EXPERIMENTAL

4.1 Black rot disease occurrence under natural conditions

Black rot disease is caused by *Corticium theae* and *Corticium invisum*. A survey was conducted to record the occurrence of black rot in various tea gardens of the Dooars of Jalpaiguri district , foot hills (Terai) and hills of Darjeeling district. Black rot disease incidence was recoded for consequtive three years from five tea gardens such as Tiriannah Tea Estate and Hansqua Tea Estate (Terai) Kailashpur Tea Estate and Chinchula Tea Estate (Dooars) and Margarate Hope Tea Estate (Hills) Highest black rot disease incidence (63%) was observed in Kailashpur Tea Estate (Plate – 1 & 3; fig. A) , and Tiriannah Tea Estate. First appearance of the disease was during early April and continued up to August. Maximum disease incidence was recorded during mid July. Disease was always noticed in the plain but rare in the hills. It attacks all teas from their seedling stage upwards. The disease persisted in the same areas for years, causing gradual deterioration in the health of the tea and loss in crop. In Kailashpur Tea Esate, it thrived for years in the badly ventilated places, mainly where air movements were prevented by overdense shade as well as the particular section was surrounded by forest areas.

Black rot is more prevalent on tea which has been cut across without any cleaning out and unskiffed and unpruned tea. *C. theae* produces on the leaves large patches covering about half and sometimes the entire leaf area. Colour on the uppersurface of the affected area at the early stage is reddish-brown, similar to sun-scroch damage, later it is a mixture of brown, yellowish-brown and grey; the undersurface is light brown or greyish-white and usually covered with a net work or cream to brown mycelium and finally leaf becomes rotten (Plate –3, figs B&E). Diseased leaves often remain attached, to other leaves and stems, held together by small cushions or films of pinkish-white or cream coloured mycelium. The fungus produces on the stem, thick crods of mycelium, up to about 3 mm across, dark purplish-brown on the older portions of the stem and dull white to light brown on the green portions at the top.The fungus produces minute resting bodies (sclerotia) in the cracks and crevices of the stem towards the end of the rainy season. Fructifications appear, during the rainy season, as white, dusted patches on the undersurface of mature, green leaves.

Plate 3 (Figs. A-E) : Natural black rot disease symptoms and causal organism. (A) Black rot disease in Kailashpur Tea Estate; (B & E) Detached leaves showing disease symptoms; (C & D) *Corticium theae* isolates (Ct-1 & Ct -2).

4.2 Correlation of weather conditions with occurrence of Black rot disease

Weather conditions have a direct influence on disease development. The organism get favourable conditions for successful establishment of disease. Keeping this mind, monthly meterological data were collected for three years (1999-2001) which included maximum and minimum temperature, relative humidity and average rainfall. Black rot disease incidence were recorded throughout the year (Table – 1).

Table 1 : Meterological data and black rot disease occurrence .

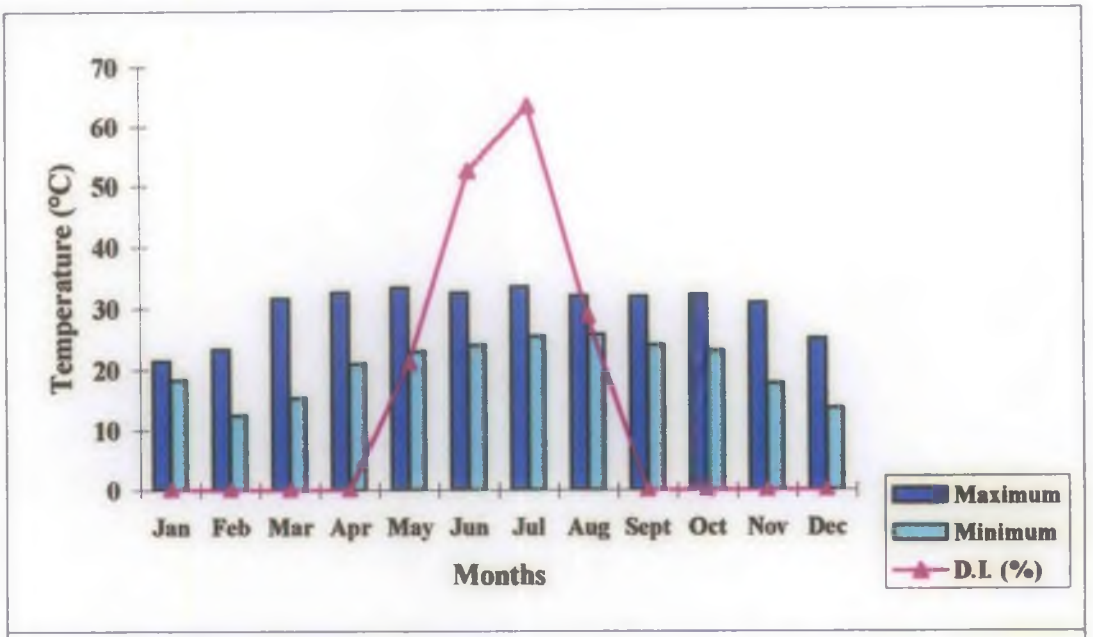
Month	Temperature (*C)		RelativeHumidity(%)		Rainfall (mm)	Disease Incidene (%)
	Maximum	Minimum	Morning	Evening		
Jan	21.4±0.32	18.2±0.22	92.3± 0.76	52.3± 2.31	22.8± 5.62	0
Feb	23.3±0.62	12.4±0.78	89.2± 1.11	50.5± 2.52	7.5± 2.32	0
Mar	31.5±0.56	15.2±0.63	88.6± 0.62	46.8± 1.45	69.6±25.37	0
Apr	32.5±0.65	20.9±0.67	89.7± 0.65	56.7± 0.61	98.2±26.57	0
May	33.2±0.59	22.8±0.61	87.3± 1.12	61.9± 1.36	260.2±54.83	21.1±0.16
Jun	32.4±0.68	23.8±0.32	90.8± 0.43	65.2± 1.76	573.2±106.89	52.3±0.10
Jul	33.4±0.79	25.2±0.54	91.6± 0.48	71.7± 2.98	998.3±90.6	63.2±0.32
Aug	31.8±0.56	25.6±0.76	92.1± 0.72	67.2± 2.12	872.5±154.98	28.6±0.56
Sep	31.7±0.76	23.8±0.31	93.5± 0.76	64.6± 1.32	400.9±76.67	0
Oct	31.9±0.19	22.8±0.60	93.6± 0.65	62.7± 1.21	157.86±19.6	0
Nov	30.6±0.68	17.6±0.59	88.9± 1.98	48.9± 2.67	37.76±6.68	0
Dec	24.8±0.63	13.5±0.47	93.8± 0.57	41.6± 1.52	14.2±13.8	0

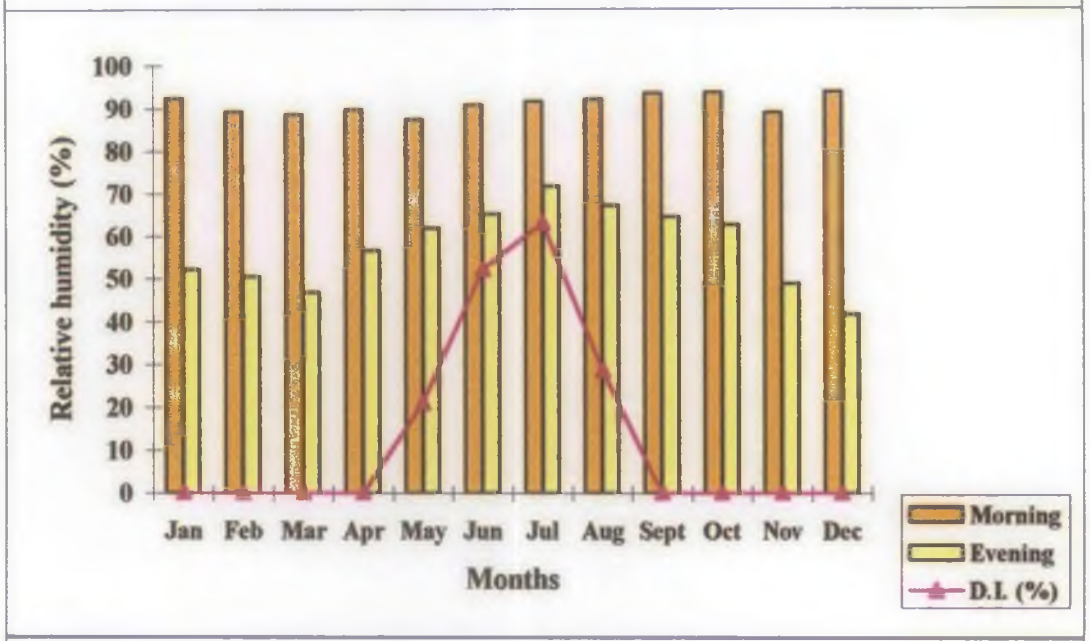
± Standard error
A Average of three years (1999-2001)
B Mean occurrence of black rot disease for three years (1999-2001)

Table 2 : Correlation matrix showing correlation between black rot disease and environmental factors during three years (1999-2001). (2-tailed significance)

	Disease
Disease[a]	1.00
Max temp	0.4583
Min temp	0.6220**
RH1[b]	- 0.0198
RH2[c]	0.7087*
Rainfall	0.8624*

Significant at P=0.01 ** Significant at P=0.05, Rest insignificant
[a] Disease computed as % incidence of mean of all varieties
[b] RH1 = % Relative humidity (Morning); [c] RH2 = % Relative humidity (afternoon)


Temperature (°C)
70
60
50
40
30
20
10
0
Jan Feb Mar Apr May Jun Jul Aug Sept Oct Nov Dec
Months
Maximum
Minimum
D.I. (%)


Relative humidity (%)
100
90
80
70
60
50
40
30
20
10
0
Jan Feb Mar Apr May Jun Jul Aug Sept Oct Nov Dec
Months
Morning
Evening
D.I. (%)

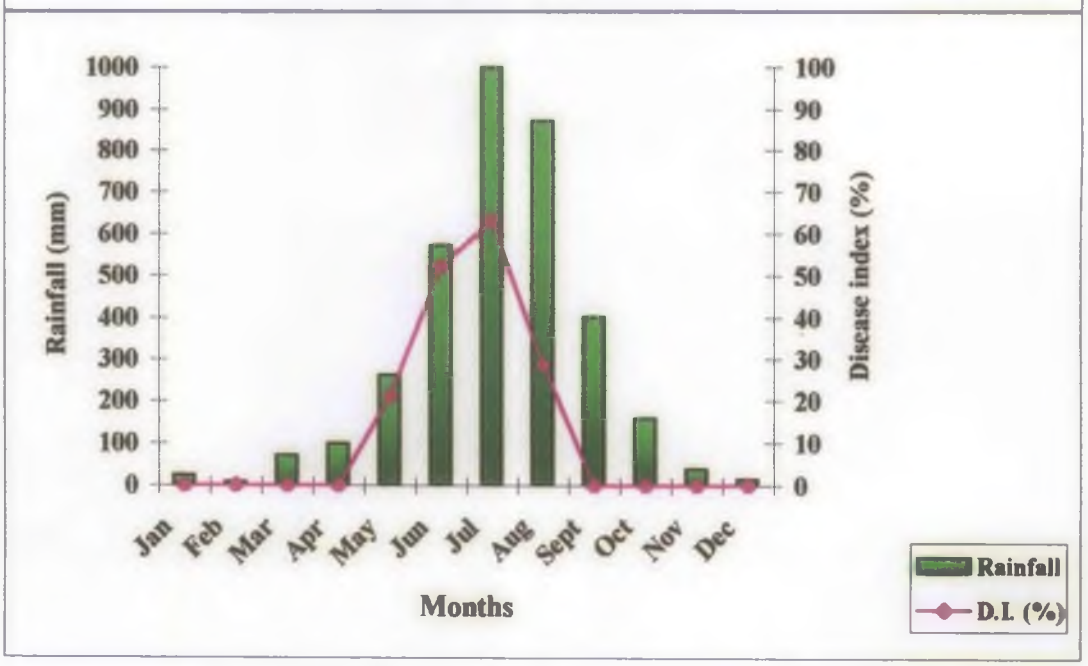

Rainfall (mm)
1000
900
800
700
600
500
400
300
200
100
0
Disease index (%)
100
90
80
70
60
50
40
30
20
10
0
Jan Feb Mar Apr May Jun Jul Aug Sept Oct Nov Dec
Months
Rainfall
D.I. (%)

Fig.1

In order to determine whether disease development in nature is correlated with different environmental factors, Karl Pearson's correlation coefficient was calculated in respect to black rot disease occurrence and the various factors. Mean data of the three years (Table –1) was used in all cases. Results have been presented in Table – 2 and Fig. 1. When mean disease incidence was correlated with the different factors, it was found that disease incidence showed positive significant correlation with rainfall and relative humidity.

