Serological detection of *Sphaerostilbe repens* B. & Br. causing violet root rot disease of tea and its management

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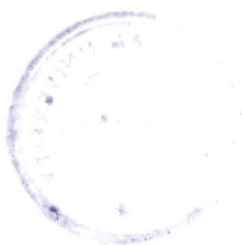

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This is to certify that Ms Jyotsna Das has carried out her research work under our supervision. Her thesis entitled **"Serological detection** of *Sphaerostilbe repens* **B. and Br. causing violet root rot disease of tea and its management"** is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

(Professor B.N.Chakraborty)

(Dr. Usha Chakraborty) Co-Supervisor

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CONTENTS

Introduction

The tea plant [*Camellia sinensis* (L.) O Kuntze] was grown for many centuries in China as a commercial crop. Tea drinking possibly spread to India from China around the 17th century and the Assam cultivar was discovered in the North-Eastern region of the undivided India in 1834 (Agnihotrudu, 1995). Today India occupies the foremost position in the world among all tea growing countries with respect to tea production, consumption and export. Tea industry is one of the most important organized sectors in the country and more than 70% of the country's population comes in contact with tea in India's economy and society both directly and indirectly. The tea bush like any other living plant is susceptible to attack by diseases more so as it has been subjected to various cultural treatments which are widely at variance with the natural conditions of growth. Practice of tea cultivation which necessitates the growing of parental crop in a pure stand extending over vast areas affords a happy hunting ground for pest and diseases of all kinds. Some of the existing soil conditions in the tea plantations predispose the plants to attacks by specific fungi causing some of the important diseases. One of the most common such diseases in Dooars and Terai region is the violet root rot caused by *Sphaerostilbe repens* B & Br. which is found to occur in all soils but is more common on stiff clay soil with water logging and poor aeration. The disease is practically unknown in hills. The pathogens attacks all tea plants from above one year upwards but the characteristic symptoms are produced on plants two year and above. When the bark of affected roots is peeled off, the wood surface is found to be covered with thick, irregular, white to orange and mauve to purplish-black, flattened strands, the rhizomorphs (Plate 1).

In nature unlike animals plants can not move and hence they can not side step or run away from attacking pathogen. They are bound to stand , wait and then face the attackers. In order to suit the above situations plants have evolved primarily localized type of defence or immune system, where each individual cell/tissue/ organism is required to defend itself without much help from distant plant parts. The pathogen is recognized and restricted by the few individual plant cells. An organism is recognized at the level of surface to surface interaction as either incompatible or compatible. Recognition or interaction as compatible depends on some kinds of molecular similarities between the host and pathogens (Chakraborty, 1988; Protsenko and Ladyzhenkaya, 1989; Chakraborty and Saha, 1994; Chakraborty et.al. 1995 and Chakraborty et.al.2002). Close serological similarity between host and pathogen is therefore one of the prerequisites for the successful establishment of the pathogen in the host.

This serological relationship between host and pathogen has been exploited for development of pathogen detection systems in the host using antisera raised against the pathogen. Such disease detection and diagnostic kits have the advantage over conventional methods by being specific and having the ability to detect even minute amounts in the tissue. Commercial diagnostic kits have been offered in recent years for the rapid diagnosis of several fungi in plant tissues and in soil and water (Werres and Steffens, 1994) Most common among these techniques are the imuunosorbent assays such as Enzyme Linked Immunosorbent Assays (ELISA) , Dot-blot, Western blot etc. ELISA is now routinely incorporated in various large scale disease indexing programmes particularly in perennial and vegetatively populated crops (Clark, 1981).

Timely detection of disease specially root diseases combined with proper management practices can lessen crop loss to a great extent. Among the current management practices for control of root diseases, the most environmental, friendly and effective one is the introduction of biocontrol agents in the soil , thus minimizing the use of fungicides. Among the available biocontrol agents *Trichoderma* sp has been tested in a large number of cases. Biomass production, their suitable formulations and delivery systems are the limiting factors for the commercialization of antagonists. Now a days a number of *Trichoderma* formulations such as Biofungus (Belgium), Bineb-T (Sween, U.K.) , Rootshield, Biotrek-T22G, Planterbox(USA), Rootpro, Trichodex, Trochoderma2000 (Israel), Trichopel, Trichodowels, Trichosean (Newzealand), Supersivit (Denmark) etc. are available in world market. In tea plantations the use of biocontrol agents assumes all the more important because of the restrictions of the use of fungicides.

Considering all the above , the present study was undertaken with the following objectives: (a) to screen various tea clones resistant to *S repens*; (b) to prepare mycelial and cell wall antigens from *S. repens* and raise polyclonal antiserum against the antigen preparations; (c) purification of antigen and antisera and analysis by immunoblotting; (d) to determine serological cross reactivity between tea roots and *S.repens* using ELISA and immunofluorescence (e) to detect the pathogen in soil and root tissues by ELISA and immunoenzymatic staining; (f) *In vitro* interaction studies of *S. repens* with *Trichoderma harzianum* and *T. viride* ; (g) To apply *T. harzianum* and *T. viride* in soil for biological control of violet root rot and (h) to determine the changes in the population of the root pathogen after *Trichoderma* infestation in soil by ELISA and immunoblotting.

Literature Review

It has long been known that most plant pathogens possess, as part of their structure, specific antigenic determinants in the form of proteins or other antigenic moiety. Recognition of the diagnostic potential of such determinants for both experimental and applied investigations in plant pathology has resulted in a bewildering array of techniques being developed referred to as immunoassays. Immunassays are mainly used for identification of plant pathogens, diagnosis and detection of pathogenic organisms associated with plant disease, either within or isolated from affected plant tissues and direct quantitation of pathogens in plant tissues. Immunological techniques are also being used for various other purposes such as localization 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 has been presented below.

Serological detection of plant pathogenic fungi

Serological techniques such as immunodetection, immunoelectrophoresis, enzyme linked immunosorbent assays (ELISA) and immunofluorescence (IF) for detecting fungal pathogens have met with variable success. Various formats of ELISA have been developed, some of which are more appropriate for diagnostic laboratories. Available formats include multiwell, flow through, dipstick, dot-blot and tissue print assays. Multiwell ELISA has been used successfully to detect wide range of plant pathogenic fungi.

Phytophthora sp.

Species of the genus *Phytophthora* were differentiated by Burrell & Lilly (1966) using gel diffusion and immunofluorescence. The type and concentration of the nitrogen source, the amount of inoculum, and the age of mycelium greatly affected the antigenic efficacy of the preparations. The antigens were found to be localized in the young growing tips of the hypae. Species-specific sera were obtained and proved useful and efficient for the identification of *P. cactorum. P. cinnamomi* and *P. erythroseptica.*

Studies on antigenicity of *Phytophthora* sp. by Merz *et. el.* (1968) revealed 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 increased by the addition of ethylenediamine tetraacetate 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. They used these methods to study the taxonomic relations, of six heterothallic species of *Phytophthora. P. cinnamoni* was serologically distinct, whereas two serological groups were evident among five closely related species when tested with antiserum to *P. arecae. 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.*

Gill and Zentmyer (1978) reported that species and isolates of *Phytophthora* species could be differentiated on the basis of soluble protein pattern. Soluble proteins from the mycelia of 30 isolates of *Phytophthora* were collected from 17 different host and from widely separated geographical location, and of five isolates of *P. cactorum,* when fractionated by disc electrophoresis, yielded 22 and 26 bands with different densities. The two species differed markedly and each exhibited its distinct, characteristic protein pattern enabling the authors to identify them. With one exception, there was little or no variation in the protein patterns within the isolates of *P. cinnamomi.* Also, identical or nearly identical protein patterns of each species were obtained regardless of date of isolation, host or geographic locality. No differences in protein patterns were seen between the mating types of *P. cinnamomi.*

In a similar study, it was shown that soluble proteins from 34 isolates representing 16 species of the genus *Phytophthora,* collected from different geographic locations and host plants when analysed by two-dimentional electrophoresis, were resolved into 100-200 protein spots with different densities. Although slight differences were occasionally observed in patterns of faintly-stained minor protein spots, those of densely-stained major protein spots were highly similar, if not identical, among isolates of the same species regardless of their geographic origin, original host plant, mating

type, or virulence race. These results suggest that the two-dimensional electrophoretic patterns are species-specific and may be a useful measure to differentiate and identify species of the genus *Phytophthora* (Masago *et. al.* 1989).

The extent of serological diversity in isolates of three species of *Phytophthora* viz. *P. palmivora, P. capsici* and *P. citrophthora* causing black pod disease of cocoa in India was also studied by immunodiffusion test (Chowdappa and Chandramohan, 2000). The three species of *Phytophthora* exhibited serological variation in their reaction with antiserum to either *P. capsici* or *P. citrophthora* in the agar double diffusion test. The serological reactions revealed that all the isolates of *P. palmivora, P. capsici* and *P. citrophthora* shared at least one common antigen. Two subgroups were distinguished among the isolates of *P. capsici* whereas all the isolates of *P. citrophthora* formed a homogeneous serological group. Thus, the results showed that serological technique can be used as additional criterion in distinguishing the three species of *Phytophthora* in support of morphological criteria.

Pscheidt (et.al. (1992) tested the sensitivity of a *Phytophthora* specific immunoassary on 17 species of *Phytophthora* collected throughout the world, including 18 isolates each of *P. cinnamomi* and *P. cactorum.* Kits were also used in the diagnosis of plant specimens with symptoms characteristic of *Phytophthora* infection. All *Phytophthora* isolates tested produced a positive reaction with the immunoassay kit. The lowest absorbance relative to other species were obtained from *P. cinnamomi* and *P. megasperma.* Variation in absorbance was high among isolates of *P. cinnamomi* but low among *P. cactorum.* Clinic samples with typical symptoms of *Phytophthora* infection produced a positive reaction with the immunoassay, as did pure cultures of *Phytophthora* sp. isolated from these samples. Cross reactions occurred with several *Phythium* sp. isolated from clinic samples and with several specimens infected with *Peronospora* sp. Others produced a positive reaction with the immunoassay. Cross-reactivity with some *Pythium* sp. made interpretation difficult, but when kit results were combined with field histories and symptomology, the immunoassays proved to be a useful tool in clinical diagnosis.

Hardham et. al. (1986) raised monoclonal antibodies to components on the surface of glutaraldehyde-fixed zoospores and cysts of an isolate of the pathogenic

fungus *Phytophthora cinnamomi.* Hybridoma supernatants were screened using an immuno fluorescence assay, and of 35 cell lines producing antibodies that reacted with the *P. cinnamomi* cells, 10 were selected and their specificities examined in detail. The monoclonal antibodies were found to possess a valuable spectrum of taxonomic specificities, and according to the authors, have revealed, for the first time, the presence of isolate-specific antigens on the surface of *P. cinnamomi* cells. The monoclonal antibodies were tested against six isolates of *P. cinnamomi,* six species of *Phytophthora,* and three species of *Pythium.* In addition to the isolate-specific monoclonal antibodies, species-specific and genus specific markers which are unambiguous in tests conducted so far have been obtained. The monoclonal antibodies have also revealed the presence of spatially restricted antigen on the surface of the zoospores. Some of these segregated antigens are species-specific and others are more general, occurring in all *Phytophthora* and *Pythium* species examined. All of the monoclonal antibodies promise to be of great assistance in investigations of the biology and taxonomy of *P. cinnamomi.*

In continuation of the previous study, Hardham and Suzaki (1989) studied the glycoconjugates on the surface of zoospores and cysts of *Phytophthora cinnamomi* using fluorescence isothiocyanate labelled lectins for fluorescence microscopy and flow cytometry, and ferritin-and gold labelled lectins for ultrastructural analysis. Of the five lectins used, only concanavalin A (Con A) binds to the surface of the zoospores, including the flagella and water expulsion vacuole. This suggested that the accessible saccharides, glycosyl or mannosyl residues predominate on the outer surface of the zoospore plasma membrane, vesiculate. These and other small peripheral vesicles quickly disappear. After the induction of ensystment, Con A was no longer localised close to the plasmamembrane but binds to the materials loosely associated in the cell surface. Quantitative measurements by flow cytometry indicated that the ConA-binding material was gradually lost from the cell surface. The cyst wall was weakly labelled, but the site of germ tube emergence stained intensely. During the first 2 min. after the induction of encystment, material that binds soybean agglutinin, *Helix pommatia* agglutinin, and peanut agglutinin appeared on the surface of the fungal cells. The distribution of this material, rich in galactosyl or N-acetyl-D-galactosaminosyl residues, was initially patchy, but by 5min the material evenly coated most of the cell surface.

Labelling of zoospores in which intercellular sites were accessible indicated that the soybean agglutinin binding material was stored in vesicles that lie beneath the plasma membrane. Quantitation of soybean agglutinin labelling showed that maximum binding occurs 2-3min. after the induction of encystment.

A set of five carbohydrate specific monoclonal antibodies (MAbs) were further used to probe the ultrastructure of the walls of the soybean pathogen *Phytophthora megasperma* f.sp. *glycinea* using a combination of immunoflurescence and immuno gold labelling techniques (Wycoff & Ayers, 1991). Results with β-1,3 glucan-specific antibodies suggested that β-1,3 glucans were present throughout the walls of both germ tubes and cysts, but were more prevalent in the outer portion. In addition, β-1,3 glucans on the surface of hyphal walls, but not cysts, were closely associated with other material, most likely protein, that sterically hinders antibody binding except to non-reducing terminal residues. 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 were present on the exterior of *P. megasperma* f.sp. *glycinae* wall as well. Staining with another antibody indicated that changes in wall composition occurred over 50-100μm from the hyphal tip, a greater distance than previously supposed.

Ten monoclonal antibodies were selected by Devergne *et. al.* (1994) from mice immunized with a highly purified elicitin secreted by *Phytophthora cryptogein*, termed cryptogein. This antibodies could be classified into five groups according to their cross-reactivity to heterologous elicitins from other *Phytophthora* species, from strict specificity (reacting solely with cryptogine) to broad reactivity (reacting with all four elicitins under study). When examined on BIA core (a real-time biospecific interaction analyser), these monoclonal antibodies were found to recognize at least 3 different epitopes on the cryptogein molecule. Their use in elecitin detection and quantification was optimized in several ELISA protocols. A mixed monoclonal-polyclonal antibody indirect DAS-ELISA procedure detected as little as 20pg. of purified elicitin per well (100μl). The four elicitins could be detected with the aid of one of couple of polyvalent reagents, whilst each one could be detected separately using appropriate monoclonal antibodies. These protocols have been used to detect elicitin secreted by *Phytophthora* spp. into culture medium as well as in plants following plant inoculation.

A dipstick immunoassay that is specific for *Phytophthora cinnamomi* was developed by Cahill and Hardham (1994) for use in soils. Azo dye detection of monoclonal antibody-labelled cysts attached to a nylon membrane provided a rapid, sensitive assay suitable for the field use. There was no cross-reaction with other *Phytophthora* and *Pythium* species in controlled environment assays or with soil or other organic matter that adhered to the membrane. The assay was as sensitive as a *Eucalyptus sieberi* baiting assay and when run together with the biting assay, was quantified for an infested soil water suspension from $2.5x10^2$ to $5x10^5$ zoospores per milliliter. The assay was used successfully to detect *P. cinnamomi* in a wide range of soil samples collected from beneath a diverse range of soil samples from a range of host species. According to the authors, there are several advantages to using the dipstic assay compared with traditional procedures; familiarity with *Phytophthora* taxonomy is not required, the assay can be performed by unskilled personnel; and soil rather than infected plant tissues can be assayed. Field testing of the assay showed that in kit form, it could be used as a reliable diagnostic tool to replace or augment current isolation and detection methods. The authors suggested that the dipstick assay should find broad use for the detection of *P. cinnamomi* in soil from forests and plant communities and in the horticultural and ornamental crops affected by this pathogen.

Alba and Devay (1985) detected cross reactive antigens in crude preparations from mycelia of *Phytophthora infestans* 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 concentratrated 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, mycelia preparation was assayed with antisera for King Edward and Pentland Dell.

Amouzou-Alladaye et. al. (1988). raised antisera by injecting rabbits with mycelial protein extracts of one strain of *Phytophthora fragariae* which had a dilution end point of 1/64 in double diffusion and 1/512,00 in indirect enzyme-linked immunosorbent assay (ELISA). This serum could detect 11 different strains of *P.*

fragariae in pure culture and in naturally infected or inoculated roots. Although the sensitivities of direct double antibody sandwich and indirect ELISA were comparable, the direct double antibody sandwich ELISA was more specific for the detection of *P. fragariae* in strawberry roots. The antiserum failed to react with 18 fungal species isolated from underground parts of strawberry but reacted with some strains of *P. cactorum*, which parasitized only rhizomes but not roots, and *P. fragariae* was detected reliably by ELISA several days before zoospores were found and before symptoms developed. Thus direct double antibody sandwich ELISA may be useful for early detection of infection and for the detection of latent infections of strawberry plants by *P. fragariae*.

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

Mohan (1989) also reported that antiserum (anti-PfM) raised against mycelial suspensions of *Phytophthora fragariae* isolates reacted strongly with antigens from several *Phytophthora* species. Some cross-reactions with antigens from *Pythium* species were decreased by fractionating on an affinity column of Sepharose 4B bound to extracts of *Fragaria vesca* roots infected with *P. fragariae*. The affinity-purified anti-PfM retained its high cross reactivity with the various *Phytophthora* species tested. It also detected infection of raspberry and strawberry roots by some

Phytophthora species. This antiserum could, therefore, prove useful as a broad spectrum *Phytophthora*-detecting antiserum. Anti-PfM could not be made specific for *P. fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However immunoblotting with the affinity purified anti-PfM produced district patterns for *P. fragariae, P. erythroseptica* and *P. cactorum* : three serotypes were identified for the latter species. This antiserum might therefore prove useful in classifying *Phytophthora* species. Polyclonal antibody, raised in rabbit immunized with a mycelium extract of *Phytophthora infestans,* reacted in an enzyme-linked immunosorbent assay (ELISA) with mycelial extracts of two *Phytophthora* species but not with those of 10 other micro organisms found on potato (Harrison et. al. 1990) *P. infestans* mycelium in leaf tissue was readily detected by ELISA using either the plate-trapped antigen or $F(ab^1)_2$ 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.

Benson (1991) compared two commercial serological assay kits to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from inoculated azaleas. Both the multiwell E kit and the rapid assay F kit detected *P. cinnamomi* in azalea roots beginning 1 wk after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P. cinnamomi* was most consistent beginning 3-4 wk after inoculation. Root symptoms, but not foliar symptoms, of *Phytophthora* root rot were evident during this period. There was a positive corelation between root rot severity in green house trials and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immunoassay results. Although colour reactions in the rapid assay detectors became increasingly darker after completion of the test, results after 5min were as reliable as those after 60min, since readings for uninoculated controls used to determine test thresholds also increased with time. The mutiwell 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 value that were unconfirmed by culture plate. The rapid assay kit detected *P. cinnamomi* was easy to use, and gave results in short time.

A polyclonal, enzyme-linked immunosorbent assay of *Phytophthora infestans* has been developed by Beckman *et. al.* (1994) for use in the determination of fungal biomass during the early stages of infection of tuber disc of *Solanum tuberosum.* By optimizing the dilution of sample extracts and the dilution of primary anti *P. infestans* antiserum, quantification of the biomass of *P. infestans* in zoospore inoculated tuber disc could be achieved by 8-18 h after inoculation. Differences in growth between avirulent and virulent isolates of *P. infestans* on the resistant potato cv. kennebec were quantified by 32-48h after inoculation. Together with a comparison of growth of the same isolates on the susceptible cv. King Edward, these results comprised on ELISA of the Quadratic check. On the resistant host, the growth of the avirulent isolate was essentially arrested by 16h. after inoculation, whereas that of the virulent isolate continued throughout the time course. On the susceptible host, however, the avirulent isolate appeared more aggressive than the virulent isolate. These results demonstrated that ELISA, which is often simpler to perform than other procedures for estimating fungal growth, may be used to complement biochemical studies of rapidly induced plant defence response.

MacDonald and Duniway (1979) investigated the use of fluorescent antibodies to study the survival of *Phytophthora megasperma* and *P. cinnamomi* zoospores in soil. For this they added zoospore cyst of the pathogens to non-sterile soil and its matric potential was adjusted to predetermined values. The infested soils were held under laboratory and field conditions and periodically sampled to determine cyst viability and soil water potential. During sampling soil containing cyst 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, zoospores cysts of *P. megasperma* survived no longer than 3 wk at water ranging from 0 to –15 bars, and under field conditions they survived no longer than 2 wk. Zoospores cyst of *P. cinnamomi* survived no more than 3 wk 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, zoospores cysts of *P. cinnamomi* survived upto 6wk in the laboratory. In a nonirrigated field plot, in which soil water potentials ranged from –7 to less than –100 bars, cysts of *P. cinnamomi* survived for 8-10wk. Under more controlled humidity

conditions, zoospore cysts of *P. cinnamomi* proved relatively tolerant of dessication; many survived for 1 wk in soils 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 1 wk in soil dried to water potentials of –20 bars or less.

The utility of immunofluerescent antibody technique for detecting *Phytophthora* in soil was also demonstrated by Mitsuo (1990). But the autofluorescence and the nonspecific staining of soil particles interfere with the detection of the fungi in soil. They reported that the pretreatment of the samples with gelatin-rhodamine conjugate prevented the samples from the autfluorescence and the non specific staining and therefore permitted the immunofluorescent antibody staining in soil. Stained *Phytophthora* was easily detected on yellow orange background.

***Ceratocystis* sp.**

Using the serological techniques of agglutination, gel diffusion and immunofluorescence, Amos and Burrell (1966) identified eight species of the genus *Ceratocystis* : *C. fagacearum, 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 absorb selectively cross-reacting antibodies firm the sera to make them species-specific. The immunofluorescence technique proved to be the most useful in differentiating among these species. Although all of the species could be shown to have serological differences, no such differences could be seen between the A and B compatibility types of *C. fagacearum.*

***Fusarium* sp.**

A strain-specific antiserum was raised by Hornok and 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 the homologous *F. culmorum* strain and those of heterologous *F. graminearum* and *F. culmorum* strains. All the other *Fusarium* sp. tested showed no detectable fluorescence.

The heterologous strains of *F. culmorum* and *F. graminearum* could not be distinguished. No significant difference was found between the "culmorum" strains from different host plants. The serum specific for *F. culmorum* and *F. graminearum* was suitable to separate these species from other fungi. Morphological examination and culturing processes were not necessary because mycelia sufficient for fluorescent antibody staining usually grew out from infected plant material on to a microscope slide n 48-72 hours.

Iannelli *et. al.* (1982) differentiated four formae speciales of *Fusurium oxysporum* (*dianthi, melonis, pisi, lycopersici*) and three physiological races (1, 2, 3) of *F. oxysporum* f.sp. *melonis* using specific antisera and 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 appropriate antisera reacted the same within each race.

Competitive types of two novel enzyme-linked immunosorbent assays (ELISA) for *Fusarium* species were developed by Kitagawa *et. al.* (1989). Antiserum against a strain (F504) of *F. oxysporum* was elicited in rabbits, and a highly specific, sensitive, and accurate ELISA for the homologous strains was developed by using the antiserum with β-D-galactosidase-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* F501 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.

Rataj-Guranuwska and Wolko (1991) compared the extracts from *Fusarium oxysporum* (F.o.) and *F. oxysporum* var. *redolens* (F.o.r.) isolates by means of electrophoresis and crossed immunoelectrophoresis. The polymorphism of five isozyme systems allowed a distinction between F.o.and F.o.r. isolates. The isozyme patterns of three other isozyme systems did not allow this distinction between F.o. and F.o.r. to be made. Both fungi appeared 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.o.r. isolates. Although the results obtained indicate a strong similarity between F.o. and F.o.r., they are not sufficient for an unequivocal statement that the fungi belong to the same species.

An enzyme-linked immunosorbent assay (ELISA) for isomarticin, a naphthazarin toxin produced by *Fasarium solani* was developed. A carbodiimide procedure was used to couple the hapten isomarticin to bovine serum albumin for the immunogen and to alkaline phosphatase for the enzyme-linked tracer. The resulting assay had a detection limit of 2 ng/ml for isomarticin; other naphthazarin toxin were detectable at less than 10 ng/per well in ELISA plates. The assay was specific for napthazarins. The cross reactivity for a number of phenolic compounds, including the closely related naphthoquinones, was three orders of magnitude less sensitive (Phelps *et. al.* 1990).

Naphthazarin toxins of *Fusarium solani* were also detected by competitive ELISA analysis in xylem fluid of roots rotted by *F. solani* and symptomless scafold roots and branches of healthy-appearing and diseased citrus trees in ridge and flatwood Florida groves by Nemee and Charest (1991). Studies concentrated on blight, a wilt disease with an undefined cause and etiology, to determine if *F. solani* is a causal factor of the disease. Healthy-appearing roots of trees with blight symptoms in six groves contained up to 11.4 times toxin than roots of healthy trees in the same groves. In blight-diseased trees from these groves, median toxin values per root and percentage of roots positive for toxin were higher than for healthy trees. Rotted roots from blight-diseased trees in the two groves contained 112 and 3.4 times more toxin than healthy appearing roots from the 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 root infected by *F. solani,* immunocytochemical localization of Naphthazarins reveled it to be 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 non-membrane-bound

electron-lucent areas. The presence of naphthazarin toxins in blight-diseased trees as well as those with other diseases suggests the non-specificity 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.

Preparations of antigens from four varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species were compared for common antigen by Charudattan and DeVay (1972). At least one antigenic substance 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 nonpathogenic 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 present in heterologous reactions between the fungal and cotton preparations. The common antigenic determinant shared by cotton and fungal isolates does not appear to be related to the severity of wilt symptoms, but it may affect host pathogen compatibility during the process of root infection.

A common antigen from conidia of *F. oxysporum* sp. *vasinfectum* that cross-reacted with antiserum to cotton root tissue antigens, was further purified by Charudattan and Devay (1981). They reported that in agar-gel double diffusion tests, one precipitin band was formed when antiserum to cotton antigens was reacted with crude fungal antigens, or when antiserum to crude fungal antigens was reacted with cotton antigens. The cross reactive antigen from fungal condia was isolated, purified, and partially characterized. The CRA migrated as a single band in polyacrylamide or agar-gel electrophoresis, and sedimented as a single band during analytical ultracentrifugation. It was antigenic in rabbits, and was a protein-carbohydrate complex.

In a further study, major cross reactive antigenic substance (CRA), common to cotton (*Gossypium hirsutum*) and certain fungal parasites of cotton roots, was isolated and purified by DeVay and Wakeman (1981) to homogenity from conidial cultures of

Fusarium oxysporum f.sp. *vasinfectum* (F.O.V). Agar gel double diffusion tests indicated the presence of CRA not only in F.O.V. 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 plasmalemma. Treatment of conidia and mycelia of F.O.V. 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 *et. al.* (1997) also determined the presence of CRA between soybean and *Fusarium oxysporum*. They performed pathogenecity test of *Fusarium oxysporum* on ten varieties of soybean which revealed Soymax and Punjab-I to be most resistant while JS-2 and UPSM-19 were most susceptible. Antigens were prepared from the root of all the ten varieties of soybean and mycelium of *F. oxysporum*. Polyclonal antisera were raised against the mycelial suspension of *F. oxysporum* and root antigen of the susceptible cutivar UPSM-19. Cross reactive antigens shared by the host and pathogen were detected first by immunodiffusion. The immunoglobulin fractions were used for detection of cross-reactive antigens by enzyme-linked immunosorbent assay. Antigens of susceptible cultivars showed higher absorbance values 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 fluoreciene isothiocyanate indicated that in cross reaction 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, macroconidia and chlamydospores of the fungus.

Antibodies 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 antibody reaction. Extracts of total

ribosomes from pith of infected corn could be separated into host, pathogen and other organism(s) fractions by the use of antibodies and sucrose density gradient centrifugation. (Marshall and Partridge, 1981).

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

Disease resistance in pea (*Pisum sativum* L.cv Alcan) tissue inoculated with macroconidia of the incompatible fungal strain *Fusarium solani* f.sp. *phaseoli* was correlated with increased levels of mitochondrial DNA (mRNA) homologous to cloned disease resistance response (DRP) 49 gene sequences (Allaire and Hadwiger, 1994). Antiserum developed against a fusion protein containing segments of native DRR 49 was used to study spatial and temporal localization of the protein in the inner parenchyma layers of immature pods inoculated with either *F. solani* f.sp. *phaseoli* or the compatible f.sp. *pisi*. The protein corresponding to DRR 49 was localized primarily in the nucleus in all treatments.

***Penicillium* sp.**

Aspergillus and *Penicillium* spp. were the predominant fungi isolated by Warnock (1973) in cultures from 5 samples of stored grains. Using inmmunofluorescent method, the presence of these fungi in most grains was confirmed but the amounts of mycelium involved were shown to be small. *Alternaria* spp. were not isolated from 3

of the samples but small amount of *Alternaria* mycelium were detected in grains of all the samples studied.

Fuhrmann *et. al.* (1989) raised antisera from a rabbit immunized with *Penicillum verrucosum* var. *verrucosum*. These antisera were characterised by immunofluorescence and by indirect enzyme-linked immunosorbent assay for their reactivity with 44 strains of moulds. Antigenically, *P. verrucosum* var. *verrucosum* (subgenus *Penicillium*) appeared to be similar to strains belonging to subgenus *Furcatum,* but strongly different from *Penicillium frequentans* (subgenus *Aspergillides*). Specific absorption of antibodies to antigens confirmed the existence of similar biochemical structures on *Penicillium frequentans, Aspergillus versicolor* and *Aspergillus fumigatus.* Immunological procedures may thus significantly contribute to refine the taxonomic classification of moulds.

***Verticillium* sp.**

Since ELISA enables quantitative measurement of antigen Casper and Mendgen (1979) used it to estimate *Verticillium lecanii* at different stages of infection in leaves of wheat heavily infected with yellow rust (*Puccinia striiformis*).

Phytotoxic protein-lipopolysaccharide (PLP) complexes were isolated from dialyzed culture fluids of a pathogenic strain of *Verticillium dahlie;* PLP complexes which lack phytotoxic activity were isolated from a mutant non-pathogenic strain of the fungus (Nachmlas *et. al.* 1982). A comparison of profiles of the wild-type and mutant PLP eluting from an Agarose A-5 column revealed quantitative differences between the two major protein peaks of each strain. The corresponding peaks of the 2 strains had similar molecular of weights and in gel immunodiffusion 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 extracts of *Verticillium* infected potato plant tissue which was apparently identical to a moiety produced by the pathogen in culture.

Geric *et. al.* (1987) detected *Verticillium dahliae* in cotton root tissue with indirect enzyme-linked immuno assay. A soluble protein extract of *V. dahliae* was

used to prepare a specific antiserum. The reaction of this antibody to the hyphae of *V. dahliae* was detected with an alkaline phosphatase antirabbit IgG conjugant that hydrolysed the substrate. Napthol. AS phosphate to a product that reacted with a diazonium salt, yielding a colored precipitate outlining fungal hyphae. The hyphae were readily observed on and in the root cortex of the host using a dissecting microscope. This technique allows for rapid determination of the presence and location of *V. dahliae* in the root systems and may be applicable to other fungal species for which a specific antiserum can be produced.

Gerik and Huisman (1988) further studied the colonization of field-grown cotton roots by *Verticillium dahliae* by using a specific immunoenzymatic staining technique. Colony densities at distances more than 1cm from the tip were relatively constant. The mean colony length of *V. dahliae* was 7.3 mm, and increased colony length was correlated with distance from root apices. Hyphae of *V. dahliae* were present through the entire depth of the cortex, and were greatest in the interior of the root cortex at the surface of the vascular cylinder. The colony appearance was consistent with growth of hyphae from the root surface towards the stele. Hyphae of *V. dahliae* also were found within numerous cortical cells. Colonies of *Fusarium oxysporum,* similarly stained, were found to be mostly confined to the root surface and the outer cortex.

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

***Sporothrix* sp.**

Ishizaki *et. al.* (1981) investigated the serological cross-reactivity of *Sporothrix schenkii* serum with various unrelated fungi by use of immunodiffusion tests. A rabbit

anti *S. schenkii* serum was obtained, which related with *Cladosporium werneckii, C. carrionii, C. bantianum, Coccidioides immitis, Phialophora jeanselmei, P. gougerotii, P. dermattidis, Fonsecaea pedrosoi, Aspergillus fumigatus, Histoplasma capsulatum* and *Trichophyton mentagrophytes,* but not with *Saccharomyces cerevisiae* antigens. The serological determinantes responsible for the cross-reactions were suggested to be D-galactosyl residue.

***Epichloe* sp.**

An anitiserum, prepared to homogenates of washed *Epichloe typhina* mycelium grown in a liquid medium was used in an enzyme-linked immunosorbent assay (ELISA) to detect antigens of the fungus in tall fescue (*Festuca arundinacea*) tissue sample by Johnson *et. al.* (1982). With ELISA they could detect as little as 100 ng of freeze-dried *E. typhina* mycelium per millilitre, and could detect *E. typhina* in individual seeds of tall fescue. Of 14 fungal genera tested including *Acremonium, Claviceps, Helminthosporium, Pythium, Rhizoctonia, Sclerotium,* all showed reactivity less than 0.1% that of *E. typhina.*

***Eutypha* sp.**

Antisera were made by Gendloff *et. al.* (1983) to both a whole cell and cell wall preparation of *Eutypa armeniacae.* Rhodamine isothiocyanate (RITC) conjugated antisera were tested for reactivity with various fungi on glass slides. Both antisera showed low specificity, but specificity was improved by cross adsorption of the RITC conjugated cell wall antiserum with *Phomopsis longicolla.* Woody cross section from concord grapevines inoculated with *E. armeniacae* and also inhabited by various other fungi were stained directly with the conjugated anti-*Eutypa* rabbit serum. In an indirect staining procedure, sections were treated with anti-*Eutypa* rabbit serum then stained with RITC-labeled goat anti-rabbit gamma globulin. Both procedure specifically stained hypae in wood sections. Hyphae stained indirectly in woody vine sections showed a much brighter fluorescence than the analoges hyphae stained by the direct method. Fungi of some species that restained' hyphae strongly with the direct method on glass slides did not always react when stained indirectly in woody vine sections in which they were growing.

***Macrophomina* sp.**

Chakraborty and Purkayastha (1983) raised antibodies against *Macrophomina phaseolina* (isolate MP.) antigens and roots of soybean cultivars Soymax and UPSM-19 which were susceptible and resistant respectively to charcoal rot disease. These antisera were used in agar gel double diffusion tests for the presence of common antigens between isolates of *M. phaseolina* and soybean cultivars. Immunoelectrophoretic tests revealed that four antigenic substances were common between the susceptible soybean cultivars and isolates of *M. phaseolina* but no common antigens were detected between resistant cultivars and *M. phaseolina.*

Antigens from two isolates of *Macrophomina phosiolina* (Tassi) Gold, a pathogen of groundnut (viz. *Cortieium sasakii* Shivai (Matsumoto), *Colletotrichum lindemuthianum, C. corchori* (Pagvi and Singh) and five cultivars of *Arachis hypogea* L. were also compared by immunodiffusion, immunoclectrophoretic, and crossed-immunoclectrophoretic techniques against *M. phaseolina* antisera 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 nonpathogens 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-I and cv. TMV-2, and cv. Kadiri 71-1 and isolates of *M. phaseolina.*(Purkayastha and Ghosal, 1987).

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* (Chakraborty and Purkayastha, 1987). 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.

Detection and quantification of *Macrophomina phaseolina,* causal agent of charcoal/ dry root rot disease in many crop plants, was carried out by Srivastava and

Arora (1997) 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 ciceri.* Mycelial antigens of *M. phaseolina* on chick pea roots were detectable with DAS-ELISA at a minimum concentration of 10 ng 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 potential antagonist (*Trichoderma harzianm* -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. phaseolina* under different environmental contidions.

***Ganoderma* sp.**

Antisera was prepared against the antigen of the pathogen *Ganoderma lucidum* causing foot rot of betelnut. (Reddy and Ananthanarayanan, 1984). This antiserum obtained appeared to be specific to the *G. lucidum.* Stained preparations of the G. *lucidum* isolates of betelnut fluoresced brilliantly on glass slide and no such fluorescence was observed with the other fungi studied. Root sections of all the affected palms and 5% of apparently healthy betelnut palms showed mycelim which emitted flurorescence. Authors suggested that the presence of *Ganoderma lucidum* in roots of betelnut can be detected by the induction of fluorescent antibodies.

***Phoma* sp.**

Antisera to *Phoma exigua* var. *foveata* and var. *exigua* were prepared by injecting rabbits and mice with protein solutions from mycelium (Aguelon and Dunez, 1984) Specific antibodies were isolated and immuno enzymatic techniques (double antibody sandwich ELISA and indirect ELISA) were used to test for the fungus in inoculated tubers and sprouts and stems grown from these tubers. The fungus was detected in these different tissues, with var. *foveata* being more aggressive, demonstrated the applicability and sensitivity of the technique. The antibodies produced to the two varieties of the fungus were not specific to their own varieties. They also reacted with

Phoma tracheiphila but did not react with antibodies from mouse ascite liquid which suggest the possibility of producing specific monoclonal antibodies.

***Phaeolus* sp.**

In liquid culture 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 dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focussing (Dewey *et. al.* 1984). 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 extracellular culture filtrates 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. This makes possible identification of the kind of propagule most likely to be the source of field isolates of the organism. This information, which can not be obtained by using selective media, strongly suggests that the pathogen can survive saprophytically in the soil.

***Acrocylindrium* sp.**

Immunodiffusion, immunoelectrophoretic and cross immunoelectrophoretic analyses of rice antigens in relation to sheath rot disease revealed a serological relationship between susceptible rice cultivars and isolates of the causal organism of sheath rot, *Acrocylindrium oryzae* (Purkayastha and Ghosal, 1985). One precipitin band was observed when the antigenic preparation of *A. oryzae* was cross-reacted with its own antiserum or against the antisera of four susceptible rice cultivars. No precipitin band was detected between the antiserum of the resistant cv. Mahsuri and antigen preparations from three isolates of *A. oryzae* or between the antigens of the resistant cultivars Mahsuri and Rupsail and the antiserum of *A. oryzae.* Cross-immunoelectrophoretic tests confirmed that there was a common antigen between Mahsuri and Jaya, and between Mahsuri and CR-125-42-1. The precipitin band between the antigen preparation of Jaya and *A. oryzae* was found to be similar.

***Botrytis* sp.**

Ala El-Dein and El-Kady (1985) used crossed immunoelectrophoresis (CIE) with an intermediate gel and CIE with antibody absorption in situ techniques for comparison of antigenic structures of *Botrytis cinerea, B. tulipae, B. paeoniae* and *B. allii* isolates. Antisera against antigen of these isolates gave 24, 15, 20 and 15 precipitin peaks, respectively, when analysed in homologous reactions, CIE with an intermediate gel and CIE with antibody absorption *in situ* revealed that each isolate was serologically different from the other and had species-specific antigens. Eight antigens distinguished *Botrytis cinerea* from the other species of *Botrytis. Botrytis alli* had less common antigens than the other species.

Thin section of leaves of *Vicia faba,* uninfected were infected with two antisera, one prepared against the surface components of *B. cinerea* and the other against the timbriae of the smut fungus *Ustilago violacea.* Then a suspension containing protein A gold complexes was applied to the infected and uninfected sections. A full range of serological control treatments included use of preimmune serum. Treatment with either antiserum resulted in heavy gold labelling of host cells in infected tissue but not of cells in healthy tissue. In many cases the labelled host cells were not penetrated by fungus and were some distance from the nearest hypha. Treatment of infected host cells with preimmune serum did not result in gold labelling. Two hypotheses were proposed for these results. First fimbrial protein, either as intact fimbriae or as dissociated subunits, enters host cells well ahead of the hypae. Second infection by the fungus may stimulate a response by the host that includes synthesis of host proteins with antigenic regions similar to those of surface proteins of *B. cinerea* and those of fimbriae of *U. violacea.*(Svirecv *et. al.*, 1986).

Ricker *et. al.* (1991) detected water soluble antigens produced by *Botrytis cinerea* in spiked and naturally infected grape juice by using an enzyme immuno assay with an indirect format of antibody horseradish-peroxidase conjugate bound to polyclonal rabbit antibodies directed against *B. cinerea* (anti Bc IgG). Protein A purified gamma globulin from an early-bled antiserum (803-7), which reacted primarily with low molecular compounds present only in extracts of *B. cinerea* was used to specifically detect *B. cinerea* and quantify levels of infection in juice from infected

rape berries. Late bled, higher titer antiserum (803-19), which cross reacted with proteins and carbohydrate present in extracts from species of *Botrytis, Aspergillus, Penicillium* and *Uncinula* was used to quantify levels of rot caused by the presence of multiple fungi. Minimum detectable levels of infection, based on mixtures of clean and infected juice, were 0.25–0.5%, with 803-7 IgG, and 0.02% with 803–19 IgG. Affinity purification of 803–19 IgG by using antigens from *Aspergillus niger* coupled to sepharose beads improved specificity of anti-Bc IgG to *B. cinerea* but decreased detection sensitivity to approximately 0.5% infection. Cross reactivity of all anti Bc IgG collection was consistently low with juice extracted from uninfected grape berries. In contrast, cross-reactivity of anti-Bc IgG with water soluble antigens extracted from sterile and reproductive structures of several fungi was negligible in early-bled antiserum and increased in subsequent collections. The increase in cross reactivity in late-bled antisera corresponded with an increase in the overall serum titres for anti-Bc IgG to antigens from *B. cinerea.* Nonspecific binding of 803-19 IgG was high with extracts from *A. niger* and an unidentified species of *Penicillium,* suggesting numerous epitopes common to antigens from these fungi.

Salinas and Schots (1994) used whole conidia, their extracellular material and a putative cutin esterase isolated from conidia of *Botrytis cinerea* as antigen to raise monoclonal antibodies (MAbs) for the detection of condia of *B. cinerea.* Using immunofluorescence, three selected MAbs recognized conidia of 43 isolates of *B. cinerea* from hosts representing six countries. The percentage of conidia that fluoresced ranged from 50 to 100%. Intensity of fluorescence was related more to the MAbs than to the *Botrytis* isolate tested. MAbs showed no reaction with healthy gerbera flowers or to spores produced by other common airborne fungi and bacteria. Cross reaction with conidia of four other species of *Botrytis* occurred, but their fluorescence patterns differed from those of conidia of *B. cinerea.*

***Colletotrichum* sp.**

It was reported that agar gel double diffusion test confirmed the existence of common antigenic relationship between the susceptible jute cultivar and the foliar pathogen *Colletotrichum corchori.* Pathogenicity test of the isolate of *C. corchori* also revealed that it is virulent on cv. JRC 212 while JRC 321 is moderately resistant.

The precipitin reaction was observed within 24 hr. In case of jute, however, the reaction occurred only after 72 hr. Bhattacharya and Purkayastha (1985) suggested that the delayed response may be attributed to the slow rate of diffusion of the host antigen because precipitation was observed within 24hr. When reactivity was tested between the fungal antigen and its own antiserum.

