Materials & Methods

3.1. Plant materials

3.1.1. Host plant

3.1.1.1. Selection of suitable varieties

Several tea varieties (21 Tocklai varieties, 2 garden series clones approved by Tocklai Experimental Station, Jorhat, Assam and 1 clonal variety of UPASI) were used during the present study. All the varieties except the one of UPASI, mentioned above were selected for plantation in the experimental garden, Department of Botany, University of North Bengal, based on their growing suitability as observed under field conditions and environmental aspects, over the years at Tocklai Experimental Station, Jorhat, Assam, India and was recommended by Bezbaruah and Singh (1988). Amongst them 21 Tocklai varieties, used during the work are enlisted in the Table 1 with their general characteristics.

3.1.1.2. Procurement of selected varieties

The nursery tea plants of selected varieties were procured from tea nurseries (Plate II) of Kharibari, Siliguri, West Bengal and were planted in the experimental garden. Twigs of different tea varieties used for cut shoot experiment, were collected from the nursery tea plants of the clone house of Mohurgong and Gulma tea estate, Siliguri, West Bengal. Some twigs were also collected from the experimental garden during the work.

3.1.1.3. Plantation of selected varieties

In order to suppress the weed from the experimental plot/field, Simazine (75 g/20 lt water) as pre-emergent and Glyphosate (1:200) against thatch type of grasses, as described by Barbora (1988) were applied before plantation of selected tea varieties. Several pits of 40 cm^3 were dug at approximately 60 cm and 90 cm intervals between plant-to-plant and row-to-row respectively. Planting mixtures per pit were formulated in the following ratios: well-rotten dry cattle manure: super phosphate: rock phosphate: thimate :: 4000:25:25:2.5. Procedures for mixing are as follows. Rock phosphate was placed at the bottom of the pit following which half portion was covered with cattle manure-soil mixture. Thimate was mixed with a portion of excavated soil and was applied with upper part of the pit. Super phosphate was placed approximately 5 cm below the ground level. Finally, plantation

Table 1

Tocklai released clonal cuttings used in the study and their general characteristics*

Clone no.	Leaf style /size	Pube-scence	Rooting	Yield rating	Quality	Drought resistance
TV-1	Erect/Medium	Medium	Very good	Good	Good	Good
TV-2	Erect/Medium	High	Good	Above average	Above average	Poor
TV-3	Erect/Medium	Low	Fair	Average	Above average	Poor
TV-6	Erect/Medium	High	Fair	Above average	Above average	Poor
TV-8	Erect/Medium	Low	Good	Good	Average	Poor
TV-9	Erect/Medium	Low	Very good	Very good	Average	Good
TV-11	Flat/Medium	High	Good	Above average	Above average	Fair
TV-12	Flat/Medium	Medium	Good	Above average	Above average	Fair
TV-16	Erect/Medium	Medium	Good	Above average	Good	Good
TV-17	Erect/Medium	Medium	Good	High	Good	Good
TV-18	Erect/Medium	Low	Very good	High	Average	Good
TV-19	Flat/Large	Low	Very good	High	Above average	Good
TV-20	Flat/Large	Low	Good	High	Good	Good
TV-22	Flat/Large	Low	Good	High	Above average	Poor
TV-23	Flat/Large	Low	Good	High	Average	Poor
TV-25	Erect/Medium	Low	Very	High	Average	Fairly resistant
TV-26	Erect/Medium	Low	Very good	High	Average	Fairly resistant
TV-27	Erect/Small	Medium	Very good	Above average	Above average	Tolerant
TV-28	Erect/Small	Medium good	Very average	Above average	Above	Tolerant
TV-29	Erect/Small	Low	Excellent	Very high	Average	-
TV-30	Erect/Small	Low	Excellent	Very high	Average	-

*Characteristics noted received from Tocklai Experimental Station source and our field studies. - = Not known.

Plate II

Fig. 1: Different tea clonal varieties are being raised in the nurseries.
Fig. 2: Maintenance of nursery tea plants.
Fig. 3: Nursery tea plants ready for plantation (in plastic sleeves) and/or experimental purpose.

was done with a total of 120 young tea plants of all 24 varieties. Some of the young tea plants of selected varieties were also grown in earthen pots (1 plant per pot, 25 cm diameter) each containing 4.5 kg of soil: planting mixture (1:1).

3.1.1.4. Clonal propagation

Cuttings of tea plants approximately 10 cm long having one mature leaf and one auxillary leaf bud was introduced in soil in plastic sleeves. The cuttings were kept for few days with light watering. The buds developed to form twigs that in turn produced new leaves. The original mature leaves served as maintenance leaves. After the development of new leaves and roots the plants grew normally. The mature leaves were either shed or remain attached until nine months.

3.1.1.5. Maintenance of planted varieties

During the work, the tea plants in the experimental garden were maintained with great care. Plants were grown in the garden under the natural conditions of daylight with mean monthly temperature ranging from maximum 31.0 ^{0}C to minimum 10.4 ^{0}C (Jain, 1991). All the plants were watered as and when required using ordinary tap water. The average rainfall in the region of our study is 3956.7 mm (Jain, 1991).

3.1.2. Collection of plants for extraction of botanicals

Twenty-one plant materials (leaf, bark, stem, root, rhizome etc. as applicable) were collected from foothills of Eastern Himalayas (sub Himalayan West Bengal). The plants were selected on the basis of easy availability in the growing areas of sub Himalayan West Bengal. The plants were collected, identified and voucher specimens have been deposited in the Departmental herbarium of the Department of Botany, University of North Bengal.

