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 (c) United Planter's Association of South India(UPASI) Tea Research Station, Valparai, Tamilnadu.

Thirteen TV clones (TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-29, TV-30, TS-449 and Teenali 17/1/54) released by Tocklai Experimental Station were collected from the clone house of Mohurgaon and Gulma Tea Estate, Sukna, W.Bengal.

Fresh clonal cuttings of 15 Darjeeling vatrieties (BS/7A/76, B668, P312, B777, T-78, P-1258, B-157, TTV_1, CP-1, BT-15, AV-2, RR-17, HV39, K1/1 and T-135) generally grown in hilly regions were collected from Darjeeling Tea Research Centre, Kurseong, W.Bengal.

Cuttings of 6 clonal varieties (UPASI 2, UPASI 3, UPASI 8, UPASI 9, UPASI 17 and UPASI 26) and seeds of 3 varieties (BSS1, 2 and 3) were collected from UPASI Tea Research Station, Valparai, Tamilnadu.

3.1.2. Propagation

Tea plants are usually propagated either by cuttings or by seeds.

3.1.2.1 Cutting

The 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 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°C to 80°C on a metal sheet with fire below to kill eelworms, if any, present in the soil.

Polythene sleeves (6") were filled up with the prepared soil and stacked in rows in a bed and then watered thoroughly. All cuttings were allowed for rooting in sleeves after dipping them in hormone obtained from UPASI Tea Research Centre mixture. These cuttings were kept in a polythene cloche and watered every 4th day, until new leaves were appeared. (Plate II, figs. A-D).

3.1.2.2 Seed germination

For the seed germination, pre treated (heat treated) seived sand was used to prepare beds of about 15 cm thickness. Sand in the bed was slightly compacted and watered thoroughly prior to putting out the seed. The seeds were treated with a 0.03% suspension of Dithane M45 and then sown in rows at a spacing of 5.0x5.0 cm triangle. Seeds were pressed down in the bed with the sear directed downwards; the dome of the seed was projected slightly above the level of the bed. The seed beds were then covered with a thick polythene sheet and watered when necessary.

At the "cracking stage" (between 25 and 35 days) the seeds were transplanted into the sleeves. Then all these sleeves were covered by polythene cloch and watered as and when necessary. At the four leaved stage the cloch was removed.(Plate III, figs. A-D)

3..1.3. Plantation

Before planting, simazine @ 75g/20 litre water and Glyphospate @ 1:200 were used in the experimental plots for the suppression of weeds (Borpujari & Banerjee; 1994). Then pits ($1^1/_2$' x $1^1/_2$' x $1^1/_2$') were dug at the intervals of 2 ' between plant and 3.5' between row to row. Planting mixture was prepared in the ratio of 4.5 kg well rotten dry cattle manure , 30 g rock phosphate, 30g super phosphate and 2.5 g phorate [0,0- diethyl S- (ethylthiomethyl) phosphoro dithioate]. At the botom 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 5 cm below the ground level.

Following soil conditioning, plants were inspected, selected and brought to the experimental garden and planted in the prepared soil. Pits were refilled with conditioned soil.

Tea plants of all the varieties were also grown in earthen pots (one plant/pot 30 cm dia) each containing 5 kg soil mixture (soil: planting mixture - 1:1). All these plants were maintained inside the Glass house under natural condition. (Plate IV, figs. A-D; Plate-V, fig. A-D)