In a further study, Purkayastha and Banerjee (1989) used six antibiotics as foliar spray on a susceptible soybean cultivar (Soymax) to induce resistance against anthracnose. In addition, common antigenic relationships between seven soybean cut tivars, their pathogens and non-pathogen were also studied using immunodiffusin, immunoelectrophoresis and indirect ELISA technique. Among the six antibiotics tested, cloxacillin and penicillin induced maximum resistance against anthracnose. 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 *C. 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 the two at a very low level.

Immunofluorescence studies of cutinase secretion by phytopathogenic fungi *Fusarium solani* f.sp *pisi* and *C. gloeosporiodes* were made to determine targeting of the enzyme in germinating spores (Podila *et. al.* 1994). Results indicated that the secretion of cutinase was directed towards the region that penetrates the host. In *F. solani* f.sp. *pisi*, which penetrates plant tissue without appressorium formation, cutinase was targeted to the growing tip of germinating spores. In *C. gloeosporioides,* cutinase secretion was directed to the infection peg that arises from the appressorium. Monensin, the carboxylic ionophore, inhibited secretion of cutinase and caused intracellular accumulation of cutinase in *F.* sp. *pisi*. Subcellular fractionation of monensin-treated cells indicated involvement of the Golgi in the secretion and targeting of cutinase by germinating spores of *F.* sp. *pisi.*

A study was conducted by Viswanathan (2000) on the possibility of identifying

resistance to red rot pathogen *C.falcatum* Went in sugarcane based on serological technique. Large number of genotypes were screened for red rot resistance at an early stage. A new serological technique, immunosorbent assay was developed based on antiserum developed against the fungus. Early detection of the pathogen well before the symptom development was possible in this technique. When different tissues in the infected sugarcane plants were screened for antigen titre, root eyes and bud in the nodal region and internodal samples from pith and white spot gave higher values showing more pathogen colonization. When 20 different sugarcane varieties were subjected to ELISA test after pathogen inoculation, it showed a clear variation in disease resistance among them as in field testing. Further, this technique was found reliable to screen sugarcane genotypes for red rot resistance at an earlier stage.

***Mycena* sp.**

Antigenic determinants responsible for a precipitation line specific for *Mycena galopus* were partially purified using salt fractionation (with ammonium sulphate) and ion exchange chromatography by Chard *et. al.* (1985a). Fractionations were assessed by immunodiffusion and immunoelectrophoresis for the presence of the specific line. Proteins, with associated lipid and carbohydrate moieties, were detected in the antigenic fraction, which was used for further antiserum production. The new (F^{-}) antiserum was tested for its specificity to *M. galopus* by immunodiffusion.They further carried out immunofluorescence tests, involving 34 species of fungi, on an anti-*Mycena galopus* serum raised against a partially purified antigenic fraction. Cross reacting fluorescence was produced primarily by *Mycena* deuteromycete and ascomycete species, non-*Mycena* basidiomycetes generally showing less fluorescence. Absorption of the antiserum with mycelium from cross-reacting fungi resulted in a reduction in fluorescence of cross reacting species, mostly to an acceptable control level(Chard *et. al.* 1985b).

***Sclerotinia* sp.**

The adaptation of enzyme-linked immunosorbent assay for the detection of *Sclerotinia 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}.

Jamaux and Spire (1994) also reported a serological test that allows the early detection of infection of young petals by *S.selerotiorum* an important pathogen of rapeseed. Two steps were required to obtain an antiserum sufficiently specific for *S. sclerotiorum.* Soluble mycelial extracts of *S. sclerotiorum* were used to produce the first generation polyclonal antiserum. This was not specific for *S. sclerotiorum* in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and allowed the screening of cross reacting fungal species such as *Botrytis cinerea,* a pathogen commonly present on rapeseed petals. Using a polyclonal anti *B. cinerea* serum enabled the absorption, by serial cycles of *S. sclerotiorum* antigens common to *B. cinerea.* Residual antigens were then used as immunogens for the production of two second generation antisera (S1 and s2), which were then tested by DAS-ELISA. Cross reaction with *B. cinerea* decreased with purification cycles of the immunogen whereas cross reaction with some unrelated fungi slightly increased *S. sclerotiorum* and *B. cinerea* were distinguished using antiserum S2.

***Ustilago* sp.**

Sections of leaves of *Nicotiana tabacum* L. infected with *Peronospora hyoscyami* De Bary f.sp. *tabacina* (Adam) Skalicky and of *Erythronium americanum* Ker. infected with *Ustilago heufleri* Fuckel were treated 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 complexes 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 indicating the presence of fimbrial antigens, whereas the control preparations showed only a low background level of labelling. Gold particles were detected on the sections of hyphae, on haustoria and on the nearby plant cells. The intensity of labelling was much higher for *P. hyoscyami* sp. *tabacina* than for *U. heufleri* and was particularly high on the walls of the former species. Relatively high levels of labelling occurred over the cells of infected hosts, but little or none occurred over the cells of uninfected host tissues, or of the infected host tissues treated with a range of serological controls. This high level of labelling was not associated with specific host structures in *P. hyocyami,* but was frequently associated with the

chloroplasts in *U. heufleri.* The antigens detected inside the host plant cells appear to indicate that fungal fimbrial protein, either as polymerized fimbrial 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 suggest the possibility that fungal fimbriae may play an important role in the molecular interaction between pathogen and host.

Cross-reactivity between antisera produced against fimbriae of *Ustilago violacea* (Pers) Fuck. (AU) or of *Rhodotorula rubra* (Demme) Lodder (AR) and cell surface proteins of two ascomycete fungi, *Aococalyx abietina* (Lagerberg.) Schlaepfer-Bernhard and *Ophistoma ulmi* (Buism.) Nannf., was revealed by means of dot-immunobinding and immunocytochemical methods (Benthamou *et.al.* 1986). 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 labelling intensity was always found to be greater over the plasma membrane. The fibrillar sheath surrounding cells of *A. abietina* reacted with antiserum A.U. while all other structures did not. No significant labelling with this antiserum occured over cells of *O. ulim* indicating that they either lacked these antigens or that those were more easily removed during the fixation process.

***Phomopsis* sp.**

Antiserum to freezed-dried powdered mycelium of *Phomopsis longicolla* soybean seed decay pathogen, was used by Gleason *et. al.* (1987) in an idirect ELISA and an immunoblot assay for detecting seed borne infections. Antigen of *P. longicolla* was detected by indirect ELISA in as little as 250 ng of dried mycelium per milliliter of extract. The antiserum reacted with mycelial preparations of *Diaporthe phaseolorum* var. *sojae* var. *caulivora* but showed comparatively little or no reaction when tested against seven other seed borne fungi. Extracts of whole seeds, or of seed coats, produced a non-specific background reaction that showed the specific reaction. *P. longicolla* could be detected in individual variety of symptomless infected seeds. A single infected seed in 20 could be readily detected by indirect ELISA of extracts of

seed coats. An immunoblot assay designated the seed immunoblot assay was developed to overcome problems with nonspecific interference in indirect ELISA. Mycelium of *P. longicolla* growing onto nitrocellulose paper from infected soybean seeds produced a conspicuous coloured blotch after the paper was assayed. Results of seed immuoblot assay (SIBA) for incidence of *P. longicolla* and *D.P.* var. *sojae* in halved seeds from 10 seed lots correlated ($P<0.001$) with agar plate bioassarys of the corresponding seed halves but not with incidence of symptomatic seeds. Indirect ELISA absorbance values for bulked samples of seed coat halves from the same 10 seed lots correlated weakly ($0.10>P>0.05$) with agar plate bioassays but strongly ($P<0.01$) with incidence of symptomatic seeds. Because SIBA detects only viable *P. longicolla* and ELISA does not discriminate between live and dead fungus, SIBA should be better indicator of pathogen activity on seeds after planting. This two types of serological assays apparently measure different aspects of this disease however and both may be useful for evaluating soybean seed lot quality.

Brill *et. al.* (1994) produced polyclonal antibodies in New Zealand white rabbits with cultiture filtrate and mycelial extract immunogen preparations from the soybean (*Glycine max*) fungal pathogen *Phomopsis longicolla.* The PAbs were purified to the immunoglobulin fraction and tested in indirect enzyme-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 the mycelial extract immunogen preparation. DAS ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PAbs was lower in DAS-ELISA. Compared with indirect ELISA. Immunization of one 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 members of the complex at the same time. Reactivity to same cultures of *P. longicolla* was undetectable following the fourth and fifth immunizations, whereas reactivity to all of their other cultures of the complex remained high.

***Ophiostoma* sp.**

The surface deposition and accumulation of crato-ulmin by the aggressive isolates *Ophiostoma ulmi* were demonstrated by Svirecv *et. al.* (1988) using polyclonal

antiserum directed against cerato-ulmin, 100-150 nm protein A-gold particles, and a scanning electron microscope. The protein A-gold complex was present on the fungal surface in areas containing the toxin cerato-ulmin (CU). The gold level was present on the surface of the vegetative hyphae, synnemata, synnematal spores, perithecia and ostiolar hair of the aggressive isolates of *O. ulmi.* The protein A-gold level was either evenly distributed in the fungal surface or in the form of large surface aggregates. The non-aggressive isolate Q412 of *O. ulmi* had a low concentration of protein A-gold label on its fungal structures. When the specific CU antiserum was replaced by preimmune serum, a lack of the protein-A gold. label was evident on all fungal structures of the aggressive isolate VA of *O. ulmi.*

***Pseudocercosporella* sp.**

Unger and Wolf (1988) presented an indirect ELISA for quantitative detection of *P. herpotrichoides* infections in wheat. All tested isolates of the virulent varieties *P. herpotrichoides infections, P. h.* var. *acuformis* or the W and R-type react on a high level in the test, while the less virulent *P. anguioides* is assessed only with 40% and avirulent *P. asestiva* with 20% of the homologous reaction. No cross reactions occur with extracts of 11 other species of *in vitro* cultivated fungi nor with plant material infected with other pathogens. The infection profile through the leaf-sheaths was clearly reflected by ELISA. The examination of 24 stembase samples from the field showed that the values assessed by ELISA correlate well also with the disease indices of naturally infected plant material.

A double-antibody-sandwich ELISA test has been developed by Priestly and Dewey (1993) for the detection of *Pseudocercosporella herpotrichoides* using a highly specific monoclonal antibody pH-10 as the capture antibody and genus specific rabbit polyclonal antiserum as the detector antibody. The assay recognizes extracts from plants both artificially and naturally infected with *P. hypotrichoides* givining at least three-fold higher absorbance values with extracts from *Pseudocercosporella* infected tissue than with extracts from healthy tissues or from tissues naturally infected with *Microdochium nivale, Rhizoctonia cerealis* or material artificially inoculated with *P. anguioides.* The assay tested positively against all isolates of *P. herpoterichoides,* including both W-type and R-type isolates. In this assay system, extraction of the

antigen from the stem bases of infected plants is a one-step process not requiring any dilution procedures. The assay can detect the pathogen in presymtomatic infected seedlings. The immunogen used to generate the specific monoclonal antibody and the rabbit antiserum was a mycelial extract from which the high-molecular-weight proteins and glycoproteins had been removed by ammonium sulphate precipitation. The high-molecular weight fraction was shown to contain cross-reactive antigens, it induced antiserum in mice that cross-reacted with the other stem-base fungi even at high dilutions. The monoclonal antibody pH-10 is an IgM antibody. Heat and periodate treatment of the antigen indicate that it is glycoprotein and that the epitope recognized by the antibody is a protein.

***Plasmodiophora* sp.**

An antiserum was made against *Plasmodiophora* brassicae, the causal agent of club root of cabbage (Lange *et. al.* 1989). A semipurified suspension of spores of *P. brassicae* was used as antigen, obtained by filtration and percoll gradient centrifugation of infected roots. Crude root suspension samples were bound to a nitrocellulose membrane 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. brassicae,* race 7, appeared smooth, while the dot immunobinding processed spores had a heavy, irregular coating, which was demonstrated to originate from incubation in the primary antiserum. Normal serum did not give rise to a coating on the resting spores. The antiserum of *P. brassicae* did not react with surface antigens of resting spores of *Polymyxa.* Further, no cross reaction with other common root pathogen such as *Pythium ultimum, Rhizoctonia solani,* and *Fusarium oxysporum* was observed. With antiserum prepared against spore suspension antigenic determinants the dot immunobinding technique can be used as a routine test for detection of infection of *P. brassicae* in the host plant and in bait plants (used as indicator of soil infestation). The sensitivity obtained was within the range permissible for a routine test.

Polyclonal antisera were raised by Wakeham and White (1996) to whole (Coded : 16.2), and sonicated (Coded : 15/2) resting spores of *Plasmodiophora brassicae,* and to soluble components prepared by filtration and ultracentrifugation

(Coded SF/2). Cross reactivity of all three antisera with a range of soil fungi, including *Spongospora subterranea* was low. Test formats including western blotting, dip-stick, dot-blot, indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence were assessed for their potential to detect resting spores of *P. brassicae* in soil. Dot-blot was least sensitive, with a limit of detection level of $1x10^7$ resting spores g^{-1} in soil. With western bloting the lower limit of detection with antiserum 15/2 was 1x10. This antiserum showed the greatest sensitivity in a dipstic assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of 1x10. The indirect ELISA was successful only after the substitution of alkaline phosphatase by protein A conjugated horseradish peroxidase. Of the assays tested, indirect immunfluorescence appears to be the most rapid and amenable assay for the detection in soil of low levels of resting spores of *P. brassicae*.

***Armillaria* sp.**

Antibodies to three isolates each of *Armillaria mellea, A. ostoyae, A. tabescens* and *Lentinula edodes* were isolated from eggs of immunized laying hens. The reactivity of each antibody preparation with the isolates was examined using an enzyme-linked immunosorbent asay (ELISA). The cross-reactivity of the antibody preparations to a given *Armillaria* species varied considerably when tested against isolates of other *Armillaria* species. Several antibody preparations were capable of distinguishing isolates of homologous species from isolates of heterologous species. The specificity of the antibodies present in eggs was dependent on time elapsed since immunization. Eggs laid 3 weeks after immunication with an *Armillaria* species isolate possessed antibodies that were most specific for isolates of that species. The enterogeneric cross-reactivity was found to be smallest with antibodies from egg laid 5 weeks or more after immunization. Preliminary results suggest that ELISA using chicken egg yolk antibodies will provide useful information in the taxonomy of *Armillaria* (Burdsall *et. al.* 1990).

***Leptosphaeria* sp**

Monoclonal antibody (MAB) LKe50 was developed against *Leptosphaeria* Korrae strain ATcc 56289. The antibody was capable of detecting *L. korrae* from culture and in naturally infected Kentucky bluegrass samples from three states (Nemeth

et. al.,(1990). In cross-reactivity testes using indirect ELISA, MAB LKc50 reacted positively to all 24 isolates of *L. korrae* screened, including strains from six states, from both Kentucky bluegrass and burmudagrass. MAb LKc50 reacted negatively to 38 of 42 isolates of related and nonrelated fungi and negatively to apparently healthy plants. The limit of detection was less than 2μg/ml of lyophilized mycelial homogenates. MAb LKc50 provides a means for rapid detection of *L. korrae* an ectotrophic root invading fungus that is difficult to identify using conventional methods.

***Myrothecium* sp.**

Antigens were extracted by Ghosh and Purkayastha (1990) 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. Result 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.

***Venturia* sp.**

An immunocytochemical stain was developed by Young and Andrews (1990) 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. bombacina,* incubated outside from November 1986 to May 1987, and sampled monthly. Sections stained immunocytochemically showed that *A. bombacina* grews endophytically and epiphytically. The antagonist prevented neither growth of hyphae of *V. inaequalis* into the interior of leaves, nor initiation of pseudothecia. There was no particular spatial association between hyphae of the two fungi, nor any sign of direct parasitism of hyphae or pseudathecia of *V. inaequalis.*

Pseudothecia in leaves with the antagonist did not mature further than the stage. of producing pseudoparaphyses, reaching on average of 84 μm in length. Pseudothecia in leaves without *A. bombacina* developed as in normally and were 108 μm long by 1 May. These data were confirmed by results from an abbreviated sampling by results from an abbreviated sampling scheme of McIntosh apple leaves during 1987-1988 and by observation of pseudothecial inhibition in crab apple leaves following delayed application of the antagonist.

***Acremonium* sp.**

Distribution of endophytic fungus, *Acremonium coenophialum,* with tissues of infected tall fescue (*Festuca arvudinacea*) can be determined with tissue print-immunoblot (TPIB); however, the accuracy of this method in determination of endophyte infestation levels of pastures and seed losts has not been evaluated (Gwinn *et. al.* 1991) Authors conducted a study to compare TPIB to other methods used for endophyte detection. Seed stocks 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 pasture. Therefore, accuracy of TPIB is comparable to other endophyte detection techniques and can be used for routine detection of endophyte in tall fescue tissue.

***Phialophora* sp.**

Daniel and Nilsson (1991) raised polyclonal antiserum against mycelial extracts from wood degrading soft rot fungus *Phialophora mutabilis.* In enzyme linked immunosorbent assay (ELISA), the antiserum reacted strongly to moderately with six soft rot *Phialophora* species. With exception of *Cerotocystis albida,* the serum reacted weakly or not at all with 11 other mold, and rot fungi occurring frequently in or on wood. The antiserum was cross-reacted strongly with antigens in extracellular filtrates from *P. mutabilis* cultures that contained about 40ng/ml of protein. Utrastructural and immunocytochemical studies on wood degraded by *P. mutabilis* showed specific localization of the fungal cell wall and certain intracellular structures. Extracellular labelling within soft rot cavities and sites of erosion decay of wood also

were noted. The antiserum was assessed by ELISA for detecting the presence of fungus and soft rot in untreated and preservative treated wood blocks of pine and birch degraded for periods of 1-12 months. *P. mutabilis* was detected in samples from all wood blocks degraded to low or high weight loss. Highest ELISA reading were recorded for wood blocks with highest substrate losses and vice versa.

***Pythium* sp.**

A panel of monoclonal antibodies (MAbs) designated PA1 to PA8 has been raised by Estrada-gracia *et. al.* (1989) against cell surface components of zoospores and cysts of the pathogenic fungus *Pythium aphanidermatum.* The antibodies were selected on the basis of binding assays using indirect immunofluorescence. Four binding patterns were observed; PA1 labeled the entire zoospore surface including both flagella, in PA2 binding was restricted to the anterior flagella, PA3-PA6 bound to the adhesive cell coat secreted by zoospres during ensystment, and PA7 and PA8 labeled zoospores and the cyst cell wall. Electron microscopic immunogold labelling of zoospores showed that PA2 bound to the mastigonemes on the interior flagellun. The MAbs were tested for binding to zoospores and cysts of several isolates of *P. aphanidermatum,* and to zoospores and cysts of several species of *Pythium, Phytophthora, Aphanomyces,* and *Saprolegnia.* This results showed that the antigens recognized by MAbs PA1-PA6 were restricted to *P. aphanidermatum,* whereas those recognized by PA7 and PA8 occured on all species tested.

Shane (1991) compared methods for sampling turfgrass tissue for their effectiveness in monitoring *Pythium* blight epidemics with enzyme-linked immunosorbent assay (ELISA). Sample area consisted of marked strips on golf course fairways and tees with bentgrass and annual bluegrass naturally infested by *Pythium aphanidermatum.* Samples consisted of (1) whole plants picked by hand and assayed as whole plants, (2) whole plants sectioned into lower, middle and 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 vary little change in ELISA readings for the two lower strata. Several episodes of

Pythium antigen increase were detected by ELISA assay of mowed samples, although signs and symptoms of *Pythium* blight were not evident. However, increase in ELISA readings for *Pythium* coincided with, but did not generally precede, the onset of blight symptoms with a 2 to 3 day sampling interval. Antibody aided detection is useful for varification of diagnosis and determination of general *Pythium* population fluctuations, but current methodology is not satisfactory for advanced detection of blight epidemics.

Takenaka (1992) utilized ribosomal protein differences among snow mold fungi and wheat to detect the causal fungi in infected wheat leaves by indirect enzyme linked immunosorbent assay (ELISA) and Western blot analysis. Polyclonal antisera were raised against the ribosomes of *Pythium paddicun, 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 antisera, ribosomes of *P. paddium* and *P. iwayamai* were serologically identical; the ribosomes of *T. incarnata* and *T. ishikariensis* had partial common antigenic determinants, but there were apparent serological differences among *Pythium* spp. *Typhula* spp. *M. invale* and wheat. With indirect ELISA, ribosomes of *Pythium* species 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 rotten wheat leaf homogenates diluted upto 1:1000 or 1:10000. The indirect ELISA could not differentiate *P. paddicum* from *P. iwyamai* 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. paddicum* from *P. iwayamai* but could differentiate wheat leaves infected with *T. incarnata* from those infected with *T. ishikariensis*. These immunological methods with antiribosome sera would be useful to evaluate wheat plants for infection by *Pythium* spp., *T. incarnata T. ishikariensis* and *M. nivale*.

Conventional methods indicated that *P.violae* was most commonly isolated from carrot cavity spot samples from 14 UK sites. For one site the most frequently isolated species was *P. sulcatum.* Results of similar isolation work were compared with the assay of cavity spot lesions using polyclonal antibodies, raised to *P. violae* or

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

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

***Bipolaris* sp.**

Chakraborty and Saha (1994) reported that TV-18 was most susceptible and TV-26 most resistant among the 14 Tocklai varieties of tea tested against *Bipolaris carbonum.*Antigens from tea varieties, isolates of *B. carbonum* and nonpathogens of tea (*B. tetramera* and *B. setaria*) were compared by immunodiffusion, immunoelectrophoresis 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 (TV-9, 17, and 18(and isolates of *B. carbonum* (BC-1, 2, 3 and 4). Such antigens were not detected between isolates of *B. carbonum* and resistant varieties (TV-16, 25 and 26), non pathogens and tea varieties, as well as nonpathogens and isolates of *B. carbonum.* Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and conidia of *B. carbonum* with antisera to leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia.

***Sclerotium* sp.**

Serological differences among three strains (266, 23, M) of *Selerotium rolfsii* were studied by Purkayastha and Pradhan (1994) along with strain pathogenicities and antigenic relationship with five groundnut cultivars. Changes in antigenic patterns of one of its host evs. AK-12-24 and disease intensity were noted after treatment with a systemic fungicide Kitazin. Results revealed 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). 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) altered the antigenic pattern of roots of a susceptible cv. AK-12-24 and also reduced disease markedly.

***Thielaviopsis* sp.**

Field isolates of *Thielaviopsis basicola,* the causal agent of black root rot of cotton (*Gossypium hirsutum*), were grown in Czapek-Dox broth amended with dialysed carrot extract (Holtz, 1994). Soluble protein extracts of chlamyolospores and mycelium were used to raise polyclonal mouse ascites antibodies. The immunoglobulin G antibody fraction was purified and biotin-labeled to devise a fungal capture sandwich enzyme-linked immunosorbent assay (ELISA). ELISA detected both brown and gray cultural types of *T. basicola* and had negligible cross-reactivity with other soilborne fungi commonly found in the San Joaquin Valley of Callifornia cotton field soils. The minimum detection limit of ELISA was between 1 and 20ng of *T. basicolla* protein depending on the assay. *T. basicolla* could be detected in cotton roots 2 days after inoculation. At this time, initial symptoms were apparent. The antibody also used to observe *T. basicola* on cotton roots with immunofluorescence microscopy.

***Exobasidium* sp.**

Blister blight of tea caused by *E. vexans* is one of the most destructive fungal diseases in Darjeeling hills. It causes enormous losses to Indian Tea Industry. Polyspecific antisera were raised against a plant cell supernatant fraction from

homogenized naturally blister infected leaf tissues (Variety AV-2) and subsequently the sensitized antisera were immunoprecipitated with healthy leaf antigens of AV-2 in order to separate antibodies unique to *E. vexans*. Immunoglobulin (IgG) was purified following ammonium sulphate fractionation and chromatography on DEAE-Sephadex Cross reactive antigens (CRA) were detected between the pathogen and susceptible Darjeeling tea varieties in immunodiffusion tests and enzyme-linked immunosorbent assay. Cellular location of CRA in cross-section of leaves was determined using indirect staining of antibodies with FITC-labelling. Specific and sensitive serological assays have been developed for early detection and diagnosis of the disease in Darjeeling and UPASI varieties of tea. Such rapid detection prior to the development of foliar symptoms will be helpful for making decision on management of blister blight which is the most threatening foliar fungal disease of tea (Chakraborty *et. al.* 1995).

***Pestalotiopsis* sp.**

Among the 12 varieties of tea tested against three isolates of *Pestalotiopsis theae,* causal agent of gray blight disease, Tean Ali-17/1/5A and TV-23 were found to be highly susceptible while CP-1 and TV-26 were resistant under identical conditions (Chakraborty *et. al.* 1995). Leaf antigens were prepared from all the tea varieties, three isolates of *P. theae* and a non-pathogen of tea (*Bipolaris tetramera*). Polyclonal antisera were raised against mycelial suspensions of *P. theae* (isolate Pt-2) and leaf antigens of Teen Ali-17/1/54 and CP-1. These were compared by an immunodiffusion test and enzyme-linked immunosorbent assay to detect cross reactive antigens (CRA) shared by the host and parasite. CRA were found among the susceptible varieties and 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 reaction 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 raised by Chakraborty *et. al.* (1996), against the mycelial extract of *Pestalotiopsis theae* fractions were further purified by immonium sulfate frationation and chromatography on DEAE-Sephadex and the ammunoglobin fractions used for further tests. In enzyme-linked immunosorbent assay, antiserum dilution

upto 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 of 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 upto 1:125, the pathogen could be detected in inoculated leaf extracts up to antigen concentration of 2mg/L. The antiserum reacted with two other isolates of *P. theae* tested but not with the antigens from mycelial extracts of *Glomerella cingulata* and *Corticium invisum* or with extracts of tea leaves inoculated with these pathogens. The results domonstrated that ELISA can be used for early detection of *P. theae* in leaf tissues even at a very low level of infection.

***Glomerella* sp.**

Chakraborty *et. al.* (1996) conducted varietal screening of tea (*Camellia sinensis*) with *Glomerella cingulata,* causal agent of brown blight disease with sixteen varieties released by Tocklai Experimental Station, Jorhat, Assam, following detached leaf, cut shoot and whole plant inoculation techniques. Among the tested varieties, TV-18 and Teenali 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 of *G. cingulata* (isolata GC-1). These antisera were used in immunodiffusion and immunoelectrophoretic tests as well as in enzyme liked immunosorbent assay (ELISA) to detect cross reactive antigen (CRA) shared between tea varieties and isolate 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 fluorescein isothiocyanate (FITC) indicated that in cross section of tea leaves (TV-18), the CRA was concentrated in epidermal cells, mesophyll tissue and vascular elements. Among a number of foliar fungal pathogens of tea *G cingulata* (Stoneman) Spauld and Schrenk which causes brown blight disease, is an important one. Polyclonal antisera were raised in separate white male rabbits against antigens prepared from

mycelia and cell wall of *G. cingulata* by Chakraborty *et. al.* (2000). Antigens were prepared from healthy artificially inoculated with *G. cingulata* and normally infected leaves of 5 UPASI varieties and DAC-ELISA performed. Pathogen could be detected in infected leaf extract at a concentration as low as 1mg/ml. Absorbance values of extracts of leaves inoculated with *G. cingulata* were higher than those inoculated with other pathogens and healthy extracts. Pathogenicity of *G cingulata* (Stoneman) Sparld and Schrenk, causal agent of brown blight disease, tested Chakraborty *et. al.* (2002) 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-17/1/5A were susceptible. Polyclonal antisera were raised separately against antigen preparations from mycelia and cell wall of *G. cingulata* 9 isolate Gc-L) 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* (G c-1, 3 and 3). Such antigens were not detected between resistant varieties of tea and isolats of *G. cingulata* (Gc-1, 2 and 3); non-pathogen (*Fusarrium oxysporum*) and tea varieties; isolates of *G. cingulata* and *F. oxysporum* and between non-host (*Glycine max*, *Cicer arietinum* and *Camellia japonicum*) and *G. cingulata.* Antisera raised against cell wall preparations gave better recognition than that against mycelial preparations as observed in ELISA test with antigens of tea leaves of different ages.

***Spongospora* sp.**

Walsh *et. al.* (1996) produced a polyclonal antiserum against spore balls of *Spongospora subterranea* f.sp. *subterranea* prepared from potato tubers which 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. However, the different spore ball contamination levels were discriminated better in ELISA tests at concentration above 2000 spore balls per g soil than at lower concentration. In contrast, a bioassay test based on 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 in spiked soils containing >2000 spore balls per g soil. Tests on a limited number of field soils suggested ELISA may be capable of predicting disease levels on tubers grown in such soils better than

the bioassy. The antiserm 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 equally well. It also detected spores from different geographical origins. An attempt to improve the sensitivity of the serological detection through concentrating spore balls from field soils by sieving was unsucessful. Cross absorption of the antisera with uncontaminated field soils 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 uncontaminated soil was much improved by using the gamma-globulin fraction or cross absorbed serum. 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 autofluorescence of spore balls, allowing immunofluorescence studies to be carried out. Using this technique in conjunction with indirect FITC immunofluorescence, discrete bright fluorescent spots were visualized using the specific serum. With the non-specific serum, only a very dull back ground fluoresecence was evident.

***Spodoptera* sp.**

Polyclonal antibodies were produced in mice against *Spodoptera exigua* (beet armyworm) larval hemolymph and hemocystes and against cell wall surfaces of hyphal bodies and hyphae of entomopathogenic hyphomycete *Nomuraea rileyi* (Pendland and Boucias, 2000). In addition to exhibiting strong activity against their original antigenic substrates, all of the antibodies cross-react extensively with other substrates. The hemolymph antibody binds to hemocytes and vice versa, and both antibodies cross-react extensively with other substrates. The hemolymph antibody binds to hemocytes and vice versa, and both antibodies cross react to the insect fat body basement membrane (extracellular matrix (ECM). Likewise, the antifungal antibodies cross-react with *S. exigua* hemolymph and hemocytes, especially the granutes that may contain ECM components, and with fat body basement membrane. These cross-reactivities are specific as indicated by negative controls in the microscopy and western blotting assays. Parallel labeling experiments using conA suggest that reactive epitopes contain mannose; however none of the antibodies bind to mannose residues of nonentomopathogenic *Candida albicans* or *Saccharomyces cerevisiae* yeast cells.

Thus, these cross-reactivities suggest the host minicry expressed by surface components of entomopathogenic fungi represents and important pathogenic determinant.

***Fomes* sp.**

Polyclonal antibody (PAb) was raised in New Zealand male rabbits against immunogen preparations from mycelial extract of *Fomes lamaoensis* (Murr.) Sacc and Trott., causal agent of brown root rot disease of tea (Chakraborty *et. al.* 2001). Eight blood samples were collected by marginal ear vein puncture from rabbits and IgG were purified using DEAE cellulose. Immunodiffusion tests were performed inorder to check the effectiveness of mycelial antigen preparations of *F. lamaoensis* for raising PAbs. Optimization of PAbs were done using indirect enzyme linked immunoassay (ELISA) Increased activity of PAbs against *F. lamaonensis* could be noticed from second bleeding, which continued upto fourth bleeding. Root antigens prepared from healthy and artificial inoculated (with *F. lamaoensis*) tea plants (TeenAli-17, TV-18, TV-22, TV-26, TV-27, TV-28, TV-30, S-449, BSS-2) were analysed following direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA). Such format was also used to detect the pathogen in infested soil. Young mycelial of *F. lamaoensis* gave bright fluorescence in indirect immunofluorescence tests using PAbs and FITC-conjugates of goat specific for rabbit globulin. Such immunological assays developed for detection of *F. lamaoensis* in rhizosphere of tea plantation can enable disease prevention at an early stage.

***Ustulina* sp.**

Chakraborty *et. al.* (2001) raised polyclonal antibodies (Pabs) against mycelial and cell wall antigens of *Ustulina zonata* (Lev.) Sacc. causing charcoal stump rot of tea root. PAbs were purified in DEAE-cellulose column. The effectiveness of antigens preparation for raising PAbs were checked following immunodiffusion 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 specific 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. Such immunological methods developed for early detected of *U.zonata* from soil and tea root tissue will be useful for the proper management of this disease.

Wide range of immunoassays available to the plant pathologist have been illustrated and factors to be considered in selecting the most appropriate technique have been indicated. Each has a role to play but there is no doubt that the development and adoption of highly sensitive assays such as ELISA, dot blot, immunofluorescence have transformed serology over the last two decades and revolutionized the plant pathologist's approach to the immunological identification of pathogens and the diagnosis of plant disease.

Materials & Methods

3. Materials and Methods

3.1. Plant Material

3.1.1. Collection

Twenty five tea varieties collected from three experimental station of India viz. (a) Darjeeling Tea Research Centre, Kurseong, West Bengal (b) Tocklai Experimental Station, Jorhat Assam and (c) United Planter's Association of South India (UPASI) Tea Research Station Valparai, Tamilnadu, being maintained in the Tea Germplasm Bank of the Department of Botany, University of North Bengal were used for experimental purposes.Among these, seven were Darjeeling varieties (S-449, HV-39, T-135, AV-2, CP-1, BS/7A/76 and P-1258), eleven Tocklai varieties (Teen Ali 17/1/54, TV-9,22, 23, 25, 26, 27,28, 29 and six UPASI varieties (UP-2,UP-3, UP-8, UP-9, UP-26 AND BSS-2).

3.1.2. Propagation

Tea plants were propagated by cutting. Soil preparation is most important in propagation technique and hence, care was taken to prepare the soil well. Sandy soil (75% sand and 25% soil) with a pH ranging 4.5-4.9 was used . Soil pH was adjusted applying 2% aluminium sulphate solution followed by leaching with water to remove excess aluminium sulphate.

Polythene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in bed and watered thoroughly. Beds were arranged in two rows, eleven in each row. Two hundred and fifty cuttings of 25 varieties were allowed for rooting in individual sleeves after dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloche and the whole setup was kept under a green agro house (Plate 2,figs A-D).

3.1.3. Maintenance of tea sleeves in nursery

After the removal of the polythene cloche, the sleeves were treated with nursery mixture (nutrient) as suggested by Ranganathan and Natesan (1987). The composition of the nursery mixture is as follows :

Ammonium Sulphate	–	8 parts by weight.
Ammonium Phosphate-Sulphate(16:20)	–	35 parts by weight
Potassium Sulphate	–	15 parts by weight
Magnesium Sulphate	–	3 parts by weight
Zinc Sulphate	–	3 parts by weight

Thirty grams of the above mixture was dissolved in 1 litre of water. Each sleeve was treated with 50ml of the nutrient mixture upto 12 months once in 15 days.

3.1.4. Plantation

In the experimental plot simazine @75g / 20 l. water and Glyophosphate @1:200 were used for weed control (Borpujari and Banerjee, 1994). Then pits (1½' x 1½' x 1½') were dug at the intervals of 2' between plants and 3.5' between row to row. Planting mixtures were prepared in the ratio of 4.5 kg of well rotten dry cattle manure, 30 kg. rock phosphate, 30 kg. super phosphate and 2.5g phorate [O,O-diethyl S- (ethylthiomethyl) phosphor dithoioate]. 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 the soil preparation, the plants were inspected, selected and brought to the experimental garden and planted in the prepared soil. For experimental purposes, seedlings were also transferred to earthen pots (12" dia). Soil mixture was prepared in the same ratio as above and each pot was filled with the mixture. Ten month old seedlings with well-developed shoot and root system were transferred from the sleeves to the pots. These were then maintained in the pots with regular watering.

The mature plants (1 year above were maintained by applying a soluble mixture of N,P,K consisting of 10kg urea (46%N),20 kg 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.

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

Plate 2 (Figs. A-E) : Nursery grown tea saplings

3.2. Fungal Cultures

3.2.1. Source of Cultures

Sphaerostilbe repens B & Br. 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 mentioned table 1.

Table 1 : List of fungal isolates used

Species	Host	Source
Sphaerostilbe repens	*Camellia sinensis* (L).O. Kuntze	Tocklai Experimental Station Jorhat, Assam
Ustulina zonata	*Camellia sinensis*	Tocklai Experimental Station Jorhat, Assam
Fomes lamaoensis	*C. sinensis*	Tocklai Experimental Station, Jorthat,Assam
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
Metar hizium 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		
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

Mycelial growth was assessed both on solid media and liquid media for various experimental purposes.

3.2.2.1. Solid media

To assess the growth of *S. repens* in solid media, the fungus was first grown in petridishes, each containing 20ml of PDA and incubated for 8 days at room temperature. Agar Block (6mm dia) containing the mycelia was cut with a sterile cork borer from advancing zone of mycelial mat and transferred to each petridish containing 20ml of sterilized solid media. The different solid media used for assessment of growth were prepared following the method of Dhingra and Sinclair (1985).

- Potato dextrose agar (PDA)

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

- Patato sucrose agar (PSA)

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

- Richards agar (RA)

KNO_3	–	1.00gn
KH_2PO_4	–	0.50g
$MgSO_4, 7H_2O$	–	0.25gn
Sucrose	–	3.00g
Agar	–	2.00g
Distilled water	–	100ml

- Carrot Juice Agar (CJA)

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

- Elliot's Agar (EA)

KH_2PO_4	–	.136g
$MgSO_4, 7H_2O$	–	0.050g
$Na_2 CO_3$	–	.106g
Dextrose	–	.500g
Asparagine	–	.10g
Agar	–	1.5g.
Water	–	100ml.

- Czapek Dox agar (CDA)

$NaNO_3$	-	0.20gn
K_2HPO_4	-	0.10g
Kcl	-	0.05g
$FeSo_4. 7H_2O$	-	0.05g
Sucrose	-	30.00g
Agar	-	2.00g
Distilled water	-	100ml

- Yeast extract dextrose agar (YDA)

Yeast extract	–	0.75g
Dextrose	–	2.0g
Agar	–	1.5g
Distilled water	–	100ml

All the petriplates were then incubated at 30^0C and colony diameter and sporulation behaviour of the fungi were studied at 3 days interval for 12 days.

3.2.2.2. Liquid media

The fungus was first allowed to grow in petridishes containing 20ml of PDA and were incubated at 30^0C for 7 days. From the advancing zone, the mycelial block (6mm) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50 ml of sterilized liquid midia. For assessment of growth in liquid media Richard's medium was used in all experiments with desired modification. And in all cases mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60^0C for 96h, cooled in dessicator and weighed.

3.3. Inoculation technique

3.3.1. Preparation of Inoculum

(a) Pathogen

The inoculum of *S. repens* was prepared in sand maize meal media and in tea root pieces. Sand maize meal (SMM) media was prepared following the method described by Biswas and Sen (2000). SMM medium (150 gm) in autoclavable plastic bag (maize meal: washed sterilized soil : water = 1:9:1.5) was sterilized at 120 lb for 20 min. Inoculum in tea root pieces was prepared following the method of Hazarika *et. al.* (2000). First root and rootlets were cut into small pieces (1½"). Root pieces (6-7) were taken in 250 ml flask and pieces were dipped into water for 2h and autoclaved in the same way. On the next day water was decanted. All media were inoculated with *S. repens.*

(b) Biocontrol agents

Inoculum of *Trichoderma* species prepared in several media- wheat bran media (wheat-bran : sand-1:1,and 25ml of water for 150g of inoculum in each poly packet), saw dust media (saw dust and water), tea waste media (tea waste and water).Media were autoclaved and inoculated as above.

3.3.2. Inoculation of healthy tea plants

3.3.2.1. Pot grown plants

Pathogen

Two year old tea plants were planted in earthen pots (12") containing 5 kg soil and allowed to be established. Regular watering was done for two weeks and then 100 g of *S. repens* inoculum was added carefully in the rhizosphere of each plant. Disease assessment was done after 10, 20 and 30 days of inoculation.

Biocontrol agents

Inoculation with *Trichoderma* sp. was also done as described above, but atleast 10 days prior to inoculation with *S repens*. Experiments were designed considering different treatments as as follows : a) pathogen(*S repens*) only b) *T. harzianum*, c) *T. viride*, d) *S repens*+ *T. harzianum* e) *S repens* + *T. viride*, f) healthy plants. Two year old tea plants (Teen Ali-17/1/54) were taken in this experiment. For each treatment three replications were taken.

3.3.2.2. Field grown plants

Inoculation and different treatments of field grown plants was same as described for potted plants, except that, in this case, 300 g inoculum was added in each pit, After inoculation plants watered and maintained. Disease assessment was done after 15,30 and 45 days of inoculation.

3.4. Disease assessment

Disease intensity of violet root rot was assessed on the basis of above ground and underground symptoms together on a scale of 1-6: 0-no disease; 1-plants look sick and root surface starts roughening in patches; 2-most of the leaves withered or looking yellow,light black patches with rough surface appear on root; 3-defoliation starts with random yellowing, roots inky black with random patches; 4-random defoliation, upto 70% roots become black; 5-total defoliation,70-85% blackening of roots and 6-total defoliation with drying of shoots, 85-100% blackening and drying of roots.

3.5. Soluble protein

3.5.1. Extraction

3.5.1.1. Mycelia

Mycelial protein was prepared following the method of Chakraborty and Saha (1994). Initially the inoculum (6mm disc containing mycelium) was transferred to 250 ml Ehrlenmeyer flask each containing 50ml of sterilized liquid Richard's medium and incubated for 7 days at 30 $\pm 1^0$C. For extraction of soluble protein, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (25g fresh wt.), were homogenized with 0.05 M sodium phosphate buffer (7.2) supplemented with 10mM sodium metabisulphite and 0.05mM magnesium chloride in mortar with pestle in the presence of sea sand. Cell homogenates were kept overnight at 4^0C and then centrifuged (10000rpm) for 15 min. at 4^0C. This was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. Different fraction of mycelial protein, 0-20%, 20-40%, 40- 60 , 60-80% and 80-100% was also collected following the method Jayaraman (1996). After this period the mixture was centrifuged (10000rpm) for 15 min at 4^0C, the precipitate was dissolved in 5ml 0.05 M sodium phosphate buffer (pH=7.2). The preparation was dialysed for 72h. through cellulose tubing (Sigma chemical Co.USA) against 1L of 0.005 M sodium phosphate (pH=7.2) with ten changes. Then the dialysed material was stored at -20^0C and used as antigen for the preparation of antiserum and other experiments.

3.5.1.2. Root protein

Roots collected from the experimental garden were washed thoroughly and cut into small pieces. Roots were then weighed and crushed in mortar with pestle using sample buffer (1.M tris (pH6.8) 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 10000 rpm for 30min at 4^0C. The supernatent was immediately used for SDS-PAGE analysis.

3.5.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et. at.* (1951). To 1ml of protein sample 5ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml of 2% sodium potassium tartarate added to 50ml of 2% Na_2CO_3 in 0.1(N) NaOH)

and incubated at room temperature; and then 0.5ml of Folin Ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for colour development following which absorbance was measured at 720nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.6.3. SDS-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 solutions were prepared :

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

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly deaminated 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 Dodecyl Sulphate (SDS)

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

C. Tris buffer

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

(b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer (pH adjusted to 6.8 with concentrated 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 running buffer consists of 25mM Tris base, 250mM glycine (pH-8.3) and 0.1% SDS. A 1xSolution 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 contains 50mM Tris HCl (pH-6.8), 10mM β mercaptoethanol, 2% SDS, 0.1% bormophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5 ml of β mercaptoethanol (14. 4M), 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8ml of distilled water.

