Studies on tea seed mycoflora and resistance of young tea plants against *Rhizoctonia solani,* a soil borne root pathogen of germinating tea seedlings

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Abbreviations

PDA	Potato Dextrose Agar	RBA	Rose Bengal Agar
CDA	Czapek Dox Agar	Rh Tr	*Rhizobium* and *Trichoderma* mixture
Az Tr	*Azotobacter* and *Trichoderma*	Tr	*Trichoderma*
Ben	Benomyl	Con	Control
Rh	*Rhizobium*	Az	*Azotobacter*
Rh Az	*Rhizobium* and *Azotobacter*	H	Hybrid
LC	Local Check	MEA	Malt Extract Agar
WA	Water Agar	2,4-D	2,4-Dichloro phenoxy acetic acid
TS	Tocklai seed Variety	AG	Anastomosis Group
DNA	Deoxy ribonucleic acid	RNA	Ribonucleic acid
CIE	Crossed Immuno Electrophoresis	ELISA	Enzyme Linked Immunosorbent Assay
Pf	*Phytophthora fragariae*	Pf M	Antiserum of *Phytophthora fragariae*
CRA	Cross Reactive Antigens	KDa	Kilodalton
TV	Tocklai Variety	FITC	Fluorescein Isothiocyanate
IgG	Immunoglobulin G	FO	Fusarium Oxysporum
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay	Anti-Bc IgG	Anti- Botrytis cinerea Immunoglobulin G

BC	Bipolaris carbonum	PAbs	Polyclonal antibodies
PTA-ELISA	Plate Trap Antigen Enzyme Linked Immunosorbent Assay	MLO	Mycoplasma Like Organism
ID-ELISA	Indirect Enzyme Linked Immunosorbent Assay	RBSDV	Rice Black-Streaked Dwarf Virus
PVY	*Potato Virus* Y	PVA	Potato Virus A
PVX	*Potato Virus* X	PVM	Potato Virus M
PVS	*Potato Virus* S	ECM	Extra Cellular Matrix
PNL	*Pinus nigra* ARN lectin	TEM	Transmission Electron Microscopy
PGPR	Plant Growth Promoting Rhizobacteria	HCN	Hydrogen Cyanide
ISR	Induced Systemic Resistance	TV2	Trichoderma viride isolate-2
ITCC	Indian Type Culture Collection	Pf	Pseudomonas fluroscence
MALDI TOF	Matrix-Assisted Laser Desorption/ Ionization Time of Flight	BCA	Bio Control Agent
TLC	Thin Layer Chromatography	ATCC	American Type Culture Collection
$CHCl_3$	Chloroform	CH_3OH	Methanol
RF	Retention Factor	MIC	Minimum Inhibitory Concentration
NCIM	National Collection of Industrial Microorganisms	Foc	*Fusarium oxysporum* f. sp *chrysanthemi*
MA	*Mentha arvensis*	TP	*Tagetus patula*
ES	*Eucalyptus* sp.	DS	*Datura stramonium*
CP	Calotropis procera	LS	*Lantana* sp.

RC	Ricinus communis	CR	*Catharanthus roseus*
PP	Partially Purified	CP	Completely Purified
SBM	Standard Blotter Method	APM	Agar Plate Method
ISTA	International Seed Testing Association	IARI	Indian Agriculture Research Institute
PDB	Potato Dextrose Broth	OMA	Oat Meal Agar
REA	Root Extract Agar	MEA	Malt Extract Agar
PCA	Potato Carrot Agar	RM	Richard's Medium
R.A	Richard's Agar	YEMA	Yeast Extract Mannitol Agar
NA	Nutrient Agar	lb	Pound
p.s.i.	Per Square Inch	g	Gram
mm	Milimeter	°C	Degree Centigrade
ml	Mililitre	Kg	Kilogram
cm	Centimeter	PVP	Polyvinyl Pyrrollidone
M	Molar	BSA	Bovine Serum Albumin
mAmp	Miliampere	V	Volt
h	Hour	PBS	Phosphate Buffer Saline
µl	Micro litre	TMB	Tetramethylbenzidine
N	Normal	nm	Nano meter
h	Hour	µm	Micro meter
SPSS	Statistical Packages for the Social Sciences	Scl	Sclerotia
d	Day	OMB	Oat Meal Broth

mg	Miligram	Wt	Weight
Mwt	Mycelial Dry Weight	RsA	*Rhizoctonia solani* Antisera
Rsa	*Rhizoctonia solani* antigen	520a	520 root antigen (520- Tocklai seed variety)
449a	449 root antigen	449A	Antisera of root variety 449
520A	Antisera of root variety 520	OD	Optical Density
µg	Micro gram	PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism	RAPD	Random Amplified Polymorphic DNA
ITS	Internal Transcribe Spacer	APMC	Agriculture Produce Market Committee
MYA	Malt Yeast Agar	CZA	Czapkl Dox Agar

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1. Introduction

Tea, the most important and popular non-alcoholic beverage, is obtained from the leaves and leaf buds of the plant *Camellia sinensis* (L.)O. Kuntze and belonging to the family Theaceae. Chinese first used tea as medicinal drink as well as beverage Eden (1958) reported that. They also reported that Chinese had been using tea for the past 3000 years. Scaly (1958) included 82 species under the genus but Mondal *et al.* (2002) mentioned about 325 species. However (i) *Camellia sinensis* (L.) O.Kuntz. (ii) *Camellia assamica* sub spp. *Lasiocalyx* and to an extent *Camellia irrawadiensis* have mainly made the genetic pool of present day tea. So the term "tea" indicates the progenies of these taxa and the hybrids there of or between them (Islam *et al.*, 2005). Although tea originated in South and Southeast Asia, today it is cultivated across the world in tropical and sub tropical regions (Wight, 1959). Tea plants are grown either from seeds or by vegetative means *i.e.* from vegetative clones. Clonal plants are genetically alike, and, therefore, have less adaptive characteristics for different agro-climatic environment and soil conditions. The Clonal plants show susceptibility like their parents to diseases/pests and become vulnerable due to their genetic homogeneity. On the other hand as the progenies of seed plants are genetically different, they do not suffer from the same problems as that of the parental plants (Barbora *et al.*, 1996). Seed planting is comparatively less labour intensive than raising clonal cuttings (Rawat, 1980).

Tea seeds are indispensible in tea cultivation as well as for other industrial purposes. Seeds of the genus *Camellia* produce edible oil in West Bengal, Himachal Pradesh, Assam and in the Northern region of Indo-China (Owuor *et al.*, 1985; Sengupta *et al.*, 1976; Facciola, 1990; Duke, 1983). Five saponins including "Theasaponin" have been detected in tea seeds (Yoshioka *et al.*, 1970; Singh *et al.*, 1992). Tea seed cakes are used as fertilizer, crude drug, for curing skin diseases in Thailand and also as fodder (Roberts and

Desilva, 1972; Sekine *et al.*, 1991). Seeds like any other parts of plants are also vulnerable to several pathogen attacks. It is necessary to control the seed borne pathogens to get healthy tea seeds which in turn produce healthy tea seedlings. Substantial works has been done for control of the pathogens of tea plants but relatively less work has been done on the seed borne diseases and their control. Many fungal pathogens, like *Fusarium solani*, *Nigrospora* sp., *Aspergillus niger*, *Pestalozzia theae*, *Penicillium* sp. and *Verticillium* sp. have been reported to cause serious diseases of tea seeds and/or tea seedlings (Barthakur *et al.*, 1998; Sarmah and Bezbaruah, 1988). Phukan (1967) and Barua (1983) reported that *Rhizoctonia bataticola* and *Rhizoctonia* sp. cause secondary root and leaf diseases of tea respectively. In the present study, while screening the seed borne pathogens *Rhizoctonia solani* Kuhn [Teleomorph: *Thanatephorus cucumeris* (Frank) Donk] was found to be one of the major pathogen of tea seedlings (Mandal *et al.*, 2006). *Rhizoctonia solani* also cause diseases in shed trees (*Albizzia chinensis*) in tea gardens (Barua and Dutta, 1986).

Rhizoctonia solani is cosmopolitan with a very wide host range including crop plants and weeds (Adams, 1988; Ou, 1985). *Rhizoctonia solani* has an ability to survive in soil and it affects the cropping system as a whole rather than an individual crop. Therefore it is necessary to collect all the information on the behavior of this pathogen to plan management strategies in a cropping or planting system (Biswas and Samajpati, 2007).

Rhizoctonia solani is a species complex and have been divided into 14 anastomosis groups (Ags). Many Ags have been sub-divided into intra specific groups depending on cultural, virulence, molecular, biochemical, immunological and other characteristics (Ogoshi, 1975, 1987; Carling *et al.*, 1994; Carling, 1996; Carling *et al.*, 1999 a, b; Singh *et al.* 2002; Biswas and Samajpati, 2007). According to Carling (1995) it has become a routine work to characterize the strains of this fungus through anastomosis grouping

systems. Some scientists have also characterized the several isolates of this fungus and confirmed its taxonomic position through PCR-RAPDs, mitochondrial DNA RFLPs and ITS sequencing (Welsh *et al.*, 1990; Williams *et al.*, 1990; Duncan *et al.*, 1993; Banniza *et al.*, 1996; Yang *et al.*, 1996; Bounou *et al.*, 1999; Toda *et al.*, 1998; Leclerc *et al.*, 1999; Pascual *et al.*, 2000; White *et al.*, 1991; Carling *et al.*, 2002; Singh *et al.*, 2002; Cardinale *et al.*, 2006).

Resistance screening of plant varieties against any fungal pathogen is a prerequisite for management of any disease of plants. Resistant and susceptible plant cultivars can be detected by the levels of common antigens present in both host and pathogen (Charudattan and Devay, 1972; Alba *et al.*, 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994; Ghosh and Purkayastha, 2003; Dasgupta *et al.*, 2005; Saha *et al.*, 2010). Common antigenicity can be detected by performing immunodiffusion, immuno electrophoresis and indirect ELISA between susceptible varieties and pathogen (Alba and Devay, 1985; Mohan, 1988; Chakraborty, *et al.*, 1995; Croft, 2002; Dasgupta *et al.*, 2005; Saha *et al.*, 2010).

In the recent years, interest is growing successively in the field of biological control of pests and diseases replacing the chemical control techniques. The actual reasons behind this fact are chemicals cause health hazards and sometimes perform non selective action against harmful and beneficial microorganisms in the rhizosphere. More over pathogens and pests develop resistance against these chemicals. Most importantly, bio-diversity should be maintained through minimizing the use of toxic plant protectants (Dennis and Webster, 1971; Papavizas and Lumsden, 1980; Papavizas, 1985; Mukhopadhya and Chandra, 1986; Barthakur, 1999; Barthakur *et al.*, 2002). Among the bio-agents *Trichoderma viride, Gliocladium* sp., *Bacillius subtilis* and *Pseudomonus* sp. have been reported as effective biocontrol agents against some root and stem diseases of tea and other plants (Dennis and Webster,1971a,b,c; Papavizas,1985; Barthakur and Dutta 1992;

Barthakur,1994,1999 ; Barthakur *et al.*, 1993,2002; Chandramouli, 1993). On the other hand it has been experimentally proved that various diseases of plants caused by *Rhizoctonia solani* isolates are effectively checked by the bio-agents *Trichoderma harzianum, T. virens, T. longibrachiatum, T. aureoviride, Bacillus subtilis* and *Pseudomonas fluorescens* through the production of volatile and non-volatile antibiotics and by plant extracts like *Xanthium strumarium, Blumea* sp., *Parthenium hysterophorus, Mentha piperita, Cymbopogon citrates* and *Cyperus scariosus* etc. (Banker and Mathur, 2001; Upmanyu *et al.*, 2002; Saikia and Gandhi, 2002; Meena *et al.*, 2003; Sharma and Gupta, 2003; Gautam *et al.*, 2003; Dhaliwal *et al.*, 2003).

On the basis of the above reports it has been observed that there is a necessity to control the seed borne pathogens of tea seeds of North East India (Plate I). Among the seed borne pathogens *Rhizoctonia solani* was found to cause severe damage to the seedlings, resulting to mortality of seedlings even up to 30 percent in susceptible varieties. The present study proposes the following objectives to be done to control seedling diseases of tea.

OBJECTIVES

1. Screening of tea seeds of different varieties for isolation of seed-borne pathogens.
2. Identification of major pathogen (*Rhizoctonia solani*) following Koch's postulates.
3. Pathogenicity of the fungus against seedlings of different tea varieties.
4. Studies on physiological characteristics of the pathogenic fungus.
5. Studies on the resistance of tea against *Rhizoctonia solani* following serological techniques.
6. Control of the pathogen (*Rhizoctonia solani*) by antagonistic microorganisms and botanicals.

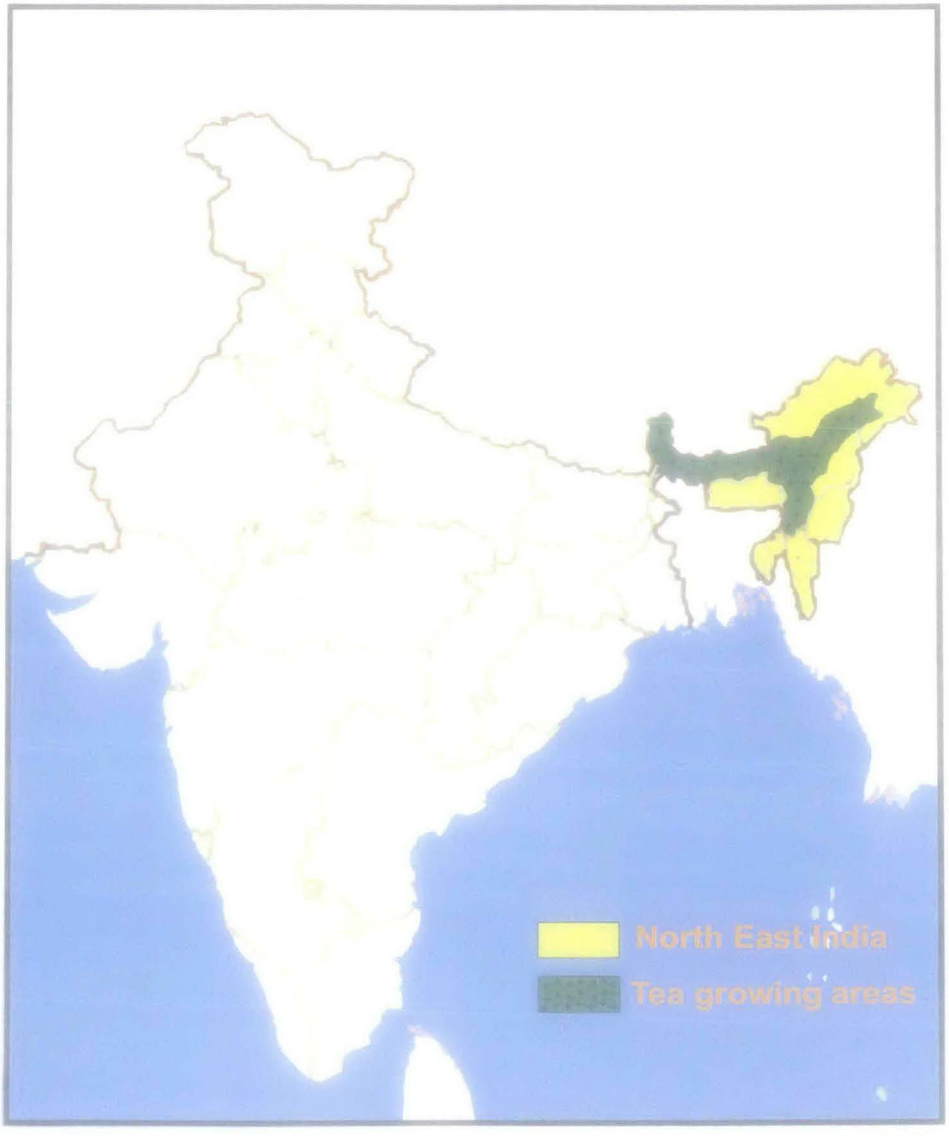

Plate I: Tea growing areas of North-East India and adjoining areas

2. Literature Review

Tea is an important commodity in Indian economy. It is one of the sources of revenue of India. Like many other cultivated crops tea plant is also attacked by many fungal pathogens and pests. 190 fungal pathogens and 125 pests were recorded to cause diseases of tea plants in North-East India. Losses have been estimated at 67,000,000 pounds (30,000,000 kilograms) from these pests and fungal diseases. Therefore it is an important task to control these diseases effectively. Tea plant diseases can be categorized as leaf diseases, stem diseases and root diseases. In the present study, attentions have been paid to control one of the serious root (collar rot) diseases of tea seedlings that are caused by the fungus *Rhizoctonia solani* Kukn. [Teleomorph: *Thanatephorus cucumeris* (Frank) Donk] (Eden, 1978).

Root rot diseases generally occur due to the lack of proper drainage system. The root rot causing fungus can be spread by the flow of water or by the movement of the soil. Tea seeds may be attacked by the pathogens when it remains attached to the plant or when it falls on the ground from the tree. Soil borne pathogens enter into the tea seeds after its fall from the tea plants. It is important to know about the physiology, morphology and growth patterns of a causal organism of a disease and also the host parasite interaction of the disease. Biological control methods of the plant diseases by antagonistic microorganisms and botanicals are now-a-days, increasingly popular and well accepted methods to eradicate any diseases of cultivated crop plants as they are environment friendly.

At the onset of the present study, it was considered to review the works of the previous workers in the following paragraphs. For the convenience, the observations have been divided into several sub groups, which are as follows:-

- Seed mycoflora and seed diseases of tea plants.
- Root diseases of tea plants.

- Diseases caused by *Rhizoctonia Solani.*
- Characteristics of *Rhizoctonia solani* as a pathogen.
- Studies on growth and physiology of the pathogens.
- Antigenic relationship in host and pathogen.
- Disease control by antagonistic organisms.
- Disease control by botanicals.

Seed mycoflora and seed diseases of tea plants

Rothe and Wadekar (2011) studied the seed born mycoflora of *Aegle mamelos* (L.) Corr., *Basella rubra* L., *Limonia acidissima* L., *Nyctanthus arbortristis* L. and *Tectona grandis.* They recorded total sixteen fungi using blotter technique and agar plate method. Maximum number of mycoflora was recorded in *N. arbortristic* which are *Cladosporium, Phytophthora, Brachysporium, Fusarium, Aspergillus, Trichoderma* and *Physarum.* On the other hand only two fungi (*Erysiphe* and *Ustilago*) were reported from *B. rubra.*

Javaid *et al.*, (2010) reported the presence of four fungal species of *Aspergillus* from twelve varieties of shisam (*Dalbergia sisso* Roxb.) seeds from Lahore. Pods of ten seed varieties of S-4, R-1, R-2, US-1, US-2, US-5, US-6, US-7, US-9, US-10, US-11 and US-12 were collected and seeds were incubated on a moist sterilized filter paper bed in petriplates for seven days to isolate the mycoflora. The most occurring fungal species were *A. flavus, A. fumigates, A. japonicas* in comparison to *A. teerius.* They concluded that all the twelve shisham varities were equally susceptible to the fungal pathogens and did not show any germination up to 20 days.

Mamatha *et al.*, (2000) screened the seeds of *Dandrocalamus strictus, Phyllanthus emblica, Hardwickia binata* and *Dalbergia latifolia* for presence of mycoflora and found the presence of both field and storage fungi. They tested the dominant mycoflora on quality aspects like germination and vigour and significant decreases in germination and seedling vigour was shown by the dominant fungi. From their experiment they also concluded

that Trichoderma spp was most effective both in reducing the incidence of mycoflora and in enhancing the germination and vigour.

Utobo *et al.*, (2011) studied the association of seed borne fungi with eight hybrid (H) and three local check (LC) varieties and their effects on grain germination and seedling vigour during harvesting seasons. Among nine fungal genera *Trichoconis padwickii, Helminthosporium oryzae* and *Fusarium moniliforme* were found most abundant.

Singh *et al.*, (2011) isolated sixteen fungal species namely *Alternaria alternata, Alternaria solani, Aspergillus terreus, Curvularia lunata, Fusarium roseum, Fusarium semitechtum, Penicillium citrinum, Penicilium rubrum, Rhizopus stolonifer, Trichoderma harzianum,* Dark sterile mycelium and white sterile mycelium from two cultivars of wheat (Kundan and HUW-234) after treatment with potassium nitrate and examination with agar plate and blotter method.

Afzal *et al.*, identified thirteen phytopathogenic fungi which includes *Alternaria alternata* and *A. helianthi, Aspergillus flavus, A. fumigatus A. niger, Curvularia lunata, Drechslera tetramera, Fusarium solani and F. moniliforme, Macrophomina phaseolina, Mucor mucedo, Penicillium* and *Rhizopus* spp from seeds of seven cultivars of sunflower by using agar and blotter paper methods. The isolated fungi were responsible for reduced seed germination and seedling mortality. They also concluded that Topsin and Bayleton (systemic fungicide) were found to be significantly effective in the elimination of seed-borne fungi and antifungal activity could be achieved by extract of *Azadirachta indica* (neem) and *Allium sativum* (garlic) at the concentration of 0.015%.

Bokhary *et al.*, (1986) reported thirty-seven species of seed-borne fungi from five varieties of wheat namely *Triticum cinnamon* (wheat X rye), T. vulgare (Host. Mex-Mx Paw/ Ono-III-Conch), *T. vulgare* (Host. 78/2), *T. vulgare* (Host. Var. Jori), *T. vulgare* (Host. Super X) using standard blotter and agar plate (PDA, PDA plus yeast, MEA and CZA) methods.

Fakhrunnisa *et al.*, (2006) studied seed-borne mycoflora of 19 samples of wheat, 27 samples of sorghum and 14 samples of barley using standard blotter and deep freezing method. Fungi most frequently isolated and identified by them were *Absidia* sp., *Alternaria alternata, Aspergillus* sp., *A. candidus, A. flavus, A. niger,A.sulphureus, Cephalosprium* sp., *Chaetomium globosum, Cladosporium herbarum, Curvularia lunata, Drechslera dematioidea, D. halodes, D. hawaiiensis, D. tetramera, Fusarium moniliforme, F. oxysporum, F. pallidoroseum, F. subglutinans, Nigrospora oryzae, Penicillium* spp., *Piptocephalis* sp., *Rhizoctonia solani, Rhizopus* sp., *Stemphylium* sp., *Syncephalastrum racemosum, Trichoderma hamatum, Trichothecium roseum* and *Ulocladium* sp. They also reported that deep freezing method was better for isolation of *Alternaria alternata, Cladosporium herbarum, Drechslera* spp., and *Fusarium* spp.

Nagarja *et al.*, (2009) analyzed 185 seed samples of castor from agro climatic regions of Karnataka during *kharif*. They collected seeds from fields (49), farmers (73), retail shops (16) and APMC markets (47) and isolated forty seven fungal species belonging to seven genera by using standard blotter method and samples were tested by potato dextrose agar (PDA), water agar (WA) and 2,4-Dichloro phenoxy acetic acid (2,4-D) methods. The fifteen predominant fungal species they isolated from the castor seeds were *Fusarium oxysporum* f. sp. *ricini, Alternaria ricini, A. alternata, Curvularia lunata, Macrophomina phaseolina, Sclerotinia sclerotiorum, Cladosporium herbarum, Chaetomium globosum, Botryodiplodia acerina, Stachybotrys chartarum, Aspergillus ochraceous, A. niger, A. flavus, A. versicolor* and *Rhizopus stolonifer.*

Butt *et al.*, (2011) isolated four fungal species namely *Fusarium moniliforme, Alternaria sp., Helminthosporium sp.* and *Curvularia* sp. from stored grains of five varieties of rice (*Oryza sativa* L.). They tested four chemical fungicides namely antracal, topsin, mencozeb and derosal against the isolated fungi.

Mahamune and Kakde (2011) isolated thirteen seed-borne fungi from Waghya variety of French bean and nine fungi from Varun varity by using Glucose Nitrate Agar and Rose Bengal Agar method. According to them on

both varieties Macrophomina phaseolina showed its quantitative dominance which were followed by *Aspergillus niger* and *Fusarium oxyporum* and Waghya variety showed maximum incidence of fungi as compared to Varun variety. They also studied the antagonistic activity of Trichoderma harzianum against dominant fungi.

Patil *et al.*, (2012) screened seed mycoflora from untreated and treated seeds of pigeonpea and chick pea using agar plate method. Highest percentage of fungi was found to be *Aspergillus flavus* (30%) followed by *A. niger, Penicillium notatum, Cladosporium herbarum* in untreatedseeds.

Mali *et al.*, (2008) isolated *Alternaria alternata, Aspergillus flavus, A. niger, Botrytus sp., Chaetomium globosum, Cladosporium herbarum, Curvularia lunata, Fusarium oxyporum, F. moniliforme, F. roseum, Macrophomina phaseolina, Penicillium notatum, Phytophthora sp., Rhizoctonia solani, Rhizopus stolonifer* from green gram (*Phaseolus aureus* Roxb.) and black gram (*Phaseolus mungo* L.) by using agar plate method. The untreated seeds of wild variety showed highest percent incidence of seed mycoflora than treated seeds.

Mogle and Mane (2010) reported maximum eleven genera and twelve species of fungi from untreated tomato seeds collected from local market by using potato dextrose agar (PDA), rose bengal agar (RBA) and czapek dox agar (CDA) media. They reported that the untreated seeds were found to be associated with highest percent incidence of mycoflora and minimum population was recorded in the treatment of Rhizobium and Trichoderma (RhTr) mixture followed by Azotobacter and Trichoderma (AzTr), Trichoderma (Tr) alone, Benomyl (Ben), Rhizobium and Azotobacter and control (Con). According to them seed germination percentage was highest in the treatment of AzTr followed by RhTr, RhAz and Az and minimum in the treatment of Rh, Tr, Con and Ben. From their experiment they concluded that Trichoderma and Rhizobium was found to be beneficial to reduce the pathogenic fungi and increase of germination percentage.

Rathod *et al.*, (2012) reported the presence of seed mycoflora from different cultivars of legumes using standard blotter paper, agar plate and seed washed methods. Agar plate method was reported by them as suitable

method as in less incubation there was higher percent incidence of seed mycoflora and they isolated sixteen fungi.

Mittal (1983) isolated 26 fungal species belonging to 13 genera were isolated from seeds of *Cedrus deodara* Loud by using standard moist blotters, potato–dextrose–agar plates, and moist sterilized sand. The isolated fungi were *Aspergillus flavus, A. luchuensis, Epicoccum purpurascens, Fusarium moniliforme, Pénicillium canadense,* and*Rhizopus oryzae. Aspergillus flavus, Penicillium canadense* and *Rhizopus oryzae.* According to Mittal (1983) RH-2161 and Dithane M-45 was most effective fungicides against the isolated fungi.

. Prochazkova (1990) identified 50 species of fungi identified from batches of seeds of 9 native species of broadleaved forest trees (beech [*Fagus sylvatica*], ash [*Fraxinus excelsior*], sycamore [*Acer pseudoplatanus*], lime [*Tilia* sp.], rowan [*Sorbus aucuparia*], elm [*Ulmus* sp.], hornbeam [*Carpinus betulus*], alder [*Alnus* sp.] and birch [*Betula* spp.]) in Czechoslovakia.

Pande and Gupta (2011) reported *Fusarium solani, Aspergillus niger, Aspergillus nidulens, Penicillium* sp., *Trichoderma harzianum, Alternaria solani, Alternaria alternata, Curvolaria lunata, Stachybotryis chartarum, Acremonium* sp., *Rhizoctonia solani, Chaetomium globosum, Cladosporium cladoporoides* and *Torula allii* from the seeds of *Oroxylum indicum* (L.) Vent of Kumaun region of Central Himalaya in India.

Safai and Mehrotra (1982) studied seed mycoflora of forest trees seeds of *Quercus, Cupressus, Sapium, Pyrus, Melia, Casuarina,* and *Thuja.* They studied mycoflora in fallen seeds as well as in plucked seeds. They isolated nearly forty-two species of fungi which includes *Mucor, Rhizopus, Aspergillus, Penicillum, Epicoccum, Pithomyces, Cladosporium, Paecilomyces, Fusarium, Gliocladium, Trichothecium, Trichoderma, Cephalosporium, Alternaria, Ulocladium and Curvularia.* They reported that frequency of occurrence of moulds in the fallen-off seeds was generally more in comparison to that in the plucked seeds.

Barthakur *et al.* (1998) reported that some varieties of tea TS-491 and TS-520 and S_3A_1 were damaged by the attack of the fungus *Fusarium*

solani. He also described that the fungi produced prominent black spots (about 0.75 cm × 0.50 cm in size) on the bark of the immature fruits during July-August. The hard seed coat under the cracked fruit carp turned black and the premature dropping of seed took place. The imperfect stage of the fungus i.e. the white cottony mycelial growth of Fusarium Solani and the perfect stage i.e. orange perithecial structures of Nectria were produced on fruit carps when cracks were developed on fruit carps.In the later stage of infestation by the fungus, the seeds became pinkish due to presence of powdery spores of the fungi.

Root diseases of tea plant:

Any disease symptoms of plants occur due to the attack by the pathogens, such as fungi bacteria, viruses or nematodes and also by the nutrient deficiency. Tea plants are no exception. 300 species of fungi are reported to affect different parts of the tea plant. (Chen and Chen, 1990; Agnihothrudu, 1964) Besides foliar diseases of tea, root rot is the most harmful and damaging disease to a *Camellia*. Even the healthiest plant of *Camellia* can wilt and die in just a few days when affected with root rot (Shinholster, 2009).

Chandra Mouli (1996) reported several root rot diseases of tea caused by the fungi, bacteria, and nematodes. The names of the root rot diseases and their causal organisms have been given in the following table.

Chandra Mouli (1996) reported that *Rhizoctonia solani* Kuhn causes collar rot disease of tea. Santyanarayana (1973) and Barthakur (1999) reported that *Ustulina zonata* (causal agent of charcoal stump rot), *Fomes lamaoensis* (causal agent of brown rot), *Rosellinia arcuata* and *Armillaria mella* cause serious root rot diseases of Assam (north-east India). Barthakur (1999) also reported the involvement of the pathogen *Poria hypolateritia* in causing red root rot disease of tea in Assam.

Barthakur (1999) also reported that root disease causing fungi were not host specific. They dissaminate either by direct root contact from

diseased woody remains in the soil after uprooting the diseased bushes or by the soil borne spores.

Table 2.1: Root rots of tea and their causal organisms

Name of the Disease	Causal Organism
Armillaria root rot	*Armillaria mellea* (Vahl:Fr.) Kummer.
Black root rot	*Rosellinia arcuata* petch & *Rosellinia bunodes* (Berk. & Broome) sacc.
Botryodiplodia root rot	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.
Poria root rot	*Poria hypobrunnea* Petch
Purple root rot	*Helicobasidium compactum* (Boedijn) Boedijn
Red Root rot	*Ganoderma Philippii* (Bresad & P. Henn) Bresad *Poria hypolateritia* (Berk.) Cooke
Root rot	*Cylindrocarpon tenue* Bugnicourt *Cylindrocladiella camelliae*(Venkataramani &Venkata ram)Boesewinkel *Fomes Lamaoensis* (Murr.) Sacc. & Trott. *Ganoderma applanatum* (Pers.) Pat
Tarry root rot	*Hypoxylon asarcodes* (Theiss.) Mill
Violet root rot	*Sphaerostibe repens* Berk & Broome
White root rot	*Rigidoporus microporus* (Sw.) Overeem
Xylaria root rot	*Xylaria* sp.
Pale brown root rot	*Pseudophacolus baudonii* (Pat.) Ryu
Root-Knot nematode	*Meloidegyne* sp.
Root lesion nematode	*Pratylenchus* sp.

Armillaria root rot caused by the fungus *Armillaria heimii* and *Armillaria mellea* were also reported to cause serious tea root rot diseases in African countries like Tanzania and Kenya etc. (Onsando *et al.*, 1997; Ndunguru, 2006). Kile *et al.* (1991) reported that *Armillaria* spreads primarily through the formation of rhizomorphs or by mycelial growth directly from the diseased roots to healthy one. Tea plants of all ages are susceptible to the disease caused by *Armillaria.* Charcoal stump rot caused by the fungus *Ustulina deusta* (Fr.) Petrak was also reported to cause serious root diseases in Africa (Muraleedharan and Baby, 2007).

Four most common tea root diseases have been reported by Muraleedharan and Baby from Srilanka (Muraleedharan *et al.*, 1997; Baby *et al.*, 2004). The diseases are red root rot (caused by the fungus *Poria hypolateritia*), black root rot (caused by the fungus *Rosellinia* sp.), brown rot (caused by the fungus *Ustulina zonata*), and chorcoal stump rot (caused by the fungus *Ustulina zonata*). *Fomes applanatus, Polyporus mesotalpae, Polyporus interruptus* and *Irpsubvinosus* were also reported to attack tea roots in Srilanka (Petch, 1923).

In China, most important root rot diseases of tea, caused by the fungus were reported as red root rot (caused by the fungus *Poria hypolateritia* Berk), brown root rot (caused by the fungus *Phellinus noxius* (Corner) G. H. Cunningam (*Fomes noxius*) and charcoal stump rot (caused by the fungus *Ustulina zonata* (lev) sacc)(Premkumar *et al.*, 2006).

Damping off caused by the fungus *Hypochnus centrifugus* (Lev.) Tul was found to cause diseases in tea nurseries in South-Eastern China. Crown gall caused by *Agrobacterium tumefaciens* Smith was also found on tea cuttings in China (Chen and Chen, 1982).

In Taiwan the serious soil borne diseases of tea shrubs are white root rot caused by the fungus *Rosellinia nectatrix* (Sun *et al.*, 2007) and brown root rot caused by the fungus *Phellinus noxius* (Ann *et al.*, 2002).

Gabner *et al.* (2004) reported the white root rot disease of Sabah tea caused by the fungus *Poria hypolateritia* in Malaysia. *Poria hypolateritia* is a soil borne pathogen. In highland areas of Malaya the root of tea plants are affected by the fungus *Ganoderma Pseudoferreum* and in low land areas the root of the plants are attacked by the *Poria hypolateritia* like fungus. Both of the fungus causes the red root rot disease of the plantation. The common symptom of this disease is the – production of red rhizomorphs on the outside of the roots.

Charcoal stump rot caused by the fungus *Ustulima deusta* (Fr.) Petrak, Red root diseased caused by the fungus *Poria hypolateritia* and

Armillaria root rot caused by the fungus *Armillaria mellea* were also reported from Java of Indonesia (Muraleedharan and Baby, 2007).

The major tea root disease reported from Japan is white root rot caused by the fungus *Rosellinia necatrix* (Hartig) Berl. (Ezuka *et al.*, 1973).

Disease caused by *Rhizoctonia solani*:

Rhizoctonia solani is a soil borne Basidiomycetes fungus and it occours worldwide (Lehtonen, 2009). According to Agrios (1997) soil borne pathogenic fungi can cause disease on roots and other underground plant parts i.e. stolons, tubers and basal parts of the stems. Though *Rhizoctonia solani* is a soil borne pathogen it can attack stem and leaf of plants (Sneh *et al.*, 1991). Soil borne plant diseases increase during relatively cool and wet weather, while air borne pathogens spread better during dry conditions (Lehton, 2009). *Rhizoctonia solani* causes significant damage on crop quantity and quality of many crop species annually. (Weinhold *et al.*, 1982; Ban ville, 1989; Martin and Loper, 1999; Green and Jenson, 2000; Botton *et al.*, 2006; Wagacha and Muthomi, 2007).

Sharma and Tripathi (2001) reported that thirty three plant species would be attacked by *Rhizoctonia solani* experimentally. The plants were of families like Leguminosae (11 plants), Graminae (5 plants), Solanaceae (11 plants), Brassicaceae (5 plants), Malvaceae (3 plants), Cyperaceae (2 plants) and one each of Cucurbitaceae, Commelinaceae and Chenopodiaceae.

Host range of *Rhizoctonia solani* is very broad and several diseases have been reported by several scientists till date. A list of plants with disease symptoms along with the references has been tabulated in the following table.

Characteristics of *Rhizoctonia solani* as a pathogen

Rhizoctonia Solani is not an obligate parasite and it can stay in the soil as saprophyte for long period. Due to the lack of conidia and the scarcity of sexual spores *Rhizoctonia solani* remain as vegetative hyphae or

in sclerotial form. *Sclerotium* is an encapsulated, tightly compact hyphal clump that gives the fungus protection from environmental stress.

Table 2.2: Diseases caused by *Rhizoctonia solani* in different hosts as reported by different authors

Host name	Disease symptoms	Reference
Buck wheat	Damping off	Herr and Fulton, 1995
Barley	Root rot	Rush *et al.*, 1994
Broad bean	Reduced growth	Valkonen *et al.*, 1993
Cotton	Root rot Root canker Minor pathogen	Rothrock, 1996 Baird and Carling, 1997 Carling *et al.*, 2002 a
Corn	Leaf blight Root rot	Tomaso-Peterson and Trevathan, 2007;Mazzola *et al.*, 1996
Common bean	Leaf blight, Web blight & Root rot Reduced growth	Muyolo *et al.*, 1993 Valkonen *et al.*,1993
Cabbage	Bottom rot	Tu *et al.*, 1996
Carrot	Damping off	Grisham and Anderson, 1983
Clover	Damping off & Root rot	Wong and Sivasithamparam, 1985
Cereals	Bare patch	Mazzola *et al.*,1996
Egg plant	Brown spot	Kodama *et al.*, 1982
Flower bulbs	Root rot	Dijst and Scneider, 1996
Lettuce	Damping off	Herr, 1993
Lupin	Late emergence Minor pathogen	Valkonen *et al.*, 1993 Mac Nish *et al.*, 1995
Mycorrhiza	Mycorrhizal	Carling *et al.*, 1999
Onion	Damping off	Erper *et al.*, 2005
Oil seed rape	Damping off	Kataria *et al.*, 1991a
	Basal rot	Verma, 1996
Potato	Stem canker Stem canker & Black scurf Stem canker Minor pathogen	Chand and Logan, 1983 Bandy *et al.*, 1988 Anguiz and Martin, 1989 Carling *et al.*,1994
Pea	Stem rot & Root rot	Hwang *et al.*,2007
Rice	Sheat blight Web blight	Sayler and Yang, 2007 Hashiba and Kobayashi, 1996
Raddish	Root rot	Grisham and Anderson, 1983

Contd....

