# MATERIALS AND METHODS

## 3.1 Plant material

### 3.1.1 Selection

Thirty-seven tea varieties (Tocklai, UPASI and Darjeeling) released by three experimental stations viz. (i) Tocklai experimental station, Jorhat, Assam; (ii) UPASI Tea Research Centre, Valparai, Tamilnadu and (iii) Darjeeling Tea Research Centre, Kurseong, Darjeeling are being maintained in Tea Germplasm Bank, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal sponsored by the department of Biotechnology, Ministry of Science and Technology, Govt. of India. Based on the growing suitability of tea plants (*Camellia sinensis* (L) Kuntz) as observed under the field conditions over the year, the following twenty-three varieties were selected for the present study.

| | Tea varieties | Origin |
|---|---|---|
| TOCKLAI | | |
| | TV-9 | II |
| | TV-18 | III |
| | TV-20 | III |
| | TV-22 | III |
| | TV-23 | III |
| | TV-25 | III |
| | TV-26 | III |
| | TV-27 | III |
| | TV-28 | III |
| | TV-29 | I |
| | TV-30 | III |
| UPASI | | |
| | UP-2 | I |
| | UP-3 | I |
| | UP-8 | I |
| | UP-9 | II |
| | UP-26 | III |
| | [a]BSS-1 | I |
| | [b]BSS-2 | II |
| | [c]BSS-3 | III |
| DARJEELING | | |
| | HV-39 | V |
| | T-135 | V |
| | BS/7A/76 | V |
| | T-17/1/54 | IV |

I – Assam, II – China, III – Combod, IV – Assam x China, V – Darjeeling, BSS -Biclonal Seed Stock; [a]UPASI-10 X TRI-2025, [b]UPASI-2 X Tri-2025, [c]UPASI-9 X TRI-2025

### 3.1.2 Growth and maintenance

The selected tea varieties were propagated by cutting as recommended by Banerjee (1993) Sandy soil (sand 75% and soil 25%) with *p*H ranging from 4.5 - 4.8 was used for propagation of tea plants by cutting. Soil *p*H was adjusted by treating with 2% aluminium sulphate. Excess aluminium sulphate was removed by watering. Polythene sleeves (8"X6") were filled up with prepared soil and stacked in rows in a bed and watered thoroughly. All the cuttings were allowed for rooting in sleeves after dipping them in hormone. These cuttings were kept in a polythene cloche, arranged in two rows, with 10 beds in each row. The complete set up was kept under a green Agronet House. Each bed was watered regularly, initially by spraying but later on by pipe. After about ninety days i.e when all the cuttings had rooted and new shoots had come, the polythene cloche was removed gradually. The Young tea plants were maintained using nutrients (2% NPK :- 2:1:2 and 2% urea) and Tricontanol as foliar spray at an interval of 3 months. The tea sleeves were then transferred to pots and maintained in the glass house (Plate-3). Besides plants were also transferred to the Phytopathological Experimental Garden of the Botany Department, North Bengal University. Two months prior to inoculation the main stem and side shoots were trimmed in order to obtain new shoots. The plants were grown under natural condition of day light and temperature and watered on alternate days with ordinary tap water by sprinklers.

## 3.2 Fungal cultures

### 3.2.1 Source

*Alternaria alternata* (W8053) isolated from naturally infected leaves of tea variety T-17 and another isolate of *A. alternata* (W8055) from TV-22 were identified by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience UK. These were used after completion of Koch's postulate.