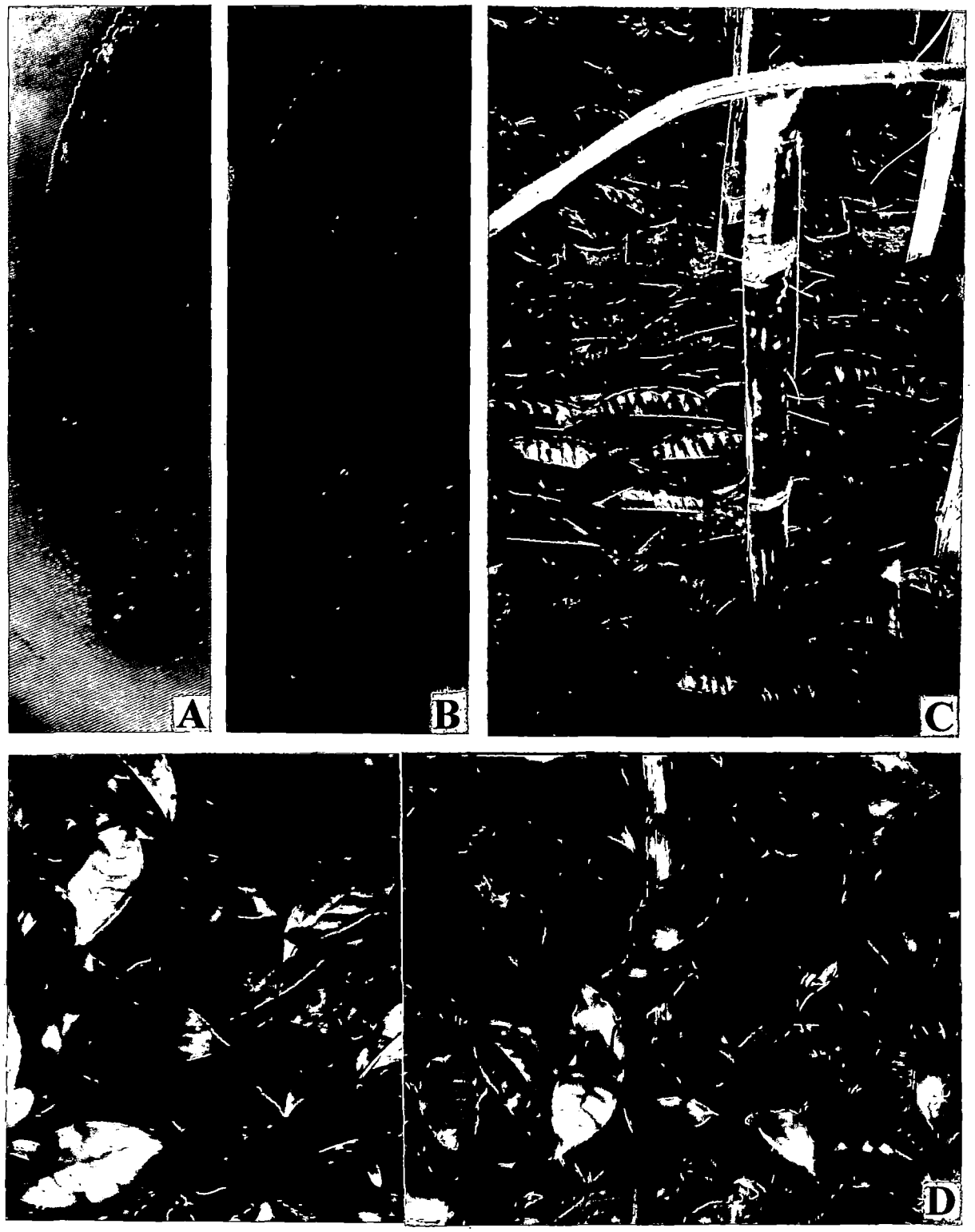

Plate II(figs. A-D) . Stages in propagation of tea (UPASI 9) by cuttings .
A- nodal cutting with excess callus ; **B**-nodal cutting after rooting ;
C- cuttings in plastic sleeves ; **D**-shoot development from cutting

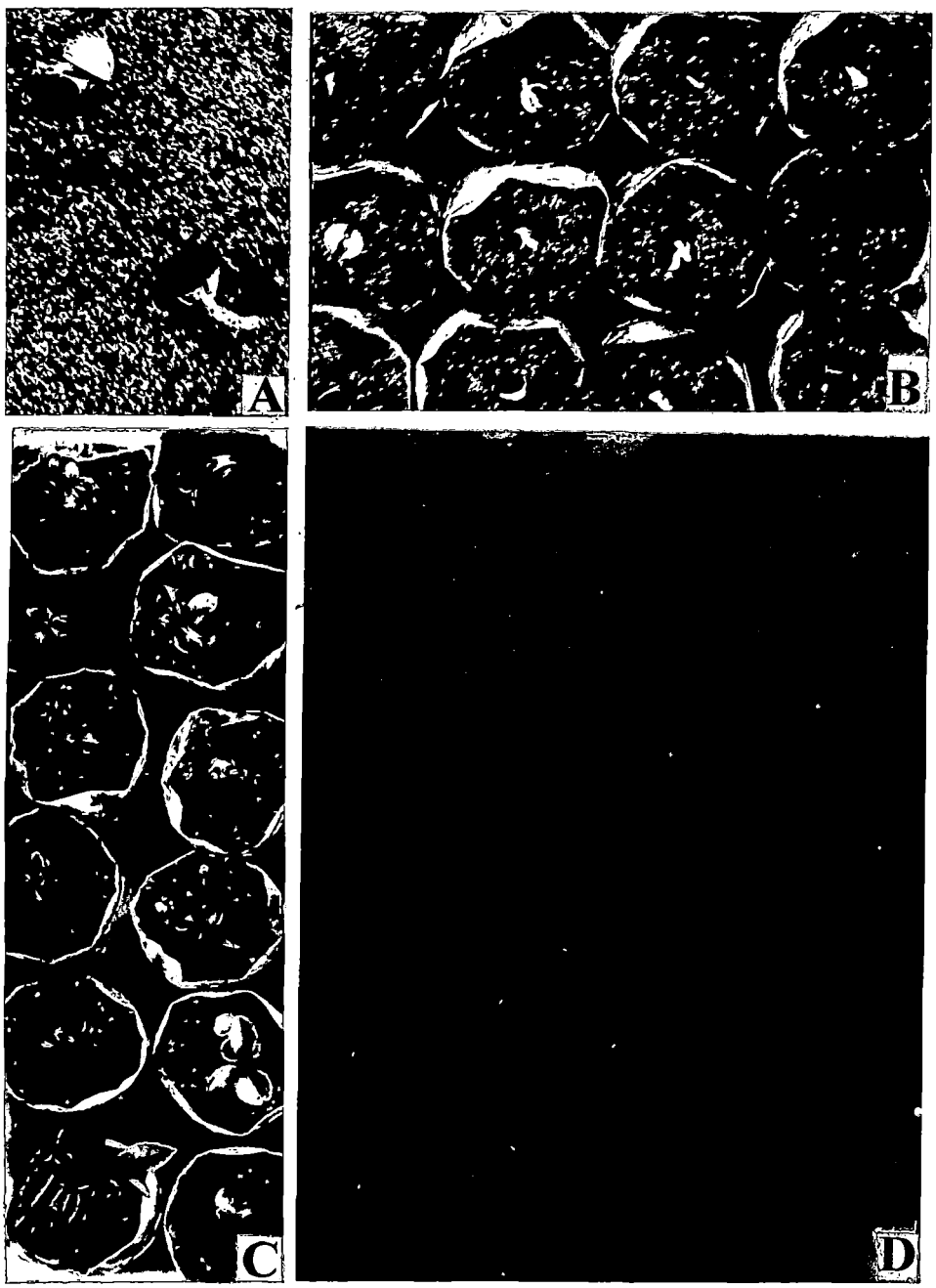

Plate III (figs. A-D). *Stages in propagation of tea by seed germination (BSS-3).* **A** - seed cracking in the bed ; **B** - seeds after cracking transferred to polythene sleeeves; **C**- seedlings (3- 4 leaved stage) ; **D**-seedling after being transferred to pot

PLATE IV (figs.A-D). Cuttings of tea in plastic sleeves **(A&B)** and after transfer to the field **(C&D)**.
A&C - UPASI 26 ; **B&D** - UPASI 9

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

3.1.4. Maintenance

In case of young plants manuring (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.) was done after rooting following the method by Ranganathan and Natesan (1987) and continued upto 12 months once only in 15 days. The mixture was dissolved @ 30 g in 1 L of water and applied @ 50 ml/plant.

The mature plants (1 year & above) were maintained by applying a soluble mixture of N,P,K consisting of 10 kg Urea-46% N, 20 kg ammonium phosphate- 11% P_2O_5, 8 kg muriate of potash-60% K_2O in the soil. Grenol (Triacontanol) was sprayed at regular intervals for good growth of bush.

Only tipping was done once to promote lateral branching in young plants (3 years) but in case of mature plants 2-year deep pruning cycle was maintained.

Besides three other plants 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 Institure for Semi Arid Tropics (ICRISAT), Hyderabad respectively, while *C.japonicum* plants were obtained from the Botanical Garden, Darjeeling.

