3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

3.1.1 Selection

Based on the growing suitability of tea plants (Camellia sinensis (L.) O. Kuntze) as observed under the field conditions over the years by Tocklai Experimental Station, Jorhat, Assam and recommendation made by Bezbaruah and Singh (1988) ten varieties were chosen for plantation in the Phytopathological Experimental Garden of the Botany Department, North Bengal University. A brief note on these tea varieties are given in Table 1.

3.1.2 Source

Eight clonal varieties of tea saplings (18-20 months old) released by Tocklai Experimental Station, Jorhat, Assam were collected from Mohurgong and Gulma Tea Estate, Sukna, Siliguri. These were TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27 and Teen Ali 17/1/54. Two more seed varieties (TS-449 and CP-1) were obtained from Darjeeling Tea Research Centre, Kurseong, Darjeeling.

3.1.3 Plantation

Initially for the suppression of weeds in the experimental plots Simazine @ 75g/20 litre water and Glyphosate @ 1 : 200 were used as suggested by Barbora (1988). Following his recommendation at the intervals of 2 ft between plant to plant and 3.5 ft between row to row, pits (1½' x 1½' x 1½') were dug. Planting mixture was prepared in the ratio of 4.5 kg well rotten dry cattle manure, 30g superphosphate, 30g rock phosphate and 2.5g phorate [0,0-diethyl S-(ethylthiomethyl) phosphoro dithioate]. At the bottom of each pit, rock phosphate was placed following which half portion was covered with cattle manure soil mixture. Phorate was mixed with a portion of excavated soil and was applied to the upper half of the pit in order to control eelworm. Superphosphate was placed approximately 5 cm below the ground level. Finally, 20 tea plants each of ten varieties as mentioned earlier were

Table 1 : Growth characteristics of tea clones released by Tocklai Experimental Station, Assam

Origin	Clone No.	Category	Frame	Leaf type	Shoot size	Pubescence	Yield	Quality	Draught tolerance
Cambodian	TV-18	Yield	Spreading	Medium	Medium	Low	High	CTC Orthodox	Good
	TV-22	Yield	Spreading	Large	Large	Low	High	CTC Orthodox	Good
	TV-23	Yield	Spreading	Large	Large	Low	High	CTC Orthodox	Good
	TV-25	Yield	Compact	Medium	Medium	Low	High	CTC Orthodox	Good
	TV-26	Yield	Compact	Medium	Medium	Low	High	CTC Orthodox	Good
	TV-27	Standard	Compact	Medium	Medium	Medium	Above average	Orthodox CTC	Good
Assam	TV-20	Standard	Spreading	Large, light leaf	Large	Low	High	Orthodox CTC	Good
	TS-449	Standard	Spreading	Medium	Medium	Medium	High	Orthodox CTC	Good
Assam x Chinese Hybrid	Teen Ali 17/1/54	Standard	Medium	Medium	Medium	High	High	Orthodox CTC	Good
	CP-1	Standard	Compact	Small	Small	High	Average	Orthodox CTC	Fair

planted in these pits. The plants of all these varieties were also grown in earthen pots (one plant/pot, 1 ft diameter), each containing 5 kg soil mixture (soil : planting mixture = 1:1).

3.1.4 Growth and maintenance

The young tea plants were maintained using nutrients (2% NPK - 2:1:2 and 2% urea) and Tricontanol as foliar spray at an intervals of 3 months. 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 light and temperature (20°-30°C) and watered on alternate days with ordinary tap water.

3.2 FUNGAL CULTURE

3.2.1 Source

A virulent strain of Pestalotiopsis theae (Sawada) Stey. was isolated from naturally infected (grey blight) tea leaves (TV-23) and was used after completion of Koch's postulate. The identification was also confirmed by Dr. J.E.M. Mordue, International Mycological Institute, Kew, Surrey, U.K. P. theae (IMI number 356807) has been kept in the IMI culture collection.

Besides, two more leaf pathogens of tea viz., Glomerella cingulata (Stoneman) Spauld. and Shrenk (IMI number 356805) and Bipolaris carbonum (Syn. Helminthosporium carbonum) anamorph of Cochliobolus carbonum Nelson (IMI number 298762) were obtained from the culture collection of Mycology and Plant Pathology Laboratory, Department of Botany, University of North Bengal.

3.2.2 Completion of Koch's postulate

Fresh, young tea leaves (TV-23) were collected from the Phytopathological Experimental Garden and inoculated with conidial

suspension of P. theae following detached leaf inoculation technique. After 72h of inoculation, infected leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ for 2-3 mins., washed several times with sterile distilled water and transferred aseptically into Potato-Dextrose-Agar (PDA) slants, and incubated at 28±1°C. After 15 days of incubation the isolated fungal culture was examined, compared with the stock culture and identification was confirmed as P. theae.

3.2.3 Maintenance of stock culture

The fungus thus obtained was subcultured on PDA slants, incubated at 28°C for two weeks and finally the sporulated culture was stored at 5°C and 28°C. Either detached leaf or cut shoot inoculation with P. 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 in liquid media

To assess mycelial growth of P. theae in liquid media, the fungus was first 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 advancing zone of 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 Richard's medium for a desired period at 28±1°C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60°C for 96h, cooled in a desiccator 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 placed in plastic trays (37.5 cm x 30 cm) 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 scalpel. On either side of the midrib 2 to 4 such wounds were made in each leaf which were immediately inoculated with 20 µl droplets of conidial suspension (at least $5x10^5$ conidia/ml) of P. theae (prepared from 14-day old culture). Fifty leaves were inoculated in each treatment. In control sets wounds were made on the leaves as described and 20 µl 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 3 to 4 leaves) of tea plants grown in the experimental garden were cut carefully with a sharp blade and immediately introduced into conical flasks containing sterile distilled water and transferred to the laboratory. Leaves were inoculated by making 2 to 4 light scratches (2 mm. dia.) with the point of a fine scalpel on the upper surfaces of leaves as described by Cook (1989). Plugs (2 mm. dia.) of mycelium bearing conidia of P. theae were taken from 14-day-old culture and were aseptically placed on the inoculation site and wrapped with cotton wool moistened in sterile distilled water. Sterile PDA plugs were used as control. For each treatment 50 cut shoots were inoculated. After the inoculation, the cut shoots were placed into the holes of styrofoam board which was floated on the Hoagland and Knop's solution kept in a glass chamber (70 cm x 32 cm) for one week with aeration.

