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Pathogenesis-related proteins of tea triggered by *Exobasidium vexans*

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Abstract
The defense strategy of tea plants against *Exobasidium vexans* are multifold and include accumulation of pathogenesis-related (PR) proteins. A study on the association of defense enzymes with resistance in tea plants triggered by *E. vexans* revealed significant changes in the level of β-1.3-glucanase (PR 2) and chitinase (PR 3) exhibiting antimicrobial activity. Accumulation of defense proteins differed in time and magnitude. Time course studies points towards accumulation of PR-2 and PR-3 in the early hours, PR-9 later on and finally the antifungal metabolites that confer resistance to the plants. Treatment with salicylic acid (SA) stimulates a multicomponent defense response in tea leaves which was confirmed by immuno-localization of PR 2 and PR 3 in tea leaf tissues following induction of resistance. Induction of PR-3 in suspension-cultured tea cells following SA treatment was confirmed immunologically using antibody probes (PAb-chitinase). Subcellular localization of PR-3 and PR-2 in tea leaves were also confirmed by indirect immunogold labeling. Marked increase in frequency of gold particles following elicitation by SA treatment was evident. Cell defense responses associated with systemic acquired resistance induced by SA against *E.vexans* has been discussed in relation to the possible role of PR-proteins in immunizing tea plants.

Keywords: Blister blight, PR- 2, PR -3, defense enzymes, immunogold labeling.

Tea [*Camellia sinensis* (L.) O. Kuntz], the preferred beverage in the world is at a threat of quality deterioration due to the persistent attack of pests and pathogens it faces all year around. When tea plant succumbs to blister blight (Plate1,figA) caused by *Exobasidium vexans* (Plate 1,fig.B), crop loss is severe and tea produced from such leaves is of inferior quality. The disease become disastrous on tea recovering from pruning which subsequently alleviates the quality of beverage production drastically and thus the tea industry suffer enormously crop loss (Chandra Mouli,2003). Higher plants protect themselves from biotic stresses by producing wide array of defense compounds, which are either induced or preformed. One of the way 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 (Chakraborty and Sharma, 2008). In the present investigation, biochemical and immunological studies have been made to elucidate defense responses of tea plants triggered by E. vexans with special reference to the involvement of PR protein, their induction for development of SAR and their immunocytochemical localization in leaf tissues.

Materials and Methods

Plant material. Nine tea varieties (BS-7A/76, BSS-1, BSS- 3, UP- 26, TV- 20, TV- 22, TV- 26, TV- 29 and TV- 30) were collected from Tea Germplasm Bank, Department of Botany, University of North Bengal. Healthy and naturally blister-blight infected tea leaf samples were collected from Tea Estates of Darjeeling hills.

Fungal material. *E. vexans* is an obligate pathogen so its growth in artificial medium is not possible. Tea shoots with well developed sporulating lesions of blister blight were freshly collected from the garden and dipped in conical flask containing 2% sucrose solution, kept at 25 °C for the collection of basidiospores and stored at 4 °C for further use.

Artificial inoculation and disease assessment. Intact tea plants were artificially inoculated with basidiospores of *E. vexans* following the method of Chakraborty et al. (1997). The spore suspension was brushed on either surface of the first, second and third leaves of each plants and incubated at 23 ± 2 °C for 16h photoperiod. Disease symptoms developed within 11 d of inoculated plants and percentage infection in each case was determined on the basis of the number of infected shoots out of 50 shoots examined at random, as suggested by Venkataram (1979). 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).

Elicitor treatment. For induction of resistance in tea plants against E. vexans, salicylic acid (Himedia) was used. Plants were treated with salicylic acid (15mM), supplemented with Tween -20, thrice at 7-d-intervals

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separately. Control plants were sprayed with distilled water with Tween-20.

Production of polyclonal antibodies. Polyclonal antibodies were raised against chitinase (CHT) and β-1,3 glucanase (GLU) after purification, in white male rabbit.