Table 2.2: (Contd.) Diseases caused by *Rhizoctonia solani* in different hosts as reported by different authors

Host name	Disease symptoms	Reference
Soybean	Bud rot Damping off Root rot Rot	Hwang *et al.*,1996 Nelson *et al.*,1996 Liu and Sinclair, 1991. Yang *et al.*,1990
Sugar beet	Root rot, Damping off & Leaf blight	Herr, 1996
Turf grass	Brown patch Large patch	Herr and Fulton, 1995 Burpee and Martin, 1996
Tree seedlings	Damping off Root rot	Hietala and Sen, 1996 Hietala and Sen, 1996
Tobacco	Target spot	Ogoshi, 1987
Tomato	Leaf blight Fruit rot	Date *et al.*,1984 Strashnov *et al.*,1985
Wheat	Root rot	Rush *et al.*, 1994

The fungus *R. solani* generally spread through selerotia, plant materials contaminated with this fungus, soil spread by wind, water or during agricultural activities such as tillage and seed transportation. Keijer(1996) reported that during initiation of infection the hyphae from the germinating selerotium starts to grow towards a suitable host by the signaling of chemical exudates, e.g. organic acids, sugars, amino acids and phenols from the plants. The unattached hypha which do not attach to the plant after the first contact, starts to grow over the plant and within a short time the hypha flattens and initiation of directional growth over the epidermal cells takes place. The T-shaped hyphal branches produce thick infection cushions which strongly attach to the host epidermis. After the

formation of infection cushions the actual active penetration of the host take place, (Keijer, 1996).

According to Lehtonen (2009) the fungus identifies the appropriate host by its surface structure. Weinhold and Sinclair (1996) reported that the fungus can take entry into the plant through a weak spot on the surface by breaking down the protecting layer.

Demirci and Doken (1998) reported that during infection the swollen hyphal tips on infection cushions produce infection pegs which penetrate the cuticle, epidermal cell wall of epidermal tissues and the outer layer of the cortex. Though the penetration occurs through the degrading enzymes such as cutinases (Baker and Bateman, 1978), Pectinases (Bertagnolli *et al.*, 1996; Jayasinghe *et al.*, 2004) and xylanases (Peltonen, 1995), it also occurs through the establishment of hydrostatic pressure. According to Demirci and Doken (1998) also reported that when the fungi grow inside the host, it spreads inter and itracellularly by degrading the tissues. As a result necrotic lesions form on the epidermal tissue of the shoots, roots and stolons. Damping off of the young seedlings was also found to take place.

Studies on growth and physiology of the Pathogen:

The genus *Rhizoctonia* is a species complex of highly heterogeneous group of filamentous fungi. These fungi have similarities in their sterile as well as anamorphic state. The fungus do not produce any sexual spores i.e. conidia. Sexual spores i.e. basidiospores occur only rarely. The asexual stages of these fungi are known as anamorphic state and the sexual stages of this fungus are known as teleomorphic state. Therefore the genus *Rhizoctonia* includes a heterogeneous group of fungi which differ in their anamorphic, teleomorphic stage and also in morphology (Vilgalys and Cubeta, 1994).

Multinucleate *Rhizoctonia solani* isolates were grouped into fourteen anastomosis group (AG). [AG-1 to AG-10 including AGBI (Sneh *et al.*,

1991), AG-II (Carling *et al.,* 1994), AG-12(Carling *et al.,*1999) and AG-13(Carling *et al.,*2002a)] based on their anastomosis behavior. Multinucleate species of *Rhizoctonia* include *R. solani, R. oryzeae* and R. zeae and R. Oryzeae isolates are classified into one AG group each, WAG-2 and WAG-O respectively. (Sneh *et al.* 1991; Carling *et al.* 1994; 1999; 2002a). Binucleate *Rhizoctonia* isolates were grouped into different AGs by various authors. Sneh *et al.* (1991) grouped binucleate *Rhizoctonia* isolates into AG-A to S on the basis of hyphal pairings. Lipps and Herr (1982) decided seven ceratobasidium anastomosis groups (CAG -1, -2, -3, -4, -5, -6, and -7). Nineteen AGs of binucleate *Rhizoctonia* have been reported by various Japanese authors (Ogoshi *et al.,* 1979), including AG -A, -B, -Ba, -Bb, -C, -D, -E, -F, -G, -H, -I, -J, -K, -L, -M, -N, -O, -P and -Q. (Carling and Summer, 1992).

Rhizoctonia solani produces thread like hyphae. Colour of the hyphae is white to brown. Immature hyphae are white in colour, as the hyphae mature it turns into brown or dark brown in colour. Dolipore septum is present within the cross wall of the hyphae. Each cell is multinucleate, though binucleate Rhizoctonia is also present. Branches produce from the main hypha at right angles. Asexual spores are not formed by the mycelium. Small, oval cells are produced in branched chains or clusters. These cells are called monilioid cells. Monilioid cells have slightly thicker walls than the mycelium. When there monilioid cells are aggregate in large amounts, they form a resting structure called sclerotia. Sclerotia are black to brown in colour and 3 to 5 mm long. Main runner hyphae are usually wider than 7 µm. Very often sexual spores i.e. basidiospores are formed after invading the host cells. Basidia are formed when the environmental condition remains moist. Sufficient growth of the fungus also occurred in moist condition. Four spores are produced on each basidium. Each basidiospore has a single nucleus. The basidiospores are not enclosed in a fleshy, fruiting body or mushroom (Uchida, 2011).

Ritchie *et al.* (2009) observed that the mycelium of *Rhizoctonia solani* isolates from potato (AGs-2-1 and AG-3) grew best between 20°C and 25°C

on all media (Potato dextrose agar, Malt yeast extract agar, water agar, soil extract agar) tested. Mycelial growth of all isolates occurred between pH4 – pH9 with an optimum pH 5.6.In AG-3 isolates sclerotia formation occurred between pH4 – pH8 and in case of AG2-1 isolates the sclerotia formed between pH5 - pH6. Sclerotia germination took place in between 20-30°C and pH 5-6 in case of AG3 on all media tested. AG 2-1 isolates grew significantly slower compared to AG-3 in soil. Mycelia grew best in soil between 20-25°C regardless of anastomosis group. Germination of sclerotia of AG3 isolates in soil took place between 10°C and 30°C. Among all the artificial media tested greatest sclerotium yields were obtained on MYA for aG3 and PDA for AG 2-1.

Gottlie (1971) observed that mycelial growth of all the fungi was not indefinite. He indicated that an age dependent, growth regulating mechanism exist in at least some fungi and is responsible for restricted growth. According to him the growth rate of *R. solani* increased to maximum at 4-5 days then decreased until growth ceased at 8-9 days. The respiration of the peripheral hyphae of mycelia that have ceased growth is greater than that of the older parts of the thallus.

According to Kliejunas and Ko (1975) *Rhizoctonia solani* grew continuously at a steady growth rate on agar media but its' growth ceased on liquid media after a certain time. Like agar media the growth of *Rhizoctonia solani* also occurred continuously in autoclaved soil media.

Elarosi (1957) stated that the optimum pH value for *Rhizoctonia solani* growth is approximately 5.9. He also observed that the growth of *R. solani* is poor in pectin media when used as a sole source of carbon. But in pectin-agar medium the growth rate of *R. solani* increased.

According to Kumar *et al.* (1999) AG-II and AG-8 of *R. solani* causes bare patch of grain crops including lupin. AG-II grew faster than AG-8 on Potato Dextrose Agar media at several temperatures (10, 15, 20, 25 or 30°C). AG-II also grew best within pH range of 4 to 7. Growth of AG-8 was

best at PH-7. At 10°C there was no difference in the linear growth of both the AGs in soil but AG-II grew at a significantly faster rate at 20°C.

Gottlieb and Etten (1966) observed that the various contents of the mycelium of *R. solani* i.e. soluble amino nitrogen, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), ergosterol and protein are decreased with the age of the fungi. Total lipids and fatty acids remained constant in *R. solani.* Total carbohydrate increased with age in *R. solani. R.solani* contained myristic, Palmitic, Palmitoleic, stearic, oleic, linoleic and Pentadecanoic acids. In *R. solani* the percentage of linoleic acid per total fatty acids decreased slightly when oleic acid increased.

Harikrishnan and Yang (2001) examined the sclerotial production of three R. solani isolates (AG -1, Ag -2 -2 and AG – 4) in the presence of the three soyabean (*Glycine max*) herbicides (Glyphosphate, imazerhapyr and pendimethalin). They observed that the growth of all the 3 isolates of *R. solani* was significantly reduced in pendimethalin where as in imazerhapyr and Glyphosphate the growth reduction was not significant. AG-1 and AG-2-2 produced sclerotia both invitro and in vivo where as AG-4 isolate did not produce sclerotia in vitro. In the presence of herbicide AG-1 showed a decrease sclerotial production and AG-2 -2 showed an increased sclerotial production in vitro. AG-1 and AG-2-2 isolated showed reduction in sclerotial production in vivo compared to AG-4 isolate. AG-4 isolate showed an increase in sclerotial production in the presence of herbicide. Production of sclerotia was generally higher in vivo than in vitro.

Tiwari and Khare (2002) reported that *Rhizoctonia solani* causing diseases in different plant parts of mungbean produces both imperfect (Hyphae, sclerotia) and perfect (basidiospore) stage in soil. In their experiment hyphal stage was successfully produced at 25°C in solid Richard's medium and sclerotia was produced at 30-35°C in Czapek's liquid medium. The perfect stage i.e. basidiospores was produced by soil method at 26 to 29°C and 95-100 % relative humidity in dark.

Webb *et al.* (2011) worked with the long term preservation of *R. solani* isolates and from their experimental result they reported that cryogenic methods (storage in liquid nitrogen) are suitable for the preservation or storage of *R. solani* cultures. They also reported that efficiency may vary in different isolates.

Antigenic relationship in host and pathogen

Plant disease can be checked effectively if control measures are taken at an early stage of disease development. The early and accurate detection of plant disease plays a vital role in any disease management programme. During the last four decades much attention has been paid on the phenomenon of common antigenic relationship among the closely related organisms or among the more distantly related organisms. When both the animal and plant hosts and their parasites or pathogens come in contact with each other they establish a serological resemblance between one another involving one or more antigenic determinants. In plants, several studies have concluded that the possibility of susceptibility is greater when antigenic similarity is greater. Thus the concept of common antigens between a plant and a pathogen is a notable feature in determining resistance or susceptibility. It is believed that the degree of compatibility and susceptibility of a plant cultivar to a pathogen is correlated to levels of common antigens present in both host and pathogen (Alba *et al.*, 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005; Eibel *et al.*, 2005; Dasgupta *et al.*, 2005, Chakraborty and Sharma, 2007; Saha *et al.*, 2010).

Botrytis cinerea were serologically different and some antigens were specific for each isolate. Isolate no.1 of *Botrytis cinerea* had four specific antigens; although these antigens were absent in other isolates. At least sixteen antigens were common in the isolates tested. Some isolates were serologically similar when tested by double gel diffusion test while they were distinguishable when CIE techniques were used. Numbers of

precipitin peaks obtained with CIE techniques were more than double the number of precipitin lines detected with double gel diffusion test. Results revealed that crossed immunoelectrohoresis (CIE) techniques could be used as valuable analytical tools in resolving the spectrum of antigens present, in *Botrytis cinerea* isolates. By using CIE techniques antigenic structures of *B. cinerea, B. tulipae, B. paeoniae* and *B. allii* isolates were also compared. Antisera against antigens of these isolates gave 24, 15, 20 and 14 precipitin peaks respectively, when analyzed in homologous reactions.CIE with an intermediate gel and CIE with antibody absorption *in situ* revealed that each isolate was serologically different from the other and has species-specific antigens. *B. cinerea* has eight distinct antigens which distinguished them from the other species of *Botrytis* (Ala-El-Dein and El-Kady, 1985).

Cross-reactive antigens and lectin were the determinants of symbiotic specificity in the Rhizobium-clover association. Cross-reactive antigens of clover roots and *Rhizobium trifolii* were detected on their cell surfaces by tube-agglutination, immunofluorescent, and radioimmunoassay techniques. Anti-clover root antiserum had a higher agglutinating titer with infective strains of *R. trifolii* than with noninfective strains. The root antiserum previously adsorbed with noninfective *R. trifolii* cells remained reactive only with infective cells, including infective revertants. Radioimmunoassay indicated twice as much antigenic cross-reactivity of clover roots and *R. trifolii* 403 (infective) than *R. trifolii* Bart A (noninfective). Immunofluorescence with anti-R. trifolii (infective) antiserum was detected on the exposed surface of the root epidermal cells and diminished at the root meristem (Dazzo and Hubbell, 1975).

Evaluation of antisera raised against pooled mycelial suspensions from five isolates (Pf-1, Pf-2, Pf-3, Pf-10 and Pf-11) representing five physiologic races of *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA) were performed by Mohan (1988). Cross-reactivity of antiserum raised against *P. fragariae* with other *Phytophthora* as a genus detecting

antiserum has been reported. Antiserum of *P. fragariae* isolates (Anti-PfM) reacted strongly with antigens from several *Phytophthora* species. Some cross-reaction with antigens from *Phythium* species was decreased by fractionating on an affinity column of sepharose 4 B bound to extracts of *Fragaria vesca* roots infected with *P. fragariae.* The affinity purified anti PfM retained its high cross-reactivity with the various *Phytophthora* species. Anti-PfM could not be made specific for *P. fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However, immunoblotting with the affinity purified anti-PfM produced distinct patterns for *P. fragariae, P. erythroseptica* and *P. cactorum.*

Amouzon-Alladaye *et al.* (1988) reported that antiserum obtained against the mycelial proteins of a strain of *Phytophthora fragariae* could detect 11 different strains of *P. fragariae* in pure culture and pathogen in naturally infected or inoculated roots. The antiserum failed to react with 18 fungal species isolated from underground parts of strawberry but reacted with some strains of *P. cactorum,* which parasitized only rhizomes but not roots. In inoculated strawberry roots, *P. fragariae* was detected reliably by ELISA several days before oospores were found and before symptoms developed.

The possible involvement of cross-reactive antigens in host-parasite interactions between pea and some fungal plant pathogens were analyzed by Scala *et al.* (1994). Antiserum to pea was used to analyse cross-reactive antigens (CRA) between pea and some fungal plant pathogens with different levels of specificity towards this host by using both double diffusion and immunoblotting techniques. Non pathogens of pea were also included in the study. The three *f. sp. of Nectria haematococca* MPVI (Viz. *dianthi, lycopersici* and *pisi*) of *Fusarium oxysporum* and *Ascochyta pisi* produced strong reactions in both techniques. No CRA was observed in the non-specific pathogens *Rhizoctonia solani, Sclerotium rolfsii* and *Sclerotinia sclerotiorum,* as well as in the non-pathogen *Phytophthora capsici.* The immunoblotting patterns of the most reactive fungi showed common bands

with molecular weights of 84, 75 and 62 kDa. Some bands were present only in the specific pathogens *N. haematococca* MPVI and *F. oxysporum* f.sp. *pisi.*

Chakraborty *et al.* (1995) discussed the detection of grey blight of tea caused by *Pestalotiopsis theae* through cross reactive antigen between *P. theae* antigen of tea leaves. Among the 12 varieties of tea tested against three isolates of *Pestalotiopsis theae*, causal agent of grey blight disease, Teen Ali-17/1/54 and TV-23 were found to be highly susceptible while CP-1 and TV-26 were resistant under identical conditions. Leaf antigens were prepared from all the tea varieties, three isolates of P. theae and a non-pathogen of tea (*Bipolaris tetramera*). Polyclonal antisera were raised against mycelial suspensions of *P. theae* (isolate Pt-2) and leaf antigens of Teen Ali-17/1/54 and CP-1. These were compared in immunodiffusion test and enzyme-linked immunosorbent assay to detect cross reactive antigens (CRA) shared btween the host and the parasite. CRA were found among the susceptible varieties and isolates of P. theae (Pt-1, 2 and 3). Such antigens were not detected between isolates of *P. theae* and resistant varieties, B. tetramera and tea varieties or isolates of *P. theae.* Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves, the CRA was concentrated in the epidermal cells and mesophyll tissues. CRA was present in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae.*

Kitagawa *et al.* (1989) developed competitive types of two novel enzyme-linked immunosorbent assays (ELISA) for specific detection of *Fusarium oxysporum* f. sp. *cucumerinum* as well as for general detection of ten strains of common *Fusarium* species. Antiserum against a strain of *Fusarium oxysporum* f. sp. *cucumerinum* (F 504) was raised in rabbits and a highly specific, sensitive and accurate ELISA for the homologous strain was developed by using the antiserum with β-D-galactosidase-labelled anti-rabbit IgG as a secondary antibody and cell fragments of the strain attached to amino-Dylark balls as the solid-phase antigens. This assay was specific for strain F 504 and showed little cross-reactivity with nine other

strains of *Fusarium* species including strain F 501 of *F. oxysporum* f. sp. *cucumerinum* (FO). F 501 possesses pathogenicity against cucumber similar to that of strain F 504, although slight differences have been observed between these two strains regarding their spore formation and pigment production. Cell fragments of strain F 501 absorbed on amino-Dylark balls possessed sufficient immune activity against anti-FO antibody to use in a heterologous ELISA for general detection of ten *Fusarium* species with high sensitivity.

Cross-reactive antigens were detected by immunodiffusion, immunoelectrophoresis and indirect ELISA technique between susceptible soybean cultivars and the virulent strain of *C. dematium* but no cross-reactive antigen was detected between soybean cultivars and avirulent pathogen (*C. dematium*) or non-pathogen *C. corchori.* Results of immunodiffusion and immunoelectrophoresis showed absence of common antigen between resistant cultivars (UPS M-19) and the pathogen, while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. They compared antigenic patterns of untreated and cloxacillin treated soybean leaves which induced resistance of soybean against anthracnose disease. The disappearance of one antigen from cloxacillin treated leaves of susceptible soybean cv. "Soymax" was correlated with alteration of disease reaction (Purkayastha and Banerjee, 1990).

Polyclonal antiserum of mycelial proteins of *Verticillium dahliae* reacted positively with 11 of 12 isolates of *V. dahliae* from potato, cotton and soil but negatively with one isolate from tomato in indirect ELISA (Sundaram *et al.*, 1991). He also found positive results in detecting, *V. dahliae* and *V. albo-atrum* from infected roots and stems of potato in a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA).

With the help of an indirect ELISA technique, Ricker *et al.* (1991) showed that increase in cross-reactivity in late bled antiserum (anti-Bc lgG), raised against water soluble antigens from *Botrytis cinerea*

corresponded with an increase in the overall serum titers for anti-Bc IgG to antigens of *B. cinerea.*

Daniel and Nilsson (1991) raised polyclonal antiserum against mycelial extracts of the rot fungus *Phialophora mutabilis* which reacted strongly with its homologous antigen and cross-reacted strongly to moderately with six other *Philosophora* soft rot spp. in ELISA.

Lyons and White (1992) compared results of conventional isolation techniques for *Pythium violae* using polyclonal antibodies raised to *P. violae* or *P. sulcatum* in competition ELISA.

A double antibody sandwich ELISA test was developed for the detection of *Pseudocercosporella herpotrichoides* using a highly specific monoclonal antibody pH 10 as the capture antibody and genus specific polyclonal rabbit antisera as test antibody by Priestley and Deway (1993). The assay recognized extracts from plants both artificially and naturally infected with *P. herpotrichoides,* at least three-fold higher absorbance values with extracts of *P. herpotrichoides* infected tissue than with extracts from healthy tissues. The high molecular weight fraction of immunogen (mycelial extracts) was shown to contain cross-reactive antigens; it induced antiserum in mice that cross-reacted with the other stem base fungi even at high dilution.

A antigens obtained from tea varieties, isolates of *Bipolaris carbonum* and non-pathogens of tea (*Bipolaris tetramera* and *Bipolaris setariae*) were compaired by immunodiffusion, immunoelectrophoresis and enzyme linked immunosorbent assay to detect cross reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties (TV 9, 17 and 18) and isolates of *B. carbonum* (BC-1, 2, 3 and 4). Such antigens were not found between isolates of *B. carbonum* and resistant varieties (TV 16, 25 and 26), non-pathogens and tea varieties, as well as non-pathogen and *B. carbonum.* CRA were also found concentrated mainly around the epidermal cells of leaves of TV-18 in cross section following indirect staining of antibodies using fluorescein isothiocyanate (FITC). They indicated the presence of CRA in the young growing hyphal tips and

conidia following treatment with antisera of leaves (TV-28) and indirect staining with FITC (Chakraborty and Saha, 1994).

Polyclonal antibodies (PAbs) were produced against culture filtrates and mycelial extracts immunogen from the soybean (*Glycine max*) and fungal pathogen *Phomopsis longicolla* (Brill *et al.*, 1994). They purified the polyclonal antibodies to the immunoglobulin fraction and tested in indirect ELISA and in direct DAS-ELISA the PAbs raised to culture filtrate were more specific but less active in binding to members of Diaparthe-*Phomopsis* complex than were those to mycelial extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PAbs was lower in DAS-ELISA compared to indirect ELISA.

A extensive cross reaction was found when two monoclonal and three polyclonal antisera, raised against the cell wall/membrane fractions of *Pythium violae* and *P. sulcatum* screened with a collection of 40 isolates of the genus *Pythium* including 20 species and the H-S group. However, when the binding of the antibodies was assessed in an enzyme-linked immunosorbent assay (ELISA) using cytoplasmic fraction antigens, the combined recognition patterns produced profiles unique to each species White *et al.* (1994).

Polyclonal antibodies against prehelminthosporol, a phytotoxin produced by the plant pathogenic fungus *Bipolaris sorokiniana* were raised in rabbits immunized with a prehelminthosporol–hexon conjugate by Akesson *et al.* (1996). The IgG was isolated from the serum and the specificity of the purified antibodies was investigated with indirect ELISA. The antibodies bound both to free prehelminthosporol and to a prehelminthosporol-bovine serum albumin conjugate bound to micro titer wells. The antibodies showed less affinity to structurally related compounds from the fungus. No cross-reactivity was shown for proteins extracted from mycelium of *B. sorokiniana*. Low-temperature preparation techniques for electron microscopy were used in combination with immunogold labeling for localization of prehelminthosporol in hyphae and

germinated conidia of *B. sorokiniana.* A low level of labeling was obtained throughout the cytoplasm, and the main labeling was seen in membrane-bound organelles identified as Woronin bodies.

Polyclonal antisera against whole (coded: 16/2) and sonicated (coded: 15/2) resting spores of *Plasmodiophora brassicae were* raised by (Wakeham and White, 1996). They also raised antisera against filtered and ultracentrifuged soluble component. Cross reactivity of all these antisera were tested against a range of soil fungi including *Spongospora subterranean* was low Test formats including western blotting, dipstick, dot blot, indirect ELISA and indirect immunofluorescence were assessed for their potential to detect resting spores of *P. brassicae* in soil. Dot blot was least sensitive, with a limit of detection level of $1x10^7$ resting spores/ g in soil. With western blotting, the lower limit of detection with antiserum 15/2 was $1x10^5$. This antiserum showed the greatest sensitivity in a dipstick assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of $1x10^2$. Of the assays tested, indirect immunoflourescens appears to be the most rapid and amenable assay for the detection in soil low levels of resting spores of *P. brassicae* in soil.

Kratka *et al.* (2002) prepared four polyclonal and two monoclonal antibodies and tested to detect *Colletotrichum acutatum,* a quarantine pathogen of strawberry. They observed that only one polyclonal antibody was sensitive enough to recognize the pathogen. The antibody was genus specific that did not cross react with several other fungal pathogens of strawberry. They also detected *C. acutatum* by Plate trap antigen enzyme linked immunosorbent assay (PTA-ELISA), dot blot and immunoprint in roots, crowns, petioles and fruits in the latent age of the disease after artificial infection of strawberry (cvs. Elsanta, Vanda and Kama).

Ghosh and Purkayastha (2003) used polyclonal antibodies and antigens of ginger and *Pythium aphanidermatum,* a causal organism of rhizome rot disease for early diagnosis of rhizome rot disease of ginger. They detected *P. aphanidermatum* in ginger rhizome after eight weeks of

inoculation by agar gel double diffusion and immuoelectrophoretic tests, but only one week after inoculation by indirect ELISA.

Polyclonal antibodies were raised against mycelium from the logarithmic growth phase of a shake culture of *Ustilago nuda*, and developed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed with biotinylated detection antibodies. Other species of *Ustilago* reacted with the antibodies. Cross-reactivity was highest with *U. tritici.* No signal was obtained with the tested isolates of *Tilletia, Rhizoctonia, Pythium* and *Fusarium.* With naturally infected barley seeds, the results of the ELISAs were always in good agreement with those obtained with the routinely used seed embryo test. They suggested that potential fields of application of the ELISA include the early prediction of the efficacy of protection agents, e.g. in screenings for seed treatments, the elucidation of the biology of the fungus and characterization of resistance mechanisms (Eibel *et al.* 2005).

Virus (Petrunak *et al.*, 1991; Abou-Jawdah *et al.*, 2001; Hema *et al.*, 2001; Devaraja *et al.*, 2005; Chen *et al.*, 2005) and bacterial (Mazarei and Kerr, 1990) pathogens of plants could also be successfully detected by various ELISA formats.

Indirect ELISA was used to monitor the distribution of Mycoplasma like organism (MLO) in the experimental host *Vicia faba.* Post-embedding colloidal gold indirect immunolabelling was developed to identify, without ambiguity, the various forms of MLO cells in the different infected parts of the plant by transmission electron microscopy. Silver enhancement of the gold probe gave accurate histological and cellular localization of MLOs in tissue sections, by light microscopy. Both ELISA and immunolocalization first detected MLO in roots 17 days after inoculation with infectious leafhoppers (Lherminier *et al.*, 1994).

Hema *et al.* (2001) detected *Sugarcane streak mosaic virus* (SCSMV-AP) following Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and direct antigen coating (DAC)-ELISA. The virus was detected up to 1/3125 and 1/625 dilutions in infected sugarcane leaf, 5 µl

and 10 µl per well in sugarcane juice, 1/3125 and 1/625 dilutions in infected sorghum leaf and 10 ng and 50 ng/ml of purified virus in DAS-ELISA and DAC-ELISA tests, respectively.

Wang *et al.* (2006) showed that an indirect enzyme-linked immunosorbent assay (ID-ELISA) protocol is capable of detecting *Rice black-streaked dwarf virus* (RBSDV) in very dilute wheat leaf extracts. Based on the results, Wang *et al.* (2006) concluded that efficient and economic detection of RBSDV can be performed routinely using polyclonal antiserum against outer capsid protein (P10) expressed in prokaryotic cells.

Several viruses were detected by Abou-Jawdah *et al.* (2001) following ELISA. Potato virus Y (PVY), potato virus A (PVA), potato virus X (PVX), potato virus M (PVM), potato virus S (PVS) and potato leaf roll virus (PVX), potato virus M (PVM) potato virus S(PVS) and potato leaf roll virus (PLRV) from main production areas of Lebanon, the Bekaa and Akkar plains.

Shahriyari *et al.* (2011) developed a rapid and efficient method for detection of witches' broom disease of lime cuased by *Canditus* Phytoplasma *aurantifolia.* They employed a sensitive seriological technique (DAS-ELISA) along with recombinant DNA technology.

Immunolocalization is a powerful tool for cellular location of different proteins or antigens. This method has been utilized for location of CRA in tissues of the host and also in pathogen. In a study, DeVay *et al.* (1981) inoculated young cotton (Acala 2) roots with antiserum to *Fusarium oxysporum* f. sp. *vasinfectum* and stained with FITC conjugated, antirabbit globulin-specific goat antiserum. Strong fluorescence was observed at the epidermal and cortical cells, and the endodermis and xylem tissues that indicated a general distribution of the CRA determinants in roots. Chakraborty and Saha (1994) labelled polyclonal antiserum with FITC and found that CRA between tea leaves and the pathogen *Bipolaris carbonum* was present mainly around the epidermal cells and mesophyll tissues of leaves of the host and in hyphal tips and in patch like areas on conidia and mycelium of the pathogen. Dasgupta *et al.* (2005) also studied the location of CRA in tea leaves that were treated with antiserum raised against the pathogen *C. gloeosporioides.* Indirect labelling of the antibodies with FITC

showed that CRA was concentrated mainly in the epidermal cells and also spread throughout the cortical cells.

Immunolocalization is a powerful tool for cellular location of different proteins or antigens. Now a days immunolocalization studies are performed using immunogold labelling which is successfully used for electron microscopy (Lee *et al.*, 2000; Trillus *et al.*, 2000; Nahalkova *et al.*, 2001; Kang and Buchenauer, 2002 and Wang *et al.*, 2003). For light microscopy, silver enhancement is done after gold labelling (Santen *et al.*, 2005; Saha *et al.*, 2006, 2010).

A gold sol was found which was able to localize the ECM (Extra cellular matrix) of *C. gloeosporioides* very well. In the case of *C. gloeosporioides*, the ECM secreted out from conidium just before germination took place. The area that ECM covered was wide-spread and could reach up to several times the spore width. With gold sol, the composition and nature of the ECM could be easily identified using cytochemical and biochemical approaches (Kuo, 1999).

Immunogold labelling showed specific labelling of chitinase in the interaction of pepper stems with *Phytophthora capsici*. Chitinase was found on the cell wall of the oomycete in both compatible and incompatible interactions at 24 h after inoculation. In particular, numerous gold particles were deposited on the cell wall of *P. capsici* with a predominant accumulation over areas showing signs of degradation in the incompatible interaction. Chitinase labelling was also detected in the intercellular space and the host cytoplasm. However, healthy pepper stem tissue was merely free of labelling (Lee *et al.* 2000).

Immunolocalization experiments were performed by Nahalkova *et al.* (2001) for locating *Pinus nigra* ARN lectin (PNL). They observed that the protein was mainly located on the cytoplasmic membranes and on the primary cell walls. In infected seedlings (infected by *Heterobasidium annosum* and *Fusarium avenaceum*), a strong labelling of hyphal materials with PNL antisera was recorded only at the early stages of infection but not at the later stages of hyphal invation.

Two antisera against acidic β-1,3-glucanase and acidic chitinase of tobacco was raised by Kang and Buchenauer (2002). They investigated the subcellular localization of the two enzymes in *Fusarium culmorum*-infected wheat spike by means of the immunogold labelling technique. These studies demonstrated that the accumulation of the enzymes in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars.

Immunogold labelling technique was used by Wang *et al.* (2003) for localization of PB90 which is a novel protein elicitor secreted by *Phytophthora boehmeriae*. The anti-90 kDa protein antiserum was used for immunocytolocalization studies of PB90 elicitor, on the mycelium and encysting zoospores of *P. boehmeriae* grown *in vitro* in liquid culture and also in solid medium. In liquid culture, immunogold labelling was located mainly in the cell wall. In solid medium gold particles were observed not only in the cell wall, but also in the solid medium near the hypha.

The location of CRA in tea (*Camellia sinensis*) leaves treated with antiserum raised against *Exobasidium vexans*, causal agent of blister blight of tea have been studied by Chakraborty and Sharma (2007). Indirect staining of antibodies using FITC indicated cross reactive antigens (CRA) were concentrated mainly around epidermal and mesophyll cells in susceptible tea variety (T-78). This finding was substantiated by ultrastructural studies using gold labelled antibodies through transmission electron microscopy (TEM) which shows specific localisation in the chloroplast and host cytoplasm.

Saha *et al.*, (2010) showed that the level of CRA was less in susceptible plants in comparison to the resistant eggplants. They used light microscope to observe the specially treated plant tissues. To visualize the CRA they labelled the tissues by indirect immunogold technique and the labelled tissues were subjected to silver enhancement for visualization in the light microscope. They also correlated the level of CRA with the pathogenicity of *Colletotrichum gloeosporioides* in different eggplant varieties. They also substantiated their results with indirect ELISA.

Disease control by antagonistic organisms

Application of chemical fungicides leads to destroy beneficial microbes on the crop milieu and thus alters the crop scenario and also causes toxicity to human and natural biota (Patro *et al.* 2008). Biological control of plant diseases involves the use of one nonpathogenic organism to control or eliminate a pathogenic organism. Biological control is benign to environment. Biological control has attracted a great interest in plant pathology (Goto, 1990) and it becomes importent to develop cheaper management practices to control disease and obtain higher yield. To develop biological control strategies for controlling any disease, a thorough knowledge of life cycle of the pathogen(s), their mode of survival, the plant-pathogen interaction processes, the physical relationship of the pathogen to its host during pathogenesis, the time of infection, factors leading to infection and disease development are needed. Several authors have reported antagonistic activity of microorganisms in different crops (Droby *et al.*, 1992; Prasad *et al.*, 1999; Meena *et al.*, 2000; Dwivedi and Johri, 2003; Jadeja, 2003; Kohli and Diwan, 2003; Vestberg *et al.*, 2004; Brewer and Larkin, 2005; Sudha *et al.*, 2005; Singh and Sinha, 2005; Evueh *et al.*, 2008; Zivkovic *et al.*, 2010; Akrami *et al.*, 2011; Parizi *et al.*, 2012; Ajith *et al.*, 2012).

Plant growth promoting rhizobacteria (PGPR) can suppress pathogen and reduce disease incidence by several ways like competition for nutrient and space, production of antibiotics, production of HCN, production of siderophores, increase in salicylic acids, excreation of lytic enzymes, enhancement of plant defense through Induced systemic resistance (ISR), plant growth promotion by production of auxins and gibberalins etc. In *Trichoderma*, the production of secondary volatile and non-volatile metabolites is one of the criterions to assess its potential as biological agent (Umamaheswari *et al.* 2008)

Rhizosphere microorganisms such as *Trichoderma* spp. were found to be antagonistic against *Fusarium solani*, the causal agent of root disease of

eggplant (Hundoo and Dwivedi, 1993). Bucki *et al.* (1998) observed the presence of some biocontrol microorganisms viz., isolates of actinomycetes, fluorescent *Pseudomonads* and *Trichoderma* sp. in the soil which prevent the damping off of egg plant caused by *Fusarium* sp., *Pythium* sp. and *Rhizoctonia* sp.

Trichoderma harzianum has antagonistic effect against four fungal pathogens (viz. *Phytophthora parasitica, Colletotrichum capsici, Sclerotium rolfsii* and *Rhizoctonia solani*) of betel vine (D'souza *et al.,* 2001). Ramamoorthy and Samiyappan (2001) suggested that *Pseudomonas flourescens* isolates were effective bacterial antagonist for the management of fruit rot of chilli caused by *Colletotrichum capsici.* Jadeja (2003) observed that fungal antagonists like *Trichoderma* spp. were highly effective for inhibiting mycelial growth and retarding pycnidial formation of *Phomopsis vexans* causing disease in brinjal. *T. koningii* exhibited the maximum antagonistic activity. Bacterial antagonists, e.g. *Bacillus* spp. and *Pseudomonas fluorescens* were also highly effective against the pathogen (Meena *et al.,* 2000).

Pseudomonas, an antibiotic and siderophore producing strain from virgin soils (with forest trees) which displayed *in vitro* antibiosis against many plant pathogenic fungi was isolated by Baruah and Kumar (2002). They noticed that seed bacterization improved germination, shoot height, root length, fresh and dry mass, enhanced yield and chlorophyll content of leaves in the five test crop plants under field conditions. Seed bacterization also reduced the number of infected brinjal plants grown in soil infested with *Rhizoctonia solani.*

Management of anthracnose in french bean caused by *C. Gloeosporioides* was studied by Gupta *et al.* (2005). On the basis of *in vitro* studies they found *Trichoderma viride* isolate (Tv2), neem extract, carboxin and carbendazim as best treatments in inhibiting the growth of the pathogen. In field the most effective combinations comprised of seed treatement with carboxin and *T. viride* followed by foliar spray of neem

extract and carbendazim. This combination treatment resulted in the least disease incidence (1.45%) and severity (0.50%) and maximized yield (126 q/ha).

Effect of antagonistic microbes and extracts of botanicals on *Sclerotium rolfsii,* incitant of collar rot of brinjal was investigated by Jadon *et al.* (2005). Efficacy of isolates of *Trichoderma* spp., *Pseudomonas fluorescens,* and *Gliocladium virens* in suppressing the growth of the pathogen was tested by dual culture technique. *T. viride* isolate was found to be superior to other isolates in reducing colony diameter and sclerotial production of the pathogen.

Mycostop, a biofungicide that has been effectively used to control a number of soil and seed-borne pathogens like *Botrytis cinerea, Rhizoctonia solani* etc. and seed borne foot rot disease of wheat and barley (Tahvonen and Lahdenpera, 1988; Tahvonen and Avikainen, 1990). The active component of mycostop was the spores and mycelium of *Streptomyces griseoviridis.* The product has been used successfully in seed treatment, soil drench, drip irrigation and as a transplant dip to control various disease causing fungi (Lahdenpera, 1987; Lahdenpera *et al.,* 1990 and Mohammadi, 1992). Mycostop when used at the rate of 0.35 g/l or greater reduced spore germination, plasmolysed germlings and reduced sporulation of *C. radicicola.* In essence, it reduced the inoculum potential of *C. radicicola* (Suleman *et al.*, 2002).

Raju (1991) and Vinod *et al.* (1991) reported that *Trichoderma* resulted in dieback and disintegration of *Pythium* spp by hyphal coiling and by producing inhibitory substances. Several other works had shown considerable potential of *Trichoderma* and *Gliocladium* in controlling disease caused by *Sclerotium rolfsii* in snap bean, sugar beet, tomato, chickpea and cotton in greenhouse and field studies (Elad *et al.,* 1983; Upadhyay and Mukhopadhyay, 1983; Punja, 1985; Wokocha, 1990; Ciccarese *et al.,* 1992 and Latunde-Dada, 1993). Efficient control of

chickpea wilt complex was found when seeds were treated with *Gliocladium virens* (10^7conidia/ml) and carboxin 0.1% (Mukhopadhyay *et al.*, 1992).

Different isolates of *Trichoderma harzianum* showed differential antagonistic potential as biocontrol agent against *Sclerotium rolfsii* (Maity and Sen, 1985; Biswas, 1999).

Filonow (1998) observed that three antagonistic yeasts competed successfully for sugars since their uptake was faster and higher than that of *Botrytis cinerea* where competitiveness plays a central role in antagonism.

Modified granular formulation containing powdered wheat bran, kaolin, acacia powder and biomass of isolates of *Trichoderma harzianum* (PDBCTH 10 and PDBCTH 8), *T. virens* (PDBCTV$_S$ 3 and ITCC 4177) and *Gliocladium deliquescens* (ITCC 3450) for their effect on the reduction of chickpea damping off caused by *Rhizoctonia solani* was evaluated by Prasad and Rangeshwaran (1999). Granules with all isolates of bioagents significantly reduced damping off. The above two *T. harzianum* isolates were more effective in reducing saprophytic growth of the pathogen compared to other bioagents.

From a comparative study of chemical, biological and integrated control of wilt of pigeon pea caused by *Fusarium udum*, it was found that bavistin was highly effective, while *Trichoderma viride* and *T. harzianum*-C isolates were found best among biocontrol agents. Integration of biocontrol agents with bavistin was not beneficial. However, integration of the bioagents with thiram reduced wilt incidence significantly. Thus, seed coating with bioagents proved better and safe for the management of wilt of pigeon pea (Pandey and Upadhyay, 1999).

