MATERIALS AND METHODS

3.1. Plant Material

3.1.1. Collection

Tea varieties released by three experimental stations viz. (a) Darjeeling Tea Research Centre, Kurseong, Darjeeling; (b) Tocklai Experiemental Station, Jorhat, Assam, and (c) United Planters Association of South India, (UPASI), Valparai, Tamil Nadu, are being maintained in Tea Germplasm Bank, Department of Botany, University of North Bengal. Thirty one tea varieties were collected from the Germplasm Bank, propagated in the nursery and planted in the experimental field as well as grown in the earthern pots. The varieties are as follows:

Table 1 : Tea varieties and their origins.

Source	Tea varieties	Origin
Tocklai Experiemental Station	TV - 9	B
Jorhat, Assam	TV - 18	C
	TV - 20	C
	TV - 22	C
	TV - 23	C
	TV - 25	C
	TV - 26	C
	TV - 27	C
	TV - 29	A
	TV - 30	A
	Teen Ali – 17/1/54	D
UPASI,	UP - 2	A
Valparai, Tamil Nadu	UP - 3	A
	UP - 8	A
	UP - 9	B
	UP - 17	C
	UP - 26	C
	BSS - 2	A
	BSS - 3	B
Darjeeling Tea Research Centre	AV - 2	B
Kurseong, Darjeeling	B - 157	B
	B - 777	A
	Takdah - 78	A
	TS - 449	C
	P1258	C
	CP - 1	B
	K1/1	D
	HV -39	B

A - Assam, **B** - China, **C** - Cambod, **D** - Assam x China.

3.1.2. Propagation by Cutting

Sandy soil (sand 75% and soil 25%) with PH ranging from 4.5 – 4.8 was used for propagation of tea plants by cutting. Soil PH was adjusted by treating with 2% Aluminium sulphate. Excess Aluminium sulphate was removed by watering.

Polythene sleenes (8"x6") were filled up with prepared soil and stacked in rows in a bed and watered thoroughly. All the cuttings were allowed for rooting in sleeves after dipping them in hormone. These cutting were kept in a polythene cloche, arranged in two rows, with 11 beds in each row. The complete set up was kept under a green Agro net house prepared with pretreated bamboo. Each bed was watered regularly, initially by sprayer and later in with pipe until the appearance of new shoots.

After about ninety days i.e. when all the cuttings had rooted, the polythene cloche was removed gradually over a period of 10-15 days, each day exposing the cuttings to the external atmosphere for brief duration. (Sudden removal may lead to shock and casualty).

3.1.3. Maintenance of tea sleeves in nursery

After the removal of the polythene cloche, the sleeves were treated with nursery mixture (nutrient). The composition of the fertilizer mixture is as follows :

Ammonium Sulphate	—	8 parts by weight.
Ammonium Phosphate	—	35 parts by weight
Potassium Sulphate	—	15 parts by weight
Magnesium Sulphate	—	3 parts by weight
Zinc Sulphate	—	3 parts by weight

Thirty grams of the above mixture was dissolved in 10 litres of water. Each sleeve was treated with 50ml of the nutrient mixture and the sleeves were treated with 100ml of the nutrient mixture. For nursery plants, a low level of shade is given with green Agro net supported with bamboos (Plate-2). Irrigation was done by means of hand sprinkler twice a day. Weeds were removed physically. Some times a dilute spray of Ankush (insecticide) at a concentration of 0.6ml/lit. was given to remove insects and pests. Disbudding or tipping was done once in 15 days to increase lateral shoots.

The tea sleeves (6-7 months old) were then transferred to pots and maintained in the glass house and also transferred to the field. Field planting is a most crucial operation

Plate 2. Tea varieties being maintained in the Phytopathological Experimental Garden

as it basically determines the development and productive level of tea throughout its economic life.

3.1.4. Maintenance of germplasm in glasshouse

Tea plants of all varieties were also grown in earthern pots (1 plant/ pot, 30cm diameter) each containing 5kg soil mixture (soil planting mixture 1:1). All these plants were maintained inside the Glasshouse under natural conditions.

