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.

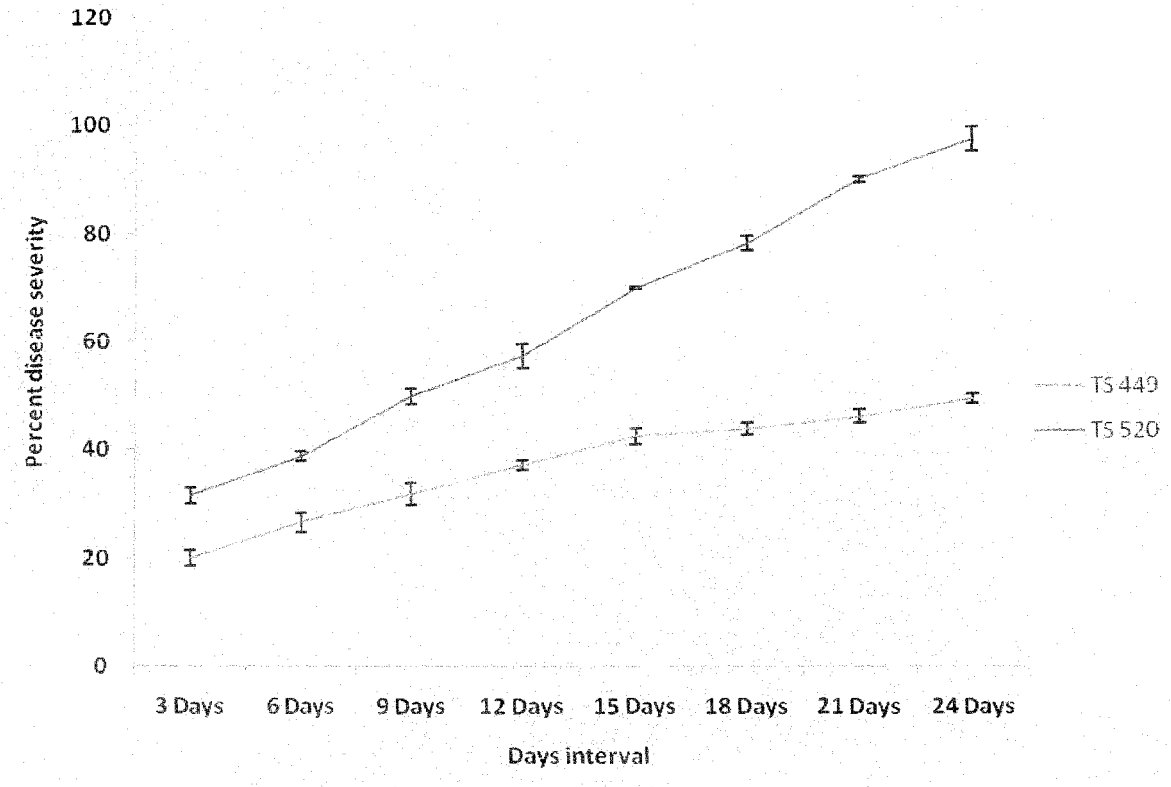

Fig. 4.1: Percent disease severity in two tea varieties (most resistant and most susceptible) tested

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.

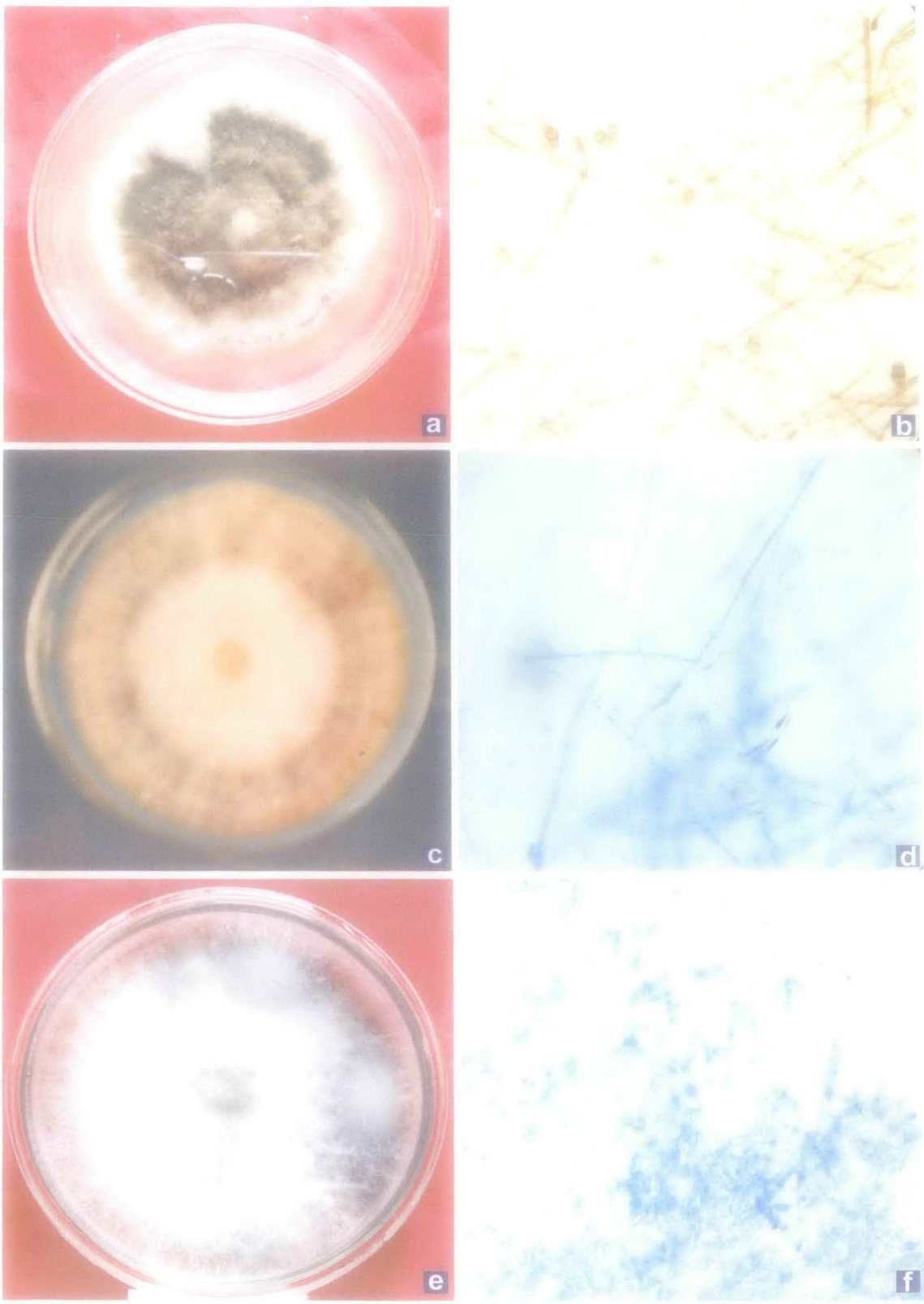

Plate VI: Various fungi isolated from tea seeds (a) *Curvularia lunata* in petriplate, (b) *C. lunata* under light microscope (mycelia and spores are visible) (c) *Rhizoctonia solani* in petriplate (d) Mycelia of *R. solani* under light microscope (e) *Fusarium* sp. in petriplate (f) Mycelia and spores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

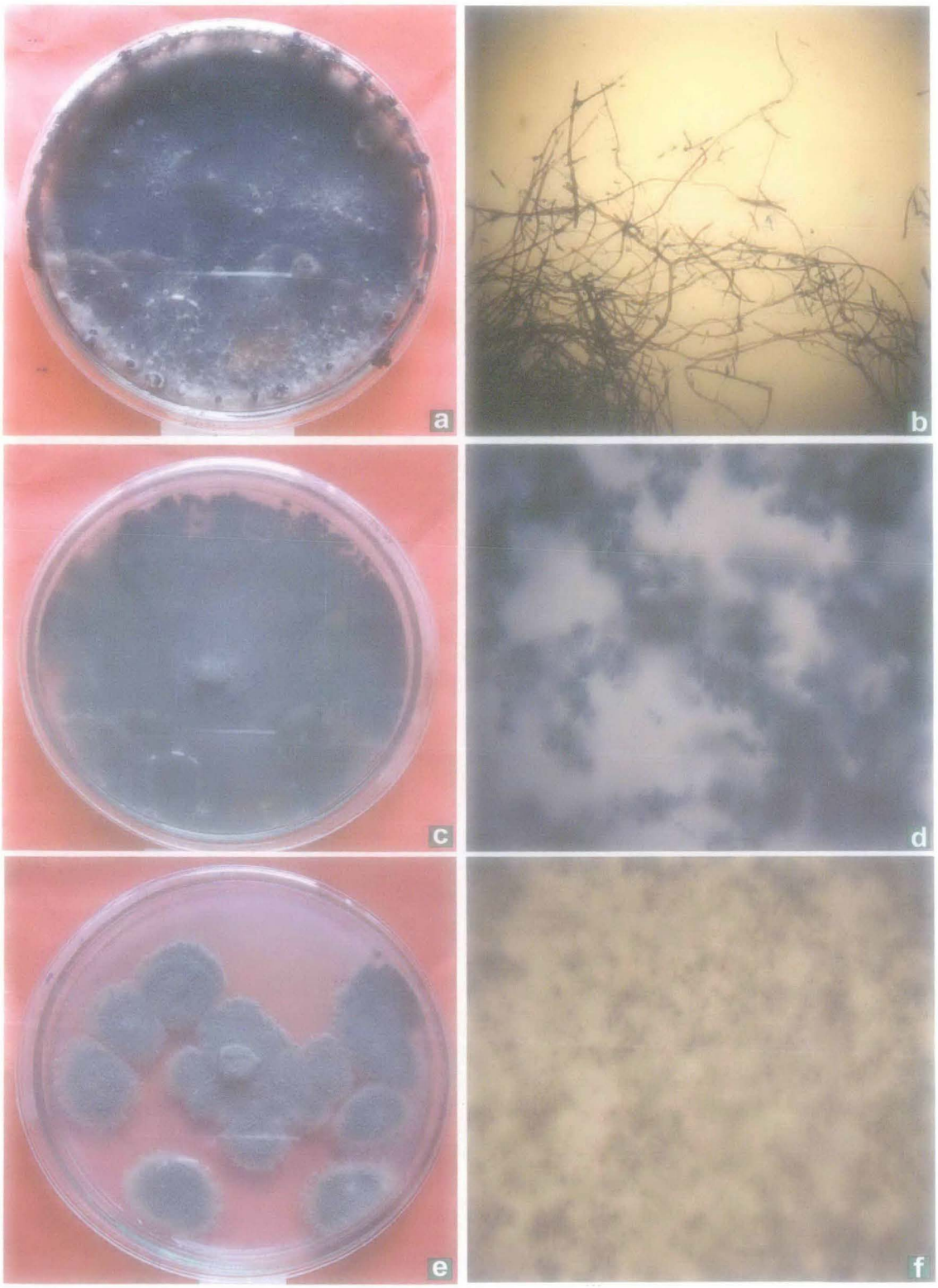

Plate VII: Various fungi isolated from tea seeds (a) *Alternaria* sp. (b) Mycelia of *Alternaria* sp. (c) *Aspergillus* sp. (Isolate I) (d) Spores of *Aspergillus* sp. (e) *Aspergillus* sp. (Isolate II) (f) Spores of *Aspergillus* sp. (Isolate II)