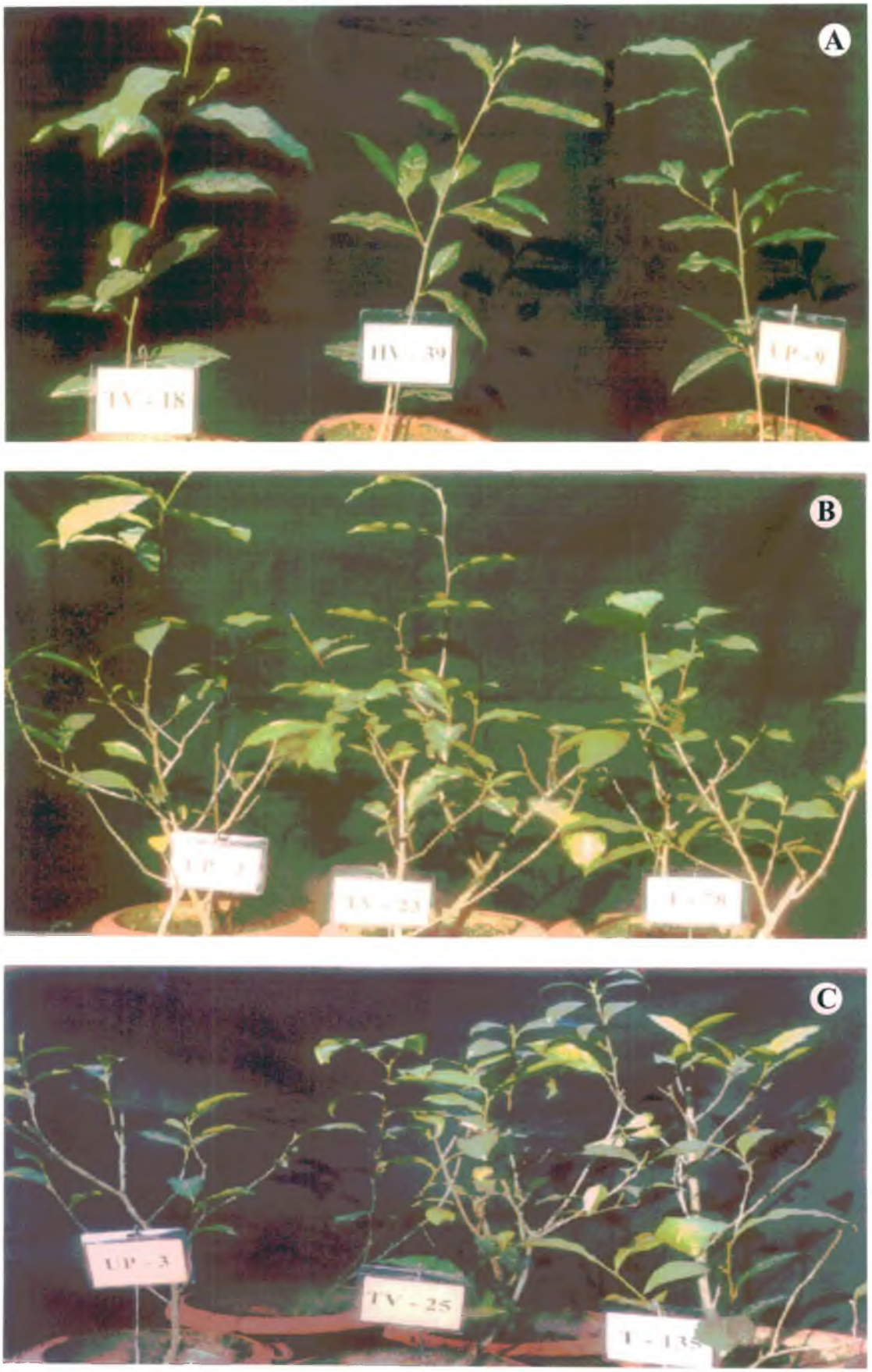


**Plate 3 (figs. A-C):** Tea varieties maintained in Glass House. (A)-TV-18, HV-39, UP-9; (B)-UP-3, TV-23, T-78; (C)-UP-3, TV-25, T-135

Black rot causing fungal pathogen (*Corticium invisum*) of tea was obtained from Tocklai Experimental Station, Jorhat, Assam. Another foliar fungal pathogen of tea - *Curvularia pallescens* (W7657), the entemogenous fungi - *Baeuveria bassiana* (MTCC-984) and *Metarhizium anisopliae* (MTCC-892) were obtained from stock culture maintained in Immuno Phytopathology Laboratory, Department of Botany, N.B.U.

### 3.2.2 Completion of Koch's postulate

Fresh, young tea leaves were collected from Phytopathological Experimental garden and inoculated with the conidial suspension of *A. alternata* and *C. invisum* ($5X10^5$ conidia/ml) separately following detached leaf inoculation technique Dickens and Cook (1989). After 72 h of inoculation, infected leaves were, washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ for 2-3 min, washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants, and incubated at 28 $\pm 1^0$C. After 7 days of incubation the isolated fungal culture was examined, compared with the respective stock cultures and identification was confirmed.

### 3.2.3 Maintenance of Stock culture

*A. alternata, C. invisum* and *C. pallescence* were grown in potato-dextrose-agar (PDA) medium where as *B. bassiana* and *M. anisopliae* were grown in yeast-glucose-agar and PDA, incubated at 28 $\pm$ 2$^0$C for two weeks and finally the cultures were stored at 5$^0$C and 28$^0$C. Re-isolation of the pathogens (*A. alternata* and *C. invisum*) were done every six months following detached leaf or cut shoot inoculation of tea leaves in order to maintain the virulence of cultures and were used for experimental purpose.

### 3.2.4 Assessment of mycelial growth

To assess mycelial growth of *A. alternata* and *C. invisum*, the fungus were grown in petriplates (9 cm.dia.), each containing 20ml. of PDA medium and incubated for 7 days at 28$\pm 1^0$C. From the mycelial mat agar block (4 mm.dia.) containing the mycelia

was cut with a sterilized cork borer and transferred to each Ehrlenmayer flask (250 ml.) containing 50 ml. of sterilized Potato Dextrose Broth for two weeks at 28±1$^0$C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60$^0$C for 96 h, cooled in a desiccators and dry weight was noted.

## 3.3 Inoculation technique

### 3.3.1 Detached leaf

The method as described by Dickens and Cook (1989) was used for artificial inoculation of tea leaves. Fully expanded young leaves were detached from plants and placed in aluminium trays (37.5cm. x 30cm.) lined with moist blotting paper. Their upper surfaces were wounded as suggested by Cook (1989). The wounds consisted of light scratches on the upper epidermis made with the point of a fine sterilized scalpel. On the either side of the midrib, two of four such wounds were made in each leaf which was immediately inoculated with the inoculum block (2 mm dia) of *C. invisum* and conidial suspension (5X10$^5$ conidia/ml) of *A. alternata*. Fifty leaves were inoculated in each treatment. In control sets wounds were made on the leaves as described and droplets of sterile distilled water was placed. Each tray was covered with a glass lid and sealed with petroleum jelly to minimize the drying of drops during incubation.

### 3.3.2 Cut shoot

Cut shoot inoculation technique was followed by Yanase and Takeda (1987). Twigs with three to four leaves of tea plants grown in the experimental garden were cut carefully with sharp blade and immediately introduced into the glass chamber (3ft x 2ft x 1.5ft) with the twigs dipped in a floating thermocol. Leaves were inoculated by making two light scratches with point of a fine scalpel on the upper surface of leaves as described by Cooks (1989). Inoculum blocks (2 mm. dia.) of 10-day-old culture of *C. invisum* were taken from the advancing zone aseptically and placed on the inoculation site and then covered with absorbent cotton wool moistened with

sterile distilled water. Sterile PDA was used as control. For each treatment 50 cut shoots were inoculated.

### 3.3.3 Whole plant

Well established and branched tea plants (2 yr.old) grown in pots were inoculated with *A. alternata* following the method of Dickens and Cook (1989). Inoculation was done by spraying conidial suspension ($5X10^5$ conidia/ml) prepared from 15-days old culture of *A. alternata* grown on PDA. In control sets, the plants were sprayed with sterile distilled water. Inoculated as well as control plants were placed in polythene covered frames in order to maintain relative humidity near 80% for 48h. Subsequently the polythene was removed.

## 3.4 Assessment of disease caused by fungal pathogens

### 3.4.1 Detached leaf

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

### 3.4.2 Cut shoot

At the onset, the number of lesions that developed on the artificially inoculated tea twigs by mycelial blocks of *C. invisum* was counted. Diameter of the individual lesion was measured and they were graded into four groups and a value was assigned to each group for small restricted lesion diameter of 2-4mm (0.1), 4-6mm with sharply defined margin (0.25), slow sprayed beyond 6mm (0.50), and spreading lesions of variable size, with diffused margin (1.0). Finally number of lesion of each group was multiplied by the value assigned to it and the sum total of each values were noted and disease index was computed as the mean of observations on 50 cut shoots per treatment. Data was taken after 24, 48 and 72h after inoculation.

