Immunodetection of *Fomes lamaoensis* (Murr.) Sacc. & Trott. in soil and tea root tissues and its management

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This is to certify that Mr.Debabrata Deb has carried out his research work under our supervision. His thesis entitled **"Immunodetection of *Fomes lamaoensis* (Murr.) Sacc. & Trott. in soil and tea root tissues and its management"** is based on his 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.

(Professor B.N.Chakraborty)

(Dr. Usha Chakraborty)

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Contents

INTRODUCTION

Tea [*Camellia sinensis* (L) O. Kuntze], which originated in China, boasts the world's longest history among beverages. Offering its own individual flavour as well as soothing and refreshing qualities, it is drunk all over the world. The tea industry has spread from China to the far corners of the world, currently encompassing countries ranging from 30° latitude in the southern hemisphere to 45° latitude in the northern hemisphere, and from 150° longitude in the east to 60° longitude in the west. In India, tea is one of the most important cultivated crops and is grown in two 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). Bramhaputra valley of Assam located 100m,above sea level is the largest flood plains of the world growing tea which accounts for more than half of Indian production. Darjeeling produces the world's finest quality teas in the steep slopes of Eastern Himalayas up to an elevation of 2000m.The extensive riverine flat plains at the base of Himalayas are the tea districts of the Terai and Dooars. Tea is also grown in the slopes of Nilgiris and Annamalai hills of peninsular South India.

The tea bush, like any other living plants is susceptible to attacks by pathogen, more so as it has been forced to grow, under varying climatic and soil conditions remote from its natural environment. For the benefit of mankind it has also been subjected to varied cultural treatment which are widely at variance with its natural conditions of growth. Diseases of the tea bushes and its ancillary crops may be caused by one or more of several agencies viz.,(a)vegetable organisms, such as fungi, algae and bacteria, (b) animal parasites, (c) adverse conditions of soil and climate, (d)mechanical damage and (e)virus.

Brown root rot disease of tea caused by *Fomes lamaoensis* (Murr.) Sacc. & Trott. is one of the primary root diseases found on all soils but more common on sandy soils. The pathogen attacks the tea plants from about 3years upward but younger plants may easily be attacked and killed if their roots happen to come in contact with diseased material in the soil. The above ground symptoms appear gradually, which includes

withering and bending of leaves and young branch-tips and losing of moisture content. Diseased plants die suddenly at advanced stages and their dried leaves remain attached for some time. Green colour of leaves changes gradually towards pale or ash green.

Roots of affected bushes are encrusted with soil, sand and stone particles held firmly by a brown mycelium which is difficult to remove by a good washing or rubbing and it sometimes forms blackish sheet on root surface. Brown or soft mycelium grows on collar region, extending on the main stem to a few inches. A thin film of white or brownish mycelium may develop on the surface of affected wood underneath the bark. In advance stages irregular rings and reticulations are formed by hard brown or blackish lines on/in the affected wood. Colour of wood is light yellow or brown and wood becomes soft. Sometimes the soft wood inside the brown rings decays and slices out, giving a honey-comb-like structure (Plate 1).

Plants respond to infection by pathogens in a number of ways, which are triggered by the initial recognition phenomenon. The initial recognition triggers the activation of immune systems of plants, which though different from that of animals, is functionally similar. Immune system in plants involves several mechanisms, some of which are known to us, but many of them are still unknown. An important area of immunological studies of disease 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 is gaining ground incase of fungal diseases (Chakraborty and Chakraborty, 2002). The recent diagnostic techniques for pathogen detection include enzyme linked immunosorbent assay, dot immunobinding assay, western blot analysis, 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. Such detection techniques makes it possible to detect microquantities of the pathogen within a few hours of infection, which is much more advantageous than the conventional techniques involving pathogen inoculation, visible symptoms and microscopy. These have tremendous potential for plant disease control measures since detection of a pathogen at the initial stages of infection can lead to

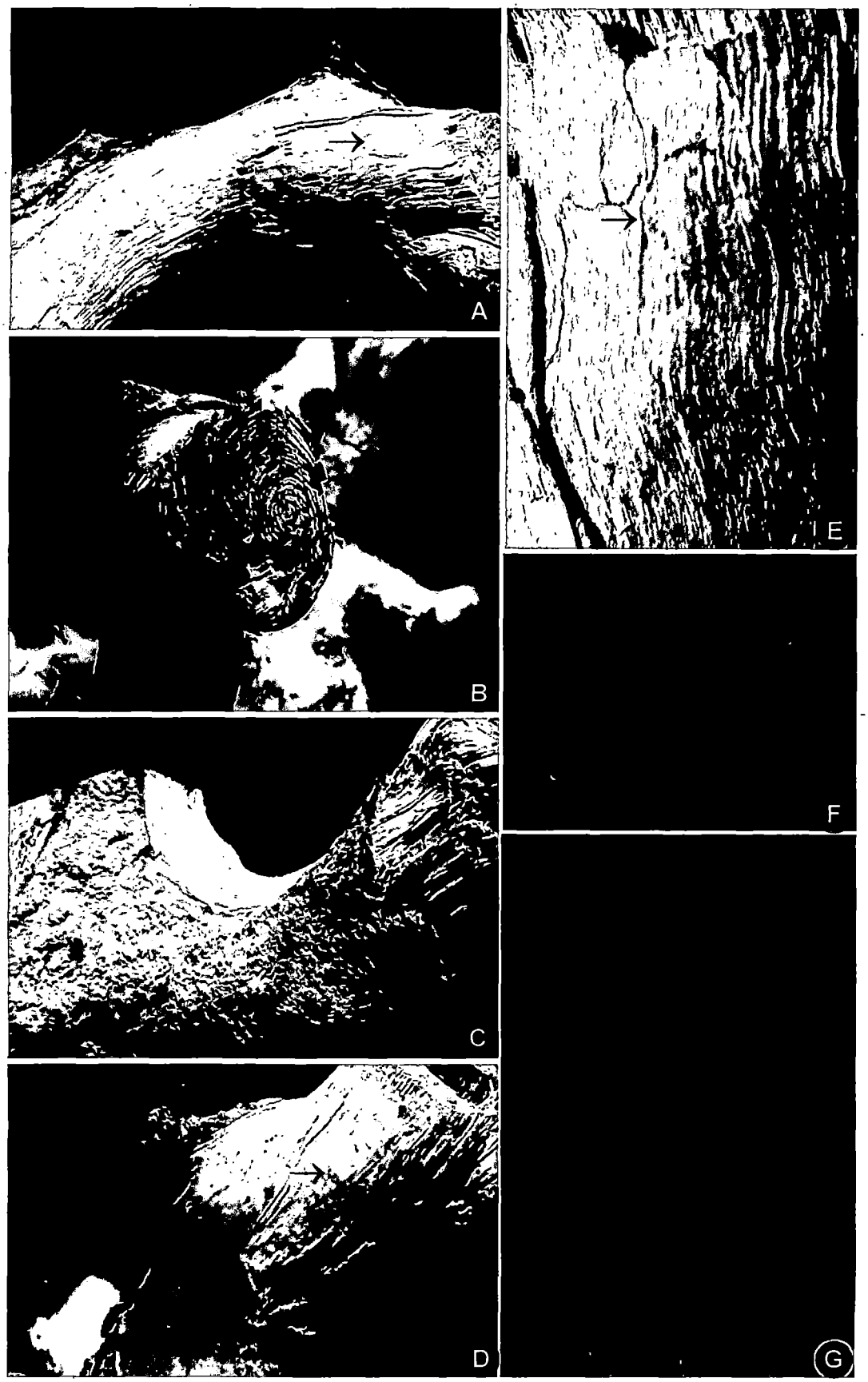


Plate 1 (A-G): Naturally infected tea root showing symptoms of brown root rot disease

formulation of control measures before much harm has been done. Taking advantage of the serological relationship between host and pathogen, the antiserum raised against the pathogen is being used for the detection of the pathogen in the host tissues beginning from the early stages of host pathogen interaction (Mohan, 1988; Linfield, 1993; Chakraborty *et.al.* 1995), as well as from soil (Wakeham and White, 1996).

Indian agriculture is now revolving under bio-prospective bioscience orbit. Biofertilizers, biopesticides, biological control and bioremediation are considered the four pillars of bioprospective bioscience. Adopting biological practices for a preventive approach of control diseases is at present on focus. So, for control of root diseases of tea, the most environmental friendly and effective one is the introduction of biocontrol agents in the soil among the current management practices and thus minimizing the use of fungicides.

Trichoderma spp. is a potential biocontrol agent for control of soil-borne diseases and is the common antagonist found in all the soils. The biological control of important root diseases of crop plants like tea and others like rice, millet, sunflower and maize can effectively be achieved using *Trichoderma* spp (Chakraborty et al.2002a). The efficacy of *Trichoderma* spp. as biocontrol agents of groundnut stem rot and root rot diseases was reported (Sreenivasaprasad and Manibhushanrao, 1993; Biswas and Sen,2000). Sunflower seed pathogen could also be controlled by *T. harzianum* (Janardhana, 1994).

For effective biological control, efficient and well-designed formulations are essential to reach the target of successful disease control. Agricultural wastes such as saw dust, tea wastes and wheat bran proved to be the best medium for sporulation and mass application. Sivan et al., (1984) found that a mixture of peat and wheat bran was a much better substrate.In another study, application of wheat bran saw dust (WBSD) preparation of *Trichoderma* spp. has been useful to control several soil-borne, root-infecting pathogens under field conditions (Baby and Manibhushanarao, 1993).

Considering the importance of tea as a plantation crop in this area, along with

all the above, the present study has been undertaken with the following objectives:

(a) Varietal resistance tests of tea clones against *Fomes lamaoensis* causing brown root-rot disease.

(b) Preparation of mycelium and cell wall antigens of *Fomes lamaoensis* and production of polyclonal antibody.

(c) Purification of antigens and antisera and analysis by immunoblotting.

(d) Detection of serological cross reactivity among tea roots and fungal isolates.

(e) Detection of *F. lamaoensis* in soil and tea root tissues using ELISA, immunofluorescence tests and immunocytochemical staining.

(f) Quantification of fungal biomass in tea root tissues and soil using various format of ELISA, Dot Blot and Western Blot.

(g) *In vitro* interaction studies with *Trichoderma harziamun*, *Trichoderma viride* and *F. lamaoensis.*

(h) *In vivo* test with *T. harzianum* and *T. viride* for management of brown root-rot disease.

The materials used and methods applied to achieve the above objectives have been outlined in the following pages along with the results achieved. In the beginning a review of the literature along lines pertaining to the present work has also been presented.

LITERATURE REVIEW

An advancing area in the study of host-pathogen interaction is the case of serological techniques to determine host pathogen interactions. With the introductio of ELISA for assaying plant viruses, serological methods are being used regularly in agricultural research an practice (Clark and Adams, 1977). Though, application of immunological technique for detection of fungi and bacteria came later then viruses, diagnostic kits are now available for detection of specific fungi in diseased tissues and soil. At present, Immunoassay are also being used for various other purposes such as ocalization of pathogens within tissues (Brlansky *et. al.,* 1982; Hansen and Wick, 1993; Werres and Steffens, 1994) identification of specific antigens in electrophoretically separated components (Townsend and Archer 1983, Wakeham and White, 1996), quantitation of defense related proteins such as PR-proteins (Antoniw *et. al.* 1985, Kemp *et. al.,* 1999).

A short comprehensive review on the various serological techniques used in detection of plant pathogenic fungi in different plant tissues and soil has been presented below.

A. Root tissue

Preparations of antigens from four varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species were compared for common antigens by Charudattan and DeVay (1972). At least one antigenic subtance was common among the varieties of cotton and isolates of *Fusarium oxysporum* f. sp. *vasinfectum, 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 non-pathogenic isolates of *F. oxysporum* f. sp. *vasinfectum* shared the common antigen. The common antigen was not shared between *F. moniliforme* (= non pathogenic) and cotton. In gel-diffusion tests, five to eight precipitin 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 does not appear related to the severity of wilt symptoms, but it may affect host-pathogen compatibility during the process of root infection. An antigen from conidia of *Fusarium oxysporum* f. sp. *vasinfectum* that cross-reacted with antiserum to cotton root tissue antigens was also purified and partially characterised by 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. Cross reactive antigen (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.

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

DeVay *et. al.* (1981) also reported that 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 epidermal 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 plasma lemma. Treatment of conidia and mycelia of *F. oxysporum* f. sp. *vasinfectam* 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) found serological relationship between *Macrophomina phaseolina* and soybean cultivars. Using antisera raised against antigens of *Macrophomina phaseolina* (isolate MP_1) and roots of soybean cultivars Soymax and UPSM-19 which were susceptible and resistant respectively to charcoal rot disease, agar-gel double diffusion test were carried out for the presence of common antigens between isolates of *M. phaseolina* and soybean cultivars. Immunoelectrophoretic tests revealed that four antigenic subtances were common between the susceptible soybean cultivars and isolates of *M. phaseolina* but no common antigens were detected between resistant cultivars and the fungus.

Pathogenicity of *Macrophomina phaseolina* was also tested on ten cultivars of soybean by Purkayastha and Chakraborty (1983). Soymax and UPSM-19 were most susceptible and resistance cultivars, respectively. Infected resistant cultivars produced significantly more glyceollin (phytoalexin) than the infected susceptible ones. Agar-gel double diffusion tests exhibited common antigenic relations between susceptible soybean plants and *M. phaseolina.* Immunoelectrophoretic studies revealed that four common antigenic substances were present between pathogen and susceptible cultivars, whereas no common antigenic substance was observed between resistant cvs and the pathogen (three isolates tested).

Dewey *et. al.* (1984) reported that in liquid culture, the fungus *Phaeolus schweinitzii,* which causes a root-and butt-rot of conifers, secretes a number of species-specific and strain-specific polypeptides which are detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. Indirect immunofluorescence microscopy was used to detect the binding of species-specific antisera to these antigens to extracellular macromolecules secreted by the fungus, to the cell surface of basidiospores and chlamydospores, and to the cell surface and cross walls of mycelia. Common antigenic determinants were present in extracullular culture filtrate material and walls of mycelia, chlamydospores and basidiospores. Indirect immunofluorescence, performed by using antisera to culture filtrate molecules has been used to demonstrate the presence of mycelium, and on occasions chlamydospores, in naturally and artificially infested soil samples. Authors suggested that this method could be used for identification of the kind of propagule most likely to be the source of field isolates of the organisms; this information, which cannot be obtained by using selective media, strongly suggests that the pathogen can survive saprophytically in the soil. In contrast, isolated mycelial cell wall preparation did not prove to be a suitable source of immunogenic material for these studies.

The presence of *Ganoderma lucidum* in roots of betelnut is detectable by the induction of fluorescent antibodies. This provides an interesting practical application of the technique which is developed by Koti Reddy & Ananthanarayanan (1984).

Sodium azide was found to be most effective of the six metabolic inhibitors tested in reducing charcoal rot disease of soybean (cv. soymax) caused by *Macrophomina*

phaseolina. Glyceollin production also increased significantly after induction of resistance by sodium azide treatment. Cross-reactive antigens were detected in purified preparations from mycelia of *M. phaseolina* with antisera of soybean roots by immunodiffusion and immunoelectrophoretic tests. An antigenic disparity was noticed in the susceptible cultivar (cv. soymax) after chemical induction of resistance (Chakraborty and Purkaystha, 1987). Purkayastha and Ghosh (1987) also prepared the antigens from two isolates of *Macrophomina phaseolina* (Tassi) Gold, a pathogen of groundnut, four non-pathogens of groundnut (viz. *Corticium sasakii* Shirai (Matsumoto), *Colletrichum lindemuthianum, C. corchori* (Pagvi and Singh), and *Botrytis ailii*), and five cultivars of *Arachis hypogea* L. These were compared by immunodiffusion, immunoelectrophoretic, and crossed-immunoelectrophoretic techniques for the presence of cross-reactive antigens. Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M. phaseolina,* but not between non pathogens and groundnut cultivars. No antigenic similarity was found between non-pathogens and *M. phaseolina* isolates. Crossed-immunoelectrophoretic tests confirmed that at least one antigen was common between cv. J-II and cv. TMV-2, cv. Kadiri 71-1 and cv. TMV-2 and cv. Kadiri 71-1 and isolates of *M. phaseolina.*

Working with another pathogen of cotton, Gerik and Huisman (1988) studied colonization of field-grown cotton roots by *Verticillium dahliae* by using a specific immunoenzymatic staining technique. Colony density of *Verticillium 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 7.3mm., 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 stcle. Hyphae of *V. dahliae* also were found with in numerous cortical cells. Colonies of *Fusarium oxysporum,* similarly stained, were found to be mostly confined to the root surface and the outer cortex.

Antiserum was raised against pooled mycelial suspensions from five isolates (designated Pf1, Pf2, Pf3, Pf10 and Pf11) representing five physiologic races of

Phytophthora fragariae (Mohan, 1988). In enzyme-linked immunosorbent assay (ELISA), this antiserum detected homologous soluble antigens at protein concentrations as low as 2ng/ml. 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. ananssa* 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, which is resistant to Pf1, 2 and 3 but susceptible to Pf10 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. Cultivars Hapil, Ostara and Providence were found to be susceptible to all isolates in this study : the corresponding root extracts were also positive in ELISA. The antiserum also detected *P. cactorum* infections. Nevertheless, the authors concluded that the ELISA test described should prove valuable in screening certified strawberry stocks.

Yuen *et. al.* (1988) developed a double antibody sandwich indirect enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of *Pythium ultimum.* A polyclonal antibody produced to cell walls of *P. ultimum* was used as the capture antibody, while a *P. ultimum* specific monoclonal antibody (MAbE5) was used for recognition of the fungus. In the ELISA, culture extracts of 7 isolates of *P. ultimum* exhibited strong positive reactions, where as none of the 37 isolates of other *Pythium* spp. and fungal genera had positive reactions. *P. ultimum* was detected by ELISA in roots of bean, cabbage, and sugar beet seedlings grown in pathogen infested soil. ELISA optical density readings for infected bean and sugar beet root samples were highly correlated ($r>0.9$) with infection levels determined by culturing the samples on water agar. The correlation between the two methods of testing cabbage roots was low, but all cabbage roots in which *P. ultimum* was detected by culturing were strongly positive in the ELISA. Samples of roots infected with *P. irregulare* and those with no *Pythium* infection did not react in the ELISA. The ELISA was highly sensitive; the fungus was detected in culture extracts diluted 1:5,000,000 and in roots with less than 1 infection per 100 cm root.

An antiserum was made against *Plasmodiophora brassiceae* the causal agent club of root of cabbage. A semipurified suspension of spores of *P. brassiceae* was used as antigen, obtained by filtration and percoll gradient contrifugation of infected roots. Crude root suspension samples were bound to a nitrocellulose nembrane and tested by a dot immunobinding assay. The individual steps of the serological procedure were examined with a scanning electron microscope. The surface of the resting spores of *P. brassiceae,* 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. Normal serum did not give rise to a coating on the resting spores. The antiserum of *P. brassiccae* did not react with surface antigens of resting spores of *Polymyxa.* Further, no cross reaction with other common root pathogens such as *Pythium ultimum, Rhizoctonia solami,* and *Fusarium oxysporum* was observed. With antiserum prepared spore surface antigenic determinants the dot immunobinding technique can be used as routine test for detection of infection of *P. brassiceae* in host plant and in bait plants (used as indicators of soil infestation). The sensitivity obtained was within the range permisssible for a routine test (Lange *et. al.* 1989).

Two commercial serological assay Kits were compared to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from inoculated azaleas (Benson, 1991). Both the multiwell E Kit and the rapid assay Kit detected *P. cinnamomi* on azalea roots beginning 1 wk after inoculation. Agreement between immunoassay Kits and culture plate results for detection of *P. cinnamomi* was most 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 greenhouse trials and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immuno assay results. Although color reactions in the rapid assay detectors became increasingly darker after completion of the test, results after 5min were as reliable as those after 60 min since readings for uninoculated controls used to determine test thresholds also increased with time. 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 at 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.

Naphthazarin toxins of *Fusarium solani* were detected by Nemec *et. al.* (1991) using competitive ELISA analysis in xylem fluid of roots rotted by *F. solani* and symptomless scaffold roots and branches of healthy-apearing and diseased citrus trees in ridge and flatwoods Florida groves. Studies concentrated on blight, a wilt disease with an undefined cause and etiology, to determine if *F. soloni* is a causal factor of the disease. Healthy-appearing roots of trees with blight symptoms in six groves contained upto 11.4 times more toxin than roots of healthy trees in the some groves. In blight-diseased trees from these groves, mediam toxin values per root and the percentage of roots positive for toxin were higher than for healthy trees. Rotted roots from blight-diseased trees in two groves contained 112 and 3.4 times more toxin than healthy appearing roots from the diseased trees. In two groves, one containing tristeza-diseased trees and the other foot rot diseased trees, toxin concentrations were greater in diseased compared with healthy trees only in the foot rot site. Toxin concentrations were not different in healthy appearing roots of healthy tangerine and sweet orange trees on *Citrus limon* 'Milam' in adjacent groves in a burrowing nematode site. Significantly more toxin was present in branches of blight than in healthy trees in two of three groves. In fibrous roots infected by *F. solani,* immunocytochemical localization of naphthazarins was present in fungal cell walls and associated electron-dense substances on the outer surface of the hyphae. In the fungal cytoplasm, the toxin was localized in nonmembrane-bound electron-lucent areas. The presence of naphthazarin toxins in blight-diseased trees as well as those with other disease suggests the nonspecificity of *F. solani* pathogenic activity on various rootstocks. Therefore, in situ toxin concentrations high enough to trigger pathogenic effects in susceptible rootstocks may be required to cause blight.

Field isolates of *Thielaviopsis basicola,* the causal agent of black root rot of cotton (*Gossypium hirsutum*), were grown in Czapek-Dox broth amended with dialyzed carrot extract. Soluble protein extracts of chlamydospores and mycelium were used to raise polyclonal mouse as cites antibodies. The immunoglubulin G antibody fraction was purified and biotin-labelled to devise a fungal capture sandwich enzyme-linked immunosorbent

assay (ELISA). ELISA detected both brown and grey cultural types of *T. basicola* and had negligible cross-reactivity with other soil brone fungi commonly found in the San Joaquin valley of California cotton field soils. The minimum detection limit of ELISA was between 1 and 20ng of *T. basicola* protein depending on the assay. *T. basicola* could be detected in cotton roots 2 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).

Purkayastha and Pradhan (1994) further studied serological differences among three strains (266, 23,M) of *Sclerotium rolfsi* along with their pathogenecities and antigenic relationship with five groundnut cultivars. Changes in antigenic patterns of one of its cvs. AK-12-24 and disease intensity were noted after treatment with a systematic fungicide Kitazin. Results reveal that significant variation exists among the strains of the pathogen. The strain 266 was most virulent having antigenic relationship with susceptible cvs. (Gangapuri, J-11 and AK-12-24) of groundnut. The strain 23 also exhibited common antigenic relationship with cv. AK-12-24. Resistant cvs. JL-24 and ICGS-26, however, showed no antigenic relationship with fungal strains in either immunodiffusion or immunoelectrophoretic test. A systemic fungicide Kitazin EC 48% (500μg ml^{-1}) altered the antigenic pattern of roots of a susceptible cv. AK-12-24 and also reduced discase markedly.

Polyclonal antibodies were prepared against crude cell wall fractions of *Pythium* isolate with filamentous non-inflated sporangia, associated with root rot of tomato in soilless cultures. In ELIA tests, the antiserum showed sensitivity and specificity against original antigens and those from closely related isolates with filamentous sporangia (such as *Pythium aquatile* and *Pythium coloratum*). The antiserum reacted weakly with mycelial preparations of several fungi commonly found on tomato roots such as *Fusarium* spp. or *Penicillium* spp. An immunocytochemical staining test was developed in order to study the colonization by filamentous noninflated sporangial isolates of *Pythium* in the presence of other fungi in soilless cultures. (Rafin *et. al.*, 1994).

Pathogencity test of *Fusarium oxysporum* on ten cultivars of soyabean revealed soymax and Punjab-1 to be most resistant while JS-2 and UPSM-19 were most susceptible

(Chakraborty *et. al.* 1997). Antigens were prepared from the roots of all ten varieties of soybean and the mycelium of *F. oxysporum.* Polyclonal antisera were raised against mycelial suspension of *F. oxysporum* and the root antigen of the susceptible cultivar UPSM-19, cross reactive antigens shared by the host and the pathogen were detected first by immunodiffusion. The immunoglobulin fraction of the antiserum was purified by ammonium sulphate precipitation and DEAE-sephadex column chromatography. The immunoglubulin fractions were used for detection of cross reactive antigens by enzyme linked immunosorbent assay. In enzyme-linked immunosorbent assay, antigens of susceptible cultivars showed higher absorbance when tested against the purified anti *F. oxysporum* antiserum. Antiserum produced against UPSM-19 showed cross reactivity with the antigens of other cultivars. Indirect staining of antibodies using fluorescein isothiocyanate indicated that in cross-sections of roots of susceptible cultivar (UPSM-19) cross-reactive antigens were concentrated around xylem elements, endodermis and epidermal cells, while in the resistant variety, fluorescence was concentrated mainly around epidermal cells and distributed in the cortical tissues. CRAs were also present in microconidia and chlamydospores of the fungus.

Srivastava and Arora (1997) detected and quantified *Macrophomina phaseolina,* causal agent of charcoal / dry root rot disease in many crop plants using the ELISA serological technique. Polyclonal antisera raised against soluble extracts of mycelium, the residual water insoluble mycelial materials or ribosomal proteins were evaluated for specificity and cross-reactivity with 16 common soil fungi by ODD and DAS-ELISA. Soluble and cell wall antisera exhibited strong cross reactivity with most of the fungal isolates. Ribosomal antibodies were less reactive to common soil fungi except *Fusarium oxysporum* f. sp. *ciceri.* Mycelial antigens of *M. phaseolina* on chickpea roots were detectable with DAS-ELISA at a minimum concentration of 10ng g^{-1} at 1:100 root: buffer dilution. Quantitative estimation of *M. phaseolina* on roots was evaluated by ELISA under different temperatures and moisture conditions, and in soil amended with a patential antagonist (*Trichoderma harzianum* 25-92). A significant reduction in ELISA values was observed in *T. harzianum* amended treatments. This method may be useful for detection and rapid screening of *M. phaseoline* under different environmental conditions.

Polyclonal antibodies (PAbs) were raised in white male rabbits immunizing separately with mycelia and cell wall antigens prepared from ten day old culture of *Ustulina zonata*

and emulsified with complete / incomplete adjuvants (Chakraborty *et. al.* 2001). Five blood samples were collected by marginal ear vein puncture from rabbits and IgG were purified DEAE-cellulose. The effectiveness of antigen preparations for raising Pabs were checked following immunodiffision test. Optimization of Pabs were done using indirect enzyme linked immunosorbent assay (ELISA). Two different ELISA formats such as direct antigen coated (DAC) and double antibody sandwich (DAS) were tested to detect the pathogen in soil and artificially inoculated tea root tissues. Indirect immunofluorescence using PAbs and FITC-conjugates of goat species for rabbit globulin were assessed for their potential to detect mycelia and spores in soil. Of the assays tested indirect immunofluorescence appears to be most rapid assay for detection of pathogen in soil. The authors suggested that such immunological methods developed for early detection of *Ustulina zonata* from soil and tea root tissue will be useful for the proper management of this disease.

B. Root and Leaf

Alba *et. al.* (1983) detected common antigens in extracts of urediniospores of *Hemileia vestatrix* and in leaf and root extracts of coffee plant prepared by different methods. An antigenic disparity was observed between coffee plants of physiologic groups D and E. The occurrrence of common antigens in coffee plants and urediniospores of *H. vestatrix* and their possible involvement in such interaction are diseased.

A commercial *Rhizoctonia* specific ELISA was compared with isolation on antibiotic amended water agar for the detection of *Rhizoctonia solani* and other *Rhizoctonia* spp. in asymptomic creeping bent grass near active brown patch disease centres. A greater number of positive samples were indicated by ELISA than by isolation in tests of foliage and root tissues. Over 75% agreement was found between ELISA and isolation with foliage, but the presence of *Rhizoctonia* spp. in root tissues could not be confirmed by isolation from any of the ELISA positive samples. There was poor agreement between the two methods in assays of organic matter from thatch. Greater numbers of thatch samples were positive for *Rhizoctonia* spp. by isolation than by ELISA. Pathogenic *R. solani* was found by both methods in asymptomatic foliage as far as 30 cm from the margins of brown

patch disease areas. There was a more restricted distribution of the fungus in the thatch. Detection of the fungus in root tissues from asymptomatic areas by ELISA could not be confirmed by isolation (Yuen *et. al.* 1984).

C. Stem tissue

Aguelon and Dunez (1984) developed an immuno enzymatic technique for the detection of *Phoma exigua* in infected potato tissues. Antisera to *Phoma exigua* var. *foveata* and var. *exigua* were prepared by injecting rabbits and mice with protein solutions from mycelium. Specific antibodies were isolated and immunoenzymatic techniques (double antibody sandwich ELISA and indirect ELISA) were used to test for the fungus in inoculated tubers and sports 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 sepcific 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 as cite liquid suggest the possibility of producing specific monoclonal antibodies.

Polyclonal antiserum was elicited against a strain of *Fusarium oxysporum* f.sp. *narcissi* (GCRI 80/26) and a specific and sensitive enzyme-linked immunosorbent assay developed by Linfield (1993). Antiserum raised to cell wall fractions give 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. oxysporium* 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 the pathogen on selective medium.

D. Leaf tissues

Purkayastha and Ghosal (1985) analysed the cross-reactive antigns of *Acrocylindrium oryzae* and rice in relation to sheath rot disease. Immunodiffusion, immunoelectrophoretic and crossed immunoelectrophoretic analysis of rice antigens in relation to sheath rot disease revealed a serological relationship between susceptible rice cultvars and isolates of the causal organism of sheath rot, *Acrocylindrium oryzae.* One precipitin 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. Mashuri and antigen preparations from three isolates of *A. oryzae* or between the antigens of the resistance cultivars Mashuri and Rupsail and the antiserum of *A. oryzae.* Crossed-immunoelectrophoretic tests confirmed that three was a common antigen between Mashuri and Jaya, and between Mashuri and CR-126-42-1. The precipitin band between the antigen preparation of Jaya and *A. oryzae* was found to be similar.

Sections of leaves of *Nicotiana tabacum* L. infected with *Peronospora hyoscymi* De Bary f. sp. *tabacina* (Adam) Skalicky and *Erythronium americanum* Ker. infected with *Ustilago heufleri* Fuekel were trreated with an antiserum directed against the fimbriae of *U. violacea* Fuckel and other fungi (Day *et. al.* 1986). The sections were then treated with protein A-gold complex to detect the presence and location of fimbrial antigens following transmission or scanning electron microscopy. Control preparations involved sections of uninfected leaves, as well as a range of serological control treatments. The infected leaf sections were heavily labelled with gold particles indication the presence of fimbrial antigens, where as the control preparations showed only a low background level of labelling. Gold particles were detected on the section of hyphae, on haustoria, and on the nearby plant cells. The intensity of labelling was much higher for *P. hyocyami* 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 hosts, but little or none occured over the cells of uninfected host tissues, or of the infected host tissue treated with a range of serological controls. This high level of labelling was not associated with specific host structures in *P. hyoscyami,* but was frequently associated with the chloroplasts in *U. heufleri.* The antigens detected inside the host plant cells appear to indicate that fungal

fimbriae protein, either as polymerized fibrils or as isolated subunits, can penetrate the host plasma membrane and therefore enter the host cytoplasm. An alternative possibility is that these antigens derive from host produced proteins synthesized as a result of infection. These results suggested the possibility that fungal fimbriae may play an important role in the molecular interaction between pathogen and host.

Though lesion formation by spore of *Botrytis cinerea* on *Vicia faba* occurred within 15h after inoculation, Gardiner *et. al.* (1989) reported that simultaneous application of various antisera delayed formation until up to 65h after inoculation. As the dilution of antiserum was increased, the length of the inhibitory period decreased and the fraction of the leaf surface that eventually became covered in lesion increased. Antisera of different antigen-binding specificity or different class of heavy chain (IgA, IgG, IgM) inhibited lesion formation, though IgM was the most effective. Antisera purified from other serum proteins were effective, but serum proteins such as albumins were not effective in causing inhibition of lesion formation. $F(ab^1)_2$ fragments of IgG molecules were as effective as the complete IgG molecule while Fc and light chain fragments were ineffective. Germination of *B. cinerea* spores on glass slides was inhibited by the same range of immunoglubulin molecules and fragments. The authors concluded that some constant portion of the antigen binding region of immunoglubulins inhibits lesion formation by inhibiting spore germination.

Ghosh and Purkayastha (1990) analysed the host parasite cross-reactive antigens in relation to *Myrothecium* infection of soybeam. For this purpose, antigens were extracted from two resistant, three susceptible soybean cultivars and three strains of *M. roridum,* a causal organism of leaf spot disease. Rabbit antisera were also raised against all three strains (M-1, ITCC-1143, ITCC-1409) of *M. roridum,* two susceptible cultivars (DS-74-24-2 and PK-327) and a resistant cultivar (UPSM-19) of soybean for analysis of cross reactive antigens. Results of immunodiffusion revealed that common antigens were present only between the virulent strain and susceptible host cultivars. But no cross reactive antigen was detected in case of resistant cultivars (UPSM-19 and DS-73-16). Immunoelectrophoretic analysis showed that one common antigen was shared by susceptible hosts and the virulent strain (M-1). This was further confirmed by both crossed and rocket-immunoelectrophoresis.

A polyclonal antiserum prepared in a rabbit immunized with a mycelium extract of *Phytophathora 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).

In a further study Purkayastha & Banerjee (1990) used six antibiotics as foliar spray on a susceptible soybean cultivar (Soymax) to induce resistance against anthracnose. In addition, common antigenic relationship between seven soybean cultivars, their pathogens and non-pathogen were also studied using immunodiffusion, immunoelectrophoresis and indirect ELISA technique. Among the six antibiotics tested, cloxacillin and Penicillin induced maximum resistance against anthraenose. Cloxacillin (100µg/ml) but not penicillin also altered the antigenic pattern of treated leaves. Cross reactive antigens were detected between susceptible soybean cultivars and the virulent strain of *Colletotrichum dematium* var. *truncata* but no cross reactive antigens were detected between soybean cultivars and an avirulent pathogen (*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 two at a very low level.

An immunocytochemical stain was developed to detect the basidiomycete *Athelia bombacina* in apple leaf litter. The polyclonal antibodies for *A. bombacina* were sufficiently specific that only hyphae of this fungus were detected in immunocytochemically treated sections of dead leaves inoculated with *A. bombacina.* Apple leaves naturally infected with *Venturia inaequalis* were inoculated with *A. bombabacina,* incubated outside from movement 1986 to may 1987, and sampled monthly. Sections stained immunocytochemically showed that *A. bombacina* grew endophytically and epiphytically. The antagonist prevented neither growth of hyphae of *V. inaequalis* into the interior of leaves, nor initiation of psuedothecia. There was no particular spatial association between hyphae of the two

fungi, nor any sign of direct parasitism of hyphae or pseudothecia of *V. inaequalis.* Pseudothecia in leaves with the antagonist did not mature further than the stage of producing pseudoparaphyses, reaching an average of 84 μm in length. Pscudothecia in leaves without *A. bomabacina* developed asci normally and were 108μm long by 1may. These data were confirmed by results from an abbreviated sampling scheme of McIntosh apple leaves during 1987-1988 and by observations of pscudothecial inhibition in crab apple leaves following delayed application of the antagonist (Young and Andrews, 1990).

Ribosomal protein differences among snow mold fungi and wheat were utilized to detect the causal fungi in infected wheat leaves by indirect enzyme linked immunosorbent assay (ELISA) and western blot analysis (Takenaka, 1992). Polyclonal antisera were raised against the ribosomes of *Pythium paddicum, P. iwayamai, Typhula incarnata* and *Microdochium nivale,* and tested for sensitivity and specificity with ribosomes of these four pathogens, *Typhula ishikariensis,* and wheat. Using polyclonal antiserums, ribosomes of *P. paddicum* and *P. iwayamai* were serologically identical; the ribosomes of *T. inacarnata* and *T. ishikariensis* had partial common antigenic determinants, but there were apperent serological differences among *Pythium* spp. *Typhula* spp., *M. nivale* and wheat. With indirect ELISA, ribosomes of *Pythium* spp. were detectable at a concentration of 69 ng/ml and ribosomes of *T. incarnata* and *M. nivale* at a concentration of 210 ng/ml. The ribosomes of each target pathogen were detected from completely rotted wheat leaf homogenates diluted up to 1:1,000 or 1:10,000. The indirect ELISA could not differentiate, *P. paddicum* from *P. iwayamai*-infected plants or *T. incarnata* from *T. ishikariensis*-infected plants but could detect and differentiate snow mold fungi at the genus level in wheat plants. Western blot analysis with these antisera also could not differentiate *P. paddicam* from *P. iwayamai* but could differentiate wheat leaves infected with *T. incarnata* from those infected with *T. ishikariensis.* These immunological methods with antiribosome serums would be useful to evaluate wheat plants for infection by *Pythium* spp. *T. incarnata, T. ishikariensis* and *M. nivale.*

A new monoclonal antibody (MAb) was raised against haustorial complexes isolated from pea leaves infected with the powdery mildew fungus *Erysiphe pisi* by Mackie *et. al.* (1993). The antibody disignated UB10 recognises a 45 kDa N-linked glycoprotein which

is specially located in the haustorial plasmamembrane. This glycoprotein is clearly distinct from a previously characterized 62 kDa glycoprotein (identified with MAb UBB) which is alos specially located in the houstorial plasma membrane. These antibodies were used, along with MAb UB7 which binds to a major 62kDa glycoprotein in the cell wall plasma membrane of both haustoria and surface hyphae, to label haustoria with in epidermal strips from infected pea leaves using indirect immunofluorescence. Different stages of haustorial development can be observed using this procedure and the results showed that all three glycoproteins recognised by the MAbs are expressed early in haustorial development.

Two new monoclonal antibodies (MAbs) were further described which recognise glycoprotein components of the extrahaustorial membrane (ehm) which surrounds the haustorium formed during the infection of pea leaves by the powdery mildew fungus *Erysiphe pisi* (Roberts *et. al.* 1993). One of the MAbs, UB9, raised against enriched pea leaf plasma membranes, recognises a large (200kDa) glycoprotein in this membrane with in infected and uninfected epidermal cells. It does not label haustoria at early stages of development showing that at least one plasma membrane glycoprotein is excluded from the elm. However, UB9 binds to the elm of a subpopulation (approx 20%) of HCs late in the infection process and it is posible that the insertion of this glycoprotein into the ehm may represent a breakdown of biotrophy during senescence of haustoria or could be a late defence response by plant. A second MAb, UB11, was raised against isolates haustorial complexes derived from the pea powdery mildew system. This antibody recognises a glycoprotein (250kDa) which is present in the ehm of the haustorial complex and is expressed very early in haustorial development. UB11 does not bind to plant membranes of infected or uninfected plants and doesnot cross-react with other fungal components. This antibody therefore indentifies a glycoprotein specifically located to the elm of haustorial complexes and shows that there is molecular differentiation of this key interface between the biotrophic partners.

Among the Tocklai varieties of tea tested against *Bipolaris carbonum,* TV-18 was found to be most susceptible and TV-26 most resistant under identical conditions. Antigens obtained from tea varieties, isolates of *B. carbonum* and nonpathogens of tea (*Bipolaris tetramera* and *Bipolaris setariae*), were compared by immunodiffusion,

immunoelectrophoriesis and enzyme-linked immunosorbent assay to detect the cross-reative 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), nonpathogens and tea varieties, as well as nonpathogens and isolates of *B. carbonum.* Indirect staining of antibodies using fluoresein isothiociyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and condia 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 condia (Chakraborty and Saha, 1994).

Chakraborty *et. al.* (1995) reported that 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 ten varieties, three isolates of *P. theae* and a non-pathogen of tea (*Bipolaris tetramera*). Polyclonal antisera were raisd 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 immunodiffusion 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* and resistant varieties, *B. tetramera* and tea varieties or 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.*

Polyclonal antiserum was raised against the mycelial extract of *Pestalotiopsis theae* and immunoglobulin fractions were purified by ammonium sulfate fractionation and chromatography on DEAE - Sephadex. In enzyme linked immunosorbent assay, antiserum dilution up to 1:16000 detected homologous antigen at a 5mg/L concentration, and at 1:125 antiserum dilution fungal antigens could be detected at a concentration as low as

25μg/L. In fifteen varieties of tea tested, originating from Darjeeling, UPASI and Tocklai breeding stations, absorbance values of infected leaf extracts were significantly higher than those healthy extracts at a concentration of 40mg/L. in indirect ELISA. ELISA positive material was detected in tea leaves as early as 12h after inoculation with *P. theae*. At antiserum dilution 1:125, the pathogen could be detected on inoculated leaf extracts upto antigen concentration of 2mg/L. The antiserum reacted with two other isolates of *P. thae* tested but not with the antigens from mycelial extracts of *Glomerella cingulata* and *Corticium invisum* or with extracts of tea leaves inoculated with pathogen. The results demonstrated that ELISA can be used for early detection of *P. theae* in leaf tissues even at a very low level of infection (Chakraborty *et. al.* 1996a).

Varietal screening of tea (*Camellia sinensis*) with *Glomerella cingulata,* causal agent of brown blight disease was conductd with sixteen varieties released by Tocklai Experimental station, Jorhat, Assam, following detached leaf, cut shoot and whole plant inoculation techniques (Chakraborty *et. al.* 1996b). Among the tested varieties, TV-18 and Teen Ali-17/1/54 were found highly susceptible, while CP-1 and TV-26 were resistant under identical conditions. Plant antigens were prepared from healthy leaves of five selected varieties and fungal antigens were prepared from three isolates of *G. cingulata* (GC-1, 2&3) as well as non - pathogens of tea (*Bipolaris tetramera* and *Fusarium graminearum*). Rabbit antisera were raised against leaf antigens of TV-18, Teen Ali 17/1/54 and CP-1 and fungal antigens of *G. cingulata* (isolate GC-1). These antisera were used in immunodiffusion and immunoelectrophoretic tests as well as in enzyme linked immunosorbent assay (ELISA) to detect cross reactive antigen (CRA) shared between tea varieties and isolates of *G. cingulata.* CRA were found among the susceptible varieties and *G. cingulata* isolates. Such antigens were not detected between *G. cingulata* and resistant varieties of tea, non-pathogens and tea varieties as well as *G. cingulata* and non-pathogens. Indirect staining of antibodies using fluoresein isothiocyanate (FITC) indicated that in cross section of tea leaves (TV-18), the CRA an concentrated in epidermal cell, mesophyll tissue and vascular elements.

The initial infection process involving adhesion / recognition events between plants and fungal pathogens is essential for the establishment of pathogenesis (Laxalt *et. al.* 1998).

The basis of the specificity between *Phytophthora infestans* and potato is not yet known. It could lie in the recognition between extracellular matrix and plasma membrane components of the penetrating structures of the fungus and host cells during the earliest stages of infection process. Author used polyclonal antibodies (AZ and AC) obtained after immunizing rabbits with either 10^7 zoospores (Z) or geminating cysts (C) of *P. infestans* to detect putative fungal components located on the surface of invasive structures. AZ recognized several bands, while AC recognized three polypeptides of approximately 87, 73 and 45 kDa in western blots. Zoospore suspensions preincubated either with AZ or AC were unable to infect potato leaves. Since it was already known that some anti-idiotypic (anti-IT) antibodies may resumble the antigen, thus mimicking its structure, anti-IT antibodies (a-AZ and a-Ac) were generated in mice and used as inducers on potato tuber discs. In northern blots a-AZ treatments increased 1.5-fold the glyceraldehyde-3-P dehydrogenase (GAPDH) and 1.8 fold the phenylalanine ammonia-lyase (PAL) transcript levels, while an increase of 1.8 and1.7 fold for GAPDH and PAL, respectively, was observed when the inducer was a-AC. Both PAL and GAPDH transcript levels have previously been shown to be modulated by fungal and elicitor treatments. These results show that the anti-idiotypic antibodies are able to mimic the pathogen components, leading to the accumulation of transcripts related to defence responses in potato *P. infestans* interactions.

Pathogenecity of *Glomerella cingulata* (Stoneman) Spauld and Schrenk, causal agent of brown blight disease, tested under identical conditions following detached leaf and cut shoot inoculation techniques against fourteen varieties of tea [*Camellia sinensis* (L.) O. Kuntze] revealed that CP-1 and TV-26 were resistant, while TV-18 and Teen Ali-17/1/54 were susceptible. Polyclonal antisera were raised separately against antigen preparations from mycelia and cell wall of *G. cingulata* (isolate GC-1), mycelia of *F. oxysporum* and leaf antigens of TV-18 and CP-1. Cross reactive antigen (CRA) were found among the susceptible varieties of tea and isolates of *G. cingulata* (GC-1, 2 and 3). Such antigens were not detected between raistant varieties of tea and isolates of *G. cingulata* (GC-1, GC-2 & GC-3); non-pathogen (*Fusarium oxysporum)* and tea varieties; isolates of *G. cingulata* and *F. oxysporum* and between non-host (*Glycine max, Cicer arientinum,* and *Camellia japonicum*) and *G. cingulata.* Antisera raised against cell

wall preparations gave better recognition from that against mycelial preparations as observed in ELISA test with antigens of tea leaves of different ages. (Chakraborty *et. al.* 2002).

E. Fruit

Antibody raised to the ribosomes of *Fusarium moniliforme* and corn (*Zea mays* L.) were shown to be specific by the ouchterlony double-diffusion test and sucrose density gradient analysis of the antigen-antigody 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. Marshall, and Partridge, (1981) identified immunochemically *Fusarium moniliforme* ribosomes from diseased corn (*Zea mays* L.) stalk tissue.

F. Seed

Distribution of the endophytic fungus, *Acremonium coenophialum,* with in the tissues of infected tall fescue (*Festuca arundinacea*) can be determined with tissue print-immunoblot (TPIB), however, the accuracy of this method in the determination of endophytic infestation levels of pastures and seed lots has not been evaluated (Gwinn *et. al.* 1991). The purpose of this study was to compare TPIB to other methods used for endophytic detection. Seed lots of known endophyte infestation levels were tested with TPIB. Percentage endophyte infestation did not differ significantly from the expected values. Also, no significant differences were found between results of protein A-sandwich ELISA and TPIB tests when both were used to estimate endophyte infestation levels in pastures. Therefore, accuracy of TPIB in comparable to other endophyte detection techniques and can be used for routine detection of endophyte in tall fescue tissues.