Extraction and estimation of defense enzymes

Chitinase (EC 3.2.1.14.). Chitinase was extracted in 0.1M sodium-citrate buffer pH 5 with sea sand and insoluble PVPP in a mortar with pestle on ice as suggested by Boller and Mauch (1988).Enzyme activity was measured at A585 nm and expressed as mg GlcNAc g^{-1}tissue h^{-1}

β-1,3-glucanase (EC 3.2.1.39). β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method as suggested by Pan et al. (1991). Enzyme activity was determined at A500 nm and expressed as µg glucose g^{-1} tisstie min^{-1}.

Immunofluorescence. Indirect immunofluoscence staining of tea leaf tissues using PAb of CHT labeled with anti-rabbit goat IgG conjugated with FITC was performed following the protocol as described by Chakraborty and Chakraborty (2003). Observations were made under UV light in a microscope (Leitz) equipped with I3 filter ideal for FITC and photographed with a Leica Wild MPS 48 camera on Kodak film (800ASA).

Western blotting. Proten samples were electrophoresed on 10% SDS-gel and electrotransfereed to nitrocellulose sheets using semi-dry Trans-blot unit (BioRad,USA) and probed with PAb-CHT. Hybridization was done using alkaline phosphatase conjugate and 5-Bromo-4-chloro-3 -indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins were visualized as violet colored bands on nitrocellulose sheets.

Electron Microscopy . Transmission electron microscopy of healthy as well as salicylic acid treated tea leaves was performed following the method of Tahiri -Alaoui et al (1993). After fixing in phosphate buffer glutaraldehyde for overnight the samples were embedded in LR White and polymerized at 60°C for 2 days. Ultra-thin sections of the samples were cut with a diamond knife and collected on Pioliform coated 300 mesh nickel grid for immunogold labeling with antirabbit IgG (whole molecule) gold conjugate (10nm). After contrasting with uranyl acetate and lead citrate, the sections were examined with a Tecnai 12 Bio Twin transmission electron microscope (Philips, The Netherlands) at 75 kV with Megaview III soft Imaging System.

Results

Screening of resistance of tea towards E. vexans. Nine tea varieties (BS-7A/76, BSS 1, BSS 3, UP 26, TV 20, TV 22, TV 26, TV 29 and TV 30) were screened for resistance towards E. vexans. Results have been presented in Table 1. Darjeeling varieties BS-7A/1/76 were highly resistant. Among the UPASI varieties BSS-3 was found to be resistant while others were found to be either moderately susceptible (MS) or moderately resistant (MR). Most of the Tocklai varieties were either moderately susceptible or moderately resistant.

Table 1: Incidence of blister blight caused by *E. vexans* on artificially inoculated tea plants

Ten varieties		Percentage Infection (%)[a]	Disease score[b]
Darjeeling	BS-7A/1/76	15.9 ± 0.04	R
UPASI	BSS-1	21.3 ± 0.06	MR
	BSS-3	18.2 ± 0.07	R
	UP-26	46.5 ± 0.07	MS
Tocklai	TV-20	22.4 ± 0.04	MR
	TV-22	44.3 ± 0.06	MS
	TV-26	38.2 ± 0.14	MR
	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 %).

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. 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 β chitinase, and β-1,3-glucanase upon artificial inoculation with E. vexans under controlled conditions was recorded for nine different varieties. Control plants were also maintained under identical conditions. 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, 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 2). It was seen that the chitinase activity was more or less constant at the control value.

β-1,3-glucanase activity show a rapid rise in the resistant varieties in comparison to susceptible ones (Table 3). Enzyme activity started to increase within 24 h of inoculation and was maximum at this time interval.

Table 2: 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	I	C	I	C	I	C	I
BS-7A/I/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

