Studies on Multicomponent Coordinated Defense Strategies in Tea against Foliar Fungal Pathogens and *Helopeltis theivora*

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This is to certify that Ms. Rakhee Das Biswas has carried out her research under my supervision. Her thesis entitled " **Studies on multicomponent coordinated defense strategies in tea against foliar fungal pathogens and *Helopeltis theivora*** " is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

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INTRODUCTION

Tea drinking is an ancient custom with origins in 2737 BC China. Today approximately 3,000 varieties of tea [*Camellia sinensis* (L.) O. Kuntze] are made from this single plant. Tea offers more beneficial than being a soothing beverage to be served at social gathering, or to ward off the chill of a winter's night. There is mountain evidence to suggest that drinking tea may also reduce the risk of developing cardiovascular disease and many forms of cancer (Chakraborty and Chakraborty 1998). Tea plants are abundant source of flavonoids, a group of compounds with antioxidant properties of which specific interest are the flavonoids catechins and flavonols which prevent the synthesis of peroxidase and free radicals, agents that can invade cell membrane and damage genetic material. Certain chemicals found in the molecular structure of these beneficial flavonoids, collectively known as phenolic groups. Since polyphenol are major constituents of tea leaves, their involvement in the defense mechanism either as preformed or induced chemicals seemed highly probable.

Tea is also one of the most important plants from the economic viewpoint and being a perennial is always challenged by pests and pathogens, which provides a stable microclimatic conditions as well as supply of food for rapid build up of insects (Muraleedharan and Chen 1997). Among the insects, tea mosquito bug *Helopeltis theivora* Wat. (Hemiptera : Miridae) has assumed the status of major pests in several tea growing areas in West Bengal and Assam (Somchowdhury *et. al.*, 1993). Both adult and nymph of *H. theivora* suck the sap from the young leaves, buds and tender stem. Feeding by *H. theivora* is seemed primarily on the leaves. A circle is formed around the feeding spot within 2-3 hour of damage, in 24 hour the inside portion of the ring becomes translucent light brown in color and within a few days the spot turns dark brown. The tissue around the affected spots first turn brown and then black and subsequently they dry up (Plate 1). The dry leaf tissue in the long run dropped resulting in numerous perforations (shoot holes) on the leaf surface. A single leaf may have innumerable puncture marks. Severely damaged leaves also curl up apart from

PLATE 1 : Tea leaves naturally infested by *Helopeltis theivora* (inset)

ceasing to grow. The typical feeding damage by *H. theivora* appears as a discolored necrotic area around the point of entry. They feed at early morning, late evening and night hours.

The major fungal diseases of tea have been classified under the leaf, root and stem diseases. Among the foliar diseases of tea blister blight, black rot, brown blight and grey blight are important fungal diseases caused by *Exobasidium vexans*, *Corticium invisum*, *Glomerella cingulata* and *Pestalotiopsis theae* respectively (Agnihothrudu, 1995). The pests and blights which pray on the leaves are of vital importance, since any damage to the leaves defeats the whole purpose of its cultivation. Blister blight disease is very common in humid and foggy regions whereas black rot is common in doors and terai of North Bengal capable of causing significant depreciation in tea yield and quality. Serious infection causes defoliation starting from the center of the bush. Black rot affected leaves are characteristically greyish brown, but at a later stage of infection they become black particularly during the rains. The disease persists in the same areas for years, if not controlled, causing gradual deterioration in the health of the tea and loss of crop. Colour of the upper surface of the affected area at the early stage become reddish-brown, similar to sum-scroch damage, later it turne to a mixture of brown, yellowish-brown and grey the undersurface become light brown of grayish white and usually covered with a net work or cream to brown mycelium. The fungus produces on the stem, thick cords of mycelium, up to about 3 mm across, dark purplish-brown on the older portions of the stem and dull white to light brown on the green portions at the top. The fungus spread not only by the direct contact from bush to bush but also by wind, bird as well as by workers of the tea garden. Alternaria blight, another foliar disease of tea (Plate 2) caused by *Alternaria alternata* (Fr.) Keissler is very common in the nursery grown plants. Disease symptoms appear as greyish brown patches on the young leaves. Older leaves were less susceptible. Symptoms first appear in the tip region and the margin of the leaves, which extend towards the midrib following which the leaves curl, and die. It causes

Plate 2 : - Tea plant (Teen Ali 17/1/54) showing symptoms of leaf blight disease caused by *Alternaria alternata* (inset)

serious infection leading defoliation of leaves. It causes considerable damage to the plants maintained in the nursery as well as in the field (Chakraborty *et.al*, 2005).

The defense strategies of plants against their pests and pathogens are manifold and include the use of antifungal chemicals. On the other hand, pests and pathogens have evolved mechanisms to evade these chemicals. In such relationship it has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid cell death. Numerous studies suggest that low molecular weight phenols, such as benzoic acid and phenylpropanoids are formed in the initial response to infection. Most research on resistance mechanisms has shown that the plant uses defenses that are activated after infection to stop pathogen development. Isolated mycelial walls of many fungi possess potent elicitor activity to induce phytoalexin accumulation in plants (Mansfield, 2000).

Natural products from plants continue to be the subject of novel and straightforward application of pathogen and pest control agents. It is noteworthy that theses agents are perhaps most often isolated from plants or associated microorganisms that grow in the humid tropics where the competition to survive and even thrive in intense. Therefore there is the expectation that those compounds may be specially fit to compete. As the development of diverse use of these and succeeding agents continue to evolve, populations, animals and crops will increasingly be protected from a broad spectrum of pest by more selective technologies. These agents may also serve as lead compounds for the development of economically superior agents against pests and pathogens.

Botanical pesticides have received renewed interest because of poor public opinion of synthetics and the increase in organic growing practices. Besides their natural origin, botanicals are valued for their low environmental persistence and alternate mode of action, although their instability and lower efficacy compared to synthetics are berried to development. Active phytochemicals from new botanicals remain important leads for the development of new synthetic pesticides with clear

commercial advantage. Phytochemicals with insecticidal properties have been isolated and studied from many plant families such as Annonaceae, Araceae, Asteraceae, Guttiferae, Meliaceae and Piperaceae for the development of botanical insecticides an important source of new agro-chemicals.

The ability to induce resistance and utilize it optimally in agriculture depends on fundamental knowledge of biochemical changes and on the specificity and compatibility of the signaling systems that regulate their expression (Chakraborty, 2005). It is generally believed that plants defend themselves against pathogenic fungi by producing fungitoxic substances such as phytoalexins (Purkayastha and Daniel, 1995), pathogenesis related proteins (Castro *et. al.*, 2004), oxidized phenols (Arendse *et. al.*, 1999) and several other components. Usually a plant responds to a pest or pathogen by mobilizing a complex network of active defence mechanism. The success of the plant in warding-off the pest and pathogenic attack depends upon the coordination among the different defence strategies and the rapidity of the response. But in most cases the role of a single defence component has been reported at a time while working on disease resistance of a host-pathogen system.

In the present investigation attempt was made to elucidate the defense strategies in tea plants against pest (*H. theivora*) and foliar fungal pathogens (*A. alternata* and *C. invisum*) with the following objectives:

(i)Screening of resistance of tea varieties towards pest and pathogens, (ii) immunodetection of fungal pathogens in tea leaves, (iii) determination of the level of phenolics and ascertaining their antifungal activity associated with differential host response to infection aginst pest and pathogens, (iv) association of defense enzymes (phenyl alanine ammonia lyase, polyphenol oxidase, peroxidase, β-1,3-glucanase and chitinase) in tea varieties triggered by pest and pathogens (v) accumulation of pyrocatechol and catechins in tea following infestation by *H. theivora* and inoculation with *A. alternata* (vi) induction of resistance in tea plants against pest and pathogen using bioresources and (vii) cellular localization of defense enzyme in tea tea leaves following induction.

LITERATURE REVIEW

Resistance or immunity of a plant against pests or pathogens is related to multiple factors. Disease resistance can also be described on several levels such as non-host resistance, parasite - and race-specific resistance, plant age - and organ-specific resistance, and acquired resistance. In fact, if one considers the multitude of microorganisms to which plants are being continuously exposed in nature, the significance of specificity becomes more apparent (Chakraborty, 1988). The physiological/biochemical basis of resistance of plants to fungal and bacterial pathogens has been associated with both preformed and infection induced antimicrobial compounds. Phenolics are significant components of the host response which involve the isolation, identification from substantially large amount of tissues following inoculation. Keeping this in mind the following review is presenting briefly the observation of previous workers in concord with the present line of investigation. Two major objects such as (A) Biochemical defense strategies of plants against pests and pathogens and (B) Efficacy of plant extract in plant defense response have been discussed in the following pages.

(A) Biochemical defense strategies of plants against pests and pathogens

Defense response of a plant depends on the speed and extent of phenols. Some occur constitutively and are thought to function as preformed inhibitors associated with non-host resistance (Millar and Higgins, 1970; Schonbeck and Schlosser, 1976; Mansfield 1983; Stoessal, 1983). Others which are formed in response to ingress of pathogens and their appearance is considered as part of an active defence response. Since the phenolic intermediates have a role in the active expression of resistance, an underlying problem in ascertaining that such secondary metabolites are of primary (rather than secondary) importance has been the localization and timing of the host response (Nicholson and Hammerschmidt, 1992).

Tea, one of the important plantation crops of North East India provides a stable microclimate and a continuous supply of food for rapid build up of more than 300 insects. Despite crop loss, pest infestation also adversely affects the quality of processed tea. Damage by sucking pest like stripes and mites resulted in dull appearance of tea. Tea made from flushworm infested shoots have low levels of extractable solids and high crude fibre content. Liquors obtained from such tea are 'flat' and the presence excreta of the larvae perhaps responsible for the deterioration in quality. The serve infestation of flushworm, pink mite, strips and tea mosquito bug adversely affected flavour and decrease in polyphenolic contents. An increase in polyphenol oxidase activity and total polyphenols was associated with high pigment formation in these teas. The tea mosquito bug *Helopeltis theivora* Waterhouse, popularly known as 'tea mosquitoes' is one of he major pest causing extensive damage in Darjeeling tea plantation. The nymphs and adults of *Helopeltis* are the active sucker of sap from buds, young leaves, tender stems and shoots. They feed at early morning, late evening and night hours. On sunny and worm day, *Helopeltis* takes shelter in lower layer of bush canopy. The insect injects toxic saliva through their needle like rostrum which causes necrosis of the tissues and turn brown first then black and they dry up subsequently. Eggs are laid in tissue of tender stem, mid-rib and petiole of leaves. It was reported that a single first instar nymph of *H. theivora* could make as many as eighty feeding lesions in 24 hours. In order to determine how the tea plant reacts to the attack by the insect, change in activities of antioxidant enzymes - peroxidase, ascorbate peroxidase as well as defense related enzymes - phenyl alanine ammonia lyase, chitinase and β-1,3 glucanase was determined in both healthy and infested tea leaves (Chakraborty *et. al.*, 2005). Protein, carbohydrate, chlorophyll, total phenol and free amino acid contents were found to be reduced in the *H. theivora* infested leaves, while a slight increase in ortho-dihydroxy phenol content and a sharp increase in proline content were noticed in infested leaves (Chakraborty and Chakraborty, 2003). In addition biochemical response of tea to

attack by *H. theivora* with special reference to oxidative enzymes and flavonoid flavor components was determined by Chakraborty and Chakraborty (2005).

Phenols are considered as one of the significant components of the host response following fungal infection. Paschenko (1978) demonstrated the role of phenols in resistance of *Nicotiana didebta* to *Peronospora tabacina.* From a study of the effects of pyrogallol, pyrocatechol and hydroquinone and aqueous extracts from leaf tissues of *N. didebta* and leaf washing on conidial growth, no direct relationship was found between their quantity and the resistance of mature plants to *P. tabacina.* Pyrocatechol and hydroquinone showed extremely high fungitoxicity in relation to *P. tabacina.* Spore growth was more strongly inhibited by extracts from tissue of receptive cultivars. Pyrogallol somewhat stimulated conidial growth. Polyphenoloxidase of resistance cultivars were highly activated during infection. In potato tubers chlorogenic acid was reported to accumulate slower following inoculation with *P. infestants* than in non-inoculated controls, regardless of cultivar resistance (Gans, 1978). In contrast, in some susceptible cultivars chlorogenic acid accumulates at an accelerated rate after inoculation (Henderson and Friend, 1979). The differentiation of the responses of plants to pathogens based on host and non-host interactions also has been argued by Health (1980).

Friend (1981) showed that the accumulation of chlorogenic acid might represent a general rise in phenolic biosynthesis. Chlorogenic acid act as a reservoir for the caffeoyl moiety that, as an activated phenylpropanoid, could be shunted to the synthesis of other phenolics possibly involved in containment of the pathogen. Such synthesis can ultimately result in the accumulation of compounds with sufficient toxicity to be involved in resistance. When carrot root slice is infected with *Botrytis cinerea*, the infection leads to the production of inhibitors such as 6-methoxymellein, p-hydroxybenzoic acid and falcarinol (Harding and Heale, 1981). According to Mayama *et. at.*, (1981) oat produces nitrogen containing phenolic phytoalexins, the

avenalumins, and these compounds accumulated only in incompatible host pathogen interactions.

UV-absorbance and autofluorescence spectra of the avenalumins were used microspectrophotometry to reveal the presence of intense fluorescence only in cells immediately associated with the infection site (Mayama and Tani 1982) where as rapid accumulation of phenols may result in the effective isolation of the pathogen (or non-pathogen) at the original site of ingress (Legrand, 1983; Ride, 1983). For most plants it are low molecular weight phenols, especially the phenyl propanoid, that are involved in the initial response to stress. In potato, phenols accumulate as an initial response to infection (Hammerschmidt, 1984; Hachler and Hohl, 1984). The accumulation of polymerized phenols also occurs as a rapid response to infection. Farmer (1985) and Bolwell *et. al.*, (1985) took hydroxycinnamic acids and their derivatives to reveal the contribution of the discoloration and autofluorescence of host tissues at the site of infection. In maize leaves change in phenolics after inoculation with *Bipolaris zeicola* and their antifungal activity was demonstrated by Werdes and Kern (1985). Maize inbreds Prl (resistant) and Pr (susceptible) to *B. zeicola* race 1 were inoculated and phenolic material was extracted from maize leaf tissue. The components were than analyzed and resistance was studied with respect to phenol metabolism and accumulation of fungitoxic compounds. Host responses could be differentiated by changes in content of phenolic compounds. The pattern of changes of total phenolic content (hydrolyzed and unhydrolyzed ethylacetate soluble phenols) of resistant and susceptible inbreds did not differ much between 0 hr. and 96 hr. after inoculation. However, phenolics content in the resistant inbred increased between 96 and 120 hr. after inoculation to a level two to three times higher than that of susceptible and non-infected control in breds. They isolated four antifungal compounds, A, B, C and D from hydrolyzed maize leaf extracts. All four compounds were fungitoxic to *B. zeicola* in spore germination and chromatographic bioassays. Compounds A and B were inhibitory to *B. zeicola* only in high concentrations. The

investigators suggested a role of the phenol metabolism in the resistance of maize to *B. zeicola* based on different content of total phenolics in resistance and susceptible inbreds. The compounds C and D were supposed to play a role in the resistance mechanism as fungitoxic component.

Saxena *et. al.*, (1986) reported the change in phenolics of two each of resistant and susceptible varieties of wheat leaves in response to *Puccinia recondita* causing brown rust were evaluated by They found that resistant varieties exhibited higher concentration of phenolics than the susceptible one. Esterification of phenols to cell-wall materials has been considered as primary theme in the expression of resistance (Fry, 1986; 1987). Parashar and Sindhan (1987) showed biochemical analysis of pea varieties resistant and susceptible to *Erysiphe polygoni* causing powdery mildew disease revealed that the quantity of total phenol and orthodihydroxyphenol was high in stem and leaves of resistant varieties as compared to susceptible ones which decreased as the age of plant increased in all the varieties.

According to Cuypers *et. al.*, (1988) the temporal and spatial differences in the accumulation of phenylalanine ammonia-lyase (PAL) mRNA occurred as a response to infection which was rapidly elevated in interactions involving an incompatible race of fungus, where as a significantly different profile of mRNA accumulation occurred in interactions involving a compatible race The kinds of phenolic compounds that accumulate prior to the active defence response as well as their origin has been addressed by Matern *et. al., (*1988) using parsley leaves with *P. megasperma* f. sp. *glycinea* (Pmg) or treatment of parsley cell suspensions with a Pmg elicitor results in the accumulation of substantial concentrations of coumarin phytoalexins as well as esterification of phenylpropanoids, in particular ferulic acid, to cell walls. Treatment of parsley cells with the Pmg. elicitor cause the synthesis of the coumarin phytoalexins isopimpinellin, psoralen, bergapten, xanthotoxin and graveolone. The healthy leaves of *Morinda tomentosa* contained the two methoxyflavonols 4'-OMe Kaempferol and 3', 4'- di OMe quercentin, and the four phenolic acids-vanillic,

syringic, gentisic and ferulic. The *Colletotrichum gloeosporoides* infected leaves contained the hydroxyflavonols kaempferol and quercetin along with four phenolic acids found in healthy leaves. The diffusates of both the pathogen and non-pathogen (*F. solani*) treated leaves contained quercetin and kaempferol (Abraham and Daniel, 1988).

The defensive strategy of plants exists in two stages. The first is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow (or even halt) the growth of the pathogen and to allow for the activation of "secondary" strategies that would more thoroughly restrict the pathogen (Matern and Kneusel 1988). Secondary responses would involve the activation of specific defenses such as the de-novo synthesis of phytoalexins or other stress-related substances. They argue that the initial defense response must occur so rapidly that it is unlikely to involve de novo transcription and translation of genes, which would be characteristic of the second level of defence. The sequence of events in a defence response can be thought to include-host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions or papillae, and, finally, the synthesis of specific antibiotics such as phytoalexins.

Phytoalexins represent one component of a battery of induced defence mechanisms used by plants, including the important formation of physical barrier to invasion by alteration plant cell wall, the transient generation of antimicrobial active oxygen species (such as H_2O_2) which are generated by the oxidative burst, and release of biologically active lipids such as a result of lipid peroxidation (Ride, 1986; Graham and Graham, 1991; Croft *et. al.*, 1993; Levine *et. al.*, 1994; Low and Merida, 1996; Bestwick *et. al.*,1997). It is important to recognize that phytoalexins accumulation may be part of co-ordinated defence strategies, in which any one factor may alone be unable to account for restriction of the potential pathogen. There are some

interactions in which the speed of accumulation of the inhibitors and their high level of toxicity argue strongly that they are the principal cause of restriction of microbial growth. Such examples are the accumulation of phaseollin in *Phaseolus vulgaris* hypocotyls and wyerone derivatives in cotyledons of *Vicia faba* (Mansfield *et. al.*, 1980; Bailey, 1982).

Total phenol increased after infection in green and ripe tomato fruits in course of rotting due to *Sclerotium* rolfsii (Prasada *et. al.*, (1988). There is often a greater increase in phenolic biosynthesis in resistant host species than in susceptible host and it is sometimes postulated that the inscrease in phenolic compounds is part of the resistance mechanism. Some of these compounds are toxic to pathogenic and nonpathogenic fungi and have been considered to play an important role in disease resistance (Vidyasekharan, 1988). Changes in phenol contents was also demonstrated by Oke (1988) in young, matured, healthy and *Cassicola corynospora* and *Colletotrichum nicotianae* infected leaves of tobacco. After infection the quantity of total pehnols and orthodihydroxyphenol increased in both stem and leaves of susceptible and resistant varieties. Tore and Tossi (1989) investigated the changes in phenolic and nitrogen metabolism in healthy and infected with *(Thielaviopsis basicola)* tobacco roots and leaves. The chlorogenic acid content increased in infected root and leaves compared with the control beginning on the 8th day after inoculation. Polyphenol content in sweet cherry bark was drastically changed after infection by *Cercospora personii* (Bayer, 1989). Infected tissue and closely neighbouring areas were characterised by the appearance of phenolic aglycons which inhibited growth of both the pathogen. Mechanically wounded bark tissue showed different phenolic patterns than infected ones.

Glycoproteins were extracted from isolated cell walls of *Phytopthora megasperma* f. sp. *glycinea* (formerly *P. megasperma* var. *sajae*) with 0.1 N NaOH at 0°C and elicited glyceollin in soybean hypocotyles with the same specificity as the fungus races from which they were obtained. Fraction of the crude extracts on DEAE

Bio-Gel and Bio-Gel A-5m columns showed that specific elicitor activity of glycoproteins was not diminished by boiling at 100^0C or pronase treatment, but was destroyed by periodate, thus indicating that the carbohydrate proteins are important for activity. The glycoproteins were the only concanavalin A reactive species detected in the crude cell wall extracts, and fluorescein labeled concanavalin A was hepten-specifically bound to living hyphae of the fungus and to native but not NaOH-extracted isolated cell walls. Therefore it was concluded that the glycoproteins are present at the surface of fungal cell wall. Tunicamycin, which inhibit the glycoprotein of eukaryote glycoproteins, was a potent inhibitor of mycelial growth of the fungus. The data supported the hypothesis that race specificity in the soybean *P. megasperma* f. sp. *glycinea* system may be determined by specific plant recognition of fungus surface glycoproteins. (Keen *et. al.*, 1980)

Quantitative changes in phenolic compound at different time intervals on barley varieties inoculated with *Puccinia hordei* detected by Etenbarian (1989). Luthra (1989) determined the levels of total phenol in sorghum leaves, resistant and susceptible to *Ramulispora sorghicola* at 15 days interval after 25 days of sowing. Resistant varieties exhibited high phenol content in comparison to susceptible ones at all stages of growth. Phenolic compounds inhibitory to the germination of spores of *Colletotrichum graminicola* were shown to leach from necrotic lesions on corn leaves caused by the fungus. Primary components of the phenolic mixture were identified as esters and glycosides of p-coumaric and ferulic acids as well as the free compound themselves. Spores of *C. graminicola* produced in acervuli of infected leaves were shown to be surrounded by a mucilaginous matrix as in the case when the fungus is cultured *in vitro*. It is suggested that the mucilage protects spores from the inhibitory effects of the phenols by the presence of proline rich proteins that have been shown to have a high binding affinity for a variety of phenols (Nicholson *et. al.*, 1989). The relatively non-specific disruptive effects on cells that result from wounding lead almost immediately to a variety of physiological changes, including oxidation of secondary metabolites. The accumulation of these esters preceded the onset of visible

necrosis of infection sites, the concentration of the compounds fell substantially after the onset of necrosis both of which strengthen the argument for their involvement in the browing response (Bostock and Stermer, 1989). Toxic phenylpropanoids, such as ferulic acid, can form rapidly without the involvement of the traditionally accepted route of phenylpropnoid synthesis and conversion to CoA esters (Hahlbrock and Scheel, 1989). It has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death (Fernandez and Heath, 1989). Baker *et. al.*, (1989) examined specific race interaction with clones of resistant and susceptible genotypes and they found greater accumulation of phenolic compounds in resistant reaction than in susceptible reaction. They suggested that accumulation of phenolics might play a role in natural and induced interaction involving *Colletotrichum trifolii* and *Medicago sativa.*

Biochemical changes in the pearl millet shoots infected with downy mildew pathogen (*Sclerospora graminicola*) (Kumar *et. al.*, (1990) The estimation revealed that the total phenol and free amino acids content were found to be low both in diseased shoot and roots of pearl millet (*Pennisetum glaucum*). In maize there is a marked accumulation of two caffeic acid esters after inoculation with *Glomerella graminicola* or *C. heterostrophus* in both compatible and incompatible combination (Lyons *et. al.*, 1990). One compound was identified as caffeoyl glucose, whereas the other was a caffeoyl ester of an unknown organic acid moiety. Although neither compound was fungitoxic, a pattern of rapid accumulation followed by a sharp decrease in the amount of both compounds in the tissue suggested that they may serve as a pool of phenols required for diversion to other products. Mansfield (1990) has proposed that cell death results from irreversible membrane damage that may occur in response to pathogen recognition or as a result of activated host response.

Niemann *et. al.*, (1991) analysed the low molecular weight phenols, such as benzoic acids and the phenylpropanoids, are formed in the initial response to infection. Early after infection, low molecular weight phenols accumulate in both

incompatible (resistant) and compatible (susceptible) interactions. Whether these compounds, are significant in the ultimate host response presents a perplexing problem. Bruzzese and Hasan (1991) demonstrated that accumulation of phenols at the infection site occurred as early as 3 hr. after inoculation, indicating an association of phenols with the initial stages of the response. The contents of phenols, O-dihydroxyphenols and peroxidase activity in healthy and *Curvularia andrepogonis* infected leaves of *Java citronella (Cymbopogan winterianus)* were determined by Alam *et. al.*, (1991). As a result of infection the content of phenols and peroxidase increased two and fourfold, respectively in necrotic lesions compared to healthy leaves. . It has been suggested by Permulla and Heath (1991) that the accumulation of phenolics as an initial response to infection may reflect a general increase in host metabolism as well as an accumulation of relatively non-toxic secondary metabolites, which could ultimately serve as precursors for compounds essential to expression of resistance. In the interaction of potato tubers with *Verticillium dahliae*, hypersensitive browning and suberization are characteristic of the initial events in resistance rather than production and accumulation of phytoalexins (Vaughn and Lulai 1991). The *Fusarium* sp infected leaves of *Trianthema portulacastrum* contained 6,7, dimethoxy-3, 5, 4'- trihydroxy flavone, vanillic acid, p-hydroxybenzoic acid, quercetin and ferulic acid. By using drop diffusate technique it was found that the pathogen induces the formation of quercetin and ferulic acid (Darshika and Daniel, 1992). Changes in carbohydrates, amino acid and phenolic contents in jute plant on inoculation with Macrophomina phaseolina, *Colletotrichum corchori* and *Lasiodiplodia theobromae* were studied by Shabuddin and Anwar (1992). Total sugars, non-reducing sugars, starch and total free amino acids were found to decrease on inoculation with all the three test pathogens of jute, while reducing sugars, total phenols and orthodihydric phenols increased.

Chakraborty *et. al.*, (1994) reported that among fourteen varieties of tea tested separately against *Glomerella cingulata, Pestalotiopsis theae* and *Bipolaris carbonum,* TV-18 and TV-26 were highly susceptible and resistant respectively to *G.*

cingulata and *B. carbonum.* While TV-23 and CP-1 were found to be highly susceptible and resistant to *P. theae.* Twelve separate phenolics were detected on thin layer chromatograms after extraction from healthy tea leaves and some were identified as gallic acid catechol, caffeic acid and p-coumaric acid. Total phenol level decrease by 4.5, 1.2 and 8.5% in the susceptible varieties TV-18, TV-9 and TV-17 respectively after inoculation with *B. carbonum*, whereas in case of resistant varieties TV-26, TV-25 and TV-16 total phenol level increased by 11.1, 5.7 and 12.2% respectively after inoculation. Similar pattern was observed for O-dihydroxy phenol content in healthy and inoculated leaves of resistant and susceptible varieties. The healthy leaves of *Tectona grandis* contained two flavones: 4'-O Me-apigenin and luteolin. The phenolic acids present were syringic, sinapic, vanillic, melilotic and gentisic acid. The other constituents of the leaves were quinones (lepachol and tectaquinon), proanthocyanidins, iridoids, alkoloids and tannins. The infected leaves did not contain any flovone but a flovonol 3', 4'- dimethoxyquercetin instead and phenolic acid such as ferulic, vanillic, melilotic and gentisic acids. They contained the same quinones as at healthy leaves as well as proanthocyanidins, iridoids, alkoloids and tannins. There was no significant chemical differences between the diffusate of control and treated leaves when the healthy leaves were treated with the spore suspension of *Curvularia clavata.* But when the leaves were treated with a non-pathogen *Fusarium solani,* the diffusate contained p-hydroxybenzoic acid. Mycelial growth, spore germination and germ tube growth of *F. solani* and *C. clavata* is strongly inhibited by P-hydroxybenzoic acid (Daniel, 1995).

One of the best and longest-studied defense responses of plants to the infection is the induced accumulation of antimicrobial, low-molecular-weight secondary metabolites known as phytoalexins. Since the phytoalexins hypothesis was first proposed in 1940, a role for the compound in defense has been revealed through several experimental approaches. Support has come, for example, though studies on the rate of phytoalexins in relation to cessation of pathogen development, quantification of phytoalexins at the infection site, and the relationship of pathogen

virulence to the phytoalexins tolerance. Evidence in support of phytoalexins in resistance as well some recent advances in phytoalexins biosynthesis are reviewed. Criteria for evaluating a role for phytoalexins in disease resistance are also discussed (Hammerschmidt, 1999).

Two antifungal compounds isolated from healthy and *Bipolaris carbonum* infected tea leaves exhibited clear inhibition zones at Rf 0.8 and 0.65, respectively in a chromatographic bioassay. On the basis of their colour reaction on TLC and UV-spectra these were identified to be catechin and pyrocatechol, respectively. Resistant varieties accumulated 439-510 μg/g fresh weight tissue of catechol in comparison to 187-212 μg/g fresh weight tissue in susceptible varieties after inoculation with *B. carbonum.* Low concentration of this compound was also detected in healthy leaf tissues (Chakraborty and Saha, 1994, 1995). Phenolic contents in pea genotypes in relation to powdery mildew disease was studied by Sharma *et. al.,* (1998). Guleria *et. al.,* (2001) demonstrated increased levels of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols in salicyclic acid sprayed leaves in comparison to untreated control plants. The multicomponent phytoalexin response that is found in many plants is clearly demonstrated by bioassay carried out on thin layer chromatograms. After chromatography, the TLC plate was sprayed with suspension of spores of *Clasporium herbarum* in nutrient solution. White zone of inhibition occurred where the dark green fungus failed to grow. No activity was detected in healthy leaves using the assay technique.

Complex drug cerbiden has been studied in vitro for the antifungal activity of its components. It has been established that the spectrum of antifungal effect and activity of cerbiden, with respect to fungi conditionally pathogenic for people, is determined by antibiotic compounds--aromatic carbohydrate phenylheptatryin and sesquiterpene phenol cernusol. They process a spectrum of antifungal activity analogous to cerbiden and activity close to Candida spp., some basidial yeast, dermatophytes, a number of Mucorales. (Stoessl, 1982)

In some host parasite interactions phenolics have been associated with phytoalexin accumulation (Mansfield *et. al.*, 1974; Langcake and Pryce, 1976; Langcake and Macarthy, 1979; Holliday *et. al.*, 1981; Pierce and Ersenberg, 1987; Baker *et. al.*, 1989). Phytoalexin accumulation is believed to be an important early defence response in several plant pathogen interactions. A lot of work has been done and several comprehensive reviews have appeared on phytoalexins and their role in disease resistance (Daniel and Purkayastha 1995; Hammerschmidt, 1999). A fundamental aspect of the definition of phytoalexins is that they are synthesized from remote precursors. Thus, simple labeling studies have demonstrated that the amino acid phenylalanine is used for the synthesis of a complex isoflavonoid such as phaseollin .Activation of defence responses usually lead to a massive, albeit transient and local diversion of normal metabolism into synthesis of groups of secondary products. The interaction between primary and secondary metabolism leading to biosynthesis of chemically diverse phytoalexins. The long term goal of their research is to define target for genetic manipulation to enhance disease resistance in plants. Phenylpropanoids include several classes of phytoalexin including furanocoumarins, flavonoids, isoflavonoids and stilbenes, all of which are ultimately derive from *p*-coumarate. For certain compounds such phytoalexin from common metabolites, whereas synthesis of glyceollin requires the co-ordinate activity of a multienzyme pathway. as the stilbenes resveratrol, a single enzymic step is required to produce the (Dixon *et. al.*, 1992; Nicholson and Hammerschmidt, 1992).

Purkayastha, (1995) analysed phytoalexins constitute a chemically heterogeneous group of substances belonging to various classes of natural products which include isoflavonoids, sesquiterpenoids, polyacetylenes and stilbenoids. Many phytoalexins are absent in healthy, unchallenged plants. It was originally believed that phytoalexins were host specific. With the evidences accumulated so far, concerning the wide spread occurrence, isolation and characterization of phytoalexins during the past 50 years, it is now clear that more than one phytoalexin could occur in a single host species of which one may be dominated Again, similar phytoalexins

may also occur in different host species. Plant organs including roots, stem, leaves and fruits have been shown to respond to infection with the formation of phytoalexins. During incompatible host-parasite interaction, phytoalexin is synthesized rapidly and accumulates at the infection site. In contrast in the compatible host parasite interaction the plant also synthesis the phytoalexin but relatively slowly and in reduced concentration. The degree of stimulation of phytoalexin biosynthesis depends on several factors such as quantity of elicitors, presence or absence of receptors in the host cell membrane, if present, strong or weak response of receptor, duration of treatment, and environmental conditions. Some selected observations in this line of research have been incorporated in the following paragraphs. An elicitor used with great success in biochemical analysis of alfalfa is derived by heat treatment of yeast cell walls; plants are unlikely to be exposed to the complex product under natural conditions (Dixon *et. al.*, 1995). Having used such elicitors to unravel the fascinating biochemistry underlying biosynthesis it will, therefore be necessary to extend experiments to live pathogens and whole plants to determine if the same control operate within infected tissue. A glucan was isolated from the cell wall extracts of *Fusarium oxysporum* f. sp. *lycopersici* (Anderson, 1980) and a polypeptide (monilicolin A), from mycelia of *Monilina fructicola* (Cruckshank and Perrin, 1968). Both compounds elicited phaseollin production. An elicitor of phaseollin was isolated from the mycelial walls and culture filtrates of *Colletotrichum lindomuthianum*, which was identified as a polysaccharide. The molecular weight varied between 1 million and 5 million Da, and consisted predominantly of 3-and 4- linked glycosyl residue (Anderson and Albersheim, 1975). An amount equivalent to 100 ng of glucose elicited a similar response in the bean tissue.

Daniels and Hadwiger, (1976) isolated *Fusarium solani* which differed in their pathogenicity also should differential pisatin-eliciting potential. It was confirmed when their culture filtrates were tested on pea There was a difference in the concentration of elicitor in the culture filtrates of isolates. The elicitor was fairly heat-stable and also stable in freezing, but eliciting activity was reduced significantly by

pronase digestion. This strongly suggests that some of the activities were due to proteinaceous components.

Sojae by Ebel *et. al.*, (1976) isolated an elicitor of glyceollin from the mycelial wall of *Phytophthora megasperma* var. This elicitor stimulated the activity of phenylalanine ammonialyase and also induced glyceollin production in soybean cell cultures. They concluded that the action of elicitors is not species or variety specific but is a part of the general defence response of plants. Shiraishi *et. al.*, (1978) detected both elicitor and suppressor of pisatin in the pycnospore germination fluid of *Mycosphaerella pinoides.*

In order to examine the control of biosynthesis pathway leading to phytoalexin production in detail, it has been necessary to use artificial model systems and in particular the use of cell culture which produce phytoalexins after treatment with various elicitors. Experiments with cell cultures have shown the induction of phenylpropanoid synthesis is typically the results of increased transcription of gene encoding the corresponding biosynthetic enzymes (Kneusel *et. al.*, 1989; Dixon and Paiva, 1995). The regulation system of phaseollin synthesis in cell suspension cultures of dwarf French bean *(Phaseolus vulgaris)* was studied by Dixon and Christopher (1979). Considerable amount of phaseollin accumulated when french bean was treated with an elicitor from the cell wall of *C. lindemuthianum.* But the elicitors isolated from the cell walls of *P. sojae* and *Botrytis cinerea* were less effective.

Bruegger and Keen, (1979) extracted elicitors from the cell walls of *Saccharomyces crevisiae* and were identified as structural glucans. These are able to stimulate glyceollin accumulation in soybean. Specific elicitors of glyceollin were also detected in the cellular envelops of incompatible races of *Pseudomonas syringe* pv. *glycinea.* However, elicitor activity could not be detected in lipopolysaccharide preparation of exopolysaccharide fraction, or the culture fluids of various races of *P. glycinea.* elicitors were solubilized with sodium dodecyl sulfate and then preparations from five bacterial races expecting one had similar specificity for elicitation of

glyceollin in cotyledons of two soybean *(Glycine max)* cultivars These observations suggests that elicitors are not always race specific. Glycoproteins were extracted from isolated cell walls of *Phytophthora sojae* with 0.1 N NaOH at 0°C and elicited glyceollin in soybean hypocotyls with the same specificity as the fungus races from which they were obtained (Keen and Legrand, 1980). Fraction of the crude extracts on DEAE Bio-Gel and Bio-Gel A-5 m columns showed that specific elicitor activity was associated with the presence of high molecular weight glycoproteins detected by SDS gel electrophoresis. The glycoproteins appeared to contain only glucose and mannose as neutral sugar. The elicitor activity of the glycoproteins was not diminished by boiling at 100°C or pronase treatment, but was destroyed by periodate, thus indicating that the carbohydrate portions are important for activity. The glycoproteins were the only concanavalin A reactive species detected in the crude cell wall extracts, and fluorescein labelled concanavalin A was hapten-specifically bound to living hyphae of the fungus.

Elicitor activity of fresh mycelial wall extract of *Myrothecium roridum* was reported by Purkayastha and Ghosh (1983). Spores were suspended in mycelial wall extract, drops placed on leaf surfaces of soybean and incubated for 48 hr. The results of bioassay test revealed that the spores suspended in mycelial wall extract were inhibitorier than the spores suspended in mycelial wall extract were inhibitorier than the spores suspended in sterile distilled water and incubated on leaf surfaces for a similar period. Mycelial wall extract induced greater production of glyceollin in soybean leaves. Yomoto *et. al.*, (1986) demonstrated that pisatin could be induced in pea leaves by elicitors from *Mycosphaerella pinoides, M. melonis* and *M lingulicola.* Accumulation of pisatin increased after removal of epidermis and application of elicitors from germination fluid of the fungus.

Tepper and Anderson (1986) reported a carbohydrate rich extracellular component from a race of *C. lindemuthianum* showed a high level of phytoalexin activity on a resistant cultivar "Dark Red" of kidney bean but not on the susceptible cultivar "Great Northern." Other extracellular components were also recognised as

elicitors by both cultivars. It is noteworthy that the two cultivars of *Pheseolus vulgaris* displayed a differential response to extracellular components. These observations support the hypothesis that both general and specific mechanisms exist in race cultivar interaction.

Gusine *et. al.*, (1990) reported that the metabolites and viable cells of *Pseudomonas corrugata* from liquid culture medium elicited biosynthesis of the phytoalexin medicarpin in ladino white clover *(Trifolium repens)* leaflets and callus. The biologically active elicitors components were soluble in 80% ethanol. They were partially purified by removing components greater then 3,500 Da by dialysis and fractionating by preparative reversed phase HPLC. None of the four fractions separated by HPLC elicited appreciable quantities of medicarpin in callus, but fraction 1 combined with fraction 4 elicited high concentrations of medicarpin. Any combination of fractions 2, 3 and 4 synergistically elicited medicarpin in callus. Elicitor activity was concentration - dependent. The active fractions were acidic in solution, but their elicitor activity was not dependent on low *p*H. Fraction 1 contained primarily uncharacterized reducing carbohydrate and phosphate. Fractions 2 and 3 were composed primarily of two related, unidentified fluorescent compounds and fraction 4 contained another unidentified fluorescent compound *Phytophthora*, the phytopathogenic fungi subspecies elicit hypersensitive-like necrosis on their nonhost tobacco *(Nicotina tabacum)*, with the exception of the tobacco pathogen *Phytophthora nicotianae*. In culture, these fungi except *P. nicotianae* secrete proteins, called elicitins, that cause these remote leaf necrosis and are responsible for the incompatible reaction. These proteins protect tobacco against invasion by the agent of the tobacco black shank, *P. nicotianae*, which is unable to produce such an elicitor. Cryptogein secreted by *P. cryptogea,* has been purified, sequenced and characterized by terce-Laforgue (1992) as an elicitin, a novel family of 10k da holoproteins. The secretion of cryptogein began later than its synthesis and stopped earlier, simultaneously with mycelium growth, when the nitrogen source in the culture medium was nearly exhausted. Electrophoretic patterns of total protein from

mycelium extracts and N-terminal sequence analysis showed that cryptogein accumulated in the mycelium in its natural form. Cryptogein was synthesized as a preprotein. Fifteen isolates of *Phytophthora parasitica*, nine from tobacco (causing black shank disease) and six from other host plants were compared by root inoculation with regard to their pathogenicity to young tobacco plants. A progressive invasion of the aerial parts over 1 week was observed only with the black shank isolates, while the non-tobacco isolates induced leaf necrosis within 2 days. Similar necrosis occured when the roots of tobacco plants were dipped in diluted culture filtrates from non-tobacco isolates, but not in those from tobacco isolates. The necrosis inducing filtrates were shown contain a c 10K Da protein band which was not present in the other filtrates. This protein (named parasiticein) was purified by ion exchange chromatography to homogeneity in SDS-PAGE and reverse phase HPLC. Parasiticein was serologically related to cryptogein, a member of the elicitin family of proteinaceous elicitors. Like the other elicitins, parasiticein induced necrosis in tobacco plants and protected them against black shank. It most closely resembled little leaf necrosis. Ricci *et. al.*, (1992) suggested that the absence of parasiticein production by the black shank isolates might be a factor involved in their specific pathogenicity to tobacco.

Coleman *et. al.*, (1992) showed that a glycoprotein elicitor of phytoalexin accumulation in leaves of *Phaseolus vulgaris* produced well before lysis in the medium of cultures of *Colletotrichum lindemuthianum* was purified to homogeneity. The glycoprotein was a monomer of M.W.28k Da with a pI of 4.25. The glycosyl side chains which accounted for 43% of the weight of the holoprotein, were composed principally of galactose, mannose and rhamnose exhibited a minimum degree of polymerization of eight and were apparently O-linked to abundant serine and/or threonine residues of the peptide backbone. In a *P. vulgaris* leaf infection bioassay the purified glycoprotein had activity easily detectable at nanomolar concentrations and inducing browning of the treated tissue and the accumulation of both phenylalanine ammonia-lyase and the isoflavanoid phytoalexins phaseollinisoflavin.

For these three linked defence responses, sub optimal concentrations of the glycoprotein induced respectively 4.2,7.6 and 9.7 fold more activity in the cultivar resistant to race delta (cv. Kievit) than in a cultivar susceptible to that race (cv. Pinto). Protein integrity was not required for elicitor activity and glycosyl side-chains isolated from the protein were shown to be active elicitor. The effects of an elicitor (CG-elicitor) from *Colletotrichum graminicola* was studied by Ransom *et. al.*, (1992). Roots of sorghum (*Sorghum bicolor*) accumulated 3-deoxyanthocyanidin phytoalexins in response to CG elicitor. Elicitation of the phytoalexins prior to treatment with the elicitor did not prevent infection and development of milo disease symptoms in susceptible seedlings inoculated with conidia of *Periconia circinata.* However, treatment of roots with the CG elicitor enhanced the synthesis of 16k Da proteins in both resistant and susceptible genotypes without expression of disease symptoms. Cruciferous has long been known to contain a family of secondary metabolites termed as glucosinolates, which are sulphur containing glucosides (Fenwick *et. al.*, 1983). Following cellular damage, glucosinolates undergo hydrolysis catalysed by the enzyme myrosinase to produce glucose, sulphate and a variety of low molecular weight products possessing diverse chemical and biological properties. Some of the degradation products, notably isothiocyanates and oxazolidine-2-thiones have been shown to possess antifungal activity as well as acting a stimuli for feeding and egg deposition in insects (Mithen *et. al.*, 1986; Chew, 1988; Mithen 1992; Mari *et. al.*, 1993).

According to Toyoda *et. al.*, (1992) the effects of the elicitor and the suppressor from a pea pathogen, *Mycospharella pinodes,* on polyphosphoinositide metabolism in pea plasma membranes were examined *in vitro* by Lipid phosphorylation in the isolated pea plasma membrane was drastically stimulated by the elicitor, but markedly inhibited by the suppressor. A similar inhibitory effect was observed by the treatment with orthovanadate or K-252a that blocked pisatin production induced by the elicitor. Neomycin, an aminoglycoside antibiotic that interacts with the

polyphosphoinositide metabolism, also affected the lipid phosphorylation *in vitro* and blocked the elicitor induced accumulation of pisatin *in vivo*. Rapid changes of poly-phosphoinositide metobolism in pea plasma membranes in one of indispensable process during the elicitation of defence responses. Cell walls of germ tubes from wheat stem rust (*Puccinia graminis* f. sp. *tritici*) contain a glycoprotein with a molecular mass of about 67 KD referred to as the Pgt elicitor. This glycoprotein induces a hypersensitive-like response in wheat leaves. In elicitor active intercellular washing fluid (IWF) from compatible wheat stem rust interactions, several elicitor-active glycoproteins were detected. One of these glycoproteins had an electrophoretic mobility identical to the Pgt elicitor. This IWF glycoprotein exhibited elicitor activity upon elution from SDS gels. It was recognized by anti Pgt elicitor antiserum suggesting partial structural identity between Pgt and IWF elicitors. As with Pgt elicitors, the elicitor activity of the IWF glycoprotein residues in the carbohydrate moiety because periodate, but not trypsin or pronase destroyed activity. These results suggest that the Pgt elicitor is released from hyphal cell walls into the wheat apoplast during stem rust infection Beissmann *et. al.*, (1992).

Phenylpropanoid derivatives induced elicitor incorporation into the cell wall and the secretion of soluble coumarin derivatives (phytoalexins) by parsley (*Petroselinum crispum* L.) suspension cultures can be potentiated by pretreatment of the cultures with 2, 6-di chloroisonicotinic acid or derivatives of salicylic acid. The cell walls and an extra cellular soluble polymer were isolated by Kauss *et. al.*, (1993) from control cells or cells treated with an elicitor from *Phytophthora megasperma f. sp. glycinea.* After alkaline hydrolysis, both fractions from elicited cells showed a greatly increased content of 4-coumaric, ferulic, and 4- hydorxybenzoic acid, as well as 4-hydroxybenzaldehyde and vanillin. Two minor peaks were identified as tyrosol and methoxy tyrosol. The pretreatment effect is most pronounced at a low elicitor concentration. Its specificity was elaborate for coumarin secretion. When the parsley suspension cultures were preincubated for 1 day, with 2, 6-dichloroisonicotinic, 4-or 5- chlorosalicylic, or 3, 5-dichlorosalicylic acid, the cells exhibited greatly

increased elicitor response. Pretreatment with isonicotinic, salicylic, acetylsalicylic, or 2, 6-dihydroxybenzoic acid was less efficient in enhancing the response, and some other isomers were inactive This increase in elicitor response was also observed for the above mentioned monomeric phenolics, which were liberated from cell walls upon alkaline hydrolysis and for "lignin-like" cell wall polymers determined by the thioglycolic acid method. It was shown for 5-chlorosalicylic acid that conditioning most likely improves the signal transduction leading to the activation of genes encoding phenylalanine ammonia lyase and 4- coumarate: coenzyme A ligase. The conditioning thus sensitizes the parsley suspension cells to respond lower elicitor concentration. If a similar mechanism were to apply to whole plants treated with 2, 6-dichloroisonicotinic acid, a known inducer of systemic acquired resistance, one can hypothesize that fungal pathogens might be recognized more readily and effectively.

Yoshikawa and Sugimoto (1993) identified the putative receptor like target sites for glucanase-released elicitor in soybean membranes. The binding was dependent on the pH of the incubation chamber, as well as on the duration and temperature of the incubation. The binding of the glucanase released elicitor to membranes was abolished by both heat and proteolytic enzymes. Therefore, the binding site was probably composed of proteinaceous molecules. The elicitor molecules that function *in vivo* for phytoalexin elicitation in soybean *(Glycine max)* infected with *Phytophthora megasperma f. sp. glycinea* have been identified as β-1, 6-and β-1, 3-linked glucans that are released from fungal cell walls by β-1, 3- endoglucanase contained in host tissue.

An interesting interaction phenomenon was noticed by Kumar *et. al.*, (1995) that resistance or virulence are modelled by multiple biochemical components of two living organisms. *Costus speciosus* a major sapogenin bearing medicinal plant was severely affected by *Drechslera rostrata* causing leaf blight disease. The HPLC analysis indicated the accumulation of glyceollin II and III as potent phytoalexins by *C. speciosus* in response of nonpathogenic *D. longirostrata.* Further the presence of a

polysaccharide elicitor, or mycelial wall component seems to be detrimental cause of phytoalexin accumulation. The same elicitor was also present in mycelial wall of pathogenic *D. rostrata* but in much lower concentration. Additionally it was associated with another polysaccharide component with different identity. The bioassay method of elicitor preparation was expressed in terms of antimicrobial activity mediated through glyceollins. It was determined to be 88.6% in incompatible which was considerably low (13.7%) in pathogenic reaction. During the pathogenesis of *D. rostrata* the susceptibility was not only exercised with low concentration of elicitor but also being mediated with the association of additional carbohydrate component of mycelial wall hence expressing the involvement of multiple biochemical components to regulate susceptibility.

The inductions of phytoalexin production by nonspecific elicitors (which include proteins, glycoproteins, various types of oligosaccharides, and unsaturated fatty acids) are more difficult to assign. (Hahn, 1996). A race specific elicitor has been isolated from *Uromyces vigna*. This elicitor can induce phytoalexin production in cowpea resistant to this race of the pathogen based on hypersensitive response (HR)-like symptoms induced by treatment of resistant cowpea leaves with the elicitor (D'Silva and Heath, 1997). The relative roles of glyceollin, lignin and the hypersensitive response (HR) in pathogen containment and restriction were investigated in soybean cultivars that were inoculated with *Phytophthora sojae*. Incompatible interactions in leaves and hypocotyls were characterized by HR, phenolic and lignin deposition and glyceollin accumulation. The uncoupling of glyceollin synthesis from the HR and phenolic and lignin deposition by ABA treatment showed that glyceollin is a major factor in restriction of the pathogen during these interactions (Mohr and Cahill, 2001). The presence of phenolic acids in cell walls- esterified p-coumaric acid and ferulic acids bound to cell wall polysaccharides are widespread in gramineae. Cell wall bound phenolics in resistance to rice blast disease was demonstrated by Kumar *et. al.*, (1997).

To understand the coordination functions of the different classes of defense related gene expression in plant defense resistance, the expression pattern of pathogenesis related protein (PR) genes and genes involved in antooxidation and the production of secondary metabolites were examined by Kong moonkyung *et. al.*, (1998). Northern blot analysis showed that PR genes such as β-1, 3 glucanase and chitinase were strongly induced in tobacco leaves upon salicylic acid treatment. Phenylalnine ammonia lyase (PAL), involved in phenylpropanoid biosynthesis was mildly induced during latter strategies of normal hypersensitive response (HR) or after salicylic acid treatment. However in acute HR they were strongly expressed during early stage. The expression of the antioxidative genes, anionic peroxidase and ascorbate peroxidase were inversely expressed following salicylic acid treatment. Differential expression of 3 groups of gene involved in plant defense responses were discussed in relation to different signal transduction pathway.

Zhang *et. al.*, (1998) demonstrated that a boicontrol agent for field compost maize, suppressive to several disease caused by soil born pathogens, induced systemic acquired resistance (SAR) in cucumber against anthracnose caused by *Colletrotrichum orbicular* and in Arabidopsis against bacterial speck caused by *P. maculocola.* A peat mix conducive to soil born disease did not induce SAR. The population size of *P. syringae* Pv. *maculicola* was significantly lower in leaves of Arabidopsis plants grown in the compost mix compared with those grown in the peat mix. Autoclaving destroyed the SAR-inducing effect of the compost mix, and inoculation of the autoclaved mix with nonautoclaved compost mix. Topical spray with salicylic acid (SA) induced the severity of bacterial spick on plants in the peat mix but did not further reduce the severity of symptoms on plants in the compost mix.

The mechanism of pathway of plant systemic acquired resistance are reviewed by Cai-Xin Zhay *et. al.*, (1999). After a plant is inoculated with a necrotizing pathogen or treated with some chemicals, the uninoculated or untreated parts of the plant demonstrate resistance to the infection of second pathogens. This is plant systemic acquired resistance (SAR). SAR is systemic, long lasting resistance to wide range of

pathogens. The inoculated or untreated parts immediately produce a systemic signal and induce the expression of SAR gene. Salicylic acid (SA) is one of the signal molecules inducing SAR. They react more rapidly and efficiently to a challenge infection wide range of pathogens. A series of SAR mutant were selected, several genes encoding components of SAR signal transduction pathway were cloned, and their function were analyzed.

Hill *et. al.*, (1999) dealt with identification of disease response genes expressed in *Gossypixm hirsutum* upon infection with the wilt pathogen caused *Verticillium dahliae* which cause disease known as verticillium wilt. To begin to understand the molecular mechanism of the disease response in cotton cultivars that display superior wilt tolerance. Two signaling pathways, one involving salicylic acid and another involving jasmonic acid participate in the expression of plant resistance to pathogens and insect herbivores. In this study Thailer *et. al.*, (1999) shown the stimulation of systemic acquired resistance in field grown tomato plants with the salicylate mimic, benzothiadiazole, attenuated the jasmonate induced expression of the anti-herbivore defense related enzyme polyphenol oxidase (catechol oxidase) and compromise host plant resistance to larvae of the beet annyworm, *Spodoptera exigua.* Conversely, treatment of plant with jasmonic acid at concentration that induce resistance to inset reduced pathogenesis related protein gene expression induced by benzothiadiazole and partially reversed the protective effects of benzothia-diazole against bacterial speck disease by *Pseudomonas syringae* Pv. *tamato.* It was concluded that the effective utilization of induced plant resistance to the multiple pest typically encountered in agricultural wilt require understanding potential signaling conflicts in the plant defense responses.

A multi-component coordinated defense response in rice plants against fungal attack was demonstrated by Bera *et. al.*, (1999). Some selective defense components such as momilactone A (a rice phytoalexin) β-1,3 glucanase and exo-chitinase (both pathogenesis related proteins) and phenylalnine ammonia lyase (PAL) were employed as biochemical parameter for evaluating the degree of resistance of rice

plants to *R. solani* the causal agent of sheath blight disease. A systemic fungicide which reduces disease significantly also concomitantly activated biosynthesis of momilacton A, induced PR-proteins and increased PAL activity. Treatment of rice leaf sheath with PR protein inhibitor increased disease but inhibited β-1,3 glucanase and exo-chitinase activity in treated plants. Similarly amino oxyacetic acid (PAL-inhibitor) enhanced disease intensity and inhibited PAL activity in plants treated with amino oxyacetic acid and inoculated with *R. solani*.

Experiment by Caroline *et. al.*, (2000) have shown that, plant developed an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of nonpathogenic bio-control bacteria. In *Arabidopsi thaliana*, this induced systemic resistance (SR) functions independently of the salicylic acid but requires an intact response to the plant hormones jasmonic acid (JA) and ethylene.

The growth coffee orange rust fungus (*Hemileia vastatrix* Berk and Br.) isolated and the sequence of response is induced in leaves if resistant *Coffee arabica* L. and *C. congesis* Froehner as well as on a susceptible *C. arobica* were investigated cytologically and biochemically by Silva *et. al.*, (2000). The first signs of incompatibility detected 2 days after inoculation, were cytologically expressed by hypersensitive host cell death (HR), host cell wall autofluorescence and haustoria encasement with callose and β-1,4 glucans. Biochemically two peak of phenylalnine ammonia lyase (PAL) activity were detected by 2 and 5 days after inoculation. The hypertrophy of the host cells in the infection area were also observed around 12 days after inoculation corresponding macroscopically to the infection site.

Soybean phenylpropanoid defense responses to the wall glucan elicitor(WGE) from *Phytophthora sojae* include the accumulation of phenolic polymers and glyceolline in cells immediately proximal to the point of treatment and accumulation of conjugate of the isoflavones, daidzein and genistein, in distal cells. It is demonstrated by Park *et. al.*, (2002) that the WGE is indeed highly effective in protecting cell distal to the point of treatment from infection by *P. sojae*.

Mycolaminaran, jasmonic acid (JA), methyle jasmonate and ethylene precursor, 1-amino-cyclopropane carboxylic acid (ACC) are also effective, while salicylic acid (SA) is not. Methyle jasmonate, WGE and mycolaminaran are most effective, resulting in early complete protection against the pathogen even in the universally susceptible line.

Biochemical study on peroxidase and polyphenol oxidase activity; reducing nonreducing and total sugar; total phenoland potash content before and after powdery mildew infection in seven mungbean genotype was carried out by Gawande *et. al.*, (2002) to know the role of different biochemicals a plant defense in host parasite interaction. Resistant genotype had higher activities of peroxidase and polyphenoloxidase, total phenol and potash content before and after infection and lower level of sugars than observed in susceptible genotype. Activity of enzymes total phenols and potash content were positively associated with resistance, whereas sugars had negative association with disease resistance.

In winter cereals, low temperature hardening plant age and genotype are known to influence the expression of resistance to snow mould diseases. A study was undertaken by Gaudet *et. al.*, (2003) to determine the effect of genotype,plant age and duration of cold hardening on the temporal expression of the PR-protein and other defense related protein. The results demonstrate that the temporal expression of cold induced, plant defense related transcripts in winter wheat is differently regulate among genotypes and during different plant development stages, and are the first to implicate lipid transfer protein in the expression of genotypic based snow mould resistance in wheat. Potential plant defense signaling pathway involved in snow mould resistance induced at low temperature during natural acclimation of winter wheat.

A study was carried out by Chakraborty *et. al.*, (2004) on the association of defense enzymes with resistance in tea plants triggered by *Exobasidium vexans* revealed significantly changes in the level of enzymes mainly β-1, 3 glucanase and chitinase exhibiting antimicrobial activity. A wide variety in the activities of the

enzymes involved in phenol metabolism including phenylalanine ammonia lyase, peroxidase and polyphenol oxidase were seen in compatible and incompatible interactions. The possibility of including resistance in susceptible varieties of tea was worked out following inoculation with salicylic acid and results established its potential in immunizing tea plants which was confirmed by immunoassays and localization of chitinase was established in tea leaf tissues after induction of resistance by employing polyclonal antibodies raised against chitinase and labeled with FITC.

Multicomponent coordinated responses of tea plants under biotic stress with special reference to a pest (*Helopeltis theivora*) and a fungal pathogen (*Exobasidium vexans* causing blister blight disease of tea) have been demonstrated by Chakraborty *et. al.*, (2004). Involvement of defense enzymes mainly chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in developing an immune response in tea plants against pests and *E. vexans* were evident in naturally growing healthy plants as inherent immunity. Induction of these enzymes was noticed when plants showed hypersensitive responses. Various elicitors, mycopesticide bio-formulations like Metabass and Salicylic acid, Hexaconazole were studied, that seem to work in activating defense networks. However, resistant tea varieties after challenge inoculation with *E. vexans* revealed four new protein bands of ca. 61, 42, 22 and 14 kDa when probed with PAb of chitinase. Immunocytochemical localization of defense enzyme in tea leaves following induction of resistance was visualized as apple green fluorescence when FITC was coupled with PAb of chitinase. Activities of POD and PPO were significantly high in compatible interaction whereas CHT, βGLU and PAL were found to be high always in resistant reactions. Time course accumulation of CHT, βGLU and POD in tea plants triggered by *E. vexans* showed a general increase in POD activity after 48 h post-inoculation whereas activities of CHT and βGLU increased within 24 h of inoculation. Systemic accumulation of these enzymes was also evident. Salicylic acid (SA), a known to SAR inducer, elicited defense responses by accumulation CHT and βGLU as early as 24 h of post-treatment

whereas increased in activity of POD was noticed after 48 h. the accumulation of defense enzymes in tea plants in response to SA treatment suggests its role in the cellular protection mechanism which was also confirmed (Chakraborty *et.al.*, 2005).

(B) Efficacy of plant extract in plant defense response

The literature on pest control is dominated by report on chemical control. Broad spectrum pesticides offered powerful incentives in the form of excellent pest control, increased yield and reliable economic returns, but they have significant limitations. However, there are welcome efforts to adopt non-chemical strategies and evolve integrated pest management system.

Integrated plant disease management was proposed in the mid seventies and this programme should be considered as a holistic approach keeping in view the agroecological system and the overall situation of agricultural production. It includes the rotational application of cultural, biological and chemical control methods, as well as the coordination and integration of various procedures for the purpose of controlling the damage due to disease.

In the recent years there has been an increased interest in the use of eco-friendly technologies for plant disease control. Botanical pesticides are considered as potential alternatives to chemical agents, which can be hazardous to human and animal health (Mukhopadhaya, 1996). Many plant-extracts have been found to possess antifungal properties. Botanicals, however, are yet to be exploited as anti-infective or anti-infestive agents on a chemical scale. The present investigation reports on evaluation of plant extracts against fungal contamination. Fungal pathogens cause severe damage to the forest nursery stocks at seedling stages resulting in considerable loss in plantation activity. Hence, an experiment was carried out to manage the disease with bio-agents, natural products, and emission for the sustainable management of foliar diseases. The presence of antifungal compounds in higher plants has long been recognized as an important factor to disease control.

Such compounds being biodegradable and selective in their toxicity are considered valuable for controlling some plant diseases (Singh and Dwivedi, 1987). In this context, a variety of plant product having antibacterial and antifungal properties, in different parts of the plants have been reported.

Plant extracts of a *Dibymocarpus oblonga* and *Piper nigrum* were tested on sclerotia forming pathogens such as *Sclerotium rolfsii* and *Rhizoctonia solani* and observed fungitonic properties (Choudhury and Sen, 1981). The fungicidal activity of the benzene extract of *P. nigrum* was inhibitorier on mycelial growth than on sclerotia germination. The essential oils of *Caesulia oxillaris* (compositae) and *Hyptis sauveolens* exhibited strong fungitoxicity against the test organism *Helminthosporium oryzae*. The oil showed a broad fungitoxic spectrum besides superiority over 8 synthetic fungicides and prevented the appearance of leaf spot disease of paddy inhibited by *H. oryzae*.

Antifungal substance with methanol, acetone and ethylether from the epidermis and periderm region of *Morus alba* were extracted and tested against *Biolaris leersiae* and other phytopathogenic fungi by (Shirata and Takashashi, 1982). Each acetone extract from root and shoot showed 3-7 active substance, the amount and number varying the different cultivars. They concluded that this antifungal substance in epidermis of root may be prohibitions and one of the resistance factors to pathogenic soil fungi. A preformed antifungal compound (1-acetoxy 2-hydroxy–4–oxo–heneicosa – 12, 15diene) was isolated from peels of unripe avocado fruits which inhibited the vegetative growth of *Colletotrichum gloesporioides* and totally inhibited the spore germination (Prusky *et. al.*, 1982). Coxon *et. al.*, (1982) took two compounds (2, 5–dihydroxy-4-methoxy–9 and 10-dihydrophenanthrene) were also isolated by from the peel of yums (*Dioscorea rotundata*) which inhibited *Cladosporium cladosporiodes* and variety of yam soft rot pathogens.

Pan *et. al.*, (1983) tested antifungal activity of some naturally occurring coumarin compound isolated from *Limonia acidissima* against *Drechslera oryzae, Fusarium solani, Alternaria solani, Sclerotium rolfsii,* and *S. hydrophyllum*. They reported that

the compounds possessed low to high inhibitory activity towards spore germination and *D. oryzae* was highly sensitive to all 4 coumarins. Seed extracts of *Iberis amara* showed fungicidal activity against *Helminthosporium oryzae* at the minimum inhibitory concentration (2%) and exhibited a broad range of activity and non–phytotoxicity. The fungitoxic principle of the seed was thermostable up to 120^0C. The oil of *Ageratun honstoniarium* was found to possess broad mycotoxic spectrum exhibitory strongest toxicity against *Fusarium lateritium* f. sp. *cajani* (Tripathi *et. al.*, 1981).

Effect of some plant extracts and oils on inoculum density of *Crysipha polygoni* on different nodal leaves of *Pisum sativum* was reported by Singh and Pathak (1984). Among the various extracts (garlic bulb, garlic oil, neem leaf and ginger extracts) tested, best results were observed with ginger extracts and oil which reduced the disease (powderymildew of pea) intensity and increased the seed yielding capacity. Leaf extracts of *Anagallis arvensis, Caesalpinia pulcherima, Psidium guajava* etc. were totally fungitoxic to *Ustilago maydis* and *U. nuda* essential oils from *Ocimum basillicum* and their components showed different inhibitory effect against *Fusarium oxysporum* f.sp. *vasinfectum* and *Rhizopus nigricans* which was depended on the percentage of main components: lineol, linaleol, methyl chavical and eugenol. Effect of 49 indigenous plants on 11 phytopathogenic fungi belonging to the genes *Phytophthora ceratocystis, Phoma* etc. were studied by *Chesne et. al.*, (1984).

Tripathi *et. al.*, (1985) isolated oil from the leaves of *Ocimum qratissium* which showed fungitoxicity against *Alternaria alternata, Colletotrichum capsici* and *Sclerotium rolfsii.* They also reported that eugenol was the major fungitoxic principle that was isolated from the oil *Ocimum* sp. five oils of coconut groundnut, pure vegetable and liquid paraflin were tested against spore germination of *Rhizopus oryzae, Curularia lenata, Phoma sorghina* and *Fusarium equiseti* by Adisa (1985). Post-harvest tomato fruit could not be controlled in Nigeria by dipping in 75% palmkernel oil and storing at 15^0C. Pan *et. al.*, (1985) reported that some naturally occurring flavonoids isolated from *Pangaria globra* showed antifungal activity

against some test fungi. Of these Pongenethyl ether was found to be most promising. Leaves and petal extracts of *Camellia japonica* and *C. grandhamiana* showed antifungal properties and inhibited the growth of hyphae of many fungi when mixed potato – sucrose liquid medium. Almost no condium of *Pyricularia oryzae* or *Cochlioblus miyabeamos* germinated, germtubes or conidia swelled resembling balloons and no normal hypae of *Pestatotie longiseta, Gloeosporium theae-sienensis* and *Botrytis cinerea* grew. Two triterpenoid saponins as the antifungal compounds from aqueous extracts of *Camellia* leaf was isolated which was designated as "Camellidin –I" and "Camellidin –II". Camellidin showed antifungal activity against *Pestalotia longiseta* and *Cochliobolus miyabeanus* (Nagata *et. al.*,1985).

The leaf extracts of *Eucalyptus oceidentalis* and *E. brockwayi* exhibited strong fungitoxicity against the mycelial growth of *Pestalotiopsis mangiferae* in Potato Dextrose Agar (PDA) medium (EL-Sayed *et. al.*, 1985). Levey *et. al.*, (1986) isolated a medicagenic acid from alfalfa roots, which exhibited the mycelial growth of *Aspergillus niger*, *Sclerotium roulfsii* and *Fusarium oxysporum* f,sp..*Lycopercisi*. The leaf extract of *Polyalthia longifolia* in neutral phosphate buffer were also inhibitory against mycelial growth of *Rhizoctonia solani Sclerotium rolfsii* and *Sclerotium oryzae* than in distilled water. The inhibitory principle of this leaf extract was also thermostable (Naidu and John, 1986).

The antifungal activity of the leaf extract of *Lawsonia inermis* on *Drechslera oryzae* was tested at 1:40 dilution (Ec_{50} concentration) by measuring the growth, protein, DNA, RNA synthesis and oxygen uptake. The oxygen uptake was inhibited more than the other metabolic process like protein, DNA and RNA synthesis. The antifungal factor contained in leaf identified as 2-hydroxy-1, 4-naphthoquinone (Lawsone). Under *in vivo* condition, foliar spray of the leaf extract effectively controlled disease then the seed treatment (Natrajan and Lalitha Kumari, 1987).

Late leaf spot (*Phaeoisariopsis personata*) and rust (*Puccinia archidis*) of groundnut were partly controlled by using plant extracts of *Tridex procumbens*, *Pongamia glabra, Lawsonia alaba* along with carbendazim plusmaneozed and

N.C.P.75. Simultaneously, the yield also increased (Ghewande, 1987) Vapours of aqueous extracts of rhizome of *Alpinia carinata* showed strong activity against *Rhizoctonia solani.* They also isolated the essential oil from the rhizome of *A. carinata* which possessed fungicidal activity and broad fungitoxic spectrum. It did not show any phytotoxicity on seed germination and seedling growth of the host plant *Phaseolus aurens* (Kishore *et. al.,* 1987).

