Materials and Methods

3.1. Plant Material

3.1.1. Collection

Tea clones were collected from three Experimental Stations from different geographical locations of India- (*a*) Tocklai Experimental Station, Jorhat, Asssam, (*b*) Darjeeling Tea research Centre, Kurseong, West Bengal, (*c*) United Planters' Association of South India (UPASI) Tea Research Station, Valparai, Tamilnadu.

Ten tea clones released by Tocklai Experimental Station i.e., TV- 9, TV - 18, TV- 20, TV - 22, TV - 23, TV - 25, TV - 26, TV - 27, TV - 28, and Teenali 17/1/54 were collected from the Mohur Gaon and Gulma tea Estate, Sukna, while TV - 29 & TV - 30 were collected from Hansqua Tea Estate. These are bieng maintained in the Phytopathological Experimental Garden of the Department of Botany, NBU, following 2 years deep pruning cycle and also in the glass house.

Fifteen Darjeeling clones generally grown in hilly regions were collected and were planted in the Experimental Garden and in the glass house. The 15 tea clones collected are BS/ 7A/176, B-668, B-777, P-312, T- 78, P-1258, B-157, TTV-1, BT-15, AV-2, HV-39, RR-17, K-1/1, T-135 and CP-1.

From UPASI Tea Research Station, Valparai, Tamilnadu, fresh clonal cuttings of 6 varieties viz, UPASI-2, UPASI-3, UPASI-8, UPASI-9, UPASI-17, and UPASI-26, and seeds of 3 varieties viz, BSS-1, BSS-2, BSS-3, were collected.

3.1.2. Propagation

Tea plants were mainly propagated by means of cuttings and seeds. Two methods followed for the propagation of tea clones are as follows -

3.1.2.1. Cutting

Initially sand and soil mixture (3:1) was used for rooting of clonal cuttings. For the removal of eelworms etc. soil was heated to 80°C on a metal sheet. The pH was adjusted to 4.5 - 4.9 using 2% solution of aluminium sulphate. Excess aluminium sulphate was removed by 2 or 3 waterings. Polythene sleeves (15 cm), were then filled with this prepared soil for raising cuttings straightaway. Soil filled sleeves were stacked into rows. All fresh cuttings were dipped into hormone mixture obtained from UPASI Tea Research Centre and then placed one each into sleeves. These were covered with polythene sheets for retention of moisture content inside and allowed for rooting. Watering was done every fourth day till new leaves appeared(Plate II, figs. A-C).

3.1.2.2. Seed germination

Pre-treated (heat treated), sieved sand was used to prepare beds of about 150 cm x 100 cm x 15 cm. Sand in the beds was compacted and watered throughly, prior to putting out the seed. The seeds were treated with Dithane M45 (0.03%) just before sowing. The seeds were sown at a spacing of 5.0 x 5.0 cm, triangle. The seeds were pressed down in the bed with the scar directed down wards; the dome of the seed was projected slightly above the level of the bed. The seed beds were then covered with thick black polythene sheets and watered as and when necessary, till cracking started. The seeds started cracking between 25 and 35 days followed by the protrusion of root (radicle), and at this "cracking" stage, they were transplanted into sleeves. In case where the radicle has protruded, a hole of an appropriate depth to accommodate the root was punched in the soil and the seeds were planted by inserting the radicle in the hole. Sleeves were covered with polythene cloche and watered as and when necessary. The cloche was removed after the seedlings were at four leaved stage (Plate III, figs. A-D).

3.1.3. Plantation

All the tea plants raised, either by cuttings or seedlings were planted in the Phytopathological Experimental Garden as well as transferred to earthen pots and maintained inside the Glass House (Plate IV, figs. A-D and Plate V, figs. A-D). For plantation, in the field, Simazine @ 75 g / 20 L water as pre-emergent and Glyphosphate @ 1:200 against thatch type of grasses was applied as described by Barpujari and Banerjee (1994). Pits (45 cm x 45 cm x 45 cm) were filled with soil mixed with cattle manure (dry) 4.5 kg, 30 g super phosphate, 30 g rock phosphate and 2.5 g phorate [0,0 - diethyl s- (ethyl thiomethyl) phosphoro dithioate]. Rock phosphate was used at the bottom of each pit, then the pits were half filled with soil-cattle manure super phosphate mixture and phorate was mixed with the excavated soil to check the population of eelworms. Ultimately, clonal sapling and seedlings of all the tea varieties collected, were planted in these pits. For earthen pots, soil and planting mixture was used at 1:1 ratio; after planting of saplings and seedlings in these pots they were kept inside the glass house.

3.1.4 Maintenance

Manuring of nursery plants were done after roots and one pair of leaves were formed in case of cuttings and seedlings respectively following recommendation made by Ranganathan and Natesan (1987). The nursery mixture of following composition was used:

Ammonium Sulphate - 8 parts by weight

Ammonium phosphate sulphate(16:20) - 35 parts by weight

Potassium Sulphate - 15 parts by weight

Magnesium Sulphate - 15 parts by weight

Zinc Sulphate - 3 parts by weight

Manuring was done upto 12 months once in 15 days. The mixture was dissolved at the rate of 30 g in 10 L of water and applied @ 50 ml per plant.

In case of mature (1 year and above) plants, in the field, a fertilizer mixture (10 Kg, Urea; 46% N, 20 Kg ammonium phosphate - 11% P_2O_5, 8 kg muriate of potash - 60% K_2O) was applied on the top of the soil, just after the first shower at the onset of monsoon. This application was repeated after 6 months interval. Tricontanol, (Greenol) was sprayed at a concentration of 2% just at the advent of new flush.

Only the tips were removed once in a year in case of plants upto the age of three years. In case of mature aged plants in the experimental garden, a two year deep pruning cycle was maintained.

Besides, three other plant species such as *Glycine max* (cv. Soymax), *Cicer arietinum* (cv. JG-62) and *Camellia japonicum* were grown in earthen pots and maintained in the glass house. Seeds of *G.max* (cv. Soymax) and *C.arietinum* (cv. JG-62) were collected from Pulses and Oil seed Research Station, Berhampore and International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad respectively, while *C.japonicum* plants were obtained from Botanical Garden, Darjeeling.

3.2. Fungal Culture

3.2.1. Source

A virulent strain of *Pestalotiopsis theae* (Sawada) Stey. was isolated from naturally infected (grey blight) tea leaves (TV 23) and was used after completion of Koch's postulate. This isolate was designated as Pt-2 (IMI number 356807). Other four isolates were obtained from different sources, and they were designated as Pt-1 (from Tocklai Tea Research Institute, Jorhat, Assam), Pt-3 (from naturally infected tea leaves of TV 27), Pt-4 (naturally infected tea leaves of Teenali -17/1/54), and Pt-5 (from naturally infected tea leaves of B-157).

