Discussion

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Tea [*Camellia sinensis* (L.) O. Kuntze.] is a non-alcoholic beverage having medicinal importance and popularity worldwide. The economy of north east India largely depends upon the cultivation of tea. Tea occupies a substantial part in Indian agro-export. In north east India It is grown in the hills as well as in the foothills. Darjeeling hills, foothills and other states of north east India produces substantial quantity of tea. In addition to the quantity, the quality of Darjeeling tea is also well known. In India, cultivation of tea started from the seeds of wild variety during 1830 AD. Presently, tea plants are grown from clonal cuttings as well as raised from seeds. Seed varieties are of great importance for their well developed tap root system. In the hilly slopes, seed varieties can well adapt due to their long tap root system. Some of the elite seed varieties are well known for their quality and production. Several seed varieties have been recognized and certified by Tocklai Experimental Station, a premier tea research institute of north east India. To get healthy plants healthy seedlings are also required. A main factor limiting the yield and quality of horticultural crops is their susceptibility to diseases (Rizza *et al.*, 2002).

Although, several fungi have been reported to attack young tea plants in the tea seed nurseries but not all were of much importance. Two different fungi (*Lasiodiplodia theobromae* and *Rhizoctonia* solani) were found to produce diseases in the nurseries in the foot-hills of Darjeeling district (West Bengal, India). The fungus, *Lasiodiplodia theobromae* was consistently found to be associated with the young tea plants. On the other hand *Rhizoctonia* solani was found to be associated with tea roots and seeds. *Lasiodiplodia theobromae* produce diplodia disease in tea and the disease is of considerable importance because it can attack any part of the tea plant (root, stem and leaf) (Sarmah, 1960) and of all ages (Sarmah, 1960; Chandramouli, 1999; Singh, 2005).

Presently, an alternative to chemical fungicides, environment-friendly disease control measures are being given utmost importance. Proper disease management are possible only when basic knowledge on pathogenesis of disease agents in the host as well as the role of different defence related enzymes are known. In the present study an attempt has been made to study the role of different defence related enzymes and their induction by different

inducers, if any. However, before induction, resistance and susceptibility of the tested varieties were also determined following conventional pathogenicity and serological tests (on the basis of the level of cross reactive antigens). It is likely that the results will broaden the scientific base upon which total control of fungal diseases of tea in nursery and tea garden may be established through an integrated disease management programme.

The present study was performed to control diplodia disease caused by *Lasiodiplodia theobromae*. *Lasiodiplodia theobromae* (Pat.) Griffon & Mauble (=*Botryodiplodia theobromae* Pat.) was originally isolated from naturally infected tea leaves in the nursery of taipoo tea estate, Bagdogra, West Bengal. After verification of Koch's postulations the fungus was identified in the laboratory and was also sent to Indian Type Culture Collection, IARI, New Delhi for identification. *Lasiodiplodia theobromae* play a major role for the failure of seedlings production in the tea nurseries.

Degree of susceptibility or resistance of a particular variety to a pathogenic fungus is determined through its differential pathogenicity to different varieties. However, pathogenicity of different fungi to a particular plant variety gives us information about different infecting ability of different pathogens. Pathogenicity of *Lasiodiplodia theobromae* was tested following two different techniques, viz. root inoculation and leaf inoculation technique. Results obtained from both the techniques were in agreement with each other. Dickens and Cook (1989) used leaf inoculation technique to detect resistance and susceptibility of *Camellia* plants against *Glomerella cingulata*. Kobriger *et al.*, 1998 used root inoculation technique to assess root rot of snap bean in Wisconsin fields. Brennan *et al.* (2003) reported pathogenicity of five different fungal pathogens viz. *Fusarium areenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Macrodochium nivale* on coleoptile growth rate of wheat seedlings (cv. Falstaff) *in vitro*. Yanase and Takeda (1987) also detected the resistance of tea varieties to grey blight disease of tea caused by *Pestalotiopsis longiseta* in Laboratory condition in Japan.

From the results it was found that pathogenicity of *L. theobromae* clearly showed TS-449 as the most susceptible and TS-491 as the most resistant among the seven different tea seed varieties tested. The results also indicated the differential resistance and susceptibility of the other five varieties. Saha *et al.*,

2001 also reported TV-12 and TV-25 as most susceptible and resistant clonal varieties respectively against *C. eragrostitis*. Chakraborty and Saha (1994a) reported TV-26, TV-25 and TV-16 as resistant and TV-18, TV-9 and TV-17 as succeptable varieties against *Bipolaris carbonum*, a foliar fungal pathogen of tea. Pathogenicity of other foliar tea pathogens like *Pestalotiopsis theae* and *Colletotrichum cammelliae* were studied by Chakraborty *et al.* (1995). They reported TV-18 as highly susceptible and TV-9 as moderately resistant among different clonal and seed varieties of tea. Hu-Shu Xia (1996) reported the pathogenicity of *P. theae* in 18 tea cultivers in Anhui province, China and found that two cultivers as highly resistant. Raja and Reddy (2007) showed disease index of some solanaceus plants by conventional pathogenicity test of *Alternaria* spp, a pathogen of brinjal. The results of the present study are in conformity with that of the studies of earlier workers. Therefore, the identities of different tea seed varieties allow us to know about their resistance or susceptibility towards root and foliar fungal pathogens. The knowledge of resistance or susceptibility might be helpful in integrated disease management practice in tea especially in cases of multiple pathogen attack.