### 3.4.3 Whole plant

Disease intensity was assigned following whole plant inoculation technique at 5 days of intervals after inoculation up to 25 days as described by Chakraborty and

Saha (1994). On the basis of visual observation lesions were graded into four size groups, viz., very small, small, medium and large with respective values of 0.1, 0.25, 0.50, and 1.0 assigned to give an appropriate idea of their relative size. Number of lesions in each size group was multiplied by the values assigned. The sum total of such values for all the leaves gave the disease index for a plant. Results were always computed as the mean of observation of 23 plants (50 young leaves randomly picked up from each plant) per treatment.

### 3.5 Assessment of incidence of attack by *Helopeltis theivora*

Disease assessment was determined on 10-year-old tea bushes of 23 different tea varieties maintained in open field condition following the method of assessment as described by Somchowhdury *et. al.*, (2001) with modification. Incidence was recorded regularly every week throughout the year and monthly averages were computed for three consecutive years, from 2002 to 2004. Incidence was calculated as the percentage of infested shoots on each of the twenty-three tea trees.

### 3.6 Inducing agents and their application.

#### 3.6.1 Plant extract

Mature leaves (400g) each of *Azadirachta indica*, *Catharanthus roseus*, and *Diplazium esculentum* were homogenized separately in a electric blender. After centrifugation leaf extracts were diluted (1:10) with DW. TWEEN-80 was mixed with all extracts (1:10) and sprayed on tea plants (10 year-old) with the help of sprayer. The control plants were sprayed with distilled water mixed with TWEEN-80. Spray was done four times at 15 days interval in the after noon. Both treated and untreated plants were inoculated with *A. alternata* following whole plant inoculation methods and disease assessment was made. Leaves from control and treated plants were sampled for biochemical analyses and immunological assays.

### 3.6.2 Biocrop

A liquid formulation with plant extracts namely 'Biocrop', an organic fertilizer and known plant growth inducer marketed by AKS Bio and Herbals Pvt. Ltd., Mumbai was used for foliar application in tea plants in order to assess their effect on the incidence of pest attack by *H. theivora*. The liquid product was diluted with distilled water (100ml/liter) mixed with TWEEN-80 before spraying in the evening. Four sprays were done at 15 days interval. The control plants were sprayed with distilled water mixed with TWEEN-80. Leaves from control and treated plants were sampled for biochemical analysis and immunological assays.

### 3.6.3 Metabass

The entomogenous fungi - *M. anisopliae* (MTCC-892) and *B. bassiana* (MTCC-984) were grown on liquid medium. The conidia were harvested by filtering through a double layered mesh. Conidial count was determined microscopically by means of Neubauer haemocytometer. These were serially diluted in distilled water and 0.2% Tween 80 was added to the solution. Metabass formulation (liquid) as outlined by Gurusubramanian *et. al.,* (1999) was prepared and foliar application on tea plants (10- years old) were made four times at 15 days interval in order to assess their effect on the incidence of pest attack by *H. theivora* in relation to the control plants which were sprayed with distilled water mixed with TWEEN-80.

### 3.6.4 Salicylic acid

Salicylic acid (SA)which plays a critical signaling role in the activation of defense responses after pathogen attack was also applied exogenously on the tea plants as foliar spray. A specific concentration (15mM) of SA supplemented with Tween-80 was used for spraying at intervals of 15 days. The control plants were sprayed with distilled water mixed with TWEEN-80. Indirect immunofluorescence and immunogold localization of defense enzyme was done with treated and untreated leaves.

## 3.7 Extraction of total soluble proteins

### 3.7.1 Leaf protein

Soluble proteins were extracted from healthy naturally infested and artificially inoculated tea leaves following the method of Chakraborty *et al.* (1995). Leaf tissues (1g) were homogenized with 0.05 M sodium phosphate buffer (*p*H 7.2) containing 10 mM- $Na_2S_2O_5$; 0.5 mM - $MgCl_2$ ; 2mM polyvinyl pyrrolidone (PVPP 10,000 M) and 2 mM poly methyl sulphonyl fluoride (PMSF) in mortar with pestle at $4^0$C with sea sand and PVPP 40,000 M. The homogenate was centrifuged at $4^0$C for 20 min. at 10,000 r.p.m and the supernatant was used as crude protein extracts and immediately stored at $-20^0$C for further use.

### 3.7.2 Mycelial protein

Extraction of mycelial protein of *A. alternata* and *C. invisum* was done following the method of Chakraborty and Purkayastha (1983). Fungus were grown in sterilized Potato Dextrose Broth (PDB) for 14 days at 28 $\pm 1^0$C. Mycelia were collected, washed with 0.2% NaCl solution, rewashed with sterile distilled water, strained through cheese cloth and then crushed with sea sand and 0.05 M sodium phosphate buffer (*p*H 7.2) using mortar and pestle at $4^0$C. The slurry was centrifuged at 10,000 r.p.m for 20 min. at $4^0$C. The supernatant was used as crude protein extract and stored at $-20^0$C for further use.

## 3.8 Estimation of protein content

Protein estimation was done following the method of Bradford (1976). The Bradford's reagent was prepared in the following way; 100 mg of Comassie Brilliant Blue $G_{250}$ (Biorad) was dissolved in 50 ml. of 95% ethanol, followed by addition of 100 ml. of concentrated phosphoric acid and deionized water up to a volume of 200 ml. This stock solution was diluted 5 times with distilled water and filtered through Whatman No. 1 filter paper during estimation. A standard curve was prepared with Bovin Serum Albumin (Sigma). The standard curve showed linearity from 20-150 μg.

of protein sample in 100 µl. To the 100 µl. of test protein sample 5 ml. of Bradford's reagent was added, mixed in a cyclomixture and incubated at room temperature for 5 min. for blue color development, following which O.D was measured at 595 nm. By UV-spectrophotometer (SICO, model Digispec 200 GL). The O.D values were potted on the standard curve prepared for the purpose and the quantity of protein determined from this.

**3.8.1 SDS-PAGE analysis of total soluble protein**

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

**3.8.1.1 Preparation of stock solutions**

For the preparation of gel following stock solutions were prepared:

- Acrylamide and N'N'- methelane 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 *p*H of the solution was kept below *p*H 7.0 and the stock solution was filtered through Whatman No.1 filter paper and was kept in dark bottle, stored at $4^0$C and used within one month.

- Sodium Dodecyl Sulphate (SDS) :

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

- Tris buffer:

**I** 1.5 M tris buffer was prepared for resolving gel. The *p*H of the tris was adjusted to 8.8 with concentrated HCl and stored at $4^0$C for use.