Plate II(figs. A-C) . Stages in propagation of tea (UPASI 9) by cutttings .**A**- nodal cutting with excess callus ; **B**-cuttings in plastic sleeves ; **C**- development of shoots from cuttings

Plate III (figs. A-D). Stages in propagation of tea by seed germination (BSS-3) . **A** - seed cracking in the bed ; **B** - seeds after cracking transferred to polythene sleeeves; **C**- seedlings (3- 4 leaved stage) ; **D**-seedling after being transferred to pot

PLATE IV (figs.A-D). Potted plants of Tocklai varieties of tea maintained in glass house

PLATE V (figs.A- D). Potted plants of UPASI varieties of tea maintained in the glass house

Besides two more leaf pathogens of tea viz., *Glomerella cingulata* (Stoneman) Spauld and Schrenk (IMI number 356805) and *Corticium invisum* Petch. were obtained from the culture collection of Mycology and Plant Pathology Laboratory, Department of Botany, University of North Bengal. A non-pathogen of tea *Fusarium oxyspor um Schlecht* (ITCC No. 2389) was collected from Division of Mycology and Plant Pathology, IARI, New Delhi.

3.2.2. Completion of Koch's postulate

Fresh young tea leaves of TV-23 were collected from the Phytopathological Experimental Garden and inoculated with conidial suspension of *P.theae* following detached leaf inoculation technique. After 72 h of inoculation, infected leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ for 3 mins., washed several times with sterile distilled water and transferred aseptically into Potato - Dextrose - Agar (PDA) slants, and incubated at 25 ± 1° C. After 15 days of incubation the isolated fungal culture was examined, compared with the stock culture and identification was confirmed as *P.theae.*

3.2.3. Maintenance of Stock Culture

The fungus thus obtained was subcultured on PDA slants, incubated at 25° C for two weeks and finally the sporulated culture was stored at 5° C and 25° C. Either detached leaf or cut shoot inoculation with *P.theae* and subsequent reisolation of the pathogen was done at the same interval in order to maintain its virulence. All the fungal cultures were kept at the above conditions for experimental use.

3.2.4. Assessment of mycelial growth

3.2.4.1 Solid Media

To assess the growth of different isolates (Pt-1 to Pt-5) in solid media, mycelial block (4mm dia.) of the the different isolates were allowed to grow in petridishes (9 cm dia.), each containing 20 ml of different media. Following solid media were used for this purpose. The plates were incubated at 25° C. The diameter of the advancing zones of the mycelia were measured after specific period of incubation.

Potato dextrose agar

Peeled potato	-	40.0 g
Dextrose	-	2.0 g
Agar	-	2.0 g
Distilled water	-	100 ml

Richard's medium

KNO_3	-	1.0 g
K_2HPO_4	-	0.5 g
$MgSO_4, 7H_2O$	-	0.25 g
$FeCl_3$	-	0.002 g
Sucrose	-	3.0 g
Agar	-	2.0 g
Distilled water	-	100 ml.

Flentze's soil extract agar

Soil extract	-	12.0 ml
Sucrose	-	1.0 g
KH_2PO_4	-	0.2 g
Dried yeast	-	0.1 g
Agar	-	25.0 g

Carrot juice agar

Grated carrot	-	20.0 g
Agar	-	2.0 g
Distilled water	-	100 ml

Czapek-dox agar

$NaNO_3$	-	0.2 g
K_2HPO_4	-	0.1 g
$MgSO_4,7H_2O$	-	0.05 g
KCl	-	0.05 g
$FeSO_4,7H_2O$	-	0.05 g
Sucrose	-	0.001 g
Agar	-	3.0 g
Distilled water	-	100 ml.

3.2.4.2 Liquid media

To assess the growth of mycelia of isolate Pt-2 in liquid media, the fungus was first grown in petridishes containing 20 ml PDA and incubated for 7 days at 25°C . From the advancing zones of the mycelial mat, agar blocks (4 mm dia.) containing the mycelia, were cut using sterilized cork borer and transferred to Ehrlenmeyer flasks (250 ml) each containing 50 ml of Richard's medium for a desired period at desired temperature. Finally, the mycelia were strained through muslin cloth, collected in aluminium foil cups of known weight, dried at 60°C for 96h, cooled in a desccator and weighed.

3.3 Inoculation technique

Conidial suspension of *P.theae* (from 14 day old culture) at a concentration of $6x10^6$ conidia/ml was used as inoculum in case of detached leaf. For cut shoot inoculations, inoculum was prepared from mycelial blocks (4 mm dia.) of the fungus grown in petridishes for 14 days at 25° C.

3.3.1 Detached leaf

The method as described by Dickens and Cook (1989) was used for artificial inoculation of tea leaves. Fully expanded young tea leaves were detached from plants and placed in plastic trays (38 cm x 30 cm) lined with moist blotting paper. Light scratches were made on the adaxial surface of the leaves with the point of a sharp scalpel (Cook, 1989). On either side of the midrib 2-4 of such scratches were made in each leaf which were immediately inoculated with droplets of conidial suspension (20 µl). Fifty leaves were inoculated in each treatment. In control sets, sterile distilled water was used instead of conidial suspension. Each tray was covered with glass lid and sealed with petroleum jelly to minimize drying of droplets.

3.3.2 Cut shoot

Cut shoot inoculation technique as described Yanase and Takeda (1987) was followed. Twigs (with 3 to 4 leaves) of tea plants grown in the experimental garden were cut carefully with a sharp blade and were immediately dipped into distilled water contained in flasks. Plugs of mycelium (2 mm dia.) bearing conidia of *P.theae* taken from 14 day old culture were aseptically placed on previously made scratches (2-4 per leaf) on the upper side of leaves and were covered with cotton wool moistened in sterile distilled water. Sterile PDA

plugs were used as controls. For each treatment 50 cut shoots were inoculated. After inoculation, the twigs were placed into holes of a thermocule sheet floated on the Hoagland and Knop's solution kept in a glass chamber (72 cm x 33 cm x 30 cm) for one week with aeration.

3.4. Assessment of disease intensity

3.4.1. Detached leaf

Assessment of inoculation infectivity and symptom development were done on the basis of percent drops that resulted in lesion production after 24, 48, 72 and 96 h of inoculation as described by Chakraborty and Saha (1994a).

3.4.2. Cut Shoot

At first, the number of lesions that developed following artificial inoculation with myceliel plugs of *P. theae* were counted. The lesions were graded into four groups according to the size of the lesion. A certain value was assigned to each group viz., 0.1, 0.25, 0.5, and 1.0, respectively for small lesions up to diameter of 2-4 mm, 4-6 mm with sharply defined margins, lesion with slow spread beyond 6 mm and spreading lesion of variable size with diffused margin. Finally, the number of lesion in each group was multiplied by the value assigned to it and the sum total of such value were noted and disease index was computed as the mean of observations on 50 cut shoots per treatment. Data were taken after 48, 72 and 96h of inoculation.