A thorough knowledge on the morphological and physiological characteristics becomes necessary after a fungal pathogen is isolated. Plant pathogens exhibit considerable variation in cultural as well as in pathogenic characters mostly due to genetic recombination during sexual reproduction (Shaner *et. al.*, 1992).To culture the fungus in artificial media and to produce inoculum in the form of spore is important for different laboratory studies. Several workers have recognized the importance of spores as inoculum and studies have been conducted on the effects of various media components along with important physiological parameters that lead to maximum sporulation (Kim *et al.*, 2005; Saxena *et al.*, 2001). During our studies with the pathogen, it was found that the fungus sporulates very poorly in commonly used media like potato dextrose agar (PDA) and oat meal agar (OMA).

Ten different liquid media (PDB, PDB-RE, OMB, OMB-RE, PCB, PCB-RE, YEG, YEG-RE, MEM and MEB-RE) were used to study the growth and sporulation of the *L. theobromae*. From the results it was evident that PDB-RE (potato dextrose broth supplemented with root extract) was best for both growth and sporulation of *L. theobromae* after 25 days. In PDB, growth was good but sporulation was poor. Next to PDB-RE, MEB-RE also showed good growth and

sporulation. Non-supplemented PCB recorded the least mycelia growth and sporulation. The results revealed that in general, sporulation was remarkably high when *L. theobromae* was grown in root extract supplemented media in comparison to the non-supplemented media. Mycelial growth also increased marginally when PDB, OMB and PCB were supplemented with root extract. However, YEGB and MEB did not show any difference in mycelia growth between supplemented and non-supplemented media, but there was no sporulation in the non-supplemented media. Addition of root extract increased sporulation significantly especially in MEB-RE media. Thus the results indicated that root extract supplementation is not necessary for *in vitro* mycelial growth of *L. theobromae*, but it remarkably enhanced the sporulation. Alam *et al.* (2001) reported that highest mycelial growth and sporulation of *B. theobromae* was observed on PDA, which was in agreement to our findings. Several other workers also stated that PDA was the best media for mycelia growth (Xu *et al.*, 1984; Maheshwari *et al.*, 1999). Other workers (Kumar and Singh, 2000) also stated that *L. theobromae* grew well in potato dextrose medium. Our observations also related with that reported by Karlatti and Hiremath (1989), who observed that the best mycelial growth of *Alternaria zinniae* was on leaf extract dextrose agar and potato dextrose agar media. They noticed higher sporulation on leaf extract dextrose agar medium.

Light had no significant influence on mycelial growth, which was found to be equally good under complete light, complete dark and alternate 12 h light and dark conditions. Sporulation was excellent and noticed after 10 days when the fungus was grown under complete light condition. However, under complete dark conditions, sporulation was poor and was delayed until 20 days. Overall results indicated that there was little variation in mycelia growth under different light conditions, but light induced sporulation. This result confirmed the findings of Alam *et al.* (2001), who showed that light was not necessary for growth of *L. theobromae*, but it enhanced the sporulation. *L. theobromae* was capable of growing at temperatures that range between 8^0-36^0 C. Best growth was recorded at 28^0C while no growth was observed at temperatures 40^0C and above. These results were in agreement to those reported by Alam *et al.* (2001), who observed that *L. theobromae* grew and sporulated at 10^0-40^0C, the optimum being 25-30^0C. In another study, Eng *et al.* (2003) reported similar observations when he studied the effect of temperature on growth characteristics of *Botryodiplodia*

theobromae. He stated that the growth density and radial velocity was affected at temperatures above 40^0 C. *L. theobromae* was able to grow within a wide range of pH, from 3.5 to 8.0. The fungus however, failed to grow in alkaline environment, beyond pH 8.0. The optimum pH for growth was recorded at the range of pH 5.5 to 6.5. The results indicated that slightly acidic pH to neutral pH was optimum for the growth of the organism. Similar results were also shown in case of *Bipolaris carbonum* by Saha and Chakraborty (1990). They showed germ tube growth of the fungus was optimum at pH 7.2. Thakare and Patil (1995) observed that the optimum pH for growth of *Colletotrichum gloeosporioides* was 4.1 to 6.8. Kang *et al.* (2003) also observed that optimum growth of the phytopathogenic fungus *Colletotrichum gloeosporioides* was around the pH 6.0. Amborabé *et al.* (2005) observed that *Eutypa lata*, a vineyard pathogen grow in a large temperature range (2-30^0C) but a higher temperature (35^0C) presented inhibitory effects on mycelial growth. Gock *et al.* (2003) studied on the influence of temperature, water activity and pH on growth of some xerophilic fungi and observed that the optimum growth occurred at 25^0C for *P. roqueforti* and *W. Sebi*; at 30°C for *Eurotium* species, *A. penicillioides* and *X. Bisporus*; at 37^0C for *C. xerophilum*. Similar results were also obtained by Lin and Sung, 2006 and Winder, 2006).

