Biochemical and Serological Studies on Charcoal Stump Rot Disease of Tea and its Management

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN SCIENCE (BOTANY) OF THE UNIVERSITY OF NORTH BENGAL

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This is to certify that Ms P. Subbalakshmi has carried out her research under my supervision. Her thesis entitled **"Biochemical and Serological Studies on Charcoal Stump Rot Disease of Tea and its Management"** is based on her original work and is being submitted for the award of Doctor of Philosophy in Science degree in Botany in accordance with the rules and regulations of the University of North Bengal.

(B.N. Chakraborty)

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INTRODUCTION

Tea [*Camellia sinensis* (L) O.Kuntze], is one of the most important plants from the economic viewpoint and being a perennial is always challenged by pests and pathogens, which provides a stable microclimatic conditions as well as supply of food for rapid build up of insects. Tea boasts the world's longest history among beverages and offers more beneficial than being a soothing beverage to be served at social gathering. Offering its own individual flavor as well as soothing and refreshing qualities, it is drunk all over the world. There is mountain evidence to suggest that drinking tea may also reduce the risk of developing cardiovascular disease and many forms of cancer. The tea industry has spread from China to the far corners of the world, currently encompassing countries ranging from 30° latitude in the southern hemisphere to 45° latitude in the northern hemisphere, and from 150° longitude in the east to 60° longitude in the west. In India, tea is one of the most important cultivated crops. Darjeeling produces the world's finest quality teas in the steep slopes of Eastern Himalayas up to an elevation of 200m. The extensive reverine flat plains at the base of Himalayas are the tea districts of the Terai and Dooars.

The tea bush, like any other living plants is susceptible to attacks by pathogen, more so as it has been forced to grow, under varying climatic and soil conditions, remote from its natural environment. For the benefit of mankind it has also been subjected to varied cultural treatment which are widely at variance with its natural conditions of growth. Diseases of the tea bushes and its ancillary crops may be caused by one or more of several agencies like (i) vegetable organisms, such fungi, algae and bacteria, (ii) animal parasites, (iii) adverse conditions of soil and climate (iv) mechanical damage and (e) virus. Charcoal stump rot disease of tea caused by *Ustulina zonata* (Lev.) Sacc.. is one of the primary root diseases occurs on all soils. It is found in all the tea growing areas and is probably the commonest of all the primary root diseases of tea in North-east India. It attacks tea bushes preferably more than three years old. The plants being perennials harbour the pathogen in root tissues and rhizosphere, over a long time. Diseased bushes, especially the young ones, die suddenly but their withered leaves remain attached to the main plant for sometime. Sometimes older bushes may be dead and rotten one side while other side remains apparently healthy for a long time. The disease results in crop losses. The fungus develops a characteristic fructification, at first white changing to charcoal-like black,

Plate 1: Tea plantations in the North Bengal University Campus.

brittle encrustation, which is wavy on the surface. Charcoal like, brittle wavy encrustations on the bark and exposed wood at the collar region appear as external symptoms.

The root surface usually bears small, white or black, isolated, cushions or lumps. Fan-like felts of dull white, silky mycelium grow on the surface of diseased wood, underneath the bark. Colour of the wood become dull-white, almost normal and permeated by irregular, single or double, black bands or lines (Plate 2). The disease spreads mainly by direct contact between infected and non-infected roots. Bits of left over infected root pieces after uprooting serve as potential sources of infection .

There is cell surface interaction between the host and pathogen, either as compatible or incompatible. Recognition or interaction as compatible depends on some kinds of molecular similarities, between the host and pathogen (Chakraborty, 1998). Close serological similarity between host and pathogen has been found to be one of the prerequisites for the successful establishment of the pathogen in the host. This serological assays have also been exploited for the development of immunodiagnostic kits for pathogen detection systems in the host. 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 tissues. Commercial diagnostic kits have been offered for the rapid diagnosis of several fungi in plant tissues, soil and water (Werres and Steffens, 1994, Chakraborty and Chakraborty, 2003, Gawande *et.al*, 2006). Most common among these techniques are ELISA, Dot-blot, Western blot, indirect immunofluorescence, immunocytochemical staining used in large scale disease indexing programmes in perennial and vegetatively propagated crops. Timely detection of disease especially root diseases combined with proper management practices can lessen crop loss to a great extent.

The defense strategies of plants against their pathogens are manifold and include the use of antifungal chemicals. On the other hand, pathogens have evolved mechanisms to evade these chemicals. In such relationship it has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid cell death. Numerous studies suggest that low molecular weight phenols, such as benzoic acid and phenylpropanoids are formed in the initial response to infection. Most research on resistance mechanisms has shown that the plant uses defenses that are activated after infection to stop pathogen development. Tea plants

Plate 2: Naturally infected tea root showing symptoms of charcoal stump rot disease.

are abundant source of flavonoids, a group of compounds with antioxidant properties of which specific interest are the flavonoids catechins and flavonols which prevent the synthesis of peroxidase and free radicals, agents that can invade cell membrane and damage genetic material. Since polyphenol are major constituents of tea leaves, their involvement in the defense mechanism either as preformed or induced chemicals seemed highly probable.

Biocontrol agents are the most environmental friendly and effective. Among the available biocontrol agents *Trichoderma harzianum and Trichoderma viride* have been tested in a large number of cases. Biomass production, their suitable formulation for commercialization of antagonists to check chemical fungicides usage are being developed.

Biocontrol agents in soil amendments using oil cakes and dung manure found effective in controlling root diseases in French bean root rot and Lentil wilt complex .In that context, the possibility of disease control through integrated management (Biocontrol agents and organic residue materials), assumes much greater significance. Considering all the above the present study has been undertaken with the following objectives antibiosis.

The basic objectives of the present investigation are (a) screening of tea varieties for resistance to *Ustulina zonata;* (b) estimation of host parasite proteins before and after infection; (c) determination of the level of phenolics in tea roots following inoculation with *U. zonata;* (d) ascertaining the antifungal activity of phenolics associated with differential host response to infection; (e) assay of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase activities in tea roots following inoculation with *U. zonata*; (f) raising polyclonal antibody against mycelia and cell wall antigens of *U. zonata* and tea roots; (g) detection of serological

cross reactivity between *U. zonata* and tea varieties using immunoassays (h) detection of *U. zonata* in artificially inoculated tea roots by PTA-ELISA,DAS-ELISA, Competetion ELISA, dot immunobinding assay, indirect immunofluorescence and immunocytochemical staining; (i) *in vitro* studies of *U. zonata* with biocontrol agents; (j) developing effective integrated management strategies against charcoal stump rot disease of tea.

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

LITERATURE REVIEW

Different kind of pathogens are capable of infecting various types of crop plants and induce diseases that cause considerable economic loss. The effective plant disease management lies in rapid diagnosis of the disease and detection of its causative agent. The prevalence, the extent of spread and damage is assessed through surveys on the basis of symptoms followed by detection of pathogen in laboratories. This also helps us to assess, alter and modify the effectiveness of plant protection measures that are being followed. Apart from the role of detection and diagnosis in plant disease management, detection of pathogen(s) can act as important tool to localize and prevent the spread of the disease(s). In the era of globalization and WTO regime, detection of plant pathogens has immense role to play because of free movement of materials across the countries creates chances for introduction of new pathogens in to the important countries. Therefore, we need to have techniques that are rapid, accurate, sensitive, robust and easy to adopt. The emerging technologies, both serological and molecular, satisfy many of these requirements.

Serological detection of plant pathogen in diseased tissue and soil is based on study of antibody-antigen interaction. The different forms of serological assay include gel diffusion, enzyme linked immunosorbent assay (ELISA), immunofluorescence (IF), immuno-fluorescent colony staining, dot immunobinding assay (DIBA) and western blot analysis. Serological methods are being used regularly in agricultural research practices such as localization of pathogens within tissues; identification of specific antigens in electrophoretically separated components (Clark and Adams, 1977; Hansen and Wick, 1993; Werres and Steffens, 1994; Chakraborty and Chakraborty, 2003; Gadewar and Singh, 2006) as well as for quantification of defense related proteins such as PR- proteins (Chakraborty, 2005).

A short comprehensive review on the various serological techniques used in detection of plant pathogenic fungi and biochemical changes in plants following fungal infection has been presented below.

Immunodetection of plant pathogens

Charudattan and DeVay(1972) compared common antigenic relationship between four cotton varieties and isolates of *Fusarium* and *Verticillium* species. One antigenic substance was found to be common among the varieties of cotton and isolates of

V.nigrescens. Cotton varieties which were resistant or susceptible to *Fusarium* wilt as well as pathogenic and non-pathogenic isolates of *F.oxysporum f.sp. vasinfectum* shared the common antigen. The common antigen was not shared between *F.moniliforme* (non pathogenic) and cotton. In gel-diffusion tests, five to eight precipitin bands were observed in the homologous reactions; of these, only one or two bands were common in heterologous reactions between the fungal and cotton preparations. The common antigenic determinants shared by cotton and the fungal isolates does not appear related to the severity of wilt symptoms, but it may affect host-pathogen compatibility during the process of root infection.

MacDonald and Duniway (1979) used the fluorescent antibody to study the survival of *Phytophthora megasperma and P. cinnamomi* zoospore in soil. Zoospore cysts of *P. megasperma and P. cinnamomi* were adjusted to non sterile soil and its 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 cysts was suspended in water and aliquots were placed on a selective agar medium to allow germination of viable cysts. After germination, the cysts were stained by an immunofluorescence detection technique and counted.

Duncan (1980) detected *Phytophthora fragariae* by a "root-tip bait" test in certified commercial stock strawberry plants, supposedly free of disease. Charudattan and DeVay (1981) further purified and partially characterized antigenic preparation from conidia of *F. oxysporum f.sp. vasinfectum* that cross-reacted with antiserum of cotton root tissue. When antiserum to cotton antigens was reacted with fungal antigens or when antiserum to fungal antigens was reacted with cotton antigens in immunodiffusion tests, one precipitin band was formed. Cross reactive antigen (CRA) migrated as a single band in polyacrylamide or agar-gel elcetrophoresis, and sedimented as a single band during analytical ultracentrifugation. DeVay *et.al.*(1981) also indicated the presence of CRA not only in *F.oxysporum f.sp. vasinfectum* and in cotton roots and seed but also in *Thielaviopsis basicola.* Indirect staining of antibodies using flourescein isothiocyanate (FITC) indicated that in cross-sections of roots, cut near or just below the root hair zone the CRA was concentrated mainly around xylem elements, the endodermis and epidermal

cells and was present throughout the cortex tissue. Protoplasts prepared from cross-sections of young cotton roots also contained the CRA which was concentrated in the region of the plasma lemma. Treatment of conidia and mycelia of *F. oxysporum f.sp vasinfectam* with antiserum to cotton and using indirect staining with FITC indicated that the CRA was mainly present in hyphal tips and in patch-like areas on conidia.

The occurrence of common antigens in leaf and root extracts of coffee plants and urediniospores of *Hemileia vestatrix* and their possible involvement in such interaction has been discussed by Alba *et.al.* (1983). Using antisera raised against antigens of *Macrophomina phaseolina* and roots of soybean cultivars, Chakraborty and Purkayastha (1983) demonstrated serological relationship between *M. phaseolina* and soybean cultivars following immunodiffusion and immunoelectrophoretic tests. Four antigenic substances were found to be common between the susceptible soybean cultivars and isolates of *M. phaseolina* but no such common antigens were detected between resistant cultivars and the fungus (Purkayastha and Chakraborty ,1983).

Aguelon amd Dunez (1984) developed an immuno enzymatic technique for the detection of *Phoma exigua* in infected potato tissues. Specific antibodies were raised against *P. exigua* solated and DAS-ELISA was used to test for the fungus in inoculated tubers and spores and in stems grown from these tubers. The fungus was detected in these different tissues demonstrating the applicability and sensitivity of the techniques. The presence of *Ganoderma lucidum* in roots of betelnut was detectable by the induction of fluorescent antibodies as developed by Koti Reddy and Ananthanarayanan (1984). *Phaeolus schweinitzii*, which causes a root-and butt-rot of conifers, secretes a number of species-specific and strain-specific polypeptides which are detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). Indirect immunoflourescence microscopy was used by Dewey *et.al* (1984) 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 also present in extracellular culture filtlrate material and walls of mycelia, chlamydospores and basidiospores. Indirect immunofluorescence, performed by using antisera to culture filtrate molecules has been used to demonstrate the presence of mycelium, and on

occasions chlamydospores, in naturally and artificially infested soil samples. Authors suggested that this method could be used for identification of the kind of propagule most likely to be the source of field isolates of the organisms; this information, which cannot be obtained by using selective media, strongly suggests that the pathogen can survive saprophytically in the soil. In contrast, isolated mycelial cell wall preparation did not prove to be a suitable source of immunogenic material for these studies.

Cross-reactive antigens were detected by Alba and DeVay (1985) in crude preparations and in purified preparations from mycelia of *Phytohthora infestans* Race 4. and Race 1.2.3.4.7. with antisera for potatoes cv. King Edward and cv. Pentland Dell by using an indirect ELISA technique. They suggested that the fungal mycelia do not easily release cross- reactive antigens (CRA) into synthetic media where they grow. Most of *P. infestans* CRA are thermo labile and can be concentrated by precipitation in the presence of 40% saturated ammonium sulphate (SAS). An antigenic disparity was also evident when 40% SAS from *P. infestans* Race 4 mycelial preparation was assayed with antisera for King Edward and Pentland Dell. The adaptation of ELISA assay for the detection of *Sclerotiania sclerotiorum* from both artificially and naturally infected host plants was described by Walez *et.al.* (1985).

Major CRA shared by soybean roots and *M. phaseolina* were evident in comaptable interaction. However, an antigenic disparity was noticed in the susceptible cultivar (cv.soymax) after induction of resistance by sodium azide (Chakraborty and Purkayastha,1987). Using immunodiffusion, immunoelectrophoresis and crossed imminoelectrophoretic techniques, antigens prepared from roots of five cutivars of groundnut and two isolates of the antigens from two pathogen isolates (*M. phaseolina*), four non-pathogens of groundnut (*Corticium sasakii, Colletotrichum lindemuthianum, C.corchori* and *Botrytis alii* were analysed for the presence of cross-reactive antigens Purkayastha and Ghosh (1987). Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M. phaseolina*, but not between non pathogens and groundnut cultivars. No antigenic similarity was found between non-pathogens and *M. phaseolina* isolates.

Colonization of cotton roots by *Verticillium dahliae* was studied by Gerik and Huisman(1988) using immunoenzymatic staining technique. Colony density of *V. dahliae*

increased with distance from the root tip, with the maximal density occurring more than 1cm from the root apex. Colony densities at distances more than 1cm from the tip were relatively constant. *Hyphae of V.dahliae* were present through the entire depth of the cortex, and were greatest in the interior of the root cortex at the surface of the vascular cylinder. The colony appearance was consistent with growth of hyphae from the root surface toward the stele. Colonies of *Fusarium oxysporum*, similarly stained, were found to be mostly confined to the root surface and the outer cortex. Yuen *et.al.*(1988) developed a double antibody sandwich indirect enzyme-linked immunsorbent assay (ELISA) for the detection and quantification of *Pythium ultimum.* A polyclonal antibody produced to cell walls of *P. ultimum* was used as the capture antibody, while a *P. ultimum* specific monoclonal antibody (MAbE5) was used for recognition of the fungus. In the ELISA, culture extracts of 7 isolates of *P. ultimum* exhibited strong positive reactions, where as none of the 37 isolates of other *Pythium spp.* and fungal genera had positive reactions. *P. ultimum* was detected by ELISA in roots of bean, cabbage, and sugar beet seedlings grown in pathogen infested soil. Mohan (1988), raised polyclonal antibody against mycelial antigens of five isolates of *Phytophthora fragriae* (Pf1, Pf2, Pf3, Pf10 and Pf11) to detect the pathogen in extracts of strawberry plants infected with *P. fragariae* following enzyme linked immunosorbent assay (ELISA). Root extracts prepared from the alpine strawberry (*Fragaria vesca* and *F. ananssa*) infected with any of the five isolates produced strong reactions in ELISA. In *F. vesca*, ELISA-positive material could be detected before macroscopic symptoms appeared. The cultivar Red Gauntlet, resistant to Pf1, 2 and 3 but susceptible to Pf10 and 11, reflected this differential response in ELISA. Cultivars (viz. Hapil, Ostara and Providence) were to all the isolates were also positive in ELISA. The antiserum also detected *P. cactorum* infections.

Polyclonal antibody was raised against semipurifed spore suspension of *Plasmodiophora brassiceae* causing club root of cabbage by Lange *et.al.* (1989). Crude root suspension samples were tested following dot immunobinding assay. The individual steps of the serological procedure were examined with a scanning electron microscope. The surface of the resting spores of *P. brassiceae*, race 7, appeared smooth, while the dot immunobinding processed spores had a heavy, irregular coating, which was demonstrated to originate from incubation in the primary antiserum. Normal serum did not give rise to

aphanidermatum. Samples consisted of (a) whole plants picked by hand and assayed as whole plants ; (b) whole plant sectioned into lower,middle, upper strata components; and (c) 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. Fluctuation in detectable *Pythium* antigens were most pronounced on the uppermost stratum compared with moderate to very little change in ELISA reading for the two lower strata. Two commercial serological assay Kits (multiwell E Kit and the rapid assay Kit) were compared to a cultlure plate method for detection of *Phytophthora cinnamomi* in root samples from inoculated azalea (Benson, 1991). Both the kits detected *P. cinnamomi* on azalea roots beginning 1 wk after inoculation. There was a positive correlation between root rot severity in greenhouse trails and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immunoassay results. Although color reactions in the rapid assay detectors became increasingly darker after completion of the test, results after 5 min were as reliable as those after 60 min since readings for uninoculated controls used to determine test thresholds also increased with time. The multiwell Kit detected *P. cinnamomi* in root samples containing as little as 1% infected root tissue. In a commercial nursery survey, 5 and 15% of the azalea root samples at two nurseries had positive ELISA values that were unconfirmed by culture plate. The rapid assay kit detected *P. cinnamomi*, was easy to use and gave results in a short time.

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

Ribosomal protein differences among snow mold fungi and wheat were utilized to detect the causal fungi in infected wheat leaves by indirect enzyme linked immunosorbent assay (ELISA) and western blot analysis (Takenaka, 1992). Polyclonal antisera wera raised against the ribosomes of *Pythium paddicum, P. iwayamai, Typhula incarnate and Microduchium nivale,* and tested for sensitivity and specificity with ribosomes of these four pathogens, *Typhula ishikariensis*, and wheat. Using polyclonal antiserums, ribosomes of *P. paddicum and P.iwayamai* were serologically identical ; the ribosomes of *T. incarnate and T. ishikariensis* had partial common antigenic determinants, but there were apparent serological differences among *Pythium spp. Typhula spp., M. nivale* and wheat. With indirect ELISA, ribosomes of *Pythium* spp. were detectable at a concentration of 69 ng/ml and ribosomes of *T. incarnate* and *M. nivale* at a concentration of 210 ng/ml. The ribosomes for each target pathogen were detected from completely rotted wheat leaf homogentates diluted up 1:1,000 or 1:10,000. The indirect ELISA could not differentiate, *P. paddicum* from *P. iwayamai*-infected plants or *T. incarnate* 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. incrnata* form those infected with *T. ishikariensis.*

Linfield (1993) developed a specific and sensitive enzyme-liked immunosorbent assay using polyclonal antiserum raised against *Fusarium oxysporum f.sp. narcissi.* Antiserum raised against cell wall fractions gave better recognition than that to cytoplasmic fractions. Recognition was equally good in artificially and naturally infected bulbs. Nine isolates of *F.oxysporium f.sp.narcissi* from a wide geographic area gave similar results in an indirect ELISA test, although some cross-reactivity was observed with two other *Fusarium spp.* 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 ELISA and recovery of the pathogen on selective medium. Purkayastha and Ghosal(1993) analysed the cross-reactive antigens of *Acrocylindrium oryzae* and rice in relation to sheath rot disease. Immunodiffusion, immunoelectrophoresis and crossed immunoelectrophoretic analysis of rice antigens in

relation to sheath rot disease revealed a serological relationship between susceptible rice cultivars and isolates of *A. oryzae*. One precipitin band was observed when the antigen preparation of *A.oryzae* was cross-reacted with its own antiserum or against the antisera of four susceptible rice cultivars. No precipitin band was detected between the antiserum of the resistant cv. Mashuri and antigen preparations from three isolates of *A.oryzae* or in reciprocal cross reaction between the antigens of the resistant cv. Mashuri and the antiserum of the pathogen.

Monoclonal antibody (MAb) was raised against haustorial complexes isolated from pea leaves infected with the powdery mildew fungus *Erysiphe pisi* by Mackie *et.al.* (1993). The antibody designated UB10 recognises a 45 kDa N-linked glycoprotein which is specially located in the haustorial plasma membrane. This glycoprotein is clearly distinct from a previously characterized 62 kDa glycoprotein (indentified with MAb UBB) which is also specially located in the houstorial plasma membrane. These antibodies were used, along with MAb UB7 which binds to a major 62kDa glycoprotein in the cell wall plasma membrane of both haustoria and surface hyphae, to label haustoria with in epidermal strips from infected pea leaves using indirect immunofluorescence. Different stages of haustorial development can be observed using this procedure and the results showed that all three glycoproteins recognized by the MAbs are expressed early in haustorial development.

Roberts *et.al.*(1993) further described two new monoclonal antibodies (MAbs) which recognize glycoprotein components of the extrahaustorial membrane (ehm) that surrounds the haustorium formed during the infection of pea leaves by the powdery mildew fungus *Erysiphe pisi.* One of the MAbs, UB9, raised against enriched pea leaf plasma membranes, recognizes a large (200kDa) glycoprotein in this membrane within infected and uninfected epidermal cells. It does not label haustoria at early stages of development showing that at least one plasma membrane glycoprotein is excluded from the elm. However, UB9 binds to the elm of a subpopulation (approx 20%) of HCs late in the infection process and it is possible that the insertion of this glycoprotein into the ehm may represent a breakdown of biotrophy during senescence of haustoria or could be a late defence response by plant. A second MAb, UB11, was raised against isolates haustorial complexes derived from the pea powdery mildew system. This antibody recognises a

glycoprotein (250kDa) which is present in the elm of the haustorial complex and is expressed very early in haustorial development. UB11 does not bind to plant membranes of infected or uninfected plants and does not cross-react with other fungal components. This antibody therefore identifies a glycoprotein specifically located to the elm of haustorial complexes and shows that there is molecular differentiation of this key interface between the biotrophic partners.

Polyclonal antibodies (PAbs) were produced in New Zealand white rabbits with culture filtrate and mycelial extract immunogen preparations from the soybean (*Glycine max*) fungal pathogen *Phomopsis longicolla* (Brill *et.al.* 1994). The PAbs were purified to the immunoglobulin fraction and tested in indirect ELISA and in double antibody sandwich (DAS) -ELISA . The PAbs raised to culture filtrate were more specific but less active in binding than those raised to mycelial extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in pathogen detection than 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 member of the complex at the some time. PAb raised against *Bipolaris carbonum* was cross reacted with antigens obtained from tea varieties, isolates of *B.carbonum* and nonpathogens of tea (*Biolaris tetramera and Bipolaris setariae*), following immunodiffusion, immunoelectrophoresis and ELISA in order to detect 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 *B. carbomum* and resistant varieties (TV-16,25 and 26), nonpathogens and tea varieties, as well as nonpathogens and isolates of *B. carbonum.* Indirect staining of antibodies using fluoresein isothiociyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and condia of *B.carbonum* with antisera to leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia (Chakraborty and Saha,1994).

Purkayastha and Pradhan (1994) studied serological differences among three strains (266, 23, M) of *Sclerotium rolfsii* and their antigenic relationship with five

groundnut cultivars. Changes in antigenic patterns of one of its cvs. AK-12-24 and disease intensity were noted after treatment with a systematic fungicide Kitazin. Results reveal that significant variation exists among the strains of the pathogen. The strain 266 was most virulent having antigenic relationship with susceptible cvs. (Gangpuri, J-11 and AK-12-24) of groundnut. The strain 23 also exhibited common antigenic relationship with cv. AK-12-24. Resistant cvs. JL-24 and ICGS-26, however, showed no antigenic relationship with fungal strains in either immunodiffusion or immunoelectrophoretic test. Alteration of antigenic pattern and reduction in disease incidence in susceptibl cultivar was evident following application of kitazin, a systemic fungicide. Polyclonal antibodies were prepared against crude cell wall fractions of *Pythium* isolate with filamentous non-inflated sporangia, associated with root rot of tomato in soilless cultures. In ELISA tests, the antiserum showed sensitivity and specificity against original antigens and those from closely related isolates with filamentous sporangia (such as *Pythium aquatile and Pythium coloratum*). The antiserum reacted weakly with mycelial preparations of several fungi commonly found on tomato roots such as *Fusarium spp.* or *Penicillium spp.* An immunocytochemical staining test was developed in order to study the colonization by filamentous noninflated sporangial isolates of *Pythium* in the presence of other fungi in soil less cultures. (Rafin *et.al.,* 1994). Soluble protein extracts of chlamydospores and mycelium of *Thielaviopsis basicola*, the causal agent of black root rot of cotton were used to raise polyclonal antibodies. The immunoglobulin G antibody fraction was purified and biotin-labelled to divise a fungal capture sandwich enzyme-linked immunosorbent assay (ELISA). ELISA detected both brown and grey cultural types of *T. basicola* and had negliglible cross-reactivity with other soil borne fungi commonly found in cotton field soils. The minimum detection limit of ELISA was between 1 and 20mg of *T.basicola* protein depending on the assay. *T. basicola* could be detected in cotton roots 2 days after inoculation when initial symptoms were apparent. The antibody also was used to observe *T. basicola* on cotton roots with immunofluorescence microscopy (Holtz *et.al.*, 1994).

Leaf antigens prepared from ten tea varieties, three isolates of *Pestalotiopsis theae* and a non-pathogen of tea (*Bipolaris tetramera*) were cross reacted with polyclonal antibody(PAb) raised against mycelial suspension of *P.theae* and reciprocal cross reaction was made with PAb raised against leaf antigens of Teen Ali-17/1/54 and CP-1

following immunodiffusion test and enzyme linked immunosorbent assay. CRA were found among the susceptible varieties and isolates of *P. theae* (Pt-1,2 and 3). Such antigens were not, detected between isolates of *P. theae* and resistant varieties, *B. tetramera* and tea varieties or isolates of *P. theae*. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves, CRA was concentrated in the epidermal cells and mesophyll tissues. CRA was present in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae* (Chakraborty *et.al.*,1995)

Optimization of purified immunoglobulin fractions of PAb raised against *Pestalotiopsis theae* was done using ELISA formats. Antiserum dilution up to 1:16000 detected homologous antigen at a 5 mg/L concentration, and at 1:125 antiserum dilution fungal antigens could be detected at concentration as low as 25 µg/L. Absorbance values of infected leaf extracts of fifteen tea varieties were significantly higher than those healthy extracts at a concentration of 40 mg/L. in indirect ELISA. ELISA positive material was detected in tea leaves as early as 12h after inoculation with *P. theae*. At antiserum dilution 1:125, the pathogen could be detected on inoculated leaf extracts upto 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 pathogen. The results demonstrated that ELISA can be used for early detection of *P. theae* in leaf tissues even at a very low level of infection (Chakraborty *et.al.* 1996a).

Wakeham and White (1996) raised polyclonal antisera were raised to whole (Coded:16/2),and sonicated (Coded: 15/2) resting spores of *Plasmodiophora brassicae*, and soluble components prepared by filtration and ultracentrifugation (Coded : SF/2) (). Cross- reactivity of all three antisera with range of soil fungi, including western blotting, dipstick, dot-blot, indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescene 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 $1x\ 10^7$ resting spores g^{-1} in soil. With western blotting the lower limit of detection with antiserum 15/2 was $1\ x10^5$. This antiserum showed the greatest sensitivity in a dip-stick assay, indirect ELISA and indirect immunoflurescence, for all of which there was a limit

of detection of $1x10^2$. The indirect ELISA was successful only after the substitution of alkaline phosphatase by protein A conjugated horse radish peroxidase. Of the assays tested, indirect immunofluorescence appears to be the most rapid and amenable assay for the detection in soil of low levels of resting spores of *P. brassicae.*

It was reported by Walsh *et.al.*(1996) that a polyclonal antiserum produced against spore balls of *Spongospora subterranean f.sp.subterranea* prepared from potato tubers was able to detect as little as 0.02 spore balls in an enzyme-linked immunosorbent assay (ELISA). In spiked soil samples, the antiserum detected 100 spore balls per g soil. How ever, the different spore ball contamination levels were discriminated better in ELISA tests at concentration above 2000 spore balls per g soil than at lower concentrations. In contrast, a bioassay test based baiting soils with tomato seedlings gave good discrimination of spore ball contamination levels in spiked soils containing <1000 spore balls per g soil and poor discrimination of levels in spiked soils containing >2000 spore balls per soil. Tests on a limited number of field soils suggested, ELISA may be capable of predicting disease levels on tubers grown in such soil better than the biossay. The antiserum did not react with 30 other micro-organisms tested, including many that are saprophytes or pathogens on potatoes and resting spores of the taxonomically related *Plasmodiaophora brassicae.* It detected spore balls of different cultivar origin equally well. It also detected spores from different geographial origins. An attempt to improve the sensitivity of the serological detection through concentrating spore balls from field soils by sieving was unsuccessful. Cross absorption of the antiserum with uncontaminated field soil increased the sensitivity of detection of spore balls in spiked soil samples four-fold. The ability of the antiserum to discriminate contaminated field soils from an uncontaminated soil was much improved by using the gamma-globulin fraction of cross-absorbed screen. Western blot analysis revealed that the antiserum detected a number of different proteins the most distinct of which had a molecular weight of slightly less than 6.5 kDa. A technique was developed to suppress autoflurescence of spore balls, allowing immunofluoresence studies to be carried out. Using this technique in conjunction with indirect FITC immunofluoreserence, discrete bright fluorescent spots were visualized using the specific serum. With the non-specific serum, only a very dull background fluorescence was evident.

Plant antigens prepared from healthy leaves of five selected tea varieties and fungal antigens prepared from three isolates of *Glomerella cingulata* (GC-1, 2 & 3) as well as non- pathogens of tea (*Bipolaris tetramera and Fusarium graminearum*) were cross reacted with PAb raised against *G.cingulata* (isolate GC-1). Reciprocal cross reaction with PAb raised against leaf antigens of TV-18, Teen Ali 17/1/54 and CP-1 were also made using immunodiffusion, immunoelectrophoresis as well as PTA-ELISA formats in order to detect CRA shared between tea varieties and isolates of *G. cingulata.* CRA were found only among the susceptible varieties and *G. cingulata* isolates but 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 FITC indicated that in cross section of tea leaves, CRA is concentrated in epidermal cell, mesophyll tissue and vascular elements (Chakraborty *et.al,* 1996b)

Population of *Phytophtora spp.* were determined by Miller *et.al.* (1997) using ELISA in field soils used for pepper and soybean production in Ohio. Srivastava and Arora (1997) detected and quantified *Macrophomina phaseolina,* causal agent of charcoal rot disease in many crop plants following PTA-ELISA formats. 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 DAS-ELISA . Cell wall antisera exhibited strong cross reactivity with most of the fungal isolates. Ribosomal antibodies were less reactive to common soil fungi except *Fusarium oxysporum f,so.ciceri.* Mycelial antigens of *M. phaseolina* on chickpea roots were detectable with DAS-ELISA at a minimum concentration of 10ng g^{-1} at 1:100 root: buffer dilution. Quantitative estimation of *M. phaseolina* on roots was evaluated by ELISA under different temperatures and moisture conditions, and in soil amended with a potential antagonist (*Trichoderma harzianum*). A significant reduction in ELISA values was observed in *T. harzianum* amended treatments. This method proved to be useful for detection and rapid screening of *M. phaseolina* under different environmental conditionsPolyclonal antisera raised against mycelial suspension of *Fusarium oxysporum* and the root antigen of the susceptible soybean cultivar (UPSM-19) were cross reacted with root antigens of ten soybean cultivars and mycelial antigen of pathogen in order to detect cross reactive antigens (CRA) shared by the host and the pathogen. The purified immunoglobulin fractions were used for detection of CRA by

ELISA. Antigens of susceptible cultivars showed higher absorbance when tested against the purified anti *F.oxysporum* antiserum. Antiserum produced against UPSM-19 showed cross reactivity with the antigens of other cultivars. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross-sections of roots of susceptible cultivar, CRA 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 microconida and chlamydospores of the fungus (Chakraborty *et.al.*1997).

The initial infection process involving adhesion/recognition events between plants and fungal pathogens is essential for the establishment of pathogenesis. The basis of the specificity between *Phytophthora infestans* and potato may lie in the recognition between extracellular matrix and plasma membrane components of the penetrating structures of the fungus and host cells during the earliest stages of infection process. Laxalt *et.al.*(1998) used polyclonal antibodies (AZ and AC) obtained after immunizing rabbits with either 10^7 zoospores (Z) or germination cysts (C) of *P. infestans* to detect putative fungal components located on the surface of invasive structures. AZ recognized several bands, while AC recognized three polypeptides of approximately 87,73 and 45 kDa in western blots. Zoospore suspensions pre-incubated either with AZ of AC were unable to infect potato leaves. Since it was already known that some anti-idiotypic (anti-IT) antibodies may resemble the antigen, thus mimicking its structure, anti- IT antibodies (a-AZ and a – Ac) were generated in mice and used as inducers on potato tuber discs. In northern blots a –AZ treatments increased 1.5- fold the glyceraldehyde-3-Pdehydrogenase(GAPDH) and 1.8 fold the phenylalanine ammonia-lyase (PAL) transcript levels, while an increase of 1.8 and 1.7 fold for GAPDH and PAL, respectively, was observed when the inducer was a –AC. Both PAL and GAPDH transcript levels have previously been shown to be modulated by fungal and elicitor treatments. These results show that the anti-idiotypic antibodies are able to mimic the pathogen components, leading to the accumulation of transcripts related to defense responses in potato *P. infestans* interactions.

Immunodetection of teliospores of Karnal bunt (*Telletia indica*) of wheat using fluorescent staining test was done by Gupta *et.al.*(2000). Polyclonal antibodies were

raised against intact teliospores in Newzeland white rabbits for the immunodetection of Karnal bunt (KB) teliospores. The indirect immunofluorescence (IIF) test was developed using anti-teliospores serum and binding was monitored by goat-rabbit antibody conjugated to fluorescein (FITC) label. The standardization of IIF test was carried out by optimization of dilutions of anti-teliospores antibodies, florescent probe and exposure time. The teliospores of *T.indica* showed bright green, patchy and ring shaped fluorescence around the teliospore. The spore exhibited uniform distribution in discrete regions of spore probably in spore episporium, Similar fluorescence pattern in the teliospores of KB isolated from infected wheat seeds of cultivars HD 2328, UP 2328,PBW 393, WH 542, as well as RR 21 (Susceptible cultivars) respectively, is an indication of the presence of similar antigenic configuration to teliospores. Again, they did not exhibit variation in the expression of teliospore associated molecular pattern (TAMP) during previous and subsequent years of infection.

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

Viswanathan *et al.* (2000) performed ELISA using PAb raised against *Colletotrichum falcatum* to detect pathogen before the symptom development. 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. Chakraborty *et al.* (2000) had demonstrated immunological detection of *Sphaerostilbe repens, Trichoderma viride* and *T. harzianum* using DAC-ELISA formats to develop strategies for management of violet root rot of tea. Polyclonal antibody based immunoassay for

detecting *Fomes lamaoensis,* causing brown root rot disease of tea has also been developed (Chakraborty *et al.,* 2001). Eight blood samples were collected and IgG were purified using DEAE cellulose. Immunodiffusion tests were performed in order to check the effectiveness of mycelial antigen preparations of *F. lamaoensis* for raising PAbs. Optimization of PAbs were done using indirect ELISA. Increased activity of PAbs against *F. lamaoensis* could be noticed from second bleedings, which continued up to fourth bleeding. Root antigens prepared from healthy and artificially inoculated (with *F. lamaoensis*) tea plants (Teen Ali 17/1/54, TV-18, TV-22, TV-26, TV-27, TV-28, TV-30, S-449, BSS-2) were analysed following DAC-ELISA format. Such format was also used to detect the pathogen in infested soil. Young mycelia 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 plantations can enable disease prevention at an early stage.

Polyclonal antibody raised separately against antigen preparation from mycelia and cell wall of *G. cingulata* (isolate GC-1) were evaluated for early detection of the pathogen in tea leaf tissue by Chakraborty *et. al.* (2002). PAb raised against cell wall preparations gave better recognition from that against mycelial preparations as observed in ELISA test with antigens of tea leaves of different ages.