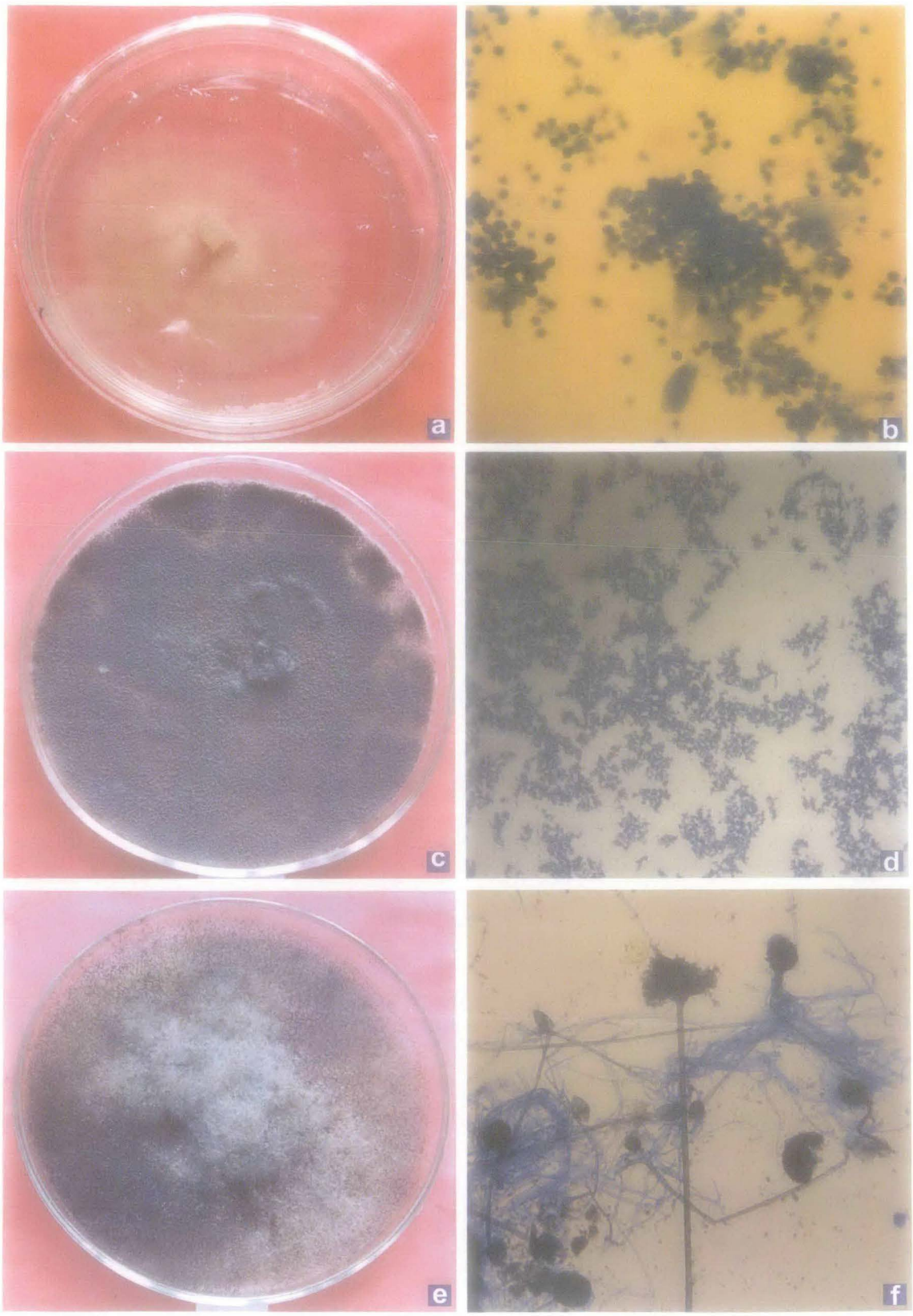

Plate VIII: Various fungi isolated from tea seeds (a) *Penicillium* sp. (b) Conidia and conidiophores of *Penicillium* sp. (c) *Aspergillus* sp. (Isolate III) (d) Spores of *Aspergillus* sp. (Isolate III) (e) *Rhizopus* sp. (Isolate I) (f) Sporangia, sporangiophores and mycelia of *Rhizopus* sp. (Isolate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

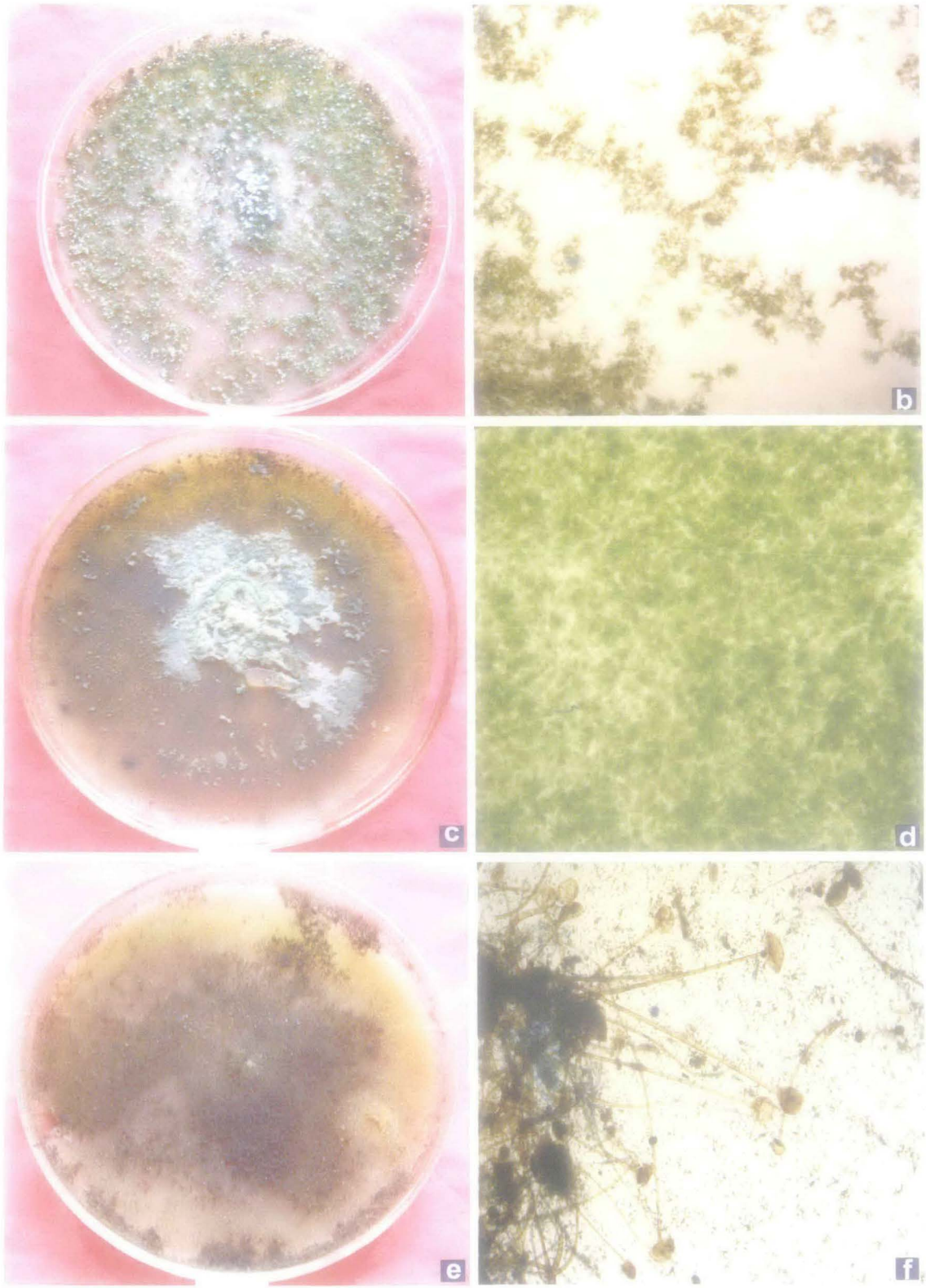

Plate IX: Various fungi isolated from tea seeds (a) *Aspergillus flavus* (b) Spores of *Aspergillus flavus* (c) *Trichoderma pseudokoningii* (d) Spores of *Trichoderma pseudokoningii* (e) *Rhizopus* sp. (Isolate II) (f) Sporangia, sporangiophores and mycelia of *Rhizopus* sp. (Isolate II)

