# Materials and Methods

## 3.1. Plant material

Eighteen tea varieties which includes five Tocklai varieties (TV-18, TV-22, TV-25, TV-26 and TV-30), six UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2) and seven Darjeeling varieties (Teen Ali -17/1/54, AV-2, HV-39, T-78, T-135, K-1/1 and B-157) being maintained in Tea Germplasm Bank at the Department of Botany, North Bengal University were used for experimental purposes. These were originally collected from Tocklai Experimental Station, Jorhat, Assam, UPASI Tea Research Station, Valparai, Tamilnadu and Darjeeling Tea Research Centre, Kurseong, Darjeeling, respectively.

### 3.1.2. Growth and maintenance

Seedlings of tea varieties were propagated by cutting in the nursery under a green agro house. Soil mixture was prepared using sand (75%) and soil ( 25%) and pH was adjusted ranging between 4.5- 4.9. Polythene sleeves (9"x 6") were filled up with the prepared soil and stacked in rows in beds and watered thoroughly. Beds were arranged in two rows, fifteen in each row. One hundred and fifty cuttings of each of the 18 varieties were allowed for rooting in individual sleeves subsequent to dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloches and maintained by pouring water frequently at an interval of two days. Sleeves were further treated with nutrient mixture as recommended by Ranganathan and Natesan (1987). The composition of this mixture is as follows:

| | | |
|---|---|---|
| Ammonium phosphate sulphate (16:20) | - | 35 parts by weight |
| Ammonium sulphate | - | 8 parts by weight |
| Magnesium sulphate | - | 3 parts by weight |
| Potassium sulphate | - | 15 parts by weight |
| Zinc sulphate | - | 3 parts by weight . |

Thirty grams of the above mixture was dissolved in 1 litre of water and 50 ml was given in each sleeve once in 15 days up to 12 months. Tea plants were then transferred from sleeves to earthen pots and maintained [Plates 1 and 2] in glass