3.5. Preparation of antigen

3.5.1. Fungal antigen

3.5.1.1. Mycelia

Mycelial antigens were prepared following the method of Chakraborty & Saha, (1994b). Discs of fungal mycelia (*P. theae*), 4 mm dia. were allowed to grow in 250 ml Eherlenmayer flasks containing sterilized liquid Richard's medium (g/l distilled water : sucrose, 30; KNO_3, 10; KH_2PO_4,5; $MgSO_4$, $7H_2O$, 2.5 and $FeCl_3$, 0.02). The flasks were incubated at 25°C for 10 days. After the incubation period, for extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl solution and rewashed with sterile distilled water several times. Washed mycelia (50 - 60 g fresh weight) were crushed with 0.05 M sodium phosphate

buffer, pH 7.2, supplemented with 10 mM sodium metabisulphate, 0.5 mM magnesium chloride and 0.85% NaCl, mortar and pestle using sea sand. Homogenates were kept at 4°C overnight, followed by centrifugation at 12000 g for 30 mins. at 4°C. The supernatant was subjected to 100% saturated ammonium sulphate precipitation, kept overnight with constant stirrring at 4°C and further centrifuged at 12000 g for 30 min. The precipitate was dissolved in 10 - 15 ml of 0.05 M sodium metabisulphate, 0.5 mM $MgCl_2$. The dissolved precipitate was dialysed against 0.005 M sodium phosphate buffer for 72 h with 6 hourly changes in dialysis tubing (Sigma corp. USA). The dialysate was stored at -20°C and was used as mycelial antigen for raising antiserum and other experiments.

3.5.1.2. Cell wall

Cell wall was isolated from *P. theae* following the procedure of Keen and Legrand (1980). Mycelia of 10 day old log phase fungus culture were collected on a filter paper in a Buchner funnel and 50 g of fresh mycelial mass were ground for 1 min in a National Super Blender mixer cup (at full speed) with 200 ml distilled water. The resulting slurry was then disrupted in a homoginizer for 1 min.at 5°C. The mixure was centrifuged for 1 mi. at 1500g, the supernatant fluid was discarded, and the sedimented walls were rewashed with distllled water (100ml/g) and was again pelleted by centrifugation at 1500g. The washing was continued till the supernatant became visually clear (at least six times). Finally, the isolated cell walls were dissolved in 10ml 0.05 M phosphate buffer saline, pH 7.2 and stored at -20°C until further use.

3.5.2. Leaf antigen

3.5.2.1. Healthy leaf

Healthy leaf antigens were prepared following the method of Chakraborty and Saha (1994*b*) Fresh young tea leaves were plucked from the Experimental Garden and dipped immediately in water. Leaves were thoroughly washed with tap water and were weighed.After weighing, leaves were crushed in morter with pestle at 40°C using sea sand and insoluble poly vinyl poly pyrroidone (PVPP, Sigma Corp. USA), in 0.05 M sodium phosphate buffer, pH 7.2 containing 10mM sodium metabisulphite, 0.5 mM magnesium chloride and 2 mM soluble PVPP, 10000. (Sigma Corpn. USA). The homogenates were strained through maslin cloth and kept overnight at 4°C. Then the homogenetes ware centrifuged at 12000g for 30 mins. (at 4°C). The supernatant served as healthy leaf antigen and were kept at -20°C until required.

3.5.2.2. Artificially inoculated leaf

Freshly plucked young and healthy tea leaves were inoculated either following the method of detached leaf inoculation or cut shoot inoculation. Infected leaves were harvested after 72h of inoculation. Infected zones were cut into pieces with sharp scalpel and weighed. After weighing antigen was prepared as described previously.

3.5.2.3. Naturally infected leaf

Natually infected tea leaves of different varieties were collected from Experimental Garden and Glass House. The infected zones were cut with sharp scalpel into pieces, healthy zones were discarded. Those infected zones were washed with sterile distilled water, weighed and antigen preparation was made as before.

3.6. Purification of mycelial antigen

3.6.1. Saturated ammonium sulfate fractionation

Freshly harvested mycelium (100g) of *P.theae* grown in RM for 10 days was crushed in a mortar with pestle at 4 C using sea sand and 100ml of 0.1(M) Tris HCl buffer, pH 7.0. The slurry was strained through muslin cloth and centrifuged at 12000 g for 30min. Finally, into the supernatant finely ground ammomium sulphate crystals were added slowly with constant stirring at 4°C. Final sequential saturations of ammonium sulfate of 20,40,60,80 and 100% were obtained. In each case, stirring was done for 6h. and kept overnight at 4° C for precipitiation. Precipitates were then recovered by centrifugation at 12000 g for 1h. Precipitates, thus recovered were dissolved in 2-3ml 0.05 Tris-HCl buffer, pH 8.0, for each saturation level. Dissolved precipitates were then dialyzed using cellulose dialysis tubing (Sigma Co. USA) against 0.01 M Tris Hcl buffer pH 7.0 at 4°C for 72h with 6 hourly changes. Dialysates were collected and stored at -20°C until further use.

3.6.2. Ion exchange chromatography

Ammonium sulfate precipitate of *P. theae* mycelium extract were further purified by ion exchange chromatography. Ammonium sulphate precipitates, previously dissolved in the requisite buffer and dialysed, were applied to an ion exchange column. Prior to use, the column material, DEAE Sephadex was suspended in distilled water for 24h, then resuspended in 0.05 M Tris-Hcl, pH 8.0 and equilibrated in the same buffer after loading into a 10 ml column. 2ml of sample was applied and the column was washed with 0.05 M Tris-HCl bufer, pH 8.0 until no proteins were detected in the eluant. A sodium chloride wash was further

applied (0.05 M Nacl in 0.05 M Tris HCl, pH 8.0) until no proteins were detcted which was then followed by a sodium chloride gradient (0.05 M-0.25 M Nacl in 0.05 M Tris-HCl, pH 8.0). In each case, 20-25 fractions (2ml) were collectd from the column and analysed for their protein content by measuring their optical density at 280nm in a UV-spectrophotometer (SICO Model).

3.7. SDS - PAGE of proteins

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) with modifications.