All the clonal and seed stocks are being maintained in the Tea Germplasm Bank (Glass house) and also in the nursery. In case of young plants manuring was done with nursery mixture consisting of — Ammonium sulphate - 8 parts by weight; Ammonium phosphate - Sulphate (16 : 20)-35 parts by weight ; potassium sulphate-15 parts by weight Magnesium sulphate, 15 parts by weight and zinc sulphate-3 parts by weight following the method by Ranganathan and Natesan (1987) and continued upto 12 months once only in 15 days. The mixture was dissolved @ 30g in 1 litre of water and applied @ 50ml/ plant. The mature plants (1 year and above) were maintained by applying a soluble mixture of N,P,K consisting of 10kg Urea 46% N, 20kg Ammonium phosphate-11% P_2O_5, 8kg murate of potash, 60% K_2O in the soil. Hormonal spray was done at regular intervals for good growth of bush.

3.1.5. Plantation

Before planting, simazine @ 75g/20 litre water and Glycophosphate @1:200 were used in the experimental plots for the suppression of weeds (Borpujari & Banerjee, 1994). Then pits (1½ x 1½ x 1½) were dug at the intervals of 2' between plants and 3.5' between row to row. Trenches were dug 30cms wide 45cms deep along rows. Planting mixture was prepared in the ratio of 4.5kg well rotten dry cattle manure, 30g rock phosphate, 30g super phosphate and 2.5g phorate [0,0 – diethyl S – ethyl thiomethyl phosphoro dithioate]. At the bottom of each pit, rock phosphate was placed following which half portion was covered with cattle manure soil mixture. Following soil conditioning, plants were inspected (generally 9 to 12 months), selected and brought to the experimental garden and planted in the prepared soil. Pits were refilled with conditioned soil. Plants raised in nursery were also planted directly with a ball of soil enclosing the roots. After the young plant was established in the field, regular hormone spray twice in 30 days and irrigation by sprinklers (Plate-3) were done. Pruning was done during the winter once in a year.

Plate 3 - Tea Plantation

3.2. Collection of blister infected tea leaves

The blister blight epidemic was generally observed between July to October in the hills, and December to February in the plains. Taking this into consideration two Tea Estates of different locations (Hills and Plains) were chosen for the collection of healthy and infected tea leaves.

(i) Healthy and blister infected tea leaves were collected from Castleton Tea Estate, Kurseong (Darjeeling Hills) from the month of July to October. Healthy and infected leaves were collected separately in polythene packets, properly sealed and brought to the laboratory. For the collection of spores, infected twigs were cut with the help of a sharp blade and immediately dipped in water and brought to the laboratory. This method of collection was done twice a week continuously for 4 months.

(ii) Healthy and blister infected tea leaves were collected from Hansqua Tea Estate (Plains) from mid December till the end of February. This method of collection was the same as above.

3.3. Inoculation technique

Naturally blister infected twigs were collected from Tea gardens for in vitro basidiospore collection. These spores were used to inoculate healthy tea sleeves of different varieties.

3.3.1. Collection of spores from infected leaves

Blister infected twigs were collected from Castleton Tea Estate, Kurseong (Sec 4, China Variety) and immediately immersed in water. The twigs were brought to the laboratory. A set-up was prepared in order to trap the basidiospores by dipping one twig each in 2% sucrose solution kept in 50 ml sterile conical flasks. 15 flasks (50ml) were placed in one set up. The leaves containing the infected blister zone was placed horizontally (ventral side) on the mouth of sterile beakers (50ml size) and covered with glass slides (5cms x 5cms). Several such sets were incubated at 25°C (BOD). Basidiospores were deposited at the bottom of the beakers after 48hrs. These beakers containing basidiospores were then properly covered with parafilm and stored at 4°C until required. The collection of basidiospores from hills could be possible from July to October, when the appearance of blister was maximum.

3.3.2. Spore germination test

For spore germination, method of Trivedi and Sinha (1976) was followed with modifications. Slides (2.5cm x 7.5 cm) were degreased with alcohol and dried. Spore suspension was preapred by adding few drops of sterile distilled water into the beaker containing basidiospores. One drop of spore suspension was placed on the slides and kept at 25°C in BOD in moist chamber. Later on the slide was observed under microscope with cotton blue and lactophenol stain.

