Biochemical and Immunological Characterization of Pathogenesis-related Proteins of Tea Triggered by *Exobasidium vexans* Massee

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This is to certify that Ms Monica Sharma has carried out her research under my supervision. Her thesis entitled " **Biochemical and Immunological Characterization of Pathogenesis-related Proteins of Tea Triggered by *Exobasidium vexans* Massee**" 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.

(B. N. Chakraborty)

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CONTENT

INTRODUCTION

Plants are often simultaneously challenged by pathogens and insects capable of triggering an array of systemic responses that may be beneficial or detrimental to plant health and productivity. Inducible defenses in plants against pathogens and insect herbivores can be strongly influenced by the mix of signals generated by external biotic factors as well as by abiotic stresses such as drought, nutrient limitation, or high soil salinity. 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.

Plants protect themselves from stressful situations by changing their physiological conditions. This reaction is known as the defense response of higher plants and a series of proteins actively synthesized with this reaction is called defense-related proteins. In addition to specific defense responses, based on so called R-genes against certain strains of a pathogen (Ellis *et al.*, 2000), plants have broad spectrum defense responses which are pre-formed such as waxes or the responses that can be induced locally or systemically by biotic or abiotic agents in nature (Oostendorp *et al.*, 2001; Liu *et al.*, 2005). In due course plants have developed ingenious molecular strategies to defend themselves against the biotic and abiotic stresses they may be confronted with and the ability of plants to survive an attack by pathogens is generally related to the expression of defenses. However, even in those cases where a species is susceptible to a specific pathogen, it is likely that certain cultivars or developmental stages of the species successfully resist infection (Heath, 2000). Hence, it is not surprising that there are potentially multiple means by which plants detect the presence of pathogens and effectively respond to the infection (Hammerschmidt, 2003).

As a consequence to the threats from invasion by pathogenic microorganisms in the outside environment plants produce a series of proteins that exert direct antimicrobial activity. Such proteins are specifically termed antimicrobial proteins or AMPs which directly interfere with the growth, differentiation, the multiplication and/ or spread of microbial organisms (Broekaert *et al.*, 2000). By far the most studied group of AMPs, the pathogenesis-related (PR) proteins that are protective plant proteins specifically induced in pathological or related situations, was discovered

through the pioneering works of Gianinazzi *et al.* (1970); Van Loon and Van Kamen (1970).

Information is emerging on how these AMPs interfere with growth of microorganisms, fungi in particular. Examples of some are, hydrolases such as PR-3 type chitinases, PR-2 type glucanases and possibly PR-4 type proteins, affect fungal growth by disturbing the integrity of their cell wall. Thionins, 2S albumins, lipid transfer proteins and puroindolines have been demonstrated to partially lyse artificial phospholipid vesicles and are therefore believed to interfere with the phospholipid bilayer of the microbial plasma membrane. Other, AMPs such as PR-5 type proteins and plant defensins are proposed to affect plasma membrane receptors. Defense-related proteins involved in the synthesis or transport of metabolites that are inhibitory to microorganisms, are not considered as AMPs, although expression of such genes often play a very similar role in host defense (Broekaert *et al.,* 2000). PRs are synthesized *de novo* and commonly found in plants responding hypersensitively to pathogen infection. The sequence similarities and serological relationships are the basis of the classification. PRs are constitutively present in tissues vulnerable to pathogen attack (Jangid *et al.*, 2004). They have been discovered in every plant species that has been studied so far, accumulate not only in response to microbial pathogens but also to nematodes, insects or herbivores, in healthy plants during flowering and senescence and can be present in virtually any organ. Most importantly, the occurrence of these novel proteins is not pathogen-specific but is determined by the type of reaction of the host plant, indicating that they are of host origin.

Pathogenesis-related proteins have been best described in the model plant tobacco and have progressively been extended to numerous other plant-pathogen combinations since its discovery. A great deal of research has focused on the isolation, characterization and regulation of expression of this unique class of defense proteins in a wide host range including tobacco, tomato, cucurbits, pepper stems, cotton, mustard, barley, wheat, rice, bean, groundnut, gram and many more. Yet, till date, there is no information in this line for tea. Tea which is cultivated under widely different geographical regions (Plate 1,figs. A & B) like any other plant suffers from a variety of damaging pests and fungal pathogens which prey on the leaves.

Plate 1 (figs. A & B). Tea plantations. [A] Hills and [B] plains.

The interactions between plants and potential pathogens maybe of the non-host type, compatible type or incompatible type. Non-host interactions occur when the attacked species does not belong to the specific host-range of the pathogen and the plant is not infected. Compatible interactions take place when the pathogen is able to multiply and spread through the host which is not able to adequately switch on its active defense mechanisms. In incompatible interactions the host recognizes the invading pathogen leading to a defense response which prevents the pathogen from infecting the plant any further usually leading to a hypersensitive reaction of the plant which impedes pathogen spread through the plant. This is followed by enhanced resistance in the uninfected parts of the plant against further pathogen attack, a phenomenon called 'induced' or 'acquired resistance'. During the hypersensitive response in plants many biochemical alterations are coordinately induced that can contribute to, or may even determine the resistance attained. PR-proteins may function as the first or last line of defense against pathogens and pests consequently representing a generalized plant defense response. PR-like proteins, which are expressed in apparently healthy tissues during normal plant growth, may have additional unsuspected roles in morphogenesis or symbiosis. Plants that are apt to intensively induce such proteins are certainly agriculturally valuable.

Tea is used as a drink made from the apical two leaves and a bud of *Camellia sinensis* (L.) O.Kuntze. The leaves contain in addition to the normal constituents, high levels of polyphenols (30-40%) and caffeine (5-6%). The beneficial health effects of tea are well known (Das *et al.*, 2005) and the terminal bud of tea contains the maximum caffeine, which contributes to the medicinal value of tea (Ramarethinam and Rajalakshmi, 2004). Tea is grown in more than 50 countries, mostly in plantation as a monocultural crop. It prefers a warm humid climate with well distributed rainfall and long sunshine hours. India with around 4,40,000 ha area under tea cultivation, is globally the largest producer and consumer of tea. Darjeeling produces the world's finest quality tea on the steep hill slopes of the eastern Himalayas up to an elevation of 2000 m (Chakraborty *et al.*, 2002a). Tea plants growing in and around the Darjeeling hills are seriously affected by pests and pathogens which is one of the acute problems of the tea industry. Of the various foliar fungal diseases 'Blister blight' (Plate 2), caused by the biotrophic fungal pathogen

Plate 2. Blister blight disease of tea.

Exobasidium vexans, is considered as one of the key factors in restricting the production of high-quality tea (Chandra Mouli, 2003). The pathogen attacks harvestable tender shoots, inflicting enormous yield loss (30-40%) and quality deterioration even below the 35% disease threshold (Radhakrishnan and Baby, 2004). *E. vexans* has no known alternate host and as such it completes its life cycle on tea itself. Under severe infection the affected leaves curl up. When the tender stem is attacked the entire shoot withers and falls. It has been noticed during appearance of blister blight that some of the tea bushes are not affected by the pathogen. This indicates that tea plants have an inherent immune system.

The aim of this discourse was to characterize the defense-related proteins in tea plants triggered by *E. vexans* and to elucidate the strategies of the defense mechanism in this host-parasite interaction. Biochemical and immunological characterization of the proteins which accumulate during compatible and incompatible interactions of tea plants against *E. vexans* was undertaken in the present investigation with the following major objectives,

(a) Biochemical analyses of proteins from tea varieties showing compatible and incompatible reaction towards *E. vexans.*

(b) Evaluation of defense related enzymes - chitinase, β-1,3-glucanase and peroxidase in tea plants following inoculation with *E. vexans.*

(c) Determination of time course accumulation of pathogenesis related (PR) proteins in tea – *E. vexans* interaction and assessment of their antimicrobial activities *in vitro.*

(d) Immunological characterization of pathogen-induced (PI) and pathogenesis-related (PR) proteins in tea varieties triggered by *E. vexans.*

(e) Analyses of PR-proteins in suspension cell cultures induced by *E. vexans* and abiotic elicitors.

(f) Induction of resistance in tea plants against *E. vexans* and associated changes in defense enzymes.

(g) Elucidation of defense responses of tea plants stimulated by *E. vexans* with special reference to antifungal phenolics.

(h) Immunocytochemical localization of the pathogen in compatible interactions and cellular localization of defense enzymes induced in leaf tissues and cell cultures of tea.

(i) Immunogold localization of *E. vexans*, chitinase and β-1,3-glucanase in tea leaves.

At the onset, a brief review of literature on Pathogenesis-related (PR) proteins in conformity with the present work has been presented. In the following pages, the materials used and the methods applied to achieve the above objectives have been outlined along with a description of the results achieved.

LITERATURE REVIEW

Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility. Higher plants protect themselves from various stresses, such as pathogen attack, wounding, application of chemicals including phyto-hormones and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions. Such constraints lead to production of a wide array of defense compounds, which are either induced or preformed. One of the ways in which plants respond to biotic and/or abiotic stress factors are the accumulation of various novel proteins collectively referred to as pathogenesis-related proteins or PR-proteins. Such protective plant proteins specifically induced in pathological or related situations have been intensively studied from the agricultural interest. On the other hand, many of the reserve proteins accumulated in seeds and fruits take the constitutive defense function against microbial pathogens and invertebrate pests in addition to their storage function. These inducible or constitutive defense mechanisms of higher plants are relatively conserved in the course of evolution. Accordingly, most plants produce or accumulate similar proteins under certain situations irrespective of their morphological differences. These proteins were first observed in tobacco, *Nicotiana tabacum*, cultivars reacting hypersensitively to tobacco mosaic virus (Van Loon and Van Kammen, 1970; Gianinazzi *et al.*, 1970). The occurrence of these proteins was not pathogen specific but determined by the type of reaction of the host plant, indicating that these proteins were of host origin. Since the proteins were induced under specific pathological conditions, they were named 'pathogenesis-related proteins' abbreviated as PRs. PRs were found in small amounts in senescing leaves of flowering plants and in relatively larger quantities when necrosis was more severe. This led to the assumption that these polypeptides were stable proteolytic breakdown products of larger leaf proteins.

By definition, these proteins abbreviated as PRs, were described as "proteins coded for by the host plant but induced specifically only in pathological or related situations" (Van Loon and Van Kammen, 1970). PRs are most often of low molecular weight, selectively extractable in low pH, highly resistant to proteolytic degradation/ or endogenous proteases and localized predominantly in the intercellular space (Kassanis *et al.*, 1974). PRs have been found in several plant species belonging to various families (Table 1).

Table 1: Plant species in which pathogenesis-related proteins have been identified

Family	Plant species
Amaranthaceae	*Gomphrena globosa*
Chenopodiaceae	*Chenopodium amaranticolor, C. quinoa, Beta vulgaris*
Compositae	*Gynura aurantiaca, Helianthus annuum*
Cruciferae	*Arabidopsis thaliana, Brassica nigra, B. juncea, B. napus, B. rapa, Raphanus sativus*
Cucurbitaceae	*Cucumis sativus, C. melo, Cucurbita maxima, C. pepo*
Gramineae	*Hordeum vulgare, Zea mays, Avena sativa, Oryza sativa, Triticum aestivum*
Malvaceae	*Gossypium hirsutum*
Papilionaceae	*Medicago sativa, Phaseolus vulgaris, P. lunatus, Cicer arietinum, Vigna unguiculata, V. radiata, Arachis hypogea, Lablab purpureus, Pisum sativum, Glycine max*
Rutaceae	*Citrus sinensis*
Solanaceae	*Capsicum annuum, Petunia, Solanum demissum, S. nigrum, S. tuberosum, S. dulcamara, Nicotiana debneyi, N. glutinosa, N. rustica, N. sylvestris, N. langsdorfii, N. plumbaginifolia, N. tomentosiformis, N. tabacum, Lycopersicon esculentum*
Umbelliferae	*Petroselinum crispum, Apium graveolens*
Vitaceae	*Vitis vinifera*
Pinaceae	*Picea abies*

The introduction of polyacrylamide electrophoresis by Davis (1964) and Ornstein (1964) by which proteins could be separated on the basis of their combination and charge was a chief innovation in unearthing PRs – the new protein components induced by TMV in hypersensitively reacting tobacco. The protein extracts from *N. tabacum* revealed thirty bands and additional separation was achieved with varying concentrations of the acrylamide in the gels and the electrophoretic pattern was represented by densitometric tracings (Van Loon and Van Kammen, 1970). Moreover, it was observed that the same proteins were induced in tobacco by different viruses, whereas different proteins appeared upon infection in *N. glutinosa*. Aided by selective extraction methods (Van Loon, 1976; Gianinazzi *et al*, 1977; Parent and Asselin, 1984) it was established that not only viruses but also

fungi and bacteria were able to induce similar protein components in various plant species particularly in incompatible combinations resulting in hypersensitive necrosis. Proteins that are induced exclusively during disease development in compatible host-pathogen combinations have hardly been considered as PRs. PRs have been grouped into families on the basis of similarities in molecular weights, amino acid composition and serological properties confirmed by nucleotide sequencing of corresponding cDNAs (Van Loon, 1975).

Nomenclature

The classification has set a convenient standard for other plant species, in which these family numbers now similarly designate PRs with properties homologous to tobacco PRs. Initially the PRs were grouped in to five main classes consisting of the 10 major acidic PRs of tobacco characterized both by biochemical and molecular biological techniques and designated as PR-1 to -5 (Gianinazzi *et al.*, 1977; Van Loon 1985).

A unifying nomenclature was proposed based on their grouping into eleven families, classified for tobacco and tomato, sharing amino acid sequences, serological relationships and/ or enzymatic or biological activity. The criteria for inclusion of new families of PRs were (i) protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and (ii) induced expression must have shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories (Van Loon and Van Strien, 1999). Each PR family is numbered and the individual family members are assigned lower case letters in the order in which they are described. In accordance with the recommendations of the Commission for Plant Gene Nomenclature, PR-genes are designated as *ypr*, followed by the same suffix as of the family. Later on three more peptides, which were capable of inducing defense responses of plants, were identified. These three families (PR-12, -13 and -14) comprise the pathogen induced plant defensins (PR-12) (Fraser, 1981) thionins (PR-13) (Antoniw *et al.*, 1981) and lipid transfer proteins or LTPs (PR-14) (Garcia-Olmedo *et al.*, 1995). So far, 17 families of PRs have been recognized (Table 2). However, the properties of all these proteins have not yet been elucidated.

Table 2. Recognized families of pathogenesis-related proteins

Families	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	antifungal	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	chitinase type I, II	*Ypr4, Chid*
PR-5	Tobacco S	thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	proteinase-inhibitor	*Ypr6, Pis (Pin')*
PR-7	Tomato P_{69}	endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	chitinase type III	*Ypr8, Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase	*Ypr9, Prx*
PR-10	Parsley 'PR1'	'ribonuclease-like'	*Ypr10*
PR-11	Tobacco 'class V' chitinase	chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	thionin	*Ypr13, Thi*
PR-14	Barley LTP4	lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	'oxalate oxidase-like'	*Yrp16*
PR-17	Tobacco PR27	unknown	*Yrp17*

Besides proteins newly defined mRNAs (cDNAs) are often considered as additional members of the existing families where shown to be induced by pathogens or specific elicitors. However, because PRs are generally defined by their occurrence as protein bands on gels, and classified within each family once the protein has been characterized, cDNA or genomic sequences without information on the corresponding protein cannot be fitted in to the adopted nomenclature. Thus for naming it is necessary to gather information at both the nucleic acid and the protein level when dealing with a stress-related sequence falling within the definition of PRs. Conversely, homologies at the cDNA or genomic level may be encountered without information on the expression or characteristics of the encoded protein. Such sequences obviously belong to the PR-type families, but yet cannot, be considered to correspond to pathogen-induced PRs and named accordingly. In more than a few situations, it is difficult to distinguish PRs from related proteins/ mRNAs that are

present in some organs or appear during specific developmental stages. Homologous proteins/ mRNAs in healthy tissues in which no induction by pathogen infection has yet been demonstrated, are to be termed PR-like proteins (PRLs) and their genes *yprl* (Van Loon, 1999).

Structure

Anther-specific proteins form *Brassica napus* and *Arabidopsis* similar to β-1,3-glucanases but with a 114-amino acid carboxy-terminal extension and 37% identity to Tag1 might from a sixth class of β-1,3-glucanase. The only non-plant proteins reported so far to display similarity to plant PR-2-type proteins are fungal β-1,3-glucanase (Hird *et al.*, 1993). β-1,3-glucanases exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization, and pattern of regulation. The most detailed sequence information for these isoforms is available from cDNA and genomic clones of tobacco β-1,3-glucanase, which form a multigene family. Based on amino acid sequence identity, the various β-1,3-glucanase of the genus *Nicotiana* have been classified into three structural classes (Payne *et al.*, 1990).

The amino-terminal residue of the basic tobacco PR-2 most likely is a glutamine, an observation consistent with the presence of a 21-residue signal peptide in the preproteins. The three-dimensional structure of the barley β-1,3-glucanase has been determined by X-ray crystallography (Varghese *et al.*, 1994). The β-glycosyl residues are not bound equally tight to the individual sub sites and a slight repulsion rather than a binding is observed for the interaction between the third and the fourth sub site and the β-1,3 -glucan substrate. This might facilitate the hydrolytic cleavage catalyzed by amino acids located between the same third and fourth β-glycosyl-interacting sites.

The approximately 33 kDa class I enzymes (βGlu1) of *Nicotiana tabacum*, which constitute the PR-2e subgroup of the tobacco PRs are basic proteins localized in the cell vacuole. β-1,3-glucanase I is produced as a proprotein with an N-terminal hydrophobic signal peptide, which is cotranslationally removed, and a C-terminal

extension N-glycosylated at a single site. The proprotein is transported from the endoplasmic reticulum via the Golgi compartment to the vacuole where the C-terminal extension is removed to give the mature, approximately 33 kDa enzymes, which is not glycosylated (Sticher *et al.*, 1992).

Inspection of the amino acid sequences deduced from cDNAs or genes encoding PR-3-type proteins from different plants reveals that they all have in common a core domain harbouring the catalytic site and hence called the chitinolytic or catalytic domain. In addition, all PR-3-type proteins can be further divided in three subgroups, which are historically designated as class I, class II and class IV chitinases. Class IV chitinases mainly differ from the class I chitinases by four deletions, one occurring in the chitin-binding lectin domain, the other three in the catalytic domain. They also lack a carboxy-terminal propeptide (Collinge *et al.*, 1993).

The class II tobacco isoforms are at least 82% identical in amino acid sequence and differ from the class I enzymes at a minimum of 48.8% of the positions. The acidic, approximately 35 kDa, PR-2d is the sole representative of tobacco class III β-1,3-glucanase and differs in sequence at least by 43 % from the class I and class II enzymes. Two highly homologous cDNA clones for class III β-1,3-glucanase have been isolated from tomato plants infected with a viroid (Leubner-Metzger and Meins, 1999).

The PR-8-type protein family accommodates the chitinases, which were previously designated as class III chitinases (Van Loon *et al.*, 1994). This new nomenclature reflects the fact that PR-8-type chitinases are structurally unrelated to PR-3-type chitinases. Mature PR-8-type chitinases are monomeric proteins with a molecular weight of about 28 kDa and can be either acidic or basic. In most cases, PR-8-type chitinases have been reported to exhibit both chitinase and lysozyme activities but at least a sugar beet PR-8 chitinase exhibits endo- and exochitinase activity but no lysozyme activity (Nielsen *et al.*, 1993), indicating that bifunctional chitinase/ lysozyme activity is not a general property of this enzyme family.

A three-dimensional fold supposedly shared by all members of the PR-1-type protein family has been determined by NMR for the tomato P14a protein (Fernandez *et al.*, 1997). This protein features an α–β–α sandwich structure in which α-helices are tightly packed on both sides of a central β-sheet. The heptapeptide sequence HYTQ (hydrophobic residue) VW, which is conserved among plant and animal PR-1 like proteins, constitutes an α-helix that is largely buried inside the protein core and thus appears to play a structural role, possibly as a folding nucleus.

Occurrence

The occurrence of almost all types of PRs in various floral tissues also suggests specific physiological functions during flower development rather than a role in general defense against pathogen invasion. This notion is supported by the presence in floral organs of additional PR-like proteins, glucanases (Coté *et al.*, 1991) and thaumatin-like proteins (Richard *et al.*, 1992). In petunia flowers, chitinase activity is localized in the petals (about 15%) and stigma (about 85%). In the stigma it increases about five-fold following anther dehiscence, strongly suggesting that the chitinase has a specific function in reproduction (Neuhaus, 1999). Demonstration that the PR-2 family are β-1,3-endoglucanases and the PR-3,-4,-8, and -11 families consist of chitinases with or without lysozyme activity, immediately suggested that these PRs are directed against cell walls of fungi and bacteria. Homology of the thaumatin-like proteins of the PR-5 family with a bifunctional α-amylase/trypsin inhibitor from maize seeds seemed consistent with a role in protection against phytophagous insects. However, no proteinase inhibitor activity has been demonstrated for PR-5 proteins. Resistance to insect attack is taken to be conferred primarily by wound-inducible proteinase inhibitors, which have now been grouped into the family PR-6 (Heitz *et al.*, 1999).

A role of PRs as specific internal signal generating enzymes would be consistent both with their occurrence in specific organs and with their induction during the development and in response to stressful pathogen infections. The major chitinase of bean leaves first described by Boller *et al.* (1983) to be induced by ethylene and located in the vacuole, appears to be also induced in abscission zones at

the stem petiole-junction together with a PR-1-like protein, two isoforms of β-1,3-glucanase, other chitinases, and a thaumatin-like protein (Staehelin *et al.*, 1994). However, the natural substrates for chitinases in higher plant cell walls remain to be determined. PR-3-type proteins are monomeric enzymes ranging from about 25 to 35 kDa in size. They act as endochitinases and release chitooligosaccharides of varying degrees of polymerization, of which the smallest are usually chitobiose and chitotriose. A PR-3-type chitinase was first isolated from bean leaves but similar enzymes have now been purified from about 20 different monocot and dicot plants.

Elucidation of the biochemical properties of the major, pathogen-inducible PRs of tobacco and subsequent cloning of their cDNAs and/ or genes revealed that proteins with substantial similarity to the classical PRs, which are mostly acidic and extracellular proteins, the homologous counterparts are mostly basic and localized intracellularly in the vacuole. As far as it has been possible to deduce, they possess the same type of enzymatic activities, but their substrate specificity and specific activity may be rather different (Linhorst, 1991). Newly defined mRNAs (cDNAs) may be added to existing families when shown to be induced by pathogens or pathogen specific elicitors, like are the, thionins (Bohlmann, 1994) and defensins (Broekaert *et. al.*, 1995) both families are of small basic, cysteine-rich polypeptides, and qualify for inclusion as new families of PRs.

Biosynthesis

Comparison of the determined amino-terminus of a mature acidic PR-1 protein from tobacco and a basic PR-1 protein from tomato with the amino acid sequence derived form their respective cDNA sequences reveals that both proteins, as well as other PR-1-type proteins, are synthesized as preproteins consisting of an amino terminal hydrophobic signal peptide followed by the mature PR-1 domain. Some, but not all, basic PR-1-type proteins feature short (6 to 19 amino acids) carboxy-terminal segment that is absent in most other PR-1-type proteins. This carboxy-terminal extension may function as vacuolar targeting determinant in analogy to the proven role of the carboxy-terminal propeptide of some basic PR-2, PR-3 and PR-5 proteins (Melchers *et al.*, 1993).

Complementary DNA clones with sequences similar to tobacco *PR-1* sequences have been isolated from *Brassica napus*, *Solanum tuberosum*, *Medicago trunculata* and maize and have been purified from tomato, barley and *Arabidopsis* as well as from tobacco (Bryngelsson *et al.*, 1994). PR-1-type proteins have also shown significant homology to some proteins of non-plant origin.

PR-8-type chitinases display homology to concanavalin B, an enzymatically inactive protein from jack bean (*Canavalia ensiformis*). More intriguingly, plant PR-8-type proteins show sequence homology with a chitinase from baker's yeast (*Saccharomyces cerevisiae*). The overall identity between these proteins is 18% including 6 invariant cysteines, with an additional 43% of homologous amino acids in at least five out of the six aligned sequences (Broekaert *et al.*, 2000).

A single endochitinase from tobacco constitutes the class V chitinases, recently reclassified as PR-11 protein (Heitz *et al.*, 1994; Van Loon *et al.*, 1994). This enzyme is synthesized as a preprotein with an amino-terminal prepeptide and a carboxy-terminal propeptide loosely homologous to the tobacco basic β-1,3-glucanase carboxy-terminal extensions, although an N-glycosylation site is missing. The tobacco PR-11 chitinase shows significant homology to bacterial and nematode chitinases and to a lesser extent also to PR-8-type chitinases. Also remarkable is the fact that some fungal chitinases are more homologous to PR-8-type chitinases (e.g. Kuranda and Robbins, 1991), while other fungal chitinases are more related to PR-11-type chitinases (Hayes *et al.*, 1994).

Functional properties

PRs are, as such, a collective set of novel proteins which a plant produces in reaction to a pathogen mainly in incompatible interactions and thus impedes further pathogen progress. The "related situations" in which PRs were found to be induced, seem to prove the point. Application of chemicals that mimic the effect of pathogen infection or induced some aspects of the host response, as well as wound responses that give rise to proteins that are also induced during infections, can induce both PRs and acquired resistance. The occurrence of homologous PRs as small multigene

families in various plant species belonging to different families, their tissue-specificity during development and consistent localization in the apoplast as well as in the vacuolar compartment and their differential induction by endogenous and exogenous signaling compounds suggest that PRs may have important functions extending beyond their apparently limited role in plant defense.

During the hypersensitive reaction cellular damage and death is a major stress to the plant, as exemplified by high increases in abscisic acid and ethylene (Van Loon, 1997). It is possible, therefore, that PRs are stress proteins directed to alleviate harmful effects of cellular degradation products on thus far untouched neighboring cells. Both acidic and basic PRs may be induced by physiological necrosis or wounding or by high concentrations of ethylene (Brederode *et al.*, 1991). Such induction in the absence of pathogenic attack might be taken to indicate protection of cellular structures, either physically to stabilize sensitive membranes or macromolecules, or chemically to keep potentially harmful saprophytic microorganisms on tissue surfaces or in intercellular spaces in check. In virtually any natural stress condition e.g., heat, cold, drought, osmotic stress, water logging, anaerobiosis, metal toxicity, etc., plants are known to react by the synthesis of novel, and sometimes partly overlapping, sets of proteins (Wasternack and Parthier, 1997).

The various conditions under which PRs occur are reminiscent of those under which heat-shock proteins (HSP) are induced. These proteins are ubiquitous in living organisms and associated with the acquisition of thermotolerance to otherwise lethal temperatures, but a causal connection is not evident. Interestingly, the promoters of all three tobacco PR-1 genes that are expressed, as well as of a different type of PR in parsley, contain a heat shock regulatory element, but the proteins are not induced to detectable levels by heat shock. Nevertheless, PRs might have an analogous function, though quite different, chaperonin-like function unlike PRs, HSP are intercellular proteins that do not accumulate during heat shock. However, the specific occurrence of individual PRs in some floral organs, but not in others, points to other, more specific roles (Dutta and Muthukrishnan, 1999).

The relative ineffectivenes's of PRs in determining resistance to pathogens does not preclude an involvement in defense. As first proposed by Mauch and Staehelin (1989) acidic, extracellular PRs might be involved in recognition processes, releasing defense-activating signal molecules from the walls of invading pathogens. This would hold particularly for chitinases and glucanases that could liberate elicitor-type carbohydrate molecules from fungal and bacterial cell walls. Thus, a β–1,3–glucanase induced in soybean seedlings by infection or chemical stress releases elicitor-active fragments from cell wall preparations of the fungus *Phytophthora megasperma* f.sp. *glycinea*. Such elicitors cold help stimulate defense responses in adjacent cells and thus accelerate and enhance these reactions, as well as induce acquired resistance to further infection (Ham *et al.*, 1991).

PR-1 the most abundant of the PR proteins, is induced 10,000-fold in infected tissue and accumulates to 1 to 2 % of the total leaf protein. PR-1-type proteins are monomeric proteins of about 15 kDa with no known catalytic activity. They were first detected and purified from tobacco leaves infected with tobacco mosaic virus (Antoniw *et al.*, 1980). Tobacco PR-1-type proteins are encoded by a small gene family comprising at least 8 members. The acidic PR-1 proteins remain soluble at pH 3, whereas most other plant proteins are denatured under these conditions. The relative resistance to the action of endogenous and exogenous proteases, suggest that PR-proteins have a low turnover rate. The estimated half-life of the PR-1 proteins is 40 to 70 hours.

There are indications for the existence of special proteolytic enzymes that are responsible for the turnover of PR-proteins. The acidic *PR-1* genes do not encode a known targeting peptide sequence for vacuolar targeting. Therefore, the synthesis and accumulation within crystal idioblasts of the acidic PR-1 proteins, indicates that these cells sort the proteins in the unique manner Rodrigo *et al.*, (1991) described an extracellular aspartyl protease in tomato, which is able to degrade PR-proteins.

In other plants, extracellular proteins corresponding to the acidic PR-1 proteins of tobacco do not necessarily have a low pI. For example, the tomato P4 and P6 proteins purified from the apoplastic fluid are basic (Joosten *et al.*, 1990).

PR-1 proteins do not seem to function as antiviral factors despite the high production of PR-1a and -1b proteins after virus infection. Niderman *et al.* (1995) found that several PR-1-type proteins inhibit zoospore germination of the Oomycete *Phytophthora infestans*, the causal agent of potato late blight. In comparative tests, basic PR-1 proteins from either tobacco or tomato were significantly inhibitorier than acidic isoforms. Much investigation has not been done on the activity of PR-1-type proteins on other microorganisms, neither is much known on how they may affect growth processes of Oomycetes.

Plants contain several types of enzymes that have the ability to hydrolyze polysaccharides from microbial cell walls. Endo-β-1,3-glucanases, for instance, are enzymes that specifically hydrolyze β-1,3-ether linkages in β-1,3-glucans and β-1,6-glucans, resulting in a range of oligosaccharides down to disaccharides (Yoshikawa *et al.*, 1993). Most endo-β-1,3-glucanases are structurally related and form a family known as PR-2-type proteins which catalyze endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans. The β-1,3-glucanses are abundant highly regulated enzymes widely distributed in seed plants (Høj and Fincher, 1995).

Although the major interest in β-1,3-glucanases stems from their possible role in the response of plants to microbial pathogens, there is strong evidence that these enzymes are also implicated in diverse physiological and developmental processes in the uninfected plant, including cell division, microsporogenesis (Bucciaglia and Smith, 1994), pollen germination and tube growth, fertilization (Ori *et al*, 1990) embryogenesis, fruit ripening, seed germination (Leubner-Metzger *et al.*, 1995), mobilization of storage reserves in the endosperm of cereal grains (Fincher, 1989), bud dormancy, and responses to wounding, cold, ozone and UV-B (Hincha *et al.*, 1997).

Basic glucanases belong to class I, whereas class II accommodates acidic β-1,3-glucanases including the two acidic 41 kDa stylar β-1,3-glucanase isoforms (Ori *et al.*, 1990) and class III β-1,3-glucanases harbor an acidic tobacco enzyme slightly

different from the class II enzyme. The products of three tandemly arranged *Arabidopsis* β-1,3-glucanase genes also fall into this class (Dong *et al.*, 1991).

Barley β-1,3- and structurally related β-1,3-1,4-glucanases are classified in a fourth class of PR-2-type proteins. *Tag1*, the PR-2-type protein, or a PR-like protein, specifically expressed in tobacco anthers forms the fifth class of β-1,3-glucanases (Bucciaglia and Smith, 1994). The tobacco PR-2 glucanases vary 250-fold in specific activity on laminarin and their relative activities on different substrates vary greatly, suggesting that their normal actions may be diverse. Expression studies of PR-2 in transgenic tobacco suggest that this protein functions developmentally in seed germination by weakening the endosperm, thus allowing the radicle to protrude (Vögeli-Lange *et al.*, 1994a).

There is considerable indirect evidence that, in analogy to tobacco class I chitinases and barley lectin, the C-terminal extension contains a signal for targeting to the vacuole. Results obtained with cultured tobacco cells provide strong evidence that vacuolar chitinases can be secreted into the medium via a novel pathway (Melchers *et al.*, 1993).

In contrast to β-1,3-glucanase I, the class II and III members of the PR-2 family are secreted into the extracellular space (Simmons, 1994). The tobacco class II β-1,3-glucanase PR-2a, PR-2b, PR-2c and the class III β-1,3-glucanase PR-2d, also known as PR-2, PR-N, PR-O, PR-Q′, respectively, are acidic proteins without the C-terminal extension present in the class I enzymes ranging in apparent size from approximately 34 to 36 kDa in denaturing gels (Van Loon *et al.*, 1994).

The specific enzymatic activities and substrate specificities of different β-1,3-glucanase vary considerably. The β-1,3-glucanase I and class II PR-2c appear to be 50 to 250 times more active in degrading the β-1,3-glucan substrate laminarin than the class II PR-2a and PR-2b and the class PR-2d enzymes (Linthorst, 1991).

Induction of β-1,3-glucanases I has been observed during germination. β-1,3-glucanases is exclusively induced in the micropylar region of the endosperm where the radicle will penetrate but prior to endosperm rupture. This induction of β-1,3-glucanase is not a classical defense response as chitinases and the known acidic II and III β-1,3-glucanases are not induced. So, it has been hypothesized that β-1,3-glucanase weakens the endosperm by digestion of cell wall material which promotes radicle protrusion to facilitate germination (Leubner-Metzger and Meins, 1999).

Gibberellins which can substitute for light in releasing dormancy in tobacco seeds, induces β-1,3-glucanase in the dark in association with germination. It is well established that 1,3;1,4-β-glucanases, which are structurally related to β-1,3-glucanase but differ in substrate specificity, hydrolyze the cell walls of the starchy endosperm during the germination of cereals (Høj and Fincher, 1995). Less is known about the function of cereal β-1,3-glucanase, which are present in ungerminated grains and rise markedly in concentration during germination. However it is proposed that high levels of β-1,3-glucanase are part of a strategy to protect the grain against microbial attack (Leubner-Metzger *et al.*, 1995). Cordero *et al.*, (1994) found that one β-1,3-glucanase isoforms and three chitinase isoforms are induced in germinating maize kernels infected by *Fusarium moniliforme*. In contrast, a second β-1,3-glucanase isoform is expressed in embryo and radicle tissues but is not induced by fungal infection. These findings support the view that cereal β-1,3-glucanase, in addition to their possible role in pathogen defense, might also be involved in embryogenesis and seed germination.

Later, it was shown that the PR-proteins include β-1,3-glucanase the PR-2 family and chitinase the PR-3 family. There is now compelling evidence that β-1,3-glucanase and chitinase, acting alone and particularly in combination, can help defend against fungal infection. It has been proposed that these glucanohydrolases act in at least two different ways: directly, by degrading the cell walls of the pathogen, and, indirectly by promoting the release of cell-wall derived materials that can act as elicitors of defense reactions (Boller, 1995). Several studies have been made in which different β-1,3-glucanase and chitinase isoforms were tested for *in vitro* antifungal

activity. In contrast, the class II β-1,3-glucanase PR-2a, PR-2b, and PR-2c did not exhibit antifungal activity alone or in any combinations tested. Similar studies with tomato β-1,3-glucanase and chitinase have shown that the vacuolar class I isoforms, but not the secreted class II isoforms, inhibit growth of *Alternaria solani*, *Trichoderma viride*, and *Phytophthora infestans* (Lawrence *et al.*, 1996; Kim and Hwang, 1997). Plant β-1,3-glucanase can release oligosaccharides from cell walls of the pathogens, which can then act as elicitors of defense reactions (Boller, 1995). This is well documented for interactions between soybean and the β-glucan elicitor from the pathogenic oomycete *Phytophthora megasperma* f.sp. *glycinea*. Following fungal attack, soybean β-1,3-glucanase releases β-glucans from fungal cell walls, which can then induce accumulation of the phytoalexin glyceollin.

The smallest β-glucan released with elicitor activity was a β-1,3-β-1,6-hepta-1,3-glucoside and the structural requirements for the elicitor activity of these oligosaccharides have been investigated. Umemoto *et al.* (1997) isolated cDNA for a β-glucan elicitor binding protein (GEPB), which is localized in the plasma membrane of soybean root cells. Expression of soybean GEPB gene has shown to confer β-glucan binding activity to *Escherichia coli* and to tobacco cells cultured in suspension, suggesting that GEPB might be an elicitor receptor. Soybean β-1,3-glucanase have been purified that are able to release active β-glucan elicitors from fungal cell walls. Ham *et al.* (1997) presented evidence that fungal pathogens secrete proteins that can selectively inhibit plant β-1,3-glucanase. Fungal pathogenesis appears to involve a complex interplay between host and pathogen β-1,3-glucanase. It is now recognized that species of higher plants produce a broad range of β-1,3-glucanase differing in primary structure, cellular localization, and catalytic activity. The available evidence suggests that different classes of β-1,3-glucanase have different functions in plant microbe interactions.

The activity of β-1,3-glucanase and β-D-glucosidases increased in the leaves of potato plants infected by species of the root parasite genus *Globodera* (Rahimi *et al.*, 1996). Enzyme assays revealed the appearance of both the extracellular and intracellularly targeted β-1,3-glucanase. The reactions of the cultivars to nematode

infection differed, indicating that different pathotypes of the same species or different species of potato cyst nematode elicited the appearance of different classes of β-1,3-glucanase. Kim and Hwang (1997) isolated a basic β-1,3-glucanase with inhibitory activity against *Phytophthora capsici* and found a close serological relationship between the pepper 34 kDa β-1,3-glucanase and tomato β-1,3-glucanase. A basic β-1,3-glucanase (34 kDa) induced in pepper (*Capsicum annuum*) stems by mercuric chloride treatment was purified by CM-cellulose cation exchange chromatography. The 34 kDa β-1,3-glucanase inhibited hyphal growth of the chitin-negative fungus *Phytophthora capsici*, but did not show antifungal activity against the chitin-containing fungi *Alternaria mali, Colletotrichum gloeosporioides Magnaporthe grisea,* and *Fusarium oxysporium* f.sp. *cucumerinum.* Treatment with higher amounts of the β-1,3-glucanase caused lysis of the hyphal and zoospore cells of *P. capsici.* Moreover the 34 kDa β-1,3-glucanase acted synergistically with a pepper basic 32 kDa chitinase in inhibiting hyphal growth of *F. oxysporium* f.sp. *cucumerinum* and *P. capsici.*

Ji and Kuc (1997), found that non-host resistance to *Colletotrichum lagenarium* in pumpkin and squash is not primarily associated with β-1,3-glucanase and chitinase activities. Disease symptoms of anthracnose were observed in cucumber, pumpkin and squash after infiltrating leaves with a conidial suspension of *Colletotrichum lagenarium*, but symptoms developed only in cucumber when droplets of the conidial suspension were applied to the leaf surface. There was no difference in the germination of conidia or in appressorium formation on leaf surfaces of cucumber, pumpkin or squash plants; however, penetration was markedly reduced into pumpkin and squash with or without systemic acquired resistance (SAR) and into cucumber with SAR. Little β-1,3-glucanase and chitinase activities were detected in challenged pumpkin and squash leaves without symptoms even after 5 days after inoculation in leaf surfaces. However, the enzymes were detected in pumpkin and squash leaves with symptoms, and activities of the enzymes were greater than in cucumber. These results suggest that β-1,3-glucanase and chitinase activities are not primarily initial defense compounds associated with non-host resistance of pumpkin and squash to *C. lagenarium.*

The localization of systemically induced chitinase activity was examined (Boller and Metraux, 1988) in the second leaves of cucumber plants that had been infected on the first leaf with tobacco necrosis virus. Leaf pieces were infiltrated with a buffer sodium-chloride solution, and the intercellular fluid was subsequently extracted by centrifugation. chitinase had a much higher specific activity in extracts of the intercellular fluid than in leaf homogenates. The specific activity of chitinase in protoplasts obtained from the leaves was only 5% of that in tissue homogenates. These data indicate that most of the chitinase activity is located in the extracellular space. Few of the inducible acidic PRs associated with SAR have been shown to possess significant anti-pathogenic activity. It could be that PRs make cells less conducive, but any such evidence is lacking. Moreover, PRs do not determine the resistance response, and at most have only an accessory role (Van Loon, 1997).

Characterization

The high resistance of PR-proteins to acidic pH and to proteolytic attack appears to be good adaptation to the conditions of the extracellular environment. Acidic PR-1 proteins were detected in extracellular spaces and xylem elements of TMV-infected tobacco leaves, using biochemical and immuno-localization experiments (Dumas *et al.*, 1988). In addition, (Dixon *et al.*,1991) described the accumulation of PR-1a, -1b, and -1c after TMV infection of *N. tabacum* Xanthi in the central vacuoles of specialized leaf cells, known as crystal idioblasts. Genomic clones and cDNAs corresponding to basic proteins, which are approximately 65% similar to the acidic PR-1a, -1b and -1c proteins, have also been characterized from the TMV-infected leaves (Cornelissen *et al.*, 1987; Payne, *et al.*, 1989). Apart from tobacco PR-1 proteins exist in other organism too. Immunoblot analysis using antibodies against the acidic tobacco PR-1b revealed the existence of the cross-reacting proteins in a number of infected dicots and monocots, including tomato, potato, cowpea, maize, sunflower, barley, *Gomphrena globosa, Chenopodium amaranticolor* and *Solanum demissum.* So far there are indications for the two basic PR-1 proteins in tobacco although hybridization studies revealed that the genome contains at least eight (pseudo) genes. (Cornelissen *et al.*, 1987) Antibodies against the tobacco acidic PR-1 proteins did not react with the basic proteins.

β-1,3-glucanases were first purified from leaves of bean and tobacco in the early 1970s (Abeles *et al.*, 1971). Kauffmann *et al.* (1987) reported that four pathogenesis related proteins induced in tobacco upon infection were in fact β-1,3-glucanase. Comparison of data from barley β-1,3-glucanases purified from various monocot and dicot plant species indicates that they are all monomers with a molecular weight of about 30 kDa (Boller, 1988). These proteins and their homologs are now known as PR-2-type proteins (Van Loon *et al.*, 1994). Phylogenetic analysis of PR-2-type proteins derived from cDNA or genes isolated from several plants yields a dendogram with at least five clusters of related proteins (Bucciaglia and Smith, 1994).

The induction of β-1,3-glucanase as a part of the hypersensitive reaction is a stereotypic response i.e., the pattern of induction is similar for viral, bacterial and fungal pathogens. β-1,3-glucanase show developmental regulation in response to treatment with hormones or infection with pathogens. More recently, specific β-1,3-glucanase proteins have been measured immunologically and their mRNAs have been measured semi-quantitatively by RNA-blot hybridization. In a limited number of cases, regulation of transcription has been studied using plants transformed with Gus reporter genes under the control of the promoter region of β-1,3-glucanase genes. β-1,3-glucanase accumulates at high concentrations in the roots and in lower leaves of mature, healthy tobacco plants. The β-1,3-glucanase content of leaves decreases toward the top of the plant. Within leaves, they are localized in the vacuole of epidermal cells (Keefe *et al.*, 1990).

Further, Metraux *et al.* (1988), found a PR in both the infected and uninfected leaves of cucumber plants inoculated on the first true leaf with a fungal, a bacterial or a virus pathogen. This host-coded protein was detected up to 5 leaves above the infected leaf. The protein was purified from the intercellular fluid by ion-exchange chromatography and by high performance liquid chromatography on ion-exchange and phenyl-sepharose columns. The purified PR was shown to be a chitinase with a molecular mass of 28000 Da as determined by SDS-polyacrylamide gel electrophoresis and by gel filtration.

Biochemical and immunological characterization of tomato PR proteins was elucidated by Fischer *et al.* (1989). Acid soluble apoplastic proteins were isolated from leaves of *Lycopersicon esculentum* (Mill) plants infected by *Phytophthora infestans* and separated by two-dimensional gel electrophoresis. Two groups of proteins with either basic (pI $\geq$ 8) or acidic (pI $\leq$ 6) pIs were distinguished. Characterization of defense related proteins that exhibit properties of chitinase and β-1,3-glucanase has also been demonstrated in the wax of "Carnauba" (*Copernica cerifera*), an economically important palm tree, by Cruz *et al.* (2002).

Induction

Usually the most effective inducers of PRs are pathogens that cause a necrotic reaction; the largest amounts of PRs occur in tissues adjacent to the necrotic areas. However, some pathogens can induce large amounts of PR without causing necrosis, for example potato virus Y infection of tobacco induces the systemic production of high concentrations of PRs 1a, 1b, 1c and 2 (Kassanis *et al*, 1974) and *Burkholderia solanacearum* induces large amounts of PR1 proteins in infiltrated tobacco leaf, neither producing any necrotic symptoms. The suppression of mannitol induces PR1 accumulation in tobacco by antibiotic (White *et al.*, 1996).

Many plant species react to treatment with the stress hormone ethylene with induction of β-1,3-glucanase I activity, protein and mRNA in leaves (Beffa *et al.*, 1995). Although ethylene increases the β-1,3-glucanase I content of epidermal cells slightly, its inductive effect is most pronounced on mesophyll cells of the tobacco leaf. Treatment of plants with ethephon results in no detectable induction, or very weak induction of class II and class III β-1,3-glucanase in leaves of tobacco and tomato.

Tobacco cultivars carrying the dominant *N* gene from *N. glutinosa* show a local lesion, HR response to TMV rather than systemic symptoms of mosaic disease. These plants also show decreased disease symptoms in response to secondary infection with certain other viral, bacterial and fungal pathogens. This is an example of a type of induced long lasting, broad-spectrum resistance called systemic acquired

resistance (SAR). Systemic accumulation of salicylic acid (SA) is associated with the HR of tobacco, *Arabidopsis thaliana*, and certain other plants (Delaney *et al.*, 1995). Treatment of mature wild-type tobacco plants with SA strongly induces accumulation of mRNAs of PR related class II and III β-1,3-glucanase and certain other PRs. While SA is probably not the long distance systemic signal for SAR activation, it is required for transduction of this signal in leaves distal to the primary infection site.

Investigations on the stress response in *Brassica* species susceptible and resistant to *Phoma lingam* was carried out by Dixelus (1994). Experiments were set up to study the effects of fungal isolates of different virulence on both a resistant and a susceptible *Brassica* host species. Two isolates of *Phoma lingam*, the fungal toxin sirodesmin PL, and salicylic acid were used to induce stress in plantlets of *Brassica napus* and *B. nigra*, which are susceptible and resistant to the fungal pathogen, respectively, *B. nigra* showed a very rapid response to antisera raised against PR-2, PR-Q and PR-S in tobacco. In contrast, in *B. napus* stressed by the fungus, sirodesmin PL or salicylic, the production of the three PR-proteins was delayed or absent.

Spraying plants with a solution of salicylic acid mainly induces the acidic PR-1 and acidic β-1,3-glucanase genes (Bol *et al.*, 1990). Salicylic acid is a naturally occurring compound in tobacco plants and the levels of endogenous salicylic acid rise after TMV infection, not only in the infected leaves but also in the uninfected parts of the plant, which show induced resistance (Malamy *et al.*, 1990). In many plant species, resistance can be induced against a range of pathogens by pre-treating the plant with certain chemicals or compounds of biological origin, ranging from inorganic salts to cell wall fractions from fungi. Pre-inoculation with pathogens or non-pathogens stimulate the same signal transduction pathways. Resistance can be local and restricted to the treated tissue or be induced systemically. The induction of resistance usually coincides with the accumulation of pathogenesis related proteins and their transcripts in resistant tissue (Kuc, 1982). Of various chemicals and biological compounds known to induce resistance in different species of plants, only 2,6-dichloroisonicotinic acid (INA) possessed the ability to induce resistance in sugar beet. Nielsen *et al.* (1994b) demonstrated that repeated spraying with low

concentrations of INA during the week prior to challenge inoculation with the fungus induced complete local and systemic resistance.

Eleven acid soluble proteins with apparent molecular masses ranging from 13-82 kD increased in tomato (*Lycopersicon esculentum* Mill.) leaves infected by *Phytophthora infestans* or *Fulvia fulva*. The most prominent changes in the protein pattern were also detectable in the untreated leaves of infected plants indicating systemic effects of the infection. Similar changes in the proteins were induced by moderate irradiations of the leaves with UV-light (254 nm) and by injecting the leaves with indole-3-acetic acid, 2-chloroethyl- phosphoric acid, ethephon, fusicoccin or an elicitor preparation from *Phytophthora megasperma* f.sp. *glycinea*. Acetyl salicylic acid, aspirin, kinetin and abscisic acid did not induce detectable changes in protein pattern nor did they induce resistance. Some of the changes in specific proteins might be useful markers of induced resistance (Christ and Mösinger, 1989).

Biosynthesis of PR-proteins in sugar beet has been intensively investigated both on plants infected by pathogens and treated with synthetic inducers of SAR: salicylic acid and its function derivates 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH). BTH was shown to induce resistance to a number of fungal and viral pathogens, e.g. *Arabidopsis*, wheat, tobacco. Analysis of extracellular fluid isolated from BTH-treated sugar beet leaves revealed the accumulation of acidic and basic proteins displaying both chitinase and β-1,3-glucanase activities indicating the ability of BTH to activate defence reactions in sugar beet (Burketova *et al.*, 2003). However, in contrast there was no increase in accumulation of transcripts encoding three chitinase isozymes (including Ch4) and β-1,3-glucanase in sugar beet leaves following the treatment with INA, compound similar to BTH, even though the INA pretreatment completely inhibited the development of *Cercospora beticola* (Nielsen *et al.*, 1994a). Recently, the BTH capability of inducing SAR to root pathogens was shown on cucumber plants against *Pythium* damping-off as well as to *Phytophthora* root rot (Ali *et al.*, 2000) and even to root-parasitic weed *Orobanche cumana* and root-knot nematodes, suggesting that BTH-induced or potentiated defence mechanism might be of more general character. Similarly, the cross-activity of defence responses against diverse pathogens has been

demonstrated on rhizomania-diseased sugar beet and *Heterodera schachii* root nematodes

Activation of natural weapons before infection, called systemic acquired resistance (SAR) is initiated by pathogens, pathogen- or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding PRs. The association of PRs with SAR, but not with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon, 1997). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the infected plants. The observations indicate that individual PRs are induced to various extents by these different signals. Consequently, the mixture of signals released or produced upon microbial stimulation appears to determine the magnitude of the plant's response and its effectiveness to inhibit further infection. A salicylate-inducible *PR-10* gene subclass from apple, designated *Apa*, was highly activated by wounding and ethephon as well as differentially expressed during infection with a compatible and incompatible race of *Venturia inaequalis* (Poupard *et al.*, 2003).

Induction of PRs has since been found to be invariably linked in necrotizing infections giving rise to SAR, and has been taken as a marker of the induced state (Ward *et al.*, 1991; Uknes *et al.*, 1992; Kessman *et al.*, 1994). This notion has been reinforced by the characterization in *Arabidopsis* of mutants that either are comprised in both the production of PRs and the induction of SAR (*npr1*; Cao *et al.*, 1994, *nim1*; Delaney *et al.*, 1995), or are constitutive expressors of PR genes as well as SAR (*cpr1*; Bowling *et al.*, 1994).

Systemic acquired resistance (SAR) of plants induced upon infection with a necrotizing pathogen, is characterized by an enhanced capacity to induce cellular defence responses to subsequent pathogen attack. Pre-treating the first leaves of cucumber plants with the synthetic SAR activator acibenzolar-S-methyl (*S*-methylbenzol[1,2,3] thiadiazole-7-carbothiate) protects whole plants from infection

with the virulent fungal pathogen *Colletotrichum arbiculare* (Cools and Ishii, 2002). Nakashita *et al.* (2002) elucidated the SAR signaling pathway in tobacco using peobenazole and its active metabolite 1,2-benzisothiazole-1,1-dioxide which induce SAR in *Arabidopsis* through salicylic acid accumulation.

Immunolocalization

Localization of the major, acidic PRs in the intercellular space of the leaf seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. In localization studies *in planta*, labelling for β-1,3-glucanases and chitinases was especially pronounced over fungal cell walls confirming their role in plant defence. Most of the investigations were devoted to leaf tissues. In roots, expression of defence genes was studied on infection by pathogens, arbuscular mycorrhizal fungi, antagonistic fungus *Trichoderma harzianum* or non-pathogenic bacterium *Pseudomonas fluorescens* and differences in the expression of distinct classes of chitinases and β-1,3-glucanases were reported in dependence on the particular microbial inducer. Similar results of localization of PR-3 proteins have been reported in potato leaves infected by *Phytophthora infestans* (Garcia-Garcia *et al.*, 1994). Subcellular localization of PR-1 proteins was studied in roots of resistant *Nicotiana tabacum* cv. *Xanthi* uninfected or infected *in vitro* by the black root rot fungus *Chalara elegans*, using polyclonal or monoclonal antibodies raised against PR-1 protein. In healthy tobacco roots, the PR-1 proteins were found to be present in low amounts in intercellular space material, over cell walls and over secondary thickening of xylem vessels. All these cell compartments were significantly enriched in the PR-1 proteins in infected tobacco root tissues. Their accumulation over the cell walls of inter- and intracellular hyphae of *C. elegans* colonizing tobacco roots may reflect an eventual role of these proteins, in association with other PRs like β-1,3-glucanases and chitinases in directly hindering hyphal growth of the pathogen (Tahiri-Alaoui *et al.*, 1993).

Two antisera raised against acidic β-1,3-glucanase and acidic chitinase from tobacco were used to investigate the subcellular localization of the two enzymes in *Fusarium culmorum*-infected wheat spike by means of the immunogold labelling technique (Kang and Buchenauer, 2002). The studies demonstrated that the

distribution of β-1,3-glucanase and chitinase were localized mainly in the cell walls of different tissues including the lemma, ovary and rachis of the wheat spike, while the cytoplasm and the organelles of cells in these tissues showed almost no labelling. However, the accumulation of β-1,3-glucanase and chitinase in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars.

Immunohistological localization of chitinase and β-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar roots (Burketova *et al.*, 2003) revealed accumulation of plant basic β-1,3-glucanase and basic class IV chitinase cell walls and extracellular spaces. The deposition of the enzymes was predominantly found in endodermis, cortical cells adjacent to endodermis, and xylem vessels. Similar to the pathogens, benzothiadiazole (BTH) induced deposition of both chitinase and β-1,3-glucanase systemically in cortex and endodermis cells.

In *in situ* localization studies of chitinase mRNA and protein in compatible and incompatible interaction triggered by *Phytophthora capsici* in pepper stems Lee *et al.* (2000) showed that induction of chitinase mRNA (*CAChi2*) mRNA started as early as 6 h after inoculation and gradually increased in the incompatible interaction of pepper stems with *P. capsici*. Another interesting finding of the authors was specific immunogold labelling of chitinase on the cell wall of the oomycete as early as 24 h after inoculation. In particular they showed that numerous gold particles were deposited on the cell wall of *P. capsici* with a predominant accumulation over areas showing signs of degradation in the incompatible interaction and that healthy pepper stem tissues were nearly free of labelling.

Transgenics

Plant protection is a major challenge to agriculture worldwide. One of the effective strategies for disease resistance in plants has been the incorporation of disease resistant genes into commercially acceptable cultivars. The most attractive initial candidates for manipulation of the single gene defense mechanisms approach are genes encoding chitinase and β-1,3-glucanase because these two enzymes hydrolyze chitin and β-1,3glucans which are structural components of the cell walls

of several fungi. chitinase gene from *Rhizopus oligosporus* has been shown to operate as an antifungal system in transgenic tobacco. Transgenic cucumber harboring the rice chitinase genes exhibited enhanced resistance against gray mold, *Botrytis cinerea.* While it is clear that it is possible in several cases to alter the expression of chitinase transgenes to generate plants with increased resistance to the pathogen, it is not clear whether constitutively expressed chitinase alone is responsible for the reduction of disease symptoms as observed in the case of tobacco and canola. Introduction of bacterial chitinase gene from *Serratia marcescens* in transgenic tobacco cells showed up to an eightfold increase in amount of chitinase protein in the plants and conferred resistance to *Rhizoctonia solani.* Expression of β-1,3-glucanase in transgenic tobacco plants was shown to result in enhanced resistance to *Alternaria alternata.* Combined expression of PR-2 (β-1,3-glucanase) and PR-3 (chitinase) gives effective protection against fungal infection as they have been shown to act synergistically.

Major interest has been devoted to plant hydrolases, β-1,3-glucanases (E.C.3.2.1.39) (PR-2) and chitinases (EC 3.2.1.14) (PR-3), as they are capable of cleaving fungal cell walls resulting in pathogen growth inhibition (Neuhaus, 1999), and moreover, the products of the hydrolysis can act as elicitors of further defence responses (Boller *et al.*, 1995). Both β-1,3-glucanases and chitinases are highly abundant proteins in plant involved in diverse physiological and developmental processes. They can act either alone or in combination strengthening their antifungal activity. Their accumulation is not restricted only to resistant plants but is often observed in compatible plant-pathogen interactions or even non-pathogenic combination.

Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals *et al.*, 1994). Significant suppression of disease symptoms caused by the soil-borne fungus *Rhizoctonia solani*was demonstrated in tobacco or canola expressing vacuolar class I chitinase from bean (Broglie *et al.*, 1991), the basic tobacco chitinase PR-3c (Vierheilig *et al.*, 1993), tobacco or cucumber PR-8 (Lawton *et al.*, 1993), or the (class II) barley chitinase (Jach *et al.*, 1995), but enhanced chitinase levels caused no significant protection

against *Cercospora nicotianae* (Zhu *et al.*, 1994) or *Fusarium oxysporum* (Van den Elden *et al.*, 1993).

Antifungal activity of chitinases can be synergistically enhanced by β-1,3-glucanses, both *in vitro* and *in vivo*. Thus co-expression of chitinase and glucanase genes in tobacco enhanced resistance against *C. nicotianae* (Jach *et al.*, 1995). In tobacco simultaneous expression of the basic tobacco chitinase PR-3d and glucanase PR-2e afforded substantial protection against *F. oxysporum* f.sp. *lycopersici*, whereas transgenic plants expressing either one of these genes were not protected (Jongedijk *et al.*, 1995). An additional 'SAR gene' in tobacco, SAR 8.2. when expressed constitutively in transgenic tobacco, was also found to reduce disease caused by *P. parasitica*, but the protein has not been characterized (Van Loon, 1997).

Proteins serologically related to the tobacco PR-1 proteins were not only detected in tomato, but in a variety of other plant species as well, including dicotyledonous plants such as cowpea and potato, and monocotyledonous species like maize and barley (Nassuth and Sänger, 1996). Genes encoding PR-1-like proteins were isolated from *Arabidopsis thaliana* (Metzler *et al.*, 1991) and maize (Casacuberta *et al.*, 1991). The maize PR-1-like mRNA is expressed in germinating seeds and further induced by infection with the fungus *Fusarium monoliforme*.

Transgenic plants with chimeric genes containing promoter fragments from a chitinase (class III) gene from *Arabidopsis* fused to the GUS reporter gene were studied by Samac and Shah (1991) and was found that the proximal 192 bp from the transcription start site contains elements necessary for salicylate-inducible expression. Naderi and Berger (1997) have observed the presence of potato virus Y coat protein (PVY-CP) inside chloroplasts of infected tobacco cells. Induction of symptoms reminiscent of virus infection was observed only in transgenic plants accumulating CP in the chloroplast but not in the cytoplasm. These results suggested that PVY-CP plays a role in symptom development. Plants with high levels of CP in their chloroplasts were more susceptible to PVY, suggesting that there was a host response to the presence of PVY-CP in the chloroplasts.

There is strong evidence that expression of β-1,3-glucanase transgenes alone or in combination with chitinase transgenes regulated by the strong CaMV ^{35}S RNA promoter can reduce the susceptibility of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean β-1,3-glucanase can elicitor releasing β-1,3-glucanase or the tobacco class II β-1,3-glucanase PR-2b show reduced symptoms when infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Perenospora tabacina* (Lusso and Kuc, 1996). β-1,3-glucans are the major components of the cell walls of oomycetes, a group of fungi that do not contain chitin. Wubben *et al.* (1996) reported on the tissue-specific expression of genes encoding the hydrolytic enzymes, β-1,3-glucanase and chitinase, as studied by means of *in situ* hybridization. Only temporal differences in gene transcript accumulation were observed for each isoform studied. Expression of the acidic chitinase gene was observed primarily near leaf vascular tissue. Expression of the basic chitinase and basic and acidic β-1,3-glucanase genes was less confined to particular tissues. No preferential accumulation of gene transcripts in tissue near penetrating hyphae was observed in compatible or incompatible interactions. Roulin *et al.*, (1997) studied the expression of β-1,3-glucanase genes in leaves of near-isogenic resistant and susceptible barley lines infected with the leaf scald fungus, *Rhynchosporium secalis*. The difference in expression of β-1,3-glucanase between the resistant backcross lines clearly showed that there are physiologically distinct modes of resistance to the scald fungus in barley, and that at least one of these is accompanied by β-1,3-glucanase induction.

Activation of natural weapons before infection, called systemic acquired resistance (SAR) is initiated by pathogens, pathogen- or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding PRs. The association of PRs with SAR, but not with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon and Van Strien, 1997). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the

infected plants. The observations indicate that individual PRs are induced to various extents by these different signals. Consequently, the mixture of signals released or produced upon microbial stimulation appears to determine the magnitude of the plant's response and its effectiveness to inhibit further infection.

In *Arabidopsis* SA-dependent expression of PR-1, PR-2 and PR-5 is required for increased protection against the biotrophic fungus *Perenospora parasitica*, whereas SA-independent but JA-dependent induction of the plant defensin gene *pdf1·2*, as well as of PR-3 and PR-4, is associated with the induced resistance against the necrotrophic fungi *A. brassicicola* (Pennickx *et al.*, 1996), *Botrytis cinerea* (Thomma *et al.*, 1998) and *Fusarium oxysporium* f.sp. *matthiolae* (Epple *et al.*, 1997; Epple *et al.*, 1998). These results suggest that the SA- and JA-dependent defense pathways in *Arabidopsis* contribute to resistance against distinct microbial pathogens. As a result, PRs and similarly induced antimicrobial proteins appear to contribute differentially to the induced resistance against different pathogens.

Klessig *et al.* (2000) have identified several potential components of the SA signaling pathway, including the members of the TGA/OBF family family of bZIP transcription factors. These bZIP factors physically interact with NPR1 and bind the SA-responsive element in promoters of several defense genes, such as the PR-1 gene. Additionally, they showed that nitric oxide might activate PR-1 expression via an NO-dependent pathway. Barwe *et al.* (2002) demonstrated that zeatin induces the accumulation of β-1,3-glucanase gene transcripts. However, the accumulation of transcription was blocked by protein kinase inhibitor staurosporine, the protein synthesis inhibitor cyclohexamide, and the calcium channel blocker verapamil.

Gene-specific expression studies (Ko *et al.*, 2003) and characterization of a tissue-specific and developmentally regulated β-1,3-glucanase gene family including two newly isolated basic β-1,3-glucanase genes, *PpGns2* and PpGns3, linked in tandem array has in *Prunus persica* revealed that alternate members of members of β-1,3-glucanase genes were expressed at different stages of development. Transgenic tomato plants overexpressing the prosystemin transgene accumulate a soluble 87 kDa cystatin constitutively (Siqueira-Junior *et al.*, 2003). This protein of the PR-6 family

has been demonstrated to have a role in defense response against pests and pathogens. Proteinase inhibitors, have also been identified in rice compatible host-blast pathogen interactions (Agrawal *et al.*, 2002)

Ergon *et al.* (1998) opined that cold hardening in wheat had a conditioning effect on snow mold (*Microdochium nivale*) induced expression of PR-proteins. Gaudet *et al.* (2003) demonstrated that the temporal expression of cold-induced, plant defense-related transcripts in winter wheat is differentially regulated among genotypes and during different developmental stages, and are the first to implicate LTPs in resistance induced at low temperatures during natural acclimation of winter wheat. Two PR-5-like genes were isolated (Campos *et al.*, 2002) from black nightshade (*Solanum nigrum* L. var. *americanum*) genome. Kruger *et al.* (2003) demonstrated that transcription of defense response genes chitinase IIb, PAL and peroxidase is induced by the barley powdery mildew fungus and is only indirectly modulated by R genes.

Despite major advances in the field of plant disease resistance, the precise molecular mechanisms of plant pathogen recognition and the detailed dissection of R-gene mediated signaling networks remain elusive. In the years ahead, new genomic and proteomic technologies will assist in the identification of signaling components and in the investigation of the biochemical functions of R-proteins and other signaling molecules. Beyond gene identification, proteomic approaches will provide insights into the biochemical properties of proteins involved in disease resistance (Martin *et al.*, 2003).

MATERIALS AND METHODS

3.1. Plant Material

Tea, *Camellia sinensis* (L.), belongs to the family Theaceae. The cultivated taxa comprise of three main natural hybrids, which are: *C. sinensis* (L.) O. Kuntze or China type, *C. assamica* (Masters) or Assam type and *C. assamica* sub spp. *lasiocalyx* (Planchon ex Watt.) or Cambod or Southern type (Mondal *et al.*, 2004). Tea is an evergreen, perennial and leaf is the main criterion by which three types of tea are classified, briefly, Assam type has biggest leaves, China type has smallest leaves and Cambod leaves size are in between of Assam and China type.

3.1.1. Collection

Thirty tea varieties were collected from the Tea Germplasm Bank, Department of Botany, University of North Bengal. The tea plants being maintained comprise of varieties released by Darjeeling Tea Research Centre (DTRC), Kurseong, West Bengal; The United Planters' Association of Southern India (UPASI), Valparai, Tamil Nadu and Tocklai Experimental Station, Jorhat, Assam. These (Table 3) were propagated in the nursery.

Table 3.Tea varieties selected from Tea Germplasm Bank for the present study

Source	Varieties
DTRC	AV-2, BS-7A/1/76, CP-1, HV-39, P-1258, RR-17, T-17/1/54, T-135, T-78, TS-449
UPASI	UP-2, UP-3, UP-8, UP-9, UP-17, UP-26, BSS-1, BSS-2, BSS-3
TOCKLAI	TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-29, TV-30

3.1.2. Clonal propagation

The most successful method adopted for vegetative propagation of tea varieties was single-node cutting. Fresh clonal cuttings of the tea varieties were propagated in sleeves containing sandy soil (3:1 of sand and soil) with a pH ranging from 4.5 – 4.8 and were maintained in a green Agro-net house. The tolerable light limit was maintained at 25 % in the early stage and 50 % once rooting progressed as suggested by Banerjee (1993).

Plate 3 (figs. A – H). Tea varieties for experimentation. [A] Tea Germplasm Bank. [C] Cuttings in sleeves. [B, D - H] Pot grown plants.

3.1.3. Maintenance in Glass House

Eighteen month-old tea plants of thirty varieties, as mentioned in Table 3, were transferred to 6 cm and 12 cm diameter plastic pots as well as 24 cm earthen pots containing soil supplemented with 20 % green manure and were maintained in the glass house for experimental purposes (Plate 3, figs. A - H). Optimum day length (11 h), relative humidity (55 - 75 %) and temperature (30 - 35 °C), at which the photosynthetic rate was maximum, were provided to these plants as suggested by Banerjee (1993).

3.2. Callus induction

For callus induction from young internodal stem segments of tea varieties, MS basal medium (Murashige and Skoog, 1962) was used. The medium was supplemented with 3 % sucrose, 0.8 % agar (Difco), 4 mg L^{-1} indole-3-butyric acid (IBA) and 2 mg L^{-1} of 6-benzylaminopurine (BA) (Kato, 1989). The pH was adjusted to 5.8 with 0.1 N HCL or 0.1 N NaOH before autoclaving and then sterilized at 121 °C (15 1b in^{-2}) for 30 min. Fresh young shoots of tea plants of twenty two different varieties *viz.*, Darjeeling: AV-2, BS-7A/1/76, RR-17, T-135, HV-39, T-78, T-17/1/54 and TS-449; UPASI: UP-2, UP-8, UP-9, UP-17, BSS-1, BSS-2, and BSS-3; Tocklai: TV-9, TV-18, TV-20, TV-22, TV-23, TV-25 and TV-30) bearing 3-4 leaves were taken and stem segments (2-3 mm long) were cut and sampled. Before sterilization, explants were agitated with 0.7 % PVP-10 solution for 60 min and washed in running tap water for another 20 min to remove phenolic substances. Stem segments were surface sterilized with 2 % sodium hypochlorite solution for 5 min and washed five times with sterile distilled water for the removal of hypochlorite. After final washing, explants were transferred into MS medium slants. Appearance of friable callus was observed after 2-4 wk of inoculation, which gradually increased in size.

3.2.1. Preparation of cell suspension cultures

Suspension cell cultures of the tea varieties were initiated from fragile callus of internodal sections developed on MS medium (26 °C and photoperiod of 16 h). The friable callus tissue was transferred to liquid MS medium supplemented with the same amounts of the phytohormones (IBA and BA) and vitamins. The suspension cultures (100 ml) were maintained in 250 ml flasks which were covered with foil to suppress

anthocyanin production and incubated at 25 ±3 °C on a rotary shaker (CIS-24, Remi) at 110 r.p.m. For subculture, the cells were sedimented out of the liquid medium at 10 to 15-day intervals to a 30 ml volume and 70 ml of fresh culture medium added. The amount of the cells for inoculation was 5.0 g on the basis of wet weight. Four to six day-old cell suspension cultures were treated with a range of different abiotic and biotic elicitors.

3.3. Fungal material

3.3.1. Causal organism (*Exobasidium vexans*)

Exobasidium vexans causing blister blight of tea is an obligate pathogen. Hence, blister blight diseased tea shoots were always freshly collected from tea gardens for the collection of basidiospores. Taking into consideration the peak season of blister blight incidence, certain tea estates of Darjeeling hills and plains were preferred for collection of such experimental material.

Blister infected leaf samples were collected during July through October mainly from two tea estates of hills, *viz.*, Castleton and Margaret's Hope, and from Hansqua Tea Estate in the plains from mid December to end of February.

3.3.1.1. Collection of blister spores

Basidiospores of *E. vexans* were trapped as illustrated in Plate 4, fig. A. Flush shoots with well-developed sporulating lesions were selected and dipped in conical flasks containing sterile 2 % sucrose solution and incubated in a BOD (Remi) at 25 °C for collection of spores. The leaves with the infected blister zone(s) were placed horizontally (ventral side) over the base of sterile Petri plates (2" dia) and roofed with the lid. After 48 - 72 h of incubation the basidiospores deposited on the surface of the Petri plate underneath the leaf (Plate 4, figs. B & C). The Petri plates containing basidiospores of *E. vexans* (Plate 4, fig. D) were sealed with parafilm, labeled with source details (Table 4) and used for inoculating the plant material.

Plate 4 (figs. A – D). Spore trapping from blister infected twig. [A] Set-up. [B - D] Spore prints, [C & D] enlarged view.

Table 4. Isolates of *Exobasidium vexans* obtained from hills and plains

Organism	Isolate Code	Source
Exobasidium vexans Massee	EV1	Castleton Tea Estate
	EV2	Hansqua Tea Estate
	EV3	Margaret's Hope Tea Estate

3.3.2. Other microorganisms

Virulent fungal cultures of *Curvularia pallescens* (1/L 0411A), *Glomerella cingulata* (Stoneman) Spauld. and Shrenk (IMI no. 356805), *Fusarium oxysporum* Schlecht (2389) and one bacterial isolate *Bacillus megaterium* were obtained from the Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Fungal cultures were maintained by subculturing on PDA medium at 28 °C while *B. megaterium* was subcultured on nutrient agar medium and incubated at 37 °C before use in *in vitro* bioassays.

3.4. Artificial inoculation

3.4.1. Spore suspension

The basidiospores of *E. vexans* settled at the bottom of the Petri dishes were lifted from the surface by gentle brushing. Sterile distilled water (100 - 200 µl) was pipetted out over the area to ease the lifting process. The spores were rubbed off the surfaces and collected in Eppendorf tubes and the spore concentrations were determined by haemocytometer counting.

3.4.2. Inoculation technique

Intact tea plants were artificially inoculated with basidiospores of *E. vexans* following the method of Chakraborty *et al.* (1997). The spore-suspensions prepared in sterile distilled water (1.6×10^5 spores ml^{-1}) were used to inoculate healthy intact tea plants. For the experimentation, the intact healthy potted tea plants of different varieties were brought to the laboratory. The leaves of each plant were thoroughly cleaned with tap water followed by sterile distilled water. Prior to brushing, the spore-suspension was supplemented with a few drops of Tween-20. The spore suspension was brushed (brush no. 00) on either surface of the first, second and third leaves of

each plant. The pots were positioned in a cooling incubator (CIS-24). The individual pots were covered with plastic bags. The open end was tied with string to retain moisture in the enclosed chamber and incubated at 23 ±2 °C. The chamber was frequently sprayed with sterile distilled water to maintain a moisture level of 80- 90 %. The plastic covers were removed after 24 h. The inoculated tea plants kept under such controlled conditions of light and temperature curled within 12 days after inoculation. However, sporulation was not induced under these conditions.

3.4.3. Disease assessment

Disease incidence was assessed by calculating the percentage of infection following the method of Venkata Ram and Chandra Mouli (1984). The plants of all selected varieties were artificially inoculated as above and shoots and leaves observed up to 30 d. The third leaf of tea shoots is considered to be the index leaf for blister blight disease assessment accordingly a representative sample of 50 shoots (per variety) consisting of three leaves and a bud were collected. Percentage infection for different categories of leaf i.e., 1st leaf, 2nd leaf and 3rd leaf from the top bud was determined with the following formula,

$$\text{Percentage leaf infection} = \frac{\text{No. of leaves infected}}{\text{No. of leaves examined}} \text{ X } 100$$

Table 5. Categories for scoring blister blight disease incidence

Leaves	Code	Range (%)
Resistant	R	0-20
Moderately resistant	MR	21-40
Moderately susceptible	MS	41-70
Susceptible	S	71-100

3.5. Abiotic treatment

3.5.1. Preparation of test solutions

Nine selective inducers such as salicylic acid, jasmonic acid, di-potassium hydrogen orthophosphate, mercuric chloride, hexaconazole, calixin, biocrop and phytoextracts of *Azadirachta indica* 'neem' and *Catharanthus roseus* 'periwinkle' were tested as foliar applicants on tea plants grown in earthen pots as well as in the experimental field.

Fresh mature leaves (400g) each of *Azadirachta indica* (neem) and *Catharanthus roseus* (periwinkle) were homogenized in a mixer (Philips HL3294) and diluted with distilled water as suggested by Paul and Sharma (2002). Biocrop, an organic fertilizer and known plant growth inducer obtained from AKS Bio and Herbals Pvt. Ltd., Mumbai, was used as recommended by the manufacturer. Rest of the inducers were mixed in proportions as tabulated below (Table 6).

Table 6. Test solutions applied to tea plants for induction of resistance

Test solutions	Strength	Product source
Salicylic acid	15 mM	Himedia
Jasmonic acid	0.5 µM	Sigma
Di-potassium phosphate	50 mM	Himedia
Mercuric chloride	5 mM	Himedia
Hexaconazole	0.1 %	Rallis India
Calixin	0.1 %	BASF
Biocrop	10 %	AKS Herbals
Azadirachta indica	0.4 %	Phytoextract
Catharanthus roseus	0.4%	Phytoextract

3.5.2. Foliar application

The tea plants were pre-treated with inducers (Table 6) prior to inoculation with *E. vexans*. The solutions were supplemented with a few drops of Tween-20 to ensure adhering. Control plants (untreated) were sprayed with distilled water plus Tween-20. Around 100 ml of the solutions were sprayed using a hand sprayer as to

wet the leaves completely on both the ventral and dorsal surfaces. The treated potted plants were labeled appropriately and allowed to stay in the glass house until required and the field grown treated plants were also earmarked.

3.6. Protein extraction and estimation

3.6.1. Soluble leaf protein

Soluble proteins were extracted from tea leaf tissues as described by Chakraborty *et al.* (1995). Leaf tissues (3 g) were homogenized with 0.05 M sodium phosphate buffer, pH 7.2 (5 ml g^{-1}), containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM PVP-10 (Sigma) and 2 mM polymethyl sulphonyl fluoride (Sigma) in a pre-chilled mortar with a pestle. The brei was centrifuged for 20 min at 4 °C and 10,000 r.p.m. The pellet was discarded and the supernatant was collected and used immediately.

3.6.2. Acid soluble proteins

PR-proteins were extracted from the tea leaf tissues following the procedure adapted from Ye *et al.*, (1990) with slight modifications. The harvested leaves were homogenized in an extraction medium consisting of 0.1 M citrate phosphate buffer, pH 2.8 (3 ml g^{-1} leaf tissue) and 2 mM PVP-10 in a mortar with pestle at 4 °C. The resultant slurry was centrifuged for 20 min at 4 °C and 10,000 r.p.m. The supernatant was decanted and dialysed against two changes of deionized water at 2 - 4 °C for 24 h and then against two changes of 0.05 M sodium acetate buffer, pH 5.2 for 2 h. The dialysate was centrifuged again at 10,000 r.p.m. for 20 min. The resulting supernatant was used for analyses immediately.

3.6.3. Intercellular fluids

The intercellular fluid of the leaves was extracted by the method of De Wit and Spikman (1982) with some modifications. Leaves (10 g) were vacuum infiltrated under cool conditions (5 - 10 °C) for 30 min in a 250 ml beaker filled with distilled water. The leaves were blotted gently on filter paper discs, put in tubes and centrifuged for 15 min at 4 °C and 10,000 r.p.m. The supernatant was pipetted into Eppendorf tubes and used immediately.

3.6.4. Protein estimation

Bradford's (1976) method was adopted, for protein quantification. The reagent contained 0.01 % Coomassie Brilliant Blue G250 (w/v), 4.7 % ethanol (w/v), and 8.5 % phosphoric acid (w/v). Protein dilutions were made with 0.15 M phosphate buffered saline, pH 7.2 and 100 µl of protein sample was pipetted in to test tubes. Five milliliters of protein reagent was added to the test tube and the contents mixed by inversion. The absorbance at 595 nm was measured after 2 min against a reagent blank prepared from 0.1 ml of the dilution buffer and 5 ml of protein reagent. The concentration was determined using BSA as standard. Assays were performed in triplicates and each experiment was repeated three times.

3.7. Extraction and assay of defense enzymes

3.7.1. Chitinase (E.C. 3.2.1.14.)

The assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988) using crustacean chitin (Sigma) as substrate with a few modifications. One gram of the leaf sample was extracted with 3 ml of 0.1 M sodium citrate buffer, pH 5.0 and 2 mM PVP-10 in pre-chilled mortars with pestles. The homogenates were centrifuged for 30 min at 4 °C and 10,000 r.p.m. Supernatants were used as enzyme source. One ml of the enzyme extract was incubated with 1 ml of colloidal chitin (10 mg ml^{-1}) at 37 °C for 1 h on a shaker. The solution was centrifuged for 3 min at room temperature to remove any unreacted colloidal chitin. An aliquot of supernatant (0.3 ml) was added to 30 µl of 1 M potassium phosphate buffer, pH 7.1 and incubated with 20 µl of (w/v) desalted snail gut enzyme Helicase (Sigma) (3 %) (w/v) for 1 h. After 1 h, the pH of the reaction mixture was brought to 8.9 by addition of 70 µl of 1 M sodium borate buffer, pH 9.8. The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled by plunging in ice. After addition of DMAB reagent [10 g 4-dimethylaminobenzaldehyde in 12.5 ml 10 N HCl and 87.5 ml glacial acetic acid], the mixture was incubated for 20 min at 37 °C. Immediately thereafter, absorbance values at 585 nm were measured using a UV-VIS-spectrophotometer (Digispec 200GL). A standard curve of serial dilutions (0.5 – 2.5 mg ml^{-1}) of N-acetyl-D-glucosamine was prepared. The enzyme activity was expressed as mg GlcNAc g^{-1} leaf tissue h^{-1}.

3.7.2. β-1,3-glucanase (E.C. 3.2.3.39.)

β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan *et al.*, 1991) with modifications. One gram of the leaf sample was extracted with 3 ml 0.05 M sodium acetate buffer, pH 5.0 supplemented with 2 mM PVP-10 by grinding in pre-chilled mortars and pestles. The extract was then centrifuged for 30 min at 4 °C and 10,000 r.p.m. and the supernatant was used as crude enzyme extract. To 62.5 µl of the crude enzyme extract, 62.5 µl of laminarin (4 %) was added and then incubated for 10 min at 40 °C. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent (1 g of DNSA dissolved in 200 mg crystalline phenol in 1 % NaOH and 0.05 g sodium sulphite) and heated for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of water, vortexed and absorbance at 500 nm was determined in a UV-VIS-spectrophotometer (Digispec 200 GL). The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. A standard curve of glucose (50 - 250 µg ml^{-1}) was prepared. The enzyme activity was expressed as µg Glucose g^{-1} leaf tissue min^{-1}.

3.7.3. Peroxidase (E.C. 1.11.1.7.)

Peroxidase was assayed following the method outlined by Chakraborty *et al.* (1993) with a few modifications. One gram of leaf sample was extracted in 0.1 M sodium phosphate buffer, pH 8.8 (5ml g^{-1}) containing 2 mM β-mercaptoethanol and 2 mM PVP-10. The homogenate was immediately centrifuged for 20 min at 4 °C and 10,000 r.p.m. For the assay, 100 µl of the supernatant, 1 ml of 0.2 M sodium borate assay buffer, pH 5.4, 100 µl of 4 mM H_2O_2, 100 µl of o-dianisidine (5 mg ml^{-1} methanol) and 1.7 ml of double distilled water were mixed and the activity was measured at 460 nm in a UV-VIS-spectrophotometer at 60 s intervals. The activity was expressed as Δ A460 nm g^{-1} leaf tissue min^{-1}.

For peroxidase isozyme analysis tea leaf samples were crushed in 2 mM sodium phosphate buffer, pH 7.0 on ice as described by Davis (1964) and used for native polyacrylamide gel electrophoresis.

