 h of inoculation as described by Chakraborty and Saha (1994 a), diameter of the lesion were noted. Observations were based on 50 inoculated leaves for each treatment and average of three separate experiments.

3.4.2. Cut shoot

In laboratory experiments, the actual number of lesion that developed on the artificially inoculated shoots were counted after 24, 48, 72 and 96h. Diameter of the individual lesions were measured. They were graded into 4 groups and a value was assigned to each group, Very small restricted lesion, 1-2 mm dia. = 0.1; 2-4 mm dia. with sharply defined margin=0.25; lesion,with slow spread beyond 4mm=0.5 and spreading lesion,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 Preparation of antigen

3.5.1. Fungal antigen

3.5.1.1. Mycelia Mycelial antigen was prepared following the method of Chakraborty & Saha (1994). Initially the fungal mycelium (4mm disc) were transferred to 250 ml Ehrlenmeyer flask each containing 50 ml of sterilized liquid Richard's medium (g/l distilled water, sucrose, 30; KNO_3, 10; KH_2PO_4, 5; $MgSO_4$, $7H_2O$, 2.5 and /$FeCl_3$, 0.02) and incubated for 10 days at 30 $\pm$ 1°C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (50 g fresh wt.), were homogenized with 0.05 M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5mM magnesium chloride and 0.85 NaCl in mortar and pestle in the presence of sea sand. Cell homogenates were kept overnight at 4°C and then centrifuged (15000g) for 30 min at 4°C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overight at 4°C. After this period the mixture was centrifuged (15000g) for 30 min at 4°C, the precipitate was dissolved in 10 ml 0.05 M sodium phosphate buffer (pH 7.2). The preparation was dialysed for 72 h. through cellulose tubing (Sigma Chemical Co. USA) against 1 L of 0.005 M sodium phosphate (PH 7.2) with ten changes. Then the dialysed material was stored at -20oC and used as antigen for the proeparation of antiserum and other experiments.

3.5.1.2. Cell Wall

Isolation of cell wall was done following the method of Keen & Legrand (1980). Mycelium of 8 day old log phase fungus culture was collected on filter paper in Buchner funnel and 50g of fresh packed cells were ground for 1 min in a high speed blender with water (4 ml/g). The resulting slurry was then disrupted in a homogenizer for 1 min at 5° C. The mixture was centrifuged for 1 min at 1500 g, the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10ml/g) and pelleted by several centrifugations until the supernatant fluids were visually clear. Finally, the isolated cell walls were dissolved in 0.05 M phosphate buffer saline (pH 7.2) and kept at -20° C until further requirement.

5.2 Leaf Antigen

3.5.2.1. Healthy Leaf

Antigens from healthy leaves were prepared following the method of Chakraborty & Saha (1994b). Fresh, young healthy leaves were collected from the experimental garden and kept at 4°C. Then the leaves were weighed & crushed in mortar & pestle with 0.05 M sodium phosphate buffer supplemented with 10 mM sodium metabisulphite, 2 mM PVPP 10,000 (soluble) and 0.5 mM magnesium chloride (pH 7.2). At the time of crushing with sea sand, insoluble PVPP of equal weight was used. The leaf slurry was strained through a muslin cloth and then centrifuged (15,000g) for 30 min at 4°C. The supernatant was used as healthy leaf antigen and was kept at -20°C until required.

3.5.2.2. Artificially inoculated leaf

Antigen from the *G.cingulata* inoculated leaves were extracted following the method of Alba & 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 inoculated with drops of conidial suspension (1.2 x 10^6 spores) of *G.cingulata.* Control sets were prepared by mounting the leaves with drops of sterile distilled water. Antigens were prepared from inoculated leaves as described earlier. The prepared antigens were stored at -20°C until further experimental purposes.

3.5.2.3. Naturally infected leaf

For the extraction of naturally infected leaf antigen, the infected leaves were collected from the experimental garden and kept at 4°C. Then the infected portion of leaf was cut into small pieces, weighed and antigens were prepared as before.

3.6. Purification of mycelial antigen

3.6.1. Saturated ammonium sulphate fractionation

Freshly harvested mycelium (150g) of *G.cingulata* was crushed in a mortar with pestle at 4°C using sea sand and homogenized with 150 ml of 0.1(M) Tris HCl buffer (pH -7.0). The slurry was strained through muslin cloth and the filtrate was centrifuged at 15,000 g for 30 min. Finally into the supernatant finely ground ammonium sulphate crystals were added slowly with constant stirring at 4°C. Final saturations of ammonium sulphate of 20,40,60,80 and 100% were obtained. In each case, stirring was done for 6h and then it was kept overnight at 4°C for precipitation. After centrifugation at 15000g for 1h the precipitates were dissolved in 2-3ml of 0.1 M Tris-HCl buffer, pH 7.0, for each saturation level. Dissolved precipitates were then dialysed by using cellulose dialysing tubing (Sigma Co., U.S.A.) against 0.01M Tris-HCl buffer, pH-7.0 at 4°C for 72h with 6 hourly changes.

3.6.2. Ion exchange Chromatography

Ammonium sulphate precipitate of mycelial extract of *G.cingulata* was also purified by ion exchange chromatography following the method as described by Chard et. al (1995). Here ammonium sulphate precipitate of desired saturation was resuspended in 0.05M Tris-HCl buffer, pH 8.0, dialysed against this buffer for 72h with 6 hourly changes and then applied to an ion exchange column. The column material, DEAE Sephadex was first suspended in distilled water for 24h, then resuspended in 0.05M Tris-HCl, pH 8.0 and equilibrated in the same buffer after loading the column material into a 10ml column. 2ml of protein sample was loaded and the column was washed with 0.05M Tris-HCl buffer, pH 8.0, until no proteins were detected in the eluant. A sodium chloride

wash was applied (0.05 M NaCl in 0.05 M Tris-HCl , pH-8.0) until no proteins were detected and then was followed by a sodium chloride gradient (0.05-0.25M NaCl in 0.05 M Tris-HCl, pH-8.0). In each case, 20-25 fractions (3 ml) were collected from the column and analysed for their protein content (Optical density at 280 nm).

3.7. SDS-Polyacrylamide Gel Electrophoresis of Protein

3.7.1. Preparation of Gel (Tube & Slab)

Stock Solutions:

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970).

(A) Acrylamide

Acrylamide	... 30 g
Bis-acrylamide	... 0.8 g
Distilled water	... 100 ml.

(filtered and stored at 4^0C).

(B) Lower gel buffer (LGB)

(1.5 M Tris)

Tris	... 18.18 g
Distilled water	... 100.0 ml

pH was adjusted to 8.8

Sodium dodecyl sulphate (SDS) .. 0.4 g

(C) Upper gel buffer (UGB)

(0.5 M Tris)

Tris	... 6.06 g
Distilled water	... 100.0 ml

pH was adjusted to 6.8

Sodium dodecyl sulphate(SDS)... 0.4 g.

(D) Ammonium peroxidisulphate (APS)

Ammonium Peroxidisulphate (APS)	... 0.1 g
Distilled water	... 1.0 ml
(freshly prepared each time)	

Tube gel preparation

The tubes (12 cm long and 4 mm dia) were washed thoroughly, dried and rewashed with diethyl ether. Resolving gel solution was first prepared with acrylamide and lower gel buffer, degassed for about 20 min and then TEMED and APS solution were added. The composition of the solution was as follows :

Solution A	... 10.0 ml
Solution B	... 7.5 ml
Distilled water	... 12.5 ml
TEMED	... 12 μl
Solution D	... 36.0 μl

Finally the gel was cast slowly upto a height of 9 cm in a tube, overlayered with water and left for polymerization overnight. After polymerization water overlay was blotted off by blotting paper and washed with distilled water 2-3 times. Stacking gel solution was then prepared with acrylamide solution and upper gel buffer, degassed for about 20 min, TEMED and APS solution were added to the solution. The composition of the stacking gel solution was as follows :

Solution A	... 3.0 ml;
Solution C	... 5.0 ml
Distilled water	... 12.0 ml
TEMED	... 10.0 μl
Solution D	... 500.0 μl

Stacking gel solution was cast similarly as before upto a height of 1.5 cm with the help of Pasteur pipette and then overlayed with water. Finally, the gel tubes were kept 30 min for polymerization.

Slab gel preparation

For slab gel preparation, two glass plates (17 cm x 19 cm) were washed with dehydrated alcohol & dried. Then 1 mm thick spacers were placed between the glass plates at the 2 edges and the 2 sides of glass plates were sealed with gel sealing tape and kept in the gel casting unit. Resolving gel solution was prepared as described in the tube gel and cast very slowly and carefully upto a height of 12 cm by a syringe. The gel was overlayered with water and kept overnight for polymerization. Then stacking gel solution was prepared as mentioned above. After polymerization of resolving gel, water overlay was decanted off and of a 13 well 1mm thick comb was placed. Stacking gel solution was poured carefully upto a height of 4 cm over the resolving gel and overlayered with water. Finally the gel was kept for 30 min for polymerization.

3.7.2. Sample Preparation

Sample was prepared by mixing the sample protein with sample buffer whose composition was as follows:

Solution B	:	12.5 ml
Sodium dodecyl sulphate (SDS)	:	2.3g
Glycerol	:	13.0 g
β-mercaptoethanol	:	5.0 ml
Distilled water	:	100.0 ml
Bromophenol blue	:	0.005 g

First of all, 50 μl of each sample protein was taken in each eppendrof tube and 20 μl of sample buffer was mixed in each tube. All the tubes were floated in boiling water bath for 3 min. After cooling 50 μl of samples were applied per gel tube/per well in case of slab gel. Along with the samples, protein marker consisting of a mixture of six proteins (carbonic anyhydrase, egg albumin, bovine albumin, phosphorylase b, β - galactosidase and myocin of molecular weight 29, 45, 66, 97, 116 and 205 KD respectively was also taken in a separate tube, prepared as above and loaded.

3.7.3. Electrophoresis

For electrophoresis the electrode buffer was prepared as follows:

(0.025 M Tris, 0.192 Glycine)

Tris	...	18.15 g
Glycine	...	72.0 g
Distilled water	...	5.0 lit.
pH was adjusted to	..	8.3
Sodium dodecyl Sulphate(SDS)	...	5.0 g

Tube gel : Electrophoresis was performed at 1.5 mA per gel until the samples penetrated the resolving gel and then at 2.5 mA per gel for 4-5 h i.e. until the dye reached the bottom of the gel column.

Slab gel: In case of slab gel 2 mA current was applied per well upto the resolving gel and then 3 mA was applied for 3 h until the dye reached at the bottom of the slab gel.

3.7.4 Fixing

For fixing, the fixer solution was prepared as follows :

Isopropanol	... 250.0 ml
Acetic acid	... 100.0 ml
Distilled water	... 650.0 ml

Tube gel: In case of tube gel, all the gels were removed from the tube and soaked in fixer solution for 20 h.

Slab gel: In case of slab gel, the entire gel was removed from the glass plates & then the stacking portion was cut off from the resolving gel. After that the gel was soaked for 20 h for fixing.

3.7.5 Staining

The staining solution was prepared as follows :

Coomassie blue R250	...	0.1 g
Methanol	...	300 ml
Acetic acid	...	100 ml
Distilled water	...	600 ml.

At first, the gels were stained by staining solution for 1h with shaking and finally soaked with destaining solution (methanol : acetic acid : distilled water - 3:1:6) until the background became clear.

3.8 Cell wall characterization

3.8.1 Extraction

Mycelial wall extract was prepared from the isolated cell wall of *G.cingulata* following the method of Brown & Kimmins (1977). Isolated cell walls (2 g) were suspended in 80 ml ice cold 0.1 N NaOH by blending in a chilled mixer cup at full speed for 30 seconds. The suspension was stirred in ice bath for 15h and then centrifuged at 8,000g for 10 min. After centrifugation, the precipitate was washed with 50 ml ice cold water and the supernatant was neutralized to 7.0 by adding 1N HCl slowly with continued stirring at 0° C. The neutralized supernatant was finally dialysed against distilled water for 48 h with 12 hourly changes and concentrated with polyethylene glycol 6000 (PEG-6000), which was then used as crude mycelial wall extract (MWE) for SDS-PAGE analysis.

3.8.2 Protein estimation

Soluble proteins were estimated following the method as described by Lowry et.al. (1951). To 1 ml of protein sample 5 ml of alkaline reagent (0.5ml of 1% CuSO4 and 0.5ml of 2% Potassium sodium tartarate, dissolved in 50 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of Folin-Ciocalteau's reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for color development following which optical density (OD) was measured at 750 nm. Using bovine serum albumin (BSA) as standard, the protein concentrations were computed.

3.7.3 Carbohydrate estimation

Carbohydrate in the extracted cell wall was estimated following Anthrone method. To 8 ml of cell wall extract 1 ml each of 0.3 M $Ba(OH)_2$ and 5% $ZnSO_4$ was added. The mixture was incubated for 10 min. Following centrifugation, the supernatant was taken. Into the supernatant (2.0 ml) 1.8 ml of distilled water and finally 6 ml of anthrone reagent (200 mg anthrone powder dissolved in 100 ml of concentrated H_2SO_4) was added. Then it was kept in a boiling water bath for 15 min, cooled and absorbance was noted at 620 nm. Glucose was used as a standard during the estimation of carbohydrate.

3.8.4 SDS PAGE Analysis

3.8.4.1 Gel preparation

Separation gel with 10% acrylamide and stacking gel with 5% acrylamide was prepared following the method of Laemmli (1970) as described earlier for SDS - Polyacrylamide gel electrophoresis.

3.8.4.2 Sample preparation

Mycelial wall extract was dissolved in a solution of 1% SDS and 1% β mercaptoethanol and heated at 50°C for 2h. Then they were diluted by the addition of 1 volume of 4M urea containing 5% sucrose and 25-50 μl of samples were applied per gel tube. Along with the sample, protein marker consisting of a mixture of six proteins ranging in molecular weight from 29 to 205 KD was also taken in a separate tube, boiled and loaded as above.

3.8.4.3 Electrophoresis

Electrophoresis was performed at 1.5 mA per gel tube until the samples penetrated the resolving gel and then at 2.5 mA per gel tube for 5h i.e. until the dye front reaches the bottom of the gel.

3.8.4.4. Fixing

The gels were removed as described earlier and soaked either in fixer solution I (25% isopropanol in 10% acetic acid) or in fixer solution II (40% ethanol in aqueous 5% acetic acid) for protein and carbohydrate staining respectively for 20 h.

3.8.4.5 Staining

Gels from fixer solution I were stained with Coomassie blue R250 and then destained as described earlier. Gels from fixer solution II were stained with periodic acid - Schiffs (PAS) reagent as described by Segrest and Jackson (1972) with modification. Details of PAS staining solutions and the procedure was as follows : At first, five following solutions A-E were prepared.

Solution A :

Anhydrous sodium acetate (0.89 g) and hydroxylamine hydrochloride (10 g) were dissolved in 90 ml distilled water. Glacial acetic acid (0.54 ml) was added to it and final volume was adjusted to 100 ml with distilled water.

Solution B :

Periodic acid (1.0 g) and anhydrous sodium acetate (0.82 g) were dissolved in distilled water and the final volume was made 100 ml.

Solution C :

1.5g basic fuchsin was dissolved in 500 ml boiling distilled water, filtered at 55°C, cooled to 40°C, 25ml 2N HCl was added and finally 3.75 g $Na_2S_2O_5$ (Sodium metabisulphite) was added, agitated rapidly and allowed to stand stoppered in refrigerator for 6h. 1.2g charcoal was mixed to it vigorously for 1 min, filtered rapidly and stored stoppered in refrigerator.

Solution D (Prepared before use):

10%(w/v) sodium metabisulphite (5 ml) and 2N HCl (5 ml) was dissolved in 90 ml distilled water before use.

Solution E (Prepared before use) :

10% (w/v) sodium metabisulphite (5 ml) and 2N HCl (5 ml) was dissolved in 90 ml distilled water and 20 ml glycerol was added to it before use.

Procedure :

Gels were soaked in solution `A' for 15 min. then washed in running tap water. Next the gels were soaked in solution `B' for 15 min, washed in running tap water for 10 min. and then transferred to solution `c' (diluted 1:1 with distilled water just before use) for 5 min. This step was repeated thrice before washing in solution `D' for 2 min and finally washed three times for 1h each time in solution `E'.

3.8.5. Binding of FITC labelled concanavalin A

Binding of fluorescent labelled concanavalin A to mycelia as well as isolated cell wall was done by the method as described by Keen & Legrand (1980). Initially mycelia or isolated cell walls were incubated for 20 min in 0.85% NaCl in 0.01M potassium phosphate buffer, pH 7.4 containing 1mg/ml fluorescein isothiocyanate (FITC) labelled concanavalin (Con A, Sigma Chemicals). The fungus or walls were then washed thrice with saline solution by repeated low speed centrifugation and resuspension. For control sets these were incubated with lectin supplemented with 0.25M α–methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluorescence optics (BP 450-490 exciting filter, RKP 520 Beam splitting mirror, 515 suppression filter). Photographs were taken by Leica WILD MPS 48 camera on Konica 400 ASA film.

3.9 Agglutination response of conidia

The agglutination response of conidia was performed following the method of Lis and Sharon (1986) and Cristinzio *et.al,* (1988). Concanavalin A (ConA), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutin - I (UEA-I) and wheat germ agglutinin(WGA) of Sigma Chemical, USA, were diluted (1 mg/ml) with 50mM phosphate buffered saline (PBS), pH 7.2, and were used for agglutination reactions. ConA solution contained 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

3.9.1 Preparation of conidial suspension

Agglutination tests were done with ungerminated spores. For this, fungus was allowed to grow in liquid Richard's medium for 7 days at 30°C. Conidia were washed off the mycelial surface with 5 ml of sterile distilled water and the resulting suspension was centrifuged at 3500 g for 15 min at 4°C. The pellet was washed thrice with cold PBS and resuspended in the PBS to a concentration of approximately $5x10^6$/ml. The conidial suspensions were used immediately after preparation.

3.9.2 Agglutination test

Ungerminated conidial suspension (10 μl) was taken in a slide and incubated with diluted lectin solution (10µl) in a moist chamber at room temperature for various incubation times (upto 2h). During incubation, slides were gently swirled several times to ensure maximal cellular contact. Agglutination of conidia was observed under Leica Leitz Biomed microscope in bright field and arbitrarily scaled from `O' (no-agglutination) to `4" (maximum agglutination).

3.10. Antisera production

3.10.1 Rabbits and their maintenance

For the production of antisera against different fungal and leaf antigens, New Zealand white, male rabbits were used. Before immunization, the body weights of rabbits were recorded and were observed for at least one week inside the cages. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they

were also given 50-75 g of gram seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Cages were cleaned everyday in the morning for better hygeinic conditions.

3.10.2 Immunization

Antisera were raised in separate rabbits against antigen preparation of mycelia and cell wall of *G. cingulata* (isolate GC-1); mycelia of *P.theae* (isolate PT-2) and *F. oxysporum* (non pathogen of tea) as well as healthy leag antigen of TV-18 and CP-1. Sera collected before immunization were used as controls. After preimmunization bleeding, immunogen (1 ml) emulsified with an equal volume of Freund's complete adjuvant (Difco) followed by incomplete adjuvant were injected intramascularly at weekly intervals, upto 20 weeks.

3.10.3 Bleeding

Blood was collected from the marginal ear vein puncture 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding, rabbits were placed on their backs on a wooden board after taking them out from the cage. The board was fixed at a 60^0 angle. The neck of the rabbit was held tight in the triangular gap at the edge of the board, and the body was fixed in such a way that the rabbits could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of a sharp sterile blade and blood samples (2-5 ml) were collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. For clotting, the blood samples were kept at 30^0C for 1h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000g for 10 min. Finally, blood samples were distributed in 1 ml vials and stored at -20^0C until required.

3.11 Purification of IgG

3.11.1 Precipitation

IgG was purified following the method of Clausen (1988). The antiserum (5 ml) was diluted with two volumes of distilled water and an equal volume of 4.0 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22°C. Then it was centrifuged at 10,000g for 1 h at 22°C and the precipitate was dissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.11.2 Column preparation

Initially, DEAE Sephadex (Sigma Co. USA) was suspended in distilled water overnight after which the water was decanted off and the gel was suspended in 0.005 M phosphate buffer, pH 8.0. The buffer washing was repeated 5 times. The gel was next suspended in 0.02M phosphate buffer pH 8.0 and was applied to a column (2.6 cm in dia, 30 cm high) and allowed to settle for 2h. After that 25 ml of 0.02M phosphate buffer (pH 8.0) was applied to the gel material.

3.11.3 Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.3 M. The initial elution buffer (1) was 0.02M sodium phosphate buffer pH 8.0 (diluted from a 0.10 M sodium phosphate buffer pH 8.0 containing 16.86 g Na_2HPO_4, $2H_2O$ + 0.731g NaH_2PO_4, H_2O/L). The final elution buffer(2) was 0.30 M sodium phosphate buffer pH 8.0.

The buffer(1) was applied in a flask in which one rubber connection from its botton was supplying the column. Another connection above the surface of buffer(1) was connected to another flask with buffer (2). During the draining of buffer (1) to column, buffer (2) was sucked into buffer (1) thereby producing a continuous rise in molarity. Finally, 40x5 ml fractions were collected and the optical density (OD) values were recorded by UV-spectrophotometer at 280 nm.

3.12 Immunodiffusion tests

3.12.1 Preparation of agar slides

The Glass slides (5cm x 5 cm) were degreased successively in 90% (v/v) ethanol; ethanol:di-ethyl ether (1:1 v/v) and ether, then dried in hot air oven & sterilized inside the petridish each containing one slide. A conical flask containing Tris-barbiturate buffer (pH 8.6) was placed in a boiling water bath; when the buffer was hot, 0.9% agarose was mixed to it & boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The molten agarose was poured in glass slides (5 ml/slide) and kept 15 min for solidification. After that 3-7 wells were cut out with a sterilized cork borer (4 mm dia.) at a distance of 5 mm. from the central well.

3.12.2 Diffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50 μl/ well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48-72h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigens were present.

3.12.3 Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Then slides were stained with 0.5% amido black (0.5g amido black, 5g $HgCl_2$, 5ml glacial acetic acid 95ml distilled water) for 10 min. at room temperature. After staining slides were washed thrice in distaining solution [2% (v/v) acetic acid] for 5 h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3h at 50° C.

3.13 Immunoelectrophoresis

3.13.1 Preparation of agarose slides

The slides (7.5 x 2.5 cm) were degreased, dried and sterilized as described earlier. Then thin and uniform layer (2 mm thickness) of fluid agarose medium (0.9% agarose,

0.1% NaN_3 dissolved in 0.05 M barbitol buffer (pH 8.6) was poured on each slide taking care that no air bubble was present in the agarose medium. This was necessary in order to avoid any irregularity which may cause asymmetrical migration and diffusion during electrophoretic separation or the immunodiffusion. The slides were kept in petridishes and stored at 4° C until use.

3.13.2 Electrophoresis

Two central wells (3mm dia.) were cut out from the agarose plate of each slide following the conventional method (Ouchterlony, 1967). Slides were placed in the middle compartment of the electrophoretic box. The anode and cathode chambers were filled with barbitol buffer (0.05 M pH 8.6). Antigens (40µl) were introduced into the wells. Filter paper strips (Whatman) were soaked in buffer and laid on both ends of the slides which connected the buffer solution in the anode and cathode compartments with the agar surfaces. An electric current (2.5 mA/slide; 10v/cm) was passed through the slides for 3h at 4°C. After electrophoresis the current was discontinued.

3.13.3 Diffusion

A longitudinal trough parallel to the long edge of the slide was cut in the agarose plates in between two wells and the undiluted antiserum (400µl) was pipetted into the trough. Diffusion was allowed to continue in a moist chamber for 48-72h at 25°C.

3.13.4. Washing, drying and staining of slides

After immunodiffusion, slides were washed, stained and destained as mentioned earlier. Then all slides were dried in hot air oven for 3h at 50°C.

3.14 Rocket Immunoelectrophoresis

3.14.1 Preparation of agarose slides

Initially the slides (7.5 x 2.5 cm) were degreased with ethanol : diethyl ether (1:1 v/v) and then dried, and sterilized as mentioned earlier. To 10 ml of fluid agarose medium (1% agarose, 0.1% NaN_3 dissolved in 0.05 M barbitol buffer pH 8.6) 1 ml of undiluted antiserum was added at 50° C (Clausen, 1988). Then this agarose gel was shaken thoroughly and poured on each slide taking care that no air bubble was present in the agarose medium. The slides were kept in petridishes and stored at 4° C..

3.14.2 Electrophoresis

2-3 wells (3 mm dia.) were cut out from one end of the agarose plate of each slide and then all slides were placed in the electrophoretic box. Antigens (50 µl) were loaded in each well. Then electrophoresis was performed for 4h at 4°C as mentioned earlier. After electrophoresis the current was discontinued.

3.14.3 Washing, staining and drying of slides

After immunoelectrophoresis, slides were washed with sterile distilled water followed by aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 24-48h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose gel. After washing, rocket-shaped sharp precipitin line of antigen-antibody complexes was found.

Slides were stained with 0.5% amido black (0.5 g amido black, 5g $HgCl_2$, 5 ml glacial acetic acid, 95 ml distilled water) for 10 min at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v)] for 5h to remove excess stain. At last, all slides were washed with distilled water and dried in hot air oven for 3h at 50° C.

3.15. Enzyme linked immunosorbent assay

For ELISA following buffers were prepared following the method as described by Chakraborty et.al., (1995).

1. Antigen coating buffer (Carbonate-bicarbonate buffer 0.05M, pH -9.6).

Stocks		
A.	Sodium Carbonate	= 5.2995 g
	Distilled water	= 1000 ml.
B.	Sodium bicarbonate	= 4.2 g
	Distilled water	= 1000 ml.

160 ml of stock solution "A" was mixed with 340 ml of stock solution "B" pH of the mixed solution was adjusted to 9.6.

2. Phosphate Buffer Saline (0.05 M PBS, pH 7.2)

Stock		
A.	Sodium dihydrogen phosphate	= 23.40 g
	Distilled water	= 1000 ml
B.	Disodium hydrogen phosphate	= 21.2940 g
	Distilled water	= 1000 ml.

With 280 ml of stock solution "A", 720 ml of stock solution "B" was mixed and the pH of the resulting solution was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15 M Phosphate buffer saline - Tween (0.15 M PBS-Tween, pH 7.2).
   To 0.15 M Phosphate buffer saline, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking reagent (Tris buffer saline, pH 8.0).
   (0.05 M Tris, 0.135 M NaCl, 0.0027 M KCl).
   Tris = 0.657 g
   NaCl = 0.81 g
   KCl = 0.223 g.
   Distilled water was added to make up the volume upto 100ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% bovine serum albumin (BSA) were added.

5. Antisera dilution buffer (0.15 M PBS-Tween, pH 7.2)
   In 0.15 M PBS-Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinyl polypyrrolidone, 10,000 (Pvpp, 10,000) and 0.03% sodium azide (NaN_2) was added.

6. Substrate
   Sigma Fast pNPP substrate tablet sets were used. Each tablet set yields the following when dissolved in 20 ml of distilled water.
   pNPP = 1.0 mg/ml
   Tris buffer = 0.2M.

7. Stop solution.
   0.3N NaOH solution was used to stop the reaction.

3.15.1. Indirect ELISA

Direct antigen coated ELISA (DAC ELISA)

This ELISA was performed following the method as described by Chakraborty et.al. (1995). Plant and fungal antigens were diluted with coating buffer and the diluted antigens were loaded (200µl/well) in a Nunc 96 well ELISA plate. After loading plate was incubated at 25°C for 4h. Then plate was washed four times under running tap water and once with PBS-Tween and each time, plate was shaken dry. Subsequently, 200µl of blocking

agent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1 h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 ml/well) to each well and incubated at 4°C overnight. After a further washing 200 ml of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma Chemicals, USA) was added & incubated at 37°C for 2h. Plate was washed, dried & loaded with 200 ml of p-nitrophenyl phosphate substrate in each well and incubated in dark at room temperature for 30-45 min. Colour development was stopped by adding 50 ml/well of 3N NaOH solution and absorbance was determined in an ELISA reader (Cambridge Tech. Inc. USA) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.15.2 Direct ELISA

Double antibody sandwich((DAS) ELISA

Conjugation of alkaline phosphatase with γ -globulin :

Alkaline phosphatase was conjugated with γ-globulin following the glutaraldehyde method. For this, initially 5 mg of alkaline phosphatase enzyme crystals (Sigma Chemical) was dissolved in 1 ml of 1/2 strength phosphate buffer saline (PBS, pH 7.2). Then 25 ml of 2.5% glutaraldehyde was added and incubated for 4h at room temperature with occasional shaking. After incubation it was dialysed against 1/2 strength PBS with three changes of 30 min each. To the dialysate, 1 ml of purified IgG of *G. cingulata* was added and incubated overnight at 4°C. After incubation, 20 mg of BSA was added and stored at 4°C until required.

Assay:

DAS-ELISA was performed following the method of Brill ***et.al*** (1994). Antisera (IgG) of *G. cingulata* was diluted in coating buffer and loaded (200 μl /well) in each well of a 96 well ELISA plate. The plate was incubated for 4h at 37°C and washed five times by flooding the wells with PBS - Tween. Then test samples were added to empty well 200 μl / well) and incubated overnight at 4°C. After incubation, plate was washed as before and alkaline phosphatase tagged rabbit IgG diluted in PBS, pH 7.2, was added (200 μl /well) to each well and incubated for 6 h at 25°C. Then the plate was washed five times as before and 200 ml pNPP substrate (0.3 mg/ml) was added to each well and incubated for 30 min. at 25°C in dark. Colour development was stopped by adding 50 ml/well of 3N NaOH solution. Absorbance values were recorded at 405 nm in an ELISA reader (Cambridge Tech. Inc. USA).

3.15.3 Competition ELISA

Competition ELISA was carried out on a 96 well ELISA plate (Nunc, Maxisorp TM, Sweden) following the method as described by Lyons & White (1992). All the wells of one plate received 100µl of *G. cingulata* antigen extract diluted in PBS, and antigen of *P. theae* was similarly loaded into each well of another plate. Following incubation at 4°C overnight, the plates were washed five times in running tap water and once with PBS. Between this and all subsequent steps the plates were inverted and slapped downwards onto absorbent paper towelling to ensure the wells were completely emptied. Blocking of non-specific binding was achieved with 5% BSA in PBS, 200 µl/well for 1h. The plates were washed and dried as before and stored at 4oC until required.

For the test, a doubling dilution series was prepared from which 75 µl of the leaf extract (healthy, naturally infected & artificially infected with *G.cingulata & P.theae*) in PBS were added in each well of the blank plates using the same paired well format of the test plates. To each well 75 µl of either *G.cingulata* or *P.theae* antiserum (IgG) diluted 1:125 with PBS was added. The plates were incubated at 37°C for 1h on a shaker. (The shaker was also used for all subsequent incubation stages). At the end of this period 100 µl of the leaf extract/antiserum mixture was transferred to the corresponding wells on the pre-prepared test plates. Unreacted antiserum was added to control wells and the plates were incubated for 45 min. The plates were washed and 100 µl antirabbit IgG alkaline phosphatase conjugated antiserum was added to all wells of the plates. Following 30 min. incubation at 37°C, plates were washed and 100 µl of p-nitrophenyl phosphate substrate (1 mg/ml) was added to each well. After a further 30 min. incubation, in the dark without shaking, the absorbance values were read at 405 nm in an ELISA reader (Cambridge Technology, Inc. USA).

3.16. Estimation of fungal mycelium in leaf tissues

For the estimation of fungal mycelium in leaf tissues, the method of Beckman *et al.* (1994) was followed with modifications. Healthy tea leaf extracts were prepared and varying concentrations of mycelia of *G.cingulata* (.4-2.0 mg fresh wt.) were added to these extracts. Indirect ELISA was performed with these extracts and absorbance values were noted at 405 nm as described previously Absorbance values of healthy extract was subtracted from those of the extract containing the mycelia. Standard curve of mycelial frest wt. versus absorbance was prepared. While estimating the amount of fungal mycelium in infected leaf extracts, the difference in absorbance between healthy and infected leaf extract were plotted in the standard curve and the amount determined. Amount of fresh wt. of mycelium/g leaf tissue was finally calculated.

3.17. Establishment of Callus

3.17.1 Culture media

For the callus induction, MS basal media (Murashige and Skoog, 1962) was used. Initially, following stocks solutions was prepared :

MS-1 (20x)

a) KNO_3 - 38.0 g
NH_4NO_3 - 33.0g
$MgSO_4,7H_2O$ 7.4 g
KH_2PO_4 - 3.4 g.
Double distilled water - 1 L.

b) $CaCl_2, 2H_2O$ - 8.82 g.
Double distilled water - 500 ml.

Solution (a) and (b) were mixed and the volume was adjusted to 2L with double distilled water and stored at 4°C.

MS-II (100x)

$MnSO_4$ - 2.23 g
$ZnSO_4, 7H_2O$ 860 mg
H_3BO_3 - 620mg
KI - 83 mg
$Na_2MO_4, 2H_2O$ 25 mg
$CuSO_4, 5H_2O$ 2.5 mg
$CoCl_2, 6H_2O$ 2.5 mg
Double distilled water - 1L.
Stored at 4°C.

MS-III(20x)

$Na_2EDTA, 2H_2O$ - 746 mg.
Boiling double distilled water - 80 ml.
$Fe_2SO_4, 7H_2O$ - 556 g
Double distilled water - 80 ml.

$FeSO_4$, $7H_2O$ solution was added to Na_2EDTA solution with vigorous stirring and volume was adjusted to 200 ml with double distilled water. Stored at 4°C.

MS-IV (100x)

Myo-inositol	-	100 mg
Thiamine HCl	-	0.5 mg
Nicotinic acid	-	0.5 mg
Pyridoxin HCl	-	0.5 mg
Glycine	-	2 mg.
Double distilled water	-	10 ml

MS-I, II, III, IV were mixed together in the following ratio :

MS-I	-	100 ml
MS-II	-	10 ml
MS-III	-	10 ml
MS-IV	-	10 ml.

Then the media was supplemented with 3% sucrose, 0.8% agar and 2 mg/L IBA, 4 mg/L BA (Kato, 1989). Final volume was made upto 1 litre. pH was adjusted to 5.8 using 0.1N HCl or 0.1 N NaOH before autoclaving and then sterilized at 121°C (15 lb/in^2) for 30 min.

Medium was distributed in culture tubes and flasks. For the prevention of browning of explants, following substances were used in media before autoclaving.

(a) Activated charcoal	-	100 mg/L
(b) Ascorbic acid	-	150 mg/L
(c) Ca-Pantothenate	-	200 mg/L.

3.17.2 Preparation of fragile callus

Shoots of fresh young tea plants with 3-4 leaves were taken from glass house and stem segments (2-3mm long) were cut and sampled. Before sterilization, explants were washed in running tap water for 20 min. to remove phenolic sustances. Stem segments were surface sterilized with 2% sodium hypochlorite solution for 5 min. and washed five times with sterile distilled water for the removel of hypochlorite. After final washing, explants were transferred into semi-solid media contained in flasks and culture tubes. They were incubated under 16h photoperiod at 26°C and observed regularly. The fragile callus obtained above were shaken gently in liquid MS medium at 100 rpm and loose cells were used for fluorescence studies.

3.18. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of cross sections of tea leaves, fungal spores and mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994b).

3.18.1. Loosened Cells

Loosened cells were allowed to grow in liquid MS medium with shaking for a period of 10 days. For fluorescent staining, cells were taken in microcentifuge tube by a sterile Pasteur pipette and was once with PBS (pH 7.2) by centrifuguation at slow speed. Diluted (1:125) antiserum or normal serum was added into the washed cells and incubated for 30 minutes at 27°C. Then cells were washed thrice with PBS Tween (pH 7.2) and incubated for d30 m. in dark at 27°C with goat antirabbit IgG conjugated with FITC (Sigma, USA), diluted 1:40 with PBS (pH 7.2). After incubation, cells were washed thrice by repeated centrifugation and mounted in 10% glycerol. A cover slip was placed and observed under UV light using I3 filter in a Leica microscope and photographed in a wild MPS camera on 400 ASA Konica film (Leitz).

