# MATERIALS AND METHODS

## 3.1 Plant Material

### 3.1.1. Source

Eighteen tea varieties were obtained from Tea Germplasm Bank, Department of Botany, University of North Bengal, for experimental purpose. Tea plants used were the varieties released by Tocklai Experimental Station of TRA (Tea Research Association), Jorhat, Assam; Darjeeling Tea Research Center (DTRC), TRA, Kurseong, West Bengal and United Planters' Association of South India (UPASI) Tea Research Station, Valparai, Tamilnadu. In total, seven Tocklai varieties, six UPASI and five Darjeeling varieties (Table 1) were chosen for the present study.

**Table 1. Tea varieties selected from Tea Germplasm Bank for the present study**

| Source | Varieties |
|---|---|
| Tocklai | TV-18, TV-22, TV-25, TV-26, TV-29, TV-30, T-17/1/54 |
| DTRC | CP- 1/1, BS/7A/76, P-312, AV-2, TS-449 |
| UPASI | UP-2, UP-3, UP-9, UP- 26, BSS-2, BSS-3 |

### 3.1.2. Clonal Propagation.

All the eighteen selected tea varieties, as mentioned above, were propagated by cuttings made from shoots of the mother bush. The standard form of tea cutting is a piece of stem 2.5 cm to 3.5 cm long, with a good mature leaf.

Sandy soil (sand 75% and soil 25% with pH ranging from 4.5 to 4.8) was used for clonal propagation. Soil pH was adjusted to 4.8 - 4.9 by treating with 2% aluminium sulphate solution. It was followed by two waterings to remove excess aluminium sulphate. The treated soil was heated to 60°C - 80° C on a metal sheet with fire below to kill eelworms, if any, present in the soil. Polythene sleeves (6"x 9") were filled up with the prepared soil and stacked in rows in a bed and watered thoroughly. All cuttings were

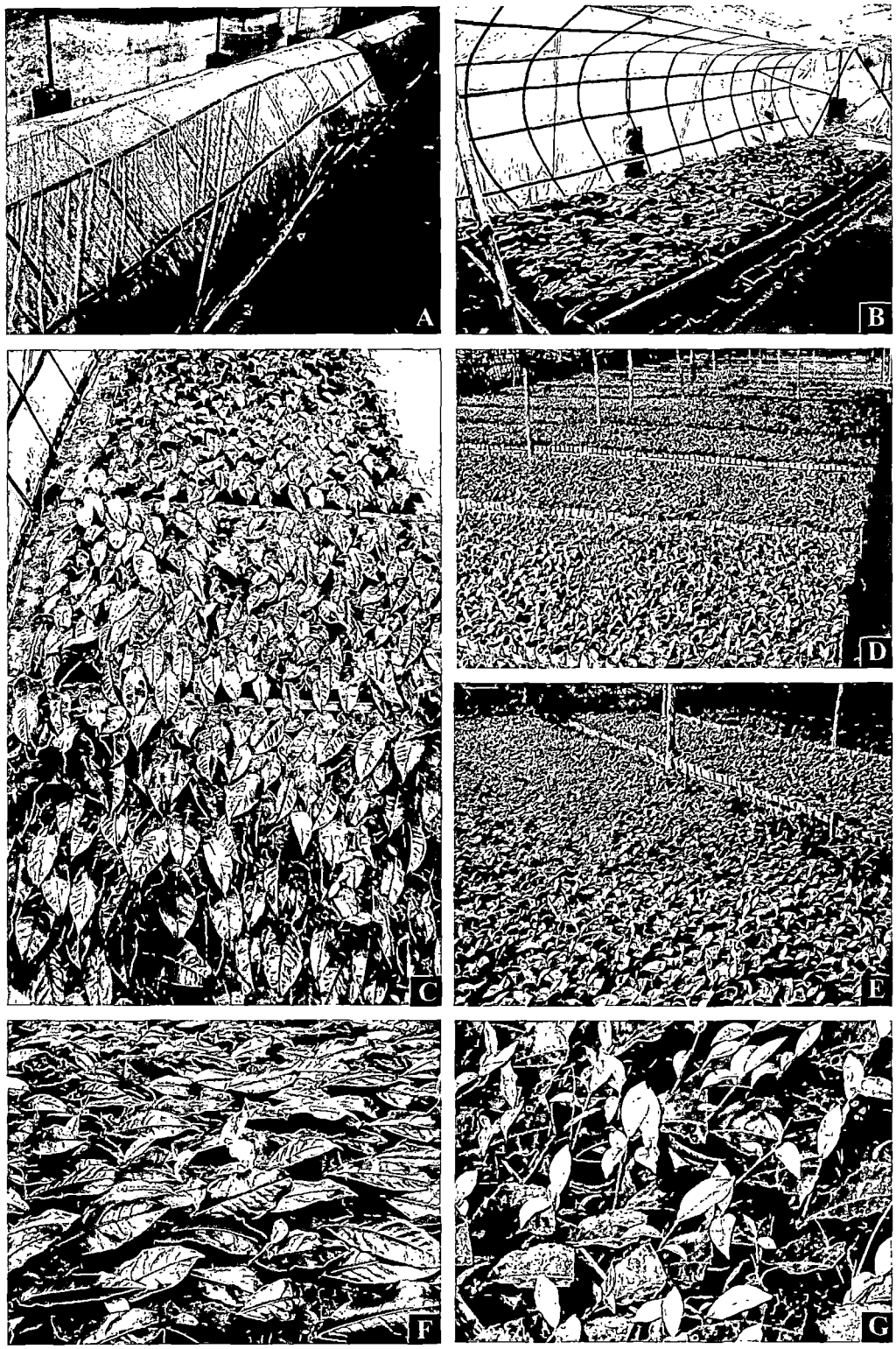


**Plate 2 (A-G) :** Propagation of tea by cuttings.

allowed for rooting in sleeves after dipping them in a rooting hormone (Ceradex). These cuttings were kept in a polythene cloche and watered every 3rd or 4th day, until new leaves appeared (Plate 2, figs. A-G). All beds with polythene cloche were arranged in 2 rows and maintained under a green agronet in the nursery of Department of Botany, University of North Bengal.

### 3.1.3. Maintenance of tea saplings in nursery

After the new shoots reached a height of 6" or more, the polythene cloche was removed from every bed, and the sleeves were treated with nursery mixture as suggested by Ranganathan and Natesan (1987). Composition of the nursery mixture - 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, and zinc sulphate – 3 parts by weight. The mixture was dissolved @ 30g in 1 L of water and applied @ 50ml $plant^{-1}$. The manuring was done after rooting and continued up to 12 months once only in 15 days. Saplings (4 to 6 months old) were shifted from under the agronet to direct sunlight for hardening.

### 3.1.4. Plantation

Eighteeen months old hardened saplings were used for planting in the field. Saplings were planted in pits (1.5' x 1.5' x 1.5') which were dug at a spacing of 2' between plants and 3.5' between rows. For experimental purposes, tea plants of the selected varieties were also grown in earthen pots. These were then maintained in glass house under controlled conditions of 30 ± 5°C, relative humidity 60-80% and 16h photoperiod (Plate 3).

### 3.1.5. Maintenance of mature plants

The mature plants were maintained in the field by applying a soluble mixture of N, P, K consisting of 10 kg Urea - 46% N, 20 kg ammonium phosphate - 11% $P_2O_5$, 8kg muriate of potash - 60% $K_2O$ in the soil. Miraculan (n-triacontanol) was sprayed at regular intervals @ 0.5ml / L of water for good growth of bush. Tipping was done to promote lateral branching in young plants (3 years), but in case of mature plants 2-year deep pruning cycle was maintained.