Biochemical changes in plants following fungal infection

Disease develops in individual plants by a series of sequential steps beginning with the arrival of inoculum at the plant surface and ending with the terminal stages of pathogens. One of the well documented host responses is the biochemical changes following infection. Phenols in plants occur constitutively and function as preformed inhibitors associated with non host resistance. Since the phenolic intermediates have a role in the active expression of resistance, an underlying problem in ascertaining that such secondary metabolites are of primary importance has been the localization and timing of the host response. Resistance of maize to *Helminthosporium carbonum* and subsequent changes in host phenolics and their antifungal activity have been discussed by Werder and Kern (1985). Host responses could be differentiated by changes in content of phenolic compounds. Phenolics content in the resistant inbreed increased between 96 and

120 h after inoculation to a level two to three times higher than that of susceptible and non-infected control in breeds. However, the pattern of changes of total phenolic content (hydrolyzed and unhydrolyzed ethylacetate soluble phenols) of resistant and susceptible inbreeds did not differ much between 0 h and 96 h. after inoculation. They isolated four antifungal compounds, A, B, C and D from hydrolyzed maize leaf extracts which were found to be fungitoxic to *B. zeicola* in spore germination and chromatographic bioassays.

Saxena *et al.* (1986) evaluated the changes in phenolics of resistant and susceptible wheat varieties in response to *Puccinia recondite.* Resistant varieties exhibited higher concentration of phenolics than the susceptible ones. Biochemical analysis of pea varieties resistant and susceptible to *Erysiphe polygoni* causing powdery mildew disease revealed that the quantity of total phenol and ortho-dihydroxyphenol was higher in stem and leaves of resistant varieties as compared to susceptible ones which decreased as the age of plant increased in all the varieties (Parashar and Sindhan, 1987).

Matern and Kneusel (1988) have proposed that the defensive strategy of plants exists in two stages. The first is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow (or even has) the growth of the pathogen and to allow for the activation of "secondary" strategies that would more thoroughly restrict the pathogen. Secondary responses would involve the activation of specific defenses as the *de novo* synthesis of phytoalexins or other stress-related substances. They argue that the initial defense response must occur so rapidly that it is unlikely to involve *de novo* transcription and translation of genes, which would be characteristic of the second level of defence. The sequence of events in a defence response can be thought to include – host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions or papillae, and finally, synthesis of specific antibiotics such as phytoalexins. Changes in phenol contents was also determined by Oke (1988) in healthy and *Colletotrichum nicotianae* infected leaves of tobacco. After infection the quantity of total phenols and ortho dihydroxy phenol increased in both stem and leaves of susceptible and resistant varieties.The changes in phenolic and nitrogen metabolism were investigated by Tore and Tossi (1989) in healthy and infected (with *Thielaviopsis basicola*) tobacco roots and leaves.

Etenbarian (1989) detected quantitative changes in phenolic compounds at different time intervals on barley varieties inoculated with *Puccinia hordei.* Luthra (1989) detemined the levels of total phenol in sorghum leaves, resistant and susceptible to *Ramulispora sorghicola* at 15-day-intervals after 25 day of sowing. Resistant varieties exhibited high phenol content in comparison to susceptible ones at all stages of growth. Phenolic compounds inhibitory to the germination of spores of *Colletotrichum graminicola* were shown to leach from necrotic lesions on corn leaves caused by the fungus. Primary components of the phenolic mixture were identified as esters and glycosides of p-coumaric and ferulic acids as well as the free compound themselves. Spores of *C. graminicola* produced in acervuli of infected leaves were shown to be surrounded by a mucilaginous matrix as in the case when the fungus is cultured *in vitro.* It is suggested that the mucilage protects spores from the inhibitory effects of the phenols by the presence of proline rich proteins that have been shown to have a high binding affinity for a variety of phenols (Nicholson *et al.,* 1989). It has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death (Fernandez and Heath, 1989).

Accumulation of two caffeic acid esters after inoculation of maize with *Glomerella graminicola* was reported in both compatible and incompatible combinations (Lyons *et al.,* 1990). One compound was identified as caffeoyl glucose, whereas the other was a caffeoyl ester of an unknown organic acid moiety. Although neither compound was fungitoxic, a pattern of rapid accumulation followed by a sharp decrease in the amount of both compounds in the tissue suggested that they may serve as a pool of phenols required for diversion to other products. Kumar *et al.* (1990) analysed certain biochemical changes in the pearl millet shoots infected with downy mildew pathogen (*Sclerospora graminicola*). The estimation revealed that the total phenol and free amino acids content were found to be low both in diseased shoot and roots of pearl millet (*Pennisetum glaucum*). Seasonal changes in the phenolic constituents of jack pine seedlings (*Pinus banksiana*) in relation to the purpling phenomenon was studied by Nozzolillo *et al.* (1990).

Mansfield (1990) has proposed that cell death results from irreversible membrane damage that may occur in response to pathogen recognition or as a result of activated host response. Niemann *et al.,* (1991) demonstrated that low molecular weight phenols, such as benzoic acids and the phenyl propanoids, are formed in the initial response to infection. Early after infection, low molecular weight phenols accumulate in both incompatible (resistant) and compatible (susceptible) interactions. Whether these compounds, are significant in the ultimate host response presents a perplexing problem. Bruzzese and Hasan (1991) demonstrated that accumulation of phenols at the infection site occurred as early as 3h after inoculation, indicating an association of phenols with the initial stages of the response. The contents of phenols, o-dihydroxy phenols and peroxidase activity in healthy and *Curvularia andrepogonis* infected leaves of *Java citronella* (*Cymbopogan winterianus*) were determined by Alam *et al.* (1991). As a result of infection the content of phenols and peroxidase increased two and four fold respectively, in necrotic lesions compared to healthy leaves. It has been suggested by

Changes in carbohydrates, amino acid and phenolic contents in jute plant on inoculation with *Macrophomina phaseolina, Colletotrichum corchori* and *Lasiodiplodia theobromae* were studied by Sahabuddin and Anwar (1992). Total sugars, non-reducing sugars, starch and total free amino acids were found to decrease following inoculation with all the three test pathogens of jute, while reducing sugars, total phenols and orthodihydroxy phenols increased. The *Fusarium* sp. infected leaves of *Trianthema portulacastrum* contained 6, 7, dimethoxy-3, 5, 4' – trihydroxy flavone, vanillic acid, p-hydroxybenzoic acid, quercetin and ferulic acid. By using drop diffusate technique it was found that the pathogen induces the formation of quercetin and ferulic acid (Darshika and Daniel, 1992).

Fifteen isolates of *Phytophthora parasitica,* nine from tobacco (causing black shank disease) and six from other host plants were compared by root inoculation with regard to their pathogenicity to young tobacco plants. A progressive invasion of the aerial parts over 1 week was observed only with the black shank isolates, while the non-tobacco isolates induced leaf necrosis within 2 days. Similar necrosis occurred when the roots of tobacco plants were dipped in diluted culture filtrates from non-tobacco isolates, but not in those from tobacco isolates. The necrosis inducing filtrates contain 10 kDa protein

band which was not present in the other filtrates. This protein (named parasiticein) was purified by ion exhange chromatography to homogenicity in SDS-PAGE and reverse phase HPLC. Parasiticein was serologically related to cryptogein, a member of the elicitin family of proteinaceous elicitors. Like the other elicitins, parasiticein induced necrosis in tobacco plants and protected them against black shanks. It most closely resembled little leaf necrosis. Ricci *et al.* (1992) suggested that the absence of parasiticein production by the black shank isolates might be a factor involved in their specific pathogenicity to tobacco.

A glycoprotein elicitor of phytoalexin accumulation in leaves of *Phaseolus vulgaris* produced well before lysis in the medium of cultures of *Colletotrichum lindemuthianum* was purified to homogeneity by Coleman *et al.* (1992). In a *P. vulgaris* leaf infection bioassay the purified glycoprotein had activity easily detectable at nanomolar concentrations and induced browning of the treated tissue and also the accumulation of both PAL and the isoflavanoid phytoalexins. The effects of an elicitor from *Colletotrichum graminicola* was studied by Ransom *et al.* (1992). Roots of sorghum (*Sorghum biocolor*) accumulated 3-deoxyanthocyanidin phytoalexins in response to CG elicitor. Elicitation of the phytoalexins prior to treatment with the elicitor did not prevent infection and development of disease symptoms in susceptible seedlings inoculated with conidia of *Periconia circinata.* However, treatment of roots with the CG elicitor enhanced the synthesis of 16 kDa proteins in both resistant and susceptible genotypes without expression of disease symptoms.

Yoshikawa and Sugimoto (1993) identified the putative receptor like target sites for glucanase-released elicitor in soybean membranes. The elicitor molecules that function *in vivo* for phytoalexin elicitation in *Glycine max* infected with *Phytophthora megasperma* f. sp. *glycinea* have been identified as β-1, 6-and β-1, 3-linked glucans that are released from fungal cell walls by β-1,3-endoglucanase contained in host tissue. The elicitor induced incorporation of phenylpropanoid derivatives into the cell wall and the secretion of soluble coumarin derivatives by parsley suspension cultures can be potentiated by pretreatment of the cultures with 2, 6-dichloroisonicotinic acid or derivatives of salicylic acid. The cell walls and an extra cellular soluble polymer were isolated by Kauss *et al.* (1993) from control cells or cells treated with an elicitor from

Phytophthora megasperma f. sp. *glycinea*. When the parsley suspension cultures were preincubated for 1 day, with 2, 6-dichloroisonicotinic, 4- or 5-chlorosalicylic, or 3, 5-dichlorosalicylic acid, the cells exhibited greatly increased elicitor response. Pretreatment with isonicotinic, salicylic, acetylsalicylic, or 2, 6-dihydroxybenzoic acid was less efficient in enhancing the response, and some other isomers were inactive. This increase in elicitor response was also observed for the above mentioned monomeric phenolics, which were liberated from cell walls upon alkaline hydrolysis and for "lignin like" cell walls polymers determined by the thioglycolic acid method. It was shown for 5-chlorosalicylic acid that conditioning most likely improves the signal transduction leading to the activation of genes encoding PAL and 4-coumarate: coenzyme A ligase.

Resistance or virulence are modelled by multiple biochemical components of two living organisms. *Costus speciosus* a major sapogenin bearing medicinal plant was severly affected by *Drechslera rostrata* causing leaf blight disease. An interesting interaction phenomenon was noticed by Kumar *et al.* (1995). The HPLC analysis indicated the accumulation of glyceollin II and III as potent phytoalexins by *C. speciosus* in response of non pathogenic *D. longirostrata*. Further the presence of a polysaccharide elicitor, or mycelial wall component seems to be detrimental cause of phytoalexin accumulation. The same elicitor was also present in mycelial wall of pathogenic *D. rostrata* but in much lower concentration. Additionally it was associated with another polysaccharide component with different identity. A race specific elicitor has been isolated from *Uromyces vigna*. This elicitor can induce phytoalexin production in cowpea resistant to this race of the pathogen based on hypersensitive response (HR) – like symptoms induced by treatment of resistant cowpea leaves with the elicitor (D'Silva and Heath, 1997). The presence of phenolic acids in cell wall - esterified p-coumaric acid and ferulic acids bound to cell wall polysaccharides are widespread in Gramineae. Cell wall bound phenolics in resistance to rice blast disease was demonstrated by Kumar *et al.* (1997).

The response of bavistin on disease incidence, phenolic compounds and their oxidative enzymes, non-structural carbohydrates, different forms of nitrogen and mineral content in cowpea roots susceptible to *Rhizoctonia solani* and *R. bataticola* was reported by Kalim *et al.* (2000). Bavistin (0.2%) as seed treatment significantly reduced the

incidence of root rot of cowpea to the extent of 57.5 and 58.9 percent in case of *Rhizoctonia solani* and *R. bataticola,* respectively. Reduction in disease incidence has been attributed to the increased activities of polyphenol oxidase (PPO) and peroxidase (PO) along with higher amounts of total phenols. PO activity was several times more as compared to PPO specific activity. Contrary to PPO and PO the specific activity of catalase declined sharply. Bavistin seed treatment also caused on increase in reducing sugars, Cu, Zn and Mn but a decrease in o-dihydric phenols, flavanols, total soluble sugars, non-reducing sugar and Fe contents (Kali *et al.,* 2000).

The relative roles of glyceollin, lignin and the hypersensitive response (HR) in pathogen containment and restriction were investigated in soybean cultivars that were inoculated with *Phytophthora sojae.* Incompatible interactions in leaves and hypocotyls were characterized by HR, phenolic and lignin deposition and glyceollin accumulation. The uncoupling of glyceollin synthesis is a major factor in restriction of the pathogen during these interactions (Mohr and Cahill, 2001). The effect of phenolics and related compounds on pectinolytic enzymes of *Sclerotinia sclerotiorum,* a phytopathogenic fungus causing white rot in pea (*Pisum sativum*) had been studied by Sharma *et al.* (2001).Biochemical study on peroxidase (PO) and polyphenol oxidase (PPO) activity; reducing, non reducing and total sugar; total phenol and potash content before and after powdery mildew infection in seven mungbean genotype was carried out by Gawande *et al.* (2002) to know their role in host parasite interaction. Resistant genotype had higher activities of PO and PPO, total phenol and potash content before and after infection and lower level of sugars than observed in susceptible genotype. Activity of enzymes total phenols and potash content were positively associated with resistance, whereas sugars had negative association with disease resistance.

Accumulation of total and o-dihydroxy phenols in three maize varieties (Malan, Ganga-5 and VL-42) infected with *Helminthosporium maydis* and *H. turcicum* was recorded as compared to their healthy counterparts. Reaction of these varieties to both the pathogens varied significantly in terms of accumulation of phenolics. Ganga-5 showed three-fold increase in phenolic contents due to infection by *H. maydis* while double amount of total phenols was recorded in VL-42. *H. turcicum* induced maximum accumulation of phenolics in variety VL-42 followed by Ganga-5 and Malan. An

increase in the activity of peroxidase, polyphenol oxidase and IAA-oxidase was noticed in all the three varieties of maize under infection of *H. maydis* and *H. turcicum.* The results have suggested that the accumulation of phenolics was higher in resistant varieties like 'Ganga-5' and "VL-42' as compared to susceptible Malan. Corresponding increase in the activities of oxidative enzymes suggested active metabolic reaction of the host to the pathogenesis and their possible role in an increased level of phenolics (Sukhwal *et al.,* 2003). Ten cultivars of soybean were tested for their disease reactions against *Fusarium oxysporum* Schlecht the causal agent of root rot disease. The different cultivars exhibited varying degrees of susceptibility with Soymax being the most and JS-2 and UPSM-19 being the least resistant. Seed bacterization with *Bradyrhizobium japonicum* reduced root rot intensity significantly. Application of *Trichoderma harzianum* to soil also reduced root rot intensity. Combined application of *B. japonicum* and *T. harzianum* gave the most significant disease reduction. *B. japonicum* did not exhibit any antagonistic reaction against *F. oxysporum in vitro*, whereas *T. harzianum* inhibited growth of *F. oxysporum.* Phenylalanine ammonia layse and peroxidase activities were assayed in both resistant and susceptible cultivars following the different treatments. Activities were significantly higher in the infected roots in comparison to healthy ones. PAL activity was higher in the resistant cultivar but bacterization with *B. japonicum* prior to inoculation with *F. oxysporum* enhanced PAL activity in both the cultivars. Persoxidase activity did not show any increase following pre-inoculation with *B. japonicum.* Glyceollin accumulation which was significantly higher in the resistant cultivar also registered a marked increase due to pre-inoculation with *B. japonicum. T. harzianum* did not affect enzyme activities or glyceollin accumulation (Chakraborty *et al.,* 2003).

Six apple rootstocks [M7, M9, M25, MM103, MM104 and MM115] showed different reactions to *Pythium ultimum* causing collar rot of apple. The maximum amount of total and ortho-dihydroxy (OD) phenols and high activity of phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and polyphenol oxidase (PPO) were detected in highly resistant apple rootstock (MM115) and minimum in highly susceptible ones (MM103, MM104). The peroxidase activity was, however, maximum in M9 (susceptible) and minimum in M25 (resistant). On infection, the levels of total phenols and activities of their synthesizing (PAL, TAL) and oxidizing enzymes (PPO, PO) increased rapidly in resistant root stocks (MM115, M25) at the initial stages of pathogenesis and subsequently

declined rapidly. The activity of these enzymes also continued to increase gradually with pathogenesis (up to 20th or 25th day of inoculation) in highly susceptible root stocks (MM103, MM104). In resistant root stocks, the level of phenols and activities of enzymes (except peroxidase) remained higher during pathogenesis in comparison to that of susceptible ones (Sharma, 2003).

Joshi *et al,*(2004) attempted to quantify biochemical changes in highly susceptible (IC 116835) and moderately resistant (IC 116903) genotypes of cluster bean against *Alternaria cucumerina* var. *cyamopsides* . The catalase activity decreased with the increase in disease intensity in both genotypes. Activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) as well as quantity of phenols and lignin increased with the increase in disease intensity, indicating thereby that these enzymes play important roles in the defense mechanism against *Alternaria* blight in clusterbean.

During cavity spot disease of carrot (*Daucus carota*), the surface of the root is penetrated by the fungus *Pythium violae* causing surface lesions and cell breakdown. Commercial varieties range from the very susceptible Bertan, to the less susceptible Bolero with Narbonne intermediate while the gene bank cultivar Purple Turkey was much less susceptible. Examination of the colonization process *in vitro* by scanning electron microscopy of Narbonne showed that fungal proliferation occurred in the first 2 days of colonization but this species had disappeared from lesions by day 7. No lesions were evident on Purple Turkey although the fungus had penetrated the root which itself was composed of small regularly arranged cells. Examination of the activity of defence related enzymes during *in vitro* colonization showed that phenylalanine ammonia lyase and chitinase activities remained low throughout the first 7 days of infection of commercial cultivars, Bolero and Bertan. Peroxidase and β-glucosidase activity in Bolero increased briefly on day 3 but otherwise were uniformly low. Enzyme activities were generally higher in Purple Turkey. The small cell size within the root and higher constitutive levels of the enzymes may constitute the basis for resistance in Purple Turkey. Potentially this cultivar may provide a source of germplasm for improving the resistance of commercial carrots to cavity spots (Copper *et al.*, 2004).

Association of defense enzymes with resistance in tea plants against *Exobasidium vexans* was demonstrated by Sharma and Chakraborty (2004). Results revealed significant changes in the level of enzymes mainly □-1, 3 glucanase and chitinase exhibiting antimicrobial activity. A wide variety in the activities of the enzymes involved in phenol metabolism including PAL, POX and PPO were seen in compatible and incompatible interactions. Multicomponent coordinated responses of tea plants under biotic stress with special reference to *E. vexans* causing blister blight disease of tea have also been demonstrated by Chakraborty *et al.* (2004a). The possibility of inducing resistance in susceptible tea varieties was achieved following foliar spray with salicylic acid and results established its potential in immunizing tea plants which was confirmed by immunoassays and immunolocalization of chitinase in tea leaf tissues by employing polyclonal antibodies raised against chitinase and labeled with FITC after induction of resistance. The accumulation of defense enzymes in tea plants in response to salicylic acid treatment suggests its role in the cellular protection mechanism which was also confirmed (Chakraborty *et al.*, 2005a).

MATERIALS AND METHODS

3.1 Plant Material

3.1.1 Collection

Twenty five tea varieties collected from Tocklai Experimental Station, Jorhat, Assam (TV-9, TV-18, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-30 and Teen Ali 17/1/54); Darjeeling Tea Research Centre, Kurseong, West Bengal (BS/74/76, CP-1, AV-2, HV-39, T-78, T-135, S-449, P-1258, and K1/1) and United Planter's Association of South India (UPASI) Tea Research Station, Valparia, Tamilnadu (UPASI-2,UPASI-3, UPASI-8, UPASI-9, UPASI-26 and BSS-2) being maintained in the Tea Germ Plasm Bank of the Department of Botany, University of North Bengal have been used for the present investigation.

3.1.2. Propagation by Cutting

Polythene sleeves (6") which were filled up with the sandy soil (3:1) with a pH ranging from 4.8 to 4.9 adjusted by treating with 2% aluminium sulphate solution followed by watering twice in order to remove excess aluminium sulphate was used for propagation by cutting. Three hundred cuttings each of the above mentioned tea varieties were allowed for rooting in sleeves, after dipping them in the hormone mixture and maintained in the nursery.

3.1.3. Maintenance of tea sleeves in nursery

Underneath the polythene cloche, new shoots came out from cuttings. After growth of 6" or more the polythene cloche was removed from every bed and the sleeves were treated with nursery mixture [ammonium sulphate-8 parts by wt., ammonium phosphate- sulphate (16:20), 35 parts by wt., potassium sulphate-15 parts by wt., magnesium sulphate-15 parts by wt. and zinc sulphate-3 parts by wt.] as suggested by Ranganathan and Natesan (1987). The manuring was done after rooting and continued up to 12 months once only in 15 days. The mixture was dissolved @ 30g in 1L of water and applied @50ml/plant.manure.

3.1.4. Potted plants

Young tea plants transferred in soil in earthen ware pots (one plant/ pot of 30Cm dia) each containing 5 Kg soil mixture (soil: panting mixture-1:1). For maintenance of young tea plants, nursery mixture (30g) was dissolved in 1L of water and applied @ 50 ml / plant, once in 15 days and continued up to 12 months. The mature plants (one year and above) were maintained by applying a soluble mixture of N,P,K consisting of 10 Kg urea, 20 Kg ammonium phosphate, 8 Kg muriate of potash in the soil.

Plate 3: (Figs. A-F): Nursery propagation of tea varieties by cuttings.

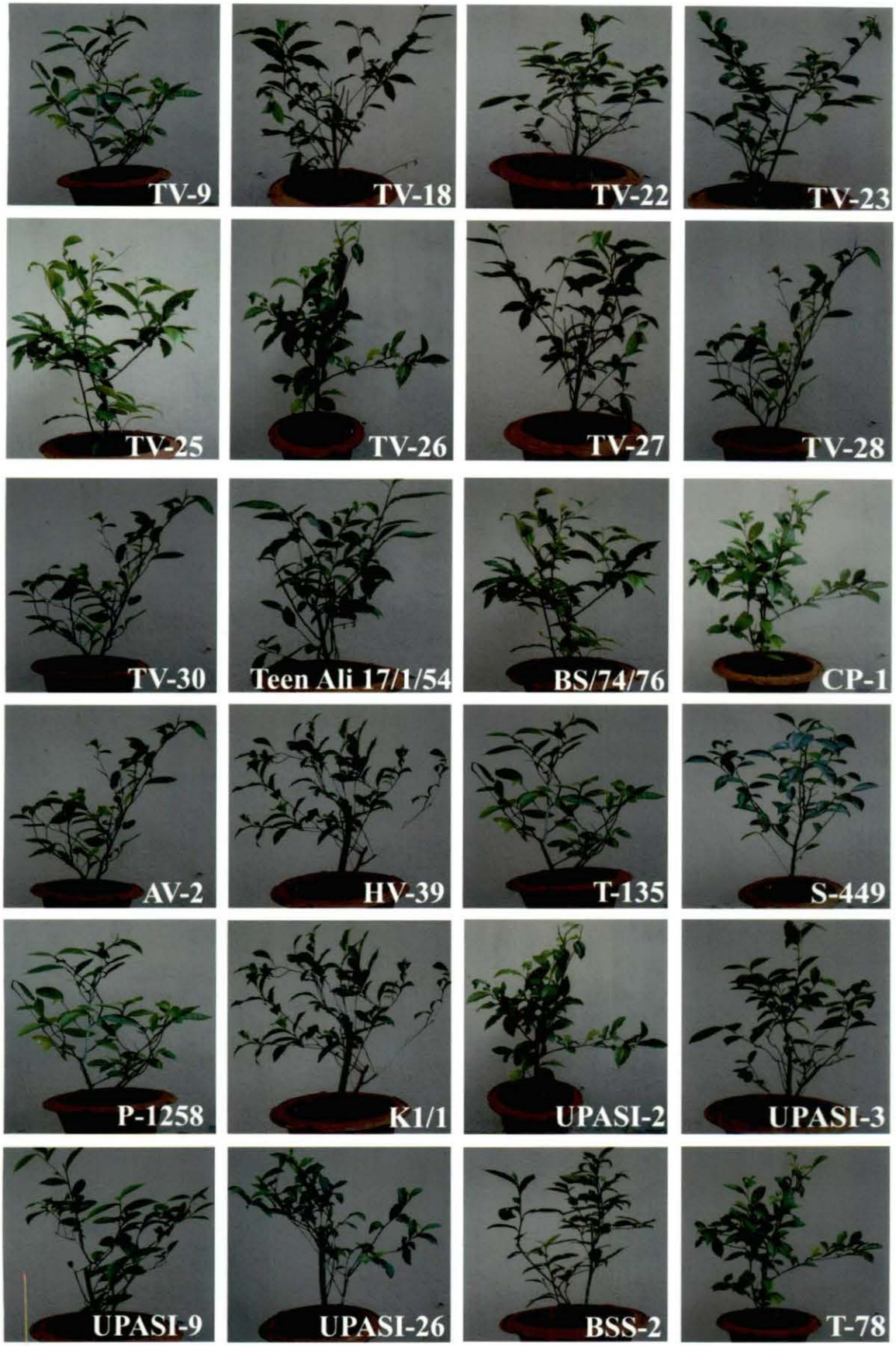


Plate 4: Tocklai, Darjeeling and UPASI varieties of tea (*Camellia sinensis*) being maintained in Tea Germplasm Bank.

3.2. Fungal culture

3.2.1. Source of culture

U. zonata (Lev.) Sacc. was obtained from Tocklai Experimental Station, Jorhat, Assam. The culture was maintained on PDA (potato dextrose agar) medium by regular subculturing. Other fungal pathogens used for the experimental purposes are mentioned below.

Species	Host	Source
Ustulina zonata	Tea	Tocklai Experimental Station, Jorhat, Assam
Sphaerostilbe repens	Tea	Tocklai Experimental Station, Jorhat, Assam
Fomes lamaoensis	Tea	Tocklai Experimental Station, Jorhat, Assam
Rosellina arcuata	Tea	UPASI Tea Research Station, Valparai
Sclerotium rolfsii	Tea	Immuno-Phytopathology Laboratory
Fusarium oxysporum	Soybean	Indian Agricultural Research Institute, New Delhi.
Trichoderma viride		Tea rhizosphere (Matigara Tea Estate)
Trihoderma harzianum		Tea rhizosphere (Hansqua Tea Estate)

3.2.2. Assessment of Mycelial Growth

3.2.2.1. Solid media

To assess mycelial growth of *U. zonata* in solid media, the fungus was first grown in Petri dishes, each containing 20ml of PDA and incubated for seven days at 25±1°C. Agar block (6mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each Petri dish containing 20ml of sterilized solid media. The different solid media were prepared following the method of Dhingra and Sinclair (1985) and used for assessment of growth. The media were as follows. All petri dishes were incubated at 25°±1°C for the desired period. Finally diameter of mycelia was measured at 2 days intervals for 8 days.

1. ***Potato dextrose agar (PDA)*** Peeled potato - 40.00g Dextrose - 2.00g Agar - 2.00g Distilled water - 100 ml	2. ***Richards Agar (RA)*** KNO_3 - 1.00g KH_2PO_4 - 0.50g $MgSO_4$ $7H_2O$ - 0.25g $FeCl_3$ - 0.002g Sucrose - 3.00g Agar - 2.00g Distilled water - 100 ml
3. ***Carrot Juice Agar (CJA)*** Grated carrot - 20.00g Agar - 2.00g Distilled water - 100 ml	4. ***Czapek– dox agar (CDA)*** $NaNo_3$ - 0.20g K_2HPO_4 - 0.10g $MgSO_4$' $7H_2O$ - 0.05g KCI - 0.05g $FeSO_4$· $7H_2O$ - 0.05g Sucrose - 3.00g Agar - 2.00g Distilled water - 100 ml
5. ***Flentze's soil extract agar (FSEA)*** Soil extract - 1 L Sucrose - 1.00g KH_2PO_4 - 0.20g Dried yeast - 0.10g Agar - 25.00g	6. ***Potato Sucrose agar (PSA)*** Peeled potato - 40.00g Dextrose - 2.00g Agar - 2.00g Distilled water - 100 ml
7. ***Malt extract Peptone agar (MPA)*** Malt extract - 20.00g Peptone - 1.00g Dextrose - 20.00g Agar - 20.00g Distilled water - 1L	8. ***Yeast extract dextrose agar (YDA)*** Yeast extract - 7.50 g Dextrose - 20.00g Agar - 15.00g Distilled water - 1 L.

3.2.2.2 Liquid media

To assess the mycelial growth of *U. zonata* in liquid medium, the fungus was first allowed to grow in Petri dishes containing 20ml of PDA and was incubated at 25°±1°C for 7 days. From the advancing zone, the mycelial block (6mm dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized Richards medium for the desired period at 25°±1°C. Finally the mycelia were

strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60°C for 96h, cooled in desiccators and weighed

3.3.1. Preparation of Inoculum

3.3.1.1 Pathogen

U. zonata was grown in PDA plates. Sand maize meal medium was prepared by mixing riverbed sand and maize meal in the ratio of 9:1 respectively as described by Biswas and Sen. (2000). This mixture (50g) was taken in each 250ml flask, 10 ml distilled water was added and the medium was autoclaved at 20 lbs pressure for 20 min. After cooling, these were inoculated with *U. zonata* and incubated for 2 weeks at 25°±1°C.

3.3.1.2. Biocontrol agents

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

3.3.2. Inoculation techniques

3.3.2.1. Pathogen

Mature tea plants (3year old) grown in earthen ware pots were selected for artificial inoculation, with *U. zonata*. For the pilot experiment ten tea varieties viz. TV-25, TV-18, TV-26, TV-22,TV-23, TenAli-17/1/54, T-78, S-449, UPASI -3, UPASI -26 were selected for standardization of inoculation technique with the pathogen. Then rest of the tea varieties were artificially inoculated with the pathogen for assessment of disease index. Prepared inoculum (50g) of 15-day old fungus soil mixture added with the rhizosphere of each plant after removing the top soil carefully without damaging the taproot system. Finally the roots were covered with soil, pots were watered and kept for observation. Disease assessment was done after 20d, 40d and 60days after inoculation.

3.3.2.2. Biocontrol agents

For biocontrol experiments, inoculation with *Trichoderma sp.* was also done as described above but at least 10 days prior to the inoculation with *U. zonata* biocontrol agents were applied to the soil. The different treatments for this experiment were as follows- (a) pathogen (*U. zonata*) only (b) *T. harzianum* (c) *T. viride* (d) *U. zonata* + *T. harzianum* (e) *U. zonata* + *T. viride* (f) healthy plants.

3.3.2.3. Field grown plants

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

3.4. Disease assessment

The inoculated plants were examined at an interval of 20 days for a period of 60 days for pathogenicity test and 10 days intervals for bio-control test. Disease intensity of charcoal stump rot was assessed on the basis of above ground and under ground symptoms together on a scale 1-6. (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, 50% roots become inky black with random patches; (4) = random defoliation up to 70% roots become black; (5) = total defoliation, 70-85% blackening of roots;(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 50 ml of sterilized potato-dextrose broth (PDB) and

incubated for 10 days at 25°±1°C. For extraction of antigens, mycelial mats were harvested washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand in a chilled mortar and pestle and homogenized with cold 0.05M sodium phosphate buffer (pH-7.2) supplemented with 0.85% NaCl, 10mM sodium metabisulphite and 0.5mM magnesium chloride in ice-bath. The homogenate was kept for 2h or overnight at 4°C and then centrifuged at 10,000rpm for 30 min at 4°C to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice-bath and kept overnight at 4°C. After this period, the mixture was centrifuged (10,000 rpm) for 30min at 4°C, the precipitate was dissolved in the same buffer (pH-7.2).The preparation was dialyzed for 72 h through cellulose tubing (Sigma chemical Co., USA) against 1 L of 0.005 M sodium phosphate buffer (pH.7.2) with six changes. The dialysate was stored at - 20°C and used as antigen for the preparation of antiserum and other experiments.

3.5.1.2 Root

Root protein was extracted following modified method of Chakraborty and Purkayastha (1983). The roots of tea plants were collected and washed with cold water and kept at -20°C for 1hr. These roots were cut into small pieces, ground for 1min. in a high speed blender with insoluble polyvinyl pyrrolidone (PVPP) (Sigma) of equal weight. The ground root powder was suspended in cold 0.05M sodium phosphate buffer (pH 7.2) containing 0.85% NaCl, 0.02% KCl, 10mM sodium metabisulphite, 2mM PVP-10 (polyvinyl pyrrolidone-10,000), 0.5mM magnesium chloride and 1mM phenylmethyl-sulphonyl fluoride (PMSF) and the mixture was kept at 4°C for over night. The mixture was then crushed in chilled mortar with pestle using sea-sand in an ice bath. The slurry was strained through muslin cloth and then centrifuged at 10,000 rpm for 30 min at 4°C. A portion of the supernatant was saved for serological assay and the rest was equilibrated to 100% saturated ammonium sulphate (SAS) under constant stirring and kept over night at 4°C. After this period, mixture was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was discarded and the precipitate dissolved in the same buffer and dialyzed against 0.0005M sodium phosphate buffer (pH 7.2) for 72 hr at 4°C with six changes. The dialysate was used as antigen for immunization of rabbits and stored at -20°C for further requirements. For better resolution of protein on SDS PAGE, roots pieces were weighed,

pulverized and crushed with insoluble PVPP of equal weight in mortar with pestle using sample buffer [1.M tris (pH 6.8) 0.5ml; 10mM β-mercaptoethanol-0.5ml; 10% SDS-2ml and 7ml H_2O]. The root slurry was centrifuged at 10,000 rpm for 30 min at 4°C, the supernatant was immediately used for SDS-PAGE analysis.

3.5.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et.al.*(1951).To 1ml of protein sample (taking10^{-1}or 10^{-2} dilution), 5ml of alkaline reagent (0.5mlof 1% $CuSO_4$ and 0.5ml of 2% sodium potassium tartarate added to 50ml of 2% Na_2CO_3 in 0.1(N) NaOH) was added and incubated for 15-20 min at room temperature. Then 0.5ml of Folin ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min and colour was developed following absorbance values was measured at 700nm. Quantity of protein was measured using bovine serum albumin (BSA) as standard.

3.6. SDA-PAGE analysis of total soluble protein

SDS-PAGE was performed for the detailed analysis of protein profile following the method of Laemmli (1970)

3.6.1. Preparation of stock solution

The following stock selections were prepared:

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

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly 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°C and used within one month.

B. Sodium Dodecyle sulphate (SDS)

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

C. Tris buffer

(i) 1.5 M Tris buffer was prepared for resolving gel (pH 8.8) and stored at 4°C.

(ii) 1.0M Tris buffer was prepared for use in the loading buffer (pH 6.8) and stored at 4°C.

D. Ammonium persulphate (APS)

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

E. Tris-Glycine electrophoresis buffer

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

F. SDS loading buffer

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

3.6.2. Preparation of Gel

Slab gels (plate size 8cm x 10 cm) were prepared for protein analysis on SDS-PAGE .Two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at the three sides and were sealed with high 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 (comb + 1 cm) for the stacking gel.

10% resolving gel : Composition of resolving gel solution (7.5ml)

Solutions	Amount
Distilled water	2.95 ml
30% Acrylamide mixture	2.50 ml
1.5 M Tris (pH 8.8)	1.90 ml
10% SDS	0.075 ml
10% APS	0.075 ml
TEMED	0.003 ml

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

5 % stacking gel : Composition of stacking gel solution.

Solutions	Amount
Distilled water	2.10 ml
30% Acrylamide mixture	0.50 ml
1.0 M Tis (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% APS	0.03 ml
TEMED	0.003 ml

Stacking gel solution was poured over the resolving gel and comb was inserted immediately leaving a space o 1 cm between resolving gel and comb and overlayered with water. The gel kept for 30 minutes. After polymerization of stacking gel the comb was removed and washed thoroughly. The gel was then mounted in the electrophoresis apparatus. 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 was prepared by mixing the sample protein (34µl) with 1 x SDS gel loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath

for 3 min to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the well with T-100 micro titer syringe. Along with the samples, protein marker was loaded in separate well.

3.6.4. Electrophoresis

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

3.6.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R250) in 45 ml methanol. When the stain was completely dissolved 45ml of distilled water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper. The gel was removed from fixer and stained in this staining solution for 4h at 37°C with constant shaking at a very low speed. After staining, the gel was finally distained in distaining solution containing methanol, distilled water and acetic acid (4.5:4.5:1) at 40°C with constant shaking until background became clear.

