MATERIALS AND METHODS

3.1 Plant material

3.1.1 Selection

Thirtyseven 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, Department of Botany, University of North Bengal sponsored by the Department of Biotechnology, Ministry of Science & Technology, Govt, of India. Based on the growing suitability of tea plants (*Camellia sinensis* (L) O Kuntze) as observed under the field conditions over the years by the Research Scientists of Immuno-Phytopathology Laboratory, the following 15 varieties were selected for the present study.

Source	Tea varieties	Origin
Tocklai Experimental Station, Jorhat, Assam	TV-9	B
	TV-18	C
	TV-20	C
	TV-22	C
	TV-23	C
	TV-25	C
	TV-26	C
	TV-29	A
	Teen Ali-17/1/54	D
UPASI, Valparai, Tamil Nadu	UP-2	A
	UP-3	A
	UP-9	B
	UP-26	C
	BSS-2	A
	BSS-3	B

A- Assam, B- China, C- Cambod, D- Assam x China; BSS – Biclonal seed stock

3.1.2. Growth and maintenance

The selected tea varieties were propagated by cutting as recommendation made by Bezbaruah and Singh (1988). Sandy soil (sand 75% and soil 25%) with pH ranging from 4.5-4.8 was used for propagation of tea palnts by cutting. Soil pH was adjusted by treating with 2% aluminium sulphate. Excess aluminium suplhate was removed by watering. Polythene sleeves (8"X6") were filled up with prepared soil and and stacked in rows in a bed and watered thoroughly. All the cuttings were allowed for rootng 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 Agro-net House. Each bed was watered regularly, initially by sprayer and later on with 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 (Plate 2 A) 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 (6-month-old) were then transferred to pots and maintained in the glass house. Besides plants were also transferred to the Phytopathological Experimental Garden (Plate 2,B-F) of the Botany Department, North Bengal University. Three 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 hight and temperature and watered on alternate days with ordinary tapwater by sprinklers (Plate 2G).

3.2 Fungal culture

3.2.1. Source

A virulent strain *Corticium theae* Bernard (Ct-1) was obtained from Tocklai Experimental station, Jorhat, Assam. Another strain (Ct-2) of *C.theae* was also isolated from naturally infected (black rot) tea leaves from Kailash Pur Tea Eastate, Jalpaiguri. These two strains of *C.theae* (Plate 3,C&D) were used after completion of Koch's postulate.The black rot disease are generally observed between May to July. Infected tea leaves were collected from Kailash Pur Tea Estate in the month of July in polythene packets, properly sealed and brought to the laboratory. *Glomerella cingulata* (Stoneman) Spauld and Schrenk was obtained from culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengl. A non-pathogen of tea

Fusarium oxysporum Schlecht was collected from Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi.

3.2.2. Completion of Koch's postulate

Fresh, young tea leaves (TV-9) were collected from Phytopathological Experimental garden and inoculated with the inoculum (grown on PDA petriplates) of *C. theae* following detached leaf inoculation technique. After 72 hr. of inoculation, infected leaves were, washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ for 2-3 minutes, washed serveral times with sterile distilled water and transferred aseptically into Potato-Dextrose-Agar (PDA) slants, and incubated at 28+1°C. After 7 days of incubation the isolated fungal culture was examined, compared with the stock culture and identification was confirmed as *Corticium theae.*

3.2.3. Maintenance of Stock culture

The fungus thus obtained was subcultured on PDA slants, incubated at 28°C for 2 weeks and finally the culture was stored at 5°C and 28°C. Either detached leaf or cut shoot inoculation with *C theae* and subsequent reisolation of the pathogen was done at some interval in order to maintain its virulence. These were kept at above conditions for experimental use.

3.2.4. Assessment of mycelial growth

To assess mycelial growth of *C theae*, the fungus was grown in Petri dishes (9 cm.dia) , each containing 20 ml of PDA medium and incubated for 7 days at 28+1°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 Potota Dextrose Broth for two weeks at 28 $\pm$ 1°C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60°C for 96 h, cooled in a desicator and weighed.

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 tea leaves were detached from plants and

Plate 2 (Figs. A-G) : Tea plants in the nursery and field. (A) Nursery grown tea saplings; (B-F) Tea Germplasm Bank; (C-F) UPASI varieties in the field; (G) Tea plantation in North Bengal University.

