3. Materials and methods

3.1 Plant materials

3.1.1 Host plants

3.1.2 Selection of suitable tea seed varieties

Fourteen biclonal seed varieties of the tea plants (*Camellia sinensis* (L.) O. Kuntze) have been released by Tocklai experimental station (Jorhat, Assam, India). Among the 14 varieties some are suitable for cultivation in Darjeeling hills and some varieties are cultivated extensively in the foot hills of sub-Himalayan West Bengal and Assam.

3.1.2.1 Collection of suitable varieties of tea seeds and storage

Tea seeds were collected from Taipoo Tea Estate, Siliguri, West Bengal and also from the seed farm of Gayaganga Tea Estate (Plate-II), Siliguri, West Bengal. Tea seeds of seven varieties (TS 520, TS 462, TS 463, TS 449, Ts464, TS491 and Ts 506) were collected during the month of November and December. After procurement, the seeds were kept in plastic bags up to two months in refrigerator at 4° C.

3.1.2.2 Raising of seedlings

Freshly harvested seeds were used or seeds from refrigerator were first soaked in water for 24 hours and then the seeds were in a germination bed inside the nursery. The seeds were sown in rows. Distance of 5 cm was kept between row to row and plant to plant. Finally the seed bed was covered with thick sheet made up of jute. Watering was done as and when required to maintain optimum humid condition. Seeds germinated after 21 to 30 days. Finally, seedlings were transplanted in pots or in fields according to the requirement of experimental design. A model nursery where tea plants have been transplanted have been shown in plate-III, a. A tea flower (Plate-III, b) and seed (Plate-III, c) have also been shown.

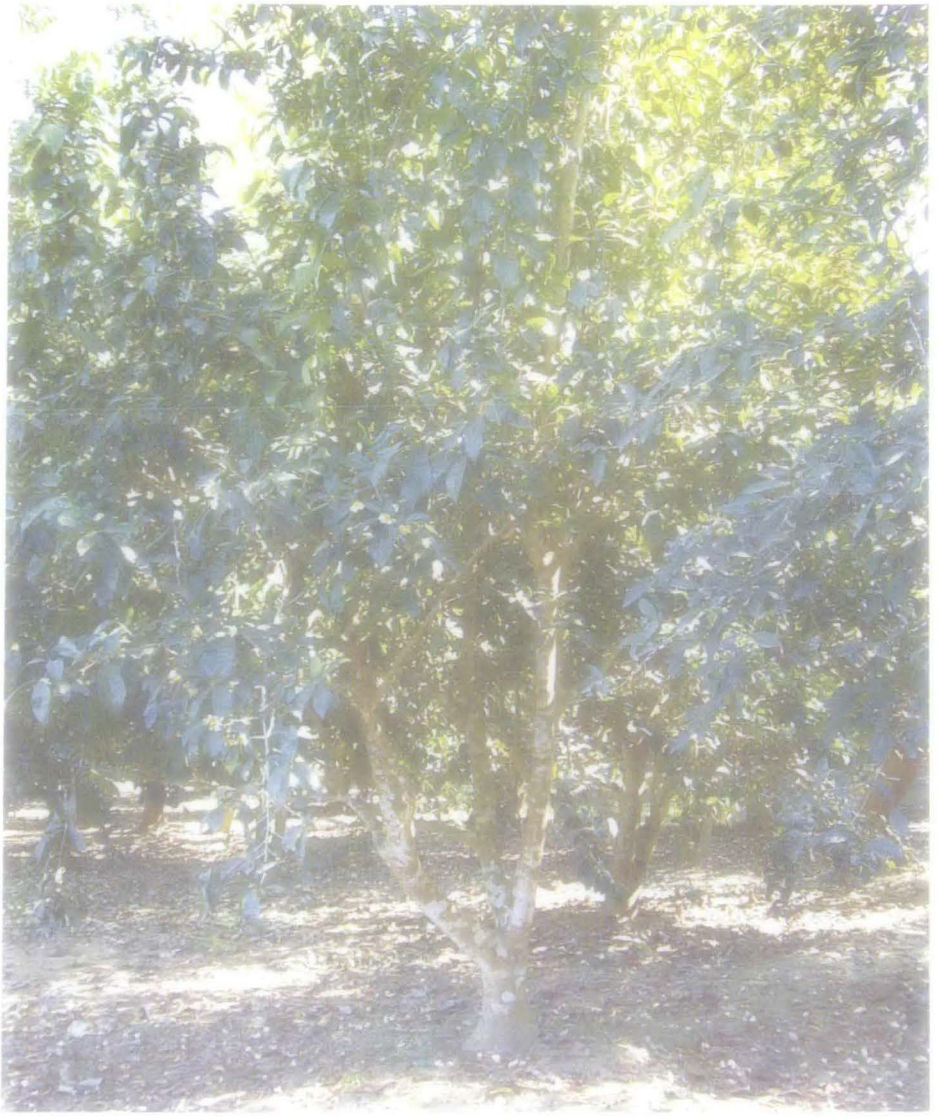

Plate II: Seed farm of Gayaganga tea estate.

Plate III: (a) Model nursery of tea (*Camellia sinensis)* plants; (b) A flower of tea plant (c) Seed of tea plant.

Plate IV: Germination, transplantation and maintenance of tea seeds; (a) Seeds purchased from seed farm (b) Germinated seeds in seed bed (c)Germinated seeds taken out from germination bed for transplantation (d) Transplanted seedling.

3.1.2.3 Transplantation and maintenance of the seedlings

The cracked seeds (germinated) were transplanted in polythene tubes or sleeves (20cm length and 10cm width) filled with a mixture of 3 parts of soil and 1 part of sand (Plate-IV) or in the experimental field. The optimum (4.8 to 5.0) soil pH was maintained. Thimate was used to remove root knot nematodes from the soil. Watering was done as and when required.

3.1.2.4 Collection of infected seeds for study of seed mycoflora

For seed mycoflora study, seeds were collected from different seed producing farms of different tea Estates of sub-Himalayan West Bengal and Assam. Seeds were collected from two places of Assam (Jorhat and Tinsukia) and six places of West Bengal (Siliguri, Jalpaiguri, Alipurduar, Malbazar, Banarhat and Madarihat).

3.1.3 Screening of tea seed samples for seed mycoflora

Fungal organisms were isolated from the seed surfaces or from the cotyledons of the seeds. For isolation of the fungi Standard Blotter Method (SBM) and Agar Plate Method (APM) were performed following the method of ISTA (1996).