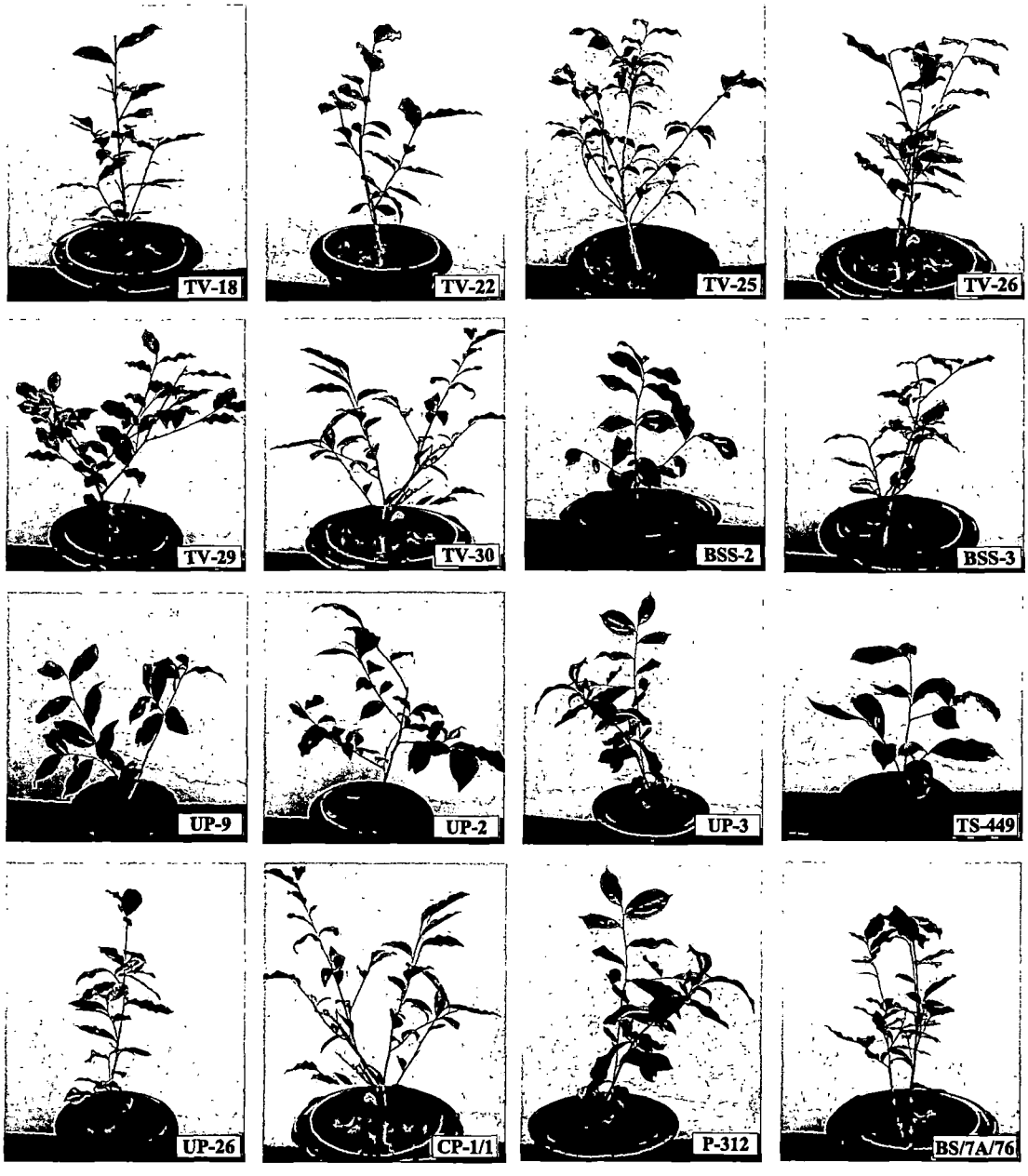


**Plate 3:** Two year old hardened tea saplings of different varieties grown in earthen pots are being maintained in glasshouse condition.

## 3.2. Fungal material

### 3.2.1. Collection

*Glomerella cingulata* (Stoneman) Spauld and Schrenk was isolated from naturally infected tea plants (TV-22) in Phytopathological Experimental Garden and subsequently was identified (W7659) from the Diagnostic and Advisory Service, CABI Bioscience UK Center and this isolate was considered as GC-1. Two more isolates (GC-2 and CG-3) obtained from the naturally infected tea plants TS-449 and TV-26 respectively, were identified by comparing with the reference isolate.

### 3.2.2 Completion of Koch's postulates

Fresh, young tea leaves were collected from Phytopathological Experimental Garden and inoculated with conidial suspension of *G.cingulata* following detached leaf inoculation technique. After 96 hours of inoculation, the infected tea leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 minutes, washed several times with sterile distilled water and transferred aseptically into Richard's medium Agar (RMA) slants. These isolates were examined after 15 days of inoculation at regular intervals to test their pathogenicity.

### 3.2.3. Maintenance of stock culture

The fungus thus obtained was subcultured on Oat Meal Agar (OMA), Richard's Medium Agar (RMA) and Potato Dextrose Agar (PDA) slants. After 2 weeks the culture was stored under 3 different conditions (5°C, 20°C and at 25±2°C). Apart from weekly transfer for experimental work, isolates of *G. cingulata* (GC-1, GC-2 and GC-3) 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* in solid media, the fungus was first grown in Petri dishes (90 mm diameter), each containing 20ml of RMA and incubated for 7 days at 30°C. Agar block (4mm diameter), containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each Petri dish containing 20ml of sterilized solid media. All Petri dishes were incubated at 25°±2°C for 6 - 10 days. The colony growth was assessed at 1 day interval by measuring colony

diameter using a ruler on the reverse side of the Petri plate held against light source. The colony morphology was recorded. Following solid media were used:

Potato Dextrose Agar (PDA)

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

Richard's Medium Agar (RMA)

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

Oat Meal Agar (OMA)

| | |
|---|---|
| Oat meal | 3.00g |
| Agar | 2.00g |
| Distilled water | 100ml |

### 3.2.4.2. Liquid media

To assess the mycelial growth of *G. cingulata* in liquid medium, the fungus was first allowed to grow in Petri dishes containing 20 ml of RMA incubated at 25°±2°C for 7 days. From the advancing zone, the mycelial block (6mm diameter) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized Richards's medium for 20 days at 30°C. At regular intervals of 4 days, fresh and dry weight of mycelium was assessed. The mycelia were strained through muslin cloth, collected in aluminium foil of known weight and weighed for fresh weight after drying at 60°C for 96h.

### 3.2.5. Assessment of sporulation in solid media

Sporulation ability of each isolate was determined in 14 day-old cultures by removing the agar plugs (0.05 cm diameter) from three linear spots across the center of a colony, suspending these in 10ml sterile distilled water in a glass test tube, and agitating

twice for about 20 sec each time, on a vortex mixer to dislodge the conidia. The number of conidia in the resultant suspension was determined, using a haemocytometer, and expressed as number of conidia $cm^{-2}$ of medium.