3.3.3 Whole plant

Well established and branched tea plants (2 yr. old) grown in pots were inoculated with P. theae following the method of Dickens and

Cook (1989). Inoculation was done by spraying conidial suspension ($5x10^5$ conidia/ml) prepared from 14-day-old culture of P. theae grown on PDA. In control sets, the plants were sprayed with sterile distilled water. Inoculated as well as control plants were placed in polythene covered frames in order to maintain relative humidity near 80% for 2-day. Subsequently the polythene was removed. Twenty plants were inoculated in each treatment.

3.4 DISEASE ASSESSMENT

3.4.1 Detached leaf

Assessment of inoculation infectivity and symptom development were done on the basis of percent drops that resulted in lesion production after 48, 72 and 96h 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 plugs bearing conidia of P. 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 96h of inoculation.

3.4.3 Whole plant

Disease intensity was assessed following whole plant inoculation technique at 7 days of intervals after inoculation upto 28 days as

described by Chakraborty and Saha (1994b). On the basis of visual observation lesions were graded into four size groups, viz., very small, small, medium and large with respective values of 0.1, 0.25, 0.50 and 1.0 assigned to give an appropriate idea of their relative size. Number of lesions in each size group was multiplied by the values assigned. The sum total of such values for all the leaves gave the disease index for a plant. Results were always computed as the mean of observation of 20 plants (50 young leaves randomly picked up from each plant) per treatment.

3.5 COMPOSITION OF MEDIA

Carboxymethyl cellulose-Yeast extract (CMC-Yeast extract)

Carboxymethyl cellulose	..	1.5%
Yeast extract	..	0.1%
$MgSO_4,7H_2O$	..	0.05%
NH_4NO_3	..	0.1%
KH_2PO_4	..	0.1%
Agar	..	2.0%

Citrate broth

$Na(NH_4)HPO_4,4H_2O$	..	0.15%
KH_2PO_4	..	0.1%
$MgSO_4,7H_2O$	..	0.02%
$C_6H_5Na_3O_7,2H_2O$	..	0.3%
pH	..	6.7

Czapekdox-agar

$NaNO_3$	..	0.2%
K_2HPO_4	..	0.1%
$MgSO_4,7H_2O$	..	0.05%
KCl	..	0.05%
$FeSO_4,7H_2O$	..	0.001%
Sucrose	..	3.0%
Agar	..	2.0%

Dextrose broth

Beef extract	..	0.3%
Peptone	..	0.5%
Dextrose	..	0.5%
pH	..	7.0

Esculin agar

Esculin	..	0.1%
$FeCl_3$	..	0.05%
Peptone	..	0.05%
Yeast extract	..	0.1%
Agar	..	2.0%
pH	..	7.0

Glucose broth

Beef extract	..	0.3%
Peptone	..	0.5%
Glucose	..	0.5%
pH	..	7.0

Glucose-mineral salt

Glucose	..	3.0%
K_2HPO_4	..	0.1%
$MgSO_4,7H_2O$	..	0.05%
KNO_3	..	0.2%
KH_2PO_4	..	0.9%
$MnSO_4,4H_2O$	..	0.005%
$ZnSO_4,7H_2O$	..	0.005%
$CuSO_4,5H_2O$	..	0.0016%
Agar	..	2.0%

Glucose-peptone broth

Glucose	..	0.5%
Peptone	..	0.5%

K_2HPO_4	..	0.5%
pH	..	7.0

Hoagland and Knop's solution

KNO_3	..	0.061%
$Ca(NO_3)_2,4H_2O$	..	0.095%
$MgSO_4,7H_2O$	..	0.049%
$NH_4(H_2PO_4)$	..	0.012%
$MnSO_4,4H_2O$	..	0.3%
$ZnSO_4,7H_2O$	..	0.05%
$CuSO_4,5H_2O$	..	0.0025%
$Na_2MoO_4,2H_2O$	..	0.0025%
H_3PO_3	..	0.05% (v/v)
H_2SO_4(sp.gr. 1.83)	..	0.05% (v/v)
$FeC_6O_5H_7,5H_2O$	..	0.02%

Kerr's medium

$NaNO_3$	..	0.2%
KCl	..	0.05%
KH_2PO_4	..	0.1%
$MgSO_4,7H_2O$	..	0.05%
$FeSO_4,7H_2O$	..	0.001%
Yeast extract	..	0.05%
Sucrose	..	3.0%
Agar	..	2.0%

Lactose broth

Beef extract	..	0.3%
Peptone	..	0.5%
Lactose	..	0.5%
pH	..	7.0

Malt extract agar

Malt extract	..	2.0%
Dextrose	..	2.0%

Malt extract agar (Contd.)

