DISCUSSION

In host-parasite interaction both the plant and the pathogen possess and use battery of enzymes, toxins and other molecules for fighting their way out. The specific interaction between host and pathogen is crucial for the success of the plant's resistance or the pathogen invasion corresponding to incompatible or compatible reactions respectively. Disease is an exception rather than rule in this battle. The constitutive defense responses (pre-formed barriers) of the plants are non-specific. On the other hand, the inducible defenses are highly specific, especially the biochemical and molecular events. Thus, passive mechanisms, coupled with rapid active responses and slower follow-up defenses provide a broad defense front to the plant. The success of defense responses is enhanced if activated in combination. The plant signaling molecules play an important role in this network of the pathways (Shetty, 2002). These signaling molecules may be cell wall fragments, active oxygen species like nitric oxide and hydrogen peroxide or SAR activators like BTH (benzothiadiazole) and SA (salicylic acid).

In the present study, brown blight infection of tea has been used as a convenient model for plant-pathogen interaction with special emphasis on the involvement of defense-related enzymes. Induction of resistance with the help of some of these signaling molecules i. e. nitric oxide and hydrogen peroxide and BTH was also attempted in the present discourse.

At the outset, symptomatology and etiology of brown blight disease was studied. The symptoms of brown blight in the form of dry brown patches with minute dots have been observed to appear on the stressed tea bushes. The symptoms described along with the photographic evidence fit perfectly into the well-accepted version of brown blight, which has been described similarly by numerous workers (Agnihotrudu, 1995; Chakraborty and Chakraborty, 2002; Keith *et al*, 2006).

Micromorphology of the healthy and naturally brown blight infected tea leaves was conducted with the help of scanning electron microscopy (SEM) in the present study. SEM of healthy dorsal surface showed ornamentations similar, but not identical to that of healthy *Curcuma amada* leaves as reported by Das *et al* (2004). However, stomata and trichomes were absent on dorsal surface of the tea leaves. Prominent absence of stomata on dorsal surface of tea leaves was emphasized by the studies

conducted by Kumar *et al* (2004). Surprisingly, except for the presence of wax deposits and oil ducts, the ornamentation was rugate with rugae forming a compact network, just like on the seed surface of *Flemingia congesta* and *Phaseolus angularis* as worked out by Sahai and Singh (2001). Damage caused by brown blight on the dorsal surface has been worked out in detail in the present investigations. At the initial stage, the disease did not affect the ornamentation, but conspicuous presence of *G. cingulata* spores was evident. In the later stage, there was shrivelling and disorganization of the epidermal cells and total destruction of the pattern, ultimately giving rise to acervuli in the dried up tissues of the tea leaf. SEM of apple fruit surface infected with *Marssonia coronaria* was perfomed by Sharma and Sharma (2006) by a similar procedure, where spores of the pathogen were distinctly observed. On the other hand, observations of SEM of healthy ventral tea leaf surface in the present study, were similar to that reported by Ghosh Hajra and Kumar (2002) in case of tea leaves. The trichome morphology was similar to those of *Bauhinia variegate* in the study conducted by Banerjee *et al* (2002) – non-glandular, simple and unbranched. The brown blight infected tea leaves had totally disorganized trichomes and stomatal guard cells, with the spores of *G.cingulata* present along the guard cells.

Variations among the isolates of many fungi is a common feature, especially so in *Colletotrichum gloeosporioides*, anamorph of *G. cingulata* . Therefore, it was decided to study at least three isolates of *G. cingulata* (GC-1, GC-2 and GC-3) obtained from the brown blight infected leaves in order to select a single best isolate for the present study. In case of *Colletotrichum graminicola* studied by Mathur *et al* (2001) and Mathur and Totla (2001), just as in present investigation, results reported were based on single-lesion isolates, in spite of the isolates being heterogenous in nature. Differences regarding morphology and pathogenicity were reported in the present investigation. Highest growth rate was found in Potato Dextrose Agar (PDA) and greatest spore production in Oat Meal Agar (OMA) at a temperature of 25°C for 12 days which were found to be consistent with those observations made by Manaut *et al* (2001) and Mello *et al* (2004), in order to obtain maximum inoculum for experimentation purposes.

In the present study beaked perithecia with spores were observed only in GC-1 isolate when cultured on OMA for 30 days. Mendes-Costa (1996) also demonstrated

formation of perithecia of *G. cingulata* f. sp. *phaseoli* spontaneously in culture medium and not only by mating. The anamorph described in the present work is most certainly that of C. *gloeosporioides* and not *C. acutatum* as its colony colour was grey and the conidial ends were rounded when grown in PDA . This character is useful to distinguish the two genera, often with overlapping characters **(Wharton and Dieguez-Uribeondo, 2004).** The morphological descriptions regarding conidiomata, conidiogenesis and pycnidia are well in agreement to accepted identifying features of *G. cingulata* as described by Domsch and Gans (1980). Size of the conidia was within the range for *Colletotrichum gloeosporioides* as reported by Sutton (1980) (9–24 × 3–4·5μm). The length of conidia depends on media used. Therefore, only PDA was used for characterizing the spore, since it is reported to be producing more consistent conidial size **(Adaskaveg and Forster, 2000).** Appressoria produced directly from germ tubes as also reported by Abang *et al* (2002). Conidial shape and width together with appressorial morphology were found to be the important morphological characters useful in distinguishing the five clinical species of *Colletotrichum* (Cano *et al*, 2004). The appressorial morphology and size were worked out in germinating conidia on host surface and found to be well in agreement to the descriptions of *G. cingulata* by Domsch and Gans (1980). It has been noted by Henson *et al* (1999) that melanin-deficient mutants of phytopathogenic fungi that utilize appressoria as their sole means of penetrating host surfaces, such as *Colletotrichum spp.*, are avirulent. Appressorial melanin limits wall permeability, facilitating osmolyte accumulation and turgour generation within the cell (Davis, 1999; Kubo, 1999). Without this turgour, the infection peg that protrudes from the adhesive surface of this appressorium cannot mechanically penetrate the underlying host tissues. In the present discourse, size of appressoria were found to be comparatively smaller in one of the isolates (GC-2).