3.2. Fungal culture

3.2.1. Source of fungal culture

Fungal cultures used during the work were either isolated from the field or procured were also collected from Indian Type Culture Collection, IARI, Pusa, New Delhi. The details of the source of the fungal cultures are given in the Table 2 and Table 3.

Table 2
List of fungal cultures isolated from tea nurseries of North Bengal

Fungal Culture	Source	Identified By	Identification No., if any
Botryodiplodia theobromae *Patoulliard.**	Naturally infected leaves and collar regions of nursery tea plants of Kharibari, Siliguri, West Bengal.	Dr.P.N.Chowdhury, IARI, New Delhi	4151.2K
Curvularia eragrostidis (P. Hennings) Meyer.	Naturally infected leaves of nursery tea plants of Matigara tea estate, Siliguri, West Bengal.	Dr.P.N.Chowdhury, IARI, NewDelhi	4150.2K
Pestalotiopsis theae (Saw.) Stey.	Naturally infected leaves of nursery tea plants of Mohurgong and Gulma tea estate, Siliguri, West Bengal.	Identified by Dr. A. Saha, Department of Botany, University of North Bengal.	–
Colletotrichum camelliae Mass.	Naturally infected leaves of nursery tea plants of Mohurgong and Gulma tea estate, Siliguri, West Bengal.	Identified by Dr. A. Saha, Department of Botany, University of North Bengal.	–
Corticium theae Bernard.	Naturally infected leaves of nursery tea plants of Kharibari, Siliguri, West Bengal.	Identified by Dr. A. Saha, Department of Botany, University of North Bengal.	–
Fusarium oxysporum Schlecht.	Naturally infected leaves of nursery tea plants of Mohurgong and Gulma tea estate, Siliguri, West Bengal.	Identified by Dr. A. Saha, Department of Botany, University of North Bengal.	–

**Botryodiplodia theobromae* presently known as *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.

Table 3
List of cultures of fungal antagonists used during the study

Name of the fungal antagonists	Source	Number
Trichoderma viride Persoon Ex S. F. Grey.	IARI, New Delhi	ITCC-2109
Trichoderma koningii Oudemans.	IARI, New Delhi	ITCC-2170
Trichoderma harzianum Rifai.	IARI, New Delhi	ITCC-4572
*Gliocladium virens** (Isolate I)	*IARI, New Delhi*	*ITCC-4177*
Gliocladium virens (Isolate II)	*Dr.Apurba Chowdhury,* UBKV, West Bengal	_

* *Gliocladium virens* presently known as *Trichoderma virens*

3.2.2. Verification of Koch's Postulates

In order to fulfill the Koch's postulations fresh young tea leaves of nursery tea plants collected from Kharibari, Siliguri, West Bengal and Mohurgong and Gulma tea estate, Siliguri, West Bengal were used. At first, the leaves were inoculated with conidial suspensions of *B. theobromae*, *C. eragrostidis*, *P. theae*, *C. camelliae*, *C. theae* and *F. oxysporum* separately following detached leaf inoculation technique (Dickens and Cook, 1989) and after 72 hours of inoculation, the pathogen in each case was reisolated from infected tea leaves. Before isolation, the infected portions were cut into small pieces, washed thoroughly with sterile distilled water, surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 1-3 minutes, washed several times again with sterile distilled water and finally transferred aseptically into sterile PDA slants. These isolates were examined after two weeks of inoculation and the identity of the organisms were confirmed after comparing them with the respective stock cultures.

3.2.3. Maintenance of stock cultures

Freshly prepared sterile slants of PDA medium were used for maintenance and preservation of fungal cultures. After two weeks of subculturing, the fungal pathogens grown on sterile PDA slants were stored in two different conditions, viz. at low temperature in refrigerator (5 ^{0}C) except the culture of *C. theae* and at room temperature. Apart from weekly transfer for experimental purpose, the cultures were also examined at regular intervals to test their pathogenicity. The cultures of three different species of *Trichoderma* (*T. viride*, *T. koningii* and *T. harzianum*) and two isolates (isolate I and isolate II) of *Gliocladium virens* were also maintained at 5 ^{0}C and at room temperature.

3.3. Major Chemicals used

In addition to the common laboratory reagents, following chemicals were used during the work :

Chemicals	Company
Acetic acid glacial	E. Merck (India) Ltd., Mumbai, India
Adjuvant complete Freund	Difco Laboratories, Detroit, Michigan, USA
Adjuvant incomplete Freund	Difco Laboratories, Detroit, Michigan, USA
Agarose	SRL Pvt. Ltd., Mumbai, India
Asparagine	SRL Pvt. Ltd., Mumbai, India
Barbituric acid	HiMedia Laboratories Ltd., Mumbai, India
Bovine serum albumin	Sigma Chemicals Co., USA
Bromophenol blue	HiMedia Laboratories Ltd., Mumbai,
Cadmium chloride	SRL Pvt. Ltd., Mumbai, India
Chloroform	E. Merck (India) Ltd., Mumbai, India
Coomassie brilliant blue	SRL Pvt. Ltd., Mumbai, Indi
Di ethyl ether	SRL Pvt. Ltd., Mumbai, India
Folin ciocalteau reagent	Ranbaxy Laboratories Ltd., India
Goat anti-rabbit IgG FITC conjugate	Bangalore Genei Pvt. Ltd., Bangalore, India
Goat anti-rabbit IgG horseradish peroxidase conjugate	Bangalore Genei Pvt. Ltd., Bangalore, India
Indole-3- acetic acid	E. Merck (India) Ltd., Mumbai, India