3.18.2. Cross sections of tea leaves

Initially, fresh cross sections of tea leaves were cut and immersed in phosphate buffer saline (PBS) pH 7.2 co ntaining 0.8% NaCl and 0.02% KCl. Then good sections were selected and treated with normal serum or antiserum diluted (1:125) with PBS pH 7.2

and incubated for 30 min. at 27°C. After incubation, sections were washed thrice with PBS-Tween pH 7.2 for 15 min transferred to 100 μl l of diluted (1:40) goat antiserum specific to rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min at 27°C. All operations with FITC labelled antibodies were made in darkness or very low light. After that, sections were washed thrice with PBS Tween, (pH 7.2) as mentioned above and then mounted on a grease free slide with 10% glycerol. A cover slip was placed on the section and sealed. Fluorescence of the leaf section were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) filter set I3. Tissue sections were photographed under both phase-contrast and UV fluorescent conditions for comparison of treatment.

3.18.3. Mycelia

Fungal mycelia were grown in liquid Richard's medium as described earlier. After four days of inoculation young mycelia were taken out from the flask and kept in grooved slide. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted (1:125) with PBS, pH - 7.2 and incubated for 30 min. at 27°C. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27°C. After incubation, mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. Then slides were observed and photographed as mentioned earlier.

3.18.4. Conidia

Fungal conidia were collected from 15 day-old culture and a suspension of this was prepared with PBS, pH 7.2. Conidial suspensions were taken in micro-centrifuge tubes and centrifuged at 3000 g for 10 min and the PBS supernatant was discarded. Then 200 μl of diluted (in PBS pH 7.2) (1:125) was added into the microcentrifuge tube and incubated for 2 h at 27°C. After incubation, tubes were centrifuged at 3000 g for 10 min. and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween pH - 7.2 by centrifugation as before and 200 μl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26°C for 1 h. After the dark incubation excess FITC - antisera was removed by repeated washing with PBS - Tween pH 7.2 and the spores were mounted on glycerol jelly and observed under Leica microscope, equipped with I-3 UV-fluorescence filter. Photographs were taken as described before.

Experimental

4.1. Pathogenicity test of *Glomerella cingulata* on different tea varieties

Varietal resistance test of tea against *Glomerella cingulata* was carried out with thirty two clonal varieties and five seed varieties released by Tocklai Experimental Station, Jorhat, Assam; Darjeeling Tea Research Centre, Kurseong, Darjeeling and UPASI Tea Research Centre, Valparai, Tamilnadu, following detached leaf and cut shoot inoculation techniques. Methods of inoculation, incubation conditions and disease assessment procedures have been described in detail under Materials and Methods and results are given in Tables 2-7, Figs. 1 & 2 and Plate VI (figs. A, B & C).

4.1.1. Detached leaf

Detached leaf inoculation of 13 Tocklai varieties, 15 Darjeeling varieties and 9 UPASI varieties was carried out. Disease assessment and symptom development were done after 48, 72 and 96h of inoculation on the basis of percent drop that resulted in lesion production. Fifty leaves of each variety were inoculated for each experiment. Results revealed that, in case of Tocklai varieties, TV-18 was most susceptible, followed by Teenali 17/1/54, TV-20, TV-25 and TV-23 while, TV-26 and TV-29 were found to be most resistant followed by TV-30, TV-9, TV-28, TS-449, TV-27 and TV-22 . After 96h of inoculation 67% lesion production was obtained in TV-18 while in TV-26 approximately 36% lesion production was observed (Table - 2, Fig.1). On the basis of significance tests (t-test), the varieties were grouped into highly susceptible (TV-18, Teenali 17/1/54 and TV-20), moderately susceptible (TV-25 and TV-23) and resistant (TV-26, TV-29, TV-30, TV-9, TV-28, TS-449, TV-27 and TV-22). Among the UPASI varieties, UPASI 9 and UPASI 3 were found to be most susceptible followed by UP-26, UP-8, BSSI, BSS 2 and BSS3 which were moderately susceptible, while UPASI 2 was resistant and UPASI-17 was moderately resistant (Table-3). Maximum lesion production among UPASI varieties was 93% in (UPASI-9) and minimum was 17% in UPASI-2 after 96h of inoculation. In case of Darjeeling varieties, B-157 and T-78 were found to be most susceptible, followed by TTV_1, KI/I, B777, BS/7A/76, HV-39 and B668 while BT-15, and CP-I were most resistant followed by P-312, T-135, AV-2, RR-17 and P-1258 (Table-4). In the most resistant vartiety BT-15, only 16% lesion production was obtained after 96h of inoculation, as compared to 90% in the most susceptible variety, B-157.

Among all the 37 varieties of tea tested, maximum and minimum susceptibilities were exhibited by UPASI-9 and BT-15 respectively.

Table 2 :Pathogenicity test of *G.cingulata* on Tocklai varieties of tea following detached leaf inoculation

Variety	Percentage lesion production[a] Hours after inoculation		
	48	72	96
TV-9	28.06 ± 1.2	34.77 ± 2.3	*41.20[c]±1.8
Teenali 17/1/54	34.66 ± 1.7	47.20 ± 3.5	57.66[b] ± 1.9
TV-18	53.33 ± 1.9	59.20 ± 3.9	66.45[b] ± 1.9
TV-20	45.00 ± 0.6	57.65 ± 2.1	57.30[b] ± 3.7
TV-22	35.90 ± 0.8	42.20 ± 3.2	47.31[c] ± 2.5
TV-23	30.55 ± 0.8	41.66 ± 1.7	50.90 ± 1.5
TV-25	45.45 ± 3.3	54.36 ± 0.9	51.60 ± 1.8
TV-26	26.08 ± 1.3	32.60 ± 3.3	36.20[c] ± 3.6
TV-27	31.25 ± 0.7	36.30 ± 0.8	45.05[c] ± 1.2
TV-28	29.30 ± 1.7	35.00 ± 2.1	41.42[c] ± 2.8
TV-29	26.85 ± 2.1	34.29 ± 2.6	40.00[c] ± 2.2
TV-30	26.99 ± 3.1	35.79 ± 1.8	40.60[c] ± 3.7
TS-449	25.90 ± 3.0	37.2 ± 2.0	42.33[c] ± 2.1

a Average of three separate trials;
50 leaves inoculated in each trial.
± Standard error.
*Difference in values with same superscript insignificant at P=0.01.

Table 3: Pathogenicity test of *G.cingulata* on UPASI varieties following detached leaf inoculation.

Variety	Percentage Lesion Production[a] Hours after inoculation		
	48	72	96
UP-2	0	5.55 ± 3.2	*16.66 ± 2.9
UP-3	75.00 ± 2.9	86.20 ± 2.5	88.60[b] ± 2.8
UP-8	36.84 ± 2.9	52.10 ± 3.1	57.36[c] ± 2.2
UP-9	85.71 ± 2.2	86.20 ± 2.7	92.85[b] ± 2.9
UP-26	50.00 ± 3.1	61.21 ± 2.1	69.14[c] ± 2.4
UP-17	14.39 ± 1.6	25.00 ±3.2	30.18 ± 2.1
BSS-1	49.12 ± 2.9	53.66 ± 2.7	57.22[c] ± 2.8
BSS-2	42.79 ± 3.2	46.77 ± 2.1	50.34[c] ± 2.3
BSS-3	35.19 ± 2.4	40.80 ± 2.9	42.93[c] ±3.1

a Average of three separate trials;
50 leaves inoculated in each trial.
± Standard error.
* Difference in values showing similar superscript in significant at p=0.01.

Table 4 : Pathogenicity test of *G. cingulata* on Darjeeling varieties of Tea following detached leaf inoculation.

Variety	Percentage lesion Production[a]		
	Hours after inoculation		
	48	72	96
B-668	33.33 ± 2.8	45.83 ± 3.2	*58.33 ± 2.9
B-777	41.37 ± 3.1	48.27 ±2.7	65.51 ± 3.1
P-312	7.40 ± 2.5	11.11 ± 2.7	18.51[c] ± 2.9
T-78	48.88 ± 3.2	62.22 ± 2.1	82.22[b] ± 2.8
P-1258	35.71 ± 2.1	42.85 ± 3.3	50.00 ± 2.7
B-157	77.27 ± 2.9	79.54 ± 2.2	90.90[b] ± 2.9
TTV[1]	55.00 ± 2.5	60.00 ± 2.4	75.00 ± 2.7
BT-15	0.00	5.28 ± 1.0	16.00[c] ± 1.0
AV-2	17.24 ± 2.6	20.68 ± 2.3	24.13[c] ± 3.1
BS/7A/76	34.48 ± 2.8	55.17 ± 2.7	62.06 ± 3.2
RR-17	9.67 ± 2.3	19.35 ± 3.1	32.25 ±2.4
HV-39	35.48 ± 2.7	51.61 ± 2.3	61.29 ± 2.9
K1/1	65.21 ± 3.2	69.56 ± 2.7	73.19 ± 3.1
T-135	13.79 ± 3.1	17.24 ± 2.8	20.68[c] ± 2.4
CP-1	6.18 ± 2.9	10.89 ± 1.4	17.34[c] ± 2.9

a Average of three separate trails
50 leaves inoculated in each trial.
± Standard error.
* Difference in values with same superscript insignificant at P = 0.01.

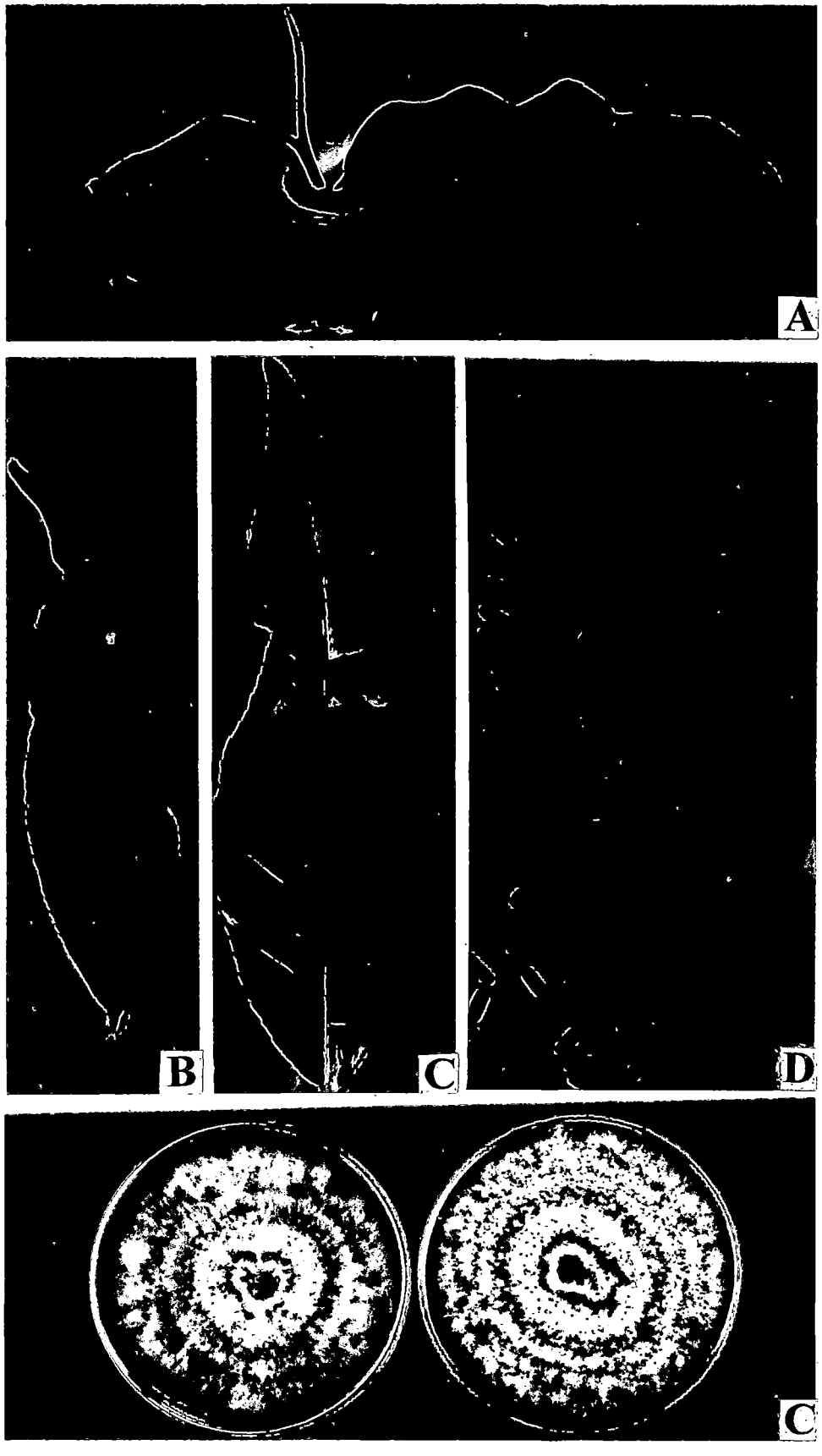


PLATE VI (figs.A-E). **A**- Twig of UPASI 9 artificially inoculated with *G. cingulata* ; **B & C**- detached leaves after artificial inoculation ; **D** -germinated conidia *G .cingulata* with appressoria ;**E**-*G.cingulata* grown on RM showing orange acervuli

Pathogenicity test of *Glomerella cingulata* on different varieties of tea (Detached leaf inoculation)

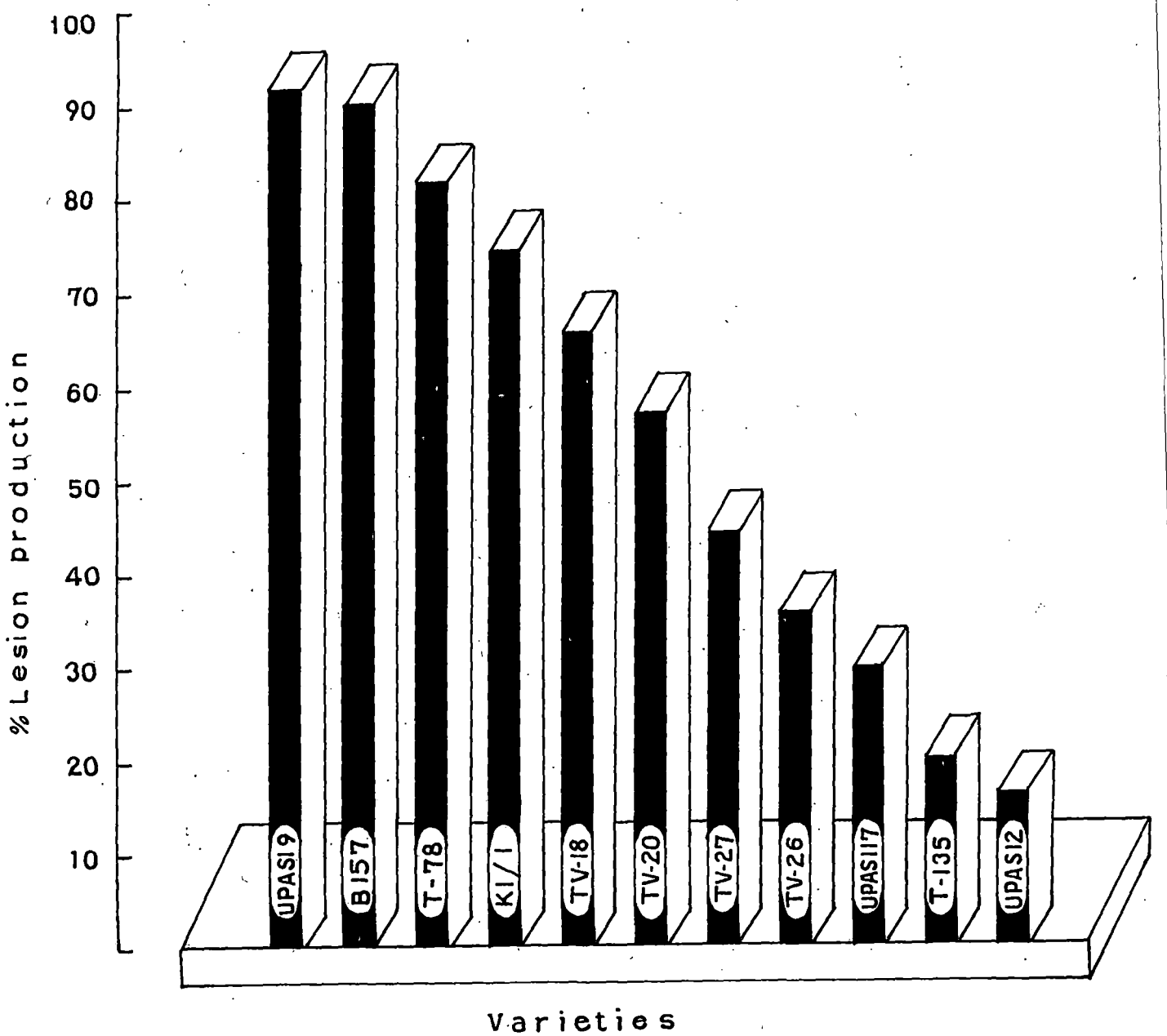


Fig. 1

4.1.2. Cut Shoot

Pathogenicity of *G cingulata* was also tested on 37 varieties of tea by cut shoot inoculation as described in Materials and Methods. Disease was assessed on the basis of mean number of lesion per shoot from which mean disease index per shoot was calculated. Disease symptoms appeared as early as 24h after inoculation. Results (Tables 5-7) confirmed the trend shown in detached leaf inoculation technique. Among the Tocklai varieties tested, highest disease index was evident in TV-18 and lowest in TV-26 (Table-5). In case of UPASI varities, UPASI-2 exhibited lowest disease index value, as revealed in Table-6 (5.90 and 1.00) respectively. A maximum disease index value of 6.6 was exhibited by B-157 and minimum of 0.75 by BT-15, among the different Darjeeling varieties tested (Table-7).

Among all the 37 varieties tested, UPASI 9 wasthe most susceptible, followed by B157 and TV-18 while BT-15 was the most resistant followed by CP-1, UPASI 2, and TV-26 on the basis of cut shoot inoculation (Fig. 2). This was in confirmity with result of detached leaf inoculation.

Table 5 : Pathogenicity test of *G.cingulata* on UPASI varieties of tea following cut shoot inoculation.

Variety	Hours after inoculation							
	24		48		72		96	
	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
UPASI 2	0	0	1.0	0.4	1.3	0.6	1.9	1.0
UPASI 3	5.0	3.0	6.0	3.9	6.5	4.5	7.0	5.2
UPASI 8	3.0	1.9	3.9	2.8	4.4	3.4	5.0	3.8
UPASI 9	6.6	4.0	7.0	4.2	7.5	4.8	8.0	5.9
UPASI 17	3.5	2.2	4.5	3.1	5.2	3.6	5.5	3.8
UPASI 26	4.0	2.0	5.0	3.0	5.6	3.8	6.0	4.0
BSS 1	3.7	2.6	4.8	3.3	5.6	3.9	5.9	4.0
BSS2	3.3	2.1	4.4	3.0	4.9	3.3	5.0	3.1
BSS3	3.1	2.0	4.3	2.9	4.8	3.2	4.9	3.0

* 50 shoots per treatment

Table 6 :Pathogenicity test of G. cingulata on Tocklai varieties of tea following cut shoot inoculation

Tea varieties	Hours after inoculation							
	24		48		72		96	
	Mean No. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean No. of lesion/ shoot	Mean dis-ease index/ shoot	Mean No. of lesion/ shoot	Mean dis-ease index/ shoot	Mean No of lesion/ shoot	Mean dis-ease index/ shoot
Teenali 7/1/54	6.0	3.0	7.4	3.6	8.0	4.2	9.2	5.2
TV-18	8.0	4.0	8.8	5.0	10.0	5.4	12.5	5.5
TV-20	5.0	2.7	6.5	3.2	7.0	3.8	8.2	4.5
TV-22	4.5	2.00	6.0	3.0	6.6	3.5	6.9	4.2
TV-23	6.2	3.5	8.0	4.3	8.3	5.0	7.9	6.3
TV-25	5.8	2.9	6.4	3.3	7.8	4.1	9.0	5.0
TV-26	2.1	0.5	2.5	0.8	4.0	1.0	5.3	1.2
TV-27	4.2	1.9	6.0	2.8	7.0	3.2	7.5	4.3
TV-28	3.5	1.2	4.1	1.7	5.7	2.0	6.0	2.6
TV-29	2.9	0.9	3.2	1.0	5.0	1.2	5.8	1.4
TV-30	3.0	1.0	3.5	1.2	5.2	1.5	6.0	2.0
TV-9	2.9	0.8	3.3	1.1	5.3	1.9	6.2	2.5
TS-449	3.1	1.2	3.3	1.5	4.9	2.1	5.2	2.5

* 50 shoots per treatment.

Table 7 : Pathogenicity test of *G.cingulata* on Darjeeling varieties of tea following cut shoot inoculation.

Variety	Hours after inoculation							
	24		48		72		96	
	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot
B668	2.9	1.5	3.5	1.9	4.0	2.5	4.6	2.8
B777	4.2	2.2	5.0	2.8	5.6	3.0	6.0	3.3
P312	1.2	0.5	2.0	0.8	2.5	1.0	3.0	1.2
T-78	5.2	3.03	6.0	4.0	6.8	4.8	7.2	5.4
P1258	2.5	1.3	3.0	2.0	3.9	2.4	4.0	2.9
B157	6.0	3.9	7.5	4.5	8.2	5.4	9.0	5.6
TTV_1	5.0	3.0	5.5	3.8	6.0	4.3	6.3	5.0
BT-15	0.8	0.3	1.1	0.4	1.5	0.5	1.9	0.75
AV-2	1.9	0.9	2.2	1.4	3.0	2.0	3.2	2.6
BS/7A/76	3.7	2.0	4.0	3.0	4.6	3.5	5.0	3.8
RR17	2.0	1.1	2.8	1.9	3.0	2.3	3.4	2.9
HV-39	3.0	1.9	3.5	2.5	4.0	2.8	4.2	3.3
K1/1	4.6	2.6	5.0	3.0	5.6	3.6	6.0	4.0
T-135	1.6	0.7	2.0	0.9	2.6	1.2	3.0	1.5
CP-1	1.0	0.4	1.5	0.5	2.0	0.7	2.2	0.9

* Average of 50 shoots

Pathogenicity test of *Glomerella cingulata* on Darjeeling varieties of tea (Cut shoot inoculation)

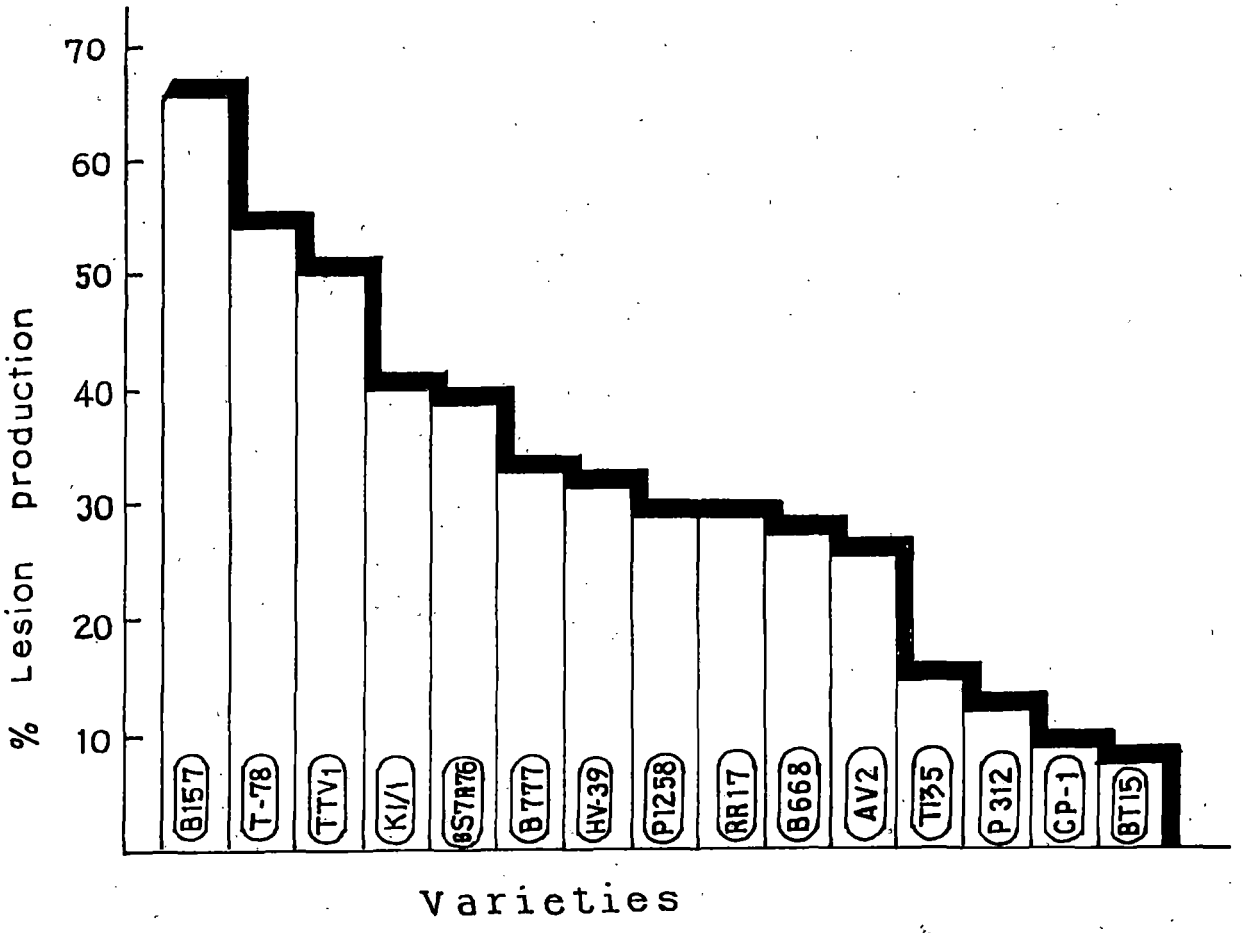


Fig. 2.

4.2. Cultural conditions affecting growth of *G.cingulata.*

Growth, sporulation and infectivity of any pathogen both **in vivo** and **in vitro** is dependent on several factors. *Glomerella cingulata* (isolate GC-1) usually had white mycelial growth which sometimes turned to brown or grey depending on the medium. Orange acervuli were characteristic of this fungus (Plate VI, Fig.E) but accrvuli production was dependent on light and temperature. Conidial morphology was studied under bright field and phase contrast of Leica Microscope (Plate VI, fig. D). Conidia appeared scantily on solitary phialides but normally in orange sporodochia; a basal stromatic cushion in covered with dense layer of cylindrical, slightly tapering phialides upto 20 μm long; sometimes these are interpersed with dark brown, tapering, blunt, septate setae. Conidia cylindrical with a rounded apex and slightly truncated base, hyaline, 12-18 μm x 3.5 μm; forming orange-red slimy masses, germinating by irregularly rounded brown appressoria.

4.2.1. Solid media

The fungus was grown in five different media, such as potato dextrose agar, Richard's agar, Flentze's soil extract agar, carrot juice agar and Czapekdox agar - medium and their growth and sporulation behaviour studied. Mycelial growth rate was recorded after 4,6,8 and 10 days of inoculation at 30$\pm$1°C. Maximum growth occurred in RMA (89.0 mm), followed by CJA (80.1 mm) as revealed in Table-8. Mycelial growth was fluffy and white in colour in RM, showing deep orange on the reverse side of the petridish. In PDA and CDA, grey white mycelia were observed on the upper side but black concentric rings with orange acervuli was prominent on reverse side of the petridish. Sporulation was very abundant in RMA and CJA, moderate in PDA and CDA while in FSEA sporulation was rare.

4.2.2. Liquid media

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* studied.

Table 8: Effect of different media on mycelial growth and sporulation of *G.cingulata*

Medium	Diameter of mycelia (mm)[a] Incubation Period (days)				
	4	6	8	10	Sporulation[b]
Potato dextrose agar	30.1 ±1.25	42.6 ±1.22	62.3 ±1.69	88.9 ±1.23	+
Richard's medium	35.6 ±2.21	48.9 ±1.08	69.9 ±2.00	89.0 ±1.33	++
Czapekdox-agar	10.0 ±1.16	15.5 ±2.01	28.9 ±1.80	44.2 ±1.43	_
Flentze's soil extract-agar	22.5 ±2.5	35.9 ±1.23	49.6 ±2.8	58.6 ±2.6	_
Carrot juice agar	32.9 ±1.23	40.6 ±2.3	56.6 ±2.6	80.1 ±1.89	+

Temperature - 30 ± 1°C

a Average of three replicates

b Excellent (++) ; Good (+) ; No(-)

± Standard error.

4.2.2.1 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 (591 mg) of *G.cingulata* was recorded after 10 days of incubation (Table-9). There was a steady decline in growth after 10 days. After 5 days of incubation there was a growth of only 61 mg. Therefore, the mycelial growth of 10 days was about 10 times that of 5 days. sporulation was abundant at 10 days' growth. Spores were also observed at 5 days' growth.

4.2.2.2 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

Table 9 : Effect of incubation period on mycelial growth of *G.cingulata*

Incubation period(days)	Mean mycelial dry wt.[a] (mg)
5	61.0 ± 2.21
10	591.0 ± 3.24
15	395.2 ± 3.21
20	365.5 ± 4.21
25	285.2 ± 3.8
30	262.8 ± 4.22

a Average of five replicates
± Standard error.
Temperature 30 ± 1°C.

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 30 ± 1°C. Results (Table-10) revealed that *G.cingulata* grew to a lesser or greater extent in all the pH tested. Maximum growth was recorded at pH 6.5, while minimum growth occurred at 4.0. Sporulation was very less in high pH 8.0; pH 6.0 and 6.5 supported good sporulation.

Table 10 : Effect of pH on mycelial growth of *G.cingulata.*

pH of medium	Mean mycelial dry wt.[a] (mg)
4.0	301.0 ± 3.61
5.0	362.5 ± 4.20
5.5	381.6 ± 2.71
6.0	421 5 ± 2.7
6.5	453.0 ± 4.2
7.0	369.0 ± 3.5
8.0	340.0 ± 3.41.

a Average of three replicates
± Standard error
Temperature 30 ± 1°C.
Incubation period 15 days.

4.2.2.3 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.* These were added separately to the basal, medium (0.2% as paragine, 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-11 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 sucrose, glucose and mannose. Among the tested carbohydrates least growth occurred in mannitol. There was little growth in absence of any carbohydrate.

4.2.2.4. Nitrogen Source

Nitrogen is undoubtedly the most important single nutrient necessory 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 wellas 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 sources, both inorganic and organic (Table-12). Among the organic sources beef extract supported 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.

Table 11 : Effect of different carbon sources on mycelial growth of *G.cingulata*

Carbon Source wt.(mg)	Incubation period					
	3 days		6 days		9 days	
	Dry[a] wt.(mg.)	Sporulation[b]	Dry[a] wt.(mg)	Sporulation[b]	Dry[a] wt.(mg.)	Sporulation[b]
Fructose	35.1 ±2.9	-	157.6 ±4.1	-	315.5 ± 2.6	±
Sorbase	38.4 ±4.21	-	117.2 ±2.21	-	270.2 ± 2.8	-
Glucose	42.2 ±3.39	±	150.8 ± 3.29	++	382.1 ±3.7	+++
Gallactose	35.9 ±3.3	-	212.6 ±2.21	-	350.2 ± 1.9	-
Mannose	32.2 ±1.29	±	225.6 ±3.2	++	340.5 ±2.9	+++
Mannitol	29.6 ±2.65	-	95.6 ±2.25	-	145.2 ±2.9	-
Sucrose	35.6 ±2.61	±	272.6 ±3.26	++	385.9 ±3.6	+++
Starch	25.6 ±2.91	-	190.3 ±3.26	-	230.9 ±3.8	-
Maltose	40.6 ±2.33	++	300.0 ±3.2	+++	416.9 ±2.6	++++
Control (without carbon)	11.2 ±2.3	-	18.5 ±3.3	-	22.4 ±2.7	-

a Average of three replicates.

b - = Nil; ± = Poor; ++ = Fair; +++ = Good; ++++ = Excellent.

Temparature 30 ± 1°c

Table 12 :Effect of different nitrogen sources on mycelial growth of *G. cingulata.*

Nitrogen Source	Incubation period					
	3 days		6 days		9 days	
	Dry[a] wt.(mg.)	Sporulation[b]	Dry[a] wt.(mg.)	Sporuloation[b]	Dry[a] wt.(mg.)	Sporulation[b]
Inorganic						
Calcium nitrate	23.3 ±3.3	-	256.0 ±1.9	+	430.6 ±2.1	+
Sodium nitrate	21.0 ±2.1	-	305.6 ±2.6	+	430.6 ±2.9	++
Ammonium sulphate	17.5 ±3.1	-	189.2 ±3.9	-	237.8 ±2.8	-
Ammonium nitrate	20.1 ±2.1	-	285.6 ±4.2	-	335.6 ±3.9	-
Potassium nitrate	25.6 ±2.5	-	321.5 ±3.8	++	435.6 ±4.2	+++
Organic						
Peptone	13.6 ±2.9	-	195.6 ±3.1	-	269.6 ±3.1	-
Urea	9.0 ±4.1	-	160.7 ±2.9	-	213.5 ±3.2	-
Yeast extract	15.6 ±2.6	-	172.5 ±5.2	-	205.7 ±4.0	-
Beef extract	12.3 ±2.6	-	190.9 ±5.2	+	293.6 ±4.0	++
Control (without nitrogen)	7.7 ±2.1	-	9.7 ±1.9	-	11.2 ±1.7	-

a Average of three replicates Temperature 30±1°C.

b - = nil ; ± = poor ; ++ = fair ; +++ = good; ++++ = excellent

4.3. Detection of cross reactive antigen between *G. cingulata* and tea varieties

The presence of cross reactive antigens among hosts and pathogenic organisms is a well documented phenomenon. Existing studies on plant hosts and pathogens suggests that whenever an intimate and continuing association of cells of hosts and pathogens occurs, partners of this association have a unique serological resemblences to one another involving one or more antigenic determinants.

Various method have been generally used to detect the presence of cross reactive antigens between hosts and parasite. Earlier techniques like immunodiffusion and immuno-electrophoresis are being currently replaced by more advanced techniques like ELISA and immunofluorescence. In the present investigation cross reactive antigens between *G. cingulata* and tea varieties have been detected using immunodiffusion, immunoelectrophoresis and ELISA. Series of experiments performed and results obtained have been presented below.

4.3.1. Immunodiffusion tests

The effectiveness of antigen preparation from tea leaves (TV-18 and CP-1) and *G. cingulata* (isolate GC-1) in raising antibodies was checked by homologous cross reaction following agar gel double diffusion technique.Control sets involving normal sera andantigens of both the host and parasite were all negative. The results are presented in Tables 13 to 15 and Plate VII, figs. A-D. Strong precipitin reactions occured when antiserum raised against mycelia of *G. cingulata* (GCIA) was reacted against its own antigen and the antigens of other isolates (Table-13; Plate VII,fig-B). When anti - *G. cingulata* antiserum was crossreacted with leaf antigens prepared from 37 tea varieties including 13 Tocklai, 15 Darjeeling and 9 UPASI varieties, 16 varieties (TV-18,TV-20, TV-22, TV-23, TV-25, TV-27, Teenali-17/1/54, UPASI 3, UPASI 9, UPASI 26, T-78, B157, TTV_1, K1/1, B668, B777) exhibited precipitin band in immunodiffusion test (Table-13). However, weak precipitin reactions were observed with antigens of 13 other varieties (TV-9, TV-28, TV-29, TV-30, TS-449, UPASI 8, UPASI 17, BSS 1, BSS 2, BSS 3, P-1258, BS/7A/76 and HV-39). No such precipitin reactions were observed in case of antigens of 8 varieties tested (TV-26, UPASI 2, P312, BT-15, AV-2, RR-17, T-135 and CP-1) as well as antigen preparations from three non host species viz. *Glycine max* (cv. soymax), *Cicer arietinum* (cv. JG-62) and *Camellia japonicum* and one non pathogen (*Fusarium oxysporum*).

To confirm the presence of common antigens between isolates of *G.cingulata* and tea varieties reciprocal cross reaction with antiserum of TV-18 was carried out with leaf antigens of host and non host as well as mycelial antigens of pathogen and non pathogen. Results are presented in Table-14. Strong precipitin reactions were observed in most of the cases with the antigens of Tocklai varieties except- TV-26 and TS-449. Whereas, antiserum of TV-18 exhibited weak precipitin band when reacted with the leaf antigens prepared from UPASI

Table 13 : Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti *G.cingulata* antiserum.

