MATERIALS AND METHODS

3.1. Plant Material

Tea, *Camellia sinensis* (L.), belongs to the family Theaceae. The cultivated taxa comprise of three main natural hybrids, which are: *C. sinensis* (L.) O. Kuntze or China type, *C. assamica* (Masters) or Assam type and *C. assamica* sub spp. *lasiocalyx* (Planchon ex Watt.) or Cambod or Southern type (Mondal *et al.*, 2004). Tea is an evergreen, perennial and leaf is the main criterion by which three types of tea are classified, briefly, Assam type has biggest leaves, China type has smallest leaves and Cambod leaves size are in between of Assam and China type.

3.1.1. Collection

Thirty tea varieties were collected from the Tea Germplasm Bank, Department of Botany, University of North Bengal. The tea plants being maintained comprise of varieties released by Darjeeling Tea Research Centre (DTRC), Kurseong, West Bengal; The United Planters' Association of Southern India (UPASI), Valparai, Tamil Nadu and Tocklai Experimental Station, Jorhat, Assam. These (Table 3) were propagated in the nursery.

Table 3.Tea varieties selected from Tea Germplasm Bank for the present study

Source	Varieties
DTRC	AV-2, BS-7A/1/76, CP-1, HV-39, P-1258, RR-17, T-17/1/54, T-135, T-78, TS-449
UPASI	UP-2, UP-3, UP-8, UP-9, UP-17, UP-26, BSS-1, BSS-2, BSS-3
TOCKLAI	TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-29, TV-30

3.1.2. Clonal propagation

The most successful method adopted for vegetative propagation of tea varieties was single-node cutting. Fresh clonal cuttings of the tea varieties were propagated in sleeves containing sandy soil (3:1 of sand and soil) with a pH ranging from 4.5 – 4.8 and were maintained in a green Agro-net house. The tolerable light limit was maintained at 25 % in the early stage and 50 % once rooting progressed as suggested by Banerjee (1993).

Plate 3 (figs. A – H). Tea varieties for experimentation. [A] Tea Germplasm Bank. [C] Cuttings in sleeves. [B, D - H] Pot grown plants.

3.1.3. Maintenance in Glass House

Eighteen month-old tea plants of thirty varieties, as mentioned in Table 3, were transferred to 6 cm and 12 cm diameter plastic pots as well as 24 cm earthen pots containing soil supplemented with 20 % green manure and were maintained in the glass house for experimental purposes (Plate 3, figs. A - H). Optimum day length (11 h), relative humidity (55 - 75 %) and temperature (30 - 35 °C), at which the photosynthetic rate was maximum, were provided to these plants as suggested by Banerjee (1993).

3.2. Callus induction

For callus induction from young internodal stem segments of tea varieties, MS basal medium (Murashige and Skoog, 1962) was used. The medium was supplemented with 3 % sucrose, 0.8 % agar (Difco), 4 mg L^{-1} indole-3-butyric acid (IBA) and 2 mg L^{-1} of 6-benzylaminopurine (BA) (Kato, 1989). The pH was adjusted to 5.8 with 0.1 N HCL or 0.1 N NaOH before autoclaving and then sterilized at 121 °C (15 1b in^{-2}) for 30 min. Fresh young shoots of tea plants of twenty two different varieties *viz.*, Darjeeling: AV-2, BS-7A/1/76, RR-17, T-135, HV-39, T-78, T-17/1/54 and TS-449; UPASI: UP-2, UP-8, UP-9, UP-17, BSS-1, BSS-2, and BSS-3; Tocklai: TV-9, TV-18, TV-20, TV-22, TV-23, TV-25 and TV-30) bearing 3-4 leaves were taken and stem segments (2-3 mm long) were cut and sampled. Before sterilization, explants were agitated with 0.7 % PVP-10 solution for 60 min and washed in running tap water for another 20 min to remove phenolic substances. Stem segments were surface sterilized with 2 % sodium hypochlorite solution for 5 min and washed five times with sterile distilled water for the removal of hypochlorite. After final washing, explants were transferred into MS medium slants. Appearance of friable callus was observed after 2-4 wk of inoculation, which gradually increased in size.

3.2.1. Preparation of cell suspension cultures

Suspension cell cultures of the tea varieties were initiated from fragile callus of internodal sections developed on MS medium (26 °C and photoperiod of 16 h). The friable callus tissue was transferred to liquid MS medium supplemented with the same amounts of the phytohormones (IBA and BA) and vitamins. The suspension cultures (100 ml) were maintained in 250 ml flasks which were covered with foil to suppress

anthocyanin production and incubated at 25 ±3 °C on a rotary shaker (CIS-24, Remi) at 110 r.p.m. For subculture, the cells were sedimented out of the liquid medium at 10 to 15-day intervals to a 30 ml volume and 70 ml of fresh culture medium added. The amount of the cells for inoculation was 5.0 g on the basis of wet weight. Four to six day-old cell suspension cultures were treated with a range of different abiotic and biotic elicitors.

3.3. Fungal material

3.3.1. Causal organism (*Exobasidium vexans*)

Exobasidium vexans causing blister blight of tea is an obligate pathogen. Hence, blister blight diseased tea shoots were always freshly collected from tea gardens for the collection of basidiospores. Taking into consideration the peak season of blister blight incidence, certain tea estates of Darjeeling hills and plains were preferred for collection of such experimental material.

Blister infected leaf samples were collected during July through October mainly from two tea estates of hills, *viz.*, Castleton and Margaret's Hope, and from Hansqua Tea Estate in the plains from mid December to end of February.

3.3.1.1. Collection of blister spores

Basidiospores of *E. vexans* were trapped as illustrated in Plate 4, fig. A. Flush shoots with well-developed sporulating lesions were selected and dipped in conical flasks containing sterile 2 % sucrose solution and incubated in a BOD (Remi) at 25 °C for collection of spores. The leaves with the infected blister zone(s) were placed horizontally (ventral side) over the base of sterile Petri plates (2" dia) and roofed with the lid. After 48 - 72 h of incubation the basidiospores deposited on the surface of the Petri plate underneath the leaf (Plate 4, figs. B & C). The Petri plates containing basidiospores of *E. vexans* (Plate 4, fig. D) were sealed with parafilm, labeled with source details (Table 4) and used for inoculating the plant material.