Chemicals	Company
Indole-3-butyric acid	E. Merck (India) Ltd., Mumbai, India
Jasmonic acid	Sigma Chemicals Co., USA
Mannitol	Qualigens, Glaxo India Ltd., Mumbai, Indi
Mercury (II) chloride	E. Merck (India) Ltd., Mumbai, India
Methanol	SRL Pvt. Ltd., Mumbai, India
Nickel chloride	Sigma Chemicals Co., USA
Polyethylene glycol (PEG)	SRL Pvt. Ltd., Mumbai, India
Polyvinyl pyrrollidone	SRL Pvt. Ltd., Mumbai, India
Salicylic acid	SRL Pvt. Ltd., Mumbai, India
Sea sand	Hi Media Laboratories, Mumbai, India
Silica Gel G	E. Merck (India) Ltd., Mumbai, India
Sodium azide	Loba Chemie Pvt. Ltd., Mumbai, India
Sodium molybdate	E. Merck (India) Ltd., Mumbai, India
Sodium selenite	SRL Pvt. Ltd., Mumbai, India
Tetramethyl benzidine/hydrogen peroxide	Bangalore Genei Pvt. Ltd., Bangalore, India
Tris (Tri hydroxy methyl methylamine)	Qualigens, Glaxo India Ltd., Mumbai, India
Tween 20	Hi Media Laboratories, Mumbai, India
Zinc sulphate	E. Merck (India) Ltd., Mumbai, India

3.4. Chemical fungicides used

Trade name	Chemical name
Bavistin	Carbendazim [2-(methoxycarbamoyl)-benzimidazole]
Baynate	Thiophanate methyl
Calixin	Tridemorph-tridecyl-2, 6-dimethyl morphine]
Captan	Cis N-trichloromethylthio-4-cyclohexen-1, 2-dicarboximide
Indofil	Mancozeb
Roko	Thiophanate methyl (70% WP)

3.5. Composition of media and solutions used

Some of the media/solutions used during the work are listed below along with their standard compositions and/or modifications, if any.

POTATO DEXTROSE BROTH (PDB)

Peeled potato	:	40 g
Dextrose	:	2 g
Distilled water	:	100 ml

(Required amount of peeled potato was boiled in distilled water. The potato broth was taken by straining through cheesecloth and required amount of dextrose was added. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

POTATO DEXTROSE AGAR (PDA)

Potato dextrose agar was prepared by adding 2% agar powder to the final potato dextrose broth solution. The agar was melted by heating the media before sterilization.

MALT EXTRACT AGAR (MEA)

Malt extract	:	20 g
Agar agar	:	20 g
Distilled water	:	1000 ml

(Required amount of malt extract was dissolved by boiling in distilled water. Then, required amount of agar powder was added and boiled with constant shaking till the agar was dissolved. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

OAT MEAL AGAR (OMA)

Oat meal	:	40 g
Agar agar	:	15 g
Distilled water	:	1000 ml

(Powdered oat was boiled in distilled water in a water bath, occasionally stirred and strained through cheese cloth. Then required amount of agar powder was added to it and boiled with constant shaking till the agar was dissolved. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

POTATO CARROT AGAR (PCA)

Grated Potato	:	20 g
Grated Carrot	:	20 g
Agar agar	:	20 g
Distilled water	:	1000 ml

(Grated potato and grated carrot were mixed and boiled with distilled water. After cooling, the broth was strained through cheese cloth and required amount of agar powder was added and boiled to dissolve the agar. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

CZAPEK DOX AGAR (CDA)

$NaNO_3$	:	3 g
K_2HPO_4	:	1 g
KCl	:	0.5 g
$MgSO_4$, $7H_2O$	:	0.5 g
$FeSO_4$	:	0.01 g
Sucrose	:	30 g
Agar agar	:	15 g
Distilled water	:	1000 ml.

(Initially, all the ingredients except agar and K_2HPO_4 were dissolved. Next, agar was added and dissolved by steaming. Finally K_2HPO_4 was added to the molten solution, mixed thoroughly and sterilized at 15 lb p.s.i. for 15 minutes).

RICHARD'S SOLUTION / MEDIUM (RM)

KNO_3	:	10 g
KH_2PO_4	:	5 g
$MgSO_4$, $7H_2O$	:	2.5 g
$FeCl_3$	:	0.02 g
Sucrose	:	50 g
Distilled water	:	1000 ml

(Required amount of all the constituents were taken and mixed with required distilled water. All the constituents were dissolved by stirring and sterilized at 15 lb p.s.i. for 15 minutes).

HOAGLAND AND KNOP'S SOLUTION

KNO_3	:	0.61 g
$Ca(NO_3)_2, 4H_2O$	:	0.95 g
$MgSO_4, 7H_2O$	:	0.49 g
$NH_4(H_2PO_4)$	:	0.12 g
$MnSO_4, 4H_2O$	:	3.0 g
$ZnSO_4, 7H_2O$	:	0.5 mg
H_3PO_3	:	0.5 ml
$CuSO_4, 5H_2O$	:	0.025 mg
$Na_2MoO_4, 2H_2O$	:	0.025 mg
H_2SO_4	:	0.5 µl
$FeC_6O_5H_7, 5H_2O$	:	0.2 g
Distilled water	:	1000 ml

(Required amount of all the constituents were taken and they were mixed thoroughly in distilled water)