**II** 1.0 M tris buffer was prepared for use in the stacking and loading buffer. The *p*H of this tris adjusted to 6.8 with concentrated HCl and stored at $4^0$C.

- Ammonium Persulphate (APS) :

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

- Tris – Glycine eletrophoresis buffer:

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

- SDS gel loading buffer:

This buffer contains 50 mM tris HCl (*p*H 6.8), 10 mM β Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was made by dissolving 0.5 ml. of 1M tris buffer (*p*H 6.8), 0.5 ml. of 14.4 M β mercaptoethanol, 2 ml. of 10% SDS, 10 mg. bromophenol blue, 1 ml. glycerol in 6.8 ml. of distilled water.

**3.8.1.2 Preparation of gel**

Slab gel of two sizes were prepared for the analysis of protein pattern by SDS-PAGE i.e. big gel (plate size 17cm. X 19cm.) and mini gel (8cm. X 10cm.). For both types of slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm. thick spacers were placed between the glass plates at the three sides and the three sides of glass plates were sealed with high vacuum grease and clipped thoroughly to prevent and leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order by pasture pipette leaving sufficient space for comb the stacking gel (comb + 1cm.).

Composition of solutions for 10% resolving gel :

| Name of compounds | Mini gel (ml.) | Big gel (ml.) |
|---|---|---|
| Distilled water | 2.85 | 11.90 |
| 30% Acrylamide mix | 2.55 | 10.00 |
| 1.5 M Tris (*p*H 8.8) | 1.95 | 7.50 |
| 10% SDS | 0.075 | 0.30 |
| 10% APS | 0.075 | 0.30 |
| TEMED | 0.003 | 0.012 |

Composition of solutions for 5% stacking gel :

| Name of compounds | Mini gel (ml.) | Big gel (ml.) |
|---|---|---|
| Distilled water | 6.8 | 2.1 |
| 30% Acrylamide mix | 1.7 | 0.5 |
| 1.5 M Tris (*p*H 6.8) | 1.25 | 0.38 |
| 10% SDS | 0.10 | 0.03 |
| 10% APS | 0.10 | 0.03 |
| TEMED | 0.01 | 0.003 |

After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for 2 hrs. for polymerization. After polymerization of the resolving gel was complete over lay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30 min. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris – glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

**3.8.1.3 Sample preparation**

Sample (32µl.) was prepared by mixing the sample protein with 1 X SDS gel-loading buffer (16µl) in cyclomixture. All the samples were floated in boiling water bath for 3 min. to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the wells with a fin pepatte. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low molecular weight (Phosphorylase b - 97,400; Bovin Serum Albumin – 68,000; Ovalbumin – 43,000; Carbonic Anhydrase 29,000;

Soyabean Trypsin inhibitor – 20,000; Lysozyme – 14,300) was treated as the ather samples and loaded in a separate well.

**3.8.1.4 Electrophoresis**

Electrophoresis was performed at constant 15 mA current for a period of 3 hrs. in case of mini gel and a constant 30 mA for a period of about 6 hrs. for large gel until the dye front reached bottom of the gel.

**3.8.1.5 Fixing and staining**

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

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

The gel was removed from fixer and stained in this staining solution for at least 4 hrs. at $37^0$C with constant shaking at very low speed. After staining the gel was finally de-stained with de-staining solution containing methanol, water and acetic acid (45:45:10) at $40^0$C with constant shaking until the back ground become clear.

## 3.9 Extraction and assay of defense enzymes

Among the defence enzymes Phenylalanine ammonia lyase, Polyphenol-oxidase, Peroxidase (PR-9), Chitinase (PR-3) and β-1,3-glucanase (PR-2) were considered for both pest and pathogen attack.

### 3.9.1 Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5)

For the extraction of Phenylalanine ammonia lyase (PAL) the methods of Chakraborty *et. al.*, (1993) was followed. Leaves (1gm) were crushed in a mortar with pestle in 5ml of 0.1M sodium borate buffer *p*H 8.8 containing 2mM β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 rpm for 20 min. at $4^0$C.

The supernatant was collected and after recording its volume was used immediately for assay or stored at –20$^0$C.

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture containing 0.3 ml 300μM sodium borate (*p*H 8.8); 0.3 ml. 30μM L-Phenyl alanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1hr. at 40$^0$C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as μg cinnamic acid $g^{-1}$ tissue $min^{-1}$.

**3.9.2 Tyrosine ammonia lyase (TAL)**

For the extraction of Tyrosine ammonia lyase (TAL) leaves (1gm) were crushed in a mortar with pestle in 5ml of 0.1M sodium borate buffer *p*H 8.8 containing 2mM β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 rpm for 20 min. at 4$^0$C. The supernatant was collected and after recording its volume was used immediately for assay or stored at –20$^0$C.

TAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-tyrosine spectrophotometrically. The reaction mixture containing 0.3 ml 300μM sodium borate (*p*H 8.8); 0.3 ml. 30μM L-tyrosine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1hr. at 40$^0$C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as μg cinnamic acid $g^{-1}$ tissue $min^{-1}$.

**3.9.3 Polyphenol-oxidase (PPO) (EC 1.10.3.2)**

For the extraction of polyphenoloxidase (PPO) the method of Mahadevan and Ulaganathan (1991) was followed with a little modification. Leaf tissue were cut into pieces and then crushed in mortar with pestle in ice with 5ml of 0.2M sodium-phosphate buffer *p*H 6.6. The slurry was immediately centrifuged at 10,000 rpm for 20min at 4$^0$C. The supernatant was collected and after recording its volume was used immediately for assay or stored at –20$^0$C.

For the determination of PPO activity 1ml of freshly prepared enzyme extract was mixed with 2ml of 0.2M sodium-phosphate buffer (*p*H 6) containing 0.01M pyrogallol in the dark. Initial absorbance was noted at 495nm immediately. The reaction mixture was incubated at room temperature in the dark for the prevention of photo-oxidation of the enzyme. Further reading was taken after 30min at 495nm. The blank was set with only 3ml of phosphate buffer. PPO activity was expressed as $A_{495}$ $g^{-1}$tissue $min^{-1}$, when the substrate pyrogallol was oxidized due to the enzyme activity from 1gm of tissue.

**3.9.4 Peroxidase (PO) (EC 1.11.1.7)**

To extract Peroxidase (PO) the methods of Chakraborty *et. al.*, (1993) was followed with modification. Tea leaves samples were crushed with 0.1M sodium borate buffer *p*H 8.8 containing β-mercaptoethanol in a mortar with pestle on ice. The homogenate was centrifuged immediately at 15,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and its final volume was measured and used immediately for assay or stored at –20°C.