Fourteen isolates of *Trichoderma* and *Gliocladium* speices were tested *in vitro* against *Sclerotium rolfsii*, the causal organism of root/ collar rot of sunflower. Two isolates of *T. viride*, four isolates of *T. harzianum*, one each of *T. hamatum*, *T. koningii*, *T. polysporum*, *G. virens*, *G. deliquescens* and *G.*

roseum inhibited mycelial growth of the pathogen significantly. Complete inhibition of sclerotial germination was obtained with the culture filtrates of *T. harzianum* (PDBCTH 2, 7 and 8), *T. pseudokoningii* and *G. deliquescens*. The three *T. harzianum* isolates and the *T. viride* isolate (PDBCTV4) were superior under greenhouse conditions with PDBCTH 8 showing maximum disease control (66.8%) followed by PDBCTH 7 (66.0%) PDBCTV 4(65.4%), PDBCTH 2 (61.6%) and were even superior to fungicide captan. *G. deliquescens* gave maximum (55.7%) disease control among *Gliocladium* spp. (Prasad *et al.*, 1999).

Ahmed *et al.* (2000) studied the effect of pepper seed and root treatments with *Trichoderma harzianum* spores on necrosis caused in stems by *Phytophthora capsici* inoculation and on the course of capsidiol accumulation in the inoculated sites. They suggested that the treatments significantly reduced stem necrosis, which fell by nearly a half compared with the values observed in plants grown from non-treated seeds. Necrosis was also reduced in plants whose roots were drenched with various doses of *T. harzianum* spores.

Etebarian *et al.* (2000) reported *T. harzianum* isolate T39 and *T. virens* isolate DAR 74290, as potential biological agents, controlled the rot disease in potato and tomato caused by *Phytophthora erythroseptica*.

Twenty isolates of fluorescent pseudomonads were evaluated for their ability to control damping-off in tomato (*Lycopersicon esculentum*) and hot pepper (*Capsicum annuum*). Among these isolates, *P. fluorescens* isolate Pf1 showed the maximum inhibition of mycelial growth of *Pythium aphanidermatum* and increased plant growth promotion in tomato and hot pepper. *P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper in greenhouse and field conditions. Moreover, the isolate Pf1 induced the production of defense related enzymes and chemicals in plants (Ramamoorthy *et al.*, 2002).

Weller *et al.* (2002) reported the microbial basis of specific suppression to four diseases, *Fusarium* wilts, potato scab, apple replant

diseases and take-all disease. One of the best-described examples occurs in take-all decline soils. In Washington State, take-all decline results from the buildup of fluorescent *Pseudomonas* spp., that produces the antifungal metabolite 2, 4-diacetylphloroglucinol. The authors suggested that producers of this metabolite may have a broader role in disease-suppressive soils worldwide.

The potential of *Trichoderma harzianum, Trichoderma aureoviride* and *Trichoderma koningii* as biocontrol agents were evaluated by Perelló *et al.* (2003). Dual cultures in petridishes containing potato dextrose agar showed that the isolates of *Trichoderma* spp. tested inhibited significantly the mycelial growth of *D. tritici-repentis* between 50% and 74%. The results of the greenhouse tests indicated that seven strains of *Trichoderma* spp. significantly reduced the disease severity on wheat plants compared with untreated plants.

Perello *et al.* (2006) also evaluated six isolates of *Trichoderma harzianum* and one isolate of *T. koningii* on the incidence and severity of tan spot (*Pyrenophora tritici-repentis*) and leaf blotch of wheat (*Mycosphaerella graminicola*) under field conditions and noticed significant differences between wheat cultivars, inoculum types and growth stages. Three of the isolates tested showed the best performance in controlling leaf blotch and tan spot when coated onto seed or sprayed onto wheat leaves at different growth stages, with significant severity reduction up to 56%. In some experiments, the biocontrol preparation (T2 and T5) gave a level of disease control similar to that obtained with Tebuconazole (70 and 48%, respectively).

A novel indigenous *Pseudomonous aeruginousa* strain was isolated from industrial waste water by Roy *et al.* (2007) following dilution plate technique in nutrient agar (pH 7) medium. They used the *Pseudononas* strain as biocontrol agent against several species of *Phytophthora* (viz. *P. nicotianea, P. capsici, P. colocasiea* and *P. melonis*) and effectively controlled their growth.

Some biocontrol agents like the isolates of *Pseudomonous fluorescence* (PfC6 and PfCIAH- 196), *Bacillus subtilis* (BSW1and BST1), Trichoderma isolates-CIAH 175 and *Trichoderma harzianum* were tested against *Alternaria alternata* in watermelon. Their result showed that the antibiotics produced by *B. subtilis* caused swelling of the germ tube while *P. fluorescence* modified hypha into a chain of knotted cells. Volatile metabolites of *Trichoderma* isolate (CIAH-175) caused a maximum reduction in growth of *A. alternata* (92.2%) (Umamaheswai *et al.*, 2008).

Four *Bacillus* spp isolated from mango fruit surface and tested against *Lasiodiplodia theobromae*, causing stem end rot disease of mango fruit. The application of any of the four Bacillus species on fruits resulted in reductions by more than 50% of the natural incidence of stem end rot (Jadeja and Bhatt, 2008).

Patro *et al.* (2008) reported that *Pseudomonous fluorescence* (*Pf* -1@ 0.6%) can be effectively used as a seed treatment and foliar spray for the management of blat in finger millet in addition to the edifenphos (0.1 %).

Zivkovic *et al.* (2010) reported on the antagonistic activity of *Trichoderma harzianum* and *Gliocladium roseum* against the pathogen *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*, the causal agents of anthracnose disease in fruit crops. They showed that *T. harzianum* and *G. roseum* was promising biocontrol agent to prevent anthracnose disease of fruits.

Akrami *et al.* (2011) evaluated the effect of three isolates of *Trichoderma* named (*T. harzianum*) T1, (*Trichoderma asperellum*) T2 and (*Trichoderma virens*) T3, against the *Fusarium* disease, caused by *Fusarium oxysporum*. They found that T1 and T2 isolates and their combination were more effective than other treatments in controlling the disease, such that it reduced disease severity from 20 to 44% and increased the dry weight from 23 to 52%.

Monteiro *et al.* (2010) reported that *Trichoderma harzianum* ALL42 were capable of overgrowing and degrading *Rhizoctonia solani* and *Macrophomina phaseolina* mycelia, coiling around the hyphae with formation of appressoria and hook-like structures. They also analyzed the extracellular proteins secreted by *Trichoderma harzianum* using two-dimensional electrophoresis and MALDI TOF mass spectrometry. Endochitinase, β-glucosidase, α-mannosidase, acid phosphatase, α-1,3-glucanase, and proteases were identified in the gel and also detected in the supernatant of culture.

Parizi *et al.* (2012) analyzed the inhibitory effect of *Trichoderma viride* in vitro against Roselle pathogens i.e. *Phoma exigua, Fusarium nygamai* and *Rhizoctonia solani* using the dual culture technique. Maximum inhibition occurred against *P. exigua*, (reduced mycelial radial growth to 71.76%). Volatile and non-volatile inhibitors of *Trichoderma* were also evaluated for this purpose and non-volatile metabolites were tested against the pathogens. Maximum inhibition occurred against *R. solani* (73.95% mycelial growth inhibition), followed by *P. exigua* (37.17% inhibition).

Ajith *et al.* (2012) reported a new fungal antagonist *Zygosporium masonii* against *Colletotrichum capsici* incitant of anthracnose on bellpeppers. Formation of clear inhibition zone in dual culture and decrease in mycelial growth of pathogen were observed when treated with volatiles and non volatile compounds from the antagonist. *Z.masonii* treated seeds showed significant increase in seed germination, shoot length, root length and dry weight of the plant. They showed that *Z .masonii* was a potential antagonist to control anthracnose and could be used as a biocontrol agent (BCA).

Evueh *et al.* (2008) evaluated the effect of phylloplane fungi i.e *Aspergillus* sp., *Trichophyton* sp., *Trichocladium* sp. and *Gliocladium* sp. against *Colletotrichum* leaf disease of rubber (*Hevea brasiliensis* Muell. Arg.). Their finding showed that *Trichocladium* sp. and *Trichophyton* sp. exhibited the highest antagonistic effects on *C. gloeosporioides*.

Diseases control by botanicals:

Several plants have shown potential antifungal activity against pathogens of crop plants. Some plant products commonly known s botanicals contain antifungal molecules that are harmless and benign to environment. In addition there are certain advantages in the deployment of botanical pesticides. These are biodegradable, safe to non-target organisms, renewable and suit to sustainability of local ecology and environment. Moreover, the need for repeated application of fungicides to attain desirable level of disease control discourages the extensive adoption of chemical control by most of the farmers (Singh and Singh, 2005).

Terras *et al.* (1993) noticed synergistic enhancement of antifungal activity of wheat thionins by 2- to 72- folds when combined with 2 S albumins of radish or rape and being effective against filamentous fungi and some gram-positive bacteria. Permeabilization of the hyphal plasmalemma of thionins has been shown to be the mode of action. Soil amendments with crop residues lead to build up of allelochemicals and plant nutrients. In a comparative study, it was shown that incorporation of straw was found more effective than burning of straw in containing the symptoms of eye spot disease (*Pseudocercosporella herpotrichiodes*) and sharp eye spot disease (*Rhizoctonia cerealis*) of wheat (Prew *et al.*, 1995).

During evaluating of rape and Indian mustard as companion crop showed that the latter was more effective in minimizing the incidence not only of take-all disease of wheat but also *Rhizoctonia solani, Pythium* and *Cochliobolus sorokiniana* (Kirkegaard *et al.*, 1996). Singh *et al.* (1997) reported that the tissue extract of Indian mustard was equally effective and hence the role of volatile isothiocyanates is implied. Certain phytochemicals like gallic acid and abscicic acid have been shown to be antifungal. For instance, abscicic acid was shown to inhibit mycelial growth and sporidial formation and also germination of teliopores.

Garlic extracts inhibited inhibited mycelial growth of *Fusarium solani, Colletotrichum lindemuthianum, Pythium ultimum* and *Rhizoctonia*

solani i. Aueous extract of powdered oven-dried (35 °C) garlic bulbs were incorporated into the growth medium. The hyphae of *R. solani* and *C. lindemuthianum* found to collapse hyphae of *F. solani* appeared thinner than in controls (Bianchi *et al.*, 1997).

Ali *et al.* (1999) screened hexane and methanol extracts of sixteen plants of the family Caesalpiniaceae, collected around Karachi, Pakistan and were tested for their antibacterial and antimicrobial activity. As compared to hexane extracts, the methanol extracts of all the examined plants showed stronger growth inhibition against bacteria and fungi, *Cassia* species being the biologically more active plant. Ethanolic extract of *Melia azadirachta* rip fruit showed fungistatic (MIC 50-300 mg/ml) a fungicidal (MFC60-500 mg/ml) activity against *Aspergillus flavus, Fusarium moniliforme, Microsporum canis* and *Candida albicans* (Carpinella *et al.*, 1999).

The antimicrobial activities of Valex (the extract of Valonia), the extracts of mimosa bark, gallnut powders, *Salvia ancheri* Benthum var. *ancheri* and *Phlomis bourgei* Boiss were studied by Digrak *et al.* (1999). The results of the study indicated that mimosa bark extracts had the greatest antibacterial activity, followed by the Valex, gullnut powders, *Salvia ancheri* var. *ancheri* and *Phlomis bourgeie* extracts, respectively. Furthermore, it was found that gullnut powders and the extracts of mimosa bark contained high amounts of tannins and showed antifungal activity.

Two hundred and four species of traditional Chinese herbal medicines belonging to 80 families from Yunnan Province in People's Republic of China were tested for antifungal activities using a *Pyricularia oryzae* bioassay. Twenty-six herbal medicines form 23 families were active against *P. oryzae* and the ethanol extract of *Dioscorea camposita* (dioscoreaceae) exhibited the most bioactivity among the entire tested sample (Ke *et al.*, 1999).

Three thiosulfinates with antimicrobial activity were isolated from oil-macerated garlic extract and their structures were identified by them as

2-propene-1-sulfinothioic acid S-(Z,E)-1-propenyl ester [AIIS(O)SPn-(Z,E)], 2-propenesulfinothioic acid S-methyl ester [AIIS (O)SMe] and methane sulfinothioic acid S-(Z,E)-1-propenyl ester [MeS(O)SPn-(Z,E)]. Antimicrobial activities of AIIS (O) SPn-(Z, E) and AIIS (O) SMe against gram positive and gram negative bacteria and yeasts were compared with 2-propene-1-sulfinothioicacids- 2-propenylester [AIIS(O)SAII, allicin]. Antimicrobial activity of AIIS(O) S Me and All S(O)S Pn-(Z,E) were comparable and inferior to that of allicin, respectively. In another study, Yoshida *et al.* (1999b) isolated and identified an organosulfur compound from oil-macerated garlic extract by silica gel column chromatography and preparative TLC. The antimicrobial activity of isoE-10-DA was inferior to those of similar oil-macerated garlic extract compounds such as E-ajoene, Z-ajoene and Z-10-DA (Yoshida *et al.*, 1999a).

Leaves of five *Betula* species, *B. pendula, B. browicziana, B. medwediewii, B. litwinowii* and *B. recurvata* were collected from different parts of Turkey. The leaves were hydro distilled to yield the consequent essential oils. The essential oils showed antifungal activity against various phytopathogenic fungi like *Cephalosporium aphidicola, Drechslera sorokiniana, Fusarium solani* and *Rhizoctonia cereals* (Demirci *et al.*, 2000).

Limonene is the major constituent of essential oil of exocarpic part of *Citrus sinensis* which possessed strong and broad-spectrum antifungal activity against important fungal pathogens of sugarcane (Rao *et al.*, 2000). The mycelial growth of *Ceratocystis paradoxa* at 2000 ppm and that of *Fusarium moliniforme* and *Curvularia lunata* at 3000 ppm concentration of limonene were completely inhibited. It proved fungistatic at minimum inhibitory concentration and exhibited non-phytotoxicity on germination and growth of sugarcane.

Methanol extracts from leaves, stem bark, root bark, fruits and seed kernels of *Butyrospomum pradoxum* (*Vitellaria paradoxa*) were analyzed. It was revealed that the presence of alkaloids (in leaves and stem barks), flavones (in stem and root bark), saponins (in root bark), steroids (in stem

bark, fruits and seed kernels) and tannins (in leaves and root bark) had antimicrobial activity against bacteria (*Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Staphylococcus aureus, Ralstonia solanacearum* and *Bacillus cereus*) and fungi (*Fusarium oxysporum* and *Candida albicans*) (Ogunwande *et al.*, 2001).

Control of *Botrytis cinerea* Pers. leaf colonization and bunch rot in grapes with oils were studied by Jaspers *et al.* (2002) in laboratory and field condition. In detached lateral experiments, the essential oils from thyme (*Thymus vulgaris* L.) and clove (*Syzygium aromaticum* L.), as well as massoialactone (derived from the bark of the tree *Cryptocarya massoia* R.Br.) were not phytotoxic on leaves at concentrations of 0.33% or less. *B. cinerea* sporulation on artificially induced necrotic leaf lesions was significantly reduced by thyme (Thyme R) and masoialactone oils at 0.33%. A single application at veraison (1997/98) of either compound at concentrations of 0.33% controlled bunch rot and necrotic leaf lesion colonization by *B. cinerea* compared with *Botrytis* control treatments. Spray applications of Thyme R oil (0.33%) at 8-10 day intervals (1998/99) from flowering to harvest controlled *B. cinerea* bunch rot but also caused floral tissues to senesce.

Antifungal activities of four polymmethoxylated flavons, isolated from cold-pressed orange oil were tested against *Colletotrichum gloeosporioides*, a major plant pathogen of fruits that causes significant damage to crops in tropical, sub-tropical and temperate regions. They noticed that methoxylated flavones were effective in inhibiting mycelial growth of the fungus. Complete inhibition of the growth of the pathogenic fungus *C. gloeosporioides* was observed at a concentration of 100 μg ml^{-1}(Almada-Ruiz *et al.*, 2003)

Garlic extract showed activity against the plant pathogenic bacteria *Agrobacterium tumefaciens, Erwinia carotovora, Pseudomonas syringae* pv. *maculicola,* P.s. pv. *phaseolicola,* P.s. pv. *tomato, Xanthomonas campestris* pv. *campestris,* the fungi *Alternaria brassisicola, Botrytis cinerea,*

Plectosphaerella cucumerina, *Magnaporthe grisea*, and the oomycete *Phytophthora infestans* (Curtis *et al.*, 2004).

Seven Yucatecan plant extracts were screened to look for fungicidal activity for the control of *C. gloeosporioides*. Bioassay-directed purification of the root extract of one of the most active plants, Acacia pennatula, resulted in the isolation of the new compound 15,16-dihydroxypimar-8(14)-en-3-one (1), which showed inhibition of growth, sporulation and germination (Peraza-Sánchez *et al.*, 2005).

Methanolic extracts of forty Indian plant species were screened for antisporulant activity against *Sclerospora graminicola*, the causative organism of pearl millet downy mildew. The methanolic extracts of nine species did not show any effect, whereas the activity of the extracts of *Clematis gouriana, Evolvulus alsinoides, Mimusops elengi, Allium sativum* and *Piper nigrum* were commensurable to that of the marketed botanical fungicides. The extracts of 11 species (*Agave americana, Artemisia pallens, Citrus sinensis, Dalbergia latifolia, Helianthus annus, Murraya koenigii, Ocimum basilicum, Parthenium hysterophorus, Tagetes erecta, Thuja occidentalis* and *Zingiber offinale*) exhibited remarkable antisporulant effect even after 10-fold dilution of the crude extracts while in the case of remaining 15 plants the crude extracts loosed activity after 10-fold dilution. The antisporulant activity of commercialised *Azadirachta preparation* (Nutri-Neem) was more pronounced than that of *Reynutria* based on (Milsana) and Sabadilla (Veratrin), however, these botanical preparations held off the extracts of *C. gouriana* and *E. alsinoides* and synthetic fungicides (Deepak *et al.*, 2005).

In the search for bioactive compounds, direct bioautography of lipophilic leaf extracts of medicinal plants used by Himalayan people was used in antifungal screening by Guleria and Kumar (2006b). *Alternaria alternata* and *Curvularia lunata* were used as test organism in bioautography. The results, evaluated by the diameter of the inhibition zone of fungal growth, indicate that five plant species, among the 12

investigated, and had shown antifungal activity. They used $CHCl_3$: CH_3OH (1:9, v/v) as a solvent to develop silica gel TLC plates. Clear inhibition zones were observed for lipophilic extracts of Vitex negundo (RF value 0.85), *Zantoxylum alatum* (RF value 0.86), *Ipomea carnea* (RF value 0.86), *Thuja orientalis* (RF value 0.80) and *Cinnamomum camphora* (RF value 0.89). The best antifungal activity was shown by lipophilic leaf extract of *T. orientalis.*

Thirty plant extracts (aquous extract) were screened against the pathogen *Sclerotium rolfsii* in vitro to examin the inhibitory effect on mycelial growth and sclerotial production. Maximum inhibition (74%) of mycelial growth was recorded at 10% concentration of plant extract (*Prosopis juliflora*). Other two antifungal plant extracts were from *Agave americana* (showed 68% overall inhibition) and *Nerium indicum* (showed 54% overall inhibition). The inhibition (94%) of sclerotial production was exhibited by *Agave americana* and almost similar inhibition was shown by *Clerodendron inerme* leaf extracts. Leaf extract of *Riccinus communis* and fruit extract of *Riccinus communis* also gave well results (showed 72%) inhibition (Kiran *et al.*, 2006).

Two mosses viz. *Entodon plicatus* C. Muell and *Rhynchostegium vagans* jaeg showed their antimicrobial activity against *Bipolaris sorokiniana, Fusarium solani* and *Pseudomonas sclanacearum, Xanthomonas oryzae.* Aquous extracts of the two mosses were found to be ineffective. Ethanolic extracts of *E. plicatus* showed maximum inhibition (42%) of *B. sporokiniana* and petroleum ether extract of *R.vagans* exhibited max. inhibition (45%) of *B. sporokiniana.* Extract of *R. vagans* were found to be more effective inhibitors of *F. solani* than those of *E. plicatus.* Ethanolic extract of *R. vagans* showed maximum inhibition (44%) of *F. solani* whereas alcoholic extracts of both the mosses showed more effective antimicrobial activity (Mewari *et al.*, 2007).

Phyton-T, an extract of seaweed (*Sargassum wightii*) reduced disease incidence, induces defense enzymes against late blight of potato caused by

Phytophthora infestants and enhances quality of potato. Siddagangaiah *et al.* (2008) reported that tuber soaking and foliar spray in combination with Phyton-T (0.4%) and mancozeb (0.3%) for thrice at 15 days interval reduced the disease incidence up to 80%.

Malabadi and Vijoy-Kumar (2007) evaluated the antifungal activities of aceton, hexane, dichloromethen and methanol extracts of leaves of four plant species (*Acacia pennata, Anaphylis wightiana, Capparis pepiaria* and *Catunaregum spinosa*) against pathogen viz. *Candida albicans, Kluyeromyces polysporus, Aspergillus niger, Aspergillus fumigantus.* High antifungal activity was observed with methanolic extract of *Anaphylis wightiana* against all the test pathogens with the MIC values ranging from 0.02 to 0.06. Methanolic extract of *Acacia pennata, Anaphylis wightiana, Capparis pepiaria* have very stroung antifungal activity against tested pathogens particularly *C. albicans* and *K. polysporus.*

Yasmin *et al.* (2008) reported that the varied degrees of inhibitory effects of fifty five aqueous extracts of angiospermic plants on vegetative growth of *Fusarium moniliforme* Sheldon *in vitro.* They reported the antifungal properties of *Andrographis paniculata* leaves and *Lagerstroemia speciosa* against bakanae disease or the first time .The leaf extract of *Lawsonia inermis* showed maximum inhibition (60.65 %) followed by roots of *Asparagus racemosus* (50.59 %) and also suggested that the possibility to control bakanae disease of rice using these plant extracts in seed treatment.

Bhosale *et al.*(2008) reported that the mancozeb (0.3%) controled 51 per cent leaf blight disease of onion followed by *Trichoderma viride* (38.84%). They also reported that highest onion bulbs were produced (10.57 t/ha and 10.31 t/ha) following application of the mancozeb and *Trichoderma viride* respectively. Two botanicals (produced from *Lantana camara* and *Psorelea pinnata*) were effective and controlled disease (27.08 % and 22.44 % respectively).

Parekh and Chanda (2008) evaluated the methanol extract of 9 Indian medicinal plants of different families, for in vitro antifungal activity against some yeasts including *Candida albicans* (1) ATCC2091, *C. albicans* (2) ATCC18804, *Candida glabrata* NCIM3448, *Candida tropicalis* ATCC4563, *Cryptococcus luteolus* ATCC32044, *Cryptococcus neoformans* ATCC34664, *Trichosporon beigelli* NCIM3404, and some moulds such as *Aspergillus candidus* NCIM883, *Aspergillus flavus* NCIM538, *Aspergillus niger* ATCC6275 and *Mucor heimalis* NCIM873. The *in vitro* antifungal activity was evaluated at three different concentrations by agar disc diffusion method and the activity obtained was not concentration dependent. A. flavus was the most susceptible fungal strain while *C. glabrata* was the most resistant one.

Aslam *et al.* (2010) reported the antifungal activity of plant diffusates from 5 indigenous medicinal plant species (*Adhatoda zeylanica, Azadirachta indica, Capparis decidua, Dodonaea viscosa* and *Salvadora oleoides*) of Potohar region. They tested the antifungal activity against 3 pathogens (*Alternaria solani, Rhizoctonia solani* and *Macrophomina phaseolina*) attacking commercial crops. All selected medicinal plants exhibited considerable reduction in radial growth of mycelia of the pathogens tested. They also observed that radial growth of selected pathogens reduced with the increase of concentration of plant diffusates. Among 5 concentrations of plant diffusates, the highest inhibition in radial growth of all 3 pathogens were observed at 200g/l. Minimum concentration found to control mycelial growth was 10g/l.

Al-Askar *et al.* (2010) investigated antifungal activity of ethanol-water extracts of four medicinal plants, cinnamon (*Cinnamomum verum* Presl.), anise (*Pimpinella anisum* L.), black seed (*Nigella sativa* L.) and clove (*Syzygium aromaticum* L. Merr. & Perry.) against pea (*Pisum sativum* L.) root-rot fungus *Rhizoctonia solani* and the efficacy of clove extract (concentration 4%) on disease incidence of *Rhizoctonia* root-rot of pea in the pot experiment in greenhouse. The highest antifungal activity (zero percent disease) was recorded in case of 1% clove extract application.

Singh and Kumar (2011) isolated seven *Trichoderma harzianum* coded as T1, T2, T3, T4, T5, T6 and T7 for their biocontrol potential against highly virulent *Fusarium oxysporum* f. sp *chrysanthemi* (Foc) isolate FO-10 and also evaluated Eight botanicals namely *Mentha arvensis* (MA), *Tagetus patula* (TP), *Eucalyptus sp* (ES), *Datura stramonium* (DS), *Calotropis procera* (CP), *Lantana sp* (LS), *Ricinus communis* (RC) and *Catharanthus roseus* (CR) for their biocontrol potential against Foc using food poison technique. Based on the performance of *Trichoderma harzianum* isolates T3, T4, T5 and botanical MA, TP and DS were selected for the biological control trials in pot conditions. Maximum disease control was recorded by the treatment of soil with botanicals [MA (70.0%), TP (61.0%) and DS (50.0%)].

Nisha *et al.* (2011) investigated the antifungal activity of aqueous extract of *Cannabis sativa*, *Parthenium hysterophorus*, *Urtica dioeca*, *Polystichum squarrosum* and *Adiantum venustum* against *Alternaria solani*, *Alternaria zinniae*, *Curvularia lunata*, *Rhizoctonia solani* and *Fusarium oxysporum* at different concentrations (5, 10, 15 and 20%). The extract of *C. Sativa* at 20% concentration showed maximum antifungal activity 100% and 59.68% against *C. lunata* and *A. zinniae* respectively. Leaf extract of *P. hysterophorus* controlled growth of *A. solani* upto 50%.

Abu-Taleb *et al.* (2011) reported that the extracts of *Rumex vesicarius* L. and *Ziziphus spina-christi* (L.) Willd. were antifungal compounds against two root rot pathogens (*Drechslera biseptata* and *Fusarium solani*) *in vitro*. Eight flavonoid subfractions (rutin, quercetin, myricetin, apigenin, quercetin-3-O-galactoside, luteolin, kaempherol and kaempherol-3-O-robinoside) and six flavonoid subfractions (apigenin-7-O-glucoide, quercitrin, quercetin, isovitexin, rutin and quercetin-3-O lucoside-7-O-rhamnoside) were isolated from the remaining aqueous layer fraction of *R. vesicarius* and *Z. spina-christi*, respectively. *F. solani* failed completely to produce spores when treated with ethanolic extract of *Z. spina-christi* at the concentration of 20% but plant extracts were more effective against to *D. biseptata*.

Seema *et al.* (2011) evaluated the antifungal effect of 10 plant extracts viz., *Thevetia peruviana*, *Ocimum basilicum*, *Piper betel*, *Murraya*

koenigii, *Chrysanthemum coronarium*, *Polyalthia longifolia*, *Catharanthus roseus*, *Pelargonium graveolens*, *Moringa officinalis* and *Lawsonia inermis* by poisoned food technique against *Rhizoctonia solani* Kuhn, the causal organism of sore shin disease of tobacco. Among them only four plants (*Lawsonia inermis*, *Piper betel*, *Polyalthia longifolia* and *Pelargonium graveolens*) showed significant antifungal activity against *Rhizoctonia solani*. Organic solvents viz., n-hexane, ethyl acetate and methanolic extracts of four plants of *Lawsonia inermis*, *Piper betel*, *Polyalthia longifolia* and *Pelargonium graveolens* showed 100% inhibition of mycelial growth and sclerotia formation of *Rhizoctonia solani* at 1000 ppm concentration.

Sasode *et al.* (2012) evaluated the effectiveness of some botanicals (viz., Neem, Eucalypts, *Datura*, Pudina, Tulsi and Lantanas). They tested crude as well as 10% extract against *Alternaria brassicae* under *in vitro* condition by poisoned food technique. Neem and *Eucalyptus* were also evaluated in the oil forms and boiled extract of Neem showed the minimum radial growth of the pathogen. The oil extract (Neem and *Eucalyptus*) were found less effective as compared to crude and boiled extracts.

Adejumo *et al.* (2012) reported that the antimicrobial activity of Partially (Pp) and completely purified (Cp) methanolic extracts of leaves of *Cassia alata*, *Cymbopogon citratus* (lemongrass), *Mangifera indica* (mango), *Carica papaya* (pawpaw), *Citrus limon* (lemon), fruits of *Xylopia aethiopica*, seeds of *Aframomum melegueta* (alligator pepper), *Citrus aurantifolia* (lime), *Garcinia kola* (bitter kola), *Piper guineense* (brown pepper), and rhizome of *Zingiber officinale* (ginger) using the poisoned medium and disc diffusion assay techniques on maize mycotoxigenic fungus: *Fusarium verticillioides*. *P.* guineense, *G. kola* and *A. melegueta* showed consistent higher growth inhibitions while *C. papaya* and *M. indica* had the least activities. They reported that *P. guineense*, *G. kola* and *A. melegueta* could be successfully used as environmentally-friendly, cheap, available, effective and sustainable alternative biopesticides.

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.

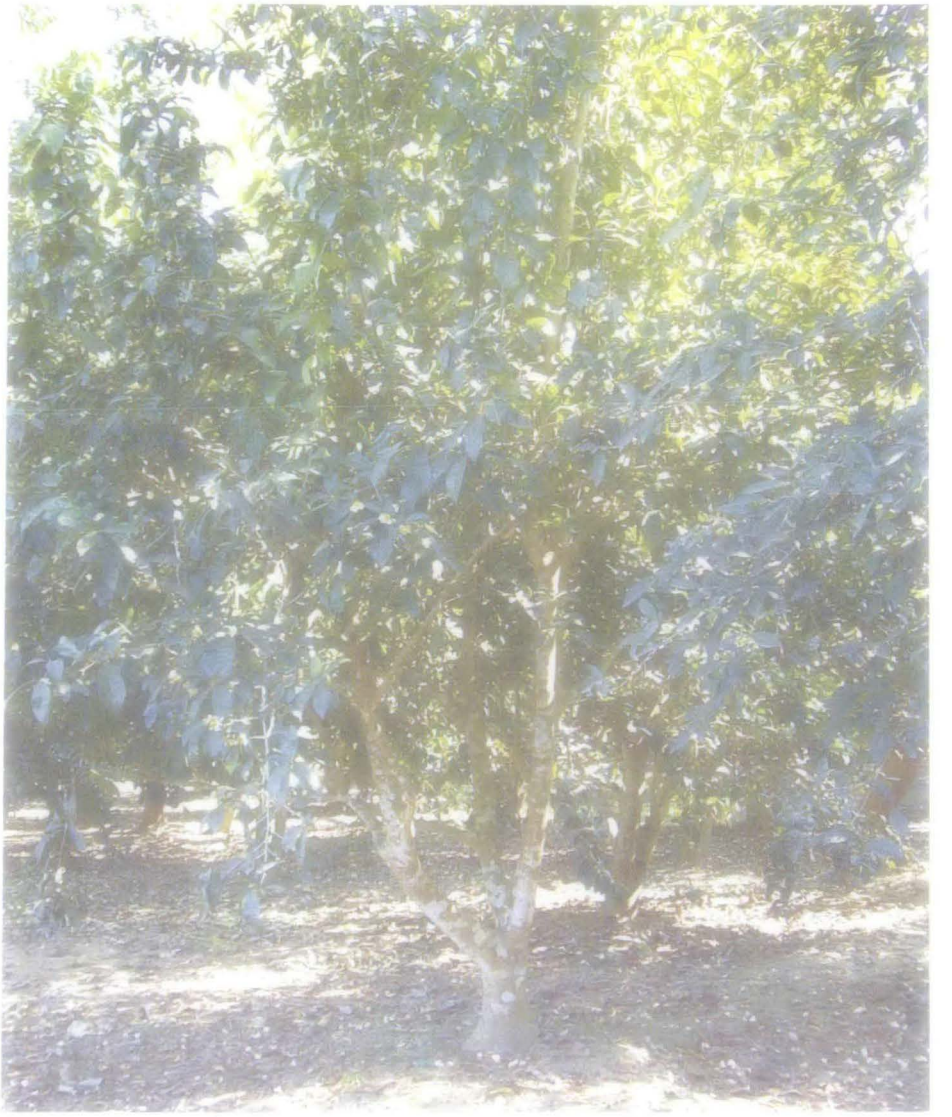

Plate II: Seed farm of Gayaganga tea estate.

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

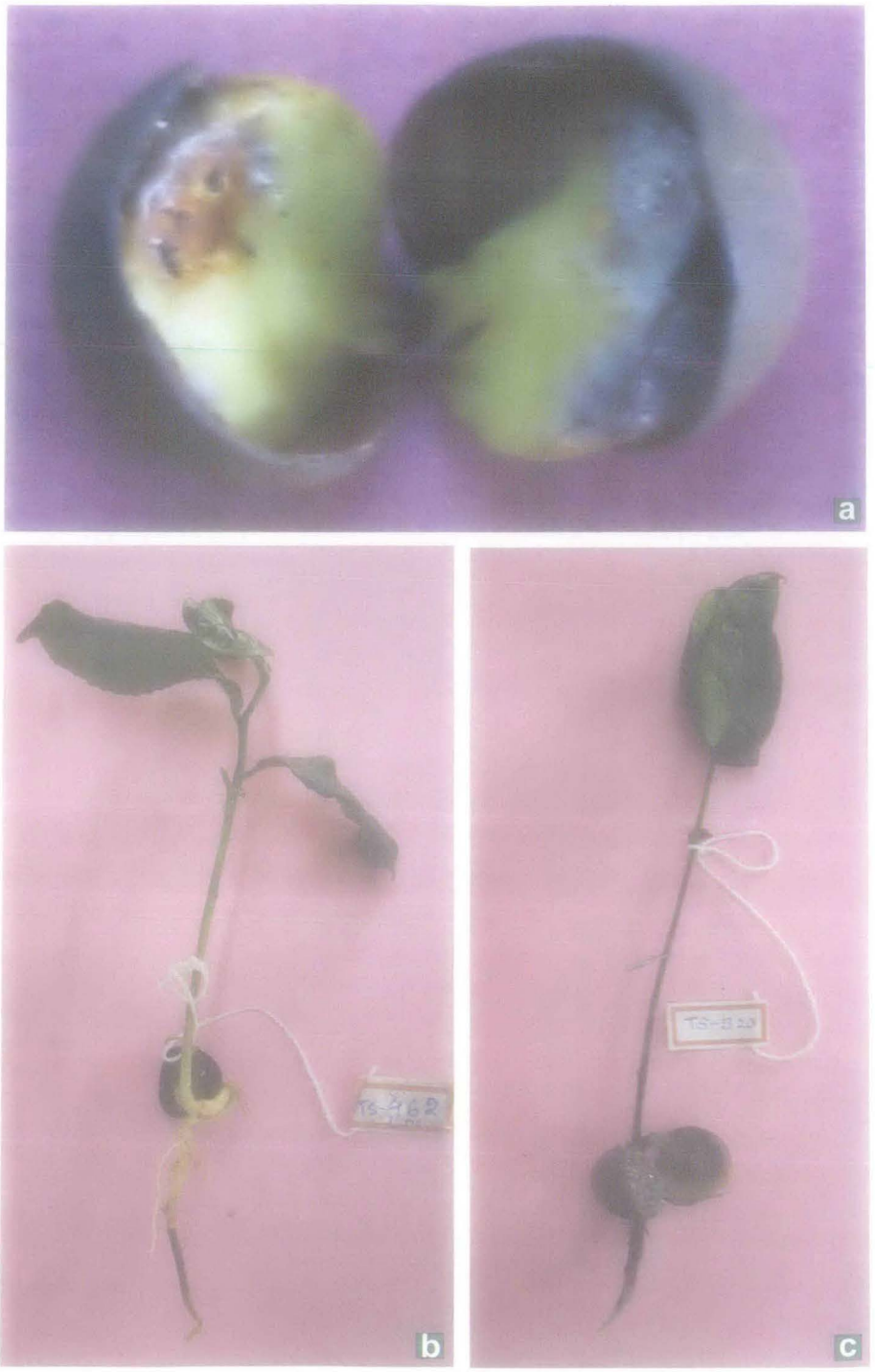

Plate V: (a) Cracked tea seed with brown patch.
(b) & (c) Artificially infected (by *Rhizoctonia solani*) tea seedling after 24 days.

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.

4. Experimental

Chapter I: Studies on tea seed mycoflora and pathogenicity of *Rhizoctonia solani*

4.1. Introduction

Tea plants are grown from seeds as well as from clonal cuttings. Plants raised from seeds are important because of their well developed tap root system. Germination of seeds is important for development of tea plantations with seed varieties. Germination percentage of seeds varies in different varieties. Low percentage of germination is often correlated with seed borne pathogens in different crops as well as trees (Sahai and Mehrotra, 1982; Mittal, 1983; Anderson, 1986; Bhardwaj *et al.*, 1988; Prochazkova, 1990; Uniyal and Uniyal, 1996; Mehrotra *et al.*, 2000; Kirti *et al.*, 2004; Singh and Sukla, 2005; Afzal *et al.*, 2010; Singh *et al.*, 2011; Utobo *et al.*, 2011; Pandey and Gupta, 2011). In tea nurseries, several growers sink tea seeds in water before placing them in germination beds. They discard the seeds which float on the surface of water as they are considered to be damaged or pathogen infected. It has been observed that pathogen associated with seeds affect the growth and productivity of several crop plants, shrubs and trees (Kubiak and Korbas, 1999; Weber *et al.*, 2001; Dawson and Bateman, 2001; Nagaraja *et al.*, 2009; Singh *et al.*, 2011; Pande and Gupta, 2011). Externally and internally associated seed borne pathogens may cause abortion, seed rot, seed necrosis, reduction of germination capacity as well as seedling damage resulting in development of disease at stages of plant growth by systemic or local infection (Bateman and Kwasana, 1999; Khanzada *et al.*, 2002).