Helle and Briner (1988) isolated phenylpropanoid glucoside isolated from *Plantago major* which inhibited bacterial growth. Minimal inhibition concentration value has been evaluated for 7 plant pathogenic bacteria and for *E. coli* (ML.30) and *Staphyllococcus aurens* (502A) after preliminary investigation following immunodiffusion test. Gourinath and Monoharachary (1988) also tested the effect of latex collected from different host plants e.g. *Calotropis Ipomoea, Carica* etc. on conidial germination and mycelial dry weight of 4 pathogenic fungi viz .*Curvularia Iunata, Fusarium solani, Cylindrocarpan lichencola* etc. Among 100 species in 54 families of plant tested, leaf extracts from *Allium cepa, Allium sativum, Malus sieboldii Reysontria japonica* and *Rheum coreanum* were inhibitory on mycelial growth of *Phytophthora* sp. However, *A. sativum* and *Malus sieboldi* were strongly inhibitory (Paik *et. al.,* 1989).

Antibacterial screening of the crude extracts of leaves and stem barks of *Cassia alata* and 2 pure compound isolated from leaves was done against 15 pathogenic bacteria by Choudhury *et. al.*, (1989). All the crude extracts and pure compounds were found to be active against both gram positive and negative bacteria, but the menthol extracts were relatively active against wide range of bacteria methanol extracts of the stem barks and kaempfrol showed MIC value against *Shigella dysenteriae* , and *Staphyllococcus aurens* .

Taking 46 plant species by (Peshney and Moghe, 1990) evaluated crude leaf extract of quite a few of them indicated presence of inhibitor of tobacco mosaic virus. The inhibitor was also present in different parts of *Polianthus tuberosa, Capsicum annum* and *Abrus precatorius* which showed 90-100% infectivity inhibition *in vitro.*

Inhibitors from these plants were systemic in nature when tested on *Chenopodium quinoa* as locaol lesion and *Capsicum annuum* cv. 'Jwala' as systemic host for the virus. Chillies were symptomless for period of 30 days with single pre-inoculation treatment with crude leaf extract of these plant species Kumar and Tripathi (1991) screened leaf juices of 18 different species, out of these only *Eupatorium cannabinum* exhibited complete toxicity against *Pythium debaryanum, Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii* shade drying of the leaves had no adverse effect, while oven drying produced an adversed effect on the fungitoxicity of the leaves of *E. cannabinum.* The crude leaf juice of *E. cannabinum* successfully inhibited (*F. oxysporum* infection of *Pisum sativum*) seeding.

Chauhan and Singh (1991) reported *in vitro* effect of garlic, onion bulb, ginger, tulsi leaves extract on germination of zoospores of *Phytophthora drechsleri* f.sq. *cajani* has been observed. Extracts inhibited spore germination at 5000 and 10000 ppm. For garlic and onion and controlled *Phytophthora* blight of pegion pea in the field. Dubey and Dwivedi (1991) reported that distilled water extract (1:1w/v) of *Acacia arabica, Allium cepa and A. sativum* inhibited the growth of *Macrophomina phaseolina* in PDA culture. Mangamma and Sreeramulu (1991) examined several plant species to explore the possible production of antibacterial compounds by plants. Of these garlic bulb extract (*Allium sativum* L.) was toxic to *Lycopersicon esculentum* and *Xanthomonas compestris*, the leaf spot pathogen of tomato. Three concentrations of extracts were prepared 10g/100ml, 20g/100ml and 30g/ml. Among these 30g/100ml concentration had larger inhibitation zone when the aqueous extracts were assayed against *X. campestris* by paper disc method on potato dextrose agar medium. Garlic extracts were also reported to be inhibitory to fungal pathogen of rice namely *Pyricularia oryzae, Drechslara oryzae* and *Corticium sasatiu.*

Effects of antiblue and biocide at various concentration and times of immersion on the growth of fungi were studied by Masuka (1991). Antiblue (active ingredient, sodium pentachloride) was the better chemical providing complete control at the lowest concentration of 1.5%. Biocide a chlorinated derivative and local substituted

for antiblue provided 55% control at 4 and 8% concentration and 74% control, at the high concentration (12%). The time of immersion did not have a significant effect on the incidence of stain and mould.

Four phenolic compounds viz. apigenin, apigenin-7-0-glucoside, echinacin, *echinaticin*, were isolated by Singh *et. al.*, (1991) from the whole plant of *Echinops echinatus*. The latter 2 compound were isolated for the first time. All these compound were assayed against germination of conidia of *Alternaria tenuissina* which incite leaf blight of *Cajanas cajan*. All showed high efficacy against the pathogen at concentration ranging from 25-150μg ml^{-1}. This compound has been suggested to use as a control measure against *Alternaria* blight of Pigeon pea. Leaf extract of five plant species *Capsicum annum, Acacia arbica, Datura metel, Azadirachta indica* and *Spinacia oleraceae* were tested *in vitro* on to *Chenopodium amaranticolor* at 3 (three) concentration) (1:10, 1:100 and 1:1000). Among them *D. metel* at 1:1000 dilution of sap produced maximum inhibition (Sawant, 1992). Aqueous, ethyl acetate and methanol extracts of *Funaria hygrometrica,* a bryophyte, were tested against a few phytopathogen and human pathogens (*Xanthomonas oryzae, X. campestris, Klebsiella, Protens, Salmonella, Fusarium oxysporum* f. sp. *lycopersisi* and *Rhizoctonia solani*). Organic (Ethanol, methanol) extract showed antimicrobial activity whereas aqueous extract did not show any inhibitory activity (Gnanagura, 1992).

Sarvamangala (1993) reported antifungal activity of leaf extracts of *Azadirachta indica, Calotropis gigantica, Catharanthus, Eucalyptus* sp., *Parthenium lysterophorus, Pongamia pinnata* was tested against *Cerotelium fici* and *Cercospora moricola* causing leaf rust and leaf spot disease in mulberry, respectively. *A. indica* was more effective in inhibiting spore germination of *C. fici* by 91.2% where as extracts of *Eucalyptus* and *C. gigantica* proved highly toxic to inhibit the conidial germination of *C. moricola.* Under field condition they showed promising result.

Gohil and Vala (1995) investigated different phytoextracts on sugar cane wilt pathogen (*Fusarium moniliforme*).The extract of the following plants were evaluated. Almond (*Allamanda cathartica* L.), Ardusi (*Adhatoda vasaca* Ness), Piludi (*Salvadora persica*), Popti (*Physalis minima* L.), Ratanjogia (*Jatropha curcas* L.), Sargava (*Moringa oleifera Lam*), Saru (*Casuarina equisetifolia* L.), Soap-nut (*Sapindus trifoliata* L.), Sweet neem (*Myrraya Koenigii*), Tulsi (*Ocimum sanctum* L.) and Trumeric (*Curcuma longa* L.). The extracts were prepared in cold distilled water (1:1). Among 33 phytoextract studied the extracts of garlic, soap-nut were found to be inhibitory against *F. moniliforme*. Spores of *F. oxysporum* f.sp. *ciceri* (race-1) was significantly inhibited by the root exudates of the wilt-resistant chick pea cultivars (CPSI and WR 315) in comparison to untreated spores and spires treated with root exudates from susceptible cultivars (Stevensen *et. al.*, (1995). The effect was concentration dependent, such that exudates from 1gm of root in 2ml. of water almost completely inhibited spore germination, whereas exudates form 1gm of root in 20ml. water did not do so. Ethyl acetate fraction of root exudates of CPSI and WR315 strongly inhibited germination and hyphae growth.

Different plant extracts against 3 major diseases of Mulberry proposed by Biswas and Das (1995). They reported that 10% alcoholic water extracts of fresh plant parts from 20 different species were studied on the development of powdery mildew (*Phyllactinia corylea* (Pers.) Karst.). Leaf spot (*Pseudocercospora mori*) and leaf rust (*Cerotelium fici*) diseases in mulberry during 1992-1993 and 1993-1994. The pooled data of these two years revealed that the extracts of *Azadirachta indica, Launea coromandelica* and *Oxalis cormiculata* which significantly minimized 2 disease, namely, powdery mildew and leaf rust, while those of *Calosia argentia* and *Eupatorium odoratum* reduced leaf rust diseases. Extracts from several other species exhibited ability to reduce either leaf rust or powdery mildew. Ansari (1995) tested antifungal activity of Ajwain (*Trachispermum ammi*), Lemon grass (*Cymbopogon citrates*), Tulsi (*Oscimum*), *Mentha, Rauwolfia, Lawsonia inermis, Vitex trifolia*

against *Rhizoctonia solani,* causal organism of sheath blight of rice. Extract from seed of ajwain and leaves of tulsi showed fungicidal activity and others were fungistatic. The spray of ajwain and tulsi leaf extracts to plants at 1:20 dilution reduced the disease by 72-25% and 69.58 respectively.

Kiregard, (1996) pointed out that superior growth of wheat following *Brassica* crops compared to that following non-Brassica crops may be due to the suppression of soil borne fungal pathogen by volatile isothiocyanates (ITCS) released in the soil during hydrolysis of glucosinolates contained in *Brassica* tissue. Investigation was made on the effects of volatile compounds released from the root, shoot and seed meal tissue of canola (*Brassica napus*) and Indian mustard (*Brassica juncea*) on the mycelial growth of soilborne pathogen of cereal *Gaeumannomyces graminis* var. *tritici, Rhizoctonia solani, Fusarium graminearum, Pythium irregulae* and *Bipolaris sorokiniana.* Three isolates of each species, originally collected from the roots of wheat (*Triticum aestivum*) and barley grass (*Hordeum leposinum*) in South Australia. The root and shoot tissue of both *Brassica* species were more suppressive of flowering than maturity and matured tissue were generally more suppressive than canola. The degree of fungal suppression by various *Brassica* tissues was related to the concentration and type of isothiocyanulate released, which varied with *Brassica* species, tissue age and tissue type. There were significant differences in the sensitivity of the fungal species and among isolates of each species, *Rhizoctonia* and *Fusarium* were generally the most sensitive to the volatiles released whereas *Pythium* and *Bipolaris* was found to be the less sensitive.

The efficacy of light anti-viral substance was tested against summer squash mosaic disease by Sandhu *et. al.,* (1996). All the antiviral products used were found inhibitory against the mosaic disease. The extract of Sorghum was much superior to the other treatments in controlling the mosaic disease followed by Thuja at both

chelldonium used. The least disease control was in plants sprayed with *Bougainvellea* extract. All the treatments increased the yield of the plants as compared to control.

The effect of aqueous leaf extracts of *Azadirachta indica, Pinus roxburghii* and *Targetes erecta* and water soluble fraction of mustard oil cake on lignin content of barley leaves in relation with incidence and development of leaf stripe disease were reported by Varshney and Sharma (1996). Concentrated (100gm of plant material/ 250ml. distilled water) and diluted (1:1000 and 1:500) extracts/fraction were used. A conventional systemic fungicide carbendazim (trade name Bavistin) used in control of leaf stripe disease of barley was also tested. As compared to control, lignin content was higher in *A. indica* and *T. erecta* treated leaves. On the contrary, treatment wilt *P. roxburghii* and mustard oil cake fraction reduced lignin content of barley leaves. A direct correlation between lignin content and incidence of leaf stripe disease was also found.

Foliar application of leaf extracts of *Azadirachta indica* and *Dryopteris filix-mas* on tea plants affecting resistance to *Pestalotiopsis theae* has been elucidated by Deb and Chakraborty (1998). There is also evidence that leaf extracts of *Lantana camara* could induce resistance in tea plants against brown blight pathogen *Glomerella cingulata* causing brown blight disease. (Chakraborty and Chakraborty, 1998).

Singh and Majumdar (2001) took an attempt to develop botanical fungicides for suppression of *Alternaria* fruit rot of pomegranate, water, ethanol and acetone extracts of leaves of neem, datura and tulsi and rhizome and bulb extracts of ginger, turmeric, garlic and onion were tested *in vitro* by poison food method at 5, 10, 15 and 20% concentrations. The five extracts found effective *in vitro* were evaluated on pomegranate fruits as pre and post inoculation treatments. All the plant extracts at 20% concentration resulted in significant disease reduction, but maximum reduction was observed with garlic extract followed by turmeric.

Paul and Sharma (2002) investigated the effects of aqueous leaf extracts of neem in inducing resistance against leaf stripe pathogen of barley (*Drechslera gsaminia*). They also recorded phylloplane micoflora of treated and untreated leaf to observe the changes, if any brought about by the treatment of leaves by neem extract. There is also evidence Imran *et. al.*, (2002) that aqueous extracts of *Argemone mexicana* L. greatly suppressed the growth of root infecting fungi *Rhizoctonia solani* and *Fusarium solani,* and at low concentrations also promoted the growth of the tomato plants.

Soil application of Karanj (Pongamia glabra) and groundnut (*Arachis hypogaea*) takes and foliar spray of karanj and subabul (*Leucaena leucocephala*) leaf extracts were evaluated separately (10 and 5%) and in integration (5 and 2.5%) against web blight of urb and mung bean caused by *Thanatephorus cucumeris*. Soil application of karanj cake (2.5%) with spraying of karanj leaf extract (2.5%) followed by karanj cake (2.5%) application with. Spraying subabul leaf extract (2.5%) and only soil application of karanj cake (5%) showed the best performance as they increased seed germination and grain yield and mung bean and decreased seedling mortality and disease intensity. Karanj leaf extract showed superiority over subabul leaf extract in all respect. Groundnut cake at 10% dose inhibited the seed germination and caused maximum mortality where as at low dose (5 and 2.5%) its performance was satisfactory, but it was inferior to karanj cake. In general, lower dose of soil cake showed superiority over higher dose (Dubey, 2002).

Accumulation of total and O-dihydroxy phenols in three maize varieties (MalaN, Ganga-5 and VL-42) infected with *Helminthosporium maydis* and *H. turcicum* was recorded as compared to their healthy counterparts. Reactions of these varieties to both pathogens varied significantly in terns of accumulation of phenolics. Ganga-5 showed three fold increases in phenolics content due to infection by *H. maydis* while double amount of total phenol was recorded in VL-42. *H. turcicum* induced

maximum amount of phenolics in variety VL-42 followed by Ganga-5 and Malan. An increase in the activity of peroxidase, polyphenol oxidase, IAA-oxidase were noticed in all the three varieties of maize under infection of *H. maydis* and *H. turcicum*. The results have suggested that the accumulation of phenolics were higher in resistant varieties like 'Ganga-5' and 'VL-42' as compared to susceptible Malan. Corresponding increase in the activity of oxidative enzyme suggested active metabolic reaction of the host to the pathogenesis and their possible role in a increased level of phenolics. (Sukhwal and Purohit 2003)

The chemical composition of ethanol extracts from a Brazilian (Et-Br) and a Bulgarian (Et-Blg) propolis, were found to have microbicidal activity against a number of bactria and fungi such as *Candida albicans, Sporothrix schenckii* and *Paracoccidioides brasiliensis* (Salomao *et. al.*, (2004). Ether and chloroform extracts and oils of *Curcuma longa* have antifungal effects. Crude ethanol extract also possesses antifungal activity. Turmeric ail is also active against *Aspergillus flavus, A. parasiticus, Fusarium moniliforme* and *Penicillium digitatum* Salomao, *et. al.*, (2004).

Meena *et. al.*, (2004) reported that aqueous bulb extract 1% (w/v) of *Allium sativum* and leaf extract of *Acacia nilotica* caused significant reduction in mycelial growth of *Alternaria brassicae.* Application of bulb extract of *Allium sativum* in mustard plant resulted in highest seed yield at Sewar in 2001-2002. Application of bulb extract of *Allium sativum* at 45 and 75 d.a.s. resulted in lowest blight severity on leaves and pods and also resulted in highest seed yield.

Plant extract from *Aloe barbadensis* provided a total inhibition of *Phaecidiopycnis pili* (Fuckle) Weindlmayr. Extracts from *Tagetes minuta, Mentha piperita* and *Pelargonium graveolens* were significantly effective. Post-harvest treatment of pears in tea extracts had a significant effect in reducing stem end rot by *P. piri.* Plant extracts from *A. barbadensis* was superior to rest of the test botanicals both as pre and post inoculation treatment (Sharma *et. al.*, 2004). Pears treated either

at 6 h before or after inoculation provided better protection and further delay in dip treatment or inoculation resulted in marked increase in rotting. Test plant extracts proved better as protectant rather than eradicants.

Zope and Thrimurty (2004) reported that botanical pesticides Neemazal (0.3%), Wanis (0.3%), Achook (0.5%), Neem gold (0.3%) increase the percent seed germination of rice varieties, Mahamaya, IR 36 and Chapti. Significant increase in root and shoot lengths were induced (except Wanis 0.5%) by these botanicals. The seedling vigour index also increased in Neemzal (0.3%), Achook (0.5%), Wanis (0.3%), and Neem gold (0.3%) and in control fungicide carbendazim (0.1%) treatment.

Walnut (*Juglance regia* L.) hull extract was evaluated dry walnut kernels viz., *Aspergillus flavus*, *Penicillium citrinum*, *Cladosporium cladosporiodes*, *Alternaria alternata*, *Fusarium maniliforme* and *Curvularia lunata* adopting. Poisoned food technique using potato dextrose agar medium at 25 ± 2^0C. Mycelial growth assessed in terms of colony diameter after 21 days of incubation in darkness was totally incubated in *C. cladosporiodes*, *F. maniliforme*, and *C. lunata* by walnut exreact at 1500 ppm and in the other three species At 2000 ppm. Mean conidial germination of *A. alternata* assessed by adopting hanging drop method at ambient temperature after 12 h was at least at 15000 ppm (1%), and at 1000 ppm (8%) in walnut extract, when compared to check (70%). Effect of three concentration of walnut hull extract 1000, 1500 and 2000 ppm, boric acid 500 ppm and sulphuric acid 1000 ppm in preventing kernel rot under artificial inoculation with *A. flavus* as test fungus was evaluated as pre and post treatment applications at 25 ± 2^0C. A similar and significant effect of walnut extract (2000 ppm) and sulphuric acid (1000 ppm) was observed in preventing rot symptoms, when applied at pre inoculation stage with 4% kernels developing rot compared to 84% in check; the other best treatment was walnut extract applied at 1500 ppm. In case of post application treatment (2000 ppm), walnut extract and

sulphuric acid (1000 ppm) recorded just 7% rot compared to 77% incidence in check (Wattal and Puttoo, 2004).

Aqueous extract of mustard cake (5%), neem cake (1%), pine needles (5%), deodar needles (3%) and neem oil (3%) respectively, led to reduce *in vitro* germination of sclerotia of test pathogen *Sclerotium rolfsii* Sacc. causing seedling blight disease in apple nurseries as compared to control have been reported by Sonali and Gupta (2004). Combination of mustard cake (5%) with neem oil (3%), neem cake (1%) with deodar needles (3%) and neem oil (3%); and mustard cake (5%) with neem cake (1%), pine needles (5%) and neem oil (3%) resulted total inhibition of sclerotial germination.

According to Bhatnagar *et. al.*, (2004) cumin wilt, a serious disease induced by *F. oxysporum* f. sp. *cumini* causes heavy losses to the crop. A few compound of plant origin have been provided to be possible alternatives to pesticides use. Out of 17 species tested plant extract from Datura (1.3 cm) and Isabgol (1.5 cm) were effective in reducing the radial growth of *F. oxysporum* f. sp. *cumini*. Four commonly used fungicides, two bio-agent, two phyto-extracts and two physical seed treated agents were evaluated both *in vitro* and *in vivo* conditions for fungitoxicity against *F. oxysporum* f. sp. *cumini*, the incitant of cumin wilt (Ghasolia and Jain, 2004). Carbendazim (0.2%), thiram (0.25%), captan (0.25%), tebuconazole (0.2%), *Trichoderma viride*, *Euphorbia antiquorum* and hot water gave higher sed germination and vigour index and minimum pre – and post – emergence seedling mortality over check. Before maturity, all treatments showed reduce number of seedlings showing wilt symptoms in the field.

A number of plant species (*Azadirachta indica, Lantana camera, Dryopteris filix-mas*, *Echhornia* sp.) have been reported to possess some natural substances in their leaves which were toxic to foliar fungal pathogens (*Pestalotiopsis theae,*

Glomerella cingulata) of tea causing brown blight grey blight disease. Attempt have also been made to use aqueous extract of selected plants (*A. indica* and *Catharanthus roseus*) on tea plants for induction of resistance against *Alternaria alternata*, a new recorded foliar fungal pathogen causing leaf blight disease of tea as well as *E. vexans* causing blister blight of tea with special reference to involvement of defense enzymes such as β-1,3 glucanase, chitinase and phenylalanine ammonia lyase and antifungal phenols. These extracts enhance the level of defense enzymes, developed acquired resistance in tea plants and reduce blister blight disease incidence (Chakraborty *et. al.,* 2004). Tea varieties treated with aqueous leaf extracts of *A. indica* exhibited high level of all three defense enzymes along with rapid and distinct accumulation of antifungal phenolics in comparison with *C. roseus*. Reduction in disease incidence by application of these extracts was also evident. Plant extract from *A. indica* seem to act at various points in the defense activating networks and mimic all or part of the biological activities of resistance. The result support the hypothesis that neem extract may act indirectly by inducing plant defense reactions and it may be useful in integrated management of foliar disease of tea (Chakraborty *et. al.*, 2005).

MATERIALS AND METHODS

3.1 Plant material

3.1.1 Selection

Thirty-seven tea varieties (Tocklai, UPASI and Darjeeling) released by three experimental stations viz. (i) Tocklai experimental station, Jorhat, Assam; (ii) UPASI Tea Research Centre, Valparai, Tamilnadu and (iii) Darjeeling Tea Research Centre, Kurseong, Darjeeling are being maintained in Tea Germplasm Bank, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal sponsored by the department of Biotechnology, Ministry of Science and Technology, Govt. of India. Based on the growing suitability of tea plants (*Camellia sinensis* (L) Kuntz) as observed under the field conditions over the year, the following twenty-three varieties were selected for the present study.

	Tea varieties	Origin
TOCKLAI		
	TV-9	II
	TV-18	III
	TV-20	III
	TV-22	III
	TV-23	III
	TV-25	III
	TV-26	III
	TV-27	III
	TV-28	III
	TV-29	I
	TV-30	III
UPASI		
	UP-2	I
	UP-3	I
	UP-8	I
	UP-9	II
	UP-26	III
	[a]BSS-1	I
	[b]BSS-2	II
	[c]BSS-3	III
DARJEELING		
	HV-39	V
	T-135	V
	BS/7A/76	V
	T-17/1/54	IV

I – Assam, II – China, III – Combod, IV – Assam x China, V – Darjeeling, BSS -Biclonal Seed Stock; [a]UPASI-10 X TRI-2025, [b]UPASI-2 X Tri-2025, [c]UPASI-9 X TRI-2025

3.1.2 Growth and maintenance

The selected tea varieties were propagated by cutting as recommended by Banerjee (1993) Sandy soil (sand 75% and soil 25%) with *p*H ranging from 4.5 - 4.8 was used for propagation of tea plants by cutting. Soil *p*H was adjusted by treating with 2% aluminium sulphate. Excess aluminium sulphate was removed by watering. Polythene sleeves (8"X6") were filled up with prepared soil and stacked in rows in a bed and watered thoroughly. All the cuttings were allowed for rooting in sleeves after dipping them in hormone. These cuttings were kept in a polythene cloche, arranged in two rows, with 10 beds in each row. The complete set up was kept under a green Agronet House. Each bed was watered regularly, initially by spraying but later on by pipe. After about ninety days i.e when all the cuttings had rooted and new shoots had come, the polythene cloche was removed gradually. The Young tea plants were maintained using nutrients (2% NPK :- 2:1:2 and 2% urea) and Tricontanol as foliar spray at an interval of 3 months. The tea sleeves were then transferred to pots and maintained in the glass house (Plate-3). Besides plants were also transferred to the Phytopathological Experimental Garden of the Botany Department, North Bengal University. Two months prior to inoculation the main stem and side shoots were trimmed in order to obtain new shoots. The plants were grown under natural condition of day light and temperature and watered on alternate days with ordinary tap water by sprinklers.

3.2 Fungal cultures

3.2.1 Source

Alternaria alternata (W8053) isolated from naturally infected leaves of tea variety T-17 and another isolate of *A. alternata* (W8055) from TV-22 were identified by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience UK. These were used after completion of Koch's postulate.

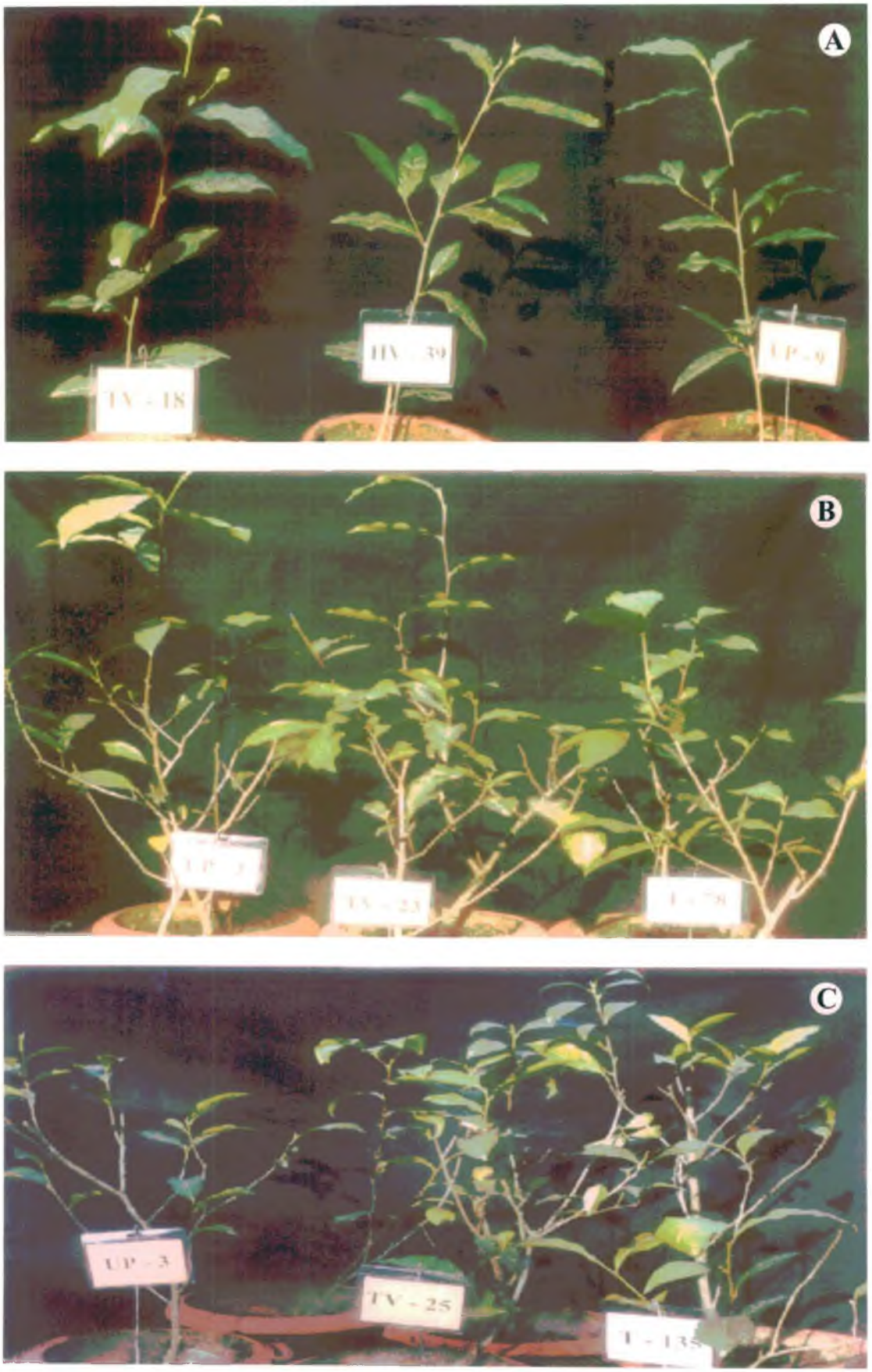


Plate 3 (figs. A-C): Tea varieties maintained in Glass House. (A)-TV-18, HV-39, UP-9; (B)-UP-3, TV-23, T-78; (C)-UP-3, TV-25, T-135

Black rot causing fungal pathogen (*Corticium invisum*) of tea was obtained from Tocklai Experimental Station, Jorhat, Assam. Another foliar fungal pathogen of tea - *Curvularia pallescens* (W7657), the entemogenous fungi - *Baeuveria bassiana* (MTCC-984) and *Metarhizium anisopliae* (MTCC-892) were obtained from stock culture maintained in Immuno Phytopathology Laboratory, Department of Botany, N.B.U.

3.2.2 Completion of Koch's postulate

Fresh, young tea leaves were collected from Phytopathological Experimental garden and inoculated with the conidial suspension of *A. alternata* and *C. invisum* ($5X10^5$ conidia/ml) separately following detached leaf inoculation technique Dickens and Cook (1989). After 72 h of inoculation, infected leaves were, washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ for 2-3 min, washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants, and incubated at 28 $\pm 1^0$C. After 7 days of incubation the isolated fungal culture was examined, compared with the respective stock cultures and identification was confirmed.

3.2.3 Maintenance of Stock culture

A. alternata, C. invisum and *C. pallescence* were grown in potato-dextrose-agar (PDA) medium where as *B. bassiana* and *M. anisopliae* were grown in yeast-glucose-agar and PDA, incubated at 28 $\pm$ 2^{0}C for two weeks and finally the cultures were stored at 5^{0}C and 28^{0}C. Re-isolation of the pathogens (*A. alternata* and *C. invisum*) were done every six months following detached leaf or cut shoot inoculation of tea leaves in order to maintain the virulence of cultures and were used for experimental purpose.

3.2.4 Assessment of mycelial growth

To assess mycelial growth of *A. alternata* and *C. invisum*, the fungus were grown in petriplates (9 cm.dia.), each containing 20ml. of PDA medium and incubated for 7 days at 28$\pm 1^0$C. From the mycelial mat agar block (4 mm.dia.) containing the mycelia

was cut with a sterilized cork borer and transferred to each Ehrlenmayer flask (250 ml.) containing 50 ml. of sterilized Potato Dextrose Broth for two weeks at 28±1^{0}C. Finally the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60^{0}C for 96 h, cooled in a desiccators and dry weight was noted.

3.3 Inoculation technique

3.3.1 Detached leaf

The method as described by Dickens and Cook (1989) was used for artificial inoculation of tea leaves. Fully expanded young leaves were detached from plants and placed in aluminium trays (37.5cm. x 30cm.) lined with moist blotting paper. Their upper surfaces were wounded as suggested by Cook (1989). The wounds consisted of light scratches on the upper epidermis made with the point of a fine sterilized scalpel. On the either side of the midrib, two of four such wounds were made in each leaf which was immediately inoculated with the inoculum block (2 mm dia) of *C. invisum* and conidial suspension (5X10^5 conidia/ml) of *A. alternata*. Fifty leaves were inoculated in each treatment. In control sets wounds were made on the leaves as described and droplets of sterile distilled water was placed. Each tray was covered with a glass lid and sealed with petroleum jelly to minimize the drying of drops during incubation.

3.3.2 Cut shoot

Cut shoot inoculation technique was followed by Yanase and Takeda (1987). Twigs with three to four leaves of tea plants grown in the experimental garden were cut carefully with sharp blade and immediately introduced into the glass chamber (3ft x 2ft x 1.5ft) with the twigs dipped in a floating thermocol. Leaves were inoculated by making two light scratches with point of a fine scalpel on the upper surface of leaves as described by Cooks (1989). Inoculum blocks (2 mm. dia.) of 10-day-old culture of *C. invisum* were taken from the advancing zone aseptically and placed on the inoculation site and then covered with absorbent cotton wool moistened with

sterile distilled water. Sterile PDA was used as control. For each treatment 50 cut shoots were inoculated.

3.3.3 Whole plant

Well established and branched tea plants (2 yr.old) grown in pots were inoculated with *A. alternata* following the method of Dickens and Cook (1989). Inoculation was done by spraying conidial suspension ($5X10^5$ conidia/ml) prepared from 15-days old culture of *A. alternata* grown on PDA. In control sets, the plants were sprayed with sterile distilled water. Inoculated as well as control plants were placed in polythene covered frames in order to maintain relative humidity near 80% for 48h. Subsequently the polythene was removed.

3.4 Assessment of disease caused by fungal pathogens

3.4.1 Detached leaf

Assessment of inoculation infectivity and symptom development were done on the basis of percent drops that resulted in lesion production after 24, 48 and 72h of inoculation as described by Chakraborty and Saha (1994).

3.4.2 Cut shoot

At the onset, the number of lesions that developed on the artificially inoculated tea twigs by mycelial blocks of *C. invisum* was counted. Diameter of the individual lesion was measured and they were graded into four groups and a value was assigned to each group for small restricted lesion diameter of 2-4mm (0.1), 4-6mm with sharply defined margin (0.25), slow sprayed beyond 6mm (0.50), and spreading lesions of variable size, with diffused margin (1.0). Finally number of lesion of each group was multiplied by the value assigned to it and the sum total of each values were noted and disease index was computed as the mean of observations on 50 cut shoots per treatment. Data was taken after 24, 48 and 72h after inoculation.

3.4.3 Whole plant

Disease intensity was assigned following whole plant inoculation technique at 5 days of intervals after inoculation up to 25 days as described by Chakraborty and

Saha (1994). On the basis of visual observation lesions were graded into four size groups, viz., very small, small, medium and large with respective values of 0.1, 0.25, 0.50, and 1.0 assigned to give an appropriate idea of their relative size. Number of lesions in each size group was multiplied by the values assigned. The sum total of such values for all the leaves gave the disease index for a plant. Results were always computed as the mean of observation of 23 plants (50 young leaves randomly picked up from each plant) per treatment.

3.5 Assessment of incidence of attack by *Helopeltis theivora*

Disease assessment was determined on 10-year-old tea bushes of 23 different tea varieties maintained in open field condition following the method of assessment as described by Somchowhdury *et. al.*, (2001) with modification. Incidence was recorded regularly every week throughout the year and monthly averages were computed for three consecutive years, from 2002 to 2004. Incidence was calculated as the percentage of infested shoots on each of the twenty-three tea trees.

3.6 Inducing agents and their application.

3.6.1 Plant extract

Mature leaves (400g) each of *Azadirachta indica*, *Catharanthus roseus*, and *Diplazium esculentum* were homogenized separately in a electric blender. After centrifugation leaf extracts were diluted (1:10) with DW. TWEEN-80 was mixed with all extracts (1:10) and sprayed on tea plants (10 year-old) with the help of sprayer. The control plants were sprayed with distilled water mixed with TWEEN-80. Spray was done four times at 15 days interval in the after noon. Both treated and untreated plants were inoculated with *A. alternata* following whole plant inoculation methods and disease assessment was made. Leaves from control and treated plants were sampled for biochemical analyses and immunological assays.

3.6.2 Biocrop

A liquid formulation with plant extracts namely 'Biocrop', an organic fertilizer and known plant growth inducer marketed by AKS Bio and Herbals Pvt. Ltd., Mumbai was used for foliar application in tea plants in order to assess their effect on the incidence of pest attack by *H. theivora*. The liquid product was diluted with distilled water (100ml/liter) mixed with TWEEN-80 before spraying in the evening. Four sprays were done at 15 days interval. The control plants were sprayed with distilled water mixed with TWEEN-80. Leaves from control and treated plants were sampled for biochemical analysis and immunological assays.

3.6.3 Metabass

The entomogenous fungi - *M. anisopliae* (MTCC-892) and *B. bassiana* (MTCC-984) were grown on liquid medium. The conidia were harvested by filtering through a double layered mesh. Conidial count was determined microscopically by means of Neubauer haemocytometer. These were serially diluted in distilled water and 0.2% Tween 80 was added to the solution. Metabass formulation (liquid) as outlined by Gurusubramanian *et. al.,* (1999) was prepared and foliar application on tea plants (10- years old) were made four times at 15 days interval in order to assess their effect on the incidence of pest attack by *H. theivora* in relation to the control plants which were sprayed with distilled water mixed with TWEEN-80.

3.6.4 Salicylic acid

Salicylic acid (SA)which plays a critical signaling role in the activation of defense responses after pathogen attack was also applied exogenously on the tea plants as foliar spray. A specific concentration (15mM) of SA supplemented with Tween-80 was used for spraying at intervals of 15 days. The control plants were sprayed with distilled water mixed with TWEEN-80. Indirect immunofluorescence and immunogold localization of defense enzyme was done with treated and untreated leaves.

3.7 Extraction of total soluble proteins

3.7.1 Leaf protein

Soluble proteins were extracted from healthy naturally infested and artificially inoculated tea leaves following the method of Chakraborty *et al.* (1995). Leaf tissues (1g) were homogenized with 0.05 M sodium phosphate buffer (*p*H 7.2) containing 10 mM- $Na_2S_2O_5$; 0.5 mM - $MgCl_2$; 2mM polyvinyl pyrrolidone (PVPP 10,000 M) and 2 mM poly methyl sulphonyl fluoride (PMSF) in mortar with pestle at 4^0C with sea sand and PVPP 40,000 M. The homogenate was centrifuged at 4^0C for 20 min. at 10,000 r.p.m and the supernatant was used as crude protein extracts and immediately stored at -20^0C for further use.

3.7.2 Mycelial protein

Extraction of mycelial protein of *A. alternata* and *C. invisum* was done following the method of Chakraborty and Purkayastha (1983). Fungus were grown in sterilized Potato Dextrose Broth (PDB) for 14 days at 28 $\pm 1^0$C. Mycelia were collected, washed with 0.2% NaCl solution, rewashed with sterile distilled water, strained through cheese cloth and then crushed with sea sand and 0.05 M sodium phosphate buffer (*p*H 7.2) using mortar and pestle at 4^0C. The slurry was centrifuged at 10,000 r.p.m for 20 min. at 4^0C. The supernatant was used as crude protein extract and stored at -20^0C for further use.

3.8 Estimation of protein content

Protein estimation was done following the method of Bradford (1976). The Bradford's reagent was prepared in the following way; 100 mg of Comassie Brilliant Blue G_{250} (Biorad) was dissolved in 50 ml. of 95% ethanol, followed by addition of 100 ml. of concentrated phosphoric acid and deionized water up to a volume of 200 ml. This stock solution was diluted 5 times with distilled water and filtered through Whatman No. 1 filter paper during estimation. A standard curve was prepared with Bovin Serum Albumin (Sigma). The standard curve showed linearity from 20-150 μg.

of protein sample in 100 µl. To the 100 µl. of test protein sample 5 ml. of Bradford's reagent was added, mixed in a cyclomixture and incubated at room temperature for 5 min. for blue color development, following which O.D was measured at 595 nm. By UV-spectrophotometer (SICO, model Digispec 200 GL). The O.D values were potted on the standard curve prepared for the purpose and the quantity of protein determined from this.

3.8.1 SDS-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

3.8.1.1 Preparation of stock solutions

For the preparation of gel following stock solutions were prepared:

- Acrylamide and N'N'- methelane bis-acrylamide :

A stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water. As both of them are slowly deaminated to acrylic and bis-acrylic acid by alkali and light the *p*H of the solution was kept below *p*H 7.0 and the stock solution was filtered through Whatman No.1 filter paper and was kept in dark bottle, stored at 4^0C and used within one month.

- Sodium Dodecyl Sulphate (SDS) :

A 10% stock solution of SDS was prepared in worm water and stored at room temprature.

- Tris buffer:

I 1.5 M tris buffer was prepared for resolving gel. The *p*H of the tris was adjusted to 8.8 with concentrated HCl and stored at 4^0C for use.

II 1.0 M tris buffer was prepared for use in the stacking and loading buffer. The *p*H of this tris adjusted to 6.8 with concentrated HCl and stored at 4^0C.

- Ammonium Persulphate (APS) :

Fresh 10% APS solution was prepared with distilled water each time before use.

- Tris – Glycine eletrophoresis buffer:

This running buffer consists of 25 mM tris base, 250 mM glycine (*p*H 8.3) and 0.1% SDS. A 1X solution can be made by dissolving 3.02 g tris base, 18.8 g glycine and 10 ml. SDS in 1L of distilled water.

- SDS gel loading buffer:

This buffer contains 50 mM tris HCl (*p*H 6.8), 10 mM β Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was made by dissolving 0.5 ml. of 1M tris buffer (*p*H 6.8), 0.5 ml. of 14.4 M β mercaptoethanol, 2 ml. of 10% SDS, 10 mg. bromophenol blue, 1 ml. glycerol in 6.8 ml. of distilled water.

3.8.1.2 Preparation of gel

Slab gel of two sizes were prepared for the analysis of protein pattern by SDS-PAGE i.e. big gel (plate size 17cm. X 19cm.) and mini gel (8cm. X 10cm.). For both types of slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm. thick spacers were placed between the glass plates at the three sides and the three sides of glass plates were sealed with high vacuum grease and clipped thoroughly to prevent and leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order by pasture pipette leaving sufficient space for comb the stacking gel (comb + 1cm.).

Composition of solutions for 10% resolving gel :

Name of compounds	Mini gel (ml.)	Big gel (ml.)
Distilled water	2.85	11.90
30% Acrylamide mix	2.55	10.00
1.5 M Tris (*p*H 8.8)	1.95	7.50
10% SDS	0.075	0.30
10% APS	0.075	0.30
TEMED	0.003	0.012

Composition of solutions for 5% stacking gel :

Name of compounds	Mini gel (ml.)	Big gel (ml.)
Distilled water	6.8	2.1
30% Acrylamide mix	1.7	0.5
1.5 M Tris (*p*H 6.8)	1.25	0.38
10% SDS	0.10	0.03
10% APS	0.10	0.03
TEMED	0.01	0.003

After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for 2 hrs. for polymerization. After polymerization of the resolving gel was complete over lay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30 min. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris – glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.8.1.3 Sample preparation

Sample (32µl.) was prepared by mixing the sample protein with 1 X SDS gel-loading buffer (16µl) in cyclomixture. All the samples were floated in boiling water bath for 3 min. to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the wells with a fin pepatte. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low molecular weight (Phosphorylase b - 97,400; Bovin Serum Albumin – 68,000; Ovalbumin – 43,000; Carbonic Anhydrase 29,000;

Soyabean Trypsin inhibitor – 20,000; Lysozyme – 14,300) was treated as the ather samples and loaded in a separate well.

3.8.1.4 Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 hrs. in case of mini gel and a constant 30 mA for a period of about 6 hrs. for large gel until the dye front reached bottom of the gel.

3.8.1.5 Fixing and staining

After eletrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250 mg of coomassie brilliant blue (Sigma R_{250}) in 45 ml. of methanol. After the stain was completely dissolved, 45 ml. of distilled water and 10 ml. of glacial acetic acid were added. The prepared stain filtered through Whatman No.1 filter paper.

The gel was removed from fixer and stained in this staining solution for at least 4 hrs. at 37^0C with constant shaking at very low speed. After staining the gel was finally de-stained with de-staining solution containing methanol, water and acetic acid (45:45:10) at 40^0C with constant shaking until the back ground become clear.

3.9 Extraction and assay of defense enzymes

Among the defence enzymes Phenylalanine ammonia lyase, Polyphenol-oxidase, Peroxidase (PR-9), Chitinase (PR-3) and β-1,3-glucanase (PR-2) were considered for both pest and pathogen attack.

3.9.1 Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5)

For the extraction of Phenylalanine ammonia lyase (PAL) the methods of Chakraborty *et. al.*, (1993) was followed. Leaves (1gm) were crushed in a mortar with pestle in 5ml of 0.1M sodium borate buffer *p*H 8.8 containing 2mM β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 rpm for 20 min. at 4^0C.

The supernatant was collected and after recording its volume was used immediately for assay or stored at –20^{0}C.

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture containing 0.3 ml 300μM sodium borate (*p*H 8.8); 0.3 ml. 30μM L-Phenyl alanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1hr. at 40^{0}C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as μg cinnamic acid g^{-1} tissue min^{-1}.

3.9.2 Tyrosine ammonia lyase (TAL)

For the extraction of Tyrosine ammonia lyase (TAL) leaves (1gm) were crushed in a mortar with pestle in 5ml of 0.1M sodium borate buffer *p*H 8.8 containing 2mM β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 rpm for 20 min. at 4^{0}C. The supernatant was collected and after recording its volume was used immediately for assay or stored at –20^{0}C.

TAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-tyrosine spectrophotometrically. The reaction mixture containing 0.3 ml 300μM sodium borate (*p*H 8.8); 0.3 ml. 30μM L-tyrosine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1hr. at 40^{0}C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as μg cinnamic acid g^{-1} tissue min^{-1}.

3.9.3 Polyphenol-oxidase (PPO) (EC 1.10.3.2)

For the extraction of polyphenoloxidase (PPO) the method of Mahadevan and Ulaganathan (1991) was followed with a little modification. Leaf tissue were cut into pieces and then crushed in mortar with pestle in ice with 5ml of 0.2M sodium-phosphate buffer *p*H 6.6. The slurry was immediately centrifuged at 10,000 rpm for 20min at 4^{0}C. The supernatant was collected and after recording its volume was used immediately for assay or stored at –20^{0}C.

For the determination of PPO activity 1ml of freshly prepared enzyme extract was mixed with 2ml of 0.2M sodium-phosphate buffer (*p*H 6) containing 0.01M pyrogallol in the dark. Initial absorbance was noted at 495nm immediately. The reaction mixture was incubated at room temperature in the dark for the prevention of photo-oxidation of the enzyme. Further reading was taken after 30min at 495nm. The blank was set with only 3ml of phosphate buffer. PPO activity was expressed as A_{495} g^{-1}tissue min^{-1}, when the substrate pyrogallol was oxidized due to the enzyme activity from 1gm of tissue.

3.9.4 Peroxidase (PO) (EC 1.11.1.7)

To extract Peroxidase (PO) the methods of Chakraborty *et. al.*, (1993) was followed with modification. Tea leaves samples were crushed with 0.1M sodium borate buffer *p*H 8.8 containing β-mercaptoethanol in a mortar with pestle on ice. The homogenate was centrifuged immediately at 15,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and its final volume was measured and used immediately for assay or stored at –20°C.

For the determination of PO activity 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2M sodium-phosphate buffer (*p*H 5.4), 100 µl of 4mM hydrogen peroxide (H_2O_2), 100µM of O-dianisidine (5mg/ml methanol) and 1.7ml of distilled water. PO activity was assayed spectrophotometrically at 460nm oxidation of O-dianisidine in presence of H_2O_2. Specific activity was expressed as the Δ A_{460} g^{-1} tissue min^{-1}.

3.9.5 Chitinase (CHT) (EC 3.2.1.14.)

To extract CHT 1gm of sample was crushed in 5ml of 0.1M sodium-citrate buffer *p*H 5 with sea sand and insoluble PVPP in a mortar with pestle on ice as suggested by Boller *et al.*, (1988). The homogenate was centrifuged immediately at 12,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording the final volume was used immediately for assay or stored at -20°C.

For the determination of CHT activity 1ml of enzyme extract and 1ml of substrate (chitin10mg/ml) and phosphate buffer were incubated and shakened at 37°C for 1 hr.

Then the solution was centrifuged at 12,000 rpm for 10 min. After centrifugation 0.5ml of the supernatant were added to 3ml of DNSA (Dinitro salicylic acid). The reaction mixtures were incubated in boiling water bath for 10min. CHT activity was assayed spectrophotometrically at 540nm.The blank was prepared by enzyme and substrate with zero hr of incubation. Absorbance value was measured at 585nm and N-acetyl-D-glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as mg GlcNAc g^{-1} tissue h^{-1}.

3.9.6 β-1, 3-glucanase (βGLU) (EC 3.2.1.39)

β-1, 3-glucanase activity was assayed by the laminarin-dinitrosalicylate method as suggested by Pan *et al.* (1991). Leaf samples were homogenized in 0.05M sodium acetate buffer, *p*H 5.0. The homogenate was centrifuged immediately at 12,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and its final volume was measured and used immediately for assay or stored at –20°C.

For the determination of βGLU activity the crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4%) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexes and the absorbance was determined at 500nm in a spectrophotometer and enzyme activity was expressed as µg glucose g^{-1} tissue min^{-1}.

3.10 Extraction of phenolics

Total phenol and orthodihydroxy phenol content of healthy artificially inoculated with *A. alternata, C. invisum* and naturally infested with *H. theivora* tea leaves of resistant and susceptible varieties were extracted following the method of Mahadevan Ulaganathan (1991). In case of artificial inoculation detached leaf inoculation technique as described earlier as followed. In case of control, sterile distilled water was mounted on the adaxial surface of leaves. Phenols were extracted from 1 gm each of healthy and infected leaves separately in boiling 80% ethanols (4 ml. ethanols/g fresh weight leaf tissue) for 10 min. cooled, and crushed thoroughly passed through

two layers of cheese cloth and then filtered through filter paper. Final volume was adjusted with 80% ethanol (5ml/g fresh weight of leaves).

3.11 Estimation of phenol content

3.11.1 Total phenol

The total phenol was estimated by Folin ciocalteau's reagent as described by Mahadevan and Ulaganathan (1991). One ml. Of the alcohol extract was taken in a test tube, 1 ml. of Folin ciocalteau's reagent following by 2 ml. of 20% Na_2CO_3 solution was added. The tube was shaken and heated on a boiling water bath for 1 min. and volume was raised to 25 ml. Absorbance was measured in a Systronics photoelectric colorimeter Model-101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.11.2 Orthodihydroxy phenol

The orthodihydroxy phenol was estimated as described by Mahadevan and Ulaganathan (1991). One ml. of the alcohol extract was taken in a test tube 2 ml. of 0.05 N HCl, 1 ml. of Arnow's reagent ($NaNO_3$ -10g ; Na_2MoO_4 –10g ; distilled water 100 ml.) and 2 ml. 0f 1 N NaOH were added, following which the volume was raised to 25 ml. Absorbance was recorded by Systronics photoelectric colorimeter Model-101 at 515 nm. Quantity of orthodihydroxy phenol was estimated using caffeic acid as standard.

3.12 Extraction of antifungal phenolics

The extraction method used was adopted from the Daayf *et. al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Tea leaves were collected from the experimental garden and detached leaf inoculation with spore suspension of *A. alternata* and sclerotial suspension of *C. invisum* was followed for artificial inoculation as well as natural infested leaves by *H. theivora.* Both the healthy and inoculated leaves were harvested separately after 48 h of inoculation. Infested leaves were collected for experiment. Leaf samples (10 g) were

mixed with 80% methanol at 10ml./g tissue and homogenized by blending for about 1 min. Samples were extracted for 48 hrs. on a rotary shaker in a conical flask at 40 r.p.m. covered with aluminium foil for protection from light. Methanolic extracts were then collected by filtration on a Whatmann No. 1 filter disc and concentrated by evaporation to a final volume of 20 ml. (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethylether three times. The ether fraction were stored and termed as Fraction I. The aqueous fraction was portioned secondly against equal volume of ethyl acetate also for three times and the ethyl acetate portioned was considered as Fraction II.

Acid hydrolysis, with 4N HCl, of the remaining aqueous fraction (yielding phenolic aglycones) was performed according to the method of Daaf *et al.*, (1997). Aglycones were recovered by partitioning hydrolysates against equal volume of ethyl acetate also for three times and labeled as fraction III. All the fractions were evaporated to the dryness and finally dissolved in 3 ml. of respective solvent.

3.12.1 Chromatographic analysis

Ethyl acetate fractions of both healthy and infected tea leaves were analysed by thin layer chromatography (TLC) on silica gel G. The development of the chromatograms was carried out at room temperature and using a chloroform:methanol solvent system (9:1 v/v) as suggested by Chakraborty and Saha (1994). Following evaporation of the solvent, thin layer plates were observed under UV light and sprayed separately either with diazotized p-nitroaniline (Van Sumere *et al.* 1965), vanillin – H_2SO_4 (Stahl, 1967) or Folin-ciocalteau's phenol reagent (Harborne, 1973). Color reactions and Rf values were noted.

3.12.2 Bioassays of antifungal phenols

3.12.2.1 TLC plate bioassay

All the fractions derived from the extraction procedure were analysed by TLC on silica G. All the three fractions were spotted on TLC plates and the development of the chromatogram was carried out at the room temperature using a ethyl acetate : chloroform (9:11) solvent mixture as suggested by Chakraborty and Saha (1994).

After development chromatogram inhibition assay was performed as devised by Hoffmans and Fuchs (1970) using *Curvularia pallescence* as the test organism. Spore suspension in 2% sucrose supplemented with Richard's Medium were sprayed on the developed TLC plates and incubated in a sterile humid chamber at 25^0C for 6 days. Fungitoxicity was ascertained by the appearance of inhibition zone, which was visualized as white spots surrounded by a deep black background of mycelia. Diameter of inhibition zones and Rf values were noted.

3.12.2.2 Radial growth

Radial growth inhibition assay as described by Van Etten (1973) was followed. Ethyl acetate fraction of healthy, infected (with fungal pathogens) and infested (with *H. theivora*) extracts (0.2 ml.) were taken separately in each of the sterile Petriplate (0.2 ml.) was initially taken and allowed to evaporate. In control sets, only ethyl acetate (0.2 ml.) was initially taken and allowed to evaporate. Subsequently 10 ml. sterilized PDA was poured in each petriplates, thoroughly mixed and allowed to solidify. Agar block (3 mm.dia.) was cut with a sterilized cork borer from the advancing zone of 7 days old culture of *C. invisum* and *A. alternata* grown in PDA and transferred to each Petriplate. Radial growth of *C. invisum* and *A. alternata* were recorded after 3 days of inoculation at 28 ± 2^oC.

3.12.2.3 Spore germination

The regions of thin layer chromatograms corresponding to the inhibitory zones were scrapped and eluted again. The eluants were tested for antifungal activities following spore germination test with *A. alternata* (at least $5x10^5$ spores/ml.) described by Werder and Kern (1985).

3.12.2.4 UV- spectrophotometric analysis

For spectral analysis of antifungal compounds extracted from healthy, *A. alternata* inoculated as well as *H. theivora* infested leaves, initially ethyl-acetate fraction were plotted on TLC plates and developed in chloroform-methanol (9:1 v/v) solvent, silica gel from corresponding antifungal zones as detected in chromatogram inhibition assay as well as in spore germination test were scrapped off and eluted

separately in methanol. These were re-spotted on TLC plates and developed in the same solvent and again scrapped and eluted in spec. methanol. The purified eluents were examined by UV-spectrophotometer (Simadzu model 160) and the maximum absorption was determined.

3.12.3 Extraction of catechins from tea leaves

Catechin was extracted from tea leaf tissue artificially inoculated with conidial suspension of *A. alternata* and natural infested leaves by *H. theivora* following the method of Obanda and Owuor (1994) with modification. Leaf samples (10g) were extracted with 100 ml. of 80% acetone at 45^0C in water bath for 30 min. Extracts were decanted and filtered through Whatman No. 1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 20 ml. distilled water. Water solution was extracted with equal volume of chloroform for four times. The *p*H of the water layer was adjusted to 2 by 2 drops of 2 N HCl and finally dissolved in 3 ml. of 2% acetic acid. The samples were finally filtered through milipore filter (Milipore 0.4 μm HA filter paper).

3.12.3.1 HPLC analysis of catechins

Catechin analysis of the extract was carried out on C 18 hypersil column using linear gradient elution system as follows: mobile phase A 100% acetonitrile; mobile phase B 2% acetic acid in water; elution; 88% B for 6min and then linear gradient to 75% B over 5min; the elution was complete after a total of 25min. Flow rate 1 μl/ min, sensitivity 0.5 aufs, injection volume 40ml monitored at 278nm (Shimadzu, Japan). Antifungal phenols extracted from healthy and infected tea leaves Fraction III were also quantified at 280nm using HPLC grade methanol.

3.13 Preparation of antigen

3.13.1 Mycelial antigen

Mycelial antigens were prepared from the mycelia of *C. invisum* and *A. alternata* grown in liquid medium following the method of Chakraborty and Saha (1994). The mycelial content was stained through muslin cloth dried and its dry weight taken. The

mycelia (6.9g and 6.2g respectively) were homogenized in mortar and pestle with 0.05M sodium phosphate buffer supplemented with 10mM Sodium meta bisulphate, 2mM soluble PVPP 10,000 and 0.05mM $MgCl_2$ (*p*H 7.2). At the time of crushing insoluble PVPP and sea sand was used. The crushing was done in cold. The slurry was centrifuged at 10,000 rpm for 30min. at 4^0C. The supernatants were used as mycelial antigens and stored at -20^0C.

3.13.2 Leaf antigen

Antigens were prepared from healthy tea leaf tissue variety TV-18 following the Alba and DeVay (1985) with modification. Healthy leaves (1g) were crushed in a mortar and pestle with 0.05M sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5mM $MgCl_2$, 2mM soluble polyvinyl pyrrolidone (PVPP 10,000M) and 2mM poly methyl sulphonyl fluoride (PMSF) at 4^0C for 20 min. at 10,000 rpm. The supernatants were used as leaf antigens and stored at -20^0C.

3.14 Production of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against the fungal mycelia of *Alternaria alternata*, and *Corticium invisum* and also against tea leaf antigens. Besides, polyclomanal antibody against one of the defense enzyme chitinase (product no.) was prepared from *Serratia marcescens* (Sigma) (PAb-CHT). The concentration of chitinase in each injection was 50μg/ml.

Initially, body weights were recorded and were observed for at least one week inside the cages before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetables etc., in morning and evening. After each bleeding they were given saline water for 3 consecutive days, cages were cleaned everyday in the morning for better hygenic conditions.

3.14.1 Immunization

Before immunization, normal serum was collected from the rabbit. The rabbit was subcutaneously injected once a week at 7 days interval with 1ml antigen mixed with

1 ml of Freund's complete adjuvant (Difco, USA) for first two injections and the next emulsified with incomplete adjuvant for 4 to 5 weeks.

3.14.2 Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board fixed at an angle of 60°. The neck of the rabbit was held tight in triangular gap at the edge of the board, and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 to 10ml of blood samples were collected in sterile graduated glass tube.

After collection, all the precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were incubated at 37°C for 1hr for clotting. After clotting, the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000g for 10 mins at room temperature) and distributed in 1ml vials and stored at – 20°C, as crude antisera.

3.15 Purification of IgG

3.15.1 Precipitation

IgG was purified as described by Clausen (1988). The polyclonal crude antiserum of leaf, mycelium and enzymes (chitinase & laminarionase) (2ml each) was first diluted with two volumes of distilled water and an equal volume of 4M ammonium sulphate. The *p*H was adjusted to 6.8 and the mixture was stirred for 16hrs at 22°C. The precipitate thus formed was collected by centrifugation at 10,000g at 22°C for 1hr. Then the precipitate was dissolved in 2ml of 0.02 M sodium phosphate buffer, *p*H 8.0.

3.15.2 Column preparation

Eight grams of DEAE-cellulose (Sigma Co. USA) was suspended in distilled water and kept overnight. The water was poured off and the gel was suspended in 0.005 M sodium phosphate buffer *p*H 8.0. Washing with buffer was repeated 5 times. The gel was then suspended in 0.02 M sodium phosphate buffer, *p*H 8.0 and was applied to a 2.6 cm diameter and 30 cm high column and allowed to settle for 2 h. After the column material had settled, 25 ml of buffer (0.02 M Sodium Phosphate buffer, *p*H 8.0) washing was given to the gel material.

3.15.3 Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant *p*H and with a continuous change in molarity from 0.02 M to 0.3 M. The initial elution buffer was 0.02 M sodium phosphate buffer *p*H 8.0 (I) and the final elution buffer was 0.3 M sodium phosphate buffer *p*H 8.0 (II).

The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of the buffer (I) was connected to another flask with buffer (II) to the column, buffer (II) was sucked into buffer (I) thereby producing a continuous rinse in mobility. Ultimately 40 X 5 ml. fractions were collected and optical density values were recorded by means of UV spectrophotometer at 280 nm. The fractions showing >2 reading were stored as purified IgG.

3.16 Immunodiffusion tests

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). Initially glass slides (5cm x 5cm) were degreased in 90% (v/v) ethanol, ethanol: diethyl ether (1:1) and then dried in a hot air oven. After drying, plates were sterilized in an autoclave at 15 lb for 20min. Agarose gel was prepared in Tris-barbiturate buffer, *p*H 8.6, at 90^{0}C; 0.9% agarose (Sigma, USA) was added into the buffer and placed on a water bath and stirred till the agarose solution became

clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added. For gel preparation, 10ml of molten agarose was added per slide; after pouring, it was kept for solidification and then wells were cut (8mm. diameter). The antigens and undiluted antisera (100µl/ well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 72 h at 25°C. After immunodiffusion, the slides were initially washed with sterile distilled water for 2 hrs. and then aqueous sodium chloride solution (0.9% NaCl + 0.1% NaN_2) for 72 hrs. with 6 hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. Then slides were stained with Coomassie blue (R_{250}) for 10 min at room temperature. After staining, slides were de-stained with 5% acetic acid solution until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3 hrs. at 50°C.

3.17 Enzyme linked immunosorbent assay (ELISA)

The following buffers were prepared following the method as described by Chakraborty *et al,* (1995), with modifications.

- Antigen coating buffer : Carbonate Bicarbonate buffer 0.05 M *p*H-9.6.

  I. Sodium Carbonate - 5.2 g in 1000ml distilled water

  II. Sodium bicarbonate - 4.2 g in 1000ml distilled water.

160 ml of stock **I** was mixed with 360 ml of stock **II.** and *p*H was adjusted 9.6.

- Phosphate Buffer Saline : 0.15 M PBS *p*H-7.2.

  I. Sodium dihydrogen phosphate - 23.40 g in 1000ml distilled water

  II. Di-Sodium hydrogen phosphate - 21.29 g in 1000ml distilled water

  280 ml of stock **I** was mixed with 720 ml of stock **II.** and the *p*H was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

- 0.15 M Phosphate buffer Saline - Tween (0.15 M PBS - Tween, *p*H 7.2).

  To 0.15 M PBS, 0.05% Tween 20 was added and the *p*H was adjusted to 7.2.

- Blocking reagent (Tris buffer saline, *p*H 8.0)

  0.05 M Tris, 0.135 M NaCl, 0.0027 M KCl

  Tris - 0.657g, NaCl - 0.81g, KCl - 0.223g

Distilled water was added to make up the volume to 100ml. Then *p*H was adjusted to 8.0 and 0.05% Tween 20 and 1% BSA were added.

- Antisera dilution buffer (0.15 M PBS – Tween, *p*H 7.2).

In 0.15 M PBS – Tween, *p*H 7.2, 0.2% BSA, 0.02% Polyvinylpyrrolidone 10,000 and 0.03% sodium azide was added.

- Substrate

p-Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in 100ml of 1% diethanolamine, *p*H 9.8.

- Stop solution - 3 N NaOH

This ELISA was performed following the method as described by Chakraborty *et. al.*,(1995) with modifications. Plant and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl / well) in 8 welled ELISA plates (Costar EIA/RIA strip plate, USA), arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25°C for 4 h. Then the plate was washed 4 times under running tap water and twice with PBS-Tween and each time shaken to dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200µl / well) to each well and incubated at 4°C overnight. After a further washing, anti-rabbit IgG goat antiserum labeled with Alkaline Phosphatase diluted 10,000 times in PBS, was added to each well (100µl / well) and incubated at 37°C for 2 h. The plate was washed, dried and each well was loaded with 100µl of p-Nitrophenyl phosphate substrate and kept in dark for 60 mins. Colour development was stopped by adding 50µl/ well of 3 N NaOH solution and the absorbance was determined in an ELISA Reader (Multiskan EX, Labsystems) at

405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.18 Fluorescence Antibody Staining and Microscopy

Indirect fluorescence staining of fungal sclerotia of *Alternaria alternata*, *Corticium invisum* and also cross sections of healthy and infected tea leaves were done using FITC labelled goat anti-rabbit IgG following the method of Chakraborty and Saha (1994), with modifications.

3.18.1 Fungal mycelia:

Fungal mycelia were harvested in PBS. The mycelial suspension was centrifuged at 2000 r.p.m. for 1 min. The supernatant was discarded and the pellet was re-dissolved in PBS (0.5 ml). Then the suspension was re-centrifuged as before followed by two more washings in PBS. Supernatant was discarded and the material was incubated in an antibody dilution (1:10) for 60 min. Washing in PBS by centrifugation was repeated as before at 2000 r.p.m. for 1 min. To the material 50µl FITC (Fluorescein Iso-Thiocyanate) - conjugate diluted in PBS (1:40) was added and incubated for 30 min. It was then washed thrice in PBS and centrifuged as before. The supernatant was discarded. The pellet was mounted in 10% glycerol. It was covered with cover glass, sealed and observed under a Leica Leitz Biomed microscope.

3.18.2 Tea leaves:

Fresh cross section of healthy tea leaves were cut and immersed immediately in PBST. The sections were washed thrice in PBS on grooved slides. After washing the sections were incubated in an antibody dilution (1:10) for 60 min. Washing in PBS was repeated as before. To the material 50µl FITC (Fluorescein Iso-thiocyanate) - conjugate diluted in PBS (1:40) was added and incubated for 30 min. After incubation sections were washed thrice in PBS and mounted in 10% glycerol. Each section was covered with a cover glass, sealed and observed under a Leica Leitz Biomed microscope.

3.19 Dot-immuno binding assay

Dot-blot was performed following the method suggested by Lange & Heide (1986). Following buffers were used for dot-blot:

- Carbonate-bicarbonate buffer (0.05 M, *p*H-9.6 coating buffer).
- Tris buffer saline (10 mM, *p*H-7.4) with 0.9% NaCl and 0.05% Tween - 20 for washing,
- Blocking buffer - 10% Casein hydrolysate in 0.05 M Tris, 0.5 NaCl, 0.5% Tween-20, *p*H-10.3.

Nitrocellulose membrane (BioRad, USA) was first cut carefully into the required size and placed inside the template. 2µl of coating buffer (Carbonate-bicarbonate buffer) was loaded in each well of the template over the nitrocellulose membrane (NCM) and kept for 25 min to dry. Following this 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 h at room temperature. The template was removed and blocking of the NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30 min. Polyclonal antibody of *A. alternata*(1: 40) was added directly in the blocking solution and further incubated at 4°C overnight. The membrane was then washed several times in TBS-Tween (*p*H -7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline Phosphatase conjugate (1:10,000) for 2 h at 37°C. This was followed by washing for 25 min in TBS-Tween. The substrate (66µl Nitro Blue Tetrazolium Chloride + 33µl 5-Bromo-4-Chloro-3-Indolyl phosphate di-sodium salt in 10ml of Tris buffer saline *p*H 7.4) was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water.

3.20 Western blotting

Blot transfer was done in three steps, following the method as described by White *et. al.*, (1994) with modifications. Soluble proteins were extracted from healthy, artificially inoculated with *A. alternata* and natural infested by *H. theivora* and resolved on SDS-PAGE. Following gel run, it was transferred to Towbin buffer(25

mM Tris, 192 mM glycine in 20% Reagent grade methanol, *p*H 8.3) and equilibrated for 1h. The transfer unit was attached to a power pack. The presoaked filter paper was placed on the platinum anode and air bubbles were rolled out with a glass rod. The pre-wetted NCM was over the filter paper followed by the gel and finally on top again another presoaked filter paper was placed. The cathode was placed on the sandwich and pressed. The unit was run for 45 min at 15V (constant). After the run the membrane was dried for 1 h and preceded for immunological probing. Initially membrane was placed in a heat sealable plastic bag containing blocking solution (5% non fat dried milk and 0.02% sodium azide in 0.15 M PBS *p*H 7.2 with 0.02% Tween-20) for 1 h with occasional shaking. Antibody was added (1:40) to the blocking solution and incubated in plastic bag at 4°C overnight. All the processes were done by gentle shaking. The nitrocellulose membrane was washed properly in 200 ml of washing buffer (150 mM NaCl, 50mM Tris HCl) *p*H 7.5 to remove azide and phosphate from the filter before enzyme coupled reactions. Enzyme was added (1:10,000 in alkaline phosphatase buffer) and kept for 1h at room temperature.

The membrane was washed in washing buffer as before and the substrate was added (66µl NBT + 33 µl BCIP + 10ml of Alkaline phosphatase buffer *p*H 9.5). The reaction was monitored carefully and when bands of the desired intensity were observed the filter was transferred to a tray of 200 µl of stop solution (0.5M EDTA, *p*H 8.0 in 50 ml of 0.15M PBS).

3.21 Scanning Electron Microscope (SEM)

The main purpose of SEM observation is to study the surface morphology. The basic requirement for the study in SEM is that electron should pass through the specimens. High vacuum in the microscope column is necessary for biological material. Dried material was mounted on metallic stubs with the help of conducting paints. The mounted medium was heat resistant. Gold was used for coating. Spores, healthy and infected leaf samples were first gold coated under high vacuum pressure for 30min and then observed under electron microscope.