3.6.1. Preparation of gel column or slabs

Following stock solutions were prepared for SDS-PAGE

Stock solutions

(A) Acrylamide 30.0g
Bis acrylamide 0.8 g
Distilled water 100.0 g
(filltered and stored at 4^0C)

(B) Lower gel buffer (LGB)
Tris.....18.18g
Distilled water 100.0ml
pH was adjusted to 8.8 with 0.1 (N) HCl.
Sodium dodecyl sulfate (SDS) 0.4g

(C) Upper gel buffer (UGB)
Tris 6.06g
Distilled water ..... 10.0 ml
pH was adjusted to 6.8 with 0.1 (N) HCl.
Sodium dodecyl sulfate 0.4g

(D) Ammonium Peroxodisulphate (APS).
Ammonium peroxodisulphate (APS) 0.1g
Distilled water ..... 1.0 ml
(freshly prepared each time)

Tube gel peparation

The gel tubes (12 cm long and 4 mm dia.) were washed with "Extran" (E.Merck) to make them grease free, dried and then rewashed with diethyl either. Resolving gel solution was prepared by mixing acryl amide stock solution and lower gel buffer, degassed for about 20 mins. and then TEMED and APS solutions were added. The composition of resolving gel solution was as follows

Solution A	10.0 ml
Solution B	7.5 ml
Distilled water	12.5 ml
TEMED	12.0 µl
Solution D	36.0 µl

Dried tubes were capped at one end and fixed on a stand. Resolving gel solution was poured into each tube upto a height of 9 cm, overlayered with distilled water (200 µl/tube) and left overnight for polymerization. After the polymerization of resolving gel unpolymerized solution mixed with distilled water was removed by blotting. The stacking gel solution was prepared with acrylamide stock solution and upper gel buffer, degassed for 20 mins, TEMED and APS solutions were added to the solution. The composition of the stacking gel solution was as follows :

Solution A	3.0 ml
Solution C	5.0 ml
Distilled water	12.0 ml
TEMED	10.0 µl
Solution D	500 µl

Stacking gel solution was poured into each tube over the polymerized resolving gel upto a height of 1.5 cm. over relsolving gel, overlayered with distilled water and allowed for polymerization for about 30 mins.

Slab gel preparation

Two glass plates (17 cm x 19 cm) were first washed with Extran (E.Merck) solution and then rewashed with dehydrated alcohol to make the plates grease free. 1 mm spacer was used between the plates and the sides of the plates were sealed. Resolving gel solution of the same

composition as mentioned earlier (30 ml) was poured in bewteen the plates upto a height of 14 cm. The gel solution was overlayered with distilled water, and it was kept for polymerization overnight. After the polymerization, unpolymerized gel solution mixed with water used for overlayering was decanted off and a13 well, 1 mm thick comb was placed over the polymerized resolving gel. After placing the comb stacking gel solution of the same composition as mentioned earlier was poured upto a height of 4 cm. over the resloving gel, overlayered with distilled water and kept for polymerization for 30 mins.

3.7.2. Sample preparation

Sample buffer of the following composition was used for sample preparation.

Solution B	12.5 ml
Sodium dodecyl sulfate	2.3 g
β- mercaptoethanol	5.0 ml
Distilled water	100 ml
Bromophenol blue	0.005g

Protein sample mixed with sample buffer was boiled in a water bath for 2 minutes, following which, 50-80μl were loaded in each tube or well in case of slab gel. Along with the samples, protein marker (Sigma, USA) consisting of a mixture of six proteins (carbonic anhydrase, egg albumin, bovine serum albumin, phosphorylase b, β - galactosidase and myosin of molecular weights 29, 45, 66, 97, 116 and 205 KD respectively) was also loaded in a tube or well , which was prepared as above.

3.7.3. Electrophoresis

For electrophoresis, Tris-Glycine electrode buffer (0.025 M Tris, 0.192 M Glycine) was prepared as follows:

Tris ..... 18.15 g
Glycine 72.0 g
Distilled water 5.0L
pH was adjusted to 8.3
Sodium dodecyl sulfate - 5.0 g

Tube gel : In case of tube gel, after the application of the samples, electrophoresis was performed by applying 1.5 mA current at 300 V per until the samples penetrated resolving gel and then 2.5 mA per tube for 4-5 h until the dye reached the bottom of gel the column.

Slab gel : After application of the samples electrophoresis was performed by applying 2 mA current per well until the protein samples penetrated into the resolving gel and then 3 mA per well at 300 V for 2-3h until the dye reached the bottom of the slab.

3.7.4. Fixing

Fixer solution was repared as follows :

Isopropanol - 250 ml
Acetic acid - 100 ml
Distilled water - 650 ml.

Tube gel : After electrophoresis, gels were removed from the tubes and were kept in fixer solution for 20h at room temperature.

Slab gel : After the removal of the entire gel from glass plates, stacking portion was cut off with sharp scalpel and the resolving gel was soaked in fixer solution for 20h at room temperature.

3.7.5. Staining

Staining solution was prepared as follows :

Comassie blue R250 - 0.1g
Methanol --- 300 ml
Acetic acid --- 100 ml
Distilled water --- 600 ml.

Gels were stained with staining solution for 1h at room temperature with shaking and then destained in destaining solutions (Methanol : Acetic acid: distilled water ::3:1:6) until the protein bands became clearly visible against a clear background.

3.8. Cell wall characterization

3.8.1. Extraction

Mycelial wall extract was prepared from the isolated cell wall of *P.theae* following the method of Brown and Kimmings (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 secs. The suspension was stirred in ice bath for 15h. After centrifugation at 8,000 g for 10 min, 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 48h with 12 hourly changes and concentrated with poly etheylene glycol 6000 (PEG-6000), which was then used as crude mycelial wall extract (MWE) for SDS-PAGE analysis.

3.8.2. Protein estimation

For the estimation of soluble proteins, method of Lowry *et al.*, (1951) was followed. To 1 ml of protein sample 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% Na-K-tartarate and 50 ml of 2% Na_2Co_3 in 0.1N NaOH) was added. This was incubated for 15 min.at room temperature and then 0.5 ml of Folin-Ciocalteau's reagent (diluted 1:1 with distilled water) was added and again incubated for 15min. for colour development. Optical density was measured at 750 nm in a spectrophotometer. Using bovine serum albumin as standard the protein concentrations were computed.

3.8.3. Carbohydrate estimation

Carbohydrate in the extracted cell wall was estimated following Anthrone method. Initially, 1 ml each of 0.3M $Ba(OH)_2$ and 5% $ZnSo_4$ was added to 8 ml of cell wall extract. The mixture was incubated for 10 min.and after centrifugation, the supernatent was taken. Into the supernatant (2.0 ml), 1.8 ml of distilled water and 6 ml of anthrone reagent (200 mg anthrone powder dissolved in 100 ml of conc.H_2So_4) was added. Then it was kept in boiling water bath for 15 min, cooled and absorbance was noted at 620 nm in a spectrophotometer. Glucose was used as a standard during the estimation of carbohydrate.