3.1.3.1 Agar Plate Method

At first 400 seeds of each variety were taken. Unsterilized seeds with/without seed coats and sterilized seeds (treated with 0.1% $Hgcl_2$ followed by 3 washings with sterilized H_2O) with/without seed coats were plated in Petriplates (90 cm in diameter) containing Potato Dextrose Agar (PDA) medium. Then the plates containing the seeds were incubated at 28±1°C for seven days. Approximately 5 to 8 seeds were placed in a petriplate of 9 cm diameter.

3.1.3.2 Standard Blotter Method

One hundred seeds from each variety were taken. Unsterilized seeds with seed coats and sterilized seeds (treated with 0.1% $Hgcl_2$ followed by 3 washings with sterilized H_2O) without seed coat were placed on three layers of moistened blotting papers placed in a sterile Petriplate. Four to five seeds were placed in each Petriplate. The plates were incubated at 28±1°C in an incubator with 12 hours alternate light and dark.

The occurrence of different fungi in different samples was recorded and their percentage incidences were tabulated. The seed borne fungi were preliminarily identified on the basis of sporulation, conidial structures, spores or fruiting structures as apparent on seed in blotter method under stereo-binocular microscope, whereas the identification of the fungi in agar plate method was done on the basis of the colony characters developed on the agar medium, the morphology of the individual fungal species and light microscopic studies. The pure cultures of some fungal species were sent to Indian Type Culture Collection, IARI (Indian Agricultural Research Institute, New Delhi, India) for identification. Thus some of our identifications were confirmed with the identification results received from ITCC (IARI) New Delhi.

3.2 Source of Fungal culture

Fungal cultures were isolated from damaged tea seeds collected from different areas of North Bengal and Assam. While all isolated cultures were initially identified in the laboratory, identity of some of the cultures including a strain of *Rhizoctonia solani* which is the main fungus used during the present study were authenticated from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi. Three other cultures, *Trichoderma harzianum, T. viride* and *Gliocladium virens* (Isolate I) were collected from ITCC, IARI, New Delhi. Another strain of *G. virens* (Isolate II) was kindly donated by Dr. A. Chowdhury, UBKV, West Bengal.

3.2.1 Antagonists used during the study

Table 3.1: List of other fungal cultures used during the study

Name of the fungal Antagonists	Source	Identification No./ Identified by
***Trichoderma harzianum.* Rifai**	IARI, New Delhi	ITCC-4572
***Trichoderma viride.* Person**	IARI, New Delhi	ITCC2109
***Trichoderma virens* (Isolate - I)**	IARI, New Delhi	ITCC-4177
***Trichoderma virens* (Isolate – II)**	Dr. Apurba Chowdhury, Uttar Banga Krisi Viswa Vidyalaya, West Bengal	

(*Gliocladium virens* is presently known as *Trichoderma virens*)

3.2.2 Cultures obtained from seed mycoflora

Fifteen different fungi were isolated and identified during the study. List of those cultures have been shown in the following table (Table 3.2).

3.3 Detection of major pathogen from seed mycoflora

Fifteen different fungi including *Rhizoctonia solani* have been isolated from seeds of seven different varieties (TS 520, TS 462, TS 463, TS 449, TS 464, TS 491 and TS 506). All the fifteen fungi were studied microscopically and photographs were taken by camera (model D 3000; make: Nikon).

Table 3.2: List of identified fungal cultures from tea seed mycoflora

Name of the Fungal cultures with code in parenthesis	Identification No.	Identified by
Curvularia lunata **(F1)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Rhizoctonia solani **(F2)**	ITCC 5995.05	IARI, New Delhi
Alternaria **sp. (F3)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Aspergillus **sp. (isolate-1) (F4)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Aspergillus **sp. (isolate-2) (F5)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Aspergillus **sp. (isolate-3) (F6)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Aspergillus flavus **(F7)**	ITCC 7537.09	IARI, New Delhi
Aspergillus niger **(F8)**	ITCC 7540.09	IARI, New Delhi
Botryodiplodia **sp. (F9)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Rhizopus **sp (isolate-1) (F10)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Rhizopus **sp (isolate-1) (F11)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Penicillium **sp. (F12)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Trichoderma pseudokoningii **(F13)**	ITCC 7538.09	IARI, New Delhi
Fusarium sp. **(F14)**	-	Dr. A. Saha, Molecular Plant Pathology Laboratory, Univ. of North Bengal.
Sterile fungi (F15)	ITCC 7539.09	IARI, New Delhi