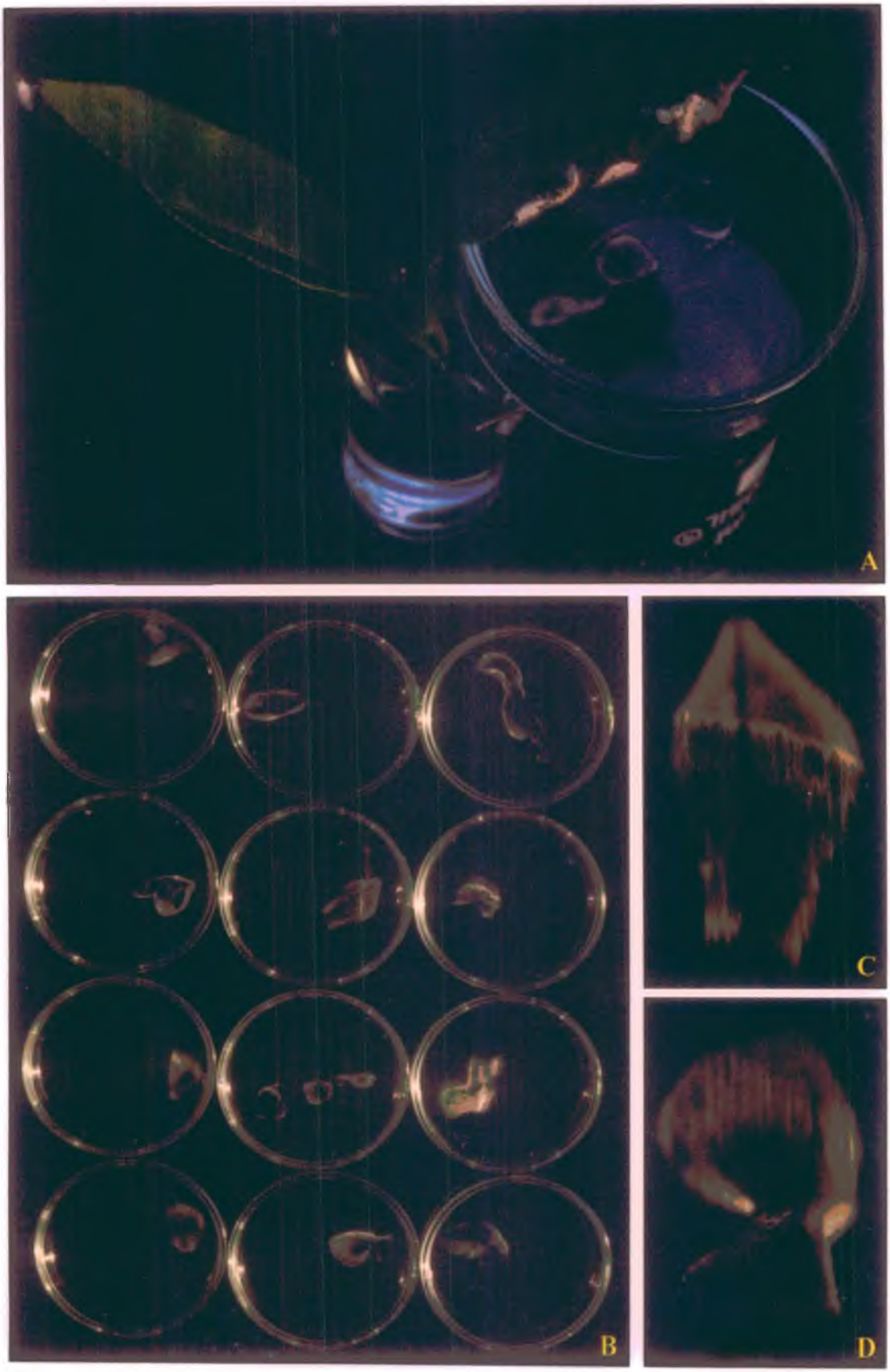

Plate 4 (figs. A – D). Spore trapping from blister infected twig. [A] Set-up. [B - D] Spore prints, [C & D] enlarged view.

Table 4. Isolates of *Exobasidium vexans* obtained from hills and plains

Organism	Isolate Code	Source
Exobasidium vexans Massee	EV1	Castleton Tea Estate
	EV2	Hansqua Tea Estate
	EV3	Margaret's Hope Tea Estate

3.3.2. Other microorganisms

Virulent fungal cultures of *Curvularia pallescens* (1/L 0411A), *Glomerella cingulata* (Stoneman) Spauld. and Shrenk (IMI no. 356805), *Fusarium oxysporum* Schlecht (2389) and one bacterial isolate *Bacillus megaterium* were obtained from the Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Fungal cultures were maintained by subculturing on PDA medium at 28 °C while *B. megaterium* was subcultured on nutrient agar medium and incubated at 37 °C before use in *in vitro* bioassays.

3.4. Artificial inoculation

3.4.1. Spore suspension

The basidiospores of *E. vexans* settled at the bottom of the Petri dishes were lifted from the surface by gentle brushing. Sterile distilled water (100 - 200 µl) was pipetted out over the area to ease the lifting process. The spores were rubbed off the surfaces and collected in Eppendorf tubes and the spore concentrations were determined by haemocytometer counting.

3.4.2. Inoculation technique

Intact tea plants were artificially inoculated with basidiospores of *E. vexans* following the method of Chakraborty *et al.* (1997). The spore-suspensions prepared in sterile distilled water (1.6×10^5 spores ml^{-1}) were used to inoculate healthy intact tea plants. For the experimentation, the intact healthy potted tea plants of different varieties were brought to the laboratory. The leaves of each plant were thoroughly cleaned with tap water followed by sterile distilled water. Prior to brushing, the spore-suspension was supplemented with a few drops of Tween-20. The spore suspension was brushed (brush no. 00) on either surface of the first, second and third leaves of

each plant. The pots were positioned in a cooling incubator (CIS-24). The individual pots were covered with plastic bags. The open end was tied with string to retain moisture in the enclosed chamber and incubated at 23 ±2 °C. The chamber was frequently sprayed with sterile distilled water to maintain a moisture level of 80- 90 %. The plastic covers were removed after 24 h. The inoculated tea plants kept under such controlled conditions of light and temperature curled within 12 days after inoculation. However, sporulation was not induced under these conditions.

3.4.3. Disease assessment

Disease incidence was assessed by calculating the percentage of infection following the method of Venkata Ram and Chandra Mouli (1984). The plants of all selected varieties were artificially inoculated as above and shoots and leaves observed up to 30 d. The third leaf of tea shoots is considered to be the index leaf for blister blight disease assessment accordingly a representative sample of 50 shoots (per variety) consisting of three leaves and a bud were collected. Percentage infection for different categories of leaf i.e., 1st leaf, 2nd leaf and 3rd leaf from the top bud was determined with the following formula,

$$\text{Percentage leaf infection} = \frac{\text{No. of leaves infected}}{\text{No. of leaves examined}} \text{ X } 100$$

Table 5. Categories for scoring blister blight disease incidence

Leaves	Code	Range (%)
Resistant	R	0-20
Moderately resistant	MR	21-40
Moderately susceptible	MS	41-70
Susceptible	S	71-100

3.5. Abiotic treatment

3.5.1. Preparation of test solutions

Nine selective inducers such as salicylic acid, jasmonic acid, di-potassium hydrogen orthophosphate, mercuric chloride, hexaconazole, calixin, biocrop and phytoextracts of *Azadirachta indica* 'neem' and *Catharanthus roseus* 'periwinkle' were tested as foliar applicants on tea plants grown in earthen pots as well as in the experimental field.

Fresh mature leaves (400g) each of *Azadirachta indica* (neem) and *Catharanthus roseus* (periwinkle) were homogenized in a mixer (Philips HL3294) and diluted with distilled water as suggested by Paul and Sharma (2002). Biocrop, an organic fertilizer and known plant growth inducer obtained from AKS Bio and Herbals Pvt. Ltd., Mumbai, was used as recommended by the manufacturer. Rest of the inducers were mixed in proportions as tabulated below (Table 6).

Table 6. Test solutions applied to tea plants for induction of resistance

Test solutions	Strength	Product source
Salicylic acid	15 mM	Himedia
Jasmonic acid	0.5 µM	Sigma
Di-potassium phosphate	50 mM	Himedia
Mercuric chloride	5 mM	Himedia
Hexaconazole	0.1 %	Rallis India
Calixin	0.1 %	BASF
Biocrop	10 %	AKS Herbals
Azadirachta indica	0.4 %	Phytoextract
Catharanthus roseus	0.4%	Phytoextract

3.5.2. Foliar application

The tea plants were pre-treated with inducers (Table 6) prior to inoculation with *E. vexans*. The solutions were supplemented with a few drops of Tween-20 to ensure adhering. Control plants (untreated) were sprayed with distilled water plus Tween-20. Around 100 ml of the solutions were sprayed using a hand sprayer as to

wet the leaves completely on both the ventral and dorsal surfaces. The treated potted plants were labeled appropriately and allowed to stay in the glass house until required and the field grown treated plants were also earmarked.

3.6. Protein extraction and estimation

3.6.1. Soluble leaf protein

Soluble proteins were extracted from tea leaf tissues as described by Chakraborty *et al.* (1995). Leaf tissues (3 g) were homogenized with 0.05 M sodium phosphate buffer, pH 7.2 (5 ml g^{-1}), containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM PVP-10 (Sigma) and 2 mM polymethyl sulphonyl fluoride (Sigma) in a pre-chilled mortar with a pestle. The brei was centrifuged for 20 min at 4 °C and 10,000 r.p.m. The pellet was discarded and the supernatant was collected and used immediately.

3.6.2. Acid soluble proteins

PR-proteins were extracted from the tea leaf tissues following the procedure adapted from Ye *et al.*, (1990) with slight modifications. The harvested leaves were homogenized in an extraction medium consisting of 0.1 M citrate phosphate buffer, pH 2.8 (3 ml g^{-1} leaf tissue) and 2 mM PVP-10 in a mortar with pestle at 4 °C. The resultant slurry was centrifuged for 20 min at 4 °C and 10,000 r.p.m. The supernatant was decanted and dialysed against two changes of deionized water at 2 - 4 °C for 24 h and then against two changes of 0.05 M sodium acetate buffer, pH 5.2 for 2 h. The dialysate was centrifuged again at 10,000 r.p.m. for 20 min. The resulting supernatant was used for analyses immediately.