3.8.4. SDS - PAGE

3.8.4.1. Gel preparation

Resolving gel and stacking gel was prepared with 10% & 5% acrylamide respectively following the method of Laemmli (1970) as described earlier.

3.8.4.2. Sample preparation

Mycelial wall extracts were first dissolved in a solution of 1% SDS and 1% mercaptoethanol and were heated at 50°C for 2h. Then into the mixture equal volume of 4M urea containg 5% sucrose was added. 50 µl of samples were applied per gel tube and 75 µl per well (in case of slab gel). Along with the samples a protein marker consisting of a mixture of six proteins of molecular weights ranging from 29 to 205 KD was also taken separately, boiled for 1 min in water bath and loaded 20-25 µl per tube well.

3.8.4.3. Electrophoresis

Electrophoresis was performed by applying 1.5 mA current per tube or 0.5 mA per well, until the samples reach the resolving gel and then 2.0 mA of current per tube or 2.5 mA per well was applied for 5h until the dye front reach the bottom of the gel.

3.8.4.4. Fixing

The gels were removed as described earlier and soaked either in fixer solution - I (25% isopropanol in 10% acetic acid) for protein staining or in fixer solution II (40% ethanol in aqueous 5% acetic acid) for carbohydrate staining for 20h in each case.

3.8.4.5. Staining

Gels from fixer solution-I were stained with coomassie blue R250 and then destained as described earlier for protein staining. Gels fixed in fixer solution-II i.e., for carbohydrate staining were stained with periodic acid Schiff's (PAS) reagent as described by Segrest and Jackson (1972) with modifications. Compositions of PAS staining solutions were as follows :

First, solutions A-E were prepared:

Solution A

Anhydrous sodium acetate (0.89g) and hydroxylamine hydrochloride (10.0g) were dissloved in 90 ml distilled water; glacial acetic acid (0.54 ml) was added and the final volume was adjusted to 100 ml with distilled water.

Solution B

Periodic acid (1.0g) and anhydrous sodium acetate (0.82g) were dissolved in distilled water and the final volume was adjusted to 100 ml.

Solution C

1.5g basic fuchsin was dissolved in 500 ml boiling distilled water, filtered at 55°C, cooled to 40°C, 25 ml 2 N HCl was added and finally 3.75g $Na_2S_2O_5$ (sodium metabisulphite) was added., the solution was agitated rapidly, allowed to stand stoppered in refrigerator for 6h. 1.2g charcoal was mixed to it with continuous shaking for 1 min, filtered rapidly and stored stoppered in a refrigerator.

Solution D

10% w/v sodium metabisulphite (5 ml) and 2 N HCl (5 ml) was dissolved in 90 ml distilled before use.

Solution E (prepared before use)

10% (w/v) sodium metabisulphite (5ml) and 2 N HCl (5ml) were dissolved in 90 ml distilled

water and 20 ml glycerol was added to it before use.

Staining procedure

Gels were soaked in solution 'A' for 15 min, then washed under running tap water. Next, the gels were soaked in solution 'B' for 15 min, washed under running tap water and then transferred to solution 'C' (diluted1:1 with distilled water before use) for 5 min. This step was repeated thrice before washing in solution 'D' for 2 min. and finally washed thrice in solution 'E' for 1h each time.

3.8.5. Binding of FITC labelled Concenavalin A

Binding of fluorescein labelled concanavalin-A to mycelia and cell walls (isolated) of fungus was followed as described by Keen & Legrand (1980). Initially, mycelia or isolated cell walls were incubated for 20 min in 0.85% NaCl in 0.01 M potassium phosphate buffer, pH 7.4 containing 1mg/ml fluorescein isothiocyanate (FITC) labelled concanavalin A (ConA, Sigma chemical, USA). Mycelia or walls were then washed three times with saline solution by repeated low speed centrifugation and resuspension. For control sets, these were incubated with lectins supplemented with 0.25M α-methyl mannoside. All these preparations were observed under `Leica' microscope equipped with epi-fluorescence optics (BP-450-490 excitation filter, RKP 520, beam splitting mirror, 515 suppression filter). Photographs were taken by Leica WILD MPS 48 camera on KONICA 400 ASA film.

3.9. Agglutination response of conidia

The test was performed following the method of Lis and Sharon (1986), and Christinzio *et al.*, (1988). Conca avalin A (Con A), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutinin-I (UAE-I) and wheat-germ agglutinin (WGA) were obtained from Sigma, USA. A lectin solution of 1 mg / ml in 50 mM phosphate buffer saline (PBS), pH 7.2, was used for agglutination reactions. Con A solution contained 1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

3.9.1. Preparation of conidial suspension

Ungerminated conidia were used for agglutination tests. These were obtained by growi the fungus on liquid Richard's medium for 10 days at 27°C. Conidia were washed off the mycelial surface with 5ml of sterile distilled water. The resulting suspension was centrifuged at 3500 rpm/min for 15 min at 4°C. The pellet was washed three times with cold PBS and resuspended in the PBS to a concentration of approximately $5x10^6$ / ml. The c onidial suspension was used immediately after preparation.

3.9.2. Agglutination test

Conidial agglutination was performed on microscope slides. Ungerminated conidial suspension (10ml) was incubated with 10ml of lectin solution in a humid chamber at room temparature for incubation. During incubation, slides were gently swirled several times to ensure maximal cellular contact. Agglutinations were examined under a liight microscope and arbitrarily scaled from 'o' (no agglutination) to 4 (maximum agglutination).

3.10. Antisera production

3.10.1. Rabbits and their maintenance

To produce or raise antisera against different fungal and leaf antigens NewZealand white, male rabbits were used. Initially, the body weights were recorded and were observede for at least 1 week inside the cages before giving them injections. They were regularly fed with green grass, 500g each time in the morning and evening. Every alternate day they were also goven 50-75g of gram seeds soaked in water. After each bleeding they were given saline water for 3 consecutine days. Cages were cleaned every day in the morning for better hygenic conditions.

3.10.2. Immunization

Before Immunization, normal sera were collected from each rabbit (2 ml). Separate rabbits were intramascularly injected once a week at 7 days interval either with host or pathogen antigens (1 ml), mixed with 1 ml of Freund's complete adjuvant (Difco, USA), for the first 2 injections, and then followed by injections with incomplete adjuvant (mixed at 1:1 ratio) for 8 to 16 weeks. Antisera were raised against antigen preparations of mycelia and cell wall of *P. theae* (isolate Pt-2) ; mycelia of *G. cingulata* (isolate Gc-1) and *F. oxysporum* (non pathogen of tea) as well as healthy leaf antigens of Teenali 17/1/54 and CP-1.