Antigen of host and parasite	Antiserum of *G. cingulata.* Pricipitin band	Antigen of host and parasite	Antiserum of *G. cingulata* Pricipitin band
Tocklai varieties		**Darjeeling varieties**	
TV 9	±	B 668	+
TV18	+	B777	+
TV20	+	P312	-
TV 22	+	T-78	+
TV 23	+	P-1258	±
TV 25	+	B157	+
TV 26	-	TTVI	+
TV 27	+	Bt-15	-
TV 28	±	AV2	-
TV 29	±	BS/7A/76	±
TV 30	±	RR17	-
TS 449	±	Hv39	±
Teenali 17/1/54	+	K 1/1	+
UPASI varieties		T-135	
UPASI-2	-	Cp-1	-
UPASI-3	+		
UPASI-8	±	**Pathogen:**	
		G.cingulata Isolates:	
UPASI-9	+	GC-1	+
UPASI-17	±	GC-2	+
UPASI-26	+	GC-3	+
BSS-1	±	GC-4	+
BSS-2	±	GC-5	+
BSS-3	±	GC-6	+
Non host		GC-7	+
Glycine max(Cv.Soymax)	-	GC-8	+
Cicer arietinum(Cv.JG-62)	-	GC-9	+
Camellia japonicum		**Non pathogen**	
		F.oxysporum	-

+ Common precipitin band present;

(±) weak precipitin band; (-) Common precipitin band absent.

Table 14 : Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti TV-18 antiserum.

Antigen of host and parasite	Antiserum of TV-18 Precipitin band	Antigen of host and parasite	Antiserum of TV-18 Precipitin band
Tocklai varieties		**Darjeeling varieties**	
TV 9	+	B 668	±
TV 18	+	B 777	±
TV 20	+	P 312	-
TV 22	+	T 78	+
TV 23	+	P 1258	±
TV 25	+	B 157	+
TV 26	±	TTV_1	±
TV 27	+	BT -15	-
TV30	+	AV2	±
TS-449	±	BS/7A/76	±
Teenali 17/1/54	+	RR 17	±
		HV 39	+
		K1/1	±
		T-135	±
		CP-1	±
UPASI varieties		**Pathogen :**	
UPASI 2	-	*G.cingulata*	
UPASI 3	±	*Isolates*	
UPASI 8	±	GC-1	+
UPASI 9	+	GC-2	+
UPASI 17	±	GC-3	+
UPASI 26	±	GC4	±
BSS-1	±	GC-5	±
BSS-2	±	GC-6	±
BSS-3	±	GC-7	+
Non host		GC-8	±
G. max(Soymax)	-	GC-9	+
C. arietinum(JG-62)	-	**Non Pathogen**	
C. japonicum	-	*F. oxysporum*	-

(+) Common precipitin band present;

(±) Weak precipitin band; (-) Common precipitin band absent.

Table 15: Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti-CP-1 antiserum.

Antigen of host and parasite	Antiserum of Cp-1 Precipitin band	Antigen of host and parasite	Antiserum of CP-1 Precipitin band
Tocklai varieties		**Darjeeling varieties**	
TV-9	+	B 668	-
TV-18	+	B 777	±
TV-20	+	P 312	-
TV-22	+	T 78	±
TV-23	+	P 1258	±
TV-25	+	B 157	±
TV-26	+	TTV_1	±
TV-28	+	BT-15	+
TV-29	+	AV-2	±
TV-30	±	BS/7A/76	+
TS-449	-	RR17	±
Teenali 17/1/54	-	HV-39	+
		K1/1	±
		T135	±
UPASI varieties		CP-1	+
UPASI-2	-	**Pathogen:**	
UPASI-3	±	*G.cingulata* isolates	
UPASI 8	-	GC- 1	-
UPASI 9	±	GC-2	-
UPASI 17	±	GC-3	-
UPASI 26	-	GC-4	-
BSS1	±	GC-5	-
BSS2	±	GC-6	-
BSS3	±	GC-7	-
		GC-8	-
Non host		GC-9	-
G. max(Soymax)	-	**Non pathogen**	
C. arietinum (JG-162)	-	*F. oxysporum*	-
Camellia japonicum	-		

(+) Common precipitin band present, (±) Weak precipitin band;

(-) Common precipitin band absent.

and Darjeeling varieties. In repeated trials with anti- TV-18 antiserum no such precipitation reaction could be observed in immunodiffusion test with antigens prepared from UPASI-2, P312, BT-15 (Plate VII, fig. A) and also non host species. It is interesting to note that among 9 isolates of *G. cingulata* tested, 4 isolates (GC-1, GC-2, GC-3, and GC-7) exhibited common precipitin band, but rest of the 5 isolates gave weak precipitin reaction in agar gel double diffusion test. (Table-14 & Plate VII, fig. C).

Reciprocal cross reaction using antiserum of CP-1 and antigens prepared from leaves of 37 tea varieties, 3 non host species, 9 isolates of *G. cingulata* (pathogen) and *F. oxysporum* (non pathogen) were also carried out. Results (Table-15) revealed that none of the isolates of *G. cingulata* could develop any precipitin reaction with anti CP-1 antiserum (Plate VII, fig. D). Non host species and non pathogen also failed to develop any precipitin line.

4.3.2. **Immunoelectrophoresis**

Results of immunodiffusion tests revealed the presence or absence of cross reactive antigens between isolates of *G.cingulata* and tea varieties. Many of the cross reactions in the immunodiffusion tests gave diffused precipitin bands which could not be clearly distinguished. It was also not clear whether precipitin reactions in all cases were due to single or several antigenic substances. Therefore, further resolution was attempted by subjecting the antigens to electrophoresis before exposing them to antisera. Details of immunoelectrophoretic technique have been described under Materials and Methods.

In this experiment, antigenic comparison among 6 selected varieties of tea in which 3 susceptible varieties (TV-18, UPASI-9 and B-157) and 3 resistant varieties (TV-26, UPASI-2 and CP-1), 4 isolates of *G.cingulata* (GC-1, 2, 3 and 4), 3 non host species (*G. max, C. japonicum and C. arietinum*) and one non pathogen (*F.oxysporum*) using antisera of host (TV-18, CP-1) and parasite (*G. cingulata*) were done following conventional set up of immunoelectrophoresis. Results are shown in Table-16.

Effectiveness of each antigen extract of TV-18 and CP-1 and isolate of *G.cingulata* (GC-1) in raising antibodies was checked by homologous cross reactions. Six precipitin arcs were formed by TV-18, CP-1 and GC-1 in homologous reaction. When anti TV-18 antiserum was cross reacted with the leaf antigens of 5 other tea varieties, they were antigenically very close to each other. In this reaction antigens of UPASI 9 and B 157 gave rise to 4 precipitin arcs, while UPASI-2 and CP1 and showed 2 precipitin arcs but TV-26 exhibited 3 arcs. No such precipitation was observed when antigens of non pathogen and non-hosts were cross

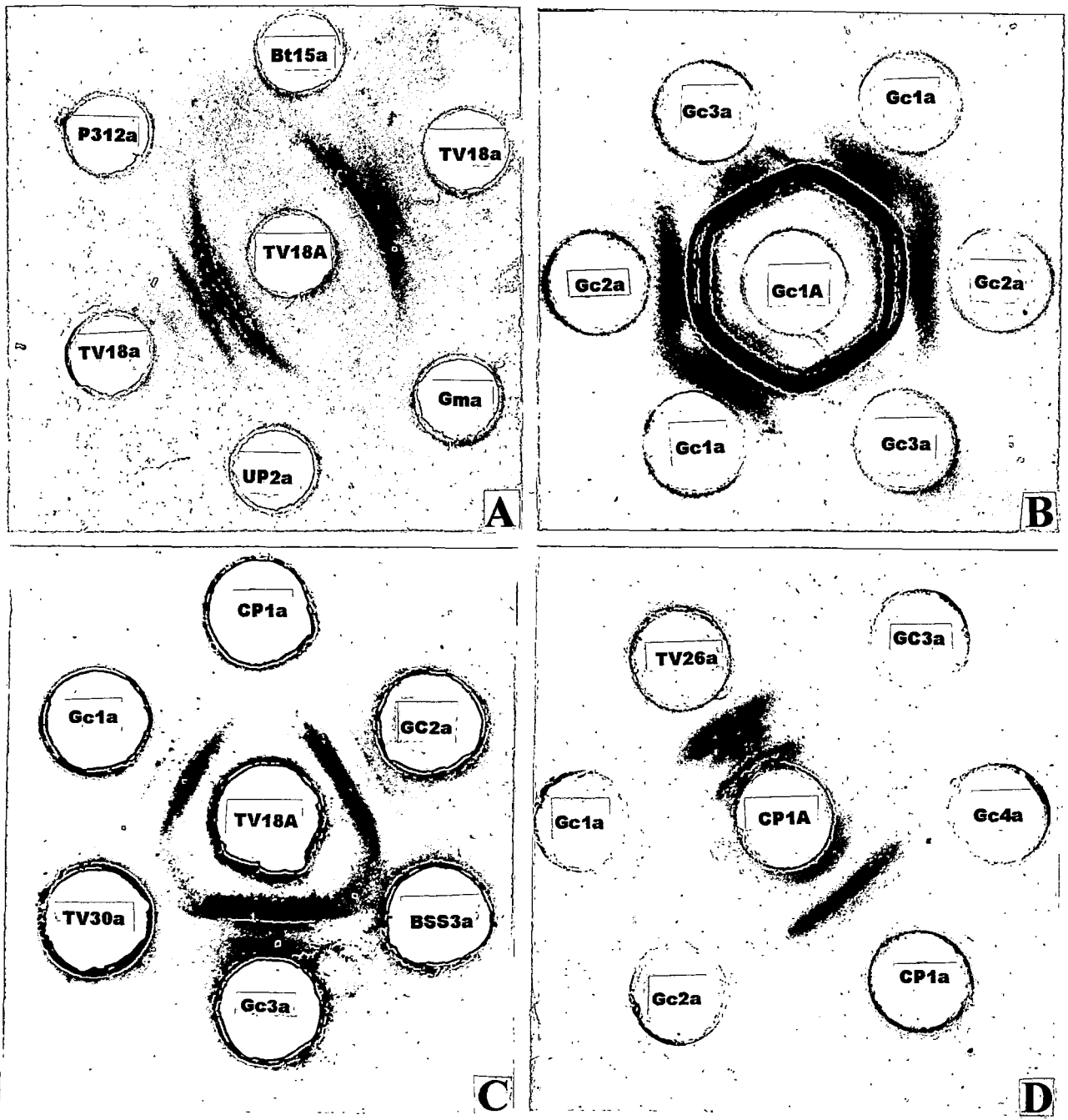


PLATE VII (figs.A-D). Agar gel double diffusion tests. Central wells contain antisera to *G.cingulata* **(B)**, TV-18 **(A&C)** and CP1 **(D)**. Peripheral wells contain antigens of *G.cingulata* (**B & C**), tea leaves **(A,C & D)** and non host (**A**)

reacted with antiserum of TV-18. It is interesting to note that all three isolates of *G.cingulata* (GC-1, 3 and 4) were antigenically more related to TV-18 by sharing 2 precipitin lines in common. Isolate GC-2 shared only 1 line in common.

Similarly, anti CP-1 antiserum was cross reacted with antigens of five different tea varieties. Antigens of UPASI 9, B 157 and UPASI 2 exhibited 3 precipitin arcs each, while 2 precipitin arcs were formed with antigens of TV-18 and TV-26. When antigens of non-pathogen (*F.oxysporum*) and non-host (*G.max, C.japonicum* and *C.arietinum*) were cross reacted with antiserum of CP-1, no precipitin arcs could be observed.

Reciprocal cross reactions with anti *G.cingulata* antiserum and antigens of all three susceptible varieties (TV-18, UPASI-9 and B 157) formed two precipitin lines but antigens of resistant varieties (TV-26, UPASI 2 and CP-1), non host and non pathogen failed to develop any precipitin lines.

4.3.3 Rocket immunoelectrophoresis

The results of some immunoelectrophoretic tests were verified by rocket immunoelectrophoresis. In this experiment antiserum of *G.cingulata* was cross reacted with its homologous antigens and leaf antigens of TV-18 and TV-26. Gel containing anti *G.cingulata* antiserum and homologous antigens resulted in 3 immunoprecipitate line while a single immunoprecipitin line was discerned for the susceptible variety (TV-18). No such line was observed in case of resistant variety (TV-26).

4.3.4. Direct antigen coated enzyme linked immunosorbent assay (DAC ELISA)

Enzyme linked immunosorbent assay in one of the most sensitive serological techniques for detection of cross reactive antigens between host and pathogen as well as for detection of pathogen in diseased tissue. In indirect or DAC ELISA antigens are bound to the microtitre plates after which the antibody is allowed to bind to the antigen. To this antigen antibody complex the conjugate (an antibody conjugated to enzyme) is added. Finally, the non-coloured substrate is added which is converted to a coloured end product which is generally detected by a reader.

Table 16:Antigenic comparison of tea varieties, pathogens, non-pathogen and non hosts.

Antigen of host and parasite	Total no. of precipitin lines		
	Antisera of host and parasite		
	TV-18	**CP-1**	***G.cingulata***
Susceptible varieties			
TV-18	6	2	2
UPASI 9	4	3	2
B-157	4	3	2
Resistant varieties			
TV-26	3	2	0
UPASI-2	2	3	0
CP-1	2	6	0
Isolate of *G.cingulata*			
GC-1	2	0	6
GC-2	1	0	4
GC-3	2	0	5
GC-4	2	0	5
Non pathogen			
F. oxysporum	0	0	0
Non hosts			
G. max (Cv. Soymax)	0	0	0
C. japonicum	0	0	0
C. arietinum (Cv JG-62)	0	0	0

In the present investigation DAC ELISA has been used in most of the experiments. Since ELISA depends on a number of factors and this varies from system to system it was considered essential to optimize these conditions in the particular host pathogen system to be studied. Hence, initially a number of experiments were performed for optimization.

4.3.4.1. Optimization of ELISA

The optimum conditions for ELISA reaction were determined initially as a large number of experiments in this investigation have been carried out using ELISA. Optimization of ELISA was done using IgG fraction of antisera raised against antigen preparations from both mycelia and cell wall of *G. cingulàta*. In all cases experiments were repeated thrice.

4.3.4.1.1. Antiserum raised against antigen preparation from mycelia of *G.cingulata*

Optimization of ELISA was done by considering three variables, dilution of the enzyme, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity. In all cases, homologous ELISA reactions using antigen of *G.cingulata* was carried out as described under Materials and Methods.

4.3.4.1.1.1.Enzyme dilution

In this experiment, keeping the antigen and antiserum dilutions constant, different dilutions of alkaline phosphatase was used. Dilutions ranged from 1:10,000 to 1:40,000. Results (Table 17 . Fig.3) revealed that absorbance values in ELISA decreased with increasing dilution of the enzyme. Absorbance value at 1:10,000 dilution was approximately 3 times that at 1:40,000 dilution (1.9 and 0.65 respectively). 1:10,000 dilution was selected for further experiments.

Effect of enzyme dilution on ELISA reaction of anti - *G.cingulata* antiserum with homologous antigen

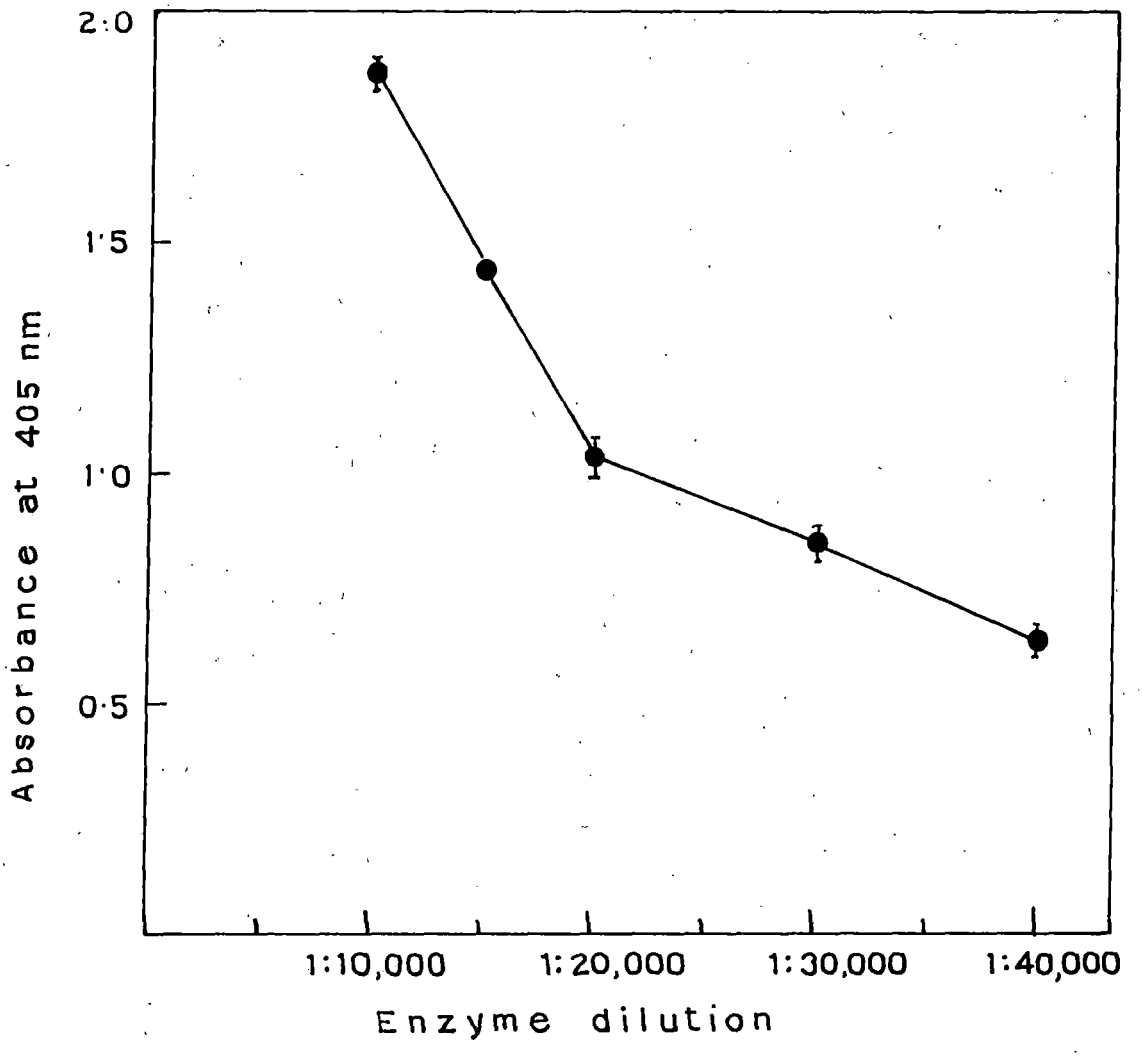


Fig. 3.

Effect of dilution of anti - *G.cingulata* antiserum on ELISA reaction with homologous mycelial antigen

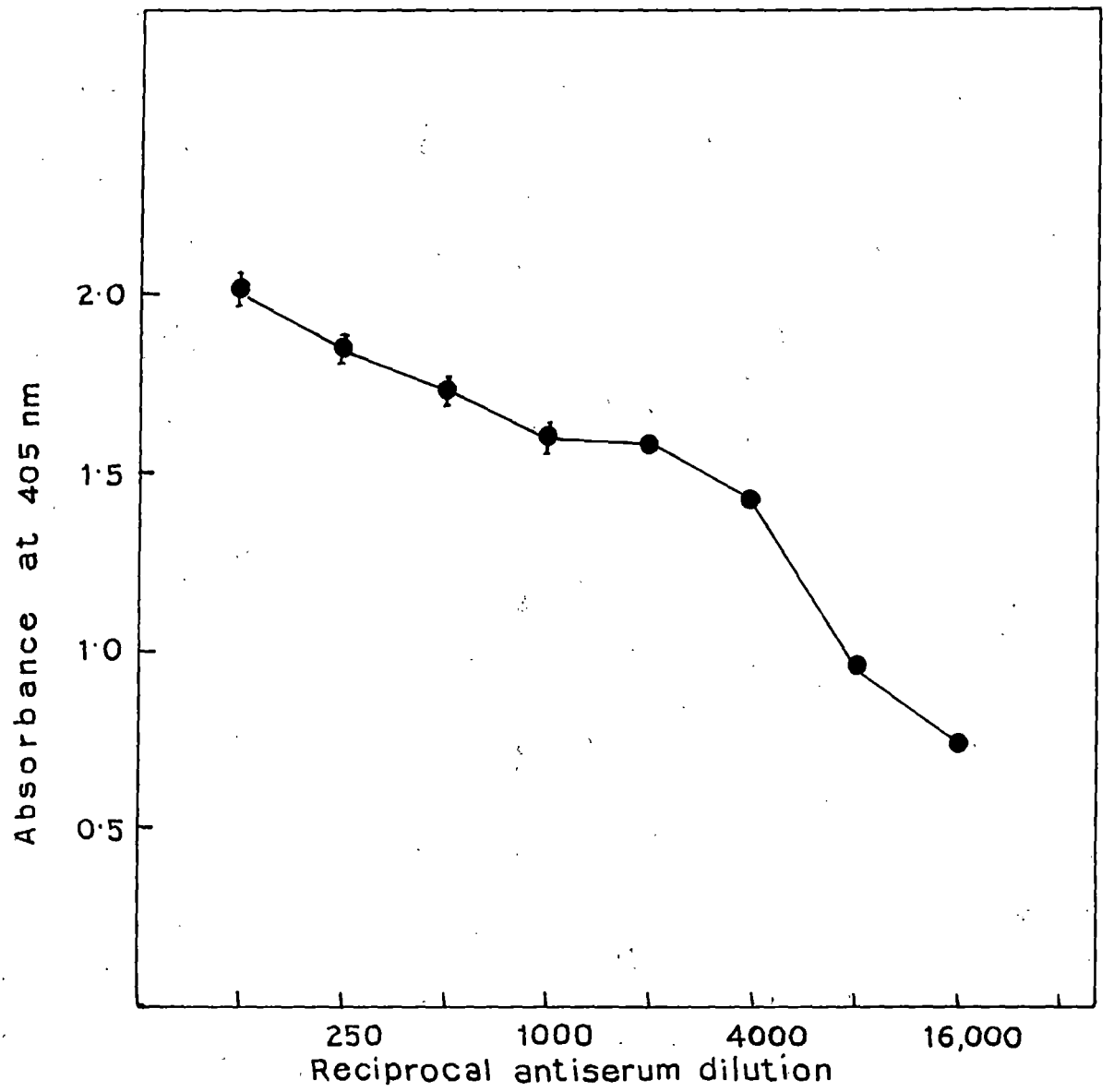


Fig. 4.

Table 17 : ELISA reaction of anti *G.cingulata* antiserum and homologous antigen with various dilution of alkaline phosphatase.

Enzyme[a] dilution	Absorbance of 405 nm			
	Anti *G.cingulata* antiserum (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
1:10000	1.890	1.900	1.892	1.894±.002
1:15,000	1.430	1.482	1.405	1.439±.022
1:20,000	1.002	1.098	1.011	1.037±.03
1:30,000	0.843	0.892	0.824	0.853±.022
1:40,000	0.623	0.679	0.645	0.649±.016

Mycelial antigen concentration - 5 μg/ml

a Alkaline phosphatase

± Standard error.

4.3.4.1.1.2 Antiserum dilution

Antiserum dilutions ranging from 1:125 to 1:16,000 were tested against homologous antigen at a concentration of 2μg/ml. Absorbance values in ELISA decreased with increasing dilution of the antiserum. At 1:125 dilution absorbance values were above 2 and it decreased to 0.743 in 1:16,000 dilution (Table 18, fig. 4). 1:250 dilution was selected for further experiments.

Table 18 :ELISA reaction with various dilution of anti *G. cingulata* antiserum(mycelia) and homologous antigen.

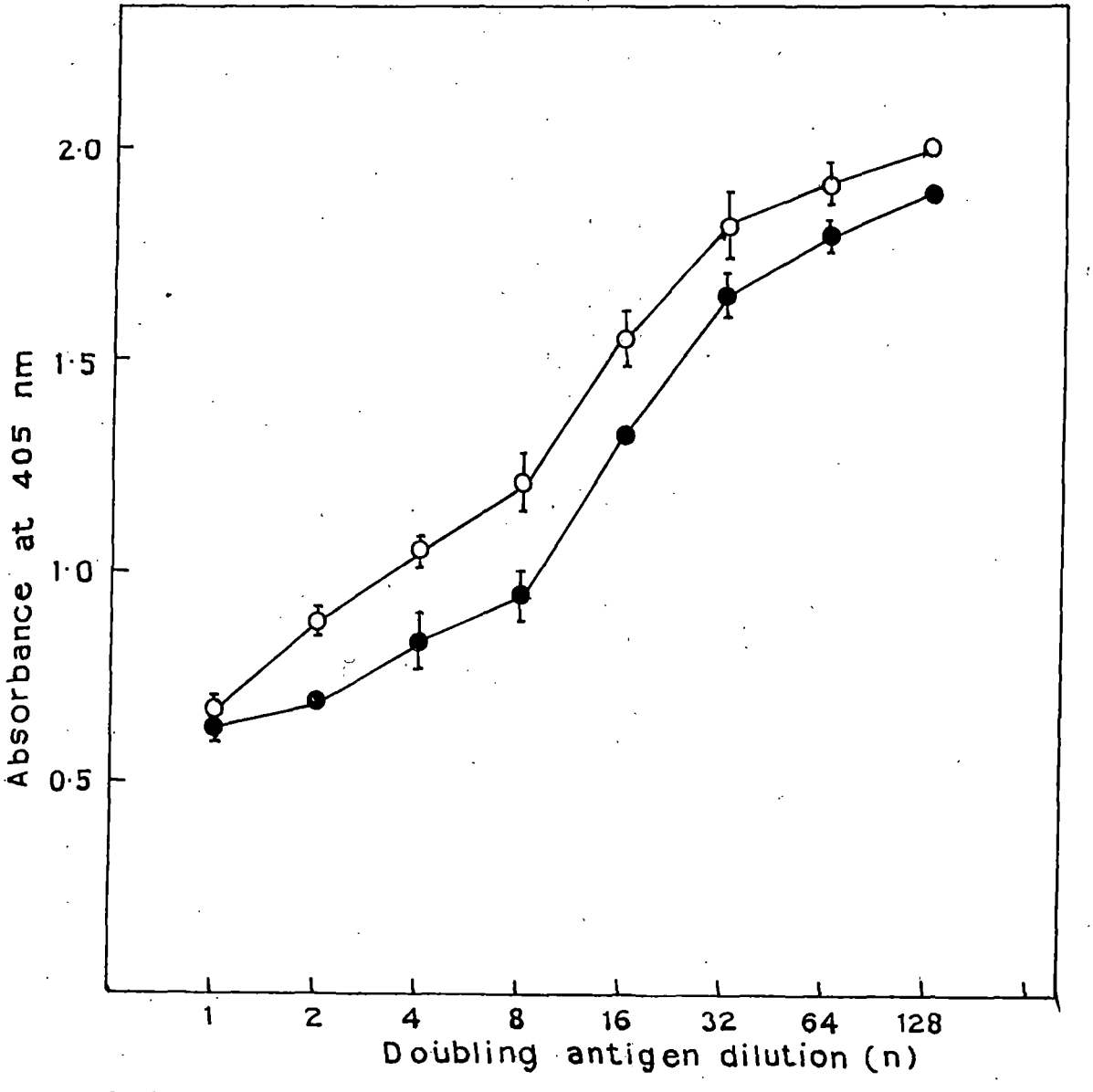
Antiserum dilution	Antigen concentration (2 μg/ml)			
	Expt.1	Expt.2	Expt.3	Mean
1:125	>2	>2	>2	>2
1:250	1.866	1.834	1.850	1.850 $\pm$ .009
1:500	1.730	1.779	1.750	1.750 $\pm$ .014
1:1000	1.624	1.607	1.599	1.610 $\pm$ .004
1:2000	1.616	1.601	1.604	1.607 $\pm$ .002
1:4000	1.410	1.419	1.413	1.414 $\pm$ .002
1:8000	0.975	0.926	0.949	0.950 $\pm$ .014
1:16000	0.722	0.764	0.743	0.743 $\pm$ .012

$\pm$ Standard error

4.3.4.1.1.3 Antigen dilution

To determine the effect of antigen dilution a doubling series of dilution of *G.cingulata* mycelial antigen from 3200 ng to 25 ng was made and tested against two antiserum dilutions (1:125 and 1:250). Results (Table 19, fig.5) revealed that ELISA values decreased with increasing dilution of antigen. However, concentration as low as 25 ng/ml could also be detected by ELISA. At a concentration of 3200 ng/ml and antiserum dilution of 1:125 absorbance values of above 2 was recorded.

Effect of dilution of mycelial antigen of *G.cingulata* on ELISA reaction with homologous antiserum



Antiserum o—o 1:125 , ●—● 1:250
n = from 25 ng/ml (1) to 3200 ng/ml (128)

Fig. 5.

Table 19 :ELISA reaction with various concentration of mycelial antigen of*G.cingulata* and homologous antiserum.

Antigen concentration (ng/ml)	Antiserum dilution	
	1:125	1:250
3200	>2	1.906 ± .001
1600	1.901 ± .034	1.804 ± .021
800	1.821 ± .046	1.663 ± .05
400	1.545 ± .035	1.349 ± .011
200	1.200 ± .032	0.949 ± .048
100	1.050 ± .021	0.838 ± .059
50	0.888 ±.03	0.682 ± .017
25	0.687 ± .029	0.646 ± .015

± Standard error.

4.3.4.1.2. Antiserum raised against antigen preparation from cell wall of *G. cingulata.*

Optimization of ELISA was also done using antiserum raised against cell wall preparations of *G. cingulata* and antigen prepared from cell wall. The effect of two variables, antiserum dilutuion and antigen dilution on ELISA reactivity, were determined.

4.3.4.1.2.1. Antigen dilution

Doubling dilution of *G. cingulata* cell wall antigen ranging from 3200 to 25 ng/ml were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with increasing concentration of antigen. Absorbance value of 0.9 was obtained at 25 ng/ml concentration and 1:125 antiserum dilution, and hence such low concentration was also detectable. At this antiserum dilution and antigen concentration of 3200 ng/ml absorbance value of >2 was obtai ned (Table 20 & Fig. 6).

Table 20 : ELISA reaction with various concentration of cell wall antigen of *G. cingulata* and homologous antiserum.

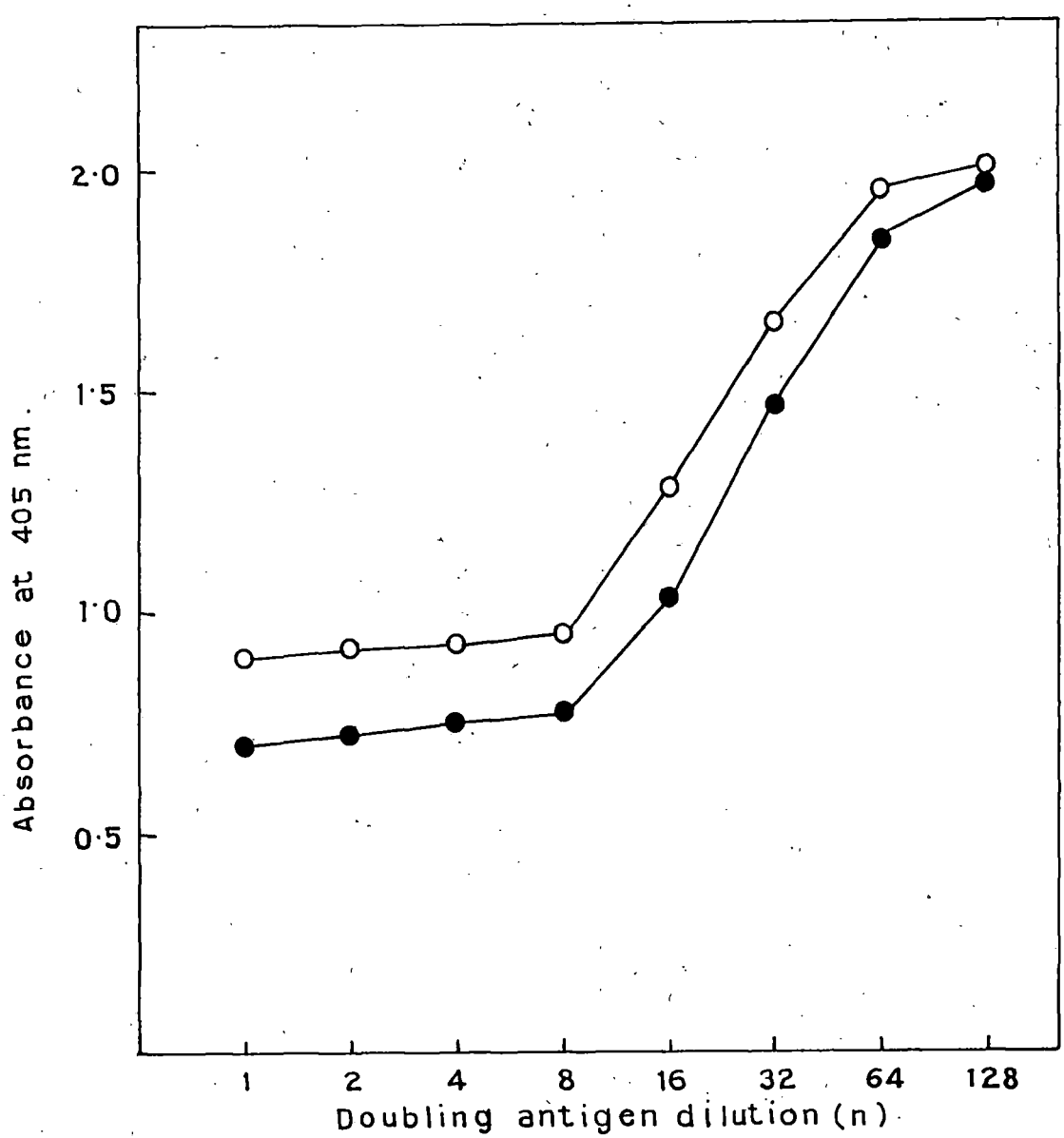
Antigen concentration	Absorbance at 405 nm[a] Antisera dilution	
(ng/ml)	1:125	1:250
3200	>2	1.984 ± .062
1600	1.952 ± .052	1.851 ± .018
800	1.662 ± .041	1.496 ± 029
400	1.292 ± .038	1.021 ± .072
200	0.952 ± .002	0.782 ± .063
100	0.932 ± .039	0.750 ± .039
50	0.917 ± .062	0.723 ± .018
25	0.906 ± .032	0.702 ±.042

a Average of three replicates
± Standard error.

4.3.4.1.2.2. Antiserum dilution

ELISA reaction with different dilution of antiserum ranging from 1:16,000 were determined at an antigen concentration 2 μg/ml. Absorbance values decreased with increasing dilution. At 1:125 dilution value of above 2 were obtained while at 1:250 dilution the value was 1.95. These value decreased to 0.88 at 1:16,000 dilution (Table-21, fig. 7). For further experiments 1:250 dilution was selected.

Effect of dilution of cell wall antigen of *G.cingulata* on ELISA reaction with homologous antiserum



Antiserum o—o 1:125 , ●—● 1:250
n = from 25 ng/ml (1) to 3200 ng/ml (128)

Fig. 6.

Table 21: ELISA reaction with various dilution of anti *G. cingulata* antiserum (cell wall) and homologous antigen.

Antiserum dilution (cell wall)	Absorbance at 405 nm			
	Antigen concentration (2μg/ml)			
	Expt. 1	Expt. 2	Expt. 3	Mean
1:125	>2	>2	>2	>2
1:250	1.952	1.912	1.975	1.946 ± 0.18
1:500	1.849	1.811	1.830	1.830 ± 0.01
1:1000	1.776	1.798	1.742	1.772 ± 0.016
1:2000	1.561	1.540	1.551	1.550 ± 0.006
1:4000	1.492	1.462	1.461	1.471 ± 0.010
1:8000	1.005	1.001	1.00	1.002 ± 0.001
1:16000	0.878	0.869	0.896	0.881 ± 0.038

± Standard error.

4.3.4.2. Comparison of ELISA reactivity among antigens of different tea varities against antiserum of *G. cingulata.*

Among 37 tea varieties tested for their resistance against *G. cingulata,* differential responses were obtained. Certain varieties exhibited high susceptibility, others high resistance while still others were either moderately susceptible or resistant. Conventional techniques for determination of host resistance or susceptibility are being replaced by more rapid and sensitive modern serological techniques. It was therefore considered worthwhile to determine the ELISA reactivity of different tea varieties against antiserum of the pathogen and compare the results obtained with those of the pathogenicity tests.