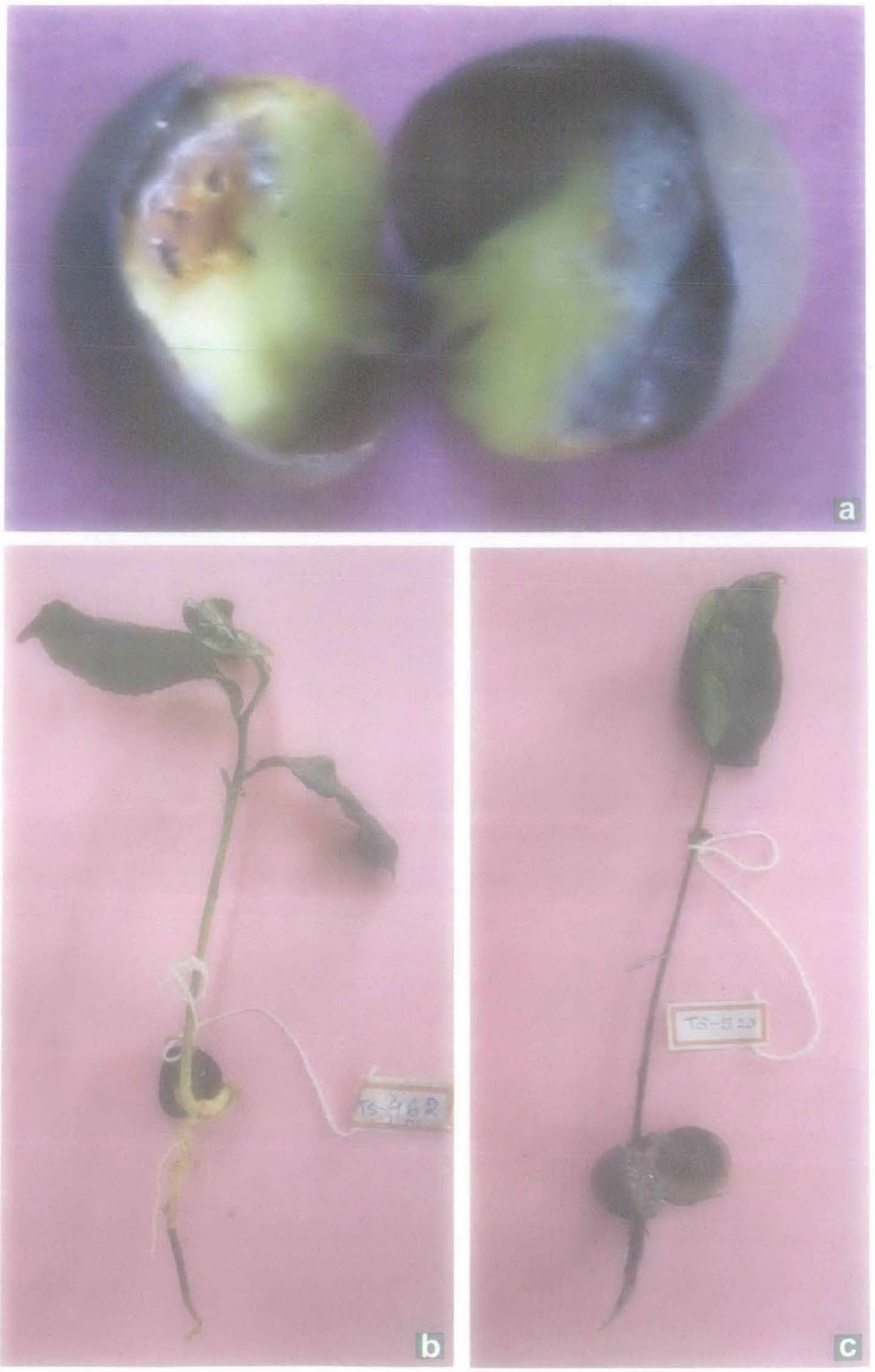

Plate V: (a) Cracked tea seed with brown patch.
(b) & (c) Artificially infected (by *Rhizoctonia solani*) tea seedling after 24 days.

3.4 Verification of Koch's Postulates by seed coating method

About 200 seed samples from each tea seed-varieties were sterilized with 0.1% $HgCl_2$ solution and then subjected to washing with sterile distilled water. The washing was done thrice. Finally, the seeds were kept on sterile trays and the trays were kept on the platform of a laminar air flow. Sterile air was blown through the seeds kept in the trays until the seed surface dried. Then, mycelia and sclerotial suspension was made following the method of Sharma *et al.* (2003). The mycelial suspension of *R. solani* was prepared by harvesting the mycelial mats from a 10-day old culture grown on PDA medium. The mats were suspended in sterilized distilled water and homogenized in a warring blender for one minute and strained through double layered muslin cloth and diluted with sterile distilled water, in such a manner, so that it contained about 15-20 mycelia-bits per-microscopic field (200 x). Inoculated seeds were transferred to a humid chamber having more than 90% relative humidity. The whole procedure was done in aseptic condition to avoid the external contaminations.

After 15 days of inoculation, the seeds were cracked and brown patches were observed (Plate-V, a). The portion of brown patches were cut from the cotyledons and transferred to a Petriplate containing PDA medium. After, growth of the fungi, the fungi was re-identified. The whole procedure was done in the month of December when the atmospheric temperature remains at 10° C, which was helpful to maintain the viability of the seeds.

In case of *Aspergillus* and *Rhizopus* sp. spore suspension was used instead of sclerotial suspension (as used in case of *Rhizoctonia solani).* Spore suspension was made separately from seven day-old cultures of the fungi.

After 15 days of inoculation, the seeds were cracked and observed for symptoms if any.

3.5. Pathogenicity of *Rhizoctonia solani* by sclerotial disc method and assessment of disease severity

Germinated tea seedlings with 2-3 leaves were sterilized with 0.1% $HgCl_2$. The seedlings were then washed thrice with sterilized distilled water. Plastic Pots of 10 cm in diameter were filled in autoclaved sand-soil mixture (1:1). One seedling per pot was planted. Two 5 mm mycelia discs with sclerotia were placed one inch below the surface of the soil and adjacent to the seedlings. The pots were transferred to a growth chamber maintaining at 25±1°C and the disease symptoms were observed at three days intervals up to 24 days (Plate-V, b & c). Disease severity was calculated on 1-5 scale (Carson *et al.*, 1991). Percent disease severity was calculated according to the method of Ahuja and Payak (1983). Twenty plants were used for the treatment and a separate control set of 20 plants were maintained.

The percent disease severity formula of Ahuja and Payak, 1983 has been given below:

$$P = \left\{ \frac{\sum (n \times v)}{Z \times N} \right\} \times 100\ \%$$

P = Disease severity
n = number of samples in each category
v = numerical value of each category.
Z = the highest numerical value of scale.
N = total number of sample

1-5 scale of Carson *et al.*, 1991 has been given in the following lines.
1 = no symptoms.
2 = < 20% root tissue discolored with scattered lesions, root system intact.
3 = 20 – 50 % discoloration, coalescing lesions some loss of root system.
4 = 50 – 75 % of root system discolored, few lateral roots left.
5 = tap root disintegrated and nonfunctional with little or no lateral root development.

3.6 Maintenance of stock cultures

For maintenance and preservation of fungal culture freshly prepared sterile PDA slants were used. After two weeks of sub culturing, the fungal pathogens grown on sterile PDA slants were stored in two different conditions, viz. at low temperature in refrigerator (at 5°C) and at room temperature. Fortnightly transfer of the cultures was done for experimental purposes. Pathogenicity of the selected cultures was examined at regular intervals. The cultures of four different antagonists viz. *T. harzianum, T. viride, G. virens* (Isolate-I), *G. virens* (Isolate-II) were also maintained at 5°C and at room temperature. The cultures isolated from the seed surfaces were also maintained at 5° C in a refrigerator.

3.7 Major chemicals used

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

Table 3.3: List of major chemicals used

Chemicals	Company/make
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
Antifungal assay agar	HiMedia Laboratories 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, India
Chloroform	E. Merck (India) Ltd., Mumbai, india
Coomassie brilliant blue	SRL Pvt. Ltd., Mumbai, India
Diethyl ether	SRL Pvt. Ltd., Mumbai, India
Folinciocalteau reagent	Ranbaxy Laboratory Ltd, India
Goat anti-rabbit IgG horseradish Peroxide conjugate	Bangalore Genei Pvt. Ltd., Bangalore, India
Immunogold reagent [Affinity Isolated aqueous glycerol Suspension of antirabbit IgG (whole molecule)-gold(5nm) from goat]	Sigma Chemicals Co., USA

Contd...