For the determination of PO activity 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2M sodium-phosphate buffer (*p*H 5.4), 100 µl of 4mM hydrogen peroxide ($H_2O_2$), 100µM of O-dianisidine (5mg/ml methanol) and 1.7ml of distilled water. PO activity was assayed spectrophotometrically at 460nm oxidation of O-dianisidine in presence of $H_2O_2$. Specific activity was expressed as the $\Delta$ $A_{460}$ $g^{-1}$ tissue $min^{-1}$.

**3.9.5 Chitinase (CHT) (EC 3.2.1.14.)**

To extract CHT 1gm of sample was crushed in 5ml of 0.1M sodium-citrate buffer *p*H 5 with sea sand and insoluble PVPP in a mortar with pestle on ice as suggested by Boller *et al.*, (1988). The homogenate was centrifuged immediately at 12,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording the final volume was used immediately for assay or stored at -20°C.

For the determination of CHT activity 1ml of enzyme extract and 1ml of substrate (chitin10mg/ml) and phosphate buffer were incubated and shakened at 37°C for 1 hr.

Then the solution was centrifuged at 12,000 rpm for 10 min. After centrifugation 0.5ml of the supernatant were added to 3ml of DNSA (Dinitro salicylic acid). The reaction mixtures were incubated in boiling water bath for 10min. CHT activity was assayed spectrophotometrically at 540nm.The blank was prepared by enzyme and substrate with zero hr of incubation. Absorbance value was measured at 585nm and N-acetyl-D-glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as mg GlcNAc $g^{-1}$ tissue $h^{-1}$.

**3.9.6 β-1, 3-glucanase (βGLU) (EC 3.2.1.39)**

β-1, 3-glucanase activity was assayed by the laminarin-dinitrosalicylate method as suggested by Pan *et al.* (1991). Leaf samples were homogenized in 0.05M sodium acetate buffer, *p*H 5.0. The homogenate was centrifuged immediately at 12,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and its final volume was measured and used immediately for assay or stored at –20°C.

For the determination of βGLU activity the crude enzyme extract of 62.5 µl 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 reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexes and the absorbance was determined at 500nm in a spectrophotometer and enzyme activity was expressed as µg glucose $g^{-1}$ tissue $min^{-1}$.

**3.10 Extraction of phenolics**

Total phenol and orthodihydroxy phenol content of healthy artificially inoculated with *A. alternata, C. invisum* and naturally infested with *H. theivora* tea leaves of resistant and susceptible varieties were extracted following the method of Mahadevan Ulaganathan (1991). In case of artificial inoculation detached leaf inoculation technique as described earlier as followed. In case of control, sterile distilled water was mounted on the adaxial surface of leaves. Phenols were extracted from 1 gm each of healthy and infected leaves separately in boiling 80% ethanols (4 ml. ethanols/g fresh weight leaf tissue) for 10 min. cooled, and crushed thoroughly passed through

two layers of cheese cloth and then filtered through filter paper. Final volume was adjusted with 80% ethanol (5ml/g fresh weight of leaves).

### 3.11 Estimation of phenol content

#### 3.11.1 Total phenol

The total phenol was estimated by Folin ciocalteau's reagent as described by Mahadevan and Ulaganathan (1991). One ml. Of the alcohol extract was taken in a test tube, 1 ml. of Folin ciocalteau's reagent following by 2 ml. of 20% $Na_2CO_3$ solution was added. The tube was shaken and heated on a boiling water bath for 1 min. and volume was raised to 25 ml. Absorbance was measured in a Systronics photoelectric colorimeter Model-101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

#### 3.11.2 Orthodihydroxy phenol

The orthodihydroxy phenol was estimated as described by Mahadevan and Ulaganathan (1991). One ml. of the alcohol extract was taken in a test tube 2 ml. of 0.05 N HCl, 1 ml. of Arnow's reagent ($NaNO_3$ -10g ; $Na_2MoO_4$ –10g ; distilled water 100 ml.) and 2 ml. 0f 1 N NaOH were added, following which the volume was raised to 25 ml. Absorbance was recorded by Systronics photoelectric colorimeter Model-101 at 515 nm. Quantity of orthodihydroxy phenol was estimated using caffeic acid as standard.

### 3.12 Extraction of antifungal phenolics

The extraction method used was adopted from the Daayf *et. al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Tea leaves were collected from the experimental garden and detached leaf inoculation with spore suspension of *A. alternata* and sclerotial suspension of *C. invisum* was followed for artificial inoculation as well as natural infested leaves by *H. theivora.* Both the healthy and inoculated leaves were harvested separately after 48 h of inoculation. Infested leaves were collected for experiment. Leaf samples (10 g) were

mixed with 80% methanol at 10ml./g tissue and homogenized by blending for about 1 min. Samples were extracted for 48 hrs. on a rotary shaker in a conical flask at 40 r.p.m. covered with aluminium foil for protection from light. Methanolic extracts were then collected by filtration on a Whatmann No. 1 filter disc and concentrated by evaporation to a final volume of 20 ml. (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethylether three times. The ether fraction were stored and termed as Fraction I. The aqueous fraction was portioned secondly against equal volume of ethyl acetate also for three times and the ethyl acetate portioned was considered as Fraction II.

Acid hydrolysis, with 4N HCl, of the remaining aqueous fraction (yielding phenolic aglycones) was performed according to the method of Daaf *et al.*, (1997). Aglycones were recovered by partitioning hydrolysates against equal volume of ethyl acetate also for three times and labeled as fraction III. All the fractions were evaporated to the dryness and finally dissolved in 3 ml. of respective solvent.

### 3.12.1 Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea leaves were analysed by thin layer chromatography (TLC) on silica gel G. The development of the chromatograms was carried out at room temperature and using a chloroform:methanol solvent system (9:1 v/v) as suggested by Chakraborty and Saha (1994). Following evaporation of the solvent, thin layer plates were observed under UV light and sprayed separately either with diazotized p-nitroaniline (Van Sumere *et al.* 1965), vanillin – $H_2SO_4$ (Stahl, 1967) or Folin-ciocalteau's phenol reagent (Harborne, 1973). Color reactions and Rf values were noted.