Effect of dilution of anti - *G.cingulata* antiserum on ELISA reaction with homologous cell wall antigen

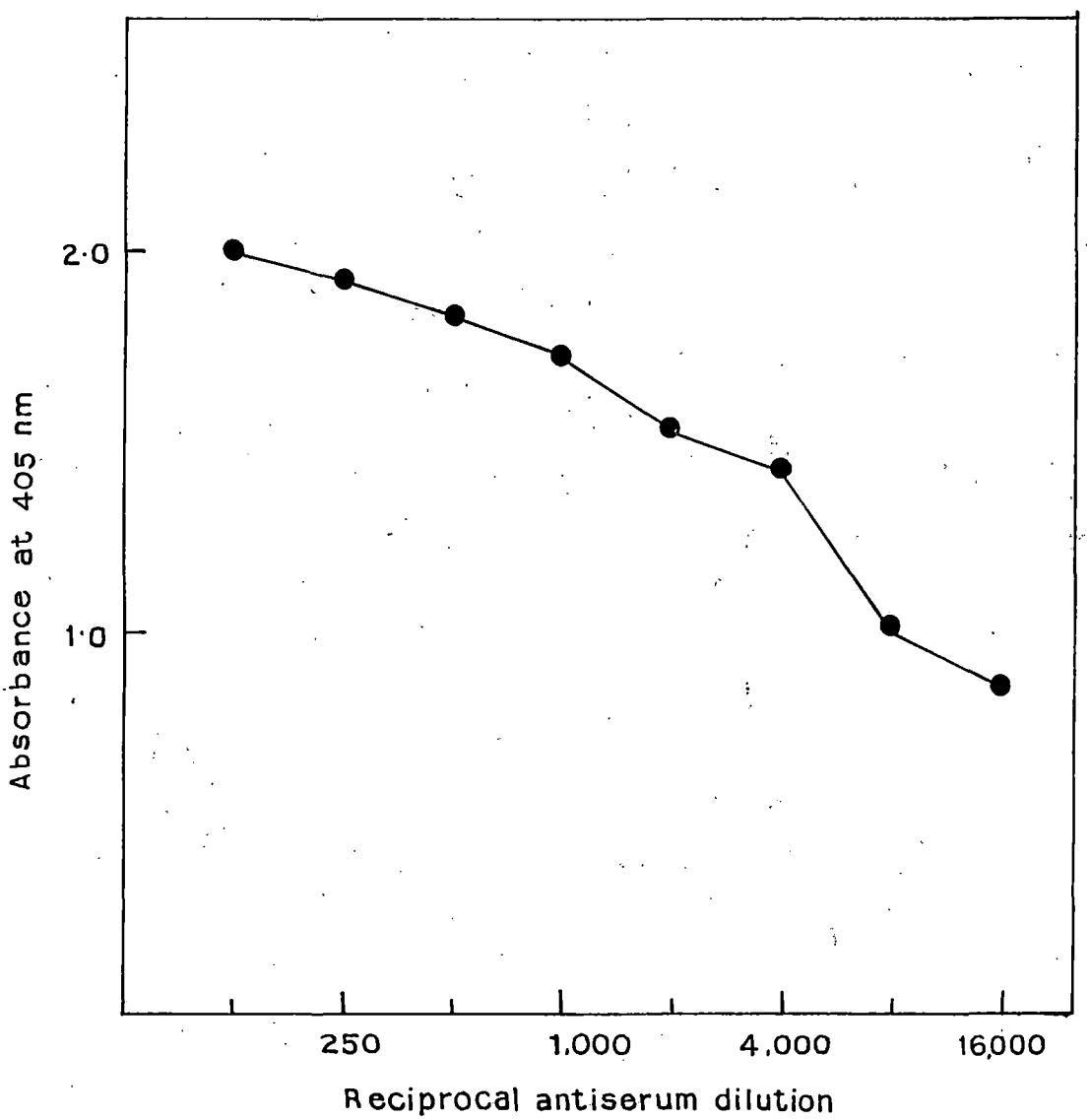


Fig. 7.

4.3.4.2.1. Mycelia

Antigens were prepared from tea leaves of all the 37 varieties as well as from 3 nonhost species (*G. max, C. arietinum, C. japonicum*), 9 isolates of the pathogen (*G. cingulata*) and 1 nonpathogen (*F. oxysporum*). Antigens at a concentration of 40 μg/ml were testedby DAC ELISA against purified mycelial antiserum of *G. cingulata* except incase of *G. cingulata* isolates, where a concentration of 5 μg/ml was used. In all cases experiments were repeated thrice under same condition. Results (Tables 22-24) revealed that absorbance value in ELISA varied with the different varieties. Among the Tocklai varieties tested TV-18 showed highest

Table 22: Indirect ELISA values (A_{405}) of tea leaf antigens (Darjeeling varieties) reacted with antiserum raised against mycelial antigen of *G.cingulata*

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
B 668	0.999	0.998	0.998	0.998±0.000
B 777	1.034	1.032	1.033	1.033±0.000
P 312	0.785	0.782	0.782	0.783±0.001
T-78	1.146	1.161	1.129	1.145±0.009
P-1258	0.967	0.921	0.944	0.944±0.013
B-157	1.350	1.481	1.408	1.413±0.037
TTV_1	1.117	1.190	1.123	1.143±0.023
BT-15	0.701	0.701	0.722	0.708±0.007
AV-2	0.833	0.827	0.833	0.831±0.002
BS/7A/76	0.757	1.012	1.034	1.023±0.088
RR 17	0.853	0.848	0.849	0.850±0.001
HV 39	1.034	1.003	1.017	1.018±0.008
KI/I	1.099	1.045	1.072	1.072±0.015
T-135	0.796	0.777	0.785	0.786±0.005
CP-1	0.738	0.804	0.801	0.781±0.021

± Standard error

A_{405} for normal serum 0.007

Table 23: Indirect ELISA values (A_{405}) of tea leaf antigens of UPASI varieties, non hosts, and mycelial antigens of *G.cingulata* isolates and *F.oxysporum* (non pathogen) reacted with anti-*G. cingulata* antiserum (mycelia).

Antigen concentration(40 μg/ml)	Antiserum of *G.cingulata* (GCIA) (1.250 dilution)			
	Expt.1	**Expt.2**	**Expt.3**	**Mean**
Tea varieties				
UPASI 2	0.729	0.768	0.747	0.748±0.011
UPASI 3	1.222	1.278	1.247	1.249±0.014
UPASI 8	0.945	1.008	0.975	0.976±0.025
UPASI 9	1.570	1.562	1.566	1.566±0.002
UPASI 17	0.864	0.824	0.844	0.844±0.011
UPASI 26	1.070	1.033	1.050	1.051±0.010
BSS1	0.947	1.004	0.974	0.975±0.016
BSS 2	0.899	0.958	0.954	0.937±0.019
BSS 3	0.860	0.905	0.881	0.882±0.013
Isolates of G.cingulata[a]				
GC-1	>2	>2	>2	>2
GC-2	1.885	1.927	1.825	1.879±0.040
GC-3	1.727	1.812	1.804	1.781±0.027
GC-4	1.779	1.751	1.736	1.755±0.0126
GC-5	1.892	1.801	1.877	1.856±0.028
GC-6	1.721	1.739	1.746	1.735±0.007
GC-7	1.776	1.712	1.801	1.763±0.026
GC-8	1.763	1.702	1.791	1.752±0.026
GC-9	1.733	1.756	1.800	1.763±0.0196
Non pathogen				
F. oxysporum	0.353	0.341	0.289	0.327 ± 0.019
Non host				
G.max	0.373	0.368	0.356	0.365 ± 0.005
C. arietinum	0.364	0.339	0.382	0.361 ± 0.010
C. japon icum	0.411	0.445	0.404	0.420 ± 0.012

A405 for normal sera = 0.005

± Standard error

a Concentration of antigen - 5μg/ml

absorbance followed by Teenali 17/1/54 & TV-20. Lowest absorbances were obtained in TV-26, TV-29 and TV-30 (Table-24). Similarly in the Darjeeling varieties highest and lowest absorbances were obtained in B157 and BT-15 respectively (Table-22). Of the 9 UPASI varities tested UPASI 9 exhibited highest reactivity while UPASI 2 exhibited lowest reactivity. Reactivity of the non host and non pathogen were low as evidenced by low absorbance values (Table-23). The different isolates of the pathogen tested also showed reactivity with the antiserum of one particular isolate (GC-1). Highest absorbance value(>2), however, was obtained in the homologus reaction. Absorbance values for normal serum controls were below the corresponding test values. Results of the ELISA tests revealed that the highest reactivitywas shown by UPASI 9 and the lowest by BT-15. These results were in confirmity with the results of the pathogenicity tests.

Table 24: Indirect ELISA values (A_{450}) of tea leaf antigens of Tocklai varieties reacted with antiserum of *G. cingulata* (Mycelia)

Antigens of (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
TV-9	0.883	0.892	0.808	0.861 ± 0.026
TV-18	1.464	1.425	1.443	1.444 ± 0.011
TV-20	1.028	0.937	0.918	0.982 ± 0.026
TV-22	0.895	0.897	0.890	0.894 ± 0.001
TV-23	0.963	0.963	0.963	0.963 ± 0.000
TV-25	0.781	0.997	0.937	0.967 ± 0.061
TV-26	0.826	0.829	0.861	0.838 ± 0.011
TV-27	0.899	0.880	0.888	0.889 ± 0.005
TV-28	0.898	0.821	0.874	0.864 ± 0.020
TV-29	0.882	1.000	0.834	0.858 ± 0.040
TV-30	0.858	0.866	0.889	0.860 ± 0.009
Teenali 17/1/54	0.950	1.042	0.785	0.996 ± 0.070
TS-449	0.830	0.917	0.894	0.880 ± 0.026

± Standard error; A_{405} for normal sera = 0.007

4.3.4.2.2. Cell wall

Since cell walls of pathogen also play a major role in the recognition phenomenon antiserum raised against cell wall preparations of *G.cingulata* was also tested against leaf antigens of all the 37 varieties. As in the case with antiserum raised against mycelial antigen here also absorbance values in ELISA varied with the different varieties. Results have been presented in Tables 25-27. Among all the 37 varieties tested with the antiserum against cell wall preparation highest absorbance value was exhibited by UPASI-9 and lowest by BT-15. Low absorbance values were obtained with non hosts and non pathogen.

The overall trend in ELISA reactivity with the different tea varieties and antiserum of mycelia or cell wall preparations of *G.cingulata* were similar. However, absorbance values were higher in most cases with antiserum raised against cell wall preparations than those of antiserum raised against mycelial preparation of *G.cingulata* as evidenced in fig.8.

4.3.4.3. Comparison of ELISA reactivity among antigens prepared from tea leaves of various ages against antiserum of *G. cingulata.*

Since tea is a perennial plant being maintained year after year, individual tea bushes having a large number of leaves of various size and position created an interest to investigate whether serological resemblances among tea varieties and the isolates of *G. cingulata* as evidenced in earlier experiments also existed in case of the leaves of all ages. In order to perform this experiment, 11 Tocklai varieties (Teenali 17/1/54, TV-18, 20, 22, 23, 25, 26, 27, 28, 29 and 30) were selected. Leaf samples were collected from individual variety considering the position and size of the leaves and they were categorised into three groups (A, B and C). Uppermost young leaves (10-15 mm length x 6-10 mm breadth), mid-branches middle aged leaves (40-45 length x 25-30 mm breadth) and lower branches mature leaves (65-72 mm length x 35-40 mm breadth) were designated as A, B and C respectively. Antigens were prepared from the categories of leaves of each of 11tea varieties and using anti *G. cingulata* antisera raised against both mycelia and cell wall DAC - ELISA was performed.

4.3.4.3.1. Mycelia

Anti *G. cingulata* antiserum (1;250 dilution) raised against antigen preparations from mycelia were cross reacted with 40 μg/ml antigens prepared from various category of 11 varieties following DAC-ELISA. Homologous reaction involving mycelial antigen and antisera gave A_{405} value of >2, while highest absorbance values in heterologous reactions were in case of TV-18 and TV-23 followed by TV-29 and Teenali 17/1/54.However , TV-26, TV-25 and TV-20 showed absorbance values <1. It is interesting to note that leaf antigens prepared from all 3 categories gave positive response in ELISA reactions under identical condition (Table - 28)

Table 25: Indirect ELISA values (A_{405}) of tea leaf antigens of UPASI varieties,non host and mycelial antigen of *G. cingulata* and *F. oxysporum* reacted with cell wall antiserum of *G. cingulata*.

Antigen Concentration (40 μg/ml	Antiserum of *G. cingulata* (GCIA) (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
Teavarieties				
UPASI 2	1.272	1.259	1.252	1.261 ± 0.005
UPASI 3	1.776	1.774	1.775	1.775 ± 0.005
UPASI 8	1.545	1.576	1.554	1.558 ± 0.009
UPASI 9	1.849	1.834	1.840	1.841 ± 0.004
UPASI 17	1.326	1.376	1.377	1.359 ± 0.016
UPASI 26	1.727	1.709	1.718	1.718 ± 0.005
BSS 1	1.574	1.577	1.643	1.568 ± 0.022
BSS 2	1.611	1.498	1.553	1.554 ± 0.032
BSS 3	1.483	1.569	1.526	1.526 ± 0.024
Isolates of *G. cingulata*[a]				
GC-1	>2	>2	>2	>2
GC-2	1.929	1.973	1.981	1.961 ± 0.021
GC-3	1.834	1.817	1.821	1.824 ± 0.042
GC-4	1.969	1.901	1.922	1.930 ± 0.020
GC-5	1.929	1.962	1.956	1.949 ± 0.010
GC-6	1.825	1.812	1.834	1.820 ± 0.006
GC-7	1.861	1.869	1.883	1.871 ± 0.006
GC-8	1.902	1.911	1.879	1.897 ± 0.009
GC-9	1.803	1.872	1.889	1.854 ± 0.026
Non pathogen				
F. oxysporum	0.378	0.329	0.386	0.364 ± 0.017
Non host				
G. max	0.399	0.378	0.351	0.376 ± 0.013
C. arietinum	0.311	0.363	0.351	0.376 ± 0.015
C. japonicum	0.412	0.426	0.404	0.414 ± 0.005

A_{405} for normal serum = 0.009.

± Standard error

a Antigen concentration 5 μg/ml

Table 26: Indirect ELISA values (A_{405}) of tea leaf antigens of Darjeeling varieties reacted with cell wall antiserum of *G. cingulata* .

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt. 1	Expt. 2	Expt. 3	Mean
B668	1.592	1.657	1.618	1.622 + 0.018
B777	1.635	1.692	1.653	1.660 ± 0.016
P312	1.343	1.222	1.281	1.282 ± 0.050
T-78	1.771	1.761	1.748	1.760 ± 0.006
P-1258	1.573	1.534	1.543	1.550 ± 0.011
B-157	1.803	1.849	1.826	1.826 ± 0.013
TTV_1	1.750	1.793	1.716	1.753 ± 0.022
BT-15	1.118	1.196	1.171	1.161 ± 0.023
AV-2	1.299	1.363	1.256	1.306 ± 0.031
BS/7A/76	1.668	1.645	1.686	1.666 ± 0.011
RR17	1.432	1.417	1.423	1.424 ± 0.004
HV-39	1.663	1.603	1.684	1.650 ± 0.024
K1/1	1.734	1.772	1.753	1.753 ± 0.010
T-135	1.363	1.269	1.247	1.293 ± 0.028
CP-1	1.365	1.205	1.285	1.285 ± 0.046

± Standard error.

A_{405} for normal sera = 0.007

Table 27: Indirect ALISA values (A_{405}) of tea leaf antigens of Tocklai varieties reacted with antiserum of *G. cingulata* (cell wall).

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt. 1	Expt. 2	Expt. 3	Mean
TV-9	1.458	1.502	1.502	1.487 ± 0.014
TV-18	1.672	1.680	1.676	1.676 ± 0.002
TV-20	1.556	1.560	1.582	1.566 ± 0.008
TV-22	1.538	1.568	1.553	1.553 ± 0.008
TV-23	1.560	1.569	1.563	1.564 ± 0.002
TV-25	1.586	1.626	1.527	1.570 ± 0.015
TV-26	1.382	1.306	1.344	1.344 ± 0.021
TV-27	1.515	1.502	1.579	1.532 ± 0.023
TV-28	1.535	1.503	1.519	1.519 ± 0.009
TV-29	1.433	1.407	1.435	1.425 ± 0.009
TV-30	1.493	1.506	1.498	1.499 ± 0.003
Teenali 17/1/54	1.578	1.528	1.645	1.583 ± 0.017
TS-449	1.484	1.555	1.518	1.519 ± 0.019

± Standard error.

$A_{(405)}$ for normal serum = 0.005.

ELISA responses of leaf antigens of different tea varieties against cell wall and mycelial antisera of *G.cingulata*

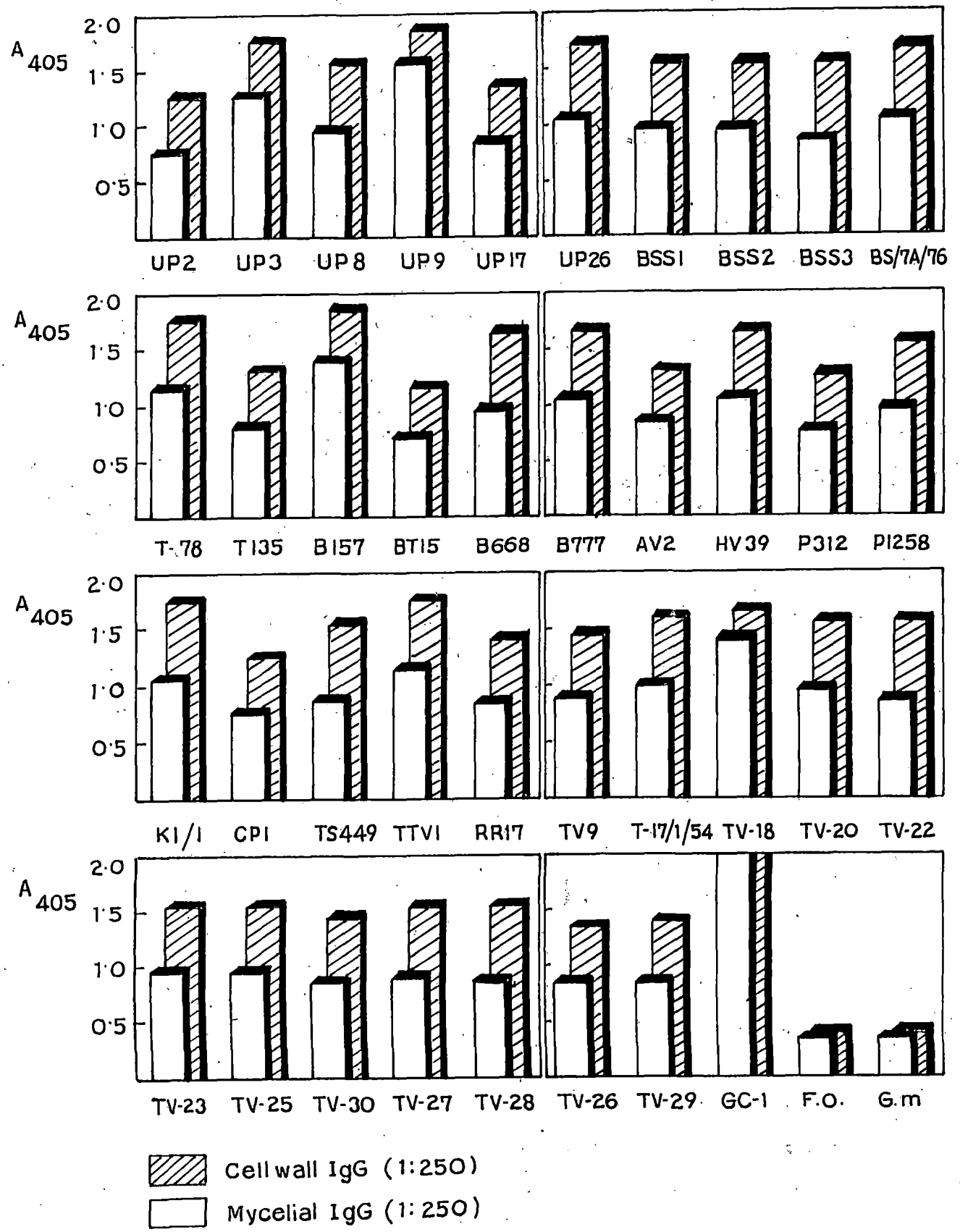


Fig. 8.

Table 28: Indirect ELISA values (A_{405}) of antigens prepared from tea leaves of various ages against antiserum of *G. cingulata* (Mycelia)

Antigen concentration 40 μg/ml	Mycelial antiserum of *G. cingulata* (1:250 dilution)		
	Position and size of tea leaves*		
	A	B	C
Teenali 17/1/54	1.151 ± 0.016	1.100 ± 0.013	1.126 ± 0.013
TV-18	1.225 ± 0.029	1.512 ± 0.027	1.565 ± 0.014
TV-20	0.996 ± 0.002	1.101 ± 0.025	1.210 ± 0.011
TV-22	1.015 ± 0.013	0.955 ± 0.018	1.109 ± 0.003
TV-23	1.214 ± 0.031	1.160 ± 0.047	1.109 ± 0.017
TV-25	0.996 ± 0.014	1.095 ± 0.015	1.078 ± 0.031
TV-26	0.726 ± 0.013	0.980 ± 0.012	1.065 ± 0.021
TV-27	1.063 ± 0.012	1.082 ± 0.006	1.145 ± 0.020
TV-28	1.096 ± 0.013	1.051 ± 0.021	1.100 ±0.012
TV-29	1.151 ± 0.024	1.075 ± 0.013	1.232 ± 0.017
TV-30	1.068 ± 0.008	1.113 ± 0.010	1.152 ± 0.016

Note: $A_{(405)}$ valueof homologous reaction >2; for normal serum = 0.006

*A = Uppermost young leaves;

B = Mid branches middle aged;

C = Mature old leaves; lower branches;

± Standard error.

4.3.4.3.2. Cell wall

When antiserum of *G. cingulata* (1:250 dilution) raised against cell wall antigens were cross reacted with three categories of leaf antigens (40 μg/ml) prepared from 11 Tocklai varieties, young leaves of TV-18 followed by TV-23, TV-20, TV-28, TV-22, showed greater absorbance values than Teenali 17/1/54, TV-25 and TV-27. Here also lowest A_{405} value was obtained in case of TV-26 (Table-29). Recognition of leaf antigens with cell wall antiserum of *G. cingulata* was always better than the antiserum of the pathogen raised against mycelial preparation. In this experiment, It was notised that there was no correlation among the antigen preparation either from young or middle or mature leaves with respect to the individual variety tested. However, irrespective of verieties or categories A_{405} values were always higher with cell wall antiserum of the pathogen than the anti *G. cingulata* antiserum raised against the mycelial antigen (Fig. 9).

4.3.4.4. Reciprocal cross reaction of antisera of tea varieties and non pathogen with leaf antigens (host and non host) and mycelial antigens (pathogen and non pathogen).

In previous experiments following DAC-ELISA serological relationship between tea leaves and *G.cingulata* have been detected using antiserum of the pathogen raised against mycelia and cell wall. Indirect ELISA could also readily detect major cross reactive antigens when reciprocal cross reaction with host antisera was performed. In this experiment, antisera raised against leaf antigens prepared from two varieties of tea TV-18 and CP-1 which were found to be most susceptible and resistant respectively in pathogenicity test, were cross reacted with leaf antigens prepared from 14 Tocklai varieties (CP-1, TS-449, Teenali 17/1/54, TV-18, 20, 22, 23, 25, 26, 27, 28, 29 and 30), 3 non-host species (viz. *G.max, C.arietinum* and *C. japonicum*) as well as mycelial antigen prepared from pathogen (*G.cingulata*, isolate GC-1) and non pathogen (*F.oxysporum*). Simultaneously, antiserum raised against *F.oxysporum* (non-pathogen) was also tested against the antigens of the above materials. Results have been presented in Table-30. In this experiment, A_{405} values in ELISA reaction involving antigens and antisera of CP-1, TV-18 and *F.oxysporum* were 1.642, 1.712 and 1.821 respectively. When anti CP-1 antiserum was cross reacted with various antigens of host, non host, pathogen and non pathogen, TV-9, TS-449. Teenali 17/1/54 and TV-23 gave higher absorbance than the other 10 varieties of tea tested. Anti TV-18 antiserum gave higher absorbance in TS-449, TV-23, TV-28 and Teenali 17/1/54 than the other varieties tested. It is interesting to note that in cross reaction with anti TV-18 antiserum and mycelial antigen of *G.cingulata* higher absorbance (1.132) was obtained. But anti CP-1 antiserum gave lower

Table 29: Indirect ELISA values (A_{405}) of antigen prepared from leaves of various ages against antiserum of *G. cingulata* (Cell wall)

Antigen concentration 40 μg/ml	Cell wall antiserum of *G. cingulata* (1:250 dilution)		
	Position and size of tea leaves*		
	A	B	C
Teenali 17/1/54	1.615 ± 0.004	1.765 ± 0.009	1.771 ±0.008
TV-18	1.750 ± 0.05	1.912 ± 0.070	1.862 ±0.029
TV-20	1.725 ± 0.005	1.692 ± 0.030	1.751 ± 0.015
TV-22	1.721 ± 0.004	1.728 ± 0.007	1.730 ± 0.004
TV-23	1.728 ± 0.015	1.750 ± 0.016	1.738 ± 0.012
TV-25	1.635 ± 0.005	1.729 ± 0.031	1.600 ± 0.009
Tv-26	1.002 ± 0.006	1.065 ± 0.070	1.231 ± 0.020
Tv-27	1.612 ± 0.006	1.803 ± 0.012	1.609 ± 0.014
Tv-28	1.725 ± 0.023	1.731 ± 0.043	1.800 ± 0.021
Tv-29	1.310 ± 0.025	1.415 ± 0.009	1.611 ± 0.017
Tv-30	1.211 ± 0.016	1.226 ± 0.017	1.512 ± 0.024

Note: A_{405} valueof homologous reaction >2; for normal serum = 0.006 ; ± Standard error.
*A = Uppermost young leaves; B = Mid branches middle aged; C = Mature old leaves;

absorbance value (0.673) when reacted with the mycelial antigen of the pathogen. Antiserum raised against non pathogen (*F.oxysporum*) did not react significantly with the tea leaf antigens, as lower absorbance values were detected in all cases. However, A_{405} value of 0.621 was obtained, when this antiserum was reacted with the antigen of *G.max* since, *F.oxysporum* is pathogenic to *G.max* (cv. Soymax). Non host and non pathogen antigens also did not react with anti TV-18 and anti CP-1 antiserum (Table-30).

ELISA reactivity among antigens prepared from tea leaves of various ages against cell wall and mycelial antisera of *G.cingulata*

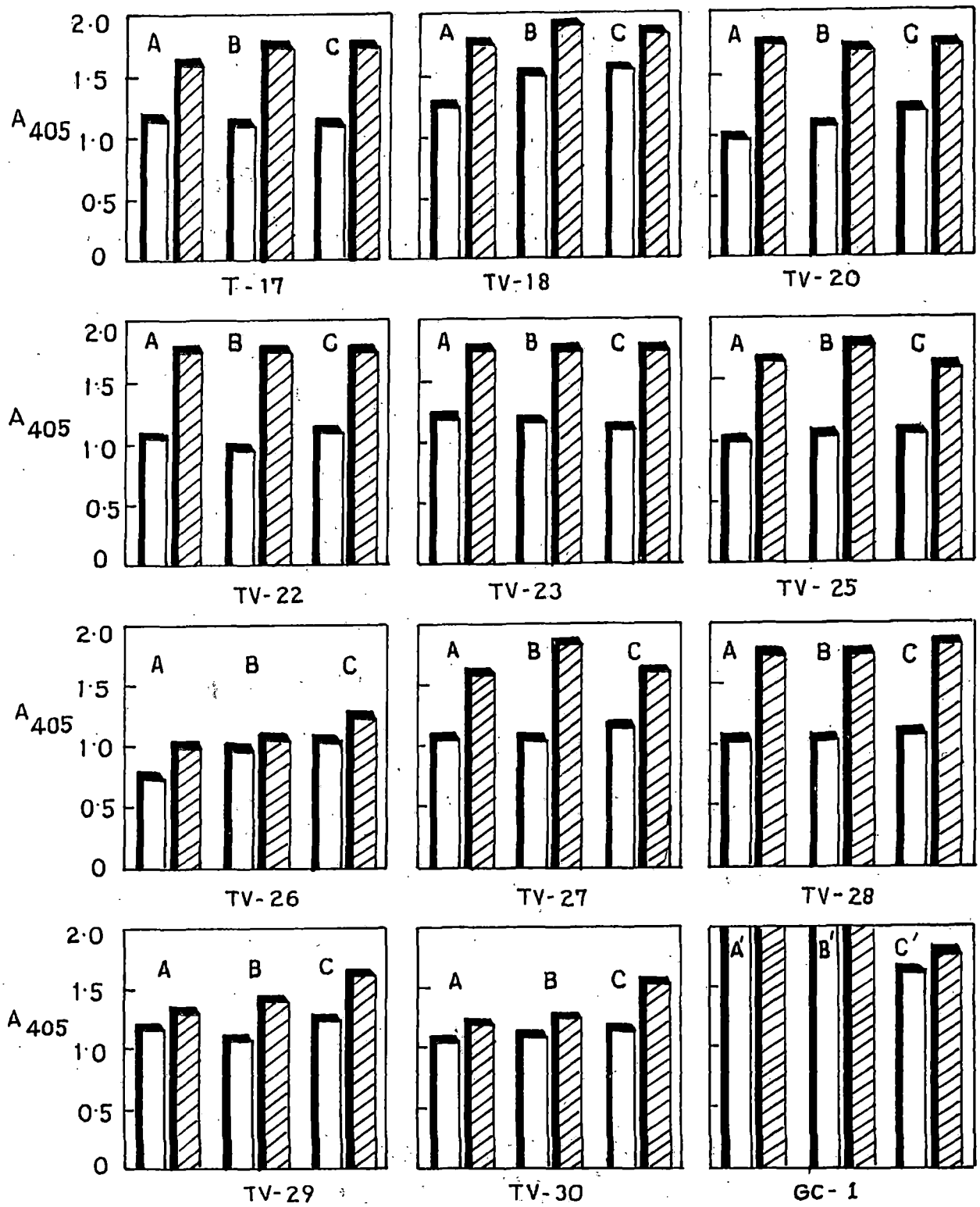


Fig.9

Table 30: Indirect ELISA values (A_{405}) of leaf antigens (host and non host) and mycelial antigens (pathogen and non pathogen) reacted with antisera of tea varieties, resistant (CP-1) and susceptible(TV-18) to *G.cingulata* and non-pathogen (*F.oxysporum)*

Antigens of host and parasite	Antisera dilution (1:250)		
	Tea varieties		Non-pathogen
	(CP1A)	(TI8A)	*F. oxysporum*
Tea varieties			
CP-1	1.642±0.001	0.895±0.001	0.301±0.001
S-449	1.055±0.016	1.122±0.016	0.312±0.016
TV-9	1.109±0.021	0.968±0.021	0.326±0.021
Teen Ali-17/1/54	1.015±0.008	1.060±0.020	0.346±0.009
TV-18	0.985±0.016	1.712±0.041	0.311±0.021
TV-20	0.848±0.008	0.935±0.032	0.319±0.036
TV-22	0.977±0.002	0.896±0.061	0.349±0.006
TV-23	1.069±0.019	1.102±0.047	0.361±0.029
TV-25	0.782±0.016	0.865±0.016	0.329±0.006
TV-26	0.993±0.021	0.981±0.023	0.261±0.011
TV-27	0.977±0.002	0.827±0.006	0.299±0.061
TV-28	0.812±0.061	1.004±0.019	0.301±0.070
TV-29	0.796±0.002	0.809±0.016	0.326±0.042
TV-30	0.818±0.018	0.874±0.009	0.316±0.039
Pathogen			
G.cingulata(GC-1)	0.673±0.006	1.132±0.019	0.381±0.006
Non pathogen			
F.oxysporum	0.198±0.031	0.140±0.011	1.821±0.042
Non host			
G.max	0.245±0.016	0.270±0.036	0.621±0.002
C.arietinum	0.267±0.021	0.259±0.029	0.269±0.019
C.japonicum	0.258±0.031	0.176±0.019	0.312±0.022

± Standard error

A_{405} for normal serum = 0.007

4.3.5. Double antibody sandwich (DAS) ELISA

Detection of cross reactive antigens shared between isolates of *G. cingulata* and tea varieties by indirect ELISA using the format of DAS ELISA was further confirmed by using another format of direct ELISA i.e., double antibody sandwich (DAS) ELISA. In this experiment, antigens were prepared from 5 selected varieties of tea - (viz., Teenali 17/1/54, TV -18, TV-23, CP-1 and TV-26), 3 non host species (*G. max, C. japonicum and C. arietinum*), an isolate of *G. cingulata* (GC-1) and *F. oxysporum* (non pathogen). Anti *G. cingulata* antiserum at 1:250 dilution was reacted with the antigen preparations (40 μg/ml) as mentioned

Table 31: Reaction of anti *G. cingulata* antiserum against tea leaf antigens, non hosts, mycelial antigen of *G. cingulata* (isolate GC-1) and *F. oxysporum* (non pathogen) using double antibody sandwich (DAS) ELISA.

Antigen concentration (40 μg/ml)	Anti-*G. cingulata* antiserum (at 1:250 dilution) Absorbance at 450 nm			
	Expt. 1	**Expt. 2**	**Expt. 3**	**Mean**
Pathogen				
G. cingulata	1.409	1.415	1.407	1.410 ± 0.002
Host				
Teenali-17/1/54	1.105	1.054	1.101	1.086 ± 0.016
TV-18	1.254	1.255	1.217	1.242 ± 0.012
TV-23	0.982	0.923	0.975	0.960 ± 0.018
CP-1	0.738	0.746	0.797	0.760 ± 0.018
TV-26	0.866	0.706	0.676	0.749 ± 0.050
Non hosts				
C. japonicum	0.391	0.372	0.392	0.385 ± 0.006
G. max	0.377	0.477	0.411	0.421 ± 0.029
C. arietinum	0.328	0.315	0.312	0.318 ± 0.004
Non pathogen				
F. oxysporum	0.396	0.426	0.361	0.394 ± 0.018

above following DAS ELISA. Details of the method have been described under Materials and Methods. Results have been presented in Table 31. Antigen and antisera of *G. cingulata* were highly reactive. Mean A_{405} values for TV-18 and Teenali 17/1/54 were 1.242 and 1.086, while, for TV-26 and CP-1 these were 0.749 and 0.760 respectively. Anti *G. cingulata* antiserum had a significantly higher absorbance mean reaction ($P < 0.01$) to susceptible varieties (TV-18 and Teenali 17/1/54) than to resistant varieties (TV 26 and CP-1). Antigen prepared from non host and non pathogen gave lower absorbance values.

4.4. Detection of *G.Cingulata* in infected leaf tissue by indirect ELISA

ELISA is one of the recent successful techniques for pathogen detection in the host where antiserum raised against the pathogen reacts with antigens of infected material to give high absorbance values. Differences in ELISA readings between infected and healthy antigens indicates the measure and extent of infection. In the present investigation initially it has been established that cross reactive antigens are present between tea leaves and *G.cingulata.* In the next series of experiments attempts have been made to detect *G.cingulata* in infected leaf tissues of different varieties as well as after different hours of inoculation.

4.4.1 Artificially inoculated leaves

Leaves of all the tea varieties used in previous studies were inoculated with *G.cingulata* as described in Materials and Methods. Antigens were extracted after 72 h of inoculation as by this time symptoms were very well established on the leaves. Antigens prepared from inoculated leaves as well as from corresponding healthy leaves,at concentrations of 40μg/ml were reacted with anti *G.cingulata* antiserum at 1:250 dilution. Results (Tables 32-34, Figs. 10 and 11) revealed that absorbance values for inoculated leaf antigen preparation of all varieties were significantly higher than their respective healthy leaf extracts. However, healthy leaf extracts also showed quite high absorbance values due to the presence of cross reactive antigens.

Table 32: ELISA values showing reaction of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Tocklai varieties (absorbance at 405 nm)

Tea varieties	Antigen concentration (40 μg/ml)	
	Healthy	Inoculated[a]
TV-9	0.942±0.012	1.233±0.011
Teenali 17/1/54	0.999±0.011	1.342±0.023
TV-18	1.020±0.018	1.469±0.012
TV-20	0.899±0.029	1.062±0.011
TV-22	0.927±0.020	1.116±0.018
TV-23	0.989±0.021	1.217±0.039
TV-25	0.971±0.008	1.116±0.013
TV-26	0.729±0.017	0.929±0.017
TV-27	0.853±0.031	1.002±0.001
TV-28	0.796±0.003	0.989±0.035
TV-29	0.763±0.016	0.962±0.028
TV-30	0.792±0.-012	1.020±0.008
TS-449	0.783±0.007	1.006±0.027

Anti *G.cingulata* antiserum used at 1:250 dilution

[a]3d after inoculation with *G.cingulata*

Difference between healthy and inoculated significant at p = 0.01 in all varieties

± Standard error.