3.6.2. Preparation of Gel

Slab gels (mini, plate size 8cm x 10cm) were prepared for the analysis of protein patterns by SDS-PAGE. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5mm thick spacers were placed between the glass plates at the three sides, and were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing stock solutions in the following order and poured leaving sufficient space of the stacking gel (Comb+1cm). Composition of solutions were as follows :

	Solutions	Amount
1.	Distilled water	2.95 ml
2.	30% Acrylamide mix	2.50 ml
3.	1.5 M Tris (pH 8.8)	1.90 ml
4.	10% SDS	0.075 ml
5.	10% APS	0.075 ml
6.	TEMED	0.003 ml

12% resolving gel was prepared mixing the stock solutions in following order.

	Solutions	Amount
1.	Distilled water	2.45 ml
2.	30% Acrylamide mix	3.00 ml
3.	1.5 M Tris (pH 8.8)	1.9 ml
4.	10% SDS	0.09 ml
5.	10% APS	0.07 ml
6.	TEMED	0.003ml

The gel was immediately overlayered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel, isobutanol was poured off and washed with water to remove any unploymerized acrylamide. Then stacking gel solution (5%) was prepared by mixing the stock solutions in the following proportion. Composition of 5% stacking gel was as follows:

Solutions		Amount
1.	Distilled water	2.1 ml
2.	30% acrylamide mix	0.5 ml
3.	1M Tris (pH 6.8)	0.38 ml
4.	10% SDS	0.03 ml
5.	10% APS	0.03 ml
6.	TEMED	0.003 ml

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. The gel was kept for 30 minutes. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then mounted in electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom was removed very carefully with a bent syringe.

3.6.3 Sample preparation

Sample (34µl) was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 3min, the denature the protein sample. The samples were immediately loaded in a pre-determined

order into the bottom of the wells with a 100 T micropippet syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low (Phosphorylase b-97,400; Bovine serum Albumin-68,000 Ovalbumin-43,000; Carbonic Anhydrase 29,000 Soybean tripsin 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 of 2 hrs 15min. until the dye front reached the bottom of the gel.

3.6.5. Fixing and staining

After 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 R 250) in 45ml methanol. After the stain was completely dissolved, 45ml of 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 37^0C with constant shaking at a very low speed. After staining, the gel was finally destined in destainer solution containing methanol, water and acetic acid (4.5:4.5:1) at 40^0C with constant shaking until back ground became clear.

3.7. Preparation of antigen

3.7.1. Fungal antigen

3.7.1.1. Mycelia

Mycelial antigen was prepared following the method of Chakraborty and Purkayastha (1983) described earlier in soluble protein extraction procedure.

3.7.1.2. Cell wall

Isolation of cell wall from *S. repens* was done following the method of Keen & Legrand (1980). Mycelial mat of 9 day old culture was collected on filter paper using Buchner funnel and 40g of fresh packed cells were ground with water (4ml/g) for 1min in a high speed blender The resulting slurry was then disrupted in homogenizer for 1 min at

5^0C. The mixture was centrifuged for 1 min at 2000 rpm, the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10ml/g) and pelleted by several centrifugation until the supernatant fluids were visually clear. Finally, the isolated cell walls were dissolved in 0.05M phosphate buffer (pH) and kept at -20^0C until further requirement. This cell wall antigen was used for immunization.

3.6.1.3. Spore antigen

Twelve day old cultures of *S. repens* growing in Richard's broth were shaken well and culture filtrate strained with muslin cloth. Culture filtrates were then centrifuged at 5,000 r.p.m. (4^0C) for 5min. Spores were washed 3 times in PBS (0.05M, pH 7.0) and stored inchilled at 4^0C for 30min. and then spores were crushed in mortar with pestle at 4^0C and mixed again in cyclomixer, and after centrifugation at 10,000 rpm (4^0C) for 10 min supernatant was used as spore antigen for immunization.

3.7.1.4. Culture filtrate

Twelve days old culture filtrate free from spore was saturated with 100% ammonium sulphate under constant stirring and kept overnight at 4^0C. Further steps of culture filtrate protein preparation was as in case of mycelia.

3.7.2. Root antigen

Antigens from healthy and infected roots were prepared following the method of Chakraborty and Purkayastha (1983. with modification. Roots were collected from the experimental pots and field and thoroughly washed in water and cut into pieces. Root pieces were weighed and homogenised in grinder with 0.05 M sodium phosphate buffer containing 2mM PVP-10 (Soluble), 10mM sodium metabisulphite and 0.5 mM magnesium chloride. Insoluble PVPP was also added during homogenization. The homogenate was then kept at 4^0C overnight. Following this, the slurry was once again crushed in mortar with pestle and centrifuged at 10,000 r.p.m. at 4^0C and supernatant was used as antigen.

3.7.3. Soil antigen

Soil antigen for microplate trapping and blotting were prepared following the method of Walsh *et. al.* (1996). 1gm of soil was crushed in 2 ml of 0.05µl sodium carbonate-bicarbonate buffer (pH 9.6) in mortar with pestle and kept overnight at 4^0C. Next day it

was centrifuged at 10.000 rpm for 10min. Supernatant was used as antigen for experimental purposes.

3.8. Cellwall characterization

3.8.1. Preparation of cell wall extract

To get mycelial wall extract from isolated cell wall, method described by Brown & Kimmins (1977) has been followed. Isolated cell walls (2g) were suspended in 80ml ice cold 0.1 (N) NaOH by blending in a chilled mixer cup at full speed for 30 seconds. The suspension was stirred in ice bath for 15h and then centrifuged at 8000g for 10min. After centrifugation, the precipitate was washed with 50ml ice cold water and the supernatant was neutralized to 7.0 by adding 1(N) HCl slowly with continued stirring at 0^0C. The neutralized supernatant was finally dialysed against distilled water for 48h. with 12 hourly changes and concentrated with polyethylene glycol 6000 (PEG-6000), which was then used as crude mycelial wall extract for SDS-PAGE analysis followed by Western blotting.

3.8.2. Protein estimation

Protein content of cell wall extracted from *S. repens* mycelia was estimated following the method as describe above in 3.5.2.

3.8.3. Carbohydrate estimation

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

3.8.4. SDS-PAGE analysis

All the steps for SDS-PAGE analysis were same as described above , only fixing and staining were done in different ways. After electrophoresis gel containing mycelial and cell was protein with replica one was fixed in solution 1 (25% isopropanol in 10% acetic acid) or in fixer solution II (40% ethanol in aqueous 5% acetic acid) for protein and

carbohydrate staining overnight. Gels from fixer soulution 1 were stained with commassie blue R250 and then destained as describe earlier. Gels from fixer solution II were stained with periodic acid-Schiffs (PAS) reagent as described by Segrest and Jackson (1972) with modification. Details of PAS staining solution and the procedure was as follows :

At first five following solution A-E were prepared.

Solution A : Anhydrous sodium acetate (0.84gm and hydroxylamine hydrochloride (10g) were dissolved in 90 ml distilled water. Glacial acetic acid (0.54 ml) was added to it final volume was adjusted to 100ml with distilled water.

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

Solution C : 1.5g basic Fuschin was dissolved in 500ml boiling distilled water, filtered at 55^{0}C, cooled to 40^{0}C, 25ml 2N HCl was added and finally 3.75g $Na_2S_2O_5$ (Sodium metabisulphite) was added, agitated rapidly and allowed to stand stoppered in refrigerator for 6h, 1.2g charcoal was mixed to it vigorously for 1min. filtered rapidly and stored in refrigerator.

Solution D (Prepared before use) : 10% (w/v) sodium metabi sulphite (5ml) and 2N HCl (5ml) was dissolved in 90ml distilled water before use.

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

Procedure : Gels were soaked in solution 'A' for 15min then washed in running tap water. Next the gels were soaked in solution B for 15 min, washed in running tap water for 10 min. and then transferred to solution 'C' (diluted 1:1 with distilled water just before use) for washed three times for 1h. each time in solution 'E'.

3.8.5. Binding of FITC labelld concanavalin - A

Binding of fluorescent labeled concanavalin A to mycelia, cell wall as well as spore was done by the method as described by Keen and Legrand (1980). Initially mycelia and spores were incubated for 20 min. in 0.85% Nacl in 0.01M potassium phosphate buffer,

pH 7.4 containing 1mg/ ml. fluorescein isothiocyanate (FITC) labeled concanavalinA (ConA Sigma Chemicals). The hyphae or the spores were then washed thrice with saline solution by repeated low speed centrifugation and resuspension. For control sets these were incubated with lectin supplemented with 0.25M α-methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluorescence optics (BP450-490 exciting filter, RKP-520 Beam splitting mirror, 515 suppression filter) Photographs were taken by Leica WILD MPS 32 camera 800 ASA film.

3.9. Agglutination response of spores

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

3.9.1. Preparation of spore suspension

Agglutination tests were done with ungerminated spores. For this, fungus was allowed to grow in liquid RM medium for 12 days at 28 ± 2°C . Spores were collected from the above grown culture as described before (3.6.1.3) in PBS to a concentration of approximately $5x10^6$/ml. The spore suspensions were used immediately after preparation.

3.9.2. Agglutination test

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

3.10. Serology

3.10.1. Rabbits and their maintenance

New Zealand white male rabbits were used to raise antisera separately against initially, the body weights were recorded and were observed for at least one week inside the cages

before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetables etc. twice a day. After each bleeding they were given saline water for 3 consecutive days and cages were cleaned everyday in the morning for better hygenic conditions.

3.10.2. Immunization

Before immunization, normal sera were collected from each rabbit. Separate rabbits were intramascularly injected once a week at 7 days interval with 1ml antigen mixed with 1ml of Freund's complete adjuvant (Difco, USA) for first two injections followed by incomplete adjuvant upto 12 weeks.

3.10.3. Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then after every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board fixed at a angle of 60^0. The neck of the rabbit was held tight in 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 from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 to 10ml of blood samples were collected in sterile graduated glass tube.

After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were kept as such at 37^0C for 1hr for clotting, following which, the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000g for 10min at room temperature) and distributed in 1ml vials and stored at- 20^0C.

3.10.4. Purification of IgG

3.10.4.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2ml) was first diluted with two volume of distilled water and an equal volume of 4M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22^0C. The precipitate

thus formed was collected by centrifugation at 10,000g at 22^0C for 1hr. Then the precipitate was dissolved in 2ml of 0.02M sodium phosphate buffer, pH 8.0.

3.10.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02M phosphate buffer, (pH 8.0) and was transferred to a column (2.6cm in diameter and 30cm high) and allowed to settle for 2hr. After the column material had settled, 25ml of buffer (0.02M sodium phosphate, pH8.0) washing was given to the column material.

3.10.4.3. Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02M to 0.3M. The initial elution buffer(1) was 0.02M sodium phosphate buffer (pH 8) whereas final elution buffer(2) was 0.3M sodium phosphate buffer(pH 8.0) . The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was soacked into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV VisSpectrophotometer (DIGISPEC-200GL) .

3.10.4.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1996). Absorbance was taken at 280nm and 260nm and then concentration of IgG was calculated by the following formula : Protein concentration (mg/ml) = 1.55XO.D. 280nm – 0.76X O.D. 260nm.

3.11. Immunodiffusion tests

3.11.1. Preparation of agarose slides

Glass slides (5cm x 5cm) were degreased in 90% (v/v) ethanol, ethanol: diethylether (1:1) and then dried in hot air oven. After drying, plates were sterilized. Agarose gel was

prepared in Tris barbiturate buffer, pH 8.6 at 90^0C, 0.9% agarose (Sigma, USA) was added into the buffer and placed on a water bath and stirred till the agarose solution became clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added. For gel preparation, 10ml of molten agarose was added per slide, after pouring, it was kept for solidification and then wells were cut with a sterilized cork borer (6mm dia).

3.11.2. Diffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (100µl/well) were pipetted directly into the appropriate wells and diffusion allowed to continue in moist chamber for 27hr at 25^0C.

3.11.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water for 2h and then aqueous NaCl solution (0.9% NaCl + 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. Then slides were stained with coomassie blue (R 250) for 10 min at room temperature. After staining, slides were destained with destain solution (methanol : water : acetic acid (45:45:10) with changes until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3h at 50^0C.

3.12. Enzyme linked immunosorbent assay (ELISA)

The following buffers were prepared following the method as described by Chakraborty *et. al.* (1995).

1. Antigen coating buffer – Carbonate Bicarbonate buffer(0.05M, pH 9.6)

Stocks

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

B. Sodium bicarbonate – 4.2g in 1000ml 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 (0.15M PBS pH - 7.2).

Stocks

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

B. Disodium hydrogen phosphate – 21.2940 in 1000ml dist water.

280ml of stock A was mixed with 720 ml of stock B and the pH was adjusted 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 0.15M PBS, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

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

0.05M Tris, 0.135M NaCl, 0.0027M 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 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% Polyvinylpyrrolidone, 10,000 (PVPP 10,000) and 0.03% Sodium azide (NaN_2) was added.

6. p-Nitrophenyl phosphate (Himedia) 1mg/ml in substrate buffer (1.0% [w/v] diethanolamine, 3mM NaN_3 ph 9.8).

3.12.1. Direct antigen coated (DAC) ELISA

ELISA was performed following the method as described by Chakraborty *et. al.* (1995). Plant and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl/well) in 96 welled ELISA plate (Costar EIA/RIA, strip plate USA) arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25^0C for 4 hrs. Then the plate was washed 4 times under running tap water and twice with PBS Tween and each time shaken to dry Subsequently, 200µl of blocking reagent was added and incubated at 25^0C for 1hr. After incubation, the plate was washed as mentioned earlier. Puriified IgG was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4^0C overnight. After a further washing, antirabbit IgG goat antiserum labelled with Alkaline Phosphatase (diluted 10,000 times in PBS) was added to each well (200µl/well) and incubated at 37^0C for 2hrs. The plate was washed, dried and loaded with 200µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50µl/well of 3N NaOH solution and

the absorbance was determined in an ELISA Reader (Labsystem, Multiskan) at 405mm. Absorbance values in wells not coated with antigens were considered as blanks.

3.12.2. Double antibody sandwich 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 enzyme was thoroughly mixed with 2mg of IgG. The total volume was made upto 5ml with PBS. It was kept at room temperature for 30min. with occasional shaking. Following this, 0.2% gluteraldehyde was added to the mixture and was again incubated for 2h with gentle stirring. Finally it was dialysed 3 times against 500ml ½ strength of PBS. After dialysis, bovin serum albumin (about 10mg/ml) + 0.02% NaN_3 was added and stored at 4^0C until required.

DAS ELISA was performed following the method of Brill *et. al.* (1994). Antisera of *S. repens* was diluted in coating buffer and loaded (200µl/well) in each well of ELISA plate. The plate was incubated for 4h at 37^0C and washed 4 times by flooding the wells with PBS-Tween, plate was shaken to dry. Then 200µl of blocking agent (1% BSA and 3mM NaN_3) was added to each well to overcome the nonspecific binding and plate was incubated at 25^0C for 1h. After blocking, plate was washed as mentioned earlier. Then test samples were added to empty well (200µl/well) and incubated overnight at 4^0C. On the next day plate was washed as before and alkaline phosphatase tagged IgG diluted in PBS, pH 7.2 was added to each well and incubated for 4h at 25^0C. Then plate was washed and 200µl pNPP substrate (1mg/ml) was added to each well and incubated for 90min. at 25^0C in dark. Colour development was stopped by adding 50µl/well of 3N NaOH solution. Absorbance values were recorded at 405 nm in an ELISA reader (Labsystems, Multiskan).

3.12.3. Competition ELISA

Competition ELISA was carried out in 96 well ELISA plate (Labsystems Finland) following the method as described by Lyons &White (1992). 3 sets were prepared each with 3 replicas for *S. repens, Trichoderma harzianum* and *T. viride*. All wells of one set was loaded with 100µl *S. repens* antigen, another with *Trichoderma harzianum* antigens and the 3rd set with *T. viride* antigen. The antigens were diluted in PBS before loading. Following incubation at 4^0C overnight, the plates were washed four times in running tap

water and twice in PBS-Tween, and were dried. Blocking of non-specific binding was achieved with 5% BSA in PBS, 200µl/well for 1h. The plates were washed and dried as before and stored at 4^0C until required. Test antigens were prepared from tea rhizosphere soils inoculated with *S. repens, T. harzianum* and *T. viride*, either simply or in different combination. These samples were added (75µl/well) to wells of 3 ELISA plate sets. To each of these wells, 75µl of *S. repens* or *T. harzianum* or *T. virde* IgG (40µg/ml) was added. The plates were incubated at 37^0C for 1h on a 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 antirabbit IgG alkaline phosphatase conjugated antiserum was added to all wells of the plates. Following 30min. incubation at 37^0C plates were washed and 100µl of p-Nitrophenyl phosphate substrate (1mg/ml) was added to each well. After a further 40 min. incubation in dark absorbance values were read at 405nm in an ELISA reader (Labsystems, Multiskan).

3.13. Immunoblotting

3.13.1. Dot-Blot

Dot-blot was performed following the method suggested by Lange *et. al.* (1989). 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 0.05% Tween-20 for washing

(iii) Blocking buffer 10% Casein hydrolysate in 0.05M Tris, 0.5 NaCl, 0.5% Tween-20, pH-10.3

Nitrocellulose membrane (NCM; millipore, H5SMO 5255, 7cmx10cm, Pore size-0.45µm, Millipore Corporation, Bedford) was first cut carefully into the required size and placed inside the template. 2µl of coating buffer (carbonate -bicarbonate buffer) was loaded in each well of the template over the NCM and kept for 25 mins to dry. Following this 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 hrs at room temperature. Template was removed and blocking of the NCM

was done with 10% non-fat dry milk (casein) prepared in TBS for 30 min. Polyclonal antibody (IgG) of *S. repens* (1:500) was added directly in the blocking solution and further incubated at 4^0C for overnight. The membrane was then washed several times in TBS-Tween-20 [(pH-7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline Phosphatase Conjugate (1:7500) for 2h a 37^0C. This was followed by washing for 25 min. in TBS-Tween. Substrate [1 tablet each of Tris buffer and Fast Red (Sigma chemicals)or NBT/BCIP tablet (Sigma) dissolved in 10 ml double distilled water] was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water. Colour development was categorized on the intensity of the dots.

3.13.2. Western blotting

Western blotting has been performed following the method of Sambrook *et. al.* (1989).

Stock Solutions

[A] All the solutions for SDS-Gel preparation for western blotting were as mentioned earlier in SDS-Gel electrophoresis

[B] Transfer buffer (Towbin buffer)

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

Tris – 3.03g.

Glycine – 14.4 g.

200ml Methanol (adjusted to 1 lit. with dist. water)

[C] Phosphate buffer saline (0.15M) pH 7.2) PBS was made as mentioned previously.

[D] Blocking solution :

Casein hydrolysate – 5% in PBS

Sodium Azide – 0.02%

Tween -20 – 0.02%

[E] Antibody dilution : PBS = 14 ml; Blocking solution = 6 ml.

[F] Washing buffer (50mM Tris Cl, 150mM NaCl) pH-7.5.

Tris – 6.07 gm

NaCl – 8.78 gm

Distilled water upto 1 lit. made.

[G] Alkaline phosphatase buffer (100mM Tris -HCl, 100mM NaCl, 5mM $MgCl_2$ pH-9.5)

Tris – 12.14gm pH adjusted

NaCl – 5.84gm

$MgCl_2$ – 1.015gm

Double distilled water made upto 1 lit.

[H] Enzyme

Alkaline phosphatase tagged with antirabbit goat IgG alkaline phosphatase buffer enzyme diluted in (1:10000).

[I] Substrate :

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

2.5mg BCIP in 50µl of 100%, N,N, dimethyl formamide

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP

10ml alkaline phosphatase buffer

[J] Stop solution : (0.5M EDTA in PBS) pH 8.0.

EDTA sodium salt - 0.0372gm in 200µl water PBS - 50 ml.

Procedure

Extraction of protein : Protein extraction was done as described earlier.

3.13.2.1. SDS-PAGE

SDS-PAGE was carried out as mentioned earlier.

3.13.2.2. Transfer process

Following the gel run, it was transferred to Towbin buffer and equilibrated for 1h. The filter paper (Bio-RAD, 2.5mm thickness) and nitrocellulose membrane (BIO-RAD, 0.45µm) were cut as same size of the gel and soaked in Towbin buffer for 20min. 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 and prewetted membrane was placed over the filter paper. Bubbles between the filter paper and membrane were rolled out with a glass rod. Gel was carefully placed on the membrane and bubbles were removed as above and finally another presoaked

filter paper was placed on the top. The cathode was placed on the sandwich and pressed. The unit was run for 45min at 14 volt. After the run the membrane was dried for 1h and proceeded for immunological probing.

3.13.2.3. Immunoprobing

Blocking was done by 5% non fat dried milk in a heat sealable plastic bag and kept for 90min. with occasional shaking. Subsequently the membrane was put in antibody solution (diluted in PBS and blocking solution, 1:40) and incubated in plastic bag at 4^0C overnight. All the processes were done with occasional shaking. Next day the membrane was washed thrice in 250ml PBS. Final washing was done in Tris-Cl (50mM Tris, 150ml NaCl pH=7.5) to remove azide and phosphate from the filter before enzyme coupled reactions. Enzyme was added (1:1000 in alkaline phosphatase buffer) and kept 1h at room temperature. After enzyme incubation, membrane was washed thrice again in Tris-buffer saline, and 10ml substrate was added. The reaction was monitored carefully and when bands were observed up to the desired intensity the filter was transferred to a tray of stop solution.'

3.14. Fluorescence antibody staining and microscopy

3.14.1. Mycelia

Fungal mycelia were grown in liquid Richard's medium as described earlier. After 4 days of inoculation young mycelia were taken out from the flask and kept in eppendrof tube. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted (3:10) PBS, (pH 7.2) and incubated for 1h. at room temperature. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45min at room temperature. After incubation, mycelia were washed thrice in PBS (ph 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. Then slides were observed and photographed under both phase-contrast and UV fluorescence condition using Leica Leitz Biomed microscope with flourescence optics equipped with ultra violet (UV) filter set 1-3.

3.14.2. Spores

Fungal spores were collected from 12 days old culture and a suspension of this was prepared with PBS, pH-7.2. Spore suspensions were taken in micro-centrifuge tubes and

centrifuged at 3000g for 10min and the PBS supernatant was discarded. Then 60µl of IgG diluted in PBS (1:1) was added to the spores and incubated for 90 min at room temperature. After incubation, tubes were centrifuged at 3000g. 10min. and supernatant was discarded. The spores were rewashed 3 times with PBS-Tween (pH 7.2) by centrifugation as before and 40µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and incubated in dark for 1h. After the dark incubation excess FITC antisera was removed by repeated washing with PBS Tween pH 7.2 and the spores were mounted on 10% glycerol jelly and observed under Leica microscope, equipped with 1-3 UV-fluorescence filter. Photographs were taken as described before.

3.14.3.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 in PBS (1:40) and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antiserum specific of rabbit globulins and 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. Flourescence of the root sections were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with UV-filter set 1-3. Tissue section were photographed under both phase contrast and UV-fluorescent conditions for comparison of treatment.

3.15. Immunocytochemical staining

The combinatin of immunochemical and histochemical techniques is feasible for the development of a staining procedure capable of locating specific fungi in host tissue. Subsequent to location of CRA in tea root tissues by immunocytochemical studies, and detection of *S. repens* by ELISA and immunoblotting, immuonocytochemical staining was performed to determine the specific location of *S.repens* in tea root tissues. For this, the method of Young and Andrew (1990) was followed. In this process the root sectinos of infected roots were made and incubated in 1% BSA solution for 15 minutes to prevent nonspecific binding of antibodies to root tissue. The root sections were rinsed (30s) three times with washing solution. Then sections were incubated for 1hr.at 37^0C on a rotary

shaker in *S. repens* PAb at a dilutin of 1:100. After incubation the root segments were washed as above, then incubated for 1hr. at 37^0C on a rotary shaker in goat antirabbit IgG (1:5000) dilution in direct ESLISA buffer containing 0.1% BSA) conjugated with alkaline phosphatase (Sigma). After incubation, the roots were washed as before, incubated in naphthol-AS-phosphate plus fast blue BB substrate solution for 40 minutes at 37^0C in the dark. Substrate solution consisted of 0.15 gm naphthol-AS-phosphate (Sigma) dissolved in 2.5ml of N-N-dimethyl formamide (Sigma), which was added to 500ml of Tris buffer (17g of Tris in 500ml distilled water, pH 9.1), this solution considered as stock solution. The staining solution, prepared immdiately before use, consisted of 1mg of fast blue (Himedia) and 5µl of 0.1M $MgCl_2$ added per ml of stock solution and filtered through Whatman No.1 filter paper. After washing the sections in PBS, were mounted in glycerol jelly and observed under bright field of the microscope (Leica Leitz Biomed) and photographs were taken in bright field.

Experimental

4.1. Violet root rot incidence

Violet root rot disease of tea caused by *Sphaerostilbe repens* is often observed in bushes subjected to water logging or poor soil aeration. Roots with enlarged lenticels are main targets of infection. Bushes at first turn yellow and leaves drop. The root tips turn inky black and the barks peel away. Just below the bark violet coloured rhizomorphs the fungus are observed (Plate 3, figs. B-D). In extreme cases roots give out a putrid odour. On peeling off the bark it is observed that the pathogen proliferates in between the bark and healthy portions without any deeper penetration. Characteristic dark ramification was seen with embedding of rhizomorphs.

A survey of the Tea estates in the Terai and Dooars revealed the occurrence of disease in water logged sections with poor drainage. It was common in certain sections of Bijoynagar Tea estate, Matigara Tea estate, Chandmani Tea estate, Bentaguri Tea estate and Hansqua Tea estate. In some cases it was also observed in nurseries (Plate3, fig. A).

4.2. Cultural Conditions affecting growth of *S. repens*

The growth of *S. repens* and its sporulation behaviour *in vitro* was studied.*S. repens* generally exhibited white fluffy growth. In solid media within a few days submerged rhizomorphs started forming. These are at first white coloured, gradually changing to orange and later to brown. Rhizomorphs are clearly visible from the media and on the surface these project out as small orange projections (Plate 4, fig. A-F). In liquid media rhizomorphs aggregates into small groups (Plate 4, fig. D & E).

Large number of hyaline, single celled conidia were visible with range in size 6.9µm x 4.8µm, 3.8µ x 2.1 µm and 2.2 x 1.1 µm in dia metre. Smaller spores were rod shaped, while larger ones ellipsoid (Plate 5).

4.2.1. Media

Growth of *S. repens* was studied in seven different solid media i.e. potato dextrose agar (PDA), potato sucrose agar (PSA), carrot juice agar (CJA), Richard's agar (RA) Elliot agar (EA), Czapek-Dox agar (CDA)and Yeast dextrose agar (YDA). In each medium mycelial growth rate was recorded at 3, 6, 9, and 12 days of incubation at

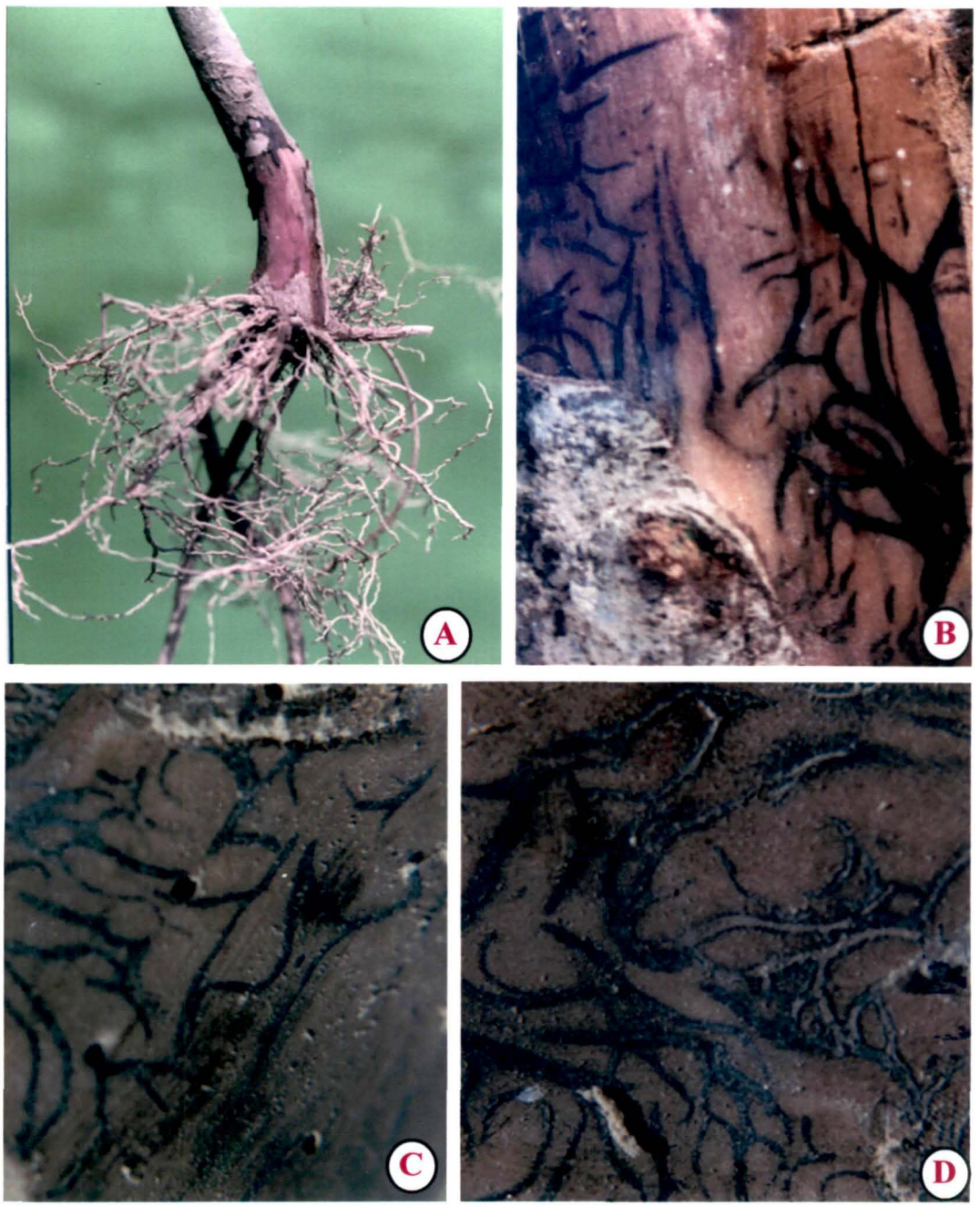


Plate 3 (Figs A-D) : Field grown tea plants infected with *Sphaerostilbe repens.* (A) Tea plants (24 months old) showing symptoms on collar region; (B-D) 20 year old tea roots showing imprint of rhizomorph beneath the bark tissue.

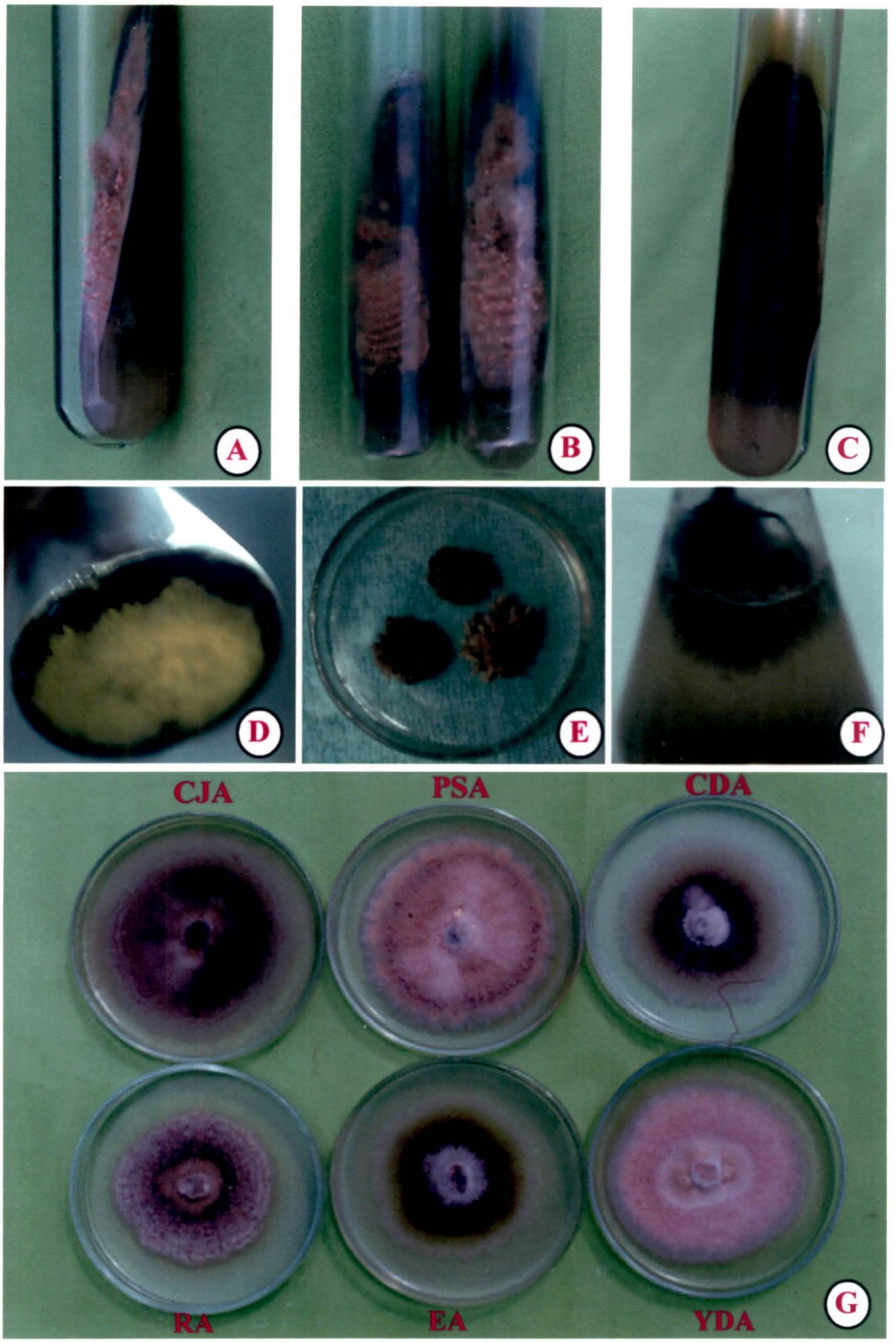


Plate 4 (Figs A-G) : Growth of *Sphaerostilbe repens* (A-C) *S.repens* grown on PDA slants; (D) Growth in potato dextrose broth; (E) Rhizomorphs; (F) Tea roots artificially inoculated with *S.repens;* (G) Growth on different media [CJA- Carrot Juice Agar; PSA- Potato Sucrose Agar; CDA- Czapek Dox Agar; RA- Richards Agar; EA-Elliot's Agar; YDA- Yeast Extract Dextrose Agar]

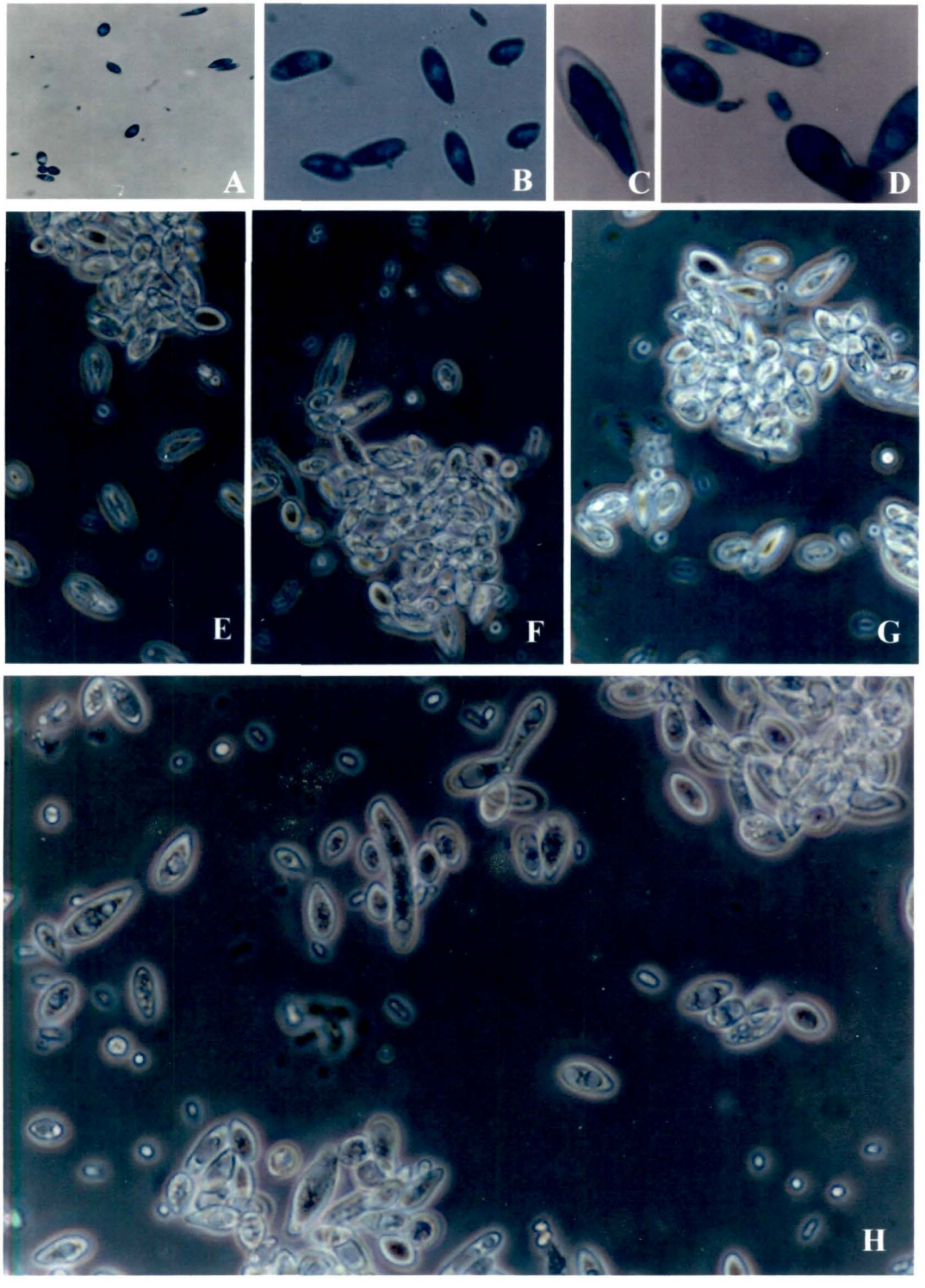


Plate 5 (Figs A-H) : *S.repens* conidia stained with cotton blue observed under bright field (A-D) and phase contrast (E-H) microscope.(A) x 40 ; (B) x 175 ;(C) x 500;(D) x 300; (E & F) x125 (G) x 225 ; (G) (H) x 300.

28 + 2°C. Results revealed that the fungus grew well in all media with the maximum growth recorded in CJA (Table-2, Plate-4, fig. G) EA supported minimum growth. Rhizomorph formation was observed in PDA, PSA and YDA media but not in RM and EA. In CJA and CDA very few rhizomprphs were observed. While in most of the media fluffy growth was seen, in RA, EA and CDA crustaceous growth was observed. Profuse sporulation was observed in YDA, followed by PSA. All other media also supported sporulation to a lesser or greater degree.

Table 2 : Effect of different media on growth of *S. repens*

Media	Diameter of growth[a] (cm)			
	Incubation period (days)			
	3	6	9	12
PDA	2.6 ± 0.22	4.46 ± 0.19	6.23 ± 0.28	8.0 ± 0.19
PSA	2.4 ± 0.12	4.7 ± 0.29	6.7 ± 0.24	8.2 ± 0.23
RA	2.1 ± 0.22	4.1 ± 0.031	5.6 ± 0.26	6.8 ± 0.39
CJA	2.7 ± 0.28	5.0 ± 0.15	7.2 ± 0.18	8.6 ± 0.29
EA	2.3 ± 0.32	4.2 ± 0.29	5.26 ± 0.28	6.0 ± 0.13
CDA	1.9 ± 0.20	4.4 ± 0.26	6.2 ± 0.26	7.2 ± 0.19
YDA	2.3 ± 1.8	4.2 ± 0.19	5.7 ± 0.22	7.2 ± 0.15

[a]Average of three replicates
± Standard error
Temperature 28 ± 2°C

4.2.2. Incubation period

S. repens was grown in Richard's medium for a period of 33 days. Mycelial growth of the fungus was recorded after 3, 6, 9, 12 15, 18, 21, 24, 27 30 and 33 of incubation at 28 ± 2°C. Results presented in Table-3 and Fig.1 revealed that maximum growth occurred after 24 days of incubation after which it declined.

4.2.3. pH

It is known that the pH of the medium influences the growth of microganisms. The utilization of nutrients depends partially upon the pH of the medium. To determine the effect of pH, buffer systems have to be used to stabilize the pH. Initially buffer solutinos with pH values ranging from 4 to 9 (4.0, 5.0, 6, 7.0, 8.0 and 9) were prepared at 0.03M concentration. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. Richard's medium and phosphate buffer were sterilized separately by autoclaving for 15min at 15 lb p.s.i. pressure and equal parts of the buffer solution and medium were mixed before use.

Table 3 : Effect of incubation period on the growth of *S. repens*

Incubation period	Mycelial dry weight (mg)			
	Expt.1	Expt.2	Expt.3	Mean
3	21	29	26	25 ± 1.2
6	60	68	70	66 ± 1.3
9	121	123	144	129 ± 1.4
12	206	197	180	194 ± 1.1
15	236	248	241	242 ± 1.2
18	236	248	241	242 ± 1.4
21	301	307	300	303 ± 1.1
24	433	430	442	435 ± 1.4
27	420	411	415	415 ± 1.1
30	398	407	403	402 ± 1.2
33	363	375	369	369 ± 1.4

± Standard error
Temperature 28 ± 2°C

Each flask (250ml) containing 50ml of the medium was then inoculated with mycelial block (4mm dia) of *S. repens* and incubated for 20 days at 28 ± 2°C. Results (Table-4, Fig. 1) revealed that *S. repens* grew over a wide range of pH (4.0-8.0) and showed optimum growth at pH 5.0 . Mycelial growth increased upto pH 6.5 and then gradually declined.