First the leaf samples (healthy, *H. theivora* punctured portion as well as Alternaria blight infected portions) were cut into small pieces (2mm aprox.) and samples were then air dried completely due to their bulk size. Dried samples were mounted on metallic stubs with the help of conducting paints. As the secondary electrons are used in the image formation of SEM, the samples need to generate sufficient secondary electrons to give good quality image. For this reason, the samples were coated using a thin layer of a metal. Coating also protects the samples from the effect of electron beam. Gold was used for coating the samples. Then the samples were observed through Electron microscope.

3.22 Transmission Electron Microscope (TEM)

Healthy as well as salicylic acid treated tea leaves were fixed in 1% (v/v) glutaraldehyde in phosphate buffer (*p*H 6.8) for 60 min at 4^0C. Then the tissues were transferred to 3% phosphate buffer glutaraldehyde for overnight. After that the samples were washed thoroughly three times in PBS (0.1M phosphate buffer saline) Samples were then dehydrated in a 50% graded ethanol for 10min. first and then in 70% ethanol for 45 min. and embedded in LR White and polymerized at 60^0C for 2 days. Ultra-thin sections of the samples were cut with a diamond knife and collected on Pioliform coated 300 mesh nickel grid for immunogold labeling.

Rabbit polyclonal antisere raised against chitinase and laminarinase were used for innumogold labeling. Sections were incubated in blocking solutions containing 1% (w/v) of BSA in Tris Buffer Saline (TBS). Then incubation of the section in primary antibody dilution 1:200 in the blocking solution for 1h at room temperature. After washing the sections were incubated in with secondary antibody diluted at 1:40. Rinsing was done with PBS for 3-4 times. After contrasting with uranyl acetate and lade citrate, the sections were examined with a Leica EM.

EXPERIMENTAL

4.1 Assessment of damage potentiality on different tea varieties following *H. theivora* infestation

Tea a popular beverage made from the leaves of evergreen shrub or tree (*Camellia sinensis*). It is predominantly an agro-based export-oriented evergreen crop and a perennial crop grown as a monoculture on large contiguous areas. Under natural conditions, a tea plant grows to a small tree but it is configured into a bush by sequential pruning and other silvicultural practices, viz. tipping, plucking and by harvesting the optimum vegetative produce.

Helopeltis spp. are pests of various cultivated plants in tropics. The damaging effect of these insects on tea plants was documented over a century ago. On sunny and warm day, *Helopeltis* takes shelter in lower layer of bush canopy. It was in these early accounts that the common names 'tea bug' and 'tea mosquito' were established and its damage was reported as 'tea blight', 'mosquito blight' and 'spot blight'. Subsequently, the species responsible for causing damage on tea was identified as *Helopeltis theivora* Waterhouse. The insect injects toxic saliva through their needle like rostrum which causes necrosis of the tissues and turn brown first then black and they dry up subsequently. It was reported that a single first instar nymph of *H. theivora* could make as many as eighty feeding lesions in 24 hours. Infestation by *H. theivora* become destructive during the cropping season. Incidence of attack by *H. theivora* was determined on ten year old tea bushes of twenty-three different varieties maintained in the experimental plots (Plate 4 figs. A-C) of Department of Botany, University of North Bengal. Incidence of attack by *H. theivora* was assayed. The nymphs and adults of *H. theivora* (Plate 5 fig. E) are the active suckers of sap from buds, young leaves, tender stems and shoots. Feeding by *H. theivora* is seemed primarily on the leaves. A circle is formed around the feeding spot within 2-3 hour of damage, the inside portion of the ring becomes translucent light brown in colour (Plate 5 fig. B) within 24 hour in relation to healthy control (Plate 5 fig. A) and

Plate 4 (figs. A-C): Tea plants maintained in the Tea Germplasm Bank (A) and natural infestation by *H. theivora* in field condition (B & C).

within a few days the spot turns dark brown. The tissues around the affected spots first turn brown and then black and subsequently they dry up (Plate 5 figs. C & D). The dry leaf tissue in the long run dropped resulting in numerous perforations (shoot holes) on the leaf surface.

A single may have innumerable puncture marks. Severely damaged leaves also curl up apart from ceasing to grow. They feed at early morning, late evening and night hours. The typical feeding damage by *H. theivora* appears as a discoloured necrotic area around the point of entry of the labial style and the fourth instar nymph causes maximum damage.

Using Scanning Electron Microscopy (SEM) healthy as well as *H. theivora* punctured tea leaf surface was scanned. The infested portion of tea leaves become thinner and fragile. Healthy leaf surface exhibited continuity (Plate 6 figs.A&B) whereas infested leaf surfaces were not continuous (Plate 6 figs. C-F). Infested leaf surface showed a number of holes due to puncture made by *Helopeltis* (Plate 6 figs. C&E). The content of the cells were empty in the feeding lesions (Plate 6 fig. F).

Damage potentiality was calculated as the percentage of infested shoots on each of the 23 tea varieties determined weekly throughout the year for four consecutive years (2002-2004). Incidence was calculated as means of 3 years and was further grouped into four quarters of the year beginning in January to March (first quarter), April to June (second quarter), July to September (third quarter) and lastly October to December (fourth quarter). Results (Table 1) revealed that incidence was highest in the second and third quarters (50% to 80%) followed by the fourth quarter, and last in the first quarter. Among the different varieties, the UPASI series was most susceptible showing 70%-80% damage in second quarter, incidence being significantly greater than the two other groups, followed by Tocklai which showed 50%-70% damage and lastly Darjeeling varieties. Analysis of variance of data presented in Table 1 was computed and presented below.

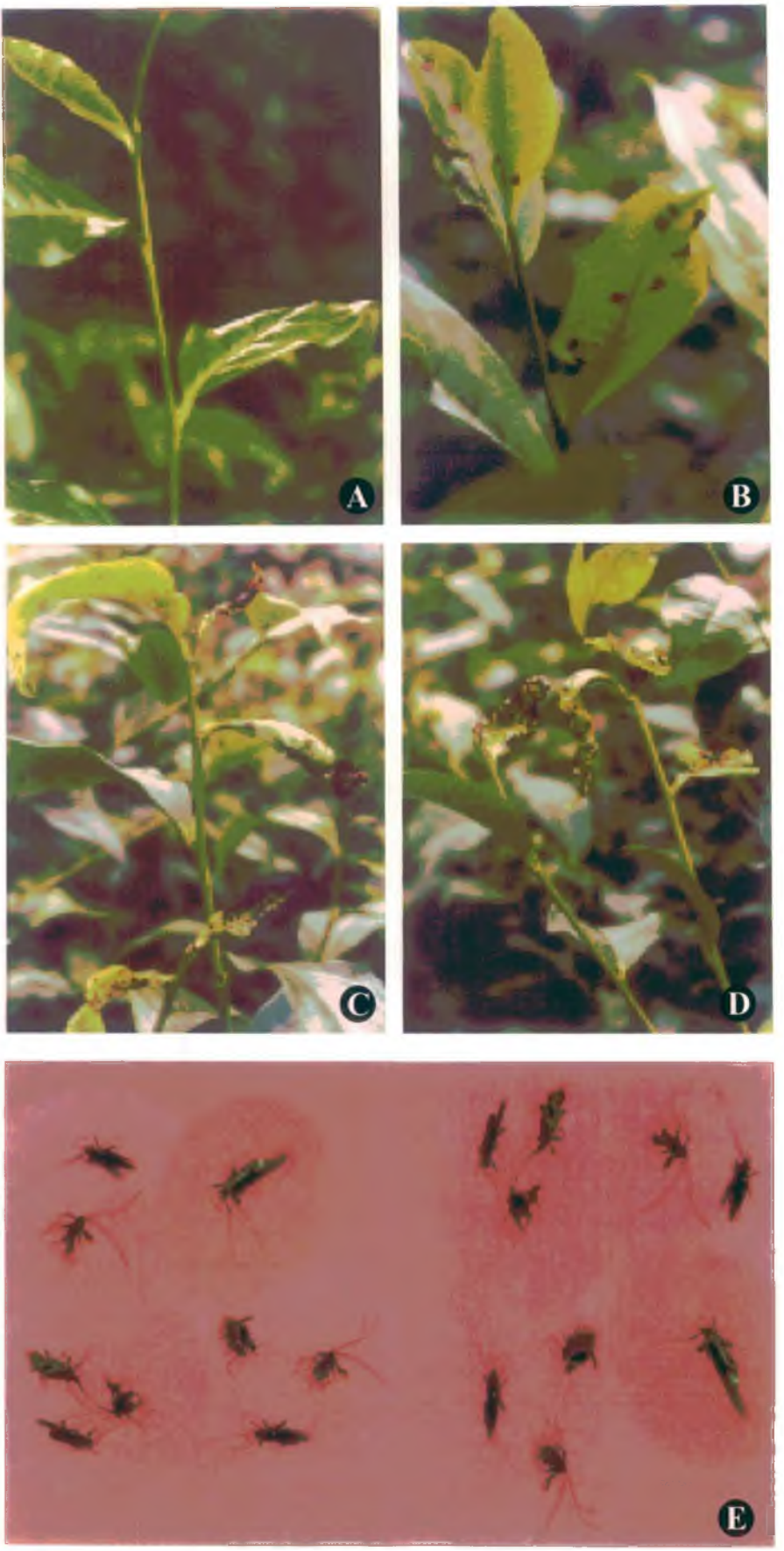


Plate 5 (figs. A-E): Healthy (A) & *H. theivora* infested tea plants (B-D) [TV-22(A&B) : UP-3 (C) and HV-39 (D)] Tea Mosquito Bug *H. theivora* (E)].

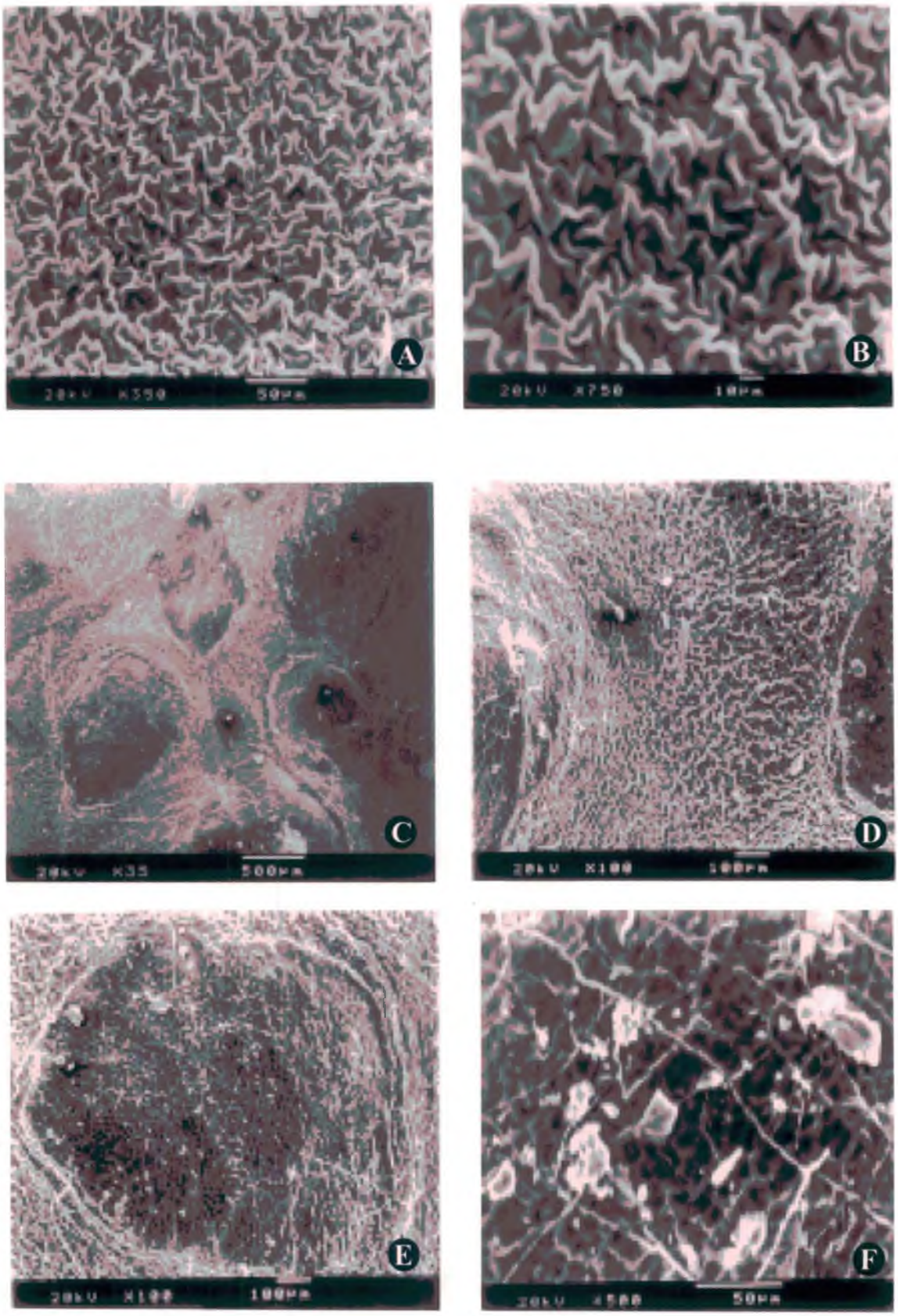


Plate 6 (figs. A-F): Scanning electron microscopic observation of healthy (A&B) and feeding lesions of *H. theivora* (C-F).

Table 1: Assessment of damage potential of *H. theivora* on different tea varieties over 3 years, 2002 - 2004.

Tea varieties	Incidence of attack			
	A[1]	B[2]	C[3]	D[4]
TV-9	12.45 ± 1.2	56.78 ± 2.3	66.75 ± 1.9	39.42 ± 1.1
TV-18	16.23 ± 1.7	68.94 ± 2.7	64.12 ± 1.8	44.23 ± 1.8
TV-20	14.74 ± 1.9	67.86 ± 3.2	67.45 ± 2.1	34.12 ± 1.7
TV-22	22.13 ± 1.1	75.11 ± 3.5	69.23 ± 2.1	39.42 ± 1.4
TV-23	28.77 ± 1.8	71.73 ± 4.1	68.12 ± 2.4	41.43 ± 2.1
TV-25	11.95 ± 1.4	65.23 ± 3.2	66.45 ± 1.9	35.46 ± 2.3
TV-26	26.23 ± 1.3	76.24 ± 4.1	75.49 ± 2.2	41.76 ± 1.7
TV-27	21.56 ± 1.9	63.79 ± 3.5	65.89 ± 3.7	36.48 ± 1.3
TV-28	16.78 ± 1.6	55.47 ± 2.9	59.46 ± 2.4	40.13 ± 1.1
TV-29	24.69 ± 1.4	69.45 ± 3.7	50.13 ± 1.9	43.12 ± 0.9
TV-30	29.48 ± 1.3	75.69 ± 3.8	56.89 ± 1.2	39.13 ± 1.4
T-17/1/ 54	23.77 ± 1.4	66.41 ± 3.1	51.22 ± 1.2	35.17 ± 1.6
UP- 2	26.23 ± 1.1	78.35 ± 3.2	75.43 ± 2.5	44.76 ± 1.4
UP-3	24.79 ± 1.3	82.75 ± 3.1	64.23 ± 2.9	49.45 ± 1.2
UP-8	29.45 ± 1.2	77.68 ± 2.5	79.85 ± 3.1	44.56 ± 1.5
UP-9	29.45 ± 1.7	75.35 ± 2.3	75.47 ± 2.2	48.12 ± 1.9
UP-26	34.11 ± 1.6	76.23 ± 1.4	73.14 ± 1.8	56.12 ± 2.0
BSS-1	27.59 ± 1.2	78.45 ± 1.8	74.13 ± 1.7	48.12 ± 1.1
BSS-2	23.77 ± 1.2	83.73 ± 3.7	68.12 ± 2.6	51.43 ± 1.6
BSS-3	28.77 ± 1.4	81.73 ± 2.8	78.12 ± 1.9	46.43 ± 1.5
HV-39	18.47 ± 1.7	68.13 ± 1.4	62.16 ± 1.3	39.67 ± 1.4
T-135	19.57 ± 1.2	69.45 ± 2.1	57.11 ± 1.1	36.16 ± 1.5
BS/7A/76	13.67 ± 1.4	65.84 ± 1.7	60.49 ± 1.7	31.17 ± 1.9

Year divided into four quarters, Data are % shoot affected
A[1] - January – March B[2] - April – June ± SE
C[3] - July – September D[4] - October – December

Analysis of variance of data presented in Table 1

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	22	2945.938	133.906	5.9787	7.96450
Time interval	3	35633.410	11877.800	530.3254	
Error	66	1478.215	22.397		
Total	91	40057.560			

4.2 Changes in the level of phenols and proteins in *H. theivora* infested tea leaves

Polyphenols are major constituents of tea leaves and defense response of a plant depend on the speed and extent of phenols. Their involvement in the defence mechanism either as preformed or induced chemicals seemed highly probable. Host responses could be differentiated by changes in content of phenolic compounds. Total phenols and orthodihydroxy phenols are also known to play definite role in a plant defence. In many cases there is an increase and decrease in phenolics biosynthesis during host defence mechanism. At the onset the level of phenolics in healthy as well as *H. theivora* infested tea leaves were determined quantitatively. Natural infested leaf samples were collected in the early morning as infestation by mosquito bug has been recorded always in the night. Healthy leaves were also collected during that period.

4.2.1 Total phenol

Total phenol from healthy and *H. theivora* infested tea leaves of twenty-three tea varieties were extracted and estimated using caffeic acid as standard. Both the healthy and infested leaf samples were collected from the experimental garden. Results (Table 2) revealed that quantity of total phenol has decreased in the infested tea leaves in comparison to healthy tea leaves. All the varieties showed changes in phenolics due to *Helopeltis* infestation.

4.2.2 Orthodihydroxy phenol

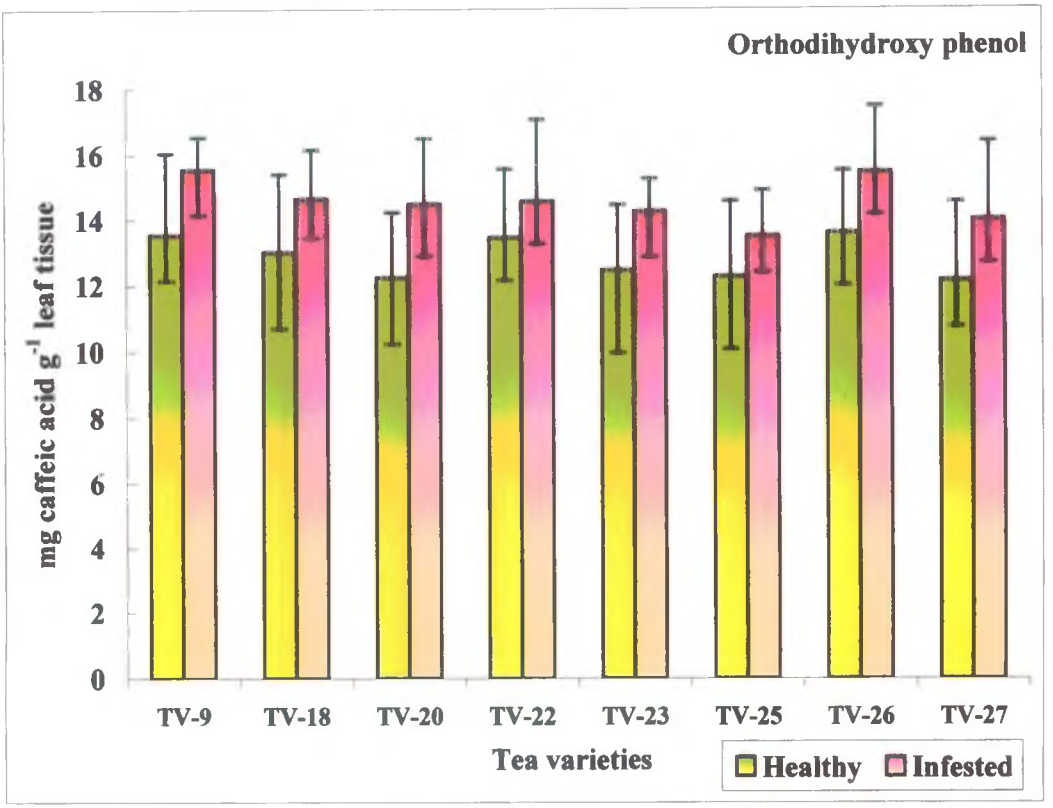
Healthy and *H. theivora* infested tea leaves from twenty-three tea varieties were also examined (Table 2). In case of orthodihydroxy phenol pattern was not similar. It has been found that as a result of infestation quantity of orthodihydroxy phenols have increased in all the varieties tested. The accumulation of phenolics due to infestation may reflect a general increase in host metabolism. Increases in the orthodihydroxy-phenol content of some of the Tocklai varieties have been depicted in Fig. 1.

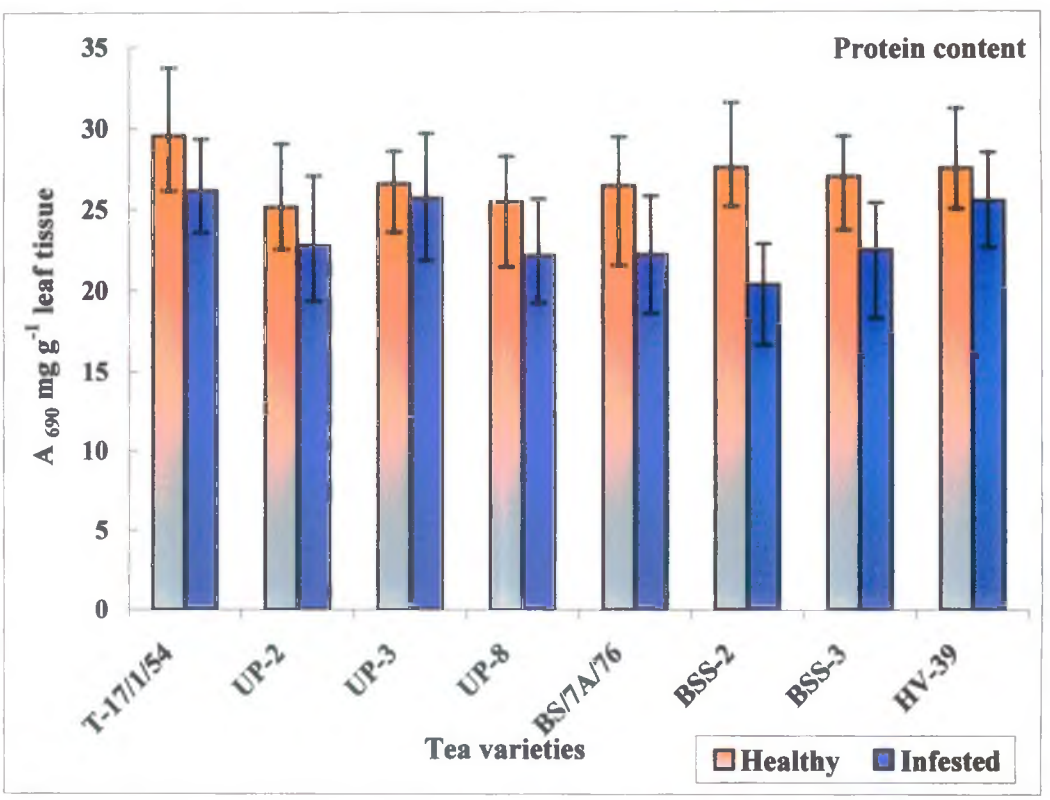
Table 2: Phenolic content in healthy and *H. theivora* infested tea leaves of different tea varieties.

Variety	Phenol content (mg caffeic acid g^{-1} leaf tissue)			
	Total		Orthodihydroxy	
	Healthy	infested	Healthy	infested
Tocklai –				
TV-9	52.12 ± 0.61	47.32 ± 0.46	13.54 ± 0.21	15.45 ± 0.45
TV-18	48.23 ± 0.52	45.26 ± 0.62	16.48 ± 0.34	17.45 ± 0.41
TV-20	49.26 ± 0.41	42.69 ± 0.47	11.23 ± 0.35	*15.49 ± 0.38
TV-22	51.47 ± 0.34	46.25 ± 0.38	10.28 ± 0.42	*14.75 ± 0.35
TV-23	48.57 ± 0.38	44.27 ± 0.42	14.56 ± 0.33	14.56 ± 0.22
TV-25	56.47 ± 0.30	48.29 ± 0.21	10.25 ± 0.37	11.25 ± 0.29
TV-26	46.48 ± 0.54	42.38 ± 0.11	12.12 ± 0.41	14.25 ± 0.36
TV-27	48.59 ± 0.41	47.56 ± 0.13	15.45 ± 0.36	15.48 ± 0.62
TV-28	45.78 ± 0.36	40.25 ± 0.19	10.25 ± 0.52	11.25 ± 0.43
TV-29	44.59 ± 0.37	39.48 ± 0.17	11.38 ± 0.29	13.47 ± 0.29
TV-30	46.59 ± 0.29	40.78 ± 0.18	13.56 ± 0.38	14.56 ± 0.35
T-17/1/54	50.46 ± 0.38	43.11 ± 0.21	11.78 ± 0.22	*16.25 ± 0.38
UPASI -				
UP-2	38.23 ± 0.24	38.11 ± 0.22	09.15 ± 0.29	*14.48 ± 0.25
UP-3	36.48 ± 0.45	40.12 ± 0.34	12.14 ± 0.48	13.45 ± 0.52
UP-8	40.16 ± 0.29	34.89 ± 0.43	08.60 ± 0.18	*12.24 ± 0.30
UP-9	41.26 ± 0.46	40.12 ± 0.14	10.23 ± 0.26	11.24 ± 0.68
UP-26	39.42 ± 0.53	37.42 ± 0.53	12.75 ± 0.22	14.25 ± 0.53
BSS-1	39.77 ± 0.58	40.58 ± 0.54	11.45 ± 0.12	12.89 ± 0.44
BSS-2	35.78 ± 0.34	32.56 ± 0.26	10.48 ± 0.34	11.36 ± 0.31
BSS-3	40.26 ± 0.31	37.25 ± 0.13	09.47 ± 0.16	12.48 ± 0.26
Darjeeling -				
HV-39	43.59 ± 0.45	42.15 ± 0.22	15.48 ± 0.13	16.47 ± 0.41
T-135	45.46 ± 0.29	40.15 ± 0.49	14.77 ± 0.51	15.47 ± 0.35
BS/7A/76	40.16 ± 0.34	32.49 ± 0.52	13.25 ± 0.61	14.27 ± 0.27

*Significant at 5% level
Means of three replicates;
± Standard error.

Changes in the level of orthodihydroxy phenol and protein in *H. theivora* infested tea leaves





4.2.3 Protein

There is evidence that changes in protein configuration in the host induce host's accessibility. The defense of plants to the pest and pathogen depend on the speed and extent of protein synthesis induced in the host. Alteration in protein synthesis in the plants can lead to the development of local resistance of immune layer around infection sites. In this study, the changes in protein contents of tea leaves following *H. theivora* infestation have been determined. Twenty-three varieties were considered for the quantification of protein from healthy and *H. theivora* infested tea leaves. Variable decrease in protein content was evident (Table 3 and Fig 1). Changes in the protein content varied between 4-14% among the varieties tested. . Significant change in level of proteins were evident in BSS-2 of UPASI variety, TV-22 and TV-25 of Tocklai varieties whereas HV-39 of Darjeeling variety.

In order to evaluate the changes in protein profile total soluble proteins extracted from healthy and *H. theivora* infested tea leaves of nine tea varieties (TV-18, TV-23, TV-29, TV-9, UP-3, UP-2, BSS-2, T-135 and HV-39) resolved in SDS-polyacrylamide gel electrophoresis along with protein markers. Results have been presented in Table 4 and Plate 7 (figs.A&B). Higher molecular weight proteins (98.5, 97.5, 95.5, 88.4, and 77.5 KDa) present in healthy leaf preparations were absent in *H. theivora* infested leaf proteins.

4.3. Determination of activity of defense enzymes in healthy and *H. theivora* infested tea leaves

Involvement of defense enzymes mainly phenylalanine ammonia lyase, polyphenol oxidase, peroxidase, chitinase and β-1,3-glucanase in developing resistance in tea plants against *H. theivora* was determined. These five enzymes were extracted and assayed from twenty-three varieties of plants infested with *H. theivora.*

Table 3: Level of proteins in different tea varieties following *H. theivora* infestation

Tea varieties	Protein content (A_{690} mg g^{-1} leaf tissue)		
	Healthy	*H. theivora infested*	
Tocklai			
TV 9	27.33 ± 0.21	25.19 ± 0.14	(-7.83)*
TV 18	28.88 ± 0.41	26.57 ± 0.16	(-7.99)
TV 20	29.56 ± 0.49	27.46 ± 0.13	(-7.10)
TV 22	31.44 ± 0.32	28.12 ± 0.13	(-10.55)
TV 23	28.45 ± 0.18	28.36 ± 0.17	(-0.31)
TV 25	25.87 ± 0.12	23.22 ± 0.23	(-10.24)
TV 26	27.33 ± 0.37	25.86 ± 0.24	(-5.37)
TV-27	29.53 ± 0.42	27.45 ± 0.31	(-7.04)
TV 28	27.88 ± 0.25	25.47 ± 0.19	(-8.64)
TV 29	28.27 ± 0.28	26.17 ± 0.32	(-7.42)
TV 30	30.02 ± 0.31	28.45 ± 0.29	(-5.22)
T 17/1/54	30.11 ± 0.21	27.66 ± 0.21	(-8.13)
UPASI			
UP 2	26.54 ± 0.11	24.15 ± 0.20	(-9.00)
UP 3	24.14 ± 0.13	22.76 ± 0.17	(-5.71)
UP 8	27.58 ± 0.14	25.68 ± 0.19	(-6.88)
UP 9	24.46 ± 0.19	22.13 ± 0.23	(-9.52)
UP 26	27.44 ± 0.21	25.19 ± 0.14	(-8.19)
BSS 1	21.56 ± 0.23	20.34 ± 0.20	(-5.65)
BSS 2	24.98 ± 0.11	21.46 ± 0.17	(-14.09)
BSS 3	27.48 ± 0.19	25.47 ± 0.18	(-7.31)
Darjeeling			
HV 39	26.87 ± 0.23	24.16 ± 0.22	(-10.08)
T 135	25.22 ± 0.14	24.01 ± 0.23	(-4.79)
BS/7A/76	28.77 ± 0.11	26.23 ± 0.16	(-8.82)

*Values in the parenthesis indicates percentage decreased in the protein content against healthy
Means of three replicates
± Standard error.

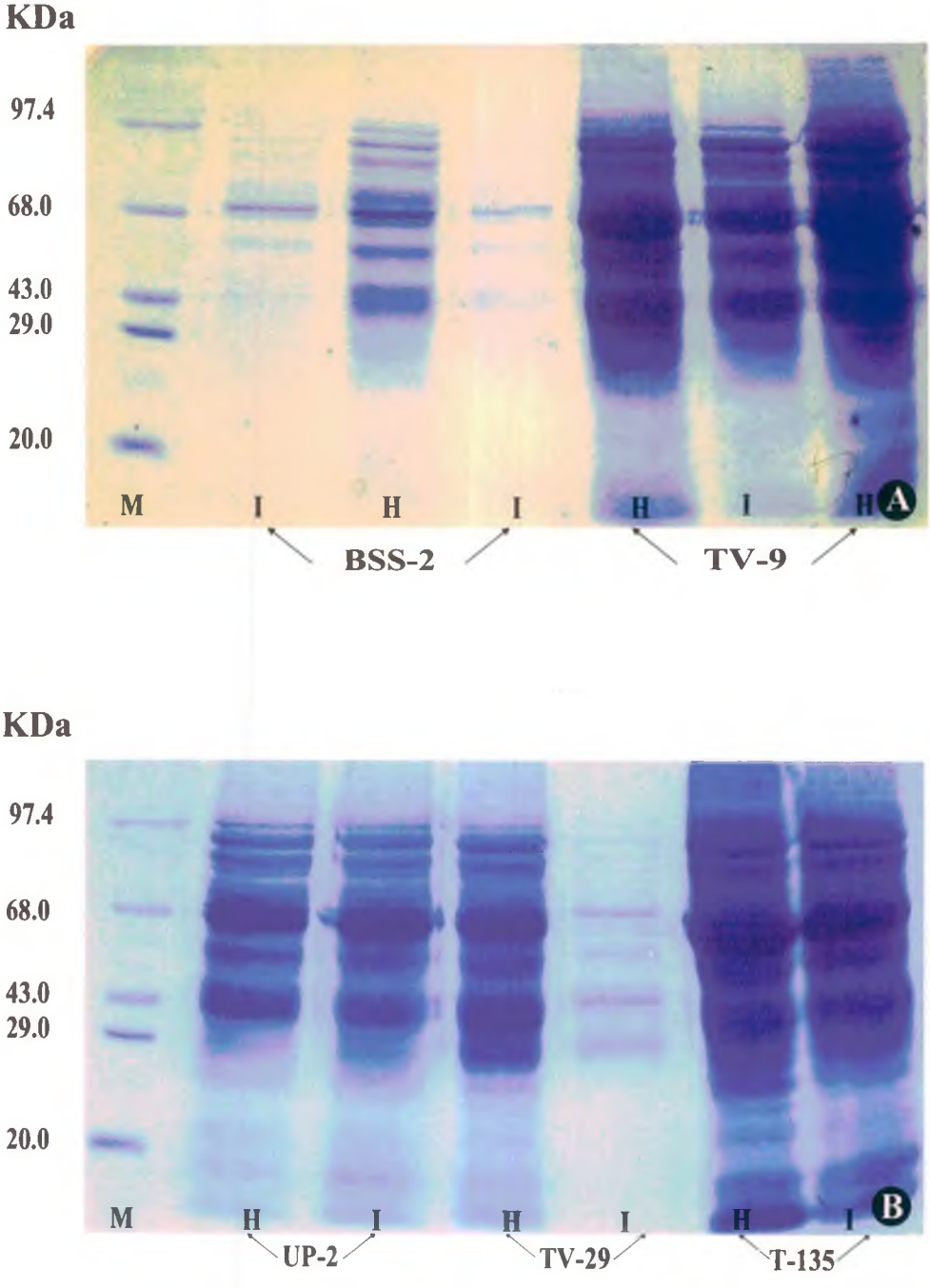


Plate 7 (figs. A& B): SDS-PAGE analysis of healthy and *H. theivora* infested tea leaf proteins; (A&B) [(M) - Molecular marker ; (H) - healthy; (I) leaf infested with *H. theivora*]

Table 4: SDS-PAGE analysis of protein extracts from healthy and *H. theivora* infested tea leaves

Tea varieties		Protein molecular weight (KDa)
TV-18	H	98.7, 97.5, 88.4, 86.4, 82.7, 79.7, 77.5, 66.7, 53.4, 45.2, 34.2, 28.7, 25.6, 20.4, 16.4, 13.2 (16)
	I	98.5, 82.4, 77.4, 65.7, 53.4, 32.2, 29.4, 21.1, 16.4, 13.7 (10)
TV-23	H	98.5, 95.4, 92.1, 89.4, 81.2, 76.4, 65.2, 52.7, 45.7, 45.2, 39.5, 33.4, 23.4, 20.2, 18.7, 12.4, 9.4 (17)
	I	86.4, 76.8, 65.4, 45.7, 39.4, 30.4, 26.4, 23.4, 18.7, 12.4 (10)
UP-3	H	96.7, 86.4, 79.7, 77.5, 69.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 16.4, 13.7, 12.4, 10.4 (18)
	I	75.4, 62.7, 59.7, 43.4, 40.1, 34.7, 28.4, 20.4, 16.7, 13.7, 10.2 (11)
HV-39	H	96.5, 94.4, 90.4, 88.7, 86.2, 79.2, 77.5, 76.2, 68.3, 60.1, 57.4, 52.7, 43.4, 39.5, 34.2, 28.7, 20.7,11.2, 10.4, 9.4 (20)
	I	98.7, 97.5, 88.4, 86.4, 82.7, 79.7, 77.5, 66.7, 53.4, 45.2, 34.2, 28.7, 25.6, 20.4, 16.4, 13.2 (16)
BSS-2	H	88.4, 86.4, 82.7, 79.7, 77.5, 66.7, 53.4, 49.7,45.2, 34.2, 28.7, 25.6, 20.4 (13)
	I	68.9, 66.7, 53.4, 45.2, 34.2, 28.7, 25.6, 20.4, 16.4, 13.2 (10)
TV-9	H	95.7, 87.4, 74.7, 71.5, 67.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 16.4, 14.8, 13.9 (17)
	I	82.6, 72.9, 69.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 15.8 (13)
UP-2	H	96.7, 86.4, 79.7, 77.5, 69.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 16.4, 13.7, 11.6 (17)
	I	74.2, 71.5, 69.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 16.4, 13.7, 12.4, 10.4 (15)
TV-29	H	96.5, 94.4, 90.4, 88.7, 86.2, 79.2, 77.5, 76.2, 68.3, 60.1, 57.4, 52.7, 43.4, 39.5, 34.2, 28.7, 20.7,11.2, 10.4, 9.4 (20)
	I	85.4, 79.4, 68.4, 61.4, 57.8, 52.8, 39.4, 34.7, 20.7 (9)
T-135	H	96.5, 94.4, 90.4, 88.7, 86.2, 79.2, 77.5, 76.2, 68.3, 60.1, 57.4, 52.7, 43.4, 39.5, 34.2, 28.7, 20.7,11.2, 10.4, 9.4 (20)
	I	96.7, 86.4, 79.7, 77.5, 69.4, 62.4, 59.4, 56.7, 52.6, 43.4, 40.7, 34.2, 28.7, 20.1, 16.4, 13.7, 11.6 (17)

H- Healthy leaf protein bands, I- *H. theivora* infested leaf protein bands.
Values in the parenthesis indicates number of total protein bands.

4.3.1 Phenylalnine ammonia lyase (PAL)

Phenylalnine ammonia lyase (PAL) is the first enzyme of phenylpropanoid metabolism in higher plants and it has been suggested that it play a significant role in regulating the accumulation of phenolics, phytoalexins and lignins, three key factors responsible for disease resistance. Keeping this in mind the present study, PAL activity in twenty-three different tea varieties following *H. theivora* infestation was determined and compared with healthy plants. Results have been presented in Table 5 and Fig. 2. It is interesting to note that PAL activity increased due to *H. theivora* infestation in all the varieties tested. However UPASI varieties exhibited significant increase in PAL activity due to infestation (Fig. 2).

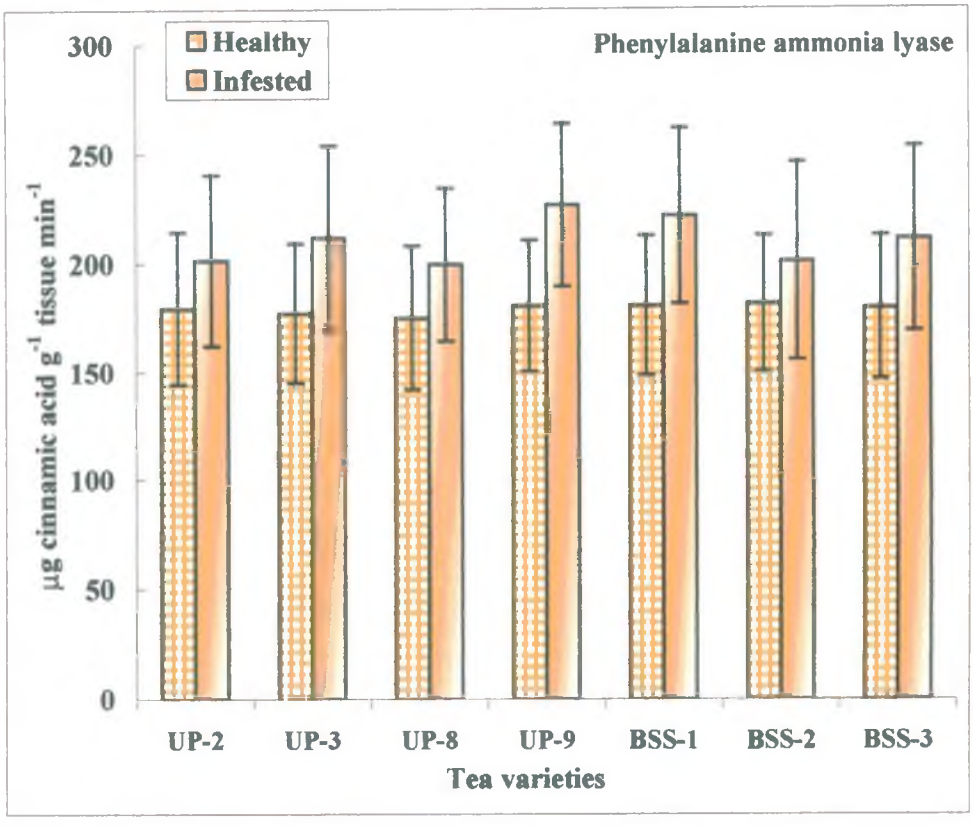
4.3.2 Peroxidase (PO)

Peroxidase (PO) activities have been assayed from healthy as well as naturally infested by *H. theivora* tea leaves. Results revealed that sharp increase in PO activity in *H. theivora* infested tea leaves in relation to healthy plants (Table 6 and Fig. 2). About three-fold increase in such enzyme activity was evident in infested leaves than the healthy leaves. Increased in the level of PO activity in Tocklai varieties have been presented in Fig 2.

4.3.3 Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) is also an important enzyme in the phenol metabolism of tea plants and plays a major role in the tea fermentation. PPO activity was assessed in twenty-three tea varieties. PPO activity was higher in the *H theiovora* infested leaves in comparison to the healthy one in all the varieties (Fig. 3). Sharp increase in PPO activity was noticed in *H. theivora* infested tea leaves in relation to healthy control plants (Fig. 3).

Activity of defense enzyme in healthy and *H. theivora* infested tea leaves



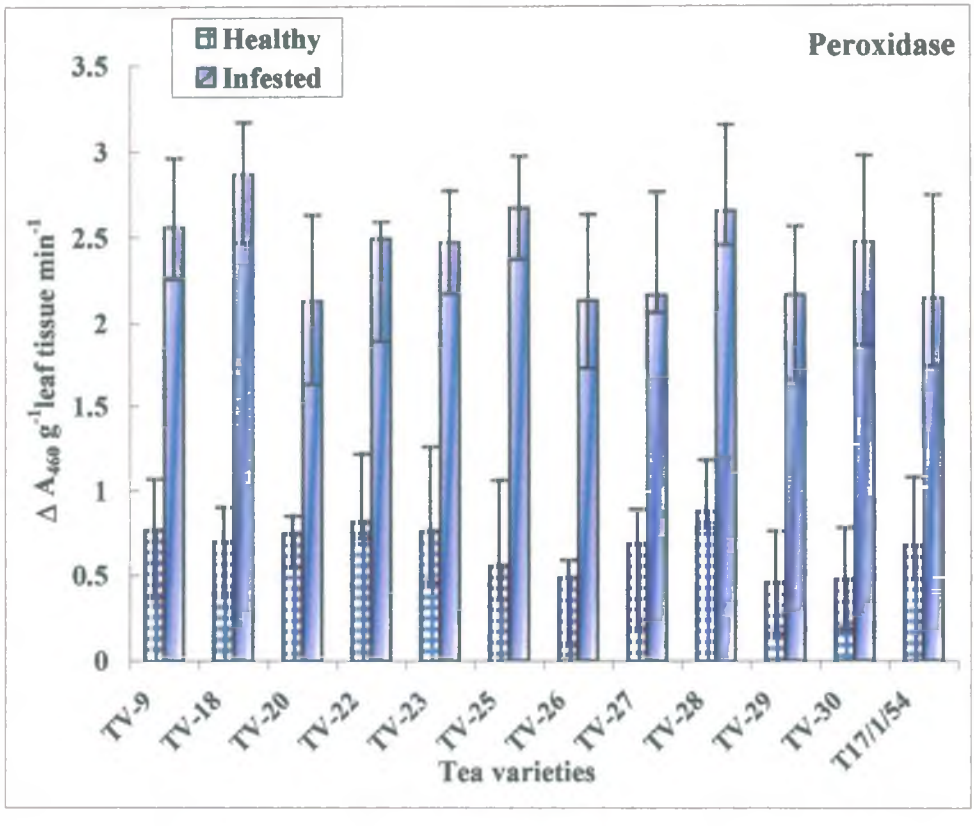


Table 5 : Activities of phenylalanine ammonia lyase in healthy and *H. theivora* infested tea leaves

Variety	PAL activity (μg cinnamic acid g^{-1} leaf tissue min^{-1})	
	Healthy	*H. theivora* infested
TV-9	194.8 ± 13.9	269.6 ± 16.2
TV-18	190.5 ± 14.8	285.6 ± 12.1
TV-20	196.4 ± 10.6	241.8 ± 09.4
TV-22	194.8 ± 12.8	231.1 ± 10.2
TV-23	186.7 ± 11.9	219.9 ± 11.1
TV-25	182.4 ± 12.3	221.9 ± 14.3
TV-26	187.7 ± 11.7	240.7 ± 13.2
TV-27	194.5 ± 16.3	233.4 ± 11.4
TV-28	199.8 ± 15.1	285.6 ± 14.7
TV-29	200.8 ± 16.2	259.4 ± 12.3
TV-30	197.4 ± 13.2	271.3 ± 11.4
T-17/1/54	159.4 ± 14.1	275.8 ± 12.4
UP-26	198.7 ± 16.7	295.7 ± 16.7
HV-39	190.4 ± 14.1	241.5 ± 11.4
T-135	194.5 ± 12.2	237.4 ± 12.1
BS/7A/76	195.7 ± 11.9	234.1 ± 12.3

Means of three replicates,
± Standard error.

Table 6: Activities of peroxidase in healthy and *H. theivora* infested tea leaves

Variety	Peroxidase activity - (ΔA_{460} g^{-1}leaf tissue min^{-1})	
	Healthy	*H. theivora* infested
Tocklai varieties		
TV-9	0.77 ± 0.02	2.58 ± 0.02
TV-18	0.54 ± 0.01	2.76 ± 0.01
TV-20	0.82 ± 0.02	2.69 ± 0.03
TV-22	0.56 ± 0.01	2.19 ± 0.01
TV-23	0.88 ± 0.02	2.26 ± 0.02
TV-25	0.53 ± 0.01	2.36 ± 0.02
TV-26	0.64 ± 0.02	2.59 ± 0.03
TV-27	0.52 ± 0.03	2.14 ± 0.01
TV-28	0.65 ± 0.02	2.54 ± 0.02
TV-29	0.88 ± 0.01	2.39 ± 0.01
TV-30	0.53 ± 0.03	2.34 ± 0.02
T-17/1/54	0.64 ± 0.02	2.84 ± 0.01
UPASI varieties		
UP-2	0.58 ± 0.02	2.35 ± 0.01
UP-3	0.55 ± 0.03	2.17 ± 0.01
UP-8	0.68 ± 0.01	2.42 ± 0.03
UP-9	0.56 ± 0.02	2.14 ± 0.02
UP-26	0.57 ± 0.03	2.59 ± 0.09
BSS-1	0.84 ± 0.01	2.57 ± 0.02
BSS-2	0.75 ± 0.02	2.46 ± 0.01
BSS-3	0.64 ± 0.01	2.36 ± 0.01
Darjeeling varieties		
HV-39	0.68 ± 0.08	1.86 ± 0.06
T-135	0.73 ± 0.04	2.04 ± 0.04
BS/7A/76	0.72 ± 0.06	1.93 ± 0.08

Means of three replicates, ± Standard error.

Polyphenol oxidase activity in healthy and *H. theivora* infested tea leaves

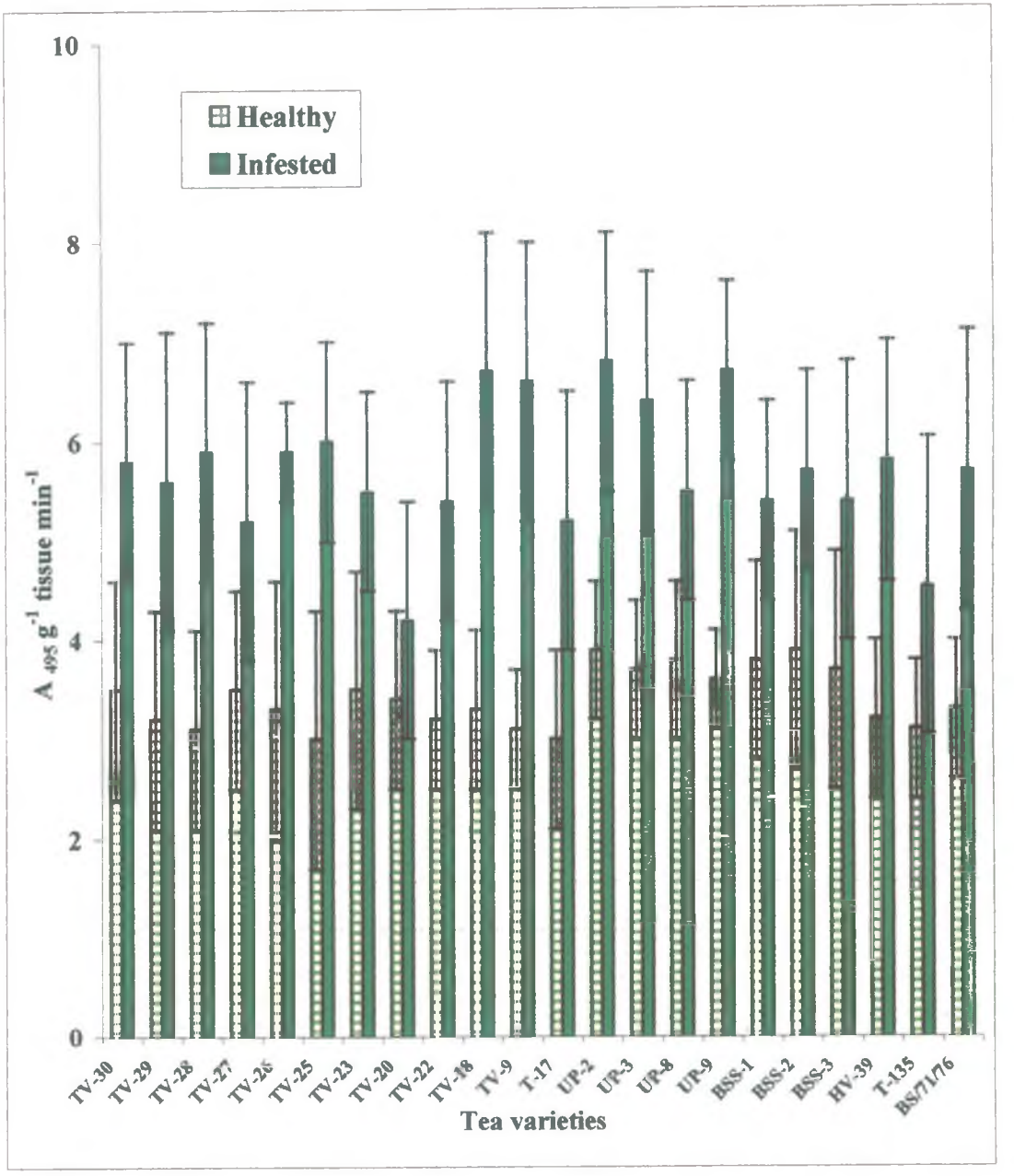


Fig. 3

4.3.4 β-1,3-glucanase (βGLU)

βGLU activities have been assayed from healthy as well as *H. theivora* infested tea leaves of twenty-three tea varieties growing in the Experimental Garden of the Department and results have been presented in Table 7 and Fig. 4. In all cases, *H. theivora* infested tea leaves showed greater enzyme activities in relation to healthy control. Significant increase observed in UPASI varieties (Fig.4) followed by Darjeeling and Tocklai varieties.

Table 7: Activities of β-1,3-Glucanase in healthy and *H. theivora* infested tea leaves

Variety	β-1,3-glucanase activity (μg glucose g^{-1} leaf tissue min^{-1})	
TV-9	47.12 ± 1.32	58.68 ± 1.16
TV-18	44.69 ± 1.24	76.03 ± 1.13
TV-20	42.68 ± 1.12	62.69 ± 1.46
TV-22	46.68 ± 1.16	69.45 ± 1.14
TV-23	48.57 ± 1.23	66.73 ± 1.18
TV-25	43.08 ± 1.32	60.73 ± 1.21
TV-26	44.03 ± 1.26	59.12 ± 1.19
TV-27	42.67 ± 1.17	62.64 ± 1.23
TV-28	45.58 ± 1.19	54.02 ± 1.24
TV-29	48.66 ± 1.23	62.69 ± 1.16
TV-30	48.69 ± 1.39	64.03 ± 1.14
T-17/1/54	44.35 ± 1.47	54.35 ± 1.26
UP-26	39.86 ± 1.56	59.75 ± 1.67
HV-39	38.68 ± 1.32	52.44 ± 1.29
T-135	36.22 ± 1.18	44.48 ± 1.34
BS/7A/76	34.68 ± 1.29	54.32 ± 1.38

Means of three replicates, ± Standard error.

4.3.5 Chitinase (CHT)

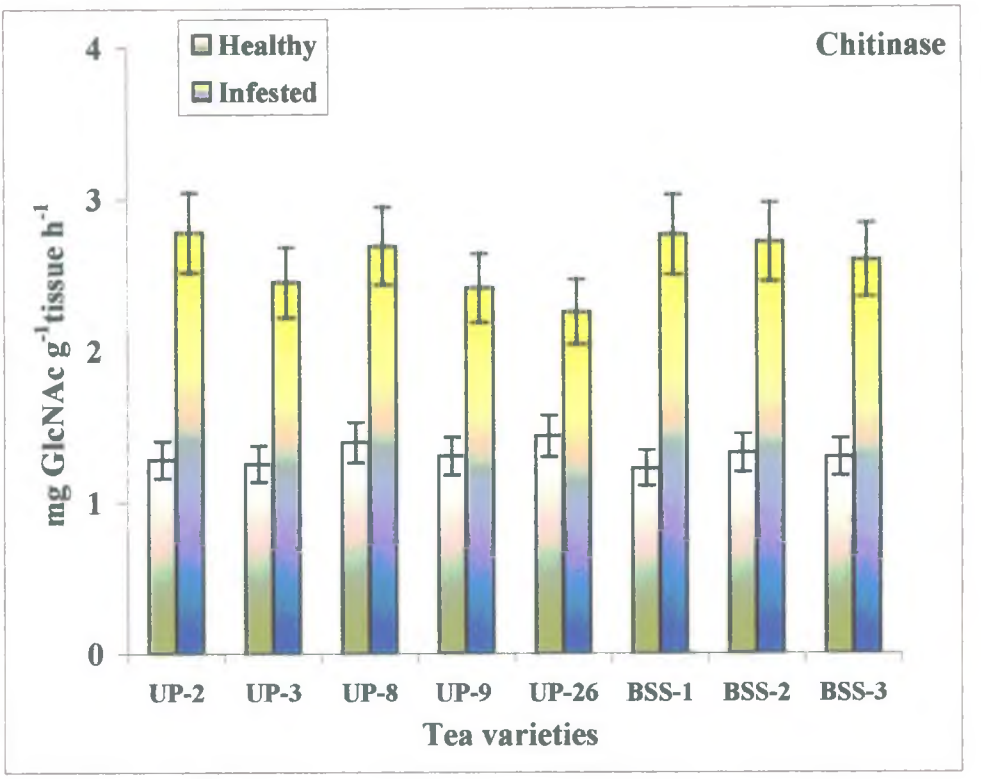
Chitinase (CHT) was extracted and assayed from healthy as well as *H. theivora* infested twenty-three different tea varieties. Activity of CHT increased in all the twenty-three varieties in comparison to healthy plants. Chitinase activity of healthy and infested tea leaves of Tocklai and Darjeeling varieties have been presented in Table 8 while CHT activites of UPASI varieties have been illustrated in Fig 4.

Table 8: Activities of chitinase in healthy and *H. theivora* infested tea leaves of Tocklai and Darjeeling varieties.

Variety	Chitinase activity (mg GlcNAc g^{-1}leaf tissue h^{-1})	
	Healthy	***H. theivora* infested**
TV-9	0.53 ± 0.03	1.52 ± 0.04
TV-18	0.64 ± 0.04	1.61 ± 0.03
TV-20	0.54 ± 0.04	1.72 ± 0.01
TV-22	0.57 ± 0.02	1.53 ± 0.03
TV-23	0.59 ± 0.03	1.42 ± 0.02
TV-25	0.67 ± 0.02	1.56 ± 0.02
TV-26	0.61 ± 0.03	1.46 ± 0.04
TV-27	0.67 ± 0.02	1.93 ± 0.01
TV-28	0.57 ± 0.06	1.21 ± 0.02
TV-29	0.54 ± 0.04	1.94 ± 0.05
TV-30	0.62 ± 0.02	1.64 ± 0.03
T-17/1/54	0.61 ± 0.05	1.89 ± 0.03
HV-39	0.59 ± 0.03	1.83 ± 0.04
T-135	0.60 ± 0.04	1.58 ± 0.02
BS/7A/76	0.54 ± 0.02	1.67 ± 0.01

Means of three replicates ± Standard error.

Activity of defense enzyme in healthy and *H. theivora* infested leaves



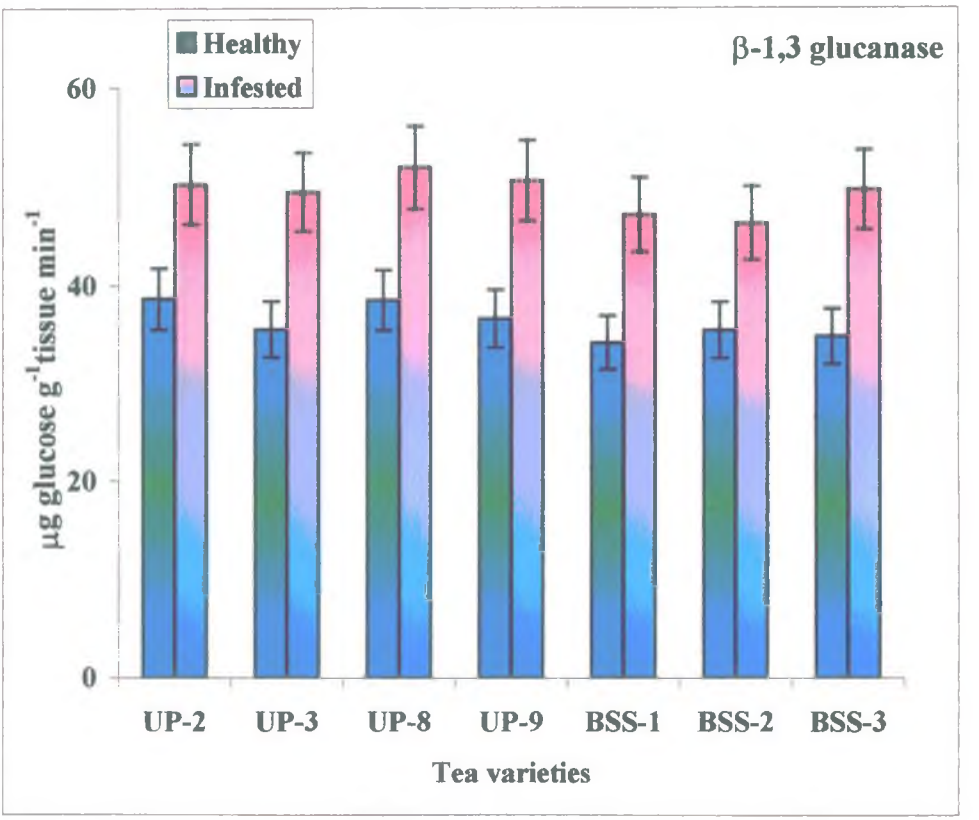


Fig. 4

Percentage increase in enzyme activities of all twenty-three tea varieties tested were assessed greater accumulation of PO, PPO, CHT and GLU for each varieties have been illustrated in Fig. 5&6. It was noticed that accumulation of PPO was higher in *H. theivora* infested in comparison to healthy plants followed by PAL, CHT, PO and GLU activity.

4.3.5.1 Dot blot

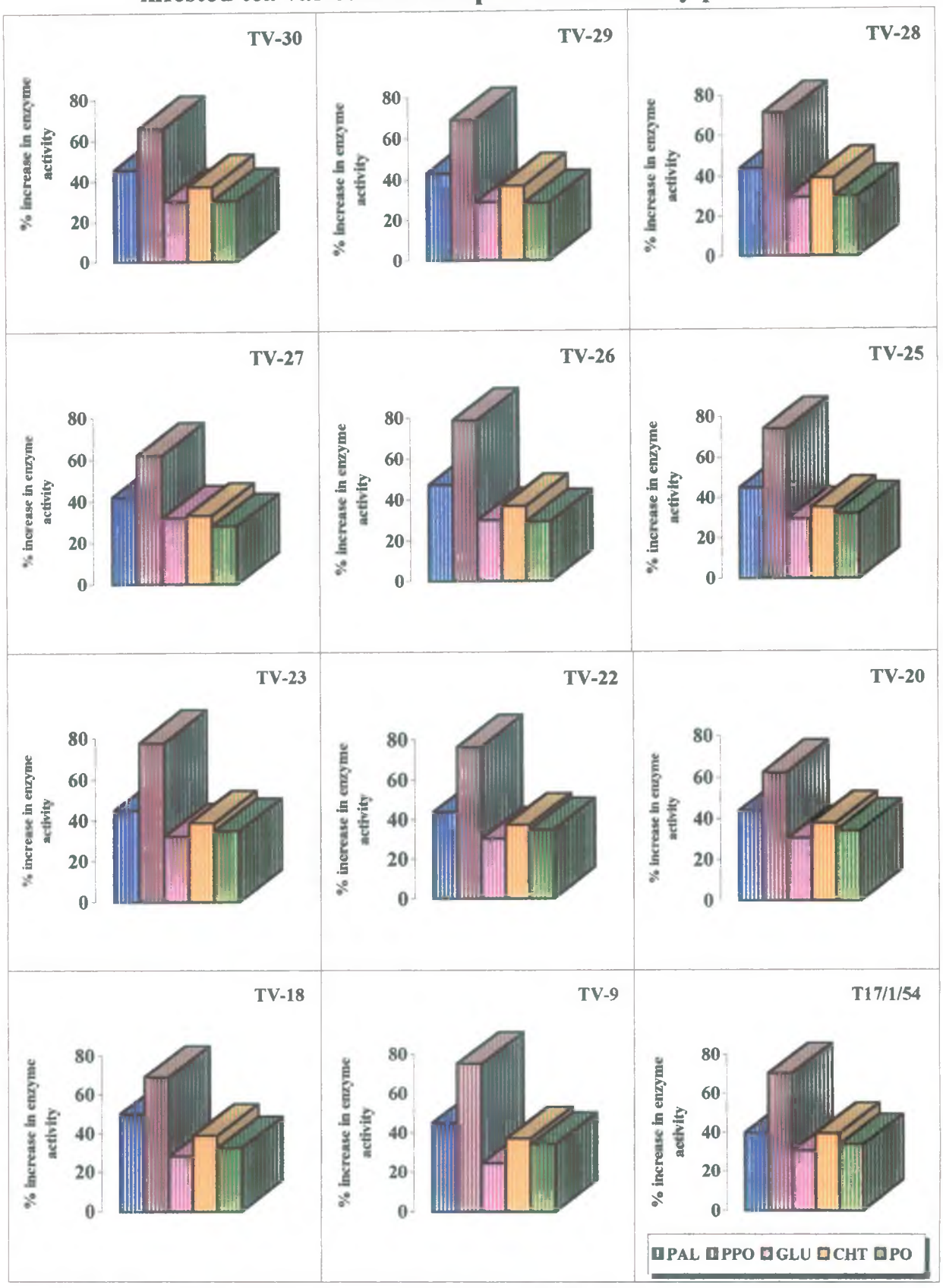
Chitinase extracted from healthy and *H. theivora* infested leaves six of tea varieties in which less infestation in the field were characterized were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAbs raised against chitinase (Sigma chemical). In dot blot maximum color intensity was observed when enzymes prepared from infested leaves were probed with PAb of chitinase. Results have been summarized in Table 9. Clear and intense color reactions were observed in the enzyme preparation of *H. theivora* infested tea leaves.

Table 9: Dot immuno binding assay of enzyme preparation of healthy and *H. theivora* infested tea leaves probed with PAb raised against chitinase.

Antigen	Colour intensity	
	Healthy	*H. theivora* infested
TV-9	+ +	+ + +
TV-30	±	+
TV-23	+ +	+ + +
TV-20	+ +	+ + + +
UP-3	+	+ +
UP-9	±	+ +

Colour intensity of dots
+ + light violet ; + + + violet ; + + + + deep violet ; ± insignificant ;
NBT/BCIP used as substrate ; PAb (60µg/ml)

Assessment of accumulation of defense enzymes in *H. theivora* infested tea varieties in comparison to healthy plants

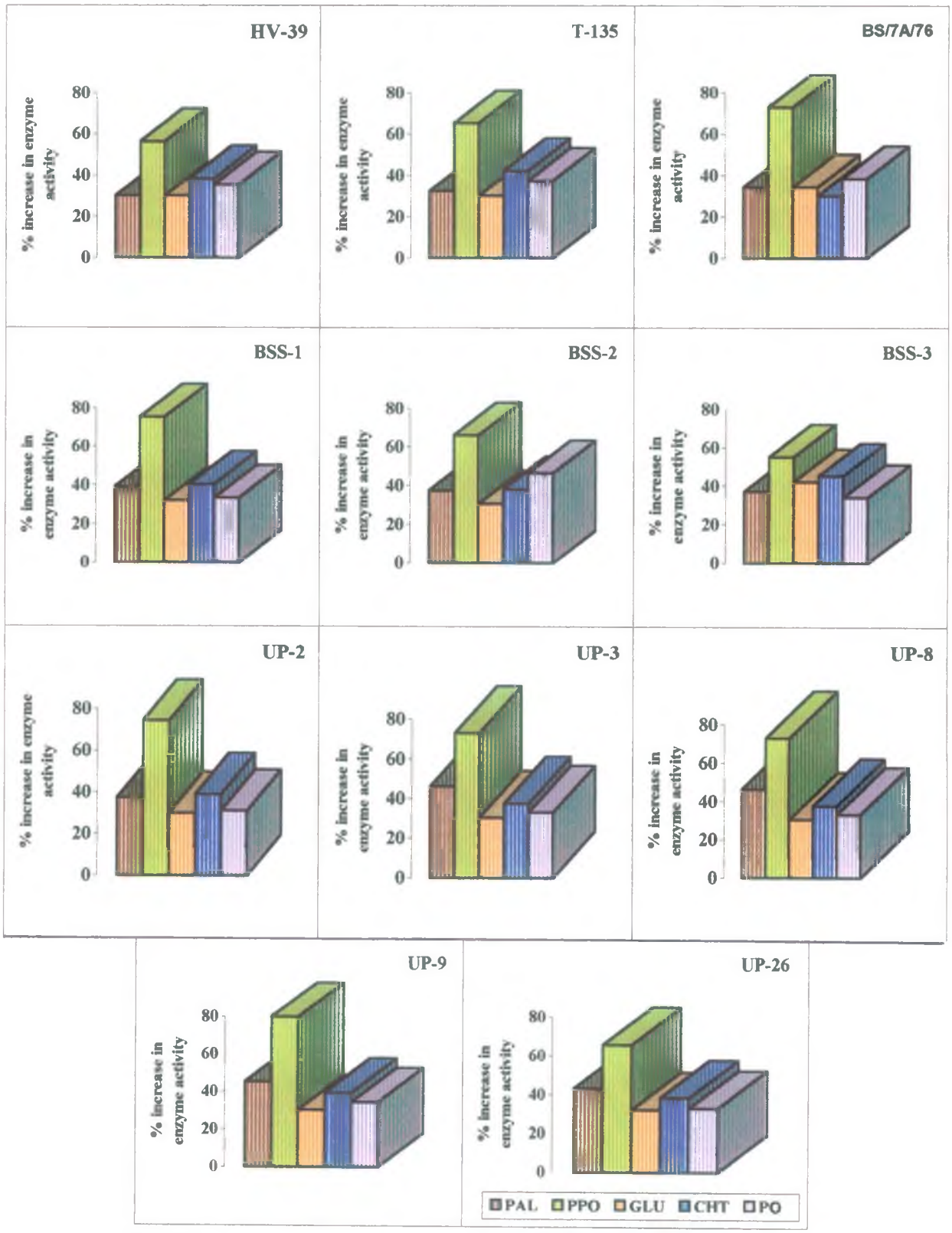


PO - Peroxidase **GLU - β,1-3 glucanase**

PPO - Polyphenoloxidase **CHT - Chitinase**

PAL - Phenylalanine ammonia lyase

Fig. 5



PO - Peroxidase
PPO - Polyphenoloxidase
PAL - Phenylalanine ammonia lyase
GLU - β-1,3 glucanase
CHT - Chitinase

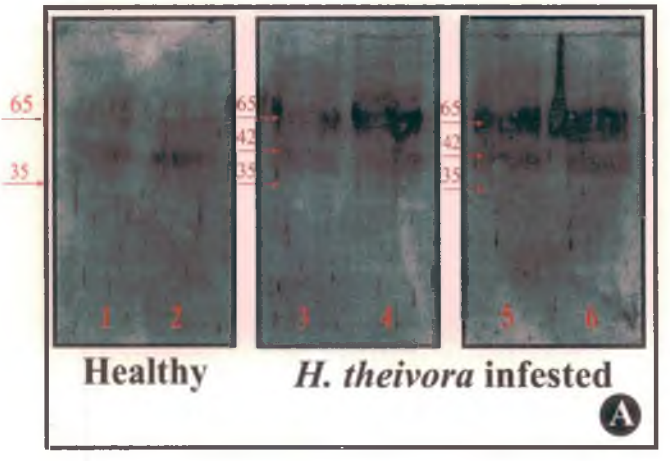
Fig. 6

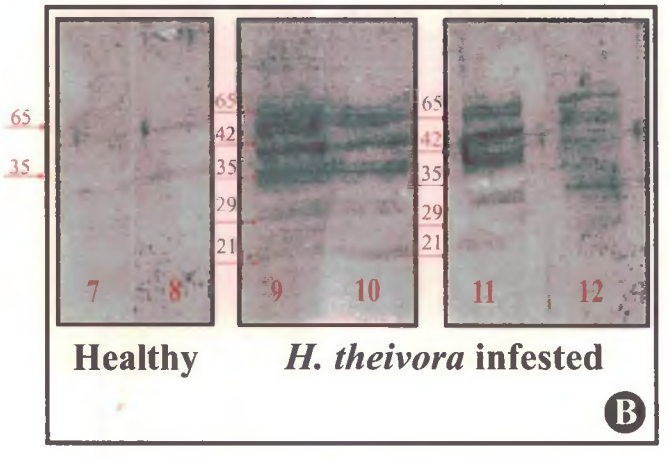
4.3.5.2 Western blotting

Enzyme preparations from Soluble proteins extracted from healthy and *H. theivora* infested tea leaves of four (TV-30, TV-23, UP-2 and HV-39) in which less infestation by *H. theivora* was recorded in the field were resolved on 10% SDS-PAGE then transferred on nitrocellulose membrane and the blots were probed with PAb of chitinase. Western blots of 1D gels were analysed. Two bands of ca. molecular weights 65 and 35 kDa were found to be common in both healthy and *H. theivora* infested leaves, while a new band of ca. molecular weight 42 kDa was evident in *Helopeltis* infested leaves of TV-30 and TV-23. However UP-2 and HV-39 yielded another two bands in low molecular weight of 29 and 21 kDa (Plate 8 fig.A&B).