3.7.4. Phenylalanine ammonia lyase (E.C. 4.3.1.5.)

Phenylalanine ammonia lyase was extracted from tea leaf tissues following the method of Chakraborty *et al.*, (1993). One gram of leaf tissue was crushed in a mortar at 4 °C in 5 ml of 0.1 M sodium borate buffer, pH 8.8 containing 2 mM β-mercaptoethanol in ice. The slurry was centrifuged for 20 min at 4 °C and 10,000 r.p.m.. The supernatant was collected and after recording its volume, was used immediately for assay. The enzyme was assayed as described by Moerschbacher *et al.*, (1986) with a few modifications. A 500 µl sample of crude extract was allowed to react with 600 µl of borate buffer containing 6 µmol L^{-1} L-phenylalanine, at 40 °C for 1 h. A blank was prepared without L-phenylalanine. The reaction was stopped by addition of 6 mol L^{-1} HCl and 1 ml chloroform was added to each tube by vortexing and centrifuged at for 10 min at 10,000 r.p.m.. From the lower organic phase 0.5 ml aliquots were withdrawn and chloroform was allowed to evaporate, the residue being redissolved in 1 ml borate buffer. The absorbance of the samples was read at 290 nm and expressed as µg Cinnamic acid g^{-1} fresh tissue min^{-1}.

3.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For protein profiling sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). The polyacrylamide gels of the proteins were evaluated using the Electrophoretic Documentation and Analysis System (Kodak EDAS 290). To perform the experiment the following stock solutions were prepared,

3.8.1. Preparation of stock solutions

Solution A: Acrylamide solution

A stock solution containing 29 % acrylamide and 1 % N,N'-methylene-bis-acrylamide was prepared in luke warm double distilled water. As both compounds undergo deamination to acrylic and bisacrylic acid, respectively, by alkali and light, the pH of the solution was kept below 7.0. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution B: Sodium dodecyl sulphate (SDS)

A 10 % stock solution of SDS was prepared in luke warm double distilled water and stored at room temperature.

Solution C: Tris buffer

(i) Resolving buffer

1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4 °C for further use.

(ii) Stacking and loading buffer

1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of was adjusted to 6.8 with concentrated HCl and stored at 4 °C.

Solution D: Ammonium persulphate (APS)

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

Solution E: Tris - glycine electrophoresis buffer (reservoir buffer)

The reservoir or tank buffer consisted of 25 mM Tris base, 250 mM glycine, pH 8.3 and 0.1 % SDS. A 1x solution was made by dissolving 3.02 g Tris base, 18.8 g glycine and 10 ml of 10 % SDS in 1 L of double distilled water.

Solution F: SDS-gel loading buffer

This buffer contained 50 mM Tris-HCL, pH 6.8, 10 mM β–mercaptoethanol, 2 % SDS, 0.1 % bromophenol blue and 10 % glycerol. A 1x solution was made by dissolving 10 mg bromophenol blue in 0.5 ml of 1 M Tris buffer, pH 6.8, 0.5 ml of 14.4 M β–mercaptoethanol, 2 ml of 10 % SDS, 1 ml glycerol and 6.8 ml of distilled water.

3.8.2. Preparation of gel

Slab gels (8 cm x 5 cm) were prepared for the analysis of protein patterns by SDS-PAGE. For the preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with high vacuum

grease. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. Resolving and stacking gels were prepared by mixing compounds in the order mentioned as follows,

Composition	10 % Resolving gel (ml)	5 % Stacking gel (ml)
Double distilled water	2.85	2.10
30% Acrylamide	2.55	0.50
Tris buffer *	1.95	0.38
10 % SDS	0.075	0.030
10 % APS	0.075	0.030
TEMED **	0.003	0.003

* For 1.5 M Tris pH 8.8 in resolving gel and 1 M Tris pH 6.8 in stacking gel;

* * N, N, N', N' -Tetramethyl ethylene diamine

The mixture was poured in to the set-up by a pasture pipette leaving sufficient space for the well-comb (comb + 1 cm). After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 1 h. After polymerization of the resolving gel was complete the water overlayer was poured off and the gel was washed with water to remove any unpolymerized acrylamide. The stacking gel solution was poured over the resolving gel followed by immediate insertion of the comb and overlayering with water. The gel was kept for 30 min. After polymerization of the stacking gel, the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in to the electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

3.8.3. Sample preparation

Prior to loading, the sample proteins (35 µl) were mixed with 1x SDS-gel loading buffer (15 µl) in a cyclomixer. All the samples were floated on a boiling water bath for 3 min to denature the proteins. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microlitre syringe. Along

with the samples, protein markers consisting of a mixture of thirteen proteins of known molecular weight (myosin – 205, β-galactosidase 116, phosphorylase b – 97, fructose-6-phosphate kinase 84, albumin – 66, glutamic dehydrogenase – 55, ovalbumin – 45, glyceraldehyde 3-phosphate dehydrogenase – 36, carbonic anhydrase 29, trypsinogen – 24, trypsin inhibitor 20, α-lactalbumin – 14.2 and aprotinin – 6.5 kDa) was similarly treated as the other samples and loaded in a separate well.

3.8.4. Electrophoresis

Electrophoresis was performed at constant 18 mA current (PowerPac 1000, Bio-Rad) for a period of approximately 3 h for mini gel until the dye front reached the bottom of the gel.

3.8.5. Fixing

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

3.8.6. Staining and destaining

The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue R250 (Sigma) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman no.1 filter paper. The gel was removed from the fixer and stained in the staining solution for 4 h at 37 °C with constant shaking at a very low speed.

After staining, the gel was finally destained in methanol, water and acetic acid (9:9:2) at 37 °C with constant shaking until the background became clear.

3.9. Native polyacrylamide gel electrophoresis (PAGE)

For isozyme analysis, polyacrylamide gel electrophoresis under non-denaturing conditions was performed following the protocol of Davis (1964). The stock solutions were prepared as follows,

3.9.1. Preparation of stock solutions

Solution A: Acrylamide (for resolving gel)

For the preparation of acrylamide stock solution 'A' used for the resolving gel, 28 g acrylamide and 0.74 g N, N'- methylene-bis-acrylamide were dissolved in 100 ml of luke warm double distilled water. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution B: Acrylamide (for stacking gel)

Similarly, for the preparation of acrylamide stock solution 'B' used for the stacking gel, 10 g acrylamide and 2.5 g N, N'- methylene-bis-acrylamide were dissolved in 100 ml of luke warm double distilled water. The stock solution was filtered through Whatman no.1 filter paper and stored in an amber bottle, at 4 °C.

Solution C: Tris-HCl (for resolving gel)

Tris base (36.6 g) was dissolved in double distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with concentrated HCl. The final volume of the solution was made up to 100 with double distilled water and was stored at 4 °C for further use.

Solution D: Tris-HCl (for stacking gel)

Tris base (5.98 g) was dissolved in double distilled water and 0.46 ml of TEMED was added. The pH was adjusted to 6.7 with concentrated HCl. The final volume of the solution was made up to 100 with double distilled water and was stored at 4 °C for further use.

Solution E: Ammonium persulphate solution

A fresh solution of ammonium persulphate was prepared by dissolving 0.015 g of it in 10 ml of double distilled water.

Solution F: Riboflavin solution

A fresh solution was prepared by dissolving 0.004 g of riboflavin in 10 ml of double distilled water. The solution was stored in an amber bottle to protect it from light.

Solution G: Electrode buffer (for reservoir)

The reservoir buffer was freshly prepared by dissolving 0.6 g Tris base and 2.9 g glycine in 1 L of chilled double distilled water.

3.9.2. Preparation of gel

A mini slab gel (8 cm x 5 cm) was prepared for isozyme analysis by PAGE. For the preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with high vacuum grease. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 7.5 % resolving gel was prepared by mixing the solutions - A, C, E and double distilled water in the ratio, 1:1:4:1 by a pasture pipette leaving sufficient space for the stacking gel (for comb + 1cm). After pouring the resolving gel solution, it was immediately overlayered with water and allowed to polymerize for 2 h.

After polymerization of the resolving gel was complete the water overlayer was poured off and the gel was washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions - B, D, F and double distilled in the ratio, 2:1:1:4 poured over the resolving gel followed by immediate insertion of the comb and overlayering with water. The gel was kept for 30 min in strong sunlight. After polymerization of the stacking gel, the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in to the electrophoresis apparatus. Chilled tris-glycine running buffer was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

3.9.3. Sample preparation

During sample preparation, ice-cold conditions were maintained throughout the entire process. The samples were prepared by mixing the enzyme extracts (40 µl) and gel loading dye (25 µl) in a cyclomixer. The loading dye consisted of 40 % sucrose and 1 % bromophenol blue in double distilled water. The samples (52 µl) were immediately loaded in a pre-determined order in to the bottom of the wells with a microlitre syringe.

3.9.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current (PowerPac 1000, Bio-Rad) at 4 °C for a period of approximately 4 h for mini gel until the dye front reached the bottom of the gel.

3.9.5. Fixing and staining

After electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally stained. The gel was incubated in an aqueous solution of benzidine (2.08 g), acetic acid (18 ml), 3 % H_2O_2 (100 ml) for five min. The reaction was stopped with 7 % acetic acid after the appearance of clear blue colored bands. Analysis of isozymes was done immediately.

3.10. Preparation of antigens

Plant antigens of healthy, naturally blister infected, and artificially inoculated (with *E. vexans*) tea leaves as well as *Exobasidium vexans* were prepared as suggested by Chakraborty and Saha (1994a).

3.10.1. Leaf antigen

Leaf antigens were prepared from healthy, blister infected, and artificially inoculated tea leaves 0.05 M sodium phosphate buffer (pH 7.2). For the preparation of naturally-infected antigen only the infected areas (blister spots) were cut and taken. For artificially inoculated, the entire inoculated leaf with even a single translucent spot was considered. Antigens of *L. leucocephala*, *O. sativa* and *G. max* (non-hosts) were prepared in a similar manner.

3.10.2. Spore antigen

Antigens were prepared by sonicating basidiospores of *E. vexans* suspended in 0.05 M sodium phosphate buffer (pH 7.2), kept at –20 °C for 4 h, freeze-thawed followed by sonication (Sonics Vibra Cell) at 35 % amplitude for 9 min. Following sonication, the spores were further freeze-thawed and homogenized for 15 min at 4 °C. The suspension was centrifuged at 10,000 r.p.m. for 30 min. The supernatant was stored in 2 ml vials at -20°C and used for immunization process. Antigens from conidia of *F. oxysporum* (non-pathogen) were prepared in a similar manner.

3.10.3. Antimicrobial protein

3.10.3.1. Protein purification

Crude preparations of chitinases and β-1,3-glucanases of healthy tea leaves (500 g) were subjected to gel filtration using a Sephadex G-50 column (Pharmacia) according to the method described by Joosten *et al.* (1995). Fourteen fractions of 15 ml were collected, dialysed against deionized water, freeze-dried and dissolved in 1 ml of deionized water. The fractions were filter-sterilized (Millipore, 0.2 μm) and the filter was flushed with 1 ml of sterile water, resulting in a final volume of 2 ml per fraction. The fractions were analysed for their protein content and chitinase and β-1,3-glucanase activity.

The purified fractions of chitinase and β-1,3-glucanase were further used for polyclonal antibody production. Besides, *in vitro* antifungal assays were also performed with the purified extracts of different combinations of treatments following the same method of purification.

3.11. Polyclonal antibody production

3.11.1. Rabbits and maintenance

Polyclonal antibodies (PAb) were raised against fungal antigens, plant antigens and defense enzymes. Long-term immunization was performed on white male New Zealand rabbits of approximately 2 kg body weight (Chakraborty and Purkayastha, 1983) being maintained under hygienic conditions in the animal house - Antisera Reserves for Plant Pathogens, Immuno-Phytopathology Laboratory, Department of Botany, N. B. U. The rabbits were fed green grass, soaked *Cicer* seeds, carrots and lettuce (during season) in the mornings and evenings and given saline water after each bleeding for three consecutive days.

3.11.2. Immunization

Immunization was performed by the macro-technique in which 250 - 500 μl of antigen solution, of optimum concentration, was injected subcutaneously into the rabbits. Prior to immunization, pre-immune (normal) antisera were collected from the rabbits. For raising PAbs, the rabbits received six injections, for six consecutive weeks, of antigen emulsified with an equal amount of Freund's complete adjuvant

(Difco) seven days after the pre-immunization bleeding followed by Freund's incomplete adjuvant (Difco) at seven-day intervals up to 10 - 14 consecutive weeks as required.

3.11.3. Bleeding

Bleeding was performed by marginal ear vein puncture. For easy handling of the rabbits during blood collection, a simple apparatus consisting of a wooden board (100 x 30 x 1cm) with a triangular gap cut from top, fixed at a 60° position to a vertical plate equipped with four screws at the top and bottom of the board was used. The board was covered by a plastic and the rabbit placed on its back with the neck in the triangular gap and the head below the board with the legs tied to the screws, thus fixing the body. When placed as described the rabbit could be bled at ease as it could not move and thus was not harmed. The upper side of the ear was disinfected with rectified spirit and shaved. After irritation of the ear with xylene, the marginal vein of the ear was punctured by a sterile razor blade. The first bleeding was taken six weeks after the first injection and three times more every fortnight. The blood samples (5 - 15 ml) were collected in sterile graduated glass tubes (Borosil). After collection, all precautionary steps were taken to stop the blood flow from the puncture. Any sort of ear infection was avoided, however in case of occurrence, it was cured by dermal application of 10 % (w/v) methylene blue.

3.11.4. Preparation of serum from whole blood

The blood samples were kept for 1 h at 30 °C. The clots were loosened and stored at 4 °C. The antisera were then clarified by centrifugation and stored at –20 °C until required.

3.11.5. Purification of IgG

IgGs were purified by affinity chromatography on a DEAE-cellulose column following the protocol of Clausen, 1988.

3.11.5.1 Precipitation

The crude antiserum (2 ml) was diluted with two volumes of distilled water and to it an equal volume of 4.0 M ammonium sulphate was added. The pH was adjusted to 6.8 and the mixture stirred for 16 h at 22 °C. The precipitate thus formed

was collected by centrifugation for 1 h at 22 °C and 10,000 r.p.m. Then the resultant pellet was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.11.5.2. Column preparation

Four grams of DEAE-cellulose (Sigma) was suspended in double distilled water overnight. The water was drained off and the gel was suspended in 0.005 M sodium phosphate buffer, pH 8.0. Washing in the same buffer was repeated 5 times. The gel was then suspended in 0.02 M sodium phosphate buffer, pH 8.0 and was applied to a column (2.6 cm dia and 30 cm high) and allowed to settle for 2 h. After the column material had settled, 25 ml of 0.02 M sodium phosphate buffer, pH 8.0 buffer was run through the gel material to give it a final wash.

3.11.5.3. Fraction collection

Two milliliters of the ammonium sulphate precipitated IgG was applied at the top of the column and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.3 M. The initial elution buffer was (1) 0.02 M sodium phosphate buffer, pH 8.0 and the final elution buffer was (2) 0.3 M sodium phosphate buffer, pH 8.0. The buffers were applied to the column through flasks connected via rubber tubes. The flask containing buffer-(2) was open at one end and connected to the flask containing buffer-(1) at the other. During draining of buffer-(1) in to the column, buffer-(2) was sucked into buffer-(1) thereby producing a continuous rise in molarity. Finally, 40 x 5 ml fractions were collected and the optical density values were recorded at 280 nm by means of UV-Spectrophotometer. The fractions showing >2 reading were pooled and stored as purified IgG after estimation of its concentration.

3.11.5.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1981). Absorbance value was taken for selected fractions at 280 nm and 260 nm. The IgG concentration was calculated by the following formula,

Protein concentration (mg/ ml) $= 1.55 \times A_{280} - 0.76 \times A_{260}$

3.11.5.5. Storage of IgG

Serum samples were obtained at regular intervals as per schedule and stored at –20 °C. To eliminate background binding and to restore the specificity of the antisera lost during storage they were adsorbed with kaolin (Shillitoe, 1982). To 0.1 ml of antiserum (= 3 mg protein) was added 0.8 ml of 0.15 M phosphate buffered saline, pH 7.2 (PBS) with 4 % B.S.A. and 1 ml of a 25 % suspension (w/v) of kaolin (hydrated aluminum silicate, particle size 0.1 – 4.0 μm, Sigma) in PBS. The mixture was allowed to stand at room temperature for 20 min with regular mixing. The kaolin was then removed by centrifugation at 10,000 r.p.m. for 10 min and the supernatant diluted to 3 ml with PBS to give a final antibody concentration of 1 mg ml^{-1}. Besides this, serum fractions were diluted with 1 vol of glycerol, and 0.01 % sodium azide was added before storage at –10 °C.

3.12. Immunodiffusion

The agar gel double diffusion method developed by Ouchterlony (1967) was adopted to test the effectiveness of the raised antibodies.

3.12.1. Agarose gel plates

The gel plates were prepared by coating square glass slides (6 cm x 6 cm x 4 mm) with agarose. The glass plates were initially degreased with 90 % (v/v) ethanol and diethyl ether (1:1) and dried in a hot air oven. After drying the plates were sterilized in an autoclave at 121 °C (15 lb in^{-2}) for 20 min in Petri plates. For preparation of agarose gel, 0.9 % agarose was added to warm 0.05 M Tris-barbiturate buffer, pH 8.6 contained in a flask and boiled in a hot water bath. The flask was repeatedly shaken until a clear solution was obtained. Prior to plating 0.1 % (w/v) sodium azide was added to the stock solution. All sterilized materials were exposed to UV-light for 10 min in a laminar airflow bench. The hot molten agarose was then pipetted (10 ml per slide) out on to the sterilized glass slides and allowed to solidify. Following this seven wells were cut out with a sterilized cork borer (6 mm dia). The usual set-up was the radial well type in which antibody wells were arranged circularly around a central antigen well with the holes about 1.5 cm from each other.

3.12.2. Diffusion

The antigen and undiluted antisera (60 µl well^{-1}) were pipetted directly in to the wells in a pre-determined manner. All steps were carried out in a laminar chamber. The antigen and antiserum was allowed to diffuse in a moist chamber for 72 h at 25 ±2°C (BOD, Remi). Precipitation lines were observed in cases where common antigens were present.

3.12.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9 % NaCl and 0.1 % NaN_3) for 72 h with 6 h changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. The slides were then stained with Coomassie blue R 250 (250 mg of Coomassie brilliant blue in 45 ml of methanol, 45 ml of water and 10 ml of glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol, water and acetic acid in the ratio 9:9:2) with changes until the background was clear. Finally the slides were washed with distilled water and dried in a hot air oven for 3 h at 50 °C.

3.13. Plate trapped antigen - enzyme linked immunosorbent assay (PTA-ELISA)

The plate trapped antigen form of ELISA (PTA-ELISA) was performed essentially as described by Chakraborty *et al.* (1995a). Flat bottom 96-well polystyrene microtitre plates (Nunc) were used for the assays. Test samples (antigen extracts) were diluted in coating buffer [50 mM carbonate buffer, pH 9.6 (a) sodium carbonate – 5.2 g in 1 L double distilled water, (b) sodium hydrogen carbonate – 4.2 g in 1 L double distilled water; 160 ml of (a) was mixed with 360 ml of (b) and the pH was adjusted 9.6] and added in to each of three wells (200 µl well^{-1}) and then incubated at 25 °C for 4 h. The wells were then emptied and washed three times by flooding wells with 0.15 M phosphate buffered saline, pH 7.2 [PBS, washing buffer - (a) sodium dihydrogen phosphate - 23.4 g in 1 L double distilled water, (b) disodium hydrogen phosphate – 21.3 g in 1 L double distilled water; 280 ml of (a) was mixed with 720 ml of (b) and the pH was adjusted 7.2. To the final mixture 0.8 % NaCl and 0.02 % KCl were added] containing 0.05 % Tween-20 (PBS-T). After each washing the plates were shaken dry.

Subsequently, 200 µl of blocking reagent [0.05 M Tris buffered saline, pH 8.0 (TBS): Tris - 0.657 g, NaCl- 0.81 g, KCl - 0.223 g, 0.05 % Tween-20 and 1 % bovine serum albumin (BSA) in 100 ml double distilled water] was added to each well for blocking the unbound sites to eliminate background binding and the plates were incubated at 25 °C for 1 h. After incubation, the plate was washed as mentioned earlier. Purified IgG was serially diluted in antisera dilution buffer (PBS-T) containing 2 % soluble PVPP, 0.2 % BSA and 0.03 % NaN_3, and added to the wells, the plates were placed at 4 °C overnight (16 h). After a further washing, anti-rabbit IgG goat (whole molecule) labelled with alkaline phosphatase diluted 10,000 times in PBS, was added to each well (200 µl $well^{-1}$) and incubated at 37 °C for 2 h. The plate was washed, dried and each well was loaded with 200 µl of the enzyme substrate p-nitrophenyl phosphate (10 mg ml^{-1}) in alkaline phosphatase buffer [0.05 M Tris-HCl buffer, pH 9.8] containing 1 % diethanolamine and kept in the dark for 60 min. The reaction was stopped by adding 50 µl per well of 3 N NaOH solution. The rate of color development was monitored on a Multiskan EX (Thermo Electron) ELISA reader interfaced with a Windows 98 computer and the rates of the reaction were recorded at A405 nm. Optical absorbance values in wells not coated with antigens were considered as blanks. Triplicate wells were used for each sample and the mean absorbance at 405 nm was calculated after subtracting the absorbance given by the substrate wells not coated with antigens. The experiments were repeated three times.

3.14. Dot immunobinding assay (DIBA)

The dot immunobinding technique has been found to be a rapid and sensitive method of detection. The method used is a modification of the method described by Lange *et al.*, 1989.

3.14.1. Immunoblotting

Pre-cut (Pall Gelman, 0.2 µm) or custom-cut (Bio-Rad, 0.45 µm) nitrocellulose membranes (8 cm x 12 cm for 96 welled-template) were carefully placed in the template of a Bio-Dot apparatus (Bio-Rad) with the help of flat forceps (Millipore). Hand gloves were always worn while carrying out all procedures related to nitrocellulose membranes (NC). Prior to placement of the nitrocellulose in the

template it was pre-soaked in antigen coating buffer (50 mM carbonate buffer, pH 9.6) and air-dried for 30 min. Crude protein samples (antigen) were pipetted into each well of the apparatus at the rate of 100 µl per well and allowed to filter for 1 h. Following a single wash with TBS-Tween-20 (200 µl per well under vacuum) the membrane was removed and immunoblotted.

3.14.2. Immunoprobing

The sheets were incubated in the blocking solution 10 % (w/v) skimmed milk powder (casein hydrolysate, SRL) in 0.05 M Tris-HCl, pH 10.3 containing 0.5 M NaCl, 0.5 % Tween-20 (TBS-T) for 2 h on an orbital shaker (CIS-24, Remi) at 37 °C and 90 r.p.m. Antiserum of desired dilution was then directly added to the blocking solution for either 3 h at 37 °C and 90 r.p.m or overnight at 4 °C. After incubation, the solution was drained and the sheets were washed gently with running tap water for 3 min followed by 3 x 250 ml 5 min washes in TBS-T (10 mM Tris buffer, pH 7.4 - with 0.9 % NaCl and 0.05 % Tween-20) (Wakeham and White, 1996). The membranes were then incubated in alkaline phosphatase conjugate goat anti-rabbit IgG (Sigma) diluted in alkaline phosphatase buffer (0.05 M Tris-HCl buffer, pH 9.8) reaching a final concentration of 1:10,000. Conditions for incubation and washing were as described for the primary antiserum. Finally, the membrane was immersed in NBT-BCIP substrate solution [5 mg Nitroblue-tetrazolium chloride (NBT, Himedia) in 100 µl of 70 % N, N, - dimethyl formamide (DMF, Sigma) and 2.5 mg 1-bromo,2-chloro,3-indolyl phosphate (BCIP, Himedia) in 50 µl of 100 % DMF were prepared freshly and added to 10ml alkaline phosphatase buffer (0.05 M Tris-HCl buffer, pH 9.8) Or, 1 tablet of NBT-BCIP (Sigma) was dissolved in 10 ml of DDW] prepared for staining and the color development was noted. On appearance of the desired color intensity, the staining reaction was terminated by washing the membranes in distilled water. The dot formed on the membrane was scored by eye.

3.15. Western blot

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed according to the method devised by Towbin *et al.*, 1979. Following this procedure a replica of the original band pattern was obtained on nitrocellulose sheets from sodium dodecyl sulphate gels. The immobilized proteins

were further analyzed by modified immunological procedures (Wakeham and White, 1996). The proteins were clearly detectable as violet colored bands on nitrocellulose.

3.15.1. Extraction and estimation of proteins

Protein extraction and estimation was performed by Bradford's (1976) method as described in section 3.6.

3.15.2. SDS-PAGE of proteins

The different protein preparations were separately mixed with sample buffer subjected to electrophoresis in the presence of sodium dodecyl sulphate in one-dimensional slab gels (Laemmli, 1970). For each trial, two gels loaded with identical protein samples were run out of which one gel was stained with Coomassie Brilliant Blue R250 and the other electrotransferred.

3.15.3. Electrophoretic blotting procedure

On completion of gel run the protein SDS-gels were removed from the glass slabs and immediately immersed in pre-chilled Towbin/ transfer buffer [25 mM Tris, with 192 mM Glycine and 20 % Methanol (AR), pH 8.3] and equilibrated for 1 h. In a separate container a pre-cut or custom-cut nitrocellulose membrane (Bio-Rad, 0.45 μm) along with two pieces of blotting filters (Bio-Rad, 2 mm thickness) of the size of the SDS-gel, were soaked in Towbin buffer for 30 min. The electrotransfer process was carried out in a Mini-Protean II SD Trans-blot unit (Bio-Rad). A pre-soaked filter paper was placed on the platinum anode of the blotting unit, followed by pre-wetted nitrocellulose, equilibrated SDS-gel and then another filter paper. The gel sandwich was very gently rolled over with a glass rod to remove any trapped air bubbles. The cathode was carefully placed over the gel-sandwich and pressed to engage the latches with the guideposts without disturbing the filter stack. The unit was set at a constant volt (15 V) for 45 min. After the transfer the membrane was removed, clipped to a filter paper (Whatman No. 1) and allowed to air dry for at least an hour.

3.15.4. Immunoprobing

Following drying, the membrane was blocked in 5 % (w/v) skimmed milk powder (casein hydrolysate, SRL) in 0.15 M, phosphate-buffered saline, pH 7.2 (PBS) supplemented with 0.5 % Tween-20 in a heat sealable bag and incubated for 2 h on an

orbital shaker (CIS-24, Remi) at 37 °C and 90 r.p.m. Subsequently, the membrane was incubated with antiserum [1:1 (v/v) blocking solution : PBS (containing IgG, diluted as per requirement)]. The bag was re-sealed leaving space for a few air bubbles and incubated at 4 °C. Next, the membrane was washed 3 x 250 ml PBS followed by a single wash in 50 mM Tris-HCL, pH 7.5 containing 50 mM NaCl (TBS) for 20 min with gentle agitation to assure removal of azide and phosphates from the membrane prior to the enzyme coupled reaction step. The enzyme, alkaline phosphatase tagged with anti-rabbit goat IgG diluted 1:10,000 in alkaline phosphatase buffer [0.05 M Tris-HCl buffer, pH 9.8] and incubated for 90 min at room temperature. Finally the membrane was washed 5 x 200 ml TBS and transferred to a bag containing 10 ml of NBT-BCIP substrate. The reaction was monitored carefully. On visualization of the bands up to the desired intensity the membrane was transferred to a tray flooded with stop solution.

3.16. Immunocytochemical staining

Immunocytochemical staining of cross sections of tea leaves was done using a non-fluorescent stain based immunoenzymatic format following the method of Young and Andrews (1990) with a few modifications. Fresh, naturally blister-infected, healthy and artificially inoculated (TV-30, T-78 and UP-2) tea leaves were used as the experimental material. Cross sections were cut through the leaves and incubated with PBS containing 1 % BSA and 2 mM PVP-10 for 20 min at room temperature to prevent non-specific binding of antibodies. The tissues were then reacted with PAb of *E. vexans* (PAb-EV1) diluted 1:10 in PBS with 1 % BSA (PBS-BSA) at 37 °C for 2 h on a rotary shaker. Following incubation the sections were washed with three 5 min-changes of PBS-T and incubated in a 1:10,000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in PBS-BSA for 2 h at 37 °C on a rotary shaker and again washed with PBS-T as described above. Immunocytochemical staining with the Fast blue BB salt substrate [1 mg Fast blue BB salt (Sigma) and 5 µl of 0.1 M $MgCl_2$ added per ml in a stock solution of - 0.15 g napthol-AS-MX-phosphate (Sigma) dissolved in 2.5 ml of N,N-dimethyl formamide (Sigma) added to 17 g of Tris base (Sigma), pH 9.1, in 500 ml of double distilled water] was carried out in the dark. The substrate solution was filtered through Whatman no. 1 immediately before application to the cross sections. Incubation in the stain was for no longer than

40 min followed by rinsing in PBS-T, as above. The sections were mounted in 10 % glycerol and observed under bright field illumination using a Biomed microscope (Leitz) and photographs were taken by Leica Wild MPS 48 camera on Kodak 200 ASA film.

3.17. Immunofluorescence

Indirect fluorescence staining of cross sections of tea leaves and basidiospores of *E. vexans* was performed following the method of Chakraborty and Saha (1994a) with polyconal antibodies labelled with anti-rabbit goat IgG conjugated with FITC and observed under UV light in a microscope (Leitz) equipped with I3 filter block ideal for FITC fluorescence. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

3.17.1. Cross sections of leaves

Cross sections were cut through tea leaves and immediately immersed in 0.15 M, phosphate-buffered saline, pH 7.2 (PBS) supplemented with 0.5% Tween-20, 0.01% PVP-10 and 1 % BSA. Good sections were selected, washed in PBS, treated with polyconal antibodies (1:10) and incubated for 1 h at 24 °C in grooved slides. After incubation, sections were washed thrice with PBS followed by treatment with 50 µl of FITC-conjugate (1:40). The sections were incubated for 30 min at 24 °C, washed and mounted in 10% glycerol on grease free slides. The slides were observed and photographed.

3.17.2. Basidiospores of *E. vexans*

Basidiospores, collected from fresh naturally blister-infected tea leaves were suspended in 100µl PBS and centrifuged, with three changes (of PBS), at 1000 r.p.m. for 1 min each. The PBS supernatant was discarded and to it antiserum (1:10) was added. The spores were incubated for 1 h at 37 °C. After incubation, the samples were again centrifuged & washed thrice for 1 min at 1000 r.p.m. The supernatant was discarded and the spores were incubated in 50 µl of FITC-conjugate diluted with PBS (1:40) in the dark at 37 °C for 30 min. After incubation, FITC was removed by repeated washings with PBS and the spores were mounted in 10% glycerol on grease free slides and observed and photographed.

3.17.3. Binding of FITC labelled concanavalin A

The method as described by Keen and Legrand (1980) was followed for binding of fluorescein labelled concanavalin A to basidiospores of *E. vexans*. Initially the basidiospores were incubated for 20 min in 0.85 % NaCl in 0.1 M potassium phosphate, pH 7.4 containing 1 mg ml^{-1} fluorescein isothiocyanate (FITC) labelled concanavalin A (Sigma). The spores were then washed thrice with a saline solution by repeated low speed centrifugation and re-suspension. Finally these were viewed under the microscope and photographs were taken.

3.18. Bioassay for antimicrobial activity of proteins

3.18.1. Spore germination bioassay

The effects of purified chitinases and β-1,3-glucanases on spore germination of *E. vexans* was assayed. Basidiospores were freshly collected from blister blight naturally infected leaves (as described earlier). The spores were suspended at a density of 1.6 x 10^5 spores ml^{-1} in sterile water. Glass micro slides were degreased with alcohol and dried in a hot air oven (80 °C, 30 min). The effect on spore germination *in vitro* was determined using microscopic cavity slides. A droplet (20 µl) of the spore suspension was placed centrally on each slide. The enzyme solutions (40 µl) of β-1,3-glucanase and chitinase at various concentrations were added. The microscopic slide was put into a Petri plate with approximately 100% humidity and incubated for 16-20 h in an incubator at 25 ±2 °C BOD (Remi) in the dark. Basidiospores were considered germinated if they had a germ tube or appressorium at least twice the length of the spore (Ji and Kuc, 1996). Two sets of spore suspensions in sterile distilled water and in buffer solution were included as controls and incubated under identical conditions. On germination the spores were stained with 0.1 % cotton blue in lactophenol [phenol crystals: lactic acid: glycerol in the ratio 1:1:1 (w/w)]. Lengths of 100 individual hyphae were measured under bright field illumination in a microscope (Leitz) and photographs of the germlings were taken by Leica Wild MPS 48 camera on Kodak 200 ASA film.

3.18.2. Microtitre dish assay

Assays for inhibition of fungal growth were conducted essentially as described by Broekaert *et al.* (1989). Three hundred microlitres of 1.5 % Bactoagar (Difco Lab.)

containing 3.5 % Czapek Dox broth were added to the wells of a microtitre plate (Costar Co.). After the medium solidified, 10 µl of a spore suspension (2 x 10^5 spores ml^{-1}) of *Glomerella cingulata* were added to each well and the spores were allowed to germinate at 22 ±2°C in the dark. Twelve hours later purified chitinase and β-1,3-glucanase (40 µl) and intercellular wash fluids from induced or control plant leaves were added to the wells. Fungal growth and development were monitored visually as well as microscopically, after which the plates were photographed. Buffer containing denatured enzyme was used as an additional control.

3.18.3. Agar cup bioassay

Antifungal activity of purified proteins was also tested using agar-cup bioassay techniques. Richard's agar medium was poured in Petri dishes and four wells were made in each plate. Aliquots (40 µl) corresponding to 1.5 g of fresh leaf tissue of individual fractions and ethyl acetate (in control sets) were dispersed in each of the four wells. Five-millimeter agar blocks of 7-day old cultures of *Glomerella cingulata*, cut from the advancing zone, were centrally deposited in each plate at equal distance from the wells and incubated at 30 °C for 7 days. Fungal growth and development was monitored and inhibitions were recorded in relation to control, when the organism had fully grown.

3.19. Extraction of antifungal compounds

3.19.1. Phenolics

For extraction of antifungal phenolics the method of Daayf *et. al.* (1995) was adopted with a few modifications for the determination of free and glycosidically linked phenolics. For the experiment, healthy, blister blight diseased as well as leaves showing hypersensitive reaction towards *E. vexans* were collected from tea gardens. In addition tea plants artificially inoculated with the pathogen were also considered. Leaves were harvested 48 h after inoculation. Leaf samples (10 g) were mixed with 80 % methanol (10ml g^{-1} leaf tissue) and homogenized for blending for about 1 min. Samples were extracted for 48 h on a rotary shaker, in conical flasks covered with aluminium foil for protection from light, at 50 r.p.m. The methanolic extracts were then filtered through Whatman no. 1 filter paper and concentrated to dryness by evaporation up to 20 ml (1/ 5^{th} of the initial volume). Concentrates (aqueous fraction)

were first partitioned against an equal volume of anhydrous diethyl ether three times. The ether fraction was stored and termed Fraction I. The aqueous fraction was secondly partitioned against an equal volume of ethyl acetate (three times) and the ethyl acetate fraction was termed Fraction II.

Acid hydrolysis with 4 N HCl of the remaining aqueous fraction (yielding phenolic aglycones) was performed as per the protocol of Daayf *et al.* (1997). Aglycones were recovered by partitioning hydrolysates against an equal volume of ethyl acetate (three times) and termed Fraction III. All the fractions were evaporated to dryness and finally dissolved in spec. methanol for UV-analysis and in HPLC grade methanol for HPLC analysis. These fractions were also used for bioassays.

3.19.2. Catechins

Tea leaf catechins were extracted from healthy, blister blight infected and artificially inoculated (with *E. vexans*) leaves according to the method of Obanda and Owuor (1994) with slight modifications. Leaf samples (10 g) were extracted in 80 % acetone (HPLC grade) (10 ml g^{-1} tissue) by boiling at 45 °C in a water bath for 45 min followed by homogenization in a mortar with pestle. The extracts were decanted and filtered through Whatman no. 1 filter paper. The acetone extracts were concentrated to dryness and the residue was finally dissolved in 20 ml water (HPLC grade). The water solution was fractionated four times with an equal volume of chloroform (HPLC grade). The pH of the water fraction was adjusted to 2 with 2 N HCl and then dissolved in 3 ml of 2 % acetic acid. The samples were finally filtered through Millipore filter paper (0.45 μm) and analysed by HPLC.

3.20. Analyses of antifungal compounds

3.20.1. Thin Layer Chromatography (TLC)

For TLC, clean, grease free glass plates were coated evenly with Silica Gel G (E Merck). After air drying, the plates were activated at 80-100 °C before spotting the respective compounds. Antifungal compounds were analyzed on the chromatograms prior to UV-spectrophotometry. The test compounds were spotted on replicate TLC plates and developed in chloroform : methanol (9:1 v/ v) solvent system (Chakraborty and Saha, 1994b). Following evaporation of the solvent, one of the thin-layer plates were observed under UV-light and sprayed with Folin-Ciocalteau's phenol reagent.

Colour reactions and R_F values were noted. The silica gel from unsprayed replicate TLC plates of corresponding R_F zones were scraped off, eluted separately with methanol, rechromatographed with authentic pyrocatechol, compared and eluted with methanol (Spec. grade) for spectral analysis.

3.20.2. UV-spectrophotometry

The purified eluants of antifungal compounds were analysed in a UV-spectrophotometer (Shimadzu, 160) at a range of 200 - 400 nm and the maximum absorption was determined. Quantification of the antifungal compound (pyrocatechol) was done from UV-spectrophotometric curve by considering the molar extinction coefficient of 6000 at 274 nm and expressed in $\mu g\ g^{-1}$ leaf tissue.

3.20.3. High Performance Liquid Chromatography (HPLC)

Catechin analyses of the compounds were 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 6 min and then linear gradient to 75 % B over 5 min. The elution was complete after a total of 25 min. Flow rate was fixed as 1ml min^{-1}, sensitivity 0.5 aufs, injection volume 20 µl and monitored at 278 nm (Shimadzu Advanced VP Binary Gradient).

3.21. Bioassay of antifungal phenolics

3.21.1. Spore germination bioassay

Antifungal activity of all antifungal phenolic fractions was tested by spore germination bioassay (Ji and Kuc, 1996) against *E. vexans*. The basidiospores were suspended at a density of 1.6 x 10^5 spores ml^{-1} in sterile water. The effect on spore germination *in vitro* was determined using microscopic cavity slides. Ten microlitres of the spore suspension were added per depression. Ten microlitres of the different fractions of antifungal phenolics, were then added to the spore suspension. The microscopic slide was put into a moist Petri plate (100 % humidity) and incubated for 16-20 h in an incubator at 25 ±2 °C BOD (Remi) in the dark. Two sets of spore suspensions in sterile distilled water and in the respective solvents were included as controls and incubated under identical conditions. Spores were considered germinated if they had a germ tube or appressorium at least twice the length of the spore, stained

with lacto-phenol cotton blue stain, observed under bright field illumination and photographed.

3.21.2. Radial growth bioassay

Radial growth inhibition bioassay was performed for determining antifungal activity of phenolics extracted from tea leaves as described by Van Etten (1973). The fractions (50 µl) were taken in sterile Petri dishes (2" dia) and allowed to evaporate. Subsequently 10 ml sterilized PDA medium was poured in each Petri dish, thoroughly mixed and allowed to solidify. Agar block (4 mm dia) containing mycelia of *Curvularia pallescens* (7-day old culture) were taken from the advancing zone and transferred to each Petri dish these were incubated at 28 ±2 °C, until inhibition of mycelial growth was observed and photographed.

3.21.3. Agar-cup bioassay

Sterilized nutrient agar medium (NA) was warmed to liquefy, cooled to 42 – 45 °C, seeded with *B. megaterium*, poured on Petri dishes (2" dia) and allowed to solidify. A well (4 mm dia) was made with a sterilized cork borer and 50 µl of each test solvent fraction was added to each plate and incubated at 37 ±2 °C. Controls sets were also prepared at the time and kept under identical conditions. The diameter of the inhibition zones was determined.

3.22. Electron microscopy

3.22.1. Scanning electron microscopy (SEM)

Basidiospores of *E. vexans* collected in Petri plates were lifted with a brush and prefixed following the method of Shetty *et al.* (2003) prior to mounting on the sample stub. Similarly, healthy and infected tea leaves were trimmed to the appropriate size so as to accommodate them on the stubs and fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 6.8 in vacuum. After 24 h, the leaf pieces were washed in buffer and dehydrated in a graded series of acetone. The tissue was dried in a Critical Point Dryer and mounted with silver paint. Subsequently the samples were coated with a 20 nm gold-palladium alloy in a Sputter Coater and examined in a JEOL JSM 5200 Scanning electron microscope (Tokyo, Japan).

3.22.2. Transmission electron microscopy (TEM)

Transmission electron microscopy of blister blight infected tea leaves as well as salicylic acid induced tea plants was performed following the method of Tahiri-Alaoui *et al.*, (1993) with a few modifications.

3.22.2.1. Tissue processing

Segments (1 to 2 mm) from control, blister blight infected and salicylic acid treated tea leaves were excised in 0.1 M sodium-phosphate buffer, pH 7.4 (PB). They were immediately transferred to 1.5 % phosphate-buffered glutaraldehyde in Eppendorf tubes for 60 min and fixed in 3 % glutaraldehyde (EM grade) for 16 h at room temperature. After rinsing 3 x 10 min in PB, specimens were dehydrated in ascending grades of ethanol in the series 2 x 10 min 50 % ethanol and 2 x 45 min 70 % ethanol, followed by infiltration in LR White resin (Sigma) in 70 % ethanol (2:1) for 60 min. Embedding of the specimens was done in 100 % LR White resin in air tight gelatine capsules (Agar, size 0). The specimens were transferred to absolute resin (60 min) and then embedded in fresh resin and polymerized at 60 °C in a fume hood for 2 days. The cured resin blocks were trimmed (Leica EM Trim) to remove the excess resin before sectioning. The cutting plane was trimmed to the shape of a trapezoid. Glass knives were made (Leica EM KMR2) for cutting semi-thin sections. The trimmed blocks were set in an ultramicrotome (Leica Ultracut, UCT M26 equipped with M26 viewer) so as to get the material in the entire cutting face. Semi-thin sections (2 µm) were stained with 0.5 % aqueous toluidine blue solution and observed under light microscopy (Leica Biomed). Ultrathin sections (50 nm) of the same selected blocks were cut with a diamond knife. A ribbon of about 6-7 sections were picked up on Pioloform coated nicked grids (300 mesh) for immunogold labelling. A grid of each sample containing ultrathin sections was stained with 2 % aqueous uranyl acetate at room temperature for morphological observation. The grids were floated on a drop of the stain for 45 min followed by a wash with double distilled water. Observations were carried out in a Tecnai 12 Bio Twin transmission electron microscope (Philips, The Netherlands).

3.22.2.2. Immunogold labeling

Immunogold labeling was carried out by floating the grids on drops of the solutions pipetted (50 µl) on to parafilm. Ultrathin sections were incubated with blocking solution containing 1 % (w/v) of BSA in 0.1 M phosphate buffered saline, pH 7.4 (PBS) for 15 min. Incubation of the sections with the primary antibody (PAb) diluted at 1:5 in the blocking solution for 60 min was followed by washing in 4 x 10 min baths in PBS. In the secondary immunolabelling step, the sections were incubated with goat anti-rabbit IgG (whole molecule) gold conjugate, 10 nm (Sigma, G7402) diluted at 1:10 in PBS for 60 min at room temperature. After rinsing with PBS the sections were stained with 2% aqueous uranyl acetate for 10 min. The sections were washed in double distilled water, post stained in 0.2 % lead citrate for 3 min, and finally washed again in double-distilled water. After contrasting with uranyl acetate and lead citrate, ultrastructural analysis of the sections was performed with a Tecnai 12 Bio Twin TEM at 75 kV with Megaview III Soft Imaging System. A minimum of six samples was examined using an average of 3 replicates per immunogold treatment. Specificity of labelling was assessed by the following control tests (i) incubation of the ultrathin sections with the rabbit pre-immune serum instead of the primary antibody and (ii) incubation with the secondary antibody and omitting the primary antibody step.

EXPERIMENTAL

4.1. Influence of environmental conditions on development of blister blight of tea

Blister blight of tea, a foliar fungal disease, incited by *Exobasidium vexans* Massee is the most important leaf disease problem in tea cultivation. This disease has been reported to occur in a number of countries with India being no exception. The disease is most prevalent in the hilly terrains of Darjeeling, upper Assam, Himachal Pradesh and the tea tracks of southern India. It has been documented that the disease is more serious in bushes recovering from pruning as they have greater proportions of succulent and tender material that makes it more susceptible to the disease. Blister blight is a multiple cycle disease spread through windborne basidiospores and the pathogen completes its life cycle in a short span. Thus, in a single disease season several generations of the pathogen are completed. A relative humidity higher than 80% is essential for basidiospore formation, ejection and germination and maximum penetration occurs at 90% where leaf wetness is the most important factor. As the fungus invades only tender foliage which forms the main harvest, severely affected leaves when processed produce tea of poor quality. The fungus infects tender leaves and stems and thereby causes direct crop losses (Plate 5, figs. A & B). Crop loss could go up to 50% and the damage, results in both quantitative and qualitative reduction – and made tea is eventually weak, lacking in strength and of poor quality. At the same time as tea is a perennial plant sufficient susceptible leaves are present all through the year. The crop loss however varies depending on the geographic area. The pathogen is a biotroph with no known alternate host; hence it completes its life cycle on the tea plant itself where occurrence has been evidenced in nursery plants (Plate 5, figs. C - E) and tea bushes.

Keeping in view the relationship between relative humidity on spore ejection and germination, the influence of weather conditions on appearance of blister blight disease was explored. Meteorological data for five years (2000-2004) of hills and plains was recorded and correlated with disease incidence all through the year at two tea estates Castleton (hills) and Hansqua (plains) (Figure 1). In the hills, disease incidence was noticed during July through October whereas in the plains incidence was recorded only from December through February during the entire study period.

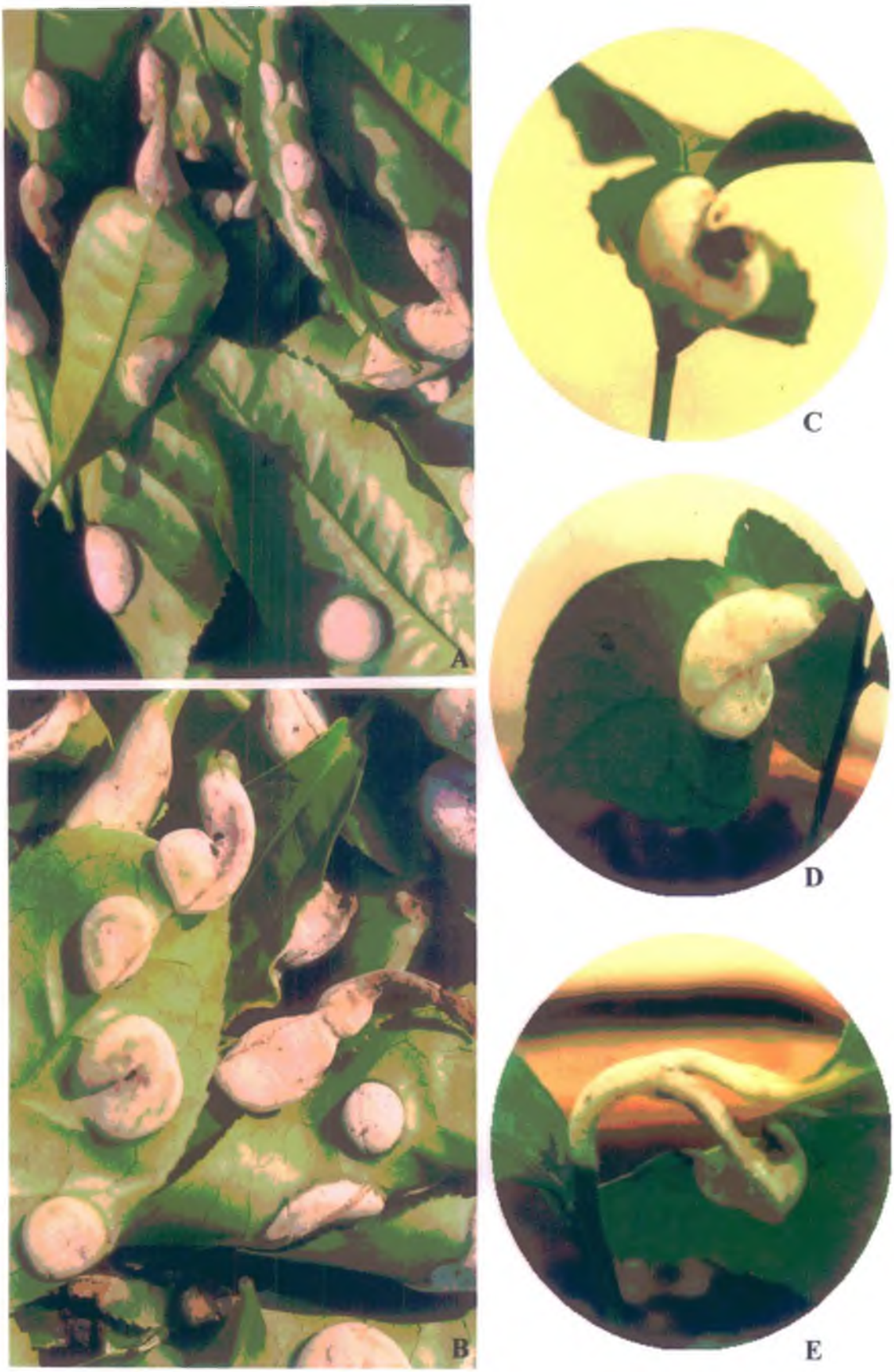


Plate 5 (figs. A – E). Symptoms of blister blight disease. [A & B] Naturally infected leaves from tea gardens. [C - D] Nursery grown plants.

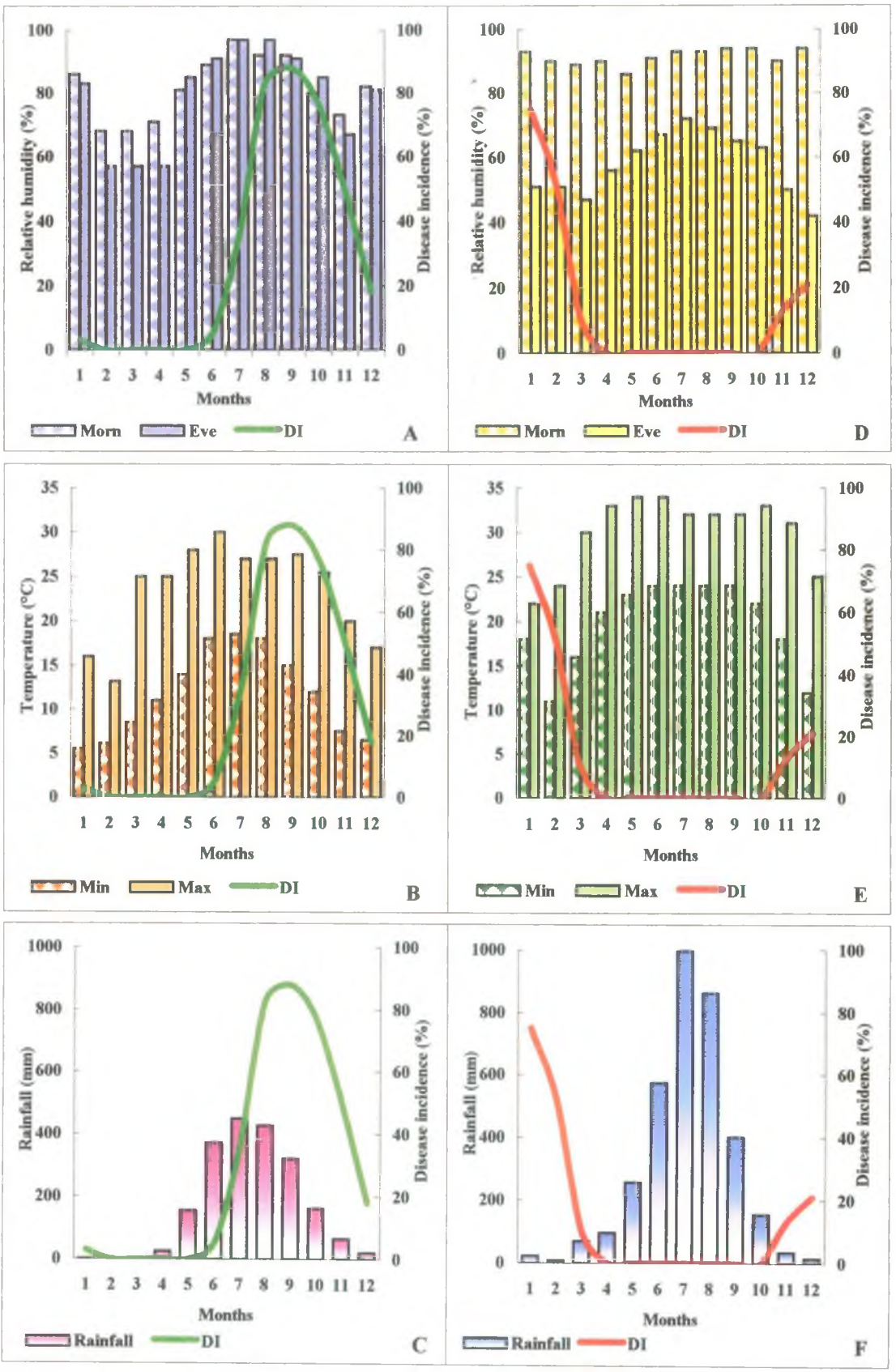


Figure 1. Correlation of weather conditions of hills (A-C) and plains (D-F) with appearance of blister blight of tea. [1-12 indicate months Jan-Dec.]

As weather has a profound influence on disease incidence it was attempted to determine whether or not disease development in nature is correlated with different environmental parameters. Karl Pearsons' correlation coefficient of disease incidence was calculated with morning and evening relative humidity, maximum and minimum temperatures and average rainfall. When mean blister blight incidence of the hills was correlated with relative humidity (Figure 1 A), a correlation coefficient of 0.72 was obtained among disease and relative humidity (R.H.). On the contrary, when mean blister blight incidence of plains was correlated with the R.H. (Figure 1 D) a value of 0.43 was obtained. In the hills temperature showed a positive correlation with maximum (0.59) and minimum temperature (0.67) (Figure 1 B), but a negative correlation with maximum (-0.87) and minimum temperature (-0.85) of the plains (Figure 1 E). As temperature, rainfall could also be correlated (0.88) to the disease incidence in the hills (Figure 1 C), while, in the plains a negative correlation (-0.675) was obtained with average rainfall and disease incidence (Figure 1 F).

The first symptom of blister blight is a small, pale green, pale yellow or pinkish translucent spot or may merely be of a lighter shade than the rest of the leaf, which can be easily discernible when the leaf is held against light. In exceptional cases the spot is red on both sides (Plate 6, fig. B). The circular spots gradually enlarge until they reach a diameter of 3 - 12.5 mm. The upper side of the leaf of the lesions sunken and at the same time on the lower surface they become correspondingly convex, thus, forming a typical blister under conducive climatic conditions (Plate 6, fig. D). The upper concave surface of the lesion is smooth, while the lower convex surface is initially dull, then grey, and finally becomes pure white, dense and velvety on which the spores are produced (Plate 6, fig. E). The final outcome of an infection resulting from a compatible interaction is a series of variable infection stages starting from appearance of a translucent spot which gradually, in 11-28 d, develops in to a white velvety sporulating blister on exposure to UV light. Under severe infection the affected leaves curl up. When the tender stem is attacked, the entire shoot withers and falls (Plate 5, fig. E). For the present study leaf samples showing natural symptoms were used. Healthy (H) (Plate 6, figs. A & C), infected (S) and resistant (R) leaves exhibiting hypersensitive reaction (Plate 6, fig. F) were collected from fields. The diseased samples were sorted in to three stages of infection (Table 7).

Plate 6 (figs. A – F). Stages of disease development. [A] Healthy twig. [C] Healthy leaves. [B] Initial symptoms on twigs (two and a bud). [D] Moderate infection. [E] Severe infection (blister postules on ventral surface). [F] Necrotic lesions (hypersensitive).

Table 7. Categories of stages of infection of blister blight diseased tea leaves

Stages of infection	Visible symptoms
Stage 1 - Initial	Appearance of small, pale green, pale yellow or pinkish translucent spots
Stage 2 - Moderate infection	Appearance of notches on the leaves with slight concavity
Stage 3 - Severe infection	Appearance of mature blister

Three isolates of *E. vexans* (EV1, EV2 and EV3) obtained from flush shoots with well-developed sporulating lesions were observed under bright field illumination. Basidiospores were hyaline, elliptical, clavate and two celled at maturity. However the isolates differed in spore size. Average size of spores measured for the isolates were EV1 – 9.95 x 3.25 μm (Plate 7, fig. B); EV2 – 5.55 x 3.55 μm (Plate 7, fig. A) and EV3 – 7.20 x 3.25 μm. The spores readily germinated by producing one or two germ tubes in a pH range of 5.0 – 8.0 at 25 ±2°C. Within 24 h of growth, the tips of the germ tubes enlarged to form appressoria observed as round thickenings (Plate 7, fig. D). Basidiospore morphology was also observed under scanning electron microscope (SEM). The white spore mass of *E. vexans* was coated with a gold-palladium alloy in order to eliminate the charging effect of the electron beam by the sputtering method in an ion coater. Photomicrograph of SEM has been presented in Plate 7, fig. C.

Blisters formed following infection were generally observed on the ventral surface of the leaf. To observe the morphology of the superficial extent of damage, scanning electron microscopic observations of the ventral surface of healthy leaves and blister affected postules were made. Several stomata were observed on the ventral surface of the healthy leaf (Plate 8, figs. A - F). On scanning the ventral surface of blister postules, extensive damage was observed. Epidermal cells close or in contact with the hyphae exhibited hypertrophy with collapsed epidermal cells. The leaf surface had lost its morphological orientation. The surface was completely distorted and stomata were not visible as clearly seen on the healthy leaf surface (Plate 9, fig. A).

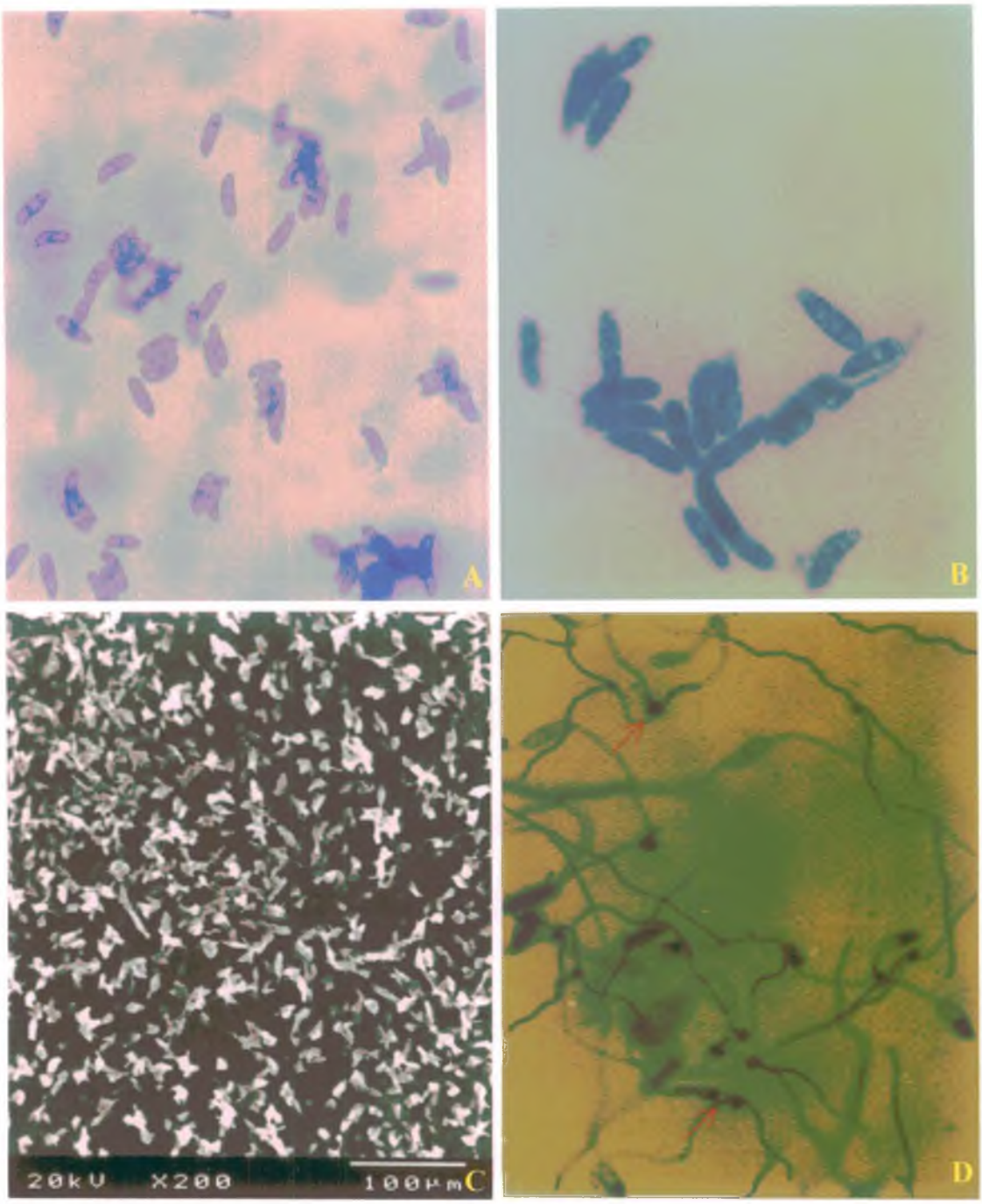


Plate 7 (figs. A – D). Basidiospores of *E. vexans*. [A & C] Isolate EV-2. [B & D] Isolate EV-1. [A & B] Bright field (40x). [C] Scanning electron microscopic micrograph (200x). [D] Germinated spores with appressoria (40x).

The pathogen appears to be of aggressive nature causing damage of the highest degree (Plate 9, figs. B - F). Significant morphological differences were observed between healthy and infected leaf surfaces.

Besides, a histological assessment of blister blight infection was also made. Transverse cross sections were cut through the blister blight infected zones of the tea leaf and observed under bright field illumination (Plate 10, figs. A & B). The blister comprised of numerous compact bundles of hyphae that formed the hymenial layer. Basidia bearing basidiospores were also observed. Fungal penetration through the epidermis in to the mesophyll tissues was also evident. Dense growths of the hyphae directed towards the lower surface were obvious.

4.2. Screening of resistance of tea towards *E. vexans*

Thirty tea varieties (AV-2, BS-7A/1/76, CP-1, HV-39, P-1258, RR-17, T-17/1/54, T-135, T-78, TS-449, UP-2, UP-3, UP-8, UP-9, UP-17, UP-26, BSS-1, BSS-2, BSS-3, TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-27, TV-28, TV-29 and TV-30) were screened for resistance towards *E.* vexans. Methods of basidiospore collection, artificial inoculation and disease assessment were followed as mentioned in materials and methods. Percentage infection was recorded 20 d after inoculation by examining the number of infected shoots and leaves. Disease incidence was scored and on the basis of the percentage of infection, the varieties were grouped as 0-20 % - resistant (R), 21-40 % - moderately resistant (MR), 41-70 % - moderately susceptible (MS) and 71-100 % - susceptible (S). Results have been presented in Table 8. Among the Darjeeling varieties T-17/1/54 and BS-7A/1/76 were highly resistant, T-78 and T-135 were susceptible, while the rest were moderately susceptible. Most of the UPASI varieties (BSS-2, UP-2, UP-3, UP-9 and UP- 17) were highly susceptible (S), BSS-3 was found to be resistant while others were found to be either moderately susceptible (MS) or moderately resistant (MR). As for Tocklai varieties, the varieties TV-9, TV-18 and TV-28 were highly susceptible (S) and the rest were either moderately susceptible or moderately resistant.

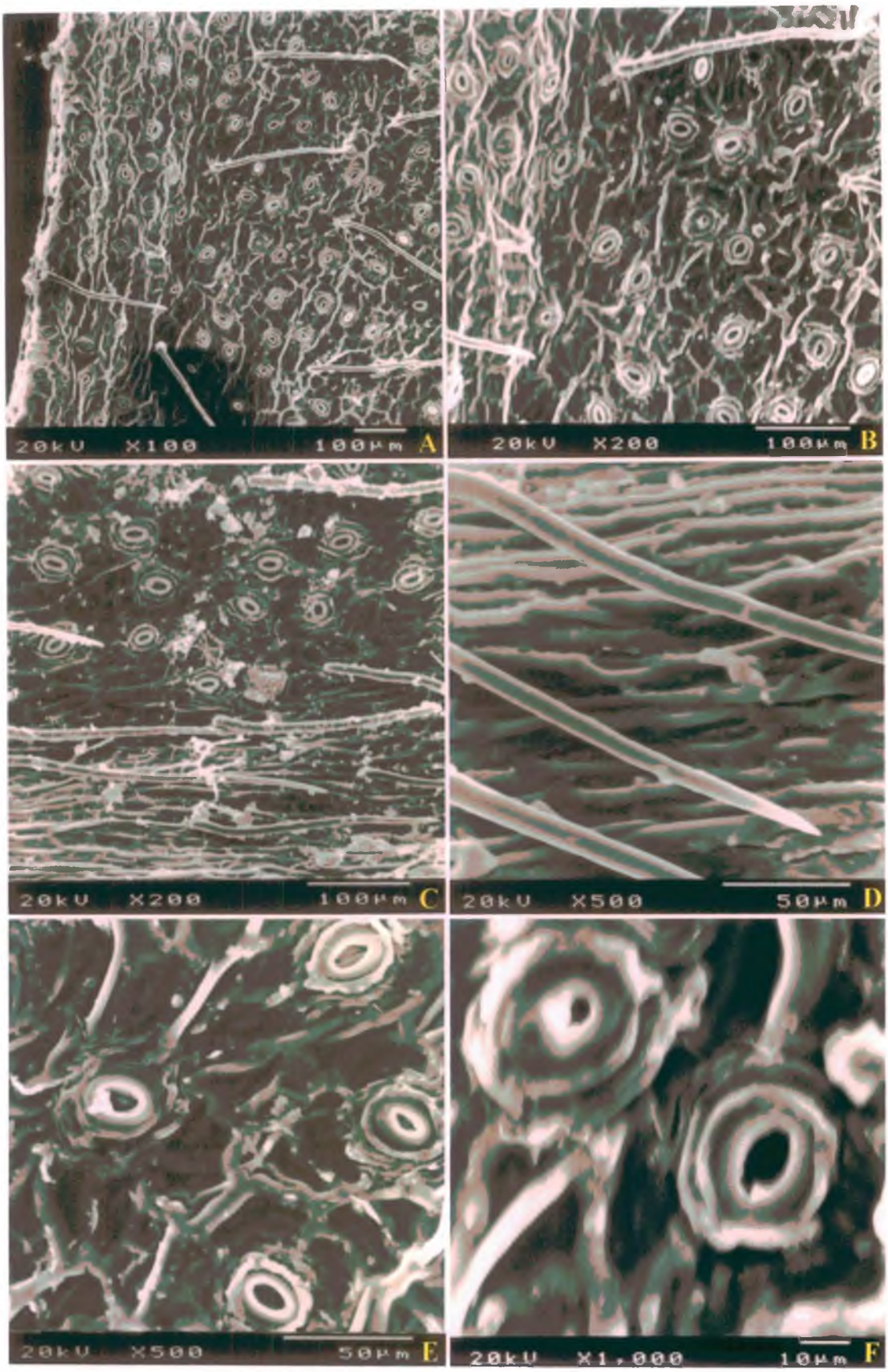


Plate 8 (figs. A – F). Scanning electron micrographs of healthy tea leaf (ventral surface).

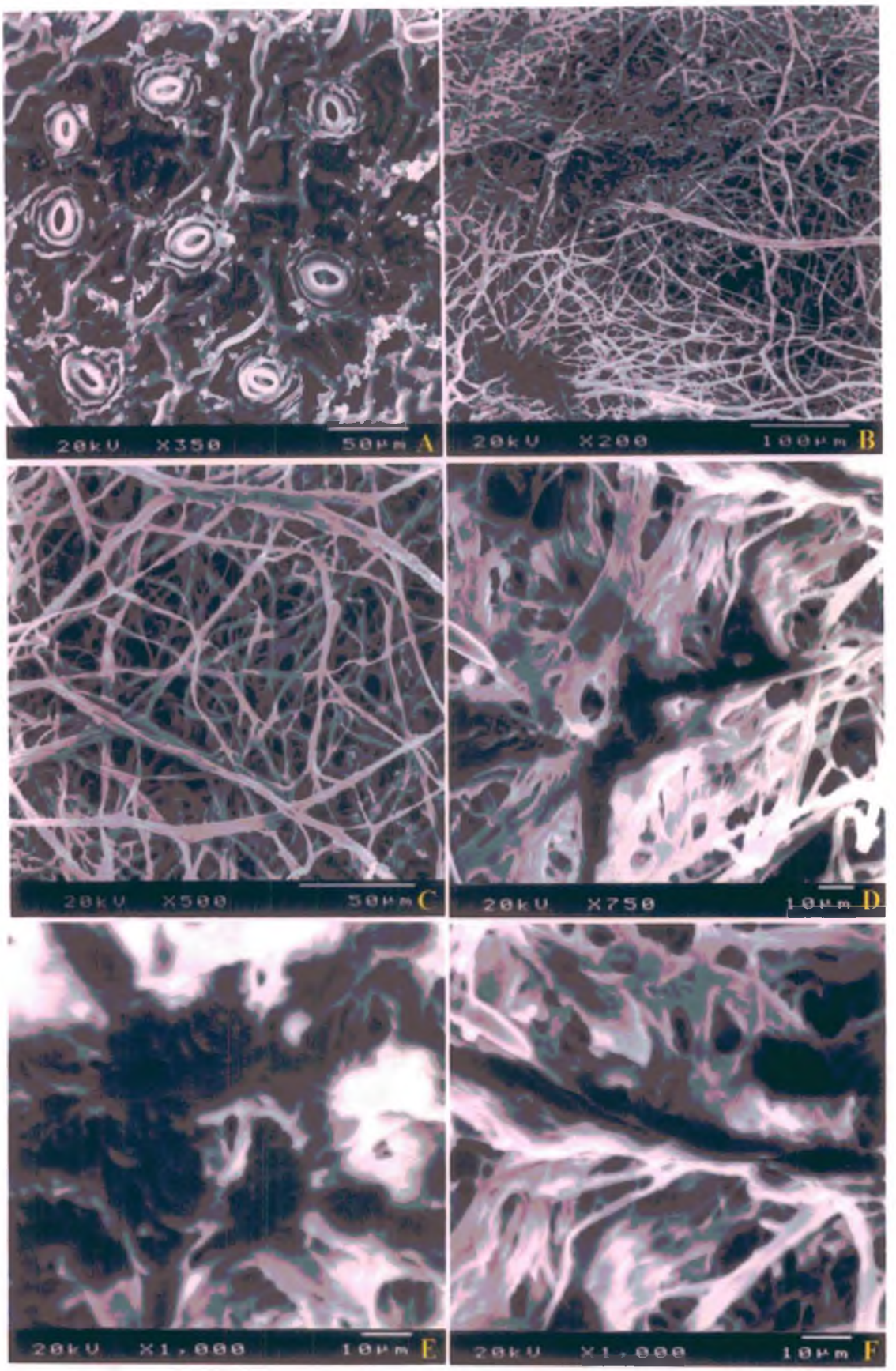


Plate 9 (figs. A – F). Scanning electron micrographs of blister blight infected tea leaves (ventral surface). [A] Healthy leaf. [B - F] Infected leaf.

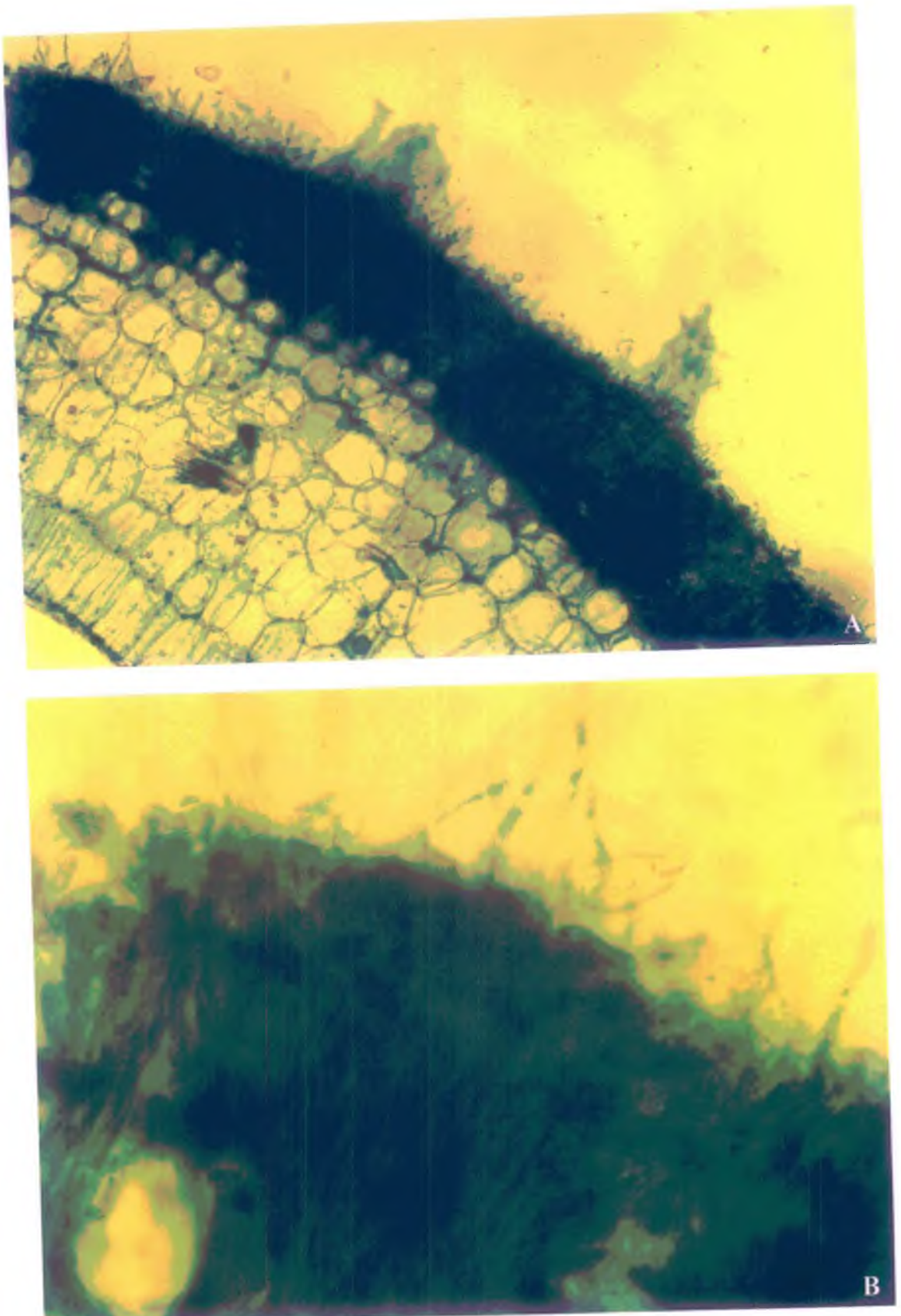


Plate 10 (figs. A & B). Histopathology of *E. vexans*-infected tea leaf.

Table 8. Incidence of blister blight caused by *E. vexans* on artificially inoculated tea plants

Tea varieties		Percentage infection (%)[a]	Disease score[b]
Darjeeling	AV-2	61.7 ± 0.18	MS
	BS-7A/1/76	15.9 ± 0.04	R
	CP-1	58.5 ± 0.09	MS
	HV-39	55.3 ± 0.18	MS
	P-1258	51.6 ± 0.13	MS
	RR-17	67.5 ± 0.19	MS
	TS-449	62.8 ± 0.09	MS
	T-135	91.5 ± 0.08	S
	T-17/1/54	11.0 ± 0.03	R
	T-78	96.2 ± 0.06	S
UPASI	BSS-1	21.3 ± 0.06	MR
	BSS-2	84.6 ± 0.10	S
	BSS-3	18.2 ± 0.07	R
	UP-2	81.3 ± 0.08	S
	UP-3	74.8 ± 0.05	S
	UP-8	48.2 ± 0.03	MS
	UP-9	89.8 ± 0.11	S
	UP-17	72.7± 0.08	S
	UP-26	46.5 ± 0.07	MS
Tocklai	TV-9	78.3 ± 0.06	S
	TV-18	93.9 ± 0.05	S
	TV-20	22.4 ± 0.04	MR
	TV-22	44.3 ± 0.06	MS
	TV-23	50.1 ± 0.07	MS
	TV-25	53.5 ± 0.02	MS
	TV-26	38.2 ± 0.14	MR
	TV-27	51.6 ± 0.09	MS
	TV-28	71.1± 0.12	S
	TV-29	28.6 ± 0.05	MR
	TV-30	34.4 ± 0.03	MR

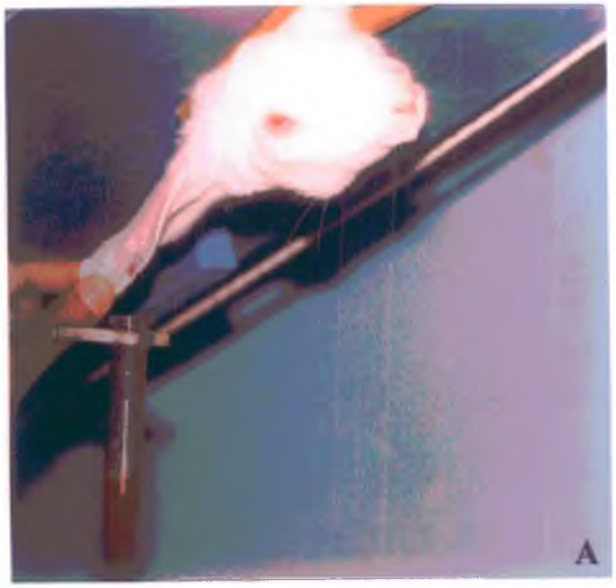
Note: Data average of three experiments; ± Standard error; [a] 50 shoots were screened per variety 20 days after inoculation with *E. vexans*, [b] R - resistant (0 - 20 %), MR - moderately resistant (21 - 40 %), MS - moderately susceptible (41 - 70 %), S - susceptible (71-100 %).

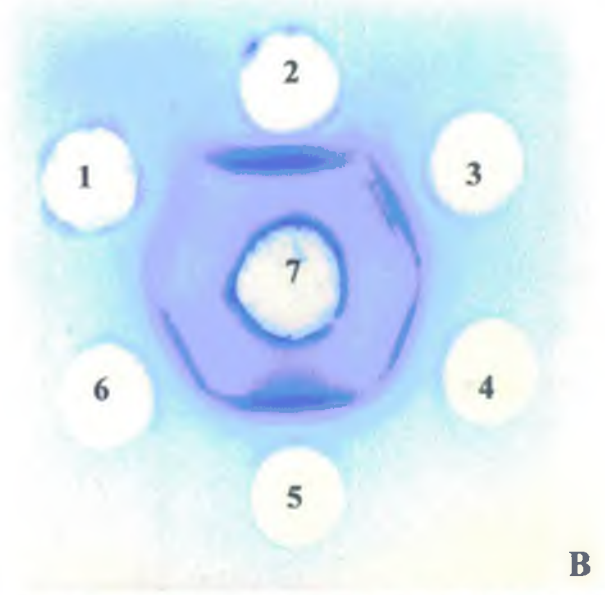
4.2.2. Immunoenzymatic assays

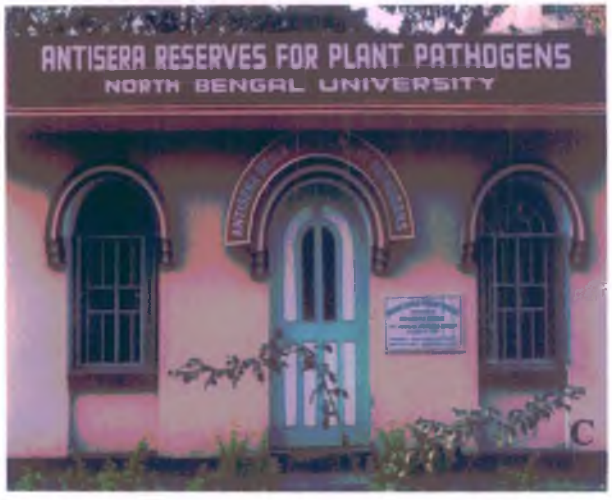
Plants respond to infection by pathogens in a number of ways. The initial recognition triggers the activation of the immune system of plants, which though different from that of animals, is functionally similar. An important area of immunological studies involves the use of pathogen antiserum for screening resistance of host plants beginning from the very early stages of host pathogen interaction. Hence, for immunological screening of resistance of tea plants against *E. vexans* were performed using enzyme linked immunosorbent assay (PTA-ELISA) and dot-immunobinding assay (DIBA). For this purpose, polyclonal antibodies (PAb) were raised against blister blight pathogen (*E. vexans*), non-pathogen of tea (*F. oxysporum*) and tea varieties (T-17/1/54 and T-78). Blood samples were collected by marginal ear vein puncture (Plate 11, fig. A). The PAbs of *E. vexans* (PAb-EV1), T-17/1/54 (PAb-T17), T-78 (PAb-T78) as well as *F. oxysporum* (PAb-FO) were stored in the Antisera Reserves (Plate 11, fig. C). The effectiveness of five bleeds was tested initially using agar gel double diffusion tests. Strong precipitation bands were observed with PAb-EV1 (Plate 11, fig. B), PAb-T17, PAb-T78 and PAb-FO in their respective homologous reactions.