3.3.3. Inoculation of healthy tea plants

Inoculation of healthy tea plants were carried out following the method of Chakraborty *et. al.* (1996) with modifications. Young nursery seedlings (12 months old) were selected for artificial inoculation. The sleeves were placed on a set up tray measuring (30cm x 30cm). Sixteen sleeves were placed on one tray. The 1st and 2nd leaves of each sleeve were thoroughly cleaned with tap water and then with sterile distilled water. Spore suspension ($1.65x10^5$ spores / ml approximately) was prepared in the beakers. The spore suspension was brushed on both sides of the leaf surfaces. The complete set up was then covered with a transparent polythene sheet and tightened enough to retain the moisture in the enclosed chamber. The chamber was frequently moistened by spraying sterile distilled water, and incubated at 25°C (BOD). Similarly potted young tea plants were also inoculated inside the glass house under controlled temperature (25°C) and moisture (80%- 90%) conditions.

3.4 Disease assessment

Assessment of disease was done by calculating percentage infection both in naturally grown tea bushes in field and artificially inoculated nursery plant following the method of Venkata Ram and Chandra Mouli (1983).

3.4.1. Natural infection of field grown plants

The natural infection of blister blight disease was studied in Castleton Tea Estate, Hansqua Tea Estate and also in the nursery of Immunophyto Pathology Laboratory, Department of Botany, North Bengal University. One hundred shoots from each bush were selected at random and examined individually for lesions. A shoot was considered to

be infected even if a single transluscent spot occurred on it. The average percentage of blister blight infection was thus recorded.

3.4.2. Artificial inoculation of nursery plant

Percentage infection was determined by the no. of infected shoots and leaves examined (Chakraborty *et al.* 1996) with modifications. Artificial inoculation of tea sleeves has been explained in Materials and Methods **3.3.3**.

3.5. Obtaining meteriological data

Monthly record of meteriological data for a period of five years (1996 to 2000) was obtained from the Gangaram Meteriological Station, Tea Research association, Terai branch, Bengdubi. W.B., and Darjeeling Tea Research Institute, Kurseong, Darjeeling. Meteriological data included maximum and minimum temperature, Relative humidity (morning and evening) and Average rainfall. Hours of observation were 0635 and 1335 ISD.

3.6. Foliar Spray

Foliar application of systemic fungicide as well as botanical pesticides were screened for blister blight control.

3.6.1. Systemic fungicide

Hexaconazole is one of the most common and effective systemic fungicide which is used in commercial scale for the control of blister blight disease.

The product name is contaf 5% EC, Hexaconazole being the Generic name. This is a Tata Rallis product. The fungicide was diluted in water @ 1:1000 as prescribed and sprayed in field grown tea plants and also tea plants grown in nursery .

3.6.2. Biocide

Plant extracts were prepared from two selected plant species viz. *Catharanthus roseus* and *Azadirecta indica* (Neem), following the method of Varshney, 2001 with modifications. Mature leaves were collected and crude extracts were prepared by grinding 250g of leaves. The leaves were thoroughly washed with distilled water and a fine slurry prepared from these leaves with 100ml of distilled water. Distilled water was then added

to the crude extract to make its final volume to 1000ml. The homogenate was strained and sprayed in the field grown plants and also tea plants grown in the nursery, fortnightly for two months and harvest was done once after two sprays.

3.7. Soluble leaf protein

3.7.1. Extraction

Soluble proteins were extracted from healthy and infected tea leaves following the method of Chakraborty *et al.* (1995). Leaf tissues (5g) were homogenised with 0.05M Sodium phosphate buffer (PH 7.2). containing 10mM $Na_2S_2O_5$, 0.5mM $MgCl_2$, 2mM soluble Polyvinyl pyrrolidone (PVPP 10,000M) and 2mM Polymethyl sulphonyl fluoride (PMSF) in morter & pestle at 4°C with sea sand and insoluble PVPP. The homogenate was centrifuged at 4°C for 20min. at 10,000 r.p.m. and the supernatant was used as crude protein extract and immediately stored at –20°C for further use.