4.2. Isolation of tea seed mycoflora

For observation and isolation of fungal organisms associated with seeds externally and internally, the standard blotter method and agar plate method was followed. Detailed methods have been mentioned in the materials and

methods (sections 3.1.3.1 and 3.1.3.2 respectively). While the standard blotter method enabled observation of fungal colonies, the agar plate method was useful in isolating the fungi present in the seeds. Preliminary identification were based on sporulation, conidial structures, sclerotial structures, spores and fruiting structures as apparent on seeds. Colony characteristics were observed as formed on PDA plates. Combined studies with seeds of seven important tea seed varieties (TS 449, TS 501, TS 491, TS 463, TS 464, TS 462, and TS 520) led to the isolation and identification of fifteen different types of fungal colonies. All the fifteen colonies were again studied well and the distinct differences in morphology, texture, colour and sporulation-behaviour were taken in to consideration for their identity. All the cultures were purified and their growth behavior has been studied. Important fungal cultures growing in petridishes along with their microscopic views have been represented in the plates VI, VII, VIII, IX and X. All the fifteen fungi were coded as F1 to F15. The fungi were identified as *Curvularia lunata*, *Rhizoctonia solani*, *Fusarium* sp., *Alternaria* sp., *Aspergillus* sp (isolate F5, F6 and F7), Aspergillus *flavus*, Aspergillus *niger*, Botryodiplodia sp., *Rhizopus* sp. (isolate F11 and F12), *Penicillium* sp., Trichoderma *pseudokoningii* and a sterile fungi. The percent incidence of each fungus was recorded as:

= (No. of incidence of a particular fungi ÷ Total no. of seeds observed) ×100

4.3. Percent incidence of different fungi in the seven seed varieties

Percent incidence of the fungal colonies was studied in case of all the seven seed varieties of the present study. Percent incidence was calculated in four different set of experiments. In the first set unsterilized seeds with seed coat were used. In second set unsterilized seeds without seed coat were used. In the third set surface sterilized seeds with seed coat were used. In the last (fourth) set, surface of the seeds were sterilized and then seed coats were removed aseptically before placing in the PDA plates.

4.3.1 Percent incidence of fungi in unsterilized seeds with seed coat

In this case unsterilized seeds with seed coat were used to isolate different fungi associated with the seed coat of the seven tea seed varieties. The results of the study have been presented in table 4.1. The fungi were identified as *Curvularia lunata* (isolate F1), *Rhizoctonia solani* (isolate F2), *Fusarium* sp. (isolate F3), *Alternaria* sp. (isolate F4), *Aspergillus* sp (isolate F5), *Aspergillus* sp.(isolate F6), *Aspergillus* sp.(isolate F7), *Aspergillus flavus* (isolate F8), *Aspergillus niger* (isolate F9), *Botryodiplodia* sp. (isolate F10), *Rhizopus* sp. (isolate F11), *Rhizopus* sp.(isolate F12), *Penicillium* sp. (isolate F13), *Trichoderma pseudokoningii* (isolate F14) and a sterile fungi (isolate F15). Nine different fungi (F1, F2, F5 –F9, F11 and F12) were found to be associated with seeds of all the seven tea varieties tested. However, the incidence of *Rhizoctonia solani*, *Aspergillus* sp. and *Rhizopus* sp. was more than the other fungi found in the experiment.

Table 4.1: Percent incidence of fungi found from unsterilized seeds with seed coat following agar plate method

Name of fungi with code in parenthesis	Percent incidence of fungi in seeds* (un sterilized seeds-with seed coat)						
	Seed varieties						
***Curvularia lunata* (F1)**	TS 449	TS 506	TS 463	TS 491	TS 464	TS 462	TS 520
***Rhizoctonia Solani* (F2)**	4	4	8	4	8	16	20
***Fusarium* sp. (F3)**	16	16	20	20	24	32	48
***Alternaria* sp. (F4)**	-	-	-	4	-	4	8
***Aspergillus* sp (F5)**	-	8	-	-	16	16	24
***Aspergillus* sp (F6)**	36	24	20	20	32	28	36
***Aspergillus* sp (F7)**	28	12	12	20	16	12	24
***Aspergillus flavus* (F8)**	40	40	44	36	32	36	48
***Aspergillus niger* (F9)**	8	12	8	4	8	16	20
***Botryodiploidia* sp. F10)**	40	36	36	32	40	48	48
***Rhizopus* sp. (F11)**	-	-	-	-	4	-	8
***Rhizopus* sp. (F12)**	36	28	24	24	32	40	40
***Penicillium* sp. (F13)**	44	32	20	28	36	36	48
***Trichoderma pseudokoningii* (F14)**	-	-	-	-	-	-	4
Sterile fungi (F15)	8	-	8	4	-	12	16

*percentage was calculated on observation of 400 seeds

4.3.2 Percent incidence of fungi in unsterilized seeds without seed coat

Another experiment was done, where seed coat of the seeds were removed and then placed in plates containing sterile potato dextrose agar medium. Studies on colony characteristic and microscopic observation revealed that eleven fungi which were found to be present externally were also present internally. These were F2, F3, F4, F5-F9, F11, F12 and F14 (Table 4.2). The other four fungi (F1, F10, F13 and F15) which were present on the surface of the seeds were not present internally. Out of the 11 fungi eight fungi were found in all the seven tea varieties tested. Importantly, two fungi *Rhizoctonia solani* and *Alternaria* sp., which are well known pathogens of different crops, were present in all the seed varieties tested. Incidence of *Rhizoctonia solani* was more than that of *Alternaria* sp.

Table 4.2: Percent incidence of fungi in unsterilized seeds without seed coat

Name of the fungi with code in parenthesis	Percent incidence of fungi in seeds (Un sterilized Seed-without seed coat)						
	Seed varieties						
	TS 449	TS 506	TS 463	TS 491	TS 464	TS 462	TS 520
***Curvularia lunata* (F1)**	-	-	-	-	-	-	-
***Rhizoctonia solani* (F2)**	4	8	8	12	24	28	36
***Fusarium* sp. (F3)**	-	-	4	-	-	-	8
***Alternaria* sp. (F4)**	8	12	16	16	4	4	12
***Aspergillus* sp. (F5)**	24	20	16	16	24	20	28
***Aspergillus* sp. (F6)**	24	8	8	16	12	6	20
***Aspergillus* sp. (F7)**	36	32	28	28	24	20	28
***Aspergillus flavus* (F8)**	-	8		4	4		8
***Aspergillus niger* (F9)**	24	20	16	8	8	12	20
***Botryodiploidia* sp. (F10)**	-	-	-	-	-	-	-
***Rhizopus* sp. (F11)**	28	20	24	16	28	36	36
***Rhizopus* sp. (F12)**	32	24	20	20	32	28	40
***Penicillium* sp. (F13)**	-	-	-	-	-	-	-
***Trichoderma pseudokoningii* (F14)**	4	-	-	-	-	4	8
Sterile fungi (F15)	-	-	-	-	-	-	-

*percentage was calculated on observation of 400 seeds

4.3.3 Percent incidence of fungi in sterilized seeds with seed coat

In another experiment, seeds were surface sterilized and were allowed to incubate in plates containing sterile potato dextrose agar medium. After 10 days, the colonies were observed and the percent incidence of the different fungi was recorded (table 4.3). From the results, it was evident that *Rhizoctonia solani*, *Aspergillus* sp. and *Rhizopus* sp. were associated with all the seven varieties.

Table 4.3: Percent incidence of fungi in surface sterilized seeds with seed coat

Name of the fungi with code in parenthesis	Percent incidence of fungi in seeds (Surface sterilized Seed-with seed coat)						
	Seed varieties						
	TS 449	TS 506	TS 463	TS 491	TS 464	TS 462	TS 520
***Curvularia lunata* (F1)**	-	-	-	-	-	-	-
***Rhizoctonia solani* (F2)**	4	8	16	16	20	24	28
***Fusarium* sp. (F3)**	-	-	4	-	-	-	-
***Alternaria* sp. (F4)**	4	-	-	-	-	-	8
***Aspergillus* sp. (F5)**	20	12	8	8	16	12	24
***Aspergillus* sp. (F6)**	20	4	4	8	4	4	16
***Aspergillus* sp. (F7)**	32	24	12	8	4	4	12
***Aspergillus flavus* (F8)**	-	-	-	-	-	-	-
***Aspergillus niger* (F9)**	24	16	8	8	4	4	12
***Botryodiploidia* sp. (F10)**	-	-	-	-	-	-	-
***Rhizopus* sp. (F11)**	24	16	20	20	16	12	16
***Rhizopus* sp. (F12)**	20	16	16	16	28	24	28
***Penicillium* sp. (F13)**	-	-	-	-	-	-	-
***Trichoderma pseudokoningii* (F14)**	-	-	-	-	-	4	-
Sterile fungi (F15)	-	-	-	-	-	-	-

*percentage was calculated on observation of 400 seeds

4.3.4 Percent incidence of fungi in surface sterilized seeds without seed coat

In the fourth experiment (table 4.4) seeds were surface sterilized and the seed coats were removed, prior to placing in the potato dextrose agar plates. Six different fungi were found to be associated with the seeds of all the

tested seed varieties. *Alternaria* sp. was found in the seeds of three varieties (TS491, TS449 and TS520) only. *Aspergillus* sp. (F6) was not found in seeds of two varities (TS506 and TS463).

From the above four experiments it was found that fungi from three different genus (*Rhizoctonia, Aspergillus* and *Rhizopus*) was associated internally with the seeds of all the tested varieties and affected the seeds in different ways.

Table 4.4: Percent incidence of fungi in pre-surface sterilized seeds without seed coat

Name of the fungi with code in parenthesis	Percent incidence of fungi in seeds* (Pre surface sterilized Seed-without seed coat)						
	Seed varieties						
	TS 449	TS 506	TS 463	TS 491	TS 464	TS 462	TS 520
***Curvularia lunata* (F1)**	-	-	-	-	-	-	-
***Rhizoctonia solani* (F2)**	4	4	8	12	16	16	20
***Fusarium* sp. (F3)**	-	-	-	-	-	-	-
***Alternaria* sp. (F4)**	4	-	-	4	-	-	4
***Aspergillus* sp. (F5)**	12	4	4	8	12	20	20
***Aspergillus* sp. (F6)**	16	-	-	4	16	4	12
***Aspergillus* sp. (F7)**	28	20	16	12	8	8	16
***Aspergillus flavus* (F8)**	-	-	-	-	-	-	-
***Aspergillus niger* (F9)**	20	12	4	4	8	8	16
***Botryodiploidia* sp. (F10)**	-	-	-	-	-	-	-
***Rhizopus* sp. (F11)**	20	16	4	8	4	8	12
***Rhizopus* sp. (F12)**	16	8	8	16	4	4	12
***Penicillium* sp. (F13)**	-	-	-	-	-	-	-
***Trichoderma pseudokoningii* (F14)**	-	-	-	-	-	-	-
Sterile fungi (F15)	-	-	-	-	-	-	-

*percentage was calculated on observation of 400 seeds

4.4. Koch's postulates and establishment of *Rhizoctonia solani* as pathogen of tea seeds

After the seed mycoflora studies it was considered to verify the Koch's postulates for all the three species of fungi (*Rhizoctonia, Aspergillus* and

Rhizopus) isolated from seed mycoflora. Detailed procedure of verification of 'Koch's postulates' have been presented in the section 3.4 of Materials and methods.

Results indicated that *Rhizoctonia solani* was a pathogen of tea seeds because in all cases of re-isolation, *R. solani* was consistently found to be associated with the infection of the seeds. However the results failed to establish the different species *Aspergillus* and *Rhizopus* as pathogens, as no symptoms were observed even after 15 days of inoculation and after the seeds were cracked. The presence of these fungi in all the seed varieties, found during seed mycoflora studies, was probably due to their secondary infection capacity (*i.e.* they could attack only when some other fungi primarily damaged the seeds).

4.5. Pathogenicity of *Rhizoctonia solani* in different varieties of tea seedlings

Virulence of a fungal pathogen varies in different varieties of a host. Differential pathogenicity to different varieties gives us information about the degree of susceptibility or resistance of a particular variety to a particular pathogen. In the present study, pathogenicity of *Rhizoctonia solani* was tested separately on seedlings of seven different tea seed varieties (TS 449, TS 501, TS 491, TS 463, TS 464, TS 462, and TS 520).

Pathogenicity was studied following seedling inoculation method as described in materials and methods (section 3.5). Twenty seedlings of each variety (raised through seed germination) were artificially inoculated with *Rhizoctonia solani.* Disease symptoms were recorded at three day intervals up to 24 days. Data were computed following 1-5 scale of Carson *et al.*, (1999) and disease severity was calculated as proposed by Ahiza and Payk, (1993). Details of the techniques have been presented in section 3.5. The experiment was repeated thrice and the results were averaged.

From the results (table 4.5 and figure 4.1) it was found that TS-520 was most susceptible while TS 449 was most resistant among the tested varieties. However, no variety was observed to be completely resistant. After 24 days of inoculation, TS 449 showed 49.66 percent disease severity while all other plant varieties showed more than 87 percent disease severity. Percent disease severity of the most susceptible and most resistant variety recorded at 3 day intervals until 24 days have been represented graphically in Fig. 4.1.

Table 4.5: Percent disease severity in different tea varieties

Tea seed variety	Percent Disease Severity* (days after)							
	3	6	9	12	15	18	21	24
TS 449	20.00± 1.52	26.67± 1.81	32.00± 2.08	37.33± 0.96	42.67± 1.44	44.00± 1.13	46.33± 1.20	49.66± 0.92
TS 506	30.00± 1.80	38.33± 1.33	48.33± 0.65	57.50± 0.80	67.50± 1.05	76.66± 1.20	86.66± 0.94	87.50± 1.05
TS 463	27.50± 0.86	39.17± 0.92	46.67± 1.8	55.83± 1.01	65.83± 1.36	77.50± 1.15	85.00± 1.73	92.50± 1.27
TS 491	25.83± 1.47	38.33± 2.12	44.17± 1.83	56.67± 1.76	62.50± 1.62	75.00± 0.57	80.80± 0.83	93.33± 1.62
TS 464	26.66± 0.95	38.33± 1.42	45.00± 1.15	56.67± 0.66	63.33± 2.05	75.00± 1.04	81.67± 0.66	93.33± 1.66
TS 462	28.33± 1.03	39.17± 1.48	47.50± 2.08	58.33± 1.85	66.67± 2.25	77.50± 2.25	85.83± 1.96	96.67± 2.60
TS 520	31.67± 1.45	38.83± 0.92	50.00± 1.52	57.50± 2.25	70.00± 0.15	78.33± 1.42	90.00± 0.57	97.50± 2.29
CD @ 5%	2.61	1.33	1.55	2.90	2.41	2.45	2.53	3.57

*Calculated following the methods of Ahuza and Payak (1993), Carson *et al.* (1999).
For each treatment 20 plants of each variety were used.

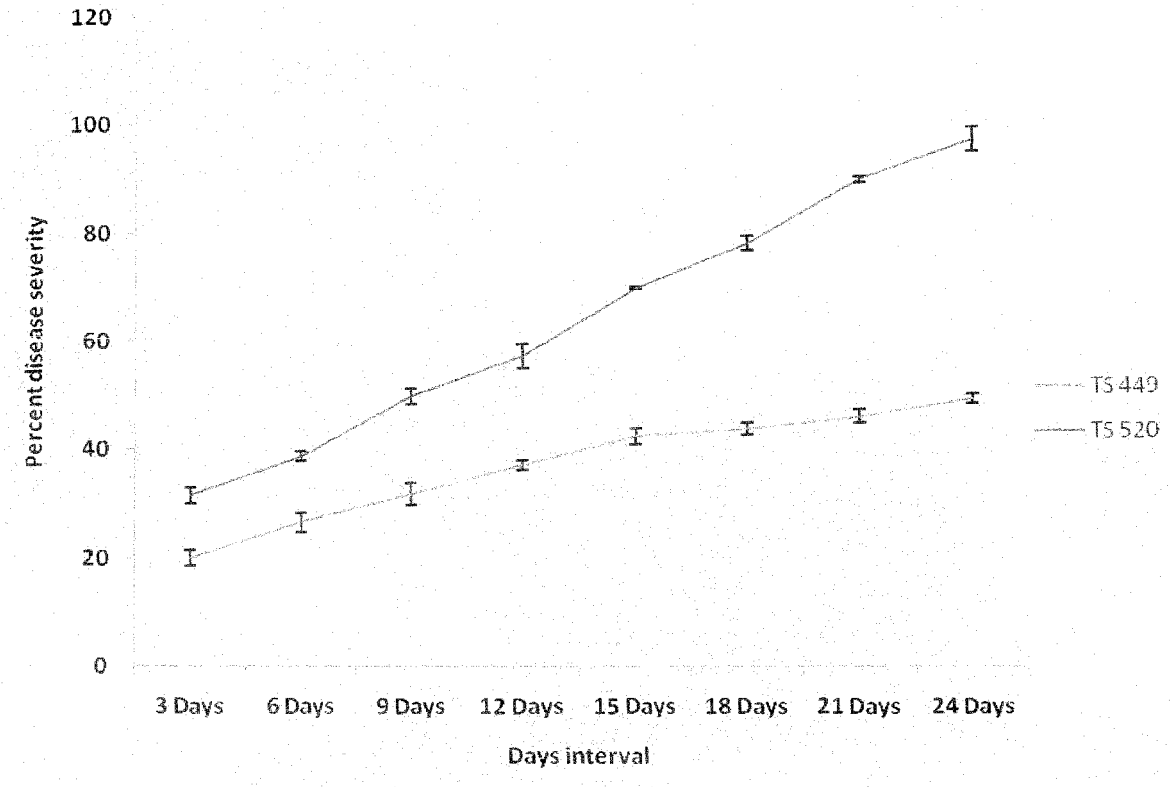

Fig. 4.1: Percent disease severity in two tea varieties (most resistant and most susceptible) tested

Chapter II: Studies on morphology and physiology of *R. solani*

4.6 Introduction

After the establishment of the fungi (*R. solani*) as pathogen of tea roots, it was considered worthwhile to study the morphological and physiological aspects of the fungi. *Rhizoctonia solani* have been found to affect tea seed varieties in their seedling stage. Sometimes the spread of the disease is so severe that the plants hardly could grow and severe necrotic symptoms on the tender roots and stems became evident. During the present study, we consistently found that the fungus attacks generally by sclerotial propagules. Sclerotia produced several branched mycelia and these mycelia entered the host through the epidermal layer. Mycelia were mostly superficial on the medium. After a certain period, shiny brown mycelia intermingled with black dots of sclerotial mass were visible on the surface of the medium. For conducting studies on host-parasite interaction and also to control a pathogen, it is important to know about the morphology and physiology of a fungus. Hence, it was necessary to culture the fungus in different artificial media. In addition, productions of inocula both in the form of sclerotia as well as vegetative mycelia are important for experimental purpose. Hence, to meet all this requirements we standardized a medium with optimized growth conditions of the fungus. The main aim of the present work was to study the environmental and nutritional requirements of culture media, temperature, pH and light for the optimal growth of mycelia and sclerotium formation of *Rhizoctonia solani.* The fungus was grown in several liquid and solid media. Different morphological characters of the fungus were also recorded.

4.7 Observation of the morphology of *R. solani* and other fungi

The colour of mycelia was initially white in PDA, OMA, and PDB media. It gradually turned yellowish and then brown with time. The growth of mycelia was profuse in PDA but less in OMA. However, radial growth was faster in

OMA than PDA. Brown coloured sclerotia was found to be scattered on the surface of PDA medium.

Microscopic observations of *R. solani* were made along with all the other fungi isolated and purified from the tea seed surfaces. For microscopic observations, mycelia were taken in microscopic slides from pure culture and stained using cotton-blue in lactophenol. The slides were mounted with cover glass, sealed and observed under microscope. Photographs of the microscopic fields (stained/unstained) have been shown in plates VI(b,d&f), VII(b,d&f), VIII(b,d&f), IX(b,d&f) and X(b,d&f). Photographs were taken in light microscope equipped with photo micrographic camera. The hyphae of *R. solani* produced branches at right and acute angles to the main hypha. The branch hypha was slightly constricted at the branch origin and often a septum was present near the branch origin. The *fungus* also produced specialized hyphae composed of compact cells called monilioid cells. The monilioid cells fuse together to produce hard structures called sclerotia. The diameter of the mature hypha was between 4-5µm. No asexual or sexual spores were observed (Plate XI, a-c).

4.8 Growth and Sclerotia formation of *R. solani* in solid media

In order to evaluate the vegetative growth and sclerotia formation of *R. solani* in solid media, nine different media viz. Potato dextrose agar (PDA), Oat meal agar (OMA), Root extract agar (REA), Czapek dox agar (CDA), Richard's agar (RA), Yeast extract mannitol agar (YEMA), Malt extract agar (MEA), Potato carrot agar (PCA) and Nutrient agar (NA) were used. Initially, petriplates with sterile medium were inoculated with the test fungus and incubated up to 12 days at 28±1°C for mycelia-inoculum production. Finally, mycelia blocks of 4 mm diameter were cut from the advancing zone of hyphae and placed in sterile petriplates (9 cm in diameter) containing 20 ml of different solid medium and were incubated for the required period. Experiments were performed with three replications.

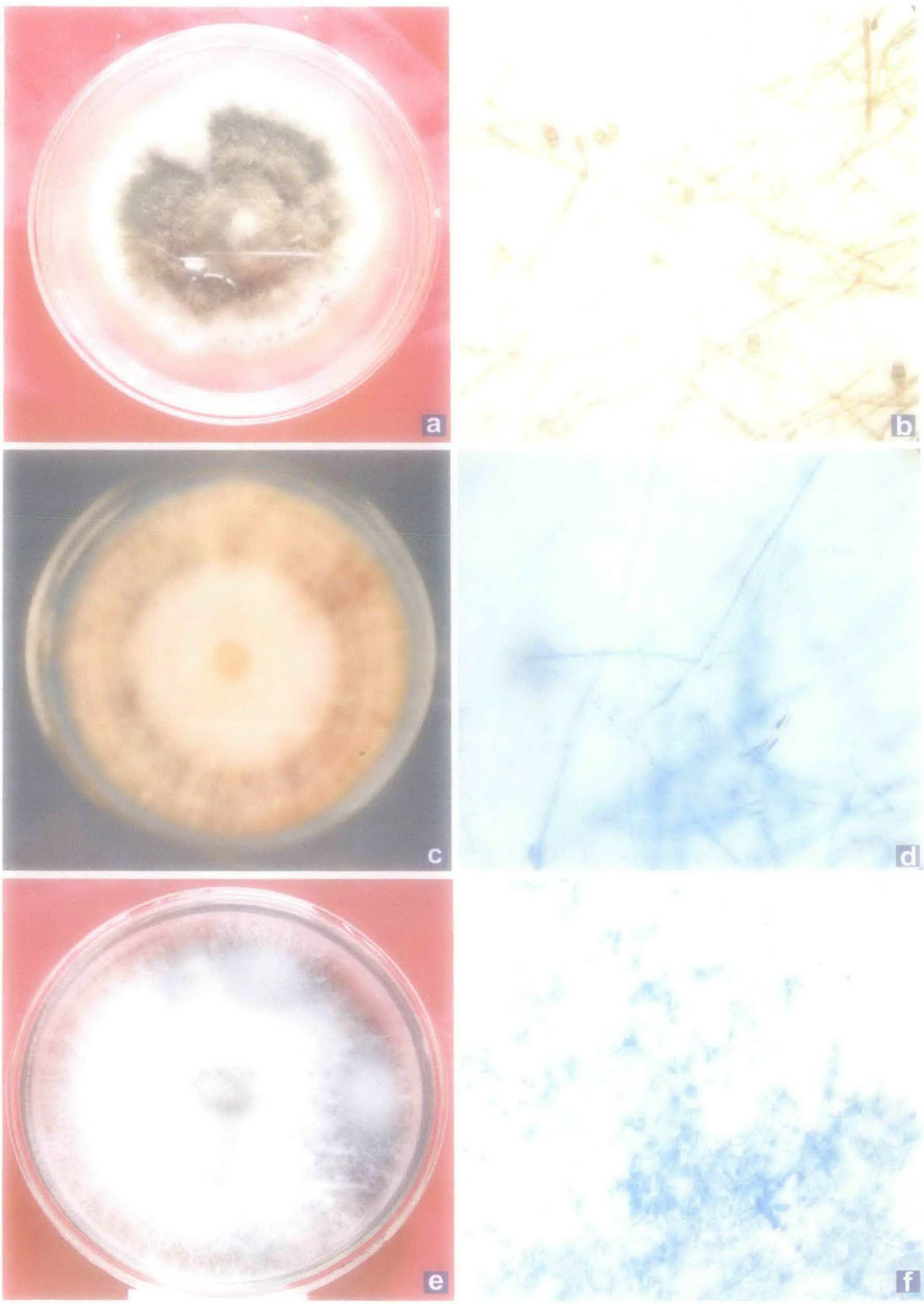

Plate VI: Various fungi isolated from tea seeds (a) *Curvularia lunata* in petriplate, (b) *C. lunata* under light microscope (mycelia and spores are visible) (c) *Rhizoctonia solani* in petriplate (d) Mycelia of *R. solani* under light microscope (e) *Fusarium* sp. in petriplate (f) Mycelia and spores of *Fusarium* sp.

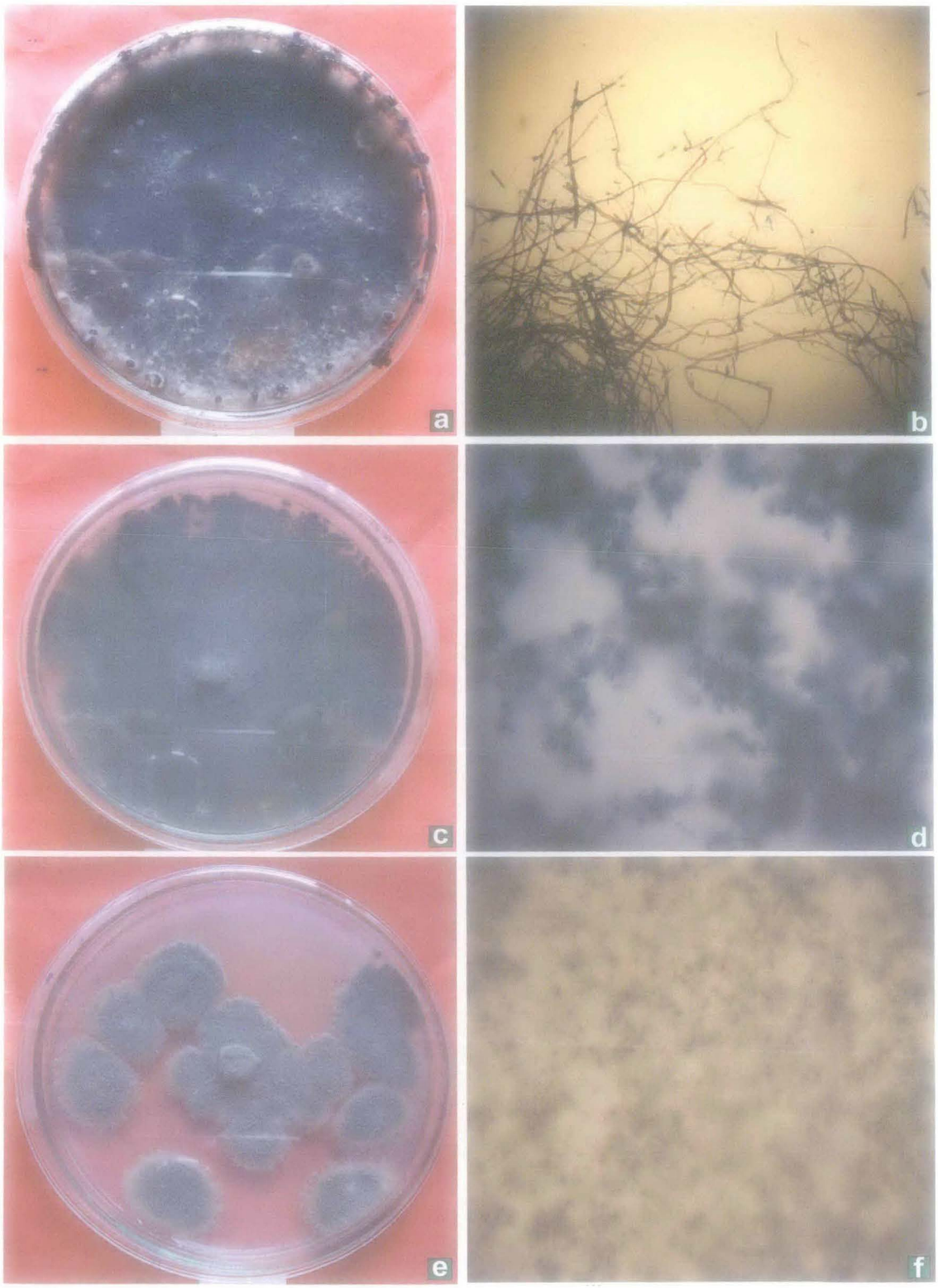

Plate VII: Various fungi isolated from tea seeds (a) *Alternaria* sp. (b) Mycelia of *Alternaria* sp. (c) *Aspergillus* sp. (Isolate I) (d) Spores of *Aspergillus* sp. (e) *Aspergillus* sp. (Isolate II) (f) Spores of *Aspergillus* sp. (Isolate II)

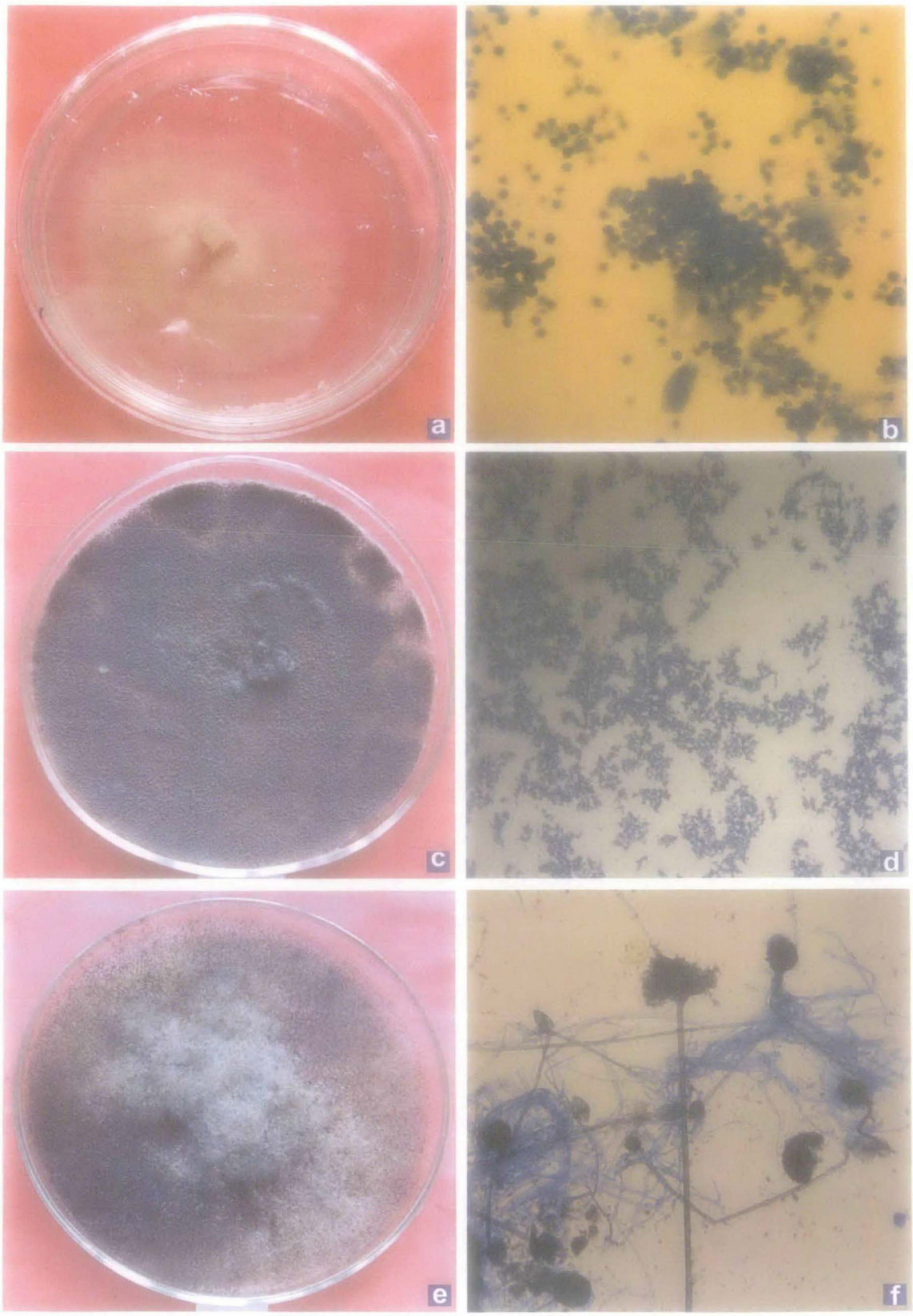

Plate VIII: Various fungi isolated from tea seeds (a) *Penicillium* sp. (b) Conidia and conidiophores of *Penicillium* sp. (c) *Aspergillus* sp. (Isolate III) (d) Spores of *Aspergillus* sp. (Isolate III) (e) *Rhizopus* sp. (Isolate I) (f) Sporangia, sporangiophores and mycelia of *Rhizopus* sp. (Isolate I)

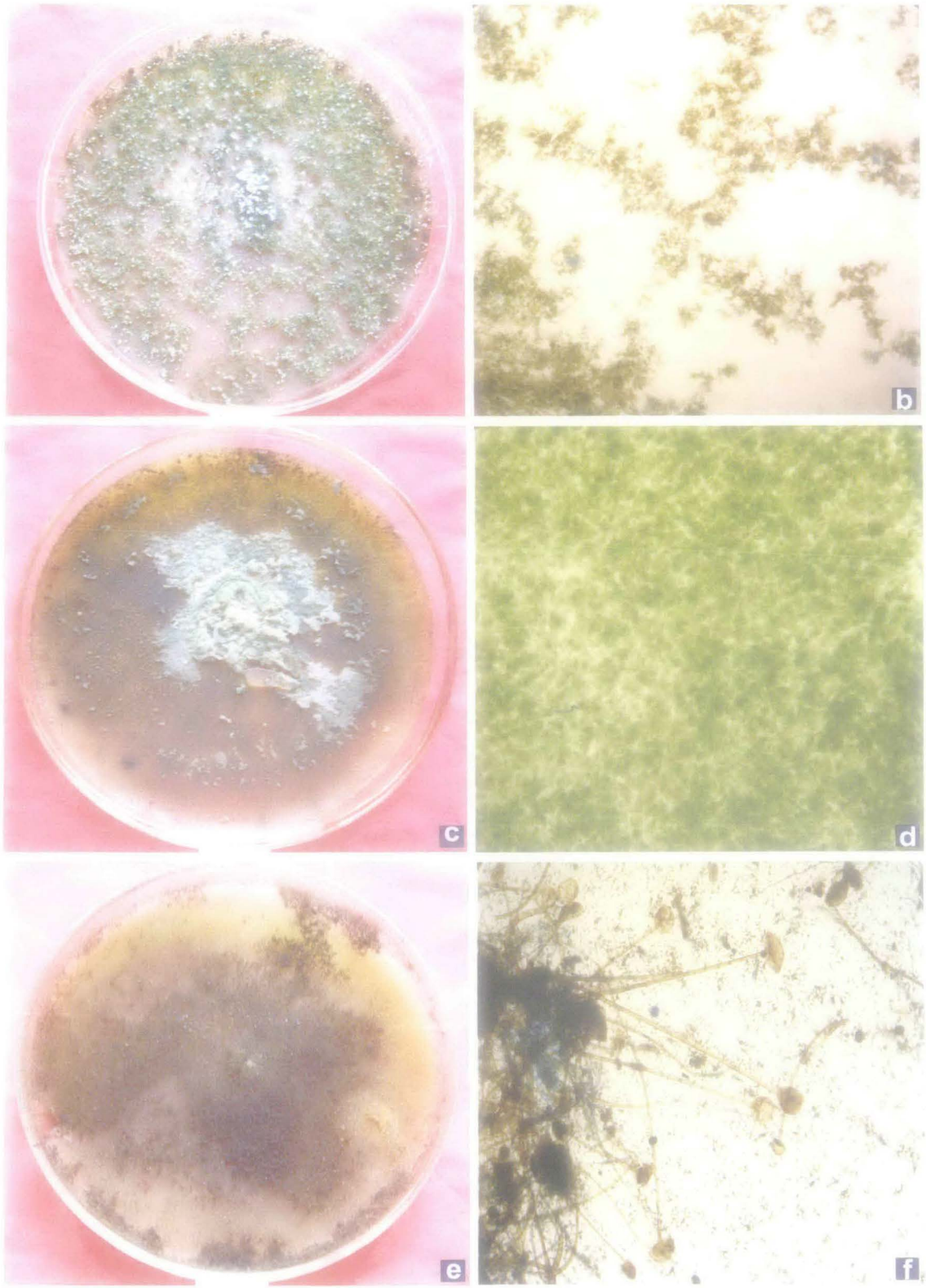

Plate IX: Various fungi isolated from tea seeds (a) *Aspergillus flavus* (b) Spores of *Aspergillus flavus* (c) *Trichoderma pseudokoningii* (d) Spores of *Trichoderma pseudokoningii* (e) *Rhizopus* sp. (Isolate II) (f) Sporangia, sporangiophores and mycelia of *Rhizopus* sp. (Isolate II)

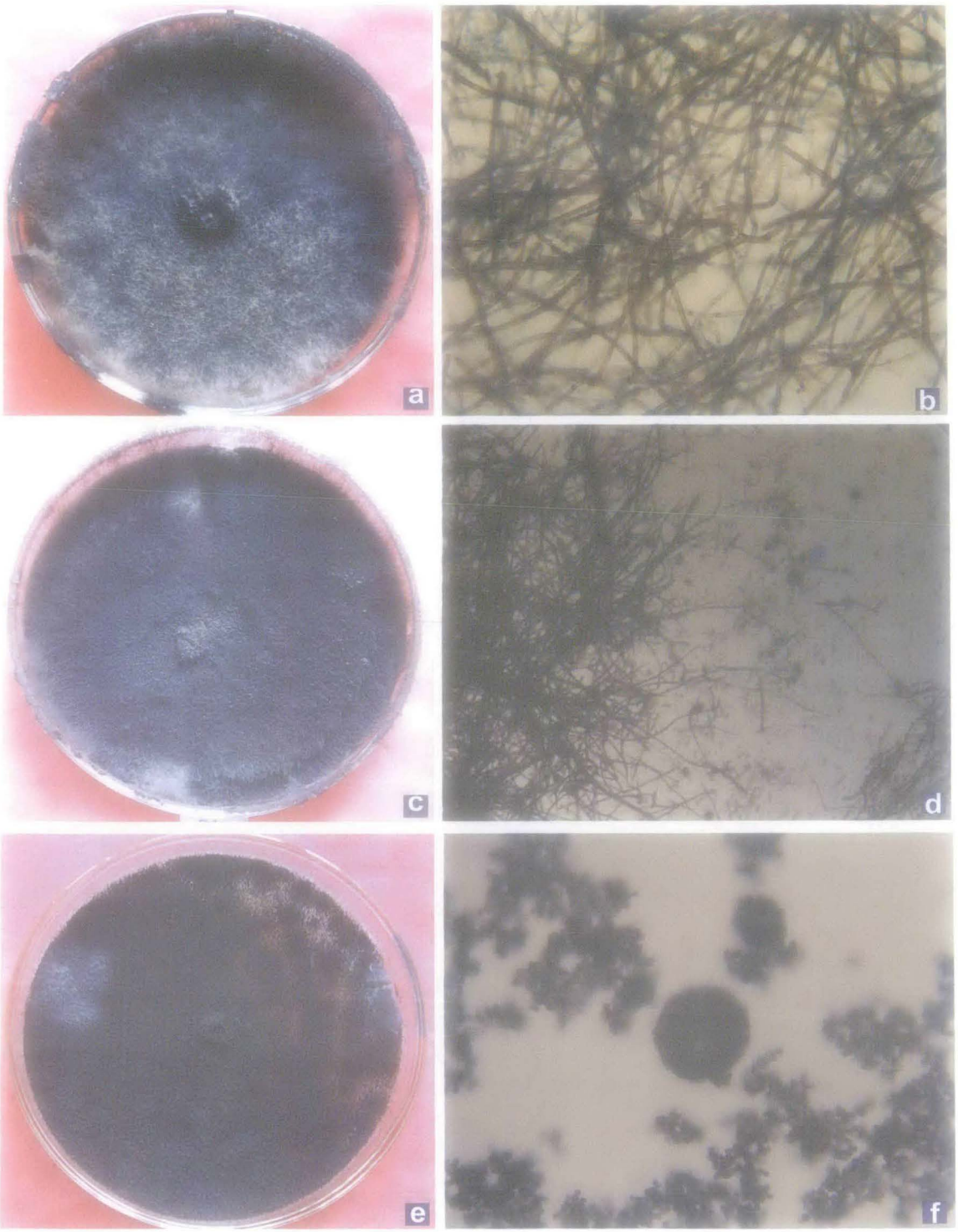

Plate X: Various fungi isolated from tea seeds (a) *Botryodiplo dia* sp. (b) Mycelia of *Botryodiplo dia* sp. (c) Sterile fungi (d) Mycelia of sterile fungi (e) *Asperg llus niger* (f) Spores of *Asperg llus niger*