Peptone	..	0.1%
Agar	..	2.0%

Milk agar

Powdered milk	..	0.2%
Agar	..	2.0%

Nitrate broth

Beef extract	..	0.3%
Peptone	..	0.5%
KNO_3	..	0.1%
pH	..	7.0

Nutrient agar (NA)

Beef extract	..	0.3%
Peptone	..	0.5%
NaCl	..	0.5%
Agar	..	2.0%
pH	..	7.0 to 7.2

Peptone-arginine agar

Peptone	..	0.1%
Arginine	..	1.0%
NaCl	..	0.5%
K_2HPO_4	..	0.03%
Phenol red	..	0.001%
Agar	..	2.0%
pH	..	7.2 to 7.4

Peptone-dextrose agar

KH_2PO_4	..	0.1%
$MgSO_4,7H_2O$	..	0.05%
Peptone	..	0.5%
Dextrose	..	1.0%
Agar	..	2.0%

Potato dextrose agar (PDA)

Peeled potato	..	40.0%
Dextrose	..	2.0%
Agar	..	2.0%

Protein agar or
Nutrient gelatin agar

Beef extract	..	0.3%
Peptone	..	0.5%
NaCl	..	0.5%
Gelatin	..	0.4%
Agar	..	2.0%

Richard's medium (RM)

KNO_3	..	1.0%
K_2HPO_4	..	0.5%
$MgSO_4,7H_2O$	..	0.25%
$FeCl_3$	..	0.002%
Sucrose	..	3.0%
Agar	..	2.0%

SIM agar

Peptone	..	3.0%
Beef extract	..	0.3%
Ferrous ammonium sulfate	..	0.02%
Sodium thiosulfate	..	0.0025%
Agar	..	0.3%
pH	..	7.3

Starch agar

Tryptone	..	1.0%
Yeast extract	..	1.0%
K_2HPO_4	..	0.5%
Soluble starch	..	0.3%
Agar	..	2.0%

Tryptone broth

Tryptone	..	1.0%
NaCl	..	0.5%
pH	..	7.0

Urea broth

Medium A :

K_2SO_4	..	9.5%
Na_2HPO_4	..	1.0%
Yeast extract	..	0.01%
Phenol red	..	0.001%
Agar	..	2.1%
pH	..	7.0

Medium B :

Urea	..	40.0%

950 ml of medium A and 50 ml of medium B were sterilized separately by autoclaving at 15 lbs p.s.i. (121°C) for 15 min., cooled to 55°C and mixed well.

3.6 EXTRACTION OF TOTAL SOLUBLE PROTEIN

3.6.1 Leaf protein

Soluble proteins were extracted from both healthy and P. 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 P. theae inoculated leaves (30g) were crushed separately in a mortar and pestle with 30g polyvinyl pyrrolidone in cold (4°C), kept at -20°C for 1h and homogenized with 0.05M sodium phosphate buffer, pH 7.0 containing 0.85% NaCl and 0.02M ascorbic acid. The leaf slurry was strained through cheese cloth and centrifuged at 12,100g for 60 min at 4°C. The supernatant was subdivided and stored at -20°C before use.

3.6.2 Mycelial protein

Extraction of mycelial protein of P. theae was done following the method of Chakraborty and Purkayastha (1983). P. theae was grown in sterilized Richard's medium (RM) for 14 days at 28±2°C. Mycelia were collected, washed with 0.2% NaCl solution and rewashed with sterile distilled water. Washed mycelia (50g fresh weight) were then crushed with sea sand using a mortar and pestle at 4°C and stored at -20°C for 2h and then soluble protein was extracted with 0.05M Tris-HCl buffer (pH 7.4). The slurry was strained through cheese cloth and then centrifuged at 12,100g for 20 min at 4°C, the supernatant being used as the crude extract.

3.7. ESTIMATION OF TOTAL SOLUBLE PROTEIN CONTENT

Method of Lowry et al. (1951) was considered for the estimation of soluble proteins. To 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% Potassium sodium tartarate dissolved in 50 ml of 2% Na_2CO_3 in 0.1N NaOH) 1 ml of protein sample was added, incubated for 15 min, after that 0.5 ml of Folin-Ciocalteau's reagent (dilute with distilled water in the ratio 1:1) was added and once again incubation was allowed for 15 min for colour development. Optical density was measured at 750 nm in Systronics Photoelectric Colorimeter (Model 101). Using bovine serum albumin (BSA) as standard the protein concentration was computed.

3.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF SOLUBLE PROTEIN

3.8.1 Preparation of gel solution and gel column

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°C)		

(B) Lower gel buffer (LGB)

1.5M Tris	..	18.18g
Distilled water	..	100.0 ml
pH was adjusted to 8.8		
Sodium dodecyl sulfate (SDS)		0.4g

(C) Upper gel buffer (UGB)

0.5M Tris	..	6.06g
Distilled water	..	100.0 ml
pH was adjusted to 6.8		
Sodium dodecyl sulfate (SDS)		0.4g

(D) Ammonium peroxodisulphate (APS)

Ammonium peroxo-disulphate (APS)	..	0.1g
Distilled water	..	1.0 ml
(freshly prepared in each time)		

The tubes (12 cm long and 4 mm dia.) were washed thoroughly, dried and rewashed with diethyl ether. 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 was as follows :

Solution A	..	10.0 ml
Solution B	..	7.5 ml
Distilled water	..	12.5 ml
TEMED	..	12.0 µl
Solution D	..	36.0 µl

Finally the gel was cast slowly upto a height of about 9 cm in a tube (12 cm long and 4 mm dia.), overlayered with water and left to polymerization for about 2 to 3h. 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 stacking gel solution was as follows :

Solution A	..	3.0 ml
Solution C	..	5.0 ml
Distilled water	..	12.0 ml
TEMED	..	10.0 µl
Solution D	..	500.0 µl

After the polymerization of the resolving gel, water overlay was decanted off and replaced with a layer of stacking gel solution with the help of a pasteur pipette. Finally the gel tubes were kept for 30 min for polymerization.