Host-parasite interaction is an important aspect during the early stages of disease development. Establishment of a disease depend on disease triangle of compatible host, pathogen and environmental interactions. The compatibility is determined by several factors contributed by host, pathogen and also by environment. However, many workers have reported about the presence of some unique antigenic determinants, so called, cross reactive antigens (CRA) between pathogen and compatible host. Thus the concept of cross-reactive antigens between host and pathogen is a notable feature in determining resistance or susceptibility. It is believed that the degree of susceptibility of plant cultivars to a pathogen is correlated to levels of cross-reactive antigens present in both the organisms (Bom and Boland, 2000; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005; Eibel *et al.*, 2005; Dasgupta *et al.*, 2005; Babitha *et al.* 2006). In the present investigation antigens of resistant and susceptible varieties and pathogenic isolate of *L. theobromae* were cross reacted separately with antisera of resistant and susceptible host. Reciprocal cross reaction was also carried out with antisera of *L. theobromae* and antigens of both resistant and

susceptible varieties. One non-pathogen of tea viz. *Trichoderma harzianum* was also included in the immunological comparison. The basic immunological techniques that are in use are radial immunodiffusion, immunoelectrophoresis and agar gel double diffusion. These techniques were successfully utilized by several workers while demonstrating cross reactive antigens.

In agar gel double diffusion test no antigenic substance was found to be common in between *L. theobromae* and resistant tea varieties TS-491 and TS-463 but susceptible tea seed varieties (TS-449, TS-464 and TS-520) shared common antigen (cross reactive antigens) with the isolated pathogen *L. theobromae* . Antigens of non-pathogen *T. harzianum* (Tha) did not show any precipitation band.

Dasgupta *et al.* (2005) were also able to detect CRA only between susceptible varieties and the pathogen *Curvularia eragrostidis*. 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, 1994b). In soybean cultivars similar results were obtained by Purkayastha and Banerjee (1990) when they conducted immunodiffusion between antigen of host and antisera of the pathogen causing anthracnose (*Colletotrichum dematium* var. *truncata*). They were able to detect precipitin bands in cross reaction between the pathogen's antisera and the antigen of susceptible host and *vice versa*, which indicated presence of CRA between susceptible host and pathogen combinations only and not between resistant host and pathogen combinations. Therefore, the results of the present study are in conformity with those obtained by previous workers.

In immunoelectrophoretic studies, antigen of susceptible tea variety (TS-449 and TS-464) shared three precipitin bands with antisera of *L. theobromae* (LtA). Antisera of susceptible varieties (TS-449) shared two precipitin bands against antigen of *L. theobromae*. No precipitin band was found between antisera of *L. theobromae* and antigen of resistant tea variety (TS-491).

The results of Immunoelectrophoretic studies confirmed the results of immunodiffusion. The advantage of immunoelectrophoresis over immunodiffusion is that complex antigenic mixtures are separated from each other due to the additional resolving power of the electrophoretic step. 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. In another study by 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 the results, they observed that each isolate was serologically different from the other and had species-specific antigens. 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. Our results were in good agreement with that of several earlier workers.

In immunoelectrophoresis a number of precipitin arcs could be separated but the quantity of the common antigens could not be determined. 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 compatibility and incompatibility among the host and pathogen. For detecting CRA at a very low concentration indirect enzyme-linked immunosorbent assay (ELISA) is one of the most specific and rapid methods for identifying fungal diseases (Sundaram *et al.*, 1991; Chakraborty and Saha, 1994b; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005). 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. 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. Several other workers have used ELISA for early detection of pathogens (Chakraborty *et al.*, 1996 and Ghosh and Purkayastha, 2003).

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 also called compatible and incompatible reaction was in conformity with the results of pathogenicity tests. The three concentrations of the antigens of *L. theobromae* showed higher absorbance values when tested with antisera of susceptible tea variety (TS-449) than when tested with antisera of resistant tea variety (TS-491). Higher absorbance values were also observed in reciprocal test of this combination i.e. in case of the antigens of susceptible tea varieties (TS-449 and TS-464) tested with the antisera of *L. theobromae* than in case of the antigens of resistant tea varieties (TS-491 and TS-463) tested with the same antisera. The results thus clearly indicated the presence of maximum cross reactivity between susceptible varieties (TS-449 and TS-464) and *L. theobromae*. ELISA values were higher in homologous reactions in all combinations.

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. Purkayastha and Banerjee (1990) 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 significantly higher level of antigens was present in compatible host-pathogen interactions than in incompatible interactions.

In the present study, CRA could not be detected between resistant host and pathogen by immunodiffusion and immunoelectrophoresis. But ELISA showed presence of common antigens in cross reactions between antisera of the pathogen and antigens prepared from seven different tea varieties, both susceptible and resistant. The levels of common antigens between resistant varieties and the pathogen were, however, significantly lower. ELISA values, in cross reactions, revealed a direct correlation with results of pathogenicity test and established the degree of susceptibility or resistance of a particular variety. Thus ELISA may be used to determine the pathogenicity of a strain in different cultivars accurately. As the gradient of similarity or disparity of CRA is the indicator of

susceptibility and resistance respectively, it would help in selecting resistant varieties for cultivation and contribute towards long term disease control.

Immunocytolocalization techniques (Immunofluorescence and Immunogold labelling-silver enhancement) have been used in susceptible and resistant tea varieties for determining cellular location of CRA. Both techniques are powerful tools to detect and locate specific CRA with great accuracy by utilizing antisera as probe. In the present work these techniques have been used to determine cellular locations of CRA in leaf and stem sections of susceptible and resistant tea varieties using antisera of pathogen. 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, in case of Immunogold labelling-silver enhancement technique. But in case of immunofluorescence polyclonal antibodies indirectly labelled with fluorescein isothiocyanate conjugate was used to locate CRA in the root sections of tea and mycelial cells of the fungal pathogens. Several authors have used immunofluorescence technique for cellular location of CRA (Chakraborty and Saha, 1994b; Wakeham and White, 1996; Chakraborty *et al.*, 1997; Kratka *et al.*, 2002; Dasgupta *et al.*, 2005). Some workers (Kuo, 1999; Lee *et al.*, 2000; Trillus *et al.*, 2000; Nahalkova *et al.*, 2001; 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 (as suggested in manufacturer's kit Sigma, USA) which is a new approach for studying cellular location of CRA.