3.7 Extraction and Estimation of phenolics

3.7.1 Extraction

Phenols were extracted and assayed as described by Mahadevan and Sridhar (1982), with modification. Root tissue (1 gm) was cut into small pieces and immediately immersed in 4 ml absolute alcohol , kept in a boiling water bath for 5-10 minutes. After cooling, the tissues were crushed with mortar and pestle using 80% alcohol. Extracts were stored at 4°C in separate vials, covered with brown paper. The whole extraction was done in dark in order to prevent light induced degradation of phenol.

3.7.2 Estimation

3.7.3 3.7.2.1. Total phenol

Total phenol was estimated by Folin-Ciocalteau's method as described by Mahadevan and Ulaganathan (1991). To 1 ml of phenolic extract, 1 ml of Folin –

Ciocalteau's reagent and 2 ml 20% Na_2CO_3 was added, shaken properly and heated on a boiling water bath for 1 min and the volume was raised to 25 ml with double distilled water. Absorbance was measured in a Systronics photoelectric colorimeter Model-101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.7.2.2. Ortho-dihydroxy phenol

Ortho-dihydroxy phenol was estimated as described by Mahadevan and Ulaganathan (1991). In 1 ml of each phenolic extract, 2 ml of 0.5 (N) HCl, 1 ml Arnow's reagent (Na_2CO_3 – 10g; distilled water 100 ml) and 2 ml of 1 (N) NaOH was added. These were then diluted with distilled water up to 25 ml. The tubes were shaken well and absorbance was recorded by Systronics photoelectric colorimeter Model -101 at 515 nm. Quantity of ortho-dihydroxy phenol was estimated using caffeic acid as standard.

3.8. Extraction of antifungal phenolics

Antifungal phenolics from root samples were extracted following the method as described by Daayf *et al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Root samples (10 g) were mixed with 80% methanol at 10 ml / g tissue and homogenized by blending, kept on a rotary shaker at 40 rpm for 48 h, then methanolic extracts were collected by filtration on a Whatman No. 1 filter disc and concentrated by evaporation to a final volume of 20 ml (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethyl ether three times which was treated as Fraction I. The aqueous fraction was partitioned secondly with equal volume of ethyl acetate three times and the ethyl acetate fraction was considered as Fraction II. Acid hydrolysis of the remaining aqueous fraction was done with 4(N) HCL to yield phenolic aglycones assuggested by Daayf *et al.*(1997).

Aglycones were recovered by partitioning hydrolysates against an equal volume of ethyl acetate (three times), which was treated as Fraction III. All the fractions were evaporated to dryness and finally dissolved in 3 ml of the respective solvents.

3.8.1. Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea roots were analysed by thin layer chromatography (TLC) on silica gel G. The development of the chromatograms

was carried out at room temperature using a solvent system (Chloroform: methanol:; 9:1v/v) as suggested by Chakraborty and Saha (1994a). Following evaporation of the solvent, thin layer plates were observed under UV light and sprayed separately with Folin-Ciocalteau's phenol reagent (Harborne, 1973). Colour reactions and Rf values were noted.

3.8.2. Bioassay of antifungal phenolics

Radial growth inhibition assay was performed as described by Van Etten (1982). Ethyl acetate fractions of healthy and infected extracts (0.2ml) were initially taken separately in sterile Petri plates and allowed to evaporate. In control sets, only ethyl acetate (0.2 ml) was taken and allowed to evaporate. Subsequently 10 ml sterilized PDA was poured in each Petri plate, thoroughly mixed and allowed to solidify. Agar blocks (4 mm dia) were cut with a sterilized cork borer from the advancing zone of a 6-day-old culture of *U. zonata* grown in PDA and was placed in the center of each Petri plate. Radial growth of *U. zonata* was recorded after 5 days of incubation at 25± 2°C

3.8.3. UV – spectrophotometric analysis

For spectral analysis of antifungal phenols extracted from healthy and *U. zonata* inoculated roots, initially ethyl-acetate fractions were plotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent. Silica gel from corresponding antifungal zones as detected in chromatogram inhibition assay were scrapped off, eluted separately in spec methanol, re-spotted on TCL plates and developed in the same solvent. Finally UV-spectrophotometric analysis at a range of 200-400 nm were done.

3.9 Extraction of enzymes

Three enzymes – phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase(PPO) involved in phenol metabolism were extracted from healthy and *U. zonata* infected tea plants to determine their activities.

3.9.1. Phenylalanine ammonia lyase

For the extraction of phenylalanine ammonialyase (PAL), the method of Bhattacharya and Ward (1987) was followed with modifications. Tea root tissue (1 g) was crushed in a mortar with pestle in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2 mM ß- mercaptoethanol in ice. The crushed material was centrifuged at

12,000 g for 20 min at 4°C. The supernatant was collected, volume recorded and then immediately used for assay.

3.9.2. Peroxidase

To extract peroxidase (POX), the method of Chakraborty *et al.*(1993) was followed with modification. Tea root tissue (1 g) was crushed with 0.1 M sodium borate buffer (pH 8.8) containing 2 mM ß- mercaptoethanol in mortar with pestle on ice. The crushed material was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was collected and its final volume measured and used immediately used for assay.

3.9.3. Polyphenol oxidase

For the extraction of polyphenol oxidase (PPO), the method of Mahadevan and Sridhar (1982) was followed with modification. Root tissue (1 g) was cut into pieces. The pieces were then crushed with mortar and pestle in ice cold condition with 5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The slurry was immediately centrifuged at 12,000 g for 20 min at 4°C. The supernatant was decanted and after recording its volume immediately used for assay.

3.10. Assay of enzyme activities

Enzyme activities were assayed following specific procedure in each case.

3.10.1. Phenylalanine ammonia lyase (PAL)

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture (total volume 3 ml) contained 0.3 ml 300 µM sodium borate (pH 8.8), 0.3 ml 30 µM L-phenylalanine and 0.5 ml of enzyme extract and 1.9 ml of double distilled water. Blank was prepared in same way but with water instead of enzyme extract. Then the tubes were incubated at 37°C for 1 h in water bath. After 1 h absorbance was noted at 295 nm in UV-VIS-spectrophotometer against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid / min.

3.10.2. Assay of peroxidase (POX)

For the assay of POX activity, freshly prepared crude enzyme was added to the reaction mixture containing 1 ml of 0.0 (M) sodium phosphate buffer pH 5.4, 100 µl of 4 mM H_2O_2, 100 µl of orthodianisidine (5mg /ml methanol) and 1.7 ml of double distilled water. POX activity was assayed spectrophotometrically at 495 nm by monitoring the oxidation of o-dianisidine in the presence of H_2O_2. Specific activity was expressed as the increase in absorbance at 495 nm / g tissues / min.

3.10.3. Polyphenol oxidase (PPO)

For the determination of PPO activity, 1.9 ml of 0.1 (M) sodium phosphate buffer pH 6.5, 0.1 ml enzyme extract and 0.1 ml of 0.025 (M) catechol solution (0.014 g in 5 ml sodium phosphate buffer pH 6.5). The reaction mixture was incubated at room temperature in the dark for the prevention of photo-oxidation of the enzyme. Initial absorbance was noted at 495 nm at 0 min.Further reading was taken at 1 min intervals. PPO activity was expressed as the increase in absorbance at 495nm / g tissue / min, when the substrate catechol was oxidized due to the enzyme activity from 1 g of tissue.

3.11. Preparation of Antigen

3.11.1. Fungal antigen

3.11.1.1. Mycelia

Mycelial antigen of tea root rot pathogens [*Ustulina zonata, Sphaerostilbe repens, Poria hypobrumea, Fomes lamaoensis, Rosellinia arcuata* and *Sclerotium rolfsii]*, two potential biocontrol fungi [*Trichoderma harzianum* and *Trichoderma viride*], two entomopathogenic fungi [*Metarhizium anisopliae* and *Beauvaria bassiana*]and one soil fungi [*Fusarium oxysporum*] were prepared using sea sand and 0.05M Sodium phosphate buffer (pH7.2) supplemented with 10mM Sodium metabisulphate and 0.5mM Magnesium chloride following the method as described by Chakraborty and Purkayastha (1983). Soluble proteins of each samples were estimated following Lowry's method and using BSA as standard.

3.11.1.2. Cell wall antigen

Isolation of cell wall was done following the method of Keen and Legrand (1980). Mycelial mat (10 day old culture) was collected on filter paper using a Buchner funnel

and 40g of fresh packed cells were ground for 1 min in a high speed blender with water (4ml/g). The resulting slurry was then disrupted and homogenized for 1 min at 4°C . The mixture was centrifuged for 1 min at 2000 rpm, the supernatant fluids discarded and the sedimented wall washed with sterile chilled distilled water (10ml/g) and pelleted by centrifugation several times until the supernatant fluids were visually clear. Finally the isolated cell walls were dissolved in 0.05M phosphate buffer saline (pH 7.2) and kept at -20°C until further requirement. This cell wall antigen was also used for immunization.

3.11.1.2.1 Cell wall extract preparation

Cell wall extract from isolated cell wall was prepared using the, method of Brown and Kimmins (1977). Isolated cell walls (2g) were suspended in 80 ml ice cold 0.1(N) NaOH by blending in a chilled mixer cup at full speed for 30 seconds. The suspension was stirred in ice bath for 15 h and then centrifuged at 8000g for 10 min. After centrifugation, the precipitate was washed with 50 ml ice-cold water and the supernatant was neutralized to 7.0 by adding 1 (N) HCl slowly with continued stirring at 0°C. The neutralized supernatant was finally dialyzed against distilled water for 48 h with 12 hly 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.

3.11.2. Root antigen

Antigens from healthy, artificially inoculated as well as naturally infected tea root tissues were prepared separately following the method of Chakraborty and Purakayasta (1983), with modification. Roots were collected from the experimental plots and field, thoroughly washed in water and cut into pieces. Root pieces were weighed and homogenized in grinder with 0.05 M sodium phosphate buffer containing 2mM PVPP soluble, 10 mM sodium metabisulphite and 0.5mM magnesium chloride. Insoluble PVPP was also added during homogenization. The homogenate was then kept at 4°C overnight and then the slurry was once again crushed in mortar with pestle and centrifuged, at 10,000 rpm at 4°C, supernatant was used as antigen.

3.11.3. Soil antigen

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

3.12. Binding of FITC labeled concanavalin- A

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

3.13. Serology

3.13.1. Rabbits and their maintenance

Polyclonal antibody (i.e. antisera) for fungal and plant antigens were produced in New Zeland white male rabbits. Approxmiately 2kg of body weight of the rabbit is needed (Alba and DeVay, 1985) for immunization. So, before immunization, the body weights of rabbits were recorded and were observed for at least one week inside the cage. Rabbits were being maintained in Animal House (Antisera Reserves for Plant Pathogens), Immuno-Phytopathology Laboratory, Department of Botany, N.B.U.. Foods used for rabbit are green grass, soaked gram seeds., green vegetables like cabbage, carrots (specially at the time of bleeding schedule). Rabbits were regularly fed in the morning and evening providing proper washed and cleaned utensils. 90-100g/day gram seeds (soaked in water), alternately with 500g green grass were given for each rabbit. Besides this, they were given saline water after each bleeding for three consecutive days. Cages

and floor were cleaned with antimicrobial agents every day in the morning for maintaining the hygienic condition.

3.13.2. Antisera production

Following the method of Chakraborty and Saha (1994), before immunization, normal sera were collected from rabbit. For raising antisera, intramuscular injection of 1 ml antigens(1mg/ml protein) emulsified in equal volume of Freunds' complete adjuvant (Difco) were given into each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Difco) at 7 day intervals, up to 10-14 consecutive weeks as required. Blood samples were collected by marginal ear vein puncture. First bleeding was taken 3 days after sixth week of immunization and subsequently seven times more every fortnight. For clotting the blood samples were kept at 37°C for h and then stored overnight at 4°C. Then the clot was slightly loosened with sterile needle and antiserum was taken in a sterile centrifuge tube and clarified by centrifugation at 2000g for min. at room temp. Finally antisera was stored at -20°Cuntil required.

3.13.3. Purification of IgG

3.13.3.1. Precipitation

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

3.13.3.2. Column preparation

Approximate 8g of DEAE cellulose (Sigma Co. USA) was suspended over in distilled water overnight. The water was drained off and the gel was suspended in 0.005M sodium phosphate buffer, pH 8.0 and the buffer washing was repeated for 5 times. The gel was then suspended in 0.02M sodium phosphate buffer, pH 8.0 and was applied to a column (2.6cm, 30cm high) in diameter and allowed to settle for 2h. After the column material had settled, 25ml of 0.02M sodium phosphate buffer, pH 8.0 was applied to the gel material.

3.13.3.3. 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-0.3M. The initial elution buffer-(1) was 0.02M sodium phosphate buffer pH.8.0. The final elution buffer-(2) was 0.3M sodium phosphate buffer pH.8.0.The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above surface of the buffer (1) was connected to upper flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to the column, buffer (2) was sucked into buffer-(1) thereby producing a continuous rise in molarity. Finally, 40 x 5 ml fractions each of 5 ml were collected and the optical density values were recorded at 280nm by means of UV spectrophotometer (DIGISPEC-200GL).

3.13.3.4. Estimation IgG concentration

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

3.14. Immunodiffusion test

3.14.1. Preparation agarose slides

Glassslides (5cm x 5cm) were degreased in 90% (v/v) ethanol; ethanol: diethylether (1:1, v/v) and then dried in hot air oven. After drying, plates were sterilized. Agarose gel was prepared in 0.05M Tris-barbiturate buffer (pH-8.6) at 90°C, 0.9% agarose (Sigma, USA) was added into the buffer and placed in a water bath and stirred till the agarose solution became clear. Into the clear agarose solution 0.1% (w/v) sodium azide (antibacterial agent) was added. For the preparation of agarose gel, 10 ml of molten agarose was poured on sterilized glass slides (10ml/side) in laminar flow chamber and kept 15 min for solidification. After that 3-7 wells were cut out with a sterilized cork borer (6/m.m.dia) at a distance of 1.5-2.0 cm from the central well. and 2.0-2.5 from well to well (peripheral).

3.14.2. Diffusion

Agar gel double diffusion test were carried out using antigen and antiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera (100µl/well) were pipetted directly into the appropriate wells in a laminar chamber and diffusion was allowed to continue in moist chamber for 27 hr at 25°C.

3.14.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then slides were stained with coomassie blue (Rs. 250, Sigma; 0.25g coomassie blue + 45ml distilled water + 10ml glacial acetic acid) for 10 min. at room temperature. After staining, slides were washed in distaining solution (methanol : distilled water and acetic acid in 45;45;10 ratio)) with changes until the background became clear. Finally, slides were washed with distilled water and dried in hot air oven for 3h at 50°C.

3.15. Enzyme linked immunosorbent assay (ELISA)

ELSA test as mentioned by Chakraborty *et.al.* (1995) was carried out using following buffers.

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

Stocks

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

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

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

2. Phosphate Buffer saline (0.15 M PBS pH -7.2)

Stocks

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

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

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

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

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

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

   0.05 M Tris, 0.135 M NaCl, 0.0027 M KCI

   Tris - 0.657g

   NaCl - 0.81g

   KCl - 0.223g

Distilled water was added to make up the volume to 100ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% bovine serum albumin (BSA) were added.

5. Antisera dilution buffer (0.15M PBS- Tween, pH 7.2)

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

6. Substrate

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

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

3.15.1. Plate trapped Antigen Coated (PTA)-ELISA

PTA-ELISA was performed following the method as described by Chakraborty *et.al.* (1995). Plants and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl /well) in ELISA plate. After loading, the plate was incubated at 25°C for 4h. Then the plate was washed 4 times under running tap water and twice with PBS-Tween and shaken to dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1h. After incubation, the plate was washed as mentioned earlier. Purified IgG was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4°C

overnight. After further washing, antirabbit IgG goat antiserum, labeled with alkaline phosphatase (Sigma Chemicals, USA, diluted to 10000 times in PBS) was added to each well (200µl/well) and incubated at 37° for 2h. Plate was washed, dried and loaded with 200µl of p-Nitrophenyl phosphate substrate in each well and incubated in dark at room temperature for 45-60 min. Colour development was stopped by adding 50µl/well of 3N NaOH solution and absorbance was determined in an ELISA Reader (Multiscan Model 352) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.15.2. Double antibody sandwich (DAS) - ELISA

3.15.2.1. Conjugation of alkaline phosphatase with γ globulin

Labeling of γ globulin with alkaline phosphatase has been done following glutaraldehyde one-step method. 5mg of alkaline phosphatase (Sigma Chemicals) was added to 2mg of immunoglobulin (IgG). The total volume was made up to 5 ml with PBS and kept at room temperature for 30min with occasional shaking. Following this 0.2% glutaraldehyde was slowly added to the mixture, and was again incubated for 2 hr with gentle stirring. Finally it was dialyzed 3 times against 500 ml ½ strength of PBS. After dialysis, bovine serum albumin (10 mg/ ml) + 0.02% NaN_3 was added and stored at 4°C.

3.15.2.2. Assay

DAS- ELISA technique was performed following the method of Brill *et al.* (1994). Antisera of *U. zonata* was diluted in coating buffer and loaded in each well (100µl/well) of ELISA plate. The plate was incubated for 4h at 37°C and washed 4 times by flooding the wells with PBS- Tween, plate was shaken to dry. Then 200µl of blocking reagent (1.0% BSA and 3mM NaN_3) was added to each well to overcome the nonspecific binding and plate was incubated at 25°Cfor 1hr. After blocking, plate was washed as mentioned earlier. Then test samples were added to empty wells (200µl/well) and incubated for over night at 4°C. On the next day plate was washed as before and alkaline phosphatase tagged IgG, diluted (1:10,000) in PBS, pH 7.2 was added (100µl/well) to each well and incubated for 4h at 25°C The plate was again washed and 200µl pNPP substrate (1mg/ml) was added to each well and incubated for 90 min at 25°C 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.

3.15.3. Competition ELISA

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

Test antigens were prepared in PBS from tea rhizosphere soils (healthy and inoculated with *U. zonata, T. harzianum and T. viride*) either singly or in different combinations. These samples were added (75µl/well) to well of 3 ELISA plate sets. To each of these wells, 75µl of *U. zonata* or *T. harzianum or T. viride* IgG (40µl/ml) was added. The plates were incubated at 37°Cfor 1h on shaker. The shaker was also used for all subsequent stages. At the end of this period 100µl of antigen / antiserum mixture was transferred to the corresponding wells on the pre-prepared test plates. Un reacted antiserum was added to control wells and the plates were incubated for 45 min. The plates were washed and 100µl anti rabbit IgG alkaline phosphatase conjugate was added to all wells of the plates. Following 30 min. incubation at 37°C plates were washed and 100µl of P-nitro phenyl phosphate substrate (1 mg/ml) was added to each well. After further 45 min incubation in dark absorbance values were recorded at 405 nm in an ELISA reader (ELISA-5 Trans Asia model).

3.16. Immunoblotting

3.16.1. Dot Blot

Dot Blot was performed following the method suggested by Lange *et. al.*, (1986).

Following buffers were used for dot-blot.

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

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

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

Assay: Nitrocellulose membrane (NCM) (Millipore, 7cm x 10cm, Pore size -0.45μm) was cut according to required size and placed inside the template. Coating buffer (2μl) was loaded in each well of the template over the NCM and kept for 30 mins to dry. Following this 2μl of test samples (antigens) were loaded into the template wells over the NCM and kept for 3h. At room temperature. Template was removed and blocking of NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30min on a shaker. Polyclonal antibody of *U. zonata* (IgG, 1:500) was added directly in the blocking solution and further incubated at 4°C for overnight. The membrane was then washed gently with running tap water for 3 min., following three times 5 min washes in TBS (pH-7.4) as suggested by Wakeham and White (1996). The membrane was then incubated in alkaline phosphatase – conjugated goat antirabbit IgG (Sigma Chemicals) diluted 1:10,000 in TBS-Tween for 2h at 37°C. The membrane was washed as before. Substrate (1 tablet each of Tris buffer and Fast Red (Sigma Chemicals) or 1 BCIP/NBT tablet (Sigma Chemicals) dissolved in 10ml double distilled water) was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water. Colour development was categorized on the intensity of the dots

3.16.2. Western Blotting

Western Blotting was performed using the protocol as described by Wakeham and White (1996). The following buffers were used for Western blotting.

Stock solutions

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

(ii) Transfer buffer (Towbin buffer)

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

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

(iv) Phosphate buffer Saline (PBS) (0.15M, pH 7.2)- Preparation was as mentioned in ELISA.

(v) Blocking solution

Casein hydrolysate -5% in PBS; Sodium azide -0.02%; Tween -20 -0.02%.

(vi) Washing buffers :

(a) Washing buffer -1 : PBS

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

Tris-6.07 gm; NaCl – 8.78gm; made up to 1 lit with distilled water.

(vii) Alkaline phosphatase buffer :(100mM NaCl, 5mM $MgCl_2$, Tris-HCl, pH9.5)

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

(vii) Substrate: NBT: 5mg NBT in 1ooµl of 70% N, N-dimethyl formamide

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

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer or 1 tablet of NBT/BCIP in 10ml of double distilled water

(viii) Enzyme. (Alkaline phosphatase tagged with antirabbit goat IgG) Alkaline phosphatase buffer; enzyme (1;10,000).

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

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

3.16.2.1. Blot transfer process

Following SDS-PAGE of antigen, gel was transferred to pre-chilled Towbin buffer and equilibrated for 1h. The nitrocellulose membrane (BIO-RAD, 0.45 µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15min. The transfer process was done in Trans-Blot SD Semi-Dry Transfer cell (Bio-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the Semi-dry cell and the pre-wetted

membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out with a glass rod. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed. The blot unit was run for 45 min at a constant volt (15v). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1 h. and preceded for immunological probing.

3.16.2.2. Immunoprobing

After proper drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min. with gentle shaking on a platform shaker at room temperature. Subsequently the membrane was incubated with antibody (IgG) solution (blocking solution: PBS [1:1, v/v + IgG, diluted as 1:100 or as per requirement]. The bag was sealed leaving space for few air bubbles and incubated at 4°C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250 ml PBS (washing buffer -1). Final washing was done in 200ml washing buffer -2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed for 3 times in washing buffer-2.Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.17. Immunofluorescence.

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

3.17.1. Fungal mycelia

Young fungal mycelia grown for 4 days in liquid Richard's medium were taken out from flask. 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 1hr at room temperature. The

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 Chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45min at room temperature. After incubation, mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase-contrast and UV fluorescence condition using Leica Leitz Biomed microscope with fluorescence optics equipped with ultraviolet (UV) filter set1-3.

3.17.2. Cross section of tea roots

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

3.18. Immunocytochemical staining

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

3.18.1. Substrate stain solution

A stock substrate solution consisted of 0.15g of naphthol-AS-phosphate (Sigma) dissolved in 2.5ml of N-N dimethyl formamide (Sigma) and added to 17.0g of Tris base (Sigma), pH 9.1, in 500ml of distilled water. The staining solution, prepared immediately before use, consisted of 1mg of Fast blue (Himedia) and 5µl of 0.1 M $Mgcl_2$ added per ml of stock selection and filtered through Whatman No.1 filter paper.

Cross-section of infected tea roots were cut and incubated with PBS containing 1% BSA for 20 min at room temperature to prevent nonspecific binding of antibodies to root tissue and then stained on grooved slides or watch glasses immunocytochemically.

The sections were then treated with antiserum diluted 1:100 in PBS with 1% BSA(PBS-BSA) at 37°C for 2h on a rotary shaker and washed with three changes (5min each) of PBS with 0.1% Tween-20. Following this sections were incubated in a 1:10,000 dilution of Goat antirabbit IgG alkaline phosphatase conjugate (Sigma) in PBS-BSA for 2h at 37°C on a rotary shaker and washed again as described above. Staining was carried out in the dark at room temperature in staining solution which was filtered through Whatman No.1 filter paper immediately before being applied to the slides and was incubated with the sections for no longer than 40min before slides were rinsed in PBS. Sections were then mounted in glycerol jelly and observed under bright field of the microscope (Leica Leitz Biomed) and photographs were taken in bright field.

3.19. Inducing agents and their application

3.19.1. *In Vitro* test

3.19.1.1. Biocontrol agents

Antagonistic properties of *T. harzianum* and *T. viride* against *U. zonata* were studied through dual plate method. Mycelial blocks (4 mm dia) cut from the margin of 3-day old cultures of both the test pathogen (*U. zonata*) and biocontrol agents (*T. harzianum* and *T. viride*) were placed opposite to each other on PDA in Petri plates (10 cm dia). The distance between inoculum blocks was 7 cm. Inoculated Petri plates were incubated at 25°C. To examine the inhibitory effect of culture filtrates of *T. harzianum* and *T. viride* against *U. zonata*, initially these were grown in potato dextrose broth (PDB) at 25°C with vigorous shaking on a platform shaker at 175 rpm for 7 days. Mycelia were harvested and culture filtrates were centrifuged at 12,000 g for 20 min and the supernatants were filter sterilized separately by passing through millipore filter. Then 45 ml of PDB media and 5 ml of culture filtrate were taken in 250 ml conical flask, while in control set 45 ml PDB media and 5 ml of sterile distilled water was taken. Each flask was then inoculated with 4 mm dia agar block of *U. zonata* and incubated ant 28°C for 10 days. Finally mycelial dry weights.were taken.

3.19.1.2. Organic additives

To evaluate the survival of *U. zonata* in organic amendments, the sand-maize meal media was prepared with 2% saw dust. The different organic amendments (cow dung, chicken manure, rabbit manure, mustard oil cake and neem cake) were powdered in

a grinder separately. The sand maize meal media and amended substance were mixed in 1:3 ratio and water added as required and sterilized. The mixture was plated in 9 cm dia Petri dishes and inoculated with 4 mm mycelial discs of *U. zonata* and incubated at 25°C. The growth of the fungus was recorded and compared.

3.19.2 *In vivo* test

Mustard oil cakes and neem cakes were allowed to decompose separately for a week in a clay pot covered with polythene. After decomposition, 100 ml of decomposed oil cake solution was added in each tea seedlings pots. The pots were then inoculated with *U. zonata*. Untreated control was kept for comparison. Growth behavior also observed up to two month. Organic additives (cow dung, rabbit manure and chicken manure); 100 gm of each were taken separately and mixed in 1 kg of soil. These soil mixtures were separately kept in each pot. Tea seedlings were planted in each pot containing different organic components. After one week, 100 gm of pathogen (*U. zonata*) inoculum was added in the rhizosphere of each tea seedling.

Mass cultures of *T. harzianum* and *T. viride* were prepared on carrier medium comprising of wheat bran and sawdust (WBSD) in 3:1 ratio. Five hundred grams of the contents of carrier medium moistened with 20 percent (w/w) distilled water was filled in each bag. These polythene bags were sterilized at 15 lb pressure for 1 h for 2 consecutive days. Each polythene bag was then inoculated with 4-6 days old bits (0.3 cm) of pure culture either of *T. harzianum* and *T. viride* and incubated at 25±1°C. During incubation, these bags were gently hand shaken to promote uniform sporulation over the carrier medium and to avoid clusters. Addition of biocontrol agents in soil was done 10 days prior to inoculation with *U. zonata*.

EXPERIMENTAL

4.1. Charcoal stump rot incidence in tea of Terai and Dooars

Charcoal stump rot disease caused by *Ustulina zonata* occurs widely in Terai and Dooars region. In order to determine the incidence of this disease in different Tea Estates, a survey of ten tea gardens was carried out. The survey of the tea gardens revealed the occurrence of charcoal rot most commonly in four gardens i.e. Hansqua Tea Estate, Matigara Tea Estate (Plate 5, fig.A), Bijoynagar Tea Estate (Plate 5, fig.B) and Trihana Tea Estate (Plate 5, fig.C) . In severe cases, plants in specific areas had died off. Diseased plant root samples submitted by the planters to Nagrakata Tea Research Station were collected (Plate 6,fig.A-E) and fungal pathogen was isolated (Plate 6, fig.F&G) for further experiment.

4.2 Cultural conditions affecting growth of *U. zonata*

The study of the growth of *U. zonata in vitro* showed variation depending on different factors like medium, pH, temperature and seasonal changes. The young mycelia of *U. zonata* were white or hyaline initially, which turned into grayish black brittle crust gradually. The mycelial growth was generally superficial with fan shaped dull silky white mycelia or small black or white cushion like growth was found depending on the medium

4.2.1. Media

U. zonata was grown in eight different media i.e. potato dextrose agar (PDA), potato sucrose agar (PSA), Richard's Agar (RA), carrot juice agar (CJA) Czapek-Dox agar (CDA), Flentze's soil extract agar (FSEA), malt extract peptone dextrose agar (MPDA), yeast extract-dextrose agar (YDA). Results revealed that the maximum growth was recorded in PDA followed by PSA and RA but minimum growth was recoded in CDA. The fungus shows submerged, translucent mycelia to surfaced thick white growth which gets shriveled and turn dark, with black grey sporulation in oval patches.

4.2.2. Incubation period

U. zonata was grown in PDA medium for a period of 30 days, Mycelial growth was recorded after 5,10,15, 20, 25 and 30 days of growth and incubated at 25 $\pm$ 1°C. Maximum growth was recorded after 20 days of incubation (Table 1) after which it declined. After 5 days of incubation the growth was negligible.

Plate 5 (Fig. A-C): Naturally infected tea plants showing above ground symptoms of charcoal stump rot. (A) Hansqua Tea Estate (B) Matigara Tea Estate. (C) Bijoynagar Tea Estate.

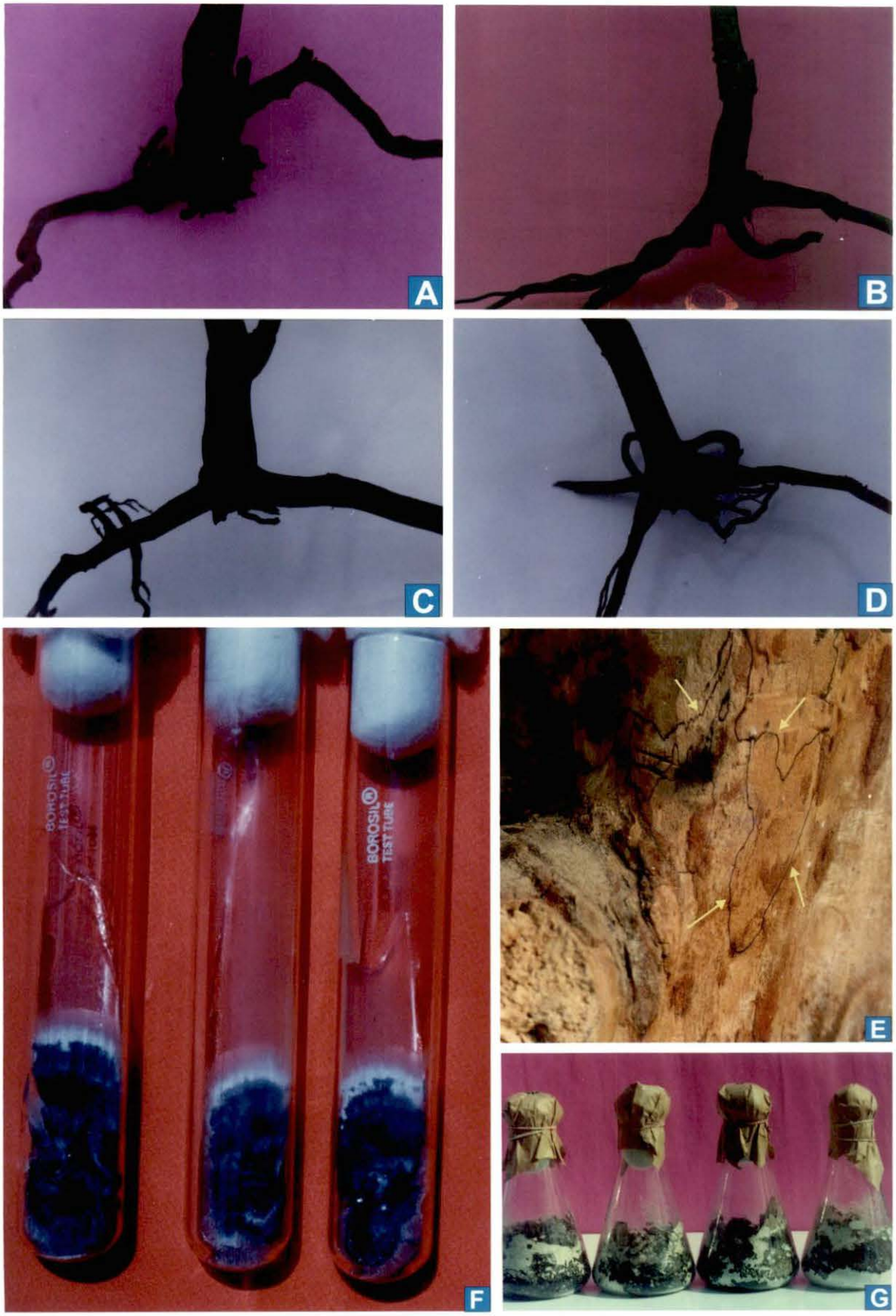


Plate 6 (Fig. A-G): Uprooted tea plants showing symptoms of charcoal stump rot disease (A-E), *Ustulina zonata* (Causal organism) grown in PDA (F) and sand maize meal medium (G).

Table 1 : Effect of incubation period on mycelial growth of *U. zonata*

Incubation Period (days)	Mycelial dry wt (mg)			
	Expt. 1	Expt.2	Expt.3	Mean
5	07.0	07.3	06.0	06.76±0.39
10	21.8	20.5	23.4	24.15±0.83
15	42.6	42.0	41.6	42.00±0.29
20	79.7	78.2	78.9	78.93±0.43
25	53.7	52.8	52.5	53.00±0.36
30	47.0	49.5	48.1	48.20±0.72

± Standard error; Temperature 25±1°C

4.2.3. pH

pH of the medium plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH, Initially buffer solution with pH values ranging from 3.0 to 8.0 (3.0,3.5, 4.0, 4.5,5.0, 5.5, 6.0, 6.5, 7.0, 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of 0.03M. The pH finally adjusted using N/10 HCl or N/10 NaOH in each case. Potato dextrose medium and phosphate buffer was sterilized separately by autoclaving for 15min. at 15 lb p.s.i pressure and equal parts of the buffer solution and medium were mixes before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 20 days after which dry wt. was taken as described previously. Results (Table 2) revealed that *U. zonata* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 3.5 - 4.0 and then growth gradually declined.

4.2.4. Carbon source

Like the pH of the surrounding medium the growth of fungus is greatly influenced by available nutrients. The ability of fungi to grow in different media depends on their capacity to utilized by the available nutrients, of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In the present investigations, eight different carbon sources (dextrose, fructose, lactose, mannitol, maltose, sorbose, starch and sucrose) were

tested for their effect on the growth of *U. zonata*. These were added separately to the basal medium. PDA medium without sugar was used as the basal medium which served as control set. Data were recorded after 20 days of incubation. Results (Table 3 , Fig.1) revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth.