4.4 Detection of antifungal compounds in tea leaves following *H. theivora* infestation

Tea plants are capable of triggering an array of systemic responses while challenged by pest attack that may be beneficial or deterimental to plant health and productivity. Inducible defense in tea plants against *H. theivora* can be strongly influenced by the mix signals generated by external biotic factors as well as by abiotic stresses. Previous attempts have clearly indicated that polyphenol oxidase and phenylalanine ammonia lyase activity followed by peroxidase, chitinase and glucanase enhanced in as many as twenty three varieties tested against *H. theivora* infestation. In these varieties a strategy may consist in identifying natural molecules with antibiotic properties, which can be transported over long distance towards the target. Considering their known chemical and biological properties, phenolics might fulfill the requirements of such a strategy.





(TV-30 - Lane 1, 3 & 4) (TV-23-Lane 2,5 & 6) (UP-2 - Lane 7,9 & 10) (HV-39-Lane 8,11 & 12)

Plate 8 (figs. A & B): Western blot analysis of healthy and *H. theivora* infested leaves of tea probed with PAbs raised against chitinase

4.4.1 Antifungal phenolics

Production of antifungal phenolics in tea plants is known to be one of the conferral mechanisms of disease resistance against foliar fungal pathogen. However, as information in this line with pest attack is not available, present investigation was undertaken with a view to determine the changes in pyrocatechol (an antifungal phenolic of tea) level in five selected tea varieties (TV-18, TV-20 ,TV-22, UP-8 and BSS-3) showing variable degree of infestation by *H. theivora.* Facilitated diffusion technique was adopted for the detection of antifungal substances from freshly harvested healthy and *H. theivora* infested tea leaves. Three fractions such as diethyl ether fraction (I), ethyl acetate fraction (II), and ethyl acetate fraction after hydrolysis (III) were obtained separately from healthy and infested leaves. These were bioassayed and then analysed by HPLC.

4.4.1.1 Radial growth bioassay

At the onset, all the three fractions (I, II and III) were bioassayed following radial growth assay against *C. invisum.* Inhibition of mycelial growth of *C. invisum* against each fractions obtained from TV-18, one of the selected varieties have Results have been presented in Table 10. Results revealed that mycelial growth of *C. invisum* was inhibited markedly in the medium supplemented with the extracts of the *H. theivora* infested leaves in relation to their respective control (media supplemented with healthy extract). Mycelial growth was measured in each treatment, when *C. invisum* covered full petridishes grown in Potato Dextrose Agar (PDA) medium without any supplementation. Maximum inhibition (74%) was noted in fraction III of *H. theivora* infested leaf extract. Where as fraction I and fraction II of *H. theivora* infested leaf extracts showed 20 and 56% inhibition respectively.

Table 10: Effect of the solvent extract from healthy and *H. theivora* infested leaves (TV-18) on radial growth of *C. invisum*.

Solvent extracts	Mycelial growth of *C. invisum* (cm.)	Inhibition of mycelial growth (%.)[a]
PDA control	5.0	0
Di-ethyleether control	5.0	0
Ethyl acetate control	5.0	0
Fraction I – H[b]	4.5	10
Fraction I – I[c]	4.0	20
Fraction II –H	2.5	50
Fraction II – I	2.2	56
Fraction III – H	1.5	70
Fraction III – I	1.3	74
Water fraction –H	3.2	36
Water fraction – I	3.9	22

[a]Values indicates percentage inhibition against solvent control
[b] Healthy leaf extract
[c]*H. theivora* infested leaf extract

4.4.1.2 Slide germination bioassay

Fractions from each solvent extract were used after partial purification following preparative TLC for spore germination assay of *A. alternata*. In this case also percentage spore germination and germ tube length of *A. alternata* against each fractions obtained from TV-18, one of the selected varieties have been presented in Table 11. Results revealed that fraction III of *H. theivora* infested leaf extract completely inhibited the spore germination. Infested leaf extract fractions in all cases markedly inhibited spore germination in relation to healthy leaf extracts.

Table 11: Spore germination bioassay of partially purified fractions of healthy and *H. theivora* infested leaves (TV-18)

Solvent extract	Spore germination[a] (%)	Length of germ tube (μm)[a]
Sterile distilled water	74.24	65.4 ± 1.21
Di-ethyle ether	60.42	62.7 ± 1.78
Ethyl acetate	62.47	63.5 ± 1.45
Fraction I – H[b]	33.33	49.7 ± 0.95
Fraction I – I[c]	29.41	35.7 ± 0.82
Fraction II –H[b]	21.25	32.4 ± 1.12
Fraction II – I[c]	11.09	12.7 ± 1.03
Fraction III – H[b]	12.45	0.8 ± 0.09
Fraction III – I[c]	0	---
Water fraction –H[b]	61.44	56.4 ± 1.12
Water fraction – I[c]	45.23	42.3 ± 0.85

[a] *A. alternata*
[b] Healthy leaf extract
[c] *H. theivora* infested leaf extract

4.4.1.3 HPLC analysis

Radial growth bioassay and spore germination bioassay indicated that the fraction III compounds were more effective than the other two fractions (I and II). Hence further analysis of fraction III obtained both from healthy as well as *H. theivora* infested leaf extracts of TV-18, one of the selected varieties were done by HPLC. Results have been presented in Table 12 and Fig.7. The UV spectra from both the compounds had a sharp peak at 280nm. A sharp peak at retention time 2.6 was present in both the samples but in the healthy leaf extract the peak height was much smaller than the *H. thievora* infested leaf extract.

HPLC elution profiles of pyrocatechol from healthy (H) and *H. theivora* infested (I) tea leaves

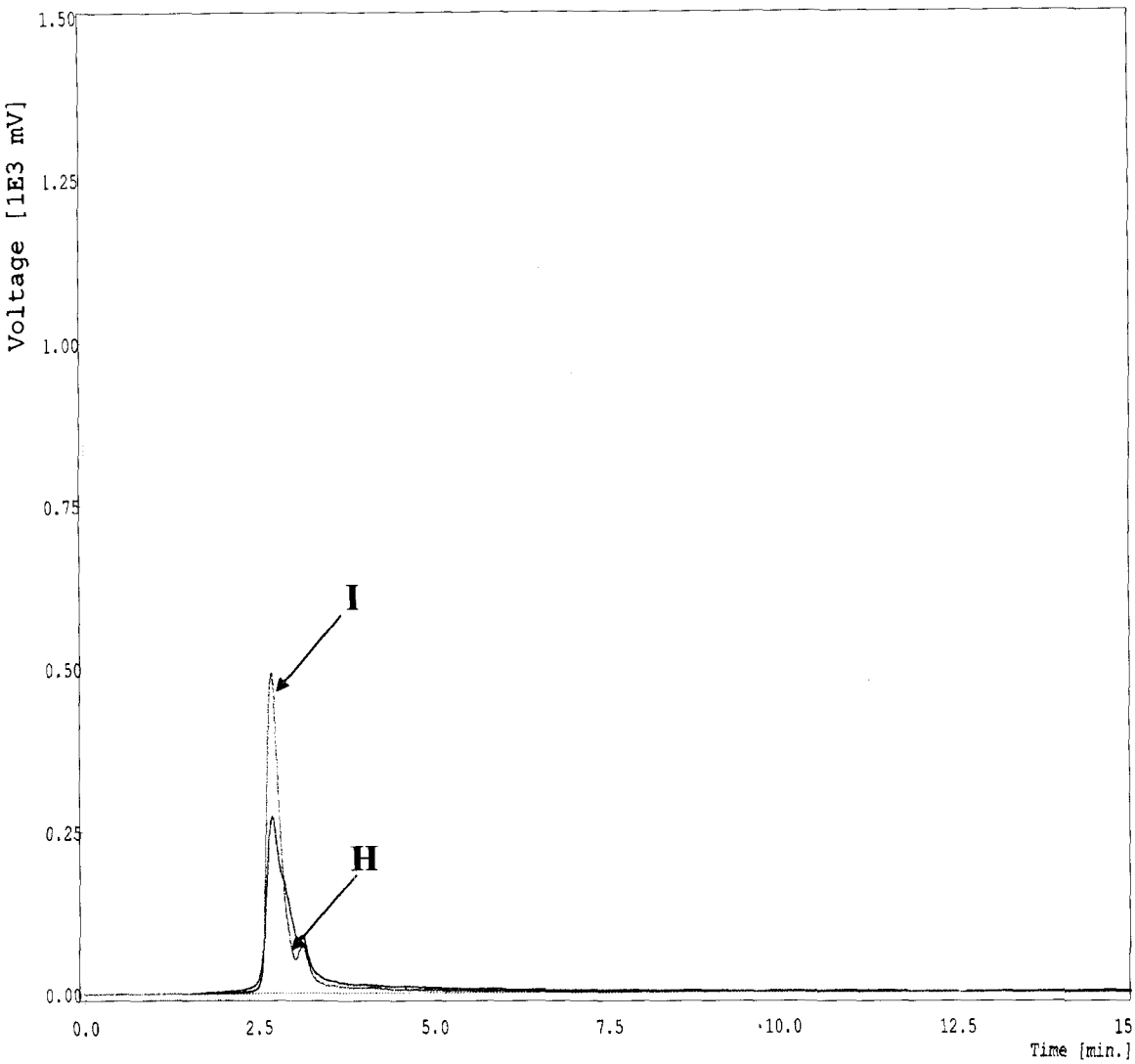


Fig. 7

Table 12: HPLC analysis of pyrocatechol from healthy and *H. theivora* infested tea leaves (TV-18)

Leaf extract	Peak No.	Reten. time (min)	Height (mV)	Height (%)
Healthy	1.	2.690	272.348	94.380
	2.	4.570	9.542	3.307
H.theivora infested	1.	2.680	493.068	98.486
	2.	4.910	7.411	1.611

Maximum absorption peak measured at 280 nm corresponded with the authentic pyrocatechol. Hence, quantification of pyrocatechol was done from UV-spectrophotometric curve by considering molar extinction coefficient of authentic pyrocatechol 6000 at 280 nm. Pyrocatechol accumulated within 24 h following infestation was estimated from three resistant varieties (TV-18, TV-20 and TV-22) and two susceptible varieties (UP-8 and BSS-3). Results have been presented in Table 13. It appears from the result that in *H.theivora* infested leaves greater amount of pyrocatechol accumulated within 24h following infestation. Higher level of accumulation was evident in all three resistant varieties tested than other two susceptible varieties.

4.4.2 HPLC analysis of Catechin

Catechins were extracted from healthy and *H. thievora* infested tea leaves of TV-18, one of the varieties which showed less attack by this pest in the field condition where other twenty two varieties were being grown. In this case, infestation by *H. theivora* on tea leaves was categorized as three different developmental stages (Plate 9 figs. B,C and D) in comparison with healthy non infested tea leaves (Plate 9 fig A).

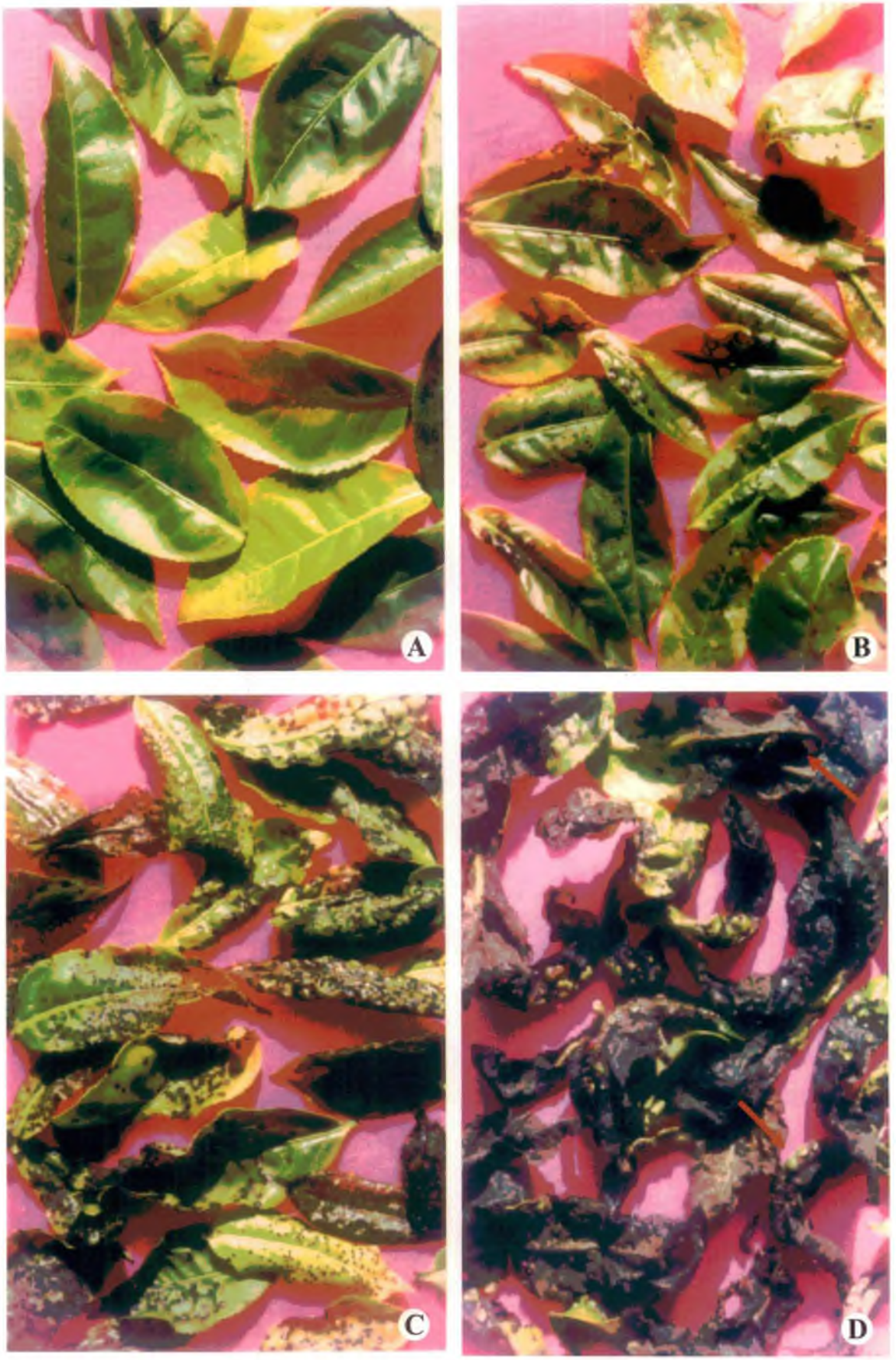


Plate 9 (figs. A-D): Healthy tea leaves (A) and stages of infestation by *H. theivora* (B-D). [Initial puncture (B); maximum puncture (C); advanced stage (D)].

Table 13: Quantitative estimation of pyrocatechol in healthy and *H. theivora* infested resistant and susceptible tea varieties

Tea varieties	Pyrocatechol concentration (μg/g fresh wt. of leaves)	
Resistant varieties	Healthy	*H. theivora* infested[a]
TV-18	102	389
TV-20	95	295
TV-22	87	280
Susceptible varieties		
UP-8	77	198
BSS-3	75	186

[a]24 h following infestation by *H.theivora*

These infested samples along with healthy leaves were analyzed by HPLC. In susceptible reaction leaf samples exhibited less isoforms of catechin than healthy control. In *H. theivora* infested leaves three peaks P4, P6 and P8 increased markedly in the initial stage of infestation (sateg-1) in relation to healthy leaves (Fig.8). P4 and P6 corresponded with authentic EGC and EGCG respectively. Tea plants amplified catechin production in the second stage of infestation where maximum peak heights were noticed. Four isoforms of catechin such as EGC, EC, EGCG, CG corresponding with the peaks - P4, P5, P6, P9 increased sharply during stage 2 infestation when *H. theivora* punctured maximum leaf areas. During stage 3, the advanced stage resulting due to infestation by *H. theivora,* levels of EGC, EGCG , GCG and ECG respectively corresponding with peaks - P4, P6 , P7 and P11 decreased markedly. However, some of the peaks (P2 and P10) did not affected due to infestation at this stage.

HPLC elution profiles of catechins from healthy and *H. theivora* infested tea leaves

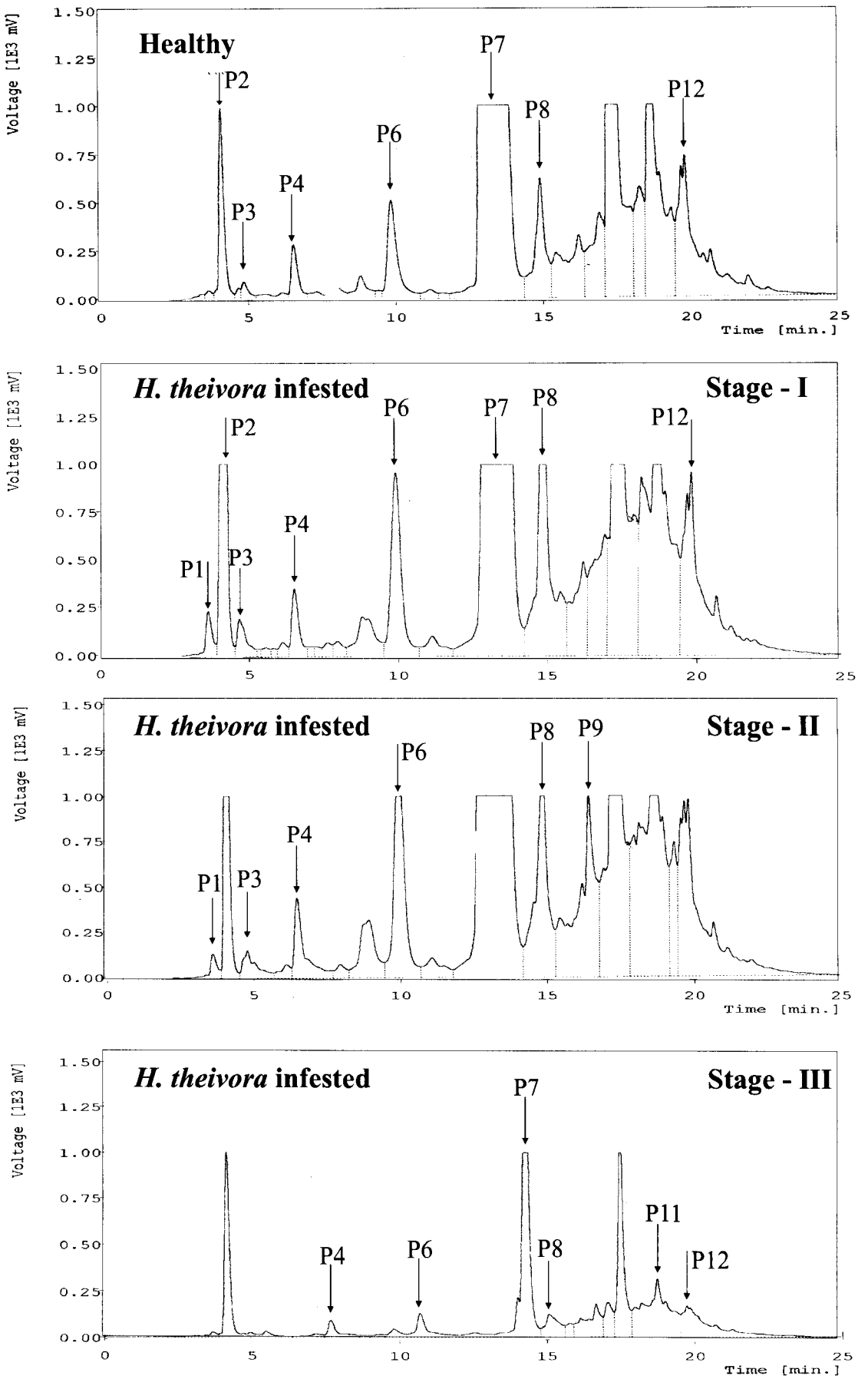


Fig. 8

Table 14: HPLC analyses of catechins extracted from healthy and variable stages of infestation by *H. theivora* on tea leaves (TV-18) in comparison with authentic catechin isomers

Peak No.	Corresponding with authentic isomers of catechins	Reten. time (min)	Height (mV)			
				H. theivora infested		
			Healthy	Stage I	Stage II	Stage III
P1	--	3.560	49.545	234.262	134.391	85.478
P2	GC	4.070	994.753	1008.527	1008.774	1009.648
P3	--	4.660	63.156	194.587	151.042	44.382
P4	EGC	6.530	288.124	315.558	443.379	128.776
P5	EC	11.160	47.272	103.614	107.431	23.037
P6	EGCG	13.860	998.981	999.263	999.912	1005.000
P7	GCG	14.910	620.086	998.154	998.892	191.738
P8	--	16.200	324.215	488.357	997.431	131.101
P9	CG	16.910	441.357	630.927	996.499	150.485
P10	--	17.530	995.224	995.631	995.386	1003.201
P11	ECG	18.740	994.005	994.441	740.305	411.953
P12	--	19.800	731.972	956.228	976.354	182.053
P13	--	24.420	5.072	4.883	6.603	44.933

GC – Galo-catechin

EGC – Epigalo-catechin

EC – Epicatechin

EGCG – Epigalo-catechingalate

GCG – Galo-catechingalate

CG – Catechin-galate

ECG – Epicatechin-galate

4.5 Application of bioresources for induction of resistance against *H. theivora*

During recent years, there has been a growing recognition that many of the synthetic compounds display a variety of adverse effect, and their use is often circumscribed. The need to control pest is even greater today, and attention is being refocused on natural products because they often have a reduced impact on the host and the environment, they may have desired specificity, and they may serve as prototype models for industrial involvement. In the present investigation biological resources involving metabass- a mycopesticide formulation, biocrop, plant product and as well as plant extract were considered for application in order to achieve pest management. Besides, Phenylalanine ammonia lyase (PAL), Tyrosine ammonia lyase (TAL) and polyphenol oxidase activities were also assayed following the application of above bioresources on seven selected UPASI varieties (UP-2, UP-3, UP-8, UP-9, BSS-1, BSS-2 and BSS-3) which were found to be more susceptible towards *H. theivora* showing 70-80% damage.

4.5.1 Metabass

The fungal biomass *M. anisopliae* and *B. bassiana* were isolated after 15 days of inoculation and blended in an electric mixture. It was strained through muslin cloth and mixed with TWEEN-20 to increase the potentiality and stability of the liquid formulation. Formulated mycopesticide (1:25 in distilled water) were used. Combination was sprayed at an interval of 15 days. The spraying was done only in the evening hours. After spraying metabass formulation disease incidence were recorded (Fig. 9). Incidence of pest attack reduced markedly following metabass application (Table 15). Activities of PAL, TAL and PPO following application of metabass were determined in untreated healthy (UH), treated healthy (TH), untreated infested (UI) and treated infested (TI) for all seven varieties. Results have been

presented in Figs.10 and 11; Table 17. In all the varieties maximum activity of PAL was noted in treated as well as treated infested samples. Trends in the TAL and PAL

Table 15: Incidence of attack by *H. theivora* in different tea varieties after treated with bioresources.

Varieties	Incidence of attack (% shoots affected)					
	DW[1]	MB[2]	BC[3]	AZ[4]	CA[5]	DE[6]
UP-2	44.76	31.12	35.45	41.45	38.45	39.11
UP-3	49.45	29.14	31.22	40.41	37.11	38.24
UP-8	44.56	27.12	30.32	37.17	35.45	37.19
UP-9	48.12	29.44	32.11	46.14	39.59	41.72
UP-26	46.38	32.11	35.44	42.79	35.66	38.59
BSS-1	51.43	33.65	39.55	46.82	42.79	44.11
BSS-2	52.48	29.14	33.44	46.12	40.23	43.19
BSS-3	56.12	30.12	32.45	49.74	42.75	45.67

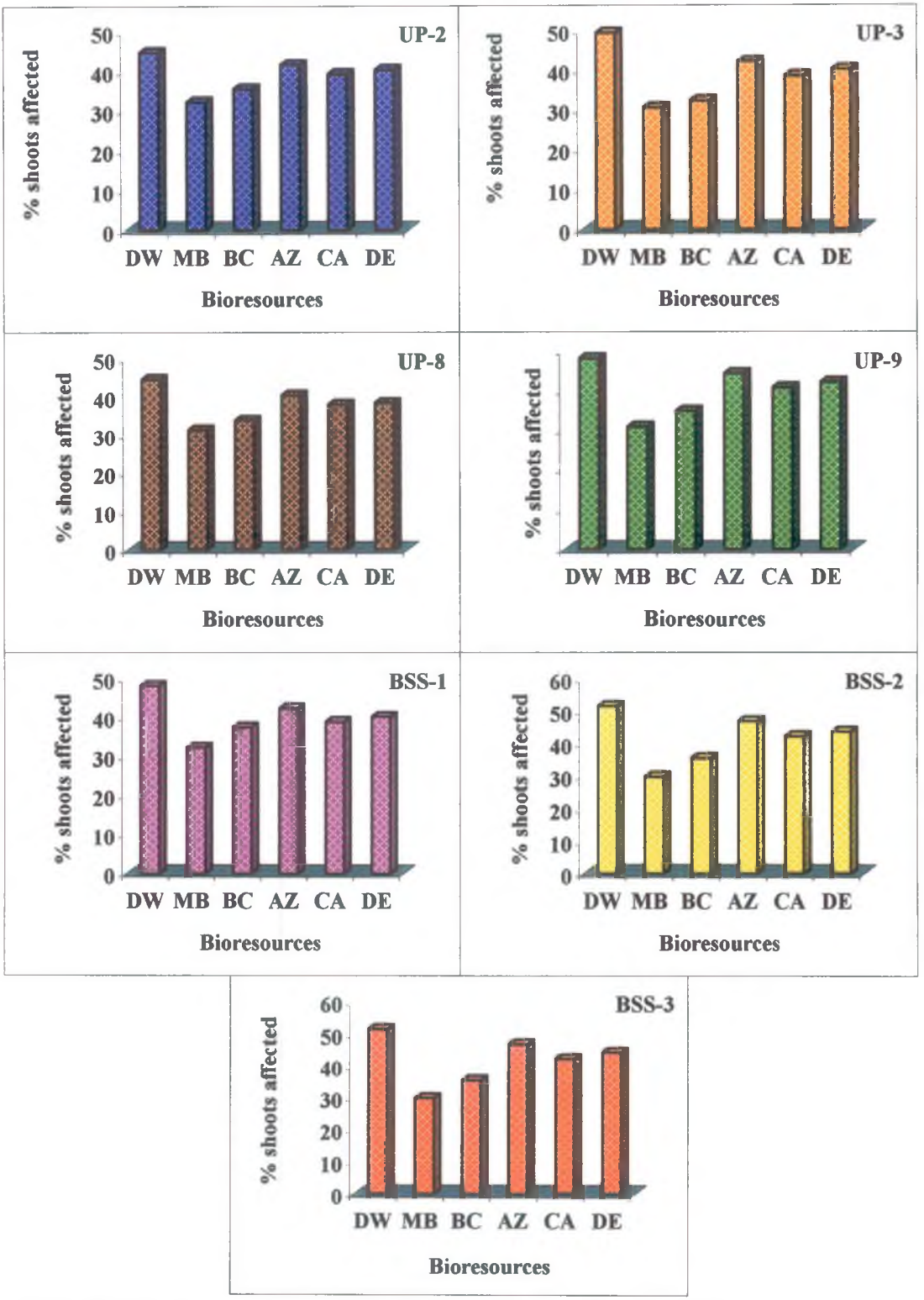
[1]Distilled water, [2]Metabass, [3]Boi-crop, [4]*A. indica* , [5]*C. roseus*, [6] *D. esculentum*

Analysis of variance of data presented in Table 15

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	7	293.349	41.907	9.4518	2.96631
Time interval	5	1897.016	379.403	85.5712	
Error	35	155.182	4.434		
Total	47	2345.547			

activities after metabass application were similar. PPO activity markedly enhanced following infestation. However, metabass application induced accumulation of PPO in the treated plants which further increased following infestation with *H. theivora* (Fig.11)

Incidence of attack by *H. theivora* in different UPASI varieties following treatment with bioresources



DW - Distilled water
MB - Metabass formulation
BC - Biocrop formulation
AZ - *Azadirachta indica*
CA - *Catharanthus roseus*
DE - *Diplazium esculentum*

Fig. 9

4.5.2 Biocrop

It is a very complex mixture of organic constituents of which has their counterparts in biological tissue of plants. Before spraying it was also mixed with TWEEN-20 and its application dose was 500ml to 5 liter of water and 40% of foliar application. Disease incidence were also noted (Table 15) and plotted (Fig. 9). Activities of PAL (Fig.10), TAL (Table 17) and PPO (Fig.11) following treatment of plants (UP-2, UP-3, UP-8, UP-9, BSS-1, BSS-2 and BSS-3) after treatment biocrop were increased. Pest infestation in the field test was also reduced. (Fig. 9).

4.5.3 Plant extract

Four hundred grams of mature leaves of *Azadirachta indica*, *Catharanthus roseus* and *Diplazium esculentum* were homogenized in water. After centrifugation the crude leaf extract were diluted as 1:10 in distilled water mixed with TWEEN-20 and sprayed on tea plants (UP-2, UP-3, UP-8, UP-9, BSS-1, BSS-2 and BSS-3). The control plants were sprayed with distilled water mixed with TWEEN-20. Leaves from control and treated plants were sampled. Incidence of attack by *H. theivora* was noted.. The results of disease incidence have been presented in Table 15 and Fig. 9. PAL activity following treatment with *A. indica* and *D. esculentum* have been incorpoarted in Table 16 while the result with *C. roseur* were plotted in fig.10. PAL and TAL activities (Table 18) increased following application with these plant extracts. Analyses of polyphenol activity in UPASI varieties following treatments with three plant extracts revealed that all the varieties exhibited increased level of PPO following infestation. When compared with the application of plant extract, the level increased in relation to healthy control. Whereas a significant increase in the level of PPO was noticed in plant extract treated plants when exposed to *H.theivora* infestation in field condition. Among three plant extracts tested, *C.roseus* exhibited better response against pest attack. UP-3, UP-9 showed highest level of PPO activity followed by UP-8, BSS-1, UP-2, BSS-2 and BSS-3 (Fig 12).

Table 16: Activities of phenylalanine ammonia lyase (PAL) in plant extracts treated healthy and *H. theivora* infested tea leaves.

Varieties	PAL activity (µg cinnamic acid g^{-1} leaf tissue min^{-1})					
	Untreated DW (C)[a]		Treated			
			A. indica		*C. roseus*	
	H	I[b]	T[c]	I[b]	T[c]	I[b]
UP-2	159.4 ±14.1	241.5 ±11.4	276.4 ±12.4	337.1 ±14.3	297.3 ±10.3	334.8 ±13.3
UP-3	164.7 ±13.4	234.7 ±11.6	286.9 ±16.2	356.9 ±11.4	326.7 ±12.7	386.7 ±16.3
UP-8	156.7 ±11.2	235.7 ±16.4	279.4 ±14.2	308.7 ±12.4	286.7 ±13.1	324.8 ±14.6
UP-9	146.7 ±11.2	243.8 ±10.3	225.4 ±11.4	256.7 ±13.6	276.9 ±12.9	353.7 ±13.6
BSS-1	153.7 ±12.3	192.7 ±11.2	246.7 ±13.7	286.7 ±14.1	234.7 ±13.7	302.7 ±13.8
BSS-2	168.7 ±14.7	204.3 ±16.3	235.7 ±12.3	286.9 ±14.4	246.7 ±13.7	296.2 ±11.2
BSS-3	164.9 ±11.9	199.3 ±13.3	210.4 ±14.4	226.4 ±12.9	256.7 ±11.9	296.7 ±10.8

± Standard error.

[a]Distilled water control

[b]Infested with *H. theivora*

[c]Treated with plant extracts

Phenylalanine ammonia lyas activity in tea varieties following treatment with bioresources against *H. theivora* attack

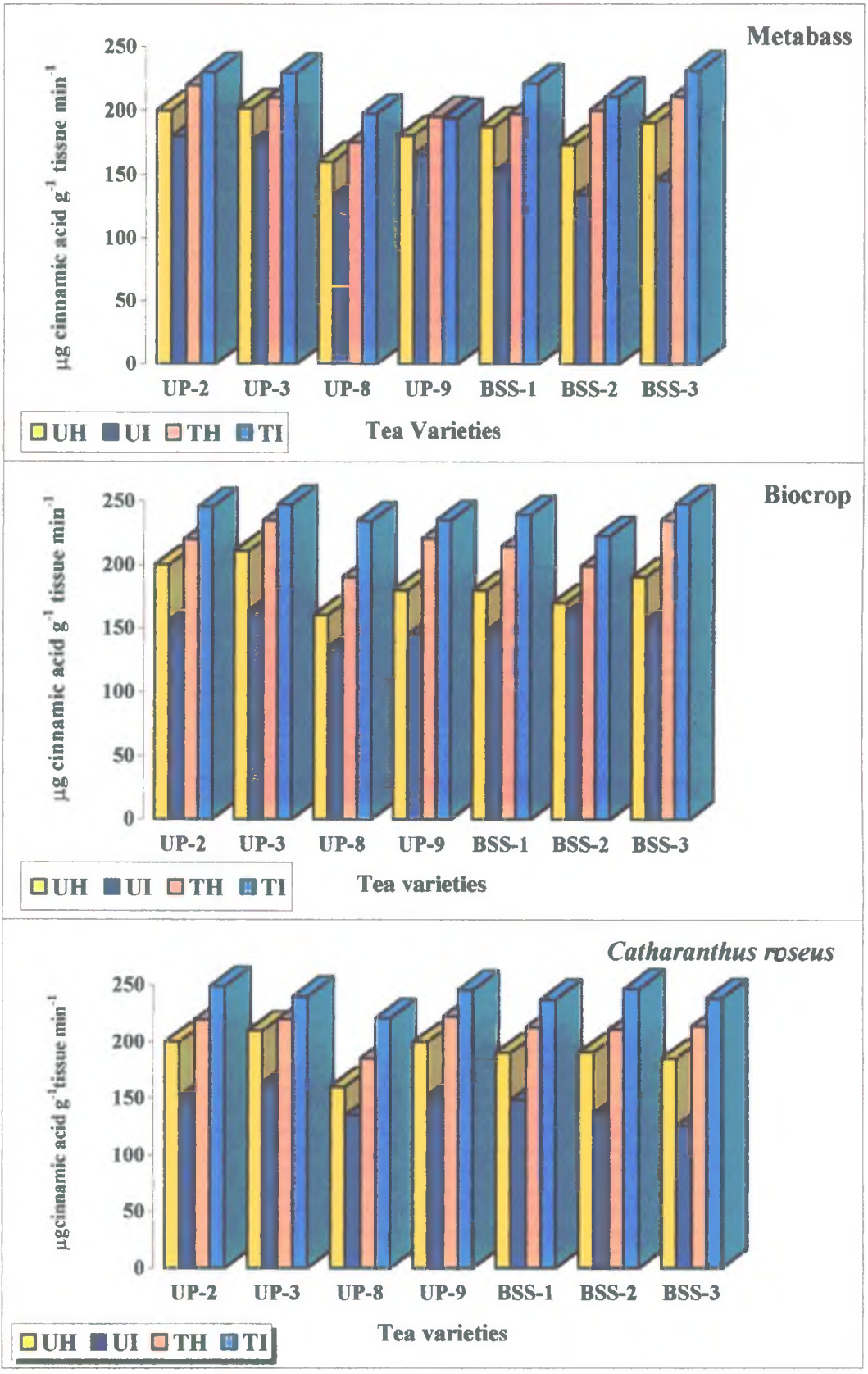


Fig. 10

Table 17: Activities of tyrosine ammonia lyase (TAL) in bioresources treated healthy and *H. theivora* infested tea leaves

Varieties	TAL activity (µg cinnamic acid g^{-1} leaf tissue min^{-1})					
	Untreated DW (C)[a]		Treated			
			Metabass		Biocrop	
	H	I[b]	T[c]	I[b]	T[c]	I[b]
UP-2	223.5 ± 12.5	135.7 ± 13.7	243.8 ± 11.2	286.4 ± 11.4	234.8 ± 12.3	267.9 ± 14.1
UP-3	215.4 ± 10.2	153.8 ± 11.8	234.7 ± 13.4	286.8 ± 14.3	234.8 ± 14.2	256.7 ± 13.1
UP-8	189.4 ± 13.4	135.4 ± 11.7	234.7 ± 16.1	294.2 ± 14.4	221.4 ± 15.6	253.9 ± 18.9
UP-9	213.7 ± 12.3	134.7 ± 11.9	235.7 ± 13.3	273.8 ± 13.1	216.7 ± 13.9	253.8 ± 11.7
BSS-1	192.7 ± 12.3	126.7 ± 13.7	234.5 ± 11.5	286.7 ± 14.8	243.7 ± 12.7	256.7 ± 13.6
BSS-2	199.2 ± 11.2	134.7 ± 13.7	234.8 ± 17.1	279.4 ± 11.4	227.9 ± 12.7	258.4 ± 13.6
BSS-3	210.6 ± 12.3	129.7 ± 11.9	235.7 ± 13.5	286.7 ± 14.1	231.8 ± 13.6	267.8 ± 14.8

± Standard error.

[a]Distilled water control

[b]Infested with *H. theivora*

[c]Treated with bioformulation

Table 18: Activities of tyrosine ammonia lyase (TAL) in plant extract treated healthy and *H. theivora* infested tea leaves

Tea varieties	Treatment	TAL activity (μg cinnamic acid g^{-1} leaf tissue min^{-1})		
		A. indica	*C. roseus*	*D. esculentum*
UP-2	UH	284.3 ± 12.3	274.8 ± 10.8	269.8 ± 12.9
	UI	159.4 ± 15.4	167.4 ± 11.8	143.7 ± 13.7
	TH	295.3 ± 11.2	286.9 ± 12.6	286.7 ± 14.7
	TI	315.7 ± 12.8	307.9 ± 13.9	310.8 ± 11.8
UP-3	UH	222.2 ± 13.2	211.5 ± 13.2	227.6 ± 10.6
	UI	167.2 ± 14.2	156.2 ± 12.2	153.2 ± 12.2
	TH	235.4 ± 10.9	235.7 ± 14.8	246.7 ± 13.7
	TI	256.8 ± 11.6	249.7 ± 16.2	286.7 ± 12.1
UP-8	UH	189.1 ± 12.7	199.3 ± 13.3	199.3 ± 11.8
	UI	153.6 ± 12.6	164.9 ± 11.9	164.9 ± 11.9
	TH	210.5 ± 16.5	225.8 ± 13.8	210.4 ± 12.4
	TI	236.8 ± 13.6	276.8 ± 14.8	226.4 ± 14.7
UP-9	UH	213.7 ± 11.8	219.7 ± 10.7	226.4 ± 10.4
	UI	173.8 ± 13.8	179.4 ± 12.4	180.4 ± 12.4
	TH	225.4 ± 11.4	236.4 ± 12.5	234.9 ± 11.9
	TI	256.7 ± 16.1	264.7 ± 13.7	276.4 ± 13.4
BSS-1	UH	226.4 ± 10.4	221.5 ± 14.5	224.7 ± 14.7
	UI	155.6 ± 12.6	165.7 ± 11.7	152.4 ± 13.4
	TH	246.8 ± 10.8	235.7 ± 12.2	245.5 ± 11.5
	TI	285.7 ± 11.7	258.9 ± 13.9	279.4 ± 12.4
BSS-2	UH	200.7 ± 13.9	195.4 ± 10.4	192.7 ± 12.7
	UI	160.4 ± 12.4	153.4 ± 11.4	153.7 ± 11.7
	TH	213.7 ± 13.7	225.7 ± 13.7	234.7 ± 16.8
	TI	256.4 ± 12.4	256.8 ± 12.1	286.7 ± 13.2
BSS-3	UH	210.9 ± 10.9	213.7 ± 10.7	211.4 ± 11.4
	UI	159.5 ± 11.4	145.6 ± 12.6	162.4 ± 12.8
	TH	225.7 ± 13.7	226.7 ± 13.7	234.7 ± 13.7
	TI	259.8 ± 12.8	249.7 ± 11.7	259.7 ± 12.9

UH – Untreated Healthy; UI – Untreated Infested;
TH – Treated Healthy; TI – Treated Infested.
Means of three replicates
± Standard error.

Polyphenol oxidase activity in tea varieties following treatment with bioformulation against *H. theivora* attack

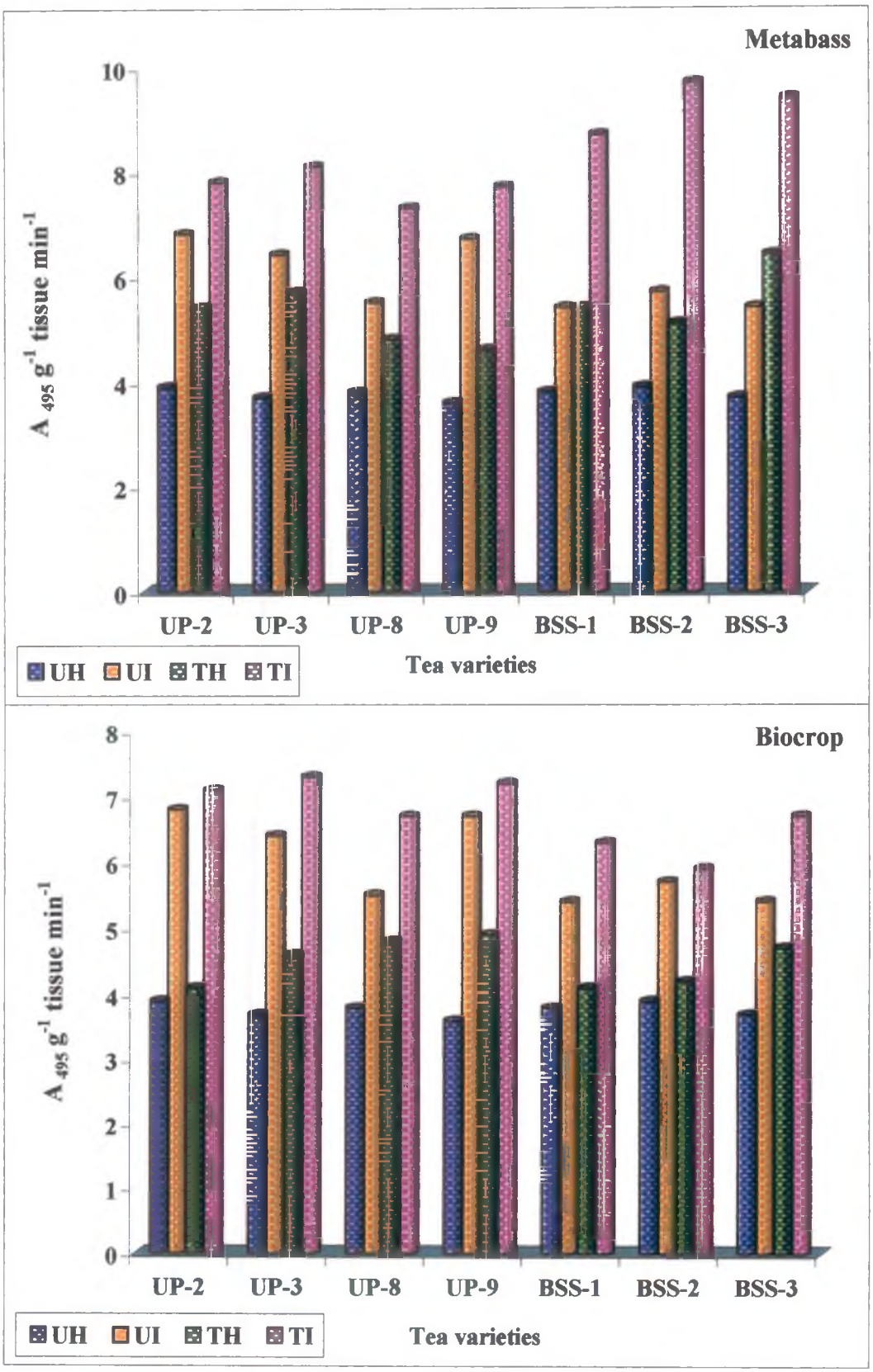


Fig. 11

Polyphenol oxidase activity in tea varieties following treatment with plant extracts against *H. theivora* attack

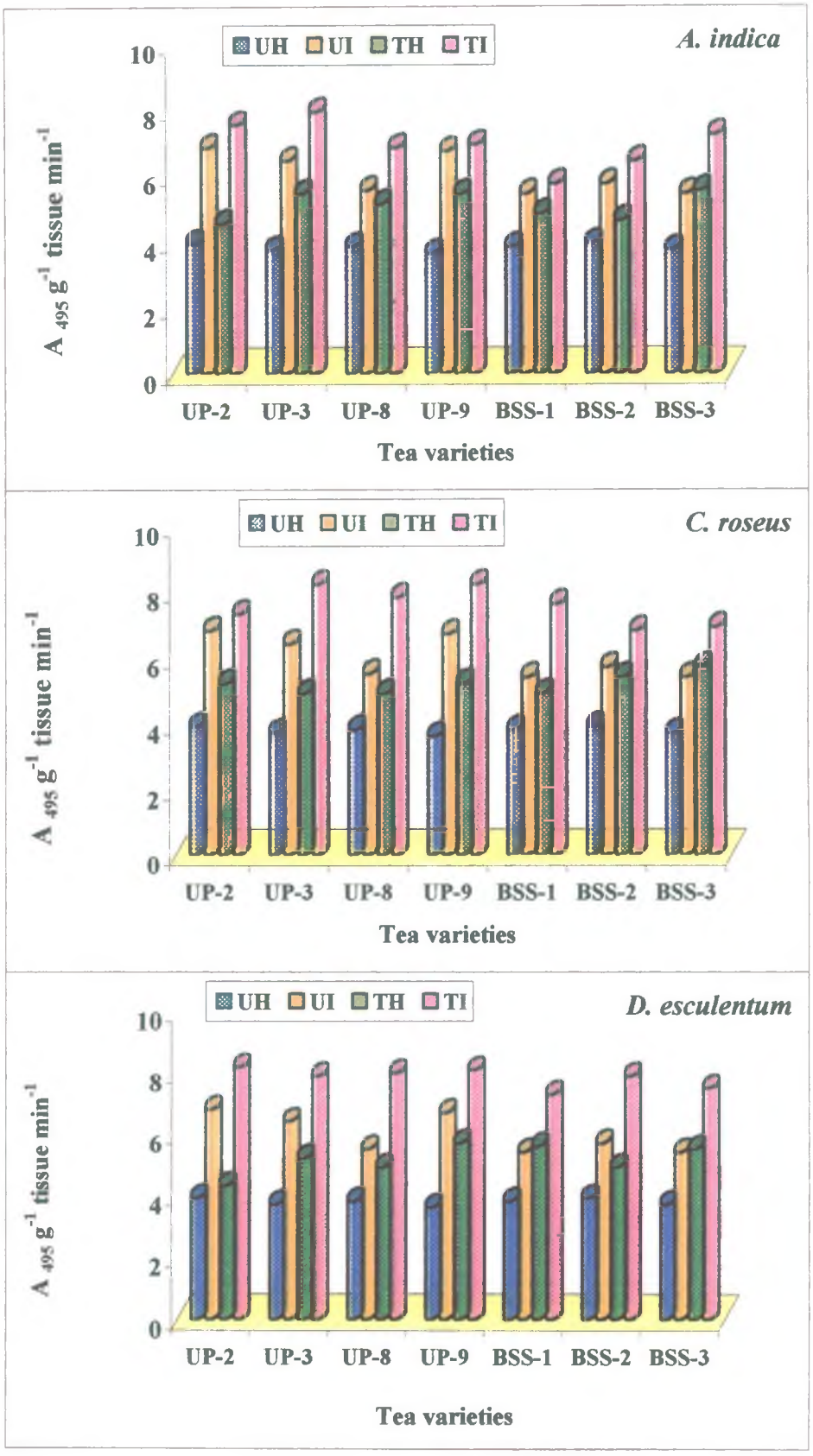


Fig. 12

4.6 Screening of resistance of tea plants against foliar fungal pathogens

In the present investigation two important foliar fungal diseases viz. leaf blight caused by *Alternaria alternata* and Black rot caused by *Corticium invisum* which are prevalent in the Doors, have been selected. Alternaria leaf blight symptoms appear as greyish brown patches on the younger leaves. Older leaves were found less susceptible. Symptoms first appear in the tip region and the margin of the leaves, which extend towards the midrib (Plate 10 figs.B&C) following which the leaves curl and die. It causes serious infection leading defoliation of leaves. It causes considerable damage to the plants maintained in the nursery (Plate 11 figs. A-E) as well as in the field. The plants in the nursery where disease was found were growing under ideal conditions (i.e. not stressed) and were not affected by any other pathogens. Overall 70% loss in the nursery has been noticed which crossed the threshold level. The fungus which was consistently isolated from the margins of these lesions, onto potato dextrose agar (PDA) amended with streptomycin sulphate, maintained at 28°C identified as *Alternaria alternata* (W8053) and was confirmed by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience Centre UK.

Hyphae and conidiophores were brown and septate. Conidia (23 –34 x 7-10 μm) were usually solitary, rarely in chains of two with transverse and longitudinal septation. Germination of spores is mainly from the apex or from any septation. Germ tubes sometimes branch just after formation. (Plate 12 fig. A). Another isolate(W8055) produced hyaline hyphae which turn olive-buff to deep olive buff on maturity. Conidiophores of the same colour, measuring 17-28 X 3-6μ, are produced through the stomata, bearing conidia produced acrogenously, single, smooth, irregular oval, both ends rounded, ellipsoidal, gradually tapering into a beak (Plate 12 fig.B). They are light brown to dark olive-buff, multicellular, with 1-5 transverse septa and 1-3 longitudinal septa, and measure 15-89 X 7-30μ. Scanning electron microscopical observations of these conidia have been presented in Plate 12 fig.C.

Plate 10 (figs. A-C): Healthy (A) and natural infection by *A. alternata* (B&C). [T-17 (A&B); TV-22 (C)].

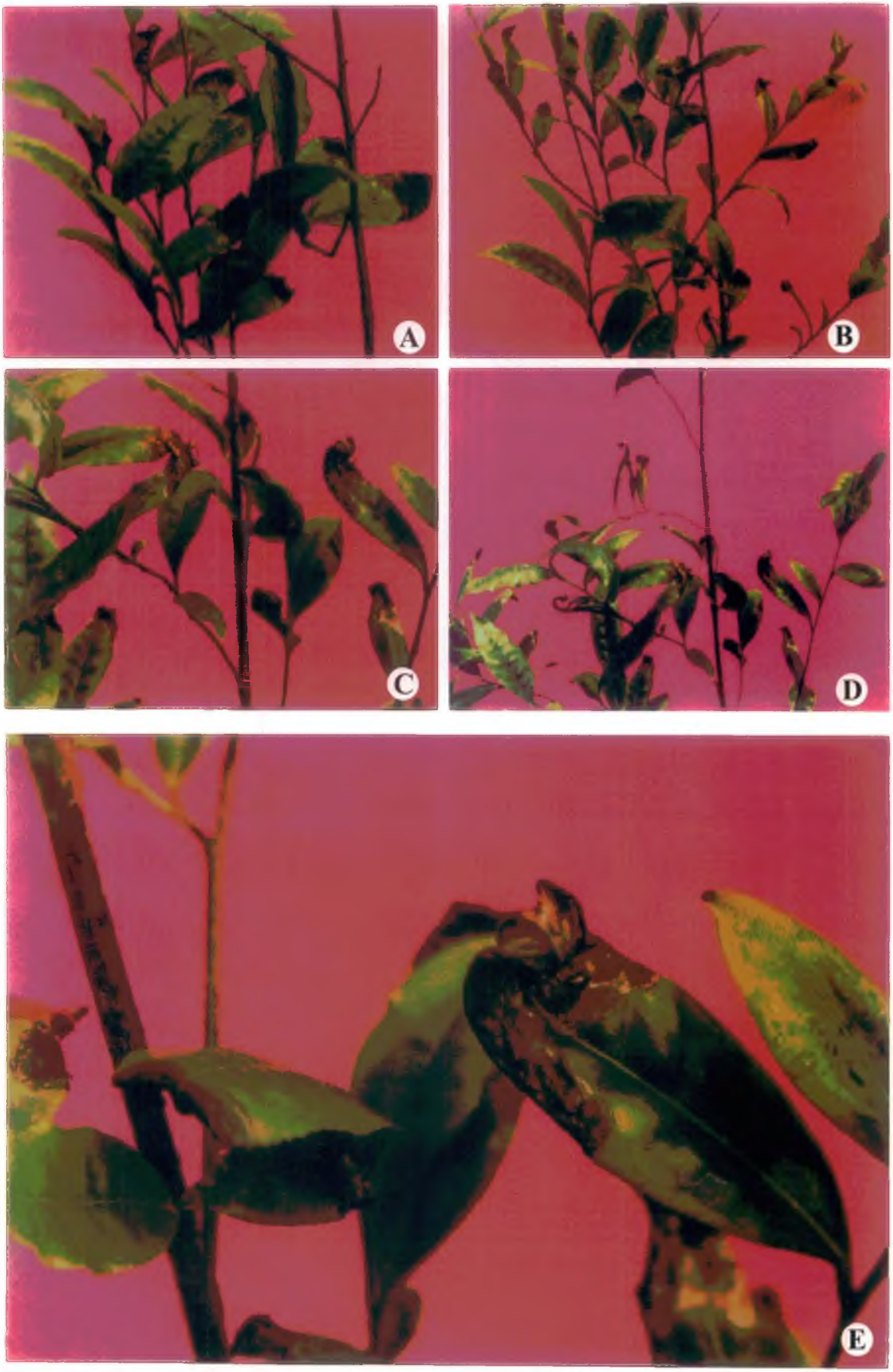


Plate 11 (figs. A-E): Natural infection caused by *A. alternata* on different tea varieties [(A) TV-20; (B) TV-25; (C) UP-8; (D) BSS-1 and (E) BS/7A/76].

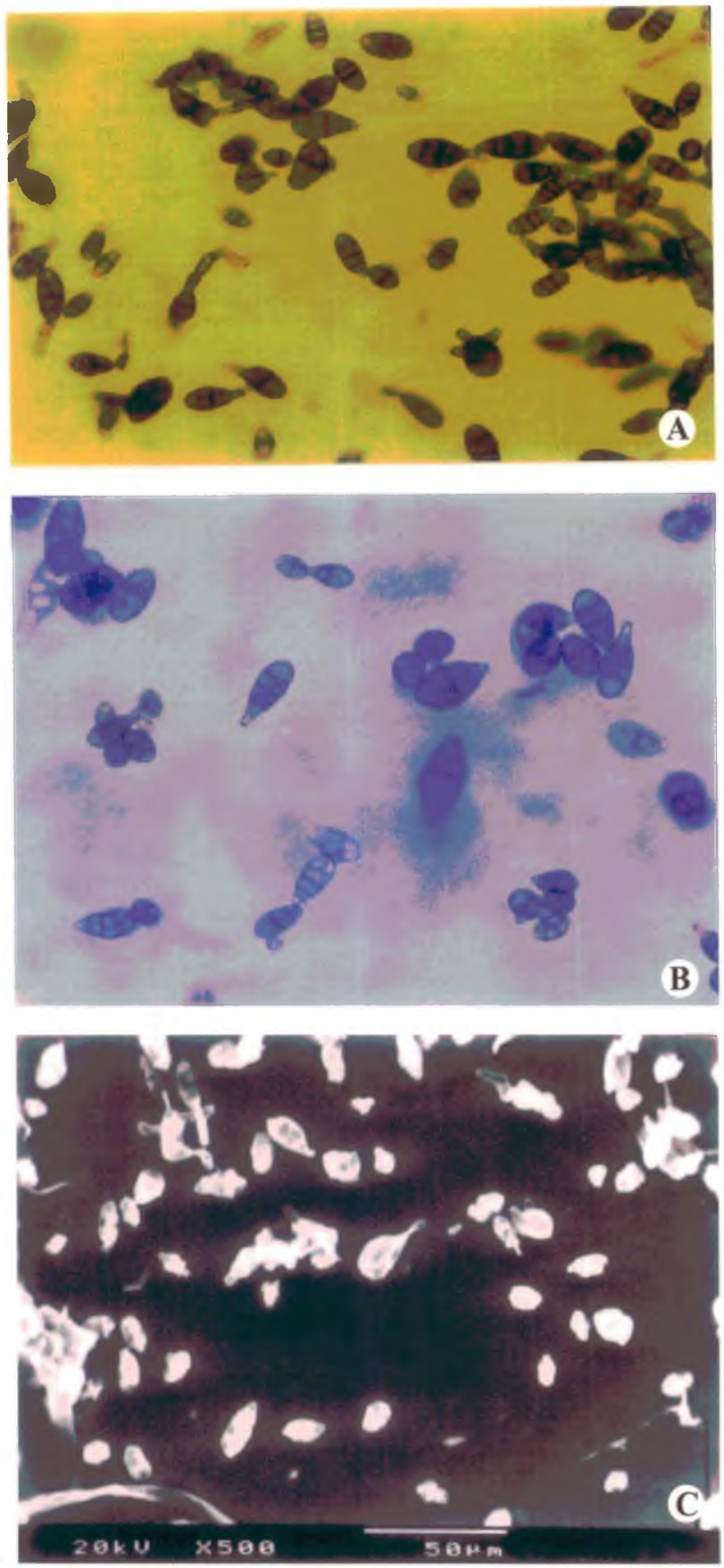
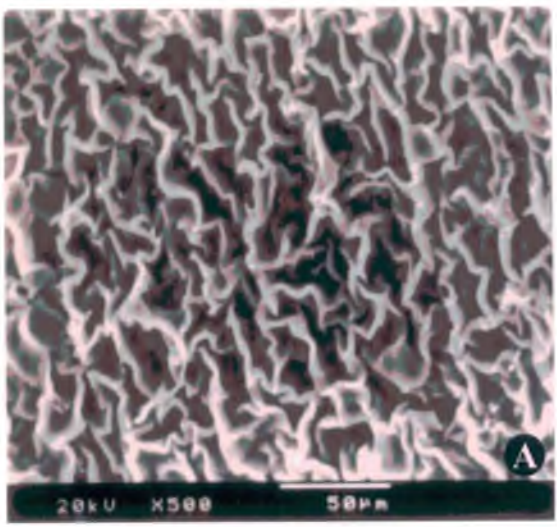


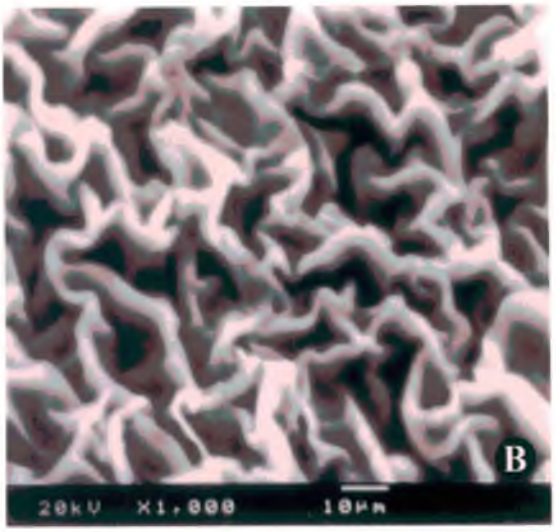
Plate 12 (figs. A-C): Conidia of *A. alternata.* (A&B) Bright field (C) scanning electron microscopy [(A&C) Isolate - W8053 (B) Isolate - W8055].

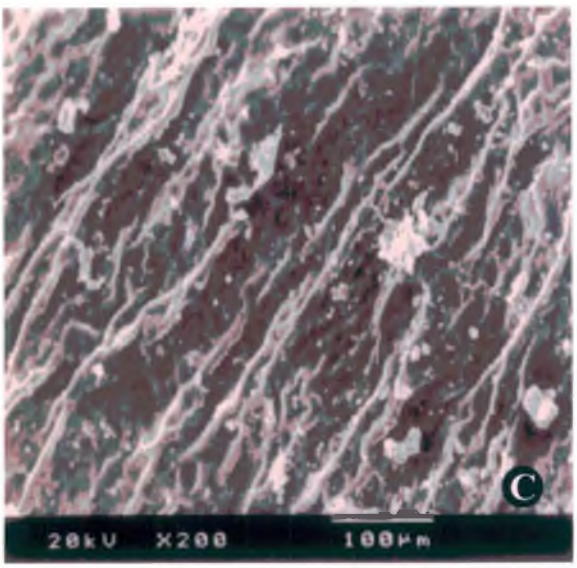
Alternaria blight infected areas were observed under scanning electron microscopically and compare with healthy leaf surface. Microphotographs were taken at different magnifications. The ornamentation patterns revealed by SEM have been presented in Plate 13 (fig.A-F). Ornamentation present in healthy leaf surface was continuous (Plate 13 figs.A&B) while in blight areas such ornamentation was distorted. Fungal mass was also visible (Plate 13 figs. C-F).

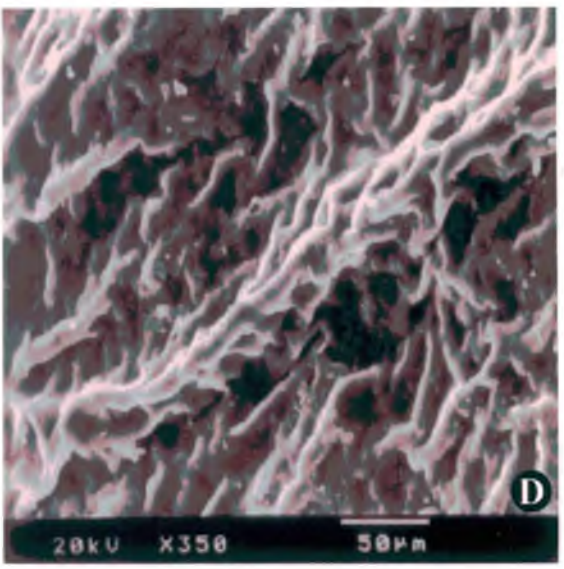
Blight refer to diseases that kill suddenly. In a diseased plant, due to severity of infection the tissues are rapidly killed, resulting in death of foliage, blossom or other above-ground plant parts. *Alternaria* enter the host by direct penetration, and in such cases they prefer young developing leaf tissue. The disease symptoms may be considered as the signs of reaction of the host to infection by the pathogen. Young leaves are more rapidly penetrated and infected by the pathogen than the mature parts. Histopathological studies of naturally blight infected tea leaves revealed that the pathogen mainly affect the palisade and spongy parenchymatous tissue. Browning reactions were evident in mesophyll tissues of infected leaves which were not found in healthy leaves (Plate 14 fig. A).

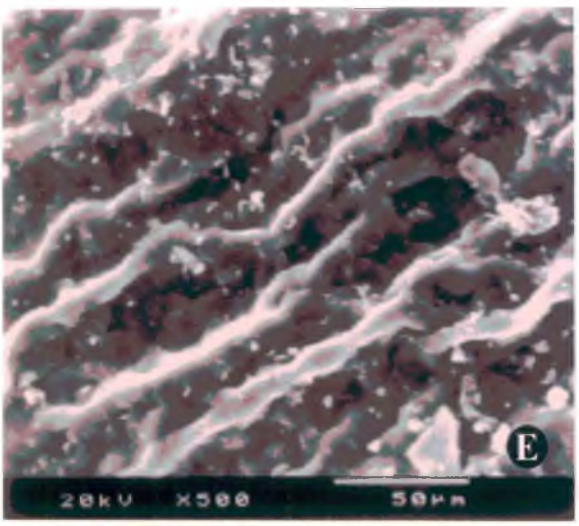
Black rot disease caused by *Corticium invisum* is more prevalent on tea which has been cut across without any cleaning out, unskiffed and unpruned tea. *C. invisum* produce on the tea leaves large patches covering about half and sometimes the entire leaf area (Plate 15 fig.A). Colour on the upper surface of the affected area at the early stage is reddish-brown, similar to sunscroch damage, later it is a mixture of brown, yellowish-brown and grey (Plate 15 fig. D&E); the undersurface is light brown or greyish-white and usually covered with a network or cream to brown mycelium and finally leaf become rotten. Diseased leaves often remain attached to other leaves and stems, held together by small cushions or films of pinksh-white or cream coloured











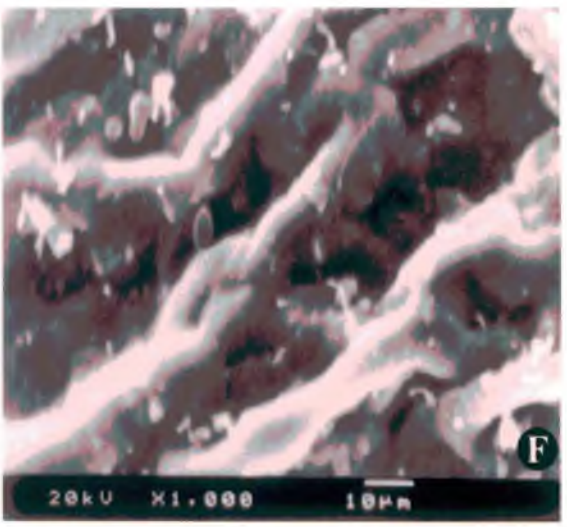


Plate 13 (figs. A-F): Scanning electron microscopic observation of adaxial surface of healthy tea leaf (A&B) and Alternaria blight infected leaf surface(C-F).