Table 3.3 (contd.): List of major chemicals used

Chemicals	Company/make
Mannitol	Qualigens, Glaxo India Ltd., Mumbai, India
Mercury (II) chloride	E. Merck (India) Ltd., Mumbai, India
Polyvinyl pyrrollidone	SRL Pvt. Ltd., Mumbai, India
Polyethylene glycol (PEG)	SRL Pvt. Ltd., Mumbai, India.
Tris (Tri hydroxyl methyl Methylamine)	Qualigens, Glaxo India Ltd., Mumbai, India
Tween 20	HiMedia Laboratories Ltd, Mumbai, India

3.8 Media and solutions used

A number of culture media and solutions were used during the present study. The name and compositions of these media and solutions are given below.

POTATO DEXTROSE BROTH (PDB)

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

Peeled potato in required amount was boiled in distilled water. The potato broth was collected by straining through cheese cloth and then required amount of dextrose was added. Finally, the medium was sterilized at 15lb p.s.i for 15 minutes.

POTATO DEXTROSE AGAR (PDA)

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

OAT MEAL AGAR (OMA)

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

Required amount of powdered oat was boiled in distilled water in a water bath stirred occasionally and strained through cheese cloth. Then agar powder was added to and melted by heating before the medium was sterilized at 15 lb p.s.i for 15 minutes.

ROOT EXTRACT AGAR (REA)

Tea root	:	20 g
Agar agar	:	2 g
Distilled water	:	100 ml

Fresh tea root of 20 g were boiled in distilled water. Root decoction was collected by straining through cheese cloth. Required amount of agar powder was then added and melted by boiling. Finally, the medium was sterilized at 15 lb p.s.i for 15 minutes.

MALT EXTRACT AGAR (MEA)

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

Malt extract was dissolved in distilled water by boiling. Then, required amount of agar powder was added. Finally the solution was boiled with constant shaking till the agar was dissolved. Sterilization was done at 15 lb p.s.i for 15 minutes.

POTATO CARROT AGAR (PCA)

Grated Potato	:	20 g
Grated Carrot	:	20 g
Agar agar	:	20 gm

Distilled water : 1000 ml

Required amount of grated potato and grated carrot were mixed and boiled with distilled water. The broth was strained through cheese cloth and agar power was added to the filtered broth. Finally, the medium was boiled to dissolve agar before sterilization at 15 lb p.s.i for 15 minutes.

RICHARD'S SOLUTION / MEDIUM (R.M)

Potassium nitrate (KNO_3)	:	10 g
Potassium Dihydrogen Phosphate (K_2HPO_4)	:	5 g
Magnesium sulphate ($MgSO_4$, $7H_2O$)	:	2.5 g
Sucrose	:	50 g
Distilled water	:	1000 ml

All the constituents were mixed with required amount of distilled water. Constituents were then dissolved by stirring and sterilized at 15 lb p.s.i. for 15 minutes.

RICHARD'S AGAR (RA)

2% agar powder was added to the final Richard's solution to prepare Richard's agar. The agar was melted by heating the media before sterilization at 15 lb p.s.i. for 15 minutes.

CZAPEK DOX AGAR (CDA)

Sodium Nitrate ($NaNO_3$)	:	3 g
Potassium hydrogen phosphate (KH_2PO_4)	:	1 g
Potassium Chloride (KCl)	:	0.5 g
Magnesium sulphate ($MgSO_4$, $7\ H_2O$)	:	0.5 g
Ferrous Sulphate ($FeSO_4$)	:	0.01 g
Agar agar	:	15 g
Distilled water	:	1000 ml

All the ingredients except agar and KH_2PO_4 were dissolved. Then agar was added and dissolved by boiling. Finally KH_2PO_4 was added to the molten solution, mixed thoroughly and sterilized at 15 lb p.s.i. for 15 minutes.

YEAST EXTRACT MANNITOLAGAR (YEMA)

Yeast extract	:	2 g
Mannitol	:	10 g
Potassium Dihydrogen Phosphate (KH_2PO_4):		0.5 g
Magnesium sulphate ($MgSO_4$, 7 H_2O)	:	0.2 g
Sodium Chloride (NaCl)	:	0.1 g
Agar agar	:	20 g

All the ingredients except agar were dissolved in distilled water. Finally, agar was added and dissolved by boiling before the medium was sterilized at 15 lb p.s.i for 15 minutes.

NUTRIENT AGAR (NA)

Beef extract	:	3 g
Peptone	:	10 g
Agar	:	15 g
Distilled water	:	1000 ml
Carbohydrate (if desired):		10 g

Required amount of beef extract and peptone were dissolved in distilled water. Agar was then added to the solution and dissolved by heating. Carbohydrate may be added if required before adding agar. Finally the medium was sterilized at 15 lb p.s.i. for 15 minutes.

3.9 Morphology and physiology of the pathogen

3.9.1 Microscopy

Rhizoctonia solani was sub cultured in PDA for ten days. A bit of fungal mycelia was taken from PDA slant, placed on a clean grease free slide and stained with lacto phenol and cotton blue. The slides were

observed under light microscope (Olympus, India). Length and breadth of mycelia were measured by ocular micrometer standardized by stage micrometer. The details of the morphology of the fungus were noted.

3.9.2 Assessment of mycelial growth

Nine solid and three liquid media were used to assess the mycelial growth of the fungal pathogen. Initially, petriplates with sterile PDA medium were inoculated with test fungus and incubated for 7 days at 28±1°C for mycelial inoculum production. Mycelial blocks (4-5mm) were cut from the 7 day old culture. The advancing zone of hypha were considered for the purpose and placed in sterile petriplates (70 mm diameter) containing 10 ml of different sterile medium and were incubated for 5 days at 28±1°C. Radial growth of mycelia was measured after regular intervals to assess the mycelial growth in different solid medium.

To assess the mycelial growth in liquid media, mycelial agar discs (4-6 mm diameter) were obtained similarly as mentioned above and were transferred to conical flasks of 250ml, each containing 50ml of different sterilized liquid medium. The flasks were incubated at 28±1°C. Mycelial mat were strained through double-layered cheese cloth after 5, 10, 15, 20 and 25 days of incubation. Mycelial mat of each flask was then blotted on a blotting paper. The blotted mycelia mat was wrapped in aluminum foil paper and was kept in a hot air oven at 60° C. The dried mycelia mat of each flask was weighed.