Table 2 : Effect of different pH on mycelial growth of *U. zonata* Mycelial dry wt (mg)

pH of Medium[a]	Expt. 1	Expt.2	Expt.3	Mean
3.0	41.6	41.0	39.9	30.83± 0.49
3.5	58.5	58.1	58.9	58.50±0.23
4.0	54.8	53.8	54.5	54.36±0.29
4.5	51.3	51.0	51.6	51.30±0.17
5.0	44.5	43.7	43.9	44.03± 0.24
5.5	38.9	38.2	38.1	38.40±0.25
6.0	35.1	35.5	35.6	35.40±0.15
6.5	28.9	27.4	26.9	27.73±0.60
7.0	24.8	24.6	24.0	24.46±0.24
8.0	19.2	19.7	18.9	19.26±0.23

[a] PDA ; ± Standard error; Temperature 25 ±1°C ; Incubation period 20 days

Table 3 : Effect of different carbon sources on mycelial growth of *U. zonata*

Carbon Sources	Mycelial dry wt (mg)			
	Expt. 1	Expt.2	Expt.3	Mean
Fructose	56.8	56.0	48.2	53.66±2.746
Sorbose	01.7	01.5	01.3	01.50±0.057
Dextrose	47.4	46.9	47.9	47.40±0.289
Mannitol	32.0	31.0	27.6	30.20±1.333
Sucrose	41.1	43.2	38.4	40.90±1.390
Starch	26.0	24.8	21.6	24.13± 1.314
Maltose	30.4	27.3	29.6	29.10±0.930
Lactose	60.1	62.5	64.4	62.33±1.245
Control	01.8	01.5	1.8	01.70±0.173

± Standard error; Temperature 25 ±1°C; Incubation period 20 days

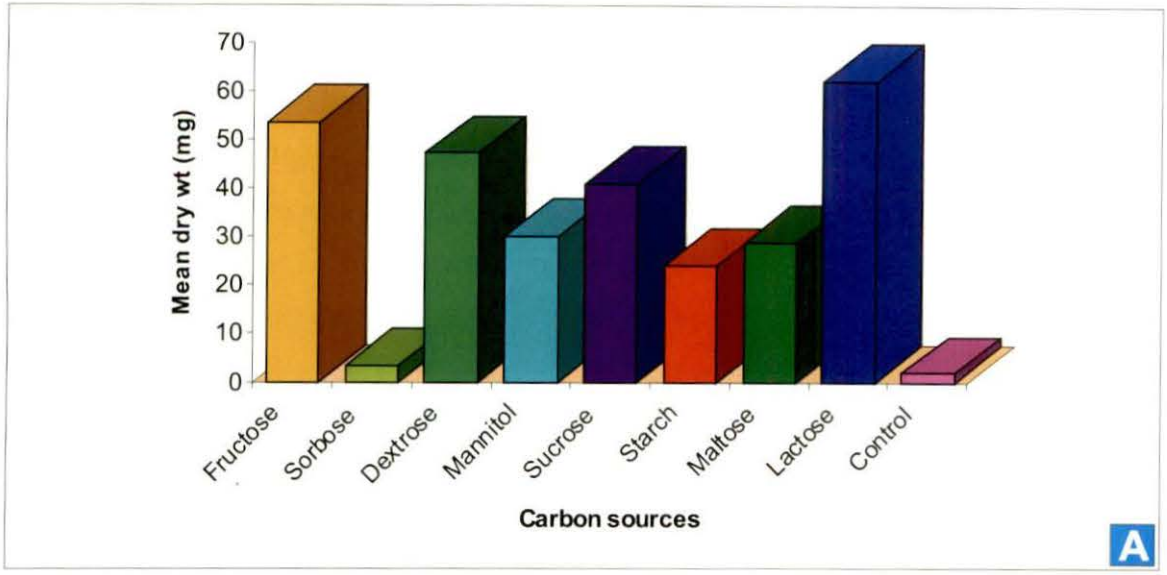
4.2.5. Nitrogen source

The availability of nitrogen for growth of the organism depends on the form in which it is supplied. Hence the most suitable medium for any particular microorganism can only be determined by testing a number of sources including both organic and inorganic. The effect of inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate and sodium nitrate) as well as complex organic sources (casein hydrolysate, beef extract, peptone, urea and yeast extract) on the mycelial growth of *U. zonata* was tested. A basal medium without any nitrogen source was considered as control. Results (Table 4, Fig.1) revealed maximum growth in beef extract followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

Table 4 : Effect of different Nitrogen sources on mycelial growth of *U.zonata*

	Dry weight of fungal mass (mg)			
Nitrogen sources	Expt. 1	Expt.2	Expt.3	Mean
Inorganic				
Potassium nitrate	29.6	35.8	25.6	30.3±2.97
Sodium nitrate	34.5	30.8	28.6	31.3±1.72
Ammonium sulphate	32.6	31.7	32.0	32.1±0.26
Ammonium nitrate	43.2	46.0	48.7	45.9±1.58
Calcium nitrate	127.5	112.9	118.6	119.6±4.25
Organic				
Urea	-	-	-	-
Peptone	201.5	210.6	205.4	205.8±2.63
Casein hydrolysate	190.5	192.0	186.7	189.7±1.57
Yeast extract	230.0	215.0	213.9	219.6±5.19
Beef extract	256.0	290.0	310.6	285.5±5.19
Control (without nitrogen)	3.2	4.1	2.9	3.4±0.36

± Standard error; Temperature 30 ±1°C ; Incubation period 20 days



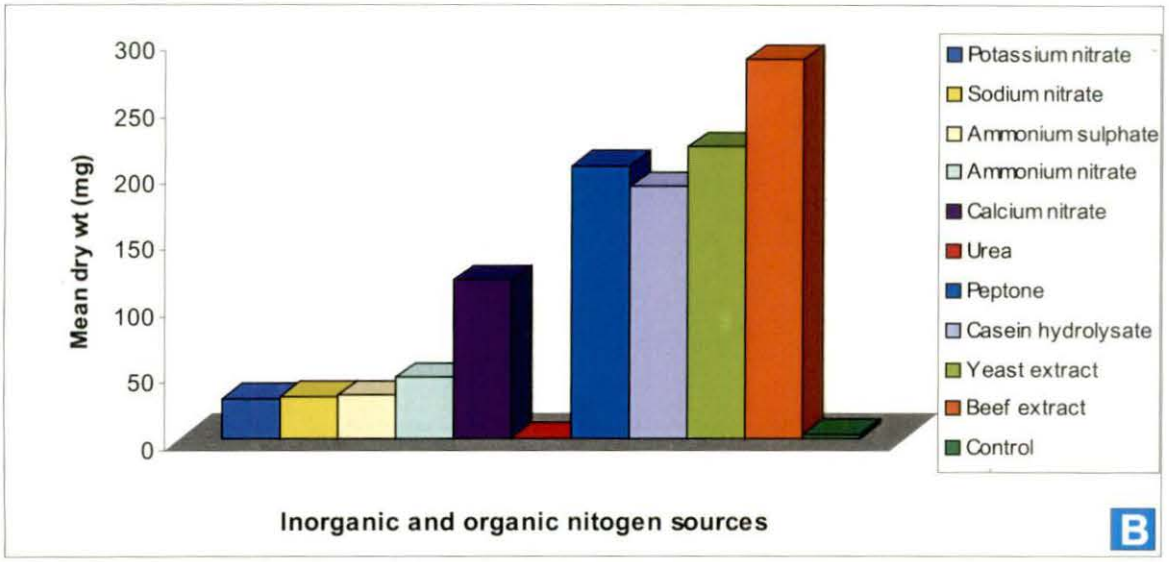


Figure 1: Effect of different carbon sources (A), inorganic and organic sources on mycelial growth of *U. zonata.*

4.3. Varietal resistance test of tea varieties against *U. zonata*

Naturally infected tea roots, showing symptoms of charcoal stump rot (Plate 2) was collected and the pathogen (*U. zonata*) was isolated from the naturally infected roots and then compared with the fungal culture received from Tocklai Experimental Station, Jorhat and was used for further studies involving different tea varieties. Varietal resistance test of tea against *U. zonata* was carried out with twenty four tea varieties. Three year old plant roots were inoculated with *U. zonata* and disease assessment was done on the basis of visual observation of symptoms and disease index (ranging from 1-6) was calculated after 20, 40 and 60 days of inoculation. Among the tested tea varieties BSS-2, BS/74/76 and AV-2 were found to be highly susceptible, while UPASI-9, UPASI-2, UPASI-3 were most resistant (Table 5; Fig. 2).

Table 5 : Varietal resistance test of tea varieties against *U. zonata*

	Disease Index		
Tea varieties [a]	20 days	40 days	60 days
TV-9	1.0	2.0	2.0
TV-18	1.1	1.9	2.0
TV-22	0.8	1.3	2.4
TV-23	0.5	2.0	2.8
TV-25	0.8	2.7	3.0
TV-26	0.5	2.0	2.9
TV-27	0.6	2.4	3.1
TV-28	1.0	2.1	2.6
TV-30	0.5	2.2	3.5
Teen Ali 17/1/54	0.9	1.6	1.9
BS/74/76	0.9	2.8	4.5
CP-1	0.4	1.5	2.6
AV-2	0.8	2.9	4.3
HV-39	0.8	0.9	2.5
T-135	0.4	1.3	2.2
S-449	1.1	2.0	2.2
P-1258	0.2	1.4	1.9
K1/1	0.4	1.6	2.3
UPASI-2	-	0.3	1.1
UPASI-3	-	0.2	1.3
UPASI-9	-	0.2	0.8
UPASI-26	0.9	2.1	3.6
BSS-2	1.9	3.7	4.6

Average of 30 separate inoculated plants of each variety ; ± Standard error

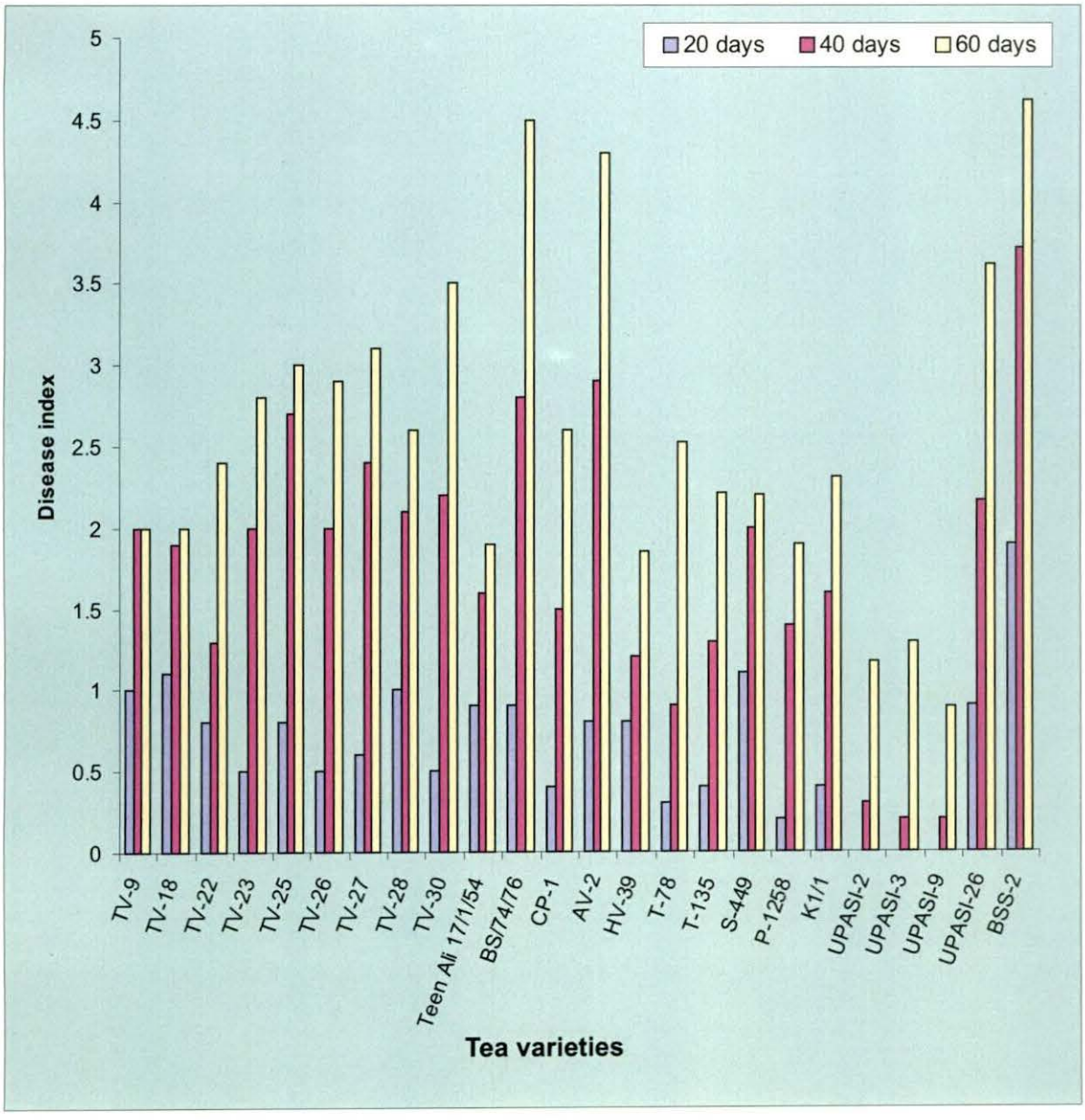


Figure 2: Varietal resistance test of tea against *U. zonata.*

4.4 Analysis of proteins in mycelia and tea roots

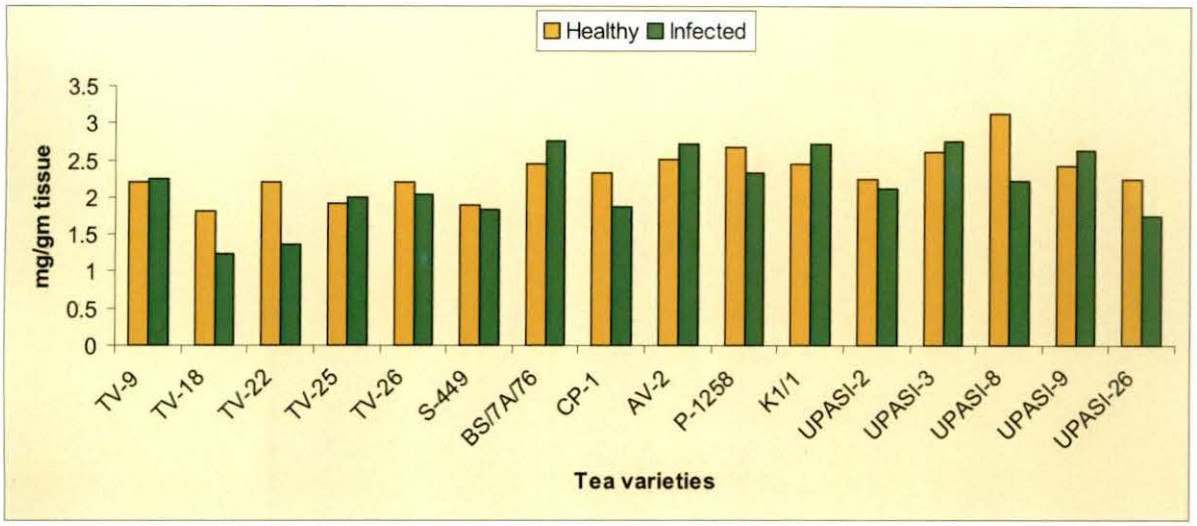
Soluble proteins of mycelia and cell wall of the pathogen as well as tea roots before and after inoculation with *U. zonata* was analysed . For this purpose estimation of protein content as well as SDS-PAGE was carried out with fungal proteins as well as proteins of healthy and infected tea roots.

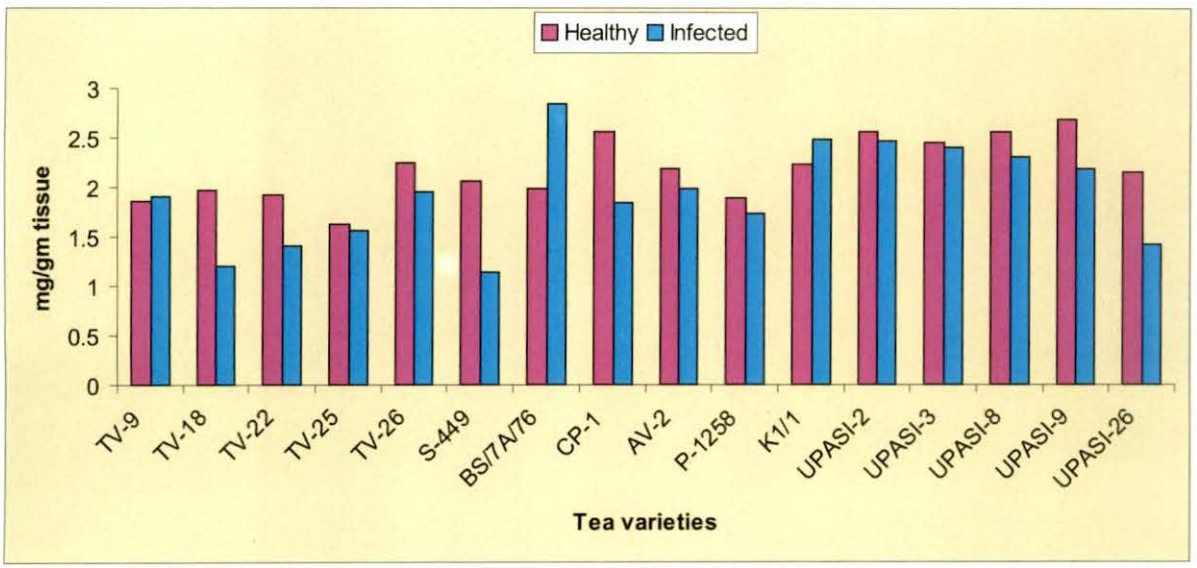
4.4.1. Protein content

Mycelial protein content of *U. zonata* was around 6.0mg/gm fresh weight tissue. Protein content of cell wall preparations was 5.8mg/g fresh weight of mycelial wall. Protein content of tea roots of tested varieties following artificial inoculation with *U. zonata* was estimated after 10,20 and 30days. Results (Table 6) revealed that protein content decreased following inoculation in most of the varieties tested (Fig 3). There was no relationship with susceptibility or resistance. In older plants (5yr old) protein content of susceptible varieties, which were greatly affected, showed a significant decrease in relation to control.

Table 6 : Protein content of healthy and infected tea root tissues.

	Protein content (mg/gm)					
	Days after inoculation					
Tea variety	10		20		30	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	2.20	2.24	1.86	1.90	1.52	1.55
TV-18	1.82	1.24	1.97	1.21	2.42	1.12
TV-22	2.21	1.35	1.92	1.41	1.96	1.36
TV-25	1.92	1.99	1.62	1.57	1.73	1.71
TV-26	2.21	2.03	2.25	1.95	2.08	1.70
S-449	1.90	1.84	2.07	1.14	2.12	1.23
BS/7A/76	2.46	2.76	1.98	2.84	2.54	2.63
CP-1	2.32	1.88	2.56	1.84	2.57	1.52
AV-2	2.51	2.72	2.19	1.99	1.92	1.64
P-1258	2.68	2.32	1.89	1.74	2.25	1.44
K1/1	2.46	2.72	2.23	2.48	2.32	2.47
UPASI-2	2.24	2.12	2.56	2.47	2.63	1.69
UPASI-3	2.62	2.75	2.46	2.41	2.24	2.02
UPASI-8	3.12	2.23	2.57	2.32	1.95	1.97
UPASI-9	2.42	2.64	2.69	2.18	2.89	2.99
UPASI-26	2.24	1.74	2.16	1.42	2.21	1.32





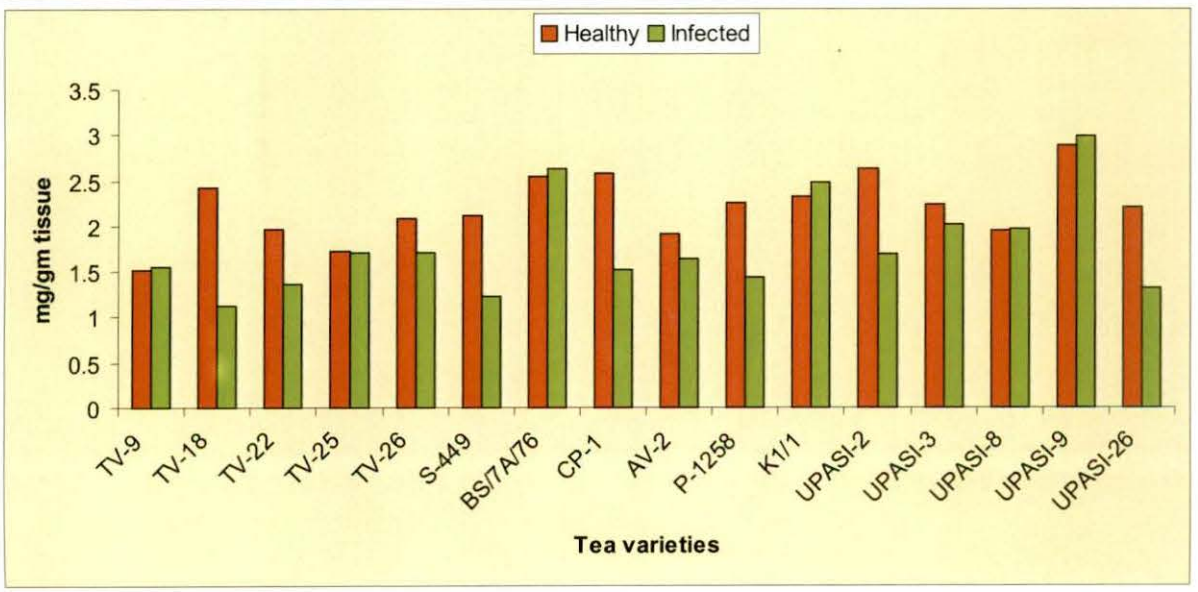


Figure 3: Soluble protein content of healthy and *U. zonata* inoculated tea roots.

4.4.2. Protein pattern

Proteins extracted from different sources were further analysed by SDS-PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker.

4.4.2.1 Mycelial protein of *U. zonata*

Mycelial protein exhibited 19 bands in SDS-PAGE ranging in molecular weight (Ca.97.4 kDa to 12.7 kDa) and bands were of varying intensities and more proteins of lower molecular were present (Plate 7,fig.A). Maximum number of bands were found in 10 and 15 days of incubation following which a decline in number of bands was found. High intensity of bands were found in extracts from 10-15 days. In case of 5 days on incubation one deep and thick band (ca. 22 kDa) was found which gradually became and less intense in following days.

4.4.2.2. Cell wall protein of *U. zonata*

Cell wall preparation of *U. zonata* were resolved in SDS-PAGE as described earlier, fixed in fixer solution and stained with coomassie blue. Gel exhibited 15 protein bands ranging from ca.97.4 to 15 kDa of which 7 bands were of higher molecular weight and 8 of low molecular weight (Plate 7, fig.B).

4.4.2.3. Tea root protein

Since protein content of tea roots had decreased following infection, it was decided to analyse the changes in protein pattern by SDS PAGE. In the healthy roots of the tea varieties number of bands ranged from 10-18 of molecular wts. 70-13 kDa. Following inoculation number of bands decreased. In infected roots of Tocklai varieties number of bands ranged from 9-11, whereas in infected roots of UPASI varieties number of bands ranged from 10-12.

4.4.3. Con A-FITC binding

Mycelia and isolated cell wall of *U. zonata* were treated with FITC labeled Con A. Strong apple green fluorescence were evident in both mycelia and cell wall which confirmed glycoprotein nature of the cell wall.

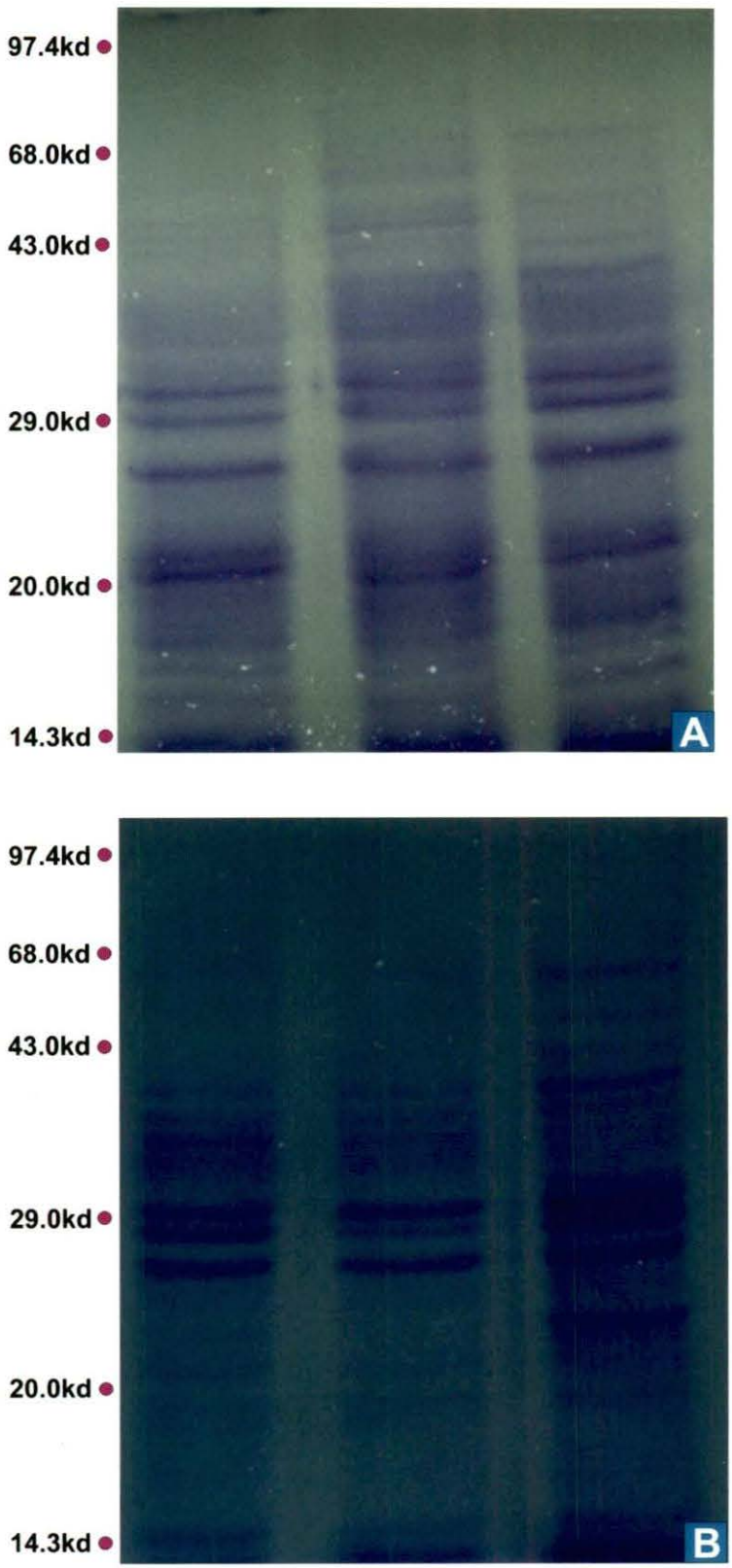


Plate 7 (Figs. A&B): SDS-PAGE analysis of soluble proteins of mycelia (A) and cell wall (B) of *Ustulina zonata*

4.5. Determination of levels of phenolics in tea varieties following inoculation with *U. zonata*

As polyphenols are the major constituents of tea roots it was decided to compare the level of total phenol and ortho-dihydroxy phenol in tea roots of resistant and susceptible varieties following inoculation with *U. zonata.* Sixteen tea varieties (TV-9, TV-18, TV-22, TV-25, TV-26, S-449, BS/7A/76, CP-1, AV-2, P-1258, K1/1, UPASI-2, UPASI-3, UPASI-8, UPASI-9 and UPASI-26) were selected for this experiment.

4.5.1 Total phenols

Total phenols from healthy and *U. zonata* inoculated tea roots of five tocklai varieties, six Darjeeling varieties and five UPASI varieties were extracted after 7 days of inoculation and estimated. Results (Table-7) revealed that total phenol decreased following inoculation with *U. zonata* in the susceptible varieties. However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested UPASI-varieties showed maximum increase in total phenol following inoculation with the pathogen.

4.5.2 Ortho-dihydroxy phenols

Ortho-dihydroxy phenols were also extracted from healthy and *U.zonata* inoculated tea roots of five each of Tocklai and UPASI varieties and six Darjeeling varieties after 7 days of inoculation with the pathogen and estimated. Results (Table -8) revealed that ortho-dihydroxy content decreased in susceptible varieties and increased in resistant varieties following inoculation with *U. zonata* . Response of UPASI varieties against the pathogen were towards increasing the level of orthodihydroxy phenol.

4.5.3. Analysis of antifungal compound in tea roots following inoculation with *U.zonata*

Further experiments were carried out to detect the antifungal phenolics from relatively large samples of freshly harvested tea roots following artificial inoculation with *U.zonata* using facilitated diffusion technique. Antifungal compounds were extracted separately from healthy and *U. zonata* inoculated tea roots of two resistant varieties (UPASI-9 and UPASI-3) and two susceptible varieties (BS/74/6 and BSS-2) after 96 h of inoculation.

Table-7: Total phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	Total phenol (mg/g Tissue)[a]	
	Healthy	**Inoculated [b]**
TV-9	5.3	4.8
TV-18	4.5	4.1
TV-22	4.9	3.7
TV-25	4.6	4.0
TV-26	6.2	5.7
S-449	6.5	3.1
BS/7A/76	5.9	2.8
CP-1	5.7	2.8
AV-2	4.9	3.0
P-1258	5.3	3.4
K1/1	5.8	2.6
UPASI-2	4.7	5.2
UPASI-3	4.9	5.0
UPASI-8	5.1	5.6
UPASI-9	5.4	5.9
UPASI-26	4.3	4.8

a = average of three replicates

b =7 days following inoculation with *U. zonata*

4.5.3.1. Bioassay

Ethyl acetate fractions of both healthy and *U. zonata* inoculated tea root extracts were loaded on TLC plates, developed in chloroform : methanol (9:1, v/v) and sprayed with Folin-Ciocalteau's reagent. Colour reaction was noted at Rf 0.56. Crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *U. zonata* inoculated roots of four varieties were bioassayed following radial growth inhibition assay. Results (Table -9) revealed that mycelial growth of *U. zonata* was inhibited markedly in the medium supplemented with the extracts of inoculated roots of resistant varieties (UPASI-9 and UPASI-3) than those of susceptible varieties (BS/74/6 and BSS-2) tested in relation to their respective control (media supplemented with healthy root extract). Mycelial growth was measured in each treatment, when *U. zonata* covered full Petridish grown in PDA without any supplementation.

Table-8: Level of Ortho-dihydroxy phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	Ortho-dihydroxy phenol(mg/g Tissue)[a]	
	Healthy	Inoculated [b]
TV-9	2.1	1.8
TV-18	2.7	2.5
TV-22	3.7	3.2
TV-25	4.1	3.8
TV-26	4.3	3.6
S-449	2.3	1.6
BS/7A/76	1.8	0.7
CP-1	1.7	0.9
AV-2	2.3	1.2
P-1258	2.4	1.1
K1/1	1.7	0.6
UPASI-2	1.3	2.4
UPASI-3	1.8	2.0
UPASI-8	0.9	2.1
UPASI-9	1.4	1.9
UPASI-26	1.2	1.8

a = average of three replicates

b =7 days following inoculation with *U.zonata*

Table 9 : Effect of antifungal compounds extracted from healthy and inoculated tea root extracts on radial growth of *U. zonata*

Variety	Diameter of mycelial growth (mm) [a]	
	Healthy	Inoculated [b]
Resistant		
UPASI-9	15.1	4.6
UPASI-3	13.2	5.3
Susceptible		
BS/74/76	19.6	10.4
BSS-2	18.4	12.8
Distilled water control	30 mm	

[a] Average of three experimental sets ; [b] Inoculated with *U. zonata*

4.5.3.2 UV -spectrophotometric analysis

Results of the bioassay revealed the presence of antifungal compounds in inoculated tea roots. Partially purified compound (Rf 0.56) from extracts of healthy and inoculated tea roots of UPASI-9 were examined in a UV-spectrophotometer. It is interesting to note that extracts from *U. zonata* inoculated root tissues gave a peak at 272nm. Maximum absorption peak measured at 272 nm was identical to an authentic sample of pyrocatechol. Hence quantification of pyrocatechol was done from UV-spectrophotometric curve by considering molar extinction coefficient of authentic pyrocatechol 6000 at 272 nm. Pyrocatechol accumulation in two resistant and two susceptible varieties of tea after 96 h of inoculation was estimated and compared with healthy controls. It appears from results that in inoculated roots, greater amount (428-512 µg/g fresh wt). Concentration of this compound in healthy root tissues were very low (54-73 µg/g fresh wt).

4.6. Determination of enzyme activity in healthy and *U. zonata* inoculated tea roots

4.6.1. Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenyl propanoid metabolism in higher plants and it has been suggested to play a significant role in regulating the accumulation of phenolics, and phytoalexins as well as lignins, three key factors responsible for disease resistance. In the present study, PAL activity of was assayed in sixteen tea root varieties following inoculation with *U. zonata*. PAL activity was assayed in each case after 2, 4 and 8 days after inoculation. Results have been presented in Table 10 and Figure 4. It showed that PAL activity increased after 4 days of inoculation markedly in all the varieties except TV-9 and UPASI-26. However, after 8 days of inoculation PAL activity decreased .

4.6.2. Peroxidase (PO)

PO activity was assayed as increase in absorbance when o-dianisidine was oxidized by the oxygen released from H_2O_2 which was oxidised by the enzyme. Peroxidase was extracted from healthy and *U. zonata* inoculated tea roots of sixteen varieties and their activity was also assayed after 2, 4 and 8 days of inoculation. Results

have been presented in Table 11. Peroxidase activity also increased in all the varieties tested following inoculation. Time course accumulation of peroxidase was highest after 4 days of inoculation and after this period peroxidase activity started declining. However, increased POX activity was noticed in TV-26, BS/74/76, CP-1, AV-2, K1/1 and UPASI-8 even after 8 days of inoculation with the pathogen (Fig.5).

Table 10 : Changes in phenylalanine ammonia lyase activity in tea roots following inoculation with *U.zonata*

	PAL activity in tea roots (µg cinnamic acid $g^{-1}m^{-1}$) [a]					
	Days after inoculation					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	160.1	35.9	163.4	89.5	170.4	101.8
TV-18	73.9	79.2	105.3	127.1	119.3	87.6
TV-22	74.8	23.9	76.0	141.9	93.2	68.7
TV-25	89.0	149.2	81.0	158.6	79.5	132.5
TV-26	69.5	57.0	75.4	173.8	81.6	98.3
S-449	71.8	89.3	73.1	129.9	76.3	101.2
BS/7A/76	138.0	153.7	143.2	247.0	136.8	59.4
CP-1	106.3	67.5	189.2	292.7	89.9	104.5
AV-2	33.2	77.4	41.6	120.9	41.8	101.1
P-1258	102.5	140.1	123.0	201.9	126.6	63.0
K1/1	135.7	170.6	133.8	231.9	139.4	53.9
UPASI-2	105.7	73.8	134.2	241.9	103.1	39.1
UPASI-3	107.5	69.8	137.3	240.8	101.7	39.2
UPASI-8	83.03	69.1	87.3	142.6	90.1	54.5
UPASI-9	56.7	70.3	68.2	89.7	49.7	28.9
UPASI-26	160.5	71.2	150.4	142.3	189.7	48.8

[a] Average of 3 replicates.