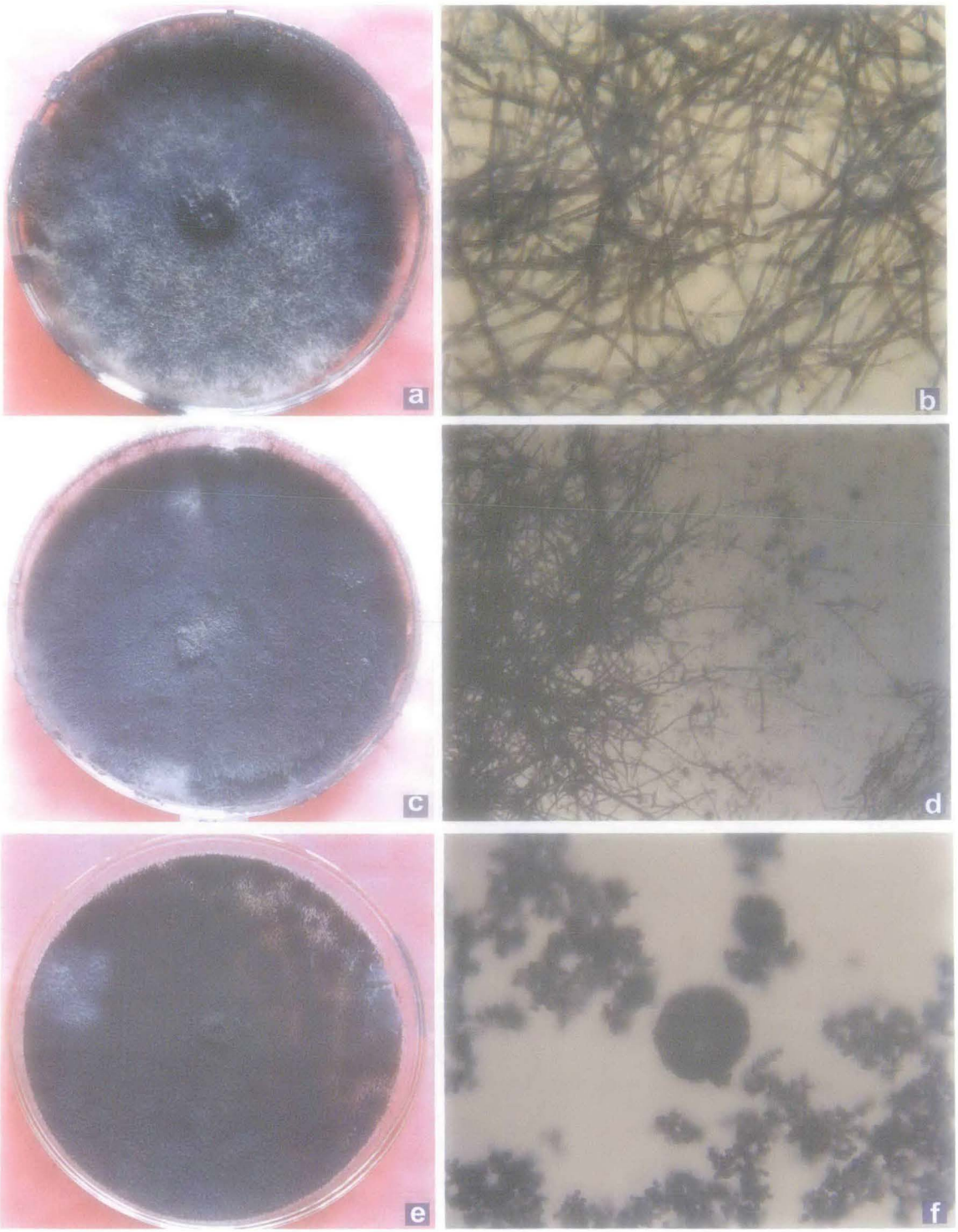

Plate X: Various fungi isolated from tea seeds (a) *Botryodiplo dia* sp. (b) Mycelia of *Botryodiplo dia* sp. (c) Sterile fungi (d) Mycelia of sterile fungi (e) *Asperg llus niger* (f) Spores of *Asperg llus niger*

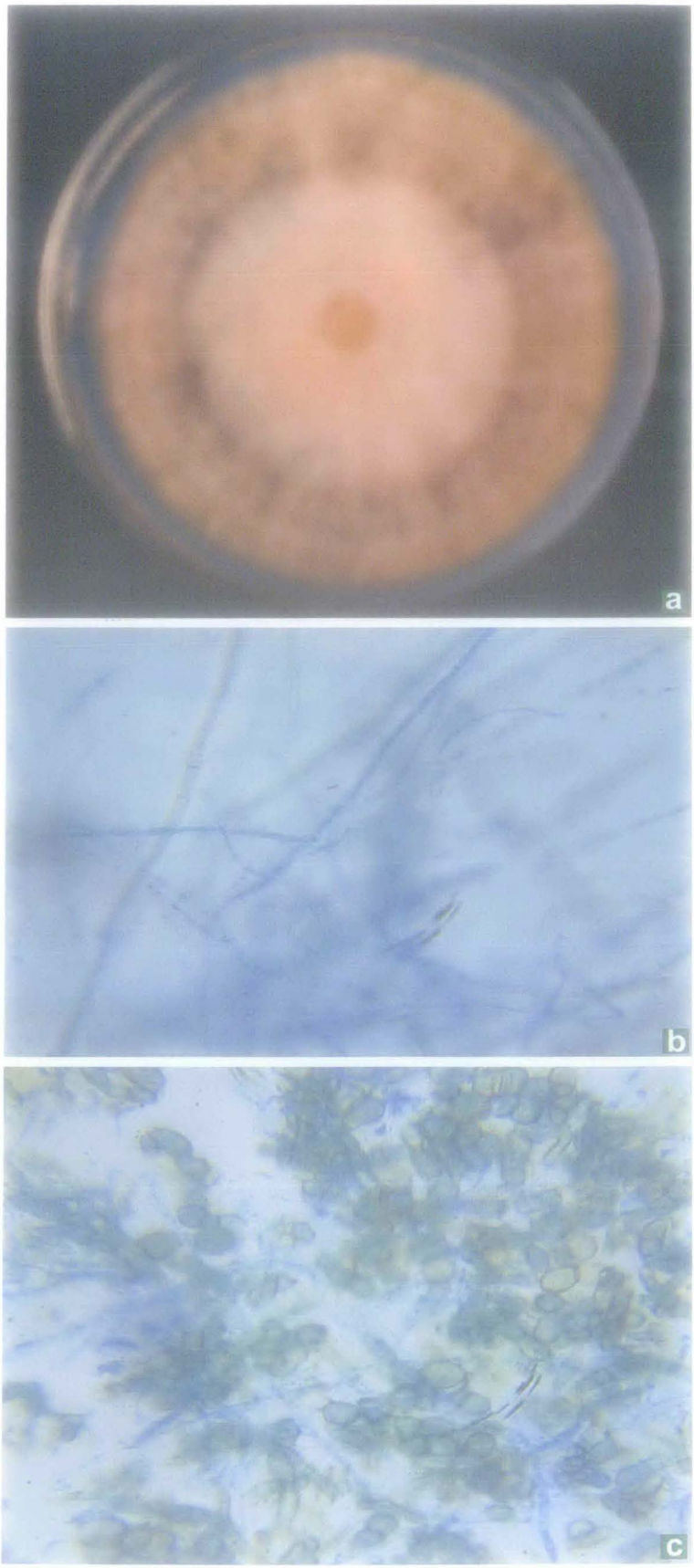

Plate XI: *Rhizoctonia solani* (a) *Rhizoctonia solani* in petri plate (b) Mycelia of *Rhizoctonia solani* (c) Moni loid cells of *Rhizoctonia solani*

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

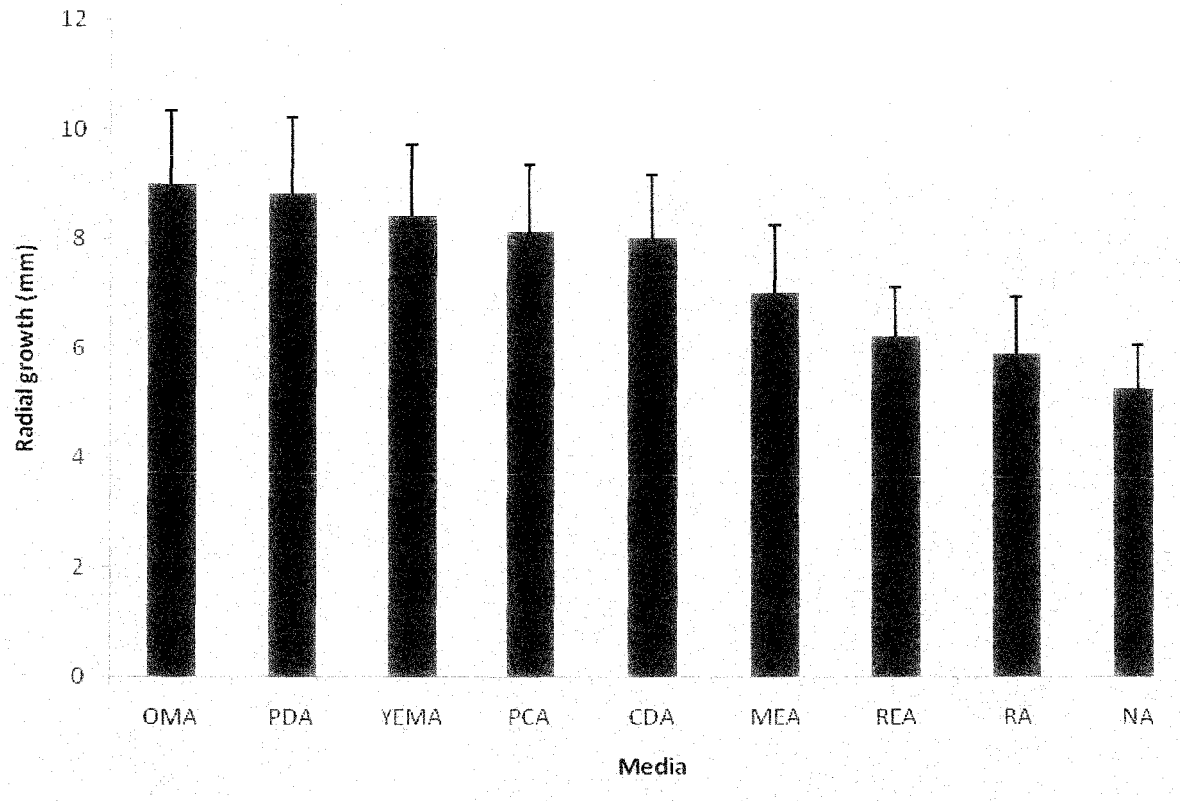

Fig.4.2 Radial mycelial growth of *R. solani* in different solid media

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.*

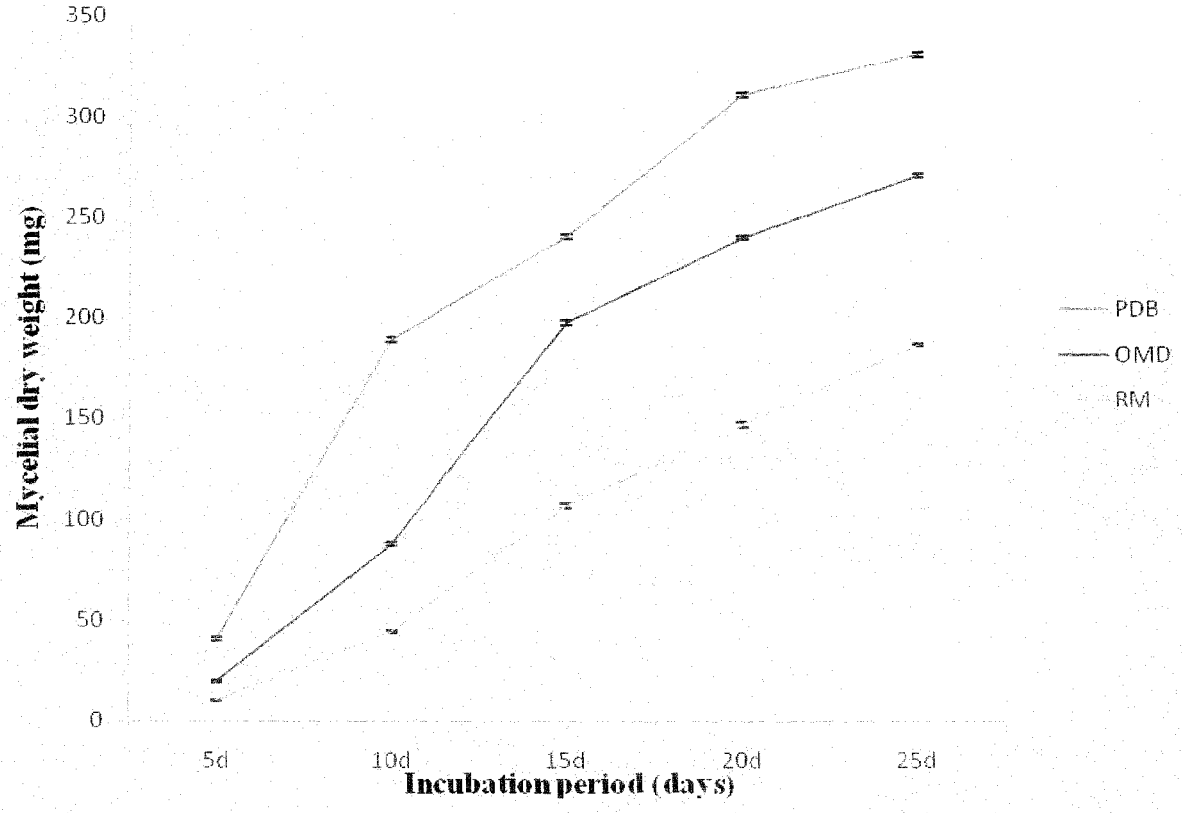

Fig 4.3: Growth of *R. solani* after different incubation periods in different liquid media

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.