3.10.3. Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then after every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board after taking them out from the cage, the board was fixed at a 60° angle. The neck of the rabbit was held tight in the triangular gap at the edge of the board, and the body was fixed in such a way that the rabbits could not move during the bleeding. The hairs from the upper side of the ear were removed with the help of a razor and disinfected with rectified spirit. After irritating the ear vein with the application of xylene an incision was made with the help of a sharp sterile blade and 5 ml of blood samples 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. The blood samples were kept as such at 30°C for 1h for clotting. After clotting, the clot was loosened with a sterile needle. Finally, normal sera as well as antisera were clarified by centrifugation (2000g for 10 min at 4°C) and distributed in 1 ml vials for storing at -20°C.

3.11. Purification of IgG

3.11.1 Precipitation

IgG was purified as described by Clausen (1988). The immune serum (5 ml) was first diluted with two volume of distilled water and an equal volume of 4.0 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22°C. The precipitate thus formed was collected by centrifugation at 10,000g at 22°C for 1h. Then the precipitate was dissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 8.0

3.11.2. Column preparation

DEAE Sephadex (Sigma Co. USA) 10g was suspended in distilled water for overnight. The water was poured off and the gel was suspended in 0.005 M phosphate buffer, pH 8.0 and the buffer washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, pH 8.0 and was applied to a column 2.6 cm in diameter, 30 cm high and allowed to settle for 2 h. After the column material had settled, 25 ml of buffer (0.02 M sodium phosphate, pH 8.0) washing was given to the gel material.

3.11.3. Fraction collection

At the top of the column, 1 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.30 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer pH 8.0 (diluted from a 0.10 M sodium phosphate buffer pH 8.0 containing 16.86 g Na_2HPO_4, $2H_2O$ + 0.731g NaH_2PO_4 ; H_2O/L) The final elution buffer (2) was 0.30 M sodium phosphate buffer pH 8.0.

The buffer 1 was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer 1 was connected to another flask with buffer (2). Buffer 2 had also connection to the open air. During the draining of buffer 1 to column, buffer 2 is sucked into buffer 1 thereby producing a continuous rise in molarity. Ultimately 40 x 5 ml fractions were collected and the optical density values were recorded by means of a UV spectrophotometer at 280 nm.

3.12. Immunodiffusion tests

3.12.1. Preparation of agarose slides

The glass slides (5 cm x 5 cm) were degreased in 90% (v/v) ethanol; ethanol: diethylether (1:1) and then dried in hot air oven. After drying, plates were sterilized. Agarose gel was prepared in Tris barbiturate buffer, pH 8.6, at 90°C ,0.9% agarose (Sigma, USA) was added into the buffer placed on a water bath and stirred until the agarose solution became clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added. For gel preparation, 5ml of molten agarose was used per slide ; after pouring, it was kept in 4°C for 1h for solidification and then wells were cut (4 mm dia.).

3.12.2. Immunodiffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50ml/well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48 - 72h at 25°C.

3.12.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water for 2h 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 antiserum widely dispersed in the agarose. Then slides were stained with 0.5% amido black (0.5g amido black, 5g $HgCl_2$, 5 ml glacial acetic acid, distilled water 95 ml) for 10 min at room temperature. After staining, slides were destained with 2% (v/v) acetic acid solution 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.

3.13. Immunoelectrophoresis

3.13.1. Preparation of agarose slides

The slides (7.5 x 2.5 cm) were first degreased with `Extran' (E. merck) solution, then dried and sterilized. Then 2 mm thick uniform agarose layer was prepared (0.9% agarose, 0.1% sodium azide, dissolved in 0.05 M barbital buffer, pH-8.6) on each slide carefully so that there were no air bubble in the agarose gel which may cause asymmetrical migration and diffusion. After solidification slides were stored at 4°C until used.

3.13.2. Electrophoresis

Two central wells were cut (3 mm dia) on the agarose film. Excess water from the wells were soaked out with a blotting paper. Slides were placed in the middle compartment of the electrophoretic chamber. The anode and cathode chambers were filled with 0.05 M barbital buffer, pH-8.6. Wells were loaded with 40-50 µl of antigens. Filter paper strips (Whatman) were soaked in buffer and were placed touching both ends of the agarose film. These strips connected the buffer solutions in anode & cathode chambers with agarose surfaces. An electric current was passed through (2.5 mA/slide ; 10 v/cm) the slides for 3h at 4°C. After electrophoresis, current was stopped and strips were removed.

3.13.3. Diffusion

A longitudinal trough parallel to the long axis of the slide was cut in the agarose gels in between the two wells and undiluted antiserum was loaded (400 µl) into trough. Diffusion was allowed to continue in a moist chamber for 48-72h at 25°C.

3.13.4. Washing, drying and staining

After immunodiffusion, all the slides were washed, stained and destained as mentioned in case of immunodiffusion. Slides were dried in hot air oven for 3h at 50°C.

3.14. Rocket immunoelectrophoresis

3.14.1. Preparation of slides

Initially the slides (7.5 x 2.5 cm) were washed with 'Extran' (E. merck) and then degreased with ethanol : diethyl ether (1:1 v/v) solution and then dried and sterilized as mentioned before. To 10 ml of liquid agarose medium (1% agarose, 0.1% NaN_3 dissolved in 0.05 M barbital buffer pH 8.6) , 1 ml of undiluted antiserum was added at 50°C (Clausen, 1988) and mixed thoroughly. Then this medium was poured on each slide to form a unifrom 2 mm agarose gel taking care that no air bubble is formed. After pouring, slides were kept at 4°C for solidification until use.

3.14.2. Electrophoresis

Two to three wells (3 mm dia) were cut out from one end of the agarose gel and then slides were placed in electrophoretic chamber. 50-60 µl of antigens were loaded in each well. Then electrophoresis was performed for 4h at 4°C (2.5 mA/slide, 10 v/cm) by connecting the two ends of agarose gels with buffer soaked filter paper as mentioned earlier. After electrophoresis, paper strips were removed and the current was discontinued.

3.14.3. Washing, staining and drying of slides

After immunoelectrophoresis, slides were first washed with sterile distilled water and then with saline solution (0.9% NaCl & 0.1% NaN_2) for 48h with 6 hourly changes to remove unreacted antigen and antisera widely dispersed in the agarose gel. After washing rocket shaped sharp precipitin arcs were found. Slides were stained with 0.5% amido black solution (0.5g amido black, 5h $HgCl_2$, 5 ml glacial acetic acid, 95 ml distilled water) for 10 min. Then the slides were destained [2% (v/v) acetic acid] until the background became clear and arcs became prominant. Finally, slides were washed with distilled water and dried at 50°C for 3h.

3.15. Enzyme linked immunosorbent assay

For different types of ELISA following buffers were first prepared as described by Chakraborty *et al.*, (1995).

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

   Stocks

   A: Sodium Carbonate = 5.2995 g
   Distilled water = 1000 ml.