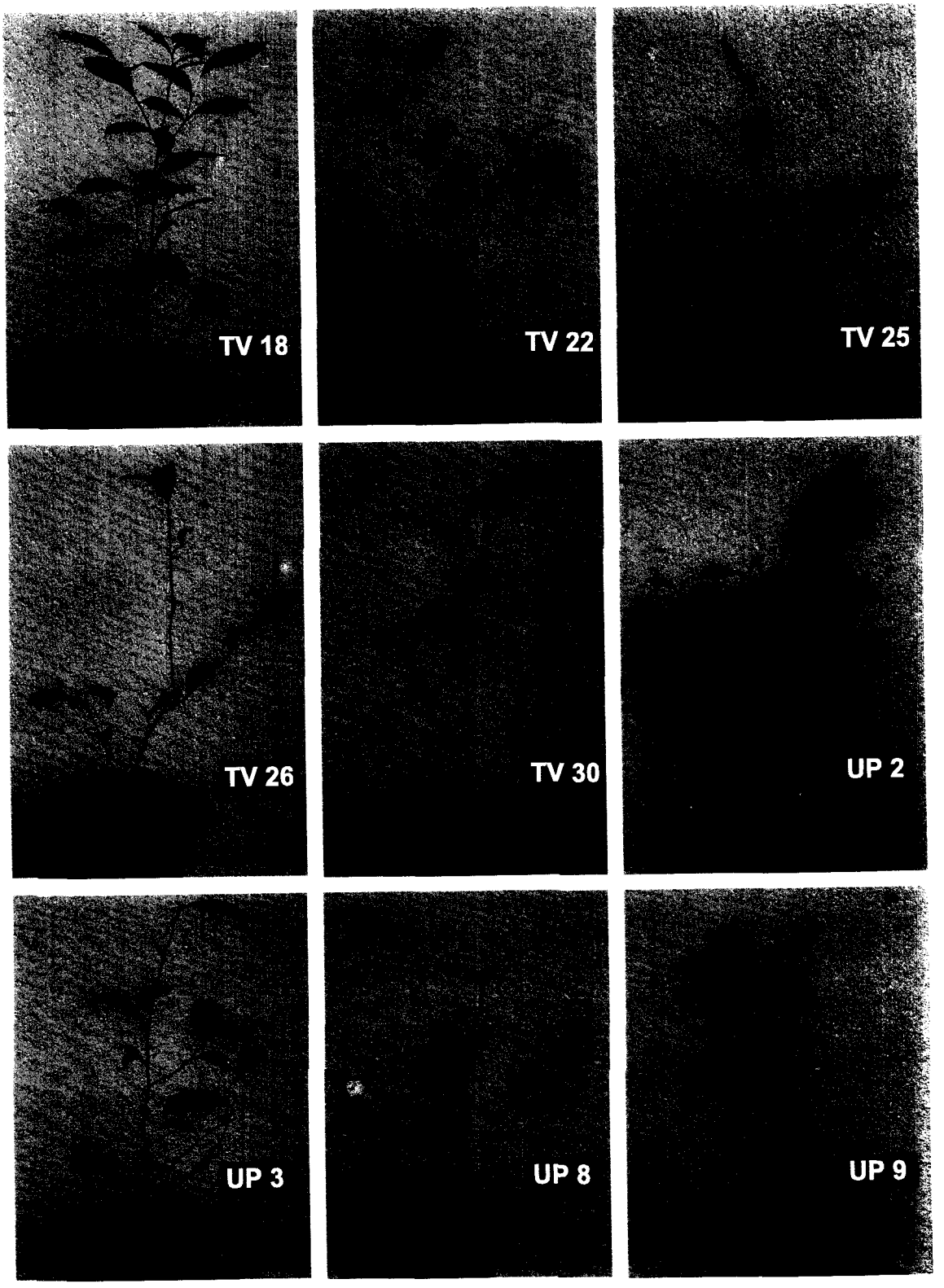


**Plate 1.** Tocklai and UPASI varieties of tea *(Camellia sinensis)* being maintained in Tea Germplasm Bank.

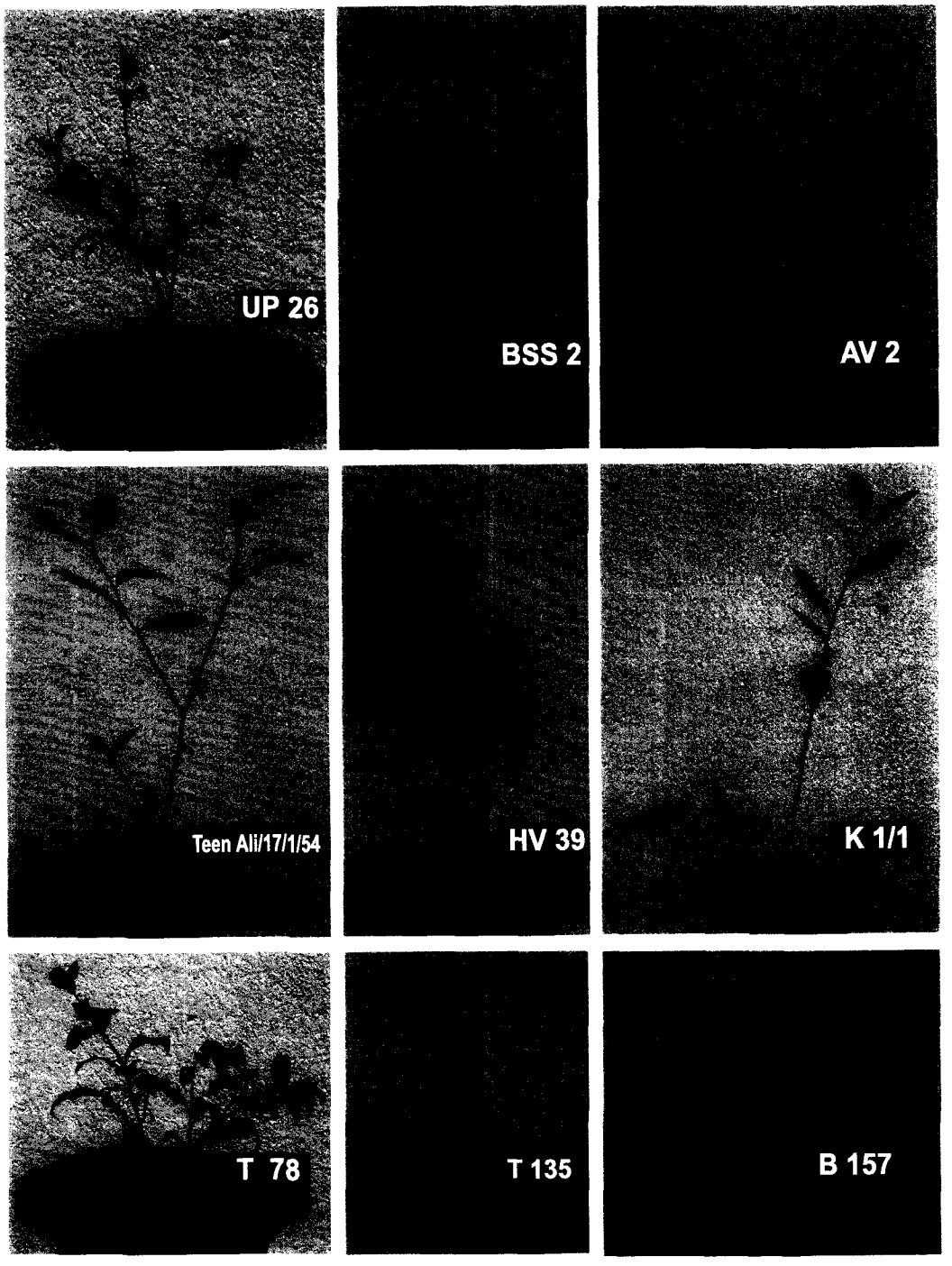


**Plate 2.** UPASI and Darjeeling varieties of tea (*Camellia sinensis*) being maintained in Tea Germplasm Bank.

house under natural condition of day light and temperature and watered on alternate days with ordinary tap water by sprinklers.

## 3.2. Fungal culture

### 3.2.1. Source of cultures

Virulent culture of *Sclerotium rolfsii* Sacc (*Corticium rolfsii* Curzi) was obtained from Immuno-Phytopathology Laboratory, Department of Botany, North Bengal University. This was originally isolated from Teen Ali-17/1/54 and after completion of Koch's postulate, the organism was identified by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience UK and designated as Sr-1. Besides, two more isolates (Sr-2 and Sr-3) of *S.rolfsii* which were used in this investigation were isolated from infected tea roots of TV-25 and UP-8 respectively. Cultures of *Fusarium graminearum* (a non pathogen of tea), *Trichoderma harzianum* and *Trichoderma viride* (biocontrol agents) were also obtained from the laboratory, mentioned above.

### 3.2.2. Completion of Koch's postulates

Tea seedlings (1-year-old ) were inoculated with 5 ml suspensions of the pathogen (*S. rolfsii*) prepared in sterilized distilled water from 15-day-old cultures grown on PDA containing one sclerotium per ml as described by Sonali and Gupta (2004). Infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water and transferred to PDA slants. After 7 days, the isolated organism was examined, compared with the original stock culture of *S. rolfsii* and its identity was confirmed.

### 3.2.3. Maintenance of stock cultures

The fungus thus obtained was sub-cultured on PDA slants. After two weeks the culture was stored under three different conditions ($5^0$C, $20^0$C and at room temperature $25^{\pm}$ $3^0$C). Apart from weekly transfer for experimental work, the culture of *S. rolfsii* was also examined at regular intervals to test its pathogenicity.

### 3.2.4. Assessment of mycelial growth

The mycelial growth on both solid and liquid media for various experimental purposes were as follows -

#### 3.2.4.1. Solid media

To assess the growth of *S. rolfsii* in solid media, the fungus was first grown in petridishes, each containing 20 ml of PDA and incubated for 7 days at room temperature. Agar blocks (6 mm dia) containing the mycelia were cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20ml of sterilized solid media. Growth of *S. rolfsii* was studied in six different solid media *i.e.*, potato dextrose agar (PDA), potato sucrose agar (PSA), carrot juice agar (CJA), Richard's agar (RA), Czapek-Dox agar (CDA) and yeast extract agar (YDA) as described by Dhingra and Sinclair (1985).