3.6. Inoculation technique

3.6.1. Detached leaf inoculation

Pathogenicity test was done by artificial inoculation of detached leaf with test pathogens following the detached leaf inoculation technique proposed by Dickens and Cook (1989). To perform the experiment fresh young fully expanded and detached nursery tea leaves were placed on plastic trays lined with moist blotting papers. The leaves in the trays were inoculated with mycelial plugs as well as spore suspensions of the two pathogens separately. Initially two wounds (light scratch of 2 mm length) were made on the adaxial surface of each leaf with the help of a sterile, sharp needle. Mycelial plugs (2 mm in diameter) bearing the conidia of the test pathogens of 15 d old cultures were then aseptically placed on the wounds. Sterile PDA plugs were used to inoculate control sets. Similarly, 20 µl (24 drops/leaf) of conidia or spore suspensions bearing approximately $1X10^6$ conidia/ml of test pathogens (prepared from 15 d old cultures) were placed on the wounds of each leaf with a hypodermic syringe in a separate set of experiment. In control sets, drops of sterile distilled water were placed on the leaves. Each tray was covered with a glass lid and sealed with petroleum gelly in order to maintain

the required moistures inside the trays during incubation. Fifty leaves of each variety were inoculated in each treatment.

3.6.2. Cut shoot inoculation

The inoculation technique described by Yanase and Takeda (1987) was followed for cut shoot inoculation. Twigs with 3-4 leaves of nursery tea plants grown in the clone house of Mohurgong and Gulma Tea Estate and of Taipoo Tea Estate were cut carefully with a sharp blade and immediately introduced into glass vials containing sterile tap water and transferred to the laboratory. Some of the twigs were also collected from the Experimental garden, Department of Botany, University of North Bengal. Leaves were inoculated with the mycelial plugs (2 mm diameter) bearing conidia of test pathogens as described by Dickens and Cook (1989). The mycelial plugs were wrapped with absorbent cotton wool moistened with sterile distilled water and sterile PDA plugs were used as controls. For each treatment sixteen cut shoots of a variety were placed into the holes of Styrofoam board, and the board was floated on the modified Hoagland and Knop's solution in a glass chamber. The glass chambers were incubated for a week. The chambers were covered with transparent cellophane papers. The cellophane papers were perforated with a needle for aeration.

3.6.3. Nursery tea plant inoculation

Following the whole plant inoculation method of Dickens and Cook (1989), nursery tea plants (raised through clonal cuttings) were treated with the test pathogens. Inoculation was done by spraying conidial suspensions ($1X10^6$ conidia/ ml) prepared from 15 d old cultures of test pathogens grown on sterile PDA slants. In control sets, plants were sprayed with sterile distilled water. The plants were kept for 48 hours in transparent polythene chamber to maintain high humidity. Twenty plants of each variety were inoculated in each treatment.

3.7. Disease assessment

3.7.1. Detached leaf

Symptoms (brown necrotic lesions) produced at the inoculation sites were termed 'lesions' and the number of lesions produced out of the total number of spore suspension drops/mycelial plugs multiplied by 100 gave the percentage of

lesions produced. Percentage of lesion production and diameter of each lesion were calculated after 24, 48 and 72 hours of inoculation. All calculations were based on the average of three separate treatments, each treatment comprising of 50 inoculated leaves.

3.7.2. Cut shoot

The number of lesions developed on the leaves after 24, 48 and 72 hours of inoculation were counted and diameters of each lesion were measured. The results were computed following the method of Sinha and Das (1972). The diameters were categorized into four groups and a value was assigned to each group as follows:

- Very small-restricted lesions of 1-2 mm diameter : 0.1
- Lesions with sharply defined margins of 2-4 mm diameter : 0.25
- Slow spreading lesions of 4-6 mm diameter : 0.5
- Spreading lesions of variable size (beyond 6 mm in diameter) with diffused margin : 1.0

The 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 sixteen cut shoots per treatment.

3.7.3. Nursery tea plant

Both number and sizes of lesions produced on the leaves were assessed after 4, 8, 12 and 16 days of inoculation as described by Sinha and Das (1972). On the basis of visual observations, lesions were graded into four size groups viz. very small, small, medium and large with respective values of 0.1, 0.25, 0.5 and 1.0 assigned to them to give an approximate idea of their relative size. Number of lesions in each group was multiplied by the value assigned to it and 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 observations on twenty plants per treatment.

3.8. Growth and physiology of the pathogens

3.8.1. Assessment of mycelial growth

A number of different solid and liquid media were used to assess the mycelial growth of isolated fungal pathogens. Initially, each fungus was grown in sterile petriplates containing 20 ml of different sterile test medium and was incubated for a maximum of 8 days at 28±2 °C. Radial growth of mycelia were measured after regular intervals to assess the mycelial growth in solid medium.

To assess the mycelial growth in different liquid media, similar petriplates with sterile PDA medium were inoculated with the test fungi and incubated for only 4 days at 28±2 °C. From the advancing zone of the cultures, agar blocks of 4 mm diameter containing the mycelia were cut aseptically by a cork borer and transferred to Ehrlenmayer flasks of 250 ml, each containing 50 ml of sterilized different liquid medium and incubated at 28±2 °C. After a minimum of 25 days of incubation, the growing fungal mycelia were strained through double-layered cheese cloth, blotted and dried at 60 °C. Finally, they were cooled and weighed.

3.8.2. Assessment of germination of spores

3.8.2.1. Preparation of spore suspension

Fifteen days old sporulated fungal culture was taken and approximately 3-5 ml sterile distilled water was poured in the culture tube aseptically, Gentle scrapping was done by an inoculating needle on the agar surface. After the scrapping, the tube was shaken and the resultant mixture was strained through cheese cloth. The filtrate was used as spore suspension. The concentration of the spores in the suspension was adjusted by adding sterile distilled water following hemocytometer count.