Immunodetection of teliospores of Karnal bunt (*Telletia indica*) of wheat using fluorescent staining Test was done by Gupta *et. al.* (2000). Polyclonal antibodies were raised against intact teliospores in Newzeland white rabbits for the immunodetection of Karnal bunt (KB) teliospores. The indirect immunofluorescence (IIF) test was developed using anti-teliosperes serum and binding was monitored by goat-rabbit antibody conjugated to fluorescein (FITC) label. The standarization of IIF test was carried out by optimization of dilutions of anti-teliospores antibodies, fluorescent probe and exposure time. The

teliospores of *T. indica* showed bright green, patchy and ring shaped fluorescence around the teliospore. The spore exhibited uniform distribution indiscrete regions of spore probably in spore episporium. Similar fluorescence pattern in the teliospores of KB isolated from infected wheat seeds of cultivars HD 2328, UP 2338, PBW 393, WH 542, as well as RR 21 (susceptible cultivars) respectively, is an indication of the presence of similar antigenic configuration of teliospores. Again, they did not exhibit variation in the expression of teliospore associated molecular pattern (TAMP) during previous and subsequent years of infection.

Polyclonal antiserum were produced in albine white rabbits against intact teliospores. The immunoprove was used for the development of Immunoblot binding assay for detective infections in wheat seed samples. The antiserum reacted strongly with intact teliospores of *T. indica,* pantnagar isolate in agglutination reaction. The wheat grains with different grades of infection could be readily detected by Seed Immunoblot Binding Assay (SIBA). The teliospores of Karnal bunt infected wheat seeds when kept for vigour testing on nitrocellulose paper, formed a coloured imprint after the paper was assayed. The SIBA developed should not only be a better indication of teliospores load on seed but also quality of seed in terms of vigour. The developed immunodetection method appearently proves to be usefull in routine monitoring of wheat lots for the presence of K.B. pathogen (Kumar *et. al.*, 2000).

G. Whole plant

Methods for sampling turf grass tissue were compared by Shane (1991) for their effectiveness in monitoring Pythium blight epidemics with enzyme linked immunosorbent assay (ELISA). Sample areas consisted of marked strips on golf course fairways and tees with bent grass and annual blue grass naturally infested by *Pythium aphanidermatum.* Samples consisted of (1) whole plants picked by hand and assayed as whole plants; (2) whole plant sectioned into lower, middle, upper strata components; and (3) leaf clipping collected with a reel mower set at a 1.2cm cutting height. ELISA readings for mowed samples generally matched those for whole-plucked samples (r^2 values ranging from 0.457 to 0.601). Fluctuation in detectable *Pythium* antigens were most pronounced on the uppermost stratum compared with moderate to very little change in ELISA reading 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 reading for *Pythium* coincided with, but did not generally precede, the on set of blight symptoms with a 2-to 3-days sampling interval. Antibody-aided detection in useful for verification of diagnosis and determination of general *Pythium* population fluctuations, but current methodology in not satisfactory for advanced detection of blight spidemics.

H. Soil

MacDonald & Duniway (1979) used the fluorescent antibody to study the survival of *Phytophthora megasperma* and *P. cinnamomi* zoospore in soil. Zoospore cysts of *Pythophthora megasperma* and *P. cinnamomi* were adjusted to nonsterile soil and its matric potential was adjusted to predetermined values. The infested soils were held under laboratory and field conditions and periodically sampled to determined cyst viability and soil water potential. During sampling, soil containing cysts was suspended in water and aliquots were placed on a selective agar medium to allow germination of viable cysts. After germination, the cysts were stained by an immunofluorescence detection technique and counted. Under laboratory conditions, zoospore cysts of *P. megasperma* survived no longer than 3wk at water ranging from 0 to –15 bars, and under field conditions they survived no longer than 2wk. Zoospore cysts of *P. cinnamomi* survived no more than 3wk in soil at water potentials of 0 to –1 bar under either field or laboratory conditions. However, at water potentials of –5 or –15 bars, zoospore cysts of *P. cinnamomi* survived upto to 6wk in the laboratory. In a nonirrigated field plot, in which soil water potentiols ranged from –7 to less than –100 bars, cysts of *P. cinnamomi* survived for 8-10 wk. Under more controlled humidity conditions, zoospore cysts of *P. cinamoni* proved relatively tolerant of desication; many survived for 1wk in soil that dried to water potentials as low as –75 bars. Compared to *P. cinnamomi,* zoospore cysts of *P. megasperma* were much more sensitive to desiccation; most were killed within 1wk in soil dried to water potentials of –20 bars or less.

Polyclonal antisera were raised to whole (Coded :16/2), and sonicated (Coded : 15/2) resting spores of *Plasmodiophora brassicae,* and soluble components prepared by

filtration and ultracentrifugation (Coded : SF/2) (Wakeham & White, 1996). Cross-reactivity of all three antisera with range of soil fungi, including western blotting, dipstick, 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 $1x10^7$ resting spores g^{-1} in soil. With western blotting the lower limit of detection with antiserum 15/12 was $1x10^5$. This antiserum showed the greatest sensitivity in a dip-stick assay, indirect ELISA and indirect immunoflurescence, for all of which there was a limit of detection of $1x10^2$. 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.*

It was reported by Walsh *et. al.* (1996) that a polyclonal antiserum produced against spore balls of *Spongospora subterranea* f. sp. *subterranea* prepared from potato tubers was able to detect as little as 0.02 spore balls in an enzyme-linked immunosorbent assay (ELISA). In spiked soil samples, the antiserum detected 100 spore balls per g soil. How ever, the different spore ball contamination levels were discriminated better in ELISA tests at concentrations above 2000 spore balls per g soil than at lower concentrations. In contrast, a bioassay test based baiting soils with tomato seedlings gave good discrimination of spore ball contamination levels in spiked soils containing <1000 spore balls per g soil and poor discrimination of levels inspired soils containing >2000 spore balls per soil. Tests on a limited number of field soils suggested, ELISA may be capable of predicting disease levels on tubers grown in such soil better than the biossay. The antiserum did not react with 30 other micro-organisms tested, including many that are saprophytes or pathogens on potatoes and resting spores of the taxonomically related *Plasmodiophora brassicae.* It detected spore balls of different cultivar origin eqully well. It also detected spores from different geographial origins. An attempt to improve the sensitivity of the serological detection through concentrating spore balls from field soils by sieving was unsuccessful. Cross-absorption of the antiserum with uncontaminated field soil increased the sensitivity of detection of spore balls in spiked soil samples four-fold. The ability of the antiserum to discriminate contaminated field soils from an uncontaminated soil was much improved by

using the gamma-globulin fraction or cross-absorbed screen. Western blot analysis revealed that the antiserum detected a number of different proteins the most distinct of which had a molecular weight of slightly less than 6.5 kDa. A technique was developed to suppress autoflurescence of spore balls, allowing immunofluoresence studies to be carried out. Using this technique in conjunction with indirect FITC immunofluoreserence, discrete bright fluorescent spots were visualized using the specific serum. With the non-specific serum, only a very dull background fluorescence was evident.

Population of *Phytophtora* spp. were determined by Miller *et. al.* (1997) using enzyme-linked immunosorbent assay (ELISA) in field soils used for pepper and soybean production in Ohio Soybean fields were sampled extensively (64 fields, n = 6 samples per field over 2 years) and intensively (4 fields, n = 64 samples per field in 1 year) to assess heterogenecity of *P. sojae* populations. Four pepper fields (n = 64), three of which had a history of *Phytophthora* blight caused by *P. capsici*, also were sampled intensively during a 6-month period. Mean (m), variance (v), and measures of aggregation (e.g., variance-to-mean ratio, v/m) of immunoassay values, translated to *Phytophthora* antigens units (PAU), were related to the disease history in each of the pepper and soybean fields. Mean PAU values for fields in which *Phytophthora* root rot (soybean) or blight pepper had been moderate to severe were higher than in fields in which disease incidence had been low or not observed. A detection threshold value of 11.3 PAU was calculated with values for 64 samples from one pepper field. All of which tested negetive for *Phytophthora* by bioassay and ELISA. Seven of the eight intensively sampled fields contained at least detectable *Phytophthora* propagules, with the percentage of positive samples ranging from 1.6 to 73.4. Mean PAU values ranged from 1 to 84 (extensive soybean field sampling), 6 to 24 (intensive soybean field sampling), and 4 to 30 (intensive pepper field sampling); however, various ranged from 0 to 7,744 (extensive sampling), 30 to 848 (intensive soybean field sampling) and 5 to 2, 401 (intensive pepper field sampling). Heterogenecity of PAU was high in most individual soybean and pepper fields, with values of v/m greater than 1, and log (v) increasing with log (m), with a slope of about 2.0. Spatial autocorrelation coefficients were not significant, indicating there was no relationship of PAU values in neighbouring sampling units (i.e., field locations) of the intensively sampled fields. Combined results for autocorrelations and v/m values indicate that *Phytophthora* was highly

aggregated in these fields but that the scale of the aggregation (e.g., average focus size) was less than the size of the sampling units. Because of the observed variability, authors calculated that sample sizes of 20 or more would be needed to estimate precisely the mean density of *Phytophthora* in most causes.

Soil borne disease caused by such pathogens as *Fusarium oxysporum* and *Plasmodiophora brassicae* are often difficult to be controlled by using soil fumigants and / or conventional fungicides (Aric-Tsutomu 1998). This is mostly due to the survival powers of the pathogens in soil and the mode of infection through the plant root system. For integrated pest management (IPM) method, researchers recently recommended the combined application of agrochemicals, resistant cultivars, biocontrol agents, and soil amendment, to guarantee plant health. Although importance of intial diagnostics for soilborne pathogens in field soil and young nursery seedlings should not be ignored to build an IPM program and obtain the expected results, no practical diagnostic methods, have been reported other than the isolation of the pathogens on selective media. Current immunology provides rapid, specific and sensitive tools for the detection of pathogens. In fact, various immunological procedures have been commonly applied to detect human pathogens and plant viruses. With some modifications / improvements, these procedures can be used to diagnose plant disease, caused by various pathogens.

I. Organism

The species of the genus *Phytophthora* was differentiated serologically by the means of gel diffusion and immunofluorescence (Burrell *et. al.* 1965). 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. cinnamomi,* and *P. erythrosepticca.*

Using the serological techniques of agglutination, gel diffusion, and immuno fluorescence, identification studies were performed by Amos & Burrell (1966) on eight species within the genus *Ceratocystis : C. fagacearum, C. pluriannulata, C. variospora, C. radicicola, C. pilifera, C. adiposa, C. ulmi,* and *C. ips.* All three serological techniques

proved to be applicable in the identification of these fungi. It was found necessary to adsorb selectively cross-reacting antibodies from the sera to make them species-specific. The immunofluorescent technique proved to be the most useful in differentiating among the species. Although all of the species could be shown to have serological differences, no such differences could be seen between A and B compatibility types of *C. fagacearum.*

Merz *et. al.* (1968) further reported that cell-free extracts of mycelium grown on a glucose-$(NH_4)_2 SO_4$– fumaric acid medium containing β-sitosterol were more effective antigens than previous preparations. The stability of the cell-free antigens was increased by the addition of ethylenediamine tetracetate to the extraction buffer. Improvements and refinements were also made with the serological techniques by defining more precisely the buffers and templates in immunodiffusion tests. These methods were used to study the taxonomic relations of six heterothallic species of *Phytophthora. Phytophthora cinnamomi* was serologically distinct, whereas two serological groups were evident among five closely related species when tested with antiserum to *P. arecae. Parecae, P. citrophthora,* 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 identical when tested with antisera to either *P. meadii or P. palmivora;* therefore, these five species might best be incorporated into one, *P. citrophthora.*

Immunological comparison of three strains of *Fusarium vasinfectum,* differing in their pathogenicity and a strain of *F. cubense* revealed close identity between pathogenic strains of *F. vasinfecum* (Kalyansundaram & Charudattan 1969). The three strains of *F. vasinfectum* had however, certain major antigenic constituents in common with *F. cubense.* According to Charudattan (1970) the pathogenic and non-pathogenic strains of *F. vasinfectum* can be differentiated by the production of the wilt toxin, fusaric acid. Higher toxin production was seen in a medium with nitrate as the source of nitrogen than with ammonical or organic sources of nitrogen. With peptone, there was maximum growth but minimum of toxin synthesis. No strict correlation was noted between growth and toxin production. The non-pathogenic strain (p_1) produced uniformly higher quantity of toxin than the two pahogenic strains (P_1 and A_1). No strict correlation was noted between the chemical presence of the toxin and its biological activity. The American pathogenic strain

A_1 produced maximum pectin methylesterase and very little polygalacturonase while the reverse was tree with the pathogenic Indian strain P_2. Perhaps, this quantitative difference accounts for the specificity of the two strains to produce disease in their respective hosts. Using agar double diffusion technique, a comparison of the antigenic components of the strain revealed a very close antigenic relationship between the two pathogenic strains P_2 and A_1 obtained from two different countries geographically much separated. Although, the pathogenic strains P_2 and A_1 possessed many antigens in common with those of non-pathogenic strain P_1, they both had a specific antigen which was lacking in P_1. However, the host specificity of the 2 pathogenic strains could not be explained by those findings. Non-pathogenic strains (P_1) not only lacked the above common antigen of P_2 and A_1, but also had its own specific antigen. Since this strain was pathogenic on original isolation, the present protein pattern might have evolved due to mutation arising out of prolonged maintenance in cultures.

A strain-specific antiserum was prepared by Horrok & Jagicza (1973) against the mature hyphae of *Fusarium culmorum* (W.G. Smith) Sacc. Both the direct and indirect methods of the fluorescent antibody technique resulted in a specific yellow-green immunofluorescence; however, indirect staining was always more intense. There was a significant difference in the intensity of fluorescence between the mycelia of 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* is suitable to separate these species from other fungi. According to the the authors morphological examination and culturing processes are not necessary because mycelia sufficient for fluorescent antibody staining usually grow out from infected plant on to a microscope slide within 48-72 hours.

The serological cross-reactivity of *Sporothrix schenckii* with vaious unrelated fungi was investigated by use of immunodiffusion tests. A rabbit anti *S. schenckii* serum was obtained, which reacted with *Cladosporium wereneckii, C. carrionii, C. bantianum, Coccidioides immitis, Phialophora jeanselmei, P. gougerotii, P. dermatitidis,*

Fonsecaea pedrosoi, Aspergillus fumigatus, Histoplasma capsulatum and *Trichophyton mentagrophytes,* but not with *Saccharomyces cerevisiae* antigens. The serological determinants responsible for the cross-reactions were suggested by Ishizaki *et. al.* (1981) to be D-galactosyl residues.

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

Nachmlas *et. al.* (1982) isolated a phytotoxin protein-lipopolysaccharide (PLP) complexe from dialysed culture fluids of a pathogenic strain of *Verticillium dahliae;* PLP complexes which lack phytotoxic activity were isolated from a mutant non-pathogenic strain of the fungus. A comparison of profiles of the wild-type and mutant PLP eluting from an Agarose A-5m Column revealed quantitative differences between the 2 major protein peaks of each strain. The corresponding peaks of the 2 strains had similar molecular weights and gel imunodiffusion were antigenically indistinguishable, but differences between them were seen in their chemical composition when analysed for protein, lipid and carbohydrate content. Antiserum prepared against the components of peak 1 from the pathogenic strain reacted with an antigen in extract of *Verticillium*-infected potato plant tissue which was apparently indentical to a moiety produced by the pathogen in culture.

Cross-reactive antigens were detected by Alba & Devay (1985) 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. Results 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 are thermolabile and that they can be concentrated by precipitation in the presence of 40% saturated ammonium sulphate (SAS). The results also revealed an antigenic disparity when 40% SAS from *P. infestans* Race 4. mycelial preparation was assayed with antisera for King Edward and Pentland Dell.

The adaptation of enzyme-linked immunosorbent assay for the detection of *Sclerotiania sclerotiorum* from both artificially and naturally infected host plants was described by Walcz *et. al.* (1985). The ELISA technique showed good specificity and sensitivity in detecting '*Sclerotinia* antigen' at a concentration as low as 10 ng ml^{-1}.

Cross-reactivity between antisera produced against fimbriae of either *Ustilago violacea* (Pers.) (AU) or of *Rhodotorula rubra* (Demme) Lodder (AR) and cell surface proteins of two ascomycete fungi, *Ascocalyx abietina* (Lagerberg.) Schlaepfer-Bernhard and *Ophiostoma ulmi (Buism)* Nannf., was revealed by dot-immunobinding and immunocytochemical methods. Following treatment with antiserum AR, the walls, septa, and plasma membrane of *A. abietina* and *O. ulmi* cells (the latter either in culture or grown in elm wood sections) were appreciably labeled by gold particles, but the labeling 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 labeling with this antiserum occurred over cells of *O. ulmi* indicating that they either lacked these antigens or that they were more easily removed during the fixation process (Benhamou *et. al.* 1986).

The agglutination effect of five lectins having different sugar-binding specifities on the conidia of a variety of strains from eight *Fusarium* species was examined by Cristinzio *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-accyl-D-galactosamine inhibited the agglutination induced by concavalin A and *Helix pomatia* agglutinin, respectively. Condia of the other species of *Fusarium* examined (*F. culmorum, F. sambucinum, F. graminearum, F. avenaeum, F. moniliforme* and *F. xylarioides*) were not agglutinated by either lectin. No conidia form the *Fusarium* species were agglutinated by wheat-germ agglutinin, potato lectin and *Ulex europaeus* agglutinin-I. These results have provided some insight into the chemical nature of the outermost layer of *Fusarium* conidia and this information may be of taxonomic significance.

Fuhrmann *et. al.* (1989) immunologically differentiated the *Penicillium* species. Anisera were obtained from a rabbit immunized with *Penicillium verrucosum* var. *verrucosum*. These antisera were characterized by immunofluorescence and by indirect

enzyme-linked immunosorbent assay for their reactivity with 44 strains of moulds. Antigenically, *P. verrucosum* var. *verrucosum* (subgenus *Penicillium)* appears 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.*

Competitive types of two novel enzyme-linked immunosorbent assays (ELISA) for *Fusarium* species were developed. Antiserum against strain F 504 of *F. oxysporum* was elicited in rabbits, and a highly specific, sensitive and accurate ELISA for the homologous strain was developed by using the antiserum with β-D-galactosidase-labeled anti-rabbit IgG as the secondary antibody and cell fragments of the strain attached to Amino-Dylark balls as the solid phase antigen. All other microorganisms tested, including nine other strains of *Fusarium,* showed little cross reactivity, when cell fragments of *F. oxysporum* F 501 attached to the balls were used as a solid-phase antigen in a heterologous competitive ELISA. The modified system was a general assay for 10 strains of four *Fusarium* species with a high sensitivity. The specificity of the heterologous competitive ELISA was shown to be limited to *Fusarium* species (Kitagawa *et. al.* 1989).

Soluble proteins from 34 isolates representing 16 species of the genus *Phytophthora,* collected from different geographic locations and host plants, when analysed by two dimensional electrophoresis, were resolved into 100 to 200 protein spots with diferent densities. 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 highy similar, if not identical, among isolates of the same species regardless of their geographic origin, original host plant, mating type, or virulence race. These reslts suggested that the two-dimensional electrophoretic patterns are species-specific and may be useful measure to differentiate and identify species of genus *Phytophthora* (Masago *et. al.* 1989).

Glycoconjugates on the surface of zoospores and cysts of the pathogenic fungus *Phytophthora cinnamomi* have been studied by Hardham & Suzaki (1990) using

fluorescein isothocyanate labelled lectins for fluorescene microscopy and flow cytometry, and ferritin-and gold-labelled lectins for ultrastructural analysis. Of the five lectins used, only concanavalin A (ConA) binds to the surface of the zoospores, including the flagella and water expulsion vacuole. This suggested that of accessible saccharides, glucosyl or mannosyl residues predominate on the outer surface of the zoospore plasma membrane. Early in encystment, a system of flat disc-like cisternae which underlie the zoospore plasma membrane, vesiculate. These and other small peripheral vesicles quickly disappear. After the induction of encystment, ConA is no longer localised close to the plasma membrane but binds to material loosely associated on the cell surface. Quantitative measurement by flow cytometry indicate that the ConA binding material is gradually lost from the cell surface. The cyst wall is weakly labelled, but the site of germ tube emergence stain intensely. Dering the first 2min. after the induction of encystment, material that binds soybean agglutinin, *Helix pomatia* agglutinin, and peanut agglutinin appears on the surace of the fungal cells. The distribution of this material, rich in galactosyl or N-acetyl-D-galactosaminosyl residues, in initially patchy, but by 5 min the material evenly coats mostly of the cell surface. Labelling of zoospores is which intracellular sites are accessible indicates that the soybean agglutinn binding material is stored in vesicles that lie beneath the plasma membrane. Quantitation of soybean agglutinin labelling shows that maximum binding occurs 2-3min after the induction of encystment.

Harold *et. al.* (1990) isolated antibodies to three isolates each of *Armillaria mellea, A. ostoyae, A. tabescens,* and *Lentinula edodes* from eggs of immunized laying hens. The reactivity of each antibody preparation with the isolates was examined using an enyme-linked immunosorbent assay (ELISA). The cross reactivity of the antibody preparations to a given *Armillaria* species varied considerably when tested against antibodies of other *Armillaria* species. Several antibody preparations were capable of distinguishing isolates of a homologous species from isolates of heterologous species. The specificity of the antibodies present in eggs depended on time elapsed since immunization. Eggs laid 3 weeks after immunization with a *Armillaria* specis 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.

Extracts from *Fusarium oxysporum* (F.O.) and *F. oxysporum* var. *redolens* (F.O.R) isolates were compared by means of electrophoresis and cross immunoelectrophoresis (Rataj-Gunanowska and Wolko 1991). The polymorphism of five isozyme systems allowed a distinction between *F. oxysporum* and *F. oxysporum* var. *redolens* isolates. The isozyme patterns of three other isozyme systems did not allow this distinction between *F. oxysporum* and *F. oxysporum* var.*redolens* to be made. Both fungi apeared almost identical serologically. Relative amounts of their corresponding proteins differed but the qualitative patterns of the proteins were nearly the same with the anti-F.O.R. serum, only one specific antigen was detected in the extracts from *F. oxysporum* var. *redolens* isolates. Although the results obtained indicate a strong similarity between *F. oxyporum* and *F. oxysporum* var. *redolens* they are not sufficient for an unequivocal statement that the fungi belong to the same species.

Wycof & Ayer (1991) used a set of five carbohydrate-specific monoclonal antibodies (mAbs) to probe the ultrastructure of the walls of the soybean pathogen *Phytophthora megasperma* f.sp. *glycinea,* using a combination of immunofluorescence and immunogold labelling techniques. Results with β-1, 3 glucan-specific antibodies suggest that β-1,3 glucans are present throughout the walls of both germtubes and cysts, but are more prevalent in the outer portion. In addition, β-1,3 glucans on the surface of the hyphal walls, but not cysts, are closely associated with other material, most likely protein, that sterically hinders antibody binding except to non-reducing terminal residuces. An antibody whose epitope involved both β-1,4 and β-1,3 glucosyl linkages bound predominantly to the inner portion of the hyphal wall. However, fluorescent labelling with the antibody suggested that β, 1-4 linkages are present on the exterior of *P. megasperma* f. sp. *glycinea* walls as well. Staining with another antibody indicates that changes in wall composition occur over 50-100μm from the hyphal tip, a greater distance than previously supposed. The role of the antigens recognised by these mAbs in the plant pathogen interaction is not known, but potential uses of these and other mAbs are discussed.

Polyclonal antibodies (PAbs) were produced in New Zealand white rabbits with culture filtrate and mycelial extract immunogen preparations from the soybean (*Glycine max*) fungal pathogen *Phomopsis longicolla* (Brill *et. al.* 1994). The PAbs were purified to the immunoglobulin fraction and tested in indirect ezyme linked immunosorbent assay

(ELISA) and in double antibody sandwich-ELISA (DAS-ELISA). The PAbs raised to culture filtrate were more specific but less active in binding to members of the *Diaporthe-Phomopsis* complex than were those raised to 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 wih indirect ELISA. Immunization of one rabbit with culture filtrate over an extended time resulted in maximum anti-*P. longicolla* activity after three immunizations, and the activity became constant against most member of the complex at the some time. Reactivity to same cultures of *P. longicolla* was undetectable following the fourth and fifth immunizations, whereas reactivity of all of other cultures of the complex remain high.

MATERIALS AND METHODS

3. 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, West Bengal (c) United Planter's Association of South India (UPASI) Tea Research Station, Valparia, Tamilnadu and maintained in the Tea Germ Plasm Bank of the Department of Botany, University of North Bengal. For present investigation twentyfive tea varieties were used for experimental purposes. These tea varieties included ten TV varieties (TV-9, TV-18, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-30, and Teen Ali 17/1/54), nine Darjeeling varieties (BS/74/76, CP-1, AV-2, HV-39, T-78, T-135, S-449, P-1258, and K1/1) and 6 UPASI varieties (UPASI-2, UPASI-3, UPASI-8, UPASI-9, UPASI-26 and BSS-2).

3.1.2.Propagation by Cutting

Tea plants are usually propagated by cuttings or by seeds. In case of propagatin by cuttings, plants are raised by planting cuttings made from shoots of the mother bush. The standard form of a tea cuttings is a piece of stem 2.5cm to 3.5cm long with a good mature leaf.

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 to 4.8-4.9 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^{0}C to 80^{0}C on a metal sheet with fire below to kill eelworms, if any, present in the soil.

Cuttings were planted directly into the sleeves (6"x9") which were filled up with the prepared soil and stacked in rows in a bed and sprinkled with water thoroughly. For rooting, lower ends of freshly made cuttings were dipped in the hormone powder, lightly tapped to remove excess powder and immediately planted. These cuttings were covered in a polythene cloche and watered every 2nd or 3rd day as required until new leaves appeared.All beds with polythene cloche were arranged in two

rows and maintained under a green agro house (Plate-2) in the nursery of Department of Botany, University of North Bengal.

3.1.3. Maintenance of tea sleeves in nursery

Underneath the polythene cloche, new shoots came out from cuttings. After growth of 6" or more the polythene cloche was removed from every bed and the sleeves were treated with nursery mixture (nutrient) as suggested by Ranganathan and Natesan (1987). The composition of the nursery mixture-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. The manuring was done after rooting and continued upto 12 months once only in 15 days. The mixture was dissolved @30g in 1L of water and applied @50ml / plant.

3.1.4. Plantation

For the plantation of tea varieties in the experimental plots pits were prepared for each plant. Before that, simazine @75g/20 litre water and glyphosphate @1:200 were used in the experimental plots for the suppression of weeds (Borpujari & Banerjee; 1994). Then pits (1½' x 1½' x 1½') were dug at the intervals of 2' between plant and 3.5' between row to row. Planting mixture was prepared in the ratio of 4.5kg of well rotten dry cattle manure, 30g rock phosphate, 30g super phosphate and 2.5g phorate [0,0-diethyl S-(ethylthiomethyl) phosphoro dithioate]. At the bottom 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 5cm below the ground level.

Following soil conditioning, plants were inspected, selected and brought to the experimental garden and planted in the prepared soil and pits were refilled upto ground level with conditioned soil. For experimental purposes tea plants of all the varieties were also grown in earthen pots (one plant/pot, 30 cm dia) each containing 5kg soil mixture (soil:planting mixture 1:1). Ten month old seedlings with well developed shoot and root system were transferred from the sleeves to the pots. These

Plate 2 (Figs A-F) : Stages of tea propagation by cuttings in nursery.

were then maintained both in glass house and experimental field under natural condition with regular watering.

3.1.5. Maintenance of mature plants

The mature plants (1 year and above) were maintained by applying a soluble mixture of N,P,K consisting of 10Kg Urea-46% N, 20kg ammonium phosphate-11% P_2O_5, 8kg murate of potash-60% K_2O in the soil. Miraculin (7ml/10L) was sprayed at regular intervals for good growth of bush and regular watering was maintained as required. Only tipping was done once to promote lateral branching in young plants (3years) but in case of mature plants 2-year deep pruning cycle was maintained.

3.2. Fungal culture

3.2.1. Source of culture

Fomes lamaoensis (Murr.) Sacc. & Trott. was obtained from Tocklai Experimental Station Jorhat, Assam. The culture was maintained on PDA (potato dextrose agar) medium by regular subculturing. Other pathogens used for the experimental purposes are mentioned Table 1.

Table 1 : List of fungal isolates used

Species	Host	Source
Fomes lamaoensis	*Camellia sinensis* (L).O. Kuntze	Tocklai Experimental Station, Jorhat, Assam
Sphaerostilbe repens	*C.sinensis*	Tocklai Experimental Station Jorhat, Assam
Ustulina zonata	*C. sinensis*	Tocklai Experimental Station Jorhat, Assam
Armillaria mellea	*C. sinensis*	UPASI Tea research Station,Valparai
Rosellinia arcuata	*C. sinensis*	UPASI Tea research Station,Valparai
Poria hypobrumea	*C. sinensis*	UPASI Tea research Station,Valparai
Fusarium oxysporum	*Glycine max*	Indian Agricultural Research Institute New Delhi
Matarhizium anisopliae 892	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.
M. anisopliae-140	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.

Beauveria bassiana-2028	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.
B. bassiana-135	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.
Trichoderma viride	Rhizosphere soil of *C. sinensis*	Isolated from Matigara Tea Estate
T. harzianum	Rhizosphere soil of *C. sinensis*	Isolated from Hansqua Tea Estate
Sclerotium rolfsii	*Glycine max*	UBKV, Cooch Behar
S.rolfsii-1	*Glycine max*	UBKV, Cooch Behar
S.r.-2	*Cajanus cajan*	UBKV, Cooch Behar
S.r. 3	*Pisum sativum*	UBKV, Cooch Behar
S.r. 4	*Tagetes patula*	UBKV, Cooch Behar

3.2.2. Assessment of Mycelial Growth

For assessment of mycelial growth of *F. lamaoensis* various experiments were set up.

3.2.2.1. Solid media

To assess mycelial growth of *F. lamaoensis* in solid media, the fungus was first grown in petridishes, each containing 20ml of P.D.A. and incubated for seven days at $30^0 \pm 1^0$C. Agar block (6mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20ml of sterilized solid media. The different solid media were prepared following the method of Dhingra and Sinclair (1985) and used for assessment of growth. The media were as follows.

1. Potato dextrose agar (PDA)

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

2. Richards Agar (RA)

KNO_3	-	1.00g
KH_2PO_4	-	0.50g
$MgSO_4, 7H_2O$	-	0.25g
$FeCl_3$	-	0.002g
Sucrose	-	3.00g
Agar	-	2.00g
Distilled water	-	100ml

3. Carrot Juice Agar (CJA)

Grated carrot	-	20.00g

	Agar	-	2.00g
	Distilled water	-	100ml
4.	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	-	3.00g
	Agar	-	3.00g
	Disttilled water	-	100ml
5.	Flentze's soil extract agar (FSEA)		
	Soil extract	-	1L
	Sucrose	-	1.00g
	KH_2PO_4	-	0.20g
	Dried yeast	-	0.10g
	Agar	-	25.00g
6.	Potato Sucrose agar (PSA)		
	Peeled potato	-	40.00g
	Sucrose	-	2.00g
	Agar	-	2.00g
	Distilled water	-	100ml
7.	Malt extract Peptone agar (MPA)		
	Malt extact	-	20.00g
	Peptone	-	1.00g
	Detrose	-	20.00g
	Agar	-	20.00g
	Distilled water	-	1L
8.	Yeast extract dextrose agar (YDA)		
	Yeast extract	-	7.50g
	Dextrose	-	20.00g
	Agar	-	15.00g
	Distilled water	-	1L.

All petridishes were incubated at $30^0 \pm 1^0$C for the desired period. Finally diameter of mycelia was measured at 2 days intervals for 8 days.

3.2.2.2. Liquid media

To assess the mycelial growth of *F. lamaoensis* in liquid medium, the fungus was first allowed to grow in petridishes containing 20ml of PDA and were incubated at $30^0 \pm 1^0$C for 7 days. From the advancing zone, the mycelial block (6mm dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized Richards medium for the desired period at 30^0C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60^0C for 96h, cooled in a desiccator and weighed.

3.3. Inoculation techniques

3.3.1. Preparation of Inoculum

3.3.1.1. Pathogen

The inoculum of *F. lamaoensis* was prepared for inoculation of healthy tea plants in media of sand maize meal, tea root pieces and tea waste. Following preparations were used for experimental purpose.

(i) Sand maize meal media (SMM) : The medium was prepared following the method described by Biswas and Sen (2000). SMM medium (maize meal : washed sterilized sand : water = 1:9:1.5, w:w:v) in autoclavable plastic bag (150g) was sterilized at 20lb for 20min.

(ii) Tea root pieces : Inoculum in tea root pieces was prepared following the method of Hazarika *et. al.* (2000). First root and root lets were cut into small pieces (3cm x1.5cm x 1.5cm). Root pieces (22-25) were taken in 250ml flask and pieces were dipped in water for 2h and autoclaved as described above. On the next day water was decanted in a steriled chamber and inoculated with *F. lamaoensis.*

In another method (Dhingra and Sinclair, 1985) the root pieces were boiled for 30min and sterilised in same way. 12-15 sterilized root pieces were then transferred in Ehrlenmeyer flask (250ml) contaiing 20 days old *F. lamaoensis* culture on MPA.

(iii) SMM + root pieces : In this medium SMM along with 6-8 root pieces were sterilized.

(iv) Tea waste : In this medium tea waste and sand were mixed in ratio 1:1, wetted with water and sterilized in polythene packet.

In all cases media were inoculated with *F. lamaoensis* and incubated for 15-20 days at 30 ± 1^{0}C.

3.3.1.2. Biocontrol agents

Trichoderma species were introduced into various media for biological control experiments. Media were wheat bran media (wheat-bran : sand 1:1 and 25ml of water in poly packet, each of 150g of inoculum), saw dust media (saw dust: sand 1:1

and water), tea waste media (tea wastes : sand 1:1 and water). Media were autoclaved and inoculated as above.

3.3.2. Inoculation of healthy tea plants

3.3.2.1. Pot grown plants

(a) Pathogen

In case of potted plant, either 2-5 yr old potted plants were used or tea plants were planted in earthen pots (30cm) containing 5kg soil and allowed to be established for two weeks with regular watering. Then 100g of *F. lamaoensis* inoculum was added carefully in the rhizosphere and ensured that root pieces of inoculum were attached with healthy tea roots. Disease assessment was done after 15, 30 and 45 days after inoculation.

(b) Biocontrol agents

For biocontrol experiments, inoculation with *Trichoderma* sp. was also done as described above but atleast 10 days prior to the inoculation with *F. lamaoensis.* The different treatments for this experiment were as follows; (i) pathogen (*F. lamaoensis*) only (ii) *T. harzianum* (ii) *T. viride* (iv) *F. lamaoensis* + *T. harzianum* (v) *F. lamaoensis* + *T. viride* (vi) healthy plants.

3.3.2.2. Field grown plants

Tea plants of desired ages from varieties to be tested were selected from plots in the Experiemental Garden. The inoculation technique was same as described for potted plants except that 200g inoculum was added in each pit. For biological control, experiments were designed considering different treatments same as potted plants. After inoculation maintenance was done with regular watering.

3.4. Disease assessment

The inoculated plants were examined at an interval of 15 days upto a period of 45 days for pathogenicity test and 10 days intervals for biocontrol test. Disease intensity of brown root rot was assessed on the basis of above ground and under ground symptoms together on a scale 1-6. 0- no symptoms; 1-small roots turn brownish and start to rot; 2-leaves start withering and 20-40% of root turn brown; 3-Leaves withered

with 50% of roots affected; 4-shoot tips start withering 60-70% roots affected; 5-shoots withered with defoliation of lower withered leaves, 80% roots affected; 6-whole plant died, with upper withered leaves still remaining attached; roots fully rotted.

3.5. Soluble protein

3.5.1. Extraction

3.5.1.1. Mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250ml Ehrlenmeyer flasks each containing 50ml of sterilized potato-dextrose broth (PDB) and incubated for 10 days at 30 $\pm$ 1°C. For extraction of antigens, mycelial mats were harvested washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05M sodium phosphate buffer (pH-7.2) supplemented with 0.85% NaCl,10mM sodium metabisulphite and 0.5mM magnesium chloride in ice-bath. The homogenated mixture was kept for 2h or overnight at 4°C and then centrifuged at 10,000rpm for 30 min. at 4°C to eliminate cell debris.

The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice-bath and kept overnight at 4°C. After this period, the mixture was centrifuged (10,000 rpm) for 30min at 4°C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72 h. through cellulose tubing (Sigma chemical Co., USA) against 1L of 0.005 M sodium phosphate buffer (pH.7.2) with six changes. The dialysate was stored at –20°C and used as antigen for the preparation of antiserum and other experiments.

3.5.1.2. Root

Root protein was extracted following modified method of Chakraborty and Purkayastha (1983). The roots of tea plants were collected and washed with cold water and kept at - 20°C for 1hr. These roots were cut into small pieces, ground for 1min. in a high speed blender with insoluble polyvinyl pyrrolidone (PVPP) (Sigma)

of equal weight. The ground root powder was suspended in cold 0.05M sodium phsophate buffer (pH 7.2) containing 0.85% NaCl, 0.02% KCl, 10mM sodium metabisulphite, 2mM PVP-10(Polyvinyl pyrrolidone- 10,000), 0.5mM magnesium chloride and 1mM phenylmethyl-sulphonyl fluoride (PMSF) and the mixture was kept at 40°C for over night. The mixture was then crushed in chilled mortar with pestle using sea-sand in an ice bath. The slurry was strained through muslin cloth and then centrifuged at 10,000 rpm for 30 min. at 4°C. A portion of the supernatant was saved for serological assay and the rest was equilibrated to 100% saturated ammonium sulphate (SAS) under constant stirring and kept overnight at 4°C. After this period, mixture was centrifuged at 10,000 rpm for 30 min. at 4°C. The supernatant was discarded and the precipitate dissolved in the same buffer and dialysed against 0.005M sodium phosphate buffer (pH 7.2) for 72 h at 4°C with six changes. The dialysate was used as antigen for immunization of rabbits and stored at –20°C for further requirements.

Another root protein extraction procedure was developed specifically for better SDS-PAGE resolution. In this process roots pieces were weighed, pulverized and crushed in mortar with pestle using sample buffer [1.M tris (pH6.8) 0.5ml; 10mM β-mercaptoethanol- 0.5ml; 10% SDS-2ml and 7ml H_2O]. At the time of crushing sea sand and insoluble PVPP of equal weight was used. The root slurry was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatent was immediately used for SDS-PAGE analysis and not stored for further any experiments.

3.5.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et. al.* (1951). To 1ml of protein sample (taking 10^{-1} or 10^{-2} dilution) 5ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml of 2% sodium potassium taratrate added to 50ml of 2% Na_2CO_3 in 0.1(N) NaoH) was added and incubated for 15-20min. at room temperature. Then 0.5ml of Folin ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min and colour was developed following absorbance values was measured at 700nm. Quantity of protein was measured from standard curve made with bovine serum albumin (BSA).

3.6. SDA-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

3.6.1. Preparation of stock solutions

The following stock selections were prepared :

A. Acrylamide and N'N'-methylene bis acrylaminde

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light, the pH of the solution was kept below 7.0 and the stock solution was filtered through Whatman No. 1 filter paper and was kept in brown bottle, stored at 4^0C and used within one month.

B. Sodium Dodecyle sulphate (SDS)

A 10% stock solution of SDS was prepared in water and stored at room temperature.

C. Tris buffer

(a) 1.5 M Tris buffer was prepared for resolving gel (pH adjusted to 8.8 with concentrated HCl and stored at 4^0C for use).

(b) 1.0M Tris buffer was prepared for use in the stacking and loading buffer. (pH adjusted to 6.8 with HCl and stored at 4^0C).

D. Ammonium persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris-Glycine electrophoresis buffer

This is a running buffer and consists of 25mM Tris base, 250mM glycine (pH-8.3) and 0.1% SDS; A 1x solution can be made by dissolving 3.02g Trisbase, 18.8g glycine and 10ml of 10% SDS in IL of distilled water.

F. SDS loading buffer

This buffer consists of 5mM Tris HCl (pH 6.8), 10mM β-mercaptoethanol, 2% SDS; 0.1% bromophenol blue, 10% glycerol. A 1 x solution was made by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4M β-mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8. ml of distilled water.

3.6.2. Preparation of Gel

To analyse the protein pattern through SDS-PAGE slab gels (mini) were prepared. Two glass plates (size 8cmx10cm) were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at the three sides and were sealed with high vaccum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving gels were prepared by mixing stock solutions in the following order and poured leaving sufficient space (comb + 1cm) for the stacking gel.

10% resolving gel : Composition of resolving gel solution of 7.5ml was as follows:

	Selections	**Amount**
1.	Distilled water	2.95 ml
2.	30% Acrylamide mixture	2.50ml
3.	1.5 M Trise (pH 8.8)	1.90ml
4.	10% SDS	0.075ml
5.	10& APS	0.075ml
6.	TEMED	0.003ml

The gel was immediately overlayered with isobutanol so that surface of gel remain even after polymerization. The solution was kept for 1h for polymerization of resolving gel. After polymerization isobutanol was poured off and washed with distilled water to remove any unpolymerized acrylamide. Then stacking gel (5%) was prepared by mixing the stock solutions.

5% stacking gel : Composition of gel solution was as follows:

	Solution	Amount
1.	Distilled water	2.10ml
2.	30% acrylamide mixture	0.50ml
3.	1.0M Tis (pH 6.8)	0.38ml
4.	10% SDS	0.03ml
5.	10% APS	0.03ml
6.	TEMED	0.003ml

Stacking gel solution was poured over the resolving gel and comb was inserted immediately leaving a space of 1cm between resolving gel and comb and overlayered with water. The gel kept for 30 minutes. After polymerization of stacking gel the comb was removed and washed thoroughly. The gel was then mounted in the electrophoresis apparatus after removing the spacer at the bottom. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Precaution was taken to remove any bubble trapped at the bottom of gel.

3.6.3. Sample preparation

Sample was prepared by mixing the sample protein (34µl) with 1 x SDS gel loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 3min to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the well with T-100 micropippete syringe. Along with the samples, protein marker consisting of a mixture of six proteins ranging in molecular weight from high to low (phosphorylase-b-97,400; bovine serum albumin-58,000; ovalbumin-43,000; carbonic anhydrase-29,000; soybean trypsin inhibitor-20,000; lysozyme-14,300 daltons) was treated as the other samples and loaded in separate well.

3.6.4. Electrophoresis

Electrophoresis was performed at constant 18mA current for a period about 3h until the dye front reached the lower end of gel.

3.6.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol : water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250mg of coomassie brilliant blue (Sigma R250) in 45ml methanol. When the stain was completely dissolved 45ml of distilled water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

The gel was removed from fixer and stained in this staining solution for 4h at 37°C with constant shaking at a very low speed. After staining, the gel was finally destained in destaining solution containing methanol, distilled water and acetic acid (4.5:4.5:1) at 40°C with constant shaking untill background became clear.

3.7. Preparation of Antigen

3.7.1. Fungal antigen

3.7.1.1. Mycelial

Preparation of mycelial antigen following the method of Chakraborty and Purkayastha (1983)/ Chakraborty and Saha (1994) described earlier in soluble protein extraction procedure.

3.7.1.2. Cell wall

Isolation of cell wall was done following the method of Keen and Legrand (1980). Mycelium of 10 day old log phase fungus cultures was collected on filter paper using a Büchner funnel and 20g of fresh packed cells were ground for 1min. in a high speed blender with water (4ml/g). The resulting slurry was then disrupted in a homogenizer at a high speed for 1min at 5°C. The mixture was then centrifuged for 1min at 2000 rpm, the supernatant fluids discarded and the sedimented walls washed with sterile chilled distilled water (10ml/g) and pelleted by centrifugation several times until the supernatant fluids were visually clear. Finally the isolated cell walls were dissolved in 0.05M phosphate buffer saline (pH 7.2) and kept at –20°C until further serological use.

3.7.2. Root antigen

Antigens were prepared from healthy, artificially inoculated as well as naturally infected tea root tissues following the modified technique as suggested by Chakraborty and Purakayasta (1983), Alba and DeVay (1985) and Chakraborty and Saha (1994) as described earlier in soluble protein extraction.

3.7.3. Soil antigen

Soil antigens were prepared following the method of Walsh *et. al.* (1996). Soil samples were collected and 1gm of soil was crushed in 2ml of 0.05M sodium carbonate-bicarbonate buffer (pH 9.6) in mortar with pestle and kept overnight at 4^0C. Next day it was centrifuged at 10,000rpm for 10min. Supernatant was collected and used as antigen for microplate trapping and blotting purposes.

3.8. Binding of FITC labelled concanavalin-A

Binding of fluorescent labelled concanavalin A to mycelia as well as cell wall was done by the method as described by Keen and Legrand (1980). Initially mycelia and cell wall were incubated for 20min. in 0.85% NaCl in 0.01M potassium phosphate buffer, pH 7.4 containing 1mg/ml fluorescein isothiocyanate (FITC) labelled concanvalin (ConA Sigma Chemicals). The hyphae or the cell wall fragments were then washed thrice with saline solution by repeated low speed contrifugation and resuspension. For control sets these were incubated with lectin supplemented with 0.25 M α-methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epifluorescence optics (BP 450-490 exciting filter, RKP-520 Beam splitting mirror, 515 suppression fitte). Photographs were taken by Leica WILD MPS 32 camera 800 ASA film.

3.9. Serology

3.9.1. Rabbits and their maintenance

Polyclonal antibody (i.e. antisera) for fungal and plant antigens were produced in New Zeland white male rabbits Approxmiately 2kg of body weight of the rabbit is needed (Alba & Devay, 1985) for immunization. So, before immunization, the body

weights of Rabbits were recorded and were observed for at least one week inside the cage. Rabbits were being maintained in Animal House (Antisera Reserve for Plant Pathogens), Immuphytopathology Laboratory, Dept. of Botany, N.B.U.. Foods used for rabbit are green grass, soaked gram seeds., green vegetables like cabbage, carrots (specially at the time of bleeding schedule). Rabbits were regularly fed in the morning and evening providing proper washed and cleaned utensils.90-100g / day gram seeds (soaked in water), alternately with 500g green grass were given for each rabbit. Besides this, they wre given saline water after each bleeding for three consecutive days. Cages and floor were cleaned with antimicrobial agents every day in the morning for maintaining the hygenic condition.

3.9.2. Immunization

Following the method of Alba and DeVay (1985) and Chakraborty and Saha (1994), before immunization, normal sera were collected from rabbit. For raising antisera, intramuscular injections of 1ml antigens (1mg/ml protein) emulsified in equal volume of Freunds' complete adjuvant (Difco) were given into each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7day intervals for consecutive week followed by Freund's imcomplete adjuvant (Difco) at 7 day intervals, upto 10-14 consecutive weeks as required.

3.9.3. Bleeding

Blood samples were collected by marginal ear vein puncture. First bleeding was taken 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding rabbit was placed on their backs on a wooden board, fixed at a 60^0 angle. The neck of the rabbit was held tightly in the triangular gap at the edge of the board and the body was fixed in such a way that the rabbit 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 sharp sterile blade and blood samples (4-10ml) 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 37^0C for 1h

and then stored overnight at 4°C. Then the clot was slightly loosened with sterile needle and the antiserum was taken another sterile centrifuge tube and clarified by contrifugation at 2000g for 10min. at room temperature. Finally, antisera were stored at –20°C until required.