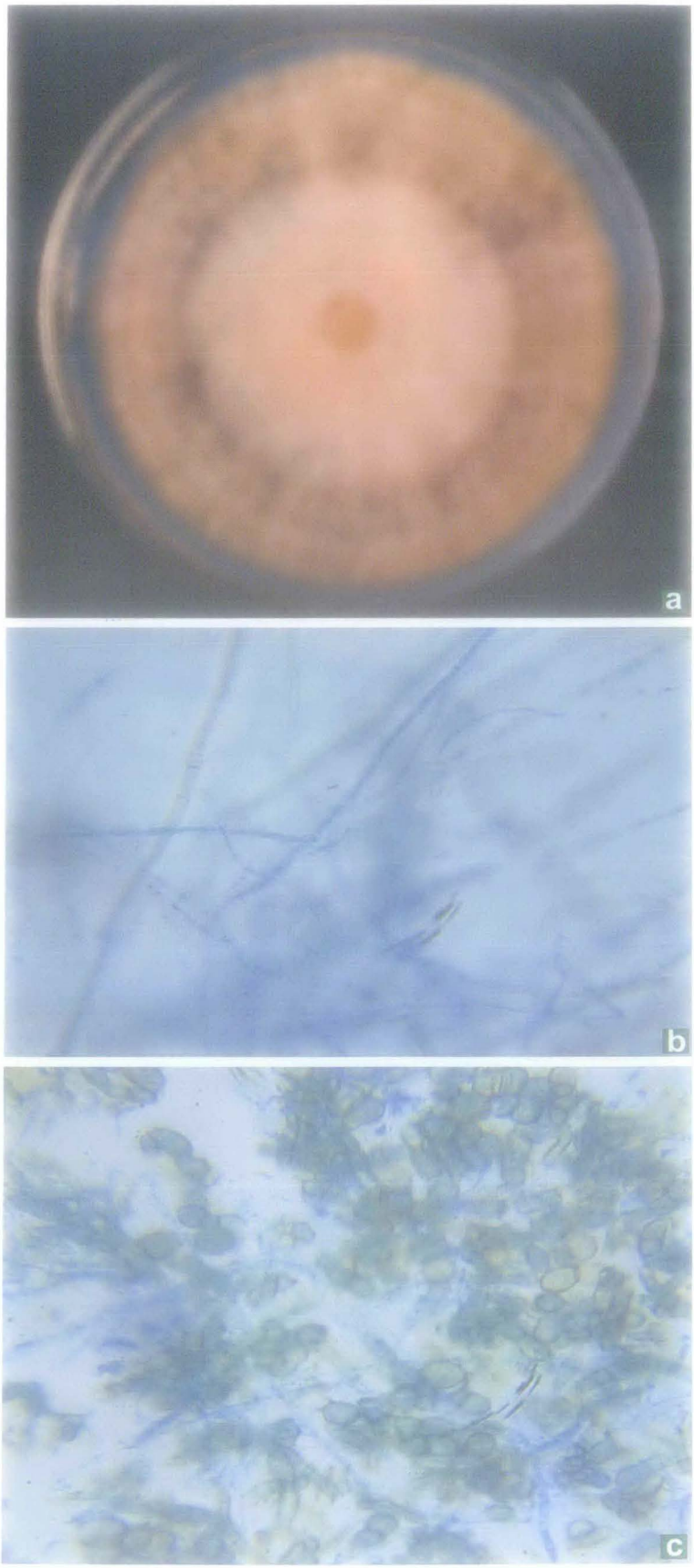

Plate XI: *Rhizoctonia solani* (a) *Rhizoctonia solani* in petri plate (b) Mycelia of *Rhizoctonia solani* (c) Moni loid cells of *Rhizoctonia solani*

From the results (Table 4.6 & Fig 4.2), it was evident that OMA (oat meal agar) was best for both growth and sclerotia formation of *R. solani*. After 10 days of incubation on OMA, radial growth of mycelia was 8.2 cm in diameter and sclerotia formation was also good. Radial growth of mycelia was 7.2 cm in PDA, after 10 days of incubation. No sclerotium formation was observed in PCA. In NA, RA and REA, sclerotia formation was comparatively less and lesser growth of mycelia was evident. Excellent sclerotium formation was observed in PCA and CDA with moderate growth of mycelia (7.2 and 7.06 cm in diameter respectively). Good mycelia growth was also observed in PCA, YEMA, and CDA. NA recorded lowest radial growth and sclerotia formation was also scarce.

4.9 Mycelia growth of *R. solani* in different liquid media

From the results (Table 4.7 and Fig. 4.3), it was evident that PDB was best for growth of *R. solani*. Mycelial dry weight was recorded as 330.23 mg after 25 days of inoculation in PDB medium. In OMB, mycelial dry weight was found 270.4 mg after 25 days of incubation but in RM mycelial growth was poor. Increasing trend of growth was observed from 5 days after inoculation and it continued till 20 days. Growth was found to decline after 25 days in all the three media tested (data not shown).

Table 4.6: Radial growth of mycelial and sclerotia formation of *R. solani* in different solid media

Medium of growth	Radial growth and sclerotia formation (days)											
	2 d		4 d		6 d		8 d		10 d		12 d	
	Rg(cm)	s	Rg(cm)	s	Rg(cm)	s	Rg(cm)	s	Rg(cm)	s	Rg(cm)	s
PDA	0.95± 0.12	-	2.20± 0.27	-	4.73± 0.60	++	6.33± 0.85	+++	7.2± 0.88	+++	8.28± 1.40	++++
OMA	0.58± 0.09	-	2.90± 0.11	-	5.08± 0.81	++	7.03± 0.81	++	8.2± 1.01	++	9.0± 1.35	+++
REA	0.45± 0.02	-	1.20± 0.13	-	3.02± 0.51	-	4.36± 0.57	+	5.60± 0.76	+	6.21± 0.91	+
CDA	0.90± 0.05	-	1.70± 0.06	-	4.00± 0.30	++	5.85± 0.72	+++	7.06± 1.19	+++	8.00± 1.15	++++
RA	0.40± 0.09	-	1.00± 0.03	-	2.61± 0.43	-	4.01± 0.62	+	5.21± 0.83	+	5.90± 1.04	+
YEMA	1.025± 0.04	-	2.50± 0.39	-	4.25± 0.46	+	6.70± 0.98	++	7.38± 1.11	++	8.40± 1.31	++
MEA	1.05± 0.03	-	2.30± 0.49	-	4.00± 0.51	++	5.20± 1.3	++	5.95± 1.12	++	7.00± 1.25	+++
PCA	0.94± 0.16	-	2.00± 0.47	-	4.98± 0.84	-	6.64± 1.09	-	7.15± 1.12	-	8.1± 1.22	-
NA	0.36± 0.05	-	0.96± 0.13	-	2.09± 0.38	-	3.87± 0.57	+	4.98± 0.94	+	5.25± .0.82	+
CD @ 5%	0.14		0.51		0.99		1.49		1.16		2.09	

Data represents mean of three replications. Data after ± represent standard error values. Rg=Radial growth, S = Sclerotia formation, - = Nil, + = Poor, ++ = Fair, +++ = Good, ++++ = Excelent

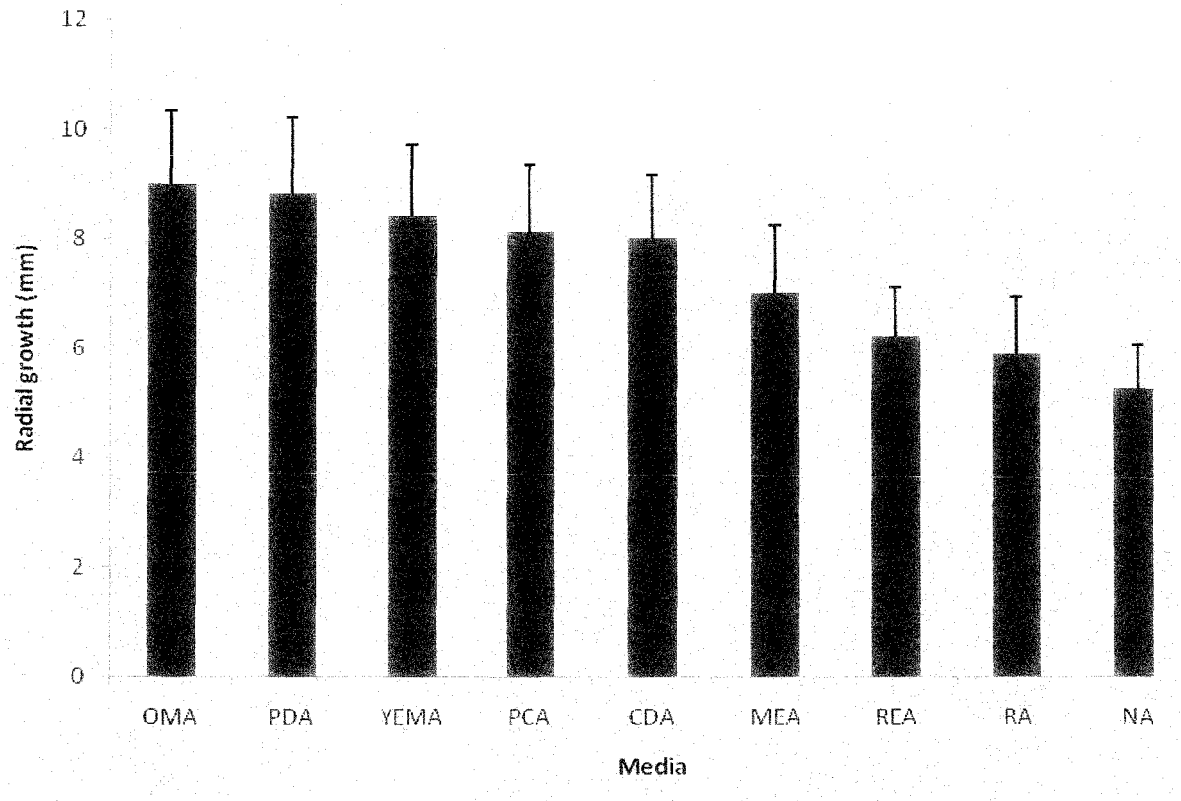

Fig.4.2 Radial mycelial growth of *R. solani* in different solid media

Table 4.7: Mycelia growth of *R. solani* after different incubation periods in different liquid media

Medium of growth	Mycelial dry weight (mg) after incubation period (days)				
	5 d	10 d	15 d	20 d	25 d
PDB	40.66±0.73	189.2±1.02	240.23±1.15	310.33±1.12	330.23±1.17
OMB	20.0±0.58	88±1.04	197.6±1.15	240.0±1.00	270.4±0.84
RM	10.0±0.40	44.5±.58	107.0±1.04	147.1±1.25	186.7±0.70
CD @ 5%	2.48	3.25	3.42	9.65	14.21

Data represents mean of three replications. Data after ± represent standard error values.

4.10 Effect of different pH on mycelia growth of *R. solani*

Potato dextrose broth (PDB) was adjusted to pH 4, pH 5, pH 5.5, pH 6, pH 6.5 pH 7 and pH 8 by adding 1(N) NaOH or 1(N) HCL drop-wise into the medium before sterilization. After adjusting the pH in PDB the media was sterilized. Each 250 ml Erlenmeyer flask contained 50 ml sterilized medium. Media of different pH were inoculated separately by 4-5 mm mycelial discs of *R. solani* and incubated at 28±1°C. Mycelial dry weight was recorded after 5, 10, 15, 20, and 25 days of inoculation.

Experimental results revealed that *R. solani* was able to grow within a wide range of pH, from 4.0 to 8.0 (table 4.8 and Fig. 4.4). The fungus however, failed to grow in alkaline environment, beyond pH 8.0 (data not shown). The optimum pH for growth was recorded at the pH 6.5. At this pH, the mycelial growth was observed to be 316.3 mg after 25 days of inoculation. Moderate growth was observed at pH 5.5, 6.0 and 7.0. Poor growth was observed at pH 5.0 and at pH 8.0. The results indicated that slightly acidic to neutral pH was optimum for the growth of *R. solani.*

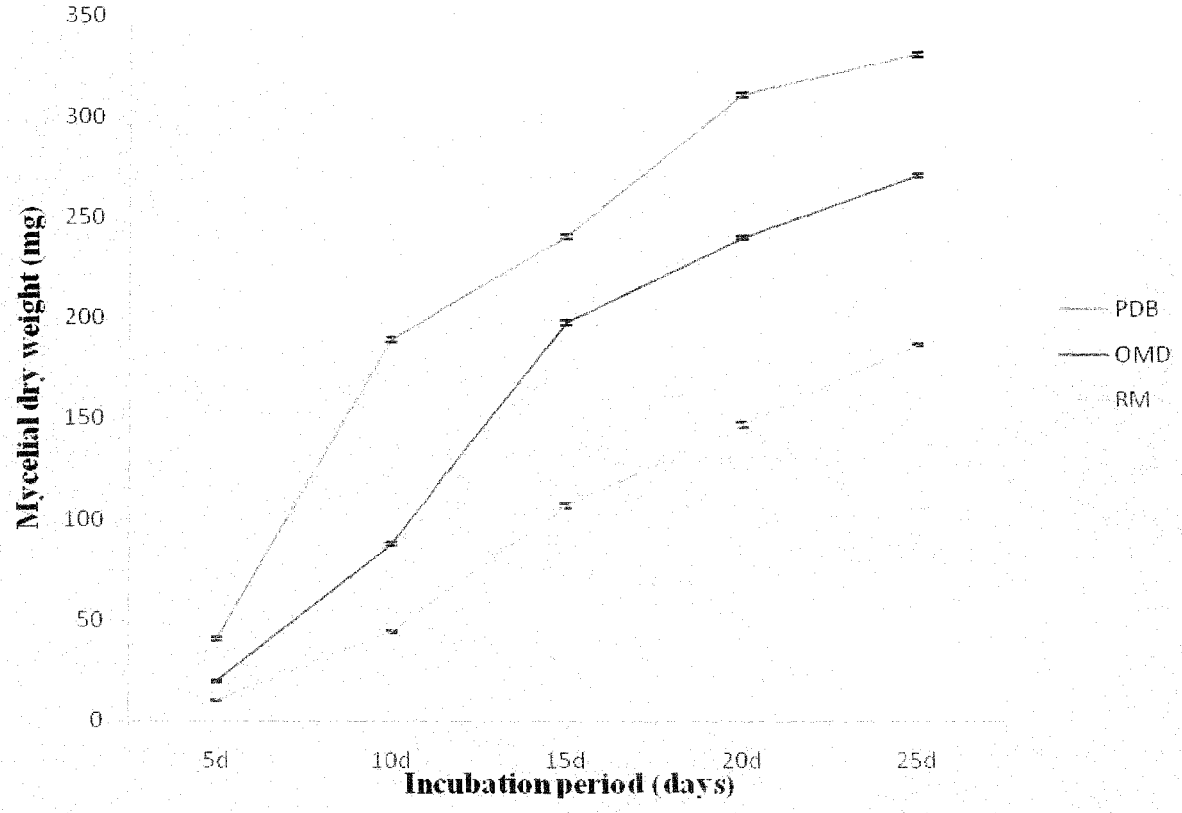

Fig 4.3: Growth of *R. solani* after different incubation periods in different liquid media

Table 4.8: Effect of different pH on the growth of *R. solani* in PDB

pH	Mycelial dry weight (mg) after incubation period(days)				
	5	10	15	20	25
4.0	5.3±1.42	57.3±2.09	127.2±3.25	176.7±3.15	160.7±3.41
5.0	7.8±0.68	61.3±2.9	132.3±2.97	183.5±3.21	171.0±3.51
5.5	14.3±1.44	67.8±4.02	140.3±3.31	193.0±3.04	180.2±4.41
6.0	24.1±1.8	73.4±2.02	147.5±1.32	190.7±4.16	201.6±3.2
6.5	38.3±1.2	164.8±1.86	223.7±2.43	271.5±3.34	316.3±2.9
7.0	12.5±0.98	51.6±2.06	119.4±1.02	165.9±2.63	152.3±2.34
8.0	1.7±0.44	48.2±1.76	114.7±2.75	159.2±2.23	145.7±2.2
CD@5%	1.8	4.6	3.8	2.5	5.3

Data represents mean of three replications. Data after ± represent standard error values.

4.11 Effect of different incubation temperatures on mycelial growth of *R. solani*

The growth of *R. solani* was assessed in OMB at different temperatures and the results have been summarized in table 4.9 and Fig 4.5. Dry weights of fungal mycelium were recorded after five days intervals up to 25 days. Results revealed that 28°C was optimum for maximum growth of fungal mycelia. At this temperature, the mycelia dry weight recorded after 5, 10, 15, 20 and 25 days were 2.5 mg, 51.6 mg, 119.4 mg, 165.9 mg and 196.7 mg respectively. Very poor growth was recorded when culture flask was incubated at 8°C. However, the overall results presented in Fig 4.5 indicate that *R. solani* was capable of growing at temperatures that ranged between 13°C to 38°C.

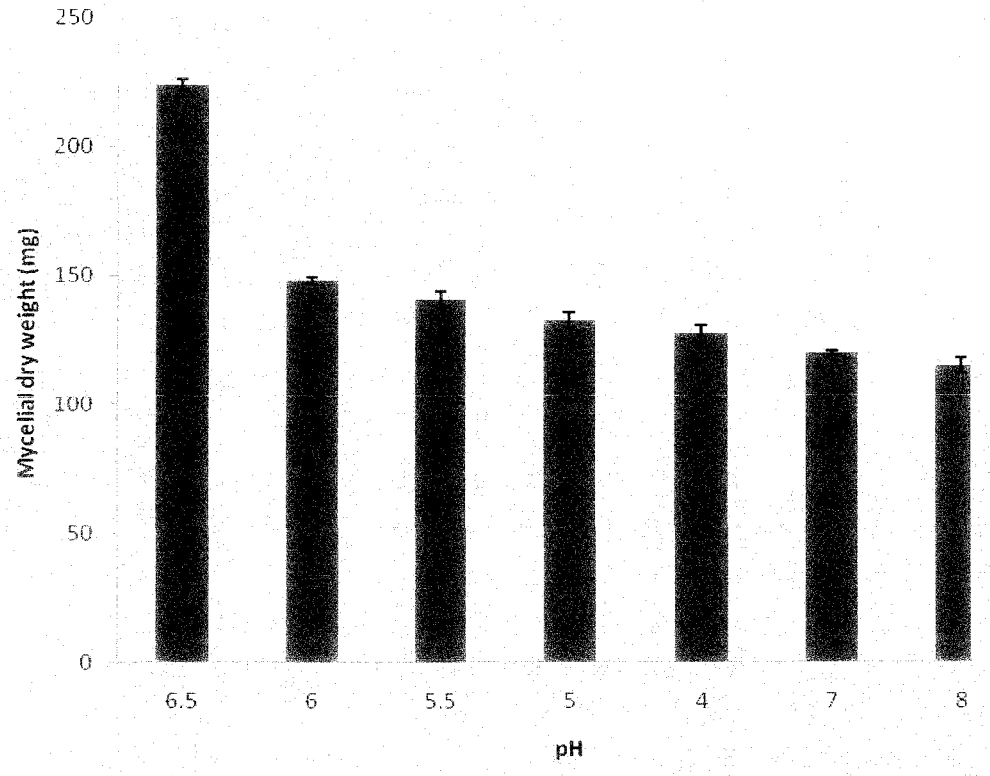

Fig 4.4: growth of R. solani at different pH conditions

Table 4.9: Effect of different temperatures on mycelia growth of *R. solani*

Temperatures (°C)	Mycelial dry weight(mg) after incubation period(days)				
	5	10	15	20	25
08	0.2±0.03	13.2±0.28	35.7±1.79	55.2±1.55	66.2±1.90
13	0.5±0.08	21.8±0.15	52.8±2	77.8±2.21	91.8±2.16
18	1.2±0.11	37.7±0.17	77.9±2.13	111.9±2.26	131.9±2.05
23	1.9±0.17	43.9±0.15	102.4±1.58	144.5±2.36	171.0±1.81
28	2.5±0.2	51.6±1.9	119.4±2.30	165.9±3.10	196.7±2.27
33	2.3±0.15	42.8±1.28	102.6±3.21	143.6±2.22	172.4±3.75
38	2.0±0.15	40.5±1.44	89.0±1.52	122.6±2.08	143.1±2.61
CD @ 5%	0.25	1.83	3.32	4.02	1.91

Data represents mean of three replications. Data after ± represent standard error values.

4.12 Assessment of mycelia growth and Sclerotia formation of *R. solani* on different carbon sources

From the results (Table 4.10 and Fig.4.6) it was evident that *R. solani* showed a gradual increase in growth until 25 days in all cases. Mycelial dry weight was maximum (525.2 mg) after 25 days of incubation when mannitol was used as carbon source. With sorbitol as carbon source mycelial growth was 496.4 mg 25 days of incubation. Minimum growth (217.2 mg) was recorded after 25 days of incubation. Mannitol was also found as the best among the different carbon sources tested when overall growth pattern of *R. solani* was observed. Sclerotia formation was excellent in all cases after 20 days of incubation.

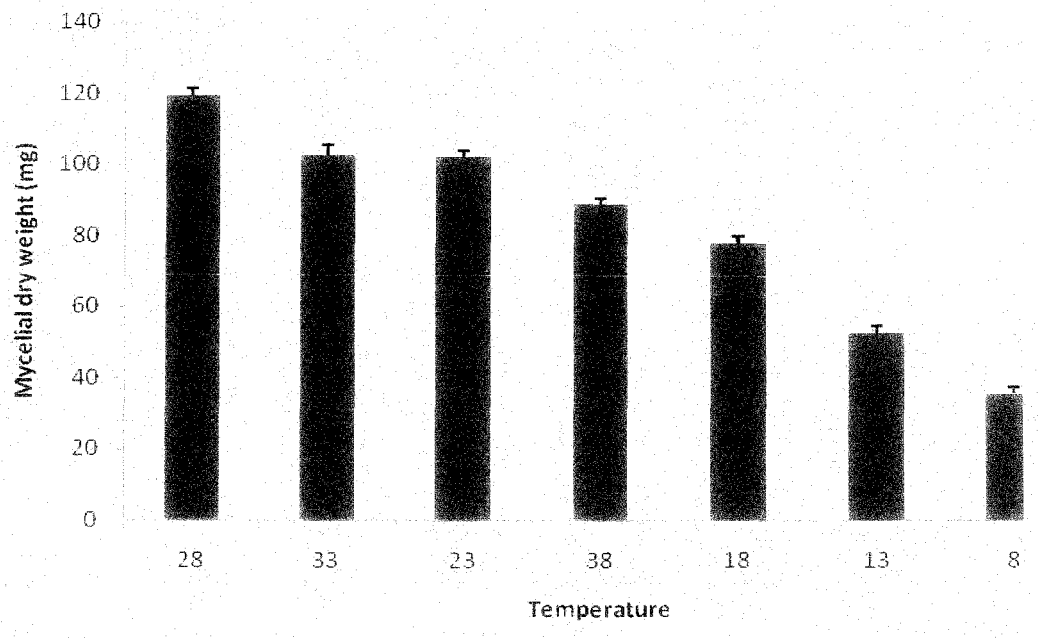

Fig 4.5: Effect of different temperature on mycelial growth of *R. solani*

Table 4.10: Effect of different carbon sources on mycelia growth of *R. solani*

Carbon source		Incubation period(days)				
		5 d	10 d	15 d	20 d	25 d
Glucose	Mycelial Dry wt (mg)	105.8±1.52	164.8±1.33	244.6±2.6	306.1±1.67	352.6 ±1.44
	Sclerotia formation	+	++	+++	++++	++++
Sucrose	Mycelial Dry wt	100.9±1.15	156.5±1.32	233.3±1.17	290.5±1.60	331.5 ±0.76
	Sclerotia formation	+	++	+++	++++	++++
Mannitol	Mycelial Dry wt	208.5±0.5	283.0±0.72	378.1±1.05	459.6±0.87	525.2 ±1.60
	Sclerotia formation	+	++	+++	++++	++++
Lactose	Mycelial Dry wt	7.6±0.55	59.6±1.22	130.6±0.90	181.6±0.83	217.2 ±0.8
	Sclerotia formation	+	++	+++	++++	++++
Sorbitol	Mycelial Dry wt	203.6±0.83	273.4±1.22	363.1±0.58	436.6±1.44	496.4 ±1.73
	Sclerotia formation	+	++	+++	++++	++++
Inositol	Mycelial Dry wt	109.9±0.95	173.4±1	257.9±1.59	325.1±1.90	379.6 ±2.08
	Sclerotia formation	+	++	+++	++++	++++
Control	Mycelial Dry wt	2.5±0.25	51.6±1.13	119.4±1.02	165.9±1.06	196.3 ±0.88
	Sclerotia formation	–	++	++	++	++
CD @ 5%		24.53	2.30	3.03	2.92	2.76

Data represent mean of three replications. Data after ± represent standard error values

Sclerotia formation: – = Nil, + = poor, ++ = fair, +++ = good, ++++ = excellent. Control= basal medium without carbon source.

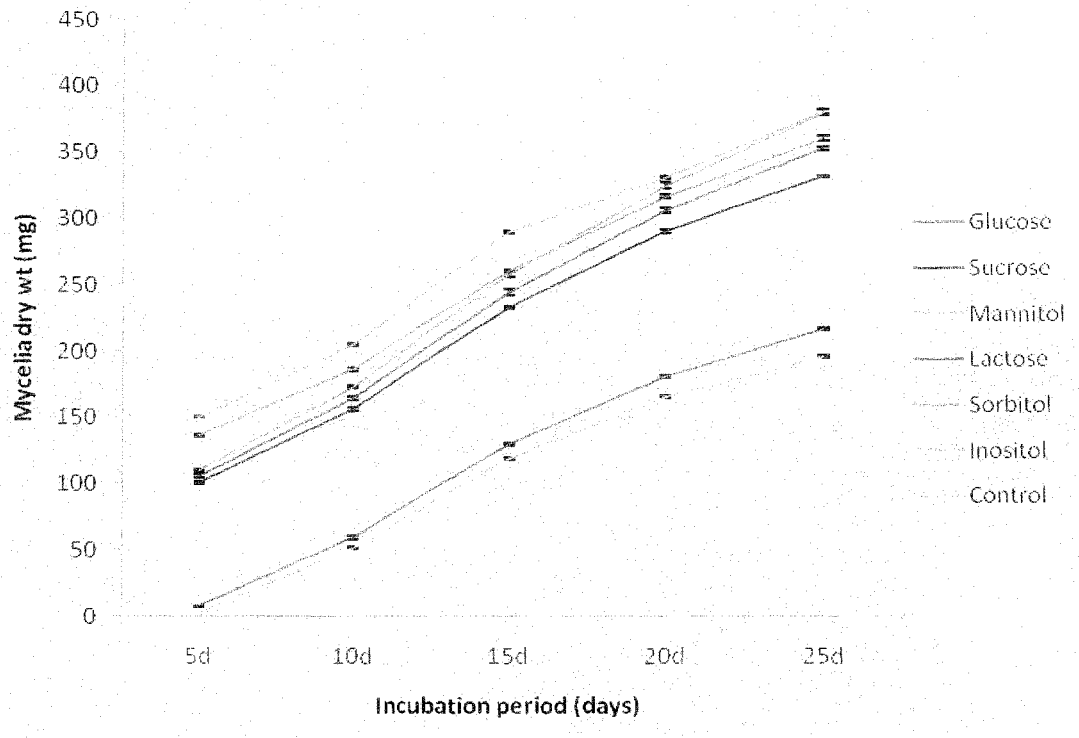

Fig 4.6: Effect of different carbon sources on mycelial growth of *R. solani*

4.13 Assessment of mycelial growth and sclerotia formation of *R. solani* on different nitrogen sources

From the results (Table 4.11 and Fig. 4.7) it was evident that *R. solani* showed highest growth (360.5 mg) in beef extract after 25 days of incubation. The other two organic nitrogen sources, peptone and trypton also showed satisfactory growth (345.8mg and 338.1mg respectively) after 25 days of incubation. Among the inorganic nitrogen sources tested, sodium nitrate and potassium nitrate showed best results (337.0mg and 308.0mg respectively) after 25 days of incubation. Least mycelial growth (253.9 mg) was produced by media containing ammonium sulphate as nitrogen source. In all cases, maximum increase in growth was observed up to 25 days of incubation and it declined there after (data not shown). Sclerotia formation was found good after 15 days of incubation and continued to increase until 25 days. Insignificant growth without any sclerotia formation was observed in control set.

Table 4.11: Effect of different nitrogen sources on the growth and sclerotia formation of *R. solani*

Nitrogen source	Incubation periods (Days)									
	5d		10d		15d		20d		25d	
	Mwt. (mg)	S	Mwt. (mg)	S	Mwt. (mg)	S	Mwt. (mg)	S	Mwt. (mg)	S
Organic										
Peptone	38.2±1.2	-	115.4±1.02	+	190.0±1.52	++	312.6±0.87	+++	345.8±1.17	+++
Yeast extract	32.1±1.64	-	104.5±1.46	+	166.5±1.56	++	297.3±0.77	+++	314.1±0.95	+++
Beef extract	35.1±0.75	-	107.2±2.02	+	174.0±1.00	+	322.7±1.80	++	360.5±0.70	++++
Glycine	28.0±1.52	-	104.0±1.0	-	167.7±1.12	+	284.4±1.49	++	304.2±2.25	++
Trypton	33.1±1.20	-	110.2±0.23	-	164.2±0.55	+	276.5±0.60	++	338.1±0.81	++
Inorganic										
Sodium nitrate	29.3±0.41	-	108.2±0.47	+	207.7±1.12	+	287.4±0.28	++	337.0±0.64	++
Potassium nitrate	21.0±0.11	-	94.1±0.37	+	188.6±0.35	++	263.8±0.70	++	308.0±0.64	++
Ammonium nitrate	14.2±0.30	-	82.0±0.65	+	169.6±0.70	+	239.5±0.60	+	279.4±1.31	+
Ammonium sulphate	9.5±0.34	-	72.3±0.40	+	153.8±0.61	+	218.3±0.88	+	253.9±0.58	++
Control	2.5±0.40	-	5.1±0.6	-	11.9±0.83	-	16.5±0.5	-	19.6±0.9	-
CD @ 5%	1.18		1.06		1.74		1.56		1.34	

Data represent mean of three replicates ± standard error. Mwt = Mycelia dry weight; S = Sclerotia formation, – = Nil, + = poor, ++ = fair, +++ = good, ++++ = excellent. Control set contained basal medium without nitrogen source.

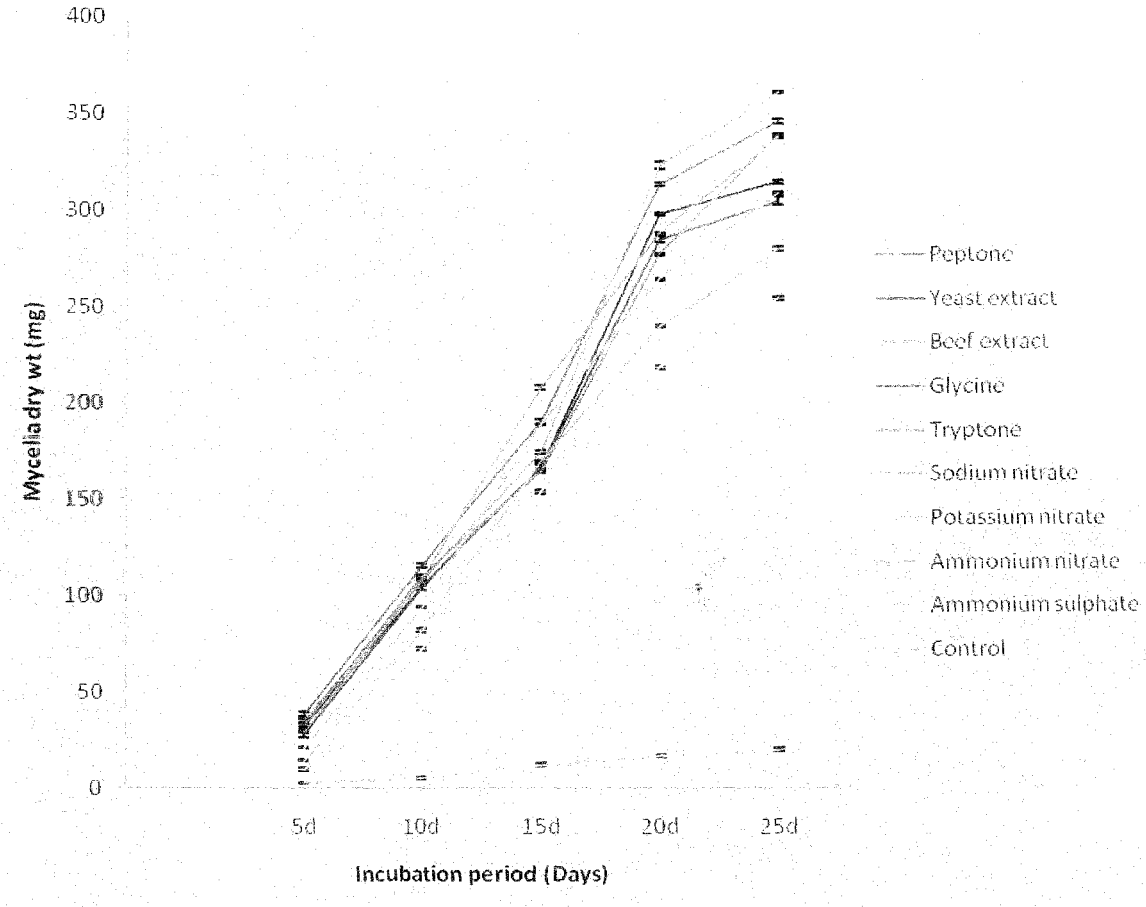

Fig 4.7: Graphical representation of different nitrogen sources on mycelial growth of *R. solani*.

Chapter III: Serological studies for detection of level of cross reactive antigens, responsible for determining susceptibility in different varieties of tea

4.14 Introduction

Serology plays an important role in the study of plant pathology. Pathogenicity of a microorganism can be detected by this method indirectly by detecting cross reactive antigens (CRA). Thus, whether a pathogen is virulent or avirulent or whether a cultivar is susceptible or resistant can be detected by determining the level of CRA. The presence of CRA at significant level between a fungal pathogen and a plant host cultivar is the determining factor of susceptibility and resistance of a cultivar. CRA can be detected by serological experiments like immuno-diffusion, immuno-electrophoresis, ELISA etc. In the present study serological experiments which included immuno diffusion, immuno electrophoresis and ELISA experiments were done to determine the presence of common antigens, among the tea seed/seedling varieties and the fungal pathogen *Rhizoctonia solani.* Antigens were prepared from the virulent isolate of *R. solani* and also from the root of different tea seed varieties (TS-449, TS-506, TS-463, TS-491, TS-462 and TS-520). Polyclonal antisera were raised in three separate male white rabbits against the antigens of one comparatively resistant tea seedling variety (TS-449), one susceptible tea seedling variety (TS-520) and the pathogen *R. solani.* Normal sera were collected before immunization by puncturing the marginal vein of the ear of the rabbit.

4.15 Relationship between different tea varieties and *Rhizoctonia solani* by agar gel double diffusion

The serological relationship between host and pathogen was determined following the standard method as described by Ouchterlony (1958) through agar gel double diffusion test. Initially, the antibody activity of all types of raised antisera against their respective homologous antigens was determined

semi quantitatively i.e. the titre values of the antigens TS-449, TS-520 and *R. solani* against their homologous antisera were determined. Results have been presented in Table 4.12. For determining common antigenic relationship, each of the raised antiserum was allowed to react with root antigens of all together six test varieties of tea (TS-449, TS-506, TS-463, TS-491, TS-462 and TS-520) and the antigen of the pathogen *R. solani.* A non pathogen (*Alternaria porri*) was included as negative control. The detailed methods of antigen and antisera preparation have already been discussed in the materials and methods section 3.10. The results have been shown in table 4.13.

From the results presented in Table 4.13 and Plate XII, it was observed that common antigenic relationship were present not only in cases of homologous reactions i.e. between antisera (RsA) and antigen (Rsa) of *R. solani* (Plate XII, iv), but also in cross reactions between antisera of *R. solani* and antigens from the root of TS-520 (520a).There was no precipitation band in reaction of 449a (root antigen of resistant variety TS-449) and RsA (antisera raised from mycelia of *R. solani*) (Plate XII, iii). Common precipitation bands were also observed when root antigens TS-462, TS-463, and mycelia antigen of *R. solani* were reacted with antisera of TS-520. Antisera of TS-449(449A) also reacted with antigens, 462a & 463a respectively of the varieties, TS462 and TS463. Similar reactions were also performed with the antigens of the two varieties TS-506 and TS-491 against the three antisera RsA, 449A and 520A.

Presence or absence of common antigens between host and pathogen were clearly determined by imunodiffusion test. However, it was not clear whether single or multiple antigens were present in a precipition line produced in an 'antigen-antisera' reaction. To overcome this electrophoresis were performed to separate the antigens before exposing them to antisera.