Immunofluorescence study was used for determining cellular location of CRA in tea, in the present study; when the root sections were treated with the antisera of respective seed varieties i.e. with homologous antisera indirectly labeled with fluorescein isothiocyanate (FITC), bright fluorescence was observed. Fluorescence was observed in the epidermal regions and xylem elements of the root. Root sections in heterologous treatments i.e. when the root sections were treated with antisera of *L. theobromae* and then reacted with FITC showed comparatively more fluorescence in susceptible tea varieties than the resistant one. The CRA observed in heterologous reactions, were mainly concentrated around the epidermal cells. In homologous treatments of the fungal mycelia,

intense fluorescence was observed around the mycelia. In cross-reactions, when the fungal mycelia were treated with antisera of susceptible varieties indirectly labeled with FITC, fluorescence was observed in the hyphal tips but the fluorescence was not so strong when the mycelia were treated with the antisera of resistant varieties with FITC label. Similar tissue and cellular location of CRA in cross-sections of cotton roots (DeVay *et al.*, 1981a), potato leaves (DeVay *et al.*, 1981b) and tea leaves (Chakraborty and Saha, 1994b; Chakraborty *et al.*, 1995; Chakraborty *et al.*,1997) using fluorescein isothiocyanate (FITC) labeled antibodies have been reported. Maximum precipitation of silver upon immunogold labels were found in leaf sections treated with homologous antisera, in the epidermal regions, mesophyll tissues and vascular bundle elements of the leaves. When leaf section of susceptible variety (TS-449) was treated with antisera of *L. theobromae* and labelled with immunogold particles followed by silver enhancement, CRA was observed mainly in the epidermal regions as strong precipitations. Mesophyll tissues and vascular bundle elements also showed marginal darkening which indicate presence of CRA in those areas also. When leaf section of resistant variety (TS-491) was treated with the antisera of pathogen, no such strong precipitations were observed. Similar results were also found in root and stem sections. However, maximum precipitation was found in the epidermal regions cortical tissues and vascular bundle elements of the roots and stems.

Studies on defense related enzymes were performed for the proper understanding of the mechanism of defense operating in the tea plants following a localized infection with a necrotizing pathogen (*Lasiodiplodia theobromae*) or treatment with elicitors. For the purpose of induction of resistance, susceptible tea variety, TS-449 was selected. Some abiotic inducers (salicylic acid, jasmonic acid and nickel chloride), plant extracts (*Azadirachta indica*, *Acalypha indica*, *Jasminum jasminoides* and *Catharanthus roseus*) and biotic inducers (*Trichoderma harzianum* and *Trichoderma virens*) were used for the treatment of the test plants. Five different defense related enzymes (phenylalanine ammonia-lyase, polyphenol oxidase, chitinase, β-1,3-glucanase and peroxidase) were studied. The induction and accumulation of PR-proteins against pathogen attack and chemical treatments are well documented (Van Loon and Kammen, 1970; Van Loon and Van Strien, 1999). Tea plants of several susceptible varieties have been induced by abiotic inducers including salicylic acid, a very common abiotic

inducer of PR proteins (Chakraborty *et al.*, 2005). Major interest have been devoted to plant hydrolases such as β-1,3-glucanases (PR-2) and chitinases (PR-3), as they are capable of cleaving fungal cell walls resulting in pathogen's growth inhibition (Wessels and Sietsma, 1981; Mauch *et al.*, 1988; Neuhans, 1999; Arlorio *et al.*, 1992; Bishop *et al.*, 2000). Several authors (Paul and Sharma, 2002; Ghosh and Purkayastha, 2003; Saha *et al.*, 2007) have used phyto-extracts for the induction of PR-proteins in a variety of crops. Ghosh and Purkayastha (2003) tested 12 elicitors for inducing systemic protection against rhizome rot of ginger caused by *Pythium aphanidermatum*. Among six phyto-extracts tested, 10% leaf extract of *Acalypha indica* showed maximum reduction in disease with associated increase of defense-related proteins. Meena *et al.* (2001) applied SA at the concentration of 1mM as foliar spray and observed significant increase of the activity of phenylalanine ammonia-lyase (PAL), chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase and phenolic content in groundnut. Induction of chitinase by treatment of inducers in plants was reported by several workers. Meera *et al.* (1994) reported that plant growth-promoting fungi (PGPF) belonged to the genera *Fusarium*, *Penicillium*, *Phoma*, *Trichoderma* and sterile fungi can induce several defense related enzymes like chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase. Phenylalanine ammonia lyase (PAL) is a key enzyme of phenylpropanoid pathway which leads to the deposition of lignin, phytoalexins and phenolic compounds and form structural and chemical barriers of the plants to the pathogens (Ramamoorthy *et al.*, 2002).

