EXPERIMENTAL

4.1. Blister blight disease occurrence under natural conditions

Blister blight disease is caused by *Exobasidium vexans*. The pathogen, is a basidiomycetous fungus, an obligate parasite, without any known alternative host. The disease symptom initially appears as pale yellow translucent spot on young expanding leaves. The spot gradually enlarges to a circular lesion of 3-12.5mm or even more in diameter. As the disease progresses inside the leaf tissue, the upper surface of the developing lesion depresses into a shallow cavity, while on the lower side, it correspondingly becomes convex and thus forms the blister. The upper convex surface of the lesion is smooth and shiny, while the lower convex surface become white and powdery as it sporulates. Under severe infection, the affected leaves curl up. The disease also affects young growing stems and the damage becomes more severe under such situations. When the tender stem is affected, the entire shoot withers, causing die-back. The disease spreads through windborne basidiospores. Environmental conditions have a great influence on the development of the pathogen and disease incidene. A relative humidity higher than 80% is essential for basidiospore formation, ejection and germination. Leaf wetness is the most important factor for the germination of spores. Occurrence of the disease has been noted from the nursery seedlings. to the tea bushes.

4.1.1. Nursery

In tea planting, nursery occupies an important position as properly raised nursery plants survive well, when planted in the field and contribute to growth and productivity of tea. Planting of the cutting in the nursery was done twice a year (i) during June-July (ii) during October-November. Nursery plants right from the inception of striking the cuttings to the stage at which they were transferred (18-month old seedlings) to field were prone to attack by blister blight pathogen (*Exobasidium vexans*). Under highly conducive climatic conditions the pathogen infected the emerging foliage as a result of which the tender leaves get blighted and drop off in severe cases of infection. In order to observe the occurrence of blister blight in nursery grown plants under natural conditions, two set ups at (i) Phytopathological Experimental Station, NBU and (ii) Tea Research Station, Darjeeling, were considered.

Blister blight incidence was recorded in tea seedlings (12months old) raised in the nursery of the phytopathological experimental station, N.B.U. Among 9 tea varieties

(T78, S449, AV2, UP26, UP3, UP8, TV18, TV22 and TV27) grown in the nursery, TV-18 showed maximum disease symptoms during the month of January (Plate-4 Figs A & B). However, almost all the nine varieties responded to the attack of the pathogen, in the nursery grown tea seedlings at Darjeeling hills AV2 and TV18 showed maximum disease incidence during August-October (Fig.1).

4.1.2. Tea gardens

4.1.2.1. Plains

A survey was conducted to record the occurrence of blister blight in various tea gardens of the plain and hill regions. Data was recorded from five tea gardens of plain (viz Diana, Lankapara, Subhasini, Simulbarie and Hansqua Tea Estates) and it was found that the highest disease incidence (50-70%) of blister blight occurred in Hansqua Tea Estate (Plate 5, Fig. A). It was also observed that recently pruned bushes and those in the shade had higher levels of disease. Accordingly, the highest disease prone area of this tea garden was choosen as experimental field. Based on the topographical, map of "Survey of India" the altitude of the Experimental field was found to be 106m above sea level. Maximum disease incidence was recorded during mid December to February end, when high relative humidity was accompanied with low temperature and regular formation of fog. Spores were collected from flush shoots with well developed lesions, *in vitro* conditions.

When observed under the microscope, the basidiospores were hyaline, elliptical and clavate in nature. (Plate-6). The spore size was found to be 9.81x3.11µm. The basidiospores readily germinated in the pH range of 5 to 8.8, pH 5.5 being the most conducive range and temperature of 25^{0}C.

4.1.2.2. Hills

The epidemic of blister blight has caused maximum crop loss in the hills, due to the prolonged compatible weather conditions in these region. After surveying about five tea gardens (Makaibari, longview, Margarete's Hope, Castleton and Happy Valley) it was observed that the incidence of the disease was maximum (70-90%) in Castleton Tea Estate, (Plate 5B and 7A). The altitude of the experimental field in Castleton Tea Estate was found to be 1505m above sea level. The blister postules were found to be comparatively smaller in diameter, but the spore size were bigger. (Plate-7, Fig. B & C) The basidiospore size

Blister blight incidence of tea under natural conditions

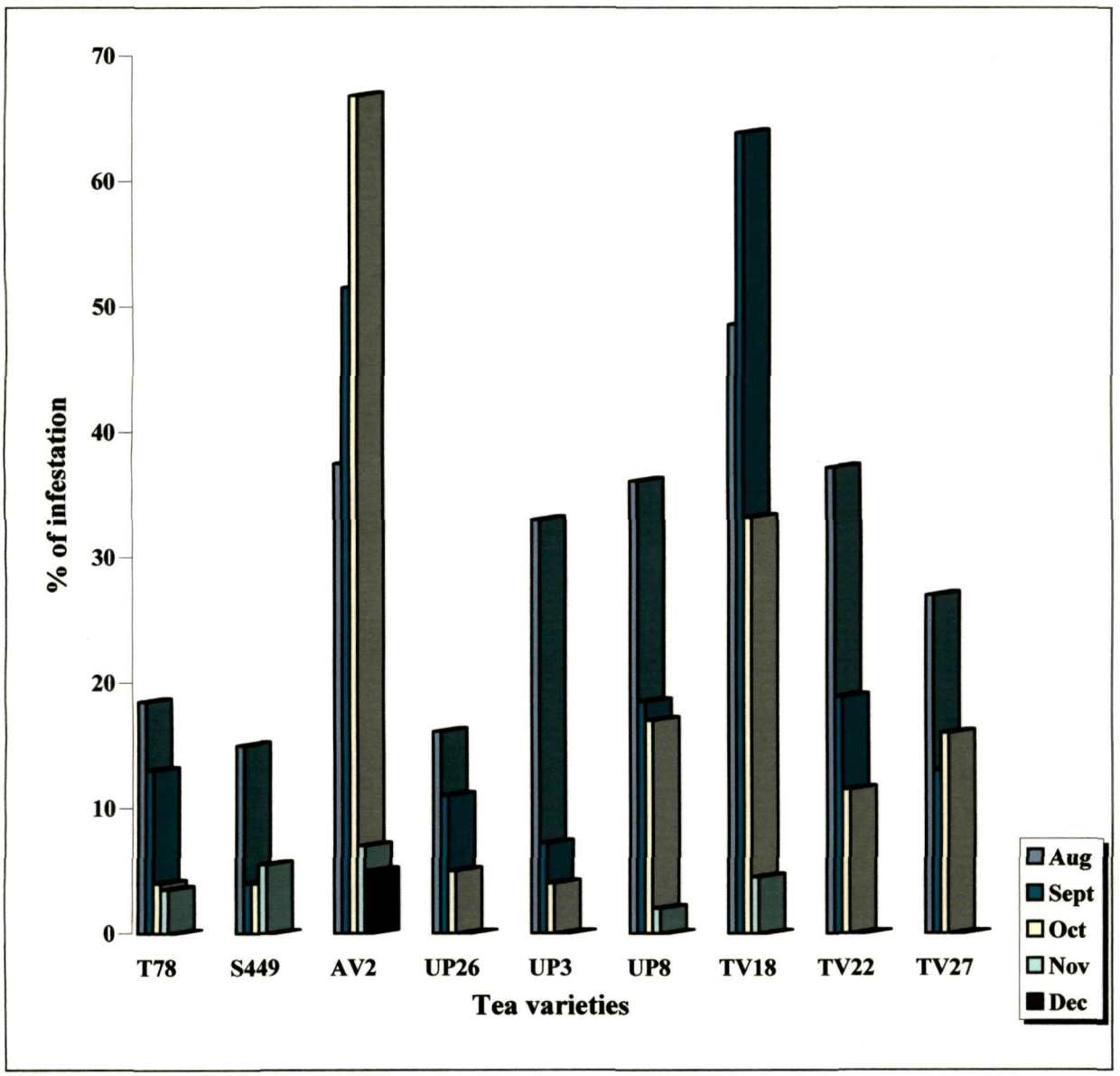


Fig.1

Plate 4 (Figs. A & B): Nursery grown Tea plants (TV-18)showing Blister blight symptoms. **A** -Dorsal view; **B**-Ventral view

Plate 5 (Figs. A & B): Blister infected tea leaves from (A) Hansqua and (B) CastletonTea Estates

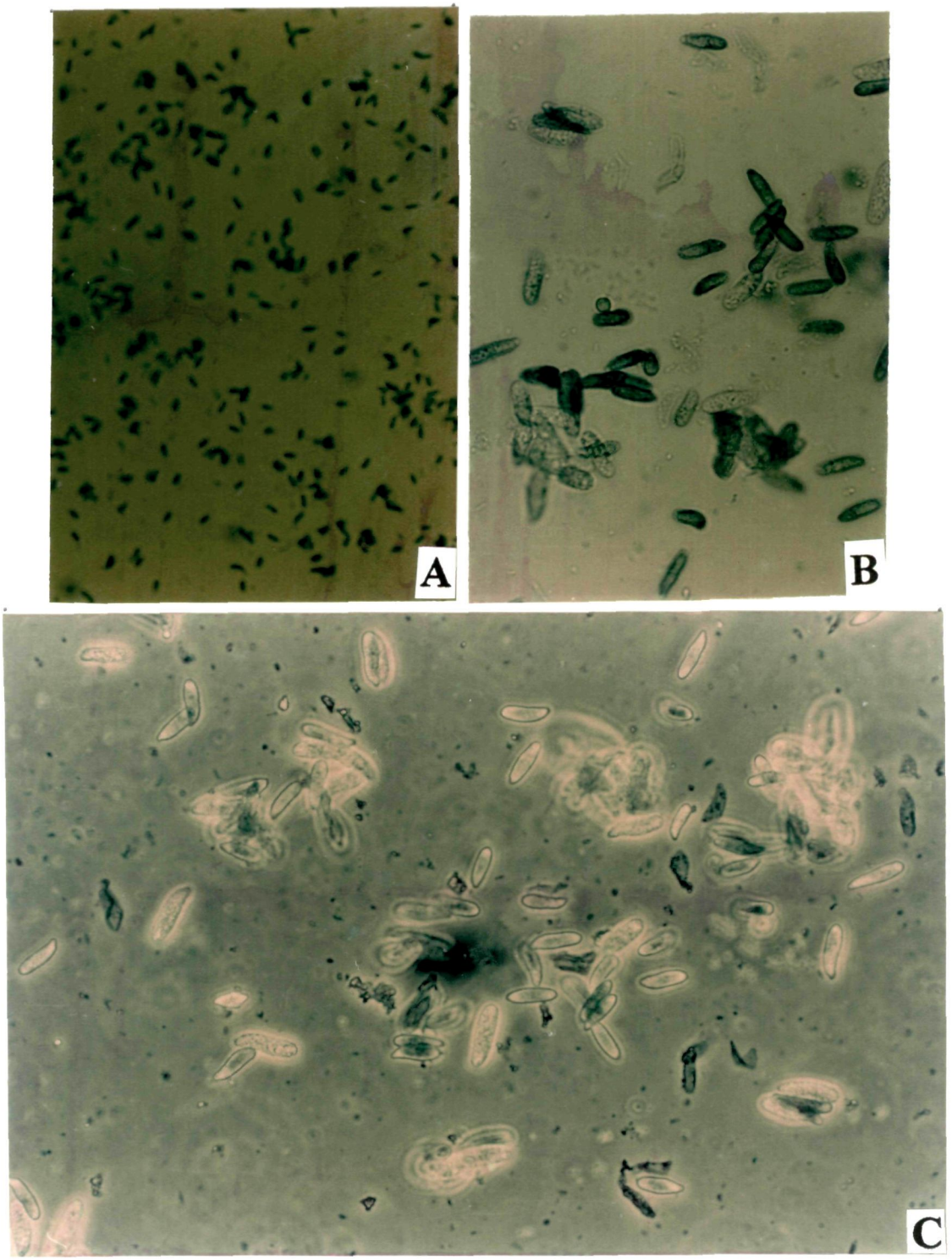


Plate 6 (Figs.A–C): Basidiospores of *Exobasidium vexans* from blister infected tea leaves of Hansqua Tea Estate observed under bright field (**A&B**) and phase contrast (**C**); **A**- Low power (10X); **B&C**- High power (40X)

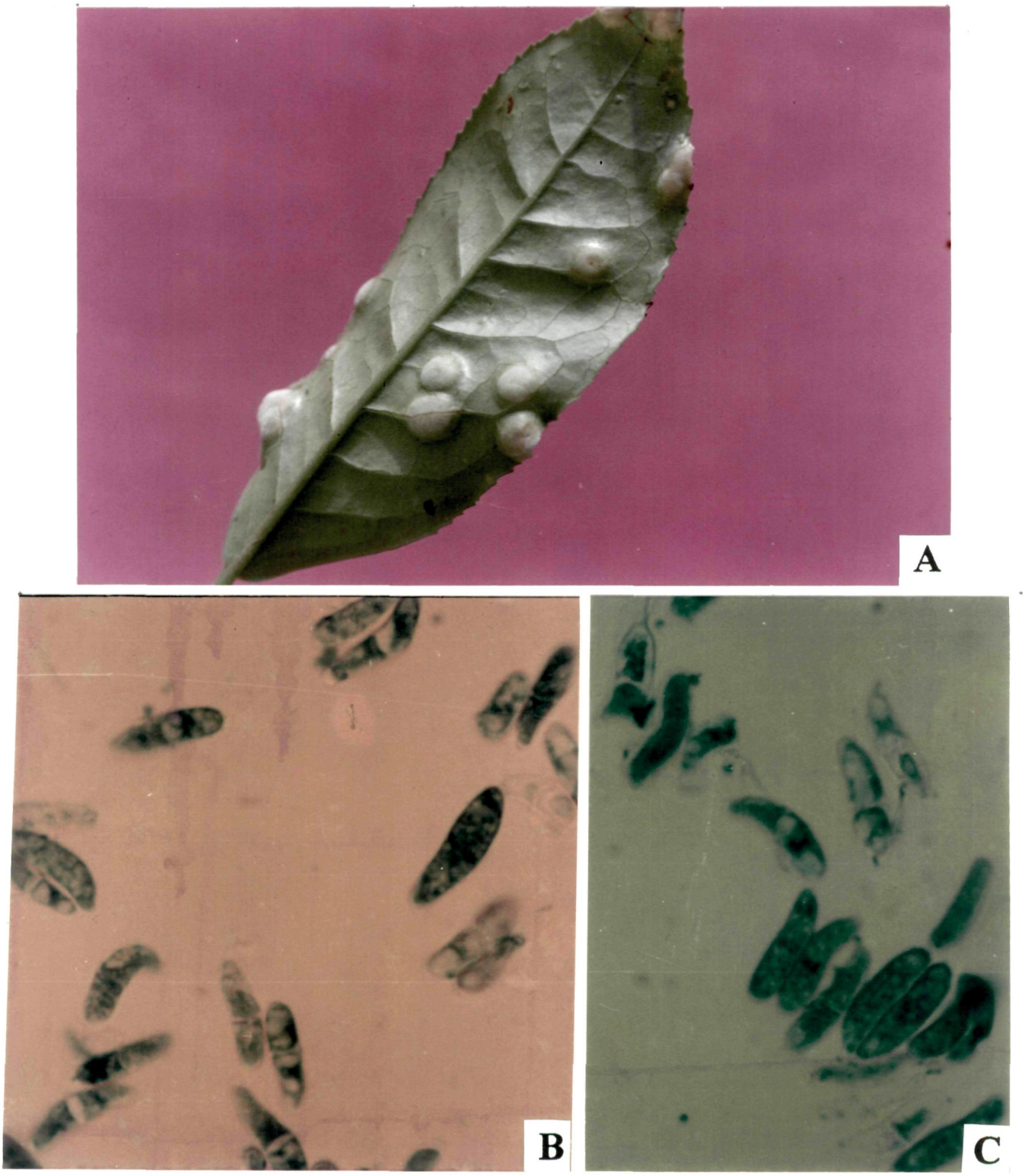


Plate 7(Figs. A-C): Blister infected tea leaf of Castleton Tea Estate (**A**); Basidiospores of *Exobasidium.vexans* (**B&C**) under bright field (40X)

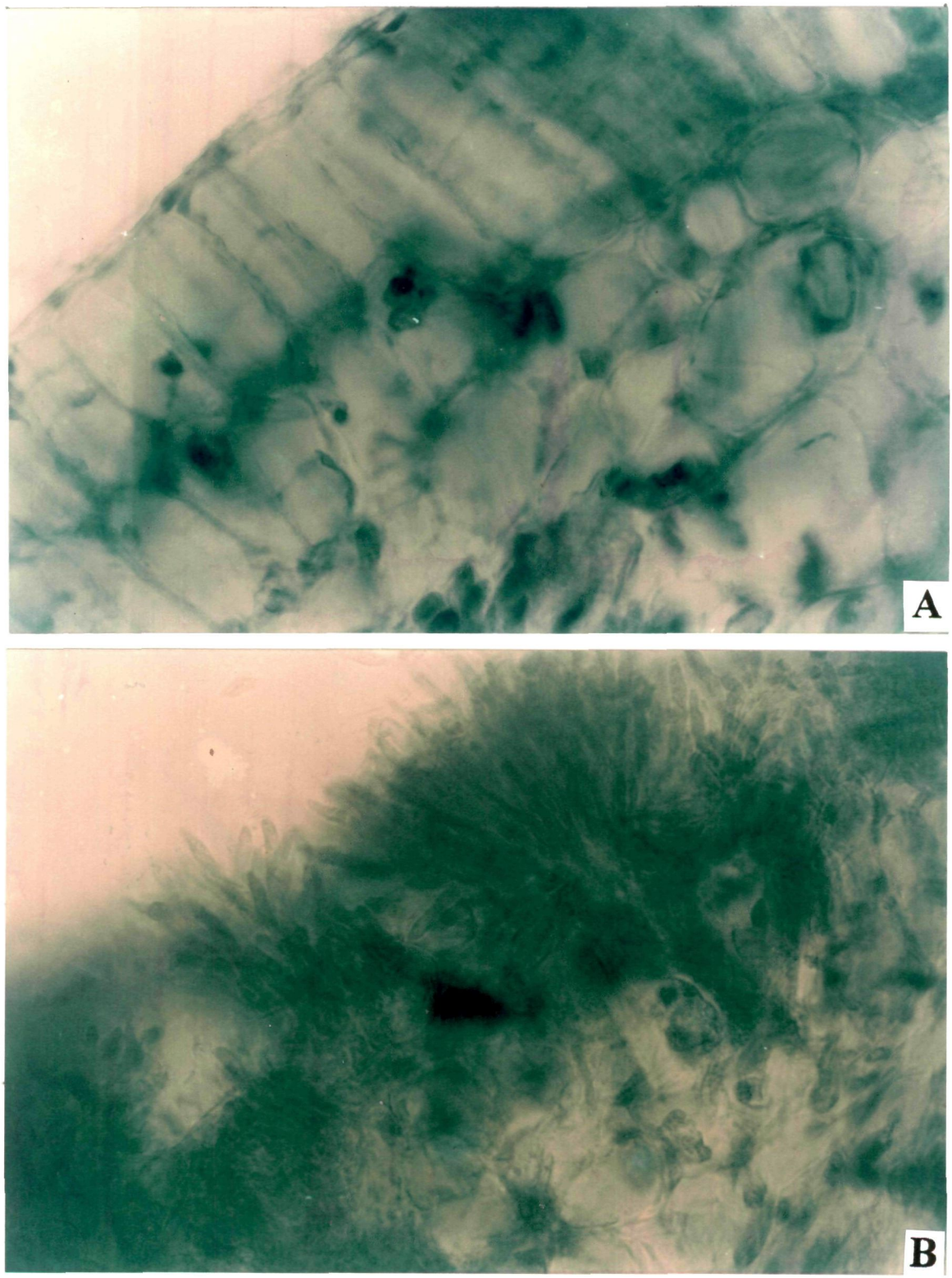


Plate 8 (Figs. A&B): Cross sections of Blister infected tea leaf

was found to be 15.51x4.22µm. The basidiospores readily germinated in the pH range of 5 and 8.8, maximum germination was noted in pH 5.5 and 7.0, and temperature 25°C.

Cross section of blister infected tea leaf were prepared and observed under microscope (bright field). Basidiospores attached to the epidermal tissue, fungal penetration into the mesophyll tissues were also evident (Plate-8 Fig., A and B).

4.2. Meterological Data (Temperature, Relative humidity and Rainfall)

Weather conditions have a profound influence on the development of the pathogen and disease incidence. Keeping this in mind, monthly meterological data were collected for five years (1996-2000) which included temperature (maximum and minimum), relative humidity (morning and evening), and average rainfall. All the datas are shown in Figs2 to7.

4.2.1. Correlation of weather conditions with occurrence of blister blight disease.

Blister blight disease incidence were recorded throughout the year at two Tea Estates, (one each of hill and plain). From the the hill, Castleton Tea Estate, Darjeeling and from the plain Hunsqua Tea Estate, Siliguri were considered for such observation. Results are given in Tables 1 and 2.

Maximum incidence of the disease was noticed during July-October in hill station while in plain disease incidence was recorded only during December to February throughout the study period of 5 years. Correlation of weather conditions (temperature, relative humidity and average rainfall) with the occurrence of blister blight disease are given in Figs. 8 and 9.

In order to determine whether disease development in nature is correlated with different environmental factors, Karl Pearsons' correlation coefficient was calculated in respect to disease occurrence and the various factors. Mean data of the five years was used in all cases. When mean blister blight incidence of hills (Castleton Tea Estate, Kurseong, Darjeeling) was correlated with the different factors, it was found that disease incidence showed positive significant correlation with rainfall and relative humidity. A correlation coefficient of 0.87 was obtained with disease and rainfall and a correlation coefficient of 0.71 was obtained between disease and relative humidity. On the contrary, when mean blister blight incidence of plains (Hansqua Tea Estate, Siliguri) was correlated with the

Monthly Maximum and Minimum Temperature (Plains)

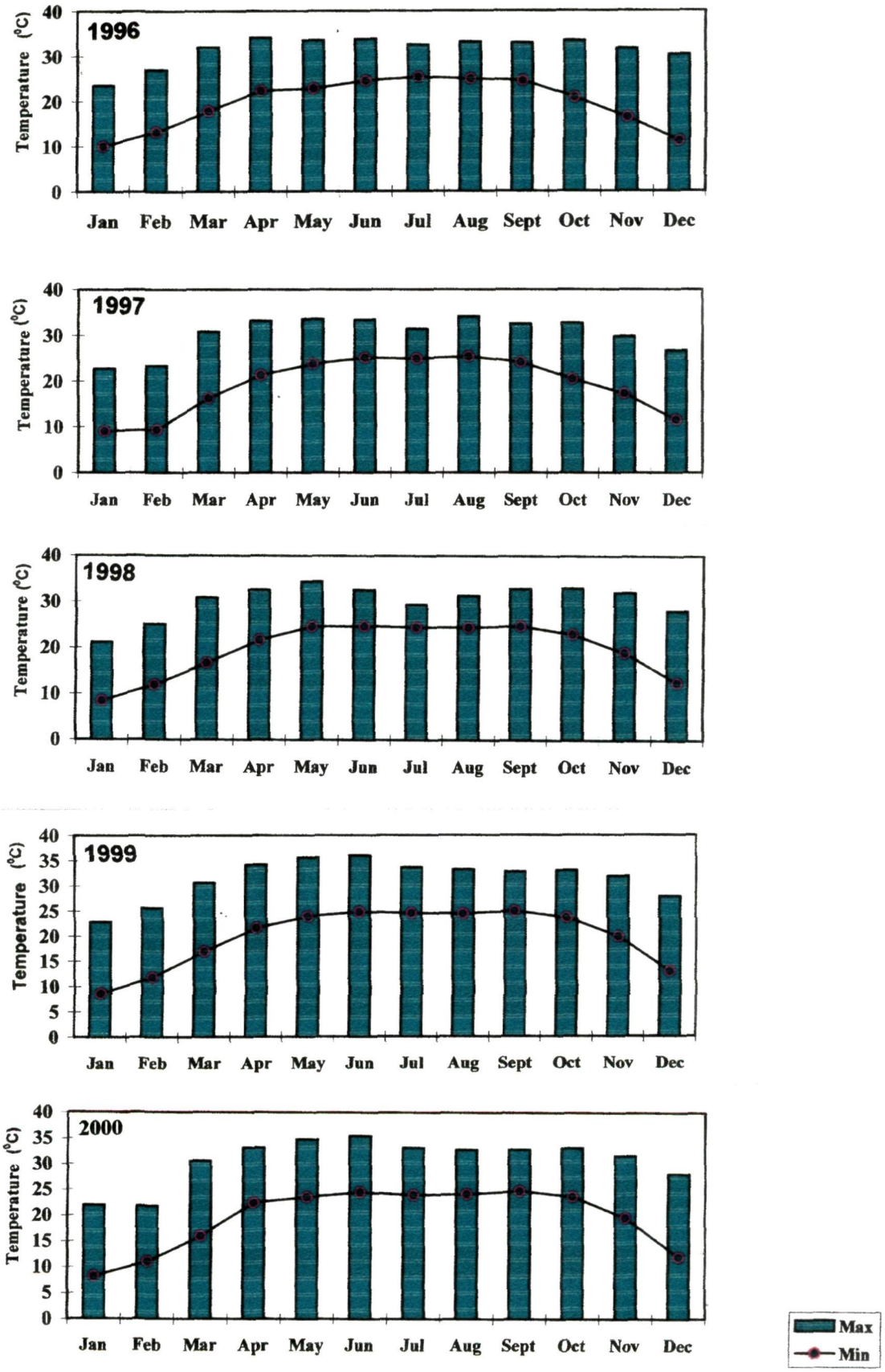


Fig. 2

Monthly Relative Humidity (Plains)

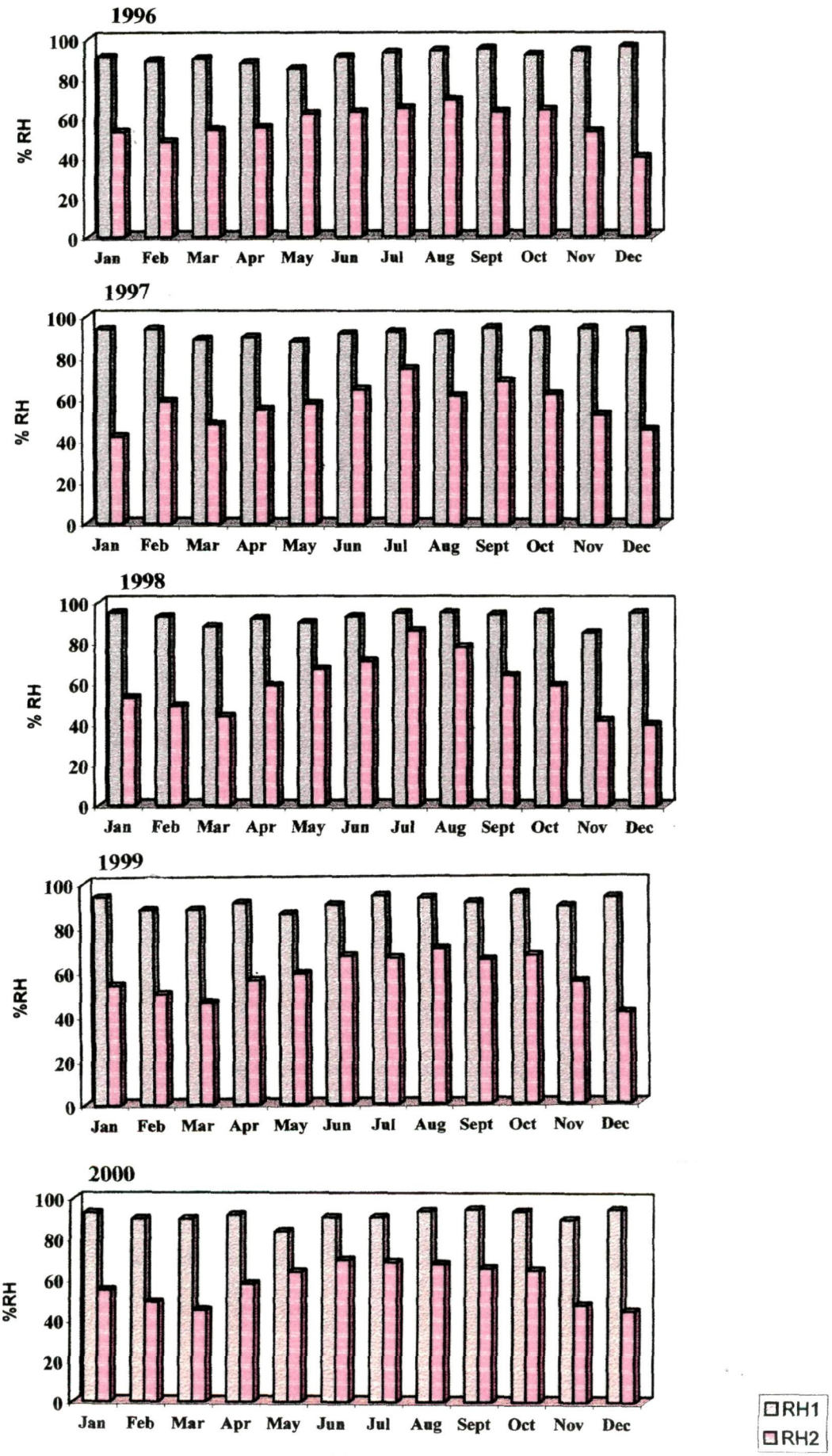


Fig. 3

Average Monthly Rainfall (Plains)