4.2.2.I. Optimization of PAbs and antigen concentrations

Optimization tests were performed separately with all PAbs raised against pathogen (*E. vexans*), non-pathogen (*F. oxysporum*) and host (T-17/1/54 and T-78). Purified IgG fractions of all of the PAbs were used for each experiment. Optimization of PTA-ELISA was done considering two variables i.e., concentration of the antigen and concentration of PAb. Reactions were done with PAb obtained from different bleedings. The protein concentration of purified IgGs obtained from different bleeds of the different sources was pre-determined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10,000) and p-nitrophenyl phosphate (10 mg ml^{-1}) were used for PTA-ELISA as enzyme-substrate The substrate (pNPP) reaction was terminated after 60 min and absorbance values were means of five adjacent wells measured at 405nm.







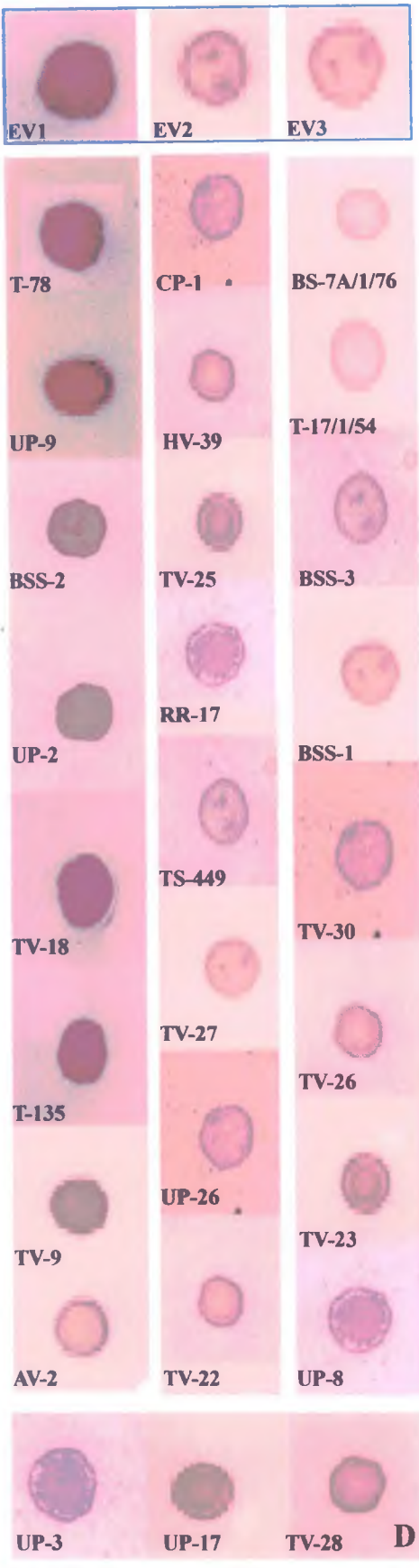


Plate 11 (figs. A – D). Immunological assays. [A] Collection of blood samples from rabbit by marginal ear vein puncture. [B] Agar gel double diffusion tests – antigens of *E. vexans* (1-6) and PAb-EV1 (7). [C] Antisera reserves for plant pathogens. [D] Dot immunobinding assay on nitrocellulose membrane using antigens of isolates of *E. vexans* and healthy leaves of tea varieties probed with PAb-EV1 and reacted with NBT-BCIP.

PAb-EV1 (Pathogen): Different concentrations of IgG (ranging from 0.312 - 40 μg ml^{-1}) from PAb-EV1 were tested against homologous antigens at a concentration of 20 μg ml^{-1}. Absorbance values in PTA-ELISA increased with increase in concentration of IgG with a maximum of 3.213 in 40 μg ml^{-1} (Figure 2 A). This concentration of IgG was selected for further experiments. Antigen concentrations ranging from 0.156-20 μg ml^{-1} were tested against PAb-EV1 at an IgG concentration of 40 μg ml^{-1}. Results (Figure 2 B) revealed that ELISA values increased with the increase of antigen concentration. However antigen concentrations as low as 0.156 μg ml^{-1} could also be specifically detected by PTA-ELISA.

PAb-T17 and PAb-T78 (Host): A series of IgG concentrations ranging from 0.312-40 μg ml^{-1}, prepared from PAb-T17 and PAb-T78 were tested against their homologous antigens at a concentration of 20 μg ml^{-1}. Absorbance values increased with increase in concentration of IgG with maximum values of 3.112 and 2.925 with PAb-T17 and PAb-T78 respectively in 40 μg ml^{-1} (Figures 2 C & E). PTA-ELISA reaction with different concentrations of tea leaf antigens (T-17/1/54 and T-78) from 0.156 - 20 μg ml^{-1} were determined at an IgG concentration of 40 μg ml^{-1} with all respective bleedings. Results are depicted in Figures 2 D & F. PAb-T17 and PAb-T78 at IgG concentrations of 40 μg ml^{-1} were used in all experiments.

PAb-FO (Non-pathogen): Optimization of ELISA was done considering two variables - dilution of antigen and antiserum was done in this case also. Dilutions of antigen concentrations were tested against PAb-FO at an IgG concentration of 40 μg ml^{-1}. ELISA could, easily detect concentrations as low as 0.156 μg ml^{-1} of the antigen. Similarly, antiserum dilutions ranging from 0.312 – 40 μg ml^{-1} were tested against a homologous antigen concentration of 20 μg ml^{-1}. Results reveal a fall in ELISA values with concomitant decrease in antiserum concentration. The highest value obtained was 2.956 at 40 μg ml^{-1} IgG concentration. This concentration of IgG was used for all experiments.

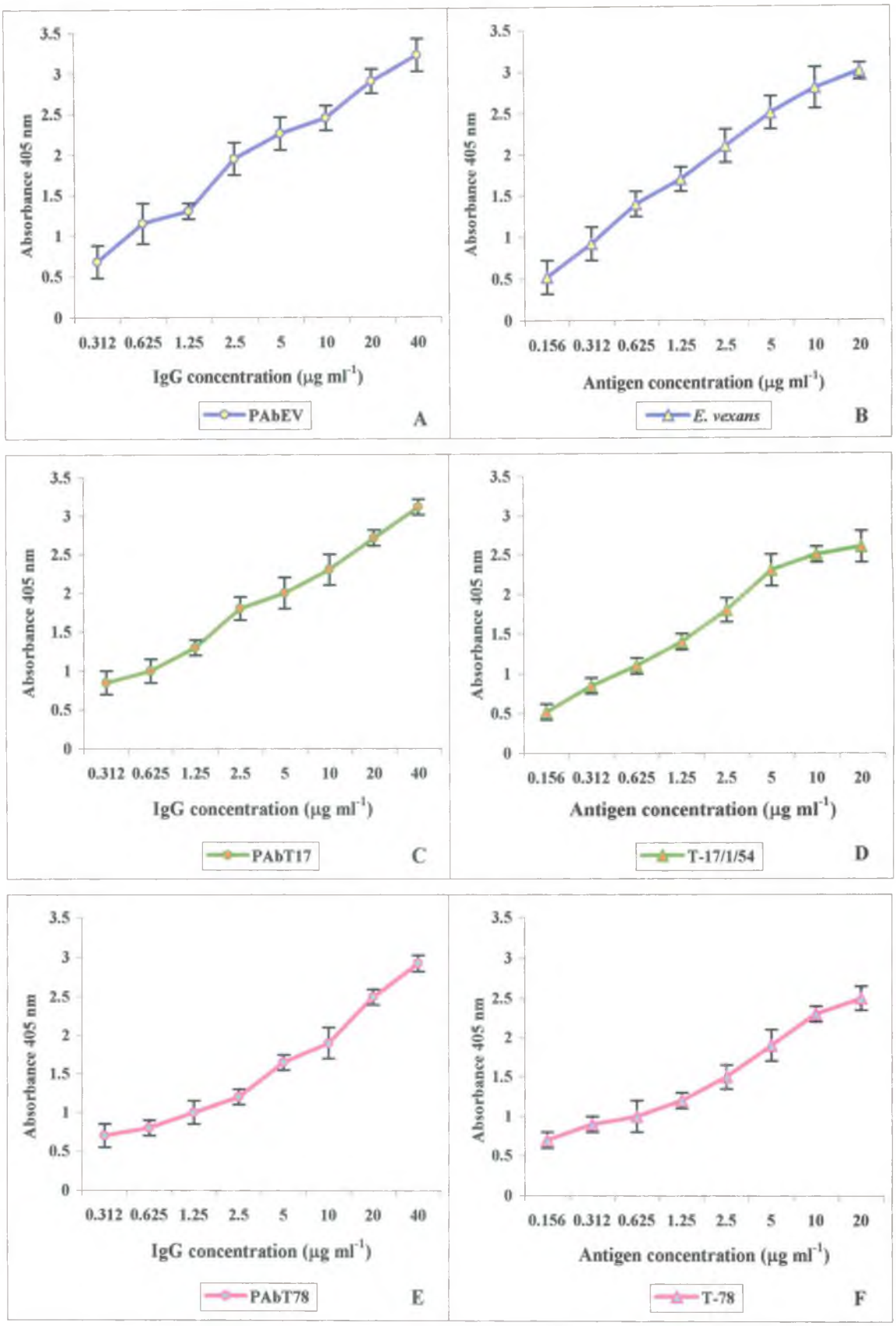


Figure 2. Optimization of PAbs raised against pathogen (*E. vexans*) and tea varieties (T-17/1/54 & T-78) reacted against homologous antigen using PTA-ELISA formats.

4.2.2.2. Screening by PTA-ELISA and DIBA

Varieties such as T-78, TV-18, T-135, UP-9, BSS-2, UP-2, RR-17, TV-28, UP-3, TS-449, AV-2, CP-1 and HV-39 showed higher ELISA values whereas for TV-25, TV-27, TV-23, UP-8, TV-22, TV-26, TV-30, TV-20, BSS-1, BS-7A/1/76 and T-17/1/54 the values decreased (Table 9), indicating T-78 to be most susceptible and T-17/1/54 most resistant with absorbance values of 0.775 and 0.225 respectively. These results of PTA-ELISA were confirmed by Dot-blot assay (Table 9) in which intensity of the dots widely differed (Plate 11, fig. D) among the varieties showing compatible and incompatible reaction against the pathogen when artificially inoculated in the experimental field. Colour intensity of dots were scored by eye and denoted as: + insignificant, + + light violet, + + + violet, + + + + deep violet. NBT/ BCIP was used as substrate in dot blot assay. Finally, percentage infection in thirty tea varieties following artificial inoculation were compared with their PTA-ELISA values as illustrated in Figure 3.

4.2.2.3. Determination of cross reactivity

Reciprocal reactions involving PAbs of resistant and susceptible tea varieties (T-17/1/54 and T-78) and antigens of host (nine varieties), pathogen (*E. vexans;* isolate EV1, EV2 and EV3), non-pathogen (*F. oxysporum*) and non-hosts (*L. leucocephala*, *O. sativa*, *G. max*) were also carried out using PTA-ELISA formats. Results have been presented in Table 10. PAb of susceptible variety (T-78) gave higher absorbance values than that of resistant variety (T-17/1/54) when tested with antigen of *E. vexans.* Very low values were recorded in reactions involving antigens of *F. oxysporum* (non-pathogen) and other plant species.

Table 9. Screening of resistance towards *E. vexans* among tea varieties using plate trapped antigen enzyme linked immunosorbent assay and dot immunobinding formats

Antigen of host and pathogen (50 μg ml^{-1})		PTA-ELISA values A405 nm with SE*	Dot blot assay
Host [Tea varieties]			
Darjeeling	AV-2	0.522 ± 0.01	+ + +
	BS-7A/1/76	0.275 ± 0.04	+
	CP-1	0.510 ± 0.03	+ + +
	HV-39	0.495 ± 0.02	+ + +
	P-1258	0.463 ± 0.03	+ + +
	RR-17	0.585 ± 0.00	+ + +
	TS-449	0.532 ± 0.04	+ + +
	T-135	0.704 ± 0.12	+ + + +
	T -7/1/54	0.255 ± 0.08	+
	T-78	0.775 ± 0.01	+ + + +
UPASI	BSS-1	0.327 ± 0.10	+ +
	BSS-2	0.644 ± 0.07	+ + + +
	BSS-3	0.331 ± 0.02	+ +
	UP-2	0.631 ± 0.06	+ + + +
	UP-3	0.555 ± 0.09	+ + +
	UP-8	0.448 ± 0.03	+ + +
	UP-9	0.675 ± 0.02	+ + + +
	UP-17	0.575 ± 0.14	+ + +
	UP-26	0.435 ± 0.14	+ + +
Tocklai	TV-9	0.620 ± 0.02	+ + + +
	TV-18	0.755 ± 0.05	+ + + +
	TV-20	0.335 ± 0.10	+ +
	TV-22	0.422 ± 0.03	+ + +
	TV-23	0.452 ± 0.12	+ + +
	TV-25	0.482 ± 0.12	+ + +
	TV-26	0.375 ± 0.04	+ +
	TV-27	0.475 ± 0.07	+ + +
	TV-28	0.575 ± 0.05	+ + +
	TV-29	0.355 ± 0.04	+ +
	TV-30	0.366 ± 0.07	+ +
Pathogen	(***E. vexans*** **isolates)**	1.023 ± 0.12	+ + + +
	EV1	2.435 ± 0.10	+ + + + + +
	EV2	1.892 ± 0.10	+ + + + +
	EV3	1.756 ± 0.09	+ + + + + +

Note: * ± Standard error; PAb-EV1, IgG concentration 40 μg ml^{-1}; Absorbance values were means of three separate experiments; 50 plants were screened per variety, Colour intensity of dots: + insignificant, + + light violet, + + + violet, + + + + deep violet, + + + + + deeper violet, + + + + + + deepest violet; NBT/BCIP was used as substrate in dot blot assay.

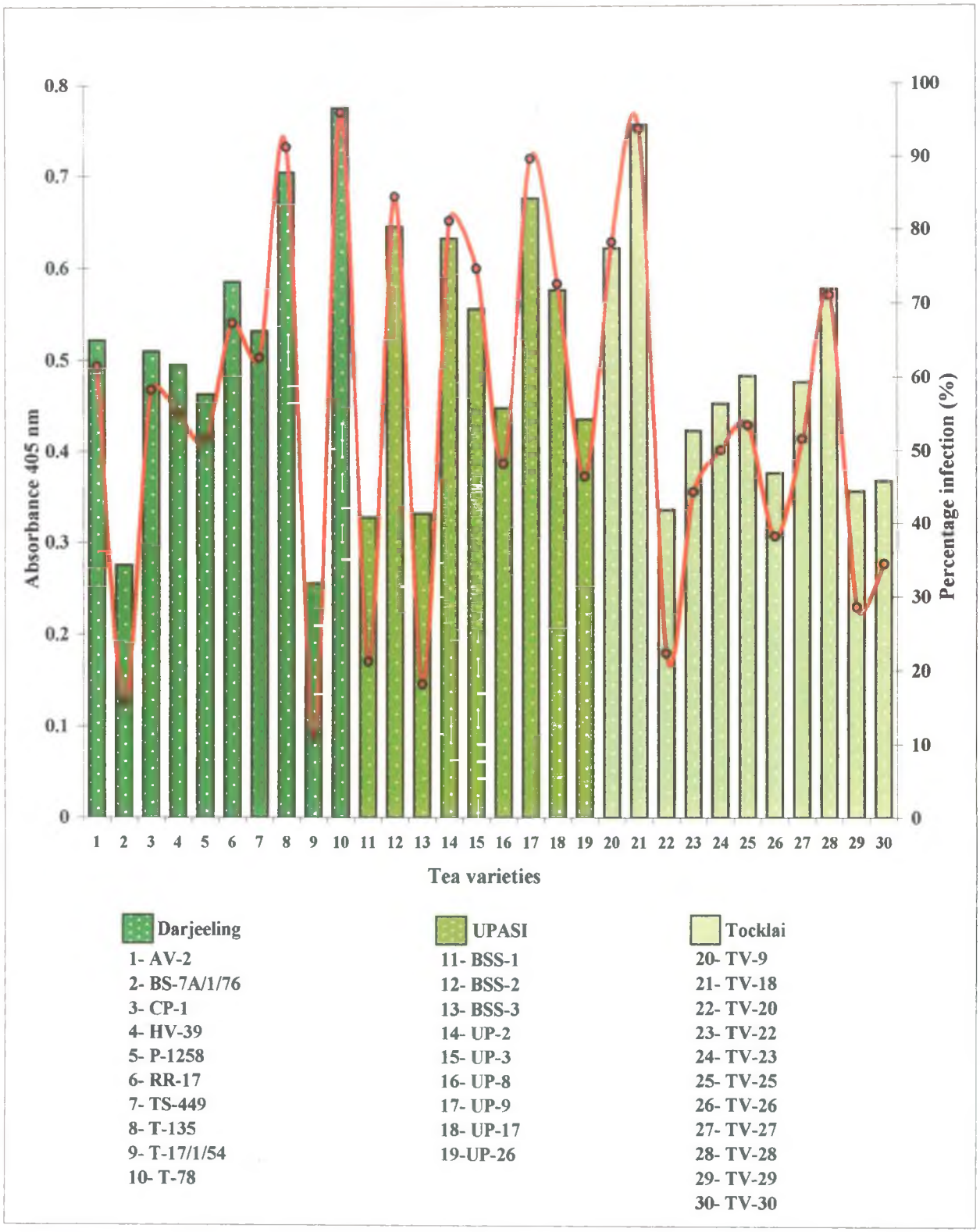


Figure 3. Comparison of PTA-ELISA values (□) of tea leaf antigens reacted with PAb-EV1 and percentage infection (o—o) on tea varieties artificially inoculated with *E. vexans*

Table 10. PTA-ELISA values of host, non-host, pathogen and non-pathogen antigens reacted with PAbs of tea varieties (T-17/1/54 and T-78), *E. vexans* (pathogen) and *Fusarium oxysporum* (non-pathogen)

Antigen (50 μg ml^{-1})	PTA-ELISA values A405nm with SE*			
	Polyclonal antiserum			
	T-17/1/54	T-78	*E. vexans* [a]	*F. oxysporum*
Host [Tea varieties]				
Darjeeling				
T-17/1/54	1.689 ± 0.06	0.567 ± 0.03	0.245 ± 0.03	0.158 ± 0.04
T-78	0.690 ± 0.04	1.184 ± 0.02	0.777 ± 0.09	0.195 ± 0.03
TS-449	0.609 ± 0.03	0.695 ± 0.02	0.539 ± 0.02	0.156 ± 0.02
UPASI				
BSS-1	0.699 ± 0.03	0.649 ± 0.02	0.321 ± 0.05	0.103 ± 0.02
UP-8	0.669 ± 0.08	0.707 ± 0.02	0.444 ± 0.03	0.113 ± 0.09
UP-2	0.625 ± 0.06	0.575 ±0.05	0.641 ± 0.04	0.189 ± 0.06
Tocklai				
TV-30	0.712 ± 0.02	0.667 ± 0.03	0.333 ± 0.08	0.160 ± 0.01
TV-18	0.581 ± 0.06	0.778 ± 0.07	0.751 ± 0.05	0.167 ± 0.04
TV-25	0.777 ± 0.03	0.745 ± 0.06	0.480 ± 0.03	0.198 ± 0.01
Pathogen (*E. vexans* isolates)				
EV1	0.420 ± 0.05	0.778 ± 0.01	1.972 ± 0.02	0.199 ± 0.08
EV2	0.395 ± 0.04	0.750 ± 0.01	1.887 ± 0.07	0.129 ± 0.03
EV3	0.350 ± 0.08	0.700 ± 0.03	1.728 ± 0.04	0.111 ± 0.02
Non-pathogen				
F. oxysporum	0.149 ± 0.08	0.177 ± 0.01	0.187 ± 0.04	1.245 ± 0.03
Non-host				
G. max	0.222 ± 0.03	0.283 ± 0.05	0.211 ± 0.06	0.432 ± 0.08
L. leucocephala	0.226 ± 0.02	0.251 ± 0.08	0.199 ± 0.07	0.346 ± 0.06
O. sativa	0.274 ± 0.04	0.230 ± 0.06	0.192 ± 0.02	0.239 ± 0.03

Note: * ± Standard error; IgG concentration 40 μg ml^{-1}; Absorbance values were means of three separate experiments.

4.3. Cellular localization of major cross reactive antigens shared by *E. vexans* and *Camellia sinensis*

In phytopathological studies it is also important to learn the host parasite relationship at the cellular level. The presence of cross-reactive antigens (CRA) between plant host and parasite that in some instances reflect degrees of compatibility in the parasite association is well known. The unique presence of CRA in hosts and parasites continues to suggest a regulatory role of CRA in host-parasite specificity. To achieve this antibodies labeled with fluorescein isothiocyanate (FITC) were used to determine location of CRA in sections of tea leaves and fungal cells, which provided positive results.

4.3.1. Indirect immunofluorescence

4.3.I.1. Healthy leaf

Indirect immunofluorescence using PAb-EV1 labeled with FITC were used to determine the location of CRA in leaf tissues. Fresh cross-sections of healthy tea leaves of susceptible (T-78, T-135 and UP-9) as well as resistant varieties (T-17/1/54, BS-7A/1/76 and BSS-3) were cut through the midrib and treated with normal, homologous (PAb-T17) and heterologous (PAb-EV1) antibody separately, labelled with FITC conjugate and observed under UV fluorescence conditions. The leaf sections exhibited a natural bright yellow autofluorescence (Plate 12, fig. A) mainly on the cuticle and sclereids present in the mesophyll tissue. Similar results were noted when the leaf sections were treated with normal antiserum and labelled with FITC. On the other hand leaf sections treated PAb-EV1, reacted much more strongly to FITC and exhibited apple green fluorescence. Leaf sections treated with PAb raised against leaf antigen and then reacted with FITC developed bright fluorescence, which was distributed throughout the leaf tissue. PAb-EV1 was most reactive with leaf sections of susceptible tea varieties recorded by visual estimation compared with resistant ones. Interestingly, occurrence of phenolic compounds was distinctly visible in sections of BS-7A/1/76, a resistant variety (Plate 12, fig. C) indicating existing constitutive phenolics as defense components. Of much significance was the strong reaction of PAb-EV1 with sections of T-135, a susceptible variety (Plate 12, fig. E) compared to all treatment combinations. Here reaction with FITC developed bright

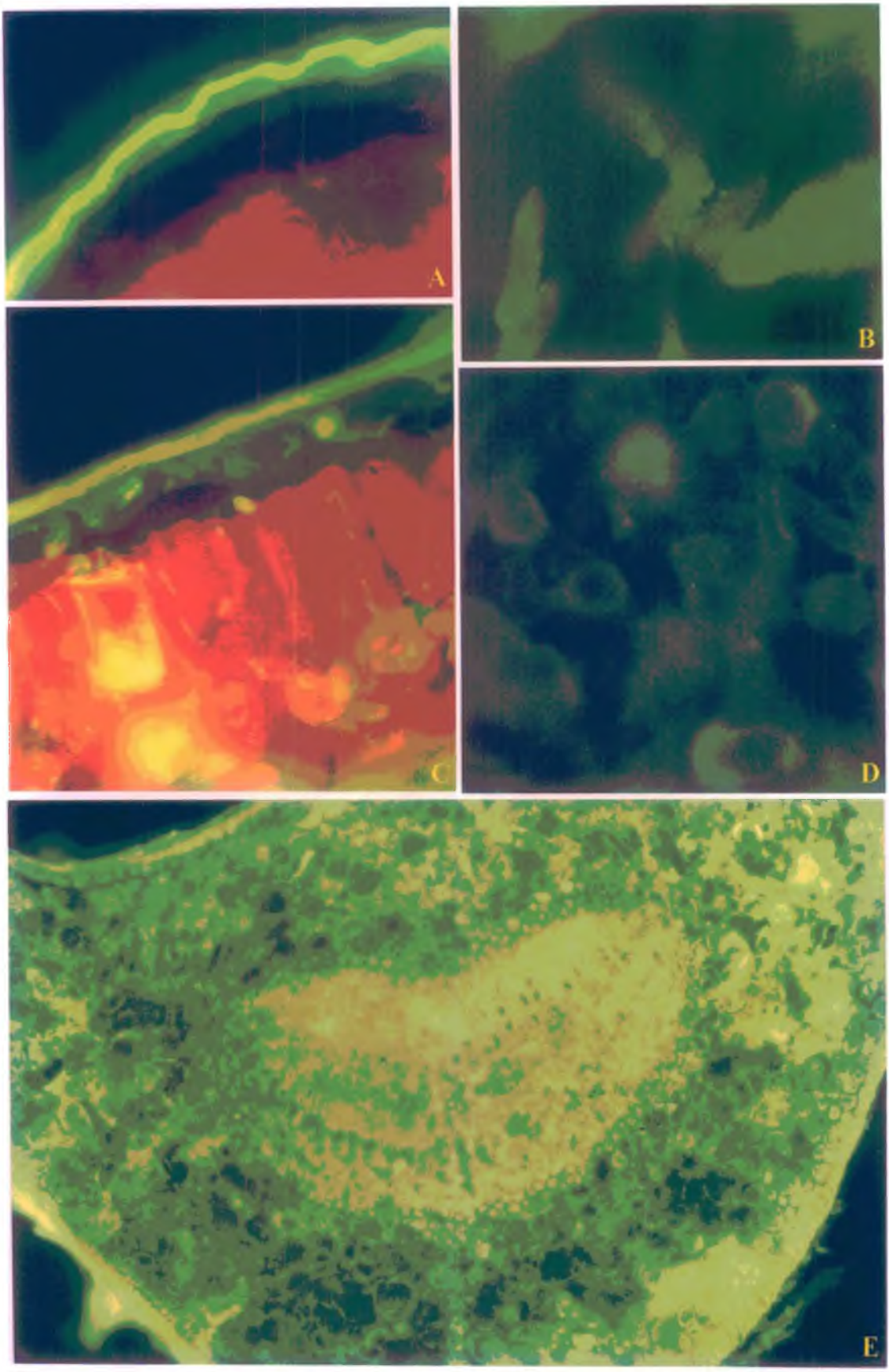


Plate 12 (figs. A – E). Cellular localization of major cross reactive antigens shared by *E. vexans* and *C. sinensis*. Autofluorescence of tea leaf tissues treated with normal serum [A]. PAb-EV1 treated and FITC labeled cross sections of healthy tea leaves of resistant variety – BS-7A/1/76 [C], susceptible variety – T-135 [E] and spores of *E. vexans* [B & D].

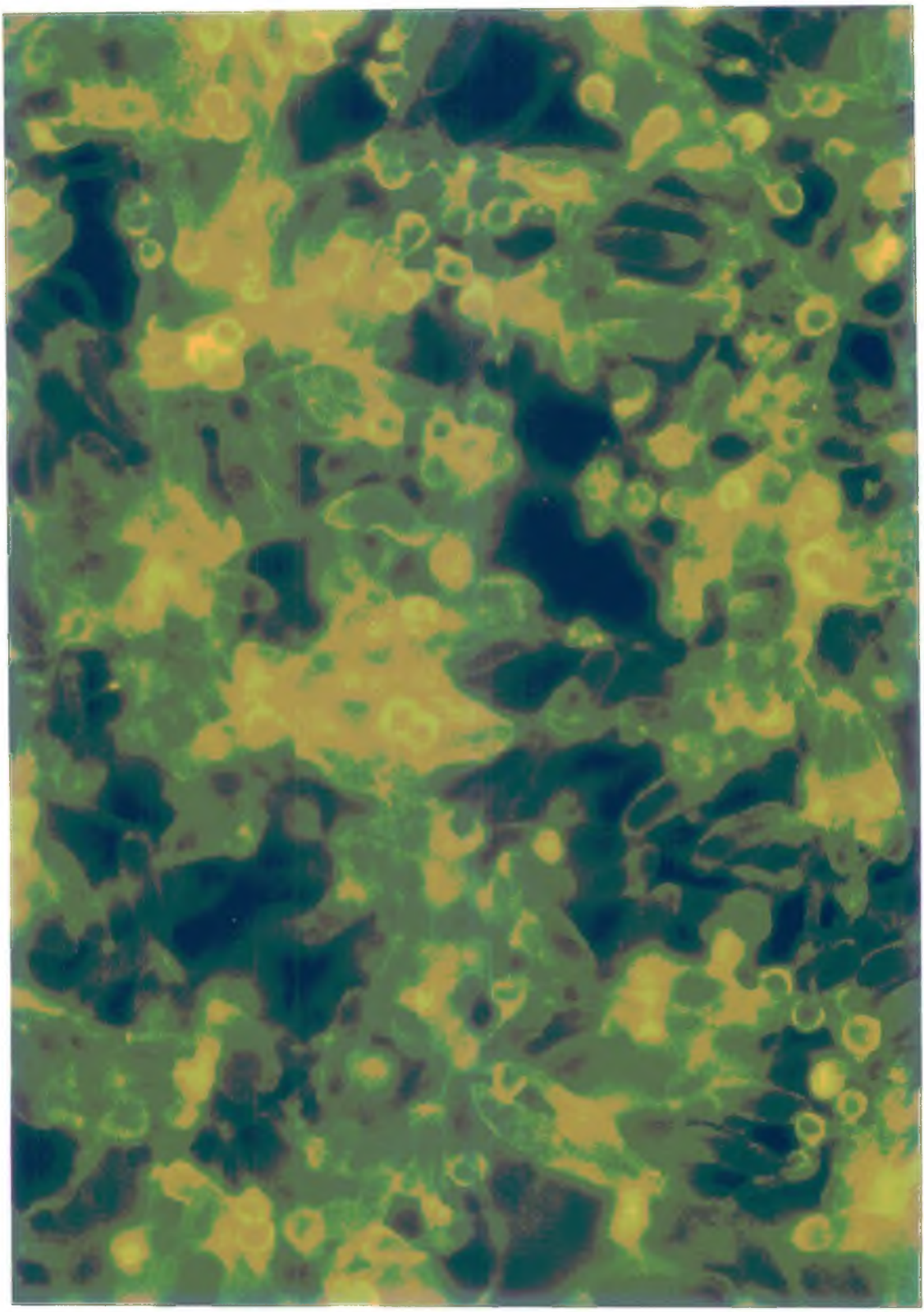


Plate 13. Concanavalin-A FITC binding of basidiospores of *E. vexans*.

fluorescence that was distributed throughout the leaf tissues but mainly over the epidermal cells, mesophyll tissues and xylem elements. It appears that CRA may form a continuum between cells of host and parasite, which favours the growth and establishment of the parasite.

4.3.I.2. Basidiospores of *E. vexans*

Indirect fluorescent antibody tests using PAb-EV1 were conducted with isolates – EV1, EV2 and EV3. The spores were observed both under bright field illumination and UV-light for comparison. The spores were not autofluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of the spores with homologous antiserum showed a general fluorescence along the spore walls indicating presence of macromolecules with recognisable antigenic epitopes, however cell definition was absent. Hence, the antibody proved it's specificity and based on the color reaction, the isolates of *E. vexans* could be identified (Plate 12, figs. B & D).

4.3.1.3. Fluorescent-labelled Concanavalin-A binding

Lectins have proved to be useful tools for locating and identifying lectin-recognizable glycoconjugate components on cell surfaces. In an attempt to determine whether the glycoproteins were present at the spore surface of *E. vexans*, FITC Con-A was used as the probe. Basidiospores intensely bound FITC Con-A, which was visible under UV- illumination (Plate 13).

4.3.2. Indirect immunogold labeling

The purpose of the ultrastructural immunocytochemical studies was to locate cross-reactive antigenic sites in tea leaf tissues shared by *E. vexans*. Ultrathin sections of healthy leaves were treated separately with normal antiserum and PAb-EV1 followed by uranyl-acetate staining. Tissue cross sections incubated with normal serum instead of primary antibodies did not show any labelling (Plate 14, figs. A - D). Electron microscopic observations of tea leaf tissues treated with PAb-EV1 and labeled with antirabbit IgG (whole molecule) gold conjugate (10 nm) showed specific localization of the antibody in certain intercellular structures (Plate 14, figs. E - H). Extracellular labeling was also noted.

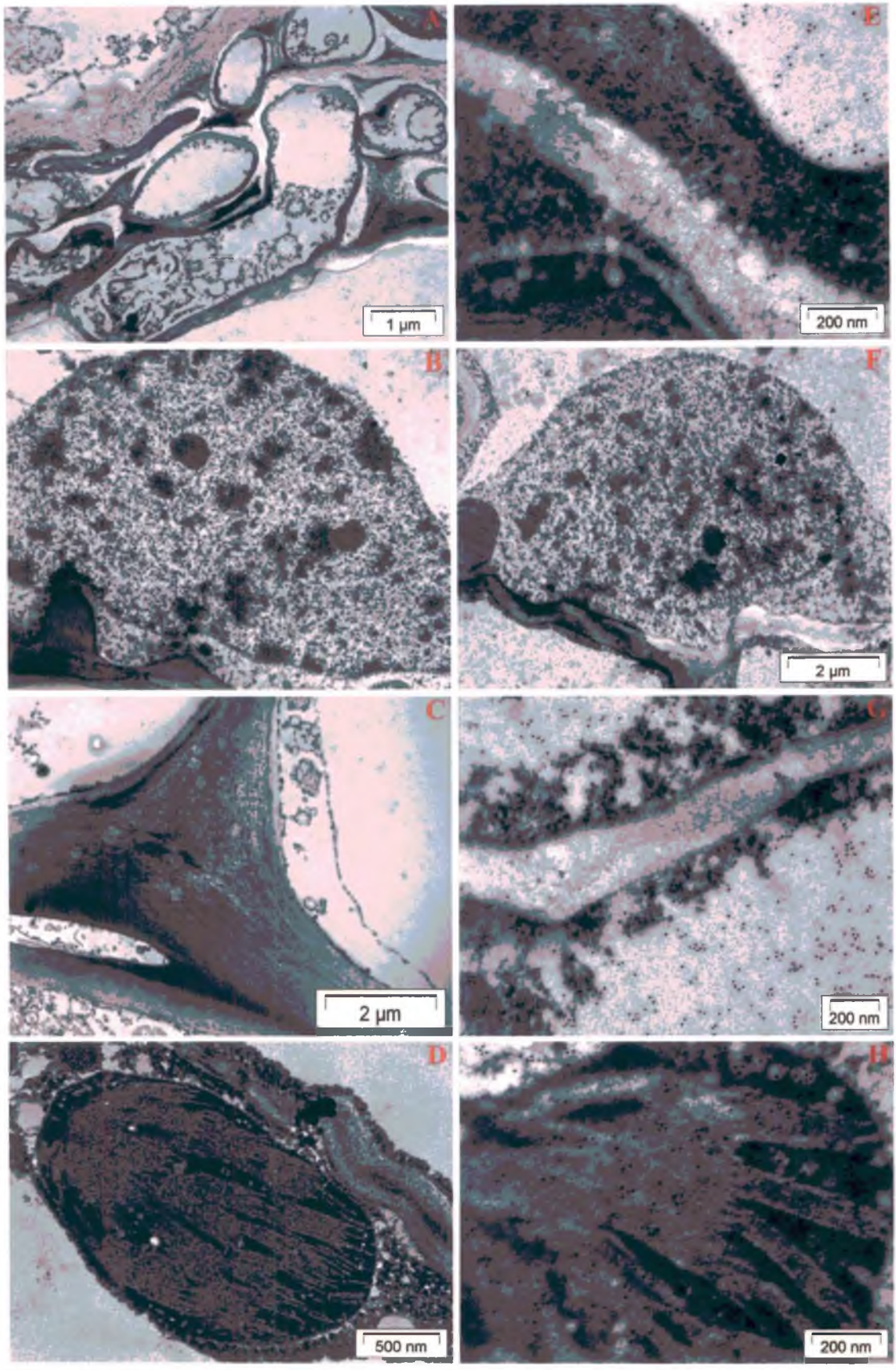


Plate 14 (figs. A - H). Transmission electron micrographs of healthy tea leaf tissues stained with uranyl acetate [A - D] and immunogold labelled [E - H] probed with PAb of *E. vexans*.

4.4. Immunodetection of *E. vexans* in tea leaf tissue

The PTA-ELISA is very sensitive and has proved valuable in detecting infection before macroscopic symptoms appear. Such detection techniques make it possible to detect microquantities of the pathogen within a few hours of infection, which is much more advantageous than the conventional techniques involving pathogen inoculation, visible symptoms and microscopy. These have tremendous potential for plant disease control measures since detection of a pathogen at the initial stages of infection can lead to formulation of control measures before much harm has been done. At present, immunoassays are also being used for various other purposes such as localization of pathogens within tissues and identification of specific antigens in electrophoretically separated components. It was of interest to determine whether *E. vexans* infections were detectable in tea leaf tissues using immunoassays such as PTA-ELISA, Dot-blot and Western blot.

4.4.1. Natural infection

Natural blister blight infected tea leaves were collected from two different tea gardens of Darjeeling hills - Margaret's Hope and Castleton; and one of the plains - Hansqua. Six different sections and three different months (July through September for hills and December through February for plains) were considered for sampling. Percentage infection of blister blight disease in those sections during the collection periods was also recorded.

4.4.1.1. PTA-ELISA

Antigens prepared from blister infected tea leaves of the different sections and corresponding healthy samples were tested in PTA-ELISA at antigen coating concentrations of 50 μg protein ml^{-1} and probed with PAb-EV1. PTA-ELISA values for blister infected extracts were higher than the healthy controls at the same coating concentrations and at all antiserum dilutions (Table 11).

Table 11. Detection of *E. vexans* in natural blister infected tea leaves using PTA-ELISA format

Tea Estate	Section	PTA-ELISA values A405 nm Leaf antigens (50 μg ml^{-1})		Percentage infection (%)
		Healthy	Infected	
Margaret's	1	0.247 ± 0.3	0.992 ± 0.7	35.7
Hope	2	0.299 ± 0.3	1.389 ± 0.3	73.9
	3	0.329 ± 0.4	0.826 ± 0.7	42.8
	4	0.281 ± 0.6	0.794 ± 0.8	96.6
	5	0.297 ± 0.8	1.456 ± 0.5	84.6
	6	0.484 ± 0.0	1.652 ± 0.4	32.4
Castleton	1	0.478 ± 0.0	1.700 ± 0.4	88.3
	2	0.456 ± 0.3	0.888 ± 0.3	72.5
	3	0.401 ± 0.6	1.473 ± 0.7	87.2
	4	0.347 ± 0.0	1.351 ± 0.1	78.9
	5	0.414± 0.3	0.851 ± 0.3	65.2
	6	0.459 ± 0.8	1.111 ± 0.3	71.1
Hansqua	1	0.209 ± 0.3	0.812 ± 0.8	43.7
	2	0.349 ± 0.4	1.442 ± 0.3	77.9
	3	0.299 ± 0.4	1.312 ± 0.5	74.8
	4	0.227 ± 0.8	0.707 ± 0.1	33.6
	5	0.279 ± 0.7	1.296 ± 0.7	70.2
	6	0.354 ± 0.3	0.801 ± 0.8	30.9

Note: * ± Standard error; PAb-EV1, IgG concentration 40 μg ml^{-1}; Absorbance values were means of three separate experiments.

4.4.1.2. Dot immunobinding assay

Healthy tea leaf antigens and antigens prepared from the different stages of infection were coated on nitrocellulose membranes and reacted with PAb-EV1 following the protocol of Dot-blot assay. Employing NBT/BCIP as substrate the reaction produced violet colored dots. Results revealed a variation in the color intensity of all stages of infection. The antigens obtained from Stage-3 had the deepest intensity, followed by Stage 1 and 2 that were more or less in the same order of strength with a moderate intensity. The healthy samples had lighter dots when compared with those of infected and homologous samples (Table 12).

Table 12. Detection of *E. vexans* in natural blister infected tea leaves by dot-immunobinding assay

Tea Estate	Stage of disease development	Dot-blot assay
Margaret's Hope	Healthy	+ + +
	Infected	
	Initial (S1)	+ + + +
	Moderate (S2)	+ + + +
	Severe (S3)	+ + + + + +
	Hypersensitive reaction (HR)	+ +
Castleton	Healthy	+ + + +
	Infected	
	Initial (S1)	+ + + + +
	Moderate (S2)	+ + + + +
	Severe (S3)	+ + + + + +
	Hypersensitive reaction (HR)	+ +
Hansqua	Healthy	+ + +
	Infected	
	Initial (S1)	+ + + + +
	Moderate (S2)	+ + + + +
	Severe (S3)	+ + + + + +
	Hypersensitive reaction (HR)	+ +

Note: PAb-EV1, IgG concentration 40 μg ml^{-1}; Colour intensity of dots: + insignificant, + + light violet, + + + violet, + + + + deep violet, + + + + + deeper violet, + + + + + + deepest violet; NBT/BCIP was used as substrate in dot blot assay.

4.4.2. Time course detection of pathogen

Leaf antigens were extracted from nine tea varieties (Darjeeling – T-78, T-135; UPASI – UP-9, BSS-2, UP-2, UP-3, UP-17; and Tocklai – TV-18, TV-9) after artificial inoculation with *E. vexans* at 1-day intervals for 12 days. These antigens (50 μg ml^{-1}) along with healthy leaf antigens were reacted with PAb-EV1 (40 μg ml^{-1} IgG concentration) using PTA-ELISA formats. Plants inoculated with *E. vexans* reacted first after 24 h of inoculation (T-135, T-78 and UP-9) where ELISA values were slightly higher than for healthy controls (Table 13). At 48 h after inoculation, ELISA values significantly increased for all nine varieties tested at the same time in comparison to healthy controls. At that time point no apparent disease symptoms were visible on leaves in any of the varieties inoculated with *E. vexans*. Detection of pathogen was possible by ELISA much before the symptoms appeared on inoculated leaves. Symptoms generally appeared 11 d after inoculation with *E. vexans* and their ELISA response with PAb-EV1 was demonstrated as early as 24 - 48 h of entry of the pathogen in the host tissues (Figure 4). The format proved to be sensitive, reliable and reproducible to detect the pathogen in tea leaf tissues.

4.4.3. Characterization of pathogen induced protein in tea leaf tissues

Tea varieties showed clear differential responses in developing either a compatible or an incompatible reaction in response to infection by *E. vexans*. Soluble proteins were extracted from healthy and artificially inoculated (with *E. vexans*) plants of three tea varieties (T-78, UP-2, TV-18) after 20 days when visible symptoms appeared and separated by SDS-PAGE. Protein profiles were analysed and their molecular weights were determined. Upon electrophoretic comparison, proteins from these tea varieties displayed similar but not identical ranges of mobility (Plate 15, fig. A). Protein bands ca. 94, 65, 45, 28 and 19 kDa were present in homogenates from both compatible and incompatible interactions, while protein bands 58 and 15 kDa were present in compatible interactions only. At this stage of infection fungal growth was significantly more advanced than in incompatible interactions as confirmed by PTA-ELISA formats.

Table 13. PTA-ELISA values obtained with extracts of different tea varieties at different times after inoculation with *E. vexans*

Days after inoculation	PTA-ELISA values A405nm with SE* Leaf antigen (50 μg ml^{-1})					
	T-135		UP 9		T-78	
	H	I	H	I	H	I
1	0.756 ± 0.05	0.819 ± 0.05	0.635 ± 0.02	0.704 ± 0.04	0.731 ± 0.04	0.799 ± 0.03
2	0.723 ± 0.03	0.859 ± 0.01	0.647 ± 0.07	0.813 ± 0.02	0.743 ± 0.07	0.837 ± 0.02
3	0.745 ± 0.02	0.893 ± 0.05	0.659 ± 0.09	0.899 ± 0.03	0.742 ± 0.02	0.948 ± 0.03
4	0.728 ± 0.02	0.942 ± 0.02	0.661 ± 0.03	0.911 ± 0.04	0.743 ± 0.05	0.976 ± 0.05
5	0.739 ± 0.07	0.999 ± 0.03	0.624 ± 0.05	0.936 ± 0.05	0.752 ± 0.06	0.990 ± 0.07
6	0.747 ± 0.03	1.195 ± 0.05	0.663 ± 0.07	0.968 ± 0.03	0.768 ± 0.01	1.141 ± 0.01
7	0.749 ± 0.09	1.228 ± 0.02	0.699 ± 0.01	0.999 ± 0.08	0.771 ± 0.04	1.278 ± 0.07
8	0.752 ± 0.03	1.348 ± 0.08	0.612 ± 0.08	1.104 ± 0.02	0.747 ± 0.06	1.377 ± 0.03
9	0.753 ± 0.09	1.432 ± 0.07	0.642 ± 0.02	1.251 ± 0.06	0.739 ± 0.08	1.479 ± 0.03
10	0.750 ± 0.05	1.542 ± 0.03	0.683 ± 0.06	1.384 ± 0.02	0.726 ± 0.04	1.582 ± 0.04
11	0.767 ± 0.03	1.627 ± 0.06	0.689 ± 0.02	1.500 ± 0.06	0.748 ± 0.06	1.733 ± 0.04
12	0.757 ± 0.08	1.746 ± 0.02	0.672 ± 0.02	1.696 ± 0.03	0.780 ± 0.03	1.897 ± 0.05

Note: * ± Standard error; PAb-EV1, IgG concentration 40 μg ml^{-1}; Absorbance values were means of three separate experiments; 50 plants were inoculated per variety.

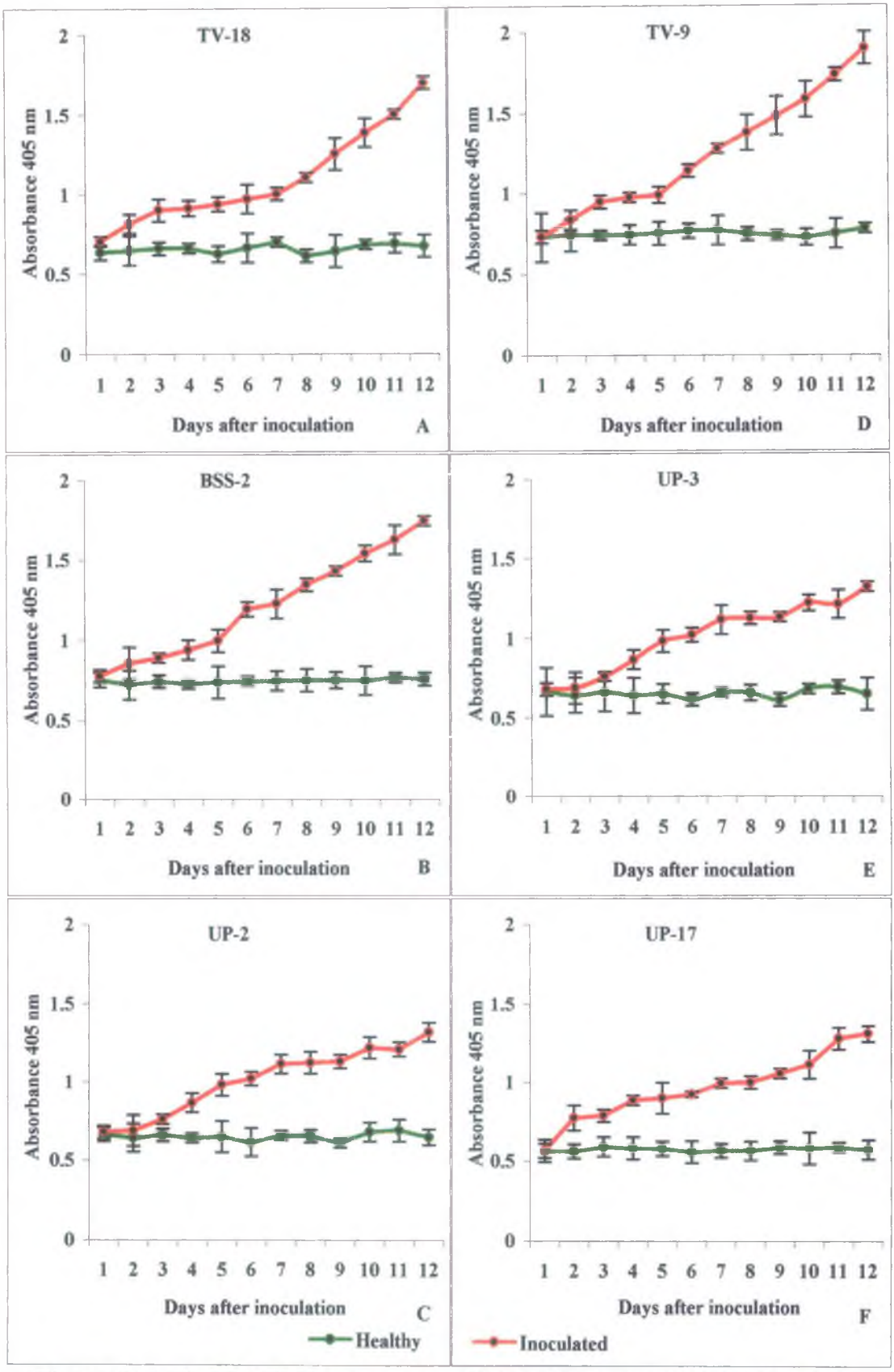


Figure 4 (A-F). PTA-ELISA responses of PAb of *E. vexans* with healthy and artificially inoculated tea leaf antigens at different intervals.

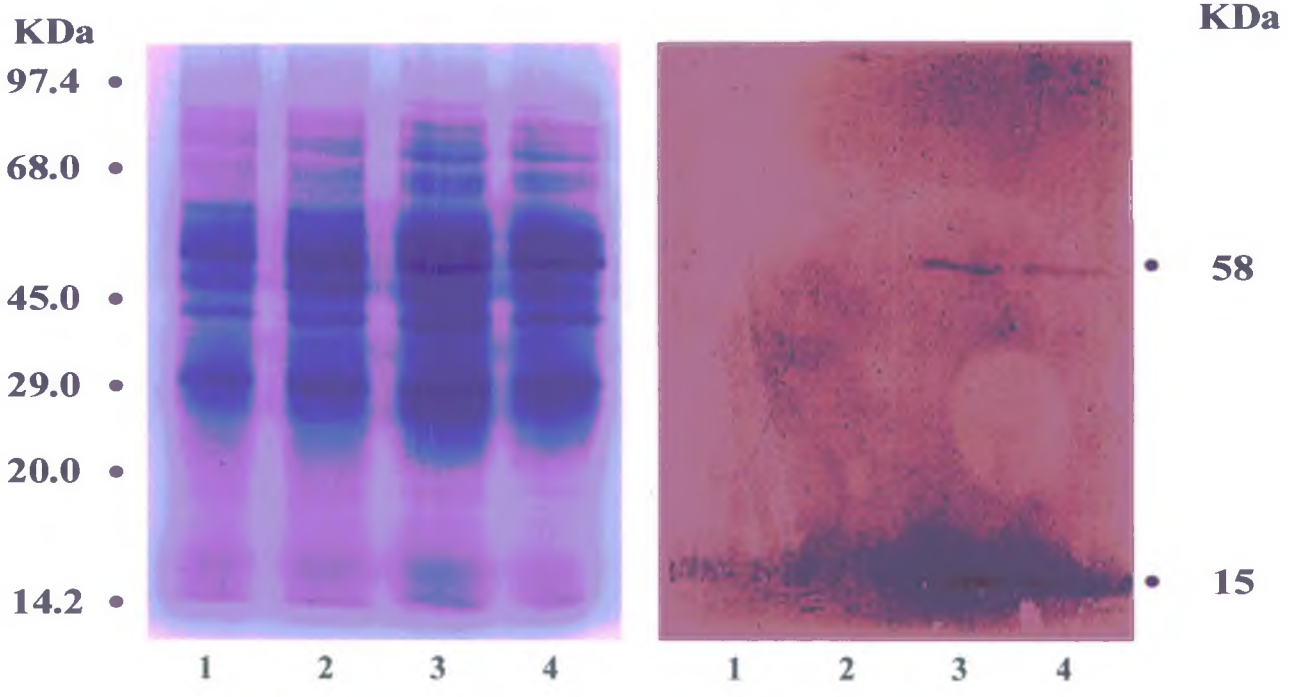


Plate 15 (figs. A & B): Pathogen–induced proteins. [A] SDS-PAGE, [B] Western blot. Lanes (1 & 2) healthy leaves; lanes (3 & 4) artificially inoculated tea varieties. T - 78 (1 & 3), UP - 2 (2 & 4).

In an attempt to determine if any of the proteins were of pathogen origin, immunological characterization of such proteins was done following western blots using the probe of *E. vexans* as well as the probe of tea leaf (PAb-T17). Accordingly one blot was incubated with PAb-EV1 and one with PAb-T17. On probing with PAb-EV1 recognition of two proteins of approximately 58 and 15 kDa was observed only in the *E. vexans*-inoculated leaf samples, which were absent in healthy leaf samples (Plate 15, fig B). These proteins were unique to the compatible interaction and were not recognized by PAb-T17. The proteins recognized by PAb-EV1 corresponded to protein bands revealed in SDS-gels.

4.5. Immunolocalization of *E. vexans* in blister infected tea leaf tissues

4.5.1. Non-fluorescent immunocytochemical staining

One of the unique formats for detection of pathogen in host tissues includes the use of a non-fluorescent immunoenzymatic stain based on specific antibodies. This immunoenzymatic assay has several advantages over conventional bioassays in determining the colonization pattern of fungi in host tissues. It allows direct detection of the hyphae present in the tissue and specificity of the stain is based on specificity of the serum. This technique could be more advantageous than fluorescent antibody staining techniques, as it does not require the use of fluorescence microscopes. The sensitivity and specificity of the antibody raised against *E. vexans* (PAb-EV1) has already been established by PTA-ELISA in the previous chapters. In the present study one resistant (TV-30) and two susceptible varieties (T-78 and UP-2) were selected for development of a specific immunocytochemical staining procedure for detection and cellular localization of pathogen in tea leaf tissues.

4.5.1.1. Growth of *E. vexans* in artificially inoculated plants

Colonization by the pathogen in cross sections of leaf tissues of artificially inoculated resistant and susceptible plants was studied employing PAb-EV1 following a specific immunoenzymatic-staining format with Fast Blue BB salt. Hyphae of *E. vexans* were detected growing within the palisade and across the mesophyll tissues of inoculated leaves, which were absent in leaf sections of healthy samples (Plate 16, fig. A).

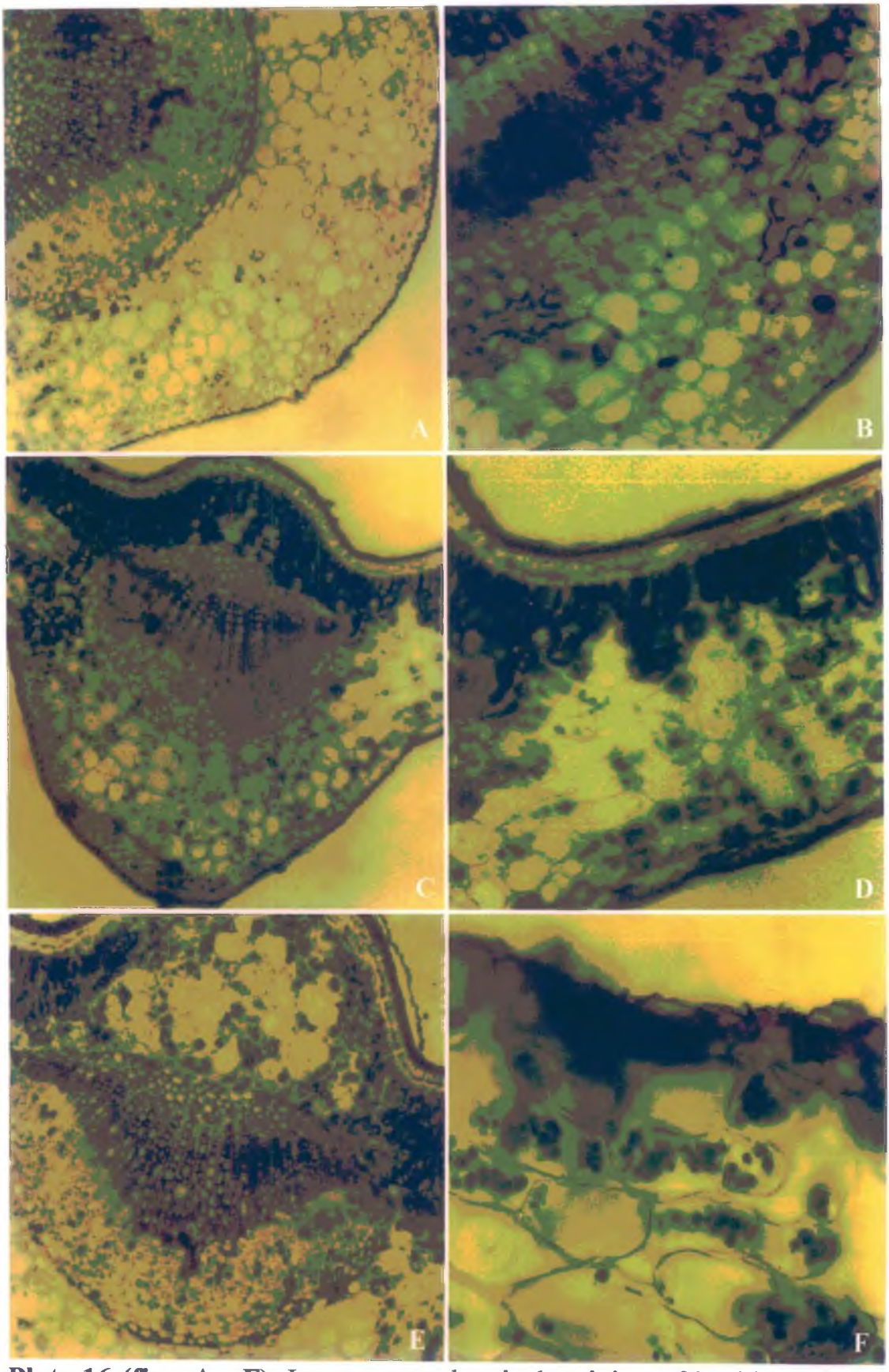


Plate 16 (figs. A - F). Immunocytochemical staining of healthy [A] and *E. vexans* inoculated [B - F] leaf tissues with Fast Blue BB salt probed with PAb of *E. vexans*. TV-30 (A & B); T-78 (C & D); UP-2(E & F).

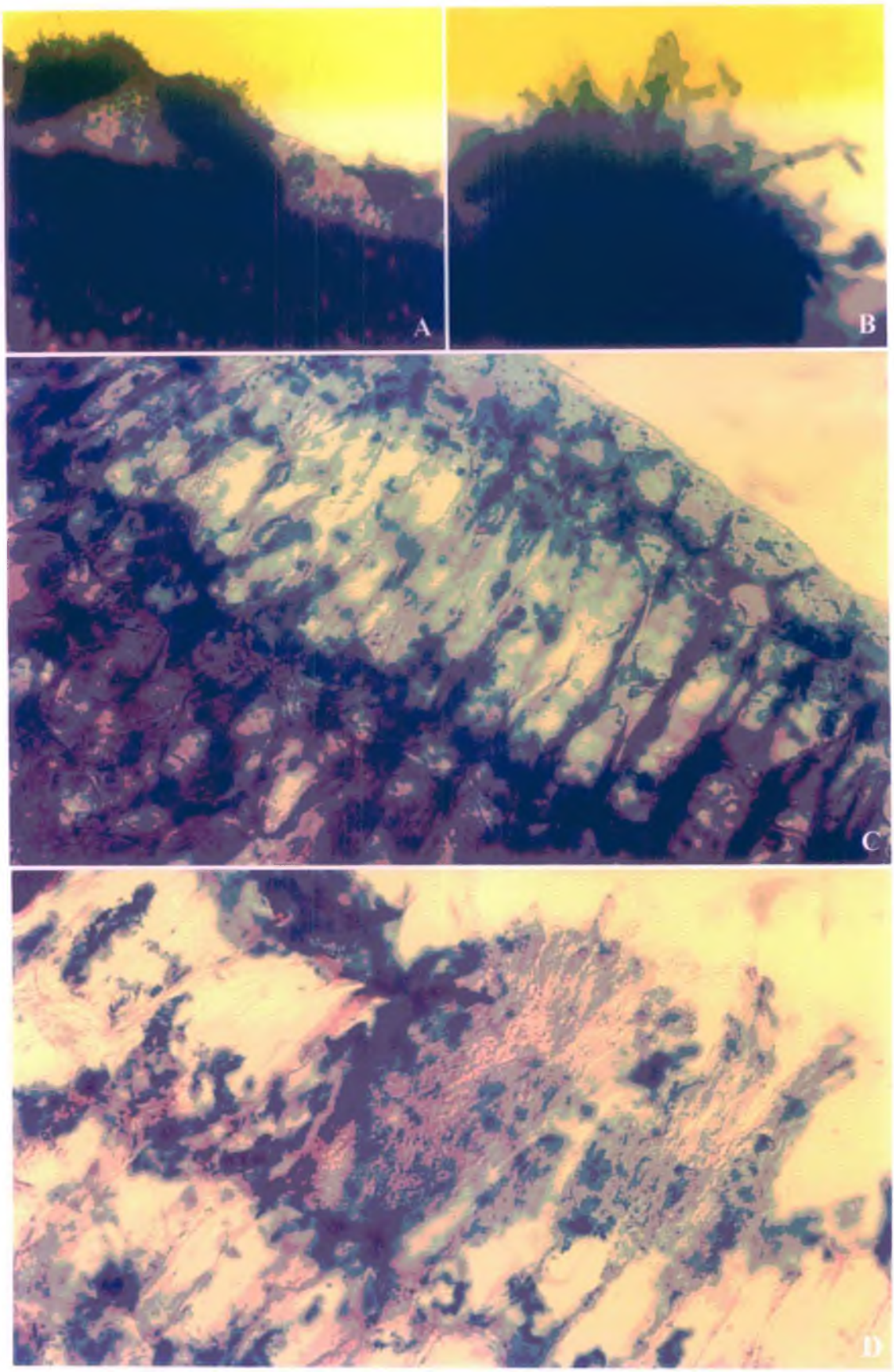


Plate 17 (figs. A - D). Cross sections of blister blight infected tea leaf probed with PAb of *E. vexans* stained with Fast blue BB salt.

In cross sections obtained from leaves after 1 d of inoculation a few mildly stained cells were observed. Intense staining reactions in inoculated samples after 8 d of incubation gave an indication of successful penetration of the fungus in the leaf tissues. There always appeared to be more fungal mass in susceptible varieties (Plate 16, figs. C - F) than in the resistant ones (Plate 16, fig. B).

4.5.1.2. Colonization of *E. vexans* in natural infection

Colonization of *E. vexans* in field grown tea leaves was also studied by using PAb-EV1 following immunoenzymatic staining with Fast Blue BB salt. Blue coloration of the colonies in the foliar tissues revealed its localization in cellular compartments (Plate 17, figs. A - D). Fungal mycelium and spores stained with Fast blue BB salt was quite distinct on the epidermal cell in infected samples (Plate 17, figs. A & B) with lack of stain in healthy (control) ones. Pathogen was well established and had ramified through the intercellular spaces of the mesophyll tissues (Plate 17, figs. C & D).

4.5.2. Indirect immunofluorescent antibody staining of infected leaves

Naturally blister infected tea leaves were considered for this experiment. Cross sections of leaves were treated with normal antisera and PAb-EV1 separately and labelled with FITC. Healthy cross sections were autofluorescent along the cuticle (Plate 18, fig. A). Normal antisera did not show reactivity with healthy or infected samples. Blister infected leaf samples colonized with *E. vexans* showed strong fluorescence in the palisade and spongy parenchymatous tissues (Plate 18, figs. B - F). Basidia with basidiospores exhibited bright green apple fluorescence (Plate 18, figs. E & F).

4.5.3 Immunogold labelling of blister infected leaves

Ultrastructural and immunocytochemical studies on leaves affected by the blister blight pathogen (*E. vexans*) showed specific localization of the antibody on the fungal cell wall and certain intercellular structures. Extracellular labelling was also noted.

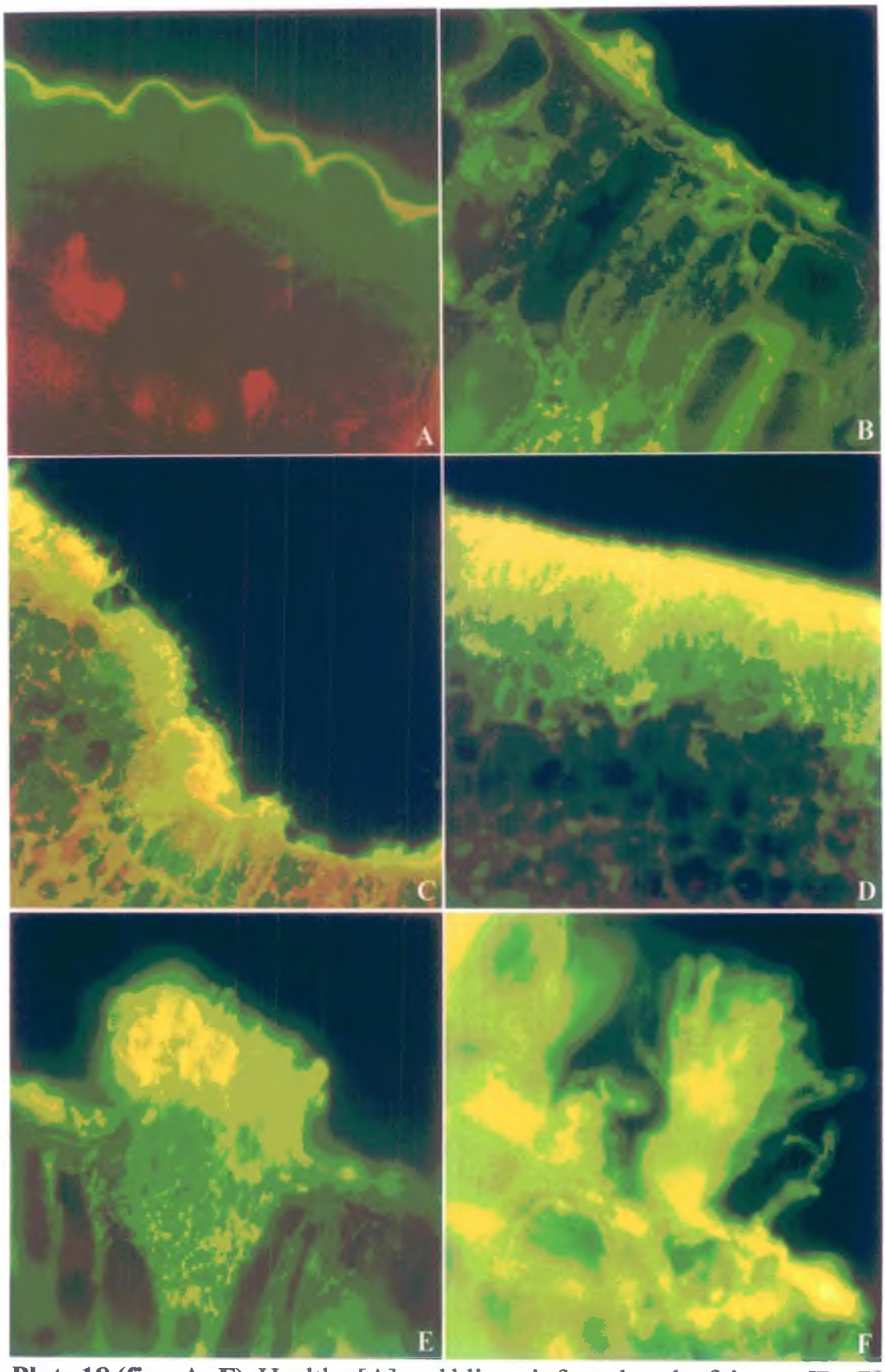


Plate 18 (figs. A- F). Healthy [A] and blister infected tea leaf tissues [B - F] treated with PAb of *E. vexans* and labelled with FITC.

The purpose of the ultrastructural immunocytochemical studies was to determine sites of antibody binding on the fungus and also to determine whether extracellular binding of the antibody occurred in tea leaf tissues infected by *E. vexans*. Fixation had an important effect on the ultrastructural quality and antigenic response. Post embedding immunocytochemical labelling of healthy and blister infected leaf segments was performed on sections of LR-White embedded tissues, previously fixed with 0.1 M sodium phosphate buffered –glutaraldehyde (3 %) and using PAb-EV1 and labeled with antirabbit-IgG (whole molecule) gold conjugate (10 nm).

Fungal mass in the cellular compartments was intensely labelled by PAb-EV1 Spore and hyphal cell walls reacted intensely. Labelling also occurred on hyphal intracellular membrane structures including invaginations of the outer cell membrane. Hyphae lacking cellular components and apparently dead also reacted strongly. Extracellularly the antisera reacted with leaf cells undergoing degradation and were apparent as the cells were deformed and lost their orientation (Plate 19, figs. A - H). Infected leaves contained *E. vexans* hyphae in the cell walls. In the host cells different degrees of labelling were observed. However, in infected tissue, gold particles were predominantly localized (Plate 20, figs. A – H). No labelling was detected in any other host cellular compartments. The gold particles observed on the surface appeared as either individual spherical particles covering the fungal surface varied in an even distribution or as clusters of particles (Plate 21, figs. A & B). Sections of host cells severely blister infected were strongly labelled confirming the presence of fungus in the cell while those of uninfected leaves of *C. sinensis* treated with *E. vexans* antibody (PAb-EV1) and then colloidal gold showed little gold labelling (Plate 14). Gold labelling in the sections showed a high amount of labelling in chloroplats and host cytoplasm and lesser amount in vacuoles, mitochondria and walls. Serological controls treated yielded negative results; i.e., gold labelling was absent or at uniformly low background levels. Thereby, specificity for labelling for *E. vexans* was demonstrated by control tests that showed complete abolition of gold labeling.

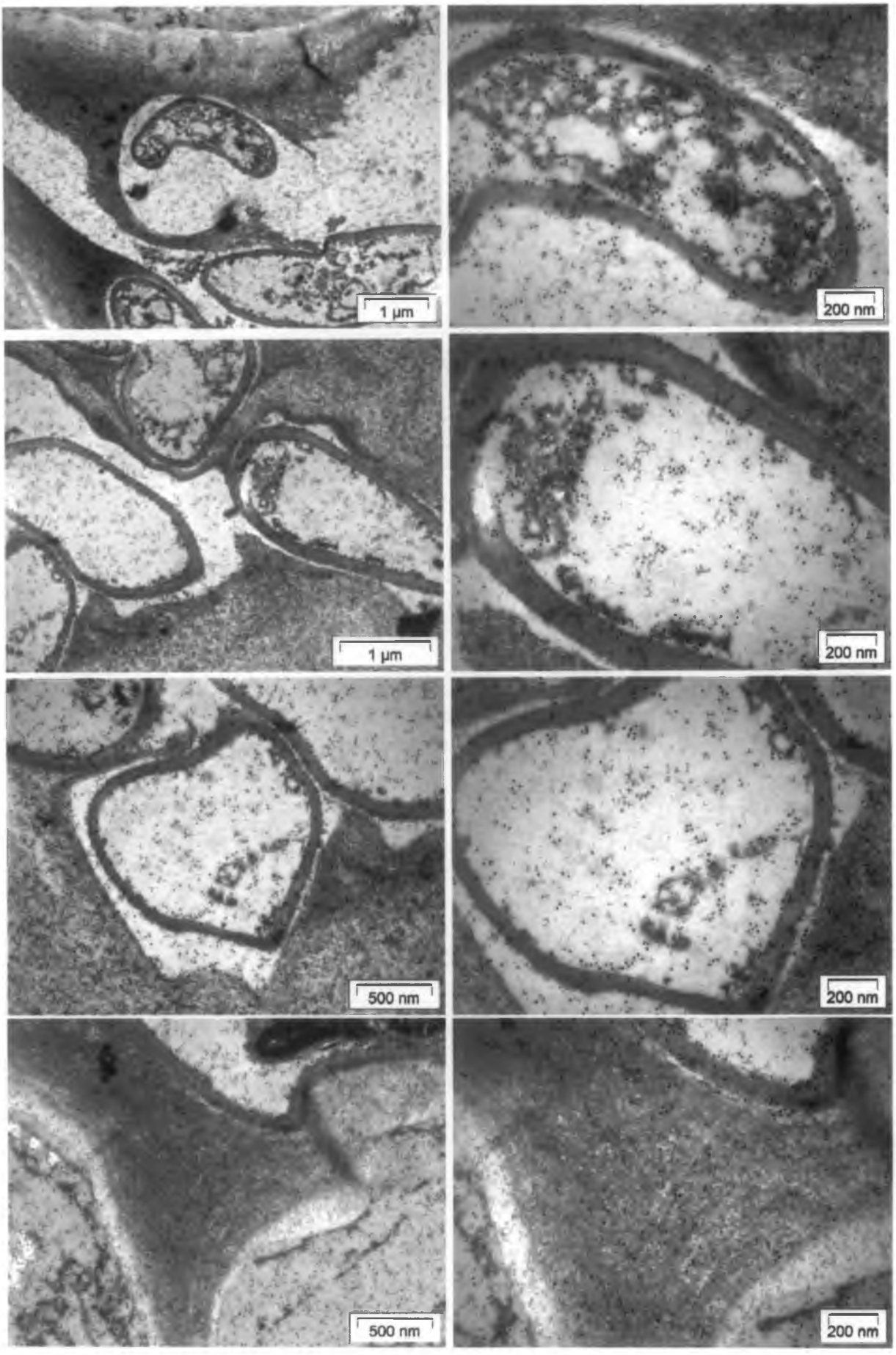


Plate 19 (figs. A - H). Transmission electron micrographs of immunogold labelled blister blight infected tea leaf tissues probed with PAb of *E. vexans*. [B, D, F & H- magnified views of A, C, E and G respectively].

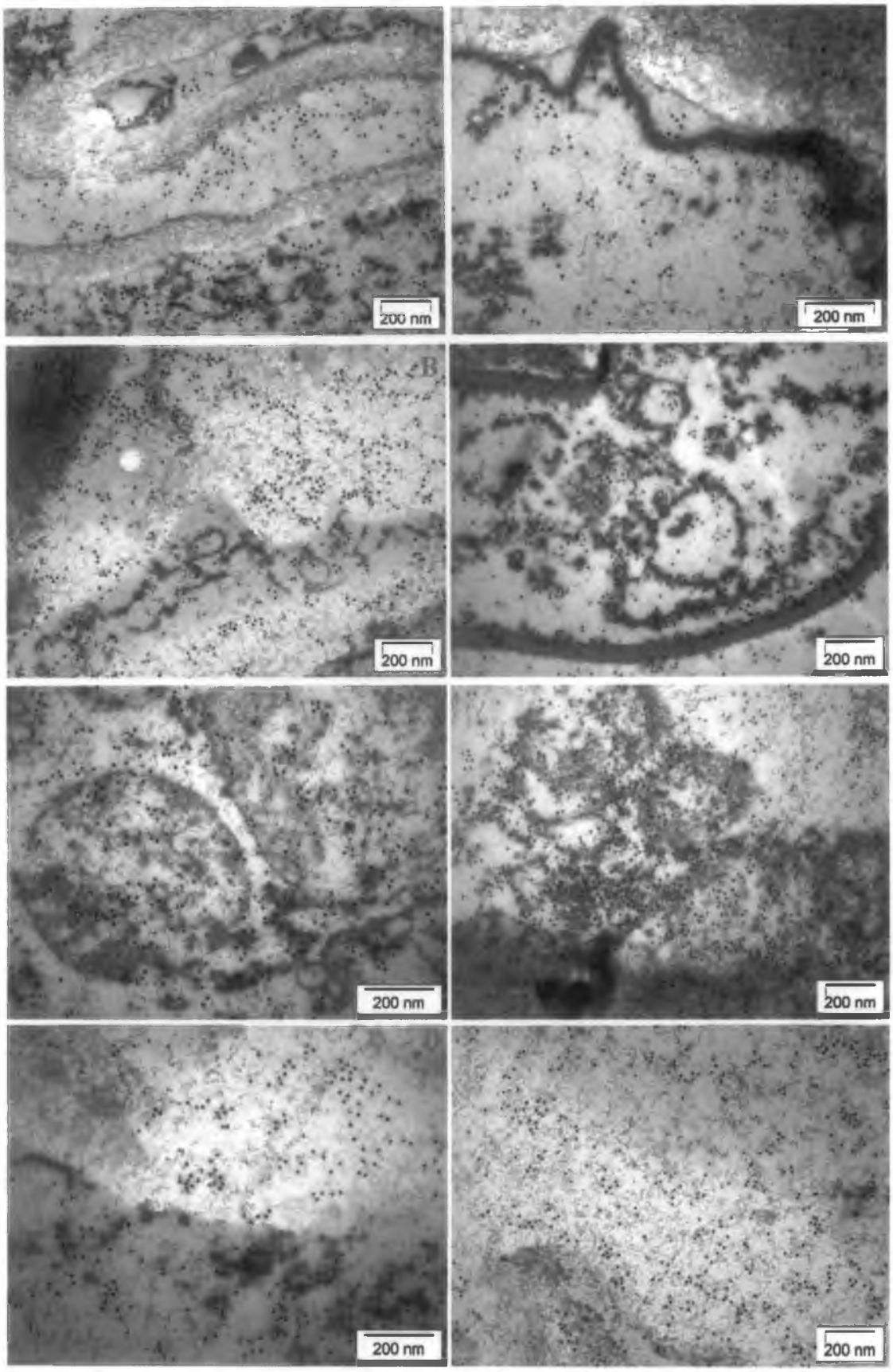


Plate 20 (figs. A - H). Transmission electron micrographs of immunogold labelled blister blight infected tea leaf tissues probed with PAb of *E. vexans*.

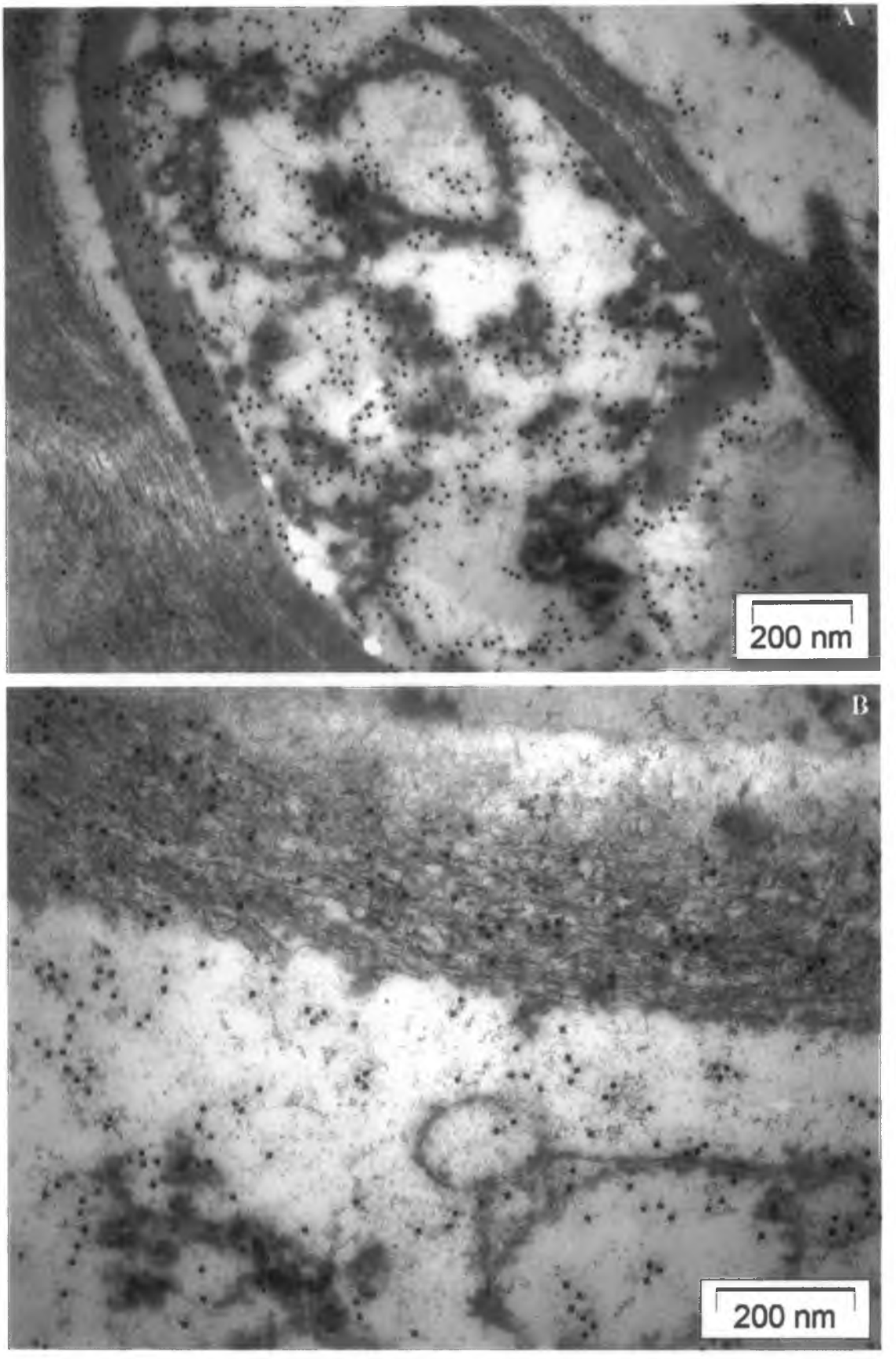


Plate 21 (figs. A & B). Transmission electron micrographs of immunogold labelled blister blight infected tea leaf tissues probed with PAb of *E. vexans* (magnified view).

4.6. Analyses of tea leaf proteins

The immune systems operating in tea plants were described by analyzing their protein profiles. Proteomic techniques were applied to study the protein patterns of tea varieties on SDS-PAGE gels followed by their immunological characterization.