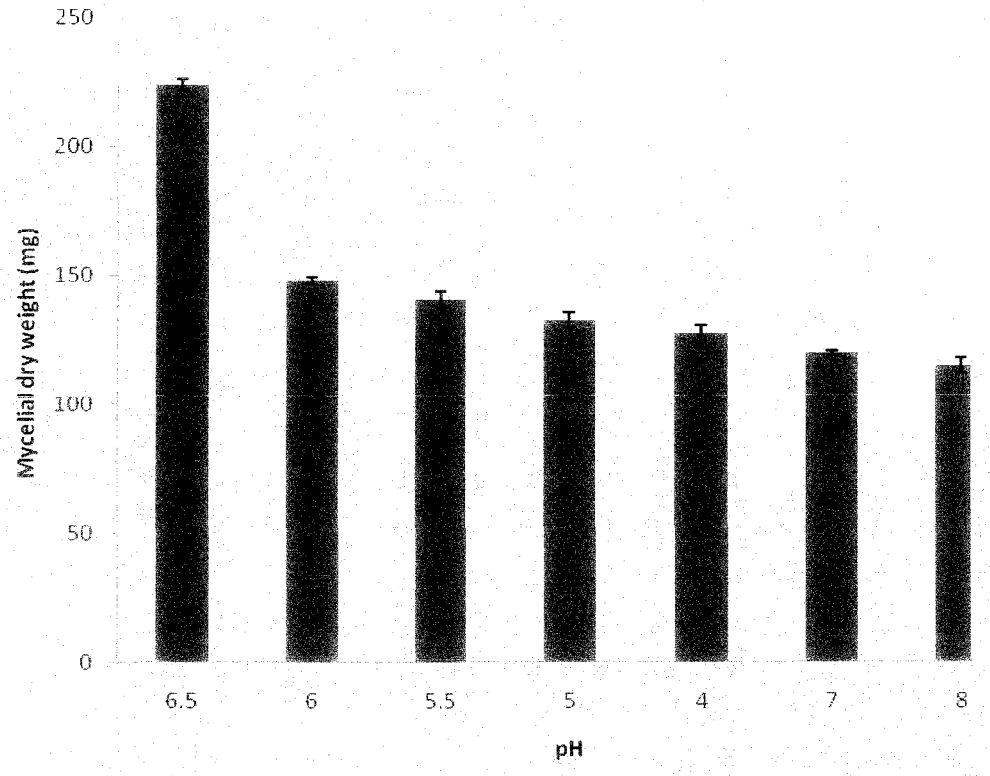

Fig 4.4: growth of R. solani at different pH conditions

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.

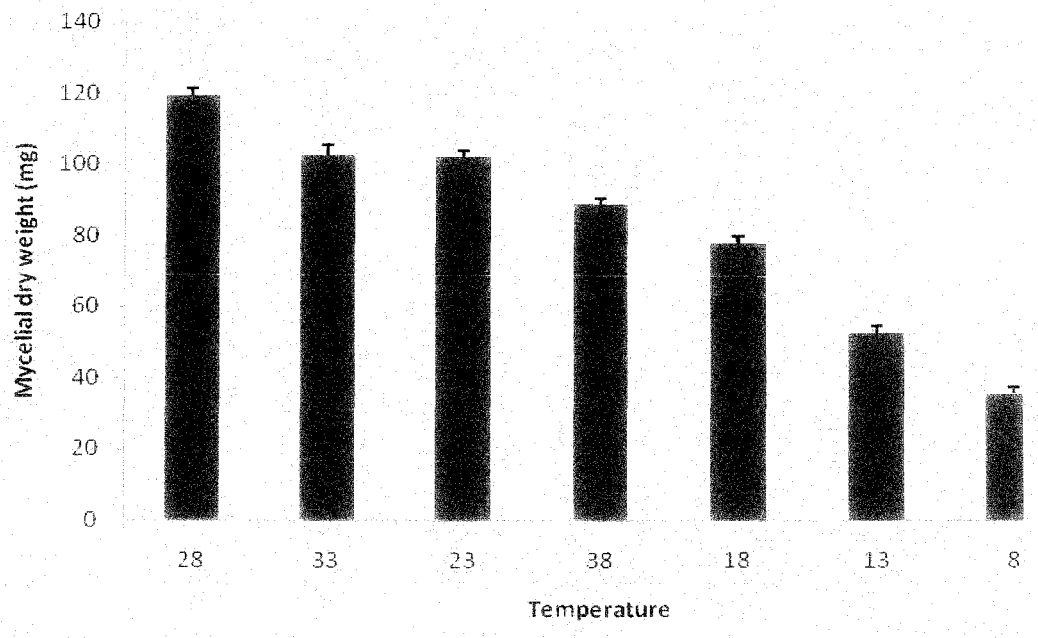

Fig 4.5: Effect of different temperature on mycelial growth of *R. solani*

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.

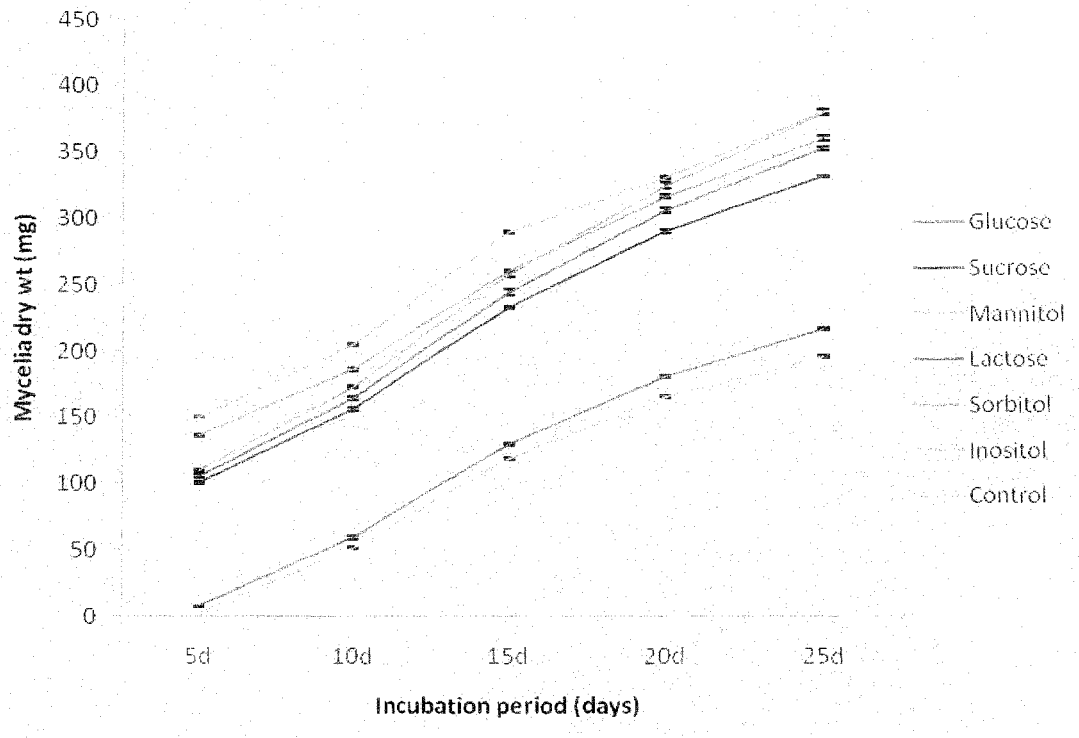

Fig 4.6: Effect of different carbon sources on mycelial growth of *R. solani*

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.

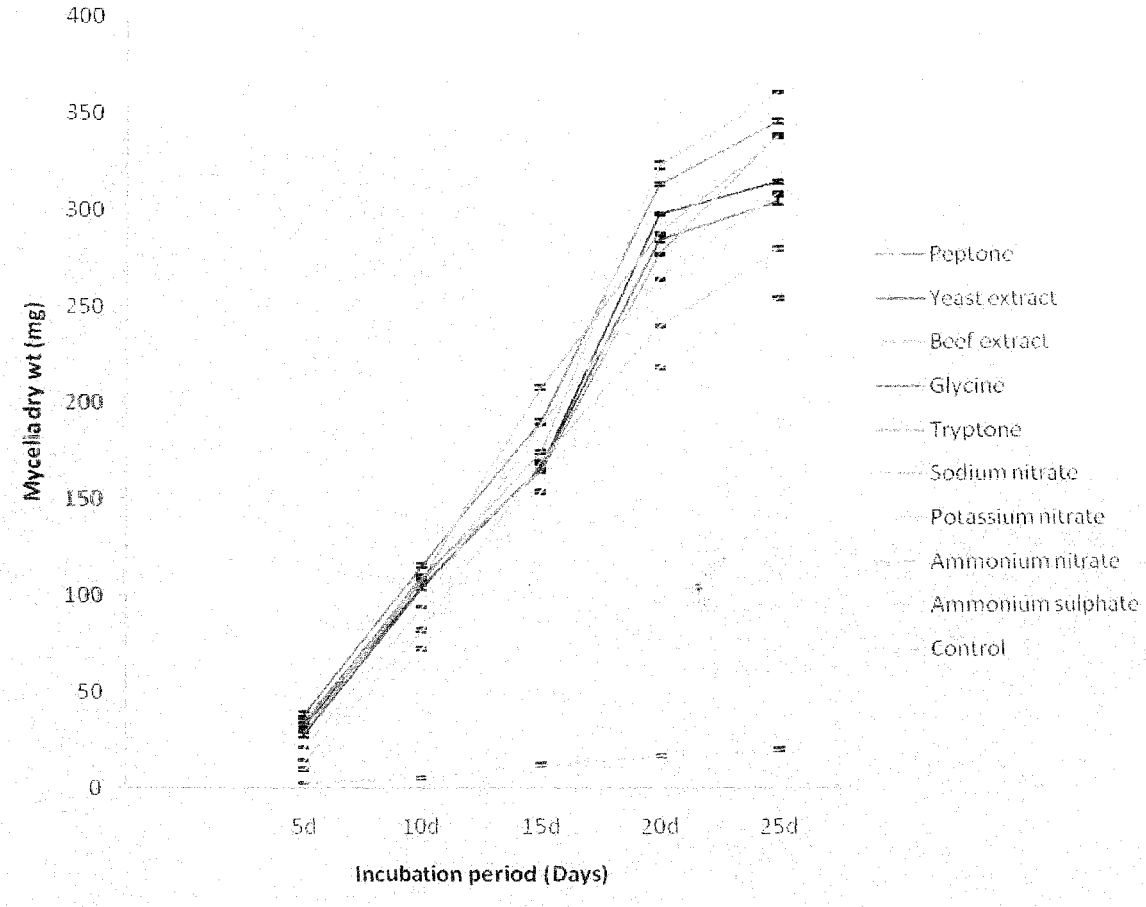

Fig 4.7: Graphical representation of different nitrogen sources on mycelial growth of *R. solani*.