Differences in the strains of pathogenic fungi are common. From the present study, it was clearly observed that the three isolates of *G. cingulata* differed regarding their growth curve (accumulation of dry weight), protein content and SDS-PAGE profiles. Considering these characteristics, isolate GC-1 was found to be the superior of the three isolates. It accumulated greatest biomass and total protein in 8 day-old liquid cultures. Also, it exhibited maximum number of protein bands when resolved on SDS-PAGE. Therefore, in order to study the serological relatedness among the isolates,

polyclonal antibody was raised against mycelial antigen of GC-1 isolate. The antisera obtained were purified by ion-exchange chromatography to minimize non specific binding. These were then tested separately for different isolates by immunodiffusion. The techniques like PTA-ELISA and DIBA (Dot Immmuno-binding Assay) were standardized with the homologous antigen. In the beginning, sensitivity of the assay was optimized. Homologous soluble antigens at concentration as low as 81.25ng/ml could be detected by PTA-ELISA. Absorbance values decreased with increase in dilutions. Chakraborty *et al* (1996a) reported that antiserum raised against *Pestalotiopsis theae* could detect homologous antigen at 25ng/ml. Antiserum dilution of upto 1:16,000 was effective for detecting the mycelial antigen of *G. cingulata*. Under such conditions, GC-1, GC-2 and GC-3 isolates were compared serologically by DIBA and ELISA. ELISA values were highest for the isolate against which PAb was raised. Similar reaction occurred in case of Dot blot. GC-1 and GC-3 seemed to be similar to each other, while GC-2 differed. For detailed analysis, Western Blot of the proteins of 8 days-old cultures of these isolates was performed. The number and intensity of bands was the highest in GC-1. Western blot of the mycelial protein of *G.cingulata* with homologous IgG indicated presence of several bands of ca 40, 38.5, 35 and 24 kDa that were present prominently in the three isolates studied. The 24 kDa protein gave the strongest recognition signal.

In the present work, GC-1 was found to be the most virulent, while GC-2 – least virulent. Besides, GC-1 was found to possess larger appressoria, higher growth rate and greater sporulation potential than the other two isolates, as mentioned earlier. All three isolates of *G. cingulata* were tested separately on eighteen tea varieties. Isolate GC-1 was found to be the most virulent, while GC-2 least virulent in both detached leaf and whole plant inoculation methods used for screening resistance of tea varieties against *G. cingulata* . Enzyme linked immunosorbent assay (ELISA) is now routine for detection and diagnosis of plant pathogens (Chakraborty and Chakraborty, 2000, 2002). Therefore, the results of pathogenicity tests were also confirmed by immunological means viz. PTA-ELISA found to be very quick and efficient means of screening for resistant tea varieties. Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after few days of infection, by which time the pathogen would be well established in the host tissues. In phytopathological studies, therefore, it is necessary to

have techniques by which pathogen can be detected at a very early stage. Recent trends have developed highly specific techniques for the detection of pathogen at a very early stage. Various formats of ELISA using polyclonal antiserum has found widespread application in plant pathology and are routinely used for detection and identification purposes (Clark, 1981; Lyons and White, 1992; Chakraborty *et al*, 1995, 1996a, Chakraborty *et al*, 2002). In the present study, the differential response of eighteen tea varieties to *G. cingulata* has been observed through Indirect ELISA following artificial inoculation of tea leaves. Indirect ELISA readily detected *G. cingulata* in tea leaf tissues. Among the 18 tea varieties tested with PAb of *G. cingulata,* significantly high absorbance values were obtained in case of TV-22, T-17/1/1, UP-26 and CP-1/1, which were found to be the most susceptible varieties as detemined by the detached leaf and whole plant inoculation, while TV-30 and BS/7A/76 were the most resistant. These varieties were identified for further studies.