3.8.2.2. Spore germination bioassay

Assessment of spore germination of the test fungus was done under various conditions as required. Spore suspensions of test pathogens were prepared and placed (30 µl) on clean, grease free glass slides with sterile distilled water/test solution (30 µl). The slides were incubated at 28±2 °C in a humid chamber. After the desired incubation period, the slides were stained with cotton blue-lactophenol and observed under microscope. Finally, the percent spore germination and average germ tube length in each case were calculated.

3.9. Collection and maintenance of Rabbits

Six New Zealand male white rabbits were used in the study for raising polyclonal antibody (Plate III : 1). The rabbits were bought from the local rabbit seller and brought to the laboratory at least a month before starting any immunization programme for proper acclimatization. The body weight of the rabbits was approximately 1.5 kg and their age was around 10 months. The rabbits were kept in separate cages (60 cm X 60 cm X 70 cm) attached with metal trays at the bottom and placed in a well-ventilated room. The room was cleaned regularly with permitted room freshening solutions. Each rabbit was supplied with sufficient quantities of vegetables like carrot (*Daucus carrota* L.), soaked gram (*Cicer arietinum* L.), common grass [*Cynodon dactylon* (L.) Pers.], lettuce leaves (*Lactuca sativa* L.), Cabbage leaves (*Brassica oleraceae* L. var. *capitata*), Cauliflower leaves (*B. oleraceae* L. var. *botrytis*) etc. as food along with clean water. The quantity of food was adjusted only after a thorough consultation with local veterinary doctor. Routine health checking of the rabbits was also done. After one month, the immunization program was started after ascertaining their complete body fitness.

3.10. Preparation of antigens

3.10.1. Antigen from tea leaf

The protein extraction procedure of Alba and DeVay (1985) and Chakraborty and Saha (1994) was followed in order to extract leaf antigens from young tea leaves. Fresh, young leaves of the required varieties of tea were collected from the clone house of Mohurgong and Gulma Tea Estate, Siliguri, West Bengal; washed thoroughly with cold water and kept for 2 hours at -20 ^{0}C. The cold leaves (20 g fresh weight in each case) were grounded in prechilled mortar at 4 ^{0}C with 20 g of insoluble polyvinyl pyrrollidone (PVP). The leaf paste was suspended in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride and 0.02 M ascorbic acid. The leaf slurry was strained through muslin cloth and centrifuged at 4 ^{0}C for 30 minutes at 12,100 g. The supernatants were collected and ammonium sulphate was added at 4 ^{0}C to 100% saturation under constant stirring, kept overnight 4 ^{0}C and finally centrifuged at 4 ^{0}C for 15 minutes at 12,100 g. The precipitate obtained was dissolved in cold 0.05 M sodium phosphate buffer (pH

7.0) and was dialysed against 0.005 M sodium phosphate buffer (pH 7.0) for 24 hours at 4 °C with 10 changes. After dialysis, the preparation was centrifuged at 4 °C for 15 minutes at 12,100 g and supernatant was stored at -20 °C until required.

3.10.2. Antigen from fungal mycelia

The mycelial antigens were prepared following the method as described by Chakraborty and Saha (1994) with some modifications. Mycelial discs (4 mm diameter) were transferred to 10 Ehrlenmayer flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28±2 °C. After 15 days, the fungal mycelia were harvested, washed with 0.2% sodium chloride and rewashed with sterile distilled water. Washed mycelia (25 g fresh weight) were homogenized with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% sodium chloride in a mortar and pestle with sea sand and kept overnight at 4 °C. The homogenates were then centrifuged at 4 °C for 30 minutes at 12,100 g. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and again kept overnight at 4 °C. Next, the mixtures were centrifuged at 4 °C for 30 minutes at 12,100 g. The supernatants were discarded and precipitates were dissolved in cold 5ml 0.05 M sodium phosphate buffer (pH 7.4). The preparations were dialyzed for 24 hours at 4 °C against 0.005 M sodium phosphate buffer (pH 7.4) with 10 changes. After dialysis, the preparations were centrifuged at 4 °C for 15 minutes at 12,100 g and supernatant was stored at -20 °C until further use.

Protein content of both the plant and fungal antigens were determined following the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

3.11. Raising and collection of blood sera through immunization

3.11.1. Raising of antisera

Before immunization, normal sera were collected from each rabbit (Plate III : 3). Antisera against antigens of host (*C. sinensis*) and pathogens (*B. theobromae and C. eragrostidis*) were raised in separate rabbits by giving intrarmuscular injections (1 ml) of antigens emulsified with equal volume of Freund's complete adjuvant (Plate III : 2). The doses were repeated at 7 days intervals with Freund's

Plate III

Fig. 1 : New Zealand white male rabbit.(Approximately 10 month old &1.5 kg.body weight)
Fig. 2 : Administration of intramuscular injection in rabbit.
Fig. 3 : Collection of blood from rabbit following marginal ear vein puncture.

incomplete adjuvant for 6 consecutive weeks. Final protein concentration was 2 mg/ml in the emulsion. On the 4th day after the last injection, the blood samples were collected and antisera were prepared.

3.11.2. Collection of sera

Blood from rabbits was taken by puncturing the marginal ear vein (Plate III: 3). At first the rabbits was taken out from the cage, placed on a table and the hairs were removed from the vein of the ear with a sterilized blade and was disinfected with rectified spirit. After irritation of the ear with xylene, an incision was made with a sharp sterilized blade on the border vein of the ear and about 10 ml blood was collected in a sterile glass graduated tube. After taking the desired quantity of blood precautions (adhesive surgical tapes etc.) were taken to stop the flow of the blood from the punctured area of the ear. The blood samples were kept undisturbed for an hour at 35-37 ^{0}C for clotting. In order to avoid the loss of serum included within the clot, it was loosened from the glass surface by turning a sterile wooden stick around the glass near the glass wall. Finally normal sera (pre-bleed sera) as well as antisera were clarified by centrifugation at 4 ^{0}C for 10 minutes at 3000 g and were distributed in sterile cryo vials and stored at -20 ^{0}C until required.