## 3.3. Scanning Electron Microscopy (SEM)

### 3.3.1. Pathogen (fungal spore)

Spores were lifted from the sporulating culture in Petri plate with a brush and pre-fixed as suggested by Shetty *et al* (2003). In brief, the spores were smeared on glass surface, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8 in vacuum. After 24h, they were washed in buffer and dehydrated in graded series of acetone, dried in a Critical Point Dryer (CPD) and mounted with silver paint on specimen stubs. These were then coated with gold at 15-20mA current and vacuum in the range of $10^{-1}$ Torr using an indigenous Sputter Coater for 5-6 times for 1min. each. The samples were then examined in a scanning electron microscope (Model JSM-5200, JEOL, Tokyo, Japan) in vacuum range of $10^{-5}$-$10^{-6}$ Torr in secondary electron mode at an accelerating voltage of 20kV. Photographs were taken using black and white Nova film (135mm).

### 3.3.2. Host (leaf tissue)

Healthy and infected tea leaves were trimmed to the appropriate size so as to accommodate them on the stub and treated similar to the procedure described for spore. They were fixed, dehydrated and dried in CPD prior to mounting on specimen stubs. Gold coating and observation procedures were also done as mentioned earlier for the spore.

## 3.4. Leaf clearing and staining

In order to visualize infection process on the leaf surface, samples of leaves, at appropriate time interval, were cleared for a minimum of 24 h in a 1:1 solution of glacial acetic acid and 95% ethanol (Muchovej and Couch, 1987). The leaf samples were then stained with 0.25% aniline blue in lactophenol. Leaf tissue was then examined with Leica Leitz Biomed microscope. Photographs were taken by Leica Wild MPS 48 camera on Kodak 100 ISO film.

## 3.5. Assessment of disease caused by *G.cingulata* on *Camellia sinensis*

### 3.5.1 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 detached from plants were placed in trays lined with moist blotting paper. Wounds were made on the adaxial surface of each leaf with 26 G $_{1/2}$ needle and inoculated with 20µl droplets of spore suspension (1.2 x $10^6$ conidia $ml^{-1}$) of the fungus (prepared from 14 day-old culture in RMA) . Spore suspension was placed (2-4 drops $leaf^{-1}$) 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 wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation.

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

### 3.5.2. Whole plant

Whole plant inoculation was carried out essentially as described by Mathur *et al* (2000) with minor modifications. The fungus was grown in RMA for 14 days at 30°±2°C and spore suspension was prepared (1.2 x $10^6$ conidia $ml^{-1}$). Tween-20 was added @ 2ml $l^{-1}$ to facilitate adhering of the spores to leaf surface. 2 year-old plants were spray-inoculated with an atomizer @ 100 ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with polythene bags so as to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glasshouse benches and maintained at 30°±2°C.

Disease intensity was assessed as described by Chakraborty *et al* (2007) 25, 30 and 35 days after inoculation of the whole plants. Diameter of individual lesions was measured. They were graded into 4 groups and a value was assigned to each group. Very small, restricted lesions, 1-2mm diameter = 0.1; 2-4mm diameter with sharply defined margin = 0.25; lesion with slow spread beyond 4mm = 0.5; and spreading lesion variable in size with diffuse margin = 1.0. Number of lesions in each group was multiplied by the value assigned to it and the sum total of such values for 50 leaves randomly picked up

from each plant gave the disease index for a plant. Results were always computed as the meant of observations of 25 well-established and branched 2 year-old tea plants in average of three separate experiments.

## 3.6. Analysis of fungal protein

### 3.6.1. Extraction

Mycelial protein was extracted according to the method of Chakraborty and Saha (1994). Mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$ and 0.05mM $MgCl_2$ at 4°C with sea sand in mortar and pestle. The homogenate was centrifuged at 10,000rpm for 15min. at 4°C and the supernatant obtained was used for protein analysis immediately and stored at -20°C for future use.

### 3.6.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et al* (1951).To 1ml of protein sample (taking $10^{-1}$ or $10^{-2}$ dilution) 5ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% sodium potassium 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 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for 30 min., following which optical density was measured at 700nm.Quantity of protein, was estimated from the standard curve made with bovine serum albumin (BSA).

### 3.6.3. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile of soluble mycelial protein following the method of Sambrook *et al* (1989).

#### 3.6.3.1. Preparation of stock solutions

For the preparation of gel the following stock solutions were prepared:

**A. Acrylamide and N'N'-methylene bis acrylamide**

A stock solution containing 29% acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below 7.0 and the stock solution was

filtered through Whatman No.1 filter paper, kept in brown bottle and stored at $4^0$C and used within one month.

**B. Sodium Dodecyl Sulphate (SDS)**

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

**C. Tris buffer**

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

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

**D. Ammonium Persulphate (APS)**

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

**E. Tris-Glycine electrophoresis buffer**

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

**F. SDS gel loading buffer**

This buffer contains 50mM Tris-HCl (pH 6.8), 10mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution can be prepared by dissolving 0.5 ml of 1M Tris buffer (pH 6.8), 0.5 ml of 14.4M β-mercaptoethanol, and 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

**3.6.3.2. Preparation of Gel**

Mini slab gel (plate size 8cm x 10cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5mm thick spacers were placed between the glass plates at the three sides and the three sides of the glass plates were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order mentioned as follows:

| Composition | 10% Resolving gel (ml) | 5%Stacking gel (ml) |
|---|---|---|
| Double distilled water | 2.85 | 2.10 |
| 30% Acrylamide | 2.55 | 0.50 |
| Tris buffer* | 1.95 | 0.38 |
| 10% SDS | 0.075 | 0.030 |
| 10% APS | 0.075 | 0.030 |
| TEMED** | 0.003 | 0.003 |

*For 1.5M Tris pH 8.8 in resolving gel and 1M Tris pH 6.8 in stacking gel;

** N, N, N', N''– tetramethyl ethylene diamine

After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Then the stacking gel (5%) was prepared by mixing the stock solutions.

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

**3.6.3.3 Sample preparation.**

Sample was prepared by mixing the sample protein (34µl) with 1 x SDS gel loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 3 minutes to denature the protein sample.The samples were immediately loaded in a pre-determined order into the bottom of the wells with T-100 micropipette. Along with the samples, protein marker (Genei) consisting of a mixture of six proteins ranging from high to low molecular weight ( Phosphorylase b – 97,400 ; Bovine Serum Albumin – 66,000 ; Ovalbumin – 43,000 ; Carbonic Anhydrase – 29,000 ; Soybean Trypsin inhibitor – 20,100 ; Lysozyme – 14,300) was treated as the other sample and loaded in a separate well.

### 3.6.3.4. Electrophoresis

Electrophoresis was performed at constant 18 mA current (Power Pac 1000, Bio-Rad) until the dye front reached the bottom of the gel.

### 3.6.3.5. Fixing and staining

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

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

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

## 3.7. Analysis of fungal cell wall

### 3.7.1. Isolation

Cell wall was isolated from *Glomerella cingulata* following the procedure of Keen and Legrand (1980). Mycelium of 10-day-old actively growing fungal culture was collected in Buchner funnel and dried by vacuum filter. 20g of fresh packed cells were ground for 1 min in a high speed blendor with 80ml ice-cold water. The fungus was then disrupted in a homogenizer at 5°C for 1min. The mixture was centrifuged for 1 min. at 1500g the supernatant fluids discarded, and the sedimented walls washed with 200ml ice-cold water and pelleted by centrifugation at least 6 times or until the supernatant fluids become visually clear. Finally, the isolated cell walls were frozen and kept at -20°C.