Differential expressions of PAL in pre-treated and challenge-inoculated tea plants were studied. Three different inducers were used to treat plants. From the results it was found that the two abiotic inducers (10^{-2}M Jasmonic acid and 10^{-2}M salicylic acid) were able to induce PAL activity. A higher level of PAL activity was found after 4 days following which the level of the enzyme was found to decline. Nickel chloride (10^{-4}M) treated plants showed a certain level of PAL activity after 4 days and then, it also declined. Similar results were observed by several authors. Cao *et al.* (2006) reported enhanced accumulation of PAL when SA was applied to the trees of *Pyrus bretschneideri* around 30 days after full flowering. Akinwunmi and John (2001) reported transient increase of PAL in cowpea after pre-treatment with BTH following challenge-inoculation by *Colletotrichum destructivum*. Trotel-Aziz *et al.* (2006) reported that treatment of

grapevine leaves with chitosan led to marked induction of PAL and strong reduction of *Botrytis cinerea* infection. Qian *et al.* (2005) reported the presence of novel synthesized pentafluoropropyle jasmonate (PFPJA) to induce plant defense response, including an oxidative burst and activation of PAL. Basha and Chatterjee (2007) used two non-conventional chemicals like zinc sulphate and oxalic acid for the induction of PAL in wheat against *Sclerotinia sclerotiorum.* Thus, our results of induction of PAL by abiotic inducers are very much in conformity with that of earlier workers.

Plant growth promoting rhizofungi (PGPF) were used as biotic inducers to trigger defense in tea plants. It was found that *Trichoderma virens* pre-treated plants showed increased level of PAL activity after 4 days in comparison to treated-uninoculated and untreated-uninoculated controls. *Trichoderma harzianum* pre-treated plants also showed a certain level of PAL activity after four days. These results are also in agreement with the observations made by several earlier workers. Duiff *et al.* (1998) reported that non-pathogenic *Fusarium oxysporum* was able to suppress *Fusarium* wilt partially by inducing resistance. Ramamoorthy *et al.* (2002) reported PAL activity in tomato after pre-treatment with *Pseudomonas fluorescens* isolate Pf1 against wilt disease causing pathogen *Fusarium oxysporum* f. sp. *lycopersici.* Maximum PAL activity was found after 4 days following challenge-inoculation. Chen *et al.* (2000) reported that root and crown rot of cucumber caused by *Pythium aphanidermatum* could be suppressed by increasing PAL activity in treated (with PGPR) - inoculated (by *P. aphanidermatum*) plants. Viterbo *et al.* (2007) used *Trichoderma virens* strain Gv29-8 to increase PAL activity in cucumber cotyledons for control against leaf pathogens. Two PGPR strains viz. *Pseudomonas fluorescens* (Pf 4) and *P. aeruginosa* (Pag) were found to induce PAL activity in wheat against *Sclerotinia sclerotiorum* (Basha and Chatterjee, 2007). Ushamalini *et al.* (2008) reported that when *Trichoderma viride* strain of MG 6 was applied to turmeric plants it induced the activity of various plant defense enzymes including PAL against rhizome rot caused by *Pythium aphanidermatum.*

Among the four different phyto-extracts used to induce plants for expression of PAL activity, *Azadirachta indica* leaf extract treated plants showed significantly higher level of PAL activity after 4 days. Other three phyto-extracts (viz. *Acalypha indica*, *Jasminum jasminoides* and *Catharanthus roseus*) also showed PAL activity, however, at a lower level. Our results are in the same line

as reported by Paul and Sharma (2002). They reported significantly higher PAL activity in pre-treated (with aqueous leaf extract of *Azadirachta indica*) and challenge-inoculated (by *Drechslera graminea*) barley plants.

In plants, the enzyme polyphenol oxidase (PPO) is involved in the synthesis of defense chemical like tannin which is toxic to pathogenic microorganisms (Mahadevan and Sridhar, 1996; Chen *et al.*, 2000). Differential expression of PPO was determined in susceptible tea variety (TS-449) following pre-treatment (with three different inducers) and challenge-inoculation (by *L. theobromae*). From the results it was evident that tea plants pre-treated with salicylic acid (SA) and jasmonic acid (JA) showed higher level of PPO expression in comparison to treated-uninoculated and untreated-uninoculated controls. Plants pre-treated with nickel chloride showed low level of PPO expression in tea plants. Meena *et al.* (2001) reported increase of PPO activity in groundnut after pre-treatment with salicylic acid against late leaf spot caused by *Cercosporium personatum*. Thus our results are similar to that of Meena *et al.* (2001).

In the present study, higher level of PPO activity was found after four days in the plants when pre-treated with *Trichoderma harzianum*. Chen *et al.* (2000) reported increasing PPO activity in cucumber after pre-treatment with plant growth promoting rhizobacteria (PGPR) following challenge-inoculation with *Pythium aphanidermatum*. Koike *et al.* (2001) reported five different fungal isolates (*Trichoderma*, *Fusarium*, *Penicilliun*, *Phoma* and a sterile fungus) to induce systemic resistance in cucumber plants against *Colletotrichum orbiculare*. Zheng *et al.* (2005) also reported increased activity of PPO in pepper plants, pre-treated with mycorrhizal fungus of *Glomus intraradices* and challenge-inoculated by *Phytophthora capsici*. Reuveni and Reuveni (2000) reported that pre-treatment of cucumber plants with non-pathogenic isolates of *Alternaria cucumarina* or *Cladosporium fulvum* following challenge-inoculation by *Sphaerotheca fuliginea* showed induced systemic protection against powdery mildew.