3.10 Serological studies

3.10.1 Immunization of Rabbit for raising antisera

Polyclonal antibody was raised, in the present study. To raise polyclonal antibody, three New Zealand male white rabbits were used. The rabbits were procured from animal farm in Siliguri. Rabbits were brought to the laboratory at least a month before initiation of any immunization

programme for proper acclimatization. The body weights of rabbits were approximately 1.2 kg and their age was around 9 months. The rabbits were kept in separate cages (75 cm x 75 cm x 90 cm) attached with plastic trays at the bottom and were placed in a well-aerated room. The room was cleaned regularly with permitted room freshening solutions. Rabbits were fed with sufficient quantities of vegetables like soaked gram (*Cicer arietinum* L.), carrot *(Daucus carrota* L.), common grass (*Cynodon dactylon* (L) Pers.), cabbage leaves (*Brassica oleraceae* L. var. *capitata*), cauliflower leaves (*B. Oleraceae* L. var. *botrytis*), lettuce leaves (*Lactuca sativa* L) etc. and clean water. Routine heaths checking of rabbits were done. The immunization program was initiated after one month of acclimatization and after ascertaining complete body fitness of the rabbits.

3.10.2 Antigen preparation

3.10.2.1 Antigen from tea root tissue

Roots of young tea seedlings were extracted following the protein extraction procedures of Alba and DeVay (1985) and Chakraborty and Saha (1994). Fresh young roots of the required varieties of tea were collected from the seed germination bed and washed thoroughly with cold water and kept for 2 hours at -20°C. The cold leaves (20 g fresh weight in each case) were grounded in pre-chilled mortar at 4°C with 10 g insoluble polyvinyl pyrrollidone (PVP). The root 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. Then the root slurry was strained through cheese cloth and centrifuged at 4°C for 30 min at 12,000 g. The supernatants were collected and ammonium sulphate was added at 4°C to 100% saturation under constant stirring. It was allowed to stand overnight at 4°C and finally centrifuged at 4°C for 14 min at 12,000 g. The precipitate obtained was dissolved in cold 0.05 M sodium phosphate buffer (pH 7.0).The resultant solution was then subjected to dialysis. Dialysis was done using dialysis

bag kept in sodium phosphate buffer of much lower molar concentration (0.005 M) and pH 7.4. Dialysis was completed within 24 hours with 12 changes.

Finally, the solution within the dialysis bag was collected and kept in small eppendorf tubes at -20°C until required.

3.10.2.2 Antigen from fungal mycelia

Fungal antigen was prepared from fungal mycelia. At the onset mycelial discs (4 mm diameter) from 5 day old PDA culture plates were transferred to 10 conical flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28±1°C. Extraction was done following the method as described by Chakraborty and Saha, 1994 with some modifications. The fungal mycelia were harvested after 15 days, washed with 0.2% NaCl and rewashed with sterile distilled water. Mycelia (25 g fresh weight) were homogenized in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl in a mortar and pestle with sea sand and kept overnight at 4°C. Centrifugation of homogenates was done at 4°C for 30 minutes at 12,000 g. The supernatants were collected and equilibrated to 100% saturated $(NH_4)_2SO_4$ under constant stirring and again kept overnight at 4°C. Then, the mixtures were centrifuged at 4°C for 30 minutes at 12,000 g. Precipitates were dissolved in 5 ml cold sodium phosphate buffer (0.05 M, pH 7.4) after discarding the supernatants. The preparations were dialyzed for 24 hours at 4°C against 0.005 M sodium phosphate buffer (pH 7.4) with 12 changes. Following dialysis, the preparations were centrifuged at 4°C for 15 minutes at 12,000 g and supernatant was then stored at -20°C until further use.

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

3.10.3 Antisera

3.10.3.1 Raising of antisera

Normal sera were collected from each rabbit before immunization. Antisera against root antigens of resistant and susceptible host varieties of *C. sinensis* and that of pathogen (*R. solani*) were raised in separate rabbits by giving intramuscular injections (1 ml) of antigens emulsified with equal volume of Freund's complete adjuvant. The doses were repeated at 7 days intervals with Freund's incomplete adjuvant for 6 consecutive weeks. The final protein concentration was adjusted to 2-4 mg/ml in the emulsion. Blood samples were collected and antisera were prepared on the 4th day after the last injection.

3.10.3.2 Collection of sera

Blood from rabbits was taken by marginal ear vein puncture and blood was collected in tubes. For this, the rabbits were first taken out from the cage, placed on a table and the hairs were removed from the vein of the ear with a sterilized blade. Ear vein was disinfected with rectified spirit. An incision was made with a sharp sterilized blade on the border vein of the ear after irritation of the ear with xylene. About 10 ml blood was collected in a sterile glass graduated tube. Adhesive surgical tape was affixed on the incision to stop the flow of the blood from the punctured area of the ear. The blood samples were kept undisturbed for an hour at 36°C for clotting. In order to avoid the loss of serum included within the clot, it was carefully loosened from the glass surface by turning a sterile wooden stick around the glass near the glass wall. Normal sera (pre-bleed sera) as well as antisera were clarified by centrifugation at 25°C for 10 minutes at 3000 g and were distributed in sterile cryovials and were stored at -20°C until required.

3.10.3.3 Optimization of titre value

Following immuno-diffusion technique as described by Ouchterlony (1967) and Clausen (1969), titres of antisera against the homologous antigens and titres of antigens against homologous antisera were determined. A constant amount (5µl) of undiluted antiserum or antigen was placed in the central well of immuno-diffusion plates, 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 pipetted into the outer wells. Diffusion was allowed for 48-72 hours at 25°C in a humid chamber. The highest dilution of antiserum or antigen that reacted with antigen or antiserum giving precipitin lines was determined as titre value.

3.10.4 Immunodiffusion

Double diffusion test on agar gel was performed following the method of Ouchterlony (1958).

3.10.4.1 Coating of slides with agarose

For preparation of agarose coated slide, barbital buffer (50 ml, 0.05m, pH 8.6) was taken in a 100 ml Ehrlenmeyer 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. To this, 0.1g (w/v) sodium azide (a bacteriostatic agent) was mixed and the agarose solution was dispensed carefully in clean, dry square glass plates of 6 cm x 6 cm so that no air bubble remained trapped in the agarose medium to avoid asymmetrical diffusion. Before dispensing the molten agarose solution, 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.