The isolated cell walls were extracted by the method of Chakraborty *et al* (1996). Isolated cell walls were suspended in ice-cold 0.1 (N) NaOH @ 40ml/g cell walls by blending in a chilled mixer-cup at full speed for 20sec. Then the suspension was slowly stirred in an ice bath for 15h. Following centrifugation at 8,000g for 10min., the residue was washed with ice-cold water and the pooled supernatants were carefully neutralized

to pH7.0 with 1(N) HCl and finally dialysed against double distilled water and concentrated with PEG. This was used as crude mycelial wall extract (MWE).

### 3.7.2. Estimation

#### 3.7.2.1. Protein content

Protein content of mycelial wall extract (MWE) was estimated as described earlier for the estimation of mycelial protein.

#### 3.7.2.2. Carbohydrate content

The carbohydrate content was estimated according to the method of Plummer (1978) with Anthrone Reagent. 8ml of MWE was pre-treated with 1ml of 0.3(M) $Ba(OH)_2$ and 1ml of 5%$ZnSO_4$ . After centrifugation at 3000g for 10min., supernatant was collected. 1ml of supernatant was mixed with 4ml of Anthrone reagent (0.2g of Anthrone powder in 100ml of concentrated $H_2SO_4$). The mixture was kept in boiling water bath for 10min. After cooling, absorbancy was measured in a colorimeter at 620nm. The carbohydrate content was estimated using glucose as a standard.

### 3.7.3. Bioassay

For bioassay, crude neutralized extracts were placed on the adaxial surface of leaves kept in a humid chamber and were collected after 48h. These were assayed for fungitoxicity by spore germination test as described by Rouxel *at al* (1989).

### 3.7.4. SDS – PAGE

All the steps were same as described earlier for SDS-PAGE analysis of mycelial protein, only fixing and staining were different. After electrophoresis, gel with mycelial and cell wall protein with replica was fixed overnight in solution I [glacial acetic acid : methanol : water (10 : 20 : 70 ) ] or in fixer solution II (40% ethanol, 5% glacial acetic acid, aqueous ) for protein and carbohydrate respectively. Gels from fixer solution I were stained in coomassie brilliant blue (Sigma $R_{250}$) and then destained as above. Gels from solution II were stained with Periodic acid-Schiff's (PAS) regent as described by Segrest and Jackson (1972) with modifications.

The gel for carbohydrate staining, after fixative, was treated with 0.7% Periodic acid solution (0.7g periodic acid in 100ml of 5% acetic acid ) for 2-3h. The gel was then washed with 0.2% $Na_2S_2O_5$ solution (0.2g $Na_2S_2O_5$ in 100ml of 5% acetic acid ) for 2-3 hours with change of 30min. each. After addition of Schiff 's reagent (10g of Basic

Fuchsin dissolved in 2L of hot double distilled water, cooled and 200ml of 1N HCl and 17g of $Na_2S_2O_5$ was mixed to the solution until it is decolorized), bands appear after 12-18 hours at Room Temperature. The gel was stored at 4°C.

## 3.8. Analysis of host protein

### 3.8.1. Extraction

Soluble proteins were extracted from healthy and infected tea leaves following the method of Chakraborty *et al* (1995).Leaf tissues (1g) were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.05mM $MgCl_2$, 2mM soluble polyvinyl pyrrholidone (PVP10,000) and 2mM polymethyl sulfonyl fluoride (PMSF) in mortar with pestle at 4°C with sea sand and insoluble PVPP (PVP 40,000).The homogenate was centrifuged for 20 min. at 10,000 r.p.m. and the supernatant was used as crude protein extract for SDS-PAGE immediately or stored at -20°C future used within 24h.

### 3.8.2. Estimation

Estimation of protein content was done by the method of Lowry *et al* (1951) after appropriate dilution.

### 3.8.3. SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile as described earlier for mycelial protein.

## 3.9. Collection of leaf diffusates and fungitoxic assay

Diffusible compounds from tea leaves were collected following drop diffusate technique of Muller (1958) with modifications. Leaves were collected from tea plants, washed in sterile distilled water and blotted dry with blotting paper. Fifty leaves were placed on moist blotting paper in each plastic tray (30cm x 30cm). Each leaf was slightly wounded with a sterile needle. 20µl droplets (2-4 per leaf) of sterile distilled water or conidial suspension of *G.cingulata* ($1.2 \times 10^6$ conidia $ml^{-1}$) prepared from 10 days old cultures with sterile distilled water were placed on the dorsal surface of each leaf. Each tray was covered with a glass lid and sealed with a smear of white petroleum jelly to maintain the necessary moisture. Drops were collected from healthy leaf surfaces,

combined and centrifuged. This supernatant was treated as diffusate. Finally, diffusates were passed through millipore filter and then used for experimental purpose.

Method of Werder and Kern (1985) was followed for bioassay of diffusates. A clean grease free slide was taken and a drop of 25µl of fungal spore suspension was placed on it. The spores were allowed to settle and adhere to the slide for at least 1h. The water was then carefully removed with highly absorbent blotting paper. Next, 25µl of test solution was added on the settled spores and incubated in humid Petri dishes for 18h. At the end of incubation period, the test solution was removed with blotting paper. Finally, 1 drop of lactophenol cotton blue was added on to the germinated spores for fixation. Slide was observed under the microscope and the percentage of spore germination, germ tube length and appressoria formation was determined from at least 200 spores in each treatment from ten different microscopic fields under high power (x450). Measurements were done with the help of stage and ocular micrometer.

## 3.10. Extraction and estimation of Phenolics

Total phenol and orthodihydroxy phenols were extracted and estimated following the method of Mahadevan and Ulanganathan (1992). Detached leaf inoculation technique as described earlier was followed. In case of control, sterile distilled water was mounted on the adaxial surface of leaves. Phenols were extracted from 1g each of healthy and inoculated leaves separately in boiling ethanol (4ml/gleaf tissue) for 10 min., crushed using 80% ethanol (5ml/ g tissue) and filtered in the dark through filter paper. Final volume was adjusted to 5ml with 80% ethanol.

### 3.10.1. Total phenol

For estimation of total phenol, to 1 ml of alcohol extract 1ml of 1N Folin-ciocalteau's reagent and 2ml of 20% $Na_2CO_3$ solution was added. The test tube was shaken and heated on a boiling water bath for 1 min. and volume was raised to 25ml with distilled water. Absorbance was measured in a Systronics photoelectric colorimeter Model – 101 at 650nm.Quantity of total phenol was estimated using caffeic acid as a standard. Phenol content was expressed as mg caffeic acid $g^{-1}$ leaf tissue.

### 3.10.2. Orthodihydroxy phenol

For estimation of orthodihydroxy phenol , 1ml of alcohol extract was taken in a test tube, to which 2ml of 0.5 N HCl, 1ml Arnow's reagent ($NaNO_3$ – 10g; $Na_2MoO_4$ – 10g; distilled water 100ml) and 2ml of 1N NaOH were added, following which the

volume was raised to 10ml with distilled water. Absorbance was recorded using a Systronics Photoelectric Colorimeter model-101 at 515nm.Quantity of orthodihydroxyphenol was estimated using caffeic acid as standard. Phenol content was expressed as mg $g^{-1}$ leaf tissue.