3.7.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et al.* (1957). To 1ml of protein sample 5ml of alkaline reagent (0.5ml of 1% Cu SO_4 and 0.5ml of 2% sodium potassium, tartarate dissolved in 50ml at room temperature and then 0.5ml of Folin Ciocalteaus reagent (diluted 1:1 with distilled water) was added and again incubated for 15 mins for colour development following which optical density (OD) was measured at 720nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.8. SDS-PAGE analysis of total soluble protein

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

3.8.1. Preparation of stock solutions

The following stock solutions were prepared :

A. Acrylamide and N'N' - methelene bis acrylamide.

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

filtered through whatman No.1 filter paper and was kept in brown bottle, stored at 4°C and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

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

C. Tris buffer

(a) 1.5M Tris buffer was prepared for resolving gel, The PH of the Tris was adjusted to 8.8 with conc. HCl and stored at 4°C for use.

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

D. Ammonium Persulphate (APS)

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

E. Tris - Glycine electrophoresis buffer

This running buffer consists of 25mM Tris base, 250mM glycine (PH-8.3) and 0.1% SDS. A 1 x Solution can be made by dissolving 3.02g Trisbase, 18.8g glycine and 10ml of 10% SDS in IL of distilled water.

F. SDS Loading buffer

This buffer contains 50mM Tris CL (PH-6.8), 10mM β Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5ml of 1M tris buffer (PH 6.8), 0.5 ml of 14.4M β Mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8ml of distilled water.

3.8.2. Preparation of Gel

Slab gel was prepared for the analysis of protein patterns by SDS-PAGE i.e. mini gel (8cm x 10cm). For slab gel preparation, two glass plates were thoroughly cleared with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at the three sides, and the three sides of glass plates were sealed with high vaccuum 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 polymerization of the stacking gel, the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.8.3. Sample preparation

Sample (34µl) was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in cyclomixture. All the samples were floated in boiling water bath for 3mins. to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microtiter syringe. Along with the samples, protein markers consisting of a mixture of Six proteins ranging in molecular weight from high to low molecular wt. (Phosphorylase b- 97, 400; Bovine serum Albumin - 68000 ovalbumin-43,000; Carbonic Anhydrase — 29,000, soybean trypsin inhibitor-20,000, lysozyme-14,300 daltons was treated as the other samples and loaded in separate well.

Composition of solution for 10% resolving gel :

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

Composition of solutions for 5% stacking gel

Name of the Compound		Minigel (7.5ml)
1.	Distilled water	2.1 ml
2.	30% acrylamide mix	0.5 ml
3.	IM Tris (PH 6.8)	0.38 ml
4.	10% SDS	0.03 ml
5.	10% APS	0.03 ml
6.	TEMED	0.003 ml

3.8.4. Electrophoresis

Electrophoresis was performed at constant 17 mA current for a period of 3 hrs in case of mini gel until the dye front reached the bottom of the gel.

3.8.5. Fixing and Staining

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

The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45ml methanol. After the stain was completely dissolved, 45ml of water and 10ml of 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 4hrs at 37^{0}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^{0}C with constant shaking until back ground became clear.

3.9. Preparation of antigens.

3.9.1. Leaf Antigen

Leaf Antigens were prepared from healthy and blister infected tea leaves following the method of Chakraborty & Saha (1994).

Leaves (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 10,000 rpm for 30mins at 4^0C. The supernatant was used as leaf antigen and stored at -20^0C. For the preparation of infected antigen only the infected areas (Blister Spots) were cut and taken.

3.9.2. Spore Antigen

Spores were collected from freshly infected blister areas of tea leaves in small sterilized beakers. The spores were first crushed by sea sand and 0.05M sodium phosphate buffer in a mortar and pestle and then homogenized in a homogenizer for 15 mins. The suspension was then centrifuged at 10,000 rmp for 30 mins. The supernatant was stored in 2ml vials at -20^0C as spore antigen for further use.

3.10.Serology

3.10.1. Rabbits and their maintenance

New Zealand white male rabbits were used to raise antisera against healthy & infected leaf antigens and spore antigens. Initially, body weights were recorded and were observed for at least one week inside the cages before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetable 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.

3.10.2. Immunization

Before immunization, normal sera were collected from each rabbit. Separate rabbits were intramascularly injected once a week at 7 days interval with 1ml antigen mixed with 1 ml of Freund's complete adjuvant (Difco, USA) for first two injections and the next with incomplete adjuvent for 8 to 16 weeks.

3.10.3. Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board fixed at an angle of 60^0. The neck of the rabbit was held tight in triangular gap at the edge of the board, and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 to 10ml of blood samples were collected in sterile graduated glass tube.