4.3. Varietal resistance test of tea against *Corticium theae*

Fifteen varieties of tea, of which 6 released by UPASI Tea research. Centre, Valparai, Tamilnadu and 9 released by Tocklai Experimental Station, Jorhat, Assam, were used for varietal resistance tests against *Corticium theae* following detached leaf and cut shoot inoculation techniques. Methods of inoculation, incubation conditions and disease assessment procedures have been described in detail under Materials and Methods and results are given in Tables 3 & 4 ; Figures 2 & 3 and Plate 4 (figs A-F)

4.3.1 Detached leaf

Artificial inoculation of detached tea leaves 9 Tocklai varieties, and 6 UPASI varieties were carried out separately as described previously. After 48, 72 and 96h of inoculation, assessment of inoculation infectivity and symptiom development were done on the basis of percent drops that resulted in disease production, which were determined from 50 leaves of each variety after every interval separately experiments were repeated thrice. Results revealed that, in case of Tocklai varieties, TV-22, TV-23, TV-18 and UPASI-2 were most susceptible followed by BSS-2, TeenAli-17/1/54 , UPASI-9 while TV-25, TV-26, TV-20 and TV- 9, UPASI-26, and BSS-3 were found to be moderately resistant. After 96h. of inoculation, 87% and 74% lesion production were observed in TV-22 and TV-23 respectively, while in TV-25, TV-26 and TV-9 approximately 20-30 % lesion production was observed (Table 3). On the basis of significance tests (t-test) the varieties were

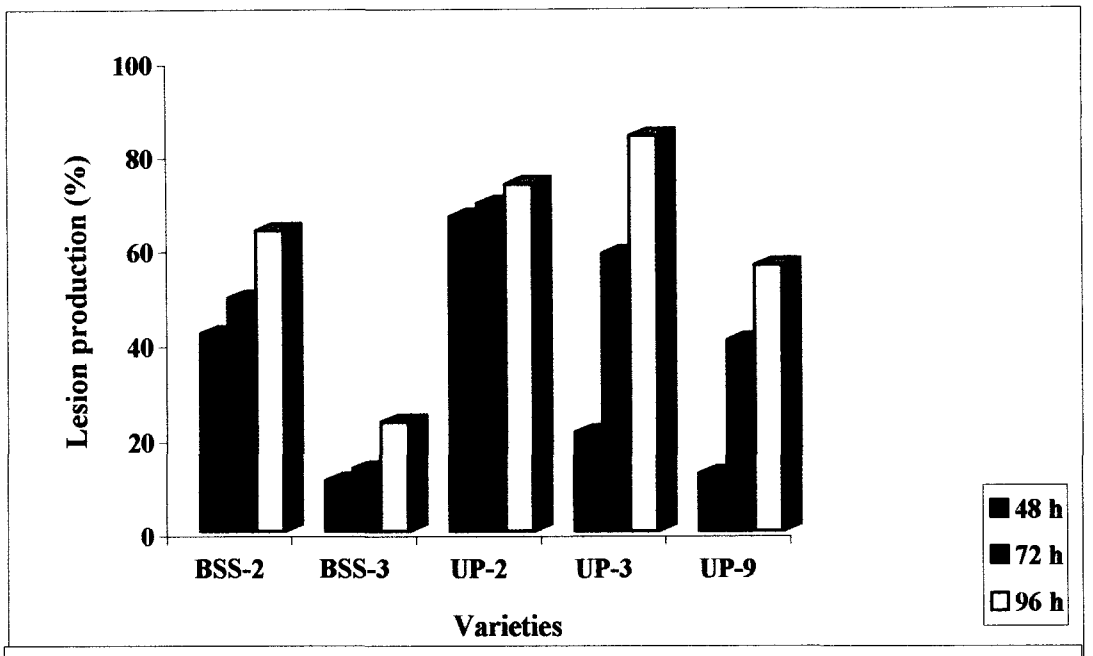
grouped into highly susceptible (TV-22, TV-23, UPASI-3 and TV-18), moderately resistant (TV-25, TV-26 and TV-9) and moderately susceptible.

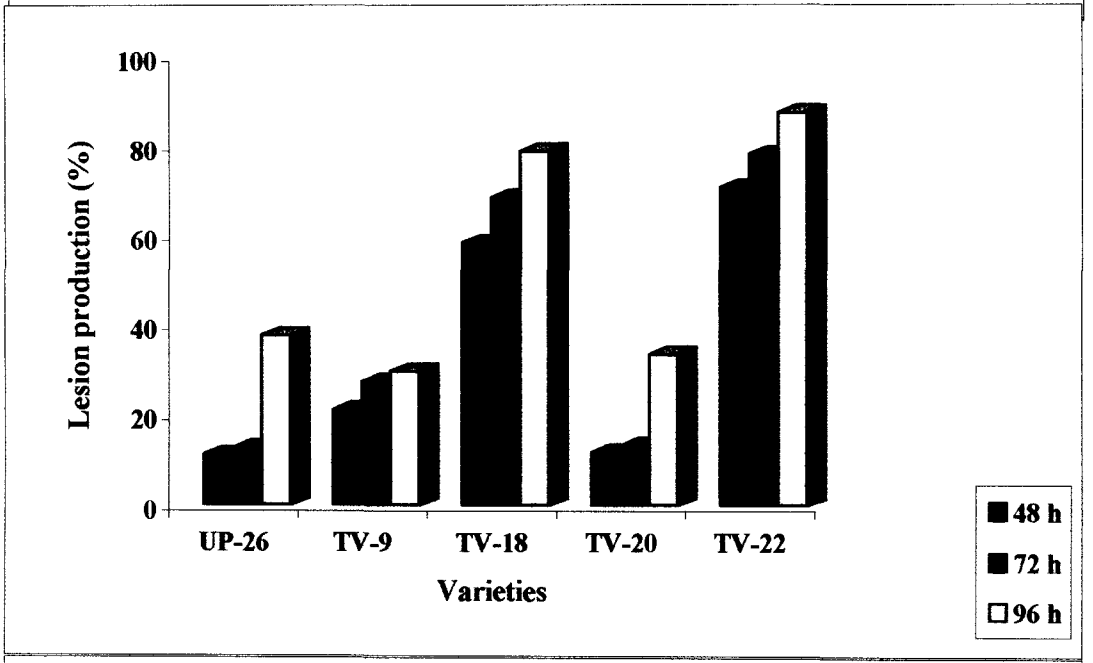
Table -3: Pathogenicity test of *Corticium theae* on different tea varieties following detached leaf inoculation

Tea Varieties	Percentage lesion production[a] Hours after inoculation		
	48	72	96
BSS-2	41.6±1.6	49.1±2.1	63.3±3.1
BSS-3	10.6±0.7	13.2±0.6	23.0±0.9
U–2	66.2±1.7	69.0±1.3	73.1±1.3
U-3	20.8±1.3	58.3±2.6	83.3±1.2
U-9	12.0±0.7	40.0±2.6	55.7±2.1
U-26	11.1±0.6	12.5±0.7	37.5±1.1
TV-9	21.0±1.2	27.2±1.0	29.5±2.1
TV-18	58.2±2.6	68.4±2.8	78.6±2.8
TV-20	11.6±1.1	13.1±1.0	33.3±1.1
TV-22	70.6±3.3	77.9±2.8	87.2±2.9
TV-23	68.7±2.6	72.4±3.2	74.1±3.0
TV-25	13.1±1.0	16.2±0.8	20.0±1.1
TV-26	16.1±1.6	19.2±1.7	22.3±1.3
TV-29	31.2±2.1	33.2±2.8	36.1±1.8
T-17/1/54	17.2±1.3	43.1±2.0	58.0±2.6

a Average of three separate trials, 50 leaves inoculated in each trial
± Standard Error

Pathogenicity test of *Corticium theae* on different tea varieties
(Detached leaf inoculation)





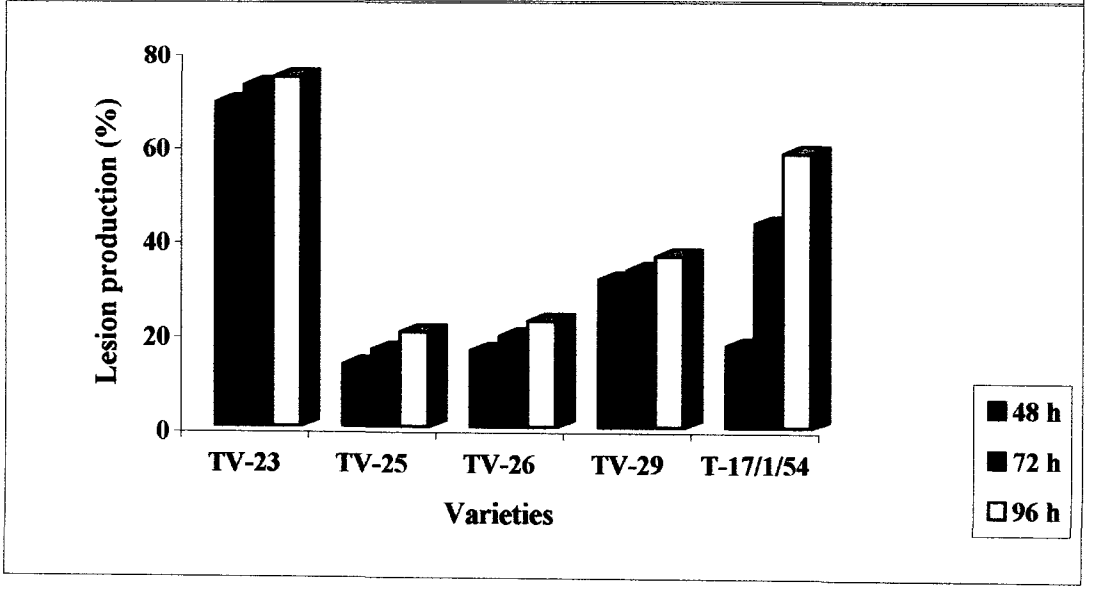


Fig 2

Among the UPASI varieties UPASI-3 was found to be most susceptible followed by BSS-2, UPASI-2, UPASI-9, while UPASI-26 were moderately resistant and BSS-3 was found to be most resistant. Maximum lesion production among UPASI varieties was 83% (UPASI-2) and minimum was 23% (BSS-3) after 96h of inoculation.

4.3.2. Cut Shoot

Pathogenicity of *Corticium theae* on 15 varieties of tea including 9 Tocklai and 6 UPASI were also tested by cut shoot inoculation (Plate – 4 ,figs C&D) as described in Materials and Methods Disease was assessed on the basis of mean number of lesions per shoot from which mean disease index per shoot was calculated after 48, 72 and 96h. of inoculation. Results (Table 4) confirmed the trend shown in detached leaf inoculation technique. Among the Tocklai varieties tested, highest disease index was evident in TV-23 , UPASI-2, TeenAli-17/1/54, BSS-2 and TV-23 and lowest in TV-20. (Table -4). Cut shoots of TV-22 and TV-23 exhibited lesions variable in size (2-3 mm diam) which appeared on third and fourth leaves within 48h. of inoculation, in case of Tocklai varieties. Spreading of these lesions (10-15 mm dia) with diffused margins were evident on the leaves after 96h. of inoculation. In contrast, at this interval cut shoots of TV-20, TV-9 and TV-25 exhibited restricted lesions. In case of UPASI varieties, UPASI-2 showed the highest index and BSS-3 exhibited the lowest disease index value.Among all the 15 varieties tested, TV-23 and UPASI-2 were found to be most susceptible followed by TV-22 and UPASI-3, while TV-20 was the most resistant followed by UPASI-26, BSS-3 and TV-9 on the basis of cut shoot inoculation (Fig. 3). This was in conformity with results of detached leaf inoculation.

Table-4 : Pathogenicity test of *Corticium theae* on different tea varieties following cut-shoot inoculation.