3.10.4.2 Diffusion of slides in humid chamber

In order to perform the immunodiffusion experiment, each agarose coated glass plate was placed in a petridish. Four to six wells of 4 mm diameter were cut by a sterile cork borer. The distances of the peripheral wells from the central wells were 5 mm. The antigens, normal sera and undiluted antisera were placed with a micropipette directly into the appropriate wells and diffusion was allowed to continue in humid chamber at 25°C for 48-72 hours. After proper staining of the slides, the precipitation reaction was observed only in cases where common antigens were present.

3.10.4.3 Staining of slides after diffusion

After immuno-diffusion the glass slides were washed with 0.9% aqueous NaCl carefully for 48 hours to remove all the unreacted antigens and antisera widely dispersed in the agarose surface. Next, the slides were washed with distilled water for three hours to remove the NaCl and dried at 40°C for 30 min. Then, the slides were stained either with 0.5% coomassie blue or 0.5% amido black (0.5 g 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. Following that, the slides were washed thrice with 2% v/v acetic acid for 3 hours (one hour each time) to remove the excess stain. Finally the slides were washed with distilled water and dried at 40°C for 30 min.

3.10.5 Immunoelectrophoresis

3.10.5.1 Slide preparation

For immunoelectrophoresis rectangular glass pieces (8 cm x 3.5 cm) were made grease free as mentioned earlier in section (3.10.4.1). Dry and grease free slides were placed on a clean surface. Thin and uniform layers (2 mm thick) of molten agarose medium (0.8%), containing 0.1% sodium

azide in 0.05 M barbital buffer (pH 8.6) were dispensed on each slide. Precautions were taken so that 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. The slides were stored at 4°C in petridishes until use.

3.10.5.2 Electrophoresis

For electrophoresis 2 central wells of 4 mm diameter were dug out from each agarose-coated slide. The slides were placed in the middle compartment of the electrophoretic platform. The anode and cathode chambers were filled with 0.05 M barbital buffer (pH 8.6). Different antigens were placed into separate wells. To trace the electrophoretic movement of the antigens, bromophenol blue was used as a marker. Barbital buffer soaked filter paper strips (Whatman No.1) were 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 (10V/cm) was passed through the slides for two hours at 4°C. The current was discontinued and the glass slides were taken out when the bromophenol blue marker reached near the short edge of the glass slides.

3.10.5.3 Diffusion of slides in humid chamber

After the electrophoretic part, 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 h in a moist chamber at 25°C. Precipitation lines (arcs) if formed were recorded. Finally, washing, drying and staining of the slides were performed as mentioned in the section (3.10.4.3).

3.10.6 Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Combining the methods of Koeing and Paul (1982) and Talbot (2001) indirect ELISA was performed. All the antigens were diluted with coating buffer [carbonate buffer (0.1M), pH 9.6] and 100 µl of each diluted antigen was 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 for 6 hours at 4°C in refrigerator. After incubation, the plate was taken out and each well of the plate was flooded with PBS – Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20) and thoroughly washed for four to five times. The plate was dried in air after washing. Following this, 100 µl of PBS-BSA (0.15M PBS containing 1% BSA) was added to each well to coat all the unbound sites and incubated for 2h at room temperature. The plate was again washed with PBS-Tween, air-dried and 100 µl of diluted antisera (diluted with PBS – Tween) was added to each well except the blank. In the control wells normal sera was added (serially diluted with PBS-Tween containing 0.5% BSA). The plate was incubated overnight at 4°C. 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 h at 30±1°C. After incubation, the plate was again washed with PBS-Tween and shakened dry. Then 100 µl (1:20) tetramethylbenzidine / hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. 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 1[N] H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm.

3.10.7 Immunogold labeling followed by silver enhancement and light microscopy

This technique was done following the method of Saha *et al.* (2010). Fresh healthy roots of Tea varieties were collected and washed thoroughly. The plant parts were kept at 4°C before use. Cross sections of roots were cut and placed on clean grease free slides. In case of fungus, the mycelia as well as spores were taken by a needle and placed on slides containing Myer's albumin for proper fixing. Water drops (100 µl) were mounted on each section. The slides were incubated at 2-5°C for 30 min and excess water surrounding the sections was blotted off. 30 µl of blocking buffer (0.15M PBS pH 7.2 containing 5% normal sera of goat) was placed on the cross sections and incubated for 10 min. Excess solution was wiped off and primary polyclonal antibody (1:50 dilution) rose in rabbit against target antigens were applied on the sections and incubated overnight at 25°C. After incubation, the sections were carefully rinsed in 0.15 M PBS (pH 7.2) for 4 min. Excess buffer was poured off by tilting the slide slightly. Next 100 µl of diluted (1:50) immunogold reagent containing 0.5 nm gold particle (Sigma, USA) was applied on the sections. Following incubation for 1h the sections were again rinsed for 4 minutes with PBS. Excess buffer was poured off and the sections were fixed in 200 µl of PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS) for 15 min. The sections were rinsed in distilled water and placed on slides for silver enhancement. For this, silver enhancement kit of Sigma (Product No. SE-100) were used. Initially solution A (silver salt) and solution B (and initiator) were mixed (1:1) according to the manufacturer's instruction. Mixed solution (100 µl) was used to flood each section. After 5 min of incubation, the cross sections were washed with distilled water. Distilled water was poured off and 100 µl of sodium thiosulphate solution (2.5% aqueous) was placed on the sections and allowed to incubate for 3 minutes. The sections were again washed in distilled water and mounted on slides with a drop of distilled water.

Immediately after the staining, photographs were taken in a binocular light microscope (Unicon, India) using digital camera (Canon, A310) with appropriate attachment system.

3.11 *In vitro* Evaluation of biocontrol agents against *R. solani*

3.11.1 *In vitro* antagonistic activity of fungal bioagents against *R. Solani* following dual culture technique

The various antagonistic fungi were screened under in vitro conditions against *R. solani* for their antagonistic activity by using dual culture method. The methods of Saikia *et al.* (2002) and Upmanyu *et al.* (2003) were followed with some modifications.

Five millimeter culture discs of each of the fungal antagonists and of the pathogen were taken from the margin of the actively growing cultures and transferred to Potato Dextrose Agar medium contained in Petri plates of 90 mm diameter. The culture discs were placed on opposite sides so that approximately 1 cm is left towards the wall of petridish having the test pathogen only was also kept for comparison. The petriplates were subsequently incubated at 25±1°C till the check plate (control) was completely covered by *R. Solani* colonies. Each treatment was replicated thrice. Radial growth of the pathogen of test plate and of control plate was measured at 24 h interval till the control plates are covered by the pathogen. Percent growth inhibition and sclerotial production of *R. solani* in dual cultures as well as control culture were calculated.