PPO activity was also studied in tea plants following pre-treatment with four different phyto-extracts. Among the phyto-extracts, *Acalypha indica* pre-treated tea plants showed maximum level of PPO activity after 4 days following challenge-inoculation. Other pre-treated (with *Azadirachta indica*, *Janminum jasminoides* and *Catharanthus roseus* leaf extracts) tea plants showed lower

level of PPO expression than *Acalypha indica* pre-treated tea plants. Baysal *et al.* (2002) reported an effect of induced resistance in the ornamental *Cotoneaster salicifolius* root stock M26, against fire blight caused by *Erwinia amylovara* by using plant extract of *Hedera helix*. Increased activity of PPO was reported in that ornamental plant by the workers. Hence, our results are in agreement with several previous workers.

Isozyme analysis showed the expression of four different types of PPO isozyme with R_f values of 0.09, 0.30, 0.40 and 0.50. Among four PPO isozymes, two isoforms of R_f 0.09 and 0.05 were expressed constitutively in all treatments including control. But plants pre-treated with *Acalypha indica* aqueous leaf extract and salicylic acid (separately) showed higher levels of the isozyme whose R_f was 0.09. Other isozymes (R_f= 0.40 and 0.30) were also expressed in pre-treated (with *Acalypha indica* aqueous leaf extract and salicylic acid) tea plants. A faint (very low intensity) band of R_f 0.40 were expressed in tea plants treated with *Acalypha indica* aqueous leaf extract and salicylic acid separately without challenge-inoculation. Several workers have reported similar results in different plants. A unique PPO isozyme was found in tomato after pre-treatment with *Pseudomonas fluorescens* isolate Pf1 against *Fusarium* wilt (Ramamoorthy *et al.*, 2002). Zheng *et al.* (2005) reported PPO isozyme in pepper plants, pre-treated with mycorrhizal fungus of *Glomus intraradices* and challenge-inoculated by *Phytophthora capsici*. Several other workers also indicated that PPO isozymes were induced by various inducer treatments in cucumber and tobacco (Piyada *et al.*, 1995; Ray *et al.*, 1998; Chen *et al.*, 2000).

The defense related enzyme, β-1-3-glucanase is an important plant enzyme having hydrolysing capability. Fungal cell wall is made up of chitin polymers which contains β-glucan in the matrix (Sivam and Chet, 1989). In the present study, enzyme β-1-3-glucanase was induced in tea plants by different inducers in order to control fungal diseases. From the results it was evident that as abiotic inducers, salicylic acid (SA) pre-treated plants showed higher level of β-1-3-glucanase expression in susceptible tea plants. Nickel chloride and JA pre-treated plants showed much lower level of β-1,3-glucanase activity after four days. Bargabus *et al.* (2002) reported increased activity of β-1,3-glucanase and reduction of cercospora leaf spot of sugar beet after pre-treatment of acibenzolar-S-methyl following challenge-inoculation of *Cercospora beticola*. Emmanuel *et al.* (2001) reported rapid induction of defense resistance in susceptible lettuce plants

after treatments of DL-β-amino butyric acid (BABA) and Phytogard against downy mildew.

It was found in our study that both the biotic inducers (*Trichoderma virens* and *T. harzianum*) were able to induce β-1,3-glucanases in tea plants at a higher level than the control plants. Increased activity of β-1,3-glucanase and reduction of cercospora leaf spot of sugar beet after pre-treatment with a non-pathogenic microorganism *Bacillus mycoides* following challenge-inoculation of *C. beticola* was reported by Bargabus *et al.* (2002). Ahmed *et al.* (2000) reported that the pepper seed and root treated with *Trichoderma harzianum* reduces necrosis and induces a systemic defense response against *Phytophthora capsici.* Ishimoto *et al.* (2004) showed that two root colonizing *Fusarium* strains, isolated from roots of Brassicaceae plants, induced the resistance in *Lepidium sativum* seedlings against *Pythium ultimum.* Thus, our study is in concurrence with that of several other workers.

On the other hand, when leaf-extracts were used, *Acalypha indica* pre-treated plants showed higher level of β-1,3-glucanases activity. *Jasminum jasminoides* and *Catharanthus roseus* leaf extracts showed induction of β-1,3-glucanase comparatively at a lower level as evidenced by its activity. Paul and Sharma (2002) reported *Azadirachta indica* leaf extract to induce defense in barley. Ghosh and Purkayastha (2003) used six different plant extracts viz. *Catharanthus roseus*, *Acalypha indica*, *Spinacea oleracea*, *Andrographis paniculata*, *Centella asiatica* and *Curcuma longa* for systemic protection against rhizome rot disease of ginger caused by *Pythium aphanidermatum.* They found higher systemic protection by using plant extract of *Acalypha indica* in ginger.

Isozyme patterns revealed the expression of three different β-1,3-glucanase isozyme bands with R_f values of 0.1, 0.35 and 0.45. Two β-1,3-glucanase isoforms of R_f 0.1 and 0.35 were found as constitutive. But they were expressed at higher levels in treated-inoculated plants in comparison to untreated-uninoculated and treated-inoculated plants. An unique β-1,3-glucanase isozyme of R_f 0.45 was also found in treated-uninoculated and treated-inoculated plants. Bargabus *et al.* (2002) showed two unique isoforms in sugar beet after pre-treatment with a non-pathogenic microorganism, *Bacillus mycoides,* following challenge-inoculation by *Cercospora beticola.* He also reported that one isoform was available when sugar beet was pre-treated by acibezolar-S-methyl and

challenge-inoculated. Lawrence *et al.* (1996) reported two isozymes of β-1,3-glucanase (33 and 35kDa) in all genotypes of tomato.