After collection, all the precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were incubated at 37^0C for 1hr for clotting. After clotting, the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000g for 10 mins at room temperature) and distributed in 1ml vials and stored at – 20^0C, as crude antisera.

3.11. Antisera Production

3.11.1. Polyspecific

Polyspecific antisera was prepared following the method of Alba, 1981 with modifications.Blister infected tea leaves were collected separately from Castleton Tea Estate, Darjeeling and Hansqua Tea Estate, Siliguri and polyspecific antisera were raised from the antigen of these two samples separately. The antigens were further purified by 100% Ammonium Sulphate precipitation, dialysed and preserved at – 20^0C till further use. Crude antisera (2ml) of different bleedings were divided into two appendroff tubes of 1ml each. To 1ml of antisera 500µl of 100% SAS healthy leaf antigen was added and mixed thoroughly. All the tubes were incubated at 4^0C for 24hrs, after which the tubes were centrifuged at 10,000 rpm for 30mins at 4^0C. The supernatant was treated as polyspecific antisera. Antisera was raised in 2 batches viz. Batch-I and Batch-II. Batch-I was raised by immunizing the rabbit with Blister infected leaf antigens collected from Castleton Tea Estate, Darjeeling Hills; Batch-II was raised from blister infected leaf antigen collected from Hansqua Tea Estate, (Plain).

For Batch-I antisera were collected four times at an interval of 6, 8, 10, 12 injection schedule and IgG was purified. These were coded as polyspecific-I, PSI (PS-I/A, PS-I/B, PS-I/C and PS-ID).

For Batch-II antisera were collected five times at an interval of 6, 8, 10, 12, 14 injection schedule and IgG was purified. These were coded as polyspecific-II, PSII (PS-II/A, PS-II/B, PS-II/C, PS-II/D, PS-II/E).

3.11.2. Polyclonal

Polyclonal antibody was raised by immunizing white male New Zealand rabbits with *E. vexans* spore antigen following the method of Chakraborty and Saha, 1994, and was coded as Batch III. Initially, 0.5ml of spore antigen was mixed with 0.5ml of complete adjuvent in a vial and was properly mixed till milky white colour appeared. This was injected intramuscularly in the rabbit. For Batch-III antisera were collected four times at an interval of 6, 8, 10, 12, injection schedule and IgG was purified. These were coded as polyclonal-I, PCI (PC-I/A, PC-I/B, PC-I/C and PC-I/D).

3.12. Purification of IgG

3.12.1. Precipitation

IgG was purified as described by Clausen (1988). The polyspecific / 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 16hrs at 22^{0}C. The precipitate thus formed was collected by centrifugation at 10,000g at 22^{0}C for 1hr. Then the precipitate was dissolved in 2ml of 0.02M Sodium Phosphate buffer, pH 8.0.

3.12.2. Column preparation

Eight gram 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. pH 8.0 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 2.6cms in diameter 30cm high and allowed to settled for 2hr. After the column material had settled, 25ml of buffer (0.02M Sodium Phosphate buffer, pH 8.0) washing was given to the gel material.

3.12.3. 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 buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was sucked into buffer (1) thereby producing a continuous rinse in molarity. Ultimately, 40x5ml 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.

3.13.Immunodiffusion tests

3.13.1. 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^{0}C; 0.9% agarose (Sigma, USA) was added into the buffer and placed on a water bath and stirred till the agarose solution became clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added.For gel preparation, 10ml of molten agarose was added per slide; after pouring, it was kept for solidification and then wells were cut (8mm diameter).

3.13.2. 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 72hrs at 25^{0}C.

3.13.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water for 2hr and then aqueous Sodium chloride solution (0.9% NaCl + 0.1% NaN_2) for 72hr with 6 hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. Then slides were stained with Coomassie blue (R 250) for 10 min at room temperature. After staining, slides were destained with 5% acetic acid solution with changes until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3hrs at 50°C.

3.14. 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 Dist water

B. Sodium bicarbonate — 4.2g in 1000ml Dist water.

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

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

Stocks

A. Sodium dihydrogen phosphate — 23.40g in 1000ml Dist water

B. Di-Sodium hydrogen phosphate — 21.2940 in 1000ml Dist water

280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2.

Then 0.8% NaCL and 0.02% KCI was added to the solution.