3.11.2 Evaluation of inhibitory effect of culture filtrates of different antagonistic fungi tested against growth of *R. solani in vitro*

Four antagonistic organisms [*Trichoderma harzianum, T. viride, Gliocladium virens*, (Isolate I and isolate II)] were cultured in Ehrlenmeyer flask containing 50ml PDB. The cultures were incubated for 14 days at 28±1°C. Finally, the culture filtrates were collected and filter sterilized. All

the culture filtrates were mixed separately with fresh PDB in ratio of 1:3 v/v. Hence, the final concentration of the culture filtrates in culture filtrate supplemented media becomes 25%. To evaluate the inhibitory effects of culture filtrates against *R. solani* the method of Chowdhury *et al.* (2003) was followed.

Four millimeter mycelial discs of *R. solani* were cut from a PDA plate containing 7days old culture of *R. solani.* The discs were placed in conical flasks containing culture filtrate supplemented PDB medium. Dry weights of *R. solani* in different treatments were determined by weighing. Results of different treatments were recorded and compared with control. Percent reduction of dry weight were also calculated and recorded.

3.12 In vitro evaluation of botanicals against *R. Solani*

3.12.1 Collection of plants for extraction of botanicals

Twenty one plant materials (root, bark, stem, root, rhizome as applicable) were collected from the foothills of sub-Himalayan West Bengal. The plants were selected on the basis of easy availability in the tea growing areas of sub Himalayan West Bengal. Plants were collected, identified and voucher specimens were deposited in the NBU herbarium, Department of Botany, university of North Bengal. A list of plants used for screening of antifungal botanicals has been given in the following table.

Table 3.4: List of plants used for extraction of botanicals with their traditional use

Name of the plant	Family	Parts used	Traditional uses
Polyalthia longifolia Sonnerat	Annonaceae	Leaf	Antibacterial, antifungal activity, and antitumor activity
Tridax procumbens L.	Asteraceae	Leaf	Antidiarrhoeal; antidysentric and controls bleeding wounds.
Xanthium strumarium L.	Asteraceae	Leaf	Antisyphilitic, astringent and diuretic.
Ocimum sanctum L.	Lamiaceae	Leaf	Fresh leaves used for curing ring worm and other skin diseases
Datura metel L.	Solanaceae	Leaf	Narcotic, Hypnotic, Hallucinogenic
Allium sativum L.	Liliaceae	Bulb	Juice used as rube facient in skin disease, atomic dyspepsia, flatulence, and colic
Azadirachta indica L.	Meliaceae	Leaf	Useful as stomachic febrifuge, antihelmintic, in dysentery and dyspepsia
Zingiber officinale Rosc.	Zingiberaceae	Rhizome	Used in dyspepsia and flatulent colic.
Catharanthus roseus (L.) G. Don	Apocynaceae	Leaf	Diabetes, Haemostatic, Treatment for leukemia.
Lantana camara L.	Verbenaceae	1.Root 2.Flower 3.Fruit	Leaf juice is used as antimicrobial in skin disease.
Adhatoda vasika L.	Acanthaceae	Leaf	Antihelmintic, antiseptic, antispasmodic, expectorant and sedative
Murraya koenigii (L.) Spreng	Rutaceae	Leaf	Bruised and applied locally to eruptions and poisonous bite.
Cannabis sativa L.	Cannabaceae	Leaf	Stimulant, decreased blood pressure and antiulcer activity
Boerhavia intermedia L.	Nyctaginaceae	Whole plant	Cancerous wounds and related complications
Curcuma longa L.	Zingiberaceae	Rhizome	Acne, wounds, boils, bruises, blistering, ulcers, eczema, insect bites, parasitic infections, heamorrhages and skin diseases like herpes zoster and pemphigus.

Contd...

Table 3.4 (contd.): List of plants used for extraction of botanicals with their traditional use

Name of the plant	Family	Parts used	Traditional uses
Croton bonpandainum Bail.	Euphorbiaceae	Leaves	Antihelmintic
Bidens alba L.	Asteraceae	Leaves	food or medicine
Calotropis procera (Aiton) W.T. Aiton	Asclepiadaceae	1.Leaves 2.Flowers 3.Root bark	Analgesic, antipyretic and neuromuscular blocking activity with negligible anti-inflammatory activity.
Dryopteris filixmas Adams.	Dryopteraceae	Leaf	anthelmintic
Calendula officinalis L.	Cavendulaceae	1.Whole flower head 2.Petals	Minor inflammations of the skin (such as sunburn) and as an aid in healing of minor wounds.

3.12.2 Extraction of Botanicals

Plant extracts were prepared as outlined by Sharma *et al.* (1999) with modifications. Fruit, flower and leaves of *Lantana camara* and leaves of other plants were crushed with cold sterile distilled water in a mortar and pestle. One gram of tissue in one mililitre of distilled water was taken for crushing. After crushing the plant materials were centrifuged at 3000 rpm for 10 min and the supernatant was used as stock solution. The stock solutions (100%) were diluted to the desired concentrations like 5%, 10% and 20% .The supernatants of the extracts were sterilized by passing through a Millipore filter (0.2µm). All extracts were stored at 4°C until use.

3.12.3 Agar Cup Bioassay

For screening of inhibitory effect of botanicals against the test pathogen, Agar Cup Bioassay technique of Suleman *et al.* (2002) and Saha *et al.* (2005) was followed.

At first, 2 ml of mycelia suspension was poured in petridish and subsequently 18ml of molten PDA medium was poured in the petridish. The medium and the mycelia suspension were mixed by rotating the petridish carefully. After solidification, by means of a cork borer (5mm in diameter) 4 to 5 cups or wells were made keeping sufficient distance between the wells and also from the end of the petridish. Plant extracts prepared from one gram tissue in one milliliter distilled water was poured into the cups. Each cup contained fifty micro liters of plants extracts. Finally, the plates were incubated at 28±1°C for 48 hours or as desired. In control set, sterile distilled water was poured into the well. In the first phase agar cup method was followed for screening of antimicrobial activity of the plant extracts. In the second phase poison food technique was followed.