3.8.2. Sample preparation

500 µl sample protein was mixed in a tube with equal amount of sample buffer whose composition was as follows :

Solution B	..	12.5 ml
Sodium dodecyl sulfate (SDS)	..	2.3g
Glycerol	..	13.0g
β-mercaptoethanol	..	5.0 ml
Distilled water	..	100.0 ml

A pinch of bromophenol blue was added to the sample buffer. The tubes of different sample proteins thus prepared were then warmed in boiling water bath for 3 min and 25-30 µl of samples were applied per gel tube. Along with the samples, protein marker consisting of a mixture of six proteins (Carbonic anhydrase, Egg albumin, Bovine albumin, Phosphorylase

b, β-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.

3.8.3 Electrophoresis

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

0.025M Tris	..	18.15g
0.192M Glycine	..	72.0g
Distilled water	..	5.0 lit.
pH was adjusted to		8.3
Sodium dodecyl sulfate (SDS)		5.0g

Electrophoresis was performed at 1.5 mA per gel until the samples penetrated the resolving gel and then at 2.5 mA per gel for 5h i.e. until the dye reaches the bottom of the gel column.

3.8.4 Fixing

The gels were removed from the tubes and soaked in fixer solution (Isopropanol - 250 ml, Acetic acid - 100 ml, Distilled water - 650 ml) for 20h.

3.8.5 Staining

The gels were then stained either with Coomassie blue R250 (0.01% Coomassie blue in destaining solution) or with amido black (0.5% amido black in destaining solution) for 1 h and finally treated with destaining solution(Methanol - 300 ml, Acetic acid - 100 ml, Distilled water - 600 ml) until the background became clear.

3.9 TOTAL AND ORTHODIHYDROXY PHENOL CONTENTS OF HEALTHY AND *P. theae* INOCULATED TEA LEAVES

3.9.1 Extraction

Total phenol and orthodihydroxy phenol content of healthy and *P.*

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 30g each of healthy and inoculated leaves separately in boiling 80% ethanol (4 ml ethanol/g fresh weight leaf tissue) for 30 min, cooled, and crushed thoroughly, passed through two layers of cheese cloth and then filtered through filter paper. Final volume was adjusted with 80% ethanol (5 ml/g fresh weight of leaves).

3.92. Estimation

Total phenol

The total phenol was estimated by Folin-ciocalteau's reagent as described by Farkas and Kiraly (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 25 ml. Absorbance was measured in a Systronics Photoelectric Colorimeter Model-101 at 515 nm. Quantity of total phenol was estimated using caffeic acid as standard.

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 0.05N HCl, 1 ml of Arnow's (1933) reagent ($NaNO_3$ - 10g; Na_2MoO_4 - 10g; Distilled water - 100 ml) and 2 ml of 1N NaOH were added, following which the volume was raised to 25 ml. Absorbance was recorded by a Systronics Photoelectric Colorimeter Model-101 at 515 nm. Quantity of orthodihydroxy phenol was estimated using caffeic acid as standard.

3.10 COLLECTION OF LEAF DIFFUSATES AND FUNGITOXIC ASSAY

Leaf diffusates 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 plastic tray (37.5 cm x 30 cm). Their upper surfaces were wounded as described by Cook (1989). Twenty µl droplets (2-4 drops per leaf) of either conidial suspension ($5x10^5$ conidia/ml) prepared from 14-day-old culture or sterile distilled water were placed on the adaxial surface of each leaf. Fifty leaves were taken for each treatment. Each tray was covered with a glass lid and sealed with a smear of white petroleum jelly in order to minimize the drying of the drops during incubation. After 48h of incubation, drops of spore suspension or 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 diffusates was mixed with 0.1 ml of known concentration of spore suspension of *P. theae*. Two drops (0.02 ml/drop) of suspension were placed separately at two ends of a grease-free glass slide. The slides were incubated in moist Petri dishes for 24h at 25±1°C. Finally, germinated and ungerminated spores were stained with cotton blue in lactophenol and examined under the microscope. Percentage germination was calculated in each case.

3.11. DETECTION OF ANTIFUNGAL COMPOUND FROM HEALTHY AND *P. theae* INOCULATED TEA LEAVES

3.11.1 Extraction

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 conidial suspension of *P. theae* while the other half was

maintained as control in water. Both healthy and inoculated leaves were harvested separately 48h after inoculation, weighed, kept in Ehrlenmeyer flasks and vacuum infiltrated with 40% aqueous ethanol (15 ml/g fresh mass tissue) following the facilitated diffusion technique of Keen (1978). The flasks containing the plant tissue immersed in the ethanol solution were stoppered and placed on a rotary shaker (110 cycles/min. at 25°C). Shaking for 12h 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 times with ethyl acetate and the organic layers pooled and dehydrated with $MgSO_4$. Ethyl acetate fraction was then concentrated in vacuo to dryness, dissolved in methanol (50 µl/g) and analysed by TLC.

3.11.2. Chromatographic analysis

Ethyl acetate fractions of both healthy and P. 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 (1994a). 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 R_F values were noted.

3.11.3. Bioassay

3.11.3.1 Radial growth

Radial growth inhibition assay as described by Van Etten (1973) was followed. Ethyl-acetate fraction of healthy and P. theae inoculated extract (0.2 ml) were taken separately in each of the sterile Petri dishes (5 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 Richard's agar medium was poured in each Petri dishes, thoroughly mixed and allowed to solidify. Agar block (4 mm dia.) was cut with a sterilized cork borer from the advancing zone of 7-day-old culture of P. theae grown in Richard's medium (RM) and transferred to each Petri dishes. Radial growth of P. theae was compared.