3.2 Fungal culture

3.2.1 Source of culture

Glomerella cingulata (Stoneman) Spauld & Schrenk isolated from naturally infected tea plants (TV-18) was identified at the International Mycological Institute, U.K., and was designated as GC-1 (IMI number-356806). Three more isolates of *G.cingulata* (GC-2,3 and 4) were collected from naturally infected leaves of TV-23, 27 and Teenali 17/1/54 respectively. Two other isolates (GC-5 and 6) were collected from naturally infected leaves of TV-9 of Chandmani Tea Estate. From Tirrihana Tea Estate, GC-7 was collected. GC-8 and GC-9 was collected from naturally infected leaves of UPASI-3.

Besides this a non-pathogen of tea, *Fusarium oxyporum* Schlecht (ITC number -2389) was obtained from Indian Type Culture Collection Centre, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. Five different species of *Colletotrichum* were also collected from IARI, New Delhi and used in different experiments.(Table-1)

Table 1: List of species of *Colletotrichum* and their isolates used.

Organism	ITCC number	Host
Colletotrichum gloesporioides	1726	*Musa* sp.
Colletotrichum gloesporioides	1809	*Capsicum annuum*
Colletotrichum papayae	1269	*Carica papaya*
Colletotrichum lindemuthianum	1119	*Phaseolus vulgaris*
Colletotrichum lindemuthianum	1764	*Phaseolus vulgaris*

A virulent strain of *Pestalotiopsis theae* Sawada (IMI No. 356807). and *Corticium invisum* Petch was collected from culture collection of Mycology and Plant Pathology Laboratory of the Department

3.2.2. Completion of Koch's postulate

Fresh, young tea leaves were collected from Phytopathological Experimental Garden and inoculated with conidial suspension of the isolated *Glomerella cingulata* following detached leaf inoculation technique. After 96h of inoculation, the infected tea leaves were washed thoroughly, cut into small pieces, disinfected with 0.1%. $Hgcl_2$ solution for 3-5 min washed several times with sterile distilled water and transferred aseptically into Richard's medium (RM) slants. These isolates were examined after 15 days of inoculation at 30°C and the identity of the organism was confirmed by comparing with the stock culture.

3.2.3. Maintenance of Stock Culture

The fungus thus obtained was sub-cultured on RMA (Richard's Medium Agar) slants. After two weeks the culture was stored under three different conditions (5°C, 20°C and at room temperature - 30± 2°C). Apart from weekly transfer for experimental work, all the isolates of *Glomerella cingulata* were also examined at regular intervals to test their pathogenicity.

3.2.4. Assessment of Mycelial Growth

3.2.4.1. Solid media

To assess mycelial growth of *G. cingulata* (GCI to GC 9) in solid media, the fungus was first grown in petridishes, each containing 20 ml of RM and incubated for 7 days at 30°C. Agar block (4mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. Following solid media were used for assessment of growth.

Potato dextrose agar (PDA)

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

Richards medium (RM)

KNO_3	-	1.00g
K_2HPO_4	-	0.50g
$MgSO_4, 7H_2O$	-	0.25g
$FeCl_3$	-	0.002g
Sucrose	-	3.00g
Agar	-	2.00g
Distilled water	-	100 ml

Carrot Juice Agar (CJA)

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

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	-	0.001g
Agar	-	3.00g
Distilled water	-	100 ml

Flentze's soil extract agar (FSEA)

Soil extract	-	1L
Sucrose	-	1.00g
KH_2PO_4	-	0.20g
Dried yeast	-	0.10g
Agar	-	25.00g

All these petridishes were then incubated at 30°C for the desired period. Finally the mycelia were strained through muslin cloth, collected in aluminium foil of known weight , dried at 60°C for 96h , cooled in a desiccator and weighed.

3.2.4.2. Liquid media

To assess the mycelial growth of *G. cingulata* (GC-1) in liquid medium, the fungus, was first allowed to grow in petridishes containing 20 ml of RMA and were incubated at 30°C for 7 days. From the advancing zone, the mycelial block (4mm-dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50 ml of sterilized Richards medium for the desired period at 30°C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60°C for 96h , cooled in a desiccator and weighed.

3.3. Inoculation techniques

3.3.1. Detached leaf

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded tea leaves were detached form plants were placed in plastic trays lined with moist blotting paper. Wounds were made on the adaxial surface of each leaf and inoculated with 20ml droplets of spores suspensions (1.2 x 10^6 conidia/ml) of the fungus (prepared from 10-day old culture). Spore suspensions were placed (2-4 drops/leaf) on the adaxial surface of each leaf with a hypodermic syringe on the wounds. In control sets drops of sterile distilled water were placed on the leaves.

Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize the drying of drops during inoculation.

3.3.2. Cut Shoot

Cut shoot inoculation technique was followed as described by Yanase and Takeda (1987). Twigs (with 3-4 leaves) of tea plant grown in the Experimental garden were cut carefully and immediately introduced into glass vials containing sterile tap water and taken to the laboratory. Leaves were inoculated by making 2 mm light scratch with a sharp sterilized needle on the adaxial surface of the leaf (Cook, 1989) and placing mycelial plugs on the scratches. Mycelial plug inoculated cut shoots were inserted into the holes of styrofoam board which was floated on modified Hoagland and Knops solution and kept in a glass chamber (72cmx33 cmx 30 cm) for one week with aeration.

3.4. Assessment of disease intensity

3.4.1. Detached leaf

Percent drops that resulted in lesion production was calculated after 24, 48, 72 and 96 h of inoculation as described by Chakraborty and Saha (1994 a), diameter of the lesion were noted. Observations were based on 50 inoculated leaves for each treatment and average of three separate experiments.

3.4.2. Cut shoot

In laboratory experiments, the actual number of lesion that developed on the artificially inoculated shoots were counted after 24, 48, 72 and 96h. Diameter of the individual lesions were measured. They were graded into 4 groups and a value was assigned to each group, Very small restricted lesion, 1-2 mm dia. = 0.1; 2-4 mm dia. with sharply defined margin=0.25; lesion,with slow spread beyond 4mm=0.5 and spreading lesion,variable in size with diffused margin = 1.0. Number of lesion, in each group was multiplied by the value assigned to it and the sum total of such values were noted and disease index were computed as the mean of observation of 50 cut shoot per treatment.