Table 4.12: Semiquantitative estimation of antigens and antisera of tea varieties and *R. solani*

Host and pathogen	Titre of antigen against homologous antiserum	Titre of antiserum against homologous antigen
Host variety		
TS 449	8	16
TS 520	8	16
Pathogen		
R. solani	16	32

Incubation time-72h; Temperature-25±1°C

Table 4.13: Common antigenic relationship between tea varieties and *R. solani* (based on agar gel double diffusion)

Antigen of pathogen, host and non-pathogen with their code in parentheses	Antisera of pathogen and host		
	Fungal pathogen	Tea varieties	
	Rhizoctonia solani (RsA)	TS 449 (449A)	TS 520 (520A)
Pathogen			
R. solani (Rsa)	+	-	+
Resistant variety			
TS 449 (449a)	-	+	+
Susceptible varieties			
TS 506 (506a)	+	+	+
TS 491 (491a)	+	+	+
TS 463 (463a)	+	+	+
TS 462 (462a)	+	+	+
TS 520 (520a)	+	+	+
Non-pathogen			
Alternaria porri (Apa)	-	-	-

Common precipitation band present=+; Common precipitation band absent= -

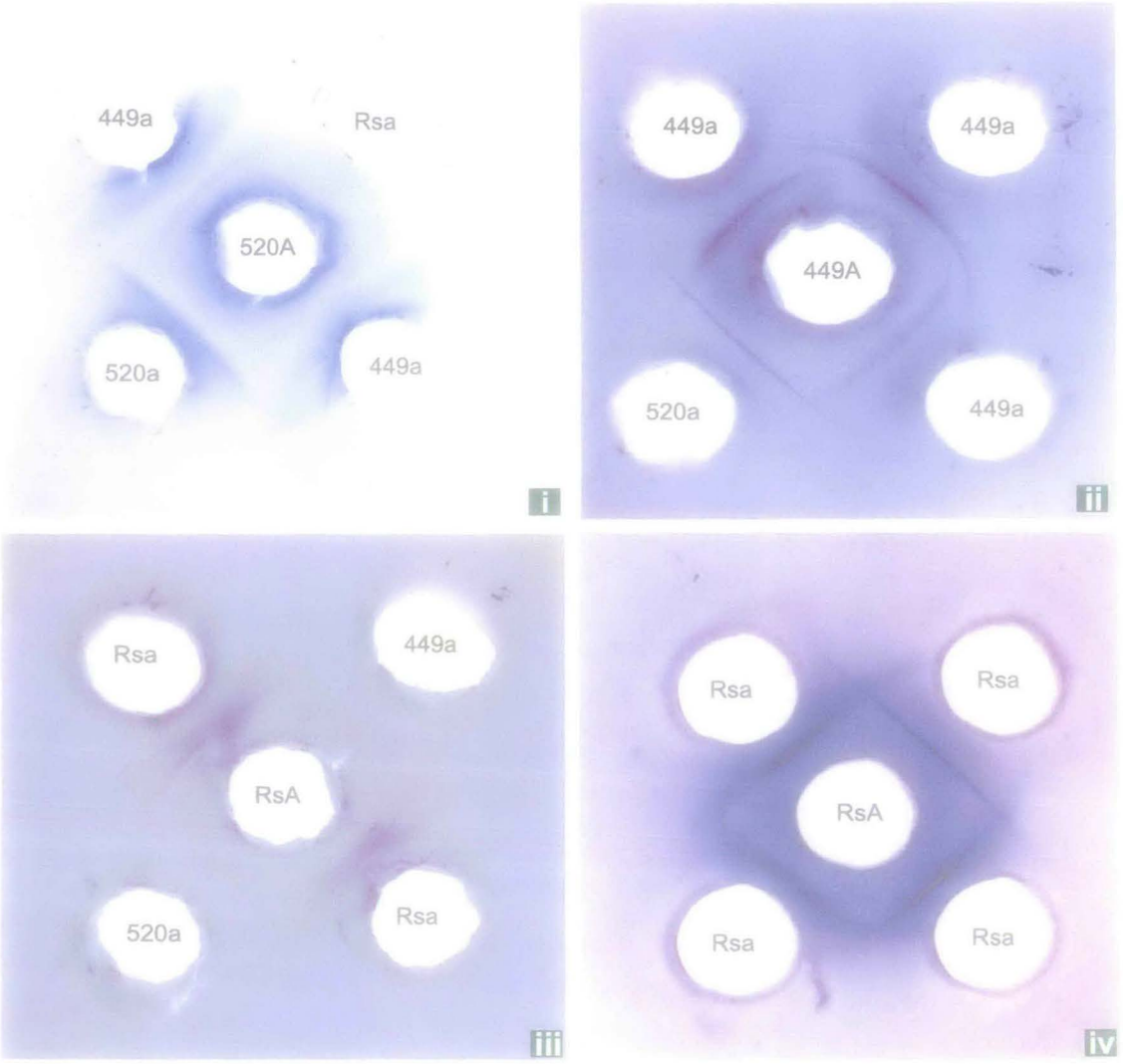

Plate XII: Agar gel double diffusion test using different antigens & antisera. Codes of antisera & antigen are shown in the wells. Numericals used to indicate respective tea varieties and suffix 'A' indicate antisera and 'a' indicate antigen. 'Rs' indicate *Rhizoctonia solani* mycelia. (i)Root antisera of TS-520 (520A) reacted with root antigens of TS-520(520a), TS449 (449a) and of mycelia antigen (Rsa). (ii) Root antisera of TS-449 (449A) reacted with antigens of TS-449 (449a), TS-520 (520a) and of mycelia antigen (Rsa). (iii) Mycelia antisera of *R.solani* (RsA) reacted with mycelia antigen of *R.solani* (Rsa) and with root antigens of TS-520 (520a) and TS-449 (449a). (iv) Mycelial antisera of *R. solani* (RsA) reacted with antigen of *R.solani* (Rsa).

Plate XIII: Agar gel double diffusion test using different antigens & antisera (i) Root antigen of TS-462(462a), root antigen of TS-520(520a), reacted with root antisera of TS-520 (520A). (ii) Root antigen of TS-463(463a), antigen of *R.solani* (Rsa), root antigen of TS-449(449a) and root antigen of TS-462(462a) reacted with root antisera of TS-449(449A). (iii) Root antigen of TS-449(449a), root antigen of TS-462(462a), Root antigen of TS-449(449a) and normal srea (NS) reacted with root antisera of TS-449 (449A).

4.16 Serological relationship between different tea varieties and pathogen *R. solani* by immunoelectrophoresis

Immunoelectrophoresis includes both electrophoresis and immunodiffusion in gel. In this technique of serology, antigens are first electrophoresed in agar gel and then antisera are allowed to diffuse through the agar gel. In agar gel, movement of molecules in an electric field is similar to that in liquid medium, with the advantage that free diffusion during and after electrophoresis is lessened (Clausen, 1969).

In immunoelectrophoresis experiments, three antisera i.e. antisera of *R. solani,* TS-449 and TS-520 were used. Antigens were prepared from mycelia of *R. solani* and roots of two different tea varieties. The detailed procedure has already been discussed in the materials and method (section 3.10). The results of the experiments have been presented in table 4.14 and table 4.15.

From table 4.14, 4.15 and Plate XIV, it was evident that antigen of *R. solani* shared three precipitation arcs with its homologous antisera and two precipitation arcs with the antisera of TS-520 but no precipitation arc was visible with the antisera of TS-449. The antigen of TS-449 shared three precipitation arcs with the antisera of TS-449 and no precipitation arc was found when reacted with the antisera of the pathogen *R. solani.* Precipitation arcs were also observed when the antisera of TS-520 and antigen of TS 449 was reacted. The antisera of *R. solani* produced atleast one precipitation arc with the root antigens of tea varieties like, TS-506, TS-463, TS-491, TS-464 and TS-462 separately but produced two precipitation arcs with the antigen of TS-520 variety. The antisera of TS-520 shared two precipitation arcs with the mycelia antigen of the pathogen *R. solani* and produced four arcs with its own homologous antigen. No precipitation arcs were observed was antigen of a non pathogen (*Alternaria porri*) when reacted with the tea root antisera.

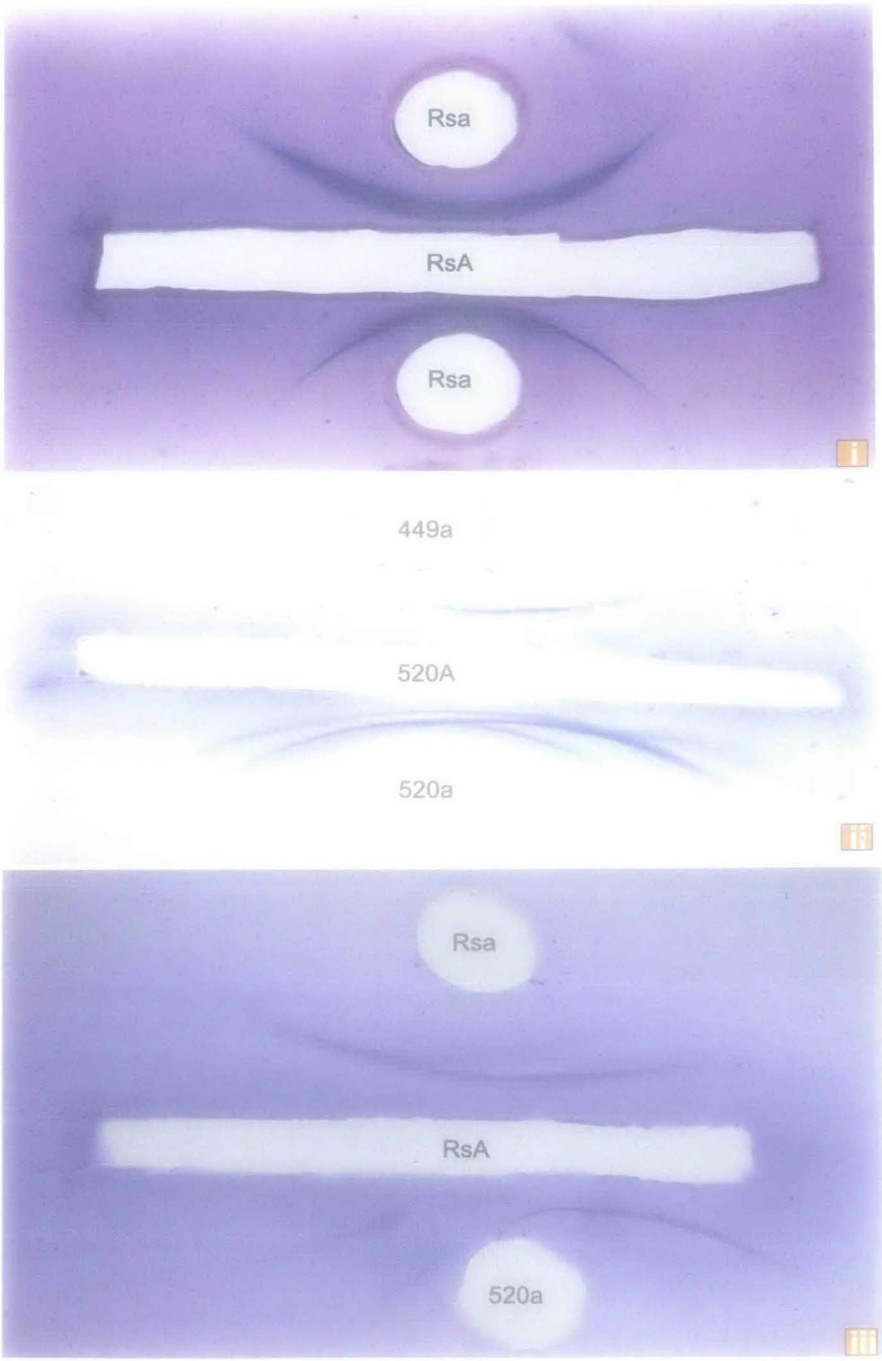

Plate XIV: Immuno electrophoresis (i) Antisera of *Rhizoctonia solani* (RsA) reacted with antigen (Rsa) of *Rhizoctonia solani.* (ii) Antisera of tea variety TS-520(520A) reacted with antigens (449a) and (Rsa) respectively of tea variety (TS-520) and *Rhizoctonia solani.* (iii) Antisera of *Rhizoctonia solani* (RsA) reacted with antigens (Rsa) and 449a respectively of *Rhizoctonia solani* mycelium and tea roots of variety TS-520.

Table 4.14: Comparison of precipitation arcs found in immune electrophoresis of tea plant varieties (susceptible, resistant and pathogen)

Antigen of pathogen, host and non pathogen with their code in parentheses	Total number of precipitation arcs		
	Antisera of pathogen and host		
	Fungal pathogen	Tea varieties	
	Rhizoctonia solani (RsA)	TS 449 (449A)	TS 520 (520A)
Pathogen			
R. solani (Rsa)	3	-	2
Tea plant variety			
TS 449 (449a)	-	3	2
TS 506 (506a)	1	1	2
TS 463 (463a)	1	1	2
TS 491 (491a)	1	1	2
TS 464 (464a)	1	1	2
TS 462 (462a)	1	1	2
TS 520 (520a)	2	1	4
Non-pathogen			
A. porii (Apa)	-	-	-

Table 4.15: Immuno electrophoretic test of antigens and antisera of tea plant varieties and *R. solani*

Antigen of pathogen and host	Antisera of R. solani				Antisera of TS 449				Antisera of TS 520			
	Precipitation arcs				Precipitation arcs				Precipitation arcs			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Pathogen												
R. solani (Rsa)	+	+	+	-	-	-	-	-	+	+	-	-
Plant variety												
TS 449 (449a)	-	-	-	-	-	+	+	+	-	-	+	+
TS 506 (506a)	+	-	-	-	-	+	-	-	-	-	+	+
TS 463 (463a)	+	-	-	-	-	+	-	-	-	-	+	+
TS 491 (491a)	+	-	-	-	-	+	-	-	-	-	+	+
TS 464 (464a)	+	-	-	-	-	+	-	-	-	-	+	+
TS 462 (462a)	+	-	-	-	-	+	-	-	-	-	+	+
TS 520 (520a)	+	-	-	-	-	+	-	-	+	+	+	+

Common precipitation band present (+); Common precipitation band absent (-)

4.17 Indirect enzyme linked immunosorbent assay (Indirect ELISA) between *R. solani* and different tea varieties

Enzyme linked immunosorbent assay is an important tool through which one can detect the presence of very small amount of antigen / antibody in a sample. Though there are various processes of ELISA i.e. Indirect ELISA, direct/Sandwich ELISA, competitive ELISA, multiple and portable ELISA. Many scientists successfully used indirect ELISA technique for their experiments. In plant pathology this technique has been used to screen the different plant varieties for resistance or susceptiblity towards a particular plant pathogen (Chakraborty and Saha, 1994; Kratka *et al.*, 2002; Dasgupta *et al.*, 2005). Through the OD reading (Optical density) in the ELISA reader, the amount of antigen/antibody can be determined quantitatively.

In cross reactions of antibody and antigens, the higher optical values indicate similarity of antigens which is also indicative of the susceptibility of a cultivar or a plant variety. In opposite sense, when the antigen and antibody in cross reactions show lower optical densities, it can be correlated to dissimilarity of the antigens, leading to resistance of the plant variety.

The root antigens of seven tea plant varieties (TS-449, TS-506, TS-491, TS-463, TS-464, TS-462 and TS-520) and the mycelial antigen of the fungal pathogen *R. solani* were used as antigen while antisera raised from the tea plant varieties (TS-449 and TS-520) and the fungal pathogen *R. solani* were used to perform indirect ELISA. All the antisera and the normal sera were diluted in 1/125 dilution and were tested against three different concentrations (5µg/ml, 10µg/ml and 20µg/ml) of each antigen separately. The detailed procedure of indirect ELISA and the procedure of antigen and antisera preparation have already been discussed in the materials and methods (Section 3.10). An ELISA reader determined the absorbance of all the combination at 492 nm and the results have been presented in table 4.16 and Fig. 4.8

From table 4.16, it was clear that all the three concentrations of the antigen *R. solani* showed higher absorbance values when tested with the antisera of the susceptible variety TS-520 (0.585 at antigen concentration 20 µg/ml) than when tested with antisera of the resistant TS-449 variety (0.152 at antigen concentration 20 µg/ml). The reciprocal cross of this combination also showed higher absorbance values produced by antigens of TS-520 (0.593 at antigen concentration 20 µg/ml) than produced by antigens of TS-449 variety (0.161 at antigen concentration 20 µg/ml) when tested with antisera of *R. solani*. From this result, it was concluded that cross-reactivity was higher between pathogen and susceptible variety than between pathogen and resistant variety. Results obtained from all the combinations proved that the absorbance values of normal serum control were lower than the corresponding test values.

Some of the ELISA values (absorbance values) of the root antigens of seven tea seed varieties and of the pathogen *R. solani* against antisera of *R. solani* have been presented graphically (Fig. 4.8). From the results, it is clear that the variety TS520 is the most susceptible towards the pathogen and TS449 was the most resistant variety, among the tested varieties. The other varieties tested were also found to moderately susceptible.

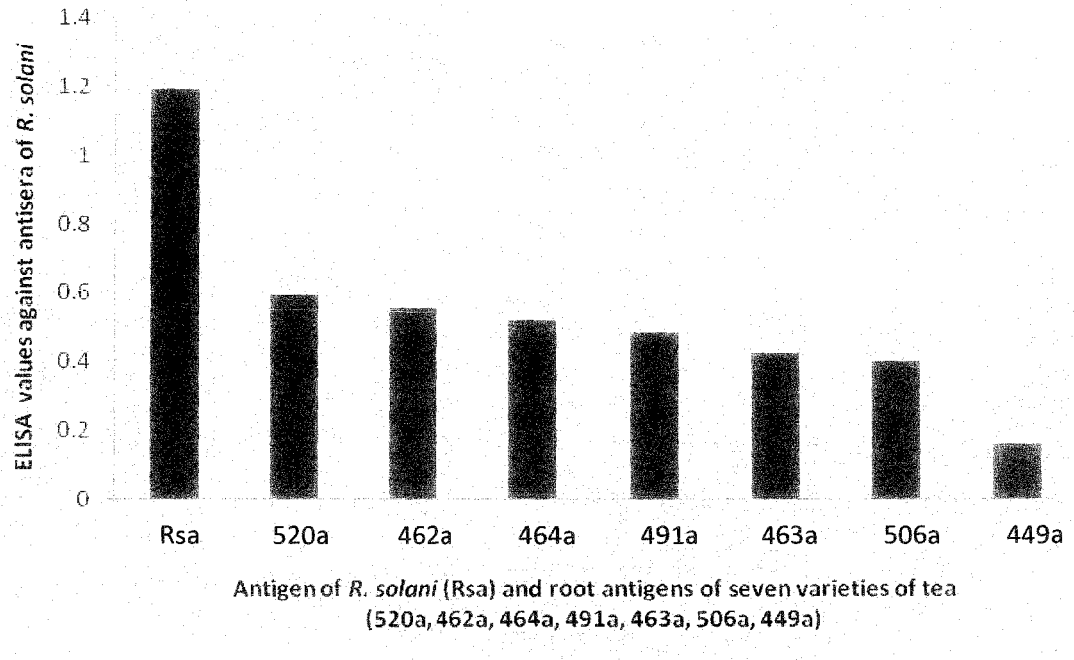

Fig. 4.8: Indirect ELISA results (A_{492}) of different antigens at concentration of 20mg protein/ml against antisera of *R. solani* (RsA) at 1/125 dilution

Table 4.16: Indirect ELISA (A492) results of different combination of antigens (antigens of seven tea plant varieties and *R. solani*) against antiserum (polyclonal) raised against *R. solani* (RsA)

Antigen of host/pathogen	mg protein/ ml	TS 449 (449A)		TS 520 (520A)		*R. solani* (RSA)	
		NS 125	AS 125	NS 125	AS 125	NS 125	AS 125
TS 449 (449a)	20	0.030	1.243	0. 030	0.376	0.030	0.161
	10	0.02	0.869	0.02	0.285	0.025	0.088
	5	0.02	0.805	0.02	0.200	0.022	0.072
TS 506 (506a)	20	0.026	1.158	0.026	0.776	0.026	0.401
	10	0.024	0.810	0.024	0.682	0.024	0.302
	5	0.021	0.785	0.021	0.592	0.021	0.273
TS 463 (463a)	20	0.025	0.989	0.025	0.789	0.025	0.426
	10	0.024	0.920	0.024	0.690	0.024	0.357
	5	0.022	0.722	0.022	0.599	0.022	0.298
TS 491 (491a)	20	0.027	0.904	0.027	0.791	0.027	0.486
	10	0.025	0.856	0.025	0.698	0.025	0.392
	5	0.021	0.733	0.021	0.603	0.021	0.301
TS 464 (464a)	20	0.028	0.865	0.028	0.806	0.028	0.519
	10	0.025	0.786	0.025	0.756	0.025	0.432
	5	0.022	0.710	0.022	0.671	0.022	0.320
TS 462 (462a)	20	0.030	0.801	0.030	0.820	0.030	0.556
	10	0.026	0.735	0.026	0.778	0.026	0.459
	5	0.023	0.685	0.023	0.692	0.023	0.356
TS 520 (520a)	20	0.030	0.158	0.030	1.410	0.030	0.593
	10	0.027	0.082	0.027	1.262	0.027	0.476
	5	0.023	0.065	0.023	1.017	0.023	0.362
R. solani (Rsa)	20	0.029	0.152	0.029	0.585	0.029	1.193
	10	0.026	0.086	0.026	0.431	0.026	1.84
	5	0.023	0.067	0.023	0.326	0.023	1.158

Values given in the parenthesis represents code of the respective antigen/antisera.

NS=normal serum; AS=anti serum

4.18 Immunogold labeling for cellular location of antigens and cross-reactive antigens

Immunogold labeling is an important staining technique which is used to localize different intracellular sites of specific proteins or antigens (Downs *et al.*, 1998). This technique was first used in 1971 by Faulk and Taylor to identify *Salmonella* antigens. The gold particles are easily detectable by transmission electron microscopy but it is difficult to visualize by bright field light microscopy. But during silver enhancement the colloidal gold particles act as a nucleation site and as a result silver is deposited on to the particle (Faulk *et al.*, 1971; Clifton, 2010). In this technique the colloidal gold particles attach to the secondary antibodies which in turn attach to the primary antibodies. The primary antibodies bind to the specific proteins or antigens of the cells. The primary antibodies may also bind to protein A or Protein G instead of a secondary antibody (Roth *et al.* 1978). The silver enhancer enhances the colloidal gold level by precipitation on the gold particles and thus increases the visibility which can be detected by light microscope (Saha *et al.* 2010).

In the present study, serological experiments like immunodiffusion, immunoelectrophoresis and indirect enzyme linked immunosorbent assay (indirect ELISA) clearly indicated the presence of cross reactive antigens (CRA) between tea varieties and *R.solani.* But, to find out tissue and cellular location of CRA shared by pathogen and tea roots the "Immunogold labeling studies followed by silver enhancement" were performed. Root sections of susceptible (TS 520) and resistant (TS 449) tea varieties and mycelia and *of R.solani* were used as antigens. The antisera of TS 520 (susceptible variety), TS 449(resistant variety) and that of pathogen (*R. solani*) were used in the experiment. To determine the exact location of CRA, both leaf section and fungal mycelia were treated with antisera and subsequently immunogold labeling and silver enhancement of the test samples was performed. The

procedure was discussed in the materials and methods in details (section 3.10).

When immunogold labeling and silver enhancement were performed on root sections that were treated with normal sera, no precipitation was observed on the cells (not shown). Root sections of TS 449, when treated with homologous antisera and subsequently immunogold labeling followed by silver enhancement, showed maximum precipitation in the epidermal regions and vascular bundle elements of the roots. Similar result was observed when root sections of TS 520 were treated with homologous antisera (Plate XV, d). Heavy precipitation was also observed when root sections TS 520 were treated with antisera of TS 449 (Plate XV, c).

In heterologous reactions, when the root section of susceptible variety (TS 520) was treated with antisera of *R. solani* and labeled with immunogold particles enhanced by silver precipitation, darkening was observed mainly in the epidermal regions. Some precipitation was also found distributed in vascular bundle elements but these were comparatively less dark than observed for homologous reaction (Plate XV, b), indicating the presence of CRA. When root section of resistant variety (TS-449) was treated with the antisera of pathogen (*R. solani*) faint precipitation was observed after immunogold labeling and silver enhancement (Plate XV, a) indicating the resistance of the variety towards the pathogen. Root section of TS-462 and TS-463 were also treated with immunogold label using antisera of *R. solani* (RsA), and moderate presence of the lebels were observed when enhanced with silver precipitation (Plate XVI, a & b).

Immunogold labeling and silver enhancement of the mycelia of pathogen (*R. solani*) showed that these were grayish in normal condition (Plate XVII, a). When treated with antisera of resistant variety (TS-449) mycelia showed very less blackening indicating antigenic dissimilarity (Plate XVII, b). When mycelia and propagules of the pathogen were treated with homologous

antiserum followed by immunogold lebelling-silver enhancement using antisera of the pathogen mycelia, darkest black silver deposition was observed on both the surface of the mycelia and propagules (Plate XVII, c)

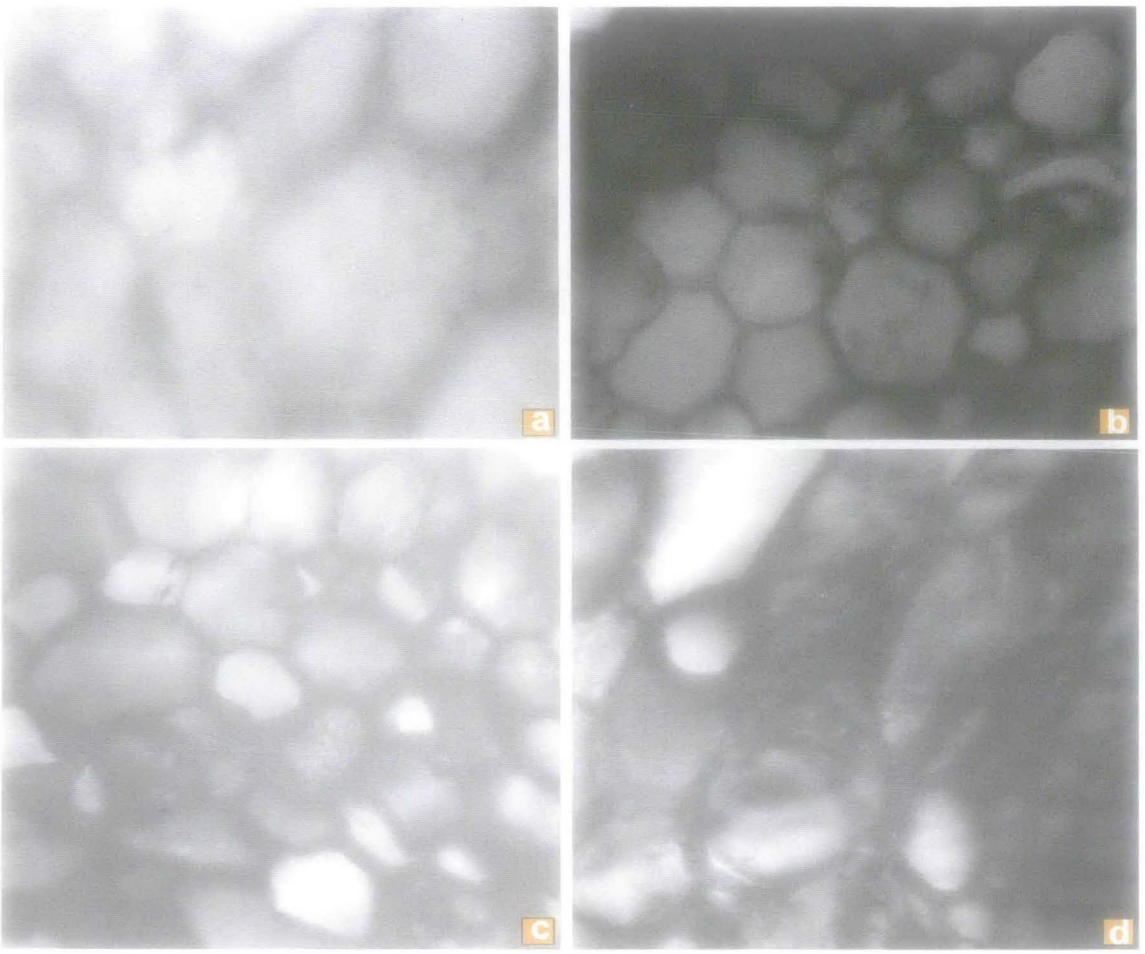

Plate XV: Silver enhancement of immunogold labelled tea root tissues for detection of crossreactive antigens and homologous antigens (a) Root section of TS-449 treated with Rsa (antisera of *R.Solani*); (b) Root section of TS-520 treated with antisera of *R.solani* (RsA); (c) Root section of TS-520 treated with the antisera of TS 449 (449A); (d) Root section of TS-520 treated with antisera of TS-520 (520A).

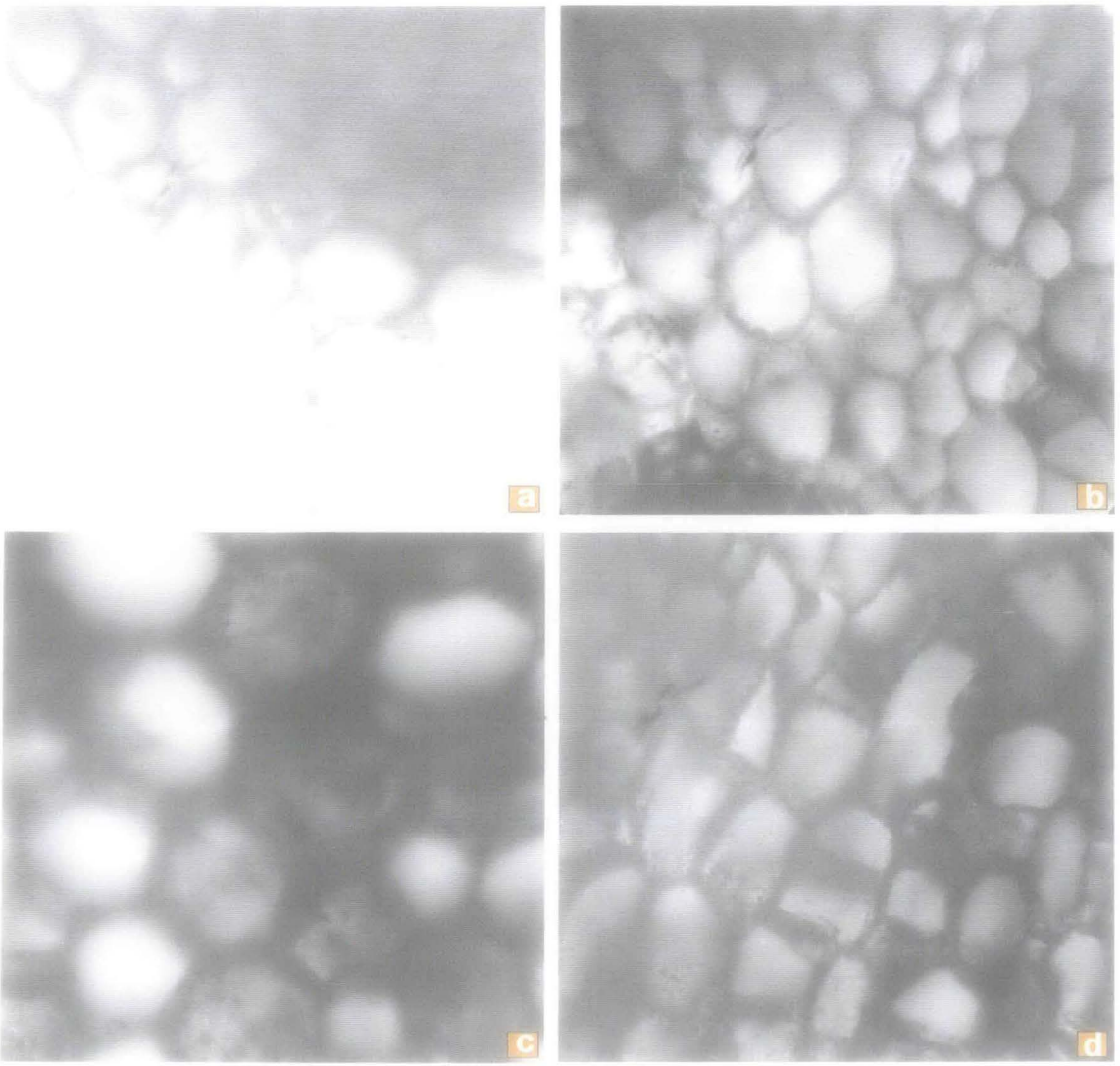

Plate XVI: Immuno-gold labeling and silver enhancement of tea root tissues for detection of crossreactive antigens and homologous antigens (a) Root section of TS-462 treated with antisera of *R.solani* (RsA); (b) Root section of TS-463 treated with antisera of *R.solani* (RsA); (c) Root section of TS-449 treated with the antisera of TS-449 (449A); (d) Root section of TS-462 treated with antisera of TS-520 (520A).

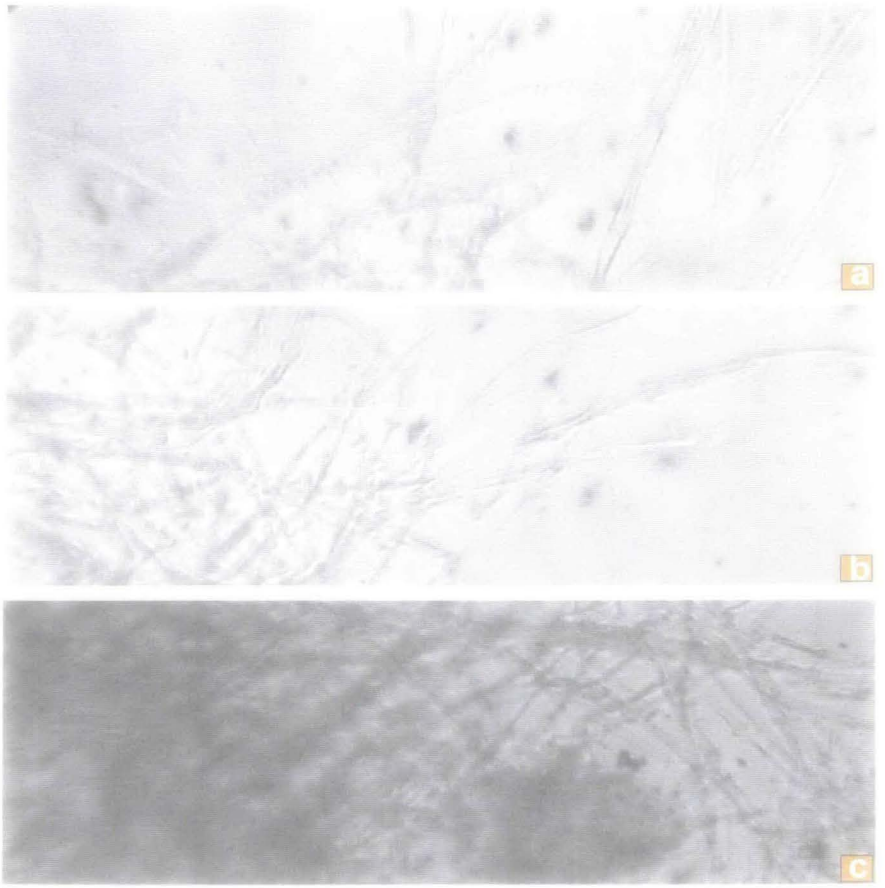

Plate XVII: Immuno-gold labeling and silver enhancement of mycelia of *R.solani* for detection of cross-reactive antigens and homologous antigens (a) Mycelia of *R.solani* (untreated); (b) Mycelia of *R. solani* treated with antisera of TS-449 (449A); (c) Mycelia of *R. solani* treated with antisera of *R. solani* (RsA).

Chapter IV: Evaluation of some known fungal biocontrol agents for controlling *Rhizoctonia solani*, a pathogen of tea seedlings

4.19 Introduction

Tea seedlings of susceptible seed-varieties are prone to attack by *Rhizoctonia solani*, if the seeds are infested with the fungi. It is important to check the fungi externally by seed surface sterilization. Even if the seeds are sterilized, there is possibility of presence of the fungi internally and during germination the seedlings become vulnerable to attack. Hence, there is a need to check the fungi during germination of the seeds and also during the initial stages of seedling growth. In the last decade or so, considerable research have been done on microbial inoculants that may be used for controlling plant diseases. Microbial inoculants are environment friendly alternative to hazardous fungicides. Several scientists (Bucki *et al.*, 1998; Meena *et al.*, 2000; Ramamoorthy and Samiyappan, 2001; Jadeja, 2003; Parello *et al.*, 2006) have identified different microorganisms that may be utilized as antagonist to fungal pathogens. Volatile and non-volatile substances are released by the many biocontrol fungi. These compounds have inhibitory effect on the growth of the pathogen (Meena *et al.*, 2003, Chowdhury *et al.* 2003). Different species of *Trichoderma* are very commonly used antagonists. In the present study four species of *Trichoderma* have been used for evaluation of their efficacy against the tea pathogen *Rhizoctonia solani*.

4.20 *In vitro* evaluation of some known antagonistic fungi against the control of *R. solani*

In the present study four different antagonistic organisms have been used in dual culture in petriplate. The detailed method of dual culture technique to control the pathogen *R. solani in vitro*, has been described in materials and methods (section 3.11.1)

The results presented in table 4.17, Plate XVIII and in Fig.4.9, indicated that the isolate *T. harzianum* was the most effective fungi against *R. solani* in dual culture technique. Strong inhibition (86.66%) of mycelial growth of *R. solani* over control was recorded with complete inhibition of sclerotia production. *G. virens* (Isolate –II), *G. virens* (Isolate-I) and *T. viride* inhibited 78.89%, 65.56% and 60.56% of mycelial growth respectively. No sclerotia was also produced when *G. virens* (Isolate-II) was used as antagonist but when *G. virens* (Isolate-I) and *T. viride* were used separately against *R. solani*, poor sclerotia development was noticed.