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= -

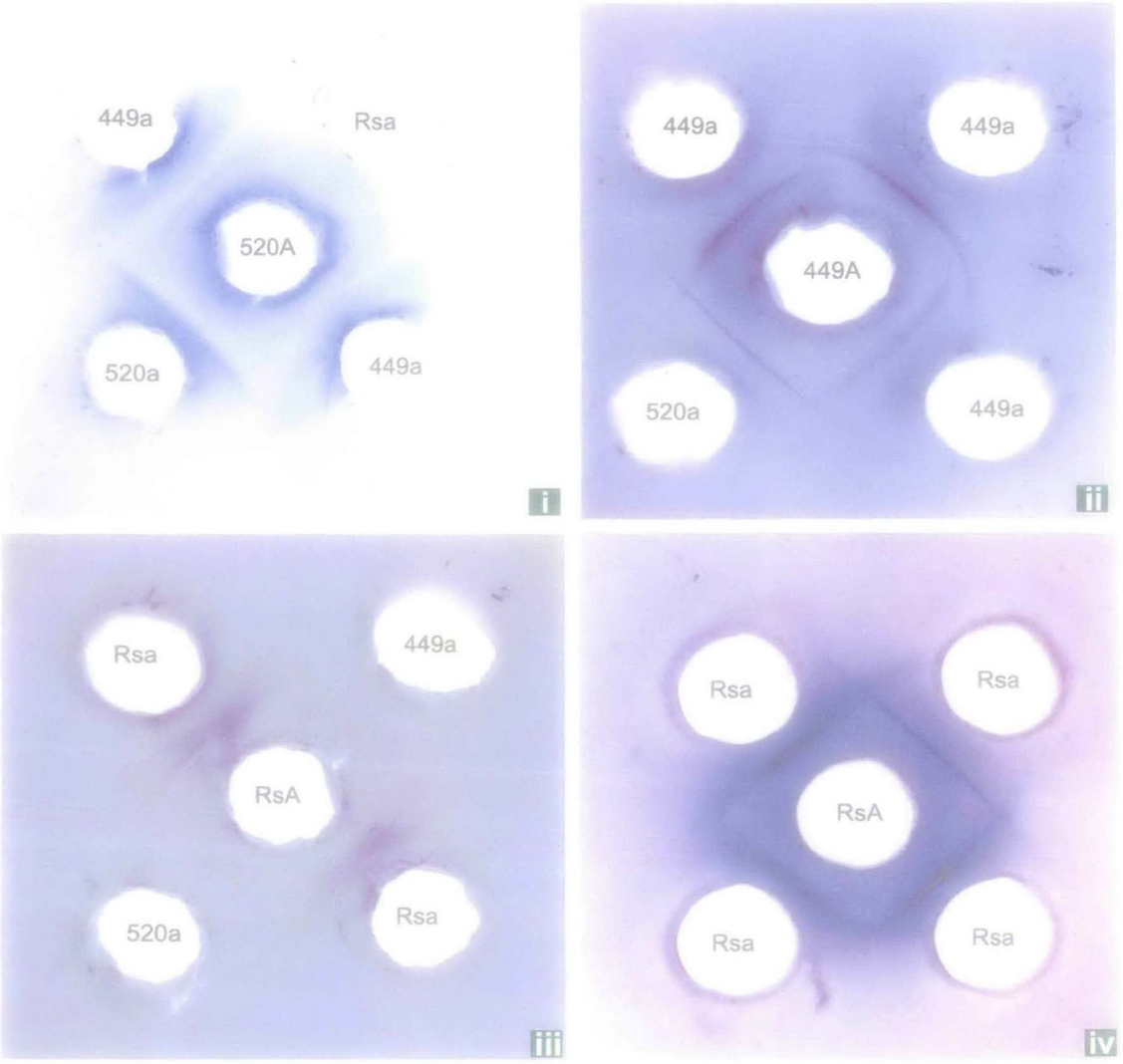

Plate XII: Agar gel double diffusion test using different antigens & antisera. Codes of antisera & antigen are shown in the wells. Numericals used to indicate respective tea varieties and suffix 'A' indicate antisera and 'a' indicate antigen. 'Rs' indicate *Rhizoctonia solani* mycelia. (i)Root antisera 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.

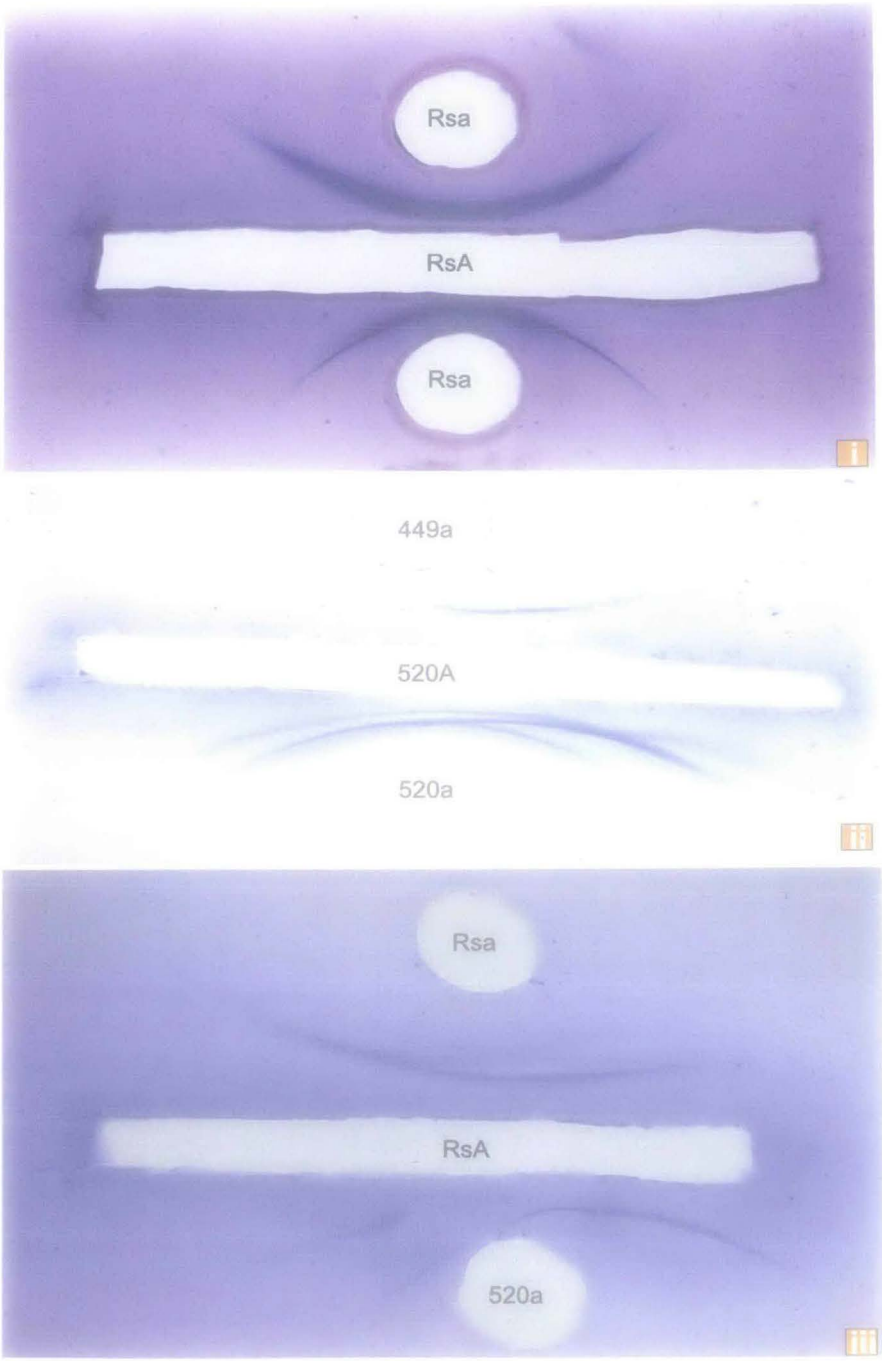

Plate XIV: Immuno electrophoresis (i) Antisera of *Rhizoctonia solani* (RsA) reacted with antigen (Rsa) of *Rhizoctonia solani.* (ii) Antisera of tea variety TS-520(520A) reacted with antigens (449a) and (Rsa) respectively of tea variety (TS-520) and *Rhizoctonia solani.* (iii) Antisera of *Rhizoctonia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