3.5 Preparation of antigen

3.5.1. Fungal antigen

3.5.1.1. Mycelia Mycelial antigen was prepared following the method of Chakraborty & Saha (1994). Initially the fungal mycelium (4mm disc) were transferred to 250 ml Ehrlenmeyer flask each containing 50 ml of 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) and incubated for 10 days at 30 $\pm$ 1°C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (50 g fresh wt.), were homogenized with 0.05 M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5mM magnesium chloride and 0.85 NaCl in mortar and pestle in the presence of sea sand. Cell homogenates were kept overnight at 4°C and then centrifuged (15000g) for 30 min at 4°C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overight at 4°C. After this period the mixture was centrifuged (15000g) for 30 min at 4°C, the precipitate was dissolved in 10 ml 0.05 M sodium phosphate buffer (pH 7.2). The preparation was dialysed for 72 h. through cellulose tubing (Sigma Chemical Co. USA) against 1 L of 0.005 M sodium phosphate (PH 7.2) with ten changes. Then the dialysed material was stored at -20oC and used as antigen for the proeparation of antiserum and other experiments.

3.5.1.2. Cell Wall

Isolation of cell wall was done following the method of Keen & Legrand (1980). Mycelium of 8 day old log phase fungus culture was collected on filter paper in Buchner funnel and 50g of fresh packed cells were ground for 1 min in a high speed blender with water (4 ml/g). The resulting slurry was then disrupted in a homogenizer for 1 min at 5° C. The mixture was centrifuged for 1 min at 1500 g, the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10ml/g) and pelleted by several centrifugations until the supernatant fluids were visually clear. Finally, the isolated cell walls were dissolved in 0.05 M phosphate buffer saline (pH 7.2) and kept at -20° C until further requirement.

5.2 Leaf Antigen

3.5.2.1. Healthy Leaf

Antigens from healthy leaves were prepared following the method of Chakraborty & Saha (1994b). Fresh, young healthy leaves were collected from the experimental garden and kept at 4°C. Then the leaves were weighed & crushed in mortar & pestle with 0.05 M sodium phosphate buffer supplemented with 10 mM sodium metabisulphite, 2 mM PVPP 10,000 (soluble) and 0.5 mM magnesium chloride (pH 7.2). At the time of crushing with sea sand, insoluble PVPP of equal weight was used. The leaf slurry was strained through a muslin cloth and then centrifuged (15,000g) for 30 min at 4°C. The supernatant was used as healthy leaf antigen and was kept at -20°C until required.

3.5.2.2. Artificially inoculated leaf

Antigen from the *G.cingulata* inoculated leaves were extracted following the method of Alba & DeVay (1985) with modification. Fresh, young leaves (first, second and third) were collected from the experimental garden and kept in plastic trays as described in detached leaf inoculation technique. Leaves were inoculated with drops of conidial suspension (1.2 x 10^6 spores) of *G.cingulata.* Control sets were prepared by mounting the leaves with drops of sterile distilled water. Antigens were prepared from inoculated leaves as described earlier. The prepared antigens were stored at -20°C until further experimental purposes.

3.5.2.3. Naturally infected leaf

For the extraction of naturally infected leaf antigen, the infected leaves were collected from the experimental garden and kept at 4°C. Then the infected portion of leaf was cut into small pieces, weighed and antigens were prepared as before.

3.6. Purification of mycelial antigen

3.6.1. Saturated ammonium sulphate fractionation

Freshly harvested mycelium (150g) of *G.cingulata* was crushed in a mortar with pestle at 4°C using sea sand and homogenized with 150 ml of 0.1(M) Tris HCl buffer (pH -7.0). The slurry was strained through muslin cloth and the filtrate was centrifuged at 15,000 g for 30 min. Finally into the supernatant finely ground ammonium sulphate crystals were added slowly with constant stirring at 4°C. Final saturations of ammonium sulphate of 20,40,60,80 and 100% were obtained. In each case, stirring was done for 6h and then it was kept overnight at 4°C for precipitation. After centrifugation at 15000g for 1h the precipitates were dissolved in 2-3ml of 0.1 M Tris-HCl buffer, pH 7.0, for each saturation level. Dissolved precipitates were then dialysed by using cellulose dialysing tubing (Sigma Co., U.S.A.) against 0.01M Tris-HCl buffer, pH-7.0 at 4°C for 72h with 6 hourly changes.

3.6.2. Ion exchange Chromatography

Ammonium sulphate precipitate of mycelial extract of *G.cingulata* was also purified by ion exchange chromatography following the method as described by Chard et. al (1995). Here ammonium sulphate precipitate of desired saturation was resuspended in 0.05M Tris-HCl buffer, pH 8.0, dialysed against this buffer for 72h with 6 hourly changes and then applied to an ion exchange column. The column material, DEAE Sephadex was first suspended in distilled water for 24h, then resuspended in 0.05M Tris-HCl, pH 8.0 and equilibrated in the same buffer after loading the column material into a 10ml column. 2ml of protein sample was loaded and the column was washed with 0.05M Tris-HCl buffer, pH 8.0, until no proteins were detected in the eluant. A sodium chloride

wash was applied (0.05 M NaCl in 0.05 M Tris-HCl , pH-8.0) until no proteins were detected and then was followed by a sodium chloride gradient (0.05-0.25M NaCl in 0.05 M Tris-HCl, pH-8.0). In each case, 20-25 fractions (3 ml) were collected from the column and analysed for their protein content (Optical density at 280 nm).

3.7. SDS-Polyacrylamide Gel Electrophoresis of Protein

3.7.1. Preparation of Gel (Tube & Slab)

Stock Solutions:

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970).

(A) Acrylamide

Acrylamide	... 30 g
Bis-acrylamide	... 0.8 g
Distilled water	... 100 ml.

(filtered and stored at 4^0C).

(B) Lower gel buffer (LGB)

(1.5 M Tris)

Tris	... 18.18 g
Distilled water	... 100.0 ml

pH was adjusted to 8.8

Sodium dodecyl sulphate (SDS) .. 0.4 g

(C) Upper gel buffer (UGB)

(0.5 M Tris)

Tris	... 6.06 g
Distilled water	... 100.0 ml

pH was adjusted to 6.8

Sodium dodecyl sulphate(SDS)... 0.4 g.