3.9.4. Purification of IgG

3.9.4.1. Precipitation

IgG was purified by affinity chromatography on a DEAE cellulose column following the method of Clausen (1988). The crude antiserum (2ml) diluted with two volumes of distilled water and then an equal volume of 4.0M ammonium sulphate was added. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22°C. Then it was centrifuged at 10,000 rpm for 1h at 22°C and the precipitate was dissolved in 2ml of 0.02M sodium phosphate buffer, pH 8.0.

3.9.4.2. Column preparation

Approximate 4g of DEAE cellulose (Sigma Co. USA) was suspended over in distilled water overnight. The water was drained off and the gel was suspended in 0.005M sodium phosphate buffer, pH 8.0 and the buffer washing was repeated for 5 times. The gel was then suspended in 0.02M sodium phosphate buffer, pH 8.0 and was applied to a coloumn (2.6cm, 30cm high) and allowed to settle for 2h. After the column material had settled, 25ml of 0.02M sodium phosphate buffer, pH 8.0 was applied to the gel material.

3.9.4.3. Fraction collection

2ml of ammonium sulphate precipitate was applied at the top of the column and the elution was performed at a constant pH and a molarity continuously changing from 0.02M–0.3M. The initial elution buffer-(1) was 0.02M sodium phosphate buffer pH. 8.0. The final elution buffer –(2) was 0.3M sodium phosphate buffer pH. 8.0.

The buffer-(1) was applied in a lower flask (or tank) in which one rubber tube connection from its bottom was supplying the column. Another connection from its top was connected to upper flask (or tank) containing buffer - (2). The buffer - (2) had also connection to the open air. During the draining of buffer - (1) to the column,

buffer - (2) was sucked into buffer-(1) thereby producing a continuous rise in molarity. Finally, 40x5 fractions each of 5ml were collected and the optical density values were recorded at 280nm by means of UV spectrophotometer (DIGISPEC-200GL).

3.9.4.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1996). Absorbance was taken for selected fractions at 280 nm and 260nm and then concentration of IgG was calculated by the following formula : protein concentration (mg/ml).= 1.55 x A. 280 – 0.76 x A 260.

3.10. Immunodifusion

3.10.1. Preparation of agarose slides

Glass slides (6cm x 6cm) were degreased in 90% (v/v) ethanol; ethanol: diethylether (1:1, v/v) and ether, then dried in hot air oven. After drying plates were sterilized inside the petridish each containing one plate. Agarose gel was prepared in 0.05M Tris-barbiturate buffer (pH-8.6). The buffer was heated within a conical flask placed in a boiling water bath. 0.9% agarose was mixed to the hot buffer and boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare clear molten agarose and 0.1% (w/v) sodium azide (antibacterial agent) was added into it. For the preparation of agarose gel, the molten agarose was poured on sterilized glass slides (10ml/slide) in laminar flow chamber and kept 15min for solidification. After that 3-7 wells were cut out with a sterilised cork borer (6/m.m.dia) at a distance of 1.5-2.0 cm from the central well. and 2.0 -2.5 from well to well (peripheral).

3.10.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera (50µl/well) were pipetted directly into the appropriate wells in a Laminar chamber. The diffusion was allowed to continue in a moist chamber for 72h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigen were present.

3.10.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initally washed with steriled 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 antigens and antisera widely dispersed in the agarose gel. Then slides were stained with coomassie blue (R 250, sigma; 0.25g coomassie blue + 45ml methanol + 45ml distilled water + 10ml glacial acetic acid) for 10 min. at room temperature. After staining, slides were washed in destaining solution [90 ml methanol : distilled water (1:1) and 10 ml acetic acid) with changes untill the background become clear. Finally, slides were washed with distilled water and dried in hot air oven for 3h at 50°C.

3.11. Enzyme linked immunosorbent assay (ELISA)

ELISA tests as out lined by Chakraborty *et. al.* (1995) was carried out using following buffers.

1. Antigen coating buffer – Carbonate-Bicarbonate buffer 0.05M pH 9.6

Stocks

A. Sodium carbonate – 5.2995g in 1000ml dist. water.

B. Sodium bicarbonate - 4.2g in 100 ml dist water.

160ml of stock A was mixed with 360 ml of stock B and pH was adjusted to 9.6.

2. Phosphate Buffer saline : 0.15 M PBS pH-7.2.

Stocks

A. Sodium dihydrogen phosphate - 23.40g in 1000ml dist, water.

B. Disodium hydrogen phosphate - 21.294g in 1000ml Dist water.

280 ml of stock A was mixed with 720ml of stock B and the pH was djusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15M phosphate buffer Saline-Tween (0.15M PBS-Tween, pH 7.2)

   To 0.15 M PBS, 0.05% Tween-20 was added.

4. Blocking reagent (Tris buffer saline, pH 8.0)

0.05 M Tris, 0.135M NaCl, 0.0027 M KCl

Tris - 0.657g
NaCl - 0.81g
KCl - 0.223g

Distilled water was added to make up the volume to 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 (0.15M PBS - Tween, pH 7.2)

In 0.15M PBS - Tween, pH 7.2, 0.2% BSA, 0.02% polyvinylphyrrolidone, 10,000 (PVPP 10,000) and 0.03% sodium azide (NaN_3) was added.

6. Substrate

P-Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in substrate buffer (1.0%, w/v, diethanolamine, 3mM NaN_3, pH 9.8).

7. 3 N NaOH solution was used to stop the reaction.

3.11.1. Direct antigen coated (DAC) ELISA

This ELISA was performed following the method as described Chakraborty *et. al.* 1995. Plants and fungal antigens were diluted with coating buffer and the antigens were loaded (200µlwell) in ELISA plate (Coastar EIA/RIA, strip plate, USA), arranged in 12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25°C for 4 h. Then the plate was washed 4 times under running tap water and twice with PBS-Tween and shaken to dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1h. After incubation, the plate was washed as mentioned earlier. Purified IgG was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4°C overnight. After a further washing, goat antirabbit IgG labelled with alkaline phosphatase (Sigma Chemicals, USA, in 1:10,000 dilution with PBS) was added to each well (200µl/well) and incubated at 37°C for 2h. Plate was washed, dried and loaded with 200µl of p-Nitrophenyl phosphate substrate in each well and

incubated in dark at room temparature for 45-60 min. Colour development was stopped by adding 50µl/well of 3N NaOH solution and absorbance was determined in an ELISA Reader (Multiskan, ThermoLabsystems) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.11.2. Double antibody sandwich(DAS) ELISA

Conjugation of alkaline phosphatase with γ-globulin

Labelling of γ globulin with alkaline phosphatase has been done following glutaraldehyde one-step method. 5mg of alkaline phosphatase (Sigma Chemicals) was added to 2mg of immunoglobulin (IgG) to a total volume of 1ml in PBS and mixed thoroughly. It was kept at room temparature for 30min. with occasional shaking. Following this glutaraldehyde was slowly added to the mixture to give a final concentration of 0.2% (e.g. for 1ml of 1gG solution, 8µl of 25% of glutaraldehyde [commercial strength] was added) while the solution was being stirried. This mixture was incubated for 2h at room temparature with very gentle stirring. The mixture was diluted with PBS to a convenient volume of 5ml and dialysed 3 times against 500ml ½ strength of PBS, and then against 0.05M tris buffer pH 8.0 at 4°C. After dialysis, bovin serum albumin (about 10mg/ml) and 0.02% NaN_2 was mixed and stored at 4°C until required.

Assay

Direct or DAS ELISA technique, described by Brill *et. al.* (1994) with modifications, was used. PAb of *F. lamaoensis* was diluted in coating buffer and loaded (100µl/well) in each well of ELISA plate. The plate was incubated for 2h at 37°C and washed as done in DAC ELISA. Then plate was shaken to dry. Blocking was done with 100µl of Tris-BSA per well (Tris buffer containing 1.0% (w/v) BSA and 3mM NaN_3) to over come the nonspecific binding and plate was incubated at 25°C for 1h. After blocknig, plate was washed as before. Then test samples were added to wells (100µl/well) and incubated for over night at 4°C. Necessary dilution was done in PBS. Following this plate was washed and alkaline phosphatase tagged IgG, diluted (1:10,000) in PBS was added (100µl/well) to each well and incubated

for 4h at 25°C. The plate was again washed and 100µl PNPP substrate (1mg/ml) was added to each well and incubated for 60 min in dark. Colour development was stopped by adding 50µl/well of 3N NaOH solution. Absorbance values were recorded at 405nm in an ELISA reader (Multiskan, ThermoLabsystems).

3.11.3.Competition ELISA

Competition ELISA was carried on a 96 well ELISA Plate (Nunc. Maxisorp TM, Sweden) following the method as described by Lyons and White (1992). 3 sets were prepared each with 3 replicas for *F. lamaoensis, Trichoderma harzianum* and *T. viride.* All wells of one set was loaded with 100µl *F. lamaoensis* antigen, the 2nd set with *Trichoderma harzianum* antigen and the 3rd set with *T. viride* antigen. The antigens were diluted in PBS before loading. Following incubation at 4°C overnight, the plates were washed four times in running tap water and twice in PBS -Tween, and it was dried. Blocking of non-specific binding was achieved with 5% BSA in PBS, 200µl/ well for 1 h. The plates were washed, dried as before and stored at 4°C until required.

For the test, a doubling dilution series was prepared from tea rhizosphere soils (healthy and inoculated with *F. lamaoensis, T. harzianum* and *T. viride*) in PBS. These samples were added (75µl/well) to wells of 3 ELISA plate sets. To each of these wells, 75µl of *F. lamaoensis* or *T. harzianum* or *T. virdie* IgG (40µg/ml) was added. The plates were incubated at 37°C for 1h on shaker. The shaker was also used for all subsequent stages. At the end of this period 100µl of antigen - 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 100µl anti rabbit IgG alkaline phosphatase conjugate was added to all wells of the plates. Following 30 min. incubation at 37°C plates were washed and 100µl of P-nitrophenyl phosphate substrate (1mg/ml) was added to each well. After a further 45 min incubation in dark absorbance values were recorded at 405nm in an ELISA reader (Multiskan, ThermoLabsystems).

3.12. Immunoblotting

3.12.1. Dot Blot

Dot Blot was performed following the method suggested by Lange *et. al.,* (1989) with modifications. Following buffers were used for dot-blot.

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

(ii) Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween-20 for washing.

(iii) Blocking solution 10% (w/v) skim milk powder (casein hydrolysate, SRL) in TBST (0.05M Tris-HCl, 0.5M NaCl, 0.5% (v/v) Tween 20, pH-10.3).

Assay

Nitrocellulose membrane (millipore, 7cm x10cm, Lot No. : H5SMO 5255, Pore size-0.45μm, Millipore Corporaton, Bedford) was first cut carefully into the required size and placed inside the template. 4μl of coating buffer was loaded in each well of the template over the NCM and kept for 30 min to dry. Following this 4μl of test samples (antigens) were loaded into the template wells over the NCM and kept for 1h. at room temparature. Template was removed and blocking of NCM was done with 10% non-fat dry milk (casein) prepared in TBST for 30-60 min on a shaker Model No., Polyclonal antibody (IgG, 1:500) of *F. lamaoensis* was added directly in the blocking solution and further incubated at 4°C for overnight. The membrane was then washed gently with running tap water for 3min., following three times 5min washes in TBST (pH-7.4) (Wakeham & White, 1996). The membrane was then incubated in alkaline phosphatase-conjugated goat antirabbit IgG (Sigma Chemicals ; diluted 1:10,000 in TBST containing 5%, w/v, Skim milk powder) for 2h at 37°C. The membrane was washed as before. Substrate (1 tablet each of Tris buffer and Fast Red (Sigma Chemicals) or 1 BCIP/ NBT tablet (Sigma Chemicals) dissolved in 10ml double distilled water) was next added and colour development noted. Colour development was stopped by washing the NCM with distilled water and colour development was categorized on the intensity of the dots.

3.12.2. Western Blotting

Immnoublotting was also determined using Western Blot technique as described by Wakeham and White (1996). The following buffers were used for Western blotting

(i) All the stock solutions and buffers used in SDS-Gel preparation for Western blotting were as mentioned earlier in SDS-PAGE protein.

(ii) Transfer buffer (Towbin buffer) :

(25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).

Tris-3.03g; Glycine-14.4g; 200ml Methanol (adjusted to 1lit. with dist. water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH7.2)

Preparation was as mentioned in ELISA.

(iv) Blocking solution

5% non fatdried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers :

(a) Washing buffer-1 : PBS

(b) Washing buffer -2 : (50mM Tris-HCl, 150mM NaCl, pH 7.5).

Tris-6.07gm; NaCl - 8.78gm; made upto 1lit with distilled water.

(iv) Alkaline phosphatase buffer :

(100mM NaCl, 5mM $mgCl_2$,

Tris-HCl, pH 9.5).

Tris-12.14gm; NaCl-5.84gm; $MgCl_2$-1.015gm; made upto 1 lit with double distilled water.

(vii) Substrate

NBT : 5mg NBT in 100µl of 70% N,N-dimethyl formamide

BCIP:5mg BCIP in 100µl of 100% N, N-dimethyl formamide.

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer.

Or, 1 lablet of NBT/BCIP (Sigma Chemical, USA) in 10ml of double distilled water

(vii) Stop solution : (0.5M EDTA solution in PBS, pH 8.0)

EDTA sodium salt-0.0372 gm in 200µl distilled water, added in 50ml of PBS.

Extraction and estimation of protein :

Protein extraction and estimation was done as described earlier.

3.12.2.1. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.12.2.2. Blot transfer process

Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter papper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15min. The transfer process was done in Trans-Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the Semi-dry cell. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The prewetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carfully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h. and proceeded for immunological probing.

3.12.2.3. Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min. with gentle shaking on a platform shaker at room temperature. Subsequently the membrane was incubated with antibody (IgG) solution (blocking solution : PBS [1:1, v/v + IgG, diluted as 1:100 or as per require ment]. The bag was sealed leaving space for few air bubbles and incubated at 4^{0}C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250ml PBS (washing buffer-1). Final washing was done in 200ml washing buffer-2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed four times in washing buffer-2. Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed upto the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.13. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungual mycelia, cross-section of tea roots and soil samples were done using FITC labelled goat antirabbit IgG following the method of Chakraborty & Saha (1994).

3.13.1. Fungal mycelia

Fungal mycelia were grown in liquid Richard's medium as described earlier. After 4 days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and was washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera dilutd (1:50) in PBS and incubated for 1h at room temperature. The myclia 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 Chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45min at room temperature. After incubation, mycelia was washed thrice in PBS and mounted in 10% glycerol. A

cover slip was placed and sealed. The slides were observed and photograph under both phase-contrast and UV fluorescence condition using Leica Leitz Biomed microscope with fluoscence optics equipped with ultraviolet (UV) filter set 1-3.

3.13.2. Cross section of tea roots

Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS), pH 7.2. These sections were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h. at room temperature. After incubation, sections were washed thirce with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30min in dark. After that sections were washed thrice with PBS-Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluorescence of the root section were observed using Leica Leitz Biomed Microscope with fluorescene optics equipped with UV-filter set-I-3 and photograph was taken.

3.13.3. Soil

20g of soil samples (Amended or uninoculated) were dipped into 50ml of steriled distilled water (for 4h) with constant stirring and centrifuged. The pellets were taken and washed with PBS. Then antibody treatment was done, which was followed by FITC treatment, washing, mounting and photographed one after another as described in fungal mycelia.

3.14. Immunocytochemical staining

Immunocytochemical staining of cross-section of root tissues was done using substrate stain solution following the method of Young and Andrews (1990).

Substrate stain solution : A stock substrate solution consisted of 0.15g of naphthol-AS-phosphate (Sigma) dissolved in 2.5 ml of N-N dimethyl formamide (Sigma) and added to 17.0g of Tris base (Sigma), pH 9.1, in 500ml of distilled water. The staining solution, prepared immediately before use, consisted of 1mg of Fast blue (Himedia) and 5µl of 0.1 M $MgCl_2$ added per ml of stock selection and filtered through Whatman No.1 filter paper.

Cross-section of infected tea roots were cut and incubated with PBS containing 1% BSA for 20min at room temparature to prevent nonspecific binding of antibodies to root tissue and then stained immunocytochemically on grooved slides or watch glasses. The sections were then treated with antiserum diluted 1:100 in PBS with 1% BSA (PBS-BSA) at 37^{0}C for 2 hr on a rotary shaker and washed with three changes (5min each) of PBS with 0.1% Tween-20. Following this sections were incubated in a 1:10,000 dilution of Goat antirabbit IgG alkaline phosphatase conjugate (Sigma) in PBS-BSA for 2h at 37^{0}C on a rotary shaker and washed again as described above. Staining was carried out in the dark at room temperature in staining solution which was filtered through whatman No.1 filter paper immediately before being applied to the slides and was incubated with the sections for no longer than 40min before slides were rinsed in PBS. Sections were then mounted in glycerol jelly. and observed under bright field of the microscope (Leica Leitz Biomed) and photographs were taken in bright field.

EXPERIMENTAL

4. Experimental

4.1. Brown root rot incidence in tea of Terai and Dooars

Brown root rot disease caused by *F. lamaoensis* occurs widely in Terai and Dooars region. In order to determine the incidence of this disease in different Tea Estates, a survey of fifteen tea gardens was carried out. The survey of the tea gardens revealed the occurrence of brown root rot most commonly in six gardens i.e. Matigara Tea Estate, Matigara, Chandmani Tea Estate, Matigara, Hansqua Tea Estate, Bagdogra, Bijoynagar Tea Estate, Naxalbari, Cooch Behar Tea Estate, Falakata and Trihana Tea Estate, Bengdubi, In severe cases, plants in specific areas had died off.

4.2. Cultural conditions affecting growth of *F. lamaoensis*

The study of the growth of *Fomes lamaoensis in vitro* showed variation depending on different factors like medium, pH, temperature and seasonal changes. The young mycelia of *Fomes lamaoensis* were white or hyaline which turned yellowish initially and then gradually to deep brown. The mycelial growth was generally submerged but sometimes superficial loose hyphal mat or rarely fluffy growth was found depending on the medium. Deep dark brown or black microsclerotia, were also found on the surface of the selected solid media. (Plate 3). In liquid media, young white submerged mycelia grew at first slowly and then compact mycelial growth formed a plate like structure with horn-beak like edges and white hyphal growth extending a few centimetre on the wall of container. As the days passed the white mycelial colour changed to light brown to dark or blackish brown.

4.2.1. Media

Fomes lamaoensis was grown in eight different media i.e. potato dextrose agar (PDA), potato sucrose agar (PSA), Richard's Agar (RA), carrot juice agar (CJA) Czapek-Dox agar (CDA), Flentze's soil extract agar (FSEA), malt extract peptone dextrose agar (MPDA), yeast extract-dextrose agar (YDA). Results revealed that the fungus grew well in all media (Plate 3, fig. C) except in CDA where the mycelial growth was very poor. Maximum growth was recorded in PDA followed by PSA and

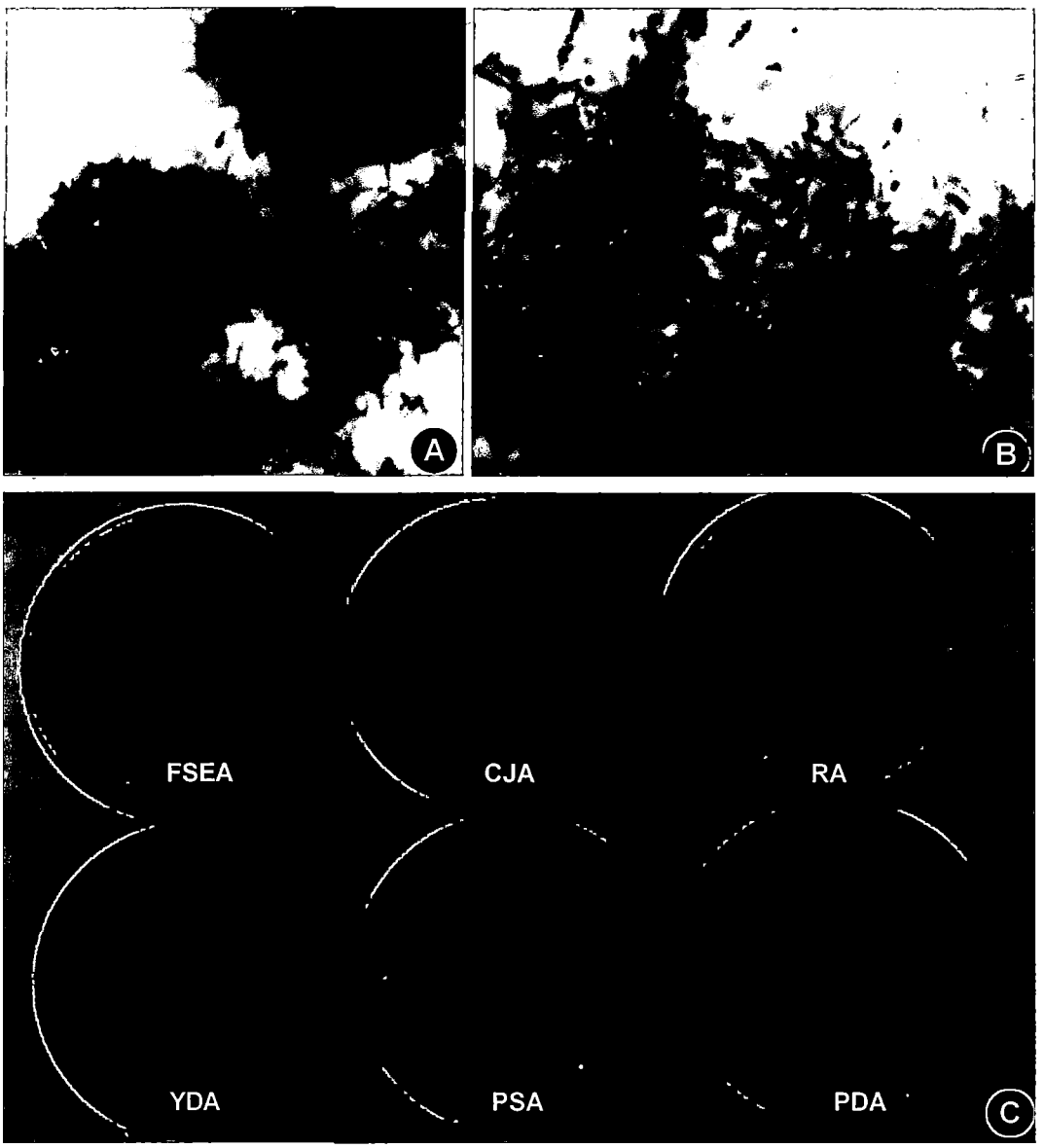


Plate 3 (Figs A-C) : Hyphae (A&B) and mycelial growth (C) of *F. lamaoensis* (A) X10, (B) X40; (C) Growth on different media (FSEA - Flentze's soil extract agar; CJA - Carrot juice agar; RA - Richard's agar; YDA-Yeast extract dextrose agar; PSA - Potato sucrose agar; PDA-Potato dextrose agar).

RA but minimum growth was recorded in CDA where hyaline, submerged hyphal growth spread very loosely was found with no compact mycelial structure; in most other media white or hyaline advancing zone was observed and mycelial colour changed from white to dark brown (white →light brown or yellow → brown → dark brown) Microsclerotia formation was observed in PDA, PSA and CJA media but not in RA, FSEA, MPDA or YDA.

Table 2 : Effect of incubation period on mycelial growth of *F. lamaoensis*

Incubation period(days)	Mycelial dry wt[a] (mg)			
	Expt.1	Expt.2	Expt.3	Mean
3	7.1	8.7	8.7	8.17 ± 0.55
6	22.5	17.7	19.1	19.77 ± 0.91
9	43.2	42.2	40.1	41.83 ± 0.73
12	65.2	64.5	66.1	65.27 ± 0.52
15	89.5	87.2	91.3	89.33 ± 0.83
18	71.5	81.0	81.6	78.03 ± 1.37
21	78.1	71.7	69.5	73.10 ± 1.22
27	68.3	72.3	67.8	69.47 ± 0.91
30	67.6	61.3	62.2	63.70 ± 1.06

± Standard error
Temperature 30 ± 1°C
Incubation period 15 days.

4.2.2. Incubation period

Fomes lamaoensis was grown in Richard's medium (RM) for a period of 30 days, Mycelial growth was recorded after 3,6,9,12,15, 18,21, 24, 27 and 30 days of growth and incubated at 30± 1°C. Maximum growth was recorded after 15 days of incubation (Table 2) after which it declined. After 3 days of incubation the growth was negligible.

4.2.3. pH

pH of the medium 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, Initially buffer solution with pH values ranging from 3.0 to 8.0 (3.0, 4.0, 5.0, 5.5, 6.0,

6.5, 7.0, 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of 0.03M. The pH finally adjusted using N/10 HCl or N/10 NaOH in each case. Richard's medium and phosphate buffer was sterilised separately by autoclaving for 15 min. at 15 lb p.s.i. pressure and equal parts of the buffer solution and medium were mixed before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 15 days after which dry wt. was taken as described previously. Results (Table 3) revealed that *F. lamaoensis* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 4.5-5.0 and then growth gradually declined.

Table 3 : Effect of different pH on mycelial growth of *F. lamaoensis*

	Mycelial dry wt. (mg)			
pH of Medium[a]	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
3.0	38.7	39.7	39.5	39.30 ± 0.41
4.0	54.2	51.0	51.5	52.23 ± 0.76
4.5	83.0	82.5	79.8	81.77 ± 0.76
5.0	79.5	83.6	81.3	81.47 ± 0.83
5.5	72.3	70.4	68.8	70.50 ± 0.76
6.0	54.5	52.3	51.0	52.60 ± 0.77
6.5	50.1	48.7	45.6	48.13 ± 0.88
7.0	47.2	44.5	43.2	44.97 ± 0.82
8.0	44.6	43.8	44.2	44.20 ± 037

[a] Richards medium
± Standard error
Temperature 30± 1°C
Incubation period 15 days

4.2.4. Carbon source

Like the pH of the surrounding medium the growth of fungus is greatly influenced by available nutrients. 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 the present investigations, eight different carbon sources (dextrose, fructose, lactose, mannitol, maltose, sorbose, starch and sucrose)

were tested for their effect on the growth of *F. lamaoensis*. These were added separately to the basal medium. Richard's medium without sugar was used as the basal medium which served as control set. Data were recorded after 15 days of incubation. Results given in Table 4 revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth.

Table 4 : Effect of different carbon source on mycelial growth of *F. lamaoensis*.

Carbon sources	Dry wt. of fungal mass (mg)			
	Expt.1	Expt.2	Expt.3	Mean
Fructose	58.0	60.5	60.1	59.53 ± 0.67
Sorbose	–	–	2.0	2.0 ± 0.00
Dextrose	52.2	41.7	45.0	46.30 ± 1.34
Mannitol	15.9	16.2	15.5	15.86 ± 0.34
Sucrose	55.3	51.2	48.9	51.80 ± 1.04
Starch	36.5	39.4	38.0	37.98 ± 0.70
Maltose	18.9	96.0	28.2	24.86 ± 1.27
Lactose	64.5	61.2	63.6	63.10 ± 0.75
Control	–	–	2.0	2.0 ± 0.00

± Standard error
Temperature 30 ± 1°C
Incubation period 15 days.

4.2.5. Nitrogen source

The availability of nitrogen for growth of the organism 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 (ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate and sodium nitrate) as well as complex organic sources (casein acid hydrolysate, beef extract, peptone, urea and yeast extract) on the mycelial growth of *F. lamaoensis* was tested. A basal medium without any nitrogen source was considered as control.

After 15 days of incubation data was recorded and result (Table 5) revealed

maximum growth in beef extract followed by yeast extract and then casein acid hydrolysate. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

Table 5 : Effect of different Nitrogen sources on mycelial growth of *F. lamaoensis.*

Nitrogen source	Dry weight of fungal mass (mg)			
	Expt.1	Expt.2	Expt.3	Mean
Inorganic				
Potassium nitrate	20.5	22.8	23.4	22.23 ± 0.71
Sodium nitrate	31.4	26.5	28.4	28.77 ± 0.91
Ammonium sulphate	36.0	35.1	41.1	37.40 ± 1.04
Ammonium nitrate	65.9	68.4	64.0	66.10 ± 0.86
Calcium nitrate	133.1	143.6	140.4	139.03 ± 1.34
Organic				
Urea	–	–	–	–
Peptone	58.4	52.9	59.6	57.98 ± 1.09
Casein hydrolysate	195.6	185.4	191.0	190.50 ± 1.55
Yeast extract	410.2	415.6	419.1	414.97 ± 1.22
Beef extract	521.7	520.4	528.2	523.43 ± 1.18
Control (without nitrogen)	4.0	6.0	5.0	5.0 ± 0.58

± Standard error
Temperature 30 ± 1°C
Incubation period 15 days.

4.3. Pathogenicity test of *F. lamaoensis* on different tea varieties

Naturally infected tea roots, showing symptoms of brown root rot (Plate 4) was collected and *F. lamaoensis* was isolated from the naturally infected roots and then compared with the sample received from Tocklai Experimental Station, Jorhat, Assam, and was used for further studies involving different tea varieties. Varietal resistance test of tea against *F. lamaoensis* was carried out with twenty five different tea varieties maintained in the phytopathological tea garden and Tea Germplasm Bank,

Plate 4 (Figs A&B) : Tea root showing brown rot symptoms with ring and irregular lines

Table 6 : Pathogenicity test of *F. lamaoensis* on tea root of Darjeeling varieties following potted plant inoculation.

Tea varieties[a]	Disease index[b]		
	Days after inoculation		
	15	30	45
Teenali 17	1.2 ± 0.20	2.0 ± 0.25	2.6 ± 0.23
TV-9	0.7 ± 0.21	1.0 ± 0.21	1.2 ± 0.20
TV-18	1.3 ± 0.25	2.4 ± 0.26	3.0 ± 0.22
TV-22	1.0 ± 0.22	1.4 ± 0.24	1.8 ± 0.14
TV-23	0.9 ± 0.19	1.2 ± 0.20	1.3 ± 0.26
TV-25	0.8 ± 0.21	1.1 ± 0.29	1.2 ± 0.21
TV-26	0.8 ± 0.21	0.8 ± 0.21	1.0 ± 0.22
TV-27	1.1 ± 0.29	1.4 ± 0.23	1.5 ± 0.27
TV-28	0.8 ± 0.25	1.3 ± 0.26	1.4 ± 0.26
TV-30	0.9 ± 0.19	1.8 ± 0.25	1.8 ± 0.21
UP-2	0.6 ± 0.23	2.0 ± 0.25	2.6 ± 0.23
UP-3	0.8 ± 0.25	1.0 ± 0.20	1.2 ± 0.25
UP-8	0.6 ± 0.26	2.4 ± 0.18	3.0 ± 0.31
UP-9	0.5 ± 0.23	1.4 ± 0.24	1.8 ± 0.30
UP-26	1.2 ± 0.18	1.2 ± 0.31	1.3 ± 0.26
BSS-2	1.0 ± 0.22	1.1 ± 0.29	1.2 ± 0.21
AV-2	0.8 ± 0.25	1.1 ± 0.27	1.3 ± 0.21
BS/7A/76	0.7 ± 0.21	1.0 ± 0.20	1.2 ± 0.21
CP-1	1.2 ± 0.18	2.0 ± 0.25	2.5 ± 0.29
HV-39	0.8 ± 0.25	1.2 ± 0.31	1.3 ± 0.26
K1/1	0.9 ± 0.19	1.3 ± 0.26	1.8 ± 0.30
P-1258	1.2 ± 0.20	2.0 ± 0.24	2.4 ± 0.23
S-449	1.1 ± 0.29	2.0 ± 0.28	2.3 ± 0.26
T-78	1.8 ± 0.21	2.4 ± 0.23	3.4 ± 0.23
T-135	0.9 ± 0.18	1.2 ± 0.18	1.6 ± 0.23

[a] Age of the plant 2yr.; Average of 10 separate inoculated plants; ± Standard error.

[b]0= No symptoms; 1= Small roots turn brownish and start to rot; 2= Leaves start withering and 20-40% of root turn brown; 3= Leaves withered with 50% of roots affected; 4= Shoot tips start withering 60-70% of roots affected; 5=shoots withered with defoliation of lower withered leaves, 80% roots affected; 6= Whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

Table 6a : Analysis of variance of the data presented in table 6

Source	D.F.	S.S.	M.S.	F
Days after inoculation	2	10.016	5.008	49,0890
Tea varieties	24	14.453	.602	5.9029
Error	48	4.897	.102	
Total	**74**	**29.367**		

Dept. of Botany, N.B.U. Rhizosphere region of potted (2 yr old) plants were inoculated with *F. lamaoensis* and disease assessment was done after 15, 30 and 45 days of inoculation. Among the twenty five varieties ten were Tocklai varieties (T-17, TV-9,18, 22, 23,25,26,27,28 and 30), nine were Darjeeling varieties (S 449, HV-39, T-78, T-135, AV-2, CP-1, BS/7A/76, P-1258 and K-1/1) and six were UPASI varieties (BSS-2, UP-2,3,8,9 and 26). Disease assessment was on the basis of visual observation of symptoms and disease index was calculated as described earlier, Disease index ranged from 1-6 and was calculated 15,30 and 45 days of inoculation (Plate 5 & 6).

Among the Tocklai, UPASI and Darjeeling varieties TV-18, UP-26 and T-78 were most susceptible, respectively, while TV-26, UP-8 and BS/7A/76 were most resistant . Among all the twenty five varieties, UP-26 was most susceptible (Table 6; Fig.1).

Since available reports indicated that plants become more susceptible with age, disease assessment in five selected varieties (four susceptible and one resistant) of different ages, under field conditions was carried out. Disease assessment was done after 40 days of inoculation. Disease intensity was observed to be maximum in 5yr. old plants and minimum in 1yr old plants (Table 7 and Fig. 2).

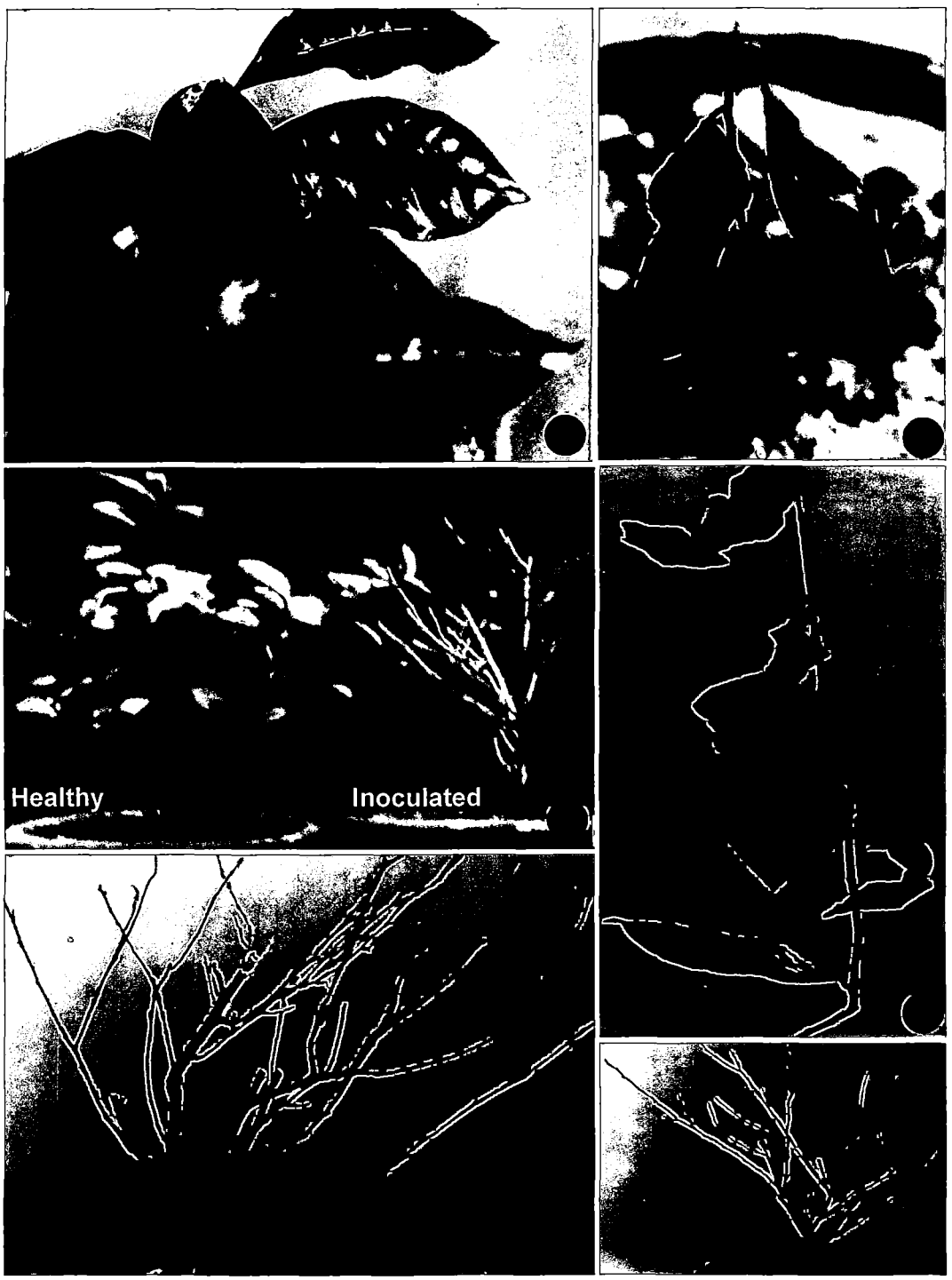


Plate 5 (Figs A-F) : Healthy and *F. lamaoensis* inoculated pot grown tea plants (A) TV-27, (B & D) UPASI-26; (C) T-17 (E) S-449; (F) TV-18.

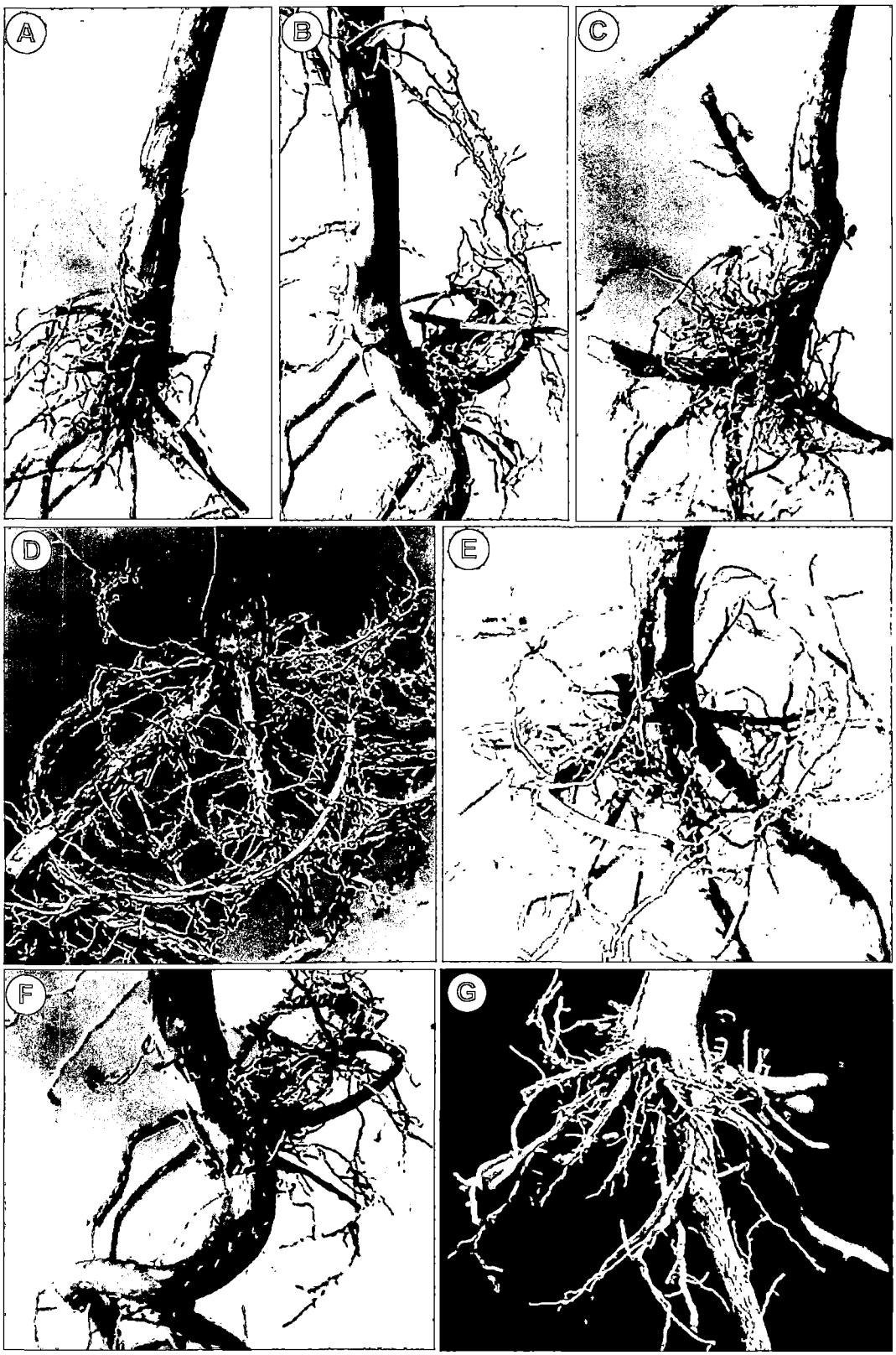


Plate 6 (Figs A-G) : Roots of uprooted tea plants showing symptoms after 40 days of inoculation with *F. lamaoensis* (A) UP-26; (B) TV-18; (C) BSS-2; (D) CP-1; (E) S-449; (F) T-17; (G) TV-30.

Table 7 : Pathogenicity test of *F. lamaoensis* on different tea varieties at different ages.

Tea varieties	Disease index[a]			
	Age of the plants (year)			
	1	2	3	5
T-17	2.0 ± 0.28	2.5 ± 0.26	3.9 ± 0.29	4.8 ± 0.26
TV-18	2.4 ± 0.23	3.4 ± 0.23	5.0 ± 0.26	5.5 ± 0.18
TV-26	0.8 ± 0.15	1.1 ± 0.13	1.6 ± 0.18	2.0 ± 0.24
UP-26	3.6 ± 0.18	3.8 ± 0.16	4.9 ± 0.24	5.6 ± 0.17
T-78	2.4 ± 0.20	4.0 ± 0.21	4.8 ± 0.29	5.2 ± 0.22

Average of separate inoculated plants
± Standard error.
After 40 days of inoculation with *F. lamaoensis.*
[a]0= No symptoms;
1= Small roots turn brownish and start to rot;
2= Leaves start withering and 20-40% of root turn brown;
3= Leaves withered with 50% of roots affected;
4= Shoot tips start withering 60-70% of roots affected;
5= Shoots withered with defoliation of lower withered leaves, 80% roots affected;
6= Whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

Table 7a : Analysis of variance of the data presented in table 7

Source	D.F.	S.S.	M.S.	F
Tea varieties	4	24.763	6.191	35.3594
Age of plants	3	17.102	5.701	32.5592
Error	12	2.102	.175	
Total	19	43.966		

4.4. Agar gel double diffusion tests with PAbs of *F. lamaoensis* and tea roots

Polyclonal antibodies (PAbs) were raised in rabbits against mycelial (100% SAS) and cell wall antigens of *F. lamaoensis,* mycelial antigens of *T. harzianum, T. viride* as well as tea root antigens (UP-26 and TV-26) as described previously and

Pathogenicity test of *Fomes lamaoensis* on different tea varieties

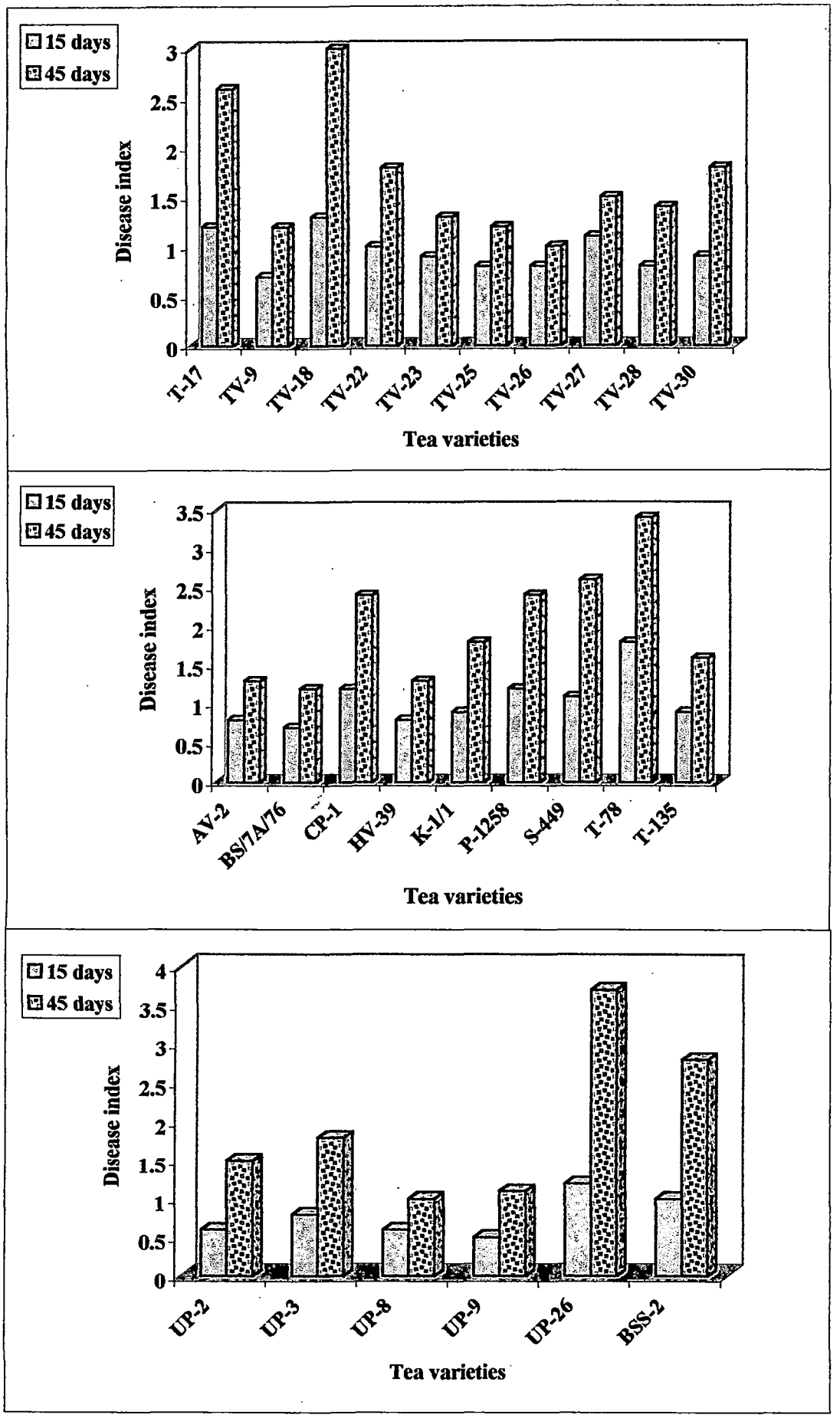


Fig.1

Development of brown root rot disease in tea varieties of different ages

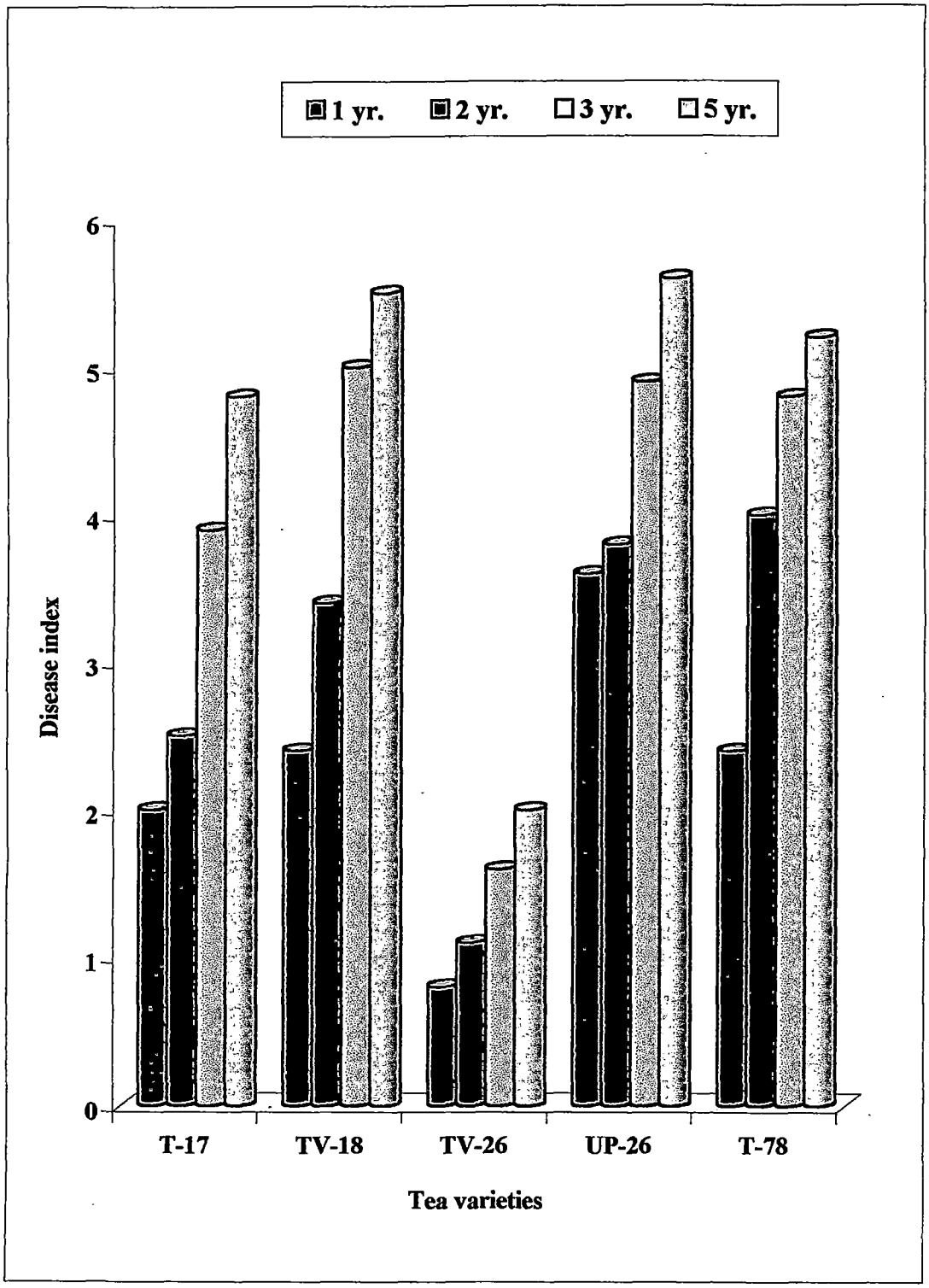


Fig.2

these were used for various experimental purposes. The PAbs obtained from different bleedings were numbered as follows.