4.6.1. Total soluble protein determinations in leaf homogenates

Proteins were extracted from thirty tea varieties of Darjeeling - T-78, T-135, AV-2, CP-1, HV-39, T-17/1/54, BS-7A/1/76, TS-449, P-1258, RR-17; Tocklai - TV-26, TV-18, TV-29, TV-23, TV-30, TV-22, TV-9, TV-25, TV-20, TV-27, TV-28 and UPASI - UP-3, UP-9, UP-26, BSS-2, UP-8, BSS-1, UP-2, BSS-3, UP-17 and were quantified (Table 14) followed by SDS-gel electrophoresis. Electrophoretic comparison, of proteins from different tea varieties displayed comparable ranges of mobility. Protein profiles on SDS-gels have been presented in (Plate 22, figs. A - C) and their molecular weights were determined by EDAS. These have been presented in Table 14. On staining the SDS-gels with Coomassie blue a large number of protein bands varying in molecular weight were revealed. Most prominent of these was a protein of approximately 48.5 kDa, which was constantly present in Tocklai as well as Darjeeling varieties and was observed in all but two of the UPASI varieties (UP-3 and UP-26). Further this band had a very high intensity in the Darjeeling varieties. A band of 66.2 kDa was common to all UPASI varieties. Another band of high molecular weight (85.2 kDa) that was dominant in most Darjeeling and UPASI varieties was not observed in the Tocklai varieties. The varieties in which this particular band was lacking revealed lack of another protein of high molecular weight (103 – 104 kDa). However a band of 100 kDa was common in Tocklai varieties (TV-25, TV-20, TV-22, TV-23, TV-27, TV-29 and TV-30). The low molecular weight proteins of the range 12.6 – 14.0 kDa were present in all varieties but the resolution of such bands was low mainly in Darjeeling varieties and in one Tocklai variety (TV-25). Thus among different varieties differences in protein patterns were evident.

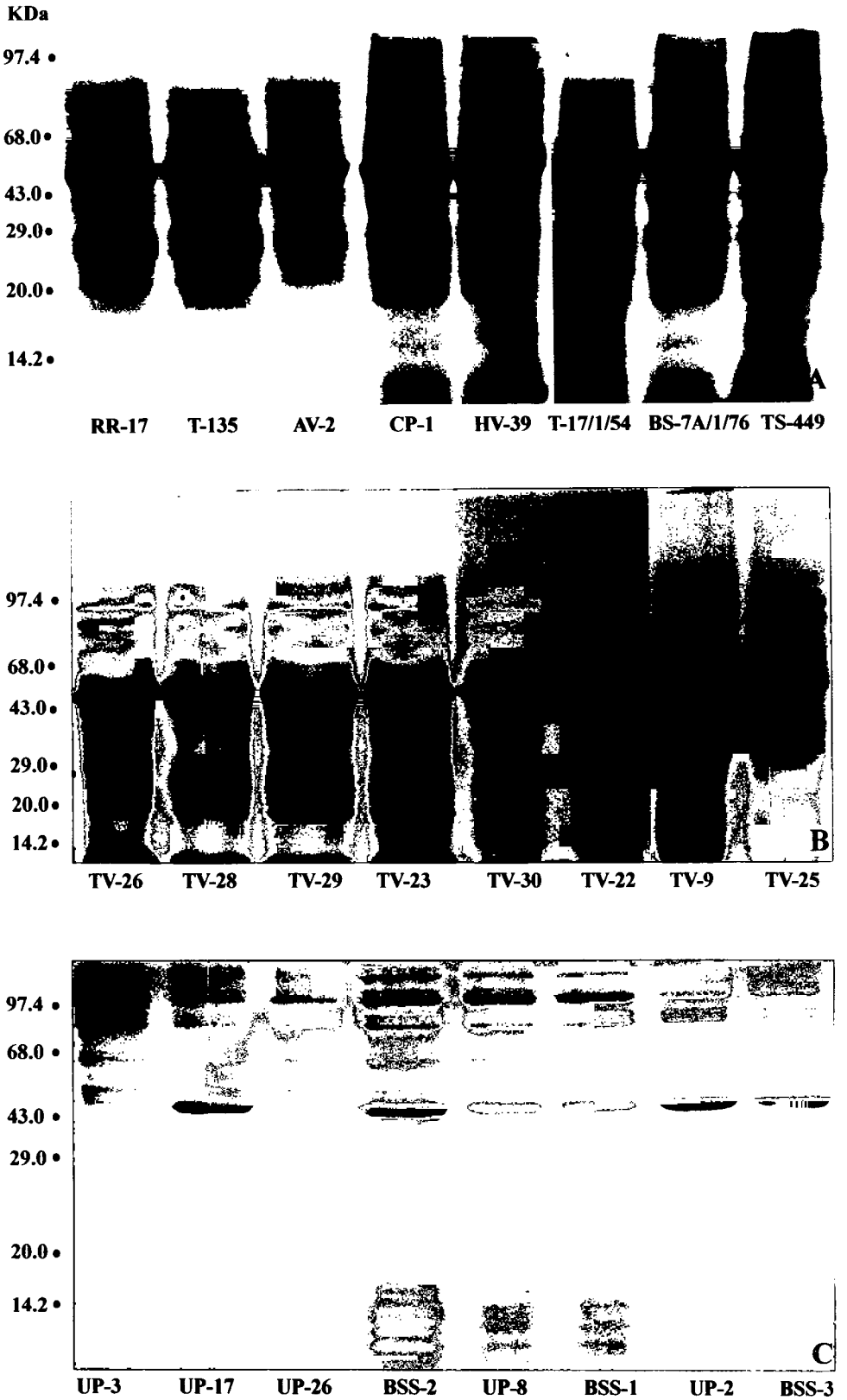


Plate 22 (figs. A - C). SDS-PAGE analysis of total soluble leaf proteins of Darjeeling [A], Tocklai [B] and UPASI [C] varieties of tea.

Table 14. Protein profiling of total soluble proteins of different tea varieties

Variety	Protein content (mg g^{-1} leaf tissue)	Molecular weight (kDa)
Darjeeling		
AV 2	42.21	103.2, 98.4, 85.2, 57.2, 52.0, 48.5, 37.2
BS 7A/1/76	34.64	103.6, 92.3, 85.2, 74.0, 68.0, 52.0, 48.5, 39.3, 37.7, 29.2, 28.2, 27.2, 26.8, 21.3
CP 1	44.26	103.8, 91.7, 85.2, 79.3, 48.5, 38.7, 37.3, 31.1, 28.9, 24.4, 19.0
HV 39	42.10	103.8, 92.3, 85.2, 60.2, 48.5, 28.0
P 1258	52.13	104.2, 97.7, 85.2, 48.5, 28.2, 15.6
RR 17	49.21	104.5, 95.5, 85.2, 58.2, 48.5, 15.8
TS 449	44.25	103.4, 91.7, 85.2, 65.8, 48.5, 15.6
T 135	35.64	103.0, 93.7, 85.2, 66.8, 58.2, 48.5, 14.2
T 17/1/54	47.88	103.8, 97.8, 68.4, 85.2, 48.5, 14.8
T 78	54.21	104.3, 94.9, 85.2, 65.6, 58.0, 45.5, 28.0, 15.2
UPASI		
BSS 1	34.36	104.1, 100.2, 85.2, 66.2, 58.3, 48.5, 15.6
BSS 2	38.26	103.0, 98.5, 89.5, 85.2, 80.7, 77.3, 66.2, 48.5,
BSS 3	45.00	103.8, 90.8, 85.2, 66.2, 48.5, 28.9
UP 2	35.25	104.8, 93.4, 85.2, 66.6, 58.5, 45.5, 28.6, 19.6, 15.6
UP 3	41.50	103.8, 89.6, 85.2, 66.2,
UP 8	37.54	103.6, 89.4, 85.2, 66.2, 48.5, 19.6, 15.6
UP 9	33.46	103.8, 94.4, 85.2, 66.2, 48.5, 19.6, 15.9
UP 17	43.25	104.2, 94.8, 85.2, 66.2, 48.2, 28.6, 15.8
UP 26	41.05	103.9, 90.0, 85.2, 66.2, 63.5, 19.0, 15.6
Tocklai		
TV 9	32.00	96.3, 67.5, 58.2, 48.5, 19.6, 15.6
TV 18	40.25	93.6, 65.9, 54.2, 48.5, 24.6, 19.0
TV 20	38.56	100.5, 91.7, 79.3, 48.5, 15.6
TV 22	37.25	100.9, 89.6, 48.5, 19.0
TV 23	34.25	100.6, 93.7, 48.5, 28.6
TV 25	40.50	100.8, 94.9, 48.5
TV 26	39.50	89.9, 58.9, 48.5, 28.6
TV 27	47.44	100.2, 66.5, 48.5, 28.6
TV 28	38.84	96.5, 48.5
TV 29	37.75	100.1, 68.2, 58.6, 48.5, 28.6
TV 30	36.40	100.0, 96.8, 87.2, 78.5, 48.5

Note: Proteins resolved in 10 % SDS-gels and molecular weights determined by Kodak EDAS 290 software

4.6.2. Acid soluble protein (PR) determination in leaf homogenates

Acid soluble PR-proteins were extracted from thirty tea varieties at pH 2.8 and subjected to electrophoresis in SDS-polyacrylamide gels. Reproducible results were obtained by extracting leaf homogenates using acidic extraction buffer. Varietal differences were observed when the gel patterns were examined (Plate 23, figs. **A** & B). The most prominent bands had molecular weights of less than 28.0 kDa. Differences were observed in number of PR-proteins in the different varieties. The appearance of at least 6 proteins was observed in both susceptible and resistant lines with the main difference between protein patterns being quantitative in nature. PR-proteins of 14 and 28 kDa were detected in all varieties with equal prominence, while a 31 kDa protein band was detected almost all varieties though the intensity varied. A 45 KDa protein was only detected in AV-2, BS-7A/1/76 and TV-30 and bands of 50-61 kDa were resolved in fifteen varieties (AV-2, BS-7A/1/76, P-1258, RR-17, TS-449, T-17, BSS-2, BSS-3, UP-2, UP-8, UP-9, TV-9, TV-18, TV-28 and TV-29) only. However bands in the range of 40-45 and 11-14 kDa protein were detected in all resistant and susceptible varieties. Slight variation was probably a reflection of genotypic differences as all plants were grown simultaneously under similar environmental conditions (Table 15).

4.6.2.1. PR-proteins in tea leaves following inoculation with *E. vexans*

It has already been established in the previous chapters that the infection process following artificial inoculation can be detected as early as 48 h. Keeping this in mind the plant material, for analysis of acid soluble PR-protein pattern, was harvested 0, 24 and 48 h after inoculation. SDS-PAGE analysis of proteins from susceptible plants (T-135, T-78, UP-9, TV-18 and CP-1) and resistant plants (T-17/1/54, BSS-3, TV-26, TV-30 and TV-29) was performed. Upon inoculation the induced plants accumulated more PR-proteins than the control plants, as shown in Plate 24, figs. A & B.

Table 15. Protein profiling of acid soluble proteins present in intercellular fluids of different tea varieties

Variety	Number of protein bands	Molecular weight (kDa)
Darjeeling		
AV-2	09	61.8, 45.2, 40.6, 37.2, 31.5, 17.2, 15.7, 14.0, 13.6
BS-7A/1/76	07	61.2, 45.4, 31.2, 19.9, 16.5, 14.2, 13.2,
CP-1	09	42.8, 39.5, 35.7, 31.7, 26.5, 14.1, 13.0, 11.4, 10.9
HV-39	05	31.9, 28.0, 19.9, 14.5, 12.7
P-1258	07	57.2, 42.5, 36.5, 31.0, 25.3, 14.6, 13.4
RR-17	07	51.6, 31.0, 28.5, 22.3, 20.4, 14.9, 13.3
TS-449	06	51.1, 31.6, 21.6, 14.0, 13.1, 12.7
T-135	09	46.7, 38.8, 33.6, 31.8, 19.3, 15.0, 14.3, 12.8, 11.2
T-17/1/54	09	54.4, 31.4, 23.1, 19.7, 16.2, 15.0, 14.8, 13.0, 10.3
T-78	09	40.1, 39.5, 36.0, 31.3, 28.7, 16.9, 14.7, 12.8, 11.6
UPASI		
BSS-1	12	48.5, 41.4, 31.0, 29.2, 25.3, 22.6, 18.9, 16.9, 15.5, 14.0, 11.7, 11.4
BSS-2	13	58.0, 36.9, 31.9, 29.2, 22.9, 20.7, 19.8, 17.5, 16.0, 14.8, 13.6, 12.1, 11.9
BSS-3	08	53.0, 31.9, 27.5, 21.4, 14.4, 13.0, 11.5, 10.9
UP-2	08	57.3, 49.8, 31.6, 28.3, 21.7, 14.5, 13.2, 11.9
UP-3	08	39.6, 31.3, 27.9, 22.6, 18.9, 14.6, 12.9, 12.3
UP-8	06	50.3, 31.3, 28.9, 19.3, 14.8, 13.3
UP-9	06	59.9, 31.6, 28.7, 19.4, 15.0, 14.0,
UP-17	09	39.3, 31.0, 28.6, 19.8, 19.0, 18.7, 18.3, 14.0, 13.2
UP-26	08	47.3, 32.7, 27.8, 19.3, 16.1, 14.2, 13.2, 11.6
Tocklai		
TV-9	08	53.5, 43.9, 31.4, 28.6, 22.0, 15.3, 14.7, 13.4
TV-18	08	53.2, 41.2, 31.5, 28.1, 20.0, 14.2, 12.6, 12.1
TV-20	11	43.8, 39.6, 35.7, 31.7, 19.3, 14.3, 13.4, 11.7
TV-22	07	44.1, 31.6, 28.0, 19.2, 16.1, 14.1, 13.5
TV-23	10	43.1, 38.8, 33.6, 31.8, 28.3, 19.3, 18.8, 14.3, 13.3, 12.4
TV-25	15	40.1, 39.6, 36.0, 31.3, 28.7, 27.9, 19.4, 19.2, 18.9, 15.5, 14.0, 13.8, 13.4, 12.2, 11.2
TV-26	06	44.8, 31.6, 28.3, 19.5, 14.6, 12.5
TV-27	07	31.0, 40.2, 28.1, 33.8, 20.0, 14.2, 12.3
TV-28	09	52.0, 40.4, 31.3, 28.0, 20.3, 14.6, 13.7, 13.2, 12.3
TV-29	08	51.5, 31.0, 28.4, 20.5, 19.0, 14.3, 13.0, 8.9
TV-30	13	45.1, 39.8, 36.8, 34.5, 31.3, 29.0, 25.4, 22.3, 19.5, 16.6, 14.9, 13.5, 11.9

Note: Proteins resolved in 10 % SDS-gels and molecular weights determined by Kodak EDAS 290 software

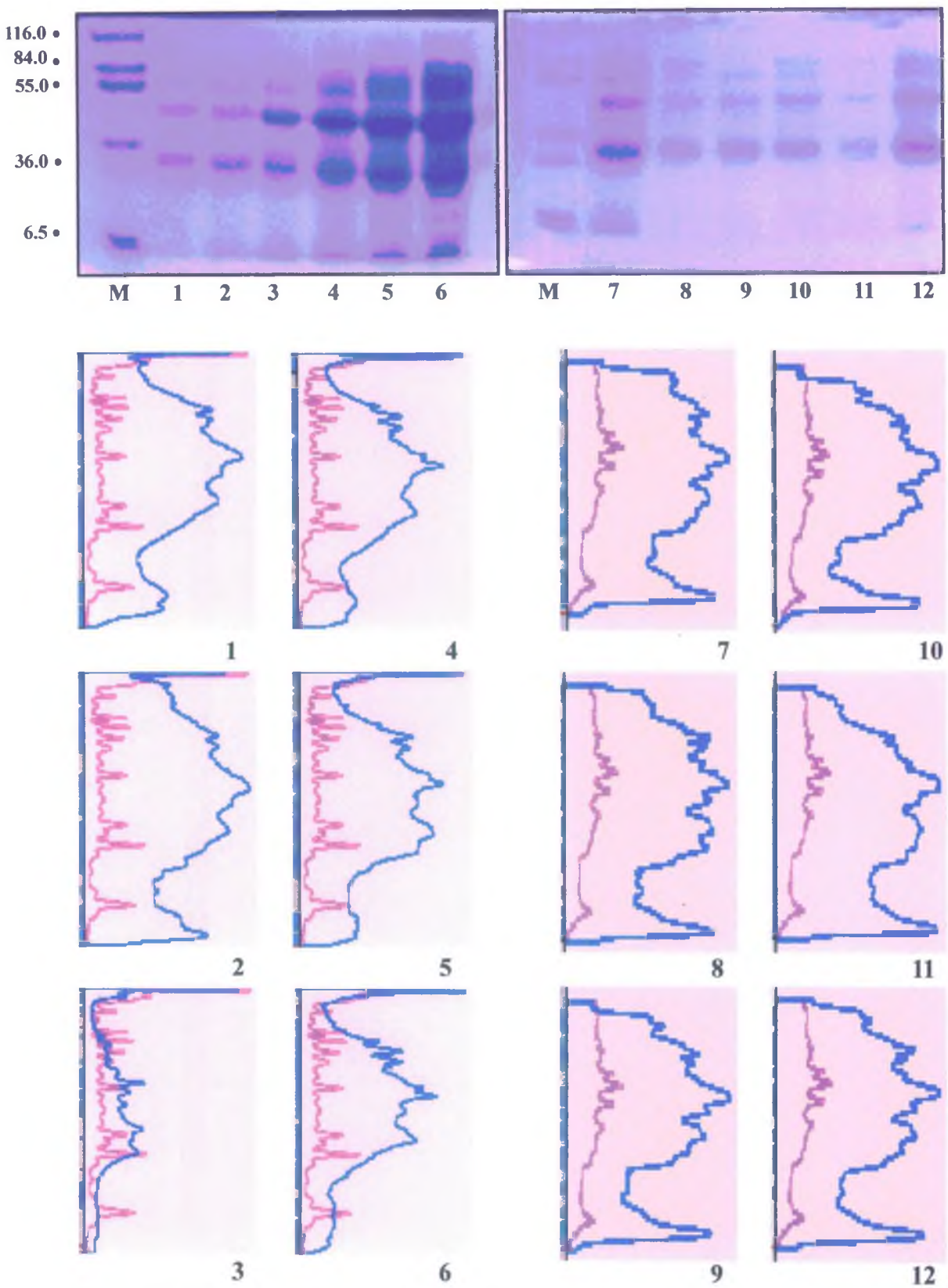


Plate 23. SDS-PAGE analysis and EDAS profiles of acid soluble leaf proteins of tea varieties. [M- molecular marker, 1- TV-18, 2- TV-26, 3- TV-27, 4- T-78, 5- T-135, 6- CP-1, 7- TV-26, 8-UP-2, 9- TS-449, 10- BSS-3, 11- T-17/1/54, 12- TV-9.]

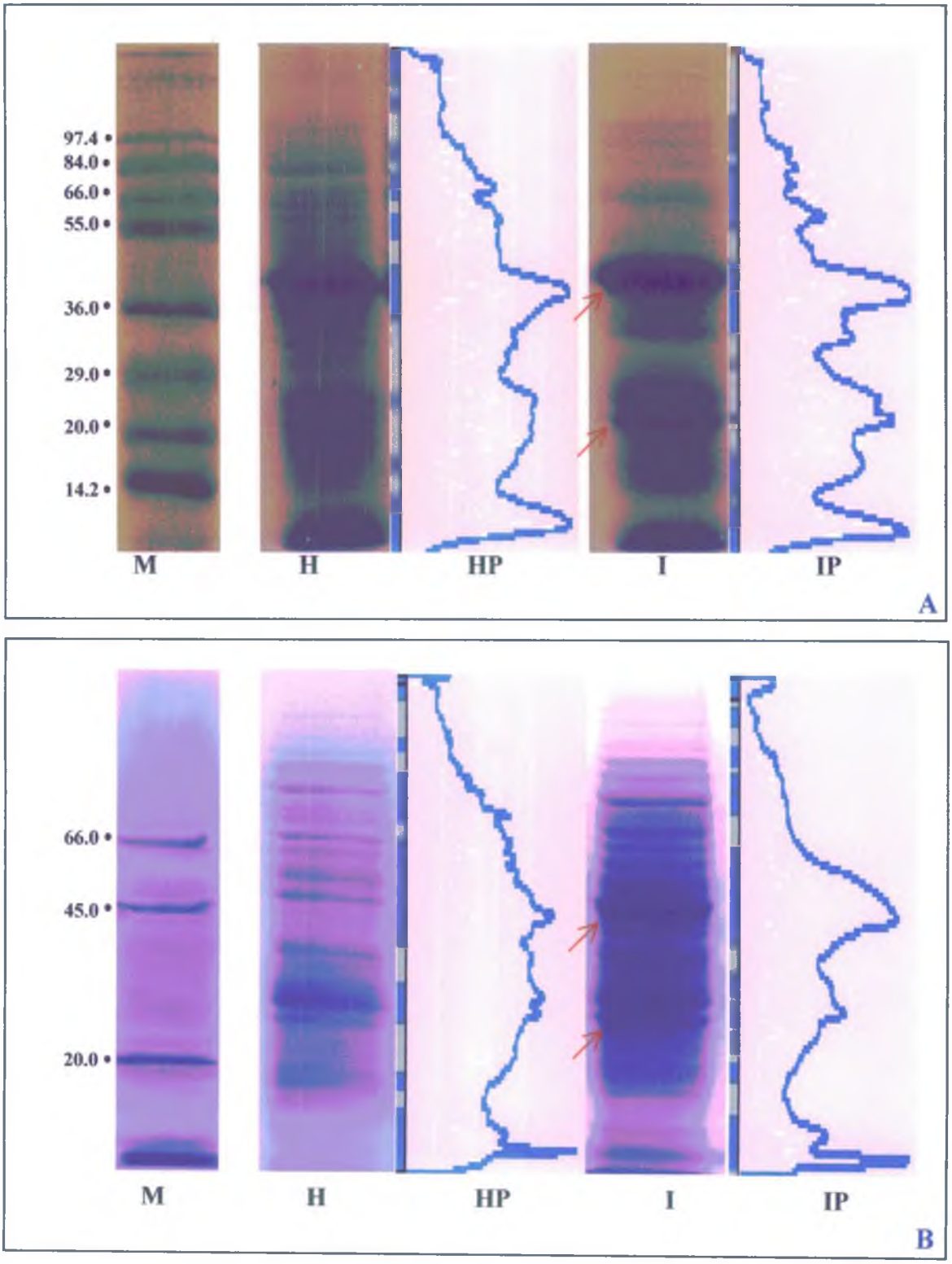


Plate 24 (figs. A & B). SDS-PAGE analysis and EDAS profiles of acid soluble leaf proteins of resistant tea varieties T-17/1/54 (A) and BSS-3 (B). [M- molecular marker, H- healthy, HP- Healthy profile, I- Inoculated with *E. vexans*, IP- Inoculated profile]

Proteins were detected in all treated material at 0 h but by 48 h had declined and were barely detected in the inoculated plants of susceptible varieties however the loss of protein bands was not so significant in resistant varieties. Appearance of new bands in the infected leaves of all varieties was observed 24 h after inoculation. The quantity of the PR-proteins accumulating was also variety and time dependent. Differences in number of bands was also observed among the control and inoculated plants. As shown in Plate 24, figs. A & B nine major protein bands were detected in extracts of *E. vexans*- inoculated plants 24 h after inoculation where two bands of 42 and 14 kDa were unique to inoculated plants. In contrast, protein bands in control tissues were fewer and progressively less intense as the symptoms appeared. However the major PR-proteins with low molecular weight decreased in quantity considerably. Hence, the electrophoretic pattern of the acidic protein extracts from tea leaves changed following inoculation with *E. vexans* where some bands disappeared, some bands increased in size and new proteins became visible. This was the case in both the resistant (T-17/1/54, TV-26, TV-29, BSS-3 and TV 30) and susceptible varieties (T-135, UP-9 and TV-18). Stronger signals for PR-proteins in the compatible reaction of the susceptible varieties than in the incompatible reaction were observed (Table 16).

Table 16: Protein profiling of acid soluble proteins present in healthy and *E. vexans* inoculated tea leaves

Variety	Protein molecular weight (kDa)	
	Healthy	Artificially inoculated with *E. vexans*
TV-26	44.8, 31.6, 28.3, 14.6, 12.5	45.2, 31.6, 28.3, 20.1, 14.6, 12.5
T-78	40.1, 39.5, 36.0, 34.3, 28.7, 12.8	40.1, 39.5, 34.3
TV-29	51.5, 31.0, 28.4, 19.0, 14.3, 13.0, 8.9	50.6, 45.6, 31.0, 28.4, 30.5, 20.5, 19.0, 14.3, 13.0, 8.9
UP-9	65.6, 43.6, 28.7, 19.4, 15.0	65.6, 28.7, 15.0
TV-18	53.2, 41.2, 35.5, 20.0, 12.6, 12.1	35.5, 20.0, 12.6, 12.1
TV-30	84.3, 78.5, 45.1, 39.8, 36.8, 34.5, 30.3, 29.0, 25.4, 22.3, 19.9, 16.6, 13.5, 11.9,	45.5, 39.8, 36.8, 34.5, 30.3, 29.0, 25.4, 22.3, 16.6, 13.5, 11.9,

4.6.2.2. *In vitro* antifungal assay of acid soluble PR-proteins

In vitro antifungal assay of acid soluble PR-proteins were performed using spore germination bioassay method. Spores of *E. vexans* were suspended at a density of 1.6 x 10^5 spores ml^{-1} in sterile water. The protein solutions (40 µl) were filter sterilized and added to the slides followed by incubation for 16-20 h at 25 ±2 °C in the dark and provided 100 % humidity. Germination of the spores was examined. Basidiospores in sterile distilled water and in buffer solutions, that was included as controls, revealed germination with germ tube or appressorium formation. Analysis of the antifungal activity of the protein fractions revealed complete inhibition of growth of *E. vexans* (Table 17). The protein solutions could be diluted substantially without losing fungicidal activity but in lower dilutions inhibition percentage was lesser.

Table 17. Bioassay of acid soluble PR-proteins against *E. vexans*

Treatment	Spore germination (%)	Germ tube length (µm)	Appressoria formation (%)
Water control	88.6	941.0	95
Buffer control[a]	56.8	665.9	83
T-17/1/54	0	0	0
Healthy	6.4	46.14	0
Inoculated[b]			
TV-18	2.5	21.2	0
Healthy	8.2	45.0	0
Inoculated[b]			

Note: [a] 0.1 M sodium-citrate buffer pH 2.8; [b] *E. vexans*; Results are average of 5 experiments and were taken after 24 h of inoculation

4.7. Association of defense enzymes in tea leaves triggered by *E. vexans* during disease development

Development of disease symptoms is the exception rather than the rule, the exception can be costly, particularly when agriculturally important plants succumb to pathogen infection. Central to the survival of higher plants has been their ability to evolve an array of defense strategies against microbial attack. One of these is the induction and accumulation of the PR group of proteins. Perhaps the most extensively studied of the many known PR-proteins are the chitinases (CHT) and β-1,3-glucanases (βGLU) of the PR-3 and PR-2 families respectively due to their antifungal property; nevertheless studies on plant peroxidases (POX) of the PR-9 family have also been carried out at length. In the present study, activities of these three PR-proteins (PR-2, -3 and -9) have been assayed from naturally blister-infected tea leaves (susceptible - S) as well as leaves showing hypersensitive reaction (resistant - R). Disease free leaves have been considered as control (H). The enzymes analyzed from three different stages of infection (S1, S2 and S3), have been compared with disease severity expressed as percentage infection (PI).

4.7.1. Chitinase

Healthy, blister infected (of all three stages of infection S1-S3) and leaves exhibiting hypersensitive reactions were collected from three different tea estates including two of the hills (Margaret's Hope and Castleton) and one of plains (Hansqua). Percentage infection of the infected leaves was recorded and activity of chitinase was assayed.

Chitinase activity was always found to be lower in the compatible interactions in comparison to resistant and healthy ones (Table 18). Highest accumulation was seen in the disease free leaf samples collected from tea bushes which have acquired immunity towards environmental stress(es) as well as the leaves showing hypersensitive reaction against the pathogen (*E. vexans*) attack resulting in incompatibility with the pathogen. Chitinase activity in healthy samples obtained

from gardens of the plains was slightly higher than that of the hills. Activity of the enzyme significantly declined with an increase in disease severity that again gradually increased in the resistant interactions, as illustrated in Figure 5 A.

Table 18. Association of the defense enzyme chitinase (PR-3) in tea leaves triggered by *E. vexans* during natural disease development

Tea Estate	Stages	Chitinase activity (mg GlcNAc g^{-1} leaf tissue h^{-1})	Percentage infection (%)
Margaret's Hope [a]	H	0.675 ± 0.07	00.0
	S1	0.448 ± 0.07	10.1
	S2	0.346 ± 0.08	43.5
	S3	0.125 ± 0.07	77.5
	R	0.688 ± 0.05	1.0
Castleton [a]	H	0.720 ± 0.01	00.0
	S1	0.558 ± 0.08	10.2
	S2	0.275 ± 0.09	40.8
	S3	0.132 ± 0.08	92.2
	R	0.619 ± 0.07	1.2
Hansqua [b]	H	0.735 ± 0.06	00.0
	S1	0.512 ± 0.07	11.5
	S2	0.225 ± 0.05	35.5
	S3	0.152 ± 0.04	78.5
	R	0.710 ± 0.07	0.9

Note: [a] hills, [b] plains; H- Healthy, S- infected (S1- initial, S2- moderate and S3- severe infection) and R - leaves exhibiting hypersensitive reaction. ± *S.E.*

4.7.2. β-1,3-glucanase

β-1,3-glucanase activity was consistently high in disease free leaf samples of all gardens with an abrupt increase in the enzyme activity in the initial stages of infection and decreased activity as the disease developed (Table 19) as well as in resistant reactions (Figure 5 B).

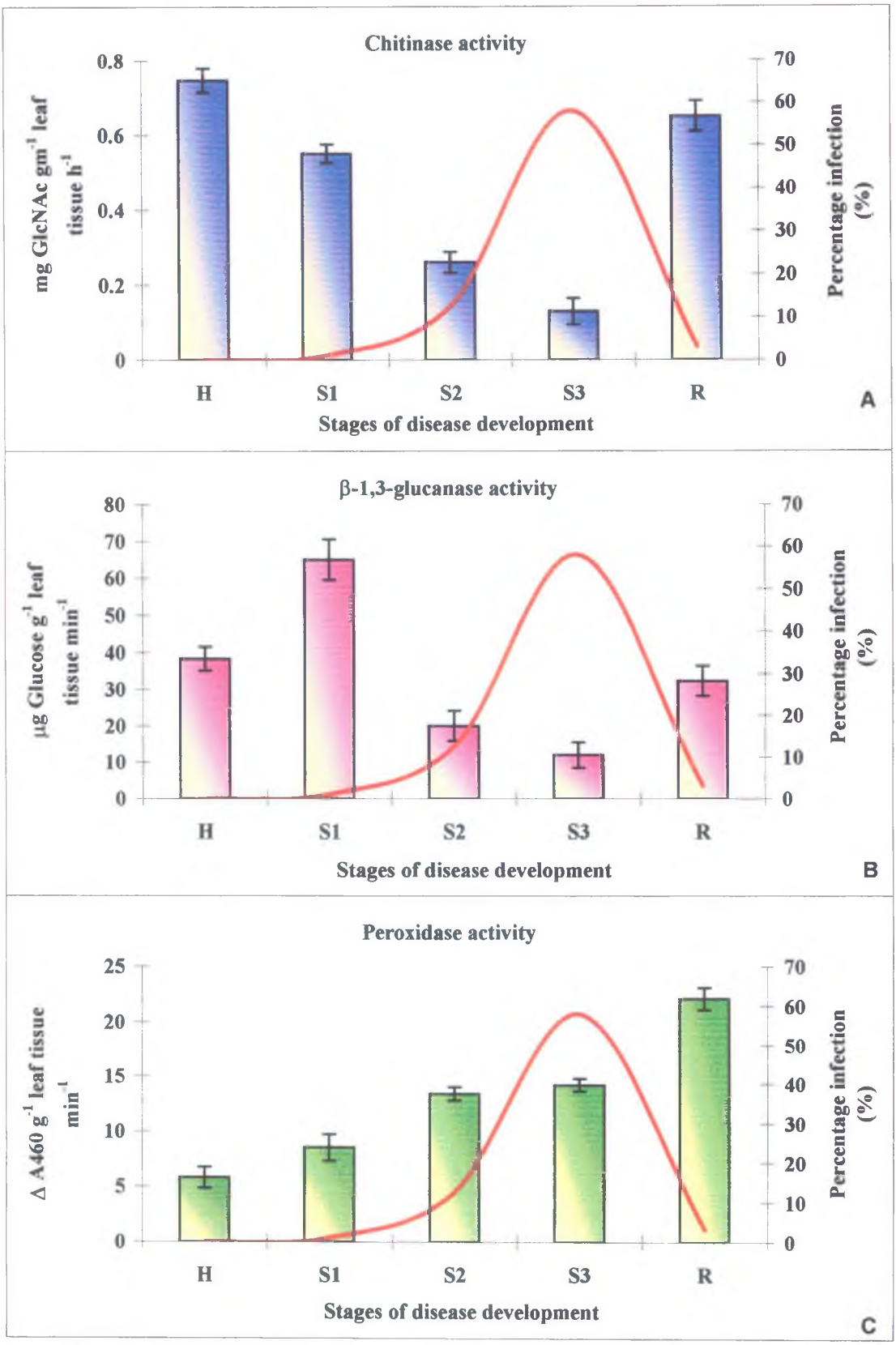


Figure 5 (A - C). Accumulation of defense enzymes in tea leaves triggered by *E. vexans* at different stages of disease development under natural conditions. [A] Chitinase, [B] β-1,3-glucanase, [C] Peroxidase. H- healthy; S1, S2, S3- Stages of infection; R- Hypersensitive reaction.

Table 19. Association of the defense enzyme β-1,3-glucanase (PR-2) in tea leaves triggered by *E. vexans* during natural disease development

Tea Estate	Stages	β-1,3-glucanase activity (μg Glucose g^{-1} leaf tissue min^{-1})	Percentage infection (%)
Margaret's Hope	H	31.25 ± 4.2	00.0
	S1	62.58 ± 4.0	10.1
	S2	20.35 ± 2.4	43.5
	S3	12.75 ± 3.7	77.5
	R	26.55 ± 1.2	1.0
Castleton	H	39.90 ± 5.2	00.0
	S1	70.54 ± 2.3	10.2
	S2	22.65 ± 4.1	40.8
	S3	15.65 ± 2.1	92.2
	R	28.33 ± 1.2	1.2
Hansqua	H	30.45 ± 2.3	00.0
	S1	68.88 ± 4.6	11.5
	S2	29.35 ± 2.3	35.5
	S3	14.05 ± 4.4	78.5
	R	21.85 ± 3.2	0.9

Note: [a] hills, [b] plains; H- Healthy, S- infected (S1- initial, S2- moderate and S3- severe infection) and R- leaves exhibiting hypersensitive reaction. ± S.E.

4.7.3. Peroxidase

Peroxidase activity was also measured in healthy, infected and leaves exhibiting hypersensitive reactions against *E. vexans* under field conditions (Table 20). The enzyme activity was measured throughout the course of infection and significant changes were observed. The peroxidase activity displayed a constant increase during the stages of infection and the values obtained for the different stages of infection were always higher than that of healthy samples. Of major significance was the increase in enzyme activity in resistant samples (Figure 5 C). Enzyme activity

was about thrice of that obtained for healthy samples. Such higher peroxidase activities may be correlated with greater resistance.

Table 20. Association of the defense enzyme peroxidase (PR-9) in tea leaves triggered by *E. vexans* during natural disease development

Tea Estate	Stages	Peroxidase activity (Δ A460nm g^{-1} leaf tissue min^{-1})	Percentage infection (%)
Margaret's Hope	H	4.44 ± 0.6	00.0
	S1	7.05 ± 0.5	10.1
	S2	9.85 ± 0.8	43.5
	S3	13.59 ± 0.4	77.5
	R	20.15 ± 0.5	1.0
Castleton	H	5.84 ± 0.7	00.0
	S1	8.61 ± 0.7	10.2
	S2	11.22 ± 0.0	40.8
	S3	14.56 ± 0.4	92.2
	R	19.58 ± 0.1	1.2
Hansqua	H	5.02 ± 0.6	00.0
	S1	7.95 ± 0.8	11.5
	S2	10.50 ± 0.4	35.5
	S3	15.65 ± 0.3	78.5
	R	22.50 ± 0.9	0.9

Note: [a] hills, [b] plains; H- Healthy, S- infected (S1- initial, S2- moderate and S3- severe infection) and R- leaves exhibiting hypersensitive reaction, ±S.E.

4.7.3.1. Isozymes of peroxidase

Isozymes of peroxidase are present in plants including tea. The isozyme pattern was revealed on native polyacrylamide gel electrophoresis. Staining of peroxidase on native PAGE gels with benzidine as the hydrogen donor demonstrated the existence of isoforms in healthy as well as infected and resistant samples. Few isozymes were constitutively present in healthy leaves. Essentially, they were induced or at least further increased in concentration after infection with *E. vexans*. Samples

collected from all three tea gardens showed differing adaptability. Relative mobility (R_m) values of the peroxizymes have been recorded in Table 21. PAGE analyses of the enzyme extracted from leaves of Castleton revealed isozymes of R_m 0.25 and R_m 0.77 in all interactions. Two new isozymes of R_m 0.125 and R_m 0.67 were visualized in compatible interactions and that of R_m 0.44 in resistant interactions only (Plate 25, fig. A) isozymes. Similarly in samples of Margaret's Hope and Hansqua an additional peroxizyme of R_m 0.44 appeared when the leaves had completely overcome the infection. A faint isozyme of R_m 0.67 was seen in all samples of Margaret's Hope.

Table 21. Relative mobility values of isozymes of peroxidase in tea leaves triggered by *E. vexans* during natural disease development

Tea Estate	Isozyme number	Relative mobility	Healthy leaves	Stages of infection			Resistant leaves [c]
				SI	S2	S3	
Margaret's Hope [a]	1	0.25	+	-	-	+	+
	2	0.44	+	-	-	-	+
	3	0.67	-	-	-	-	-
Castelton [a]	1	0.125	-	-	-	+	-
	2	0.25	+	+	+	+	+
	3	0.44	-	-	-	-	+
	4	0.67	-	-	-	+	-
	5	0.77	+	+	+	+	+
Hansqua [b]	1	0.25	+	+	+	+	+
	2	0.44	-	-	-	-	+

Note: [a] hills, [b] plains; S1- initial, S2- moderate and S3- severe infection, [c] leaves exhibiting hypersensitive reaction; (+) Deep band

4.8. Time course accumulation of defense enzymes in tea varieties following inoculation with *E. vexans*

Resistance to disease in plants is associated with inducible compounds, which may function in defense against disease. Characteristic pathogenesis-related proteins (PR-proteins) accumulate in plant cells responding to pathogen attack. In the preceding chapter we observed that their accumulation is not restricted to non-infected plants but is often observed in compatible plant-pathogen interactions as well. Timing and magnitude of PR-protein induction differs, which is important for a successful defense reaction. Therefore, as time course studies may reveal important differences in accumulation of defense enzymes such experiments were performed. Activity of the defense enzymes chitinase, β-1,3-glucanase, peroxidase upon artificial inoculation with *E. vexans* under controlled conditions was recorded for eighteen varieties (AV-2, BS-7A/1/76, RR-17, TS-449, T-17/1/54, T-78, BSS-1, BSS-3, UP-9, UP-17, UP-26, TV-18, TV-20, TV-22, TV-23, TV-26, TV-29 and TV-30). Control plants were also maintained under identical conditions.

4.8.1. Chitinase

Chitinase activity was measured in leaf extracts of control and *E. vexans*-inoculated tea plants of resistant (BS-7A/1/76, T-17/1/54, BSS-1, BSS-3, TV-20, TV-26, TV-29 and TV-30) and susceptible varieties (AV-2, RR-17, TS-449, T-78, UP-9, UP-17, UP-26, TV-18, TV-22 and TV-23) after 0, 24, 48, and 72 h of inoculation with *E. vexans*. Chitinase activity significantly increased after inoculation with *E. vexans*. The enzyme activity was higher after inoculation in both resistant and susceptible varieties. However increase in enzyme activity was much steeper in the resistant lines with highest levels in the order (BS-7A/1/76, T-17/1/54, TV-26, TV-30, TV-29, BSS-1 and BSS-3). This difference between the two lines widened and reached a maximum level 24 h of inoculation. Further comparison showed a gradual decline with time in the resistant lines whereas the decline in enzyme activity was rapid in susceptible ones. Despite fall in the activity, chitinase levels were still higher in the inoculated plants than their respective controls (Table 22, Figures 6 A & B). It was seen that the chitinase activity was more or less constant at the control value

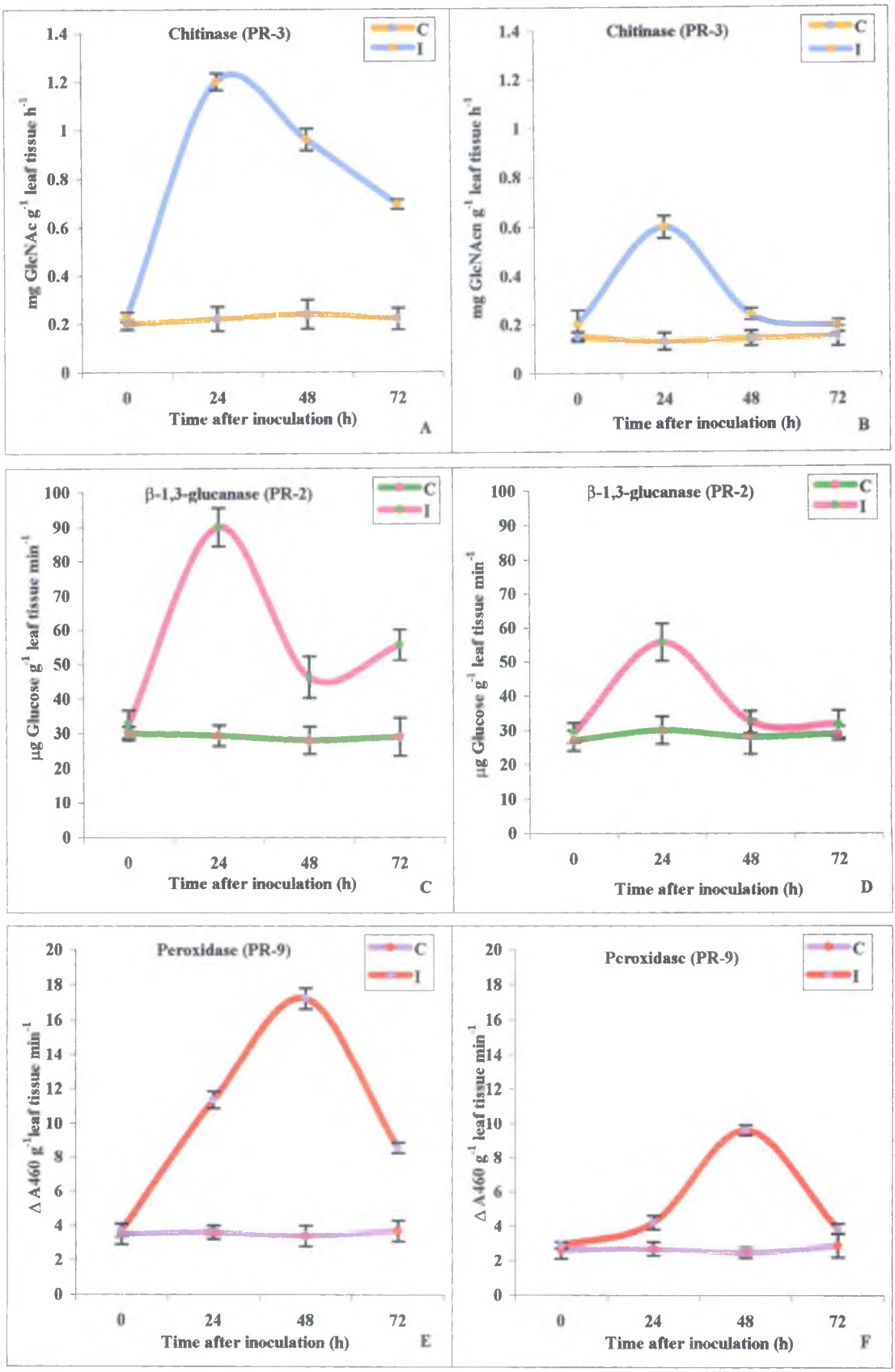


Figure 6 (A - F). Time course accumulation of PR-proteins in tea plants following inoculation with *E. vexans*. [A & B] PR-3, [C & D] PR-2, [E & F] PR-9; [A, C & E] Resistant variety (T-17/1/54); [B,D & F] Susceptible variety (TV-18).

throughout the study. On the other hand the enzyme activity always displayed an increase within 24 h post inoculation (Figure 7 A) and gradually declined thereafter up to 72 h. However even after 72 h, though low, chitinase activity was still higher in inoculated plants than controls.

Table 22. Association of the defense enzyme chitinase (PR-3) in tea leaves triggered by artificial inoculation with *E. vexans*

Variety	Chitinase activity (mg GlcNAc g^{-1} leaf tissue h^{-1})*							
	Hours after inoculation							
	0		24		48		72	
	C	Inoc	C	Inoc	C	Inoc	C	Inoc
BS-7A/1/76	0.256 ± 0.09	0.26 ± 0.05	0.221 ± 0.07	1.282 ± 0.02	0.232 ± 0.09	0.961 ± 0.08	0.241 ± 0.07	0.693 ± 0.04
BSS-1	0.148 ± 0.03	0.151 ± 0.03	0.149 ± 0.01	0.681 ± 0.04	0.151 ± 0.08	0.404 ± 0.09	0.143 ± 0.03	0.321 ± 0.02
BSS-3	0.152 ± 0.03	0.155 ± 0.05	0.158 ± 0.03	0.671 ± 0.01	0.159 ± 0.03	0.471 ± 0.08	0.160 ± 0.09	0.381 ± 0.09
UP-26	0.147 ± 0.07	0.149 ± 0.09	0.154 ± 0.09	0.662 ± 0.09	0.160 ± 0.08	0.490 ± 0.02	0.153 ± 0.07	0.298 ± 0.03
TV-20	0.188 ± 0.05	0.189 ± 0.01	0.185 ± 0.05	1.014 ± 0.07	0.191 ± 0.05	0.802 ± 0.08	0.184 ± 0.05	0.628 ± 0.05
TV-22	0.212 ± 0.04	0.231 ± 0.08	0.221 ± 0.07	0.620 ± 0.03	0.224 ± 0.05	0.472 ± 0.03	0.230 ± 0.04	0.301 ± 0.04
TV-26	0.189 ± 0.05	0.190 ± 0.04	0.178 ± 0.08	0.971 ± 0.08	0.177 ± 0.04	0.711 ± 0.06	0.170 ± 0.07	0.600 ± 0.03
TV-29	0.197 ± 0.05	0.198 ± 0.08	0.195 ± 0.07	0.891 ± 0.06	0.186 ± 0.07	0.603 ± 0.05	0.181 ± 0.06	0.492 ± 0.07
TV-30	0.238 ± 0.03	0.249 ± 0.02	0.241 ± 0.01	1.111 ± 0.02	0.223 ± 0.01	0.814 ± 0.08	0.233 ± 0.09	0.721 ± 0.05

*The results presented are the means calculated for five experiments. ± S.E.

Hence, chitinase enzyme assayed in the extracts from leaves of inoculated (with *E. vexans*) tea plants showed that chitinase was strongly stimulated by infection. In contrast, chitinase activity remained at low levels in healthy controls. In leaves at 24 h after inoculation induction of chitinase was similar in compatible and incompatible interactions. As compared to the compatible interactions, chitinase activity was higher in incompatible ones.

4.8.2. β-1,3-glucanase

β-1,3-glucanase activity was measured in leaf extracts of control and *E. vexans*-inoculated tea plants of resistant (BS-7A/1/76, T-17/1/54, BSS-1, BSS-3, TV-20, TV-26, TV-29 and TV-30) and susceptible varieties (AV-2, RR-17, TS-449, T-78,

UP-9, UP-17, UP-26, TV-18, TV-22 and TV-23) after 0, 24, 48, and 72 h of inoculation with *E. vexans*. β-1,3-glucanase activity in the resistant and susceptible tea plants was influenced by inoculation with *E. vexans* and displayed a peak rise within 24 h post inoculation (Table 23). The values obtained for the resistant varieties show a rapid rise in the β-1,3-glucanase activity in comparison to susceptible ones (Figures 6 C and D). Enzyme activity started to increase within 24 h of inoculation and was maximum at this time interval (Figure 7 B). Inoculated resistant varieties showed higher levels of the enzyme in comparison to inoculated susceptible ones. After 24 h the enzyme activity gradually decreased in all varieties, which continued till 48 h. Interestingly, though not so significant, the activity of β-1,3-glucanase in the inoculated plants had a tendency to slightly increase (72 h) after a fall in peak value (24 h). Further comparison showed that the resistant inoculated plants attained the same level of enzyme after 48 h, as that attained by susceptible varieties after 24 h. Susceptible varieties showed a faster decline in enzyme activity whereas resistant varieties still maintained much higher levels. The enzyme activities of both resistant and susceptible varieties following inoculation were higher than their respective controls throughout.

4.8.3 Peroxidase

Time course accumulation of peroxidase enzyme in susceptible (AV-2, RR-17, TS-449, T-78, UP-9, UP-17, UP-26, TV-18, TV-22 and TV-23) and resistant varieties (BS-7A/1/76, T-17/1/54, BSS-1, BSS-3, TV-20, TV-26, TV-29 and TV-30) upon inoculation with *E. vexans* was determined. During interaction between susceptible or resistant hosts peroxidase activity increased markedly in resistant varieties 48 h after inoculation. In the susceptible varieties increase in peroxidase activity could be detected after 48 h of inoculation to a lesser extent. In general, peroxidase activity in incompatible interactions was about two-fold of that in compatible interactions (Figure 6 E and F). The peroxidase activity levels in response to *E. vexans* infection rapidly increased from 24 - 48 h post inoculation. The trend was the same in resistant and susceptible varieties except for level of enhancement of enzyme activity (Table 24). The highest levels of the enzyme were obtained 48 h after inoculation, which was higher in all resistant-inoculated varieties than the susceptible-inoculated plants (Figure 7 C). Peroxidase activity was consistently higher in inoculated leaves as to compared healthy controls.

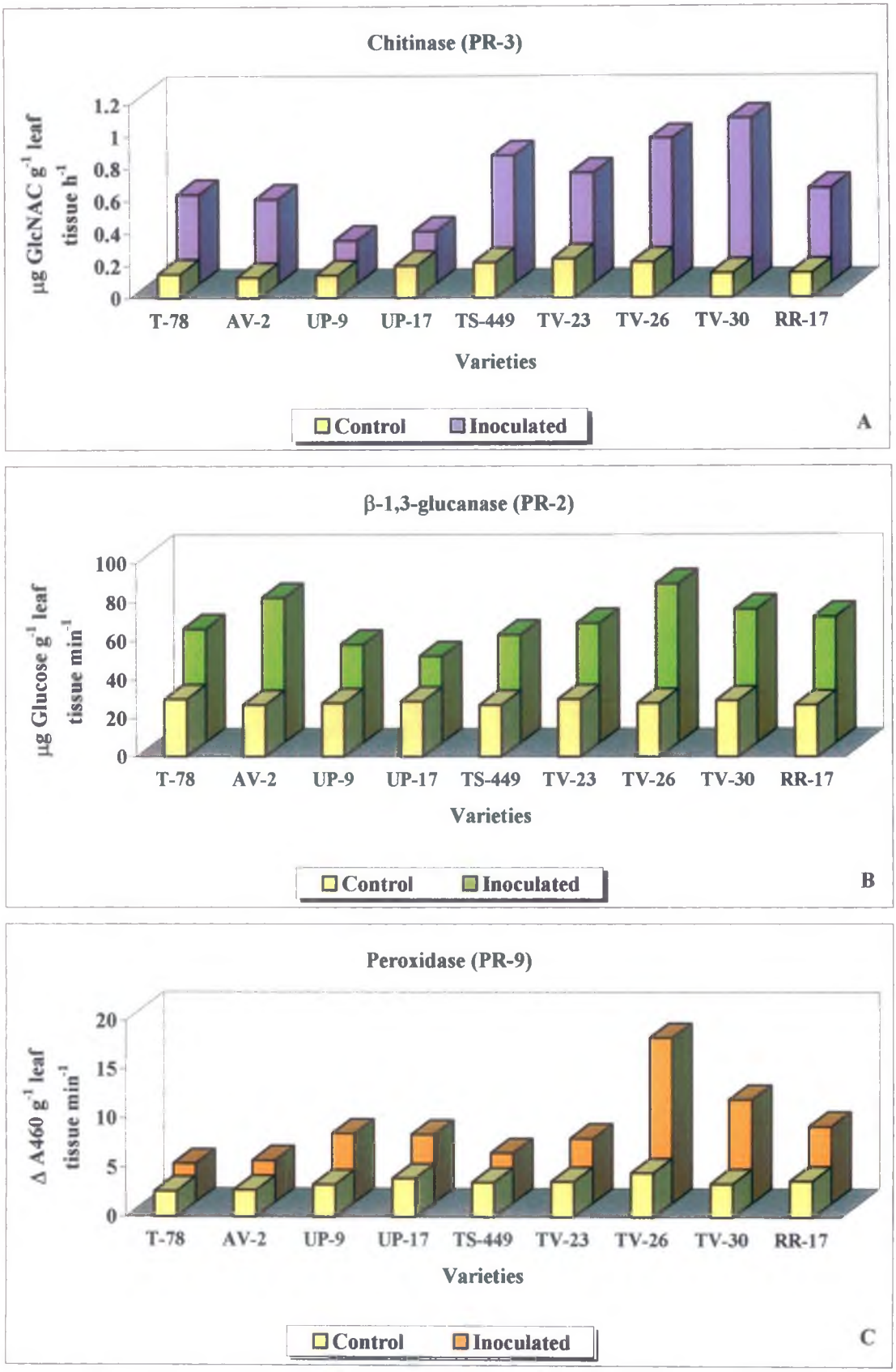


Figure 7 (A-C). Accumulation of PR-proteins in tea varieties 24 h [A & B] and 48 h [C] following inoculation with *E. vexans* [A] PR-3, [B] PR-2, [C] PR-9.

Table 23. Association of the defense enzyme β-1,3-glucanase (PR-2) in tea leaves triggered by artificial inoculation with *E. vexans*

Variety	β-1,3-glucanase activity (μg Glucose g^{-1} leaf tissue min^{-1})*							
	Hours after inoculation							
	0		24		48		72	
	C	Inoc	C	Inoc	C	Inoc	C	Inoc
BS-7A/1/76	34.2 ± 2.6	36.2 ± 1.6	32.1 ± 1.8	81.2 ± 1.5	36.1 ± 2.7	46.3 ± 3.5	32.8 ± 1.1	54.2 ± 1.9
BSS-1	30.3 ± 1.9	31.2 ± 1.3	32.9 ± 1.7	59.9 ± 1.0	30.6 ± 2.8	40.6 ± 1.5	31.4 ± 1.3	39.7 ± 1.7
BSS-3	29.4 ± 1.1	30.4 ± 2.3	30.7 ± 1.6	55.3 ± 2.8	29.8 ± 1.1	35.1 ± 1.0	29.7 ± 1.6	30.1 ± 1.3
UP-26	29.7 ± 1.3	29.1 ± 1.5	28.4 ± 1.5	47.5 ± 2.4	28.7 ± 1.5	38.2 ± 2.4	29.6 ± 2.8	32.8 ± 1.3
TV-20	41.2 ± 1.6	40.5 ± 2.4	41.4 ± 3.5	78.7 ± 3.5	39.9 ± 1.3	47.8 ± 1.1	41.3 ± 3.5	49.9 ± 1.4
TV-22	37.9 ± 2.8	38.2 ± 1.7	39.2 ± 1.0	57.8 ± 1.7	38.4 ± 1.6	40.7 ± 1.2	39.3 ± 1.3	47.8 ± 1.0
TV-26	40.2 ± 1.3	41.7 ± 1.0	41.5 ± 1.0	99.3 ± 3.4	41.0 ± 1.8	51.9 ± 1.6	41.7 ± 1.3	44.2 ± 2.7
TV-29	39.8 ± 1.4	40.7 ± 2.8	40.8 ± 2.4	82.7 ± 3.5	39.6 ± 1.2	47.2 ± 2.7	40.8 ± 2.8	41.7 ± 1.1
TV-30	36.5 ± 2.3	37.1 ± 1.4	36.0 ± 1.6	85.4 ± 2.3	37.2 ± 1.0	44.7 ± 1.1	36.1 ± 2.1	40.0 ± 1.0

*The results presented are the means calculated for five experiments. ±S.E.

Table 24. Association of the defense enzyme peroxidase (PR-9) in tea leaves triggered by artificial inoculation with *E. vexans*

Variety	Peroxidase activity (Δ A460nm g^{-1} leaf tissue h^{-1})*							
	Hours after inoculation							
	0		24		48		72	
	C	Inoc	C	Inoc	C	Inoc	C	Inoc
BS-7A/1/76	3.54 ± 0.1	3.41 ± 0.5	3.61 ± 0.6	10.52 ± 0.1	3.42 ± 0.3	15.21 ± 0.1	3.37 ± 0.1	8.52 ± 0.8
BSS-1	3.75 ± 0.1	3.69 ± 0.5	3.71 ± 0.4	9.21 ± 0.2	3.68 ± 0.4	13.82 ± 0.5	3.69 ± 0.4	7.71 ± 0.2
BSS-3	4.21 ± 0.6	4.19 ± 0.6	4.21 ± 0.2	7.37 ± 0.4	4.23 ± 0.6	15.61 ± 0.2	4.29 ± 0.3	6.32 ± 0.6
UP-26	4.40 ± 0.4	4.52 ± 0.4	4.44 ± 0.1	10.48 ± 0.5	4.58 ± 0.3	7.77 ± 0.4	4.52 ± 0.5	5.10 ± 0.2
TV-20	3.96 ± 0.1	3.91 ± 0.6	3.89 ± 0.5	9.87 ± 0.2	3.83 ± 0.5	14.70 ± 0.8	3.91 ± 0.5	6.27 ± 0.1
TV-22	4.28 ± 0.3	4.30 ± 0.6	4.27 ± 0.5	10.71 ± 0.1	4.31 ± 0.5	9.28 ± 0.3	4.29 ± 0.4	5.19 ± 0.8
TV-26	4.32 ± 0.1	4.40 ± 0.5	4.41 ± 0.4	10.00 ± 0.8	4.43 ± 0.5	16.84 ± 0.9	4.42 ± 0.5	5.22 ± 0.9
TV-29	4.46 ± 0.3	4.49 ± 0.2	4.52 ± 0.1	11.29 ± 0.1	4.55 ± 0.2	21.34 ± 0.6	4.52 ± 0.8	11.28 ± 0.5
TV-30	4.48 ± 0.2	4.50 ± 0.3	4.43 ± 0.6	12.5 ± 0.3	4.45 ± 0.8	18.92 ± 0.5	4.45 ± 0.9	10.36 ± 0.9

Note: *The results presented are the means calculated for five experiments. ±S.E.

4.8.3.I. Isozymes of peroxidase

Existence of isoforms of peroxidase was observed during natural disease development. Similarly, peroxidase enzyme extracts of control and inoculated tea plants also revealed presence of isozymes in native PAGE gels. It was also observed that peroxizymes were constitutively present in healthy leaves. Upon artificial inoculation of the different varieties (T-17/1/54, BSS-3 and TV-18) few isozymes were induced and few increased in concentration. Relative mobility (R_m) values of the peroxizymes have been recorded in Table 25.

Table 25. Relative mobility values of isozymes of peroxidase in tea leaves after artificial inoculation with *E. vexans*

Variety	Time after inoculation (h)			
	24		48	
	H	I	H	I
T-17/1/54	-	-	-	0.125
	0.250	0.250	0.250	0.250
	-	0.450	-	0.450
	-	0.670	-	0.670
	0.750	0.750	0.750	0.750
BSS-3	-	-	-	0.125
	0.250	0.250	0.250	0.250
	-	0.450	-	0.450
	-	-	-	0.670
	0.750	0.750	-	-
TV-18	-	-	-	0.125
	0.250	0.250	0.250	0.250
	-	0.450	-	0.450
	-	-	-	0.670
	-	-	-	0.750

PAGE analyses of the isoperoxidases extracted from leaves inoculated with *E. vexans* as well as healthy control revealed a common isozyme of R_m 0.25 in all varieties. Another isozyme of R_m 0.75 was consistent in Darjeeling resistant variety (T-17/1/54) in all interactions. However, the band of R_m 0.75 was observed in the UPASI moderately resistant variety also (BSS-3), in both compatible and incompatible interactions up to 24 h of inoculation which disappeared with time. Interestingly, this band of R_m 0.75 appeared in the inoculated extracts after 48 h.

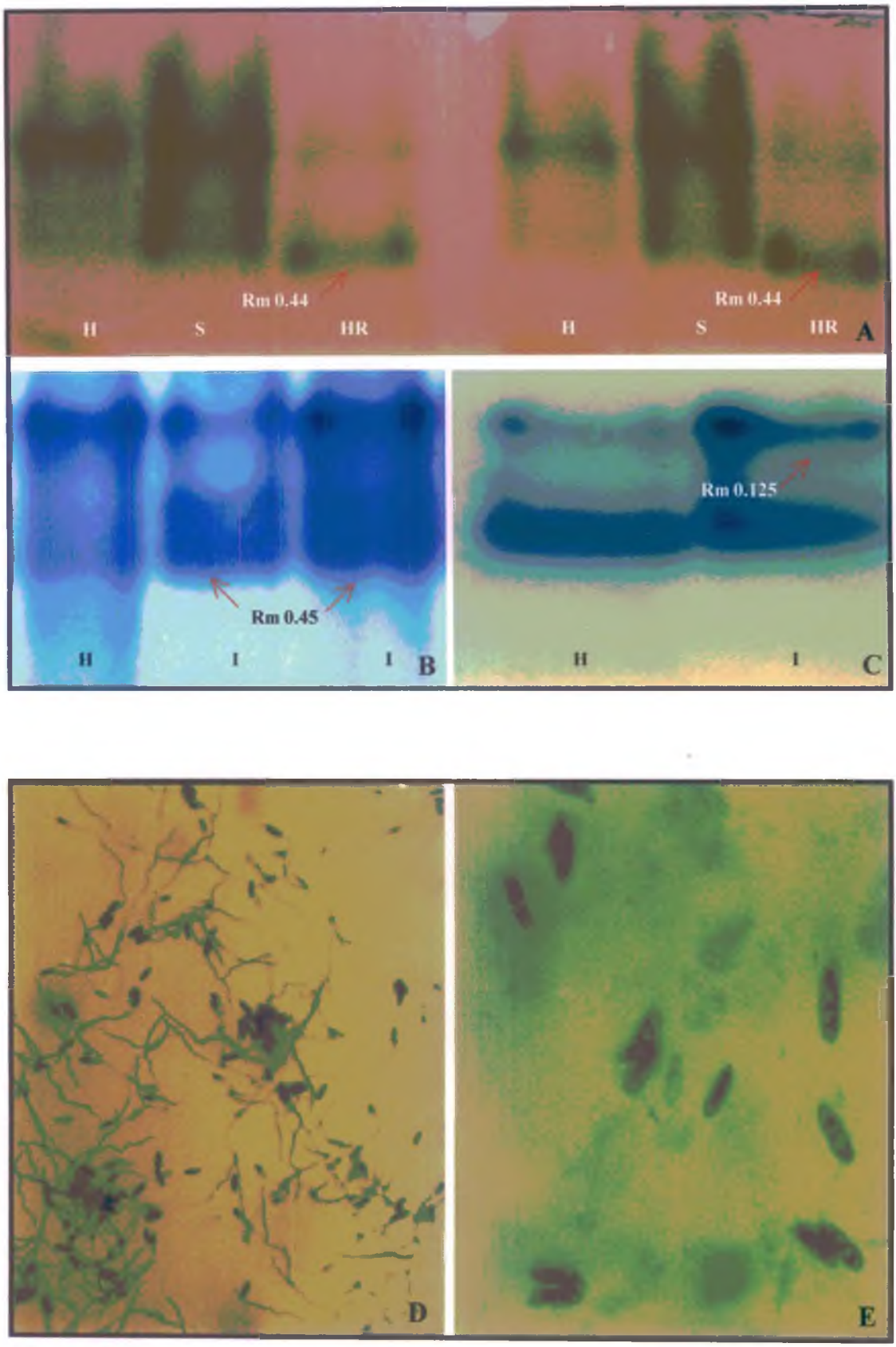


Plate 25 (figs. A - E). Peroxidase isozyme analysis of tea plants triggered by *E. vexans* [A - C] and *in vitro* antifungal bioassay of PR-proteins against *E. vexans* [D & E]. Natural infection [A], artificial inoculation [B & C]. Distilled water control [D], PR-3 treated [E].

Two new isozymes one of R_m 0.45 appeared in all inoculated extracts within 24 h and was persistent up to 48 h, while the isozyme of R_m 0.67 appeared in the resistant variety (T-17/1/54) after 24-48 h, in the moderately resistant (BSS-3) and susceptible (TV-18) varieties it appeared only after 48 h of inoculation with *E. vexans*. However, the isozyme R_m 0.67 was more intense in the moderately resistant inoculated variety than the susceptible inoculated one (Plate 25, figs. B & C).

On the whole it was seen that following artificial inoculation, inoculated plants showed better responses in comparison to control. Chitinase and β-1,3-glucanase appear as the first set of defense enzymes followed by peroxidase.

4.8.4. *In vitro* antifungal activity of chitinase and β-1,3-glucanase

The effects of purified chitinases and β-1,3-glucanases on the germination of *E. vexans* were assayed in order to verify whether these extracts were inhibitory to *E. vexans* by spore germination bioassay. Spore suspensions were added to purified chitinase and β-1,3-glucanase extracts (crude, 10^{-1} and 10^{-2}). A spore was considered germinated when its germ tube was twice its length, with or without appressoria. Chitinase significantly inhibited spore germination (Plate 25, fig. E) in relation to control (Plate 25, fig. D) but β-1,3-glucanase did not inhibit germination (Table 26). However, complete inhibition of spore germination was observed in solutions containing equal concentrations of chitinase and β-1,3-glucanase (1:1). Lysis of hyphal tips and reduced appressoria formation were observed in solutions containing a mixture (1:2) of chitinase and β-1,3-glucanase. These tests confirmed the antifungal activity of the enzyme extracts. The extracts were sterile filtered before use.

The effects of purified chitinase on the mycelial growth of *G. cingulata* was also analyzed by the microtitre dish assay. After solidification of the Czapek Dox broth added to the wells of a microtitre plate spore suspensions of *G. cingulata* were added to each well and the spores were allowed to germinate at 22 °C in the dark. Twelve hours later purified chitinase and β-1,3-glucanase and intercellular wash fluids from inoculated (with *E. vexans*) and control plants were added to the wells. Buffer containing denatured enzyme was used as an additional control.

Table 26. ***In vitro*** **antifungal activity of purified PR-proteins of tea triggered by** ***E. vexans***

Treatment		Spore germination (%)	Inhibition in spore germination (%)	Appressoria Formation (%)	Inhibition in appressoria formation (%)
Water control		82.7	-	94.5	-
Buffer control		62.3	24.6#	80.5	14.8#
Chitinase *		1.6	97.4##	3.5	95.6##
	10^{-1}	13.1	78.9##	4.2	94.8##
	10^{-2}	26.9	56.8##	45.0	44.0##
β-1,3-glucanase *		46.3	25.6##	50.2	37.2##
	10^{-1}	50.2	19.4##	58.1	27.8##
	10^{-2}	55.8	10.4##	62.8	21.9##
CHT + βGLU (1:2)*		3.6	94.2##	(lysis)	100##
CHT + βGLU (1:1)*		0	100##	0	100##

Note: * Crude enzyme; # Values indicate percentages of inhibition against water control; ## Values indicate percentages of inhibition against buffer (0.1 M sodium-citrate buffer pH 5.2) control; Results are average of 5 experiments and were taken after 24 h of incubation

Chitinase had antifungal activity and 50 % reduction of growth was observed. The intercellular fluids (ICF) from control and induced plants did not have antifungal activity. Concentrated ICF (3x) also did not have antifungal activity. However antifungal activity was obtained after adding purified chitinase and β-1,3-glucanase to the ICF. To verify the synergistic effect, the purified PR-proteins were also tested using the agar-cup bioassay technique. The purified fractions of chitinase and β-1,3-glucanase were added in combination (1:1) to the agar cups. Mycelial blocks of *C. pallescens* were placed at equidistance from the wells and observed until full growth was attained in PDA control sets. Antifungal activity of the PR-proteins was confirmed since growth of the test fungus was totally inhibited.

4.9. Evaluation of PAbs raised against chitinase (PR-3) and β-1,3-glucanase (PR-2)

Immunological techniques have become indispensable tools for the rapid purification, visualization, and quantification of proteins. Immunoassays including PTA-ELISA, Dot blot, Western blot and Indirect immunofluorescence were developed and optimized with PAbs raised against purified chitinase (PAb-CHT) and β-1,3-glucanase (PAb-βGLU).

4.9.1. Immunodiffusion

Polyclonal antibodies (PAbs) were raised in rabbits against purified chitinase (PR-3) and β-1,3-glucanase (PR-2) as described previously and these were used in various immunological formats. For each antigen source normal sera were collected before immunization. The effectiveness of the purified chitinase as well as β-1,3-glucanase antigen in raising PAbs were checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and chitinase were all negative. Strong precipitin bands occurred when PAb-CHT and PAb-GLU were reacted separately with it's own antigen (Plate 26, fig. A & B). The titre values of PAbs were checked after each bleeding and only those showing strong precipitin bands were used for subsequent immunoassays.

4.9.2. PTA-ELISA

Optimization of ELISA was done by considering two variables i.e., concentration of the antigen and concentration of the PAb. Reactions were done with PAb-CHT and PAb-βGLU after different bleedings. Enzyme concentration was 1:10,000 [Goat antirabbit IgG (whole molecule) alkaline phosphatase conjugate] while substrate (p-Nitrophenyl phosphate) was used at a concentration of 10 mg ml^{-1}. The substrate reaction was terminated after 60 min and absorbance values were means of five adjacent wells measured at 405nm.

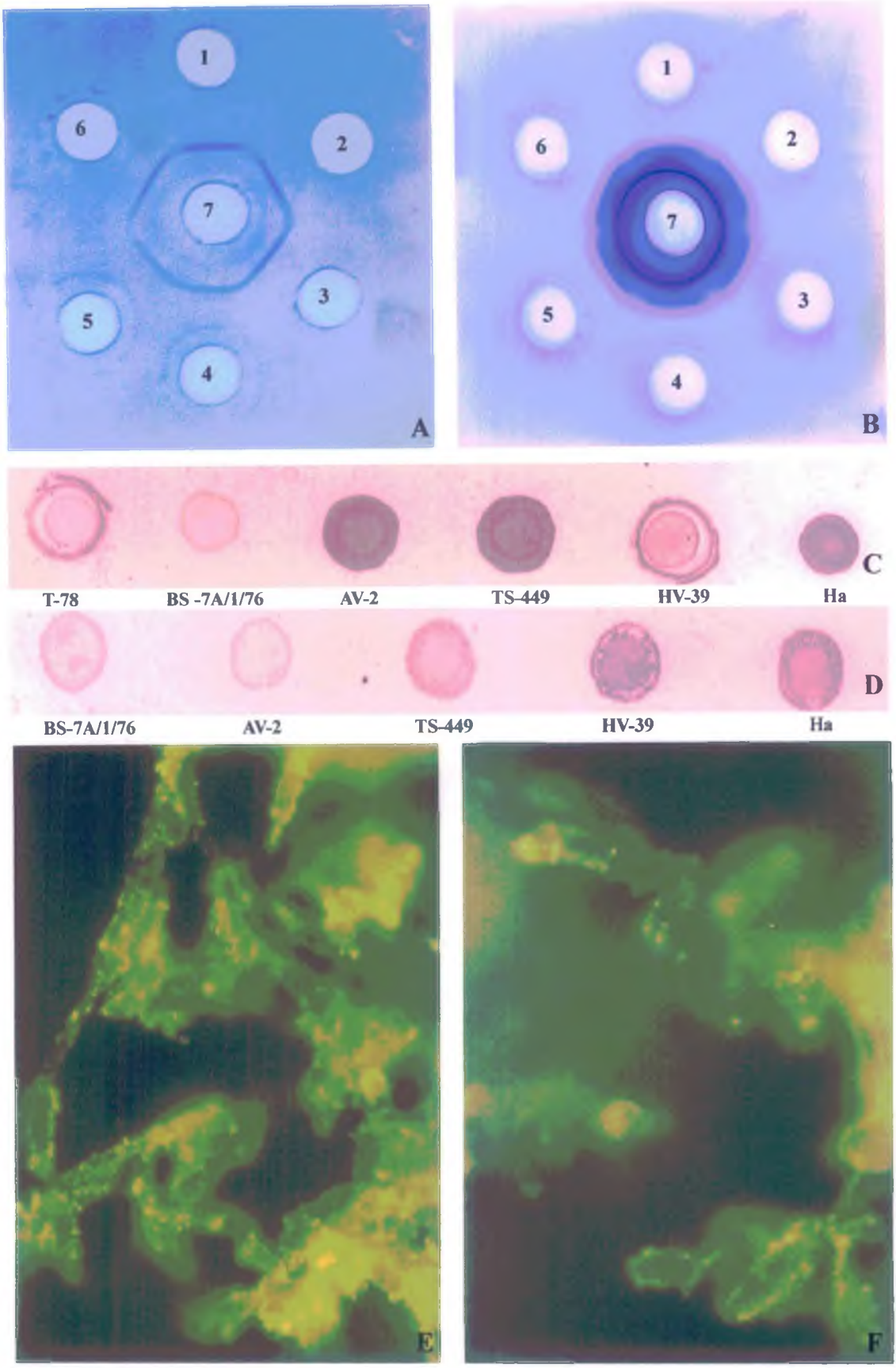


Plate 26 (figs. A - F). Immunoassays: Immunodiffusion [A & B], Immunobinding assay [C&D] of defense enzymes and Indirect immunofluorescence [E & F] of *E.vexans* with PAbs raised against chitinase [B,C & E] and β-1,3-glucanase [A, D & F]; homologous antigen [1 - 6 & Ha], PAbs [7].

Different concentrations of IgG (ranging from 0.312-40 μg ml^{-1}) from PAb-CHT and βGLU were tested against homologous antigens at a concentration of 10 μg ml^{-1}. Absorbance values in PTA-ELISA increased with increase in concentration of IgG with a maximum in 40 μg ml^{-1} IgG concentration (Figure 8 A). This concentration of IgG was selected for further experiments. Antigen concentrations ranging from 0.156-20 μg ml^{-1} were tested against PAb-CHT at a 40 μg ml^{-1} concentration of IgG. Results (Figure 8 B) revealed that ELISA values increased with the increase of antigen concentration. However antigen concentrations as low as 0.156 μg ml^{-1} could also be specifically detected by PTA-ELISA.

Absorbance values increased with increase in concentration of IgG (PAb-βGLU) with a maximum at 40 μg ml^{-1} IgG concentration (Figure 8 C). Further, antigen concentrations ranging from 0.156-20 μg ml^{-1} were also tested against PAb-βGLU at an IgG concentration of 40 μg ml^{-1}. Results are depicted in Figure 8 D. PAb-βGLU at 40 μg ml^{-1} was used in all experiments.

4.9.3. Dot Immunobinding Assay

Acid soluble proteins (PR) were extracted from healthy tea leaves of seventeen varieties (CP-1, HV-39, T-78, T-135, AV-2, BS-7A/1/76, TS-449, UP-2, UP-3, UP-8, UP-9, UP-26, BSS-2, BSS-3, TV-18, TV-22 and TV-23). These were reacted on nitrocellulose paper with PAbs raised against purified chitinase (PAb-CHT) and β-1,3-glucanase (PAb-βGLU) using dot-immunobinding formats as described earlier. Results revealed wide variations in the intensity of the dots when reacted with BCIP/NBT substrate. With PAb-CHT, antigens of CP-1, AV-2, TS-449, UP-2, UP-3, UP-9, TV-22 and TV-23 revealed deep violet coloured dots, whereas T-78, T-135, UP-8, BSS-2 and BSS-3 exhibited light violet dots and reactions with antigens of HV-39, BS-7A/1/76, UP-26 and TV-18 were insignificant with faint dots. Homologous reactions between PAb-CHT and its antigen revealed maximum intensity (Plate 26, fig. C). Similarly strong reaction were observed between PAb-βGLU and its homologous antigen (Plate 26, fig. D), however, on reaction with antigens of healthy tea leaves the reactions were weaker.

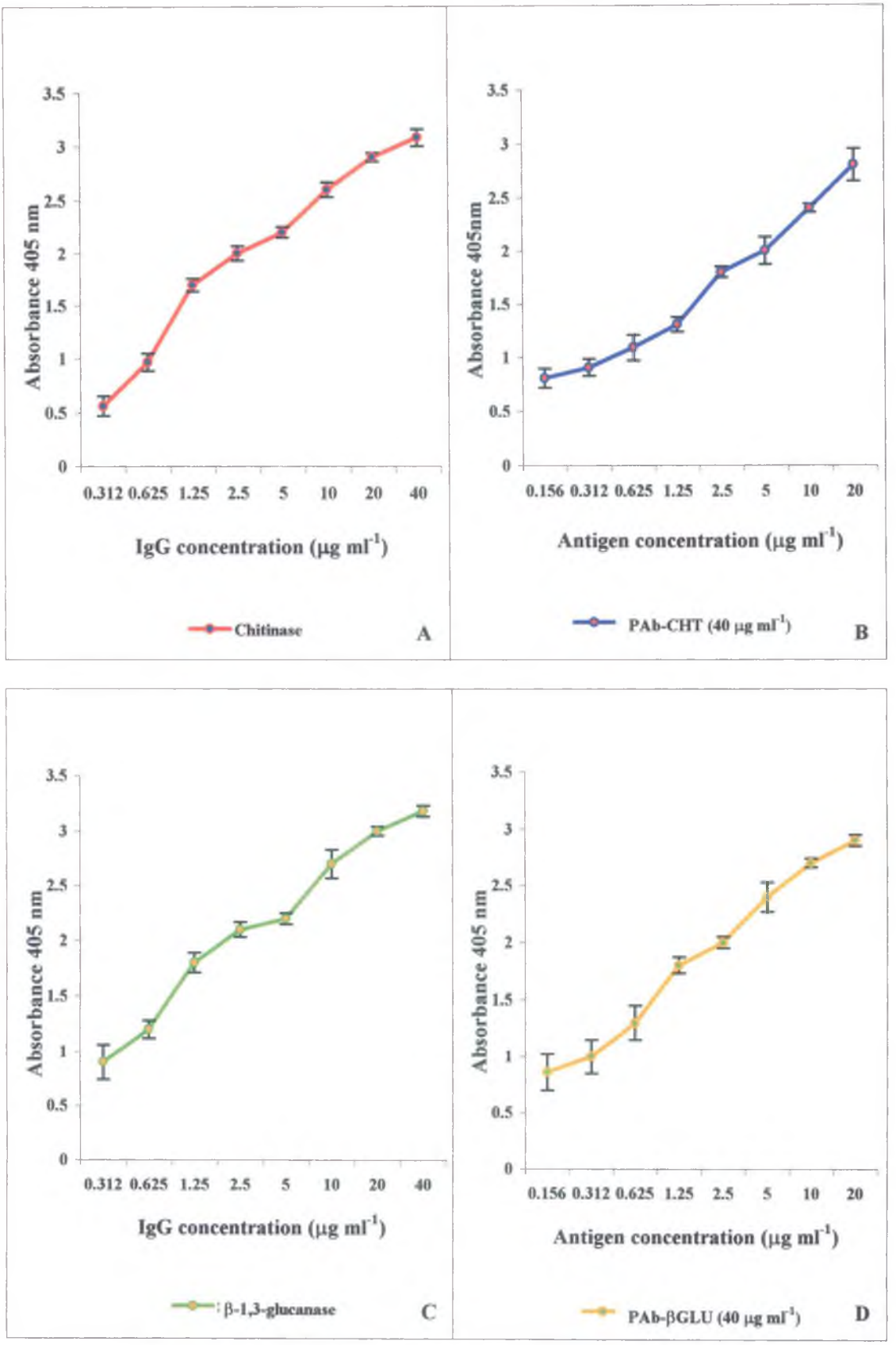


Figure 8 (A - D). Optimization of PAbs raised against chitinase and β-1,3-glucanase reacted against homologous antigens using PTA-ELISA formats.

Colour intensity of dots in reactions of leaf homogenates of HV-39, UP-26, BSS-2, TV-18 and TV-23 treated with PAb-βGLU were high TS-449, BS-7A/1/76, UP-2, UP-3, UP-9, TV-22, T-78, T-135 reacted positively but could not develop high intensity. The reactions CP-1, AV-2, BSS-3, and UP-8 were weak with very low intensity dots (Table 27).

Table 27. Immunodetection of PR-protein in healthy tea leaves by dot-immunobinding assay

Varieties	PAb-Chitinase	PAb-β-1,3-glucanase
CP-1	++++	+
HV-39	+	++++
T-78	++	++
T-135	++	++
AV-2	++++	+
BS-7A/1/76	+	++
TS-449	++++	++
UP-2	++++	++
UP-3	++++	++
UP-8	++	++
UP-9	++++	++
UP-26	+	+++
BSS-2	+++	+++
BSS-3	+++	+
TV-18	+	+++
TV-22	++++	++
TV-23	++++	+++
Chitinase[a]	++++	ND
β-1,3-glucanase[a]	ND	++++

Note: IgG concentration 40 μg ml^{-1}; Colour intensity of dots: + insignificant, + + light violet, + + + violet, + + + + deep violet; NBT/BCIP was used as substrate in dot blot assay; [a] purified homologous antigen, ND - not detected.