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 either side of the midrib, two to four such wounds were made in each leaf which were immediately inoculated with the inoculum block (made by sterile cork borer) of *C. theae*. 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 as described by Yanase and Takeda (1987). Twigs with three to four leaves of tea plants grown in the experimental garden were cut carefully with a sharp blade and immediately introduced into the glass aquarium, with the twigs dipped in a floating thermacol. Leaves were inoculated by making two light scratches with the point of a fine scalpel on the upper surfaces of leaves as described by Cooks (1989). Inoculum blocks (2 mm. dia) of *C. theae* were taken from the petriplates aseptically and placed on the inoculation site and then covered with absorbent cotton wool moistenced with sterile distilled water. Sterile PDA blocks were used as control. For each treatment 50 cut shoots were inoculated.

3.4. Disease assessment

3.4.1. Detached leaf

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

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. theae* were counted. Diameter of the individual lesions were measured and they were graded into four groups and a value was assigned to each group viz., 0.1, 0.25, 0.5 and 1.0, respectively for small restricted lesion diameter of 2-4

mm, 4-6 mm with sharply defined margin, lesions with slow spread beyond 6 mm, and spreading lesions of variable in size, with diffused margin. Finally number of lesions in each group was multiplied by the value assigned to it and the sum total of such values were noted and disease index was computed as the mean of observations on 50 cut shoots per treatment. Data were taken after 48, 72 and 96 h of inoculation.

3.5. Meteriological data

Monthly record of meteriological data for a period of three years (1999-2001) were obtained from Nagrakata Tea Research Station, Jalpaiguri. Meteriological data included maximum and minimum temperature, relative humidity and average rainfall.

3.6 Extraction of phenolics

Total phenol and orthodihydroxy phenol content of healthy and *C. theae* inoculated tea leaves of resistant and susceptible varieties were extracted following the method of Mahadevan and Sridhar (1982). Detached leaf inoculation technique as described earlier was followed. In case of control, sterile distilled water was mounted on the adaxial surface of leaves. Phenols were extracted from 1 gm each of healthy and inoculated 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 though filter paper. Final volume was adjusted with 80% ethanol (5ml/g fresh weight of leaves).

3.7. Estimation of phenol content

3.7.1 Total phenol

The total phenol was estimated by Folin ciocalteau's reagent as described by Farkas and Kir (1962). One ml of the alcohol extract was taken in a test tube, 1 ml of Folin ciocalteau's reagent followed 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 25ml. Absorbance was measured in a Systronics photoelectric colorimeter Model-101at 515 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.7.2. Orthodihydroxy phenol

The orthodihydroxy phenol was estimated as described by Mahadevan (1966). 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_2 MoO_4-10g ; Distilled water 100 ml) and 2 ml of 1 N NaOH were added, following which the volume was raised to 25 ml. Absorbance was recorded by a systronics Photo electric colorimeter model-101 at 515 nm. Quantity of orthodihydroxy phenol was estimated using caffeic acid as standard.

3.8 Extraction of enzymes

Two enzymes- phenyl alanine ammonia lyase and peroxidase- involved in phenol metabolism were extracted from tea leaves to determine their activities.

3.8.1. Phenyl alanine ammonia lyase (PAL)

For extraction of PAL, method of Chakraborty *et al* (1993) was followed. Leaves (1gm each) were crushed in a mortar in 5ml of 0.1 M sodium borate buffer (pH8..8) containing 2m M β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was collected & its final volume was measured and used immediately for assay or stored at -20°C.

3.8.2. Peroxidase (PO)

To extract PO, method of Chakraborty *et al.* (1993) was followed. Tea leaves samples were crushed with 0.1 M sodium borate buffer (pH 8.8) containing 2m M β - mercaptoethanol in mortar in ice. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was collected & its volume recorded and used for assay or stored at -20°C.

3.9 Assay of enzyme activities

3.9.1. Phenylalanine ammonia lyase (PAL)

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenyl alanine spectrophotometrically. The reaction mixture contained 0.3 ml. of 300 μM sodium borate (pH 8.8); 0.3 ml of 30 μM L- phenyl alanine and 0.5 ml of supernatant in a total volume of 3ml. Following incubation for 1h. at 40°C

the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as µg cinnamic acid produced in one minute/g fresh wt. of tissue.