3.11.3.2 Chromatogram inhibition

Ethyl-acetate fractions of healthy and P. theae inoculated tea leaf extracts were spotted on TLC plates (silica gel G), and the chromatogram inhibition assay as devised by Hofmans and Fuchs (1970) was performed using B. carbonum 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 72h. Fungitoxicity was ascertained by the presence of inhibition zone(s), which appeared as white spots surrounded by a blackish background of mycelia. Diameters of the inhibition zone(s) and R_F values were noted.

3.11.3.3 Spore germination

The regions of thin layer chromatograms corresponding to the inhibitory zones were scrapped and eluted in methanol. The eluants were rechromatographed on TLC plates to purify them and then eluted again. The eluants were tested for antifungal activities following spore germination test with P. theae, B. carbonum and G. cingulata as described by Werder and Kern (1985).

3.11.4 UV-spectrophotometric analysis

For spectral analysis of antifungal compound extracted from healthy and P. theae inoculated leaves, initially ethyl-acetate fraction were spotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent. Following evaporation of the 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 *Pestalotiopsis theae*

3.12.1 Isolation

Cell wall was isolated from *P. theae* following the procedure of Keen and Legrand (1980). Mycelium of 10-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 (4 ml/g). The resulting slurry was then disrupted in a homogenizer for 1 min at 5°C. The mixture was centrifuged for 1 min at 1,500g, the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10 ml/g) and pelleted by centrifugation at least six times or until the supernatant fluids were 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 *P. theae* following the method of Brown and Kimmins (1977). Isolated cell walls (2.0g) were suspended in 80 ml ice-cold 0.1N 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 15h. Following centrifugation at 8,000g for 10 min, the residue was washed with 50 ml ice-cold water and the pooled supernatants were carefully neutralized to pH 7.0 with 1N HCl at 0°C. 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 2.0g of cell walls in 80 ml of 0.02M sodium citrate, pH 7.0 in the National Super Blender mixer and autoclaving for 2h 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 wall extract was mixed with an antibiotic gentamycin sulfate (100 µg/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 0.3M $Ba(OH)_2$ and 5% $ZnSO_4$ was added to 8 ml of cell wall extract. The mixture was incubated for 5 to 10 mins and the supernatant was taken after centrifugation. This supernatant (0.2 ml) was mixed with 1.8 ml of distilled water and finally 6 ml of anthrone reagent (200 mg anthrone powder dissolved in 100 ml of conc. H_2SO_4) was added, kept for 15 min 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

Estimation of protein in the preparation of mycelial wall extract was done following the method of Lowry *et al.* (1951) as described earlier.

3.12.4 Bioassay of mycelial wall extract

Drops (20 µl, 2-4 drops/leaf), each of sterile distilled water, spore suspension of *P. theae*, mycelial wall extract (MWE) preparation

and MWE mixed with P. theae spore suspension were placed separately on the adaxial tea leaf surface kept in moist trays and incubated for 48h as described earlier. These drops of four different treatments were collected separately, centrifuged and assayed for their biological activities against spore germination of P. theae as described by Rouxel et al. (1989).

3.12.5 Characterization

3.12.5.1. SDS-PAGE Analysis

Gel preparation :

Separation gel with 10% acrylamide and stacking gel with 5% acrylamide was prepared following the protocol of Laemmli (1970) as described earlier for SDS-polyacrylamide gel electrophoresis.

Sample preparation :

Mycelial wall extract were dissolved in a solution of 1% SDS and 1% mercaptoethanol and heated at 50°C for 2h. They were then diluted by the addition of 1 vol. of 4M urea containing 5% sucrose and 25-50 µl of samples were applied per gel tube. Along with the sample, protein marker consisting of a mixture of six proteins ranging in molecular weight from 29 to 205 kD was also taken in a separate tube, boiled and loaded as above.

Electrophoresis :

Electrophoresis was performed at 1.5 mA per gel until the samples penetrated the resolving gel and then at 2.5 mA per gel for 5h i.e. until the dye front reaches the bottom of the gel column.

Fixing :

The gels were removed from the tubes and soaked either in fixer solution I (25% isopropanol in 10% acetic acid) or in fixer solution II

(40% ethanol in aqueous 5% acetic acid) for protein and carbohydrate staining respectively for 20h.

Staining :

Replicate gels from fixer solution I were stained with Coomassie blue R250 and then destained as described earlier. Gels from fixer solution II were stained with periodic acid - Schiff's reagent as devised by Segrest and Jackson (1972). The procedure is described below:

Initially five following solutions A-E were prepared.

Solution A

Anhydrous sodium acetate (0.89g) and hydroxylamine hydrochloride (10.0g) was dissolved in 90 ml distilled water. Glacial acetic acid (0.54 ml) was added to it and final volume was adjusted to 100 ml with distilled water.

Solution B

Periodic acid (1.0g) and anhydrous sodium acetate (0.82g) was dissolved in distilled water and the final volume was made 100 ml.

Solution C (Schiff's reagent)

1.5g basic fuchsin dissolved in 500 ml boiling distilled water, filter at 55°C, cooled to 40°C, 25 ml 2N HCl was added and finally 3.75g $Na_2S_2O_5$ (sodium metabisulphite) was added, agitated rapidly and allowed to stand stoppered in refrigerator for 6h. 1.2g charcoal was mixed to it vigorously for 1 min, filtered rapidly and stored with stopper in refrigerator.

Solution D (prepared before use)

10% (w/v) sodium metabisulphite (5 ml) and 2N HCl (5 ml) was dissolved in 90 ml distilled water before use.