## 3.11. Polyclonal antibody preparation

### 3.11.1. Rabbits and their maintenance

Polyclonal antibody (i.e. antisera) for fungal antigen was produced in New Zealand white male rabbits. Approximately 2kg body weight rabbit is needed is needed (Alba and Devay, 1985) for immunization. Thus before immunization, the body weight of rabbits were recorded and observed for at least 1 week inside the cage. Rabbits were maintained in Animal House (Antisera Reserve for Plant Pathogens), Department of Botany, University of North Bengal. Food given to the rabbits consisted of green grass, soaked gram seeds, green vegetables like cabbage and carrots (especially at the time of bleeding schedule). Rabbits were regularly fed in the morning and evening, providing properly washed and cleaned utensils. 90-100g / day of gram seeds (soaked in water), alternately with 500g green grass were given for each rabbit. Besides this, they were given saline water after each bleeding for three consecutive days. Cages and floor were cleaned with antimicrobial agents every day in the morning for maintaining the hygeinic condition.

### 3.11.2. Immunization

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

### 3.11.3. Bleeding

Blood samples were collected by marginal ear vein puncture. First bleeding was taken 3 days after $6^{th}$ week of $1^{st}$ immunization and subsequently 5 times more every fortnight. During bleeding the rabbit was placed on its back on a wooden board, fixed at $60^{\circ}$ angle. The neck of the rabbit was held tightly in the triangular gap at the edge of the

board and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a sharp 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 sample (4-10ml) was collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from puncture. For clotting, the blood samples were kept at 37°C for 1h and then stored overnight at 4°C. Then the clot was slightly loosened with a sterile needle and the antiserum was taken into another sterile centrifuge tube and clarified by centrifugation at 2000g for 10min. at room temperature. Finally, antisera were stored at -20°C until required.

## 3.12. Purification of IgG

### 3.12.1. Precipitation

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

### 3.12.2. Column preparation

4g of DEAE cellulose (Sigma Co., USA) was suspended in distilled water overnight. The water was drained off and the gel was suspended in 0.005M sodium phosphate buffer pH8.0 and the buffer washing was repeated for 5 times. The gel was then suspended in 0.02M sodium phosphate buffer, pH 8.0 and applied to a column (2.6cm wide, 30cm high) and allowed to settle for 2h. After the column material had settled, 25ml of 0.02M sodium phosphate buffer, pH 8.0 was applied to the gel material.

### 3.12.3. Fraction collection

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

The buffer-(1) was applied in a lower flask (or tank) in which one rubber tube connection from its bottom was supplying the column. Another connection from its top was connected to upper flask (or tank) containing buffer-(2). The buffer-(2) had also connection to the open air. During the draining of buffer-(1) to the column, buffer-(2) was sucked into buffer-(1) thereby producing a continuous rise in molarity. Finally, 40x5 fractions each of 5ml were collected and the optical density values were recorded at 280nm by means of UV spectrophotometer (DIGISPEC-200GL).

### 3.12.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1996). Absorbance was taken for selected fractions at 280 nm and 260nm and then concentration of IgG was calculated by the following formula: protein concentration (mg $ml^{-1}$) =1.55 x A. 280 - 0.76 x A 260.

### 3.12.5. Storage of IgG

Serum samples were obtained at regular intervals as per schedule and stored at -20°C. To eliminate the background binding and to restore the specificity of antisera lost during storage, they were adsorbed with kaolin (Shillitoe, 1982). To 0.1 ml of antiserum (=3mg protein) was added 0.15M phosphate buffer saline pH 7.2 (PBS) with 4% BSA and 1 ml of 25% suspension (w/v) of kaolin (hydrated aluminiumm silicate, particle size 0.1 – 4.0 mm, Sigma) in PBS. The mixture was allowed to stand at room temperature for 20 minutes with regular mixing. The kaolin was then removed by centrifugation at 10, 0000 rpm for 10min. and then the supernatant was diluted with 1 volume of glycerol and 0.01% sodium azide was added before storage at -10°C.

## 3.13. Immunodiffusion

### 3.13.1. Preparation of agarose slides

Glass slides (6cm x 6cm) were degreased in 90% (v/v) ethanol; ethanol:diethylether (1:1, v/v) and ether, then dried in hot air oven. After drying plates were sterilized inside the petridishes, each containing one plate. Agarose gel was prepared in 0.05M Tris-barbiturate buffer (pH 8.6). The buffer was heated in a conical flask placed in a boiling water bath. 0.9% agarose was mixed to the hot buffer and boiled for the next 15min. The flask was repeatedly and thoroughly shaken on order to prepare clear molten agarose,

after which 0.1% (w/v) sodium azide (antibacterial agent) was added into it. For the preparation of agarose gel, the molten agarose was poured on sterilized glass slides (10ml/slide) in laminar air flow chamber and kept for solidification under UV light for 30min. After that 7 wells were cut out with a sterilized cork borer (6mm dia.) at a distance of 1.5-2.0 cm from the central well and 2.0-2.5 cm well to well (peripheral).

### 3.13.2. Diffusion

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

### 3.13.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterilized distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% $NaN_3$) for 72h with 6-hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. The slides were then stained with coomassie blue ($R_{250}$, Sigma; 0.25g coomassie blue + 45ml methanol + 45ml distilled water + 10ml glacial acetic acid ) for 10min. at room temperature. After staining, slides were washed in destaining solution [90ml methanol: distilled water (1 : 1) and 10ml acetic acid ] with changes until the background becomes clear. Finally, slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

## 3.14. Immunological techniques

The immunodiagnostic techniques are based on the similarity of the antigenic determinants between the host and the pathogen. The IgG is raised against the pathogen and used to probe for the presence of the particular organism in the host. Several techniques have been used in the present study.

### 3.14.1. Plate-trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA)

PTA-ELISA was performed following the method as described by Chakraborty *et al* (1995). It has been found to be a rapid and sensitive method of detection. The following buffers were prepared at the onset :

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

**Stocks**

A. Sodium carbonate - 5.3 g in 1000ml double distilled water .

B. Sodium bicarbonate - 4.2g in 1000ml double distilled water

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

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

**Stocks**

A. Sodium dihydrogen phosophate - 23.40g in 1000ml double distilled water.

B.Disodium hydrogen phosphate - 21.294g in 1000ml double distilled water. 280ml of stock A was mixed with 720 ml of stock B and pH was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15M Phosphate Buffer Saline-Tween (0.15M PBS-T, pH 7.2)

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

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

0.05M Tris, 0.135M NaCl, 0.0027M KCl

Tris - 0.657g

NaCl - 0.81g

KCl - 0.223g

Distilled water was added to make up the volume to 100ml. 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 (0.15M PBS-Tween, pH 7.2)

In 0.15M PBS-Tween, pH 7.2, 0.2% BSA, 0.02% polyvinylpyrrolidone, 10,000 (PVPP 10,1000) and 0.03% sodium azide ($NaN_3$) were added.

6. Substrate

p-nitrophenyl phosphate (Himedia) 10mg/ml dissolved in substrate buffer (0.05M Tris-HCl buffer, pH 9.8 containing 1.0%, w/v diethanolamide, 5mM $NaN_3$, pH 9.8)

7. Stop solution

3N NaOH solution was used to stop the reaction.