### 3.12.2 Bioassays of antifungal phenols

#### 3.12.2.1 TLC plate bioassay

All the fractions derived from the extraction procedure were analysed by TLC on silica G. All the three fractions were spotted on TLC plates and the development of the chromatogram was carried out at the room temperature using a ethyl acetate : chloroform (9:11) solvent mixture as suggested by Chakraborty and Saha (1994).

After development chromatogram inhibition assay was performed as devised by Hoffmans and Fuchs (1970) using *Curvularia pallescence* as the test organism. Spore suspension in 2% sucrose supplemented with Richard's Medium were sprayed on the developed TLC plates and incubated in a sterile humid chamber at $25^0$C for 6 days. Fungitoxicity was ascertained by the appearance of inhibition zone, which was visualized as white spots surrounded by a deep black background of mycelia. Diameter of inhibition zones and Rf values were noted.

**3.12.2.2 Radial growth**

Radial growth inhibition assay as described by Van Etten (1973) was followed. Ethyl acetate fraction of healthy, infected (with fungal pathogens) and infested (with *H. theivora*) extracts (0.2 ml.) were taken separately in each of the sterile Petriplate (0.2 ml.) was initially taken and allowed to evaporate. In control sets, only ethyl acetate (0.2 ml.) was initially taken and allowed to evaporate. Subsequently 10 ml. sterilized PDA was poured in each petriplates, thoroughly mixed and allowed to solidify. Agar block (3 mm.dia.) was cut with a sterilized cork borer from the advancing zone of 7 days old culture of *C. invisum* and *A. alternata* grown in PDA and transferred to each Petriplate. Radial growth of *C. invisum* and *A. alternata* were recorded after 3 days of inoculation at 28 ± $2^o$C.

**3.12.2.3 Spore germination**

The regions of thin layer chromatograms corresponding to the inhibitory zones were scrapped and eluted again. The eluants were tested for antifungal activities following spore germination test with *A. alternata* (at least $5x10^5$ spores/ml.) described by Werder and Kern (1985).

**3.12.2.4 UV- spectrophotometric analysis**

For spectral analysis of antifungal compounds extracted from healthy, *A. alternata* inoculated as well as *H. theivora* infested leaves, initially ethyl-acetate fraction were plotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent, silica gel from corresponding antifungal zones as detected in chromatogram inhibition assay as well as in spore germination test were scrapped off and eluted

separately in methanol. These were re-spotted on TLC plates and developed in the same solvent and again scrapped and eluted in spec. methanol. The purified eluents were examined by UV-spectrophotometer (Simadzu model 160) and the maximum absorption was determined.

### 3.12.3 Extraction of catechins from tea leaves

Catechin was extracted from tea leaf tissue artificially inoculated with conidial suspension of *A. alternata* and natural infested leaves by *H. theivora* following the method of Obanda and Owuor (1994) with modification. Leaf samples (10g) were extracted with 100 ml. of 80% acetone at $45^0$C in water bath for 30 min. Extracts were decanted and filtered through Whatman No. 1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 20 ml. distilled water. Water solution was extracted with equal volume of chloroform for four times. The *p*H of the water layer was adjusted to 2 by 2 drops of 2 N HCl and finally dissolved in 3 ml. of 2% acetic acid. The samples were finally filtered through milipore filter (Milipore 0.4 μm HA filter paper).

#### 3.12.3.1 HPLC analysis of catechins

Catechin analysis of the extract was carried out on C 18 hypersil column using linear gradient elution system as follows: mobile phase A 100% acetonitrile; mobile phase B 2% acetic acid in water; elution; 88% B for 6min and then linear gradient to 75% B over 5min; the elution was complete after a total of 25min. Flow rate 1 μl/ min, sensitivity 0.5 aufs, injection volume 40ml monitored at 278nm (Shimadzu, Japan). Antifungal phenols extracted from healthy and infected tea leaves Fraction III were also quantified at 280nm using HPLC grade methanol.

## 3.13 Preparation of antigen

### 3.13.1 Mycelial antigen

Mycelial antigens were prepared from the mycelia of *C. invisum* and *A. alternata* grown in liquid medium following the method of Chakraborty and Saha (1994). The mycelial content was stained through muslin cloth dried and its dry weight taken. The

mycelia (6.9g and 6.2g respectively) were homogenized in mortar and pestle with 0.05M sodium phosphate buffer supplemented with 10mM Sodium meta bisulphate, 2mM soluble PVPP 10,000 and 0.05mM $MgCl_2$ (*p*H 7.2). At the time of crushing insoluble PVPP and sea sand was used. The crushing was done in cold. The slurry was centrifuged at 10,000 rpm for 30min. at $4^0$C. The supernatants were used as mycelial antigens and stored at $-20^0$C.

**3.13.2 Leaf antigen**

Antigens were prepared from healthy tea leaf tissue variety TV-18 following the Alba and DeVay (1985) with modification. Healthy leaves (1g) were crushed in a mortar and pestle with 0.05M sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5mM $MgCl_2$, 2mM soluble polyvinyl pyrrolidone (PVPP 10,000M) and 2mM poly methyl sulphonyl fluoride (PMSF) at $4^0$C for 20 min. at 10,000 rpm. The supernatants were used as leaf antigens and stored at $-20^0$C.

## 3.14 Production of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against the fungal mycelia of *Alternaria alternata*, and *Corticium invisum* and also against tea leaf antigens. Besides, polyclomanal antibody against one of the defense enzyme chitinase (product no.) was prepared from *Serratia marcescens* (Sigma) (PAb-CHT). The concentration of chitinase in each injection was 50μg/ml.

Initially, body weights were recorded and were observed for at least one week inside the cages before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetables etc., in morning and evening. After each bleeding they were given saline water for 3 consecutive days, cages were cleaned everyday in the morning for better hygenic conditions.

**3.14.1 Immunization**

Before immunization, normal serum was collected from the rabbit. The rabbit was subcutaneously injected once a week at 7 days interval with 1ml antigen mixed with

1 ml of Freund's complete adjuvant (Difco, USA) for first two injections and the next emulsified with incomplete adjuvant for 4 to 5 weeks.

### 3.14.2 Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board fixed at an angle of 60°. The neck of the rabbit was held tight in 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 from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 to 10ml of blood samples were collected in sterile graduated glass tube.

After collection, all the precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were incubated at 37°C for 1hr for clotting. After clotting, the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000g for 10 mins at room temperature) and distributed in 1ml vials and stored at – 20°C, as crude antisera.