3.6.3. Intercellular fluids

The intercellular fluid of the leaves was extracted by the method of De Wit and Spikman (1982) with some modifications. Leaves (10 g) were vacuum infiltrated under cool conditions (5 - 10 °C) for 30 min in a 250 ml beaker filled with distilled water. The leaves were blotted gently on filter paper discs, put in tubes and centrifuged for 15 min at 4 °C and 10,000 r.p.m. The supernatant was pipetted into Eppendorf tubes and used immediately.

3.6.4. Protein estimation

Bradford's (1976) method was adopted, for protein quantification. The reagent contained 0.01 % Coomassie Brilliant Blue G250 (w/v), 4.7 % ethanol (w/v), and 8.5 % phosphoric acid (w/v). Protein dilutions were made with 0.15 M phosphate buffered saline, pH 7.2 and 100 µl of protein sample was pipetted in to test tubes. Five milliliters of protein reagent was added to the test tube and the contents mixed by inversion. The absorbance at 595 nm was measured after 2 min against a reagent blank prepared from 0.1 ml of the dilution buffer and 5 ml of protein reagent. The concentration was determined using BSA as standard. Assays were performed in triplicates and each experiment was repeated three times.

3.7. Extraction and assay of defense enzymes

3.7.1. Chitinase (E.C. 3.2.1.14.)

The assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988) using crustacean chitin (Sigma) as substrate 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 mortars with pestles. The homogenates were centrifuged for 30 min at 4 °C and 10,000 r.p.m. Supernatants were used as enzyme source. One ml of the enzyme extract was incubated with 1 ml of colloidal chitin (10 mg ml^{-1}) at 37 °C for 1 h on a shaker. The solution was centrifuged for 3 min at room temperature to remove any unreacted colloidal chitin. An aliquot of supernatant (0.3 ml) was added to 30 µl of 1 M potassium phosphate buffer, pH 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 pH of the reaction mixture was brought to 8.9 by addition of 70 µl of 1 M sodium borate buffer, pH 9.8. The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled by plunging in ice. After addition of DMAB reagent [10 g 4-dimethylaminobenzaldehyde in 12.5 ml 10 N HCl and 87.5 ml glacial acetic acid], the mixture was incubated for 20 min at 37 °C. Immediately thereafter, absorbance values at 585 nm were 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 mg GlcNAc g^{-1} leaf tissue h^{-1}.

3.7.2. β-1,3-glucanase (E.C. 3.2.3.39.)

β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan *et al.*, 1991) with modifications. One gram of the leaf sample was extracted with 3 ml 0.05 M sodium acetate buffer, pH 5.0 supplemented with 2 mM PVP-10 by grinding in pre-chilled mortars and pestles. The extract was then centrifuged for 30 min at 4 °C and 10,000 r.p.m. and the supernatant was used as crude enzyme extract. To 62.5 µl of the crude enzyme extract, 62.5 µl of laminarin (4 %) was added and then incubated for 10 min at 40 °C. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent (1 g of DNSA dissolved in 200 mg crystalline phenol in 1 % NaOH and 0.05 g sodium sulphite) and heated for 5 min on 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 - 250 µg ml^{-1}) was prepared. The enzyme activity was expressed as µg Glucose g^{-1} leaf tissue min^{-1}.

3.7.3. Peroxidase (E.C. 1.11.1.7.)

Peroxidase was assayed following the method outlined by Chakraborty *et al.* (1993) with a few modifications. One gram of leaf sample was extracted in 0.1 M sodium phosphate buffer, pH 8.8 (5ml g^{-1}) containing 2 mM β-mercaptoethanol and 2 mM PVP-10. The homogenate was immediately centrifuged for 20 min at 4 °C and 10,000 r.p.m. For the assay, 100 µl of the supernatant, 1 ml of 0.2 M sodium borate assay buffer, pH 5.4, 100 µl of 4 mM H_2O_2, 100 µl of o-dianisidine (5 mg ml^{-1} methanol) and 1.7 ml of double distilled water were mixed and the activity was measured at 460 nm in a UV-VIS-spectrophotometer at 60 s intervals. The activity was expressed as Δ A460 nm g^{-1} leaf tissue min^{-1}.

For peroxidase isozyme analysis tea leaf samples were crushed in 2 mM sodium phosphate buffer, pH 7.0 on ice as described by Davis (1964) and used for native polyacrylamide gel electrophoresis.

3.7.4. Phenylalanine ammonia lyase (E.C. 4.3.1.5.)

Phenylalanine ammonia lyase was extracted from tea leaf tissues following the method of Chakraborty *et al.*, (1993). One gram of leaf tissue was crushed in a mortar at 4 °C in 5 ml of 0.1 M sodium borate buffer, pH 8.8 containing 2 mM β-mercaptoethanol in ice. The slurry was centrifuged for 20 min at 4 °C and 10,000 r.p.m.. The supernatant was collected and after recording its volume, was used immediately for assay. The enzyme was assayed as described by Moerschbacher *et al.*, (1986) with a few modifications. A 500 µl sample of crude extract was allowed to react with 600 µl of borate buffer containing 6 µmol L^{-1} L-phenylalanine, at 40 °C for 1 h. A blank was prepared without L-phenylalanine. The reaction was stopped by addition of 6 mol L^{-1} HCl and 1 ml chloroform was added to each tube by vortexing and centrifuged at for 10 min at 10,000 r.p.m.. From the lower organic phase 0.5 ml aliquots were withdrawn and chloroform was allowed to evaporate, the residue being redissolved in 1 ml borate buffer. The absorbance of the samples was read at 290 nm and expressed as µg Cinnamic acid g^{-1} fresh tissue min^{-1}.

3.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For protein profiling sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). The polyacrylamide gels of the proteins were evaluated using the Electrophoretic Documentation and Analysis System (Kodak EDAS 290). To perform the experiment the following stock solutions were prepared,

3.8.1. Preparation of stock solutions

Solution A: Acrylamide solution

A stock solution containing 29 % acrylamide and 1 % N,N'-methylene-bis-acrylamide was prepared in luke warm double distilled water. As both compounds undergo deamination to acrylic and bisacrylic acid, respectively, by alkali and light, the pH of the solution was kept below 7.0. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution B: Sodium dodecyl sulphate (SDS)

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

Solution C: Tris buffer

(i) Resolving buffer

1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4 °C for further use.

(ii) Stacking and loading buffer

1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of was adjusted to 6.8 with concentrated HCl and stored at 4 °C.

Solution D: Ammonium persulphate (APS)

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

Solution E: Tris - glycine electrophoresis buffer (reservoir buffer)

The reservoir or tank buffer consisted of 25 mM Tris base, 250 mM glycine, pH 8.3 and 0.1 % SDS. A 1x solution was made by dissolving 3.02 g Tris base, 18.8 g glycine and 10 ml of 10 % SDS in 1 L of double distilled water.

Solution F: SDS-gel loading buffer

This buffer contained 50 mM Tris-HCL, pH 6.8, 10 mM β–mercaptoethanol, 2 % SDS, 0.1 % bromophenol blue and 10 % glycerol. A 1x solution was made by dissolving 10 mg bromophenol blue in 0.5 ml of 1 M Tris buffer, pH 6.8, 0.5 ml of 14.4 M β–mercaptoethanol, 2 ml of 10 % SDS, 1 ml glycerol and 6.8 ml of distilled water.