Flat bottom 96-well polystyrene micro-tiltre plates (Nunc) were used for assay. Test samples were diluted with antigen coating buffer and added into each of the three wells (200µl $well^{-1}$) and then incubated at 25°C for 4h. The wells were then emptied and washed 3 times by flooding with 0.15M PBS pH 7.2 containing 0.05% Tween-20 (PBS-T). After each washing, plates were shaken dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites to eliminate background binding and the plate was incubated at 25°C for 1h. After incubation the plate was washed as mentioned earlier. Purified IgG was diluted in antisera dilution buffer (PBS-T) containing 2% soluble PVPP, 0.2% BSA and 0.03% $NaN_3$, loaded (200µl $well^{-1}$) and incubated at 4°C overnight (16h). After another washing, goat antirabbit IgG labelled with alkaline phosphatase (Sigma Chemicals, USA) diluted 10,000 times with PBS was added to each well (200µl $well^{-1}$) and incubated at 37°C for 2h. The plate was washed, dried and loaded with 200µl of enzyme substrate p-nitrophenyl phosphate (10 mg $ml^{-1}$) in alkaline phosphatase buffer containing 1% diethanolamine in each well and incubated in dark at room temperature for 60min. Colour development was stopped by adding 50µl $well^{-1}$ of 3N NaOH solution. The rate of colour development was monitored on a multiskan EX (Thermo Electron) ELISA reader interfaced with Windows 98 computer and the rates of the reaction was recorded at A405nm. Absorbance values in wells not coated with antigens were considered as antigen blanks. Triplicate wells were used for each sample.

### 3.14.2. Dot Immunobinding assay (DIBA)

Dot immunobinding assay was performed following the method as suggested by Lange *et al* (1989) with modifications. It can be used instead of PTA-ELISA in an efficient manner.

Following buffers were used for dot-blot :

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

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

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

### 3.14.2.1. Immunoblotting

Nitrocellulose membrane (Bio-Rad, 0.45µm) was first cut carefully into the required size (8cm x 12cm for 96 welled template) and were carefully placed inside the template of Bio-Dot apparatus (Bio-Rad) with the help of flat foreceps (Millipore). Hand gloves were always worn while carrying out all the procedures involving nitrocellulose membrane (NCM). 10µl of coating buffer was loaded in each well of the template over the NCM and kept for 30min. to dry.

Following this 10µl of test samples (antigens) were loaded into the template wells over the NCM and kept for 1h at room temperature.

### 3.14.2.2. Immunoprobing

Template was removed and blocking of NCM was done with 10% non-fat dry milk (casein) prepared in TBST for 30-60min. on a magnetic stirrer (Remi). Polyclonal antibody (IgG, 1:5000) of *G. cingulata* mycelia was added directly into the blocking solution and further incubated at 4°C overnight. The membrane was then washed gently with running tap water for 3 min., followed by 3 times 5 min. washes in TBST (pH 7.4) (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase-conjugated goat antirabbit IgG (Sigma Chemicals, diluted 1:10,000 in TBST containing 5% w/v, skim milk powder) for 2h at 37°C. The membrane was washed as before. Substrate (BCIP/NBT, Bangalore Genei, 10ml/blot) was next added and colour development noted. Colour development was stopped by washing the NCM with distilled water and categorized on the intensity of the dots.

### 3.14.3. Western blot analysis

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed according to the method as devised by Towbin *et al*, 1979. Two identical SDS-PAGE gels were prepared. Following this, one was stained with coomassie brilliant blue. The immobilized proteins on the other gel were transferred to the nitrocellulose membrane (NCM) and analysed immunologically. Immunoprobing

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

(i) All the stock solutions and buffers used in SDS-Gel preparation for Western Blot were prepared as described earlier.

(ii) Transfer buffer (Towbin buffer) :
200mM Tris, 192mM glycine, 20% reagent grade methanol, pH 8.3.
Tris - 3.03g; Glycine - 14.4g; 200ml methanol (adjusted to 1000ml with double distilled water)

(iii) Phosphate buffer saline, PBS (0.15M, pH 7.2).Preparation as mentioned in ELISA

(iv) Blocking solution :
5% non-fat dried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers :
(a) Washing buffer-1 : PBS
(b) Washing buffer-2 : (50mM Tris-HCl, 150mM NaCl, pH 7.5).
Tris - 6.07g; NaCl - 8.78g; made upto 1 lit with distilled water.

(vi) Alkaline phosphatase buffer :
(100mM NaCl, 5mM $MgCl_2$, 100mM Tris-HCl, pH 9.5).
Tris - 12.14g; NaCl - 5.84g; $MgCl_2$ - 1.015g; made upto 1 lit with double distilled water.

(vii) Substrate : BCIP/NBT, Bangalore Genei, 10ml/blot

(viii) Stop solution : (0.5M EDTA solution in PBS, pH 8.0)
EDTA sodium salt - 0.0372g in 200µl double distilled water added in 50ml of PBS.

**3.14.3.1. Immunoblotting**

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

Semi-dry cell. A glass rod was rolled over the surface of the filter paper to exclude all air bubbles. The pre-wet membrane was placed on the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and again the air bubbles were removed. Finally another pre soaked filter paper was placed on the top of gel, removing all the air bubbles. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant voltage of 15V. After the run the membrane was removed and dried on a clean piece of filter paper for 1 h.

### 3.14.3.2. Immunoprobing

After drying the NCM, blocking was done by 5% non-fat dried milk in a heat sealable lastic bag and incubated for 90 min with gentle shaking on a platform shaker at room temperature. Subsequently the membrane was incubated with antibody (IgG) solution. The bag was sealed leaving space for few air bubbles and incubated at 4°C overnight. All the steps were done with gentle shaking. Next day, the membrane was washed thrice in 250ml PBS (washing buffer-1). Final washing was done in 200ml washing buffer-2 to remove azide and phosphate from the membrane before enzyme-coupled reaction. The enzyme, alkaline phosphatase tagged with anti-rabbit goat IgG (Sigma Chemicals) diluted (1 : 1000) in alkaline phosphatase buffer, was added and incubated for 1 h at room temperature. After the enzyme reaction, membrane was washed four times in washing buffer-2. Then 10ml of substrate was added and the reaction was monitored carefully. When the bands appeared upto the desirable intensity, the membrane was transferred into a reservoir with 50ml stop solution.

### 3.14.4. Indirect Immunofluorescence

Indirect fluorescence staining of fungal mycelia and cross sections of tea leaves were done using RITC labeled goat anti-rabbit IgG as well as FITC labeled goat anti-rabbit IgG following the method of Chakraborty and Saha (1994).

### 3.14.4.1. Fungal mycelia and cell wall

Fungal mycelia were grown in liquid Richard's medium as described earlier. After 4 days of inoculation, young mycelia were taken out from flask, taken in Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at low speed. Mycelia and cell wall of

*G. cingulata* (isolate GC-1) were treated separately with normal sera or antisera diluted (1 : 50) in PBS and incubated for 1 h at room temperature. The mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat anti-rabbit IgG (whole molecule) conjugated with rhodamine isothiocyanate (RITC) or fluoroscein isothiocyanate (FITC) diluted 1 : 40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation, mycelia were washed thrice in PBS and mounted in 10% glycerol. After placing the coverslip carefully, it was sealed. The slides were observed under UV fluorescence conditions using Leica Leitz Biomed microscope with fluorescence optics suitably equipped with N2 or I3 filter and photographs taken using 800 ASA film.