Solution E (prepared before use)

10% (w/v) sodium metabisulphite (5 ml) and 2N HCl (5 ml) was dissolved in 90 ml distilled water and 20 ml glycerol was added to it before use.

Procedure

Gels were soaked in solution 'A' for 15 min, then washed in running tap water. Next the gels were soaked in solution 'B' for 15 min, washed in running water for 10 min and transferred to solution 'C' (diluted 1:1 with distilled water just before use) for 5 min. This step was repeated thrice before washing in solution 'D' for 2 min and finally washed three times for 1h each time in solution 'E'.

3.12.5.2. 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 mycelia as well as isolated cell wall of *P. 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 isothiocyanate (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 0.25M α-methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluoresceince 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 PHYLLOSPHERE MICROORGANISMS OF TEA

3.13.1 Isolation

Leaf washing technique as described by Dickinson (1971) was

followed for the isolation of microorganisms from phyllosphere of tea. Sixty leaves were randomly collected from the field, placed in 100 ml of sterile distilled water and shaken for 1h on a mechanical shaker (200 cycles/min). Leaves were removed and the remaining suspension was centrifuged at 15,000 r.p.m. for 10 min. The pellet was resuspended in 10 ml of sterile distilled water and dilution series (10^{-1} to 10^{-6}) were made. Aliquots of 1 ml each of these dilutions were added separately to 20 ml of either Potato-dextrose agar (PDA) supplemented with 100 µg/ml chlorotetracycline or Nutrient agar (NA) supplemented with 20 µg/ml nystatin. These were poured in sterilized Petri dishes (9 cm dia.) and incubated at 30 ± 1°C. Five replicates per dilution were taken for each of PDA and NA medium supplemented with chlorotetracycline and nystatin respectively used for isolation of fungi and bacteria. The fungal and bacterial colonies arising on the agar plates were transferred to PDA or NA slants for preservation and identification.

3.13.2. Identification

3.13.2.1 Fungi

The isolated fungi were allowed to grow in Petri dishes (9 cm dia.) containing sterile PDA medium for 6 days. Nature of mycelial growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were placed on clean grease-free glass slides, mounted with lactophenol-cotton blue, covered with coverslip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

3.13.2.2 Bacteria

Shape, motility and size

To determine the shape, motility and sporulation the test bacteria were inoculated in nutrient broth for 24h at 37°C. A loopful cell suspension of each were placed separately on a clean grease-free slide and observed under the phase contrast photomicroscope.

Endospore-staining

The smears of the test organisms were fixed by passing the slide about 20 times over the flame. The slides were placed on the edge of a Petri dish with the film side upward and covered with Malachite green (saturated aqueous solution). The slides were warmed gently over the flame till fuming and removed and allowed to react for 10 min. The slides were then rinsed with tap water for 10 sec, counter stained with safranin for 1 min, rinsed with running tap water till no colour came out and excess water blotted off. The slides were air dried and examined under the same phase-contrast microscope.

Physiological characters

Gram staining :

Smears of test organisms prepared from 24h old culture (on Nutrient Agar slant) with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, heat-fixed and flooded with crystal violet (crystal violet - 2.0g, 95% alcohol - 20 ml, ammonium oxalate 1% w/v,aqueous solution - 80 ml) stain for 1 min,washed with tap water for 5 sec, flooded with Burke's iodine solution (Iodine - 1.0g, KI - 2.0g, distilled water - 100 ml) and allowed to react for 1 min. Slides were washed for 5 sec in 95% ethanol which was poured drop by drop by holding the slides in slanting position till the smears became decolourise, rinsed with water and dried. The smears were finally counter stained with safranin (2.5% w/v safranin in 95% ethanol - 10 ml, distilled water - 100 ml) for at least thirty seconds, rinsed with water and dried. The Gram character and morphological characters were determined under oil-immmersion objective (Bartholomew, 1962).

Oxygen-requirement :

25 ml test tubes containing 20 ml yest extract tryptone agar medium were melted and held at 100°C for 10 min to expel dissolved

oxygen. These were cooled to 42°-45°C and inoculated heavily with organisms. The tubes were gently whirled to distribute the inoculum into agar, being careful not to agitate the tubes in a manner that would permit incorporation of air into agar. The agar were solidified and incubated at 37°C for 48h. The nature of bacterial growth were observed.

Utilization and Gas production from sugar :

Glucose, sucrose, dextrose and lactose broth containing Durham's tube were prepared. 0.5 ml of bacteria inoculum was inoculated into each of the sugar broth (10 ml/tube), incubated at 37°C for 24h. The formation of any gas in Durham's tube and utilization of sugar was then observed (Chambers,1950).

Catalase production :

Bacterial cultures (24h old) were flooded with 0.5 ml of 10% H_2O_2 solution and production of effervescens confirmed the presence of catalase enzyme (Norris et al., 1981).

Oxidase production :

Freshly prepared 1% aqueous dimethyl-p-phenylenediamino hydrochloride solution was poured over 24h old NA slant of bacterial cultures. The solution was poured off immediately. Reddish purple colour in 3-5 min and intense jet black colour in 30 min indicated the presence of oxidase.

Urease production :

The surface of the slants of the medium containing urea were heavily inoculated and incubated for 24h at 37°C. A positive urease reaction is indicated by a change in colour of the medium from yellow to cerise (Bornside and Kallio, 1956).

Cellulase production :

Cellulose overlay agar plates were streaked with the 24h old bacterial cultures and incubated at 37°C for several days. The plates were observed for any clearing zone around or underneath the growth.