3.8.2. Preparation of gel

Slab gels (8 cm x 5 cm) were prepared for the analysis of protein patterns by SDS-PAGE. For the preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with high vacuum

grease. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. Resolving and stacking gels were prepared by mixing compounds in the 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 1.5 M Tris pH 8.8 in resolving gel and 1 M Tris pH 6.8 in stacking gel;

* * N, N, N', N' -Tetramethyl ethylene diamine

The mixture was poured in to the set-up by a pasture pipette leaving sufficient space for the well-comb (comb + 1 cm). After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 1 h. After polymerization of the resolving gel was complete the water overlayer was poured off and the gel was washed with water to remove any unpolymerized acrylamide. The stacking gel solution was poured over the resolving gel followed by immediate insertion of the comb and overlayering with water. The gel was kept for 30 min. After polymerization of the stacking gel, the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in to the electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

3.8.3. Sample preparation

Prior to loading, the sample proteins (35 µl) were mixed with 1x SDS-gel loading buffer (15 µl) in a cyclomixer. All the samples were floated on a boiling water bath for 3 min to denature the proteins. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microlitre syringe. Along

with the samples, protein markers consisting of a mixture of thirteen proteins of known molecular weight (myosin – 205, β-galactosidase 116, phosphorylase b – 97, fructose-6-phosphate kinase 84, albumin – 66, glutamic dehydrogenase – 55, ovalbumin – 45, glyceraldehyde 3-phosphate dehydrogenase – 36, carbonic anhydrase 29, trypsinogen – 24, trypsin inhibitor 20, α-lactalbumin – 14.2 and aprotinin – 6.5 kDa) was similarly treated as the other samples and loaded in a separate well.

3.8.4. Electrophoresis

Electrophoresis was performed at constant 18 mA current (PowerPac 1000, Bio-Rad) for a period of approximately 3 h for mini gel until the dye front reached the bottom of the gel.

3.8.5. Fixing

After electrophoresis, the gel was removed carefully from the glass plates and 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.

3.8.6. Staining and destaining

The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue R250 (Sigma) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman no.1 filter paper. The gel was removed from the fixer and stained in the staining solution for 4 h at 37 °C with constant shaking at a very low speed.

After staining, the gel was finally destained in methanol, water and acetic acid (9:9:2) at 37 °C with constant shaking until the background became clear.

3.9. Native polyacrylamide gel electrophoresis (PAGE)

For isozyme analysis, polyacrylamide gel electrophoresis under non-denaturing conditions was performed following the protocol of Davis (1964). The stock solutions were prepared as follows,

3.9.1. Preparation of stock solutions

Solution A: Acrylamide (for resolving gel)

For the preparation of acrylamide stock solution 'A' used for the resolving gel, 28 g acrylamide and 0.74 g N, N'- methylene-bis-acrylamide were dissolved in 100 ml of luke warm double distilled water. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution B: Acrylamide (for stacking gel)

Similarly, for the preparation of acrylamide stock solution 'B' used for the stacking gel, 10 g acrylamide and 2.5 g N, N'- methylene-bis-acrylamide were dissolved in 100 ml of luke warm double distilled water. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution C: Tris-HCl (for resolving gel)

Tris base (36.6 g) was dissolved in double distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with concentrated HCl. The final volume of the solution was made up to 100 with double distilled water and was stored at 4 °C for further use.

Solution D: Tris-HCl (for stacking gel)

Tris base (5.98 g) was dissolved in double distilled water and 0.46 ml of TEMED was added. The pH was adjusted to 6.7 with concentrated HCl. The final volume of the solution was made up to 100 with double distilled water and was stored at 4 °C for further use.

Solution E: Ammonium persulphate solution

A fresh solution of ammonium persulphate was prepared by dissolving 0.015 g of it in 10 ml of double distilled water.

Solution F: Riboflavin solution

A fresh solution was prepared by dissolving 0.004 g of riboflavin in 10 ml of double distilled water. The solution was stored in an amber bottle to protect it from light.

Solution G: Electrode buffer (for reservoir)

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

3.9.2. Preparation of gel

A mini slab gel (8 cm x 5 cm) was prepared for isozyme analysis by PAGE. For the preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with high vacuum grease. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 7.5 % resolving gel was prepared by mixing the solutions - A, C, E and double distilled water in the ratio, 1:1:4:1 by a pasture pipette leaving sufficient space for the stacking gel (for comb + 1cm). After pouring the resolving gel solution, it was immediately overlayered with water and allowed to polymerize for 2 h.

After polymerization of the resolving gel was complete the water overlayer was poured off and the gel was washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions - B, D, F and double distilled in the ratio, 2:1:1:4 poured over the resolving gel followed by immediate insertion of the comb and overlayering with water. The gel was kept for 30 min in strong sunlight. After polymerization of the stacking gel, the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in to the electrophoresis apparatus. Chilled tris-glycine running buffer was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

3.9.3. Sample preparation

During sample preparation, ice-cold conditions were maintained throughout the entire process. The samples were prepared by mixing the enzyme extracts (40 µl) and gel loading dye (25 µl) in a cyclomixer. The loading dye consisted of 40 % sucrose and 1 % bromophenol blue in double distilled water. The samples (52 µl) were immediately loaded in a pre-determined order in to the bottom of the wells with a microlitre syringe.

3.9.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current (PowerPac 1000, Bio-Rad) at 4 °C for a period of approximately 4 h for mini gel until the dye front reached the bottom of the gel.

3.9.5. Fixing and staining

After electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally stained. The gel was incubated in an aqueous solution of benzidine (2.08 g), acetic acid (18 ml), 3 % H_2O_2 (100 ml) for five min. The reaction was stopped with 7 % acetic acid after the appearance of clear blue colored bands. Analysis of isozymes was done immediately.

3.10. Preparation of antigens

Plant antigens of healthy, naturally blister infected, and artificially inoculated (with *E. vexans*) tea leaves as well as *Exobasidium vexans* were prepared as suggested by Chakraborty and Saha (1994a).

3.10.1. Leaf antigen

Leaf antigens were prepared from healthy, blister infected, and artificially inoculated tea leaves 0.05 M sodium phosphate buffer (pH 7.2). For the preparation of naturally-infected antigen only the infected areas (blister spots) were cut and taken. For artificially inoculated, the entire inoculated leaf with even a single translucent spot was considered. Antigens of *L. leucocephala*, *O. sativa* and *G. max* (non-hosts) were prepared in a similar manner.

3.10.2. Spore antigen

Antigens were prepared by sonicating basidiospores of *E. vexans* suspended in 0.05 M sodium phosphate buffer (pH 7.2), kept at –20 °C for 4 h, freeze-thawed followed by sonication (Sonics Vibra Cell) at 35 % amplitude for 9 min. Following sonication, the spores were further freeze-thawed and homogenized for 15 min at 4 °C. The suspension was centrifuged at 10,000 r.p.m. for 30 min. The supernatant was stored in 2 ml vials at -20°C and used for immunization process. Antigens from conidia of *F. oxysporum* (non-pathogen) were prepared in a similar manner.

3.10.3. Antimicrobial protein

3.10.3.1. Protein purification

Crude preparations of chitinases and β-1,3-glucanases of healthy tea leaves (500 g) were subjected to gel filtration using a Sephadex G-50 column (Pharmacia) according to the method described by Joosten *et al.* (1995). Fourteen fractions of 15 ml were collected, dialysed against deionized water, freeze-dried and dissolved in 1 ml of deionized water. The fractions were filter-sterilized (Millipore, 0.2 μm) and the filter was flushed with 1 ml of sterile water, resulting in a final volume of 2 ml per fraction. The fractions were analysed for their protein content and chitinase and β-1,3-glucanase activity.

The purified fractions of chitinase and β-1,3-glucanase were further used for polyclonal antibody production. Besides, *in vitro* antifungal assays were also performed with the purified extracts of different combinations of treatments following the same method of purification.

3.11. Polyclonal antibody production

3.11.1. Rabbits and maintenance

Polyclonal antibodies (PAb) were raised against fungal antigens, plant antigens and defense enzymes. Long-term immunization was performed on white male New Zealand rabbits of approximately 2 kg body weight (Chakraborty and Purkayastha, 1983) being maintained under hygienic conditions in the animal house - Antisera Reserves for Plant Pathogens, Immuno-Phytopathology Laboratory, Department of Botany, N. B. U. The rabbits were fed green grass, soaked *Cicer* seeds, carrots and lettuce (during season) in the mornings and evenings and given saline water after each bleeding for three consecutive days.

3.11.2. Immunization

Immunization was performed by the macro-technique in which 250 - 500 μl of antigen solution, of optimum concentration, was injected subcutaneously into the rabbits. Prior to immunization, pre-immune (normal) antisera were collected from the rabbits. For raising PAbs, the rabbits received six injections, for six consecutive weeks, of antigen emulsified with an equal amount of Freund's complete adjuvant

(Difco) seven days after the pre-immunization bleeding followed by Freund's incomplete adjuvant (Difco) at seven-day intervals up to 10 - 14 consecutive weeks as required.