| **Potato dextrose agar** | | **Potato sucrose agar** | |
|---|---|---|---|
| Peeled potatoes | 40 g | Peeled potatoes | 40 g |
| Dextrose | 2 g | Sucrose | 2 g |
| Agar | 2 g | Agar | 2 g |
| Distilled water | 100 ml | Distilled water | 100 ml |

| **Richard's agar** | | **Carrot juice agar** | |
|---|---|---|---|
| $KNO_3$ | 1.00g | Carrot | 2g |
| $KH_2PO_4$ | 0.50g | Agar | 2g |
| $MgSO_4.7H_2O$ | 0.25g | Distilled water | 100ml |
| Sucrose | 3.00g | **Czapek Dox agar** | |
| KCl | 0.05g | $NaNO_3$ | 0.20g |
| Agar | 2.00g | $K_2HPO_4$ | 0.10g |
| Distilled water | 100ml | KCl | 0.05g |
| **Yeast dextrose agar** | | $FeSO_4.7H_2O$ | 0.05g |
| Yeast extract | 0.75g | Sucrose | 3.00g |
| Dextrose | 2.00g | Agar | 2.00g |
| Agar | 1.50g | Distilled water | 100ml |
| Distilled water | 100ml | | |

All the petriplates were then incubated at $28^0$C and colony diameter of the fungi were studied at 2 –day-intervals for 8 days.

**3.2.4.2. Liquid media**

To assess mycelial growth of *S. rolfsii* in liquid culture, the fungus was first grown in a petridish, containing 20 ml of PDA medium and incubated for 8 days at $28\pm1^0$C. From the advancing zone of the mycelial mat, agar blocks (6 mm dia) containing the mycelia, were cut with a sterilized cork borer and transferred to Erlenmeyer flasks (250 ml) each containing 50 ml of sterilized medium for the desired period at $28\pm1^0$C. Finally the mycelia were strained through muslin, collected in aluminium foil cups of known weight, dried at $60^0$C for 96 h, cooled in a desiccator and weighed.

## 3.3. Inoculation technique

### 3.3.1. Inoculum preparation

**3.3.1.1. Fungal pathogen:**

According to Chowdhury and Sinha (1995), sand maize meal medium was prepared in the ratio of 3:1 (sand : maize). In the prepared sand maize meal medium fungal pathogen (*S. rolfsii*) was inoculated and incubated at $28^0$C for 7 days. The inoculum was mixed with sterile soil at the ratio of 1:8. Fungus soil mixture (100 gm) were mixed with the top soil of earthen pots containing tea seedlings and kept for development of disease reaction.

**3.3.1.2. Biocontrol agents**

*Trichoderma* species prepared in several media *viz.*, wheat bran media (wheat-bran : sand 1:1, and 25 ml of water for 150 g of inoculum in each polythene packet); Saw dust media (saw dust and water), tea waste media (tea waste and water). Media were autoclaved and inoculated as above.

### 3.3.2. Inoculation of healthy tea seedlings in pot

One year tea seedlings were planted in earthen pots containing 1 kg soil and allowed to be established. Regular watering was done for two weeks and then 100 g

of pathogen inoculum was added carefully in the rhizosphere of each plant. Disease assessment was done after 2-week- intervals and up to 45 days of inoculation.

### 3.4. Disease assessment

The external symptoms were assessed thrice (15, 30 and 45 days) after inoculation with *S. rolfsii*. Disease intensity was assessed on the basis of above ground and underground symptoms together on a scale of 0 - 6; 0-no disease; 1 - small roots turn rotten; 2 - leaves start wilting and 10-20% of the roots turn brown; 3 - leaves wilted and 20-40% roots become dry with browning of shoot; 4 - extensive rotting at the collar region of root 60-70% of the roots and leaves wilted, browning of shoot over 60%; 5 - 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%; 6 - whole plants die.

### 3.5. Soluble protein

#### 3.5.1. Extraction

For extraction of soluble proteins from tea roots, the method of Alba and Devay (1985) was followed with modification. Roots were collected from the experimental garden and washed thoroughly. Root tissue (1 gm) was homogenized with 0.05 (M) sodium phosphate buffer pH 7.2 containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM soluble polyvinyl pyrrolidine (PVP) and 2 mM polymethyl sulphonyl fluoride (PMSF) in a mortar with a pestle using sea sand and insoluble PVP at $4^0$C. Homogenates were centrifuged at 12,000 *g* for 15 minutes at $4^0$C. The supernatant was collected and after recording its volume, was used immediately for estimation and analysis or stored at - $20^0$C for further use.

#### 3.5.2. Estimation

The protein estimation was done following by the method of Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent ( 0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% Sodium-potassium-tartarate and 50 ml of 2% $Na_2CO_3$ in 0.1 N NaOH) was added, mixed thoroughly and incubated for 15 minutes at room temperature. Then to it 0.5 ml of Folin-Ciocalteau's reagent (diluted 1:1 with distilled water) was added and again incubated for 20 min after mixing well for colour development.

Then optical density was measured at 690 nm in a colorimeter. Using bovine serum albumin as standard the protein concentrations were computed.

### 3.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total protein

This technique is used for analyzing protein mixtures quantitatively. It is useful for monitoring protein purification and also used to determine the molecular mass of proteins. Denaturation of the proteins by SDS makes all the proteins negatively charged. Electrophoresis in this leads the movement of proteins according to their molecular weight.

#### 3.6.1. Preparation of stock solutions

**Stock solutions:** For the preparation of gel the following stock solutions were prepared as described by Sambrook *et al.* (1989)

**A. Acrylamide and N-N$^{1}$-methylenebisacrylamide**

| | | |
|---|---|---|
| Acrylamide | - | 29 gm |
| N, N$^{1}$ – methylenebisacrylamide | - | 1 gm |
| Distilled water | - | 100 ml |

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in warm water. The solution was filtered in dark, kept in a brown bottle and stored at 4$^{0}$C. It was used within one month.

**B. Sodium dodecyl sulphate (SDS)**

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

**C. Tris buffer**

**(a) Lower gel buffer (1.5 M Tris)**

1.5 M Tris buffer was prepared for resolving gel (pH adjusted to 8.8 with concentrated HCl and stored at 4$^{0}$C) until use.

**(b) Upper gel buffer (1M Tris)**

1.0 M Tris buffer was prepared for stacking and loading buffer (pH adjusted to 6.8 with concentrated HCl).

**D. Ammonium per sulphate (APS)**

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

**E. Tris-glycine electrophoresis buffer (reservoir buffer)**

25 mM Tris base, 250 mM glycine (pH 8.3) and 0.1% SDS, 18.8 g glycine, 10 ml of 10% SDS in 1 L of distilled water.