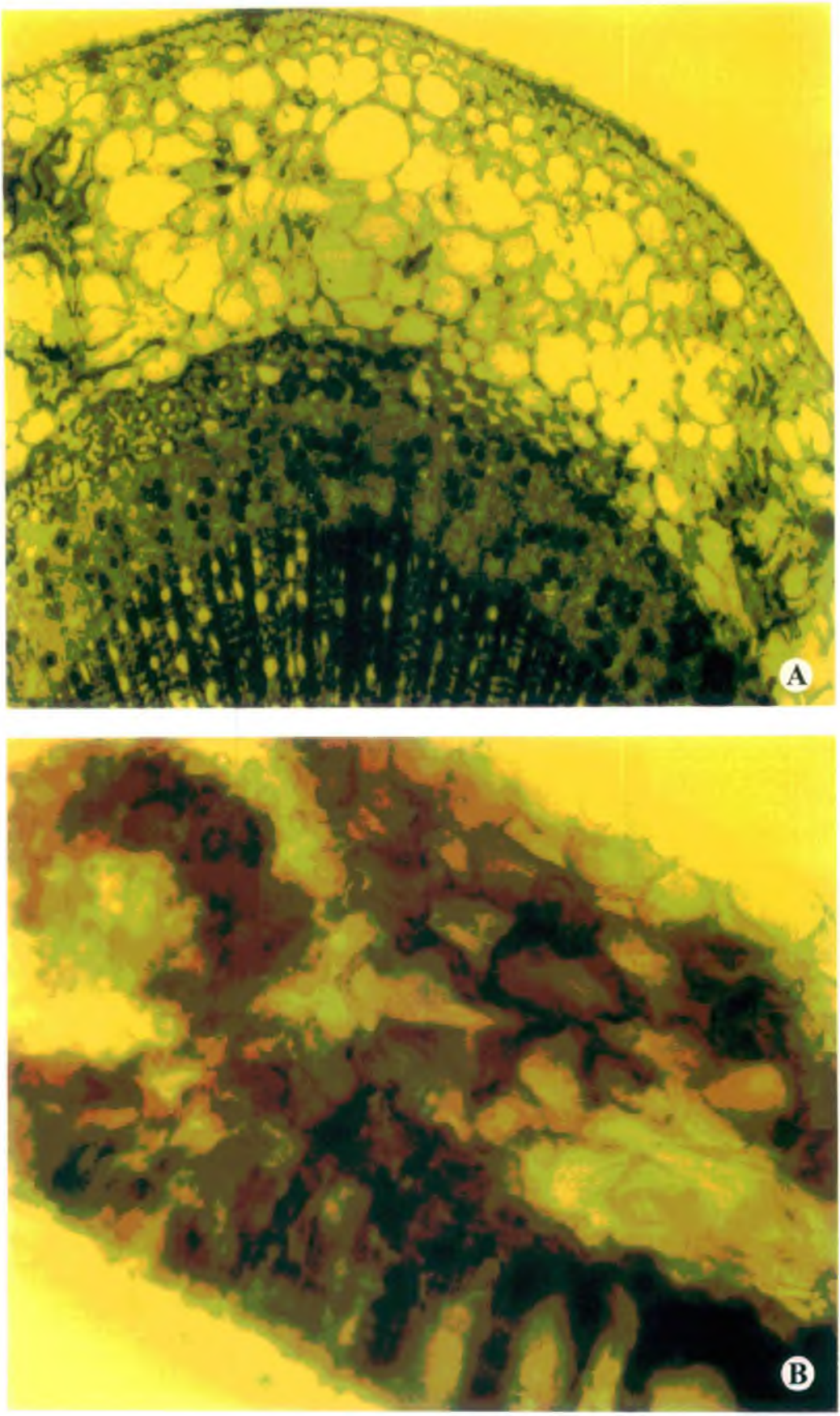


Plate 14 (figs. A&B): Histopathology of healthy (A) and *A. alternata* infected tea leaves (B)

mycelium. The fungus produced on the stem, thick crods of mycelium, up to about 3 mm across, dark purplish-brown on the older portions of the stem and dull white to light brown on the green portions at the top. The fungus produces minute resting bodies (sclerotia) in the cracks and crevices of the stem towards the end of rainy season. Fructifications appear, during the rainy season, as white, dusted patches on the undersurface of mature, green leaves. *Corticium* is characterized by smooth hymenophore and absence of cystidia or gloeocystidia. The hyphae of this species are hyaline, septate, profusely branched with numerous basidia, which arise in groups.

In early stages of growth of *C. invisum* in pure culture mycelia at first silky-white but gradually become black in appearance. Often the aerial hyphae appear as dense tufts dispersed all over the culture medium (Plate 15 Fig B&C). Sometimes the hyphae aggregate into strands of rhizomorph like structure. Individual hyphae are hyaline, thin-walled, sparsely septate when young. The cells have been reported to be 60 – 350 μm long and 2 – 8 μm wide. Sclerotial initials are formed from hyphal strands which consist of 3 – 12 hyphae lying parallel. With further hardening differentiation takes place and the sclerotium show an outer layer of polyhedral cells. Rind cortex and medulla are differentiated as the sclerotium darkens. Mature sclerotia are dark brown by variation from lighter to darker colour may found.

When the affected tissues disintegrate the symptom is rot. In advancing stages of certain leaf rot, dry and form a dark, wrinkled. *C. invisum* also infect the mesophyll tissue (Plate 16 fig.B) which shows browning reactions.

Plate 15 (figs. A-E): Black rot disease of tea (A,D&E) caused by *Corticium invisum* (B & C)

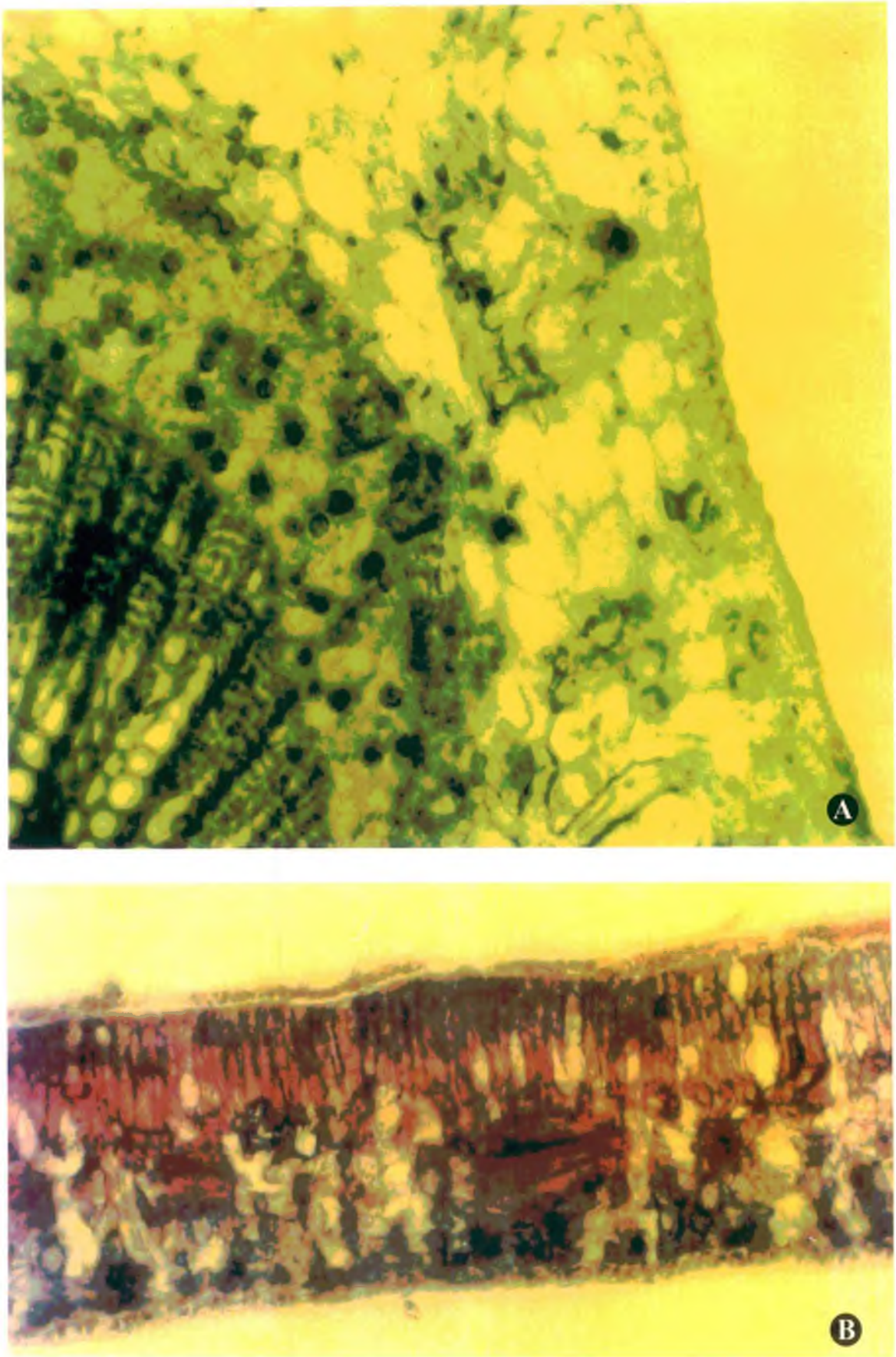


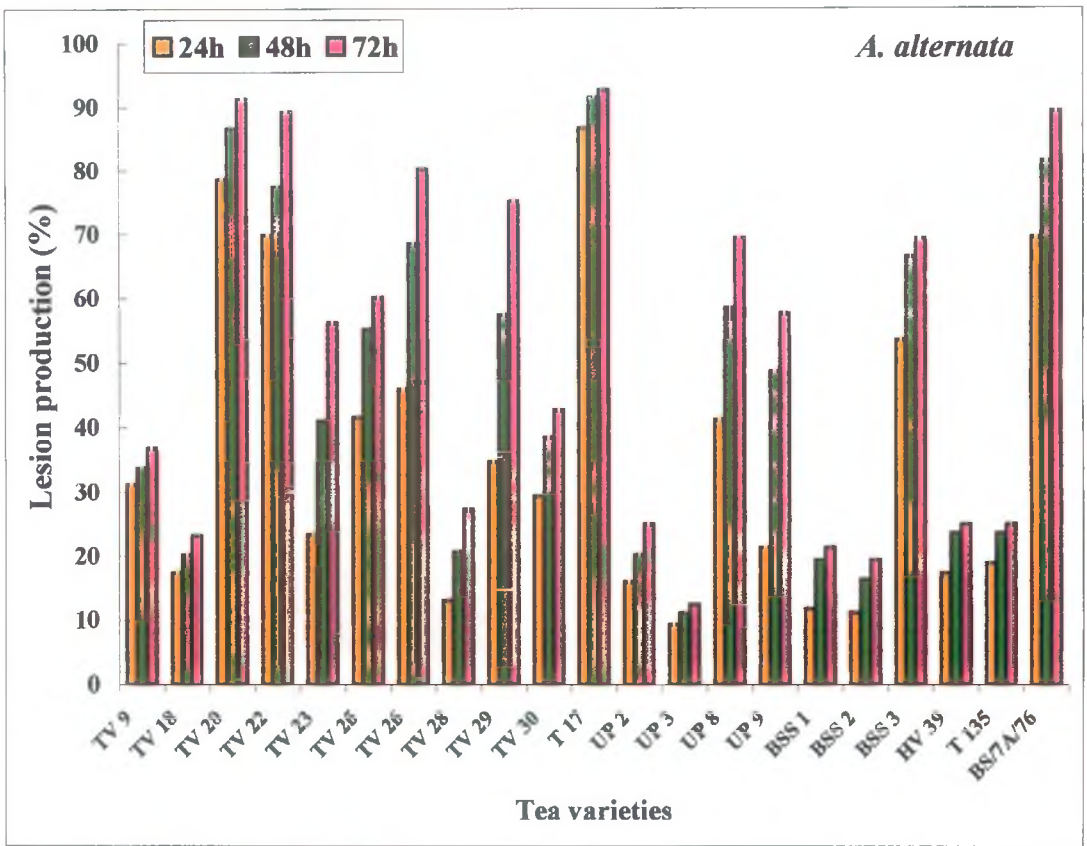
Plate 16 (figs. A&B): Histopathology of healthy (A) and *C. invisum* infected tea leaves (B)

Twenty three varieties of tea, of which twelve released by Tocklai Experimental Station, Jorhat, Assam, eight released by UPASI Tea Research Centre, Valparai, Tamilnadu and three released by Darjeeling Tea Research Centre, Kurseong, were used for screening of resistance separately against *A. alternata* and *C. invisum.* Detached leaf and Whole plant inoculation techniques were followed for screening resistance against *A. alternata*, while detached leaf and cut shoot inoculation techniques were followed for screening resistance against *C. invisum.*

4.6.1 Detached leaf

Artificial inoculation of detached leaves of twenty-three varieties were carried out separately by spore suspension of *A. alternata* and inoculum of *C. invisum* as described previously. After 24, 48 and 72 hrs of inoculation, assessment of inoculation infectivity and symptom development were done on the basis of percent drops that resulted in disease lesion production, which were determined from 50 leaves of each variety after every interval separately. Experiments were repeated thrice. Results revealed that (Fig.13), T-17, TV-20, TV-27, UPASI-9, UPASI-26 and T-78 were found to be most susceptible followed by TV-25, TV-29, T-135 and UPASI-8 which were found to be moderately resistant against *A. alternata.* On the basis of significant tests the varieties grouped into highly susceptible (T-17, TV-20, TV-22, UPASI-8, BSS-3 and T-78) moderately resistant (TV-9, TV-18, T-135 and UPASI-3) and moderately susceptible (TV-30, TV-25, and HV-39)

In case of *C. invisum*, TV-22, TV-23, TV-28, HV-39, UPASI-26, BSS-1 were categorized as most susceptible where as TV-30, TV-27, T-135, UPASI-9 were found to be moderately resistant (Fig. 13).


24h
48h
72h
A. alternata
Lesion production (%)
100
90
80
70
60
50
40
30
20
10
0
TV 9
TV 18
TV 20
TV 22
TV 23
TV 25
TV 26
TV 28
TV 29
TV 30
T 17
UP 2
UP 3
UP 8
UP 9
BSS 1
BSS 2
BSS 3
HV 39
T 135
BS/7A/76
Tea varieties

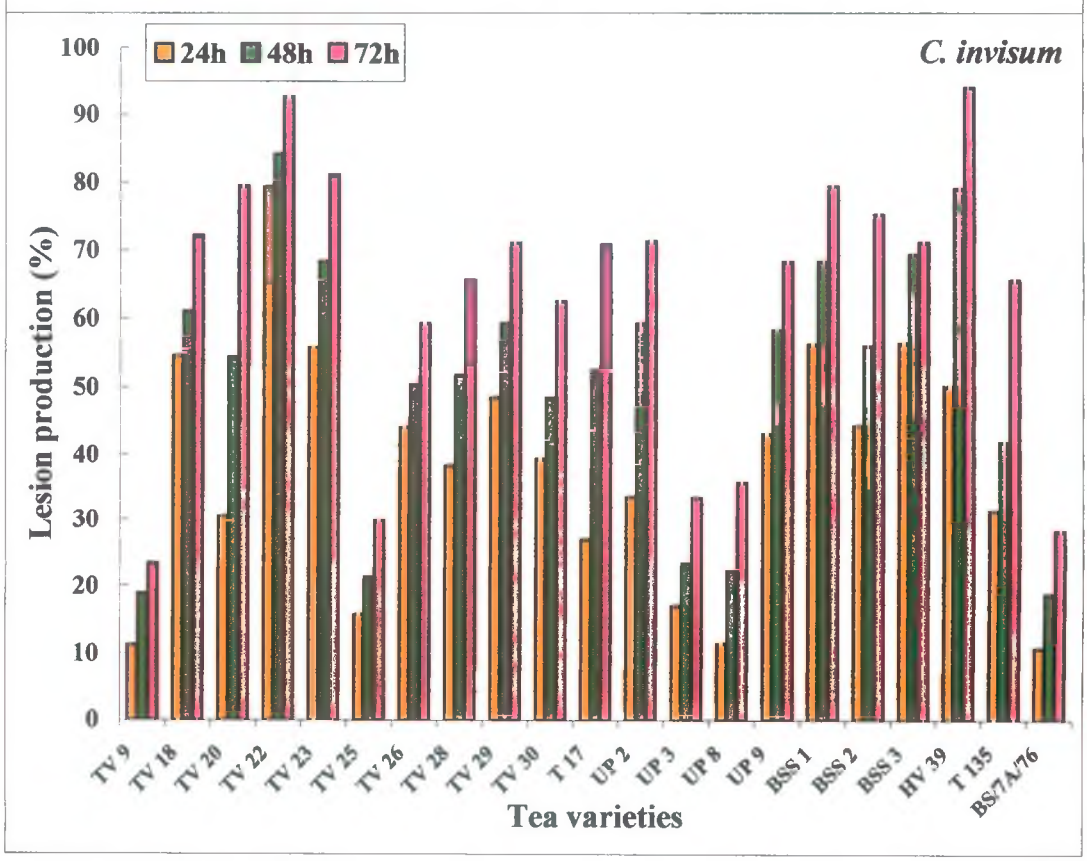

24h
48h
72h
C. invisum
Lesion production (%)
100
90
80
70
60
50
40
30
20
10
0
TV 9
TV 18
TV 20
TV 22
TV 23
TV 25
TV 26
TV 28
TV 29
TV 30
T 17
UP 2
UP 3
UP 8
UP 9
BSS 1
BSS 2
BSS 3
HV 39
T 135
BS/7A/76
Tea varieties

Fig.13

4.6.2 Cut shoot

Screening of twenty-three tea varieties were also tested against *C. invisum* by cut shoot inoculation technique. Disease was assessed on the basis of mean number of lesion per shoot from which highest mean disease index was evident in TV-22, TV-28, UPASI-26 and HV-39 and lowest in TV-9, TV-25 (Table 19). Cut shoot of TV-23, TV-28 exhibited lesions variable size (2-3 mm diam), which appeared after 48 h of inoculation. In contrast, at this interval cut shoot of TV-9, TV-25 exhibited restriction lesions. In case of UPASI varieties, UPASI-26 showed the highest index and UPASI-3 exhibited the lowest disease index value. Among the varieties tested, TV-22 and UPASI-26 were found to be most susceptible followed by TV-23, BSS-1 while TV-9 was the most resistant followed by TV-25, UPASI-8 on the basis of cut shoot inoculation. Analysis of variance of data presented in Table 19 was computed and presented below.

4.6.3 Whole plant

Twenty-two varieties of well established pot grown tea plants were inoculated with spore suspension of *A. alternata*. Disease assessment was done after 5, 10, 15, 20 and 25 days of inoculation. The degree of resistance in each variety corresponded with the other method of inoculation viz. detached leaf. In TV-22, T-78, BSS-1 and T-17 disease intensity was high at each interval in comparison to the other varieties. Restricted lesions, in contrast, which were lesser in number could be noticed in TV-9, TV-25 and TV-28. The degree of susceptibility or resistance in the other varieties against *A. alternata* was moderate.

Results obtained from varietal resistance test performed on 22 tea varieties against *A. alternata* following detached leaf and whole plant inoculation technique conclusively proved that T-17, TV-22 and T-78 were highly susceptible while TV-25 and TV-28 were resistant. These were selected for further experiment. Analysis of variance of data presented in Table 20 was computed and presented below.

Table 19: Screening of tea varieties against *C. invisum* following cut shoot inoculation.

Tea varieties	Mean disease index/shoot		
	Hour after inoculation		
	24	48	72
TocklaiTV-9	0.19 ± 0.03	0.25 ± 0.03	0.35 ± 0.02
TV-18	0.21 ± 0.01	0.68 ± 0.01	1.21 ± 0.11
TV-20	0.26 ± 0.02	0.46 ± 0.03	1.31 ± 0.12
TV-22	0.75 ± 0.01	0.98 ± 0.01	2.45 ± 0.13
TV-23	0.56 ± 0.03	0.79 ± 0.03	1.94 ± 0.03
TV-25	0.23 ± 0.01	0.46 ± 0.02	0.58 ± 0.02
TV-26	0.35 ± 0.03	0.45 ± 0.03	1.32 ± 0.11
TV-27	0.26 ± 0.02	0.31 ± 0.02	1.11 ± 0.10
TV-28	0.18 ± 0.03	0.23 ± 0.03	0.76 ± 0.02
TV-29	0.15 ± 0.01	0.56 ± 0.01	1.32 ± 0.14
TV-30	0.19 ± 0.02	0.35 ± 0.01	1.20 ± 0.13
T-17	0.26 ± 0.02	0.72 ± 0.01	1.34 ± 0.14
UPASI			
UP-2	0.38 ± 0.03	0.49 ± 0.04	1.35 ± 0.12
UP-3	0.18 ± 0.05	0.43 ± 0.01	0.96 ± 0.05
UP-8	0.27 ± 0.04	0.43 ± 0.02	0.99 ± 0.02
UP-9	0.21 ± 0.02	0.58 ± 0.01	1.26 ± 0.12
BSS-1	0.28 ± 0.06	0.63 ± 0.03	1.36 ± 0.14
BSS-2	0.25 ± 0.04	0.58 ± 0.02	1.16 ± 0.11
BSS-3	0.21 ± 0.03	0.44 ± 0.01	1.04 ± 0.13
Darjeeling			
T-78	0.38 ± 0.03	0.53 ± 0.04	1.76 ± 0.12
T-135	0.28 ± 0.02	0.43 ± 0.05	1.26 ± 0.13
HV-39	0.25 ± 0.01	0.53 ± 0.02	1.48 ± 0.14
BS/7A/76	0.08 ± 0.03	0.23 ± 0.01	0.76 ± 0.02

Average of 50 shoots/variety, ± Standard error

Analysis of variance of data presented in Table 19

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	22	3.859	0.175	3.9875	0.41443
Time interval	3	10.927	5.463	124.1956	
Error	44	1.936	0.044		
Total	68	16.721			

Table 20: Screening of tea varieties against *A. alternata* in potted grown tea plants.

Tea varieties	Mean disease index/plant				
	Days after inoculation				
	5	10	15	20	25
Tocklai					
TV-9	0.36 ± 0.03	0.49 ± 0.09	0.65 ± 0.01	0.98 ± 0.02	1.75 ± 0.19
TV-18	0.24 ± 0.09	0.56 ± 0.02	0.72 ± 0.03	0.86 ± 0.03	1.23 ± 0.17
TV-20	2.45 ± 0.17	3.45 ± 0.19	3.89 ± 0.13	6.45 ± 0.16	8.44 ± 0.21
TV-22	1.98 ± 0.17	2.45 ± 0.11	4.89 ± 0.14	5.48 ± 0.15	7.45 ± 0.16
TV-23	1.11 ± 0.15	1.89 ± 0.13	2.45 ± 0.11	3.68 ± 0.11	4.57 ± 0.14
TV-25	1.06 ± 0.09	1.85 ± 0.11	2.86 ± 0.12	3.67 ± 0.12	4.89 ± 0.13
TV-26	1.45 ± 0.14	2.49 ± 0.12	3.98 ± 0.13	5.49 ± 0.14	6.89 ± 0.16
TV-27	1.86 ± 0.16	2.86 ± 0.12	4.00 ± 0.14	5.89 ± 0.12	7.24 ± 0.11
TV-28	0.31 ± 0.03	0.64 ± 0.02	0.82 ± 0.03	0.96 ± 0.05	1.53 ± 0.10
TV-29	0.45 ± 0.05	2.05 ± 0.13	4.49 ± 0.12	5.48 ± 0.12	6.45 ± 0.16
TV-30	0.47 ± 0.07	0.59 ± 0.01	0.78 ± 0.05	0.98 ± 0.02	2.75 ± 0.17
T-17/1/54	2.35 ± 0.12	3.45 ± 0.14	4.89 ± 0.15	6.85 ± 0.16	8.64 ± 0.14
UPASI					
UP-2	0.18 ± 0.03	0.43 ± 0.03	0.56 ± 0.03	0.89 ± 0.04	0.96 ± 0.03
UP-3	0.11 ± 0.02	0.23 ± 0.02	0.43 ± 0.03	0.56 ± 0.03	0.89 ± 0.06
UP-8	1.56 ± 0.12	2.46 ± 0.11	3.45 ± 0.12	4.69 ± 0.06	6.98 ± 0.18
UP-9	1.32 ± 0.13	1.89 ± 0.12	2.89 ± 0.11	3.47 ± 0.11	5.48 ± 0.12
BSS-1	0.24 ± 0.03	0.56 ± 0.03	0.72 ± 0.01	0.86 ± 0.03	1.23 ± 0.15
BSS-2	0.21 ± 0.04	0.46 ± 0.02	0.56 ± 0.04	0.75 ± 0.05	1.11 ± 0.11
BSS-3	1.46 ± 0.14	2.49 ± 0.15	3.95 ± 0.14	4.88 ± 0.16	6.58 ± 0.12
Darjeeling					
HV-39	0.16 ± 0.01	0.46 ± 0.04	0.66 ± 0.03	0.81 ± 0.04	0.98 ± 0.03
T-135	1.34 ± 0.14	2.55 ± 0.14	3.49 ± 0.14	5.78 ± 0.16	6.47 ± 0.17
BS/7A/76	1.45 ± 0.11	2.11 ± 0.12	4.78 ± 0.15	6.55 ± 0.17	7.96 ± 0.16

Average of 15 plants/variety, ± Standard error

Analysis of variance of data presented in Table 20

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	21	317.381	15.113	16.7130	1.43140
Time interval	4	176.350	44.087	48.7537	
Error	84	75.960	0.904		
Total	109	569.691			

4.7 Immuno detection of fungal pathogens in tea leaf tissue

Soluble proteins prepared from mycelia of *A. alternata* and *C. invisum* were analysed by SDS-PAGE. Molecular weight of these proteins were determined. Results have been presented in Table 21. Total thirteen protein bands were observed when soluble proteins prepared from mycelia of *A. alternata* were resolved on SDS-PACE. Whereas mycelial protein of *C. invisum* yielded twelve protein bands.

Table 21: SDS-PAGE analysis of soluble proteins of *A. alternata* and *C. invisum*.

Soluble proteins	Molecular weight of mycelial protein (kda)
A. alternata	103.2, 92.2, 86.5, 73.8, 59.4, 41.5, 28.1, 26.2, 19.7, 16.1, 14.1, 12.9, 9.3 (13)
C. invisum	98.4, 97.0, 96.4, 68.0, 63.2, 45.5, 42.3, 34.6, 23.3, 20.8, 19.2, 14.1 (12)

Values in the parenthesis indicates total number of total protein bands.

Following SDS-PAGE analyses, these protein samples were used for immunization purpose. Each sample was emulsified with adjuvant, immunized in rabbits separately and antisera were collected from blood samples and finally IgGs were purified. Purified IgGs were prepared for *A. alternata* and *C. invisum* used for following immuno assays.

4.7.1 Plate Trape Antigen Enzyme Linked Immunosorbent Assay (PTA ELISA)

Enzyme linked immunosorbent assay (ELISA) is now routine for detection and diagnosis of plant pathogens, viruses, bacteria and fungi. This serological methods are being used in agricultural research and practices. Since ELISA depends on a number of factors and these varies from system to system, it was considered essential to optimize the conditions for PTA-ELISA.

4.7.1.1 Optimization of ELISA

Optimization of ELISA was done using IgG fraction of antisera raised against mycelial antigens of *A. alternata* and *C. invisum*. Three variables such as enzyme dilution, dilutions of the antiserum and antigens were optimized. In all cases, ELISA reactions were carried out using PAb raised against *A. alternata* and *C. invisum* and homologous antigens respectively.

4.7.1.1.1 Enzyme dilution

In both the cases, antigen concentration (10μg/ml) and antiserum dilution (1:125) were kept constant, and different dilution of enzyme (alkaline phosphatase) were used ranging from 1:10,000 to 1:40,000. On the basis of results 1:10,000 of enzyme (alkaline phosphatase) dilution was selected for further experiments.

4.7.1.1.2 Antiserum dilution

PAb raised against *A. alternata* were pooled in four batches. First bleeding, second bleeding, third bleeding and fourth bleeding were separately purified for IgG. These four batches of IgGs were diluted ranging from 1:125 to 1:16,000 and then tested against homologous antigens (mycelial antigen of *A. alternata*) at a concentration of 10μg/ml. Results are given in the Fig: 14. Absorbance values in ELISA decreased from the dilution of 1:125 to 1: 16,000 prepared from IgG of all the bleeding. Highest absorbance value (2.985) was obtained with fourth bleeding where as absorbance value of 1.675 was obtained with first bleeding at 1:125 dilution. In all serological assays IgG prepared from fourth bleeding was considered.

PAb raised against *C. invisum* were pooled in two batches. First bleeding and second bleeding were separately purified for IgG. Dilution factors were same as *A. alternata*. Results in Table 22 showed that highest absorbance value (2.754) was

Optimization of PAb raised against mycelia of *A. alternata* with homologous antigen

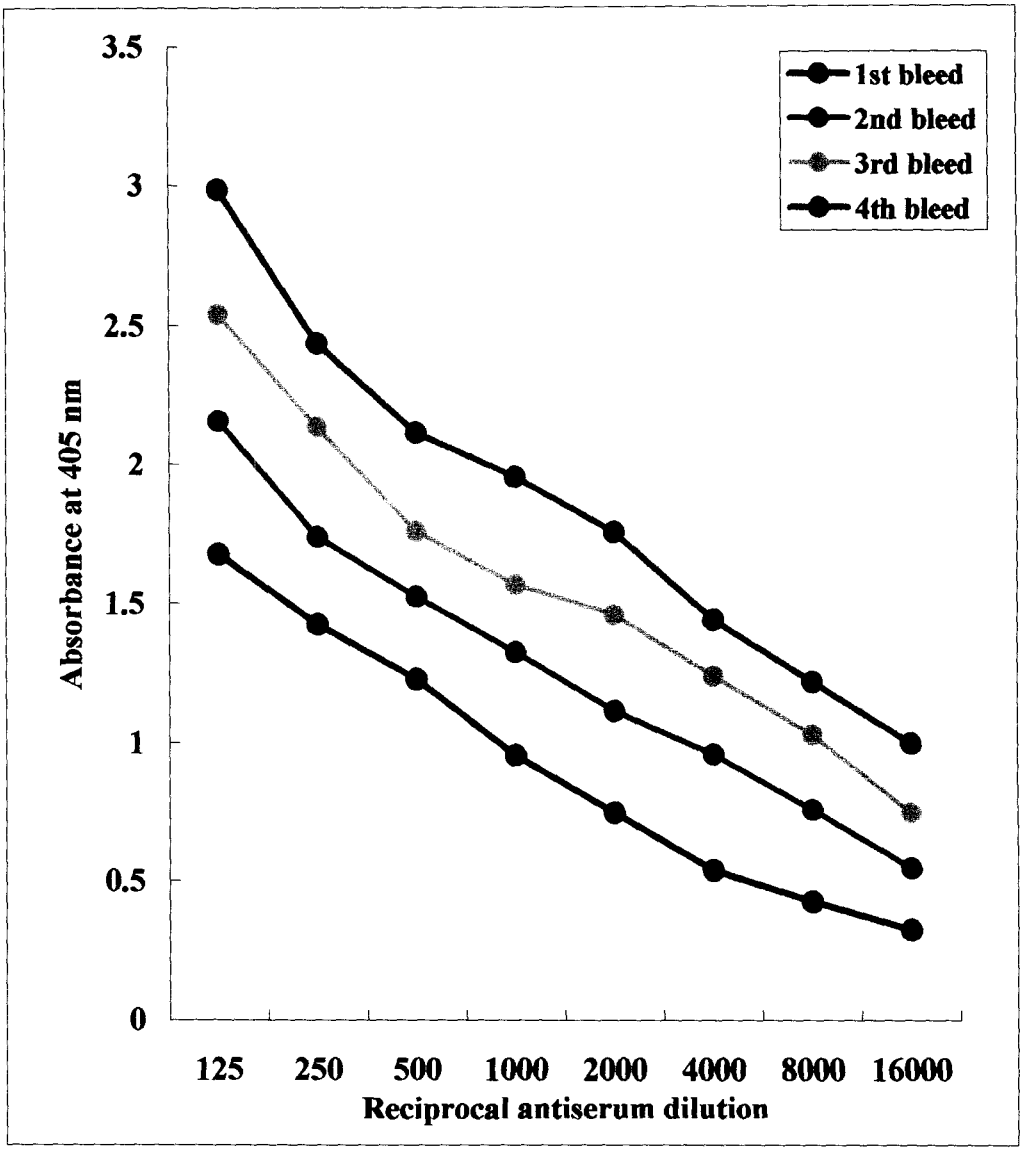


Fig.14

obtained with second bleeding where as 1.456 was obtained from the first bleeding at 1:125 dilution. So second bleeding was used for further experiment.

Table 22: ELISA reaction with various dilution of anti – *C. invisum* antiserum and homologous antigen.

Antiserum dilution	Absorbance at 405 nm	
	First bleeding	Second bleeding
1:125	1.456 ± 0.09	2.754 ± 0.03
1:250	1.322 ± 0.11	1.885 ± 0.02
1:500	1.294 ± 0.06	1.753 ± 0.04
1:1000	1.144 ± 0.05	1.551 ± 0.08
1:2000	0.965 ± 0.03	1.468 ± 0.05
1:4000	0.852 ± 0.12	1.344 ± 0.04
1:8000	0.684 ± 0.03	1.066 ± 0.06
1:16000	0.493 ± 0.07	0.775 ± 0.07

Mean of three replicates

± Standard error

4.7.1.1.3 Antigen dilution

Antigen dilution of *A. alternata* and *C. invisum* ranging from 10,000 ng/ml to 78 ng/ml were tested against two antisera dilutions (1:125 and 1:250). ELISA values increased with the concomitant increase of antigen concentration. ELISA reaction with various antigen dilution of *A. alternata* are given in Fig.15 while Table 23 presents the results of *C. invisum*. In both the cases mycelial antigen concentrations as low as 78 ng/ml could also be easily detected by ELISA at 1:125 and 1:250 dilutions.

Optimization of antigen concentrations of *A. alternata* with homologous PAbs using PTA-ELISA format

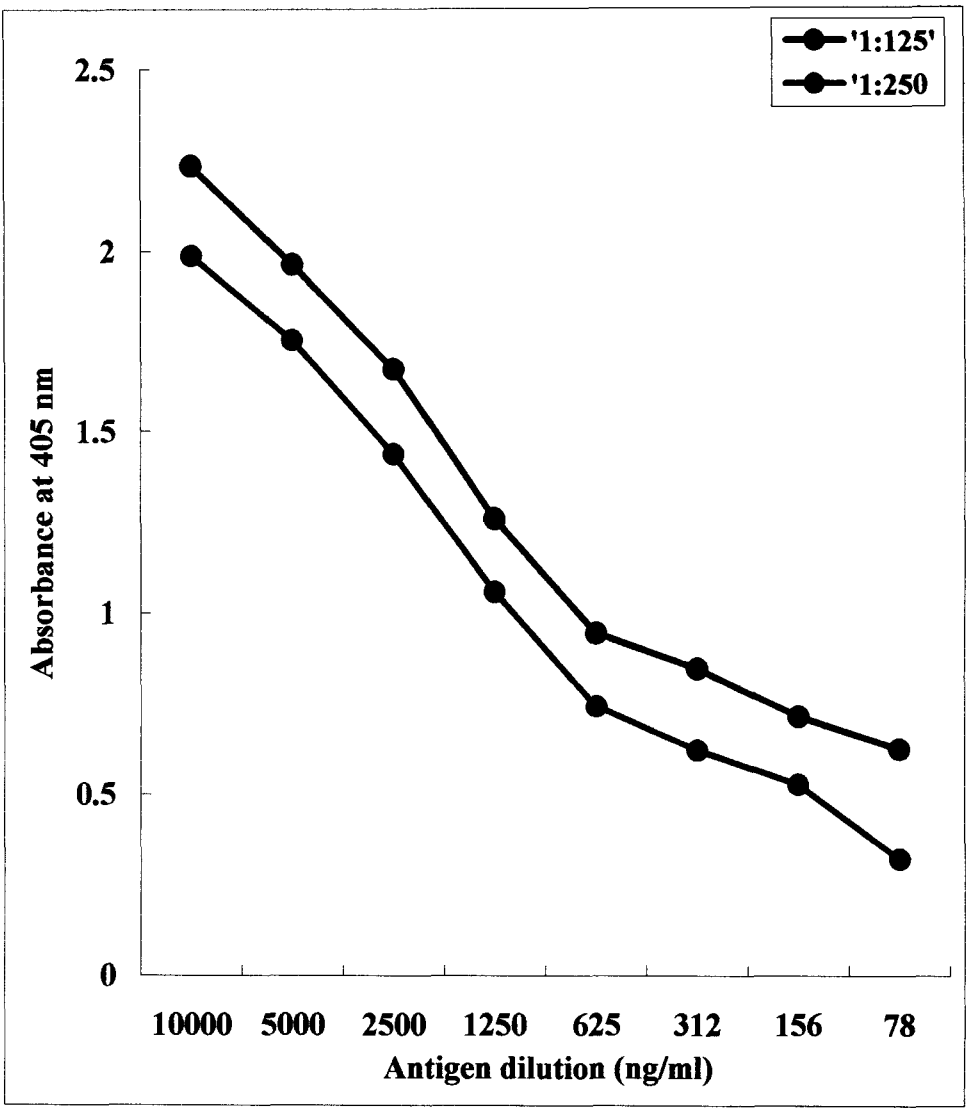


Fig. 15

Table 23: ELISA reaction with various concentration of mycelial antigen of *C. invisum* and homologous antiserum

Antigen dilution (ng/ml)	Absorbance at 405 nm Antisera dilution (Second bleeding) 1:125	1:250
10000	2.598 ± 0.05	1.852 ± 0.11
5000	1.852 ± 0.11	1.752 ± 0.05
2500	1.642 ± 0.05	1.523 ± 0.09
1250	1.431 ± 0.08	1.321 ± 0.05
625	1.227 ± 0.11	1.112 ± 0.12
312	0.852 ± 0.10	0.782 ± 0.07
156	0.643 ± 0.09	0.466 ± 0.09
78	0.553 ± 0.04	0.375 ± 0.08

Enzyme dilution = 1:10,000

Means of three replicates; ± Standard error.

4.7.1.2 Detection of fungal pathogens in tea leaf tissues

PAb raised against mycelia of *A. alternata* and *C. invisum* were tested against nineteen tea varieties following artificial inoculation with test pathogens separately. Healthy and artificially inoculated leaves were harvested after 70h for extraction. Results (Fig. 16 and Fig. 17) revealed that in all tested varieties gave higher absorbance values in PTA-ELISA format in inoculated leaf antigens than the healthy but the difference was significant in susceptible varieties. In Tocklai varieties maximum ELISA values against were obtained in TV-22 and TV-20 against both the pathogens. T-17 responded significantly against *A. alternata* where as UPASI varieties showed significant response against *C. invisum* in comparison to the other pathogen.

ELISA response of PAb of *A. alternata* with antigens of healthy and inoculated tea leaf tissues

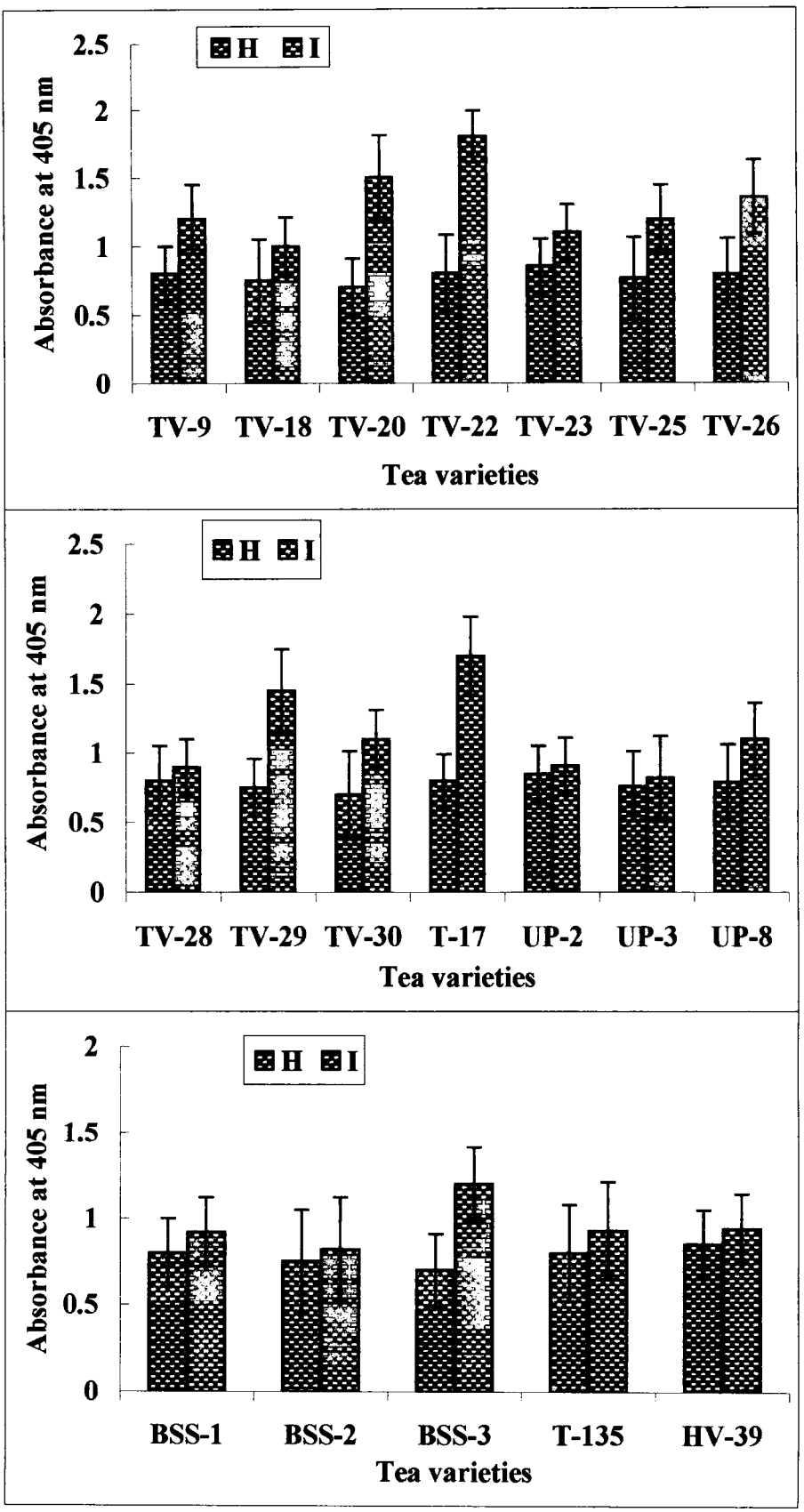


Fig 16

ELISA responses of PAb of *C. invisum* with antigens of healthy and inoculated tea leaf tissues

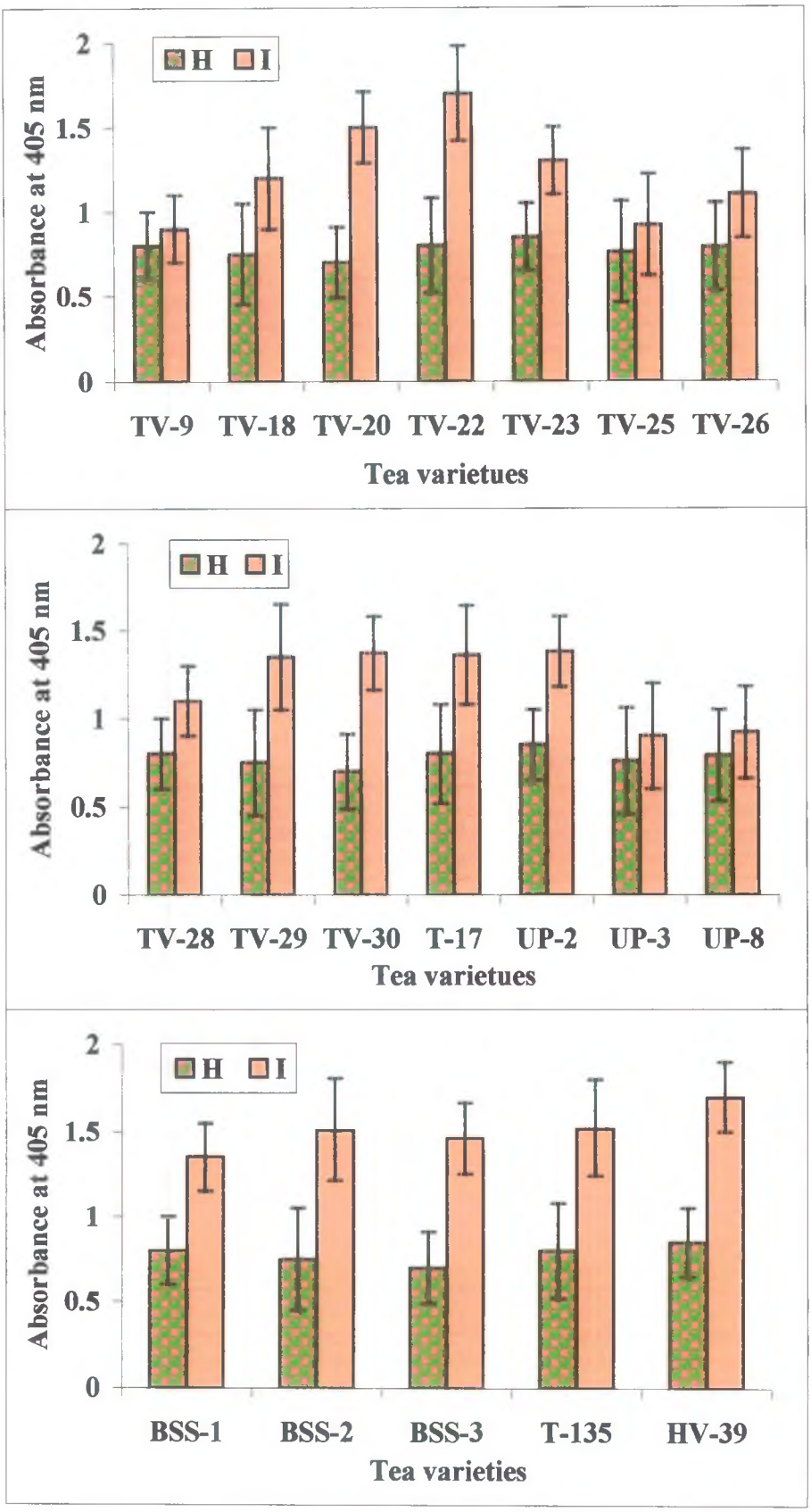


Fig 17

4.7.2 Dot immunobinding assay

Total soluble proteins were extracted from healthy and artificially inoculated leaves of seventeen different tea varieties (host). Where as leaf antigens were prepared from *Glycine max* and *Leucaena leucocephala* (non-host). Besides mycelial antigens were prepared from two foliar pathogens (*C. invisum* and *A. alternata*) of tea and one non pathogen (*Fusarium oxysporum*) of tea. Antigen preparation from non pathogen were performed from *M. anisopliae* and *B. bassiana.* Dot immunobinding assayes performed using above antigen preparation with PAb of *A. alternata* and *C. invisum.* Antigens were carefully spotted on nitrocellulose paper and probed with IgG of *A. alternata.* Results have been presented in Plate 17 (fig B) and Table 24 Clear and intense color reactions were observed in case of homologous antigen. Higher colour intensity was noticed in TV-22 and T-17 varieties which showed susceptible reactions in varietal resistant test. HV-39, UP-3 showed insignificant color and also these were resistant variety against *A. alternata.* These preparations were further assayed using PAb of *C. invisum.*

Results have been presented in Plate 18 (Fig B) and Table 25. Leaf antigens prepared from TV-9, UP-3, TV-25 showed insignificant colour intensity. These varieties also showed resistant reaction during varietal resistant test. Clear and intense color intensity were observed in case of leaf antigens prepared from inoculated susceptible tea varieties (TV-22, HV-39 and T-17). Maximum color intensity was observed in homologous reaction on nitrocellulose paper involving of mycelial antigen and respective IgG preparations.

4.7.3 Immunocytochemical staining

Immunocytochemical staining based on specific antibodies produced against *A. alternata* and *C. invisum* could produce a mean of visualizing hyphae of those fungus. Production of a specific immunocytochemical stain involves preparation of a suitable.

Table 24: Dot immunobinding assay of healthy and *A. alternata* inoculated leaves of host (tea) and non-host using PAb of *A. alternata*

Antigen preparations	PAb of *A. alternata* Colour intensity	
	Healthy	Inoculated with *A. alternata*
Leaf antigens		
Host : *C. sinensis*		
TV-9	±	+
TV-18	±	+
TV-20	+	+++
TV-22	+	+++
TV-26	+	++
TV-29	+	++
T-17	+	+++
UP-3	±	±
UP-8	+	++
BSS-1	±	+
T-135	±	++
HV-39	±	±
Non host :		
Glycine max	–	–
Leucaena leucocephala	–	–
Fungal antigens		
Pathogen		
Alternaria alternata	++++	
Corticium invisum	±	
Fusarium oxysporum	±	
Non pathogens		
Metarhizium anisopliae	–	
Beauveria bassiana	–	

Colour intensity of dots
++ light violet ; +++ violet ; ++++ deep violet ;
± insignificant ; – no colour reaction ;
NBT/BCIP used as substrate ; PAb (60µg/ml)

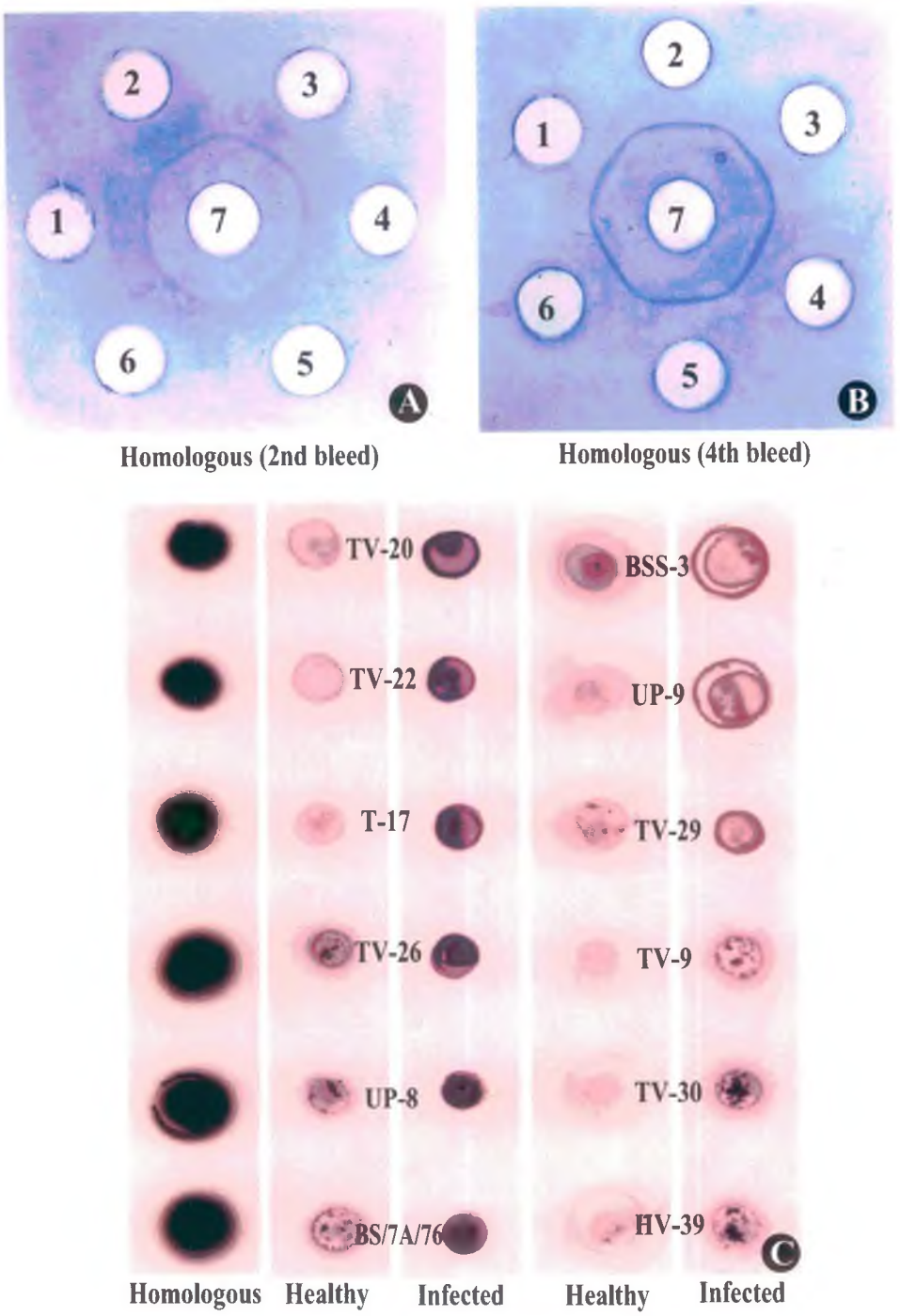
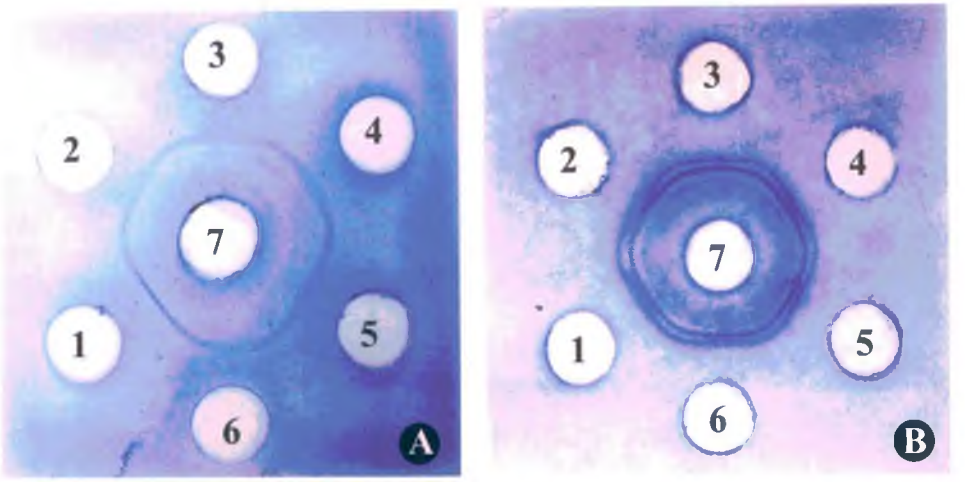


Plate 17 (figs. A-C): Immuno -diffusion (A&B) and Dot immunobinding assay (C) on nitrocellulose paper with PAb of *A. alternata* against leaf antigens of tea varieties and mycelial antigen of *A. alternata.* (A&B) Peripheral wells (1-6)-mycelial antigen; central well (7): PAb of *A. alternata* [2nd bleed (A) and 4th bleed (B)]

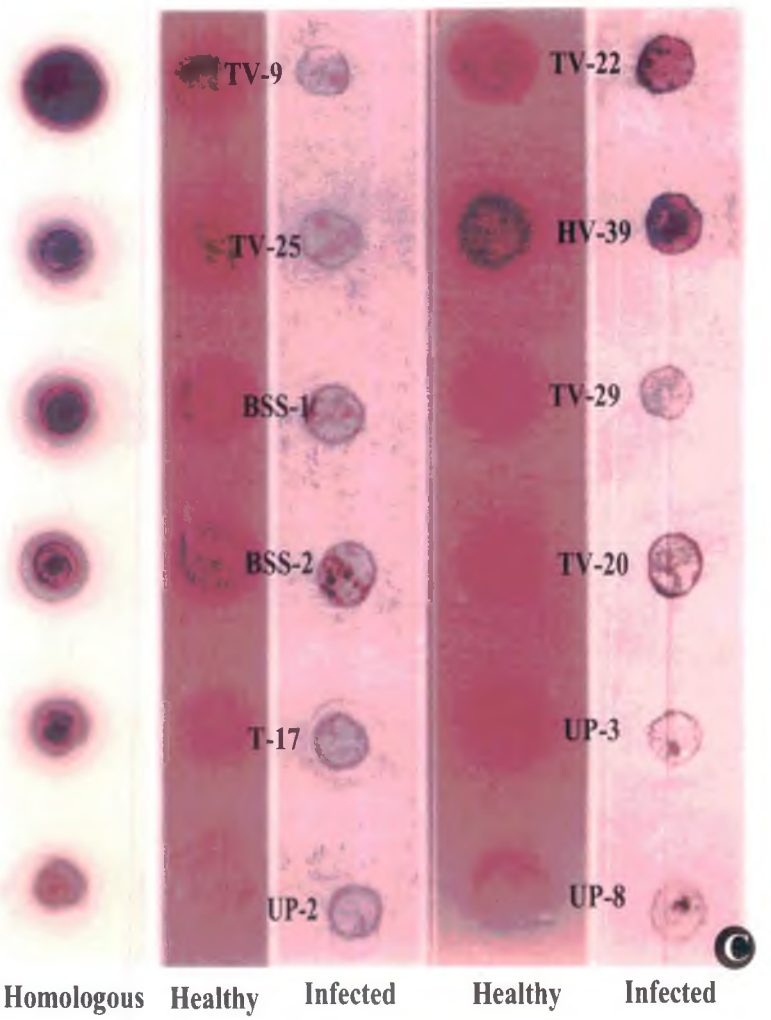
Table 25: Dot immunobinding assay of healthy and *C. invisum* inoculated leaves of host (tea) and non-host using PAb of *C. invisum*

Antigen preparations	PAb of *C. invisum* Colour intensity	
	Healthy	Inoculated with *C. invisum*
Leaf antigen		
Host : *C. sinensis*		
TV-9	±	+
TV-18	+	++
TV-20	+	++
TV-22	+	+++
TV-23	+	+++
TV-26	+	++
TV-29	+	++
TV-30	+	++
T-17	±	++
UP-2	±	++
UP-3	±	+
UP-8	±	+
UP-9	+	++
BSS-2	±	++
T-135	±	+
HV-39	+	+++
BS/7A/76	±	+
Non host :		
Glycine max	–	–
Leucaena leucocephala	–	–
Fungal antigen		
Pathogens		
Corticium invisum	++++	
Alternaria alternata	±	
Fusarium oxysporum	±	
Non pathogens		
Metarhizium anisopliae	–	
Beauveria bassiana	–	

Colour intensity of dots
++ light violet ; +++ violet ; ++++ deep violet ;
± insignificant ; – no colour reaction ;
NBT/BCIP used as substrate ; PAb (60µg/ml)



Homologous (1st bleed) Homologous (2nd bleed)



Homologous Healthy Infected Healthy Infected

Plate 18 (figs. A-C): Immuno -diffusion (A&B) and Dot immunobinding assay (C) on nitrocellulose paper with PAb of *C. invisum* against leaf antigens of tea varieties and mycelial antigen of *C. invisum.* (A&B) Peripheral wells (1-6)-mycelial antigen; central well (7) : PAb of *C. invisum.*

antigen, appropriate method evaluating specificities of the antibodies and development of the immunocytochemical staining procedure. In the present study specific refer to binding of antibodies only to the fungus against which they were produced and not to the other species of the fungus in the same habitat. The purpose of this study was to develop a specific immunocytochemical staining for *A. alternata* and *C. invisum* for detection of the hyphae of the fungus within tea leaf tissues. Different tea varieties were artificially inoculated with conidial suspension of *A. alternata* and sclerotial suspension of *C. invisum*.

In case of *A. alternata* susceptible varieties of tea (TV-20, TV-22, UP-8, BSS-3 T-17 and BS/7A/76) were artificially inoculated. After 42h of inoculation transverse sections were made from infected portion of leaf and PAb raised against *A. alternata* was used for probing the fungal hyphae, which penetrate the leaf tissue. Plate 19 illustrate the labeling pattern as observed with different tea varieties following treatment with PAb of *A. alternata* and stained with Fast blue BB salt. All these varieties showed highly susceptible reactions in varietal resistant test.

Besides, one of the susceptible variety, TV-22 was chosen for artificial inoculation with *C. invisum*. Cross sections were made from the infected portion of leaf and treated with PAb raised against *C. invisum* and finally stained with Fast blue BB salt. Hyphal penetration throughout the leaf tissue was evident (Plate 20 figs.A&B). Using PAb of *C. invisum* when detection of pathogen were done by PTA ELISA and DOT BLOT against TV-22 variety, high absorbance values were noted in ELISA as well as intense color developed in dot immunobinding assay.

4.7.4 Immunofluorescence

Fluorescence antibody labeling with FITC is known to be one of the powerful technique to determine the cell or tissue location of antigens shared by host and parasite. In the present study following immunodiffusion, PTA-ELISA as well as

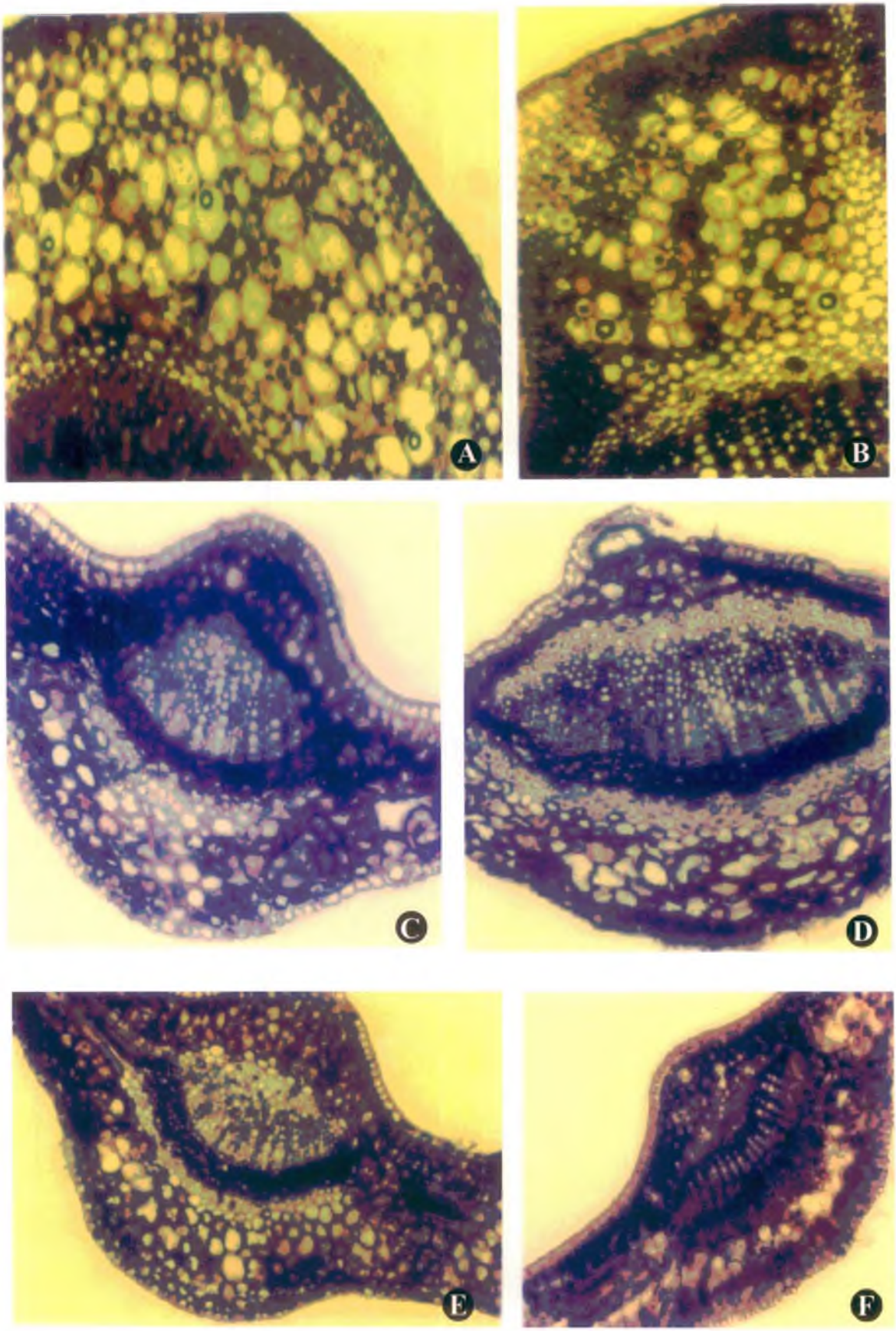


Plate 19 (figs. A-F): Immunoenzymatic staining of tea leaf tissues artificially inoculated with *A. alternata.* Cross sections of tea leaf tissues [T-17 (A); TV-22 (B); UP-8 (C); BSS-3 (D); TV-20 (E); BS/7A/76 (F)] probed with PAb of *A. alternata* and stained with fast blue BB salt.

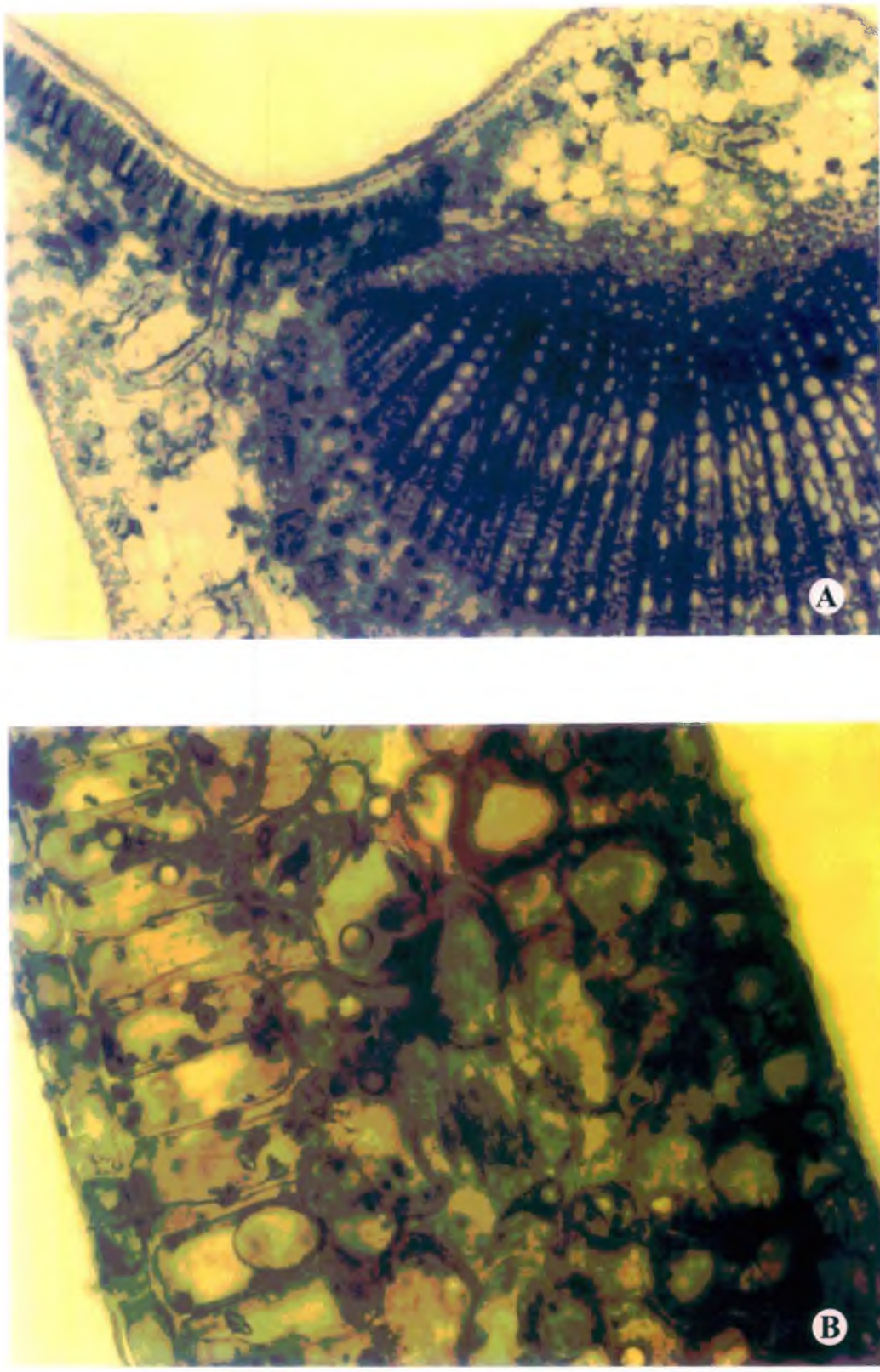


Plate 20 (figs. A&B): Immunoenzymatic staining of tea leaf tissues artificially inoculated with *C. invisum.* Cross sections of tea leaf tissues [TV-20 (A); TV-22 (B);] probed with PAb of *C. Invisum* and stained with fast blue BB salt.