Table 33: ELISA values (A_{405}) of healthy and *G.cingulata* inoculated (72h) leaf antigens of different Darjeeling varieties reacted with pathogen antiserum.

Tea variety	Leaf antigens (40 μg/ml)	
	Healthy	Inoculated[a]
B 668	0.733±0.002	0.977±0.021
B 777	0.751±0.019	1,084±0.002
P 312	0.722±0.023	0.991±0.006
T-78	0.811±0.005	1.176±0.009
P-1258	0.784±0.014	1.018±0.016
B-157	0.897±0.021	1.215±0.039
TTV_1	0.801±0.001	0.916±0.005
BT-15	0.716±0.003	0.903±0.039
AV-2	0.719±0.016	0.967±0.024
BS/7A/76	0.804±0.005	1.010±0.002
RR17	0.733±0.007	1.012±0.062
HV-39	0.735±0.019	0.914±0.003
K1/1	0.753±0.006	1.113±0.040
CP-1	0.721±0.016	0.927±0.070
T-135	0.723± 0.009	1.002±0.039

Anti *G. cingulata* antiserum used at 1:250 dilution.

[a]3 d after inoculation with *G.cingulata*

Differences between healthy and inoculated significant at p=0.01 in all varieties.

± Standard error

Table 34: ELISA values showing reaction of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of UPASI varieties (absorbance at 405 nm)

Tea variety	Antigen concentration (40 μg/ml)	
	Healthy	Inoculated[a]
UPASI-2	0.629 ± 0.002	0.759 ± 0.009
UPASI-3	0.687 ± 0.012	1.073 ± 0.019
UPASI-8	0.635 ± 0.006	0.869 ± 0.017
UPASI-9	0.739 ± 0.021	1.242 ± 0.016
UPASI-17	0.630 ± 0.022	0.763 ± 0.012
UPASI-26	0.670 ± 0.036	1.049 ± 0.020
BSS-1	0.668 ± 0.017	0.892 ± 0.010
BSS-2	0.679 ± 0.022	0.919 ± 0.021
BSS-3	0.652 ± 0.016	0.879 ± 0.006

Anti *G.cingulata* antiserum used at 1:250 dilution.

a - 3d after inoculation with *G.cingulata*.

Differences between healthy and inoculated significant at p=0.01

± Standard error.

ELISA responses of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Tocklai varieties

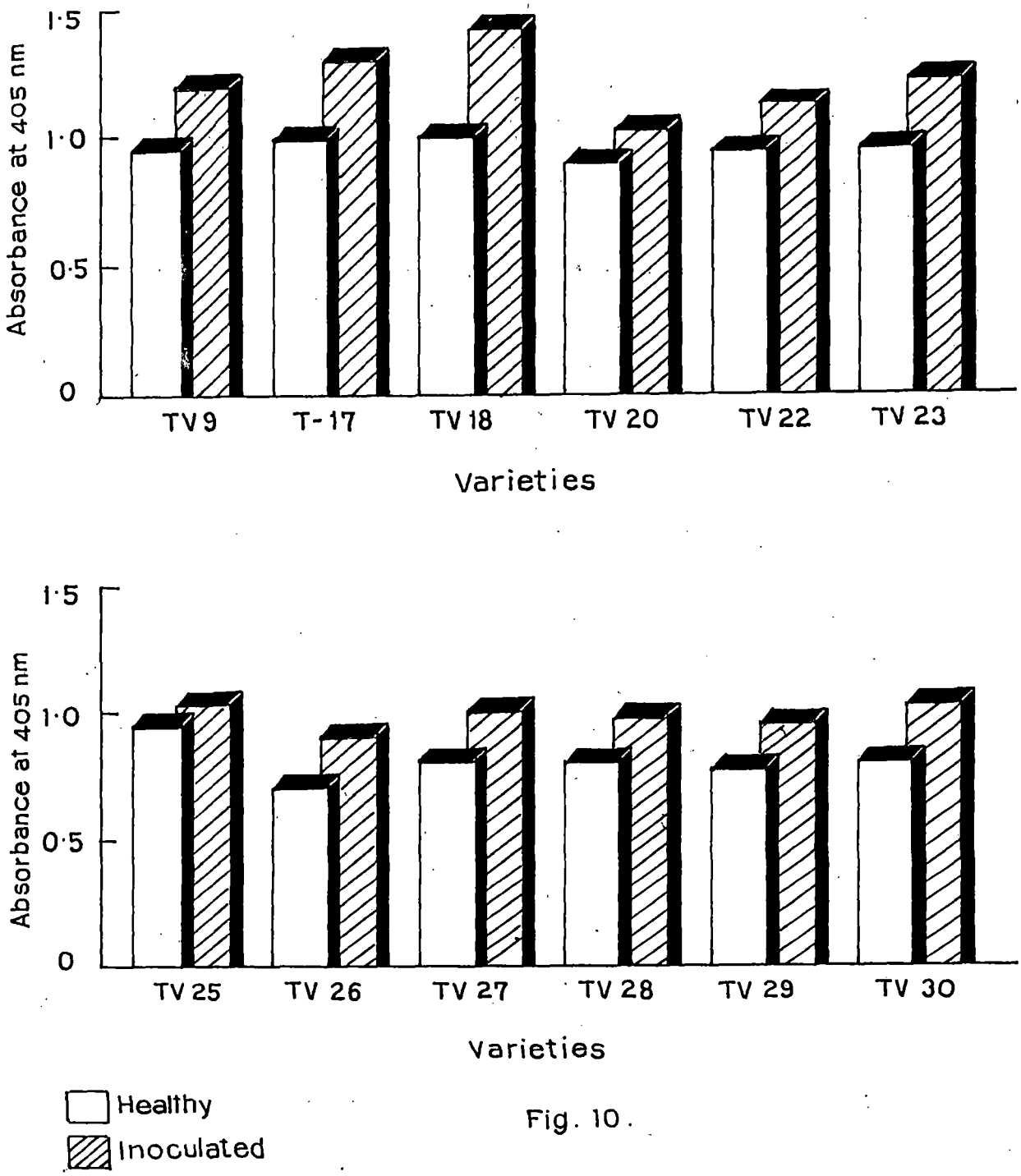


Fig. 10.

ELISA responses of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Darjeeling varieties

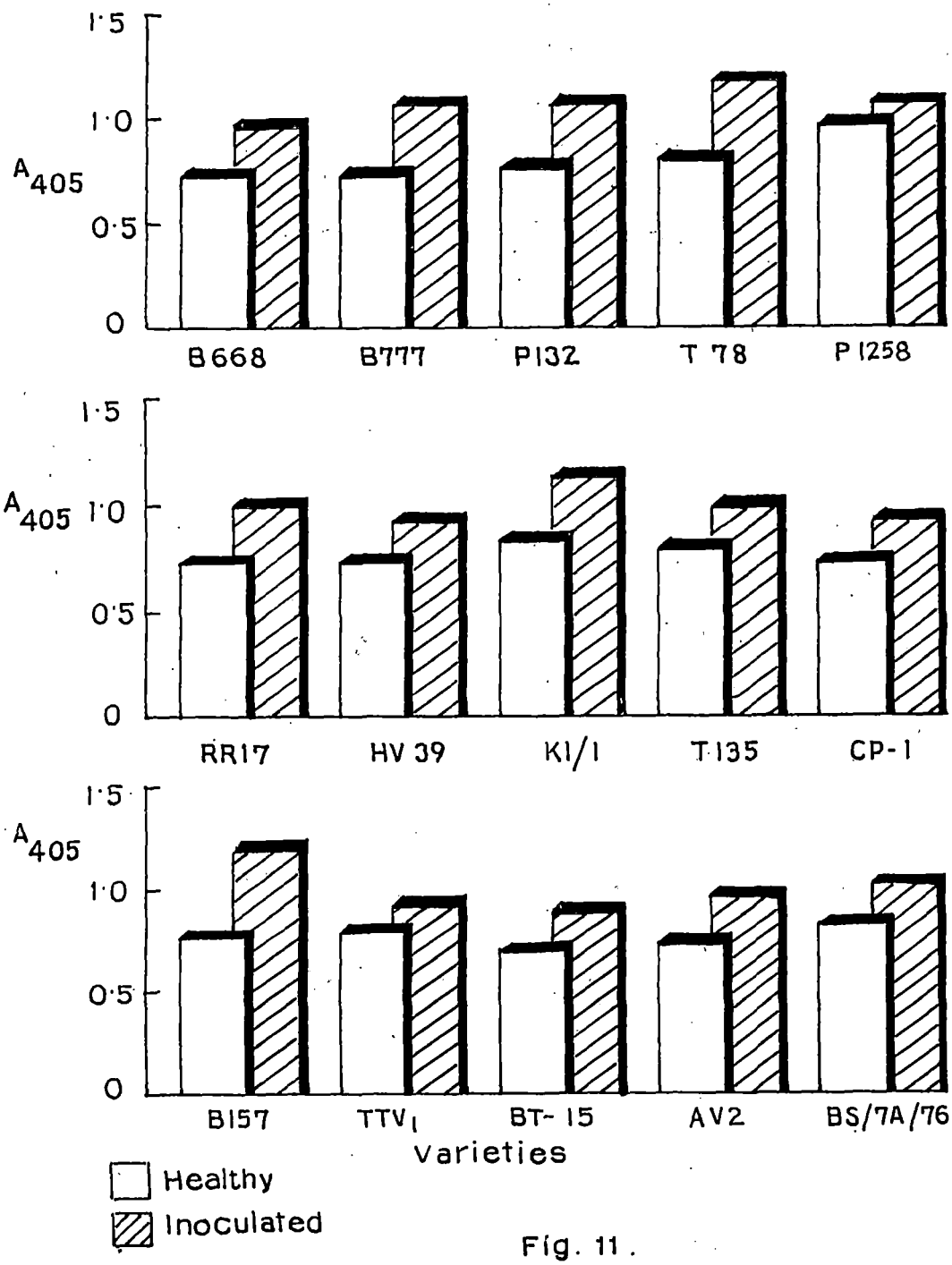


Fig. 11.

4.4.2 Naturally infected leaves

In the previous experiment anti *G.cingulata* antiserum was used to detect infection in artificially inoculated tea leaves. Subsequently in the next experiment it was decided to test whether natural brown blight infection could be detected with this antiserum. For this, tea leaves of different UPASI varieties showing brown blight infection in the field were collected and antigens were prepared from these leaves. Using the antigen extracts from healthy and naturally infected tea leaves ELISA was performed against anti *G.cingulata* antiserum. Results presented in Table-35 revealed that infected extracts of all varieties had significantly higher absorbance values in ELISA in comparison to the healthy extracts.

Table 35: ELISA values showing reaction of antiserum of *Glomerella cingulata* with antigens of healthy and naturally infected tea leaves of UPASI varieties.

Tea varieties	Antigen concentration (40 μg/ml)	
	Healthy	Infected[a]
UPASI clones		
UPASI-2	0.619 ± 0.017	0.713 ± 0.011
UPASI-3	0.690 ± 0.001	1.020 ± 0.015
UPASI-8	0.647 ± 0.012	0.964 ± 0.017
UPASI-9	0.795 ± 0.007	1.154 ± 0.029
UPASI-17	0.700 ± 0.019	1.092 ± 0.019
UPASI-26	0.675 ± 0.027	1.019 ± 0.022
Seed varieties		
BSS-1	0.592 ± 0.002	1.012 ± 0.017
BSS-2	0.612 ± 0.023	1.023 ± 0.022
BSS-3	0.724 ± 0.015	1.000 ± 0.037

Anti *G.cingulata* antiserum used at 1:125 dilution

a - Naturally infected tea leaves.

Difference in values between inoculation and healthy leaves significant at p=0.01.

± Standard error.

4.4.3 Different times after inoculation

G.cingulata was detected in infected tea leaves of different varieties using antiserum raised against *G.cingulata*. Since in these cases antigens were extracted after 72h of inoculation, symptoms were already well developed at the time of antigen preparation. As ELISA has been considered to be an important tool for early detection of pathogen in infected tissues even before appearance of symptoms it was decided to perform an experiment to determine the earliest hour after inoculation when *G.cingulata* could be detected in artificially inoculated leaf. For this experiment leaves of a susceptible and a resistant variety (B 157 and BT-15 respectively) of tea were inoculated with *G.cingulata* as described in Materials and Methods. Antigens were extracted at 6h intervals till the period of 96h. These antigens were then tested against both anti *G.cingulata* mycelial antiserum and anti *G.cingulata* cell wall antiserum.

4.4.3.1 Anti *G.cingulata* antiserum (Mycelia)

Antigens from healthy and infected leaves were tested against purified antiserum (IgG) raised against *G.cingulata* mycelial antigen. Experiments were repeated thrice in all cases. Results revealed that in ELISA, absorbance values were higher for infected extracts in comparison to healthy extracts. In the susceptible variety (B 157) differences between healthy and infected extracts were statistically significant from 6h onwards while in resistant variety (BT-15) differences were statistically significant only from 30h onwards. Hence, on the basis of significantly higher absorbance values of infected leaf extracts in comparison to healthy extracts the earliest period for detection of infection was 6h after inoculation in case of susceptible variety and 30h in case of resistant variety (Table-36). In both the varieties no lesion production was evident at the earliest time of pathogen detection.

Table 36: ELISA values obtained by reaction of anti *G.cingulata* antiserum (mycelial) and tea leaves after different hours of inoculation with *G.cingulata.*

Hours after inoculation	Anti *G.cingulata* antiserum (1:250 dilution)			
	B 157[a]		BT-15[a]	
	H	I	H	I
6	0.750±0.003	0.823±0.003	0.709*±0.030	0.795±0.022
12	0.752±0.001	0.901±0.016	0.711*±0.021	0.800±0.032
18	0.709±0.009	1.101±0.012	0.750*±0.207	0.801±0.027
24	0.765±0.003	1.150±0.017	0.761*±0.034	0.811±0.029
30	0.750±0.013	1.152±0.012	0.709± 0.009	0.820±0.017
36	0.732±0.012	1.155±0.019	0.702± 0.012	0.869±0.029
42	0.782±0.017	1.159±0.020	0.750± 0.031	1.050±0.031
48	0.785±0.016	1.195±0.009	0.761± 0.021	1.072±0.009
54	0.751±0.008	1.198±0.001	0.752± 0.051	1.082±0.021
60	0.800±0.006	1.198±0.009	0.732± 0.002	1.155±0.015
66	0.750±0.001	1.223±0.016	0.751± 0.019	1.200±0.002
72	0.762±0.002	1.229±0.012	0.795± 0.017	1.209±0.012
78	0.763±0.001	1.250±0.002	0.762± 0.024	1.216±0.032
84	0.750±0.020	1.270±0.009	0.713± 0.032	1.202±0.051
90	0.761±0.016	1.301±0.050	0.723± 0.039	1.153±0.002
96	0.732±0.012	1.253±0.034	0.750± 0.014	1.101±0.051

a Leaf antigen used at 40 μg/ml concentration.

* Difference in values between healthy and inoculated extracts not significant at p=0.01 ; all the rest significant.

± Standard error.

4.4.3.2 Anti *G.cingulata* antiserum (Cell wall)

Antiserum raised against cell wall preparations of *G.cingulata* were also used to determine the earliest period when infection could be detected in the leaf tissues. Healthy leaf extracts as well as antigen extracted at 6h intervals after inoculation with *G.cingulata* were used in ELISA. Absorbance values in infected extracts were higher than those of healthy

Table 37: ELISA values obtained by reaction of anti *G.cingulata* antiserum (cell wall) and tea leaves after different hours of inoculation with *G.cingulata.*

Hours after inoculation	Anti *G.cingulata* antiserum (1:250 dilution)			
	B 157[a]		BT-15[a]	
	H	I	H	I
6	0.672 ± 0.021	0.850 ± 0.009	0.750* ± 0.025	0.861 ± 0.016
12	0.663 ± 0.059	0.853 ± 0.022	0.756* ± 0.031	0.846 ± 0.029
18	0.701 ± 0.042	0.923 ± 0.034	0.711 ± 0.034	1.050 ± 0.017
24	0.785 ± 0.022	0.962 ± 0.032	0.752 ± 0.018	1.069 ± 0.009
30	0.750 ± 0.008	0.983 ± 0.052	0.711 ± 0.002	1.092 ± 0.060
36	0.732 ± 0.001	0.995 ± 0.051	0.709 ± 0.009	1.099 ± 0.029
42	0.783 ± 0.030	1.016 ± 0.039	0.803 ± 0.021	1.116 ± 0.032
48	0.772 ± 0.034	1.082 ± 0.034	0.761 ± 0.029	1.136 ± 0.026
54	0.762 ± 0.022	1.159 ± 0.008	0.742 ± 0.007	1.212 ± 0.021
60	0.801 ± 0.021	1.185 ± 0.016	0.750 ± 0.012	1.229 ± 0.006
66	0.750 ± 0.034	1.232 ± 0.012	0.750 ± 0.015	1.232 ± 0.014
72	0.795 ± 0.031	1.332 ± 0.019	0.762 ± 0.025	1.250 ± 0.016
78	0.782 ± 0.056	1.350 ±0.009	0.755 ± 0.032	1.261 ± 0.025
84	0.800 ± 0.021	1.369 ± 0.023	0.732 ± 0.039	1.251 ± 0.009
90	0.750 ± 0.021	1.400 ± 0.032	0.716 ± 0.024	1.226 ± 0.021
96	0.762 ± 0.003	1.342 ± 0.009	0.800 ± 0.026	1.200 ± 0.029

a Leaf antigen used at 40 μg/ml concentration.

* Difference in values between healthy and inoculated extracts not significant at p=0.01 ; all the rest significant.

± Standard error.

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens (B-157) at different periods

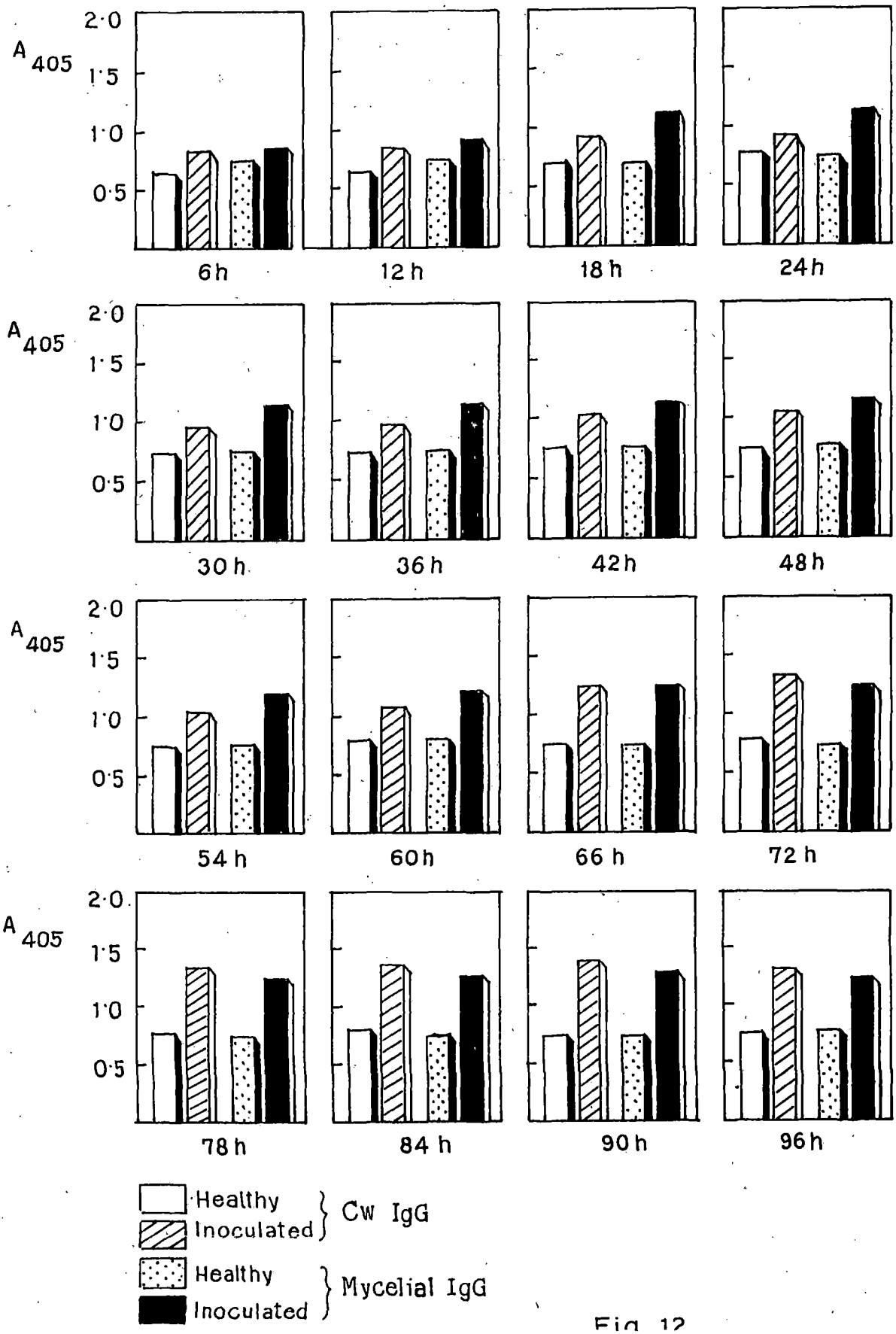


Fig. 12

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens (BT-15) at different periods

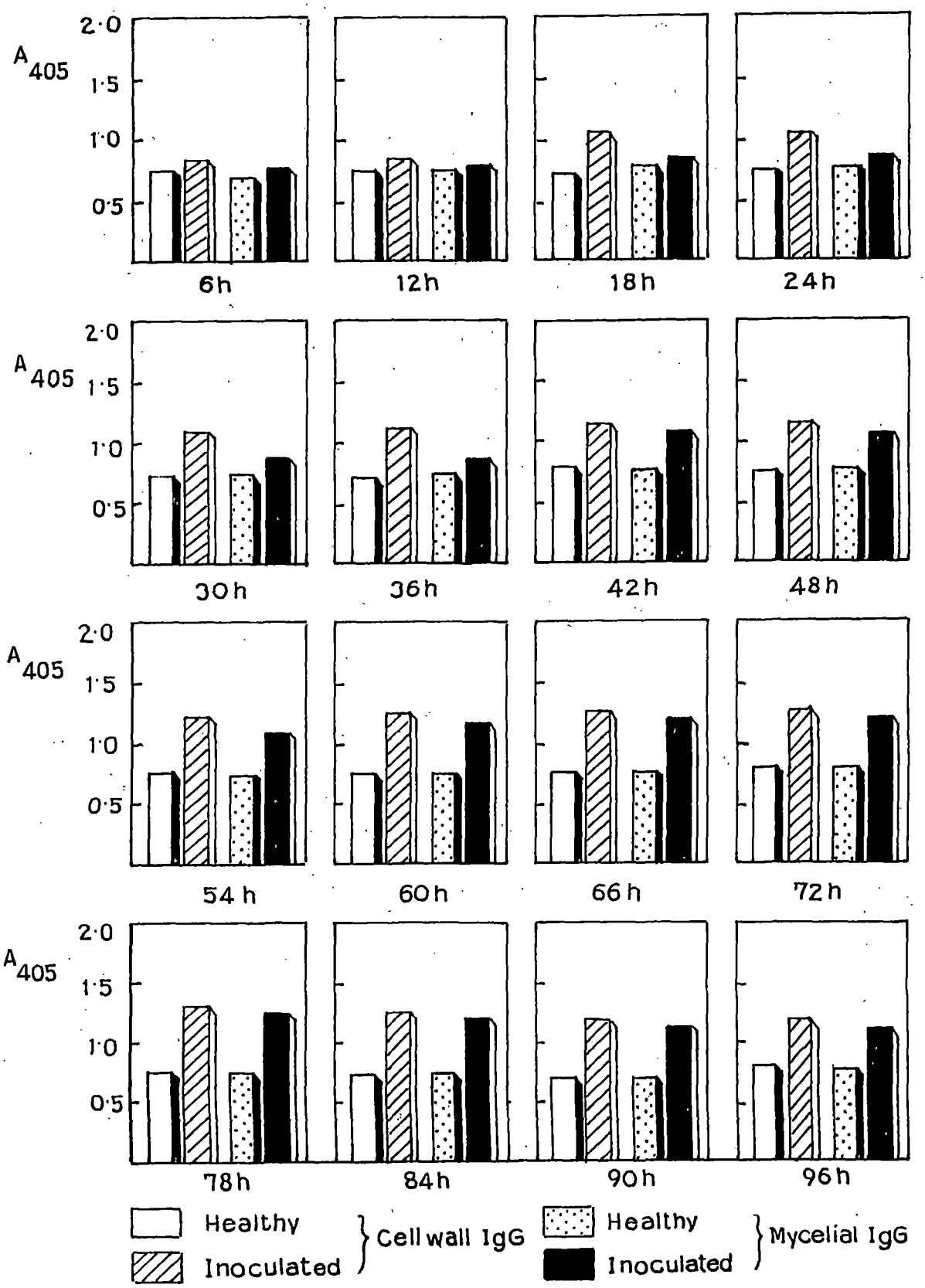


Fig. 13

extracts. In B157 the difference in values between infected and healthy extracts were significant at p=0.01 from 6h of inoculation onwards. In case of BT-15 such statistically significant difference was obtained from 18h onwards. (Table-37)

The above results indicate that *G.cingulata* can be detected in the susceptible variety as early as 6h of inoculation using antiserum raised against either mycelial or cell wall preparation. (Fig.12). In the resistant variety antiserum raised against cell wall preparation could detect *G.cingulata* from 18h onwards while antiserum raised against mycelial preparation could detect *G.cingulata* only after 30h of inoculation. (Fig. 13)

4.4.4. Different antigen concentration

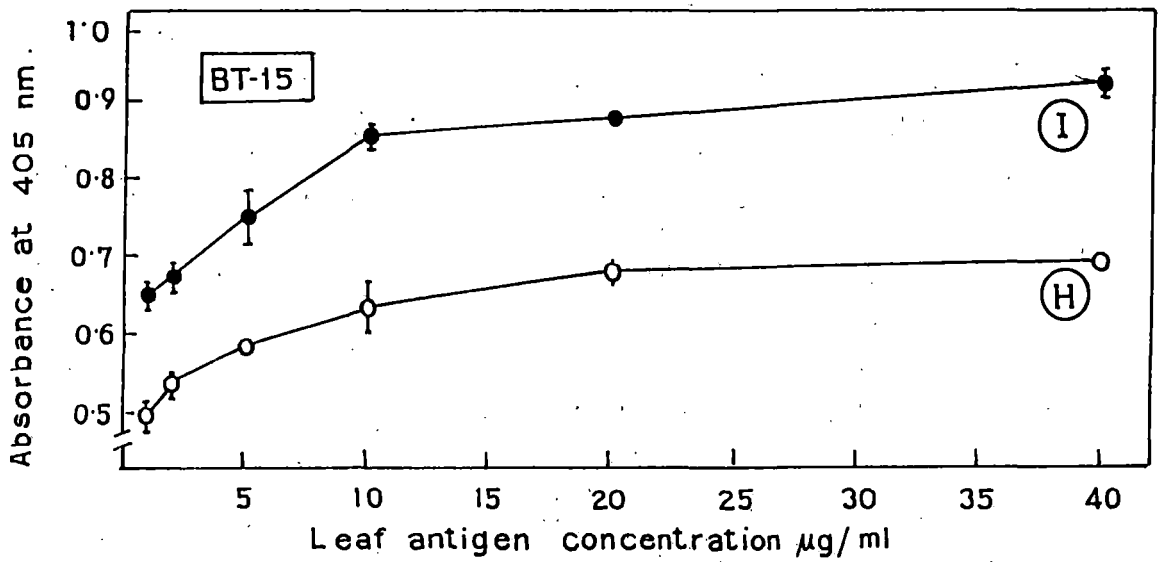
After determining the earliest period after inoculation when infection could be detected in leaf tissues it was decided to determine the lowest concentration of infected leaf extract in which infection could be detected using antiserum raised against mycelial antigen of *G.cingulata*. Infected leaf extract of two varieties (B 157 and BT-15) at dilution ranging from 40-1 μg/ml were tested in ELISA. Results (Table 38, Fig.14) revealed that differences in absorbance values between infected and healthy leaf extracts for both the varieties were significant at p=0.01 in all concentrations tested. Hence by ELISA *G.cingulata* could be detected in infected leaf extracts at a concentration as low as 1 μg/ml.

Table 38: ELISA response obtained with different concentrations of artificially inoculated leaf extracts and anti - GC antiserum.

Antigen concentration (μg/ml)	Absorbance of 405 nm			
	BT-15		B157	
	Healthy	Inoculated	Healthy	Inoculated
40	0.699±.006	0.954±.021	0.864±..012	1.159±.002
20	0.694±.017	0.854±0.008	0.840±.009	1.093±.009
10	0.634±.042	0.851±0.016	0.740±.017	1.000±.016
5	0.586±.003	0.757±.0.042	0.667±.021	0.863±.022
2	0.565±.017	0.671±.0 021	0.653±.022	0.807±.026
1	0.500±.019	0.654±.0235	0.542±.022	0.719±.031

Anti *G.cingulata* antiserum used at a dilution of 1:250. ; ± Standard error.
Difference between healthy and infected leaf extracts significant at p=0.01 at all concentrations

Effect of dilution of healthy and inoculated tea leaf antigens on ELISA responses with anti - *G.cingulata* antiserum



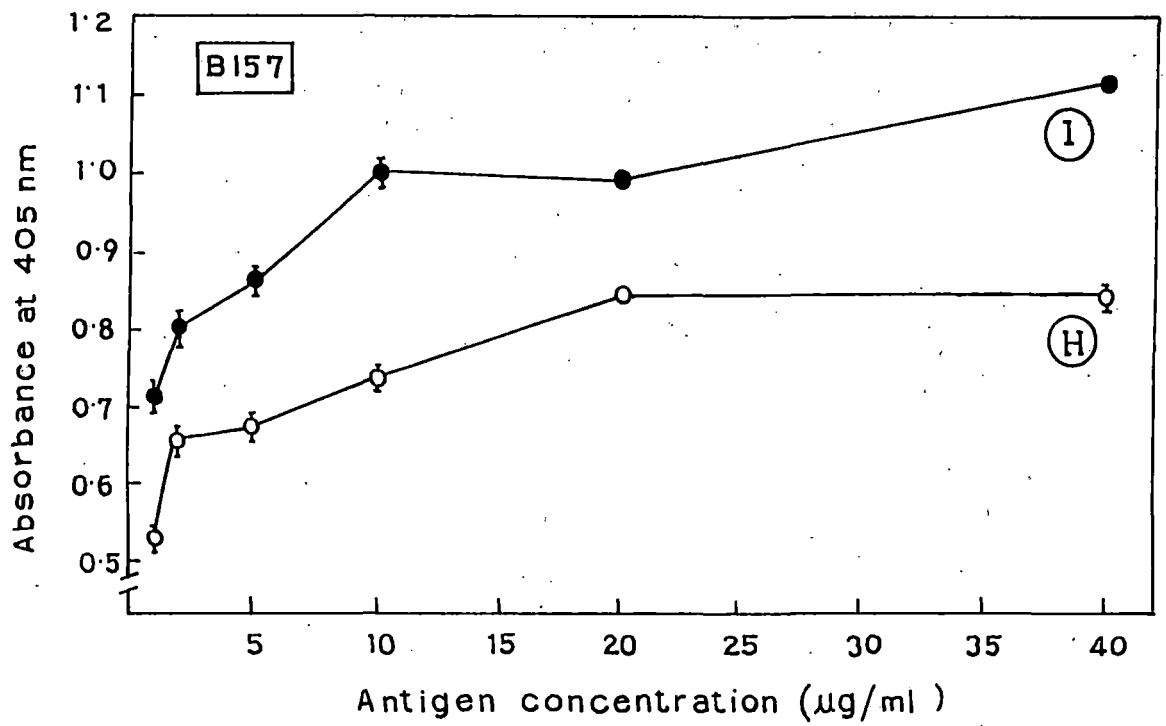


Fig. 14

4.5. Detection of infection with other pathogens using anti *G.cingulata* antiserum.

It has been observed that under field condition brown blight of tea caused by *G.cingulata* is sometimes associated with other diseases such grey blight caused by *Pestalotiopsis theae* or black rot caused by *Corticium invisum.* It was therefore, decided to test whether antiserum raised against *G.cingulata* could detect other infection. For this, two types of ELISA reactions were tested by DAC-ELISA and competition ELISA.

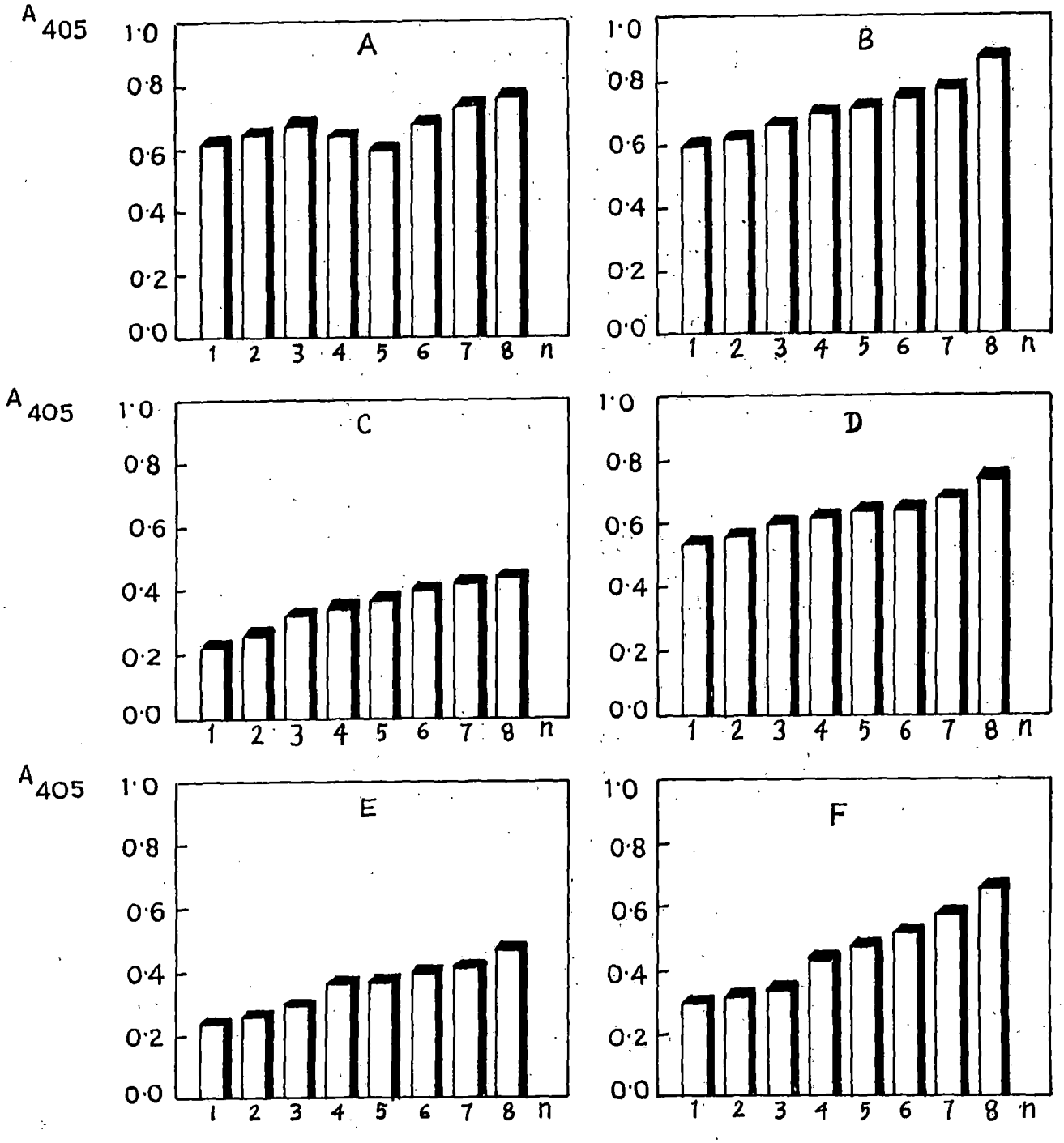
4.5.1 DAC - ELISA

Tea leaves of two varieties (AV-2 and HV-39) were inoculated with *G.cingulata, P.theae* and *C.invisum* separately and antigens were extracted after 72h of inoculation. These antigens along with corresponding healthy leaf antigens were tested against anti *G.cingulata* antiserum in DAC - ELISA. Two antigen concentrations (40 and 20μg/ml) were used and antiserum was used at a dilution of 1:250. Absorbance values in extracts of leaves inoculated with *G.cingulata* were higher than those with the other two pathogens. Significantly higher values for infected extracts in comparison to healthy extracts were only obtained when leaves were inoculated with *G.cingulata.* However, anti *G.cingulata* antigen did show a certain amount of reactivity with the extracts of leaves inoculated with other pathogens which was more or less similar to that obtained with healthy leaf extracts (Table-39).