Source	Bleed	PAb No.
F. lamaoensis - Mycelia		
100% SAS	1-6	101-106
60-80% SAS	1-3	301-303
Cell wall	1-5	201-205
T. harzianum - mycelia	1-3	601-603
T. viride - mycelia	1-3	701-703
UP-26-root tissue	1-4	401-404
TV-26-root tissue	1-4	501-503

For each antigen source normal serum (100, 200, 300, 400, 500, 600 & 700 respectively) were collected before immunization. Initially, titres of antisera were determined through immunodiffusion tests. The effectiveness of antigens preparations from mycelia, cell wall of *F. lamaoensis* and tea root antigens (UP-26 and TV-26) in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigen of pathogen and tea roots were all negatives. Strong precipitin reaction occurred when PAb of *F. lamaoensis* was reacted with its own antigen (Plate 7, fig.A & B). The different titre values of different bleedings of mycelial PAb were checked time to time after each bleeding (101-106) and also at a time in a single slide using a reciprocal set (i.e. central well of homologous antigen and peripheral wells of PAbs) in immunodiffusion tests. Result presented in Plate 7, fig. C showed strong precipitin reactions in 102-105 bleedings with maximum reaction occurring in 103 and 104 bleed. For subsequent immunoassays, 103/104 bleed PAbs were used. Only weak precipitin bands were observed in 101 and 106 bleedings.

PAbs of five bleedings (201-205) raised against cell wall immunogens of *F. lamaoensis* with homologous antigen were also tested initially in immunodiffusion tests. Strong precipitin bands were detected in all bleedings tested as shown one slide in Plate 7, fig. D.

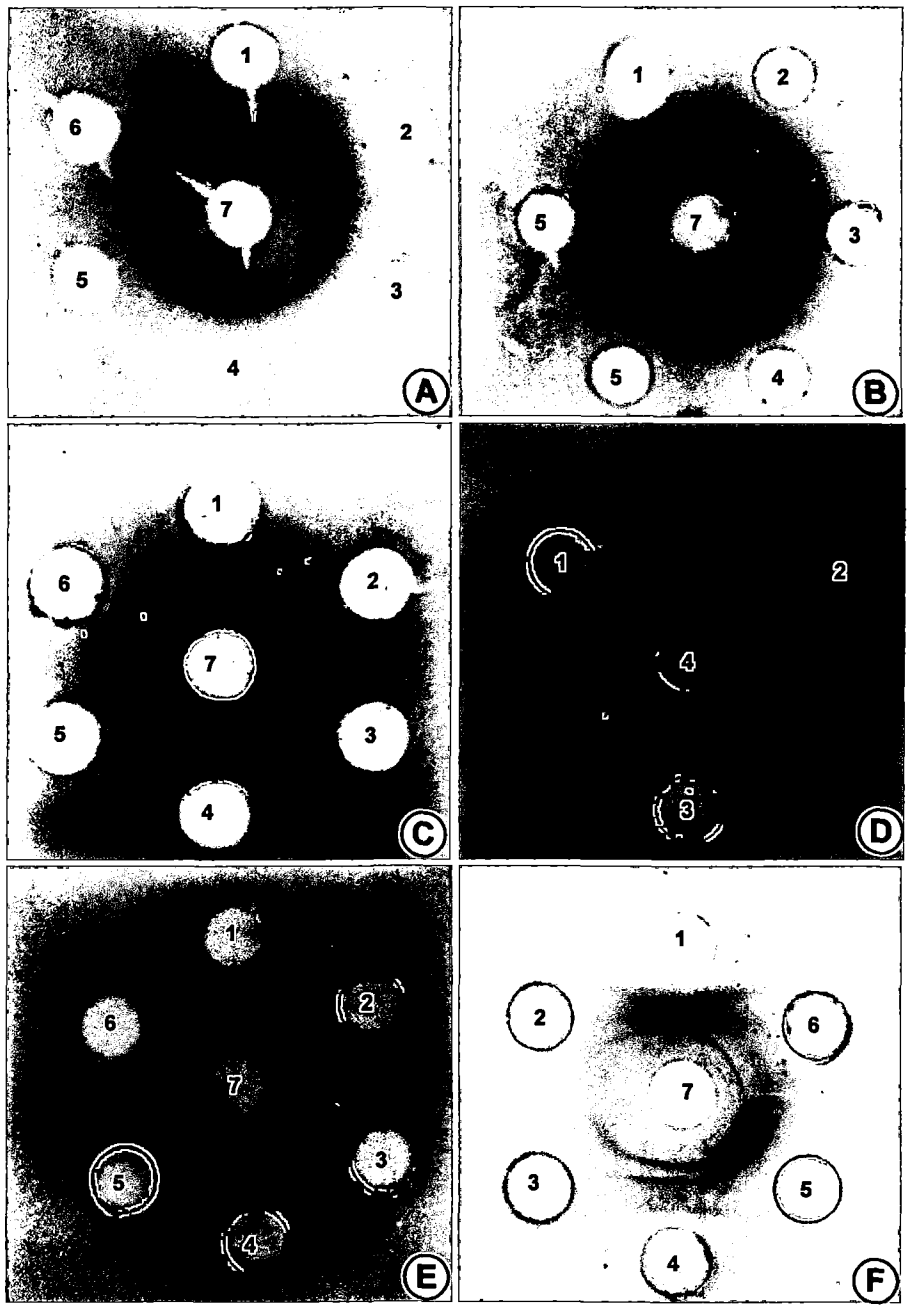


Plate 7 (Figs A-H) : Agar gel double diffusion tests with PAb of 100% SAS (A,B,C&E) ; 60-80% SAS (F) of mycelial and cell wall (D) of *F. lamaoensis*. Peripheral wells were loaded with antigens (A&B) 1-6 = mycelial antigens; (E&F) 1=100%, 2=0-20%, 3=20-40%, 4=40-60%, 5=60-80% and 6=80-100% SAS of mycilial antigens of *F. lamaoensis*; (C) 1-6 = PAb different bleedings [1=101, 2=102, 3=103, 4=104, 5=104 & 6=106], (D) 1-3 Cell wall antigen of *F. lamaoensis*. Central wells (7) were loaded with PAbs of *F.lamaoensis* in A=101, B=103, D=202, E= 103 and F=302 and mycelial antigen of *F. lamaoensis* in C.

Besides, PAb raised against tea root antigens were also checked in immunodiffusion tests. Positive reaction were noticed in 1st to 4th bleedings.

4.5. Analysis of proteins in mycelia and tea roots

Since the experiments in the present study involved antigenic proteins, it was decided to analyse the proteins both quantitatively and qualitatively. For this purpose estimation of protein content as well as SDS-PAGE was carried out with fungal proteins as well as proteins of healthy and infected tea roots.

4.5.1. Protein content

Estimation of proteins, extracted from the fungal and tea root tissues was carried out as described. Mycelia of *F. lamaoensis* had protein content generally around 6.0mg/ gm. fresh weight tissue. Cell walls were isolated from *F. lamaoensis* and protein content of cell wall preparations was 5.8mg/g fresh weight of mycelial wall. In case of protein content of roots, that, in all tested varieties, protein content decreased following inoculation increase in days of inoculation (Table 8). There was no relationship with susceptibility or resistance. In older plants (5yr old) protein content of susceptible varieties, which were greatly affected, showed a significant decrease in relation to control. The decrease was in the range 43% (T-78) - 68% (UP-26).

4.5.2. Protein pattern

Proteins extracted from different sources were further analysed by SDS-PAGE following the method described under materials and methods. The molecular weight of protein bands, visualized after staining with coomassie blue were determined from the known molecular weight marker of 6 proteins as described in materials and methods.

45.2.1. Mycelial soluble protein of *F. lamaoensis*

Mycelial protein exhibited 21 bands in SDS-PAGE ranging in molecular weight (Ca. 104 kDa to 12 kDa) and bands were of varying intensities and more proteins of lower molecular were present (Table 9 & Plate 8, fig.A). The number and intensities of bands were different in case of different incubation periods (5, 10, 15, 20, 25, 30 &

Table 8 : Protein content of healthy and infected tea root tissues.

Tea varieties	Protein content (mg/gm)					
	Days after inoculation					
	15		30		45	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
T-17	1.40 ± 0.2	1.25 ± 0.1	1.43 ± 0.2	1.08 ± 0.1	1.50 ± 0.1	0.88 ± 0.1
TV-9	1.75 ± 0.1	1.84 ± 0.1	1.56 ± 0.1	1.58 ± 0.1	1.27 ± 0.1	1.13 ± 0.1
TV-18	1.62 ± 0.1	0.81 ± 0.4	1.71 ± 0.1	0.80 ± 0.5	1.84 ± 0.1	0.60 ± 0.1
TV-22	1.80 ± 0.1	1.02 ± 0.2	1.70 ± 0.1	0.95 ± 0.2	1.45 ± 0.2	0.97 ± 0.2
TV-23	1.82 ± 0.2	1.40 ± 0.2	1.67 ± 0.1	1.39 ± 0.1	1.68 ± 0.2	1.18 ± 0.2
TV-25	1.46 ± 0.1	1.51 ± 0.1	1.38 ± 0.1	1.25 ± 0.1	1.32 ± 0.2	1.31 ± 0.2
TV-26	1.71 ± 0.1	1.53 ± 0.2	1.75 ± 0.1	1.45 ± 0.4	1.58 ± 0.1	1.35 ± 0.1
TV-27	1.42 ± 0.2	0.95 ± 0.1	1.34 ± 0.1	1.00 ± 0.4	1.30 ± 0.3	0.98 ± 0.3
TV-28	1.70 ± 0.2	1.24 ± 0.2	1.74 ± 0.1	1.08 ± 0.6	1.70 ± 0.1	1.26 ± 0.1
TV-30	1.28 ± 0.1	1.19 ± 0.1	1.46 ± 0.1	1.03 ± 0.2	1.63 ± 0.2	1.36 ± 0.1
UP-2	1.93 ± 0.1	1.82 ± 0.1	1.91 ± 0.2	1.82 ± 0.2	2.02 ± 0.2	1.37 ± 0.1
UP-3	2.19 ± 0.2	2.28 ± 0.1	2.06 ± 0.1	2.00 ± 0.1	1.95 ± 0.1	1.66 ± 0.4
UP-8	2.81 ± 0.2	1.86 ± 0.1	2.23 ± 0.2	2.10 ± 0.3	1.64 ± 0.2	1.65 ± 0.1
UP-9	1.95 ± 0.8	2.05 ± 0.1	2.24 ± 0.1	1.88 ± 0.2	2.47 ± 0.2	2.61 ± 0.2
UP-26	1.93 ± 0.1	1.36 ± 0.5	1.87 ± 0.2	1.05 ± 0.1	1.61 ± 0.3	0.99 ± 0.1
BSS-2	2.05 ± 0.1	1.47 ± 0.6	1.95 ± 0.4	0.74 ± 0.1	2.48 ± 0.4	0.73 ± 0.1
S-449	1.46 ± 0.1	1.38 ± 0.2	1.67 ± 0.1	0.74 ± 0.1	1.55 ± 0.2	0.72 ± 0.5
AV-2	2.03 ± 0.1	2.15 ± 0.1	1.73 ± 0.4	1.56 ± 0.2	1.53 ± 0.2	1.32 ± 0.7
BS/7A/76	2.16 ± 0.1	2.36 ± 0.1	1.58 ± 0.1	2.44 ± 0.2	2.14 ± 0.3	2.23 ± 0.1
CP-1	1.92 ± 0.1	1.57 ± 0.1	2.23 ± 0.1	1.41 ± 0.4	2.25 ± 0.1	1.01 ± 0.2
HV-39	1.53 ± 0.9	1.42 ± 0.1	1.62 ± 0.1	1.43 ± 0.3	1.85 ± 0.1	1.68 ± 0.1
K1/1	2.21 ± 0.2	2.36 ± 0.2	1.80 ± 0.2	1.24 ± 0.2	1.92 ± 0.1	1.99 ± 0.6
P-1258	2.43 ± 0.2	2.11 ± 0.2	1.62 ± 0.2	1.34 ± 0.1	1.84 ± 0.4	1.13 ± 0.1
T-78	1.65 ± 0.1	1.24 ± 0.1	1.49 ± 0.1	0.71 ± 0.2	1.35 ± 0.1	0.89 ± 0.3
T-135	1.74 ± 0.1	1.47 ± 0.1	1.45 ± 0.2	1.10 ± 0.2	1.33 ± 0.3	1.02 ± 0.2

35 days). Maximum number of bands were found in 10,15 and 20 days of incubation following which a decline in number of bands was found. High intensity of bands were found in extracts from 10-25 days. In case of 5 days of incubation one deep and thick band (mol. wt = ca.22 kDa) was found which gradually became and less intense in following days. In case of 5, 30 & 35 days, proteins bands of higher molecular weight were very few in number. (Plate 8).

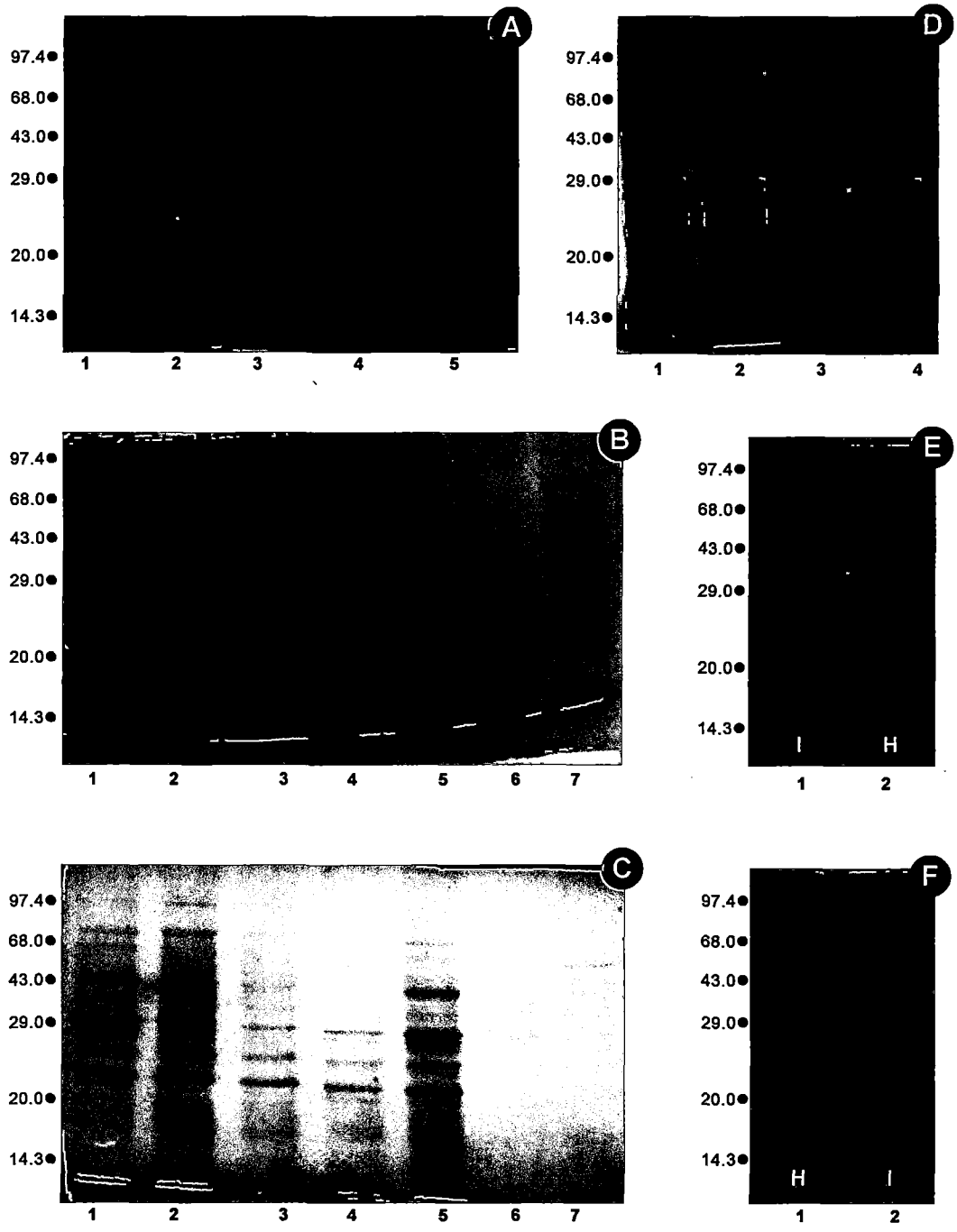


Plate 8 (Figs A-F) :SDS-PAGE analysis of *F.lamaoensis* (A-C) and tea root (D-F) proteins; [A] lanes 1-5: mycelial proteins (12 day old) ; [B] lanes 1-7: 5, 10,15,20 , 25, 30 & 35 day old mycelia , respectively; [C] lane 1 : crude , lane 2: 100% ; lane 3 : 0-20% ; lane 4: 20-40% ; lane-5: 40-60% ; lane-6:60-80% and lane-7: 8 0-100% SAS; [D] Healthy tea root proteins; lane 1&2 : UP-26; lane 3 : TV-26 & lane 4: S-449; ; [E] TV-18; [F] T-78

4.5.2.2. Cell wall protein of *F. lamaoensis*

Cell wall preparation of *F. lamaoensis* were resolved in SDS-PAGE as described earlier, fixed in fixer solution and stained with coomassie blue. Gel exhibited 15 protein bands ranging from ca. 102 to 15 kDa of which 8 bands were of higher molecular weight and 7 of low molecular weight (Table 9).

Table 9 : SDS- PAGE analysis of mycelial and cell wall proteins of *F. lamaoensis*

Protein source	No. of Bands	Molecular Weight (kDa)
Mycelia	21	104.7, 102.0, 97.4, 91.9, 79.4, 60.9, 54.5, 50.1, 43.0, 38.6, 32.2, 28.7, 27.1, 24.5, 22.2, 19,0, 18.4, 17.4, 15.3, 14.3, 12.7.
Cell wall	16	102.0, 97.4, 88.2, 83.6, 68.0, 63.5, 59.0,54.5, 43.0, 38.6, 34.5, 24.5, 19.0,18.4, 17.0, 15.0.

4.5.2.3. Tea root protein

Since protein content of tea roots had decreased following infection, it was decided to analyse the changes in protein pattern by SDS PAGE. In the healthy roots of the different varieties No. of bands ranged from 10-18 of molecular wts. 13-73 kDa. In the 2 varieties TV-18 and T-78, number of bands decreased from 14 to 2 and 10 to 3, rescpectively. In infected roots of TV-18, 2 bands of mol. wts 72.6 and 21.6 were still evident, while in T-78, 3 badns of 72.6, 24.5 and 20.0 kDa were evident (Plate 8, figs. D, E, & F).

4.5.3. Con A-FITC binding

To determine the glycoprotein nature of cell walls, mycelia and isolated cell walls of *F. lamaoensis* were treated with FITC labelled Con A and observed under microscope as described in materials and methods. Strong fluorescence was observed under the microscope in both the mycelial and cell wall (Plate 9).

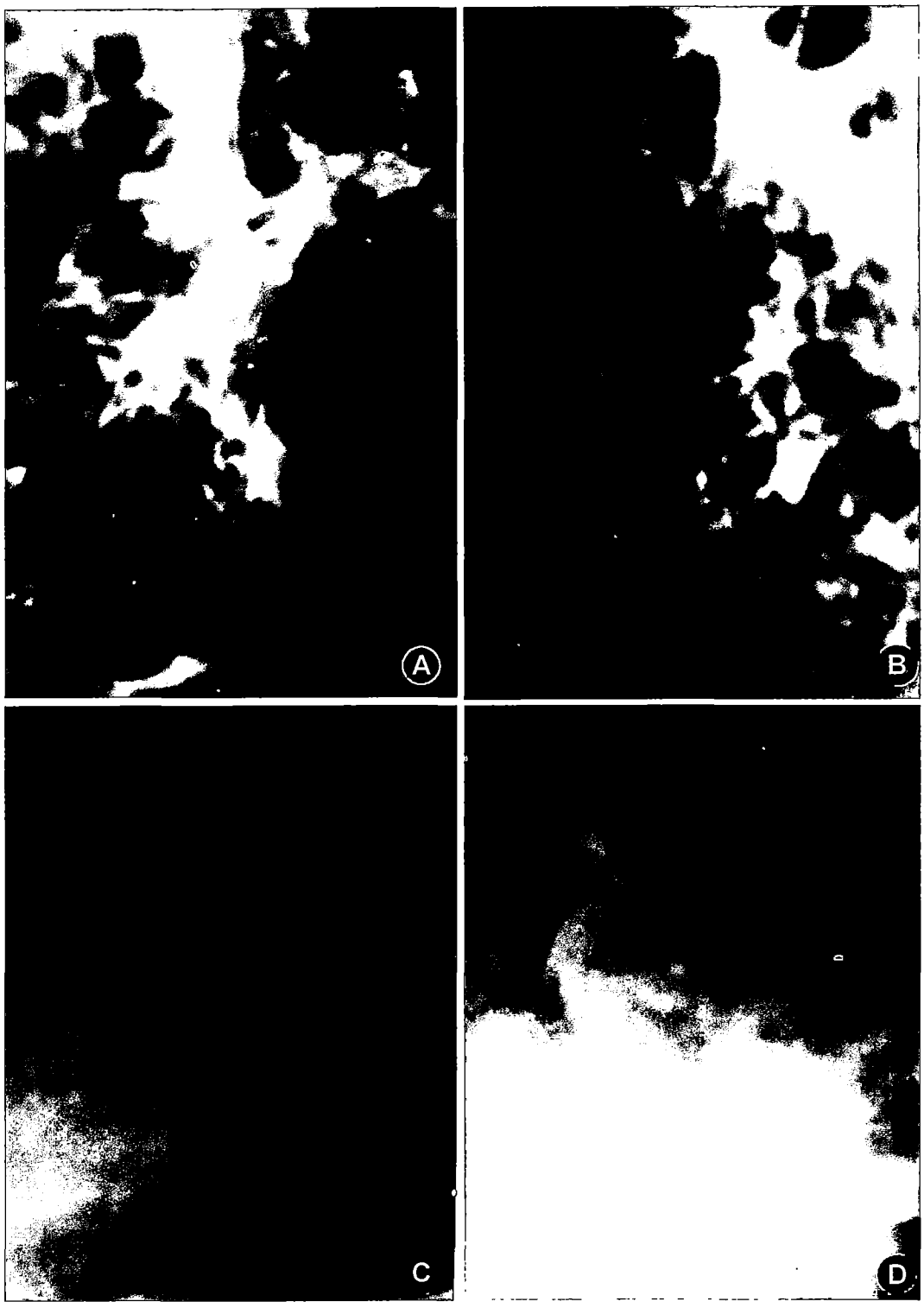


Plate 9 (Figs A-D) : Fluorescence of hyphae (A&B) and isolated cell walls (C&D) of *F. lamaoensis* after staining with FITC-ConA.

4.6. Optimization of PAb and antigen concentrations in direct antigen coated enzyme linked immunosorbent assay (DAC ELISA)

Enzyme linked immunosorbent (ELISA) assay is one of the most sensitive serological techniques for detection of cross reactive antigens between host and the pathogen as well as for detection of pathogen in diseased tissue and surrounding soil. Since ELISA depends on number of factors and this varies from system to system it was considered essential to optimize various conditions before further tests. Hence initially a number of experiments were performed for optimization. DAC or direct antigen coated ELISA is the technique where plates are first coated with antigens followed by primary antibody and finally secondary antibody labells with enzyme.

Optimization tests were performed separately with all polyclonal antibodies (PAbs) raised against mycelial and cell wall antigens of *F. lamaoensis* and tea root antigens. PAbs in each case were collected by different bleedings at definite time intervals, IgG fractions were purified following the ammonium sulphate preceipitation and fractionation through chromatography on DEAE-cellulose column as described by clausen (1988) and experiments were done with purified IgG fraction of the PAbs.

4.6.1. PAb raised against mycelia of *F. lamaoensis*

Optimization of ELISA was done by considering two variables i.e. concentration of the antigen and concentration of PAb. Reactions were done with PAb obtained after different bleedings. Enzyme concentration was 1:10,000 while substrate was used at a concentration of 1mg/ml.

4.6.1.1. IgG concentration

Different concentrations of IgG (ranging from 0.312-40 μg/ml.) from PAb (102) were tested against homologous antigens at a concentration of 10μg/ml. Absorbance values in ELISA increased with increase in concentration of IgG with a maximum value of 2.1 in 40μg/ml (Table 10 & Fig. 3). This concentration of IgG was selected for further experiments.

Table 10 : ELISA reaction with different concentration of *F. lamaoensis* PAb (mycelia) and homologous antigen.

Antisera (IgG) concentration (μg/ml)	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.519	0.525	0.520	0.521 ± 0.03
0.625	0.665	0.683	0.687	0.678 ± 0.06
1.25	0.878	0.875	0.844	0.866 ± 0.08
2.5	1.042	1.050	1.034	1.042 ± 0.05
5	1.221	1.217	1.239	1.226 ± 0.06
10	1.596	1.595	1.576	1.589 ± 0.06
20	1.855	1.843	1.861	1.853 ± 0.06
40	2.100	2.070	2.082	2.084 ± 0.07

Antigen concentration 10μg/ml
IgG source-102 PAb.
± Standard error.

4.6.1.2. Antigen concentration

Antigen concentration ranging from 0.156-20μg/ml. were tested 0.156μg/ml tested against IgG from 101 to 106 bleeding at a concentration of 40μg/ml. Results (Table 11 & Fig. 4A) revealed that ELISA values decreased with the decrease of antigen concentration. However concentration as low as 0.156μg/ml could also be well detected by ELISA and maximum ELISA value was obtained in IgG 103 followed by 104.

4.6.2. PAb raised against cell wall of *F. lamaoensis*

Antigens prepared from cell wall of *F. lamaoensis* were used to raise PAbs. For optimization of ELISA, the effect of two variables, antiserum concentration and antigen concentration on ELISA reactivity, were determined.

Table 11 : ELISA reaction of mycelial PAb of *F. lamaoensis* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (μg/ml)	Absorbance at 405 nm					
	101	**102**	**103**	**104**	**105**	**106**
0.156	0.610 ± 0.07	0.635 ± 0.05	0.882 ± 0.02	0.809 ± 0.09	0.768 ± 0.03	0.757 ± 0.04
0.312	0.823 ± 0.04	0.788 ± 0.01	0.916 ± 0.08	0.907 ± 0.09	0.883 ± 0.07	0.959 ± 0.06
0.625	0.900 ± 0.06	0.911 ± 0.03	1.114 ± 0.06	1.090 ± 0.08	1.029 ± 0.09	1.088 ± 0.09
1.25	1.121 ± 0.04	1.257 ± 0.03	1.443 ± 0.05	1.306 ± 0.06	1.249 ± 0.03	1.288 ± 0.05
2.5	1.428 ± 0.07	1.572 ± 0.04	1.850 ± 0.04	1.706 ± 0.09	1.487 ± 0.04	1.355 ± 0.06
5	1.637 ± 0.05	1.901 ± 0.04	2.235 ± 0.06	1.998 ± 0.03	1.812 ± 0.03	1.529 ± 0.02
10	1.714 ± 0.04	2.070 ± 0.06	2.317 ± 0.07	2.191 ± 0.03	2.025 ± 0.05	1.663 ± 0.05
20	1.733 ± 0.02	2.161 ± 0.02	2.384 ± 0.05	2.326 ± 0.03	2.098 ± 0.08	1.708 ± 0.09

IgG concentration 40μg/ml.

± Standard error.

4.6.2.1. IgG concentration

A series of IgG concentration tested ranged from 0.312-40μg/ml, prepared from PAb 201. Cell wall antigens were used at a concentration of 10μg/ml. Absorbance values increased with increase in concentration of IgG with a maximum value 2.2. in 40μg/ml (Table 12 & Fig.3).

4.6.2.2. Antigen concentration

ELISA reaction with different concentrations of cell wall antigen from 0.156-20μg/ml were determined at an IgG concentration 40μg/ml in respect of different bleedings. Absorbance values increased with increasing concentration

(Table 13 & Fig. 4B). Result also revealed maximum ELISA values in IgG-203 followed by IgG-204.

Table 12 : ELISA reaction of cell wall antigen of *F. lamaoensis* with different concentration of homologus PAb.

IgGconcentration (µg/ml)	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.832	0.831	0.803	0.822 ± 0.07
0.625	0.912	0.948	0.938	0.933 ± 0.08
1.25	0.998	1.050	1.018	1.022 ± 0.09
2.5	1.338	1.277	1.254	1.290 ± 0.06
5	1.496	1.500	1.477	1.491 ± 0.06
10	1.873	1.847	1.848	1.856 ± 0.07
20	2.038	2.017	2.007	2.021 ± 0.07
40	2.207	2.176	2.164	2.182 ± 0.09

Antigen concentration 10µg/ml
IgG source - 201 PAb
± Standard error.

Table 13 : ELISA reaction of cell wall PAb of *F. lamaoensis* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (µg/ml)	Absorbance at 405 nm				
	101	102	103	104	105
0.156	0.765 ± 0.10	0.786 ± 0.09	0.877 ± 0.10	0.862 ± 0.09	0.732 ± 0.07
0.312	0.891 ± 0.12	0.954 ± 0.08	1.159 ± 0.13	1.071 ± 0.13	0.041 ± 0.06
0.625	1.070 ± 0.13	1.104 ± 0.17	1.338 ± 0.11	1.298 ± 0.10	1.163 ± 0.10
1.25	1.214 ± 0.10	1.330 ± 0.08	1.838 ± 0.08	1.778 ± 0.16	1.442 ± 0.08
2.5	1.411 ± 0.08	1.623 ± 0.11	2.064 ± 0.13	2.011 ± 0.08	1.841 ± 0.12
5	1.711 ± 0.06	1.974 ± 0.13	2.491 ± 0.07	2.422 ± 0.11	2.337 ± 0.11
10	1.948 ± 0.13	2.327 ± 0.14	2.710 ± 0.15	2.653 ± 0.13	2.522 ± 0.10
20	2.251 ± 0.16	2.539 ± 0.11	3.066 ± 0.09	2.838 ± 0.10	2.630 ± 0.12

IgG concentration 40µg/ml
± Standard error.

4.6.3. PAb raised against tea root

Optimization of ELISA was also done using PAb against root antigens of UP-26. The effect of two variables, antiserum concentration and antigen concentration on ELISA reactivity, were determined in this case also.

4.6.3.1. IgG concentration

Homologous reactions were carried out using different concentration of PAb 403 ranging from 0.312-40μg/ml at an antigen concentration of 100μg/ml. A405 values decreased with decrease in concentration as revealed in Table 14 & Fig. 3.

Table 14 : ELISA reaction of tea root (UP-26) antigen with different concentration of homologous PAb.

IgG concentration (μg/ml)	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.452	0.449	0.455	0.452 ± 0.03
0.626	0.523	0.529	0.516	0.523 ± 0.05
1.25	0.592	0.601	0.581	0.591 ± 0.06
2.5	0.675	0.677	0.670	0.674 ± 0.04
5	0.713	0.708	0.725	0.715 ± 0.05
10	0.862	0.832	0.841	0.845 ± 0.07
20	1.035	1.035	1.023	1.031 ± 0.05
40	1.175	1.181	1.206	1.187 ± 0.07

Antigen concentration 10μg/ml
IgG source-403 PAb
± Standard error.

4.6.3.2. Antigen concentration

To determine the effect of concentrations antigen on ELISA reactivity, various concentrations of root antigen ranging from 0.781-100μg/ml were used. IgG was used at a concentration of 40μg/ml. Absorbance value decreased with decrease in concentration (Table 15 & Fig.4C). In both mycelial and cell wall PAb, high A405 values were obtained even with 156ng/ml, indicating that the PAb could detect even lower concentrations. Detection was possible even from the first bleed (Fig. 5).

Optimization of PAbs raised against mycelia and cell wall of *F.lamaoensis* and tea root antigens

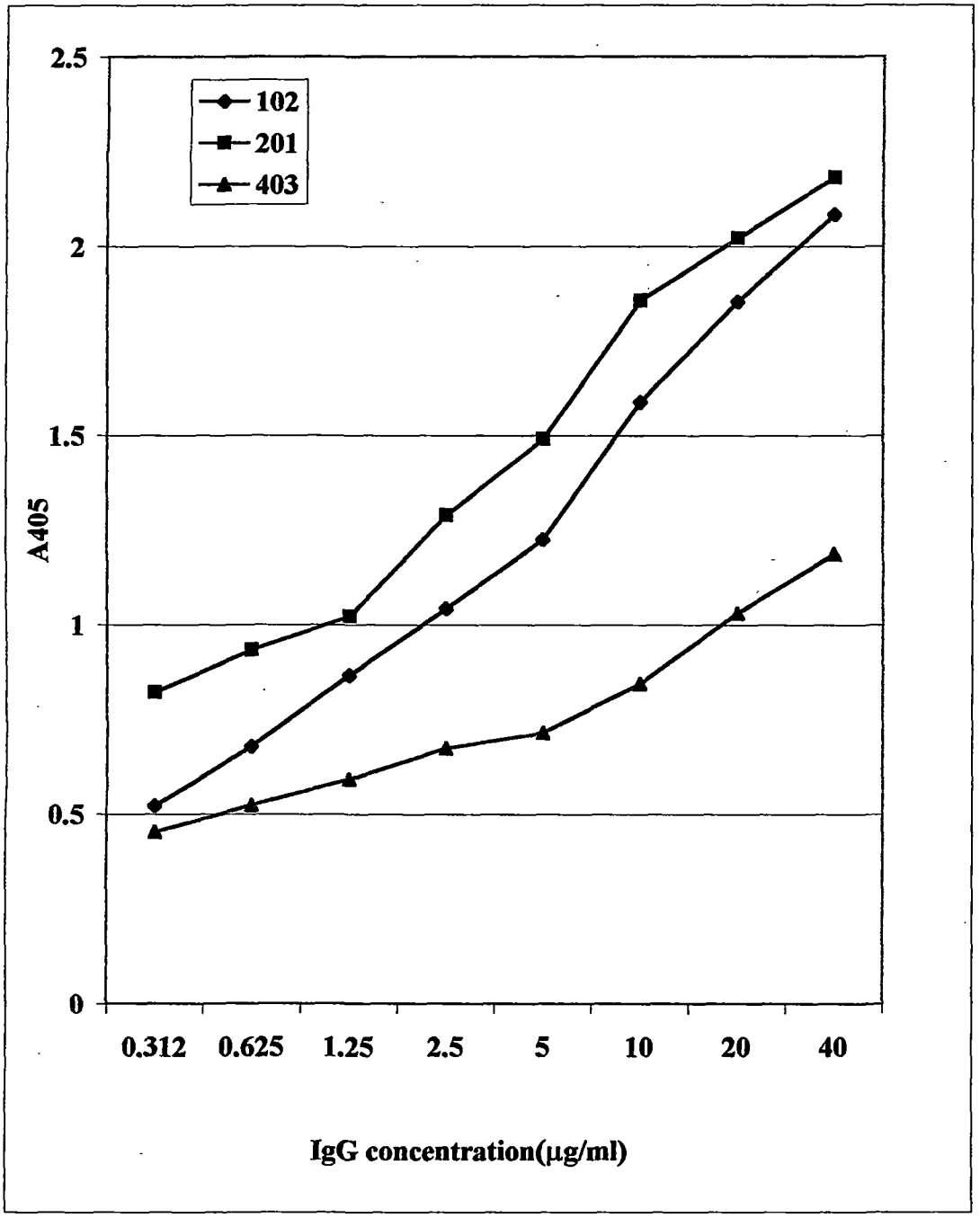


Fig.3

Optimization of antigen concentrations by DAC-ELISA with homologous PAbs

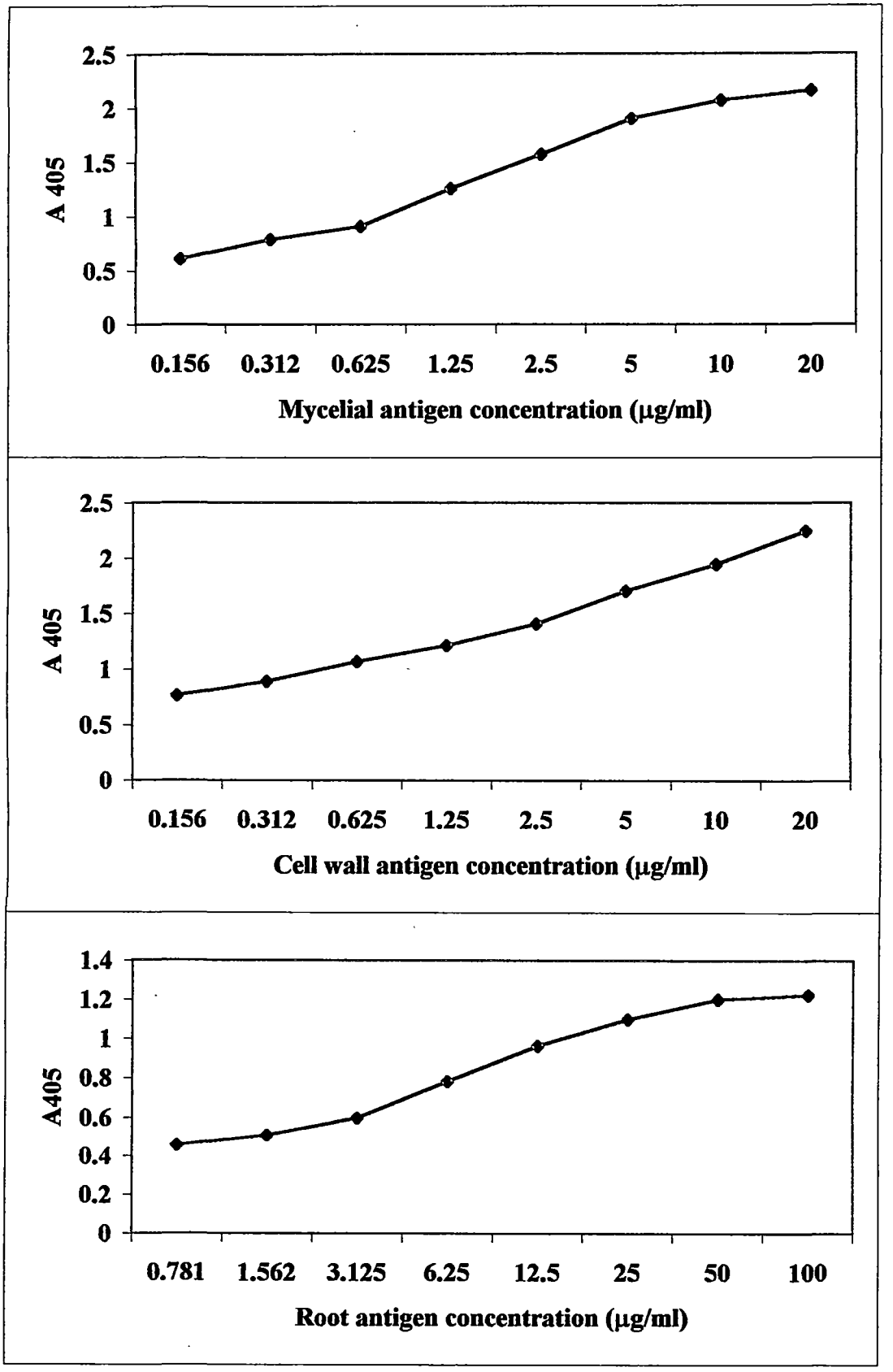


Fig.4

ELISA responses of *F.lamaoensis* mycelial and cell wall antigens with homolous PAbs from different bleedings

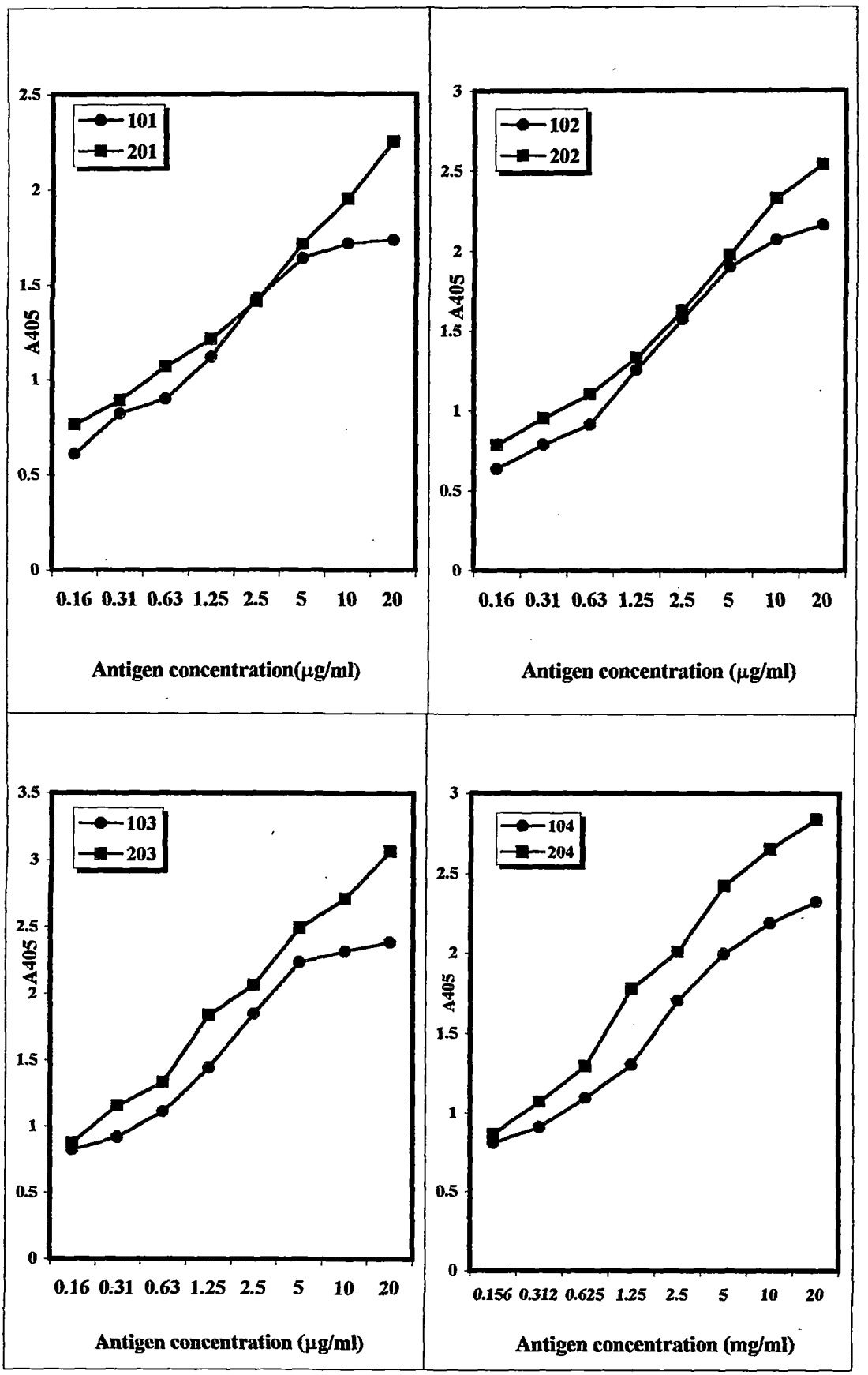


Fig.5

Table 15 : ELISA reaction of tea root (UP-26) PAb with different concentration of homologous antigen

Antigen concentration (μg/ml)	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
0.78	0.454	0.458	0.450	0.454 ± 0.04
1.56	0.511	0.515	0.501	0.509 ± 0.05
3.25	0.594	0.601	0.604	0.600 ± 0.04
6.25	0.780	0.792	0.787	0.786 ± 0.05
12.5	0.956	0.953	0.986	0.965 ± 0.08
25	1.102	1.093	1.112	1.102 ± 0.06
50	1.204	1.202	1.206	1.204 ± 0.03
100	1.227	1.229	1.227	1.228 ± 0.02

IgG source-403 PAb; concentration 40μg/ml; ± Standard error.