Tabale 4 : Effect of different pH on mycelial growth of *S. repens*

pH of the medium*	Mycelial dry weight (mg)			
	Expt.1	Expt.2	Expt3	Mean
4	348	351	346	348 ± 0.9
5	372	380	370	374 ± 1.3
5.5	292	295	301	296 ± 1.2
6	289	284	291	288 ± 1.2
6.5	284	286	270	280 ± 1.7
7	265	270	260	264 ± 1.2
8	260	268	264	264 ± 1.2
9	224	220	230	225 ± 1.2

* Richards medium
± Standard error
Temperature 28 ± 2°C
Incubation period 24 days

4.2.4. Carbon source

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 investigation, seven different carbon sources (starch, sucrose, fructose, maltose, Dextrose, Sorbitol and mannitol) were tested for their effect on the growth of *S. repens*. Richard's medium without sugar was used as the basal medium. Data was recorded after 24 days of inoculation. Results (Table 5, Fig. 1) revealed maximum growth in medium containing starch, while no growth was observed in sorbitol and mannitol which were similar to the medium without any carbon source the fungus apparently could not utilize sugar alcohols. Growth in medium containing sucrose; and maltose was also good.

Table 5 : Effect of different carbon sources on growth of *S. repens.*

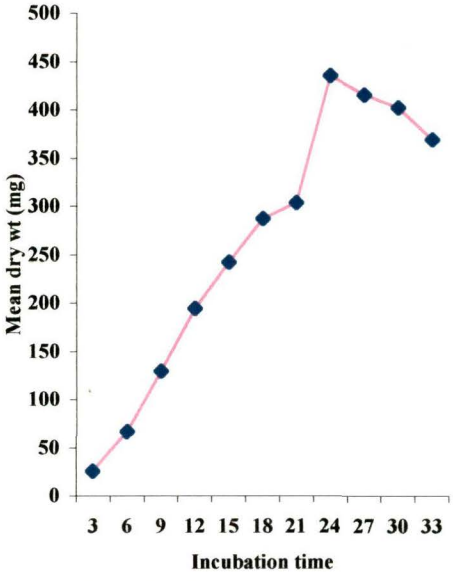
Source of carbon	Dry wt. of mycelia (mg.)			
	Expt.1	Expt.2	Expt.3	Mean
Sourose	360	354	350	354 ± 1.6
Starch	396	402	388	395 ± 1.5
Maltose	330	336	324	330 ± 1.4
Dextrose	300	284	280	288 ± 1.9
Fructose	106	111	109	109 ± 0.9

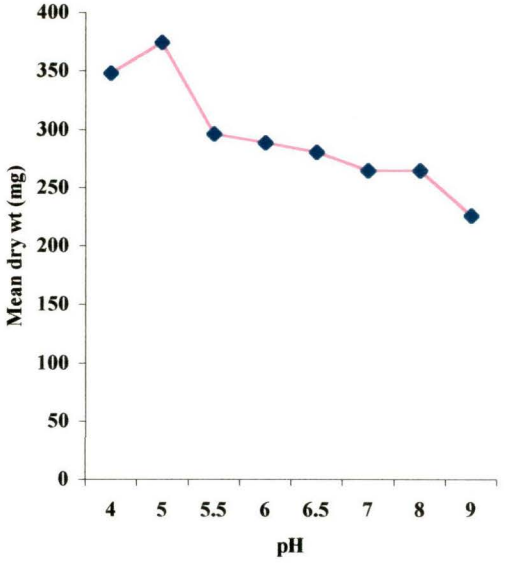
No growth was observed in media with sorbitol or mannitol as carbon source, or with out carbon source
± Standard error
Temperature 28 ± 2°C
Incubation period 24 days

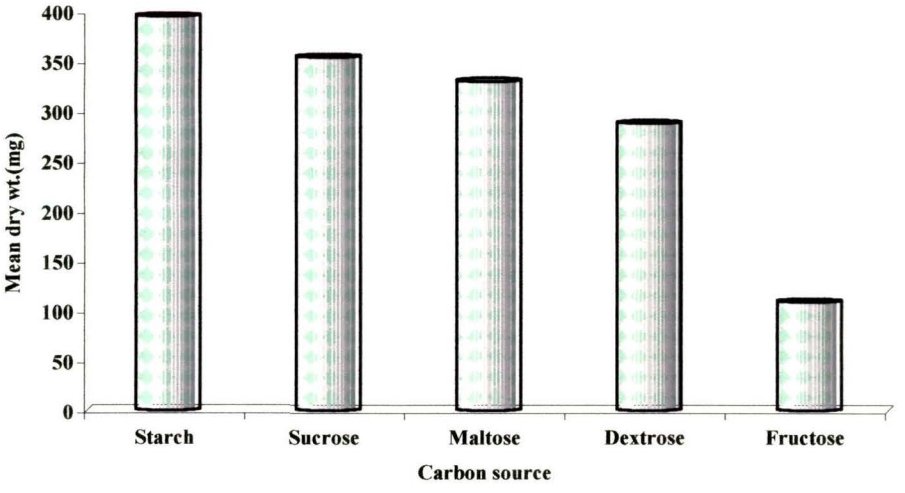
4.2.5. Nitrogen source

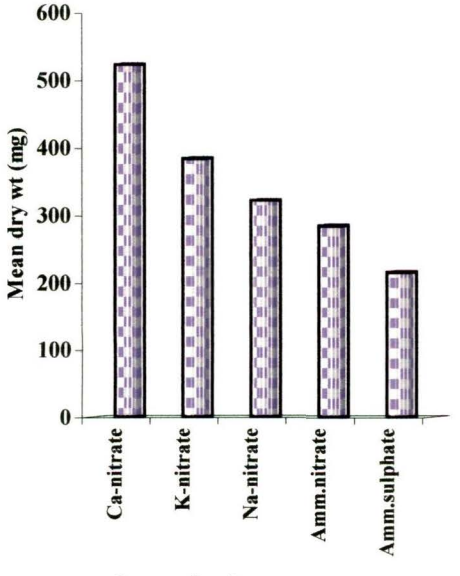
Nitrogen is undoubtedly the most single nutrient necessary for the growth of an organism. The availability of nitrogen depends to a great degree on the form in which it is supplied. Hence, the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effect of inorganic nitrogen sources (potassium nitrate, sodium nitrate, calcium nitrate, ammonium sulphate and ammonium nitrate) as well as complex organic sources (urea, peptone, beef extract, casein hydrolysate and yeast extract) on the

Cultural conditions affecting growth of *Sphaerostilbe repens*









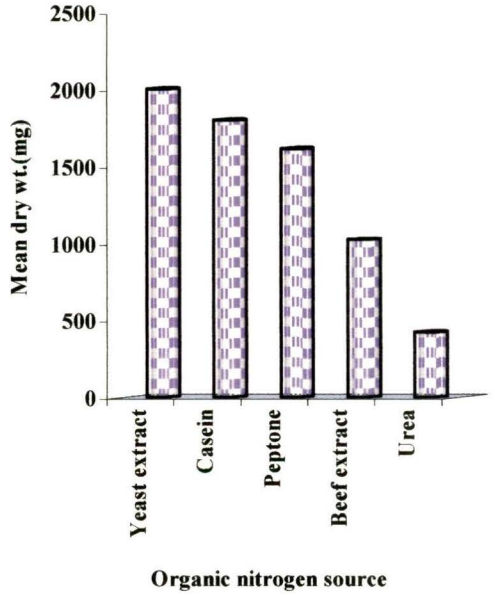


Fig.1

mycelial growth of *S. repens* was tested. A basal medium without any nitrogen sources was considered as control.

Results revealed maximum growth in yeast extract followed by casein hydrolysate (Table 6) Organic sources were better (Fig. 1) for growth than inorganic ones. Among the inorganic nitrogen sources maximum growth occurred in calcium nitrate (Fig.1).Only very less growth was observed in basal medium without nitrogen.

Table 6 : Effect of nitrogen source of growth of *S. repens*

Organic nitrogen Source	Dry wt. of mycelia (mg.)			
	Expt.1	Expt.2	Expt.3	Mean
Urea	426	418	423	422 ± 1.16
Peptone	1608	1611	1624	1614 ± 1.68
Beef extract	1030	1025	1025	1027 ± 0.93
Casein	1800	1796	1808	1801 ± 1.43
Yeast	2002	2009	1998	2003 ± 1.70
Inorganic nitrogen Source				
Potssium nitrate	380	386	382	383 ± 1.01
Sodium nitrate	316	322	325	321 ± 1.50
Calcium nitrate	520	516	531	522 ± 1.97
Ammmonium sulfate	210	215	219	215 ± 1.20
Ammonium nitrate	280	282	291	284 ± 1.40

± Standard error
Temperature 28 ± 2°C
Incubation period 24 days

4.3. Pathogenicity test of *S. repens* on different tea varieties

Pathogen isolated from the naturally infected roots was compared with the sample received from TRA Jorhat, Assam, and was used for further studies involving different tea varieties. Rhizosphere region of two year old potted plants of twenty five different tea varieties were inoculated with *S. repens* as described in materials and methods. Twenty plants of each variety were used. Among the twenty five varieties eleven were Tocklai varieties (Teen Ali-17, TV-9, 18, 22, 23, 25, 26, 27, 28, 29 and 30), eight were from Darjeeling (S449, HV-39, T-135, AV-2, CP-1. BS/7A/76, P-1258 and T-78) 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

developed as described earlier. Disease index ranged from 1-6 and was calculated after 10, 20 and 30 days of inoculation.

Results revealed that among the Tocklai varieties TV-26 and T-17 were the most susceptible while TV-23 and 25 were the most resistant (Table 7) Among the Darjeeling varieties AV-2 and BS/7A/76 showed maximum susceptibility while S449 was the most resistant followed by HV-39 and CP-1 (Table 8). In case of UPASI varieties BSS-2 and UP-9 were most susceptible (Plate 6, fig. A) and resistant respectively (Table 9). Among all the 25 varieties TV-26, and T-17 were most susceptible (Plate 6, fig. B) whereas UP-9 and UP-8 most resistant (Fig.2).

Plants of susceptible varieties were further inoculated in the field and appearance of the disease symptoms were noted in the field. Plants showed both above ground and underground symptoms ultimately leading to the death of the plants (Plates 7& 8).

Table 7 : Pathogenicity test of *S. repens* on Tocklai varieties of tea root following potted plant inoculation

Tea Varieties	Disease index[a]		
	Days after inoculation		
	10	20	30
T-17	2.23 ± 0.16	4.33 ± 0.13	5.31 ± 0.15
TV-9	1.00 ± 0.10	2.55 ± 0.11	4.1 ± 0.13
TV-18	0.93 ± 0.09	2.16 ± 0.10	3.77 ± 0.10
TV-22	0.82 ± 0.08	1.70 ± 0.11	2.9 ± 0.11
TV-23	0	0.7 ± 0.06	1.0 ± 0.09
TV-25	0	0.75 ± 0.06	1.1 ± 0.08
TV-26	2.82 ± 0.15	4.47 ± 0.10	5.55 ± 0.14
TV-27	0.87 ± 0.03	1.33 ± 0.10	2.1 ± 0.10
TV-28	0.52 ± 0.04	1.43 ± 0.09	2.25 ± 0.11
TV-29	0	0.8 ± 0.62	1.32 ± 0.14
TV-30	1.15 ± 0.11	1.95 ± 0.12	3.62 ± 0.18

Average of 20 separate inoculated plants
± Standard error;
[a]Disease index; 0= No symptoms; 1= Plants look sick and root surface started roughening in patches ; 2= Most of the leaves withered or looking yellow, light black patches with rough surface appear on roots ; 3=Defoliation starts with random yellowing , roots inky black with random patches ; 4= Random defoliation, Upto 70% roots become black ; 5=Total defoliation, 70 to 85% blackening of roots; 6 = Total defoliation with drying of shoots, 85 to 100% blackening and drying of roots.

Table 7a : Analysis of variance of data presented in Table 7.

Source	D.F.	S.S.	M.S.	F.	CD (5%)
Treatment	2	23.333	11.666	59.9203	0.91078
Varieties	10	47.397	4.740	24.3439	
Error	20	3.894	3.894	0.195	
Total	**32**	**74.624**			

Table 8 : Pathogenicity test of *S. repens* on UPASI varieties of tea root following potted plant inoculation

Tea Varieties	Disease index[a]		
	Days after inoculation		
	10	20	30
BSS-2	1.95 ± 0.06	3.72 ± 0.13	4.62 ± 0.15
UP-2	0	0.22 ± 0.02	1.17 ± 0.07
UP-3	0	0.19 ± 0.01	1.30 ± 0.06
UP-8	0	0.21 ± 0.02	0.95 ± 0.06
UP-9	0	0.18 ± 0.02	0.8 ± 0.04
UP-26	0.9 ± 0.04	2.16 ± 0.10	3.62 ± 0.12

Average of 20 separate inoculated plants
± Standard error;
[a]Disease index; 0= No symptoms; 1= Plants look sick and root surface started roughening in patches ; 2= Most of the leaves withered or looking yellow, light black patches with rough surface appear on roots ; 3=Defoliation starts with random yellowing , roots inky black with random patches ; 4= Random defoliation, Upto 70% roots become black ; 5=Total defoliation, 70 to 85% blackening of roots; 6 = Total defoliation with drying of shoots, 85 to 100% blackening and drying of roots.

Table 8a : Analysis of variance of data presented in Table 8.

Source	DF	S.S	M.S	F	C.D (5%)
Treatment	2	7.950	3.975	18.5505	1.04471
Varieties	5	25.292	5.058	23.605	
Error	10	02.143	0.214		
Total	**17**	**35.385**			

Plate 6 (Figs A & B) : Healthy and artificially inoculated pot grown tea plants showing defoliation of leaves after 30 days of inoculation with *S.repens.* (A) UPASI-26 ; (B) Teenali – 17/1/54

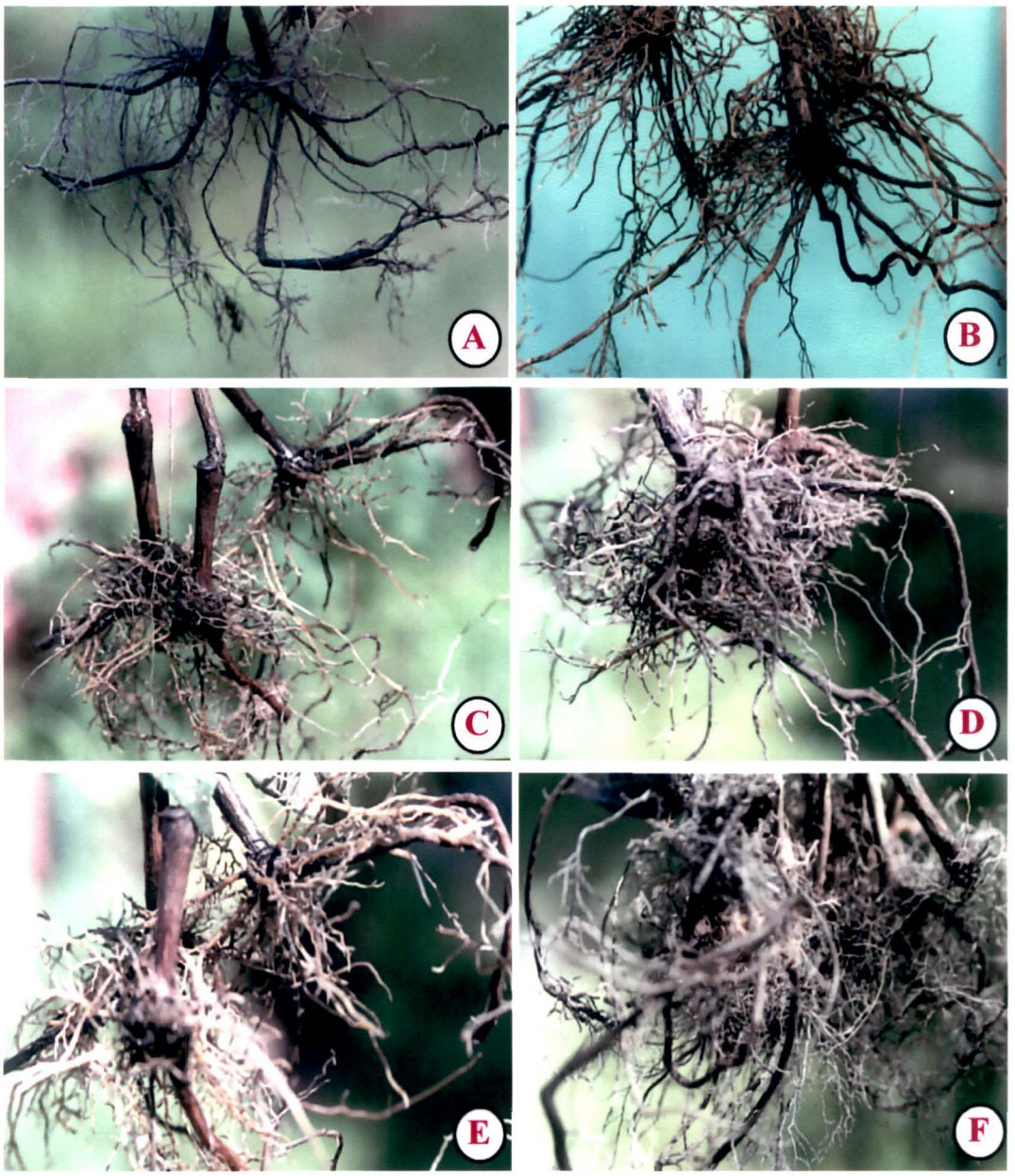


Plate 7 (Figs A-F) : Roots of uprooted tea plants showing symptoms 20 days after inoculation with *S.repens.* (A) UPASI-26; (B) P-1258 ; (C) TV-9 ; (D) TeenAli-17/ 1/54; (E) BSS-2 and (F) BS/7A/76.

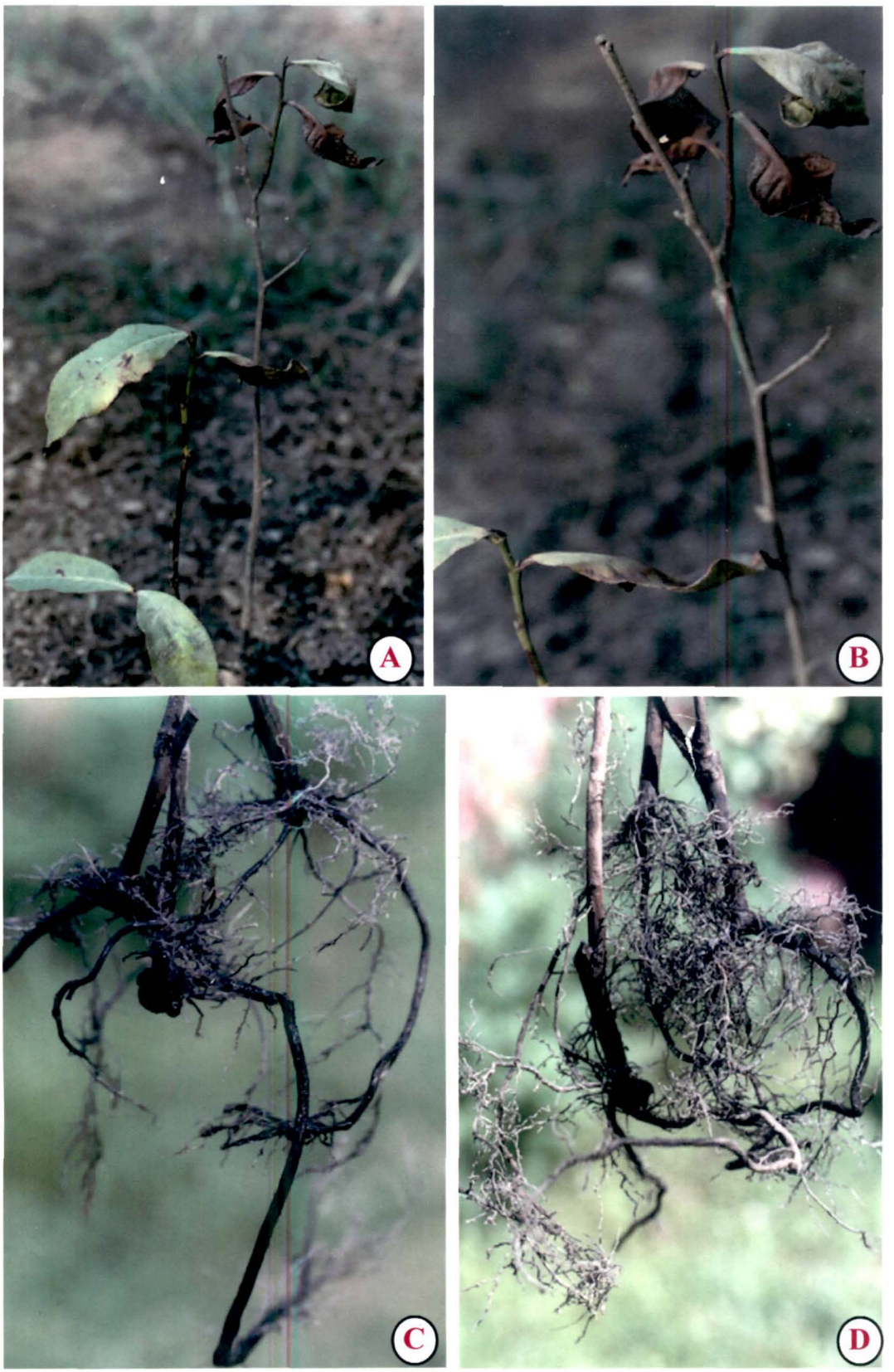
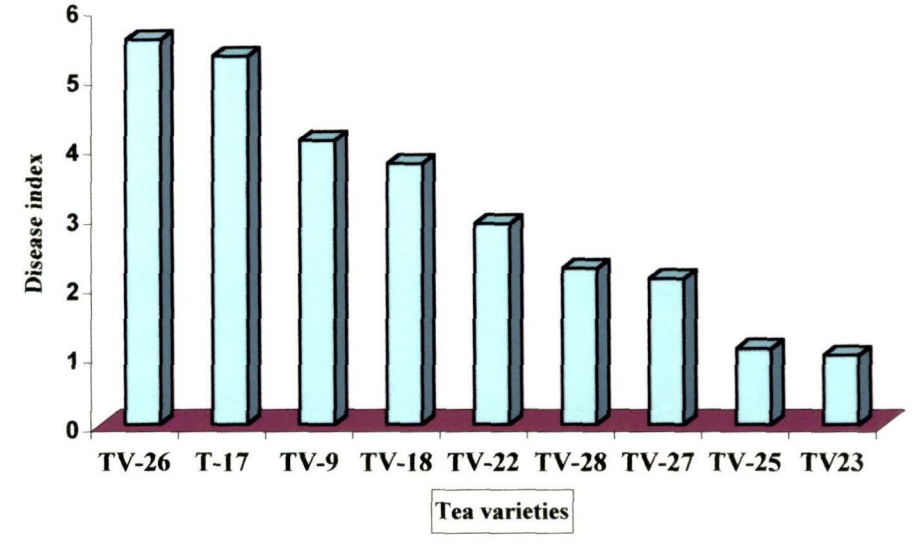
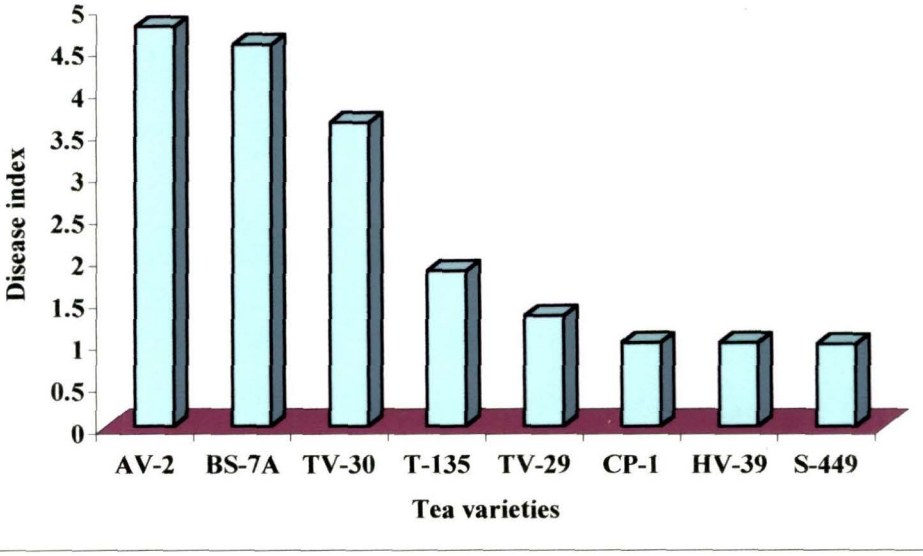


Plate 8 (Figs A-D) : Field grown tea plants (TV-26) showing aboveground (A & B) and underground (C & D) symptoms 30 days after inoculation with *S.repens*.


Disease index
0
1
2
3
4
5
6
TV-26
T-17
TV-9
TV-18
TV-22
TV-28
TV-27
TV-25
TV23
Tea varieties


Disease index
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0.5
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1.5
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2.5
3
3.5
4
4.5
5
AV-2
BS-7A
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T-135
TV-29
CP-1
HV-39
S-449
Tea varieties

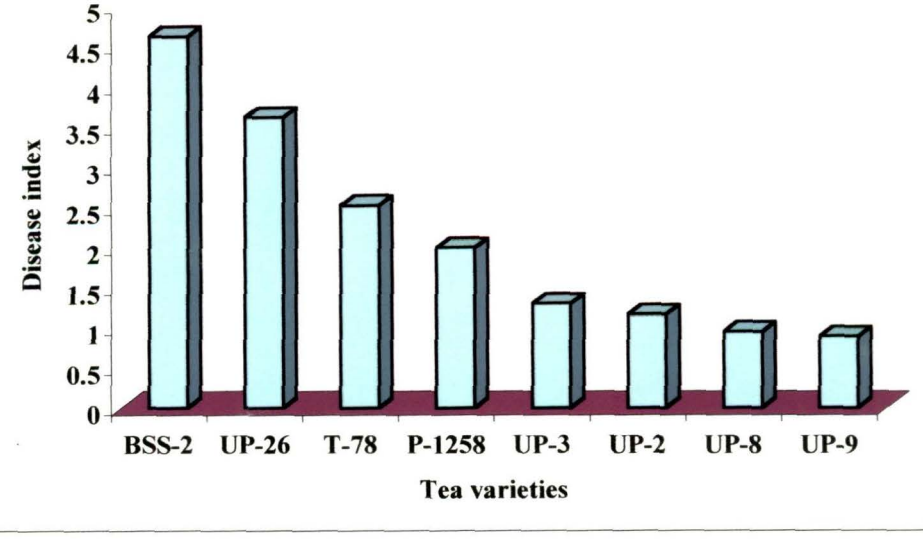

Disease index
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
BSS-2
UP-26
T-78
P-1258
UP-3
UP-2
UP-8
UP-9
Tea varieties

Fig.2

Table 9 : Pathogenicity test of *S. repens* on Darjeeling varieties of tea root following potted plant inoculation

Tea Varieties	Disease index[a]		
	Days after inoculation		
	10	20	30
S-449	0	0.22 ± 0.01	0.98 ± 0.06
HV-39	0	0.25 ± 0.31	1.0 ± 0.06
T-135	0.21 ± 0.32	0.63 ± 0.41	1.85 ± 0.10
AV-2	0.56 ± 0.06	2.32 ± 0.09	4.76 ± 0.15
CP-1	0.20 ± 012	0.54 ± 0.02	1.0 ± 0.09
BS/7A/76	0.48 ± 038	2.40 ± 0.12	4.55 ± 0.15
P-1258	0.25 ± 0.20	0.44 ± 0.05	2.0 ± 0.10
T-78	0.26 ± 0.19	0.95 ± 0.01	2.52 ± 0.16

Average of 20 separate inoculated plants
± Standard error;
[a]Disease index; 0= No symptoms; 1= Plants look sick and root surface started roughening in patches ; 2= Most of the leaves withered or looking yellow, light black patches with rough surface appear on roots ; 3=Defoliation starts with random yellowing , roots inky black with random patches ; 4= Random defoliation, Upto 70% roots become black ; 5=Total defoliation, 70 to 85% blackening of roots; 6 = Total defoliation with drying of shoots, 85 to 100% blackening and drying of roots.

Table 9a : Analysis of variance of data presented in table 9.

Source	DF	S.S	M.S	F	C.D (5%)
Treatment	2	17.973	8.987	19.1058	1.46937
Varieties	7	15.791	2.256	4.790	
Error	14	6.585	0.470		
Total	**23**	**40.349**			

4.4. Immuno diffusion

The effectiveness of antigen preparations from mycelia, cell wall and conidia of *S.repens* and tea root antigen (TV-26) for raising polyclonal antibodies (PAbs) against each preparations were checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigens of pathogen (*S.repens*) and tea roots (TV-26) were all negative. PAbs raised to mycelial extract

immunogens and purified IgG for rabbits with five bleedings were used for immunodiffusion tests. Results have been presented in Plate 9 (figs A,B,D-F). Strong precipitin reactions were observed in 1st to 4th bleedings. Preciptin reaction in agar gel double diffusion test was found to be weak in fifth bleeding. Hence, further experimental set up for optimization of enzyme immunoassays with PAbs raised against mycelial extract immunogens were conducted using 1st to 4th bleeding. On the other hand PAbs raised to cell wall and spore extract immunogens (Plate 9 fig. C) and purified IgG for rabbits with four and three bleedings respectively were tested initially in immunodiffusion tests. Strong precipitin bands were detected in all the bleedings tested. Besides antibody raised against tea root antigens were also checked in immunodifusion test. Positive reactions were noticed in 1st to 3rd bleedings which were used for further test.

4.5. Enzyme linked Immunosorbent assay

One of the most sensitive serological techniques for detection of antigenic similarity between host and pathogen as well as for detection of pathogen in diseased tissue, is enzyme linked immunosorbent assay(ELISA). Since ELISA depends on a number of factors and these vary from system to system, it was considered necessary to optimize the various conditions before further tests. Hence initially a number of experiments were performed for optimization.

4.5.1. Optimization

Polyclonal antibodies (PAbs) were raised against mycelial, cell wall and spore antigens of *S. repens* and optimization tests were done with all PAbs separately. PAbs in each case were collected by different bleedings at definite time intervals and experiments were done with the purified IgG fraction of the PAbs.

4.5.1.1. PAb raised against mycelia of *S. repens*

Optimization of ELISA was done by considering two variables i.e. concentration of the antigen and concentration of PAb (IgG). In both cases, reactions were done with PAb obtained after 1st, 2nd, 3rd and 4th bleedings. Enzyme concentration was 1:10000 while substrate was used at a concentration of 1mg/ml.

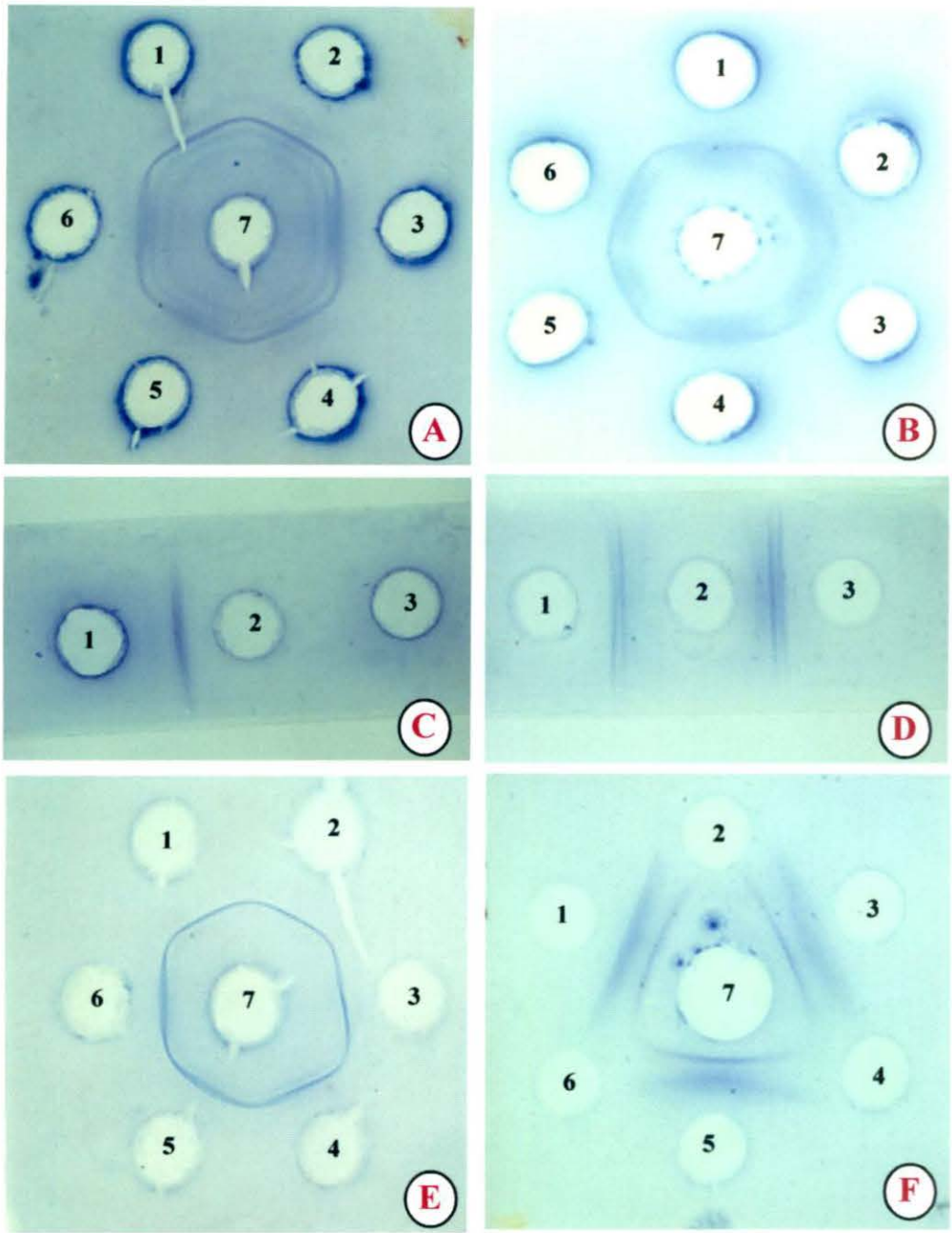


Plate 9 (Figs A-F) : Agar gel double diffusion test with PAb of *S.repens*. Peripheral wells (1-6 in A,B&E; 2 in C ; 1&3 in D and 1,3&5 in F) were loaded with fungal antigens and normal serum (3 in C; 2,4&6 in F) and central wells were loaded with PAb [1st Bleeding (D); 2nd Bleeding (F); 3rd bleeding (A &B) and 4th bleeding (E)] of *S.repens* prepared from mycelial antigen (7 in A,B,E-F& 2 in D) and PAb [2nd bleeding] prepared from spore antigen (1in C)

4.5.1.1.1. Antiserum dilution

Doubling dilution of IgG purified from PAbs obtained after 1st, 2nd, 3rd and 4th bleedings were tested against homologous antigens at a concentration of 10mg/ml. Absorbance values decreased from 40 to 0.312 µg/ml. A_{405} values on the other hand increased with the different bleedings (Table 10, Fig. 3).

Table 10 : ELISA reaction of *S.repens* mycelial antigen with different concentration of homologous Pab

IgG conc. (µg/ml)	Absorbance at 405nm			
	1st IgG	2nd IgG	3rd IgG	4rd IgG
40	1.423 ± 0.04	2.117 ± 0.05	2.144 ± 0.06	2.218 ± 0.06
20	0.934 ± 0.04	2.029 ± 0.03	2.079 ± 0.05	2.216 ± 0.05
10	0.665 ± 0.03	1.785 ± 0.04	1.876 ± 0.04	2.032 ± 0.05
5	0.471 ± 0.03	1.497 ± 0.04	1.636 ± 0.05	1.967 ± 0.04
2.5	0.310 ± 0.02	1.182 ± 0.05	1.168 ± 0.09	1.564 ± 0.05
1.25	0.290 ± 0.03	0.821 ± 0.03	0.876 ± 0.05	1.076 ± 0.04
0.625	0.284 ± 0.02	0.744 ± 0.03	0.758 ± 0.03	1.056 ± 0.03
0.312	0.249 ± 0.03	0.353 ± 0.02	0.707 ± 0.03	0.957 ± 0.04

Antigen concentration 10µg/ml
± Standard error.

4.5.1.1.2. Antigen dilution

Doubling dilution of *S. repens* mycelial antigen ranging from 40 to 0.312µg/ml tested against IgG from 1st, 2nd, 3rd and 4th bleeding at a concentration of 40µg/ml. ELISA values decreased with the decrease of antigen concentration but the values were still quite high indicating that the range of detection could be much lower (Table11, Fig.4).

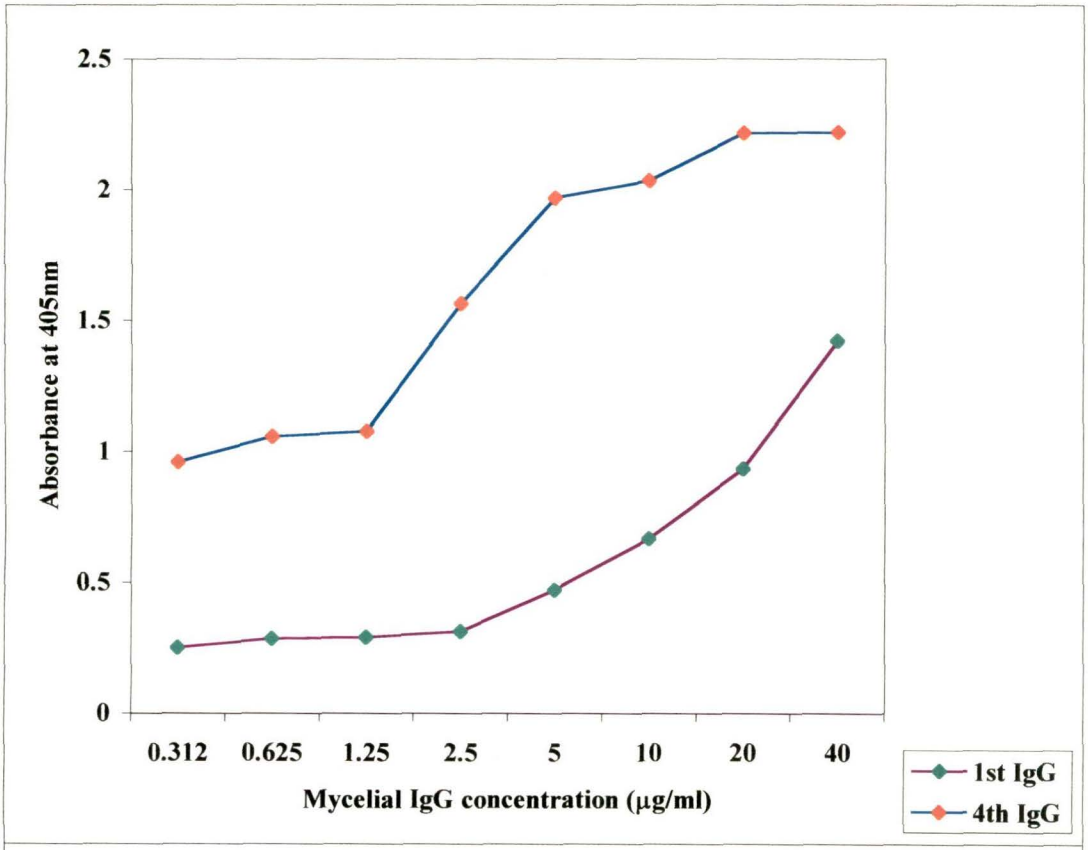
4.5.1.2. PAb raised against cell wall of *S. repens*

Homologous reactions were carried out using cell wall antigens and PAbs raised against these antigens.

4.5.1.2.1. Antiserum dilution

The concentrations of IgG tested ranged from 40-0.312µg/ml in all the bleedings. Cell wall antigens were used at a concentration of 10µg/ml. Absorbance values decreased with the decreased in concentration of IgG in all the bleedings (Table 12, Fig 3).