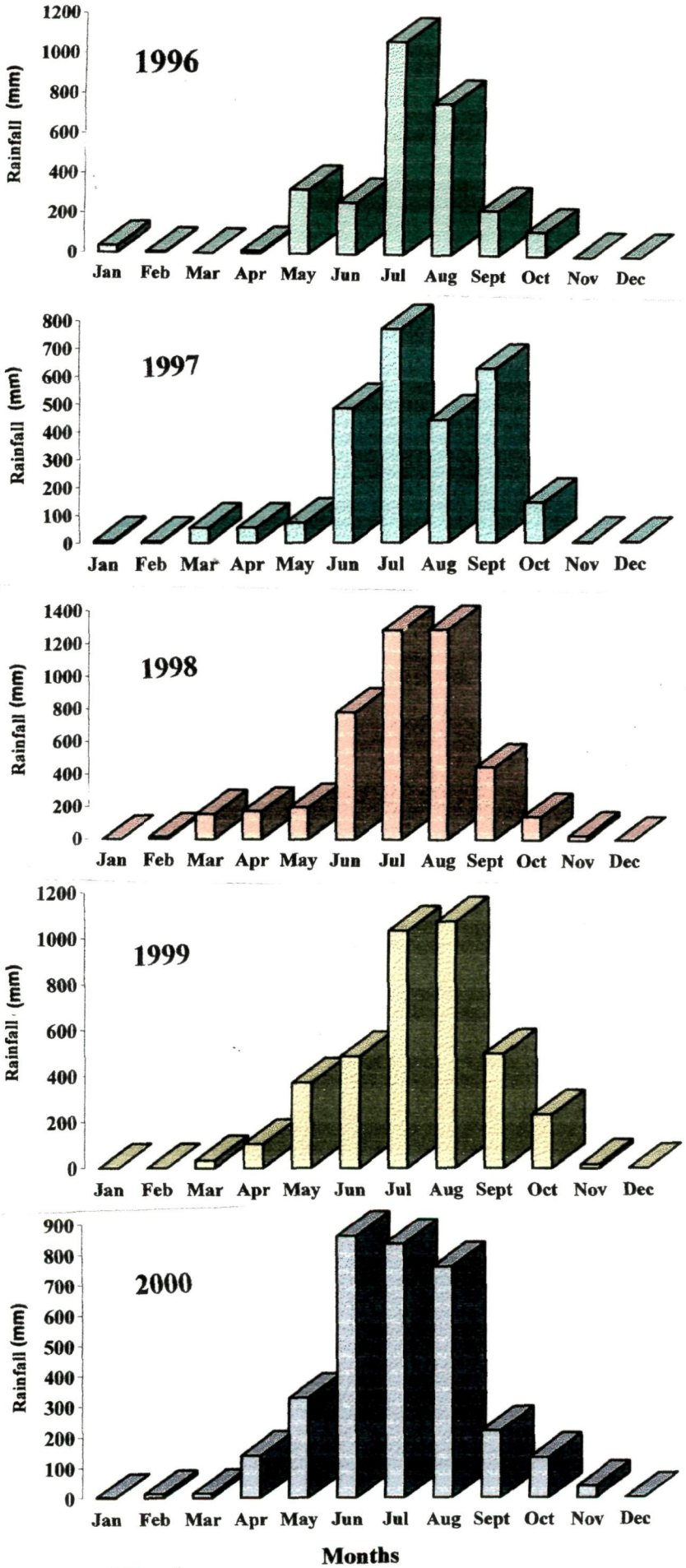


Fig. 4

Monthly Maximum and Minimum Temperature (Hills)

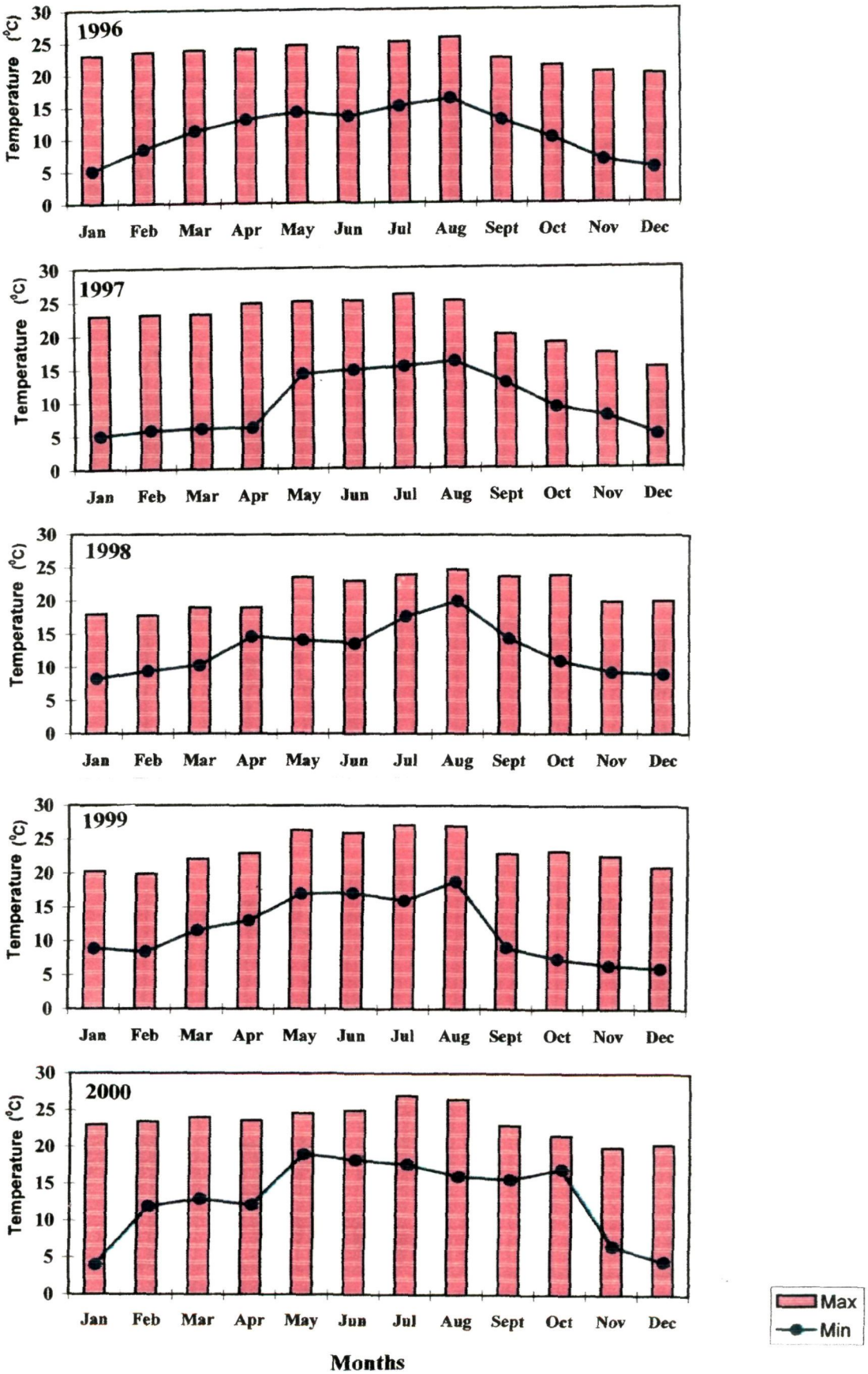


Fig. 5

Monthly Relative Humidity (Hills)

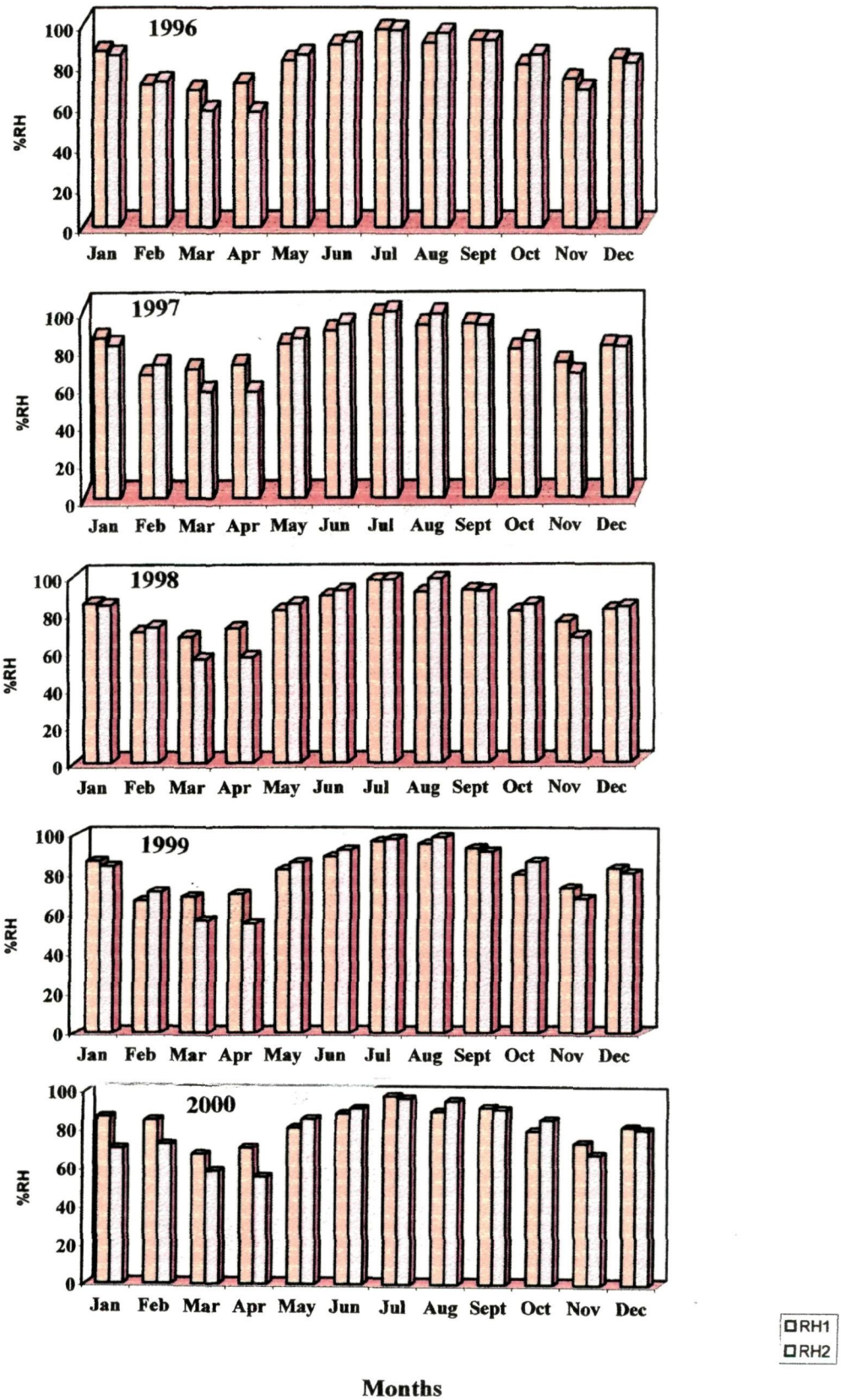


Fig. 6

Average Monthly Rainfall (Hills)

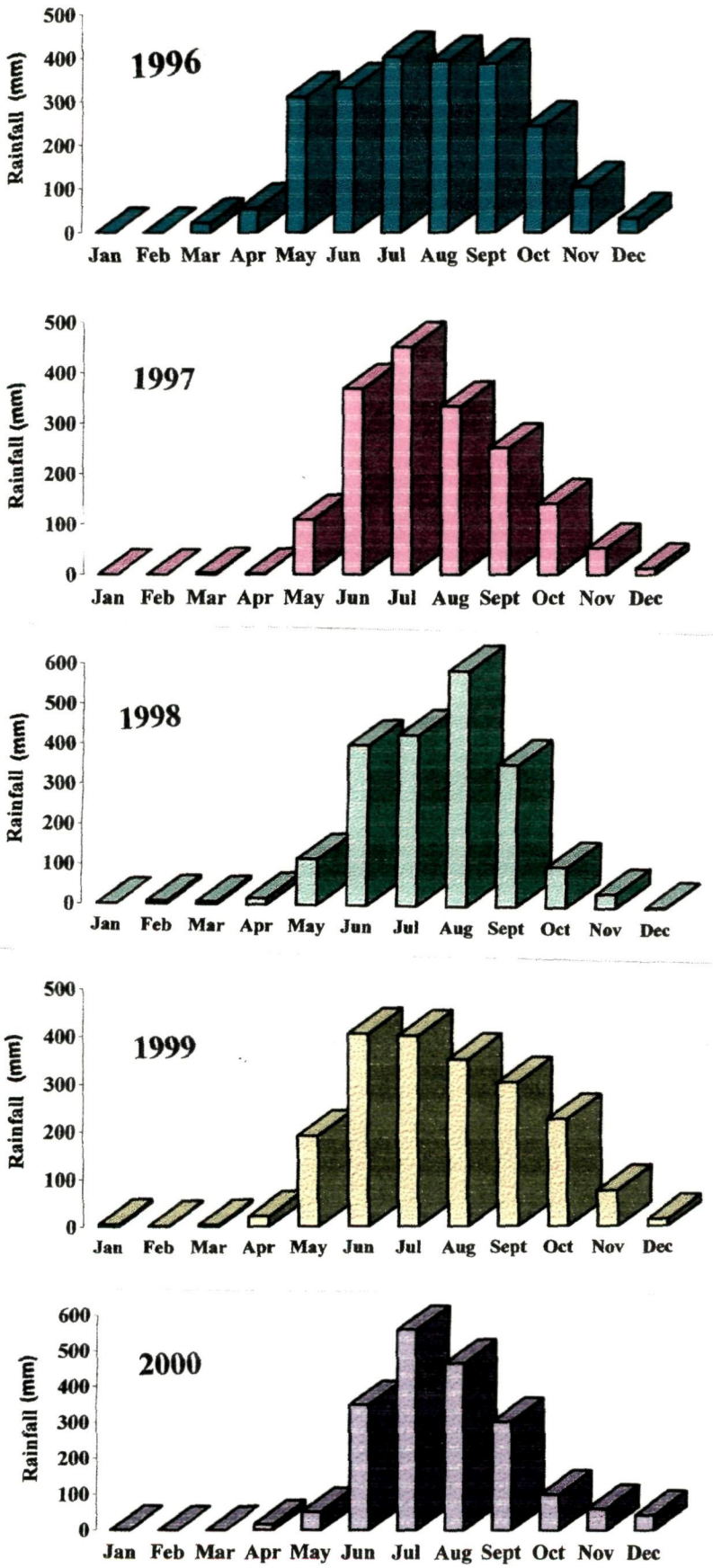


Fig. 7

different factors, it was found that disease incidence showed negative correlation with maximum temperature, minimum temperature and rainfall. Results are given in Tables 3 and 4.

Table 1 : Meterological data and blister blight disease occurrence in plains.

Month	Meterological data[a]					
	Temperature (°C)		Relative humidity (%)		Rainfall (mm)	Disease[b] incidence (%)
	Maximum	Minimum	Morning	Evening		
Jan	22.4±0.4	18.8±0.32	93.4± 0.68	51.4±2.37	23.9±6.92	75.5±0.02
Feb	24.6±0.91	11.4± 0.64	90.8±1.16	51.0±2.02	8.0±2.78	52.1±0.06
Mar	30.9± 0.26	16.7± 0.31	89.0± 0.45	47.4±1.77	70.3±27.73	10.3±0.02
Apr	33.5± 0.31	21.8± 0.22	90.6± 0.75	56.6±0.81	97.9±28.75	0
May	34.4± 0.39	23.7± 0.31	86.6±1.08	62.0±1.64	258.7±55.03	0
Jun	34.3±0.64	24.7± 0.11	91.4± 0.51	67.2±1.49	574.5±109.69	0
Jul	32.3± 0.75	24.6± 0.22	93.2± 0.66	72.7±3.86	997.5±91.9	0
Aug	32.9± 0.44	24.7± 0.21	93.6± 0.51	69.4±2.56	863.7±145.95	0
Sep	32.8±0.80	24.6± 0.13	94.0± 0.77	65.4±1.03	402.7±78.80	0
Oct	33.6±0.12	22.3± 0.70	94.0± 0.55	63.6±1.32	155.96±20.0	0
Nov	31.4±0.41	18.4± 0.67	90.6±1.80	50.2±2.35	35.9±7.75	13.2±0.08
Dec	25.6± 0.58	12.0± 0.33	94.6± 0.51	42.4±1.29	15.2±15.23	21.2±0.13

± Standard error.

[a] Average of five years (1996-2000).

[b]Mean occurrence of blister blight disease for five years (1996-2000).

Table 2 : Meterological data and blister blight disease occurrence in hills.

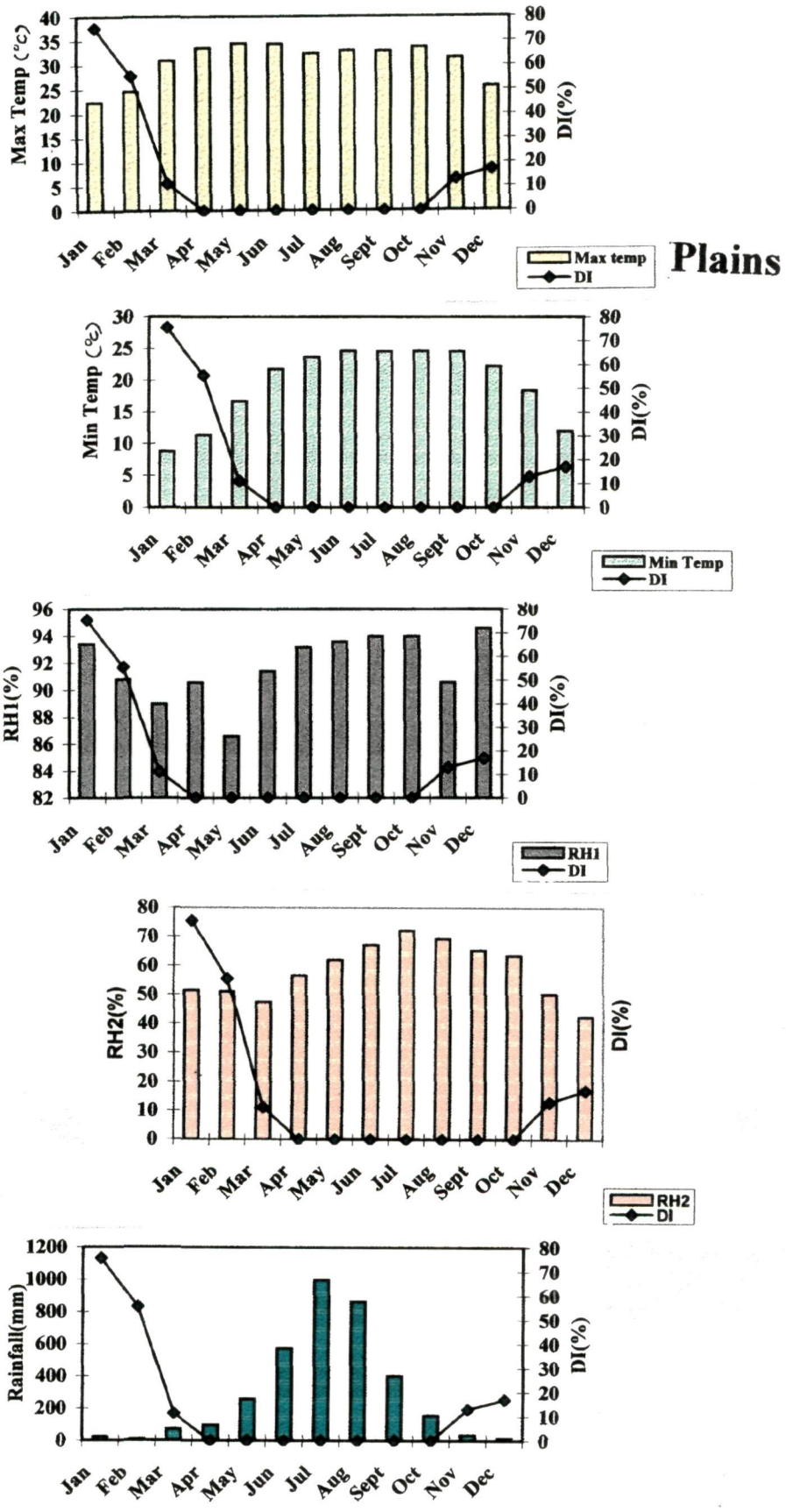
Month	Meterological data[a]					
	Temperature (°C)		Relative humidity (%)		Rainfall (mm)	Disease[b] incidence (%)
	Maximum	Minimum	Morning	Evening		
Jan	21.4±1.01	6.3±0.86	86.2±0.31	83.8±0.73	1.1±1.11	2.7±0.03
Feb	21.5±1.07	8.8±0.87	68.6±0.35	57.3±0.34	2.1±1.51	0
Mar	22.4±0.91	10.2±0.94	68.0±0.28	57.6±0.50	7.6±3.87	0
Apr	22.8±1.01	11.7±1.45	71.0±0.31	57.2±0.31	25.8±10.45	0
May	24.8±0.45	15.7±0.99	81.9±0.21	85.4±0.26	155.6±44.80	0
Jun	24.6±0.49	15.4±0.95	89.2±0.21	91.9±0.23	371.4±14.24	5.1±0.04
Jul	25.8±0.59	16.3±0.56	97.5±0.22	97.7±0.38	449.1±27.90	35.6±0.02
Aug	25.7±0.42	17.4±0.84	92.0±0.95	97.2±0.68	426.9±46.69	83.8±0.01
Sep	22.4±0.41	12.9±0.97	92.5±0.11	91.5±0.12	319.8±23.70	88.4±0.04
Oct	21.8±0.90	10.9±1.63	80.1±0.38	85.4±0.60	162.7±29.65	77.8±0.03
Nov	20.3±0.85	7.3±0.54	73.4±0.58	67.3± 0.45	66.4±11.80	50.4±0.06
Dec	19.3±1.06	5.9±0.79	82.6±0.53	81.0±0.57	21.4±7.34	18.6±0.07

± Standard error

[a] Average of five years (1996-2000)

[b] Mean occurrence of blister blight disease for five years (1996-2000).

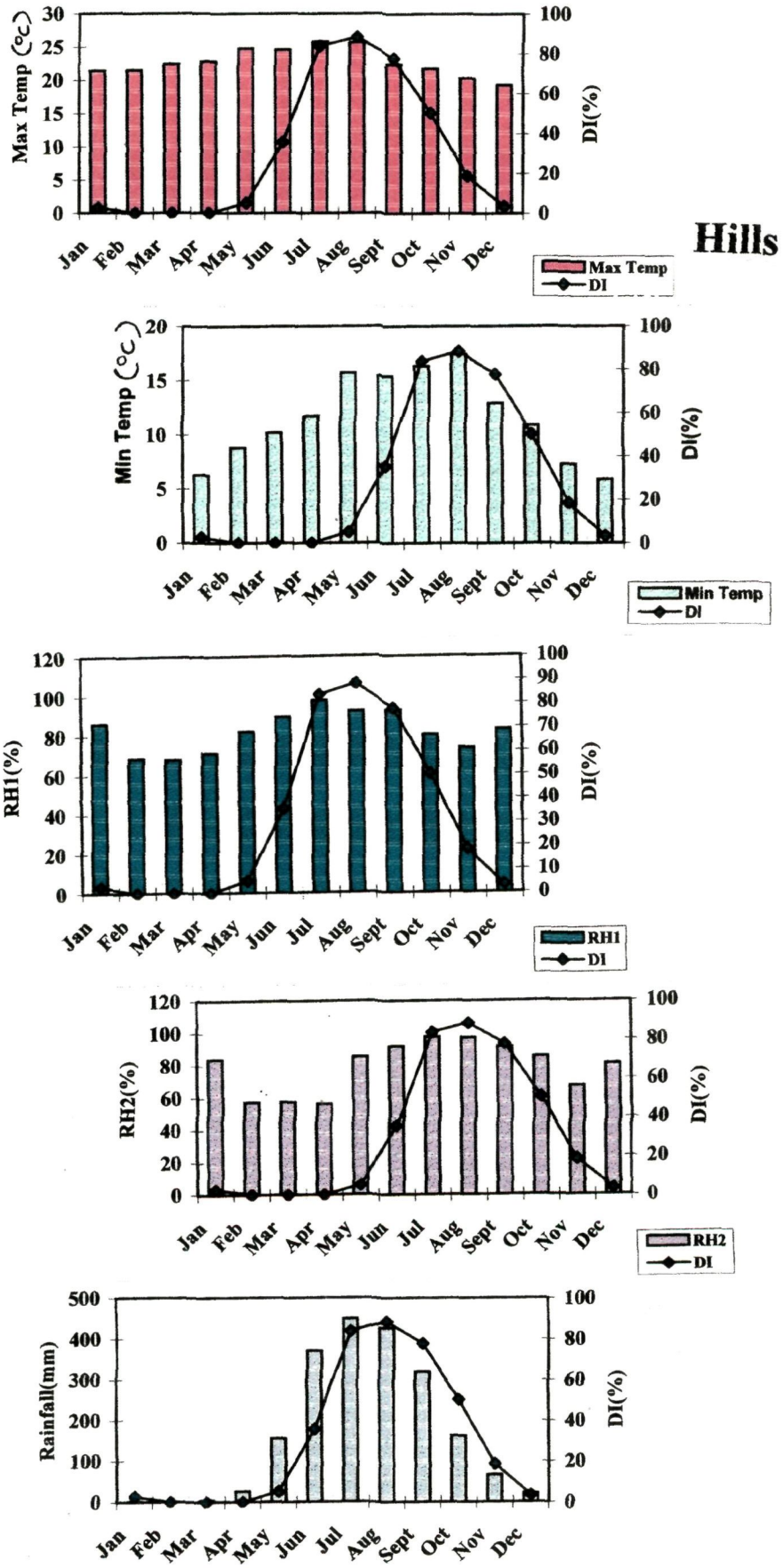
Correlation of weather conditions with occurrence of blister blight disease (Average of 5 years)



RH1:Relative humidity(morning)
RH2:Relative humidity(evening)
DI :Disease incidence

Fig. 8

Correlation of weather conditions with occurrence of blister blight disease (Average of 5 years)



RH1:Relative humidity(morning)
RH2:Relative humidity(evening)
DI :Disease incidence

Fig. 9

Table 3 : Correlation Matrix showing correlation between blister blight and environmental factors during 5 years (1996-2000), two tailed significance, (plains).

	Disease[a]	Max. Temp	Min Temp	RH 1[b]	RH2 [c]	Rain
Disease	1	– 0.872**	– 0.847	0.432	0.421	– 0.670
Max. Temp	– 0.872**	1	0.995*	– 0.688	0.021	0.717
Min. Temp	– 0.847	0.995*	1	– 0.666	0.051	0.657
RH1	0.432	– 0.688	– 0.666	1	– 0.573	– 0.809
RH2	0.420	0.021	0.051	– 0.051	1	0.064
Rain	– 0.670	0.717	0.657	– 0.809	0.064	1

'r' R values are Pearsons' Correlation coefficient

* Significant at P– 0.01;

** Significant at P – 0.05, rest insignificant.

[a]Disease computed as % incidence (mean of five years);

[b]R.H.1 – % relative humidity (morning); [c]R.H.2 – % relative humidity (evening)

4.3. Pathogenicity test of *Exobasidium vexans* on different tea varieties

Thirty one tea varieties which includes 11 Darjeeling, 11 Tocklai and 9 UPASI, obtained from Tea Germplasm collection centre were used for varietal resistance test against *Exobasidium vexans*. Methods of inoculation, incubation conditions and disease assessment procedures have been discussed under Materials and Methods and results are given in Table-5. On the basis of disease incidence, the varieties are grouped into highly susceptible (S), highly resistant (R), moderately susceptible (MS) and moderately resistant (MR), Among the Darjeeling varieties, AV2 was found to be highly susceptible while S449 and Teen Ali 17/1/54 were resistant. In case of Tocklai varieties, TV18 and TV28 were highly susceptible and TV26 followed by TV23 and TV9 were highly resistant. However, UP8 followed by UP3 and UP26 were highly susceptible among UPASI varieties tested, whereas, UP2, BSS1 and BSS3 were resistant. Among the 31 varieties tested

maximum percentage of disease incidence was 80.1% for AV2 and minimum was 3.2% (TV26). The external symptoms of the disease, occurrence on few artificially inoculated tea varieties (S449, AV2, TV18 and UP3) have been presented in plate 9 (Figs. A to D). S449 showed resistance towards blister blight while AV2, TV18 and UP3 showed high susceptibility.