The results of our findings indicate that PTA-ELISA is the more precise and dependable method for quick detection of fungal pathogen. Further, latent infection often creates problems in early detection of pathogen, a problem which can be overcome by immunological techniques. Ghosh and Purakayastha (2003) detected the pathogen (*Pythium aphanidermatum*) of rhizome rot of ginger one week after inoculation by the polyclonal antibodies, even though plants showed no visible symptoms. Early detection of *Colletotrichum falcatum* well before symptom expression was possible by Indirect ELISA in sugarcane (Viswanathan *et al*, 2000). Also, similar to the present findings, the relationship between ELISA values and pathogen concentration per gram of soil appeared to be linear in case of *Spongospora subterranea* (Welsch *et al*, 1996). Polyclonal antibodies are easier to produce than the monoclonal antibodies and in some cases have proved to be more sensitive of the two. For instance, Kratka *et al* (2002) had raised polyclonal and monoclonal antibodies to detect *Colletotrichum acutatum* by PTA-ELISA. According to their findings, only a polyclonal antibody was sensitive enough to recognize the pathogen. Several groups had demonstrated the usefulness of ELISA for detecting the *Pythium spp.* in plants (Mc Donald *et al*, 1990; Shane, 1991; Yuen *et al*, 1998). As suggested by Ghosh *et al* (2005), ELISA is the more sensitive method, but the field applicability of Dot Immunobinding Assay is much greater for the pathogen detection.

Initial events during host-pathogen interaction on leaf surface have been studied *in vitro* with the help of leaf clearing technique to remove chlorophyll for clear observation in cases where epidermal peelings are not easily obtained (Murphy *et al,* 2000). The more critical initial application of microscopy for the infection by *Colletotrichum* has been emphasized by Latunde-Dada (2001). In the present investigation, pathway of spore germination and ingress into the tissue was followed in detail. Besides, differences in penetration events of the fungus in case of resistant and susceptible varieties was worked out by this method. Germ tubes were formed 6 hours after inoculation. In case of resistant variety, percentage of germ tube formation was less and initiation of microconidia were observed. After 18-24h of inoculation, there was formation of melanized appressoria that indicates pathogenic nature of the fungus. There were differences at this point of pathogenesis also in case of resistant and susceptible varieties. Comparatively low percentage of appressoria formation was noticed in case of resistant variety. It is well-known that melanin-deficient mutants of phytopathogenic fungi such as *Magnaporthe grisea* and several *Colletotrichum* species, are avirulen (Henson *et al,* 1999). Appressorial melanin limits wall permeability, facilitating osmolyte accumulation and turgor generation within the cell (Davis *et al,* 1999; Kubo *et al,* 1999). Penetration hyphae were formed 48 hours after inoculation as observed in the present discourse. Microcyclic conidiation and formation of secondary conidia was observed in resistant variety. *Colletotrichum* spp. spore may remain dormant in this stage until favourable conditions for its proliferation are formed (Latunde-Dada, 2001). The duration of the quiescent phase depends heavily upon the circumstances (Wharton and Dieguez-Uriobeondo, 2004). The chronology of infection by *Colletotrichu acutatum* has been already established in several hosts like citrus, almond, strawbwrry and blueberry (Leandro *et al,* 2001; Curry *et al,* 2002 ; Dieguez- Uribeondo *et al,* 2003; Wharton and Schilder, 2003). These studies have shown that appressorium formation and microcyclic conidiation occur 3 to 48 h post inoculation. In the present work also all these events occur within 48 h after inoculation on the tea leaves with the conidia of *G. cingulata.*

The plant and fungal cell wall fragments are important signals in mobilizing a wide variety of biochemically different types of plant defense responses. Walker-Simmons *et al* (1984) used pure fungal *Rhizopus stolinifer* endopolygalacturonase and

citrus pectic fragments to elicit phytoalexin pisatin in pea and proteinase inhibitor I in tomato. In the present discourse, the mycelial wall extract (MWE) of *G. cingulata* was prepared and disease reaction was assessed. The MWE itself did cause very mild symptoms. However, the percentage of such lesions produced was proportional to the percentage produced by the spore suspension. Cell wall fragment elicitors have been reported to elicit defence-realated responses such as HR. Roopa *et al* (2006) purified the crude oligosaccharide extracted from mycelial mats of *Fusarium oxysporum* that induced HR in pearl millet seedlings against downy mildew disease.

Chitin oligomers have been shown to induce various defense-related responses in tomato (Baureither *et al,* 1994), wheat (Barber *et al,* 1989), melon (Roby *et al,* 1987), barley (Kaku *et al,* 1997), pepper (Ahmed *et al,* 2003) and mango (Vivekananthan *et al,* 2004). In the present study, the diffusible compounds elicited by the MWE have been bioassayed and found to be fungitoxic especially in resistant variety. Enhanced accumulation of antifungal compounds in the resistant varieties of tea has also been reported by Chakraborty *et al* (1996b) in case of *Pestalotiopsis theae*.The nature of MWE was confirmed by staining for carbohydrate and protein with Periodate- Schiff's and coomassie blue respectively, following SDS – Polyacrylamide gel electrophoresis. A single high M. W. band of 66 kDa was revealed on staining. Manjunathan and Shetty (2006) performed electrophoretic analysis of cell wall protein of *Penicillium* sp. and indicated that it contains proteins of approximately 50 kDa and 69 kDa. The glycoprotein nature of the mycelial cell wall was also reported by a number of earlier workers (Keen and Legrand, 1980; Lawton and Lamb, 1987; Chakraborty *et al,* 1996; Ndimba *et al,* 2003; Manjunathan and Shetty, 2006).