3.11.3. Bleeding

Bleeding was performed by marginal ear vein puncture. For easy handling of the rabbits during blood collection, a simple apparatus consisting of a wooden board (100 x 30 x 1cm) with a triangular gap cut from top, fixed at a 60° position to a vertical plate equipped with four screws at the top and bottom of the board was used. The board was covered by a plastic and the rabbit placed on its back with the neck in the triangular gap and the head below the board with the legs tied to the screws, thus fixing the body. When placed as described the rabbit could be bled at ease as it could not move and thus was not harmed. The upper side of the ear was disinfected with rectified spirit and shaved. After irritation of the ear with xylene, the marginal vein of the ear was punctured by a sterile razor blade. The first bleeding was taken six weeks after the first injection and three times more every fortnight. The blood samples (5 - 15 ml) were collected in sterile graduated glass tubes (Borosil). After collection, all precautionary steps were taken to stop the blood flow from the puncture. Any sort of ear infection was avoided, however in case of occurrence, it was cured by dermal application of 10 % (w/v) methylene blue.

3.11.4. Preparation of serum from whole blood

The blood samples were kept for 1 h at 30 °C. The clots were loosened and stored at 4 °C. The antisera were then clarified by centrifugation and stored at –20 °C until required.

3.11.5. Purification of IgG

IgGs were purified by affinity chromatography on a DEAE-cellulose column following the protocol of Clausen, 1988.

3.11.5.1 Precipitation

The crude antiserum (2 ml) was diluted with two volumes of distilled water and to it an equal volume of 4.0 M ammonium sulphate was added. The pH was adjusted to 6.8 and the mixture stirred for 16 h at 22 °C. The precipitate thus formed

was collected by centrifugation for 1 h at 22 °C and 10,000 r.p.m. Then the resultant pellet was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.11.5.2. Column preparation

Four grams of DEAE-cellulose (Sigma) was suspended in double distilled water overnight. The water was drained off and the gel was suspended in 0.005 M sodium phosphate buffer, pH 8.0. Washing in the same buffer was repeated 5 times. The gel was then suspended in 0.02 M sodium phosphate buffer, pH 8.0 and was applied to a column (2.6 cm dia and 30 cm high) and allowed to settle for 2 h. After the column material had settled, 25 ml of 0.02 M sodium phosphate buffer, pH 8.0 buffer was run through the gel material to give it a final wash.

3.11.5.3. Fraction collection

Two milliliters of the ammonium sulphate precipitated IgG was applied at the top of the column and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.3 M. The initial elution buffer was (1) 0.02 M sodium phosphate buffer, pH 8.0 and the final elution buffer was (2) 0.3 M sodium phosphate buffer, pH 8.0. The buffers were applied to the column through flasks connected via rubber tubes. The flask containing buffer-(2) was open at one end and connected to the flask containing buffer-(1) at the other. During draining of buffer-(1) in to the column, buffer-(2) was sucked into buffer-(1) thereby producing a continuous rise in molarity. Finally, 40 x 5 ml fractions were collected and the optical density values were recorded at 280 nm by means of UV-Spectrophotometer. The fractions showing >2 reading were pooled and stored as purified IgG after estimation of its concentration.

3.11.5.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1981). Absorbance value was taken for selected fractions at 280 nm and 260 nm. The IgG concentration was calculated by the following formula,

Protein concentration (mg/ ml) $= 1.55 \times A_{280} - 0.76 \times A_{260}$

3.11.5.5. Storage of IgG

Serum samples were obtained at regular intervals as per schedule and stored at –20 °C. To eliminate background binding and to restore the specificity of the antisera lost during storage they were adsorbed with kaolin (Shillitoe, 1982). To 0.1 ml of antiserum (= 3 mg protein) was added 0.8 ml of 0.15 M phosphate buffered saline, pH 7.2 (PBS) with 4 % B.S.A. and 1 ml of a 25 % suspension (w/v) of kaolin (hydrated aluminum silicate, particle size 0.1 – 4.0 μm, Sigma) in PBS. The mixture was allowed to stand at room temperature for 20 min with regular mixing. The kaolin was then removed by centrifugation at 10,000 r.p.m. for 10 min and the supernatant diluted to 3 ml with PBS to give a final antibody concentration of 1 mg ml^{-1}. Besides this, serum fractions were diluted with 1 vol of glycerol, and 0.01 % sodium azide was added before storage at –10 °C.

3.12. Immunodiffusion

The agar gel double diffusion method developed by Ouchterlony (1967) was adopted to test the effectiveness of the raised antibodies.

3.12.1. Agarose gel plates

The gel plates were prepared by coating square glass slides (6 cm x 6 cm x 4 mm) with agarose. The glass plates were initially degreased with 90 % (v/v) ethanol and diethyl ether (1:1) and dried in a hot air oven. After drying the plates were sterilized in an autoclave at 121 °C (15 lb in^{-2}) for 20 min in Petri plates. For preparation of agarose gel, 0.9 % agarose was added to warm 0.05 M Tris-barbiturate buffer, pH 8.6 contained in a flask and boiled in a hot water bath. The flask was repeatedly shaken until a clear solution was obtained. Prior to plating 0.1 % (w/v) sodium azide was added to the stock solution. All sterilized materials were exposed to UV-light for 10 min in a laminar airflow bench. The hot molten agarose was then pipetted (10 ml per slide) out on to the sterilized glass slides and allowed to solidify. Following this seven wells were cut out with a sterilized cork borer (6 mm dia). The usual set-up was the radial well type in which antibody wells were arranged circularly around a central antigen well with the holes about 1.5 cm from each other.

3.12.2. Diffusion

The antigen and undiluted antisera (60 µl well^{-1}) were pipetted directly in to the wells in a pre-determined manner. All steps were carried out in a laminar chamber. The antigen and antiserum was allowed to diffuse in a moist chamber for 72 h at 25 ±2°C (BOD, Remi). Precipitation lines were observed in cases where common antigens were present.

3.12.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9 % NaCl and 0.1 % NaN_3) for 72 h with 6 h changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. The slides were then stained with Coomassie blue R 250 (250 mg of Coomassie brilliant blue in 45 ml of methanol, 45 ml of water and 10 ml of glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol, water and acetic acid in the ratio 9:9:2) with changes until the background was clear. Finally the slides were washed with distilled water and dried in a hot air oven for 3 h at 50 °C.

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

The plate trapped antigen form of ELISA (PTA-ELISA) was performed essentially as described by Chakraborty *et al.* (1995a). Flat bottom 96-well polystyrene microtitre plates (Nunc) were used for the assays. Test samples (antigen extracts) were diluted in coating buffer [50 mM carbonate buffer, pH 9.6 (a) sodium carbonate – 5.2 g in 1 L double distilled water, (b) sodium hydrogen carbonate – 4.2 g in 1 L double distilled water; 160 ml of (a) was mixed with 360 ml of (b) and the pH was adjusted 9.6] and added in to each of three wells (200 µl well^{-1}) and then incubated at 25 °C for 4 h. The wells were then emptied and washed three times by flooding wells with 0.15 M phosphate buffered saline, pH 7.2 [PBS, washing buffer - (a) sodium dihydrogen phosphate - 23.4 g in 1 L double distilled water, (b) disodium hydrogen phosphate – 21.3 g in 1 L double distilled water; 280 ml of (a) was mixed with 720 ml of (b) and the pH was adjusted 7.2. To the final mixture 0.8 % NaCl and 0.02 % KCl were added] containing 0.05 % Tween-20 (PBS-T). After each washing the plates were shaken dry.