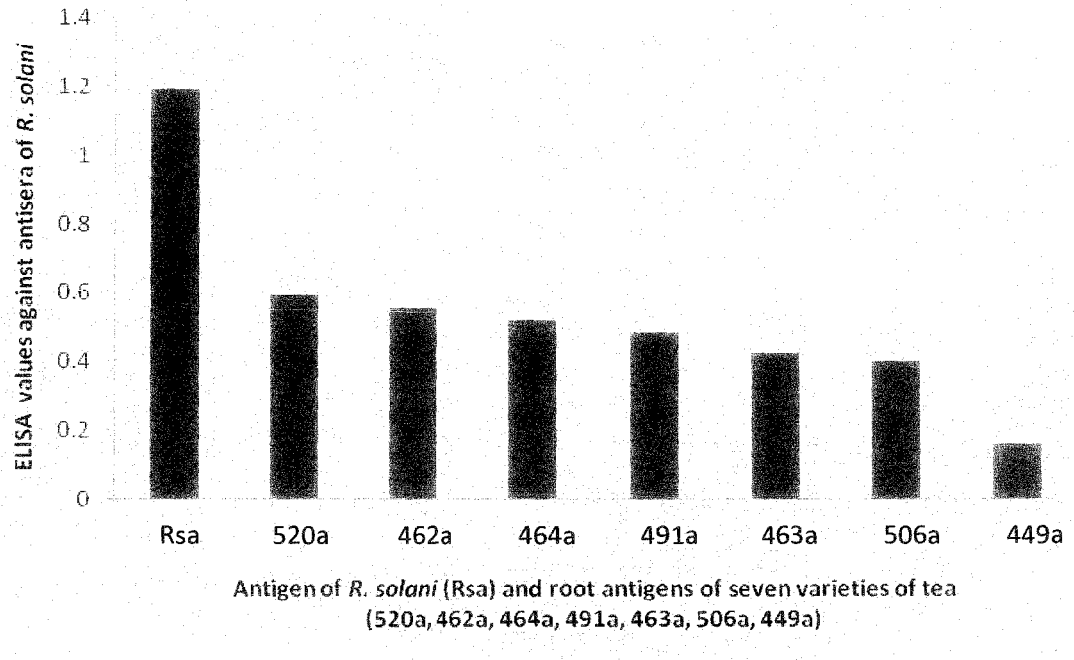

Fig. 4.8: Indirect ELISA results (A_{492}) of different antigens at concentration of 20mg protein/ml against antisera of *R. solani* (RsA) at 1/125 dilution

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)

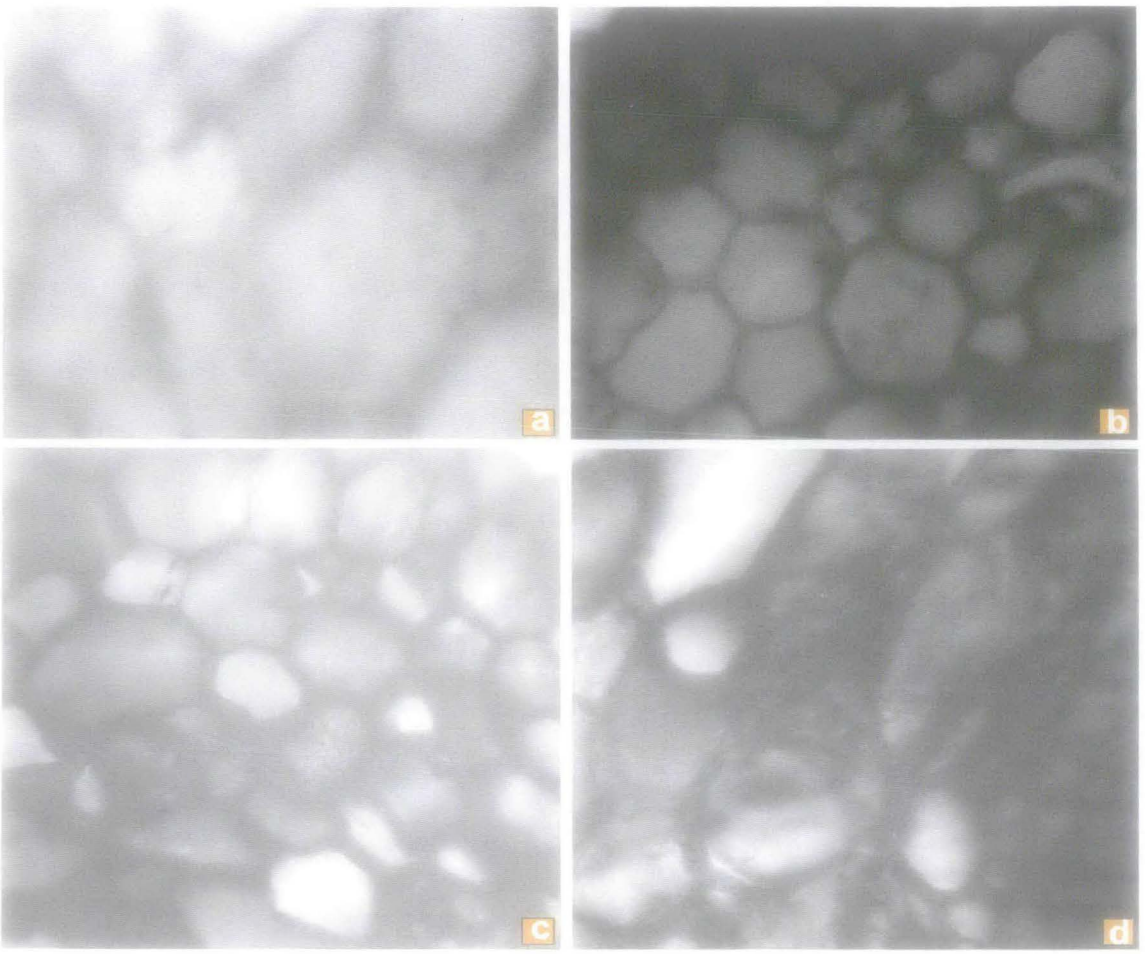

Plate XV: Silver enhancement of immunogold labelled tea root tissues for detection of crossreactive antigens and homologous antigens (a) Root section of TS-449 treated with Rsa (antisera of *R.Solani*); (b) Root section of TS-520 treated with antisera of *R.solani* (RsA); (c) Root section of TS-520 treated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

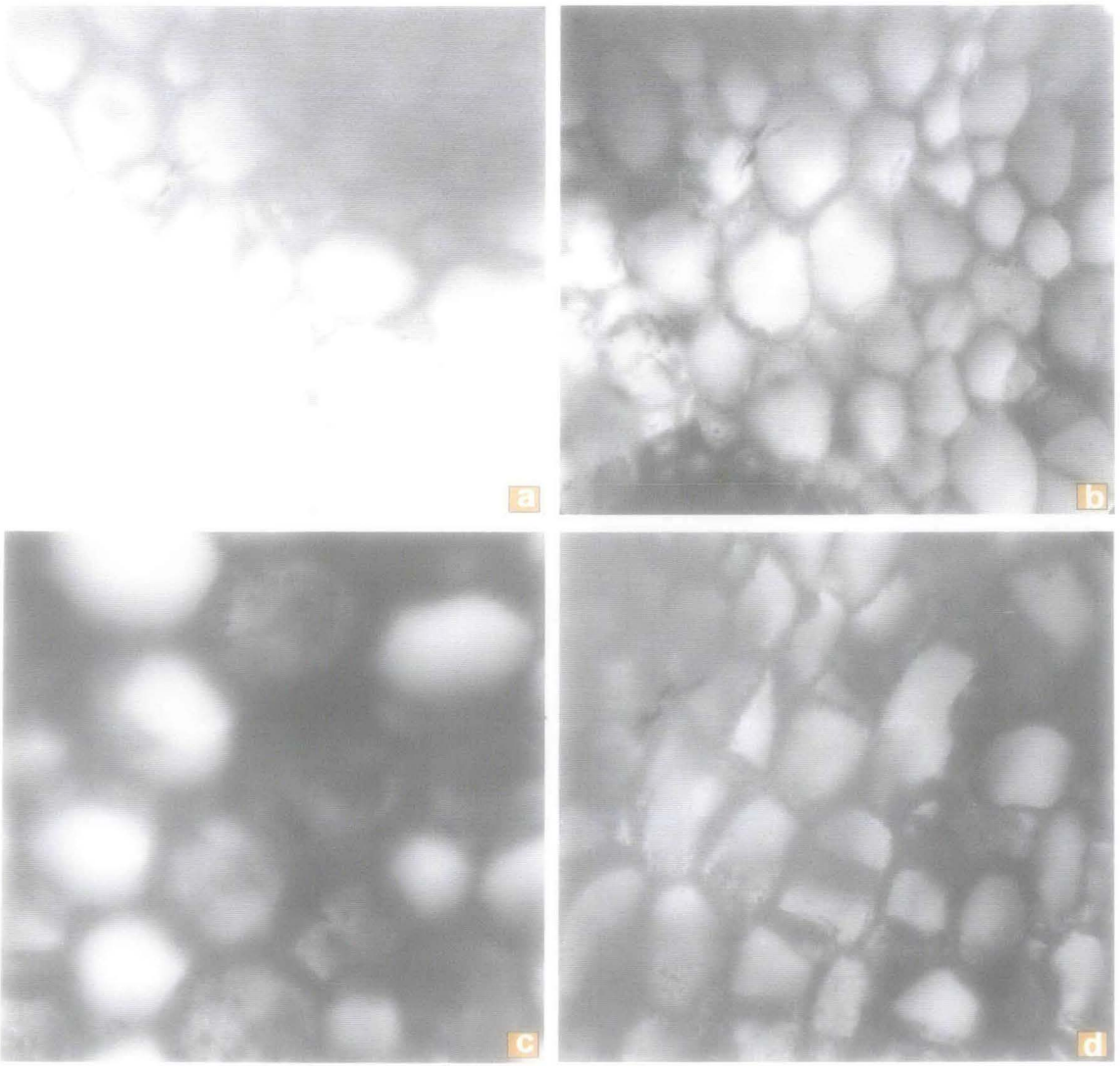

Plate XVI: Immuno-gold labeling and silver enhancement of tea root tissues for detection of crossreactive antigens and homologous antigens (a) Root section of TS-462 treated with antisera of *R.solani* (RsA); (b) Root section of TS-463 treated with antisera of *R.solani* (RsA); (c) Root section of TS-449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