Proteins are the most important constituents of living organisms, including plants. It is, therefore, expected that the total soluble protein pool will change on inoculation with pathogen. In the present study, the total protein content was measured in healthy and *G. cingulata* inoculated tea leaves 48 hours post inoculation, when the symptoms started appearing. In the present investigation significant reduction in the protein content was noted in the *G. cingulata* inoculated leaves of susceptible varieties in relation to their healthy control. These findings are similar to those reported by Jebakumar *et al* (2001), who noted that in the variety of pepper susceptible to *Phytophthora capsici*,

disintegration of many proteins was noticed in the infected tissues. However, infection with pathogen increased concentration of total proteins, which was associated with susceptibility in Citrus root stock genotypes to root rot pathogen *Phytophthora palmivora* (Albrecht and Bowman, 2007). On the other hand, Chakraborty and Sen Gupta (2001) reported that the total protein content in the non-pathogen inoculated seedlings of the susceptible cultivar of pigeonpea was 1.5 to 2 tomes higher than *Fusarium udum* inoculated susceptible cultivar. In the present discourse, however protein quantity did not alter significantly when compared to control in case of most of the resistant and moderately resistant varieties. Besides, slight increase was noticed in some cases, that was however statistically insignificant. However, it is difficult to separate the relative contribution of host and parasite to the total protein content. It is evident from the above statement that some changes occur in proteins of infected plants but these changes are not always significant. Sometimes protein content of the host remains more or less similar even after inoculation but isozyme and pattern may change.

Therefore, in order to evaluate the changes in the protein profiles of the extracts, SDS-PAGE was run for these samples and the patterns analysed. In case of incompatible reaction there was synthesis of some extra bands, especially with molecular weigh of ca. 97.4 and 94.3 kDa, which are associated with pathogen infection in resistant varieties. Involvement of PR-proteins that can be visualized on SDS-PAGE profiles, is being studied since their discovery by Van Loon (1985). In the present discourse, clear differences in profiles of different varieties were noticed. In case of two susceptible and one resistant variety, the concurrent SDS-PAGE was blotted and the proteins probed with antibody raised against *G. cingulata*. The band of M.W. 66.0 was revealed in the three varieties upon inoculation and thus it seems to be aiding in general pathogenesis. The band with 40.0kDa was found only in compatible interaction. Immunological characterization of such PIps (Pathogenesis-induced proteins) was done by Sharma and Chakraborty, 2004 in case of *Exobasidium vexan* infected tea plants, where two proteins of approximately 58 kDa and 15 kDa were revealed in compatible leaf samples. In the present investigation, a protein of 24 kDa was found in the susceptible infected TV – 22 variety only. The same band was found to be very prominently recognized by the antiserum in case of homologous hybridization. Similarly, Joosten and De Wit (1988) studied the interaction between *Cladosporium fulvum* and tomato, where the 14 kDa

protein was traced by western blot immunologically and was found to be associated with compatible interactions.

It is often difficult to trace a particular protein band on SDS-PAGE. Besides, the function of the induced proteins are a subject of purification and intense biochemical analysis. Therefore, instead of extracting total protein pool, only crude enzyme extracts can be assayed. Enzymes are the biological catalysts that are indespensible for life and especially so under biotic stress conditions. Numerous enzymes are now-a-days considered as defense enzymes. The detailed review has been presented earlier. Presently, only the four important enzymes that are especially involved in the phenolics biosynthesis [phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase(TAL)] on one hand and oxidation of phenolics on the other hand [peroxidase (POX) and polyphenoloxidase (PPO)], have been followed in the necrotrophic reaction on inoculation of tea leaf tissues with *Glomerella cingulata*. Peroxidase specific activity was found to increase abruptly in the susceptible varieties as early as 24 hours after inoculation. However, low activity was registered in these varieties 48hours after inoculation, when the symptoms of disease first started appearing. At that point the specific activity of POX in the incompatible interactions was very high and there was a negative correlation between percentage lesion formation and POX activity. At this point POX is associated with resistance. Thus, it is clear that POX being a regulatory enzyme plays a definite role in both the types of interactions – compatible and incompatible. Similarly, peroxidase activity could not be associated with resistance or susceptibility alone in numerous studies (Sulman *et al*, 2001; Som and Chakraborty, 2003; Van Pelt-Heerschap and Smit-Bakker ,2004; Yasupova *et al* ,2006). From the present investigation, it is clear that POX has a role to play in both kinds of interactions.

Multiple molecular forms of peroxidase have been reported in numerous plants, especially tea. The existence of a large group of isoforms suggests that the proteins encoded by these genes are involved in a broad spectrum of physiological processes, requiring abundant or redundant members to act efficiently during normal and stress conditions (Liu *et al*, 2005). Therefore, isozymes of POX were analysed in all such interactions to follow and identify the resistance and/or susceptibility factor within the soluble POX fractions. Definite isozymes of POX revealed by Native PAGE were

associated with resistance in the present system. One isozyme with R_m (relative mobility) value 0.83 was induced in both resistant and susceptible varieties upon inoculation with *G. cingulata*. On the contrary, the isoperoxidase with R_m=0.11 was found to be induced only in incompatible interactions. Similarly, Shivakumar *et al* (2003) used Native PAGE analysis of POX isozymes which revealed appearance of new isozymes in the incompatible interactions. Numerous studies indicated appearance of peroxizymes associated with resistance in maize (Dowd and Johnson, 2005), *Aegilops umbellata* (Maksimov *et al*, 2006), wheat (Yasupova *et al*, 2006).