## 3.15 Purification of IgG

### 3.15.1 Precipitation

IgG was purified as described by Clausen (1988). The polyclonal crude antiserum of leaf, mycelium and enzymes (chitinase & laminarionase) (2ml each) was first diluted with two volumes of distilled water and an equal volume of 4M ammonium sulphate. The *p*H was adjusted to 6.8 and the mixture was stirred for 16hrs at 22°C. The precipitate thus formed was collected by centrifugation at 10,000g at 22°C for 1hr. Then the precipitate was dissolved in 2ml of 0.02 M sodium phosphate buffer, *p*H 8.0.

### 3.15.2 Column preparation

Eight grams of DEAE-cellulose (Sigma Co. USA) was suspended in distilled water and kept overnight. The water was poured off and the gel was suspended in 0.005 M sodium phosphate buffer *p*H 8.0. Washing with buffer was repeated 5 times. The gel was then suspended in 0.02 M sodium phosphate buffer, *p*H 8.0 and was applied to a 2.6 cm diameter and 30 cm high column and allowed to settle for 2 h. After the column material had settled, 25 ml of buffer (0.02 M Sodium Phosphate buffer, *p*H 8.0) washing was given to the gel material.

### 3.15.3 Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant *p*H and with a continuous change in molarity from 0.02 M to 0.3 M. The initial elution buffer was 0.02 M sodium phosphate buffer *p*H 8.0 (I) and the final elution buffer was 0.3 M sodium phosphate buffer *p*H 8.0 (II).

The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of the buffer (I) was connected to another flask with buffer (II) to the column, buffer (II) was sucked into buffer (I) thereby producing a continuous rinse in mobility. Ultimately 40 X 5 ml. fractions were collected and optical density values were recorded by means of UV spectrophotometer at 280 nm. The fractions showing >2 reading were stored as purified IgG.

## 3.16 Immunodiffusion tests

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). Initially glass slides (5cm x 5cm) were degreased in 90% (v/v) ethanol, ethanol: diethyl ether (1:1) and then dried in a hot air oven. After drying, plates were sterilized in an autoclave at 15 lb for 20min. Agarose gel was prepared in Tris-barbiturate buffer, *p*H 8.6, at 90$^{0}$C; 0.9% agarose (Sigma, USA) was added into the buffer and placed on a water bath and stirred till the agarose solution became

clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added. For gel preparation, 10ml of molten agarose was added per slide; after pouring, it was kept for solidification and then wells were cut (8mm. diameter). The antigens and undiluted antisera (100µl/ well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 72 h at 25°C. After immunodiffusion, the slides were initially washed with sterile distilled water for 2 hrs. and then aqueous sodium chloride solution (0.9% NaCl + 0.1% $NaN_2$) for 72 hrs. with 6 hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. Then slides were stained with Coomassie blue ($R_{250}$) for 10 min at room temperature. After staining, slides were de-stained with 5% acetic acid solution until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3 hrs. at 50°C.

### 3.17 Enzyme linked immunosorbent assay (ELISA)

The following buffers were prepared following the method as described by Chakraborty *et al,* (1995), with modifications.

- Antigen coating buffer : Carbonate Bicarbonate buffer 0.05 M *p*H-9.6.

  **I.** Sodium Carbonate - 5.2 g in 1000ml distilled water

  **II.** Sodium bicarbonate - 4.2 g in 1000ml distilled water.

160 ml of stock **I** was mixed with 360 ml of stock **II.** and *p*H was adjusted 9.6.

- Phosphate Buffer Saline : 0.15 M PBS *p*H-7.2.

  **I.** Sodium dihydrogen phosphate - 23.40 g in 1000ml distilled water

  **II.** Di-Sodium hydrogen phosphate - 21.29 g in 1000ml distilled water

  280 ml of stock **I** was mixed with 720 ml of stock **II.** and the *p*H was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

- 0.15 M Phosphate buffer Saline - Tween (0.15 M PBS - Tween, *p*H 7.2).

  To 0.15 M PBS, 0.05% Tween 20 was added and the *p*H was adjusted to 7.2.

- Blocking reagent (Tris buffer saline, *p*H 8.0)

  0.05 M Tris, 0.135 M NaCl, 0.0027 M KCl

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

Distilled water was added to make up the volume to 100ml. Then *p*H was adjusted to 8.0 and 0.05% Tween 20 and 1% BSA were added.

- Antisera dilution buffer (0.15 M PBS – Tween, *p*H 7.2).

In 0.15 M PBS – Tween, *p*H 7.2, 0.2% BSA, 0.02% Polyvinylpyrrolidone 10,000 and 0.03% sodium azide was added.

- Substrate

p-Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in 100ml of 1% diethanolamine, *p*H 9.8.

- Stop solution - 3 N NaOH

This ELISA was performed following the method as described by Chakraborty *et. al.*,(1995) with modifications. Plant and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl / well) in 8 welled ELISA plates (Costar EIA/RIA strip plate, USA), arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25°C for 4 h. Then the plate was washed 4 times under running tap water and twice with PBS-Tween and each time shaken to dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200µl / well) to each well and incubated at 4°C overnight. After a further washing, anti-rabbit IgG goat antiserum labeled with Alkaline Phosphatase diluted 10,000 times in PBS, was added to each well (100µl / well) and incubated at 37°C for 2 h. The plate was washed, dried and each well was loaded with 100µl of p-Nitrophenyl phosphate substrate and kept in dark for 60 mins. Colour development was stopped by adding 50µl/ well of 3 N NaOH solution and the absorbance was determined in an ELISA Reader (Multiskan EX, Labsystems) at

405nm. Absorbance values in wells not coated with antigens were considered as blanks.

### 3.18 Fluorescence Antibody Staining and Microscopy

Indirect fluorescence staining of fungal sclerotia of *Alternaria alternata*, *Corticium invisum* and also cross sections of healthy and infected tea leaves were done using FITC labelled goat anti-rabbit IgG following the method of Chakraborty and Saha (1994), with modifications.

#### 3.18.1 Fungal mycelia:

Fungal mycelia were harvested in PBS. The mycelial suspension was centrifuged at 2000 r.p.m. for 1 min. The supernatant was discarded and the pellet was re-dissolved in PBS (0.5 ml). Then the suspension was re-centrifuged as before followed by two more washings in PBS. Supernatant was discarded and the material was incubated in an antibody dilution (1:10) for 60 min. Washing in PBS by centrifugation was repeated as before at 2000 r.p.m. for 1 min. To the material 50µl FITC (Fluorescein Iso-Thiocyanate) - conjugate diluted in PBS (1:40) was added and incubated for 30 min. It was then washed thrice in PBS and centrifuged as before. The supernatant was discarded. The pellet was mounted in 10% glycerol. It was covered with cover glass, sealed and observed under a Leica Leitz Biomed microscope.