4.7. Detection of cross reactive antigens between *F. lamaoensis* and tea roots

4.7.1. Indirect ELISA

Indirect ELISA could readily detect cross reactivity between pathogen antisera and host tissues. Cross reactive antigens have in several host pathogen combinations been shown to be the determinants of susceptibility and resistance. In the present study pathogenicity test of 25 tea varieties revealed different responses ranging from high susceptibility to high resistance. In order to determine whether resistance or susceptibility could be correlated with cross reactive antigens, ELISA tests were carried out with antigens of all varieties and PAbs raised against mycelia and cell wall. The ELISA reactivity of different tea varieties against PAbs of the pathogens was determined and the results were compared with those of the pathogenicity tests.

4.7.1.1. PAb of *F. lamaoensis*

4.7.1.1.1. Mycelial

Antigens were prepared from tea roots of 25 varieties, two non-host, and one non-pathogen as well as mycelia of *F. lamaoensis*. ELISA reaction were carried out with these antigens (at a concentration 100μg/ml for root antigen and 10μg/ml for fungal antigen) against purified mycelial PAb of *F. lamaoensis*. Maximum absorbance values were recorded in reactions with antigens of susceptible varieties (UP-26, TV-18 and T-78); but in general, absorbance values were rather low (Table 16). ELISA

Table 16 : Indirect ELISA values (A405) of antigens of tea root, *F. lamaoensis,* non-pathogen and non host reacted with mycelial PAb of *F. lamaoensis.*

Antigens	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties[a]				
UPASI - 2	0.438	0.440	0.430	0.436 ± 0.04
UPASI - 3	0.472	0.476	0.474	0.474 ± 0.03
UPASI - 8	0.448	0.443	0.444	0.445 ± 0.03
UPASI - 9	0.440	0.424	0.435	0.433 ± 0.05
UPASI - 26	0.648	0.647	0.652	0.649 ± 0.03
BSS - 2	0.613	0.608	0.601	0.607 ± 0.05
TV - 9	0.543	0.533	0.537	0.538 ± 0.04
TV - 18	0.652	0.648	0.653	0.651 ± 0.03
TV - 22	0.555	0.568	0.559	0.561 ± 0.05
TV - 23	0.535	0.535	0.529	0.533 ± 0.03
TV - 25	0.459	0.453	0.452	0.455 ± 0.04
TV - 26	0.466	0.469	0.468	0.468 ± 0.02
TV- 27	0.578	0.571	0.572	0.574 ± 0.04
TV- 28	0.542	0.547	0.555	0.548 ± 0.05
TV- 30	0.509	0.507	0.518	0.511 ± 0.04
T-17/1/54	0.624	0.623	0.634	0.627 ± 0.05
S-449	0.591	0.595	0.593	0.593 ± 0.03
AV-2	0.460	0.451	0.454	0.455 ± 0.04
BS/7A/76	0.452	0.461	0.467	0.460 ± 0.05
CP-1	0.608	0.604	0.605	0.606 ± 0.03
HV-39	0.466	0.472	0.466	0.469 ± 0.04
K1/1	0.517	0.502	0.511	0.510 ± 0.05
P-1258	0.591	0.580	0.584	0.585 ± 0.04
T-78	0.646	0.651	0.652	0.650 ± 0.03
T-135	0.558	0.556	0.561	0.558 ± 0.03
Mycelia of *F. lamaoensis*[b]	2.282	2.265	2.268	2.272 ± 0.06
Non-pathogens[b]				
Fusarium oxysporum	0.466	0.458	0.454	0.459 ± 0.05
Non-host[a]				
Oryza sativa	0.362	0.360	0.371	0.364 ± 0.04
Dracaena	0.340	0.331	0.335	0.334 ± 0.04

[a]Antigen concentration 100µg/ml

[b]Antigen concentration 10µg/ml

IgG source-104 PAb; concentration 40µg/ml

± Standard error.

reactivity of tea root antigens with PAb of *F. lamaoensis* was however seen to increase with age of plant. While in case of 1yr. old plant. A405 were around 0.43, in case of 7yr. plant, it had increased to about 0.73.

4.7.1.1.2. Cell wall

ELISA reactions were carried out with antigens prepared from root of 25 tea varieties against cell wall PAb of *F. lamaoensis*. Results, presented in Table 17 also revealed higher A405 values in case of susceptible varieties, and slightly lower in case of resistant ones.

When ELISA responses of tea root antigens with PAb of mycelia and cell wall were compared (Fig.6), it was observed that values were always higher in case of cell wall PAb.

4.7.1.2.PAb of tea root

PAb raised against healthy tea root antigen (UP-26) was tested to detect the cross reactive antigens of the root tissues of other tea varieties as well as root-pathogens. The PAb of UP-26 reacted with the antigens of all other varieties and A405 values were quite high (Table 18) A405 values were even higher where this PAb was treated with antigens of tea root pathogens PAb of the tea root reacted with the antigens of all the tested pathogens, with A405 values ranging from 0.9-1.04 (Fig.7).

4.7.2. Western blot

Cross reactivity between *F. lamaoensis* and tea root antigens were also determined by Western Blot analysis. It was observed that when healthy tea root extracts wer probed with PAb of *F. lamaoensis,* in the susceptible varieties 2-3 bands out of 4 bands (ca.79, 50, 21 and 19 kDA) were visible while in the others no bands or sometimes only 1 (ca. 50 kDa) was evident. In case of homologous reactions of mycelial and cellwall PAb showed 16 bands (ranging from ca. 104 to 13 kDa) and 11 (ranging from ca. 102 to 17 kDa) respectively (Table 19 and Plate 11, fig. A&B), were observed.

Table 17 : Indirect ELISA values (A405) of tea root antigens, cell wall antigen of *F. lamaoensis,* non-pathogen and non host reacted with PAb of *F. lamaoensis* raised against cell wall immunogens.

Antigens	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties[a]				
UPASI - 2	0.537	0.541	0.531	0.536 ± 0.04
UPASI - 3	0.543	0.557	0.549	0.560 ± 0.05
UPASI - 8	0.558	0.549	0.556	0.554 ± 0.05
UPASI - 9	0.518	0.522	0.516	0.519 ± 0.03
UPASI - 26	0.687	0.690	0.689	0.689 ± 0.02
BSS - 2	0.628	0.631	0.627	0.629 ± 0.03
TV - 9	0.569	0.568	0.563	0.567 ± 0.03
TV - 18	0.698	0.699	0.686	0.694 ± 0.05
TV - 22	0.589	0.593	0.596	0.593 ± 0.03
TV - 23	0.560	0.555	0.566	0.560 ± 0.04
TV - 25	0.537	0.541	0.532	0.537 ± 0.04
TV - 26	0.530	0.527	0.536	0.531 ± 0.04
TV - 27	0.621	0.618	0.627	0.622 ± 0.04
TV - 28	0.603	0.609	0.607	0.606 ± 0.03
TV - 30	0.559	0.555	0.564	0.559 ± 0.04
T-17/1/54	0.651	0.659	0.656	0.655 ± 0.04
S-449	0.614	0.604	0.615	0.611 ± 0.05
AV-2	0.535	0.542	0.535	0.537 ± 0.04
BS/7A/76	0.508	0.509	0.500	0.508 ± 0.02
CP-1	0.622	0.612	0.620	0.618 ± 0.04
HV-39	0.524	0.526	0.517	0.530 ± 0.05
K1/1	0.541	0.548	0.539	0.543 ± 0.04
P-1258	0.617	0.628	0.629	0.625 ± 0.05
T-78	0.702	0.708	0.699	0.703 ± 0.04
T-135	0.564	0.579	0.575	0.573 ± 0.05
Cell wall of *F. lamaoensis*[b]	2.782	2.778	2.785	2.787 ± 0.03
Non-pathogens[b]				
Beauveria bassiana	0.366	0.377	0.368	0.370 ± 0.04
Non-host[a]				
Leucaena leucocephala	0.370	0.374	0.361	0.368 ± 0.05
Phyllanthus niruri	0.352	0.343	0.355	0.350 ± 0.05

[a]Antigen concentration 100µg/ml

[b]Antigen concentration 10µg/ml

IgG source-204 PAb; concentration 40µg/ml

± Standard error.

Table 18 : Indirect ELISA values (A405) of tea root antigens, and mycelial antigens of root pathogens with PAb raised against tea (UP-26) root immunogens.

Antigens	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties[a]				
UPASI - 2	0.909	0.901	0.910	0.907 ± 0.04
UPASI - 3	0.916	0.923	0.911	0.917 ± 0.05
UPASI - 8	0.836	0.826	0.831	0.831 ± 0.04
UPASI - 9	0.809	0.804	0.803	0.805 ± 0.03
UPASI - 26	1.215	1.213	1.211	1.213 ± 0.03
BSS - 2	0.885	0.871	0.855	0.870 ± 0.07
TV - 9	0.831	0.828	0.831	0.830 ± 0.02
TV - 18	0.819	0.824	0.830	0.824 ± 0.04
TV - 22	0.826	0.827	0.821	0.825 ± 0.03
TV - 23	0.840	0.837	0.833	0.837 ± 0.03
TV - 25	0.796	0.787	0.792	0.792 ± 0.04
TV - 26	0.805	0.811	0.812	0.809 ± 0.04
TV - 27	0.865	0.881	0.867	0.871 ± 0.05
TV - 28	0.825	0.832	0.837	0.831 ± 0.05
TV - 30	0.885	0.875	0.872	0.877 ± 0.05
T-17/1/54	0.872	0.869	0.874	0.872 ± 0.03
S-449	0.741	0.745	0.750	0.745 ± 0.04
AV-2	0.704	0.712	0.713	0.709 ± 0.04
BS/7A/76	0.749	0.733	0.745	0.742 ± 0.05
CP-1	0.772	0.763	0.775	0.770 ± 0.05
HV-39	0.747	0.749	0.735	0.744 ± 0.05
K1/1	0.782	0.788	0.779	0.783 ± 0.04
P-1258	1.002	1.010	0.998	1.003 ± 0.05
T-78	0.933	0.942	0.940	0.938 ± 0.04
T-135	1.035	1.026	1.023	1.028 ± 0.05
Pathogens[b]				
F. lamaoensis	1.045	1.032	1.043	1.040 ± 0.05
P. hypobrumia	1.043	1.039	1.048	1.043 ± 0.04
S. repens	0.989	0.998	0.999	0.995 ± 0.04
Armillaria mellea	0.882	0.858	0.856	0.862 ± 0.05
Ustulina zonata	0.911	0.919	0.920	0.917 ± 0.04

[a]Antigen concentration 100µg/ml
[b]Antigen concentration 10µg/ml
Age of plant 3 yrs.
IgG source - 403 PAb ; concentration 40µg/ml
± Standard error.

Cross reactivity of tea root antigens against mycelial and cell wall PAbs of *F.lamaoensis*

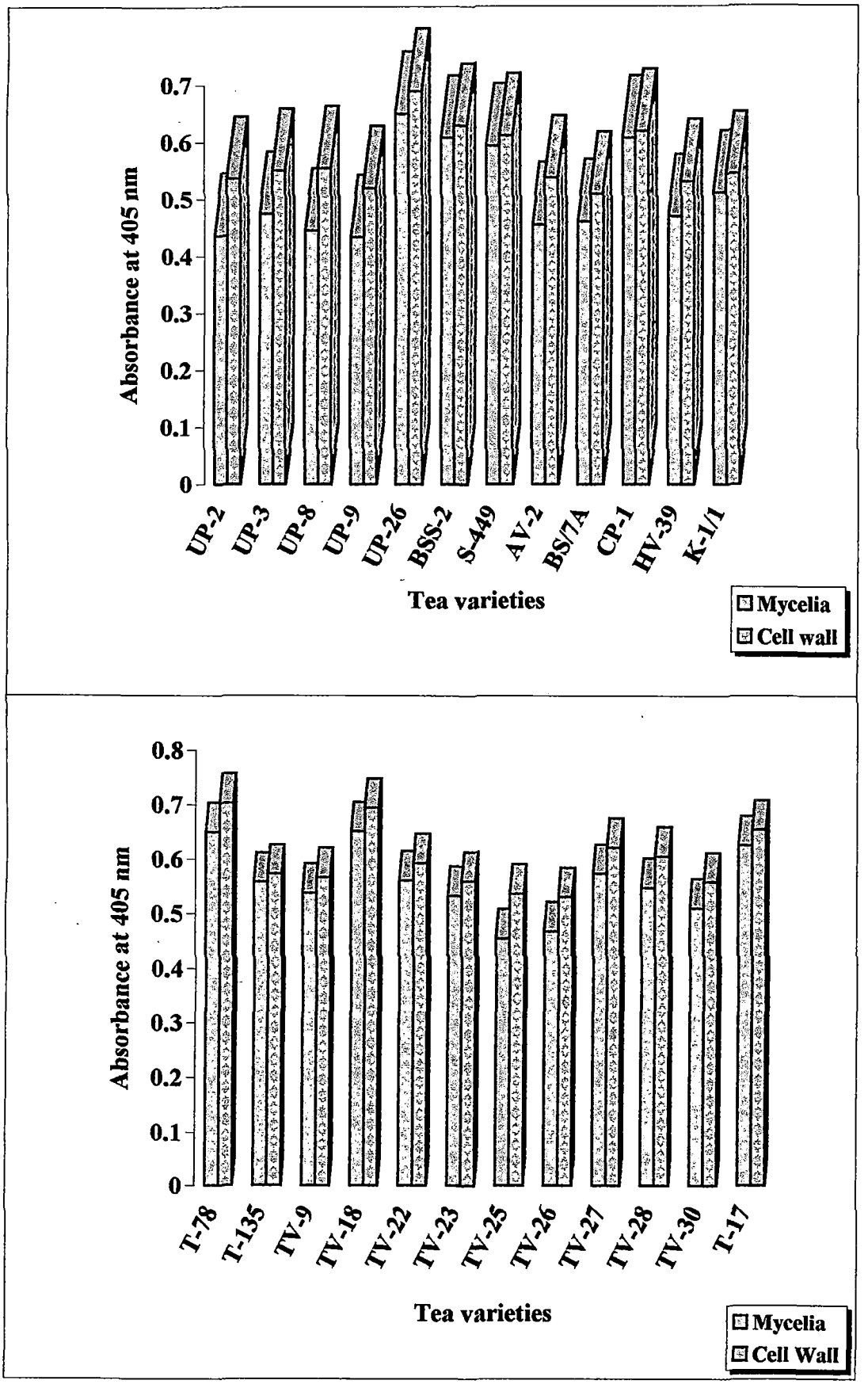
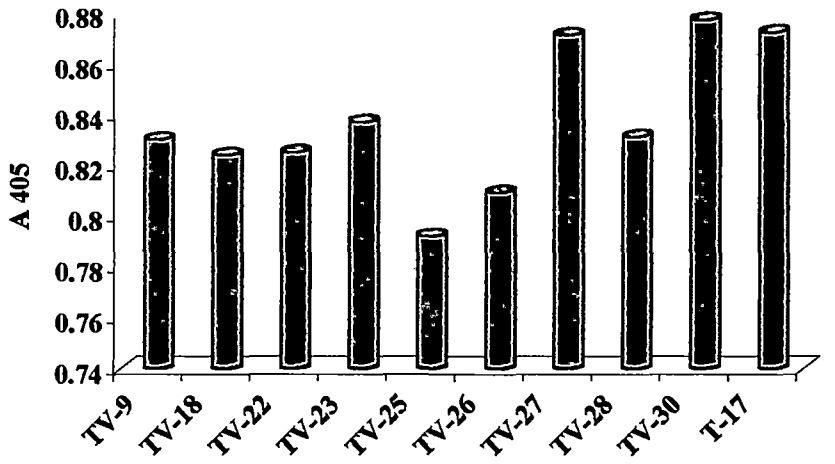
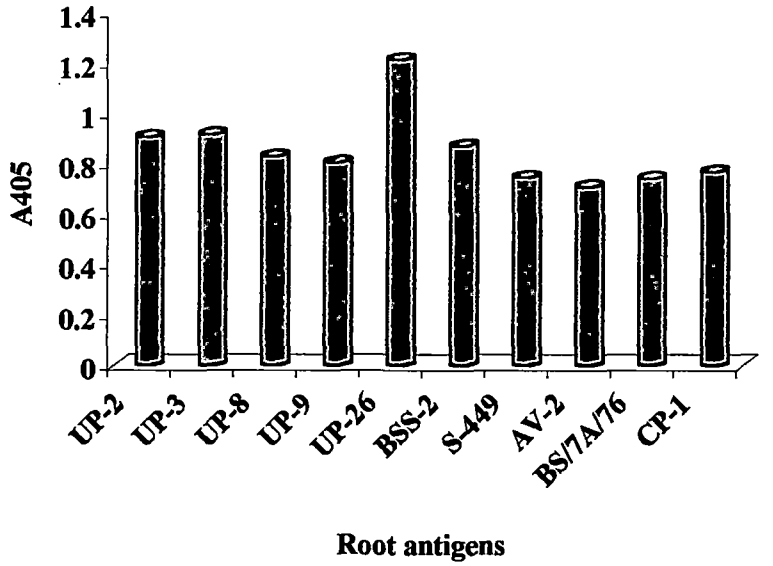


Fig.6

Cross reactivity of tea root PAb with root and pathogen antigens





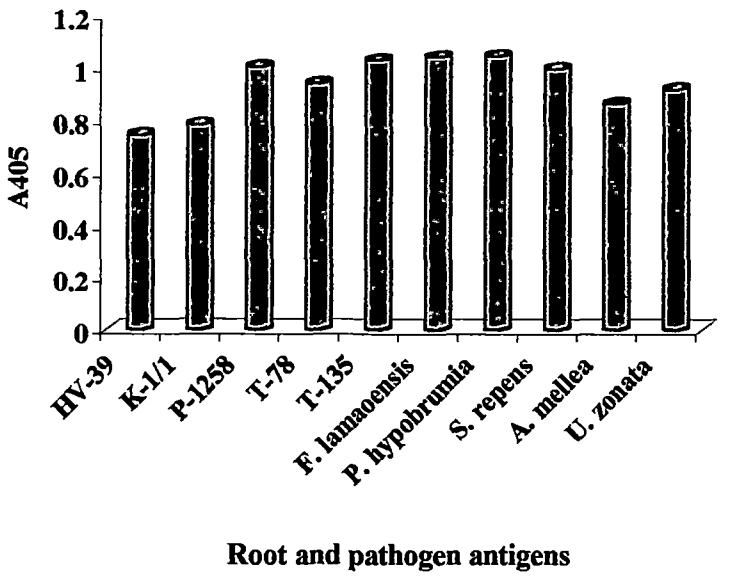


Fig.7

Table 19 : Western blot analysis of mycelial and cell wall proteins of *F. lamaoensis.*

Mycelia	16	104.7, 97.4, 79.4, 60.9, 50.1, 43.0, 38.6, 32.2, 27.1, 24.5, 19.0, 18.4, 17.4, 15.3, 14.3, 12.7.
Cell wall	11	102.0, 88.2, 83.6, 68.0, 63.5, 59.0, 54.5, 43.0, 38.6, 34.5, 17.0.

Western blotting using PAb of tea root (UP-26) revealed that the homologous antigens showed 15 bands ranging from 97.4 to 18.5kDa (Plate 11, fig.E). Antigens of seven healthy tea roots (BSS-2, UP-9, T-17, TV-18, TV-26, CP-1 and T-78) were probed with PAb-403 to determine the cross reactivity among different tea varieties. The cross reactive antigens were found in case of BSS-2 (i.e. ca. 79 and 50 kDa bands) while UP-9 showed 8 bands ranging from 97.4 to 18.5 kDa. The band patterns were more or less same in case of Tocklai varieties with appearance of 7-8 bands, most of which were of higher molecular weight and a single thick, very prominent band (ca. 70 kDa). An additional band of about 24 kDa was found in TV-18 and a band of about 97 kDa was absent in TV-26. The two Darjeeling varieties (CP-1 and T-78) tested showed 5-6 bands, of which 3 bands (ca. 97, 59 and 24kDa) were common (Plate11, fig.F).

4.8. Detection of *F. lamaoensis* in infected tea root tissues

To detect the pathogen in host tissues a number of immunodetection assays have proved effective where antisera raised against the pathogen reacted with antigens of infected material to give high absorbance values. These include ELISA, Western Blot and Dot Blot analyses. In the present study following pathogenicity test and determination of cross reactive antigens in the different varieties attempts were made to detect *F. lamaoensis* in infected root tissues of different varieties, ages as well as after different days of inoculation, by ELISA, Western Blot and Dot Blot assays.

4.8.1. ELISA

ELISA is the most routinely used detection assay as the reactivity can be measured quantitatively by difference in A405 values between healthy and infected host antigens. Two commonly used ELISA formats are DAC-ELISA and DAS-ELISA which differ in the time of PAb coating.

4.8.1.1. DAC-ELISA

4.8.1.1.1. PAb of mycelia

4.8.1.1.1.1. Artificially infected root tissues

PAb raised against mycelia of *F. lamaoensis* was reacted in ELISA against the root antigens prepared from healthy and inoculated roots of all 25 varieties (Tocklai, UPASI and Darjeeling) of 2 yr old tea plants. Antigens were prepared from healthy and inoculated root tissues at 15, 30 and 45 day intervals following the date of inoculation. Results revealed that in all tested varieties infected extracts showed higher ELISA values than the healthy extracts but the difference was not significance except in susceptible varieties. In Tocklai varieties maximum ELISA values were obtained in TV-18 and T-17 (Table 20). Among the Darjeeling varieties the infected extracts of T-78 followed by S-449 showed maximum A405 values (Table 21). Among UPASI varieties, the maximum and significant difference between infected extracts and healthy extracts was obtained in UP-26 followed by BSS-2 (Table 22). Significantly higher ELISA values in infected root extracts was obtained in the susceptible varieties (Fig 8) and over all comparison showed that the difference between infected and healthy extract in Tocklai varieties (except TV-26 and TV-9) was higher than that of other two varieties.

Subsequently, tea root antigens from healthy and infected extracts of nine selected varieties were tested with PAb of *F. lamaoensis* mycelia, obtained from 6 bleeds (101-106). Significant differences between healthy and infected tea root antigens were obtained in all varieties and against PAb from all bleedings. Maximum A405 values were obtained in 3rd and 4th bleed (103 & 104). Thus detection could be done even with PAb of 1st bleed (Table 23, Fig.9).

Table 20 : ELISA values showing reaction of PAb of *F. lamaoensis* with antigens of healthy and inoculated tea roots of Tocklai varieties.

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	0.461 ± 0.05	0.585 ± 0.04	0.516 ± 0.03	0.697 ± 0.04	0.519 ± 0.03	0.695 ± 0.07
TV-18	0.578 ± 0.03	0.743 ± 0.05	0.592 ± 0.04	0.814 ± 0.03	0.604 ± 0.04	0.841 ± 0.02
TV-22	0.557 ± 0.05	0.659 ± 0.03	0.568 ± 0.03	0.672 ± 0.04	0.560 ± 0.03	0.692 ± 0.05
TV-23	0.509 ± 0.03	0.638 ± 0.05	0.531 ± 0.04	0.645 ± 0.04	0.541 ± 0.06	0.673 ± 0.03
TV-25	0.475 ± 0.04	0.579 ± 0.03	0.498 ± 0.04	0.572 ± 0.05	0.523 ± 0.04	0.605 ± 0.06
TV-26	0.459 ± 0.05	0.544 ± 0.03	0.488 ± 0.04	0.550 ± 0.04	0.517 ± 0.05	0.592 ± 0.05
TV-27	0.559 ± 0.04	0.686 ± 0.02	0.577 ± 0.04	0.685 ± 0.05	0.569 ± 0.05	0.706 ± 0.03
TV-28	0.539 ± 0.06	0.642 ± 0.04	0.541 ± 0.03	0.665 ± 0.03	0.559 ± 0.08	0.675 ± 0.06
TV-30	0.503 ± 0.03	0.630 ± 0.03	0.501 ± 0.04	0.648 ± 0.03	0.537 ± 0.03	0.664 ± 0.06
T-17/1/54	0.580 ± 0.08	0.702 ± 0.04	0.588 ± 0.05	0.769 ± 0.05	0.587 ± 0.04	0.793 ± 0.06

Age of plant 2 yr.
Antigen concentration 100µg/ml
IgG source-104 PAb; concentration 40µg/ml
± Standard error.

Table 21 : ELISA values showing reaction of PAb of *F. lamaoensis* with antigens of healthy and inoculated tea roots of UPASI varieties.

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
UP-2	0.436 ± 0.03	0.535 ± 0.03	0.438 ± 0.04	0.545 ± 0.06	0.431 ± 0.03	0.618 ± 0.04
UP-3	0.476 ± 0.04	0.501 ± 0.04	0.465 ± 0.03	0.585 ± 0.03	0.477 ± 0.06	0.684 ± 0.02
UP-8	0.445 ± 0.04	0.515 ± 0.02	0.459 ± 0.03	0.553 ± 0.03	0.488 ± 0.05	0.571 ± 0.04
UP-9	0.440 ± 0.01	0.558 ± 0.02	0.448 ± 0.04	0.561 ± 0.04	0.448 ± 0.03	0.584 ± 0.04
UP-26	0.581 ± 0.03	0.684 ± 0.03	0.592 ± 0.04	0.806 ± 0.04	0.594 ± 0.04	0.872 ± 0.04
BSS-2	0.568 ± 0.03	0.599 ± 0.03	0.561 ± 0.04	0.750 ± 0.06	0.558 ± 0.07	0.781 ± 0.06

Age of plants 2 yr.
Antigen concentration 100µg/ml
IgG of 104 PAb; concentration 40µg/ml
± Standard error.

In a further experiment, it was decided to determine ELISA responses of healthy and inoculated tea root extracts obtained from plants of ages varying 1-5 yr. A405 values of healthy and infected extracts increased with the age of the plants. In susceptible varieties the A405 of infected extracts of plants 3 yr and above were very high, whereas in resistant varieties, they did not increase with age (Table 24). A good correlation was therefore obtained with the disease symptoms rated visually and ELISA responses (Fig.10). It seems probable that the entry of the mycelia in resistant varieties is rather restricted, whereas in susceptible varieties, the mycelia had spread into the tissues of the older plants.

Table 22 : ELISA values showing reaction of PAb of *F. lamaoensis* with antigens of healthy and inoculated tea roots of Darjeeling varieties.

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
AV-2	0.445 ± 0.04	0.480 ± 0.05	0.448 ± 0.07	0.569 ± 0.05	0.457 ± 0.03	0.618 ± 0.04
BS/7/A/76	0.392 ± 0.04	0.471 ± 0.05	0.406 ± 0.06	0.561 ± 0.05	0.419 ± 0.04	0.579 ± 0.05
CP-1	0.558 ± 0.03	0.575 ± 0.03	0.559 ± 0.05	0.673 ± 0.07	0.558 ± 0.05	0.726 ± 0.04
HV-39	0.458 ± 0.02	0.570 ± 0.04	0.448 ± 0.02	0.578 ± 0.09	0.457 ± 0.04	0.595 ± 0.04
K1/1	0.486 ± 0.09	0.498 ± 0.04	0.483 ± 0.04	0.572 ± 0.08	0.478 ± 0.03	0.591 ± 0.03
P-1258	0.562 ± 0.05	0.591 ± 0.04	0.568 ± 0.0	0.684 ± 0.07	0.581 ± 0.06	0.701 ± 0.08
T-78	0.580 ± 0.03	0.686 ± 0.02	0.580 ± 0.03	0.727 ± 0.05	0.595 ± 0.08	0.896 ± 0.07
T-135	0.481 ± 0.03	0.523 ± 0.09	0.473 ± 0.05	0.633 ± 0.04	0.477 ± 0.07	0.651 ± 0.04
S-449	0.558 ± 0.02	0.592 ± 0.07	0.552 ± 0.06	0.699 ± 0.04	0.568 ± 0.05	0.787 ± 0.09

Age of plants 2 yr.
Antigen concentration 100µg/ml
IgG of 104 PAb; concentration 40µg/ml
± Standard error.

Table 23 : DAC-ELISA values of healthy and infected tea root antigens with PAb of *F. lamaoensis* raised against mycelial antigen in respect of different bleedings.

Tea varieties	Absorbance at 405nm											
	IgG number											
	101		102		103		104		105		106	
	H	I	H	I	H	I	H	I	H	I	H	I
T-17	0.370 ±0.04	0.587 ±0.04	0.465 ±0.03	0.689 ±0.04	0.573 ±0.03	0.783 ±0.04	0.563 ±0.07	0.774 ±0.02	0.543 ±0.03	0.754 ±0.03	0.521 ±0.04	0.721 ±0.03
TV-18	0.379 ±0.02	0.609 ±0.04	0.494 ±0.05	0.701 ±0.03	0.602 ±0.04	0.812 ±0.05	0.612 ±0.04	0.825 ±0.03	0.582 ±0.03	0.783 ±0.04	0.546 ±0.05	0.745 ±0.04
TV-27	0.374 ±0.08	0.543 ±0.05	0.449 ±0.03	0.653 ±0.06	0.539 ±0.03	0.713 ±0.04	0.546 ±0.04	0.718 ±0.03	0.516 ±0.04	0.699 ±0.03	0.488 ±0.03	0.643 ±0.05
CP-1	0.378 ±0.03	0.576 ±0.03	0.478 ±0.04	0.648 ±0.04	0.524 ±0.04	0.706 ±0.04	0.554 ±0.04	0.730 ±0.05	0.514 ±0.05	0.701 ±0.03	0.504 ±0.03	0.648 ±0.05
T-78	0.382 ±0.04	0.611 ±0.03	0.492 ±0.04	0.735 ±0.06	0.593 ±0.06	0.835 ±0.07	0.603 ±0.02	0.815 ±0.07	0.579 ±0.09	0.773 ±0.09	0.559 ±0.04	0.698 ±0.03
S-449	0.364 ±0.08	0.563 ±0.09	0.451 ±0.07	0.621 ±0.05	0.543 ±0.08	0.732 ±0.06	0.546 ±0.05	0.721 ±0.07	0.523 ±0.07	0.689 ±0.03	0.594 ±0.06	0.632 ±0.05
BSS-2	0.364 ±0.06	0.572 ±0.06	0.459 ±0.04	0.665 ±0.05	0.553 ±0.06	0.726 ±0.04	0.558 ±0.06	0.713 ±0.05	0.528 ±0.06	0.682 ±0.03	0.487 ±0.04	0.637 ±0.03
UP-3	0.347 ±0.07	0.503 ±0.05	0.426 ±0.05	0.557 ±0.04	0.476 ±0.07	0.612 ±0.09	0.472 ±0.06	0.615 ±0.04	0.419 ±0.02	0.585 ±0.03	0.402 ±0.07	0.558 ±0.03
UP-26	0.379 ±0.05	0.642 ±0.05	0.486 ±0.06	0.702 ±0.04	0.591 ±0.03	0.812 ±0.05	0.595 ±0.02	0.805 ±0.04	0.577 ±0.06	0.787 ±0.08	0.550 ±0.03	0.739 ±0.04

Age of plants 2 year
Antigen concentration 100µg/ml
IgG concentration 40µg/ml
40 days after inoculation
± Standard error.

ELISA responses of PAb of *F.lamaoensis* with antigens of healthy and inoculated tea root tissues

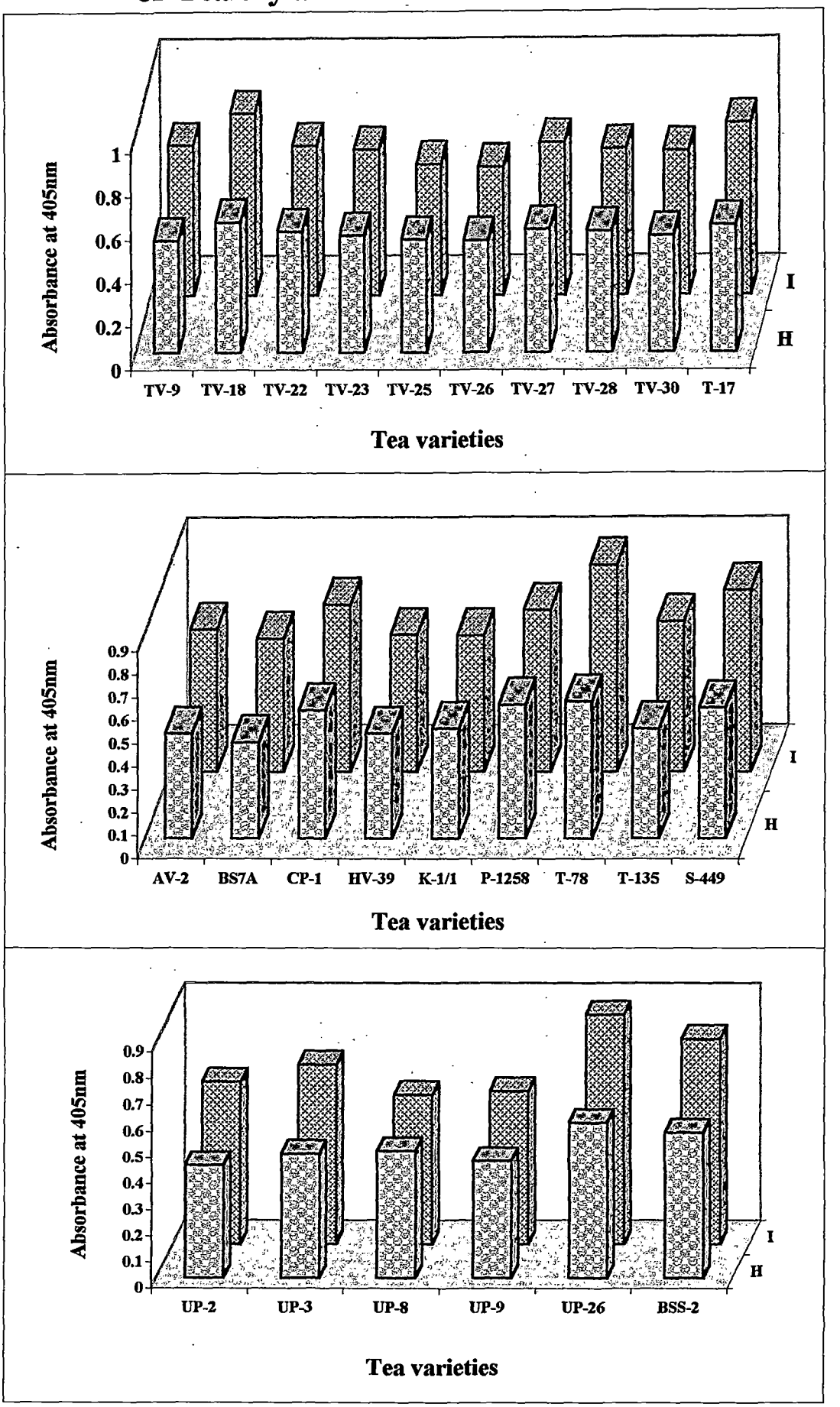


Fig.8

ELISA responses of healthy and inoculated tea root antigens with PAb of *F.lamaoensis* (mycelia)

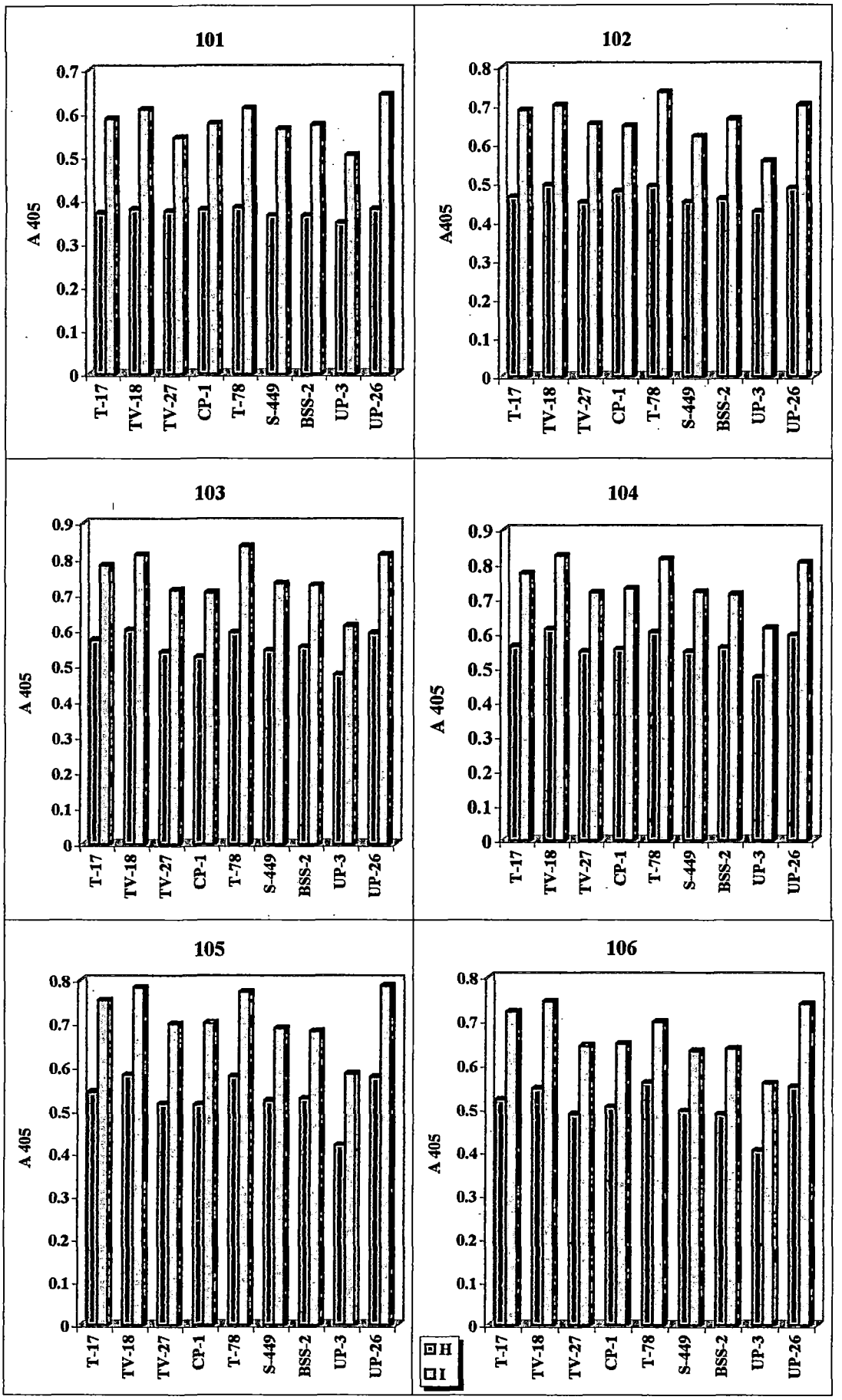


Fig.9

Table 24 : Indirect ELISA (A405nm) of healthy and inoculated roots of a few selected tea varieties of different ages reacted with PAb of *F. lamaoensis*

Tea varieties	Absorbance at 405 n							
	Age of the plants (year)							
	1 Yr		2 Yr		3 Yr		5 Yr	
	H	Inf	H	Inf	H	Inf	H	Inf
T-17	0.506 ±0.04	0.712 ±0.03	0.522 ±0.05	0.807 ±0.04	0.574 ±0.04	0.988 ±0.02	0.649 ±0.04	1.107 ±0.03
TV-18	0.572 ±0.03	0.743 ±0.05	0.574 ±0.03	0.845 ±0.03	0.642 ±0.04	1.147 ±0.03	0.658 ±0.04	1.324 ±0.04
TV-26	0.468 ±0.05	0.530 ±0.03	0.450 ±0.04	0.542 ±0.06	0.471 ±0.05	0.534 ±0.03	0.478 ±0.04	0.625 ±0.05
UP-26	0.584 ±0.03	0.696 ±0.05	0.592 ±0.05	0.866 ±0.06	0.649 ±0.05	1.102 ±0.05	0.661 ±0.02	1.418 ±0.05
T-78	0.568 ±0.04	0.709 ±0.04	0.562 ±0.03	0.883 ±0.05	0.655 ±0.03	1.211 ±0.04	0.683 ±0.05	1.546 ±0.02

Antigen concentration 100 µg/ml
IgG source-103 PAb; concentration 40µg/ml
After 40 days of inoculation
± Standard error.

4.8.1.1.1.2. Naturally in infected root tissues

As the PAb of *F. lamaoensis* was used to detect infection in artificially inoculated root tissues it was decided to test whether natural brown root rot infection could be detected with this PAb. For this, infected tea roots were collected from different tea gardens showing symptoms of brown root rot infection and antigens were prepared from these roots. Using antigen extracts from healthy and naturally infected tea roots ELISA was performed against mycelial PAb of *F. lamaoensis*. Results presented in Table 25 revealed the infected extract of TV-18 varieties had significantly higher absorbance values in comparison to the healthy extracts.

DAC-ELISA response of healthy and inoculated tea roots and disease development

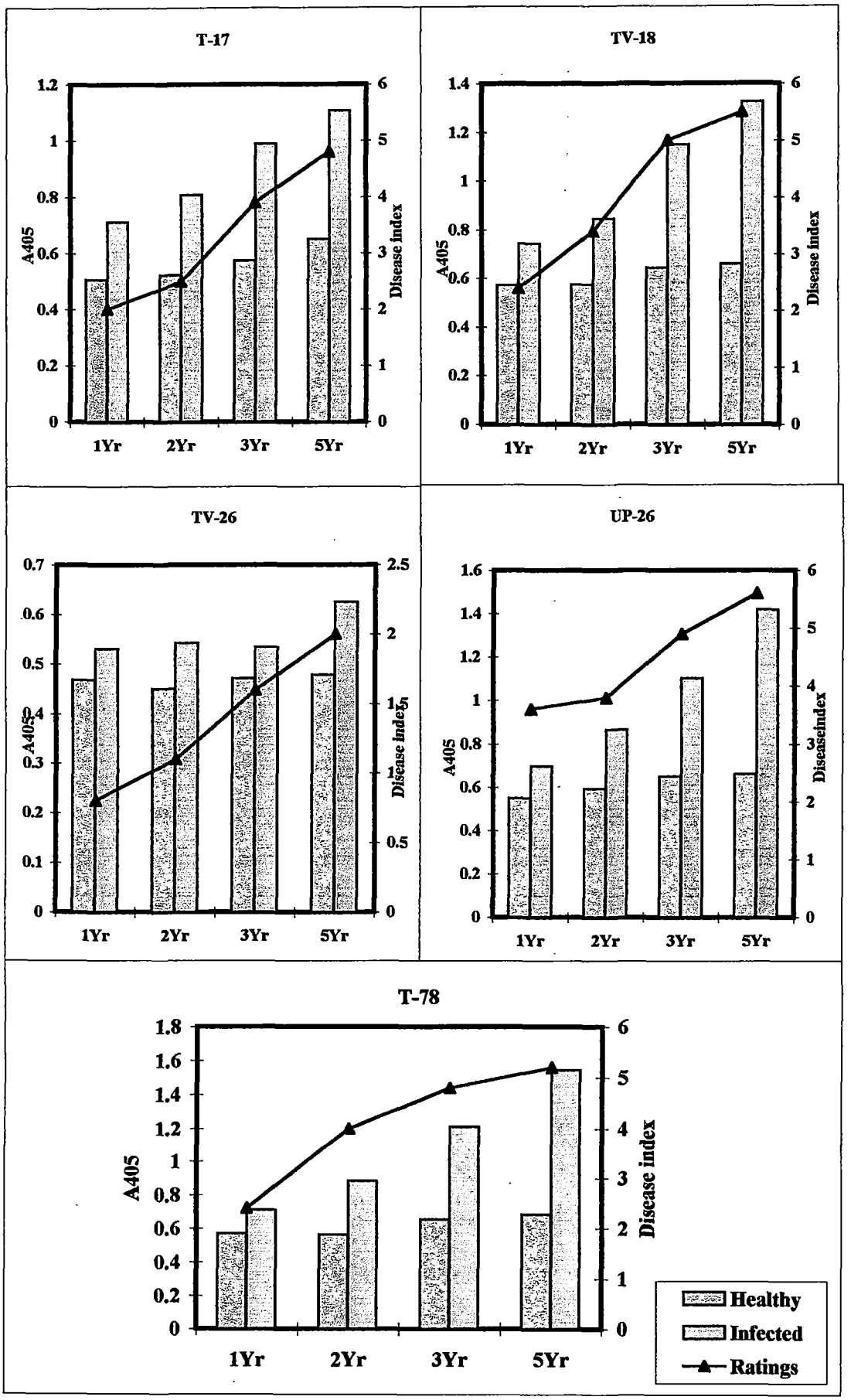


Fig.10

Table 25 : DAC-ELISA values of healthy and naturally infected tea root antigens with PAb of *F. lamaoensis*

Tea gardens	Tea varieties	Absorbance at 405 nm	
		Healthy	Infected
Bijoynagar Tea Estate	T-17/1/54	0.588 ± 0.03	0.799 ± 0.05
	TV-18	0.606 ± 0.04	1.028 ± 0.09
	TV-30	0.531 ± 0.04	0.778 ± 0.04
	TV-28	0.546 ± 0.02	0.784 ± 0.06
Matigara Tea Estate	T-17	0.588 ± 0.03	0.813 ± 0.06
	TV-18	0.606 ± 0.04	0.900 ± 0.05
	TV-27	0.564 ± 0.05	0.709 ± 0.07
Trihana Tea Estate	TV-18	0.606 ± 0.04	0.988 ± 0.09
	TV-30	0.531 ± 0.04	0.610 ± 0.08
Cooch Behar Tea Estate	T-17	0.588 ± 0.03	0.778 ± 0.06
	TV-18	0.606 ± 0.05	0.849 ± 0.06
	TV-22	0.551 ± 0.06	0.681 ± 0.03

Age of plants 3yr.
Antigen concentration 100µg/ml
IgG source-103 PAb; concentration 40µg/ml
± Standard error

4.8.1.1.2. PAb of cell wall

In this experiment, healthy and inoculated root antigens were prepared from nine tea varieties and tested against the PAb raised against cell wall *F. lamaoensis* and DAC-ELISA reactions were carried out using 201, 202, 203, 204, 205 PAbs. ELISA values in general were higher in this case than mycelial PAb. Infected root antigens gave higher reactivity than healthy ones (Table 26 & Fig.11) with results similar as in case of mycelial PAb.