PPO, on the other hand, showed a definite and well-defined response 72 hour after inoculatiion. There was a significant increase in specific activity of PPO in the incompatible interactions and thus a negative and statistically significant correlation was established between the specific activity and percentage lesions produced at that point. Similar to the present findings, PPO activity was definitely associated with resistance in many studies (Sharma *et al*, 1994; Renault *et al*, 1996; Raj *et al*, 2006). Overexpression of polyphenoloxidase in transgenic tomato plants results in enhanced bacterial disease resistance (Li and Steffens, 2002). PPO, just like POX, possesses multiple molecular forms. Therefore, Native PAGE of PPO isoforms was conducted for the present pathosystem. The particular isopolyphenoloxidase with R_m=0.55 was found to be associated with incompatible reactions only. Recently PPO isozymes have been studied and particular isoforms were related to resistance in cocoa (Omokolo *et al* ,2003) and pearl millet (Raj *et al*, 2006).

PAL plays an active role in the biosynthesis of cinnamic acid from phenylalanine; cinnamic acid is closely associated with biosynthetic pathways of some isoflavonoid phytoalexins (Lamb and Dixon, 1997). The activity of this important enzyme was found to be significantly increasing as early as 24 hp.i. in the resistant tea varieties. However, in the susceptible varieties the increase was very insignificant. Therefore, the present findings confirm the earlier reports on positive involvement of PAL in the incompatible reactions (Cui *et al*, 1996 ; Chen *et al*, 2000; Chakraborty *et al*, 2000; Jebakumar *et al* 2001; Geetha *et al*, 2005). Previous reports also indicated that oxidative enzymes such as PPO and PO, as well as those involved in phenolic biosynthesis such as PAL are involved in defense reaction in plants (Chen *et al*, 2000 ; Chakraborty *et al*, 2002,2005).

TAL is a subsidiary enzyme in the phenylpropanoid pathway and it catalyses conversion of L-tyrosine to p-coumaric acid, which in turn is converted to caffeic acid, a phenolic precursor. The importance of TAL as a defense enzyme has often been overlooked. Besides, its activity is not well studied in dicots and according to Reglinski *et al* (2001) its activity was not demonstrated in dicots till 2001. In the present discourse, TAL was found to be induced only 72 hours after inoculation in *G. cingulata* inoculated tea leaf tissues . Its specific activity was especially high in the resistant varieties and quite low, though an enhanced activity in the susceptible varieties. In maize TAL catalyses deamination of tyrosine rather than phenylalanine to produce p-coumaric acid (Roster *et al*, 1997). Presumably, the demonstration of TAL activity even in the healthy tea leaves may account for the high amounts of phenolics in tea plants.

Phenolics are major constituents of tea leaves and it is expected that they would be affected by the different abiotic and biotic stresses (Chakraborty *et al*, 2005). Thus, it was considered imperative to study the total as well as ortho-dihydroxy phenol content changes in the tea-brown blight pathosystem. There is often a greater increase in phenolic biosynthesis in resistant host species than in susceptible hosts and it is postulated that the increase in phenolic compounds is part of the resistance mechanism. Some of these compounds are toxic to pathogenic and non-pathogenic fungi and have been considered to play an important role in disease resistance. The involvement of phenol in the defence strategies of tea plants against foliar fungal pathogens e.g *Bipolaris carbonum*, *Pestalotiopsis theae*, *Glomerella cingulata* has been described by Chakraborty *et al.*, (1995, 1996). Biochemical responses to tea plants growing in Darjeeling hills exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* in the levels of phenols and enzyme activities were studied by Chakraborty *et.al* (2002). In the present study, levels of phenolics in leaves of resistant and susceptible tea varieties were estimated after 48h of inoculation with *G. cingulata.* Host responses could be differentiated by changes in content of phenolic compounds. Total phenol and orthodihydroxy phenol content significantly increased in resistant varieties but decreased in susceptible varieties in comparison to their healthy controls. There are ample evidences that an increased production of phenolic compounds are involved in phytoalexin accumulation (Mansfield, 2000), which are the important components of defense machinery.

Leaf surface after coming in contact with the fungal spore, may be rendered more accessible to the infection if the spore is covered with a film of water. However, in resistant genotypes, secretion of antifungal compounds on leaf surface will not allow the spore to germinate. Fungitoxicity of leaf diffusates has been implicated on natural defense mechanism of plants against attack by fungal pathogens in several instances (Chakraborty and Saha, 1989; Chakraborty *et al*, 1996). Although the drop diffusate method has often been criticised as biologically unnatural, the advantage it has over other techniques is that a relatively pure phytoalexin preparation can be obtained without maceration of the plant tissues. Recently, Prats *et al* (2007) demonstrated that constitutive coumarin accumulation on sunflower leaf surface prevents rust germ tube growth and appressorium differentiation.