3.9.2. Peroxidase (PO)

For PO determination 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture, containing 1ml of 0.2M sodium phosphate buffer (pH 5.4), 100 µl of 4 mM hydrogen peroxide, 100µl of O-dianisidine (5mg/ml of methonal) and 1.7 ml of distilled water. PO activity was assayed spectrophotometrically at 460nm by monitoring oxidation of O-dianisidine in presence of hydrogen peroxide. Specific activity was expresed as the increase in absorbance at 460 nm/g tissue/minute.

3.10. Colllection of leaf diffusates and fungitoxic assay

Leaf diffusate of resistant and susceptible varieties of tea were collected following the drop diffusate procedure as described by Chakraborty and Saha (1989). Fresh tea leaves were detached from plants and placed on moist blotting paper kept in a plastic tray (37.5 cm x 30 cm). Their upper surfaces were wounded as described by cook (1989). Twenty droplets (2-4 drops per leaf) of sclerotial suspension prepared from 14 day old culture were placed on the adaxial surface of each leaf. In case of control, sterile distilled water was used. Fifty leaves were taken for each treatment. The tray was covered with a glass lid and sealed with a smear of white petroleum jelly. After 24 h and 48 h. of inoculation, drops of sclerotial suspension and water were collected separately from the leaf surfaces and centrifuged. These were passed through sintered glass filter, and finally their biological activities, were assayed following the slide germination procedure as described by Rouxel et al., (1989). Usually, 1.9 ml of diffusate was mixed separately with 0.1ml of known concentration of spore suspension of *Glomerella cingulata* and sclerotial suspension of *C. theae* respectively. Single drop (0.02 ml/drop) of suspension was placed at the centre of a grease-free class slide. The slide was incubated in moist Petri dishes for 24 h at 25 $\pm$ 1°C. Finally, spores/sclerotia were stained with cotton blue in lactophenol and examined under the microscope. Percentage germination was calculated in each case.

3.11. Extraction of antifungal compound

Tea leaves were collected from the experimental garden and detached leaf inoculation technique was followed for artificial inoculation. In this case half of the total number of leaves were inoculated with the sclerotial suspension of *C. theae* while the other half was maintained as control in water. Both healthy and inoculated leaves were harvested separately after 24 h and 48 h inoculation, weighed, kept in Erlenmeyer flask and vacuum infiltrated with 40% aqueous ethanol (15ml/g fresh mass tissue) following the facilitated diffusion technique of keen (1978). The flask, containing the plant tissue immersed in the ethanol solution were stoppered and placed in rotary shaker (110 cycles/ min at 25°C). Shaking for 12 h removed most of the extractable antifungal compounds from tea leaves, these were separated by filtration and the filtrates were concentrated in vacuo to approximately one half volume at 45°C. The concentrated solution was extracted three time with ethyl acetate and the organic layers pooled and dehydrated with Mg SO_4. Ethyl acetate fraction was then concentrated in vacuo to dryness, dissolved in methanol (50ml/g) and analyzed by TLC.

3.11.1. Chromatographic analysis

Ethyl acetate fractions of both healthy and *C. theae* inoculated tea leaves were analyzed 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 a). Following evaporation of the solvent, the 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).Colour reactions and Rf values were noted.

3. 11. 2. Bioassay

3. 11. 2.1. Radial growth

Radial growth inhibition assay as described by Van Etten (1973) was followed. Ethyl-acetate fraction of healthy and *C theae* inoculated extract (0.2ml) were taken separately in each of the sterile Petri dishes (3 cm dia.) 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 Petridishes, thoroughly mixed

and allowed to solidify. Agar block (3 mm dia.) was cut with a sterilized cork borer from the advancing zone of 7 day old culture of *C theae* and *G.cingulata* grown in PDA and transferred to each Petri dish. Radial growth of *C theae* and G.cingulata were compared.