Chitinase belongs to the category of pathogenesis-related (PR) protein which is the key hydrolytic enzyme in plant defense induction. Chitinase plays a distinct role in plant defense by degrading chitin, a β-1,4-linked polymer of N-acetyl D-glucosamine, a major fungal cell wall component (Lawrence, *et al.*, 1996). For the induction of chitinases in tea plants, three different inducers were used in the present study. From the results it was found that the abiotic inducer, nickel chloride showed higher level of chitinase activity in tea plants. On the other hand salicylic acid and jasmonic acid showed lower level of chitinase activity after four days. Our results are in agreement to that of Kozlowski *et al.* (1999), who reported increased activity of chitinase after pre-treatment by methyl jasmonate (MeJA) in *Picea abies* seedlings against *Pythium ultimum*.

Similarly, higher level of chitinase activity was found in tea plants pre-treated with *Trichoderma harzianum*, used as a biotic inducer. Plants pre-treated with *T. virens* showed comparatively lower level of chitinase induction under similar conditions. Abd-El-Kareem (2007) reported that integrated treatments of *Trichoderma harzianum* and humic acid in bean seeds reduced the root rot and leaf spot incident. He observed increased chitinase activity in pre-treated bean seeds against *Rhizoctonia solani*, *Fusarium solani* and *Alternaria alternata*. Oostendorp *et al.* (2001) reported that plants could be induced locally and systemically to make the plants more resistant to disease through various biological inducers including necrotizing pathogens, non-pathogens and root colonizing bacteria. Schweizer *et al.* (1999) reported *Pseudomonas syringae* as biotic inducer against the rice blast fungus *Magnaporthe grisea*. Hence, our results are in agreement with that of other workers.

Plant extracts (*Acalypha indica* and *Azadirachta indica*) were used for induction of chitinase activity in susceptible tea plants. Treated tea plants with both type of extracts showed higher level of chitinase activity following challenge inoculation after 3 days. Kagale *et al.* (2004) induced systemic resistance in rice plants following application of leaf extract of *Datura metel* and challenge-inoculation with *Rhizoctonia solani*. They showed increased accumulation of pathogenesis-related proteins (PRs) and other defense related compounds in the pre-treated and inoculated rice plants.

After colorimetric assay of chitinase, 'chitinase activity' was studied by chitin supplemented plate method. In this study, induction of chitinase activity was visualized in chitin supplemented plate by two different inducers (nickel chloride and leaf extract of *Acalypha indica*). After pre-treatments (with *Acalypa indica* leaf extract and nickel chloride separately) tea plants (both susceptible and resistant) were challenge-inoculated by *L. theobromae.* From the results it was evident that the susceptible tea variety (TS-449) pre-treated with *A. indica* leaf extract and nickel chloride showed high intensity of lytic zone (intensity grade ++++). Under similar conditions, resistant tea variety (TS-491) showed comparatively lower intensity of lytic zone (intensity grade +++). From the results it was evident that both the inducers have the ability to induce enzyme chitinase. From the study it can be concluded that *Acalypha indica* leaf extract is a potent inducer of defense response in tea plants. This plant contains cyanogenic glucosides along with other substances (Nahrstedt *et al.*, 1982), but the active component associated with increase in chitinase activity in tea is yet to be identified. Bargabus *et al.* (2002) reported specific chitinase activity after pre-treatment of acibezolar-S-methyl against *Cercospora beticola* causing cercospora leaf spot of sugar beet. Chitinase have been reported to express as multiple isozymes in several plants. Three classes of plant chitinases have been reported based upon primary protein structure (Shinshi *et al.*, 1990). The highly variable nature of chitinases, and the multiplicity of chitinase isozymes in plants suggest that plant chitinase isozymes may carry out specific and differing roles. Some of them have antifungal activity. Chitinase was isolated from tobacco (Sela-Buurlage *et al.*, 1993) and tomato (Lawrence *et al.*, 1996) and have been found to be specific for certain pathogens.

Peroxidase is a well known defense enzyme in plants which is changed under various environmental stresses such as heavy metals, salts, temperature (Kiwan and Lee, 2003) and air pollution (Lee e*t al.*, 2000). It is related with the defense reaction in plants that lead to the detoxification of the reactive oxygen species (Higa e*t al.*, 2001). In the present study, three different types of inducers were used for induction of peroxidase following challenge-inoculation in susceptible tea plants (TS-449). From the results, it was found that among abiotic inducers, nickel chloride pre-treated tea plants showed higher level of peroxidase activity. Our results are in the line of some previous workers. Stadnik and Buchenaur (2000) reported higher activity of peroxidase in wheat to *Blumeria*

graminis after pre-treatment of BTH. Prachi *et al.* (2002) reported that exogenous application of SA resulted in increased activity of peroxidase in the callus culture of *Zingiber officinale*.