Indole production :

Tryptone broth (5 ml/tube) were inoculated with bacteria and incubated for 48h at 37°C. The production of indole was performed employing the Kovac's reagent (n-amyl alcohol - 75 ml, conc. HCl - 25 ml, p-dimethylaminobenzaldehyde - 4g) on the culture.

2 ml of inoculum was poured into a clean test tube. 0.4 ml of Kovac's reagent was added and allowed to stand until the reagent had risen to the top. The alcohol layer separate from aqueous layer upon standing and reddening of the alcohol layer within a few minutes indicated the production of indole (Cimino and Bracci, 1960).

H_2S production :

Slants containing SIM agar inoculated with the test bacteria and incubated for 48h at 37°C. Darkening along the line of the slants indicated the production of H_2S by the organisms.

Esculin hydrolysis :

Test tubes containing esculin agar medium were inoculated with the organisms and incubated for 96h at 37°C. Hydrolysis of esculin by the organisms imparts dark brown colour to the medium (Facklam and Wilkinson, 1981).

Casein hydrolysis :

Dried milk-agar plates were streaked with 24h old bacterial

cultures and incubated for 96h. The plates were observed for any clear zone around and underneath the growth indicating hydrolysis of casein (Gordon et al., 1973).

Protein hydrolysis :

Gelatin agar plates were inoculated with organisms and incubated at 37°C for 48h. The plates were flooded with protein precipitating reagent (1N H_2SO_4 saturated with ammonium sulphate). Hydrolysis was indicated by clear zone around and underneath the growth, in contrast to the opaque precipitate of unchanged gelatin .

Starch hydrolysis :

Streaks of test organisms were made on the sterile starch agar medium of each Petri dish and were incubated for 48h at 37°C. Plates were flooded with fresh Gram's iodine solution (Iodine 0.33%, Potassium iodide 0.66%). The excess iodine was poured off after allowing to act for a few minutes. A clear and colourless zone in Petri dishes indicated an area where starch had been hydrolyzed and the blue area represented unhydrolyzed starch (Gordon et al., 1973).

Arginine hydrolysis :

The stabs with arginine (Peptone-arginine-agar) supplemented medium were inoculated with the test organisms and incubated for 2-3 days at 37°C. The change in colour from yellow to red was an indication of arginine hydrolysis (Thornley, 1960).

Nitrate reduction :

Sterile nitrate broth (5 ml in each case) were inoculated with test organisms and incubated for 24h at 37°C. Nitrites are colourless in acidic environment, but when reacts with α-naphthylamine give a pink colour. Few drops of sulphanilic acid (0.8g sulphanilic acid in 100 ml of 5N acetic acid) and α-naphthylamine (0.5g α-Naphthylamine in 100 ml

of 5N acetic acid) were added to it. A distinct red or pink colour indicate nitrate reduction (Norris et al., 1981).

Methyl red and Voges-Proskauer (M.R.V.P. reaction)

Glucose-peptone broth (10 ml/tube) were inoculated with bacteria and kept for 24h at 37°C. Keeping one tube containing the medium as control acetyl methyl carbinol (V.P.) and methyl red (M.R.) tests were performed.

Methyl red test : Few drops of alcoholic solution of methyl red was added to 2 ml of bacterial suspension. Red colour shows the production of acid.

Voges-Proskauer test : 2 ml of culture broth were taken in clean test tubes. About 0.5 ml of α-naphthol (5% α-naphthol in 95% alcohol) and then 0.5 ml of 40% potssium hydroxide containing 0.3% creatine were added, tubes were shaken thoroughly and allowed to stand for 30 min. The appearance of pink to red colour indicated the presence of acetyl methyl carbinol (Barry and Feeney, 1967).

Gelatin liquefaction :

The organisms were inoculated in sterile nutrient gelatin stabs and incubated for 24h at room temperature. Gelatin liquefaction is checked by refrigerating the stab inoculated tubes along with an uninoculated control tube for 20 min as gelatin melts at about 24°C and solidifies at refrigeration temperature (McDade and Weaver, 1959).

Citrate test :

Sterile citrate broth were inoculated with bacterial inoculum and incubated for 24h at 37°C. Growth was determined (O'Brien and Stern, 1969).

3.13.3 *In vitro* interaction

3.13.3.1 Solid medium

Antagonism of phyllosphere microorganisms isolated from tea leaves were tested against foliar pathogen (*P. theae*) as described by Skidmore and Dickinson (1976). Agar blocks (4 mm dia.) from the advancing zone of the mycelia of the fungi were placed 3.5 cm apart on the medium (PDA) in a Petri dish and incubated at 30±1°C . Isolated bacteria (24h old) were streaked on to the nutrient agar medium (supplemented with 0.5% dextrose) in Petri dish 3 cm away from the test fungus (*P. theae*). Growth of *P. theae* in NA medium was not found to be satisfactory and hence dextrose was supplemented with NA for the growth of both test fungus and isolated bacteria for such pairing experiment. Five replicates were taken for each treatment. The paired cultures were examined after regular intervals upto three weeks and the nature of reactions noted.

3.13.3.2 Liquid medium

The growth of *P. theae* in dual culture was assessed following the method as outlined by Chakraborty and Chakraborty (1989). For this experiment bacteria showing antagonistic reaction in solid medium were selected and their possible mutualistic antagonism between the test fungus (*P. theae*) was examined in mixed culture. In this case also dextrose supplemented nutrient broth medium was chosen as it allowed vigorous growth of both the fungus and bacterium as mentioned earlier. One agar block (4 mm dia.) from the advancing zone of 6-day-old mycelia of *P. theae* and 0.5 ml of bacterial suspension ($1x10^6$ bacteria/ml) were used as inocula for each flask (50 ml broth/250 ml flask) and incubated at 30±1°C (growth occurred without shaking). After 7 and 14 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and harvested by straining through muslin cloth and mycelial dry weights were determined. The suspension was centrifuged at 3,000 r.p.m., which removed mycelial fragments but

not the bacteria. By measuring optical density in a Photoelectric Colorimeter, Systronics Model-101 bacterial growth was determined using the medium as blank.