Table 4 : Correlation Matrix showing correlation between blister blight and environmental factors during 5 years (1996-2000), two tailed significance, (hills)

	Disease[a]	Max. Temp	Min Temp	RH 1[b]	RH2[c]	Rain
			**	**	**	*
Disease	1	0.584	0.665	0.70	20.711	0.897
Max.			*	**	*	*
Temp	0.584	1	0.953	0.688	0.811	0.839
Min.	**	*			*	*
Temp	0.665	0.953	1	0.623	0.774	0.892
	**	**	*		*	*
RH1	0.702	0.688	0.953	1	0.925	0.784
	*	*	*	*		*
RH2	0.711	0.811	0.774	0.925	1	0.826
	*	*	*	**	*	
Rain	0.867	0.839	0.892	0.784	0.826	1

'r' r values are Pearsons' Correlation coefficient

* Significant at P–0.01,

** Significant at P –0.05, rest insignificant.

[a]Disease computed as % incidence (mean of five years).

[b]RH1 –% relative humidity (morning)

[c]RH2 – % relative humidity (evening)

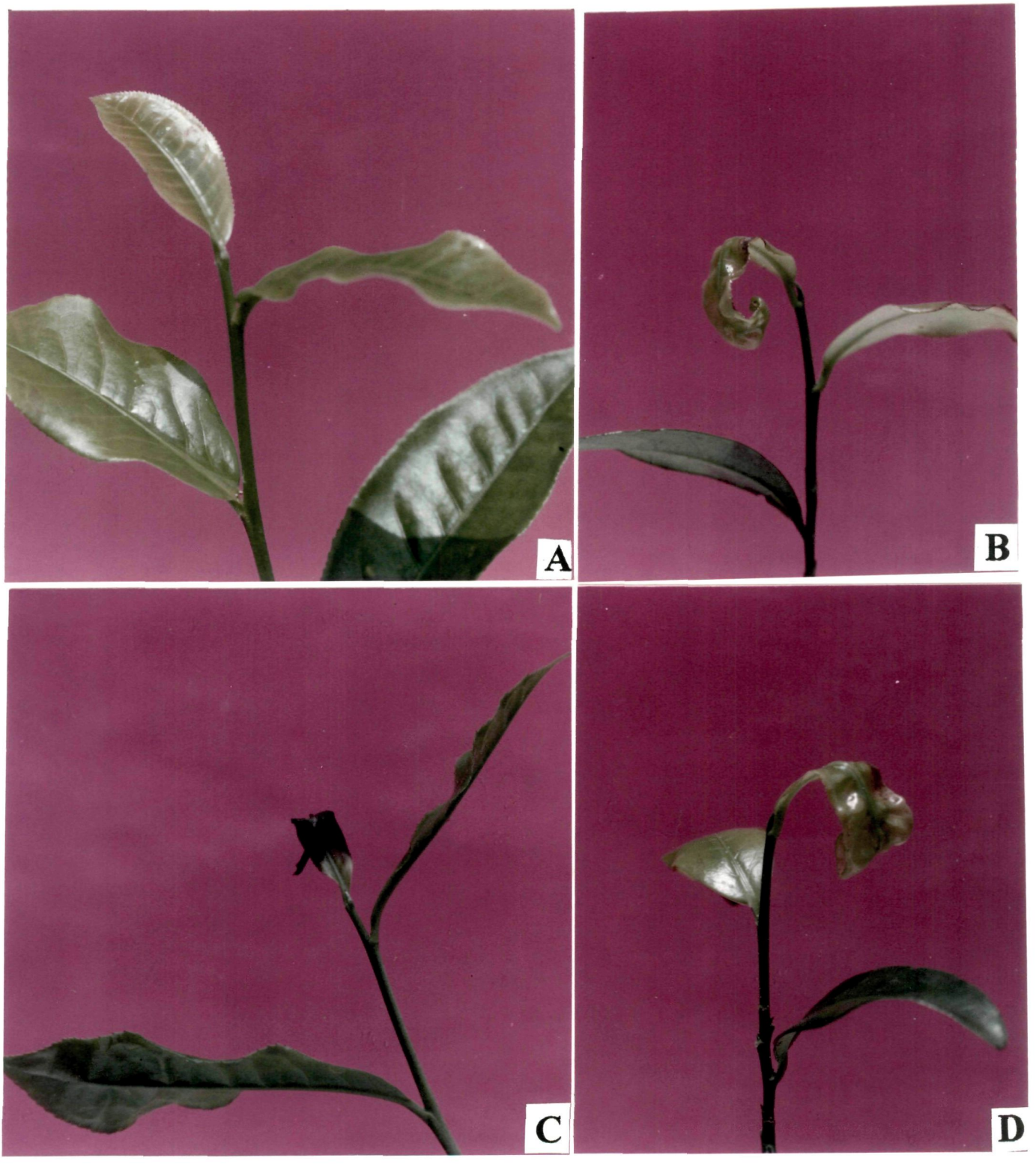


Plate 9 (Figs. A-D): Tea varieties artificially inoculated with *E.vexans*
(**A**) S-449; (**B**) AV-2; (**C**) TV-18; (**D**) UPASI-3

Table 5 : Pathogenicity test of *Exobasidium vexans* on tea varieties

Tea Varieties		Disease incidence (%)[a]	Category
Darjeeling			
	CPI	60.0 ± 0.02	S
	S449	10.0 ± 0.03	R
	K1/1	32.0 ± 0.04	MR
	T-78	40.0 ± 0.03	MR
	Teen Ali 17/1/54	10.0 ± 0.01	R
	T-135	61.6 ± 0.02	S
	P-1258	20.2 ± 0.03	MR
	BS/7A/76	58.8 ± 0.02	MS
	HV-39	17.1 ± 0.04	R
	B777	65.3 ± 0.01	S
	AV-2	80.1 ± 0.03	S
Tocklai			
	TV-9	15.5 ± 0.02	R
	TV-18	75.0 ± 0.05	S
	TV-20	51.6 ± 0.03	MS
	TV-22	42.3 ± 0.08	MS
	TV-23	15.6 ± 0.04	R
	TV-25	51.4 ± 0.02	MS
	TV-26	3.2 ± 0.05	R
	TV-27	68.5 ± 0.12	S
	TV-28	70.1 ± 0.03	S
	TV-29	55.5 ± 0.01	MS
	TV-30	26.9 ± 0.08	MR
UPASI			
	UP-2	13.1 ± 0.04	R
	UP-3	65.5 ± 0.08	S
	UP-8	70.6 ± 0.04	S
	UP-9	60.2 ± 0.07	S
	UP-17	54.1 ± 0.07	MS
	UP-26	60.0 ± 0.08	S
	BSS1	19.9 ± 0.01	R
	BSS2	22.2 ± 0.05	MR
	BSS3	17.7 ± 0.06	R

± Standard error

[a]Mean of 3 experiements.

S – Susceptible ; R-Resistant ; MR-Moderately resistant ; MS-Moderately susceptible.

4.4. Detection of cross reactive antigens between *E. vexans* and tea varieties

The complexity of the interactions that affect the selection of parasites and allow for their establishment and survival away among host cells is manifest in the frequency and variability of cell surface antigens. Antigen sharing betwen different cells has been of special interest because of its coincidence in compatible host-parasite relationships, its possible significance also has been indicated in cell to cell relationships of host parasites. The presence of cross reactive antigens (CRA) among hosts and pathogenic organisms is a well established phenomenon. Various methods have been generally used to detect the presence of CRA between host and parasite. In the present investigation, cross reactive antigens between *E. vexans* and tea varieties have been detected using immunodiffusion and enzyme linked immunosorbent assay (ELISA).

4.4.1. Immunodiffusion tests

At the onset, polyspecific antibody raised against blister infected tea leaves of Castleton Tea Estate (CTE) was used for detection of CRA using agar gel double diffusion test. In addition, polyclonal antibody raised against antigen prepared from basidiospores of *Exobasidium vexans* collected from natural blister infected tea leaves of Castleton Tea Estate (CTE) and polyspecific antibody raised against blister infected tea leaves of Hansqua Tea Estate (HTE), were used for detection of CRA by enzyme linked immunosorbent assay (ELISA) after their optimization.

The effectiveness of antigen preparations from blister infected leaves of CTE and HTE as well as basidiospores of *E. vexans* in raising polyspecific and polyclonal antibodies were checked by homologous cross-reaction in agar gel double diffusion test (Plate 10 Figs. A-F). Control sets involving normal serum (preimmune serum) and antigen of blister infected leaves and basidiospores were all negative. Effectiveness of antigen extracts from healthy tea leaves of 31 varieties were tested against polyspecific antibody (PS1/B) of *E. vexans*. Results have been presented in Table 6. Fifteen tea varieties (CP1, K1/1, T78, BS/7A/76, B777, AV2, TV18, TV25, TV26, TV27, TV28, UP8, UP9, UP17 and UP26) showed strong precipitation reactions. Weak precipitation reactions were observed when PSI/B reacted with Teen Ali 17/1/54, P1258, HV39, TV20, TV22, TV30, UP2 and UP3. However, no such precipitation was observed in cross reaction between pathogen antisera and 8 tea varieties such as BSS1, BSS2, TV29, TV23, T135 and S449, and non pathogen (*Fusarium graminearum*) and non host (*Oryza sativa*).

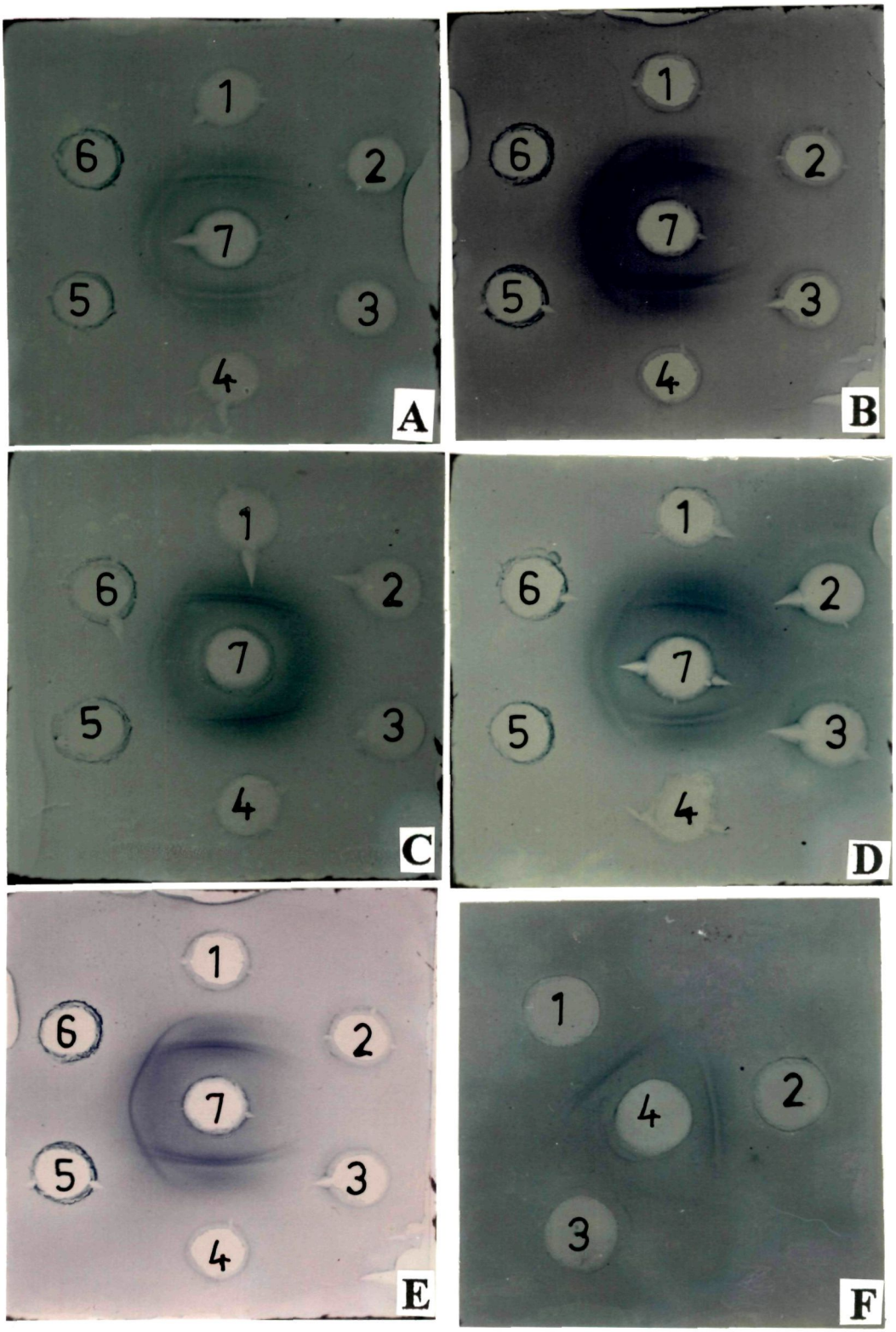


Plate 10 (Figs. A-F) : Agar gel double diffusion tests.Central wells (7 & 4) contain polyspecific (A-D)and polyclonal (E&F) antisera of *E.vexans*. Peripheral wells (1-6) contain antigens; (A,C&E)-2nd bleeding; (B,D&F)-3rd bleeding; .(A-E) Blister infected leaf (1,4,5&6); Healthy leaf (2&3). (F)- infected leaf (1),basidiospore (2),healthy leaf (3).

Table 6 :Detection of Cross reactive antigens among Tea varieties and *Exobasidium vexans* using agar gel double diffusion tests

Antigens of host	Polyspecific Antisera of *E.vexans* (PS-I/B)	Antigens of host and parasite	Polyspecific Antisera of *E.vexans* (PS-I/B)
Tea Varieties		**Tea Varieties**	
Darjeeling		**UPASI**	
CP1	+	UP2	±
S449	−	UP3	±
K1/1	+	UP8	+
T78	+	UP9	+
Ten Ali 17/1/54	−	UP17	+
T135	−	UP26	+
P-1258	±	BSS1	−
BS/7A/76	−	BSS2	−
HV39	±	BSS3	−
B777	+		
AV2	+	**Pathogen**	
Tocklai		*E. vexans*	
TV9	−	Blister postules (CTE)	+
TV18	+	Blister postules (HTE)	+
TV20	±	Basidiospores (CTE)	+
TV22	±		
TV23	−	**Non-Pathogen**	
TV25	+	*F. graminearum*	−
TV26	−		
TV27	+	**Non-host**	
TV28	+	*Oryza sativa*	−
TV29	−		
TV30	±		

PS1/B : Polyspecific antibody prepared from blister infected tea leaves of Castleton Tea Estate / 2nd bleeding ; CTE : Castleton Tea Estate ; HTE : Hansqua Tea Estate

+ : Common precipitin band present ; ± : Weak precipitin band present ;

− : Common precipitin band absent

4.4.2. Direct antigen coated enzyme linked immunosorbent asay (DAC-ELISA)

Enzyme linked immunosorbent assay is one of the most sensitive serological techniques for detection of cross reactive antigens between host and pathogen as well as for detection of pathogen in diseased tissue. In indirect or DAC-ELISA antigens are bound

to the microtitre plates after which the antibody is allowed to bind to the antigen. To this antigen antibody complex, the conjugate (an antibody conjugated to enzyme) is added.Finally the non coloured substrate is added which is converted to a coloured end product which is generally detected by a reader.

4.4.2.1. Optimization of ELISA

Optimization was done considering two variables, dilution of the antigen extract and dilution of antiserum. Enzyme dilution was kept constant at 1:10,000.

4.4.2.1.1. Polyspecific antibody (PS-I) raised against blister infected tea leaves of Castleton Tea Estate (CTE).

Immunogen (blister infected tea leaves of CTE) and purified sera (IgG) were designated as PS-I/A, PS-I/B, PS-I/C and PS-I/D with first, second, third and fourth bleedings respectively. These were used for optimization.

4.4.2.1.1.1. Antigen dilution

Doubling dilution of blister infected leaf antigen (Source-CTE) were tested against two antiserum dilutions (1:125 and 1:250). Result have been presented in Tables 7-10.

Table 7 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous antiserum (PS-I)[a]

Antigen concentration ng/ml	Antisera (PS-I/A[b]) dilution	
	1:125	1:250
62.5	0.260 ± 0.05[c]	0.201 ± 0.03[c]
125	0.265 ± 0.02	0.256 ± 0.08
250	0.379 ± 0.08	0.292 ± 0.06
500	0.484 ± 0.03	0.354 ± 0.03
1000	0.617 ± 0.03	0.472 ± 0.01
2000	0.770 ± 0.03	0.595 ± 0.04
4000	1.039 ± 0.01	0.799 ± 0.01
8000	1.303 ± 0.06	1.242 ± 0.04

± Standard error

[a]Poly specific antisera raised against blister infected tea leaves collected from Castleton Tea Estate. ; [b]1st bleeding ; [c]Mean of three experiments.

Table 8 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous antiserum (PS-1)[a]

Antigen concentration ng/ml	Antisera (PS-I/B[b]) dilution	
	1:125	1:250
62.5	0.452 ± 0.01[c]	0.292 ± 0.05[c]
125	0.483 ± 0.03	0.319 ± 0.08
250	0.582 ± 0.04	0.411 ± 0.09
500	0.745 ± 0.08	0.650 ± 0.04
1000	0.928 ± 0.01	0.704 ± 0.04
2000	1.077 ± 0.05	0.871 ± 0.08
4000	1.329 ± 0.01	1.038 ± 0.05
8000	1.531 ± 0.03	1.467 ± 0.04

± Standard error

[a]Polyspecific antisera raised against blister infected tea leaves collected from Castleton Tea Estate ; [b]2nd bleeding ; [c]Mean of three experiments.

Table 9 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous antiserum (PS-I)[a]

Antigen concentration ng/ml	Antisera (PS-I/C[b]) dilution	
	1:125	1:250
62.5	0.336 ± 0.05[c]	0.216 ± 0.03[c]
125	0.368 ± 0.01	0.257 ± 0.06
250	0.494 ± 0.02	0.349 ± 0.01
500	0.595 ± 0.02	0.532 ± 0.04
1000	0.731 ± 0.01	0.661 ± 0.04
2000	0.841 ± 0.02	0.939 ± 0.05
4000	1.064 ± 0.01	0.992 ± 0.01
8000	1.511 ± 0.09	1.495 ± 0.01

± Standard error

[a]Poly specific antisera raised against blister infected tea leaves collected from Castleton Tea Estate. ; [b]3rd bleeding ; [c]Mean of three experiments.

Table 10 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous antiserum (PS-I)[a]

Antigen concentration ng/ml	Antisera (PS-I/D[b]) dilution	
	1:125	1:250
62.5	0.332 ± 0.04[c]	0.220 ± 0.03[c]
125	0.385 ± 0.05	0.244 ± 0.06
250	0.444 ± 0.05	0.336 ± 0.02
500	0.561 ± 0.08	0.426 ± 0.05
1000	0.699 ± 0.05	0.604 ± 0.01
2000	0.781 ± 0.05	0.715 ± 0.03
4000	0.972 ± 0.01	0.920 ± 0.06
8000	1.356 ± 0.01	1.492 ± 0.06

± Standard error

[a]Polyspecific antisera raised against blister infected tea leaves collected from Castleton Tea Estate. ; [b]4th bleeding ; [c]Mean of three experiments.

4.4.2.1.1.2. Antiserum dilution

Antiserum dilutions ranging from 1:125 to 1:16000 were tested against homologous antigen at a concentration of 8µg/ml. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:16000.The reactions are performed using all four bleedings (PS-I/A, PS-I/B, PS-I/C and PS-I/D). Results are shown in Tables 11-14.

4.4.2.1.2. Polyspecific antibody (PS-II) raised against blister infected tea leaves of Hansqua Tea Estate

Immunogen (blister infected tea leaves of HTE) and purified sera (IgG) were designated as PS-II/A, PS-II/B, PS-II/C, PS-II/D, and PS-II/E with first second, third, fourth and fifth bleedings respectively. These were used for optimization.

4.4.2.1.2.1. Antigen dilution

Doubling dilution of blister infected leaf antigen (Source-HTE) were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with the increase of antigen concentrations. Results have been presented in Tables 15 to 19.

Table 11 : ELISA reaction with various dilutions of anti-*E.vexans* antiserum and homologous antigen.

Antisera dilution PS-I/A (First bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	0.961	0.969	0.977	0.969 ± 0.01
1:250	0.800	794	0.789	0.794 ± 0.02
1:500	0.512	0.502	0.514	0.509 ± 0.01
1:1000	0.478	0.478	0.479	0.478 ± 0.01
1:2000	0.362	0.372	0.382	0.372 ± 0.02
1:4000	0.330	0.336	0.370	0.330 ± 0.03
1:8000	0.333	0.326	0.331	0.330 ± 0.01
1:16000	0.217	0.217	0.214	0.216 ± 0.01

± Standard error.

Table 12 : ELISA reaction with various dilutions of anti-*E.vexans* antiserum and homologous antigen

Antisera dilution PS-I/B (Second bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	1.281	1.282	1.285	1.282 ± 0.00
1:250	0.999	0.991	0.992	0.994 ± 0.03
1:500	0.646	0.675	0.670	0.663 ± 0.02
1:1000	0.564	0.570	0.567	0.567 ± 0.02
1:2000	0.482	0.481	0.481	0.481 ± 0.04
1:4000	0.433	0.438	0.434	0.435 ± 0.02
1:8000	0.345	0.340	0.342	0.342 ± 0.02
1:6000	0.243	0.246	0.249	0.246 ± 0.02

± Standard error.

Table 13 :ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen

Antisera dilution PS-I/C (Third bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	1.224	1.235	1.246	1.235 ± 0.01
1:250	1.024	1.040	1.110	1.058 ± 0.03
1:500	0.932	0.939	0.941	0.937 ± 0.04
1:1000	0.789	0.780	0.776	0.781 ± 0.01
1:2000	0.658	0.654	0.652	0.654 ± 0.01
1:4000	0.411	0.402	0.402	0.405 ± 0.01
1:8000	0.276	0.280	0.279	0.278 ± 0.01

± Standard error

Table 14 :ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-I/D (Fourth bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	1.003	0.973	1.001	0.992 ± 0.04
1:250	0.887	0.870	0.881	0.879 ± 0.01
1:500	0.750	0.741	0.745	0.745 ± 0.03
1:1000	0.628	0.630	0.632	0.630 ± 0.02
1:2000	0.494	0.494	0.495	0.494 ± 0.00
1:4000	0.383	0.380	0.379	0.381 ± 0.02
1:8000	0.330	0.350	0.341	0.343 ± 0.01
1:16000	0.237	0.233	0.231	0.234 ± 0.02

± Standard error

Table 15 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous polyspecific antiserum (PS-II)[a].

Antigen concentration ng/ml	Antisera (PS-II/A[b]) dilution	
	1:125	1:250
62.5	0.386 ± 0.08[c]	0.299 ± 0.03[c]
125	0.408 ± 0.05	0.321 ± 0.03
250	0.426 ± 0.07	0.298 ± 0.02
500	0.564 ± 0.05	0.394 ± 0.01
1000	0.807 ± 0.08	0.586 ± 0.04
2000	0.961 ± 0.01	0.685 ± 0.03
4000	1.153 ± 0.04	1.119 ± 0.06
8000	1.265 ± 0.01	1.165 ± 0.08

± Standard error

[a]Poly specific antisera raised against blister infected tea leaves collected from Hansqua Tea Estate; [b]1st bleeding ; [c]Mean of three experiments.

Table 16: ELISA reaction with various concentrations of blister infected leaf antigen and homologous polyspecific antiserum (PS-II)[a]

Antigen concentration ng/ml	Antisera (PS-II/B)[b] dilution	
	1:125	1:250
62.5	0.397 ± 0.02	0.304 ± 0.03
125	0.484 ± 0.03	0.326 ± 0.04
250	0.478 ± 0.02	0.397 ± 0.04
500	0.609 ± 0.01	0.496 ± 0.07
1000	0.694 ± 0.03	0.578 ± 0.01
2000	0.826 ± 0.02	0.624 ± 0.03
4000	0.918 ± 0.02	0.801 ± 0.03
8000	1.108 ± 0.03	0.945 ± 0.02

± Standard error

[a]Polyspecific antisera raised against blister infected tea leaves collected from Hansqua Tea Estate; [b]2nd bleeding ; [c]Mean of three experiments.

Table 17 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous polyspecific antiserum(PS-II)[a]

Antigen concentration ng/ml	Antisera (PS-II/C[b]) dilution	
	1:125	1:250
62.5	0.507 ± 0.03[c]	0.162 ± 0.02[c]
125	0.655 ± 0.08	0.293 ± 0.03
250	0.560 ± 0.09	0.327 ± 0.01
500	0.756 ± 0.06	0.364 ± 0.09
1000	0.907 ± 0.01	0.566 ± 0.08
2000	1.283 ± 0.04	0.914 ± 0.09
4000	1.290 ± 0.06	0.948 ± 0.05
8000	1.885 ± 0.05	0.987 ± 0.06

± Standard error

[a]Poly specific antisera raised against blister infected tea leaves collected from Hansqua Tea Estate; [b]3rd bleeding ; [c]Mean of three experiments.

Table 18 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous polyspecific antiserum (PS-II)[a]

Antigen concentration ng/ml	Antisera (PS-II/D[b]) dilution	
	1:125	1:250
62.5	0.285 ± 0.01[c]	0.208 ± 0.01[c]
125	0.288 ± 0.01	0.205 ± 0.01
250	0.280 ± 0.10	0.220 ± 0.06
500	0.330 ± 0.04	0.228 ± 0.03
1000	0.426 ± 0.06	0.300 ± 0.09
2000	0.492 ± 0.04	0.384 ± 0.06
4000	0.560 ± 0.03	0.457 ± 0.01
8000	0.656 ± 0.06	0.576 ± 0.02

± Standard error

[a]Polyspecific antisera raised against blister infected tea leaves collected from Hansqua Tea Estate; [b]4th bleeding ; [c]Mean of three experiments.

Table 19 : ELISA reaction with various concentrations of blister infected leaf antigen and homologous polyspecific antiserum (PS-II)[a]

Antigen concentration ng/ml	Antisera (PS-II/E[b]) dilution	
	1:125	1:250
62.5	0.296 ± 0.02[c]	0.222 ± 0.02[c]
125	0.292 ± 0.01	0.224 ± 0.02
250	0.326 ± 0.04	0.237 ± 0.01
500	0.340 ± 0.09	0.256 ± 0.06
1000	0.345 ± 0.09	0.244 ± 0.07
2000	0.403 ± 0.05	0.291 ± 0.01
4000	0.422 ± 0.01	0.350 ± 0.01
8000	0.434 ± 0.01	0.392 ± 0.06

± Standard error

[a]Polyspecific antisera raised against blister infected tea leaves collected from Hansqua Tea Estate; [b]5th bleeding ; [c]Mean of three experiments.

4.4.2.1.2.2 Antiserum dilution

Antiserum dilution ranging from 1:125 to 1:16000 were tested against homologous antigen at a concentration of 8 µg / ml. This was tested for all the polyspecific antisera (Tables 22 to 24).

Table 20 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-II/A (First bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1: 125	1.200	1.127	1.127	1.124± 0.03
1: 250	0.956	0.991	1.025	0.990± 0.02
1: 500	0.696	0.672	0.768	0.712± 0.04
1: 1000	0.565	0.564	0.580	0.569± 0.01
1: 2000	0.439	0.389	0.440	0.422± 0.02
1: 4000	0.334	0.331	0.339	0.334± 0.02
1: 8000	0.263	0.259	0.260	0.260± 0.02
1: 16000	0.252	0.250	0.258	0.253± 0.03

± Standard error

Table 21 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-II/B (Second bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	0.850	0.795	0.762	0.802 ± 0.04
1:250	0.799	0.664	0.673	0.705 ± 0.05
1:500	0.562	0.582	0.588	0.574 ± 0.01
1:1000	0.498	0.490	0.488	0.492 ± 0.01
1:2000	0.386	0.380	0.400	0.388 ± 0.01
1:4000	0.313	0.308	0.301	0.307 ± 0.02
1:8000	0.274	0.274	0.280	0.276 ± 0.02
1:16000	0.275	0.272	0.277	0.274 ± 0.02

± Standard error.