Dot-blot the presence of antigens shared by *Camellia sinensis* and two foliar fungal pathogens *A. alternata* and *C. invisum* has been detected. It was decided to determine the tissue and surface recognition in tea leaf tissue as well as sclerotia of *A. alternata* and *C. invisum*.

4.7.7.1 Mycelia

Mycelia of both the foliar fungi were neither auto-fluorescence nor they fluorescence when treated with normal serum followed by FITC. Treatment of young mycelia of *A. alternata* with homologous antiserum and FITC showed a general fluorescence that was also more intense on young hyphal tips (Plate 21 Fig: B&C) Mycelia another strain of *A. alternata* (W8055) also showed such nature when treated with FITC and observed under microscope (Plate 22 fig A&B). Treatment of mycelia of *C. invisum* with homologous antiserum and FITC showed a general fluorescence that was more intense on young hyphal tips (Plate 23 Fig A-C).

4.7.4.2 Tea leaf tissue

Cross sections of tea leaves (T-17) were treated separately with normal serum, homologous and pathogen antisera of *A. alternata*, and then treated with FITC. Leaf sections exhibited a natural auto-fluorescence under UV light on the cuticle (Plate 24 fig A). Same observation was noted when the leaf sections were treated with normal serum and FITC. Leaf sections treated with PAb of *A. alternata* and then treated with FITC developed bright fluorescence which was distributed throughout the leaf tissue, mainly in the epidermal and mesophyll tissues (Plate 24 figs. B&C).

In case of *C. invisum* tea leaf sections of TV-22 variety were treated with pathogen antisera then treated with FITC. It has been observed that sections treated with pathogen antisera and FITC developed bright fluorescence mainly in the mesohlyll tissue region (Plate 25 figs. A&B). Fluorescence was more intense in palisade and spongy parenchyma cells.

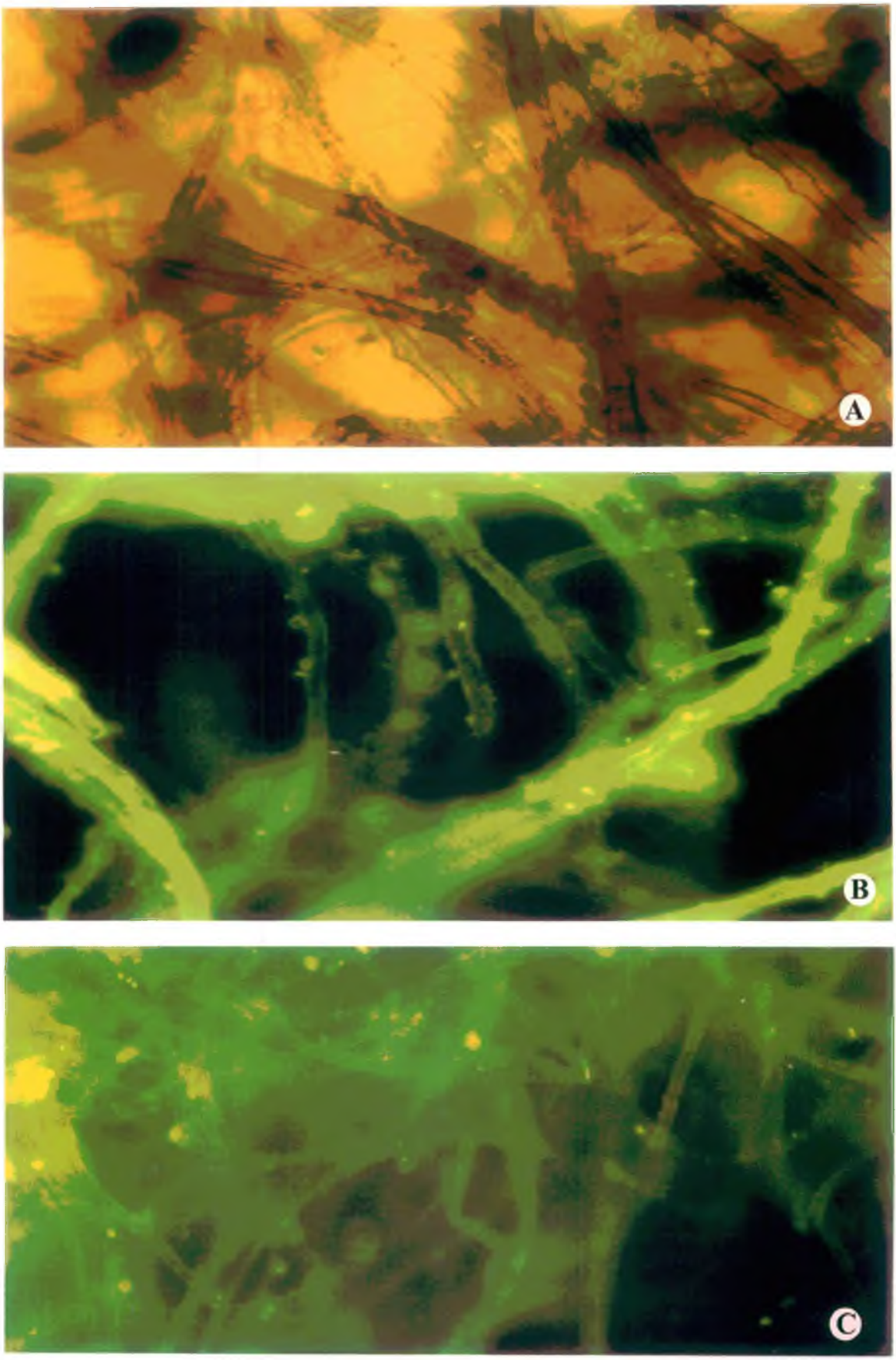


Plate 21 (figs. A-C): Indirect immunofluorescence of hyphae of *A. alternata* (Isolate-W8055). (A) under bright field. (B&C) Fluorescence of hyphae treated with PAb of *A. alternata* and reacted with FITC labelled antibodies of goat specific for rabbit globulin.

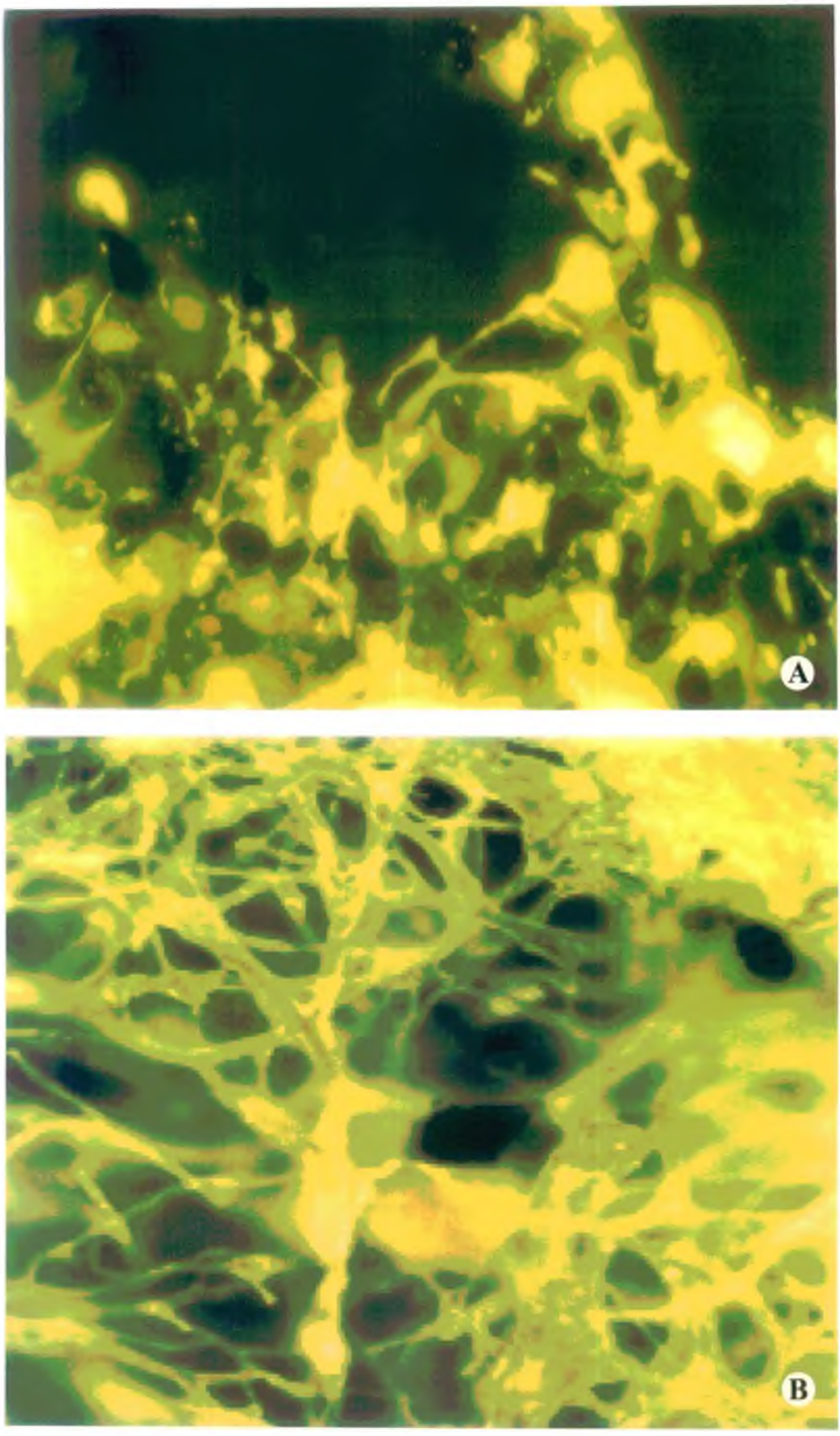


Plate 22 (figs. A&B): Fluorescence of hyphae of *A. alternata* (Isolate-W8053) treated with PAb of *A. alternata* and reacted with FITC labelled antibodies of goat specific for rabbit globulin.

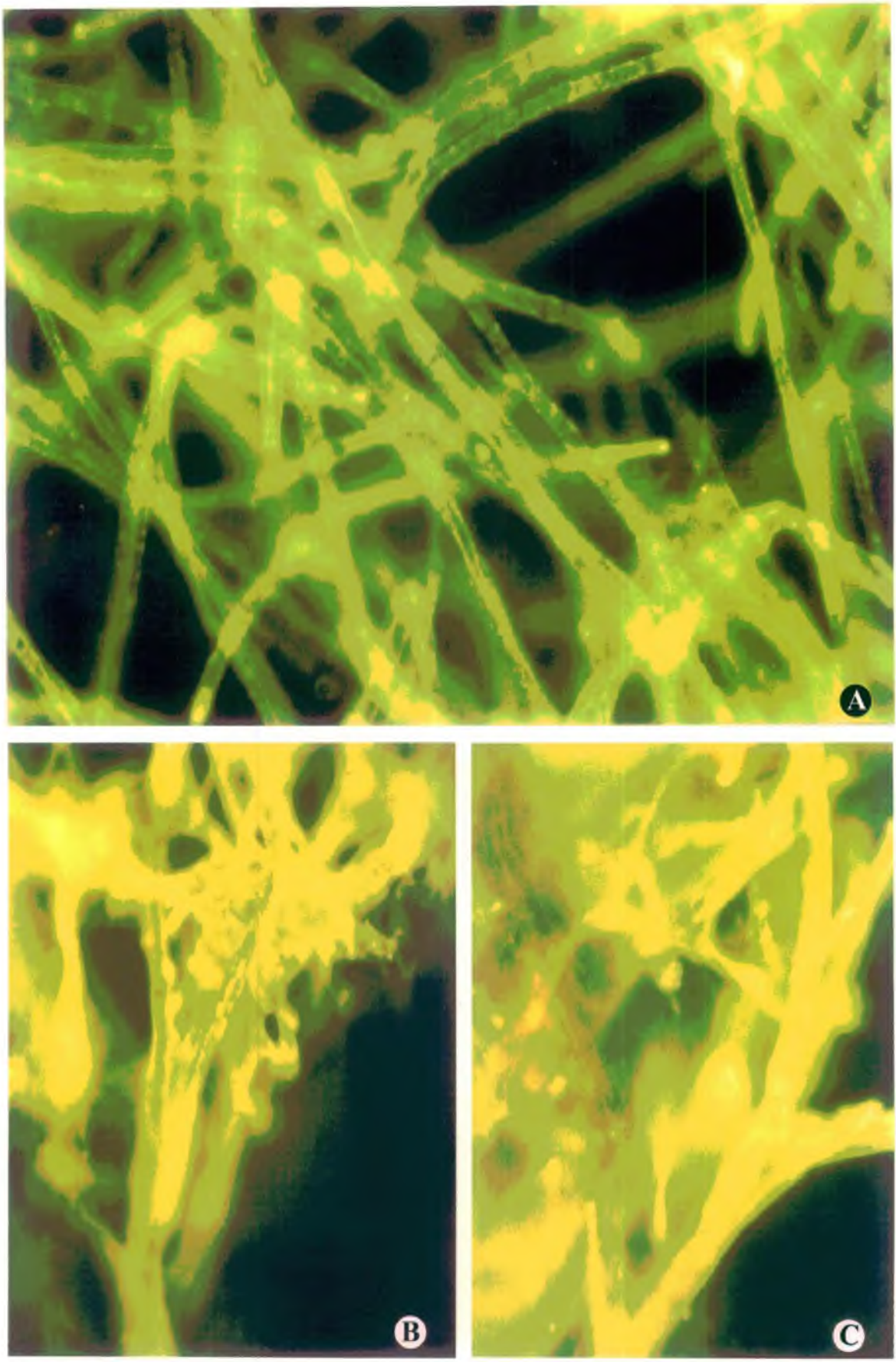


Plate 23 (figs. A-C): Fluorescence of hyphae of *C. invisum* treated with PAb of *C. invisum* and labelled with FITC antibody conjugate of goat specific for rabbit globulin [(A) 1st Bleed (B&C) 2nd Bleed]

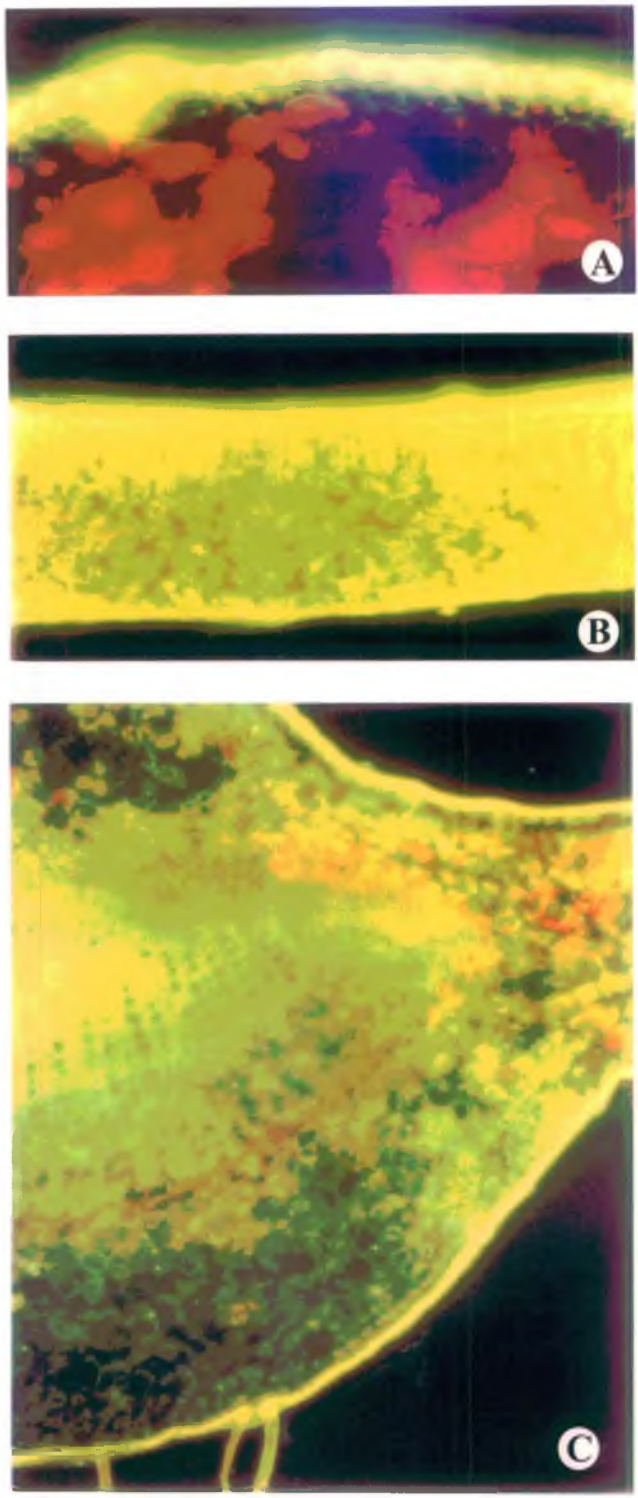


Plate 24 (figs. A-C): Cross section of healthy (A) and artificially inoculated (with *A. alternata)* tea leaf tissues (T-17) reacted with PAb of *A. alternata* and labelled with FITC (B&C)

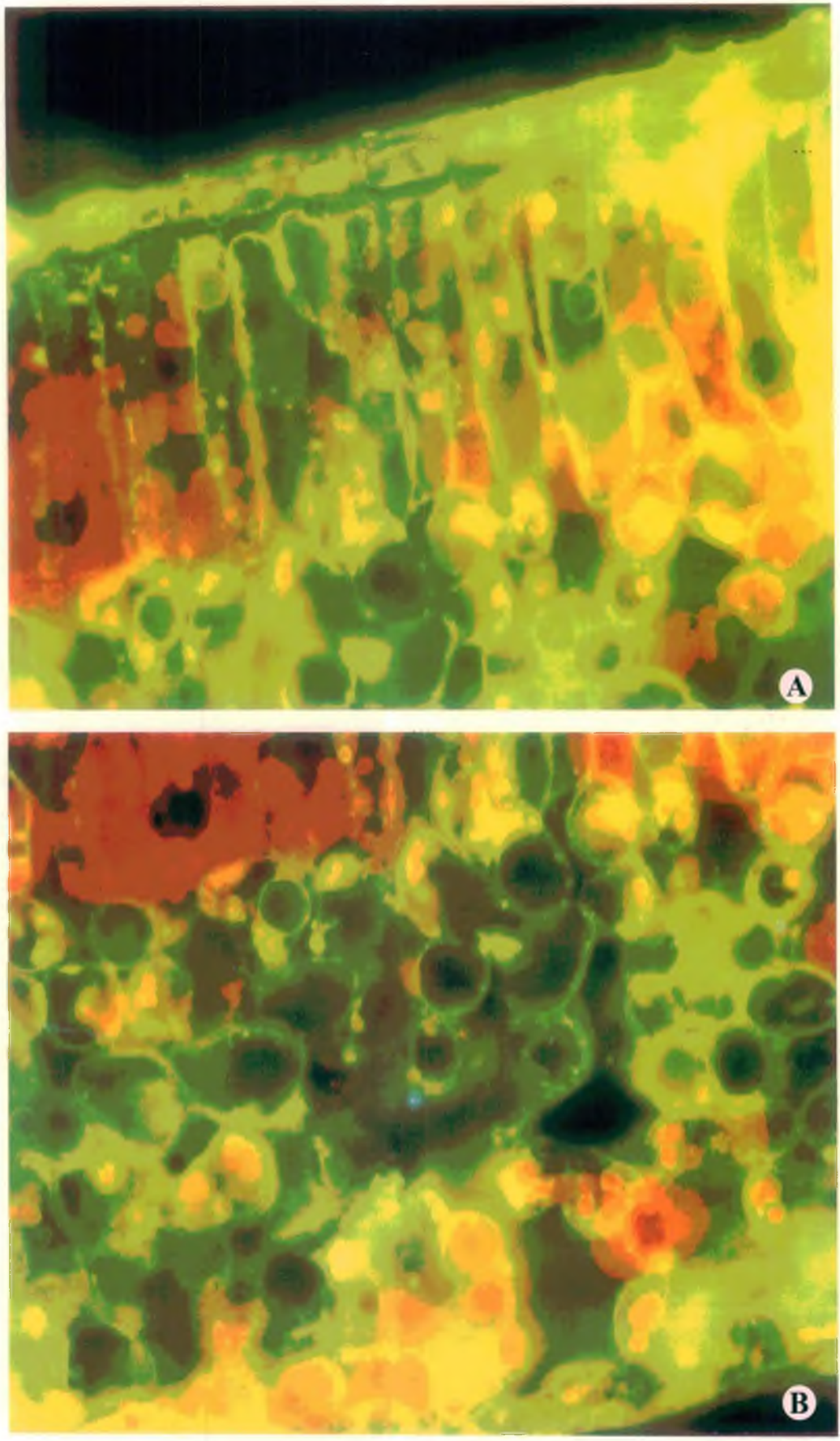


Plate 25 (figs. A&B): Cross sections of artificially inoculated (with *C. invisum*) tea leaf tissues [TV-20 (A), TV-22 (B)] reacted with PAb of *C. invisum* and labelled with FITC

4.8 Changes in the level of phenols and proteins in tea leaves following inoculation with foliar fungal pathogens

Phenolics and proteins are the major constituent of tea leaves and have an important role in defense response. Hence, accumulation of phenolics and proteins following inoculation of tea plants with both the foliar fungal pathogens were determined. Among twenty three varieties tested for screening resistance against these fungal patogens ten tea vaieties were selected for the present investigation. These includes four each resistant varieties (TV-18, TV-28, UP-3, HV-39) against *A. alternata* and against *C. invisum* (TV-9, UP-3, UP-8 BS/7A/76) two moderately resistant (UP-8, BSS-3) against *A. alternata* and T-17 and TV-28 against *C. invisum* and four highly susceptible (TV-20, TV-22, T-17, BS/7A/76) against *A. alternata* and TV-20, TV-22, HV-39 and BSS-3 against *C. invisum.*

4.8.1 Total phenols

Total phenols were extracted from tea leaves of healthy, 24 and 48h following inoculation separately with *A. alternata* and *C. invisum* of ten varieties and estimated. Results have been presented in Table 26, 27 and Fig. 18. Total phenol content decreased following inoculation with foliar fungal pathogens in the susceptible varieties. However there was an increase in the total phenol content of resistant varieties following inoculation with *A. alternata* and *C. invisum.* Among the varieties tested, TV-9 and TV-18 showed maximum increase in total phenol following inoculation with *A. alternata* while TV-9 and TV-25 showed maximum increase when inoculated with *C. invisum.*

4.8.2 Orthodihydroxy phenols

Orthodihydroxy phenols were also extracted from ten tea varieties. Tea leaves of healthy and inoculated separtely with *A. alternata* and *C. invisum* after 24 and 48h of inoculation were harvested for estimation. Results presented in Table 28 and 29 revealed that orthodihydroxy phenol content decreased in the susceptible varieties

Table 26: Level of total phenol in healthy and *A. alternata* inoculated tea leaves

	Total phenol content (mg caffeic acid g^{-1} leaf tissue)			
	24h		48h	
Varieties	**Healthy**	**Inoculated**	**Healthy**	**Inoculated**
TV-18	48.23 ± 0.42	49.24 ± 0.67	47.25 ± 0.83	50.23 ± 0.48
TV-20	49.26 ± 0.40	47.25 ± 0.23	48.59 ± 0.26	45.78 ± 0.43
TV-22	51.47 ± 0.81	48.76 ± 0.73	50.47 ± 0.62	46.25 ± 0.65
TV-28	45.78 ± 0.91	47.12 ± 0.24	43.56 ± 0.49	52.16 ± 0.59
T-17	50.46 ± 0.76	45.75 ± 0.62	51.46 ± 0.67	42.96 ± 0.32
UP-3	36.48 ± 0.42	40.12 ± 0.86	35.45 ± 0.35	43.56 ± 0.16
UP-8	40.16 ± 0.68	41.46 ± 0.79	41.25 ± 0.56	43.56 ± 0.46
BSS-3	38.77 ± 0.48	42.47 ± 0.64	36.48 ± 0.26	45.68 ± 0.32
HV-39	43.59 ± 0.87	45.58 ± 0.77	42.58 ± 0.71	54.56 ± 0.42
BS/7A/76	42.12 ± 0.21	38.79 ± 0.83	41.22 ± 0.13	36.78 ± 0.32

Average of five replicates per treatment ; Means of three replicates; ± Standard error

Table 27: Level of total phenol in healthy and *C. invisum* inoculated tea leaves

	Total phenol content (mg caffeic acid g^{-1} leaf tissue)			
	24h		48h	
Varieties	**Healthy**	**Inoculated**	**Healthy**	**Inoculated**
TV-9	42.12 ± 0.42	48.24 ± 0.29	41.13 ± 0.52	52.67 ± 0.51
TV-20	49.26 ± 0.47	47.25 ± 0.26	48.59 ± 0.21	45.78 ± 0.78
TV-22	51.47 ± 0.84	48.76 ± 0.75	50.47 ± 0.51	46.25 ± 0.52
TV-28	45.78 ± 0.76	47.12 ± 0.29	43.56 ± 0.44	51.16 ± 0.61
T-17	50.46 ± 0.71	52.75 ± 0.63	51.46 ± 0.83	54.96 ± 0.64
UP-3	36.48 ± 0.43	40.12 ± 0.23	35.45 ± 0.37	43.56 ± 0.65
UP-8	40.16 ± 0.65	43.46 ± 0.71	41.25 ± 0.80	47.56 ± 0.56
BSS-3	38.77 ± 0.48	35.47 ± 0.69	36.48 ± 0.23	32.68 ± 0.68
HV-39	43.59 ± 0.81	37.58 ± 0.74	42.58 ± 0.46	34.56 ± 0.42
BS/7A/76	48.23 ± 0.19	52.24 ± 0.39	47.25 ± 0.55	55.23 ± 0.37

Average of five replicates per treatment ; Means of three replicates; ± Standard error

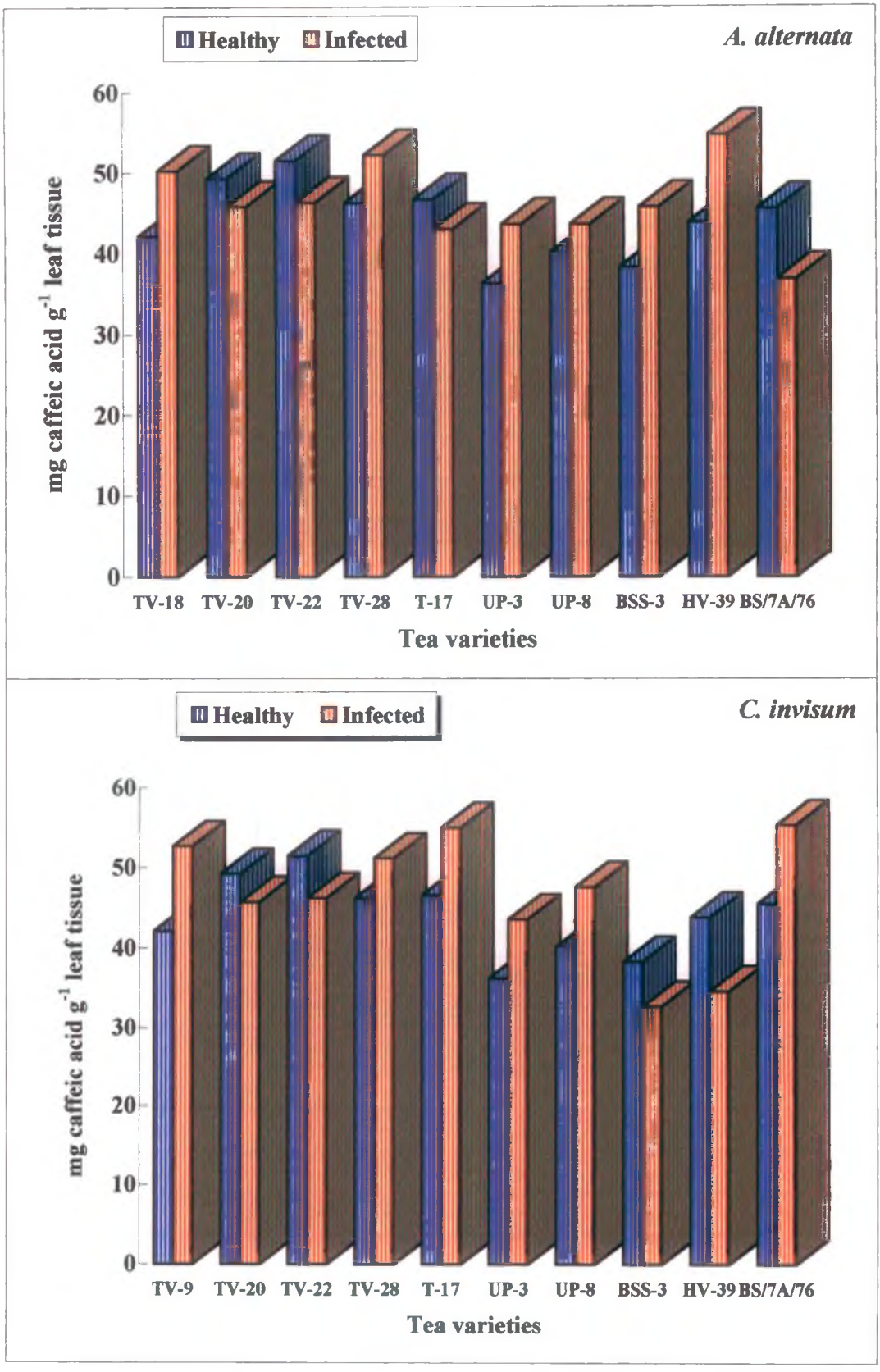

Healthy
Infected
A. alternata
mg caffeic acid g-1 leaf tissue
60
50
40
30
20
10
0
TV-18
TV-20
TV-22
TV-28
T-17
UP-3
UP-8
BSS-3
HV-39
BS/7A/76
Tea varieties
Healthy
Infected
C. invisum
mg caffeic acid g-1 leaf tissue
60
50
40
30
20
10
0
TV-9
TV-20
TV-22
TV-28
T-17
UP-3
UP-8
BSS-3
HV-39
BS/7A/76
Tea varieties

Fig. 18

Table 28: Level of Orthodihydroxy phenol in healthy and *A. alternata* inoculated tea leaves

	Orthodihydroxy phenol content (mg caffeic acid g^{-1} leaf tissue)			
	24h		48h	
Varieties	Healthy	Inoculated	Healthy	Inoculated
TV-18	16.13 ± 0.05	19.45 ± 0.05	17.21 ± 0.03	21.23 ± 0.07
TV-20	11.24 ± 0.04	10.25 ± 0.04	12.45 ± 0.01	09.74 ± 0.07
TV-22	15.28 ± 0.08	12.45 ± 0.11	16.44 ± 0.06	10.25 ± 0.03
TV-28	10.25 ± 0.10	13.12 ± 0.09	13.56 ± 0.07	19.16 ± 0.08
T-17	15.46 ± 0.05	13.75 ± 0.07	14.75 ± 0.08	12.97 ± 0.04
UP-3	12.85 ± 0.07	14.17 ± 0.10	13.45 ± 0.03	18.54 ± 0.11
UP-8	14.16 ± 0.09	16.46 ± 0.07	14.25 ± 0.12	18.45 ± 0.05
BSS-3	11.43 ± 0.04	14.23 ± 0.11	12.45 ± 0.09	15.26 ± 0.08
HV-39	13.45 ± 0.06	15.74 ± 0.05	12.75 ± 0.11	20.77 ± 0.08
BS/7A/76	12.12 ± 0.36	10.54 ± 0.03	11.13 ± 0.03	08.64 ± 0.04

Average of five replicates per treatment ; Means of three replicates; ± Standard error.

Table 29: Level of Orthodihydroxy phenol in healthy and *C. invisum* inoculated tea leaves

	Orthodihydroxy phenol content (mg caffeic $acid^{-1}$ gm leaf tissue)			
	24h		48h	
Varieties	Healthy	Inoculated	Healthy	Inoculated
TV-9	14.13 ± 0.03	18.29 ± 0.07	15.53 ± 0.09	22.59 ± 0.11
TV-20	12.51 ± 0.08	10.28 ± 0.02	12.45 ± 0.01	08.76 ± 0.10
TV-22	15.28 ± 0.08	12.45 ± 0.11	16.44 ± 0.06	10.25 ± 0.03
TV-28	10.25 ± 0.10	13.12 ± 0.09	13.56 ± 0.07	16.58 ± 0.08
T-17	15.46 ± 0.05	13.75 ± 0.07	14.75 ± 0.08	12.97 ± 0.04
UP-3	12.85 ± 0.07	14.17 ± 0.10	13.45 ± 0.03	18.54 ± 0.11
UP-8	14.16 ± 0.09	18.46 ± 0.12	14.25 ± 0.12	21.86 ± 0.13
BSS-3	11.43 ± 0.04	09.23 ± 0.05	12.45 ± 0.09	07.37 ± 0.10
HV-39	13.45 ± 0.06	11.76 ± 0.06	12.75 ± 0.11	08.75 ± 0.07
BS/7A/76	13.45 ± 0.36	17.23 ± 0.08	12.56 ± 0.03	21.46 ± 0.07

Average of five replicates per treatment ; Means of three replicates; ± Standard error.

Level of orthodihydroxy phenol in healthy and artificially inoculated tea leaves

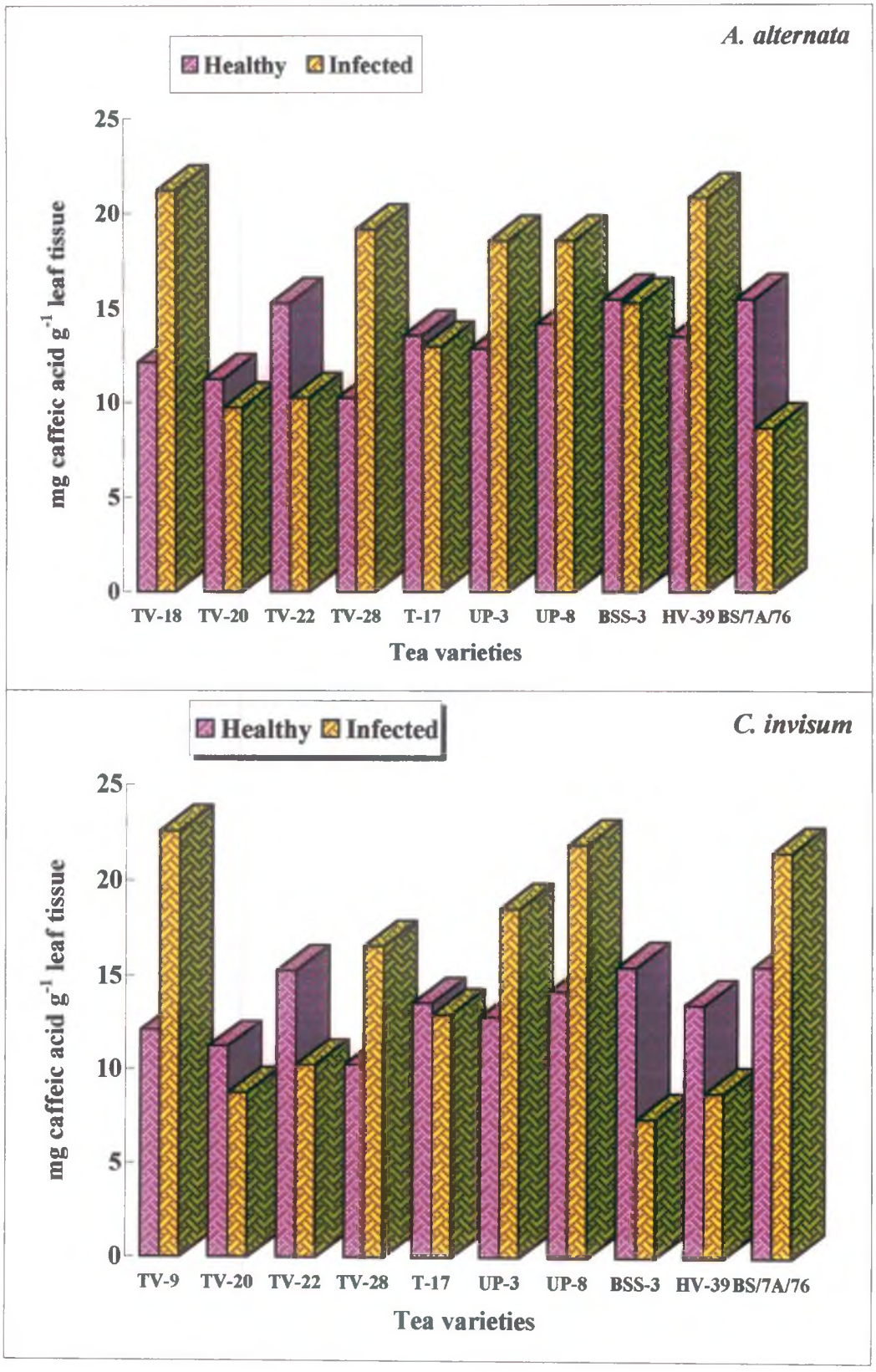


Fig. 19

(TV-20, TV-22, T-17, BS/7A/76) and increased in resistant varieties (TV-18, TV-28, UP-3, HV-39) following inoculation with *A. alternata* (Table 28 and Fig.19). Orthodihydroxy phenol content decreased following inoculation with *C. invisum* in TV-20, TV-22, HV-39 and BSS-3, (susceptible varieties) in comparison to TV-9, UP-3, UP-8 and BS/7A/76 varieties which showed resistant reaction against *C. invisum* (Table 29 and Fig 19).

4.8.3 Soluble proteins

Soluble proteins were extracted from ten tea varieties (TV-18, TV-28, TV-20, TV-22, T-17, UP-3, UP-8, BSS-3, HV-39 and BS/7A/76) of healthy and artificially inoculated with *A. alternata* and *C. invisum* and estimated. Results have been presented in Table 30 and Fig. 20. It reveals that in all the tested varieties protein content decreased following inoculation with *A. alternata* and *C. invisum.*

4.9 Determination of the level of defense enzymes in tea plants following inoculation with foliar fungal pathogens

Five defence enzymes Phenylalanine ammonia lyase, Polyphenol-oxidase, Peroxidase (PR-9), Chitinase (PR-3) and β-1,3-glucanase (PR-2) were extracted and assayed from healthy and artificially inoculated (with *A. alternata* and *C. invisum* separately) tea leaves. All the enzymes were assayed after 24, 48 and 72h of inoculation. Spore suspension of *A. alternata* and sclerotial suspension of *C. invisum* separately.

4.9.1 Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenylpropanoid metabolism in higher plants and it has been suggested to play a significant role in regulation of accumulation of phenolics, phytoalexins and lignins, three key factors responsible for disease resistance. Under such conditions Phenylalanine ammonia lyase activity were assayed from ten different tea varieties inoculated with foliar

Table 30: Level of protein in healthy and artificially inoculated tea leaves

Varieties	Protein content (mg g^{-1} leaf tissue)		
	Healthy	*A. alternata* inoculated	*C. invisum* inoculated
TV-18	25.98 ± 0.13	21.78 ± 0.09	20.58 ± 0.11
TV-28	27.89 ± 0.16	23.45 ± 0.08	18.76 ± 0.19
TV-20	24.89 ± 0.15	14.59 ± 0.12	16.56 ± 0.12
TV-22	28.79 ± 0.13	15.49 ± 0.17	19.75 ± 0.21
UP-3	26.56 ± 0.11	18.45 ± 0.12	16.78 ± 0.14
UP-8	27.58 ± 0.09	22.75 ± 0.08	20.45 ± 0.17
T-17	27.68 ± 0.13	21.48 ± 0.11	23.75 ± 0.07
BSS-3	21.56 ± 0.14	15.47 ± 0.10	18.76 ± 0.11
HV-39	26.78 ± 0.09	20.75 ± 0.09	22.75 ± 0.12
BS/7A/76	26.77 ± 0.12	19.56 ± 0.10	21.45 ± 0.09

Average of five replicates per treatment
Means of three replicates;
± Standard error.

fungal pathogens. PAL activity assayed in each case after 24, 48 and 72h after inoculation. Results following inoculation with *A. alternata* have been presented in Table 31. TV-9, TV-18, UP-3 and BSS-1 showing resistant reaction towards *A. alternata*. Results against *C. invisum* have been presented in Fig 21. In the susceptible varieties TV-20, TV-22, HV-39 and BSS-1 increase in PAL activity were not significant. But in TV-9, TV-25, UP-3 and UP-8 showing markedly increase in PAL activity as these were showed resistant reaction against *C. invisum*.

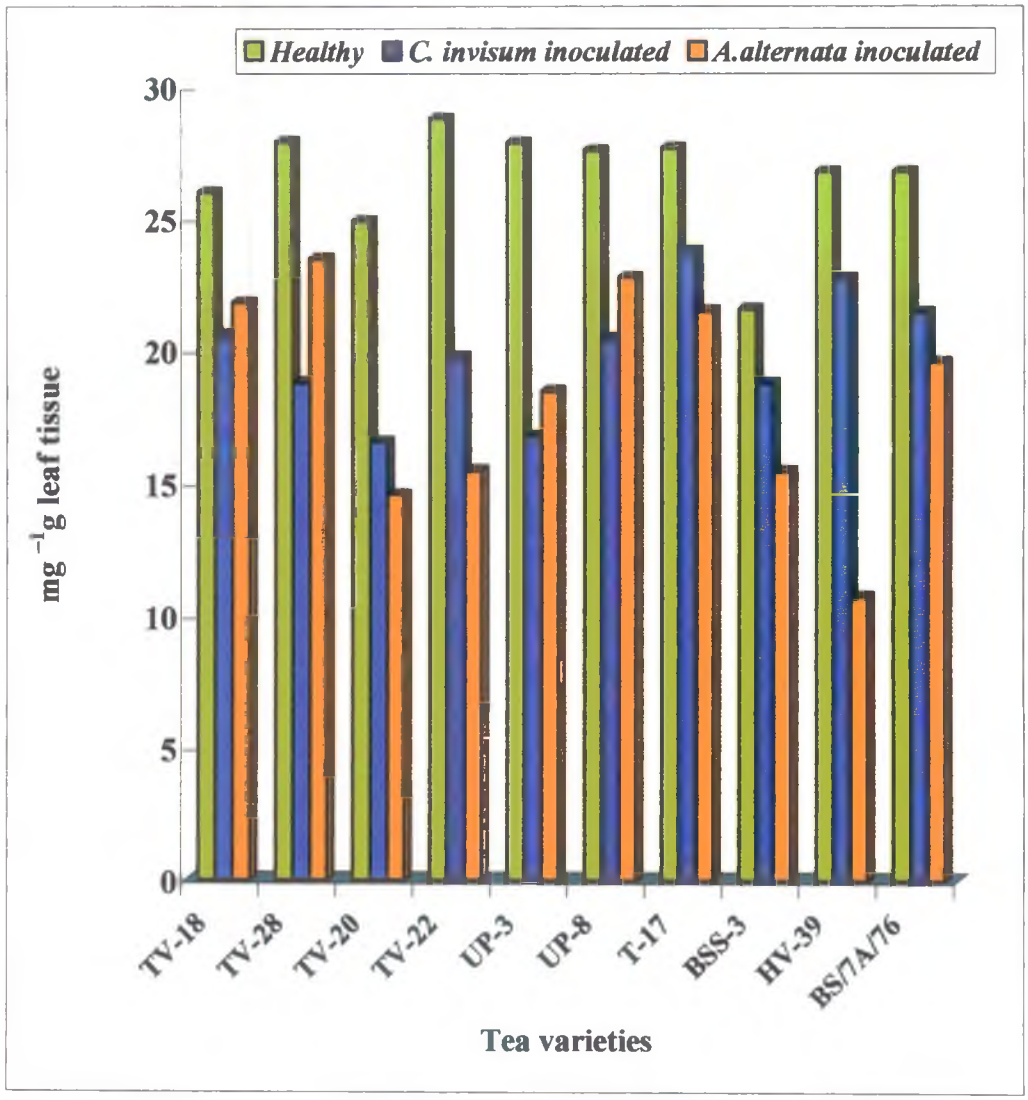

Healthy
C. invisum inoculated
A.alternata inoculated
30
25
20
15
10
5
0
mg $^{-1}$g leaf tissue
TV-18
TV-28
TV-20
TV-22
UP-3
UP-8
T-17
BSS-3
HV-39
BS/7A/76
Tea varieties

Fig. 20

Table 31: Phenylalanine ammonia lyase (PAL) activity in tea leaves following inoculation with *A. alternata*

Varieties	PAL activity (μg cinnamic acid g^{-1} leaf tissue min^{-1})					
	24h		48h		72h	
	Healthy	Inoculated	Healthy	Inoculated	Healthy	Inoculated
TV-9	269.6 ± 0.16	294.2 ± 0.12	270.5 ± 0.11	320.7 ± 0.19	276.2 ± 0.16	365.5 ± 0.18
TV-18	285.6 ± 0.21	325.8 ± 0.19	280.3 ± 0.20	386.7 ± 0.21	283.4 ± 0.17	410.5 ± 0.13
TV-20	241.8 ± 0.23	256.4 ± 0.19	234.8 ± 0.17	257.7 ± 0.14	245.7 ± 0.16	259.8 ± 0.27
TV-22	231.1 ± 0.16	237.8 ± 0.12	234.5 ± 0.15	241.4 ± 0.19	236.4 ± 0.17	244.5 ± 0.27
TV-28	233.4 ± 0.17	254.8 ± 0.12	235.4 ± 0.18	259.6 ± 0.21	237.8 ± 0.18	268.5 ± 0.21
T-17/	260.5 ± 0.23	265.4 ± 0.13	262.3 ± 0.20	270.8 ± 0.19	265.7 ± 0.16	272.7 ± 0.16
UP-3	211.6 ± 0.14	245.8 ± 0.18	220.4 ± 0.21	298.7 ± 0.15	216.7 ± 0.18	324.6 ± 0.27
UP-8	199.3 ± 0.17	213.5 ± 0.25	208.4 ± 0.19	214.5 ± 0.13	210.7 ± 0.16	223.8 ± 0.26
BSS-1	226.4 ± 0.20	265.7 ± 0.28	230.7 ± 0.14	315.7 ± 0.19	235.8 ± 0.25	385.7 ± 0.29
HV-39	241.5 ± 0.18	256.7 ± 0.21	246.8 ± 0.23	260.7 ± 0.21	253.7 ± 0.27	265.7 ± 0.25

Means of three replicates;
± Standard error.

Phenylalanine ammonia lyase activity in healthy and *C. invisum* inoculated tea leaves

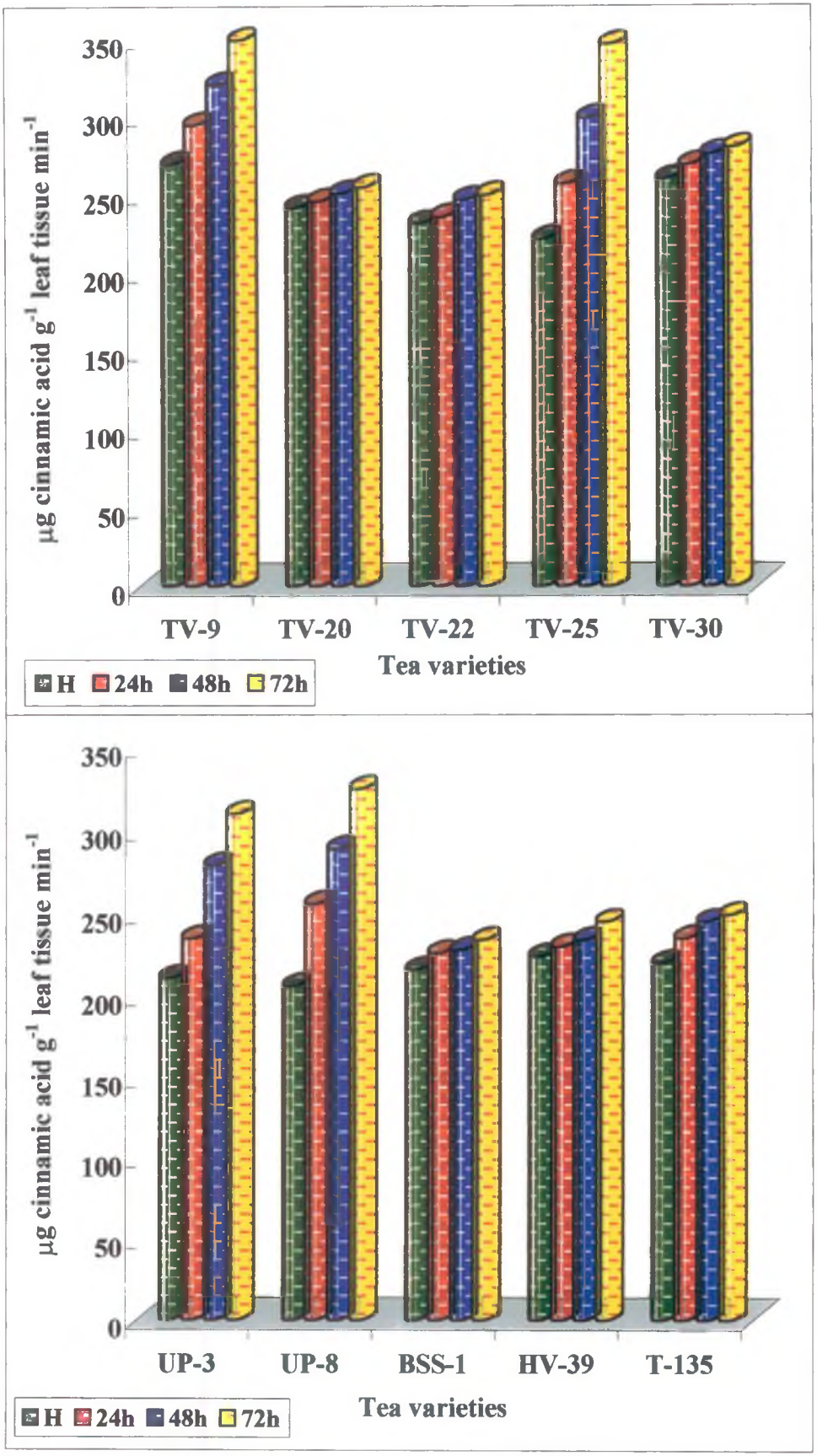


Fig. 21

4.9.2 Polyphenol oxidase (PPO)

Polyphenol oxidase also an important enzyme and have an important role in defense mechanism. PPO were extracted from healthy and inoculated leaves and assayed after 24, 48 and 72h of inoculation. Results following inoculation with *C. invisum* have been computed in Table 32. Varieties (TV-9, TV-28, UP-3 and UP-8) showing resistant reaction like showed increased PPO activity. Where as following inoculation with *A. alternata* (Fig. 22) PPO activity were not significant in TV-20, TV-22, UP-8 but significant increase in resistant varieties (TV-9, TV-18 and UP-3).

Table 32: Polyphenol oxidase (PPO) activity in tea leaves following inoculation with *C. invisum*

Varieties	PPO activity (ΔA_{465} mg^{-1} leaf tissue min^{-1})					
	24h		48h		72h	
	Healthy	Inoculated	Healthy	Inoculated	Healthy	Inoculated
TV-9	3.1 ± 0.06	4.2 ± 0.02	3.3 ± 0.09	5.2 ± 0.19	3.2 ± 0.06	5.9 ± 0.18
TV-20	3.2 ± 0.03	3.4 ± 0.09	3.1 ± 0.07	3.7 ± 0.04	3.7 ± 0.06	3.9 ± 0.07
TV-22	3.5 ± 0.06	3.9 ± 0.02	3.5 ± 0.05	4.1 ± 0.09	3.4 ± 0.07	4.5 ± 0.07
TV-25	3.0 ± 0.07	4.8 ± 0.02	3.4 ± 0.08	5.6 ± 0.01	3.2 ± 0.08	5.9 ± 0.01
TV-30	3.5 ± 0.03	3.4 ± 0.03	3.3 ± 0.02	3.8 ± 0.10	3.7 ± 0.06	4.5 ± 0.06
UP-3	3.7 ± 0.04	4.8 ± 0.08	3.4 ± 0.01	5.2 ± 0.05	3.7 ± 0.08	5.6 ± 0.07
UP-8	3.8 ± 0.07	5.3 ± 0.05	3.4 ± 0.09	5.9 ± 0.03	3.7 ± 0.06	6.2 ± 0.06
BSS-1	3.6 ± 0.03	3.7 ± 0.08	3.7 ± 0.04	3.9 ± 0.19	3.8 ± 0.05	3.7 ± 0.09
HV-39	3.1 ± 0.08	4.3 ± 0.11	3.3 ± 0.03	5.0 ± 0.01	3.4 ± 0.07	5.7 ± 0.05
T-135	3.3 ± 0.05	3.7 ± 0.08	3.4 ± 0.04	3.6 ± 0.09	3.5 ± 0.09	4.0 ± 0.06

Means of three replicates;
± Standard error.

Polyphenol oxidase activity in healthy and *A. alternata* inoculated tea leaves

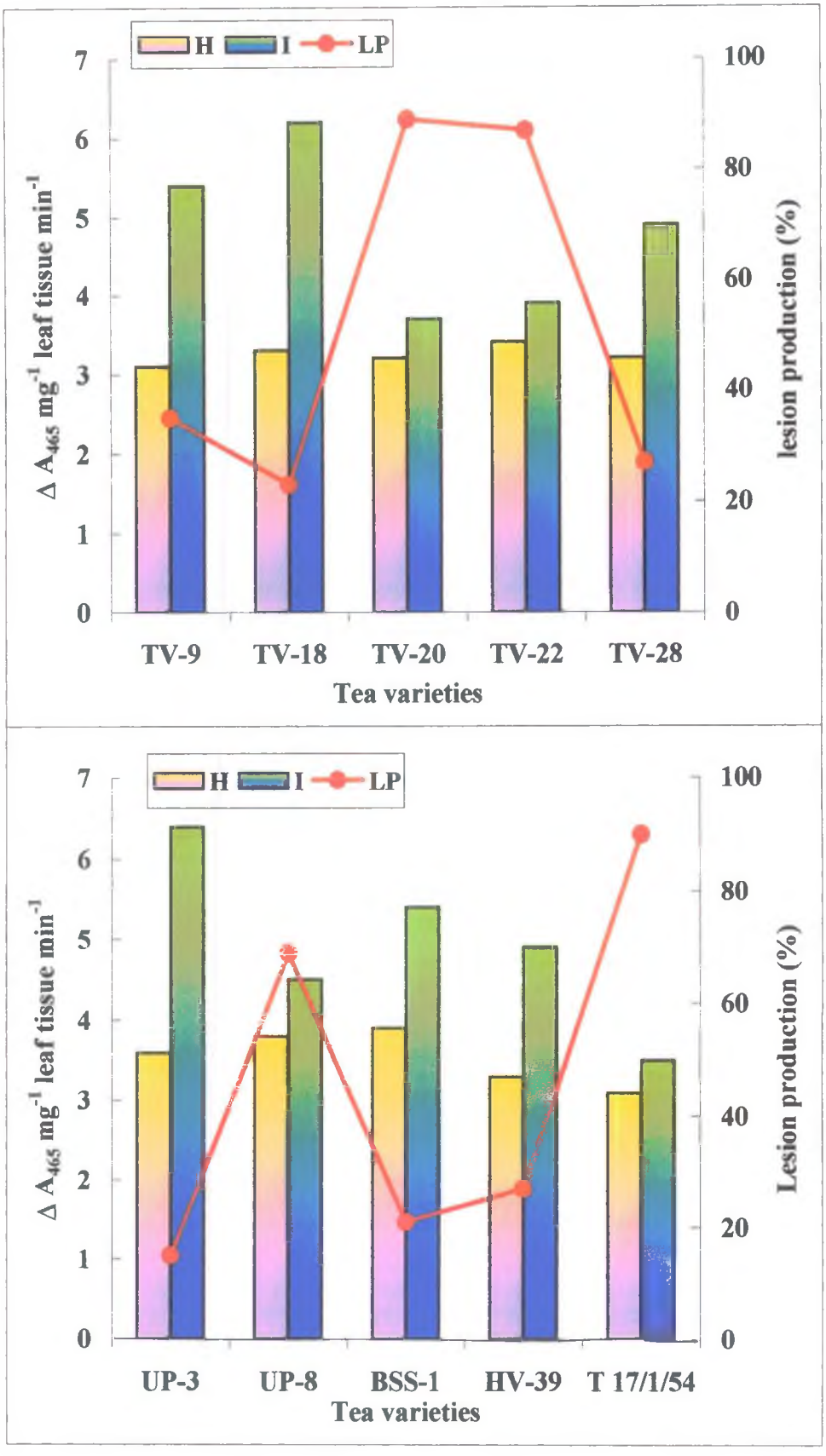


Fig. 22

4.9.3 Peroxidase (PO)

Peroxidase activity was assayed as increase in absorbance when O-dianisdidine was oxidized by the oxygen released from H_2O_2 which was oxidized by the enzyme. Peroxidase was extracted and assayed from healthy and artificially inoculated tea leaves of eleven varieties. Results have been presented in Table 33. Peroxidase activity increased markedly mainly in resistant varieties (TV-9, TV-25, UP-3).

Table 33: Peroxidase (PO) activity in tea leaves after 48h of inoculation with foliar fungal pathogens

Varieties	Peroxidase activity - (Δ A_{460} g^{-1}leaf tissue min^{-1})		
	Healthy	*C. invisum* inoculated	*A. alternata* inoculated
TV-9	0.77 ± 0.02	2.35 ± 0.09	2.65 ± 0.05
TV-18	0.54 ± 0.01	1.03 ± 0.04	2.85 ± 0.07
TV-20	0.82 ± 0.02	0.97 ± 0.07	1.03 ± 0.08
TV-22	0.56 ± 0.06	0.85 ± 0.03	0.96 ± 0.05
TV-25	0.53 ± 0.04	2.75 ± 0.08	1.59 ± 0.03
TV-30	0.59 ± 0.07	1.86 ± 0.07	2.45 ± 0.11
T17/1/54	0.64 ± 0.09	1.23 ± 0.04	0.86 ± 0.12
UP-3	0.55 ± 0.04	2.56 ± 0.07	2.79 ± 0.06
UP-8	0.68 ± 0.10	2.46 ± 0.12	0.86 ± 0.08
BSS-1	0.84 ± 0.09	0.94 ± 0.06	2.86 ± 0.13
HV-39	0.68 ± 0.11	0.86 ± 0.03	2.06 ± 0.08
T-135	0.76 ± 0.05	1.56 ± 0.05	1.06 ± 0.06

Means of three replicates;

± Standard error.

4.9.4 β-1,3-glucanase (βGLU)

βGLU, important enzyme involved in the plant's defense response against pathogen attack. Ten different tea varieties were inoculated with *C. invisum* and βGLU enzyme were extracted and assayed after 24, 48 and 72h after inoculation. Results have been presented Table 34. TV-9, UP-3, UP-8 and BS/7A/76 showed resistant reaction and in theses case enzyme activity increased markedly in comparison to the susceptible varieties (TV-20, TV-22, HV-39 and BSS-1). βGLU activity following inoculation with *A. alternata* have been presented in Fig. 23. TV-9, TV-18, TV-28, UP-3, BSS-1 varieties showed remarkable increase in glucanase activity where disease index were low.

Table 34: β-1,3-glucanase activity in tea leaves following inoculation with *C. invisum*

Varieties	β-1,3-glucanase activity (μg glucose g^{-1} leaf tissue min^{-1})					
	24h		48h		72h	
	Healthy	Inoculated	Healthy	Inoculated	Healthy	Inoculated
TV-9	43.1 ± 1.16	64.2 ± 1.12	40.3 ± 1.10	65.2 ± 1.19	43.2 ± 1.06	65.9 ± 1.18
TV-20	44.2 ± 1.13	43.4 ± 1.19	45.1 ± 1.29	46.7 ± 1.14	44.7 ± 1.16	49.9 ± 1.10
TV-22	46.5 ± 1.11	47.9 ± 1.12	44.5 ± 1.25	49.1 ± 1.09	43.4 ± 1.12	50.5 ± 1.57
TV-25	43.0 ± 1.07	52.8 ± 1.11	45.4 ± 1.18	63.7 ± 1.17	44.2 ± 1.08	69.9 ± 1.26
TV-30	48.5 ± 1.13	50.4 ± 1.09	50.3 ± 1.12	53.8 ±1.10	46.7 ± 1.16	54.5 ± 1.46
UP-3	35.7 ± 1.04	44.8 ± 1.08	33.4 ± 1.11	55.2 ± 1.25	37.7 ± 1.08	65.6 ± 1.27
UP-8	38.7 ± 1.07	45.3 ± 1.15	33.4 ± 1.29	55.9 ± 1.23	35.7 ± 1.06	62.2 ± 1.26
BSS-1	34.6 ± 1.09	38.7 ± 1.08	33.7 ± 1.14	39.4 ± 1.19	36.8 ± 1.05	40.7 ± 1.29
HV-39	38.1 ± 1.08	40.3 ± 1.11	34.3 ± 1.13	45.0 ± 1.11	32.4 ± 1.07	45.7 ± 1.25
T-135	36.3 ± 1.15	39.7 ± 1.08	35.4 ± 1.14	43.6 ± 1.39	33.5 ± 1.67	43.1 ± 1.36

Means of three replicates; ± Standard error.

β-1,3 glucanase activity in healthy and *A. alternata* inoculated tea leaves

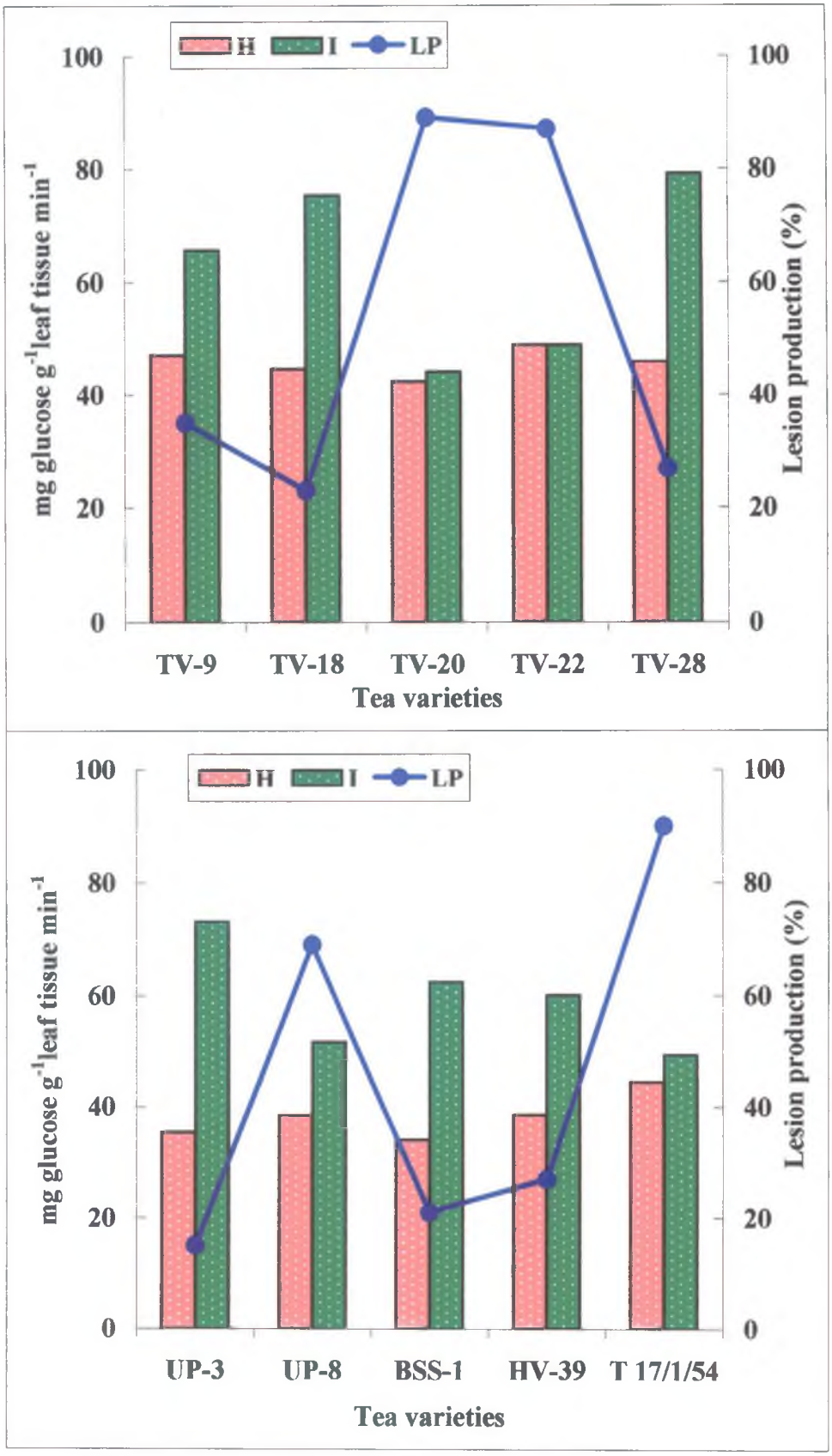


Fig. 23

4.9.5 Chitinase (CHT)

Healthy plants contain stress amount of pathogenesis related proteins that can be induced by biotic and abiotic elicitors. CHT were extracted and assayed from ten different varieties following inoculation with fungal pathogens. Enzyme activity after 24 and 48h following inoculation with *A. alternata* were presented in Table 35. Enzyme activity were high in the resistant varieties than the susceptible varieties. After challenge inoculation with *C. ĭnvisum*. CHT activity increased in three varieties (TV-9, UP-3 and UP-8) which exhibited resistant reaction towards *C. ĭnvisum* (Fig. 24). However where disease index were high (TV20, TV-22, BSS-1, HV-39) CHT activity did not increased significantly.

Table 35: Chitinase activity in healthy and *A. alternata* inoculated tea leaves

	Chitinase activity (mg GlcNAc g^{-1}leaf tissue h^{-1})			
	24h		48h	
Varieties	Healthy	Inoculated	Healthy	Inoculated
TV-9	0.64 ± 0.06	1.24 ± 0.09	0.65 ± 0.07	1.89 ± 0.05
TV-18	0.54 ± 0.03	1.56 ± 0.05	0.58 ± 0.03	2.01 ± 0.06
TV-20	0.57 ± 0.04	0.73 ± 0.06	0.54 ± 0.10	0.86 ± 0.04
TV-22	0.59 ± 0.07	0.79 ± 0.08	0.55 ± 0.07	0.92 ± 0.06
TV-28	0.57 ± 0.06	0.86 ± 0.09	0.60 ± 0.03	1.02 ± 0.04
T-17	0.60 ± 0.03	0.63 ± 0.04	0.58 ± 0.07	0.68 ± 0.03
UP-3	0.61 ± 0.07	1.43 ± 0.06	0.64 ± 0.04	1.83 ± 0.05
UP-8	0.52 ± 0.02	0.58 ± 0.07	0.50 ± 0.03	0.61 ± 0.06
BSS1	0.53 ± 0.04	1.56 ± 0.07	0.56 ± 0.09	1.75 ± 0.06
HV-39	0.59 ± 0.08	0.85 ± 0.06	0.62 ± 0.10	0.96 ± 0.11

Means of three replicates; ± Standard error.