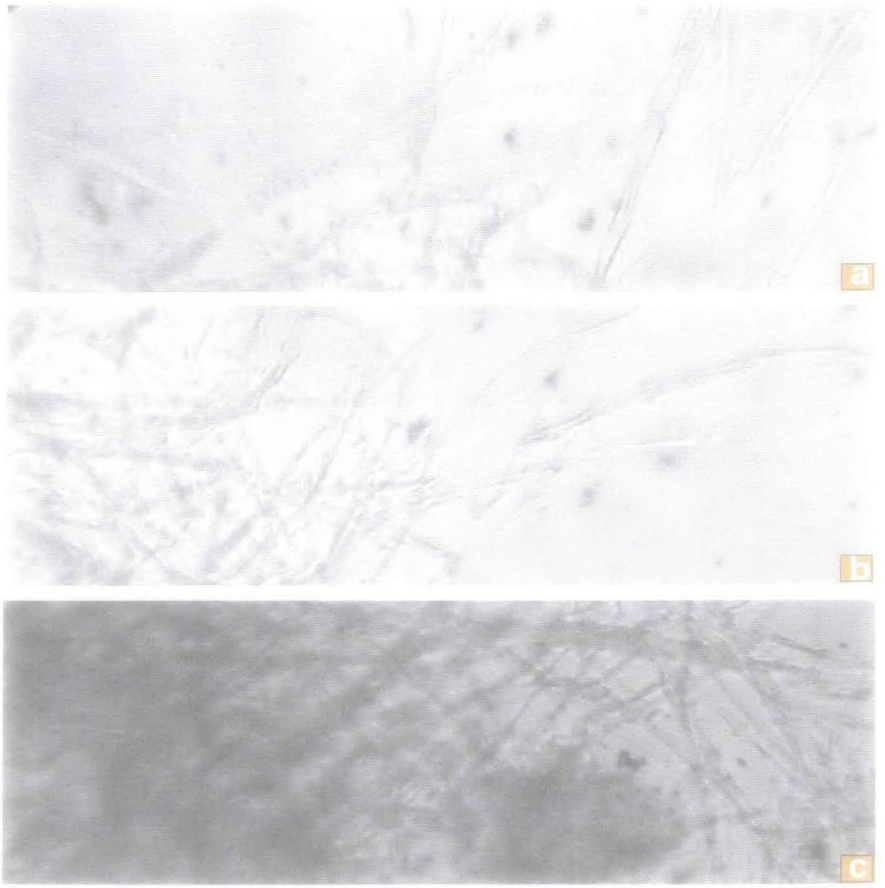

Plate XVII: Immuno-gold labeling and silver enhancement of mycelia of *R.solani* for detection of cross-reactive antigens and homologous antigens (a) Mycelia of *R.solani* (untreated); (b) Mycelia of *R. solani* treated with antisera of TS-449 (449A); (c) Mycelia of *R. solani* treated with antisera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

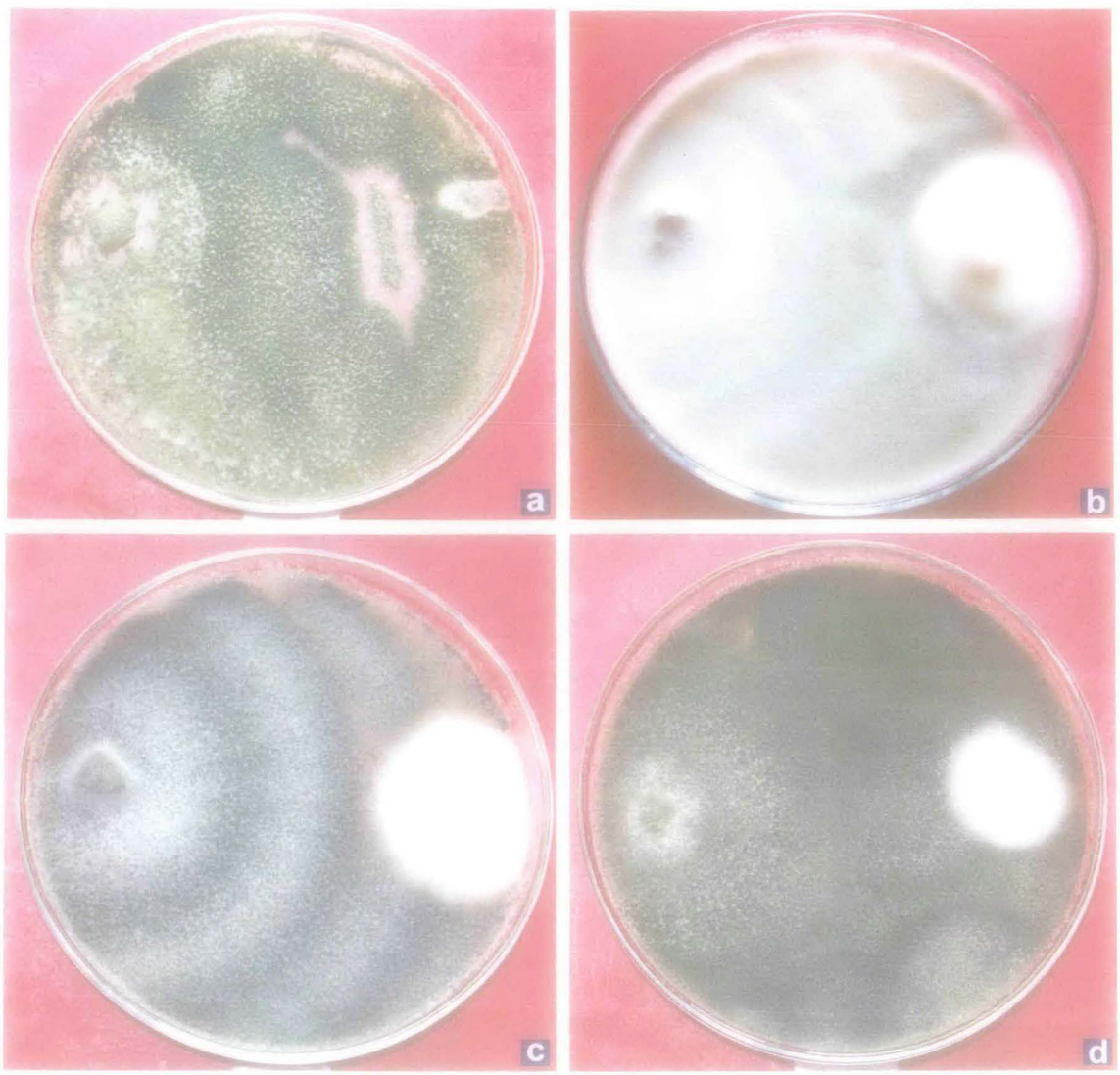

Plate XVIII: In vitro control of *Rhizoctonia solani* with four different biocontrol fungi in dual culture test (a) Control *R. solani* by *Trichoderma harzianum* (b) Control of *R. solani* by *Trichoderma viride* (c) Control of *R. solani* by *Trichoderma virens* (isolate 1) (d) Control of *R. solani*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

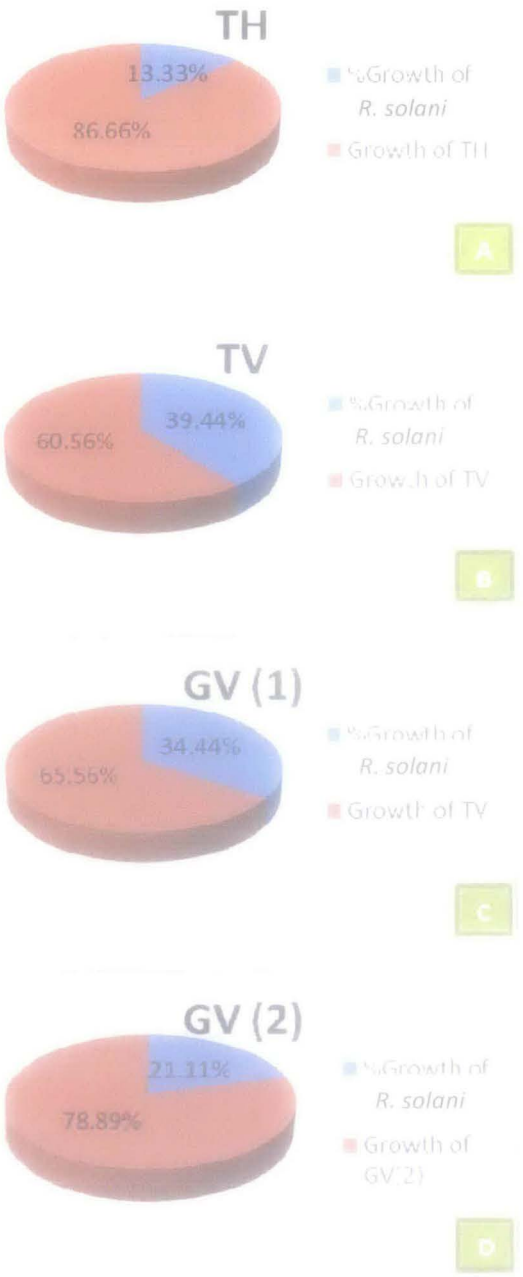

Fig 4.9: **Effect of antagonists on the radial growth of *R. solani* in PDA dual culture assay; TV=*Trichoderma viride*; TH=*Trichoderma harzianum*; GV (1) = *Gliocladium virens* isolate 1; GV (2)= *G. virens* isolate 2**

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.

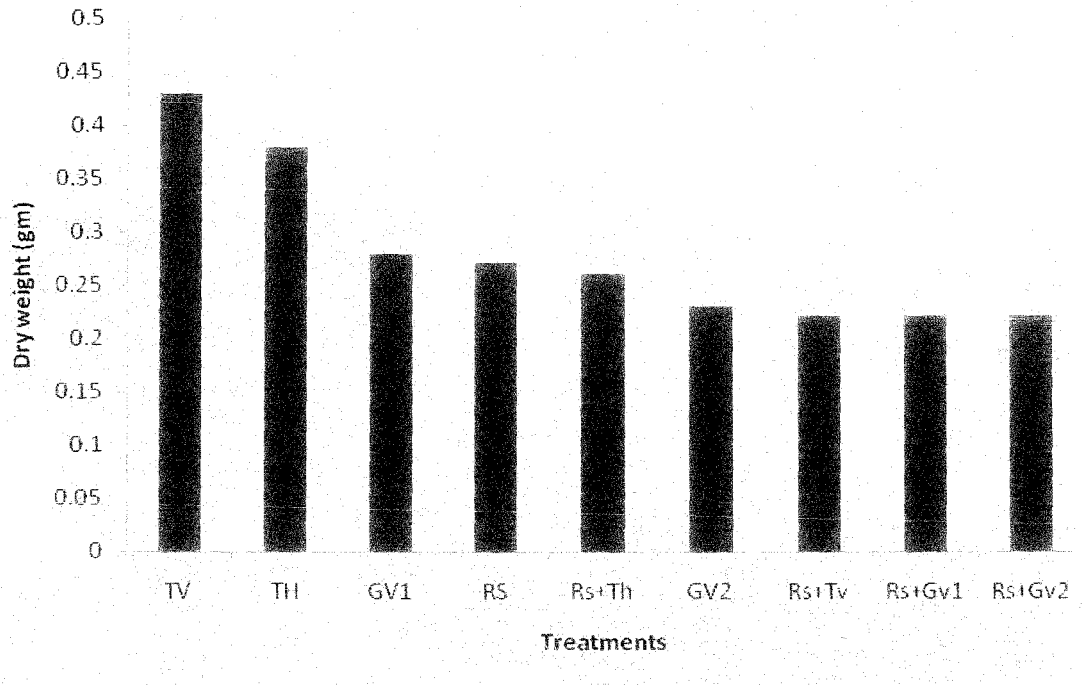

Fig 4.10: Graphical representation of mycelia growth in 25% culture filtrate supplemented PDB medium