Optimization of concentrations of PAbs raised against mycelial and cell wall antigens of *S.repens* by DAC- ELISA



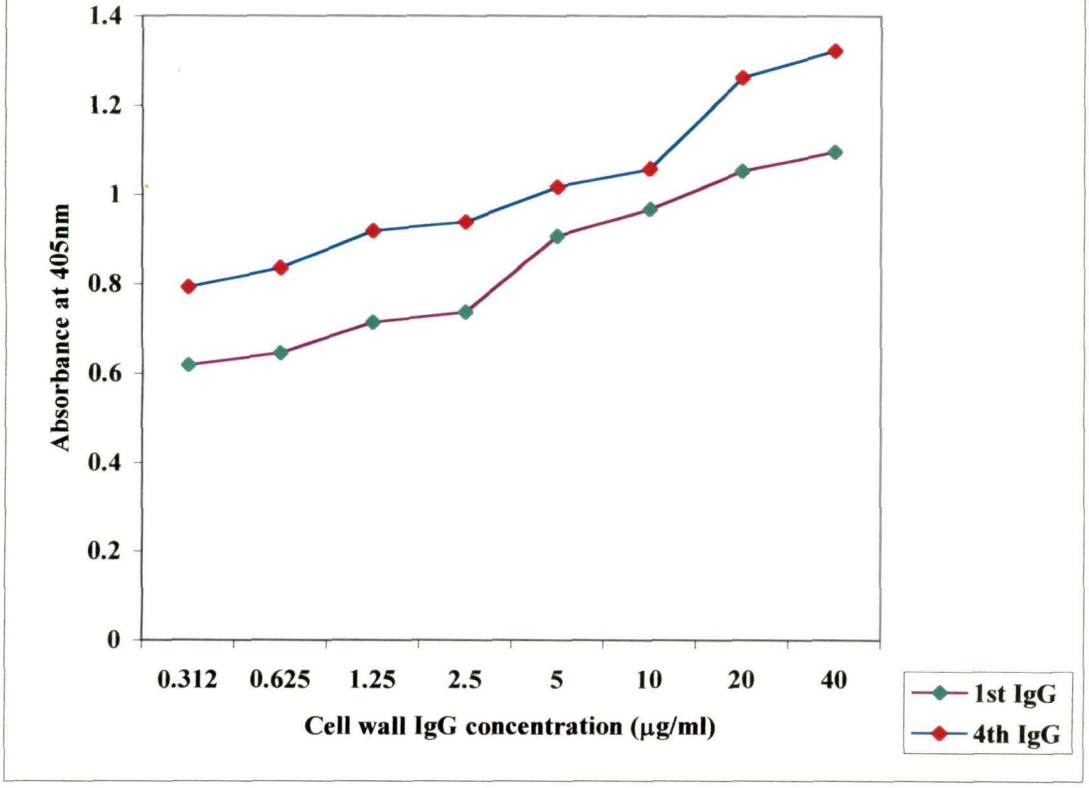


Fig.3

Optimization of mycelial and cell wall antigen concentrations of *S.repens* by DAC- ELISA

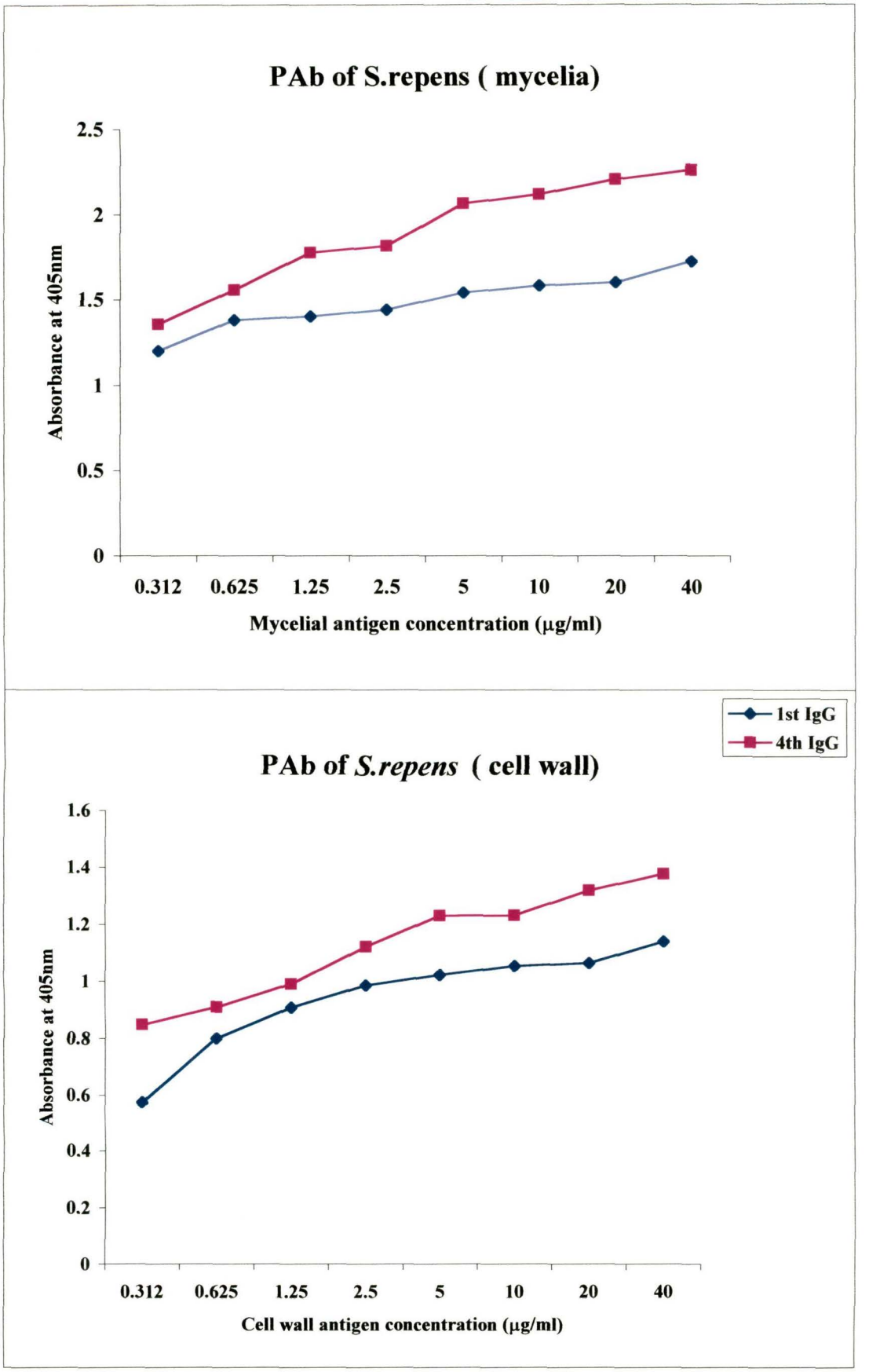


Fig.4

Table 11 : ELISA reaction of *S repens* PAb with different concentration of homologous mycelial antigen

Antigen conc. (µg/ml)	Absorbance at 405nm			
	1st IgG	2nd IgG	3rd IgG	4rd IgG
40	1.724 ± 0.05	1.967 ± 0.04	1.082 ± 0.06	2.254 ± 0.05
20	1.641 ± 0.05	1.930 ± 0.04	2.070 ± 0.06	2.203 ± 0.05
10	1.584 ± 0.04	1.874 ± 0.05	2.065 ± 0.05	2.117 ± 0.06
5	1.547 ± 0.05	1.820 ± 0.03	2.062 ± 0.04	2.063 ± 0.05
2.5	1.446 ± 0.05	1.745 ± 0.06	1.754 ± 0.05	1.815 ± 0.04
1.25	1.408 ± 0.06	1.564 ± 0.04	1.579 ± 0.05	1.776 ± 0.05
.625	1.383 ± 0.03	1.479 ± 0.03	1.482 ± 0.04	1.556 ± 0.03
.312	1.201 ± 0.04	1.333 ± 0.03	1.231 ± 0.04	1.357 ± 0.03

PAb concentration 40µg/ml
± Standard error.

Table 12 : ELISA reaction of cell wall antigen of *S repens* different concentration of homologous PAb.

Antisera conc. (µg/ml)	Absorbance at 405nm			
	1st IgG	2nd IgG	3rd IgG	4rd IgG
40	1.096 ± 0.037	1.228 ± 0.051	1.231 ± 0.052	1.322 ± 0.050
20	1.053 ± 0.046	1.072 ± 0.042	1.102 ± 0.049	1.262 ± 0.061
10	0.967 ± 0.041	0.969 ± 0.046	1.039 ± 0.051	1.058 ± 0.042
5	0.908 ± 0.048	0.914 ± 0.033	0.941 ± 0.036	1.018 ± 0.031
2.5	0.737 ± 0.036	0.755 ± 0.038	0.824 ± 0.032	0.939 ± 0.046
1.25	0.714 ± 0.039	0.723 ± 0.042	0.79 ± 0.031	0.919 ± 0.044
.625	0.646 ± 0.030	0.696 ± 0.048	0.744 ± 0.037	0.836 ± 0.032
.312	0.618 ± 0.031	0.664 ± 0.043	0.702 ± 0.038	0.793 ± 0.039

Antigen concentration 10µg/ml
± Standard error.

4.5.1.2.2. Antigen dilution

To determine the affect of antigen dilution on ELISA reactivity a doubling dilution series of *S. repens* cell wall antigens ranging from 40 to 0.312mg/ml values. IgG was used at a concentration of 40µg/ml. A 405 values decreased with dilution a (Table 13, Fig. 4).

Table 13 : ELISA reaction of of *S repens* PAbswith different concentration of homologous cell wall antigen.

Antigen conc. (µg/ml)	Absorbance at 405nm			
	1st IgG	2nd IgG	3rd IgG	4lt IgG
40	1.139 ± 0.04	1.250 ± 0.05	1.361 ± 0.04	1.378 ± 0.03
20	1.063 ± 0.03	1.126 ± 0.04	1.156 ± 0.04	1.320 ± 0.04
10	1.054 ± 0.04	1.057 ± 0.04	1.096 ± 0.05	1.239 ± 0.02
5	1.022 ± 0.04	1.024 ± 0.04	1.137 ± 0.03	1.232 ± 0.03
2.5	0.984 ± 0.04	1.008 ± 0.03	1.018 ± 0.03	1.230 ± 0.04
1.25	0.907 ± 0.03	0.921 ± 0.05	0.936 ± 0.04	0.990 ± 0.04
.625	0.800 ± 0.03	0.866 ± 0.03	0.877 ± 0.04	0.909 ± 0.03
.312	0.574 ± 0.04	0.705 ± 0.04	0.800 ± 0.04	0.847 ± 0.03

PAb concentration 40µg/ml
± Standard error.

4.5.1.3. PAb raised against spore antigen of *S. repens*

Antigens were prepared from spores of *S. repens*, and these were used to raise PAbs. IgG from PAbs collected after 2nd and 3rd bleeding were used in homologous test.

4.5.1.3.1 Antigen dilution

Doubling dilution of spore antigen was prepared from 40µg/ml to 0.312 µg/ml, which were then tested against homologous PAbs at a concentration of 40µg/ml. Absorbance values decreased with dilution (Table 14).

Optimization using PAbs raised against mycelia, cell wall and spores showed similar trends i.e. the values decreased with dilution of both antigen and PAb (Fig. 3 and 4). Reactivity increased with the time of bleeding in both mycelial and cell wall PAbs but in case of PAb raised against spore antigens decreased after 2nd bleed.

4.5.1.4. PAb raised against root antigens

Root antigen prepared from TV-26, was used to raise the PAbs. Optimization of the PAbs through ELISA was done by considering concentration of the antigen. Doubling dilutin of root antigens ranging from 40 to 0.313 µg/ml values were used. IgG concentration was 40 µg/ml. A_{405} value decreased with dilution as revealed in Table15.

Table 14 : ELISA reaction of spore PAb of *S. repens* with different concentration of homologous antigen

Antigen conc. (µg/ml)	Absorbance at 405nm	
	2nd IgG	3rd IgG
40	1.93 ± 0.042	3> ± 0.0
20	1.87 ± 0.051	2.2 ± 0.046
10	1.72 ± 0.053	2.05 ± 0.035
5	1.59 ± 0.043	2.02 ± 0.043
2.5	1.36 ± 0.046	1.89 ± 0.051
1.25	1.059± 0.039	1.72 ± 0.032
.625	0.73 ± 0.034	1.58 ± 0.039
.312	0.443± 0.036	0.87 ± 0.031

PAb concentration 40µg/ml
± Standard error.

Table 15 : ELISA reaction of PAb raised against Tea root (TV-26) with different concentration of homologous antigen

Antigen conccentration	Absorbance at 405nm			
(µg/ml)	Expt.1	Expt.2	Expt.3	Mean
40	1.238	1.229	1.232	1.233 ±0.039
20	1.231	1.235	1.231	1.232 ±0.027
10	1.218	1.225	1.225	1.221 ± 0.034
5	1.005	0.997	0.999	1.003 ± 0.037
2.5	0.798	0.82	0.824	0.814 ± 0.068
1.25	0.652	0.645	0.644	0.647 ± 0.038
.625	0.615	0.606	0.609	0.610 ± 0.039
.312	0.576	0.59	.577	0.581 ± 0.051

PAb concentration 40µg/ml
± Standard error.

4.5.2. Detection of cross reactive antigens between *S. repens* and tea roots

Cross reactive antigens have in several host pathogen combinations been shown to be the determinants of susceptibility and resistance. In the present study pathogencity test of 25 tea varieties revealed different responses ranging from highly susceptibility to high resistance. In order to determine whether resistance/ susceptibility could be

correlated with cross reactive antigens, ELISA tests were carried out with antigens of all varieties and PAbs raised against mycelia, cell wall and spore antigens.

4.5.2.1. PAb of *S. repens*

4.5.2.1.1. Mycelia : ELISA reactions were carried out with antigens of 25 tea varieties, two non-hosts (*Cicer arietinum*, *Oryza sativa*) non pathogen as well as mycelial antigens of *S. repens*. (Table 16, Fig. 5) . In all cases experiments were repeated thrice under same condition. Results revealed that among the twenty five varieties tested Teen Ali 17/1/54 showed heighest absorbance followed by TV-26 and T-135. Of the twenty five varieties five (TV-9, AV-2, P-1, BS/7A/76, and P-1258) were further selected for testing with PAbs raised against mycelial antigens obtained from different bleeding. Result revealed that the PAbs from 3rd and 4th bleedings showed more or less similar response, while those from 1st and 2nd bleeding showed lesser values (Table 17, Fig.6).

4.5.2.1.2. Cell wall

Among all twenty five varieties tested with the antiserum against cell wall preparation highest absorbance value exhibited by TV-26 followed by Teen Ali 17/1/ 54. While UP-9, UP-2, UP exhibited lowest reactivity, Reactivity of the non host and non pathogen were low as evidenced by low absorbance values (Table 18). Five selected varieties of twenty five were again tested with PAbs from 1st and 2nd 3rd and 4th bleeding did not show any significant differences among the varieties (Table 19).

4.5.2.1.3. Spore

PAbs raised against spore antigen also reacted with the tea root antigens of the varieties tested (Table 20). Response obtained was similar to that obtained with the PAbs raised against mycelia and cell wall PAb from two bleedings were also tested against 5 selected varieties. No significant differences were obtained either among the varieties or between the 2 bleedings (Table- 21).

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

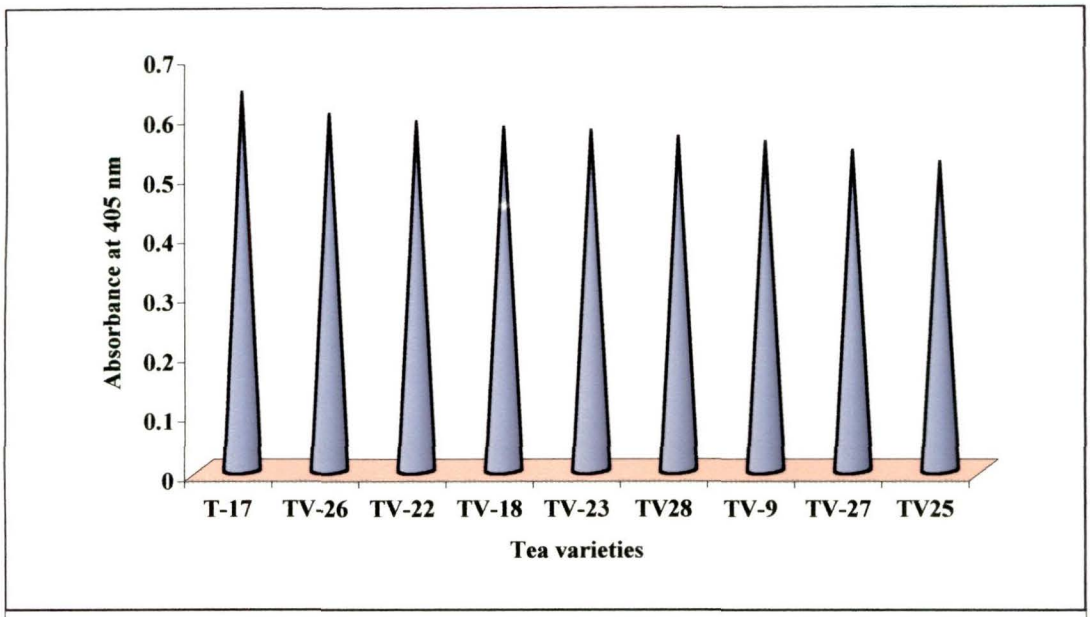
Antigen conc. (100µg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
T-17	0.630	0.635	0.640	0.635± 0.04
TV-9	0.553	0.548	0.551	0.551± 0.02
TV-18	0.570	0.576	0.579	0.575± 0.03
TV-22	0.581	0.583	0.591	0.585± 0.04
TV-23	0.569	0.573	0.568	0.570± 0.02
TV-25	0.512	0.517	0.519	0.516± 0.03
TV-26	0.597	0.596	0.600	0.598± 0.02
TV-27	0.535	0.533	0.540	0.536± 0.03
TV-28	0.557	0.558	0.561	0.559± 0.02
TV-29	0.584	0.586	0.591	0.537± 0.03
TV-30	0.575	0.579	0.577	0.577± 0.02
S-449	0.545	0.553	0.555	0.551± 0.04
HV-39	0.435	0.437	0.442	0.438± 0.03
T-135	0.597	0.593	0.594	0.594± 0.02
AV-2	0.663	0.658	0.622	0.661± 0.03
CP-1	0.460	0.455	0.456	0.457± 0.03
BS/7A/76	0.589	0.591	0.590	0.590± 0.01
P-1258	0.545	0.540	0.538	0.541± 0.03
T-78	0.507	0.510	0.520	0.513± 0.04
BSS-2	0.555	0.560	0.566	0.560± 0.02
UP-2	0.493	0.494	0.489	0.492± 0.03
UP-3	0.445	0.452	0.457	0.451± 0.04
UP-8	0.481	0.488	0.486	0.485± 0.03
UP-9	0.427	0.425	0.432	0.428± 0.03
UP-26	0.469	0.465	0.4468	0.467± 0.02
S. repens mycelia	2.128	2.128	2.129	2.126± 0.03
Non-Pathogen				
Fusarium oxysporum	0.449	0.448	0.445	0.447 ± 0.026
Non-host				
Cicer arietinum	0.381	0.385	0.382	0.383± 0.026
Oryza sativa	0.406	0.407	0.400	0.404± 0.035

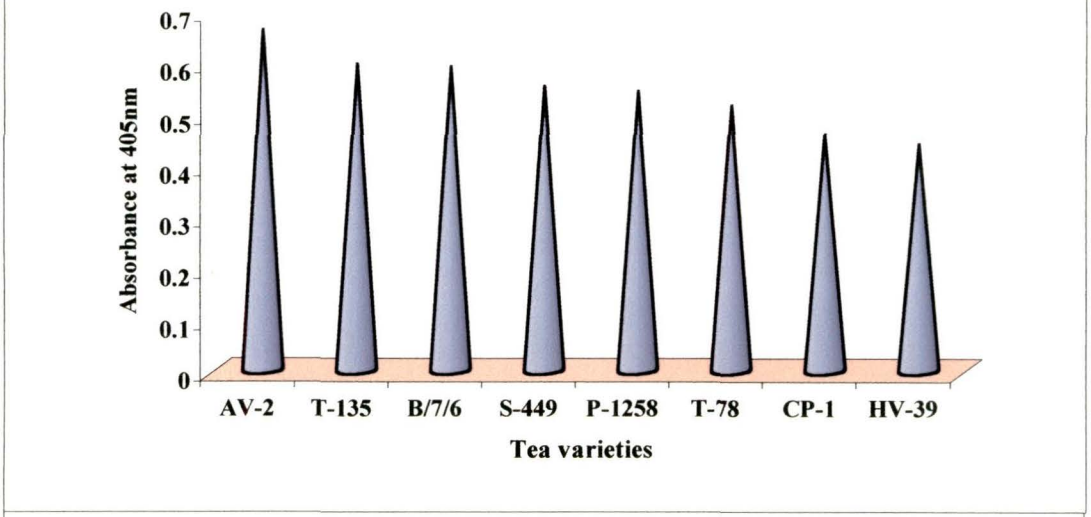
a- antigen concentration 10 µg /ml

± Standard error.

IgG concentration

Cross reactivity of tea root antigens against PAb of *S. repens*





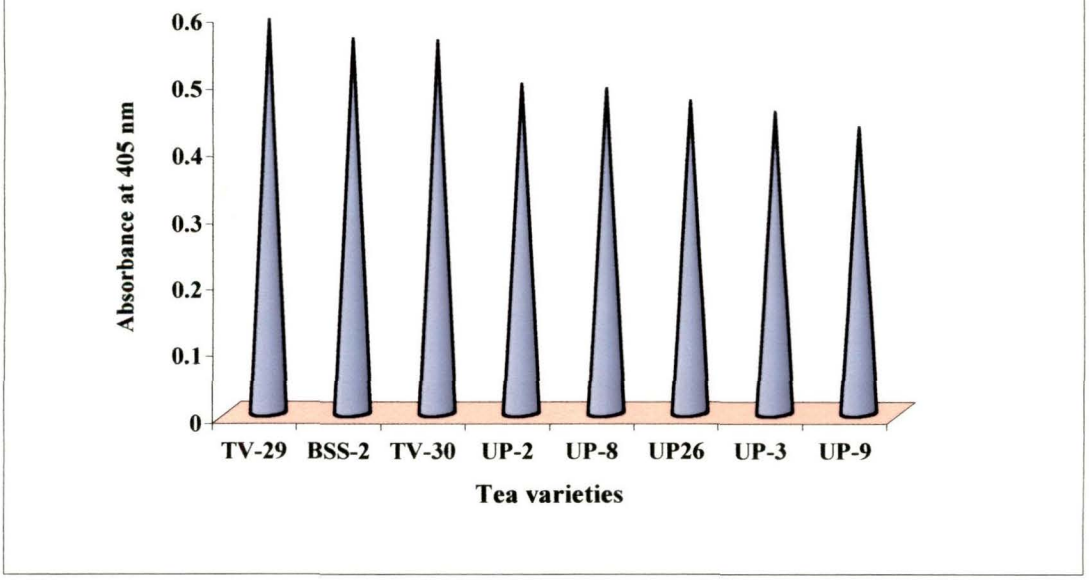


Fig.5

Table 17 : DAC- ELISA of healthy tea root antigen against PAbs of *S.repens* (mycelial) different bleedings

Tea Varieties	Absorbance at 405 nm			
	Source of IgG (Bleed)			
	1st	2nd	3rd	4 th
TV-9	0.466± 0.05	0.499± 0.03	0.522± 0.02	0.555± 0.03
AV-2	0.518± 0.05	0.562± 0.02	0.620± 0.02	0.656± 0.04
CP-1	0.451± 0.03	0.431± 0.04	0.439± 0.03	0.458± 0.03
BS/7A/76	0.401± 0.04	0.561± 0.02	0.573± 0.06	0.594± 0.04
P-1258	0.444± 0.04	0.478± 0.03	0.501± 0.04	0.535± 0.02

Age of the plants 2 years
Antigen conc. 100 µg /ml
IgG concentration 40 µg /ml
± Standard error

4.5.2.2. Detection of cross reactive antigens among tea roots.

PAb raised against tea root antigen (TV-26) was tested to detect the cross reactive antigens of the roots of other tea varieties. Homologous ELISA reaction i.e. reaction with the antigen of TV-26 showed highest absorbance followed by AV-2, Teenali 17/ 154 and T-78 and lowest absorbance value was shown by TV-9 and T-135 (Table 22).

4.5.3. Determination of cross reactivity of *S. repens* with different soil fungi

Cross reactivity of the PAb raised against *S. repens* was tested against a number of fungi some of which were pathogenic to tea (*Fomes lamaenosis*, *Poria* sp. *Ustulina zonata*) and others were non pathogenic (*Beauveria sp. Metarrhizium sp., Trichoderma harzianum, Sclerotium rolfsii*) Antigens were prepared from the mycelia of all the above fungal isolates and were tested against PAb of *S. repens* by ELISA. Result (Table 23 and Fig. 7 revealed theat among all the fungi tested PAb of *S. repens* reacted to some extent with antigen of *Beauveria bassiana* (isolate-2) and *Metarrhizium anisopliae* (isolate-1).

Table 18 : Indirect ELISA values (A405) of tea root antigens, cell wall antigens of *S. repens,* non-pathogen and non-host reacted with anti *S.repens* cell wall PAbs.

Antigen Concentration (100 µg /ml)	**Absorbance at 405 nm**				
	Expt. 1	**Expt.2**	**Expt.3**	**Mean**	
Tea varieties					
T-17	0.623	0.630	0.622	0.625	± 0.038
TV-9	0.595	0.588	0.593	0.592	± 0.034
TV-18	0.525	0.541	0.542	0.536	± 0.056
TV-22	0.595	0.597	0.597	0.596	± 0.019
TV-23	0.580	0.584	0.584	0.583	± 0.030
TV-25	0.525	0.538	0.538	0.534	± 0.051
TV-26	0.642	0.643	0.643	0.643	± 0.031
TV-27	0.557	0.568	0.565	0.563	± 0.044
TV-28	0.598	0.605	0.599	0.601	± 0.036
TV-29	0.546	0.539	0.543	0.543	± 0.034
TV-30	0.588	0.589	0.599	0.592	± 0.045
S-449	0.571	0.573	0.572	0.572	± 0.018
HV-39	0.576	0.580	0.581	0.579	± 0.029
T-135	0.623	0.616	0.618	0.619	± 0.034
AV-2	0.580	0.584	0.585	0.583	± 0.029
CP-1	0.501	0.502	0.502	0.502	± 0.013
BS/7A/76	0.599	0.601	0.606	0.602	± 0.035
P-1258	0.555	0.557	0.562	0.558	± 0.034
T-78	0.562	0.565	0.565	0.564	± 0.024
BSS-2	0.601	0.599	0.610	0.603	± 0.044
UP-2	0.480	0.485	0.479	0.481	± 0.033
UP-3	0.496	0.496	0.495	0.496	± 0.014
UP-8	0.475	0.480	0.479	0.478	± 0.030
UP-9	0.424	0.417	0.415	0.419	± 0.042
UP-26	0.480	0.482	0.481	0.481	± 0.018
S.repens cell wall	1.37 0	1.372	1.372	1.371	± 0.020
Non pathogen					
Metarhiziumanisopliae	0.414	0.411	0.418	0.414	± 0.034
Non host					
Impatiens balsamina	0.319	0.324	0.317	0.320	± 0.037
Tägetes patula	0.382	0.378	0.381	0.380	± 0.026

Age of the plants 2 years
Antigen concentration 10µg/ml
PAb concentration 40µg/ml (4th)
± Standard error

Table 19: DAC- ELISA of healthy tea root antigen against PAbs of *S.repens* (cell wall) different bleedings.

Tea Varieties	Absorbance at 405 nm			
	Source of IgG (Bleed)			
	1st	2nd	3rd	4 th
TV-9	0.529 ± 0.055	0.532 ± 0.038	0.558 ± 0.025	0.555 ± 0.027
AV-2	0.488 ± 0.048	0.513 ± 0.042	0.548 ± 0.033	0.579 ± 0.041
CP-1	0.410 ± 0.032	0.428 ± 0.051	0.471 ± 0.021	0.507 ± 0.031
BS/7A/76	0.494 ± 0.037	0.529 ± 0.042	0.532 ± 0.016	0.592 ± 0.025
P-1258	0.410 ± 0.023	0.503 ± 0.019	0.550 ± 0.036	0.557 ± 0.028

Age of the plants 2 years
Antigen conc. 100 µg /ml
PAb concentration 40µg/ml

4.5.4. Detection of *S. repens* in tea root tissues

Detection of pathogen in host tissues by the use of ELISA with the antiserum raised against the pathogen is one of the most effective and easy methods. Two types of ELISA are generally used in such experiments i.e. direct antigen coated (DAC) ELISA and double antibody sandwich (DAS) ELISA. In the present study following pathogenicity test and determination of cross reactive antigens in the different varieties attempts were made to detect the pathogen in infected tissues by DAC and DAS ELISA.

4.5.4.1. PAb raised against mycelia

In this experiment, antigens were prepared from healthy and inoculated roots of all 25 tea varieties. These were then tested by ELISA against PAb of *S. repens*. Antigens from inoculated roots were prepared after 10, 20 and 30 days of inoculation. Results revealed that in all tested varieties infected extracts showed higher ELISA values than the healthy extracts. In the Tocklai varieties maximum ELISA were values obtained in TV-26, T-17, and TV-27 (Table-24). In the UPASI varieties the infected extracts BSS-2 showed the highest ELISA value (Table 25). Among the Darjeeling varieties, the difference in ELISA values between infected and healthy extracts was maximum and significant in AV-2 and BS/7A/76 (Table 26). Significantly higher ELISA values in infected root extract was obtained in the susceptible varieties (Fig. 8).

Table 20 : Indirect ELISA values (A405) of tea root antigens, spore antigens of *S. repens*, non-pathogen and non-host reacted with anti *S.repens* spore PAbs

Antigen Concentration (100 µg /ml)	**Absorbance at 405 nm**				
	Expt. 1	**Expt. 2**	**Expt. 3**	**Mean**	
Tea varieties					
T-17	0.586	0.592	0.592	0.590	± 0.03
TV-9	0.549	0.538	0.537	0.541	± 0.05
TV-18	0.515	0.523	0.525	0.521	± 0.04
TV-22	0.548	0.549	0.554	0.550	± 0.03
TV-23	0.528	0.525	0.535	0.529	± 0.04
TV-25	0.502	0.499	0.507	0.503	± 0.03
TV-26	0.522	0.514	0.519	0.518	± 0.03
TV-27	0.511	0.514	0.516	0.513	± 0.03
TV-28	0.517	0.521	0.516	0.518	± 0.04
TV-29	0.499	0.495	0.501	0.497	± 0.03
TV-30	0.486	0.488	0.482	0.485	± 0.03
S-449	0.587	0.484	0.483	0.485	± 0.03
HV-39	0.424	0.419	0.422	0.421	± 0.03
T-135	0.476	0.466	0.472	0.471	± 0.04
AV-2	0.523	0.518	0.524	0.522	± 0.03
CP-1	0.405	0.400	0.403	0.402	± 0.03
BS/7A/76	0.525	0.519	0.522	0.523	± 0.03
P-1258	0.448	0.453	0.452	0.451	± 0.03
T-78	0.386	0.392	0.389	0.389	± 0.03
BSS-2	0.512	0.517	0.519	0.516	± 0.04
UP-2	0.381	0.386	0.384	0.383	± 0.03
UP-3	0.312	0.317	0.314	0.315	± 0.03
UP-8	0.301	0.307	0.306	0.305	± 0.03
UP-9	0.299	0.298	0.303	0.300	± 0.03
UP-26	0.4436	0.435	0.440	0.439	± 0.04
S. repens spore	2.208	2.220	2.205	2.212	± 0.05
Non pathogen					
Beauveria bassiana	0.453	0.456	0.452	0.454	± 0.04
Non host					
Yacca	.325	0.330	0.332	0.329	± 0.03
Phyllanthes niruri	0.314	0.311	0.311	0.312	± 0.02

Age of the plant 2 years

[a] Antigen concentration 10

± Standard error

Table 21: DAC- ELISA of healthy tea root against antigens PAb of *S. repens* spore.

Antigen source	Absorbance at 405nm	
	Bleeding	
Tea Varieties	2nd	3rd
TV-9	0.495 ± 0.042	0.519 ± 0.062
AV-2	0.479 ± 0.039	0.535 ± 0.035
CP-1	0.385 ± 0.034	0.402 ± 0.022
BS/7A/76	0.486 ± 0.051	0.521 ± 0.029
P-1258	0.416 ± 0.024	0.435 ± 0.048

Age of the plants 2 years ; Antigen concentration 100 µg /ml; IgG concentration 40 µg /ml ;

Table 22 : ELISA responses of different varieties of tea root antigens to PAb raised against root antigen

Antigen Concentration (100 µg /ml)	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
T-17	1.102	1.099	1.104	1.102± 0.03
TV-9	0.612	0.617	0.613	0.614± 0.03
TV-18	0.939	0.922	0.934	0.932± 0.05
TV-22	0.998	0.977	1.008	0.001± 0.07
TV-23	0.761	0.753	0.769	0.761± 0.05
TV-25	0.703	0.706	0.701	0.703± 0.02
TV-26	1.189	1.181	1.118	1.186± 0.03
TV-27	0.696	0.694	0.692	0.694± 0.02
TV-28	0.672	0.663	0.669	0.668± 0.04
TV-29	0.0651	0.648	0.650	0.650± 0.02
TV-30	0.928	0.937	0.935	0.933± 0.04
S-449	0.670	0.655	0.665	0.663± 0.05
HV-39	0.677	0.667	0.673	0.672± 0.04
T-135	0.654	0.661	0.652	0.656± 0.04
AV-2	1.114	1.118	1.121	1.118± 0.03
CP-1	0.786	0.782	0.785	0.784± 0.02
BS/7A/76	0.556	0.552	0.554	0.554± 0.03
P-1258	0.674	0.677	0.671	0.674± 0.03
T-78	1.054	1.057	1.051	1.054± 0.03
BSS-2	0.932	0.925	0.936	0.931± 0.04
UP-2	0.672	0.672	0.676	0.673± 0.02
UP-3	0.781	0.775	0.774	0.777± 0.03
UP-8	0.819	0.820	0.812	0.817± 0.03
UP-9	0.737	0.732	0.734	0.734± 0.02
UP-26	0.853	0.866	0.863	0.860± 0.04

Age of the plant - 2 years; IgG concentration 40µg /ml

Table 23 : DAC ELISA of the antigen of different soil pathogen against *S. repens* antisera

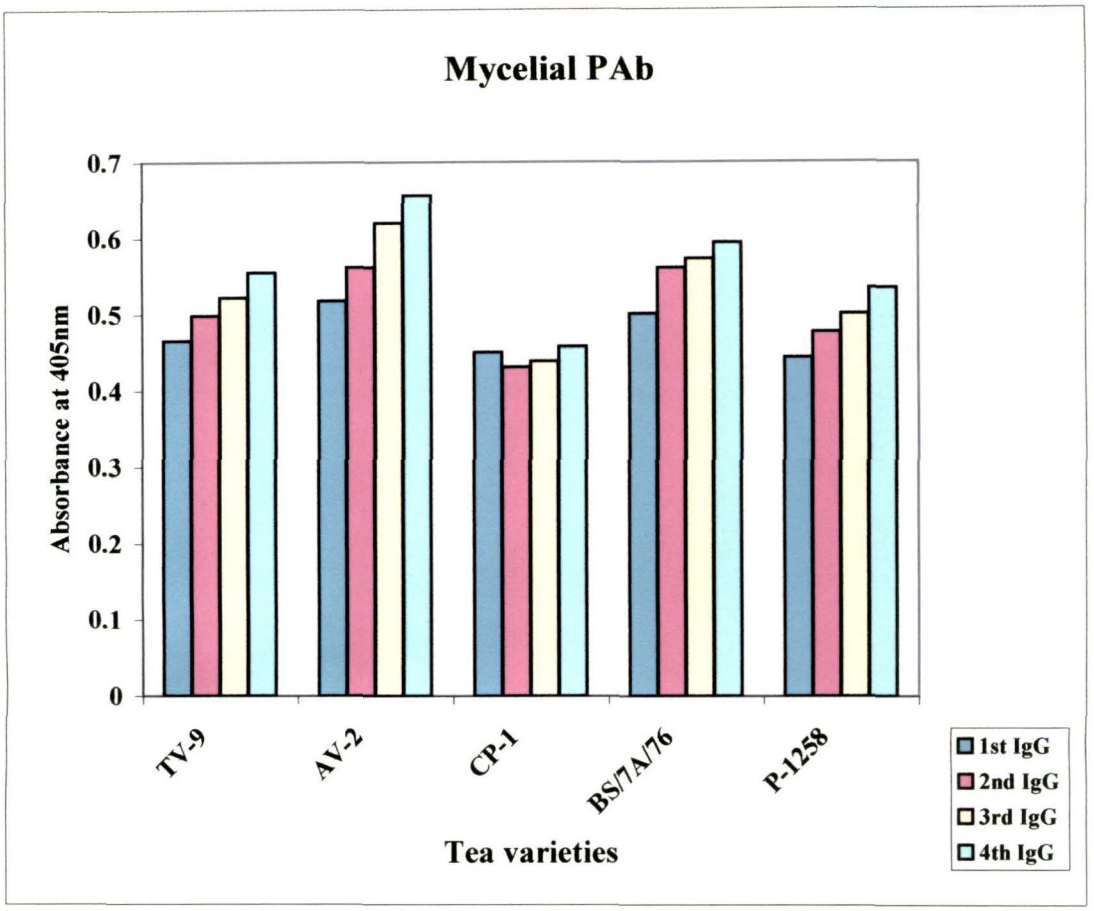
Antigen source[a]	Expt.1	Expt.2	Expt.3	Mean
S. repens	2.119	2.122	2.142	2.128 ± 0.06
Fomes lamaoensis	0.521	0.524	0.517	0.521 ± 0.03
Poria hypobrumer	0.514	0.514	0.517	0.518 ± 0.02
Ustulina zonata	0.419	0.404	0.404	0.409 ± 0.05
Armillaria melea	0.434	0.425	0.421	0.425 ± 0.05
Metarrhizium anisopliae-892	0.419	0.423	0.409	0.417 ± 0.05
M.anisopliae - 140	0.405	0.401	0.410	0.405 ± 0.04
Beauveria basiana - 135	0.472	0.463	0.468	0.468 ± 0.04
B. basiana- 2028	0.446	0.448	0.455	0.450± 0.04
Trichoderma harzianum	0.593	0.596	0.590	0.593± 0.03
T. viride	0.447	0.445	0.448	0.447 ± 0.02
Fusarium oxysporum	0.450	0.454	0.442	0.446 ± 0.05
S. rolfsii	0.316	0.327	0.323	0.322 ± 0.04
S. rolfsii -1	0.312	0.331	0.323	0.322± 0.06
S. rolfsii-2	0.318	0.320	0.312	0.316 ± 0.04
S. rolfsii-3	0.308	0.312	0.305	0.308 ± 0.03
S. rolfsii-4	0.304	0.303	0.299	0.322 ± 0.03

Antigen concentration 10µg/ ml
IgG concentration 40µg /ml
± Standard error.
Age of the plants 2 years

When the PAbs obtained from 1st, 2nd, 3rd and 4th bleedings were tested against healthy and infected root extracts of 5 selected tea varieties TV-9, AV-2, CP-1, BS/ 7A/76 and P1 258), it was observed 3rd and 4th were the most effective in disease detection (Table 27 and Fig.9).

Detection of *S. repens* in the infected roots of the 5 selected varieties was also done by DAS ELISA. This was carried out following the methods described under material and methods. Results presented in Table 27, revealed significantly higher ELISA values in the infected extracts of all the 5 varieties tested. (Fig. 10).

Cross reactivity of tea root antigens against PAb of *S.repens* (different bleedings)



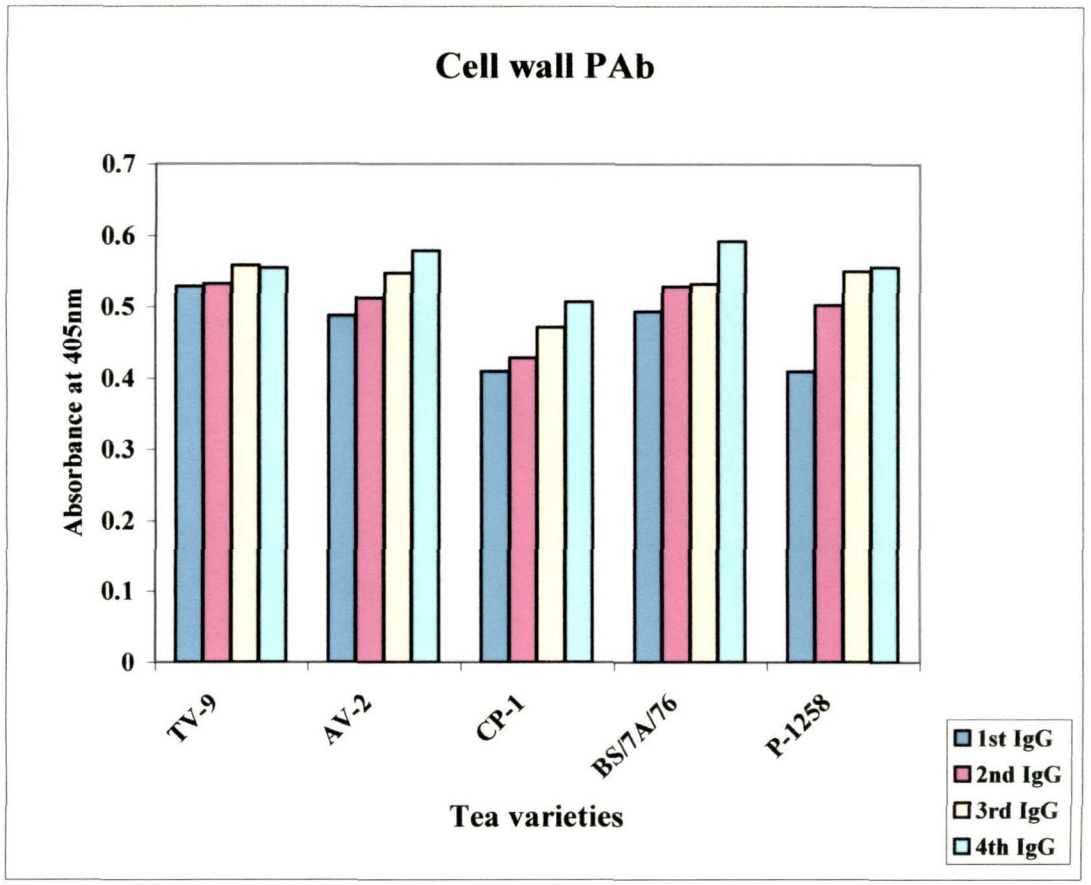


Fig.6

Cross reactivity of *S.repens* PAb with other fungi in DAC-ELISA

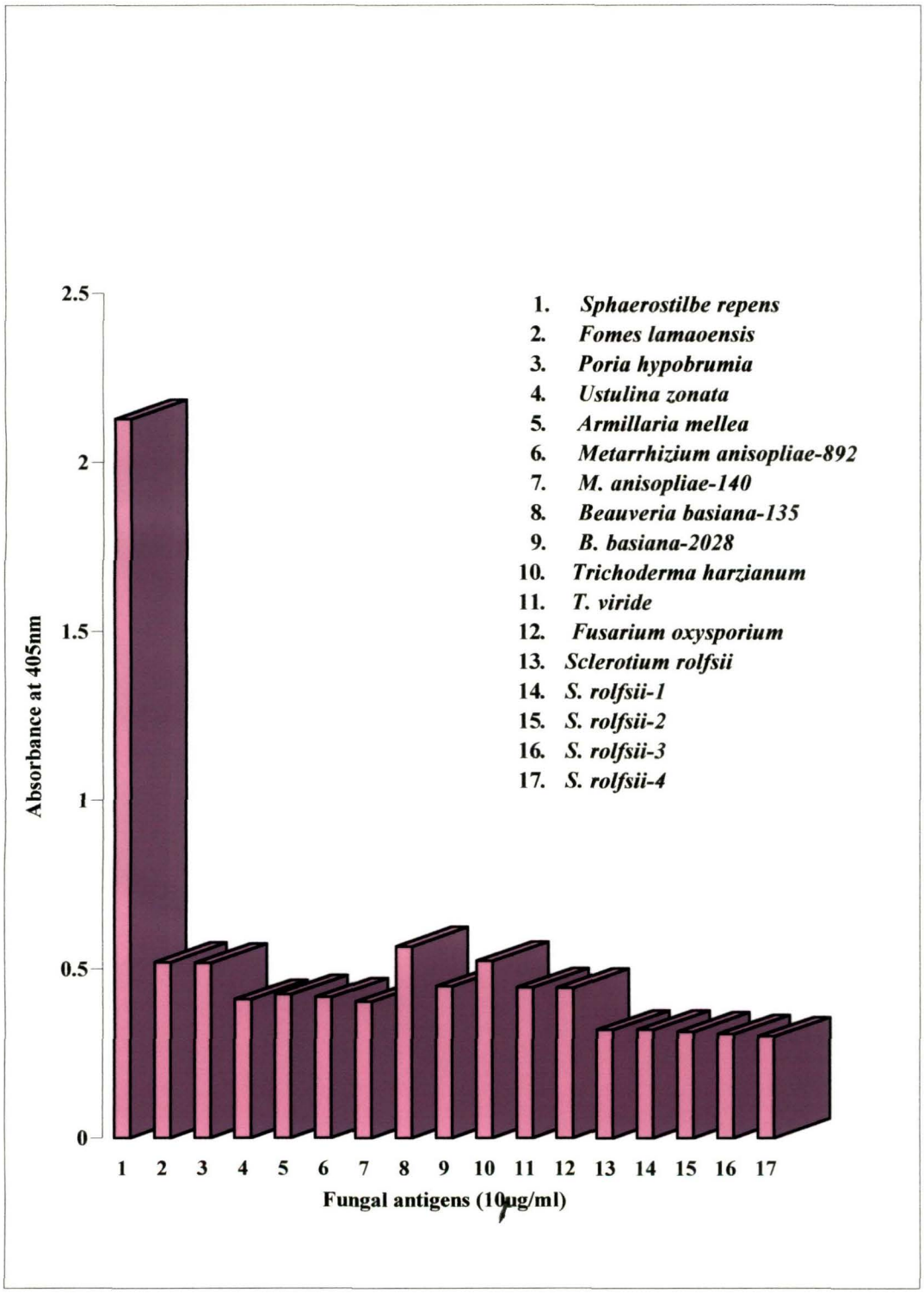


Fig.7

Table 24 : ELISA values showing reaction of PAb of *S. repens* with antigens of healthy and inoculated tea roots of Tocklai varieties

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
T-17	0.624 ± 0.036	0.829 ± 0.026	0.628 ± 0.029	0.922 ± 0.031	0.514 ± 0.026	1.043 ± 0.039
TV-9	0.513 ± 0.025	0.726 ± 0.019	0.511 ± 0.036	0.790 ± 0.026	0.489 ± 0.027	0.897 ± 0.059
TV-18	0.536 ± 0.027	0.701 ± 0.023	0.530 ± 0.041	0.768 ± 0.041	0.518 ± 0.019	0.809 ± 0.047
TV-22	0.570 ± 0.041	0.611 ± 0.041	0.566 ± 0.036	0.664 ± 0.037	0.551 ± 0.025	0.739 ± 0.032
TV-23	0.519 ± 0.023	0.534 ± 0.027	0.535 ± 0.031	0.595 ± 0.042	0.448 ± 0.027	0.680 ± 0.041
TV-25	0.517 ± 0.019	0.534 ± 0.035	0.503 ± 0.029	0.562 ± 0.052	0.440 ± 0.021	0.672 ± 0.036
TV-26	0.562 ± 0.042	0.874 ± 0.032	0.560 ± 0.044	0.938 ± 0.043	0.468 ± 0.031	1.146 ± 0.026
TV-27	0.531 ± 0.046	0.564 ± 0.048	0.547 ± 0.037	0.583 ± 0.049	0.568 ± 0.047	0.706 ± 0.031
TV-28	0.539 ± 0.027	0.573 ± 0.033	0.528 ± 0.046	0.606 ± 0.026	0.551 ± 0.049	0.739 ± 0.046
TV-29	0.516 ± 0.039	0.540 ± 0.099	0.532 ± 0.040	0.599 ± 0.021	0.435 ± 0.052	0.648 ± 0.027
TV-30	0.553 ± 0.045	0.698 ± 0.053	0.534 ± 0.029	0.725 ± 0.053	0.577 ± 0.033	0.796 ± 0.032

Antigen conc. 100 µg /ml
IgG concentration 40 µg /ml(4th bleed)
± Standard error

Table 25 : ELISA values showing reaction of PAb of *S. repens* with antigens of healthy and inoculated tea roots of Darjeeling varieties.