Chitinase activity in healthy and *C. invisum* inoculated tea leaves

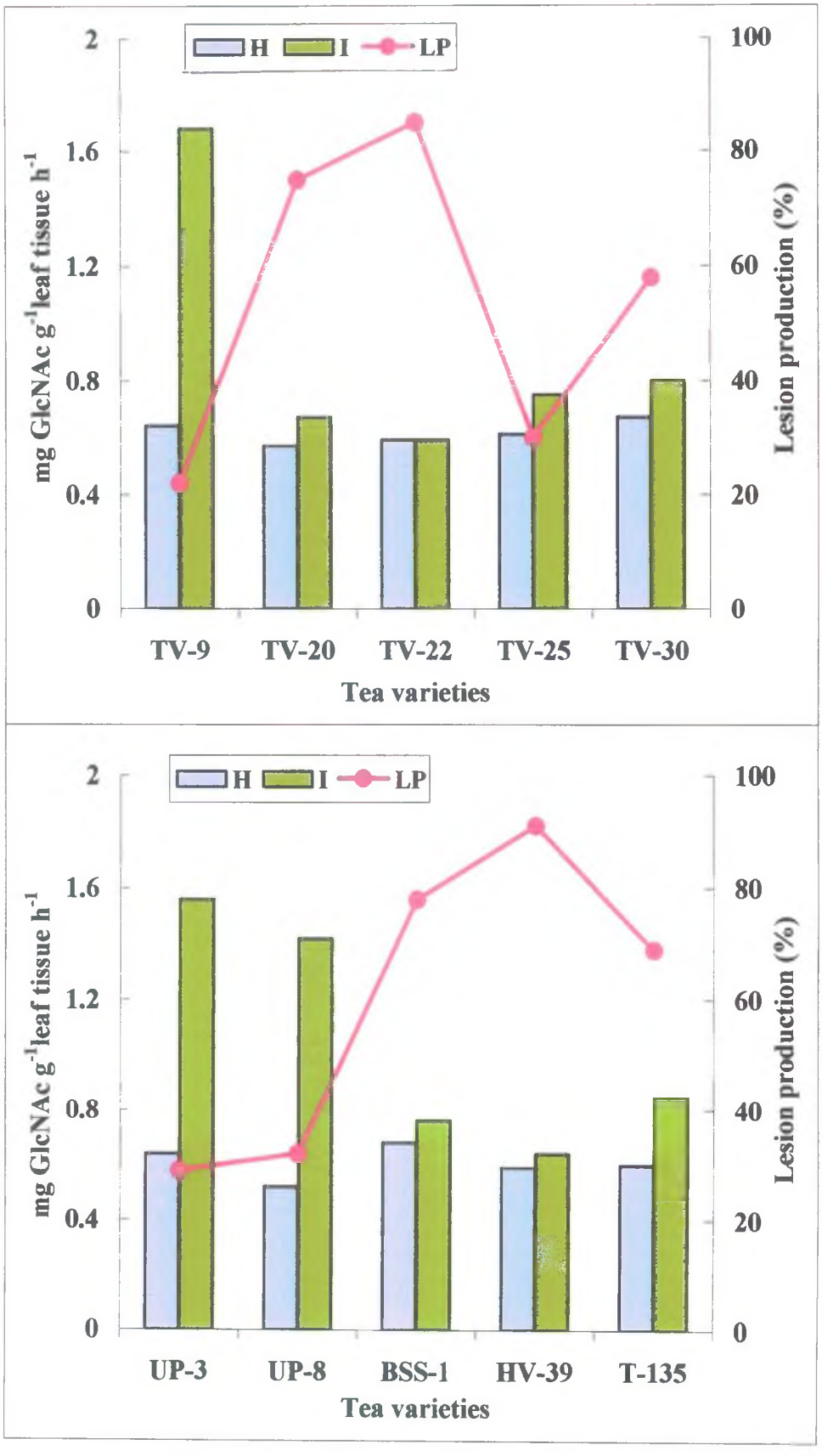


Fig. 24

4.10 Accumulation of pyrocatechol and catechin in tea leaves following inoculation with *A. alternata*

The restriction and unrestricted growth of the pathogen on resistant and susceptible hosts, respectively, clearly indicates the differential response of host cultivars to a pathogen. Disease resistance in many plant-fungal pathogen interactions has been suggested to be due to inducible production of antibiotic low molecular weight compounds, *i.e.*, phytoalexins. The involvement of antifungal compounds in tea leaves against foliar fungal pathogens are not easily applied to the large amount of tissue and the utility of this technique is diminished further due to low solubility of many phytoalexins in pure water.

In the present investigation further experiments were carried out following facilitated diffusion technique for the detection of antifungal substances (pyrocatechol) from relatively large samples of freshly harvested tea leaves inoculated with *A. alternata*. Pyrocatechol was extracted separately from healthy and *A. alternata* inoculated three resistant varieties (TV-9, TV-25 and UP-3) and three susceptible varieties (TV-20, TV-22 and T-17) after 40 h of inoculation.

Ethyl acetate fraction of both healthy and inoculated (with *A. alternata*) tea leaf extracts were loaded on TLC plates, developed in chloroform : methanol (9:1,v/v) and sprayed with Folin-ciocalteau's reagent. All the compounds gave positive colour reaction indicating the presence of phenolic compound. Healthy leaf extract showed Rf 0.63 where as *A. alternata* inoculated tea leaves showed at Rf 0.51.

4.10.1 Radial growth bioassay

Ethyl acetate extract after hydrolysis from both healthy as well as inoculated tea leaves of six varieties were bioassayed following radial growth inhibition assay. Results have been presented in Table 36. Mycelial growth was measured in each treatment, when *C. invisum* covered full petridish (3 cm. dia) grown in potato

dextrose agar medium without any supplementation. Growth of *C. invisum* was inhibited markedly in the medium supplemented with the extracts of *A. alternata* inoculated tea leaves of resistant varieties TV-9, TV-25 and UP-3 than those of susceptible varieties TV-22 and T-17 in relation to their respective control *i.e.* media supplemented with the healthy leaf extract. Maximum inhibition 59.2% in case of UP-3 variety (Plate 26 fig B).

Table 36: Effect of antifungal compounds from tea leaves extract on radial growth of *C. invisum*

Variety	Diameter of mycelial[a] growth (mm)[b]	
	Healthy	**Inoculated with *A. alternata***
Control	30	
Susceptible		
TV-20	22.5	16.9 (24.8)[#]
TV-22	23.7	15.4 (35.0)
T-17	21.7	14.7 (32.2)
Resistant		
TV-9	20.4	9.4 (53.9)
TV-25	19.7	8.6 (56.3)
UP-3	17.9	7.3 (59.2)

[a]*Corticium invisum*

[b]Average of three experimental sets

[#]Values in the parenthesis indicates inhibition against healthy

4.10.1.2 TLC plate bioassay

Fraction III of both healthy and inoculated leaves of two varieties were bioassayed for antifungal compounds using *Curvularia pallescence* as the test organism following TLC plate bioassay technique. Diameter of the inhibition zones for healthy inoculated tea leaf extracts for two varieties appeared on the chromatograms are presented in Table 37. The inhibitory compounds were presented not only in leaf extracts of inoculated resistant and susceptible varieties but also in extracts of non-inoculated healthy leaves. There was no evidence of the inhibition zone of TV-22, susceptible variety. However inhibition zone appeared in resistant variety TV-9.

Table 37 : TLC plate bioassay of antifungal compounds

Variety	Treatment	Diameter of the inhibition zone (mm)
Resistant		
TV-9	Healthy	8.4
TV-9	*A. alternata* inoculated	2.9
Susceptible		
TV-22	Healthy	9.8
TV-22	*A. alternata* inoculated	0.0

Solvent system - Chloroform : methanol (9:1 V/V)

Organism tested - *Curvularia pallescence*

Incubation period – 78 h at 25^0C

4.10.1.3 Glass slide bioassay

Biological activities of antifungal compounds were assayed following the slide germination procedure. For spore germination bioassay 20µl drop of ethyl acetate solvent extract was placed on clean, grease-free slide and allowed to evaporate following which drop of spore suspension of *A. alternata* ($5x10^5$spores/ml) was placed over it and incubated at 25 ± 1^0C for 24h. In case of control drop respective

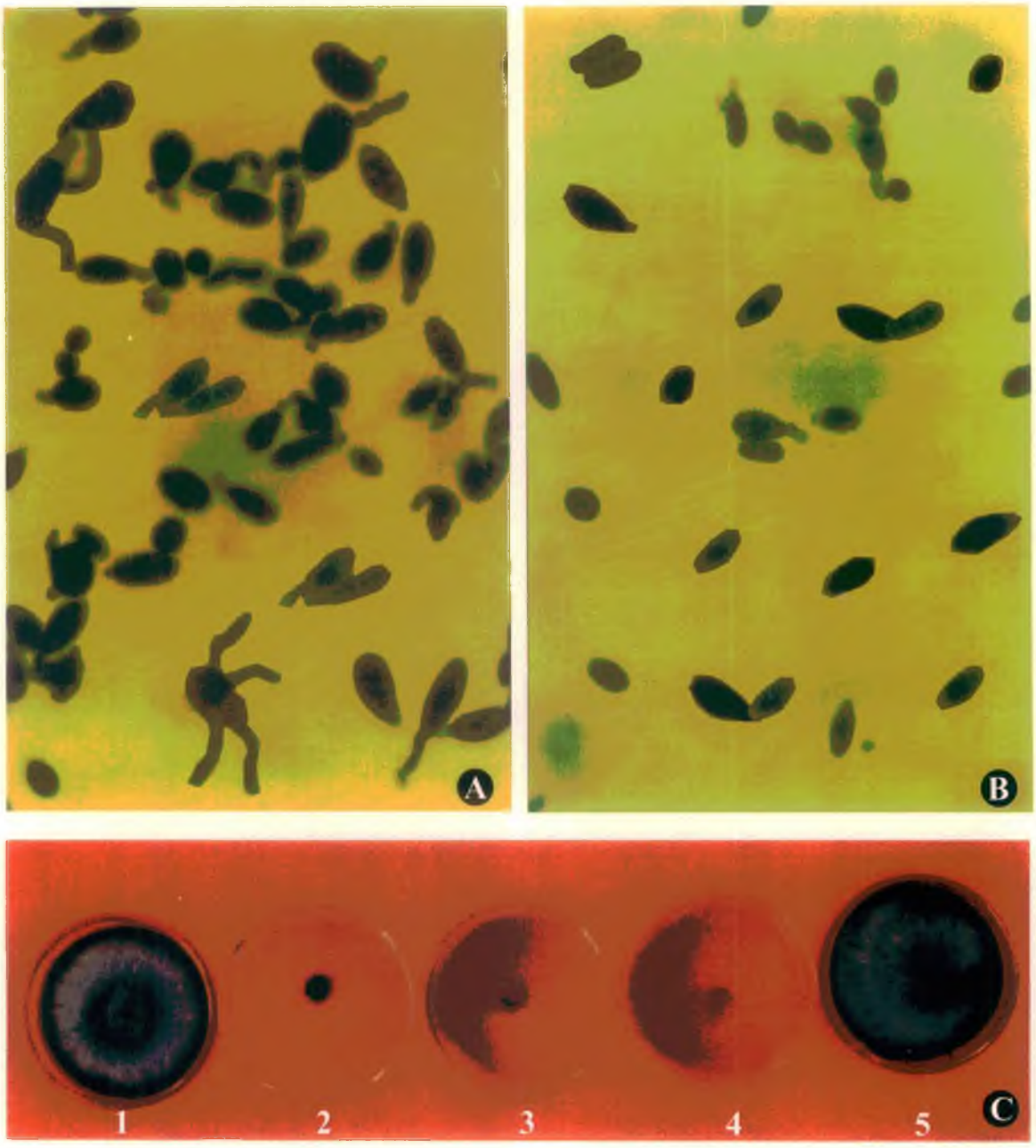


Plate 26 (figs. A-C): Spore germination (A & B) and radial growth (C) bioassay of antifungal phenolics - pyrocatechol. Distilled water control(A):pyrocatechol treated (B & C - 2,3,4) ; media control (C - 1 & 5) ; *A. alternata* (A & B); *C. pallescence* (C)

solvents were used. Percentage spore germination and germ tube length were determined of resistant and susceptible varieties. Finally germinated and ungerminated spores were stained with cotton blue in lactophenol and examined under the microscope. Percentage of germination was calculated in each case. Results presented in Table 38. Spore germination of *A. alternata* inhibited markedly by the compound extracted from resistant variety (Plate 26 figs. A&B) in relation to susceptible variety. Spore germination inhibited 8%-15% by the resistant variety in both the cases.

Table 38: Effect of antifungal compounds from tea leaves extract on slide germination bioassay of *A. alternata*.

Variety	Treatment	Percentage of germination Inhibition	
Control		75.7	
Resistant			
TV-9	Healthy	61.3	
TV-9	*A. alternata* inoculated	11.6	(81.0)[#]
Susceptible			
TV-22	Healthy	65.4	
TV-22	*A. alternata* inoculated	55.4	(15.2)

[#]Values in the parenthesis indicates inhibition against healthy

Organism tested - *A. alternata* ($5x10^5$spores/ml)

4.10.2 HPLC analysis of pyrocatechol

Results of the bioassay revealed that the presence of antifungal compounds in ethyl acetate fractions. Radial growth bioassay and spore germination bioassay indicates that the compounds were effective against the growth of fungal pathogens. In order to determine the further analysis the phenolics compounds of both the healthy as well as *A. alternata* inoculated tea leaf extracts of TV-9 compounds were analysed by HPLC. The UV spectra from both the compounds were analysed at 280 nm. A sharp peak at retention time 2.6 was present in both the compounds but in the

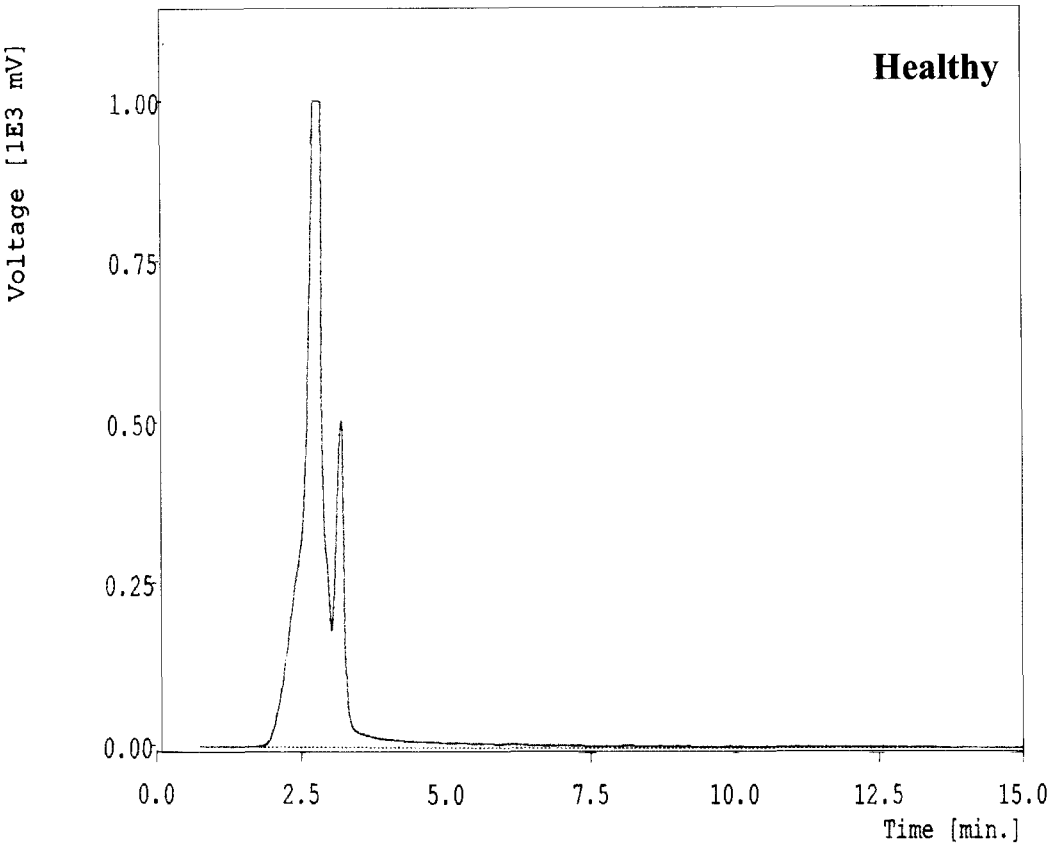
healthy extracts the peak height was much smaller than the other one (Fig 25 A & B). Other small humps and shoulders were also evident in all the cases. Results (Table 39) revealed the nature of phenolics in both the cases.

Table 39: HPLC analysis of pyrocatechol from healthy and *A. alternata* inoculated tea leaves (TV-9)

Leaf extract	**Peak No.**	**Reten. time (min)**	**Height (mV)**	**Height (%)**
Healthy	1.	2.660	999.149	99.125
	2.	8.150	2.604	0.260
A.alternata inoculated	1.	2.630	1004.400	99.740
	2.	8.150	3.252	0.321

Maximum absorption peak measured at 280 nm corresponded with the authentic pyrocatechol. Hence, quantification of pyrocatechol was done from UV-spectrophotometric curve by considering molar extinction coefficient of authentic pyrocatechol 6000 at 280 nm. Pyrocatechol accumulated within 48 h following inoculation with *A. alternata* was estimated from two resistant varieties (TV-18 and UP-3) and two susceptible varieties (TV-22 and T-17). Results have been presented in Table 40. It appears from the result that in *A. alternata* inoculated leaves greater amount of pyrocatechol accumulated within 48h following infestation. Higher level of accumulation was evident in all three resistant varieties tested than other two susceptible varieties.

HPLC elution profiles of pyrocatechol from healthy and *A. alternata* infected tea leaves



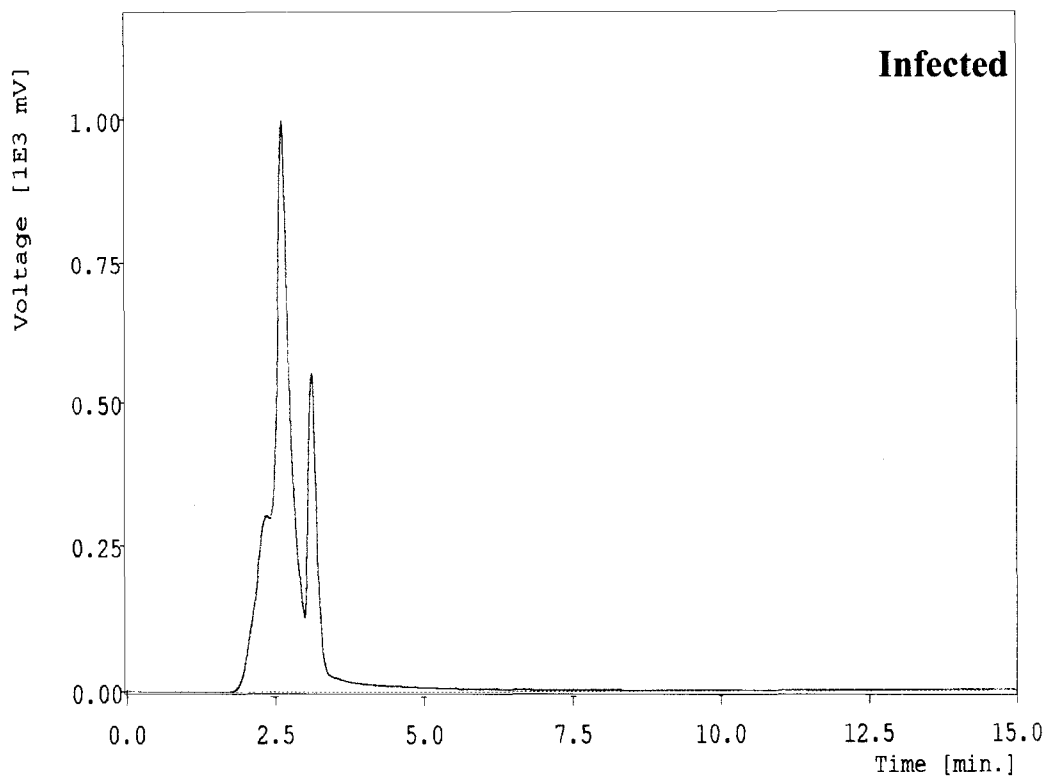


Fig. 25

Table 40: Quantitative estimation of pyrocatechol in healthy and *A.alternata* inoculated resistant and susceptible tea varieties

Tea varieties	Pyrocatechol concentration (µg/g fresh wt. of leaves)	
Resistant varieties	Healthy	*A. alternata* inoculated[a]
TV-18	110	573
UP-3	90	497
Susceptible varieties		
TV-22	70	286
T-17	69	257

[a]48h after inoculation.

4.10.3 HPLC analysis of Catechins

Fresh tea leaves (TV-9) were detached from plant and placed on moist blotting paper kept in aluminum tray. Their upper surfaces were wounded as described by Cook (1989). 20µl drops per leaf of spore suspension prepared from 14 days culture of *Alternaria alternata* ($5x10^5$spores/ml) were placed on the adaxial surface of each leaf. Each tray was covered with a glass lid and sealed with a smear of petroleum jelly in order to minimize the drying of the drops during incubation. After 40h of inoculation leaves were collected and used for catechins extract. Healthy leaves were collected from the experimental garden. Catechins extracted from both the healthy as well as inoculated tea leaves and analyzed by HPLC (Fig. 26). Due to reaction with pathogen inoculated leaf samples exhibited more isoforms of catechin than healthy control. In *A. alternata* inoculated leaves four peaks P4, P6 P8 P16 and P17 increased markedly in in relation to healthy leaves (Fig. 26). P4 and P8 corresponded with authentic EGC and EC respectively. Resulting (Table 40) due to infection by *A. alternata,* levels of EGCG corresponding with peaks – P9 decreased markedly. However, some of the peaks (P1, P3 and P10) did not affect due to infestation at this stage.

HPLC elution profiles of catechins from healthy and *A. alternata* infected tea leaves

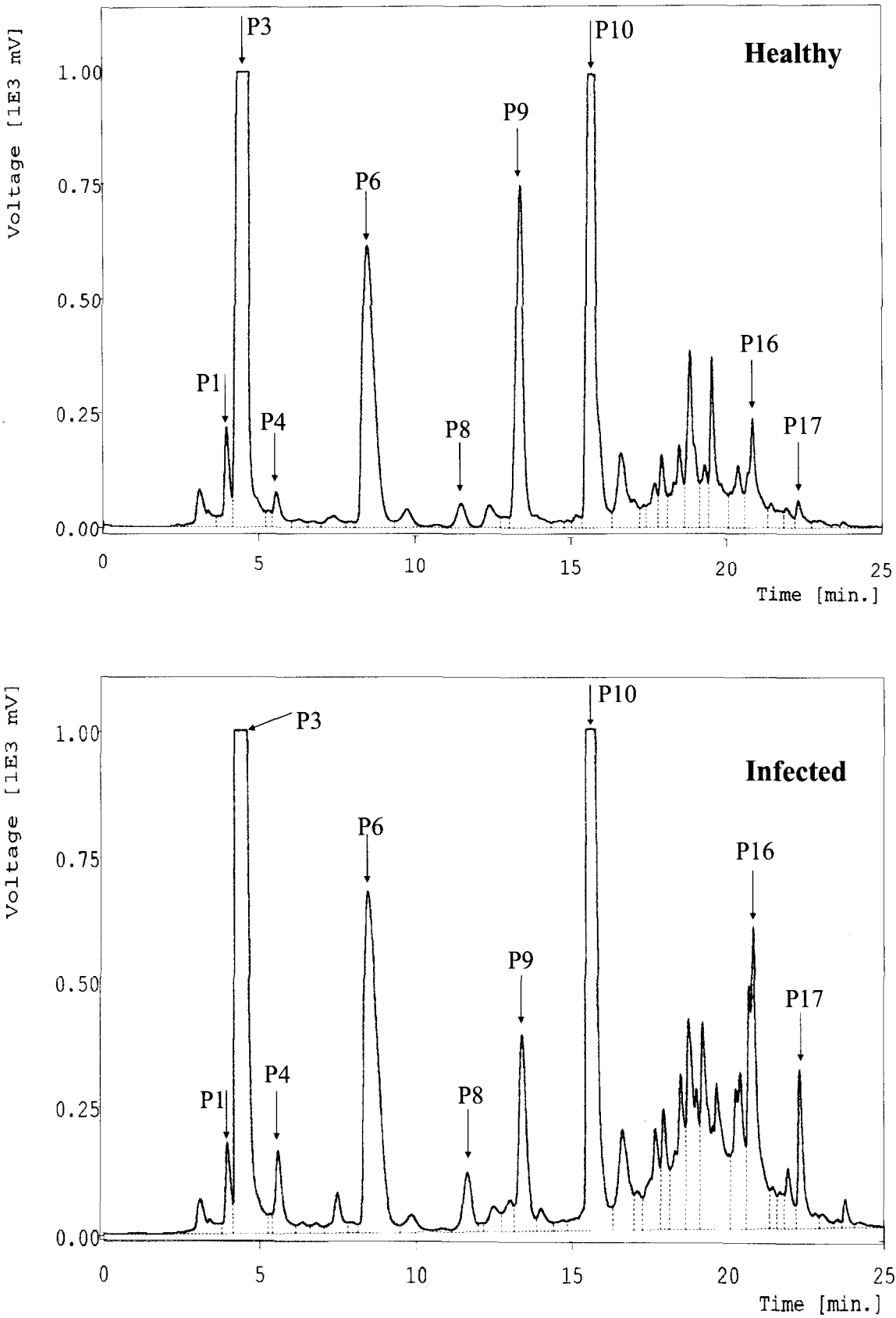


Fig. 26

Table 40: HPLC analyses of catechins extracted from healthy and A. alternata inoculated tea leaves (TV-9) in comparison with authentic catechin isomers

Peak No.	Corresponding with authentic isomers of catechins	Reten. time (min)	Height (mV)	
			Healthy	*A. alternata* inoculated
P1	--	3.070	81.152	67.412
P2	--	3.930	217.863	177.290
P3	GC	4.550	996.799	996.546
P4	EGC	5.540	75.689	159.046
P5	--	7.390	24.643	77.907
P6	--	8.380	615.645	676.347
P7	--	9.720	38.724	33.671
P8	EPC	11.460	51.874	114.638
P9	EGCG	13.290	747.499	383.684
P10	GCG	15.660	989.670	989.640
P11	CG	16.590	162.694	194.527
P12	--	17.690	95.253	194.236
P13	ECG	18.800	358.964	410.691
P14	--	19.500	371.667	405.566
P15	--	20.360	134.060	304.302
P16	--	20.820	237.275	597.060
P17	--	22.310	57.546	304.545
P18	--	23.760	12.293	54.560

GC – Galo-catechin **EGC – Epigalo-catechin**
EPC – Epicatechin **EGCG – Epigalo-catechingalate**
GCG – Galo-catechingalate **CG – Catechin-galate**
ECG – Epicatechin-galate

4.11 Application of plant extracts for induction of resistance in tea plants against *Alternaria alternata*

Leaf diseases of tea are usually controlled by conventional broad-spectrum fungicides applied as foliar sprays. However, due to growth concern about harmful effects of the chemicals on environment and human health, effects have been made to evolve environmentally friendly strategies for the control of pests and pathogens. In the present investigation, aqueous extracts of leaf have been prepared from the three plant species. These are *Azadirachta indica*, *Catharanthus roseus* and *Diplazium esculentum*.

4.11.1 *In vitro* test

An experiment was set up to determine the effect of the different concentration of leaf extracts on spore germination of *A. alternata*. Spore suspension was prepared (10^5 conidia/ml) in sterile distilled water from 12 day-old culture grown in PDA slant. Plant extracts prepared from healthy leaves of *Azadirachta indica, Catharanthus roseus* and *Diplazium esculentum*. Crude extracts (undiluted) and two more dilutions (1:10 and 1:100) were used for *in vitro* test following spore germination assay. Percentage germination of spores were determined. Maximum inhibition in spore germination was observed in crude extracts, which decrease with the decrease in concentration (Table 41). ED_{50} values were determined. Effect of *C. roseus* was found to be more potent in comparison to *D. esculentum* and *A. indica*.

4.11.2 *In vivo* test

Eight different tea varieties of which six varieties (TV-20, TV-22, T17/1/54, UP-3, BSS-3 and BS/7A/76) showed susceptible reaction and two varieties (TV-18 and UP-3) showed resistant reaction against the pathogen (*A. alternata*) in resistant screening test, were chosen for the present experiment. The leaf extracts prepared from *Azadirachta indica, Catharanthus roseus* and *Diplazium esculentum* were

sprayed on tea plants as described earlier. Leaves from treated and control plants were inoculated with spore suspension of *A. alternata.* Lesion developed after 72h of inoculation on tea leaves were recorded. The experiment was repeated thrice. Results (Table 41) revealed the alteration of disease reaction following treatment with plant extracts.

Table 41: Effect of different concentration of plant extract on inhibition of spore germination of *A. alternata*

Plant extract	% inhibition of spore germination*		
		Dilution	
	Crude	1: 10	1: 100
A. indica	91.5	68.7	10.4
C. roseus	100.0	73.4	26.7
D. esculentum	98.5	65.4	14.7

*In relation to sterile distilled water control

Highest lesion production (80-90%) was evident in three varieties (TV-20, T17/1/54 and BS/7A/76) in control sets where plants were sprayed with distilled water and then inoculated with the pathogen. It is interesting to note that in these varieties percentage lesion production were significantly reduced following treatment with plant extract. Statistical analysis confirm that the application of plant extract alter disease reaction in susceptible plants.

Six varieties (TV-20, TV-22, T17/1/54, UP-3, BSS-3 and BS/7A/76) which showed susceptible reaction were taken for further experiment. Leaf extracts were sprayed on those varieties and spore suspensions of *A. alternata* were again sprayed

on leaves of treated and untreated and incubated for 25 days. The incidence of the disease in whole plant was estimated by calculating mean disease index per plants and the mean of after treatment. Results (Fig. 27) revealed that maximum severity of the disease was recorded in control plants followed by leaf treated with *A. indica*, *D. esculentum* and *C. roseus* respectively in all the susceptible varieties.

Table 42: Effect of foliar application of plant extracts on lesion production in tea leaves following inoculation with *A. alternata*

	% of lesion production after treated with aqueous plant extract			
Varieties	**DW**	***A. indica***	***C. roseus***	***D. esculentum***
Susceptible				
TV-20	86.7 ± 2.9	61.7 ± 2.8	49.7 ± 2.1	54.3 ± 1.9
TV-22	77.4 ± 2.8	59.2 ± 1.6	40.2 ± 1.2	48.7 ± 2.1
T17/1/54	91.5 ± 3.4	69.1 ± 2.1	53.7 ± 1.3	57.1 ± 1.9
UP-8	58.4 ± 2.1	47.8 ± 1.9	39.2 ± 1.6	44.7 ± 1.8
BSS-3	66.4 ± 1.6	51.7 ± 1.5	31.7 ± 1.8	42.8 ± 2.1
BS/7A/76	81.4 ± 2.7	56.7 ± 1.9	42.1 ± 1.1	47.9 ± 1.4
Resistant				
TV-18	20.4 ± 1.2	16.8 ± 0.8	11.2 ± 0.5	14.7 ± 1.1
UP-3	11.1 ± 0.9	10.1 ± 0.7	08.4 ± 0.1	09.7 ± 0.2

Mean of three replicates
± Standard error

Analysis of variance of data presented in Table 42

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	7	14073.270	2010.467	42.8860	10.68291
Time interval	4	4885.422	1221.355	26.0532	
Error	28	1312.620	46.879		
Total	39	20271.310			

Alteration of disease reaction of Alternaria blight after treated with plant extracts

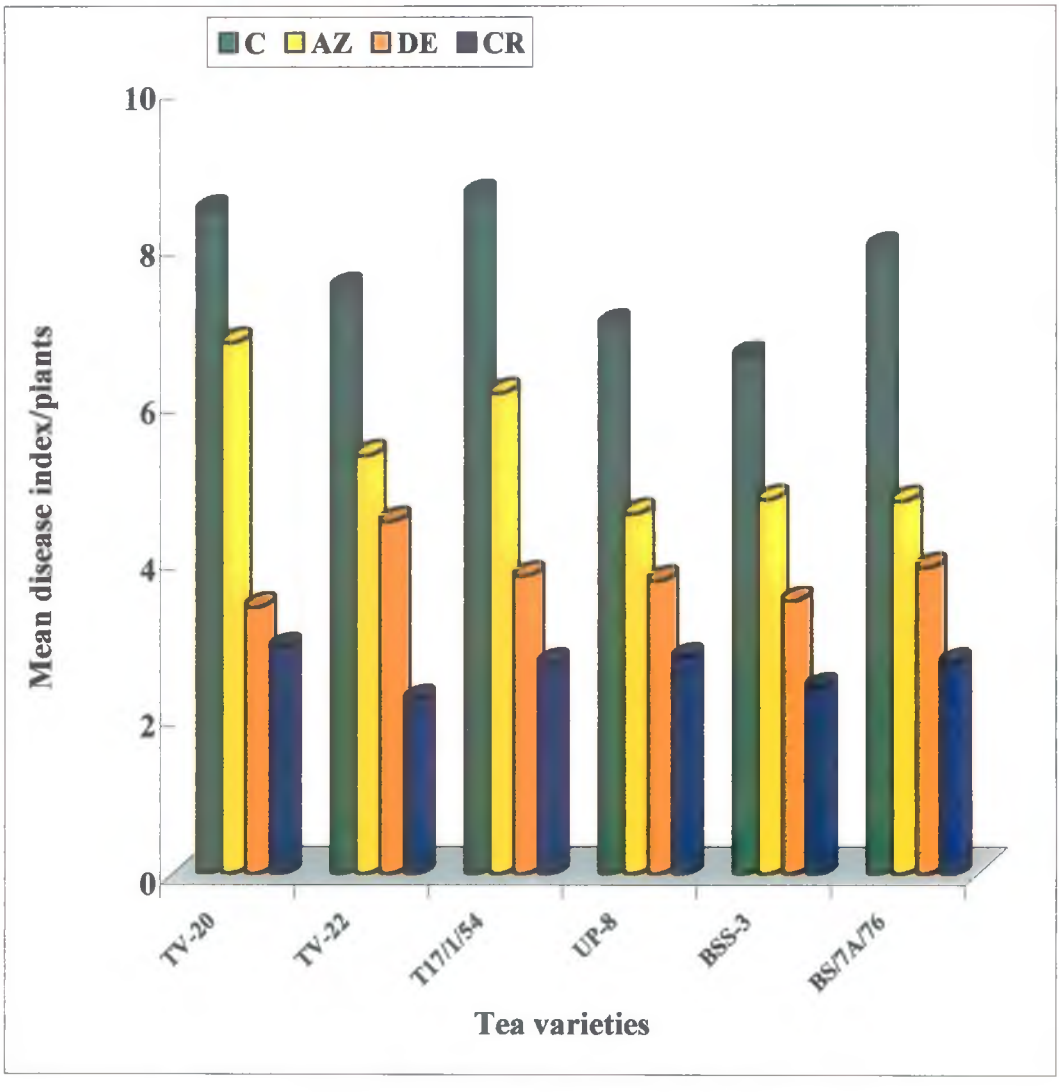


Fig. 27

4.11.3 Biochemical analysis

4.11.3.1 Phenolics

Six tea varieties (TV-20, TV-22, T17/1/54, UP-3, BSS-3 and BS/7A/76) which showed susceptible reaction in screening of resistance were choosen for biochemical analysis. Tea plants of those varieties were treated with plant extracts of *A. indica, D. esculentum and C. roseus*. Leaves were sampled after 24, 48 and 72 h after treatment. It has been found that all the extracts have significant effect on tea leaves. It is evident from Table 43 total phenol content of the treated plants has considerably increased as compared to the untreated plants. And the total phenol content is greater in the inoculated plants than in the uninoculated ones. In the same way o-phenol also increased in the treated varieties (Table 44). Tea varieties treated with *C. roseous* showing greater phenol content in comparison to the rest two.

4.11.3.2 Defence enzymes

Six varieties (TV-20, TV-22, T17/1/54, UP-3, BSS-3 and BS/7A/76) which showed susceptible reaction against *A. alternata* were considered for the assay of defense enzymes. Among the defense enzyme phenylalanine ammonia lyase (PAL), chitinase (CHT) and β-1,3-glucanase (βGLU) were studied. Tea seedlings of 10 years old were taken for the experiment. Tea plants of those varieties were treated with plant extracts of *A. indica, D. esculentum and C. roseus*. Leaves were sampled after 24, 48 and 72 h after treatment. Plants treated with distilled water were considered as control or untreated healthy (UH), only treated with spore suspension were untreated inoculated (UI), treated with plant extracts as treated healthy (TH) and treated plants again treated with spore suspension considered as treated inoculated (TI). The activity of all the enzymes (Table 45, 46, 47 and Fig.28, 29, 30) were higher in leaf extracts treated leaves of tea. However, maximum activity of all the enzymes were recorded in the leaves of treated inoculated.

Table 43: Total phenol content of different tea varieties after treated with plant extracts.

Varieties	Time (h)	Untreated DW (C)[a]		Treated with plant extracts					
				C. roseus		*A. indica*		*D. esculentum*	
		H	I[b]	T[c]	I[b]	T[c]	I[b]	T[c]	I[b]
TV-20	24	49.26 ± 0.53	47.12 ± 0.53	62.48 ± 0.36	66.48 ± 0.56	53.48 ± 0.23	59.66 ± 0.53	59.86 ± 0.36	63.78 ± 0.26
	48	48.44 ±0.35	45.89 ± 0.43	66.86 ± 0.23	70.46 ± 0.85	58.96 ± 0.26	64.89 ± 0.25	65.48 ± 0.56	69.86 ± 0.36
	72	49.76 ± 0.53	40.59 ± 0.56	70.16 ± 0.23	76.28 ± 0.23	64.59 ± 0.36	70.55 ± 0.66	69.86 ± 0.53	73.48 ± 0.33
TV-22	24	51.26 ± 0.47	48.56 ± 0.48	66.78 ± 0.45	62.49 ± 0.35	54.85 ± 0.43	59.46 ± 0.36	59.86 ± 0.36	61.23 ± 0.53
	48	50.26 ± 0.26	45.79 ± 0.56	68.96 ± 0.53	69.56 ± 0.86	58.65 ± 0.42	63.45 ± 0.23	63.59 ± 0.63	69.35 ± 0.65
	72	51.49 ± 0.86	40.59 ± 0.75	72.15 ± 0.66	76.48 ± 0.53	65.48 ± 0.25	69.86 ± 0.26	69.89 ± 0.45	73.58 ± 0.22
T17/1/54	24	48.56 ±0.26	45.86 ± 0.46	52.66 ± 0.25	56.48 ± 0.35	50.44 ± 0.66	56.89 ± 0.53	53.48 ± 0.44	59.35 ± 0.36
	48	46.75 ± 0.65	40.16 ± 0.53	59.86 ± 0.53	63.86 ± 0.66	55.48 ± 0.56	62.55 ± 0.42	56.48 ± 0.45	63.59 ± 0.25
	72	47.56 ± 0.43	38.75 ± 0.26	66.59 ± 0.36	69.85 ± 0.85	60.25 ± 0.53	69.85 ± 0.36	62.48 ± 0.32	67.25 ± 0.53
UP-8	24	40.16 ± 0.35	41.56 ± 0.56	48.56 ± 0.36	53.89 ± 0.66	44.56 ± 0.35	48.56 ± 0.56	47.89 ± 0.56	53.59 ± 0.26
	48	41.58 ± 0.45	36.47 ± 0.43	53.48 ± 0.65	59.44 ± 0.58	49.89 ± 0.59	52.46 ± 0.53	52.48 ± 0.56	59.89 ± 0.43
	72	40.86 ± 0.56	31.85 ± 0.26	59.86 ± 0.59	65.48 ± 0.56	56.48 ± 0.48	56.89 ± 0.63	56.89 ± 0.36	62.48 ± 0.56
BSS-3	24	38.77 ± 0.49	35.46 ± 0.25	52.56 ± 0.56	56.89 ± 0.53	42.56 ± 0.59	46.85 ± 0.57	48.75 ± 0.56	50.35 ± 0.45
	48	37.66 ± 0.45	30.12 ± 0.45	58.59 ± 0.23	63.86 ± 0.49	48.59 ± 0.47	53.85 ± 0.43	53.46 ± 0.38	54.85 ± 0.37
	72	39.46 ± 0.47	28.45 ± 0.43	66.79 ± 0.53	76.49 ± 0.68	53.89 ± 0.59	56.89 ± 0.73	59.86 ± 0.46	60.26 ± 0.65
BS/7A/76	24	42.12 ± 0.56	38.56 ± 0.26	56.89 ± 0.42	59.86 ± 0.66	48.95 ± 0.67	53.85 ± 0.45	52.69 ± 0.53	56.44 ± 0.55
	48	41.23 ± 0.65	32.46 ± 0.25	59.77 ± 0.55	64.58 ± 0.53	53.86 ± 0.86	59.86 ± 0.62	59.56 ± 0.66	62.99 ± 0.44
	72	42.89 ± 0.46	26.78 ± 0.36	64.86 ± 0.38	70.15 ± 0.56	59.44 ± 0.76	63.44 ± 0.86	62.48 ± 0.77	68.53 ± 0.58

± Standard error
[a]Distilled water control
[b]Inoculated with *A. alternata*
[c]Treated with plant extract

Table 44: Orthodihydroxy phenol content of different tea varieties after treated with plant extracts.

Varieties	Time (h)	Untreated DW (C)[a]		Treated with plant extracts					
				C. roseus		*A. indica*		*D. esculentum*	
		H	I[b]	T[c]	I[b]	T[c]	I[b]	T[c]	I[b]
TV-20	24	11.24 ± 0.45	10.25 ± 0.36	20.45 ± 0.23	34.56 ± 0.52	12.53 ± 0.13	20.13 ± 0.12	15.46 ± 0.23	20.86 ± 0.36
	48	12.56 ± 0.36	09.56 ± 0.19	29.53 ± 0.24	39.75 ± 0.35	16.75 ± 0.36	25.46 ± 0.23	29.56 ± 0.36	31.25 ± 0.23
	72	11.56 ± 0.35	07.86 ± 0.13	34.86 ± 0.43	42.56 ± 0.53	28.12 ± 0.23	34.16 ± 0.25	36.45 ± 0.13	39.75 ± 0.53
TV-22	24	15.28 ± 0.18	12.45 ± 0.10	20.45 ± 0.13	26.48 ± 0.25	16.45 ± 0.22	15.44 ± 0.12	18.24 ± 0.16	20.56 ± 0.25
	48	16.45 ± 0.16	10.25 ± 0.09	29.46 ± 0.19	31.55 ± 0.26	16.55 ± 0.31	19.88 ± 0.34	19.46 ± 0.23	25.45 ± 0.33
	72	15.89 ± 0.14	08.46 ± 0.11	33.45 ± 0.23	41.75 ± 0.45	24.77 ± 0.11	33.85 ± 0.65	29.22 ± 0.22	35.44 ± 0.56
T17/1/54	24	15.46 ± 0.33	13.75 ± 0.22	23.45 ± 0.33	25.66 ± 0.35	16.45 ± 0.22	20.11 ± 0.21	20.15 ± 0.33	23.56 ± 0.23
	48	14.75 ± 0.23	12.56 ± 0.24	29.86 ± 0.85	32.86 ± 0.66	19.45 ± 0.44	23.44 ± 0.46	24.55 ± 0.35	26.86 ± 0.44
	72	14.96 ± 0.32	10.26 ± 0.19	33.56 ± 0.28	42.56 ± 0.42	23.44 ± 0.33	32.86 ± 0.62	30.44 ± 0.51	37.59 ± 0.33
UP-8	24	14.16 ± 0.29	16.46 ± 0.35	18.56 ± 0.35	20.45 ± 0.23	14.75 ± 0.33	16.48 ± 0.35	16.85 ± 0.36	18.47 ± 0.52
	48	14.25 ± 0.36	18.45 ± 0.55	21.56 ± 0.33	26.75 ± 0.64	16.48 ± 0.66	18.44 ± 0.46	18.75 ± 0.45	21.45 ± 0.36
	72	15.25 ± 0.36	21.49 ± 0.36	25.48 ± 0.55	31.45 ± 0.58	20.45 ± 0.19	23.11 ± 0.47	21.45 ± 0.54	27.45 ± 0.58
BSS-3	24	11.43 ± 0.13	14.23 ± 0.26	19.86 ± 0.35	21.45 ± 0.36	14.55 ± 0.33	17.88 ± 0.65	16.45 ± 0.66	18.95 ± 0.44
	48	12.45 ± 0.22	15.26 ± 0.53	23.56 ± 0.42	25.75 ± 0.44	17.88 ± 0.53	21.48 ± 0.35	21.56 ± 0.36	22.85 ± 0.63
	72	12.85 ± 0.33	18.52 ± 0.36	29.86 ± 0.56	31.25 ± 0.55	20.47 ± 0.63	25.44 ± 0.22	24.75 ± 0.55	27.99 ± 0.54
BS/7A/76	24	12.12 ± 0.36	10.54 ± 0.35	18.44 ± 0.65	17.56 ± 0.56	14.75 ± 0.52	16.44 ± 0.77	15.48 ± 0.39	18.45 ± 0.66
	48	11.13 ± 0.11	09.85 ± 0.34	23.48 ± 0.75	21.45 ± 0.45	18.44 ± 0.33	20.15 ± 0.83	19.44 ± 0.36	21.45 ± 0.35
	72	12.89 ± 0.52	07.86 ± 0.42	27.46 ± 0.44	29.75 ± 0.53	21.66 ± 0.47	14.96 ± 0.72	23.66 ± 0.48	25.66 ± 0.45

± Standard error

[a]Distilled water control

[b]Inoculated with *A. alternata*

[c]Treated with plant extract

Time course accumulation of phenylalanine ammonia lyase in tea plant (T17/1/54) following treatment with plant extracts against *A. alternata*

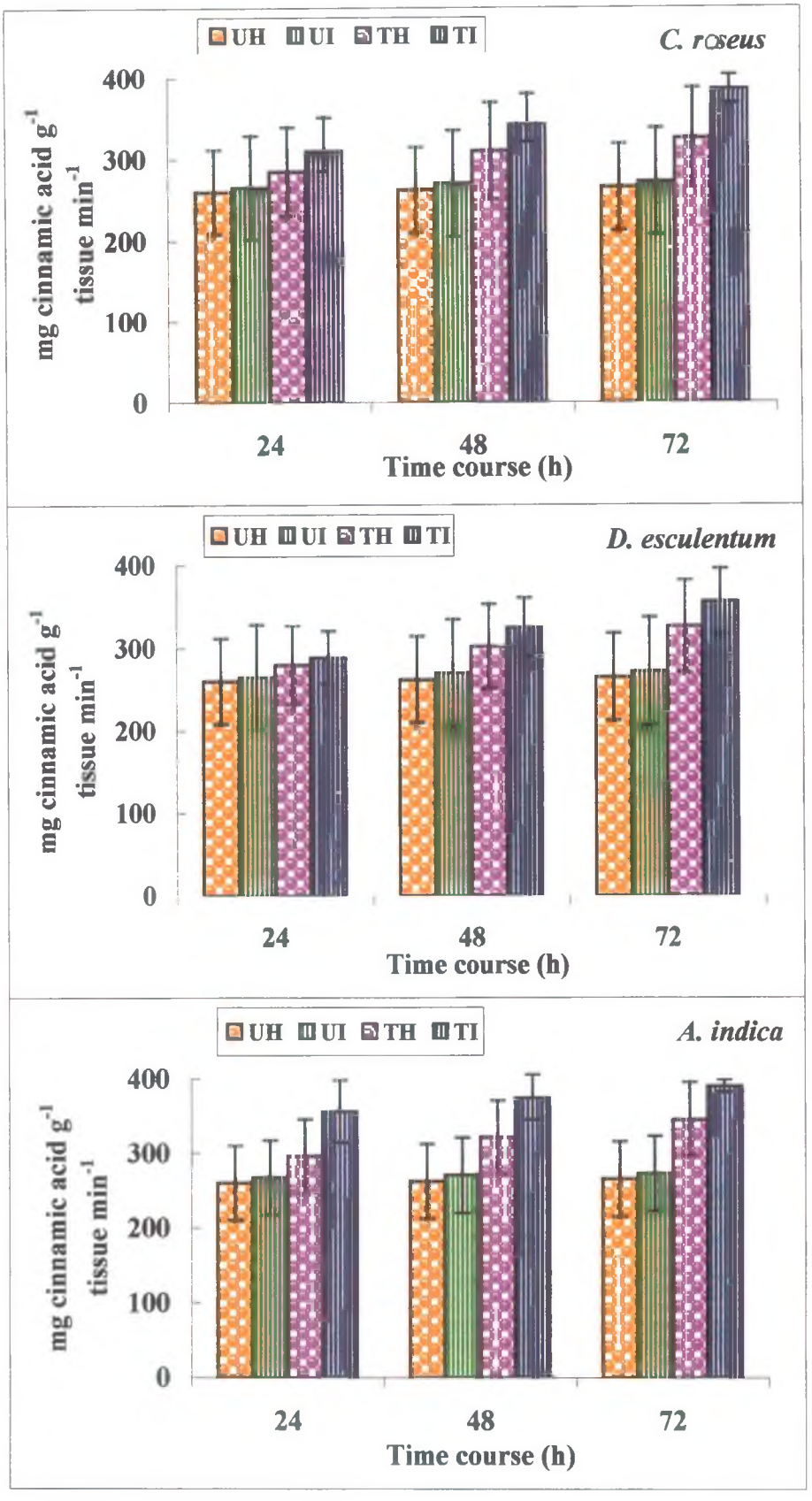


Fig. 28

Table 45. Phenylalanine ammonia lyase activity of different tea varieties after treated with plant extracts.

Varieties	Time (h)	Untreated DW (C)[a]		Treated with plant extracts					
				C. roseus		*A. indica*		*D. esculentum*	
		H	I[b]	T[c]	I[b]	T[c]	I[b]	T[c]	I[b]
TV-20	24	269.6 ±13.5	294.2 ±12.8	276.4 ±15.7	310.4 ±17.7	270.4 ±16.8	297.4 ±15.5	272.6 ±12.5	298.4 ±14.7
	48	270.5 ±15.1	320.7 ±19.7	282.7 ±15.7	326.9 ±16.4	275.8 ±18.4	309.9 ±19.7	279.8 ±14.6	312.8 ±17.8
	72	276.2 ±16.4	365.5 ±18.6	294.3 ±13.7	356.7 ±18.7	281.4 ±14.4	216.7 ±18.8	286.7 ±13.8	328.4 ±14.7
TV-22	24	231.1 ±16.7	237.8 ±12.5	243.5 ±15.7	246.7 ±14.7	235.8 ±18.5	238.4 ±17.5	240.5 ±17.3	243.2 ±14.9
	48	234.5 ±15.4	241.4 ±19.8	256.9 ±17.4	259.7 ±18.8	243.7 ±12.7	246.7 ±16.4	248.6 ±16.6	253.7 ±16.7
	72	236.4 ±17.2	244.5 ±21.2	268.7 ±19.4	275.4 ±16.8	256.7 ±17.8	254.3 ±13.7	256.7 ±19.7	268.4 ±14.3
UP-8	24	199.7 ±17.3	213.5 ±19.7	225.4 ±15.4	229.7 ±14.6	214.7 ±14.7	220.4 ±15.9	220.4 ±17.8	226.7 ±21.4
	48	208.4 ±19.3	214.5 ±13.3	236.9 ±17.6	239.7 ±16.4	224.7 ±18.8	237.8 ±16.4	229.4 ±17.5	238.9 ±19.7
	72	210.7 ±16.3	223.8 ±18.7	243.7 ±14.4	254.9 ±15.7	235.7 ±14.9	239.7 ±15.5	237.8 ±19.8	246.8 ±16.7
BSS-3	24	226.4 ±20.1	245.7 ±15.4	239.7 ±19.4	256.7 ±16.2	230.7 ±22.5	242.6 ±16.7	235.4 ±14.6	249.4 ±16.2
	48	230.7 ±14.8	255.7 ±19.2	246.7 ±17.8	263.4 ±12.8	238.4 ±15.4	250.4 ±17.6	246.7 ±16.9	253.8 ±18.4
	72	235.8 ±25.7	268.7 ±0.17	253.9 ±13.3	273.8 ±18.9	246.9 ±19.8	261.7 ±18.5	259.4 ±15.7	267.8 ±19.9
BS/7A/76	24	233.4 ±17.2	254.8 ±12.6	243.8 ±14.8	259.8 ±18.7	236.7 ±17.6	256.7 ±18.9	240.6 ±17.9	252.4 ±17.3
	48	235.4 ±18.3	259.6 ±21.5	249.8 ±16.7	262.8 ±20.1	242.7 ±15.2	260.4 ±17.5	246.8 ±16.7	259.7 ±15.8
	72	237.8 ±18.6	268.4 ±19.4	253.7 ±17.8	274.6 ±18.4	249.8 ±21.2	263.8 ±18.3	251.7 ±17.3	266.8 ±18.6

± Standard error

[a]Distilled water control

[b]Inoculated with *A. alternata*

[c]Treated with plant extract

Time course accumulation of chitinase in tea (TV-22) following treatment with plant extracts against *A. alternata*

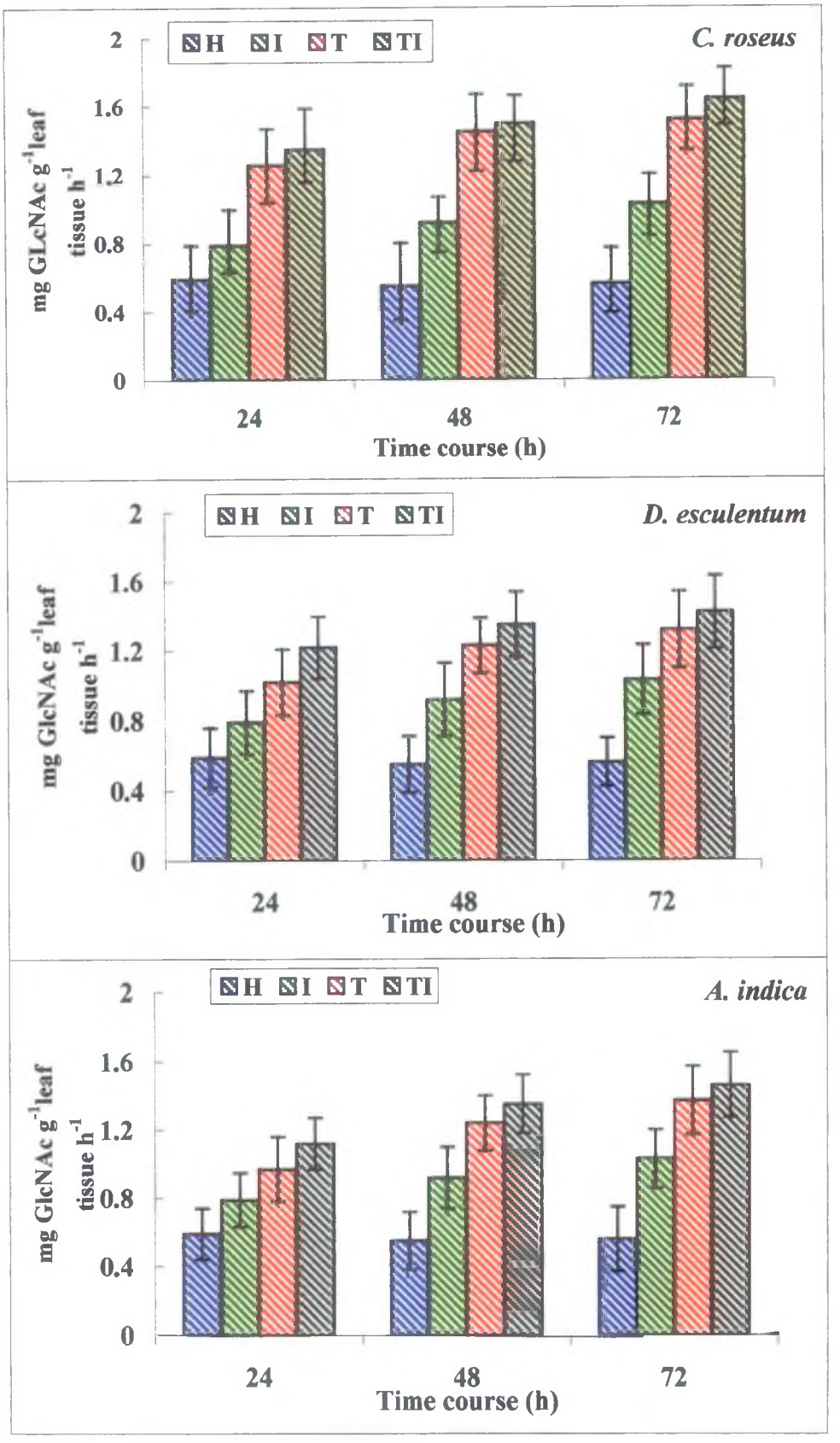


Fig. 29

Table 46: Chitinase activity of different tea varieties after treated with plant extracts.

Varieties	Time (h)	Untreated DW (C)[a]		Treated with plant extracts					
				C. roseus		*A. indica*		*D. esculentum*	
		H	I[b]	T[c]	I[b]	T[c]	I[b]	T[c]	I[b]
TV-20	24	0.64 ±0.06	0.73 ±0.09	0.75 ±0.08	0.86 ±0.06	0.68 ±0.04	0.75 ±0.06	0.69 ±0.06	0.79 ±0.07
	48	0.65 ±0.08	1.89 ±0.06	0.82 ±0.03	1.06 ±0.05	0.70 ±0.03	0.87 ±0.07	0.72 ±0.04	0.92 ±0.03
	72	0.66 ±0.07	0.86 ±0.07	1.02 ±0.07	1.21 ±0.08	0.85 ±0.07	0.98 ±0.05	0.89 ±0.03	1.07 ±0.04
T17/1/54	24	0.60 ±0.04	0.63 ±0.03	0.69 ±0.06	0.78 ±0.10	0.63 ±0.02	0.71 ±0.06	0.66 ±0.04	0.75 ±0.08
	48	0.58 ±0.07	0.68 ±0.09	0.76 ±0.08	0.86 ±0.09	0.72 ±0.03	0.82 ±0.04	0.72 ±0.08	0.89 ±0.04
	72	0.61 ±0.04	0.72 ±0.08	0.86 ±0.05	1.03 ±0.11	0.86 ±0.07	0.94 ±0.08	0.84 ±0.06	0.97 ±0.05
UP-8	24	0.52 ±0.04	1.08 ±0.06	0.63 ±0.07	1.26 ±0.09	0.56 ±0.08	1.10 ±0.07	0.59 ±0.06	1.10 ±0.08
	48	0.50 ±0.03	1.34 ±0.08	0.78 ±0.09	1.32 ±0.10	0.62 ±0.07	1.18 ±0.07	0.67 ±0.07	1.23 ±0.10
	72	0.51 ±0.04	1.45 ±0.05	0.94 ±0.05	1.46 ±0.09	0.78 ±0.07	1.26 ±0.09	0.81 ±0.07	1.34 ±0.04
BSS-3	24	0.53 ±0.04	1.56 ±0.07	0.67 ±0.05	1.69 ±0.10	0.58 ±0.07	1.55 ±0.09	0.61 ±0.05	1.59 ±0.08
	48	0.56 ±0.09	1.75 ±0.06	0.78 ±0.06	1.76 ±0.12	0.67 ±0.06	1.59 ±0.10	0.70 ±0.06	1.64 ±0.04
	72	0.55 ±0.04	1.85 ±0.07	0.93 ±0.07	1.86 ±0.09	0.73 ±0.04	1.69 ±0.07	0.86 ±0.07	1.73 ±0.07
BS/7A/76	24	0.57 ±0.06	0.66 ±0.09	0.68 ±0.04	0.78 ±0.06	0.59 ±0.05	0.68 ±0.05	0.61 ±0.04	0.72 ±0.06
	48	0.60 ±0.03	0.82 ±0.08	0.73 ±0.03	0.86 ±0.08	0.61 ±0.03	0.73 ±0.06	0.69 ±0.04	0.82 ±0.04
	72	0.59 ±0.07	0.89 ±0.07	0.81 ±0.06	1.01 ±0.05	0.70 ±0.06	0.89 ±0.04	0.76 ±0.04	0.95 ±0.07

± Standard error

[a]Distilled water control

[b]Inoculated with *A. alternata*

[c]Treated with plant extract

Time course accumulation of β,1-3 glucanase in tea (UP-8) following treatment with plant extracts against *A. alternata*

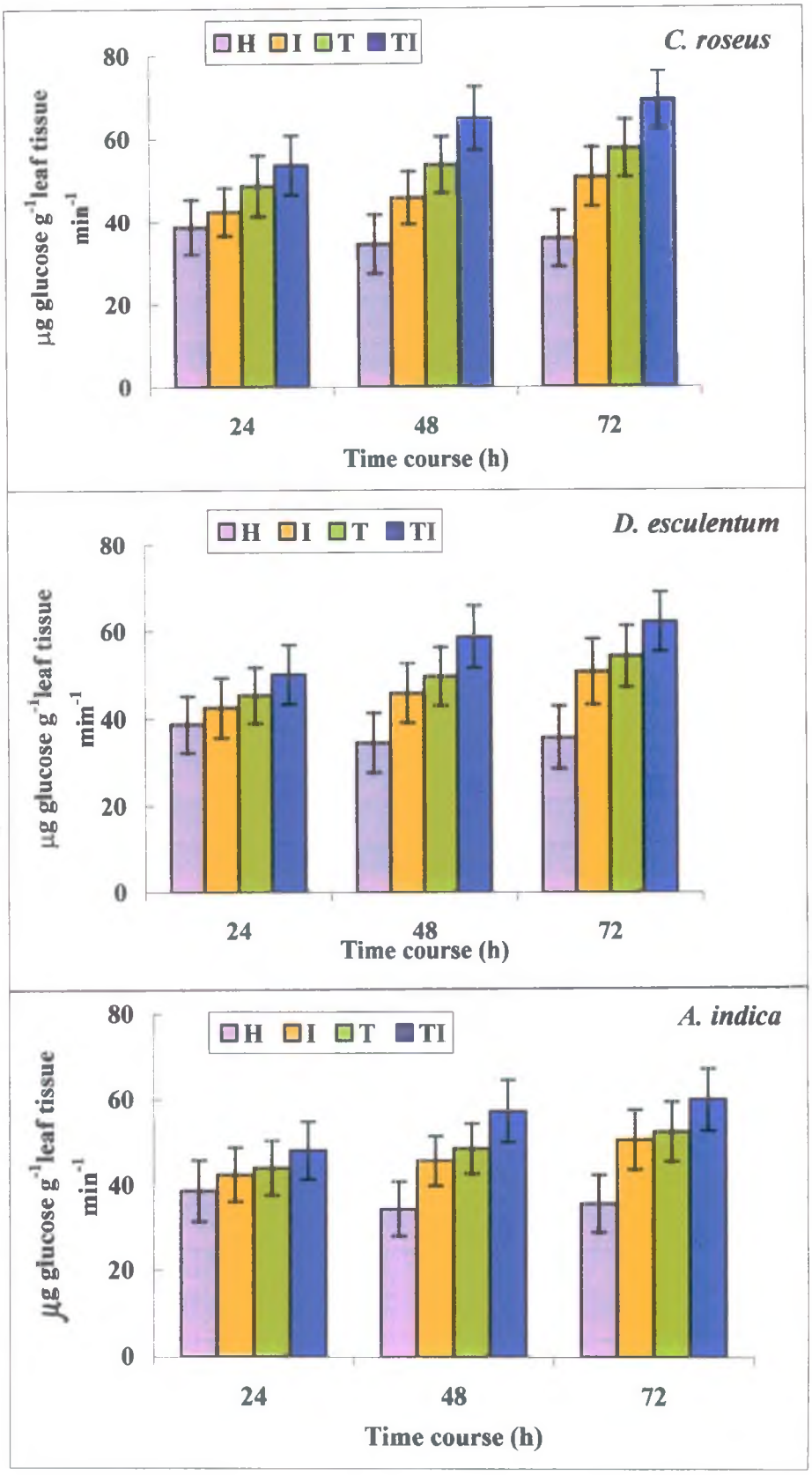


Fig. 30

Table 47: β-1,3 glucanase activity in different tea varieties after treated with plant extracts

Varieties	Time (h)	Untreated DW (C)[a]		Treated with plant extracts					
				C. roseus		*A. indica*		*D. esculentum*	
		H	I[b]	T[c]	I[b]	T[c]	I[b]	TH	I[b]
TV-20	24	46.5 ±1.23	43.7 ±1.92	49.5 ±1.56	52.4 ±1.78	44.5 ±1.67	48.7 ±1.65	46.4 ±1.46	50.4 ±1.74
	48	44.5 ±1.35	46.7 ±1.48	52.4 ±1.42	58.7 ±1.65	48.75 ±1.68	52.4 ±1.65	50.4 ±1.68	56.7 ±1.35
	72	44.7 ±1.12	49.9 ±1.10	55.48 ±1.56	63.4 ±1.48	50.48 ±1.68	58.0 ±1.42	53.8 ±1.45	60.48 ±1.68
TV-22	24	46.68 ±1.16	47.9 ±1.28	49.7 ±1.25	53.7 ±2.10	44.2 ±1.25	48.5 ±1.56	46.5 ±1.25	50.4 ±1.32
	48	45.78 ±1.12	49.1 ±1.35	54.8 ±1.68	56.7 ±1.94	48.7 ±1.65	52.4 ±1.65	50.4 ±1.23	52.4 ±1.23
	72	46.66 ±1.32	50.5 ±1.27	59.7 ±1.32	67.4 ±1.94	52.6 ±2.11	57.4 ±1.24	55.4 ±2.11	59.7 ±0.09
T17/1/54	24	44.35 ±1.47	47.5 ±1.35	49.5 ±1.57	53.7 ±1.35	43.8 ±1.56	48.9 ±1.75	46.7 ±1.22	50.4 ±1.88
	48	45.76 ±1.39	50.2 ±1.68	53.7 ±1.33	59.4 ±1.33	47.5 ±1.44	54.9 ±1.94	49.7 ±1.35	57.3 ±2.14
	72	45.75 ±1.46	52.4 ± 1.56	65.2 ±1.11	68.1 ±2.11	52.7 ±1.68	62.4 ±1.22	54.7 ±1.55	65.8 ±1.75
BSS-3	24	34.5 ±1.68	37.8 ±1.35	39.7 ±1.25	43.2 ±1.44	38.7 ±1.22	39.7 ±1.56	38.4 ±1.22	41.7 ±1.44
	48	33.7 ±1.23	39.7 ±1.94	43.7 ±1.11	48.7 ±1.35	40.1 ±1.35	44.2 ±1.53	41.5 ±1.69	46.7 ±1.75
	72	34.1 ±1.35	40.6 ±1.34	50.4 ±1.66	56.8 ±1.47	51.4 ±1.46	52.7 ±1.74	55.7 ±1.33	54.8 ±1.42
BS/7A/76	24	48.5 ±1.34	50.4 ±1.12	48.5 ±1.45	53.9 ±2.11	44.7 ±1.55	48.5 ±1.25	46.7 ±1.54	50.7 ±2.01
	48	50.3 ±1.25	51.8 ±1.37	53.7 ±1.69	59.7 ±1.35	49.7 ±1.35	51.6 ±1.45	51.7 ±1.66	54.7 ±2.11
	72	48.7 ±1.68	50.5 ±1.67	66.7 ±1.33	72.8 ±1.44	59.7 ±1.62	61.4 ±1.58	64.8 ±1.34	69.3 ±1.12

± Standard error

[a]Distilled water control

[b]Inoculated with *A. alternata*

[c]Treated with plant extract

4.11.4 Western Blot analysis

Enzyme preparations from Soluble proteins extracted from healthy and all three plant extract treated tea leaves of one variety (TV-18) in showed resistant reaction against *A. alternata* was resolved on 10% SDS-PAGE then transferred on nitrocellulose membrane and the blots were probed with PAb of chitinase. Western blots of 1D gels were analyzed. Two bands of ca. molecular weights 65 and 35 kDa were found to be common in both healthy and plant extracted treated leaves, while a new band of ca. molecular weight 46 kDa was evident in *A. indica* treated leaves. However another one bands in low molecular weight of 29 kDa was evident in *C. roseus* and *D. esculentum* treated leaves (Plate 27 fig.C).