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

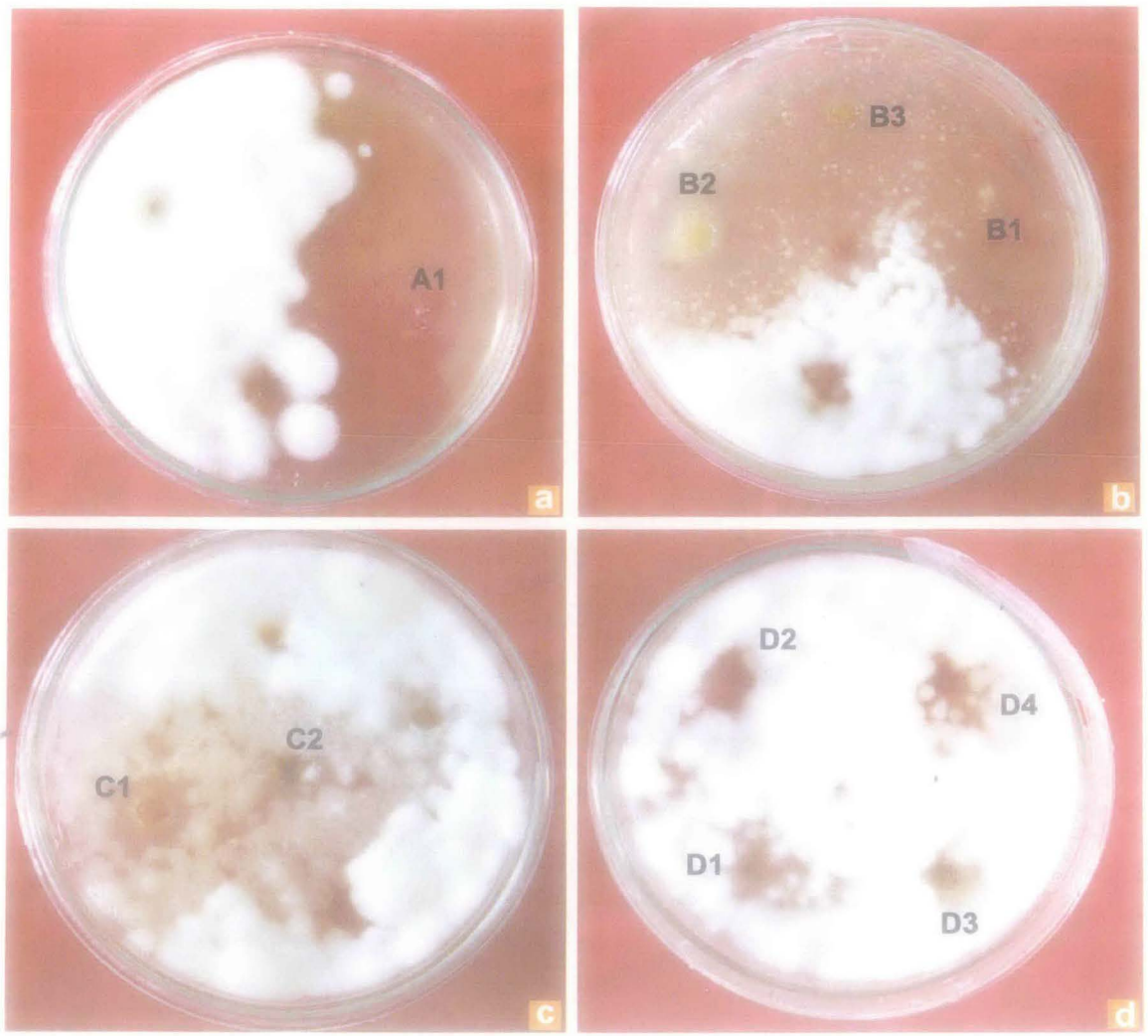

Plate XIX: Inhibitory effect of botanicals following agar cup method.
(a) A1 = *Allium sativum* L. (b) B1 = *Polyalthia longifolia* sonn., B2= *Leucus cephalotes* L., B3= *Tridex procumbens* L., (c) C1= *Xanthium strumarium* L., C2=*Occimum sanctum* L., (d) D1=*Datura metel* L., D2=*Azadirachta indica*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

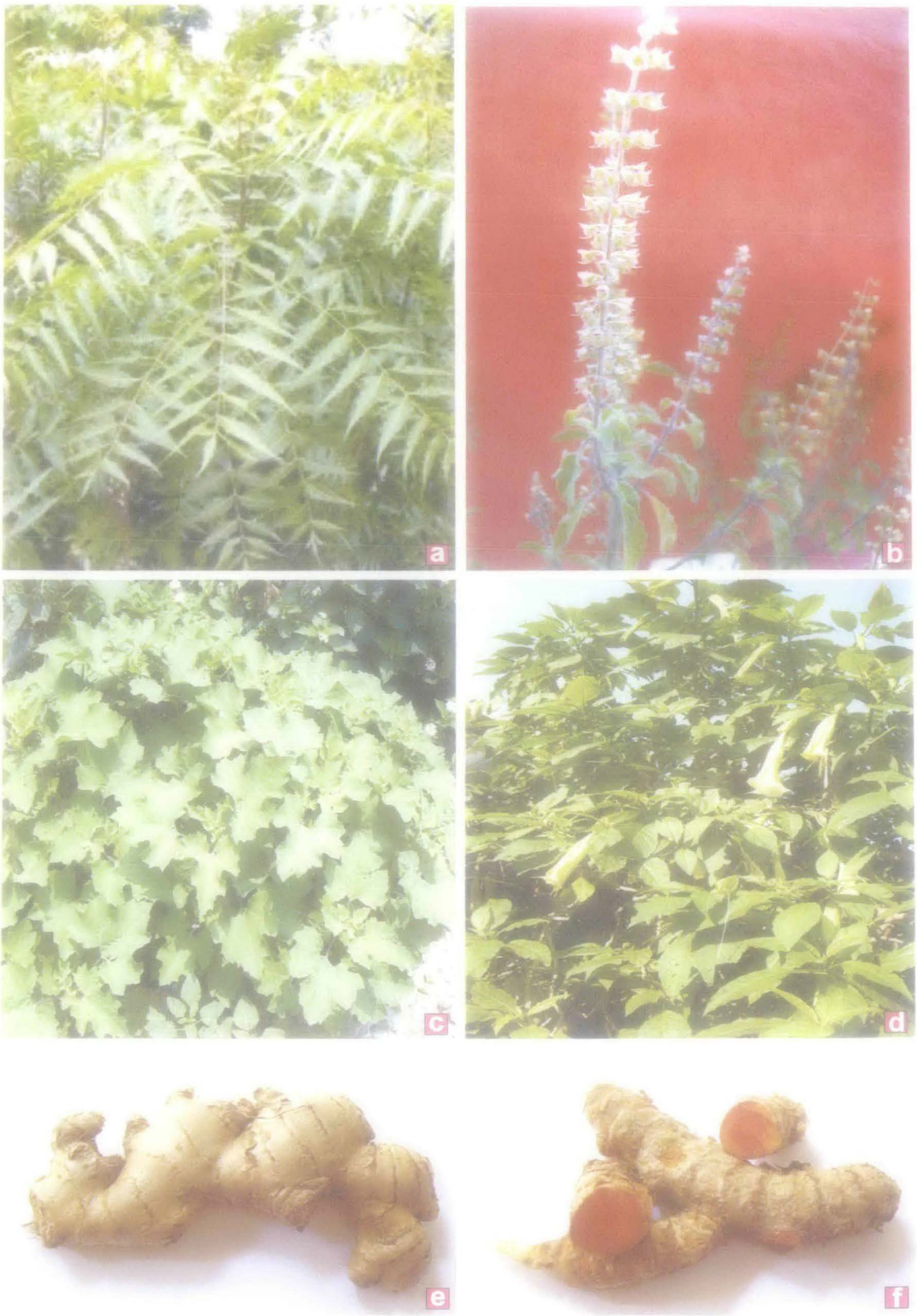

Plate XX: Photographs showing some plants used for extraction of botanicals for controlling *Rhizoctonia solani* (a) Leaves of *Azadirachta indica* A.Iuss (b)Leaves and inflorescence of *Occimum sanctum* L. (c) Leaves of *Xanthium strumarium* L. (d) Leaves and flowers of *Datura metal* L. (e) Rhizome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

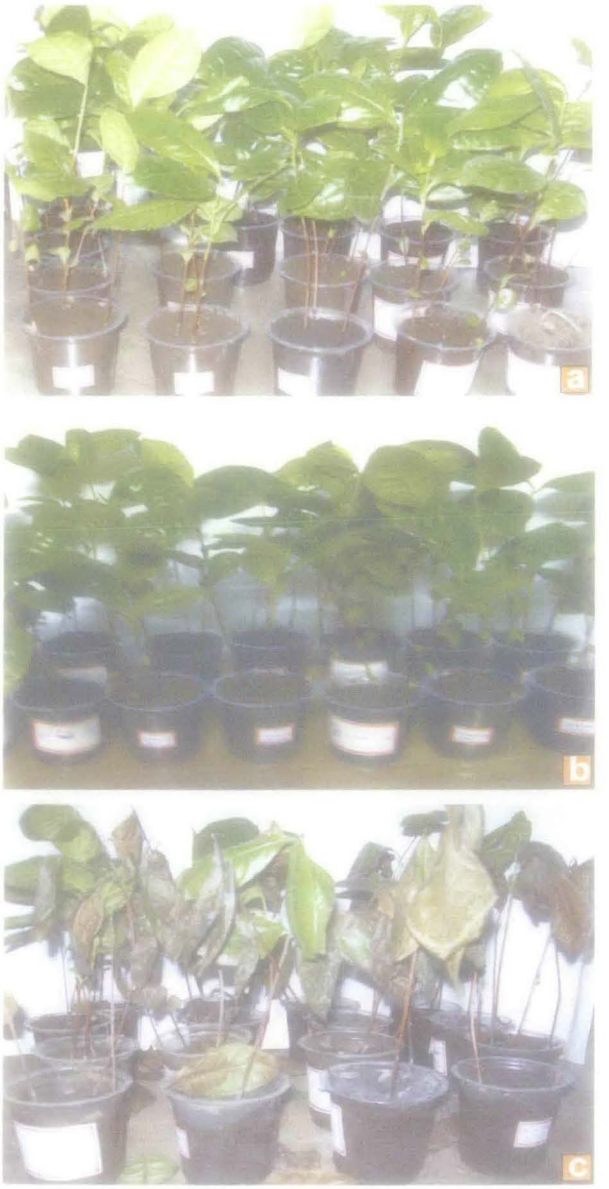

Plate XXII: *In vivo* application of *Trichoderma harzianum* for control of *Rhizoctonia solani* in tea plants (TS520): (a) Untreated healthy control (b) *Trichoderma harzianum* and *Rhizoctonia solani* inoculated plants after 10 days (c) Tea plants severely affected showing disease symptoms following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