3. 0.15 M 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 (0.15M PBS – Tween, pH 7.2).

In 0.15M PBS – Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinylpyrrolidone, 10,000 (PVPP 10,000) and 0.03% Sodium azide (NaN_2) was added.

6. Substrate

p-Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in 100ml of di ethanolamine (1.0% w/v, 3mM NaN_2) pH9.8.

7. 3N NaOH solution was used to stop the reaction.

This ELISA was performed following the method as described by Chakraborty *et.al*, 1995 with modifications. Plant and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl / well) in 8 welled ELISA 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, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1hr. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200µl / well) to each well and incubated at 4°C overnight. After a further washing, antirabbit IgG goat antiserum labelled with Alkaline Phosphatase diluted 10,000 times in PBS, was added to each well (100µl / well) and incubated at 37°C for 2hrs. The plate was washed, dried and loaded with 100µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50µl / well of 3N NaOH solution and the absorbance was determined in an ELISA Reader (LISA - 5 Trans Asia model) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.15. Establishment of Callus

3.15.1. Culture media

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

(a) KNO_3 – 38.0g

NH_4NO_3 – 33.0g

$MgSO_4.7H_2O$ – 7.4g

KH_2PO_4 – 3.4g

Double distilled water - IL

(b) $CaCl_2, 2H_2O$ – 8.82g

Double distilled water – 500ml.

Solution (a) and (b) were mixed and the volume was adjusted 2 liter with double distilled water and sotred at 4^0C.

MS II (100x)

$MnSO_4$ – 2.23g.

$ZnSO_4, 7H_2O$ – 860 mg

H_3BO_3 – 620 mg

KI – 83 mg

$Na_2MO_4, 2H_2O$ – 25 mg

$CuSO_4\ 5H_2O$ – 2.5 mg

$CoCl_2, 6H_2O$ – 2.5 mg

Double distilled water – IL.

Stored at 4^0C

MSIII (20x)

$Na_2EDTA, 2H_2O$ – 746 mg

Boiling double distilled water – 80 ml

$FeSO_4, 7H_2O$ – 556g

Double distilled water – 80ml

$FeSO_4$, $7H_2O$ solution was added to Na_2 EDTA solution with vigorous stirring and volume was adjusted to 200ml with double distilled water. Stored at 4^0C.

MS (IV) (100x)

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

MS-I, II, III, IV were mixed together in the following ratio :

MSI	–	100 ml
MSII	–	10 ml
MS III	–	10 ml
MS IV	–	10 ml

Then the media was supplemented with 3% sucrose, 0.8% agar and 2mg/L IBA, 4 mg/IBA (Kato, 1989). Final volume was made upto 1 litre. PH was adjusted to 5.8 using 0.IN HCL or 0.IN NaOH before autoclaving and then sterilized at 121^0C ($15 lb/in^2$) for 30nins.

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

(a)	Activated charcoal	–	100 mg / L
(b)	Ascorbic acid	–	150 mg / L
(c)	Ca-pantothenate	–	200 mg / L

3.15.2. Preparation of fragile callus

Shoots of fresh young tea plants with 3-4 leaves were taken from glass house and stem segments (2-3mm long) were cut and sampled. Before sterilization, explants were washed in running tap water for 20mins to remove phenolic substances. Stem segments were surface sterilized with 2% sodium hypochlorite solution for 5mins and washed five times with sterile distilled water for the removal of hypochlorite. After final washing, explants

were transferred into semi-solid media contained in flasks and culture tubes. They were incubated under 16h photoperiod at 26°C and observed regularly. The fragile callus obtained above was shaken gently in liquid MS medium at 100 rpm and loose cells were used for fluorescence studies.

3.16. Fluorescence Antibody Staining and Microscopy

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

3.16.1 Blister Spore

Basidiospores, collected from fresh naturally blister infected leaves were taken in a small beaker. A spore solution was prepared with PBS (pH 7.2). The suspension was taken in a appendroff tube and centrifuged at 3000g for 10 mins and the PBS supernatant was discarded. The 100µl of purified spore IgG was added into the appendroff tube and incubated for 2hr. at 27°C. After incubation, the tubes were again centrifuged & washed for 10mins at 3000g. The spores were rewashed 3 times with PBS Tween, and then 100µl of antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and incubated in dark at 27°C for 1hr. After incubation, FITC was removed by repeated washing with PBS-Tween and the spores were mounted on 10% glycerol and observed under Leica microscope, equipped with UV fluorescence filter & photographs taken.