   B: Sodium bicarbonate = 4.2 g
   Distilled water = 1000 ml.

   With 160 ml of stock sodium A, 340 ml of stock solution 'B' was mixed and the pH of the resulting solution was adjusted to 9.6.

2. Phosphate Buffer Saline (0.05 M PBS, pH 7.2).

   Stock

   A: Sodium dihydrogen phosphate = 23.40 g
   Distilled water = 1000 ml.

   B: Disodium hydrogen phosphate = 21.2940 g
   Distilled water = 1000 ml.

   280 ml of stock solution A was mixed with 720 ml of stock solution B & pH of the resulting solution was adjusted to pH 7.2 and 0.8% NaCl and 0.02% KCl were added.

3. 0.15(M) Phosphate buffer saline - Tween (0.15 M PBS - Tween) pH 7.2.

   In 0.15 M Phosphate buffer saline 0.05% of Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking agent (Tris buffer saline, pH 8.0 , 0.05 M Tris, 0.138 M NaCl, 0.0027M KCl)

Tris = 0.657 g
NaCl = 0.81 g
KCl = 0.223 g.

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

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

In 0.15 M phosphate buffer saline - Tween, pH 7.2 ,0.2% bovine serum albumin (BSA) , 0.02% Polyvinyl polypyrrolidone, 10,000 (PVPP , 10,000) and 0.03% sodium azide were added.

6. Substrate

Sigma Fast pNPP substrate tablet sets were used. Each tablet set yields the following when dissolved in 20 ml distilled water.

PNPP = 1.0 mg/ml
Tris buffer = 0.2 M.

7. Stop solution

0.3 N NaOH solution was used as stop solution.

3.15.1. Indirect ELISA

Direct antigen coated (DAC) ELISA

DAC ELISA was performed following the method of Chakraborty *et al.*, (1995). Plant and /or fungal antigens were diluted in coating buffer and the diluted antigens were loaded 200 µl /well into NunC 96 well-ELISA plates. The plates were incubated at 25°C for 4h. The plates were then washed four times under running tapwater and once with PBS-Tween and each time, plates were shaken dry. Subsequently , for blocking the unbound sites 200 µl/well of blocking agent was added and the plates were incubated at 25°C for 1h. After incubation, plates were washed five times as before. Purified antisera suitably diluted in antisera dilution buffer, were loaded 200 µl/well and incubated overnight at 4°C. The plates were again washed fine times as before and dried. After drying , 200 µl of diluted alkaline phosphatase conjugate anti rabbit IgG whole molecule (Sigma Chemicals, USA) enzyme was added to each well and incubated at 37°C for 2h. After a further washing and drying, 200 µl of p-Nitrophenyl Phosphate substrate was added into each well and

incubated in dark for 30 - 45 min. at room temperature. Colour development was stopped by adding 50 µl/well of 3N NaOH solution and absorbance were determined at 405 nm in an ELISA reader (Cambridge Tech. USA). Absorbance values in wells not coated with antigens were considered as blanks.

3.15.2. Direct ELISA

Double antibody sandwich (DAS) ELISA

Conjugation of alkaline phosphatase with γ - globulin

The gluteraldehyde method was performed by a two step procedure. At first , 5 mg of alkaline phosphatase enzyme crystals (Sigma) was dissolved in 1 ml half strength phosphate buffer saline (PBS-pH 7.2). Then 25 µl of 2.5% glutaraldehyde was added and incubated at room temperature for 4h with occasional shaking. After incubation, it was dialysed against half strength PBS, with three changes of 30 min each. After the removal of excess glutaraldehyde by dialysis, 1 ml of purified IgG of *P.theae* was added and incubated overnight at 4°C. After incubation, 20 mg of BSA was added and stored at 4°C until required

Assay :

DAS ELISA was performed following the method of Brill *et al.,* (1994). Rabbit IgG was diluted in coating buffer and loaded in wells (200 µl/well) of a 96 well ELISA plate. The plate was incubated at 37°C for 4h and washed 5 times by flooding the wells with PBS-Tween. Into the empty wells 200 µl of test samples were added and incubated at 4°C overnight. After incubation, wells were washed as before and 200 µl/well of alkaline phosphatase tagged rabbit IgG diluted in PBS , pH 7.2 was added and incubated at 25°C for 6h. Then the plates were washed five times as before and 200 µl pNPP substrate (0.3 mg/ml) was added to each well and incubated at 25°C for 30 min in dark. Colour development was stopped by adding 50 µl/well of 3N NaOH solution. Absorbance values were recorded as before.

3.15.3. Competition ELISA

Competition ELISA was performed following the method of Lyons and White (1992). For this purpose two 96 well ELISA plates (Nunc, Maxisorp, Sweden), were taken first. Into all the 96 wells of one plate, 100 µl of test antigen of one fungus was loaded and all the 96 wells of the other plate was loaded with antigen of other fungus (100 µg/well). All the antigens were diluted in 0.15 M PBS. Plates were incubated at 4°C overnight , then washed five times under running tap water and once with PBS. Between this and all subsequent steps, the plates were inverted and slapped downwards onto absorbent paper towelling to ensure the plates were completely emptied. Non specific binding sites were blocked with 5% BSA in PBS , 200 µl/well for 1h. The plates were washed and dried as before and stored at 4°C until required.

For the test, a doubling dilution series was prepared from which 75 µl of leaf extracts (healthy, naturally infected and artificially infected) in PBS were loaded in the blank plates using the same paired well format of the test plates. To each well, 75 µl of antiserum (IgG) raised against any one fungus, diluted 1:125 with PBS were added. The plates were incubated at 37°C for 1h on a shaker. At the end of incubation, 100 µl of the leaf extract-antiserum muxture was transferred to the corresponding wells on the pre-prepared test plates. Unreacted antiserum was added to control wells and the platees were incubated for 45 min at 37°C by constant shaking. The plates were washed after incubation and 100 µl of antirabbit IgG alkaline phosphatase conjugated antiserum, (diluted 1:10,000) , was added to all the wells of the plates. Following 30 min. incubation at 37°C on a shaker, plates were washed , dried and 100 µl of p-nitrophenyl phosphate substrate (1mg/ml) was added to each well. After a further 30 min. incubation in the dark without shaking, the color reaction was stopped using 3N NaOH solution (50 µl/well) and absorbance values were recorded at 405 nm in an ELISA reader (Cambridge Tech. USA).