Two different PGPF were used as biotic inducers for the study of peroxidise in tea plants. *Trichoderma harzianum* pre-treated tea plants showed higher level of peroxidase activity. Van Loon (2007) reported non-pathogenic soil borne microorganisms to promote plant growth as well as suppression of disease through microbial antagonism or induction of resistance in plants. Pelt-Heerschap and Smit-Bakker (1999) reported increased activity of peroxidase in stem tissue of carnation inoculated with pathogenic and non-pathogenic race of *Fusarium oxysporum*. Lavania *et al.* (2006) reported increased activity of peroxidase in *Piper betle* after pre-treatment with *Serratia marcescens* NBR11213 against foot and root rot caused by *Phytophthora nicotianae*. Yedidia *et al.* (1999) reported increased activity of peroxidase in cucumber plants within 48h of inoculation with *Trichoderma harzianum*.

Leaf extracts of *Azadirachta indica* and *Acalypha indica* were used for induction of peroxidase activity. Higher level of peroxidase activity was observed after 3 days following challenge-inoculation by *L. theobromae*. Shi *et al.* (2007) reported that the compound osthol (a natural compound extracted from dried fruit of *Cnidii* in *monnieri*), induced pumpkin plants for accumulation of peroxidase and PAL against powdery mildew caused by *Sphaerotheca fuliginea*. Baysal *et al.* (2002) reported plant extract of *Hedera helix* to induce peroxidase in *Cotoneaster salicifolius* root stock M26 against fire blight.

Studies on peroxidase isoform patterns in susceptible tea plants were performed. Tea plants pre-treated with *Azadirachta indica* and *Acalypha indica* showed prominent peroxidase isoforms with two bands of molecular mass 38KDa and 33KDa. The expression of the 33KDa isozyme did not increase much following treatment with phyto-extract but increased dramatically when treated plants were challenge-inoculated with *L. theobromae*. The results indicated the possibility of induction of peroxidase isozymes in susceptible tea plants, which in turn, shows resistance to the pathogen. Several authors reported multiple forms of peroxidase isozymes in many higher plants including Korean radish, *Arabidopsis* and rice (Lee and Kim, 1994; Lee *et al.*, 1994; Tognolli *et al.*, 2000; Lee *et al.*, 2001). Ushamalini *et al.* (2008) reported that *Trichoderma viride* strain

of MG 6 could induce the activity of peroxidase isozymes in turmeric plants against rhizome rot (caused by *Pythium aphanidermatum*).

In the present study an approach was made towards environment friendly management of diplodia disease in tea through induction of plant defense enzymes using various inducers (abiotic, biotic and phyto-extracts). Degree of susceptibility or resistance of a particular variety to a pathogenic fungus is determined through its pathogenicity. Pathogenicity is determined, ordinarily, by disease incidence. Disease incidence was assessed and compared in the differentially induced susceptible variety, TS-449. Three different abiotic inducers (10^{-2}M salicylic acid, 10^{-2}M jasmonic acid and 10^{-4}M nickel chloride), two biotic inducers (*Trichoderma harziamum* and *T. virens*) and four leaf extracts (of *Azadirachta indica, Acalypha indica, Catharanthus roseus* and *Jasminum jasminoides*) were used for induction of resistance in the susceptible variety TS-449. Assessment of diplodia disease was performed from 4th day up to 16th day at 4-days intervals. Salicylic acid and *T. virens* effectively reduced disease incidence (mean foliar disease index/plant) in tested tea plants. Disease incidence was also found to reduce in tea plants induced by three different leaf extracts (viz. *Azadirachta indica, Acalypha indica* and *Catharanthus roseus*) as evidenced by the results. Several scientists have reported similar results in different plants. Abd-El-Kareem (2007) reported that integrated treatments of *Trichoderma harzianum* and humic acid spray reduced the root rot incident of bean seeds caused by *Fusarium solani*, *Rhizoctonia solani* and *Fusarium solani.* Reuveni and Reuveni (2000) reported that the systemic resistance was expressed by a significant reduction in the number of powdery mildew colonies on cucumber leaves pre-treated with non-pathogenic isolate (of *Alternaria cucumarina* or *Cladosporium fulvum*) before challenge-inoculation with the pathogen *Sphaerotheca fuliginea.* Van Loon (2007) reported suppression of disease by non-pathogenic soil borne microorganism. Cao *et al.* (2006) reported that the Ya Li pear trees pre-treated with SA induced the activities of various defense related enzymes (phenylalanine ammonia lyase, β-1,3-glucanase, chitinase and peroxidase) in addition to reduction of disease incident and lesion diameter. Meena *et al.* (2000) also reported reduction of disease incidence in groundnut pre-treated with *Pseudomonas fluorescens* against late leaf spot caused by *Cercosporidium personatum.* Premkumar (1998) has reported systemic action of triazole compound in clonal tea plants (TES-34) which are

highly susceptible to *Exobasidium vexans* causing blister blight in tea. Thus our results are in conformity with that of earlier workers. The results were encouraging since several inducers showed significant resistance inducing capacity. Further these may be integrated with other biocontrol agents and may be used in fields as part of integrated disease management system.

All the investigations presented here have 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. Pathogenicity of *L. theobromae* has been tested in several tea seed varieties in different ways. The significance of antigenic relationship with regard to compatible interaction between *L. theobromae* and tea 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 plant and the pathogen were detected in the cells of tea and pathogen *L. theobromae* through immunofluorescence study (in fluorescence microscope) and immunogold labelling followed by silver enhancement using light microscope. Resistance was induced in susceptible tea varieties using some chemicals and plant extracts. Hence, this study has provided an insight to formulate a definite defense inducer against diplodia disease caused by *L. theobromae*. The present study would help to design suitable control measures of diplodia disease in tea using resistance inducers of different natures: abiotic, biotic and botanicals.