4.11.5 Immunohistology

Plant's own inducible defence mechanism may be utilized which is natural weapons before infection. Activation of these mechanism is initiated by pathogen, plant derived elicitors, as well as number of chemical compounds such as Salicylic acid (SA) etc. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding pathogenesis related proteins (PR). Major interest has been devoted to plant hydrolases, chitinase and β-1,3 glucanase, as they are capable of cleaving fungal cell walls resulting in pathogen growth inhibition. Keeping this mind following experiment were conducted. In the present study leaves of tea plants (Teenali 17/1/54) were first treated with leaf extracts of *C. roseus*, *A. indica* and *D. esculentum*. In localization studied in plant tissues treated with plant extracts, chitinase was specially pronounced over mesoplyll tissues with bright apple green fluorescence fungal confirming their presence and their role in plant defence. Healthy plants were treated with distilled water. Cross sections were made both from untreated and plant extracts treated leaves. Theses were separately reacted with antibody raised against chitinase (PAb-CHT), PR-3, one of the defense enzymes and then labeled with FITC. Untreated leaf sections exhibited a natural auto-fluorescence

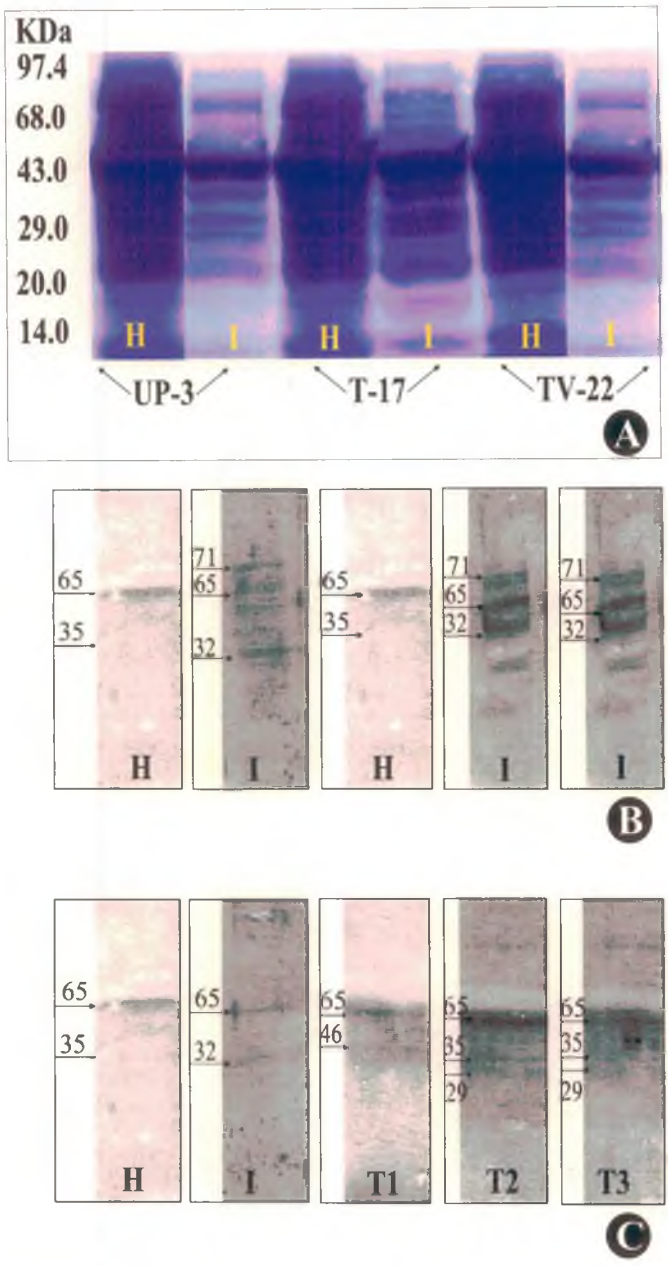


Plate 27 (figs. A-C): SDS-PAGE(A) and Western blot analysis (B & C) of tea varieties probed with PAbs raised against chitinase [(H)- healthy, (I) - Inoculated with *A. alternata*, Treated with *Azadirachta indica* (T1), *Catharanthus roseus* (T2), *Deplazium esculentum* (T3).

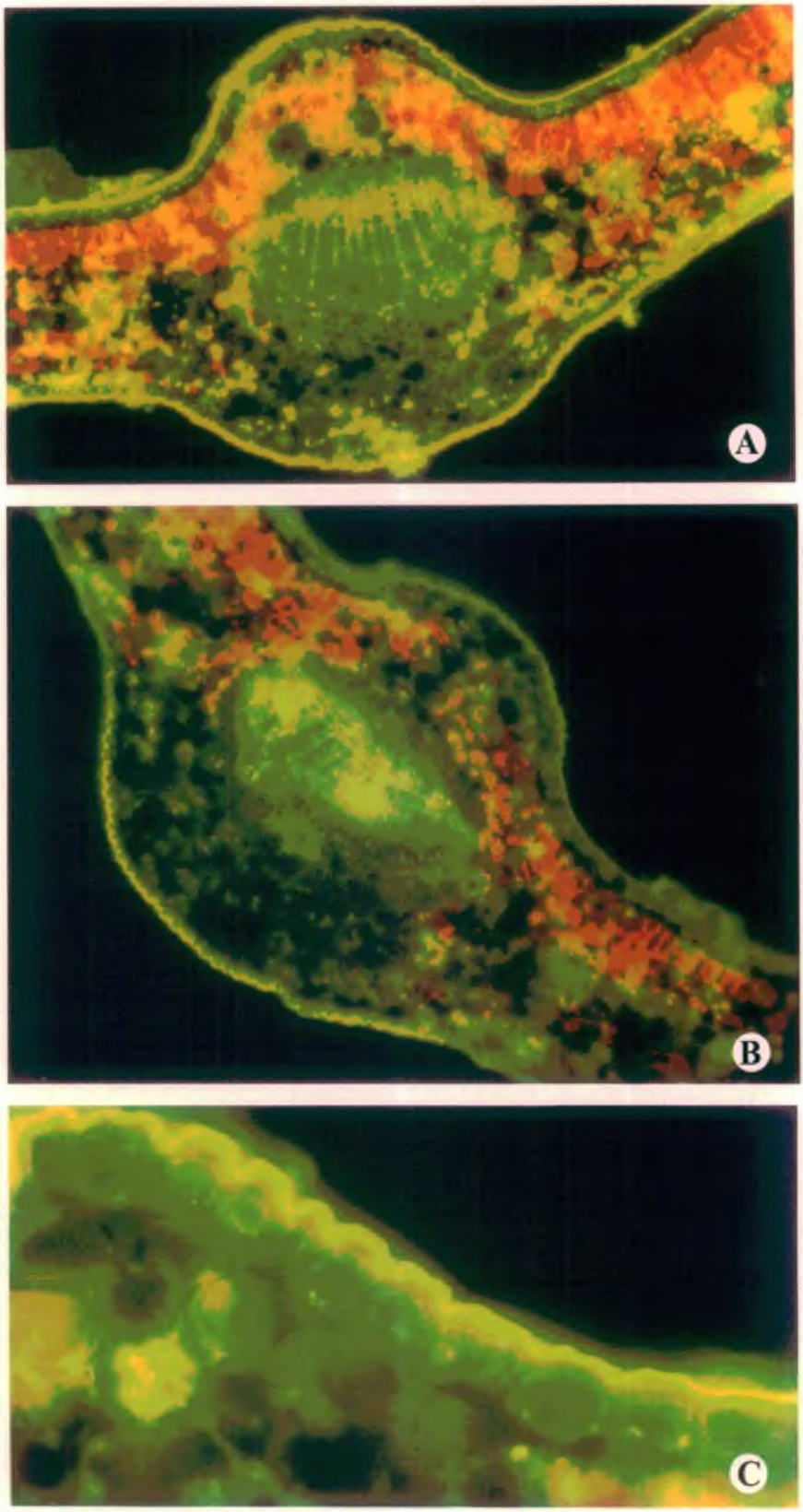


Plate 28 (figs. A-C): Indirect immunofluorescence of tea leaves treated with plant extracts of *Catharanthus roseus* (A); *Azadirachta indica* (B); *Deplazium esculentum* (C) Cross sections of leaf tissues probed with PAb raised against Chitinase (CHT) and reacted with FITC labelled antibodies of goat specific for rabbit globulin.

under UV light on the cuticle as indicated in Plate 29 (fig.A). Leaf treated with all three plant extracts developed bright apple green fluorescence which was distributed in mesophyll tissues. However, the palisade parenchymatous tissues exhibited red colouration indicating the high level of phenolics accumulated in leaf tissues following treatment with *C. roseus* (Plate 28 figs. A) and *A. indica* (Plate 28 figs. B). This was not evident in case of *D. esculentum* treated leaves.

4.12 Cellular localization of defense enzyme (chitinase) in tea leaf tissues following inoculation with salicylic acid

Salicylic acid (SA) one of the many activators of disease resistance being utilized to aid elucidation of the complex mechanism of the defense response and to assess the potential of employing systemic acquired resistance (SAR) commercially. SA has been implicated as a component of induced resistance signaling pathway. Application of SA to many plant species resulted in the accumulation of pathogenesis related proteins. The relation between SA accumulation, PR gene expression and the degree of SAR has been established in many plants. Among the PR-proteins two plant hydrolases, β-1,3 glucanase and chitinase have been intensively studied for their accumulation in the infected plant tissues and their function in the plant defense reactions in different fungal pathogen systems. Theses two enzymes are particular interest because pathogenic fungi contain β-1, 3 glucanase and chitinase are major structural cell wall components. Besides it has also been found that SA can activate other resistance mechanisms such as phytoalexin production and lignification. Endogenous level of SA also increases following inoculation with microorganisms.

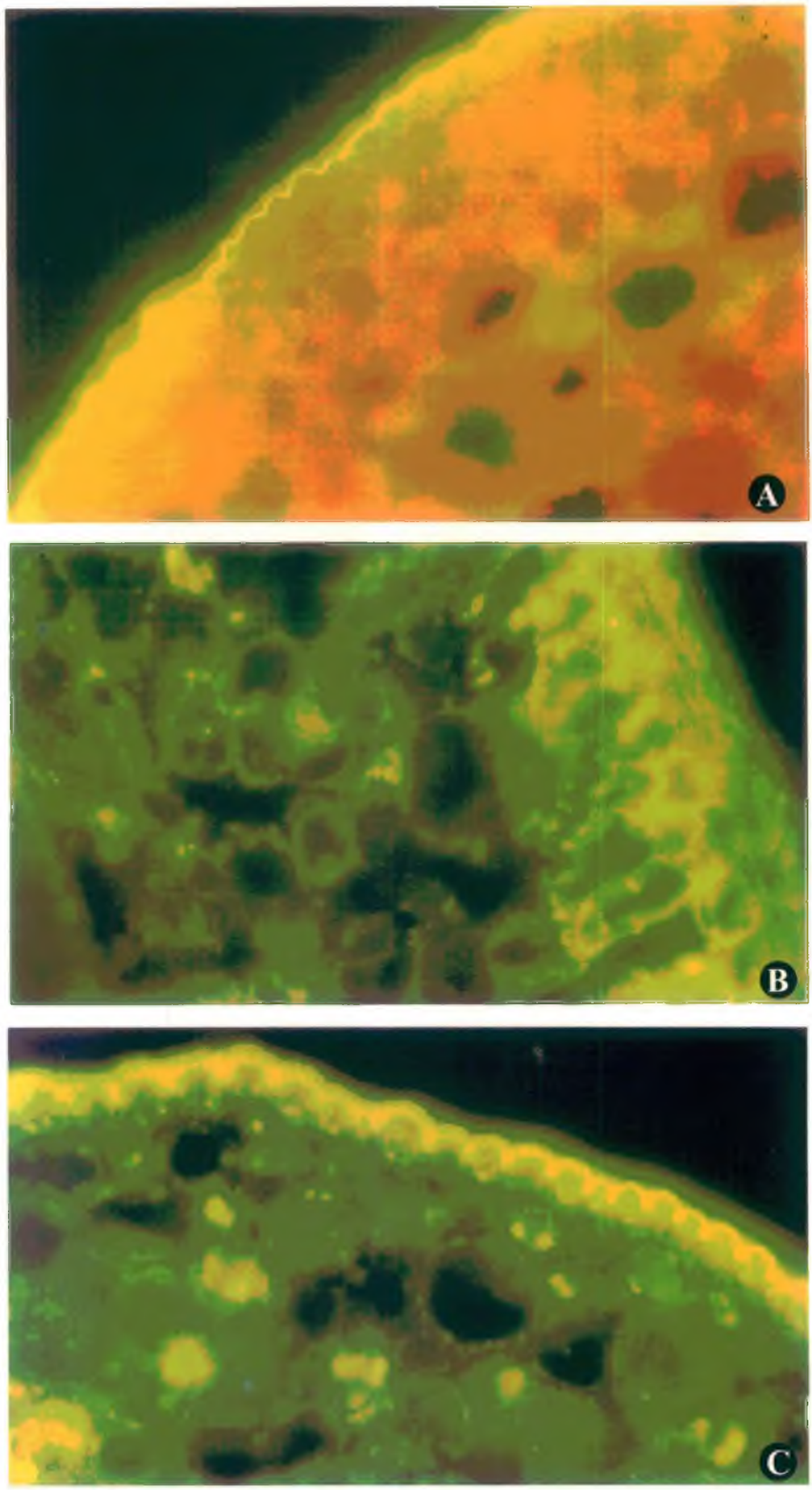


Plate 29 (figs. A-C): Cross sections of tea leaves treated with Salicylic acid showing indirect immunofluorescence. Autofluorescence of unstained leaf section (A) leaf tissues probed with PAb raised against Chitinase (CHT) and stained with FITC labelled antibodies of goat specific for rabbit globulin.

4.12.1 Indirect immunofluorescence

In this study, salicylic acid (15mM) was sprayed on the tea plants (Teenali 17/1/54) in order to induce resistance. Accumulation of chitinase, one of the defense enzymes which was evident following treatment with plant extracts both in assay and indirect immunofluorescence studies mentioned in the previous chapter was further confirmed by the exogenous application of SA on Teenali 17/1/54, one of the susceptible varieties. Untreated as well as SA treated leaves were examined microscopically. Indirect immunofluorescence studies using PAb Ctitinase and labeled with FITC conjugate revealed an excellent apple green fluorescence throughout the leaf tissues in SA treated leaves (Plate 29 figs.B&C). Untreated leaves showed only fluorescence on the cuticle region (Plate 29 figs. A).

4.12.2 Immunogold localization

The plants (Teenali 17/1/54) treated with SA was further investigated for confirming the accumulation of chitinase (CHT) in tea leaf tissues following electron microscopic observation. Healthy leaves treated with distilled water and SA treated leaves were processed for the present study as mentioned in Materials and Methods. Ultra-thin sections were prepared, reacted with PAb-CHT, labeled with gold particles and observed under electron microscope. Intense gold labeling corresponding to CHT deposition in SA treated plants were evident in comparison with untreated control plants. Deposition of CHT was predominantly found in cellular compartment. High amount of gold labeling was found in host cytoplasm and chloroplast while lesser amount in vacuoles, mitochondria and walls. In untreated healthy plants, presence of chitinase was evident constitutively. Immunohistology controls were done in parallel which were treated with pre-immune serum instead of primary antibodies where in gold deposition was absent. Uniform distribution of gold labeling was clearly evident in SA treated leaf tissues reacted with PAb-CHT (Plate....). The extracellular immunogold localization of CHT suggests the potential of SA for induction of defense enzymes.

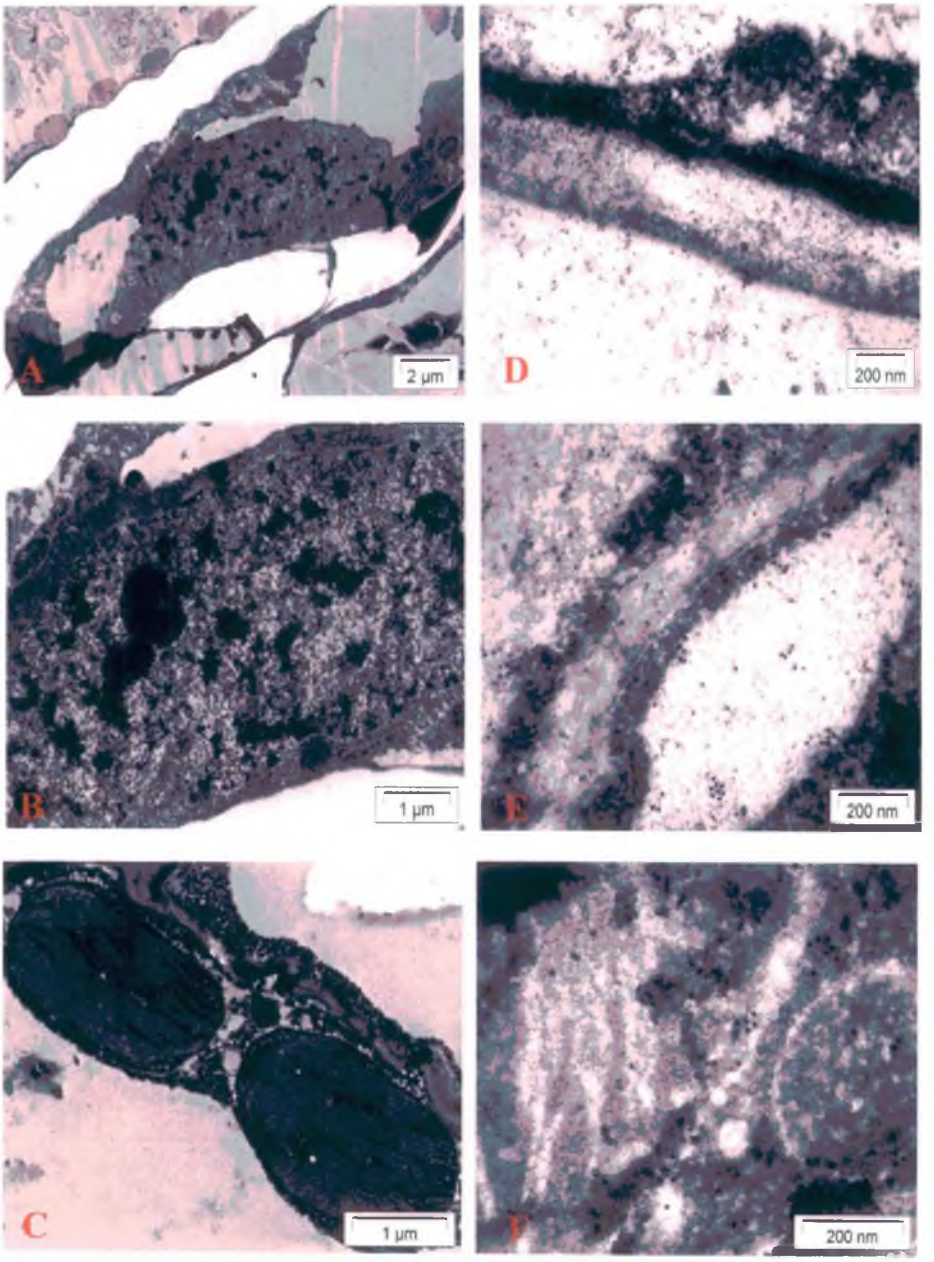


Plate 30 (figs. A-F): Transmission electron micrograph of healthy tea leaf tissues stained with uranyl acetate (A-C) and immunogold label (D-F) probed with PAb of Chitinase

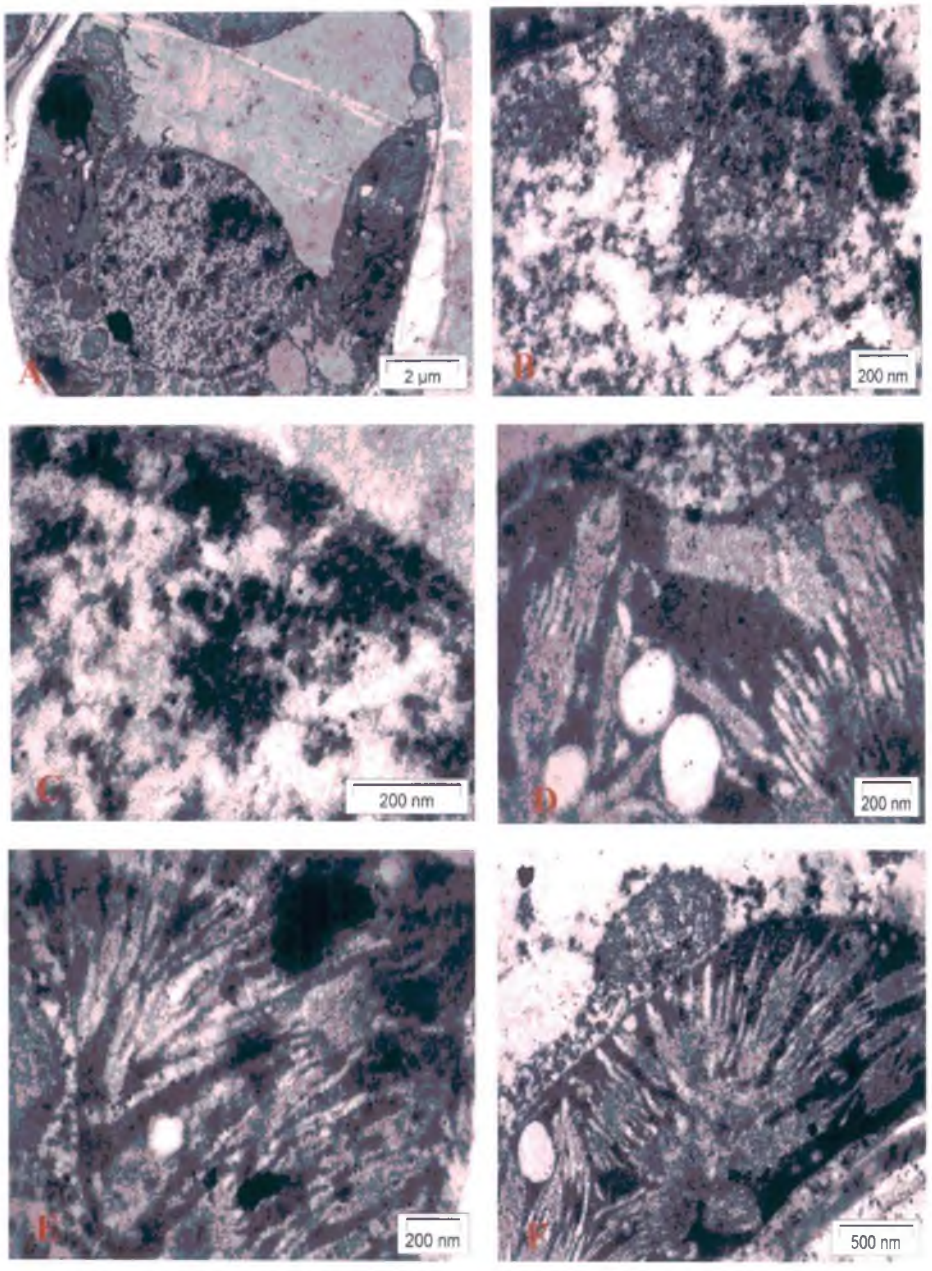


Plate 31 (figs. A-F): Transmission electron micrograph of immunogold label salicylic acid treated tea leaf tissues probed with PAb of Chitinase

DISCUSSION

One of the most complex biological processes occurring in nature is host parasite interaction both at the cellular and sub-cellular level. If the microorganism is successful, disease is the end result; but more often than not, the host emerges the winner as the invader is successfully warded off. Plants have well developed defense mechanism which enable them to defend themselves against penetration, intracellular growth and the development of parasites in their tissues. The biochemical basis for this resistance against microbial attack consist of both preformed and post-infectional ones. Preformed defenses are often regarded as general or unspecific as compared to inducible defense systems which are highly specific. The versatile multicomponent defense adequate to provide them protection against most of their potential pathogens, only a few of them can overcome this defense and cause disease. Varieties within the host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such gene. To account for the observed specificity and degree of variability of host parasite system, the fungal recepter must have high information content which involves recognition between the host and pathogen both at the cellular and subcellular level. A cell that react in a special way consequence of an association with another cell because it acquires information, which is convey through chemical or physical signals in the process or recognition. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signaling processes (Graham and Graham, 1996). In spite of lacking immune responses like animals, plants have nevertheless evolved immune mechanisms of various types by which they can account the advance of foreign organisms. The result is that disease tends to be specific, a given pathogen usually infecting distinct range of host plants.

Field grown tea plants are constantly subjected to advance environmental conditions, one of the most important of these being attack by the pest and pathogens. Besides of their immobility plants have to make necessary metabolic and structural

adjustments to cope with the stress conditions (Ho and Sachs, 2000). Environmental effects in phenolics are all the more long-lasting, as they have to cope of such conditions year after year. Tea is subjected to varying environmental conditions throughout its life and in addition to numerous attack by pests and foliar fungal pathogens, which in turn are influenced by various environmental conditions. In the present study, attack on tea leaves by *H. theivora* was observed throughout the year, except during the winter months, probably because that the period when bushes are pruned and leaves are not abundant. High temperature, combined with certain amount of humidity, were found to be most conducive to attack by *Helopeltis*. In a similar study on tea, with the fungus *Glomerella cingulata*, which causes brown blight of tea, it was reported that high humidity and rainfall were the most important factor predisposing the plants to infection (Chakraborty *et. al.* 2002). Mirid bugs of the genus *Helopeltis* are major pest of various cultivated plants including a few plantation crops such as cashew, cocoa and tea (Muralidharan, 1995). Typical feeding damage by *H. theivora* on tea leaves appears as discolored necrotic area or lesion around the point of entry into the plant tissue. These lesions can be elongated or spherical and become darker with age as the tissue around the stylet puncture reacts presumably in response to enzymatic reaction of the insect's salivary secretions (Stonedahl, 1991). Immediately, after insertion of stylet bundle, water soaked lesion appear around the site of insertion and it spread slowly as round spot on tea leaves. Such water soaked lesion also appeared in intervenal area of lamina of cashew leaf which never expanded beyond outer boundary of round spot that initially formed. Similar type of instant water soaked lesion was observed in the feeding lesion of *H. bergrithi* on mango leaves. The water soaked lesion that was initially formed indicated that the saliva of the insect was injected first and its components rapidly in filtered in the feeding region.

It is presumed that the outflow of cell contents through cell membrane rather than cell walls is aided by hydrolytic enzyme of saliva which is usually injected at water

soaked feeding lesion during initial penetration of stylet bundle in plant tissue. Hydrolytic (protease and lipase) and oxidoreductase (catechol oxidase, peroxidase and catalase) enzymes and free amino acids were detected in the salivery glands of *H. theivora* and these were implicated in detoxification of phytochemicals of cell contents and initiation of phytotoxaemia from feeding lesion. Similar type of interaction between salivary secretion of aphids and susceptible plant tissue resulted in varied reactions ranging from hyperplasia, hypertrophy and necrosis (Miles, 1990). The phytochemical may also change with the insect attack on the plant (Ananthakrishnan, 1993). The polyphenol oxidase (catechol oxidase) system gets activated after insect feeding which results in the release of phenolics compounds which are oxidized to quinines, subsequently forming nontoxic polymers causing brown discoloration (melanization) of wound tissues giving rise to hypersensitive reaction.

In the present investigation, incidence of attack by *H.theivora* was examined in the experimental field against twenty three tea varieties. Highest infestation (50-80%) by *H.theivora* was found in the month of April to June (second quarters). Among all the varieties tested, UPASI series were found to be severely damaged (70-80%) followed by Tocklai and then Darjeeling varieties. An immediate response of plants to injury, in most cases is the accelerated accumulation of oxidative enzymes required for scavenging toxic radicals (Kessler and Baldwin, 2002). In the present study, activities of all oxidative enzymes studied, namely peroxidase(PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) increased following infestation by *H.theivora.* However, Sudhakaran *et.al.* (2000) observed increased activity of PPO and PO enzyme only when infested by *H. theivora* was low. In cotton, damage by an insect pest caused activation of several of oxidative enzymes, including peroxidase, diamine oxidase and lipoxygenese within the tissue. Damaged tissue also had altered phenol metabolism (Bi *et. al.*, 1997). Results of the present study strongly support the above observation of Bi *et. al.* (1997). Induction of PPO

activity have also been reported in *Populus tremuiloides* after herbivory by the forest tent caterpillar, by wounding and by methyl jasmonate (Haruta *et. al.*, 2001).

Wounding may have been one of the primary inducer of the oxidative enzymes in the present study. 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). PPO activity in fruit tissue of apple has been found to be localized in or near the epidermis and in the vicinity of vascular tissue. The greenish healthy eruption of guava fruit form in the feeding lesion of *H. antonii* may be due to hyperplasia initiated by salivary protease which results in release of indole acetic acid (IAA) from inactive conjugate protein of the host. The above observations were found to be consistent with the hypothesis of Miles (1990) that the plant react strongly to the feeding of the insects, the outcome may be either necrosis or enhanced growth in those tissues where oxidative defense and content defense are well matched.

Besides, two major defense enzymes such as β,I,3-glucanase (GLU) and chitinase (CHT) corresponding to pathogenesis related (PR)-protèins - PR2 and PR3 respectively have been assayed from *Helopeltis* infested tea leaves of twenty three varieties. CHT and GLU activities which were found to be higher in infested leaves may be correlated with the defense reaction of tea plants in response to *H. theivora* attack (Chakraborty *et.al*, 2005). Soluble proteins extracted from healthy and early stages of *H. theivora* infested leaves showing resistant reaction were characterized on dot blot and western blots using the probe of CHT (PAb of chitinase, PR3). In Western blotting, three bands of ca. molecular weights 35, 59 and 65 kDa were similar in both healthy and insect-attacked leaves, while a new band of ca. molecular weight 42 kDa was evident in TV-30 and TV-23 infested with *H.theivora*, and two more new bands of ca. molecular weight 29 and 21 KDa were evident in UP-2 and HV-39 *H.theivora* infested tea varieties.

Results of the present study indicate the involvement of multiple enzymes in a coordinated manner in the insect-tea interaction process to develop resistance (Chakraborty *et. al.*, 2004). The induction and accumulation of pathogenesis related proteins of different classes against pest and pathogen attack is well documented (Bostock, 1999; Dutta and Muthukrisnan, 1999). Understanding the molecular genetics of insect hosts and molecular basis of biochemical and cellular defense of plants will pave the way for proper management of pest (Narayanan, 2004).

Phenols are also known to play definite roles in a plant defense. Considering this in the present study the effect of insect saliva on phenol contents of the insect-attacked leaves were determined. In the twenty-three varieties of tea plants tested, it has been found that total phenol content has decreased in the infected leaves but orthodihydroxy phenol content has increased slightly. Decreased phenol content may be attributed to the conversion of polyphenols to quinines. It has been reported previously that quinines in plant tissues react with proteins to form melanin and other tannins leading to the discoloration of damaged tea leaves (Sudhakran *et. al.*, 2000).

Many studies have demonstrated the importance of phenolic compounds in plant defense. In general, plant phenolics have a diverse range of biological activity, depending on their structure, degree of polymerization, stereoisomeric differences etc. interaction between phenolic compounds and environmental conditions determines their action. Polyphenols have distinctive ability to engage in molecular recognition, or formation of intermolecular complex with each other and with other molecules (Haslam, 1999). In the present study, total phenol content decreased with insect attack. The decrease was most significant in UPASI varieties. However, in these varieties, PAL activity increased significantly. In the present study, the level of antifungal phenolics (pyrocatechol) in leaves of healthy and *H. theivora* infested tea varieties were estimated. In resistant varieties (TV-18, TV-20 TV-22) accumulation

of pyrocatechol increased sharply (280-389 μg/g fresh wt.) following infestation by *H.theivora* in relation to healthy plants. Accumulation of pyrocatechol in susceptible varieties (UP-8, BSS-3) were not greater than the resistant one. Increased level of pyrocatechol may be associated with the host response to resistant reaction. Catechins, a class of polyphenols, are the major phenolic compounds of tea leaves. HPLC analysis revealed four isoforms of catechins such as EGC, EPC, EGCG, CG increased sharply due to *H. theivora* infestation in initial stage of puncture. In advance stage of infestation level of EGC, EGCG, GCG and ECG decreased markedly. In conclusion, while attack by *Helopeltis* led to an increased in activity of the antioxidant enzyme, the activity of PPO, PAL. As well as total phenol content decreased significantly in most of the susceptible varieties. One of the reasons for the observed tolerance of certain varieties to insect attack could be their ability to maintain higher level of phenolics in the face of attack.

The pest and blights, which prey on the leaves, are of vital importance, since any damage to the leaves defeats the whole purpose of its cultivation. The aerial surfaces of tea plants, like any other plant, are usually inhabited by a variety of microorganisms, many of which are capable of influencing the growth of foliar pathogens (Chakraborty and Chakraborty, 1997). The interactions between these microorganisms might result in the suppression of pathogen activity. Besides, it is likely that the tea plant, in the course of its adjustment to varying environments, has evolved a very effective defense mechanism, which successfully wards off most of the fungal pathogens. The common plant pathogens (fungi, bacteria, viruses) and pests (insects and other animals) induce some type of resistance in plants to subsequent challenges, both to the original and as well as to other biotic agents. In general, the defenses of higher plants against any form of stress, whether biotic or abiotic, fall under two categories: performed and induced. The main objectives have been to determine the mechanism of defense of tea plant against pest and pathogens,

with special emphasis on both performed and induced chemical defenses. In the present investigation, at the onset, pathogenicity test of *Alternaria alternata* and *Corticium invisum* was carried out on detached leaves and cut shoots of twenty one tea varieties, eleven released by Tocklai Experimental Station, Jorhat, Assam, seven released by UPASI Tea Research Centre, Valparai, Tamilnadu and three released by Darjeeling Tea Research Centre, Kurseong. Detached leaves as well as cut shoots gave satisfactory results. *C. invisum* was most virulent on TV-22, TV-23 least on HV-39 and BSS-1 Results obtained from varietal resistance test performed on twenty one tea varieties against *A. alternata* following detached leaf and whole plant inoculation technique conclusively proved that T-17, TV-20, TV-22 and BS/7A/76 were highly susceptible while TV-9, TV-18 and UPASI-2 were resistant. The results of pathogenicity tests performed on detached leaves of tea varieties corresponded to the same degree of resistance as determined by cut shoot methods. Yanase and Takeda (1987) also used cut shoot method to detect the resistance of tea plant to grey blight disease caused by *Pestalotia longiseta.*

Although less in known with certainty about the specific recognition events that predict incompatible host-pathogen interaction, considerable genetic and biochemical evidence indicates that constitutive specificity imparting molecules must exist in the incompatible pathogen and the resistant host plants that dictate the ultimate accumulation of antifungal compound at the infection site (Albersheim and Anderson-Prouty, 1975; Keen and Bruegger, 1977), Cell recognition has been defined as the initial event of cell-cell communication which elicits morphological, physiological and biochemical response (Clarke and Knox, 1978). Surface molecules of eukaryote cells have been involved in cell-cell recognition and/or adhesion and as receptors for various effects (Snary and Hudson, 1979). Many of these specificity imparting molecules are glycoproteins, and fungi are knwon to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980; Ransom *et. al.*, 1992). In

this study, both the fungal pathogens were found to elicit greater amount of antifungal compound (pyrocatechol) in the resistant varieties than the susceptible ones.

In initial stage of infection at the cellular level the exchange of molecular signals between host and parasite is considered to be one of the mechanisms resulting in the specificity of such interactions. The genetic information contained in nucleic acid is expressed in the cell via protein synthesis. Several proteins function as enzymes in the metabolic pathways, which synthesize or breakdown cellular components. When plants containing various kinds of proteins are infected by pathogens, the proteins in the penetrated plant cells are changed chemically and physically. Some enzymatic proteins are also produced in penetrated cells by the pathogens themselves (Pathogen induced - PI). Thus qualitative and quantitative changes in proteins are related to both plant and pathogen (Uritani, 1971). A protein competition model was proposed by Jones and Hartley (1999) for predicting total phenolic allocations and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis compete for the common limiting resource-phenylalanine and hence protein and phenolic allocations are inversely correlated.

In the present investigation changes in the protein content was noted in the *A. alternata* and *C. invisum* inoculated leaves of susceptible varieties in relation to their healthy control. Increased protein level was also detected after infection of susceptible bean leaves by *Uromyces phaseoli* (Staples and Stalmann, 1964). Similar findings were reported by other workers (Tomiyama, 1966; Daly, 1972; Ouchi *et al.* 1974). They suggested that in case of compatible combination, changes in protein configuration in the host may indicate the host's accessibility to the pathogen which is related to susceptibility. The greater accumulation of protein in susceptible host after inoculation could be attributed to the total proteins of both host and parasite. 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. However, these changes are not always significant. Sometimes protein content of the host remains more or less similar even after inoculation but isozyme pattern may change. Changes in protein patterns in barley leaves after infection with *Erysiphe graminis* f. sp. *hordei* was detected by polyacrylamide gel electrophoretic study, but no change could be detected in total buffer soluble protein content of mildew infected barley leaves in comparison with healthy control (Johnson et al., 1966). Similarly, Sako and Stahman (1972) also detected five new isozyme band viz., acetyl esterase acid phosphatase malate dehydrogenase, succinate dehydrogenase and peroxidase in the susceptible line of barley after infection by *Erysiphe graminis* f. sp. *hordei.*

The interaction between *Cladosporium fulvum* and tomato has been used as a model system by Joosten and Dewit (1988) to study the accumulation of host, pathogen, and interaction-specific proteins in leaf apoplastic fluids from compatible and incompatible combination. Electrophoresis of apoplastic fluids under low pH and non-denaturing conditions revealed one protein which was present in all compatible interactions studied, but not incompatible interactions nor in un inoculated controls. Purification of this protein from the apoplastic fluids from several compatible interactions was achieved by ion-exchange chromatography on CM-Sephadex followed by chromatofocusing. The purified protein migrated on SDS-polyacrylamide gels as one bund with an estimated molecular mass of 14 kDa. Antibodies obtained by injecting the purified protein, bound to nitrocellulose, into rabbits had high affinity for the protein on western blots and little or no interactions with other protein bands. In compatible *C. fulvum*-tomato interactions the protein could be detected in apoplastic fluid 8 days after inoculation. The protein was not detected in the mycelium or culture filtrates neither obtained from *C. fulvum* grown in

culture nor in apoplastic fluids from tomato leaves inoculated with the tomato strain of *Phytophthora infestans*. Furthermore, it was not detectable in old tomato leaves.

Advances made in the formulation of concepts and techniques of modern, quantitative cell biology in recent years have paved the way for a basic understanding of the physiology and biochemistry of plant host pathogen interactions. Difference in physiological responses and morphological structures of various host genotypes affect their susceptibility or resistance to invasion and its consequence while similar variation in pathogens influence their growth rate and virulence. The success or failure of infection is determined by the dynamic competition and the final out come is determined by the sum of favourable and unfavourable conditions for both the pathogen and host cells. At the same time the potential host may be able to detect or recognize a fungal pathogen and use the initial act of recognition to trigger a range of induced resistance (Purkayastha 1994). The initial cellular recognition is followed by communication between its components. This exchange of information is generally mediated by soluble antigens located on or near the cell surface (Chakraborty, 1988).

The significance of antigenic relationship between plant hosts and pathogenic organisms with regard to disease susceptibility has been recognized by many investigators. Whenever an intimate and continuing association of cells of host and pathogen occur it has been observed that partners of this association have unique serological resemblance to one another involving one or more antigenic determinants.

Enzyme linked immunosorbent assay (ELISA) is probably one of the most sensitive serological techniques for the detection of pathogen in host tissues (Chakraborty and Chakraborty, 2002). In the present study polyclonal antibody was raised against mycelia of *A. alternata* and *C. invisum*. The antisera obtained were purified to minimize non specific binding. At the beginning, the sensitivity of the

assay was optimized. Homologous soluble antigens at concentration as low as 78ng/ml could be detected in indirect ELISA in both the cases. Absorbance values decreased with increase in dilutions. Chakraborty *et. al.*, (1996) also reported that antiserum raised against *Pestalotipisis theae* could detect homologus antigen at 25ng/ml. Antiserum dilution of upto 1:16,000 was effective for detections.

Visible outcome of a compatible host pathogen interaction may be obtained in many case only after few days of infection, by which time the pathogen would be well established in the host tissues. In phytopathology 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 (Chakraborty and Chakraborty, 2002). 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; Hansen and Wick, 1993, Chakraborty *et al.*, 1995, 1996, Chakraborty *et. al*,. 2002). In the present study, the differential response of nineteen tea varieties to *A. alternata* and *C. theae* has been observed through Indirect ELISA following artificial inoculation of tea leaves. In the present study indirect ELISA readily detected (*C. invisum* and *A. alternata*) in tea leaf tissues. Among the 23 tea varieties tested with PAb of *A. alternata* and *C. invisum,* very high absorbance values were obtained in case UPASI-3 TV-22, TV-23, TV-18, UPASI-2 and BSS-2 and TV-20, TV-26, BSS-3 showed very low absorbance values.

It is also important in the studies on host parasite relationship to determine the cellular location of the pathogen. For this purpose in this study, indirect immunofluorescence tests were conducted with cross sections of healthy and artificially inoculated (separately with *A. alternata* and *C. invisum)* tea leaves, and mycelia. In case of *A. alternata* susceptible varieties of Tocklai TV-20, TV-22,

UPASI UP-8, BSS-3 and T-17, BS/7A/76 were artificially inoculated. After 42h of inoculation transverse sections were made from all the infected plant parts and PAb raised against mycelial antigens of *A. alternata* were used for probing the fungal hyphae which penetrate the leaf tissue. Bright fluorescence was observed in the cross section of all these varieties of tea leaves. DeVay *et. al.* (1981) determined the tissue and cellular location of major cross reactive antigens (CRA) shared by cotton and *F. oxysporum* f. sp. *vasinfectum*. Cellular location of CRA in tea leaf tissues shared by three foliar fungal pathogens such as *Bipolaris carbonum* (Chakraborty and Saha, 1994); *Pestalotiopsis theae* (Chakraborty *et. al.,* 1995) and *Exobasidium vexans* (Chakraborty *et. al.*, 1997) have been demonstrated. Besides detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors (Warnock, 1973; Hornok and Jagicza, (1973; Reddy and Ananthanarayan, 1984). Dewey *et. al.* (1984) suggested, on the basis of immunofluorescence studies that chlamydospores, basidiospores and mycelia of *Phaseolus Schweinitzii* contained molecules antigenically related to species specific antigens secreted by mycelia grown in liquid culture. They also demonstrated the presence of mycelium and chalmydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, Western blotting, dot blot and indirect immunofluorescence was assessed for their potential to detect resting spores of *Plasmodiophora brassica* in soil (Wakeham and White 1996).

The dot immunobinding technique has also been found to be rapid and sensitive method for detection of fungal pathogens. In the present study, antigens were prepared from black rot infected tea leaf. Healthy tea leaves and artificially inoculated (with *A. alternata* and *C. invisum*) tea leaves were tested on nitrocellulose paper. Infected and artificially inoculated leaf antigens gave intense dot when compared to the healthy control confirming the presence of fungal pathogens. Complex mixtures

of antigens can be quickly and easily separated by high-resolution techniques such as sodium dodecyl sulfate-acrylamide gel electrophoresis using discontinuous buffer systems and two dimensional techniques. However, once separated in this manner, it has been difficult to determine which of the separated species reacted with a given antiserum. Several methods have been developed previously. Towbin *et. al.* (1979) overcome these problems by electrophoretically transferring the separated mixture onto nitrocellulose. Blake *et. al.* (1984) has described a method using the alkaline phosphates substrate 5 bromo-4-chloroindoxyle phosphate (BCIP) and nitro blue tetra zolium (NBT) to detect the precipitated indoxyl group. When the substrate 5-bromo 4-chloroindoxyl phosphate is used, the phosphate is cleaved by the enzyme and the indoxyl group precipitates. The hydroxyl group of the indigo then tautomerizes forming a Ketone, and under alkaline conditions dimerization occurs, forming a dehydroindigo. In the process of dimerizing, it releases hydrogen ions and reduces the nitroblue tetrazolium which precipitates, forming an intense blue deposition of diformazan.

Accumulation of defense enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (PO), β–1,3-glucanase (β–GLU) and chitinase in tea varieties following inoculation separately with foliar fungal pathogens (*A. alternata* and *C. invisum*) were determined. Defense enzymes tested against foliar fungal pathogens increased significantly in all the resistant varieties tested than the susceptible ones. Western blotting analysis of *A. alternata* inoculated tea plants revealed two new bands of ca. molecular weight of 71 and 32 in comparison to healthy

Accumulation of chitinase and β-1,3-glucanase may be associated with the restriction of symptom development on the tea leaves on attack by *A. alternata* and *C. invisum,* as the enzyme activity was more rapidly enhanced in incompatible than

compatible interactions. It has been suggested by Ham *et al.*, (1991) that a β-1,3-glucanase, induced in soybean leaves, by infection with *Phytophthora megasperma* f.sp. *glycinea*, functions in defense by releasing a phytoalexin elicitor from the mycelial walls of the fungus. Furthermore, Kim and Hwang (1994) supported the role for β-1,3-glucanase in disease resistance by demonstrating that β-1,3-glucanase was induced and accumulated in pepper plants by *Phytophthora capsici* infection, more distinctly in resistant than susceptible tissues. The hydrolytic enzymes β-1,3-glucanases (PR-2; βGlu; E. C. 3.2.1.39.), which are capable of hydrolyzing the β-1,3-glucans found in the cell walls of several genera of fungi. Induction of βGlu has been demonstrated in many plant-fungal pathogen interactions (Kini *et. al.*, 2000) and they are thought to play several roles in plant defense. Firstly, they can degrade the cell wall of the pathogen or disrupt its deposition, contributing to pathogen death. Secondly, they can also release cell wall fragments that act as elicitors of active host defense response. The chitinases (PR-3; Cht; E.C. 3.2.1.14.) catalyze the hydrolysis of chitin, a linear homopolymer of β-1,4-linked *N*-acetylglucosamine residues.

Polyphenol oxidase usually accumulated upon wounding in plants. PPO transcript levels systemically increased in tomato when mature leaflets were injured (Thipyapong and Steffens, 1997)Increased activity of PPO and PO was demonstrated in the cucumber leaf in the vicinity of the lesions caused by some foliar pathogens or by phosphate application (Avdiushko *et al.*, 1993). Moreover PPO could be induced by jasmonic acid (Constabel and Ryan, 1998). Among all the stress related enzymes, role of peroxidase has been most thoroughly worked out. PO is a metallo-enzyme containing porphyrin bound iron. The enzyme act on a wide range of substrates including phenols, aromatic amines, amino acids and inorganic compounds (Balasimaha, 1982). These are ubiquitous to plants and are characterized by a large number of isozymes. Various naturally occurring and synthetic substances, growth regulator and environmental factors markedly influence the activity of these peroxidases. Akhtar and Garraway (1990) observed increased PO activity susceptible

cultivars compared with the resistant one when both were treated with sodium bisulphate prior to inoculation with *Botrytis maydis*. On the other hand there are also reports of increased PO activity due to induction of resistance (Ye *et al.*, 1990; Chen *et al.*, 2000). Curtis *et al.*, (1997) also reported the induction of PO activity by pathogens and methyl jasmonate. The existence of multiple molecular forms of peroxidase in tea have been reported by previous authors (Takeo and Kato 1971; Gunashekhar *et al.*, 1996)

Previous report indicate 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). Considering the importance of phenol metabolism in tea plants, those three enzymes were selected for studies. Results showed that the constitutive enzyme activities under no stress conditions of the different clones varied. Matsumato *et al.*, (1994) reported that Japanese green tea cultivars belonging to variety 'sinensis' could be divided into three groups on the basis of their PAL cDNA cloning. Assam hybrids could not be placed into any specific groups because complex patterns were produced. They confirmed the existence of many kinds of PAL gene, expressing of which varied depending on the varieties. An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plants to biotic (Southerton and Deverall, 1990; Chakraborty *et al* 1993; Shiraishi *et al* 1995) or to other environmental stresses (Kuhn *et al.*, 1984; Eckey-Kaltenbach *et al.*, 1997). It was reported by Orczyk *et al.*, (1996) that in sorghum, naturally occurring high level of PAL activity induced by light should be differentiated from the activity induced as a response of attempted fungal infection. Battacharya and Ward (1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma* f.sp. *glycinea*. Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolic compounds in general, and melanins, all of which

have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicator of the activation of defense related enzymes.

Polyphenolic 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). In case of temperature stress it was observed that there was a correlation between the inherent phenol content in the tea variety and its increase following exposure to elevated temperatures. In general, in those varieies with high inherent phenol content, accumulation of phenols kept increasing till 50^0C. A wide variation in the phenol contents in the different tea varieties was also evident. (Chakraborty *et.al.* 2001). The observed trend could be explained by the fact that phenols are considered to be involved in plant's defense to various stresses. When subjected to temperature stress, varieties with low inherent phenol content increased its accumulation while those that already had a higher content did not have to increase synthesis (Chakraborty *et.al*, 2005). In case of tea, polyphenols are also known to vary seasonally (Zakoskiva *et.al.*, 1991). Thus phenol biosynthesis seems to be well regulated to help the tea plant to overcome various stresses. Similarly, in case of drought too, phenol content increased initially up to 8 days of stress after which there was a decline (Chakraborty *et.al.*, 2001)

Alteration of phenol metabolism following fungal infection has been observed in many diseases and phenolics have been implicated in the defense reaction in several instances (Mahadevan, 1991). There is often a greater increase in phenolic biosynthesis in resistant host species than in susceptible host and it is sometimes 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, the levels of phenolics in leaves of resistant and susceptible tea varieties were estimated after 24h and 48h of inoculation with *A. alternata* and *C. invisum*. Host responses could be differentiated by changes in content of phenolic compounds. In both the cases total phenol and orthodihydroxy phenol content increased in resistant varieties but decreased in susceptible varieties in comparison to their healthy controls.

Sridhar and Ou (1974) reported diffences in total phenolics accumulation in the interaction of *Pyricularia oryzae* with rice. Hammerschmidt and Nicholson (1977) demostrated a clear difference between resistant and susceptible interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. However, no differences were found in the phenolic content in the interaction *of Helminthosporium maydis* race T (Macri *et al.*, 1974). On the other hand, a resistant cotton cultivar contained fairly high amount of total as well as orthodihydroxy phenol than susceptible cultivar. In the present study, greater accumulation of orthodihydroxy phenol in resistant interaction of *A. alternata*, *C. invisum* and tea varieties indicated that this might play a role in disease resistance mechanism. Eswaran (1971) has also considered hat orthodihydroxy phenols play a major role in disease resistance and disease development. They are easily oxidized to highly reactive quinones which are effective inhibitors of sulphydryl enzymes, thereby preventing the metabolic activities of host and parasite cells (Kalaichelvan and Mahadevan, 1988). There are ample evidences that an increased production of phenolic compounds are involved in phytoalexin accumulation (Mansfield 2000).

Fungitoxicity of leaf diffusates has been implicated on natural defense mechanism of plants against attack by fungal pathogens in several instances (Purkayastha and Ray, 1975; Hait and Sinha, 1986; Chakraborty and Saha, 1989). 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. However, unstable phytoalexins might decompose during isolation. On the other hand diffusates do not give any indication of the phytoalexin concentrations within inoculated tissue. Moreover phytoalexins which are not diffusible into the inoculum droplet also cannot be detected by this method.

Hence, in the present investigation facilitated diffusion technique as suggested by Keen (1978) was followed to detect the antifungal phenolics (pyrocatechol) from tea leaves inoculated with *A. alternata*. Healthy leaf extract showed Rf 0.63 where as *A. alternata* inoculated tea leaves showed at Rf 0.51 which was found to be fungitoxic against *Curvularia pallescence*. No such fungitoxic activity was evident on TLC plate in leaf extracts from susceptible variety (TV-22) inoculated with *A. alternata* but traces of the inhibition zone was evident in resistant varieties (TV-9) even after 48h of inoculation. Rf value and colour reaction of this antifungal compound corresponded with catechin. The UV spectra from both the healthy and *A. alternata* inoculated leaf extract compounds were analysed at 290 nm. A sharp peak at retention time 2.6 was present in both the compounds but in the healthy extracts the peak height was much smaller than the inoculated one. Other small humps and shoulders were also evident in both the cases.

It is known that catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. Sambandam *et al.*, (1982) isolated an enzyme (catechin 2-3 dioxygensis) from *Chaetomium cupreum*

which cleaved catechin into simpler phenols. It is not unreasonable to speculate that the antifungal compound cleaved to some simpler phenols in the present study. In the susceptible variety, the breakdown of catechin was almost complete while traces were evident in the resistant variety even after 48h of inoculation. Accumulation of pyrocatechol in resistant varieties increased (497-573μg/g fresh wt.) significantly after 48h of inoculation with *A. alternata*. Concentration of this compound in healthy leaf tissue is very low (90-110 μg/g fresh wt.). Accumulation of pyrocatechol in susceptible variety was not greater than the resistant ones even though complete breakdown of catechin was detected in the former case. Increased level of pyrocatechol may be associated with the differential host responses to disease production.

Two phenolic compounds in leaf diffusates and water extracts of maize leaves after inoculation with *Helminthosporium turcicum* have been found antifungal. However, Hammerschmidt and Nicholson (1977) reported accumulation of three phenolic compounds in maize leaves after inoculation with *Colletotrichum graminicola* which increased earlier in the resistant interaction than in the susceptible one. Two of these three compounds inhibited the germination of *C. graminicola* spores *in vitro*. Role of phenolic metabolism in the resistance of maize to *Helminthosporium carbonum* have been investigated by Werder and Kern (1985). Accumulation of antifungal compounds in tea leaf tissue infected with *Bipolaris carbonum* has also been discussed by Chakraborty and Saha (1994). Resistant varieties accumulated more pyrocatechol than the susceptible varieties 2 days after inoculation with *B. carbonum.*

Catechins are flavon-3 ols with two hydoxyl groups in the side ring. These include gallic acid esters with the acid moiety attached to the hydroxyl groups. Kawamura and Takeo (1989) showed the antimicrobial activity of tea catechin

towards *Streptococcus mutans*. Wang (1991) have reported the presence of four forms of catechins such as, (-) epicatechin (EC), (-) epicatechin gallate (ECG), (-) epigallocatechin (EGC) and (-) epigallocatechingallate (EGCG). It was also known that catechins is oxidatively cleaved to simpler phenols and phenolics acids like catechol, phloroglucinol and protocatechuic acid. The enzyme catechins-2,3-dioxygenase was isolated from *Chaetomium cupreum*, which cleaved catechins into simpler phenols as mentioned (Sambandam, 1982).

In the present investigation catechins extracted from both the healthy as well as *A. alternata* inoculated tea leaves and analyzed by HPLC. Due to reaction with pathogen inoculated leaf samples exhibited more isoforms of catechin than healthy control. In *A. alternata* inoculated leaves four peaks at 15.7, 18.4, 20.8 and 22.3 retention time increased markedly in relation to healthy of which two peaks corresponding to authentic EGC and EC could be identified whreas level of EGCG decreased following inoculation with *A. alternata*. Chakraborty and Saha (1994) reported the presence of antifungal catechins in healthy leaf extracts, which were broken down to catechol in the infected (with *Bipolaris carbonum*) leaves. Nagahulla *et.al.* (1996) reported the production of antifungal compounds in tea leaves following infection with blister pathogen(*Exobasidium vexans*). HPLC analysis of the catechins from healthy and blister infected tea leaves showed little qualitative differences and some quantitative changes. (Chakraborty *et.al*, 2002, 2004).

In the present investigation bioresources involving two bioformulations - 'Metabass' and 'Biocrop' and plant extracts of *Azadirechta indica*, *Catharanthus roseus* and *Diplazium esculentum* were applied in the field grown tea plants in order to reduce pest infestation by *H. theivora* and for management of Alernaria blight. Seven UPASI varieties which showed susceptible reaction towards *H. theivora* were selected for this studies. Incidence of *H. theivora* attack decreased markedly after foliar application of these bioresources. Three defense enzymes PPO, PAL and TAL

increased significantly in treated plants than the untreated control. Among the bioresources tested 'Metabass' formulation was found to be more effective against *H. theivora.* Above three plant extracts were also used as foliar spray on tea plants (six susceptible varieties) and their effects were tested for the alteration of disease reaction against *A. alternata.* Both *in vitro* and *in vivo* tests revealed that *C. roseus* was more effective than the other two plant extracts. Phenolic contents as well as defense enzymes (CHT, GLU and PAL) increased after foliar application of plant extracts. However higher activity of all the defense enzymes were noted in treated plants following inoculation with *A.alternata* than the control. Western blot analysis of acid soluble proteins from plant extracts treated tea plants using PAb-CHT revealed the presence of new molecular weight protein band. Band of ca. molecular weight 46 was noted in *A. indica* treated plant where as another new band of ca. molecular weight 29 was evident due to *C. roseus* and *D. esculentum* treated plant.

Herger *et. al.* (1990) have also demonstrated that the leaf extracts of *Reynoutria saehalinensis* induced resistance in a number of plants against powdery mildews. Protection of cucumber and tobacco plants against powdery mildews by leaf extracts of *R. saehalinensis* was also demonstrated to be accompanied by increased activities of peroxidases, β-1,3 glucanase, polyphenol oxidase and PAL. Treatment in cucumber leaves with leaf extracts of *R. saehalinensis* caused accumulation of phenols protecting them against *Sphaerotheca fuliginea* (Daayf *et. al.*1995). It is also noted that tea leaves treated with plant extracts induced an increase in the activity of chitinase, β-1,3 glucanase and phenylalanine ammonia lyase enzyme which led to an increased phenol biosynthesis rendering the leaves resistant to *A. alternata.* Increased activity of PAL, TAL, the key enzymes in phenol biosynthesis by neem product have also been demonstrated earlier by Singh and Prithviraj (1997). The results of present study provide additional evidence that host metabolic pathway altered by treatment with plant extracts can result in an effective resistance against disease. Salicylic acid

can induce resistance when sprayed exogenously. Plants treated with SA and then reacted with PAb-CHT revealed the presence of PR-3 when labeled with FITC in immunohistological studies. The presence of defense enzyme in mesoplyll tissues were also evident. Plants treated with SA were further studied for accumulation of PR-3 following EM observation. Deposition of chitinase was evident following immunogold labelling with PAb-CHT in the cellular compartment.

Any drastic change in the basic physiology of tea plant could influence the development of a disease. Inducible defenses in tea plants against foliar fungal pathogens and pests can be strongly influenced by the mix of signals generated by external biotic factors as well as by abiotic stresses such as draught, nutrient limitation, or high soil salinity (Chakraborty *et.al*, 2005). Our ability to capitalize on inducible defenses and utilize them optimally in agriculture depends, in part upon a fundamental knowledge of their biochemical nature, and of the specificity and compatibility of the signaling systems that regulate their expression. The importance of the phytohormones salicylic acid and jasmonic acid as critical signals in induced resistance responses in plants is recognized. It is well known that plants defend themselves from microbial pathogens through different mechanisms including the synthesis of antimicrobial phytoalexins, the hypersensitive response, and cell wall modification. The inductions of phytoalexins in infected plants is presumed to be mediated by an initial recognition process between plants and pathogens which involves detection of certain unique molecules of pathogen origin, termed elicitors, by recognitional receptor-like molecules in plants, thereby setting off a cascade of biochemical events leading ultimately to phytoalexin accumulation. In the present study, multicomponent coordinated defense response of tea plants to pest and pathogen has been elucidated. It is not unreasonable to speculate that a relationship might exist between the production of antimicrobial phenolics - pyrocatechol and defense enzymes since both are defense component and are regulated by host genes.
To trace the relationship between phytoalexins and PR-proteins, PAL may be considered as an important factor. 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. Salicylic acid, an inducer of PR-proteins, is also synthesized from cinnamic acid via benzoic acid and accumulation of salicylic acid may induce production of PR-proteins. The results of the present study showed the plant extract not only reduced incidence of *Helopeltis* attack as well as Alternaria blight, but also induced both antifungal phenolics and defense enzymes (PAL,TAL,CHT and GLU). 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 various applications of bioresources which is evident from the higher production of antifungal phenolics, higher activities of PAL, TAL, CHT and GLU in treated than in untreated plants. Such induced resistance to pathogen and insects using biological agents as well as bioresources can be viewed as a desirable crop protection strategy with a relatively benign environmental impact. Crucial to the success of induced resistance in agriculture is an understanding of the range and limitations of this form of pest and pathogen control, especially in different stress context. There is sufficient evidence to indicate potential constraints at the level of specific signal interaction whereby it may not be possible to simultaneously maximize defense against all kind of attackers. Indeed, induction of resistance to one pest may result in an increased vulnerability to another. These constraints derive in part from the balance of interacting regulatory signals, all of which can be strongly influenced by abiotic stresses typically encountered in agriculture. The potential for interaction among abiotic and biotic stressors provokes a number of intriguing issues that could impinge on our ability to deploy or optimize induced resistance strategies.

SUMMARY

In the present investigation attempt has been made to elucidate defense strategies in tea plants against pest (*H. theivora*) and foliar fungal pathogens (*A. alternata* and *C. incisum*). Review of literature with two major objectives – biochemical defense strategies of plants against pest and pathogens and efficacy of plant extracts in plant defense response have been discussed. Methods adopted and material used in the experimental set up has been illustrated in details.

Incidence of attack by *Helopeltis theivora* on twenty-three different tea varieties was determined highest (50% to 80%) in the second and third quarters of the year. Among these varieties, UPASI was most susceptible followed by Tocklai. Darjeeling varieties showed less incidence of damage. Using Scanning Electron Microscopy (SEM) healthy as well as *H. theivora* punctured tea leaf surface was scanned. Infested leaf surface showed a number of holes due to puncture made by *Helopeltis*.

All the varieties showed changes in phenolics due to *Helopeltis* infestation. Total phenol content decreased as a result of infestation where as of orthodihydroxy phenols have increased. The accumulation of phenolics due to infestation may reflect a general increase in host metabolism. Protein content decreased following infestation in comparison to healthy ones. Changes in the protein content varied between 4-14% among the varieties tested. Maximum decrease in protein content was noticed in UPASI variety (BSS-2). In SDS-PAGE analysis higher molecular weight proteins (98.5, 97.5, 95.5, 88.4, and 77.5 kDa) of healthy leaves were mainly absent in *H. theivora* infested leaf samples.

Defense enzymes mainly phenylalanine ammonia lyase(PAL), polyphenol oxidase(PPO), peroxidase(PO), chitinase(CHT) and β-1,3-glucanase(βGLU) were studied in healthy and *H. theivora* infested tea leaves. It is interesting to note that PAL activity decreased due to *H. theivora* infestation in all the varieties tested. However UPASI varieties exhibited significant decrease in PAL activity due to infestation. However, sharp increase (2-3 folds) in PO activity was noticed in all the varieties infested with *H. theivora*. PPO activity was higher in the *H. theivora*

infested leaves in comparison to the healthy one. β-1,3 glucanase and chitinase activities increased in *H. theivora* infested leaves when compared with healthy plants. Significant increase β-1,3 glucanase activity was observed in UPASI varieties followed by Darjeeling and Tocklai varieties. When percentage increase in defense enzyme activities of twenty-three tea varieties were compared maximum increased in PPO activity were found in infested leaves followed by PAL, CHT, PO and GLU activities in comparison to healthy. Using PAb of chitinase dot immunobinding assay and Western blots analysis were done. Three bands of ca. molecular weights of 35, 59 and 65 kDa were found to be common in both healthy and *H. theivora* infested leaves, while a new band of ca. molecular weight 42 kDa was evident only in *Helopeltis* infested leaves. Results of the present study indicate the involvement of several enzymes in the insect-tea interaction process.

Antifungal phenolic (pyrocatechol) extracted from healthy and *H. theivora* infested leaf showed antimicrobial activities which were further analyzed HPLC. Catechins extracted from different developmental stages of *H. theivora* infestation were analyzed by HPLC. Four isoforms of catechins such as EGC, EC, EGCG, CG increased sharply due to *H. theivora* infestation in initial stage of puncture. In advance stage of infestation level of EGC, EGCG, GCG and ECG decreased markedly.

Application of biological resources involving metabass - a mycopesticide formulation, biocrop - a plant product and plant extracts of *Azadirachta indica*, *Catharanthus roseus* and *Diplazium esculentum* were evaluated for management of pest attack. In these varieties maximum activity of defense enzymes (PAL, TAL) was noted in infested treated one.

Two important foliar fungal diseases viz. leaf blight caused by *Alternaria alternata* and black rot caused by *Corticium invisum* which are prevalent in the Doors, have been worked out with special reference to their histopathology. Twenty three varieties of tea were scanned for resistance towards *A. alternata* and *C. invisum.*

The varieties were grouped into highly susceptible (T-17, TV-20, TV-22, UPASI-8, BSS-3 and T-78) and moderately resistant (TV-9, TV-18, T-135 and UPASI-3) moderately susceptible (TV-30, TV-25, and HV-39) towards *A. alternata*. In case of *C. invisum,* highly susceptible (TV-22, TV-23, TV-28, HV-39, UPASI-26 and BSS-1) and moderately susceptible varieties (TV-9, TV-25, UPASI-8 and BS/7A/76) were categorized. Polyclonal antibodies were prepared from mycelial antigen of *A. alternata* and *C. invisum* and packaged for ELISA format. Detection of fungal pathogens in twenty-one tea varieties following artificial inoculation with *A. alternata* and *C. invisum* were made using PTA-ELISA format, dot immunobinding assay and indirect immunofluorescence. Young mycelia gave bright fluorescence when conjugate with FITC labeled antibodies. FITC developed apple green fluorescence in infected leaves, which was distributed throughout the leaf tissue, mainly in the epidermal and mesophyll tissues.

Total phenol content decreased following inoculation with foliar fungal pathogens in the susceptible varieties. However there was an increase in the total phenol content as well as PAL, PPO, CHT, βGLU activities of resistant varieties following inoculation with *A. alternata* and *C. invisum*. Greater accumulation of antifungal compounds (pyrocatechol) in developing resistance against *A. alternata* were detected. HPLC analysis pyrocatechol extracted from healthy as well as *A. alternata* inoculated tea leaves showed a sharp peak at retention time 2.6 in both the preparation. But the peak height was higher in inoculated one. Catechins extracted from both the healthy as well as *A. alternata* inoculated tea leaves further analyzed by HPLC. Due to reaction with pathogen inoculated leaf samples exhibited more isoforms of catechin than healthy control. In *A. alternata* inoculated leaves four peaks at 15.7, 18.4, 20.8 and 22.3 retention time increased markedly in relation to healthy of which two peaks corresponding to authentic EGC and EC could be identified where as level of EGCG decreased following inoculation with *A. alternata.*

Aqueous extracts of leaf of *Azadirachta indica, Catharanthus roseus* and *Diplazium esculentum* have been applied on six varieties (TV-20, TV-22, T17/1/54, UP-3, BSS-3 and BS/7A/76) in order to alter the disease reaction against *A. alternata*. In vitro test of crude extracts against *A. alternata* spores exhibited maximum inhibition. Effect of *C. roseus* was found to be more potent in comparison to *D. esculentum* and *A. indica*. Phenolics in treated plants has considerably increased as compared to the untreated plants which further increased in the inoculated plants than in the uninoculated ones. The activity of defense enzymes (PAL, CHT, βGLU) increased in tea leaves treated with plant extracts.

In indirect immunofluorescence leaf treated with all three plants extracts developed bright apple green fluorescence when reacted with PAb-CHT which was distributed in mesophyll tissues. However, the palisade parenchymatous tissues exhibited red colouration indicating the high level of phenolics accumulated in leaf tissues following treatment with *C. roseus* and *A. indica.* This was not evident in case of *D. esculentum* treated leaves.

SA has been implicated as a component of induced resistance signaling pathway. Untreated as well as SA treated leaves were examined microscopically. Indirect immunofluorescence studies using PAb Chitinase and labeled with FITC conjugate revealed an excellent apple green fluorescence throughout the leaf tissues in SA treated leaves. Ultra-thin sections of SA treated following reaction with PAb-CHT, labeled with gold particles exhibited intense gold labeling, corresponding to CHT deposition in comparison with untreated control plants. High amount of gold labeling was found in host cytoplasm and chloroplast while lesser amount in vacuoles, mitochondria and walls. Multicomponent coordinated defense response of tea plants to pest and pathogens has been critically discussed.

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