3.12. Determination of titre value

Titres of antisera against the homologous antigens and titres of antigens against homologous antisera were determined following immunodiffusion technique as described by Ouchterlony (1967) and Clausen (1969). A constant amount (5 µl) of undiluted antiserum or antigen was placed in the central well, while diluted antigens or antisera (diluted with normal saline with ratios 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128, respectively) were pippeted into the outer well. Diffusion was allowed for 48-72 hours at 25 ^{0}C in a humid chamber. Titre was expressed as the reciprocal of the highest dilution of antiserum or antigen that reacted with antigen or antiserum giving precipitin lines.

3.13. Immunological experiments

3.13.1. Immunodiffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1958). Barbital buffer (50 ml, 0.05 M, pH 8.6) was taken in a

100 ml Ehrlenmayer flask and was placed in a boiling water bath. Agarose (0.4g) was mixed with hot barbital buffer. The buffer-agarose mixture was carefully placed on water bath. Finally a clear molten agarose solution was prepared. The solution was then mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The agarose solution was dispensed carefully in clean and dry square and rectangular glass plates of 6 cm X 6 cm and 6 cm X 3 cm respectively so that no air bubble was trapped in the agarose medium to avoid asymmetrical diffusion. Before dispensing, the glass plates were washed with extran solution and water and then the glass plates were serially dipped in 90% ethanol, ethanol:ethyl ether (1:1, v/v) and ether for removal of grease, if any. The glass plates were placed inside sterile petridishes. When the agarose solution was solidified, 3 to 6 wells of 5 mm diameter were cut by a sterile cork borer. The distances of the peripheral wells from the central wells were 5-7 mm. The antigens, normal sera and undiluted antisera were placed with a micropippette directly into the appropriate wells and diffusion was allowed to continue in a humid chamber at 25 ^{0}C for 48-72 hours. The precipitation reaction was observed after proper staining of the slides only in cases where common antigens were present.

3.13.2. Immunoelectrophoresis

3.13.2.1. Preparation of agarose coated glass slides

For immunoelectrophoresis rectangular glass pieces (8 cm X 3.5 cm) were made grease free as mentioned earlier in section 3.13.1. The slides were dried and placed on a clean surface. Thin and uniform layers (2 mm thick) of fluid agarose medium (0.8%), containing 0.1% sodium azide in 0.05 M barbital buffer (pH 8.6) were dispensed on each slide, taking care so that no air bubbles were trapped in the agarose medium. This was necessary in order to avoid irregularity that may cause asymmetrical migration and diffusion during later stages. Finally, the slides were stored at 4 ^{0}C in petridishes until use.

3.13.2.2. Electrophoresis

The agarose coated slides were placed in the middle compartment of the electrophoretic platform and two central wells of 4 mm diameter were dug out from each slide. The anode and cathode chambers were filled with 0.05 M barbital

buffer (pH 8.6). Different antigens were placed into separate wells. Bromophenol blue was used as marker for tracing the electrophoretic movement of the antigens. Filter paper (Whatman-1) strips were soaked in buffer and placed on both ends of the slides, which connected the buffer solution of anode and cathode compartments with the agarose surface of the slides. An electric current of 2.5 mAmp/slide; 10 V/ cm was passed through the slides for two hours in cold (4 ^{0}C) conditions. When the bromophenol blue marker reached near the short edge of the glass slides the current was discontinued and the glass slides were taken out.

3.13.2.3. Diffusion

A longitudinal trough parallel to the long edge of the slides was cut between the two central wells of the agarose surface and undiluted antiserum was placed into the trough. Diffusion was allowed to continue for 24-48 hours in a moist chamber at 25 ^{0}C.Precipitation arcs if formed were recorded.

3.13.3. Washing, Drying and Staining of slides

After immunodiffusion and immunoelectrophoresis the glass slides were washed with 0.9% aqueous sodium chloride carefully for 48 hours to remove all the unreacted antigens and antisera widely dispersed in the agarose surface. Following that the slides were washed with distilled water for three hours to remove the sodium chloride and dried at 40 ^{0}C for 30 minutes. Next, the slides were stained either with 0.5% coomassie blue or 0.5% amido black (0.5g coomassie blue/amido black, 5 g $HgCl_2$ and 5 ml glacial acetic acid dissolved in 95 ml distilled water) for 30 minutes at room temperature, washed thrice with 2% v/v acetic acid for three hours (one hour each time) to remove the excess stain. Finally the slides were washed with distilled water and dried at 40 ^{0}C for 30 minutes.