4.9.4. Western blot

The occurrence of PR-proteins during the natural development of tea plants indicates existence of an inherent immune system. To visualize and identify such proteins in healthy tea leaves (CP-1, HV-39, T-78, T135, AV-2, BS-7A/1/76, TS-449, UP-2, UP-3, UP-8, UP-9, UP-26, BSS-2, BSS-3, TV-18, TV-22 and TV-23), these were analysed by the Western blot procedure. After separation of proteins they were electrotransferred to nitrocellulose membranes and probed separately with PAb-CHT and PAb-βGLU to bind the target protein on the blots. All varieties tested revealed at least two bands of ca. 45 and 42 kDa with PAb-CHT, however the intensity of the bands varied. The varieties giving weaker signals with PAb-CHT were UP-26, HV-39 and BS-7A/1/76. On the other hand, reactions with PAb-βGLU revealed a single band of ca. 25 kDa with high intensity in AV-2, BS-7A/1/76, TS-449, UP-2, UP-3, UP-8, UP-9, UP-26, BSS-2, BSS-3, TV-18, TV-22 and TV-23. In CP-1, HV-39, T-78 and T135 although the protein band of ca. 25 kDa was visible, the reaction with PAb-βGLU was weak.

4.9.5. Indirect immunofluorescence

Basidiospores of *E. vexans* were treated separately with PAb-CHT and PAb-βGLU and labelled with FITC conjugate and observed under UV-illumination. Pre-immune sera did not show any reactivity with the spores of *E. vexans* and the spores were not autofluorescent. Bright-apple green fluorescence was emitted from the spore walls following treatment with the labelled PAbs, which indicated the positive reaction. On observation in both treatments it could be seen that the reaction was rather interrupted, as the fluorescence observed was not continuous along the walls. However, though weak, reaction of PAb-CHT was stronger than PAb-βGLU (Plate 26, figs. E & F).

Finally, the polyclonal antibodies raised against purified tea chitinase (CHT) and β-1,3-glucanase (βGLU) were cross reacted with Chitinase (Sigma, C7809) and Laminarinase (Sigma, L5257) following immunodiffusion technique and PTA-ELISA formats. In addition, polyclonal antibodies were raised separately against Chitinase

(Chi, PAb-Chi) and Laminarinase (Lam, PAb-Lam) according to the standard protocol adapted in this study. After optimization, agar gel double diffusion tests were carried out. A number of combinations were set including, PAb-CHT and Chi; PAb-Chi and CHT; PAb-βGLU and Lam; and, PAb-Lam and βGLU. Strong precipitin bands were observed in all combinations indicating homology between the different sources and thus confirming the efficacy of the PAbs raised against tea chitinase and β-1,3-glucanase.

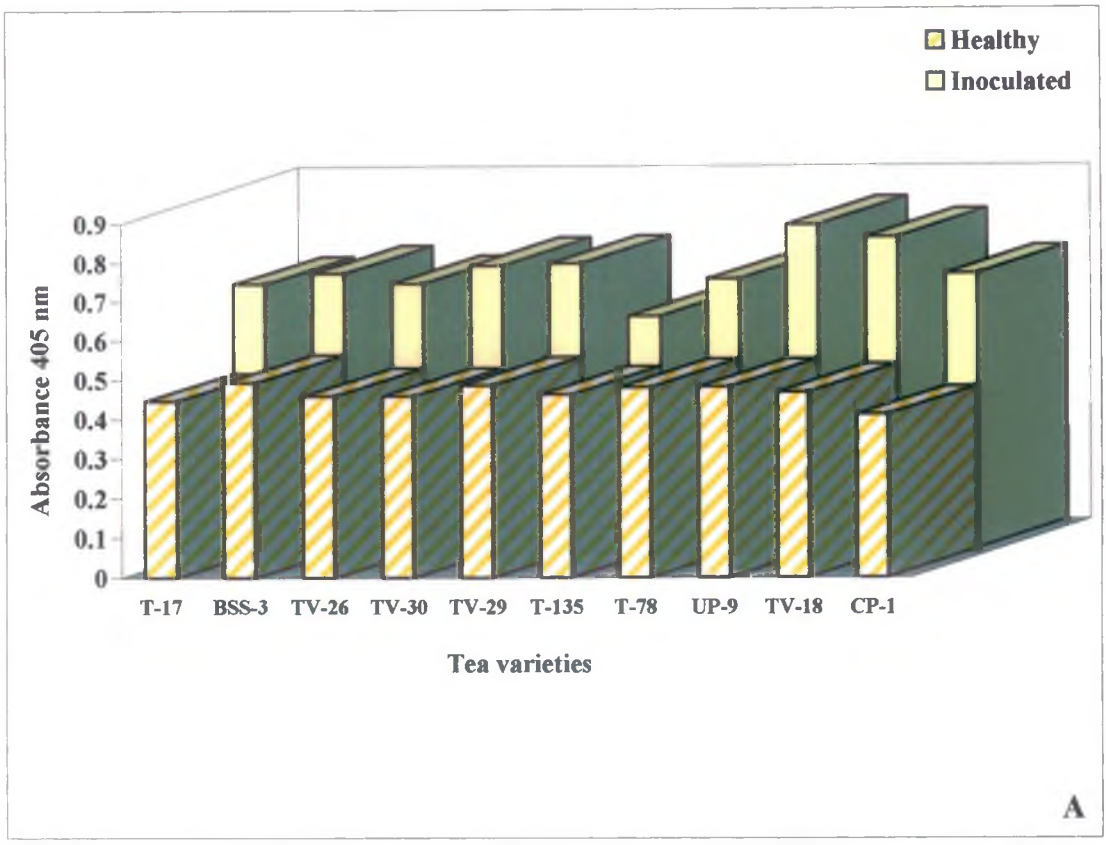
4.10. Immunological characterization of pathogenesis-related proteins in tea triggered by *E. vexans*

Association of pathogenesis-related proteins in this host-pathosystem reveals that disease resistance in tea following attack by *E. vexans* is a culminated action of many defense related enzymes such as chitinase (CHT), β-1,3-glucanase (βGLU) and peroxidase (POX). Activities of the hydrolytic enzymes were high in incompatible interactions and peroxidase activity was significantly high in leaves exhibiting hypersensitive responses. Results suggest an early specific induction of CHT and βGLU and delayed POX induction in incompatible interactions. Artificial inoculation of plants with *E. vexans* and associated changes in these three defense enzymes (CHT, βGLU and POX) revealed that accumulation was comparatively high in incompatible interactions. After a series of biochemical tests in the preceding chapters, an attempt was made to present further information on the immunological characterization of defense proteins using PAbs specific for chitinase and β-1,3-glucanase from tea, by PTA-ELISA, immunoblot, and immunogold labelling with an intention to study biotic stress responses in tea varieties susceptible and resistant to *E. vexans*.

4.10.1. Chitinase (PR-3)

4.10.1.1. PTA-ELISA

The antisera raised against the purified chitinase (PR-3) was first used to study their accumulation in ten selected tea varieties (T-17/1/54, BSS-3, TV-26, TV-30, TV-29, T-135, T-78, UP-9, TV-18 and CP-1) following artificial inoculation with *E. vexans*. Strong signals were obtained with PAb-CHT (Figure 9 A).



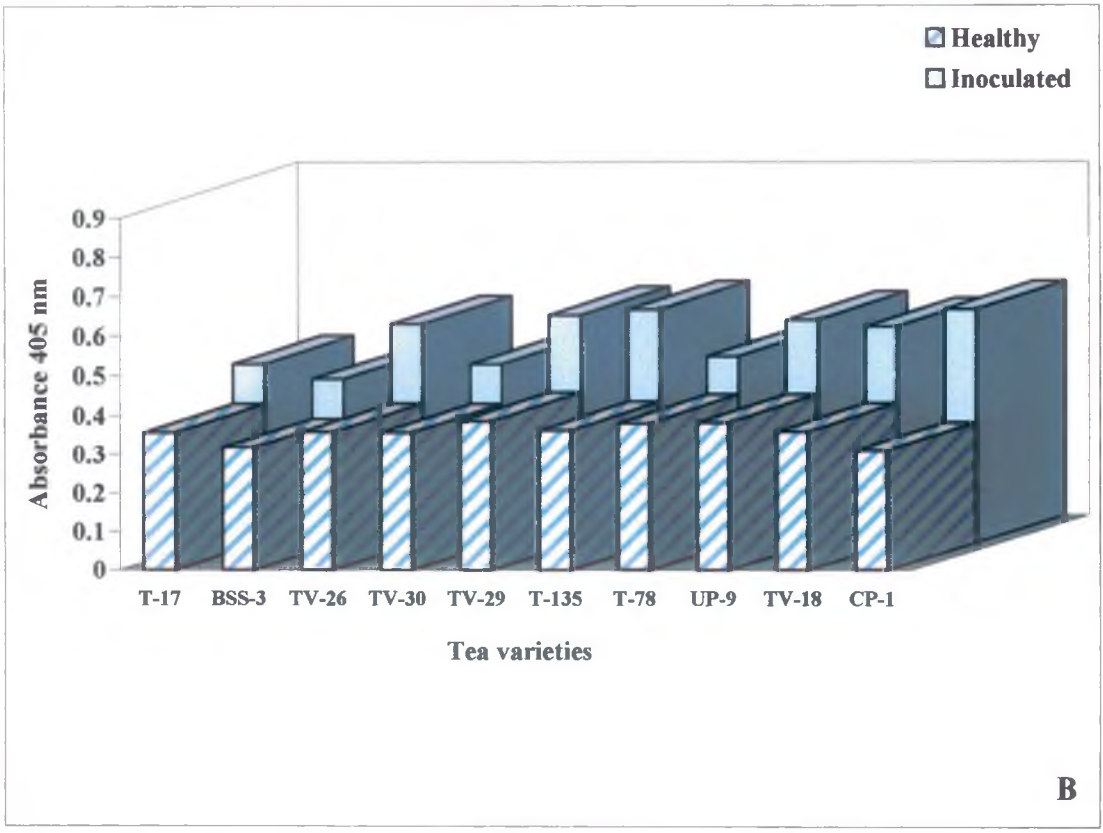


Figure 9 (A & B). PTA-ELISA responses of PAbs raised against chitinase [A] and β-1,3-glucanase [B] reacted with acid soluble leaf proteins of healthy and *E. vexans* inoculated (48 h) tea varieties.

The accumulation of chitinase appeared to start at an early stage after inoculation. Higher concentration of the induced proteins, than uninoculated healthy control, was detected following 1 d post inoculation. The concentration reached maximum levels at 2 d after inoculation.

4.10.1.2. Dot-immunobinding assay

Acid soluble protein extracts obtained from tea leaves of control and *E. vexans* inoculated tea plants of resistant and susceptible varieties were reacted with PAb-CHT in dot immunobinding assays. Results revealed that the antigens from leaves after 24 h of inoculation of resistant varieties (T-17/1/54, BSS-3, TV-26, TV-30 and TV-29) showed deep violet colour and that of susceptible varieties (T-135, T-78, UP-9, TV-18 and CP-1) was lighter in colour indicating weaker reaction (Table 28, Plate 27, fig. B) in comparison to homologous reactions.

Table 28. Dot blot reaction of PR-3 protein of different tea varieties

Varieties	PAb-CHT		PAb-βGLU	
	Healthy	Inoculated	Healthy	Inoculated
T-17/1/54	+++	+++++	++	+++++
BSS-3	+++	+++++	+	++++
TV-26	++	++++	+	+++
TV-30	++	++++	+	+++
TV-29	+	+++	+	++
T-135	++	+++	+	++
T-78	+	++	+	++
UP-9	+	++	+	++
TV-18	+	++	+	++
CP-1	++	+++	+	++

Note: IgG concentration 40 μg ml^{-1}; Colour intensity of dots: + insignificant, + + light violet, + + + violet, + + + + deep violet, + + + + + very deep violet; NBT/BCIP was used as substrate in dot blot assay.

4.10.1.3. Western blotting

Proteins were extracted from the healthy and *E. vexans* - inoculated tea plants (T-17/1/54, BSS-3, TV-26, TV-30, TV-29, T-135, T-78, UP-9, TV-18, HV-39, UP-8, BSS-2 and CP-1) and quantified. It has already been seen that the electrophoretic pattern of the acidic proteins extracted from tea leaves change following inoculation

with *E. vexans*, with stronger signals for PR-proteins in incompatible reaction of the resistant varieties than in the compatible reaction. To further investigate induction of PR-proteins that were related to PR-3 (chitinase) western blots of extracts collected from control and *E. vexans* inoculated plants were performed. Immunoblotting of the extracts separated on SDS-gels using the chitinase probe (PAb-CHT) detected two bands of ca. 42 and 45 kDa that were universal in all protein extracts of tea plants. These proteins appeared to be constitutively present. Immunoblots of healthy extracts alone contained two weaker bands of ca. 42 and 45 kDa (Plate 27, fig. D).

In a time-course experiment protein extracts of tea varieties 24, 48, 72 and 96 h after inoculation with the pathogen were analyzed. In all time bound interactions at least one protein of ca. 14 kDa was recognized in all inoculated extracts and two of ca. 45 kDa were common with healthy (Plate 27, fig. E). Of major significance were the hybridization results of extracts obtained after an interval of 24 h of inoculation (Table 29). In addition to the bands of ca. 42 and 45 kDa, and the other common band of ca. 14 kDa, hybridization with the probe produced two more bands of ca.61 and 20. The signal strength of the bands detected 24 h post inoculation using PAb-chitinase appeared to be the same. The bands of ca.61 and 20 kDa were unique to this time interval however a weak signal of the ca.42 and 14 kDa bands were sometimes revealed even after 48 h of inoculation, but never obtained 72 and 96 h post inoculation. Representative results are given in Plate 27.

Table 29. Detection of PR-proteins in healthy and *E. vexans* inoculated tea varieties by western blotting

Varieties	Molecular weight (kDa)			
	PAb-CHT		PAb-βGLU	
	Healthy	Inoculated[a]	Healthy	Inoculated[a]
TV-26	45, 42	61, 45, 42, 20, 14	25	33, 17
TV-29	45, 42	61, 45, 42, 14	25	33
T-135	45, 42	45, 42, 14	25	33, 17
T-78	45, 42	45, 42, 14	25	33, 17
UP-9	45, 42	61, 45, 42, 14	25	33, 17
TV-18	45, 42	61, 45, 42, 14	25	17
HV-39	45, 42	45, 42, 20, 14	25	33, 17
UP-8	45, 42	45, 42, 20, 14	25	33
BSS-2	45, 42	61, 45, 42, 14	25	17
CP-1	45, 42	61, 45, 42, 20, 14	25	33, 17

Note: IgG concentration 40 μg ml^{-1}; [a] 24 h after inoculation with *E. vexans*

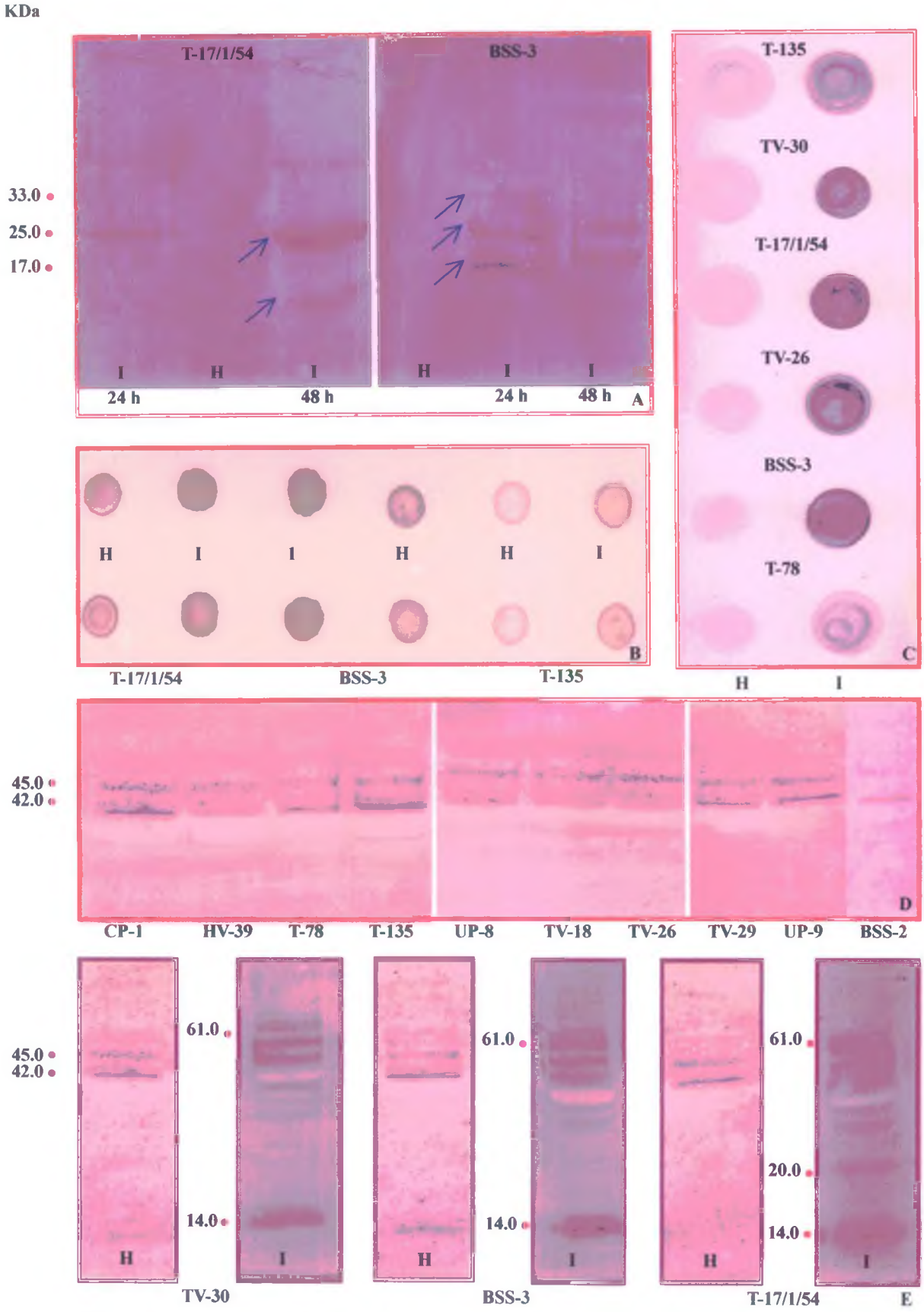


Plate 27 (figs. A - E). Dot immunobinding assay [B & C] and Western blot analyses [A, D & E] of PR-2 [A & C] and PR-3 [B, D & E] in tea plants triggered by *E.vexans*.

4.10. 1.4. Immunogold localization of chitinase (PR-3) in *E. vexans* inoculated tea leaves

Treatment of ultrathin sections from *E. vexans* – inoculated tea leaves (susceptible – T-135 and resistant – T-17/1/54) embedded in LR-white resin with PAb-CHT (PR-3) resulted in a significant increase in the number of gold particles over host cell walls and in material of intercellular spaces between parenchyma cells (Plate 28, figs. A & B), as compared to healthy leaves. The electron-translucent zone of intercellular spaces was virtually free of labelling either in the palisade parenchyma or spongy parenchyma. A heavy deposit of gold particles occurred over the cells. Immunohistology controls were run in parallel and treated with pre-immune serum instead of primary antibodies.

Microscopic examinations revealed labelling corresponding to chitinase deposition both in healthy and *E. vexans* inoculated plants. However the labelling of chitinase in leaves artificially inoculated with *E. vexans* was intense. It was established that *E. vexans*, the causal agent of blister blight disease of tea induced accumulation of chitinase in cell walls and extracellular spaces. Results indicate that accumulation of the defense protein chitinase is most likely connected with its induction in a systemic mode. In the healthy plants, the labelling was recorded, confirming that chitinase is constitutively present in healthy tissues. Considering the presence of chitinase not only in the surrounding area but also distant from infected cells it is likely that the chitinase is induced both locally and systemically.

4.10.2. β-1,3-glucanase (PR-2)

4.10.2.1. PTA-ELISA

The antisera raised against the purified β-1,3-glucanase (PR-2) was also initially used to study their accumulation in ten varieties of tea (T-17/1/54, BSS-3, TV-26, TV-30, TV-29, T-135, T-78, UP-9, TV-18 and CP-1) following inoculation with *E. vexans*. The accumulation of β-1,3-glucanase commenced at an early stage after inoculation. In comparison to control concentrations of the induced proteins higher and were detectable following 1 d post inoculation which reached maximum levels 2 d after inoculation.

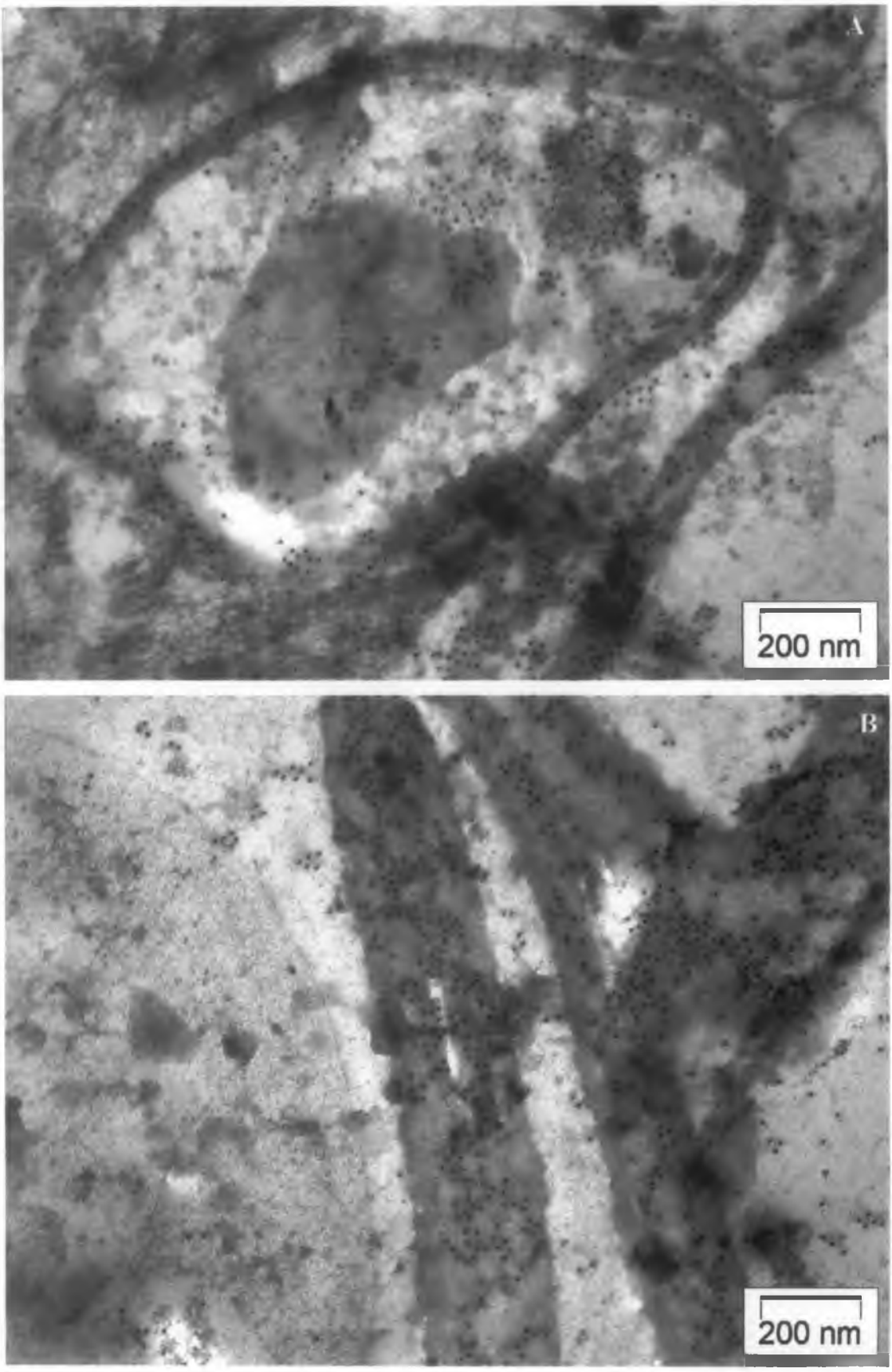


Plate 28 (figs. A & B). Transmission electron micrographs of immunogold labelled blister blight infected tea leaf tissues probed with PAb of chitinase (magnified view).

4.10.2.2. Dot immunobinding assay

Acid soluble extracts of tea plants (T-17/1/54, BSS-3, TV-26, TV-30, TV- 29, T-135, T-78, UP-9, TV-18 and CP-1) inoculated with *E. vexans* and their corresponding controls were analyzed by dot-blot. β-1,3-glucanase enzyme activity was measured in the extracts and loaded on nitrocellulose membranes. Intensity of the dots was scored by eye and variation was apparent.

4.10.2.3. Western blotting

Western blot analysis using PAb-β-1,3-glucanase (PR-2) was also performed to characterize accumulation patterns of β-1,3-glucanase isozymes in *E. vexans* inoculated tea varieties as well as their respective controls. Analysis of β-1,3-glucanase located in leaf protein extracts separated on SDS-gels using the β-1,3-glucanase probe (PAb-βGLU) revealed a band of ca. 25 kDa that was common in all protein extracts of tea plants. This protein appeared to be constitutively present. Following inoculation accumulation patterns were similar in all varieties. Additional bands of ca. 17 and 33 kDa were observed in inoculated extracts. These two isozymes, especially the ca. 25 kDa protein were induced to a much greater extent in resistant varieties during the course of pathogenesis when compared to the susceptible varieties.

Hence, new bands with β-1,3-glucanase activity as a result of inoculation with *E. vexans* compared to extracts from uninoculated plants was seen. The intensity of bands was greater in resistant inoculated plants.

4.10.2.4. Immunogold localization of β-1,3-glucanase (PR-2) in *E. vexans* inoculated tea leaves

The accumulation of β-1,3-glucanase (PR-2) was investigated on cross sections of tea leaves inoculated with *E. vexans* (susceptible – T-135 and resistant – T-17/1/54). Ultrathin sections of healthy and *E. vexans* inoculated tea leaves embedded in LR-white resin and incubated with the PR-2 antiserum (PAb-βGLU) and labelled with gold conjugate. The results were compared with uninoculated control plants. Electron microscopic observations indicate that *E. vexans*, the causal agent of

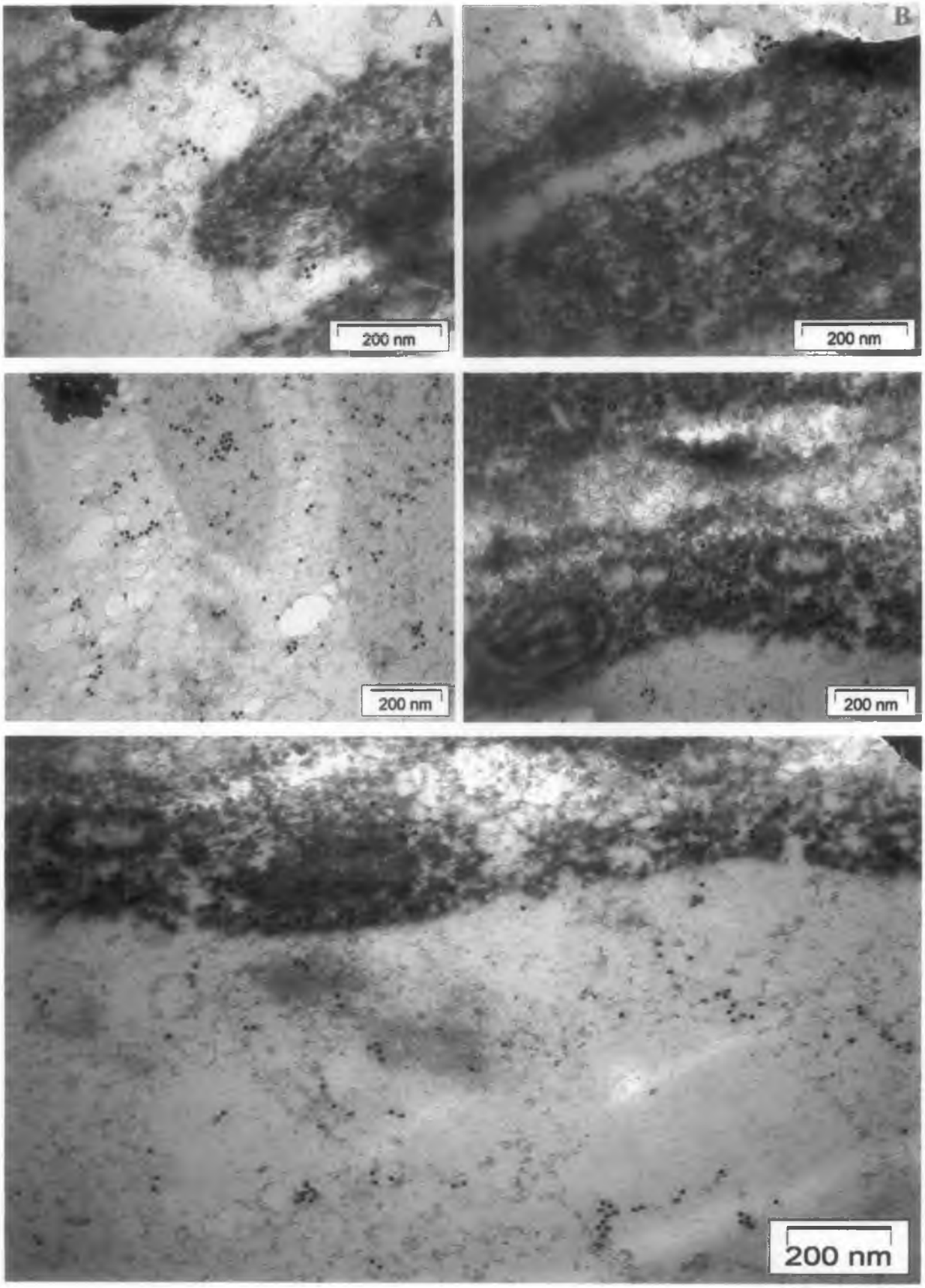


Plate 29 (figs. A - E). Transmission electron micrographs of immunogold labelled blister blight infected tea leaf tissues probed with PAb of β-1,3-glucanase

blister blight disease of tea induced accumulation of β-1,3-glucanase as well. Gold labelling corresponding to β-1,3-glucanase deposition both in inoculated and resistant plants was scattered (Plate 29, figs. A - E). In the healthy plants PAb-βGLU showed labelling to some extent and confirmed its presence in healthy tissues. Tissue cross-sections incubated with pre-immune serum instead of primary antibodies did not show any labelling. Comparing to chitinase, β-1,3-glucanase was not so strongly induced by the pathogen.

4.11. Accumulation of antifungal phenolic compounds in tea plants triggered by *E. vexans*

Histological studies have shown that during infection in both compatible and incompatible interactions, *E. vexans* germinates, develops appressoria, and penetrates the epidermis. However only in the compatible interaction does the fungus develop infection hyphae, penetrate internal cell walls and ramify throughout the host. It therefore appears that resistance is expressed prior to internal cell wall penetration and ramification of infection hyphae. Several mechanisms have been demonstrated in varied plant-pathogen systems and were suggested to account for the failure in penetration or reduced ramification of fungal development in resistant interactions. In most of these cases an increased production of phenolic compounds is apparent. This increased accumulation of phenolic compounds contributes to the fluorescent characteristic often associated with the resistant interactions. Such autofluorescence and accumulation of phenolics in cellular compartments during resistant reactions has been demonstrated in this study. Further, a method was developed in this study, to quantify extractable pyrocatechol, an antifungal phenolic compound, and to analyse catechins by high performance liquid chromatography (HPLC). Artificially *E. vexans* – inoculated and naturally blister blight infected leaf samples as well as their respective healthy controls were sampled. In addition leaves exhibiting hypersensitive resistant reactions were also considered. Leaf samples with two replicate treatments were taken for processing at one time. Experiments were repeated thrice.

4.11.1. Phenolics

Phenolic compounds could be detected in both susceptible and resistant interactions, however there were distinct differences between the interactions. Ethyl acetate fractions of both healthy and *E. vexans* inoculated leaf extracts were loaded on TLC plates, developed in chloroform - methanol (9:1, v/v) and sprayed separately with Folin-Ciocalteau's reagent, diazotized p-nitroaniline and vanillin-H_2SO_4. Compound I at Rf. 0.63 showed brown colour reaction when sprayed with vanillin-H_2SO_4, whereas compound II at Rf. 0.58 gave positive colour reaction with Folin-Ciocalteau's reagent indicating the presence of phenolic compounds.

4.11.1.1. UV-spectrophotometric analysis

Antifungal compounds extracted from the healthy, naturally blister infected tea leaf tissues as well as tea leaves exhibiting hypersensitive reactions against *E. vexans* were partially purified in preparative TLC. Theses were further analysed by UV-Spectrophotometer (Shimadzu 160). Maximum absorption peak measured at 274 nm was identical to an authentic sample of pyrocatechol. Hence quantification of the antifungal compound pyrocatechol was done from the UV-Spectrophotometric curve considering the molar extinction co-efficient of authentic pyrocatechol 6000 at 274 nm (Table 31).

$$\text{Molar extinction}^{\#}\ \text{Coefficient (E)} = \frac{\text{O.D of the tested solution}}{\text{Concentration (x) of the tested solution (moles } L^{-1})^{*} \times \text{Path length of the cell (cm)}}$$

*Moles L^{-1} converted to g L^{-1} by multiplying moles with molecular weight of catechol. Results have been expressed in $\mu g\ g^{-1}$ fresh weight of leaves.

Table 30. Quantification of pyrocatechol from tea varieties following inoculation with *E. vexans*

Varieties	Pyrocatechol (μg g^{-1} leaf tissue)	
	Healthy	Inoculated [a]
Resistant	98.4 ± 3.4	692.7 ± 5.4
T-17/1/54	74.3 ± 2.2	572.9 ± 3.1
BSS-3		
Susceptible		
T-135	64.4 ± 2.8	356.9 ± 4.9
UP-9	60.6 ± 5.2	292.8 ± 6.0

Note: Results are average of three experiments; [a] Quantification was done 48 h after inoculation with *E. vexans*, ±S.E.

4.11.1.2. HPLC analysis in leaf tissues before, after and during infection

In an attempt to analyse the extractable antifungal phenolics corresponding to the assorted incompatible and compatible interactions, HPLC analysis of tea leaf samples before, after and during infection was performed. The elution pattern of the phenolic compounds is illustrated in Figure 10 (A - D) where the significant peaks have been labelled and consecutively numbered (P1 - P3). In all samples noticeable peaks were resolved (Table 31). Accumulation of P1 at average retention time of 2.756 min was observed in all samples.

In plants inoculated with *E. vexans*, the peak height of P1 (2.7 min) and P2 (3.1 min) were higher than their corresponding peaks in control samples with greater accumulation of phenolic compounds (Figure 10 A). Similarly in naturally infected samples also P1 was higher in compatible interactions (Figure 10 C) than that in healthy leaves (Figure 10 B). Accumulation of two additional peaks – P2 and P3 at retention time 3.120 and 3.470, respectively, in the severely infected samples was of major significance (Figure 10 D). These peaks (P2 and P3) were observed in naturally infected samples undergoing compatible interactions only.

Another interesting finding was the increased production of the phenolic P1 in the leaves exhibiting hypersensitive resistant reactions (Figure 10 D). The 2-fold enhancement in the level of P1 (2.83 min) was very much significant in comparison to healthy and blister infected samples.

Table 31. HPLC analysis of antifungal phenolics in tea leaves

Sample description	Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
Artificial inoculation [a]							
Healthy	P1	2.690	7463.8584	434.978	0.290	65.886	73.840
	P2	3.120	3207.5152	138.736	0.220	28.314	23.551
Inoculated [b]	P1	2.700	9773.2254	550.275	0.300	58.996	71.220
	P2	3.110	5045.4727	202.030	0.210	30.457	26.148
Natural infection [a]							
Healthy	P1	2.870	11574.3050	319.009	0.440	100.000	100.00
Infected	P1	2.690	7627.7092	367.175	0.390	52.860	50.205
	P2	3.120	2608.3193	238.182	0.180	18.075	32.567
	P3	3.470	3578.9324	119.820	0.240	24.802	16.383
HR [c]	P1	2.830	27681.3406	752.152	0.340	100.000	100.00

Note: [a] Blister blight pathogen – *E. vexans*; [b] 48 h after inoculation; [c] HR- hypersensitive reaction

4.11.1.3. Antifungal assay

Antifungal phenolic compounds extracted from tea plants following inoculation with *E. vexans* as well as natural infection was assayed *in vitro*. Three fractions, viz., diethyl ether, ethyl acetate, and ethyl acetate hydrolysates were tested against *E. vexans* and *C. pallescens*. Using spore germination and radial growth bioassay techniques respectively. Controls were also set-up with sterile distilled water, diethyl ether and ethyl acetate. All fractions were filter sterilized before use.

All fractions were found to be inhibitory against basidiospores of *E. vexans* in the spore germination bioassay. Ethyl acetate fractions exhibited more inhibition than diethyl ether fractions. However, hydrolysed fraction of ethyl acetate was found to be more inhibitory than the non-hydrolysed one. This trend was the same for all other fractions prepared from various developmental stages (S1-S3) of the disease collected from Castleton Tea Garden (Table 32).

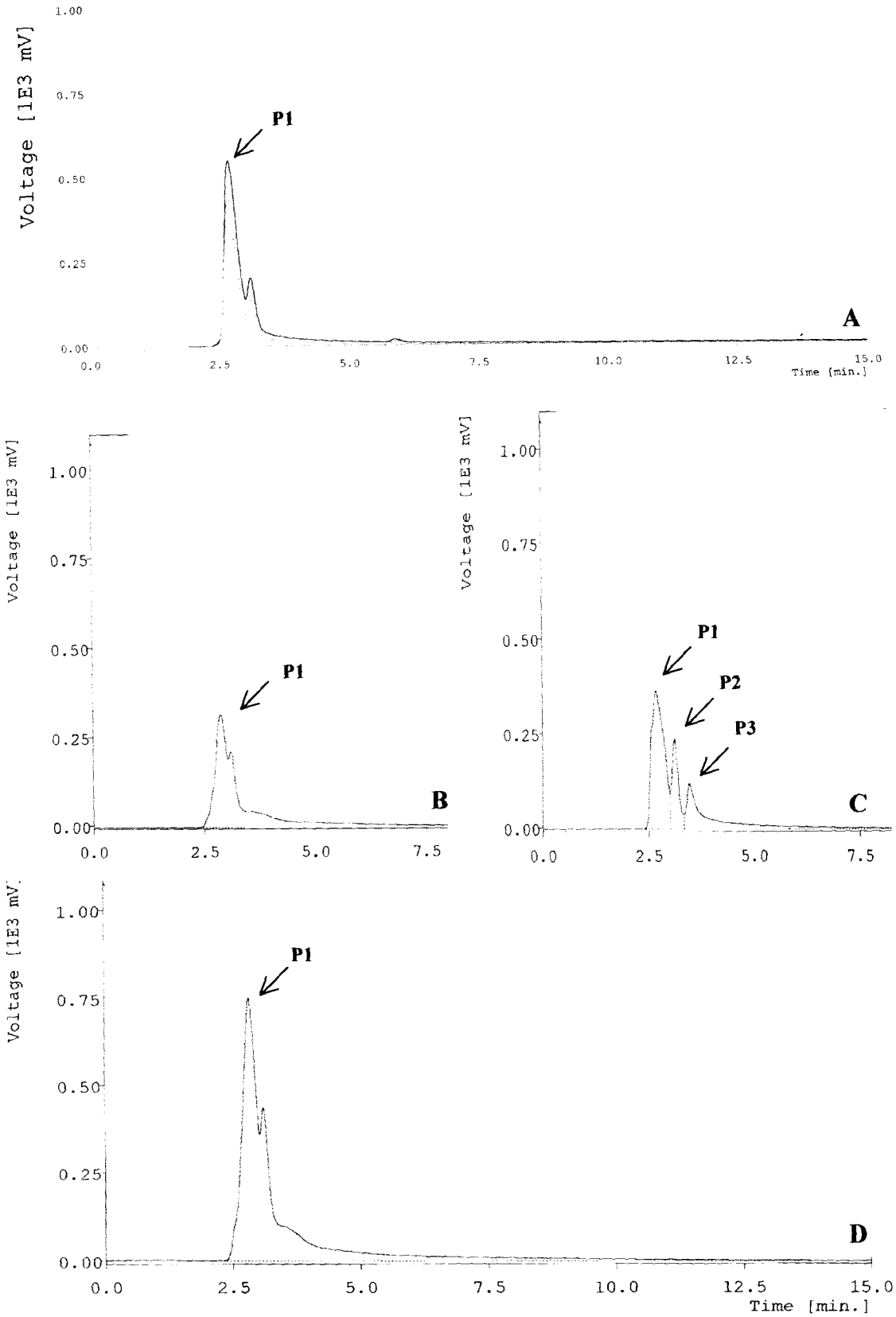


Figure 10 (A - D). HPLC elution profiles of pyrocatechol from healthy [A- green curve], *E. vexans* inoculated [A, blue curve] tea plants (T-17/1/54 - resistant variety) and leaves collected from Castleton Tea Estate exposed to blister blight infection. [B-D] Healthy plant [B], blister infected [C] and plants showing hypersensitive reaction [D].

Mycelial blocks of *C. pallescens* were inoculated on PDA medium supplemented with the phenolic extracts (50 µl) obtained from healthy and *E. vexans* inoculated tea leaves to assess their antifungal activity by radial growth bioassay. Results revealed that mycelial growth of *C. pallescens* was inhibited markedly in the medium supplemented with ethyl acetate hydrolysates (Plate 30, fig. B). Mycelial growth was inhibited by 45% in diethyl ether and 65 % in ethyl acetate (non-hydrolysed) fractions in relation to their respective controls. Interestingly, extracts prepared from leaves exhibiting hypersensitive resistant reactions completely inhibited spore germination of *E. vexans*, indicating that the plants accumulate their defensive compound in highest amount with speed and magnitude. (Plate 30 E). Hence from this we can conclude that the extracts are antimicrobial in nature with significantly inhibitory components.

Table 32: Antimicrobial activity of ethyl acetate hydrolysates of healthy and infected tea leaves

Test sample	*Exobasidium vexans* [a]		*Curvularia pallescens* [b]	
	Spore germination (%)	Inhibition in spore germination (%)	Mycelial growth (mm)	Inhibition in mycelial growth (%)
Control				
Water	84.7	-	-	-
PDA	-	-	40.0	-
Methanol	60.0	29.1#	32.0	20.0*
Diethyl ether	63.5	25.0#	36.5	8.7*
Ethyl acetate	66.5	21.4#	37.2	7.0*
Ethyl acetate hydrolysates				
Natural infection				
Healthy (H)	16.5	75.2##	0.0	100.0##
Blister infected (S)	50.0	24.8##	29.0	22.0##
Hypersensitive (HR)	12.5	81.2##	6.0	83.9##
Artificial inoculation				
Healthy (H)	0.0	100.0##	0.0	100.0##
E. vexans inoculated	28.0	57.9##	15.0	59.67##

Results (average of three experiments) recorded after [a]24 and [b]48 h of incubation
values indicate inhibition against water control; ## values indicate inhibition against ethyl acetate control; * values indicate inhibition against PDA medium control

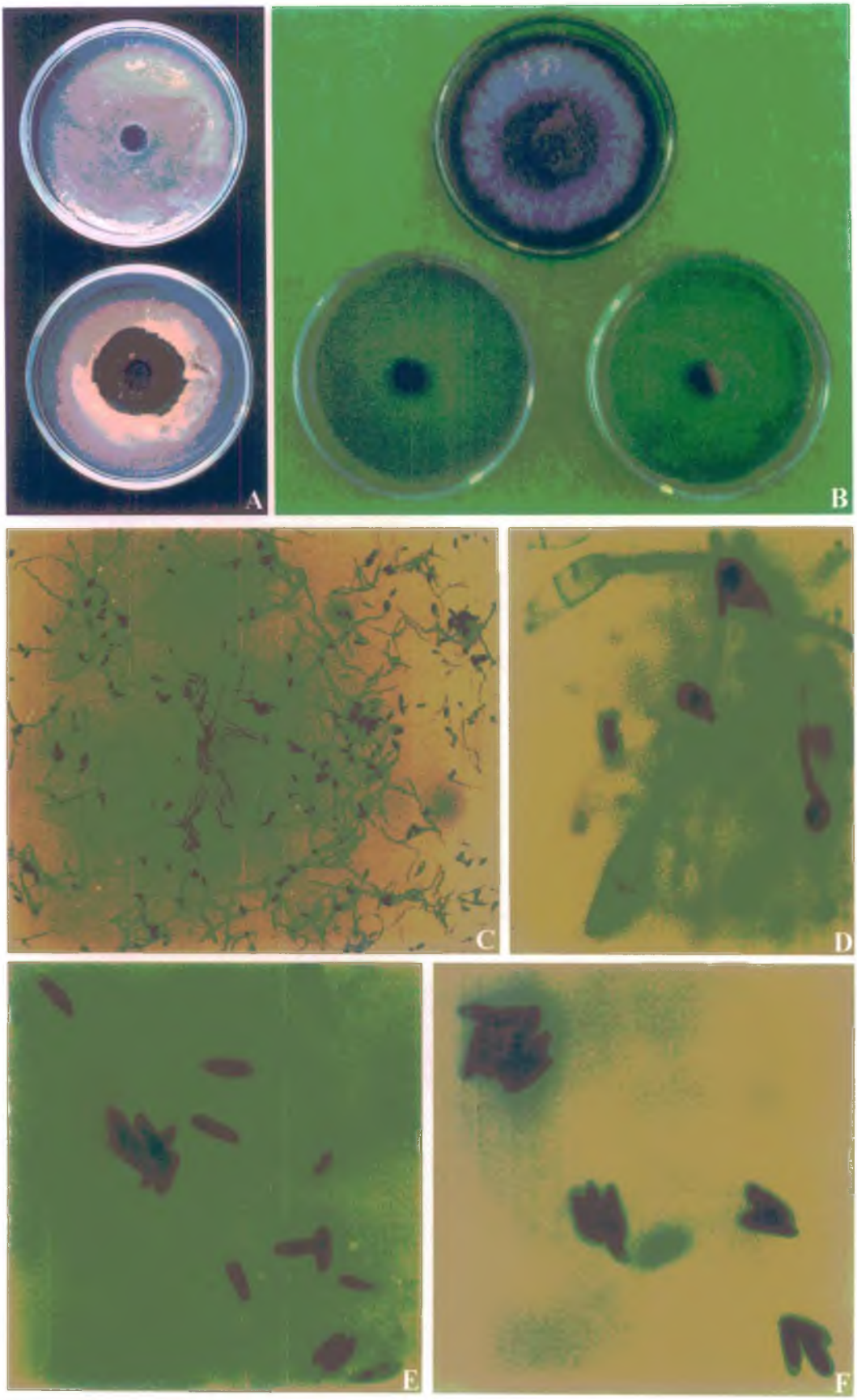


Plate 30 (A - F). Agar cup [A], radial growth [B] and spore germination [C - F] bioassay of antifungal phenolic compounds - Catechin [A & F], pyrocatechol [B & E]. Distilled water control [C & D], Medium control [B- top]. *Bacillus megaterium* [A], *Curvularia pallescens* [B], *Exobasidium vexans* [C - F].

4.11.2. Catechins

This study was carried out in order to investigate the inherent catechins present in tea plants that are stimulated during the development of blister blight disease as well as during hypersensitive resistance mechanism.

4.11.2.1. HPLC analysis in leaf tissues before, after and during infection

Involvement of catechins in both compatible and incompatible interactions of tea leaves with *E. vexans* were analysed by HPLC for a comparative analysis of the flavonoid compounds the extracts obtained from leaves of healthy plants and artificially *E. vexans* inoculated plants. Besides, healthy tea leaves from Castleton Tea Estate, naturally blister infected leaves of different stages of infection (S1-S3), as well as leaves exhibiting hypersensitive reactions were subjected to HPLC. Each sample was run in triplicate. All HPLC runs confirmed the presence of several flavonoid compounds in tea plants.

Analysis of the catechin extracts of healthy, artificially inoculated resistant tea variety (T-17/1/54) and naturally blister infected tea plants showed variant levels of the flavonoids. A number of peaks, P1-P20 (Figure 11 A & B), were prominent. The compounds that exhibited higher levels of accumulation in control plants include – P1, P2, P3, P7, P10, P11, and P14. The peaks of major significance in healthy plants were – P3, P7, P11 and P19 at retention times 4.5, 6.2, 8.3 and 19.5 min respectively (Table 33). In the inoculated (with *E. vexans*) plants (T-17/1/54), slight increase in peak height was revealed by P5, P6 and P9 noticeable increase was revealed by peaks - P13, P15, P17, P18 and P20 (Table 34). Of particular significance was the peak P18 at retention time 18.9 min, which was predominantly present, and was considerably enhanced in comparison to healthy controls. While peaks P4, P8 and P14 were of more or less similar heights, P14 at retention time 16.0 min appeared to be of very high concentration, which was revealed by the wide end of the peak. Two novel peaks, P12 and P16 at retention times 13.9 and 18.0 min respectively, were exhibited in inoculated extracts while a peak P19 at retention time 19.5 min present in control plants was absent in inoculated ones.

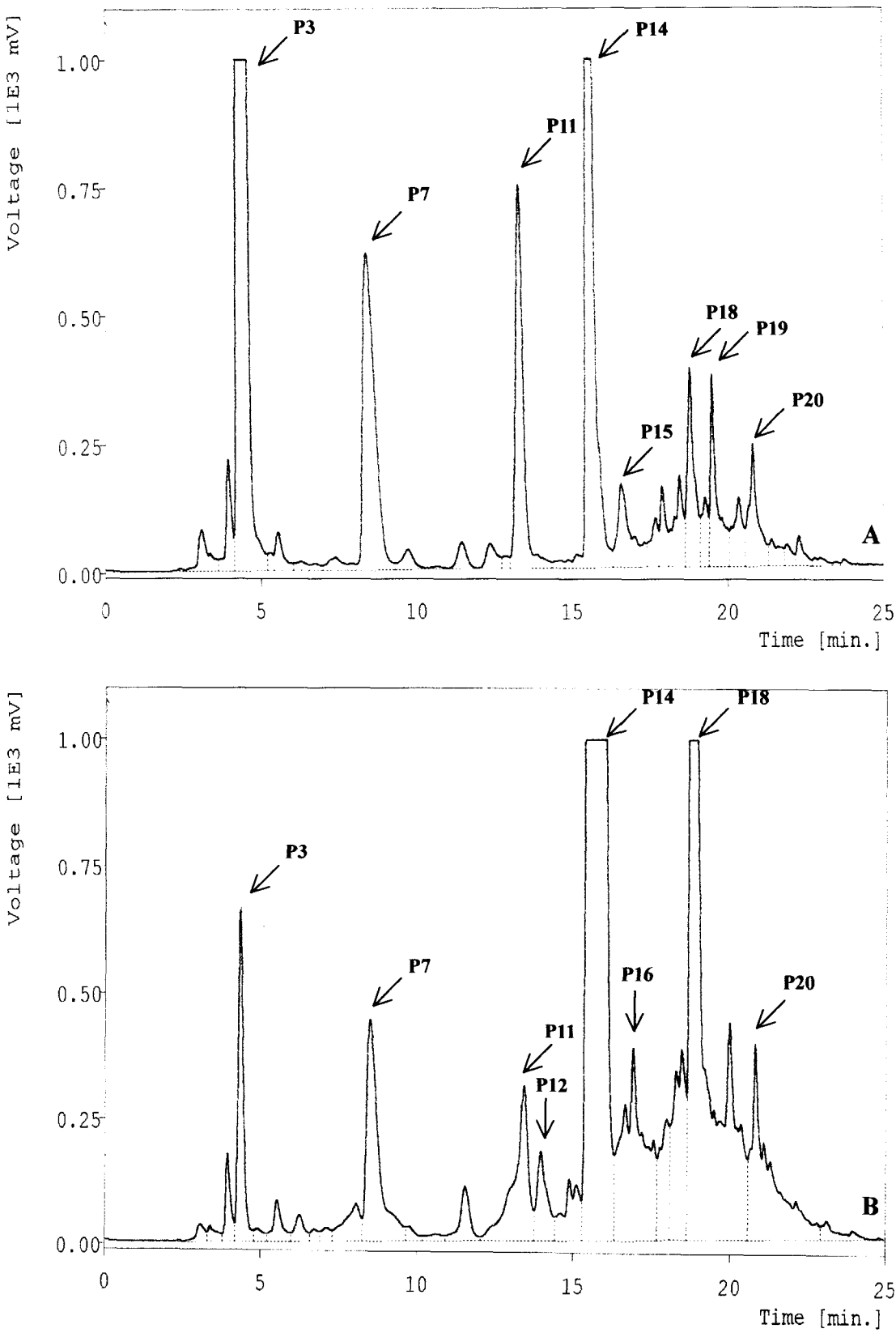


Figure 11 (A & B). HPLC elution profiles of leaf catechins of healthy [A] and *E. vexans* inoculated [B] tea plants (T-17/1/54 - resistant variety).

Table 33: HPLC analysis of tea leaf catechins before inoculation

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	3.070	1844.8569	81.152	0.270	1.400	1.320
P2	3.930	2711.0611	217.863	0.190	2.058	3.543
P3	4.550	29688.8998	996.799	0.460	22.536	16.210
P4	5.540	1371.2397	75.689	0.260	1.041	1.231
P5	6.280	438.1554	16.784	0.520	0.333	0.273
P6	6.730	265.9023	12.576	0.410	0.202	0.205
P7	8.380	16689.0414	615.645	0.440	12.668	10.012
P8	9.720	1058.7682	38.724	0.390	0.804	0.630
P9	10.670	121.0449	5.331	0.350	0.092	0.087
P10	11.460	1083.9410	51.874	0.330	0.823	0.844
P11	13.290	13048.4388	747.499	0.260	9.905	12.156
P12	-	-	-	-	-	-
P13	14.890	188.3559	14.862	0.230	0.143	0.242
P14	15.660	23070.9213	989.670	0.340	17.513	16.094
P15	16.590	4351.6828	162.694	0.330	3.303	2.646
P16	-	-	-	-	-	-
P17	18.470	3387.3730	178.528	0.370	2.571	2.903
P18	18.800	5939.0467	385.976	0.220	4.508	6.277
P19	19.500	5542.1199	371.667	0.160	4.207	.044
P20	20.820	4702.5336	237.275	0.230	3.570	3.859

Naturally blister-infected leaves exhibited less isoforms of catechins than healthy leaves (Figure 12 A). Peaks present in healthy leaves (Figure 13 A and Table 35) were mostly absent in blister infected leaves. Two peaks of 16.5 and 18.6 retention times and those with decreased height were of retention times 4.0, 13.4, 13.7, 14.5, 19.8 (Figures 13 A-C).

Though the plants showing resistant reaction reflected responses similar to healthy plants, catechins in *E. vexans*-infected samples and leaves exhibiting resistant reactions were remarkably different. Interestingly, after analysis of catechins from the plants showing hypersensitive reaction against *E. vexans* re-appearance of two major peaks at 13.9 and 18.7 retention times with recovery percentages of 92 and 86 respectively were evident (Figure12 B and Table 36).

Table 34: HPLC analysis of tea leaf catechins after inoculation with *E. vexans*

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	3.060	644.3913	34.288	0.330	0.326	0.593
P2	3.930	1986.4624	175.509	0.170	1.004	3.033
P3	4.320	7881.8791	662.793	0.190	3.985	11.454
P4	5.520	1593.7085	81.668	0.240	0.806	1.411
P5	6.240	1098.6679	52.720	0.320	0.555	0.911
P6	6.740	404.8969	23.390	0.330	0.205	0.404
P7	8.490	12262.0354	441.992	0.380	6.199	7.639
P8	9.750	610.8494	27.912	0.340	0.309	0.482
P9	10.630	481.2763	13.162	0.720	0.243	0.227
P10	11.550	2469.5137	108.681	0.300	1.248	1.878
P11	13.420	9857.3911	307.489	0.330	4.983	5.314
P12	13.970	3994.0251	178.517	0.370	2.019	3.085
P13	14.890	4035.8447	123.153	0.490	2.040	2.128
P14	16.040	48495.5080	986.625	0.790	24.517	17.051
P15	16.920	18271.1731	381.072	0.880	9.237	6.586
P16	18.000	4934.1183	240.652	0.400	2.494	4.159
P17	18.480	9925.2656	377.451	0.540	5.018	6.523
P18	18.970	47383.8776	984.243	0.420	23.955	17.010
P19	-	-	-	-	-	-
P20	20.830	15767.9145	388.384	0.240	7.971	6.712

Blister infected leaf samples of all three stages of infection (S1, S2 & S3) were analysed. Though there were no new peaks variation in the levels of the separated compounds was observed (Tables 37 - 39). The polyphenols present as inherent components were greatly enhanced in the leaves exhibiting hypersensitive reactions in comparison to susceptible ones, which was determined by peak height.

Table 35: HPLC analysis of catechins isolated from healthy tea leaves

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	4.120	7194.0844	517.449	0.190	5.282	11.184
P2	-	-	-	-	-	-
P3	6.660	1737.7937	112.834	0.270	1.276	2.439
P4	-	-	-	-	-	-
P5	-	-	-	-	-	-
P6	-	-	-	-	-	-
P7	13.950	31091.4911	1005.178	0.440	22.826	21.725
P8	16.520	8717.0287	428.563	0.240	6.400	9.263
P9	17.420	21845.5378	1003.637	0.250	16.038	21.692
P10	18.680	37654.9656	517.685	0.850	27.645	11.189

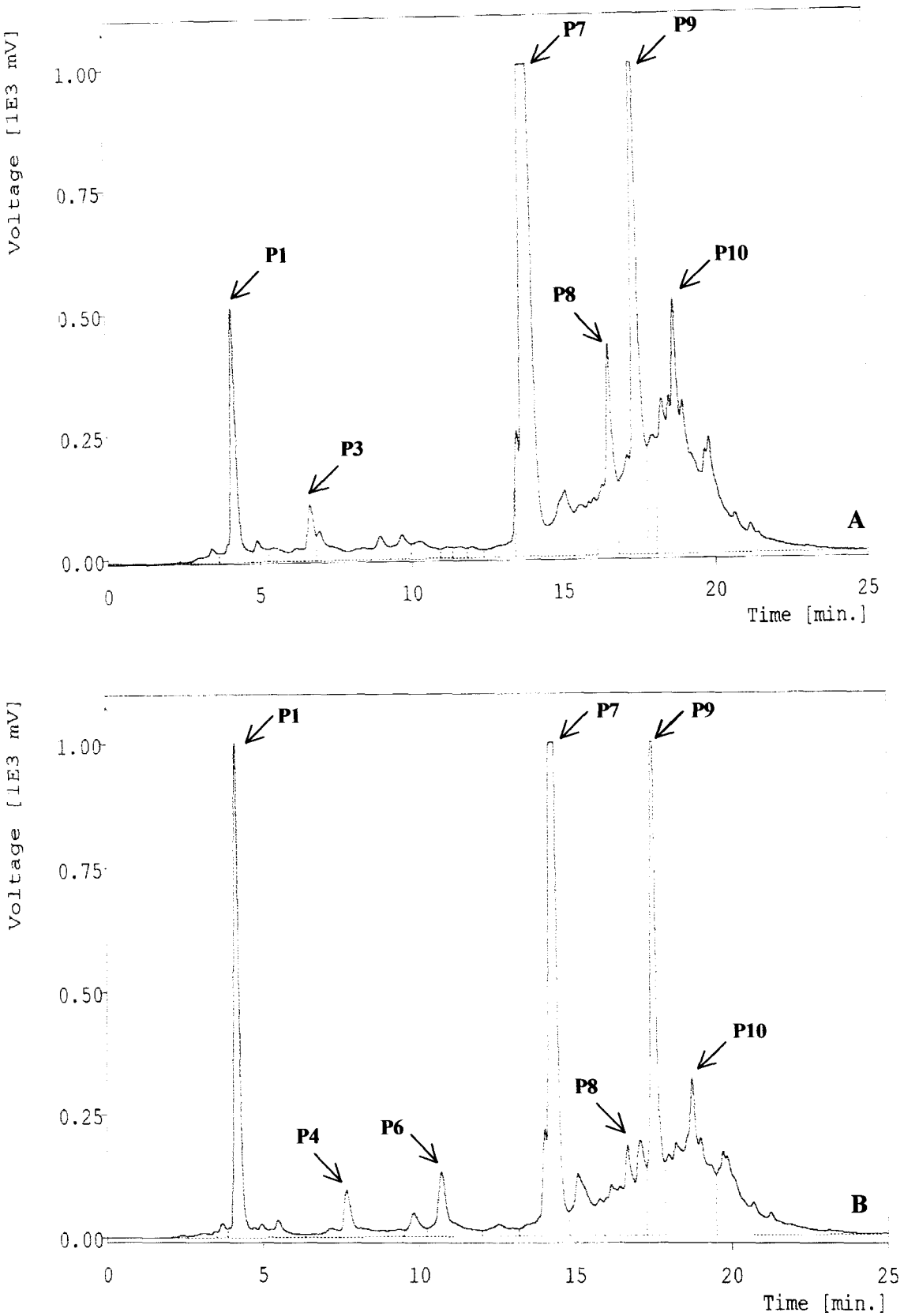


Figure 12 (A & B). HPLC elution profiles of leaf catechins of Castleton Tea Estate exposed to blister blight infection. [A] Healthy [B] Plants showing hypersensitive reactions.

Table 36. HPLC analysis of catechins isolated from leaves exhibiting hypersensitive reactions against *E. vexans*

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	4.130	13671.8302	1002.148	0.180	12.813	22.576
P2	-	-	-	-	-	-
P3	-	-	-	-	-	-
P4	7.670	2421.0155	95.154	0.250	2.269	2.144
P5	-	-	-	-	-	-
P6	10.710	3689.1413	132.140	0.270	3.457	2.972
P7	14.340	22880.9570	998.124	0.300	21.443	22.486
P8	16.680	6429.0934	183.206	0.810	6.025	4.127
P9	17.500	15055.7039	996.804	0.210	14.110	22.456
P10	18.750	17858.6707	318.997	1.000	16.737	7.186

Table 37. HPLC analysis of catechins isolated from leaves during initial stages of blister blight infection

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	4.000	6558.2640	493.190	0.180	14.774	27.531
P2	-	-	-	-	-	-
P3	-	-	-	-	-	-
P4	-	-	-	-	-	-
P5	-	-	-	-	-	-
P6	-	-	-	-	-	-
P7	-	-	-	-	-	-
P8	-	-	-	-	-	-
P9	17.410	27056.8385	867.232	0.170	60.949	48.411
P10	-	-	-	-	-	-

Table 38. HPLC analysis of catechins isolated from leaves at moderate blister blight infection stage

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	3.660	16278.8442	950.447	0.270	23.273	28.970
P2	-	-	-	-	-	-
P3	6.020	2961.9157	139.174	0.280	4.234	4.242
P4	-	-	-	-	-	-
P5	8.570	5502.1468	261.440	0.300	7.866	7.969
P6	-	-	-	-	-	-
P7	-	-	-	-	-	-
P8	-	-	-	-	-	-
P9	17.230	12092.6347	872.009	0.190	17.288	26.580
P10	-	-	-	-	-	-

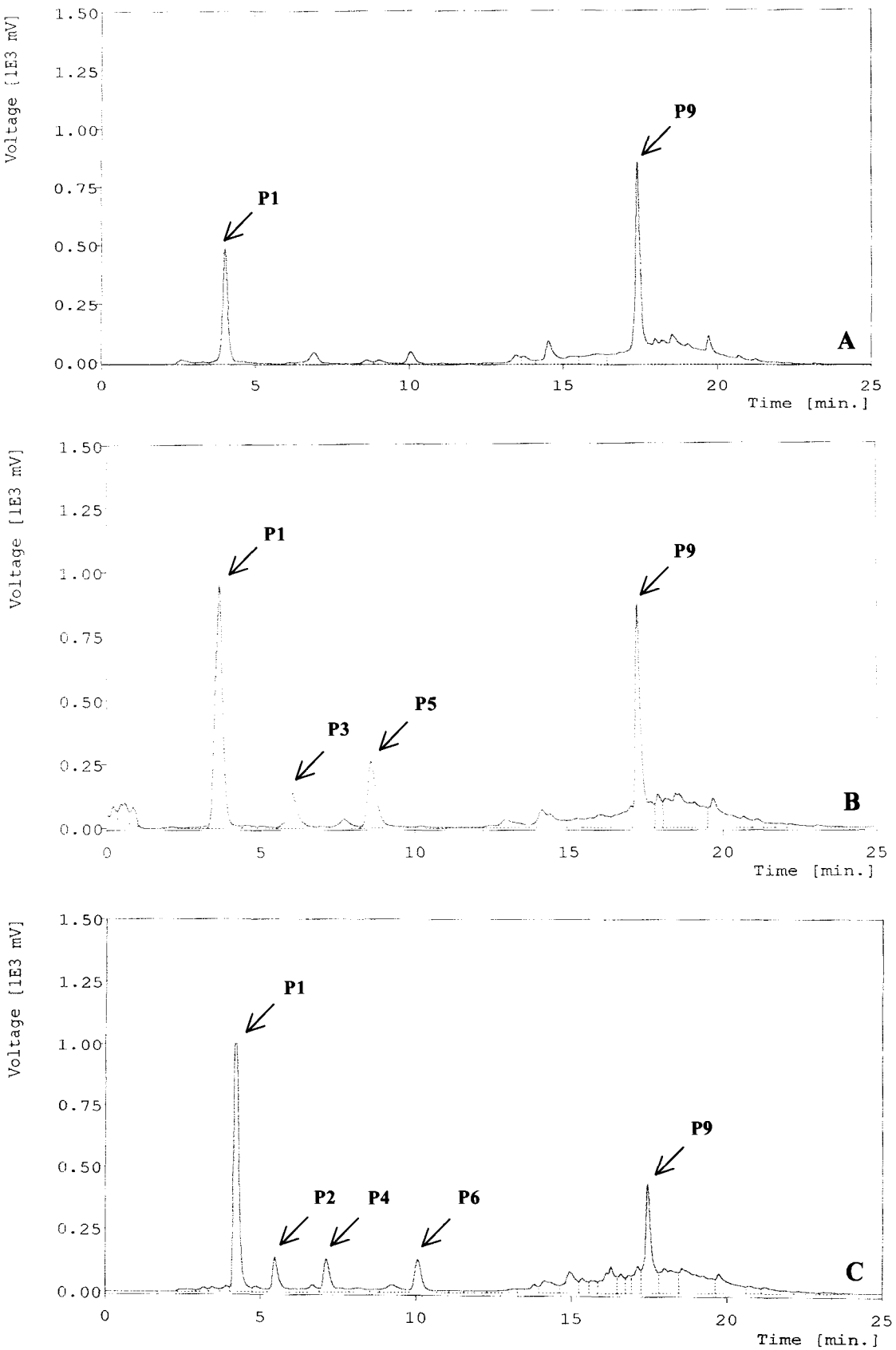


Figure 13 (A - C). HPLC elution profiles of catechins from leaves of Castleton Tea Estate exposed to blister blight infection during different stages of disease development. Stage - 1 [A], Stage -2 [B] and Stage - 3 [C].

Table 39. HPLC analysis of catechins isolated from leaves severely infected by *E. vexans*

Peak no.	Retention time	Area [mV.s]	Height [mV]	W05 [min]	Area [%]	Height [%]
P1	4.210	12618.7057	1002.681	0.200	22.344	33.223
P2	5.460	2212.5983	136.394	0.200	3.918	4.519
P3	-	-	-	-	-	-
P4	7.120	2267.0494	130.317	0.220	4.014	4.318
P5	-	-	-	-	-	-
P6	-	-	-	-	-	-
P7	-	-	-	-	-	-
P8	-	-	-	-	-	
P9	17.460	6321.5308	434.159	0.640	11.194	14.386
P10	-	-	-	-	-	-

4.11.4.2. Antifungal assay

Antimicrobial activity of catechin was tested against a bacterium, *Bacillus megaterium*. Clear inhibition zone was observed (Plate 30, fig. A) in catechin supplemented medium. In control sets initially respective solvent was given and after evaporation the medium was poured and seeded with bacterium. Antifungal activity of tea catechin extracts from healthy, *E. vexans* inoculated as well as blister infected tea leaves were also assessed using spore germination bioassay. Two separate controls sets, with water and methanol, were also set simultaneously. Germination was stopped in all sets after 24 h when spores in water control had germinated and formed appressoria (Plate 30, figs. C & D). Complete inhibition of germination of *E. vexans* was noticed in all the catechin extracts tested (Plate 30, fig. F).

4.12. Induction of resistance towards *E. vexans* in tea plants and associated changes in defense enzymes

The potential of systemic acquired resistance (SAR) as a tool in integrated disease management programs is becoming more widely recognized, especially as information regarding the occurrence of the phenomenon for an increasing number of plant-pathogen interactions become more abundant. Research concerning such defense mechanism has largely concentrated on determining the significance of the many associated pathogenesis-related proteins (PR-proteins) and investigating the nature of endogenous signal entities. The phenomenon of systemic acquired resistance (SAR) suggests that there is a signal that originates at the site of the elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns compatible plant-pathogen interactions into incompatible ones that are related to accumulation of defense compounds and PR-proteins. In this chapter an attempt has been made to induce resistance in tea varieties (T-78, UP-9 and TV-18) that have been established to be susceptible towards *E. vexans*. The plants were treated with abiotic elicitors followed by inoculation of leaves of whole plants using basidiospore suspensions of *E. vexans* under controlled conditions. Initially one of the most susceptible varieties (T-78) was considered for induction and associated changes in defence enzymes (chitinase- PR-3, β-1,3-glucanase- PR-2, peroxidase- PR-9 and phenylalanine ammonia lyase) were measured at 24, 48 and 72 h after inoculation from untreated healthy (UH), untreated inoculated with *E. vexans* (UI), treated healthy (TH) and treated inoculated tea varieties. Each experiment was repeated five times.

4.12.1. Influence of abiotic ellcitors on defense enzymes

Induced resistance may be achieved by the use of 'substances' that induce defense reactions in plants called elicitors. Resistance induction by abiotic elicitors has been observed and also suggested as an alternative control method to fungicides, which generally have toxic side affects. Of various chemical compounds known to induce resistance in different species two salts, a chloride and a phosphate, that possess the ability to induce PR-protein accumulation in plants were selected for this

study. The changes in activities of the defense enzymes induced with such abiotic elicitors before and after challenge of tea plants with *E. vexans* have been described.

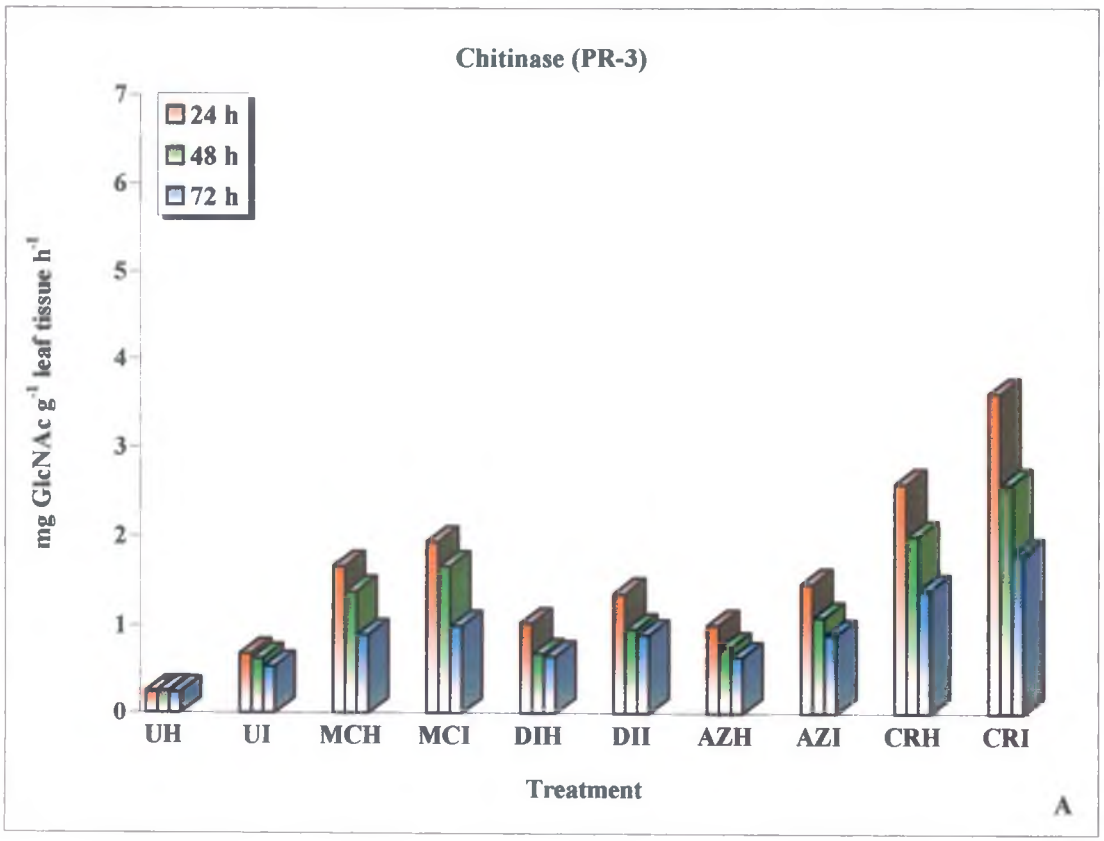
4.12.1.1. Fungicides

Several groups of organic compounds have been developed in the recent years for fungicidal effect by true systemic action with major interest in testing the different groups for systemic, eradicant and antisporulant action on *E. vexans*. Two recommended systemic fungicides Hexaconazole and Calixin offering satisfactory blister blight disease control were obtained to study their effects as inducers of resistance in tea plants towards *E. vexans* with special emphasis on the altering levels of defense enzyme.

4.12.1.1.1. Hexaconazole

Hexaconazole, a promising systemic fungicide in the management of tea blister blight caused by *E. vexans* was obtained from Rallis India Limited, Bangalore. In recent years application of hexaconazole (RS)-2-2(2,4-dichlorophenyl)-1-(1,H-1,2,4-triazol-1-1-yl)hexan-zol, an ergosterol biosynthesis inhibitor (EBI), has been common practice in tea cultivation areas. The recommended dose for foliar spray is 0.1 % at 7-d-intervals. To study the associated changes in defense enzymes, tea plants (T-78) were treated and then inoculated *E. vexans*. Samples were harvested at 24, 48 and 72 h after inoculation and levels of the defense enzymes (chitinase, β-1,3-glucanase, peroxidase); Besides, the levels of phenylalanine ammonia lyase (PAL), a key enzyme in phytoalexin synthesis were also recorded for these samples. Control plants were sprayed with distilled water and maintained under identical conditions.

Chitinase activity in hexaconazole treated plants showed higher activity levels than inoculated plants. The effect of both agents resulted in a major peak after 24 h in treated plants following inoculation and was 4-fold than untreated inoculated (Figure 14 B). After attaining a peak, fall in activity was abrupt in treated healthy plants but the decline in treated inoculated was gradual. Despite fall in the activity, chitinase levels were still higher in the treated-inoculated plants than their respective controls. β-1,3-glucanase activity was also enhanced by treatment with hexaconazole with highest activity 24 h after inoculation.



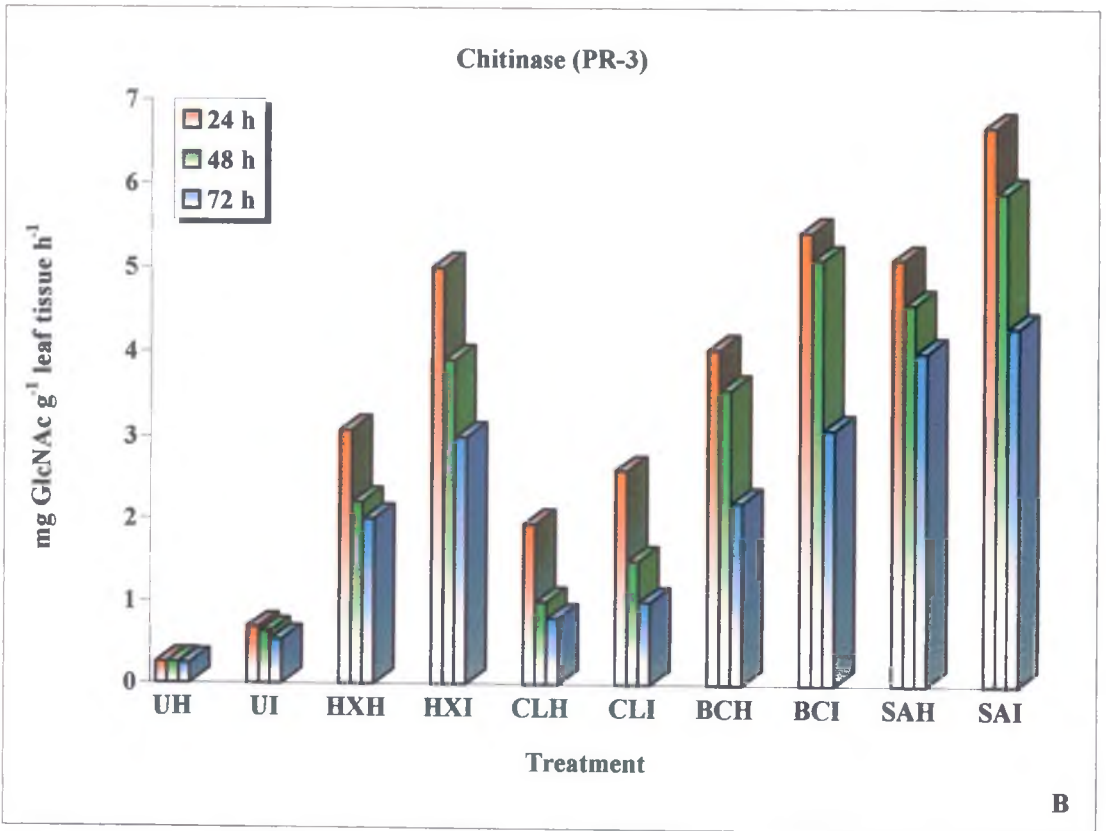


Figure 14 (A & B). Chitinase activity in tea plants (T-78) following treatment with abiotic elicitors and inoculation with *E. vexans*.

[U- untreated, H- healthy, I- inoculated, MC- mercuric chloride, DI- di-potassium phosphate, AZ- *A. indica*, CR- *C. roseus*, HX- hexaconazole, CL- calixin, BC- biocrop, SA- salicylic acid.]

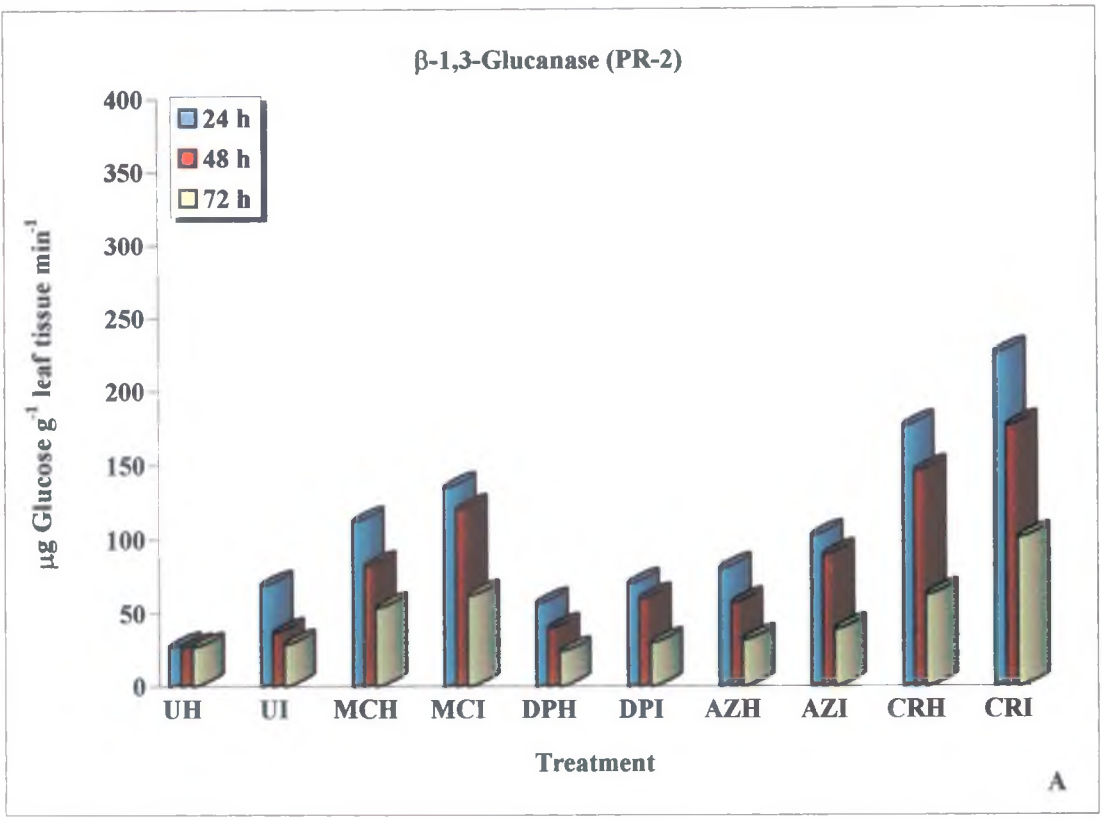
In treated inoculated plants levels were significantly high with a rapid drop in activity at 48 and 72 h time points. Contrastingly, treated healthy plants high level of activity persisted up to 48 h that abruptly declined thereafter (Figure 15 B). Peroxidase activity started increasing rapidly after 24 h of inoculation of the treated plants. However increase in enzyme activity was much steeper 48 h after inoculation (Figure 16 B).