4.5.2 Competition ELISA

One modification of the commonly used DAC-ELISA is the competition or inhibition ELISA which can detect particular antigens from mixed infections at very low concentrations. Since very often brown blight occurs as part of mixed infection specific detection of this pathogen using anti *G.cingulata* antiserum sometimes becomes difficult. In the previous experiment it was demonstrated that anti *G.cingulata* antiserum did not react significantly with antigen of leaves inoculated with other pathogens. However, such clear cut significant differences is not obtained in case of mixed natural infection. To detect the specific pathogen from mixed infection it was decided to use the competition ELISA format. Detailed procedure for competition ELISA has been outlined in Materials and Methods.

Absorbance values of doubling dilutions of healthy and infected tea leaf (UPASI-3) antigens using competition ELISA



n = antigen dilution from 1:100 to 1:12800
Left column - G. cingulata antiserum
Right column - P. theae antiserum

Fig·15

A – B = healthy leaf antigens
C – D = naturally infected leaf antigens
E – F = artificially infected leaf antigens

Table 39: Reaction of anti *G.cingulata* antiserum with antigens of tea leaves infected with other pathogens.

Tea varieties	Pathogen	Absorbance of 405 nm	
		40 μg/ml	20 μg/ml
AV-2	Uninoculated	0.660±0.031	0.418±0.012
	G.cingulata	0.960±0.031	0.718±0.029
	P.theae	0.643±0.022	0.389±0.009
	C.invisum	0.584±0.029	0.373±0.026
HV-39	Uninoculated	0.600±0.012	0.402±0.021
	G.cingulata	0.900±0.029	0.720±0.025
	P.theae	0.573±0.003	0.487±0.043
	C.invisum	0.543±0.016	0.459±0.061

Anti *G.cingulata* antiserum used at a dilution of 1:250.

± Standard error.

Results of competition ELISA are presented in (Table 40, Fig.15). Antigens of healthy tea leaves (UPASI-3), tea leaves artificially inoculated with *G.cingulata* and *P.theae* and naturally infected tea leaves were tested against anti *G.cingulata* and anti *P.theae* antisera. Test plates were initially bound with antigens of the two pathogens separately. Doubling dilution of leaf antigens ranging from 1:100 - 1:12,800 were used for binding with the two antisera. ELISA values decreased with increasing reactivity as this was a competition ELISA. Positive reactions were recorded when the absorbance value of 1:100 was less than approximately 50% of the value for the extract diluted 1:12,800. Positive reactions on the above basis was recorded in case of both naturally infected leaf extracts as well as leaves artificially inoculated with *G.cingulata* using anti *G.cingulata* antiserum. Positive responses were not recorded in healthy leaf extracts using either of the two antiserum or naturally infected extracts with anti *P.theae* antiserum. The presence of *G.cingulata* as the major pathogen in the natural infection was therefore confirmed by competition ELISA.

Table 40: Absorbance values of doubling dilutions of healthy and inoculated tea leaf antigens in competition ELISA.

Leaf antigen[a]	Antigen dilution	Antiserum (1:125 dilution)	
		G.cingulata	*P.theae*
Healthy	1:100	0.619	0.601
	1:200	0.669	0.618
	1:400	0.685	0.661
	1:800	0.647	0.688
	1:1600	0.602	0.717
	1:3200	0.672	0.747
	1:6400	0.738	0.782
	1:12800	0.770	0.867
Naturally infected			
	1:100	0.224	0.555
	1:200	0.274	0.579
	1:400	0.303	0.599
	1:800	0.342	0.622
	1:1600	0.366	0.635
	1:3200	0.397	0.633
	1:6400	0.418	0.696
	1:12800	0.433	0.756
Artificially inoculated[b]			
	1:100	0.238	0.309
	1:200	0.265	0.313
	1:400	0.298	0.353
	1:800	0.366	0.440
	1:1600	0.374	0.480
	1:3200	0.400	0.510
	1:6400	0.420	0.587
	1:12800	0.464	0.661

a Variety UPASI-3 ;

b Inoculated with *G.cingulata*

4.6. Determination of cross-reactivity of anti *G.cingulata* antiserum.

Cross reactivity of the antiserum raised against *G.cingulata* (isolate GC-1) was tested against 8 other isolates (isolates GC 2-9). Since *Glomerella cingulata* is a telomorph of *Colletotrichum gloeosporoides* it was decided to test the cross reactivity of the antiserum raised against *G.cingulata* with other species of *Colletotrichum* (2 strains of *C.gloeosporiodes,* 2 strains of *C.lindemuthianum* and one species of *C.papayae*). Cross reactivity was also tested against other foliar pathogens of tea i.e. *P.theae* and *C.invisum.*

For cross reactivity tests, antigens were prepared from the mycelia of all the above species and isolates and were tested against anti *G.cingulata* antiserum. Results revealed that among the different isolates of *G.cingulata* tested GC-5 showed 91% homology with GC-1. Of all the isolates minimum homology of 35% was exhibited by GC-8 (Table-41). Hence, all the isolates showed some homology with the isolate GC-1. In reactions involving other species of *Colletotrichum* homology ranged from 27-35% (Table-42). Hence, the different species did not cross react with the antiserum raised against *G.cingulata.* Antigens of *P.theae* and *C.invisum* exhibited homologies of 28 and 11% respectively.

Table 41: ELISA values showing reaction of antiserum of *Glomerella cingulata* (GC-1) at 1:250 dilution, with antigens of other isolates of *G.cingulata* (GC 1 to 9)

Pathogen	Isolate	Absorbance at 405 nm			
		Expt.1	Expt.2	Expt.	Mean
Glomerella cingulata	GC-1	1.894	1.899	1.920	1.904(100%)
	GC-2	0.703	0.729	0.729	0.720(37.9%)
	GC-3	1.038	1.084	1.076	1.066(55.0%)
	GC-4	1.245	1.209	1.243	1.232(64.7%)
	GC-5	1.744	1.715	1.740	1.733(91.0%)
	GC-6	0.817	0.854	0.866	0.845(44.3%)
	GC-7	1.220	1.231	1.263	1.238(65.0%)
	GC-8	0.681	0.640	0.694	0.671(35.24%)
	GC-9	0.959	0.955	0.959	0.954(50.10%)

Values in parenthesis represents homology as a percentage of that of *G.cingulata* (GC-1) which is designated as 100.

Anti *G.cingulata* antiserum used at a dilution of 1:250.

Table 42: ELISA reaction of different species of genus *Colletotrichum* against antisera of GC-1 (IgG 1:250).

Pathogens	Absorbance values at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Colletotrichum gloeosporioides (1809)	0.505	0.502	0.511	0. 506(27.26%)
C.gloeosporioides(1726)	0.604	0.598	0.627	0.609(32.8%)
C.lindemuthianum(1119)	0.643	0.662	0.667	0.660(35.5%)
C.lindemuthianum(1764)	0.501	0.506	0.505	0.504(27.15%)
C.papayae (1269)	0.672	0.634	0.664	0.656(35.34%)
G.cingulata	1.916	1.829	1.823	1.856(100%)
P.theae	0.521	0.506	0.554	0.527(28.39%)
C.invisum	0.198	0.244	0.184	0.208(11.20%)

Values in parenthesis represents homology as a percentage of that of *G.cingulata* (GC-1) which is designated as 100.

Anti *G.cingulata* antiserum used at a dilution of 1:250.

4.7 Estimation of mycelia in infected tea leaf tissues

The measurement of fungal growth in tissues cannot be effectively done by direct methods like quantitative microscopy. A recent approach to the estimation of fungal biomas is the use of serological techniques such as ELISA. In the present study, initially a standard curve of known mycelial fresh weight of *G.cingulata* against absorbance value in ELISA was prepared. Mycelial growth in leaf tissues were estimated from the standard curve.

4.7.1 Different hours after inoculation

In order to estimate the growth of *G.cingulata* in tea leaves after different hours of inoculation, ELISA was carried out using healthy and inoculated tea levves of 2 varieties (B 157 and BT-15). In case of inoculated tea leaves antigens were prepared after every 6h of inoculation till 96h. The antigens were used at a concentration of 40 μg/ml against anti *G.cingulata* antiserum at 1:250 dilution. Results revealed that the absorbance values of infected leaf extracts were always higher than those of corresponding healthy extracts (Fig. 16). Difference in ELISA values between healthy and infected extracts were plotted on the previously prepared standard curve and the mycelial fresh weight was determined in each case (Fig. 17). Results revealed that in the susceptible variety (B 157) mycelial fresh wt. within the leaf tissue increased till 90h of inoculation. However, in the resistant variety (BT-15) mycelial fresh weight decreased after 78h (Table-43).

4.7.2. Different varieties

Fungal biomass was also determined in infected leaf extracts of different tea varieties from the standard curve as mentioned above. Results (Table 44) revealed that the susceptible varieties showing higher percentage of lesion production had maxinum fungal biomass within the tissue. Among the 15 varieties tested (UPASI-2, 3, 8, 9 and 26; TV-18, 23, 26, 29 and Teenali 17/1/54; B668, T-78, AV-2, CP-1, BS/7A/76), after 72 h of inoculation, UPASI-9 showed maximum lesion production of 67%. Maximum mycelial fresh weight as determined by ELISA was also in this variety (2.5 mg/g leaf tissue). Varieties showing low percentage of lesion production (5 %) also had lower mycelial fresh weight (0.85 mg/g leaf tissue).

Table 43: Estimation of biomas s of *G. cingulata* by ELISA within tea leaf tissues after different hours of inoculation.

Hours after inoculation	Mycelial fresh wt. (mg/g)	
	B 175	BT-15
6	0.006	0.004
12	0.186	0.118
18	0.483	0.514
24	0.651	0.669
30	1.760	2.287
36	1.767	2.732
42	2.349	2.943
48	2.609	3.193
54	2.796	3.565
60	3.385	3.664
66	4.166	3.742
72	4.259	3.918
78	4.823	4.550
84	6.088	4.283
90	6.621	4.147
96	6.503	3.961

Table 44: Percentage lesion production and growth of mycelia in different varieties of tea leaves inoculated with *G. cingulata.*

Tea varieties	72h after inoculation	
	Percent lesion production (%)	mycelial fresh weight mg/g leaf tissue[a]
Tocklai varieties		
Teenali 17/1/54	49.2	2.16
TV-18	60.0	2.02
TV-23	43.2	1.78
TV-26	29.9	0.09
TV-29	33.0	1.02
Darjeeling varieties		
B668	43.2	1.05
T-78	60.1	2.28
AV2	35.0	1.05
CP1	21.0	1.22
BS/7A/76	54.4	1.07
UPASI varieties		
UPASI-2	4.8	0.84
UPASI-3	62.4	1.94
UPASI-8	51.0	1.04
UPASI-9	66.7	2.05
UPASI-26	63.4	2.03

[a] Determined on the basis of ELISA response

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens at different periods

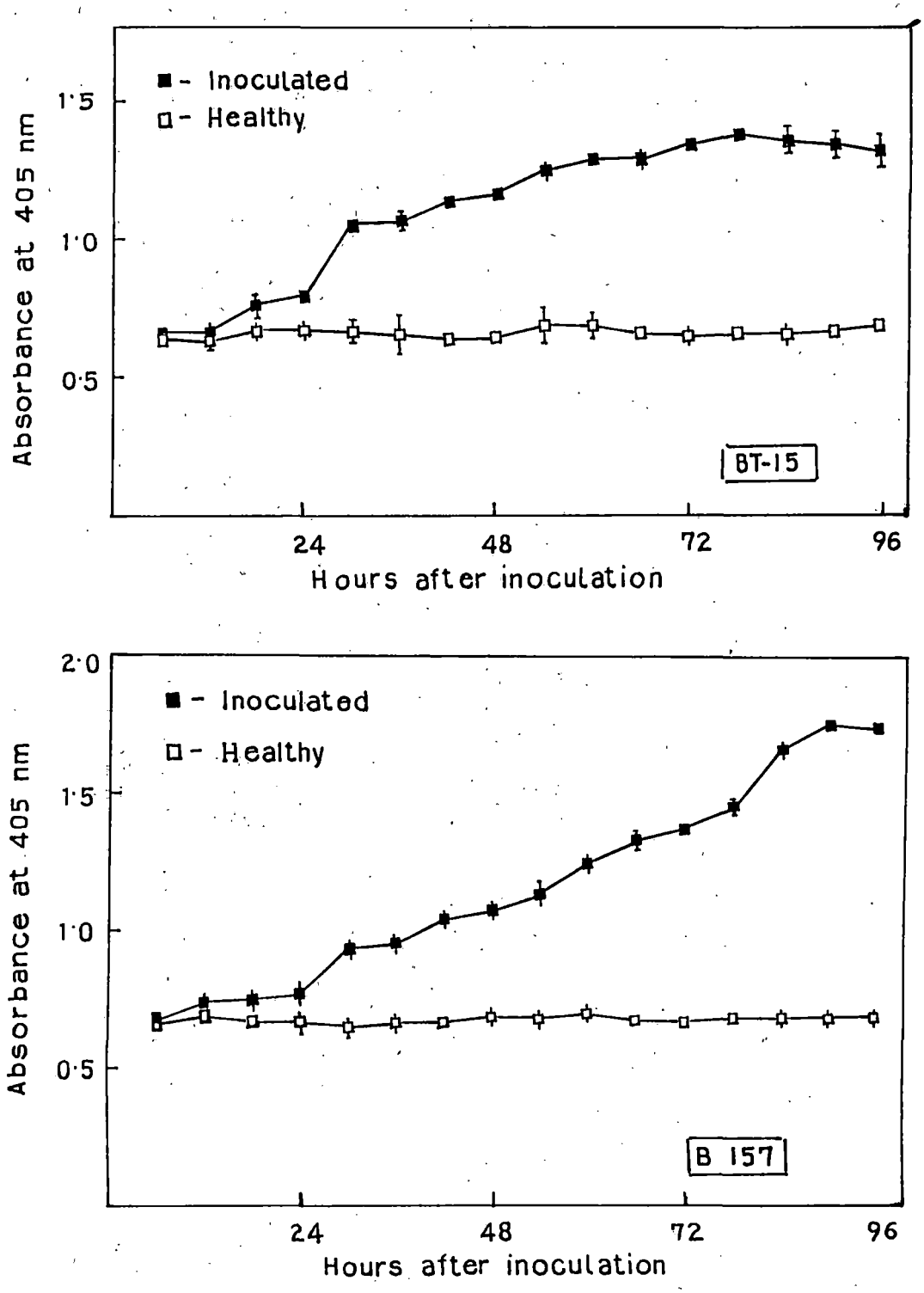


Fig. 16

Fungal biomass in tea leaves after different hours of inoculation with *G.cingulata*

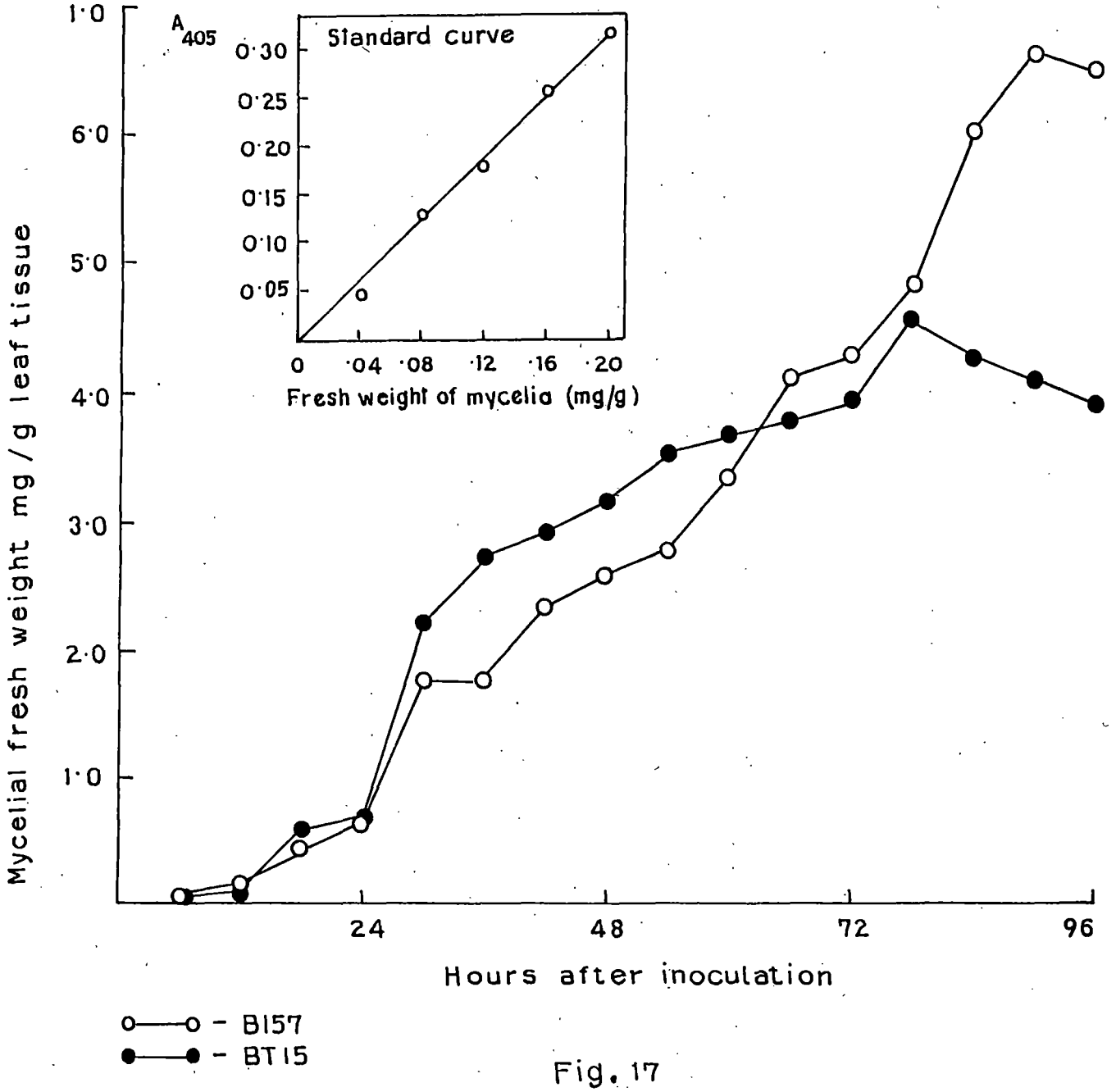


Fig. 17

4.8. Purification of cross reactive antigen (s) from mycelia of *G. cingulata.*

Having established that cross reactive antigen is present in *G. cingulata.* it was decided to purify the protein (s) from the crude extract which are actually responsible for the antibody production. A series of steps outlined in Materials and Methods were performed for this purification.

4.8.1. Ammonium sulphate fractionation

Initially from the crude mycelial protein preparation of *G. cingulata* ammonium sulphate fractionation were carried out. Each ammonium sulphate fraction after centrifugation and dialysis was tested by agar gel double diffusion and indirect ELISA, against homologous (anti *G. cingulata* antiserum) and heterologous antisera. The heterologous antiserum was raised against a susceptible tea variety, TV-18.

4.8.1.1. Agar gel double diffusion

The different ammonium sulphate saturated (0-20%, 20-40%, 40-60%, 60-80% and 80-100%) fraction were tested for cross reactivity by agar gel double diffusion tests. Tests involving antisera of either *G. cingulata* or TV-18 showed common precipitin bands with the crude extract and 80-100% saturated fraction (Plate VIII, figs. A & C). No precipitin bands were detect with any of the other fractions.

4.8.1.2. ELISA

To further confirm the result of agar gel double diffusion tests ELISA were performed with the different fractions and the two antisera as mentioned above. Results revealed that in both homologous and heterologous combinations 80-100% fraction gave highest absorbance values which was comparable to the value obtained with the total protein extract (Table-45).

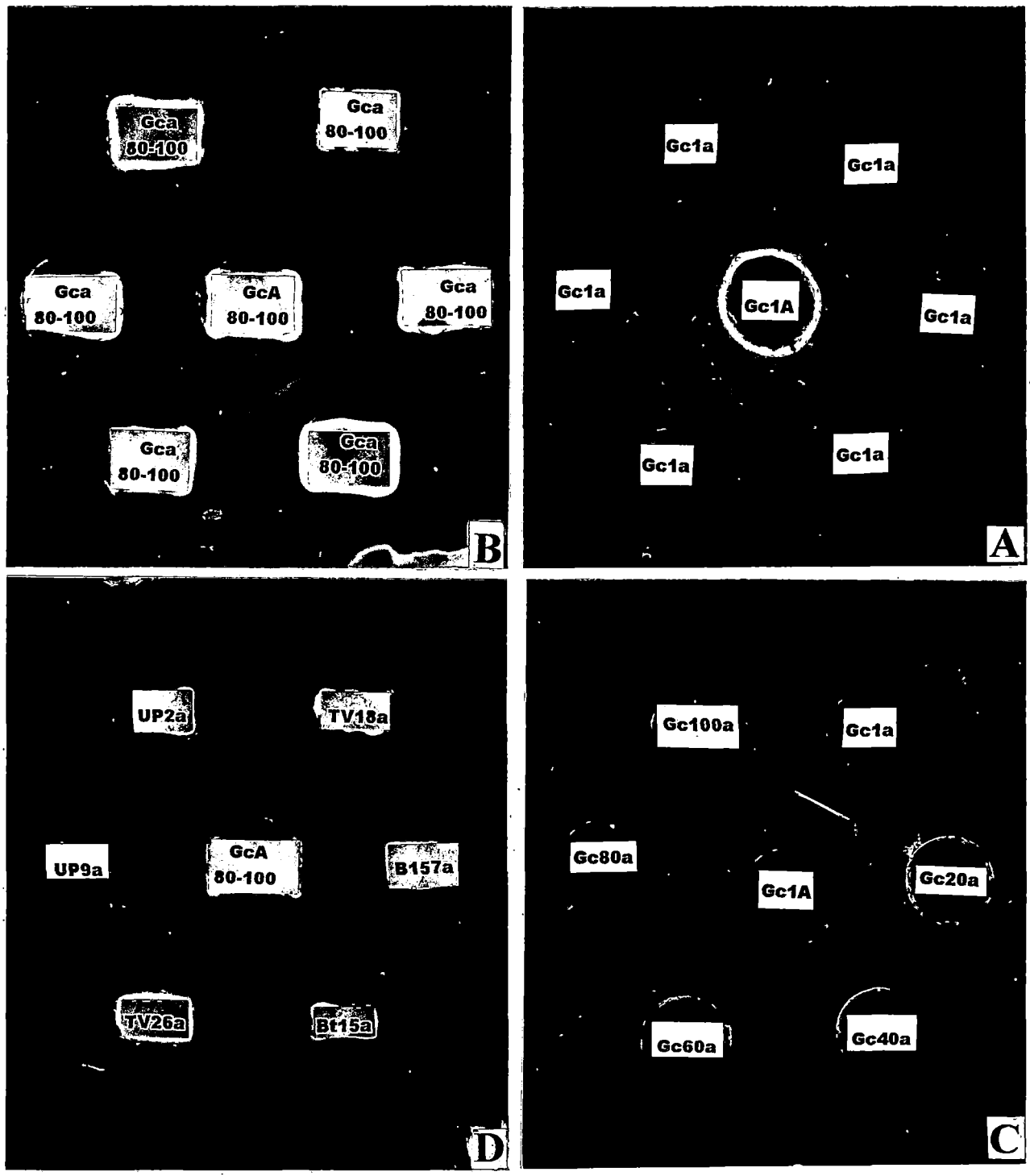


PLATE VIII (figs.A-D) . Agar gel double diffusion tests.Central wells contain antisera to crude **(A & C)** and partially purified **(B&D)** mycelial antigen (80-100% SAS) of *G.cingulata* . Peripheral wells contain *G.cingulata* crude antigen **(A)** , 80-100% SAS fraction **(B)** , ammonium sulphate fraction **(C)** and tea leaf antigens **(D)**

4.8.2. DEAE - Sephadex chromatography

Since both agar gel double diffusion and ELISA tests revealed that the major cross reactive antigens were present in 80-100% SAS of *G. cingulata* this fraction was further purified by DEAE - Sephadex chromotography. Details of the procedure used for the purification have been given in Materials nad Methods. Twenty fractions each were collected from the three buffers (0.05 MTris-HCl, 0.05 M NaCl in 0.05 M Tris-HCl and 0.05 M to 0.25 M NaCl in 0.05 M tris-HCl). Absorbance values of all the fractions were taken at 280nm and these were plotted on a graph paper. Analysis of the OD values revealed three distinct peaks corresponding to the three buffer systems (Fig. 18). In the third buffer the OD values were much lesser than the previous two.

4.8.2.1. DAC - ELISA

Indirect ELISA was performed with all the fractions obtained from DEAE Sephadex chromotography and antisera of *G. cingulata* and TV-18. When tested against antiserum of *G. cingulata* high absorbance were obtained in four fractions (5,6,7,8) of the second buffer system (0.05 M NaCl in 0.05 M Tris-HCl). Readings in all other fractions were insignificant (Table-46). Similar results (Table-47) were obtained against antiserum of TV-18 but in this case high absorbance values were recorded in two-5 and 6 (Table-47) fractions. These two purified fractions therefore contained the major cross reactive antigens.

4.8.2.2. SDS - PAGE

In order to determine the molecular weight of the purified antigen SDS-PAGE was performed as described previously. The antigenic fractions were pooled and run along with 80-100% SAS protein as well as crude protein extract and a set of molecular markers. Following electrophoresis, staining and destaining revealed a prominent band. This corresponded to a molecular weight of approximately 10 KDA (Fig. 19). Hence the antigenic protein seems to be a low molecular weight protein.

Elution pattern of 80-100% SAS fraction of *G.cingulata* antigen in different buffers by DEAE-Sephadex column chromatography

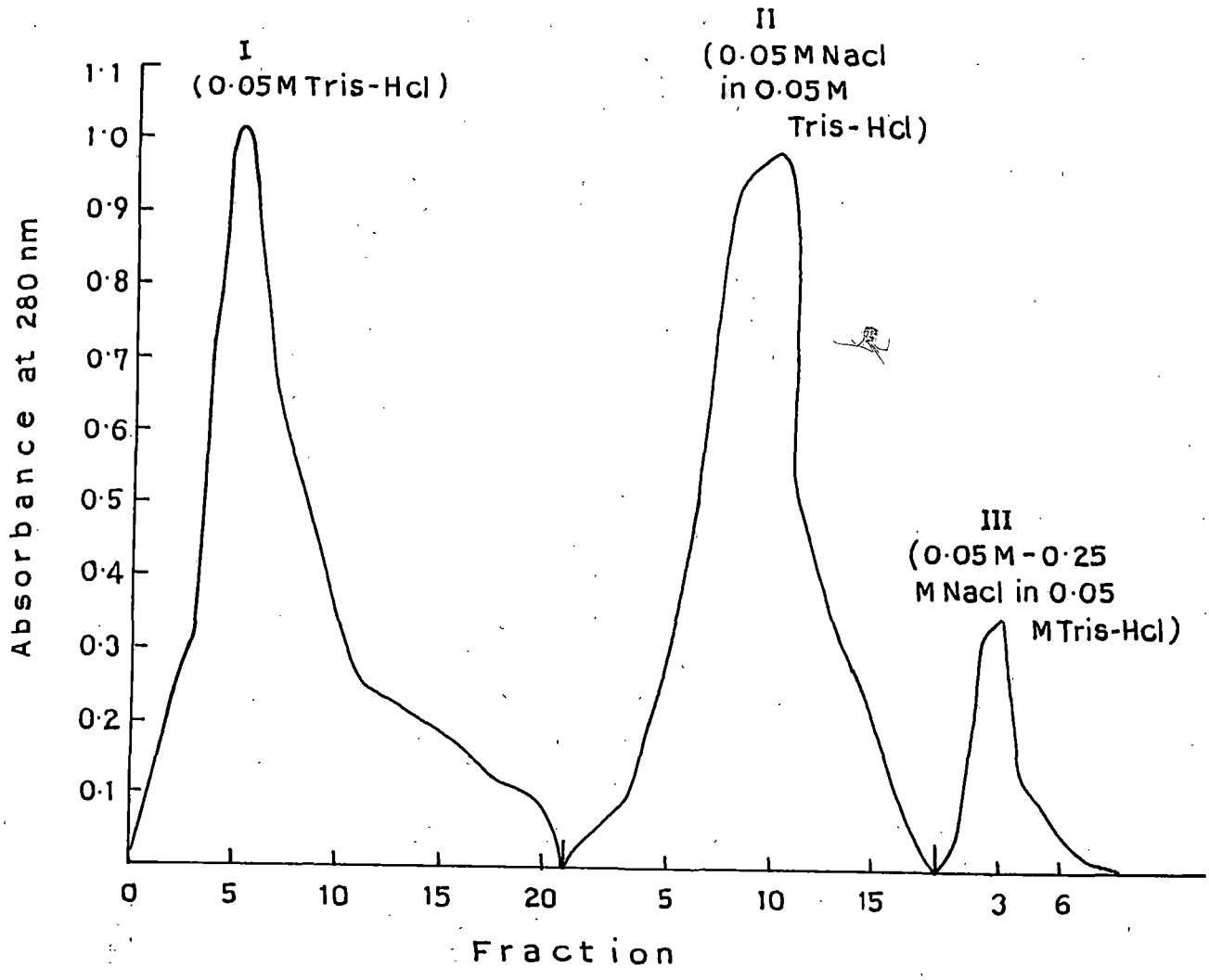


Fig. 18

Table 45: Absorbance values obtained in ELISA reactions of ammonium sulphate saturated fraction of *G.cingulata* antigen with anti *G.cingulata* and anti TV-18 antiserum.

Antigen	Absorbance at 405 nm[c]	
	Antisera (1:250 dilution)	
	G.cingulata	TV-18
G.cingulata[a] fractions:		
0-20% SAS	0.684±0.003	0.593±0.031
20-40%	0.644± 0.017	0.469±0.041
40-60%	0.607±0.003	0.547±0.003
60-80%	0.726±0.009	0.707±0.047
80-100%	1.860± 0.007	1.356±0.013
100%	1.889±0.037	1.427±0.006
TV-18[b]	ND	1.648±0.070

SAS - Saturated ammonium sulphate precipitation

a Antigen concentration - 5 μg/ml

b Antigen concentration - 40 μg/ml

c Average of three experiments.

± Standard error.

Table 46: ELISA responses obtained for reactions of different fractions from DEAE Sephadex chromatography of partially purified *G.cingulata* antigen against anti *G.cingulata* antiserum.

Antigen fractions	Anti *G.cingulata* antiserum (1:250 dilution)		
	Absorbance at 405 nm		
	Buffer 1[a]	Buffer 2[b]	Buffer 3[c]
1.	ND	ND	ND
2.	0.366	0.360	0.349
3.	0.379	0.351	0.386
4.	0.374	0.347	0.360
5.	0.369	0.918	0.371
6.	0.386	1.040	0.327
7.	0.355	1.060	0.339
8.	0.397	0.829	0.342
9.	0.360	0.785	0.361
10.	0.375	0.685	0.356
11.	0.395	0.356	ND
12.	0.366	0.304	ND
13.	0.365	0.305	ND
14.	0.376	0.367	ND
15.	0.382	0.371	ND
16.	0.393	0.363	ND
17.	0.377	0.373	ND
18.	0.368	0.323	ND
19.	0.357	0.318	ND
20.	0.325	ND	ND

a 0.05 M Tris - HCl

b 0.05 M NaCl in 0.05 M Tris HCL

c 0.05 - 0.025 M NaCl in 0.05M Tris HCl.

ND - Not detected.

Table 47: ELISA responses obtained for reactions of different fractions from DEAE Sephadex chromatography of partially purified *G.cingulata* antigen against anti TV-18 antiserum.

Antigen fractions	Antisera dilution 1:250 (TV-18 IgG)		
	Absorbance at 405 nm		
	[a] Buffer 1	[b]Buffer 2	[c] Buffer 3
1.	0.341	0.386	0.392
2.	0.348	0.378	0.327
3.	0.373	0.362	0.342
4.	0.400	0.381	0.336
5.	0.370	0.941	0.361
6.	0.362	0.952	0.351
7.	0.424	0.317	0.362
8.	0.368	0.315	0.361
9.	0.370	0.335	0.351
10.	0.329	0.372	0.341
11.	0.340	0.396	ND
12.	0.321	0.366	ND
13.	0.322	0.362	ND
14.	0.335	0.354	ND
15.	0.352	0.350	ND
16.	0.363	0.325	ND
17.	0.331	0.320	ND
18.	0.395	0.311	ND
19.	0.395	0.312	ND
20.	0.393	0.319	ND

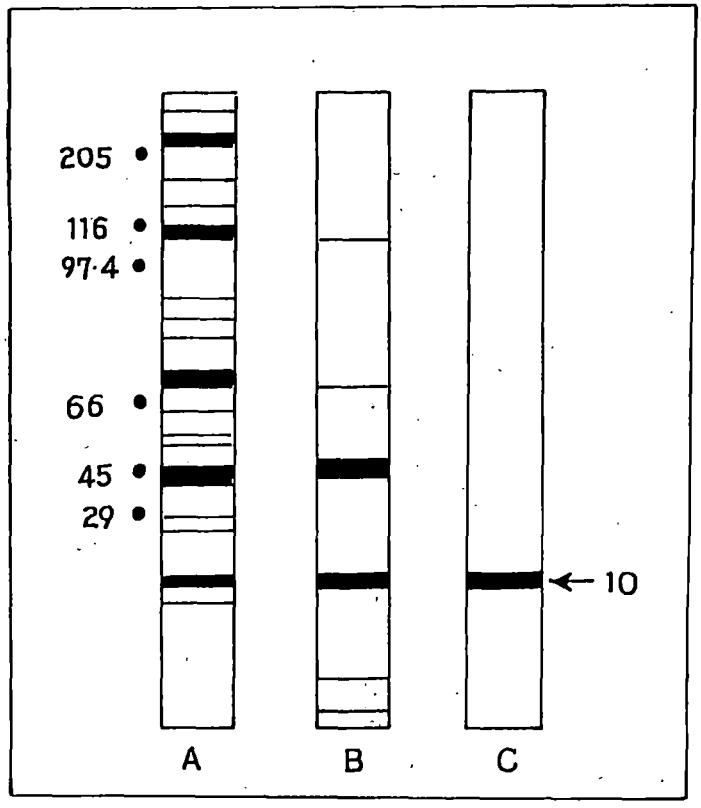
a 0.05 M Tris HCl

b 0.05 M NaCl in 0.05 M Tris-HCl

c 0.05-0.25M NaCl in 0.05 M Tris HCl.

ND Not detected

SDS-PAGE analysis of crude and purified mycelial antigen of *G.cingulata*



A = Crude mycelial antigen .

B = 80-100% SAS fraction .

C = Antigenic fraction from DEAE-Sephadex Column.

Fig. 19 .

4.9 Evaluation of antisera raised against purified mycelial antigens of *G.cingulata*

In the previous experiments antisera were raised against antigenic preparations from mycelia of *G.cingulata* and these were used for various tests. Subsequently from the crude mycelial preparations antigens were purified by ammonium sulphate fractionation and ion exchange chromatography. It was evident from immunodiffusion and ELISA tests that 80-100%. SAS fraction contained major cross reactive antigens. Hence, antiserum was raised against this fraction (80-100% SAS) following the protocol as described previously. This antiserum was further tested by immunodiffusion and ELISA against both homologous and heterologous combinations.

4.9.1. Immunodiffusion tests

In agar gel double diffusion tests the antiserum raised against 80-100% SAS was placed in the central well and in the peripheral well either homologous antigens (80-100% SAS precipitate) or heterologous antigens (TV-18, UPASI-9, B-157, UPASI-2, BT-15 and TV-26) were placed. Strong precipitation was observed in the homologous reactions (Plate VIII, fig.B). In reactions involving tea leaf antigens precipitation was evident against susceptible varieties (TV-18, UPASI-9 and B 157) while no such reactions occurred with the resistant varieties UPASI-2, BT-15 and TV-26 (Plate VIII, fig. D).