3.14 CULTURE FILTRATE OF ANTAGONISTIC MICROORGANISMS

3.14.1 Preparation

Loopful of bacteria showing antagonistic reaction were taken separately from 24h old culture grown on NA slants were inoculated separately into nutrient broth and incubated at 37°C on a Orbital shaking incubator (Model REMI) at 170 r.p.m. for 4 days. Five Ehrlenmayer flasks each containing 50 ml nutrient broth were inoculated with individual antagonistic bacteria. Cutlures were centrifuged at 15,000 r.p.m. for 20 min.

Half of the supernatant was cold-sterilized by passing through bacto-filter (G-5). Before use this was streaked on NA slants in order to confirm the presence of viable bacteria, if any, in the preparation of cold-sterilized (CS) culture filtrate. Other part of the supernatant was autoclaved at 15 lbs p.s.i. for 15 min, which served as heat-killed (HK) culture filtrate.

3.14.2 Bioassay

Both cold-sterilized and heat-killed culture filtrates were bioassayed following spore germination test against P. theae as

described by Rouxel et al. (1989). Fresh conidial suspension (500 µl) of P. theae (at least $5x10^5$ spores/ml) was suspended separately in 500 µl each of cold-sterilized and heat-killed culture filtrate and 10 µl drops were mounted on clean-grease free slides and incubated at 25°C for 24h. Sterile distilled water and uninoculated medium were used as control. Percentage germination and germ tube length were calculated in each case after 24h of incubation.

3.15 SEPARATION OF ACTIVE PRINCIPLE

3.15.1 Solvent extraction

The bacteria were grown in nutrient broth for 4 days at 37°C in shaking condition. Cell free culture filtrate was obtained after centrifugation at 15,000 r.p.m. for 20 min and the supernatant was cold-sterilized as described earlier in each case. The active principle in the cold-sterilized cell-free culture filtrate were extracted with equal volume of chloroform, diethyl ether or ethyl-acetate. The organic fractions and the corresponding aqueous fractions were evaporated at room temperature in a rotary evaporator to complete dryness and the residue in each case was redissolved in 2 ml of 80% ethanol and stored in cap vials at 4°C. For respective controls, the organic solvents were dried separately and redissolved in 80% ethanol. All the fraction pools were then bioassayed against P. theae following agar cup, radial growth and spore germination assay.

3.15.2. Thin layer chromatography

Solvents containing active principle were spotted on TLC plate and developed in benzene-ethanol (6:1 v/v). Following evaporation of the solvent, the thin layer plates were observed under UV-light and either placed separately in iodine vapour or sprayed with ninhydrin.

3.15.3 Bioassay

3.15.3.1 Agar cup

Sterilized Richard's medium (RM) was warmed to liquefy, cooled to 42°-45°C, mixed with conidial spore suspension of *P. theae*, poured on Petri dishes (5 cm dia.) and allowed to solidify. An agar cup was made on each Petri dish with a sterilized cork borer (4 mm dia.) and 50 µl of each solvent fraction was added to each plate and incubated for 7 days at 30±1°C. In control set, ethanol fractions obtained after evaporation of each solvent was added. The diameter of inhibition zone were determined.

3.15.3.2 Radial growth

Inhibition of radial growth of *P. theae* was assayed against antifungal compound obtained from cell-free cold-sterilized bacteria. Initially, 0.5 ml active fraction was mixed with 10 ml sterilized and cooled (45°C) Richard's medium and plated in Petri dishes (5 cm dia.). Mycelial block (4 mm dia.) of 7-day-old test organism was placed in the centre of each Petri dish. Control sets were prepared without the active fraction. The plates were kept for six days at 30±1°C. Inhibition in radial growth of the test organism was determined in relation to control.

3.15.3.3. Spore germination

Fresh conidial suspension (500 µl) of *P. theae* (at least $5x10^5$ spores/ml) was suspended in 500 µl of each active fraction separately and 10 µl drops were mounted on different clean grease-free slides, and incubated at 25°C for 24h following the method of Rouxel *et al.* (1989). Percentage of spore germination and germ tube length were calculated in each case after 24h of incubation.

3.15.4. UV-analysis

The UV-spectrophotometry of the active fractions were done using

UV-spectrophotometer (Shimadzu, 160).

3.16 IN VIVO ASSAY

Four-day-old culture of both Bacillus pumilus and Micrococcus sp. growing on NA slants were scrapped off and suspended in sterile distilled water, to give concentration of $1x10^6$ bcteria/ml. This was the aqueous cell suspension. The cell-free culture filtrate was prepared as described earlier. The pellets obtained after centrifuging the bacterial culture, was resuspended in sterile distilled water and served as the washed cell treatment. All treatments (aqueous cell suspension, washed cells, cell-free culture filtrate and sterile distilled water) were applied to the upper and lower leaf surface of tea plants until run-off. This treatment was repeated thrice at two days interval.

3.16.1. Detached leaf

After 72h of last spray, leaves were collected and transferred to the laboratory and inoculated with P. theae as described earlier. Disease intensity was assessed after 48, 72 and 96h of inoculation of detached leaf.

3.16.2. Cut shoot

For cut shoot inoculation the sprayed shoots (two leaf and a bud) were removed (after 72h of the last spray), brought to the laboratory and inoculated following the method as described earlier.