Subsequently, 200 µl of blocking reagent [0.05 M Tris buffered saline, pH 8.0 (TBS): Tris - 0.657 g, NaCl- 0.81 g, KCl - 0.223 g, 0.05 % Tween-20 and 1 % bovine serum albumin (BSA) in 100 ml double distilled water] was added to each well for blocking the unbound sites to eliminate background binding and the plates were incubated at 25 °C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified IgG was serially diluted in antisera dilution buffer (PBS-T) containing 2 % soluble PVPP, 0.2 % BSA and 0.03 % NaN_3, and added to the wells, the plates were placed at 4 °C overnight (16 h). After a further washing, anti-rabbit IgG goat (whole molecule) labelled with alkaline phosphatase diluted 10,000 times in PBS, was added to each well (200 µl $well^{-1}$) and incubated at 37 °C for 2 h. The plate was washed, dried and each well was loaded with 200 µl of the enzyme substrate p-nitrophenyl phosphate (10 mg ml^{-1}) in alkaline phosphatase buffer [0.05 M Tris-HCl buffer, pH 9.8] containing 1 % diethanolamine and kept in the dark for 60 min. The reaction was stopped by adding 50 µl per well of 3 N NaOH solution. The rate of color development was monitored on a Multiskan EX (Thermo Electron) ELISA reader interfaced with a Windows 98 computer and the rates of the reaction were recorded at A405 nm. Optical absorbance values in wells not coated with antigens were considered as blanks. Triplicate wells were used for each sample and the mean absorbance at 405 nm was calculated after subtracting the absorbance given by the substrate wells not coated with antigens. The experiments were repeated three times.

3.14. Dot immunobinding assay (DIBA)

The dot immunobinding technique has been found to be a rapid and sensitive method of detection. The method used is a modification of the method described by Lange *et al.*, 1989.

3.14.1. Immunoblotting

Pre-cut (Pall Gelman, 0.2 µm) or custom-cut (Bio-Rad, 0.45 µm) nitrocellulose membranes (8 cm x 12 cm for 96 welled-template) were carefully placed in the template of a Bio-Dot apparatus (Bio-Rad) with the help of flat forceps (Millipore). Hand gloves were always worn while carrying out all procedures related to nitrocellulose membranes (NC). Prior to placement of the nitrocellulose in the

template it was pre-soaked in antigen coating buffer (50 mM carbonate buffer, pH 9.6) and air-dried for 30 min. Crude protein samples (antigen) were pipetted into each well of the apparatus at the rate of 100 µl per well and allowed to filter for 1 h. Following a single wash with TBS-Tween-20 (200 µl per well under vacuum) the membrane was removed and immunoblotted.

3.14.2. Immunoprobing

The sheets were incubated in the blocking solution 10 % (w/v) skimmed milk powder (casein hydrolysate, SRL) in 0.05 M Tris-HCl, pH 10.3 containing 0.5 M NaCl, 0.5 % Tween-20 (TBS-T) for 2 h on an orbital shaker (CIS-24, Remi) at 37 °C and 90 r.p.m. Antiserum of desired dilution was then directly added to the blocking solution for either 3 h at 37 °C and 90 r.p.m or overnight at 4 °C. After incubation, the solution was drained and the sheets were washed gently with running tap water for 3 min followed by 3 x 250 ml 5 min washes in TBS-T (10 mM Tris buffer, pH 7.4 - with 0.9 % NaCl and 0.05 % Tween-20) (Wakeham and White, 1996). The membranes were then incubated in alkaline phosphatase conjugate goat anti-rabbit IgG (Sigma) diluted in alkaline phosphatase buffer (0.05 M Tris-HCl buffer, pH 9.8) reaching a final concentration of 1:10,000. Conditions for incubation and washing were as described for the primary antiserum. Finally, the membrane was immersed in NBT-BCIP substrate solution [5 mg Nitroblue-tetrazolium chloride (NBT, Himedia) in 100 µl of 70 % N, N, - dimethyl formamide (DMF, Sigma) and 2.5 mg 1-bromo,2-chloro,3-indolyl phosphate (BCIP, Himedia) in 50 µl of 100 % DMF were prepared freshly and added to 10ml alkaline phosphatase buffer (0.05 M Tris-HCl buffer, pH 9.8) Or, 1 tablet of NBT-BCIP (Sigma) was dissolved in 10 ml of DDW] prepared for staining and the color development was noted. On appearance of the desired color intensity, the staining reaction was terminated by washing the membranes in distilled water. The dot formed on the membrane was scored by eye.

3.15. Western blot

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed according to the method devised by Towbin *et al.*, 1979. Following this procedure a replica of the original band pattern was obtained on nitrocellulose sheets from sodium dodecyl sulphate gels. The immobilized proteins

were further analyzed by modified immunological procedures (Wakeham and White, 1996). The proteins were clearly detectable as violet colored bands on nitrocellulose.

3.15.1. Extraction and estimation of proteins

Protein extraction and estimation was performed by Bradford's (1976) method as described in section 3.6.

3.15.2. SDS-PAGE of proteins

The different protein preparations were separately mixed with sample buffer subjected to electrophoresis in the presence of sodium dodecyl sulphate in one-dimensional slab gels (Laemmli, 1970). For each trial, two gels loaded with identical protein samples were run out of which one gel was stained with Coomassie Brilliant Blue R250 and the other electrotransferred.

3.15.3. Electrophoretic blotting procedure

On completion of gel run the protein SDS-gels were removed from the glass slabs and immediately immersed in pre-chilled Towbin/ transfer buffer [25 mM Tris, with 192 mM Glycine and 20 % Methanol (AR), pH 8.3] and equilibrated for 1 h. In a separate container a pre-cut or custom-cut nitrocellulose membrane (Bio-Rad, 0.45 μm) along with two pieces of blotting filters (Bio-Rad, 2 mm thickness) of the size of the SDS-gel, were soaked in Towbin buffer for 30 min. The electrotransfer process was carried out in a Mini-Protean II SD Trans-blot unit (Bio-Rad). A pre-soaked filter paper was placed on the platinum anode of the blotting unit, followed by pre-wetted nitrocellulose, equilibrated SDS-gel and then another filter paper. The gel sandwich was very gently rolled over with a glass rod to remove any trapped air bubbles. The cathode was carefully placed over the gel-sandwich and pressed to engage the latches with the guideposts without disturbing the filter stack. The unit was set at a constant volt (15 V) for 45 min. After the transfer the membrane was removed, clipped to a filter paper (Whatman No. 1) and allowed to air dry for at least an hour.

3.15.4. Immunoprobing

Following drying, the membrane was blocked in 5 % (w/v) skimmed milk powder (casein hydrolysate, SRL) in 0.15 M, phosphate-buffered saline, pH 7.2 (PBS) supplemented with 0.5 % Tween-20 in a heat sealable bag and incubated for 2 h on an

orbital shaker (CIS-24, Remi) at 37 °C and 90 r.p.m. Subsequently, the membrane was incubated with antiserum [1:1 (v/v) blocking solution : PBS (containing IgG, diluted as per requirement)]. The bag was re-sealed leaving space for a few air bubbles and incubated at 4 °C. Next, the membrane was washed 3 x 250 ml PBS followed by a single wash in 50 mM Tris-HCL, pH 7.5 containing 50 mM NaCl (TBS) for 20 min with gentle agitation to assure removal of azide and phosphates from the membrane prior to the enzyme coupled reaction step. The enzyme, alkaline phosphatase tagged with anti-rabbit goat IgG diluted 1:10,000 in alkaline phosphatase buffer [0.05 M Tris-HCl buffer, pH 9.8] and incubated for 90 min at room temperature. Finally the membrane was washed 5 x 200 ml TBS and transferred to a bag containing 10 ml of NBT-BCIP substrate. The reaction was monitored carefully. On visualization of the bands up to the desired intensity the membrane was transferred to a tray flooded with stop solution.