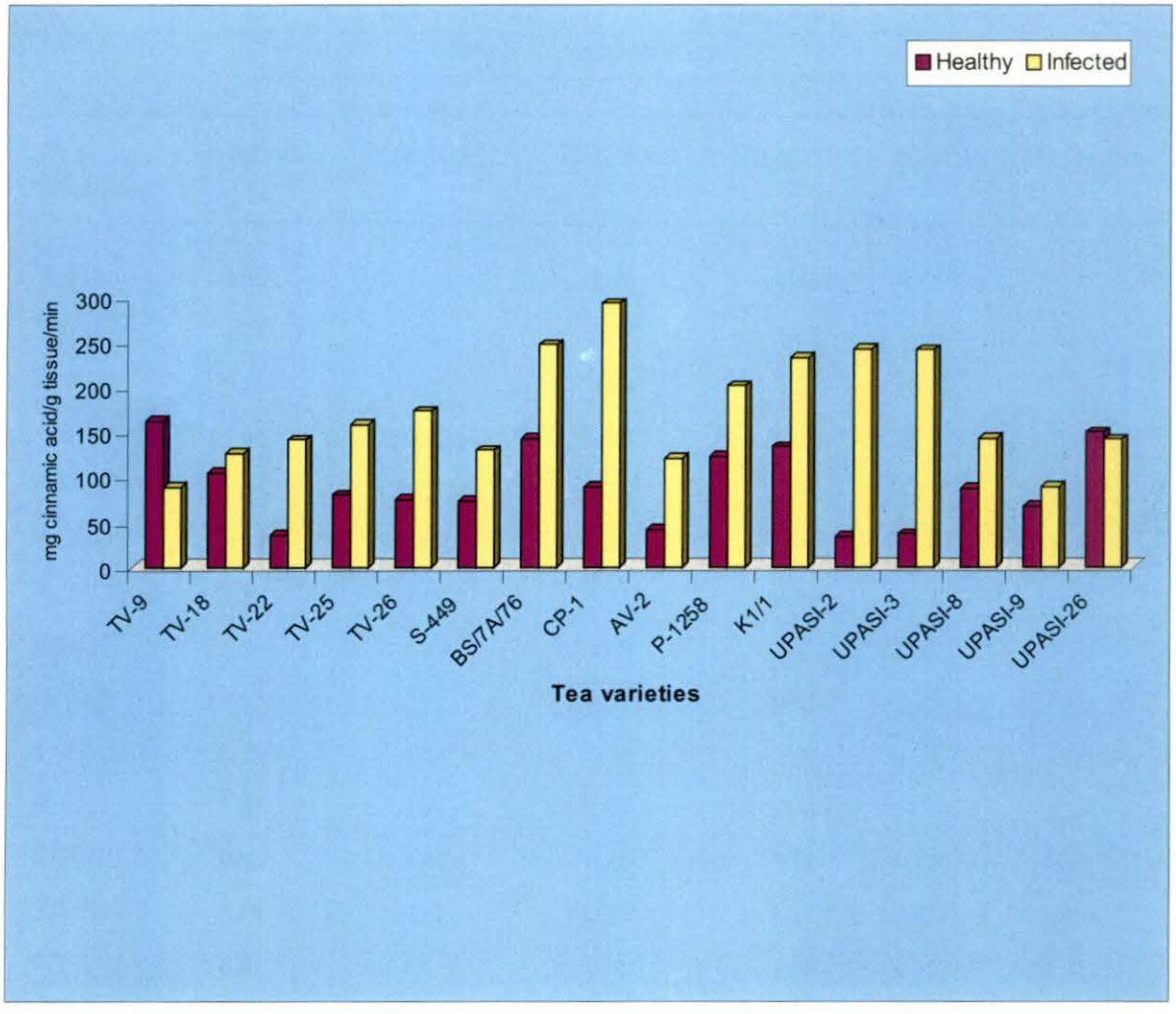


Figure 4: Phenylalanine ammonia lyase (PAL) activity in tea varieties following inoculation with *U.zonata.*

Table 11 : Changes in peroxidase activity in tea roots following inoculation with *U.zonata*

	PO activity in tea roots (ΔOD/g tissue/min) [a]					
	Days after inoculation[b]					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	1.40	1.13	1.46	1.57	1.63	1.37
TV-18	1.48	1.02	1.58	1.63	1.49	1.17
TV-22	0.65	0.16	0.64	1.80	0.65	1.14
TV-25	1.03	1.10	1.11	1.22	1.17	1.19
TV-26	1.45	1.53	1.29	1.77	0.89	1.74
S-449	0.91	0.98	1.23	1.06	1.14	1.26
BS/7A/76	1.23	1.09	1.11	2.51	1.21	1.56
CP-1	0.79	1.23	1.03	1.62	1.23	1.50
AV-2	0.23	0.81	0.52	0.91	0.22	0.81
P-1258	0.59	1.18	0.72	1.09	1.33	1.00
K1/1	1.05	1.21	1.02	2.34	1.06	1.38
UPASI-2	0.82	0.69	0.88	1.41	0.99	0.93
UPASI-3	0.68	0.87	0.52	1.21	0.65	0.83
UPASI-8	0.61	1.4	0.68	1.43	0.71	1.22
UPASI-9	0.89	1.13	1.24	1.32	1.36	1.20
UPASI-26	0.69	0.88	0.89	1.76	0.97	1.01

[a] Average of 3 replicates ; [b] Days after inoculation

4.6.3. Polyphenol oxidase (PPO)

Sixteen tea varieties were selected and enzyme activity following inoculation with the pathogen was assessed. PPO activity in tea roots increased markedly after 4 days of inoculation with *U. zonata* in all the varieties tested. Results have been presented in Table 12 and Fig.6

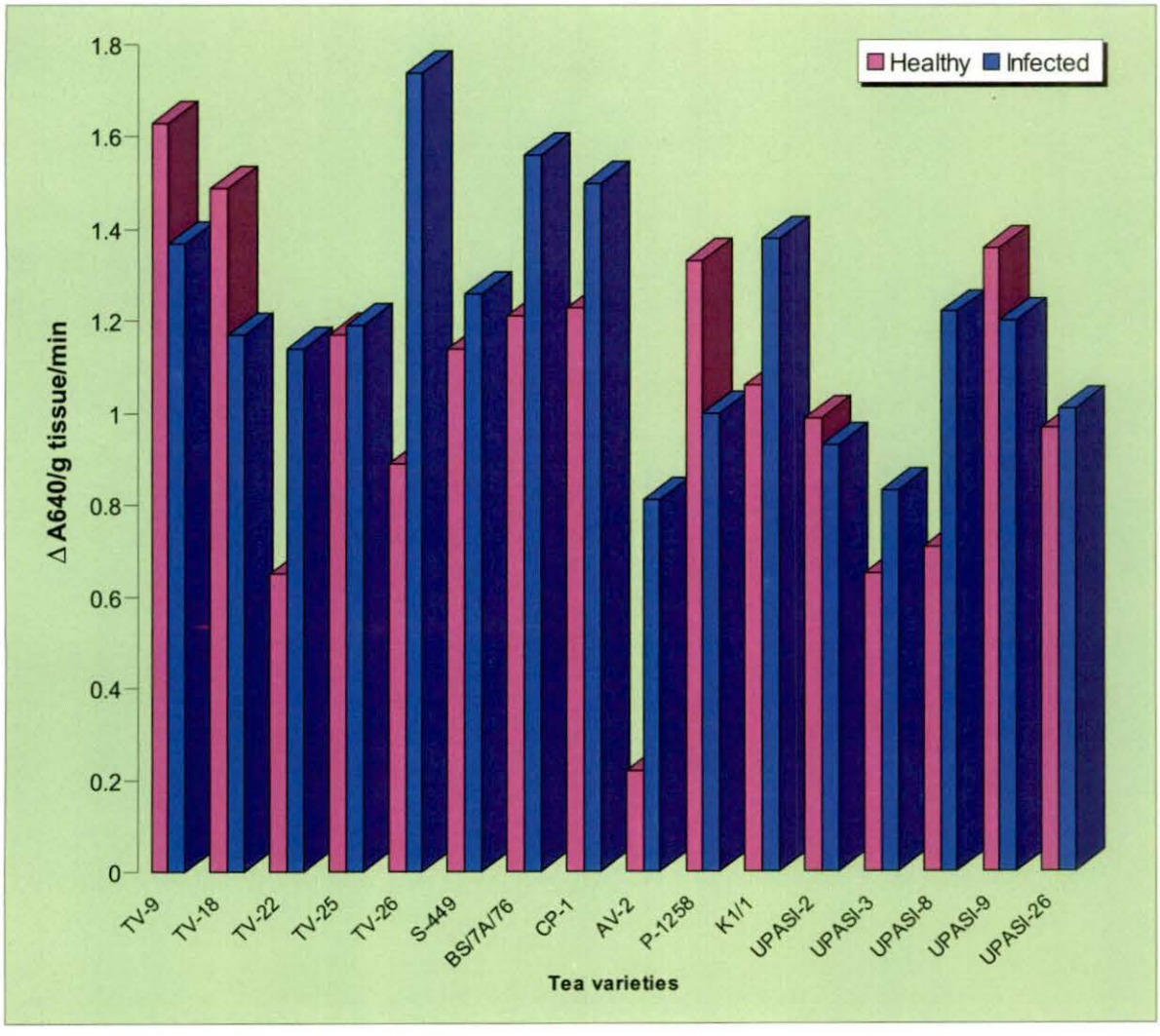


Figure 5: Peroxidase (POX) activity in tea varieties following inoculation with *U. zonata.*

Table 12 : Changes in polyphenol oxidase activity in tea roots following inoculation with *U.zonata*

	PPO activity in tea roots (ΔOD/g tissue/min) [a]					
	Days after inoculation[b]					
	2		4		8	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
TV-9	0.13	0.2	0.14	0.19	0.14	0.20
TV-18	0.15	0.16	0.15	0.19	0.17	0.21
TV-22	0.12	0.18	0.11	0.21	0.12	0.13
TV-25	0.15	0.14	0.16	0.21	0.16	0.19
TV-26	0.25	0.17	0.25	0.29	0.26	0.29
S-449	0.04	0.12	0.04	0.16	0.05	0.14
BS/7A/76	0.24	0.29	0.26	0.31	0.37	0.41
CP-1	0.11	0.08	0.13	0.28	0.15	0.21
AV-2	0.13	0.14	0.12	0.16	0.17	0.19
P-1258	0.04	0.11	0.08	0.28	0.09	0.21
K1/1	0.21	0.29	0.25	0.37	0.27	0.29
UPASI-2	0.23	0.21	0.19	0.26	0.14	0.19
UPASI-3	0.09	0.10	0.04	0.21	0.09	0.14
UPASI-8	0.24	0.25	0.18	0.22	0.25	0.09
UPASI-9	0.21	0.29	0.24	0.19	0.25	0.17
UPASI-26	0.13	0.12	0.15	0.16	0.13	0.08

[a] Average of 3 replicates ; [b] Days after inoculation

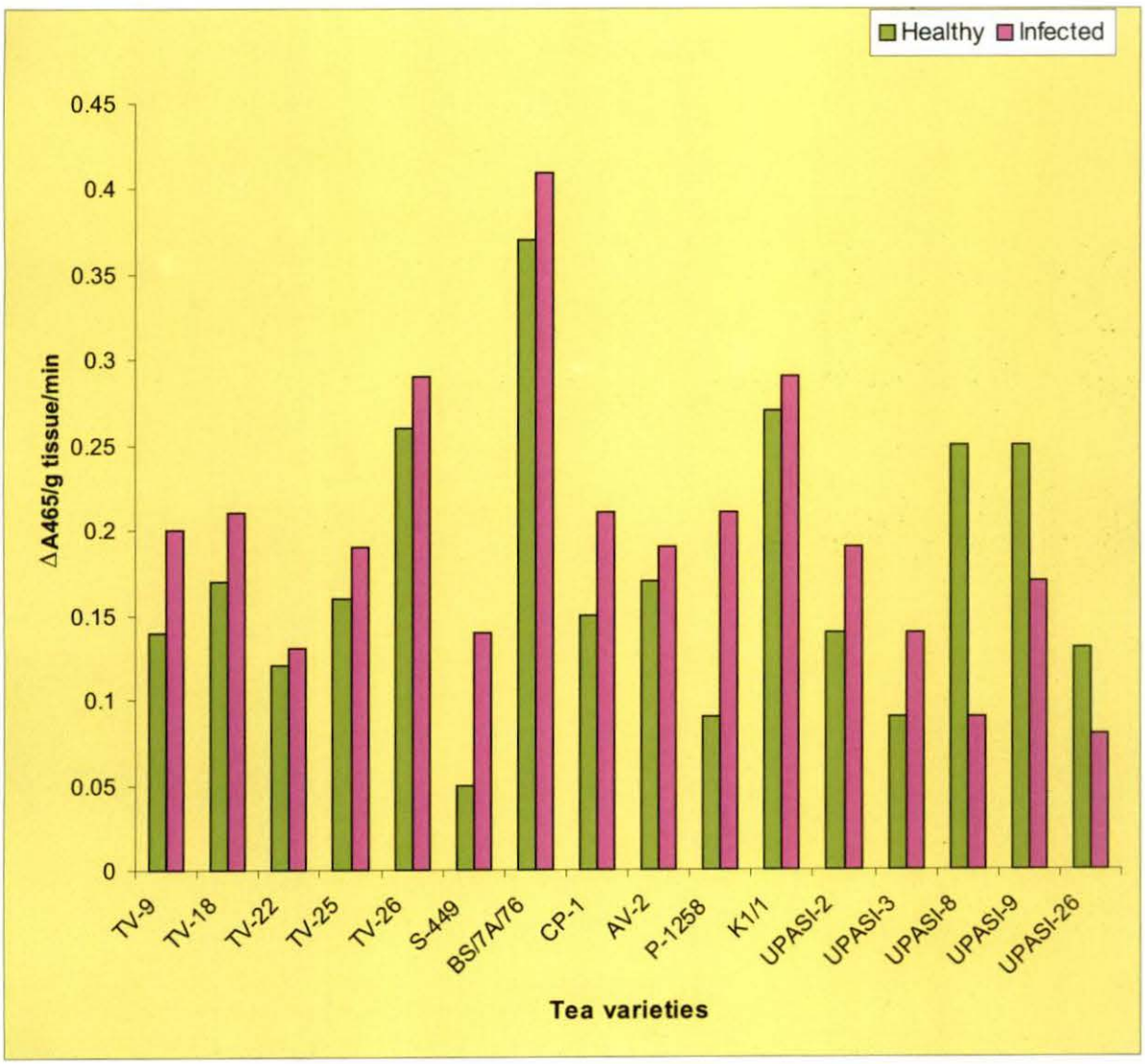


Figure 6: Polyphenol oxidase (PPO) activity in tea varieties following inoculation with *U. zonata.*

4.7. Agar gel double diffusion tests with PAbs of *U. zonata* and tea roots

Polyclonal antibodies (PAbs) were raised in rabbits against mycelial (100% SAS) and cell wall antigens of pathogen (*U. zonata*), mycelial antigens of two mycoparasites selected as biocontrol agents (*T. harzianum, T. viride*), as well as tea root antigens (UPASI-26) and these were used for development of immunodiagnostic kits using various serological assays. The PAbs obtained from different bleedings were initially checked for the effectiveness of each antigen preparation in raising PAbs by homologous cross reaction using agar gel double diffusion tests. Control sets involving normal sera and antigen of pathogen and tea roots were all negatives. Strong precipitin reaction occurred when PAb of *U. zonata* raised against mycelia and cell wall antigens were reacted with its own antigen (Plate 8 figs. A&B). Besides, strong positive reaction was also noticed when PAb raised against tea root antigen was checked in immumodiffusion test.

4.8. Optimization of PAb and antigen concentrations by ELISA

Enzyme linked immunosorbent (ELISA) assay is one of the most sensitive serological techniques for detection of cross reactive antigens between host and the pathogen as well as for detection of pathogen in diseased tissue and surrounding soil. Optimization using plate trapped antigen coated (PTA)- ELISA technique followed by primary antibody and finally secondary antibody labels with enzyme were performed separately with PAbs raised against mycelial and cell wall antigens of *U. zonata* and tea root antigens. PAbs in each case were collected by different bleedings at definite time intervals.

4.8.1. PAb raised against mycelia of *U. zonata*

Optimization of ELISA was done by considering two variables i.e. concentration of the antigen and concentration of PAb. Reactions were done with PAb obtained after different bleedings. Enzyme concentration was 1:10.000 while substrate was used at a concentration of 1mg/ml.

4.8.1.1. IgG concentration

Different concentrations of IgG (ranging from 0.312-40 µg/ml) from PAb were tested against homologous antigens at a concentration of 10µg/ml Absorbance values in PTA-ELISA increased with increase in concentration of IgG with a maximum value of 2.8 in 40 µg/ml (Table-13). This concentration of IgG was selected for further experiments.

Table 13: ELISA reaction with different concentration of PAb of *U. zonata* (mycelia) and homologous antigen

Antisera (IgG) concentration (µg/ml)	Absorbance at 405nm			
	Expt. 1	Expt.2	Expt.3	Mean
0.312	0.524	0.550	0.540	0.538± 0.007
0.625	0.721	0.743	0.738	0.734±0.006
1.25	0.892	0.889	0.868	0.883±0.007
2.5	1.064	1.067	1.021	1.050±0.014
5	1.341	1.456	1.401	1.399±0.033
10	1.567	1.621	1.501	1.563±0.034
20	2.078	2.154	2.240	2.157±0.046
40	2.871	2.909	2.91	2.896±0.012

Antigen concentration - 10 µg/ml
± Standard error

4.8.1.2. Antigen concentration

Antigen concentration ranging from 0.156-20 µg/ml were tested against IgG from 1st to 5th bleeding at a concentration of 40 µg/ml. Results (Table 14, Fig.7) revealed that ELISA values decreased with the decrease of antigen concentration. However concentration as low as 0.156 µg/ml could also be well detected by ELISA and maximum ELISA value was obtained in IgG 3rd followed by 4th bleeding.

Table 14 : ELISA reaction of mycelial PAb of *U. zonata* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (µg/ml)	Absorbance at 405nm				
	1st bleed	2nd bleed	3rd bleed	4th bleed	5th bleed
0.156	0.501	0.592	0.639	0.649	0.656
0.312	0.723	0.732	0.759	0.768	0.789
0.625	0.811	0.871	0.889	0.892	0.898
1.25	0.841	0.862	0.878	0.901	0.912
2.5	1.064	1.167	1.191	1.201	1.223
5	1.341	1.456	1.471	1.488	1.562
10	1.507	1.600	1.621	1.678	1.897
20	1.634	1.756	1.811	1.892	1.909
40	1.758	1.987	2.010	2.210	2.340

IgG concentration 40 µg/ml

4.8.2. PAb raised against cell wall of *U. zonata*

PAb raised against antigens prepared from cell wall of *U. zonata* were used to for optimization, considering two variables, antiserum concentration and antigen concentration on ELISA reactivity.

4.8.2.1. IgG concentration

A series of IgG concentration tested ranging from 0.312-40 µg/ml, prepared from PAb of cell wall preparation of the pathogen. Cell wall antigens were used at a concentration of 10 µg/ml. Absorbance values increased with increase in concentration of IgG with a maximum value 2.58 in 40 µg/ml (Table 15)

4.8.2.2 Antigen concentration

ELISA reaction with different concentration of cell wall antigen from 0.312-40 µg/ml were determined at an IgG concentration 40 µg/ml in respect of different bleedings. Absorbance values increased with increasing concentration (Table 16 & Fig.7) Result also revealed maximum ELISA values in IgG- 4th bleed followed by IgG-5th bleed raised against cell wall .

Table 15 : ELISA reaction of cell wall antigen of *U. zonata* with different concentration of homologous PAb.

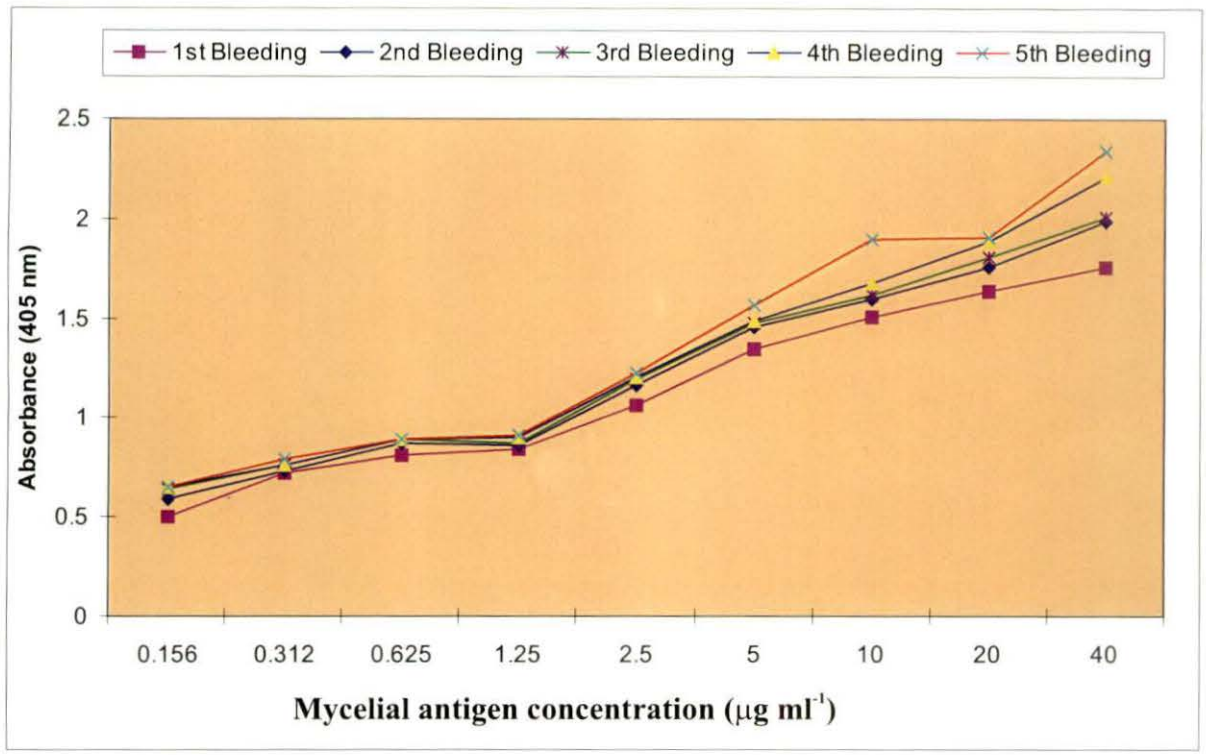
IgG concentration (µg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.841	0.839	0.840	0.858± 0.0005
0.625	0.894	0.896	0.897	0.895±0.0008
1.25	0.923	0.926	0.924	0.924±0.0008
2.5	0.923	0.926	0.924	0.981±0.0008
5	1.23	1.40	1.52	1.380±0.0842
10	1.87	1.89	1.92	1.890±0.0145
20	2.23	2.34	2.25	2.270±0.0338
40	2.56	2.61	2.59	2.580±0.0145

Antigen concentration 10 µg/ml; ±Standard error.

Table 16 : ELISA reaction of cell wall PAb of *U. zonata* obtained from different bleedings with different concentration of homologous antigen.

Antigen concentration (µg/ml)	Absorbance at 405nm				
	1st bleed	2nd bleed	3rd bleed	4th bleed	5 th bleed
0.312	0.687	0.691	0.721	0.798	0.813
0.625	0.691	0.711	0.856	0.872	0.976
1.25	0.823	0.837	0.924	0.956	0.965
2.5	0.928	0.977	0.982	0.993	1.102
5	1.223	1.488	1.512	1.545	1.684
10	1.687	1.869	1.882	1.889	1.909
20	1.923	1.934	1.995	2.102	2.198
40	2.209	2.451	2.287	2.897	2.567

IgG concentration 40 µg/ml; ± Standard error.



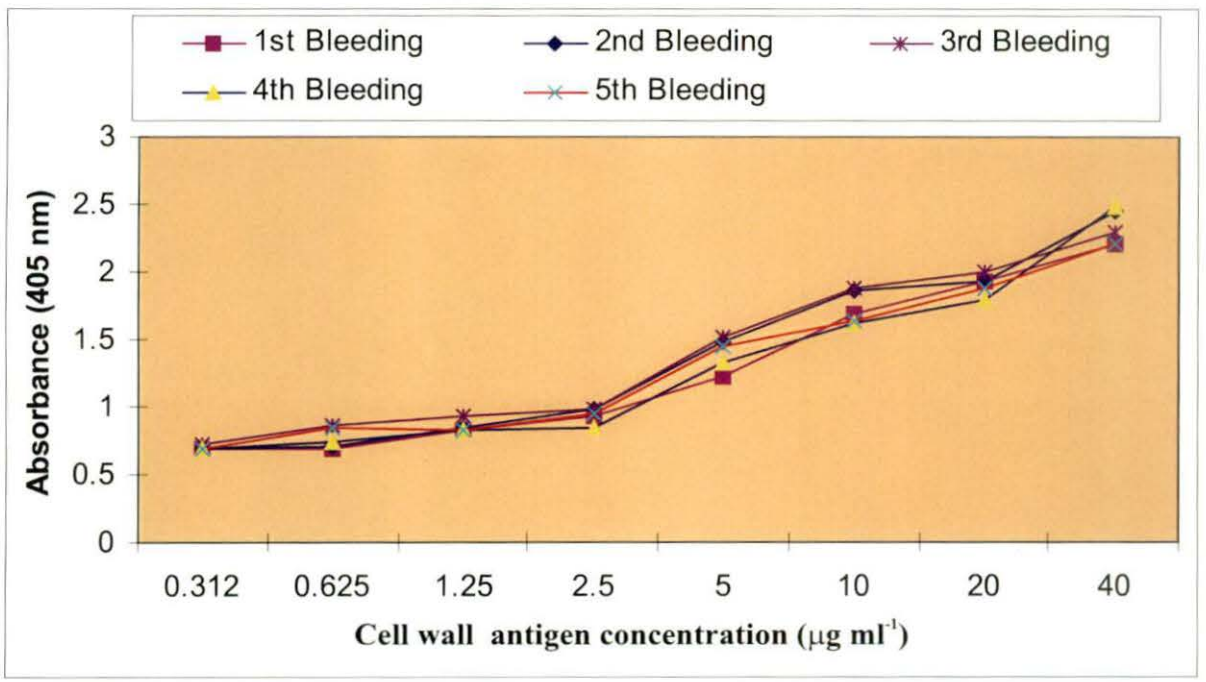


Figure 7: Optimization of mycelial and Cell wall antigen concentration of *U. zonata* using homologous PAb of the pathogen.

4.8.3. PAb raised against tea root

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

4.8.3.1. IgG concentration

Homologous reactions were carried out using different concentration of Pab raised against UP-26 root tissue ranging from 0.312 - 40μg/ml. A405 values decreased with decrease in concentration as revealed in Table 17.

Table 17 : ELISA reaction of tea root (UP-26) antigen with different concentration of homologous PAb.

IgG concentration (μg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt.3	Mean
0.312	0.543	0.560	0.547	0.550± 0.005
0.626	0.587	0.590	0.579	0.585±0.003
1.25	0.629	0.634	0.633	0.632±0.001
2.5	0.691	0.692	0.689	0.691±0.000
5	0.710	0.715	0.712	0.712±0.001
10	0.921	0.923	0.920	0.921±0.000
20	1.112	1.128	1.121	1.120±0.004
40	1.234	1.245	1.240	1.239±0.003

Antigen concentration 10 μg/ml
± Standard error.

4.8.3.2. Antigen concentration

To determine the effect of antigen concentration on ELISA reactivity, various concentrations of root antigen ranging from 0.312 – 40 μg/ml were used. IgG was used at a concentration of 40μg/ml. Absorbance value decreased with decrease in concentration (Table 18). In both mycelial and cell wall PAb, high A405 values were obtained even with 312ng/ml, indicating that the PAb could detect even lower concentrations.

Table 18: ELISA reaction of PAb of tea root (UP-26) with different concentration of root antigen

Antigen concentration (μg/ml)	Absorbance at 405nm			
	Expt.1	Expt.2	Expt. 3	Mean
0.312	0.467	0.560	0.434	0.487± 0.037
0.626	0.553	0.498	0.545	0.532±0.017
1.25	0.612	0.620	0.625	0.619±0.003
2.5	0.662	0.649	0.660	0.657±0.004
5	0.731	0.741	0.742	0.738±0.003
10	0.879	0.881	0.889	0.883±0.003
20	1.113	1.201	1.118	1.144±0.028
40	1.321	1.320	1.228	1.289±0.030

IgG concentration = 40 μg/ml
± Standard error.

4.9. Detection of cross reactive antigens between *U. zonata* and tea roots

4.9.1 Indirect ELISA

Indirect ELISA could readily detect cross reactivity between pathogen antisera and host tissues. Cross reactive antigens have been shown to be the determinants of compatible reaction in several host pathogen combinations. In the present study sixteen tea varieties revealed variable responses ranging from high susceptibility to high resistance. In order to determine whether resistance or susceptibility could be correlated with involvement of CRA, PTA-ELISA formats were designed with antigens of all tested tea varieties using PAbs raised against mycelia and cell wall. Results were compared with those of the varietal resistance test.

4.9.1.1. PAb of *U. zonata*

4.9.1.1.1. Mycelia

Antigens were prepared from tea roots of sixteen varieties, one non-host and one non-pathogen as well as mycelia of *U. zonata.* ELISA reaction were carried out with these antigens against purified mycelial PAb of *U. zonata.* Maximum absorbance values were recorded in reactions with antigens of susceptible varieties however, in

general, absorbance values were rather low (Table 19). PTA- ELISA reactivity of tea root antigens with PAb of *U. zonata* increased with age of plant.

Table 19 : Indirect ELISA values (A405) of antigens of tea root, *U. zonata*, non-pathogen and non- host reacted with mycelial PAb of *U. zonata*.

	Absorbance at 405nm			
Antigens	Expt.1	Expt.2	Expt.3	Mean
TV-9	0.567	0.561	0.559	0.562± 0.002
TV-18	0.617	0.612	0.600	0.609±0.005
TV-22	0.523	0.512	0.520	0.518±0.003
TV-25	0.462	0.437	0.431	0.443± 0.009
TV-26	0.456	0.451	0.449	0.452±0.002
S-449	0.597	0.589	0.592	0.592±0.002
BS/7A/76	0.472	0.469	0.461	0.467±0.003
CP-1	0.648	0.642	0.641	0.643±0.002
AV-2	0.478	0.471	0.456	0.468±0.006
P-1258	0.612	0.621	0.611	0.615±0.003
K1/1	0.532	0.554	0.530	0.539±0.007
UPASI-2	0.451	0.443	0.441	0.445±0.001
UPASI-3	0.475	0.471	0.477	0.474±0.000
UPASI-8	0.459	0.461	0.460	0.460±0.005
UPASI-9	0.461	0.443	0.450	0.451±0.011
UPASI-26	0.651	0.611	0.640	0.634±0.002
Mycelia of *U.zonata*	2.870	2.879	2.875	2.875±0.001
Non-pathogen				
Fusarium oxysporum	0.444	0.440	0.439	0.441±0.001
Non-host				
Oryza sativa	0.371	0.366	0.379	0.372±0.003

Antigen concentration = 20µg/ml
PAb Concentration of *U. zonata* (Mycelia) = 40µg/ml

4.9.1.1.2. Cell wall

ELISA reactions were carried out with antigens prepared from root of sixteen tea varieties against cell wall PAb of *U. zonata*. Results (Table 20) also revealed higher A405 values in case of susceptible varieties and slightly lower in case of resistant ones.

Table 20 : Indirect ELISA values (A405) of tea root antigens, cell wall antigen of*U. zonata*, non-pathogen and non host reacted with PAb of *U. zonata* (cell wall)

	Absorbance at 405nm			
Antigens	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
Tea Varieties				
TV-9	0.554	0.549	0.552	0.552± 0.001
TV-18	0.692	0.681	0.696	0.689±0.004
TV-22	0.579	0.581	0.572	0.577±0.002
TV-25	0.527	0.531	0.535	0.531±0.002
TV-26	0.515	0.520	0.511	0.515±0.002
S-449	0.601	0.592	0.605	0.599±0.003
BS/7A/76	0.519	0.522	0.525	0.522±0.001
CP-1	0.610	0.618	0.607	0.612±0.003
AV-2	0.535	0.526	0.530	0.530±0.002
P-1258	0.666	0.660	0.653	0.560±0.003
K1/1	0.523	0.519	0.528	0.523±0.002
UPASI-2	0.462	0.469	0.460	0.464±0.002
UPASI-3	0.543	0.549	0.557	0.549±0.004
UPASI-8	0.568	0.564	0.562	0.565±0.001
UPASI-9	0.512	0.509	0.511	0.511±0.000
UPASI-26	0.682	0.687	0.627	0.665±0.019
Cell wall	2.827	2.870	2.770	2.822±0.028
U. zonata				
Non-pathogens				
Beauveria bassiana	0.346	0.339	0.350	0.345±0.003
Non-host				
Leucaena leucocephala	0.356	0.370	0.377	0.368±0.006

Antigen concentration = 20µg/ml; IgG concentration of cell wall = 40µg/ml

4.9.1.2. PAb of tea root

PAb raised against healthy tea root antigen (UP-26) was tested to detect the cross reactive antigens of the root tissues with other tea varieties as well as root pathogens. The PAb of UP-26 reacted with the antigens of all other varieties and A405 values were quite high. Absorbance values were even higher where this PAb was treated with antigens of tea root pathogens. Strong reaction in ELISA reaction was noticed when PAb of the tea root reacted with pathogen antigens (Table 21).

Table 21 : Indirect ELISA values (A405) of tea root antigens, and mycelial antigens of root pathogens with PAb raised against tea (UP-26) root immunogens.

	Absorbance at 405nm			
Antigens	Expt.1	Expt.2	Expt.3	Mean
_Tea Varieties				
TV-9	0.829	0.828	0.831	0.829± 0.000
TV-18	0.820	0.819	0.809	0.816±0.003
TV-22	0.846	0.847	0.841	0.845±0.001
TV-25	0.782	0.780	0.778	0.780±0.001
TV-26	0.811	0.806	0.815	0.811±0.002
S-449	0.723	0.732	0.730	0.728±0.002
BS/7A/76	0.729	0.713	0.733	0.725±0.006
CP-1	0.760	0.769	0.777	0.769±0.004
AV-2	0.701	0.709	0.699	0.703±0.003
P-1258	1.010	1.021	1.001	1.038±0.005
K1/1	0.728	0.786	0.791	0.768±0.020
UPASI-2	0.919	0.921	0.925	0.922±0.001
UPASI-3	0.927	0.905	0.933	0.922±0.008
UPASI-8	0.821	0.825	0.819	0.822±0.001
UPASI-9	0.809	0.804	0.803	0.805±0.001
UPASI-26	1.200	1.211	1.190	1.200±0.006
Pathogen				
U. zonata	1.085	1.062	1.073	1.073±0.006

Antigen concentration 20µg/ml

PAb concentration (Mycelia)=40µg/ml

4.9.2. Western blot

Cross reactivity between *U. zonata* and tea root antigens were also determined by Western Blot analysis. It was observed that when healthy tea root extracts were probed with PAb of *U. zonata*, in the susceptible varieties 2-3 bands out of 4 bands (ca.79, 50, 21 and 19 kDA) were visible while in the others no bands or sometimes only 1 (ca.68kDa) was evident. In case of homologous reactions of mycelial and cellwall PAb showed 12bands (ranging from ca.80 to 16 kDa) and 7 (ranging from ca.68 to 18 kDa) respectively (Table 22 and Plate 8, fig. E & F) were observed.

Table 22 : Western blot analysis of mycelial and cell wall proteins of *U. zonata*.

Mycelia	12	79.4,68.9,50.1,43.0, 38.6, 29.2, 27.1, 24.5,20.0,18.4,17.4,16.3
Cell wall	7	68.9,43.0, 29.2,27.1,24.5,20.0,18.4

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

4.10. Detection of *U.zonata* in infected tea root tissues

To detect the pathogen in host tissues a number of immunodetection assays have proved effective where antisera raised against the pathogen reacted with antigens of infected material to give high absorbance values. These include ELISA, Western Blot

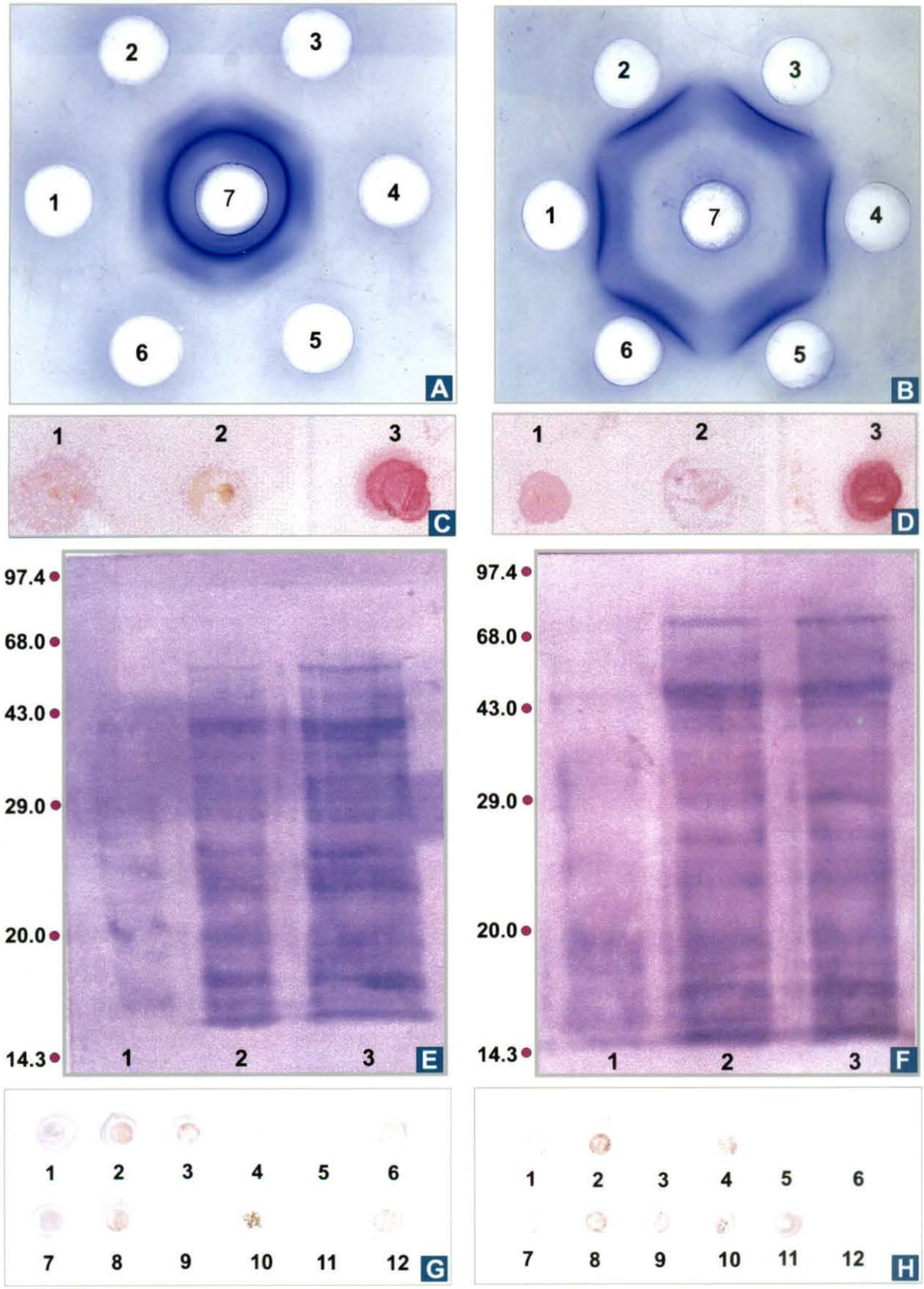


Plate 8 (Figs A-H): Serological assay of *Ustulina zonata* using(A,C,E&G) PAb of mycelia and (B,D,F&H) PAb of cell wall. (A&B) Agar gel double diffusion tests-antigens (1-6) and PAb of *U. zonata* (7). (C&D) Dot immunobinding assay-soil antigens(1&2) fungal antigen (3). (E&F) Western blot analysis- soil antigen (lane 1) fungal antigen (2&3). (G&H) Dot immunobinding assay of infested soil (1-12) collected from tea gardens.

and Dot Blot analyses. In the present study, following varietal resistance test and determination of cross reactive antigens in the different varieties attempts were made to detect *U. zonata* in infected tea root tissues by ELISA and Dot immunobinding assay.