4.8.1.2. DAS-ELISA with PAb of mycelia

DAS-ELISA, in contrast to DAC-ELISA, involves coating of ELISA plates first with the primary antibody, followed by antigens, which again is followed by secondary antibody labelled with enzyme. Detection of *F. lamaoensis* in the infected roots of selected varieties using the DAS-ELISA format was also carried out followed the

Table 26 : DAC-ELISA values of healthy and infected tea root antigens with PAb of *F. lamaoensis* raised against cell wall in respect of different bleedings.

	Absorbance at 405nm									
	IgG number									
	201		202		203		204		205	
Tea varieties	H	I	H	I	H	I	H	I	H	I
T-17	0.465 ±0.04	0.663 ±0.11	0.561 ±0.07	0.703 ±0.08	0.608 ±0.04	0.828 ±0.05	0.592 ±0.03	0.811 ±0.08	0.563 ±0.03	0.722 ±0.02
TV-18	0.482 ±0.06	0.687 ±0.02	0.598 ±0.09	0.722 ±0.06	0.642 ±0.03	0.872 ±0.10	0.651 ±0.03	0.868 ±0.09	0.592 ±0.05	0.801 ±0.09
TV-27	0.438 ±0.01	0.635 ±0.08	0.549 ±0.06	0.669 ±0.05	0.595 ±0.06	0.752 ±0.03	0.588 ±0.03	0.758 ±0.04	0.561 ±0.04	0.702 ±0.04
CP-1	0.459 ±0.07	0.633 ±0.05	0.558 ±0.04	0.691 ±0.07	0.592 ±0.06	0.763 ±0.05	0.579 ±0.06	0.761 ±0.06	0.559 ±0.02	0.715 ±0.05
T-78	0.477 ±0.07	0.672 ±0.03	0.593 ±0.03	0.728 ±0.04	0.638 ±0.07	0.886 ±0.04	0.645 ±0.04	0.871 ±0.04	0.588 ±0.03	0.812 ±0.05
S-449	0.448 ±0.03	0.612 ±0.05	0.552 ±0.05	0.636 ±0.06	0.562 ±0.02	0.719 ±0.04	0.578 ±0.07	0.711 ±0.03	0.532 ±0.09	0.685 ±0.04
BSS-2	0.440 ±0.10	0.618 ±0.05	0.548 ±0.03	0.642 ±0.05	0.568 ±0.06	0.722 ±0.07	0.561 ±0.01	0.701 ±0.08	0.548 ±0.04	0.688 ±0.05
UP-3	0.421 ±0.06	0.526 ±0.08	0.448 ±0.08	0.573 ±0.04	0.538 ±0.09	0.602 ±0.04	0.521 ±0.04	0.598 ±0.05	0.487 ±0.07	0.566 ±0.03
UP-26	0.480 ±0.04	0.699 ±0.03	0.588 ±0.03	0.789 ±0.03	0.632 ±0.03	0.918 ±0.07	0.636 ±0.05	0.903 ±0.04	0.583 ±0.03	0.881 ±0.08

Age of plants 2 year
Antigen concentration 100µg/ml
IgG concentration 40µg/ml
40 days after inoculation
± Standard error.

method described under materials and methods. IgG from 104 bleeding was used at a concentration of 40µg/ml in this experiment. Results presented in Table 27, revealed

ELISA responses of healthy and inoculated tea root antigens with PAb of *F.lamaoensis* (cell wall)

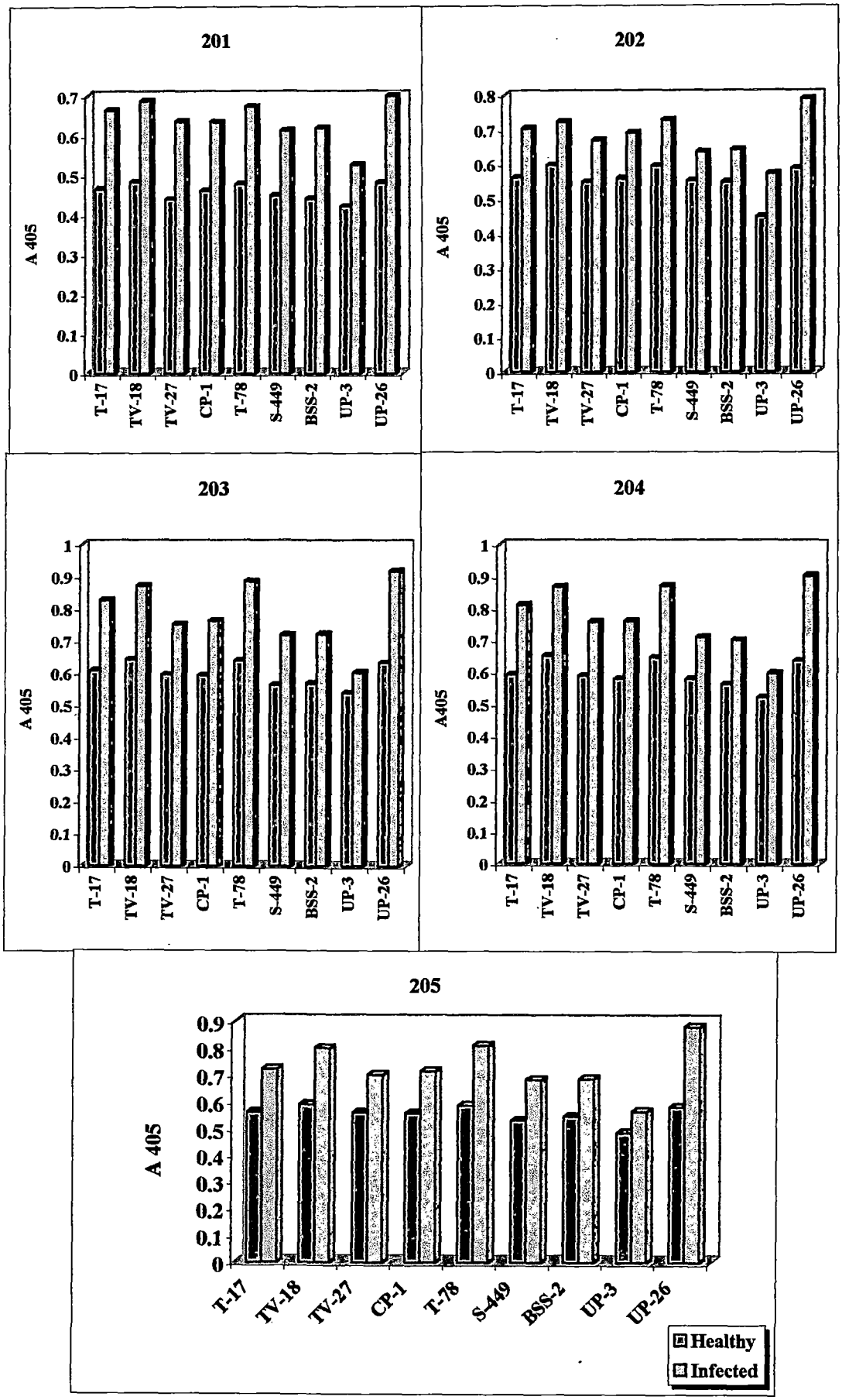


Fig.11

significantly higher ELISA values with infected extracts than healthy ones. In comparison to DAC-ELISA A405 values in both healthy and infected antigens were higher in this case.

Table 27 : DAS-ELISA values of healthy and infected tea root antigens tested against PAb of *F. lamaoensis.*

	Absorbance of 405nm	
Tea varieties	**Healthy**	**Infected**
T-17/1/54	0.873 ± 0.09	1.005 ± 0.05
TV-18	0.891 ± 0.11	1.043 ± 0.08
TV-26	0.792 ± 0.03	0.794 ± 0.07
CP-1	0.907 ± 0.06	1.021 ± 0.04
S-449	0.865 ± 0.09	0.995 ± 0.04
BSS-2	0.811 ± 0.06	0.955 ± 0.05

Antigen concentration 100µg/ml
IgG source-104 PAb; concentration 40µm/ml
Age of the plants 2yr
45 days after inoculation
± Standard error.

4.8.2. Dot-Blot

Root antigens of different tea varieties were reacted with mycelial and cell wall, PAbs of *F. lamaoensis* in dot-blot as described in material and methods. Results (Table 28) revealed that the antigens from infected roots of T-17, TV-18, UP-26, T-78 showed violet coloured (NBT/BCIP-subtrate) or pink coloured (Fast Red-substrate) dots while TV-26 and UP-9 had very light coloured dot, indicating weak reaction. These varieties had shown resistance in previous tests including pathogenicity and ELISA. WHEN antigens of healthy and infected tea roots of plants of different ages (1-5yr) were similarly reacted in dot-blot, it was observed that in healthy extracts of 1yr old plants, there was no reaction, while weak reactions were observed in root extracts from 2 and 3yr. old plants. Colour intensity of dots in reactions of root antigens from 5 yr old plants were higher. In reactions with antigens from infected tea roots, *positive reactions were obtained in all cases, though intensity increased with age* (Table 29 & Plate 10, fig. A).

Table 28 : Dot-blot analysis of healthy and inoculated antigens of different ages of tea plants with PAbs of *F. lamaoensis.*

	Colour intensity[a]			
	Mycelial PAb (103)		Cell wall PAb (203)	
Tea varieties	**Healthy**	**Infected**	**Healty**	**Infected**
T-17	–	+	–	+
TV-26	–	–	–	–
TV-27	–	+	–	±
BSS-2	–	–	–	±
UP-3	–	±	–	±
UP-9	–	±	–	–
UP-26	–	+	±	+
CP-1	–	±	–	+
T-78	–	+	–	+
Mycelia of *Fomes lamaoensis*	++++		+++	

[a] Fast red colour intensity : Pinkish red; ++++ Bright, +++ High, ++ Medium, + Low, ± Faint, – no reaction; Age of plants 2 yrs; IgG concentration 40μg/ml.

Table 29 : Dot-blot analysis of healthy and inoculated antigens of different ages of tea plants with PAbs of *F. lamaoensis.*

Antigen source		Colour intensity[a]			
		Mycelial PAb (103)		Cell wall PAb (203)	
Tea varieties	**Plant age(yr)**	**Healthy**	**Infected**	**Healty**	**Infected**
UP-26	1	–	+	–	+
	2	±	+	+	+
	3	±	++	+	++
	5	+	++	+	++
T-17	1	–	±	–	+
	2	±	+	±	+
	3	+	+	+	++
	5	+	++	+	++

[a] Fast red colour intensity : Pinkish red; ++++ Bright, +++ High, ++ Medium, + Low, ± Faint, – no reaction; Age of plants 2 yrs; IgG concentration 40μg/ml.

Healthy and infected root antigens of selected tea varieties (5yrs old) were further tested in Dot-Blot with PAbs of *F. lamaoensis* (mycelia and cell wall). as well as PAbs raised against root antigens of 2 tea varieties, one susceptible (UP-26) and other resistant (TV-26). Among the 4 selected varieties 3 were most susceptible (TV-18, UP-26, T-78) and one was resistant (TV-26). Reactions with PAbs of tea roots showed dots of high intensity in general. Besides homologous reaction, PAb of TV-26 reacted very strongly with antigens from healthy roots of TV-26 and T-78, and PAb of UP-26 reacted with antigens of UP-26 & T-78 strongly, where deep violet coloured dots appeared. In cases of reactions with PAbs of roots, infected root antigens showed dots of lesser intensity then healthy ones while with PAbs of *F. lamaoensis* (mycelia and cell wall) infected extracts showed dots of higher intensity (Table30, Plate 10, fig.D).

Table 30 : Comparison of dot-blot reaction of PAbs from different sources with healthy and incoculated root antigens of 5yr old tea varieties.

Tea varieties	Plant condition	Colour intensity[a] PAbs raised agaisnt			
		F. lamaoensis		UP-26	TV-26
		103	203	403	503
TV-18	Healthy	±	+	++	+++
	Infected	++	++	+	++
TV-26	Healthy	±	±	++	+++
	Infected	±	±	++	++
UP-26	Healthy	+	±	+++	++
	Infected	++	+++	++	+
T-78	Healthy	++	++	+++	+++
	Infected	+++	+++	++	++

[a] NBT/BCIP colour intensity: +++ Deep violet; ++ violet; + light violet; ± faint; – no reaction; IgG concentration 40µg/ml.

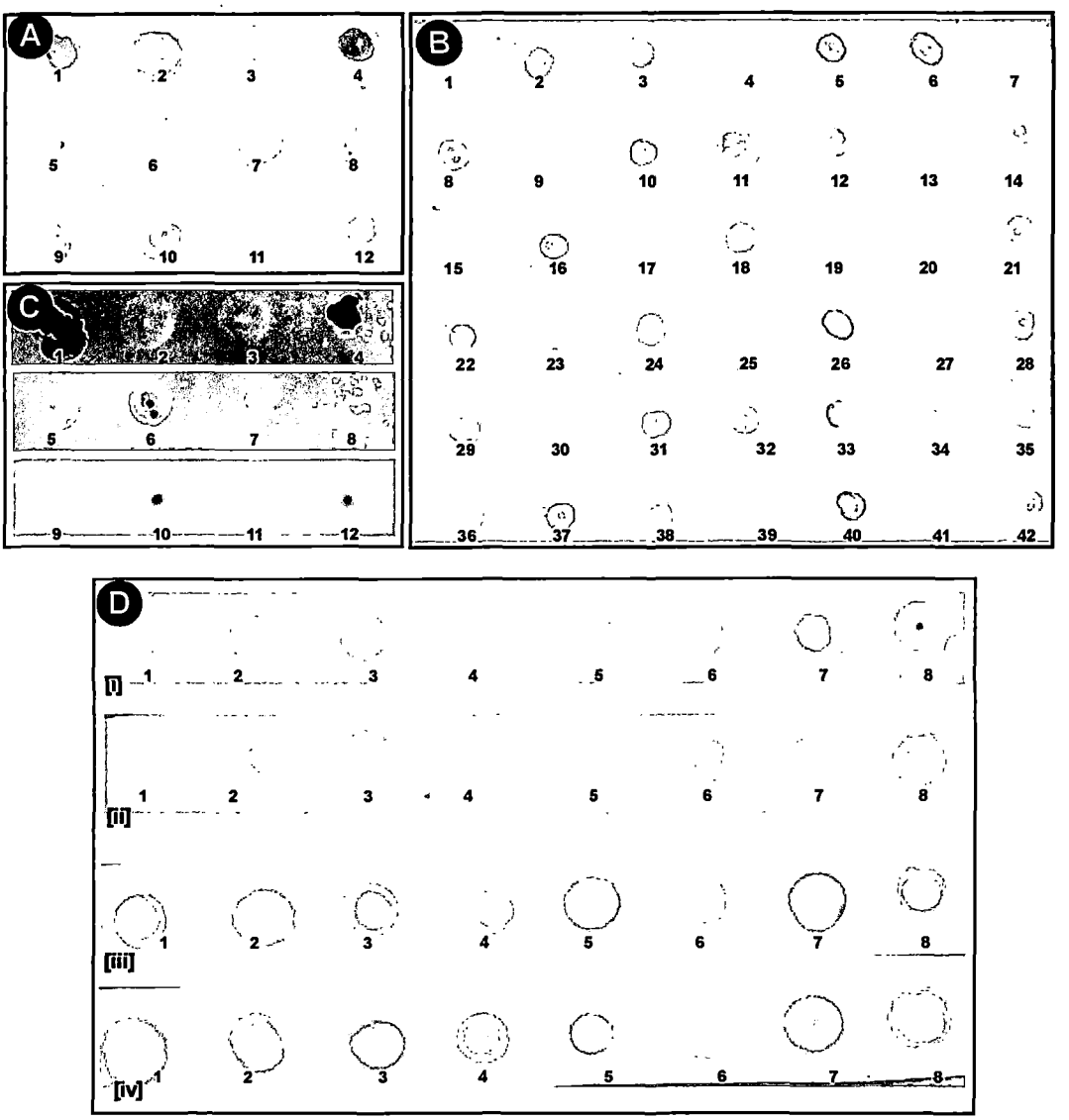


Plate 10 (Figs A-F) : Dot immunobindings of antigen and antibody on nitrocellulose paper. Antigens were: (A)1-4: *F.lamaoensis* ; 1&2-crude & purified mycelia ;3 - cell wall & 4- microsclerotia ; 5-12 : tea roots ; 5-10: UP-26;11&12 :T-17; (5,8,9 &11-healthy ; 6,7,10 &12-infected); [B]Control soil (4), Sterile soil (39), tea rhizosphere soil collected from different tea gardens (1-3,5-10,12-24,26-38,40-42); soil amended with *F. lamaoensis* (11&25) [C] 60-80% mycelial SAS (1),100% mycelia (4) of *F. lamaoensis* ; healthy tea roots of different varieties (2,3,5,7,8,9&11);infected tea root (6,10&12) ;[D] healthy (1,3,5&7) and infected (2,4,6&8) tea roots of different varieties; Blots were probed with PAbs - A,B & D-[i]:103; C: 303 ; D-[ii] : 203; D-[iii] : 403 ; D-[iv] : 503

4.8.3. Western blotting

In Western blot analysis, it was observed that when healthy root extracts were probed with PAb of *F. lamaoensis,* in susceptible varieties 2-3 bands out of 4 bands (ca. 79, 50, 21 and 19 kDa) were visible and infected root extracts from 2yr old plants, western blot probing showed 5-6 bands (ca. 61, 50, 38, 27, 17 & 13kDa) but more bands (ca.101, 97, 79, 71, 61, 54, 53, 37, 32, 28, 25 & 21) were found in case of plants from 3 yr onwards (Plate 11, fig. C).

4.9. Detection of *F. lamaoensis* in soil

4.9.1. DAC-ELISA

In order to determine whether PAb of *F. lamaoensis* could detect the pathogen in soil, samples were collected from various locations including several tea estates. Antigens were also prepared from amended soil infested with the propagules of *F. lamaoensis* either in field condition or in potted condition. In this investigation, antigens were prepared from 38 samples from root rhizosphere soil and tested against the PAb of *F. lamaoensis.* Result (Table 31) revealed only low A405 values in the range of 0.3-0.5 in most soil samples collected, except 6 samples [3 from Bijoynagar T. E., (Section A: Plot 1, 2; Section B : Plot 4), 3 from Trihna T.E. (Section C : Plot 1,4,5) and 1 from Matigara T. E. (Section A: Plot 2)] which gave high absorbance value (ranging from 0.7-0.9) in ELISA. This indicated the presence of propagules of *F. lamaoensis* only in these soils. In case of amended soils, high values were obtained after 15, 30 and 45 days of amendment but after 70 days, the values had decreased greatly. This was probably because the pathogen could not survive in the soil for such a long period. Both positive and negative controls were in expected ranges.

4.9.2. Dot blot

Identification of *F. lamaoensis* propagules in artificially infested and non infested root rhizosphere soil was carried out through dot immunobinding reaction also. Soil antigens were prepared from soil samples from *F. lamaoensis* amended soil and different locations including tea gardens and reacted with PAb of *F. lamaoensis.* Among.

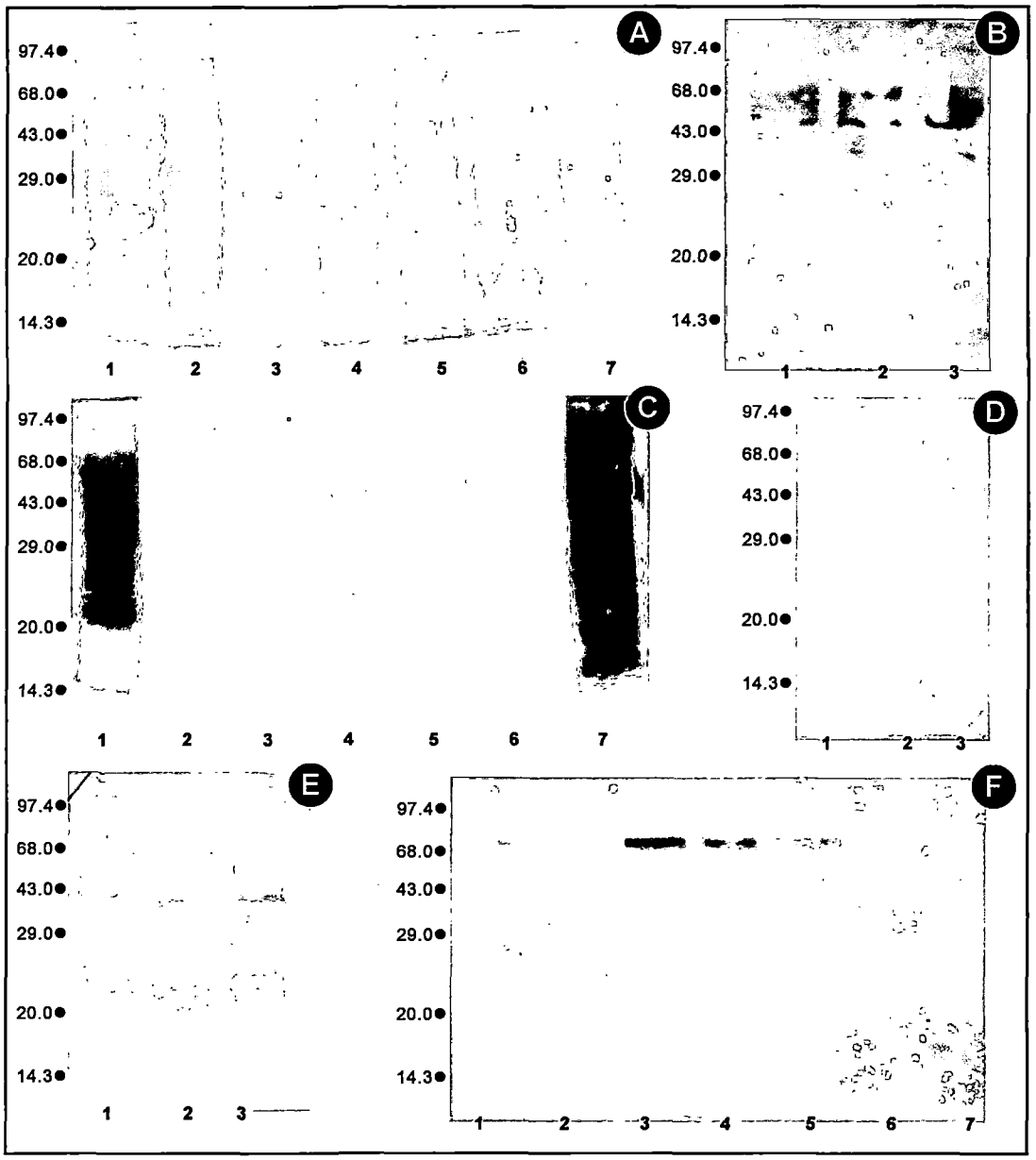


Plate 11 (Figs A-H) : Western blot analysis of *F. lamaoensis* and tea root antigens.[A] crude (lane1), 100% SAS (lane 2) and SAS mycelial fractions, 0-20% (lane-3), 20-40% (lane 4), 40-60% (lane-5), 60-80%, (lane-6) and 60-100%, (lane-7) of *F. lamaoensis;* [B] Cell wall antigens (lanes 1-3); [C] Healthy (lanes2,4,6) and *F. lamaoensis* inoculated (1,3&5) tea root antigens ; lanes (1 &2) : UP-26; (3&4): T-17, (5&6) :S-449;and (7) : crude mycelial antigen of *F. lamaoensis* ; [D] lanes 1-3: root antigens of UP-26; healthy (lane-2) and inoculated (lanes 1&3); [E] lanes 1-3 : healthy root antigens of UP-26; [F] lanes 1-7: healthy root antigens of BSS-2 (1), UP-9 (2), T-17 (3), TV-18 (4), TV-26 (5), CP-1 (6) and T-78 (7); Blots were probed with PAb 103 (A & C), 203 (B),303 (D) of *F. lamaoensis* and PAb 403 (E&F) of tea roots on nitrocellulose paper.

Table 31 : ELISA responses of different soil antigens with PAb of *F. lamaoensis* (mycelia).

Soil sample	Absorbance at	Soil sample	Absorbance at 405nm[a]
S-1	0.442 ± 0.06	S-21	0.685 ± 0.05
S-2	0.302 ± 0.05	S-22	0.460 ± 0.04
S-3	0.576 ± 0.05	S-23	0.599 ± 0.02
S-4	0.413 ± 0.04	S-24	0.498 ± 0.03
S-5	0.461 ± 0.04	S-25	0.483 ± 0.03
S-6	0.544 ± 0.07	S-26	0.496 ± 0.09
S-7	0.760 ± 0.08	S-27	0.409 ± 0.03
S-8	0.592 ± 0.02	S-28	0.334 ± 0.07
S-9	0.492 ± 0.02	S-29	0.361 ± 0.04
S-10	0.444 ± 0.05	S-30	0.732 ± 0.03
S-11	0.478 ± 0.06	S-31	0.396 ± 0.02
S-12	0.449 ± 0.04	S-32	0.345 ± 0.04
S-13	0.455 ± 0.06	S-33	0.442 ± 0.05
S-14	0.402 ± 0.06	S-34	0.363 ± 0.07
S-15	0.845± 0.08	S-35	0.361 ± 0.07
S-16	0.750 ± 0.07	S-36	0.997 ± 0.02
S-17	0.543 ± 0.05	S-37	1.542 ± 0.03
S-18	0.460 ± 0.05	S-38	2.142 ± 0.04
S-19	0.522 ± 0.03	Homologus	
S-20	0.470 ± 0.03	mycelia	2.310 ± 0.03

PAb - 104, dilution 1:500; ± Standard error,.
Soil antigen - S-1 = Control soil; S-2 Sterile soil. 3-34 = collected from different tea growing field; [Hansqua T.E.-Section B : Plot 7 (S-3), 8(S-4), 9(S-5); Trihana T.E. Section C : Plot 1 (S-6,7), 4(S-8,9), 5(S-30); Cooch Behar T.E. Plot 2(S-10), 3(S-11, 12), 4(S-13); Bijoynagar T.E. - Section A : Plot 1(S-14, 15), 2(S-16); Section B : Plot 1(S-17, 18), 3(S-19,20), 4(S-21); Matigara T.E. - Section A : Plot 1(S-22), 2(S-23) 5(S-24), 6(S-25); Section D : Plot 4(S-26, 27), 5(S-28,29); Chandmoni T.E.- Section A : Plot 2(S-31,32), 3(S-33,34), S-35-38 = Amended soil of *F. lamaoensis* (38=15 , 37= 30, 36 = 45 and 35 = 70 days after amendment).

38 collected soil samples only 4 sample [2 from Bijoynagar T.E. (Section A Plot 1; Section B: Plot 4) and 2 from Trihana T.E. (Section : Plot 1,5)] showed positive reactions, though dots were of low intensity; in all other samples, either no dots could

be detected or the reactions were very weak. Amended soil antigens gave pink coloured dots when probed with both mycelial and cell wall PAb of *F. lamaoensis* but dot intensity of amended soil decreased as the time passed (Table 32 & Plate 10, fig.B).

Table 32 : Dot-blot of different soil antigens with PAb of *F. lamaoensis.*

Soil antigens	Colour intensity[a]	Soil antigens	Colour intensity[a]
S-1	–	S-21	±
S-2	–	S-22	–
S-3	±	S-23	±
S-4	±	S-24	+
S-5	–	S-25	–
S-6	–	S-26	–
S-7	–	S-27	±
S-8	+	S-28	±
S-9	–	S-29	–
S-10	±	S-30	–
S-11	±	S-31	–
S-12	–	S-32	–
S-13	±	S-33	±
S-14	–	S-34	±
S-15	–	S-35	–
S-16	+	S-36	–
S-17	±	S-37	+
S-18	–	S-38	++
S-19	±	Homologus	
S-20	+	mycelia	++++

[a]Fast Red colour intensity - Pinkish red: ++++ Bright , +++ High, ++ Medium, + Low, ± Faint, – no colour.
IgG of 103 PAb; concentration 40μg/ml.
Soil antigen - S-1 = Control soil; S-2 = Sterile soil; S-3-36 = collected from different tea growing fields; [Cooch Behar T.E. : Plot 2 (S-3), 3 (S-4,5), 4 (S-6,7); Bijoynagar T.E.-Section A : Plot 1 (S-8,9), 2 (S-10); Section B : Plot 1 (S-11,12), 3 (S-13,14) 4 (S-15,16); Hansqua T.E. Section B : Plot 7 (S-17), 8 (S-18), 9 (S-19); Trihana T.E. Section C : Plot 1 (S-20,21), 4 (S-20,23), 5 (S-24); Matigara T.E - Section A. : Plot 1 (S-25), 2 (S-26), 5 (S-27), 6 (S-28); Section D : Plot 4 (S-29,30), 5 (32,33); Chandmoni T.E. Section A : Plot 2 (S-34,35) 3 (S-36)] S-37 & 38 = Amended soil of *F. lamaoensis* (38=20 days old and 37=45 days after amendment).

4.10. Determination of cross reactivity of *F. lamaoensis* Pab

4.10.1. ELISA

4.10.1.1. Fungal mycelial antigens of different soil fungi

Cross reactivity of the PAb raised against *F. lamaoensis* was tested against a number of soil fungi of which some were pathogenic to tea (*Poria hypobrumea, Ustulina zonata, Sphaerostilbe repens, Rosellinia arcuata*) and others were non-pathogenic (*Fusarium oxysporum, Metarhizium anisoplia, Beauveria bassiana, Trichoderma viride, T. harzianum, Sclerotium rolfsii*). Antigens prepared from the mycelia of all the above were tested against PAb of *F. lamaoensis* by ELISA. Results presented in Table 33 and Fig.12) revealed that among all the fungi tested PAb of *F. lamaoensis* reacted to some extent with antigens of *Ustulina* zonata and *Rosellinia arcuata.*

Table 33 : Indirect ELISA values (A405) of PAb of *F. lamaoensis* reacted with antigens of soil fungi.

Antigen Source	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
S. repens	0.525	0.538	0.542	0.535 ± 0.05
P. hypobrumia	0.543	0.604	0.612	0.586 ± 0.11
R. arcuata	0.658	0.668	0.649	0.658 ± 0.06
U. zonata	0.689	0.670	0.687	0.682 ± 0.06
Fusarium sp.	0.477	0.468	0.454	0.466 ± 0.06
T. viride	0.417	0.408	0.416	0.414 ± 0.04
T. harzianum	0.345	0.338	0.339	0.341 ± 0.03
S. rolfsii	0.344	0.332	0.342	0.339 ± 0.05
S.rolfsii - 3	0.378	0.378	0.380	0.379 ± 0.02
S.rolfsii -2	0.351	0.350	0.344	0.348 ± 0.03
S.rolfsii-4	0.396	0.412	0.413	0.407 ± 0.06
S. rolfsii -1	0.452	0.473	0.460	0.462 ± 0.06
M.anisopliae-892	0.316	0.326	0.311	0.318 ± 0.05
M.anisopliae-140	0.406	0.404	0.408	0.406 ± 0.03
B.bassiana-2028	0.366	0.377	0.368	0.370 ± 0.04
B.bassiana-135	0.486	0.489	0.479	0.485 ± 0.04
Fomes lamaoensis	2.013	2.028	2.043	2.028 ± 0.07

Antigen concentration 10µg/ml; IgG source-104 PAb; concentration 40µg/ml;
± Standard error.

Cross reactivity of *F.lamaoensis* PAb with other fungi in DAC-ELISA

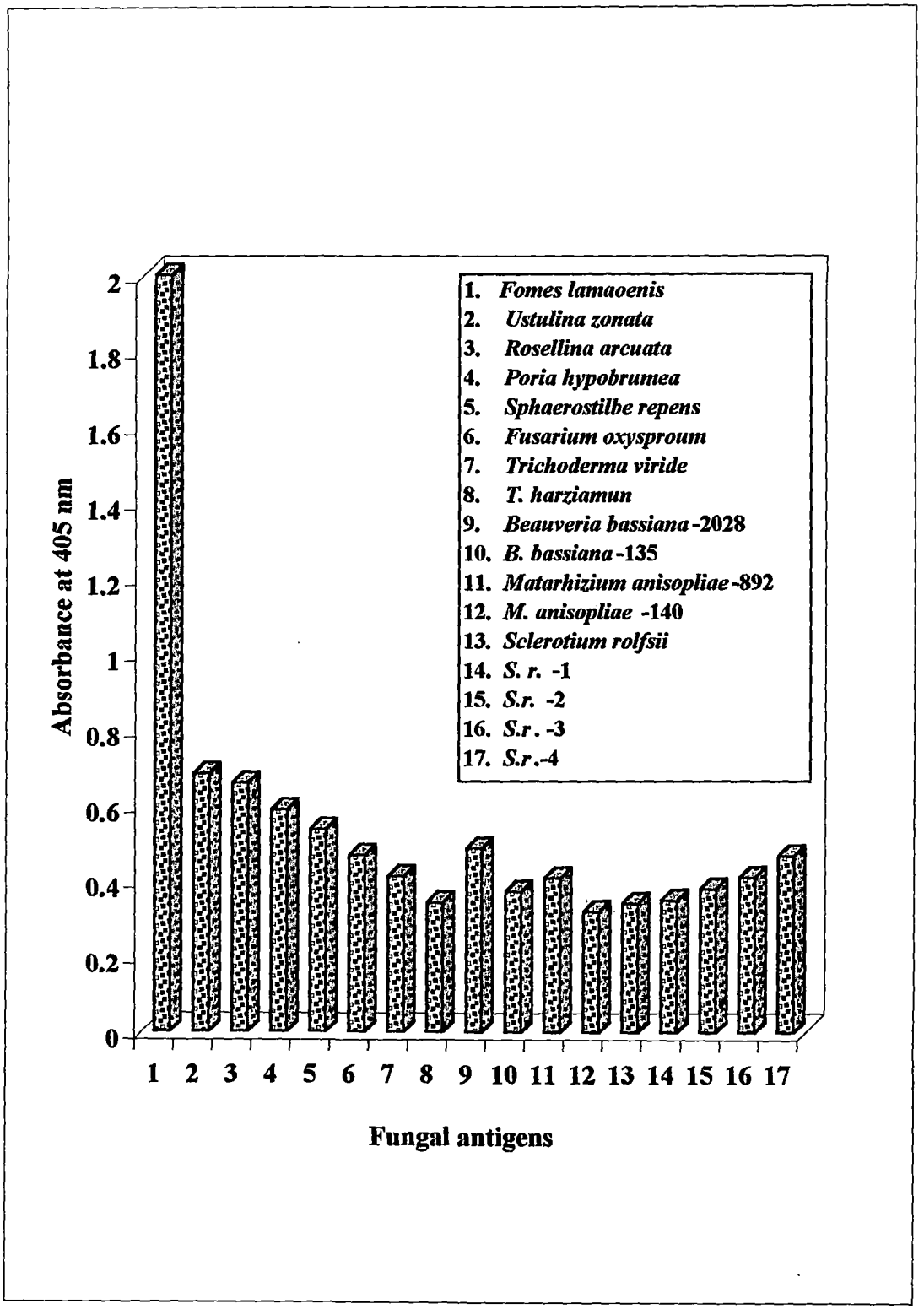


Fig.12

4.9.1.2. Fungi in tea roots infected with other root pathogens

A number of pathogens can infect tea roots causing different types of root rot diseases of tea. So, it was considered worthwhile to investigate whether PAb of *F. lamaoensis* could also react with the antigens from tea roots infected with other root pathogens. Hence, the PAb of *F. lamaoensis* was reacted by DAC-ELISA with antigens prepared from tea roots (T-17, TV-23, UP-8, BSS-2 and CP-1) infected with *Spaerostilbe repens* (violet root rot), *Rosellinia arcuata* (black root rot) an *Ustulina zonata* (charcoal stamp / root rot). Results (Table 34) revealed that reactivity of these antigens was lesser when compared with *F. lamaoensis* infected root antigens though here also infected root extracts showed higher ELISA values than the healthy ones. Thus a certain degree of cross reactivity was observed through this investigation.

Table 34 : DAC-ELISA values of healthy and infected (with different root pathogen) tea root antigens reacted with. PAb of *F. lamaoensis.*

Tea varieties	Absorbance at 405nm				
	Healthy	Infected			
		F. lamaoensis	*R. arcuata*	*S.repens*	*U.zonata*
T-17/1/54	0.599 ± 0.03	1.227 ± 0.04	0.644 ± 0.03	0.597 ± 0.03	0.662 ± 0.06
TV-23	0.499 ± 0.02	0.851 ± 0.07	0.602 ± 0.05	0.511 ± 0.06	0.611 ± 0.03
UP-18	0.478 ± 0.04	0.714 ± 0.02	0.548 ± 0.05	0.497 ± 0.08	0.570 ± 0.06
BSS-2	0.543 ± 0.09	0.986 ± 0.04	0.622 ± 0.06	0.559 ± 0.03	0.620 ± 0.04
CP-1	0.548 ± 0.08	1.077 ± 0.06	0.626 ± 0.02	0.571 ± 0.09	0.637 ± 0.04
Homologous mycelia	2.028 ± 0.03				

Antigen concentration 100 µg/ml
PAb source104 PAb; Concentration 40µg/ml
Age of plant 3 year.
40 days after inoculation
± Standard error.

4.10.2. Dot Blot

Cross reactivity of *F. lamaoensis* PAb (mycelial and cell wall) with antigens of other fungi were tested by dot-blot also using antigens of other root pathogens. Antigens of *U. zonata, R. arcuata* and *T. viride* showed slightly positive reactions, though the dots were of much lesser intensity than the homologous reaction (Table 35).

Table 35 : Dot-blot reaction of antigens of different soil fungi with *F. lamaoensis* PAbs.

Antigne source	Colour intensity[a]	
	Mycelial PAb (103)	Cell wall PAb (203)
S. repons	±	+
U. zonata	+	+
R. arcuata	+	+
P. hypobrumia	±	±
A. mellea	±	±
T. harzianum	±	±
T. viride	+	+
F. lamaoensis		
Mycelial	++++	+++
Cell wall	++	++++

Fast Red colour intensity (Pinkish red) :(++++) Bright, (+++) High, (++) Medium, (+) Low (±) Faint ; (–) no colour.

4.11. Purification of antigen by ammonium sulphate precipitation

The crude antigens preparations were purified by ammonium sulphate precipitation, as described under materials and methods. In order to determine the fractions, which contained the antigenic proteins, immunodiffusion, DAC-ELISA and Western blot were performed with PAb raised against mycelia of *F. lamaoensis* (104) the results of which are given below.

The precipitin reaction of fractionated proteins (0-20%, 20-40%, 40-60%, 60-80% and 80-100% SAS) with PAb raised against mycelial extracts (3rd bleeding, 103) was tested in immunodiffusion. Result presented in Plate 7, fig.E revealed

3 separated, strong precipitin bands in 40-60% in 60-80% fractions, 4 bands in 100% SAS while one strong band was observed in 80-100% fraction and no bands in 0-20% and 1weak band in 20-40%. The reactions of the different fractions could not be differentiated by DAC-ELISA. SDS-PAGE of the proteins from different fractions were stained by coomassie blue, and another set was used for Western Blot. In SDS-PAGE, a number of bands of different mol. wts. were found in the different fractions, with maximum bands in the fraction 40-60% SAS (Plate 8, fig.C). In case of Western blot, however, maximum bands due to antigen-antibody reaction was evident in 60-80% SAS (Table 36 and Plate 11, fig.A). Based on the results of immunodiffusion and Western blot, 60-80% SAS fraction was selected as the fraction containing the maximum antigenic proteins and was used as immunogen to raised the PAb.

Table 36 : Western Blot analysis of fractionated (SAS) mycelial proteins of *F. lamaoensis* with PAb of *F. lamaoensis.*

	Molecular weight (kDa)						
	Antigen source						
S. No.	**Crude**	**100% SAS**	**0-20% SAS**	**20-40% SAS**	**40-60% SAS**	**60-80% SAS**	**80-100% SAS**
1.	→ 104.7	→ 104.3	→ 104.3	→ –	→ 104.3	→ 104.3	→–
2.	→ 97.4	→ 97.4	→–	→ –	→–	→ 97.4	→ 97.4
3.	→ 79.0	→ 79.0	→–	→ 79.0	→ 79.0	→ 79.0	→–
4.	→–	→ 60.9	→–	→ –	→–	→–	→–
5.	→ 50.1	→ 50.1	→–	→ –	→–	→ 50.1	→–
6.	→ 43.0	→ 43.0	→–	→ –	→ 43.0	→ 43.0	→ 43.0
7.	→ 38.6	→ 38.6	→ 38.6	→ 38.6	→ 38.6	→–	→–
8.	→ 27.1	→ 27.1	→ 27.1	→ –	→ 27.1	→ 27.1	→ 27.1
9.	→–	→ 24.5	→ 24.5	→ 24.5	→–	→ 24.5	→ 24.5
10.	→ 19.0	→ 19.0	→–	→ –	→–	→–	→–
11.	→ 18.4	→ 18.4	→–	→ 18.4	→ 18.4	→ 18.4	→ 18.4
12.	→ 17.4	→ 17.4	→–	→ –	→ 17.4	→ 17.4	→–
13.	→ 15.3	→ 15.3	→–	→ –	→–	→ 15.3	→–
14.	→–	→ 14.3	→–	→ –	→–	→ 14.3	→–
15.	→–	→ 12.7	→–	→ –	→–	→ 12.7	→–

IgG source-103 PAb; concentration 40µg/ml.

4.12. Evaluation of PAb raised against purified mycelial antigen

4.12.1. Immunodiffusion

The precipitin reaction was also done with PAb raised against 60-80% fractionated protein (302) and result (Plate7, fig. F) shows 5 separated, sharp bands in 60-80% and 100% SAS, 2 strong band in 40-60%, one in 80-100%, no bands in 0-20% and 20-40% SAS. IgG fractions were purified and experiments were done with purified IgG fraction of this PAb.

4.12.2.2. Cross reactive antigens

PAbs raised against 60-80% SAS fraction of mycelial antigen of *F. lamaoensis* were also reacted with tea root antigens of all 25 varieties tested Table... ELISA responses obtained were similar trends to that obtained with PAbs raised against mycelial and cell wall (Table 37).

4.12.2.3. Detection of *F. lamaoensis* in tea root tissues

4.12.2.3.1. ELISA

Ability of PAb raised against 60-80% SAS fraction of *F. lamaoensis* (403) to detect the pathogen in root tissues was tested by ELISA, Dot-Blot and Western Blot analysis. In ELISA differences in A405 values between healthy and infected roots were highly significant in the susceptible varieties - TV-18, UP-26, T-78 (Table 38, Fig 13).

4.12.2.3.2. Dot Blot

In dot-blot, where the root antigens were reacted with the above PAb, healthy root antigenic extracts showed only faint dots, while infected showed dots which were either light violet or violet. In no case, deep coloured dots were visible (Table-39, Plate 10, fig.C).

Table 37 : Indirect ELISA values (A405) of tea root antigens, 60-80% SAS mycelial antigen of *F. lamaoensis,* non-pathogen and non host reacted with anti-60-80% SAS PAb of *F. lamaoensis.*

Antigens	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties[a]				
UPASI - 2	0.455	0.453	0.460	0.456 ± 0.035
UPASI - 3	0.496	0.498	0.504	0.499 ± 0.037
UPASI - 8	0.528	0.534	0.530	0.531 ± 0.032
UPASI - 9	0.531	0.536	0.537	0.535 ± 0.033
UPASI - 26	0.682	0.697	0.693	0.691 ± 0.051
BSS - 2	0.624	0.623	0.618	0.622 ± 0.033
TV - 9	0.551	0.568	0.557	0.552 ± 0.054
TV - 18	0.706	0.707	0.691	0.701 ± 0.055
TV- 22	0.549	0.550	0.559	0.553 ± 0.043
TV - 23	0.582	0.586	0.590	0.586 ± 0.037
TV- 25	0.468	0.465	0.465	0.466 ± 0.024
TV- 26	0.453	0.448	0.445	0.449 ± 0.037
TV- 27	0.583	0.585	0.580	0.583 ± 0.029
TV- 28	0.528	0.536	0.531	0.532 ± 0.037
TV - 30	0.490	0.499	0.480	0.490 ± 0.056
T-17/1/54	0.642	0.651	0.658	0.650 ± 0.052
S-449	0.605	0.583	0.606	0.598 ± 0.066
AV-2	0.484	0.497	0.501	0.494 ± 0.054
BS/7A/76	0.460	0.478	0.473	0.470 ± 0.056
CP-1	0.581	0.589	0.597	0.589 ± 0.050
HV-39	0.458	0.457	0.461	0.459 ± 0.026
K1/1	0.513	0.521	0.517	0.517 ± 0.037
P-1258	0.568	0.558	0.554	0.560 ± 0.049
T-78	0.678	0.680	0.681	0.680 ± 0.021
T-135	0.482	0.484	0.500	0.489 ± 0.057
60-80% SAS of *F. lamaoensis*[b]	2.869	2.872	2.867	0.869 ± 0.029
Non-pathogens[b]				
Metarhizium anisopliae	0.316	0.326	0.311	0.318 ± 0.050
Non-host[a]				
Tageties patula	0.385	0.382	0.374	0.380 ± 0.044
Impatiens balsamina	0.319	0.316	0.321	0.319 ± 0.029

[a]Antigen concentration 100µg/ml; [b]Antigen concentration 10µg/ml
IgG source-303PAb; concentration 40µg/ml
± Standard error.

Table 38 : DAC-ELISA values of healthy and inoculated tea root antigens of 4 selected varieties with anti-60-80% mycelial SAS PAb of *F. lamaoensis*

Root antigens		**Absorbance at 405 nm**			
Tea varieties	**Condition**	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
TV-18	Healthy	0.679	0.679	0.674	0.677 ± 0.031
	Infected	1.063	1.053	1.062	1.059 ± 0.043
TV-26	Healthy	0.498	0.500	0.512	0.503 ± 0.050
	Infected	0.585	0.589	0.591	0.588 ± 0.032
UP-26	Healthy	0.639	0.637	0.643	0.640 ± 0.032
	Infected	1.013	1.029	1.027	1.023 ± 0.054
T-78	Healthy	0.668	0.660	0.668	0.665 ± 0.039
	Infected	1.210	1.198	1.200	1.203 ± 0.046

Antigen concentration 100µg/ml; IgG concentration 40µg/ml
Age of the plants 5yr; 40 days after inoculation; ± Standard error.

Table 39 : Comparison of dot-blot reaction of PAbs from mycelial 100% amd 60-80% SAS of *F. lamaoensis* with healthy and incoculated root antigens of 5yr old tea varieties.

		Colour intensity[a]	
		PAbs raised agaisnt	
Tea varieties	**Plant condition**	**100% SAS (103)**	**60-80%SAS (303)**
TV-18	Healthy	±	±
	Infected	++	+
TV-26	Healthy	±	±
	Infected	±	±
UP-26	Healthy	+	+
	Infected	++	++
TV-26	Healthy	++	±
	Infected	+++	+

[a] NBT/BCIP colour intensity, violet colour: + + + Deep violet, + + Violet, + Light violet, ± Faint, – No reaction; IgG concentration 40µg/ml.