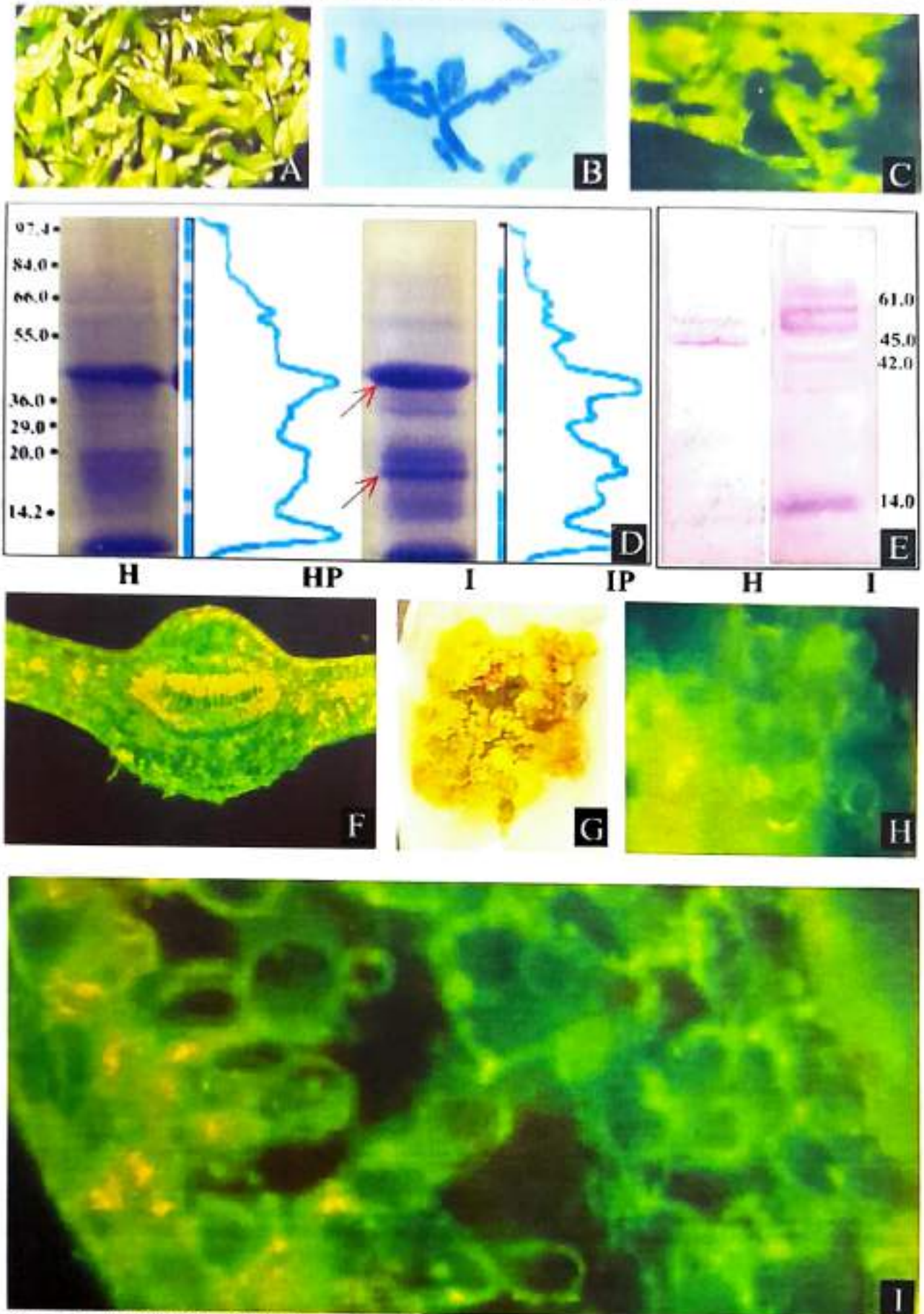
*The results presented are the means calculated for five experiments

Table 3: 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	I	C	I	C	I	C	I
BS-7A/I/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

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 (72h) after a fall in peak value (24h). Further comparison showed that the resistant inoculated plants attained the same level of enzyme after 48 h, as that attained by susceptible varieties after 24h. 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



(Plate I. fig. A-I): (A) Blister blight disense of tea; (B) basidiospores of E. vexans bright field (40x); (C) *E. vexans* treated with PAb - Chitinase labeled with FITC; (D) SDS-PAGE analysis and EDAS profiles of acid soluble tea leaf proteins of healthy and *E. vexans* inoculated; (E)Western blot analysis of PR-3 in tea leaf tissues triggered by *E. vexans*; Fluorescent antibody staining of tea leaf tissues (F& I) and friable calli (G & I) treated with salicylic acid and probed with PAb Chitinase.