4.10.1. ELISA

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

4.10.1.1. PTA– ELISA

4.10.1.1.1. PAb of mycelia

4.10.1.1.1.1. Artificially infected root tissues

PAb raised against mycelia of *U. zonata* was reacted in ELISA against the root antigens prepared from healthy and inoculated roots of sixteen varieties (Tocklai, UPASI and Darjeeling) of 2 year old tea plants. Antigens were prepared from healthy and inoculated root tissues at 15, 30 and 45 day intervals following the date of inoculation. Results revealed that in all tested varieties infected extracts showed higher ELISA values than the healthy extracts but the difference was not significance except in susceptible varieties. Results have been presented in Tables 23, 24 & 25. In Tocklai varieties maximum ELISA values were obtained in TV-18. Among the UPASI varieties the infected extracts and healthy extracts was obtained in UP-26 followed by UP-9. Significantly higher ELISA values in infected root extracts was obtained in the susceptible varieties (Fig 8).

Subsequently, tea root antigens from healthy and infected extracts of seven selected varieties (TV-9, TV-26, BSS-2, BS/7A/76, UPASI-2, UPASI-3 and UPASI-9) were tested with PAb of *U. zonata* mycelia, obtained from 4 bleeds . Significant differences between healthy and infected tea root antigens were obtained in all varieties and against PAb from all bleedings. Maximum A405 values were obtained in 3rd and 4th bleed (Table 26). However, it was observed that the detection could be done even with PAb of 1st bleed.

Table 23 : ELISA values showing reaction PAb of *U. zonata* with antigens of healthy and inoculated tea roots of Tocklai varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	**Healthy**	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
TV-9	0.441	0.565	0.506	0.687	0.509	0.690
TV-18	0.568	0.733	0.582	0.811	0.599	0.798
TV-22	0.546	0.650	0.548	0.662	0.545	0.682
TV-25	0.462	0.571	0.473	0.592	0.491	0.673
TV-26	0.449	0.489	0.461	0.620	0.493	0.670

Age of plant 2 yr.;Antigen concentration = 20µg/ml;
PAb concentration (Mycelia) = 40µg/ml

Table 24 : ELISA values showing reaction of PAb of *U. zonata* with antigens of healthy and inoculated tea roots of UPASI varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	**Healthy**	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
BSS-2	0.431	0.532	0.436	0.540	0.429	0.612
UPASI-2	0.562	0.599	0.561	0.750	0.558	0.781
UPASI-3	0.471	0.515	0.469	0.588	0.473	0.681
UPASI-8	0.440	0.511	0.453	0.559	0.480	0.576
UPASI-9	0.439	0.684	0.592	0.800	0.594	0.870
UPASI-26	0.581	0.682	0.581	0.803	0.590	0.871

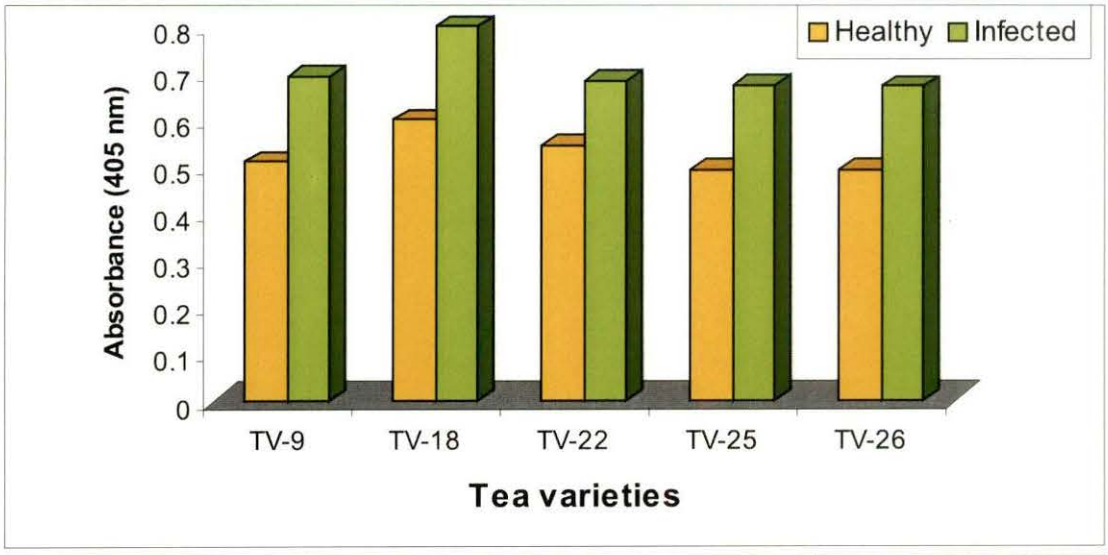
Antigen concentration 20µg/ml; PAb concentration (Mycelia)= 40µg/ml

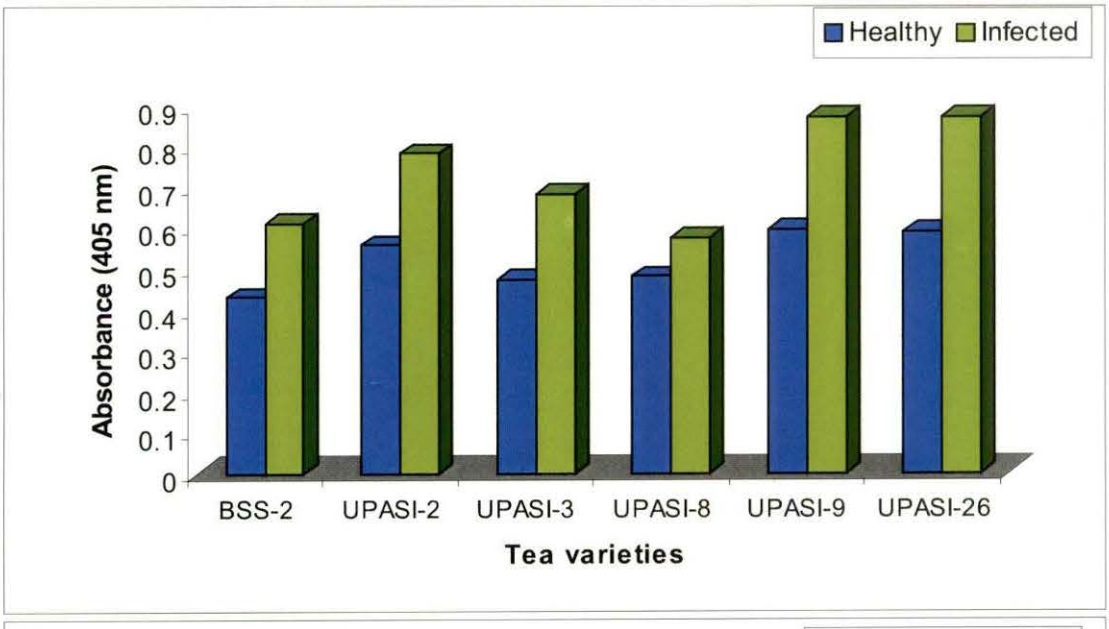
Table 25 : ELISA values showing reaction of PAb of *U. zonata* with antigens of healthy and inoculated tea roots of Darjeeling varieties.

	Absorbance at 405 nm					
	Days after inoculation					
	15		30		45	
Tea Varieties	Healthy	Infected	Healthy	Infected	Healthy	Infected
BS/7A/76	0.392	0.469	0.400	0.559	0.410	0.569
CP-1	0.548	0.562	0.552	0.670	0.554	0.722
AV-2	0.440	0.483	0.453	0.571	0.463	0.623
P-1258	0.552	0.581	0.558	0.570	0.587	0.713
K1/1	0.468	0.487	0.475	0.589	0.492	0.612

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

In a further experiment, it was decided to determine ELISA responses of healthy and inoculated tea root extracts obtained from plants of ages varying from 1 to 4 years. Absorbance values of healthy and infected extracts were found to increase with the age of the plants (Table 27). In susceptible varieties the A405 of extracts from infected plants 3 to 5 years old were very high, whereas in resistant varieties, they did not increase with age (Table 27). A good correlation was therefore obtained with disease symptoms rated visually and ELISA responses. It seems probable that the entry of the mycelia in resistant varieties is rather restricted, whereas in susceptible varieties, the mycelia had spread into the tissues of the older plants.





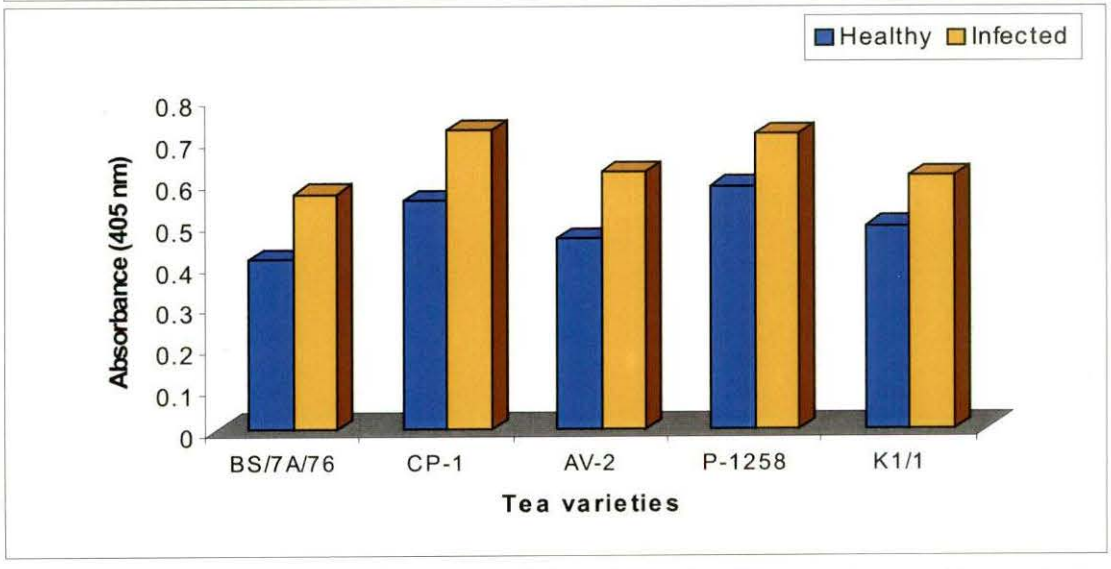


Figure 8: PTA-ELISA of healthy and artificially inoculated root antigens of tea varieties using PAb of *U. zonata*.

Table 26 : PTA-ELISA values of healthy and infected tea root antigens with PAb of *U. zonata* raised against mycelial antigen

	Absorbance at 405 nm							
	Bleeding No.							
	1st		2nd		3rd		4th	
Tea Varieties	**H**	**I**	**H**	**I**	**H**	**I**	**H**	**I**
TV-9	0.353	0.499	0.387	0.770	0.511	0.801	0.534	0.901
TV-26	0.372	0.599	0.463	0.711	0.589	0.734	0.631	0.897
BS/7A/76	0.532	0.736	0.569	0.792	0.634	0.989	0.672	1.897
BSS-2	0.469	0.660	0.475	0.675	0.482	0.892	0.521	1.439
UPASI-2	0.333	0.436	0.376	0.457	0.387	0.592	0.431	0.735
UPASI-3	0.347	0.503	0.436	0.557	0.476	0.612	0.472	0.618
UPASI-9	0.372	0.639	0.475	0.701	0.589	0.802	0.577	0.813

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

Table 27 : PTA- ELISA values of healthy and inoculated roots of a few selected tea varieties of different ages reacted with PAb of *U. zonata.*

	Absorbance at 405 nm					
	Age of the plants (years)					
	2 Yr		3 Yr		5 Yr	
Tea Varieties	**H**	**I**	**H**	**I**	**H**	**I**
BS/7A/76	0.432	0.679	0.713	0.990	0.817	1.542
BSS-2	0.469	0.660	0.570	0.871	0.938	1.835
UPASI-2	0.313	0.431	0.372	0.561	0.730	0.789
UPASI-3	0.347	0.392	0.438	0.493	0.634	0.710
UPASI-9	0.372	0.639	0.475	0.701	0.537	0.601

Antigen concentration 20µg/ml; IgG concentration = 40µg/ml

4.10.1.1.1.2. Naturally infected root tissues

It was decided to test whether natural charcoal stump rot infection could be detected with the PAb of *U. zonata* that was used to detect infection in artificially inoculated root tissues. For the purpose, infected tea roots were collected from different tea gardens showing symptoms of charcoal stump rot infection and antigens were prepared from these roots. Using antigen extracts from healthy and naturally infected tea roots PTA-ELISA was performed against mycelial PAb of *U.zonata*. Results presented in Table 28 revealed that the infected extract of Hansqua Tea Estate and Bijaynagar Tea Estate had significantly higher absorbance values in comparison to the healthy extracts.

Table 28 : PTA-ELISA values of healthy and naturally infected tea root antigens with PAb of *U. zonata*

		Absorbance at 405 nm	
Tea gardens		Healthy	Infected
Hansqua Tea Estate	Plot-1	0.582	0.793
	Plot-2	0.600	1.022
	Plot-3	0.530	0.702
Matigara Tea Estate	Plot-1	0.588	0.813
	Plot-2	0.600	0.913
	Plot-3	0.537	0.736
Trihana Tea Estate	Plot-1	0.606	0.938
	Plot-2	0.437	0.719
Bijaynagar Tea Estate	Plot-1	0.569	1.900
	Plot-2	0.598	1.002
	Plot-3	0.496	0.692

Antigen concentration =20µg/ml ; PAb concentration = 40µg/ml;

4.10.1.1.2. PAb of cell wall

In this experiment, healthy and inoculated root antigens were prepared from nine tea varieties and tested against the PAb raised against cell wall *U.zonata* by DAS-ELISA. ELISA values in general were higher in this case than those obtained using mycelial PAb. Infected root antigens gave higher reactivity than healthy ones (Table 29) with results similar as in case of mycelial PAb.

4.10.1.2. DAS-ELISA with PAb of mycelia

DAS-ELISA involves coating of ELISA plates first with the primary antibody, followed by antigens, which again is followed by secondary antibody labeled with enzyme. Detection of *U.zonata* in the infected roots of selected varieties using the DAS-ELISA format was also carried.

Table 29 : DAS-ELISA values of healthy and infected tea root antigens with PAb of *U. zonata* raised against cell wall in respect of different bleedings.

	Absorbance at 405 nm							
	Bleeding No.							
	1st		2nd		3rd		4th	
Tea Varieties	H	I	H	I	H	I	H	I
TV-9	0.430	0.489	0.382	0.779	0.516	0.841	0.54	0.910
TV-26	0.372	0.590	0.453	0.769	0.469	0.800	0.531	0.832
BS/7A/76	0.532	0.736	0.531	0.735	0.631	0.892	0.734	1.932
K 1/1	0.537	0.639	0.586	0.734	0.539	0.937	0.633	1.703
UPASI-2	0.411	0.521	0.403	0.532	0.422	0.600	0.531	0.819
UPASI-3	0.322	0.636	0.434	0.735	0.532	0.799	0.568	0.670
UPASI-9	0.431	0.536	0.451	0.707	0.492	0.790	0.613	0.745

Antigen concentration= 20µg/ml
PAb concentration =40µg/ml

Results presented in Table 29, revealed significantly higher ELISA values with infected extracts than healthy ones. In comparison to PTA- ELISA A405 values in both healthy and infected antigens were higher in this case.

Table 30 : DAS-ELISA values of healthy and infected tea root antigens tested against PAb of *U.zonata*.

	Absorbance at 405 nm	
Tea varieties	Healthy	Infected
BS/7A/76	0.870	1.201
K-1/1	0.713	1.436
UPASI-2	0.791	0.932
UPASI-3	0.643	0.790
UPASI-9	0.716	0.801

Age of plants 3year
Antigen concentration = 20µg/ml
IgG concentration = 40µg/ml

4.10.2. Dot - Blot

Root antigens of different tea varieties were reacted with mycelial and cell wall, PAbs of *U. zonata* in dot-blot as described earlier. Results (Table 31) revealed that the antigens from infected roots of BS/7A/76 and K1/1 showed pink coloured (Fast Red-substrate) dots while UP-2, UP-3 and UP-9 had very light coloured dot, indicating weak reaction. These varieties had shown resistance in previous tests including pathogenicity and ELISA. When antigens of healthy and infected tea roots of plants of different ages (1-5yr) were similarly reacted in dot-blot, it was observed that in healthy extracts of 1 yr old plants, there was no reaction, while weak reactions were observed in root extracts from 2 and 3yr. old plants. Colour intensity of dots in reactions of root antigens from 5 yr old plants were higher. In reactions with antigens from infected tea roots, positive reactions were obtained in all cases, though intensity increased with age (Table 32).

Table 31 : Dot – blot analysis of healthy and inoculated tea plants using PAb of *U.zonata*.

	Colour intensity[a]			
	Mycelial PAb (3rd bleed)		Cell wall PAb (3rd bleed)	
Tea varieties	Healthy	Infected	Healthy	Infected
BS/74/76	-	+	-	+
K-1/1	-	+	-	+
UPASI-2	-	±	-	±
UPASI-3	-	±	-	±
UPASI-9	-	±	-	±

[a] Fast red colour intensity : Pinkish red ; + + + + Bright, + + + High, + + Medium, + Low, ± Faint, - no reaction ; Age of plants 3year; IgG concentration 40µg/ml.

Table 32 : Dot-blot analysis of healthy and inoculated antigens of different ages of tea plants with PAbs of *U.zonata*.

		Colour intensity [a]			
Antigen source		Mycelial PAb (3rd bleed)		Cell wall PAb (3rd bleed)	
Tea varieties	Plant age (yr)	Healthy	Infected	Healthy	Infected
BS/74/76	2	-	+	-	+
	3	-	+ +	-	+ +
	4	±	+ +	-	+ + +
BSS-2	2	-	+	±	+
	3	-	+	-	+ +
	4	-	+ +	±	+ + +

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

Healthy and infected root antigens of selected tea varieties (5yrs old) were further tested in Dot-Blot with PAbs of *U.zonata* (mycelia and cell wall). As well as PAbs reised against root antigens of 2 tea varieties, one susceptible (UP-26) ad other resistant (TV-26). Among the 4 selected varieties 3 were most susceptible (TV-18, UP-26, T-78) and one was resistant (TV-26). Reactions with PAbs of tea roots showed dots of high intensity in general. Besides homologous reaction, PAb of TV-26 reacted very strongly with antigens from healthy roots of TV-26 & T-78, and PAb of UP-26 reacted with antigens of UP-26 & T-78 strongly, where deep violet coloured dots appeared. In cases of reactions with PAbs of roots, infected root antigens showed dots of lesser intensity then healthy ones while with PAbs of *U.zonata* (mycelia and cell wall) infected extracts showed dots of higher intensity (Table 33).

Table 33 : Comparison of dot-blot reaction of PAbs from different sources with healthy and incoculated root antigens of 5yr old tea varieties.

Tea Varieties	Plant Condition	Colour intensity [a] PAbs raised against		
		U.zonata Cell wall	BSS2	BS/7A/76
TV-18	Healthy	-	-	-
	Infected	+	±	±
BSS-2	Healthy	-	-	-
	Infected	+ +	+	+
UP-2	Healthy	±	-	-
	Infected	±	+	±
UP-9	Healthy	-	-	-
	Infected	+	±	±

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

4.10.3 Western blotting

In Western blot analysis, it was observed that when healthy root extracts were probed with PAb of *U. zonata*, in susceptible varieties 2-3 bands out of 4 bands (ca.79, 50, 21 and 19 kDa) were visible and infected root extracts from 2yr old plants. More bands (ca.101, 97, 79, 71, 61, 54, 53, 37, 32, 28, 25 & 21) were found in case of plants from 3 yr onwards.

4.11. Detection of *U. zonata* in soil

4.11.1. PTA - ELISA

In order to determine whether PAb of *U. zonata* could detect the pathogen in soil, samples were collected from various locations including several tea estates. Antigens were also prepared from amended soil infested with the propagules of *U.zonata* either in field condition or in potted condition. In this investigation, antigens were prepared from 38 samples from root rhizosphere soil and tested against the PAb of *U.zonata*. Result (Table 34) revealed only low A405 values in the range of 0.3 – 0.5 in most soil samples collected, except few samples which gave high absorbance valüe (ranging from 0.7 – 0.9) in ELISA. This indicated the presence of propagules of *U. zonata* only in these soils. In case of amended soils, high values were obtained. Both positive and negative controls were in expected ranges.

4.11.2. Dot blot

Identification of *U. zonata* propagules in artificially infested and non infested root rhizosphere soil was carried out through dot immunobinding reaction also. Soil antigens were prepared from soil samples from *U.zonata* amended soil and different locations including tea gardens and reacted with PAb of *U.zonata*. Among 38 Collected soil samples only 4 sample showed positive reactions, though dots were of low intensity ; in all other samples, either not dots could be detected or the reactions were very weak. was (Table 35).

Table 34 : ELISA responses of different soil antigens with PAb of *U. zonata* (mycelia).

Soil sample	Absorbance at 405nm	Soil sample	Absorbance at 405nm [a]
S – 1	0.489	S - 21	0.693
S - 2	0.362	S – 22	0.465
S – 3	0.552	S – 23	0.593
S – 4	0.443	S – 24	0.502
S – 5	0.456	S – 25	0.487
S – 6	0.584	S – 26	0.499
S – 7	0.792	S – 27	0.419
S – 8	0.596	S – 28	0.343
S – 9	0.498	S – 29	0.373
S – 10	0.462	S – 30	0.741
S – 11	0.479	S – 31	0.398
S – 12	0.468	S – 32	0.356
S – 13	0.472	S – 33	0.354
S – 14	0.422	S – 34	0.378
S – 15	0.856	S – 35	0.374
S – 16	0.763	S – 36	0.999
S – 17	0.560	S – 37	1.582
S – 18	0.460	S – 38	2.446
S – 19	0.532	Homologous	
S – 20	0.482	mycelia	2.567

PAb concentration= 40µg/ml.

Soil antigen – S- 1 = Control soil; S-2 Sterile soil. 3-34 = collected from different tea growing field; [Hansqua T.E. – Section B : Plot 7 (S-3), 8 (S-4), 9 (S-5); Trihana T.E. Section C : Plot 1 (S-6,7), 4 (S-8,9), 5(S-30); Cooch Behar T.E. Plot 2(S-10),3(S-11,12), 4(S-13); Bijoynagar T.E. – Section A : Plot 1 (S-14,15), 2(S-16); Section B : Plot 1 (S-17,18), 3(S-19,20), 4(S-21); Matigara T.E.-Section A: Plot 1(S-22), 2(S-23) 5 (S-24), 6 (S-25); Section D : Plot 4(S-26,27), 5(S-28,29); Chandmoni T.E. – Section A : Plot 2(S-31,32), 3(S-33,34), S-35-38 = Amended soil of *U. zonata* (60 days after amendment).

Table 35 : Dot-blot of different soil antigens with PAb of *U.zonata*.

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

[a] Fast red colour intensity : Pinkish red ; + + + + Bright, + + + High, + + Medium, + Low, ± Faint, - no colour ;

PAb concentration= 40µg/ml.

Soil antigen – S- 1 = Control soil; S-2 Sterile soil. 3-34 = collected from different tea growing field; [Hansqua T.E. – Section B : Plot 7 (S-3), 8 (S-4), 9 (S-5); Trihana T.E. Section C : Plot 1 (S-6,7), 4 (S-8,9), 5(S-30); Cooch Behar T.E. Plot 2(S-10),3(S-11,12), 4(S-13); Bijoynagar T.E. – Section A : Plot 1 (S-14,15), 2(S-16); Section B : Plot 1 (S-17,18), 3(S-19,20), 4(S-21); Matigara T.E.-Section A: Plot 1(S-22), 2(S-23) 5 (S-24), 6 (S-25); Section D : Plot 4(S-26,27), 5(S-28,29); Chandmoni T.E. – Section A : Plot 2(S-31,32), 3(S-33,34), S-35-38 = Amended soil of *U. zonata* (60 days after amendment).

4.12. Determination of cross reactivity of PAb of *U.zonata*

4.12.1. PTA- ELISA

4.12.1.1. Fungal mycelial antigens of different soil fungi

Cross reactivity of the PAb raised against *U. zonata* was tested against a number of soil fungi of which some were pathogenic to tea (*Poria hypobrumea, Ustilina zonata, Sphaerostilbe repens, Rosellinia arcuata*) and other were non-pathogenic (*Fusarium oxysporum, Metarhizium anisopliae, Beauveria bassiana, Trichoderma viride, Trichoderma. harzianum, Sclerotium rolfsii*). Antigens prepared from the mycelia of all the above were tested against PAb of *U. zonata* by PTA-ELISA. Results presented in Table 36 and Fig. 9 revealed that among all the fungi tested PAb of *U. zonata* reacted to some extent with antigens of *Rosellinia arcuata* in relation to its homologous reaction.

Table 36 : Indirect ELISA values (A405) of PAb of *U.zonata* reacted with antigens of soil fungi

Antigen Source	Absorbance at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Sphaerostilbe repens	0.562	0.559	0.423	0.515± 0.045
Poria hypobrumea	0.572	0.592	0.621	0.595± 0.014
Fomes lamaoensis	0.496	0.635	0.546	0.559± 0.040
Rosellinia arcuata	0.726	0.643	0.721	0.697± 0.026
Fusarium oxysporum	0.477	0.544	0.328	0.449± 0.063
Metarhizium anisoplia	0.392	0.342	0.401	0.378± 0.018
Beauveria bassiana	0.469	0.367	0.450	0.429± 0.031
Trichoderma viride	0.481	0.492	0.490	0.488± 0.003
Trichoderma harzianum	0.320	0.347	0.458	0.375± 0.0422
Sclerotium rolfsii	0.423	0.489	0.420	0.444± 0.0225
Ustilina zonata	2.523	2.420	2.726	2.556± 0.0899

Antigen concentration 100µg/ml

IgG source - U.zonata mycelial 4 bleed PAb; concentration 40µg/ml

± Standard error.

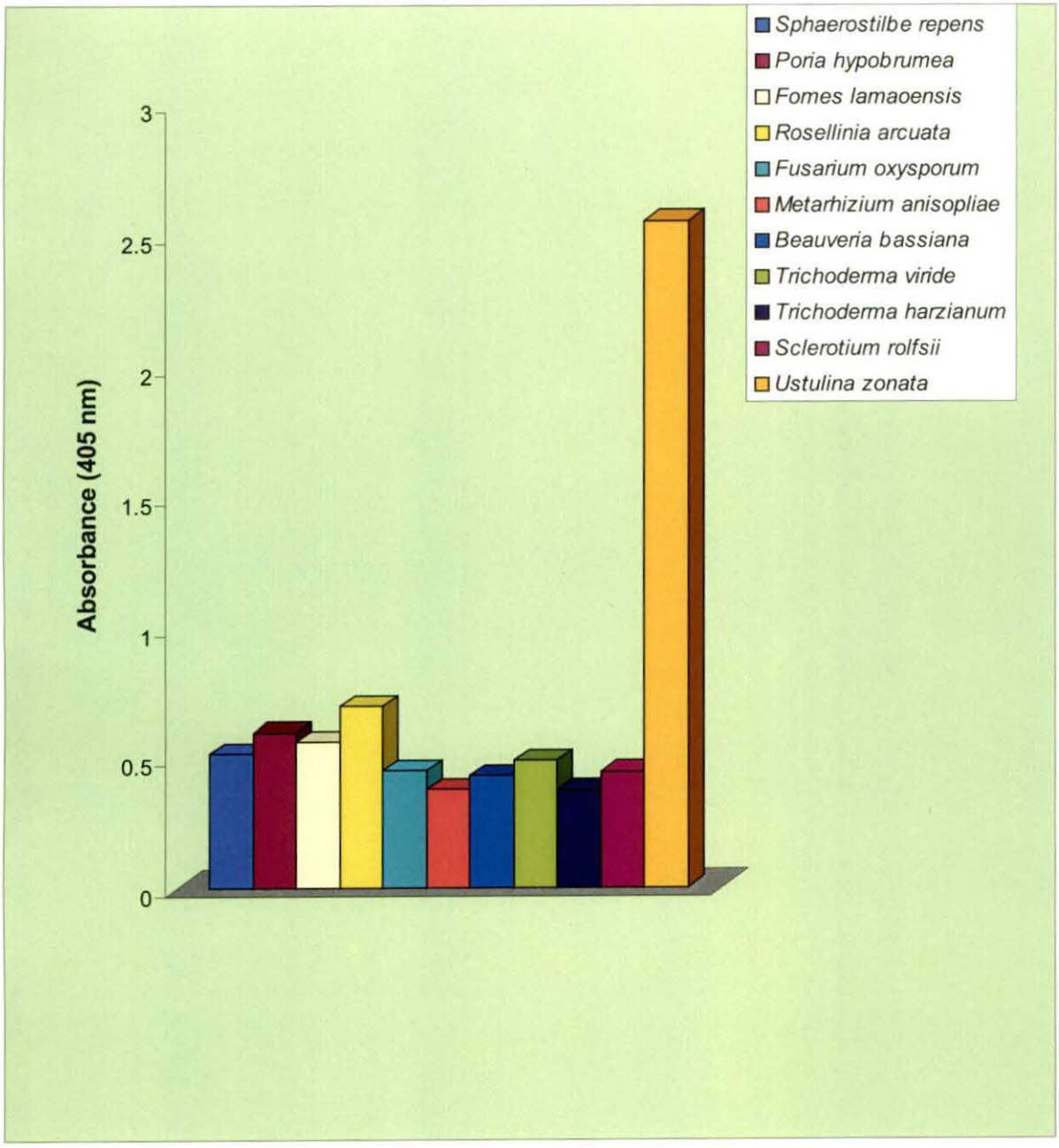


Figure 9: PTA-ELISA of mycelial antigens prepared from soil fungi using Pab of *U. zonata*.

4.12.1.2 Fungi in tea roots infected with other root pathogens

A number of pathogens can infect tea roots causing different types of root rot diseases of tea. So, it was considered worthwhile to investigate whether PAb of *U.zonata* could also react with the antigens from tea roots infected with other root pathogens. Hence, the PAb of *U.zonata* was reacted by PTA-ELISA with antigens prepared from tea roots (BSS -2, BS/7A/76, UPASI-2, UPASI 3,and UPASI-9) infected with *Spaerostilbe repens* (violet root rot). Results (Table 37) revealed that reactivity of these antigens was lesser when compared with *U.zonata* infected root antigens though here also infected root extracts showed higher ELISA values than the healthy ones. Thus a certain degree of cross reactivity was observed through this investigation.

Table 37 : PTA-ELISA values of healthy and infected (with different root pathogen) tea root antigens reacted with PAb of *U.zonata.*

Tea Varieties	Absorbance at 405 nm				
	Healthy	Infected			
		F. lamaoensis	*R.arcuata*	*S.repens*	*U.zonata*
UPASI-2	0.791	0.315	0.401	0.424	0.523
UPASI-3	0.643	0.348	0.309	0.444	0.816
UPASI-9	0.716	0.459	0.422	0.369	0.849
BSS-2	0.713	0.460	0.364	0.458	1.550
BS/7A/76	0.870	0.279	0.412	0.581	1.872
U. zonata (mycelia)					2.053

Antigen concentration = 20µg/ml
PAb concentration of *U. zonata* (mycelia) = 40µg/ml

4.12.2. Dot Blot

Cross reactivity of *U. zonata* PAb (mycelia and cell wall) with antigens of other fungi were tested by dot-blot also using antigens of other root pathogens. Antigens of *S. repens, R. arcuata* and *T. viride* showed slightly positive reaction, though the dots were of much lesser intensity than the homologous reaction (Table 38).

Table 38 : Dot – blot reaction of antigens of different soil fungi with *U. zonata* PAbs.

Antigen Source	Colour intensity[a]	
	Mycelia- PAb	Cell wall -PAb
S. repens	±	+
R. arcuata	+	+
P. hypobrumia	±	±
A. mellea	±	±
T. harzianum	±	±
T. viride	+	+
U.zonata		
Mycelia	+ + +	+ ++
Cell wall	+ ++	+ + +

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

4.13. Purification of antigen by ammonium sulphate precipitation

The crude antigens preparations were purified by ammonium sulphate precipitation, as described under materials and methods. In order to determine the fractions, which contained the antigenic proteins, immunodiffusion, PTA-ELISA and Western blot were performed with PAb raised against mycelia of *U. zonata* the results of which are given below. The precipitin reaction of fractionated proteins (0-20%, 20-40%, 40-60%,60-80% and 80 – 100% SAS) with PAb raised against mycelial extracts was tested in immundiffusion. Result (Table 39) revealed 3 separated, strong precipitin bands in 40-60% in 60 – 80% fraction, 4 bands in 100% SAS while one strong band was observed in 80 – 100 % fraction and no bands in 0 - 20% and one weak band in 20 – 40%. The reactions of the different fractions could not be differentiated by PTA-ELISA. SDS-PAGE of the proteins from different fractions were stained by coomassie blue, and another set was used for Western Blot. In SDS-PAGE, a number of bands of different molecular weights were found in the different fractions, with maximum bands in the fraction 40 – 60% SAS . In case of Western blot, however, maximum bands due to

antigen-antibody reaction was evident in 60-80% SAS. Based on the results of immunodiffusion and Western blot, 60 – 80% SAS fraction was selected as the fraction containing the maximum antigenic proteins and was used as immunogen.

Table 39 : Western Blot analysis of fractionated (SAS) mycelial proteins of *U.zonata* with PAb of *U.zonata*.

	Molecular weight (kDa)						
Antigen Source							
S.No.	**Crude**	**100% SAS**	**0-20% SAS**	**20-40% SAS**	**40-60% SAS**	**60-80% SAS**	**80-100% SAS**
1.	102.2	102.1	102.1	-	102.1	102.1	-
2.	95.2	95.2	-	-	-	95.2	95.2
3.	75.3	75.3	-	75.3	75.3	75.3	-
4.	-	59.8	-	-	-	-	-
5.	52.0	52.0	-	-	-	52.0	-
6.	45.6	45.6	-	-	45.6	45.6	45.6
7.	39.3	39.3	39.3	39.3	39.3	-	-
8.	24.5	24.5	24.5	-	24.5	24.5	24.5
9.	-	20.8	20.8	20.8	-	20.8	20.8
10.	18.2	18.2	-	-	-	-	-
11.	17.6	17.6	-	17.6	17.6	17.6	17.6
12.	16.0	16.0	-	-	16.0	16.0	-
13.	14.9	14.9	-	-	-	14.9	-
14.	-	13.7	-	-	-	13.7	-
15.	-	12.2	-	-	-	12.2	-

IgG source – 3rd bleed PAb concentration = 40µg/ml.

4.14. Evaluation of PAb raised against purified mycelial antigen

4.14.1. Immunodiffusion

The precipitin reaction was also done with PAb raised against 60-80% fractionated protein and results shows four separated, sharp bands in 60-80% and 100% SAS, two strong band in 40-60%, one in 80-100%, no bands in 0-20% and 20-40% SAS. IgG fractions were purified and experiments were done with purified IgG fraction of this PAb.

4.14.2. Cross reactive antigens

PAbs raised against 60-80% SAS fraction of mycelial antigen of *U.zonata* were also reacted with tea root antigens of all sixteen varieties tested. ELISA responses obtained were similar trends to that obtained with PAbs raised against mycelia and cell wall of the pathogen (Table 40).