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
S-449	0.547 ± 0.031	0.571 ± 0.056	0.538 ± 0.039	0.595 ± 0.029	0.549 ± 0.027	0.631 ± 0.051
HV-39	0.422 ± 0.038	0.449 ± 0.041	0.429 ± 0.043	0.442 ± 0.046	0.440 ± 0.032	0.452 ± 0.042
T-135	0.574 ± 0.029	0.603 ± 0.029	0.584 ± 0.036	0.647 ± 0.019	0.583 ± 0.052	0.712 ± 0.044
AV-2	0.651 ± 0.049	0.746 ± 0.046	0.649 ± 0.029	0.795 ± 0.023	0.657 ± 0.035	0.892 ± 0.046
CP-1	0.462 ± 0.036	0.495 ± 0.029	0.438 ± 0.019	0.490 ± 0.021	0.455 ± 0.039	0.542 ± 0.033
BS/7A/76	0.586 ± 0.042	0.679 ± 0.024	0.572 ± 0.026	0.775 ± 0.047	0.592 ± 0.044	0.896 ± 0.049
P-1258	0.531 ± 0.031	0.565 ± 0.031	0.538 ± 0.039	0.587 ± 0.048	0.540 ± 0.033	0.669 ± 0.032
T-78	0.517 ± 0.021	0.586 ± 0.042	0.509 ± 0.041	0.606 ± 0.051	0.523 ± 0.03	0.695 ± 0.041

Antigen conc. 100 µg /ml
IgG concentration 40 µg /ml (4th bleed)
± Standard error

Table 26 : ELISA values showing reaction of PAb of *S. repens* with antigens of healthy and inoculated tea roots of UPASI varieties.

Tea varieties	Absorbance at 405 nm					
	Days after inoculation					
	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
BSS-2	0.543 ± 0.021	0.613 ± 0.026	0.546 ± 0.019	0.719 ± 0.041	0.561 ± 0.033	0.776 ± 0.053
UP-2	0.479 ± 0.026	0.499 ± 0.041	0.483 ± 0.033	0.518 ± 0.048	0.494 ± 0.029	0.572 ± 0.042
UP-3	0.444 ± 0.037	0.478 ± 0.043	0.445 ± 0.026	0.498 ± 0.026	0.447 ± 0.038	0.558 ± 0.037
UP-8	0.473 ± 0.028	0.391 ± 0.026	0.478 ± 0.028	0.528 ± 0.034	0.484 ± 0.046	0.571 ± 0.036
UP-9	0.426 ± 0.032	0.465 ± 0.029	0.430 ± 0.032	0.481 ± 0.042	0.433 ± 0.028	0.499 ± 0.027
UP-26	0.460 ± 0.052	0.475 ± 0.031	0.462 ± 0.027	0.550 ± 0.045	0.469 ± 0.051	0.591 ± 0.032

Antigen conc. 100 µg /ml
IgG concentration 40 µg /ml (4th bleed)
± Standard error.

Table 27 : DAC-ELISA of healthy and infected tea root antigens with PAb of *S. repens* raised against (mycelia) obtained after different bleeding.

Tea varieties	Absorbance at 405 nm							
	Source of IgG (Bleed)							
	1st		2nd		3rd		4th	
	Healthy	Infected	Healthy	Infected	Healthy	Infecteds	Healthy	Infected
TV-9	0.465 ±0.03	0.661 ±0.04	0.497 ±0.04	0.727 ±0.04	0.510 ±0.03	0.782 ±0.05	0.550 ±0.04	0.888 ±0.03
AV-2	0.515 ±0.03	0.613 ±0.05	0.561 ±0.04	0.663 ±0.05	0.624 ±0.03	0.793 ±0.04	0.650 ±0.05	0.886 ±0.05
CP-1	0.406 ±0.04	0.428 ±0.04	0.435 ±0.02	0.457 ±0.02	0.438 ±0.04	0.492 ±0.03	0.456 ±0.05	0.497 ±0.03
BS/7A/7	0.492 ±0.03	0.527 ±0.03	0.560 ±0.05	0.636 ±0.02	0.572 ±0.05	0.571 ±0.05	0.590 ±0.04	0.893 ±0.05
P-1258	0.437 ±0.02	0.554 ±0.04	0.476 ±0.03	0.560 ±0.04	0.560 ±0.03	0.498 ±0.03	0.538 ±0.06	0.697 ±0.08

Age of the plants 2 years
Antigen conc. 100µg/ml
IgG concentration 40µg/ml (4th bleed)
± Standard error
20 days after inoculation.

ELISA responses of PAb of *S.repens* with antigens of healthy and inoculated tea root tissues

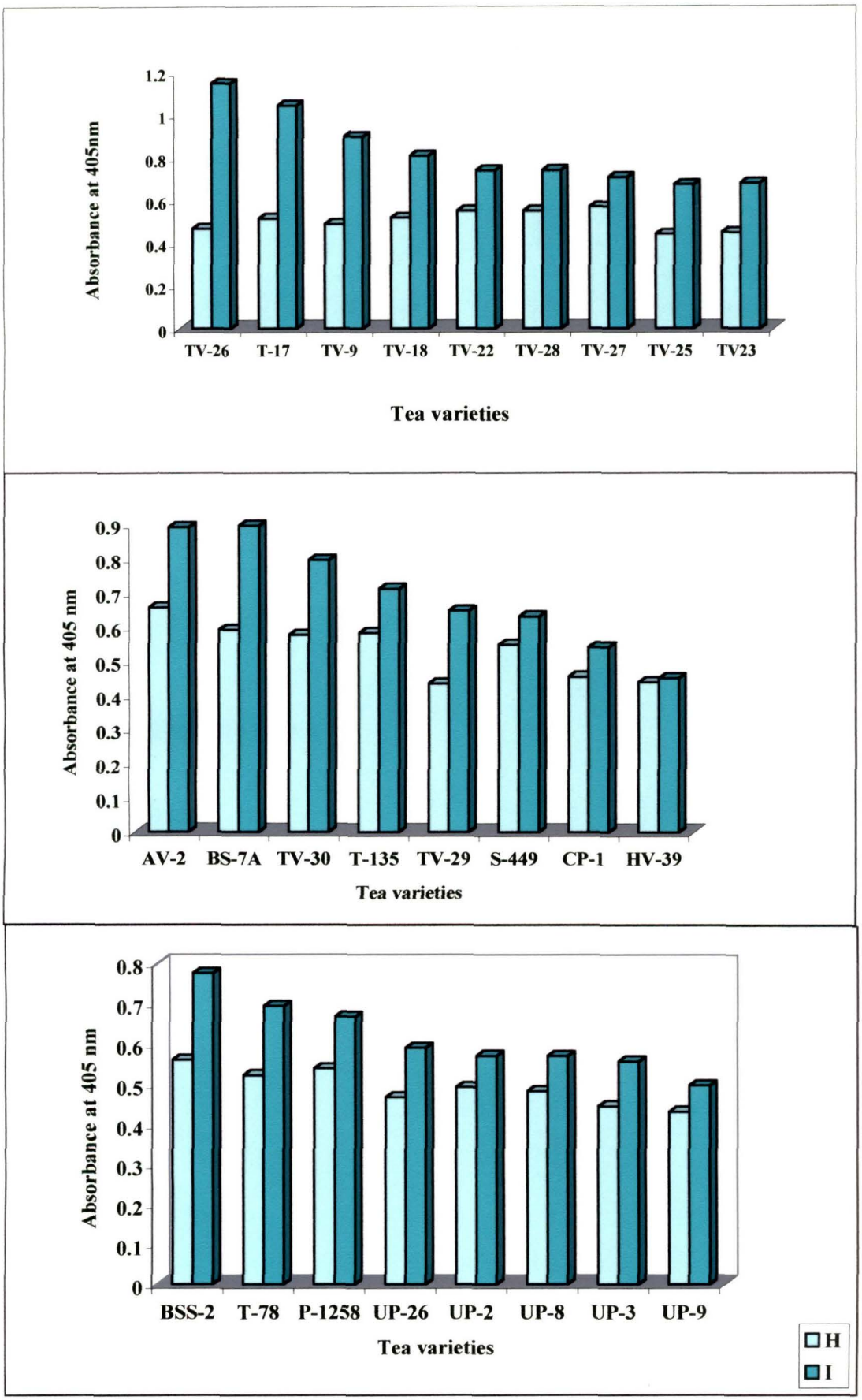


Fig.8

DAC-ELISA responses of healthy and inoculated tea root antigens against PAb of *S.repens* (mycelia)

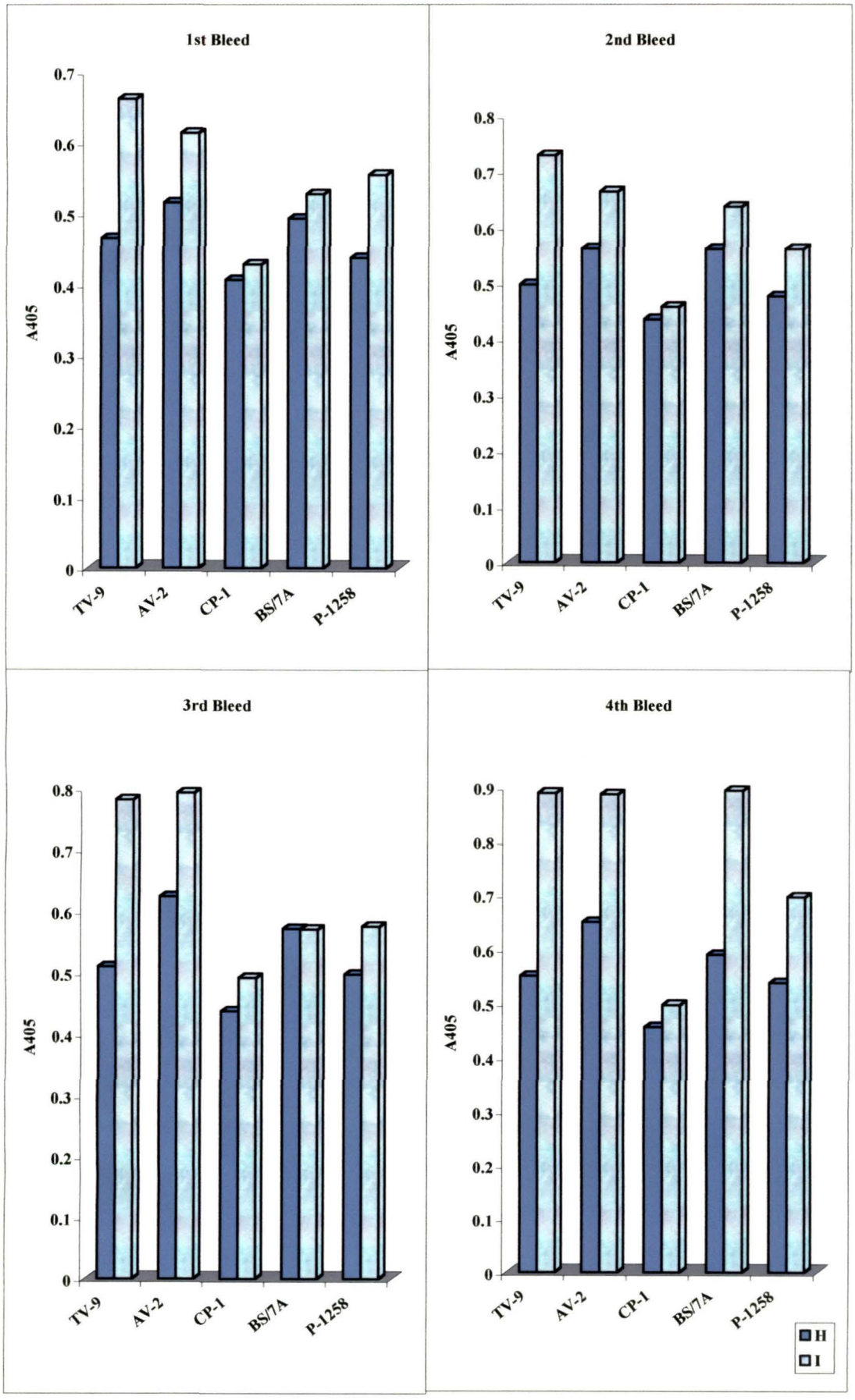


Fig.9

Table 28 : DAS-ELISA of healty and infected tea roots against PAb of *S. repens.* (raised against mycelia).

	Absorbance at 405 nm	
Variety	**Healthy**	**Infected**
TV-9	0.835 ± 0.029	1.092 ± 0.022
AV-2	0.793 ± 0.042	1.036 ± 0.036
CP-1	0.704 ± 0.023	0.867 ± 0.029
BS/7A/76	0.798 ± 0.019	1.035 ± 0.041
P-1258	0.904 ± 0.033	1.002 ± 0.017

Age of the plants 2 years; Antigen conc. 100µg/ml; IgG concentration 40µg/ml (4th bleed); 20 days after inoculation; ± Standard error.

4.5.4.2. PAb raised against cell wall

Healthy and infected tea root antigens from the five varieties were tested against PAb raised raised against *S. repens* cell wall by ELISA. DAC-ELISA reactions were carried out using PAbs from 1st 2nd, 3rd and 4th bleedings and DAS ELISA was done using IgG from 4th bleeding. In both DAC and DAS-ELISA infected root antigens showed higher reactivity than the healthy ones (Table 29 and 30, Figs 10 and 11).

Table 29 : DAC-ELISA of healty and infected tea roots antigens with PAb of *S. repens* obtained from different bleeding (raised against cell wall)

	Absorbance at 405 nm							
Tea varieties	Source of IgG (Bleed)							
	1st		2nd		3rd		4th	
	Healthy	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
TV-9	0.555 ±0.04	0.617 ±0.04	0.562 ±0.02	0.682 ±0.04	0.573 ±0.03	594 ±0.03	0.595 ±0.02	0.731 ±0.04
AV-2	0.553 ±0.04	0.663 ±0.04	0.571 ±0.03	0.691 ±0.03	0.578 ±0.03	0.719 ±0.04	0.588 ±0.02	0.781 ±0.04
CP-1	0.422 ±0.03	0.467 ±0.03	0.437 ±0.04	0.494 ±0.02	0.478 ±0.03	0.516 ±0.03	0.505 ±0.04	0.619 ±0.02
BS/7A/76	0.521 ±0.03	0.581 ±0.04	0.562 ±0.04	0.664 ±0.03	0.582 ±0.03	0.689 ±0.05	0.610 ±0.05	0.763 ±0.05
P-1258	0.440 ±0.03	0.489 ±0.02	0.466 ±0.02	0.495 ±0.03	0.497 ±0.03	0.549 ±0.02	0.554 ±0.03	0.628 ±0.04

Age of the plants 2 years; [a]20 days after inoculation; Antigen conc. 100 µg /ml ; IgG concentration 40 µg /ml; ± Standard error

DAS-ELISA responses of healthy and inoculated tea root antigens against PAb of *S.repens*

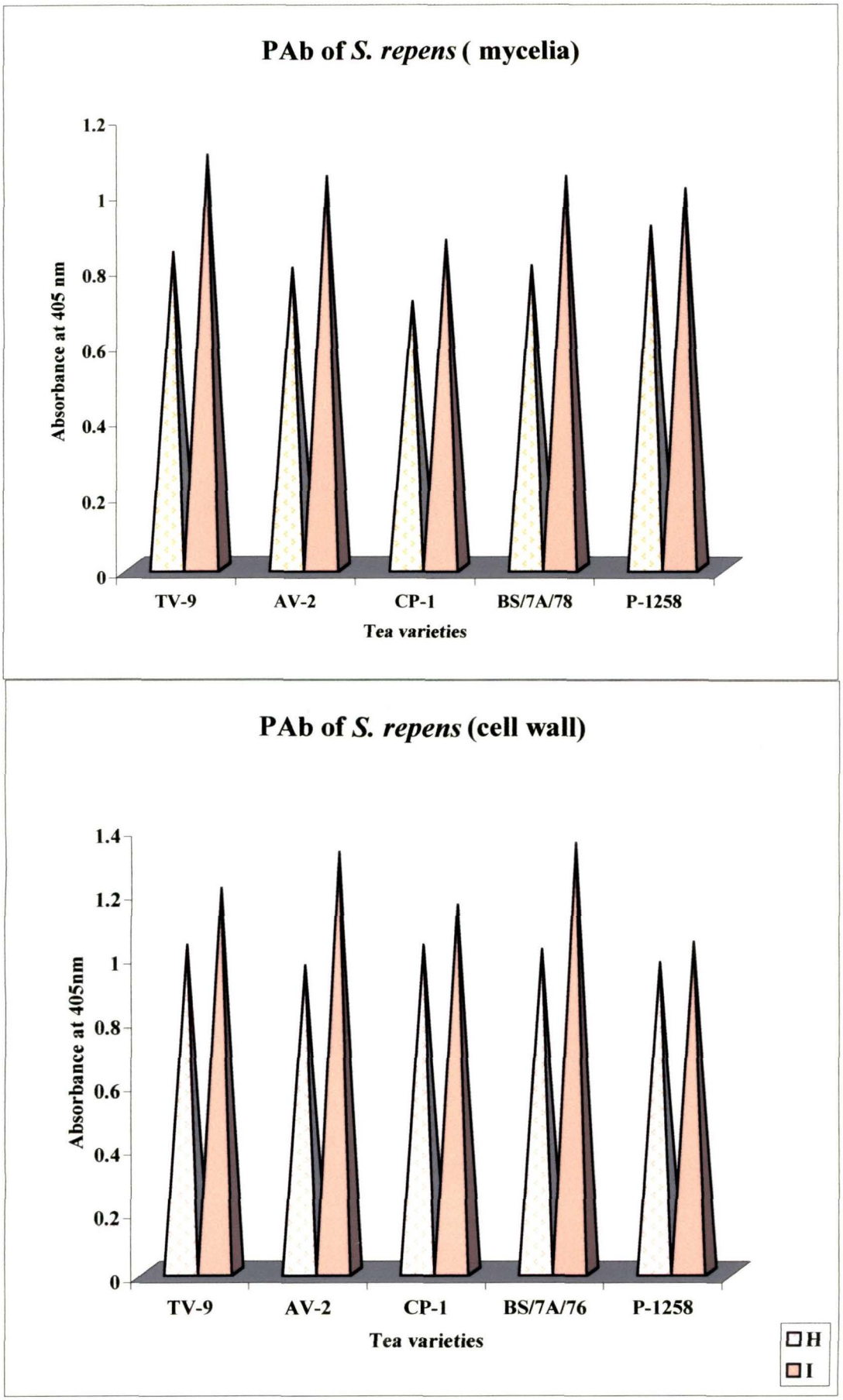


Fig.10

DAC-ELISA responses of healthy and inoculated tea root antigens against PAb of *S.repens* (cell wall)

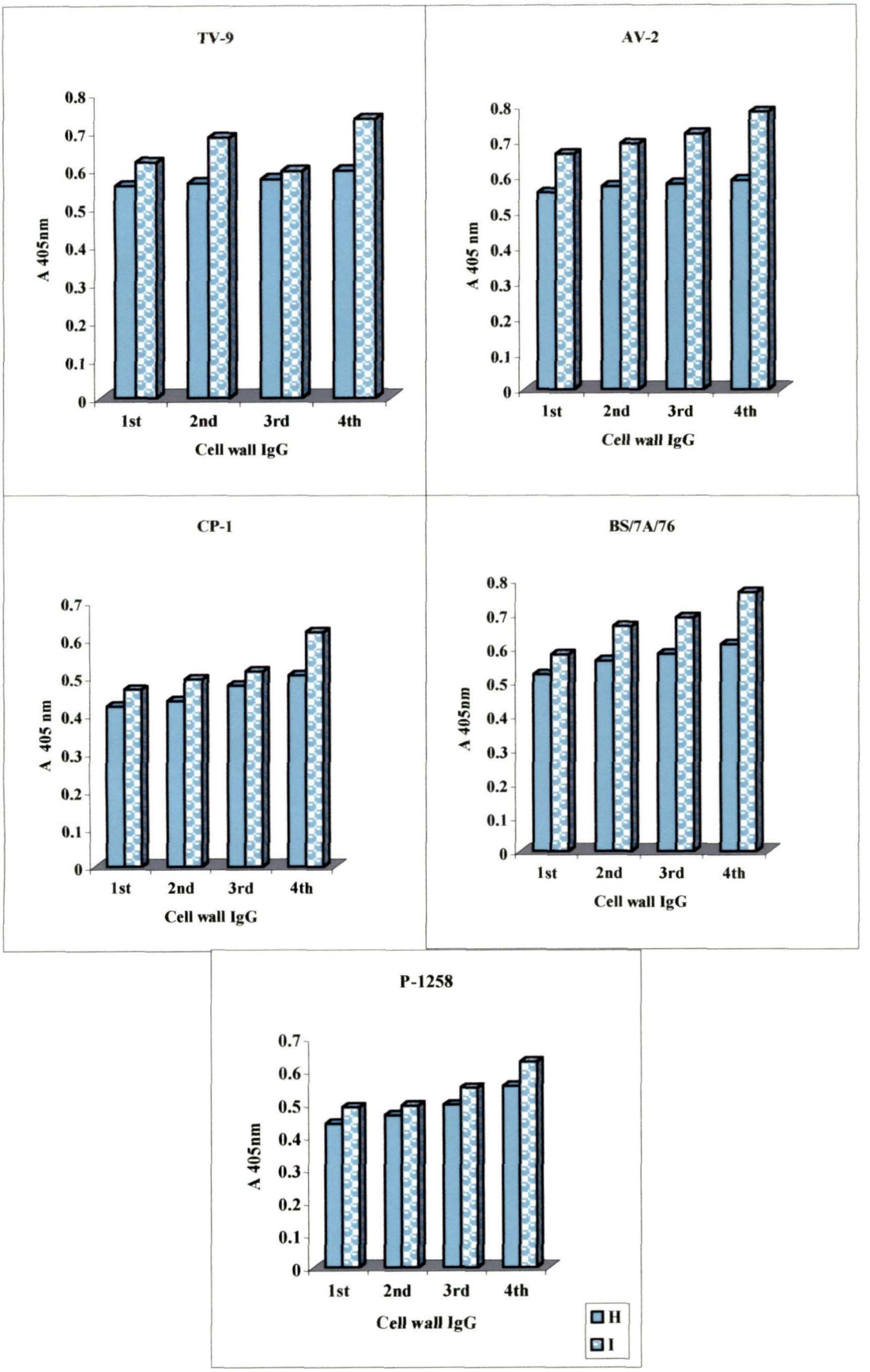


Fig.11

Table 30 : DAS-ELISA of healty and infected tea roots against PAb of *S. repens* (raised against cell wall).

	Absorbance at 405 nm	
Tea Variety	**Healthy**	**Infected[a]**
TV-9	1.027 ± 0.02	1.205 ± 0.05
AV-2	0.963 ± 0.03	1.320 ± 0.05
CP-1	1.027 ± 0.04	1.154 ± 0.04
BS/7A/76	1.015 ± 0.02	1.350 ± 0.04
P-1258	0.974 ± 0.03	1.038 ± 0.02

Age of the plants 2 years
[a]20 days after inoculation
± Standard error.

4.5.4.3. PAb raised against spores

Finally, PAbs raised against spore antigens of *S. repens* were tested against antigens from healthy and infected tea roots of the five selected varieties. Results (Table 31, Fig. 12) revealed that though the values were slightly higher in the infected tea root antigens, differences were not significant enough for detection. Hence PAbs raised against mycelia or cell wall were found to be more effective for disease detection in tea root tissues.

Table 31 : DAC- ELISA of healthy and infected tea root against the PAb of *S. repens* obtained from different bleeding (raised against spore).

Tea Varieties	Absorbance at 405 nm			
	2nd PAb		3rd PAb	
	Healthy	**Infected**	**Healthy**	**Infected**
TV-9	0.442 ± 0.03	0.472 ± 0.04	0.523 ± 0.03	0.584 ± 0.03
AV-2	0.465 ± 0.03	0.530 ± 0.03	0.531 ± 0.03	0.629 ± 0.03
CP-1	0.384 ± 0.03	0.393 ± 0.03	0.411 ± 0.04	0.446 ± 0.04
BS/7A/76	0.456 ± 0.03	0.510 ± 0.05	0.487 ± 0.03	0.592 ± 0.05
P-1258	0.416 ± 0.04	0.436 ± 0.03	0.488 ± 0.03	0.488 ± 0.04

Age of the plants 2 years
Antigen conc. 100 µg /ml
PAb concentration 40 µg /ml
20 days after inoculation.

DAC-ELISA responses of healthy and inoculated tea root antigens against PAb of *S.repens* (Spore)

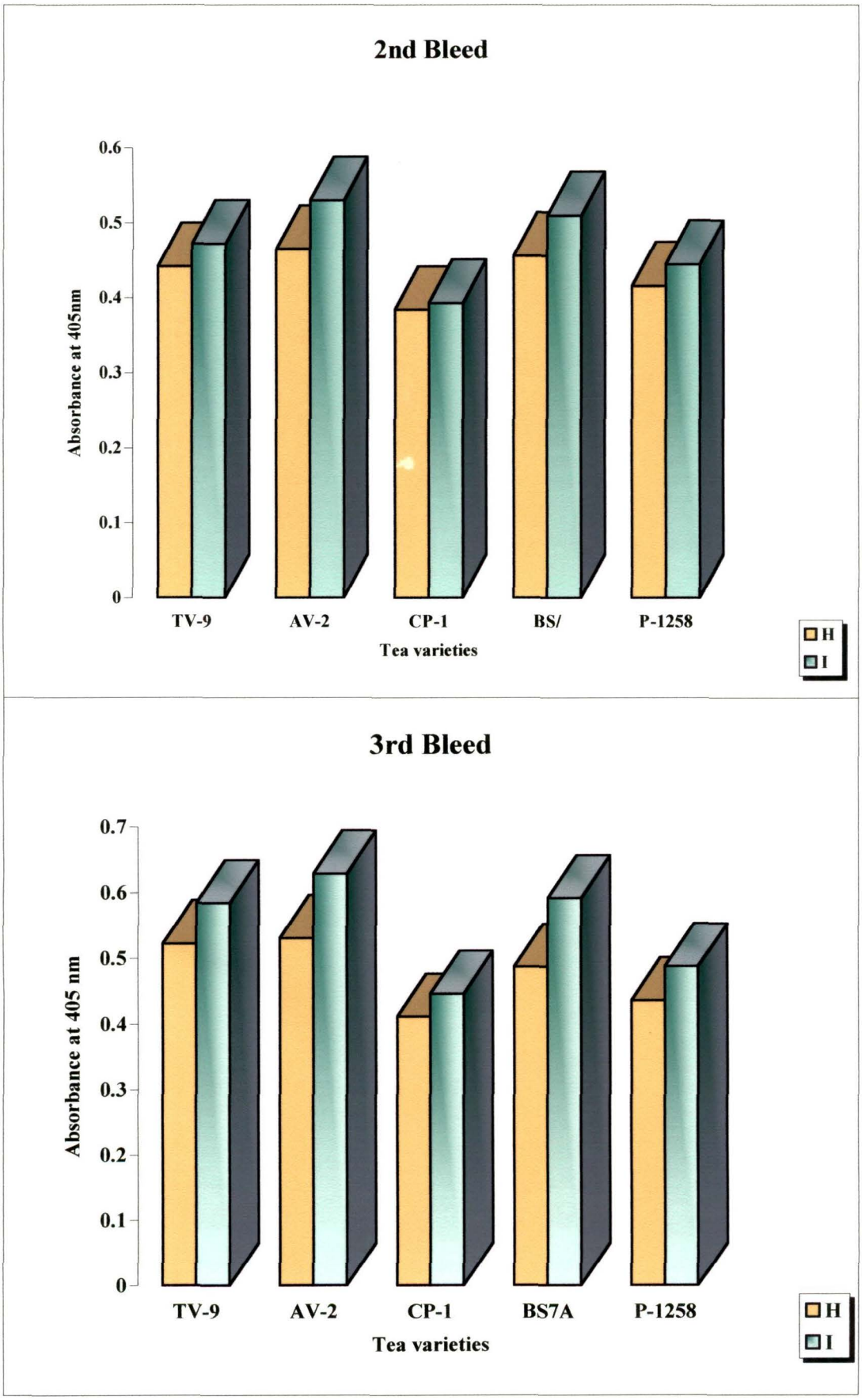


Fig.12

4.5.5. Detection of infection with other pathogens in tea roots using PAb of *S. repens*

Since tea roots are infected by a number of pathogens, it was considered worthwhile to investigate whether. PAb of *S. repens* could also react with the antigens from tea roots infected with other pathogens. Accordingly antigens from tea roots (T-17, CP-1, TV-23, UP-8 and BSS-2) infected with *Fomes lamaoensis* and *Ustulina zonata* were tested with PAb of *S. repens* by DAC ELISA. In this case also infected extract showed higher values than the healthy ones though reactivity was lesser when compared with *S. repens* infected tea root antigens. Thus a certain degree of cross reactivity was observed in this case (Table 32, Fig.13).

Table 32 : DAC- ELISA of healthy and infected (with different pathogens) tea root antigens against PAb of *S. repens*.

Tea Varieties	Absorbance at 405 nm Roots infected with			
	Healthy	***S. repens***	***F. lamaoensis***	***U.zonata***
T-7	0.654 ± 0.031	1.043 ± 0.019	0.667 ± 0.017	0.683 ± 0.036
TV-23	0.567 ± 0.046	0.671 ± 0.052	0.580 ± 0.025	0.573 ± 0.022
CP-1	0.471 ± 0.05	0.552 ± 0.042	0.481 ± 0.03	0.472 ± 0.02
UP-8	0.489 ± 0.026	0563 ± 0.034	0.495 ± 0.045	0.497 ± 0.052
BSS2	0.558 ± 0.063	0.769 ± 0.029	0.565 ± 0.046	0.561 ± 0.018

[1]Antigen concentration 100µg/ml
PAb concentration 40µg/ml
Harvesting 30 days after inoculation
± Standard error.

4.5.6. Detection of *S. repens* in soil

In order to determine whether PAb of *S. repens* could detect the pathogen in soil samples were collected from various locations including several tea estates. In one set the soil was spiked with spores of *S. repens*. Antigens from 26 soil samples were prepared and tested against PAb of *S. repens*. Spiked soils gave very high values

DAC-ELISA response with PAb of *S.repens* against antigens of tea roots infected with other pathogens

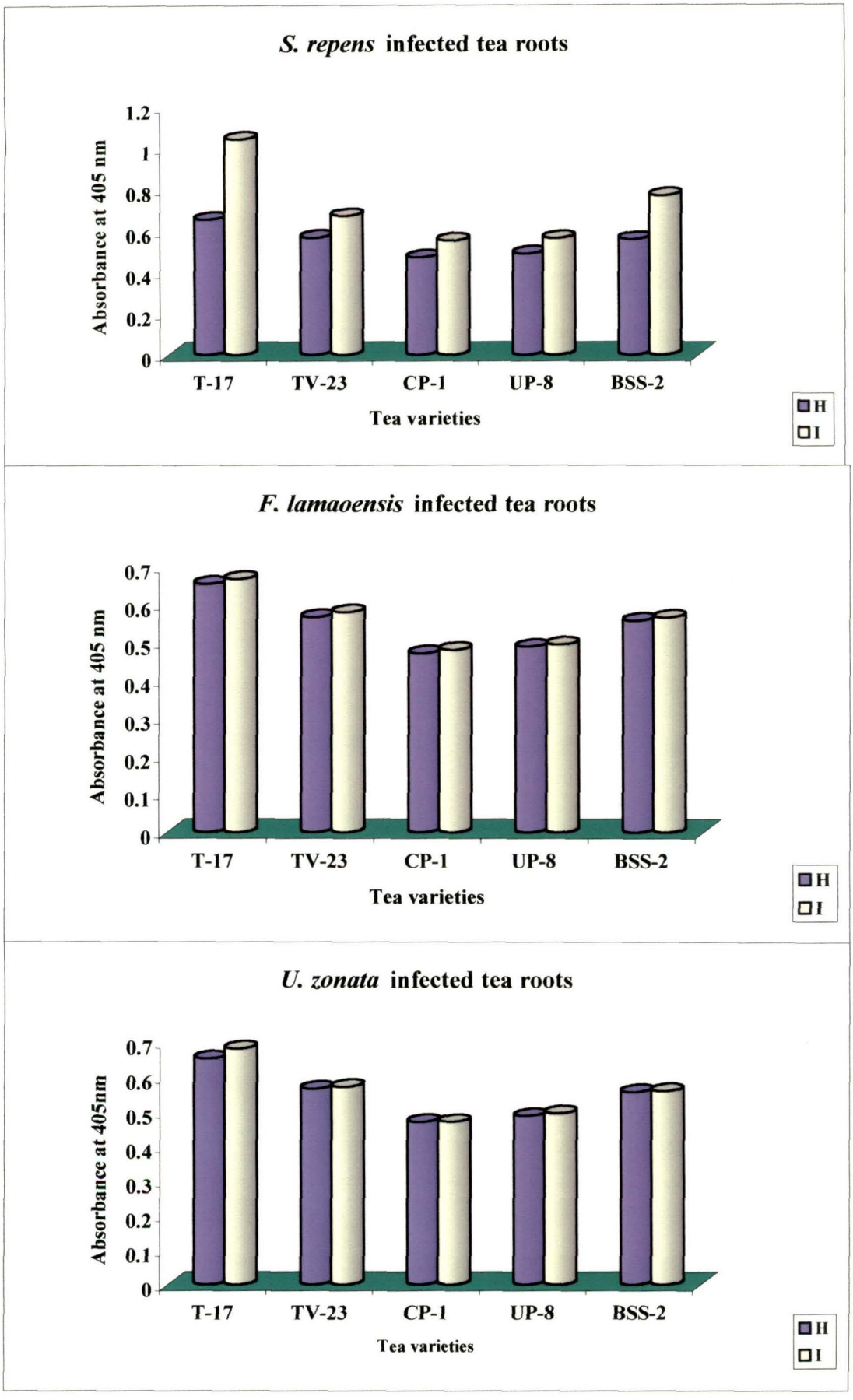


Fig.13

comparable to homologous values (Table 33). Among the different soil samples,4 samples [2 from Matigara Tea Estate (Section 11 & 12), 1 from Bijoynagar Tea Estate (Section 8) and 1 from Bentaguri Tea Estate] gave high absorbance values in ELISA (ranging from 0-9 - 1.2) indicating the presence *S. repens* propogules in those soils. Most of the other soil samples showed low A405 values in the range of 0.3-0.5.

Table 33 : ELISA responses of different soil antigens with PAb of *S. repens* mycelia.

Soil sample	**Absorbance at 405nm[a]**	**Soil sample No.**	**Absorbance at 40 5nm[a]**
1. Mycelial antigen	2.213 ± 0.0089	16. Section - 8	0.437 ± 0.053
		17 Section - 9	0.437 ± 0.061
2. Spiked soil*	2.050 ± 0.092		
Soils from Matigara T.E.		Soil from Bentaguri T.E.	
3. Section-1	0.575 ± 0.056	18. Section - 1	0.460 ± 0.026
4. Section -2	0.524 ± 0.039	19. Section - 4	0.422 ± 0.037
5. Section -3	0.708 ± 0.036	20. Sectiont -5	0.913 ± 0.042
6. Section - 4	0.565 ± 0.051	21. Section - 6	0.375 ± 0.036
7. Section -10	0.354 ± 0.032	22. Section - 7	0.418 ± 0.029
8. Section -11	1.231 ± 0.056	23. Section - 8	0.358 ± 0.024
9. Section -12	0.992 ± 0.052		
10. Section -16	0.579 ± 0.043		
Soil from Bijoynagar T.E.		Soil from Pot and field of NBU experimental garden	
11. Section -5	0.563 ± 0.029		
12. Section -8	1.206 ± 0.036		
13. Section -17	0.350 ± 0.042	24. Section - A	0.794 ± 0.041
		25. Section - B	0.356 ± 0.032
Soil from Hansqua T.E.		26. Section - C	0.414 ± 0.029
14. Section - 6	0.550 ± 0.026	27. Sterile soil	0.351 ± 0.037
15. Section - 7	0.639 ± 0.048		

PAb concentration - 1:500
± Standard error
[a] Mean value of three replicates.
A - Soil from rhizosphere of plant
B and C Sections from experimental garden
*Conc.of spore1.0 x 10^6/g soil

In order to determine whether the high values obtained amended soil is due to cross-reactivity in soil and also to determine how absorbance values in ELISA vary with different spore concentration, soil was amended with spores ranging in concentration from $7.5x10^7 - 0.1 x10^7$ spores /g soil. In another set, initially, unamended soil was cross-absorbed with PAb of *S. repens* and the serum after cross-absorption was used with amended soil to avoid the background colour. ELISA values showed decreasing values with decrease in spore concentration. Values after cross-absorption were lower, and more significantly, when spore concentration was lower. When spore concentration is low, the higher values obtained in the raw serum might be due to cross-absorption (Table 34).

Table 34 : A comparison of the detection of spores of *S. repens* in spiked soil by raw serum and same serum after cross absorption with uncontaminated soil.

Spore Concentration per g soil	**Absorbance at 405nm**[a]	
	Raw serum	**Cross absorbed serum**
$7.5x10^7$	3.319 ± 0.062	3.119 ± 0.058
$3.75x10^7$	3.100 ± 0.069	2.878 ± 0.061
$1.87x10^7$	3.00 ± 0.053	2.758 ± 0.051
$.93 x 10^7$	2.900 ± 0.043	2.469 ± 0.037
$.45 x 10^7$	2.828 ± 0.058	2.050 ± 0.049
$.22 x 10^7$	2.296 ± 0.061	1.203 ± 0.046
$.11 x 10^7$	2.02 ± 0.052	0.986 ± 0.042
Sterile soil	0.632 ± 0.039	0.607 ± 0.042
Carbonate buffer	0.272 ± 0.030	0.261 ± 0.028

[a] Mean value of three experiments.
PAb concentration 40µg/ml
4th Bleeding of mycelia

4.6. Analysis of proteins in mycelia and tea roots following infection

Proteins are major biochemical components in all plants.Proteins are generally known to either increase or decrease due to infection by pathogens and more importantly their patterns may also change. Hence for a thorough investigation into the biochemical changes in the protein patterns in tea root tissues, a number of experiments were designed.

4.6.1. Protein content

Protein was extracted from the mycelia of *S. repens* and tea root and contents were estimated. Detailed procedures for extraction and estimation have been presented under material and methods.Estimation of mycelial protein revealed that *S. repens* had protein content of 9.1 mg/gm fresh weight of tissue. Protein content of tea root tissues were estimated in the same way. Protein contents of healthy and infected tea root presented in Table 35.

Table 35 : Protein content of healthy and infected tea root (Tocklai) tissues.