Table - 22 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-II/C (Third bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	**Exp-2**	**Exp-3**	**Mean**
1:125	1.010	0.990	1.001	1.003 ± 0.02
1:250	0.907	0.901	0.910	0.906 ± 0.01
1:500	0.726	0.698	0.711	0.712 ± 0.01
1:1000	0.661	0.686	0.666	0.671 ± 0.01
1:2000	0.460	0.491	0.486	0.479 ± 0.03
1:4000	0.395	0.390	0.392	0.392 ± 0.02
1:8000	0.345	0.353	0.349	0.349 ± 0.01
1:16000	0.292	0.289	0.290	0.290 ± 0.01

± Standard error.

Table 23 :ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-II/D (Fourth bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	0.545	0.528	0.562	0.545 ± 0.01
1:250	0.456	0.532	0.550	0.542 ± 0.01
1:500	0.460	0.457	0.455	0.457 ± 0.01
1:1000	0.372	0.356	0.358	0.362 ± 0.01
1:2000	0.270	0.283	0.255	0.269 ± 0.01
1:4000	0.304	0.310	0.308	0.307 ± 0.01
1:8000	0.263	0.257	0.269	0.263 ± 0.02
1:16000	0.250	0.264	0.2789	0.264 ± 0.01

± Standard error

Table 24 :ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PS-II/E (Fifth bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	0.443	0.441	0.446	0.443 ± 0.01
1:250	0.437	0.432	0.428	0.432 ± 0.01
1:500	0.336	0.350	0.343	0.343 ± 0.02
1:1000	0.334	0.338	0.331	0.334 ± 0.01
1:2000	0.290	0.298	0.282	0.290 ± 0.02
1:4000	0.263	0.268	0.260	0.263 ± 0.01
1:8000	0.259	0.260	0.259	0.259 ± 0.02
1:16000	0.207	0.210	0.215	0.210 ± 0.01

± Standard error.

ELISA values increased with increase of antigen concentration. Concentrations as low as 62.5ng/ml could be easily detected by DAC-ELISA at both antisera dilution (1:125 and 1:250) (Fig. 10). On the other hand, absorbance values in ELISA reaction decreased from the dilution of 1:125 to 1:16000 (Fig. 11).

4.4.2.1.3. Polyclonal antibody (PC-I) raised against basidiospores collected from blister infected tea leaves (CTE)

Immunogen (basidiospores collected from blister infected tea leaves of CTE) and purified sera (IgG) were designated as PC-I/A, PC-I/B, PC-I/C and PC-I/D with first, second, third and fourth bleedings respectively. These were used for optimization.

4.4.2.1.3.1. Antigen dilution

Doubling dilution of spore antigen (CTE) were tested against two antiserum dilutions (1:125 and 1:250). Results have been presented in Tables 25 to 28.

Table 25 : ELISA reaction with various concentrations of spore antigen and homologous polyclonal antiserum (PC-I)[a]

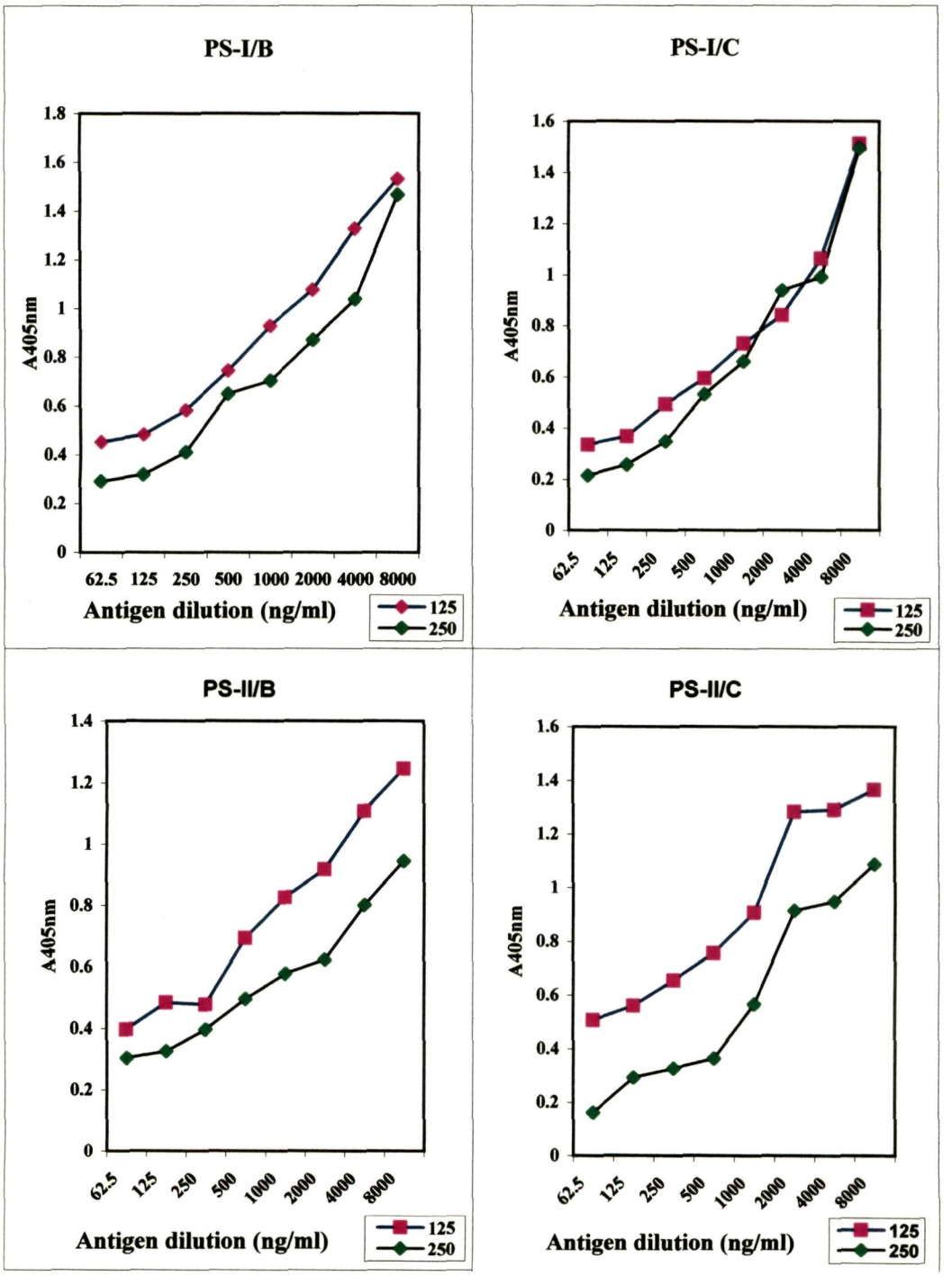
Antigen concentration ng/ml	Antisera (PC-I/A[b]) dilution	
	1:125	1:250
62.5	0.251 ± 0.04[c]	0.211 ± 0.08[c]
125	0.384 ± 0.01	0.256 ± 0.01
250	0.412 ± 0.05	0.310 ± 0.01
500	0.501 ± 0.05	0.345 ± 0.06
1000	0.588 ± 0.05	0.444 ± 0.05
2000	0.781 ± 0.01	0.587 ± 0.06
4000	0.897 ± 0.06	0.771 ± 0.06
8000	1.231 ± 0.07	1.001 ± 0.07

± Standard error

[a]Polyclonal antisera raised against basidiospores of blister infected leaf from Castleton Tea Estate.

[b]1st bleeding ; [c]Mean of three experiments.

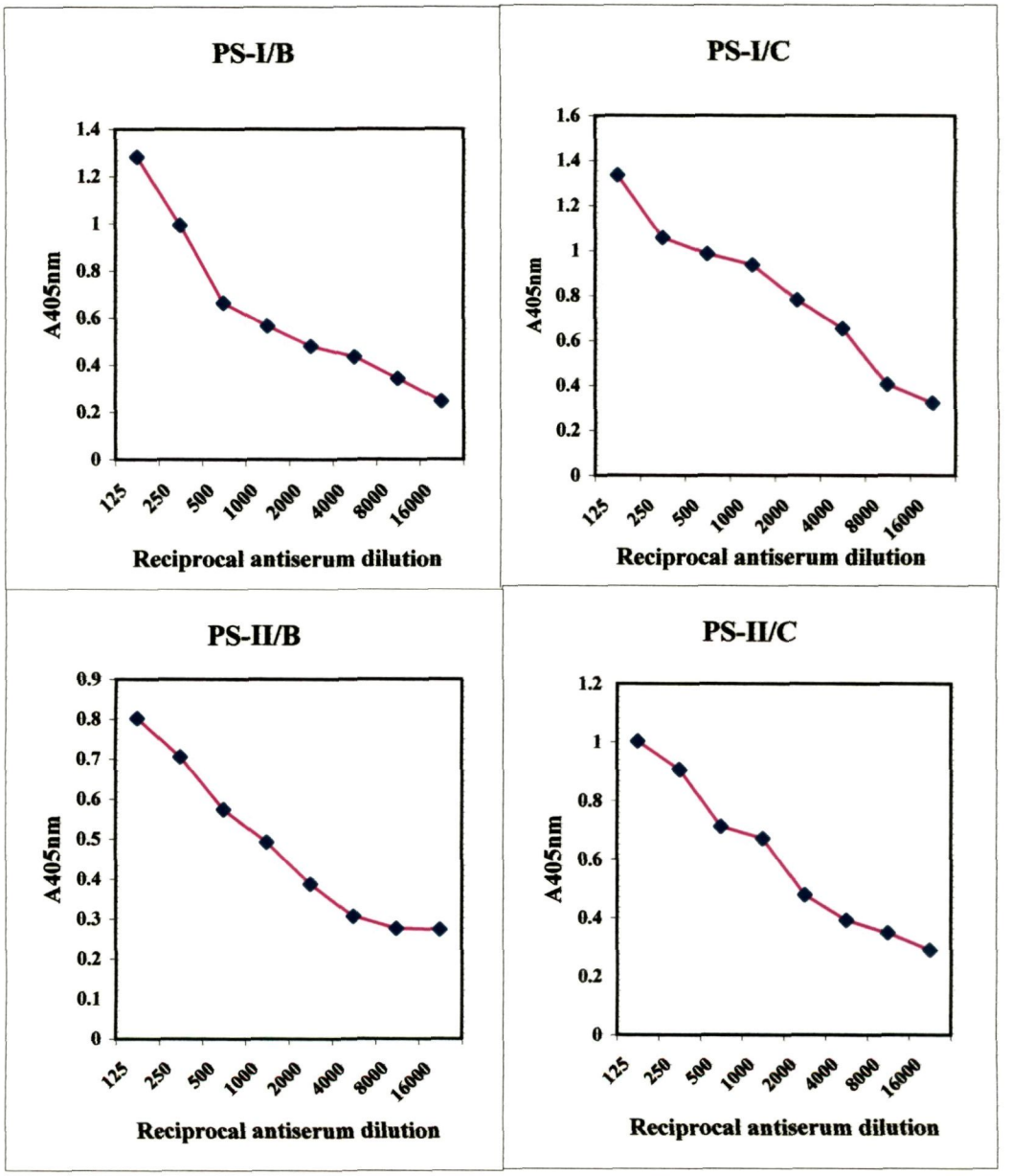
Effect of antigen dilution of *E.vexans* on ELISA reaction with homologous antisera



PS: Polyspecific antisera;I-Castleton;II-Hansqua;B-Second bleeding;C-Third bleeding

Fig.10

Effect of dilution of polyspecific antisera of *E.vexans* on ELISA reaction with homologous antigens



PS: Polyspecific antisera;I-Castleton;II-Hansqua;B-Second bleeding;C-Third bleeding

Fig.11

Table 26 : ELISA reaction with various concentrations of spore antigen and homologous polyclonal antiserum (PC-I)[a]

Antigen concentration ng/ml	Antisera (PC-I/B[b]) dilution	
	1:125	1:250
62.5	0.242 ± 0.02[c]	0.211 ± 0.03[c]
125	0.385 ± 0.06	0.266 ± 0.01
250	0.442 ± 0.05	0.311 ± 0.01
500	0.512 ± 0.06	0.361 ± 0.01
1000	0.590 ± 0.04	0.463 ± 0.07
2000	0.791 ± 0.04	0.586 ± 0.05
4000	0.880 ± 0.03	0.789 ± 0.06
8000	1.324 ± 0.02	1.213 ± 0.01

± Standard error

[a]Polyclonal antisera raised against basidiospores of blister infected leaf from Castleton Tea Estate ; [b]2nd bleeding; [c]Mean of three experiments

Table 27 : ELISA reaction with various concentrations of spore antigen and homologous polyclonal antiserum (PC-I)[a]

Antigen concentration ng/ml	Antisera (PC-I/C[b]) dilution	
	1:125	1:250
62.5	0.245 ± 0.05[c]	0.210 ± 0.02[c]
125	0.374 ± 0.05	0.232 ± 0.06
250	0.410 ± 0.05	0.310 ± 0.02
500	0.521 ± 0.02	0.354 ± 0.08
1000	0.584 ± 0.01	0.451 ± 0.07
2000	0.638 ± 0.01	0.582 ± 0.08
4000	0.781 ± 0.02	0.659 ± 0.06
8000	1.001 ± 0.01	1.984 ± 0.06

± Standard error

[a]Polyclonal antisera raised against basidiospores of blister infected leaf from Castleton Tea Estate ; [b]3rd bleeding ; [c]Mean of three experiments.

Table 28 : ELISA reaction with various concentrations of spore antigen and homologous polyclonal antiserum (PC-I)[a]

Antigen concentration ng/ml	Antisera (PC-I/D[b]) dilution	
	1:125	1:250
62.5	0.245 ± 0.05[c]	0.191 ± 0.02[c]
125	0.385 ± 0.08	0.241± 0.04
250	0.420 ± 0.04	0.321 ± 0.03
500	0.511 ± 0.02	0.512 ± 0.04
1000	0.598 ± 0.08	0.552 ± 0.02
2000	0.682 ± 0.03	0.588 ± 0.07
4000	0.795 ± 0.06	0.692 ± 0.01
8000	1.898 ± 0.09	1.791 ± 0.02

± Standard error

[a]Polyclonal antisera raised against basidiospores of blister infected leaf from Castleton Tea Estate ; [b]4th bleeding ; [c]Mean of three experiments.

4.4.2.1.3.2. Antiserum dilution

Antiserum dilutions ranging from1:125 to 1:16000 were tested against homologous antigen at a concentration of 8µg/ml.in ELISA reactions (Tables 29 to 32).

Table 29 : ELISA reaction with various dilution of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PC-I/A (First bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	1.021	1.008	1.022	1.005 ± 0.05
1:250	0.993	0.899	0.986	0.971 ± 0.04
1:500	0.872	0.805	0.866	0.847 ± 0.02
1:1000	0.776	0.713	0.618	0.702 ± 0.03
1:2000	0.581	0.556	0.582	0.573 ± 0.01
1:4000	0.441	0.458	0.419	0.439 ± 0.01
1:8000	0.401	0.336	0.382	0.373 ± 0.02
1:16000	0.209	0.218	0.199	0.208± 0.01

± Standard error.

Table 30 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen

Antisera dilution PC-I/B (Second bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	1.231	1.201	1.211	1.214 ± 0.01
1:250	0.986	0.913	0.817	0.905 ± 0.05
1:500	0.816	0.809	0.791	0.805± 0.01
1:1000	0.755	0.769	0.640	0.721 ± 0.04
1:2000	0.486	0.505	0.513	0.501 ± 0.01
1:4000	0.501	0.446	0.433	0.460 ± 0.02
1:8000	0.401	0.344	0.381	0.375 ± 0.02
1:16000	0.211	0.266	0.234	0.237 ± 0.02

± Standard error.

Table 31 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen.

Antisera dilution PC-I/C (Third bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	0.863	0.852	0.800	0.838 ± 0.04
1:250	0.711	0.732	0.713	0.718 ± 0.01
1:500	0.622	0.626	0.623	0.623 ± 0.01
1:1000	0.611	0.556	0.538	0.568 ± 0.02
1:2000	0.412	0.448	0.436	0.432 ± 0.02
1:4000	0.338	0.296	0.288	0.307 ± 0.01
1:8000	0.211	0.203	0.210	0.208 ± 0.01
1:16000	0.198	0.2012	0.189	0.196 ± 0.02

± Standard error.

Table 32 : ELISA reaction with various dilutions of anti-*E. vexans* antiserum and homologous antigen

Antisera dilution PC-I/D (Fourth bleeding)	Absorbance at 405 nm. Antigen Conc. (8µg/ ml)			
	Exp-1	Exp-2	Exp-3	Mean
1:125	0.658	0.707	0.711	0.692 ± 0.02
1:250	0.616	0.599	0.603	0.606 ± 0.01
1:500	0.561	0.512	0.498	0.523 ± 0.02
1:1000	0.468	0.488	0.471	0.475 ± 0.01
1:2000	0.403	0.411	0.412	0.408 ± 0.01
1:4000	0.358	0.336	0.321	0.338 ± 0.01
1:8000	0.228	0.215	0.195	0.212 ± 0.01
1:16000	0.162	0.168	0.153	0.161 ± 0.02

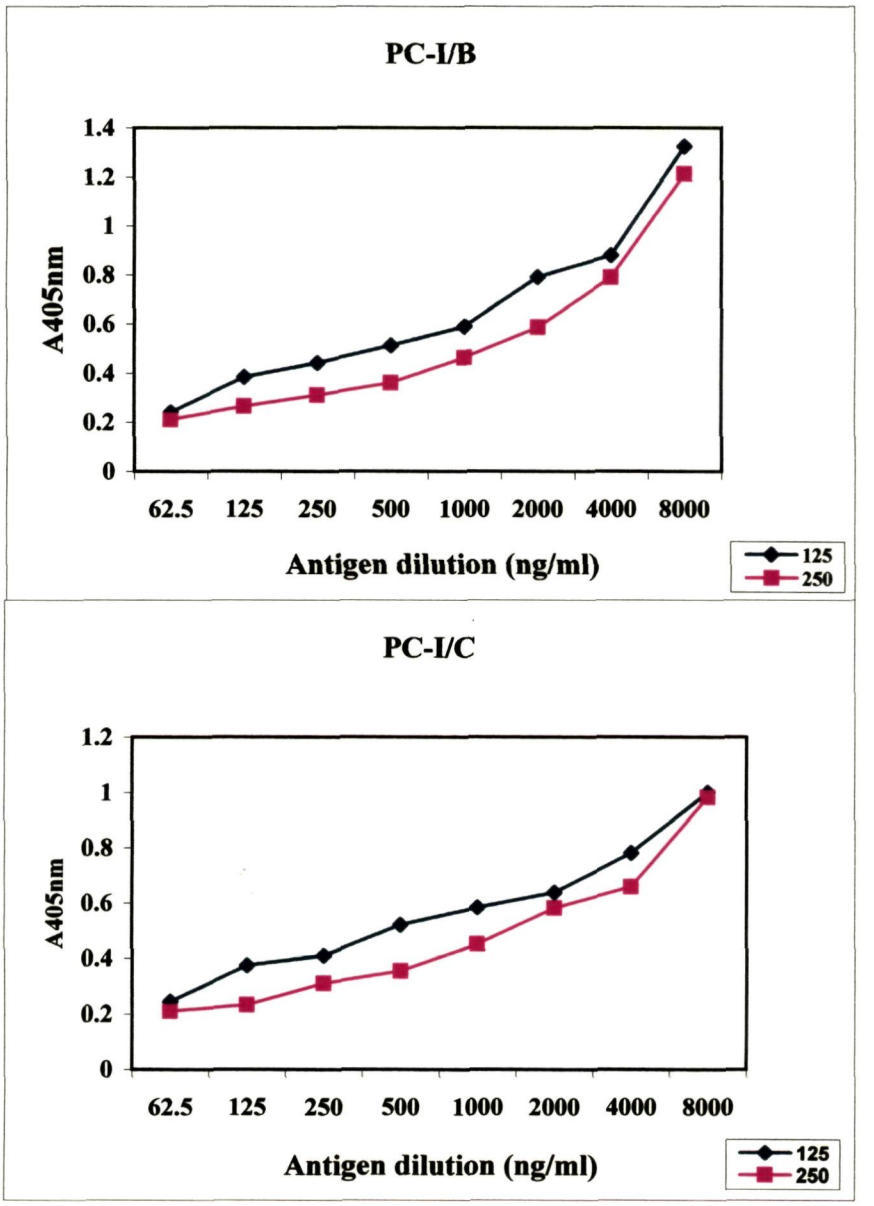
± Standard error.

ELISA values increased with increase of antigen concentration. Concentrations as low as 62.5 ng/ml could be easily detected by DAC-ELISA at both antisera dilutions (1:125 and 1:250) (Fig. 12). Where as absorbance values in ELISA reactions decreased from dilution of 1:125 to 1:16000 (Fig.13).

4.4.2.2. Comparison of ELISA reactivity among antigens of different tea varieties against antiserum of *E. vexans*.

Indirect ELISA could readily detect cross reaction between pathogen antisera and host. Healthy tea leaf antigens of 11 Darjeeling, 9 UPASI and 11 Tocklai varieties were tested separately against two antibodies (PS-I and PS-II 1st to 4th bleeding) of *E. vexans* using DAC-ELISA for detecton of cross reactive antigens shared between *E. vexans* and tea varieties. In each cases antigen concentration was 40µg/ml and antiserum dilution was made to 1:250. However, 12 varieties (4 each from Darjeeling, Tocklai and UPASI) were selected and tested against polyclonal antibody (PC-I/B) of *E. vexans* for the detection of CRA. Antigen concentration was kept as 40µg/ml and antisera dilution was made to 1:250. Experiments were repeated thrice under same conditions. Results have been presented from tables 33 to 39.

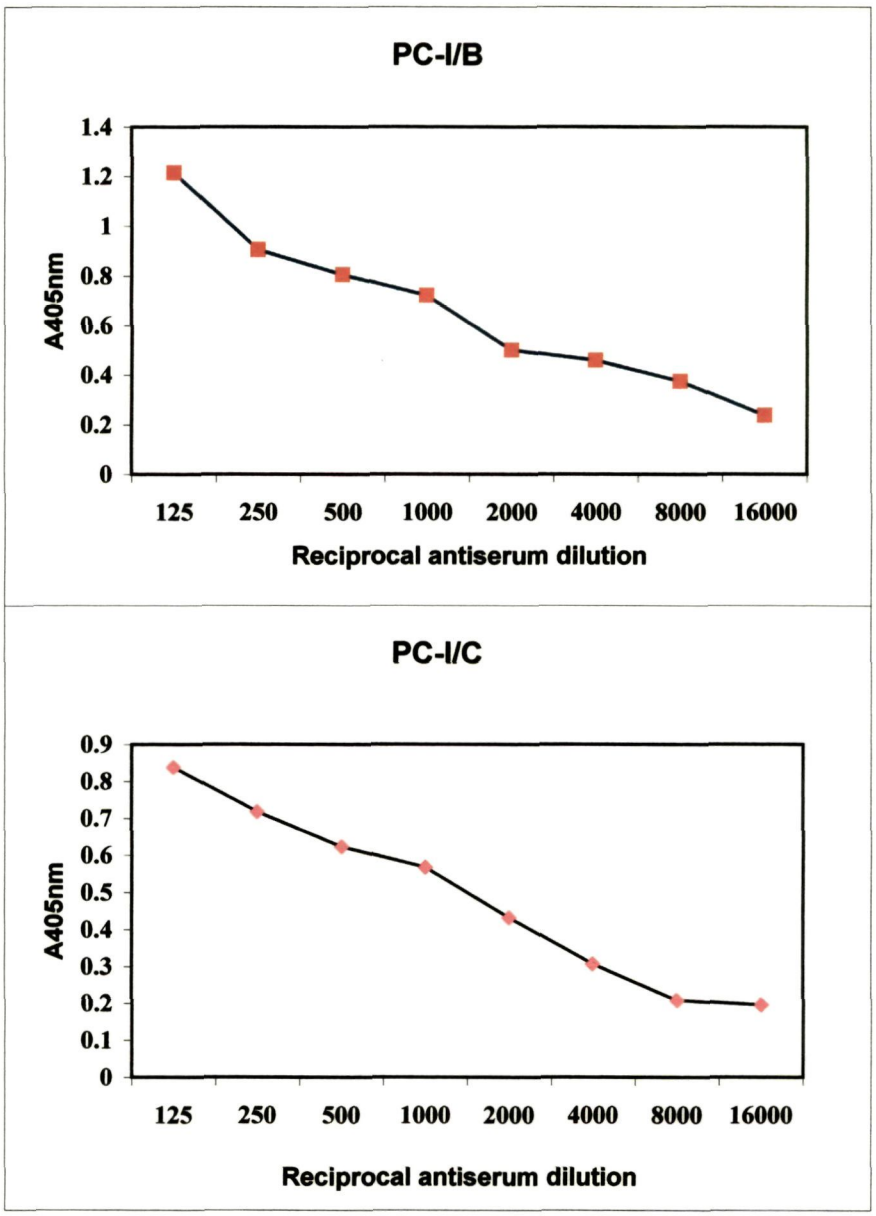
Effect of antigen dilution of *E.vexans* on ELISA reaction with homologous antisera



PC: Polyclonal antisera;I-Castleton;B-Second bleeding;C-Third bleeding

Fig.12

Effect of antisera dilution of *E.vexans* on ELISA reaction with homologous antigens



PC: Polyclonal antisera;I-Castleton;B-Second bleeding;C-Third bleeding

Fig.13

Table 33: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of Darjeeling varieties against *E.vexans* Polyspecific antiserum (PSI)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-I) antibody of *E.vexans* (1 : 250 dilution) PS-I/A	PS-I/B	PS-I/C	PS-I/D
CP1	0.568 ± 0.04	0.523 ± 0.10	0.511 ± 0.01	0.501 ± 0.01
S449	0.333 ± 0.08	0.323 ± 0.04	0.279 ± 0.03	0.200 ± 0.01
K1/1	0.635 ± 0.04	0.720 ± 0.03	0.777 ± 0.04	0.760 ± 0.01
AV2	0.872 ± 0.02	0.868 ± 0.03	0.860 ± 0.02	0.852 ± 0.02
T-78	0.652 ± 0.03	0.691 ± 0.02	0.638 ± 0.03	0.515 ± 0.02
Teen Ali-17/1/54	0.340 ± 0.02	0.310 ± 0.01	0.348 ± 0.01	0.350 ± 0.03
BS/7A/76	0.510 ± 0.02	0.558 ± 0.04	0.557 ± 0.04	0.535 ± 0.02
P1258	0.435 ± 0.01	0.480 ± 0.02	0.441 ± 0.05	0.448 ± 0.02
HV39	0.500 ± 0.06	0.551 ± 0.01	0.568 ± 0.01	0.551 ± 0.02
T135	0.245 ± 0.01	0.279 ± 0.01	0.233 ± 0.02	0.244 ± 0.03
B777	0.744 ± 0.01	0.750 ± 0.02	0.779 ± 0.02	0.771 ± 0.04
E.vexans[b]	1.220 ± 0.04	1.770 ± 0.01	1.560 ± 0.04	1.670 ± 0.04

± Standard error.

PSI – Polyspecific antibody prepared from blister infected tea leaves of Castleton Tea Estate (Hills).

PSI/A – 1st bleeding ; PS-I/B – 2nd Bleeding; PS-I/C – 3rd bleeding ; PS-I/D – 4th bleeding.

[a]Healthy tea leaves.

[b]Naturally blister infected tea leaves of Castleton Tea Estate.