Table 4.17: *In vitro* antagonistic effect of different fungal biocontrol agents against *R. solani* in dual cultures

Name of the antagonists	Average radial growth of *R. solani* (cm)	% mycelia growth inhibition over control	% of sclerotia formation
Trichoderma harzianum	1.2	86.66	-
Trichoderma viride	3.55	60.56	+
***Gliocladium virens* (isolate-I)**	3.10	65.56	+
***Gliocladium virens* (isolate-II)**	1.99	78.89	-
Control	9.00	-	++++

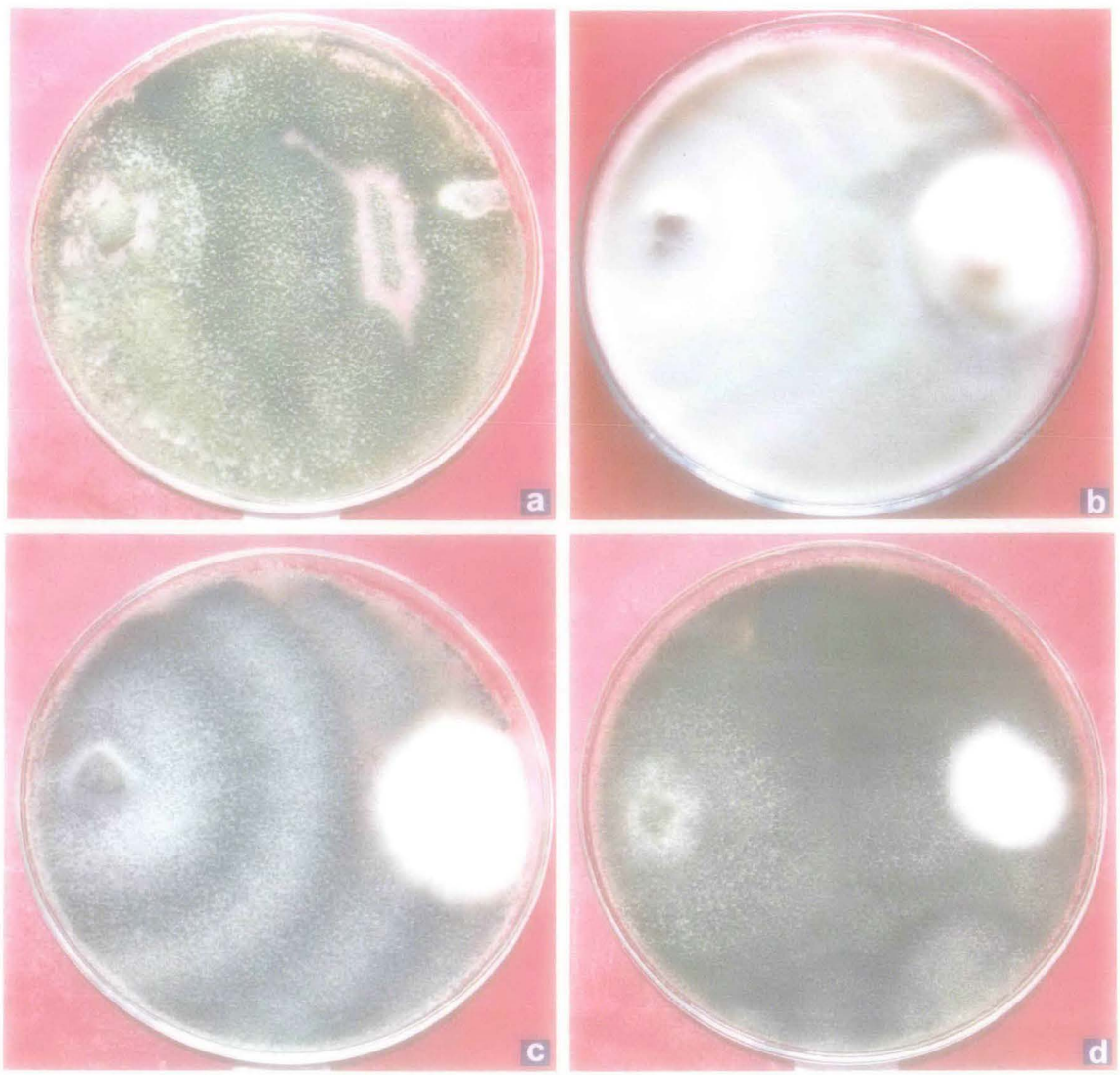

Plate XVIII: In vitro control of *Rhizoctonia solani* with four different biocontrol fungi in dual culture test (a) Control *R. solani* by *Trichoderma harzianum* (b) Control of *R. solani* by *Trichoderma viride* (c) Control of *R. solani* by *Trichoderma virens* (isolate 1) (d) Control of *R. solani* by *Trichoderma virens* (isolate 2).

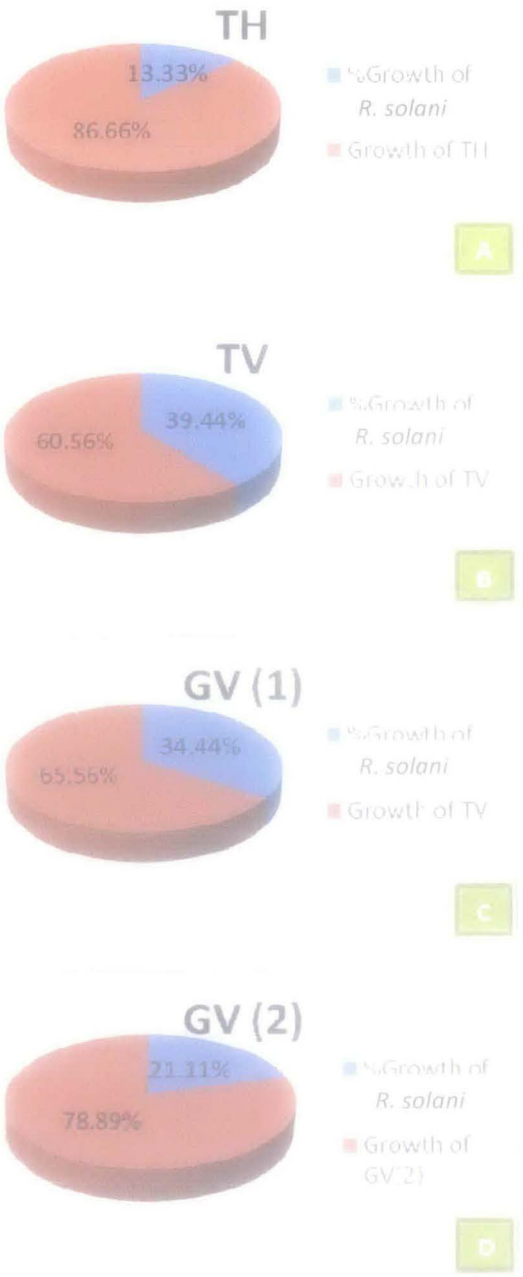

Fig 4.9: **Effect of antagonists on the radial growth of *R. solani* in PDA dual culture assay; TV=*Trichoderma viride*; TH=*Trichoderma harzianum*; GV (1) = *Gliocladium virens* isolate 1; GV (2)= *G. virens* isolate 2**

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

Following dual culture experiments four antagonistic organisms [*Trichoderma harzianum, T. viride, Gliocladium virens*, (Isolate I and isolate II)] were cultured in Ehrlenmeyer flask and 25% culture filtrate were prepared as described in section 3.11.2. From the results of Table 4.18 and figure-4.10, it was evident that the 25% culture filtrate of *T. harzianum*, *G. virens* (Isolate-II), *G. virens* (Isolate-I) and *T. viride* inhibited the mycelia weight of *R. solani* by 31.53%, 20.94%, 20.61% and 5.89% respectively. The volatile or non-volatile compounds have the inhibitory effect on the mycelia growth of *R. solani* but the inhibition rate is less than 50%. From this result, it may be concluded that the sporulating culture of the bio-agents (as found in case of dual culture in plates) are more effective than the 25% culture filtrate (as shown in the present experiment) of the fungus.

Table 4.18: *In vitro* antagonistic activity of culture filtrate of fungal bio-control agents (25% culture filtrate) against *R. solani*

Name of culture	Dry weight (gm)	% growth inhibition of mycelia
R. solani* + 25% culture filtrate of *T. harzianum	0.2620	31.53
R. solani* + 25% culture filtrate of *T. viride	0.2210	5.89
***R. solani* + 25% culture filtrate of *Gliocladium virens* (isolate-1)**	0.2210	20.61
***R. solani* + 25% culture filtrate of *Gliocladium virens* (isolate-2)**	0.2201	20.94
***Rhizoctonia solani* (PDB:water::3:1) (control*)**	0.2784	-
Trichoderma harzianum* *	0.3865	-
Trichoderma viride* *	0.4360	-
Gliocladium virens* (isolate-I) *	0.2840	-
Gliocladium virens* (isolate-II) *	0.2379	-

* Instead of culture filtrate sterile distilled water was added and it was treated as control.
** The four antagonistic organisms were separately cultured and their individual growths were noted for comparison.

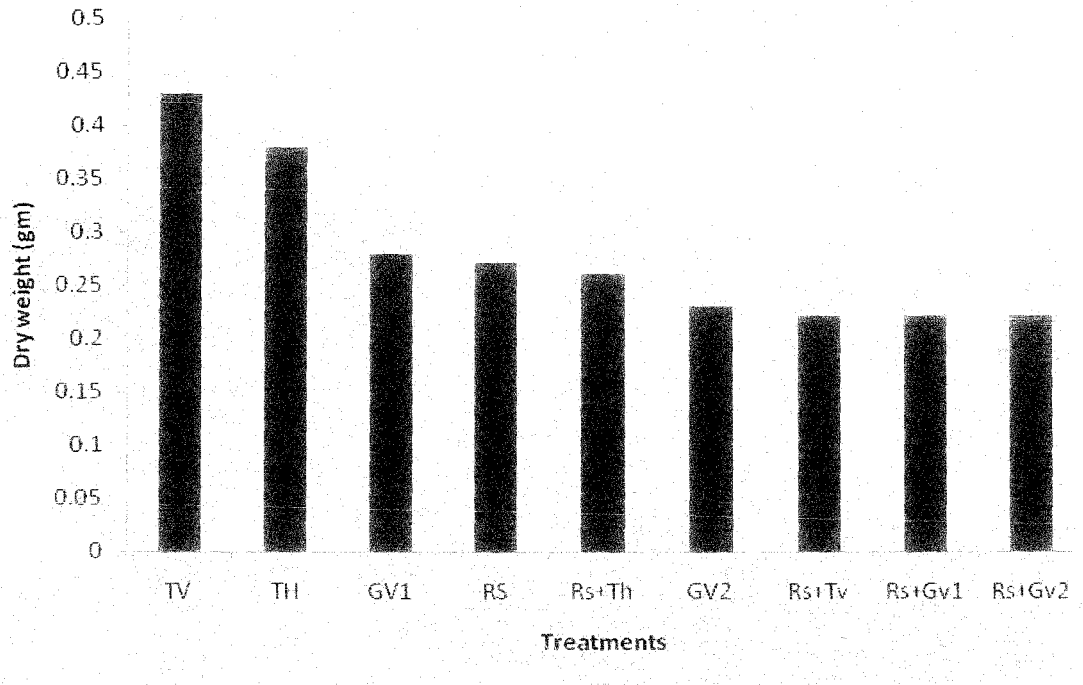

Fig 4.10: Graphical representation of mycelia growth in 25% culture filtrate supplemented PDB medium

RS=*Rhizoctonia solani* (Unsupplemented), TH=*Trichoderma harzianum* (Individual growth of the fungi), TV= *Trichoderma viride* (Individual growth of the fungi), GV= *Gliocladium virens* (isolate 1) (Individual growth of the fungi), GV= *Gliocladium virens* (isolate 2) (Individual growth of the fungi)

Treatments: Rs+Th [*Rhizoctonia solani* mycelia dry weight in 25% culture filtrate of *T. harzianum*], Rs+Tv[*Rhizoctonia solani* mycelia dry weight in 25% culture filtrate of *T. viride*], Rs+Gv1[*Rhizoctonia solani* mycelia dry weight in 25% culture filtrate of *Gliocladium virens* isolate 1], Rs+Gv2[*Rhizoctonia solani* mycelia dry weight in 25% culture filtrate of *Gliocladium virens* isolate 2].

Chapter V: *In vitro* evaluation of some botanicals against *R. solani*

4.22: Introduction

Rhizoctonia solani is not only a soil borne pathogen but also a seed borne pathogen (Almedia *et al.*, 1980). During germination, when the fungi infect the root portion of the plant it may be controlled by the application of sporulating cultures of the antagonistic fungi. But when the freshly emerged shoots come out of the seed coat and are infected with the pathogen *R. solani*, it is better to treat the seeds with the plant extracts. In certain cases, scientists have effectively utilized plant extracts to control plant pathogens.

In the present study, 23 plant extracts were evaluated for their antifungal efficacy against *R. solani in vitro* by agar cup bioassay. After selection of the suitable botanicals for controlling the pathogen (to evaluate the effect of plant extracts) poisoned food technique was also performed. The detailed methods of agar cup bioassay and poisoned food technique have been described in sections 3.12.3 & 3.12.4 respectively.

4.23: Agar cup assay of the plant extracts against *R. solani*

From the results of agar cup bioassay (table 4.19) it was evident that among 23 plant materials tested, only the ethanol extracts of 10 plant materials were effective against the pathogen *R. solani*. The 10 potential plants were *Allium sativum, Polyalthia longifolia, Leucus cephalotus, Tridex procumbens, Xanthium strumarium, Ocimum sanctum, Datura metel, Azadirachta indica, Zingiber officinale* and *Curcuma longa*. Among these 10 effective plant materials, *Allium sativum* had most inhibitory effect which developed a large inhibition zone with the diameter of 5.0 cm. Next to *Allium sativum*, there was inhibitory activity in *Leucus cephalotus* leaf extract which showed the inhibition zone of 4.8 cm diameter. The other eight plant extracts (*Tridex procumbens, Polyalthia longifolia, Xanthium strumarium, Ocimum Sanctum, Datura metel, Azadirachta indica, Zingiber officinale* and *Curcuma*

longa) showed inhibitory activity against *R. solani.* The diameter of the inhibitory zones has been presented in the table 4.19 and photographic evidences have been presented in the Plate XIX. Photographs of some of the plant materials used in the study have also been presented in the plates XX & XXI.

4.24 Evaluation on selected plant extracts following poisoned food technique

Among the 23 plant species tested in the agar cup assay, only 10 plant species exhibited inhibitory effect against the pathogen *R. solani.* The inhibitory effect of these botanicals against *R. solani* was verified by poisoned food technique. Medium of growth was supplemented with aqueous extracts of the plant materials. Final concentration of the plant extracts in the medium was maintained at 5%, 10% and 20%. The inhibition of growth was computed in percent and has been presented in table 4.20. Data were recorded after 5 and 10 days.

The inhibitory effect of *Allium sativum* and *Polyalthia longifolia* and *Leucas cephalotes* was significantly high. In comparison to the above three plant extracts, the other seven plant extracts tested showed much less inhibitory activity towards growth of *R. solani.* The fungi toxicity of the three plant extracts in comparison to control have shown that 100% inhibition was possible by 10% and 20% concentration of the aqueous extracts after 10 days of incubation.

Table 4.19: Agar cup bioassay of plant extracts against *R. solani*

Name of the plants	Plant parts used	Diameter of inhibition zone (cm)
***Adhatoda vasica* L.**	Leaf	0.0
***Allium sativum* L.**	Bulb	5.0
***Azadirachta indica* A. Jass.**	Leaf	1.2
***Bidens alba* L.**	Leaf	0.0
***Boerhavia intermedia* L.**	Leaf	0.0
***Calendula officinale* L.**	Leaf	0.0
***Calotropis procera* (Aiton) W. T. Aiton.**	Leaf	0.0
***Cannabis sativa* L.**	Leaf	0.0
***Croton bonplandianum* Baill.**	Leaf	0.0
***Curcuma longa* L.**	Rhizome	0.0
***Datura metel* L.**	Leaf	1.2
***Dryopteris filixmus* Adams.**	Leaf	0.0
Lantana camera L.	Leaf	0.0
	Flower	0.0
	Fruit	0.0
***Leucus cephalotes* L.**	Leaf	4.8
***Murrya koeningii* L.**	Leaf	0.0
***Occimum sanctum* l.**	Leaf	2.0
***Polyalthia longifolia* sonn.**	Leaf	3.0
Tridex procumbens L.	Leaf and flower	3.2
***Vinca rosia* (L.)G. Don**	Leaf	0.0
***Xanthium strumarium* L.**	Leaf	1.5
***Zingiber officinale* Ross.**	Rhizome	0.9

Data are average of three replications

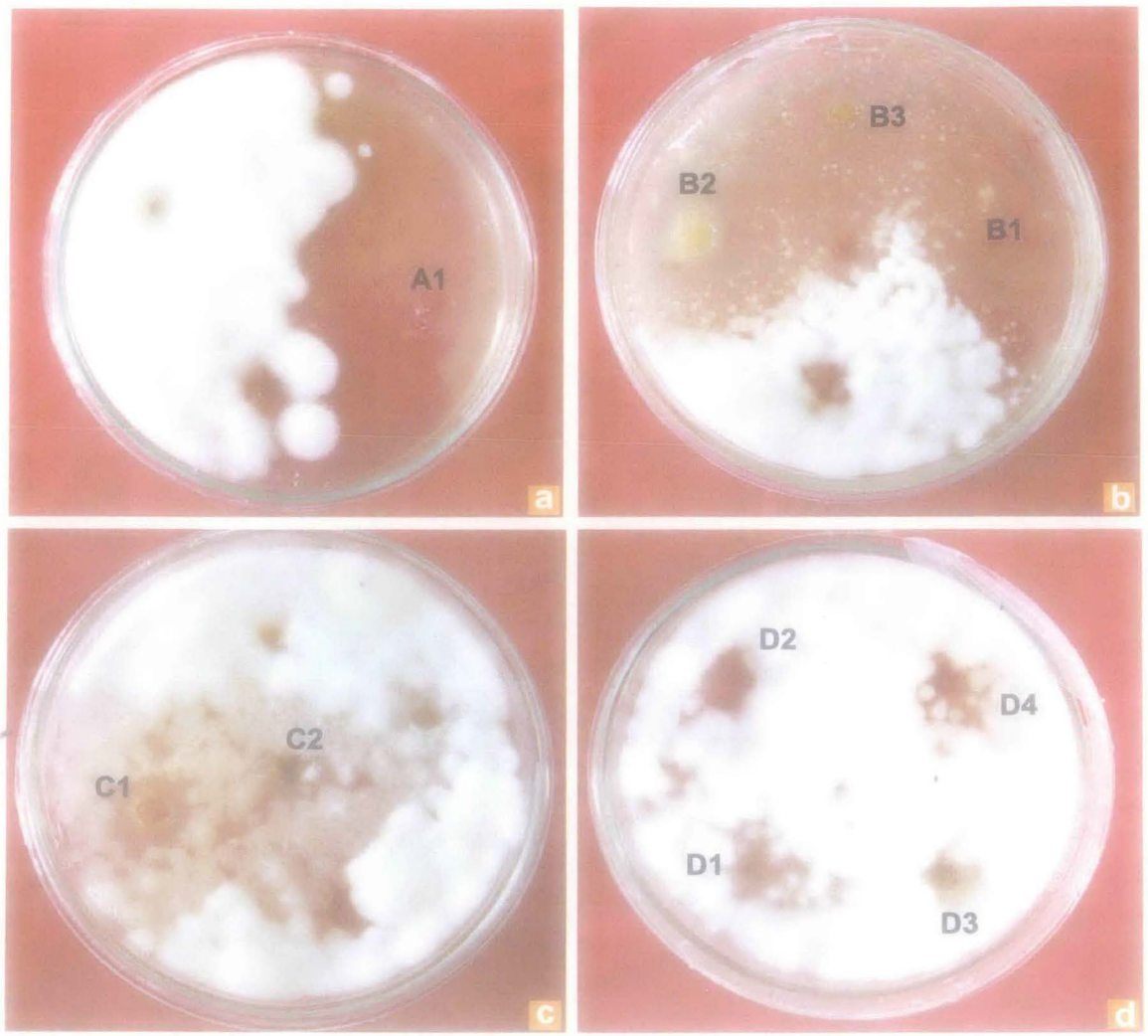

Plate XIX: Inhibitory effect of botanicals following agar cup method.
(a) A1 = *Allium sativum* L. (b) B1 = *Polyalthia longifolia* sonn., B2= *Leucus cephalotes* L., B3= *Tridex procumbens* L., (c) C1= *Xanthium strumarium* L., C2=*Occimum sanctum* L., (d) D1=*Datura metel* L., D2=*Azadirachta indica* A.Juss., D3=*Zingiber officinale* Rosc. D4= *Curcuma longa* L.

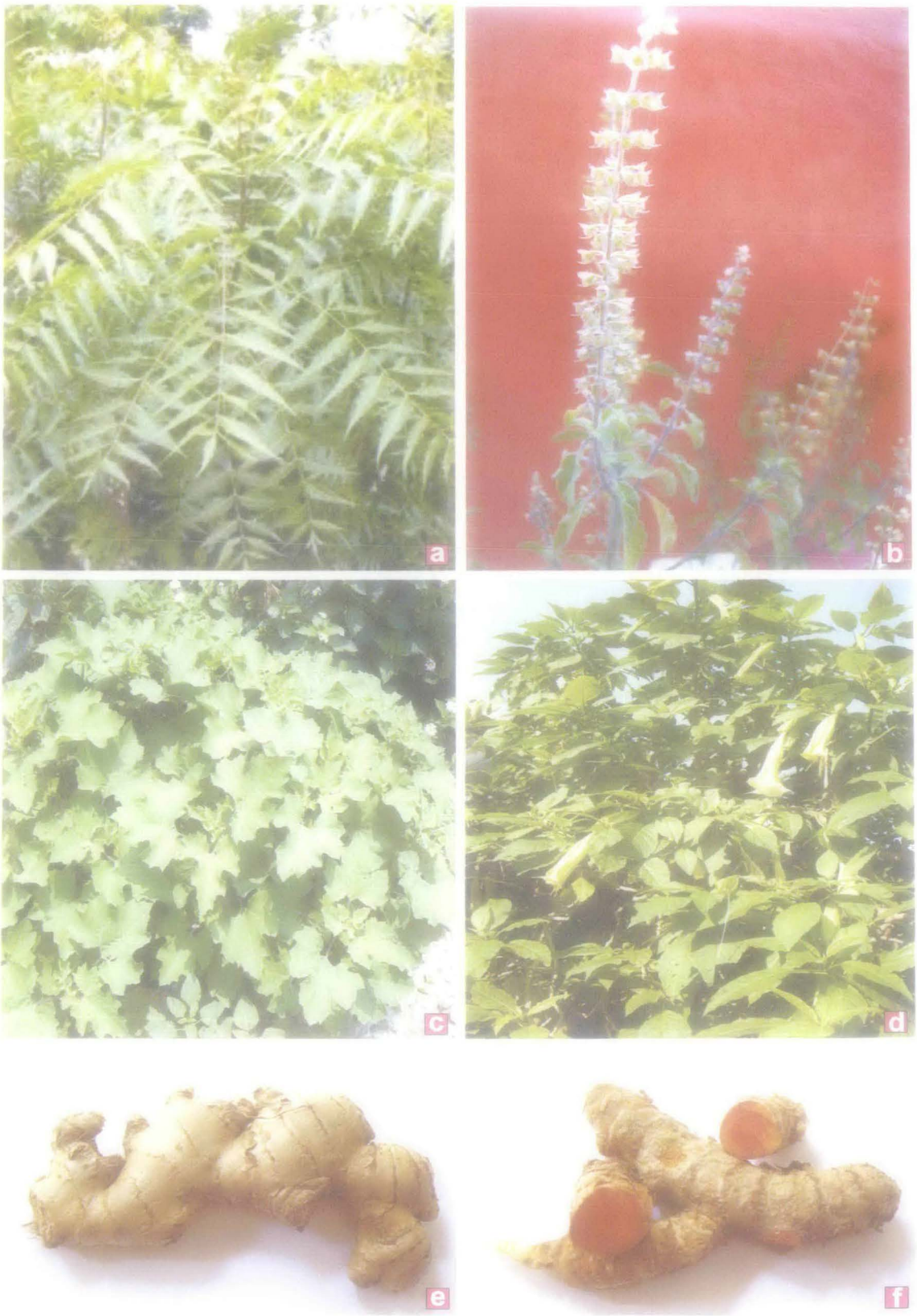

Plate XX: Photographs showing some plants used for extraction of botanicals for controlling *Rhizoctonia solani* (a) Leaves of *Azadirachta indica* A.Iuss (b)Leaves and inflorescence of *Occimum sanctum* L. (c) Leaves of *Xanthium strumarium* L. (d) Leaves and flowers of *Datura metal* L. (e) Rhizome of *Zingiber officinale* Rocs. (f) Rhizome of *Curcuma longa* L.

Plate XXI: Photographs showing some plants used for extraction of botanicals for controlling *Rhizoctonia solani* (a) *Polyalthia longifolia* sonn. (b) *Lucus cephalotes* L. (c) *Tridex procumbens* L. (d) *Allium sativum* L.

Table 4.20: Percent inhibition of *R. solani* by aqueous extracts of plant species after different time intervals (following poisoned food technique)

Name of the plants	Concentration (%)	*Percent inhibition of *R. solani* by aqueous plant extracts after different time intervals	
		5 day	10day
Allium sativum L.	5	90.90	94.44
	10	93.02	100.0
	20	95.79	100.0
Azadirachta indica A. Jass.	5	37.73	20.00
	10	39.32	22.99
	20	40.39	25.56
Curcuma longa L.	5	37.60	18.06
	10	37.05	23.56
	20	40.00	24.98
Datura metel L.	5	38.22	20.09
	10	40.05	23.06
	20	42.01	26.05
Leucus cephalotes L.	5	86.90	80.44
	10	89.89	84.06
	20	91.02	86.99
Occium sanctum L.	5	38.17	20.54
	10	41.09	22.54
	20	47.88	27.77
Polyalthia longifolia sonn.	5	90.90	94.44
	10	92.99	100.0
	20	95.66	100.0
Tridex procumbens L.	5	87.04	30.36
	10	89.73	35.53
	20	90.01	50.89
Xanthium strumarium L.	5	53.19	38.81
	10	59.02	43.07
	20	66.03	45.91
Zingiber officinale Ross.	5	37.75	19.89
	10	38.01	23.37
	20	42.22	24.27

Data are average of three replications.*Percent inhibition was calculated in comparison to growth of the pathogen in control set.

Chapter VI: *In vivo* evaluation of selected bio-control agents and botanicals

4.25 Introduction

Protective activity of bio-control agents and botanicals need to be evaluated *in vivo,* before recommendation of the products. All the effective products also need to be formulated for field application. In the present study, an attempt was made to control the fungus *R. solani in vivo* by selected biocontrol agents and botanicals.

4.26 Seed treatment and assessment of pathogen population

Seed treatment with a potential plant extract (*Polyalthia longifolia*), 25% culture filtrate of *T. harzianum*, and a mixed formulation (of the plant extract and culture filtrate) were used against *R. solani,* in different tea seed varieties. The experiment was done following the method of Mamatha *et al.* (2000). Details of the method have been presented in section 3.13. Extract of *Polyalthia longifolia* (20% aqueous) exhibited reduction in the presence of the pathogen in all the varieties in comparison to control. Similar results were also experienced when the seeds were treated with 25% culture filtrate of *T. harzianum.* In combined formulation treatment, the pathogen-population was also found to be reduced. The detailed results after 10 days of treatment have been presented in table 4.21. *T. harzianum* culture filtrate was found to be most effective as it recorded the highest inhibition (36%) of incidence of *R. solani* in tea seeds. On the other hand a combination of both *T.* harzianum and P. longifolia leaf extract was found to be less effective in controlling the pathogen. Inhibition capacity of *P. longifolia* was same as that of combined formulation.

4.27 Effect of seed treatment on percent germination and vigour index

Tea seeds of different variety (TS-449, TS-506, TS-463, TS-491, TS-464, TS-462 and TS-520) were treated with the 20% aqueous extract of *Polyalthia*

longifolia, sporulating culture of *T. harzianum* and 25% culture filtrate of *T. harzianum.* The result of germination percentage and vigour index of the seedlings was calculated as described in materials and methods section 3.14.

Table 4.21: Percent incidence of R. solani after treatment of the seed with 20% *P. longifolia* leaf extract, 25% culture filtrate of *T. harzianum* and their combined formulation (1:1)

Seeds of Tea varieties	Percent presence of *R. solani* in seeds (following agar plate method)			
	Untreated control	Treated with 20% leaf extract of *P. longifolia*	Treated with 25% culture filtrate of *T. harzianum*	Treated with combined formulation of 20% leaf extract of *P. longifolia* and 25% culture filtrate of *T. harzianum*
TS 449	51	25	36	22
TS 506	69	36	37	35
TS 463	60	37	39	37
TS 491	62	38	42	38
TS 464	70	39	54	40
TS 462	50	40	56	40
TS 520	72	40	59	41

Data given in the table were calculated on the basis of 100 seeds per treatment

Results of the experiment have been presented in table 4.22. It was evident from the results that percent germination of seeds was highest (range between 76 to 82.09%in all the tea seed varieties, when the seeds were treated with the sporulated culture of *T. harzianum.* Percent germination of *P. longifolia* leaf extract treated seeds was slightly less than the seeds treated with sporulated *T. harzianum.* The lowest percent germination was observed in different varieties when the seeds were treated with the 25% culture filtrate of *T. harzianum.* However, all the three treatments helped in controlling R. solani and as a result higher no of seeds germinated.

The vigour index was calculated according to the method of Prasad *et al.* 1999. The vigour index was determined by multiplying the percentage of germination with the sum of root length and shoot length in centimeters. Disease free seeds (surface sterilized and untreated and uninoculated) served

as control. Detailed procedure has been presented in materials and methods section 3.14.

Vigour index of the seedlings were also highest in case of seeds treated with sporulated culture of *T. harzianum* and lowest in seeds treated with 25% culture filtrate of *T. harzianum*. Among the different varieties tested in the experiment, the percent germination of seeds and seedling vigour index were highest in TS-449 (resistant variety) and lowest in TS-520 (susceptible variety).

Table 4.22: Percent germination and vigour index of different tea seeds under various treatments

Seeds of tea variety	Observation	Control		Treatments		
		Surface sterilized seed (untreated and uninoculated)	Pathogen inoculated seed	Pathogen inoculated seed + leaf extract of *P. l*	Pathogen inoculated seed + sporulated culture of *T. h*	Pathogen inoculated seed + 25% culture filtrate of *T. h*
TS 449	Germination (%)	96	40	78	82	52
	Vigour index	5070.11	464.93	3075.56	3808.98	642.82
TS 506	Germination (%)	94	40	77	80	51
	vigour index	4746.31	425.52	2767.53	3552.09	599.37
TS 463	Germination (%)	93	37	76	80	51
	vigour index	4520.29	369.88	2622.00	3416.96	531.13
TS 491	Germination (%)	92	37	75	80	51
	vigour index	4300.13	329.19	2439.28	3104.00	474.49
TS 464	Germination (%)	92	36	75	78	41
	vigour index	4423.57	335.34	2362.50	3029.89	438.6
TS 462	Germination (%)	90	36	74	78	50
	vigour index	4255.96	295.61	2162.42	2465.43	380.46
TS 520	Germination (%)	89	34	73	76	50
	vigour index	4159.10	268.60	2065.90	2167.71	295.00

Vigour index = (shoot length + root length) × germination percent; *P. l= Polyalthia longifolia*
T.h = Trichoderma harzianum

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

R. solani causes root disease in tea plants. For controlling root disease, the tea seedlings were treated with the fungus *T. harzianum* by soil inoculation method. *T. harzianum* 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 detailed method of this experiment was described in materials and methods (section 3.15). From the results of Table 4.23 it was proved that among the three methods applied (pre, post and simultaneous inoculation) seedling mortality percentage was highly reduced in pre-inoculation method than in post inoculation and simultaneous inoculation method. The bio-control micro-organism, *T. harzianum* inhibited the growth of *R. solani* and allowed us to conclude that 7-days pre-inoculation of the fungi is best for getting results (Plate XXII).

Table 4.23: Soil application of biocontrol agent (*T. harzianum*) in three different methods and two different doses against *R. solani* and evaluation of percent seedling mortality

Seedlings of Tea variety	Percent seedling mortality in Control	Percent seedling mortality at different doses of antagonist treatment (gm/kg soil) in three different methods (after 30 days)					
		7days pre inoculation		7 days post inoculation		Simultaneous inoculation	
		2g/kg*	10gm/kg*	2gm/kg	10gm/kg	2gm/kg	10gm/kg
TS 449	45.0	8.5	5.7	10.2	22.8	10.7	9.4
TS 506	87.0	11.4	8.5	31.4	22.8	17.1	11.4
TS 463	76.0	14.2	11.4	31.4	22.8	20.0	11.4
TS 491	77.0	17.1	11.4	34.2	28.5	20.0	14.2
TS 464	80.0	17.1	14.2	37.1	31.4	22.8	17.1
TS 462	80.0	20.0	14.2	37.1	31.4	22.8	17.1
TS 520	82.0	22.0	17.1	42.8	34.2	25.7	20.0

* Dose [2gm/Kg indicates 2g antagonist per kg soil & 10gm/kg indicates 10g antagonist per kg soil.] In control set seedlings in pots inoculated with *R. solani* served as control.

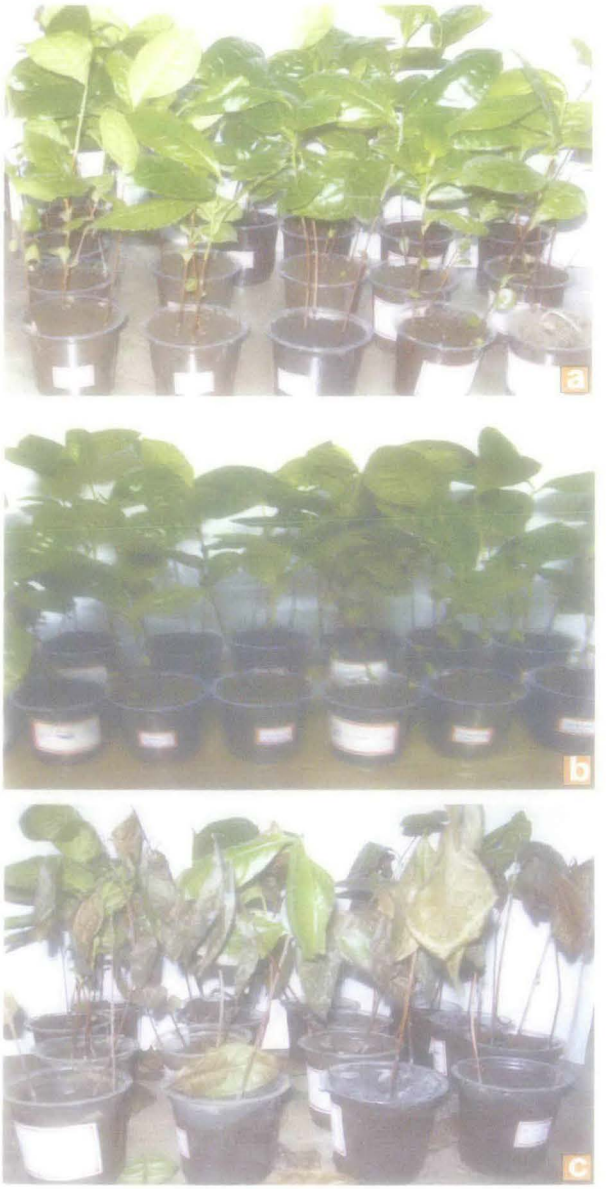

Plate XXII: *In vivo* application of *Trichoderma harzianum* for control of *Rhizoctonia solani* in tea plants (TS520): (a) Untreated healthy control (b) *Trichoderma harzianum* and *Rhizoctonia solani* inoculated plants after 10 days (c) Tea plants severely affected showing disease symptoms following infection with *Rhizoctonia solani*

5. Discussion

Tea seeds are important for raising new plants. Although several tea varieties are developed by clonal cuttings but a large number of tea varieties are grown from tea seeds. In several places of tea plantations of north east India, well developed tap root system of the seed varieties are advantageous over plants raised by clonal cuttings. Tea seeds are collected from soil and thus almost all commercial seeds are fallen seeds. The seeds are kept in bags and are transported to the place of plantations. Most of the seeds are kept for at least six months in store. And thus like any other seeds tea seeds also harbor a large number of fungi during storage. Some of these fungi cause damage by reducing or completely eliminating the germination capacity of the seeds while others are pathogens of the seedlings which infect the young plants after germination. During the present study several genera of fungi were isolated from all together 400 seeds of seven different varieties (TS520, TS462, TS463, TS 449, TS464, TS491 and TS506) of tea procured from seed farms. These were *Curvularia lunata*, *Rhizoctinia solani*, *Fusarium* sp., *Alternaria* sp., five species of *Aspergillus*, Botryodiplodia sp., two species of *Rhizopus*, *Penicillium* sp., Trichoderma *pseudokoningii* and a sterile fungus. Anderson (1986) has reported several seed borne pathogens to be associated with seeds of different forest trees.

Seeds are regarded as highly effective means for transporting plant pathogens over long distance. Spread of plant diseases as a result of the transportation of seeds that were infected or contaminated with pathogens have been reported by Agarwal and Sinclair (1996). Various microorganisms may get associated with seeds if proper post harvest storage conditions are not maintained (Mahamune and Kakde, 2011). Among these microorganisms, fungi play a dominant role in decreasing quality and longevity of the seeds (Mahamune and Kakde, 2011). In the present study, fifteen different fungi were found to be associated with the seeds of tea plants when tested both

externally or internally. The seeds were found to be attacked by nearly 11 fungi internally. The presence of *Rhizoctonia solani*, five species of *Aspergillus* and two species of *Rhizopus* was found to be associated with the cotyledons of all the seeds of seven different tea seed verities. Several workers (Khanzade *et al.*, 2002; Bateman and Kwasna, 1999) have reported that seed-borne pathogens present externally or internally or associated with the seeds as contaminant, may cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of disease at later stages of plant growth by systemic or local infection. Low germination of tea seeds are mainly due to fungal infection of seeds. The fungi invade the outer seed coat, endosperm and embryo. Janardhanan (1994) reported that seeds which carry seed-borne infection produce diseased seedlings resulting in massive destruction due to seedling blights in some plants. In the present study, similar results were found where *Rhizoctonia solani* infected the young seedlings of tea after germination and produced diseased seedlings which ultimately produced weak plants, unsuitable for plantation.

Following the isolation of *R. solani* from infected seeds, its pathogenicity was confirmed through the verification of Koch's postulates. Once the fungus was established as a pathogen, pathogenicity test was conducted which showed TS 449 variety as moderately resistant but the other varieties were susceptible to the fungi. A large number of seed-borne fungi produce toxic metabolites which often kill the embryo (Vidyasekaran *et al.*, 1970). Most of the storage fungi are species of *Aspergillus* and *Penicillium* (Neergaard, 1986). In the present study also we recovered *Aspergillus* and *Penicillium* from some tea seeds which did not lose weight but their embryo probably damaged the germinability due to toxins. Neergaard (1986) reported similar results in case of some other seeds. They correlated the presence of *Aspergillus* and *Penicillium* with decreased seed germinability, seed disclouration, toxin production and reduction in seed weight. Tea seeds are discarded which float

in water due to reduction of weight. The reduction may be correlated with the infestation of *Aspergillus* and *Penicillium* along with some other internal organisms. Rathod *et al.* (2012) mentioned that presence of pathogens in seeds of economically important crop may be disastrous if introduced into disease free areas. We also experienced similar conditions in case of tea as *Rhizoctonia solani* infected plants produced weak plants which, if planted, may introduce the pathogen in the new plantation areas. Therefore, seed must be "Substantially free" from inoculum with high level of germination and purity before sowing and the disease free seedlings only should be selected for plantation of tea plants. Several fungi have been associated with the seeds. Some of them such as *Curvularia lunata, Alternaria* sp., *Botryodiplodia* sp., *Trichoderma pseudokoningii* and a sterile fungus are being reported to be associated with the seeds for the first time. Barthakur *et al.* (1998) and Sarmah and Bezbaruah (1988) reported the presence of *Aspergillus niger, Fusarium solani* and *Penicillium* sp to be associated with tea seeds/ seedlings. In this study too, this fungi were found to be associated with tea seeds. However, the other fungi reported by those author such as *Nigrospora* sp., *Pestalozzia theae,* and *Verticillium* sp. were not detected during the current study.