Tea varieties	Protein content (mg/gm of tissue)					
	Days after inoculation					
	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
T-17	2.3±0.16	2.4±0.11	1.8±0.17	2.1±0.18	0.9±0.06	1.4±0.08
TV-9	1.3±0.11	1.5±0.01	1.1±0.08	1.3±0.094	0.9±0.56	1.2±0.07
TV-18	1.8±0.24	2.4±0.13	1.0±0.06	0.3±0.083	0.9±0.06	0.6±0.03
TV-22	1.8±0.07	2.3±0.15	1.9±0.10	1.3±0.065	0.4±0.03	1.0±0.61
TV-23	3.8±0.15	4.4±0.19	2.9±0.11	3.1±0.09	1.2±0.52	2.5±0.09
TV-25	1.2±0.08	1.5±0.11	1.0±0.05	1.3±0.11	0.9±0.05	0.9±0.63
TV-26	1.7±0.12	3.1±0.16	0.9±0.49	2.1±0.79	0.5±0.04	1.7±0.07
TV-27	1.4±0.09	2.2±0.19	1.3±0.09	1.6±0.069	1.0±0.06	1.1±0.06
TV-28	1.7±0.11	2.5±0.18	0.9±0.05	2.0±0.085	0.4±0.04	1.2±0.05
TV-29	2.9±0.15	2.2±0.13	2.1±0.07	1.3±0.085	1.2±0.05	0.9±0.06
TV-30	1.8±0.16	2.8±0.24	0.9±0.63	1.9±0.085	0.9±0.06	1.1±0.07
S-449	2.8 ±0.91	3.4 ±0.11	0.9 ±0.07	1.8 ±0.10	0.7 ±0.05	0.6±0.04
HV-39	1.7 ±0.62	3.2 ±0.12	1.3 ±0.08	1.7 ±0.12	1.0 ±0.07	1.1±0.08
T-135	1.4 ±0.68	1.7 ±0.85	1.1 ±0.07	1.4 ±0.091	0.9 ±0.07	1.0±0.07
AV-2	3.0 ±0.13	4.5 ±0.17	2.1 ±0.10	2.3 ±0.13	1.0 ±0.09	1.3±0.10
CP-1	2.9 ±0.11	5.7 ±0.22	2.2 ±0.13	3.4 ±0.16	0.92 ±0.07	1.0±0.07
BS/7A/76	2.6 ±0.85	3.6 ±0.13	1.4 ±0.11	2.0 ±0.10	0.8 ±0.07	0.9±0.06
P-1258	5.2 ±0.23	6.2 ±0.21	2.3 ±0.09	2.9 ±0.09	1.0 ±0.07	2.4±0.11
T-78	2.5 ±0.10	2.4 ±0.11	0.9 ±0.06	1.4 ±0.10	0.8 ±0.07	1.0±0.75
BSS-2	2.9±0.07	3.1±0.11	1.2± 0.07	1.4 ± 0.07	0.5±0.03	0.7±0.04
UP-2	2.0±0.08	2.0±0.09	1.7± 0.08	1.8 ±0.05	1.0±0.06	1.1±0.05
UP-3	3.8±0.11	5.8±0.23	2.0± 0.10	3.2 ±0.15	1.3±0.05	1.5±0.07
UP-8	2.5±0.11	3.0±0.13	2.0± 0.08	2.2 ±0.04	1.6±0.08	1.9±0.10
UP-9	2.9±0.09	3.0±0.11	2.2± 0.09	2.4 ±0.09	1.8±0.09	1.2±0.08
UP-26	2.1±0.08	2.3±0.10	1.2± 0.08	1.3 ±0.08	0.9±0.06	0,9±0.06

[a] Average of three replicates

4.6.2. SDS PAGE analysis

Protein extracted from different sources were further analysed by SDS-PAGE following the method described under material and methods. A number of bands were visualized in all cases following staining with coomassie from the known molecular weight mixer of 6 proteins as described in material and methods.

S. repens mycelial protein exhibited about 23 bands ranging in molecular weight from 97-14 kDa (Table 36 , Plate 10 fig.A). The bands were of varying intensities and more proteins lower molecular weight were present.SDS-PAGE analysis of fraction protein was presented in Plate 10, fig. B.

SDS-PAGE analysis of proteins from healthy root tissue revealed that it exhibited about 20 bands(Plate 10, fig.C), and lower molecular weight proteins were more in number than high molecular weight ones. In UPASI-26, infected root extracts showed very few bands, while in case of Teen Ali-17, the infected extracted showed bands of same on higher intensity than healthy ones (Plate 10, fig. D).

4.7. Characterization of mycelial and conidial wall of *S.repens*

4.7.1 Mycelial wall

Cell walls were isolated from *S.repens* and the isolated cell wall were further extracted with NaOH as described earlier. Carbohydrate and protein content of the cell wall preparation from *S.repens* were 0.75 µg/ and 7.0 µg/g respectively of fresh wt. of mycelial wall.

This preparations were further analysed by SDS-PAGE and confirmed by binding with fluoresce in labeled concanavalin A.

4.7.1.1. SDS-PAGE Analysis

In order to determie the glycoprotein nature of the cell wall preparation of *S.repens*, samples were resolved in SDS-PAGE as described earlier ,fixed in appropriate solution and stained either with Coomassie Blue R 250 or with periodic acid -Schiff's reagent respectively for protein and carbohydrate detection .Gels exhibited 11 protein bands ranging from 83 to 14 kDa of which 3 bands corresponded with high molecular weight (83,52,56 kDa) and 8 low molecular weight (33,30,29,27,23,19,17,14 kDa) when stained with periodic acid-Schiff's reagent (Table 36) These, 6 bands indicated the presence of glycoproteins in the cell wall preparation of *S.repens*.

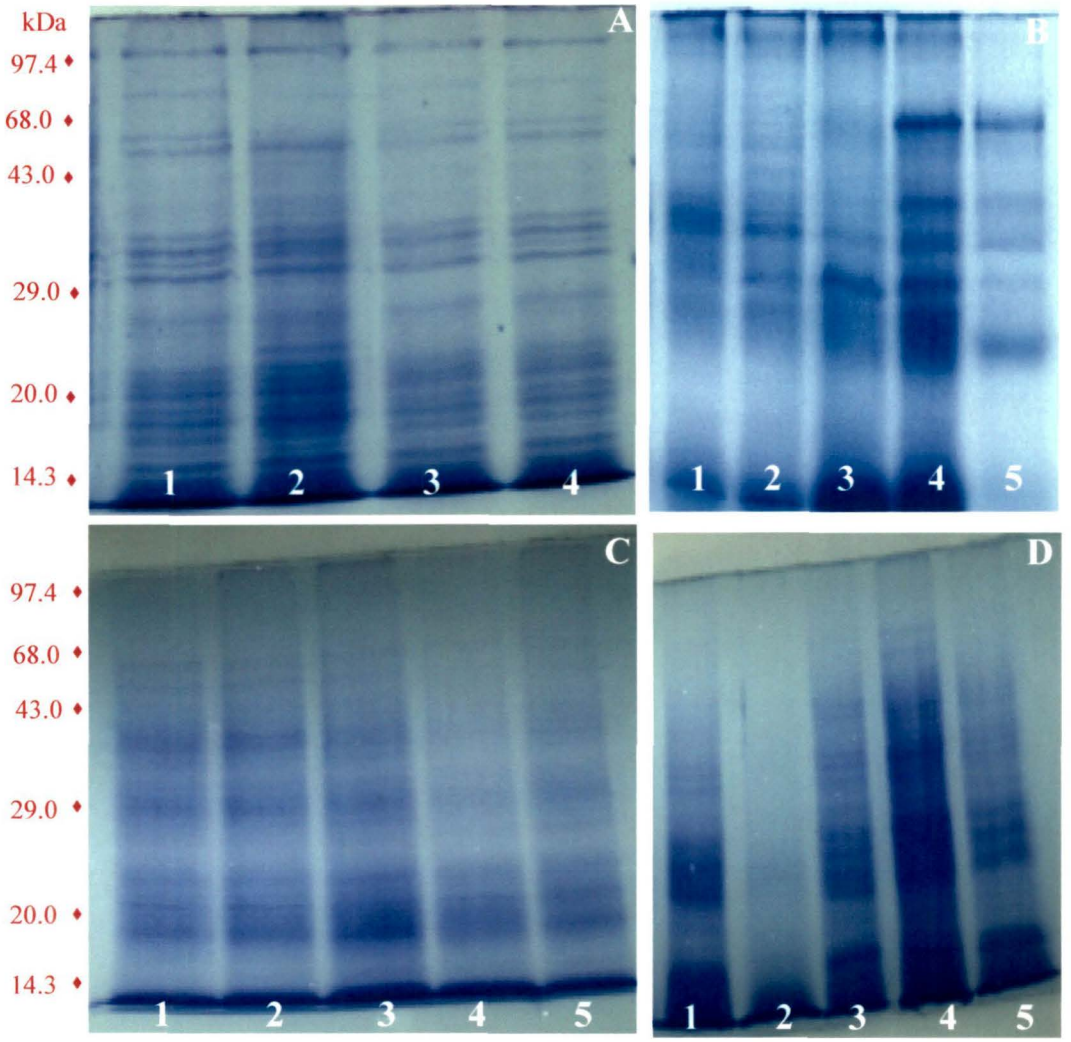


Plate 10 (Figs A-D) : SDS-PAGE analysis of mycelial and cell wall proteins of *S.repens* and tea roots. (A) Mycelial proteins [lanes 1-4]; (B) SAS fractionation of mycelial proteins [lane 1: 0-20% ; lane 2: 20-40%; lane 3: 40-60%;lane 4: 60-80% and lane 5: 80-100% SAS fractions];(C) Healthy tea root proteins (lane 1: TV-26; lane 2: TeenAli-17/1/54; lane 3: CP-1; lane 4: AV-2; lane 5: BSS-2; (D) Healthy and *S.repens* inoculated tea root proteins (lanes 1,3&5 – healthy; lanes 2&4–infected) UPASI-26 [lanes1&2]; TeenAli-17/1/54 [lanes 3-5].

Table 36 : SDS-PAGE analysis of mycelial protein and cell wall extract from *S. repens*

AntigenSource	No.of bands	Mol. wt (kDa)
		Coomassie-blue
Mycelia	23	97, 83,77, 62, 56, 50, 43, 40, 33, 30,29, 28, 27,26, 25, 23, 20, 19.5, 19, 18,17,16, 14
Cell wall	11	83, 62, 56, 33, 30, 29, 27,23, 19,17, 14
		Periodic acid-Schiff's reagent
Cell wall PAb		
Mycelia	12	62, 56, 43, 33, 30, 29, 27, 23,19,17, 14
Cell wall	6	62, 56,33, 30, 17, 14

4.7.1.2. Con A-FITC binding

To further confirm the glycoprotein nature of cel walls,mycelia or isolated cell walls of *S.repens* were treated with FITC labelled ConA and observed under the microscope as described in materials and methods. Strong fluorescence was observed under the microscope in both the mycelia and cell of *S. repens* (Plate 11). The occurrence of ConA binding substance in the cell walls further confirmed the glycoprotein nature of mycelial wall of *S. repens*.

4.7.2. Conidial wall

4.7.2.1. ConA FITC binding

Condia of *S. repens* were treated with FITC labelled ConA and observed under fluorescence microscope. Strong fluorescence was observed which reveal the glycoprotein nature of the conidial wall (Plate 12).

4.7.2.2. Agglutination

Since conidial wall plays an important role in fungal morphogencsis and development it may also be involved in recognition phenomena leading to host pathogen interaction. Lectins are proteins of non immune origin which agglutinate cells and / or precipitate glycoconjugates by specifically interacting with sugar moieties. Lectins

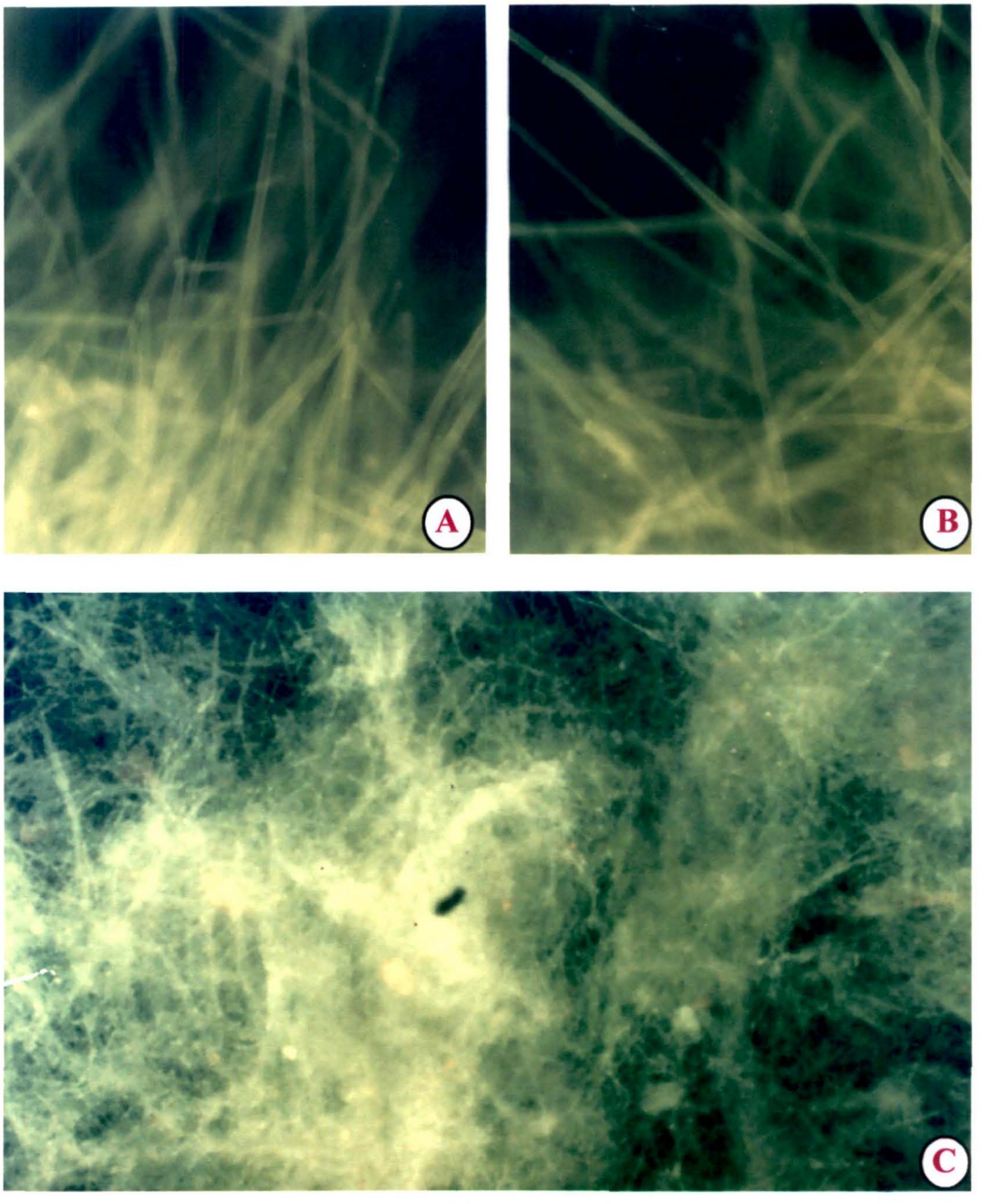


Plate 11 (Figs A-C): Fluorescence of hyphae (A&B) and isolated cell walls (C) of *S.repens* after staining with FITC-ConA

Plate 12 : Fluorescence of conidia of *S repens* after staining with FITC-ConA

have proved to be useful tools for identifying glycoconjugate components on cell surfaces. In the present investigation initially mycelial wall was characterized following which attempts were made to characterize then conidial wall by studying the agglutination effect of 4 lectins (ConA, HPA, UAE 1, WGA) on conidia. Agglutination procedure has been described in material and methods.

Agglutination were examined and arbitrarily scaled from 0 to 4 (0 = no aggutination; 1 = 1.25%; 2=26-50%; 3 = 51-75%; 4 = 76-100% agglutination) Microscopical examination revealed that conidia were strongly agglutined by conA , HPA and to slightly lesser degree by WGA.Very less agglutination was evident by UAE-II. ConA intracts specifically with α-D mannosyl and α-D glucosyl residues while HPA interacts with N-acetyl α-D galactosaminyl residues. Strong agglutination with ConA and suggests that the surface components are glycoconjugates containing α-D glucopyranoside, α-D mannopyranoside and N-acetyl galactosamine residues. Agglutination with WGA lectins which has affinity for N-acetyl glucosamine indicated that residues of this sugar was also located on the surface of conidia (Table 37).

Table 37 : Agglutination response of conidia of *S. repens* to different lectins.

Name of lectins	***Agglutination extent of condia of *S. repens****
Concanavalin - A (ConA)	4
Helix pomatia agglutinin (HPA)	4
Ulex europaeus agglutinin 1 (UEA1)	1
Wheat-germ agglutinin WGA	3

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

4.8. Immunoblotting

4.8.1. Dot-blot

Dot immunobinding technique is a rapid and sensitive method for detection of pathogen in the root and soil tissues. In the present study, dot blot was used to detect the antigen-antibody reactions in different combinations. Homologous reactions of mycelial, cell wall, spore and culture filtrate antigens showed positive racction i.e. deep pink coloured dots when fast red was used as substrate, (Table 38). When NBT/ BCIP was used as substrate, violet colour developed.

PAbs used in ths experiment were raised against mycelial, cell wall and spore antigens of *S. repens* When *S. repens* amended soil antigens were probed with PAb of *S. repens,* dots of medium colour intensity developed. Antigens of other tea root pathogens *Poria hypobrumea* and *Armillaria mellea* when reacted with *S. repens* PAb, did not show cross reactivity, but antigens from *U. zonata, Rosellinia arcuata* and *Fomes lamaoensis* showed very light coloured pinkish red dot, indicating a slight degree of cross reactivity (Table 39).

Table 38 : Dot-blot reaction of different antigen source of pathogen *S.repens* against PAb raised aginst *S. repens* mycelia.

Antigen Source	Colour intensity[a]		
	Mycelial PAb	Cell Wall PAb	Spore PAb
S. repens			
Mycelia	++++	++++	+++
Cell wall	+++	+++	+++
Spore	++++	++	+++
Culture filtrate	++++	++++	+++
Amended (*S. repens*) soil	+++	+++	+++

[a] Colour intensity of dots : – no colour; + light pink; ++ light pinkish red; +++ pinkish red; ++++ deep pinkish red
Fast red used as substrate
PAb (40µg/ml)

Table 39 : Dot-blot reaction of antigens of different pathogens with *S. repens* PAb.

Antigen Source	Colour intensity[a]
Mycelial antigen	
S. repens	++++
U. zonata	+
Povia hypolaterita	–
Armillaria melea	–
Rosellinia arcuata	+
Fomes lamaoensis	+

[a] Colour intensity of dots : – no colour; + light pink; ++++ deep pinkish red ; Fast red used as substrate ; PAb (40µg/ml)

When host root antigen dots (heathy and infected) were reacted with PAb of *S. repens,* infected root antigens showed coloured spots of different intensities. Among these, antigens from infection T-17 showed purple coloured dot (+++); while TV-26 and AV-2 had light purple coloured spots (++). Healthy tea root antigens did not react significantly with the PAb of *S. repens* (Plate 13 and Table 40)

Table 40 : Dot-blot of healthy and *S.repens* infected root antigens with *S. repens* PAb.

Antigen Source		Colour intensity[a]		
		Mycelial PAb	Cell Wall PAb	Spore PAb
Mycelia of S. repens		++++	+++	+++
Tea varities				
TV-9	Healthy	—	—	—
	Infected	+	—	+
BS/7A/76	Healthy	—	—	—
	Infected	+	+	+
CP-1	Healthy	—	—	—
AV-2	Infected	++	++	+
P-1258	Healthy	—	—	—
	Infected	+	+	—
T-17	Healthy	—	—	—
	Infected	+++	+	+
TV-26	Healthy	—	—	—
	Infected	++	+	+
TV-27	Healthy	—	—	—
	Infected	+	+	+

[a] Colour intensity of dots : – no colour; + very light violet; ++ light Violet; +++ Violet; ++++ deep violet.
NBT/BCIP used as substrate; PAb (40µg/ml)

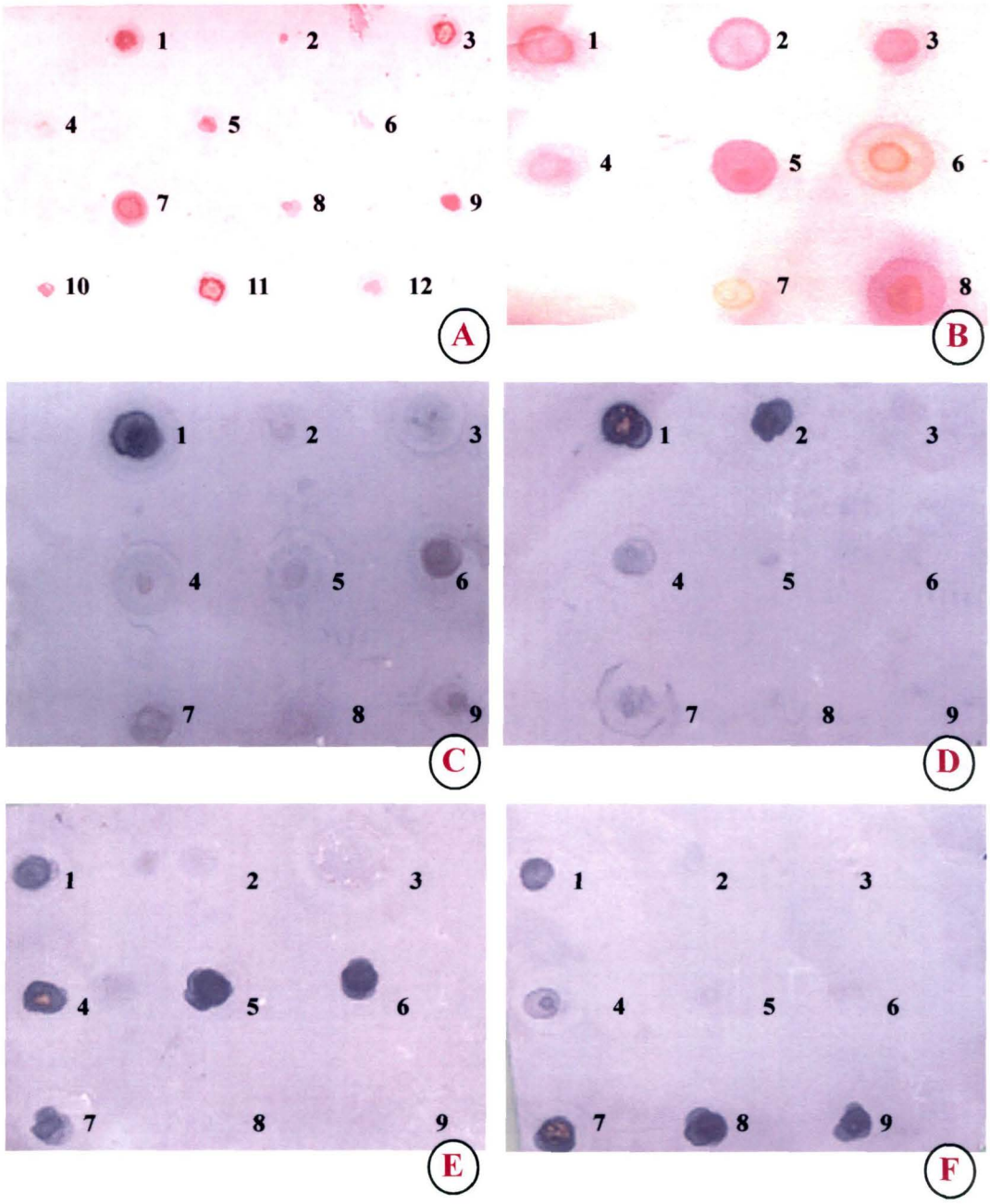


Plate 13 (Figs.A-H) : Dot immunobindings of antigens and antibody on nitrocellulose paper.Antigens were - [**A**] : mycelia (3,7,10 & 11) , cell wall (5) and culture filtrate (1) of *S.repens*; mycelia of *U.zonata* (2), *A.mellea* (4), *P.hypobrumea* (6), *R.arcuata* (8), *F.lamaoensis*.(12) ; [**B**] : *S.repens* - mycelia (1), cell wall (3), spore (5) and amended soil; *U.zonata* (4), healthy roots- TV-26 (6),CP-1(7); [**C**] *S.repens* –mycelia (1), healthy roots (2,4,5,8),*S.repens* inoculated roots (3,6,7,9) ; [**D,E&F**] : mycelia of *S.repens*(1),*T.harzianum* (4),and *T.viride* (7), amended soil (2,5,6,8,9), tea rhizosphere soil (3). Soil amended with *S.repens* (2), *T.harzianum* (5), *T.viride* (8), *S.repens*+*T.harzianum* (6), *S.repens* + *T.viride* (9). Blots were probed with PAb prepared from mycelial (A, C and D) and cell wall (B) antigens of *S. repens* ; mycelial antigens of *T. harzianum* (E) and *T. viride* (F) on nitrocellulose paper.

4.8.2. Western blotting

Molecular probing of different antigens with PAb raised against mycelial, cell wall and spore antigens of *S. repens* were also performed through western blotting. Homologous mycelial extracat exhibited 15 bands (Plate 15, fig.A, Table 41). Different fraction protein of mycelia when treated with mycelial PAb 0-20% fraction showed 7, 20-40% gave 9 bands, 40-60 gave 10 bands 60-80 showed 11 bands and 80-100 gave 10 bands (Table 41,Pate 14, fig.B). Cell wall and mycelial, each antigen showed 9 bands when probed with cell wall PAb(Table 41,Pate 14, fig.C).When spore and culture fitrate antigens were treated with mycelial PAb, spore antigens showed 7 bands ranging from 90-33 kDa and culture fitrate had 5 bands (90 to 23 kDa, Table 41, Plate14 fig.D). When the same antigens (i.e. spore and culture filtrate) probed with cell wall PAb they showed same bands as when treated with mycelial PAb but bands were more intensely stained in this case (Table 41, Plate14 fig.E). Spore and amended soil antigens were probed with cell wall and mycelial PAb separately. In the former case, 5 bands were revealed of molecular wts. between 97-15kDa (Plate 14, fig. F), whereas in the latter treatment, 4 bands of molecular wts. 80-35 kDa, were observed. Of these, 2 bands were common in both cases.

Antigens of healthy tea roots (BS/7A/76, CP-1, TV-26, Teen Ali-17 and UP-26) were probed with PAb of tea root (TV-26) and analysed by Western Blot to determine the reactivity among different varieties. Banding pattern was more or less similar in or varities, with the appearance of 3-4 bands of high molecular wt. A band of about 80 kda was absent in Tenn Ali-17 and an additional band of low molecular wt. (ca. 18kda) was present in UP-26 (Plate 14, fig. G). When healthy and infected tea root antigens were probedwith PAb of healthy tea roots (TV-26), healthy and infected extracts showed similar pattern (Plate 14, fig.H).

To detect the cross reactivity of *S. repens* with other fungi, antigens from 5 soil fungi (*U. zonata, F. lamaoensis, Fusarium oxysporum Metarrhizium anisopliae* and *Beauveria basiana*) were probed with *S. repens* PAb. It revealed no bands for any other fungus, except against homologous antigens. When healthy and infected root antigens were probed with PAb raised against 60-100% fractionated protein of *S.repens* mycelia results revealed that TV-26 infected root antigens showed 3 bands (97, 43 and 33 kDa) and infected T-17 exhibited 2 bands of 97 and 33 kDa but healthy antigens showed no band.

Table 41 : Western blotting analysis of different antigen with *S. repens* PAb

Antigen Source	No. of bands	Probed with mycelial PAb of *S. repens* Mol. wt (kDa)
Mycelia	15	97, 83, 77, 62, 50, 43, 40, 33, 30, 25, 23, 19.5, 19, 17,14
0-20% mycelial fraction	7	50, 43, 30, 19.5,19,17,14
20-40%	9	62,50, 43, 33,30, 19.5,19,17,14
40-60%	10	83, 80, 62,50, 43, 40,25, 19.5,19,17,14
60-80%	11	83, 80, 62,50, 43, 40,25, 19.5,19,17,14
80-100%	10	83, 80, 62,50, 43, 40,30 25,19,17,14 17, 14
Spore	7	97, 83, 77, 62, 48, 36, 33
Culture filtrale	5	90, 83, 62, 25, 23
Amended soil	4	77, 62, 43, 36
		Probed with Cell wall PAb of *S. repens*
Mycelia	9	97, 62, 56, 50, 43, 30, 27, 19, 14
Cell wall	9	97, 62, 50, 43, 33, 30, 25, 19, 14
Spore	7	97, 83, 77, 62, 48, 36, 33
Culture filtrate	5	83, 77, 43, 25, 14
Amended soil	5	97, 62, 43, 30, 17

4.9. Immunofluorescence

Fluorescencet antibody labelling with fluorescein isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. In the present study, to determine the tissue and cellular location of CRA in root tissues of tea varieties as well as mycelia and conidia of *S. repens* immunofluorescence was performed. Detailed methods of antibody staining of root sections and fungal cell have already been discussed under materials and methods. Root sections, mycelial, cell wall and spore preparations were photographed under UV-fluorescence.

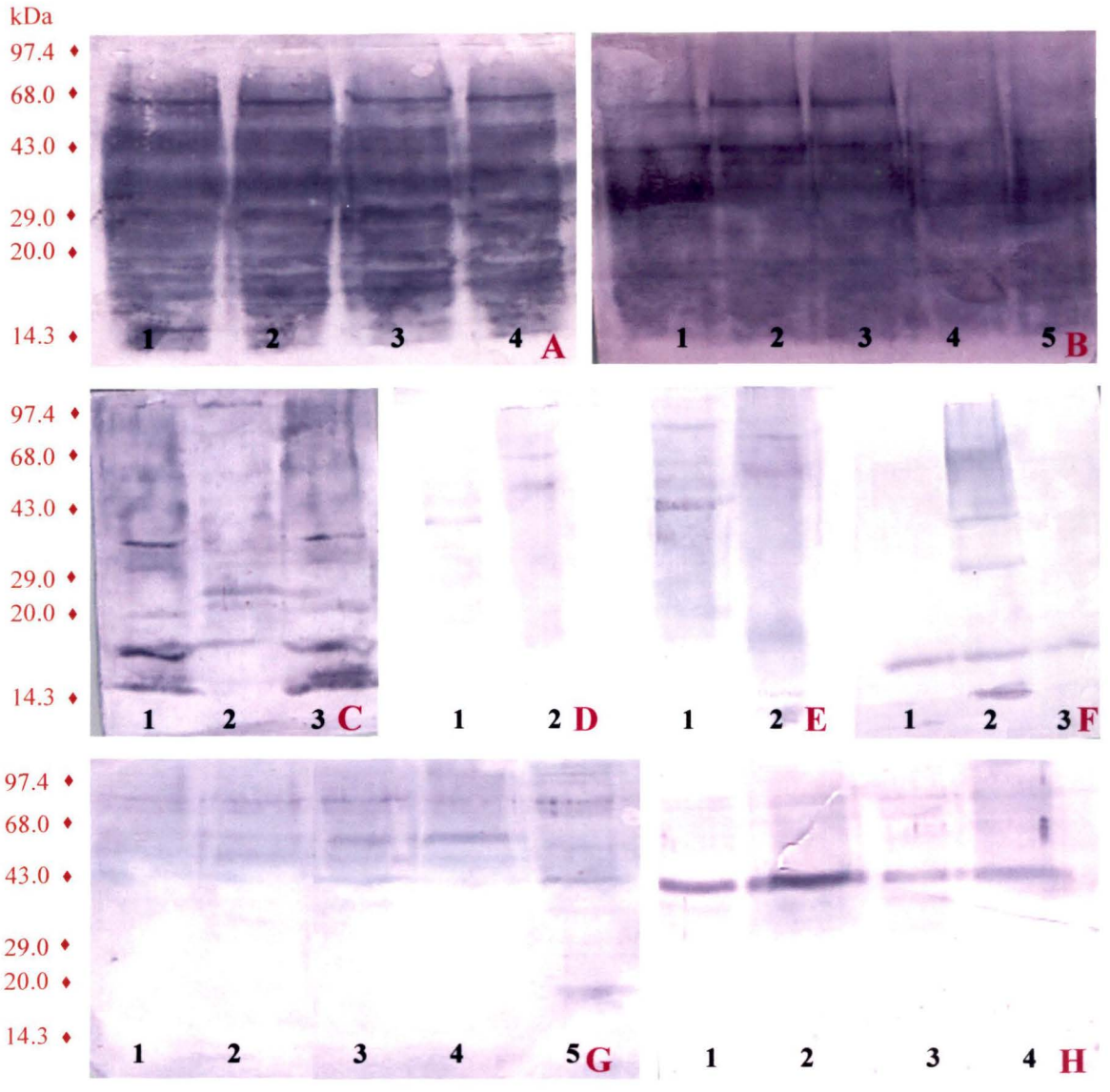


Plate 14 (Figs A-H) : Western blot analysis of *S.repens* and tea root antigens. Blots were probed with PAb raised against mycelial (**A,B&D**) and cell wall (**C,E&F**) antigens of *S.repens* and tea root (TV-26) antigens (**G&H**).(**A**) Crude mycelial antigens (lanes1-4); (**B**) SAS fractions of *S.repens* : 80-100% (lane -1), 60-80% (lane-2), 40-60% (lane- 3), 20-40% (lane- 4), 0-20% (lane-5); (**C**) lanes – 1&3 (cell wall), 100% SAS mycelia (2) of *S.repens*; (**D & E**) spore (lane-1) and culture filtrate (2) of *S.repens;* (**F**) amended soil (lane-1), soil from infected plot (lane-3), mycelia (lane-2) of *S.repens*; (**G**) healthy tea roots - BS/7A/76 (lane-1), CP-1(lane-2), TV-26 (lane-3), TV-23 (lane-4) TeenAli-17 (lane-4), UP-26 (lane-5); (**H**) Healthy tea roots– (lanes 1 & 3), *S.repens* inoculated tea roots (lanes -2 & 4) : TV-26 (lanes -1 & 2);TenAli-17/1/54 (lanes-3 & 4).

4.9.1. Mycelia

Mycelia of *S. repens* was not auto-fluorescent nor did it fluoresce when treated with normal serum followed by FITC. Treatment of mycelia of *S. repens* with homologous antiserum and FITC showed a general fluorescence that was more intense on young hyphal tips (Plate 15, fig.A&B).

4.9.2. Spore

Conidia of *S. repens* were also not auto-fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of conidia of *S. repens* with homologous antisera and FITC showed a general fluorescence throughout the surface of the conidia (Plate 15, fig.C-F).

4.9.3. Root tissue

Cross section of tea roots were treated separately with normal serum and pathogen antisera, then reacted with FITC. In sections treated with PAb of *S. repens* and then reacted with FITC, CRA was concentrated mainly arround the epiblema, cortex and endodermal cells (Plate 16, fig. A-E).

4.10. Immunocytochemical staining

Immunocytochemical staining, based on specific antibodies produced against *S.repens* could provide a means of visualizing hyphae of this fungus. Production of a specific immunocytochemical stain involves preparation of a suitable antigen, appropriate methods of evaluating specificity of the antibodies, and development of the immunocytochemical staining procedure. In the present study, specificity refers to binding of antibodies only to the fungus against which they were produced and not to other species of fungi in the same habitat. The purpose of this study was to develop a specific immunocytochemical stain for *S.repens* for detection of hyphae of the fungus within tea root tissues. Tea Plants (TeenAli-17/1/54 and TV-26) were artificially inoculated with *S.repens* in the experimental field. After 30 days of inoculation the affected plants were uprooted and washed properly. Transverse sections were made from the infected tea roots and PAb raised against mycelial antigens of *S.repens* were used for probing the fungal hyphae which penetrated the root tissues. Plate 17 (figs A-L) illustrate the labeling pattern as observed with serial sections of TV-26 (figs A,C,E,G,I&K) and TeenAli-17/1/54 (figs B,D,F,H,J&L) treated with PAb of *S.repens*

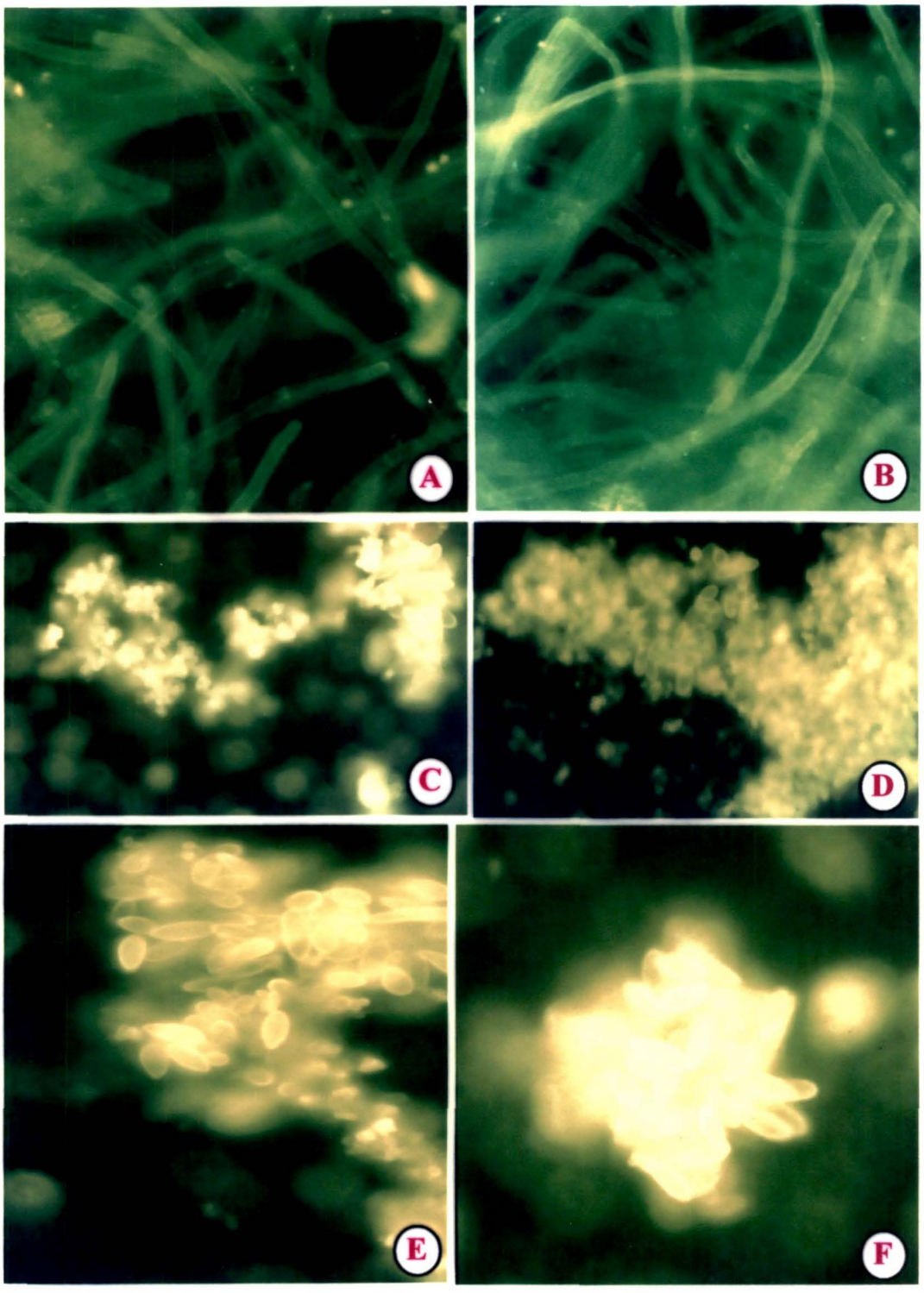


Plate 15 (Figs A-F) : Fluorescence of hyphae (A&B) and spores (C-F) of *S. repens* treated with PAb of *S.repens* (mycelia : A&B ; conidia : C-F) and FITC labelled antibodies of goat specific for rabbit globulin . A & C-E : PAb of 3rd bleeding ; B & F: 4th bleeding.

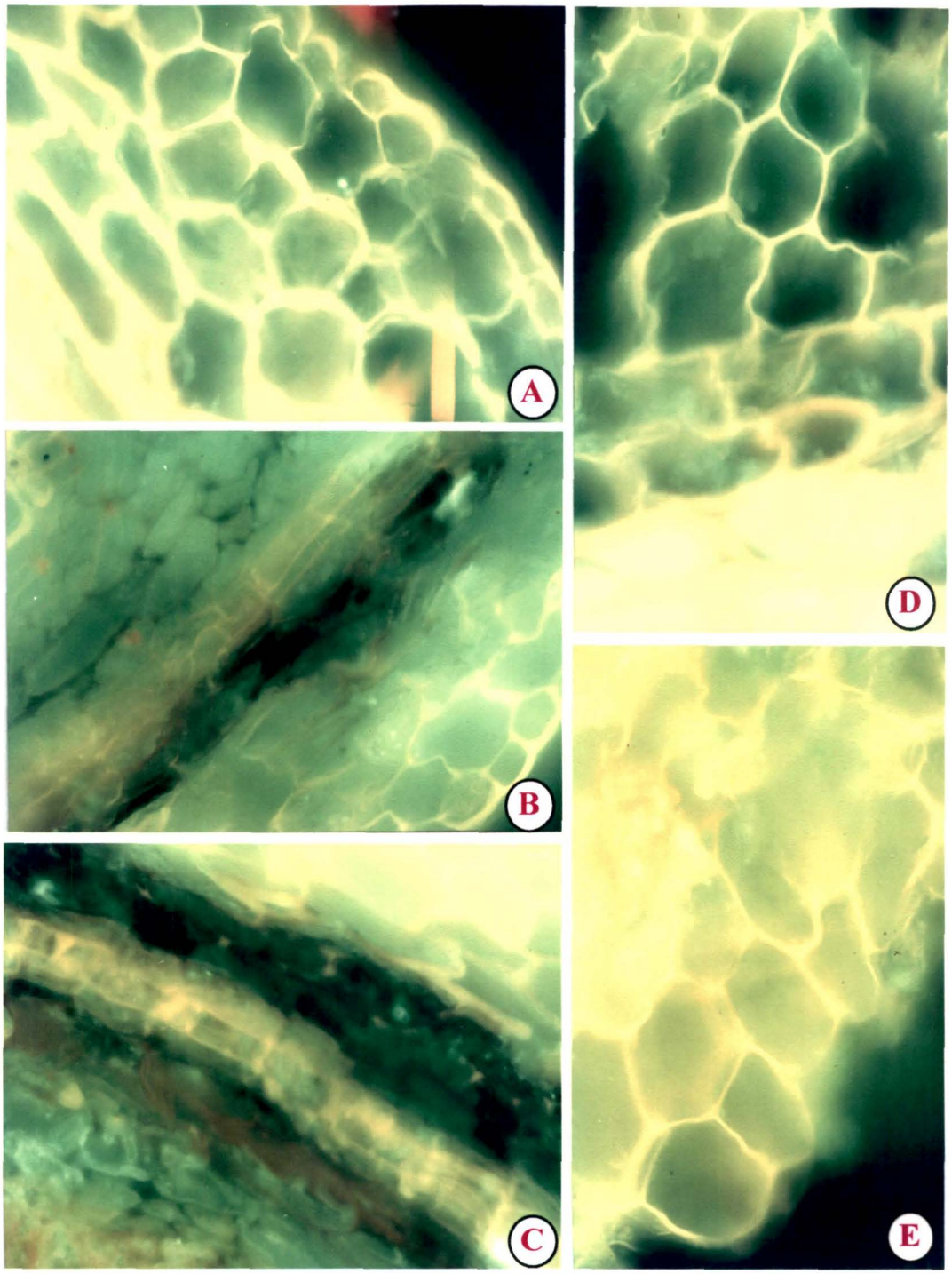


Plate 16 (Figs A-E) : Fluorescent antibody staining of tea root tissues for cross reactive antigens shared with *S.repens*. Healthy root tissues treated with PAb of *S.repens* (4^{th} bleeding) and labelled with FITC antibodies of goat specific for rabbit globulin. (A-C): TeenAli-17/1/54 ; (D-E): TV-26.

and stained with Fast blue BB salt. Both these varieties exhibited highly susceptible reactions in varietal resistance test. Using PAb of *S.repens,* when detection of pathogen in artificially inoculated tea roots were done by DAC-ELISA and dot blots , high absorbance were noticed in ELISA test as well as intense colour developed in dot blots for these two varieties. Beneath the bark tissue, rhizomorph development was clearly visible in TV-26 (Plate 17, fig A). On the other hand hyphal penetration throughout root tissue was evident.