4.9.2. ELISA

The antiserum raised against 80-100% SAS was also tested by ELISA to determine whether it could detect CRA among *G.cingulata* and tea varieties and also whether infection could be detected by this antiserum. Both DAC and DAS ELISA was performed and results are given below.

4.9.2.1 Detection of CRA among selected tea varieties

Cross reactive antigens have been detected between tea varieties and *G.cingulata* by ELISA using purified antiserum (IgG) raised against whole mycelial antigen as described earlier. Since the antiserum raised against 80-100% SAS gave positive results in immunodiffusion tests they were further tested by ELISA. For ELISA the antisera were initially purified and the IgG was used.

4.9.2.1.1 DAC ELISA

Antigenic preparations from tea leaves (varieties Teenali 17/1/54, TV-18, 23, 26, CP-1), non hosts (*G.max, C.arietinum* and *C.japonicum*), non pathogen (*F.oxysporum*) as well as mycelia of *G.cingulata* (80-100%) were tested against the above antiserum by DAC ELISA. Experiments were repeated thrice and results were represented in Table-48. High absorbance values were noted in homologous reactions followed by reactions against susceptible varieties (TV-18, 23, Teenali 17/1/54). ELISA reactions with either non host or non pathogen were marked low. Thus, the antiserum raised against partially purified antigen could detect the presence of cross reactive antigens between host and pathogen.

4.9.2.1.2 DAS ELISA

Using similar antigenic preparations as in DAC ELISA, DAS ELISA was also performed to confirm the results obtained in DAC ELISA. Procedures for DAC ELISA have been outlined in Materials and Methods. In this case also absorbances were high in reactions with homologous antigens as well as with susceptible varieties (Table 49). Low absorbances were evident against non host and non pathogen.

4.9.2.2 Detection of *G.cingulata* in artificially inoculated tea leaves of selected varieties

The efficacy of the antiserum raised against 80-100% SAS fraction of *G.cingulata* mycelial antigen was further tested by its ability to detect the pathogen in infected tissues by ELISA. For this both DAC ELISA and DAS ELISA were performed and results are given below.

4.9.2.2.1 DAC ELISA

Antigenic preparations from healthy and inoculated tea leaves of 8 UPASI varieties (UPASI 1,2,3,8,9,26; BSS 1,2,3) were tested against the above antiserum. A_{405} values of inoculated extracts were significantly higher than the corresponding healthy extracts at p=0.01 (Table-50).

Table 48: Absorbance values in DAC ELISA for reactions of selected tea varieties with antiserum raised against 80-100% SAS fraction of *G.cingulata* mycelium.

Antigen	Absorbance at 405 nm[a]			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
Teenali 17/1/54	0.850	0.934	0.824	0.869±0.033
TV-18	1.030	1.129	1.018	1.059±0.035
TV-23	0.859	0.878	0.815	0.850±0.018
TV-26	0.613	0.624	0.598	0.611±0.007
CP-1	0.676	0.620	0.665	0.653±0.015
Pathogen				
G.cingulata	1.697	1.702	1.668	1.689±0.010
Non pathogen				
F.oxysporum	0.223	0.312	0.238	0.253±0.027
Non host				
C.japonicum	0.362	0.392	0.325	0.359±0.019
G.max.	0.334	0.337	0.286	0.319±0.016
C.arietinum	0.326	0.355	0.386	0.355±0.017

a Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

± Standard error

Table 49: Absorbance values in DAS ELISA for reactions of selected tea varieties with antiserum raised against 80-100% fraction of *G.cingulata* mycelium.

Antigen	Absorbance at 405 nm[a]			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
Teenali 17/1/54	1.139	1.128	1.099	1.112±0.011
TV-18	1.239	1.244	1.284	1.255±0.014
TV-23	0.940	1.032	1.005	0.992±0.027
TV-26	0.695	0.700	0.682	0.692±0.005
CP-1	0.710	0.721	0.749	0.726±0.011
Pathogen				
G.cingulata	1.408	1.457	1.437	1.434±0.014
Non pathogen				
F.oxysporum	0.305	0.210	0.287	0.267±0.029
Non host				
C.japonicum	0.389	0.382	0.384	0.385±0.002
G.max	0.368	0.407	0.324	0.366±0.024
C.arietinum	0.371	0.390	0.393	0.384±0.016

a Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

± Standard error

Table 50: Absorbance values in DAC ELiSA for reactions of antiserum raised against 80-100% SAS of *G.cingulata* mycelia with healthy and inoculated tea leaf antigens.

Antigen (40 μg/ml)	Absorbance at 405 nm[a,b,c]	
	Healthy	Inoculated*
UPASI-2	0.616±0.011	0.763±0.016
UPASI-3	0.685±0.009	1.090±0.015
UPASI-8	0.636±0.017	0.863±0.013
UPASI-9	0.738±0.012	1.142±0.029
UPASI-26	0.668±0.002	1.046±0.020
BSS 1	0.648±0.016	1.012±0.024
BSS 2	0.633±0.021	1.001±0.017
BSS 3	0.630±0.032	0.848±0.012

* 72h after inoculation

a Average of three experiments

b Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

c Difference between healthy and inoculated extracts significant at p=0.01 in all cases.

4.9.2.2.2 DAS ELISA

Healthy and infected tea leaf extracts were tested as in DAC ELISA mentioned above. Results of ELISA reactions revealed significantly higher absorbance values for infected extracts as compared to healthy extracts in all the varieties tested (Table - 51). Both immunodiffusion tests and ELISA revealed that antiserum raised against partially purified mycelial preparation (80-100% SAS) was as effective as the antiserum raised against whole mycelial preparation in detection of cross reactive antigens as well as infection.

Table 51: Absorbance values in DAS ELISA for reactions of antiserum raised against 80-100% SAS of *G.cingulata* mycelia with healthy and inoculated tea leaf antigen.

Antigen (40µg/ml)	Absorbance at 405 nm[a,b,c]	
	Healthy	Inoculated*
UPASI-2	0.526±0.003	0.653±0.012
UPASI-3	0.670±0.032	1.015±0.009
UPASI-8	0.569±0.015	0.826±0.021
UPASI-9	0.740±0.024	1.061±0.013
UPASI-26	0.612±0.031	0.823±0.017
BSS 1	0.606±0.017	0.816±0.016
BSS 2	0.601±0.021	0.802±0.022
BSS 3	0.593±0.032	0.693±0.035

* 72h after inoculation ; a Average of three experiments.

b Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

c Difference between healthy and inoculated extracts significant at p=0.01 in all cases.

4.10. Characterization of mycelial and conidial wall of *G.cingulata*

Since, both cell walls of mycelia and conidia play important roles in host pathogen interaction phenomena and as earlier experiements also proved cell walls to be effective in raising antesera, it was decided to characterize the cell walls and to determine their chemical nature.

4.10.1. Mycelial wall

Cell walls were isolated from *G. cingulata* and the isolated cell walls were further extracted with NaOH as described earlier. Carbohydrate and protein content of cell wall preparations from *G.cingulata* were 11 mg/ml 9.25 mg/ml respectively. This preparation was further analysed by SDS-PAGE and confirmed by binding with fluorescein labelled concanavalin A.

4.10.1.1. ConA-FITC binding

In order to detect the nature of the cell walls, mycelia or isolated cell walls of *G.ingulata* were treated with FITC labelled conA and observed under the microscope as described under Materials and Methods. Strong fluorescence was observed under the microscipe in both mycelia and cell wall of *G.cingulata* (Plate IX, figs. A & B). The occurrence of conA binding substance in cell walls confirmed the glycoprotein nature of mycelial wall of *G.cingulata.*

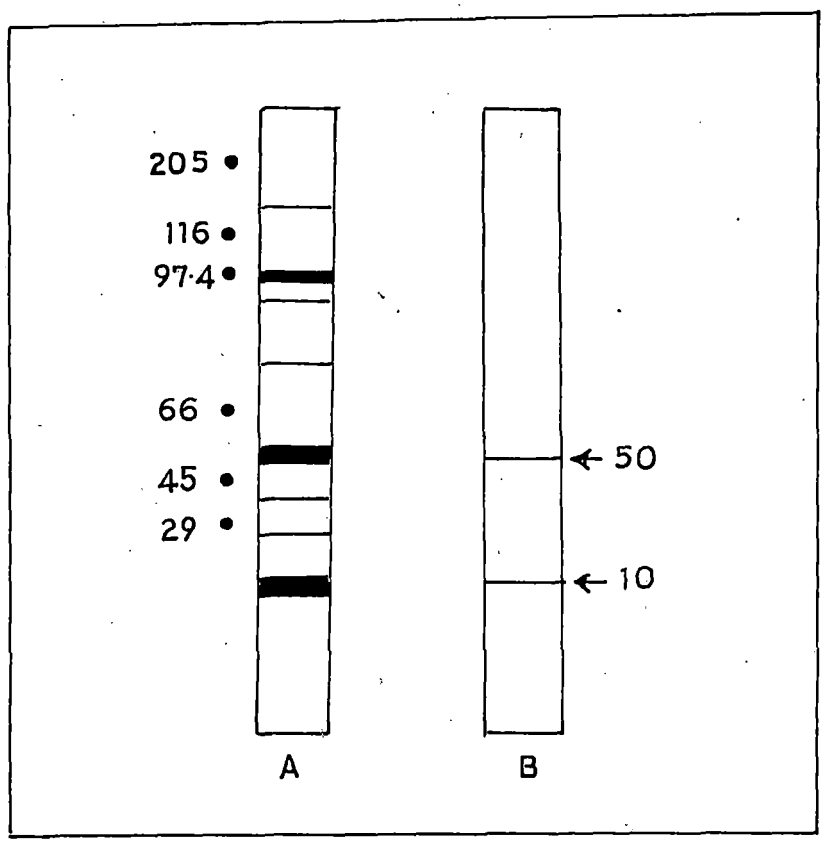
4.10.1.2. SDS-PAGE

The cell wall preparation of *G.cingulata* were further resolved in SDS-PAGE in order to confirm their glycoprotein nature. Gels were fixed in appropriate solution and stained either with coomassie blue R250 or with Periodic acid Schiff's reagent respectively for protein and carbohydrate detection. Staining of gels with coomassie blue 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 molocular weight of 10 and 50 KD in the mycelial wall extract of *G.cingulata* (Fig. 20).

4.10.2. Conidial wall

Since conidial wall plays an important role in fungal morphogenesis and development it may also be involved in recognition phenomena leading to host pathogen interaction. Lectiins are proteins of non immune origin which agglutinate cells and /or precipitate glycoconjugates by specifically inteacting with sugar moieties. Lectins have proved to be useful tools for identifying glycoconjugate components on cell surfaces. In the present investigation initially mycelial wall was characterized following which attempts were made to characterize then conidial wall by studying the agglutination effect of 7 lectins (Con A, HPA, UAE1, WGA) on conidia. Agglutination procedure has been

SDS-PAGE analysis of cell wall extract of *Glomerella cingulata*



A = Cell wall extract (protein)

B = Cell wall extract (carbohydrate)

Fig . 20 .

described in Materials and Methods. Agglutination were examineed and arbitrarily scaled from 0 to 4 (0 = no agglutination ; 1 = 1-25% ; 2 = 26-50% ; 3 = 51-75% ; 4 = 76-100% agglutination). Microscopical examination revealed that conidia were strongly agglutinated by conA and to slightly lesser degree by UAE 1. No agglutination was evident by HPA and WGA (Table 52, Plate X, figs. A-C). Con A interacts specifically with α-D mannosyl (and α-D glucosyl residues) while HPA interacts with N-acetyl α-D galactoseaminyl residues. Strong agglutination with conA suggests that the surface components are glycoconjugate containing α-D glucopyranoside and /or α-D mannopyranoside residues. Agglutination with UAE 1 lectins which has affinity for L-fucose indicated that residues of this sugar was also located on the surface of conidia.

Table 52 : Agglutination response of conidia of *G.cingulata* to different lectins

Name of lectins	**Agglutination extent of conidia of *G.cingulata* ***
Concanavalin A (conA)	4
Helix pomatia agglutinin (HPA)	0
Ulex europaeus agglutinin I (UEA 1)	3
Wheat-germ agglutinin (WGA)	0

* 0 - No agglutination
1 - 1-25%
2 - 26-50%
3 - 51-75%
4 - 76-100%.

PLATE IX (figs. A&B) . Fluorescence of hyphae (**A**) and isolated cell walls (**B**) of *G . cingulata* after staining with FITC - Con A

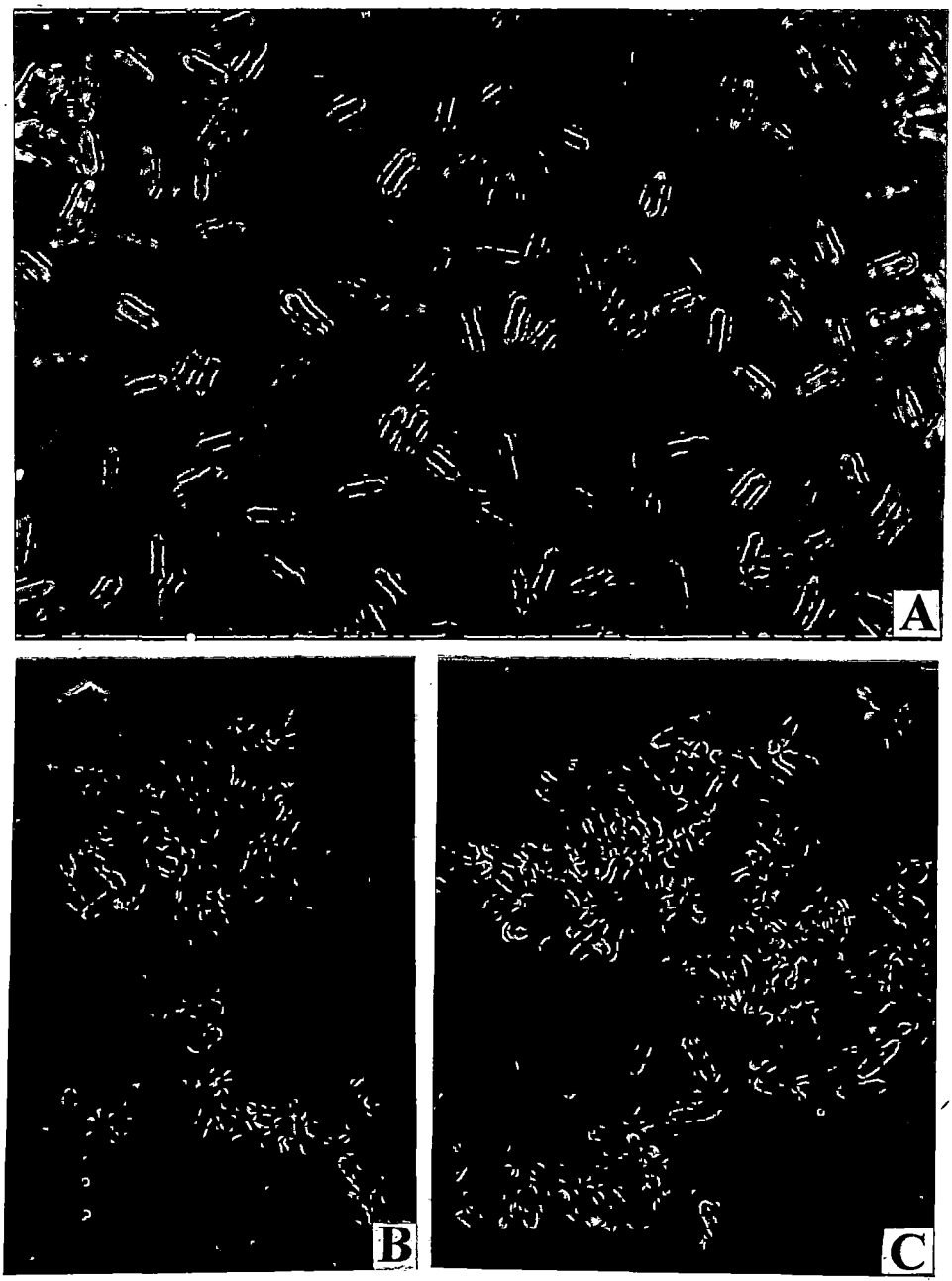


PLATE X (figs. A - C) . Agglutination of spores of *G.cingulata*;
A - control ; **B & C** - after treatment with Con A

4.11. Callus induction

Having established the presence of cross reactive antigens between tea leaves and *G.cingulata* it was decided further to detect the cellular location of the CRA by immunofluorescence test in leaf tissues, mycelia and conidia of *G.cingulata* as well as in loosened cells. For obtaining loosened cells, initially induction of callus from stem segments of susceptible variety (TV-18) was done. TV-18 was selected as it was one of the highly suceptible varieties and in field tests was the most suceptible one. It was observed that within 10 days of transferrring the stem segments to MS medium (as described in Materials and Methods) callus initiation started and well developed calli were, formed within one month (Plate XI, fig. A). Young calli thus formed were fragile and within a period of 7 days these were tranferred to liquid MS medium and agitated gently to obtained loosened cells.

4.12. Immunofluoresence

Fluorescent antibody labelling with fluorescein isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. In the present study following immunodiffusion, immunoelectrophoresis, DAC-ELISA as well as DAS-ELISA the presence of CRA shared by *C. sinensis* and *G.cingulata* have been detected. It was decided to determine the tissue and cellular location of CRA in fragile callus and leaf tissues of tea varieties as well as mycelia and conidia of *G.cingulata* Detailed methods of antibody staining of leaf sections and fungal cells have already been discussed under Materials and Methods. Leaf sections and mycelial preparations were photographed under UV- fluoresence.

4.12.1. Loosened cells

Fragile callus or loosened cells (Plate XI fiig. B&C) were prepared from the stem segments of susceptible variety (TV-18). The loosened cells were treated separately with normal serum, antiserum raised against TV-18 and *G.cingulata* and finally reacted with FITC. These cells were not autofluorescent nor did they fluoresce when reacted with normal serum, but bright fluorescence of these cells under UV-light were noticed when reacted with antiserum of TV-18. It is interesting to note that when cells were treated with anti *G.cingulata* antiserum and then reacted with FITC, bright fluorescence was noticed (Plate XII, figs. A & B).

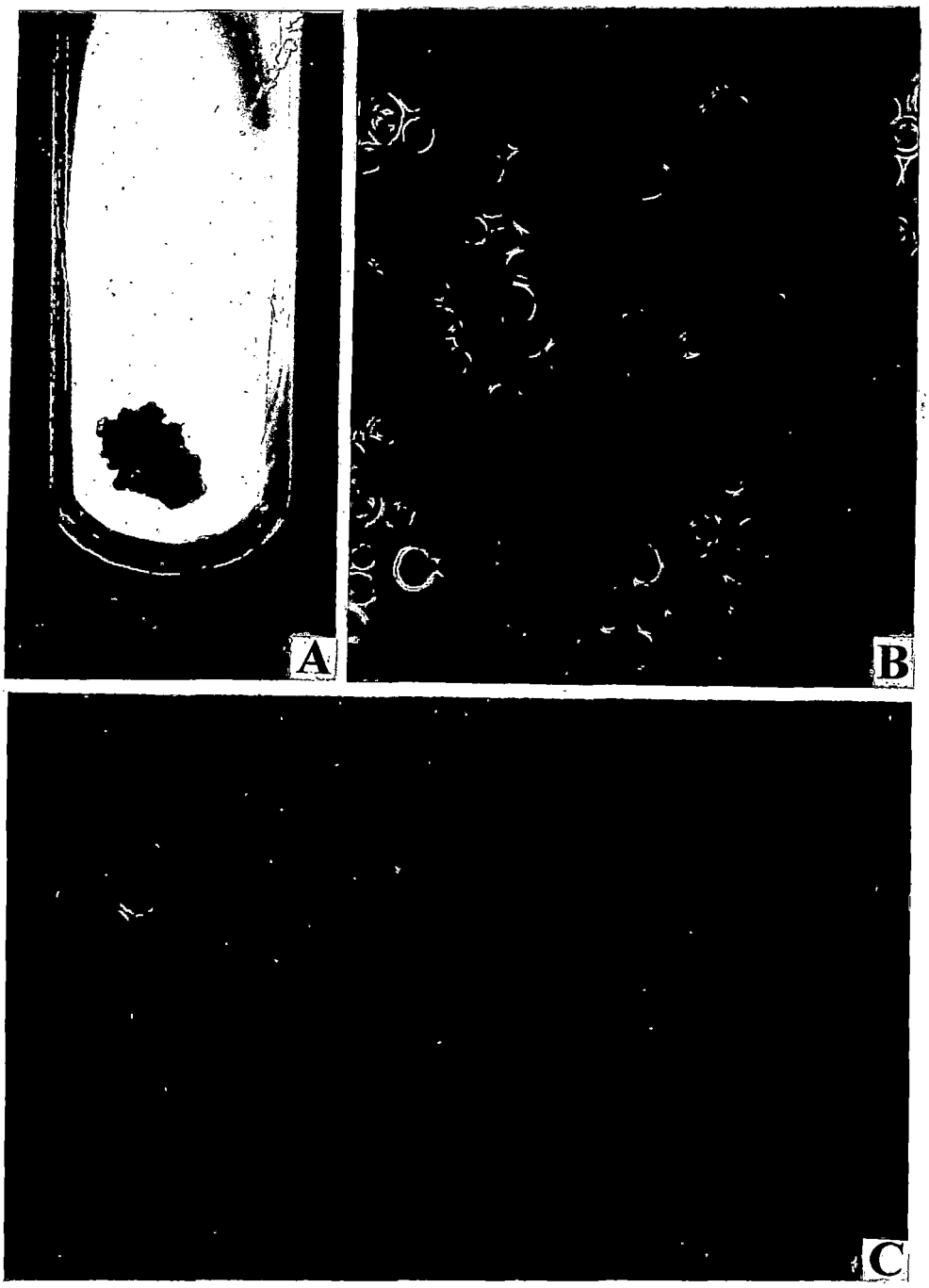


PLATE XI (figs. A-C) .Callus from stem segments of tea (TV -18); **B & C** - loosened cells from callus observed under microscope

4.12.2. Leaf tissues

Cross sections of tea leaves (Tv-18) were treated separately with normal serum, homologous and pathogen antisera, then reacted with FITC. Leaf sections exhibited a natural autofluorescence under UV-light on the cuticle (Plate XIII, fig. A). Same observations were noted when the leaf sections were treated with the normal serum and FITC. Leaf sections treated with antiserum of TV-18 and then reacted with FITC, developed bright fluorescence which was distributed throughout the leaf tissue, mainly in the epidermal cells and mesophyll tissues. Of much significance was the strong reaction of anti *G.cingulata* antiserum with leaf tissues of TV-18. CRA was concentrated mainly around epidermal cells and mesophyll tissues (Plate XIII, fig.B).

4.12.3. Mycelia and conidia

Mycelia and conidia of *G. cingulata* were not auto fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of mycelia and conidia of *G. cingulata* with homologous antisera and FITC showed a general fluorescence that was more intense on young hyphae (Plate XIV, fig. A) and throughout the surface of the conidia (PlateXIV, figs. B & C). When fungal cells were reacted with antiserum to leaves of TV-18 and treated with FITC, bright fluorescence was apparent on young hyphae and throughout the surface of some conidia.

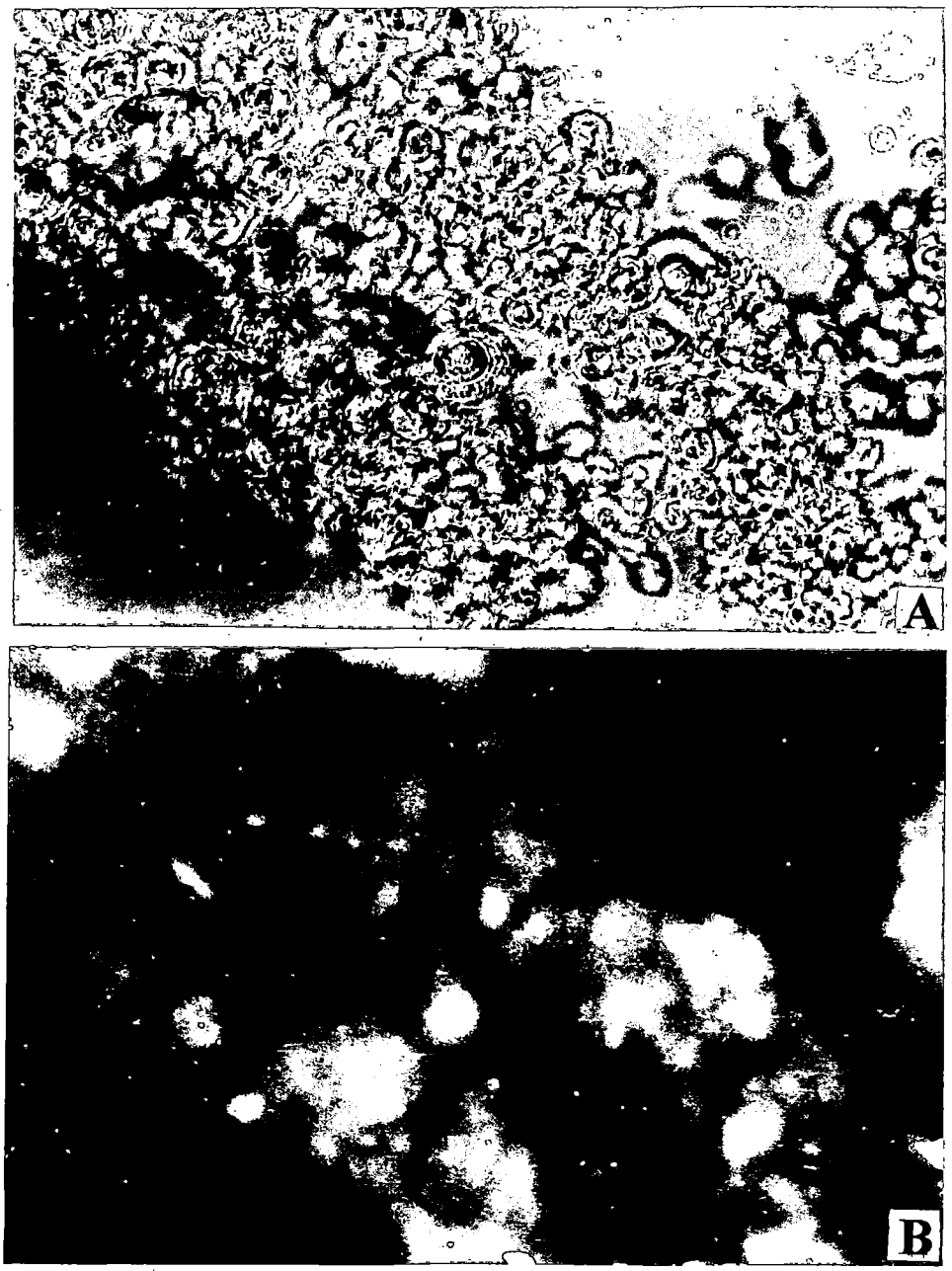


PLATE XII (figs. A & B) . Loosened cells from callus of tea (TV-18) . **A**- under bright field microscope ; **B**- after treatment with antiserum to TV-18 followed by FITC labelling

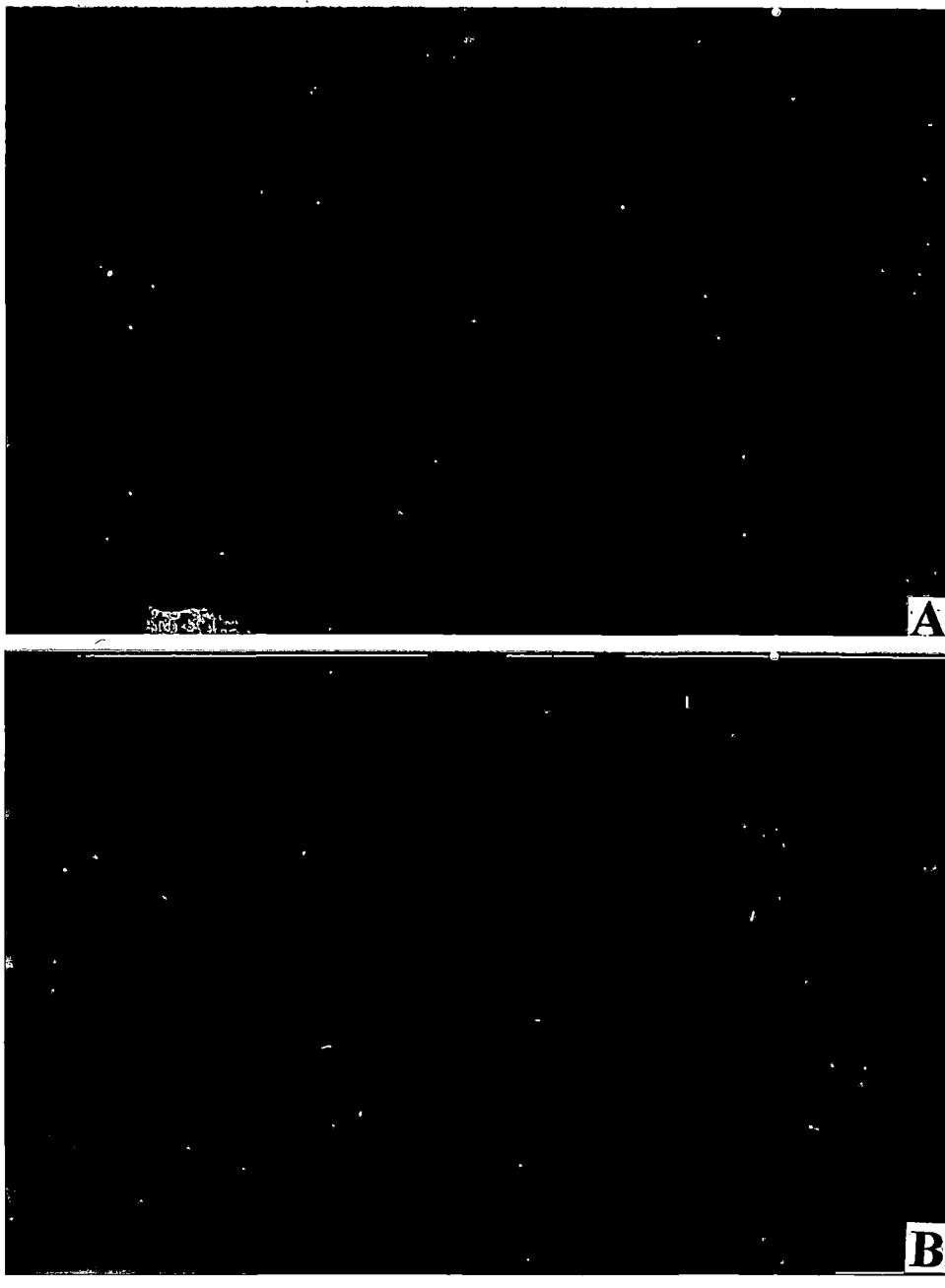


PLATE XIII (figs. A & B) . FITC antibody staining of tea leaf tissues (TV - 18) for cross reactive antigens shared with *G.cingulata* ; **A** - autofluorescence of unstained leaf section; **B** - leaf section treated with antiserum to *G.cingulata* and FITC antibodies of goat specific for rabbit globulin

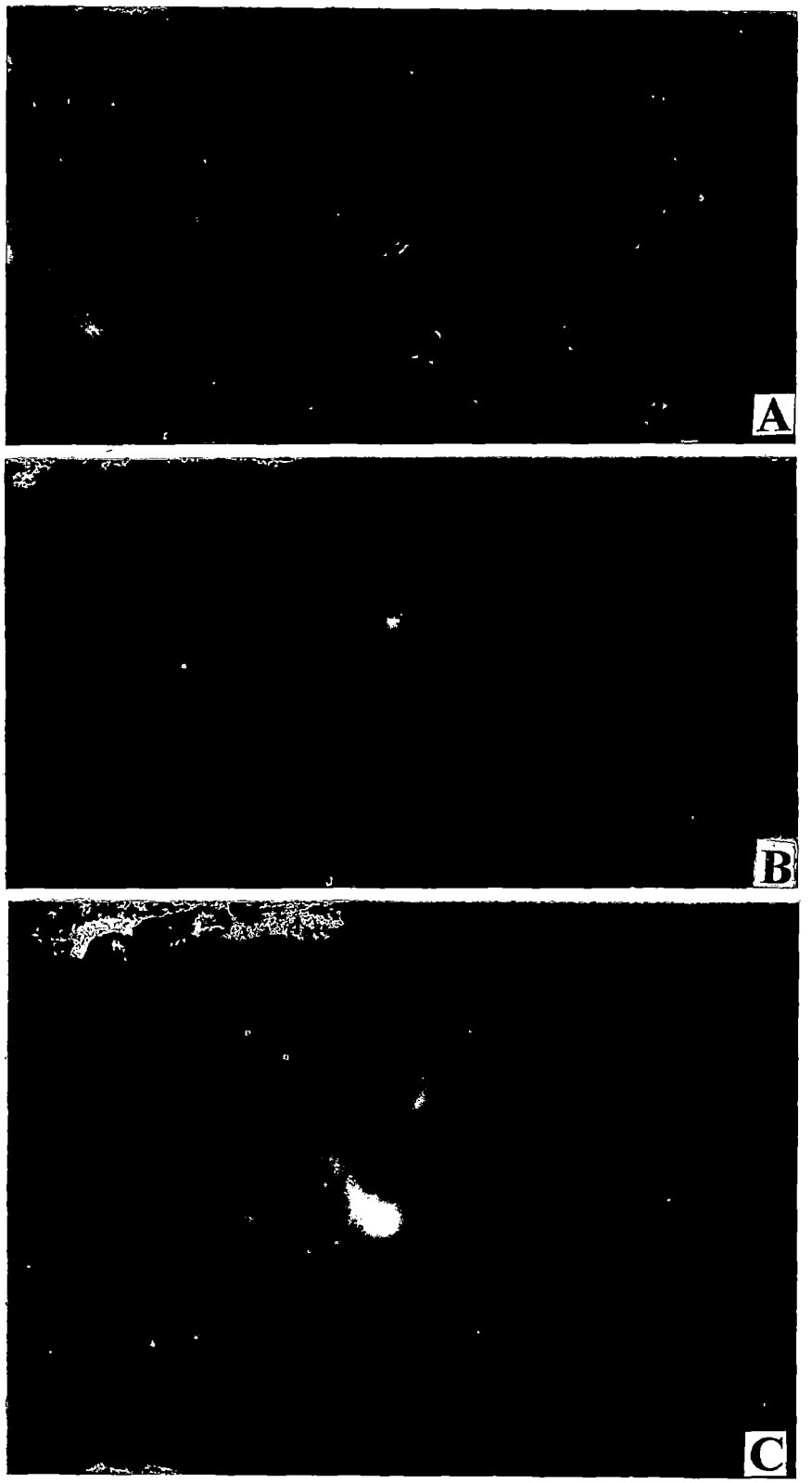


PLATE XIV (figs. A-C) . Hyphae **(A)** and conidia **(B&C)** of *G.cingulata* treated with antiserum to *G.cingulata* and FITC antibodies of goat specific for rabbit globulin

Discussion

A complex and variable developmental pattern between host and parasite is initiated by the invasion of the host tissue by pathogen which continues throughout their course of coexistance. Differences in 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 invadors are present in the plant environment. In spite of this , disease is still the exception rather than the rule. This is because a potential pathogen in order to establish a compatible interaction must recognize features of the plant which signals the suitability of that plant for parasitism. At the same time the potential host may be able to detect or recognize a potential fungal pathogen or nonself and use the initial act of recognition to trigger a range of induced resistance mechanisms (Callow, 1982, 1983; Purkayastha, 1994). Plants thus have evolved immune mechanisms of various types by which they can counter the advance of foreign organisms. The result is that, disease tends to be specific, a given pathogen usually infecting a distinct range of host plant.

In the host pathogen interaction therefore, the initial cellular recognition is followed by communication between its components, whereby information is transmitted to and received by cell to signals or instructions the cell can understand. This exchange of information are generally mediated by soluble antigens located on or near the cell surface (Chakraborty, 1988).

