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.

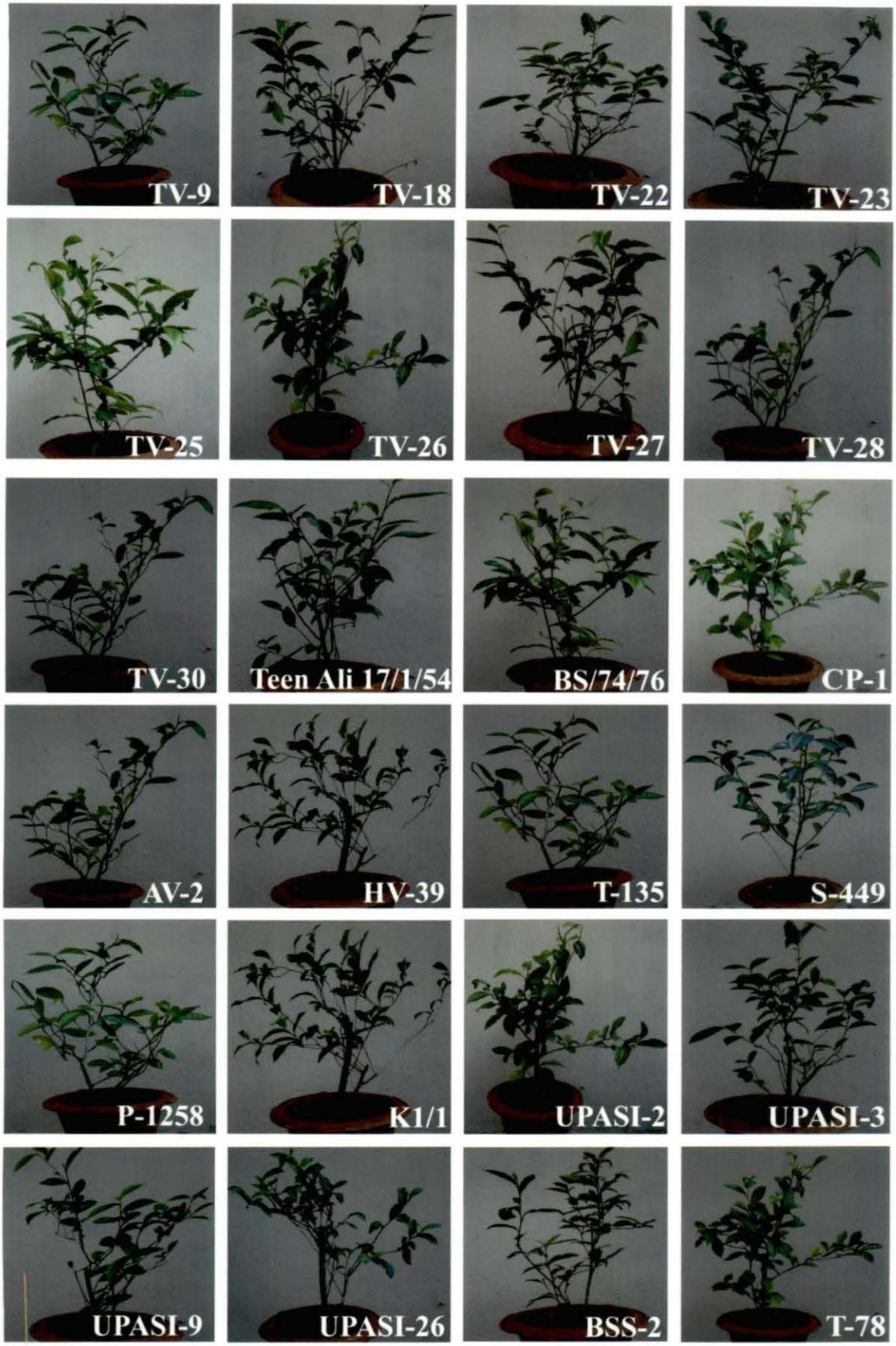

Plate 4: Tocklai, Darjeeling and UPASI varieties of tea (*Camellia sinensis*) being maintained in Tea Germplasm Bank.

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*.