4.14.3. Detection of *U. zonata* in tea root tissues

4.14.3.1. PTA- ELISA

Ability of PAb raised against 60-80% SAS fraction of *U.zonata* to detect the pathogen in root tissues was tested by PTA-ELISA, Dot-Blot and Western Blot analysis. In PTA-ELISA differences in A405 values between healthy and infected roots were highly significant in the susceptible varieties (Table 41).

4.14.3.2. Dot Blot

In dot-blot, where the root antigens were reacted with the above PAb, healthy root antigenic extracts showed only faint dots, while infected showed dots which were either light violet or violet. In no case, deep coloured dots were visible (Table 42).

4.14.3.3. Western Blot

Western Blot analysis was also carried out with PAb and root antigens. No bands were visible when healthy root extracts were reacted but four bands of ca. 23.2, 27.4, 37.6 and 40.8 kDa were visible .When compared to the reaction with PAb raised against mycelial extract, these bands were lesser in number, but these may be more specific.

Table 40 : PTA-ELISA values (A405) of tea root antigens, 60-80% SAS mycelial antigen of *U. zonata*, non-pathogen and non host reacted with anti-60-80% SAS PAb of *U.zonata.*

	Absorbance at 405 nm			
Antigens	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
Tea varieties				
TV-9	0.420	0.392	0.425	0.412± 0.010
TV-18	0.391	0.412	0.418	0.407± 0.008
TV-22	0.452	0.444	0.450	0.448± 0.002
TV-25	0.467	0.470	0.453	0.463± 0.005
TV-26	0.381	0.411	0.424	0.405± 0.012
S-449	0.756	0.882	0.869	0.835± 0.040
BS/74/76	0.943	0.890	0.949	0.927± 0.018
CP-1	0.791	0.693	0.787	0.757± 0.032
AV-2	0.821	0.820	0.825	0.822± 0.001
P-1258	0.711	0.723	0.709	0.714± 0.004
K1/1	0.801	0.793	0.805	0.799± 0.003
UPASI-2	0.550	0.559	0.553	0.554± 0.002
UPASI-3	0.573	0.579	0.600	0.584± 0.008
UPASI-8	0.582	0.573	0.585	0.580± 0.003
UPASI-9	0.566	0.549	0.554	0.556± 0.005
UPASI-26	0.590	0.587	0.580	0.586± 0.002
60-80% SAS of *U. zonata*	2.770	2.862	2.860	2.831± 0.030
Non-pathogen				
Beauveria bassiana	0.344	0.337	0.351	0.344± 0.004
Non-host				
Leucaena leucocephala	0.356	0.378	0.376	0.370± 0.007

Antigen concentration = 20µg/ml; PAb concentration= 40µg/ml

± Standard error.

Table 41: PTA-ELISA values of healthy and inoculated tea root antigens tea varieties with anti-60-80% mycelial SAS PAb of *U. zonata*

Root antigens		**Absorbance at 405nm**			
Tea varieties	**Condition**	**Expt.1**	**Expt.2**	**Expt.3**	**Mean**
K-1/1	Healthy	0.719	0.815	0.810	0.781± 0.031
	Infected	1.539	1.630	1.535	1.568± 0.031
BS/7A/76	Healthy	0.964	0.955	0.960	0.960± 0.002
	Infected	1.119	1.303	1.124	1.182± 0.060
UP-2	Healthy	0.709	0.721	0.716	0.715± 0.003
	Infected	0.915	0.922	0.932	0.923± 0.004
UP-3	Healthy	0.648	0.642	0.640	0.643± 0.002
	Infected	0.793	0.786	0.790	0.789± 0.002

Antigen concentration 20µg/ml; PAb concentration of *U. zonata* (mycelia) = 40µg/ml
± Standard error.

Table 42 : Comparison of dot-blot reaction of PAbs from mycelial 100% and 60-80% SAS of *U. zonata* with healthy and inoculated root antigens tea varieties.

		Colour intensity [a]	
		PAbs raised against	
Tea Varieties	**Plant Condition**	**100% SAS**	**60 – 80% SAS**
BS/7A/76	Healthy	-	-
	Infected	+ +	+
K-1/1	Healthy	-	+
	Infected	+ +	+
UP-2	Healthy	±	-
	Infected	±	±
UP-3	Healthy	-	-
	Infected	+	±

[a] NBT / BCIP colour intensity : + + + Deep violet; + + violet; + light violet ; ± Faint ; - no reaction ; PAb concentration = 40µg/ml.

4.15. Immunofluoresence

Fluorescent antibody labeling with fluorescein isothiocyanide (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite as well as for detection of pathogen in plant tissue. Present study reports the use of indirect immunofluorescent test using polyclonal antibodies to determine the tissue and cellular location of the pathogen in root tissue of tea varieties, tea rhizosphere soil as well as mycelia of *U. zonata*. Mycelia, cross section of root tissue as well as soil preparations were photographed under UV- fluoresecence and the intensity of bright apple-green fluorescence indicated the positive reaction.

4.15.1. Mycelia

Pre-immune sera did not show reactivity with the mycelia of *U.zonata* followed by FITC and mycelia was not auto-fluorescent. Examination of mycelia treated with homologous PAbs of mycelia, cell wall and 60-80% fractionated mycelial protein and stained indirectly with FITC indicated strong fluorescence throughout the mycelia, specially in young hyphal tips (Plate 9 Fig. E).

4.15.2. Root tissue

Cross section of tea roots were treated separately with normal and PAb of *U. zonata* and then reacted with FITC. Root sections exhibited a natural autofluoresence under UV-light. When the cross section of infected tea root tissue of susceptible varieties incubated with PAb of *U. zonata* and stained with FITC, strong fluorescence was observed in the infected root tissues. Present of fungal mycelium was evident with strong fluorescence in infected root tissue. Fluorescence was evident throughout the sections, extending upto the vascular tissues as well as outer surface (Plate 9 Figs.A-E). Healthy sections exhibited no fluorescence.

4.15.3. Soil sample

Amended soil preparation was done for immunoflurescece study as described under materials and methods. Microscopic observation under UV-fluorescence revealed that presence of strongly fluorescing mycelia. Thus immunofluorescence could be used to detect the pathogen in soil.

4.16. Immunocytochemical staining

Another approach adopted to study the interaction of *U. zonata* with root tissue was by direct observation, immunocytochemical staining, based on specific antibodies produced anainst *U. zonata*, provided a means of visualizing hyphae within root tissues of infected tea plants. Production of a specific immunocytochemical stain involves preparation of suitable antigen, appropriate methods for evaluating specificity of the antibodies, and development of the immynocytochemical staining procedure. In the present study three susceptible tea varieties were artificially inoculated with *U. zonata* in the experimental field. After 40 days of inoculation the affected plants were uprooted and washed properly. Cross-sections were made from the infected tea roots and fungal hyphae which penetrated the root tissues were probed with PAb raised against mycelial antigens of *U. zonata*. In root sections stained immunocytochemically, hyphae of *U. zonata* were growing along the epidermis and hyphal penetration throughout root tissues was evident (Plate 9 , Figs.F&G). Hyphae, which were growing horizontally in the cortical regions could be distinguished as strands, whereas those which were growing vertically either inter or intracellularly appeared as blue coloured masses (cross section of hyphae). Deep blue coloured thick layer was found on outer surface of root which was an evidence of deep brown and blackish sheet on root surface formed by fungal mycelia. This staining clearly showed the penetration of hyphae throughout the tissue.

4.17. Biological control of Charcoal stump rot disease with *Trichoderma harzianum and Trichoderma viride*

The present work was aimed at developing a management strategy to control Charcoal stump rot of tea by biological means. Antibiosis to *U. zonata* by biocontrol agents [*Trichoderma harziamum* and *T.viride*] were evaluated *in vitro* and *in vivo* condition.

4.17.1. *In vitro* test

Both the biocontrol agents tested *in vitro* were effective in causing significant suppression of growth of *U. zonata* (Plate 10). After 3-4 days of incubation *T. harzianum* and *T. viride* over grew the pathogen. But in control plate pathogen grew characteristically on PDA.

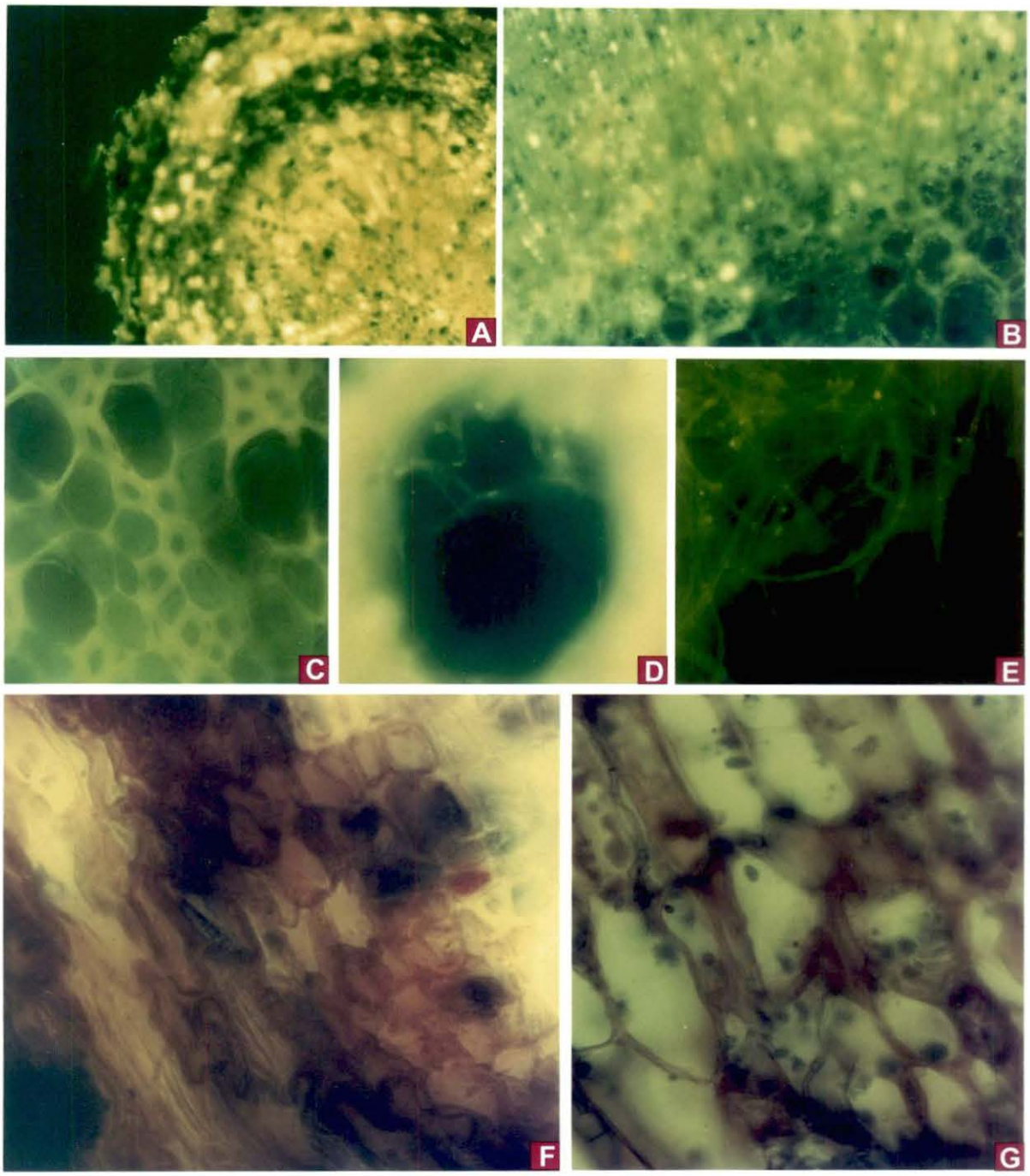


Plate 9 (Figs. A-G): Cross section of *Ustulina zonata* inoculated tea roots treated with PAb of *U. zonata* and labelled with FITC (A-D); stained with fast blue BB salt (F&G) and mycelia of *U. zonata* treated with homologous PAb and labelled with FITC (E).

4.17.2. *In vivo* test

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

4.17.2.1. Potted plants

Three years old tea plants (BSS-2, BS/7A/76) grown in potted condition were used for this purpose. Ten replicates of each treatment were taken and disease ratings was as described in materials and methods. Results revealed that treatment with either *T.harzianum* or *T.viride* reduced disease significantly (Table 43) and population of *U. zonata* also decreased significantly in rhizosphere soil.

Table 43 : Effect of *T. harzianum* and *T. viride* on Charcoal stump rot disease in potted condition.

	Disease Index [a]	
	Tea varieties [b]	
Treatments	**BSS-2**	**BS/7A/76**
U.zonata	3.58	3.87
U.zonata + *T.harzianum*	0.92	0.99
U.zonata + *T.viride*	0.87	0.76

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

[a] 0 = No symptoms;
1 = Small roots turn brownish and start to rot;
2 = Leaves start withering and 20-40% of root turn brown;

3 = Leaves withered with 50% of roots affected ;
4 = Shoot tips start withering 60-70% of roots affected;
5 = Shoots withered with defoliation of lower withered leaves, 80% roots affected;
6 = Whole plants die, with upper withered leaves still remaining attached; roots fully Rooted.

[b] Age of plants 3 yr
Average of 10 separate inoculated plants
40 days after inoculation

4.17.2.2. Field grown plants

For field experiments 4 selected varieties of 5 year old plants were taken and among them 2 varieties were susceptible (BSS-2 and BS/7A/76) and one was resistant (UP-2) as proved in earlier pathogenicity test as well as different immunoenzymatic reactions. Different treatment with bio-control agents were done and disease incidences were recorded as described in earlier experiments. In this experiment also both *T. harzianum* and *T.viride* significantly reduced the disease intensity (Table 44, Fig. 9). In case of treatment with *U.zonata* alone all the plants BSS-2 and BS/7A/76 varieties died after 40 days of inoculation.

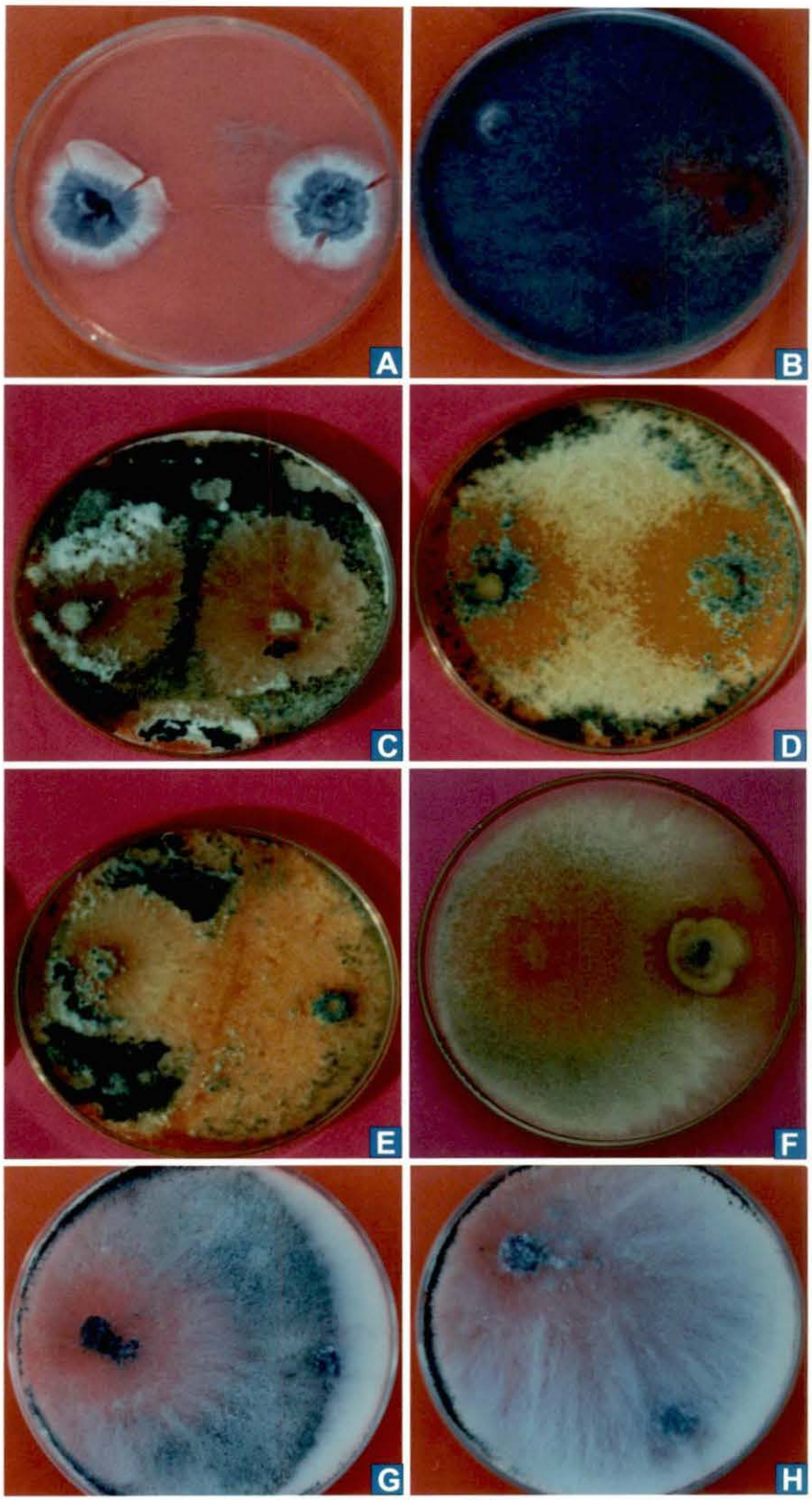


Plate 10 (Figs. A-H): *In vitro* pairing of *Ustulina zonata* with *Trichoderma harzianum* and *Trichoderma viride*. Homologous pairing of *U.zonata* (A&B); *T. harzianum* (C) *T. viride* (D). Pairing of *T. harzianum* with *T. viride* (E), *U. zonata* with *T. viride* (F) and *U. zonata* with *T. harzianum* (G&H).

Table 44 : Effect *of T. harzianum* and *T. viride* on development of Charcoal stump rot disease in field condition.

		Disease Index [a]		
		Days after inoculation		
Tea varieties [b]	**Treatments**	**10**	**20**	**30**
BS/7A/76	*U.z.*	2.78	3.62	3.95
	U.z.+ T.h	0.43	0.38	0.27
	U.z. + T.v	0.49	0.41	0.33
BSS-2	*U.z*	2.62	3.12	4.20
	U.z.+ T.h	0.45	0.32	0.25
	U.z. + T.v	0.52	0.41	0.29
UP-2	U.z.	0.72	0.81	0.99
	U.z.+ T.h	0.29	0.22	0.19
	U.z. + T.v	0.36	0.31	0.30
UP-3	U.z.	0.92	1.09	1.22
	U.z.+ T.h	0.33	0.27	0.18
	U.z. + T.v	0.47	0.38	0.32

No disease was observed in uninoculated control, or those inoculated with either *T.harz ianum* of *T. viride* alone.

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

[b] Age of plants 3 yr. Average of 10 separate inoculated plants

U.z. = U. zonata,T.h.= T. harzianum, T.v. = T. viride

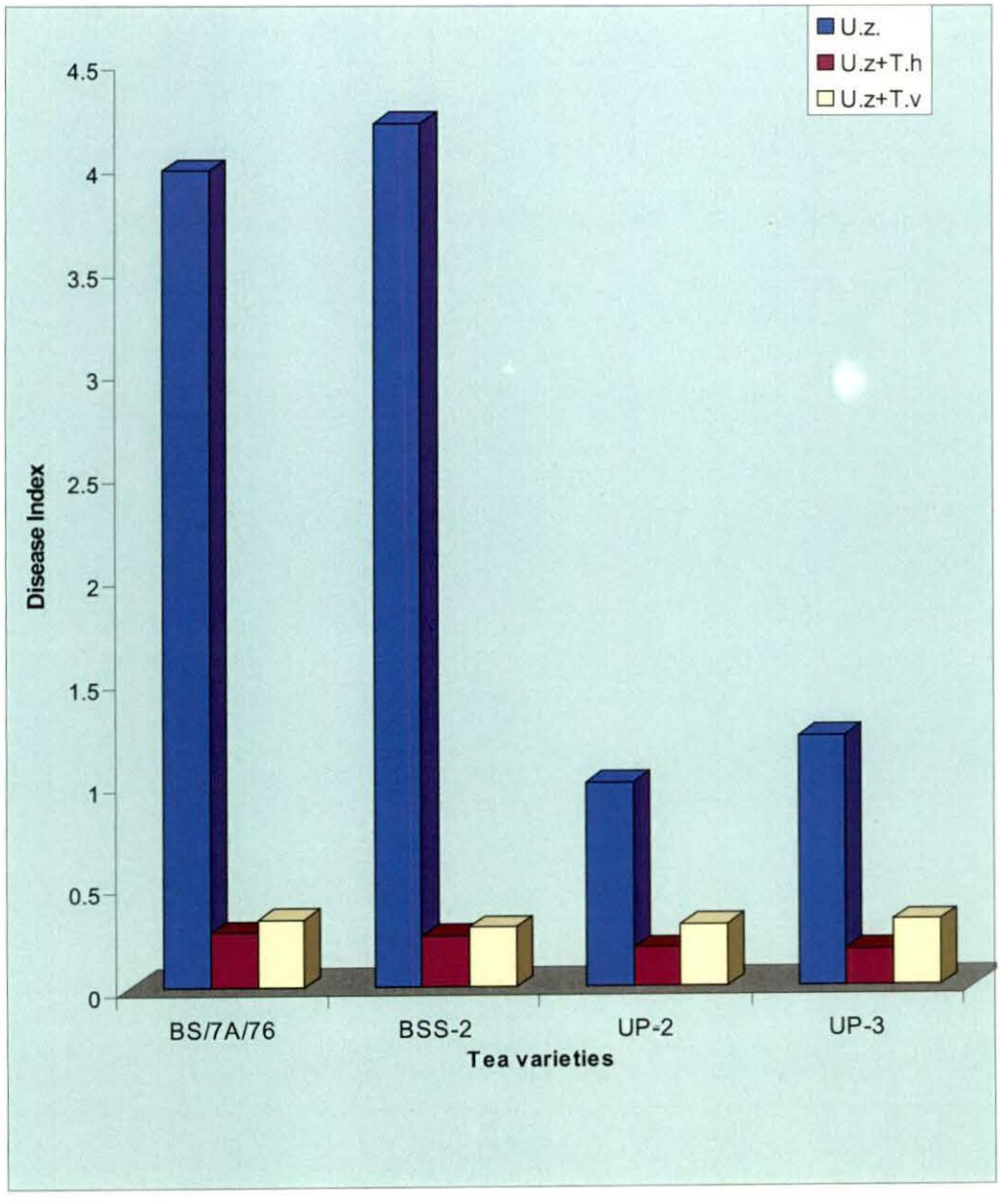


Figure 10: Charcoal stump rot disease development following application of biological control agents.

4.18. Detection of *U. zonata* in tea root and soil following treatment with biocontrol agents.

4.18.1. PTA-ELISA

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

4.18.1.1. Root tissues

Root antigens were prepared from uprooted plants (2yr old) of different treatment after 40 days of pathogen inoculation for biocontrol experiment of potted plants. These antigens were reacted in PTA-ELISA using PAb of *U. zonata*. Results showed that ELISA values of roots treated with *T. harizianum* and *T. viride* were significantly lesser than with *U.zonata* alone (Table 45). In case of 5yr old field grown plants also similar results were observed (Table 46), though A405 values were higher than the potted plants.

Table 45 : ELISA of reactions of PAb *U.zonata* with root antigens of tea varieties following treatments with Biocontrol agents.

	Absorbance at 405nm	
Antigen Source	**BSS-2**	**BS/7A/76**
Healthy Plant	0.592	0.572
Treatments		
U.zonata + T.harzianum	0.378	0.266
U.zonata + T.viride	0.312	0.343
U.zonata	1.618	1.554
T. harzianum	0.216	0.230
T.viride	0.292	0.201

Antigen concentration 20µg/ml
PAb concentration of *U. zonata* (mycelia) = 40µg/ml

Table 46 : ELISA values of reaction of PAbs with root antigens of tea varieties following treatment with biocontrol agents.

Antigen Source	Absorbance at 405nm					
	U. zonata		*T. viride*		*T. harzianum*	
	K-1/1	BS/74A/76	K-1/1	BS/74A/76	K-1/1	BS/74A/76
Healthy Plant	0571	0.582	0.349	0.368	0.378	0.369
Treatments						
U. zonata + *T. viride*	0.483	0.400	0.388	0.367	0.584	0.673
U. zonata + *T. harzianum*	0.550	0.572	0.627	0.731	0.785	0.881
U. zonata	1.543	1.538	0.410	0.428	0.520	0.529
T. viride	0.574	0.591	1.333	1.322	0.996	0.987
T. harzianum	0.423	0.419	0.327	0.321	1.237	1.226

Antigen concentration = 20µg/ml
PAb of *U. zonata* (mycelia) concentration = 40µg/ml

4.18.1.2. Soil

Soil samples of the rhizosphere of different treatments were collected at a depth of 7-9 inches from soil surface. *U. zonata* was evaluated through PTA- ELISA and competition ELISA by reacting the antigens from collected soils after 30 days of Control set was prepared from uninfested soil of control plants.

In PTA-ELISA results from soil treated with *U. zonata* and *T. harzinum* or *U. zonata* and *T. viride* reacted with PAb of *U. zonata* showed significantly lower absorbance values than that of soil antigen treated with *U. zonata* alone. This indicated that population of *U. zonata* soil had been reduced by the biocontrol agents (Table 47). When soil samples treated either with *T. harzianum* or *T. viride* were reacted with their homologous antisera, absorbance values were comparable with the soil samples treated with biocontrol agent(s) and inoculated with pathogen.

Table 47 : ELISA values of soil antigens of different treatments with PAbs of *U. zonata*, *T. harzianum* and *T. viride*.

	Source of PAbs		
Antigen Source	***U. zonata***	***T. viride***	***T. harzianum***
Unifested soil	0.301	0.253	0.212
Treatments			
U.zonata + *T.harzianum*	0.480	0.807	0.806
U.zonata + *T.viride*	0.486	0.723	0.747
U.zonata	1.282	0.335	0.432
T. harzianum	0.423	0.809	1.402
T. viride	0.367	1.328	0.873

Soil samples collected 30 days after pathogen inoculation;

The same trend of result (Table 48) was also obtained in case of competition ELISA which is an inhibition ELISA. Reduction of population in soil treated with *T.harzianum / T.viride* and *G.viriens* was confirmed using this ELISA format and detailed procedure has been outlined in materials and methods. Antigens were prepared from soils under various treatments as mentioned earlier and were used under doubling dilutions of from 1:25 to 1:400. Since competition ELISA is a double binding assay, where PAb is allowed to react to the test antigen first and the residual PAb is once again reacted with homologous antigen in separate plates, higher ELISA values in this procedure would indicate lower reactivity to a test sample.

Table 48 : Competition ELISA of various dilution of treated soil antigens with PAbs of *U. zonata*, *T. viride* and *G. virens*.

Soil antigen		Absorbance at 405nm		
		Source of PAbs		
Treatment	**Dilution**	***U. zonata***	***T. viride***	***T.harzianum***
U. zonata	1:25	0.502	1.040	1.186
	1:50	0.719	1.241	1.356
	1:100	0.754	1.298	1.445
	1:200	0.853	1.388	1.536
	1:400	0.947	1.423	1.618
U. zonata +	1:25	1.652	0.465	1.283
T. harzianum	1:50	1.693	0.718	1.475
	1:100	1.758	0.842	1.504
	1:200	1.847	0.880	1.685
	1:400	1.930	0.986	1.694
U. zonata +	1:25	1.787	0.850	0.637
T. viride	1:50	1.843	1.022	0.830
	1:100	1.880	1.051	0.942
	1:200	1.921	1.148	1.016
	1:400	1.973	1.253	1.044
T. harzianum	1:25	1.623	0.438	0.936
	1:50	1.787	0.693	1.332
	1:100	1.800	0.758	1.389
	1:200	1.875	0.813	1.416
	1:400	1.967	0.930	1.472
T. viride	1:25	1.683	0.807	0.411
	1:50	1.738	0.921	0.710
	1:100	1.778	0.979	0.872
	1:200	1.902	1.091	0.901
	1:400	1.933	1.276	1.082
Control	1:25	1.875	1.443	1.485
	1:50	1.923	1.520	1.551
	1:100	1.967	1.542	1.673
	1:200	2.083	1.545	1.725
	1:400	2.129	1.588	1.750

Sample collected 30 days after pathogen inoculation;
IgG concentration = 40µg/ml

4.18.2. Dot - Blot

Results presented in Table 49 and Plate 11 revealed that when PAb of *U. zonata* (mycelia and cell wall) was treated in the antigens from soil subjected to different treatments (described earlier), positive reaction was obtained in case of treatment with *U. zonata* alone. Similarly when these were treated with PAbs of *T. harzianum* or *T.viride* positive reactions were shown in cases where soil was amended with the particular fungus. In cases where soil was treated with both *U.zonata* and *T. harzianum /T.viride* also, positive reaction were not evident.

Table 49 : Dot-blot of soil antigens of different treatment (with combinations of *Trichoderma* sp and *U. zonata*) collected from root rhizosphere of field.

	Colour intensity [a]			
	PAbs raised against			
Antigen Source	***U. zonata*** **Mycelia**	***U. zonata*** **Cell wall**	***T.harzianum*** **Mycelia**	***T.viride*** **Mycelia**
Sterile Soil	-	-	-	-
Control Soil	-	-	-	-
Soil treated with				
U. zonata	+ +	+ +	-	-
T.harzianum	-	±	+	+
T. viride	-	-	+	+
U. zonata +	-	-	+	-
T.harzianum	-	±	+	+
U. zonata +	-	-	+	±
T. viride				
Mycelia				
U. zonata	+ + + +	+ + +	-	-
T. viride	+	+	+ + +	+ + + + +
T.harzianum	±	+	+ + + + +	+ + +

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

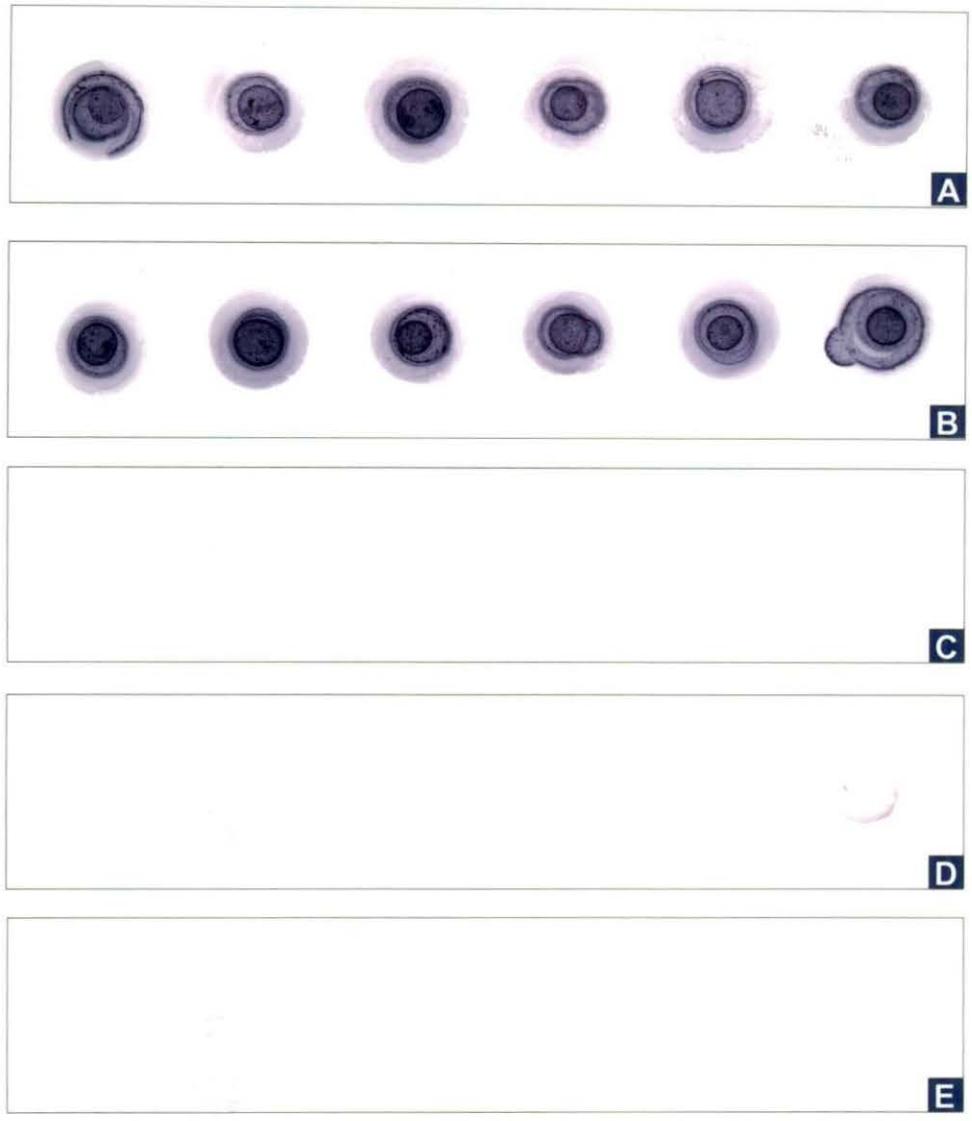


Plate 11 (Figs. A-E): Dot immunobinding assay of rhizosphere soil of tea plants following treatment with *Trichoderma harzianum* , *Trichoderma viride* and after inoculation with *U. zonata* and probed with PAb of *U. zonata.* (A) Mycelial antigen of *U. zonata*, (B) Plants inoculated with *U. zonata* (C&D) Treated with *T. harzianum* and inoculated with pathogen (E) Treated with *T. viride* and inoculated with pathogen.

4.18.3. Western Blot

Western Blot analysis of soil samples from different treatments set up using PAb of *U. zonata* revealed four bands with molecular weight of ca. 79,64,55 and 31 kDa. These four bands were also present in homologous reaction, with mycelial antigen though 18-19 bands were found in case of homologous reaction.

In case of soil from rhizosphere of plants inoculated with the biocontrol agents prior to inoculation with the pathogen, no bands were visible (Table 50). When these soil antigens were treated with PAb of *T. viride*, 4 bands of ca. mol. wt. 93, 50, 41 & 39 kDa were obtained with soil inoculated with *T. viride*, 3 bands (ca.93, 50 & 39 kDa mol. Wt.) with *U. zonata* + *T. viride* and 2 bands with *U. zonata* + *T. harzianum.*

Table 50 : Western blot analysis of different soil antigens from root rhizosphere infested with different combination of *Trichoderma* sp. and *U. zonata*

Antigen source	**IgG No.**			
	U. zonata		***T. viride***	
	No. of bands	**Mol.wt.kDa**	**No. of bands**	**Mol.wt.kDa**
Mycelia				
U. zonata	17	104.3, 97.4, 79.0, 60.9, 50.1, 43.0, 38.6, 32.2, 27.1, 24.5, 22.2, 19.0, 18.4, 17.4, 15.3, 14.3, 12.7,		NR
T.viride	NR		10	92.8, 83.6, 72.6, 68.0, 54.5, 50.0, 43.0, 40.8, 38.6, 29.8.
Soil treated with				
(a) *U.zonata*	4	79.0, 63.5, 54.5, 31.2		
(b) *T.harzianum*	Nil		Nil	
(c) *T.viride*	Nil		4	92.8, 50.0, 4.8 38.6
(d) *U.zonata* + *T. harzianum*	Nil		2	63.5, 54.5
(e) *U.zonata* + *T. viride*	Nil		3	92.8, 50.0, 38.6
(f) Uninfested soil	Nil		Nil	

IgG concentration = 40µg/ml.

4.19. Management of Charcoal stump rot disease using organic additives

In addition to biocontrol agents attempts were also made to develop effective integrated management practices for charcoal stump rot disease of tea using organic additives.

4.19.1. Growth promotion in tea plants

Two varieties of tea plants (BS/7A/76 and K 1/1) were grown in soil amended with neem cake and oil cake separately. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one-month interval and up to two months following the treatment of neem cake and oil cake and after inoculation with *U. zonata*. Results (Table 51) revealed that the growth of tea plants increased following amendment with neem and oil cakes than those plants inoculated with *U.zonata* in relation to untreated uninoculated control as recorded after two months following treatment. It has been observed that the percentage increase in shoot length with neem cake and oil cake in treated inoculated with *U. zonata* tea plants was more than the treated uninoculated one (Table 52).

Similarly three tea varieties (UP-3 BS/7A/76 and K-1/1) were grown in soil amended separately with cowdung, rabbit manure and chicken manure. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one month interval up to two months following the treatment of organic components and after inoculation with *U. zonata*. It has been observed that the growth of tea seedlings had been increased in treated uninoculated than treated inoculated tea seedlings (Table 53). Among the three treatments with organic components, rabbit manure gave very good and healthy growth the tea seedlings than chicken manure and cowdung.