Tea Varieties	Hours after inoculation					
	48		72		96	
	Mean no. of lesion/shoot	Meandisease index/shoot	Mean no. of lesion/shoot	Mean disease index/shoot	Mean no. of lesion/shoot	Mean disease index/shoot
BSS-2	1.65	0.21	2.60	0.68	3.41	1.81
BSS-3	0.63	0.07	1.12	0.15	2.00	0.33
U-2	2.58	1.05	2.61	1.15	3.68	1.83
U-3	1.55	0.20	2.10	0.64	3.10	1.31
U-9	1.10	0.26	1.47	0.31	2.12	1.11
U-26	0.96	0.19	1.32	0.25	2.06	0.35
TV-9	1.10	0.16	1.24	0.53	1.28	0.56
TV-18	1.01	0.15	1.88	0.44	2.87	1.40
TV-20	0.65	0.09	1.12	0.15	1.87	0.29
TV-22	1.75	0.11	2.25	0.18	3.75	1.32
TV-23	1.70	0.26	3.00	0.76	3.71	1.82
TV-25	1.00	0.15	1.58	0.33	2.46	1.14
TV-26	1.12	0.17	2.13	0.35	2.88	1.20
TV-29	1.12	0.17	2.13	0.35	2.88	1.20
Ten Ali 17/1/54	1.63	0.24	2.75	0.70	3.38	1.74

Average of 50 Shoots/variety

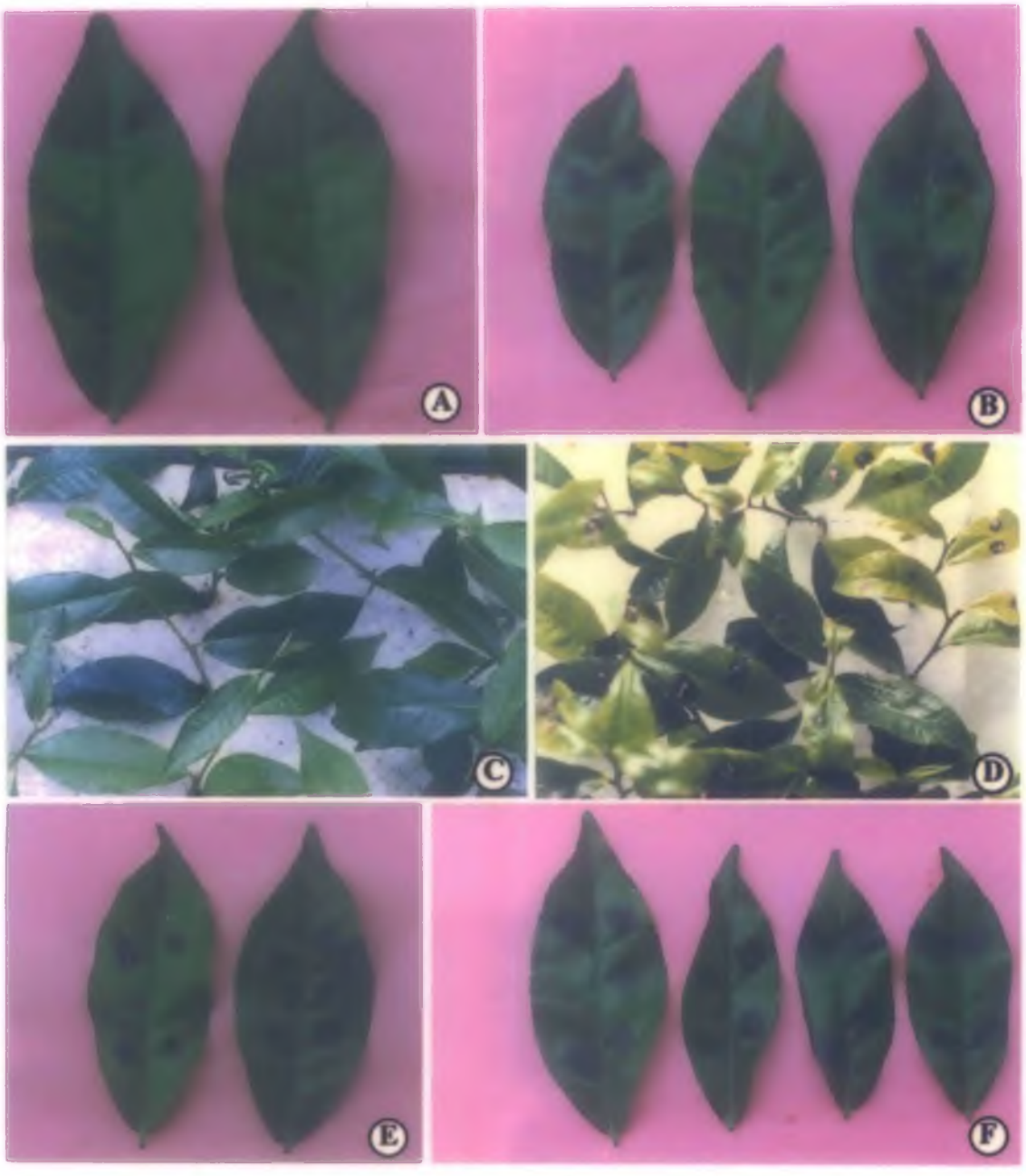
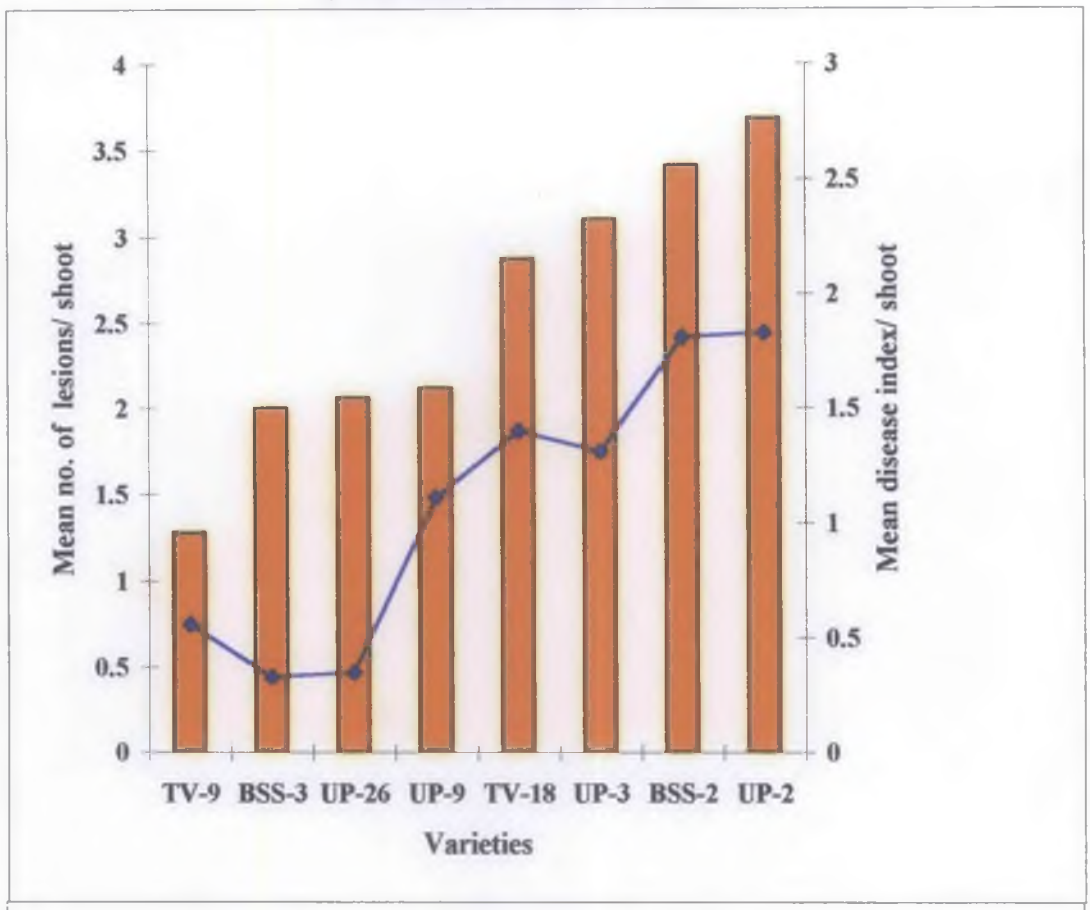


Plate 4 (Figs. A-F) : Detached leaf and cut shoot inoculation of tea leaves with *C.theae* (A&C) Healthy leaf (control); (B,D,E&F) Artificially inoculated with *C.theae*. (A&B) TV-23, (C&D) TV-22, (E&F) TV-18.

Pathogenicity test of *Corticium theae* on different tea varieties
(Cut shoot inoculation)



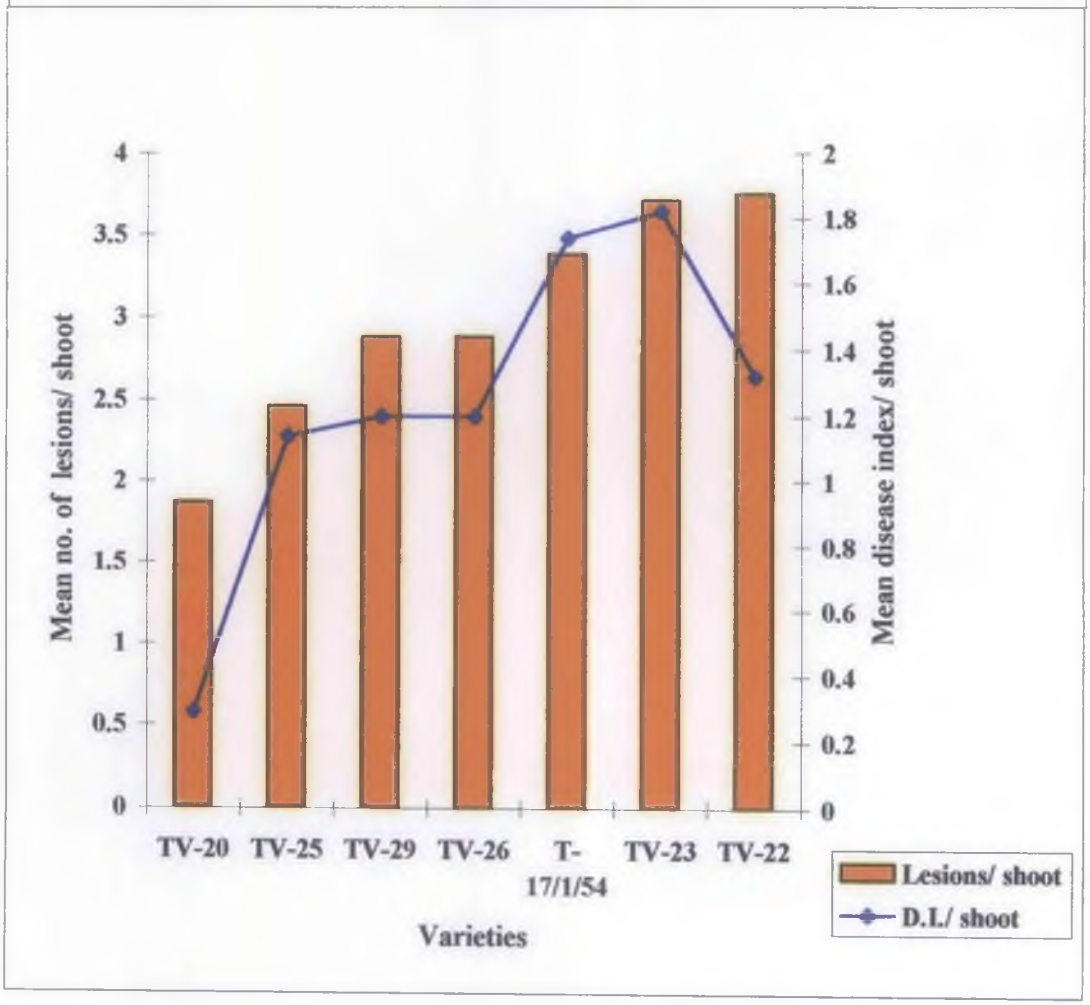


Fig 3

4.4. Determination of levels of phenolics in healthy and *C. theae* inoculated tea leaves

Phenolic compounds are known to accumulate in numerous plant species following infection with fungal pathogens and in many cases there is a greater increase in phenolic biosynthesis in resistant host species than susceptible ones (Mahadwon, 1991; Borkar and Verma, 1991). As polyphenols are the major constituents of tea leaves it was decided to compare quantitative changes in the phenolics of resistant and susceptible varieties. At the onset, the simple phenolics present in the healthy leaves were characterized following which quantitative estimation of total pehnol and arthodihydroxy phenol was done.

4.4.1. Total phenols

Total phenols from healthy and *C. theae* inoculated tea leaves of 15 tea varieties were extracted after 24 and 48h of inoculation and estimated as described in Materials and Methods. Results are given in Table 5 and Figure 4. Total phenol content decreased following inoculation with *C. theae* in the susceptible varieties. However there was an increase in the phenol contents of resistant varieties following inoculation. Among all the varieties tested, *C. theae* inoculated leaves of TV-20, showed maximum increase in total phenol.