Table 34: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of Tocklai varieties against *E.vexans* Polyspecific antiserum (PSI)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-I) antibody of *E.vexans* (1 : 250 dilution) PS-I/A	PS-I/B	PS-I/C	PS-I/D
TV9	0.471 ±.0.01	0.466 ± 0.02	0.494 ± 0.01	0.390 ± 0.03
TV20	0.500 ± 0.04	0.560 ± 0.04	0.550 ± 0.01	0.517 ± 0.04
TV22	0.442 ± 0.01	0.494 ± 0.04	0.406 ± 0.01	0.462 ± 0.01
TV23	0.380 ± 0.01	0.320 ± 0.01	0.300 ± 0.02	0.312 ± 0.03
TV18	0.622 ± 0.03	0.610 ± 0.02	0.654 ± 0.05	0.590 ± 0.03
TV25	0.511 ± 0.01	0.524 ± 0.01	0.500 ± 0.03	0.500 ± 0.02
TV26	0.210 ± 0.03	0.268 ± 0.02	0.200 ± 0.02	0.258 ± 0.02
TV27	0.666 ± 0.04	0.678 ± 0.03	0.678 ± 0.01	0.598 ± 0.03
TV28	0.649 ± 0.04	0.652 ± 0.01	0.658 ± 0.05	0.659 ± 0.01
TV29	0.325 ± 0.02	0.369 ± 0.06	0.310 ± 0.03	0.300 ± 0.02
TV30	0.440 ± 0.04	0.466 ± 0.01	0.480 ± 0.01	0.450 ± 0.01
E.vexans[b]	1.270 ± 0.04	1.750 ± 0.01	1.650 ± 0.04	1.566 ± 0.04

± Standard error.

PSI – Polyspecific antibody prepared from blister infected tea leaves of Castleton Tea Estate (Hills).

PSI/A – 1st bleeding ; PS-I/B – 2nd Bleeding; PS-I/C – 3rd bleeding ; PS-I/D – 4th bleeding.

[a]Healthy tea leaves.

[b]Naturally blister infected tea leaves of Castleton Tea Estate.

Table 35: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of UPASI varieties against *E.vexans* Polyspecific antiserum (PSI)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-I) antibody of *E.vexans* (1 : 250 dilution) PS-I/A	PS-I/B	PS-I/C	PS-I/D
UP2	0.464 ± 0.08	0.467 ± 0.02	0.428 ± 0.01	0.421 ± 0.06
UP3	0.424 ± 0.06	0.463 ± 0.02	0.440 ± 0.03	0.350 ± 0.01
UP8	0.780 ± 0.03	0.720 ± 0.01	0.728 ± 0.03	0.725 ± 0.01
UP9	0.610 ± 0.04	0.622 ± 0.04	0.667 ± 0.08	0.565 ± 0.02
UP17	0.632 ± 0.01	0.687 ± 0.04	0.640 ± 0.03	0.655 ± 0.04
UP26	0.598 ± 0.08	0.551 ± 0.01	0.560 ± 0.03	0.550 ± 0.03
BSS1	0.310 ± 0.08	0.345 ± 0.01	0.350 ± 0.07	0.348 ± 0.05
BSS2	0.430 ± 0.03	0.445 ± 0.04	0.450 ± 0.06	0.460 ± 0.06
BSS3	0.350 ± 0.06	0.340 ± 0.08	0.345 ± 0.01	0.360 ± 0.09
E.vexans[b]	1.320 ± 0.02	1.670 ± 0.05	1.560 ± 0.04	1.670 ± 0.03

± Standard error.

PSI – Polyspecific antibody prepared from blister infected tea leaves of Castleton Tea Estate (Hills).

PSI/A – 1st bleeding ; PS-I/B – 2nd Bleeding; PS-I/C – 3rd bleeding ; PS-I/D – 4th bleeding.

[a]Healthy tea leaves.

[b]Naturally blister infected tea leaves of Castleton Tea Estate.

Table 36: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of Darjeeling varieties against *E.vexans* Polyspecific antiserum (PSII)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-I) antibody of *E.vexans* (1 : 250 dilution)				
	PS-II/A	PS-II/B	PS-II/C	PS-II/D	PS-II/E
CP1	0.543 ± 0.06	0.599 ± 0.02	0.571 ± 0.04	0.362 ± 0.01	0.433 ± 0.01
S449	0.368 ± 0.02	0.461 ± 0.04	0.363 ± 0.06	0.314 ± 0.02	0.313 ± 0.03
K1/1	0.532 ± 0.05	0.561 ± 0.01	0.589 ± 0.04	0.447 ± 0.02	0.434 ± 0.02
AV2	0.849 ± 0.01	0.868 ± 0.09	0.898 ± 0.06	0.805 ± 0.08	0.712 ± 0.02
T-78	0.611 ± 0.02	0.658 ± 0.07	0.585 ± 0.01	0.571 ± 0.01	0.543 ± 0.02
Teen Ali-17/1/54	0.341 ± 0.02	0.338 ± 0.07	0.384 ± 0.03	0.340 ± 0.01	0.324 ± 0.06
BS/7A/76	0.567 ± 0.07	0.558 ± 0.02	0.566 ± 0.01	0.415 ± 0.05	0.375 ± 0.04
P1258	0.435 ± 0.02	0.406 ± 0.07	0.364 ± 0.06	0.377 ± 0.01	0.295 ± 0.05
HV39	0.408 ± 0.03	0.485 ± 0.01	0.481 ± 0.04	0.306 ± 0.01	0.276 ± 0.03
T135	0.321 ± 0.02	0.353 ± 0.03	0.349 ± 0.05	0.345 ± 0.05	0.304 ± 0.03
B777	0.713 ± 0.02	0.741 ± 0.04	0.669 ± 0.01	0.560 ± 0.01	0.539 ± 0.06
E.vexans[b]	1.509 ± 0.03	1.453 ± 0.08	1.466 ± 0.06	1.034 ± 0.02	0.956 ± 0.01

± Standard error.

PSII – Polyspecific antibody prepared from blister infected tea leaves of Hansqua Tea Estate (Plains).

PSII/A – 1st bleeding ; PS-II/B – 2nd Bleeding; PS-II/C – 3rd bleeding ;PS-II/D – 4th bleeding; PS-II/E – 5th bleeding.

[a]Healthy tea leaves; [b]Naturally blister infected tea leaves of Hansqua Tea Estate.

Table 37: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of Tocklai varieties against *E.vexans* Polyspecific antiserum (PSII)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-II) antibody of *E.vexans* (1 : 250 dilution)				
	PS-II/A	PS-II/B	PS-II/C	PS-II/D	PS-II/E
TV9	0.381 ± 0.02	0.410 ± 0.02	0.454 ± 0.03	0.333 ± 0.07	0.342 ± 0.03
TV20	0.506 ± 0.03	0.687 ± 0.07	0.556 ± 0.03	0.358 ± 0.06	0.341 ± 0.07
TV22	0.492 ± 0.02	0.447 ± 0.05	0.464 ± 0.01	0.461 ± 0.04	0.405 ± 0.01
TV23	0.389 ± 0.02	0.394 ± 0.06	0.393 ± 0.02	0.306 ± 0.07	0.279 ± 0.02
TV18	0.648 ± 0.02	0.639 ± 0.04	0.694 ± 0.02	0.593 ± 0.09	0.440 ± 0.02
TV25	0.545 ± 0.09	0.525 ± 0.05	0.562 ± 0.06	0.358 ± 0.02	0.579 ± 0.04
TV26	0.231 ± 0.01	0.238 ± 0.07	0.238 ± 0.05	0.239 ± 0.09	0.257 ± 0.01
TV27	0.706 ± 0.02	0.748 ± 0.02	0.678 ± 0.07	0.612 ± 0.06	0.724 ± 0.04
TV28	0.629 ± 0.01	0.655 ± 0.02	0.617 ± 0.04	0.607 ± 0.03	0.484 ± 0.06
TV29	0.349 ± 0.05	0.364 ± 0.01	0.400 ± 0.08	0.257 ± 0.05	0.236 ± 0.08
TV30	0.445 ± 0.05	0.461 ± 0.01	0.477 ± 0.03	0.379 ± 0.06	0.268 ± 0.01
E.vexans[b]	1.309 ± 0.03	1.461 ± 0.08	1.513 ± 0.06	1.203 ± 0.02	1.003 ± 0.01

± Standard error.

PSII – Polyspecific antibody prepared from blister infected tea leaves of Hansqua Tea Estate (Plains).

PSII/A – 1st bleeding ; PS-II/B – 2nd Bleeding; PS-II/C – 3rd bleeding ; PS-II/D – 4th bleeding; PS-II/E – 5th bleeding.

[a]Healthy tea leaves.

[b]Naturally blister infected tea leaves of Hansqua Tea Estate.

Table 38: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of UPASI varieties against *E.vexans* Polyspecific antiserum (PSII)

Antigens of host and parasite (40µg/ml) Tea varieties[a]	Polyspecific(PS-II) antibody of *E.vexans* (1 : 250 dilution)				
	PS-II/A	PS-II/B	PS-II/C	PS-II/D	PS-II/E
UP2	0.522 ±0.04	0.528 ± 0.02	0.546 ± 0.05	0.549 ± 0.01	0.459 ± 0.01
UP3	0.543 ±0.01	0.563 ± 0.04	0.563 ± 0.02	0.461 ± 0.01	0.407 ± 0.02
UP8	0.678 ±0.01	0.620 ± 0.06	0.608 ± 0.03	0.578 ± 0.05	0.552 ± 0.03
UP9	0.695 ±0.01	0.622 ± 0.08	0.667 ± 0.03	0.538 ± 0.01	0.509 ± 0.02
UP17	0.984 ±0.01	0.587 ± 0.01	0.840 ± 0.03	0.824 ± 0.02	0.626 ± 0.05
UP26	0.579 ±0.01	0.580 ± 0.02	0.516 ± 0.02	0.508 ± 0.03	0.420 ± 0.07
BSS1	0.343 ±0.05	0.357 ± 0.07	0.328 ± 0.02	0.269 ± 0.02	0.277 ± 0.05
BSS2	0.369 ±0.01	0.392 ± 0.03	0.360 ± 0.06	0.342 ± 0.01	0.309 ± 0.04
BSS3	0.348 ±0.08	0.329 ± 0.02	0.325 ± 0.06	0.353 ± 0.04	0.298 ± 0.02
E.vexans[b]	1.419 ±0.01	1.463 ± 0.08	1.369 ± 0.01	1.201 ± 0.02	1.009 ± 0.01

± Standard error.

PSII – Polyspecific antibody prepared from blister infected tea leaves of Hansqua Tea Estate (Plains).

PSII/A – 1st bleeding ; PS-II/B – 2nd Bleeding; PS-II/C – 3rd bleeding ; PS-II/D – 4th bleeding; PS-II/E – 5th bleeding

[a]Healthy tea leaves.

[b]Naturally blister infected tea leaves of Hansqua Tea Estate.

Table 39: Indirect ELISA values (A-405nm) of healthy tea leaf antigens of Darjeeling, Tocklai and UPASI varieties against *E.vexans* Polyclonal antiserum (PC-I/B)

Antigens of host and parasite (40µg/ml)		Polyclonal (PC-I/B) antibody of *E. vexans* (1:250 dilution)
Tea varieties[a]		
Darjeeling		
	CPI	0.532 ± 0.03[c]
	S449	0.387 ± 0.02
	Teen-Ali-17/1/54	0.263 ± 0.08
	AV2	0.604 ± 0.04
Tocklai		
	TV 18	0.571 ± 0.01
	TV 9	0.357 ± 0.04
	TV 22	0.360 ± 0.03
	TV 23	0.372 ± 0.02
UPASI		
	UP 2	0.312 ± 0.05
	UP 3	0.356 ± 0.01
	UP 8	0.447 ± 0.05
	UP 9	0.405 ± 0.01
Pathogen		
E. vexans[b]		1.467 ± 0.04

± Standard error.

PC-I/B = Polyclonal antibody raised against basidiospre of *E. vexans* / 2nd bleeding.

[a]Healthy tea leaves.

[b]Basidiospore antigen of *E. vexans*.

[c]Mean of three experiments.

Results reveal, that the absorbance value in ELISA varied with different varieties. Among the Darjeeling varieties AV2 and B777 showed maximum absorbance values. A slight decline in the A405nm reading has been observed in the varieties, from 1st bleeding to 4th bleeding. However, the absorbance value of AV2 was almost in a constant range (0.872 to 0.852) from 1st to 4th bleeding (Table 33). T135 showed least absorbance values.Similarly, among the Tocklai varieties TV18, TV28 and TV27 showed very high

absorbance readings, where as TV26 showed very low values. (0.210...0.258) (Table34). When the UPASI varieties were screened it was found that UP8, UP9 and UP17 showed very high CRA and BSS1, BSS2 and BSS3 showed the least absorbance values (Table 35).

Similar results were obtained when these varieties were tested with PS-II antiserum. Among the Darjeeling varieties AV2 and B777 showed high cross reactivity with *E. vexans*. The ELISA values increased from 1st bleeding to 3rd bleeding. A decline in the A 405 value was noted in 4th and 5th bleeding in all cases (Table 36). Among the Tocklai varieties, TV18, TV27 and TV28 showed high cross reactivity and TV26 the least (Table 38) and UP8, UP9 and UP17 showed high absorbance values (Table 38) and BSSI the least.

After screening the CRA reactions of all the 31 varieties against PSI and PSII (all the bleedings), twelve varieties were picked up and tested for the detection of CRA against polyclonal antibody (PC-I/B) of *E. vexans.* (Table 39). The absorbance values obtained in this case were comparatively lower than that of PSI and PSII. Among the four Darjeeling varieties AV2 showed highest CRA being (0.604), TV18 (0.571) and UP8 (0.447). Teen Ali-17/1/54, TV9 and UP2 showed very low absorbance values.

4.4.2.3. Reciprocal cross reaction of antisera of tea varieties non pathogen and leaf antigens (host and nonhost) fungal antigens (pathogen / non pathogen)

In previous experiments following DAC-ELISA, serological relationship between tea leaves and *E. vexans* have been detected using antiserum of the pathogen raised against blister infected tea leaves CTE, HTE, and basidiospores of *Exobasidium vexans.* Indirect ELISA could also readily detect major cross reactive antigens when reciprocal cross reaction with host antisera was performed. In this experiment, antisera raised against tea leaf antigens prepared from two varieties TV18 and CPI and were cross reacted with leaf antigens prepared from 12 Tea varieties (CP1, S449, Teen-Ali-17/1/54, AV2, TV18, TV9, TV22, TV23, UP2, UP3, UP8 and UP9), 4 non-host species (viz. *P. indica T. patula, L. leucearum* and *O. sativa*), as well as antigens prepared from blister postule (CTE), blister postule (HTE) and basidiospores of *E. vexans,* and non-pathogen (*Fusarium graminearum*). Simultaneously, antiserum raised against *F. graminearum* (non-pathogen) was also tested against the antigens of the above materials. Results have been presented in Table-40. In this experiment, A405 values in ELISA reaction involving

antigens and antisera of CPI, TV18 and *Fusarium graminearum* were 1.186, 1.230 and 1.112. When anti CPI antiserum was cross reacted with various antigens of host, nonhost, pathogen and nonpathogen, AV2, Teen-Ali-17/1/54 and UP3 gave higher absorbance than the other 9 varieties tested. TV18 antiserum gave higher absorbance in TV23, TV22, AV2 and Teen-Ali-17/1/54 when compared with the other 8 varieties tested. It is interesting to note that in cross reaction with anti TV18 antiserum and antigens of blister postules (CTE and HTE) and spore gave higher absorbance than CPI. Antiserum raised aginst non pathogen (*F. graminearum*) did not react significantly with tea leaf antigens, as lower absorbance values were detected in all cases.

4.5. Detection of *E. vexans* in naturally infected tea leaf tissues by indirect ELISA (PS-I and PS-II)

ELISA is one of the recent successful techniques for pathogen detection in the host where antiserum raised against the pathogen reacts with antigens of infected material to give high absorbance values. Differences in ELISA reading between infected and healthy antigens indicated the measure and extent of infection. In the present investigation, initially, it has been established that cross reactive antigens are present between tea leaves and *E. vexans*. In the next series of experiments, attempts have been made to detect *E. vexans* in infected leaf tissues of different tea varieties as well as after different hours of inoculation using DAC-ELISA formats.

For this purpose, naturally blister infected tea leaves were collected from Castleton Tea Estate and Hansqua Tea Estate and polyspecific antibodies PS-I/B and PS-II/B (2nd bleeding) raised against *E. vexans* for the respective gardens were used. Healthy leaves and blister infected leaves were collected randomly from 10 different sections of each garden. Blister incidence in those sections during collection period was also recorded. Antigens were prepared for healthy and infected leaf samples and tested using DAC-ELISA formats. Results presented in Table 41 and 42 revealed that infected leaf antigens had higher absorbance value in comparison to the healthy leaf antigens.

Table 40: Indirect ELISA values (A-405nm) of (host, nonhost) pathogen and non pathogen antigens reacted with antisera of tea varieties (CP1 and TV18) and non pathogen (*F. graminearum*)

Antigens (40µg/ml)	Antisera of host and non pathogen (1:250 dilution) CP 1	TV 18	*F. graminearum*
Host (Tea varieties)			
CPI	1.186 ± 0.04	0.611 ± 0.06	0.289 ± 0.08[a]
S449	0.653 ± 0.03	0.659 ± 0.04	0.260 ± 0.01
Teen Ali-17/1/54	0.796 ± 0.01	0.729 ± 0.03	0.258 ± 0.01
AV 2	0.730 ± 0.01	0.762 ± 0.03	0.246 ± 0.02
TV18	0.619 ± 0.02	1.230 ± 0.05	0.256 ± 0.03
TV9	0.631 ± 0.05	0.567 ± 0.05	0.260 ± 0.03
TV 22	0.695 ± 0.06	0.734 ± 0.04	0.232 ± 0.04
TV 23	0.670 ± 0.06	0.769 ± 0.03	0.293 ± 0.06
UP 2	0.635 ± 0.03	0.625 ± 0.03	0.232 ± 0.05
UP 3	0.749 ± 0.01	0.708 ± 0.01	0.256 ± 0.05
UP 8	0.655 ± 0.01	0.712 ± 0.01	0.203 ± 0.02
UP 9	0.629 ± 0.02	0.609 ± 0.08	0.283 ± 0.01
Pathogen (*E. vexans*)			
Blister postule (CTE)	0.433 ± 0.06	0.543 ± 0.01	0.338 ± 0.04
Blister postule (HTE)	0.321 ± 0.01	0.552 ± 0.08	0.363 ± 0.01
Spore	0.457 ± 0.02	0.551 ± 0.03	0.307 ± 0.02
Non pathogen			
F. graminearum	0.274 ± 0.01	0.210 ± 0.06	1.112 ± 0.05
Non host			
Phylanthus indica	0.257 ± 0.01	0.236 ± 0.05	0.243 ± 0.04
Tagetus patula	0.260 ± 0.03	0.226 ± 0.04	0.315 ± 0.08
Leucenia leucearum	0.251 ± 0.01	0.267 ± 0.07	0.287 ± 0.05
Oryza sativa	0.272 ± 0.02	0.230 ± 0.01	0.237 ± 0.04

± Standard error.

[a]Average of 3 experiments

CTE – Blister infected leaf collected from Castleton Tea Estate.

HTE – Blister infected leaf collected from Hansqua Tea Estate.

Table 41: Indirect ELISA values obtained by the reaction of anti *E. vexans* antiserum (PS-I/B) and naturally blister infected tea leaves from Castleton Tea Estate.

Antigen concentration (40µg/ml)	Polyspecific (PS-I/B)[a] Antiserum (1:250) dilution A405 nm.		Blister blight incidence (%)
Castleton Tea Estate	**Healthy**	**Infected[b]**	
Section - 1	0.328 ± 0.03	0.804 ± 0.02	42.3
Section - 2	0.334 ± 0.03	1.321 ± 0.02	75.6
Section - 3	0.347 ± 0.05	1.070 ± 0.02	51.6
Section - 4	0.247 ± 0.01	0.667 ± 0.01	31.8
Section - 5	0.295 ± 0.02	1.309 ± 0.02	71.2
Section - 6	0.384 ± 0.02	0.729 ± 0.03	29.9
Section - 7	0.405 ± 0.02	1.063 ± 0.08	53.8
Section - 8	0.432 ± 0.02	1.228 ± 0.01	66.8
Section - 9	0.389 ± 0.03	1.493 ± 0.02	80.5
Section - 10	0.250 ± 0.06	0.971 ± 0.02	55.1

± Standard error

[a]Mean of 3 experiments

[b]Samples were collected during August-October

PS-I/B – Polyspecific antisera raised against blister infected tea leaves from Castleton Tea Estate / 2nd bleeding.

Table 42: Indirect ELISA values obtained by the reaction of anti *E. vexans* antiserum (PS-II/B) and naturally blister infected tea leaves from Hansqua Tea Estate.

Antigen concentration (40µg/ml)	Polyspecific (PS-II/B)[a] Antiserum (1:250) dilution A405 nm.		Blister blight incidence (%)
Hansqua Tea Estate	**Healthy**	**Infected[b]**	
Section - 1	0.436 ± 0.08	0.846 ± 0.01	46.6
Section - 2	0.546 ± 0.04	1.237 ± 0.03	60.3
Section - 3	0.352 ± 0.02	1.085 ± 0.02	59.8
Section - 4	0.368 ± 0.02	0.621 ± 0.02	30.4
Section - 5	0.357 ± 0.04	1.986 ± 0.01	46.6
Section - 6	0.435 ± 0.02	1.038 ± 0.05	72.5
Section - 7	0.381 ± 0.02	1.379 ± 0.04	75.8
Section - 8	0.435 ± 0.02	0.850 ± 0.01	22.1
Section - 9	0.462 ± 0.07	1.523 ± 0.01	68.8
Section - 10	0.516 ± 0.02	1.081 ± 0.05	70.3

± Standard error

[a]Mean of 3 experiments ;

[b]Samples were collected during December - February

PS-II/B – Polyspecific antisera raised against blister infected tea leaves from Hansqua Tea Estate / 2nd bleeding.

4.6. Detection of *E. vexans* in artificially inoculated tea leaf tissues by indirect ELISA (PS-I, PS-II and PC-I)

Twelve tea varieties, 4 each from Darjeeling (CP1, S449, HV39 and AV2), Tocklai (TV18, TV28, TV22 and TV29) and UPASI (UP2, UP8, UP26 and BSS2) were selected for this experiment. Young leaves (1st and 2nd) of five seedlings each of 12 varieties were artificially inoculated with *E. vexans* as mentioned in Materials and Methods (3.3.3), and incubated at 25°C for 12 days. During this period, infection was optimum showing curling and external swelling of the whole leaves of some of the varieties (TV18, TV29, UP8, TV22 and TV28) as shown in plates 11 and 12. After twelve days of incubation, antigens were prepared from healthy and artifically inoculated leaves of 12 varieties and tested against polyspecific (PS-I/B & PS-II/B) and polyclonal (PC-I/B) antibodies separately using DAC-ELISA formats. In this case, 40µg/ml antigen concentration and 1:250 dilution of antiserum were used. Results have been presented in Tables 43 to 45. Response of healthy and artificially inoculated Darjeeling, Tocklai and UPASI varieties tested against polyspecific (PS-I/B and PS-II/B) and polyclonal (PC-I/B) antibodies of *E. vexans* have been summarized in Figs. 14, 15 and 16 respectively. It is interesting to note that in ELISA reaction, A405 values of artificially inoculated leaves of CP1, HV39 and AV2 were always higher than the respective healthy control against all three antibodies of *E. vexans* tested (Fig-14). Similarly, all four Tocklai varieties inoculated with *E. vexans* showed higher absorbance values (A405) than the respective healthy control (Fig-15). However, among the four UPASI varieties tested inoculated leaves of UP8 and UP26 showed higher absorbance in ELISA reaction (Fig-16).

Symptom appearence after 12 days of inoculation with *E. vexans* in TV18, TV22, TV28, TV29, UPASI-I8, UPASI-26, AV2, HV39 and CP1 and their ELISA response against pathogen antibodies clearly demonstrate the entry and establishment of the pathogen in leaf tissues.

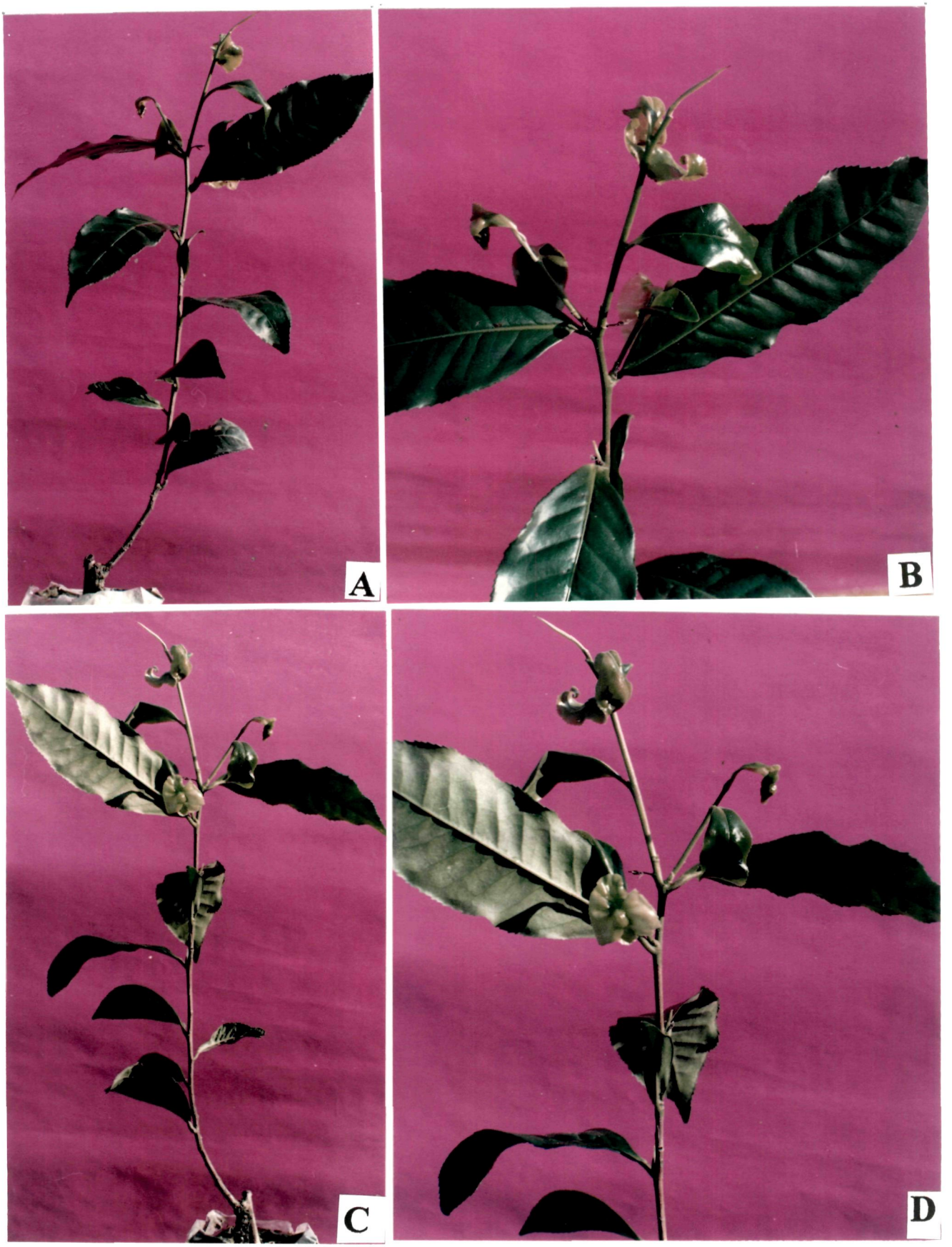


Plate 11 (Figs. A-D): Tea varieties (**A&B**) TV-22, (**C&D**) TV-28 artificially inoculated with *E.vexans* showing symptoms after 12 days of inoculation.