In the present discourse, biological activities of tea leaf diffusates from resistant and susceptible varieties were studied and their fungitoxicity assessed. The presence of spore germination inhibiting compounds in the diffusates of the two resistant varieties was demonstrated . Similarly, germination and growth of *Botrytis cinerea* was assessed after collecting the diffusates from the leaf surfaces of *Arabidopsis* of different genotypes. Diffusates from the resistant varieties were more fungitoxic (Bessire *et al*, 2007). Further, attempts were made to impart resistance to the susceptible tea varieties with three different abiotic inducers. Use of such compounds is well-documented and has been reviewed at length previously. Shetty (2002) reported that various chemical compounds like salicylic acid, acetyl salicylic acid, aminobutyric acid, benzothiadiazole, calcium chloride, hydrogen peroxide, sodium triphosphate, methionine, polyacrylic acid, jasmonic acid, isonicotinic acid showed promising results in protecting pearl millet crop from downy mildew pathogen. For the present investigation, three chemicals were selected – hydrogen peroxide, which in itself is active oxygen radical; sodium nitroprusside, NO donor, and benzothiadiazole (BTH), salicylic acid analogue and a well-known SAR inducer. All were applied as foliar sprays. However, the schedule, doze and induction period differed. These were initially standardized. Induction of resistance against *G. cingulata* was evaluated in terms of changes in the disease index per plant. Experimental designs were similar in the three cases. Level of defense-related enzymes was measured during the induction period and after challenge inoculation.

Involvement of ROS in pathogen resistance is a well-known phenomenon (Apel and Hirt, 2004; Torres *et al*, 2006). However, whether these impart resistance on their own is not clear. Most of the work on the effect of hydrogen peroxide was done using direct detection of the molecule (Vanacker *et al*, 2000; Borden and Higgins, 2002; Mellersh *et al*, 2002; Trujilo *et al*, 2004). The present investigation was conducted to test the response of direct application of hydrogen peroxide to the tea plants. This radical has been only recently tested for its efficacy to act as an inducer of various defense related molecules (Chico *et al*, 2002; Liu *et al*, 2005; Babosha, 2006). The low cost of this compound and the ability to use it as a foliar spray are its chief attractive features.

A method for control of horticulture diseases and decontamination of plant tissue using hydrogen peroxide, one of the most important ROS, has been patented by Larose and Abott (1998). Besides, it is known to be used as a constituent in preparing home made fungicide, for keeping the cut flowers fresh and for enhancing seed germination . In the present investigation, there was general increase in vigour of the tea plants when low doze (0.15% aqueous solution) of hydrogen peroxide was applied. Similar observations were done by Geetha and Shetty (2002) in case of pearl millet. In tobacco, moderate doses of H_2O_2 enhanced the antioxidant status and induced stress tolerance, while higher concentrations caused oxidative stress and symptoms resembling a hypersensitive response (Gechev *et al*, 2002). Thus H_2O_2 plays a dual role and is a likely candidate as a signal and/or regulatory molecule in signal transduction system occurring during host-pathogen interaction.

The tea plants were protected from the brown blight disease to an extent of 45.6% after treatment with this radical. Similarly, hydrogen peroxide was reported to induce the resistance in transgenic tobacco (Champongol *et al*, 1998). The antimicrobial properties of hydrogen peroxide is a debatable issue. Therefore, bioassay using the spore suspension of *G. cingulata* with the different concentrations of this compound was conducted. It was found that hydrogen peroxide affected the spore germination of *G. cingulata* spore to a great extent. In tomato- *Cladosporium fulvum* interaction also H_2O_2 plays a critical role in limiting colonization by the pathogen either affecting it directly or playing a significant role (Borden and Higgins, 2002). There was total inhibition of spore germination in 3% aqueous solution of hydrogen peroxide. Similar to these findings,

spore germination of several fungi has been shown to be inhibited by H_2O_2 (Peng and Kuc, 1992 ; Lu and Higgins, 1999). Hydrogen peroxide exhibited fungistatic rather than fungicidal action on *G. cingulata* . Interestingly, there was an enhancement in the germ tube length at 0.01% hydrogen peroxide. *Exserohilum turcicum* , necrotrophic pathogen of maize, germinates and survives in high concentrations of hydrogen peroxide. Similar to the findings with *G. cingulata* pathogen, which becomes a necrotroph at later stages of infection, the growth and development of fungus was enhanced *in vitro* at lower concentrations of hydrogen peroxide in case of *Exserohilum turcicum* (Keissar *et al*, 2002).

G. cingulata was found to be quite resistant to the hydrogen peroxide and very high amounts of this molecule would be needed to be present in the tissue for its antimicrobial property to act. There must be some other mechanism for its action in the tissues by which it limits the disease. Hence, level of the defense-related enzymes was measured after the treatment with hydrogen peroxide as well as after the challenge inoculation with *G. cingulata* spore suspension. Treatment of tea plants with hydrogen peroxide enhanced the levels of the defense enzymes peroxidase, ascorbate peroxidase, chitinase and glucanase activity with respect to control especially in the susceptible tea plants. These were thus more responsive to treatment probably because the threshold level of these defence enzymes were not reached and thus could be raised further by the inducer (hydrogen peroxide). Similarly, hydrogen peroxide was reported to increase the level of mRNA transcripts of TmPRX1 (peroxidase) in case of *Arabidopsis* (Liu *et al*, 2005). Catalase (CAT) and ascorbate peroxidase (APX) activities have also been associated with the defense response (Blilou *et al*, 2000).