**F. SDS loading buffer**

Tris buffer containing 50 mM Tris HCl (pH 6.8) 10 mM β-mercaptoethanol, 2% SDS, 10mg bromophenol blueb and 1 ml glycerol in 6.8 ml of distilled water was prepased.

**3.6.2. Slab gel preparation**

Glass plates of the same size (8 cm x 5 cm) were degreased with alcohol, wiped with blotting paper and dried. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the two sides of the glass plates were sealed with gel sealing grease uniformly and were kept in a gel casting unit. Resolving gel solutions (10% and 12%) were prepared mixing the stock solutions in the following order :

**Preparation of 10% resolving gel**

| **Solution** | **Amount** |
|---|---|
| Distilled water | 2.85 ml |
| 30% Acrylamide mixture | 2.55 ml |
| 1.5 M Tris (pH 8.8) | 1.90 ml |
| 10% SDS | 0.075 ml |
| 10% APS | 0.075 ml |
| TEMED | 0.003 ml |

**Preparation of 12% resolving gel**

| Solutions | Amount |
|---|---|
| Double distilled water | 2.45 ml |
| 30% Acrylamide mixture | 3.0 ml |
| 1.5 M Tris (pH 8.8) | 1.9 ml |
| 10% SDS | 0.07 ml |
| 10% APS | 0.07 ml |
| TEMED | 0.003 ml |

The mixture was poured into the set-up leaving sufficient space for the well-comb. After pouring the resolving gel solution it was immediately over layered with water and kept for polymerization for 45 min. After polymerization of the resolving gel the water overlayer was decanted off and a seven welled 1.5 mm thick comb was inserted.

**Preparation of 5% stacking gel**

| Solutions | Amount |
|---|---|
| Double distilled water | 2.10 ml |
| 30% Acrylamide | 0.50 ml |
| 1 M Tris (pH 6.8) | 0.38 ml |
| 10% SDS | 0.03 ml |
| 10% APS | 0.03 ml |
| TEMED | 0.003 ml |

The stacking gel solution was poured carefully up to a height of 1 cm over the resolving gel and finally overlayered with water. The gel was then kept for 30 min for polymerization.

### 3.6.3. Sample preparation

34 µl sample was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in a cyclomixer. Samples were floated in a boiling water bath for 3 min which denatured the sample.

### 3.6.4. Electrophoresis

Electrophoresis was performed at 18 mA current for a period of 2 h until the dye front reached the bottom of the gel.

### 3.6.5. Fixing and Staining

After electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R250) in 45 ml methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from the fixer and was stained by staining solution for overnight and finally soaked in destaining solution containing methanol, water and acetic acid (4.5:4.5:1).

## 3.7. Extraction and estimation of phenolics

### 3.7.1. Extraction

Phenols were extracted and assayed as described by Mahadevan and Sridhar (1982), with slight modification. 1 gm of root tissue was cut into small pieces and immediately immersed in boiling absolute alcohol which was then boiled on a boiling water bath for 5-10 minutes, using 4 ml of alcohol / 1gm of tissue. After cooling, the tissues were crushed with mortar and pestle using 80% alcohol and were filtered. Residues were re-extracted as before for 3 minutes and filtered. Both the filtrates were mixed and final volume was made up with 80% alcohol. Extracts were stored at $4^0$C in separate vials, covered with brown paper. The whole extraction was done in dark to prevent light induced degradation of phenol.

### 3.7.2. Estimation

#### 3.7.2.1. Total phenol

Total phenol was estimated by Folin–Ciocalteau's method as described by Mahadevan and Ulaganathan (1991). To 1 ml of phenolic extract, 1 ml of Folin - Ciocalteau's reagent and 2 ml 20% $Na_2CO_3$ was added, shaken properly and heated on a boiling water bath for 1 min and the volume was raised to 25 ml with double distilled water. Absorbance was measured in a Systronics photoelectric colorimeter Model-101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

#### 3.7.2.2. Ortho-dihydroxy phenol

Ortho-dihydroxy phenol was estimated as described by Mahadevan and Ulaganathan (1991). In 1 ml of each phenolic extract, 2 ml of 0.5 (N) HCl, 1 ml Arnow's reagent ($NaNO_3$ – 10 g; $Na_2MoO_4$ – 10 g; distilled water 100 ml) and 2 ml of 1(N) NaOH was added. These were then diluted with distilled water up to 25 ml. The tubes were shaken well and absorbance was recorded by Systronics photoelectric colorimeter Model-101 at 515 nm. Quantity of ortho-dihydroxy phenol was estimated using caffeic acid as standard.

### 3.8. Extraction of antifungal phenolics

Antifungal phenolics from root samples were extracted following the method as described by Daayf *et al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Root samples (10 g) were mixed with 80% methanol at 10 ml / g tissue and homogenized by blending for about 1 min. Samples were extracted for 48 h on a rotary shaker in a conical flask at 40 r.p.m covered with aluminum foil for protection from light. Methanolic extracts were then collected by filtration on a Whatman No.1 filter disc and concentrated by evaporation to a final volume of 20 ml (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethyl ether three times which was treated as Fraction I. The aqueous fraction was partitioned secondly with equal volume of ethyl acetate three times and the ethyl acetate fraction was considered as Fraction II. Acid hydrolysis of

the remaining aqueous fraction was done with 4(N) HCl to yield phenolic aglycones as suggested by Daayf *et al.* (1997). Aglycones were recovered by partitioning hydrolysates against an equal volume of ethyl acetate (three times), which was treated as Fraction III. All the fractions were evaporated to dryness and finally dissolved in 3 ml of the respective solvents.

### 3.8.1. Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea roots were analysed by thin layer chromatography (TLC) on silica gel G. The development of the chromatograms was carried out at room temperature using a solvent system (chloroform: methanol:; 9:1 v/v) as suggested by Chakraborty and Saha (1994a; 1995). Following evaporation of the solvent, thin layer plates were observed under UV light and sprayed separately with Folin-Ciocalteau's phenol reagent (Harborne, 1973). Colour reactions and Rf values were noted.