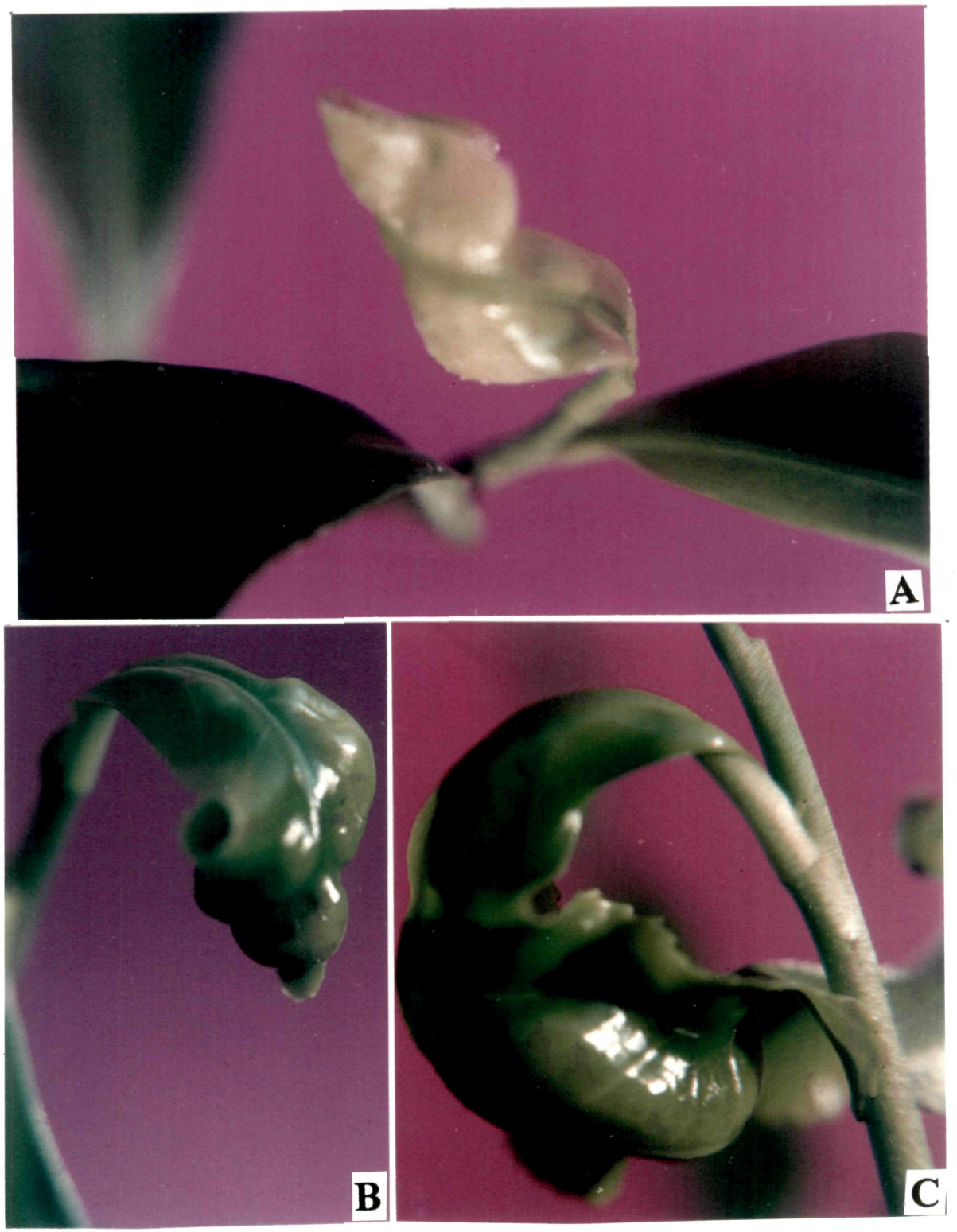


Plate 12 (Figs. A-C): Tea varieties artificially inoculated with *E.vexans* showing symptoms after 12 days of inoculation.
(**A**) TV-18; (**B**) TV-29; (**C**) UPASI-8

Table 43: Indirect ELISA values (A-405nm) showing reaction of polyspecific (PS-I/B) antiserum of *E. vexans* with healthy and artificially inoculated tea leaf antigens.

Antigen concentration (40µg/ml)	Absorbance at A405nm[a]	
	Polyspecific antibody (PS-I/B) of *E. vexans* (1:250 dilution)	
Tea varieties	Healthy	Inoculated[b]
Darjeeling		
CP1	0.548 ± 0.07	0.833 ± 0.02
S449	0.342 ± 0.03	0.385 ± 0.02
HV39	0.555 ± 0.01	0.848 ± 0.04
AV2	0.821 ± 0.01	1.918 ± 0.03
Tocklai		
TV18	0.652 ± 0.02	1.847 ± 0.05
TV28	0.633 ± 0.03	0.847 ± 0.01
TV22	0.455 ± 0.03	0.816 ± 0.01
TV29	0.328 ± 0.02	0.575 ± 0.02
UPASI		
UP2	0.464 ± 0.01	0.541 ± 0.04
UP8	0.753 ± 0.02	1.357 ± 0.04
UP26	0.531 ± 0.01	1.058 ± 0.04
BSS2	0.463 ± 0.02	0.468 ± 0.05

± Standard error

PS-I/B – Polyspecific antibody prepared from blister infected tea leaves of Castleton Tea Estate/ 2nd bleeding.

[a]Average of 3 readings

[b]12 days after inoculation

Table 44: Indirect ELISA values (A-405nm) showing reaction of polyspecific (PS-II/B) antiserum of *E. vexans* with healthy and artificially inoculated tea leaf antigens

Antigen concentration (40µg/ml)	Absorbance at A405nm[a]	
	Polyspecific antibody (PS-II/B) of *E. vexans* (1:250 dilution)	
Tea varieties	**Healthy**	**Inoculated[b]**
Darjeeling		
CP1	0.551 ± 0.03	0.789 ± 0.02
S449	0.280 ± 0.04	0.316 ± 0.05
HV39	0.456 ± 0.02	0.691 ± 0.04
AV2	0.858 ± 0.02	1.863 ± 0.02
Tocklai		
TV18	0.669 ± 0.03	1.816 ± 0.05
TV28	0.634 ± 0.03	0.785 ± 0.05
TV22	0.475 ± 0.04	0.801 ± 0.06
TV29	0.387 ± 0.05	0.502± 0.03
UPASI		
UP2	0.543 ± 0.04	0.586 ± 0.04
UP8	0.671 ± 0.03	1.281 ± 0.05
UP26	0.554 ± 0.03	1.101 ± 0.05
BSS2	0.369 ± 0.01	0.446 ± 0.04

± Standard error

PS-II/B – Polyspecific antibody prepared from blister infected tea leaves of Hansqua Tea Estate/ 2nd bleeding.

[a]Average of 3 readings

[b]12 days after inoculation.

Table 45: Indirect ELISA values (A-405nm) showing reaction of polyclonal (PC-I/B) antiserum of *E. vexans* with healthy and artificially inoculated tea leaf antigens.

Antigen concentration (40µg/ml)	Absorbance at A405nm[a]	
	Polyclonal antibody (PC-I/B) of *E. vexans* (1:250 dilution)	
Tea varieties	**Healthy**	**Inoculated[b]**
Darjeeling		
CP1	0.503 ± 0.03	0.648 ± 0.03
S449	0.378 ± 0.02	0.387 ± 0.01
HV39	0.356 ± 0.08	0.556 ± 0.05
AV2	0.588 ± 0.05	1.346 ± 0.02
Tocklai		
TV18	0.572 ± 0.06	1.220 ± 0.06
TV28	0.557 ± 0.04	1.110 ± 0.02
TV22	0.452 ± 0.01	0.811 ± 0.08
TV29	0.302 ± 0.02	0.406± 0.09
UPASI		
UP2	0.412 ± 0.03	0.426 ± 0.05
UP8	0.448 ± 0.04	1.210 ± 0.02
UP26	0.447± 0.05	0.811 ± 0.01
BSS2	0.366 ± 0.06	0.406 ± 0.09

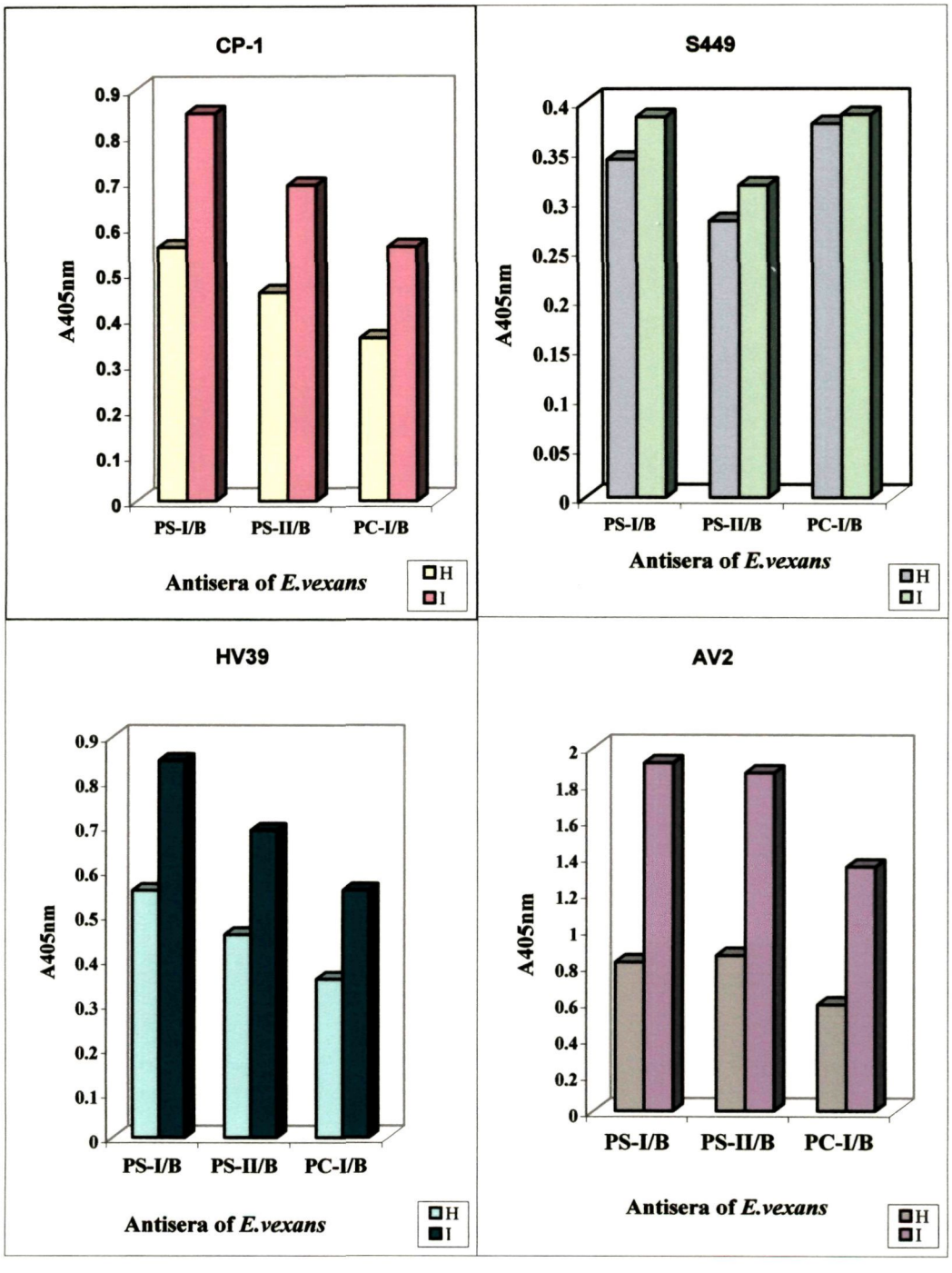
± Standard error

PS-II/B – Polyspecific antibody prepared from blister infected tea leaves of Hansqua Tea Estate/ 2nd bleeding.

[a]Average of 3 readings

[b]12 days after inoculation.

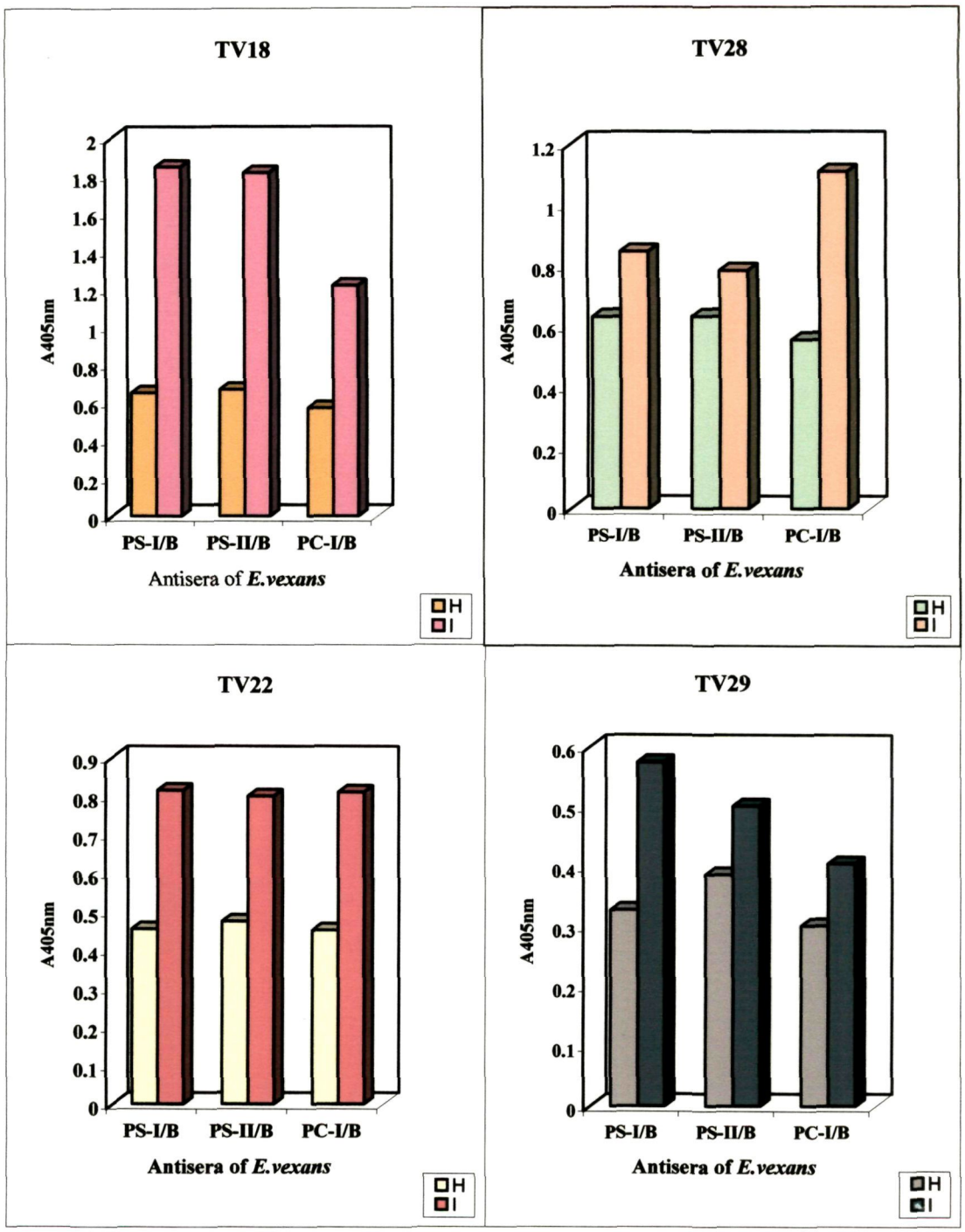
DAC-ELISA responses of E.vexans antiserum with healthy and inoculated tea leaf antigens of Darjeeling varieties



PS: Polyspecific PC-Polyclonal antisera;I-Castleton, II-Hansqua;B-Second bleeding

Fig.14

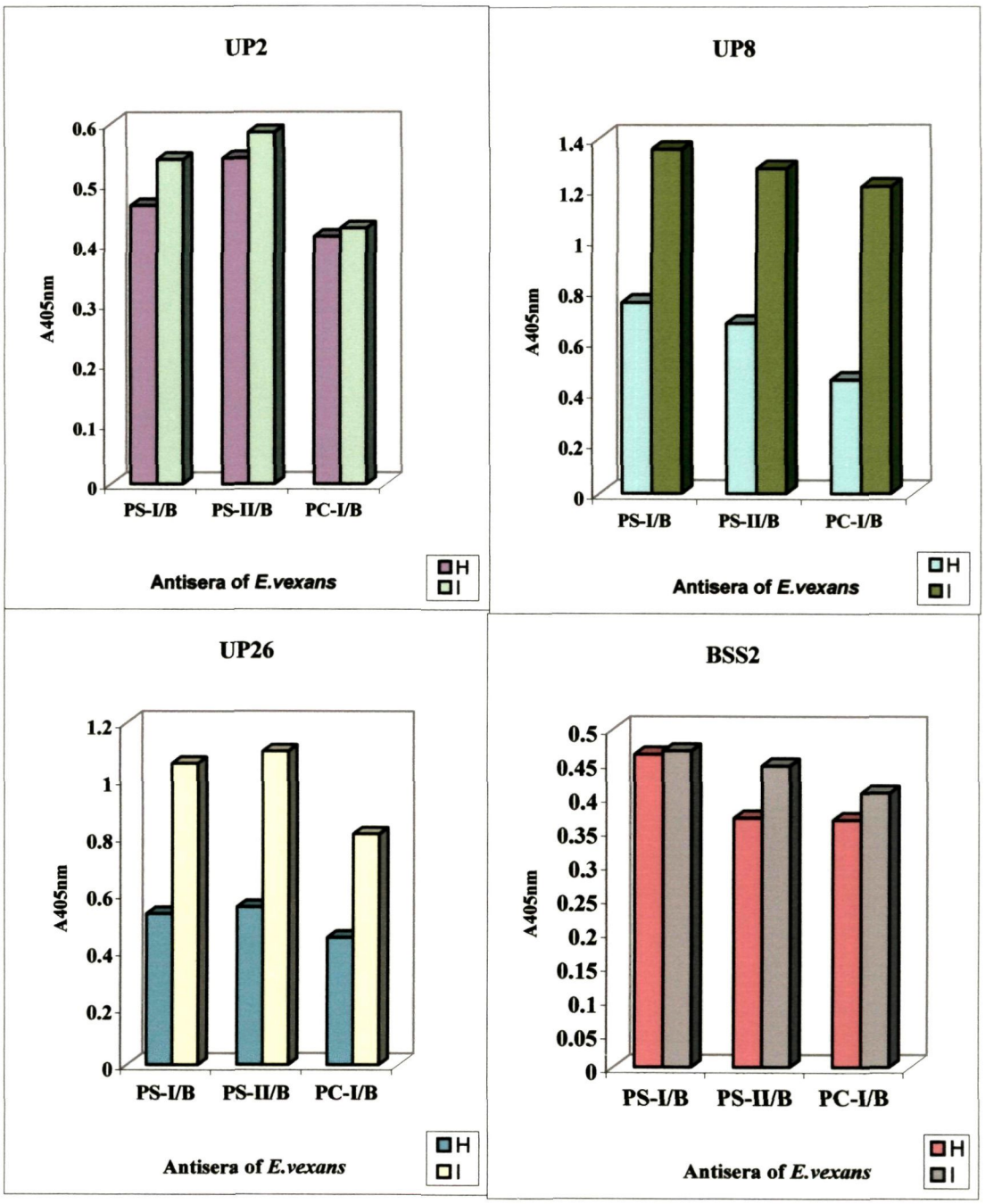
DAC-ELISA responses of E.vexans antiserum with healthy and inoculated tea leaf antigens of Tocklai varieties



PS: Polyspecific PC-Polyclonal antisera;I-Castleton, II-Hansqua;B-Second bleeding

Fig.15

DAC-ELISA responses of *E.vexans* antiserum with healthy and inoculated tea leaf antigens of UPASI varieties



PS: Polyspecific PC-Polyclonal antisera;I-Castleton, II-Hansqua;B-Second bleeding

Fig.16

4.7. Detection of *E. vexans* in artificially inoculated tea leaf tissues at different times after inoculation, by indirect ELISA (PS-I, PS-II and PC-I)

Before the appearence of the symptom, whether DAC-ELISA formats can be used for pathogen detection, following experiments were designed. In this experiment, three highly susceptible tea varieties, one each from Darjeeling (AV2), Tocklai (TV18) and UPASI (UP8) were selected. Sixty seedlings, each of three varieties were artificially inoculated as mentioned earlier, incubated at 25^{0}C and at an interval of 24h upto 12 days, healthy and inoculated leaf samples were collected, antigens were prepared and finally tested against all three antibodies [Polyspecific- (PS-I/B and PS-II/B); Polyclonal (PC-I/B)]. Results has been presented in Table 46 to 48.

When these three varieties were compared against all three antibodies, differences between healthy and infected samples were noticed after 48 h of inoculation for UP8 and TV18 while 96h of inoculation for AV2 using polyspecific antibodies (PS-I/B and PS-II/B) as evidenced in Figs. 17,18 and 19. However, using polyclonal antibody (PC-I/B) pathogen detection was possible within 24h for UP8, 48h for TV18 and 72h for AV2 (Fig.-19). Symptoms were not visible at this period.

4.8. Immunofluorescence

Fluorescent antibody labelling with fluorescein isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. In the present study CRA shared by *C. sinensis* and *E. vexans* was detected by immunodiffusion and DAC-ELISA. It was then decided to determine the tissue and cellular location of CRA in fragile callus, leaf tissues (healthy and infected) and basidiospores of *E. vexans*. Detailed methods of antibody staining of leaf sections and basidiospores of the pathogen have already been discussed under Material and Methods. Leaf sections and spore preparations were photographed under UV-fluorescence.

Table 46: Indirect ELISA reaction of anti-*E. vexans* antisera (PS-I/B,PS-II/B & PC-I/B) with healthy and artificially inoculated tea leaf (AV2) antigen at different periods.

Time after inoculation (in days)	Anti-*E. vexans* antiserum (1:250 dilution)					
	PS-I/B		PS-II/B		PC-I/B	
	H	I	H	I	H	I
1 d	0.839 ± 0.03	0.878 ± 0.08	0.839 ± 0.03	0.885 ± 0.01	0.664 ± 0.05	0.680 ± 0.06
2 d	0.838 ± 0.03	0.885 ± 0.02	0.838 ± 0.03	0.940 ± 0.04	0.642 ± 0.03	0.688 ± 0.04
3 d	0.852 ± 0.04	0.949 ± 0.08	0.853 ± 0.05	0.968 ± 0.07	0.661 ± 0.02	0.760 ± 0.02
4 d	0.822 ± 0.028	0.987 ± 0.03	0.822 ± 0.08	1.243 ± 0.05	0.641 ± 0.08	0.866 ± 0.02
5 d	0.820 ± 0.02	1.008 ± 0.02	0.820 ± 0.02	1.280 ± 0.03	0.651 ± 0.02	0.981 ± 0.05
6 d	0.830 ± 0.04	1.198 ± 0.06	0.830 ± 0.04	1.403 ± 0.08	0.615 ± 0.01	1.021 ± 0.08
7 d	0.850 ± 0.01	1.201 ± 0.02	0.850 ± 0.01	1.441 ± 0.03	0.658 ± 0.02	1.115 ± 0.03
8 d	0.838 ± 0.06	1.253 ± 0.08	0.838 ± 0.06	1.630 ± 0.01	0.655 ± 0.03	1.124 ± 0.03
9 d	0.841 ± 0.05	1.256 ± 0.04	0.841 ± 0.05	1.638 ± 0.03	0.612 ± 0.04	1.131 ± 0.01
10 d	0.855 ± 0.08	1.356 ± 0.03	0.855 ± 0.08	1.909 ± 0.01	0.680 ± 0.03	1.220 ± 0.01
11 d	0.885 ± 0.08	1.919 ± 0.04	0.885 ± 0.08	1.918 ± 0.01	0.691 ± 0.01	1.210 ± 0.06
12 d	0.831 ± 0.04	1.980 ± 0.06	0.831 ± 0.04	1.980 ± 0.01	0.648 ± 0.05	1.320 ± 0.02

± Standard error

PS-I/B – Polyspecific antibody raised from blister infected leaves of Castleton Tea Estate/2nd bleeding.

PS-II/B – Polyspecific antibody raised from blister infected leaves of Hansqua Tea Estate/2nd bleeding.

PC-I/B – Polyclonal antibody raised from basidiospores of blister infected leaves of Castleton Tea Estate /2nd bleeding.

Table 47: Indirect ELISA reaction of anti-*E. vexans* antisera (PS-I/B,PS-II/B & PC-I/B) with healthy and artificially inoculated tea leaf (TV18) antigen at different periods.

Time after inoculation (in days)	Anti-*E. vexans* antiserum (1:250 dilution)					
	PS-I/B		PS-II/B		PC-I/B	
	H	I	H	I	H	I
1 d	0.648±0.03	0.677±0.02	0.668±0.03	0.730±0.03	0.564±0.08	0.565±0.03
2 d	0.666±0.03	0.834±0.02	0.686±0.01	0.866±0.04	0.562±0.04	0.773±0.02
3 d	0.683±0.05	0.853±0.01	0.681±0.02	0.859±0.06	0.591±0.03	0.789±0.08
4 d	0.665±0.04	0.939±0.05	0.683±0.03	0.924±0.04	0.582±0.02	0.887±0.02
5 d	0.655±0.02	1.165±0.02	0.679±0.04	1.032±0.03	0.579±0.03	0.901±0.04
6 d	0.652±0.02	1.195±0.03	0.684±0.01	1.101±0.06	0.558±0.04	0.925±0.02
7 d	0.668±0.01	1.226±0.04	0.674±0.04	1.116±0.06	0.566±0.08	0.998±0.01
8 d	0.651±0.08	1.398±0.06	0.681±0.02	1.326±0.04	0.565±0.05	1.002±0.04
9 d	0.647±0.01	1.452±0.08	0.695±0.01	1.465±0.02	0.588±0.04	1.058±0.06
10 d	0.665±0.08	1.629±0.01	0.690±0.02	1.500±0.04	0.582±0.02	1.115±0.02
11 d	0.671±0.03	1.918±0.05	0.691±0.03	1.788±0.04	0.589±0.03	1.281±0.04
12 d	0.673±0.01	1.965±0.02	0.698±0.03	2.009±0.02	0.572±0.06	1.311±0.05

±Standard error

PS-I/B – Polyspecific antibody raised from blister infected leaves of Castleton Tea Estate/2nd bleeding.

PS-II/B – Polyspecific antibody raised from blister infected leaves of Hansqua Tea Estate/2nd bleeding.

PC-I/B – Polyclonal antibody raised from basidiospores of blister infected leaves of Castleton Tea Estate /2nd bleeding.