#### 3.14.4.2. Cross sections of tea leaves

Initially, cross sections of healthy and infected tea leaves were cut and immediately immersed in phosphate buffer saline (PBS), pH 7.2 containing 0.01% polyvinyl poly pyrrolidone-10 (PVP-10) and 1% BSA (Bovine Serum Albumin) . These sections were washed in PBS and treated with normal serum or antiserum diluted (1 : 50) in PBS and incubated for 3 h at room temperature in grooved slides. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1 : 40) goat antirabbit IgG labeled with RITC or FITC. The sections were incubated for 45 min in dark. After that sections were washed thrice with PBS-Tween as mentioned above and mounted on a grease free slide with 10% glycerol. Fluorescence of the leaf sections was observed using Leica Leitz Biomed microscope with fluorescence optics equipped with UV filter set (N2/ I3) and photographs were taken.

## 3.15. Application of inducers

### 3.15.1. Hydrogen Peroxide ($H_2O_2$)

The required percentage of hydrogen peroxide (30% stock) was sprayed with an atomizer @100ml per plant so as to wet both ventral and dorsal surfaces. The plants were treated overnight so that the light-sensitive hydrogen peroxide is not degraded and there is sufficient time for absorption. The concentrations (v/v) used were 3%, 0.3% and 0.15% made from the stock solution diluted with distilled water and stirred properly.

### 3.15.2. Sodium Nitroprusside (SNP)

Nitric oxide donor – Sodium Nitroprusside (NITROP,Chandra Bhagat Pharma Pvt.Ltd, Mumbai) was used in aqueous solution of appropriate molarity to test the effect of NO on tea plants. The solution was sprayed with an atomizer @100ml per plant so as to wet both ventral and dorsal surfaces. The plants were always treated during daytime, as SNP forms NO in presence of light and vascular tissue (Yamamoto and Bing, 2000). The concentrations used were 1mM, 0.1mM amd 0.01mM made from the stock solution diluted with distilled water and stirred properly.

### 3.15.3. Benzothiadiazole (BTH)

Benzothiadiazole [ benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester, HiMedia] was used in aqueous solution of appropriate concentration to test the effect of BTH on tea plants. The solution was sprayed with an atomizer @100ml per plant so as to wet both ventral and dorsal surfaces. The concentrations (v/v) used were 0.01%, 0.1% and 1% made from the stock solution diluted with distilled water and stirred properly.

## 3.16. Assay of enzyme activity

### 3.16.1. Peroxidase ( POX; E. C. 1.11.1.7)

To extract peroxidase, the method described by Chakraborty *et al* (2002) was used. Tea leaf tissue (1g) was crushed in mortar with pestle in 5ml of 0.1(M) sodium phosphate buffer ( pH 7.0) on ice with addition of a pinch insoluble PVP and sea sand. The homogenate was centrifuged immediately at 15,000 r.p.m. for 20 min. at 4°C. After centrifugation, the supernatant was collected and stored at - 20°C and used within 24h for assay and isozyme analysis. For determination of peroxidase activity,100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4 mM $H_2O_2$, 100µl of o-dianisidine (5mg $ml^{-1}$ of methanol) and 1.7 ml of distilled $H_2O$. Peroxidase activity was assayed spectrophotometrically [using UV-VIS Spectrophotometer (Digispec 200GL)]at 460nm by monitoring the oxidation of o-dianisidine in presence of $H_2O_2$. Specific activity was expressed as the increase in absorbance at 460nm $mg^{-1}$ protein $min.^{-1}$ ($\Delta A_{460}$ mg $^{-1}$ protein $min.^{-1}$).

### 3.16.2 Polyphenol oxidase (PPO; E.C. 1.14.18.1)

Extraction and assay of PPO was done according to the method of Meena *et al* (2001). 1 g of leaf tissue was crushed on ice with mortar and pestle with 5 ml of 0.1 M sodium phosphate buffer (pH 6.5). The slurry was immediately centrifuged at 4000 rpm for 30 min. at 4°C. The supernatant was collected and after recording its volume was stored at - 20°C and used within 24h for assay and isozyme analysis. For assay of PPO activity, 100 µl of freshly prepared enzyme extract was mixed with 1.9 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 1ml of 0.025 M catechol solution . The reaction mixture was incubated in dark at room temperature for preventing photooxidation of enzyme. Further readings were taken at 1 min interval at 495nm. The blank was set with 3 ml of phosphate buffer and enzyme activity expressed as $\Delta A_{495}$ mg $^{-1}$ protein min.$^{-1}$

### 3.16.3. Phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5)

For extraction of PAL method of Chakraborty *et al* (1993) was followed. One gram of leaf tissue was crushed using mortar and pestle on ice in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol. The slurry was centrifuged at 10,000 rpm for 20 min. at 0° C. The supernatant was collected and its final volume was measured and used immediately for assay. PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3ml of 300µM sodium borate buffer (pH 8.8), 0.3ml 30µM L-phenylalanine and 0.5 ml of supernatant in a total volume of 3ml. Following incubation for 1h at 40 °C, the absorbance at 290nm was read using UV-VIS Spectrophotometer (Digispec 200GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid liberated from L-phenylalanine mg $^{-1}$ protein min.$^{-1}$.

### 3.16.4. Tyrosine ammonia lyase (TAL; E.C. 4.3.1.5)

For extraction of TAL method of Paul and Sharma (2002) was followed. Leaves (1g each ) were crushed using mortar and pestle on ice in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol. The slurry was centrifuged at 15,000 rpm for 20 min. at 0°C. The supernatant was collected, its final volume was measured and the extract used immediately for assay. TAL activity in the supernatant was determined by

measuring the production of p-coumaric acid from L-tyrosine spectrophotometrically. The reaction mixture contained 0.3ml of 300μM sodium borate buffer (pH 8.8), 0.3ml 30μM L-tyrosine and 0.5 ml of supernatant in a total volume of 3ml. Following incubation for 1h at 40°C, the absorbance at 290nm was read using UV-VIS Spectrophotometer (Digispec 200GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as μg p-coumaric acid liberated from L-tyrosine $mg^{-1}$ protein $min.^{-1}$

### 3.16.5. Chitinase (CHT; E.C. 3.2.1.14)

The colorimetric assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988) with a few modifications. One gram of the leaf sample was extracted with 3 ml of 0.1 M sodium citrate buffer, pH 5.0 and 2 mM PVP-10 in pre-chilled mortar with pestle. The homogenate was centrifuged for 30 min. at 4°C and 10,000 r.p.m. The supernatant was used as enzyme source. One ml of the enzyme extract was incubated with 1 ml of colloidal chitin (1mg $ml^{-1}$ ) at 37°C for 1 h on a shaker. The solution was centrifuged for 3min. at R. T. to remove any unreacted colloidal chitin. An aliquot of supernatant (0.3ml) was added to 30 μl of 1 M potassium phosphate buffer, *p*H 7.1 and incubated with 20 μl of (w/v) desalted snail gut enzyme Helicase (Sigma) (3%) (w/v) for 1 h. After 1 h, the *p*H of the reaction mixture was brought to 8.9 by addition of 70 μl of 1 M sodium borate buffer, *p*H 9.8. The mixture was incubated in a boiling water bath for 3 min. and then rapidly cooled by dipping in ice . After addition of DMAB reagent [10g of dimethylaminobenzaldehyde in 12.5ml of 10N HCl and 87.5ml glacial acetic acid], the mixture was incubated for 20 min. at 37°C. Immediately thereafter, absorbance value at 585 nm was measured using a UV-VIS Spectrophotometer (Digispec 200GL). A standard curve of serial dilutions (0.5 – 2.5 mg $ml^{-1}$) of N-acetyl-D-glucosamine was prepared. The enzyme activity was expressed as μg GlcNAc $min^{-1}mg^{-1}$fresh tissue.

