MATERIALS AND METHODS

3. Materials and Methods

3.1. Plant Material

3.1.1 Collection

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

3.1.2.Propagation by Cutting

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

Sandy soil (sand 75% and soil 25% with a pH ranging from 4.5 to 4.8) was used for clonal propagation. Soil pH was adjusted to 4.8-4.9 by treating with 2% aluminium sulphate solution. It was followed by two waterings to remove excess aluminium sulphate. The heated soil was treated between 60^{0}C to 80^{0}C on a metal sheet with fire below to kill eelworms, if any, present in the soil.

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

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

3.1.3. Maintenance of tea sleeves in nursery

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

3.1.4. Plantation

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

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

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

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

3.1.5. Maintenance of mature plants

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

3.2. Fungal culture

3.2.1. Source of culture

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

Table 1 : List of fungal isolates used

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

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

3.2.2. Assessment of Mycelial Growth

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

3.2.2.1. Solid media

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

1. Potato dextrose agar (PDA)

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

2. Richards Agar (RA)

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

3. Carrot Juice Agar (CJA)

Grated carrot	-	20.00g

	Agar	-	2.00g
	Distilled water	-	100ml
4.	Czapek-dox agar (CDA)		
	$NaNO_3$	-	0.20g
	K_2HPO_4	-	0.10g
	$MgSO_4$, $7H_2O$	-	0.05g
	KCl	-	0.05g
	$FeSO_4$, $7H_2O$	-	0.05g
	Sucrose	-	3.00g
	Agar	-	3.00g
	Disttilled water	-	100ml
5.	Flentze's soil extract agar (FSEA)		
	Soil extract	-	1L
	Sucrose	-	1.00g
	KH_2PO_4	-	0.20g
	Dried yeast	-	0.10g
	Agar	-	25.00g
6.	Potato Sucrose agar (PSA)		
	Peeled potato	-	40.00g
	Sucrose	-	2.00g
	Agar	-	2.00g
	Distilled water	-	100ml
7.	Malt extract Peptone agar (MPA)		
	Malt extact	-	20.00g
	Peptone	-	1.00g
	Detrose	-	20.00g
	Agar	-	20.00g
	Distilled water	-	1L
8.	Yeast extract dextrose agar (YDA)		
	Yeast extract	-	7.50g
	Dextrose	-	20.00g
	Agar	-	15.00g
	Distilled water	-	1L.

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

3.2.2.2. Liquid media

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

3.3. Inoculation techniques

3.3.1. Preparation of Inoculum

3.3.1.1. Pathogen

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

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

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

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

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

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

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

3.3.1.2. Biocontrol agents

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

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

3.3.2. Inoculation of healthy tea plants

3.3.2.1. Pot grown plants

(a) Pathogen

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

(b) Biocontrol agents

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

3.3.2.2. Field grown plants

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

3.4. Disease assessment

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

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

3.5. Soluble protein

3.5.1. Extraction

3.5.1.1. Mycelia

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

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

3.5.1.2. Root

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

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

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

3.5.2. Estimation

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

3.6. SDA-PAGE analysis of total soluble protein

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

3.6.1. Preparation of stock solutions

The following stock selections were prepared :

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

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

B. Sodium Dodecyle sulphate (SDS)

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

C. Tris buffer

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

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

D. Ammonium persulphate (APS)

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

E. Tris-Glycine electrophoresis buffer

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

F. SDS loading buffer

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

3.6.2. Preparation of Gel

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

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

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

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

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

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

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

3.6.3. Sample preparation

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

3.6.4. Electrophoresis

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

3.6.5. Fixing and staining

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

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

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

3.7. Preparation of Antigen

3.7.1. Fungal antigen

3.7.1.1. Mycelial

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

3.7.1.2. Cell wall

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

3.7.2. Root antigen

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

3.7.3. Soil antigen

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

3.8. Binding of FITC labelled concanavalin-A

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

3.9. Serology

3.9.1. Rabbits and their maintenance

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

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

3.9.2. Immunization

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

3.9.3. Bleeding

Blood samples were collected by marginal ear vein puncture. First bleeding was taken 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding rabbit was placed on their backs on a wooden board, fixed at a 60^0 angle. The neck of the rabbit was held tightly in the triangular gap at the edge of the board and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of sharp sterile blade and blood samples (4-10ml) were collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. For clotting, the blood samples were kept at 37^0C for 1h

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

3.9.4. Purification of IgG

3.9.4.1. Precipitation

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

3.9.4.2. Column preparation

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

3.9.4.3. Fraction collection

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

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

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

3.9.4.4. Estimation of IgG concentration

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

3.10. Immunodifusion

3.10.1. Preparation of agarose slides

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

3.10.2. Diffusion

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

3.10.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initally washed with steriled distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then slides were stained with coomassie blue (R 250, sigma; 0.25g coomassie blue + 45ml methanol + 45ml distilled water + 10ml glacial acetic acid) for 10 min. at room temperature. After staining, slides were washed in destaining solution [90 ml methanol : distilled water (1:1) and 10 ml acetic acid) with changes untill the background become clear. Finally, slides were washed with distilled water and dried in hot air oven for 3h at 50°C.

3.11. Enzyme linked immunosorbent assay (ELISA)

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

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

Stocks

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

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

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

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

Stocks

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

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

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

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

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

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

0.05 M Tris, 0.135M NaCl, 0.0027 M KCl

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

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

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

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

6. Substrate

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

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

3.11.1. Direct antigen coated (DAC) ELISA

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

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

3.11.2. Double antibody sandwich(DAS) ELISA

Conjugation of alkaline phosphatase with γ-globulin

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

Assay

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

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

3.11.3.Competition ELISA

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

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

3.12. Immunoblotting

3.12.1. Dot Blot

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

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

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

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

Assay

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

3.12.2. Western Blotting

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

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

(ii) Transfer buffer (Towbin buffer) :

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

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

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

Preparation was as mentioned in ELISA.

(iv) Blocking solution

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

(v) Washing buffers :

(a) Washing buffer-1 : PBS

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

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

(iv) Alkaline phosphatase buffer :

(100mM NaCl, 5mM $mgCl_2$,

Tris-HCl, pH 9.5).

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

(vii) Substrate

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

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

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

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

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

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

Extraction and estimation of protein :

Protein extraction and estimation was done as described earlier.

3.12.2.1. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.12.2.2. Blot transfer process

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

3.12.2.3. Immunoprobing

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

3.13. Fluorescence antibody staining and microscopy

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

3.13.1. Fungal mycelia

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

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

3.13.2. Cross section of tea roots

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

3.13.3. Soil

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

3.14. Immunocytochemical staining

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

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

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