4.4.2. Orthodihydroxy phenols

Orthodihydroxy phenols from healthy and *C. theae* inoculated tea leaves of fifteen varieties were also extracted after 24 and 48h. of inoculation and estimated. The method of extraction and estimation have been described in detail under Materials and Methods. Results depicted in Table 6 and Figure 5 revealed that orthodihydroxy phenol content also decreased in susceptible varieties (BSS-2, UP-2, UP-3, TV-18, TV-22, TV-23) and increased in resistant varieties (BSS-3, UP-9, UP-26, TV-9, TV-20, TV-25, TV-26, TV-29 and Teenali-17/1/54) after inoculation with *C. theae.*

Table 5 Level of total phenols in healthy and *C theae* inoculated tea leaves

	Total phenol (mg/g tissue)			
	24h		48h	
UPASI Varieties	**Healthy**	**Inoculated**	**Healthy**	**Inoculated**
BSS-2	30.0 ± 0.13	28.1± 0.02	30.2 ± 0.12	27.2 ± 0.01
BSS-3	40.6 ± 0.37	48.9 ± 0.03	42.1 ± 0.16	54.2 ± 0.13
UP-2	10.8 ± 0.13	10.1 ± 0.27	11.7 ± 0.02	10.3 ± 0.40
UP-3	16.1 ± 0.53	15.2 ± 0.17	16.3 ± 0.02	14.2 ± 0.38
UP-9	34.3 ± 0.10	33.1 ± 0.33	35.0 ± 0.17	33.2 ± 0.03
UP-26	35.0 ± 0.17	34.6 ± 0.31	35.2 ± 0.09	33.1 ± 0.24
Tocklai varieties				
TV-9	27.3 ± 0.21	32.0 ± 0.61	28.6 ± 0.60	46.6 ± 0.13
TV-18	22.7 ± 0.11	22.3 ± 0.21	23.0 ± 0.23	21.2 ± 0.26
TV-20	42.6 ± 0.03	48.2 ± 0.06	43.2 ± 0.09	54.6 ± 0.13
TV-22	26.0 ± 0.08	22.1 ± 0.03	28.4 ± 0.04	21.7 ± 0.23
TV-23	28.3 ± 0.12	26.0 ± 0.13	29.9 ± 0.02	23.2 ± 0.11
TV-25	42.7 ± 0.03	48.1 ± 0.12	43.0 ± 0.17	53.2 ± 0.09
TV-26	44.8 ± 0.06	49.3 ± 0.07	45.0 ± 0.09	54.2 ± 0.06
TV-29	27.2 ± 0.03	26.8 ± 0.11	27.6 ± 0.13	25.3 ± 0.03
TeenAli 17/1/54	25.9 ± 0.01	25.1 ± 0.32	26.2 ± 0.26	25.2 ± 0.13

Total phenol content in healthy and *C.theae* inoculated tea leaves

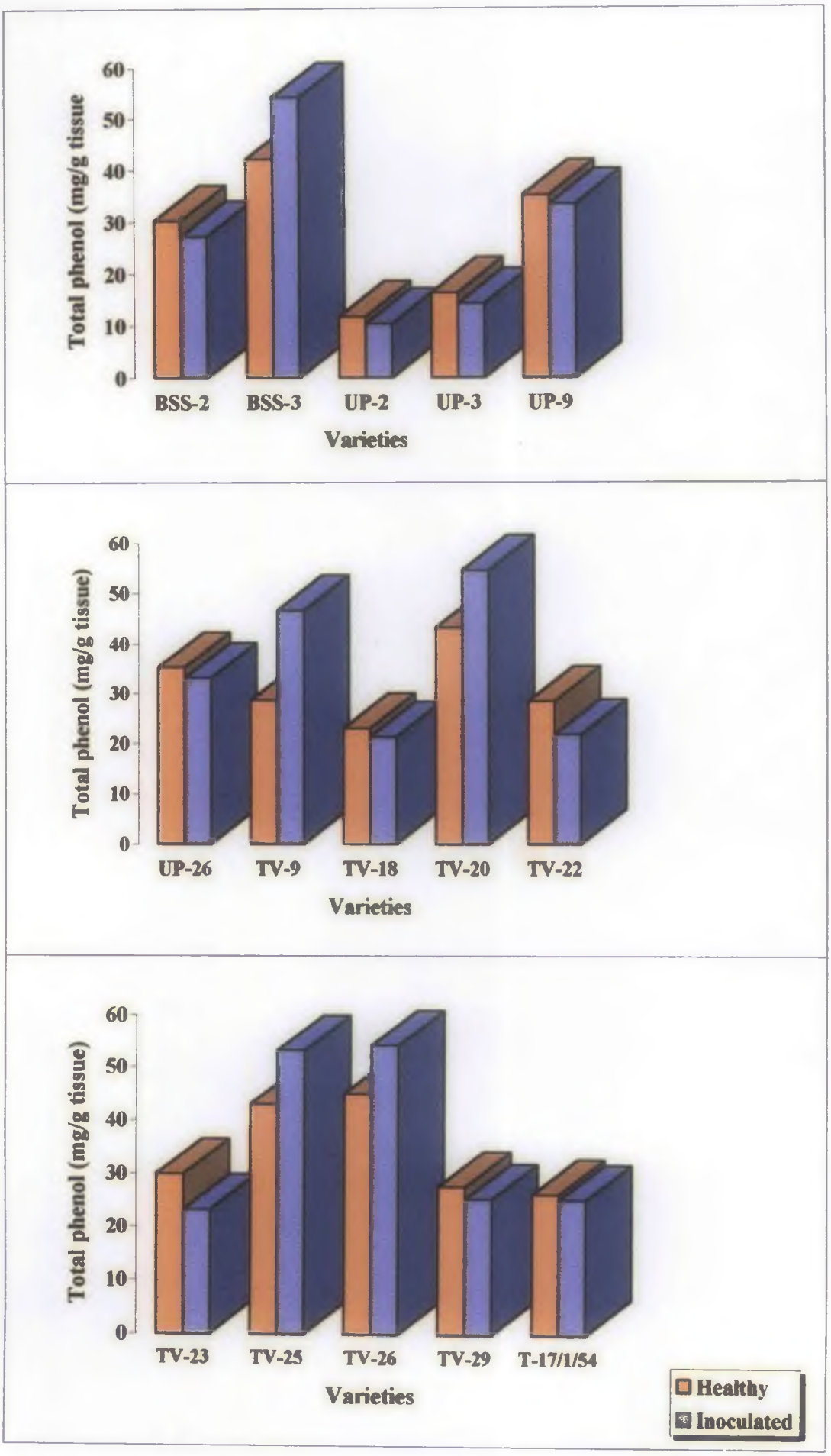


Fig.4

Table 6 : Level of Ortho-dihydroxyphenol in healthy and *C theae* inoculated tea leaves

Varieties	Ortho-dihydroxy phenol (mg / g fresh wt. of leaf tissue)			
	24h		48h	
	H	I	H	I
UPASI				
BSS-2	1.36	1.35	1.43	1.35
BSS-3	1.68	2.34	1.74	2.62
UP-2	1.51	1.47	1.52	1.43
UP-3	1.45	1.30	1.48	1.31
UP-9	1.59	1.65	1.61	1.68
UP-26	1.57	1.66	1.60	1.69
Tocklai				
TV-9	1.58	1.83	1.62	2.06
TV-18	1.39	1.30	1.41	1.30
TV-20	1.83	2.62	1.86	2.87
TV-22	1.43	1.30	1.46	1.32
TV-23	1.51	1.42	1.53	1.45
TV-25	1.69	2.13	1.78	2.26
TV-26	1.65	1.89	1.69	1.93
TV-29	1.59	1.67	1.63	1.70
TeenAli 17/1/54	1.54	1.65	1.59	1.69

H = Healthy I = Inoculated with *C theae*

Average of five replicates

Ortho-dihydroxy phenol content of healthy and *C.theae* inoculated tea leaves

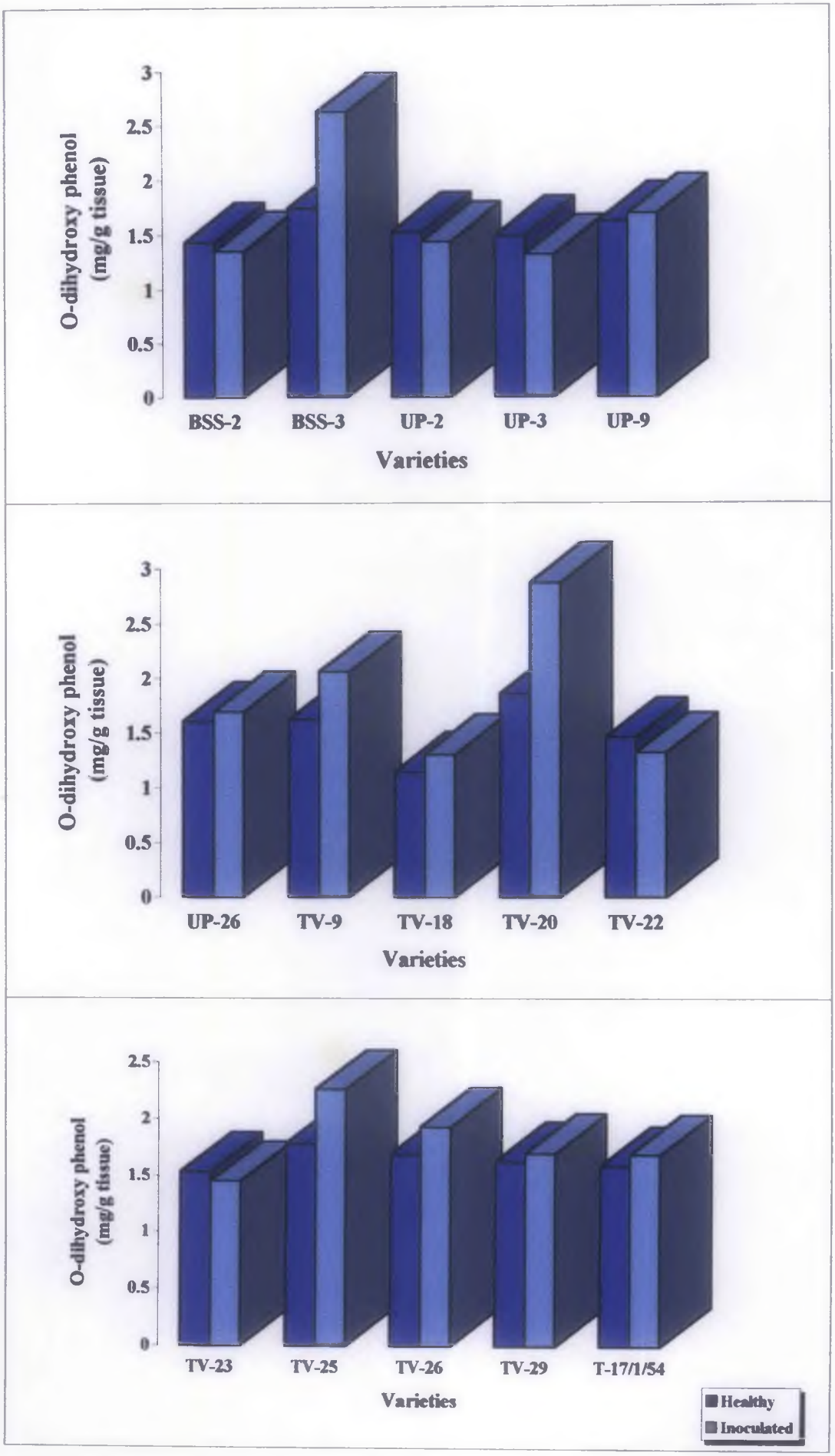


Fig.5

4.5. Determination of enzyme activity in healthy and *C.theae* inoculated tea leaves

4.5.1. Peroxidase

Peroxidase activity was assayed as increase in absorbance when o-dianisidine was oxidised by the oxygen released from H_2O_2 which was oxidised by the enzyme. Peroxidase was extracted from healthy and artificially inoculated (with *C.theae*) tea leaves of 15 varieties and their activity was assayed. Results have been presented in Table 7 and Figure 6. Peroxidase activity increased mainly in case of resistant varieties of which PO activity increased markedly in case of *C.theae* inoculated leaves of TV-26.

4.5.2. Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenylpropanoid metabolism in higher palnts and it has been suggested to play a significant role in regulating the accumulation of phenolics, phytoalexins and lignins , three key factors responsible for disease resistance. Keeping this in mind in the present study, activity of phenylalanine ammonia lyase was assayed in 15 different tea varieties subjected to biotic stress i.e. following inoculation with *C.theae* . PAL activity was assayed in each case after 24 and 48h of inoculation. Results have been presented in Table 8 and Figure 7. Results revealed that PAL activity increased after 48 h of inoculation in 6 varieties (BSS-3, TV-9, TV-18, TV-20, TV-25, TV-26) which exhibited resistant reaction towrads *C.theae*, while in other varieties increase PAL activity was not significant.

Table 7 Peroxidase activity in healthy and *C.theae* inoculated tea leaves

	Peroxidase (Δ O.D. / g tissue / min)			
	24h		48h	
	Healthy	**Inoculated**	**Healthy**	**Inoculated**
UPASI varieties				
BSS-2	8.02	8.32	8.40	8.90
BSS-3	13.20	13.62	13.22	13.73
UP-2	9.40	9.82	9.42	10.12
UP-3	8.73	9.02	8.77	9.13
UP-9	6.02	6.32	6.40	6.91
UP-26	8.00	8.12	8.10	8.28
Tocklai varieties				
TV-9	9.62	10.10	9.83	10.63
TV-18	6.42	6.87	6.48	6.89
TV-20	14.12	14.26	14.23	14.93
TV-22	5.68	6.01	5.72	6.21
TV-23	4.48	5.02	4.62	4.93
TV-25	10.12	10.32	10.26	10.41
TV-26	11.40	12.26	11.60	13.62
TV-29	8.21	8.26	8.01	8.90
TeenAli 17/1/54	7.31	7.32	7.20	7.63