3.16. Estimation of fungal mycelium in leaf tissue

For the estimation of fungal mycelium in leaf tissues, the method of Beckman et al., (1994) was followed with modifications. Healthy tea leaf extracts were prepared and varying concentrations of mycelia of *P. theae* (4-20 mg fresh weight) were added to these extracts. Indirect ELISA was performed with these extracts and the absorbance values were noted at 405 nm as described previously. Absorbance values of the healthy extracts were subtracted from those of the extracts containing the mycelia. Standard curve of mycelial fresh weight vs-absorbance was prepared. While estimating the amount of fungal mycelium in infected leaf extracts, the difference in absorbance between healthy and infected leaf extracts were plotted in the standard curve and the value determined. Amount of fresh weight of mycelium per gm. leaf tissue was finally calculated.

3.17. Establishment of callus

3.17.1. Culture media

For the initiation of callus MS basal medium was used (Murashige and Skoog 1962). Following stocks were prepared first.

MS - 1 (20X)

(a) KNO_3 - 38.0 g
NH_4NO_3 - 33.0 g
$MgSO_4, 7H_2O$ - 7.4 g
KH_2PO_4 - 3.4 g
Double distilled water - 1 L

(b) $CaCl_2,2H_2O$ - 8.82 g.

Double distilled water- 500 ml.

Solution a and b were mixed and the volume was adjusted to 2 L with double distilled water and stored at 4°C.

MS - II (100x).

$MnSO_4, 4H_2O$	- 2.23 g
$ZnSO_4, 7H_2O$	- 8.60 mg.
H_3BO_3	- 620 mg.
KI	- 83 mg.
$Na_2MO_4, 2H_2O$	- 25 mg.
$CuSO_4, 5H_2O$	- 2.5 mg.
$CoCl_2, 6H_2O$	- 2.5 mg.

Dissolved in IL double distilled water, stored at 4°C

MS - III (20x)

$Na_2EDTA, 2H_2O$ - 746 mg

Dissolved in 80 ml of near boiling double distilled water.

$FeSO_4, 7H_2O$ - 556 mg

Dissolved in 80 ml double distilled water.

$FeSO_4, 7H_2O$ solution was added to Na_2EDTA solution with vigorous stirring ans volume was adjusted to 200 ml with double distilled water stored at 4°C.

MS - IV (100x)

Myo - inositol - 100mg.

Thiamine HCl - 0.5 mg.

Nicotinic acid - 0.5 mg.

Pyridoxine HCl - 0.5 mg.

Glycine - 2 mg.

Dissolved in 10 ml of double distilled water.

MS - I, II, III, IV were mixed together in the following ratio :

MS - I	100 ml
MS - II	10 ml
MS - III	10 ml
MS - IV	10 ml.

Then the medium was supplemented with 3% sucrose, 0.8% agar, 2 mg/L IBA and 4 mg/L BA (Kato 1989). pH was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving and then autoclaved at 121°C (15 lb/in^2 for 30 min. after distributing the media in flasks and culture tubes. For the prevention of browning of tissue in the medium following substances were also used before autoclaving

(a) Activated charcoal - 100 mg/L
(b) Ascorbic acid - 150 mg/L
(c) Ca-pantothenate - 200 mg/L

3.17.2. Preparation of fragile callus

Young shoots (with 3-4 leaves) were taken from plants grown under glass house conditions. Stem segments each 2-3 mm long were cut and sampled. For the prevention of browing explants were pre-soaked in cold sterile distilled water before surface sterilization for 10 min. and then under running tap water for 20 min. For surface sterilization, stem segments were dipped in 2% sodium hypochlorite solution for 5 min and washed five times with sterile distilled water for the removal of excess hypochlorite. After final washing explants were transferred asephtically into semi-solid media contained in flasks and culture tubes. They were incubated at 26°C under 16h photoperiod and observed regularly.

The fragile callus thus obtained were transfered to sterile liquid MS media and shaken gently. Loosened cells were obtained and these were used for further studies.

3.18. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of cross sections of tea leaves, fungal spores and mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994*b*).

3.18.1. Loosened cells

Loosened cells were allowed to grow in liquid MS medium with shaking for a period of 10 days. For fluorescent staining cells were taken in microcentrifuge tubes with a sterile pipette and washed once with PBS (pH 7.2) by centrifugation at slow speed. Diluted (1:125) antiserum or normal serum was added into the washed cells and incubated for 30 min at 27°C. Then cells were washed thrice with PBS Tween, pH 7.2 and incubated for 30 min in dark at 27°C with goat antirabbit IgG conjugated with FITC (Sigma, USA), diluted 1:40 with PBS. After repeted washings cells were mounted in 10% glycerol and observed under UV light and photographed in a wild MPS camera (Leica, Germany) on 400 ASA Konica film.

3.18.2. Cross section of tea leaves

Initially, fresh cross section of tea leaves were cut and immersed in phosphate buffer saline (PBS) pH 7.2 containing 0.8% NaCl and 0.02% KCl. Then good sections were selected and treated with normal serum or antiserum diluted (1:125) with PBS pH 7.2 and incubated for 30 min at 27°C. After incubation, sections were washed thrice with PBS-Tween. pH 7.2 for 15 min, transferred to 100 µl of diluted (1:40) goat antiserum specific to rabbit globulin and conjugated with fluorescein isothiocyanate (FITC). These were incubated for 30 min at 27°C. All operations with FITC labelled antibodies were made in darkness or very low light. After that sections were washed thrice with PBS-Tween, pH 7.2 as mentioned above and then mounted on a grease free slide with 10% glycerol. A cover slip was placed on the section and sealed. Fluorescence of the leaf sections were observed using Leica Leitz Biomed microscope with flourescence optics equipped with ultra violet (UV) filter set I-3.

Tissue sections were photographed under both phase contrast and UV flourescent conditions for comparison of treatment.

3.18.3. Mycelia

Fungal mycelia were grown on liquid Richard's medium as described earlier. After four days of inoculation young mycelia were taken out from the flask and kept in grooved slide. After washing with PBS (pH 7.2) , mycelia were treated with normal sera or antisera diluted (1:125) with PBS , pH 7.2 and incubated for 30 min at 27°C. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with flourescein isothiocyanate (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27°C. After incubation , mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A coverglass was placed on mycelia and sealed. Then slides were observed and photographed as mentioned earlier.

3.18.4. Conidia

Fungal conidia were collected from 15 day old culture and a suspension of this was prepared with PBS, pH 7.2. Then conidial suspensions were taken in micro centrifuge tubes and centrifuged at 3000 g for 10 min and the PBS supernatant was discarded. 200 µl of diluted in PBS - pH 7.2) antisera (1:125) was added into the microcentrifuge tube and incubated for 2 h at 27°C. After incubation, tubes were centrifuged at 3000 g for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween pH 7.2 by centrifugation as before and 200 µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26°C for 1 h. After the dark incubation, excess FITC antisera was removed by repeated washing with PBS-Tween pH 7.2 and the spores were mounted on glycerol jelly and observed under Leica microscope, equipped with I-3 UV flourescence filter. Photographs were taken as described before.