3.16. Immunocytochemical staining

Immunocytochemical staining of cross sections of tea leaves was done using a non-fluorescent stain based immunoenzymatic format following the method of Young and Andrews (1990) with a few modifications. Fresh, naturally blister-infected, healthy and artificially inoculated (TV-30, T-78 and UP-2) tea leaves were used as the experimental material. Cross sections were cut through the leaves and incubated with PBS containing 1 % BSA and 2 mM PVP-10 for 20 min at room temperature to prevent non-specific binding of antibodies. The tissues were then reacted with PAb of *E. vexans* (PAb-EV1) diluted 1:10 in PBS with 1 % BSA (PBS-BSA) at 37 °C for 2 h on a rotary shaker. Following incubation the sections were washed with three 5 min-changes of PBS-T and incubated in a 1:10,000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in PBS-BSA for 2 h at 37 °C on a rotary shaker and again washed with PBS-T as described above. Immunocytochemical staining with the Fast blue BB salt substrate [1 mg Fast blue BB salt (Sigma) and 5 µl of 0.1 M $MgCl_2$ added per ml in a stock solution of - 0.15 g napthol-AS-MX-phosphate (Sigma) dissolved in 2.5 ml of N,N-dimethyl formamide (Sigma) added to 17 g of Tris base (Sigma), pH 9.1, in 500 ml of double distilled water] was carried out in the dark. The substrate solution was filtered through Whatman no. 1 immediately before application to the cross sections. Incubation in the stain was for no longer than

40 min followed by rinsing in PBS-T, as above. The sections were mounted in 10 % glycerol and observed under bright field illumination using a Biomed microscope (Leitz) and photographs were taken by Leica Wild MPS 48 camera on Kodak 200 ASA film.

3.17. Immunofluorescence

Indirect fluorescence staining of cross sections of tea leaves and basidiospores of *E. vexans* was performed following the method of Chakraborty and Saha (1994a) with polyconal antibodies labelled with anti-rabbit goat IgG conjugated with FITC and observed under UV light in a microscope (Leitz) equipped with I3 filter block ideal for FITC fluorescence. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

3.17.1. Cross sections of leaves

Cross sections were cut through tea leaves and immediately immersed in 0.15 M, phosphate-buffered saline, pH 7.2 (PBS) supplemented with 0.5% Tween-20, 0.01% PVP-10 and 1 % BSA. Good sections were selected, washed in PBS, treated with polyconal antibodies (1:10) and incubated for 1 h at 24 °C in grooved slides. After incubation, sections were washed thrice with PBS followed by treatment with 50 µl of FITC-conjugate (1:40). The sections were incubated for 30 min at 24 °C, washed and mounted in 10% glycerol on grease free slides. The slides were observed and photographed.

3.17.2. Basidiospores of *E. vexans*

Basidiospores, collected from fresh naturally blister-infected tea leaves were suspended in 100µl PBS and centrifuged, with three changes (of PBS), at 1000 r.p.m. for 1 min each. The PBS supernatant was discarded and to it antiserum (1:10) was added. The spores were incubated for 1 h at 37 °C. After incubation, the samples were again centrifuged & washed thrice for 1 min at 1000 r.p.m. The supernatant was discarded and the spores were incubated in 50 µl of FITC-conjugate diluted with PBS (1:40) in the dark at 37 °C for 30 min. After incubation, FITC was removed by repeated washings with PBS and the spores were mounted in 10% glycerol on grease free slides and observed and photographed.

3.17.3. Binding of FITC labelled concanavalin A

The method as described by Keen and Legrand (1980) was followed for binding of fluorescein labelled concanavalin A to basidiospores of *E. vexans*. Initially the basidiospores were incubated for 20 min in 0.85 % NaCl in 0.1 M potassium phosphate, pH 7.4 containing 1 mg ml^{-1} fluorescein isothiocyanate (FITC) labelled concanavalin A (Sigma). The spores were then washed thrice with a saline solution by repeated low speed centrifugation and re-suspension. Finally these were viewed under the microscope and photographs were taken.

3.18. Bioassay for antimicrobial activity of proteins

3.18.1. Spore germination bioassay

The effects of purified chitinases and β-1,3-glucanases on spore germination of *E. vexans* was assayed. Basidiospores were freshly collected from blister blight naturally infected leaves (as described earlier). The spores were suspended at a density of 1.6 x 10^5 spores ml^{-1} in sterile water. Glass micro slides were degreased with alcohol and dried in a hot air oven (80 °C, 30 min). The effect on spore germination *in vitro* was determined using microscopic cavity slides. A droplet (20 µl) of the spore suspension was placed centrally on each slide. The enzyme solutions (40 µl) of β-1,3-glucanase and chitinase at various concentrations were added. The microscopic slide was put into a Petri plate with approximately 100% humidity and incubated for 16-20 h in an incubator at 25 ±2 °C BOD (Remi) in the dark. Basidiospores were considered germinated if they had a germ tube or appressorium at least twice the length of the spore (Ji and Kuc, 1996). Two sets of spore suspensions in sterile distilled water and in buffer solution were included as controls and incubated under identical conditions. On germination the spores were stained with 0.1 % cotton blue in lactophenol [phenol crystals: lactic acid: glycerol in the ratio 1:1:1 (w/w)]. Lengths of 100 individual hyphae were measured under bright field illumination in a microscope (Leitz) and photographs of the germlings were taken by Leica Wild MPS 48 camera on Kodak 200 ASA film.

3.18.2. Microtitre dish assay

Assays for inhibition of fungal growth were conducted essentially as described by Broekaert *et al.* (1989). Three hundred microlitres of 1.5 % Bactoagar (Difco Lab.)

containing 3.5 % Czapek Dox broth were added to the wells of a microtitre plate (Costar Co.). After the medium solidified, 10 µl of a spore suspension (2 x 10^5 spores ml^{-1}) of *Glomerella cingulata* were added to each well and the spores were allowed to germinate at 22 ±2°C in the dark. Twelve hours later purified chitinase and β-1,3-glucanase (40 µl) and intercellular wash fluids from induced or control plant leaves were added to the wells. Fungal growth and development were monitored visually as well as microscopically, after which the plates were photographed. Buffer containing denatured enzyme was used as an additional control.

3.18.3. Agar cup bioassay

Antifungal activity of purified proteins was also tested using agar-cup bioassay techniques. Richard's agar medium was poured in Petri dishes and four wells were made in each plate. Aliquots (40 µl) corresponding to 1.5 g of fresh leaf tissue of individual fractions and ethyl acetate (in control sets) were dispersed in each of the four wells. Five-millimeter agar blocks of 7-day old cultures of *Glomerella cingulata*, cut from the advancing zone, were centrally deposited in each plate at equal distance from the wells and incubated at 30 °C for 7 days. Fungal growth and development was monitored and inhibitions were recorded in relation to control, when the organism had fully grown.

3.19. Extraction of antifungal compounds

3.19.1. Phenolics

For extraction of antifungal phenolics the method of Daayf *et. al.* (1995) was adopted with a few modifications for the determination of free and glycosidically linked phenolics. For the experiment, healthy, blister blight diseased as well as leaves showing hypersensitive reaction towards *E. vexans* were collected from tea gardens. In addition tea plants artificially inoculated with the pathogen were also considered. Leaves were harvested 48 h after inoculation. Leaf samples (10 g) were mixed with 80 % methanol (10ml g^{-1} leaf tissue) and homogenized for blending for about 1 min. Samples were extracted for 48 h on a rotary shaker, in conical flasks covered with aluminium foil for protection from light, at 50 r.p.m. The methanolic extracts were then filtered through Whatman no. 1 filter paper and concentrated to dryness by evaporation up to 20 ml (1/ 5^{th} of the initial volume). Concentrates (aqueous fraction)

were first partitioned against an equal volume of anhydrous diethyl ether three times. The ether fraction was stored and termed Fraction I. The aqueous fraction was secondly partitioned against an equal volume of ethyl acetate (three times) and the ethyl acetate fraction was termed Fraction II.

Acid hydrolysis with 4 N HCl of the remaining aqueous fraction (yielding phenolic aglycones) was performed as per the protocol of Daayf *et al.* (1997). Aglycones were recovered by partitioning hydrolysates against an equal volume of ethyl acetate (three times) and termed Fraction III. All the fractions were evaporated to dryness and finally dissolved in spec. methanol for UV-analysis and in HPLC grade methanol for HPLC analysis. These fractions were also used for bioassays.

3.19.2. Catechins

Tea leaf catechins were extracted from healthy, blister blight infected and artificially inoculated (with *E. vexans*) leaves according to the method of Obanda and Owuor (1994) with slight modifications. Leaf samples (10 g) were extracted in 80 % acetone (HPLC grade) (10 ml g^{-1} tissue) by boiling at 45 °C in a water bath for 45 min followed by homogenization in a mortar with pestle. The extracts were decanted and filtered through Whatman no. 1 filter paper. The acetone extracts were concentrated to dryness and the residue was finally dissolved in 20 ml water (HPLC grade). The water solution was fractionated four times with an equal volume of chloroform (HPLC grade). The pH of the water fraction was adjusted to 2 with 2 N HCl and then dissolved in 3 ml of 2 % acetic acid. The samples were finally filtered through Millipore filter paper (0.45 μm) and analysed by HPLC.