Phenylalanine ammonia lyase, a key phenolic enzyme, showed increased activity with peak value 48 h after inoculation (Figure 17 B). The trends in untreated healthy and inoculated plants were similar

4.12.1.1.2. Calixin

Another heterocyclic compound that has shown promise in controlling blister blight is Calixin, a product of Messrs. BASF India, Mumbai, belonging to the group morpholine, with the common name tridemorph and chemical name N-tridecyl-2,6-dimethyl morpholine. Calixin foliar spray at 0.1 % concentration applied at 7-d-intervals is the recommended dose that inhibits sporulation of *E. vexans* and thereby keeping the inoculum level low. Calixin has also been observed to exert a marked stimulation on crop production. To investigate the role of calixin in induction of resistance, 7 doses (0.1 % at 7-d-intervals) of the fungicide were applied on tea plants (T-78) and inoculated with *E. vexans* 7 d after the last dose. Samples were harvested at 24, 48 and 72 h after inoculation and levels chitinase, β-1,3-glucanase, peroxidase and PAL were recorded.

Comparison of the defense enzymes showed enhancement of chitinase (Figure 14 B) and β-1,3-glucanase (Figure 15 B) after 24 h and of peroxidase (Figure 16 B) and phenylalanine ammonia lyase (Figure 17 B) after 48 h of inoculation with a gradual decline with time. However, level of increase of β-1,3-glucanase and peroxidase was higher than the increase in chitinase and phenylalanine ammonia lyase in comparison to untreated healthy control.



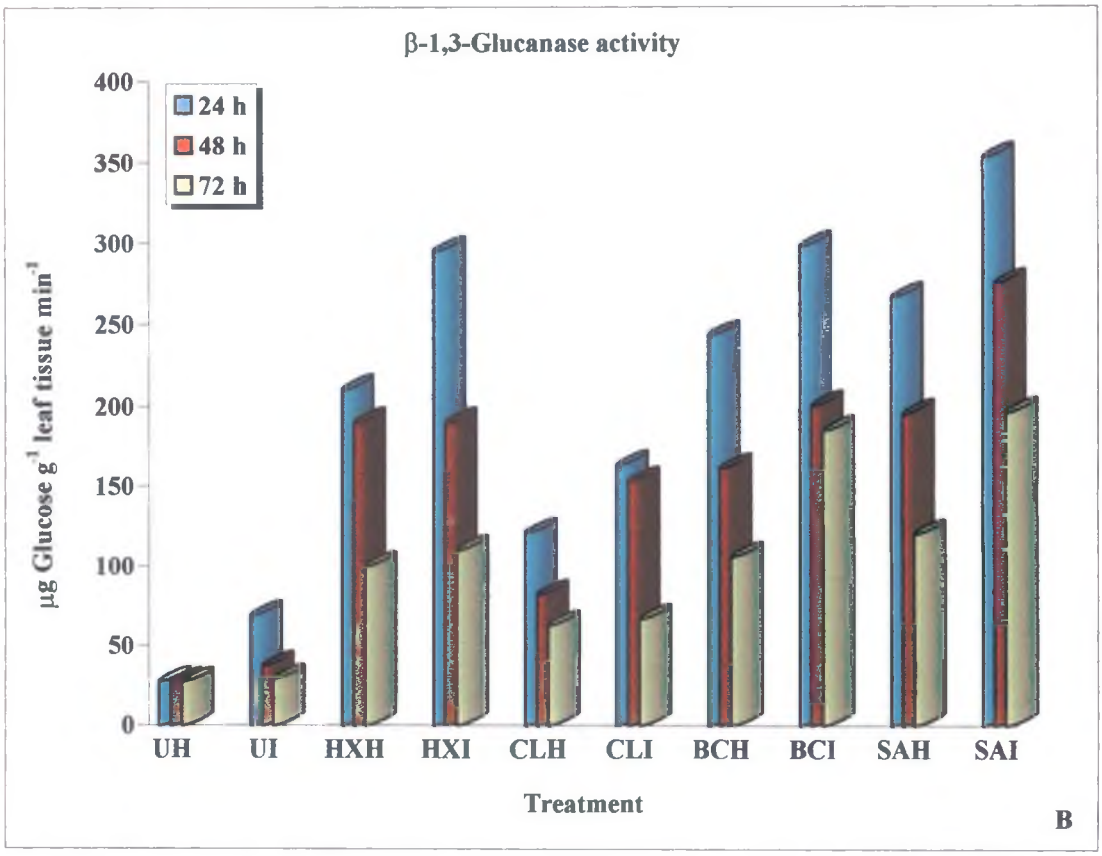


Figure 15 (A & B). β-1,3-glucanase activity in tea plants (T-78) following treatment with abiotic elicitors and inoculation with *E. vexans.*

[U- untreated, H- healthy, I- inoculated, MC- mercuric chloride, DI- di-potassium phosphate, AZ- A. indica, CR- C. roseus, HX- hexaconazole, CL- calixin, BC- biocrop, SA- salicylic acid.]

4.12.1.2. Mercuric chloride

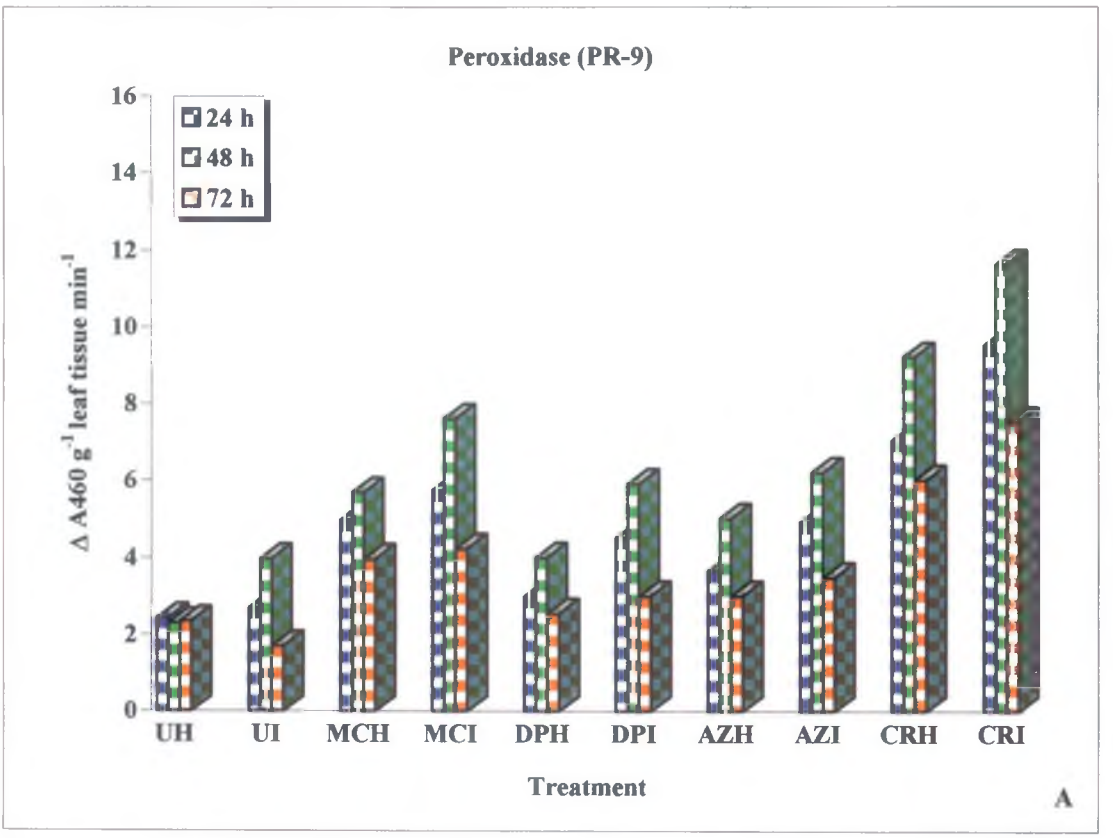
It has already been established that mercuric chloride has potential in PR-protein induction and accumulation in plants even at low concentrations. With this background mercuric chloride was selected as an inducer of defense enzymes in tea plants. A 10 mM solution of mercuric chloride was sprayed uniformly on alternate days for 7 d on to the leaves of susceptible tea plants (T-78). After applying the last dose the plants were inoculated with *E. vexans* and levels of the defense enzymes chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were measured after 24, 48 and 72 h following inoculation.

Mercuric chloride, enhanced the levels of chitinase (Figure 14 A) and β-1,3-glucanase (Figure 15 A) enzyme activity. The enzyme activities were higher in treated inoculated plants than their respective controls. The highest levels of the enzymes were obtained 24 h after inoculation. Peroxidase activity was consistently higher in leaves from inoculated plants (Figure 16 A). The peroxidase activity levels rapidly increased from 24 to 48 h following inoculation. The trend was the same in phenylalanine ammonia lyase activity following all treatments (Figure 17 A).

4.12.1.3. Di-potassium ortho-phosphate

Phosphate salts can also induce resistance in plants. An aqueous solution of di-potassium orthophosphate (50 mM) was used for resistance induction in tea plants. The solution was sprayed on the leaves of the tea plants (T-78). Plants were inoculated with *E. vexans* 7 d after treatment.

Within 24 h of inoculation the levels of chitinase enzyme in treated plants had risen in comparison to control and inoculated plants. Chitinase levels were higher in treated inoculated plants than both untreated-inoculated and treated-healthy plants (Figure 14 A). On the other hand β-1,3-glucanase activity did not increase as rapidly in the leaves induced with di-potassium ortho-phosphate as in those inoculated with *E. vexans*. β-1,3-glucanase activity rarely reached the levels observed in inoculated leaves.



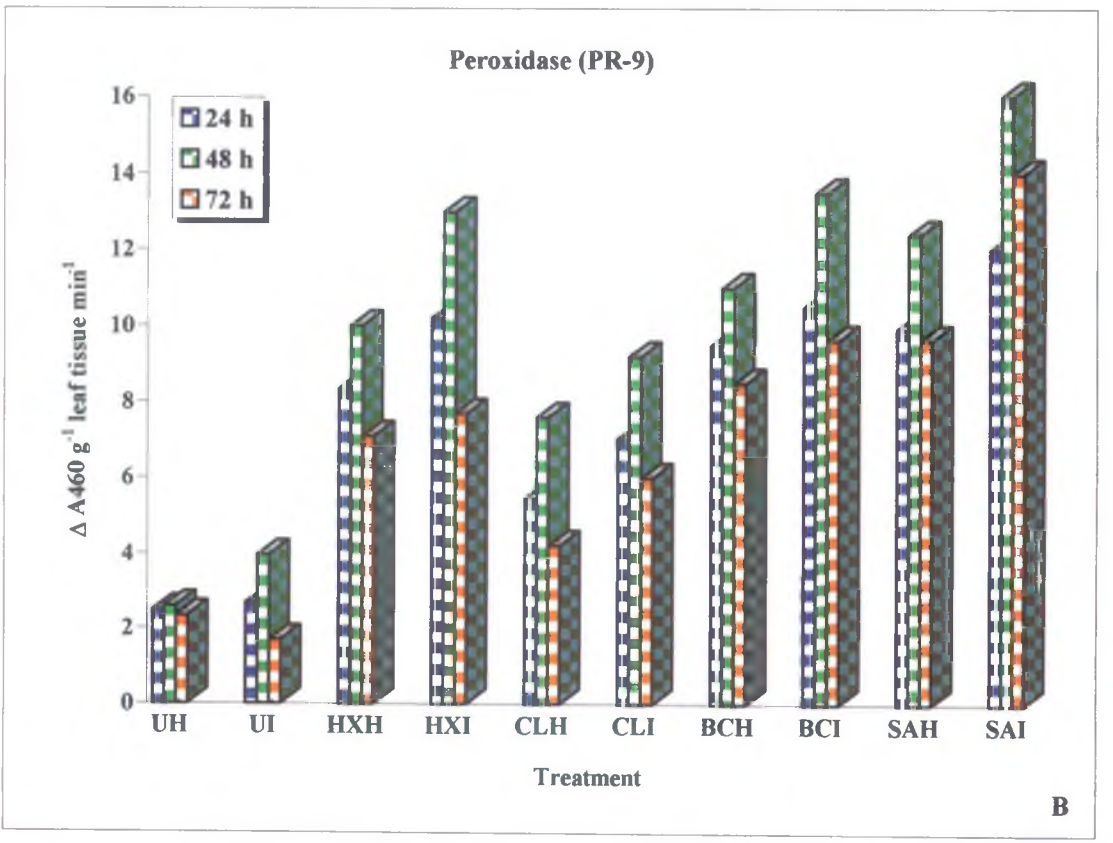


Figure 16 (A & B). Peroxidase activity in tea plants (T-78) following treatment with abiotic elicitors and inoculation with *E. vexans.*

[U- untreated, H- healthy, I- inoculated, MC- mercuric chloride, DI- di-potassium phosphate, AZ- *A. indica* , CR- *C. roseus* , HX- hexaconazole, CL- calixin, BC- biocrop, SA- salicylic acid]

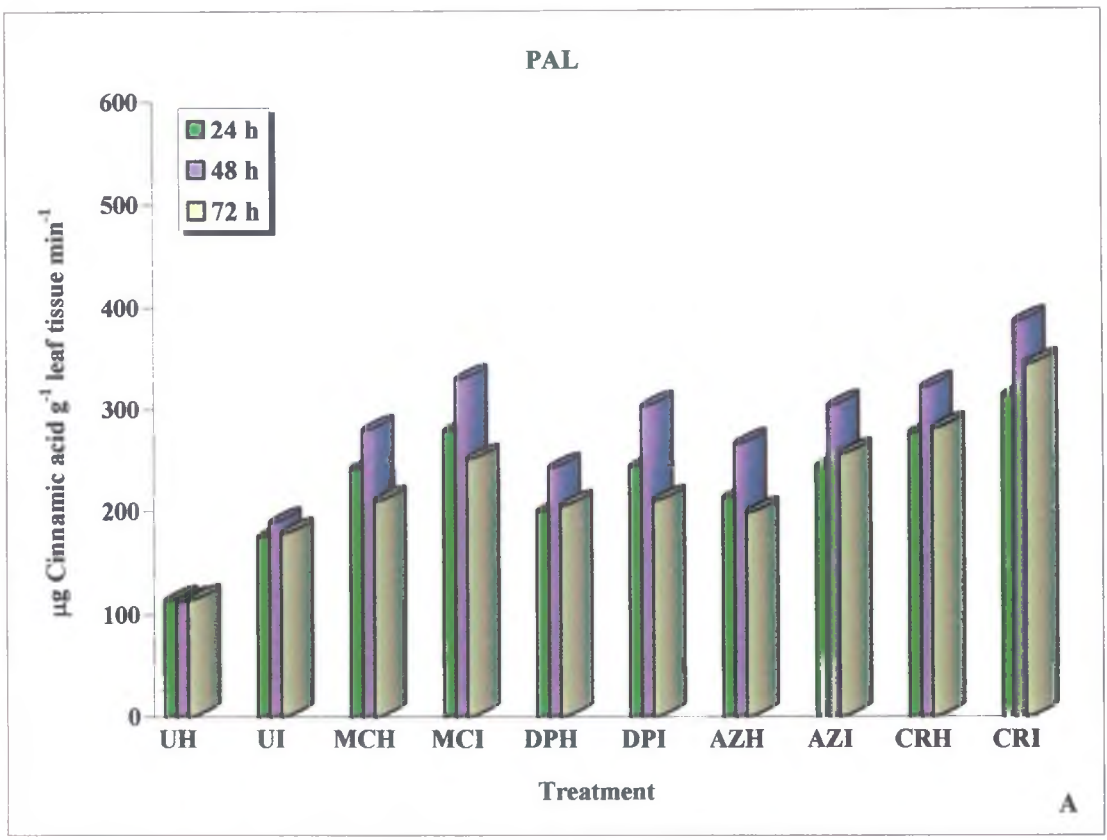
However, interestingly, the treated-inoculated leaves exhibited similar levels of β-1,3-glucanase as untreated inoculated plants (Figure 15 A). Peroxidase activity reached peak levels 48 h after inoculation. Here levels of the enzyme were similar in untreated inoculated plants (Figure 16 A).

On the contrary, a general increase in PAL activity was noticed in all treatments. Activity was nearly two fold higher in treated-inoculated plants than untreated healthy and untreated-inoculated plants 48 h following inoculation with *E. vexans* (Figure 17 A).

4.12.1.4. Salicylic acid

Although agrochemicals are competent in combating the disease, their high cost and health hazards caused by them limit their extensive use. However, chemicals like salicylic acid with low concentrations could provide an alternative to protect the crop plants by activating SAR. Salicylic acid is one of the many activators of disease resistance being utilized to aid elucidation of the complex mechanisms of the defense response and to assess the potential of employing SAR commercially. Salicylic acid has been implicated as a component of the induced resistance signaling pathway for many years. Not only does exogenous application of salicylic acid to many plant species result in the accumulation of PR-proteins but can also activate other resistance mechanisms such as phytoalexin production and lignification. Endogenous levels of salicylic acid also increase following inoculation with microorganisms. A lot of research in this line has been demonstrated in a number of plant-pathogen systems, but information regarding the *E. vexans* - *C. sinensis* is lacking. Therefore, in the current study results in this line of investigation have been presented.

Tea plants (T-78) susceptible to blister blight were sprayed with salicylic acid (15 mM) for 7 d and challenge inoculated with *E. vexans*. Samples were harvested at 24, 48 and 72 h. The extractable defense enzymes chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were obtained from untreated healthy (UH), untreated inoculated with *E. vexans* (UI), treated healthy (TH) and treated inoculated (TI) tea varieties.



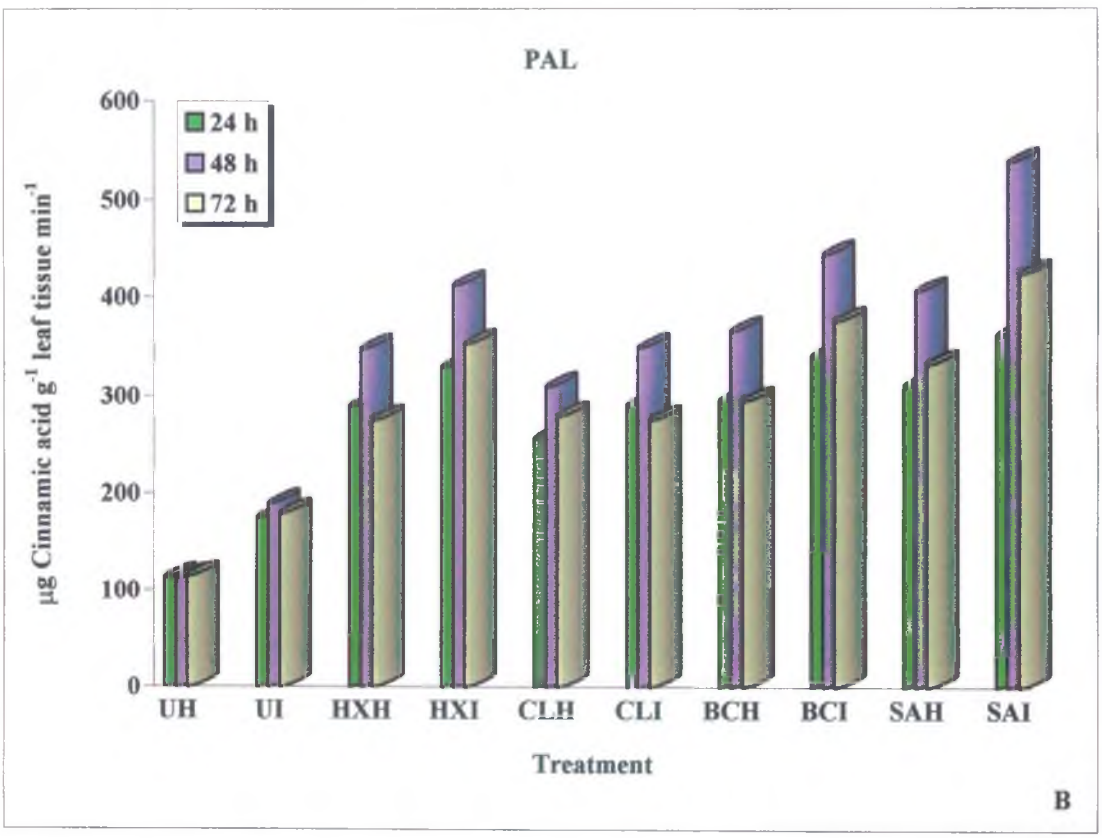


Figure 17 (A & B). Phenylalanine ammonia lyase (PAL) activity in tea plants (T-78) following treatment with abiotic elicitors and inoculation with *E. vexans.*
[U- untreated, H- healthy, I- inoculated, MC- mercuric chloride, DI- di-potassium phosphate, AZ- *A. indica*, CR- *C. roseus*, HX- hexaconazole, CL- calixin, BC- biocrop, SA- salicylic acid.]

The patterns of accumulation of all defense enzymes were of significantly high levels in treated-inoculated plants. Chitinase activity was measured in leaves following all treatments as well as control (Figure 14 B). The levels rose with a peak at 24 h after inoculation and at the later time points (48 and 72 h after inoculation) declined but the fall was steady.

β-1,3-glucanase activity in the leaf extracts was influenced by inoculation with *E. vexans* and displayed a peak rise within 24 h post inoculation (Figure 15 B). The values obtained for the treated-inoculated plants showed a rapid rise in the β-1,3-glucanase activity in comparison to treated healthy and untreated inoculated plants. After 24 h the enzyme activity gradually decreased in all treatments, which continued till 72 h.

Peroxidase activity also started to increase within 24 h of inoculation but was maximum at 48 h after inoculation. Inoculated plants (UI) showed higher levels of the enzyme in comparison to control (UH) but at all time intervals the levels were similar. Further comparison showed that the treated healthy and treated-inoculated plants (SAI) attained peak levels of enzyme after 48 h (Figure 16 B). Treated plants showed a faster decline in enzyme activity whereas treated inoculated plants still maintained much higher levels.

The phenylalanine ammonia lyase levels recorded in the treated plants were similar to peroxidase levels. Enzyme activity attained a peak 48 h after inoculation in all treatments, which gradually declined thereafter up to 72 h (Figure 17 B).

4.12.1.5. Plant products and Phytoextracts as inducers of resistance

Plant diseases are usually controlled by broad-spectrum fungicides or chemical salts applied as foliar sprays. But, the growing concern about harmful chemicals on the environment and human health, efforts have been made to evolve environmentally friendly strategies for the control of plant diseases. Plant extracts or products have been found effective against a wide range of pathogens. Therefore, experiments were designed to investigate the effect of a plant product – Biocrop, and phytoextracts of two plants – *Azadirachta indica* 'neem' and *Catharanthus roseus* 'periwinkle' on tea plants in inducing resistance against blister blight of tea.

4.12.1.5.1. Biocrop

Biocrop, an organic fertilizer, is a naturally occurring metabolic activator and plant growth enhancer. It is a mixture of organic constituents that have their counterparts in plants. It has been tried on various plantation crops and has improved the growth potential of crops without harming the soil structure, texture and environment.

Significant increases in enzyme activities were observed following induction with Biocrop. After 24 h of inoculation, chitinase activity increased in the treated plants in comparison to control. Interestingly, following treatments after attaining peaks at 24 h, the plants still maintained much higher levels up to 48 h followed by a rapid decline in enzyme activity after 72 h (Figure 14 B).

Significant increases over respective controls in activities of β-1,3-glucanase were found in all treatments after 24 h of inoculation which declined thereafter up to 72 h (Figure 15 B). An alteration in the levels of β-1,3-glucanase activity was evident following induction with Biocrop.

The levels of peroxidase (Figure 16 B) and phenylalanine ammonia lyase (Figure 17 B) after treatment following inoculation were highest after 48 h of inoculation with slight decline at 72 h. Activities of both enzymes showed similar trends except for level of enhancement of enzyme activity.

4.12.1.5.2. *Catharanthus roseus*

Aqueous extracts (4 %) of leaves of *Catharanthus roseus,* periwinkle was applied to tea plants (T-78) and levels of defense enzymes were recorded. As compared to control chitinase activity was high and attained a peak after 24 h of inoculation with *E. vexans* (Figure 14 A). Similarly, β-1,-3-glucanase activity was also enhanced during initial stages within 24 h after inoculation (Figure 15 A) followed by peroxidase which rose 48 h after inoculation (Figure 16 A). PAL activity was also enhanced after 48 h of inoculation and interestingly the levels remained high with a gradual decline in due course of time (Figure 17 A).

4.12.1.5.3. *Azadirachta indica*

Azadirachta indica, neem extracts, were also applied to tea plants (T-78) followed by inoculation with *E. vexans*. The activity of both chitinase and β-1,3-glucanase was higher in treated inoculated plants (Figures14 A and 15 A). Induction with *A. indica* changed the levels of peroxidase and phenylalanine ammonia lyase and both enzymes attained peaks 48 h after inoculation (Figures 16 A and 17A).

It can be derived from the results that both the phytoextracts, *C. roseus* and *A. indica* are effective in increasing the defense enzyme levels in tea plants. However, though is increase in activity of the enzymes was recorded in *A. indica* treated tea plants their effect is lesser than *C. roseus* treatments.

4.12.2. Evaluation of disease reaction by PTA-ELISA

After biochemical elucidation of the changes of levels of defense enzymes in susceptible tea varieties, further, acid soluble proteins were extracted from three tea varieties (T-78, UP-9 and TV-18) separately treated with all the said elicitors and immunologically analyses were made following PTA-ELISA formats. Pathogen was detected in the extracts by PAb-EV1. Similarly for detection of PR-proteins, PR-3 and PR-2, PAbs raised against Chitinase (PAb-Chi) and Laminarinase (PAb-Lam) were used. Results were recorded following pre-treatment of the plants with the abiotic elicitors and after 24 h after inoculation with *E. vexans*. Pathogen could be specifically detected by PAb-EV1 in untreated-inoculated plants with higher values in comparison to treated-inoculated (TI). Decrease in absorbance value in treated inoculated plants confirms limit of the pathogen in tea leaf tissues and hence induction of resistance which was further confirmed by increase in values of defense enzymes. Strong signals of PR-proteins were obtained with PAb-Chi and PAb-Lam in plants of the different treatments and varieties. Accumulation of both proteins started at an early stage after inoculation and higher concentrations were noticed especially in treated-inoculated (TI) in comparison to all treatments (UH, UI and TH). Results following treatment with the abiotic elicitors and inoculation with *E. vexans* have been tabulated in tables 40-47.

Table 40. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Hexaconazole treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (0.1 %)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.761 ± 0.02	0.469 ± 0.06	0.364 ± 0.08
	UI	0.878 ± 0.04	0.557 ± 0.05	0.608 ± 0.02
	TH	0.623 ± 0.05	1.509 ± 0.04	0.995 ± 0.03
	TI	0.235 ± 0.04	2.475 ± 0.02	1.802 ± 0.04
UP-9	UH	0.648 ± 0.02	0.419 ± 0.02	0.330 ± 0.02
	UI	0.857 ± 0.03	0.793 ± 0.04	0.541 ± 0.01
	TH	0.686 ± 0.08	1.569 ± 0.07	0.560 ± 0.10
	TI	0.255 ± 0.08	2.559 ± 0.08	0.793 ± 0.04
TV-18	UH	0.739 ± 0.07	0.441 ± 0.02	0.392 ± 0.04
	UI	0.877 ± 0.07	0.686 ± 0.11	0.519 ± 0.04
	TH	0.550 ± 0.08	1.453 ± 0.02	0.688 ± 0.02
	TI	0.281 ± 0.09	2.638 ± 0.05	1.475 ± 0.03
E. vexans		1.791 ± 0.04	ND	ND
Chitinase		ND	1.325 ± 0.04	ND
Laminarinase		ND	ND	1.258 ± 0.04

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans*; IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated; ND – not detected.

Table 41. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Calixin treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (0.1 %)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.784 ± 0.05	0.461 ± 0.06	0.331 ± 0.05
	UI	0.872 ± 0.05	0.550 ± 0.02	0.584 ± 0.06
	TH	0.896 ± 0.04	0.750 ± 0.03	0.771 ± 0.02
	TI	0.294 ± 0.02	1.003 ± 0.05	1.561 ± 0.05
UP-9	UH	0.688 ± 0.02	0.442 ± 0.03	0.301 ± 0.03
	UI	0.840 ± 0.05	0.718 ± 0.07	0.494 ± 0.09
	TH	0.920 ± 0.08	1.221 ± 0.06	0.462 ± 0.05
	TI	0.225 ± 0.09	2.001 ± 0.09	0.512 ± 0.02
TV-18	UH	0.750 ± 0.02	0.445 ± 0.05	0.336 ± 0.08
	UI	0.856 ± 0.01	0.685 ± 0.07	0.538 ± 0.05
	TH	0.625 ± 0.04	0.965 ± 0.06	0.864 ± 0.06
	TI	0.256 ± 0.06	1.234 ± 0.08	0.996 ± 0.05
E. vexans		1.82 ± 0.02	ND	ND
Chitinase		ND	1.45 ± 0.06	ND
Laminarinase		ND	ND	1.30 ± 0.08

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 42. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Mercuric chloride treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 μg ml^{-1})	Treatment (5 mM)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.780 ± 0.02	0.426 ± 0.06	0.311 ± 0.04
	UI	0.881 ± 0.07	0.586 ± 0.02	0.554 ± 0.01
	TH	0.712 ± 0.04	0.616 ± 0.06	0.559 ± 0.12
	TI	0.227 ± 0.06	0.785 ± 0.02	0.596 ± 0.01
UP-9	UH	0.679 ± 0.06	0.478 ± 0.07	0.333 ± 0.05
	UI	0.898 ± 0.07	0.697 ± 0.05	0.496 ± 0.04
	TH	0.899 ± 0.02	0.712 ± 0.05	0.491 ± 0.06
	TI	0.285 ± 0.04	0.794 ± 0.01	0.523 ± 0.07
TV-18	UH	0.761 ± 0.11	0.458 ± 0.04	0.352 ± 0.08
	UI	0.858 ± 0.05	0.669 ± 0.07	0.548 ± 0.07
	TH	0.898 ± 0.06	0.726 ± 0.08	0.648 ± 0.04
	TI	0.201 ± 0.02	0.896 ± 0.02	0.690 ± 0.03
E. vexans		1.900 ± 0.04	ND	ND
Chitinase		ND	1.620 ± 0.03	ND
Laminarinase		ND	ND	1.230 ± 0.07

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 μg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 43. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Di-potassium phosphate treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 μg ml^{-1})	Treatment (50 mM)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.795 ± 0.03	0.484 ± 0.01	0.323 ± 0.05
	UI	0.875 ± 0.03	0.586 ± 0.11	0.525 ± 0.04
	TH	0.816 ± 0.04	0.662 ± 0.05	0.575 ± 0.01
	TI	0.299 ± 0.05	0.772 ± 0.07	0.611 ± 0.05
UP-9	UH	0.689 ± 0.05	0.438 ± 0.01	0.329 ± 0.06
	UI	0.808 ± 0.04	0.618 ± 0.03	0.459 ± 0.07
	TH	0.789 ± 0.04	0.699 ± 0.07	0.418 ± 0.03
	TI	0.295 ± 0.07	0.869 ± 0.07	0.580 ± 0.06
TV-18	UH	0.732 ± 0.06	0.447 ± 0.06	0.313 ± 0.01
	UI	0.835 ± 0.03	0.679 ± 0.03	0.563 ± 0.03
	TH	0.726 ± 0.06	0.774 ± 0.03	0.618 ± 0.02
	TI	0.288 ± 0.04	0.989 ± 0.12	0.687 ± 0.02
E. vexans		1.690 ± 0.06	ND	ND
Chitinase		ND	1.380 ± 0.01	ND
Laminarinase		ND	ND	1.290 ± 0.07

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 μg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 44. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Salicylic acid treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (15 mM)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.725 ± 0.05	0.498 ± 0.03	0.379 ± 0.04
	UI	0.839 ± 0.06	0.595 ± 0.06	0.502 ± 0.06
	TH	0.701 ± 0.06	1.734 ± 0.04	0.982 ± 0.01
	TI	0.217 ± 0.06	2.993 ± 0.05	1.789 ± 0.05
UP-9	UH	0.675 ± 0.03	0.479 ± 0.05	0.384 ± 0.08
	UI	0.813 ± 0.05	0.867 ± 0.03	0.586 ± 0.05
	TH	0.677 ± 0.06	1.034 ± 0.05	0.601 ± 0.05
	TI	0.229 ± 0.08	2.121 ± 0.04	1.856 ± 0.04
TV-18	UH	0.715 ± 0.07	0.464 ± 0.01	0.356 ± 0.01
	UI	0.824 ± 0.01	0.759 ± 0.07	0.550 ± 0.05
	TH	0.741 ± 0.08	1.211 ± 0.01	1.082 ± 0.07
	TI	0.261 ± 0.05	2.321 ± 0.07	1.956 ± 0.10
E. vexans		1.859 ± 0.02	ND	ND
Chitinase		ND	1.435 ± 0.05	ND
Laminarinase		ND	ND	1.274 ± 0.06

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 45. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in Biocrop treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (10 %)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.783 ± 0.08	0.449 ± 0.05	0.383 ± 0.01
	UI	0.890 ± 0.10	0.553 ± 0.02	0.513 ± 0.03
	TH	0.742 ± 0.06	1.521 ± 0.08	0.956 ± 0.02
	TI	0.210 ± 0.06	2.153 ± 0.03	1.137 ± 0.10
UP-9	UH	0.635 ± 0.10	0.472 ± 0.06	0.323 ± 0.04
	UI	0.813 ± 0.02	0.621 ± 0.08	0.427 ± 0.02
	TH	0.709 ± 0.03	1.487 ± 0.03	0.854 ± 0.05
	TI	0.294 ± 0.06	2.121 ± 0.01	1.099 ± 0.04
TV-18	UH	0.764 ± 0.05	0.486 ± 0.11	0.367 ± 0.05
	UI	0.845 ± 0.10	0.691 ± 0.02	0.574 ± 0.02
	TH	0.675 ± 0.01	1.426 ± 0.05	0.896 ± 0.05
	TI	0.208 ± 0.01	2.275 ± 0.01	1.100 ± 0.03
E. vexans		1.874 ± 0.02	ND	ND
Chitinase		ND	1.465 ± 0.08	ND
Laminarinase		ND	ND	1.246 ± 0.03

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 46. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in *C. roseus* treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (0.4 %)	Absorbance at 405 nm		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.765 ± 0.01	0.494 ± 0.05	0.342 ± 0.03
	UI	0.879 ± 0.03	0.565 ± 0.03	0.589 ± 0.04
	TH	0.752 ± 0.08	1.012 ± 0.05	0.821 ± 0.03
	TI	0.203 ± 0.08	1.253 ± 0.03	0.987 ± 0.02
UP-9	UH	0.664 ± 0.08	0.487 ± 0.01	0.348 ± 0.05
	UI	0.854 ± 0.01	0.692 ± 0.03	0.480 ± 0.03
	TH	0.758 ± 0.04	1.123 ± 0.02	0.612 ± 0.01
	TI	0.201 ± 0.05	1.501 ± 0.01	0.884 ± 0.04
TV-18	UH	0.723 ± 0.05	0.446 ± 0.01	0.370 ± 0.07
	UI	0.848 ± 0.04	0.611 ± 0.04	0.598 ± 0.07
	TH	0.695 ± 0.01	0.992 ± 0.01	0.749 ± 0.01
	TI	0.256 ± 0.04	1.104 ± 0.08	0.835 ± 0.01
E. vexans		1.974 ± 0.12	ND	ND
Chitinase		ND	1.454 ± 0.04	ND
Laminarinase		ND	ND	1.383 ± 0.08

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

Table 47. Immunodetection of pathogen and PR-proteins (PR-3 and PR-2) in *A. indica* treated tea plants following inoculation with *E. vexans* using PTA-ELISA formats

Leaf antigen (50 µg ml^{-1})	Treatment (0.4 %)	Absorbance at 405 nm**		
		PAb-EV1	PAb-CHT	PAb-βGLU
T-78	UH	0.782 ± 0.01	0.488 ± 0.03	0.349 ± 0.02
	UI	0.885 ± 0.04	0.562 ± 0.08	0.504 ± 0.04
	TH	0.827 ± 0.04	0.745 ± 0.04	0.618 ± 0.06
	TI	0.298 ± 0.01	1.168 ± 0.02	0.785 ± 0.02
UP-9	UH	0.615 ± 0.06	0.402 ± 0.01	0.320 ± 0.06
	UI	0.839 ± 0.03	0.621 ± 0.01	0.460 ± 0.01
	TH	0.800 ± 0.03	0.701 ± 0.04	0.662 ± 0.04
	TI	0.213 ± 0.01	0.999 ± 0.02	0.792 ± 0.03
TV-18	UH	0.726 ± 0.08	0.491 ± 0.05	0.343 ± 0.04
	UI	0.857 ± 0.08	0.626 ± 0.08	0.593 ± 0.12
	TH	0.773 ± 0.07	0.891 ± 0.04	0.712 ± 0.04
	TI	0.203 ± 0.06	1.292 ± 0.02	0.892 ± 0.04
E. vexans		1.797 ± 0.12	ND	ND
Chitinase		ND	1.322 ± 0.11	ND
Laminarinase		ND	ND	1.254 ± 0.07

Note: Results are average of three replicates, ± S.E.; Values recorded in pre-treated plants 24 h after inoculation with *E. vexans;* IgG concentration 40 µg ml^{-1}; UH-Untreated healthy, UI-Untreated inoculated, TH- Treated healthy and TI- Treated inoculated

4.12.3. Changes in antifungal phenolics (pyrocatechol)

Following studies related to changes in levels of phenolic enzymes, extractable pyrocatechol (antifungal compound) from treated tea plants (T-78) following inoculation with *E. vexans* was analysed. Hexaconazole and salicylic acid were selected for inducer treatments. Partially purified (preparative TLC) pyrocatechol extracted from the treated and inoculated plants were further analysed by UV-Spectrophotometer (Shimadzu 160). The extracts obtained absorption maxima at 274 nm. Quantification of the antifungal compound pyrocatechol was done from the UV-Spectrophotometric curve considering the molar extinction co-efficient of authentic pyrocatechol 6000 at 274 nm (Table 48). Salicylic acid treated plants revealed a peak higher than hexaconazole treated plants, in relation to control. However following inoculation with *E. vexans*, hexaconazole treated plants displayed a high peak (Figure 18).

Table 48. Quantification of pyrocatechol from tea (T-78) triggered by *E. vexans* following treatment with abiotic inducers

Treatment		Pyrocatechol ($\mu g\ g^{-1}$ fresh tissue)	
		Healthy	Inoculated [a]
Untreated		65.8 ± 1.2	345.5 ± 5.2
Treated	Hexaconazole (0.1 %)	248.0 ± 5.8	672.2 ± 5.1
	Salicylic acid (15 mM)	121.3 ± 2.6	521.6 ± 4.8

Note: Results are average of three experiments; [a] Quantification was done 48 h after inoculation with *E. vexans*, ±S.E.

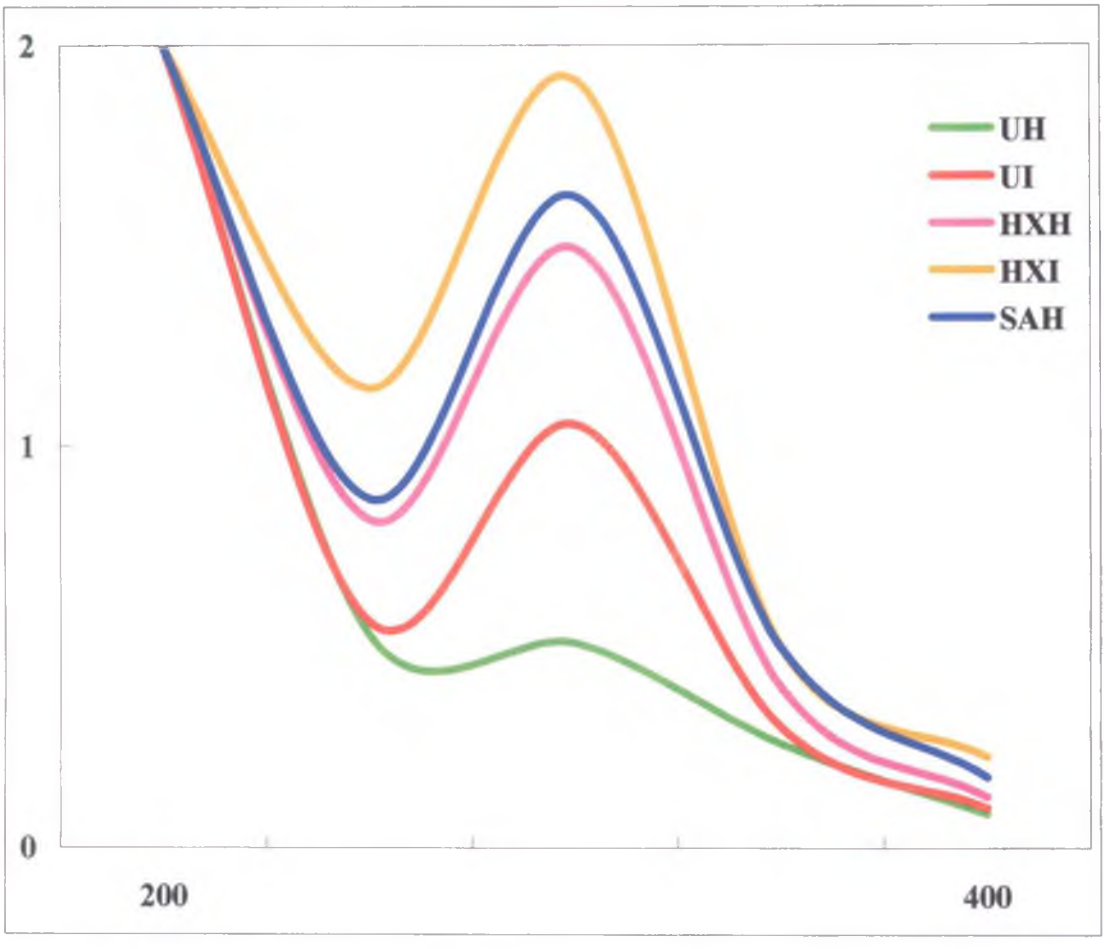


Figure 18. UV - Spectrophotometric analyses of antifungal phenolics (pyrocatechol) of tea triggered by *E. vexans* following treatment with abiotic inducers.
[U- untreated, H- healthy, I- inoculated, HX- hexaconazole, SA- salicylic acid.]

4.13. Induction of PR-proteins in suspension-cultures of tea cells by treatment with biotic and abiotic elicitors

4.13.1. Tea cell cultures

Initially, shoot tips, juvenile nodal segments with dormant axillary buds, young stem segments as well as immature cotyledons that are commonly used as explants for tea micropropagation were considered. Among these the stem segments were found to be most ideal for this study as apart from being readily available during all seasons these explants produced calli fast with very low contamination rates. The most common basal medium – Murashige and Skoog (MS) – was used for generation of calli from the young stem segments of twenty two tea varieties (AV-2, BS-7A/1/76, RR-17, T-135, HV-39, T-78, T-17/1/54, TS-449, UP-2, UP-8, UP-9, UP-17, BSS-1, BSS-2, BSS-3, TV-9, TV-18, TV-20, TV-22, TV-23, TV-25 and TV-30). Among the different growth regulators addition of 6-benzylaminopurine (2 mg L^{-1}) and indole-3-butyric acid (4 mg L^{-1}) in the culture medium suited well for tea stem callus initiation. Surface sterilization of the explants using 2 % sodium hypochlorite solution effectively checked most of the explant-borne contaminants. For reducing phenolic exudation from cultures, pretreatments like agitating with 0.7 % PVP-10 solution and rinsing in running tap water were more or less successful. Regarding semi-solid or liquid culture media, full-strength MS-medium was the most suitable. The segments were incised diagonally and inserted in the medium under sterile conditions. The cultures were incubated (26 ±2 °C and photoperiod of 16 h) and monitored carefully. The tips if the segments started swelling within a week and friable callus was observed after 2-4 wk of inoculation, which gradually increased in size (Plate 31). Both semi-solid and liquid media were effective for tea callus production.

Suspension cell cultures of the tea varieties were initiated from such fragile callus. The calli were weighed on fresh weight basis (5 g) and added to MS-liquid medium (100 ml) for propagation of suspension cultures and incubated in the dark. Thus the obtained cell suspension cultures (4 to 6 day-old) were treated with biotic and abiotic elicitors which include *E. vexans*, salicylic acid (SA) and jasmonic acid (JA) for elicitation of PR-proteins (PR-2 and PR -3).

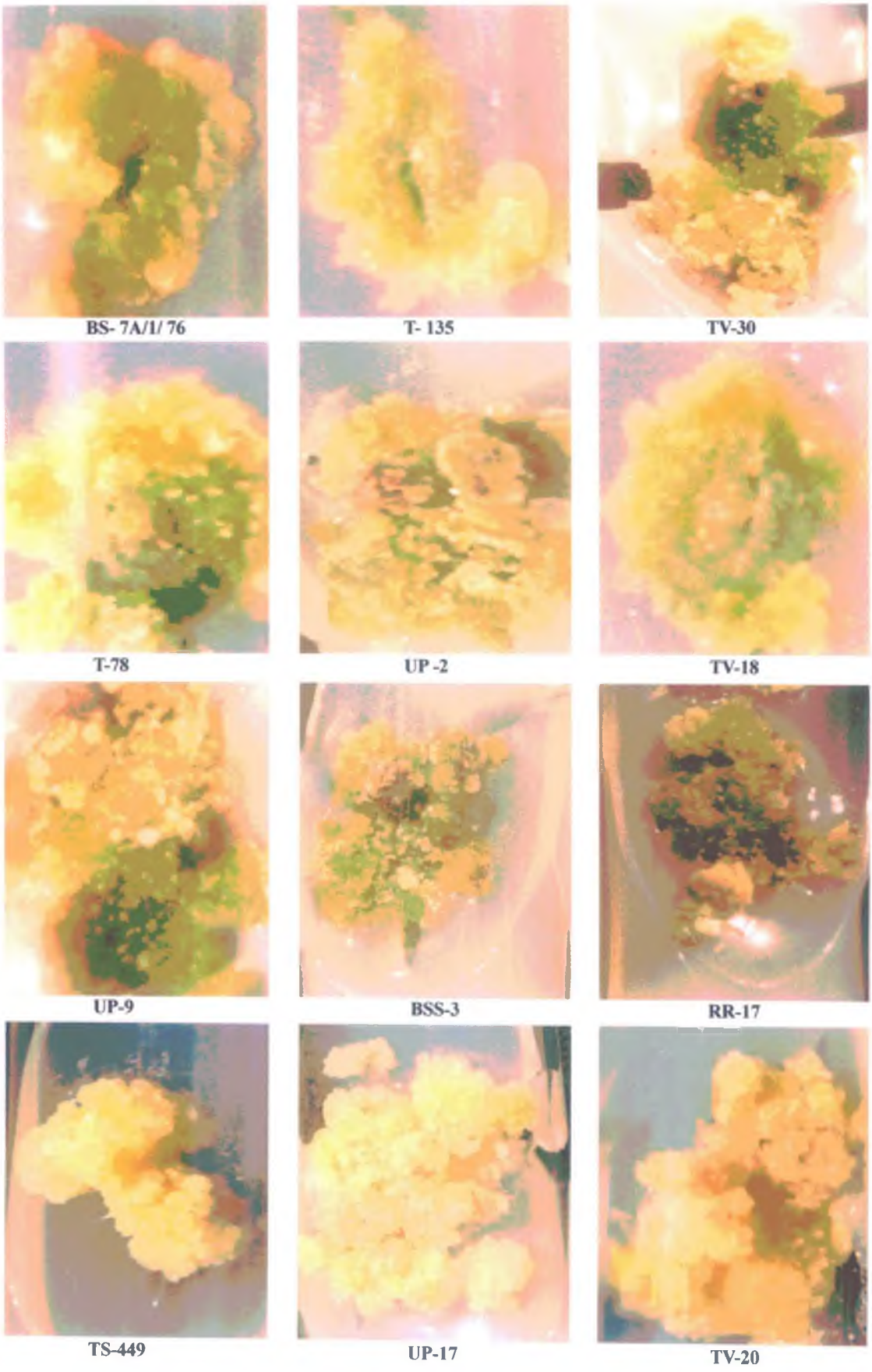
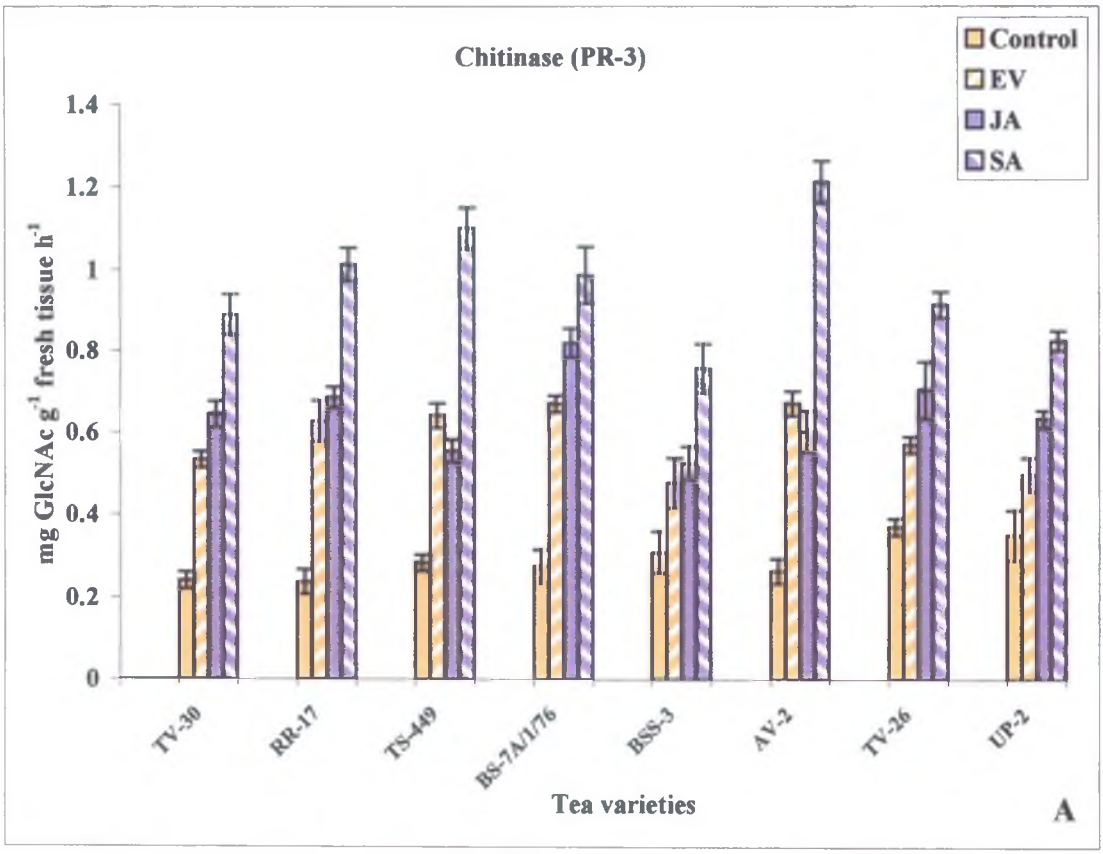


Plate 31. Friable calli from stem segments of tea varieties.

Elicitor-inducible, antifungal PR-proteins may have great potential for effective protection against pathogens. Such PR-proteins have not yet been characterized in tea cultures. Hence, attempts were made to assess whether the PR-2 and PR-3 proteins could be induced in tea by biotic (*E. vexans*) and abiotic (SA and JA) elicitors. For treatment with biotic and abiotic elicitors the cell cultures were used 3 days after subculturing. Two milliliter-fractions of the cultures were transferred to 25 ml flasks containing 10 ml of fresh liquid MS-medium supplemented with appropriate amount of the elicitor, and filter sterilized before adding to the medium. Cells were incubated in the dark on a shaker before sampling. After the desired period of incubation the cells were harvested. For extraction of PR-proteins the cultures were filtered through a nylon mesh and placed between folds of blotting paper to remove excess moisture, and then fresh weight was taken. The material was then ground in the appropriate extraction buffer (3 ml g^{-1} fresh tissue) using a mortar and pestle. Following elicitation, the enzymes chitinase (PR-3) and β-1,3-glucanase (PR-2) in the tea-culture cells were characterized biochemically as well as immunologically by probing with PAb-chitinase and PAb-β-1,3-glucanase. All experiments were repeated thrice.

4.13.1.1. Induction by *E. vexans*

Tea cell suspension cultures were treated with basidiospores of *E. vexans* for elicitation of PR-proteins. Cell cultures treated with sterile distilled water only served as control. After an interval of 12 and 24 h the cell cultures were harvested and characterized. When the suspension cultured tea cells were treated with *E. vexans*, the chitinase and β-1,3-glucanase activity increased in comparison to control (Figures 19 A & B). However, chitinase activity was more rapid in comparison to β-1,3-glucanase activity and consistently increased in the elicitor treated cells throughout the experimental period of 24 h. Enzyme levels were low in susceptible varieties whereas a five-fold increase in activity occurred 24 h after elicitor treatment in the resistant varieties (T-17/1/54, BSS-3 and TV-30). Thus, elicitation with *E. vexans* altered the levels of the enzymes by increasing their accumulation in comparison to control plants (Tables 48 & 49).



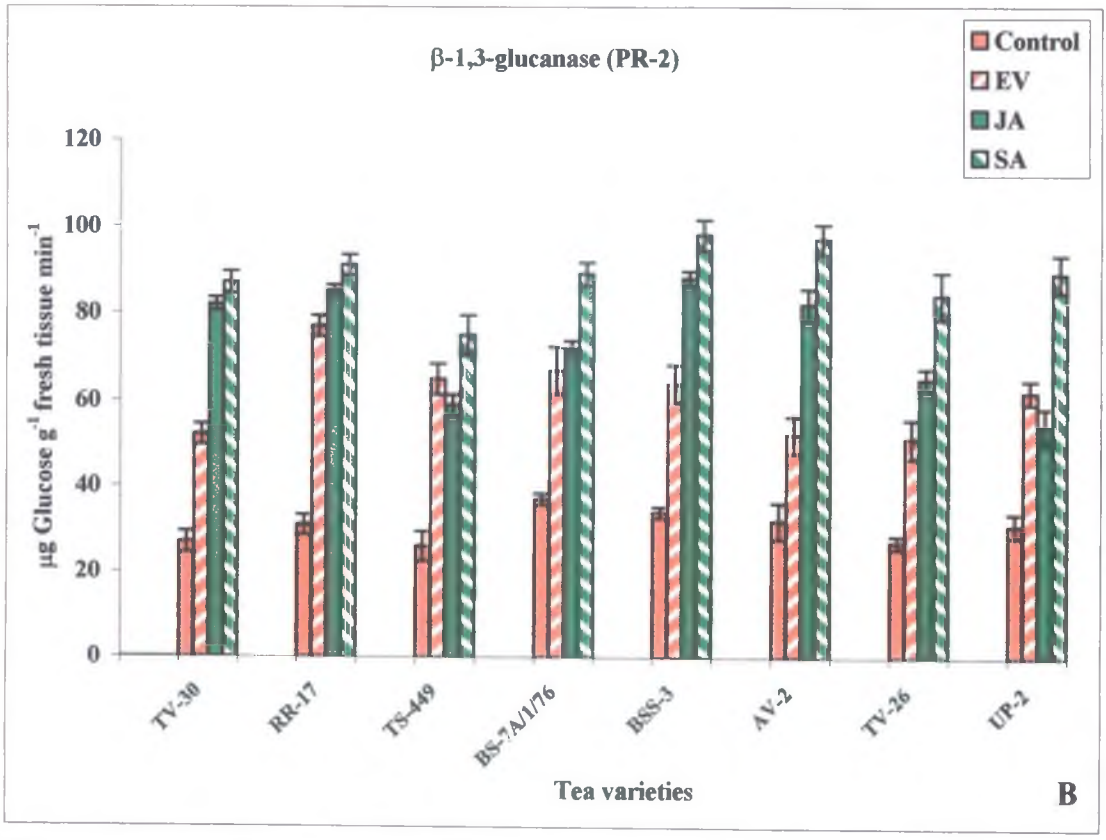


Figure 19 (A & B). Induction of PR-proteins in suspension tea cells in response to biotic and abiotic elicitor treatment.
[EV- *E. vexans* , JA- jasmonic acid, SA- salicylic acid]

4.13.1.2. Induction by salicylic acid (SA)

In the elicitor system developed for salicylic acid (15 mM) and suspension-cultured tea cells, salicylic acid was found to elicit chitinase and β-1,3-glucanase activity in tea cells. The response of suspension cells to the elicitor treatment indicated more significant chitinase activity in comparison to β-1,3-glucanase activity (Figures 19 A & B). The activities of the enzymes were assayed in cells harvested after an interval of 12 and 24 h of treatment with SA and a consistent increase was observed in the elicitor treated cells (Tables 49 & 50). Elicitation of the PR-proteins by SA was found to be significantly higher than *E. vexans* (biotic) in all varieties tested. Resistant varieties always showed higher levels of enzyme activity in comparison to the susceptible ones.

Table 49. Alteration in the levels of chitinase (PR-3) in suspension cell cultures of tea varieties following biotic and abiotic treatment

Variety	Time Course (h)	Chitinase activity (mg GlcNAc g^{-1} leaf tissue h^{-1})			
		Control	*E. vexans*	Jasmonic acid (0.5 μM)	Salicylic acid (15 mM)
TV-30	12	0.221 ± 0.02	0.401 ± 0.03	0.471 ± 0.09	0.689 ± 0.12
	24	0.241 ± 0.09	0.534 ± 0.03	0.644 ± 0.01	0.888 ± 0.07
RR-17	12	0.229 ± 0.01	0.474 ± 0.01	0.528 ± 0.03	0.712 ± 0.03
	24	0.237 ± 0.01	0.627 ± 0.01	0.685 ± 0.01	1.012 ± 0.09
TS-449	12	0.271 ± 0.02	0.510 ± 0.10	0.413 ± 0.01	0.748 ± 0.04
	24	0.284 ± 0.07	0.641 ± 0.03	0.555 ± 0.06	1.104 ± 0.12
BS-7A/1/76	12	0.264 ± 0.01	0.525 ± 0.01	0.521 ± 0.05	0.579 ± 0.04
	24	0.275 ± 0.06	0.699 ± 0.02	0.819 ± 0.05	0.985 ± 0.11
BSS-3	12	0.312 ± 0.07	0.371 ± 0.01	0.411 ± 0.02	0.644 ± 0.10
	24	0.319 ± 0.09	0.477 ± 0.04	0.525 ± 0.10	0.756 ± 0.12
AV-2	12	0.268 ± 0.04	0.469 ± 0.09	0.429 ± 0.04	0.723 ± 0.12
	24	0.264 ± 0.07	0.671 ± 0.01	0.602 ± 0.05	1.211 ± 0.12
TV-26	12	0.364 ± 0.03	0.432 ± 0.03	0.547 ± 0.01	0.590 ± 0.01
	24	0.371 ± 0.01	0.572 ± 0.07	0.702 ± 0.09	0.911 ± 0.05
UP-2	12	0.341 ± 0.08	0.331 ± 0.01	0.581 ± 0.02	0.613 ± 0.10
	24	0.352 ± 0.07	0.497 ± 0.08	0.632 ± 0.12	0.823 ± 0.09
TV-25	12	0.334 ± 0.08	0.582 ± 0.08	0.423 ± 0.05	0.656 ± 0.05
	24	0.336 ± 0.09	0.673 ± 0.10	0.697 ± 0.07	0.901 ± 0.04
TV-9	12	0.463 ± 0.07	0.511 ± 0.02	0.521 ± 0.02	0.613 ± 0.02
	24	0.475 ± 0.05	0.623 ± 0.03	0.611 ± 0.04	1.081 ± 0.07
HV-39	12	0.491 ± 0.06	0.689 ± 0.07	0.621 ± 0.10	0.881 ± 0.02
	24	0.494 ± 0.02	0.723 ± 0.07	0.716 ± 0.07	1.117 ± 0.03

Note: Results are average of three experiments, ± S.E.

4.13.1.3. Induction by jasmonic acid (JA)

The elicitation of chitinase (PR-3) and β-1,3-glucanase (PR-2) by cultured cells of tea under the influence of jasmonic acid (0.5 μM) was studied. Enzyme activities were enhanced in cultured cells after 24 h of treatment with JA compared to untreated control cells (Figures 19 A & B). Elicitation of both PR-2 and PR-3 was lesser than induction by SA in all tested varieties. Nevertheless enzyme levels in resistant varieties were higher than susceptible ones.

Table 50. Alteration in the levels of β-1,3-glucanase (PR-2) in suspension cell cultures of tea varieties following biotic and abiotic treatment

Variety	Time Course (h)	β-1,3-glucanase (μg Glucose g^{-1} leaf tissue min^{-1})			
		Control	*E. vexans*	Jasmonic acid (0.5 μM)	Salicylic acid (15 mM)
TV-30	12	25.1 ± 0.9	42.1 ± 0.5	72.2 ± 1.2	60.1 ± 0.2
	24	27.3 ± 1.0	52.7 ± 1.1	82.1 ± 0.5	87.2 ± 0.1
RR-17	12	30.4 ± 1.0	63.0 ± 0.9	69.4 ± 0.2	74.1 ± 0.7
	24	31.6 ± 1.2	77.4 ± 0.5	85.6 ± 1.5	91.3 ± 1.7
TS-449	12	24.7 ± 0.8	58.2 ± 0.8	41.7 ± 0.7	69.4 ± 0.6
	24	26.8 ± 0.5	65.7 ± 1.0	59.6 ± 0.8	75.6 ± 1.2
BS-7A/1/76	12	36.9 ± 1.3	52.1 ± 1.1	51.8 ± 0.6	68.1 ± 0.6
	24	37.1 ± 1.0	67.7 ± 0.9	72.1 ± 1.8	89.7 ± 0.9
BSS-3	12	33.3 ± 1.5	55.2 ± 0.9	60.5 ± 0.6	82.1 ± 0.8
	24	34.6 ± 0.8	64.8 ± 0.8	88.1 ± 1.3	98.8 ± 0.8
AV-2	12	32.7 ± 0.6	40.7 ± 0.6	58.1 ± 1.5	79.9 ± 0.8
	24	32.8 ± 0.8	52.8 ± 0.9	82.3 ± 0.9	97.4 ± 0.9
TV-26	12	25.1 ± 0.6	40.3 ± 0.8	48.1 ± 0.6	77.7 ± 0.9
	24	27.3 ± 1.0	51.3 ± 1.0	65.7 ± 0.8	84.5 ± 1.3
UP-2	12	30.4 ± 0.7	48.1 ± 1.0	41.2 ± 0.7	76.9 ± 1.2
	24	31.6 ± 0.6	62.7 ± 0.9	54.8 ± 0.6	89.5 ± 1.5
TV-25	12	33.7 ± 1.5	52.8 ± 1.5	57.9 ± 1.5	66.4 ± 1.4
	24	35.9 ± 0.9	67.9 ± 0.8	62.1 ± 0.9	82.1 ± 0.9
TV-9	12	27.9 ± 0.2	41.8 ± 0.7	50.3 ± 1.4	71.3 ± 1.4
	24	29.0 ± 1.0	58.1 ± 0.6	65.5 ± 0.9	87.8 ± 0.9
HV-39	12	34.1 ± 0.2	57.0 ± 0.6	53.4 ± 1.3	68.4 ± 1.1
	24	33.7 ± 0.2	66.5 ± 1.4	61.0 ± 1.0	80.2 ± 1.0

Note: Results are average of three experiments, ± S.E.

4.13.2. Western blot analysis of elicitor induced PR-proteins in cultured tea cells

PR-proteins were detected in cultured tea cells harvested after 24 h of treatment with *E. vexans* as well as with SA and JA. Western blot analysis of *E. vexans* treated tea cells (Plate 32 B) revealed presence of three proteins with apparent molecular weights of 14, 20 and 61 kDa that reacted with the chitinase antibody and two proteins of ca.33 and 17 kDa with the antibody of β-1,3-glucanase. Among them, the 20 and 61 (PAb-CHT) 33 (PAb-βGLU) bands were most prominent. In the untreated control tea cells these bands could not be detected. However, two minor bands, which were not observed in elicitor-treated cells, were seen in control lanes. A group of two PR-3 proteins was detected in all treatments, while a 20 kDa protein was only in detected treated cells.

4.13.3. Immunolocalization of defense enzymes in cultured tea cells treated with abiotic elicitors

The PR-proteins chitinase (PR-3) and β-1,3-glucanase (PR-2) were immunolocalized in the cultured tea cells (susceptible: T-135 and T-78) by probing with PAbs raised against chitinase and β-1,3-glucanase separately followed by labelling with fluorescein isothiocyanate (FITC). A 100 µl fraction each of salicylic acid (SA) and jasmonic acid (JA) treated cell. cultures were taken in Eppendorf tubes, diluted 1:10 with distilled water and treated with polyclonal antibodies- PAb-CHT and PAb-βGLU (1:10). The antibodies specifically located their respective antigens in the cells and accumulation of the enzymes was evident from the fluorescence emitted after FITC-labelling of the treated cultures. Fluorescence in the cultures was displayed along the cell walls, which was more intense in the salicylate treated cells probed with PAb-CHT (Plate 32, fig. C) than probed with PAb-GLU (Plate 32, fig. D). Cells treated with jasmonate and probed with PAb-CHT and PAb-GLU showed less recognition (Plate 33, figs. A – D). As for antibody treatment, PAb-chitinase treated cultures revealed bright apple green fluorescence whereas PAb-β-1,3-glucanase treated cultures exhibited yellow fluorescence. Enzyme-labelled accumulation was demonstrated in both resistant and susceptible varieties. Elicitation of phenolic substances exhibited as red coloured compounds in the cells was noticed more in some of the Tocklai varieties than UPASI ones.

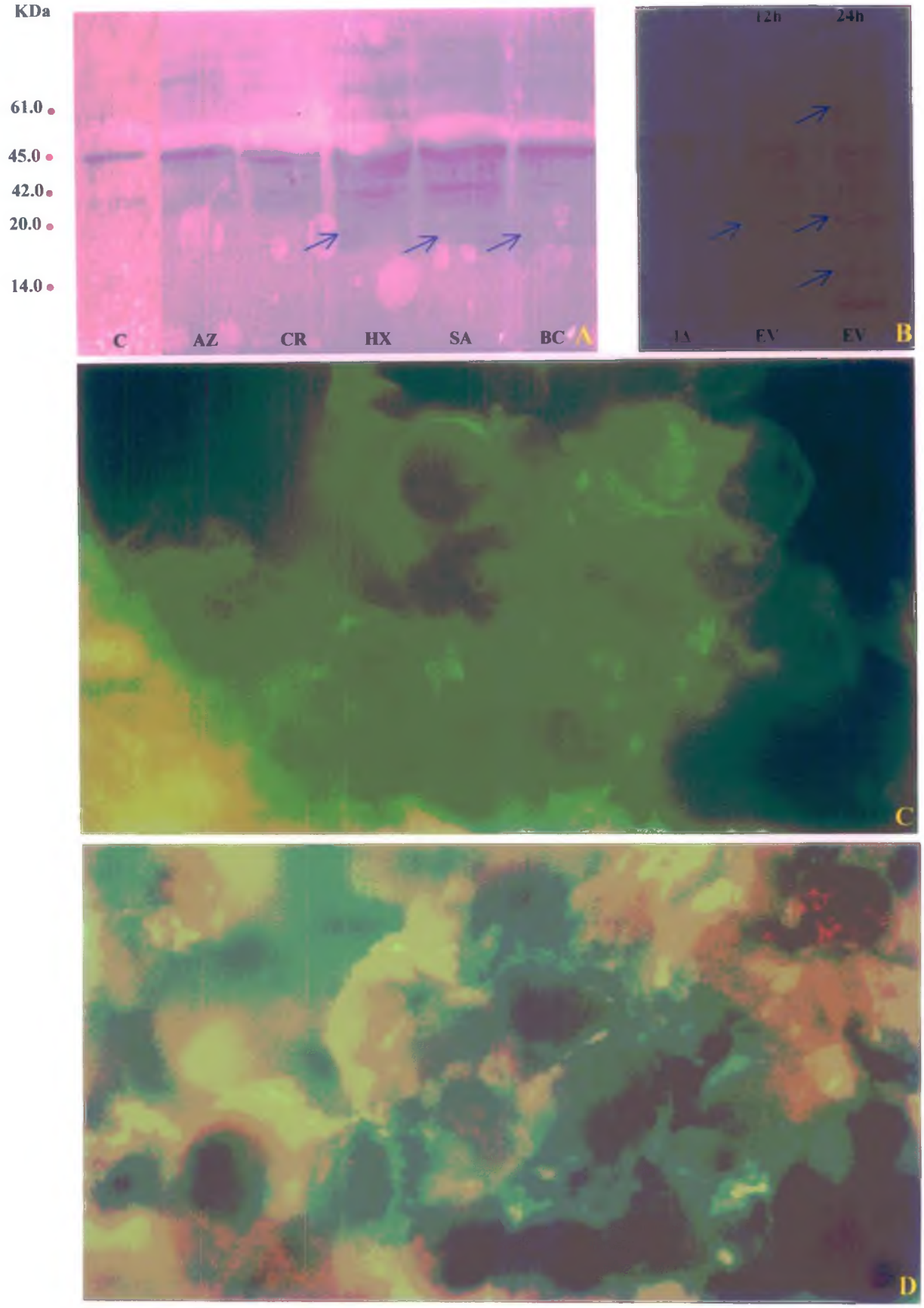


Plate 32 (figs. A - D). Western blot analyses [A & B] of elicitor induced PR-proteins and immunofluorescence [C & D] of salicylic acid treated tea (T-135) cell cultures probed with PAb-chitinase [A, B & C] and PAb-β-1,3-glucanase [D].

[C- control, AZ- *A. indica*, CR, *C. roseus*, HX, hexaconazole, SA- salicylic acid, BC- biocrop, JA- jasmonic acid, EV- *E. vexans*]

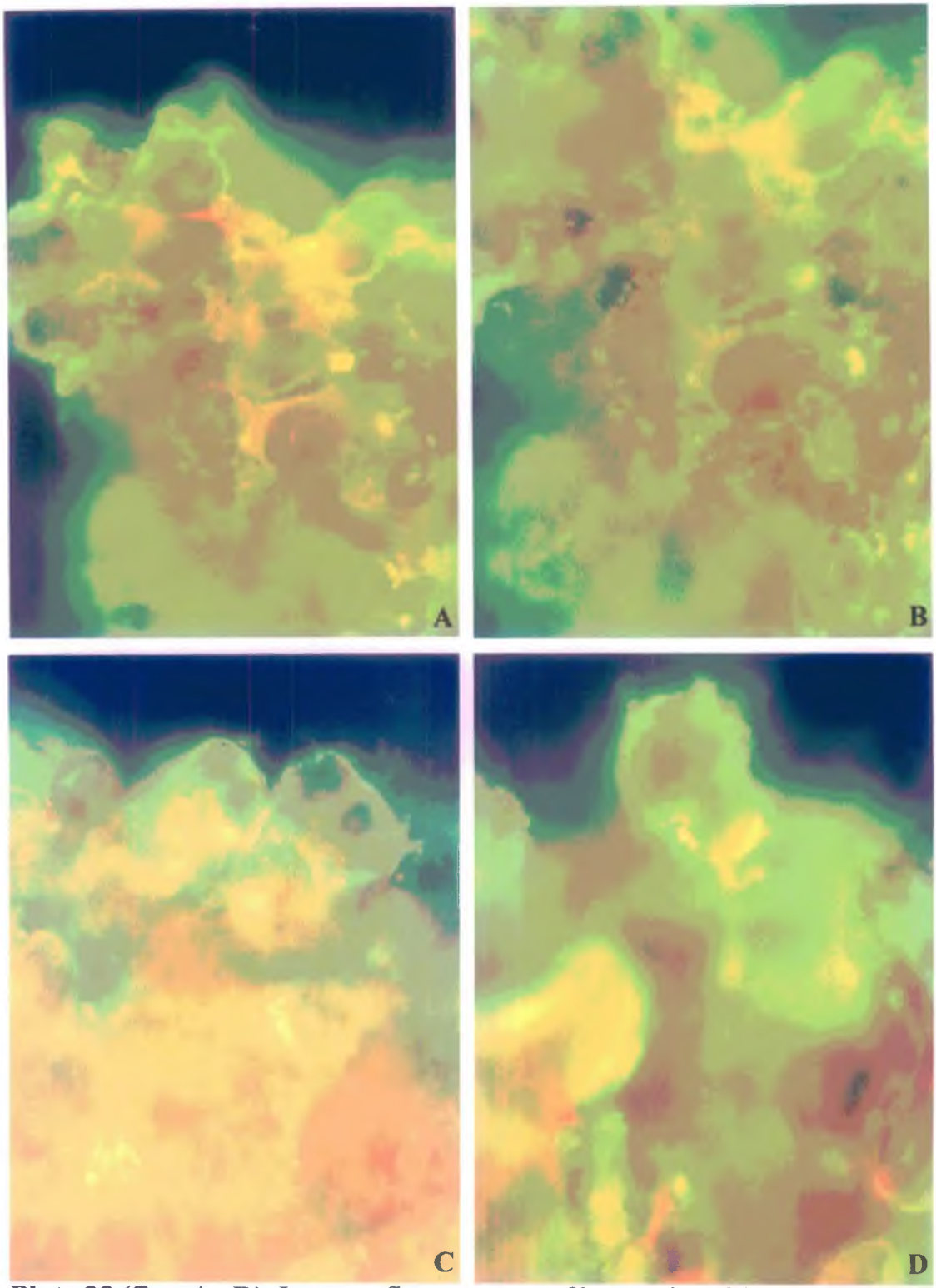


Plate 33 (figs. A - D). Immunofluorescence of jasmonic acid treated tea cell cultures probed with PAb-chitinase [A & B] and PAb-β-1,3-glucanase [C & D]. T-135 [A & C]; T-78 [B & D].

4.14. Cellular localization of chitinase in tea leaf tissues following abiotic treatments

Tea plants were induced with abiotic elicitors such as salicylic acid, hexaconazole and phytoextracts of *Azadirachta indica* and *Catharanthus roseus*. In the previous experiments it has been established that these inducers effectively enhance the levels of defense enzymes. It was noticed that chitinase was significantly enhanced in the treated plants and β-1,3-glucanase induction was not very significant as also observed in suspension cell cultures of tea. Keeping this in view immunofluorescence studies were conducted to localize chitinase (PR-3) at the cellular level in tea leaf tissues (TV-18, T-135, T-78, BSS-2) following induction.

Cross sections of the leaf tissues were treated separately with normal antiserum and PAb-chitinase. Leaf sections exhibited a natural autofluorescence under UV-light but that was not characteristic of FITC fluorescence. Observations in the treatment with normal antiserum were the same. When the cross sections of healthy and treated leaves were incubated with PAb-chitinase and labelled with FITC strong fluorescence was observed in the treated leaves. Bright apple green fluorescence was evident in the epidermal and homogenously in mesophyll tissues. PAb-chitinase was most reactive with leaf sections of salicylic acid treated plants (Plate 34, figs. A & B). In healthy leaves fluorescence was limited to the epidermis and certain elements like sclereids (Plate 35, fig. A). Fluorescence was also exhibited in hexaconazole treated plants which was distributed throughout the mesophyll tissues (Plate 35, figs. B & C). Observations also revealed enhancement of chitinase in the leaf tissues following induction with the phytoextracts which was more intense in *C. roseus* (Plate 36, fig.B) than *A. indica* (Plate 36, fig. A) induced plants. Interestingly, along with the PR-protein, chitinase, induction of phenolics, exhibited as red coloured compounds, in the cells was also evident in sections of tea leaf tissues treated with hexaconazole and the phytoextracts. So positive reaction with FITC occurred in cellular localization experiments and gave indications of the induction of chitinase (PR-3) in tea leaf tissues following induction with abiotic elicitors.

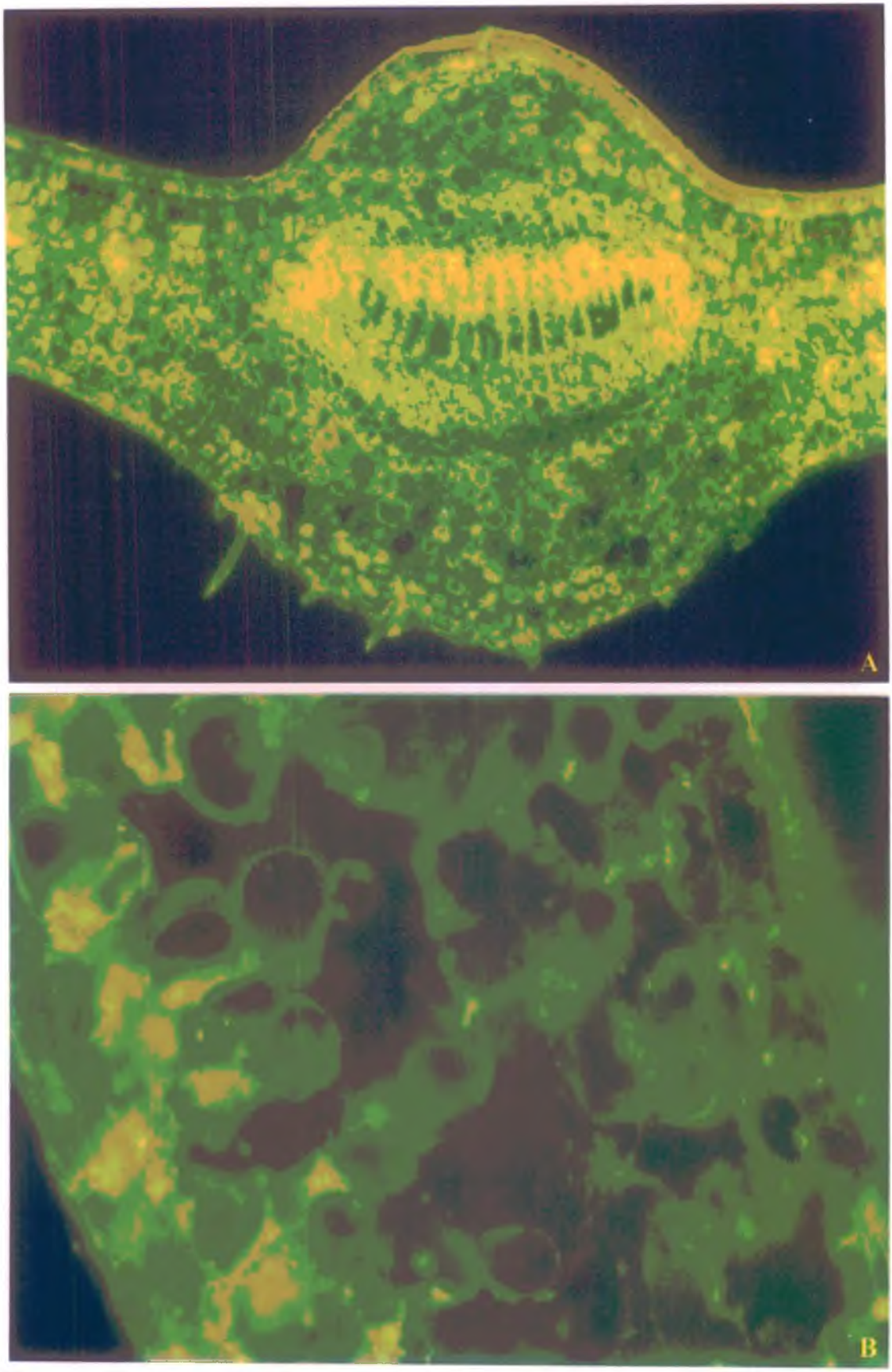


Plate 34 (figs. A & B). Fluorescent antibody staining of tea leaf tissues treated with salicylic acid and probed with PAb-chitinase. T-78 [A], T-135 [B].

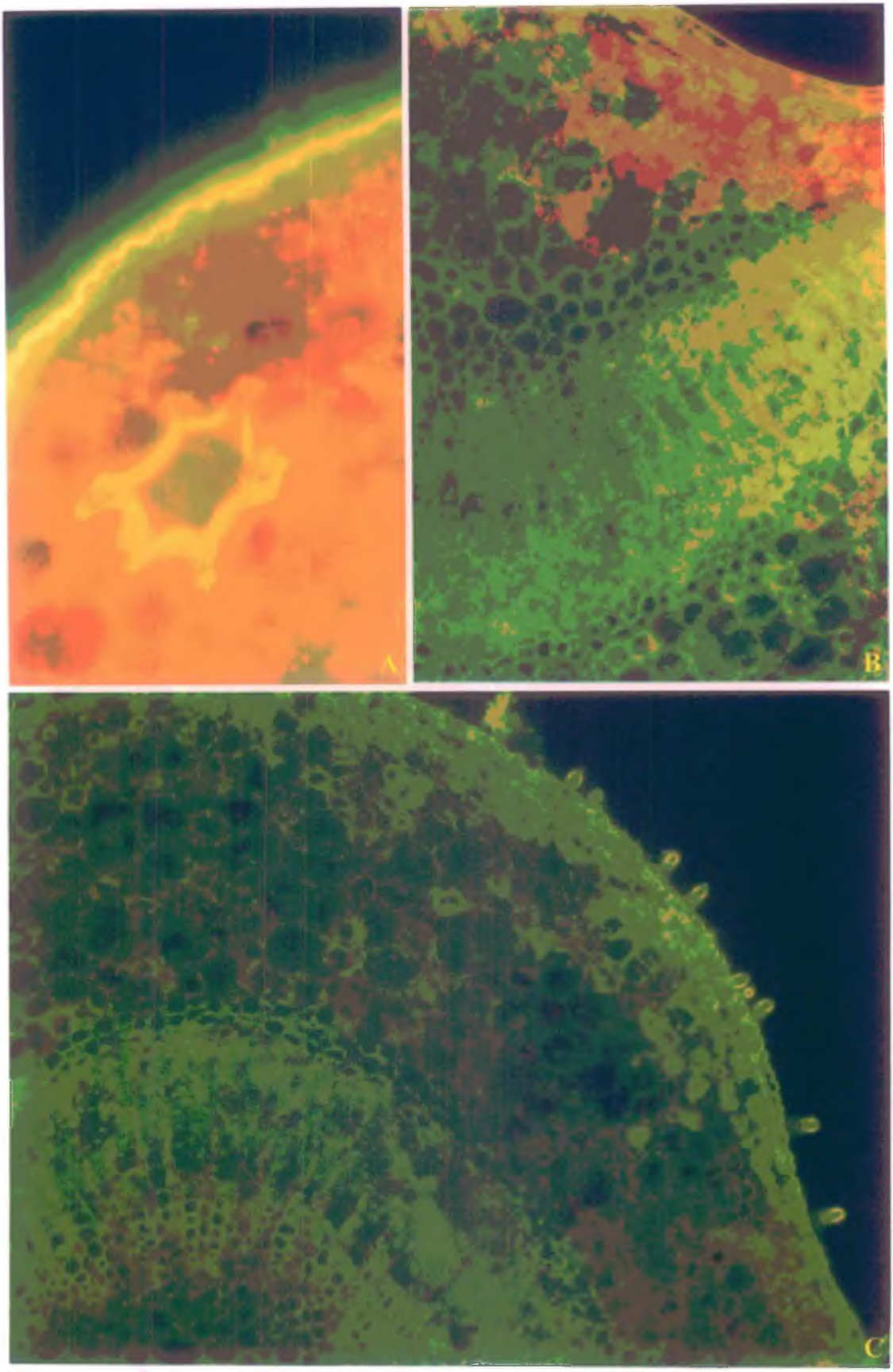


Plate 35 (figs. A & B). Fluorescent antibody staining of tea leaf tissues treated with hexaconazole and probed with PAb-chitinase. Untreated healthy [A], T-135 [B], TV-18 [C].