(D) Ammonium peroxidisulphate (APS)

Ammonium Peroxidisulphate (APS)	... 0.1 g
Distilled water	... 1.0 ml
(freshly prepared each time)	

Tube gel preparation

The tubes (12 cm long and 4 mm dia) were washed thoroughly, dried and rewashed with diethyl ether. Resolving gel solution was first prepared with acrylamide and lower gel buffer, degassed for about 20 min and then TEMED and APS solution were added. The composition of the solution was as follows :

Solution A	... 10.0 ml
Solution B	... 7.5 ml
Distilled water	... 12.5 ml
TEMED	... 12 μl
Solution D	... 36.0 μl

Finally the gel was cast slowly upto a height of 9 cm in a tube, overlayered with water and left for polymerization overnight. After polymerization water overlay was blotted off by blotting paper and washed with distilled water 2-3 times. Stacking gel solution was then prepared with acrylamide solution and upper gel buffer, degassed for about 20 min, TEMED and APS solution 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	... 500.0 μl

Stacking gel solution was cast similarly as before upto a height of 1.5 cm with the help of Pasteur pipette and then overlayed with water. Finally, the gel tubes were kept 30 min for polymerization.

Slab gel preparation

For slab gel preparation, two glass plates (17 cm x 19 cm) were washed with dehydrated alcohol & dried. Then 1 mm thick spacers were placed between the glass plates at the 2 edges and the 2 sides of glass plates were sealed with gel sealing tape and kept in the gel casting unit. Resolving gel solution was prepared as described in the tube gel and cast very slowly and carefully upto a height of 12 cm by a syringe. The gel was overlayered with water and kept overnight for polymerization. Then stacking gel solution was prepared as mentioned above. After polymerization of resolving gel, water overlay was decanted off and of a 13 well 1mm thick comb was placed. Stacking gel solution was poured carefully upto a height of 4 cm over the resolving gel and overlayered with water. Finally the gel was kept for 30 min for polymerization.

3.7.2. Sample Preparation

Sample was prepared by mixing the sample protein with sample buffer whose composition was as follows:

Solution B	:	12.5 ml
Sodium dodecyl sulphate (SDS)	:	2.3g
Glycerol	:	13.0 g
β-mercaptoethanol	:	5.0 ml
Distilled water	:	100.0 ml
Bromophenol blue	:	0.005 g

First of all, 50 μl of each sample protein was taken in each eppendrof tube and 20 μl of sample buffer was mixed in each tube. All the tubes were floated in boiling water bath for 3 min. After cooling 50 μl of samples were applied per gel tube/per well in case of slab gel. Along with the samples, protein marker consisting of a mixture of six proteins (carbonic anyhydrase, egg albumin, bovine albumin, phosphorylase b, β - galactosidase and myocin of molecular weight 29, 45, 66, 97, 116 and 205 KD respectively was also taken in a separate tube, prepared as above and loaded.

3.7.3. Electrophoresis

For electrophoresis the electrode buffer was prepared as follows:

(0.025 M Tris, 0.192 Glycine)

Tris	...	18.15 g
Glycine	...	72.0 g
Distilled water	...	5.0 lit.
pH was adjusted to	..	8.3
Sodium dodecyl Sulphate(SDS)	...	5.0 g

Tube gel : Electrophoresis was performed at 1.5 mA per gel until the samples penetrated the resolving gel and then at 2.5 mA per gel for 4-5 h i.e. until the dye reached the bottom of the gel column.

Slab gel: In case of slab gel 2 mA current was applied per well upto the resolving gel and then 3 mA was applied for 3 h until the dye reached at the bottom of the slab gel.

3.7.4 Fixing

For fixing, the fixer solution was prepared as follows :

Isopropanol	... 250.0 ml
Acetic acid	... 100.0 ml
Distilled water	... 650.0 ml

Tube gel: In case of tube gel, all the gels were removed from the tube and soaked in fixer solution for 20 h.

Slab gel: In case of slab gel, the entire gel was removed from the glass plates & then the stacking portion was cut off from the resolving gel. After that the gel was soaked for 20 h for fixing.

3.7.5 Staining

The staining solution was prepared as follows :

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

At first, the gels were stained by staining solution for 1h with shaking and finally soaked with destaining solution (methanol : acetic acid : distilled water - 3:1:6) until the background became clear.

3.8 Cell wall characterization

3.8.1 Extraction

Mycelial wall extract was prepared from the isolated cell wall of *G.cingulata* following the method of Brown & Kimmins (1977). Isolated cell walls (2 g) were suspended in 80 ml ice cold 0.1 N NaOH by blending in a chilled mixer cup at full speed for 30 seconds. The suspension was stirred in ice bath for 15h and then centrifuged at 8,000g for 10 min. After centrifugation, the precipitate was washed with 50 ml ice cold water and the supernatant was neutralized to 7.0 by adding 1N HCl slowly with continued stirring at 0° C. The neutralized supernatant was finally dialysed against distilled water for 48 h with 12 hourly changes and concentrated with polyethylene glycol 6000 (PEG-6000), which was then used as crude mycelial wall extract (MWE) for SDS-PAGE analysis.

3.8.2 Protein estimation

Soluble proteins were estimated following the method as described by Lowry et.al. (1951). To 1 ml of protein sample 5 ml of alkaline reagent (0.5ml of 1% CuSO4 and 0.5ml of 2% Potassium sodium tartarate, dissolved in 50 ml of 2% Na_2CO_3 in 0.1 N 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 15 min for color development following which optical density (OD) was measured at 750 nm. Using bovine serum albumin (BSA) as standard, the protein concentrations were computed.

3.7.3 Carbohydrate estimation

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

3.8.4 SDS PAGE Analysis

3.8.4.1 Gel preparation

Separation gel with 10% acrylamide and stacking gel with 5% acrylamide was prepared following the method of Laemmli (1970) as described earlier for SDS - Polyacrylamide gel electrophoresis.

3.8.4.2 Sample preparation

Mycelial wall extract was dissolved in a solution of 1% SDS and 1% β mercaptoethanol and heated at 50°C for 2h. Then they were diluted by the addition of 1 volume of 4M urea containing 5% sucrose and 25-50 μl of samples were applied per gel tube. Along with the sample, protein marker consisting of a mixture of six proteins ranging in molecular weight from 29 to 205 KD was also taken in a separate tube, boiled and loaded as above.