3.20. Analyses of antifungal compounds

3.20.1. Thin Layer Chromatography (TLC)

For TLC, clean, grease free glass plates were coated evenly with Silica Gel G (E Merck). After air drying, the plates were activated at 80-100 °C before spotting the respective compounds. Antifungal compounds were analyzed on the chromatograms prior to UV-spectrophotometry. The test compounds were spotted on replicate TLC plates and developed in chloroform : methanol (9:1 v/ v) solvent system (Chakraborty and Saha, 1994b). Following evaporation of the solvent, one of the thin-layer plates were observed under UV-light and sprayed with Folin-Ciocalteau's phenol reagent.

Colour reactions and R_F values were noted. The silica gel from unsprayed replicate TLC plates of corresponding R_F zones were scraped off, eluted separately with methanol, rechromatographed with authentic pyrocatechol, compared and eluted with methanol (Spec. grade) for spectral analysis.

3.20.2. UV-spectrophotometry

The purified eluants of antifungal compounds were analysed in a UV-spectrophotometer (Shimadzu, 160) at a range of 200 - 400 nm and the maximum absorption was determined. Quantification of the antifungal compound (pyrocatechol) was done from UV-spectrophotometric curve by considering the molar extinction coefficient of 6000 at 274 nm and expressed in $\mu g\ g^{-1}$ leaf tissue.

3.20.3. High Performance Liquid Chromatography (HPLC)

Catechin analyses of the compounds were 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 6 min and then linear gradient to 75 % B over 5 min. The elution was complete after a total of 25 min. Flow rate was fixed as 1ml min^{-1}, sensitivity 0.5 aufs, injection volume 20 µl and monitored at 278 nm (Shimadzu Advanced VP Binary Gradient).

3.21. Bioassay of antifungal phenolics

3.21.1. Spore germination bioassay

Antifungal activity of all antifungal phenolic fractions was tested by spore germination bioassay (Ji and Kuc, 1996) against *E. vexans*. The basidiospores were suspended at a density of 1.6 x 10^5 spores ml^{-1} in sterile water. The effect on spore germination *in vitro* was determined using microscopic cavity slides. Ten microlitres of the spore suspension were added per depression. Ten microlitres of the different fractions of antifungal phenolics, were then added to the spore suspension. The microscopic slide was put into a moist Petri plate (100 % humidity) and incubated for 16-20 h in an incubator at 25 ±2 °C BOD (Remi) in the dark. Two sets of spore suspensions in sterile distilled water and in the respective solvents were included as controls and incubated under identical conditions. Spores were considered germinated if they had a germ tube or appressorium at least twice the length of the spore, stained

with lacto-phenol cotton blue stain, observed under bright field illumination and photographed.

3.21.2. Radial growth bioassay

Radial growth inhibition bioassay was performed for determining antifungal activity of phenolics extracted from tea leaves as described by Van Etten (1973). The fractions (50 µl) were taken in sterile Petri dishes (2" dia) and allowed to evaporate. Subsequently 10 ml sterilized PDA medium was poured in each Petri dish, thoroughly mixed and allowed to solidify. Agar block (4 mm dia) containing mycelia of *Curvularia pallescens* (7-day old culture) were taken from the advancing zone and transferred to each Petri dish these were incubated at 28 ±2 °C, until inhibition of mycelial growth was observed and photographed.

3.21.3. Agar-cup bioassay

Sterilized nutrient agar medium (NA) was warmed to liquefy, cooled to 42 – 45 °C, seeded with *B. megaterium*, poured on Petri dishes (2" dia) and allowed to solidify. A well (4 mm dia) was made with a sterilized cork borer and 50 µl of each test solvent fraction was added to each plate and incubated at 37 ±2 °C. Controls sets were also prepared at the time and kept under identical conditions. The diameter of the inhibition zones was determined.

3.22. Electron microscopy

3.22.1. Scanning electron microscopy (SEM)

Basidiospores of *E. vexans* collected in Petri plates were lifted with a brush and prefixed following the method of Shetty *et al.* (2003) prior to mounting on the sample stub. Similarly, healthy and infected tea leaves were trimmed to the appropriate size so as to accommodate them on the stubs and fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 6.8 in vacuum. After 24 h, the leaf pieces were washed in buffer and dehydrated in a graded series of acetone. The tissue was dried in a Critical Point Dryer and mounted with silver paint. Subsequently the samples were coated with a 20 nm gold-palladium alloy in a Sputter Coater and examined in a JEOL JSM 5200 Scanning electron microscope (Tokyo, Japan).

3.22.2. Transmission electron microscopy (TEM)

Transmission electron microscopy of blister blight infected tea leaves as well as salicylic acid induced tea plants was performed following the method of Tahiri-Alaoui *et al.*, (1993) with a few modifications.

3.22.2.1. Tissue processing

Segments (1 to 2 mm) from control, blister blight infected and salicylic acid treated tea leaves were excised in 0.1 M sodium-phosphate buffer, pH 7.4 (PB). They were immediately transferred to 1.5 % phosphate-buffered glutaraldehyde in Eppendorf tubes for 60 min and fixed in 3 % glutaraldehyde (EM grade) for 16 h at room temperature. After rinsing 3 x 10 min in PB, specimens were dehydrated in ascending grades of ethanol in the series 2 x 10 min 50 % ethanol and 2 x 45 min 70 % ethanol, followed by infiltration in LR White resin (Sigma) in 70 % ethanol (2:1) for 60 min. Embedding of the specimens was done in 100 % LR White resin in air tight gelatine capsules (Agar, size 0). The specimens were transferred to absolute resin (60 min) and then embedded in fresh resin and polymerized at 60 °C in a fume hood for 2 days. The cured resin blocks were trimmed (Leica EM Trim) to remove the excess resin before sectioning. The cutting plane was trimmed to the shape of a trapezoid. Glass knives were made (Leica EM KMR2) for cutting semi-thin sections. The trimmed blocks were set in an ultramicrotome (Leica Ultracut, UCT M26 equipped with M26 viewer) so as to get the material in the entire cutting face. Semi-thin sections (2 µm) were stained with 0.5 % aqueous toluidine blue solution and observed under light microscopy (Leica Biomed). Ultrathin sections (50 nm) of the same selected blocks were cut with a diamond knife. A ribbon of about 6-7 sections were picked up on Pioloform coated nicked grids (300 mesh) for immunogold labelling. A grid of each sample containing ultrathin sections was stained with 2 % aqueous uranyl acetate at room temperature for morphological observation. The grids were floated on a drop of the stain for 45 min followed by a wash with double distilled water. Observations were carried out in a Tecnai 12 Bio Twin transmission electron microscope (Philips, The Netherlands).

3.22.2.2. Immunogold labeling

Immunogold labeling was carried out by floating the grids on drops of the solutions pipetted (50 µl) on to parafilm. Ultrathin sections were incubated with blocking solution containing 1 % (w/v) of BSA in 0.1 M phosphate buffered saline, pH 7.4 (PBS) for 15 min. Incubation of the sections with the primary antibody (PAb) diluted at 1:5 in the blocking solution for 60 min was followed by washing in 4 x 10 min baths in PBS. In the secondary immunolabelling step, the sections were incubated with goat anti-rabbit IgG (whole molecule) gold conjugate, 10 nm (Sigma, G7402) diluted at 1:10 in PBS for 60 min at room temperature. After rinsing with PBS the sections were stained with 2% aqueous uranyl acetate for 10 min. The sections were washed in double distilled water, post stained in 0.2 % lead citrate for 3 min, and finally washed again in double-distilled water. After contrasting with uranyl acetate and lead citrate, ultrastructural analysis of the sections was performed with a Tecnai 12 Bio Twin TEM at 75 kV with Megaview III Soft Imaging System. A minimum of six samples was examined using an average of 3 replicates per immunogold treatment. Specificity of labelling was assessed by the following control tests (i) incubation of the ultrathin sections with the rabbit pre-immune serum instead of the primary antibody and (ii) incubation with the secondary antibody and omitting the primary antibody step.