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.*