3.16.2. Cross-section of tea leaf

Initially, fresh cross-section of healthy and infected leaves were proceeded for FITC treatment.

3.16.2.1. Healthy leaf

Fresh cross-sections of Healthy leaves (AV2 and TV 18) were cut and immediately immersed in phosphate buffer saline (PH 7.2) containing 0.8% NaCl & 0.02% KCl and 0.01% PVP-10. Good sections were selected and treated with *E. vexans* antiserum (1:40). and incubated for 1hr. at 27°C. in grooved slides. After incubation, sections were washed thrice with PBS Tween (PH 7.2), and treated with 100µl of diluted (1:40) goat antiserum specific to rabbit globulins and conjugated with FITC. The sections were incubated

for 1hr at 27°C. All operations with FITC labelled antibodies were made in darkness (red light). After incubationm sections were washed thrice with PBS Tween (PH-7.2) as mentioned above and then mounted on greese free slides with 10% glycerol.A cover slip was placed on the section and sealed. Fluorescence of the leaf sections were observed using leica leitz Biomed microscope with flourescence optics equipped with U1tra violet (UV) filter set 13.

3.16.2.2. Infected leaf

Fresh, naturally blister infected leaves were collected from Tea Estates (Hills & plains) and sections were cut. The rest of the process was repeated, same as above (3.16.2.1.).

3.17. 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 membrane (Millipore, H5SMO 5255, 7cm x 10cm, Pore size-0.45µm, 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-*E. vexans* 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-Tween (pH -7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline Posphatase Conjugate (1:7500) for 2hrs at 37°C. This was followed by washing

for 25 mins. In TBS-Tween. Substrate (66µl Nitro Blue Tetrazolium Chloride + 33µl 5-Bromo-4-Chloro-3 Indolyl phosphate Di-sodium salt in 10ml of Tris buffer saline pH 7.4) was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water.

3.18. Western blotting

Blot transfer was done in three steps, following the method as described by white *et al.* (1994) with modification .

3.18.1. Extraction of Soluble Proteins :

Soluble proteins were extracted from healthy and blister infected tea leaves and basidiospores and protein content was estimated as described earlier.

3.18.2. SDS PAGE analysis of total soluble protein was performed as described previously.

3.18.3. Transfer process

Preparation of transfer buffer : (Towbin)

25mM Tris, 192mM glycine in 20% Reagent grade Methanol, pH 8.3.

(Tris-3.03g; Glycine- 14.4g; 200ml Methanol) - volume make upto 1 litre

SDS Gel electrophoresis was carried out in a mini Gel unit. Following gel run, it was transferred to Towbin buffer and equilibrated for 1hr. The transfer unit was attached to a power pack. The presoaked filter paper was placed on the platinum anode and air bubbles were rolled out with a glass rod over the pre-wetted membrane placed, followed by the gel and finally on top again another presoaked filter paper was placed. The cathode was placed on the sandwich and pressed. The unit was run for 45mins at 15 volts constant voltage. After the run the membrane was dried for 1hr and proceeded for immunological probing.

3.18.4. Immunoblotting

Blocking was done by 5% non fat dired milk and 0.02% sodium azide in 0.15M PBS pH 7.2 with 0.02% Tween-20 in a heat sealable plastic bag kept for 1hr with occasional shaking. Antibody was added (1:40) to the blocking solution and incubated in plastic bag

at 4^0C overnight. All the processes were done by occasional shaking. The nitrocellulose membrane was washed properly in 200ml of 150mM NaCl, 50mM Tris HCl, pH 7.5 to remove azide and phosphate from filter before enzyme coupled reactions. Enzyme was added (1:10,000 in alkaline phosphatase buffer) and kept for 1hr at room temperature.

The membrane was washed in 150mM NaCl, 50mM Tris HCl, pH 7.5 and substrate was added (66µl NBT + 33 µl BCIP + 10ml of Alkaline phosphatase buffer). The reaction was monitored carefully and when bands were observed of the desired intensity the filter was transferred to a try of 200µl of 0.5M EDTA, pH 8.0 in 50 ml of 0.15M PBS.