ELISA responses of healthy and inoculated tea root antigens with PAb raised against 60-80% mycelial SAS of *F.lamaoensis*

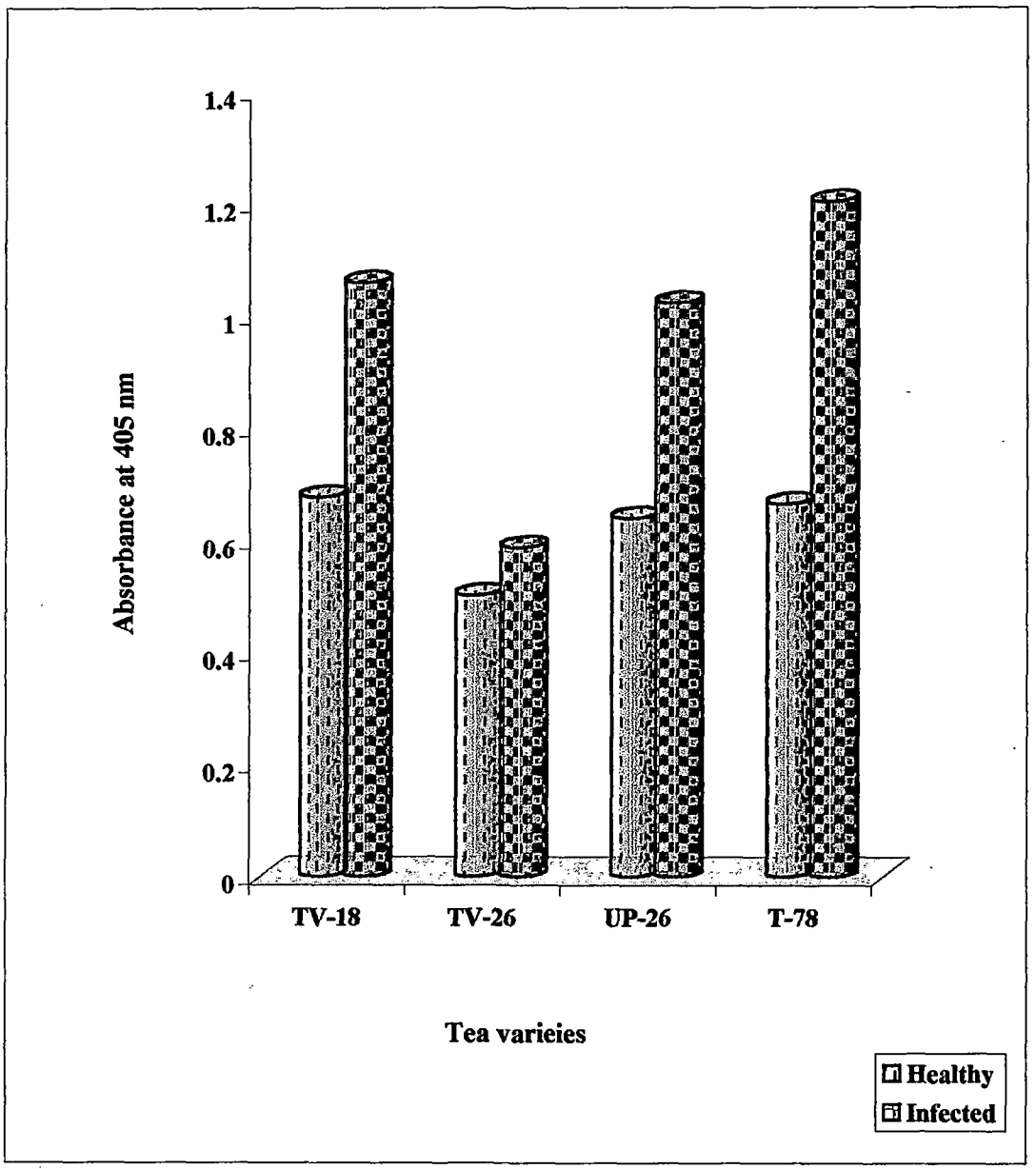


Fig.13

4.12.2.3.3. Western Blot

Western Blot analysis was also carried out with PAb 303 and root antigens. No bands were visible when healthy root extracts were reacted but 4 bands of ca. molecular wts. 23.2, 27.4, 37.6 and 40.8 kdas were visible (Plate 11, fig.D). When compared to the reaction with PAb raised against mycelial extract (103), these bands were lesser in number, but these may be more specific.

4.13. Immunofluoresence

Fluorescent antibody labelling with fluorescein isothiocyanide (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. Present study reports the use of indirect immunofluorescent test using polyclonal antibodies to determine the tissue and cellular location of CRA in root tissue of tea varieties, tea rhizosphere soil as well as mycelia of *F. lamaoensis* and detailed method for this has already been discussed under materials and methods. Mycelial, root section and soil preparations were photographed under UV- fluorescence and the intensity of bright apple-green fluorescence indicated the positive reaction

4.13.1. Mycelia

Pre-immune sera did not show reactivity with the mycelia of *F. lamaoensis* followed by FITC and mycelia was not auto-fluorescent. Examination of mycelia treated with homologous PAbs of mycelial, cell wall and 60-80% fractionated mycelial protein and stained indirectly with FITC indicated strong fluorescence throughout the mycelia, specially in young hyphal tips (Plate 12, figs. A-D).

4.13.2. Root tissue

Cross section of tea roots were treated separately with normal and *F. lamaoensis* antisera and then reacted with FITC. Root sections exhibited a natural autofluoresence under UV-light but that was not characteristic of FITC fluorescence. Observations in the treatment with normal serum and FITC were same. When the cross sections of healthy and infected tea root tissue of susceptible varieties (UP-26, T-78, TV-18)

incubated with PAb of *F. lamaoensis* and stained with FITC, strong fluorescence was observed in the infected root tissues. Present of fungal mycelium was evident with strong fluoresence in infected root tissue of susceptible varieties (TV-18, UP-26 and T-78) after immunofluorecent treatment. Fluorescence was evident throughout the sections, extending upto the vascular tissues as well as outer surface. Healthy sections exhibited only weak fluorescence. The positive reaction with FITC occurred in TV-18, UP-26, and T-78 and gave indication of the degree of susceptibility (Plate 13).

4.13.3. Soil sample

Amended soil preparation was done for immunoflurescence study as described under materials and methods. Microscopic observation under UV-fluorescence revealed that presence of strongly fluorescing mycelia (Plate 12, fig. E&F). Thus, immunofluorescence could be used to detect the pathogen in soil.

4.14. Immunocytochemical staining

Another approach adopted to study the interaction of *F. lamaoensis* with root tissue was by direct observation, Immunocytochemical staining, based on specific antibodies produced against *F. lamaoensis,* provided a means of visualizing hyphae within root tissues of infected tea plants. Production of a specific immunocytochemical stain involves preparation of suitable antigen, appropriate methods for evaluating specificity of the antibodies, and development of the immunocytochemical staining procedure. In the present study three susceptible varieties (TV-18, UP-26 and T-78) of 5yr old tea plants were artificially inoculated with *F. lamaoensis* in the experimental field. After 40 days of inoculation the affected plants were uprooted and washed properly. Cross-sections were made from the infected tea roots and fungal hyphae which penetrated the root tissues were probed with PAb raised against mycelial antigens of *F. lamaoensis*. In root sections stained immunocytochemically, hyphae of *F. lamaoensis* were growing along the epidermis and hyphal penetration throughout root tissues was evident as observed in Plate 14 (Fig A-H) with sections of TV-18 (A&E), UP-26 (D,F&G) and T-78 (B, C & H). Hyphae, which were growing horizontally in the cortical regions could be distinguished as strands, whereas those

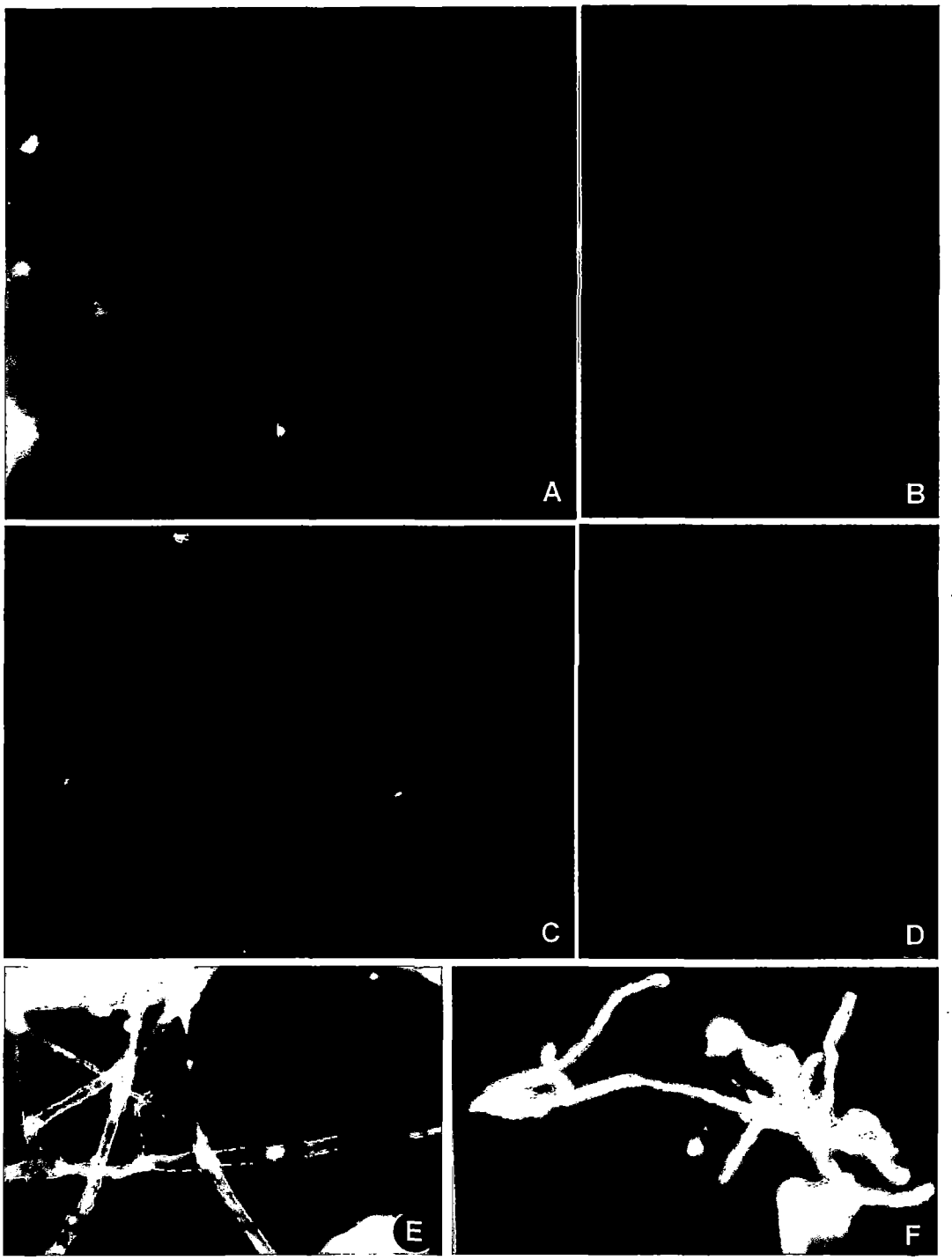


Plate 12 (Figs A-F) : Fluorescence of hyphae of *F. lamaoensis* (A-D) and amended soil (E & F) treated with PAb of *F. lamaoensis* and labelled with FITC labelled antibodies of goat specific for rabbit globulin. A-D: PAb 101-104,respectively; E&F : PAb 103.

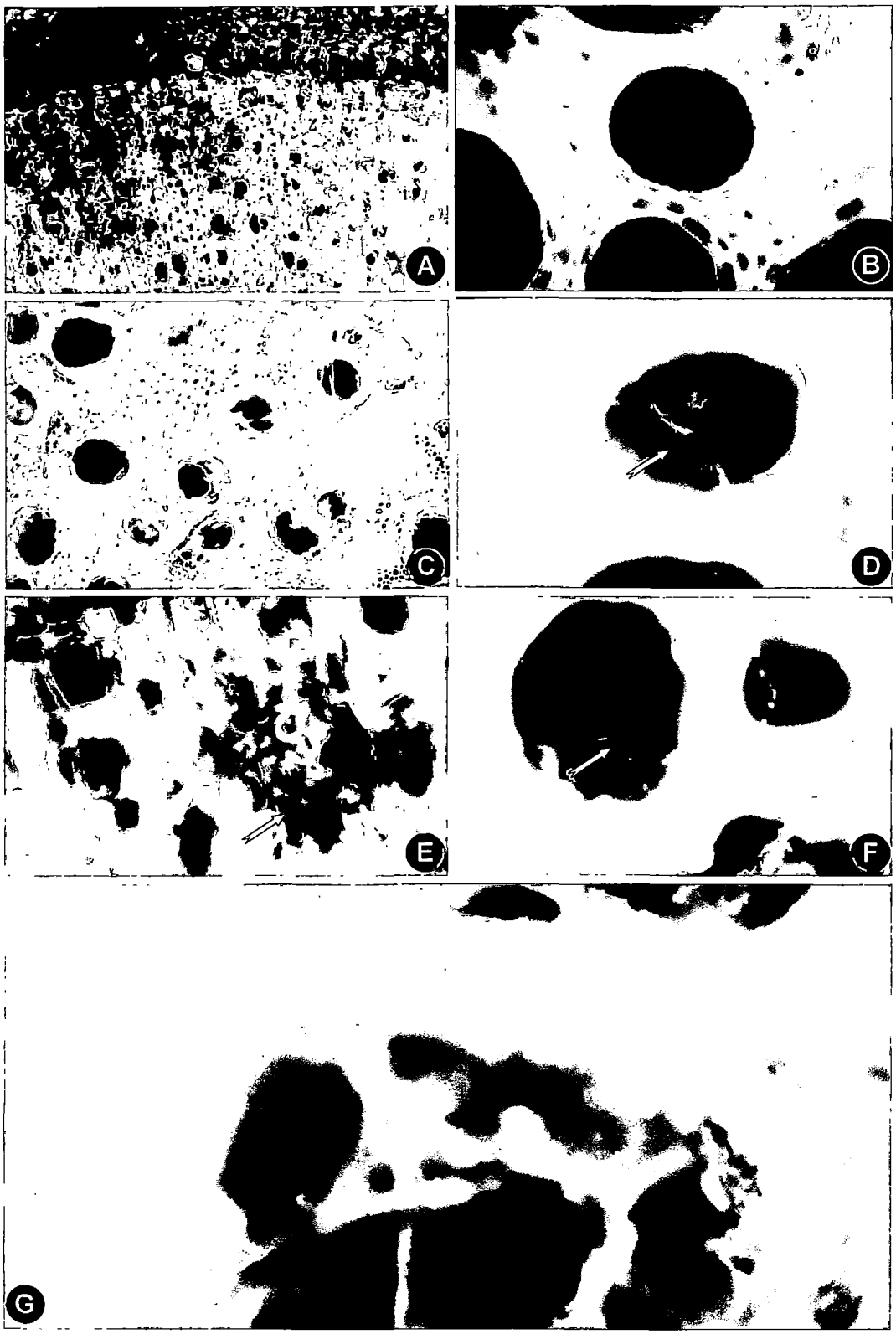


Plate 13 (Figs A-G) : Fluorescent antibody staining of tea root tissues treated with PAb (103) for detection of *F. lamaoensis.* Cross sections of roots : Healthy (B); inoculated with *F. lamaoensis* (A,C-G); UP-26 (A,B,C&E), TV-18 (D), T-78 (F & G).

which were growing vertically either inter or intracellularly appeared as blue coloured masses (cross section of hyphae). Deep blue coloured thick layer was found on outer surface of root which was an evidence of deep brown and blackish sheet on root surface formed by fungal mycelia. This staining clearly showed the penetration of hyphae throughout the tissue.

4.15. Biological control of brown root rot disease with *Trichoderma* sp.

The present work was aimed at developing a management strategy to control brown root rot of tea by biological means. Antibiosis to *F. lamaoensis* by isolates of fungal biocontrol agents, *Trichoderma harziamum* and *T. viride* were evaluated *in vitro* and *in vivo* condition.

4.15.1. *In vitro* test

Both the biocontrol agents tested *in vitro* were effective in causing significant suppression of growth of *F. lamaoensis* (Plate 15). After 3-4 days of incubation *T. harzianum* and *T. viride* over grew the pathogen. But in control plate pathogen grew characteristically on PDA.

4.15.2. *In vivo* test

To manage the brown root rot disease using biocontrol agents in potted and field conditions above two *Trichoderma* species were used. The experimental set up was carried out with following treatments : (a) pathogen (*F. lamaoensis*), (b) *T. harzianum,* (c) *T. viride,* (d) *F. lamaoensis* and *T. harzianum,* (e) *F. lamaoensis* and *T. viride* and (f) control plants i.e. healthy. Inocula were prepared in different media for mass application as described in materials and methods. Among them the most useful inculum was sand-maize meal + tea root medium for pathogen and tea waste/ wheat bran medium for biocontrol agents (Plate 16). Biocontrol agents were infested in the rhizosphere 7 days before inoculation with pathogen.

4.15.2.1. Potted plants

Two years old tea plants (TV- 18 and UP-26) grown in potted condition were used for this purpose Twelve replicates of each treatment were taken and disease

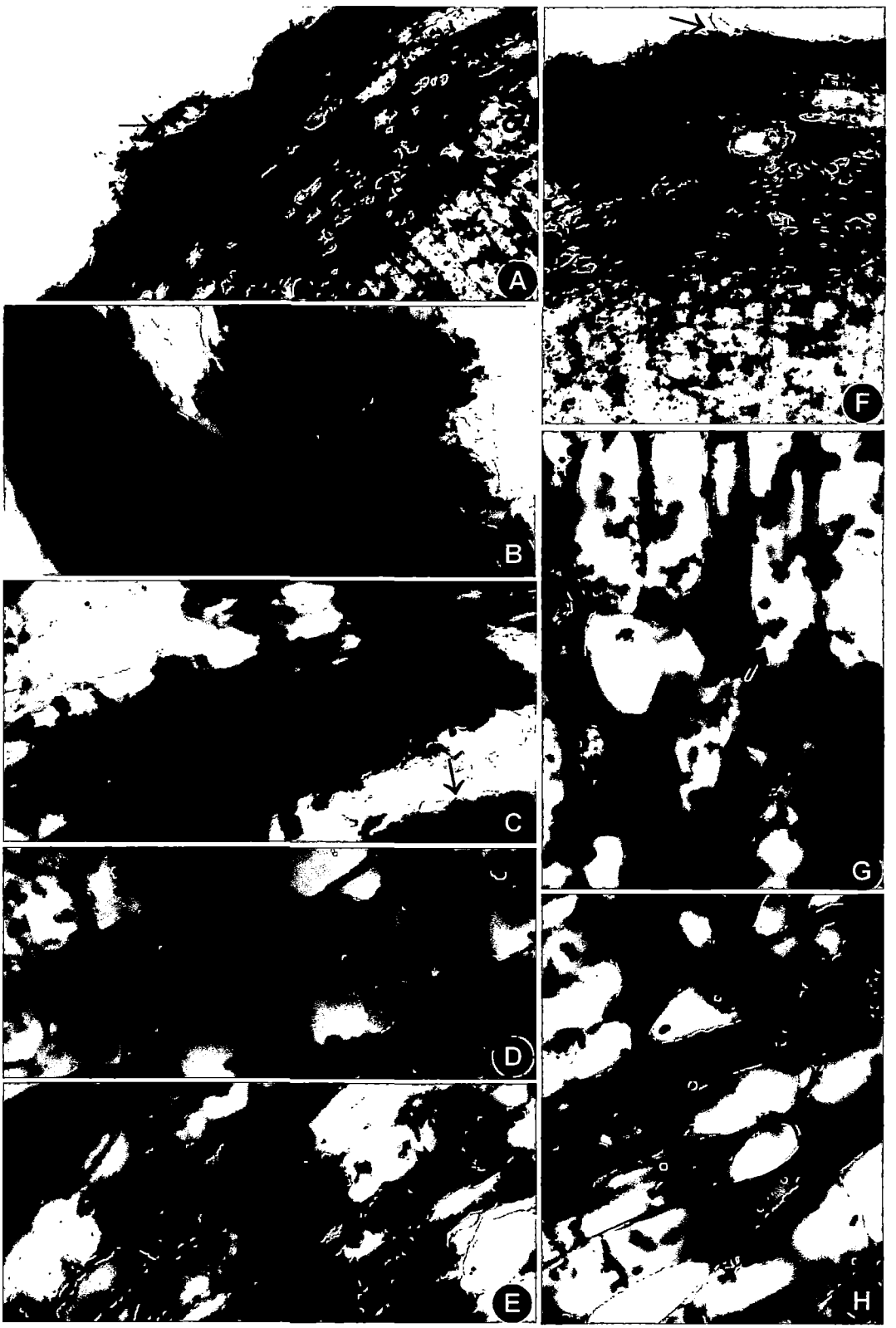


Plate 14 (Figs A-H) : Immunoenzymatic staining of tea root tissues artificially inoculated with *F. lamaoensis*. PAb of *F. lamaoensis* was reacted with root tissue [TV-18 (A & E), UP-26 (D,F&G) & T-78 (B,C&H)] and stained with fast blue BB salt showing the cellular location of fungal hyphae in the root tissue.

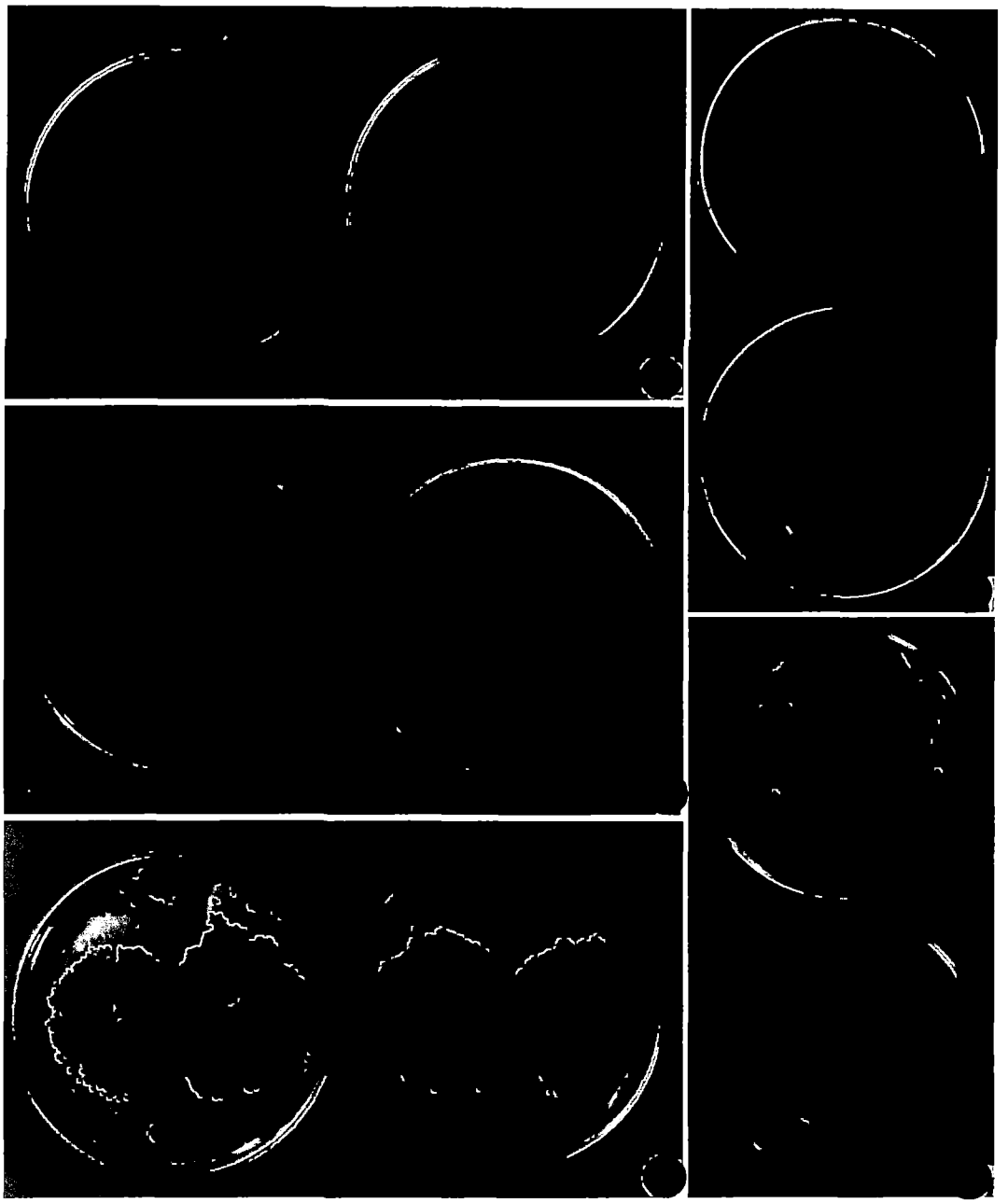


Plate 15 (Figs A-E) : Pairing of *F. lamaoensis.* with *Trichoderma harzianum* and *T. viride.* A,B & C: Homologous pairing of *F. lamaoensis, T. viride* and *T. harzianum* respectively. Pairing of *F. lamaoensis* with *T. viride* (D) and with *T. harzianum* (E).

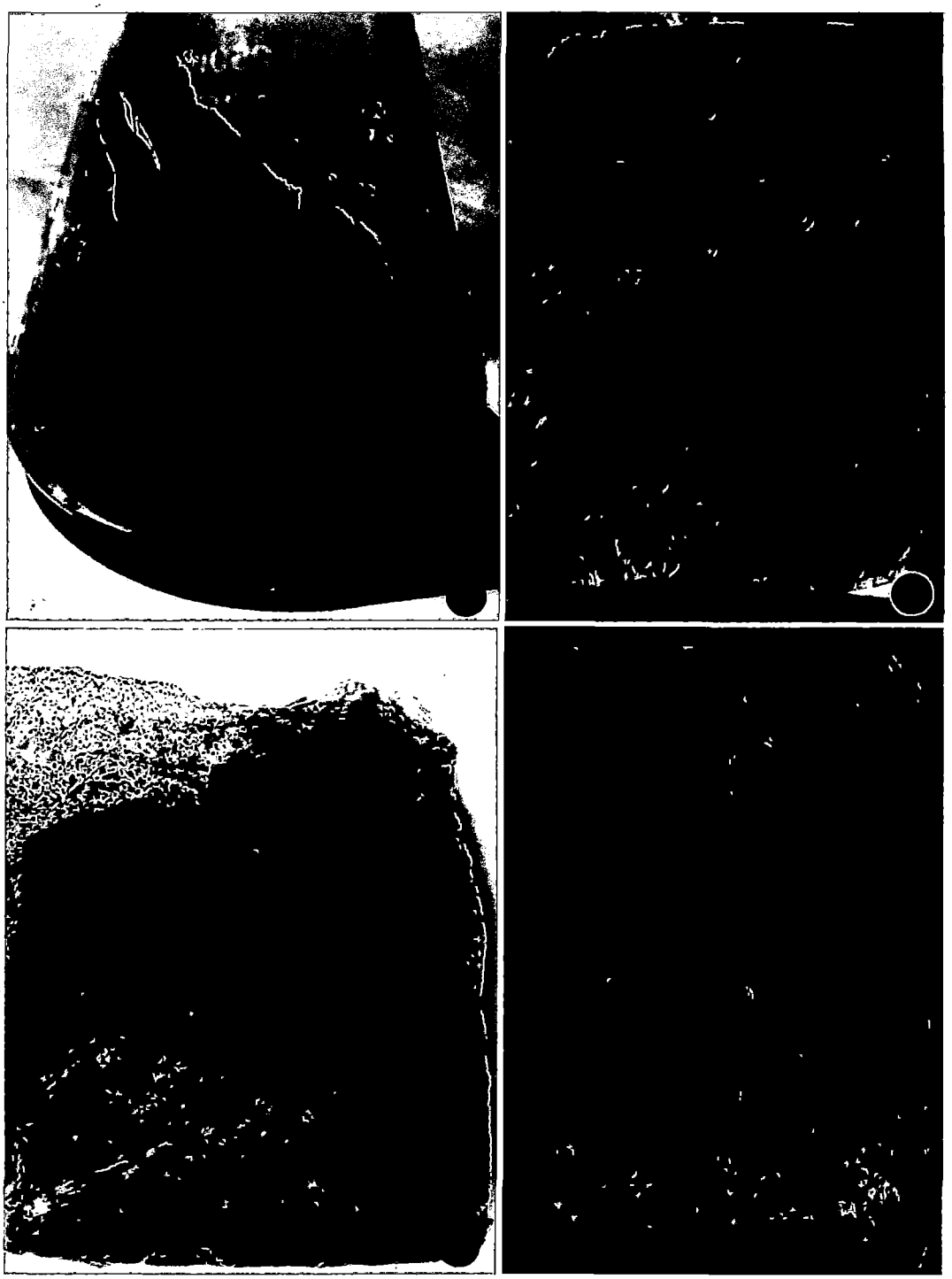


Plate 16 (Figs A-D) : Preparation of inocula. (A) *F. lamaoensis* grown in sand-maize meal medium with tea root pieces; (B &D) *T.viride* grown in tea waste medium; (C) *T. harzianum* grown in tea waste medium.

ratings was as described in materials and methods. Results revealed that treatment with either *T. harzianum* or *T. viride* reduced disease significantly (Table 40) and population of *F. lamaoensis* also increase significantly in rhizosphere soil. At the beginning when pots were inoculated with pathogen.

Table 40 : Effect of *T. harzianum* and *T. viride* on brown root rot disease in potted condition.

Treatments	Disease Index[a]	
	Tea varieties[b]	
	TV-18	UP-26
F. lamaoensis	3.50 ± 0.21	3.83 ± 0.26
F. lamaoensis + *T. harzianum*	0.66 ± 0.20	0.42 ± 0.21
F. lamaoensis + *T. viride*	0.25 ± 0.19	0.17 ± 0.18

No disease was observed in uninoculated control, or those inoculated with either *T. harzianum* or *T. viridi* alone.

[a] 0= No symptoms;
1= Small roots turn brownish and start to rot;
2= Leaves start withering and 20-40% of root turn brown;
3= Leaves withered with 50% of roots affected;
4= Shoot tips start withering 60-70% of roots affected;
5= Shoots withered with defoliation of lower withered leaves, 80% roots affected;
6= Whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

[b] Age of plants 2 yr
Average of 12 separate inoculated plants
40 days after inoculation
± Standard error.

4.15.2.2. Fields grown plants

For field experiments 4 selected varieties of 5 year old plants were taken and among them 3 varieties were susceptible (TV-18, UP-26 and T-78) and one was resistant (TV-26) as proved in earlier pathogencity test as well as different immunoenzymatic reactions. Different treatment with bio-control agents were done and disease incidences were recorded as described in earlier experiments. In this experiment also both. *T. harzianum* and *T. viride* significantly reduced the disease

intensity (Table 41 and Fig. 14) as the result of TV-18 shown in Plate17. In case of treatment with *F. lamaoensis* alone all the plants TV-18, UP-26, T-78 varieties died after 40 days of inoculation but TV-26 showed a certain degree of resistance.

Table 41 : Effect of *T. harzianum* and *T. viride* on brown root rot disease in field condition.

Tea varieties[b]	Disease Index[a]				
		Days after inoculation			
	Treatment	10	20	30	40
TV-18	F.l.	1.83 ± 0.24	2.75 ± 0.19	4.50 ± 0.21	5.33 ± 0.24
	F.l. + T.h	0.17 ± 0.18	0.33 ± 0.20	0.83 ± 0.24	1.16 ± 0.18
	F.l + T.v.	0	0	0.42 ± 0.21	0.58 ± 0.20
TV-26	F.l.	0.83 ± 0.18	1.00 ± 0.22	1.17 ± 0.21	2.33 ± 0.29
	F.l + T.h.	0	0.33 ± 0.20	0.66 ± 0.20	0.75 ± 0.28
	F.l + T.v.	0	0.17 ± 0.18	0.17 ± 0.18	0.33 ± 0.20
UP-26	F.l.	2.66 ± 0.20	3.66 ± 0.38	5.50 ± 0.32	5.83 ± 0.18
	F.l.+ T.h	0.33 ± 0.20	0.66 ± 0.20	1.33 ± 0.20	1.66 ± 0.21
	F.l. + T.v.	0.17 ± 0.18	0.33 ± 0.20	0.66 ± 0.20	0.93 ± 0.16
T-78	F.l.	2.33 ± 0.20	3.00 ± 0.18	5.00 ± 0.12	5.66 ± 0.32
	F.l. + T.h	0.17 ± 0.18	0.33 ± 0.20	0.66 ± 0.21	1.33 ± 0.20
	F.l. + T.v.	0.	0.17 ± 0.18	0.25 ± 0.19	0.83 ± 0.18

No disease was observed in uninoculated control, or those inoculated with either *T. harzianum* or *T. viridi* alone.

[a] 0= No symptoms;

1= Small roots turn brownish and start to rot;

2= Leaves start withering and 20-40% of root turn brown;

3= Leaves withered with 50% of roots affected;

4= Shoot tips start withering 60-70% of roots affected;

5= Shoots withered with defoliation of lower withered leaves, 80% roots affected;

6= Whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

[b] Age of plants 2 yr; Average of 12 separate inoculated plants; 40 days after inoculation; ± Standard error; F.l. = *F. lamaoensis,* T.h. = *T. harzianum,* T.v. = *T. viride.*

Brown root rot incidence in the field following soil treatment with *Trichoderma* sp.

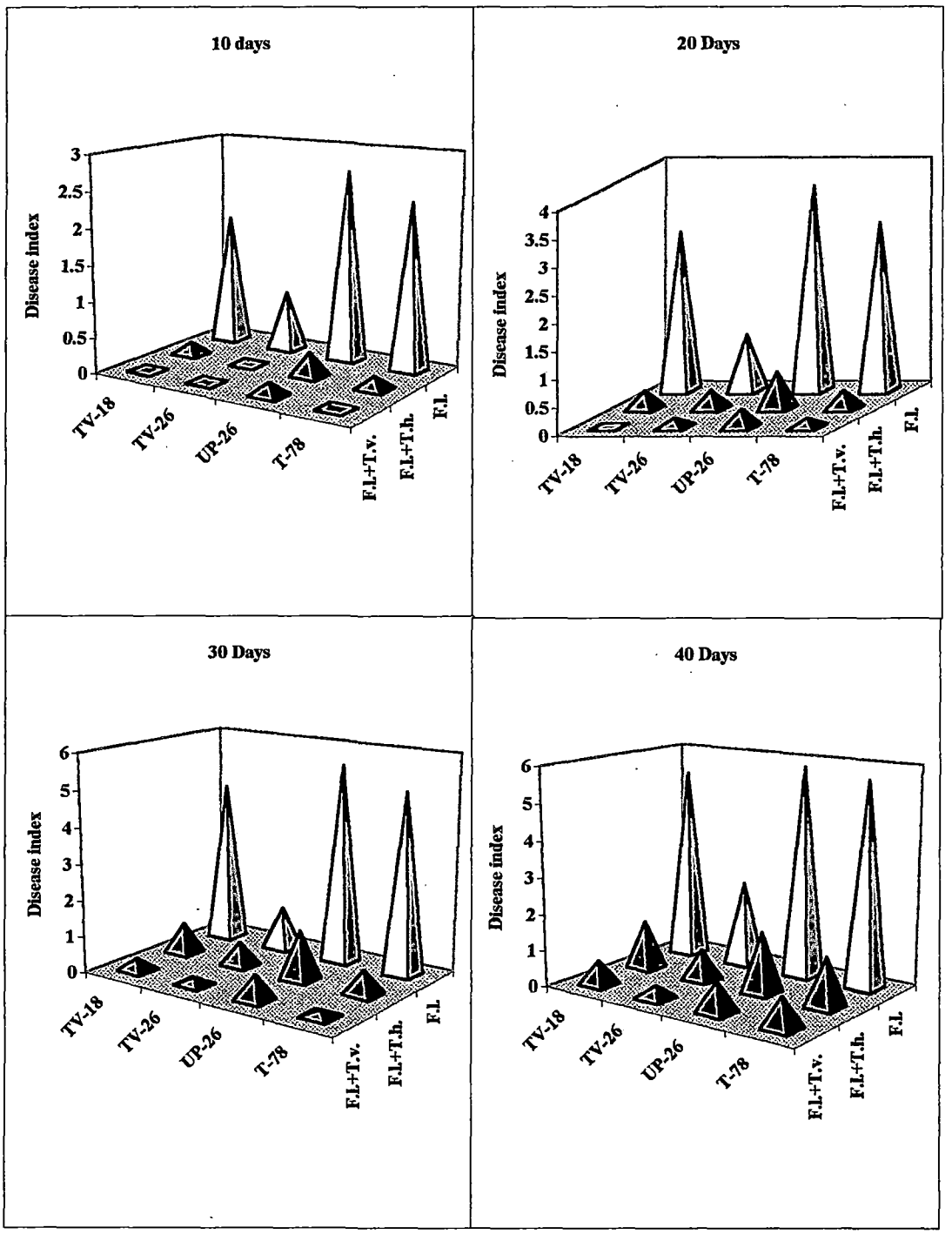


Fig.14

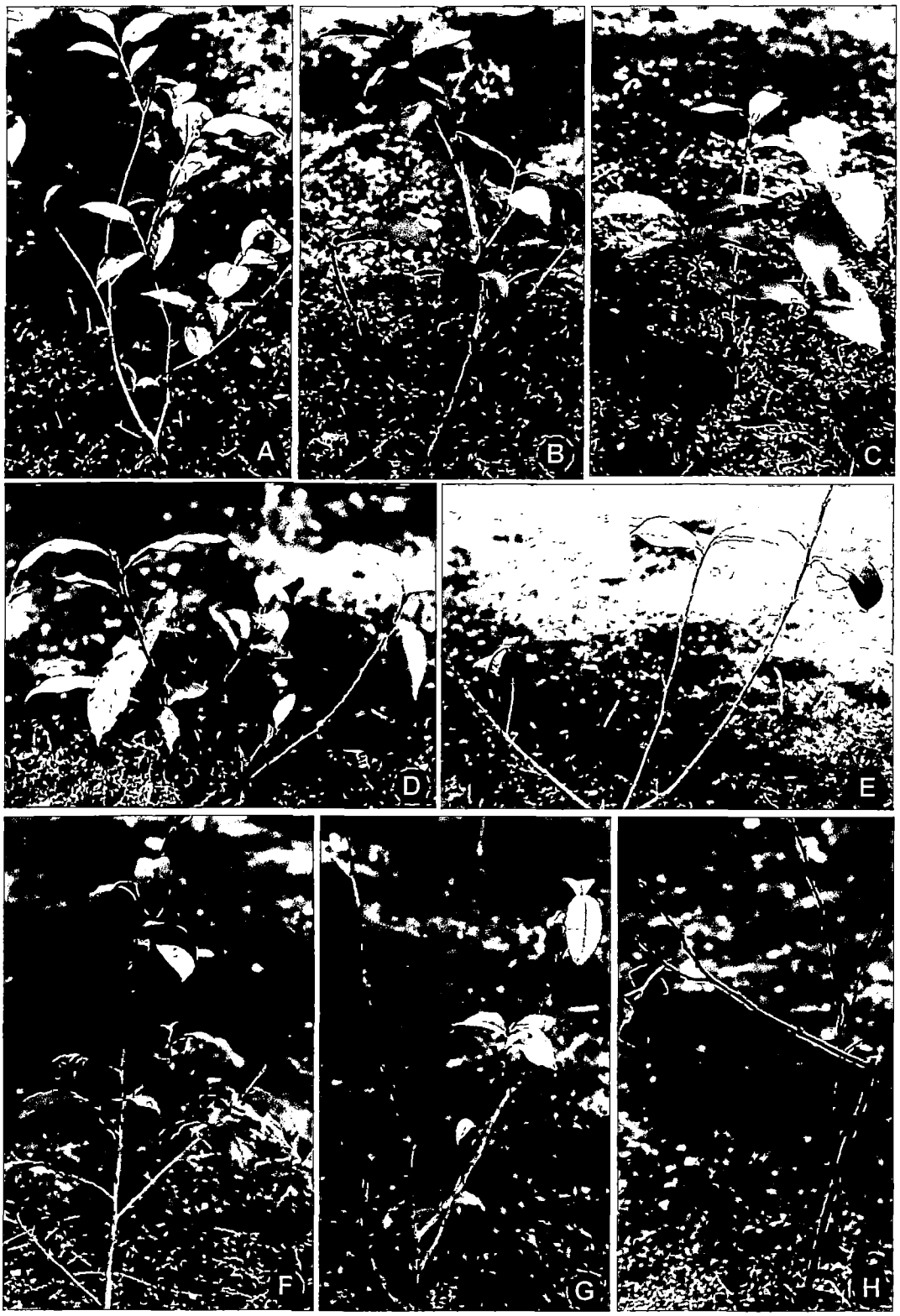


Plate 17 (Figs A-H) : Field trial of biocontrol agents for management of brown root rot disease of tea plant (TV-18). (A) Healthy , (B) *T.viride* inoculated, (C) *T.harzianum* inoculated, (D&F) *T.viride* + *F.lamaoensis* inoculated (E&G), *T.harzianum* +*F.lamaoensis* inoculated and (H) *F.lamaoensis* inoculated.

4.16. Detection of *F. lamaoensis* in tea root and soil following treatment of biocontrol agents

4.16.1. ELISA

Since the application of biocontrol agents in rhizosphere soil reduced intensity of brown root rot disease, it was decided to investigate whether this reduction could also be determined immunologically in both root tissues and soil. For this purpose DAC ELISA, and competition ELISA were carried out. ELISA reactions were performed with root antigens from different treatments as well as soil antigens.

4.16.1.1. Root tissues

Root antigens were prepared from uprooted plants (2yr old) of different treatment after 40 days of pathogen inoculation for biocontrol experiment of potted plants. These antigens were reacted in DAC-ELISA using PAb-103. Results showed that ELISA values of roots treated with *T. harzianum* and *T. viride* were significantly lesser than with *F. lamaoensis* alone (Table 42). In case of 5yr old field grown plants also similar results were observed (Table 43), though A405 values were higher than the potted plants.

Table 42 : ELISA of reactions of PAb *F. lamaoensis* with root antigens of tea varieties following treatments with Biocontrol agents.

Antigen Source	Absorbance at 405nm	
	TV-18	UP-26
Control Plant	0.576 ± 0.05	0.546 ± 0.11
Treatments		
F. lamaoensis + *T. harzianum*	0.602 ± 0.04	0.592 ± 0.02
F. lamaoensis + *T. viride*	0.592 ± 0.04	0.566 ± 0.03
F. lamaoensis	0.836 ± 0.07	0.854 ± 0.03
T. harzianum	0.582 ± 0.09	0.569 ± 0.06
T. viride	0.594 ± 0.02	0.572 ± 0.08

Antigen concentration 100µg/ml
IgG source-103 PAb; concentration 40µg/ml
40 days after pathogen inoculation
Age of plants 2yr
± Standard error.

Table 43 : ELISA values of reactions of PAbs with root antigens of tea varieties following treatment with biocontrol agents.

	Source of PAbs					
Antigen source	***F. lamaoensis* (103)**		***T. harzianum* (703)**		***T. viride* (603)**	
	UP-26	**T-78**	**UP-26**	**T-78**	**UP-26**	**T-78**
Control plant	0.572 ± 0.04	0.591 ± 0.02	0.322 ± 0.03	0.353 ± 0.04	0.372 ± 0.04	0.378 ± 0.04
Treatments						
F. lamaoensis + *T. harzianum*	0.585 ± 0.05	0.603 ± 0.06	0.390 ± 0.09	0.363 ± 0.02	0.385 ± 0.04	0.377 ± 0.03
F. lamaoensis + *T. viride*	0.554 ± 0.03	0.574 ± 0.09	0.330 ± 0.02	0.335 ± 0.03	0.387 ± 0.03	0.384 ± 0.06
F. lamaoensis	1.345 ± 0.05	1.396 ± 0.04	0.413 ± 0.04	0.436 ± 0.06	0.524 ± 0.07	0.536 ± 0.08
T. harzianum	0.567 ± 0.02	0.579 ± 0.04	0.385 ± 0.03	0.372 ± 0.05	0.356 ± 0.05	0.363 ± 0.06
T. viride	0.575 ± 0.03	0.593 ± 0.09	0.334 ± 0.08	0.329 ± 0.03	0.384 ± 0.02	0.392 ± 0.04

[a]Antigen concentration 100µg/ml
IgG concentration 40µg/ml
40 days after pathogen inoculation.
Age of plants 5 year
± Standard error.

4.16.1.2. Soil

Soil samples of the rhizosphere of different treatments were collected at a depth of 7-9 inches from soil surface. *F. lamaoensis* was evaluated through DAC ELISA and competition ELISA by reacting the antigens from collected soils after 30days of pathogen inoculation with the PAbs of *F. lamaoensis, T. harzianum* and *T. viride.* Control set was prepared from uninfested soil of control plants.

In DAC-ELISA results from soil treated with *F. lamaoensis* and *T. harzinum* or *F. lamaoensis* and *T. viride* reacted with PAb of *F. lamaoensis* showed significantly lower absorbance values than that of soil antigen treated with *F. lamaoensis* alone. This indicated that population of *F. lamaoensis* soil had been reduced by the biocontrol agents (Table 44).

Table 44 : ELISA values of soil antigens of different treatments with PAbs of *F. lamaoensis, T. harzianum* and *T. viride.*

	Source of PAbs		
Antigen source	***F. lamaoensis***	***T. harzianum***	***T. viride***
Uninfested soil	0.308 ± 0.09	0.273 ± 0.04	0.305 ± 0.03
Treatment			
F. lamaoensis + *T. harzianum*	0.483 ± 0.04	0.811 ± 0.04	0.412 ± 0.06
F. lamaoensis + *T. viride*	0.490 ± 0.04	0.454 ± 0.07	0.752 ± 0.08
F. lamaoensis	0.945 ± 0.02	0.336 ± 0.05	0.485 ± 0.06
T. harzianum	0.430 ± 0.02	0.819 ± 0.04	0.417 ± 0.04
T. viride	0.379 ± 0.06	0.339 ± 0.05	0.882 ± 0.09

Sample collected 30 days after pathogen inoculation.
PAb dilution 1:500
± Standard error.

The same trend of result (Table 45, Fig.15) was also obtained in case of competition ELISA which is an inhibition ELISA. Reduction of population in soil treated with *T. harzianum* and *T. viride* was confirmed using this ELISA format and detailed procedure has been outlined in materials and methods. Antigens were prepared from soils under various treatments as mentioned earlier and were used under doubling dilutions of from 1:25 to 1:400. Since competition ELISA is a double binding assay, where PAb is allowed to react to the test antigen first and the residual PAb is once again reacted with homologous antigen in separate plates, higher ELISA values in this procedure would indicate lower reactivity to a test sample.