Peroxidase activity in healthy and *C.theae* inoculated tea leaves

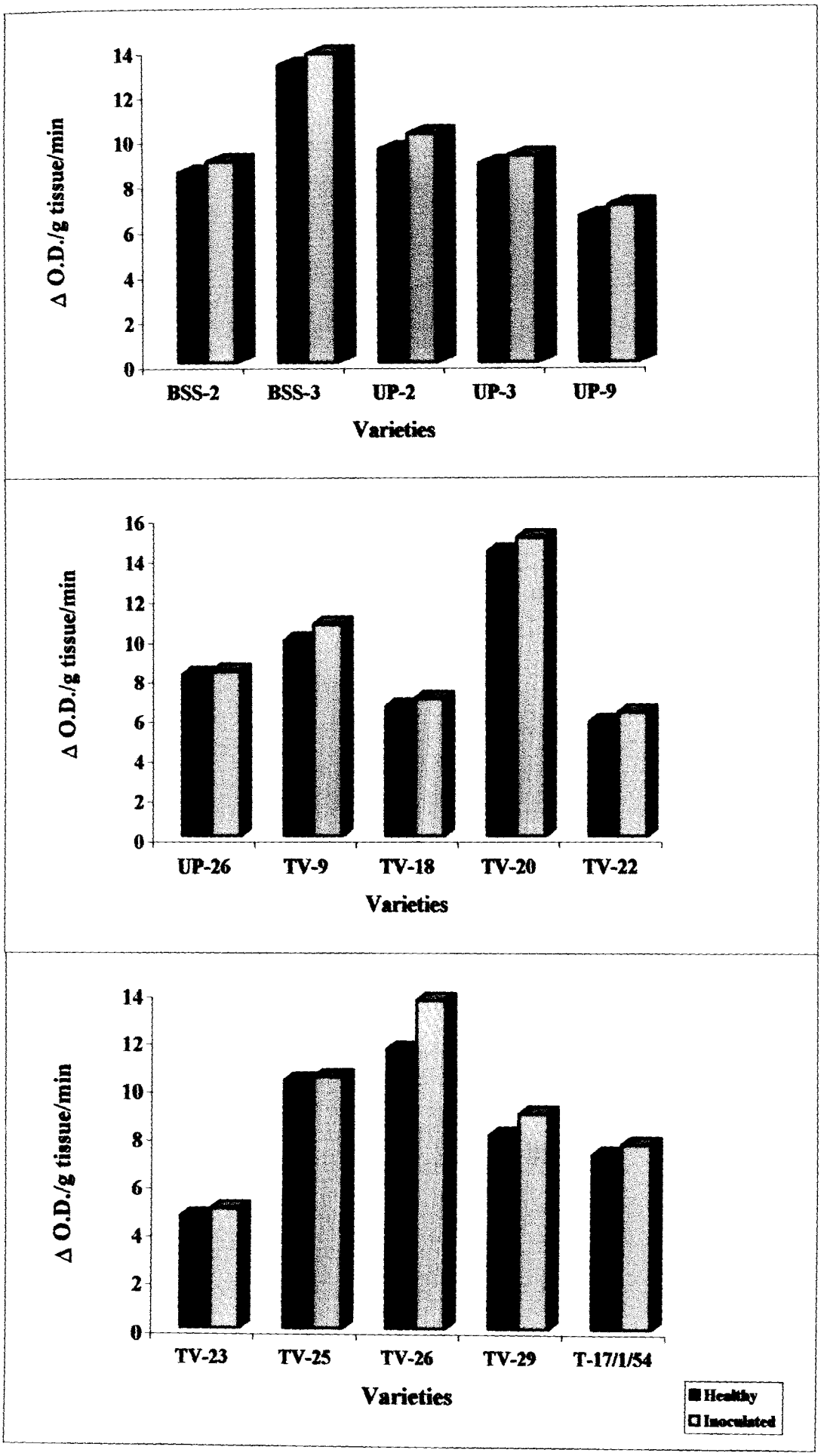
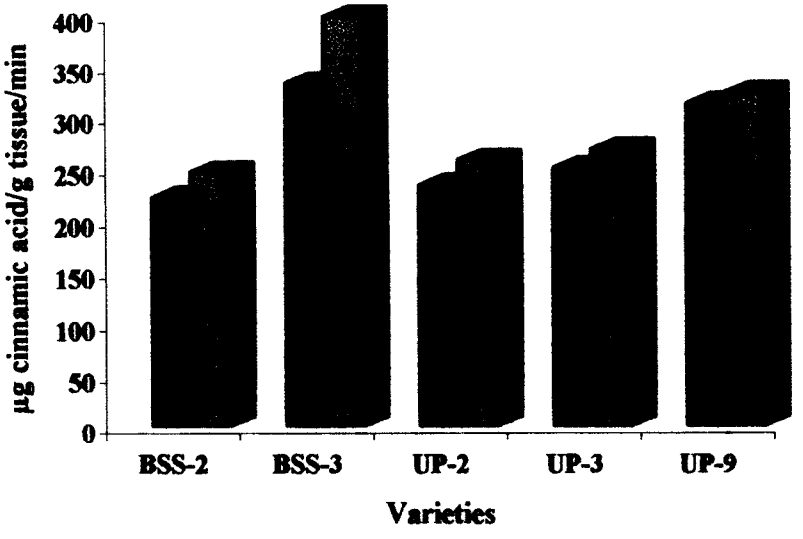


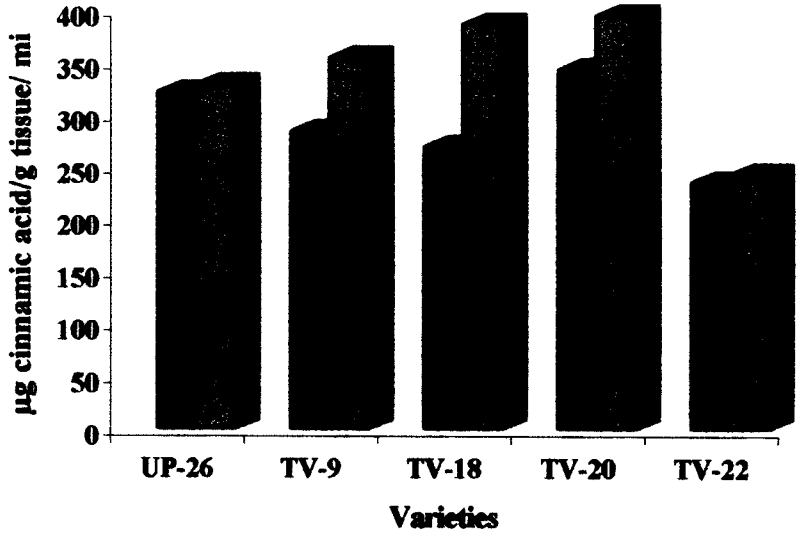
Fig.6

Table 8 Phenylalanine Ammonia Lyase (PAL) activity in tea leaves following inoculation with *C. theae*

Varieties	PAL activity (μg cinnamic acid / g tissue / min)			
	24h		48h	
	Healthy	Inoculated	Healthy	Inoculated
UPASI varieties				
BSS-2	219±5.6	236±4.3	221±8.0	245±6.8
BSS-3	329±4.3	343±1.7	332±6.0	397±2.8
UP-2	230±8.8	243±2.7	232±9.8	256±7.2
UP-3	246±1.6	253±5.3	248±6.1	266±3.5
UP-9	313±1.5	317±3.1	310±5.1	321±1.3
UP-26	319±6.7	323±6.2	321±7.6	328±2.6
Tocklai Varieties				
TV-9	280±7.2	320±6.7	283±6.8	353±3.6
TV-18	270±3.6	310±8.6	268±7.2	384±8.8
TV-20	329±5.6	346±2.3	341±4.3	392±2.0
TV-22	230±6.0	237±6.1	233±7.0	241±1.6
TV-23	255±3.0	263±3.1	268±0.3	280±1.3
TV-25	328±1.5	369±6.2	330±5.1	410±2.6
TV-26	283±5.7	332±5.9	285±7.5	385±9.5
TV-29	240±5.6	249±3.5	243±6.5	256±5.3
TeenAli 17/1/54	233±5.6	239±6.3	236±5.7	241±3.6

PAL activity in healthy and *C.theae* inoculated tea leaves





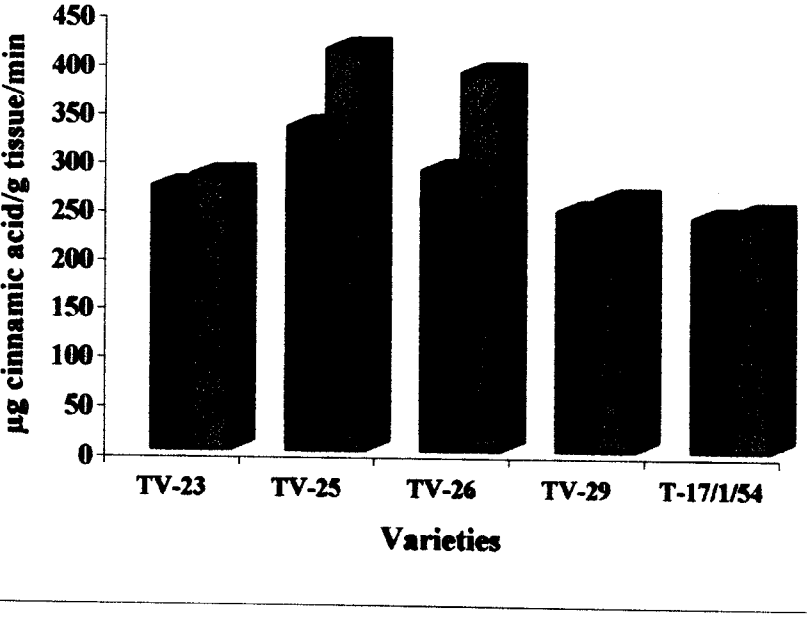


Fig.7

4.6 Studies on biological activities of leaf diffusates of tea

The differential resistance reaction of tea varieties in response to infection with *C. theae* as evidenced in the varietal resistance test may be attributed to differences in their abilities for the production of antifungal compounds. To determine this, at the onset, leaf diffusates were collected following drop diffusate method and their biological activities were tested.

Leaf diffusates were collected separately from adaxial surface of four tea varieties, viz, TV-9 TV-20, TV-22 and TV-23 after 48h. of inoculation with *C. theae* as described in Materials and Methods and their biological activities were evaluated an spore germination of *Glomeralla cingulata*. The results presented in Table-9 indicate that diffusates collected from TV-20 and TV-9 were more fungitoxic than those from TV-22 and TV-23. Percentage inhibition in spore germination was maximum (77.7%) with the duffusates collected from TV-20 and minimum (46.43%) with the diffusates of TV-23.

Table 9 : Effect of leaf diffusates of tea varieties on spore germination of *G.cingulata*

Diffusates collected from	%spore germination[a]	% inhibition in spore germination	Germ tube length (um)[b]	% inhibition in germ tube length [c]
TV-9	21.3 ± 1.14	75.5	38.4 ± 1.17	46.8
TV-20	19.4 ± 0.59	77.7	45.2 ± 0.98	47.4
TV-22	41.0 ± 0.66	52.8	49.8 ± 1.22	31.1
TV-23	46.6 ± 1.23	46.43	43.6 ± 0.89	39.6
Distilled water (Control)	87.0 ± 1.85		72.3 ± 2.06	

Diffusate collected after 48 h, Incubation temperature : 25 $\pm^0$C
[a] Average of 500 spores ; [b] Average of 60 germlings;
[c] Inhibition in relation to control

4.7. Characterization of simple phenolics in healthy tea leaves

Simple phenolics from healthy leaves of four tea varieties (TV-9, TV-20, TV-22 and TV-23) were extracted and characterized by thin layer chromatography (TLC). They were identified by comparing with authentic phenols and phenolic acids. The phenols extracts were chromatographed two dimensionally on silica gel plates. TLC plates were development first in acetic acid: chloroform (1:9) and then in ethyl acetate: benzene (9:11). The plates were sprayed with Folin-Ciocalteav's reagent and the phenols were detected as blue spots. Rf values of these spots were determined. After the spray fuming with ammonia vapour revealed the presence of some more blue to grey spots. Rf. values of these spots were also noted which are presented in Table 10. Simultaneously some authentic phenolics known to be present in tea leaves (protocatechuic acid, gallic acid, catechol, caffeic acid, p-coumaric acid) were separately run on TLC plates. Their colour reaction and Rf values were noted and compared.

Table10:Rf values and colour reaction of phenol extracts from healthy tea leaves.

	Rf (x100) Solvent I Acetic acid Chloroform (1:9)	Solvent II Ethyle-acetate Benzene (9:11)	Colour reactions with Folin Ciocalteau's reagent	Corresponded with authentic phenol and phenolic acid
1	3	15	Blue	-
2	6	32	Blue	-
3	14	38	Blue	-
4	19	44	Blue	Protocatechuic acid
5	3	46	Blue	Gallic acid
6	19	72	Blue	-
7	16	80	Blue	-
8	32	63	Blue	Catechol
9	52	80	Blue [b]	Caffeic acid
10	57	74	Blue [b]	p-coumaric acid
11	73	65	Blue b	-
12	92	97	Blue	-

a = On thin-layer chromatograms of silicagel
b = Blue colour after ammonia fumigation

4.8. Detection of antifungal compounds in tea leaves after challenge with *C.theae*

Detection of antifungal compounds in tea leaves after challenge with *C. theae* thi methods is not easily applied to large amount of tissue and the utility of this technique is diminished further due to low solubility of many phytoalexins in pure water. Phytoalexin production is known to be one of the conferral mechanisms of disease resistance in several plants (Hammerschmidt, 1999). In the present investigation further experiments were carried out following facilitated diffusion technique for the detection of antifungal substances (phytoalexin) from relatively large samples of freshly harvested tea leaves inoculated with *C. theae*. Antifungal compounds were extracted separately from healthy and *C. theae* inoculated tea leaves of two resistant varieties (TV-9 and TV-20) and two susceptible varieties (TV-22 and TV-23) after 48 h of inoculation.