### 3.8.2.Bioassay of antifungal phenols

### 3.8.2.1. Radial growth

Radial growth inhibition assay was performed as described by Van Etten (1982). Ethyl acetate fractions of healthy and infected extracts (0.2 ml) were initially taken separately in sterile Petri plates and allowed to evaporate. In control sets, only ethyl acetate (0.2 ml) was taken and allowed to evaporate. Subsequently 10 ml sterilized PDA was poured in each Petri plate, thoroughly mixed and allowed to solidify. Agar blocks (3 mm dia) were cut with a sterilized cork borer from the advancing zone of a 4-day-old culture of *S. rolfsii* grown in PDA and was placed in the center of each Petri plate. Radial growth of *S. rolfsii* was recorded after 3 days of incubation at $28\pm2^{0}C$

### 3.8.2.2. Sclerotial germination

Sample solution was placed on a clean grease free slide, and it was dried. Mature sclerotia of *S. rolfsii* were placed on the test solution. Slides were kept on bent rods in moist Petri plates (100% humidity) and incubated for 24 h . In control

sets sclerotia were placed on sterile distilled water. Slides were observed under the microscope and percentage of germination were determined. Another set up was made for determination of sclerotial germination. In Petri plates black paper was kept and sterilized. These papers were soaked either with sample solution or sterile distilled water and in each Petri plate 10-20 mature sclerotia were placed on the top of the soaked black papers and incubated for 48 h. Germination percentage was computed and photographs were taken.

### 3.8.3. UV- spectrophotometric analysis

For spectral analysis of antifungal phenols extracted from healthy and *S. rolfsii* inoculated roots, initially ethyl-acetate fractions were plotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent. Silica gel from corresponding antifungal zones as detected in chromatogram inhibition assay as well as sclerotial germination assay were scrapped off and eluted separately in spec methanol. These were re-spotted on TLC plates and developed in the same solvent, were diluted with spec. methanol and taken for UV-spectrophotometric analysis at a range of 200-400 nm.

### 3.8.4. HPLC analysis

Analysis of antifungal phenolics extracted from healthy and *S. rolfsii* inoculated tea plant roots were carried out on C 18 hypersil column using methanol as mobile phase in isocratic system. The elution was complete after a total of 15 min. Flow rate was fixed at 1 µl/min, sensitivity 0.5 aufs, injection volume 20 µl and monitored at 220 nm (Shimadzu, Japan).

## 3.9. Extraction of enzymes

Three enzymes – phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) involved in phenol metabolism were extracted from healthy and *S. rolfsii* infected tea plants to determine their activities.

### 3.9.1. Phenylalanine ammonia lyase (EC 4.3.1.5)

For the extraction of phenylalanine ammonia lyase the method of Bhattacharya and Ward (1987) was followed with modifications. Tea root tissue (1 *g*) was crushed in a mortar with pestle in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol in ice. The crushed material was centrifuged at 12,000 *g* for 20 min at $4^0$C. The supernatant was collected, volume recorded and then immediately used for assay.

### 3.9.2. Peroxidase ( EC 1.11.1.7)

To extract peroxidase the method of Chakraborty *et al.* (1993) was followed with modification. Tea root tissue (1 g) was crushed with 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol in mortar with pestle on ice. The crushed material was centrifuged at 12,000 *g* for 20 min at $4^0$C. The supernatant was collected and its final volume was measured and used immediately for assay.

### 3.9.3. Polyphenol oxidase ( EC 1.10.3.2)

For the extraction of polyphenol oxidase the method of Mahadevan and Sridhar (1982) was followed with modification. Root tissue (1 g) was cut into pieces. The pieces were then crushed with mortar and pestle in ice cold condition with 5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The slurry was immediately centrifuged at 12,000 *g* for 20 min at $4^0$C. The supernatant was decanted and after recording its volume was used immediately for assay.

### 3.10. Assay of enzyme activities

Enzyme activities were assayed following specific procedure in each case.

### 3.10.1. Phenylalamine ammonia lyase

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture (total volume 3 ml) contained 0.3 ml 300 μM sodium borate (pH 8.8), 0.3 ml 30 μM L-phenylalanine and 0.5 ml of enzyme extract and 1.9 ml of double distilled water

Blank was prepared in same way but with water instead of enzyme extract. Then the tubes were incubated at $37^0$C for 1h in water bath. After 1 h absorbance was noted at 295 nm in UV-VIS-spectrophotometer (SICO, Digispec – 200 GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid / min.

### 3.10.2. Assay of peroxidase

For the assay of PO activity, 100 µl of freshly prepared crude enzyme was added to the reaction mixture containing 1 ml of 0.2 (M) sodium phosphate buffer pH 5.4, 100 µl of 4 mM $H_2O_2$, 100 µl of orthodianisidine (5mg /ml methanol) and 1.7 ml of double distilled water. PO activity was assayed spectrophotometrically at 495 nm by monitoring the oxidatioin of o-dianisidine in the presence of $H_2O_2$. Specific activity was expressed as the increase in absorbance at 495 nm / g tissue / min

### 3.10.3. Polyphenol oxidase

For the determination of PPO activity, 1.9 ml of 0.1 (M) sodium phosphate buffer pH 6.5, 0.1 ml enzyme extract and 0.1 ml of 0.025 (M) catechol solution (0.014 g in 5 ml sodium phosphate buffer pH 6.5). The reaction mixture was incubated at room temperature in the dark for the prevention of photo-oxidation of the enzyme. Initial absorbance was noted at 495 nm at 0 min. Further reading was taken at 1 min intervals. PPO activity was expressed as the increase in absorbance at 495nm / g tissue / min, when the substrate catechol was oxidized due to the enzyme activity from 1 g of tissue.

## 3.11. Preparation of antigens

### 3.11.1. Root antigen

Root antigens were extracted from healthy and *S. rolfsii* infected tea roots following the method of Chakraborty and Saha (1994a). Healthy and infected plants were uprooted after 2-week-intervals, washed with cold water and kept at $15^0$C for 1 h. Finally, roots (20 g fresh wt.) were crushed with sea sand in mortar and pestle in

cold ($4^0$C) and kept at $-15^0$C for 1 h and homogenized with 20 ml of 0.05 M sodium phosphate buffer\ (pH 7.2) supplemented with 10 mM sodium metabisulphite and 0.5 mM magnesium chloride. Homogenate was strained through cheese cloth and then centrifuged (12,000 *g*) at $4^0$C for 1 h. A known quantity of ammonium sulphate was added to it for 100% precipitation (Green and Hughes, 1995), and was kept at $4^0$C. Precipitate was dissolved in the same extractive buffer (pH 7.4) and dialysed against 0.05 M phosphate buffer for 24 h at $4^0$C. During this period 10 changes were given. The dialysate (*i.e.* soluble protein) was used for antisera production and for gel electrophoretic studies.