Table 48: Indirect ELISA reaction of anti-*E. vexans* antisera (PS-I/B,PS-II/B & PC-I/B) with healthy and artificially inoculated tea leaf (UP8) antigen at different periods

Time after inoculation (in days)	Anti *E. vexans* antiserum (1:250 dilution)					
	PS-I/B		PS-II/B		PC-I/B	
	H	I	H	I	H	I
1 d	0.780 ±0.04	0.805 ±0.04	0.680 ±0.01	0.681 ±0.03	0.441 ±0.01	0.667 ±0.02
2 d	0.739 ±0.08	0.883 ±0.03	0.639 ±0.08	0.736 ±0.05	0.425 ±0.08	0.839 ±0.04
3 d	0.721 ±0.01	0.976 ±0.03	0.641 ±0.01	0.830 ±0.02	0.456 ±0.01	0.851 ±0.01
4 d	0.756 ±0.01	1.105 ±0.02	0.656 ±0.02	0.918 ±0.05	0.411 ±0.02	0.989 ±0.06
5 d	0.791 ±0.02	1.112 ±0.03	0.671 ±0.02	1.108 ±0.03	0.438 ±0.02	0.985 ±0.03
6 d	0.710 ±0.08	1.211 ±0.01	0.650 ±0.08	1.121 ±0.09	0.4662 ±0.02	1.013 ±0.04
7 d	0.720 ±0.03	1.202 ±0.01	0.622 ±0.03	1.201 ±0.01	0.435 ±0.01	1.021 ±0.04
8 d	0.780 ±0.04	1.314 ±0.03	0.680 ±0.04	1.212 ±0.03	0.412 ±0.01	1.118 ±0.01
9 d	0.725 ±0.04	1.320 ±0.03	0.625 ±0.04	1.321 ±0.04	0.455 ±0.04	1.120 ±0.03
10 d	0.731 ±0.03	1.341 ±0.02	0.631 ±0.03	1.351 ±0.01	0.423 ±0.04	1.122 ±0.04
11 d	0.725 ±0.03	1.336 ±0.02	0.625 ±0.03	1.356 ±0.02	0.481 ±0.04	1.211 ±0.02
12 d	0.781 ±0.01	1.351 ±0.03	0.681 ±0.08	1.366 ±0.03	0.448 ±0.04	1.210 ±0.01

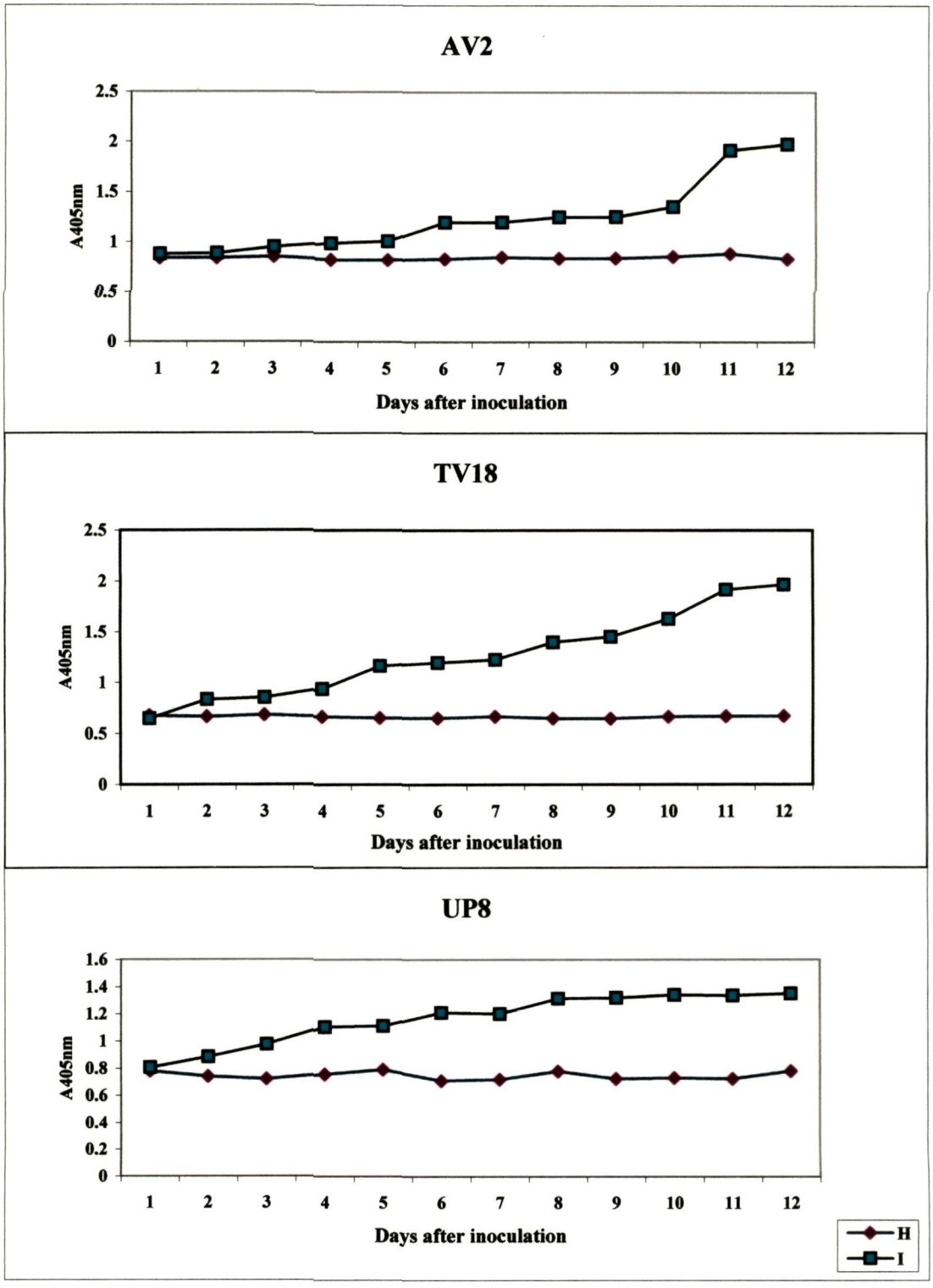
±Standard error

PS-I/B – Polyspecific antibody raised from blister infected leaves of Castleton Tea Estate/2nd bleeding.

PS-II/B– Polyspecific antibody raised from blister infected leaves of Hansqua Tea Estate/2nd bleeding.

PC-I/B – Polyclonal antibody raised from basidiospores of blister infected leaves of Castleton Tea Estate /2nd bleeding.

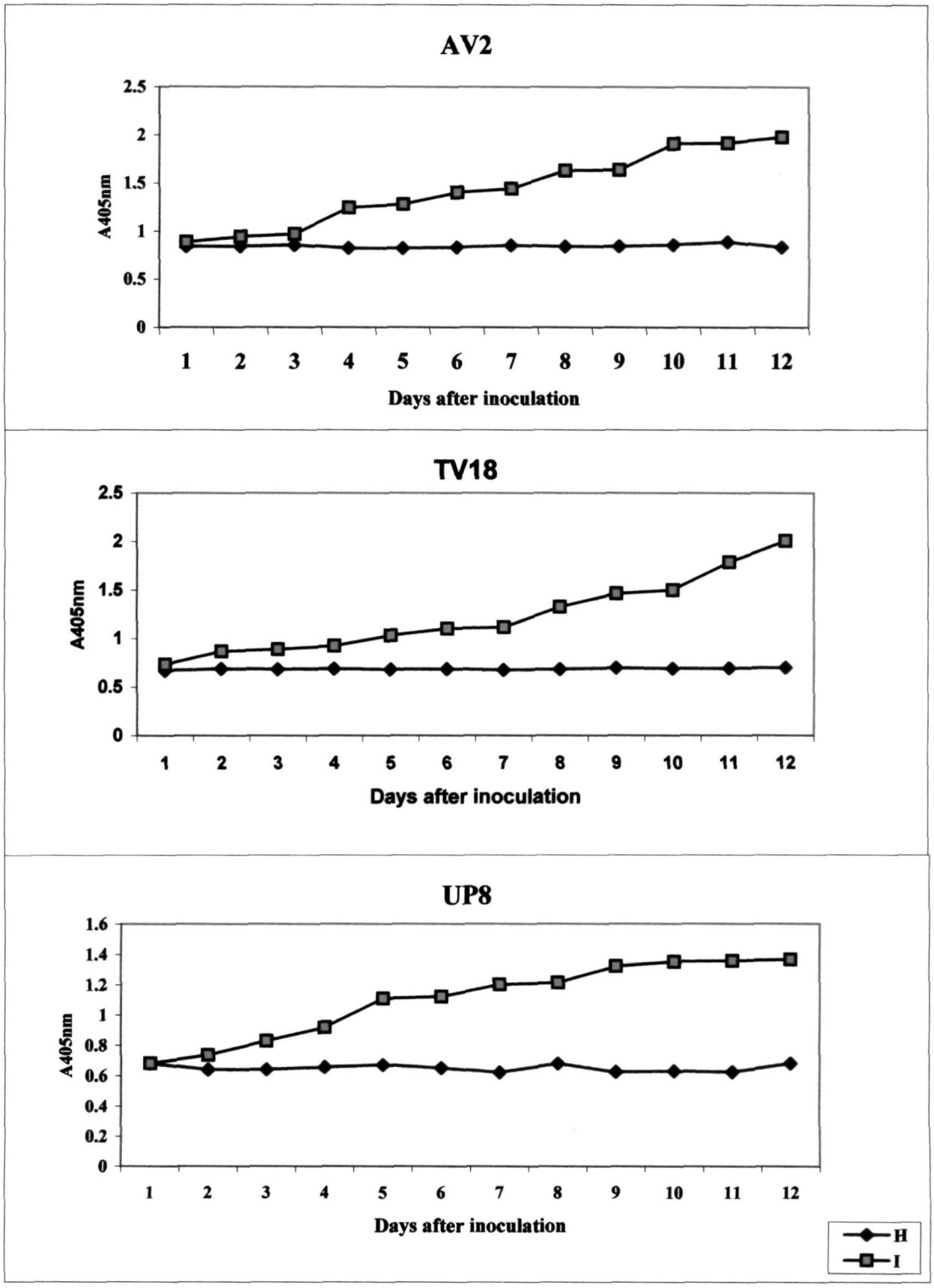
DAC-ELISA responses of *E.vexans* antiserum (PS-I/B) with healthy and inoculated tea leaf antigens at different intervals



PS: Polyspecific;
I-Castleton;B-Second bleeding

Fig.17

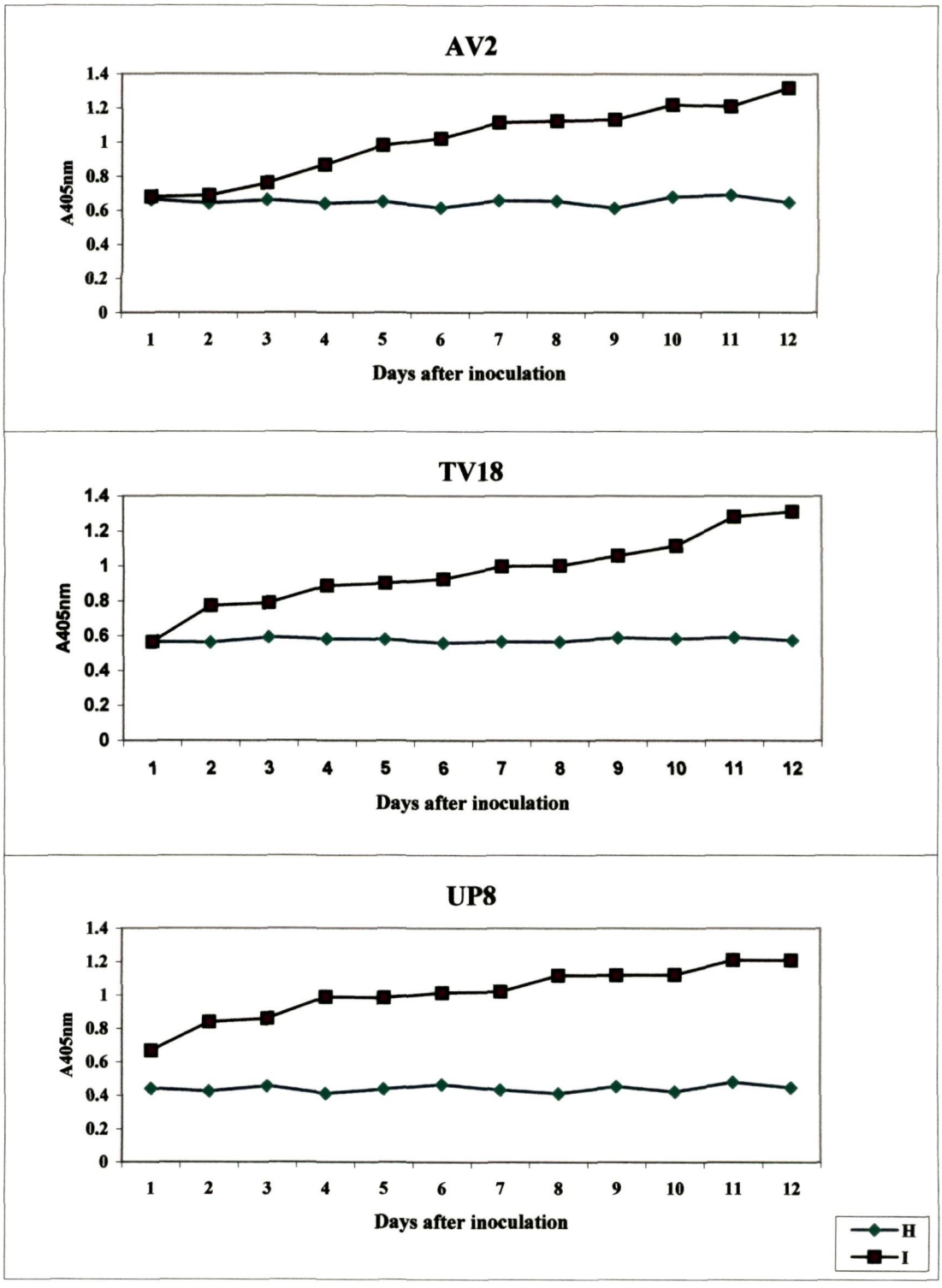
DAC-ELISA responses of *E.vexans* antiserum (PS-II/B) with healthy and inoculated tea leaf antigens at different intervals



PS: Polyspecific;
II-Hansqua;B-Second bleeding

Fig.18

DAC-ELISA responses of *E.vexans* antiserum (PC-I/B) with healthy and inoculated tea leaf antigens at different intervals



PC: Polyclonal;
I-Castleton;B-Second bleeding

Fig.19

4.8.1. Healthy leaf

Two highly susceptible varieties (TV18 and AV2) were considered for FITC staining. Cross sections of tea leaves (TV18 and AV2) were treated separately with polyclonal antisera of *E. vexans* (PC-I/B) and then FITC. Section showed bright fluorescence, mainly in the epidermal cells and mesophyll tissues. (Plate 13 Figs A to C).

4.8.2. Fragile Callus

Fragile callus or loosened cells (Plate-14) were prepared from stem segments of susceptible variety (TV18). Loosened cells were treated with polyclonal antibody of *Exobasidium vexans* and finally reacted with FITC. It is interesting to note that when these cells were treated with anti-*E. vexans* antiserum and then reacted with FITC, bright fluorescence was notice (Plate 14, Fig. A-C)

4.8.3. Blister infected leaves

Naturally blister infected tea leaves collected from CTE were considered for this experiment. Cross section of the leaves through the infected zone was stained and observed under bright field (Plate 15, Fig. A). Sections were then treated with polyclonal antibody of *E. vexans* and proceeded for FITC staining, following the same method as described earlier. Bright fluorescence was observed in the palisade and spongy parenchymatous areas, where maximum infection occurred (Plate 15, Fig. B ; Plate 16, Fig. A & B).

4.8.4. Basidiospores of *E. vexans*

Basidiospores collected from blister infected tea leaves of CTE were used for the observation of cross reactive antigens. These spores were treated with homologous polyclonal antibody raised against *E. vexans* and then labelled with FITC. Bright fluorescence was observed (Plate 17).

In previous experiments, it was noticed that basidiospores germinated better in pH 7.2. Hence, the basidiospores were initially treated in Sodium Phosphate buffer (pH 7.2) for 4 hours and then treated with polyclonal antibody of *E. vexans* followed by labelling with FITC. Spore behaviour as noticed under bright field and fluorescence were observed under microscope and the results are shown in Plate 18 (Figs A and B).

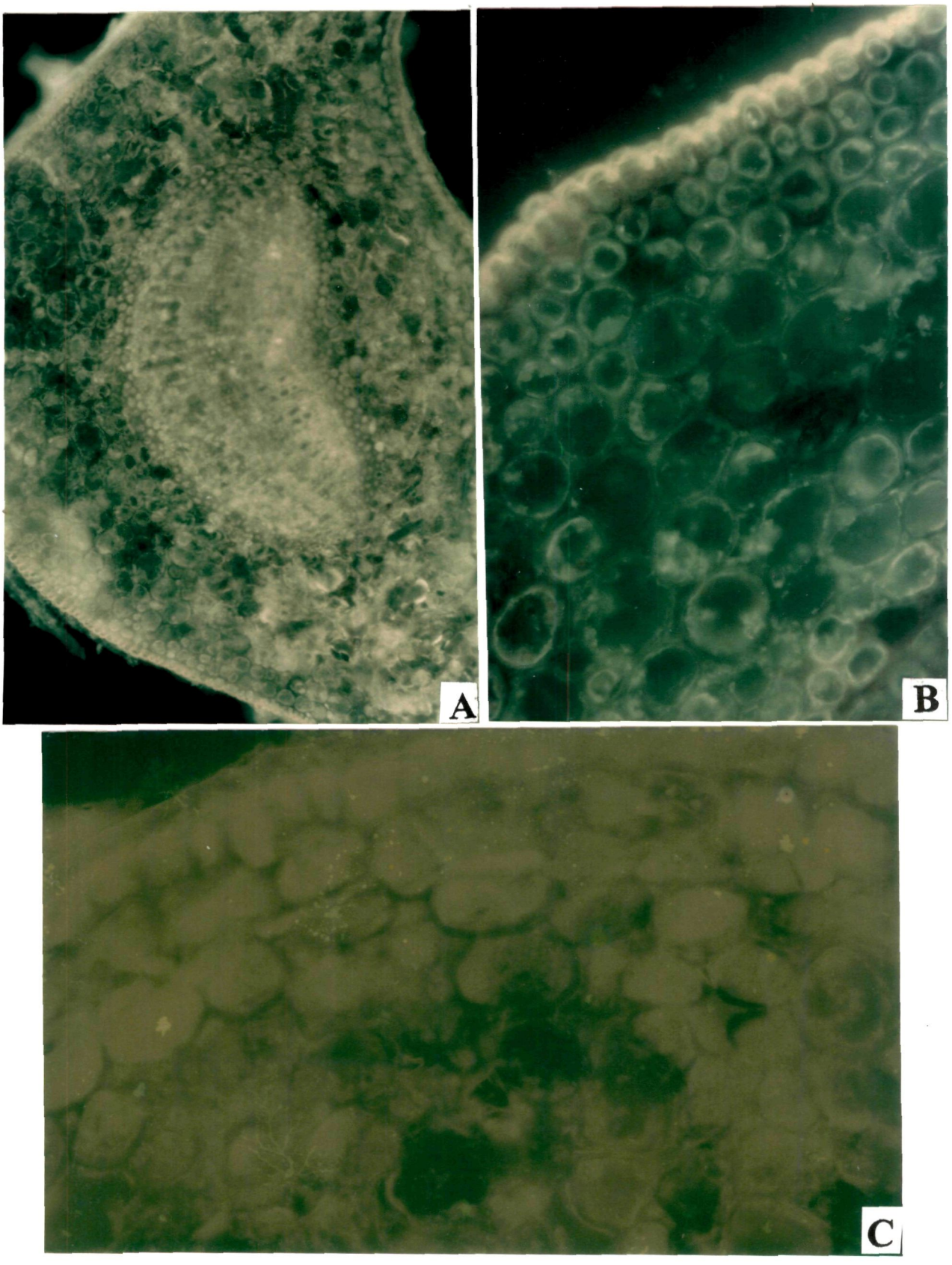
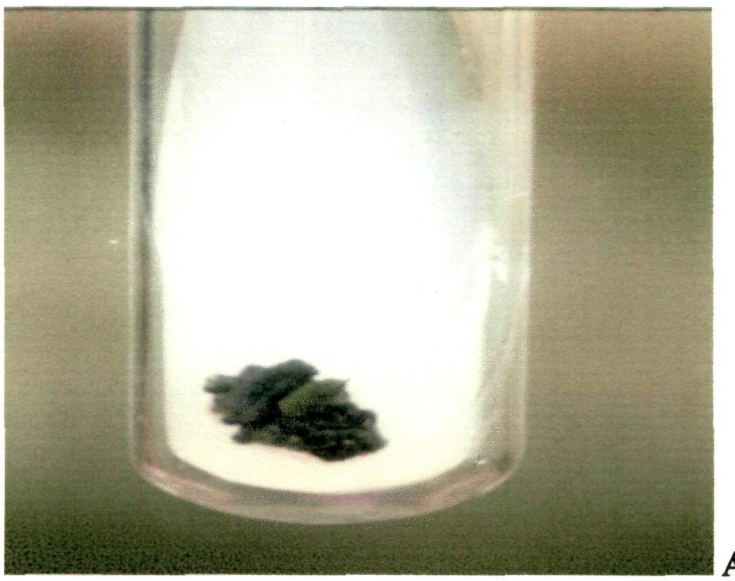
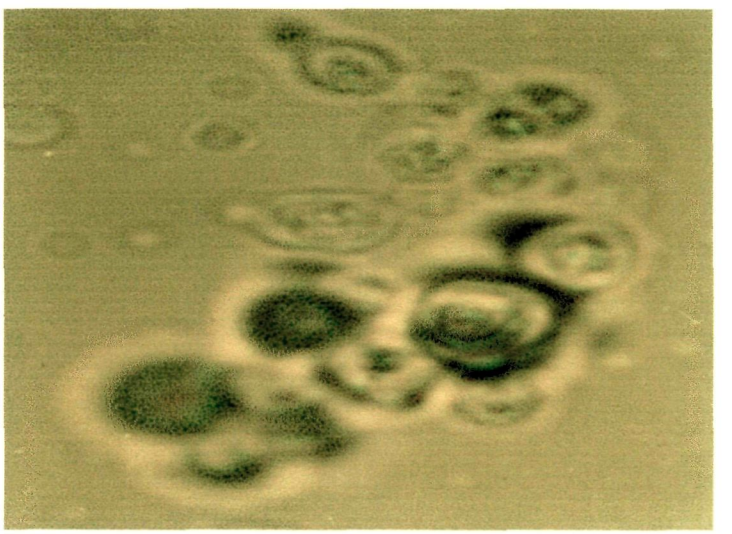


Plate 13 (Figs. A-C): FITC antibody staining of healthy tea leaf tissues (**A&B**) TV-18, (**C**) AV-2, treated with polyclonal antibody of *E.vexans* showing cellular location of cross reactive antigens



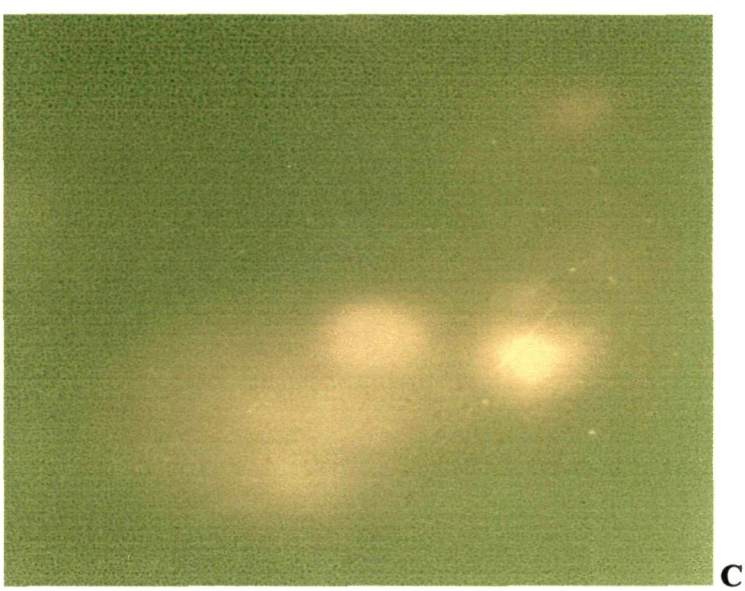
A

Fragile callus prepared from stem segments of TV-18



B

Loosened cells prepared from fragile callus of TV-18



C

Loosened cells treated with Pab of *E. vexans* and FITC

Plate 14

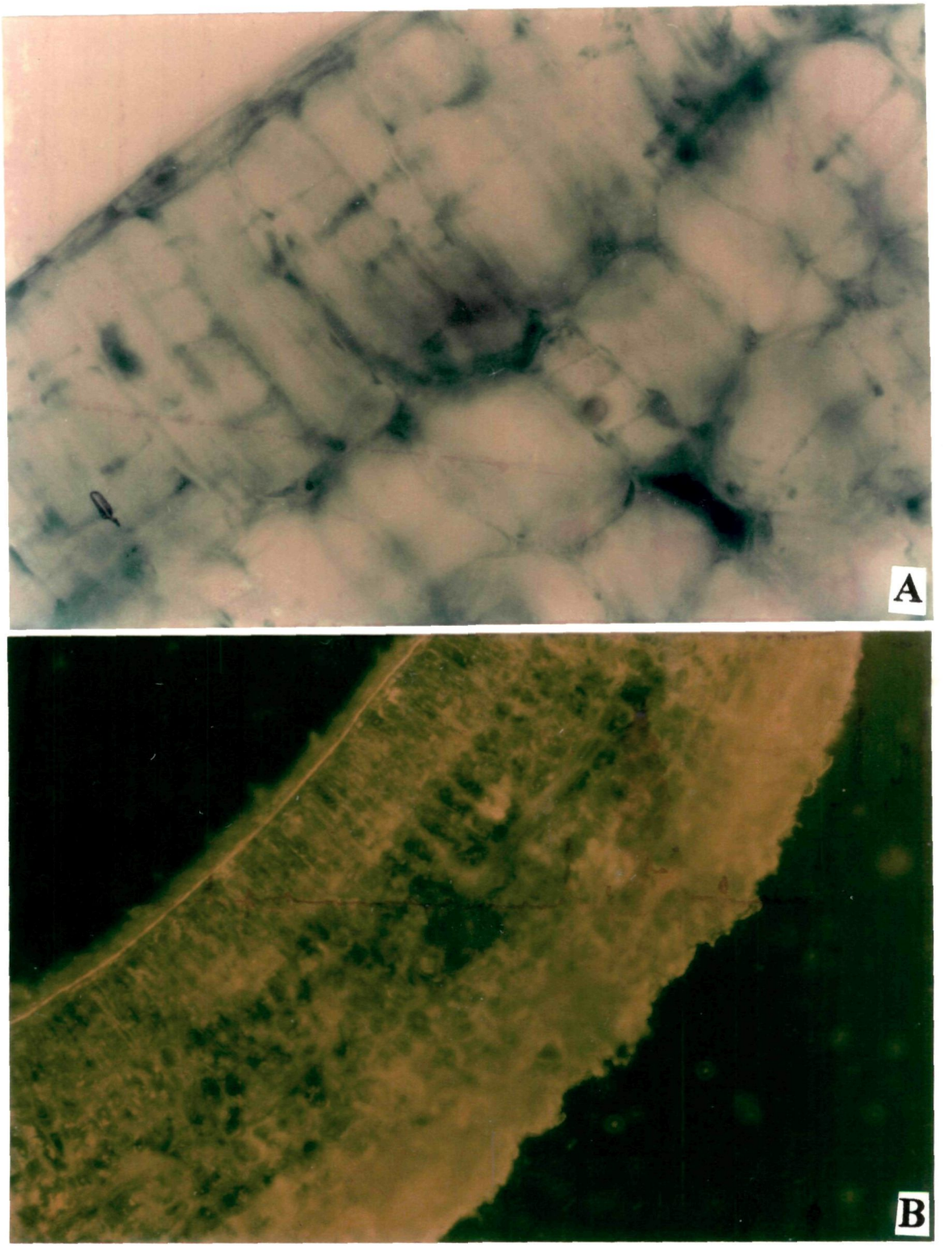


Plate 15 (Figs. A&B): T.S. of Blister infected tea leaves (A) Under bright field (B) Treated with polyclonal antibody of *E.vexans* labelled with FITC antibodies of goat specific for rabbit globulin

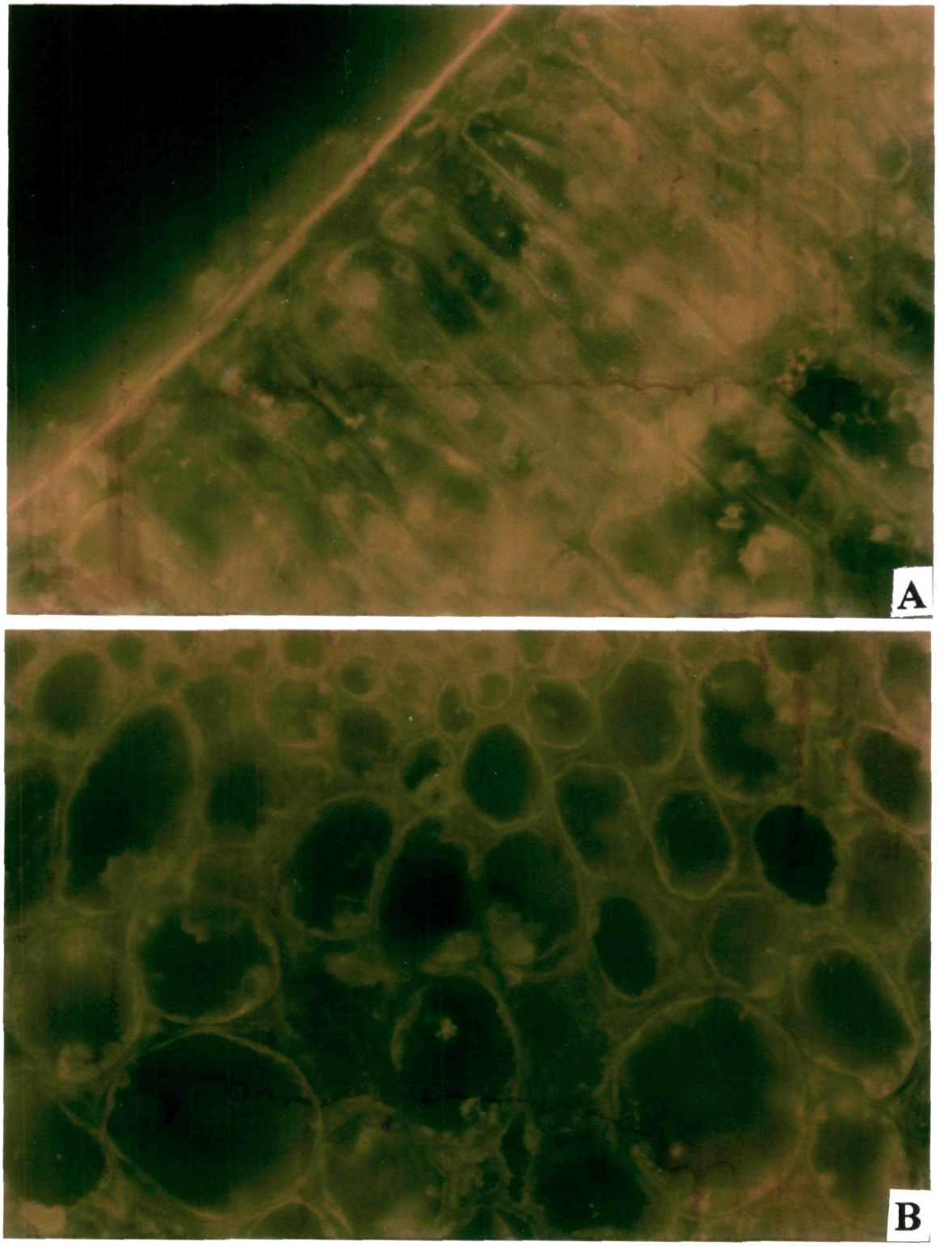


Plate 16 (Figs. A&B): Immunofluorescence study of Blister infected tea leaf tissues treated with polyclonal antibody of *E.vexans* labelled with FITC antibodies of goat specific for rabbit globulin