### 3.16.6. β-1,3-Glucanase (β-GLU; E.C. 3.2.1.39)

β-1,3-glucanase was assayed by the laminarin-dinitrosalicylate method (Pan *et al,* 1991) with modifications. One gram of the leaf sample was extracted with 3 ml of 0.05 M sodium acetate buffer, *p*H 5.0 supplemented with 2mM PVP-10 by grinding in pre-chilled mortar and pestle. The extract was then centrifuged at 10,000 g for 30 min at

4°C and the supernatant was used as crude enzyme extract. 62.5 µl of the crude enzyme extract was added to 62.5 µl of laminarin (4%) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent ( 1g of DNSA dissolved in 200mg crystalline phenol in 1% NaOH and 0.05g sodium sulphite) and heated for 5 min. in a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of water, vortexed and absorbance at 500 nm was determined in a UV-VIS Spectrophotometer (Digispec 200 GL). The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. A standard curve of glucose (50-250µg $ml^{-1}$ ) was prepared. The enzyme activity was expressed as µg Glucose $g^{-1}$ leaf tissue $min^{-1}$ .

### 3.16.7. Catalase (CAT; E.C. 1.11.1.6.)

For extraction of catalase, tea leaf tissue (1g) was crushed in mortar with pestle in 5ml of 0.1(M) sodium phosphate buffer ( pH 7.0) on ice with addition of a pinch insoluble PVP and sea sand. The homogenate was centrifuged immediately at 15,000 r.p.m. for 20 min. at 4°C. After centrifugation, the supernatant was collected and used immediately for assay. Catalase activity was determined by the method as described by Malolepsza and Rozalska(2005). The reaction mixture contained 2.9 ml 0.05 M sodium phosphate buffer (pH 7.0), 10µl of 4 mM $H_2O_2$ and 20µl of plant extract. The consumption of $H_2O_2$ was monitored spectrophotometrically at 240nm. Enzyme activity was expressed as the decrease in absorbance at 240nm $mg^{-1}$ protein $min.^{-1}$ ($\Delta A_{240}$ $mg^{-1}$ proteinmin.$^{-}$).

### 3.16.8. Ascorbate Peroxidase (APX; E.C. 1.11.1.11. )

For extraction of catalase, tea leaf tissue (1g) was crushed in mortar with pestle in 5ml of 0.1(M) sodium phosphate buffer ( pH 7.0) on ice with addition of a pinch insoluble PVP and sea sand. The homogenate was centrifuged immediately at 15,000 r.p.m. for 20 min. at 4°C. After centrifugation, the supernatant was collected and used immediately for assay. Ascorbate peroxidase activity was determined by the method as described by Zhou *et al* (2005). The reaction mixture contained 2.9 ml 0.05 M sodium phosphate buffer (pH 7.0), 10µl of 4 mM $H_2O_2$, and 50µl of ascorbic acid (0.01g ascorbic acid in 10 ml double distilled water) and 20µl of plant extract. The consumption

of ascorbic acid was monitored spectrophotometrically at 290nm. Enzyme activity was expressed as the decrease in absorbance at 290nm $mg^{-1}$ protein $min.^{-1}$ ($\Delta A_{290} mg^{-1}$ protein $min.^{-1}$).

## 3.17. Isozyme analysis by Native PAGE

The extraction of POX and PPO was conducted as described earlier for each enzyme separately. Casting and running of the Native PAGE was performed according to the method of Davis (1967), followed by staining of the gels for POX and PPO separately.

### 3.17.1. Preparation of stock solutions

Solution A. Acrylamide stock solution (Resolving gel)

28 g of acrylamide and 0.74 g of N'N' methylene bis acrylamide was dissolved in 100ml of warm double distilled water. The stock solution was filtered with Whatman No.1 filter paper and stored at $4^0$C in a dark bottle.

Solution B. Acrylamide stock solution (Stacking gel)

10 g of acrylamide and 2.5 g of N'N' methylene bis acrylamide was dissolved in 100ml of warm double distilled water. The stock solution was filtered with Whatman No.1 filter paper and stored at $4^0$C in a dark bottle.

Solution C.Tris – HCl (Resolving gel):

36.6 g of Tris base was mixed with distilled water and 0.25 ml of TEMED was added.The pH was adjusted to 8.9 with concentrated HCl.The volume of the solution was made up to 100 ml with distilled water. The solution was stored at $4^0$C for future use.

Solution D. Tris – HCl (Stacking gel):

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.8 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at $4^0$C for future use.

Solution E. Ammonium persulphate solution:

Fresh solution of ammonium persulphate was prepared by dissolving 0.015 g of ammonium persulphate in 10 ml of distilled water.

Solution F. Riboflavin solution:

Fresh solution of riboflavin was prepared by dissolving 0.4mg of riboflavin in 10ml of distilled water. The solution was kept in a dark bottle to protect from sunlight.

Solution G. Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6g of Tris base and 2.9g glycine in 1L of distilled water.

### 3.17.2. Preparation of gel

Mini slab gel was prepared. Two glass plates were thoroughly cleaned with dehydrated alcohol and then dried, 1.5mm thick spacers were placed between the glass plates on 3 sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution. 7.5% resolving gel was prepared by mixing solutions A : C : E : $dH_2O$ in the ratio 1 : 1: 4 : 1 by Pasteur pipette leaving sufficient space (comb + 1cm ) for stacking gel. The resolving gel was immediately overlayered with water and kept for polymerization for 2 hours. After polymerization of resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. 2.5% stacking gel solution was prepared by mixing solutions B : D : F : $dH_2O$ in the ratio 2 : 1 : 1 :4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water.The gel was kept for polymerization for 30 min. in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus.Tris – glycine buffer was added sufficiently in both upper and lower reservoir.

### 3.17.3. Sample preparation

Sample (32µl) was prepared by mixing the sample enzyme (20µl) with gel loading dye (40% sucrose and 1% bromophenol blue in distilled water) in cyclomixer in ice.All

the solutions for electrophoresis were cooled. The samples were immmediately loaded in a pre-determined order into the bottom of the wells with a micriliter syringe.

### 3.17.4. Electrophoresis

Electrophoresis was performed at constant 15mA current at 4°C until the dye front reached the bottom of the gel.

### 3.17.5. Staining

#### 3.17.5.1. Peroxidase

After electrophoresis, the gel was removed carefully from the glass plates and then stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was followed according to the method of Reddy and Gasber (1973). The gel was incubated in the aquaous (80ml) solution of benzidine (2.08g), acetic acid (18ml) and 3% $H_2O_2$ (100ml) for 5min.The reaction was stopped with 7% acetic acid after the appearance of bands. Analysis of POX isozymes was done immediately.

#### 3.17.5.2. Polyphenoloxidase

Staining for PPO analysis was done as described by Sadasivam and Manickam (1992). After equilibration of the gel for 30min. in 0.1% p-phenylenediamine in 0.1M potassium phosphate buffer (pH 7.0) at 4°C, it was stained in 10mM catechol solution dissolved in 0.1M potassium phosphate buffer (pH 7.0) for 1h. Analysis of bands was done immediately after their appearance.