3.13.4. Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Combining the methods of Koeing and Paul (1982) and Talbot (2001), indirect ELISA was performed. At first antigens were diluted with coating buffer [carbonate buffer (0.1 M), pH 9.6] and 100 µl of each diluted antigens were placed on the wells of a flat bottomed micro titre ELISA plate (Tarsons) except one well which was considered as blank. The plate was incubated overnight at 4 ^{0}C in

refrigerator. After incubation, the plate was taken out and each well of the plate was flooded with phosphate buffer saline (PBS)-Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20) and washed thoroughly for four to five times. After washing the plate was dried in air. Following this, 100 µl of PBS-BSA (0.15 M PBS containing 1% BSA) was added to each well to coat all the unbound sites. The plate was incubated for 2 hours at room temperature. After incubation, the plate was again washed with PBS-Tween and air-dried. After this, 100 µl of diluted antisera (diluted with PBS-Tween) were added to each well except the blank and the control wells where normal sera was added (serially diluted with PBS-Tween containing 0.5% BSA). The plate was incubated overnight at 4 °C. In the next day, thorough washing of the plate was done with PBS-Tween. After washing and drying, 100 µl (1:10000) goat-anti rabbit IgG-Horse radish peroxidase conjugate was added to each well except the blank and the plate was incubated for 2 hours at 30±2 °C. After incubation, the plate was again washed with PBS-Tween and shaken dry. Then 100 µl (1:20) of tetramethyl benzidine/ hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. After addition of substrate, a blue colour was produced due to the reaction between the enzyme and the substrate. Finally, the reaction was terminated after 30 min by adding 100 µl of 1(N) H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm.

3.13.5. Immunofluorescence

3.13.5.1. Fluorescence staining of mycelia of pathogens

The technique of Merz *et al.* (1969) was followed with some modifications. The mycelia of the test pathogens (used as antigens) were placed on sterile microscopic slides containing albumin. After adhering, the mycelia were treated with 95% ethanol-ethyl ether (1:1,v/v) for 10 minutes at room temperature for fixing. Next, the slides were treated with 95% ethanol for 20 minutes at 37 °C. The fixed mycelia were then flooded with normal sera or with ten-fold dilution (diluted with 0.01M potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride) of appropriate antisera. The slides were then incubated for 30 minutes within a moist chamber. After incubation, the slides were washed with PBS (pH 7.2) for two to

three times and air-dried. Following that, the diluted (diluted with carbonate-bicarbonate buffer, pH 9.6 in 1:40 dilution) goat-anti rabbit IgG conjugated with fluorescein isothiocyanate (FITC) was added and the slides were kept in the dark. After this all processings were done in a dark room. After 30 minutes, the slides were again washed twice with PBS and once with distilled water and air-dried. Finally, the slides were mounted in a glycerol based mounted medium (Hardham *et al.*, 1986) and a cover glass was placed on the mycelia and sealed. The slides were observed in a fluorescence microscope (Leitz, German).

3.13.5.2. Fluorescence staining of leaf sections of the host

The technique as described by DeVay *et al.* (1981) was followed. The cross sections through the midrib portions of the leaves were cut, placed into the slides and flooded with 100 µl normal serum and/or with ten-fold dilution (diluted with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride) of appropriate antisera. The slides were then incubated for 30 minutes at 27 °C. After proper incubation, the antisera treated leaf sections were washed by shaking the sections with 4 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride for 15 minutes. Following that, 5 µl of diluted (diluted with carbonate-bicarbonate buffer, pH 9.6 in 1:40 dilution) goat-anti rabbit IgG conjugated with FITC was added and the slides were kept for incubation in a dark moist chamber at 27 °C. Henceforth, all processings were done in a dark room. After 30 min the leaf sections were again washed by shaking the sections with 4 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride for 15 minutes at 27 °C. Finally, the slides were mounted in a glycerol based mounted medium (Hardham *et al.*, 1986), and observed in a fluorescence microscope as mentioned earlier (section 3.13.5.1). Photographs were taken with a Leica Wild MPS 48 camera on Fuji 800 ASA photographic film.

3.14. Application of various chemicals in selected tea varieties

Nine different chemicals (nickel chloride, salicylic acid, jasmonic acid, indole acetic acid, cadmium chloride, sodium selenite, sodium molybdate, sodium azide and indole butyric acid) were sprayed on the cut shoots of selected tea varieties at different concentrations. Fresh aqueous leaf extracts from jasmine plants at desired

concentrations were also sprayed on the cut shoots in a closed chamber. The control set was sprayed with sterile distilled water. Inoculation technique as described by Dickens and Cook (1989) was followed for inoculation of the sprayed twigs with the test pathogens. Results were taken and expressed as mean disease index/shoot following the procedure of Sinha and Das (1972).

3.15. Extraction and estimation of ortho-dihydroxy phenol and total phenol from the leaves of the twigs ofselected tea varieties after chemical treatment

3.15.1. Extraction

Tea leaves (1g) were immersed into absolute alcohol (10 ml) and boiled for 5 minutes. After boiling, the leaves were cooled and crushed with 80% alcohol, strained through cheesecloth and the final volume was made up to 5 ml.

3.15.2. Estimation of ortho-dihydroxy phenol

Sample (0.1ml) was mixed with distilled water (0.9 ml). Two milliliters of 0.5 (N) HCl, 1 ml of Arnow's reagent and 2ml of 1(N) NaOH were added sequentially in the sample. A blank containing all the reagents minus Arnow's was used to adjust the absorbance to zero. Quantity of phenol was estimated following the standard curve made from caffeic acid in a digital photoelectric colorimeter (Electronics India, Model No. 312) at 520 nm.

3.15.3. Estimation of total phenol

One-milliliter sample (0.1ml of leaf extract + 0.9 ml distilled water) was added to 1ml half-diluted folin-ciocalteau reagent and 2 ml Na_2CO_3 solution (20%). The mixture was boiled in water bath for 1 minute and cooled under running tap water. The mixture was then diluted up to 25 ml by adding distilled water. A blank was prepared with all reagents except Folin Ciocalteau's to adjust the zero reading. Quantity of total phenol was estimated with caffeic acid standard curve in a digital photoelectric colorimeter (Electronics India, Model No. 312) at 520 nm.