3.12.4 Bioassay by Poisoned food technique

Crude aqueous extracts (1 gm tissue in 1 ml distilled water) of selected plants were added to molten medium (PDA), mixed well and poured in sterile petridish (90mm diameter) under aseptic condition. Aqueous extracts were added in the medium in such a way so that final concentrations of the aqueous extract in the medium were 5%, 10% and 20%. The plates were allowed to solidify. The plates were inoculated with pathogen at the centre with 4mm disc cut from the advancing zones of the growing mycelia of the test pathogen. Control plates (without any botanicals) were also inoculated. The plates were incubated at 28±1°C. Diameter of the radial growth were measured and percent inhibition of radial growth of *R. solani* were calculated and noted at two days intervals up to 10 days. Percent inhibition was calculated following the method of Chowdhury *et al.* (2003). The computation formula of Chowdhury *et al.* (2003) has been given in the following box.

Percent inhibition = (x-y / x) x 100

Where x = diameter of control disc

3.13 Assessment of incidence of *R. solani* in seeds pretreated with botanical, with 25% culture filtrate of *T. harzianum* and with a combined formulation of the botanical and culture filtrate

Fresh healthy tea seeds were surface sterilized with 0.1% $HgCl_2$ solution for 3 minutes followed by three washings with sterile distilled water. Seeds were sprayed by mycelial suspension of *R. solani* and incubated for 24 hours. After 24 hours of incubation the seeds were soaked in the 20% leaf extracts of *Polyalthia longifolia* and 25% culture filtrate of *T. harzianum* separately an also in mixed (1:1) formulation. The seeds were soaked for 24 hours in room temperature (28 ±2°C). After 24 hours the seeds were removed from the plant extract or the culture filtrate. Similar experiment with a combined formulation of the botanical and culture filtrate (1:1) was also tested. Presence of the pathogen (*R. solani*) was measured after 20 days of inoculation. The untreated seeds inoculated with the fungus *R. solani* under similar conditions served as corresponding control.

3.14 Effect of seed treatment on germination percentage and vigour index

Tea seeds were treated with the leaf extracts of *polyalthia longifolia* and culture filtrate (25%) of *T. harzianum* and sporulating culture of *T. harzianum* to assess the germination percent and vigour index of the seedlings.

3.14.1 Seed treatment with *Polyalthia longifolia* leaf extract

The method of Mamatha *et al.* (2000) was followed with some modifications. Crude leaf extract of *Polyalthia longifolia* was diluted to 20% concentration. Tea seeds of different variety were soaked in the leaf extracts for 24 hours. After that seeds were air-dried for next 24 hours and placed

in 10 cm diameter pots. One seed was sown in one pot containing sterile and unsterile soil.

3.14.2 Seed treatment with culture filtrate (25% concentration) of *T. harzianum*

The method of Mamatha *et al.* (2000) and Singh *et al.* (2003) were followed for the purpose. Culture of *T. harzianum* was grown in PDB medium for 12 days. Fungal mass (mycelia and spore) were separated by filtering through filter paper. Culture filtrate was collected. Culture filtrate (25%) of *T. harzianum* was prepared by adding sterilized distilled water. Seeds of different varieties were soaked in that culture filtrate for 24 hours and then air dried for next 24 hours. Finally seeds were placed in 10 cm plastic pots (one seed/pot) containing sterile and unsterile soil.

3.14.3. Seed treatment with sporulating culture of *T. harzianum*

The method of Meena *et al.* (2003) was followed. Culture of *T. harzianum* was grown in PDB medium for 10 days. After that the fungal mass (mycelia and spore) was separated by filtering through filter paper and dried overnight at 25°C. Dried culture of *T. harzianum* was gently powdered and mixed with equal amount of sterilized fine clay and equal volume of sterilized water to prepare slurry. That mixture was used for seed coating. The coated tea seeds were kept overnight in moist chamber so as to enable the antagonist to establish on seeds. These seeds were placed in 10 cm plastic pots containing sterile and unsterile soil. In one pot one treated seed was planted.

After 30 days observations on seed germination, root length and shoot length were done. The vigour index was determined by multiplying the percentage of germination with the sum of root length and shoot length

in centimetres. Disease free seeds served as control. The vigour index was calculated according to the method of Prasad *et al.* (1999).

3.15 *In vivo* evaluation of antagonist against *R. solani*

R. solani is a soil borne pathogen and it causes root diseases in tea plants. Tea plant is shrub in nature. It is not possible to treat the seedlings or tea plants time to time with the leaf extracts of botanicals and 25% culture filtrate of *T. harzianum.* Only the sporulating culture of *T. harzianum* can check the root diseases of tea plants as well as seedlings. For these reasons in this experiment tea plants were treated with the sporulating culture of *T. harzianum* by soil inoculation method. Seedling mortality was tested after 30 days.

3.15.1 Preparation of inoculums of *R. solani*

The method of Chowdhury *et al.*, 2003 was followed. Overnight soaked paddy seeds were kept in the conical flasks and sterilized at 15 p.s.i. for 20 min, which were later inoculated with actively growing culture of *R. solani* and incubated at 28±2°C for 10 days.

3.15.2. Preparation of mass culture of antagonist

The method of Upmanyu *et al.* (2003) was followed. The mass culture of the antagonist *T. harzianum* was prepared on wheat bran:Saw dust:tap water (3 : 1 : 4, w/w/v) medium and autoclaved at 1.05 Kg/cm^2 for 1 hour for 2 consecutive days. The medium was poured in polypropylene bags. Finally, the polypropylene bags filled with media were inoculated by mycelia of *T. harzianum* taken from actively growing 7-days old culture. The inoculated bags were incubated at 25±1°C for 14 days. After inoculum (of *T. harzianum*) preparation, it was applied in the soil at two different doses i.e. @ 2gm/Kg and 10gm/Kg in the soil. *R. solani* was applied @ 6 gm/Kg in the soil. The antagonist was evaluated by adopting pre inoculation, post inoculation and simultaneous inoculation method. The antagonist was

evaluated by adopting pre-inoculation (antagonist applied one week before inoculation), post inoculation (pots were inoculated with the pathogen and allowed to establish for a week and then antagonist applied) and simultaneous application (Pathogen and antagonist applied in the soil simultaneously) method. The experiment was completed in fresh unsterilized garden soil. For each treatment 10 pots (each of 10cm in diameter) were maintained. The percent mortality was recorded after 30 days of treatment. The experiment was performed in Thrice and the results were computed on that basis.

3.16 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 and Statistical Packages for the Social Sciences (SPSS), version 11.0, SPSS Inc.