3.8.4.3 Electrophoresis

Electrophoresis was performed at 1.5 mA per gel tube until the samples penetrated the resolving gel and then at 2.5 mA per gel tube for 5h i.e. until the dye front reaches 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) or in fixer solution II (40% ethanol in aqueous 5% acetic acid) for protein and carbohydrate staining respectively for 20 h.

3.8.4.5 Staining

Gels from fixer solution I were stained with Coomassie blue R250 and then destained as described earlier. Gels from fixer solution II were stained with periodic acid - Schiffs (PAS) reagent as described by Segrest and Jackson (1972) with modification. Details of PAS staining solutions and the procedure was as follows : At first, five following solutions A-E were prepared.

Solution A :

Anhydrous sodium acetate (0.89 g) and hydroxylamine hydrochloride (10 g) were dissolved in 90 ml distilled water. Glacial acetic acid (0.54 ml) was added to it and final volume was adjusted to 100 ml with distilled water.

Solution B :

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

Solution C :

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

Solution D (Prepared before use):

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

Solution E (Prepared before use) :

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

Procedure :

Gels were soaked in solution `A' for 15 min. then washed in running tap water. Next the gels were soaked in solution `B' for 15 min, washed in running tap water for 10 min. and then transferred to solution `c' (diluted 1:1 with distilled water just before use) for 5 min. This step was repeated thrice before washing in solution `D' for 2 min and finally washed three times for 1h each time in solution `E'.

3.8.5. Binding of FITC labelled concanavalin A

Binding of fluorescent labelled concanavalin A to mycelia as well as isolated cell wall was done by the method as described by Keen & Legrand (1980). Initially mycelia or isolated cell walls were incubated for 20 min in 0.85% NaCl in 0.01M potassium phosphate buffer, pH 7.4 containing 1mg/ml fluorescein isothiocyanate (FITC) labelled concanavalin (Con A, Sigma Chemicals). The fungus or walls were then washed thrice with saline solution by repeated low speed centrifugation and resuspension. For control sets these were incubated with lectin supplemented with 0.25M α–methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluorescence optics (BP 450-490 exciting 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 agglutination response of conidia was performed following the method of Lis and Sharon (1986) and Cristinzio *et.al,* (1988). Concanavalin A (ConA), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutin - I (UEA-I) and wheat germ agglutinin(WGA) of Sigma Chemical, USA, were diluted (1 mg/ml) with 50mM phosphate buffered saline (PBS), pH 7.2, and were used for agglutination reactions. ConA solution contained 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

3.9.1 Preparation of conidial suspension

Agglutination tests were done with ungerminated spores. For this, fungus was allowed to grow in liquid Richard's medium for 7 days at 30°C. Conidia were washed off the mycelial surface with 5 ml of sterile distilled water and the resulting suspension was centrifuged at 3500 g for 15 min at 4°C. The pellet was washed thrice with cold PBS and resuspended in the PBS to a concentration of approximately $5x10^6$/ml. The conidial suspensions were used immediately after preparation.

3.9.2 Agglutination test

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

3.10. Antisera production

3.10.1 Rabbits and their maintenance

For the production of antisera against different fungal and leaf antigens, New Zealand white, male rabbits were used. Before immunization, the body weights of rabbits were recorded and were observed for at least one week inside the cages. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they

were also given 50-75 g of gram seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Cages were cleaned everyday in the morning for better hygeinic conditions.

3.10.2 Immunization

Antisera were raised in separate rabbits against antigen preparation of mycelia and cell wall of *G. cingulata* (isolate GC-1); mycelia of *P.theae* (isolate PT-2) and *F. oxysporum* (non pathogen of tea) as well as healthy leag antigen of TV-18 and CP-1. Sera collected before immunization were used as controls. After preimmunization bleeding, immunogen (1 ml) emulsified with an equal volume of Freund's complete adjuvant (Difco) followed by incomplete adjuvant were injected intramascularly at weekly intervals, upto 20 weeks.

3.10.3 Bleeding

Blood was collected from the marginal ear vein puncture 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding, rabbits were placed on their backs on a wooden board after taking them out from the cage. The board was fixed at a 60^0 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 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 a sharp sterile blade and blood samples (2-5 ml) 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 30^0C for 1h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000g for 10 min. Finally, blood samples were distributed in 1 ml vials and stored at -20^0C until required.

3.11 Purification of IgG

3.11.1 Precipitation

IgG was purified following the method of Clausen (1988). The antiserum (5 ml) was diluted with two volumes 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. Then it was centrifuged at 10,000g for 1 h at 22°C and the precipitate was dissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.11.2 Column preparation

Initially, DEAE Sephadex (Sigma Co. USA) was suspended in distilled water overnight after which the water was decanted off and the gel was suspended in 0.005 M phosphate buffer, pH 8.0. The buffer washing was repeated 5 times. The gel was next suspended in 0.02M phosphate buffer pH 8.0 and was applied to a column (2.6 cm in dia, 30 cm high) and allowed to settle for 2h. After that 25 ml of 0.02M phosphate buffer (pH 8.0) was applied to the gel material.

3.11.3 Fraction collection

At the top of the column, 2 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 0.3 M. The initial elution buffer (1) was 0.02M 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 in which one rubber connection from its botton was supplying the column. Another connection above the surface of buffer(1) was connected to another flask with buffer (2). During the draining of buffer (1) to column, buffer (2) was sucked into buffer (1) thereby producing a continuous rise in molarity. Finally, 40x5 ml fractions were collected and the optical density (OD) values were recorded by UV-spectrophotometer at 280 nm.

3.12 Immunodiffusion tests

3.12.1 Preparation of agar slides

The Glass slides (5cm x 5 cm) were degreased successively in 90% (v/v) ethanol; ethanol:di-ethyl ether (1:1 v/v) and ether, then dried in hot air oven & sterilized inside the petridish each containing one slide. A conical flask containing Tris-barbiturate buffer (pH 8.6) was placed in a boiling water bath; when the buffer was hot, 0.9% agarose was mixed to it & boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The molten agarose was poured in glass slides (5 ml/slide) and kept 15 min for solidification. After that 3-7 wells were cut out with a sterilized cork borer (4 mm dia.) at a distance of 5 mm. from the central well.

3.12.2 Diffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50 μl/ well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48-72h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigens were present.