4.11. Biological control of violet root rot disease with *Trichoderma* sp.

4.11.1. *In vitro* test

Antagonistic properties of *Trichoderma harzianum* and *T. viride* were studied through dual plate method (Biswas and Sen, 2000). Mycelial discs of 6mm dia cut from the margin of 5 days-old cultures of both test pathogen (*S. repens*) and antagonists were placed opposite to each other on PDA in petriplates (9 cm dia.) The distance between inoculum blocks was 6cm. The pathogens and antagonists were placed in the same day.Control plates were also prepared both for pathogen as well as for antagonists. Three replications of each were done. Inoculated petriplates were incubated at 30 $\pm$ 2^{0}C in an incubator.

After 3 days *Trichoderma viride* overgrew the pathogen and lysed it over a period of time. But *T. harzianum* did not overgrow the pathogen. In this case pathogen formed an inhibition zone around it though pathogen also was not able to grow further i.e. its growth was ceased. (Plate 18).

4.11.2. *In vivo* test

The experiments to manage the disease (violet root rot of tea) in potted and field conditions were designed by applying biocontrol agents. Two *Trichoderma* species *T. harzianum* and *T. viride* were used for this purposes. The experiments in both cases were set up with the following treatments : (a) pathogen (*S. repens*) (b) *T. harzianum* (c) *T. virde,* (d) *S. repens* + *T. harzianum,* (e) *S. repens* + *T. viride* and (f) control plants i.e. healthy. Biocontrol agents were infested in the rhizosphere ten days before inoculation with pathogen. Ten replicates of each treatment was taken, the effect of treatment on disease development was determined.

Plate 17 (Figs A-L) : Immunoenzymatic staining of tea root tissues artificially inoculated with *S.repens*. PAb of *S.repens* were reacted with root tissues and stained

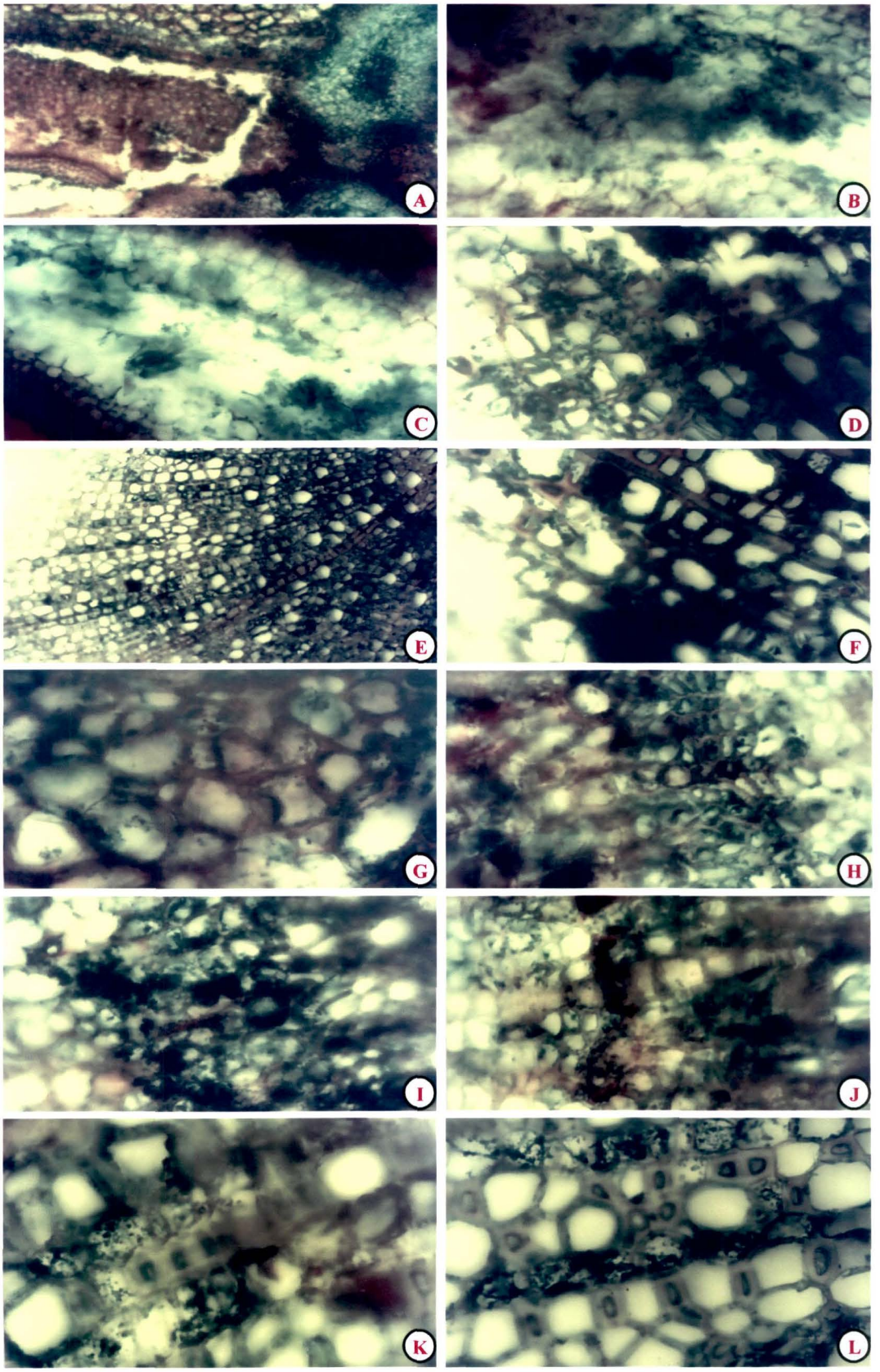


Plate 17 (Figs A-L) : Immunoenzymatic staining of tea root tissues artificially inoculated with *S.repens*. PAb of *S.repens* were reacted with root tissues and stained with Fast blue BB salt. (A,C,E,G,I & K : TV26; B,D,F,H,J &L : TeenAli-17/1/54)

4.11.2.1. Potted plants

In case of potted plants, disease rating was on the basis of both under ground and above ground symptoms as mentioned in material and methods. Results revealed that treatment of two year old plants with either *T. harzianum* or *T. viride* reduced disease significantly, of which *T. viride* was more effective (Table 42).Population of *S. repens* was also determind in the various treatment. At the begining , in all inoculated pots, concentration of *S. repens* was 2×10^6/ 100g soil. after 30 says, in the pots where *S.repens* alone was inoculated, spore concentrations had increased to 3.5×10^6/ 100g soil. However ,pots where *T. harzianum / T. viride* were added the concentration of *S. repens* was almost negligible.

Table 42 : Effect of *Trichoderma harzianum,* and *Trichoderma viride* on violet root rot of tea (Teen Ali-17/1/54) caused by *S. repens* (30 days after inoculation)

Treatments	Disease index
S. repens	5.210 ± 0.13
T. harzianum + S. repens	1.305 ± 0.05
T.viride + S. repens	0.943 ± 0.04

± Standard error

[a] 0 = No symptoms; 1 = Plants look sick and root surface started rouginening in patches 2 = Most of the leaves withered or looking yellow, light black patches with rough surface; 3 = Defoliation started / random yellowing root inky black with random patches; 4 = Random defoliation, Upto 70% become black; 5 = Total defoliation, 70 to 85% blackening; 6 = Total defoliation and shoot become dry, 85 to 100% blackening and dry.

4.11.2.2. Field grown plants

For field experiments three varieties (Teen Ali-17/1/54, TV-26 and T-78) were selected . Different treaments with the biocontrol agents were done as described previously. Disease incidence were recorded as that of potted plants.Both *T. viride* and *T. harzianum* significantly reduced disesae intensity(Table 43, and Fig. 14).

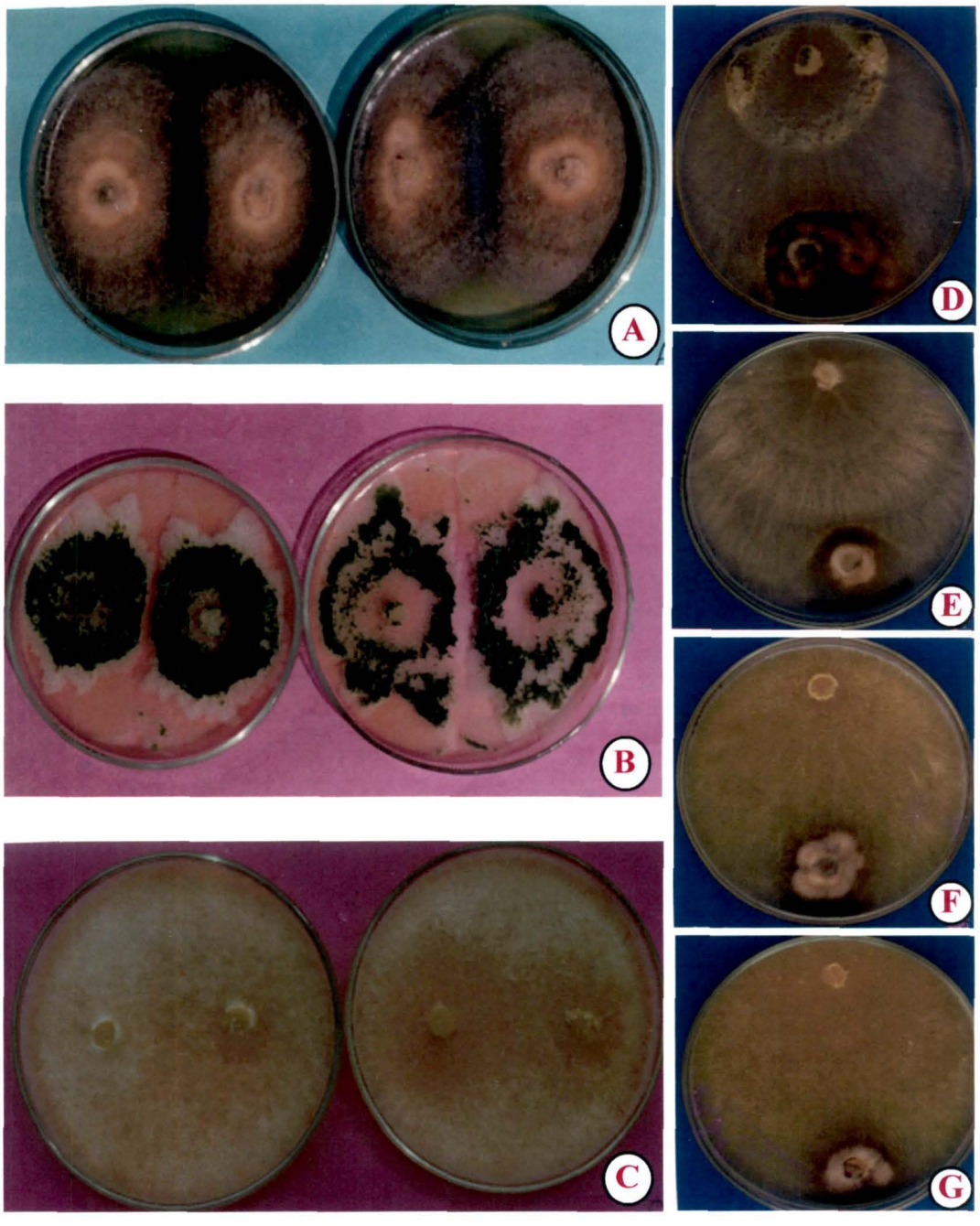


Plate 18 (Figs A-G) : Pairing of *S. repens* with *Trichoderma harzianum* and *T. viride*.A,B&C: homologous pairing of *S. repens, T. harzianum* and *T.viride,* respectively. Pairing of *S. repens* with *T. harzianum* (D & E) and with *T. viride* (F & G).

4.12. Detection of *S. repens* in tea root and soil following treatment with biocontrol agents

4.12.1. ELISA

Since *T. viride* and *T. harzianum* reduced intensity of violet root rot disease caused by *S. repens*, it ws decided to investigate whether this reduction could also be determined immunologically in both soil and root tissues. For this purpose, DAC-ELISA and competition ELISA . ELISA were performed with root antigens from different treatment as well as soil antigens.

Table 43 : Effect of *T. harzianum* and *T. viride* on *S. repens* infection in field condition

Tea varieties		Disease index[a]		
		Days after inoculation		
	Treatments	15	30	45
TV-26	*S. repens*	3.62 ± 0.17	5.51 ± 0.19	5.97 ±0.20
	S. repens + *T.harzianum*	0.35 ± 0.06	1.31 ± 0.01	1.35 ± 0.11
	S.repens+ *T.viride*	0.25 ± 0.04	0.89 ±0.05	0.90 ± 0.12
TV-18	*S. repens*	1.86 ± 0.09	3.69 ± 0.18	4.8 ±0.24
	S. repens + *T.harzianum*	0.20 ± 0.03	1.00 ± 0.08	1.0 ±0.06
	S.repens+ *T.viride*	0.18 ± 0.02	0.72 ± 0.05	0.75±0.06
T-78	*S. repens*	0.92 ± 0.06	2.41 ± 0.1	3.96 ± 0.2
	S. repens + *T.harzianum*	0.20 ± 0.05	0.80 ± 0.06	0.75 ± 0.06
	S.repens+ *T.viride*	0.20 ± 0.04	0.80 ± 0.07	0.70 ± 0.06

± Standard error; [a]0 = No symptoms; 1 = Plants look sick and root surface started rouginening in patches; 2 = Most of the leaves withered or looking yellow, light black patches with rough surface; 3 = Defoliation started / random yellowing root inky black with random patches; 4 = Random defoliation, Upto 70% become black; 5 = Total defoliation, 70 to 85% blackening; 6 = Total defoliation and shoot become dry, 85 to 100% blackening and dry.

No disease was obtained uninoculated controls or those treated with *T. harzianum/ T. viride* alone.

4.12.1.1. Root tissue

For biocontrol expriments in potted plants, Teenali - 17/1/54 (suceptible to *S.repens*) was selected. After 40 days of infestation with biocontrol agents and 30 days of pathogen inoculation, plants were uprooted and antigens were made from the roots subjected to different treatments. These antigens were used with PAb raised against mycelia of *S. repens* in DAC-ELISA. Results showed that in case of roots treated with *T. viride* and *T. harzianum* ELISA values are almost same as that of the healthy root antigen. Antigens of roots from the *S. repens* + *T. viride* and *S. repens* + *T. harzianum*- treated soil showed slightly higher absorbance than healthy, while only pathogen-treated root showed almost double absorbance value as that of healthy tissue (Table 44).

Table 44 : DAC-ELISA of tea root antigens with PAb of *S. repens* following treatment with biocontrol agents.

Antigen source (Tea root)	**Absorbance at 405 nm**			
	Expt.1	**Expt.2**	**Expt.3**	**Mean**
Healthy	0.492	0.501	0.500	0.497 ± 0.07
Inoculated with				
S. repens	0.837	0.850	0.823	0.837 ± 0.067
T. harzianum + *S. repens*	0.605	0.600	0.616	0.607 ± 0.052
T. viride + *S. repens*	0.552	0.549	0.546	0.549 ± 0.031
T. virdie	0.495	0.494	0.497	0.495 ± 0.022
T. harzianum	0.500	0.508	0.503	0.504 ± 0.036

Days after inoculation-30
Tea variety - T-17
Age of the plants - 2 years
± Standard error.

4.12.1.2. Soil

S. repens in the rhzosphere of different treatment was evaluated through DAC-ELISA and competition ELISA, by reacting the different soil antignes with PAbs of *S. repens, T. viride* and *T. harzianum.*

In DAC-ELISA, when antigens from soil treated with *S. repens* + *T. viride* or *S. repens* + *T. harzianum* were treated with PAb of *S. repens*, it showed significantly lower A_{405} values, than when antigens from soil treated with *S. repens* alone were reacted with the same PAb. This showed that population of *S repens* in soil had-been reduced by the biocontrol agents. This was also confirmed by determining the populaiton of *S. repens* is soil (Table 45, Fig. 15).

Similar results were also obtained in case of competition ELISA, which is an inhibition ELISA. Reduction in the population of *S. repens* in soil presence of *T. viride* and or *T. harzianum* was confirmed by treatement of antigens from soils under various treatments as mentioned earlier. Since competition ELISA is a double binding assay, where the PAb is allowed to react with the antigens first, and the residual PAb is once again reacted with same antigens in different plates higher ELISA values in this ELISA, would indicate lower reactivity. Results in this case showed that higher values were obtained when soil was treated with *S. repens* + *T. harzianum* / *T. viride* indicating lesser populatio of *S. repens* (Table 46).

Table 45 : DAC-ELISA of soil antigens with PAbs of *S. repens. T. harzianum and T.virde*

	Source of PAbs		
Soil antigen source	*S.repens*	*T. harzianum*	*T. viride*
Uninfested soil	0.295 ± 0.02	0.301 ± 0.02	0.316 ± 0.03
S. repens	0.637 ± 0.03	0.350 ± 0.03	0.323 ± 0.02
T. viride + *S. repens*	0.305 ± 0.02	0.400 ± 0 .03	0.516 ± 0.03
T. harzianum + *S. repens*	0.322 + 0.03	0.529 ± 0.03	0.336 ± 0.02
T. viride	0.335 ± 0.03	0.424 ± 0.04	0.697 ± 0.03
T. harzianum	0.365 ± 0.03	0.608 ± 0.04	0.403 ± 0.02

Samples collectd 30 days after inoculation with *S. repens* and 40 days after amendment with biocontrol agents
± Standard error

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

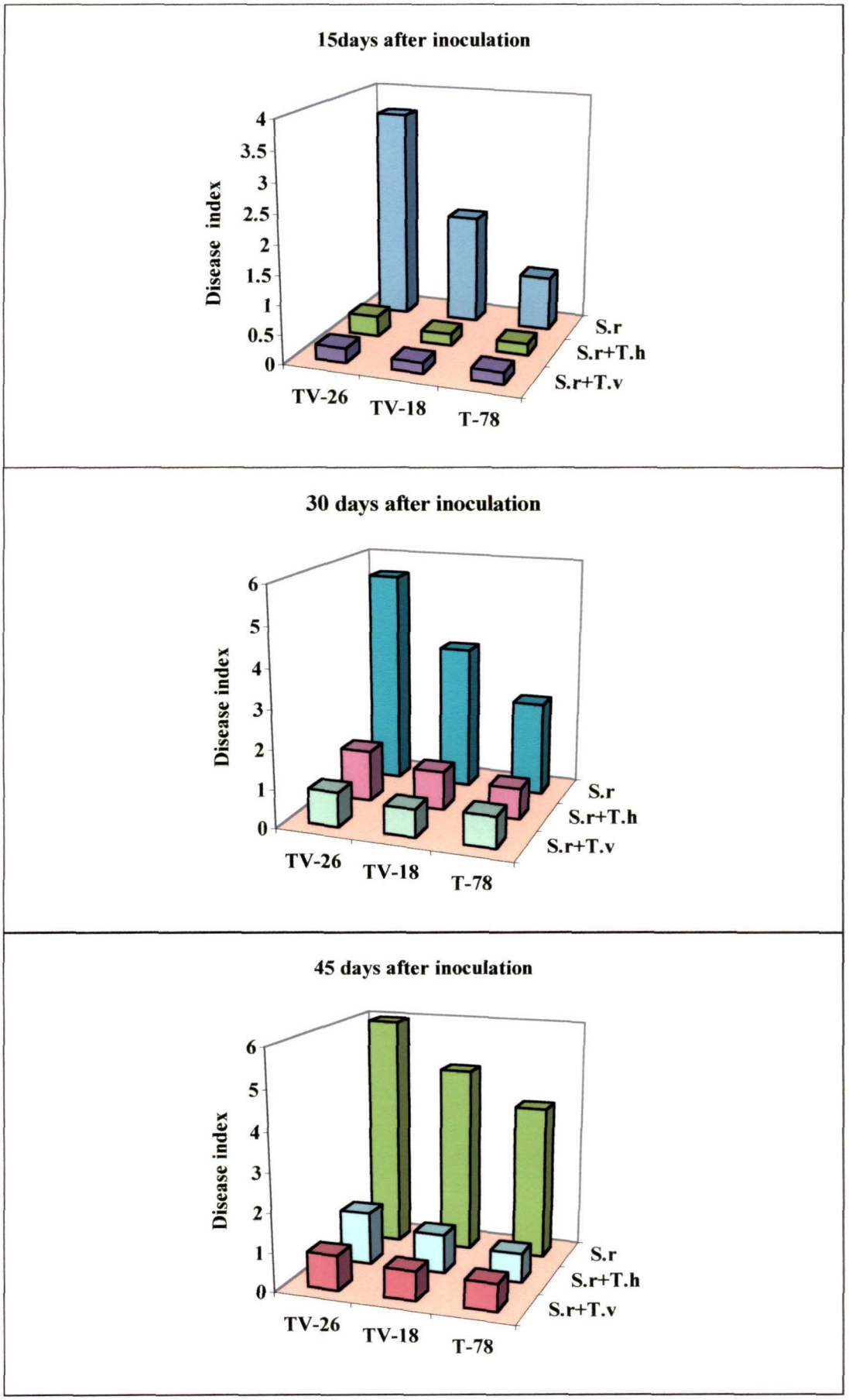


Fig.14

Table 46 : Competition ELISA of soil antigens with PAbs of *S. repens, T. viride* and *T. harzianum*

Antigen source	Absorbance at 405nm		
	Antisera source		
	S.repens	*T. harzianum*	*T. viride*
Sterile soil	1.226 ± 0.06	0.869 ± 0.04	0.746 ± 0.05
Healthy	0.602 ± 0.04	0.675 ± 0.05	0.674 ± 0.04
S. repens	0.368 ± 0.02	0.310 ± 0.03	0.627 ± 0.04
T. viride and S. repens	0.590 ± 0.04	0.406 ± 0.03	0.455 ± 0.03
T. harzianum + S. repens	0.650 ± 0.04	0.562 ± 0.04	0.411 ± 0.03
T. viride	0.415 ± 0.05	0.422 ± 0.02	0.535 ± 0.04
T. harzianum	0.673 ± 0.04	0.447 ± 0.04	0.441 ± 0.04
Homologous antigen without competition	0.059 ± 0.06	1.295 ± 0.05	1.003 ± 0.05

Soil collected 30 days after pathogen inoculation and 40 days after *Trichoderma* sp. infestation
± Stadard error.

4.12.2. Dot-blot

Dot immunoblotting technique is a rapid and sensitive method for detection of pathogen. In the present study, the presence of pathogen and biocontrol agents were detected in the soil by this technique using antigens from rhizosphere soils subjected to various treatments, and PAb of *S. repens*. Results revealed that when antigen dots reacted with anti-*S. repens* PAb only *S. repens* mycelial antigen and *S. repens* inoculated rhizosphere soil gave dark violet coloured spots (Table 47).

Detection of fungi in amended soil by DAC-ELISA

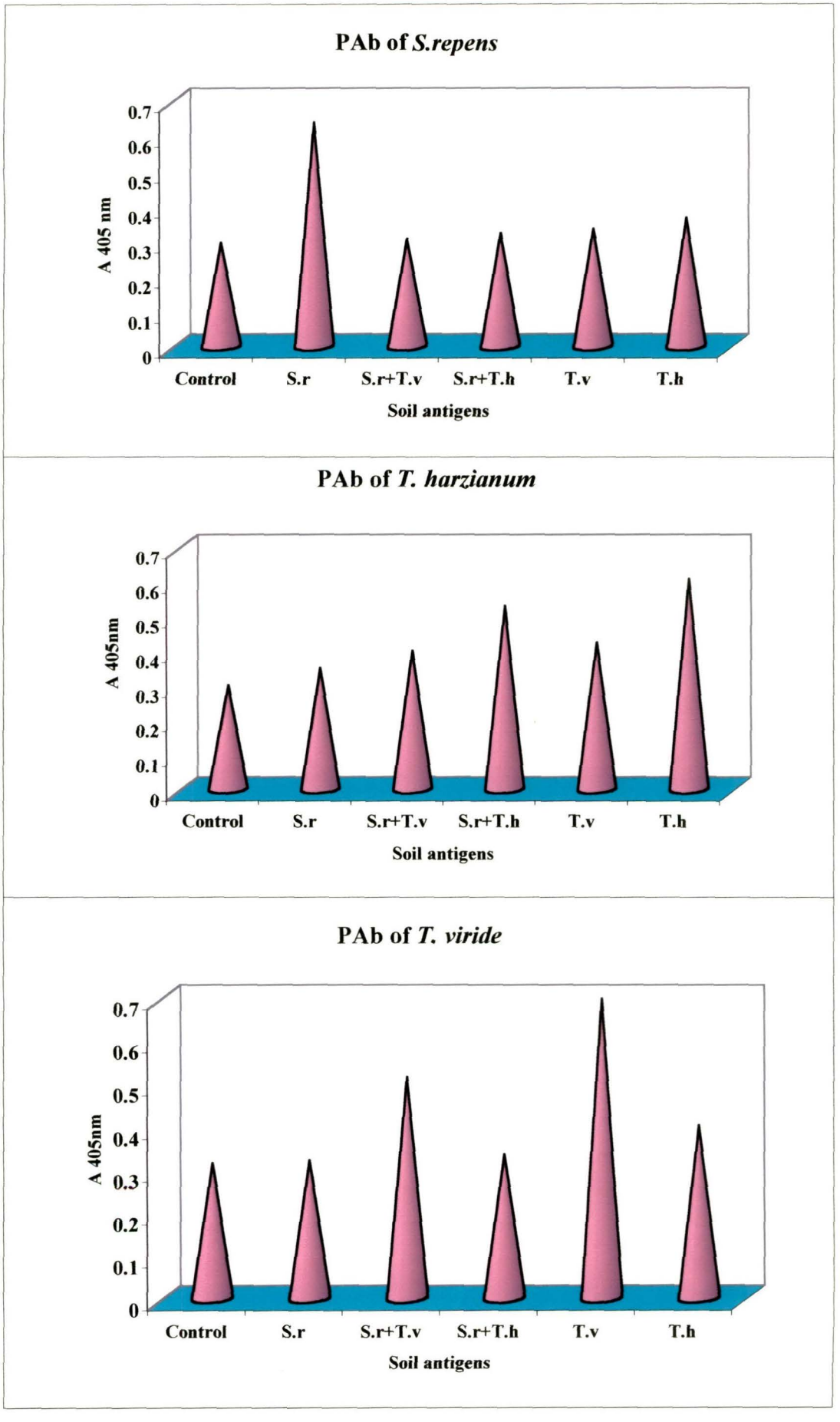


Fig.15

Table 47 : Dot-blot of different soil antigen collected from root rhizosphere (pots) infested with different combination *Trichoderma* sp. and *S. repens*.

Antigen source	Absorbance at 405nm PAbs		
	S.repens	*T. harzianum*	*T. viride*
Mycelia			
S. repens	++++	+	+
T. harzianm	++	++++	++
T. virde	++	+	++++
Soil inoculated with			
S. repens	++++	–	–
T. harzianm	–	++++	–
T. viride	–	–	++++
T. harzianm + *S. repens*	–	+++	–
T. viride + *S. repens*	–	–	+++
Uninfested soil	–	–	–

++++ Deep coloured dot
+++ Medium deep coloured dot
++ Light coloured dot
+ Very light coloured dot
– No dot
NBT/BCIP used as substrate; PAb (40µg/ml)

All antigen dots reacted with *T. harzianum* PAb and gave deep violet caloured dot in case of *T. harzianum* mycelial antigen, *T. harzianum* infested rhizosphere soil antigen and *S. repens* + *T. harzianum* only, though *S. repens* and *T. viride* mycelial antigen dot also gave middle intensity. In case of the treatment with anti *T. viride* PAb, deep coloured dots were obtained in case of *T. viride* mycelial antigen, *T. virde* , and *S. repens* + *T. virde* infested rhizosphere soil antigen. *S. repens* mycelial antigen and *T. harzianum* mycelial antigen dot also gave light coloured dots when treated with *T. viride* antiserum.

Discussion

Host pathogen interaction occurring in nature is one of the most complex biological processes and involves recognition between the host and the 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. 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 defense is adequate to provide them protection against most of their potential pathogens; only a few of them can overcome this defense 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. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signalling processes (Graham and Graham ,1996). Inspite 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. To account for the observed specificity and degree of variability of host parasite system, the fungal receptors must have a high information content. Antigens located on near 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.

Pathogenicity test of *Sphaerostilbe repens* was initially carried out on twenty five tea varieties. Among all the tested varieties, TV-26 and T-17 were most susceptible while UP-9 and UP-8 were most resistant. Studies on pathogenicity are extremely useful for several purposes, mainly in understanding more clearly the mechanism of disease development and the way and degree to which host plant performance is altered (Loomis and Adams, 1983). Violet root rot disease was generally observed to occur in water logged area with poor drainage systems. Since the growth and infectivity of the pathogen depends on a number of environmental factors, the effect of some of the factors on mycelial growth of *S. repens* was investigated *in vitro*. Optimum growth of the fungus occurred after 24 days of growth, pH 5.0 and utilising complex carbon source such as starch or sucrose and preferred organic nitrogen sources. Rhizomorph

formation which is an important event in the pathogen life cycle *in vivo*, was also found to occur *in vitro* when the fungus was grown in certain media.

Polyclonal antibodies were raised against mycelial, cell wall and spore antigens of *S. repens* and these were used for determining the presence of cross reactive antigens (CRA) between tea varieties and *S. repens* as well as for the immunodetection of the pathogen in tea root tissues and in soil. Since enzyme linked immunosorbent assay (ELISA) has proved to be one of the most sensitive serological techniques, PAbs raised against *S. repens* were used in ELISA test for pathogen detection. Since ELISA is a very sensitive technique and non-specific binding interferes with the actual antigen-antibody reaction, initially PAbs were purified and IgG fractions were used in all further tests. Prior to other tests, the sensitivity of assay was optimized and the minimum detectable antigen concentration and optimum IgG concentration were determined in homologous reactions. Positive results were obtained with very low concentration of both antigens and IgG. 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. He reported that this sensitivity was achieved because of the indirect ELISA method used; the direct double antibody sandwich form of ELISA (Voller *et. al.* 1976) detected antigens of protein concentrations greater than 50µg/ml. The indirect method used 'native' antibodies rather than antibody conjugates required in the direct assays and hence avoided masking or impairing of antigen binding sites by the conjugation procedure. Chakraborty *et. al.* (1996) also reported that antiserum raised against *Pestalotiopsis theae* could detect homologous antigens at 25ng/ml. Antiserum dilution of upto 1:16000 was effective for detection.

The presence of CRA among *S. repens* and tea varieties was evident in indirect ELISA, using PAb raised against mycelia, cell wall or spore antigen preparations of *S. repens* at a concentration of 40µg/ml with tea root antigens at a concentration of 100µgml. Though much difference was not observed in ELISA values among the different varieties T-17 the most susceptible variety exhibited the highest value. In general the reactivity of the tea varieties with the antibodies was quite less as evident from a generally low range of ELISA values. Alba & Devay (1985) also detected CRA in crude and in purified preparations from mycelia of *Phytophthora infestans*

with antisera of potato in concentration of lower than 50µg/ml protein using indirect ELISA. The presence of CRA several in host pathogen interaction has also been reported by a number of previous workers. e.g. soybean and *Myrothecium roridun* (Ghosh and Purkayastha (1990); groundnut and *Macrophomina phaseolina* (Purkayastha and Pradhan (1994); Tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994); tea and *Pestalotiopsis theae* (Chakraborty *et. al.*, 1995) and tea and *Glomerella cingulata* (Chakraborty *et. al* 2002).Cross reactivity of the Pab raised against *S. repens* was tested with othr fungal species. Results revealed that Pab of *S. repens* reacted to some extent with two entomopathogenic isolates *Beauveria* and *Metarrhizium* species. 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 in all *Phytophthora* sp. tested. Similar results with *P. fragariae* 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. Harrisen *et. al.* (1990) further reported that anit *P. infestans* γ globulin reacted strongly with extract 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.

With the advent of more sensitive techniques like ELISA, detection of plant pathogens in host tissues is now possible even when the pathogen concentration in host tissues in very low or 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 preparation of *S. repens* were used for the detection of the pathogen in tea roots and

the response was more or less similar with both antisera. Infected tea root extracts generally had higher ELISA values than the healthy ones. Pab raised against spore antigens were however not very effective in pathogen detection. Holtz *et. al.* (1994) produced polyclonal antisera to both the cell wall fraction and the soluble cytosol fraction of *Thielaviopsis basicola*. Both indirect ELISA and fungal capture sandwich ELISA revealed no difference in specificity of the cell wall and the cytosol antisera and the antibodies to each immunising 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 *S. repens* 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 of *S. repens* could also react with antigens from tea roots infected with other pathogens showing certain degree of cross reactivity.Since PAbs raised against *S. repens* 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 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 *S. repens* by DAC ELISA. Of the twenty five samples tested four samples from - plot no. 11 and 12 of Matigara Tea Estates , plot no. 8 of Bijoynagar T.E. and plot no. 5 of Bentaguri T.E. showed high A_{405} values while all the others had relatively low values. Thus it was possible to identify these soils as being contaminated with *S. repens* Wakeham and White (1996) reported the ability of polyclonal antisera of *Plasmodiophora brassicae* to detect the presence of the pathogen in soil. In another study Walsh *et. al.* (1996) reported serological detection of spore balls of *Spongospora subterranea* and its quantification in soil. They reported that the antiserum could detect about 100 spore balls/gm soil but discrimination of 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. In the present study, using spiked soil, the ELISA values decreased with decrease in concentration of spores. Thus ELISA showed potential for detection of *S. repens* in soil.

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

Results of various experiments of this study has established very definitely the importance of cross reactive antigens between host and pathogen in determining the responses of the host to pathogen. This has also been supported by the workers of several previous workers (DeVey and Adler, 1976; Chakraborty and Purkayastha, 1983; Chakraborty and Saha, 1994b). It is also important in studies on host parasite relationship to determine the cellular location of the CRA. For this purpose in this study fluorescence tests were conducted with cross section of tea roots as well as mycelia and conidia of *S. repens*. Cross sections of tea roots were treated with anti-*S. repens* PAb followed by staining with FITC conjugated anti rabbit globule specific goat antiserum. Bright fluorescence was observed in case of epiblema, cortical and endodermis region . So treatment of root sections with *S. repens* revealed that the CRA was concentrated mainly around the epiblema, cortical and endodermal zone. Treatment of mycelia and conidia of *S. repens* with homologous antiserum and FITC

showed a general fluorescence that was more intense on young hyphae and conidia. DeVay *et. al.* (1981a) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum* f.sp. *vasinfestans.* On the basis of strong fluorescence obtained at the epidermis, cortex, endodesmis 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. infeotans,* to detect the CRA in potato leaf section. It was also reported by Chakraborty and Saha (1994b) that CRA between tea and B. carbonum were mainly present in the hyphal tips and in patch like areas on the condia, mycelium and mainly around epidermal cells and mesophyll tissues of the leaf. The cellular location of CRA between *P. theae* and tea leaves was also established by Chakraborty *et. al.* (1995).

Detection of pathogen in host tissues using antibody based 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 immunofluorescence studies that chlalmydospores, basidiospores and mycelia of *Phaeolus schweinitzii* contained molecules antigenicaly 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) .

The dot immunobinding technique has been found to be rapid and sensitive method for detection of virus and plant pathogenic bacteria. Detection of fungal pathogens is a more recent application of this method. Antiserum specificity obtained against fungal pathogen varied greatly in the studies done 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, antigen of mycelial, cell wall, spore, culture filtrate, amended soil, soil from infected plot, healthy and *S. repens* inoculated tea root, mycelial antigen of other tea root pathogens were prepared and tested on nitrocellulose paper against PAbs raised against mycelial, cell wall and spore of *S. repens* using fast red of NBT / BCIP as substrate. Antigens of homologous source,

soil of infected plot 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 pathoges responded slightly or no cross reactivity with *S. repens.* Wakeham and White (1996) got positive detection of soluble components of the spore wall and whole resting spores of *P. brassicae* in PBST was 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 $1x10^5$ resting spores ml^{-1} Identification of resting spores in artificially infested soil required a minimum concentration of $1x10^8$ spores gm^{-1} soil.

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 several method have been developed previously. Towbin (1979) *et. al.* overcame these problems be 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 labeled staphylococeal protein A or corresponding anti-antibody. Blake *et. al.* (1984) have described a method using alkaline phosphatase substrate 5-bromo 4-chloroindoxyl phosphate and nitroblue tetrazolium (NBT) to detect the precipitated indoxyl group. When the substrate BCIP is used, the phosphate is cleaved 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 a intense blue deposition of dimerization. For Western blot analysis, electrophoresis of the soluble mycelia proteins from *V. dahlia* and *F. oxysporum f. sp. vasinfectum* was performed in PAGE by Sundaram *et. al.* (1991). They confirmed the specificity of the antibodies prepared for *V. dahliae* mycelium extract reacted with the purified protein of *V. dahliae* antigens, but not with the *F. oxysporum* protein. In theb present study, antigens prepared from mycelial, cell wall, spore, culture filtrate of *S. repens,* infested soil, healthy and artificially *S. repens* inoculated root and 5 different soil fungi were prepared and

probed with *S. repens* PAb. The PAb of *S. repens* was found to very specific for detection of the pathogen in the soil, infected root tissues and in different isolates of fungi. Walsh *et. al.* (1996) also performd Western blotting using the raw serum of *Spongospora subterranean* spore balls.

The immunoenzymatic 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 specificity of the staining of hyphae is dependent on the specificity of the serum. A specific 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 observation 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 (1987) used this specific serological staining procedure to detect *Verticillium dahlie* 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. dahlie* under field condition. They observed that the colony density of *V. dahlie* 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 *Sphaerostilbe repens* in tea root tissues was done using the Pab of *S. repens* reacting with alkaline phosphatase antirabbit IgG conjugate and napthol-AS-phosphate and fast blue BB salt as substrate. Rhizomorph entered through the lenticels below the bark of root-stem transition region which stained blue colour. Intense fungal colony as shown by the blue colour were also visible clearly within the cells of cortical tissue.

Consequent to the study on the detection of *S. repens* in root tissues and soil, experiments were conducted both *in vivo* and *in vitro* 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 a biocontrol agents. Both *T. harzianum* and *T. viride* inhibited the growth of *S. repens 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 oxysporum* 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* (A.P.) Studies on production of volatile compounds by *Trichoderma* species revealed that *T. virde* (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 ChandraMouli (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 against all pathogens excepting *Rosellinia. G. virens* showed high antibiosis to *Rosellinia.* Production of toxic metabolite(s) was more in *G. virens than Trichoderm.* Hazarika *et. al.* (2000) also tested the antagonistic effect of *Trichoderma harzianum* against *U. zonata,* causing claarcoal 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 their potential to suppress *Rhizoctonia solani.* Culture filtrate of *T. harzianum* and *T. virde* 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 T2 was effective against *Sclerotium rolfsii,* the causual agent of stem rot of groundnut and they overgrew the pathogen up to 92%, 85% an 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), T*richoderma 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 vitro* for their ability to reduce violet root rot intensity. Of the 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. This was observed in all tested varieties. 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 us 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 *Macrophomina 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. viirde* 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 than systemic fungicide.

In conclusion, it can be stated that violet root rot can cause severe damage to

tea plants, particularly to those growing in non-aerated soils with poor drainage. Detection, even by immunological methods, is not possible at very early stages, because the pathogen does not penetrate deep into the tissues. However, immunodetection can be used as an early tool for detection of *S.repens* in soil. Once detection is done before the appearance of symptoms , strategies for control can be formulated. In this study, effective formulations of *Trichoderma harzianum* and *Trichoderma viride* have been prepared, by which violet root rot can be controlled.

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 *Sphaerostilbe repens* was tested on twenty five varieties (11 Tocklai, 8 Darjeeling and 6 UPASI) of tea. Among these , TV-26 and TeenAli-17/1/54 were appeared to be susceptible and TV-23, TV-25, UP-9, S-449 were found to be resistant.

4. Cultural conditions affecting growth of *S.repens* were studied with special reference to their growth in different media, variable pH and five different types each of carbon, organic and inorganic nitrogen sources. Maximum growth of the pathogen occurred in carrot juice agar while minimum growth was noticed in Elliot agar. Starch was the most effective carbon source whereas yeast extract followed by casein hydrolysate yielded optimum mycelial growth. Organic nitrogen sources were found to be better than inorganic nitrogen sources.

5. Polyclonal antibodies (PAbs) were raised against antigen preparations from mycelia, cell wall and spores of *S.repens* and tea root tissues (TV-26). These were purified by ammonium sulphate precipitation followed by DEAE cellulose chromatography. IgG obtained in each case was used for immunodiffusion and ELISA tests.

6. Agar gel double diffusion tests were performed using crude antibody as well as purified IgG prepared after four different bleedings collected for the pathogen. Strong precipitin reactions were observed in each case.

7. Optimization of ELISA using PAbs of *S.repens* and antigen preparations at variable concentrations were performed. ELISA values decreased with the decrease of antigen concentrations ranging from 40 to 0.312 ug/ml . However absorbance values increased with different bleedings.

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

9. Detection of *S.repens* in artificially inoculated tea root tissues using DAC-ELISA and DAS-ELISA formats were standardized.

10. Antigens prepared from 26 soil samples were tested against PAb of *S.repens* using DAC-ELISA formats and dot blot analysis. Spiked soils gave very high values comparable to homologous values.

11. Protein content of healthy and artificially inoculated tea root tissues from 25 different tea varieties as well as mycelia and cell wall proteins of *S.repens* were estimated and analysed in SDS-PAGE. Mycelial protein of *S.repens* exhibited 23 bands ranging in molecular weight from 97kDa to 14 kDa.

12. Characterization of the cell wall of *S.repens* by ConA-FITC binding and SDS-PAGE electrophoresis revealed its glycoprotein nature, with 6 bands of 62, 56, 33, 30, 17 and 14 kDa molecular weights.

13. Agglutination test of conidia of *S.repens* with different lectins revealed strong agglutination with ConA and HPA followed by WGA and least with UAE-1. The presence of glycoconjugates containing glucose and /or mannose residues, and N-acetylgalactosamine and N-acetyl glucosamine were confirmed on the outer surface of the conidial wall.

14. Cross sections of tea roots (TeenAli-17/1/54 and TV-26) treated with PAb of *S.repens* and then labelled with FITC developed a bright fluorescence throughout, which was concentrated mainly in epidermal cells and cortical tissues.

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

16. Mycelia and conidia of *S.repens* when treated with homologous antisera followed by FITC, bright fluorescence was noticed on young hyphae and throughout the surface of conidia.

17. Specific immunocytochemical stain for detection of hyphae of *S.repens* within tea root tissues (TeenAli-17/1/54 and TV-26) were developed. Beneath the bark tissue,rhizomorph development and hyphal penetration throughout root tissue were evident.

18. *In vitro* interaction of *S. repens* with *Trichoderma harzianum* and *T. viride* was studied. Both inhibited the growth of *S.repens*. *T. viride* overgrew the pathogen while *T. harzianum* completely inhibited its growth .

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

20. DAC-ELISA of rhizosphere soil and tea root tissues as well as competition ELISA of rhizosphere soil with PAbs of *S. repens*, *T. harzianum* and *T. viride* indicated the reduction of pathogen population in rhizosphere soil and root tissues.

21. The implications of results have been discussed.

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