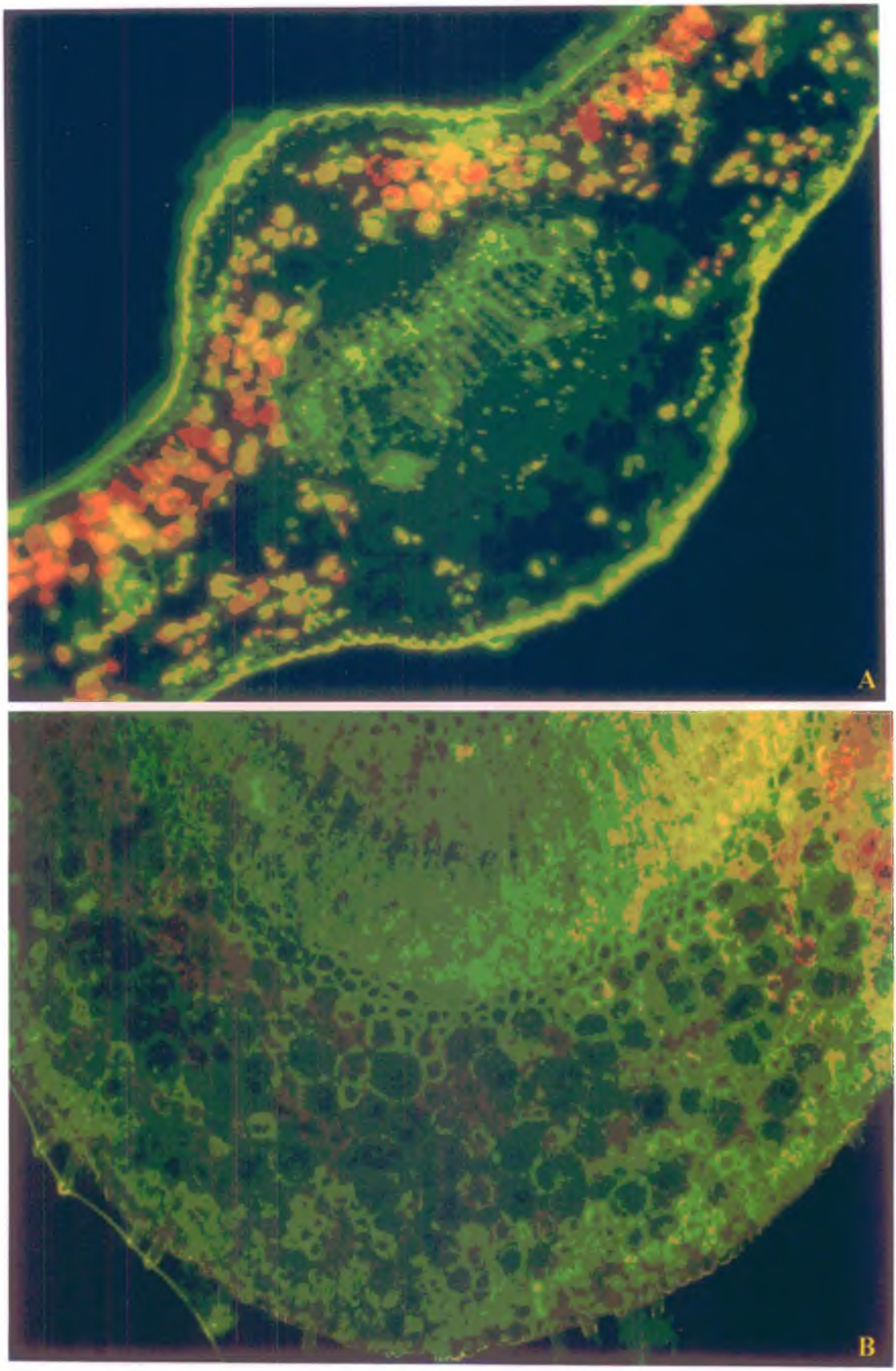


Plate 36 (figs. A & B). Fluorescent antibody staining of tea leaf (BSS-2) tissues treated with phytoextracts. *Azadirachta indica* [A] and *Catharanthus roseus* [B].

4.15. Immunogold localization of PR-2 in tea plants induced by Salicylic acid

Tea plants were treated with salicylic acid in order to induce resistance. Induction of defense enzymes in treated plants as well as treated inoculated plants was evident when these were assayed in the previous experiments. Besides, the PTA-ELISA formats also confirmed that in the salicylic acid treated plants infection was less, as absorbance values were significantly low. Indirect immunofluorescence of salicylic acid treated leaf tissues also gave bright apple green fluorescence in mesophyll tissues. Hence, it was interesting to locate these defense proteins in ultra thin sections of tea leaves treated with salicylic acid and then probed with PAb raised against β-1,3-glucanase. Healthy leaf tissues when probed with PAb-β-1,3-glucanase exhibited some deposition which has been described earlier.

Salicylic acid induced the deposition of β-1,3-glucanase (PR-2) systemically in the cells. Accumulation of β-1,3-glucanase in treated tea plants was observed in cell walls and extracellular spaces. The results were compared with untreated control plants. Microscopic examinations revealed labelling corresponding to β-1,3-glucanase deposition both in control and salicylic acid treated plants but the response was weak. Similar to pathogens, as shown earlier, salicylic acid induced accumulation of β-1,3-glucanase (Plate 37, figs. A - C.) in cell walls. After salicylic acid treatment, β-1,3-glucanase accumulated prevalently in the cells (Plate 38, figs. A - C) but the response was not so strong. Tissue cross-sections from the healthy, as well as the SA treated plants incubated with pre-immune serum instead of primary antibodies did not show any labelling.

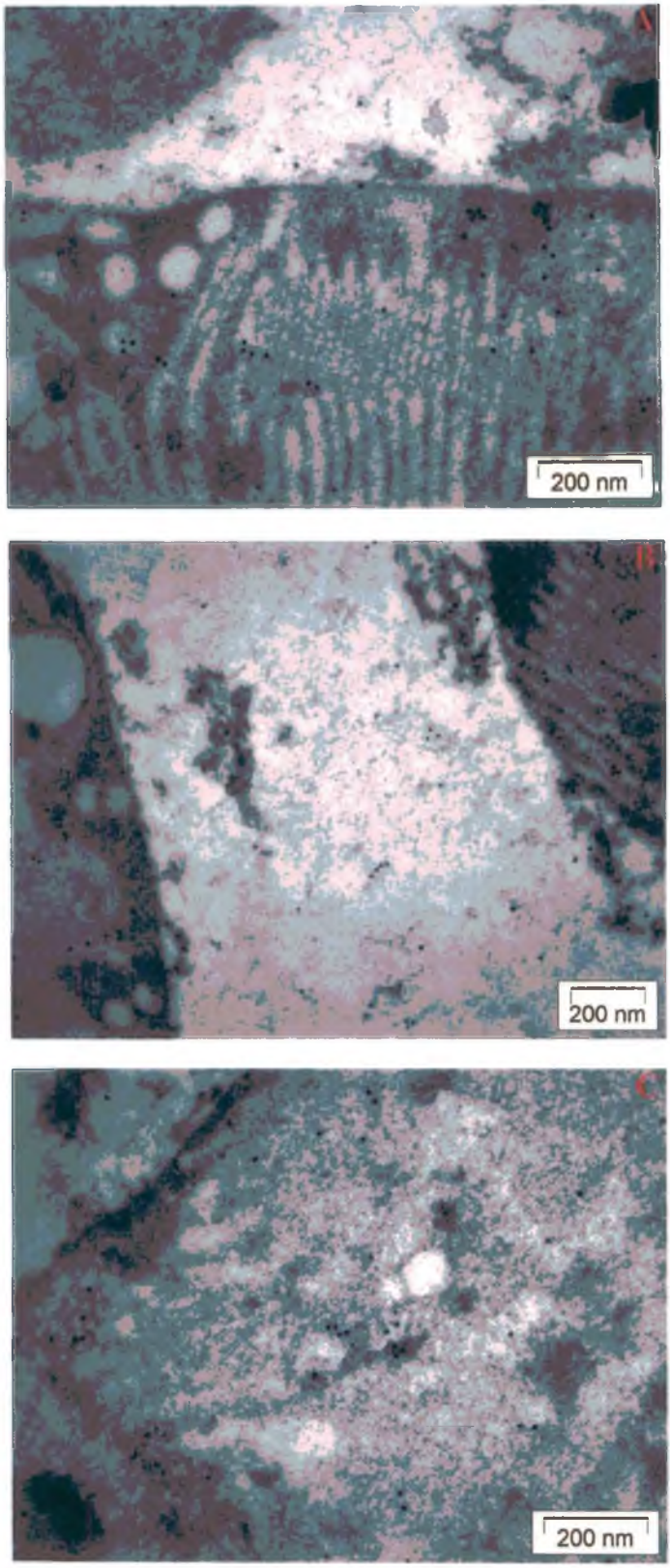


Plate 37 (figs. A - C). Transmission electron micrographs of immunogold labelled salicylic acid treated tea leaf tissues probed with PAb of β-1,3-glucanase.

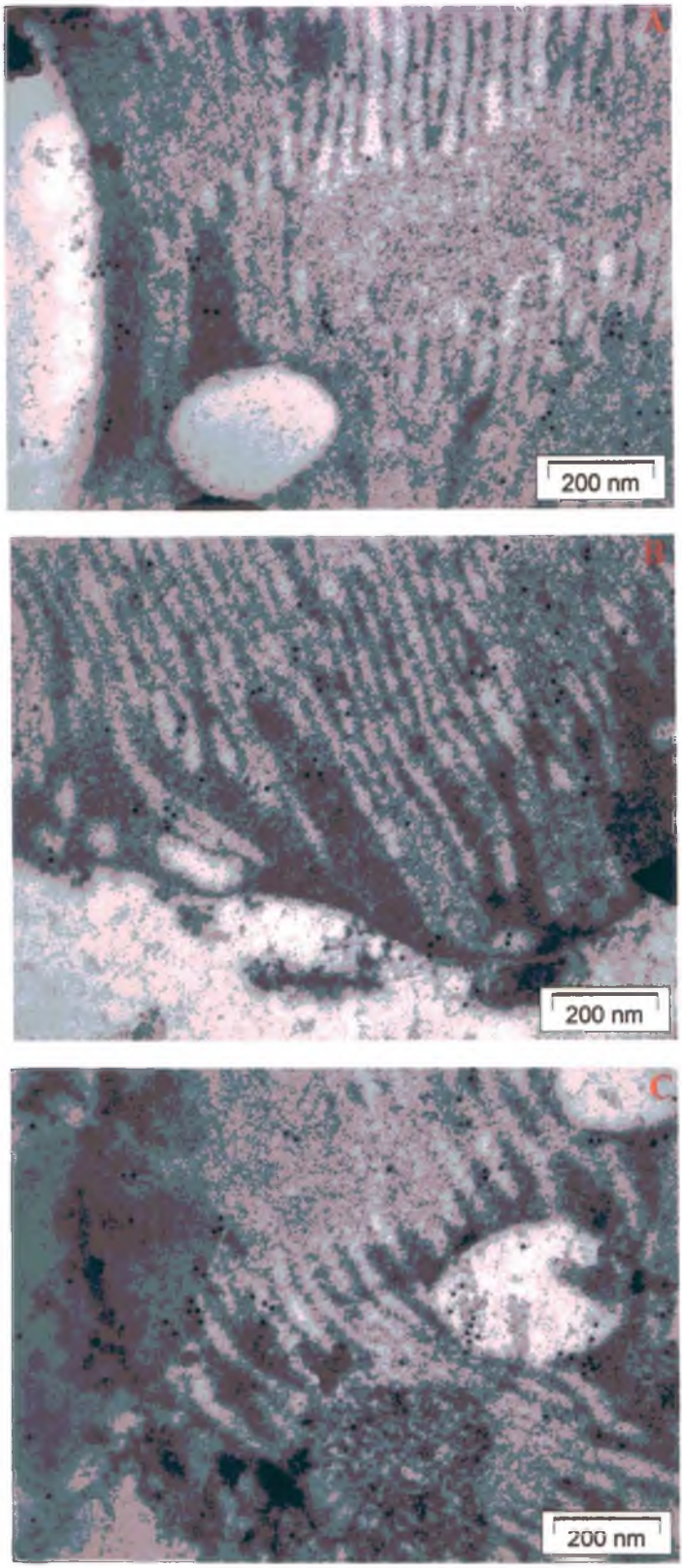


Plate 38 (figs. A - C). Transmission electron micrographs of immunogold labelled salicylic acid treated tea leaf tissues probed with PAb of β-1,3-glucanase.

4.16. Immunogold localization of PR-3 in tea plants induced by Salicylic acid

The accumulation of chitinase (PR-3) was investigated on cross sections of tea leaves of plants treated with the inducer of SAR, salicylic acid (SA). The results were compared with untreated control plants. Microscopic examinations revealed intense labelling corresponding to chitinase deposition in SA treated plants. Comparing to pathogen induced accumulation the gold labelled cells were found more regularly through the cell walls and extracellular spaces. The deposition of the chitinase, not β-1,3-glucanase, was found predominantly in cellular compartments. Gold labelling in the sections showed a high amount of labelling in chloroplasts and host cytoplasm and lesser amount in vacuoles, mitochondria and walls. Similarly to the pathogen, salicylic acid induced chitinase also in areas distant from infected cells it is likely that the chitinase is induced in a systemic manner. In healthy untreated plants, labelling studies with specific chitinase antibodies often revealed constitutive presence of chitinase. Immunohistology controls were run in parallel and treated with pre-immune serum instead of primary antibodies.

Different degrees of labelling were observed in the salicylic acid-induced plants with PR-2 and PR-3 antisera. In PR-3 treated tissues, gold particles were predominantly localized. In tissues treated with PR-3 antiserum uniform distribution of gold labelling was observed (Plates 39 and 40).

Thus, PR-2 antiserum showed lesser labelling associated with the cell wall and material filling intercellular spaces of parenchyma tissues as compared to PR-3 antiserum. A few gold particles were also deposited over secondary thickenings of xylem vessels. The extracellular localization of these hydrolases (chitinase and β-1,3-glucanase) suggests the potential of salicylic acid for protection of tea against foliar diseases. However, comparing to CHT, β-GLU was not so strongly induced by salicylic acid (or either by *E. vexans*).

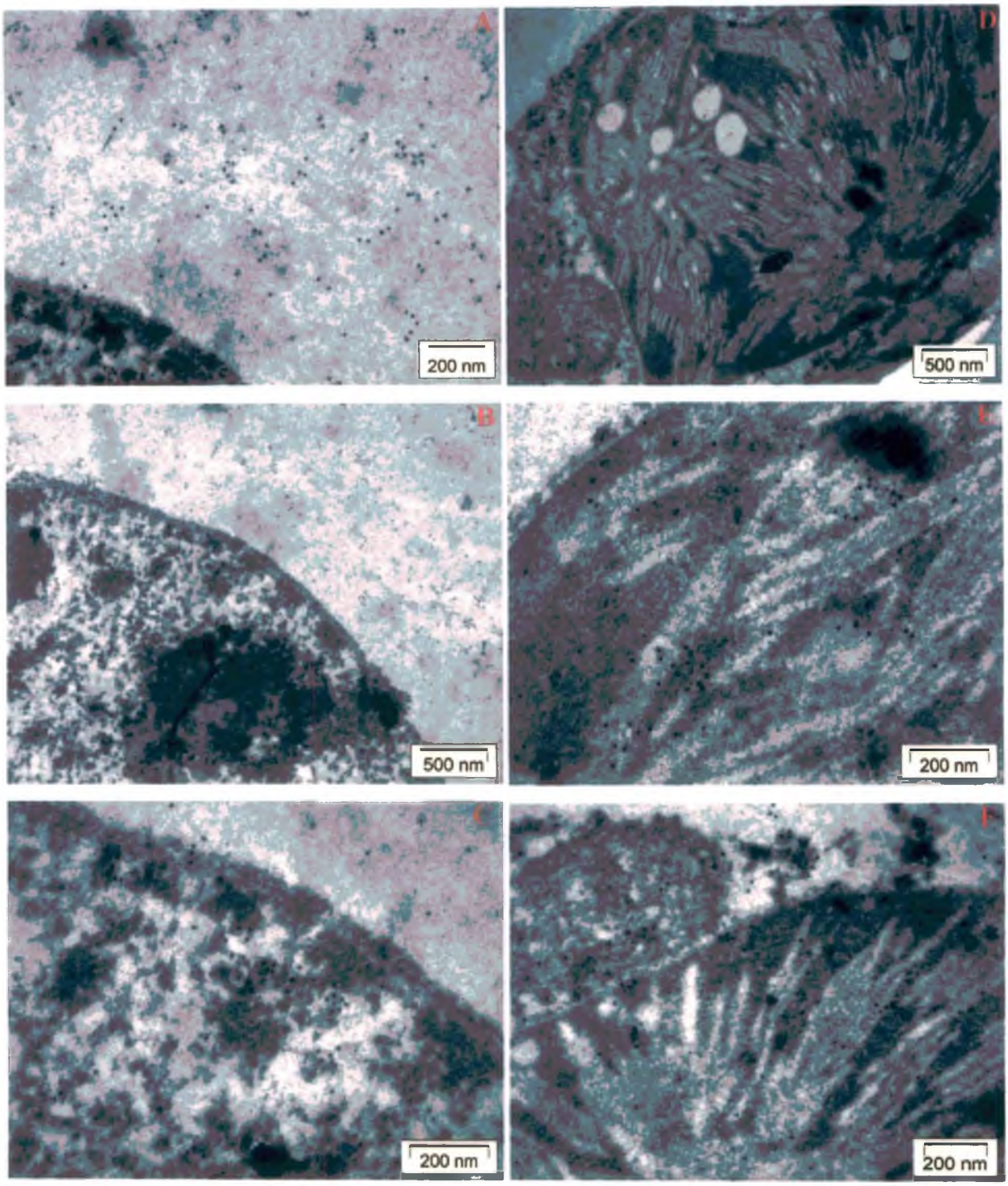


Plate 39 (figs. A-F). Transmission electron micrographs of immunogold labelled salicylic acid treated tea leaf tissues probed with PAb of chitinase. [B & C and E & F- magnified views of A and D respectively].

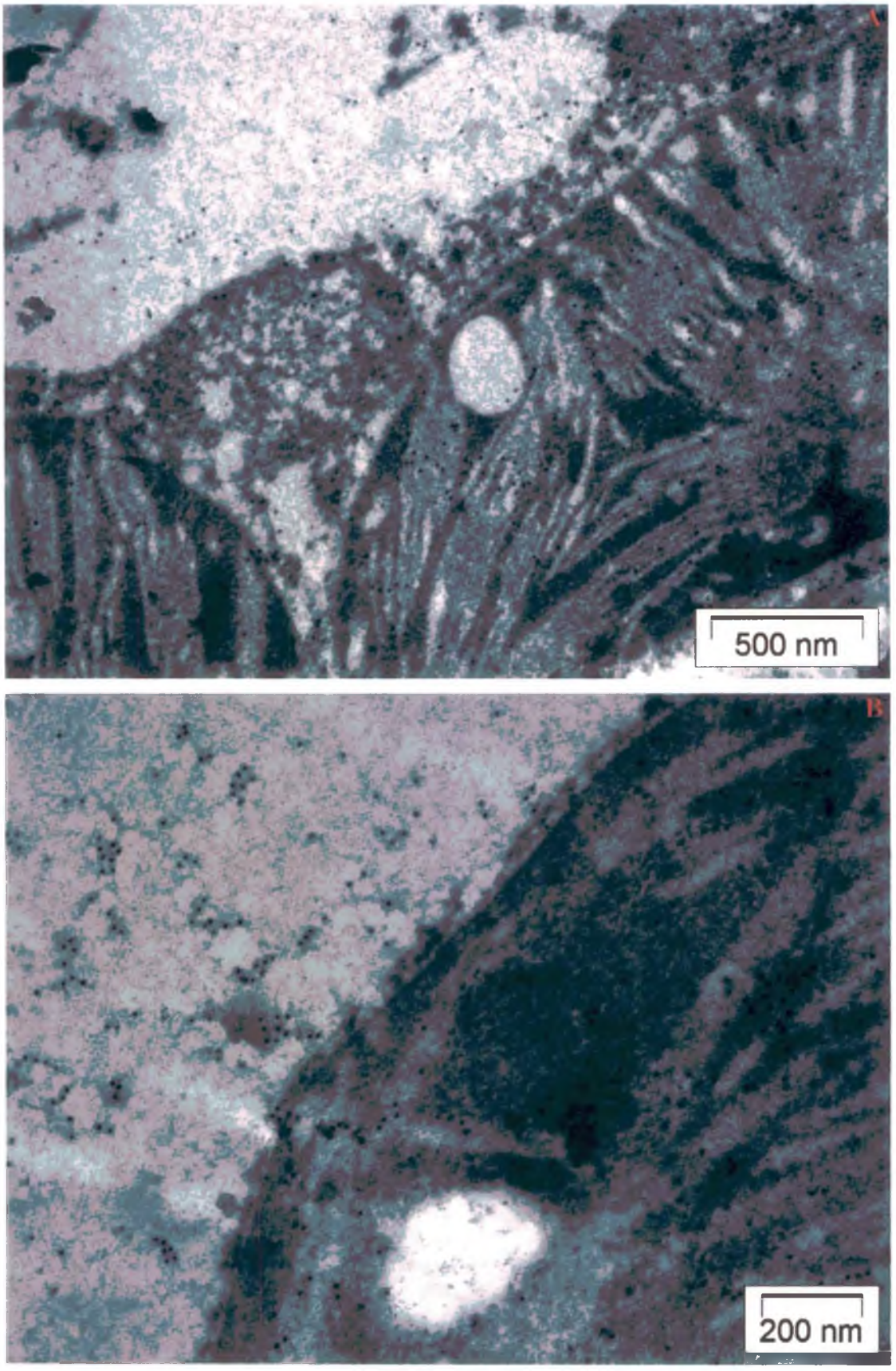


Plate 40 (figs. A & B). Transmission electron micrographs of immunogold labelled salicylic acid treated tea leaf tissues probed with PAb of chitinase.

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 as the winner as the invader is successfully warded off. Plants therefore have highly well developed defence mechanisms that enable them to defend themselves against penetration, intracellular growth and the development of parasites in their tissues. In nature plants have evolved multicomponent coordinated mechanisms by which they can defend themselves against the multitude of organisms attacking them. Resistance to disease in plants is associated with preformed and or inducible compounds, which may function in defense against disease (Broekaert, 2000; Rivera, 2002).

The versatile multicomponent defense is adequate to provide them protection against most of their potential pathogens; only a few of them can overcome this defense and cause disease (Sinha, 1995). Just before or concomitant with the appearance of a hypersensitive reaction is the increased synthesis of several families of pathogenesis-related (PR) proteins in the inoculated plants (Van Loon, 1985). Many of these proteins have been shown to exhibit antimicrobial activity either *in vitro* or *in vivo* (Datta and Muthukrishnan, 1999). Among these are 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 β-1,3-glucanases has been demonstrated in many plant-fungal pathogen interactions (Joosten and De Wit, 1989; Kim and Hwang, 1994; Kemp *et al.*, 1999; 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 (Mauch *et al.*, 1988). Secondly, they can also release cell wall fragments that act as elicitors of active host defense response (Yoshikawa *et al.*, 1993). 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. Higher plants do not contain chitin or β-1,3-glucans, or any other known endogenous substrate for chitinases and β-1,3-glucanases. However, chitin and β-1,3-

glucans are major components in the cell wall of many fungi and are target substrates for plant chitinases and β-1,3-glucanases (Tyagi *et al.*, 2001).

Another enzyme of interest with known defensive properties is peroxidase (PR-9; POX; E.C. 1.11.1.7.). The plant cell wall also constitutes one of the first lines of defense against pathogen invasion, and peroxidases are key enzymes in the wall-building processes. These processes include peroxidase-mediated oxidation of hydroxycinnamyl alcohols into free radical intermediates, phenol oxidation, polysaccharide cross-linking, crosslinking of extension monomers, lignification, and suberization. Although direct involvement of any one type of peroxidase in defense has not been demonstrated conclusively, extracellular or wall-bound peroxidases have been proposed to enhance resistance by the construction of a cell wall barrier that may impede pathogen ingress and spread. The accumulation of cell wall-strengthening materials following infection might be expected to correlate with enhanced resistance (Chittoor *et al.*, 1999).

Peroxidase activity produces the oxidative power for cross-linking of proteins and phenylpropanoid radicals resulting in reinforcement of cell walls against attempted fungal penetration (Huckelhoven *et al.*, 1999; Kristensen *et al.*, 1999). High levels of H_2O_2 trigger HR, while lower levels induce the accumulation of transcripts encoding antioxidant enzymes. Since high levels of H_2O_2 also preclude HR, peroxidase activity may modulate HR by altering the concentration of available H_2O_2 (Kruger *et al.*, 2003). In higher plants, the hypersensitive reaction occurs as an expression of incompatibility between a host plant and a specific pathogen. The occurrence of chlorotic and or necrotic flecking on disease resistant plants is usually typical of an HR; resulting from interaction between corresponding genes for resistance, plants contain genes encoding for proteins as part of the natural defense response of the plant and usually accompany HR (Bowles, 1990). In addition to the localized HR, many plants respond to pathogen infection by activating defenses in uninfected parts of the plant (systemic acquired resistance, SAR). As a result, the entire plant is more resistant to a secondary infection. SAR is long lasting and confers broad-based resistance to a variety of different pathogens. The synthesis of antimicrobial products, including phytoalexins and pathogenesis-related (PR) proteins, correlates well with the development of both HR and SAR.

Today tea is the most prominent drink of the world, and India is the world's largest producer and consumer of tea. Darjeeling tea is the best and most expensive in the world due to its superior flavor and unique aroma but when the plant succumbs to epidemics like blister blight crop loss is severe and tea produced from such leaves is of inferior quality. The obligate biotrophic fungus *Exobasidium vexans* is the sole cause of blister blight of tea, a disease characterized by white velvety blisters, developing mainly on the ventral and rarely on the dorsal leaf surfaces of tender leaves and the stem. There are no known alternate hosts and its lifecycle is completed on tea itself. The disease was described and identified as a fungus belonging to the class Basidiomycetes under the order Exobasidiales (Hawksworth *et al.*, 1983). Substantial progressive decline in theaflavins, thearubigins, caffeine and high-polymerized substances as well as brightness, briskness and total liquor colour occurs in severe infection. The total nitrogen, soluble nitrogen, and amino acid content were also affected with increasing severity of the disease (Chandra Mouli, 2003).

Research concerning the significance and expression of induced resistance in tea plants in their systemic parts is lacking. Clearly, a better understanding of the defense mechanisms underlying this basic function is the need of the hour; hence, it was found essential to initiate investigations in this line of research. In this discourse two major lines of approach were considered that included (i) application of biochemical and immunological techniques to study compatible and incompatible interactions between tea plants and *Exobasidium vexans* and (ii) elucidation of defense responses of tea plants triggered by the pathogen with special reference to pathogenesis-related proteins and their induction for development of systemic acquired resistance in tea.

At the inception, a brief study on the disease epidemiology was carried out. Disease incidence for blister blight was recorded for five years (2000-2004) in the hills and plains. In Castleton Tea Estate, the hills, disease incidence was high during July through October whereas in Hansqua Tea Estate, the plains, significant disease

appearance commenced in December and disappeared by February during the entire study period. Weather parameters such as temperature, relative humidity, rainfall have influence on plant disease development. Disease intensity may be forecast by correlating the factors that are essential for the establishment of the pathogen in the host and its dissemination, with meteorological data for a given period. In the hills, a positive correlation was obtained with rainfall and relative humidity. The impact of relative humidity, temperature, leaf wetness, light and pH of leaf surface on the development and severity of the blister blight disease has also been reported by Agnihothrudu and Chandra Mouli (1991). Leaf wetness is the most important factor for the germination of spores. Protracted leaf wetness of 11 h is optimum for infection and maximum infection occurs after 8 h of leaf wetness. In the plains even at low rainfall the disease incidence was high. Though rainfall was low, relative humidity was high. This provided a foggy environment, which is again highly favourable for the disease. A thin film of water is more favourable for germination and hence when dew is formed in the evening, the spores germinate to bring about infection. The disease is very common in areas lying above 700 m above sea level and in humid and foggy regions. Survey has shown that the actively sporulating blisters occur on tea during bushes in sheltered humid pockets alongside perennial water sources, even during the dry weather. The pathogen was also reported to survive on necrotic lesions during the off-season (Sugha, 1997; Baby *et al.*, 2001).

In the hills disease incidence showed a positive correlation with maximum and minimum temperature however higher temperatures were unfavourable for the pathogen in plains. Direct sunlight is lethal to the development of the pathogen. Venkataram (1979) found that exposure of basidiospores to UV light of 365 nm for up to 90 min did not completely inhibit their germination. At 15, 30 and 45 min of exposure, there was only a marginal difference in the rate of germination, but after 60 min of exposure the germination fell by 70 percent as compared to 98 percent in the check treatment. However the length of the germ tubes decreased markedly, when the spores were exposed to more than 30 min of UV radiation. Exclusion of UV-B radiation increased the immature stages of infection but has no effect on the sporulation of *E. vexans* (Gunasekera *et al.*,1997).

On microscopic observation, variation in size of the spores collected from the three different gardens was noticed. Accordingly, the isolates were designated EV-1 (Castleton), EV-2 (Hansqua) and EV-3 (Margaret's Hope). The spores readily germinated by producing germ tubes in a pH range of 5.0 – 8.0 at 25 ±2°C. The tips of the germ tubes enlarged to form appressoria within 24 h of growth. Pathogens are known to produce infective structures that ease the pre- and post penetration processes of which appressoria formation is the first step in establishment of disease and factors affecting this process largely decide the fate of the pathogen at initial stages (Purkayastha and Menon, 1981). The behaviour of microorganisms on the plant surface is affected by stimulants or inhibitors of fungal development in infection droplets, by the nature of the cuticle, the nature of the substrate and other environmental and cultural factors. It is well established that soluble organic and inorganic nutrients exude from uninjured plant surfaces and will either support or inhibit the growth of the microbial pathogen. Appressoria formation is also influenced to a large extent by these factors (Yang and Ellingboe, 1972; Emmett and Parbery 1975).

Adhesion of fungal spores to the plant surface is the first step in committing a pathogen to the establishment of disease. Chakraborty *et al.* (1995a) attempted to determine the role of biotic and abiotic factors influencing spore germination, appressoria formation of *Glomerella cingulata* for the development of brown blight disease in tea. Tucker and Talbot (2001) evaluated the involvement of surface-acting proteins and the existence of conserved signaling pathways that regulate appressorium formation.

In the present study, basidiospore morphology of *E. vexans* was observed under scanning electron microscope (SEM) that appeared as white spore masses. The spores have a mucilaginous sheath and adhere firmly once they lodge on to the leaf surface. One function of the adhesion of fungal propagules is to prevent their displacement from the host surface, which allows initiation of the infection process. Conidia of *Colletotrichum graminicola* that adhered to corn leaves released a material at the interface of the site of contact of the conidium with the leaf surface which has been demonstrated by SEM

(Mercure *et al.*, 1995). On the other hand, using SEM, the extent of damage on tea leaf surface by blister blight pathogen was also examined. The superficial extent of damage was evident by the dense hyphae network of *E. vexans* in the host cells. Similar studies have demonstrated the presence of a fibrous-cottony net or matrix on the surface of vegetative hyphae, and ostiolar head fluid of the aggressive isolates of *Ophiostoma ulmi* (Svircev *et al.*, 1988). Using SEM, Kruger *et al.* (2003) demonstrated the extent of *Blumeria graminis* f.sp. *hordei* development on each barley genotype through 72 h after inoculation.

Plants have mechanisms to keep a homeostatic balance with potential pathogen or non-pathogens in the immediate environment. A basic requirement for the proper functioning of even a simpler cellular system is communication between its components. In this sense, communication means transmitting information to and receiving information from the environment, then transforming it to signals or instructions, which the cell can understand and respond to. This exchange of information can be mediated by soluble agents (transmitters) or by direct cell-cell interaction. It is assumed that compatibility and incompatibility are initiated by surface-surface contact (Chakraborty, 1988). Screening for resistance of thirty tea varieties towards *E. vexans* was carried out. Among these varieties, T-17/1/54, BS-7A/1/76 and BSS-3 exhibited very low infection percentages. On the other hand in varieties like T-78, TV-18, T-135, UP-9, BSS-2, UP-2, TV-9, UP-3, UP-17 and TV-28 the infection percentage was high. Chandra Mouli (1983) has also reported that tea plants differ in their resistance to blister blight.

In recent years, studies have been taken up for detection of the pathogen in host tissues by immunological means (Chakraborty and Chakraborty, 2002) and identification of specific antigens in electrophoretically separated components (Blake *et al.*, 1984; Wakeham and White, 1996). The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseased plant tissues (Lyons and White, 1992; Werres and Steffens, 1994). These rely on the recognition of solid or soluble antigenic materials by antibodies raised against the organisms and subsequent use of an enzyme labelling system. The possible involvement

of cross reactive antigens (CRA) in determining the degree of compatibility has been reported by several workers in different host-pathogen systems, viz., potato-*Phytophthora infestans* (Alba and De Vay, 1985), cotton - *Verticillium albo-atrum* (Charudattan and De Vay, 1972), soybean - *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983), soybean - *Myrothecium roridum* (Ghosh and Purkayastha, 1990), tea - *Bipolaris carborum* (Chakraborty and Saha, 1994b), groundnut - *Macrophomina phaseolina* (Purkayastha and Pradhan, 1994) and tea - *Ustulina zonata* (Chakraborty *et al.*, 2002b). In the present study serological relationship between *E. vexans* and tea varieties were determined. The potential of ELISA, DIBA and immunofluorescence for screening tea germplasm for resistance towards *E. vexans* and also for early detection of pathogens in tea leaf tissues were developed.

Initially, effectiveness of PAbs raised against host and pathogen was confirmed by immunodiffusion. IgGs were purified before application in immunoenzymatic assays to minimize non-specific binding. Optimization of ELISA was done considering two variables - dilution of antigen and antiserum. The PTA-ELISA format was employed for the detection of CRA shared between *E. vexans* and tea. Absorbance value (A_{405}) was always higher in susceptible host pathogen combinations than resistant ones. Among the thirty varieties tested T-78 was showed highest absorbance value while T-17/1/54 obtained least. Harrison *et al.* (1990) also employed the PTA-ELISA technique in estimating the amounts of *Phytophthora infestans* mycelium in potato leaf tissues and suggest it's useful application particularly in screening for resistance programs. The results of PTA-ELISA were confirmed by Dot-immunobinding assay (DIBA) in which intensity of the dots widely differed among the varieties artificially inoculated with the pathogen. A good correlation was found between the two techniques as results of DIBA gave similar level of detection as in PTA-ELISA.

Reciprocal cross reactions involving PAbs of tea varieties (T-17/1/54 and T-78) and antigens of host, pathogen (*E. vexans*), non pathogen (*F. oxysporum*) and non hosts (*L. leucocephala*, *O. sativa*, *G. max*) were also investigated. In PTA-ELISA, little cross-reaction was found between the antiserum raised non-pathogen and the tea varieties

tested. Results were alike between antiserum raised against *E. vexans* and non-host antigen. Linfield (1993) performed similar studies between the antiserum raised to *F. oxysporum* f.sp. *narcissi* and other common unrelated fungi and found little cross reaction. Whilst this was achieved with PAb, it has also been found that cross-reactivity within fungal genera can be achieved with monoclonal antibodies (MAb). Serological profiling of the fungal genus *Pythium* has been worked out at length (Mohan, 1989; White *et al.*, 1994). MAbs and PAbs raised against *P. violae* and *P. sulcatum* exhibited little binding for the heterologous species but there was extensive, differential cross-reactions between all antibodies and the other *Pythium* spp. tested.

Early detection of disease is an important requisite for development of management strategies. Its implication has been elaborately described by previous workers in a range of plant-pathogen systems (Chakraborty *et al.*, 1996a, b; Chakraborty *et al.*, 1997; Chakraborty *et al.*, 2002b, c). The visible outcome of compatible host pathogen interactions have been obtained in many cases before the pathogen could be well established in the host tissues. A microtitre immunospore trapping device, which uses a suction system to trap air particulates directly by impaction into microtitre wells, has been used successfully for the rapid immunodetection and quantification of ascospores of *Mycospaerella brassicicola* (Wakeham *et al.*, 2000) and conidia of *Botrytis cinerea* (Kennedy *et al.*, 2000).

In accordance with established information, in this investigation also early detection of *E. vexans* could be possible using PTA-ELISA formats. Indirect immunofluorescence tests were performed in this study for cellular localization of major CRA in tea leaf tissues. Cross sections of healthy tea leaves exhibited a natural bright yellow autofluorescence mainly on the cuticle and apple green fluorescence distributed mainly over epidermal cells, mesophyll tissue and xylem elements more strongly in susceptible (T-78, T-135 and UP-9) tea varieties compared with resistant ones (T-17/1/54, BS-7A/1/76 and BSS-3). The cellular location of CRA in tea leaves shared by *Pestalotiopsis theae* (Chakraborty *et al.*, 1995a), and *G. cingulata* (Chakraborty *et al.*, 1996b) and detection of pathogen in host tissues using antibody based

immunofluorescent techniques has been reported previously (Warnock, 1973; Reddy and Anantanarayan, 1984, Werres and Steffens, 1994; Chakraborty and Chakraborty, 2002). Dewey *et al.* (1984) have reported interesting observations 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. Ascospores of *Mycosphaerella brassicicola* were also detected on artificially inoculated spore tape using immunofluorescence (Kennedy *et al.*, 1999). Thereby, the present study reports the use of indirect immunofluorescence tests using polyclonal antibodies of *E. vexans* as a suitable technique for localization of the pathogen and could be employed for immunodetection of *E. vexans* in tea leaf tissues.

Immunofluorescent studies on basidiospores of *E. vexans* was possible in the present discourse with PAbs of *E. vexans.* The spores were not autofluorescent but nevertheless showed a general fluorescence along the spore walls with homologous antiserum demonstrating specificity of the antiserum. Another motivating finding of this study was direct detection of the fungus in leaf tissues using a unique non-fluorescent staining (Gerik *et al.*, 1987; Young and Andrews, 1990) immunoenzymatic assay based on polyclonal antibodies, which has several advantages over conventional bioassays in determining the colonization pattern of fungi in leaf tissues. Additionally this technique can also be applied to sequentially stain different fungi in the same tissue using different enzymes linked to the antibodies and/ or different diazo dyes as demonstrated by Gerik and Huisman (1988). In the present study, intense fungal colonization was observed following treatment with PAb of *E. vexans* and reaction with Fast blue BB salt substrate. Deep blue coloured fungal colonies were clearly visible in naturally infected as well as artificially inoculated tea leaf tissues which was noticeably more in susceptible varieties (T-78 and UP-2) compared to resistant ones (TV-30).

With the advent of immunocytochemistry sub-cellular level studies have gained enormous importance and substantiating preliminary findings with novel and sophisticated techniques. In the current investigation, an attempt was also made to

conduct ultrastructural immunocytochemical studies to locate cross-reactive antigenic sites shared by *E. vexans* and *C. sinensis* through transmission electron microscopy (TEM). Encouraging results were obtained following immunogold cytochemical labelling of ultra thin tea leaf sections. Electron microscopic observations of healthy tissues treated with PAb-EV showed specific localization of the antibody associated with the epidermal and mesophyll cells. In addition immunogold labelling of *E. vexans* in blister infected tea leaf tissues was also performed and intense labelling was observed on fungal mass that had established. Ultrastructural observations on penetration, colonization and cytological modifications in blister blight infected tea leaf tissues showed penetration of the fungus.

In the present investigation protein pattern of healthy and *E. vexans* inoculated leaves of resistant (TV-9,TV-20), and susceptible (TV-22 and TV-23), varieties were evaluated by SDS-polyacrylamide gel electrophoresis. Biochemical and immunological characterization of pathogen induced (PI) protein in tea leaf tissues were explored. After 12 d of inoculation of tea plants with *E. vexans* visible symptoms appeared. The soluble proteins that were extracted from healthy and artificially inoculated plants (T-78, UP-2, TV-18) and separated by SDS-PAGE were blotted (western blotting) in an attempt to determine if any of the proteins were of pathogen origin. On probing with PAb-EV1 recognition of two proteins of approximately 58 and 15 kDa was observed only in the *E. vexans*-inoculated leaf samples, which were absent in healthy leaf samples.

Proteins occupy a central role in all organisms, and plants are no exception to this rule. These relatively large, diverse macromolecules account for a substantial portion of the dry weight of most living plant cells and are important constituents of plant tissues. A large number, if not most, of the biochemical, physiological and structural processes are governed and regulated by one or more of these proteins. The information for all proteins ultimately lies in the nucleotide sequence of the gene that encodes the specific protein (Ko, 1997). Tea plants, like any other plant, have to deal with numerous challenges without being able to move from their growing site. Thus, the chemical profile displayed by any particular plant at any given time and place will be broadly defined by its genetic inheritance, which has arisen from an ongoing refinement of the optimum chemistry for

survival in the face of rapidly evolving populations of both microbes and insects. Both the plant's developmental state and its environment, however, condition expression of this genetic potential (Ellis, 1997). Protein profiles of total soluble proteins as well as acid soluble proteins in leaf homogenates obtained from tea plants growing under natural conditions were examined. A number of proteins of diverse molecular masses were identified. Results obtained following analysis of total soluble proteins displayed similar proteins of ca.45, 85 and 100 kDa in most Darjeeling and Tocklai varieties and rarely in UPASI varieties. Analysis in the protein pattern of acidic extracts from thirty tea varieties revealed presence of bands mainly with molecular weights of less than 27.5 kDa. Differences were observed in number of PR-proteins in the different varieties. PR-proteins of 50-60 kDa and 10-15 kDa were detected in most varieties.

Changes in the soluble protein (pH 2.8) pattern of non-infected control barley plants was examined by Bryngelsson *et al.* (1988) who have reported to find distinct differences in the gel patterns, while during the resistant reaction to mildew, new host proteins between 13.5 to 27.5 kDa that were not present in immune or susceptible responses were induced. An attempt was also made in this study to see whether such responses could be induced in tea plants following inoculation with the pathogen, *E. vexans*. The acid soluble protein pattern of the ten artificially inoculated tea varieties clearly exhibited increase in intensity of few bands and also exhibited appearance of new bands that were not revealed in uninoculated control plants. Another very interesting investigation has been carried out by Christ and Mösinger (1989) where they have reported appearance of eleven acid soluble proteins with apparent molecular masses ranging from 13-82 kDa increased in tomato (*Lycopersicon esculentum*) leaves infected by *Phytophthora infestans* or *Fulvia fulva*. The most prominent changes in the protein patterns were also detectable in the untreated leaves of infected tomato plants indicating systemic effects of the infection. As results of the present investigation substantiate the findings of previous workers it can be inferred that the low molecular weight acid soluble proteins that appeared after inoculation of both susceptible and resistant tea plants may be pathogenesis-related proteins as they share distinctive properties with PR-proteins in dicot species. This study also provides evidence that the induced acid soluble proteins

had antifungal activity. This was demonstrated by *in vitro* tests against spores of *E. vexans*. Christ and Mösinger (1989) also observed a reduction in the size of lesions in *P. infestans* in untreated leaves of treated tomato plants which coincided with the changes in protein composition Acid soluble proteins, are strongly enhanced in tomato plants (Fischer *et al.*, 1989) infected with *P. infestans*.

In the present study, PR-protein accumulation was measured in healthy, infected and leaves exhibiting hypersensitive reactions against *E. vexans* under field conditions. Chitinase activity was always found to be lower in the compatible interactions in comparison to resistant and healthy ones with highest accumulation in the disease free leaf samples collected from tea bushes which have acquired immunity towards environmental stress(es) as well as the leaves showing hypersensitive reaction against the pathogen (*E. vexans*) attack resulting in incompatibility with the pathogen. β-1,3-glucanase activity was consistently high in disease free leaf samples of all gardens with an abrupt increase in the enzyme activity in the initial stages of infection and decreased activity as the disease developed as well as in resistant reactions. The peroxidase activity displayed a constant increase during the stages of infection and the values obtained for the different stages of infection were always higher than that of healthy samples. Of major significance was the increase in enzyme activity in resistant samples. Such higher peroxidase activities may be correlated with greater resistance.

Following infection with *E. vexans* two new isozymes of R_m 0.125 and R_m 0.67 were visualized in compatible interactions and interestingly a band of of R_m 0.44 was observed only in leaves exhibiting hypersensitive reactions. Peroxidase isozymes of *Aloe barbadensis* oxidize *p*-coumaric acid (Esteban-Carrasco *et al.*, 2002). The developmental regulation of the H_2O_2-producing system and a basic peroxidase isozyme in the *Zinnia elegans* lignifying xylem has been elucidated (Ros-Barcelo *et al.*, 2002).

The induction and accumulation of chitinase and β-1,3-glucanase however may be associated with the restriction of symptom development on the tea leaves on attack by *E. vexans* as the enzyme activity was more rapidly enhanced in incompatible than

compatible interactions. Recently it has been suggested 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 (Ham *et al.*, 1991). 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. Time course experiments in the present study revealed enhancement in activity of the defense enzymes chitinase, β-1,3-glucanase and peroxidase in plants inoculated with *E. vexans* at specific time intervals. After 24 h of inoculation chitinase and β-1,3-glucanase displayed a peak rise whereas peroxidase was high after 48 h of inoculation in both resistant (BS-7A/1/76, T-17/1/54, BSS-1, BSS-3, TV-20, TV-26, TV-29 and TV-30) and susceptible (AV-2, RR-17, TS-449, T-78, UP-9, UP-17, UP-26, TV-18, TV-22 and TV-23) varieties. Isozymes of peroxidase were constitutively present in healthy leaves. Upon artificial inoculation of the different varieties (T-17/1/54, BSS-3 and TV-18) a common band of R_m 0.25 in all varieties. Another band of R_m 0.75 was consistent the Darjeeling resistant variety (T-17/1/54). In Tocklai variety (TV-18) two new bands one of R_m 0.45 appeared in all inoculated extracts with 24 h and was persistent up to 48 h. The accumulation of lignin and phenolic compounds is correlated with resistance in a number of plant-pathogen interactions. The resistance response in wheat cultivar Prelude-Sr5 against an avirulent race of the stem rust fungus *Puccinia graminis* f. sp. *tritici* was correlated with rapid lignification in penetrated host cells. In tomato, resistance to the fungal pathogen *Verticillium albo-atrum* was correlated with rapid deposition of suberin and lignin in a resistant isoline than in a susceptible isoline Extensin, a cell wall-associated hydroxyproline-rich glycoprotein (HRGPs), also accumulates in the walls of a number of plant species during interactions with microbes. In parsley, HRGPs and peroxidase mRNA have been localized by *in situ* hybridization to the site of fungal infection. HRGPs are thought to act as a matrix for deposition of phenolic materials, and oxidative cross-linking of these proteins into the wall by peroxidases has been proposed to strengthen cell walls. Alternatively, during the process of cross-linking, microbes may be agglutinated and thus immobilized (Chitoor *et al.*,

1999). Because peroxidases are implicated in the deposition of cell wall-strengthening materials such as lignin, suberin, and extension, changes in activities of peroxidase during resistant and susceptible interactions have also been monitored in a number of plant-pathogenic race-specific interactions (Levine *et al.*, 1994). Increase in peroxidase activity that correlated with resistance between pathogen and their hosts were observed in rice, wheat, cotton, sugar cane and barley (Kerby and Somerville, 1989). In contrast, peroxidase enzyme activity in plants undergoing susceptible interactions was delayed or not induced within the time period studied.

Hence, in the present investigation it was demonstrated that defense enzymes in the extracts from leaves of inoculated (with *E. vexans*) tea plants were strongly stimulated by infection. The enzyme activities of both resistant and susceptible varieties following inoculation were higher than their respective controls throughout. Chitinase and β-1,3-glucanase appear as the first set of defense enzymes followed by peroxidase.

In vitro antifungal spore germination bioassays purified chitinase inhibited spore germination of *E. vexans*. Complete inhibition of spore germination was observed in solutions containing both chitinase and β-1,3-glucanase. In microtitre dish bioassays against *Glomerella cingulata* it was also observed that the intercellular fluids (ICF) from control and induced plants did not have antifungal activity on the other hand antifungal activity was obtained after adding purified chitinase and β-1,3-glucanase to the ICF. The synergistic effect, of the purified PR-proteins were further verified by using the agar-cup bioassay technique and antifungal activity of the PR-proteins was confirmed since growth of the test fungus was totally inhibited.

To substantiate results obtained from biochemical assays attempts were also made in this study to further characterize the PR-proteins of tea triggered by *E. vexans* by immunological means. PAbs raised against the purified chitinase (PR-3) and β-1,3-glucanase (PR-2) was tested for effectiveness by immunodiffusion which revealed strong precipitin bands. The antisera were further optimized by PTA-ELISA and the most ideal concentration of IgG was found to be 40 μg ml^{-1} for both antisera and were therefore

used in all immunological experiments. Initially the antisera were used to detect accumulation of the PR-proteins in tea plants (T-17/1/54, BSS-3, TV-26, TV-30 and TV-29; T-135, T-78, UP-9, TV-18 and CP-1) inoculated with *E. vexans* and accumulation of both proteins started at an early stage after inoculation and higher concentration, than uninoculated healthy control, of the induced proteins were detected at maximum levels 2 d post inoculation.

In the present study, it was observed that acidic proteins extracted from tea leaves change following inoculation with *E. vexans*, with stronger signals for PR-proteins in the compatible reaction of the susceptible varieties than in the incompatible reaction. In dot-immunobinding assays performed with acid soluble protein extracts and the chitinase probe (PAb-CHT) in the present study, antigens obtained from leaves after 24 h of inoculation (with *E. vexans*) of resistant varieties (T-17/1/54, BSS-3, TV-26, TV-30 and TV-29) showed deep violet colour and those of susceptible varieties (T-135, T-78, UP-9, TV-18 and CP-1) were of lighter intensity signifying weaker reaction in comparison to homologous ones.

Soluble proteins when run on SDS-gels revealed a band of ca. 45 kDa that was universal in all protein extracts of tea plants appearing to be constitutively present. In western blotting experiments, performed in the present study, with healthy and inoculated (with *E. vexans*) antigens. Hybridization results of these extracts, with PAb-CHT, revealed a common band ca. 14 kDa in addition to the main bands of ca. 42 and 45 kDa and two more bands of ca. 61 and 20 which were unique to the 24 h-interval however a weak signal of the ca. 42 and 14 kDa bands were sometimes revealed even after 48 h of inoculation, but never obtained 72 and 96 h post inoculation. While with the β-1,3-glucanase probe (PAb-βGLU) a band of ca. 33 kDa was observed in *E. vexans* inoculated plants and was induced to a much greater extent in resistant varieties during the course of pathogenesis when compared to the susceptible varieties. A band of ca. 25 kDa was common in all protein extracts of tea plants, which appeared on treatment with PAb-βGLU. In this investigation constitutive expression if β-1,3-glucanase and chitinase in tea plants was observed and were triggered by *E. vexans* to higher levels in resistant

varieties in comparison to susceptible varieties suggest that they might play a role in early stages of infection. Similar results were obtained by Salles *et al.* (2002) in *Colletotrichum trifolii* infected leaves of alfalfa and also are of the opinion same and also suggest that susceptible varieties may not release elicitors at an early stage of infection and thereby delay defense responses causing increased susceptibility.

Results obtained with *E. vexans* and *C. sinensis* in this study are indicative of induction of both the hydrolytic enzymes β-1,3-glucanase and chitinase in tea leaves. The accumulation of chitinase was much more pronounced in tea plants within 24 h of inoculation with *E. vexans* than in the control ones, suggesting that the enzymes might be involved in disease development. It was observed that the degree of enhancement of the defense enzymes was always greater in resistant tea varieties in comparison to susceptible ones. Yi and Hwang (1996) also opined that induction and accumulation of β-1,3-glucanases and chitinases in hypocotyl and leaf tissues of soybean following infection with *Phytophthora megasperma* f.sp. *glycinea* has a role in defense reactions against the pathogen where the induction was more conspicuous in the incompatible interactions in the late stages of the infection process (30 and 66 h after inoculation). In an interesting study Dalisay and Kuc, (1995) showed that cucumber plants exhibited enhanced resistance towards *C. lagenarium*, following inoculation of the first leaves of plants with fungal, bacterial or viral pathogens. From this they concluded that chitinase could be induced both locally and systemically in response to stress caused by biotic and abiotic agents. Induction of peroxidase enzyme activity was also correlated with systemically induced resistance in cucumber. Inoculation of the first leaf of cucumber with a hypersensitive reaction-inducing bacterium *Pseudomonas syringae* pv. *syringae*, resulted in systemic resistance and accumulation of peroxidase within 24 h. Activities of at least three acidic extracellular peroxidases increased with the appearance of systemic induced resistance. The same complement of peroxidases was also induced in response to a disease resistance-inducing heat shock treatment. In potato, peroxidase enzyme activity increased during wound-healing responses, but an anionic peroxidase was induced only in cells undergoing suberization (Hammerschmidt *et al.*, 1982; Rasmussen *et al.*, 1995) .

In the present investigation, *in vitro* antifungal activity of the purified chitinases was tested against few foliar fungal pathogens of tea. Inhibition of germination of basidiospores of *E. vexans* and some evidence of lysis of hyphal tips verify the antifungal nature of chitinase. To substantiate results, the effect of chitinase was tested against *G. cingulata* and *C. pallescens* where complete inhibition of mycelial growth was observed. Antifungal activity of chitinases such as lysis of isolated fungal cell walls or growth inhibition of some phytopathogenic fungi have been reported (Mauch *et al.*, 1988; Schlumbaum *et al.*, 1986; Sela-Buurlage *et al.*, 1993). Vannini *et al.* (1999) elucidated antifungal properties of chitinases from *Castanea sativa* against hypovirulent and virulent strains of the chestnut blight fungus, *Cryphonetrica parasitica.* They reported that chitinases from chestnut were able to inhibit the both fungal strains however higher levels of enzyme were required to inhibit the virulent ones. But on the contrary, apparent insensitivity of *Cladosporium fulvum* to tomato chitinase and β-1,3-glucanase in *in vitro* bioassays and the role of these enzymes in resistance of tomato against *C. fulvum* was demonstrated by Joosten *et al.* (1995). They also described the purification of a 27 kDa, extracellular chitinase and two intracellular chitinases (30 and 32 kDa, respectively), from *C. fulvum*-infected tomato leaves.

In the present study, induction of PR-proteins in suspension-cultured tea cells following inoculation with *Exobasidium vexans* or treatment with salicylic acid and jasmonic acid for elicitation of PR-proteins were demonstrated. An attempt was made to characterize the elicited PR-proteins biochemically as well as immunologically probing with PAb-chitinase and PAb-β-1,3-glucanase. The PR-proteins were induced in all interactions where chitinase activity was more rapid in comparison to β-1,3-glucanase activity. Interestingly the response of the varieties varied with the treatments nevertheless resistant varieties always showed higher levels of enzyme activity in comparison to the susceptible ones. Elicitation of the PR-proteins by salicylic acid was found to be significantly higher than jasmonic acid as well as biotic (*E. vexans*) elicitation in all varieties treated. To substantiate the results obtained through biochemical assays, immunoassays including indirect immunofluorescence as well as western blotting were performed. PR-proteins were detected in all treated material harvested after 24 h by

western blot which revealed three proteins with apparent molecular weights of 20, 42, and 45 kDa and two proteins of ca.33 and 25 kDa when probed with PAb-CHT and PAb-GLU respectively. Intense fluorescence was observed with both antisera following abiotic treatments. Production of phytoalexins and the hydrolases, β-1,3-glucanase and chitinase, occurs simultaneously in cultured cells induced with elicitors (Velazhahan *et al.*, 2000).

Jones and Hartley (1999) proposed that protein and phenol synthesis compete for the common limiting resource-phenylalanine and hence protein and phenolic allocations are inversely correlated. Polyphenolics are major constituents of tea leaves and as such it is expected that they would be affected by the different abiotic and biotic stresses. In case of temperature stress it was observed that there was a correlation between the inherent phenol content tea varieties and its increase following exposure to elevated temperatures. In general, in those varieties with high inherent phenol content, accumulation of phenols kept increasing till 50°C. 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. In case of tea, polyphenols are also known to vary seasonally. 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.*, 2002).

Alteration of phenol metabolism following infection has been observed in many diseases and phenolics have been implicated in the defense reaction in several instances. (Friends, 1977; Baker *et al.*, 1989; 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.

In the present study, the levels of antifungal phenolics (pyrocatechol) in leaves of resistant and susceptible tea varieties were estimated following inoculation with *E. vexans*. Host responses could be differentiated by changes in content of pyrocatechol. Biochemical responses of tea plants growing in Darjeeling hills exposed to biotic stress due to blister blight infection caused by *E. vexans* in the levels of total phenols, ortho-dihydroxy phenol and enzyme activities (PAL and POX) were studied by Chakraborty *et al.* (2002a). The involvement of phenol in the defence strategies of tea plants against other foliar fungal pathogens (*Bipolaris carbonum*, *Pestalotiopsis theae*, *Glomerella cingulata* has already been described by Chakraborty *et al.* (1995a) and Chakraborty *et al.* (1996b). In the present study, phenolic compounds could be detected in both susceptible and resistant interactions, however there were distinct differences between the interactions as analysed by HPLC. The elution pattern of the phenolic compounds revealed accumulation of two peaks, P1 and P2, at the respective average retention times of 2.756 and 3.116 min in all samples the peak heights were higher in *E. vexans* inoculated plants in comparison to their corresponding controls. Similar peaks were observed in naturally infected samples with accumulation of an additional peak (P3) in the severely infected leaf samples Another interesting finding was the 2-fold increased production of phenolics P1 and P3 in the leaves exhibiting hypersensitive responses. The ethyl acetate hydrolysate fractions of the antifungal phenolic compounds were found to be antimicrobial as tested in this investigation against *E. vexans*, *C. pallescens* and *B. megaterium.* Mansfield (2000) also opined that antimicrobial compounds can be recovered from both healthy and infected plants and that many compounds have been demonstrated to have striking activity *in vitro* against potential pathogens.

However compound I could be detected mainly from the healthy tea leaf extracts of all the four varieties tested that showed the highest fungitoxic activity in the spore germination assay. No such fungitoxic activity was evident on TLC plates in leaf extracts from susceptible varieties (T-135, TV-18, UP-9) inoculated with *E. vexans*, but traces of the inhibition zone were evident in resistant varieties (TV-30, BSS-3, BS-7A/1/76) even after 48 h of inoculation. Rf value and colour reaction of this antifungal compound

corresponded with catechin. 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).The chemical composition of tea leaves in general and catechins in particular depends on the variety geographical location, climatic condition and cultural practices. In tea products, it again varies with processing conditions (Chakraborty *et al.*, 1995c; Chakraborty and Chakraborty, 1998). Chakraborty and Saha (1994b) reported the presence of antifungal catechins in healthy leaf extracts, which, they averred, had been 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 (*E. vexans*). In a preliminary study of the HPLC analysis of catechins from healthy and blister infected tea leaves, little qualitative differences were pointed out (Chakraborty *et al.*, 2002a). In the present investigation the inherent catechins present in tea plants were stimulated during the blister blight disease resistance mechanism. Quantitative determination by HPLC of catechins in both susceptible and resistant interactions of tea leaves with *E. vexans* displayed a number of peaks that were either new or enhanced or lost in the different interactions. In naturally blister-infected leaves undergoing susceptible reactions leaf samples exhibited less isoforms of catechins than healthy leaves. Interestingly two major peaks at 13.9 and 18.7 retention times present in healthy leaves were found to be absent in blister infected leaves, which re-appeared during the hypersensitive interaction with recovery percentages of 92 and 86 respectively (Chakraborty *et al.* 2004a).

The biochemical mechanisms responsible for containment of fungal pathogens in the resistant interactions are undoubtedly manifold. Indirect evidence (Hammerschmidt and Kuc, 1982; Baker, 1989) suggests that phenolic metabolites play a role. In the present study results revealed that UV-absorbing compounds and fluorescent compounds accumulate in resistant interactions of *E. vexans* and tea. Some of which may be

metabolites from defence mechanisms activated by recognition of the presence of the pathogen.

The compound II showed positive colour reaction of phenolics with the chromogenic sprays on TLC plates at Rf 0.56 and exhibited prominent inhibition zone in TLC plate bioassay as well as inhibited markedly the spore germination of *E. vexans*. This compound was identical to an authentic pyrocatechol as determined by thin layer chromatography and UV-spectrophotometry. 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 catechol in resistant varieties increased significantly after 48h of inoculation with *E. vexans*. Concentration of this compound in healthy leaf tissue is very low. 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. Accumulation of antifungal compounds in tea leaf tissue infected with *Bipolaris carbonum* has also been discussed by Chakraborty and Saha (1994b). Resistant varieties accumulated more pyrocatechol than the susceptible varieties 2 d after inoculation with *B. carbonum*.

In the present study, greater accumulation of pyrocatechol in resistant interaction of *E. vexans* and tea varieties indicated that this may play a role in disease resistance mechanism. It has also been considered that ortho-dihydroxy 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. There are ample evidences that an increased production of phenolic compounds are involved in phytoalexin accumulation (Hammerschmidt, 1999).

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). Cell recognition has been defined as the initial event of cell-cell communication that elicits morphological, physiological and biochemical responses (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. Many of these specificity imparting molecules are glycoproteins, and fungi are known to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980). In this study, *E. vexans* was found to elicit greater amount of antifungal compound in the resistant varieties than the susceptible ones. The chemical nature of germinated spore walls of *E. vexans* was confirmed by Con A-FITC binding as glycoprotein. Results of this investigation along with those of other workers clearly demonstrate that the walls of *E. vexans* contain glycoprotein which have a role to play in the initial recognition leading to the activation of the defense mechanisms by accumulating the antifungal compounds. It has been reported that sweet potato infected by *Ceratocystis fimbriata* produce new proteins in both resistant and susceptible varieties. Similarly detection of five new isozyme bands viz., acetyl esterase acid phosphatase malate dehydrogenase, succinate dehydrogenase and peroxidase in the susceptible lines of barley after infection by *Erysiphe graminis* f.sp. *hordei* has been reported (Uritani, 1971). The interaction between *Cladosporium fulvum* and tomato has been used as a model system by Joosten and De Wit (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 uninoculated 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

band 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 d 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*.

Following pathogen attack, plants react by synthesizing a number of chemical defenses in which the phenylpropanoids occupy a central place. It has been shown in a wide variety of host-pathogen relationships (Mansfield, 2000) that the phenolic compounds may play a role in the resistance mechanisms of healthy plants. Their accumulation in infected tissues may also be implicated in the general mechanism of resistance against pathogens (Harborne, 1980). More precisely, a large body of evidence has accumulated suggesting a key role for salicylic acid in both SAR signaling and disease resistance (Raskin, 1992). Interestingly, not only is salicylic acid a potent inducer of resistance but is also capable of acting directly on fungal development as has been elaborately described by Amborabé *et al.* (2002) towards *Eutypa lata*.

Plant innate immunity is based on a surprisingly complex response that is highly flexible in its capacity to recognize and respond to the invader encountered. Pieterse *et al.* (2004) raised the question as to how plants are capable of integrating signals, induced by pathogens or insects, into defenses that are specifically directed against the attacker. The hormonal plant signals salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) emerged as major regulators. The signaling pathways that are activated by them cross-communicate, providing the plant with a great regulatory potential to fine-tune its defense reaction. The capacity of a plant to develop systemic acquired resistance (SAR) after infection with a pathogen is well known and its SA-dependent signaling pathway extensively studied.

In the present study, few abiotic inducers including mercuric chloride, di-potassium hydrogen phosphate and salicylic acid, were applied on tea plants as inducers of resistance. Slight changes in the levels of defense enzymes chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were observed that increased after inoculation. Results obtained are consistent with those of Irving and Kuc (1990) who demonstrated the local and systemic induction of peroxidase, chitinase and resistance in cucumber plants by di-potassium hydrogen phosphate. The induction of β-1,3-glucanase and peroxidase in groundnut leaves infected with mercuric chloride and *Cercospora arachidicola* a major foliar disease was reported by Roulin and Buchala (1995). Basak *et al.* (2000) and Chakraborty *et al.* (2002c) have previously shown that peroxidase and phenylalanine ammonia lyase increase when tea plants are subjected to abiotic stresses.

Most promising results were obtained with salicylic acid in the present investigation. Levels of enzymes chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase were highly induced and protection against the pathogen *E. vexans* was maximum. Yalpani *et al.* (1993) have already proved that salicylic acid is the likely endogenous regulator of localized and systemic disease resistance in plants. Gordon-weeks *et al.* (1997) also demonstrated the induction of PRs with salicylic acid resulting in accumulation of a novel PR-1 protein detectable by it's antiserum in whole leaf extracts. Dann *et al.* (1996) studied the effect of chemical treatment with INA on activities of chitinase and β-1,3-glucanase and accumulation of salicylic acid in leaves of green bean, *Phaseolus vulgaris* L and observed that accumulation of total SA was not enhanced by INA treatment. Agnieszka (2004) isolated the *gluB* gene coding for a novel apoplastic β-1,3-glucanase from potato (*Solanum tuberosum* cv. Désirée) and showed the presence of *gluB* mRNA at a very low level in mature leaves of uninfected plants whereas a considerable increase of expression was observed after infection with *Phytophtora infestans* and after treatment with salicylic acid or ethylene.

Zhu and Moore (2004) showed that natural plant defense can be induced by a nonpesticidal chemical, Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester

(BTH) an analogue of salicylic acid, to control blights and root rots of papaya (*Carica papaya* L.) seedlings. BTH also increased enzyme activity of two PR- proteins up to seven times of controls which indicates that PR genes are involved in disease resistance of papaya and that chemical induction of SAR has a potential for being used for crop protection. Induced resistance has been implicated as an alternative management strategy for *Plasmopara viticola* causing Downy mildew disease of grapevine (David *et al.*, 2004). Kassemeyer *et al.* (2004) studied the expression of PR-proteins in grapevine against biotrophic pathogen - *Plasmopara viticola* and the kinetic of transcript accumulations showed a maximum of transcripts of a *Vitis* chitinase class III and a *Vitis* glucanase within 48 h after infection. Besides, they have studied the induction of the phenyl propanoid pathway and the apposition of phenolics and callose around penetration sites: they observed papilla formation around the penetrating haustorium in incompatible interactions.

So far there are no reports on the effect of fungicides on PR-proteins of plants. Thereby, systemic fungicides such as hexaconazole and calixin, which are recommended for blister blight control, were also applied on tea plants in the present study. Defense enzyme activity increased with application of the fungicides, however level of protection of hexaconazole was higher in comparison to calixin sprayed tea plants.

A number of plants species have been reported to possess some natural substances (antimicrobial compounds) in their leaves that are toxic to many plant disease causing pathogens (Dixit *et al.*, 1983; Shetty *et al.*, 1989; Singh and Dwivedi, 1990). Antimicrobial compounds from plants have potential as fungicides themselves or in providing classes of compounds suitable for development of fungicides (Mansfield, 2000). In the present investigation attempts were made to use plant products including Biocrop (commercial biocide) and aqueous leaf extracts of *Azadirachta indica* and *Catharanthus roseus* for induction of resistance in tea plants. It was evident from the results that the phytocompounds effectively controlled the blister blight disease. Treatment of tea plants with the elicitors induced an increase in the defense enzymes chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase which rendered

the leaves resistant to *E. vexans*. Among the three treatments *A. indica* extracts were less effective in comparison to Biocrop and *C. roseus* extracts. Paul and Sharma (2002) demonstrated that extracts of *A. indica* leaves provided control of leaf stripe disease of barley. Leaf extracts of *Reynoutria sachalinensis* also provided protection against powdery mildew disease of tobacco and cucumber that is accompanied by increased activities of peroxidases, β-1,3-glucanases and phenylalanine ammonia lyase (Herger and Klingauf, 1990; Schneider and Ullrich, 1994; Daayf *et al.*, 1995). The results of the present study thus provide additional evidence that host metabolic pathways altered by treatment with plant extracts can result in an effective resistance against diseases.

The accumulation of the PR-proteins in *E. vexans*-infected tea leaf tissue, as compared to uninfected controls shown in the present study, is consistent with the biochemical results obtained in western blottings. The predominant accumulation of the PR-1 proteins over intercellular spaces, host primary cell walls, secondary thickenings of xylem vessels and papillae in infected tobacco roots bears some resemblance to the distribution of PR-P14 in tomato roots infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Benhamou *et al.* 1991). Interestingly, these two PR proteins are 60% homologous in their amino acid sequences (Lucas *et al.* 1985) and serologically related (Nassuth and Sanger, 1986). PR-1 proteins are considered to reflect activation of plant genes involved in plant defence systems (Bowles, 1990). Their accumulation over the cell walls of inter- and intracellular hyphae of *C. elegans* colonizating tobacco roots may reflect an eventual role of these proteins, in association with other PR-proteins like β-1,3-glucanase and chitnases, in directly hindering hyphal growth of the pathogens (Tahiri-Alaoui *et al.* 1993). The resemblance in the subcellular localization of defence-related proteins of known or unknown function, whatever the pathogen-plant interaction studied, suggests that these proteins may act synergistically when defence mechanisms are activated in plants.

Antimicrobial proteins (PR-2 and PR-3) extracted from *E. vexans* inoculated resistant tea varieties were electrophoretically resolved on SDS-gels, analysed by EDAS and characterized immunologically in the present study after probing with PAb-chitinase (Chakraborty *et al.*, 2004a) and PAb-β-1,3-glucanase. It was inferred that the enhanced

activity of the defence enzymes could contribute to resistance via their hydrolytic action. The likely involvement of these enzymes (PR-2 and PR-3) in active defense was verified by *in vitro* inhibition of microorganisms by preparations of each enzyme alone or in combination. Hoffland *et al.* (1995) have demonstrated through western blot analyses that in the healthy plants PR-2 is constitutively present in the intercellular fluid of leaves and PR-3 in the roots. The perthotrophic fungus *Drechslera teres* (Sacc.) Shoem also strongly induced the accumulation of PR-proteins in barley in the susceptible cv. Karat compared with the cvs. Ming and Zenit (Reiss and Bryngelsson, 1996). A polyclonal antibody against β-1,3-glucanase labeled enzyme bands only from intercellular fluid extracts. The analysis of infected roots showed no significant increase in β-1,3-glucanase activity (Rahimi *et al.*, 1996).

Induction of resistance in tea varieties against blister blight pathogen was attempted with a few abiotic inducers. Salicylic acid and hexaconazole treated leaves of *Camellia sinensis* (L.) O. Kuntze were reacted with PAb-CHT (Chakraborty *et al.*, 2004b) and PAb-βGLU followed by labelling with FITC, strong bright apple green fluorescence was observed in the epidermal and homogenously in mesophyll tissues. In healthy leaves fluorescence was limited to the epidermis and certain elements like sclereids. Additionally, in the present study, leaves of plants induced with the phytoextracts of *Azadirachta indica* and *Catharanthus roseus* were also immunolabelled separately with PAb-CHT (PR-3) and PAb-βGLU (PR-3) and FITC. Observations were motivating and revealed . . enhancement of chitinase and glucanase in the leaf tissues following induction, which was more intense in *C. roseus*, induced plants. Keefe et al. (1990) have also provided evidence that PR-2 and PR-3 proteins are mainly restricted to the epidermis in healthy tobacco leaves and accumulate in all leaf cells upon treatment that simulates pathogen stress. Similarly, PR-3 protein was confined to healthy epidermal cells in healthy potato leaves, but was homogenously distributed through mesophyll and epidermis area around infection sites by the Oomycete *Phytophthora infestans* (Garcia-Garcia *et al.*, 1994).

Ultrathin sections from *E. vexans* – inoculated tea leaves (susceptible – T-135 and resistant – T-17/1/54) embedded in LR-white resin were separately treated with PAb-CHT (PR-3) and PA*b* -GLU (PR-2) followed by gold labelling. Results of the present study showed a significant increase in the number of gold particles over host cell walls and in material of intercellular spaces between parenchyma cells, with the PAb-CHT probe, in comparison to healthy leaves while the electron-translucent zone of intercellular spaces was virtually free of labelling either in the palisade parenchyma or spongy parenchyma. Gold labelling corresponding to β-1,3-glucanase deposition both in inoculated and resistant plants was scattered. In the healthy plants PAb-βGLU showed labelling to some extent and confirmed its presence in healthy tissues. Comparing to chitinase, β-1,3-glucanase was not so strongly induced by the pathogen. Results obtained in the present investigation indicate that accumulation of the defense protein chitinase is most likely connected with *E. vexans* multiplication or induced in a systemic mode. In the healthy plants, the labelling was recorded, confirming that chitinase is constitutively present. Considering the presence of chitinase not only in the surrounding area but also distant from infected cells may be attributed that the chitinase is induced both locally and systemically.

The enzymes chitinase and β-1,3-glucanase, which depolymerize carbohydrates in fungal cells could be very useful as a first line of defense by inhibiting fungal growth. They may also indirectly activate plant defense systems by releasing elicitors from fungal cell walls. The studies correlating deposition of cell wall-strengthening materials and peroxidase activities are consistent with a role for this enzyme in defense through wall strengthening. However, in addition, the highly reactive oxygen species formed during the deposition of these compounds into the walls by peroxidase activity are likely toxic to pathogens. Alternatively, these active species may act as messengers to activate plant defense responses that contribute to resistance. Overall, defense related proteins have become very popular and usually provide a plant with resistance to stresses. Thereby varieties that are apt to intensively induce such proteins are agriculturally important and

are drawing much attention of plant breeders. The knowledge gained by such studies also provides a base for the development of novel agrochemicals for disease control and also for the development of disease-resistant crops by regulating the system in plants through genetic manipulation.

New advances in proteome analyses include the development of techniques for reproducible 2-D gel electrophoresis and for protein identification based on accurate mass spectrometry. In future, these techniques will assist in the analysis of differential protein expression and post translational modification, such as, phosphorylation and glycosylation, during plant pathogen interactions. In addition, the introduction of protein microarrays will boost the study of protein-protein interactions, and screens for substrates of protein kinases and for targets of small molecules (Martin *et al.*, 2003). Taken together, these investigations will significantly contribute to our understanding of the mechanism of plant-pathogen recognition and of the complex signaling networks mediating the activation of defense responses.

Elucidation of defense responses of tea plants triggered by *Exobasidium vexans* with special reference to pathogenesis-related (PR) proteins and their induction for development of systemic acquired resistance (SAR) have been achieved in the present investigation. To achieve this, the biochemical and immunological techniques which were undertaken in order to study compatible and incompatible interactions between tea plants and blister blight pathogen have been schematically summarized in **Figure 20A.**

At the onset, influence of environmental conditions such as temperature, relative humidity and rainfall on blister blight disease development in hills and plains were evaluated and compared for five years (2000-2004). Leaf surface damage during disease development was assessed by scanning electron microscope (SEM). The potential of enzyme linked immunosorbent assay (ELISA), Dot immunobinding assay (DIBA) and indirect immunofluorescence (IMF) for screening resistance of thirty tea varieties towards *E. vexans* were developed. Among these varieties, T-17/1/54, BS-7A/1/76 and BSS-3 exhibited very low infection percentages. On the other hand in varieties like T-78, TV-18, T-135, UP-9, BSS-2, UP-2, TV-9, UP-3, UP-17 and TV-28 the infection percentage was high. The PTA-ELISA format was employed for the detection of cross reactive antigens (CRA) shared between *E. vexans* and tea. Absorbance value (A_{405}) was always higher in susceptible host pathogen combinations than resistant ones. Among the thirty varieties tested T-78 was showed highest absorbance value while T-17/1/54 obtained least. Reciprocal cross reactions involving PAbs of tea varieties (T-17/1/54 and T-78) and antigens of host, pathogen (*E. vexans*), non pathogen (*F. oxysporum*) and non hosts (*L. leucocephala*, *O. sativa*, *G. max*) were also investigated.

Indirect immunofluorescence tests were performed in this study for cellular localization of major CRA in tea leaf tissues. Cross sections of healthy tea leaves exhibited a natural bright yellow autofluorescence mainly on the cuticle. Bright apple green fluorescence distributed mainly over epidermal cells, mesophyll tissue and xylem elements was exhibited more strongly in susceptible tea varieties (T-78, T-135 and UP-9) compared with resistant ones (T-17/1/54, BS-7A/1/76 and BSS-3) when probed with PAb of pathogen and labelled with FITC.

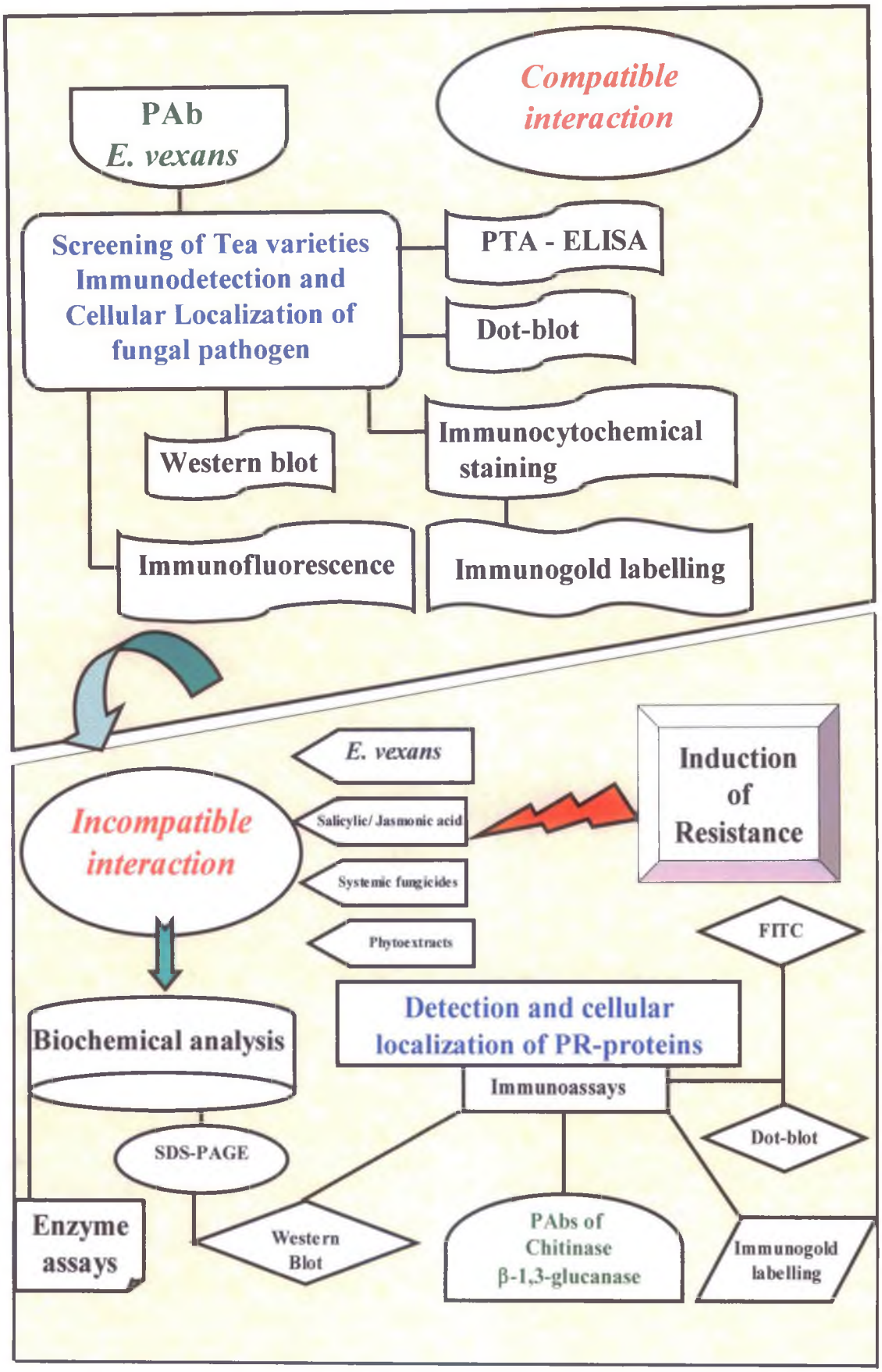


Figure 20 A. Schematic representation of methods adopted.