3.12.3 Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Then slides were stained with 0.5% amido black (0.5g amido black, 5g $HgCl_2$, 5ml glacial acetic acid 95ml distilled water) for 10 min. at room temperature. After staining slides were washed thrice in distaining solution [2% (v/v) acetic acid] for 5 h to remove excess stain. Finally, all 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 degreased, dried and sterilized as described earlier. Then thin and uniform layer (2 mm thickness) of fluid agarose medium (0.9% agarose,

0.1% NaN_3 dissolved in 0.05 M barbitol buffer (pH 8.6) was poured on each slide taking care that no air bubble was present in the agarose medium. This was necessary in order to avoid any irregularity which may cause asymmetrical migration and diffusion during electrophoretic separation or the immunodiffusion. The slides were kept in petridishes and stored at 4° C until use.

3.13.2 Electrophoresis

Two central wells (3mm dia.) were cut out from the agarose plate of each slide following the conventional method (Ouchterlony, 1967). Slides were placed in the middle compartment of the electrophoretic box. The anode and cathode chambers were filled with barbitol buffer (0.05 M pH 8.6). Antigens (40µl) were introduced into the wells. Filter paper strips (Whatman) were soaked in buffer and laid on both ends of the slides which connected the buffer solution in the anode and cathode compartments with the agar surfaces. An electric current (2.5 mA/slide; 10v/cm) was passed through the slides for 3h at 4°C. After electrophoresis the current was discontinued.

3.13.3 Diffusion

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

3.13.4. Washing, drying and staining of slides

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

3.14 Rocket Immunoelectrophoresis

3.14.1 Preparation of agarose slides

Initially the slides (7.5 x 2.5 cm) were degreased with ethanol : diethyl ether (1:1 v/v) and then dried, and sterilized as mentioned earlier. To 10 ml of fluid agarose medium (1% agarose, 0.1% NaN_3 dissolved in 0.05 M barbitol buffer pH 8.6) 1 ml of undiluted antiserum was added at 50° C (Clausen, 1988). Then this agarose gel was shaken thoroughly and poured on each slide taking care that no air bubble was present in the agarose medium. The slides were kept in petridishes and stored at 4° C..

3.14.2 Electrophoresis

2-3 wells (3 mm dia.) were cut out from one end of the agarose plate of each slide and then all slides were placed in the electrophoretic box. Antigens (50 µl) were loaded in each well. Then electrophoresis was performed for 4h at 4°C as mentioned earlier. After electrophoresis the current was discontinued.

3.14.3 Washing, staining and drying of slides

After immunoelectrophoresis, slides were washed with sterile distilled water followed by aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 24-48h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose gel. After washing, rocket-shaped sharp precipitin line of antigen-antibody complexes was found.

Slides were stained with 0.5% amido black (0.5 g amido black, 5g $HgCl_2$, 5 ml glacial acetic acid, 95 ml distilled water) for 10 min at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v)] for 5h to remove excess stain. At last, all slides were washed with distilled water and dried in hot air oven for 3h at 50° C.

3.15. Enzyme linked immunosorbent assay

For ELISA following buffers were prepared following the method as described by Chakraborty et.al., (1995).

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

Stocks		
A.	Sodium Carbonate	= 5.2995 g
	Distilled water	= 1000 ml.
B.	Sodium bicarbonate	= 4.2 g
	Distilled water	= 1000 ml.

160 ml of stock solution "A" was mixed with 340 ml of stock solution "B" pH of the mixed 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.

With 280 ml of stock solution "A", 720 ml of stock solution "B" was mixed and the pH of the resulting solution was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15 M Phosphate buffer saline - Tween (0.15 M PBS-Tween, pH 7.2).
   To 0.15 M Phosphate buffer saline, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking reagent (Tris buffer saline, pH 8.0).
   (0.05 M Tris, 0.135 M NaCl, 0.0027 M KCl).
   Tris = 0.657 g
   NaCl = 0.81 g
   KCl = 0.223 g.
   Distilled water was added to make up the volume upto 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 (0.15 M PBS-Tween, pH 7.2)
   In 0.15 M PBS-Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinyl polypyrrolidone, 10,000 (Pvpp, 10,000) and 0.03% sodium azide (NaN_2) was added.

6. Substrate
   Sigma Fast pNPP substrate tablet sets were used. Each tablet set yields the following when dissolved in 20 ml of distilled water.
   pNPP = 1.0 mg/ml
   Tris buffer = 0.2M.

7. Stop solution.
   0.3N NaOH solution was used to stop the reaction.

3.15.1. Indirect ELISA

Direct antigen coated ELISA (DAC ELISA)

This ELISA was performed following the method as described by Chakraborty et.al. (1995). Plant and fungal antigens were diluted with coating buffer and the diluted antigens were loaded (200µl/well) in a Nunc 96 well ELISA plate. After loading plate was incubated at 25°C for 4h. Then plate was washed four times under running tap water and once with PBS-Tween and each time, plate was shaken dry. Subsequently, 200µl of blocking

agent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1 h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 ml/well) to each well and incubated at 4°C overnight. After a further washing 200 ml of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma Chemicals, USA) was added & incubated at 37°C for 2h. Plate was washed, dried & loaded with 200 ml of p-nitrophenyl phosphate substrate in each well and incubated in dark at room temperature for 30-45 min. Colour development was stopped by adding 50 ml/well of 3N NaOH solution and absorbance was determined in an ELISA reader (Cambridge Tech. Inc. USA) at 405 nm. 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 :

Alkaline phosphatase was conjugated with γ-globulin following the glutaraldehyde method. For this, initially 5 mg of alkaline phosphatase enzyme crystals (Sigma Chemical) was dissolved in 1 ml of 1/2 strength phosphate buffer saline (PBS, pH 7.2). Then 25 ml of 2.5% glutaraldehyde was added and incubated for 4h at room temperature with occasional shaking. After incubation it was dialysed against 1/2 strength PBS with three changes of 30 min each. To the dialysate, 1 ml of purified IgG of *G. cingulata* 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). Antisera (IgG) of *G. cingulata* was diluted in coating buffer and loaded (200 μl /well) in each well of a 96 well ELISA plate. The plate was incubated for 4h at 37°C and washed five times by flooding the wells with PBS - Tween. Then test samples were added to empty well 200 μl / well) and incubated overnight at 4°C. After incubation, plate was washed as before and alkaline phosphatase tagged rabbit IgG diluted in PBS, pH 7.2, was added (200 μl /well) to each well and incubated for 6 h at 25°C. Then the plate was washed five times as before and 200 ml pNPP substrate (0.3 mg/ml) was added to each well and incubated for 30 min. at 25°C in dark. Colour development was stopped by adding 50 ml/well of 3N NaOH solution. Absorbance values were recorded at 405 nm in an ELISA reader (Cambridge Tech. Inc. USA).