3. 11.2.2. Chromatogram inhibition

Ethylacetate fraction of healthy and *C. theae* inoculated tea leaf extracts, were spotted on TLC plates (silical gel G), and the chromatogram inhibition assay as devised by Hofmans and Fuchs (1970) was performed using *Curvularia lunata* as the test organism. Spore suspension supplemented with Richard's medium were sprayed on TLC plates and incubated in a sterile humid chamber at 25°C for 72 h. Fungitoxicity was ascertained by the presence of inhibition zone(s) which appeared as white spots surrounded by a blackish background on mycelia. Diameters of inhibition zone(s) and RF values were noted.

3.11.2.3 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 *G. cingulata* as described by Werder and Kern (1985).

3. 11.2.4. UV- Spectrophotometric analysis

For spectral analysis of antifungal compound extracted from healthy and *C. theae* inoculated leaves, initially ethyl-acetate fraction were spotted 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 respotted on TLC plates and developed in the same solvent and again scrapped and eluted in spec methanol. The purified eluants were examined by UV-spectrophotometer (Shimadzu model 160) and the maximum absorption was determined.

3.12. Cell wall of *Corticium theae*

3.12.1. Isolation

Cell wall was isolated from *C. theae* following the procedure of Keen and Legrand (1980). Mycelium of 14 day-old log phase fungus culture was collected on filter paper in a Buchner funnel and 50g of fresh packed cells were ground for 1 min in a National Super Blender mixer cup (full speed) with water (4ml/g). The resulting sherry was then disrupted in homogenizer for 1 min at 5°C. The mixture was centrifuged for 1 min at 1500 rpm the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10ml/g) and pelleted by contrifungation at least six times or until the supernatant fluids are visually clear. Finally the isolated cell walls were frozen and kept at-20°C.

3. 12.2. Preparation of mycelial wall extract

Mycelial wall extract was prepared from the isolated cell wall of *C theae* following the method of Brown and Kimmins (1977). Isolated cell walls (2.0g) were suspended in 80ml ice-cold 0.1 N NaOH by blending in a chilled mixer-cup at full speed for 30 sec. Then the suspension was slowly stirred in an ice bath for 15 h. Following centrifungation at 8000 rpm for 10 min the residue was washed with 50 ml ice-cold water and the pooled supernatants were carefully neutralized to pH 6.0 with 1 N HCL. The pooled supernatants were finally dialysed against distilled water and concentrated which were then used as crude mycelial wall extract (MWE) for SDS-PAGE analysis.

A second extraction method involved suspending 2.0 g of cell walls in 80 ml of 0.02M Sodium Citrate, pH 7.0 in the National super blender mixer and autoclaving for 2 h at 15 lbs,. p.s.i. Following centrifugation of walls the supernatant fluids were dialysed against distilled water, concentrated and used for bioassay purpose. This mycelial walls extract was mixed with an antibiotic gentamycin sulfate (100 mg/ml) in order to avoid bacterial growth.

3.12.3.Estimation

3. 12.3. 1. Carbohydrate

Estimation of carbohydrate in the preparation of mycelial wall extract was done following Anthrone method. Initially 1 ml each of 0.3 M Ba $(OH)_2$ and 5%$ZnSO_4$ was added to 8ml of cell wall extract. The mixture was incubated for 5 to 10 mins and the supernatant was taken after centrifugation. This supernatant (0.2ml) was mixed with

1.8ml of distilled water and finally 6ml of anthrone reagent (200 mg anthrone powder dissolved in 100ml of conc. H_2SO_4) was added, kept for 15 mins in water bath, cooled and absorbance noted at 620 nm in Systronic Photoelectric colorimeter model 101. Using glucose as standard, the carbohydrate content was estimated.

3.12.3.2. Protein

Method of Lowry et al. (1951) was considered for the estimation of soluble proteins. To 5ml of Alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml of 2% Potassium Sodium tartarate dissolved in 50 ml of 2% Na_2CO_3 in 0.1 NaOH), 1 ml of protein sample (10^{-2} dilution) was added, shaken and incubated for 20 minutes. After that 0.5ml of Folin-ciocalteau's reagent (dilute with distilled water in the ration 1:1) was added shaken and incubated for another 15 min for colour development. Optical density was measured at 720nm in Systronics Photoelectric colorimeter (Model 101). Using Bovine Serum Albumin (BSA) as standard the protein concentration was computed.