4.8.1. Radial growth bioassay

At the onset, crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *C. theae* inoculated tea leaves of four varieties were bioassayed following radial growth inhibition assay as described in Materials and Methods. Results have been presented in Table 11 , Plate 5,figs A-G. Mycelial growth of *C. theae* was inhibited markedly in the medium supplemented with the extracts of inoculated leaves of resistant variety TV-9 and TV-20 than those of susceptible variety TV-22 and TV-23 in relation to their respective control ie. media supplemented with healthy leaf extract. Mycelial growth was measured in each treatment, when *C. theae* covered full petridish (3 cm dia) grown in Potato Dexlrose Agar medium without any supplementation. It is interesting to note that growth of *C. theae* was completely inhibited in the medium supplemented with extract of inoculated leaves of TV-20.

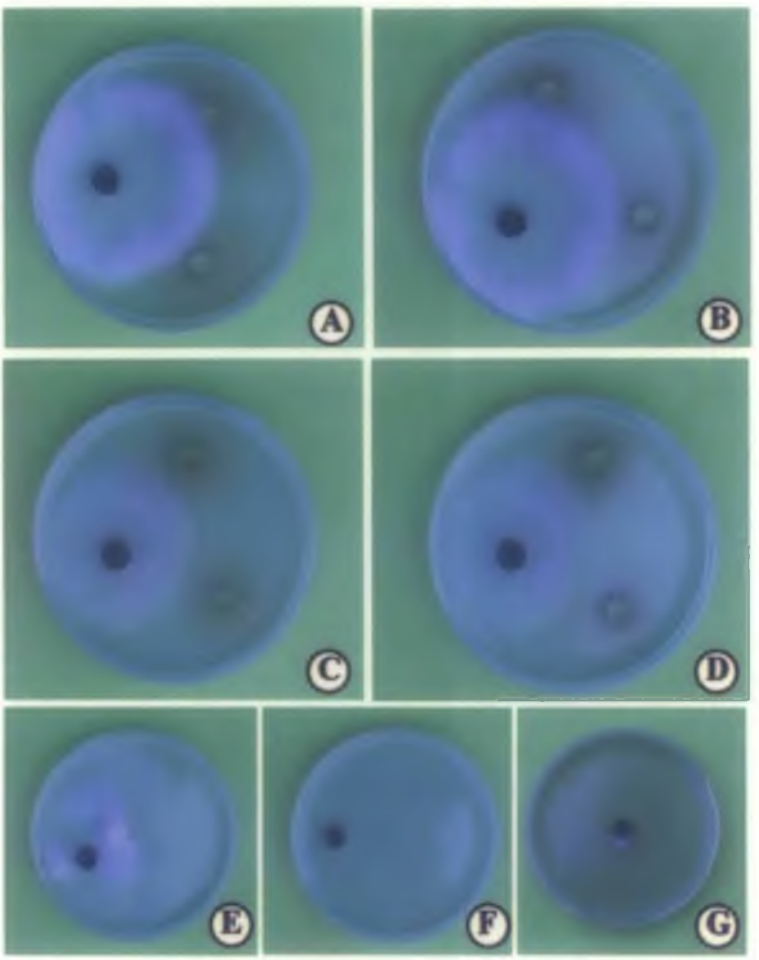


Plate 5 (Figs. A-G) :Bioassay of antifungal compound extracted from tea leaves following inoculation with *C.theae*. (A-D) Agar-cup bioassay of antifungal compound extracted from healthy leaves (A&B), 48h after inoculation with *C.theae* (C&D). TV-18 (A&C); TV-20 (B&D). (E-F) Bioassay of purified antifungal compound extracted from TV-9 using poision food technique. (E) Healthy leaf extract (control) (F&G) inoculated with *C.theae*.

Table 11 : Effect of antifungal compounds from tea leaf extracts on radial growth of *C.theae*

Variety	Diameter of mycelial growth (mm) [a]	
Susceptible	**Healthy**	**Infected with *C.theae***
TV-22	18.2	11.5
TV-23	16.5	12.8
Resistant		
TV-9	13.2	7.5
TV-20	14..8	8.0
Control	30	

[a] Average of two experimental sets.

4.8.2. Chromogenic spray

Ethyl acetate fractions of both healthy and *C. theae* inoculated tea leaf extracts were loaded on TLC plates, developed in chlorofrom : methanol (9:1,v/v) and sprayed separately with Folin-Ciocalteau's reagent, diazotized p-nitroaniline and vanillin -H_2SO_4. Compound I at Rf 0.62 showed brown colour reaction when sprayed with vanillin -H_2SO_4, whereas compound II at Rf 0.56 gave positive colour reaction indicating the presence of phenolic compounds.

Table 12 : Colour reaction of the antifungal compounds in visible light after spraying with chromogenic reagents

Chromogenic spray	Colour reaction of the antifungal compounds	
	I (Rf 0.62)	II (Rf 0.56)
Folin-Ciocalteau's reagent	No colour	Deep blue
Diazotized p-nitroaniline	No colour	Brick red
Vanillin – H_2SO_4	Brown	No colour

Solvent system : Chloroform : Methanol (9 : 1, v/v)

4.7.2. TLC plate bioassay

Ethylacetate fractions of both healthy and *C. theae* inoculated leaves of four varieties were bioassayed for antifungal compounds using Curvularia sp as the test organism following TLC plate bioassay technique as described. Two compounds, (I and II) at Rf 0.62 and 0.56 respectively exhibited inhibition zone on the chromatagram. Diameter of inhibition zones for healthy and inoculated tea leaf extracts for four varieties appeared on the chromatograms are presented in Table 13. The inhibitory compounds were present not only in leaf extracts of inoculated resistant and susceptible varieties but also in extracts of non-inoculated healthy leaves. There was no evidence of the inhibition zone at Rf 0.62 (I) in *C.theae* inoculated leaves of TV-22 and TV-23. However, inhibition zone at Rf 0.56 (II) appeared in resistant varieties.

4.8.4. Glass slide bioassay

Partially purified (by preparative TLC) pyrocatechol obtained from *C. theae* inoculated leaf extract of TV-20 was further bioassayed following spore/sclerotia germination method. In this experiment antifungal nature of pyrocatechol was tested against *G.cingulata* and *C. theae*. Sclerotia germination of *C. theae* was completely inhibited by this compound in

relation to distilled water control. However, spore germination of *G. cingulata* was inhibited by 90% in relation to distilled water control.

Table 13 : TLC –plate bioassay of antifungal compounds

Variety	Treatment	Diameter of the inhibition zone (mm)	
		Compound I	**Compound II**
		Rf 0.62	**Rf 0.56**
Susceptible			
TV-22	Healthy	7.5	0
TV-22	Infected	0	2.8
TV-23	Healthy	8.2	0
TV-23	Infected	0	2.2
Resistant			
TV-9	Healthy	10.0	2.0
TV-9	Infected	3.2	7.8
TV-20	Healthy	12.2	2.4
TV-20	Infected	3.0	6.9

Solvent system – Chloroform : Methanol (9:1 V/V)

Organism tested – *Curvularia lunata*

Incubation period – 96 h at 25^0C

4.8.5. UV- spectrophotometric analysis

Partially purified Pyrocatechol (Rf 0.56) from the extracts of healthy leaf and *C. theae* inoculated leaves were examined in a UV- spectrophotometer (Model Shimadzu 160). It is interesting to note that extracts from healthy leaf tissue following facilitated diffusion technique did not give any peak at 274 nm, while extract from healthy leaf tissue with wound exhibited absorption

peak at this wavelength. Maximum absorption peak measured at 274 nm was identical to an authentic sample of pyrocatechol. Hence, quantification of pyrocatechol was done from UV-spectro photometric curve by considering molar extinction coefficient of authentic pyrocatechol 6000 at 274 nm (Williams and Flaming, 1988). Pyrocatechol accumulated in two resistant (TV-20 and TV-9) and two susceptible (TV-22 and TV-23) varieties of tea after 48h of inoculation was estimated and compared with their healthy controls. It appears from the result (Table 14) that in inoculated leaves, greater amount (484-610 μg/g fresh weight) of antifungal compound (pyrocatechol) accumulated in the resistant varieties than in the susceptible varieties (275-346 μg/g fresh wt.) Concentration of this compound in healthy leaf tissues were very low (75-80 μg/g fresh weight).

Table 14 : Quantitative estimation of pyrocatechol in resstant and susceptible tea varieties inoculated with *C.theae*

Tea varieties	Pyrocatechol concentration (μg/g fresh wt. of leaves)	
	Healthy leaves	Leaves inoculated with *C.theae*[a]
Resistant varieties		
TV-9	80	484
TV-20	96	610
Susceptible varieties		
TV-22	71	346
TV-23	75	275

[a] 48h after inoculation

4.9. Studies on elicitors of *C. theae*

There is evidence that plants accumulate antifungal compounds or phytoalexins as a part of the inducible defence mechanism in response to pathogen invasion or treatment with biotic and abiotic elicitors

(Greyer and Kokubun, 2001). Hence, the elicitors of this pathogen (*C..theae*) was tested to determine their effect on development of disease reactions. In the present study cell wall extracts were prepared from 15-day old culture of *C. theae* and their role in elicitation of antifungal compounds were studied. Besides, their chemical nature was also determined.

4.9.1. Determination of nature of disease reaction elicited by the elicitor

To determine the nature of disease reaction elicited by the elicitor, detached tea leaves of resistant varieties (TV-9 and TV-20) were inoculated separately with four different treatment such as (a) sclerotial suspension of *C. theae*, (b) mycelial wall extract (MWE) preparation of *C. theae*, (c) sclerotial suspension mixed with mycelial wall extract and (d) sterile distilled water. Percentage lesion production was calculated after 48, 72 and 96h. inoculation results (Table 15) revealed that the disease reaction elicited both by sclerotial suspension and the mycelial wall extract were similar.

4.9.2. Bioassay of diffusible compounds elicited by the elicitor

Since the mycelial wall extract could also elicit the resistant in tea leaves (TV-9 and TV-20) it was considered imperative to determine whether it elicited any antifungal compound. Bioassay of diffusible compound collected after 48h from the adaxial surface of tea leaves of two resistant varieties (TV-9 and TV-20) following four treatments viz. distilled water, mycelial wall extract with distilled water, sclerotial suspension, and mycelial wall extract with sclerotial suspension were centrifuged and bioassayed by spore germination method. Control sets were also kept with mycelial wall extract preparation and sterile distilled water. Diffusible compound collected from leaf surface against all four treatments inhibited spore germination markedly. The mycelial wall extract was almost as effective as the sclerotial suspension in eliciting antifungal compounds on the leaf surface (Table 16). However, when the sclerotia of *C.theae* were allowed to germinate on glass slides in presence of mycelial wall extract and distilled water separately, the germination percentage was similar to that of distilled control. Therefore mycelial wall extract was as such not fungitoxic but it elicited the production of antifungal compounds in the host leaf surface.

Table 15 Comparison of lesion production by mycelial wall extract and sclerotial suspension of *C.theae* on detached leaves of resistant varieties of tea

Variety	Treatment	% lesion production [c]		
		48h	**72h**	**96h**
TV-9	Sclerotial suspension[a]	19.4±0.98	23.6±1.13	27.5±1.56
	Mycelial wall extract[b]	15.3±1.41	20.2±0.95	24..8±0.87
	Sclerotial suspension + Mycelial wall extract	18.5±0.89	24.2±1.02	29.3±1.34
	Distilled water	0	0	0
TV-20	Sclerotial suspension[a]	13.2±1.23	15..6±1.61	28.4±1.83
	Mycelial wall extract[b]	11.5±1.11	16.2±1.12	25.5±0.97
	Sclerotial suspension + Mycelial wall extract	12.8±0.95	15.8±2.11	22.9±1.13
	Distilled water	0	0	0