### 3.11.2. Mycelial antigen

Mycelial antigen was prepared following the method of Chakraborty *et al.* (1995b). Initially fungal mycelia (6 mm disc) were transferred to 250 ml Erlenmeyer flasks each containing 50 ml of sterilized liquid Richards medium and incubated for 10 days at $28\pm1^0$C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30 g fresh wt.) were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ and 0.85% NaCl with mortar and pestle and sea sand. Cell homogenates were kept overnight at $4^0$C and then centrifuged (15000 *g*) for 30 min at $4^0$C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at $4^0$C. After this period the mixture was centrifuged (15000 *g*) for 30 min at $4^0$C, the precipitate was dissolved in 10 ml of 0.05 M sodium phosphate buffer (pH 7.2). The preparation was dialysed for 72 h through cellulose tubing (Sigma) against 1 L of 0.005 M sodium phosphate buffer (pH 7.2) with 10 changes. Then the dialysed material was stored at $-20^0$C and used as antigen for the preparation of antiserum.

### 3.11.3. Soil antigen

1 gm of soil was crushed in 2 ml of 0.05 M sodium phosphate buffer (pH 7.2) in mortar with pestle and kept overnight at $4^0$C. Next day it was centrifuged at 12,000 *g* for 10 min. Supernatant was used as antigen for experimental purpose.

### 3.12. Serology

#### 3.12.1. Maintenance of rabbit

New Zealand white male rabbits (one and half kg wt.) were used for immunological works. The rabbits were observed at least for one week before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds and green vegetables in the morning and evening. Cages were cleaned everyday in the morning for better hygenic conditions. After each bleeding they were given saline water for 3 consecutive days.

#### 3.12.2. Immunization

Before immunization normal serum was collected from each rabbit. Polyclonal antibodies were raised against mycelial antigen of *S. rolfsii* and tea root antigen. The rabbit was injected subcutaneously once a week at 7-day-intervals with 1 ml antigen mixed with 1 ml of Freund's complete adjuvant (Difco, USA) for first two injections and the next emulsified with incomplete adjuvant for 5 weeks.

#### 3.12.3. Bleeding

Bleeding of the rabbits was performed by marginal ear vein puncture, 3 days after the first six injections and then after every fourth injection.The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp blade and 5 to 10 ml of the blood samples from each rabbit were collected in sterile graduated glass tubes. After collection, all the precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were kept as such for 1 h at $37^0$C for clotting then the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000 *g* for 10 min at room temperature) and distributed in 1ml vials and stored at – $20^0$C for further use.

### 3.12.4. Purification of IgG

#### 3.12.4.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2 ml) was first diluted with two volumes of distilled water and an equal volume of 4 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16 h at $22^0$C. The precipitate thus formed was collected by centrifugation at 10,000 *g* at $22^0$C for 1 h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer (pH 8.0).

#### 3.12.4.2. Column preparation

Eight grams of DEAE cellulose (Sigma Co., USA) was suspended in distilled water and kept for overnight. The water was poured off and DEAE cellulose was suspended in 0.05 M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30 cm high) and allowed to settle for 2 h. After the column material had settled, 25 ml of 0.02 M sodium phosphate buffer (pH 8.0) washing was given to the column material.

#### 3.12.4.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and with a continuous change in molarity from 0.02 M to 0.3 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer (pH 8.0) where as final elution buffer (2) was 0.3 M sodium phosphate buffer (pH 8.0). Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV Spectrophotometer (SICO, Digispec – 200GL).

### 3.13. Immunodiffusion

#### 3.13.1. Preparation of agarose gel plates

Glass plates (6 cm x 6 cm) were degreased in 90% (v/v) ethanol, ethanol: diethylether (1:1 v/v) and then dried in a hot air oven. After drying, plates were

sterilized. Agarose gel was prepared in Tris barbiturate buffer (pH 8.6) at $90^0$C using 0.9% agarose (Sigma, USA), placed on a water bath and stirred till the agarose solution became clear. Then 0.1% (w/v) sodium azide was added in it. After pouring 10 ml of molten agarose on each plate, it was kept for solidification. After that, 3-7 wells were cut with a sterilized cork borer at a distance of 6 mm dia from the central well.

### 3.13.2. Diffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony, 1967. The antigens and undiluted antisera (50 µl per well) were pipetted directly into pre-determined wells and allowed to diffuse in a moist chamber for 48-72 h at $25^0$C.

### 3.13.3. Washing, staining and drying of slides

After immunodiffusion, the plates were washed with aquous NaCl solution (0.9% NaCl and 0.1% $NaN_2$) for 72 h with six hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. After that the plates were stained with Coomassie blue (Sigma R250) for 10 min at room temperature. After staining, these were destained with methanol:water:acetic acid (45:45:10) until the background become clear. Lastly, the slides were washed with distilled water and dried in a hot air oven for 3 h at $50^0$C.

### 3.14. Enzyme linked immunosorbent assay (ELISA)

Following buffers were prepared for indirect ELISA

**(A) Antigen coating buffer** (0.05 M Carbonate-bicarbonate buffer, pH 9.6)

**Stock**

| | | |
|---|---|---|
| a. | Sodium carbonate | 5.29 g |
| | Distilled water | 1000 ml |
| b. | Sodium bicarbonate | 4.2 g |
| | Distilled water | 1000 ml. |

160 ml of stock solution "A" was mixed with 360 ml of stock solution "B" pH was adjusted to 9.6.

**(B) 0.15M Phosphate buffered saline pH 7.2 (PBS)**

**Stock**

| | | |
|---|---|---|
| a. | Sodium dihydrogen phosphate | 23.40 g |
| | Distilled water | 1000 ml |
| b. | Disodium hydrogen phosphate | 21.29 g |
| | Distilled water | 1000 ml |

With 280 ml of stock solution 'A', 720 ml of stock solution 'B' was mixed and the pH of the mixed solution was adjusted to 7.2. Then 0.8% NaCl and 0.2% KCl was added to the solution.

**(C) 0.15 M Phosphate buffered saline – Tween pH 7.2 (PBS-T)**

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

**(D) Blocking reagent** (0.05 M Tris buffer saline, pH 8.0)

| | |
|---|---|
| Tris | 0.657g |
| NaCl | 0.81g |
| KCl | 0.023g |

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

**(E) Antisera dilution buffer**

In 0.15 M PBS -Tween, pH 7.2, 0.2% BSA, 0.02% polyvinyl polypyrrolidone (PVPP-10), and 0.03% Sodium azide ($NaN_3$) was added.