Catalase is the main hydrogen peroxide scavenger in the plant tissue system. It is, therefore, expected to be greatly affected by the external application of hydrogen peroxide. In the present investigation catalase was found to increase significantly in case of the treated resistant variety only. It seems that in the resistant variety catalase scavenged the hydrogen peroxide due to its already high content in the tissues and higher levels might harm the plant. The susceptible varieties, however showed a very insignificant increase in catalase, inducating that the hydrogen peroxide applied was useful for the overall metabolism of the plant. Catalase is of paramount importance for regulating H_2O_2 homeostasis, as it can function as a cellular sink for H_2O_2. Catalase

deficiency leads to elevation of H_2O_2 levels and triggering Plant Cell Death (Gechev *et al*, 2004; Vandenabeele *et al*, 2004).Studies with exogenously applied H_2O_2 confirm the role of H_2O_2 as a cell death trigger and show that high concentrations can cause necrosis instead of Plant Cell Death (Yao *et al*, 2001). In agreement with these observations, overexpression of the H_2O_2-detoxifying enzyme ascorbate peroxidase can suppress the cell death induced by H_2O_2 or nitric oxide (Murgia *et al*, 2004).

Results by Sarowar *et al* (2005) suggested that the overproduction of ascorbate peroxidase increased peroxidase activity that enhances active oxygen scavenging system, leading to oomycete pathogen resistance. In the present findings, increase in levels of ascorbate peroxidase is followed by the increase in peroxidase activity on treatment with hydrogen peroxide. This may in turn lead to the increased resistance to the brown blight pathogen. Treatment with the signal molecule hydrogen peroxide also enhanced the level of PR-proteins usually associated with SAR (Systemic Acquired Resistance) – chitinase and β-1,3-glucanase (Tyagi *et al*, 2001; Sharma and Chakraborty, 2004; Chakraborty, 2005). In the present discourse, treatment with the hydrogen peroxide elicitor alone gave rise to more significant increase of chitinase (CHT) and β-1,3-glucanase (GLU) in the susceptible varieties. The resistant variety did not show any significant increase in these SAR-associated enzymes. However, when the resistant variety was inoculated with the pathogen, there was a greater increase in activity of these enzymes as compared to the susceptible inoculated plants. Treatment with hydrogen peroxide brought the level of these two enzymes near to the level found in the resistant plants. Thus, the resistant and induced resistant plants exhibited similar biochemical parameters.

The present finding strongly support the views of Gechev *et al* (2002), where stress tolerance induced by spraying the intact tobacco plants with H_2O_2 was indicated by higher activity of catalase, ascorbate peroxidase , glutathione peroxidase and guaiacol peroxidase. Moderate dozes of H_2O_2 enhanced the antioxidant status and induced stress tolerance. Inoculation of the treated and untreated tea plants showed that the highest increase in the activity of the antioxidant enzymes – peroxidae , catalase and ascorbate peroxidase, was in the treated inoculated tea plants. In the overall, inoculation stimulated the activity of these enzymes. Thus, inoculation was much more effective than the treatment with hydrogen peroxide. The present investigation confirm the findings of

Wang *et al* (2004), who treated avocado fruits with hydrogen peroxide one day before inoculation with *Colletotrichum gloeoporioides* and found it to exhibit increased resistance to fruit rot fungus.

Another reactive oxygen species used as inducer of resistance was nitric oxide (NO) that was produced by the well-known nitric oxide donor sodium nitroprusside (SNP). There was suppression of lesion formation due to the treatment with this molecule as observed from disease index (DI). Similarly, when tobacco plants were treated with NO donors, the lesion fomation caused by TMV (Tobacco mosaic virus) was significantly reduced (Song and Goodman, 2001). It was found that the 1mM of SNP sprayed on the tea plants invariably induced the formation of necrotic spots similar to hypersensitive reaction. Similarly, the addition of sodium nitroprusside (SNP), an NO donor, can cause cell death to soybean (*Glycine max*) suspension cultures at millimolar concentrations (Delledonne *et al.*, 1998, 2001; Durner and Klessig, 1999). Contrary to this, observations by Tada *et al* (2004) have shown that the administration of NO donors has no effect on the elicitation of the HR in infected cells in oat plants, although they are required for the onset of cell death in adjacent cells. The activities of catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) increased under SNP treatment in case of *Zea mays* (Sun *et al*, 2006). As per the findings of Dual *et al* (2007) longan fruits were dipped for 5 min in a solution containing 1 mM sodium nitroprusside (SNP), which inhibited pericarp browning and suppressed increases in membrane permeability and lipid peroxidation. These effects were associated with higher APX and CAT activities . In the present discourse also SNP treatment enhanced the activities of APX, POX and CAT . However, according to Clark *et al* (2000) nitric oxide inhibited activities of tobacco catalase and ascorbate peroxidase. Induced resistance by SNP was characterized by increased amounts of the PR-proteins such as peroxidase, chitinase and glucanase in the treated plants as well as of the antioxidant enzymes like CAT and APX. The level of these enzymes in the SNP-treated susceptible inoculated plants was similar to the levels found in the resistant inoculated plants. Therefore, it seems that the resistance induced by NO application is mediated by the said defense enzymes. Since the activity of CAT also increases, it is reasonable to think that the amount of hydrogen peroxide increases due to the treatment with SNP, which in turn leads to induced resistance.