Ultrastructural immunocytochemical studies to locate CRA shared by *E. vexans* and *C. sinensis* through transmission electron microscopy (TEM) healthy leaf tissues of susceptible variety treated with PAb-EV showed specific localization in healthy tissue, however probe signal was less. It confirmed the co-evolution of pathogen and host for their understanding to establish successfully to cause the disease.

Early detection of *E. vexans* could be possible using PTA-ELISA formats. DIBA revealed widely differed intensities among the varieties artificially inoculated with the pathogen. A good correlation was found between the two techniques as results of DIBA gave similar level of detection as in PTA-ELISA. Immunofluorescent studies on basidiospores of *E. vexans* were performed in the present discourse using PAb-EV. The spores were not autofluorescent but nevertheless showed a general fluorescence along the spore walls with homologous antiserum demonstrating specificity of the antiserum. The chemical nature of germinated spore walls of *E. vexans* was confirmed by Con A-FITC binding as glycoprotein. Direct detection of the fungus in leaf tissues using a unique non-fluorescent stain (Fast blue BB salt) in immunoenzymatic assays, revealed intense fungal colonization following treatment with PAb of *E. vexans*. Deep blue coloured fungal colonies were clearly visible in naturally infected as well as artificially inoculated tea leaf tissues which was noticeably more in susceptible varieties (T-78 and UP-2) compared to resistant ones (TV-30). In addition immunogold labelling of *E. vexans* in blister infected tea leaf tissues was also performed and intense labelling was observed on fungal mass that had established. Ultrastructural observations on penetration, colonization and cytological modifications in blister blight infected tea leaf tissues showed penetration of the fungus. Biochemical and immunological characterization of pathogen induced (PI) protein in tea leaf tissues were explored. On probing with PAb-EV1 recognition of two proteins of approximately 58 and 15 kDa was observed only in the *E. vexans*-inoculated leaf samples, which were absent in healthy leaf samples.

Protein profiles of total soluble proteins as well as acid soluble proteins (ASP) in leaf homogenates obtained from tea plants growing under natural conditions were examined. A number of proteins of diverse molecular masses were identified. Results

obtained following analysis of total soluble proteins displayed similar proteins of ca.45, 85 and 100 kDa in most Darjeeling and Tocklai varieties and rarely in UPASI varieties. Analysis in the protein pattern of acidic extracts from thirty tea varieties revealed presence of bands mainly with molecular weights of less than 27.5 kDa. Differences were observed in number of PR-proteins in the different varieties. PR-proteins of 50-60 kDa and 10-15 kDa were detected in most varieties. An attempt was also made in this study to see whether such responses could be induced in tea plants following inoculation with the pathogen, *E. vexans*. ASP of the artificially inoculated tea varieties clearly exhibited increase in intensity of few bands and also exhibited appearance of new bands that were not revealed in uninoculated control plants. This study also provides evidence that the induced acid soluble proteins had antifungal activity. In the present study, PR-protein accumulation was measured in healthy, infected and leaves exhibiting hypersensitive reactions against *E. vexans* under field conditions. Chitinase activity was always found to be lower in the compatible interactions in comparison to resistant and healthy ones with highest accumulation in the disease free leaf samples collected from tea bushes which have acquired immunity towards environmental stress(es) as well as the leaves showing hypersensitive reaction against the pathogen (*E. vexans*) attack resulting in resistant reaction towards the pathogen. β-1,3-glucanase activity was consistently high in disease free leaf samples of all gardens with an abrupt increase in the enzyme activity in the initial stages of infection and decreased activity as the disease developed as well as in resistant reactions. The peroxidase activity displayed a constant increase during the stages of infection and the values obtained for the different stages of infection were always higher than that of healthy samples. Of major significance was the increase in enzyme activity in resistant samples. Such higher peroxidase activities may be correlated with greater resistance. Following infection with *E. vexans* two new isozymes of R_m 0.125 and R_m 0.67 were visualized in compatible interactions and interestingly a band of R_m 0.44 was observed only in leaves exhibiting hypersensitive reactions.

Time course experiments revealed enhancement in activity of the defense enzymes chitinase, β-1,3-glucanase and peroxidase in plants inoculated with *E. vexans* at specific time intervals. After 24 h of inoculation chitinase and β-1,3-glucanase displayed

a peak rise whereas peroxidase was high after 48 h of inoculation in both resistant (BS-7A/1/76, T-17/1/54, BSS-1, BSS-3, TV-20, TV-26, TV-29 and TV-30) and susceptible (AV-2, RR-17, TS-449, T-78, UP-9, UP-17, UP-26, TV-18, TV-22 and TV-23) varieties. Isozymes of peroxidase were constitutively present in healthy leaves. Upon artificial inoculation of the different varieties (T-17/1/54, BSS-3 and TV-18) a common band of R_m 0.25 appeared in all varieties. Another band of R_m 0.75 was consistent in the Darjeeling resistant variety (T-17/1/54). In Tocklai variety (TV-18) a new isozyme of R_m 0.45 appeared in all inoculated extracts with 24 h and was persistent up to 48 h.

Hence, in the present investigation it was demonstrated that defense enzymes assayed in the extracts from leaves of inoculated (with *E. vexans*) tea plants were strongly stimulated by infection. The enzyme activities of both resistant and susceptible varieties following inoculation were higher than their respective controls throughout. Chitinase and β-1,3-glucanase appear as the first set of defense enzymes followed by peroxidase. *In vitro* antifungal assays with purified chitinase markedly inhibited spore germination of *E. vexans*. However, complete inhibition of spore germination was observed in solutions containing both chitinase and β-1,3-glucanase.

Attempts were also made to further characterize the PR-proteins of tea triggered by *E. vexans* by immunological means. PAbs raised against the purified chitinase (PR-3) and β-1,3-glucanase (PR-2) was tested for effectiveness by immunodiffusion which revealed strong precipitin bands and further optimized by PTA-ELISA formats. Accumulation of the PR-proteins in tea plants (T-17/1/54, BSS-3, TV-26, TV-30, TV-29, T-135, T-78, UP-9, TV-18, HV-39, BSS-2, UP-8 and CP-1) inoculated with *E. vexans* started at an early stage after inoculation with higher concentration, than uninoculated healthy control, which was detected by PTA-ELISA.

In the present study, it was observed that acidic proteins extracted from tea leaves changed following inoculation with *E. vexans*, with stronger signals for PR-proteins in the incompatible reaction than in the compatible reaction as evidenced in DIBA. Soluble proteins when run on SDS-gels revealed a band of ca. 45 kDa that was universal in all protein extracts of tea plants appearing to be constitutively present. Western blotting experiments performed with healthy and *E. vexans*-inoculated extracts hybridized with

PAb-CHT, revealed a common band ca. 14 kDa in addition to the main bands of ca. 42 and 45 kDa and two more bands of ca. 61 and 20 which were unique to the 24 h-interval however a weak signal of the ca. 42 and 14 kDa bands were sometimes revealed even after 48 h of inoculation, but never obtained 72 and 96 h post inoculation. While with the β-1,3-glucanase probe (PAb-βGLU) a band of ca. 33 kDa was observed in *E. vexans* inoculated plants and was induced to a much greater extent in resistant varieties during the course of pathogenesis. A band of ca. 25 kDa was common in all protein extracts of tea plants, which appeared on treatment with PAb-βGLU. Constitutive expression of chitinase in tea plants was observed and were triggered by *E. vexans* to higher levels in resistant varieties in comparison to susceptible varieties suggesting that they might play a role in early stages of infection. Induction of both the hydrolytic enzymes β-1,3-glucanase and chitinase were also evident. *In vitro* antifungal activity of the purified chitinases was established against few foliar fungal pathogens (*G. cingulata* and *C. pallescens*) of tea.

Induction of PR-proteins in suspension-cultured tea cells following inoculation with *Exobasidium vexans* or treatment with salicylic acid and jasmonic acid were demonstrated. The PR-proteins were induced in all interactions where chitinase activity was more rapid in comparison to β-1,3-glucanase activity. Interestingly the response of the varieties varied with the treatments nevertheless resistant varieties always showed higher levels of enzyme activity in comparison to the susceptible ones. Elicitation of the PR-proteins by salicylic acid was found to be significantly higher than jasmonic acid as well as biotic (*E. vexans*) elicitation in all varieties treated. Western blot analysis revealed three proteins with apparent molecular weights of 20, 42 and 45 kDa and two proteins of ca.33 and 25 kDa when probed with PAb-CHT and PAb-GLU respectively. Intense fluorescence was observed with both antisera following abiotic treatments.

In the present study, the levels of antifungal phenolics (pyrocatechol) in leaves of resistant and susceptible tea varieties were determined following inoculation with *E. vexans*. Host responses could be differentiated by changes in content of pyrocatechol as analysed by UV-spectrophotometer and HPLC. This was found to be antimicrobial as tested against *E. vexans*, *C. pallescens* and *B. megaterium*. Greater accumulation of

pyrocatechol in resistant interaction of *E. vexans* and tea varieties indicated that this may play a role in the disease resistance mechanism.

Catechin could be detected mainly from the healthy tea leaf extracts of all the four varieties tested that showed the highest fungitoxic activity in the spore germination assay as well as agar cup bioassay. Inherent catechins present in tea plants were stimulated during the resistant reaction with blister blight pathogen. In naturally blister-infected leaves undergoing susceptible reactions leaf samples exhibited less isoforms of catechins than healthy leaves. Interestingly two major peaks at 13.9 and 18.7 retention times present in healthy leaves were found to be absent in blister infected leaves, which re-appeared during the hypersensitive interaction with recovery percentages of 92 and 86 respectively.

In the present study, few abiotic inducers including mercuric chloride, di-potassium hydrogen phosphate and salicylic acid, were applied on tea plants as inducers of resistance. Most promising results were obtained with salicylic acid . Defense enzyme activity increased with application of the fungicides, however level of protection of hexaconazole was higher in comparison to calixin sprayed tea plants. Plant products including Biocrop (commercial biocide) and aqueous leaf extracts of *Azadirachta indica* and *Catharanthus roseus* when treated on the tea plants showed, enhancement in the levels of defense enzymes -chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase which rendered the leaves resistant to *E. vexans.* Among the three treatments *A. indica* extracts were less effective in comparison to Biocrop and *C. roseus* extracts.

Induction of resistance in tea varieties against blister blight pathogen was attempted with a few abiotic inducers. Salicylic acid and hexaconazole treated tea leaves reacted with PAb-CHT and PAb-βGLU followed by labelling with FITC, developed strong bright apple green fluorescence in the epidermal and homogenously in mesophyll tissues. In healthy leaves fluorescence was limited to the epidermis and certain elements like sclereids. Additionally, leaves of plants induced with the phytoextracts of *Azadirachta indica* and *Catharanthus roseus* were also immunolabelled separately with PAb-CHT (PR-3) and PAb-βGLU (PR-3) and FITC. Observations were motivating and

revealed enhancement of chitinase and glucanase in the leaf tissues following induction, which was more intense in *C. roseus*, induced plants.

Ultrathin sections from *E. vexans* – inoculated tea leaves followed by gold labelling indicated accumulation of the defense protein chitinase and is most likely connected with inhibition of *E. vexans* multiplication or induced in a systemic mode. In the healthy plants, the labelling was recorded, confirming that chitinase is constitutively present. Considering the presence of chitinase not only in the surrounding area but also distant from infected cells it may be attributed that the chitinase is induced both locally and systemically.

Elaborate studies of compatible and incompatible interaction between *E.vexans* - Tea provided clues for formulation of strategies of induction of resistance. It is crystal clear that chitinase and β-1,3-glucanase play a crucial role in the initial event which might be vital for tea plants to combat the blister-blight pathogen followed by elevated levels of peroxidases and expression of phenylalanine ammonia lyase leading to formation of polyphenolic compounds including antifungal phenolics and catechins. Interplay of the antifungal PRps - PR-2, PR-3 and the enzyme peroxidase – PR- 9 that is required for final polymerization of the phenolic derivatives enables tea plants to develop a long lasting broad spectrum resistance or SAR as summarized in **Figure 20 B.**

In future, immunization of tea plants with these SAR inducing agents and also those with limited expenses such as, botanical extracts can be employed in the field to boost the natural defense system of tea plants, which would provide farmers with an additional option for low risk disease control in crops that will be cost effective, as well as ecofriendly.

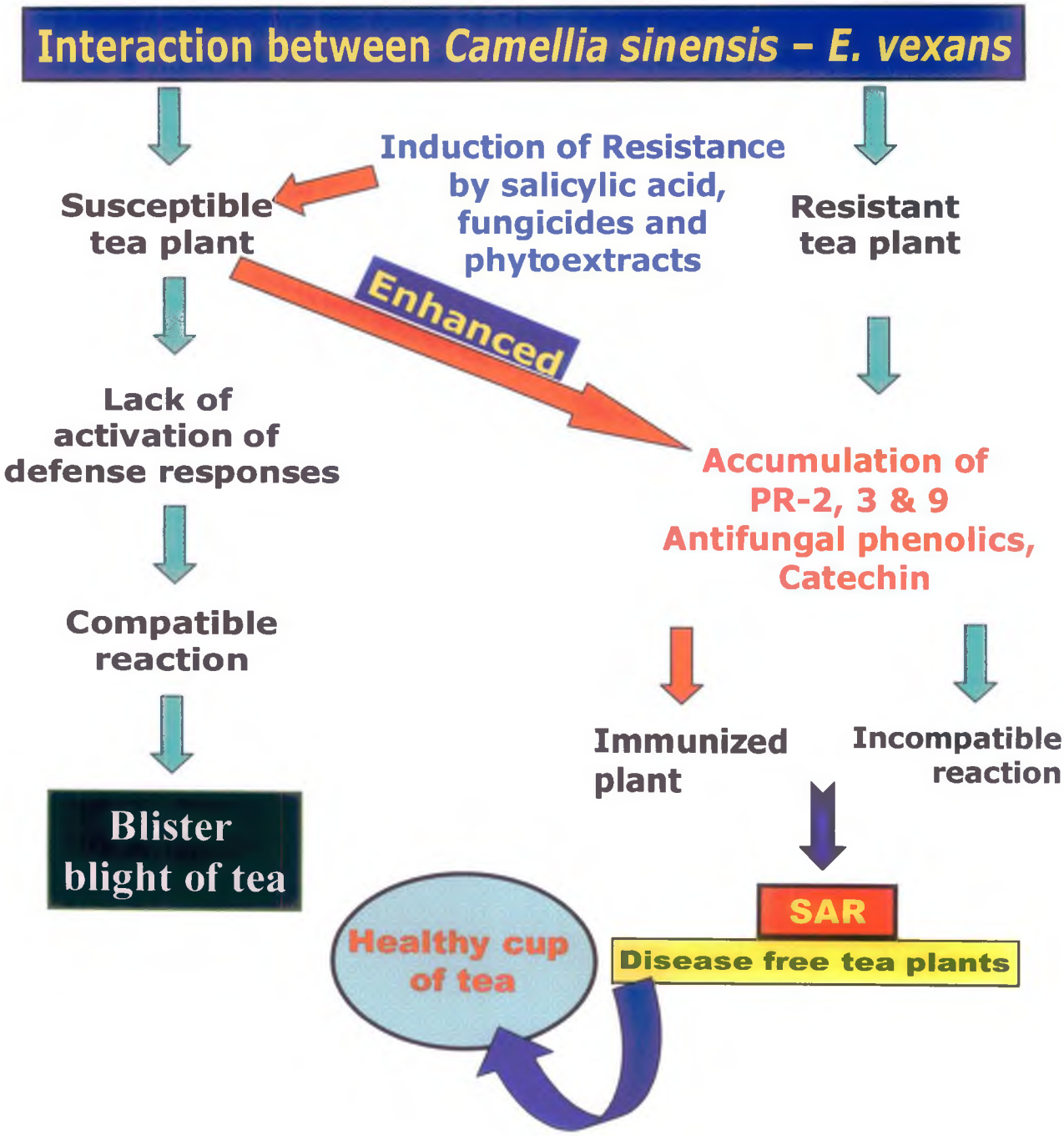


Figure 20 B. Schematic presentation of involvement of PR-proteins in developing resistance against *Exobasidium vexans*.

REFERENCES

Abeles, F. B., Bosshart, R. P., Forrence, L. E. and Habig, W. H. (1971). Preparation and purification of glucanase and chitinase from bean leaves. *Plant Physiology* **47**: 129 – 134.

Agnieszka, M., Barabasz, A., Krzymowska, M., Beiniak, B., Charzynska, M. and Hennig, J. (2004). GLUB 20-2 – A novel β-1,3-glucanase involved both in pathogenesis and development of potato (*Solanum tuberosum*). *The Joint International Workshop on PR-proteins and Induced Resistance* p.104.

Agnihothrudu, V. and Chandra Mouli, B. C. (1991). Blister blight of tea, its control and future lines of research. In: *Proceedings of the International Symposium on Tea Science.* (ed. T. Yamanishi). Shizuoka, Japan. pp 655-659.

Agrawal, G. K., Rakwal, R., Jwa, N. and Agarwal, V. P. (2002). *Oryza sativa* proteinase inhibitor gene, *OsPIN*, whose mPNA specifically accumulates in a compatible host-blast pathogen interaction. *Plant Physiological and Biochemistry* **40**: 175-182.

Alba, A. P. C. and De Vay, J. E. (1985). Detection of cross-reactive antigens between *Phytophthora infestans* (Mont.) de Bary and *Solanum* species by indirect enzyme-linked immunosorbent assay. *Phytopathologische Zeitschrift* **112**: 97-104.

Albersheim, P. and Anderson-Prouty, A. J. (1975). Carbohydrates, proteins, cell surfaces and biochemistry of pathogenesis. *Annual Review of Plant Physiology* **26**: 31-52.

Ali, Z., Smith, I. and Guest, D. I. (2002). Combinations of potassium phosphonate and Bion (acibenzolar-*S*-methyl) reduce root infection and dieback of *Pinus radiata*, *Banksia integrifolia* and *Isopogon cuneatus* caused by *Phytophthora cinnamoni*. *Australasian Plant Pathology* **29**: 59-63.

Amborabé, B., Lessard-Fleurat, P., Chollet, J. and Roblin, G. (2002). Antifungal effects of salicylic acid and other benzoic acid derivatives towards *Eutypa lata*: structure-activity relationship. *Plant Physiology and Biochemistry* **40**: 1051-1060.

Antoniw, J. F. and Pierpoint, W. S. (1978). The purification and properties of one of the 'b' proteins from virus-infected tobacco plants. *Journal of General Virology* **39**: 343 – 350.

Antoniw, J. F., Kuch, J. S. H., Walkley, D. G. A. and White, R. F. (1981). The presence of pathogenesis-related proteins in callus of Xanthi-nc tobacco. *Phytopathologische Zeitschrift* **101**: 179-184.

Antoniw, J. F., Ritter, C. E., Pierpoint, W. S. and Van Loon, L. C. (1980). Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. *Journal of General Virology* **47**: 79-87.

Baby, U. I., Premkumar, R. and Ajay, D. (2001). New trends in blister blight control. *Planters' Chronicle* **96(5-6)**: 207-210.

Baker, C. J., O'Neill, N. R. and Tomerlin, J. R. (1989). Accumulation of phenolic compounds in incompatible clone/ race interactions of *Medicago sativa* and *Colletotrichum trifolii. Physiological and Molecular Plant Pathology* **35**: 231-241.

Banerjee, B. (1993). Tea: Production and processing. Oxford & IBH, New Delhi, p336.

Barwe, S. P., Sathiyabama, M. and Jayabaskaran, C. (2002). Enhancement of β-1,3-glucanase activity and its gene expression by exogenous zeatin: involvement of calcium and staurosporine-sensitive protein kinase for signal transduction in cucumber cotyledons. *Journal of Plant Biology* **29(3)**: 337-248.

Basak, M., Sharma, M. and Chakraborty, U. (2001). Biochemical responses of *Camellia sinensis* (L.) O.Kuntze to heavy metal stress. *Jounal of Environmental Biology* **22(1)**: 37-41.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiological and Molecular Plant Pathology* **57**: 101-110.

Bednarek, S. Y. and Raikhel, N. V. (1991). The barley lectin carboxyl-terminal propeptide is a vacuolar protein sorting determinant in plants. *The Plant Cell* **3**: 1195 – 1206.

Beffa, R., Szell, M., Meuwly, P., Pay, A., Vögeli-Lange, R., Metraux, J. P., Meins, F. and Nagy, F. (1995). Cholera toxin elevates pathogen resistance and induces defense reactions in transgenic tobacco plants. *The EMBO Journal* **14**: 5753 – 5761.

Benhamou, N., Broglie, K., Broglie, R. and Chet, I. (1993). Antifungal effect of bean endochitinase on *Rhizoctonia solani:* ultrastructural changes and cytochemical aspects of chitin breakdown. *Canadian Journal of Microbiology* **39**: 318 – 328.

Benhamou, N., Grenier, J. and Asselin, A. (1991). Immunogold localization of pathogenesis-related protein P14 in tomato root cells infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici. Physiological and Molecular Plant Pathology* **38**: 237-253.

Bernasconi, P., Locher, R., Pilet, P. E., Jollès, J. and Jollès, P. (1987). Purification and N-terminal amino acid sequence of a basic lysozyme from *Parthenocissus quinquifolia* cultured *in vitro. Biochimica Biophysica Acta* **915**: 254-260.

Blake, M. S., Johnston, K. H., Russell-Jones, G. J. and Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody western blots. *Analytical Biochemistry* **136**: 175-179.

Bohlmann, H. (1994). The role of thionins in plant protection. *Critical Reviews of Plant Science* **13**: 1 – 16.

Bol, J. F., Linthorst, H. J. M. and Cornelissen, B. J. C. (1990). Plant pathogenesis-related proteins induced by virus infection, *Annual Review of Phytopathology* **28**: 113-138.

Boller T. and Metraux, J. P. (1988) Extracellular localization of chitinase in cucumber. *Physiological and Molecular Plant Pathology* **33**:11-16.

Boller, T. (1988). Ethylene and the regulation of antifungal hydrolases in plants. In: *Oxford Surveys of Plant Molecular and Cell Biology.* (eds. B. J. Miflin and H. F. Miflin) Oxford University Press, Oxford. Vol. 5. pp. 145 – 174.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance. In: *The Plant Hormone Ethylene.* (eds. A. K. Matto and J. C. Suttle) CRC Press, Boca Raton. pp 293 – 314.

Boller, T. (1995). Chemoperception of microbial signals in plant cells. *Annula Review of Plant Physiology and Molecular Biology* **46**: 189-214.

Boller, T. and Mauch, F. (1988). Colorimetric assay for chitinase. *Methods in Enzymolology* **161**: 430-435.

Boller, T. Gehri, A., Mauch, F., Vögeli, U. (1983). Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* **157**: 22 – 31.

Bowles, D. J. (1990). Defence-related proteins in higher plants. *Annual Review of Biochemistry* **59**: 873-907

Bowling, S. A., Guo, A., Cao, H, Gordon, A. S., Klessig, D. F. and Dong, X. (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *The Plant Cell* **6**: 1845 – 1857.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Brederode, F. T, Linthorst, H. J. M. and Bol, J. F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. *Plant Molecular Biology* **17**: 1117 – 1125.

Broekaert, W. F., Terras, F. R. G. and Cammue, B. P. A. (2000). Induced and preformed antimicrobial proteins. In: *Mechanisms of Resistance to Plant Diseases.*

(eds. A. Slusarenko, R. S. S. Fraser and L. C. van Loon). Kluwer Academic Publishers, The Netherlands. pp. 371-477

Broekaert, W. F., Terras, F. R. G., Cammue, B. P. A. and Osborn, R. W. (1995). Plant defensins: Novel antimicrobial peptides as components of the host defense system. *Plant Physiology* **108**: 1353 – 1358.

Broekaert, W. F., Van Parijs, J., Allen, A. K. and Peumans, W. J. (1988). Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. *Physiological and Molecular Plant Pathol.* **33**: 318 – 331.

Broekaert, W. F., Van Parijs, J., Leyns, F., Joos, H. and Peumans, W. J. (1989). A chitin-binidng lectin from stinging nettle rhizomes with antifungal properties. *Science* **245**: 1100 – 1102.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S. Mauvais, C. J. and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani. Science* **245:** 1194 – 1197.

Bryngelsson T. and Green B. (1989). Characterization of a pathogenesis-related, thaumatin like protein isolated from barley challenged with an incompatible race of mildew *Physiological Molecular Plant Pathology* **35**: 45-52.

Bryngelsson, T., Gustafsson, M., Ramos Leal, M. and Bartonek, E. (1988). Induction of pathogenesis-related proteins in barley during the resistance reaction to mildew. *Journal of Phytopathology* **123**: 193-198.

Bryngelsson, T., Sommer-Knudsen, J. Gregersen, P. L., Collinge, D. B., Ek, B. and Thordal-Christensen, H. (1994). Purification, characterization, and molecular cloning of basic PR-1-type pathogenesis-related proteins from barley. *Molecular Plant-Microbe Inter actions* **7**: 267 – 275.

Bucciaglia, P. A. and Smith, A. G. (1994). Cloning and characterization of *Tag1*, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. *Plant Molecular Biology* **24**: 903 – 914.

Burketova, L., Stillerova, K. and Feltova, M. (2003). Immunohistological localization of chitinase and β-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar beet roots. *Physiological and Molecular Plant Pathology* **63**: 47-54.

Campos, M. A., Ribeiro, S. G., Rigden, D. J., Monte, D. C. and Grossi De Sa, M. F. (2002). Putative pathogenesis-related genes within *Solanum nigrum* L. var. *americanum* genome: isolation of two genes coding for PR5-like proteins, phylogenetic and sequence analysis. *Physiological and Molecular Plant Pathology* **61**: 205-216.

Cao, H., Bowling, S. A., Gordon, A. S. and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is non-responsive to inducers of systemic acquired resistance. *The Plant Cell* **6**: 1583-1592.

Casacuberta, J. M., Puigdoménech, P. and San-Segundo, B. (1991). A gene coding for a basic pathogenesis-related (PR-like) protein from *Zea mays*. Molecular cloning and induction by a fungus (*Fusarium moniliforme*) in germinating maize seeds. *Plant Molecular Biology* **16:** 527 – 536.

Chakraborty, B. N. (1988). Antigenic disparity. In: *Experimental and conceptual plant pathology* (eds. R. S. Singh, U. S. Singh, W. M. Heiss and D. J. Weber). Oxford & IBH Publishing, New Delhi, India. pp. 477-484.

Chakraborty, B. N. and Chakraborty, U. (1998). Tea and Human Health. In: *Phytodiversification and Human Welfare* (Chapter 18) (Eds. S. K. Varma, J. V. V. Dogra and A. K. Roy) M. D. Publications, New Delhi, p.197-207.

Chakraborty, B. N. and Chakraborty, U. (2002). Immunodetection of plant pathogenic fungi. In: *Frontiers of fungal diversity in India* (eds. G. P. Rao, C. Manoharachary, D.

J. Bhatt, R. C. Rajak, and T. N. Lakhanpal). International Book Distributing Company, Lucknow. pp. 23-41.

Chakraborty, B. N. and Purkayastha, R. P. (1983). Serological relationship between *Macrophomina phaseolina* and soybean cultivars. *Physiological Plant Pathology* **23**: 197-205.

Chakraborty, B. N. and Saha, A. (1994a). Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. *Physiological and Molecular Plant Pathology* **44**: 403-416.

Chakraborty, B. N. and Saha, A. (1994b). Accumulation of antifungal compounds in tea leaf tissue infected with *Bipolaris carbonum*. *Folia Microbiologica* **39(5)**: 409-414.

Chakraborty, B. N., Basu, P., Das, R., Saha, A., and Chakraborty, U. (1995a). Detection of cross reactive antigens between *Pestalotiopsis theae* and tea leaves and their cellular location. *Annals of Applied Biology* **127**: 11-21.

Chakraborty, B. N., Chakraborty, U. and Saha, A. (1995c). Defense strategies of tea (*Camellia sinensis*) against fungal pathogens. In: *Handbook of phytoalexin metabolism and action*. (Eds) Daniel, M. and Purkayastha, R. P.; Marcel and Dekker, Inc., New York pp 485-501.

Chakraborty, B. N., Chakraborty, U., Das, R., Basu, P. and Saha, A. (1996b). Serological relationship between *Glomerella cingulata* (Stoneman) Spaulid & Schrenk and *Camellia sinensis* (L.)O. Kuntze. *Journal of Plantation Crops* **24**: 205-211.

Chakraborty, B. N., Chakraborty, U., Deb, D., Sengupta, D. and Das, J. (2002c). Immunoenzymatic detection of *Fomes lamaoensis* in soil and tea root tissues. *Proceedings of Placrosym XV* 495-503.

Chakraborty, B. N., Chakraborty, U., Saha, A., Das, R. and Basu, P. (1997). Detection and management of blister blight of tea. In: *Management of Threatening*

Plant Diseases of National Importance (eds. V. P. Agnihotri, A. K. Sarbhoy, and D. V. Singh). Malhotra Publishing House, New Delhi. pp 227-235.

Chakraborty, B. N., Chakraborty, U., Sengupta, D., Deb, D. and Das, J. (2002b). Development of immunodiagnostic kits for detection of *Ustulina zonata* in the soil and tea root tissues. *Journal of Basic Applied Mycology* **1**: 58-61.

Chakraborty, B. N., Das Biswas, R. D. and Sharma, M. (2004a). Multicomponent coordinated defence strategies in tea plants against *Helopeltis theivora* and *Exobasidium vexans*. *Journal of Plantation Crops* **32**: 289-297.

Chakraborty, B. N., Dutta, S. and Chakraborty, U. (2002a). Biochemical responses of tea plants induced by foliar infection with *Exobasidium vexans*. *Indian Phytopathology* **55(1)**: 8-13.

Chakraborty, B. N., Sharma, M. and Das Biswas, R. D. (2004b). Antimicrobial proteins of tea. *Proceedings of International Conference on O-CHA (tea) Culture and Science* 165-168.

Chakraborty, U., Basu, P., Das, R., Saha, A. and Chakraborty, B. N. (1996a). Evaluation of antiserum raised against *Pestalotiopsis theae* for the detection of grey blight of tea by ELISA. *Folia Microbiologica* **41(5)**: 413-418.

Chakraborty, U., Chakraborty, B. N. and Kapoor, M. (1993). Changes in levels of peroxidase and phenylalanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiologica* **38**: 491-499.

Chakraborty, U., Das, G. and Chakraborty, B. N. (1995b). Factors influencing spore germination, appressoria formation and disease development in *Camellia sinensis* by *Glomerella cingulata*. *Folia Microbiologica* **40(2)**: 159-164.

Chakraborty, U., Dutta, A. and Chakraborty, B. N. (2001). High temperature-induced changes in phenol metabolism in tea leaves. *Journal of Plant Biology* **28(2)**: 223-226.

Chakraborty, U., Dutta, S. and Chakraborty, B. N. (2002c). Response of tea plants to water stress. *Biologia Plantarum* **45(4):** 557-562.

Chandra Mouli, B. (1983). Blister blight of tea: Present understanding of the disease and control measures. *Journal of Plantation Crops* **11**: 14-21.

Chandra Mouli, B. (2003). Blister blight of tea: Biology, epidemiology and management. *Annual Review of Plant Pathology* **2**: 145-162.

Charudattan, R. and De Vay, J. E. (1972). Common antigens among varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species. *Phytopathology* **62(2)**: 230-234.

Chittoor, J. M., Leach, J. E. and White, F. F. (1999). Induction of peroxidase during defense against pathogens. In: *Pathogenesis-related proteins in plants* (eds. S. K. Dutta and S. Muthukrishnan) CRC Press, LLC. pp 171-193.

Christ, U. and Mösinger, E. (1989). Pathogenesis-related proteins of tomato: I. Induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlations with resistance *Physiological and Molecular Plant Pathology* **35**: 53-65.

Clarke, A. E. and Knox, R. B. (1978). Cell recognition in flowering plants. *Q. Rev. Biol.* **53**: 3-28.

Clausen, J. (1988). Immunochemical techniques for the identification and estimation of macromolecules. In: *Laboratory techniques in biochemistry and molecular biology* (Eds. R. H. Burdon and P. H. van Knippenberg). Elsevier, The Netherlands. Vol. 1 Part III. pp. 464.

Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U., Vad, K.(1993) Mini Review: Plant chitinases. *The Plant Journal* **3**: 31-40.

Cools, H. J. and Ishii, H. (2002). Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (*PAL1*) for

enhanced expression upon attack with pathogenic fungus. *Physiological and Molecular Plant Pathology* **61**: 273-280.

Cordero, M. J., Raventos, D. and San Segundo, B. (1994). Differential expression and induction of chitinases and β-1,3-glucanase in response to fungal infection during germination of maize seeds. *Molecular Plant-Microbe Interaction* 7: 23-31.

Cornelissen, B. J. C., Horowitz, J., Van Kan J. A. L., Golberg, R. B. and Bol, J. F. (1987). Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. *Nucleic Acids Research* **15**: 6799 – 6811.

Côté, F. Cutt, J. R., Asselin, A. and Klessig, D. F. (1991). Pathogenesis-related acidic β-1,3-glucanase genes of tobacco are regulated by both stress and development signals. *Molecular Plant-Microbe Interactions* **4**: 173-181.

Cruz, M. L., Gomes, V. M., Fernandes, K. V. S., Machado. O. L. T. and Xavier-Filho, J. (2002). Indentification and partial characterization of a chitinase and a β-1,3-glucanase from *Copernica cerifera* wax. *Plant Physiology and Biochemistry* **40**: 11-16.

Daayf, P., Schmidt, A. and Belanger, P. R. (1995). The effect of plant extract of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. *Plant Disease* 79: 577-580

Daayf, P., Schmidt, A. and Belanger, P. R. (1997). Evidence of phytoalexin in cucumber leaves infected with powdery mildew following treatment with leaf extracts of *Reynoutria sachalinensis. Plant Physiology* **113**: 719-727

Dalisay, R. F. and Kuc, J. A. (1995) Persistence of induced resistance and enhanced peroxidase and chitinase activities in cucumber plants. *Physiological and Molecular Plant Pathology* **47**: 315-327.

Dann, E. K., Meuwly, P., Metraux, J. P. and Deverall, B. J. (1996). The effect of pathogen inoculation or chemical treatment on activities of chitinase and β-1,3-

glucanase and accumulation of salicylic acid in leaves of green bean, *Phaseolus vulgaris* L. *Physiological and Molecular Plant Pathology* 49: 307-319.

Das, D., Mukherjee, S., Mukherjee, M., Das, A. S. and Mitra, C. (2005). Aqueous extract of black tea (*Camellia sinensis*) prevents ethanol toxicity. *Current Science* **88(6)**: 952-961.

Datta, S. K. and S. Muthukrishnan.(1999).In: S. K. Dutta and S. Muthukrishnan (eds.) *Pathogenesis-related proteins in plants*. CRC Press, LLC, p. 291

David, R., Robert, H., Philip, K. and Daryl, J. (2004). Evaluating elicitors of resistance for the management of downy mildew of grapevines. *The Joint International Workshop on PR-proteins and Induced Resistance* p.71.

Davis, B. J. (1964). Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**: 404-427

De Wit, P. J. G. M. and Spikman, G. (1982). Evidence for the occurrence of race- and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. *Physiological and Molecular Plant Pathology* **21**: 1-11.

Delaney, T., Friendrich, L. and Ryals, J. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced diseases resistance. *Proceedings of the National Academy of Sciences U.S.A.* 92: 6602-6606

Dewey, F. M., Barret, D. K., Vose, I. R. and Lamb, C. J. (1984). Immunofluorescence microscopy for the detection and identification of propagules of *Phaseolus schweinitzii* in infested soil. *Phytopathology* **74**: 291-296.

Dickinson, C. H. and Preece, T. F. (1976). *Microbiology of Aerial Plant Surfaces*. Academic Press, New York

Dixelus, C. (1994) Presence of the pathogenesis-related proteins 2, Q and S in stressed *Brassica napus* and *B. nigra* plantlets. *Physiological and Molecular Plant Pathology* **44**: 1-8.

Dixon, D. C., Cutt, J. R., and Klessig, D. F. (1991). Differential targeting of the tobacco PR-1 pathogenesis-related proteins to the extracellular space and vacuoles of crystal idioblasts. *The EMBO Journal* **10**: 1317-1324.

Dong, X., Mindrinos, M., Davis, K. R. and Ausubel, F.M. (1991). Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *The Plant Cell* **3**: 61 – 72.

Dumas, E., Lherminier, J., Gianinazzi, S., White, R. F. and Antoniw, J. F. (1988). Immunocytochemical location of pathogenesis-related b1 protein induced in tobacco mosaic virus-infected or polyacrylic acid-treated tobacco plants. *Journal of General Virology* **69**: 2687-2694.

Ellis J., Dodds, P. and Pyror, T. (2000). Structure, function and evolution of plant disease resistance genes. *Current Opinion in Plant Biology***3**: 278-284.

Ellis, B. E. (1997). Metabolism of defence and communication. In: *Plant metabolism* (eds. D. T. Dennis, D. H. Turpin, D. D. Lefebvre and D. B. Layzell). Longman Singapore Publishers (Pte) Ltd. Singapore. pp.148-160.

Emmet, R. W. and Parberry, D. G. (1975). Appressoria. *Annual Review of Phytopathology* **13**: 147-167.

Epple, P. Apel, K. and Bohlmann, H. (1997). Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum. The Plant Cell* **9**: 509 – 520.

Epple, P., Apel, K. and Bohlmann, H. (1995). An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. *Plant Physiology* **109**: 813 – 820.

Epple, P., Vignutelli, A., Apel, K. and Bohlmann, H. (1998). Differential induction of the *Arabidopsis thaliana Thi2.1* gene by *Fusarium oxysporum* f.sp. *matthiolae. Molecular Plant-Microbe Interactions* **11**: 523-529.

Ergon, A., Klemsdal, S. S. and Tronsmo, A. M. (1998) Interactions between cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat. *Physiological and Molecular Plant Pathology* **53**: 301-310.

Esteban-Carrasco, A., Zapata, J. M., Serrano-López, M., Sabater, B. and Martin, M. (2002). Purification of two peroxidase isozymes of Aloe barbadensis which oxidize *p-* coumaric acid. *Plant Physiology and Biochemistry* **40**: 127-132.

Fernandez, C., Szyperski, T., Bruyére, T., Ramage, P., Mösinger, E., and Wüthrich (1997). NMR solution structure of the pathogenesis-related protein P14a. *Journal of Molecular Biology* **266:** 576 – 593.

Fincher, G.B. (1989). Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 305 – 346.

Fischer, W., Christ, U., Baumgartner, M., Erismann, K. H. and Mösinger, E. (1989), Pathogenesis-related proteins of tomato: II. Biochemical and immunological characterization. *Physiological and Molecular Plant Pathology* **35**: 67-83.

Fraser, R. S. S. (1981). Evidence for the occurrence of the "pathogenesis-related proteins in leaves of healthy tobacco plants during flowering. *Physiological Plant Pathology* **19:** 69-76

Friend, J. (1977). Biochemistry of plant pathogens. *International Review of Biochemistry* **13**: 141-182.

Garcia-Garcia, F., Schmelzer, E. Hahlbrock, K. and Roxby, R. (1994). Differential expression of chitinase and β-1,3-glucanase genes in various tissues of potato plants. *Zeitschrift Naturforschung* **49c:** 195 – 203.

Garcia-Olmedo, F., Molina, A., Segura, A. and Moreno, M. (1995). The defensive role of non-specific lipid transfer proteins in plants. *Trends in Microbiology* **3**: 72 – 74.

Gaudet, D. A., A., Laroche, Frick, M., Huel, B. and Puchalski, B. (2003). Plant development affects the cold-induced expression of plant defence related transcripts in winter wheat. *Physiological and Molecular Plant Pathology* **62**: 175-184.

Gerik, J. S. and Huisman, O. C. (1988). Study of field grown cotton-roots infected with *Verticillium dahliae* using an immunoenzymatic staining technique. *Phytopathology* **78**: 1174-1178.

Gerik, J. S., Lommel, S. A. and Huisman, O. C. (1987). A specific serological staining procedure for *Verticillium dahliae* in cotton root tissue. *Phytopathology* **77(2)**: 261-265.

Ghosh, S. and Purkayastha, R. P. (1990). Analysis of host parasite cross-reactive antigens in relation to *Myrothecium* infection of soybean. *Indian Journal of Experimental Biology* **28**: 1-5.

Gianinazzi, S., Martin, C. and Valléé, J.C. (1970). Hypersensibilité aux virus, température et protéins solubles chez le *Nicotiana* Xanthi nc. Apparition de nouvelles macromolécules lors de la répression de la synthèse virale. *C R Academy of Science Paris* **270D**: 2883 – 2886.

Gianinazzi, S., Pratt., H. M., Shewry, P. R. and Miflin, B. J. (1977). Partial purification and preliminary characterization of soluble leaf proteins specific to virus infected tobacco plants. *Journal of General Virology* **34**: 345-351.

Gordon-Weeks, R., Sugars, J. M., Antoniw, J. F. and **White, R. F.** (1997). Accumulation of a novel protein in *Nicotiana langsdorfii* leaves in response to virus infection of treatment with salicylic acid. *Physiological and Molecular Plant Pathology* **50**: 263-273.

Gunasekhara, T. S., Paul, N. D. and Ayers, P. G. (1997). The effects of ultraviolet –B (UV-B: 290-320) radiation on blister blight disease of tea (*Camellia sinensis*). *Plant Pathology* **46**: 179-185.

Ham, K. S., Kauffmann, S., Albersheim, P. and Darvill, A. G. (1991). Host-pathogen interactions XXXIX. A soybean pathogenesis-related protein with β-1,3-glucanse activity releases phytoalexin elicitor-active heat-stable fragments from fungal cell walls. *Molecular Plant-Microbe Interactions* **4**: 545-552.

Ham, K-S, Wu S-C, Darvill, A.G. and Albersheim, P. (1997). Fungal pathogens secrete an inhibitor protein that distinguishes isoforms of plant pathogenesis-related endo-β-1,3-glucanases. *Plant Journal* **11**: 169 – 179.

Hammerschmidt, R. (1999). Phytoalexins: what have we learned after 60 years? *Annual Review of Phytopathology* **37**: 285-306.

Hammerschmidt, R. (2003). Dealing with infection: the plant responds in multiple ways. *Physiological and Molecular Plant Pathology* **62**: 191-192

Hammerschmidt, R. and Kuc, J. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. *Physiological Plant Pathology* **20**: 61-71.

Hammerschmidt, R., Nuckles, E., and Kuc, J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium. Physiological Plant Pathology* **20**: 73-82.

Harborne, J. B. (1980). Plant phenolics. In: *Encyclopedia of Plant Physiology* (eds. E. A. Bell and B. V. Charlwood) Springer-Verlag, Berlin. New series Vol. 8, pp. 329-402.

Harrison, J. G., Barker, H., Lowe, R. and Rees, E. A. (1990). Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by enzyme-linked immunosorbent assay. *Plant Pathology* **39**: 274-277.

Hawksworth, D. L., Sutton, B. C. and Ainsworth, G. C. (1983). *Ainsworth and Bisby's Dictionary of the Fungi* 7th Edition. Commonwealth Mycological Institute, Kew, Surrey, U. K. p.412.

Hayes, C. K., Klemsdal, S., Lorito, M., Di Pietro, A., Peterbauer, C., Nakas, J. P., Tronsmo, A. and Harman, G. E. (1994). Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum. Gene* **138**: 143 – 148.

Heath, M. C. (1995). Hypersensitive response-related death. *Plant Molecular Biology* **44**: 321-334.

Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology* **44**: 321-334.

Heitz, T., Geoffroy, P., Fritig, B. and Legrand, M. (1999). The PR-6 family: Proteinase inhibitors in Plant-Microbe Interactions. In: *Pathogenesis-related proteins in plants* (eds. S. K. Dutta and S. Muthukrishnan) CRC Press, LLC. pp 131-170.

Heitz, T., Segond, S., Kauffmann, S., Geoffroy, P., Prasad, V., Brunner, F., Fritig, B. and Legrand, M. (1994). Molecular characterization of a novel pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. *Molecular and General Genetics* **245**: 246 – 254.

Herger, G. and Klingauf, F. (1990). Control of powdery mildew fungi with extracts of the giant knotweed, *Reynoutria sachalinensis* (Polygoneaceae). *Mededlingen Facultet Landbouw Rijksuniversitet Gent* **55**: 1007-1014.

Hincha, D. K., Meins, F. Jr., and Schmitt, J. M. (1997). β-1,3-glucanase is cryoprotective *in vitro* and is accumulated in leaves during cold acclimation. *Plant Physiology* **114**: 1077 – 1083.

Hird, D. L., Worrall, D., Hodge, R., Smartt, S., Paul, W. and Scott, R. (1993). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to β-1,3-glucanases. *Plant Journal* **4**: 1023 – 1033.

Hoffland, E., Pieterse, C. M. J., Bik, L. and Van Pelt J.A. (1995). Induced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins *Physiological and Molecular Plant Pathology* **46**: 309-320.

Hoffland, E., Pieterse, C. M. J., Bik, L. and Van Pelt, J. A. (1995) Induced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins. *Physiological and Molecular Plant Pathology* **46**: 309-320.

Høj, P. B. and Fincher, G. B. (1995). Molecular evolution of plant β-glucan endohydrolases. *Plant Journal* 7: 367 – 379.

Huckelhoven, R., Fodor, J., Preis, C. and Kogel, K. H. (1999). Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiology* **119**: 1251-1260.

Irving, H. R. and Kuc J. A. (1990). Local and systemic induction of peroxidase, chitinase and resistance in cucumber plants by K_2HPO_4. *Physiological and Molecular Plant Pathology* **37**: 355-366.

Jach, G., Görnhardt, B., Mundy, J., Logemann, J., Pinsdorf, E. Leah, R., Schell, J. and Maas, C. (1995). Enhanced quantitative resitance against fungal diseases by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant Journal* **8**: 97 – 109.

Jangid, P. K., Mathur, P. N., Mathur, K. (2004). Role of chitinase and β-1,3-glucanase elicitation in the *Trichoderma harzianum* induced systemic resistance in *Capsicum*. *Indian Phytopathology* **57(2)**: 217-221.

Jayaraman, J. (1981). Laboratory manual in biochemistry. *New Age International Publishers, New Delhi* p.180.

Ji , C. and Kuc, J. (1997). Non-host resistance to *Collectrichum lagenarium* in pumpkin and squash is not primarily associated with β-1,3-glucanase and chitinase activities. *Physiological and Molecular Plant Pathology* **50**: 361-370.

Ji, C. and Kuc, J. (1996) Antifungal activity of cucumber β-1,3-glucanase and chitinase. *Physiological and Molecular Plant Pathology* **49**: 257-265.

Jones, C. J. and Hartley, S. E. (1999). Protein competition model of phenolic allocation. *Oikos.* **8:** 27-44.

Jongedijk, E., Tigelaar, H., Van Rockel, J. S. C., Bres-Vloemans, S. A., Dekker, I., Van den Elzen, P. J. M. Cornelissen, B. J. C. and Melchers, L. S. (1995). Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85**: 173-180.

Joosten, M. H. A. J. and De Wit, P. J. G. M. (1988). Isolation, purification and preliminary characterzation of a protein specific for compatible *Cladosporium fulvum* (syn. *Fulvia fulva*) – tomato interactions. *Physiological and Molecular Plant Pathology* **33**: 241-253.

Joosten, M. H. A. J. and De Wit, P. J. G. M. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as β-1,3-glucanases and chitinases. *Plant Physiology* **89**: 945-951.

Joosten, M. H. A. J., Bergmans, C. J. B., Meulenhoff, E. J. S. Cornelissen, B. J. C. and De Wit P. J. G. M. (1990). Purification and serological characterization of three

basic 15-kilodalton pathogenesis-related proteins from tomato. *Plant Physiology* **94**: 585 – 591.

Joosten, M. H. A. J., Verbakel, H. M., Nettekoven, M. E., Van Leeuwen, J., Van Der Vossen, R. T. M. and De Wit, P. J. G. M. (1995). The phytopathogenic fungus *Cladosporum fulvum* is not sensitive to the chitinase and β-1,3-glucanase defence proteins of its host, tomato. *Physiological and Molecular Plant Pathology* **46**: 45-59.

Kang, Z. and Buchenauer, H. (2002). Immunocytochemical localization of β-1,3-glucanase and chitinase in *Fusarium culmorum*-infected wheat spikes. *Physiological and Molecular Plant Pathology* **60**: 141-153.

Kassanis, B., Gianinazzi, S. and White, R. F. (1974). A possible explanation of the resistance of virus-infected tobacco to second infection. *Journal of General Virology.* **23:** 11-16

Kassemeyer, H., Tobias, S., Gunther, B. and Claudia, B. (2004). Early events in the host pathogen interactions and the expression of PR-proteins in grapevine. *The Joint International Workshop on PR-proteins and Induced Resistance* p.102.

Kato, M. (1989). *Camellia sinensis* L. (Tea): *In vitro* regeneration. In: *Biotechnology in Agriculture and Forestry,* Medicinal and Aromatic Plants II. (Ed. Y. P. S. Bajaj). Springer-Verlag, Berlin. Vol. 7, pp.82-98.

Kauffmann, S., Legrand, M., Geoffroy, P. and Fritig, B. (1987). Biological function of 'pathogenesis-related' proteins. Four PR-proteins of tobacco have 1,3-β-glucanase activity. *The EMBO Journal* 6: 3209 – 3212.

Kawamura, J. and Takeo, T. (1989). Antibacterial activity of tea catechin to *Streptococcus mutans. Nippon Shokukin Kogyo Gakkaishi* **36**: 463-467.

Keefe, D., Hinz, U. and Meins, F. (1990). The effect of ethylene on the cell-type specific and intracellular localization of β-1, 3-glucanase and chitinase in tobacco leaves. *Planta* **182:** 43 - 51.

Keen, N. T. and Legrand, M. (1980). Surface glycoproteins: evidence that they may function as the reace specific phytoalexin elicitors of *Phytophthora megasperma* f.sp. *glycinea*. *Physiological Plant Pathology* **17**: 175-192.

Kemp, G., Botha, A. M., Kloeppers, F. J. and Pretorius, Z. A. (1999). Disease development and β-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings. *Physiological and Molecular Plant Pathology* **55**: 45-52.

Kennedy, R., Wakeham, A. J. and Cullington, J. E. (1999). Production and immunodetection of ascospores of *Mycosphaerella brassicicola*: ringspot of vegetable crucifers. *Plant Pathology* **48**: 297-307.

Kennedy, R., Wakeham, A. J., Byrne, K. G., Meyer, U. M. and Dewey F. M. (2000). A new method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. *Appl. Environ. Microbiol.*, **66**, 2996-3000.

Kerby, K. and Somerville, S. C. (1989). Enhancement of specific intercellular peroxidases following infection of barley with *Erysiphe graminis* f.sp. *hordei*. *Physiological and Molecular Plant Pathology* **35**: 323-337.

Kessmann, H., Staub, T., Ligon, J., Oostendorp, M. and Ryals, J. (1994). Activation of systemic acquired disease resistance in plants. *European Journal of Plant Pathology* **100**: 359-369.

Kim, Y. J. and Hwang, B. K. (1994). Differential accumulation of β-1,3-glucanase and chitinase isoforms in pepper stems infected by compatible and incompatible isolates of *Phytophthora capsici*. *Physiological and Molecular Plant Pathology* **45**: 195-209.

Kim, Y. J. and Hwang, B. K. (1997). Isolation of a basic 34 kilo Dalton β-1,3-glucanase with inhibitory activity against *Phytophthora capsici* from pepper stems. *Physiological and Molecular Plant Pathology* **50**: 103-115

Kini, K. R., Vasanthi, N. S. and Shetty, H. S. (2000). Induction of β-1,3-glucanase in seedlings of pearl millet in response to infection by *Sclerospora graminicola*. *European Journal of Plant Pathology* **106**: 267-274.

Kitagawa, T., Sakamoto, Y., Furumi, K. and Ogura, H. (1989). Novel enzyme immunoassays for the detection of *Fusarium oxysporum f. sp. cucumerinum* and for general detection of various *Fusarium* species. *Phytopathology* 79(2): 162-165.

Klessig, D. F., Durner, J., Noad, R., Navarre, D. A., Wendehenne, D., Kumar, D., Zhou, J. M., Saha, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E. and Silva, H. (2000). Nitric oxide signaling in plant defense. *Proceedings of the National Academy of Sciences U.S.A.* 97 **(16)**: 8849-8855.

Ko, K. (1997). Protein synthesis in plant cells. In: *Plant metabolism* (eds. D. T. Dennis, D. H. Turpin, D. D. Lefebvre and D. B. Layzell). Longman Singapore Publishers (Pte) Ltd. Singapore. pp.148-160.

Ko, T., Lee, S., Schaefer, S. C. and Korban, S. S. (2003). Characterization of a tissue-specific and developmentally regulated β-1,3-glucanase gene family in *Prunus persica*. *Plant Physiology and Biochemistry* **41**: 955-963.

Kristensen, B. K., Bloch, H. and Rasmussen, S. K. (1999). Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens. *Plant Physiology* **120**: 501-512.

Kruger, W. M., Szabo, L. J. and Zeyen, R. J. (2003). Transcription of the defense response genes chitinase IIb, phenylalanine ammonia lyase and peroxidase is induced by the barley powdery mildew fungus and is only indirectly modulated by R genes. *Physiological and Molecular Plant Pathology* **63**: 167-178.

Kuc, J. 1982. Induced immunity to plant disease. *Biosciences* **32**: 854-859.

Kuranda, M. J. and Robbins, P. W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **266**: 19758 – 19767.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227**: 680-685.

Lange, L., Heide, M., Hobolth, L. and Olson, L. W. (1989). Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reaction by scanning electron microscopy. *Phytopathology* **79 (10)**: 1066-1071.

Lawrence, C. B. Joosten, M. H. A. J and Tuzun, S. (1996). Differential induction of pathogenesis-related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. *Physiological and Molecular Plant Pathology* **48**: 377 – 383.

Lawton, K., Uknes, S., Friedrich, L., Gaffney, T., Alexander, D., Goodman, R., Métraux, J. P., Kessmann, H., Ahl-Goy, P., Gut Rella, M., Ward, E. and Ryals, J. (1993). The molecular biology of systemic acquired resistance. In: *Mechanisms of Plant Defense Response* (eds. B. Fritig and M. Legrand). Kluwer Academic Publishers. pp 422-432.

Lee, Y. K., Hippe-Sanwald, S., Jung, H. W., Hong, J. K., Se, B. H. and Hwang, B. K. (2000). *In situ* localization of chitinase mRNA and protein in compatible and incompatible interactions of pepper stems with *Phytphthora capsici*. *Physiological and Molecular Plant Pathology* **57**: 111-121.

Leubner-Metzger, G. and Meins, F. Jr. (1999). Functions and regulation of plant β-1,3-glucanases. In: *Pathogenesis-related proteins in plants* (eds. S. K. Dutta and S. Muthukrishnan) CRC Press, LLC. pp 49-76.

Leubner-Metzger, G. Fründt, C., Vögeli-Lange, F. and Meins, F. Jr. (1995). Class I β-1,3-glucanases in the endosperm of tobacco during germination. *Plant Physiology* **109**: 751-759.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-593.

Linfield, C. A. (1993). A rapid serological test for detecting *Fusarium oxysporum* f.sp. *narcissi* in *Narcissus*. *Annals of Applied Biology* **123**: 685-693.

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Critical Reviews of Plant Science* **10**: 123 – 150.

Liu, J. J., Ekramoddoullah, A. K. M. and Zamani, A. (2005). A class IV chitinase is up-regulated by fungal infection and abiotic stresses and associated with slow-canker-growth resistance to *Cronartium ribicola* in Western White Pine (*Pinus monticola*). *Phytopathology* **95**: 284-291.

Lucas, J., Camacho-Henriquez, A., Lottspeich, F., Henschen, A. and Sanger, H. L. (1985). Amino acid sequence of the "pathogenesis-related" leaf protein p14 from viroid-infected tomato reveals a new type of structurally unfamiliar proteins. The *EMBO Journal* 4: 2745-2749

Lusso, M. and Kuc, J. (1996) The effect of sense and antisense expression of the PR-N gene for beta-1,3-glucanase on disease resistance of tobacco to fungi and viruses. *Physiological and Molecular Plant Pathology* **49**: 269-283.

Lyons, N. F. and White, J. G. (1992). Detection of *Pythium violae* and *Pythium sulcatum* in carrots with cavity spot using competition ELISA. *Annals of Applied Biology* **120**: 235-244.

Mahadevan, A. (1991). Biochemical aspects of plant disease resistance. *Post infectional defense mechanisms* Today and Tomorrow's Printers and Publishers, New Delhi. Vol.2, p.871.

Malamy J., Carr, J. P., Klessig, D. F. (1990). Salicylic acid – a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**: 1002-1004.

Mansfield, J.W. (2000). Antimicrobial compounds and resistance. The role of phytoalexins and phytoanticipins In: *Mechanisms of Resistance to Plant Diseases*. (eds. A. Slusarenko, R. S. S. Fraser and L. C. Van Loon). Kluwer Academic Publishers, The Netherlands. pp. 371-477.

Martin, G. B., Bogdanove, A. J. and Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. *Annual Review of Plant Biology* **54:** 23-61.

Mauch, F, Mauch-Mani, B. and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and β-1,3-glucanase. *Plant Physiology* **88**: 936-942.

Mauch, F. and Staehelin, L. A. (1989). Functional implications of the subecllular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. *The Plant Cell* **1:** 447-457.

Melchers, L. S., Sela-Buurlage, M. B., Vloemans, S. A., Woloshuk, C. P., Van Roekel, J. S. C., Pen, J., Van den Elzen, P. J. M. and Cornelissen, B. J. C. (1993). Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. *Plant Molecular Biology* **21**: 583 – 593.

Mercure, E. W., Kunoh, H. and Nicholson, R. L. (1995). Visualization of materials released form adhered ungerminated conidia of *Colletotrichum graminicola*. *Physiological and Molecular Plant Pathology* **46**: 121-135.

Metraux J. P., Streit, L. and Staub, T. (1988). A pathogenesis-related protein in cucumber is a chitinase. *Physiological and Molecular Plant Pathology* **33**: 1-9.

Metraux, J. P. and Boller, T. (1986). Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. *Physiological and Molecular Plant Pathology* **28**:161-169.

Metzler, M. C., Cutt, J. R. and Klessig, D. F. (1991). Isolation and characterization of a gene encoding a PR-1-like protein from *Arabidopsis thaliana. Plant Physiology* **96**: 346-348.

Moerschbacher, B., Kogel, K. H., Noll, U. and Reisener H. J. (1986). An elicitor of the hypersensitive lignification response in wheat leaves isolated from the rust fungus *Puccinia graminis* f.sp. *tritici*. I. Partial purification and characterization. *Zeitschrift fur Naturforschung* **41c**: 830-838.

Mohan, S. B. (1989). Cross-reactivity of antiserum raised against *Phytophthora fragariae* with other *Phytophthora* species and its evaluation as a genus-detecting antiserum. *Plant Patholology* **38**: 352-363.

Mondal, T. K., Bhattacharya, A., Laxmikumaran, M. and Ahuja, P. S. (2004). Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell Tissue and Organ Culture* **76**: 195-254.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 437-497.

Naderi, M. and Berger, P.H. (1997). Pathogenesis-related protein 1a is induced in potato virus Y-infected plants as well as by coat protein targeted to chloroplasts *Physiological and Molecular Plant Pathology* **51**: 41-44.

Nagahulla, S. M., Merath, N. L. and Punyasiri, P. A. N. (1996). Preliminary study of the phytoalexins produced in tea leaf in relation to the blister blight leaf disease of tea. *Sri Lanka Journal of Tea Science* **64**: 5-11.

Nakashita, H., Yoshioka, K., Yasuda, M., Nitta, Y. A., Yoshida, S. and Yamaguchi, I. (2002). Probenazole induces systemic acquired resistance in tobacco through

salicylic acid accumulation. *Physiological and Molecular Plant Pathology* **61**: 197-203.

Nassuth, A. and Sanger, H. L. (1986). Immunological relationships between "pathogenesis-related" leaf proteins from tomato, tobacco and cowpea. *Virus Research* **4**: 229-242.

Neuhaus, J. (1999). Plant chitinases. In: *Pathogenesis-related proteins in plants* (eds. S. K. Dutta and S. Muthukrishnan) CRC Press, LLC. pp 77-106.

Niderman, T., Genetet, I., Bruyére, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mösinger, E. (1995). Pathogenesis-related PR-1 proteins are antifungal: Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans. Plant Physiology* **108**: 17 – 27.

Nielsen, K. K., Bojsen, K., Roepstorff, P. and Mikkelsen, J. D. (1994a). A hydroxyproline-containing class IV chitinase of sugar beet is glycosylated with xylose. *Plant Molecular Biology* **25**: 241 – 257.

Nielsen, K. K., Mikkelsen J. D., Kragh, K. M. and Bojsen, K. (1993). An acidic class III chitinase in sugar beet: induction by *Cercospora beticola,* characterization, and expression in transgenic tobacco plants. *Molecular Plant-Microbe Interactions* **6**: 495 – 506

Nielsen, K.K., Bojsen, K., Collinge, D. B. and Mikkelsen, J. D. (1994b). Induced resistance in sugar beet against *Cercospora beticola*: induction by dichloroisonicotinic acid is independent of chitinase and β-1,3-glucanase transcript accumulation. *Physiological and Molecular Plant Pathology* **45**: 89-99.

Obanda, M. and Owuor, P. O. (1994). Effects of withering and plucking methods on the biochemical and chemical parameters of selected Kenyan tea. *Discovery and Innovation* **6**: 190-197.

Oostendorp, M., Kunz, W., Dietrich, B. and Staub, T. (2001). Induced resistance in plants by chemicals. *European Journal of Plant Pathology* **107**: 19-28.

Ori, N., Sessa, G., Lotan, T., Himmelhoch, S. and Fluhr, R. (1990). A major stylar matrix polypeptide (sp41) is a member of pathogenesis-related proteins superclass. *The EMBO Journal* **9**: 3429 – 3436.

Ornstein, L. (1964). Disc electrophoresis I. Background and theory. *Annals of the New York Academy of Sciences* **121:** 321-349.

Ouchterlony, O., Immunodiffusion and immunoelectrophoresis. In: *Handbook of Experimental Immunology* (ed. D. M. Weir). Blackwell, London, 1967, pp. 655.

Pan, S. Q., Ye, X. S. and Kuc, J. (1991). A technique for detection of chitinase, β-1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectricfocussing. *Phytopathology* **81**: 970-974.

Parent, J. G. and Asselin, A. (1984). Detection of pathogenesis related proteins (PR or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Canadian Journal of Botany **62**: 564 – 569.

Paul, P. K. and Sharma, P. D. (2002). *Azadirachta indica* leaf extract induces resistance in barley against leaf stripe disease. *Physiological and Molecular Plant Pathology* **61**: 3-13.

Payne, G. Middlesteadt, W., Desai, N., Williams, S., Dincher, S., Carnes, M. and Ryals, J. (1989). Isolation and sequence of a genomic clone encoding the basic form of pathogenesis-related protein I from *Nicotiana tabacum. Plant Molecular Biology* **12:** 595 – 596.

Payne, G., Ward, E., Gaffney, T. Goy, P.A., Moyer, M., Harper, A., Meins, F. Jr and Ryals, J. (1990). Evidence for a third structural class of β-1,3-glucanase in tobacco. *Plant Molecular Biology* **15**: 797 – 808.

Penninckx, I. A. M. A., Eggermont, K., Terras, F. R. G., Thomma, B. P. H. J., De Samblanx, G. W., Buchala, A., Métraux, J. P., Manners, J. M. and Broekaert, W. F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *The Plant Cell* **8**: 2309 – 2323.

Pieterse, C. M. J., Verhagen, B. W. M., De Vos, M., Van Oosten, V. R., Pozo, M. J., Van der Ent, S., Koornneef, A., Van Pelt, J. A., Dicke, M. and Van Loon, L. C. (2004). Rhizobacteria-induced systemic resistance and pathway cross talk to fine-tune defense. *The Joint International Workshop on PR-proteins and Induced Resistance* p.17.

Poupard, P., Parisi, L., Campion, C., Ziadi, S. and Simoneau, P. (2003). A wound-inducible and ethephon-inducible PR-10 gene subclass from apple is differentially expressed during infection with a compatible and incompatible race of *Venturia inaequalis*. *Physiological and Molecular Plant Pathology* **62**: 3-12.

Preece, T. F. and Dickinson, C. H. (1971). *Ecology of Leaf Surface Microorganisms*. Academic Press, New York

Purkayastha, R. P. and Menon, U. (1981). Factors affecting appressoria formation by Colletotrichum corchori. *Transitions of British Mycological Society* 77: 185-187.

Purkayastha, R. P. and Pradhan, S. (1994). Immunological approach to study the etiology of *Sclerotium* rot disease of ground nut. *Proceedings of National Science Academy* B-60 **2**: 157.

Radhakrishnan, B. and Baby, U. I. (2004). Economic threshold level for blister blight of tea. *Indian Phytopathology* **57**: 195-196.

Rahimi, S., Perry, R. N. and Wright, D. J. (1996) Identification of pathogenesis-related proteins induced in leaves of potato plants infected with potato cyst nematodes, *Globodera* species. *Physiological and Molecular Plant Pathology* **49**: 49-59.

Rahimi, S., Perry, R. N. and Wright, D. J. (1996). Identification of pathogenesis-related proteins induced in leaves of potato plants infected with potato cyst nematodes. *Globodera* species. *Physiological and Molecular Plant Pathology* **49**: 49-59.

Ramarethinam, S. and Rajalakshmi, N. (2004). Caffeine in tea plants [*Camellia sinensis* (L.) O. Kuntze]: *In situ* lowering by *Bacillus licheniformis* (Weigmann) Chester. *Indian Journal of Experimental Biology* **42**: 575-580.

Raskin, I. (1992). Role of salicylic acid in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**: 439-463.

Rasmussen, J. B., Smith, J. A., Williams, S., Burkhart, W., Ward, E., Somerville, S. C., Ryals, J. and Hammerschmidt, R. (1995). cDNA cloning and systemic expression of acidic peroxidases associated with systemic acquired resistance to disease in cucumber. *Physiological and Molecular Plant Pathology* **46**: 398-400.

Reddy, M. K. and Ananthanarayanan, T. V. (1984). Detection of *Ganoderma lucidum* in betelnut by the fluorescence antibody technique. *Transitions of the British Mycological Society* **82**: 559-561.

Reiss, E. and Bryngelsson, T. (1996). Pathogenesis-related proteins in barley leaves induced by infection with *Drechslera teres* (Sacc.) Shoem. and by treatment with other biotic agents *Physiological and Molecular Plant Pathology* **49**: 331-341.

Reuveni, R. and Bothma, G.C. (1985). The relationship between peroxidase activity and resistance to *Sphaerotheca fulginea* in melons. *Phytopathologische Zeitschrift* **98**: 47-52.

Richard, L. Arró, M., Hoebeke, J., Meeks-Wagner, D. R. and Van K. T. T. (1992). Immunological evidence of thaumatin-like proteins during tobacco floral different a ion. *Plant Physiology* **98**: 337 – 342.

Rivera, M. E., Codina, J. C., Francisco, O., De Vincete, A. and Perez-Garcia, A. (2002). Differential expression of β-1,3-glucanase in susceptible and resistant melon

cultivars in response to infection by *Sphaerotheca fusca*. *Physiological and Molecular Plant Pathology* **62**: 257-265.

Rodrigo, I., Vera, P., Van Loon, L. C. and Conejero, V. (1991). Degradation of tobacco pathogenesis-related proteins. Evidence for conserved mechanisms of degradation of pathogenesis-related proteins in plants. *Plant Physiology* **95**: 616-622.

Ros-Barceló, A., Pomar, F., Serrano-López, M., Martínez, P. and Pedreno, M. A. (2002). Developmental regulation of the H_2O_2-producing system and of a basic peroxidase isozyme in the *Zinnia elegans* liginifying xylem. *Plant Physiology and Biochemistry* **40**: 325-332.

Roulin, S. and Buchala, A. J. (1995). The induction of β-1,3-glucanases and other enzymes in groundnut leaves infected with *Cercospora arachidola*. *Physiological and Molecular Plant Pathology* **46**: 471-489.

Roulin, S., Xu, P., Brown, A. H. D. and Fincher G. B. (1997). Expression of specific (1→3)-β-glucanase genes in leaves of near-isogenic resistant and susceptible barley lines infected with the leaf scald fungus (*Rhynchosporium secalis*). *Physiological and Molecular Plant Pathology* **50**: 245-261.

Ryals, J., Uknes, S. and Ward. E. (1994). Systemic acquired resistance. *Plant Physiology* **104**: 1109-1112.

Salles, I. I., Blount, J. W., Dixon, R. A. and Schubert, K. (2002). Phytolaexin induction and β-1,3-glucanase activities in *Colletotrichum trifolii* infected leaves of alfalfa (*Medicago sativa* L.). *Physiological and Molecular Plant Pathology* **61**: 89-101.

Samac, D. A. and Shah, D. M. (1991). Development and pathogen-indiced activation of the *Arabidopsis* acidic chitinase promoter. *The Plant Cell* **3**: 1063-1072.

Sambandam, T. Sivaswamy, N. and Mahadevan, A. (1982). Microbial degradation of phenolic substances. *Indian Reviews of Life Science* **2**: 1-18.

Schneider, S. and Ulrich, W. (1994). Differential induction of resistacne and enhanced enzyme activities in cucumber and tobacco caused by various abiotic and biotic inducers. *Physiological and Molecular Plant Pathology* **45**: 291-304.

Sclumbaum, A., Mauch, F. Vögeli, U., Boller, T (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**: 365-367.

Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S. Van den Elzen P. J. M. and Cornelissen, B. J. C. (1993). Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. *Plant Physiology* **101**: 857 – 863.

Shetty, N. P., Kristensen, B. K., Newman, M. A., Møller, K., Gregersen, P. L. and Jørgensen, H. J. L. (2003). Association of hydrogen peroxide with restriction of *Septoria tritici* in resistant wheat. *Physiological and Molecular Plant Pathology* **62**: 333-346.

Shetty, S. A., Prakash, H. S., Shetty, H. S. (1989). Efficacy of certain plant extracts against seed borne infection of *Trichoconiella padwickii* in paddy (*Oryza sativa*). *Canadian Journal of Botany* **67**: 1956-1958.

Shillitoe, E. J. (1982). Decline in specificity of the ELISA due to storage of serum, and its recovery by adsorption with kaolin. *Journal of Virological Methods* **4**: 241-248.

Simmons, C. R. (1994). The physiology and molecular biology of plant 1,3-β-D-glucanase and 1,3,1,4-β-D-glucanase. *Critical Reviews of Plant Science* **13**: 325-387.

Singh, R. K. and Dwivedi, R. S. (1990). Fungicidal properties of neem and blue germ against *Sclerotium rolfsii* Sacc. A root rot pathogen of barley. *Acta Botanica* **18**: 260-262.

Siqueira-Júnior, C. L., Fernandes, K. V. S., Machado, O. L. T., Cunha, M., Gomes, V. M., Moura, D. and Jacinto, T. (2002). 87 kDa tomato cystatin exhibits properties

of a defense protein and forms protein crystals in prosystemin overexpressing transgenic plants. *Plant Physiological and Biochemistry* **40**: 247-254.

Somssich, I. E. (1994). Regulatory elements governing pathogenesis-related (PR) gene expression. In: *Plant Promoters and Transcription Factors* (eds. L. Nover). Springer Verlag, Berlin. pp.163-179

Somssich, I. E., Schmelzer, E., Kawalleck, P. and Hahlbrock, K. (1988). Gene structure and *in situ* transcript localization of pathogenesis-related protein 1 in parsley. *Molecular and General Genetics* **213**: 93-98.

Staehelin, C. Granado, J., Müller, J. Wiemken, A., Mellor, R. B., Felix, G., Regenass, M., Broughton, W. J. and Boller, T. (1994). Perception of *Rhizobium* nodulation factors by tomato cells and inacitivation by root chitinases. *Proceedings of National Academy of Sciences U.S.A.* **91**: 2196-2200.

Sticher, L., Hinz, U., Meyer, A. D. and Meins, F. Jr. (1992). Intracellular transport and processing of a tobacco vacuolar β-1,3-glucanse. *Planta* **188**: 559 – 565.

Sugha, S. K. (1997). Perpetuation and seasonal build up of *Exobasidium vexans*, causal agent of blister blight of tea in Himachal Pradesh. *Tropical Sciences* **37**: 123-128.

Svircev, A. M., Jeng, R. S. and Hubbes, M. (1988). Detection of cerato-ulmin on aggressive isolates of *Ophiostomi ulmi* by immunocytochemistry and scanning electron microscopy. *Phytopathology* **78(3)**: 322-327.

Tahiri-Alaoui, A., Dumas-Gadot, E. and Gianinazzi, S. (1993). Immunocytochemical localization of pathogenesis-related PR-1 proteins in tobacco root disease tissues infected *in vitro* by the black root rot fungus *Chalara elegans. Physiological and Molecular Plant Pathology* **42**: 69-82

Terras, F. R. G., Goderis, I. J., Van Leuven, F., Vanderleyden, J., Cammue, B. P. A. and Broekaert, W. F. (1992). *In vitro* antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. *Plant Physiology* **100**: 1055-1058.

Thomma, B. P. H. J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. and Broekaert, W. F. (1998). Separate jasmonate-dependent and salicylate-dependent defense response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogen. *Proceedings of the National Academy of Science U.S.A.* **95**: 15107 – 15111.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences U. S. A.* **76** (**9**): 4350-4354.

Tucker, S. L. and Talbot, N. J. (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annual Review of Phytopathology* **39**: 385-417.

Tyagi, M. Kayastha, A. M. and Sinha, B. (2001). Induction of chitinase and β-1,3-glucanase in resistant and susceptible lines following infection with *Alternaria triticina*. Journal of Biochemistry and Biotechnology **10**: 71-74.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. R. and Ryals, J. A. (1992). Acquired resistance in *Arabidopsis*. *The Plant Cell* **4**: 645 – 656.

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N. and Ishida, I. (1997). The structure and function of a soybean β-glucan-elicitor-binding protein. *Proceedings of the National Academy of Sciences U.S.A.* **94**: 1029-1034.

Uritani, L. (1971). Protein changes in diseased plants. *Annual Review of Phytopathology* **9**: 2-11.

Van den Elzen, P. J. M., Jongedijk, E., Melchers, L. S. and Cornelissen, B. J. C. (1993). Virus and fungal resistance: from laboratory to field. *Philosophical Transactions of the Royal Society of London B* **342**: 271-278.

Van Etten, H. D. (1973). Differential sensitivity of fungi to pisatin and to phaseollin. *Phytopathology* **63**: 1477-1482.

Van Kan, J. A. L., Joosten, M. H. A. J., Wagemakers, C. A. M. van den Berg-Velthius, G. C. M. and De Wit, P. J. G. M. (1992). Differential accumulation of mRNA encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum. Plant Molecular Biology* **20:** 513-527.

Van Loon, L. C. (1975). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN" IV. Similarity of qualitative changes of specific proteins after infection with different viruses and their relationship to acquired resistance, *Virology,* **67:** 566-575

Van Loon, L. C. (1977). Induction by 2-chloroethylphosphonic acid of viral-like lesions, associated proteins, and systemic resistance in tobacco. *Virology* **80**: 417-420.

Van Loon, L. C. (1985). Pathogenesis-related proteins. *Plant Molecular Biology* **4**: 111-116.

Van Loon, L. C. (1997). Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* **203**: 753-756.

Van Loon, L. C. (1999). Occurrence and properties of plant pathogenesis-related proteins; In: *Pathogenesis-related proteins in plants* (eds. S. K. Dutta and S. Muthukrishnan) CRC Press, LLC. pp 1-19

Van Loon, L. C. and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var "Samsum" and "Samsun NN" II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* **40**: 199 –211.

Van Loon, L. C. and Van Strien, E. A. (1999). Mini Review: The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology* **55**: 85-97.

Van Loon, L. C., Plerpoint, W. S., Boller, T. and Conejero, V. (1994). Recommendations for naming plant pathogenesis-related protein. *Plant Molecular Biology Reporter* **12**: 245 – 264.

Vannini, A., Caruso, C., Leonardi, L., Rugini, E., Chiarot, E., Caporale, C. and Buonocore, V. (1999) Antifungal properties of chitinases from *Castanea sativa* against hypovirulent strains of the chesnut blight fungus *Cryphonectria parasitica. Physiological and Molecular Plant Pathology* **55**: 29-35.

Varghese, J. N., Garrett, T. P.J ., Colman, P. M., Chen, L., Hoj, P. B. and Fincher, G. B. (1994). Three-dimensional structures of two plant β-glucan endohydrolases with distinct substrate specificities. *Proceedings of the National Academy of Science U.S.A.* **91**: 2785 – 2789.

Velazhahan, R., Samiyappan, R. and Vidyasekharan, P. (2000). Purification of an elicitor-inducible antifungal chitinase from suspension-cultured rice cells. *Phytoparasitica* **28(2)**: xxx-xxx. http://www.phytoparasitica.org

Venkata Ram, C. S. (1979). Factors influencing germination, penetration and lesion development on tea leaves following deposition of *Exobasidium vexans* spores. In: *Proceedings of PLACROSYM II* pp.193-204.

Venkata Ram, C. S. and Mouli, B. C. (1984). Interaction of dosage spray interval and fungicide action in blister blight disease control in tea. *Planters' Chronicle* 79: 11-16.

Vierheilig, H, Alt, M., Neuhaus, J. M., Boiler, T. and Wiemken, A. (1993). Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mossae. Molecular Plant-Microbe Interactions* **6**: 261-264.

Vögeli-Lange, R., Fründt, C., Hart, C. M., Beffa, R., Nagy, F. and Meins, F. Jr. (1994). Evidence for a role of β-1,3-glucanase in dicot seed germination. *Plant Journal* **5**: 273 – 278.

Wakeham, A. J. and White, J. G. (1996). Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiological and Molecular Plant Pathology* **48**: 289-303.

Wakeham, A. J., Kennedy, R., Byrne, K. G., Keane, G. and Dewey, F. M. (2000). Immunomonitoring air-borne fungal plant pathogens: *Mycosphaerella brassicicola*. *The EPPO Bulletin* **30**: 475-480.

Wang, Z. N. (1991). Chinese famous teas and their characteristic constituents bioformation. *World Tea, International Symposium on Tea Science* Shizuoka, Japan. pp. 23-33.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J. P. and Ryals. J. A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell* **3**: 1085 – 1094.

Warnock, D. W. (1973). Use of immunofluorescence to detect mycelium of *Alternaria*, *Aspergillus* and *Penicillium* in barley grains. *Transactions of British Mycological Society* **61(3)**: 547-552.

Wasternack, C. and Parthier, B. (1997). Jasmonate-signalled plant gene expression, *Trends in Plant Science* **2**: 302-307.

Werres, S. and Steffens C. (1994). Immunological techniques used with fungal plant pathogens: aspects of antigens, antibodies and assays for diagnosis. *Annals of Applied Biology* 125: 615-643.

White, J. G., Lyons, N. F., Wakeham, A. J., Mead, A. and Green, J. R. (1994). Serological profiling of the fungal genus *Pythium*. *Physiological and Molecular Plant Pathology* **44**: 349-361.

White, R. F., Reynolds, H. C., Sugars J. M. and Antoniw, J. F. (1996). The suppression of mannitol induced pathogenesis-related protein accumulation in tobacco by antibiotics. *Physiological and Molecular Plant Pathology* **49**: 389-393.

Wubben, J. P., Lawrence, C. B. and De Wit P. J. G. M. (1996) Differential induction of chitinase and β-1,3-glucanase gene expression in tomato by *Cladosporium fulvum* and its race-specific elicitors. *Physiological and Molecular Plant Pathology* **48**: 105-116.

Yalpani, N. Leon, J., Lawton, M. A. and Raskin I. (1993). Pathway of salicylic acid biosynthesis in healthy and virus inoculated tobacco. *Plant Physiology* **103**: 315-321.

Yang, S. L. and Ellingboe, A. H. (1972). Cuticle layer as a determining factor for the formation of mature appressoria of *Erisyphe graminis* on wheat and barley. *Phytopathology* **62**: 708-714.

Ye, X. S., Pan, S. Q. and Kuc, J. (1990). Association of pathogenesis-related proteins and activities of peroxidase, β-1,3-glucanase and chitinase with systemic induced resistance to blue mold of tobacco but not to systemic tobacco mosaic virus. *Physiological and Molecular Plant Pathology* **36**: 523-531.

Ye, Z. H. and Varner, J. E. (1991). Tissue-specific expression of cell wall proteins in developing soybean tissues. *The Plant Cell* **3**: 23-37

Yi, S. Y. and Hwang, B. K. (1996). Differential induction and accumulation of β-1,3-glucanase and chitinase isoforms in soybean hypocotyls and leaves after compatible

and incompatible infection with *Phytphthora megasperma* f.sp. *glycinea*. *Physiological and Molecular Plant Pathology* **48**: 179-192.

Yoshikawa, M., Tsuda, M. and Takeuchi, Y. (1993). Resistance to fungal disease is transgenic tobacco plants expressing the phytoalexin elicitor-releasing factor, β-1,3-endoglucanase, from soybean. *Naturwissenschaften* **80**: 417 – 420.

Young, C. S. and Andrews, J. H. (1990). Inhibition of pseudothecial development of *Venturia inaequalis* by the basidiomycete *Athelia bombacina* in apple leaf litter. *Phytopathology* **80**: 536-542.

Zhu, Q., Maher, E. A., Masoud, S., Dixon, R. A. and Lamb, C. J. (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *BioTechnology* **12**: 807-812.

Zhu, Y. J. and Moore, P. H. (2004). Pathogenesis-related (PR) proteins induced by benzothiadiazole (BTH) in *Carica papaya* improved resistance responses to *Phytopthora palmivora*. *The Joint International Workshop on PR-proteins and Induced Resistance* p.68.