Table 51 : Growth promotion in tea seedlings following soil amendment with neem cake oil cake

Tea Variety	One month				Two month			
	Healthy		Infected		Healthy		Infected	
	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves
BS/7A/76 Untreated	3	5	0	2	5	7	7.5	2
Treated Neem Cake	3	4	1	0	1.5	7	2	5
Oil cake	1	3	1.5	2	2	6	1.5	3
K 1/1 Untreated	1	2	1	0	3	1	1	3
Treated Neem Cake	2	3	2	0	1	2	0.5	2
Oil cake	1	4	1.5	0	2	3	1	1

Table 52 : Percentage increase in shoot length in tea seedlings following treatment with neem cake and oil cake

Tea Variety	Percentage increase in shoot length after two months treatment	
	Healthy	Infected
BS/7A/76 Untreated	5.5	2.3
Treated Neem cake	7.8	1.2
Oil cake	6.5	5.3
K 1/1 Untreated	8.1	4.8
Treated Neem cake	9	7
Oil cake	9.5	5.6

Table 53 : Growth promotion in tea seedlings by different organic components after inoculation with *U.zonata*

Tea Variety	One month				Two month			
	Healthy		Infected		Healthy		Infected	
	Increase in height (cm)	Increas No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves	Increase in height (cm)	Increase No. Of leaves
UP-3								
Untreated	2	0	1	0	3	2	1.5	1
Treated Cowdung	5	2	3	1	5	2	1.5	1
Rabbit Manure	9	0	6	1	5	1	4	0
Chicken Manure	4	3	2	1	3	1	2	0
BS/7A/76								
Untreated	1	0	0	0	1	2	1	0
Treated Cowdung	4	2	2	1	4	3	1.5	1
Rabbit Manure	7	4	5	1	5	3	2	2
Chicken Manure	4	0	2	1	4	3	2	1
K-1/1								
Untreated	2	0	1	0	2	1	0	0
Treated Cowdung	3	2	1	1	2.5	3	1	2
Rabbit Manure	8	5	5	2	8	2	4	3
Chicken Manure	6	3	4	0	3	3	2	1

4.19.2. Disease development

Under pot culture conditions *T. harzianum* alone and in combination with neem cake and oil cake provided best effective management practices of charcoal stump rot in

all the three modes of application viz., simultaneous, repeated and pot infection. Combination with neem cake and oil cake showed 66.2% disease incidence where as in oil cake, neem cake and *Trichoderma harzianum* in combination disease incidence were recorded 15.5% . But in combination with cowdung, neem cake, chicken manure and rabbit manure, results were insignificant as shown in (Table 54).

Table 54 : Effect of simultaneous treatments with biocontrol, fungicide, organic amendments and plant extract on development of seedling blight of tea following inoculation with *U.zonata*.

Treatment	Disease incidence (%	Disease control (%
Trichoderma harzianum	10.0	90.0
Oil Cake with Neem cake	66.2	33.8
T. harzianum, oil cake and neem cake	15.5	84.5
Cowdung, Neem cake and Oil cake	40.0	60.0
Chicken manure, Neem cake and Oil cake	44.5	54.5
Rabbit manure, Neem cake and Oil cake	42.4	55.6
Untreated Control	100	0

4.20. Biochemical changes associated with reduction of charcoal stump rot disease in tea plants

As polyphenols are the major constituents of tea plants, their role in the resistance mechanism was investigated. Changes in the levels of phenolic substances (total phenols and ortho-dihydroxy phenols) were determined in the untreated and treated varieties (K1/1 and BS/7A/76) after inoculation with pathogen (*U. zonata*). Results have been presented in Table 55 and Fig.11. It revealed that total phenol content increased in treated plants following inoculation than untreated inoculated plants. It has also been observed that total phenol levels increased in treated inoculated tea root varieties with *U.zonata* than treated uninoculated tea root varieties.

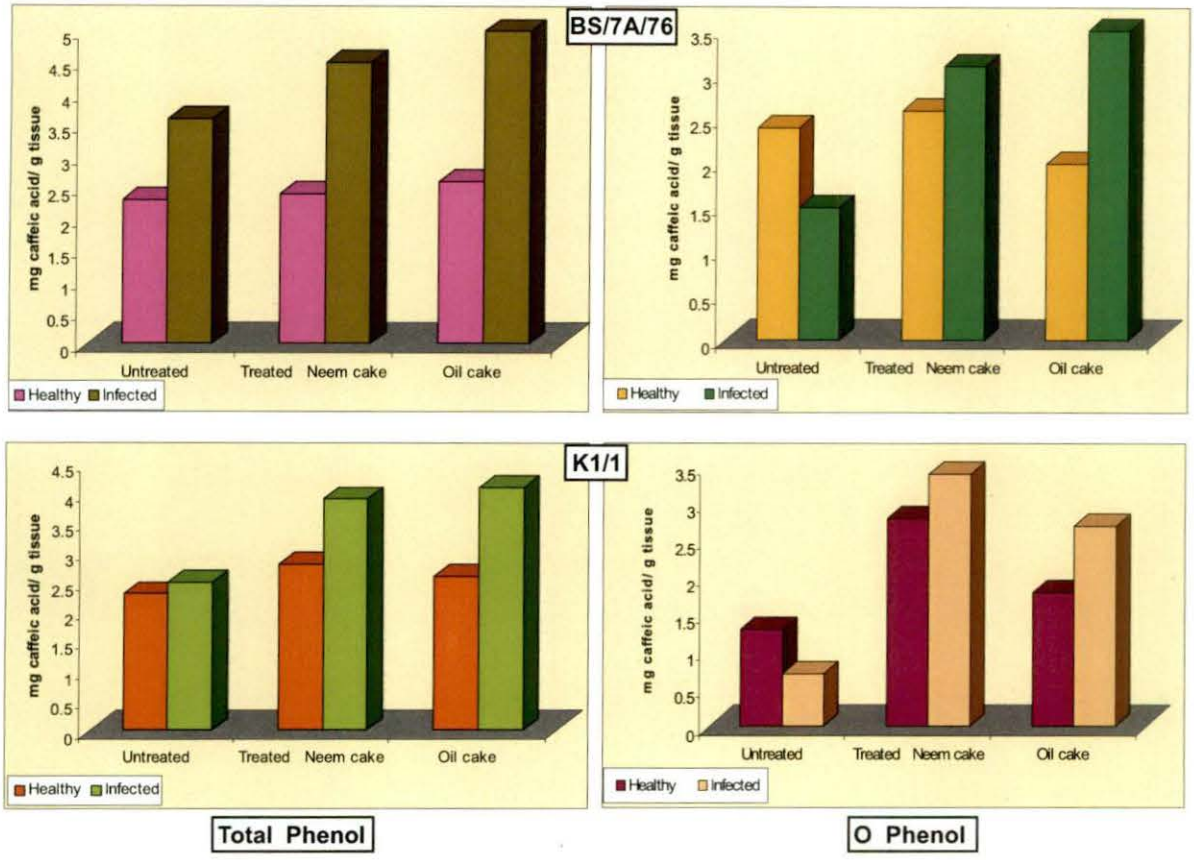


Figure 11: Total phenol and ortho-dihydroxy phenol content in tea varieties following application with organic additives and inoculation with *U. zonata*.

Level of ortho-dihydroxyphenol was also determined in these varieties (K1/1 and BS/7A/76) after treatment with neem cake and oil cake following inoculation with *U. zonata*. Results (Table 56, Fig. 11) revealed that ortho-dihydroxy phenol decreased in untreated inoculated tea root varieties in comparison to uninoculated healthy control. Ortho-dihydroxy phenol levels increased in treated roots following inoculation with the pathogen than treated healthy plants. Similar pattern was noted in case both the varieties tested. It is interesting to note that the plants grown in soil amended with neem cake and oil cake could resist the pathogen and changes in the level of total phenols as well as ortho-dihydroxy phenol can be correlated with the development of resistance in susceptible plants following such treatments.

Changes in the level of phenolics were also determined in two varieties of tea plants (UP-3, BS/7A/76 and K 1/1) grown separately in soil amended with cowdung, rabbit manure and chicken manure following inoculation with *U.zonata*. Results revealed that total phenol content decreased in untreated plants of two susceptible varieties (BS/7A/76 and K1/1) following inoculation with the pathogen in relation to healthy control, whereas the resistant variety (K 1/1) responded against inoculation with the pathogen. In this case total phenol and ortho-dihydroxy phenol content increased in comparison with untreated healthy control (Table 57 and 58; Fig. 12).

Table 55 : Total phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *U. zonata*.

Tea variety	Phenol content (mg / g) [a]	
	Healthy	**Infected**
BS/7A/76		
Untreated	2.3	3.6
Treated		
Neem cake	2.4	4.5
Oil cake	2.6	5.0
K1/1		
Untreated	2.3	2.5
Treated		
Neem cake	2.8	3.9
Oil cake	2.6	4.1

Table 56 : Ortho-dihydroxy phenol content in tea varieties after treatment with Neem cake and oil cake following inoculation with *U.zonata* in treated tea root variety

Tea variety	Ortho-dihydroxy phenol content (mg / g) [a]	
	Healthy	**Infected**
BS/7A/76 Untreated	2.4	1.5
Treated Neem cake	2.6	3.1
Oil cake	2.0	3.5
K1/1 Untreated	1.3	0.7
Treated Neem cake	2.8	3.4
Oil cake	1.8	2.7

[a] Average of 3 replicates.

It has also been observed that total phenol levels increased in all the varieties tested following treatment with organic amendments. Rabbit manure responded markedly and in this case total phenol increased following inoculation with the pathogen in relation to treated healthy as well as untreated healthy control. Level of ortho-dihydroxy phenol increased markedly in soil amended with cowdung, whereas level of ortho-dihydroxyphenol increased in plants grown in soil amended with rabbit manure following inoculation with the pathogen.

Table 57 : Changes in the level of total phenol content in tea roots grown in soil amended with organic additives following inoculation with *U.zonata.*

Tea variety	Phenol content (mg / g) [a]	
	Healthy	Infected
UP-3 Untreated	2.8	2.3
Treated Cowdung	3.0	3.3
Rabbit Manure	3.7	5.8
Chicken manure	3.5	3.9
BS/7A/76 Untreated	3.6	2.9
Treated Cowdung	2.7	3.4
Rabbit Manure	4.0	4.9
Chicken manure	4.5	5.0
K-1/1 Untreated	2.9	6.5
Treated Cowdung	5.3	8.1
Rabbit manure	4.3	8.7
Chicken manure	4.4	5.3

[a] Average of 3 replicates.

Table 58 : Changes in the level of ortho-dihydroxy phenol content in tea roots grown in soil amended with organic additives following inoculation with *U. zonata.*

Tea variety	Ortho-dhydroxy content (mg / g) [a]	
	Healthy	**Infected**
UP-3 Untreated	2.0	1.4
Treated Cowdung	2.5	2.8
Rabbit Manure	2.3	2.5
Chicken manure	2.3	2.7
BS/7A/76 Untreated	1.0	0.7
Treated Cowdung	1.9	2.3
Rabbit Manure	1.7	2.1
Chicken manure	1.9	2.4
K-1/1 Untreated	0.9	2.1
Treated Cowdung	2.1	3.4
Rabbit manure	3	4.4
Chicken manure	1.5	2.5

[a] Average of 3 replicates.

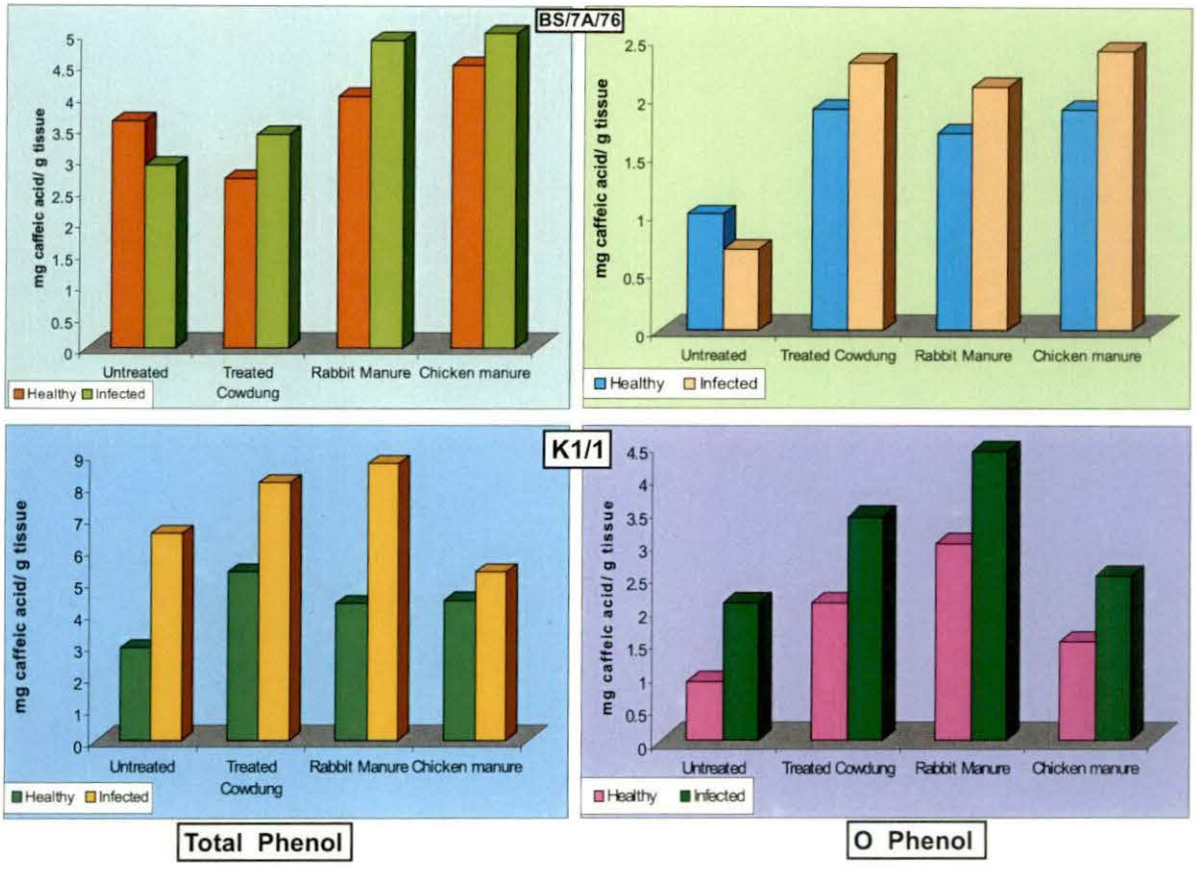


Figure 12: Total phenol and ortho-dihydroxy phenol content in treated tea varieties following application with organic manure and inoculation with *U. zonata.*

DISCUSSION

Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility. Tea, being perennial, is subjected to varying environmental conditions throughout its life as well as to numerous attacks by pest and pathogens, which in turn are influenced by various environmental conditions. Root system the foundation of the plant is weakened by many primary rot pathogens. *Ustulina zonata*, one of the soil borne fungal pathogens causes charcoal stump rot, a primary root disease. The initial infection process involving recognition events between plants and fungal pathogens is essential for the establishment. In nature plants have evolved multicomponent coordinated mechanisms by which they can defend themselves against the multitude of organisms attacking them. The art and science of plant disease control has moved in the direction of biological control of plant pathogens, including use of introduced antagonists. It is now widely recognized that biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within framework of integrated disease management systems. Integrated control is a flexible, multi-dimensional approach to disease control utilizing a range of control components such as biological, cultural and chemical strategies needed to hold diseases below damaging economic threshold without damaging the agro-ecosystem.

The rationale behind the disease control is to check pathogen's growth in the host and improve the health status of the plant. Disease resistance and susceptibility in plants do not represent any absolute values. Even susceptible variety shows resistance to its pathogen under certain cropping conditions or by treating stress situation. This would suggest that even a susceptible variety has a potentially effective defense mechanism and that by manipulating cropping conditions or by treating stress, it may be possible to elicit the expression of such latent defense potential during host parasite interaction. This constitutes the very basis of induced resistance in plants as a possible disease control measure. Since the first report of induction of resistance in plants against their fungal pathogens by prior inoculation with their less virulent form, the emphasis had been mostly on biological induction of resistance. Considerable evidence has now accumulated to that prior inoculation of susceptible plant host with an avirulent form of pathogen. Cultivars of non pathogenic races of pathogens of both homologous and heterologous

nature or non pathogen can provide it significant levels of protection form the subsequent attack of the virulent forms of pathogen (Purkayastha, 1994; Mukhopaddhya, 1994). Plants so protected develop less disease symptoms. In some cases such induced or acquired resistance is systemic in nature and persists effectively over a fairly long periods. Even though effective field level protection has been achieved by this method against many diseases of Tobacco, Bean, Cucumber and Melons, various logistical problems relation to the large scale production of the inducer biotic agent and its application to the crop under field conditions make this approach both cumbersome and uneconomic an heavily limit its utility as a measure for plant disease control particularly in the Indian agricultural perspective.

In the present investigation, sixteen tea varieties were screened against *U. zonata*. Among all the tested varieties, BSS-2, BS/7A/76 and AV-2 were found to be highly susceptible, while UPASI-9, UPASI-2 and UPASI-3 were most resistant. Although less in known with certainty about the specific recognition events that predict incompatible host-pathogen interaction, considerable genetic and biochemical evidence indicates that constitutive specificity imparting molecules must exist in the incompatible antifungal compounds at the infection site. Cell recognition has been defined as the initial event of cell-cell communication which elicits morphological, physiological and biochemical response. Surface molecules of eukaryote cells have been involved in cell-cell recognition and/or adhesion and as receptors for various effects. Many of these specificity imparting molecules are glycoproteins, and fungi are known to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980; Ransom *et al.*, 1992).

In the initial stage of infection at the cellular level the exchange of molecular signals between host and parasite is considered to be one of the mechanisms resulting in the specificity of such interactions. The genetic information contained in nucleic acid is expressed in the cell via protein synthesis. Several proteins function as enzymes in the metabolic pathways, which synthesize or breakdown cellular components. When plants containing various kinds of proteins are infected by pathogens, the proteins in the penetrated plant cells are changed chemically and physically. Thus qualitative and

quantitative changes in proteins are related to both plant and pathogen. A protein competition model was proposed by Jones and Hartley (1999) for predicting total phenolic allocations and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis compete for the common limiting resource-phenylalanine and hence protein and phenolic allocations are inversely correlated.

In the present investigation changes in the protein content was noted in the *U. zonata* inoculated leaves of susceptible varieties in relation to their healthy control. Increased protein level was also detected after infection of susceptible bean leaves by *Uromyces phaseoli.* The greater accumulation of protein in susceptible host after inoculation may also be attributed to the total proteins of both host and parasite. However, it is difficult to separate the relative contribution of host and parasite to the total protein content. It is evident from the above statement that some changes occur in proteins of infected plants. However, these changes are not always significant. Sometimes protein content of the host remains more or less similar even after inoculation but isozyme pattern may change.

Environmental effects in phenolics are all the more long-lasting, as they have to cope of such conditions year after year. In a similar study on tea with the fungus *Glomerella cingulata* which causes brown blight of tea, it was reported that high humidity and rainfall were the most important factors predisposing the plants to infection (Chakraborty *et. al.*, 2002). Phenols are also known to play definite roles in a plant defense. Considering this in the present stud phenol contents of the healthy and artificially inoculated (with *U. zonata*) plants were determined. It has been reported previously that quinones in plant tissues react with proteins to form melanin and other tannins leading to the discoloration of damaged tea leaves. Many studies have demonstrated the importance of phenolic compounds in plant defense. In general, plant phenolics have a diverse range of biological activity, depending on their structure, degree of polymerization; stereo isomeric differences etc. interaction between phenolic compounds and environmental conditions determines their action. Polyphenols have a distinctive ability to engage in molecular recognition, or formation of intermolecular complexes with each other and with other molecules (Haslam, 1999). In the present

Pestalotiopsis theae, Glomerella cingulata] has been described by Chakraborty *et. al.*, (1995). Biochemical responses to tea plants exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* in the levels of phenols and enzyme activities were studied (Sharma and Chakraborty, 2004).

In the present study, the levels of phenolics in leaves of resistant and susceptible tea varieties were estimated following inoculation with *U. zonata.* Host responses could be differentiated by changes in content of phenolic compounds. In both the cases total phenol and orthodihydroxy phenol content increased in resistant varieties but decreased in susceptible varieties in comparison to their healthy controls. Hammerschmidt and Nicholson (1977) demonstrated a clear difference between resistant and susceptible interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. Sridhar and Ou (1974) reported differences in total phenolics accumulation in the interaction of *Pyricularia oryzae* with rice. However, no differences were found in the phenolic content in the interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. On the other hand, a resistant cotton cultivar contained fairly high amount of total as well as orthodihydroxy phenol than susceptible cultivar. In the present study, greater accumulation of orthodihydroxy phenol in resistant interaction of *U. zonata* and tea varieties indicated that this might play a role in disease resistance mechanism. Orthodihydroxy phenols play a major role in disease resistance and disease development. They are easily oxidized to highly reactive quinones which are effective inhibitors of sulphydryl enzymes, thereby preventing the metabolic activities of host and parasite cells (Mansfield 2000). The UV spectra from both the healthy and *U. zonata* inoculated tea roots were analyzed at 290nm. A sharp peak at retention time 2.6 was present in both but in the healthy extracts the peak height was much smaller than the inoculated one. Other small humps and shoulders were also evident in both the cases.

It is known than catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. Sambandam el. Al., (1982) isolated and enzyme (catechin 2-3 dioxygensis) from *Chaetomium cupreum* which cleaved catechin into simpler phenols. It is not unreasonable to speculate that the antifungal compound cleaved to some simpler phenols in the present study. In the susceptible variety, the breakdown of catechin was almost complete while traces were

evident in the resistant variety even after 48 h of inoculation. Accumulation of pyrocatechol in resistant varieties increased after 48 h of inoculation with *U. zonata.* Increased level of pyrocatechol may be associated with the differential host responses to disease production.

Accumulation of defense enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (PO), in tea varieties following inoculation with *U. zonata* were determined. PPO usually accumulated following inoculation of plants. PPO transcript levels systemically increased in tomato when mature leaflets were injured. Increased activity of PPO and PO was demonstrated in the cucumber leaf in the vicinity of the lesions caused by some foliar pathogens. Among all the stress related enzymes, the role of peroxidase has been most thoroughly worked out. PO is a metallo-enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. Various naturally occurring and synthetic substances, growth regulator and environmental factors markedly influence the activity of these peroxidases. Increased PO activity in susceptible cultivars were noticed when compared with the resistant following treatement with sodium bisulphate prior to inoculation with *Botrytis maydis.* On the other hand, there are also reports of increased PO activity due to induction of resistance (Chakraborty *et. al.,* 2005). The induction of PO activity by pathogens and methyl jasmonate and existence of multiple molecular forms of peroxidase in tea has also been reported (Sharma and Chakraborty, 2004). Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosnthesis such as PAL are involved in defense reactions in plants. Considering the importance of phenol metabolism in tea plants, those three enzymes (PAL, POX and PPO) were selected in the present investigation. An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plants to biotic (Chakraborty *et. al.,* 1993) or to other environmental stresses. In sorghum, naturally occurring high levels of PAL activity induced by light should be differentiated from the activity induced as a response of attempted fungal infection. Bhattacharya and Ward (1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma.* Considering that PAL is a key enzyme in the biosynthesis,

not only of phytoalexins, but also of phenolic compounds have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicators of the activation of defense related enzymes.

There is surface to surface interaction between the host and pathogen. Recognition or interaction as compatible depends on some kinds of molecular similarities, between the host and pathogen (Chakraborty, 1998; Chakraborty and Saha, 1994; Chakraborty *et al.* 1995, 2002). One of the prerequisites for the successful establishment of the pathogen in the host is based on close serological similarity between host and pathogen. This serological relationship between host and pathogen has been exploited for development of pathogen detection systems in the host using PAb 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 tissues. Commercial diagnostic kits have been offered in recent years for the rapid diagnosis of several fungi in plant tissues, soil and water (Werres and Steffens, 1994). Most common among these techniques are ELISA, Dot- blot, Western blot etc. used in large scale disease indexing programmes in perennial and vegetatively propagated crops (Clark, 1981). Timely detection of disease especially root diseases combined with proper management practices can lessen crop loss to a great extent.

Enzyme linked immunosorbent assay (ELISA) is probably one of the most sensitive serological techniques for the detection of pathogen in host tissues (Chakraborty and Chakraborty, 2003). In the present study polyclonal antibody was raised against mycelia and cell wall of *U. zonata*. The antisera obtained were purified to minimize non specific binding. At the beginning, the sensitivity of the assay was optimized. Root antigens of sixteen tea varieties, non host and non pathogen were cross reacted separately with PAb of *U. zonata*. Presence of cross reactive antigens (CRA) between *U. zonata* and tea varieties were evident in immunodiffusion test. However, weak precipitation reaction was observed with antigens of some selected tea varieties. No common antigenic substance was found between *U. zonata* and non host and non pathogen. The presence of CRA and their involvement in various host parasite combinations have been observed. These are cotton and *Fusarium oxysporum f. sp. vasinfectum* (Charudattan, 1970); cotton and *Verticillium alboatrum* (Charudattan and DeVay, 1972); sweet potato and *Ceratocystis fimbriatae* (DeVay *et. al.,* 1972); potato and *Phytophthora infestans*

(Palmerly and Callow, 1978, Alba and DeVay, 1985); soybean and *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983); coffee and *Hemilea vastatrix* (Alba *et. al.*, 1983); jute and *Colletotrichum corchori* Bhattacharya and Purkayastha, 1985); soybean and *Colletotrichum dematum* var. *truncate* (Purkayastha and Banerjee, 1986), tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994); tea and *Pestalotiopsis theae* (Chakraborty *et. al.*, 1995); soybean and *Fusarium oxysporum* (Chakraborty *et. al.*, 1997). tea and *Glomerella cingulata* (Chakraborty *et.al.*, 2002), in the present study PTA-ELISA readily detected CRA between tea root antigens and *U. zonata* at a concentration of 1:250 antiserum dilution. Alba and DeVay (1985) also detected CRA in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* using antisera of two potato cultivars at concentrations lower than 50 µg/ml protein in indirect ELISA. Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after few days of infection, by which time the pathogen would be well established in the host tissues. Various formats of ELISA using polyclonal antibody have found widespread application in plant pathology and are routinely used for detection and identification purposes (Lyons and White, 1992; Hansen and Wick, 1993, Chakraborty *et. al.*, 1995; 1996; Chakraborty *et. al.*, 2002).

It is also important in the studies on host parasite relationship to determine the cellular location of the pathogen. For this purpose in this study, indirect immunofluorescence tests were conducted with cross sections of healthy and artificially inoculated (with *U. zonata*) tea roots and mycelia. Transverse sections from infected roots were made and PAb raised against mycelial antigens of *U. zonata* were used for probing the fungal hyphae which penetrate the root tissues. Bright fluorescence was observed in the cross sections of tea roots. DeVay *et. al.*, (1981) determined the tissue and cellular location of major cross reactive antigens (CRA) shared by cotton and *F. oxysporum f. sp. vasinfectum.* Cellular location of CRA in tea leaf tissues shared by three foliar fungal pathogens such as *Bipolaris carbonum* (Chakraborty and Saha, 1994); *Pestalotionpsis theae* (Chakraborty *et. al.* 1995) and *Exobasidium vexans* (Sharma and Chakraborty, 2004) have been demonstrated. Besides detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors (Reddy and Ananthanarayan,1984) On the basis of immunofluorescence studies, Dewey *et. al.*, (1984) demonstrated the presence of mycelium and

chalmydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence was assessed for their potential to detect resting spores of *Plasmodiophora brassica (* Wakeham and White 1996) as well as *Fomes lamaoensis* (Chakraborty *et. al.*, 2002) in soil.

The dot immunobinding assay was developed using alkaline phosphates substrate 5-bromo-4chloroindolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) to detect the precipitated hydroxyl group. When the substrate 5-bromo-4chloroindolyl phosphate is used, the phosphate is cleaved by the enzyme and the hydoxyl group precipitates. The hydroxyl group then tautomerizes forming a ketone and under alkaline conditions dimerization occurs, forming a dehydroindigo. In the process of dimerizing, it releases hydrogen ions and reduces the nitroblue tetrazolium which precipitates, forming an intense blue deposition of diformazan. The dot immunobinding technique has also been found to be a rapid and sensitive method for detection of fungal pathogens. In the present study, antigens were prepared from charcoal stump rot infected tea roots. Naturally infected tea root as well as root artificially inoculated with *U. zonata* were tested on nitrocellulose paper. Infected and artificially inoculated root antigens gave intense dots when compared to the healthy control confirming the presence of fungal pathogens. So, early detection of disease is an important requisite for development of management strategies. A microtitre immunospore trapping device, which uses a suction system to trap air particulates directly by impaction into microtitre wells, has been used successfully for the rapid immunodetection and quantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea* (Kennedy *et. al.*, 2000).

Plants have well developed defense mechanisms which enable them to defend themselves against parasites in their tissues. The biochemical basis for this resistance against microbial attack consists of both preformed and post-infectional ones. Preformed defenses are often regarded as general or unspecific as compared to inducible defense systems which are highly specific. Though 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. Varieties within the host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such gene. To account for the observed specificity and degree of variability of

host parasite system, the fungal receptor must have high information content which involves recognition between the host and pathogen both at the cellular and subcellular level. A cell reacts in a special way as a consequence of an association with another cell because it acquires information, which is conveyed through chemical or physical signals in the process or recognition. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signaling processes. In spite of lacking immune responses like animals, plants have nevertheless evolved immune mechanisms of various types by which they can account for the advance of foreign organisms. The result is that disease tends to be specific, a given pathogen usually infecting a distinct range of host plants.

In the present investigation, using PTA-ELISA formats and PAb of *U. zonata*, treated and untreated plants exposed to natural inoculum after 15 and 30 days of soil amendments were compared. The absorbance (A_{405}) values were always reduced in treated root tissues than untreated ones. It indicates clearly that in the treated root tissues the establishment of the pathogen (*U. zonata*) was not successful due to the application of biocontrol agents. Detection of *U. zonata* in tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents was also determined immunologically in both root tissues and soil. For this purpose, PTA-ELISA format was carried out. Results showed that ELISA values of root tissues treated with *T. harzianum* and *T. viride* were significantly lesser than with *U. zonata* alone. The same trend of results was obtained in infested rhizosphere soil through PTA-ELISA analysis. This result is in conformity with that planting of tea seedlings after dipping roots in spore suspension of *T. harzianum* reduced 56.6% mortality of plant due to fungal infection. However the reduction of mortality of plant increased to 62.2% when *T. harzianum* was applied to soil. Significant control of charcoal stump rot of tea with antagonistic microflora and role of *T. hazianum* and *T. viride* as biocontrol agents have been well established. In the present study, antigens prepared from mycelia of *U. zonata* amended soils and following application of *T. harzianum* and *T. viride* were prepared and tested on nitrocellulose paper using PAbs raised against mycelia of *U. zonata* and NBT/BCIP as substrate. Antigens of homologous source showed deep colored dot when compared with soil antigens prepared from treated organic amendments. Other tea root pathogens responded slightly reactivity with *U. zonata.* Walsh *et. al.* (1996) also performed western

blotting using the crude serum of *Spongospora subterranean* spore balls. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence were assayed by Wakeham and White (1996) for detection of resting spores of *Plasmodiophora brasica* in soil. In conclusion, it can be stated that charcoal stump rot can cause severe damage to tea plants, as primary root disease and such immunodetection techniques makes it possible to detect micro quantities of the pathogen within root tissue and rhizosphere soil before much damage cause by the pathogen. Therefore, an accurate, rapid and cost-effective diagnosis and rapid detection of pathogen is important to take preventive steps for disease management.

Further in this investigation, effective integrated management practices against *U. zonata* were tested *in vivo*. Biocontrol agents [*T. harzianum, T.viride*] alone and in combination with neem cake, oil cake provided control of charcoal stump rot disease in all the three modes of application viz, simultaneous, repeated and post infection. But repeated application of neem cake, oil cake with various combinations of cow dung, rabbit manure and chicken manure were found to be significant. A possible long-term benefit of increased implementation of microbial control would be reduced input into agriculture, particularly if seasonal colonization and introduction-establishment come into widespread use. Initially, inputs due to implementation of microbial control are more likely to increase than decrease. Biological control using agriculturally important microorganisms is simply one of the best potential alternatives for disease control that could be made available in a relatively short time period. A successful disease control program depends on a crop production system which is closely aligned with the goals of disease management. In this context, attempts using biocontrol agents and organic residue materials as an integrated approach for disease control assumes much greater significance. There is potential for yield increase in the near future. Biomass production, their suitable formulation for commercialization of antagonists to check chemical fungicides usage needs to be developed.

SUMMARY

1. A short comprehensive review pertaining to this investigation has been presented which deals mainly detection of plant pathogenic fungi and biochemical changes in plants following fungal infection has been presented below.

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

3. Screening of resistance on twenty five tea varieties (10 Tocklai, 9 Darjeeling and 6 UPASI) were done. Among the tested tea varieties BSS-2, BS/74/76 and AV-2 were found to be highly susceptible, while UPASI-9, UPASI-2, UPASI-3 were most resistant.

4. Cultural conditions affecting growth of *U. zonata* were studied with special reference to their growth in different media, variable pH and different types of carbon, organic and inorganic nitrogen source. Maximum growth of the pathogen occurred in Potato dextrose agar. Lactose was the most effective carbon source while no growth was observed in sorbose. Beef extract was most effective nitrogen source followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

5. Protein content of healthy and artificially inoculated tea root tissues from sixteen tea varieties as well as mycelia and cell wall proteins of *U. zonata* was estimated and analyzed in SDS-PAGE. Characterization of the cell wall of *U. zonata* by ConA-FITC binding revealed its glycoprotein nature.

6. Sixteen tea varieties (TV-9, TV-18, TV-22, TV-25, TV-26, S-449, BS/7A/76, CP-1, AV-2, P-1258, K1/1, UPASI-2, UPASI-3, UPASI-8, UPASI-9 and UPASI-26) were selected for biochemical analyses. Total phenol, orthodihydroxy phenol content as well as antifungal phenolics were quantified in resistant (428-512 µg/g fresh wt) and susceptible tea varieties (54-73 µg/g fresh wt) following inoculation with *U. zonata.*). Concentration of this compound in healthy root tissues were very low.

Time course accumulation of peroxidase , phenyl alanine ammonia lyase and polyphenol oxidase in sixteen selected tea varieties were determined.

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

8. To check the effectiveness of PAbs, Agar gel double diffusion tests were performed using crude antibody as well as purified IgG obtained from different bleeding collected from *U. zonata* . Strong precipitin reactions were observed in homologous cross reaction of each case.

9. Optimization of ELISA using PAbs of *U. zonata* and antigen preparations at variable concentrations ranging from 40 to 0.312mg/ml. However maximum absorbance values were obtained in 3rd bleeding followed by 4th bleedings.

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

11. Detection of *U. zonata* in artificially inoculated tea root tissues using PTA-ELISA and DAS-ELISA formats were standardized.

12. Antigens prepared from soil samples collected from different tea field and amended soil, were tested against PAb of *U. zonata* using PTA-ELISA formats and Dot blot analysis.

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

14. Antigenic preparation of 60-80% SAS fraction was used for raising antiserum and this antiserum was tested by immunodiffusion test and IgG was purified. CRA between *U. zonata* and tea varieties, non-host and non-pathogen was detected in PTA-ELISA. This antiserum also showed higher reactivity in the susceptible reactions as compared to resistant reaction. PAb raised to 60-80% SAS fraction could also significantly detect infections by PTA-ELISA, Dot blot and Western blot analysis.

15. Cross sections of tea roots inoculated with *U. zonata* and treated with PAb of *U. zonata* followed by labelling with FITC developed a bright fluorescence throughout the sections, extending up to vascular tissues as well as outer surface.

16. Mycelia of *U. zonata* when treated with homologous antisera followed by FITC, bright fluorescence was noticed on young hyphae.

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

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

19. *In vitro* interaction of *U. zonata* with *T. harzianum* and *T. viride* was studied. Both inhibited the growth of *U. zonata.*

20. Soil amendment of tea rhizosphere with *T. harzianum* and *T. viride* both in potted conditions and in the field reduced disease intensity significantly.PTA-ELISA of tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents, reacted with PAbs of *U. zonata*, *T.harzianum* and *T. viride* indicated the reduction of pathogen population in rhizosphere soil and root tissues.

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