Table 45 : Competition ELISA of various dilution of treated soil antigens with PAbs of *F. lamaoensis, T. harzianum* and *T. viride*.

Soil antigen		Absorbance at 405nm Source of IgG		
Treatment	**Dilution**	***F. lamaoensis* (103)**	***T. harzianum* (703)**	***T. viride* (603)**
Fomes lamoensis	1:25	0.505 ± 0.07	1.043 ± 0.06	1.188 ± 0.02
	1:50	0.721 ± 0.05	1.246 ± 0.09	1.360 ± 0.04
	1:100	0.759 ± 0.08	1.301 ± 0.07	1.447 ± 0.04
	1:200	0.855 ± 0.10	1.390 ± 0.04	1.539 ± 0.07
	1:400	0.941 ± 0.09	1.429 ± 0.03	1.610 ± 0.05
F. lamaoensis +	1:25	1.658 ± 0.05	0.477 ± 0.05	1.284 ± 0.04
T. harzianum	1:50	1.698 ± 0.03	0.720 ± 0.07	1.483 ± 0.04
	1:100	1.763 ± 0.04	0.826 ± 0.07	1.511 ± 0.08
	1:200	1.860 ± 0.05	0.887 ± 0.06	1.691 ± 0.06
	1:400	1.934 ± 0.06	0.999 ± 0.05	1.704 ± 0.04
F. lamaoensis +	1:25	1.790 ± 0.05	0.853 ± 0.06	0.640 ± 0.06
T. viride	1:50	1.845 ± 0.05	1.027 ± 0.07	0.838 ± 0.04
	1:100	1.885 ± 0.03	1.054 ± 0.05	0.947 ± 0.12
	1:200	1.923 ± 0.05	1.154 ± 0.05	1.009 ± 0.05
	1:400	1.980 ± 0.08	1.263 ± 0.06	1.056 ± 0.05
T. harzianum	1:25	1.626 ± 0.05	0.440 ± 0.03	0.941 ± 0.08
	1:50	1.792 ± 0.07	0.698 ± 0.03	1.337 ± 0.09
	1:100	1.806 ± 0.05	0.754 ± 0.05	1.384 ± 0.06
	1:200	1.872 ± 0.09	0.820 ± 0.05	1.427 ± 0.05
	1:400	1.972 ± 0.06	0.934 ± 0.07	1.476 ± 0.04
T. viride	1:25	1.683 ± 0.05	0.809 ± 0.09	0.414 ± 0.05
	1:50	1.741 ± 0.05	0.925 ± 0.05	0.715 ± 0.05
	1:100	1.782 ± 0.06	0.976 ± 0.05	0.870 ± 0.09
	1:200	1.912 ± 0.08	1.095 ± 0.06	0.909 ± 0.04
	1:400	1.943 ± 0.09	1.281 ± 0.09	1.086 ± 0.06
Control	1:25	1.879 ± 0.10	1.451 ± 0.08	1.486 ± 0.11
	1:50	1.929 ± 0.06	1.523 ± 0.05	1.554 ± 0.08
	1:100	1.971 ± 0.09	1.545 ± 0.05	1.674 ± 0.03
	1:200	2.091 ± 0.06	1.591 ± 0.08	1.731 ± 0.06
	1:400	2.121 ± 0.07	1.597 ± 0.04	1.761 ± 0.05

Sample collected 30 days after pathogen inoculation.
IgG concentration 40µg/ml
± Standard error.

Competition ELISA of treated soil antigens with PAbs of *F. lamaoensis, T. harziamun and T. viride*

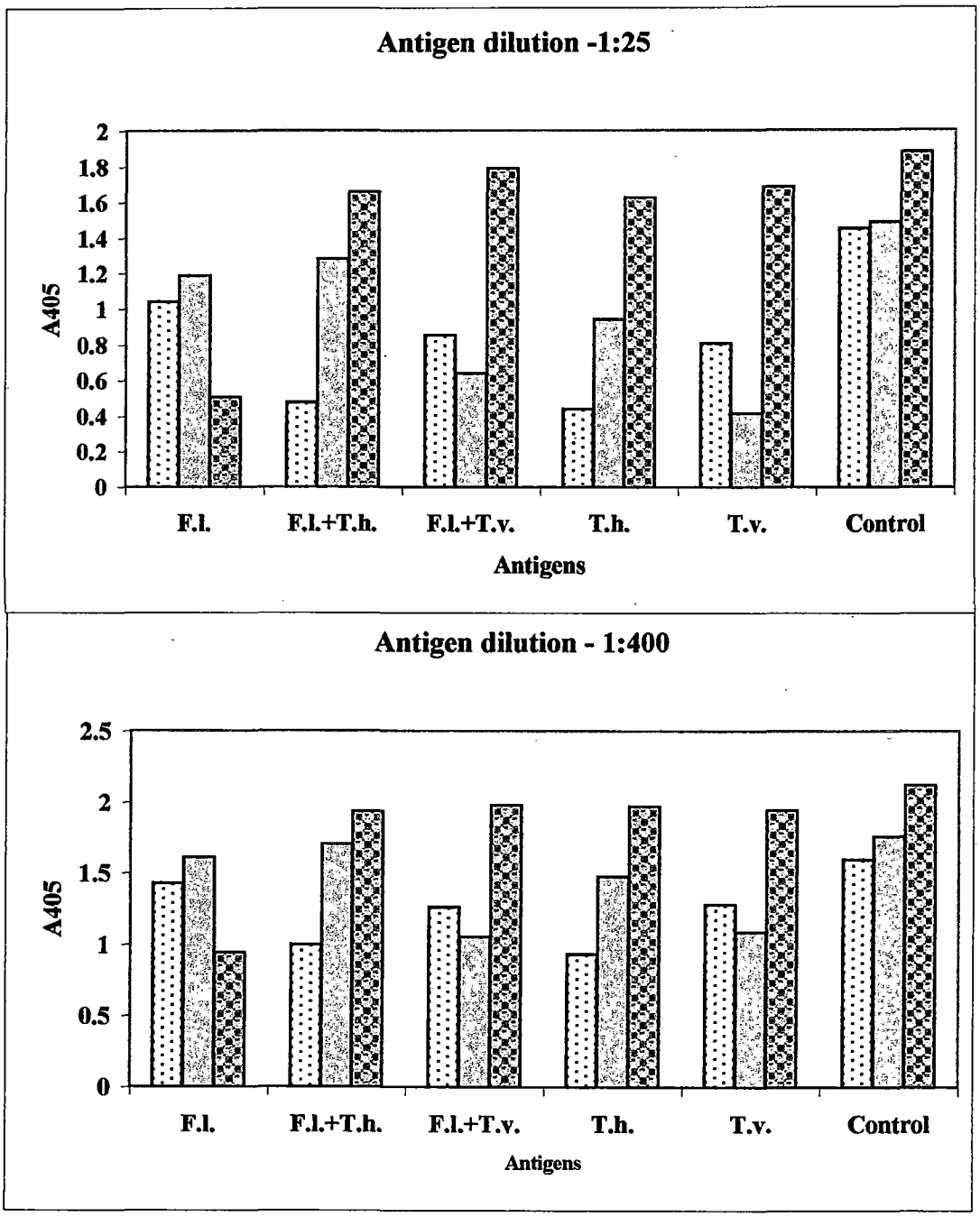


Fig.15

4.16.2. Dot-Blot

Results presented in Table 46 & Plate 18, fig.C revealed that when PAb of *F. lamaoensisi* (mycelia and cell wall) was treated in the antigens from soil subjected to different treatments (described earlier), positive reaction was obtained in case of treatment with *F. lamaoensis* alone. Similarly when these were treated with PAbs of *T. harzianum* or *T. viride* positive reactions were shown in cases where soil was amended with the particular fungus. In cases where soil was treated with both *F. lamaoensis* and *T. harzianum* or *T. viride* also, positive reaction were not evident.

Table 46 : Dot-blot of soil antigens of different treatment (with combinations of *Trichoderma* sp. and *F. lamaoensis*) collected from root rhizosphere of field.

	Colour intensity[a]			
	PAbs raised against			
Antigen source	***F. lamaoensis* Mycelial (103)**	***F. lamaoensis* Cell wall (203)**	***T. viride* Mycelial (603)**	***T. harzianum* Mycelial (703)**
Sterile soil	–	–	–	–
Control Soil	–	–	–	–
Soil treated with				
T. harzianum	–	±	–	+
T. viride	–	–	+	–
F. lamaoensis	++	++	–	–
F. lamaoensis + *T. harzianum*	–	±	±	+
F. lamaoensis + *T. viride*	–	–	+	±
Mycelial				
F. lamaoensis	++++	+++	+	±
T. harzianum	±	+	++	++++
T. viride	+	+	++++	+

[a]Fast Red colour intensity (Pinkish red) :(+ + + +) Bright, (+ + +) High, (+ +) Medium, (+) Low, (±) Faint, (–) No colour.

4.16.3. Western Blot

Western Blot analysis of soil samples from different treatments set up using PAb of *F. lamaoensis* revealed four bands with molecular weight of ca. 79, 64, 55 & 31 kDa. These four bands were also present in homologous reaction, with mycelial

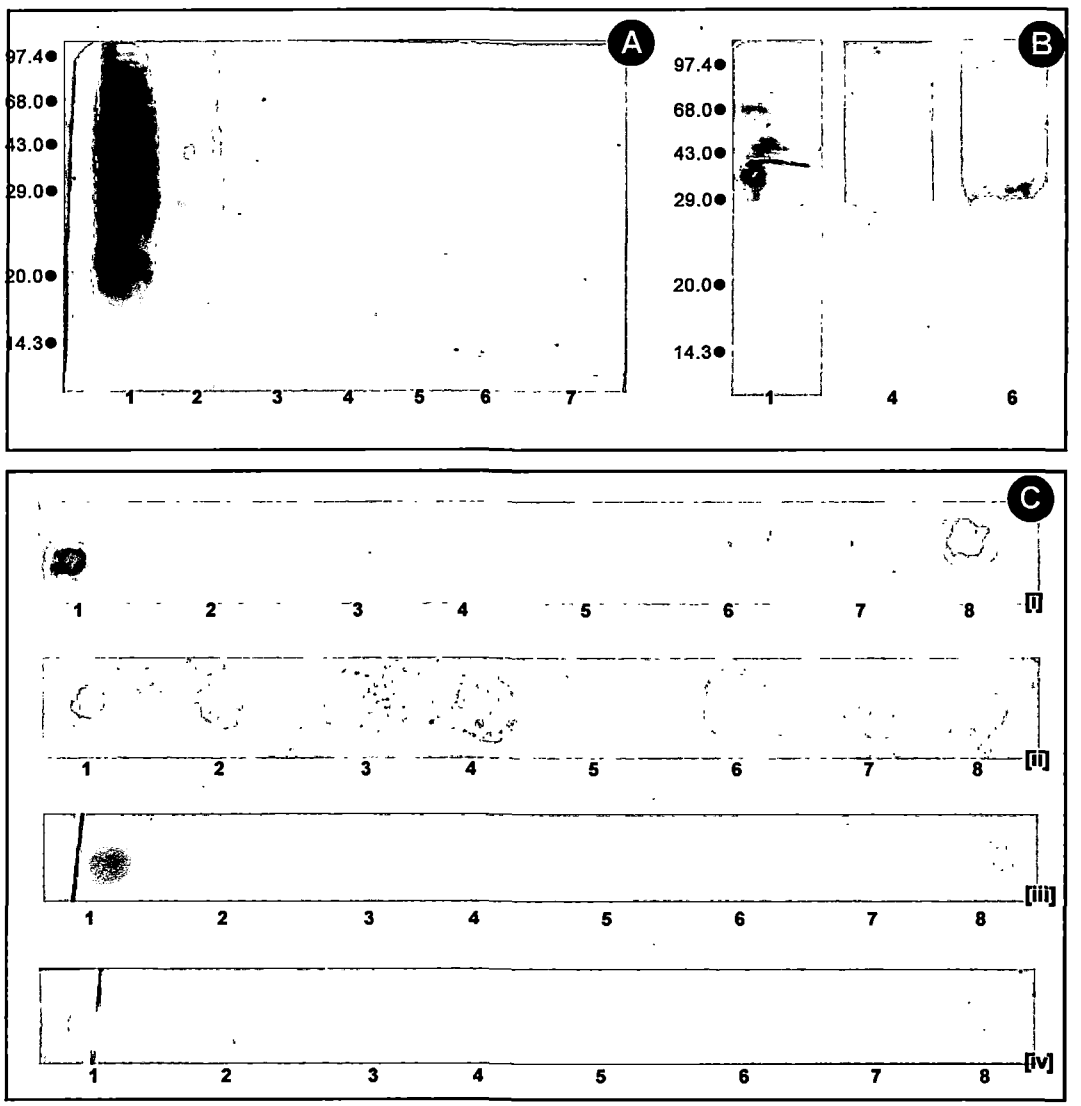


Plate 18 (Figs A-C) : A&B - Western blot analysis of tea rhizosphere soil of different treatments probed with PAb 103 (A) & PAb 603 (B); lane 1-mycelial antigens of *F. lamaoensis* (A) and *T. viride* (B) ; lanes 2-6: soils treated with *F.lamaoensis*(2), *F.lamaoensis*+ *T.harzianum*(3), *F.lamaoensis*+ *T.viride* (4), *T.harzianum* (5), *T.viride* (6);lane 7-Control soil. **C**- Dot blot analysis of tea rhizosphere soils of different treatments probed with PAbs -103 [i] , 203 [ii] , 703[iii] and 603 [iv]. Antigens were: homologous antigens (1), sterile soil (2), control soil (3), amended soils -*T.harzianum* (4), *T.viride* (5), *F.lamaoensis*+ *T.harzianum* (6), *F.lamaoensis* + *T.viride* (7) and *F. lamaoensis* (8).

Table 47 : Western blot analysis of different soil antigens from root rhizosphere infested with different combinaiton of *Trichoderma* sp. and *F. lamaoensis.*

Antigen source	IgG No.			
	103		603	
	No.of bands	Mol.wt. kDa	No.of bands	Mol.wt. kDa
Mycelia				
F. lamaoensis	17	104.3, 97.4, 79.0, 60.9, 50.1, 43.0, 38.6, 32.2, 27.1, 24.5, 22.2, 19.0, 18.4, 17.4, 15.3, 14.3, 12.7,		NR
T. viride	NR		10	92.8,83.6,72.6 68.0,54.5,50.0, 43.0,40.8,38.6, 29.8.
Soil treated with				
(a) *F. lamaoensis*	4	79.0,63.5,54.5, 31.2	Nil	
(b) *T. harzianum*	Nil		Nil	
(c) *T. viride*	Nil			92.8,50.0,40.8
			4	38.6
(d) *F. lamaoensis* + *T. harzianum*	Nil		2	63.5, 54.5
(e) *F. lamaoensis* + *T. viride*	Nil		3	92.8, 50.0,38.6
(f) Uninfested soil	Nil		Nil	

IgG concentration 40µg/ml.

antigen though 18-19 bands were found in case of homologous reaction. In case of soil from rhizospheres of plants inoculated with the biocontrol agents prior to inoculation with the pathogen, no bands were visible (Table 47 & Plate 18, fig. A&B). When these soil antigens were treated with PAb of *T. viride,* 4 bands of ca. mol. wt. 93, 50, 41 & 39 kDa were obtained with soil inoculated with *T. viride,* 3 bands (ca. 93, 50 & 39 kDa mol. wt.) with *F. lamaoensis* + *T. viride* and 2 bands with *F. lamaoensis* + *T. harzianum.*

DISCUSSION

Plants in nature have evolved a series of mechanisms by which they can defend themselves against the multitude of organisms attacking them. The versatile multicomponent defence is adequate to provide them protection against most of their potential pathogens; only a few of them can overcome this defence and cause disease (Sinha, 1995). Varieties within the host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such genes. To account for the observed specificity and degree of variability of host parasite system, the fungal receptors must have high information content. Antigens located on the cell surface are generally involved in information transfer and/ or the maintenance of membrane integrity during the cell-to-cell interaction of host and parasites. Host pathogen interaction occurring in nature is one of the most complex biological processes and involves recognition between the host and pathogen both at the cellular and subcellular level. A cell that reacts in a special way in consequence of an association with another cell or its products does so because it acquires information, which is conveyed through chemical or physical signals in the process of recognition. 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). The spatial and temporal deployment of plant defence responses involves the complex interplay of signal events, often resulting in superimposition of signaling processes (Graham and Graham, 1996). In spite of lacking immune responses like animals, plants have nevertheless 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 distinct range of host plants

The present study was undertaken to study the interaction of tea and *F. lamaoensis,* the causal organism of brown root rot disease. At the onset, pathogenicity test of the Brown root rot pathogen *Fomes lamaoensis* was carried out on 10 Tocklai, 6 UPASI and 9 Darjeeling tea varieties. Among the Tocklai, UPASI and Darjeeling varieties TV-18, UP-26 and T-78 were most susceptible, respectively, while TV-26, UP-8 and BS/7A/76 were most resistant. Among all twentyfive varieties, UP-26 was most

susceptible. Since available reports indicated that the tea plants become more susceptible from 3yr upward disease assessment of plants of all ages was carried out and disease intensity was observed to be maximum in 5yr old plants and minimum in 1yr old plants.

The growth and infectivity of the pathogen depends on a number of environmental factors like temperature, pH of medium and seasonal changes. So, the effect of different cultural conditions on growth of *F. Lamaoensis invitro* was carried out. physiological studies in relation to medium, pH, incubation period was carried out. The optimum temperature for the growth of *Fomes lamaoensis* was 30°C which was in accordance to previous reports (Barthakur and Samajpati, 1985). Maximum vegetative growth was obtained with 15 days of incubation period utilizing carbon source, lactose or fructose and calcium nitrate and beef extract as inorganic nitrogen and organic nitrogen sources respectively. The pathogen was found to withstand a wide range of pH (3.0-8.0) but the optimum was 4.5-5.0 and the pathogen grew well under acidic condition (pH 4.0-6.0), which coincides with the pH of tea soil. The wood-rotting fungi exhibit maximum metabolic activity at the acidic range (pH 3.0- 7.0) in most cases (Srivastava and Bano, 1970; Brodziac, 1980 and Hong *et al.*, 1981).

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 defense 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 antigens might be involved in determining the degree of compatibility in such interactions which has been reviewed by several authors (DeVay *et. al.*, 1972; DeVay and Alder, 1976; Kalyansundaram, 1978; Chakraborty, 1988; Purkayastha, 1989; Purkayastha et. al., 1991). In the present study, polyclonal antibodies (Pabs) raised against fungal and host tissues were used for determining the presence of cross reactive antigens (CRA)

between tea varieties and *F. lamaoensis* as well as for the immunodetection of the pathogen in the tea root tissues and soil. Effectiveness of each PAb was initially checked through immunodiffution tests. Since immunoenzymatic reactions like ELISA were very sensitive techniques and non-specific bindings interferes with the actual antigen-antibody reactions, IgG was purified from crude antiserum following ammonium sulphate precipitation and fractionation through chromatography on DEAE-cellulose column as described by Clausen (1988).

Enzyme linked immunosorbent assay (ELISA) has proved to be one of the most sensitive serological techniques. Since ELISA depends on number of factors and this varies from system to system it was considered essential to optimize various conditions before further tests. Hence sensitivity of assay was optimized considering two variables i.e. concentration of antigen and concentration of IgG using Direct antigen coated (DAC) ELISA. Enzyme dilution ,1:10,000, and substrate concentration, 1mg/ml, were kept constant. Positive results were obtained with very low concentration of both antigen and IgG and the sensitivity tests of Pabs obtained from different bleedings raised against different immunogens showed maximum ELISA values in 3rd followed by 4th bleedings. It was reported by Mohan (1988) that a concentration of Phytophthora antigens as low as 2ng/ml could be detected in indirect ELISA by antiserum raised against pooled mycelial suspensions of five *P. fragariae* races. Chakraborty *et. al.* (1996) also reported that antiserum raised against *Pestalotiopsis theae* could defect homologous antigen at 25ng/ml and antisera upto 1: 16,000 was effective for detections.

In indirect ELISA the presence of CRA among *F. lamaoensis* and tea varieties, using PAb raised against mycelia and cell wall antigen preparations of *F. lamaoensis* at a concentration of 40 mg/ml with tea root antigens at a concentration of 100 mg/ml. In the present study, TV-18, UP-26, T-78 showed maximum absorbance values in ELISA. Also in this study, though indirect ELISA readily detected CRA between tea root antigens and *F. lamaoensis*, much difference was not observed among the different tea varieties. CRA was also deteced in crude preparations and purified preparations from mycelia of *Phytophthora infestans* (races 4 and 1.2.3.4.7) with antisera of potato cvs. King Edward and Pentland Dell in concentrations lower than 50 mg/ml protein

(Alba and Devay, 1985) using indirect ELISA. The presence of CRA in several host pathogen interactions has also been reported by a number of previous workers, e.g. soybeen and *Myrotheciun roridum* (Ghosh and Purkayastha, 1990); groundnut and *Macrophomina phaseolina* (Purkayastha and Pradhan, 1994); tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994) and tea and *Ustulina zonata* (Chakraborty *et.al.* 2002b). In order to determine whether the observed cross reactivity between *F. lamaoensis* and susceptible varieties was specific, antigen preparations from non host (*Oryza sativa, Dracaena* sp., *Leucaena leucocephala, Phyllanthus niruri*) and non pathogen (*Fusarium oxysporum, Beauveria Bassiana*) were also assayed with PAb of mycelia and cell wall of *F. lamaoensis* and showed very low reactivity in ELISA. Since the indirect ELISA tests were made under the same conditions and with at least three replicates it appears that these observed antigenic disparities have same significance in the basic compatibility of host and pathogen (Chakraborty, 1988).

Cross reactivity of the PAb raised against *F. lamaoemsis* was tested with other soil fungi. Results revealed that among all the fungi tested PAb of *F. lamaoensis* reacted to some extent with two tea root pathogens *U. zonata* and *R. arcuata*. Mohan (1989) showed that antisera raised against mycelial suspension of *Phytophthora fragariae*(PfM) reacted strongly with antigens 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 to all *Phytophthora* sp. tested. Similar results with *P. frageriae* were also reported by Amouzon-alladaye *et.al.* (1988). In their studies, antibodies obtained with a strain of *P. fragariae* detected two different strains of this pathogen in pure culture by DAS and DAC-ELISA. Non-specific cross reactions with most fungi of the root flora of strawberry represented an advantage. Harrison *et. al.* (1990) further reported that anti-*P. infestans* g globulin reacted strongly withextract of *P. erythroseptica* in DAC-ELISA but not with extracts of nine unrelated fungi or a culture of bacterium *Erwinia carotovora*, all of which were saprophytes of pathogens of potato.

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. 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 law amount in or on plant tissue and therefore in many cases the pathogen can be detected in much earlier stage of disease development than that was previously possible (Hansen and Wick, 1993) and also when visible symptoms have not yet developed. This offers a definite advantage over classical techniques and is thus gaining an importance for pathogen detection purposes. Various formats of ELISA using polyclonal antisera has found wide spread application in plant pathology and are routinely used for detection and identification purposes (Clark, 1981; Lommel *et.al.* 1982; Sundaram *et.al.* 1991; Lyons &White 1992 Chakraborty *et. al.* 1996 and Viswanathan *et.al* 2000). In the present study antisera raised against mycelia or cell wall antigen preparations of *F. lamaoensis* were used for the detection of the pathogen in tea roots and the responses were in general slightly higher (about 0.1) in case of cell wall PAb than mycelial PAb. Infected tea root extracts generally had higher ELISA values than the healthy ones. In case of two years old plants the difference of absorbance values between infected and healthy root extracts was not significantly higher except in susceptible varieties. Again the difference increased with the age of the plants as evident in present investigation using plants of different ages, from 1 yr. to 5 yr ld. Results from data also showed that the degree of detection of *F. lamaoensis* significantly higher from 3 yr. upward in susceptible varieties (TV-18, UP-26 and T-78) but resistant varieties (eg. TV-26) did not show high A405 values even in case of 5 yr plants. Holtz *et.al.* (1994) raised 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. Viswanathan *et.al.* (2000) Reported that presence of *Colletotrichum falcatum* in sugarcane tissues could be detected by ELISA. They reported that when twenty different sugarcane varieties were subjected to ELISA test after pathogen inoculation

a clear variation in disease resistance was seen. They suggested that this technique could be reliably used to screen sugarcane genotypes for red rot resistance at an early stage. In the present study presence of *F. lamaoensis* in tea root tissues could be detected by both DAC and DAS- ELISA using PAb raised against either mycelial or cell wall antigens . It was observed that PAb *F. lamaoensis* also weakly reacted with antigens from tea roots infected with other pathogens showing certain degree of cross reactivity. 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), *Septoria nodorum* and *S. tricity* in wheat (Mittermier *et.al.*,1990;Peterson *et. al.* 1990) and *Sclerotinia sclerotiorum* in rapeseed (Jamaux and Spire, 1994). Mohan (1998) 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.*(1998) 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.

Using PAb prepared against mycelial and cell wall antigenic determinants the dot detection of infection in root tissue and rhizosphere soil was done. The dot immunobinding technique has been found to be rapid and sensitive method for detection of virus and plant pathogenic bacteria. This procedure was tested for the specificity of antiserum for possible cross reaction with other root inhabiting fungi by Lange *et.al.*(1989). The antiserum against *Plasmodium brassicae* used in their study showed no cross reaction with other common pathogen (*Pythium ultimum Rhizoctonia solani* and *F.oxysporum*) and did not cross react with resting spores of *Polymyxa betae*, which is also member of the Plasmodiophoraceae . In this study, PAb raised against antigen of mycelia, cell wall of *F. lamaoensis* using was tested fast red or NBT/BCIP

as substrate. Antigens of homologous source and infested soil showed deep coloured dot. Infected tea root antigens also showed deep coloured dot when compared to healthy confirming the presence of fungal pathogen. Other tea root pathogens responded slightly or not at all with *F. lamaoensis*.

Complex mixture of antigens can be separated by high resolution techniques such as sodium dodecyl acrylamide gel electrophoresis using discontinuous buffer systems and two dimensional techniques. However once separated in this way, it has been difficult to determine which of the separated species reacted with a given antiserum. Towbin (1979) et.al. overcame these problems by electrophoretically transferring the separated mixture onto nitrocellulose. Once attached to the nitrocellulose, the antigenicity of each of the separated species could be tested by treating the blot with antiserum and the bound antibody detected with radio labaled staphylococeal protein A or corresponding anti-antibody. Blake *et.al.* (1984) have described a method of using alkaline phosphatase substrate 5-bromo 4-chloroindoxyl phosphate (BCIP) and nitroblue tetrazolium (NBT) to detect the precipitated indoxyl group. When the substrate BCIP is used, the phosphate is cleared by the enzyme and indoxyl group precipitates. The hydroxyl group of the indigo then tautomerizes forming a ketone, and under alkaline conditions dimerization occurs, forming a dehydro indigo. In the process of dimerization it release hydrogen ion and reduces the NBT which precipitates, forming an intense blue / violet deposition of dimerization. For Western blot analysis, electrophoresis of the soluble mycelia proteins from *V.dahliae* mycelium extract reacted with the purified protein of *V.dahliae* antigens, but not with the *F.oxysporum* protein. In the present study, antigens prepared form mycelial, cell wall, culture filtrate of *F. lamaoensis* healthy and artificially *F. lamaoensis* inoculated root and infested soil were prepared and probed with *F. lamaoensis* PAb.

Since Pabs raised against *F. lamaoensis* could detect the presence of the pathogen in root tissues it was decided to determine the efficacy of the PAb in detecting the specific pathogen in the soil. Detection of specific soil -borne pathogen in soil is equally or more important than detecting the pathogen in the root tissues. Detection of specific pathogens in soil requires very sensitive techniques, which would make it

possible to differentiate between the various microorganisms. Use of serological techniques, most specifically ELISA are gaining importance in such studies. In the present study, initially antigens prepared from soil collected from various tea estates were tested against PAb of *F. lamaoensis* by DAC-ELISA. Of the 38-40 samples tested 7 samples from Matigara, Bijoynagar and Trihana Tea Estate gave marginally high A405 values while all the others had relatively weakly reacted with PAb of *F. lamaoensis*. In case of amended soil sample ELISA values decreased with the increase in period of inoculation after an optimum period as data showed that after 70 days of amendment the values had decreased greatly. This was probably because the pathogen could not survive in the soil for such a long period. The ability of polyclonal antisera of *Plasmodiofora brassicae* to detect the presence of the pathogen in soil was reported by Wakeham and White (1996). In another study Walsh *et.al.* (1996) Reported that the antiserum could detect about 100 spore ball; levels appear to be better for concentration greater than 2000/gm soil. There was a quantifiable relationship between concentration of spore balls and ELISA values . Thus ELISA showed potential for detection of *F. lamaoensis* in soil.

Dot Blot analysis of rhizosphere soil samples collected from different tea fields and amended soil showed same trend of result obtained in DAC-ELISA. Wakeham and White (1996) got positive detection of soluble components of the spore wall and whole resting spores of *P. brassicae* in PBST as seen at concentrations at or above $1x10^7$ and $1x10^6$ resting spores ml^{-1} resting spores ml^{-1} respectively. When the sonicated fraction of the resting spores was assayed, the limit of detection was $1x10^8$ resting spores ml^{-1}. Identification of resting spores in artificially infested soil required a minimum concentration of $1x10^8$ spores gm^{-1} soil. The PAb of *F. lamaoensis* was found to be very specific for detection of the pathogen in the soil, infected root tissues and in different isolates of fungi. Walsh *et.al.* (1996) also performed Western blotting using the raw serum of *Spongospora subterranea* spore balls. Earlier workers have used immunofluorescent assays for diagnostic tests of infection on seeds, plant parts and soil. Thornton et.al.(1993) had previously reported that dip-stick, ELISA and immunofluorescence gave greater sensitivity for detection of live propagules of *Rhizoctonia solani* in soil. These assays could also prove useful in the rapid

determination of *P.brassiceae* infestation in soils (Wakeham and White, 1996). Fluorescence of hyphal structure was observed in immunofluorescence test of infested rhizosphere soil. So, propagules of *F. lamaoensis* could also be detected in soil through this study. Results of study have led to substantially similar conclusions using ELISA, dot-blot, immunofluorescence tests.

As detection of cross reactive antigens between *F. lamaoensis* and tea roots as well as detection of the fungus within infected tissues was possible by the antiserum raised against whole mycelial antigen *F. lamaoensis*; it was decided to purify the antigenic proteins from the crude preparation. This was carried out by purification procedures involving ammonium sulphate saturation, analysis by SDS-PAGE. In order to determine the fractions, which contained the antigenic proteins, immunodiffusion, DAC-ELISA and western blot were performed with PAb of *F. lamaoensis*. Results revealed maximum cross reactivity through immunodiffusion and protein bands through Western Blot analysis in the 60-80%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, results of immunodiffusion and Western blot pointed to the 60-80% SAS fraction to be the major antigenic proteins the ability of this fraction to raise antibody was further tested. In this study evaluation of the antiserum raised against 60-80% SAS fraction of mycelial antigen of *F. lamaoensis* was also done by DAC-ELISA, Dot blot, Western blot analysis. CRAs between *F. lamaoensis* and tea varieties was detected in DAC-ELISA. This PAb behaved in a similar manner as that raised against whole mycelial preparation and hence presence of the antigenic proteins was confirmed in the 60-80% SAS fraction. Chard et.al.(1985) also raised antiserum against purified mycelial preparation of *Mycena galopus* and compared with the antiserum against whole mycelia . Both the antisera were shown to be specific to *M. galopus* by immunodiffusion tests. This PAb could also detected *F. lamaoensis* infection in inoculated tea root tissue by DAC-ELISA and showed highly significant differences in A 405 values between healthy and infected roots in susceptible varieties. In dot

blot analysis the PAb also detect *F. lamaoensis* showing violet dot with infected root antigen while faint colour dots with healthy root antigens; Western blot analysis of this PAb with infected root antigens of susceptible variety (UP-26) showed four bands while no band in case of healthy was observed. Results of all the above therefore, confirmed the presence of the antigenic proteins in 60-80% SAS fraction, since, this fraction was effective in raising specific antiserum against *F. lamaoensis.*

The importance of cross reactive antigens between host and pathogen in determining the response of the host to pathogen has been established in this study and also by previous workers (DeVay and Alder, 1976 ; Chakraborty and Purkayasatha , 1983; Chakraborty and Saha, 1994; Chakraborty *et.al.*, 1995). For this purpose in this study fluorescence tests were conducted with cross section of tea roots as well as mycelia *F. lamaoensis.* Cross section of tea roots were treated with *F. lamaoensis* PAb followed by staining with FITC conjugated anti rabbit globule specific goat antiserum. Bright fluorescence was observed throughout the sections, extending upto the vascular tissues as well as outer surface. Treatment of mycelia of *F. lamaoensis* with homologous antiserum and FITC showed a general fluorescence that was more intense on young hyphae. DeVay *et.al.*(1981a) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum f.sp.vasinfectam.* 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 of potato leaf section .It was also reported by Chakraborty and Saha (1994) 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 in tea leaves shared by *P. theae* (Chakraborty *et.al.*1995), *E. vexans* (Chakraborty *et.al.* 1995) and *G. cingulata* (Chakraborty *et.al.* 2002c) have also been previously determined.

Present study reports the use of indirect immunofluorescence test using polyclonal antibodies of *F. lamaoensis* as suitable technique for localization of fungal mycelium and could be employed immunodetection of *F. lamaoensis* present in tea root tissue.

Presence of fungal mycelium was evident with strong fluorescence in infected root tissue of susceptible varieties (TV-18, UP-26& T-78) after immunofluorescent treatment. Detection of pathogen in host tissues using antibody based immunoflurescent technique has been reported by several previous authors (Warnock, 1973; Hornok and Jagicza, 1973; Reddy and Anantanarayanan, 1984). Dewey *et.al.*(1984) suggested, on the basis of immunofluorescene 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 be detected in soil by immunofluorescence antibody technique (Watabe, 1990).

Fungal hyphae and spores have been visiualized successfully in natural substrata with specific immunofluorescent (Deway *et.al.*, 1984) or nonfluorescent immunocytochemical stains (Gerik *et.al.*, 1987) based on polyclonal antibodies. The immucoenzymatic assay has several advantages over conventional bioassays in determining the colonization pattern of fungi in roots. It allows direct detection of the hyphae present on the root; the specific of the staining of hyphae is dependent on the specificity of the serum. A specificity fungal antiserum employed with an enzymatic staining technique was developed and proved to be a valuable tool in the study of root infecting fungi, for it permits their direct, selective observations in host tissue. Moreover this staining assay offers many advantages over fluorescence antibody techniques. It does not require the use of fluorescence microscope, and specimens can be viewed with a dissecting microscope. The increased field of view of dissecting microscope allows for the screening of more specimens in less time than is required with fluorescent techniques. It may be possible to sequentially stain different fungi in the same root tissue by using different enzymes linked to the antibodies and / or different diazo dyes. Geric and Huisman (1988) used this specific serological staining procedure to detect *Verticillium dahlae* on and in the root cortex of host with an alkaline phosphatase antirabbit IgG conjugant that hydrolyzed the substrate napthol-As-phosphate to a product that reacted with a diazonium salt. In 1988, Geric and Huisman

again used this technique to determine the dynamic colonization of cotton roots by *V. dahlae* under field condition. They observed that the colony density of *V.dahlae* on roots varied with the distance of tissue from the root tip. Young and Andrews (1990) also used this immunocytochemical staining for *Athelia bombacina* for detection of hyphae of the fungus within apple leaf tissue, and used the stain to examine the effect of the antagonists on pseudothecial development of *Venturia inaequalis*. In the present study detection of *F. lamaoensis* in tea root tissues was done using napthol-AS-phosphate and fast blue BB salt as substrate .The cross sections of infected tea root tissues were treated with PAb diluted with 1:100 in PBS-BSA followed by treatment with Goat antirabbit IgG alkaline phosphatase conjugate and substrate reaction was carried out at dark. Intense fungal colony as shown by blue colour were visible clearly along the epidermis and hyphal penetration throughout root tissue was evident. Deep blue coloured thick layer on outer surface, was an evidence of brown or blackish sheet on the root surface formed by the fungal mycelia.

Consequent to the study on the detection of *F. lamaoensis* in root tissues and soil, experiments were conducted both *in vitro* and *in vivo* for the management of the disease. Since it is necessary to reduce the use of fungicide / pesticide in tea plantation it was decided to test the efficacy of biocontrol agents in disease management. For this purpose *Trichoderma harzianum* and *T.viride* were selected and experiments were conducted using these as biocontrol agents. Both *T.harzianum* and *T.viride* inhibited the growth of *F.lamaoensis in vitro*. There are several reports on the ability of *T.harzianum* and *T.viride* to inhibit the growth of pathogen under in vitro condition. Ten isolates of *Trichoderma* species were screened by Padmodaya and Reddy (1996) in in vitro for their efficacy in suppressing the growth of *Fusarium oxysporium f. sp. lycopersici. Trichoderma viride* (H) was found to be highly inhibitory to *F. oxysporum f. sp. lycopersici* in dual culture followed by *T.harzianum* sp. (A.P). Studies on production of volatile compounds by *Trichoderma* species revealed that *T.viride* (H), *T.viride* (A.P.) and *Trichoderma* sp. (D) as effective in reducing radial growth of *F.oxysporum f. sp. lycopersici* in a study on production of non-volatile compounds by

Trichoderma sp. Baby and Chandra Mouli (1996) tested antagonistic potential of *Trichoderma* sp. and *Gliocladium virens* against primary root pathogens of tea viz. *Fomes noxius, P.hypolaterita, Rosellinia arcuata* and *Armillaria mellea in vitro.* In dual culture, the mycoparasites invariably invaded the pathogen, though there was variation in the rate and quantity of invasion. *T.harzianum* was found to be the best colonizer of *Poria* and *Armillaria* and *T.viride* colonized with *Rosellinia. G.virens* colonized all the pathogens fairly well. The antagonists showed moderate to high antibiosis to *Rosellinia.* Production of toxic metabolite(s) was more in *G.virens* than *Trichoderma.* Hazarika et. al., (2000) also tested the antagonistic effect of *Trichoderma harzianum* against *U.zonata,* causing charcoal stump rot of tea in dual culture method. Both antagonists were most effective in inhibiting the mycelial growth of *U.zonata.* Assam and Tamil Nadu isolates of *T.harzianum, T.viride* and *T.virens* were tested by Hazarika and Das (1998) for the potential to suppress *Rhizoctonia solani.* Culture filtrate of *T.harzianum* and *T.viride* inhibited mycelial growth and sclerotial germination. Wheat bran substrate supported maximum growth of all isolates followed by firm yard manure and tea waste. Both *T.harzianum* and *T.viride* effectively controlled the bean rot disease when they were applied as seed and soil treatment. In dual culture of 11 isolates of *T.harzianum,* three isolates, viz. T8, T10 and T12 was effective against *Sclerotium rolfsii,* the causal agent of stem rot of groundnut and they overgrew the pathogen up to 92%, 85% and 79% respectively, in vitro. Phookan and Chaliha (2000) reported that growth of *Sclerotinia sclerotiorum* was significantly suppressed by *Gliocladium virens* and *T.viride* significantly. Amongst fungal antagonists tested by Sharma and Sharma (2001). *Trichoderma harzianum* and *T.viride* were found most effective in inhibiting mycelial growth of *Dematophora nectrix* in dual culture.

T.harzianum and *T.viride* were tested *in vivo* for the ability to reduce brown root rot intensity. Of various delivery systems tested for this biocontrol agent, tea waste formulations were found to be most effective. Disease intensity was reduced by both *T.harzianum* and *T.viride* when tested under potted condition as well as in the field condition. This was observed in all tested varieties. Detection of *F.lamaoensis*

in tea root tissues and rhizosphere soil of different treatments with pathogen and biocontrol agents was also investigated. Reduction of intensity of disease was also determined immunologically in both root tissues and soil. For this purpose DAC-ELISA, competition-ELISA, Dot-Blot and Western blot were carried out. Results showed that ELISA values of root tissues treated with *T. harzianum* and *T. viride* were significantly lesser than with *F. lamaoensis* alone. The same trend of result was obtained in infested rhizosphere soil through DAC and Competition ELISA, Dot Blot and Western blot analysis. This result is in conformity with that of Hazarika et. al., (2000) who reported that planting of tea seedlings after dipping roots in spore suspension of *T. harzianum* reduced 56.6% mortality of plant due to *U. zonata* infection. This was also obtained with *T. viride* and *G. virens*. However they observed that the reduction of mortality of plant increased to 62.2% when *T. harzianum* were applied to soil to soil drench. Significant control of charcoal stump rot of tea with antagonistic microflora obtained previously by Borthakur and Dutta (1992) and Hazarika et. al., (1999). The role of *T.harzianum* and *T.viride* as biocontrol crops is well established. Sarker and Jayarajan (1996) reported that root rot of sesamum caused by *Macrophimina phaseolina* was significantly reduced to 12% by seed treatment with antagonist (*T.harzianum*) in comparison to untreated inoculated control plants where 62% disease incidence was noticed. Prasad *et. al.*, (1999) found three *T.harzianum* isolates (PDBCTH-2, 7 and 8) and the *T.viride* isolates (PDBCTV4) highly efficient in controlling root/ collar rot of sunflower caused by *Sclerotium rolfsii*. Under green house condition PDBCTH-8 showed maximum disease control of 66.8% followed by PDBCTH-7 (66%), PDBCTV-4 (65.4%). PDBCTH-2 (61.6%) and were even superior to systemic fungicide.

In conclusion, it can be stated that brown root rot can cause severe damage to tea plants, particularly to those growing on sandy soil. Such detection techniques makes it possible to detect microquantities of the pathogen within root tissue and rhizosphere soil, which is more advantageous than the conventional techniques involving pathogen inoculation, visible symptoms and microscopy. Being perennials, plants harbor the pathogen in root tissues and rhizosphere over a long time and the

depletion of pathogen by the conventional techniques is difficult and by the time the above ground symptoms appear it is too late. So, an important area of immunological studies of diseases involves the use of pathogen antiserum for detection of infection in host at an initial stage which can lead to formulation of control measures before much harm has been done. In this study, effective formulations of *Trichoderma harzianum* and *Trichoderma viride* have been prepared, by which brown root rot can control.

SUMMARY

1. A review of literature pertaining to this investigation has been presented which deals mainly with serological techniques for the detection of plant pathogenic fungi.

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

3. Pathogenicity of *Fomes lamaoensis* was tested on twenty five varieties (10 Tocklai, 9 Darjeeling and 6 UPASI) of tea. Among these, TV-18, T-78 and UP-26 appeared to be susceptible and TV-26, BS/74/76 and UP-9 were found to be resistant.

4. Cultural conditions affecting growth of *F. lamaoensis* were studied with special reference to their growth in different media, variable pH and different types of carbon, organic and inorganic nitrogen source. Maximum growth of the pathogen occurred in Potato dextrose agar while minimum growth was noticed in Czapex-dox. Lactose was the most effective carbon source whereas Beef extract was most effective nitrogen source followed by yeast extract and casein hydrolysate. Organic nitrogen sources were found to be better than inorganic nitrogen sources, though no growth was observed in urea as organic and optimum growth in calcium nitrate as inorganic source.

5. Protein content of healthy and artificially inoculated tea root tissues from 25 different tea varieties as well as mycelia and cell wall proteins of *F. lamaoensis was* estimated and analysed in SDS-PAGE.

6. Characterization of the cell wall of *F. lamaoensis* by ConA-FITC binding revealed its glycoprotein nature.

7. Polyclonal antibodies (PAbs) were raised against antigen preparations from mycelia and cell wall of *F. lamaoensis,* mycelia of bioconrol agents (*T. harzianum* and *T. viride*) and tea root tissues (UP-26 and TV- 26). These were purified by ammonium sulphate precipitation followed by DEAE cellulose chromatography. IgG obtained in each case was used for different immunoenzymatic tests.

8. To check the effectiveness of PAbs, Agar gel double diffusion tests were performed using crude antibody as well as purified IgG obtained from different bleeding collected for the pathogen. Strong precipitin reactions were observed in homologous

cross reaction of each case.

9. Optimization of ELISA using PAbs of *F. lamaoensis* and antigen preparations at variable concentrations were performed. ELISA values decreased with the decrease of antigen concentrations ranging from 40 to 0.312mg/ml. However maximum absorbance values was obtained in 3rd bleeding followed by 4th bleedings.

10. DAC-ELISA tests were performed separately using PAbs raised against mycelia and cell wall antigens of *F. lamaoensis* against root antigens prepared from 25 different tea varieties, non-pathogen and non-host and major cross reactive antigens (CRA) shared between tea varieties and *F. lamaoensis* were detected.

11. Detection of *F. lamaoensis* in artificially inoculated tea root tissues using DAC-ELISA and DAS-ELISA formats were standardized.

12. Antigens prepared from soil samples collected different tea field and amended soil, were tested against PAb of *F. lamaoensis* using DAC-ELISA formats and Dot blot analysis. Amended soils gave very high values comparable to homologous values and ELISA values decreased with the increase in days of incubation in field condition after an optimum period.

13. Purification of antigenic protein of *F. lamaoensis* from the crude mycelial extracts by ammonium sulphate saturation was done and detection was done by immunodiffusion, ELISA and Western blot.

14. Antigenic preparation of 60-80% SAS fraction was used for raising antiserum and this antiserum was tested by immunodiffusion test and IgG was purified. CRA between *F. lamaoensis* and tea varieties, non-host and non-pathogen was detected in DAC-ELISA. This antiserum also showed higher reactivity in the susceptible reactions as compared to resistant reaction.

15. PAb raised to 60-80% SAS fraction could also significantly detect infections by DAC-ELISA, Dot blot and Western blot analysis.

16. Cross sections of tea roots (TV-18, UP-26 and T-78) treated with PAb of *F.*

lamaoensis and then labelled with FITC developed a bright fluorescence throughout the sections, extending upto vascular tissues as well as outer surface.

17. Mycelia of *F. lamaoensis* when treated with homologous antisera followed by FITC, bright fluorescence was noticed on young hyphae.

18. Reactions of various antigens (fungal and root) with PAb of *F. lamaoensis* has also been determined through dot-immunobinding as well as Western blot analysis.

19. Specific immunocytochemical stain for detection of hyphal location of *F. lamaoensis* within tea root tissues of susceptible varieties were developed. Hyphal penetration throughout root tissue was evident and presence of fungal mycelia on outer surface was also evident.

20. *In vitro* interaction of *F. lamaoensis* with *T. harzianum* and *T. viride* was studied. Both inhibited the growth of *F. lamaoensis.*

21. Soil amendment of tea rhizosphere with *T. harzianum* and T. viride both in potted conditions and in the field reduced disease intensity significantly.

22. DAC-ELISA of tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents, reacted with PAbs of *F. lamaoensis, T. harzianum* and *T. viride* indicated the reduction of pathogen population in rhizosphere soil and root tissues.

23. The implication of results have been discussed.

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