3.12. 4. Bioassay of mycelial wall extract

Drops (20 µl, 2-4 drops/leaf), each of sterile distilled water, sclerotial suspension of *C theae*, mycelial wall extract (MWE) preparation and MWE mixed with *C theae* sclerotial suspension were placed separately on the adaxial tea leaf surface kept in moist trays and incubated for 48 h as described earlier. These drops of four different treatments were collected separately, centrifused and assayed for their biological activities against (spore germination) of *C theae* as described by Rouxel et al., (1989).

3.12.5. Characterization

The method as described by Keen and Legrand (1980) was followed for binding of fluorescein labelled concanavalin A to mycelia as well as isolated cell wall of *C theae*. Initially mycelium or isolated cell walls were incubated for 20 min in 0.85% NaCl in 0.01M Potassium phosphate, pH 7.4 containing 1 mg/ml fluorescein isthiocyanate (FITC) labelled concanavalin (Con A, SIGMA chemicals) The fungus or walls were then washed thrice with saline solution by repeated low speed centrifugation and re-suspension. For control sets these were incubated lectin supplemented with 0.25 m a methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluorescence optics (BP 450-490 exciting filter, RKP 520 Beam splitting mirror, 515

suppression filter). Photographs were taken by Leica WILD MPS 48 Camera on Konica 400 ASA film.

3.13. Extraction of total soluble protein

3.13.1. Leaf protein

Soluble proteins were extracted from both healthy and *C. theae* inoculated tea leaves following the method of Alba and De vay (1985) with modification. Detached leaf inoculation technique as described earlier was followed, while control sets were prepared by mounting the leaves with drops of sterile distilled water. Healthy and *C. theae* inoculated leaves (1 gm each) were crushed separately in a mortar and pestle with 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM soluble polyvinyl pyrrolidone (PVPP 10,000 M) and 2 mM Poly methyl sulphonyl fluoride (PMSF) at 4°C with sea sand.The leaf slurry was centrifuged at 4°C for 20 mins. at 10,000 rpm. The supernatant was used as crude protein extract and immediately stored at 20°C for further use.

3.13.2. Mycelial protein

Extraction of mycelial protein of *C. theae* was done following the method of Chakraborty and Purkayastha (1983). *C theae* was grown in sterilized Potato Dextrose Broth (PDB) for 14 days at 28±2. C. Mycelia were collected, washed with 0.2% NaCl solution, rewashed with sterile distilled water, strained through cheesecloth and then crushed with sea sand and 0.05 M Tris- HCl buffer (pH7.4) using a mortar and pestle at 4^0C. The slurry was centrifuged at 10,000 rpm for 20 min at 4^0C. The supernatant was used as crude protein extract and stroed at -20°C for further use.

3.14. Estimation of total soluble protein content

Estimation of protein was done following the method of Lowry et al. (1951) as described earlier.

3.15. Polyacrylamide gel electrophoresis of soluble protein SDS-PAGE

3.15..1. Preparation of gel solution

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

Stock Solutions:

(A) Acrylamide

Acrylamide	..	30. 0g
Bis-acrylamide	..	0. 8g
Distilled water	..	100.0 ml

(filtered and stored at 4°Cin a brown bottle and used within one month)

(B) Lower gel buffer (LGB)

1.5 M tris	..	18.18 g
Distilled water	..	100.0ml

pH was adjusted to 8.8 with conc. HCl and stored at 4*C for use. 1.5 M Tris buffer was prepared for resolving gel.

(C) Upper gel buffer (UGB)

1M Tris	..	12.12g
Distilled water	..	100.0 ml

pH was adjusted to 6.8 with conc. HCl and stored at 4*C. This buffer was prepared for use in stacking and loading buffer.

(D) Ammonium peroxidisulphate (APS)

Ammonium peroxidisulphate	..	0.1 g
Distilled water	..	1.0 ml

(freshly prepared in each time)

(E) Sodium Dodecyl Sulphate (SDS)

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

(F) Tris-Glycine electrophoresis buffer

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

(G) SDS Loading buffer

This buffer contains 50mM Tris CL (pH 6.8), 10mM Beta Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1 x solution was made by dissolving

0.5ml of 1M tris buffer (pH6.8), 0.5ml of 14.4M Beta Mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1mg glycerol in 6.8ml of distilled water.