3.15.3 Competition ELISA

Competition ELISA was carried out on a 96 well ELISA plate (Nunc, Maxisorp TM, Sweden) following the method as described by Lyons & White (1992). All the wells of one plate received 100µl of *G. cingulata* antigen extract diluted in PBS, and antigen of *P. theae* was similarly loaded into each well of another plate. Following incubation at 4°C overnight, the plates were washed five times in 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 wells were completely emptied. Blocking of non-specific binding was achieved with 5% BSA in PBS, 200 µl/well for 1h. The plates were washed and dried as before and stored at 4oC until required.

For the test, a doubling dilution series was prepared from which 75 µl of the leaf extract (healthy, naturally infected & artificially infected with *G.cingulata & P.theae*) in PBS were added in each well of the blank plates using the same paired well format of the test plates. To each well 75 µl of either *G.cingulata* or *P.theae* antiserum (IgG) diluted 1:125 with PBS was added. The plates were incubated at 37°C for 1h on a shaker. (The shaker was also used for all subsequent incubation stages). At the end of this period 100 µl of the leaf extract/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 100 µl antirabbit IgG alkaline phosphatase conjugated antiserum was added to all wells of the plates. Following 30 min. incubation at 37°C, plates were washed and 100 µl of p-nitrophenyl phosphate substrate (1 mg/ml) was added to each well. After a further 30 min. incubation, in the dark without shaking, the absorbance values were read at 405 nm in an ELISA reader (Cambridge Technology, Inc. USA).

3.16. Estimation of fungal mycelium in leaf tissues

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 *G.cingulata* (.4-2.0 mg fresh wt.) were added to these extracts. Indirect ELISA was performed with these extracts and absorbance values were noted at 405 nm as described previously Absorbance values of healthy extract was subtracted from those of the extract containing the mycelia. Standard curve of mycelial frest wt. versus absorbance was prepared. While estimating the amount of fungal mycelium in infected leaf extracts, the difference in absorbance between healthy and infected leaf extract were plotted in the standard curve and the amount determined. Amount of fresh wt. of mycelium/g leaf tissue was finally calculated.

3.17. Establishment of Callus

3.17.1 Culture media

For the callus induction, MS basal media (Murashige and Skoog, 1962) was used. Initially, following stocks solutions was prepared :

MS-1 (20x)

a) KNO_3 - 38.0 g
NH_4NO_3 - 33.0g
$MgSO_4,7H_2O$ 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 2L with double distilled water and stored at 4°C.

MS-II (100x)

$MnSO_4$ - 2.23 g
$ZnSO_4, 7H_2O$ 860 mg
H_3BO_3 - 620mg
KI - 83 mg
$Na_2MO_4, 2H_2O$ 25 mg
$CuSO_4, 5H_2O$ 2.5 mg
$CoCl_2, 6H_2O$ 2.5 mg
Double distilled water - 1L.
Stored at 4°C.

MS-III(20x)

$Na_2EDTA, 2H_2O$ - 746 mg.
Boiling double distilled water - 80 ml.
$Fe_2SO_4, 7H_2O$ - 556 g
Double distilled water - 80 ml.

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

MS-IV (100x)

Myo-inositol	-	100 mg
Thiamine HCl	-	0.5 mg
Nicotinic acid	-	0.5 mg
Pyridoxin HCl	-	0.5 mg
Glycine	-	2 mg.
Double distilled water	-	10 ml

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 media was supplemented with 3% sucrose, 0.8% agar and 2 mg/L IBA, 4 mg/L BA (Kato, 1989). Final volume was made upto 1 litre. pH was adjusted to 5.8 using 0.1N HCl or 0.1 N NaOH before autoclaving and then sterilized at 121°C (15 lb/in^2) for 30 min.

Medium was distributed in culture tubes and flasks. For the prevention of browning of explants, following substances were used in media 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

Shoots of fresh young tea plants with 3-4 leaves were taken from glass house and stem segments (2-3mm long) were cut and sampled. Before sterilization, explants were washed in running tap water for 20 min. to remove phenolic sustances. Stem segments were surface sterilized with 2% sodium hypochlorite solution for 5 min. and washed five times with sterile distilled water for the removel of hypochlorite. After final washing, explants were transferred into semi-solid media contained in flasks and culture tubes. They were incubated under 16h photoperiod at 26°C and observed regularly. The fragile callus obtained above were shaken gently in liquid MS medium at 100 rpm and loose cells were used for fluorescence 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 (1994b).

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 microcentifuge tube by a sterile Pasteur pipette and was once with PBS (pH 7.2) by centrifuguation at slow speed. Diluted (1:125) antiserum or normal serum was added into the washed cells and incubated for 30 minutes at 27°C. Then cells were washed thrice with PBS Tween (pH 7.2) and incubated for d30 m. in dark at 27°C with goat antirabbit IgG conjugated with FITC (Sigma, USA), diluted 1:40 with PBS (pH 7.2). After incubation, cells were washed thrice by repeated centrifugation and mounted in 10% glycerol. A cover slip was placed and observed under UV light using I3 filter in a Leica microscope and photographed in a wild MPS camera on 400 ASA Konica film (Leitz).

3.18.2. Cross sections of tea leaves

Initially, fresh cross sections of tea leaves were cut and immersed in phosphate buffer saline (PBS) pH 7.2 co ntaining 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 l of diluted (1:40) goat antiserum specific to rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections 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 section were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) filter set I3. Tissue sections were photographed under both phase-contrast and UV fluorescent conditions for comparison of treatment.

3.18.3. Mycelia

Fungal mycelia were grown in 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 fluorescein 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 cover glass 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. Conidial suspensions were taken in micro-centrifuge tubes and centrifuged at 3000 g for 10 min and the PBS supernatant was discarded. Then 200 μl of diluted (in PBS pH 7.2) (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-fluorescence filter. Photographs were taken as described before.