**(F) Substrate solution**

This solution was prepared by addition of the substrate, p-nitrophenyl phosphate (10 mg / ml), to alkaline phosphatase buffer (0.05 m Tris – HCl buffer, pH 9.8) containing 1% diethanolamine.

**(G) Stop solution**

A 3 M NaOH solution was used to stop the reaction.

### 3.14.1. Plate trapped antigen - ELISA

PTA- ELISA was performed following the method as described by Chakraborty *et al.* (1996a). Plant and fungal antigens were diluted with coating buffer and the diluted antigens were loaded (200 µl/well) in a Nunc 96 well ELISA plate. After loading, the plate was incubated 25$^0$C for 4 h. The plate was then washed three times under running tap water and once with PBS-Tween and each time the plate was shaken dry. Subsequently, 200 µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated at 25$^0$C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 µl/well) to each well and incubated at 4$^0$C overnight. After further washings 200 µl of anti rabbit IgG goat antiserum labeled with alkaline phosphatase was added and incubated at 37$^0$C for 2 h. Plate was washed, dried and loaded with 200 µl of p-nitrophenyl phosphate substrate in each well and incubated in the dark at room temperature for 30-45 min. Colour development was stopped by adding 50 µl / well of 3 N NaOH and absorbance was determined in an ELISA reader at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

## 3.15. Immunoblotting

### 3.15.1. Dot-immunobinding assay

Dot-immunobinding assay was performed following the method suggested by Hammond and Jordan (1990) for detection of pathogen. Following buffers were used for dot-blot.

(i) **Coating buffer** - 0.05 M Carbonate – bicarbonate, pH 9.6.

(ii) **Washing buffer** - 10 mM Tris buffer saline, pH 7.4 (TBS) with 0.9% NaCl and 0.5% Tween – 20

(iii) **Blocking solution** -10% (w/v) skimmed milk powder (casein hydrolysate, SRL) in TBST (0.05 M Tris – HCl, pH 10.3, 0.5 M NaCl, 0.5% (v/v) Tween-20. Nitrocellulose membrane (NCM) (7 cm x 10 cm), pore size 0.45 µm,

Millipore) was first cut carefully into the required size and placed inside the template; 2 µl of coating buffer was loaded in each well of the template over the NCM and kept for 30 min to dry.

Following this 2µl of test samples (antigens) were loaded into the template wells over the NCM and kept for 1 h at room temperature. The template was removed and blocking was done with 1% non fat dry milk (Casein) prepared in TBS for 30-60 min on a shaker. Polyclonal antibody (IgG 1:500) was added, directly in the blocking solution and further incubated at $4^0$C for overnight. The membrane was then washed gently with washing buffer for 3 min (three times) in TBS (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase-conjugated goat antirabbit IgG (Sigma) diluted at 1:10,000 in alkaline phosphatase buffer, for 2 h at $37^0$C. The membrane was washed as before. Substrate BCIP-NBT tablet (Sigma) dissolved in 10 ml double distilled water, was added and colour development noted. Colour development was stopped by washing the NCM with distilled water and colour development was categorized on the intensity of the dots. Finally floating the NCM in deionized water stopped the reaction.

### 3.15.2. Western blotting

Immunoblotting was also determined using Western Blot technique as described by Wakeham and White (1996). The following buffers were used for western blotting.

[A] All the solutions for SDS-PAGE were prepared as mentioned earlier under 3.6

[B] Transfer buffer (Towbin buffer) - 25 mM Tris, 192 mM glycine in 20% reagent grade Methanol, pH 8.3.

[C] 0.15 M phosphate buffer saline, pH 7.2 (PBS) was made as mentioned previously.

[D] Blocking solution 5% Casein hydrolysate in PBS, containing 0.02% Sodium Azide and 0.02% Tween –20

[E] Washing buffer (50 mM Tris HCl, 150 mM NaCl pH-7.5)

[F] Alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ pH-9.5)

[G] Enzyme Alkaline phosphatase tagged with antirabbit goat IgG alkaline phosphatase buffer enzyme diluted (1:1000) in alkaline phosphatase buffer

[H] Substrate – one tablet of BCIP-NBT (Sigma) in 10 ml double distilled water

[I] Stop solution – EDTA sodium salt (0.0372 g in 200 µl distilled water) added in 50 ml of PBS.

### 3.15.2.1. SDS-PAGE of protein

Protein extraction, estimation and SDS-PAGE was carried out as mentioned earlier.

### 3.15.2.2. Blot transfer process

Following SDS-PAGE, the gel was transferred in prechilled (Towbin buffer) for 1 h. The nitro-cellulose membrane (Bio-Rad, 0.45 µm ) and the filter paper (Bio--Rad, 2 mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15 min. The transfer process was done in Trans-Blot SD Semi-Dry. Transfer cell (Bio-Rad) through a power pack (Bio-Rad). The presoaked filter paper was placed on the platinum anode of the unit. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The pre-wetted membrane was placed on top of the filter paper. Then the equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another pre-soaked filter paper was placed on the top of the gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant volt (15 V). After the run the membrane was removed and dried on a clean piece of 3 mm filter paper for 1 h and proceeded for immunological probing.

### 3.15.2.3. Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min with gentle shaking on a platform

shaker at room temperature. Subsequently the membrane was incubated with purified IgG (diluted in PBS). The bag was sealed leaving space for few air bubbles and incubated at $4^0$C overnight. All the processes were done with gentle shaking following incubation the membrane was washed thrice in 250 ml PBS. Final washing was done in 200 ml TBS to remove azide and phosphate from the membrane before the enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1 h at room temperature. After the enzyme reaction, the membrane was washed four times in PBS. Then 10 ml the substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity the membrane was transferred to a tray containing 50 ml of the stop solution.

### 3.16. Florescence antibody staining and microscopy (Immunofluorescence)

Indirect immunofluorescence staining of cross-section of tea roots, fungal sclerotia and mycelia were done using FITC labeled goat antirabbit IgG following the method of Chakraborty *et al.*(1995b).