3.15.2 Preparation of Gel

Slab gel was prepared for the analysis of protein patterns by SDS-PAGE ie.,mini gel (8cm x 10cm). For 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 the plates were sealed with high vacum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order by pasture pipette leaving sufficient space for any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. The gel was kept for 30 minutes. After polymeization 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.

Resolving gel solution was first prepared with acrylamide and lower gel buffer, degassed for about 20 min and then TEMED and APS solution was added.

The composition of the solution for 10% resolving gel was as follows.

Name of the compound	Minigel (7.5ml)
1. Distilled water	2.85ml
2. 30% Acrylamide mix	2.55ml
3. 1.5 M Tris (pH 8.8)	1.95ml
4. 10% SDS	0.075ml
5. 10% APS	0.075ml
6. TEMED	0.003ml

Stacking gel solution was then prepared with acrylamide solution and upper gel buffer, degassed for about 20 min, TEMED and APS solution were added to the solution.

The composition of the 5% stacking gel solution was as follows:

Name of the compound	Minigel (7.5ml)
1. Distilled water	2.1ml
2. 30% Acrylamide mix	0.5ml
3. 1.5 M Tris (pH 8.8)	0.38ml
4. 10% SDS	0.03ml
5. 10% APS	0.03ml
6. TEMED	0.003ml

3.15.3. Sample preparation

Sample protein (34ul) was prepared in a tube with 1 x SDS gel loading buffer (16ul) in clyclomixture. A pinch of bromophenol blue was added to the sample buffer. The tubes of different sample proteins thus prepared were then floated in boiling water bath for 3 min to denature the protein sample and 25-30 ul of samples were immediately loaded in a pre-determined order into the bottom of the wells with a microtiter syringe. Along with the samples, protein marker consisting of a mixture of six proteins (Carbonic anhydrase, Egg albumin, Bovine albumin, Phosphorylase b, B- galactosidase and Myosin of molecular weight 29, 45, 66, 97, 116 and 205 KD) was also taken in a separate tube, prepared as above and loaded .

3.15.4. Electrophoresis

For this purpose, tris-glycine electrophoresis buffer was prepared as follows:

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

Electrophoresis was performed at constant 17mA current until the samples penetrated the resolving gel for 3 h in case of minigel, until the dye reaches the bottom of the gel column.

3.15.5. Fixing

The gels were removed from the glass plates and then the sracking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water (10:20/70) for overnight.

3.15.6. Staining

The gels were then stained either with Coomassie brilliant blue (sigma R 250)in 45ml methanol after the stain was completely dissolved, 45ml of water and 10ml glacial acetic acid were added. The prepared stain was filtered through filter Whatman No. 1 filter paper. The gel was removed from fixer and stained in this staining solution for 4 hrs. 37°C with constant shaking at a very low speed. After staining, the gel was finally destained in destaining solution containing methanol, water and acetic acid (4.5:4.5:1) at 37°C with constant shaking until background becomes clear.

3.16 Preparation of Antigen

3.16.1 Mycelial antigen

Mycelial antigen was prepared from the mycelia of *C theae* grown in liquid medium following the method of Chakraborty and Saha (1994).The mycelial content was strained through muslin cloth dried and its dry weight taken. The mycelia (5.5g) were homogenized in morter and pestle with 0.05M Sodium Phosphate buffer supplemented with 10mM Sodium meta bisulphite, 2mM soluble PVPP 10,000 and 0.5mM $MgCl_2$ (pH 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 10,000g for 30mins at 4^0C. The supernatant was used as mycelial antigen and stored at -20^0C.

3.16.2. Leaf antigen

Leaf antigens were extracted from both healthy and *C. theae* inoculated tea leaves following the method of Alba and De vay (1985) with modification. Detached leaf inoculation technique as described earlier was followed, while control sets were prepared by mounting the leaves with drops of sterile distilled water. Healthy and *C. theae* inoculated leaves (1 gm each) were crushed separately in a mortar and pestle with 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM soluble polyvinyl pyrrolidone (PVPP 10,000 M) and 2 mM Poly methyl

sulphonyl fluoride (PMSF) at 4°C with sea sand.The leaf slurry was centrifuged at 4°C for 20 mins. at 10,000 rpm. The supernatant was used as leaf antigen and immediately stored at 20°C for further use.