In spite of the importance of Active oxygen species in resistance development, the amount of protection provided by hydrogen peroxide or even SNP is much less than by the SA analogue and a well-known SAR inducer, BTH. The results of study on resistance induction with BTH provide additional evidence in support of the fact that BTH can act as one of the best crop protectants (Fidantsef *et al*, 1999; Baysal *et al*, 2003; Qiu *et al*, 2004; Faoro *et al*, 2007). Exogenous application of BTH to tobacco, *A. thaliana*, wheat and cucumber has been shown to activate a number of SAR associated genes leading to enhanced plant protection against various pathogens (Gorlach *et al*, 1996; Lawton *et al*, 1996; Narusaka *et al*, 1999). In tea plants also a number of SAR associated enzymes were induced upon exogenous application of BTH. Studies on the activities of antioxidant enzymes revealed that treatment increased POX activity, while there was suppression of CAT and APX activity. Suppression of CAT and APX by BTH was also reported by Wendehenne *et al* (1998). This indicates that BTH resistance may be acting via a pathway different from that of H_2O_2 or NO in tea plants. These findings were similar to those reported by Cao and Jiang (2006) in pear trees. The ASM (similar to BTH) spray also significantly increased H_2O_2 level and glutathione reductase activity, but reduced activities of catalase and ascorbate peroxidase in young pears. The study indicated that enhancement of disease resistance in harvested Yali pear fruit could be the result of multiple effects of several factors related to plant defenses induced by ASM sprays on trees during fruit growth.

Chitinase (PR-3) was also induced on treatment with BTH in the tea plants. At the level of mRNA among the different compounds tested, BTH applied by spraying proved to be the stronger inducer of PR-8 (acidic chitinase) mRNA accumulation. BTH sprayed at a concentration of 10 μM was still able to induce *PR-8* gene expression. The protective effects of BTH on cucumber were confirmed in challenge inoculation with *C. lagenarium*. Spraying cucumber plants with BTH (10 μM or 50 μM) allowed effective local and systemic protection against *C. lagenarium* (Bovie *et al*, 2004). It therefore looks probable that BTH acts by increasing the level of endogenous hydrogen peroxide and by concurrent suppression of CAT and APX, which further increased the level of ROS. However, the return of the basal levels of these two enzymes on inoculation of the treated plants leads to hypothesise that there is buffering action of some signal compounds involved. Tight regulatory network seems to govern the endogenous levels

of the candidate signal molecule hydrogen peroxide. Increase in the PR-proteins – POX (PR-9), chitinase (PR-3) and β-1,3-glucanase (PR-2) in the BTH-induced tea plants further underline the fact that BTH acts as a SAR inducer, since it is well-accepted that SAR acts via PR-proteins (Vallad and Goodman, 2004). Increase in chitinase and β-1,3-glucanase level was especially noticed in the BTH-induced plants, which was confirmed by immunolocalization of these defense enzymes. Sharma and Chakraborty (2004) also reported accumulation of chitinase following induction of resistance in the susceptible varieties. Similarly, the induced resistance was associated with increased level of chitinase and β-1,3-glucanase by Saikia *et al* (2005). *In vivo* staining of chitinase activity in fresh root sections allowed the localization of the activity in roots treated with INA (2,6-dichloroisonicotinic acid) by Yedidia *et al* (2000). The formation of fluorescent products mainly in intercellular spaces of the induced roots provided evidence for the involvement of the plant defence system, just as in the present findings

POX, as analysed earlier, possesses a number of isozymes and some of them were found to be specific in the incompatible interactions. Besides, it has been recently proposed to be a regulatory enzyme. It acts as ROS scavenger as well as producer. Therefore, playing a very controversial role. It was decided to check whether the same isozymes were operative in the induced resistance in the tea plants. There are numerous reports of involvement of acidic isoforms of peroxidases in host resistance (Flott *et al*, 1989; Shivakumar *et al*, 2003). In the present discourse anionic (acidic) isoforms of POX were analysed. It was found that some isozymes were constitutively expressed, while others were induced upon treatment or inoculation with *G. cingulata*. The particular isozyme with R_m=0.11 was found to be associated with resistance in constitutive as well as induced resistance. It was strongly associated with induced resistance in case of the best protectant among the three i.e. in the BTH-induced tea plants. Since chitinase was also strongly associated with the induced resistance in this case, involvement of the chitin-specific isoperoxidases cannot be overruled. Such peroxidases have been reported by Maksimov *et al* (2003).

The results of present study provide additional evidence that host metabolic pathway altered by treatment with abiotic agents can result in an effective resistance against fungal disease. BTH can induce resistance when sprayed exogenously. It is likely

that the speed of defense response of susceptible tea varieties are usually slow or weak and the production of defense components are not sufficient for the total inhibition of growth of the pathogen or the synthesis of certain critical components of defense are not activated by the infection process. The delay in recognition of the pathogen and induction of the defense response, in this case, are also not unlikely. It has been possible to enhance the speed of response to some extent by the application of inducers. An induced hypersensitive response was evident in this case which substantiate the observation made by Faoro *et al* (2007). Crucial to the success of induced resistance in agriculture is an understanding of the range and limitations of this form of pathogen control. Besides, the impact of the treatment on the productivity of the crop should be taken into account. The optimum doze must be standardized for each crop separately.