#### 3.16.1. Mycelia

Fungal mycelia were grown in Richards agar media. After 4 days of inoculation young mycelia were taken out from the flask and kept in grooved slides. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted with PBS (1:125) and incubated at $27^0$C for 30 min. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with FITC (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at $27^0$C. After incubation, mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. These slides were observed and photographed under both phase contrast and UV fluorescence condition for comparison of treatment using Leica Leitz Biomed microscope with fluorescence optics equipped with Ultra Violet (UV) I3 filter block.

### 3.16.2. Cross-section of tea roots

Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS) pH 7.2. These sections were treated with normal serum or antiserum diluted in PBS (1:125) and incubated for 1 h at room temperature. After incubation, for 1 h at room temperature and transferred to 40 µl of diluted (1:40) anti-rabbit goat IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min in the dark. After that sections were washed thrice with PBS-Tween and mounted on a grease free slide with 10% glycerol. Fluorescence of the root sections was observed under UV light using Leica Leitz Biomed microscope equipped with I3 filter block ideal for FITC fluorescence. Tissue sections were photographed under both phase contrast and UV-fluorescent conditions for comparison of treatment.

## 3.17. Inducing agents and their application

### 3.17.1 *In vitro* test

**(a) Biocontrol agents:** Antagonistic properties of *T. harzianum* and *T. viride* against *S. rolfsii* were studied through dual plate method. Mycelial blocks (4 mm dia) cut from the margin of 3-day-old cultures of both the test pathogen (*S. rolfsii*) and biocontrol agents (*T. harzianum* and *T. viride*) were placed opposite to each other on PDA in petri plates (10 cm dia). The distance between inoculum blocks was 7 cm. Inoculated petri plates were incubated at $28^0$C. To examine the inhibitory effect of culture filtrates of *T. harzianum* and *T. viride* against *S. rolfsii*, initially these were grown in potato dextrose broth (PDB) at $28^0$C with vigorous shaking on a platform shaker at 175 r.p.m. for 7 days. Mycelia were harvested and culture filtrates were centrifuged at 12,000 *g* for 20 min and the supernatants were filter sterilized separately by passing through milipore filter. Then 45 ml of PDB media and 5 ml of culture filtrate were taken in 250 ml conical flask, while in control set 45 ml PDB media and 5 ml of sterile distilled water was taken. Each flask was then inoculated with 4 mm dia agar block of *S. rolfsii* and incubated at $28^0$C for 10 days. Finally mycelial dry weights were taken.

**(b) Plant extracts:** For *in vitro* evaluation of plant extracts of *Azadirachta indica* and *Catharanthus roseus*, these were separately mixed with 15 ml of molten PDA (v/v 5:15) and poured into sterilized glass Petri plates. After solidification, all the plates were inoculated individually with a 4 mm diameter culture disc taken from the advancing region of *S. rolfsii* grown on PDA . PDA plates without plant extracts but inoculated with *S. rolfsii* served as control. The plates were then incubated at $28\pm2^0$C. The colony diameter of the fungus was measured after six days of incubation and compared with the colony growth of the fungus in control.

**(c) Fungicides:** The fungitoxicity of the fungicides were tested using slide germination and poisoned food techniques. For *in vitro* fungicidal evaluation, five systemic fungicides *viz.*, thiodan, calixin, captan, carbendazim (Bavistin) and Indofil M-45 were screened at 4 different concentrations (0.1%, 0.05%, 0.025% and 0.0125%) and inhibitory effect on the mycelial growth was assessed using poisoned food technique. Three replicates of each fungicide concentration were prepared by pouring 15 ml PDA in each petri plate. After solidification of media, mycelial blocks (4 mm dia) from actively growing culture of pathogen (*S. rolfsii*) was inoculated at the center. Control plates without any fungicides were also inoculated for comparison. The inoculated plates were incubated $28^0$C till the fungus covered the PDA in control plates. The growth of the fungus was recorded.

**(d) Organic additives:** To evaluate the survival of *S. rolfsii* in organic amendments the sand-maize meal media was prepared with 2% saw dust. The different organic amendments (cow dung, chicken manure, rabbit manure, mustard oil cake and neem cake) were powdered in a grinder separately. The sand maize meal media and amended substance were mixed in 1:3 ratio and water added as required and sterilized. The mixture was plated in 9 cm dia Petri dishes and inoculated with 4 mm mycelial discs of *S. rolfsii* and incubated at $28^0$C. The growth of the fungus was recorded and compared.

### 3.17.2. *In vivo* test

Mature leaves (500 g) each of *Azadirachta indica* and *Catharanthus roseus* were harvested, washed thoroughly with running tap water, rinsed with distilled

water, air dried and macerated separately homogenized in a electric blender. The leaf extract was filtered through double – layered muslin cloth and centrifuged at 10,000g for 30 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper. Each filtrate was further filter sterilized and preserved as stock (100%) solution aseptically in bottles at $5^0$C for further use. Leaf extracts were diluted (1:10) with distilled water, drops of Tween-80 was mixed and spayed on tea plants with the help of sprayer. The control plants were sprayed with distilled water mixed with Tween 80. Spray was done four times at 7-day intervals. Both treated and untreated plants were inoculated with *S. rolfsii* and disease assessment was made.

Mustard oil cakes and neem cakes were allowed to decompose separately for a week in a clay pot covered with polythene. After decomposition, 100 ml of decomposed oil cake solution was added in each tea seedlings pots. The pots were then inoculated with *S. rolfsii*. Untreated control was kept for comparison. Growth behaviour also observed up to two months. Organic additives (cow dung, rabbit manure and chicken manure), 100 gm of each were taken separately and mixed in 1 kg of soil. These soil mixtures were separately kept in each pot. Tea seedlings were planted in each pot containing different organic components. After one week, 100 gm of pathogen (*S. rolfsii*) inoculum was added in the rhizosphere of each tea seedling.

Mass cultures of *T. harzianum and T. viride* were prepared on carrier medium comprising of wheat bran and sawdust (WBSD) in 3:1 ratio. Five hundred grams of the contents of carrier medium moistened with 20 percent (w/w) distilled water was filled in each bag. These polythene bags were sterilized at 15 lb pressure for 1 h for 2 consecutive days. Each polythene bag was then inoculated with 4-6 days old bits (0.3 cm) of pure culture either of *T. harzianum* and *T. viride* and incubated at $28\pm1^0$C. During incubation, these bags were gently hand shaken to promote uniform sporulation over the carrier medium and to avoid clusters. Addition of biocontrol agents in soil was done 10 days prior to inoculation with *S. rolfsii*.