3.17. Production of polyclonal antibody

3.17.1. Rabbits and their maintenance

New Zealand white male rabbits were used to raise antisera against mycelial antigens of *C.theae*, non-pathogen (*F.oxysporum*) of tea, and healthy tea leaf antigens. Initially body weights were recorded before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetables etc., 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.

3.17.2 Immunization

Before immunization, normal sera were collected from each rabbit. Separate rabbits were intramuscularly injected once a week at 7 days interval with 1ml antigen mixed with 1ml of Freund's complete adjuvent (Difco, USA) for the first three injections and the next emulsified with incomplete adjuvent for 4 to 5 weeks.

3.17.3 Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first five injections, and then every third injection. In order to handle the rabbits during bleeding, they were placed on their back on a woddenboard fixed at an angle of 60*C. 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 hair from the upper side of the ear was shaved 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 1h for clotting. After clotting, the clot was loosened with a sterile needle. Finally the serum

was clarified by centrifugation (2000g for 10mins at room temperature) and distributed in 1ml vials and stored at –20 ^{0}C, as crude antisera.

3.18. Purification of IgG

3.18.1. Precipitation

IgG was purified as described by Clausen(1988). The polyclonal crude antiserum(2ml) was first diluted with two volume of distilled water and an equal volume of 4M Ammonium Sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22^{0}C The precipitate thus formed was collected by centrifugation at 10,000g at 22^{0}C for 1h. Then the precipitate was dissolved in 2ml of 0.02M Sodium phosphate buffer, pH8.0.

3.18.2. Column preparation

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

3.18.3 Fraction collection

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

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 bufer (1) was connected to another flask with buffer (20. The buffer (2) had also connection to the open air. During the draining of the buffer (1) to the column, buffer (2) was sucked into buffer (1) thereby producing a continuous rinse in molarity. Ultimately 40 x 5ml fractions were collected and the optical density values were recorded by

means of UV spectrophotometer at 280nm.The fractions showing >2 reading were stored as purified IgG.

3.19. Immunodiffusion tests

3.19.1. Preparation of agarose slides

Glass slides (5cm x 5cm) were degreased in 90% (v/v) ethanol, ethanol : diethylether (1:1) and then dried in hot air oven. After drying, plates were sterilized in autoclave at 15lbs for 20mins. Agarose gel was prepared in Tris barbiturate buffer, pH 8.6, at 90*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).

3.19.2. Diffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (100µl/well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in moist chamber for 72h at 25 °C

3.19.3. Washing, staining and drying of slides

After immunodiffusions, the slides were initially washed with sterile distilled water for 2h and then aqueous Sodium Chloride solution (0.9% NaCl + 0.1%NaN2) for 72h with 6 hourly changes to remove unreacted antigens and antiserum widely dispensed in the agarose.The slides were stained with Coomassie blue(R250) for 10mins at room temperature. After staining, slides were destainedwith 5% acetic acid solution with changes untill the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3h at 50 ^{0}C.

3.20. Enzyme linked immunosorbent assay (ELISA)

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

1. Antigen coating buffer : Carbonate bicarbonate buffer 0.05M pH 9.6 Stocks
   A. Sodium Carbonate - 5.2995g in 1000ml distilled water.

B. Sodium Bicarbonate - 4.2g in 1000ml distilled water. 160ml of Stock A with 360ml of Stock B and pH was adjusted to 9.6.

2. Phosphate buffer saline : 0.15M PBS pH 7.2.

   Stocks

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

   B. Disodium hydrogen phosphate-21.29g in 1000ml distilled water. 280ml of StockA was mixed with 720ml of Stock B and pH was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

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

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

4. Blocking reagent (Tris buffer saline pH 8.0). 0.05M Tris, 0.135M NaCl, 0.0027M KCl., Tris - 0.657g, NaCl - 0.81g, KCl -0.223g

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

5. Antisera dilution buffer (.15 M PBS Tiween pH 7.2) In o.15M PBS-Tween, pH 7.2, 0.2% BSA, 0.02% Poly vinylpyrrolidone, 10,000 (PVPP 10,000) and 0.03% sodium azide (NaN_2) was added.