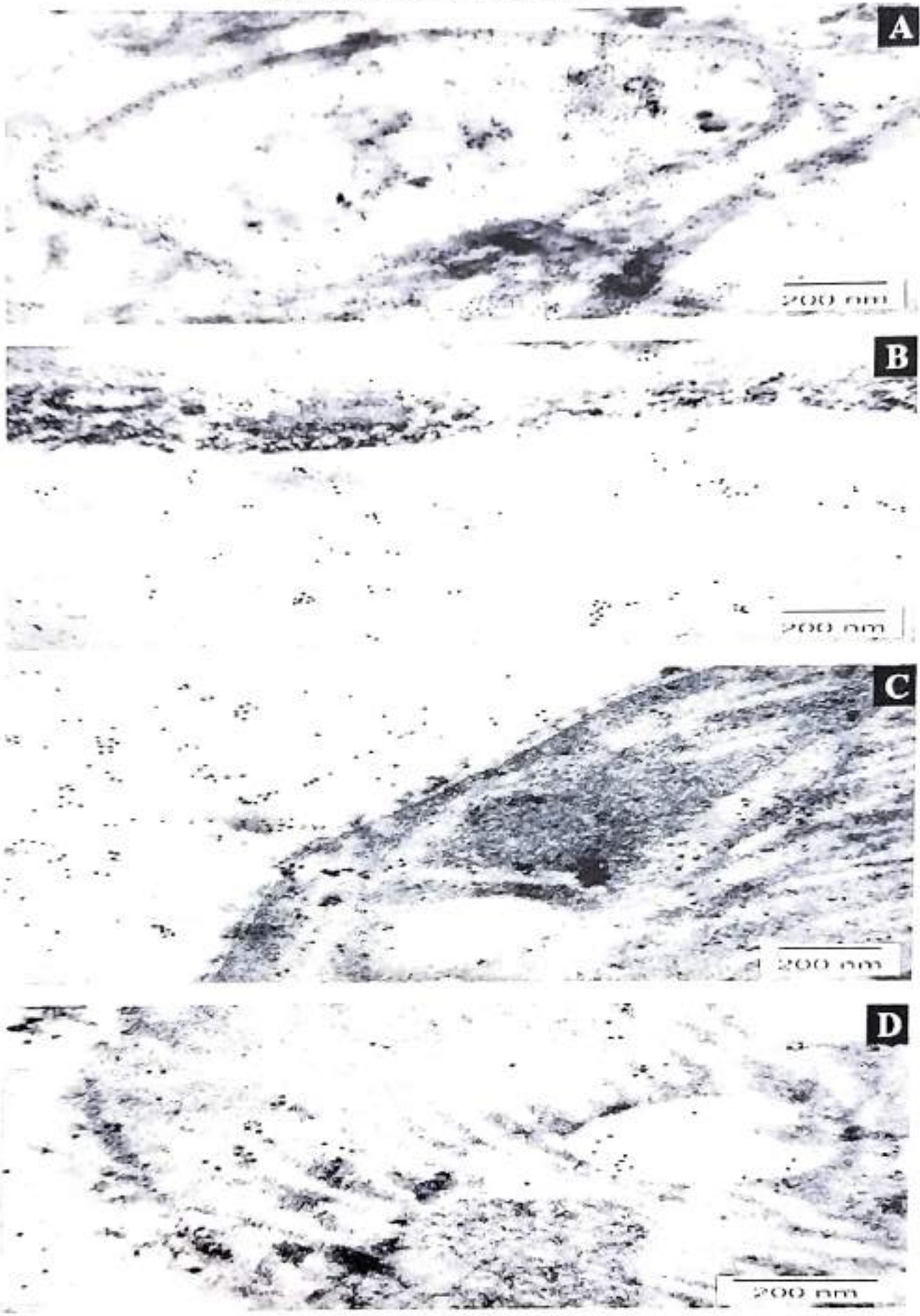


Plate 2 Fig. A-D: Transmission electron micrographs of immunogold labeled tea leaf tissue treated with SA and probed with Pab of chitinase (A&B) and β, 1-3 glucanase (C&D)

Table 4: Dot immunobinding reactions of PR-2 and PR-3 protein of tea varieties triggered by *E.vexans*