At the onset of the present study varietal resistance tests of 37 varieties of tea released by Tocklai Experimental Station, Jorhat, Assam; Darjeeling Tea Research Centre, Kurseong, Darjeeling; UPASI Tea Research Centre, Valparai, Tamilnadu, against the brown blight pathogen, *G. cingulata* was carried out by detached leaf and cut shoot inoculation techniques. Responses exhibited by the different varieties were essentially the same in both the inoculation techniques. Of the 37 varieties tested, UPASI 9 and BT-15 were the most and least susceptible respectively. Yanase and Takeda (1987) detected resistance of tea plants to grey blight disease caused by *Pestalotia longiseta* by cut shoot method.

Fungal plant pathogens invade host plant cells with a variety of specialised 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. In many rust fungi positioning of the appressorium is most critical because invasion of the host can occur only through the stomata (Staples and Macko, 1984). It has been reported by Ando and Hamaya (1987) that infection of tea by the anthracnose fungus *Gloeosporium theae-sinensis* could only occur 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). 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. Chakraborty *et. al* (1995a) studied the factors influencing spore germination, appressoria formation and disease development in *Camellia sinensis* by *Glomerella cingulata.* They reported that spore germination and appressoria formation were optimum at a temparature of 25°C, pH 5.0, 7h light/day regime and a 24h incubation period. In the present investigation optimum condition necessary for growth of *G. cingulata* was 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. Sporulution behaviour varied with the different media on which the fungus was grown.

During host parasite interaction even when coincidence of location and time is provided and supplemented with optimum conditions for the development of the pathogen, parasitic relationship can only be established if the host recognizes the pathogen on the one hand, and the pathogen can overcome the various defence mechanisms of the host, on the other hand

whenever an intimate and continuing association of cells of host and pathogen occur it has been observed that partners of this association have a unique serological resemblance to one another involving one or more antigenic determinants. The presence of cross reactive antigen (CRA) between plant host and their parasites and the concept that these antigens might be involved in determining the degree of compatibility in such interactions have been reviewed by several authors (Devay et. al, 1972; Devay and Adler, 1976; Kalyanasundaram, 1978; Chakraborty, 1988; Purkayastha, 1989; Purkayastha et. al., 1991; Purkayastha, 1994). In the present study, leaf antigens of 37 tea varieties, 3 non hosts (*G. max* , *C. arietinum* and *C. japonicum*), 9 isolates of *G. cingulata* and one non pathogen of tea (*F. oxysporum*) were cross reacted separately with anti - *G. cingulata* antiserum. The presence of cross reactive antigens among *G. cingulata* (all isolates) and 16 varieties of tea (TV-18, 20, 22, 23, 25, 27, Teenali 17/1/54, UPASI 3, 9, 26, T-78, B-157, TTV_1 K1/1, B668 and B777) was evident in immunodiffusion test. No common antigenic substance was found between *G. cingulata* and 8 other varieties (TV-26, UPASI-2, P312,BT-15, AV-2, RR17, T-135 and CP-1). However, weak precipitin reaction was observed with antigens of 13 other varieties (TV-9, 28, 29, 30, TS-449, UPASI-8,17 BSS1, 2, 3, P-1258, BS/7A76 and HV-39). Cross reaction of anti- *G. cingulata* antiserum with antigens of non pathogens and non hosts failed to develop any precipitin band. Reciprocal cross reaction was also carried out with antisera of TV-18 (susceptible variety) and CP-1 (resistant variety). Presence of common antigens between *G. cingulata* and suceptible tea varieties were confirmed in the reciprocal cross reaction with the antiserum of TV-18. None of the isolates of *G. cingulata* developed any precipitin reaction with anti CP-1 antiserum. Non host species and non pathogens also fail to develop any precipitin band with either of the two antisera.

Several earlier studies have also implicated the importance of common antigens in host pathogen compatibility. The occurrence of CRA and their involvements in various host parasite combinations have been demostrated. These are flax and *Melamspora lini* (Doubly et. al., 1960), cotton and *Verticillium alboatrum* (Charudattan and DeVay, 1972) cotton and *Fusarium oxysporum* f. sp. *vasinfectum* (Charudattan and DeVay, 1970; Kalyanasundaram *et. al.*, 1975), sweet potato and *Ceratocystis fimbriatae* (DeVay *et. al.*, 1976), potato and *Phytophthora infestans* (Palmerley and Callow, 1978; Alba and DeVay, 1985), soybean and *Macrophomina phaseolina* (Chakraborty and Purkayasta, 1983), soybean and *colletotrichum dematium* var. *truncata* (Purkayastha and Banerjee, 1986), soybean and *Myrothecium roridum* (Ghosh and Purkayastha, 1990, coffe and *Hemiliea vastatrix* (Alba *et. al.*, 1983), groundnut and *Macrophomina phaseolina* (Purkayastha and Pradhan, 1994); tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994). Present result also confirm the presence of CRA between *P.*

theae and susceptible tea varieties reported by Chakraborty *et. al.* (1995).

Results obtained in immunodiffusion tests were further confirmed by immunoelectrophoretic analysis with antigen and antisera preparations from tea leaves (CP-1 and TV-18) and *G. cingulata.* Reaction of anti - *G. cingulata* antiserum with homologous antigen revealed the presence of 6 precipitin arcs of which 2 were common with the susceptible varieties, UPASI 9, TV-18 and B- 157. No precipitin arc was formed between antiserum of *G. cingulata* and antigens of TV-26, UPASI 2 and CP-1 (resistant varieties), *G. max, C. arietinum* and *C. japonicum* (non hosts) and *F. oxysporum* (non pathogen). Following rocket immunoelectrophoresis a single precipitin line was discerned for TV-18 when reacted in the agarose gel containing *G. cingulata* antiserum. The present results are in agreement with the findings of previous workers (Chakraborty and Purkayastha), 1983; Purkayastha and Ghosal, 1987; Ghosh and Purkayastha, 1990). In the above studies Purkayastha and coworkers have examined various host pathogen/non pathogen combinations including cultivars of soybean, rice, jute, pigeon pea, bean and groundnut to find out the serological relationship with some fungal pathogens as well as non pathogens following immunodiffusion and immunoelectrophoretic tests. More than 50% combination between host and pathogen while no CRA could be detected between resistant host and respective pathogens. Chakraborty and Saha (1994b)have also reported similar findings with tea and *Bipolaris carbonum.*

In host parasite interactions failure to detect CRA by conventional serological techniques have been attributed to a number of factors including antigen treatment, method of extraction of antigen, culture of microbes and age of plants and tissue (DeVay and Adler, 1976; Chakraborty,1988). Alba *et. al.*, (1983) showed that urediospores of *Hemilia vastatrix* shared common antigenic determinants with coffee plants which was in contrast to their previous conclusions made with the same host parasite system (Alba et. al., 1973). This disagreement was attributed to the low concentration of antigenic preparations used in earlier investigation.

Among all available serological techniques for detection of CRA enzyme linked immunosorbent assay is probably the most sensitive (Alba and DeVay, 1985; Chakraborty and Saha, 1994). In the present study antisera were raised against antigenic preparation of *G. cingulata* from mycelia and cell wall separately. The antisera thus obtained were initially purified and the IgG fraction was used in all further tests. This was necessary to minimize non specific binding which may interfere with the actual antigen antibody reaction. At the onset the sensitivity of the assay was optimised. Homologous soluble antigens at a

concentration as low as 25ng/ml could be detected in indirect ELISA by both antisera. Positive results were also obtained at antisera dilutions of upto 1:16,000. Absorbance values in ELISA decreased with increased dilution. *Pythophthora fragariae* antigen at a concentration as low as 2ng/ml was detected in indirect ELISA using antiserum raised against pooled mycelial suspension of 5 *P. fragariae* races (Mohan, 1988). He suggested that this sensitivity may be because of the indirect ELISA method used; the direct double antibody sandwhich form of ELISA (Voller *et. al.*, 1976) detected antigen at protein concentration greater than 50ng/ml. The indirect method used 'native' antibodies rather than antibody conjugates required in the direct assays and hence avoided masking or impairing of antigenic binding sites by the conjugation procedure. Chakraborty et. al. (1996) also reported that antiserum raised against *Pestalotiopisis theae* could detect homologous antigen at 25 ng/ml. Antiserum dilution of upto 1:16,000 was effective for detection.

In the present study indirect ELISA readily detected CRA in semipurified antigen preparations of *G.cingulata* at a concentration of 40 μg/ml with 1:250 antiserum dilution. Alba and DeVay (1985) also detected CRA in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* (races 4 and 1.2.3.4.7) using antisera of potato cultivars King Edward and Pentland Dell at concentrations lower than 50 μg/ml protein in indirect ELISA. Among the 37 tea varieties tested with antiserum of *G.cingulata* raised against either mycelial or cell wall preparations high absorbance values were obtained in reaction with antigenic preparations from UPASI 9, B-157 and TV-18 while CP-1, UPASI-2 and BT-15 showed lowest reactivity. In order to determine the observed cross reactivity between *G.cingulata* and suceptible varieties was specific, antigen preparations from non host (*G.max, C.arietinum* and *C.japonicum*) and non pathogen (*F.oxysporum*) were also assayed with antisera of mycelia and cell wall of *G.cingulata* (isolate GC-1), resistant (CP-1) and susceptible (TV-18) tea varieties and non pathogen (*F.oxysporum*). None of the above reactions showed any reactivity in ELISA. Since indirect ELISA test gave the same results in all three repetitions it appears that this observed antigenic disparities have some significance in the basic compatibility of host and pathogen (Chakraborty, 1988). In another experimental set up of DAC ELISA when antigens prepared from 3 categories of leaves (uppermost young, middle and mature leaves) each of 11 Tocklai varieties of tea were cross reacted with anti-*G.cingulata* antisera raised against both mycelia and cell wall, all three categories of leaves gave positive response. Recognition of leaf antigen with cell wall antiserum of *G.cingulata* was always better than the antiserum of the pathogen raised against mycelial preparations. No correlation could be established between ELISA responses and age of leaves. However, irrespective of varieties or categories absorbance values were always higher for antiserum raised against cell wall than mycelia of the pathogen.

Detection of major CRA shared between *G.cingulata* and tea leaves was comparable in both the formats of ELISA tested viz. double antibody sandwich (DAS) and direct antigen coated(DAC). Both the methods showed sensitivities with leaf extracts but DAC-ELISA was more specific. It appears from the results of studies on cross reactive antigen of *G.cingulata* and tea that common antigenicity between tea leaf and *G.cingulata* may be associated with susceptibility to brown blight disease.

Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after several days of infection, by which time the pathogen would be well and truly established in the host tissues. In plant disease studies, therefore, it is necessary to have techniques by which the pathogen can be detected at a very early stage. Recent trends in detection of plant pathogenic fungi include the development of more rapid diagnostic techniques with high specificity for the target organism. These techniques can be used to detect fungi present in low amount in or on plant tissue and therefore in many cases the pathogen can be detected at a much earlier stage of disease development than was previously possible (Hansen and Wick, 1993). Various formats of ELISA using polyclonal antiserum has found widespread application in plant pathology and are routinely used for detection and identification purposes (Clark and Adams, 1977 ; Clark, 1981; Lommel *et. al.* 1982, Sundaram *et. al.*, 1991; Lyons and White, 1992).

Differential responses of the different varieties of tea to *G.cingulata* has been established by both pathogenicity test and cross reactivity test between *G.cingulata* and tea varieties. Following this, the ability of the antisera raised against both mycelial and cell wall preparations of *G.cingulata* to detect the pathogen in infected tea leaves was tested in ELISA. ELISA could detect infection in all the varieties tested irrespective of their susceptibility or resistance in other tests. Pathogen detection in the host tissue by ELISA have also been reported by a number of previous workers. These include the detection of *Pythium* species, *Rhizoctonia solani* and *Sclerotinia homeocarpa* in turfgrass (Miller *et. al.*, 1986; *Humicola laniginusa* in rice (Dewey *et.al.*, 1989), *Leptosphaeria korrae* in turfgrass (Nameth *et. al.*, 1990), *Phytophthora* species in a variety of woody ornamentals (MacDonald *et.al.*, 1990; Benson, 1991) and *Septoria nodorum* and *S.tritici* in wheat (Mittermeier *et. al.*, 1990, Peterson *et.al.* 1990). Mohan (1988) reported the ability of anti *P.fragariae* antiserum to detect infection in strawberry cultivars. *P. fragariae* infections were also detected readily in field infected samples of strawberry cultivars. Amouzou-Alladaye *et.al.* (1988) also reported the use of specific IgG of *Phytophthora fragariae* in DAS ELISA which constituted a method of early detection of the fungus in roots of inoculated plants. For the 5 varieties studied *P.fragariae* could be detected between 15 and 25 days after inoculation. In the present study natural brown blight infections could also be detected easily in ELISA.

To determine the earliest time at which infections could be detected extracts from *G.cingulata* inoculated tea leaves were prepared at 6 h intervals and tested against anti *G.cingulata* antiserum in ELISA. Infections could be detected as early as 6 h after inoculation in the susceptible variety (B 157) while in the resistant variety it could be detected either from 18 h onwards (by cell wall antiserum) or 30 h onwards (by mycelial antiserum).Thus,

ELISA could easily detect infections in the leaf tissues much earlier than the appearance of the visible symptoms which generally appeared after 48 h of inoculation with *G.cingulata.* This is in confirmity with the results of several previous authors who have reported that ELISA could detect pathogens in tissues (Linfield, 1993; Jamoux and Spire, 1994). In experiments conducted over 20 days with *Fragaria vesca,* Mohan (1988) showed that ELISA positive material was detectable 6-8 days after inoculation with *P.fragariae* when the plants were apparently still healthy.

Antisera raised against either mycelial antigen preparation or cell wall antigen preparation of *G.cingulata* could detect infections in tea leaves. In general antisera raised against cell wall preparations gave slightly higher absorbance values. El-Nashaar (1986) made polyclonal antisera to soluble hyphal antigens and to cell wall antigens. They found that the number of fungi that cross reacted was reduced considerably when the cell wall fraction was used as an immunogen instead of the soluble cytosol fraction. Gerik *et.al.* (1987) on the other hand stated that the antigen preparation containing fungal cell wall or cell wall materials could evoke antibodies to chitin or other cell wall polysaccharides common to most fungi. They obtained a more specific sera using only soluble fungal proteins as the antigen. Linfield (1993) again reported that antiserum raised to cell wall fraction of *F.oxysporum* f.sp. *narcissi,* gave better recognition than that to cytoplasmic fraction. Holtz *et.al.* (1994) produced polyclonal antisera to both the cell wall fraction and the soluble cytosol fraction of *Thielaviopsis basicola.* Both indirect ELISA and fungal capture sandwich ELISA revealed no difference in specificity of the cell wall and the cytosol antisera and the antibodies to each immunizing fraction reacted with the other fraction.

G.cingulata could be detected in ELISA upto a concentration of 2 μg/ml proteins in infected leaf extracts. Detection of infection in such low levels has also been previously reported by Mohan (1988) who showed that *P.fragariae* infected strawberry roots diluted 200-400 fold yielded positive reactions in ELISA. He suggested that infection levels 200-400 times less than those tested might be detectable. In the present study it is noteworthy that some amount of cross reactivity was always obtained between anti *G.cingulata* antiserum and healthy tea leaf extracts. This may be due to the presence, to some extent, of cross reactive antigens between the host and the pathogen.

Since the antiserum raised against *G.cingulata* could detect *G.cingulata* infections in tea leaves it was further decided to evaluate its specificity with regard to detection of the pathogen both *in vivo* and *in vitro*. Anti *G.cingulata* antiserum was shown to react positively with extracts of tea leaves artificially inoculated with *G.cingulata* but reactions with extracts of leaves inoculated with the pathogens were comparatively lower when tested in DAC ELISA.

Brown blight and grey blight symptoms are sometimes difficult to differentiate in the field since they are known to occur in close association in nature. The use of competition or inhibition ELISA for the recognition of specific microorganisms in mixed population from plant tissues has been reported (Alvarez, 1990). A competitive type of ELISA was therefore used in the present study to determine the predominant pathogen from mixed natural infections

Antiserum raised against *G.cingulata* gave positive results against antigen extracts of naturally infected tea leaves while antiserum raised against *P.theae* gave negative results in this test. This confirmed that the infection was mainly of brown blight with *G.cingulata* being the predominant pathogen. Lyons and White (1992) also used a competition ELISA to determine the predominant pathogen in cavity spot of carrots. They reported that *Pythium violae* was predominantly isolated from infected carrot tissues and competitive ELISA also confirmed *P.violae* as the causal agent and not *P. sulcatum.*

Cross reactivity of the antiserum raised against *G.cingulata* (isolate GC-1) was tested against 8 other isolates (GC-2 to 9). Since *G.cingulata* is a telomorph of *Colletotrichum gloeosporoides* the cross reactivity of the antiserum raised against *G.cingulata* with other species of *Colletotrichum* was tested. Cross reactivity was also tested with other foliar pathogen of tea. Results revealed that anti *G.cingulata* antiserum cross reacted to a greater or lesser degree with the different isolates but not with those of other species of *Colletotrichum* or other genera. Mohan (1989) showed that antiserum raised against mycelial suspension of *Phytophthora fragariae* (PfM) reacted strongly with antigen from several *Phytophthora* species. He observed that anti PfM could not be made specific for *P.fragariae* because it was raised to components shown to be antigenically similar in all *Phytophthora* species tested. Similar results with *P.fragariae* were also reported by Amouzou-Alladaye *et.al.* (1988). In their studies antibodies obtained with a strain of *P.fragariae* detected 11 different strains of pathogen in pure culture by both DAS and DAC ELISA. Harrison *et.al.* (1990) further reported that anti *P.infestans* γ-globulin reacted strongly with an extract of *P.erythroseptica* in DAC-ELISA but not with extracts of unrelated fungi or a culture of bacterium *Erwinia carotovora*, all of which were either saprophytes or pathogen of potato. Sundaram *et.al.* (1991) raised anti bodies against PAGE separated proteins obtained from an isolate of *Verticillium dahliae.* This could detect 11 different isolates of this pathogen from potato, cotton and soil. It also reacted to a lesser extent with *V.albo-atrum.* They concluded that since the antisera appeared to be specific for *V. dahliae* and *V. alboatrum* and did not cross react with other species of *Verticillium, Fusarium* and *Colletotrichum,* it could be a valuable aid in the detection and identification of *V.dahliae* and *V.albo-atrum.* In studies on the cross reactivity of different isolates of *P.theae* and other fungal genera it was also reported that the antiserum raised against one isolate cross reacted with antigenic extract from other isolates but not from other genera (Chakraborty *et.al.*, 1996). Results of the present study, as well as those of previous authors therefore point to the occurrence of cross reactivities within the isolates of the same species and to some extent within related species but not with unrelated species or genera.

Even though pathogen can be detected in infected tissues rather easily the extent of growth of a pathogen within the leaf tissue cannot be easily quantified. Measurement of lesion size, sporulation or disease rating are often used to quantify the manifestation of disease several days after inoculation (Hoffman and Hill, 1989; Desjardin and Gardner, 1989; Ward *et. al,* 1989; Shaik and Steadman ; 1989). Lesion diameter defines the limits or spread of a colony but yields little information about the mass of hyphae within the colony. Moreover, visible symptoms like lesions do not appear within the first 2-3 days after infection of healthy tissue. Though quantification of plant virus antigens in host tissues by ELISA using samples of antigen of known concentration is now well established (Clark, 1981; Barker and Harrison, 1985), quantitative estimation of mycelia within infected tissues by ELISA has not been widely reported. In the present study quantitative estimation of *G.cingulata* in artificially inoculated leaves from 6h onwards was done by indirect ELISA using anti *G.cingulata* antiserum. Mycelial fresh weight within the leaf tissue of susceptible variety (B 157) increased till 90h of inoculation while in resistant variety (BT-15) it decreased after 78h. Estimation of fungal biomass in infected leaf tissues of 15 varieties revealed that maximum biomass was obtained in the most susceptible variety UPASI-9. Harrison *et.al.* (1990) estimated the amount of *P.infestans* mycelium in potato leaf extracts by comparing the values obtained in ELISA with those for known concentration of *P.infestans* mycelium. It was further reported (Beckman *et.al.*, 1994) that by optimizing the dilution of sample extracts and the dilution of primary anti *P.infestans* antisera, quantification of the biomass of *P.infestans* in zoospore inoculated potato tuber discs could be achieved by 8-18 hours after inoculation. Differences in growth between a virulent and virulent isolates of *P.infestans* on the resistant potato cultivar Kennebec were quantified by 32-48 hours after inoculation.

Detection of cross reactive antigens between *G.cingulata* and tea leaves as well as detection of the fungus within infected tissues was possible by the antiserum raised against whole mycelial antigen of *G.cingulata.* Since antibodies are not raised to all the proteins in the extracts but rather to specific antigenic proteins, in the next step of the present investigation, it was decided to purify the cross reactive antigens from the crude preparation. This was carried out by a series of purification procedures involving ammonium sulphate saturation, DEAE Sephadex chromatography and SDS-PAGE electrophoresis. Ammonium sulphate saturation from the crude mycelial preparation resulted in a number of fraction each of which was then tested by ELISA against anti *G.cingulata* antiserum. Results revealed maximum cross reactivity in the 80-100% SAS fraction. Alba and DeVay (1985) also purified cross reactive antigens from *Phytophthora infestans* by ammonium sulphate fractionation which

was followed by detection in ELISA. They reported that most of the cross reactive antigens were precipitated at 40% SAS.

Since the 80-100% SAS fraction was found to contain the major antigenic proteins the ability of this fraction to raise antibody was further tested. Antiserum raised against this fraction was tested by both immunodiffusion and ELISA against tea leaf antigens. In agar gel double diffusion test precipitation was observed in the homologous reaction as well as in heterologous reaction with antigen of the susceptible variety (TV-18). This antiserum therefore behaved in a similar manner as that raised against whole mycelial preparation. Presence of the antigenic proteins was confirmed in the 80-100% SAS fraction. Chard *et.al* (1985) also raised antiserum against purified mycelial preparation of *Mycena galopus* and compared with the antiserum raised against whole mycelia. Both the antisera were shown to be specific to *M.galopus* by immunodiffusion tests. In this study evaluation of the antiserum raised against 80-100% SAS fraction of *G.cingulata* mycelial antigen was also done by both DAC and DAS-ELISA. CRAs between *G.cingulata* and selected tea varieties was detected in both ELISA formats using this antiserum. This antiserum could also detect *G.cingulata* infection in inoculated tea leaves by both DAC and DAS-ELISA. Results of all the above therefore confirmed the presence of the antigenic proteins in 80-100% SAS fraction since this fraction was effective in raising specific antiserum against *G.cingulata.*

For further prurification, the 80-100% SAS fraction was subjected to DEAE Sephadex chromatography and the proteins were eluted out in three different buffer systems. All the eluates were tested for cross reactivity with anti *G.cingulata* antiserum in ELISA. ELISA results revealed the antigenic proteins to be eluted out in 0.05 M NaCl in 0.05M Tris-HCl buffer. The fractions containing the antigenic proteins were further pooled, concentrated and subjected to SDS-PAGE electrophoresis. Purification of an antigen from *Mycena galopus* by ammonium sulphate precipitation and DEAE cellulose chromatography was reported by Chard *et.al.* (1985). They showed that the antigenic protein as detected by immunodiffusion tests was present in peak III corresponding to the buffer 0.05M-0.25M NaCl in 0.05M Tris-HCl. SDS-PAGE analysis of the antigenic fraction obtained by purification of *G.cingulata* mycelial extract revealed a band corresponding to 10 KDA molecular weight. No other bands were visible.

Purification of the mycelial antigen from *G.cingulata* was further followed by characterization of the cell wall since antisera raised against antigenic preparations from both mycelia as well as cell wall fractions were equally effective in detection of CRAs and pathogen

in tissues. The chemical nature of the mycelial wall extract was determined by SDS-PAGE and ConA-FITC binding both of which confirmed the cell wall to contain glycoproteins. Two glycoprotein bands of molecular weight 50 to 10 KDA were detected in the extract. It is interesting to note that molecular weight of one of the glycoproteins corresponded to the molecular weight of the purified antigenic protein from mycelial preparations. The antisera against both whole mycelial and cell wall preparations were probably raised against similar components. Previous reports on the chemical nature of fungal cell walls also support their glycoprotein nature (Keen and Legrand, 1980; Beissmann, 1992; Ransom *et.al*, 1992).

Host parasite interactions are generally initiated in nature by the fungal spores since they come in contact with the host cell at the first instance. Therefore, conidial wall also plays an important role in recognition phenomenon. Hence, in this study, the conidial wall was also characterized by agglutination test. Responses of conidia to different lectins revealed that the surface components contained mannose and glucose residues. Further, the presence of glycoconjugates containing L-fucose was also established. Studies on the conidial cell wall surface of *F.solani* and *F.oxysporum* by Cristinzio *et.al.* (1988) revealed that the surface contained α-D mannosyl and α-D galactosaminyl residues whereas other species tested such as *F.culmorum F.graminearum, F.moniliformae, F.xylarioides, F. avenaceus* and *F.sambucinum* did not contain these residues in the outermost layer of the conidial wall. Glycoprotein nature of the material released from conidial wall of *Collototrichum graminicola* was established by Mercure *et.al.* (1995) on the basis of studies with FITC conjugated lectins. They further determined glucose and/or mannose to be components of this glycoprotein.

Results of various experiments of this study has established very definitely the importance of cross reactive antigens between host and pathogen in determining the response of the host to pathogen. This has also been supported by the works of several previous workers (DeVay and Adler, 1976; Chakraborty and Purkayastha, 1983; Chakraborty and Saha, 1994b; Chakraborty et.al., 1995). It is also important in studies on host parasite relationship to determine the cellular location of the CRA. For this purpose in this study fluorescence tests were conducted with loosened cells and cross sections of tea leaves as well as mycelia and conidia of *G.cingulata*. Loosened cells were obtained from calli prepared from stem segments of TV-18. Cross sections of tea leaves (TV-18) along with the loosened cells were treated with either anti -*G.cingulata* or anti TV-18 antiserum followed by staining with FITC conjugated anti rabbit globulin specific goat antiserum. Bright fluorescence was observed in both the loosened cells and in the cross sections of tea leaves (TV-18) treated with either of

the two antisera. In homologous reactions within the tea leaf sections fluorescence was distributed throughout the leaf tissues mainly on the epidermal cells and mesophyll tissues. Treatment of leaf sections with *G.cingulata* revealed that the CRA was concentrated mainly around epidermal cells and mesophyll tissues. Treatment of mycelia and conidia of *G.cingulata* with homologous antiserum amd FITC showed a general fluorescence that was more intense on young hyphae and throughout the surface of the conidia. DeVay *et.al.* (1981a) determined the tissue and cellular location of major CRA shared by cotton and *F.oxysporum* f.sp. *vasinfectum*. On the basis of strong fluorescence obtained at the epidermis, cortex, endodermis and xylem tissues, they suggested that the CRA determinants in roots have a general distribution. DeVay *et.al.* (1981b) also used FITC labelled antibodies for races of *P.infestans* to detect the CRA in potato leaf sections. It was also reported by Chakraborty and Saha (1994b) that CRA between tea and *B.carbonum* were mainly present in the hyphal tips and in patch like areas on the conidia, mycelium and mainly around epidermal cells and mesophyll tissues of the leaf. The cellular location of CRA between *P.theae* and tea leaves was also established by Chakraborty *et.al.*, (1995).

Detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors (Warnock, 1973; Hornok and Jagicza, 1973; Reddy and Ananthanarayanan, 1984). Dewey *et.al.* (1984) suggested, on the basis of immunofluorescence studies that chlamydospores, basidiospores and mycelia of *Phaeolus schweinitzii* contained molecules antigenically related to species specific antigens secreted by mycelia grown in liquid culture. They also demonstrated the presence of mycelium and chlamydospores in naturally and artificially infested soil samples, using this technique. *Phytophthora* could also be detected in soil by immunofluorescence antibody teachnique (Watabe, 1990). Different test formats including indirect ELISA, Western blotting, dip stick, dot blot and indirect immunofluorescence was assessed by Wakeham and White (1996) for their potential to detect resting spores of *Plasmodiophora brassicae* in soil. Among the different test formats they found indirect immunofluorescence to be the most rapid and suitable assay for the detection of resting spores of *P.brassicae* in soil.

Studies on the host pathogen interaction between *G.cingulata* and tea leaves mainly on an immunological basis have revealed the major determinants of the resistant or susceptible reactions to be the cross reactive antigens specific for this reaction. Interestingly the antigenic component from both the whole mycelial as well as cell wall preparations of *G.cingulata* appeared to be similar , at least partly. Low molecular weight protein of approximately 10

KDA was purified from the mycelial preparation and the cell wall also had a glycoprotein of similar molecular weight. The observed lack of difference in specificity of the antisera raised against the two antigenic preparations may be explained by the fact that both contained similar proteins. Both proteins and polysaccharide structures have high potential informational content and play important roles in biological recognition phenomena. Thus, undoubtedly these have very important roles to play in host pathogen interactions and success or failure of infections depend to large extent on these structures. Other than the establishment of cross reactive antigens between *G.cingulata* and tea leaves, a major outcome of the present study has been the detection of brown blight infection in tea leaves at very early stages of infection and at very low amounts. Quantification of the mycelial growth in leaf tissues even before the visible symptoms have appeared is another important achievement. Such early detection of disease is of extreme importance where conventional techniques may fail to detect infection or infections can be detected only after appearance of well defined symptoms. This can be of great use in the area of disease management and control where it would be possible to take preventive measures if disease can be detected sufficiently early, before the appearence of visible symptoms. This would lead to minimisation of economic loss due to desease.

Summary

(1) A review of literature pertaining to this investigation has been presented which deals mainly with serological cross reactivity between host and pathogen, detection of plant pathogenic fungi and serological cross reactivity among fungal species.

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

(3) Pathogenicity of *Glomerella cingulata* was tested on 37 varieties of tea (13 Tocklai varieties 15 Darjeeling varieties and 9 UPASI varieties). UPASI 9, TV-18 and B-157 were found to be the most susceptible while CP-1 and BT-15 were resistant.

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

(5) Maltose was the most effective carbon source and potassium nitrate the most effective nitrogen source for optimum growth of *G.cingulata.*

(6) Antisera were raised against antigen preparations from mycelia and cell wall of *G.cingulata* (isolate GC-1), tea leaves (TV-18 and CP-1) and *F.oxysporum* (non-pathogen of tea).

(7) In agar gal double difusion tests anti *G.cingulata* antiserum was cross reacted with leaf antigens of all 37 tea varieties, 3 non host species and a non pathogen of tea. Strong precipitin reaction occurred in homologous reactions and in reactions involving susceptible varieties while, no precipitation occurred with resistant varieties, non hosts or non pathogen.

(8) Reciprocal cross reaction was performed with antisera of TV-18 (susceptible variety) and CP-1 (resistant variety) and leaf antigens of host and non host as well as mycelial antigens of pathogen and non pathogen. Precipitation was observed in reactions of most of the tea leaf antigens. Antiserum of TV-18 reacted strongly with antigen of *G.cingulata* but antiserum of CP-1 did not. No cross reactions were observed with non host species or non pathogen.

(9) Immunoelectrophoretic tests revealed cross reactivity of anti *G.cingulata* antiserum with its isolates, and susceptible tea varieties. No precipitin arcs were discerned with resistant varieties, non hosts or non pathogen.

(10) Reciprocal reaction in immunoelectrophoresis also confirmed cross reactivity in susceptible reacftions but not in resistant reactions.

(11) Rocket immunoelectrophoresis using anti *G.cingulata* antiserum showed 3 immunoprecipitin lines in homologous reactions and a single line for the susceptible reaction.

(12) All the antisera raised were purified by ammonium sulphate precipitation and DEAE Sephadex chromatography. IgG, obtained in each case was used for ELISA tests.

(13) Optimum conditions for ELISA reactions were determined. An antiserum dilution of 1:250 and an enzyme (alkaline phosphatase) dilution of 1:10,000 were optimum for antisera raised against both mycelial and cell wall preparations of *G.cingulata* in homologous reactions.

(14) Both the antisera detected homologous antigens upto a concentration of 25 ng/ml .

(15) Comparison of ELISA reactivities of all the 37 varieties of tea with anti *G.cingulata* antiserum revealed highest absorbance in the variety UPASI-9 and lowest in BT-15.

(16) In reactions with antiserum raised against cell wall preparations also, maximum absorbance was obtained for UPASI 9 and minimum for BT-15. Absorbance values in this case were generally higher than for reactions with mycelial antiserum.

(17) All the isolates of *G.cingulata* tested showed high absorbance values with antiserum raised against one of the isolates (GC-1).

(18) Absorbance values for reactions with non hosts and non pathogens were low.

(19) Indirect ELISA reactivity of antigens, with anti *G.cingulata* antiserum (mycelia and cell wall) from tea leaves of different ages did not show any significant difference.

(20) Reciprocal cross reactions with antisera raised against TV-18 and CP-1 showed high absorbance values in susceptible reactions and low values in resistant reactions as well as reactions with non host and non pathogen.

(21) Antiserum raised against a non pathogen did not react with any of the antigens as evidenced by very low absorbance values in ELISA.

(22) Direct ELISA (DAS) of anti *G.cingulata* antiserum with antigens of different tea varieties, non hosts and non pathogen revealed maximum absorbance in susceptible reactions.

(23) *G.cingulata* was detected in artificially inoculated leaves of all tea varieties on the basis of significantly higher absorbance values in ELISA of inoculated extracts as compared to healthy extracts, using anti *G.cingulata* antiserum. Similarly, natural infection could also be detected in ELISA.

(24) *G.cingulata* was detected as early as 6h after inoculation in a susceptible variety (B 157) and after 30h in a resistant variety (BT-15) using anti *G.cingulata* antiserum in ELISA reactions.

(25) Detection was done after 6 and 18h of inoculation respectively in the susceptible and resistant varieties by ELISA using antiserum raised against cell wall preparation of *G.cingulata.*

(26) In ELISA, infection could be detected till a concentration of 1 μg/ml of infected leaf extract on the basis of significantly higher absorbance values of infected extracts as compared to healthy extracts.

(27) ELISA values extracts of tea leaves inoculated with *P.theae* or *C.invisum* were low when tested against anti *G.cintulata* antiserum.

(28) The occurrence of *G.cingulata* as the predominant pathogen in a mixed natural infection was established by competition ELISA using antisera of both *G.cingulata* and *P.theae.*

(29) Antiserum of *G.cingulata* (isolate GC-1) showed cross reactivity in ELISA with antigens of 8 other isolates but not with antigens of different species of *Colletotrichum, P.theae* or *C.invisum.*

(30) Estimation of fungal biomass of *G.cingulata* was done by ELISA from infected tissues after different hours of inoculation. On the basis of standard curve of mycelial fresh wt. versus absorbance in ELISA maximum mycelial fresh weight was obtained in the susceptible variety after 96h of inoculation. Of the different varieties tested, maximum growth was estimated to be in the susceptible varieties.

(31) Purification of antigenic protein of *G.cingulata* from the crude mycelial extract by ammonium sulphate saturation and DEAE Sephadex chromatography was done and detection was done by ELISA. SDS-PAGE electrophoresis of the purified protein revealed one band at approximately 10 KDa.

(32) Partially purified (80-100% SAS fraction) antigenic preparation was used for raising antiserum and this antiserum was tested by immunodiffusion tests, DAC ELISA and DAS ELISA with homologous and heterologous antigens. This antiserum also showed higher reactivity in the susceptible reactions as compared to resistant reaction.

(33) The antiserum raised to 80-100% SAS-fraction could also detect infections by ELISA.

(34) Characterization of the cell wall of *G.cingulata* by ConA-FITC binding and SDS-PAGE electrophoresis revealed the glycoprotein nature, with 2 bands of 50 KDa and 10 KDa molecular weights.

(35) Agglutination test of conidia of *G.cingulata* with different lectins revealed strong agglutination with ConA followed by UAE I. The presence of glycoconjugates containing glucose and/or mannose residues as well as L-fucose was confirmed on the outer surface of the conidial wall.

(36) Callus was induced from stem segments of TV-18 and from this callus loosened cells were obtained. Immunofluorescence studies of these cells with homologous antiserum or anti *G.cingulata* antiserum revealed bright fluorescence after treatment with FITC.

(37) Cross sections of tea leaves (TV-18) treated with homologous antiserum and then with FITC, developed a bright fluorescence throughout, which was concentrated mainly in epidermal cells and mesophyll tissues. Cross sections of the leaf exhibited autofluorescence also.

(38) Treatment of cross sections of tea leaf with anti *G.cingulata* antiserum and FITC revealed bright fluorescence mainly around epidermal cells and mesophyll tissues.

(39) Mycelia and conidia of *G.cingulata* when treated with homologous antiserum or anti TV-18 antiserum and then with FITC revealed bright fluorescence mainly on young hyphae and throughout the surface of conidia.

(40) The implications of the results have been discussed.

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