6. Substrate

   p-Nitrophenyl phosphate (Himedia) 1mg/ml dissolned in 100ml of di ethanolamine (1.0% w/v; 3mM NaN_2) pH 9.8.

7. 3N NaOH solution was used to stop the reaction. This ELISA was peformed 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 eluded (200ul/well) in 8 welled ELISA strips (Costar EIA/RIA strip plate, USA), arranged in 12 rows in a (casette) ELISA plate. After loading, the plate was incubated at 25°C for 4 hrs. Then the plate was washed 4 times under running tap water and twice with PBS Tween and each time shaken to dry. Subsequently, 200ul of blocking reagent was added to each well for blocking the unbound sties and the plates were incubated at 25°C for 1 hr. After imabation. The plate was washed as mentioned earlier. Purified polyclonal Ig G was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4°C overnight. After further washing, antirabit IgG goat antiserum labelled with Alkaline Phosphatase diluted

10,000 times in PBS, was added to each well (100ul/well) and incubated at 37°C for 2 hrs. The and incubated at 37°C for 2 hrs. The plate was washed, dried and loaded with 100ul p-Nitro phenyl Phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50ul/well of 3N NaOH solution and the absence was determined in an ELISA reader (Model : Multiscan EX, Lab System) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.21. Fluorescence Antibody Staining and microscopy

Indirect fluorescence staining of cross sections of healthy tea leaves, and black rot infected leaves were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994), with modifications.

3.21.1. Mycelia and sclerotia

Mycelia and Selerotia, collected from fresh naturally infected black rot leaves were taken in a small beaker. A mycelial and sclerotial suspensions were prepared with PBS (pH 7.2) separately. The suspension were taken in an eppendroff tube and centrifuged at 3000 g for 10 mins and the PBS supernatant was discarded. The 100 µl of purified IgG was added into the eppendroff tube and incubated for 2 hr at 27°C. After incubation, the tubes were again centrifuged and washed for 10 mins at 300g. The mycelia / sclerotia were washed three times with PBS Tween, and the 100µl of antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and incubated in dark at 27°C For 1 hr. After incubation, FITC was removed by repeated washing with PBS-Tween and the mycelia/Selerotia were mounted on 10% glycerol and observed under leicamicroscope, equipped with UV fluorescence filter and photographs taken.

3.21.2 Cross-section of tea leaf

Fresh cross-sections of healthy leaves (TV-9 and TV23) were cut and immediately immersed in phosphate buffer saline (pH7.2) containing 8% NaCL and 0.02% KCL and 0.01% PVP 10. Good sections were selected and treated with *C. Theae* antiserum (1:40) and incubated for 1 hr at 27°C. All operations with FITC labelled antibodies were made in darkness (red light). After incubation,

sections were washed thrice with PBS Tween (pH 7.2) as mentioned above and then mounted on gresse free slides with 10% glycerol. A coverslip was placed on the section and sealed. Fluorescence of the leaf section were observed using leica leitz Biomed microscope with fluorescence optics equipped with UV filter set 13.

3.22. Dot-Blot

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

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

(ii) Tris buffer saline (10mM, pH 7.4) with 0.9% NaCl and 0.05% Tween 20 for washing

(iii) Blocking buffer- 10% casein hydrolysate in 0.05M Tris, 0.5 NaCl, 0.5% Tween-20,(pH 10.3).

Nitrocellulose membranae (Millipore, H5SMO 5255, 7cm x 10cm pore size 0.04 μm, Millipore corporation, Bedford) 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 NCM and kept for 25 mins to dry. Following this 2μl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 hrs at room temperature. Template was removed and blocking of the NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30 mins. Polyclonal antibody (IgG) of *C theae* 1:40 was added directly in the blocking solution and further incubated at 4°C for overnight.

The membrane was then washed several times in TBS for Tween (pH 7.4). Enzymatic reactions were done by treating the NCM with Alkaline phosphatase Conjugate (1:7500) for 2 hrs at 37°C. This was followed by washing for 25 mins. In TBS Tween substrate (66μl) Nitro Blue Tetrazolium chloride (NBT)+ 33μl 5,Bromo-4 chloro-3 Indolyl phosphate Di sodium salt (BCIP) in 10ml of Tris buffer saline (pH 7.4). Finally reaction was stopped by floating the NCM in deionized water.