#### 3.18.2 Tea leaves:

Fresh cross section of healthy tea leaves were cut and immersed immediately in PBST. The sections were washed thrice in PBS on grooved slides. After washing the sections were incubated in an antibody dilution (1:10) for 60 min. Washing in PBS was repeated as before. To the material 50µl FITC (Fluorescein Iso-thiocyanate) - conjugate diluted in PBS (1:40) was added and incubated for 30 min. After incubation sections were washed thrice in PBS and mounted in 10% glycerol. Each section was covered with a cover glass, sealed and observed under a Leica Leitz Biomed microscope.

### 3.19 Dot-immuno binding assay

Dot-blot was performed following the method suggested by Lange & Heide (1986). Following buffers were used for dot-blot:

- Carbonate-bicarbonate buffer (0.05 M, *p*H-9.6 coating buffer).
- Tris buffer saline (10 mM, *p*H-7.4) with 0.9% NaCl and 0.05% Tween - 20 for washing,
- Blocking buffer - 10% Casein hydrolysate in 0.05 M Tris, 0.5 NaCl, 0.5% Tween-20, *p*H-10.3.

Nitrocellulose membrane (BioRad, USA) was first cut carefully into the required size and placed inside the template. 2µl of coating buffer (Carbonate-bicarbonate buffer) was loaded in each well of the template over the nitrocellulose membrane (NCM) and kept for 25 min to dry. Following this 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 h at room temperature. The template was removed and blocking of the NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30 min. Polyclonal antibody of *A. alternata*(1: 40) was added directly in the blocking solution and further incubated at 4°C overnight. The membrane was then washed several times in TBS-Tween (*p*H -7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline Phosphatase conjugate (1:10,000) for 2 h at 37°C. This was followed by washing for 25 min in TBS-Tween. The substrate (66µl Nitro Blue Tetrazolium Chloride + 33µl 5-Bromo-4-Chloro-3-Indolyl phosphate di-sodium salt in 10ml of Tris buffer saline *p*H 7.4) was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water.

### 3.20 Western blotting

Blot transfer was done in three steps, following the method as described by White *et. al.*, (1994) with modifications. Soluble proteins were extracted from healthy, artificially inoculated with *A. alternata* and natural infested by *H. theivora* and resolved on SDS-PAGE. Following gel run, it was transferred to Towbin buffer(25

mM Tris, 192 mM glycine in 20% Reagent grade methanol, *p*H 8.3) and equilibrated for 1h. The transfer unit was attached to a power pack. The presoaked filter paper was placed on the platinum anode and air bubbles were rolled out with a glass rod. The pre-wetted NCM was over the filter paper followed by the gel and finally on top again another presoaked filter paper was placed. The cathode was placed on the sandwich and pressed. The unit was run for 45 min at 15V (constant). After the run the membrane was dried for 1 h and preceded for immunological probing. Initially membrane was placed in a heat sealable plastic bag containing blocking solution (5% non fat dried milk and 0.02% sodium azide in 0.15 M PBS *p*H 7.2 with 0.02% Tween-20) for 1 h with occasional shaking. Antibody was added (1:40) to the blocking solution and incubated in plastic bag at 4°C overnight. All the processes were done by gentle shaking. The nitrocellulose membrane was washed properly in 200 ml of washing buffer (150 mM NaCl, 50mM Tris HCl) *p*H 7.5 to remove azide and phosphate from the filter before enzyme coupled reactions. Enzyme was added (1:10,000 in alkaline phosphatase buffer) and kept for 1h at room temperature.

The membrane was washed in washing buffer as before and the substrate was added (66µl NBT + 33 µl BCIP + 10ml of Alkaline phosphatase buffer *p*H 9.5). The reaction was monitored carefully and when bands of the desired intensity were observed the filter was transferred to a tray of 200 µl of stop solution (0.5M EDTA, *p*H 8.0 in 50 ml of 0.15M PBS).

### 3.21 Scanning Electron Microscope (SEM)

The main purpose of SEM observation is to study the surface morphology. The basic requirement for the study in SEM is that electron should pass through the specimens. High vacuum in the microscope column is necessary for biological material. Dried material was mounted on metallic stubs with the help of conducting paints. The mounted medium was heat resistant. Gold was used for coating. Spores, healthy and infected leaf samples were first gold coated under high vacuum pressure for 30min and then observed under electron microscope.

First the leaf samples (healthy, *H. theivora* punctured portion as well as Alternaria blight infected portions) were cut into small pieces (2mm aprox.) and samples were then air dried completely due to their bulk size. Dried samples were mounted on metallic stubs with the help of conducting paints. As the secondary electrons are used in the image formation of SEM, the samples need to generate sufficient secondary electrons to give good quality image. For this reason, the samples were coated using a thin layer of a metal. Coating also protects the samples from the effect of electron beam. Gold was used for coating the samples. Then the samples were observed through Electron microscope.

**3.22 Transmission Electron Microscope (TEM)**

Healthy as well as salicylic acid treated tea leaves were fixed in 1% (v/v) glutaraldehyde in phosphate buffer (*p*H 6.8) for 60 min at $4^0$C. Then the tissues were transferred to 3% phosphate buffer glutaraldehyde for overnight. After that the samples were washed thoroughly three times in PBS (0.1M phosphate buffer saline) Samples were then dehydrated in a 50% graded ethanol for 10min. first and then in 70% ethanol for 45 min. and embedded in LR White and polymerized at $60^0$C for 2 days. Ultra-thin sections of the samples were cut with a diamond knife and collected on Pioliform coated 300 mesh nickel grid for immunogold labeling.

Rabbit polyclonal antisere raised against chitinase and laminarinase were used for innumogold labeling. Sections were incubated in blocking solutions containing 1% (w/v) of BSA in Tris Buffer Saline (TBS). Then incubation of the section in primary antibody dilution 1:200 in the blocking solution for 1h at room temperature. After washing the sections were incubated in with secondary antibody diluted at 1:40. Rinsing was done with PBS for 3-4 times. After contrasting with uranyl acetate and lade citrate, the sections were examined with a Leica EM.