3.16. Bioassay of common chemical fungicides by poisoned food technique

Bioassay of fungicides was done by taking five different concentrations of six different fungicides following poisoned food technique as described by Suleman *et al.* (2002). Nine ml of sterilized PDA was mixed with 1 ml of prepared fungicide solution (made in sterile distilled water), and poured into sterilized petriplates of 70 mm diameter under aseptic condition. After solidification of media, the plates were inoculated at the center with 4 mm discs from 15 days old cultures of test pathogens. Control plates (without any fungicides) were also inoculated. The plates were incubated at 28±2 °C. After the required incubation period, the radial growth of the mycelia was measured and percent inhibition over control was calculated. Minimum inhibitory concentration (MIC) values of the test fungicides were also determined.

3.17. Screening, extraction and bioassay of botanicals

3.17.1. Preparation of plant extracts

Following the method of Mahadevan and Sridhar (1982) with some modifications, the extracts of the plant parts were made. Fresh plant materials were collected and washed thoroughly with sterile distilled water and allowed to dry at room temperature. After drying the materials were weighed, ground and extracted separately with sterile distilled water and ethanol (0.5 g/ml). The extracts were filtered through double-layered cheese cloth and centrifuged at 10,000 rpm for 15 minutes. The supernatants of the aqueous extracts were sterilized by passing through a Millipore filter (0.2 µm). All extracts were stored at 4 °C. The extracts were used for spore germination bioassay and TLC plate bioassay.

3.17.2. Spore germination bioassay

For screening of inhibitory effects of botanicals against test pathogens, the technique as described by Suleman *et al.* (2002) was followed. Aqueous plant extract (30 µl) was placed on a clear grease free microscopic slides. In control, 30 µl sterile distilled water was placed. Then 30 µl of spore suspension was added to 30 µl plant extract or 30 µl distilled water. In case of ethanol extract of plants 30 µl of ethanol extract was placed and in the control 30 µl of ethanol was placed. Spore

suspension (30 µl) was added after ehanol was evaporated from both the ethanol extract and ethanol from the slides. The slides were then incubated at 28±2 ^{0}C in a humid chamber. Humid chamber was prepared inside petridishes of 90 mm diameter. At first two small glass rods (60-70 mm in length) were placed in a petridish and a slide was placed on the rods in an uniformly balanced positions. Then the petridish was filled with sterile distilled water so that the bottom of the slide remains just above the water surface. The petridish was then covered. After 24 hours, the slides were stained with lacto phenol-cotton blue mixture and observed under the microscope. Approximately, 500 spores were observed for germination and length of 50 germ tubes were measured.

3.17.3. Preparation of TLC plates

Glass plates (20 cm X 10 cm) were dipped in chromic acid and then washed in running tap water. The plates were dried in hot air oven. After cooling the plates were placed in a platform of 100 cm X 20 cm. Silica gel G Merck (70 g) was dissolved in a conical flask containing 100 ml double distilled water, shaken vigorously and poured in a TLC applicator. A coat of silica gel (1 mm thick) was applied on the glass plates. The plates were allowed to dry and kept for future use.

3.17.4. Developing of TLC plates

TLC plates were activated at 70 ^{0}C for 30 minutes in a hot air oven. After cooling, sample extracts were applied on the plates by a capillary tube, 1 cm away from the basal end and the distance of two different spots was 1.5-2 cm. The sample-loaded plates were developed in a glass chamber containing solvent (chloroform: methanol:: 9:1). The solvent was allowed to run up to 15 cm. Following this the plates were taken out, air dried and used for chromatographic analysis or bioassay.

3.17.5. TLC plate bioassay

Air dried TLC plates were sprayed with spore suspension diluted with Richard's media (spore suspension: liquid Richard's media:: 1:1). The mixture was sprayed with an automyzer and then placed in a humid chamber keeping the coated side down on two parallel glass rods placed 16 cm apart. The humid chamber was

made by pouring mild hot water in a tray (25 cm X 15 cm). Precaution was taken so that the coated side do not touch the water surface. The whole set was incubated at 25±1 °C for 3 days. After three days, the plates were taken out and the diameters of the inhibition zones were noted, if any. The R_f values of the zones were recorded.

3.18. Application of biocontrol agents

3.18.1. Dual culture technique

Screening of potential antagonist was done following the dual culture method of Johnson and Curl (1982). In this technique, 20 ml of sterilized PDA medium in 100 ml Ehrlenmeyer flask was prepared. They were then transferred to sterile petriplates of 90 mm diameter, each containing 20 ml of PDA. Discs (4 mm) were cut from 15 d old cultures of a pathogen and of a biocontrol agent. The two discs were placed aseptically into the peripheral region of the petriplates in a straight line but opposite to each other. The plates were then incubated at 28±2 °C. Radial growth of the pathogen and the biocontrol agent were measured and percent inhibition in comparison to control (where no biocontrol agent was placed) was determined for each treatment.

3.18.2. Bioassay of cell free culture filtrate by poisoned food technique

Poisoned food technique as described in section 3.16 was followed with some modifications. PDA (9 ml) was prepared in 100 ml Ehrlenmeyer flask and sterilized. Membrane filtered culture filtrate (1ml) was added to the molten PDA medium, mixed well and poured in sterile petridishes (70 mm diameter) under aseptic conditions. After solidification of the media, the plates were inoculated with the pathogens, incubated for desired period and radial growth was measured following the methods described earlier (section 3.16).

3.19. Statistical analysis

Statistical analysis was done with the help of Smith's statistical package (version 2.5), developed by Dr. Gray Smith, Pomona College, Claremont-91711,. USA.