Genus *Alternaria* has the capacity to produce toxin (Janardhanan, 1994). Similarly *Fusarium* is also known to be toxin producer (Kern *et al.*, 1972; Jin *et al.*, 1996). They may also have some function in loss of germination ability of the tea seeds. Further studies are required to know about the mode of entrance of the fungi (in the seed coat) and the extent of their effect on germination of the seeds.

After the establishment of *R. solani* as pathogen of tea seedlings and its ability to infect majority of the tea verities tested, it was considered to study the growth parameters of the fungi. Hence, nine different media viz. PDA, OMA, REA, CDA, RA, YEMA, MEA, PCA, and NA were used to study the growth of *R. solani* in the present study. Among the media tested, Oat meal

agar (OMA) and YEMA were best medium for the vegetative growth of the fungus. They showed 9.0 cm and 8.4 cm radial growth respectively after 12 days of inoculation. PDA medium was also good for growth. Sclerotia formation was very good in PDA and CDA media. Tiwari and Khare (2002) studied the efficacy of the five liquid media viz. Ashana and Hawken's medium, Czapek's medium, Mertin's rose Bengal streptomycin medium, potato dextrose broth on the production of imperfect stage of *R. solani*- mycelial growth and sclerotia formation. According to them Czapek's media was excellent for both growth of mycelium and sclerotia formation followed by PDB. The authors also reported that Czapek's agar, corn meal agar, chickpea meal agar, oat meal agar, potato dextrose agar and Richard's agar were good for vegetative growth of mycelia. They also showed that within 96 hours fungal hyphal mat touched the sides of petriplates of 9 cm diameter. The results of Tiwari and Khare (2002), Tolba and Salama (1960), Beever and Bollard (1970) and Midgley *et al.* (2006) matched with the results obtained during the present study.

Following the study of growth in different media, the fungus was grown in different temperatures. From that study it was found that the optimum temperature for mycelial growth of *R. solani* was found between 23°C and 33°C. The maximum growth was observed at 28°C. Chand and Logan (1983) reported the optimum temperature range for growth of *R. solani* in between 22°C and 25°C. The optimum growth between 20 and 25°C was reported by Ritche *et al.* (2005) and also by Anguiz and Machin (1989). The present strain of *R. solani* therefore grew at slightly higher temperature than that reported by those others.

pH has important role in the growth and propagules formation. Therefore *R. solani* was grown in media adjusted to different pH. Studies on the mycelial growth at different pH showed that the mycelial dry weight of *R. solani* was maximum at pH 6.5 and lowest at pH 8.0. According to Deacon, 1984, several fungi can grow over a wide pH range, with optimum between pH

5.5 to 8.0. Sherwood (1970) described the ability of *R. solani* to initiate growth on moderately acid or alkaline media and subsequently it can modify the pH to more favorable one, in order to grow successfully. A wide range of pH optima has been reported in growth studies of R. solani by different authors. For instant ph 4 to 8 was described by Ritchie *et al.* (2009), pH 3.5 to 7.5 was described by Bateman (1962) and pH 5 to 6 was described by Grosch and Kofoet (2003). The results of this study were grossly similar to the findings of the other researchers.

After the study of the above physical parameters, it was thought to conduct some experiments in order to know the nutritional requirements of the pathogen. Several authors (Israel and Ali, 1964; Bakshi, 1974; Smith and Read, 1997; Midgley *et al.*, 2006) have studied the utilization of carbon and nitrogen sources as factors influencing growth of fungus. In this study, different carbon sources were initially supplemented in a basal medium. It was found that mannitol was the best carbon source for optimum growth and sclerotia formation of *R. solani* among the six different carbon sources tested. Sorbitol showed second best mycelial growth and sclerotia formation next to mannitol. Lactose showed minimum growth among the carbon sources tested. Pal and Koushik (2012) studied growth response of *R. solani* (isolated from members of Orchidaceae) in six different carbon (*i.e.* glucose, glycerin, maltose, mannitol, starch and sucrose) supplemented media. They showed that glucose supplemented medium supported maximum growth of *R. solani* followed by maltose and sucrose. In our results mannitol supported maximum growth and sclerotia formation followed by sorbitol and glucose. Thus our results are slightly different from that of Pal and Koushik (2012).

Similarly, the influence of organic and inorganic nitrogen sources on growth and sclerotia formation of *R. solani* were also tested. Excellent growth and sclerotia formation was observed in beef extract supplemented medium. Peptone, trypton, sodium nitrate, yeast extract and potassium nitrate were also good for growth and sclerotia formation. Pal and Koushik (2012) utilized

five different nitrogen sources such as yeast extract, peptone, sodium nitrate, ammonium chloride and potassium nitrate. They reported sodium nitrate as best among the inorganic nitrogen sources which is also matching with our results. But in case of organic nitrogen sources their results slightly differed from our results. They used only two organic sources as supplement and among the two, yeast extract was best. But, in the present study we observed that beef extract was best supplement followed by peptone, trypton and yeast extract.

Compatible host pathogen interaction in suitable environmental conditions is required for a successful disease manifestation. The compatibility is determined by many factors contributed by both host and pathogen. Different workers (Alba and DeVay, 1985; Purkayastha and Banerjee, 1990) have noticed that there is a unique serological similarity between pathogen and compatible host involving one or more antigenic determinants. In plants, the susceptibility towards a pathogen seems to increase with increase in similarity between the antigens (Chakraborty and Saha, 1994). These antigens cross react with each other in experimental antigen-antisera reactions and produce precipitin bands (Purkayastha and Banerjee, 1990; Ghosh and Purkayastha, 2003; Saha *et al.*, 2010). The present study was undertaken to determine the presence and the level of cross reactive antigens (CRA) between the seven different tea seed varieties and the pathogen, *R. solani.* Preliminary immunological techniques that are in use today are radial immunodiffusion, immunoelectrophoresis and agar gel double diffusion. Some of these techniques were successfully utilized by several workers in demonstrating cross-reactive antigens (Alba and DeVay, 1985; Ghosh and Purkayastha, 2003; Dasgupta *et al.*, 2005).

For immunodiffusion studies, root antigens of susceptible and resistant tea seed varieties were cross reacted separately with antisera of *R. solani.* The mycelial antigen of *R. solani* was also cross-reacted with antisera of susceptible and resistant tea seed varieties. Mycelial antigen of *A. porri,* a non

pathogen of tea was also used. From the results, serological comparison was made. No common antigen could be detected in agar gel double diffusion plates when antigens of resistant tea seed-varieties were cross reacted with the antisera of *R. solani*. In contrast when susceptible tea varieties were cross reacted with the antisera of *R. solani*, common antigens were detected in the form of precipitin arcs. When antisera of both the resistant and susceptible tea seed-varieties and *R. solani* were reacted with antigen of *A. porri* (non-pathogen of tea) no precipitin arcs were found.

Similar results were obtained by Dasgupta *et al.* (2005) who conducted studies in tea varieties and cross reacted their antigen preparations with antisera of the leaf pathogen, *Curvularia eragrostidis*. They were also able to detect CRA only between susceptible varieties and the pathogen. Saha *et al.* (2010) also performed similar studies in brinjal varieties and *Colletotrichum gloeosporioides* interaction. Purkayastha and Banerjee (1990) conducted immunodiffusion between antigen and antisera of soybean cultivars and the pathogen causing anthracnose (*Colletotrichum dematium* var. *truncata*). They too were able to detect precipitin bands in cross reaction between the pathogen, antisera and the antigen of susceptible host only and vice versa which indicated presence of CRA between these combinations only and not between resistant host and pathogen. Several other authors also obtained similar results in different host parasite combinations viz., jute and *Colletotrichum corchori* (Bhattacharya and Purkayastha, 1985), soybean and *Myrothecium roridum* (Ghosh and Purkayastha, 1990) and tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994). Therefore, the results of the present study are in conformity with those obtained by previous workers.

After the immunodiffusion studies, immunoelectrophoretic studies were performed to confirm the results of the immunodiffusion and also to find out number of arcs formed in antigen-antibody reactions. In immunoelectrophoretic studies, antigen of *R. solani* shared three precipitin bands with antisera of *R. solani* (RsA). Antigens of susceptible varieties (520a,

462a, 464a and 491a) shared precipitin bands each in all the cases when reacted with antisera of susceptible variety TS 520 (520A). Antigens of susceptible varieties (520a, 462a, 464a and 491a) shared at least one precipitin band each in all the cases when reacted with antisera of *R. solani* (RsA) while antigen of resistant variety (449a) showed no precipitin band. In reciprocal cross reaction, antigen of *R. solani* shared one precipitin band with antisera (520A) of TS-520 (susceptible variety) but no precipitin band with antisera of TS449 variety (resistant variety). No common antigenic relationship was noticed between the host plant (*Camellia sinensis*) and non pathogen (*A. porri*).

The advantage of immunoelectrophoresis over immunodiffusion is that complex antigenic mixture is separated because of the additional resolving power of the electrophoretic step. Saha *et al.* (2010) showed evidence that common antigenic substances were present between *C. gloeosporioides* and different varieties of eggplant following immunodiffusion and immunoelectrophoresis. In immunodiffusion and immunoelectrophoresis the antiserum raised against *C. gloeosporioides*, successfully detected CRA between susceptible varieties and the pathogen. The absence of such common antigens between resistant varieties and the pathogen was also significantly explained. Antigens of *A. porri* (non pathogen) produced negative results in cross-reactions with host antisera. Purkayastha and Banerjee (1990) observed that the antibiotic cloxacillin when used as an elicitor of the host defense altered the antigenic patterns of soybean cultivars such that one specific precipitin band was found to be absent in immunoelectrophoretic studies between antigen of the treated leaves and untreated leaf antisera when compared with homologous reaction between antigen and antisera of untreated control. In another study by Ghosh and Purkayastha (2003) that involved ginger cultivars and *Pythium aphanidermatum* as host and pathogen respectively, both immunoelectrophoresis and cross immuno-electrophoresis (CIE) confirmed that cross reactive antigens were absent between antigens of

infected rhizome or non pathogen and antiserum of avirulent strains of *P. aphanidermatum* SR 2, but CRA was easily noted when antigens of heavily infected ginger (cv. Mahima) were cross reacted with antiserum of the pathogen. Ala-El- Dein and El-Kady (1985) used CIE techniques to resolve similarities and dissimilarities between the antigens present in *Botrytis cinerea* isolates and between antigens present in different species of *Botrytis*. From their results, they observed that each isolate was serologically different from the other and had species-specific antigens.

With a view to quantify the common antigens and to make a gradient of common antigenic similarity it was decided to perform indirect ELISA which on the basis of certain distinct values gives us a clear picture of similarity and disparity among the host and pathogen. The gradient of similarity or disparity is the indicator of susceptibility and resistance respectively as shown by Saha, *et al.* (2010) and Dasgupta *et al.* (2005). For detecting CRA at a very low concentration and identifying fungal diseases, indirect ELISA is one of the most specific and rapid methods (Purkayastha and Banerjee, 1990; Sundaram *et al.*, 1991; Chakraborty and Saha, 1994; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005; Dasgupta *et al.*, 2005). Eibel *et al.* (2005) developed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) by raising polyclonal antibodies against *Ustilago nuda* and barley plant. Dasgupta *et al.* (2005) performed ELISA between tea varieties and *Curvularia eragrostidis*, which revealed the presence of a certain minimum level of antigens for compatible host-pathogen interaction. In 2010, Saha *et al.* also showed graphically how the resistant and susceptible varieties of brinjal differed in quantity of common antigens. Several other workers have also used ELISA for early detection of pathogens (Chakraborty *et al.*, 1996 and Ghosh and Purkayastha, 2003).

In the present study, cross-reactive antigens (CRA) were detected in indirect ELISA using very low concentration of antigens and antisera. In this study, the higher ELISA values in cross reactions revealed the presence of

more CRA, which indicated the susceptibility of the variety. Similarly, lower ELISA values revealed lower amount of CRA that indicated resistance. The results obtained by indirect ELISA values i.e. the degree of susceptibility and resistance was in agreement with the results of pathogenicity tests also. The three concentrations of the antigens of *R. solani* showed higher absorbance values when tested with antisera of susceptible variety (TS-520) than when tested with antisera of resistant variety (TS449). Higher absorbance values were also observed in reciprocal tests of this combination i.e. in case of the antigens (520a, 462a, 464a and 491a etc.) of susceptible varieties tested with the antisera (RsA) of *R. solani* than in case of the antigens of resistant variety (TS-449) tested with the same antisera. The results thus clearly indicated the presence of maximum cross reactivity between susceptible varieties (520a, 462a etc.) and *R. solani*. Higher ELISA values were found in homologous reactions in all combinations.

Saha *et al.* (2010) detected detected cross reactive antigens using indirect ELISA technique between susceptible brinjal cultivars and the C. *gloeosporioides* at a very low concentration. Purkayastha and Banerjee (1990) also detected cross reactive antigens using indirect ELISA technique between susceptible soybean cultivars and the virulent strain of *C. dematium* var. *truncata* at a very low concentration. Dasgupta *et al.* (2005) also detected CRA while studying the pathogenicity of *Curvularia eragrostidis* against tea varieties by analyzing the antigenic patterns of host and pathogen. They used indirect ELISA which revealed the presence of low level of common antigens between all combinations. They observed that a certain minimum level of antigens was present for compatible host-pathogen interactions that were significantly higher than incompatible interactions. CRA was also detected in other host pathogen combinations like *Phytophthora infestans* and potato (Alba and DeVay, 1985) and *Phytophthora fragariae* and strawberries (Mohan, 1988) by indirect ELISA.

In the present study, although immunodiffusion and immunoelectrophoresis could not detect CRA between resistant host and pathogen, ELISA showed presence of common antigens in cross reactions between antisera of the pathogen and antigens prepared from all tea seed varieties tested, both susceptible and resistant. But the level of common antigens between resistant variety and the pathogen were significantly lower. A direct correlation of ELISA values (in cross reactions) with results of pathogenicity test were evident and from that result it can be established that the degree of susceptibility or resistance of a particular variety is related with the amount of cross reactive antigens. Thus ELISA may be used to determine the pathogenicity of a strain in different cultivars accurately. This would help in selecting resistant varieties for cultivation and contribute towards long term disease control.

Immunocytolocalization is a powerful tool which detects and locates specific molecules with great accuracy by utilizing an antisera as probe. In the present study, this technique has been used to determine cellular locations of CRA in root sections of tea varieties and mycelial cells and propagules of the fungal pathogen *R. solani*. Polyclonal antibodies (raised against the pathogen and the susceptible and resistant host variety) were used as antisera probes. For visualization, these were indirectly labelled with colloidal gold and subsequently enhanced with metallic silver. Several authors have used immunofluorescence technique for cellular location of CRA (Chakraborty and Saha, 1994; Chakraborty *et al.*, 1997; Wakeham and White, 1996; Kratka *et al.*, 2002; Dasgupta *et al.*, 2005). Although immunofluorescence is often used in localization studies, natural autofluorescence sometimes causes problem in locating CRA. Several workers (Kuo, 1999; Lee *et al.*, 2000; Nahalkova *et al.*, 2001; Trillas *et al.*, 2000; Kang and Buchenauer, 2002; Wang *et al.*, 2003) have used immunogold labelling for cellular location studies in electron microscope. However, in the present study, the immunogold labelling followed by silver enhancement was done specifically to study in the light microscope,

which is relatively new approach for studying cellular location of CRA. Saha *et al.* (2010) have successfully used the technique in locating CRA in brinjal tissues against antisera of *C. gloeosporioides*.

When root sections were treated with homologous antisera, maximum precipitation was observed in the epidermal regions, cortical tissues and vascular bundle elements of the roots. When root section of susceptible variety (TS-520) was treated with antisera of *R. solani* and labelled with immunogold particles enhanced by silver precipitation, CRA was observed mainly in the epidermal regions. Cortical tissues and vascular bundle elements also showed marginal darkening which indicate presence of CRA in these areas also. When root section of resistant variety (TS-449) was treated with the antisera of pathogen, no such precipitation was observed. Similar results were obtained by Saha *et al.* (2010), when they studied the presence of the common antigens in leaves of brinjal varieties. Dasgupta *et al.* (2005) studied immunolocalization of CRA between tea and the leaf pathogen *Curvularia eragrostridis* by using fluorescence labeling techniques. DeVay *et al.* (1981) studied innunolocalization of CRA in roots of succeptible young cotton plants treated with antiserum of *Fusarium oxysporum* f. sp. *vasinfectum* and observed that CRA was located at the epidermal and cortical cells as well as in the endodermis and xylem tissues.

In the present study mycelia and sclerotia of the fungal pathogen *R. solani* which were grayish in normal condition, turned dense blackish when treated with antisera of *R. solani* (i.e. homologous treatment) followed by immunogold labelling and subsequent silver enhancement. When treated with antisera of susceptible host (TS-520) followed by immunogold labelling and silver enhancement, dense blackish colour was observed mainly in the hyphal tips indicating presence of CRA. Similar treatment when done with antisera of resistant variety (TS-449), no darkening was observed indicating disparity in the antigens. Chakraborty and Saha (1994) also observed CRA in the hyphal

tips and in patch like areas on sclerotia and mycelium of the fungus by FITC labeling of the antiserum.

Immunolocalization studies also confirmed the presence of common antigens between susceptible host and pathogen. These common antigens may be involved in the invasion of pathogen and its growth and proliferation in host tissues. On the other hand, it may act as immunosuppressor and perform indirectly by not allowing the host defense machinary to successfully inhibit pathogen attack. Future research is required to clearly define the exact role of CRA in host pathogen interaction. More knowledge on susceptibility factors of the host and virulence factors of the pathogen should be able to throw light on how CRA performs in compatible interactions and further disease establishment.

Although chemical fungicides are very effective, their indiscriminate use is harmful to humans and adversely affect the microbial population present in the ecosystem. Therefore an eco-friendly approach to control the plant diseases is necessary. Biological control provides an alternative where a micro-organism that is non pathogenic to the plant but antagonistic towards plant pathogens is used.

Among fungal antagonists, *Trichoderma* spp. are most commonly used mainly due to their high efficacy in controlling several diseases. Several authors have reported the successful use of different isolates of *Trichoderma* for controlling many plant diseases (Maity and Sen, 1985; Latunda Dada 1993; Prasad *et al.*, 1999; Biswas, 1999; Jadeja, 2003; Saravanan *et al.*, 2003; Roberts *et al.*, 2005).

Rhizoctonia solani is responsible for serious damage to many economically important agricultural and horticultural crops as well as to trees worldwide (Anderson, 1982, Sneh *et al.* 1996). Grosch *et al.* (2005) reported that the importance of the pathogen *R. solani* was increased in European Condition. *R. solani* strains occur ubiquitously and are both saprophytic as well as pathogenic to more than 500 plant hosts. Although the pathogen

causes serious economical loss, no effective strategy to control the pathogen was available till 2007 as reported by Kai *et al.* (2007).

Parasitism of a plant pathogen by another microorganism like fungi, is a well known phenomenon (Alabourette *et al.* 2006). The parasitic activity of strains of *Trichoderma* spp. towards pathogens Rhizoctonia solani has been extensively studied by Chet and Baker (1981). It involves specific recognition between the antagonist and its targetpathogen and several types of cell wall degrading enzymes to enable the parasite to enter the hyphae of the pathogen.

Considering the capacity of mycoparasitism and also its ability to destroy the pathogen (hyphae) by several cell wall degrading enzymes, three different strains which included *Trichoderma harzianum*, *T. viride* and *Gliocladium virens* (isolates 1 and II) was tested in the present study to control *R. solani*, the most persistent and destructive pathogen of tea seeds. Dual culture method is widely used in antagonistic studies (Huang, 1978, Pachenan and Dix, 1980, Bell *et al.* 1982). Hence, the technique was adopted in the present study also. In the present study *T. harzianum* was the most effective fungi against *R. solani* showing 86.66% inhibition of mycelial growth of *R. solani* in comparison to control. *G. virens* (Isolate –II), *G. virens* (Isolate-I) and *T. viride* inhibited 78.89%, 65.56% and 60.56% mycelial growth respectively. Complete inhibition of sclerotium production by *T. harzianum* and *G. virens* (Isolate II) was also experienced. Poor sclerotia development was noticed when *G. virens* (Isolate-I) and *T. viride* were used separately against *R. solani*. Antagonism of *Trichoderma* species against several pathogens including *Rhizoctonia solani* has been reported by several workers (Chet and Baker, 1980; Bell *et al*, 1982; Papavizas, 1985; Elad, 2000; El-Katatny *et al.* 2001; Mahamune and Kakde, 2011).

Saikia and Gandhi (2003) used *Trichoderma viride*, *Trichoderma harzianum* and *Gliocladium virens* (presently known as *Trichoderma virens*) to control *Rhizoctonia solani*, isolated from cauliflower stem. They found

Trichoderma *viride* had higher biocontrol ability against the pathogen both in soil as well as in *in vitro* tests. A similar trend in cauliflower was also noted by Keinath (1995). Upamanyu *et al.*, (2002) also used several *Trichoderma* species to control *Rhizoctonia solani.* Among the bio-control agents tested *Trichoderma viride* showed good results to control root rot and web blight causing pathogen (*Rhizoctonia solani*) of French bean. Meena *et al.* 2003 reported that *Trichoderma harzianum* was effective in causing significant suppression of both growth and sclerotia formation of *R. solani* f. sp. *sasakii.* They also showed that *T. harzianum* and *T. viride* could inhibit the growth of the pathogen and percent inhibition of growth was about 80% and 70% respectively. In the current study our results also showed that *Trichoderma harzianum* was best effective in controlling the *Rhizoctonia solani* among the species of *Trichoderma* tested. Thus our findings are in conformity with some of the earlier observations.

Trichoderma has the capacity to reduce growth of the pathogens by cell wall degrading enzymes. In the present study, the mycoparasitism and cellular degradation has been studied together in dual culture experiments. In absence of the fungi, extracellular metabolites were also tested for their antagonistic activity in an experiment using culture filtrate of different *Trichoderma* species. The inhibition of growth was also evidenced in those experiments. Thus it can be concluded that the growth of *R. solani* could be checked partially by the fungi and also partially by the extracellular cell wall degrading enzymes. Mishra *et al.* (2011) reported the effectiveness of cell free culture filtrates of several *Trichoderma* sp. including *T. harzianum,* in controlling fungal pathogen. Among the *Trichoderma* spp., *T. harzianum* was found to be best by them in controlling *Pythium aphanidermatum.* Chowdhury *et al.* (2003) also found that cell free culture filtrate of *Trichoderma* (*T. virens* and *T. harzianum*) could control the pathogen *R. solani* causal pathogen of rice sheath blight. Thus, findings of our studies are in agreement with that of some of the earlier studies. Cell free culture filtrates of *Trichoderma* were also

used by Prasad and Kumar (2011), Chandrakala *et al.* (2012), Mishra (2010), Mishra *et al.* (2011), Shanmugam *et al.* (2008) and Perveen and Bokhari (2012). From the present study it may be concluded that *Trichoderma* may be used to control seed borne diseases of tea caused by *R. solani.*

In the last two decade or so a number of reports of antifungal activity of different plant extracts have been published (Singh *et al.*, 1995; Bhandary *et al.*, 2000; Deena and Thopil, 2000; Natrajon *et al.*, 2001; Ali *et al.*, 2001; Mittal *et al.*, 2002; Sharma *et al.*, 2002; Saxena *et al.*, 2003; Al-Howrini *et al.*, 2005; Saha *et al.*, *2005*; Obi, 2012; Barros *et al.*, 2012; Talibi *et al.*, 2012; askerne *et al.*, 2012). Most of the earlier workers prepared crude extracts prepared from the plants collected randomly or based on known ethnomedical use. Plants of different countries have been evaluated. Country or region wise the antifungal activity of the plant extracts are available from the works as given below. Plants of different regions including India was reported by Goel *et al.*, 2002; Perumal Samy, 2005; Lakshmi *et al.*, 2006; Satish *et al.*, 2009; Sheikh *et al.*, 2012 etc. Similarly, biological evaluation has been conducted on plants from different regions like Jordon (Alkofahi *et al.* 1996,1997); Mexico (Andrade-Cetto and Heinrich, 2005); Malayasia (Wiart *et al.*, 2004); Pakisthan (Ali *et al.*, 2001); Bangladesh (Rahaman *et al.*, 2001); Papua New Guniea (Rao,1996), Indonesia (Kevin *et al.*, 1999); Egypt (Khafagi and Dewedar, 2000); Thailand (Chuakul, 2000); Ghana (Konning *et al.*, 2004), Tanzania (Boer *et al.*, 2005) and Turkey (Dulger and Gonuz, 2004, Erturk, 2006).

In the present study, 23 plant extracts were evaluated for their antifungal efficacy against *R. solani in vitro* by agar cup bioassay. After selection of the suitable botanicals for controlling the pathogen (to evaluate the effect of plant extracts) poisoned food technique was also performed. The inhibitory effect of *Allium sativum* and *Polyalthia longifolia* and *Leucas cephalotes* was significantly high. In comparison to the above three plant extracts, the other seven plant extracts tested, showed much less inhibitory activity towards growth of *R. solani.* The fungitoxicity of the three plant

extracts in comparison to control have shown that 100% inhibition was possible by 10% and 20% concentration of the aqueous extracts after 10 days of incubation.

Allium sativum has been reported to possess antifungal activity by many workers (Jadeja, 2003; Curtis *et al.*, 2004 and Saha *et al.*, 2005). The antifungal activity of *A. sativum* have been shown due to compounds like allicin, E-ajoene, Z-ajoene, alliin, allitridin etc. (Ankri and Mirelman, 1999; Yoshida *et al.*,1999a,b; Miron *et al.*, 2002; Liu *et al.*, 2004; Hughes *et al.*,2005 and Baghalian *et al.*, 2006). Literature reports indicate that *P. longifolia* bark extracts are antifungal due to presence of 16-cleroda-3, 13E-dien-15-oic acid, kovavenic acid and 16B-hydroxycleroda-3, 13-dien-15, 16-olide (Rasid *et al.*,1996). Annapurna *et al.* (1983) evaluated leaf extracts of *P. longifolia* with different solvents of increasing polarity for antagonism against some pathogenic fungi and bacteria. Both the ethanol and aqueous extracts of *Datura metel* and *A. sativum* were used by Saha *et al.* (2005a) to control some pathogens of tea. Kagale *et al.* (2004) showed that leaf extracts of *Datura metel* significantly reduced the growth of *Rhizoctonia solani* and *Xanthomonas oryzae.* Thus our findings of antifungal activity of *A. sativum* and *P. longifolia* bulb and leaf extracts against the pathogen (*R. solani*) are in agreement with the observations of some of the previous workers. The leaf extract of *Datura metel* could inhibit the growth of *R. solani* as reported by Kagale *et al.*, (2004) but in our case it could control the growth of *R. solani* at a much lower efficiency than the extracts of *Allium sativum, P. longifolia* and *Leucas cephalotes.* Some earlier workers (Antariksh *et al.*, 2010; Srinivasan *et al* 2011; Das *et al.*, 2012) have reported the antifungal activity of *Leucas* species. *L. cephalotes* leaf extract has antifungal activity (Antariksh *et al.*, 2010 and Bhoria and Kainsa, 2013). *L. aspera* also has antifungal activity against *Trichophyton* and *Microsporum gypseum.* It had both fungistatic and fungicidal actions (Srinivasan *et al.*, 2011). A variety of phytoconstituents have been isolated from *Leucas* species, which include flavonoids, coumarins,

steroids, terpenes, fatty acids and aliphatic long-chain compounds. Anti-inflammatory, analgesic, anti-diarrheal, antimicrobial, antioxidant and insecticidal activities have been reported in the extracts of these plants and their phytoconstituents (Das *et al.*, 2012)

Protective activity of bio-control agents and botanicals need to be evaluated *in vivo,* before recommendation of the products. At the same time all the effective products also need to be formulated in such a way, so that it can be applied in the field suitably. In the present study, the fungus *R. solani* was controlled *in vivo* by selected biocontrol agents and botanicals. Extract of *Polyalthia longifolia* (20%) and 25% culture filtrate of *T. harzianum* was tested individually and also in combination. In all treatments, the pathogen-population was reduced. *A. sativum,* although showed good results in *in vitro* experiments was not included in the *in vivo* tests considering its high cost.

Percent germination of seeds was highest in all the tea seed varieties, when the seeds were treated with the sporulated culture of *T. harzianum.* Percent germination of seeds treated with *Polyalthia longifolia* leaf extract was slightly less than the seeds treated with sporulated culture of *T. harzianum.* The lowest percent germination was observed in different varieties when the seeds were treated with the 25% culture filtrate of *T. harzianum.* However, all the three treatments helped in controlling *R. solani* and as a result higher number of seeds germinated. Several authors (Prasad and Kulshreestha, 1999; Yamauchi and Winn, 1996; Ahuja and Payak, 1983) have assessed seedling vigour following seed germination to understand the efficacy of disease control agents. In the present study the vigour index was calculated following the method of Ahuja and Payak (1983). Vigour index of seeds treated with sporulated culture of *T. harzianum* was highest (3808.98) and Vigour index of seeds treated with 25% culture filtrate of *T. harzianum* was lowest (464.93) in case of variety, TS 449. Similar results were also observed in other seed varieties of tea of the present study.

For controlling root disease caused by *R. solani*, the tea seedlings were treated with the fungus *T. harzianum* by soil inoculation method. Two different formulations were tested by adopting pre inoculation, post inoculation and simultaneous inoculation method. Seedling mortality percentage was highly reduced in pre-inoculation method than in post inoculation and simultaneous inoculation method. This may be due to the fact that when the antagonist is applied one week before inoculation of *R. solani*, the antagonistic fungus established them in soil and did not allow *R. solani* to proliferate properly in soil. Similar results were also observed by Sharma and Gupta (2003). They used three methods of inoculation such as method I (seven day pre application of antagonist), method II (simultaneous inoculation of antagonist and pathogen) and method III (seven day post inoculation of antagonist). Best control of root rot incidence was achieved by them when they used antagonist-culture seven days prior to inoculation with *Rhizoctonia solani* which caused root rot of french bean. Thus our results are in conformity with that of Sharma and Gupta (2003).

All the investigations presented here has been confirmed and also extended some of the findings of the earlier workers. During this study, certain new facts of fundamental importance have also been revealed. A study of the tea seed mycoflora from North East India has been presented in the thesis. The role of *R. solani* in germination of tea seeds has been established. Verification of Koch's postulates has confirmed *R. solani* as a pathogen of tea seedlings. Pathogenicity test has identified the susceptible and resistant varieties against *R. solani*. The significance of antigenic relationship with regard to compatible interaction between *R. solani* and tea seed varieties has been demonstrated by various serological techniques. Correlation between pathogenicity test and different serological experiments was observed and was confirmed with indirect ELISA. Major Cross reactive antigens between the tea seed-varieties and the pathogen were detected in the cells of tea plants and pathogen *R. solani* through immunogold-silver enhancement studies using

light microscope. Some of the biocontrol agents and some of the botanicals tested, showed significant antifungal (against *R. solani*) efficacy. Thus the present study, identifies the problem of low percentage of seed germination, establishes a pathogen of the seedlings and also designs the suitable control measures of the disease using bio-control agents, botanicals and also suggests suitable formulations for controlling seed borne pathogen *R. solani.*

6. Summary

1. The present study deals with "Studies on tea seed mycoflora and resistance of young tea plants against *Rhizoctonia solani*, a soil borne root pathogen of germinating tea seedlings". The study consists of: i) Screening of tea seeds of different varieties for isolation of seed-borne pathogens. ii) Identification of major pathogen (*Rhizoctonia solani*) following Koch's postulates. iii) Pathogenicity of the fungus against seedlings of different tea varieties. iv) Studies on physiological characteristics of the pathogenic fungus. v) Studies on the resistance of tea against *Rhizoctonia solani* following serological techniques. vi) Control of the pathogen (*Rhizoctonia solani*) by antagonistic microorganisms and botanicals.
2. After a short introduction to the work, a brief review of literature related to the present line of investigation has been presented. The review is selective manner rather than comprehensive. Keeping relevance with the present works and also for convenience, the review has been grouped separately under subheadings likes seed mycoflora and seed diseases of tea plants, root diseases of tea plants, diseases caused by *Rhizoctonia Solani*, characteristics of *Rhizoctonia solani* as a pathogen, studies on growth and physiology of the pathogens, antigenic relationship in host and pathogen, disease control by antagonistic organisms and by disease control by botanicals.
3. Details of different experimental procedures and techniques have been described in the materials and methods section. The present study involves investigations on damages of tea seeds due to fungal infection. Initially, tea seed surface fungal flora and seed borne fungal flora were established following isolation and identification of the organisms. Altogether 400 seeds of seven different varieties (TS520, TS462, TS463, TS 449, TS464, TS491 and TS506) of tea were studied and the associated fungal species identified were *Curvularia lunata*, *Rhizoctinia*

solani, *Fusarium* sp., *Alternaria* sp., five species of *Aspergillus*, *Botryodiplodia* sp., two species of *Rhizopus*, *Penicillium* sp., *Trichoderma pseudokoningii* and a sterile fungus.

4. Following isolation, pathogenicity of the fungal isolates was confirmed by verification of Koch's postulates. Degree of pathogenicity of a selected pathogen, *Rhizoctonia solani* was determined by pathogenicity test in the selected varieties of tea which showed TS 449 as moderately resistant and the other varieties as susceptible to the fungus.
5. Growth and sporulation of *R. solani* have been studied on nine different media viz. Potato dextrose agar (PDA), Oat meal agar (OMA), Root extract agar (REA), Czapek dox agar (CDA), Richard's agar (RA), Yeast extract mannitol agar (YEMA), Malt extract agar (MEA), Potato carrot agar (PCA) and Nutrient agar (NA). Important physiological parameters have also been studied. Among these OMA and YEMA were best medium for the vegetative growth of the fungus. PDA medium was also good for growth. Sclerotia formation was very good in PDA and CDA media.
6. Optimum temperature for mycelial growth of *R. solani* was found between 23°C and 33°C. Maximum growth was observed at 28°C. Optimum pH was found to be 6.5.
7. Optimization of nutrient requirements was done where different carbon sources were supplemented in a basal medium. It was found that mannitol was the best carbon source for optimum growth and sclerotia formation of *R. solani* among the six different carbon sources tested. Sorbitol showed second best mycelial growth and sclerotia formation next to mannitol. Lactose showed minimum growth among the carbon sources tested.
8. Similarly, the influence of organic and inorganic nitrogen sources on growth and sclerotia formation of *R. solani* were also tested. Excellent growth and sclerotia formation was observed in beef extract

supplemented medium. Peptone, trypton, sodium nitrate, yeast extract and potassium nitrate were also good for growth and sclerotia formation.

9. The presence and the level of cross reactive antigens (CRA) between the seven different tea seed varieties and the pathogen, *R. solani* was determined in order to study the correlation between host-pathogen compatibility and CRA levels by immunodiffusion, immunoelectrophoresis and ELISA. Common antigens were detected in the form of precipitin arcs in immunodiffusion plates when antigens of susceptible tea varieties (520a, 462a, 464a and 491a) were cross reacted with the antisera of *R. solani* but were absent during similar cross-reactions with resistant variety.
10. In immunoelectrophoretic studies, antigens of susceptible varieties shared at least one precipitin band each in all the cases when reacted with antisera of *R. solani* while antigen of resistant variety (449a) showed no precipitin band. In reciprocal cross reaction, antigen of *R. solani* shared one precipitin band with antisera (520A) of TS-520 (susceptible variety) but no precipitin band with antisera of TS449 variety (resistant variety).
11. Indirect ELISA technique was followed for detecting CRA at very low concentrations. The higher ELISA values in cross reactions revealed the presence of more CRA, which indicated the susceptibility of the variety. Similarly, lower ELISA values revealed lower amount of CRA that indicated resistance. The results obtained by indirect ELISA values i.e. the degree of susceptibility and resistance was in agreement with the results of pathogenicity tests also.
12. Immunocytolocalization studies were conducted to determine cellular locations of CRA in root sections of tea varieties and mycelial cells and propagules of the fungal pathogen *R. solani.* In the present study, immuno-gold labeling followed by silver enhancement was done

specifically to study CRA level in the light microscope. When root section of susceptible variety (TS-520) was treated with antisera of *R. solani* and labelled with immunogold particles enhanced by silver precipitation, CRA was observed mainly in the epidermal regions. Cortical tissues and vascular bundle elements also showed marginal darkening which indicate presence of CRA in these areas also. When root section of resistant variety (TS-449) was treated with the antisera of pathogen, no such precipitation was observed. When the fungal pathogen, *R. solani* was treated with antisera of susceptible host followed by immunogold labelling and silver enhancement, dense blackish colour was observed mainly in the hyphal tips indicating presence of CRA. When similar treatment was done with antisera of resistant variety, no darkening was observed indicating disparity in the antigens.

13. Considering the capacity of mycoparasitism and also its ability to destroy the fungal pathogens by several cell wall degrading enzymes, three different strains which included *Trichoderma harzianum*, *T. viride* and *Gliocladium virens* (isolates 1 and II) was tested in the present study to control *R. solani*. Results revealed that *T. harzianum* was the most effective fungi by *in vitro* dual culture tests. Similarly 23 different plant extracts were also tested for their ability to control the growth of the pathogen by agar cup assay which showed that *Allium sativum*, *Polyalthia longifolia* and *Leucas cephalotes* were 100% effective in inhibiting the growth of *R. solani*.
14. Extract of *Polyalthia longifolia* (20%) and 25% culture filtrate of *T. harzianum* was tested individually and also in combination in various seed treatment studies for determining the effect of the extracts *in vivo*. In all treatments, the pathogen-population was reduced. Vigour index of seeds treated with sporulated culture of *T. harzianum* was highest and

that of seeds treated with 25% culture filtrate of *T. harzianum* was lowest in TS 449 variety.

15. For controlling root disease caused by *R. solani*, the tea seedlings were treated with the fungus *T. harzianum* by soil inoculation method. Two different formulations were tested by adopting pre inoculation, post inoculation and simultaneous inoculation method. Seedling mortality percentage was highly reduced in pre-inoculation method than in post inoculation and simultaneous inoculation method.
16. Thus the present study, identifies the problem of low percentage of seed germination, establishes a pathogen of the seedlings and also designs the suitable control measures of the disease using bio-control agents, botanicals and also suggests suitable formulations for controlling seed borne pathogen *R. solani.*

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