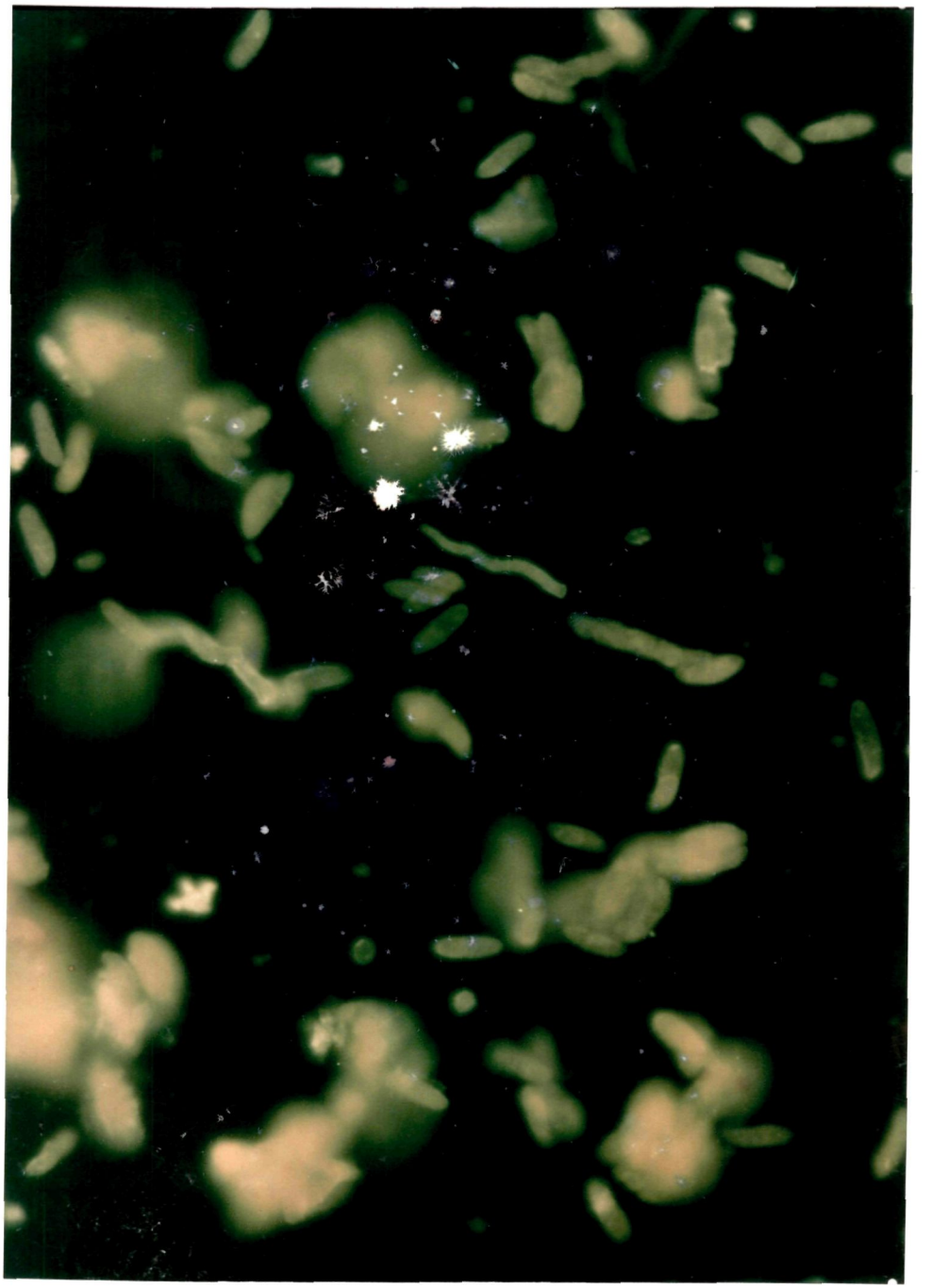


Plate 17 : Basidiospores of *E.vexans* treated with homologous polyclonal antiserum and FITC antibodies of goat specific for rabbit globulin

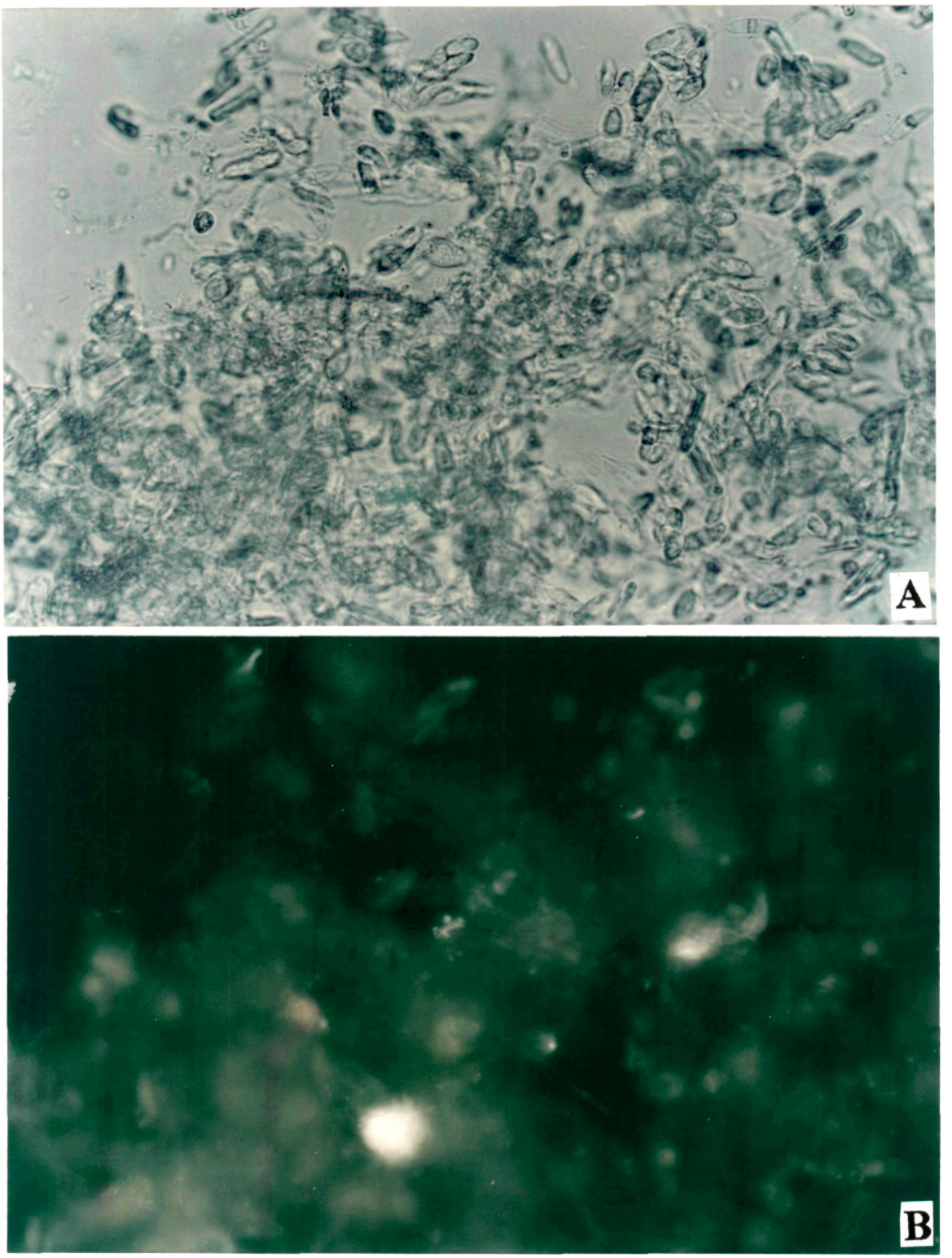


Plate 18 (Figs. A&B) : Basidiospores of *E.vexans* pretreated in phosphate buffer (pH 7.2) (A) Under bright field (B) Treated with homologous polyclonal antiserum and FITC antibodies of goat specific for rabbit globulin

4.9. Dot immunoblotting

Detection of fungal pathogens is a more recent application of dot immunoblotting. In this experiment, healthy and artificially blister infected leaf antigen, antigens from basidiospores as well as antigens from naturally blister infected leaves were loaded (5µl/ well) on nitrocellulose paper and treated with polyspecific (PS-I/B) antibody and polyclonal (PC-I/B) antibody of *Exobasidium vexans* followed by staining with BCIP and NBT. Results have been presented in plate 20 (Fig. C&D). Protein responses in different varieties have been depicted by the intensity of dots on the nitrocellulose paper. Antigen dots of, blister infected tea leaves (CTE) and basidiospores (Plate 20, Fig. D-1 & 2 and Fig. C-1&2) respectively showed deep colouration confirming the presence of the pathogen. Similar results have been depicted when hybridized by PC-I/B of *E. vexans*, (Fig. C-1&2). Healthy and artificially inoculated tea varieties (UP3, TV22, TV18 and AV2) results are shown in [Plate 20, Fig. C&D (3,4,5,6)] respectively, when treated with polyspecific and polyclonal antibodies, similar results were obtained. The dots of artificially inoculated TV22, TV18 and AV2 were higher intensity then their respective healthy dots conforming the presence of infection. However, UP3 healthy as well as inoculated leaf antigens did not respond to the *E. vexans* antibody. The result clearly shows the high susceptibility of these varieties (TV18, AV2 and TV22) to blister blight disease which confirms the pathogenecity test of *E. vexans* also.

4.10. Western blotting

Apart from ELISA, western blotting has proved to be a very sensitive method for the detection of infection in the host tissues. Details of the method has been already discussed in Material and Methods. To determine the sensitivity of this assay, SDS electrophoresis gel containing 5µg of purified protein antigens of healthy and blister infected tea leaves were run accross slab gel and transferred on nitrocellulose paper. After treating the nitrocellulose with the target polyspecific antibody (PS-I/B), it was stained with BCIP and NBT. Bands were visible within 15 minutes of the treatment with substrate. The results are presented in plate 19 (Fig. A and B). SDS PAGE of healthy and infected tea leaves stained with coomassie blue is presented in (Plate 19 Fig. A). Many Bands were visible between the Molecular weights of 14.3 to 97.4 daltons (Plate 19 Fig. A). Immunoblotting on the Nitrocellulose paper when probed with polyspecific antibody (Plate 19 Fig.B) showed selected bands with intense staining. It is interesting to note that the lane with

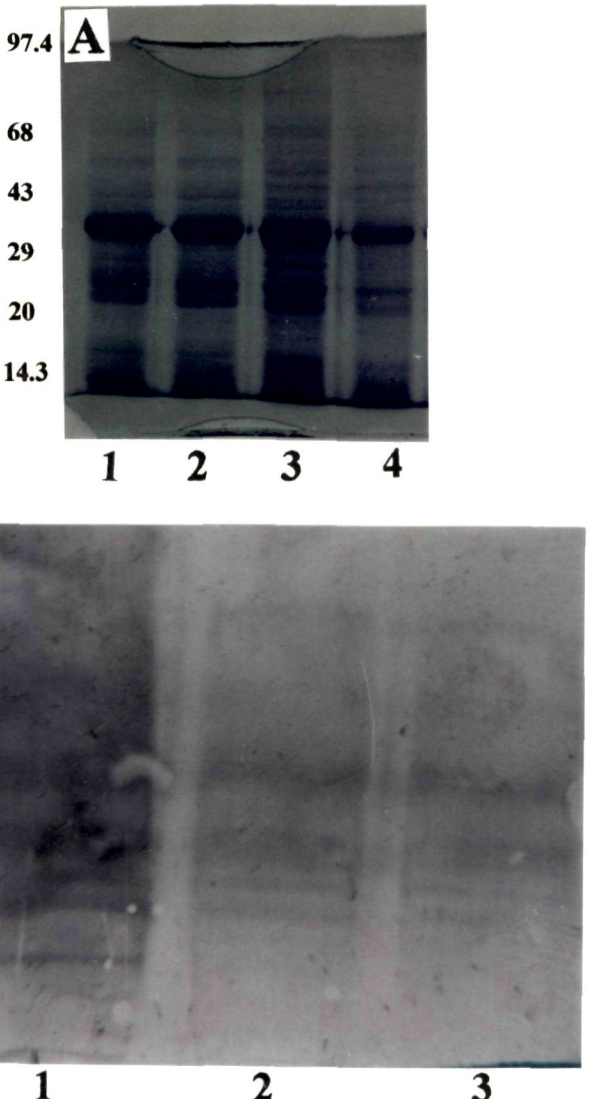


Plate 19 (Figs. A&B) : SDS-PAGE (A) and Western blot (B) of soluble proteins of healthy and blister infected tea leaf
(A) stained with commassie blue,
Lanes 1&3 – Healthy leaf; Lanes 2&4 – Blister infected
(B) probed with polyspecific antisera of *E.vexans*
Lanes 2&3 – Healthy leaf; Lane 1- Blister infected

blister infected leaf antigen showed deeper bands with more intensity when compared to the healthy leaf antigens.

This clearly shows the presence of infection in the leaf tissues. This result confirms the ELISA reactions performed by healthy and blister infected leaf antigens. Similar results were observed when the nitrocellulose paper with the transferred gel was probed with polyclonal antibody (PC-I/B) (Plate 20 Fig. A). Lanes 1 and 3 consists of infected tea leaf antigens where as lane 2 contains healthy antigen. In this case there was no colour reaction in lane no.2 showing more specificity with the pathogen. Epitopes were observed having molecular wts of 38.24, 32.54 and 23.42 KDA.

4.11. Disease management

In the earlier chapters, the response of different tea varieties towards *E. vaxans* and their detection by various methods were discussed. It is therefore important to discuss about the control of the disease. Foliar application of Hexaconazole (systemic fungicide) as recommended by UPASI, has proved to be effective control, but has toxic side effects. The present few experiments have been designed to test the effect of Hexaconazole as well as few eco-friendly biocides for the management of blister blight.

4.11.1. Systemic fungicide

Hexaconazole, a product of "Rallis India Ltd." has been proved to be very effective, systemic fungicide for the control of the disease. The recommended proportion (1:1000 dilution) was used for the spray schedules. It has already been established by pathogenecity tests that TV18 is highly susceptible to blister blight disease specially in the plains. Hence, it was decided to treat TV18 seedlings in pilot scale with Hexaconazole, in the experimental station, N.B.U. The foliar sprays were done once in 15 days for one month. The leaves (1st and 2nd) were artificially inoculated with basidiospores of *E. vexans* and incubated at 25^{0}C and 80% humid conditions. Antigens were prepared from inoculated and their corresponding healthy leaves at 3 different intervals (4 days, 8 days and 12 days) after inoculation. Untreated healthy and untreated infected were considered as two controls. SDS-PAGE analysis with coomasie blue stain of these protein samples are shown in plate 21 (Fig. A-C). Many proteins of molecular weights between 14.3 to 97.4 KDA were visible. It is interesting to note that in the samples from all the 3 harvests, the concentration of protein as well as the number of bands have increased in blister infected (untreated

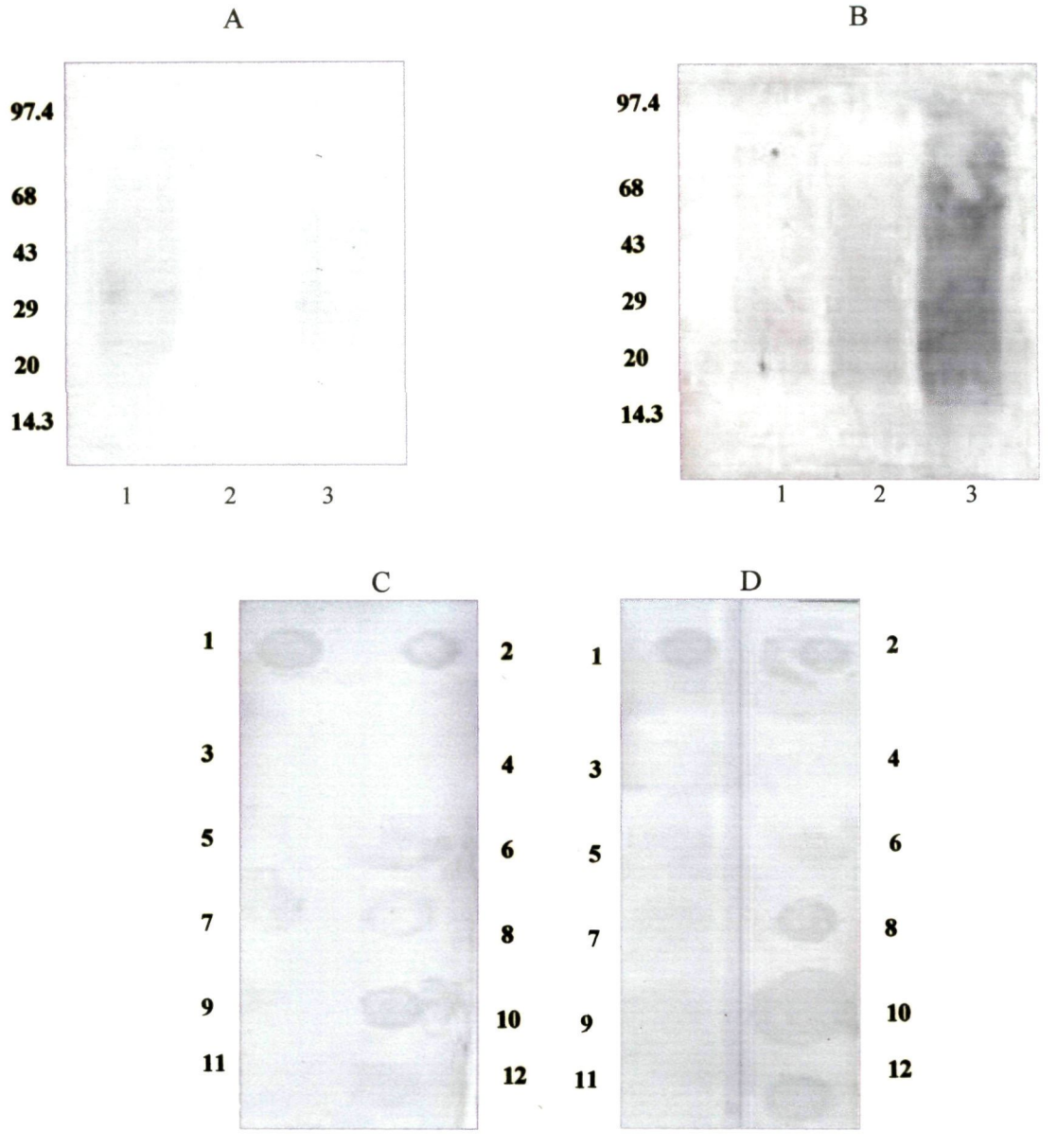


Plate 20 : Western Blot (A&B) and Dot Blot (C&D) of antigens from healthy and blister infected tea leaves probed with polyspecific (A&C) and polyclonal antisera of *E.vexans* - Basidiospores (B&D). A: Lane-1&3- Blister infected and 2-Healthy leaf antigens; B: 1- Healthy; 2 – Antigens from young blisters and 3- Antigens from mature blisters; C & D : Tea leaf antigens ;Naturally blister infected (1) ; Basidiospores- of *E.vexans* (2); Healthy leaves of UP3 (3), TV-22 (5), TV-18(7), AV-2 (9) & Castleton(11); Artificially inoculated leaves of UP3 (4), TV-22(6), TV-18 (8), AV-2 (10) and 100% SAS of naturally blister infected leaves (12).

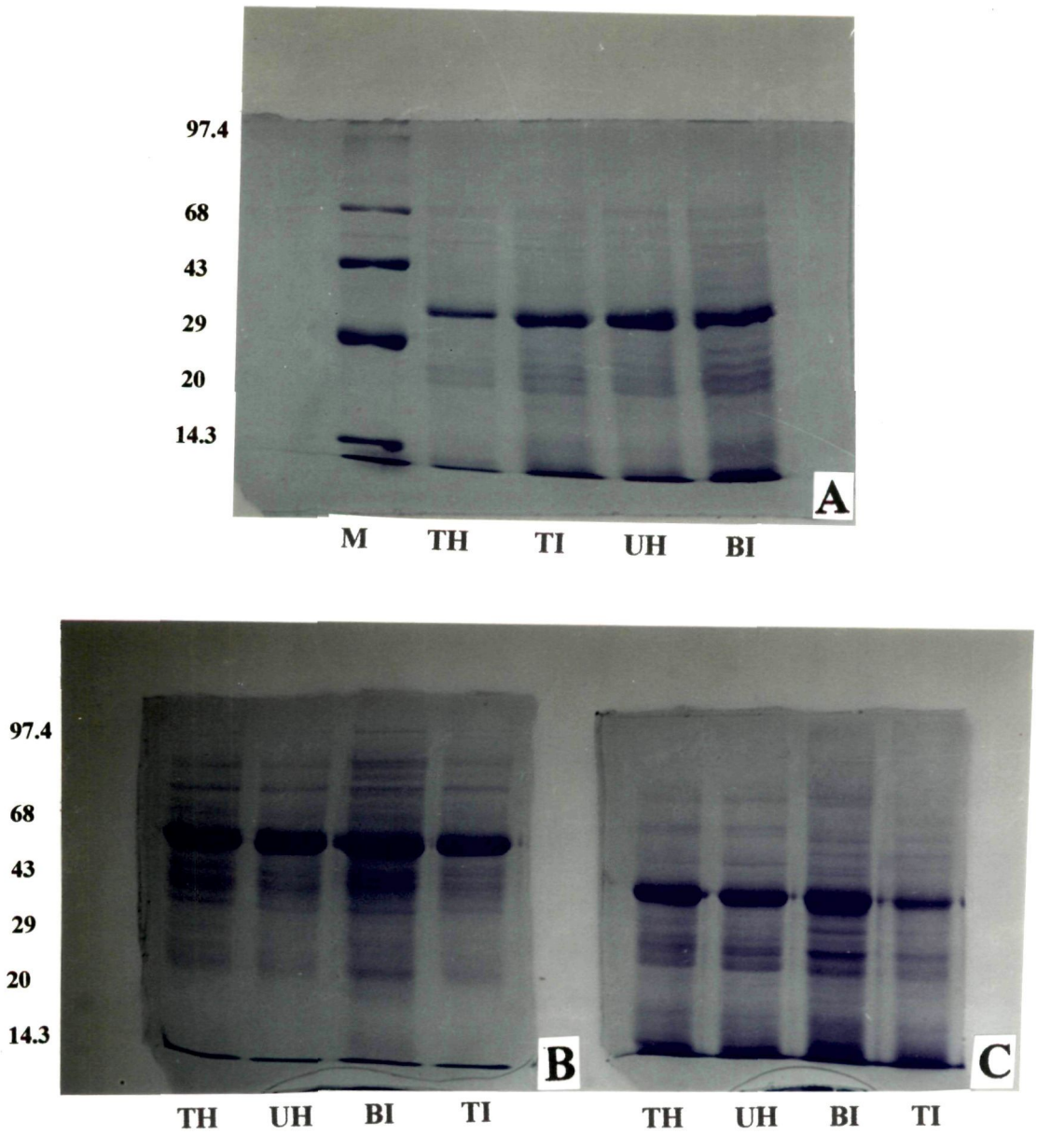


Plate 21(Figs. A-C) : SDS-PAGE analysis of soluble proteins of untreated healthy (UH),blister infected (BI),treated (with hexaconazole) healthy (TH) and treated inoculated (TI) - 4 days (A); 8 days (B) and 12 days (C) after inoculation Molecular marker - M

inoculated) plants. After discussing the *in vitro* analysis it is necessary to study the effect of systemic fungicide in field conditions (Tea garden) and also comparison with botanical pesticides. For this, an experimental section was marked in Castleton Tea Estate (CTE), Darjeeling.

4.11.2. Biocide

In the present study, few selective botanical pesticides were screened out of which *Azadirecta indica* (Neem) and *Catharanthus roseus* were selected for further field applications. Foliar applications of plant extracts (20g/litr) and Hexaconazole (1:1000 dilution) were given twice a month for two months.

The percentage of blister blight incidence was calculated by counting infected shoots out of 100 shoots. Results of two harvests are shown in Table 49. It is remarkable to note that the occurrence of the disease is markedly reduced in all the three cases, Hexaconazole being the highest (19%), *Cantharanthus* spray (41.3%) and Neem spray (50.3%) after the second harvest.

Table 49 : Blister blight incidence after the foliar treatments with systemic fungicide and biocide, in field condition

	No. of infected shoots (Out of 100 shoots)			
Treatment	**R1**	**R2**	**R3**	**Mean**
1st harvest[a]				
Untreated	90	85	90	88.3 ± 1.66
Catharanthus	50	45	42	45.6 ± 2.33
Neem	57	57	45	53.0 ± 2.49
Hexaconazole	19	23	25	22.3 ± 2.49
2nd harvest[b]				
Untreated	86	89	91	92.3 ± 2.95
Catharanthus	47	45	42	41.3 ± 3.48
Neem	52	48	51	50.3 ± 4.92
Hexaconazole	17	15	25	19.0 ± 3.05

± Standard error.

[a]Harvest was done after 4 sprays ; [b]Harvest was done after 8 sprays

"t" tests for paired samples were statistically analysed. 2 tail sig. at 0.017 was found in case of Neem; 0.001 in case of *Cantharanthus* and 0.000 in case of Hexaconazole.

4.12. DAC-ELISA response of tea leaves after treatment with systemic fungicide and biocides

DAC-ELISA format was developed using polyclonal antibody (PC-I/B) raised against *E. vexans* basidiospores in order to screen the infection. Healthy, untreated infected and treated tea leaf antigens were prepared and tested on DAC-ELISA format. The results and means of three experimental sets are shown in Table 50.

It is very interesting to note that untreated and blister infected leaves show very high absorbance (A 405) values when compared to the treated leaf antigens. Treatment with systemic fungicides gave the lowest O.D. value (0.512) followed by *Catharanthus roseus* and *Azadirecta indica*. This result has definitely opened new horizons for testing various other ecofriendly biocides for the management of the disease.

Table 50 : Indirect ELISA detection of treated (systemic fungicide and biocide) and untreated tea leaves of Castleton Tea Estate, against *E. vexans* Polyclonal antiserum (PC-I/B)

Antigen conc. (40µg/ml)	*E. vexans* (PC-I/B) Antiserum (1:250 dilution) OD at A405nm[b]					
Treatment[a]	1st harvest			2nd harvest		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Untreated						
UH	0.644±.016	0.728±.031	0.711±.044	0.605±.066	0.697±.051	0.659±.044
UI	0.837±.008	0.817±.019	0.769±.061	0.761±.006	0.869±.061	0.790±.033
Treated (Hexaconazole)						
TH	0.668±.011	0.630±.055	0.623±.038	0.574±.089	0.512±.009	0.661±.055
TI	0.670±.066	0.692±.019	0.658±.032	0.578±.051	0.555±.065	0.657±.011
Neem extract						
TH	0.655±.065	0.617±.001	0.572±.022	0.670±.054	0.701±.036	0.618±.014
TI	0.657±.022	0.731±.081	0.569±.056	0.668±.099	0.762±.098	0.616±.012
Catharanthus extract						
TH	0.629±.041	0.550±.032	0.627±.056	0.654±.021	0.647±.066	0.652±.041
TI	0.802±.032	0.708±.046	0.638±.055	0.738±.032	0.762±.046	0.662±.055

± Standard error ;

[a]Treatment was done with (systemic fungicide) at a dilution of 1:1000, to 50 bushes. Similiarly 50 bushes each was treated separately with 25% Neem extract and 25% *Catharanthus* extract ;

[b]Each experiment is mean of 3 replicates and 3 experiments were performed for each treatment per harvest ; UH –Untreated healthy ; UI – Untreated infected ; TH – Treated healthy ; TI – Treated infected.