Varieties	PAb-CHT		PAb-βGLU	
	Healthy	Inoculated	Healthy	Inoculated
BS/7A/76	+ + +	+ + + + +	+ +	+ + + + +
BSS-3	+ + +	+ + + + +	+	+ + + +
TV-26	+ +	+ + + +	+	+ + +
TV-30	+ +	+ + + +	+	+ + +
TV-29	+	+ + +	+	+ +
TV-22	+	+ +	+	+ +
UP-26	+	+ +	+	+ +
TV-20	+	+ +	+	+ +
BSS-1	+ +	+ + +	+	+ +

IgG concentration 40 mg 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

respective controls throughout. Association of PR proteins in this host-pathosystem reveals that disease resistance in tea following attack by E. vexans is a culminated action of chitinase (CHT) and β-1,3-glucanase (bGLU).

Polyclonal antibodies (PAbs) raised against purified β-1,3-glucanase (PR-2) and chitinase (PR-3) were used in various immunological formats. 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 its own antigen. The titre values of PAbs were checked after each bleeding and only those showing strong precipitin bands were used for subsequent immunoassays. In vitro treatment of basidiospores of *E.vexans* with chitinase enzymes (PR-3) purified from leaf samples and labelled with PAb CHT and FITC gave clear inhibition of spore wall (Plate 1,fig. C).

Acid soluble protein extracts obtained from tea leaves of control and *E. vexans* inoculated tea plants of resistant and susceptible varieties were resolved in SDS-PAGE (Plate 1,fig.D). These were reacted with PAb-CHT and PAb-GLU in dot immunobinding assays (Table-4). Results revealed that the antigens from leaves after 24 h of inoculation of resistant varieties (BS/7A/76, BSS-3, TV-26, TV-30 and TV-29) showed deep violet colour whereas susceptible varieties showed weaker reactions. When healthy and *E.vexans* inoculated proteins were probed with PAb of CHT in western blot analysis, new band yielded following challenge inoculation with *E.vexans* in resistant varieties (Plate 1,fig. E).

Induction of resistance towards E. vexans in tea plants and associated changes in defense enzymes. An attempt was made to induce resistance in tea variety (UP -26) that has been established to be susceptible towards *E. vexans*. The plant was treated with salicylic acid (abiotic elicitor), 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. Plants were sprayed with 15 mM salicylic acid for 7 d and challenge inoculated with *E. vexans*. Samples were harvested at 24, 48 and 72 h. The extractable defense enzymes - chitinase and β-1,3-glucanase were obtained from untreated healthy (UH), untreated inoculated with *E. vexans* (UI), treated healthy (TH) and treated inoculated (TI) tea plants. 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. 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. 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.

Immunogold localization of PR-2 and PR-3 in tea plants induced by Salicylic acid. Indirect immunofluorescence of salicylic acid treated tea leaf tissues gave bright apple green fluorescence in mesophyll tissues when probed with PAb-CHT and labeled with FITC (Plate 1, figs.F&I). Cell suspension culture (Plate 1,fig.G) also gave bright fluorescence following treatment with PAb CHT and labeled with FITC (Plate 1, fig. H). 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 CHT as well as PAb GLU. Salicylic acid induced the deposition of β-1,3-glucanase (PR-2) and Chitinase (PR-3) systemically in the cells. The deposition of the chitinase, 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 (Plate 2, figs A&B). 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 (Plate 2, figs C&D). 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. 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. 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, bGLU was not so strongly induced by salicylic acid (or either by *E. vexans*).

Discussion

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 et.al. 2000; Rivera et.al, 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. 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). Induction of β-1,3-glucanases has been demonstrated in many plant-fungal pathogen interactions (Kemp et al., 1999; Kini et al., 2000) and they are thought to play several roles in plant defense.

Studies have been taken up for detection of the pathogen in host tissues by immunological means (Chakraborty and Chakraborty, 2003) and identification of specific antigens in electrophoretically separated components (Blake et al., 1984).The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseased plant tissues (Lyons and White,1992). 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. Yi and Hwang (1996) also opined that induction and accumulation of β-1,3-glucunases 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). 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. In tea plants the SA-dependent defence pathway induces resistance to *E. vexans*. The phenomenon of induced resistance by SA as an activator to enhance resistance in tea plants against a broad range of foliar fungal pathogens could be an attractive and an alternative disease control practice.

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