[a] Sclerotial suspension of *C.theae*

[b] Mycelial wall extract of *C.theae*

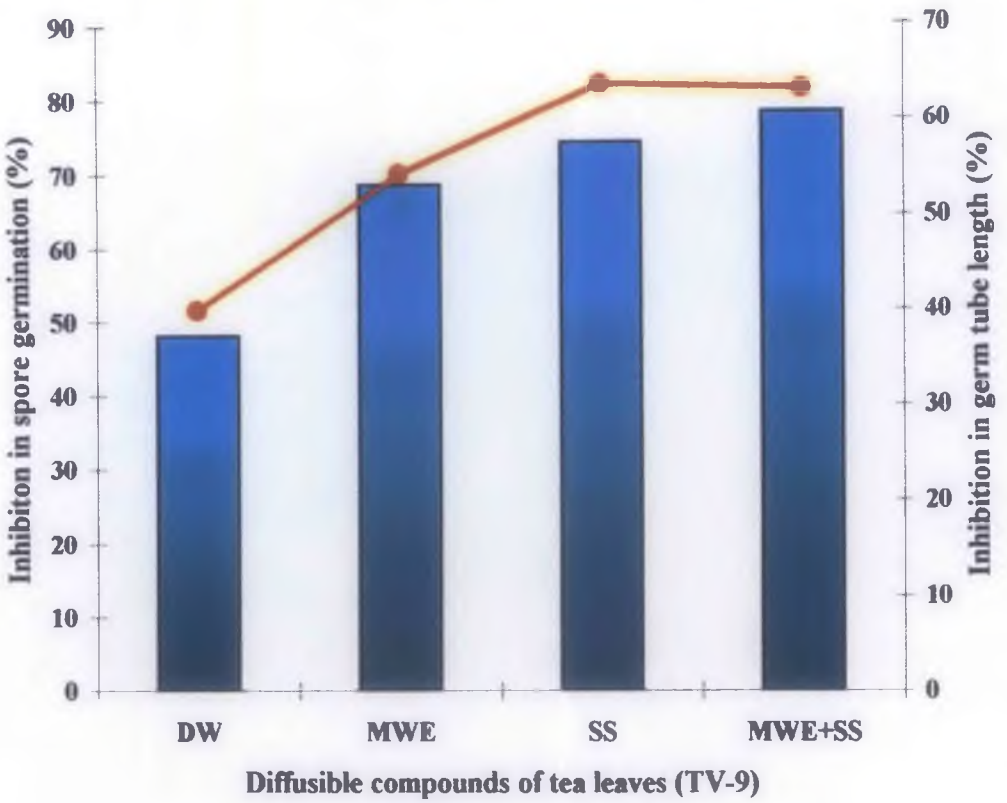
[c] Average of 50 leaves / treatment

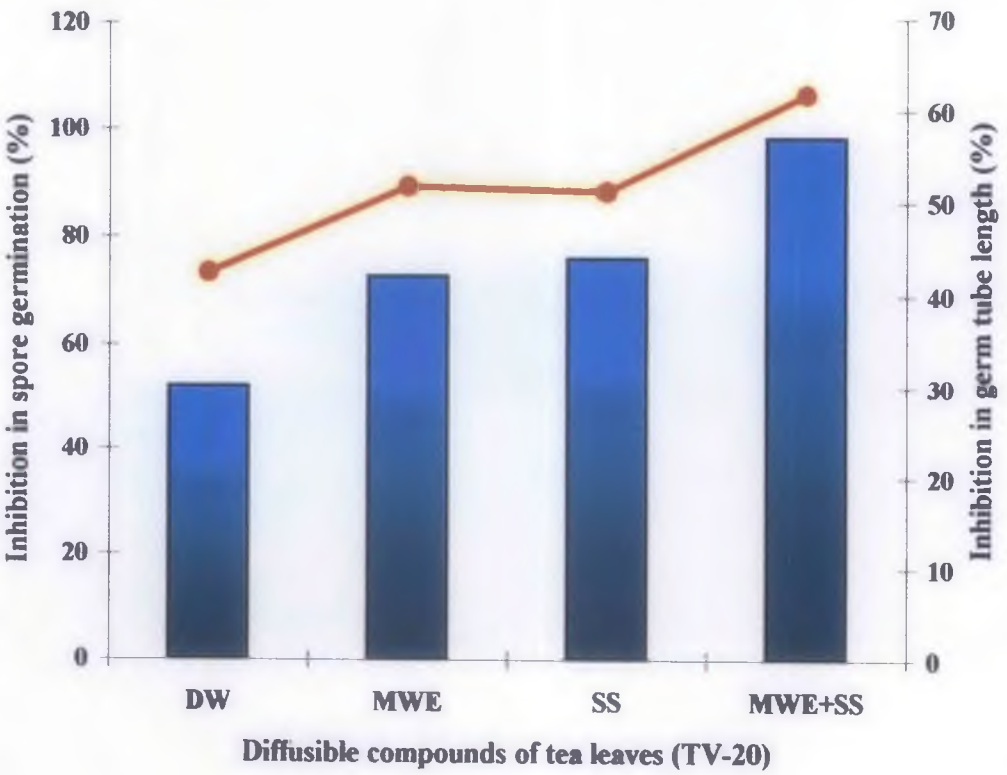
Table 16 : Spore germination bioassay of diffusible compounds elicited by the mycelial wall extract of *C.theae*

Treatment	Variety	%spore germination	Germtube length
Diffusible compounds collected from adaxial surfaces of tea leaves			
Distilled water	TV-9	43.8 ± 2.4	55.2 ± 1.62
Distilled water	TV-20	40.5 ± 1.85	52.7 ± 1.33
Mycelial wall extract	TV-9	26.5 ±1.03	42.0 ± 1.85
Mycelial wall extract	TV-20	23.1 ± 0.92	44.3 ± 0.98
Sclerotial suspension	TV-9	21.8 ± 1.26	33.4 ± 0.85
Sclerotial suspension	TV-20	20.5 ± 1.54	37.9 ± 1.11
Mycelial wall extract + Sclerotial suspension	TV-9	18.4 ± 0.88	33.9 ± 0.78
Mycelial wall extract + Sclerotial suspension	TV-20	17.9 ± 1.18	35.2 ± 2.21
Control			
Distilled water		84.2 ± 2.56	92.0 ± 2.87
Mycelial wall extract		86.7 ± 1.46	89.5 ± 2.01

[a] Average of 500 spores ; [b] Average of 60 germlings

Bioassay of diffusible compounds collected from tea leaves after treatment with mycelial wall extract of *C.theae*





DW: Distilled water; MWE: Mycelial wall extract; SS: Sclerotial suspension

Fig.8

4.9.3. ConA-FITC binding of mycelia and cell wall

Cell walls were isolated from *C. theae* and the isolated cell walls were further extracted with NaOH as described earlier. This preparation was further analysed by SDS- polyacrylamide gel electrophoresis and confirmed by binding with fluorescein labelled concanavalin A. Mycelia and isolated cell walls of *C. theae* were treated with FITC-labelled con A and observed under the microscope. Strong fluorescence was observed under the microscope in both the mycelia and cell wall of *C. theae* (Plate 6, figs A&B). The occurrence of Con A binding substances in the cell walls confirmed the glycoprotein nature of mycelial wall of *C. theae*.

4.10. Analysis of host-parasite protein

4.10.1. Determination of levels of soluble proteins in healthy and inoculated tea leaves

Soluble proteins were extracted from healthy and artificially inoculated tea leaves of fifteen varieties and estimated Results have been presented in Table 17 and Figure 9. It reveals that in all the tested varieties protein content decreased following inoculation after 24 and 48h. Analysis of protein pattern by SDS-PAGE of four selected varieties (TV-9, TV-20, TV-22 and TV-23) were done. Results have been presented in Plate 7 (Figure B). Protein pattern of healthy and infected leaves were more or less similar. No specific extra protein bands were visible. The molecular weights (kDa) of the proteins on gel were 98.0, 97.8, 87.6, 77.8, 40.2, 34.6, 29.0, 24.5, 21.8,16.1,16.6,13.5,12.2 for healthy leaves whereas for infected leaves the molecular weights were 97.8, 86.7, 77.4, 40.2, 29.0, 21.8, 16.8, 16.2, 16.1, 15.0, 13.8 and12.4.

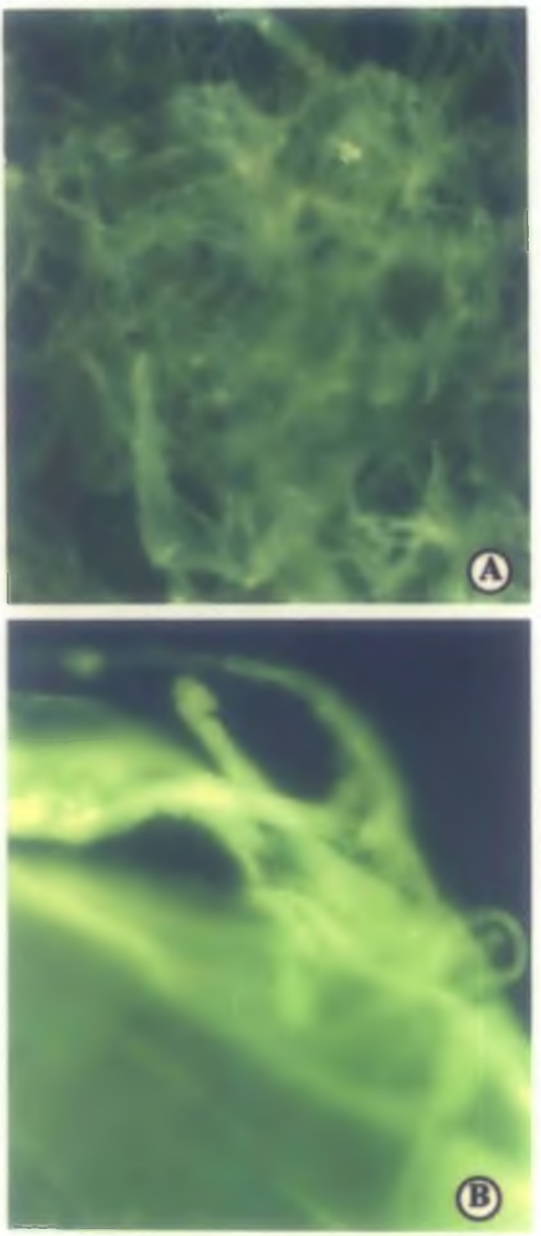


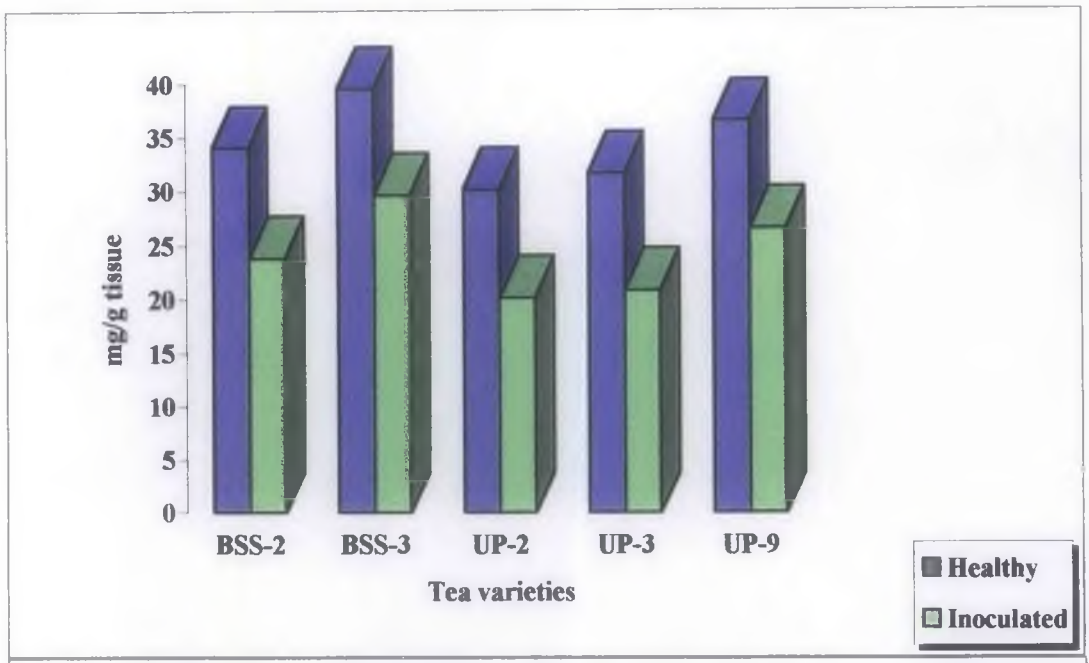
Plate 6 (Figs. A&B) : Fluorescence of hyphae (A) and isolated cell walls (B) of *C.theae* after staining with FITC-ConA

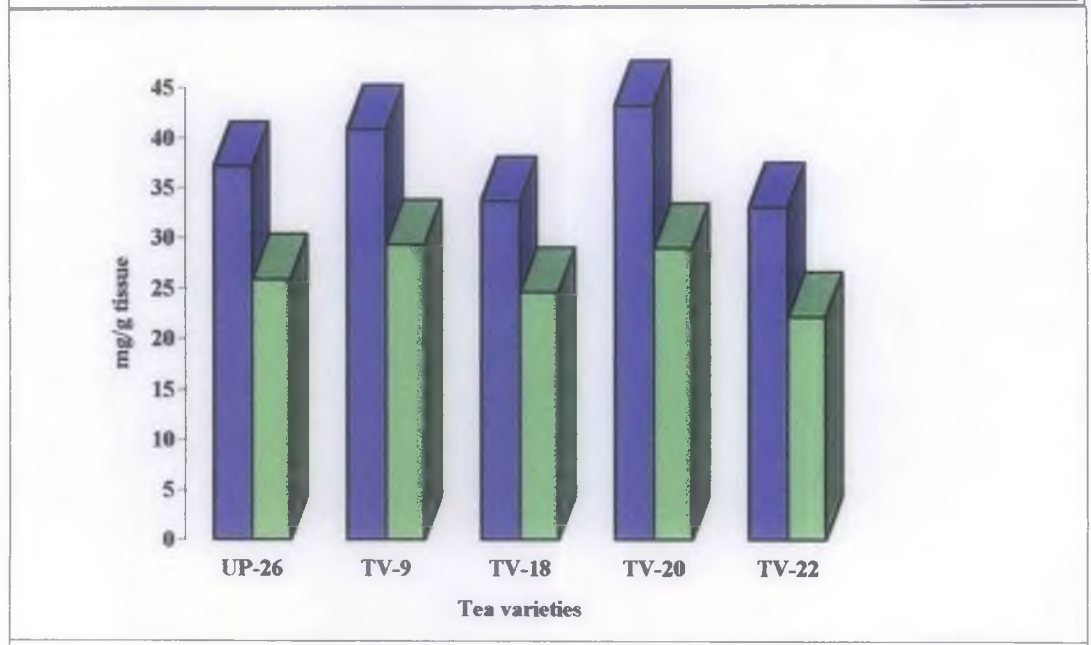
Table 17 : Level of protein in healthy and *C. theae* inoculated tea leaves

Varieties	Protein content (mg / g tissue)			
	24h		48h	
	H	I	H	I
UPASI				
BSS-2	32.13±1.3	21.52±1.2	34.07±1.1	23.80±2.6
BSS-3	37.06±2.1	24.80±1.1	39.60±2.1	29.60±2.3
UP-2	27.30±3.1	20.20±2.7	30.08±1.0	20.10±1.8
UP-3	30.23±1.4	20.20±0.3	31.60±0.3	20.77±1.2
UP-9	35.15±1.1	24.50 ±1.0	36.51±2.9	26.46±2.0
UP-26	35.91±1.1	27.00±3.1	37.14±0.9	25.88±3.8
Tocklai				
TV-9	37.57±3.2	25.17±1.9	40.75±1.1	29.30±2.2
TV-18	32.10±1.6	23.50±2.1	33.60±1.4	24.50±2.1
TV-20	41.26±2.3	27.64±1.1	43.10±1.3	28.87±0.3
TV-22	32.10±0.9	21.50±0.7	32.95±2.1	22.07±1.6
TV-23	34.21±1.2	23.90±0.2	35.25±2.3	24.07±1.5
TV-25	37.20±1.3	25.90±1.2	38.53±1.2	27.81±3.1
TV-26	41.10±0.5	29.53±1.6	41.51±1.6	31.47±2.1
TV-29	35.11±1.1	24.51±0.1	36.22±1.3	26.25±2.1
TeenAli 17/1/54	32.13±2.5	23.52±0.7	34.33±1.6	25.90±1.3

H = Healthy I = Inoculated with *C. theae*
Average of five replicate/treatment

Protein content of healthy and *C.theae* inoculated tea leaves





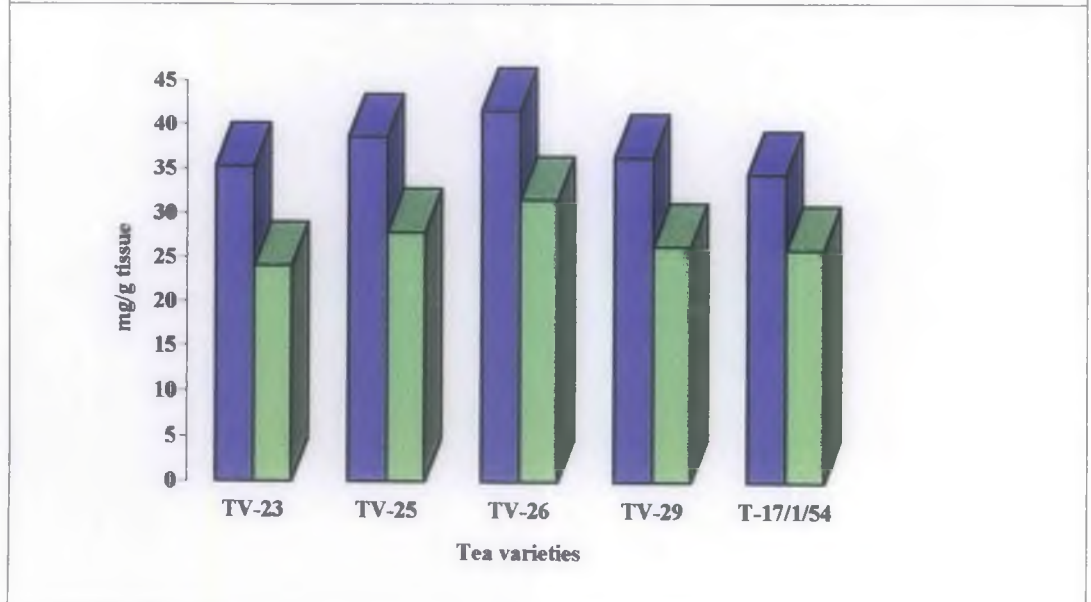


Fig.9

4.10.2. SDS-PAGE analysis of mycelial and cell wall proteins of *C.theae*

Mycelial proteins as well as proteins from the cell wall of *C.theae* were also analysed by SDS-PAGE . Results have been presented in Table 18 and Plate 7 (Figure A). Total 12 protein bands were observed in when soluble proteins prepared from mycelia of *C.theae* were analysed in gel. Whereas 9 protein bands were visible when soluble protein preparation from cell wall of *C.theae* were resolved in SDS-PAGE.

Table 18 : Molecular weight of soluble proteins of mycelia and cell wall of *C.theae*

Molecular weight (kDa)	
Mycelial protein of *C.theae*	**Cell wall proteins of *C.theae***
99.0	68.0
98.5	60.5
97.8	55.5
97.0	45.5
68.0	38.4
63.0	25.7
55.5	23.3
48.0	17.7
38.4	
29.1	
20.8	
19.2	

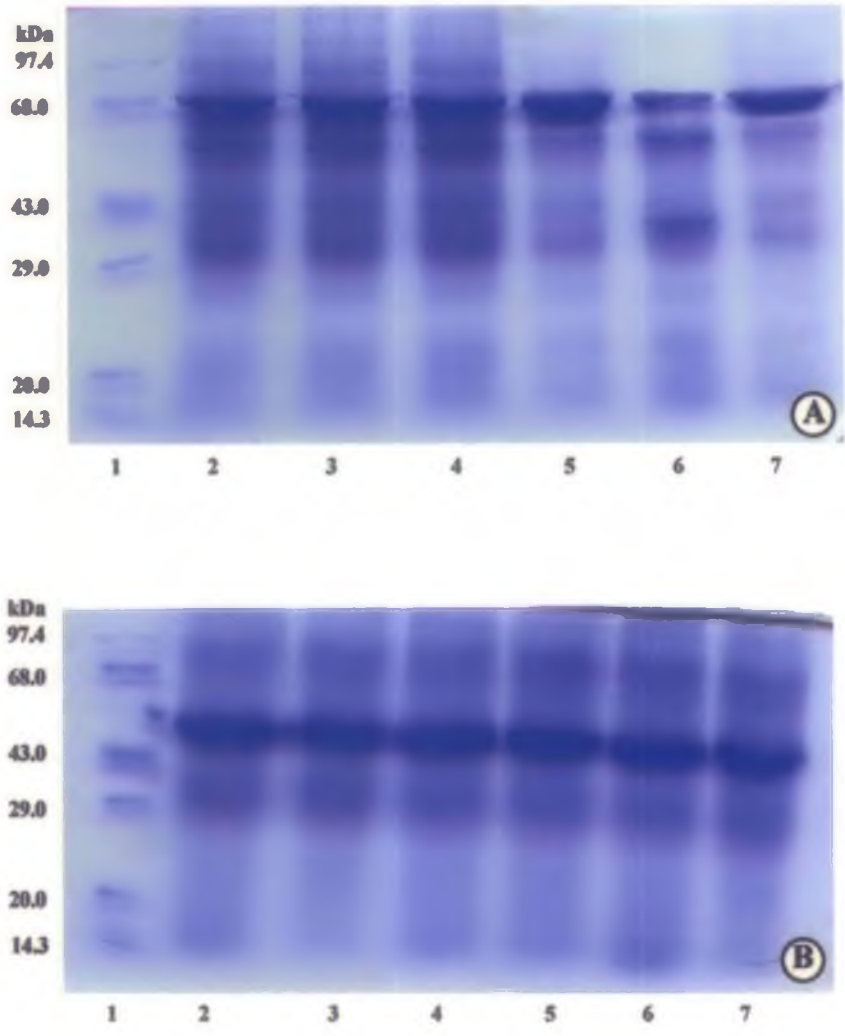


Plate 7 (Figs. A & B) : SDS-PAGE analysis of mycelial, cell wall proteins of *Corticium theae* and tea leaf proteins (A) *C. theae*; lane 1 Molecular marker; lanes 2-4 Mycelia ; lanes 5-7 Cell wall (B) Tea leaf; lane 1 Molecular marker; lanes 2,4 &6 Healthy ; lanes 3,5&7 Inoculated with *C.theae*

4.11. Detection of cross reactive antigens between *Corticium theae* and tea varieties

The presence of cross reactive antigens among plant host and pathogenic organisms is a well documented phenomenon. Existing studies on immuno-phytopathology suggests that whenever, an intimate and continuing association of host and pathogen occurs, partners of this association have a unique serological resemblance to one another involving one or more antigenic determinants. Antigen sharing between different cells has been of special interest because of its coincidence in compatible host-parasite relationship. It's possible significance also has been implicated in cell to cell relationships of host and parasites. The presence of major cross reactive antigens (CRA) among plants and pathogenic organisms is a well established phenomenon. Using various immunological methods presence of CRA has been demonstrated between host and parasite. Earlier techniques like immunodiffusion and immunoelectrophoresis are being currently replaced by more advanced techniques like enzyme linked immunosorbent assay (ELISA) and immunofluorescence. In the present study, major cross reactive antigens (CRA) shared beween *Corticium theae* and Tea varieties (Tocklai and UPASI) have been detected using immunodiffusion , ELISA and immunofluorescence. Series of experiments performed and results obtained have been presented below.

4.11. 1 Immnodiffusion tests

The effectiveness of antigen preparations from *C.theae* (isolates Ct-1) and tea leaves (TV-18 and TV-26) for raising polyclonal antibodies were checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigens of pathogen (*C.theae* isolates, Ct-1 and Ct-2) and tea leaves (TV-18 and TV-26) were all negative. When polyclonal antibody (PAb) raised against mycelial antigens of *C.theae* (Ct-1) was reacted with its own antigen and antigen of another isolate (Ct-2), strong precipitin reaction occurred.

(Table 19, Plate 8 fig A-C) . When PAb of *C.theae* was cross reacted with leaf antigens prepared from 15 tea varieties (6 UPASI and 9 Tocklai) , 7 varieties (UPASI-2, UPASI-3, UPASI-9, BSS-2, TV-18, TV-22 and TV-23) exhibited strong precipitin bands in immunodiffusion tests. However, weak precipitin reactions were observed with antigens of other 6 varieties (UPASI-26, BSS-3, TV-25, TV-26, TV-29 and TeenAli-17/1/54). No such preciptin bands were observed in case of leaf anigens prepared from 2 specific varieties (TV-9 and TV-20) as well as leaf antigen preparation from one non-host plant viz. *Oryza sativa* and one non-pathogens of tea (*Fusarium oxysporum*).

Reciprocal cross reaction using PAb raised against TV-26 and antigens prepared from tea leaves of 15 variieties, one non-host and one non-pathogen species and two isolates of *C.theae* were also carried out. Results (Table 20) revealed that none of the isolates of *C.theae* could develop any precipitin reaction with anti-TV26 antiserum. Non-host species and non pathogen also failed to develop any precipitin band. Serological cross reactivity among UPASI and Tocklai varietes revealed by the appearance of precipitin bands in diffusion tests. However, strong bands were observed in case of UPASI-3, UPASI-9, TV-9, TV-18, TV-22, TV-23, and TV-25, while weak preciptin reactions were observed in case of UPAS-2, UPASI-26, BSS-2, BSS-3, TV-20, TV-26, TV-29 and Teenali-17/1/54.

To confirm the presence of common antigens between *C.theae* islates and tea varieties reciprocal cross reaction with PAb raised against TV-18 was also cariied out with leaf antigens of host and non-host as well as with mycelial antigens of pathogen isolates and non-pathogen. Results are presented in Table 20. Strong precipitin recations were observed in homologous reactions with most of the Tocklai varieties (TV-9, TV-18, TV-20, TV-22, TV-23, TV-26, TV-29) and four UPASI varieties (UPASI-2, UPASI-3, UPASI-26 and BSS-2). However, weak precipitin reactions were noticed in cross reactions between PAb of TV-18 and leaf antigens of UPASI-9, BSS-3, TV-25, and TeenAli-17/1/54. It is interesting to note that in this reciprocal cross reactions *C. theae* isolates Ct-1 gave strong precipitin bands while weak precipitin bands were noticed in reactions with mycelial antigens of isolate Ct-2 and PAb of TV-18. No precipitin reactions were observed when non-host and non-pathogen antigens were reacted with PAb of TV-18 in immunodiffusion test.

Table 19 Detection of cross reactive antigens among Tea varieties and *Corticium thea* using agar gel double diffusion tests

Antigen of host and parasite	PAb of *Corticium theae*
UPASI varieties	
UPASI-2	+
UPASI-3	+
UPASI-9	+
UPASI-26	±
BSS-2	+
BSS-3	±
Tocklai varieties	
TV-9	-
TV-18	+
TV-20	-
TV-22	+
TV-23	+
TV-25	±
TV-26	±
TV-29	±
TeenAli-17/1/54	±
Pathogen	
C.theae isolate-Ct1	+
C.theae isolate-Ct2	+
Non -pathogen	
Fusarium oxysporum	-
Non-host	
Oryza sativa	-

\+ Common precipitin band present
± Weak precipitin band present
\- Common precipitin band absent

Table 20 . Serological cross reactivity among different tea varieties and *C.theae* isolates following immunodiffusion test

Tea leaf antigens	Polyclonal antibody raised against tea leaf antigens	
	TV-26	TV-18
UPASI varieties		
UPASI-2	±	+
UPASI-3	+	+
UPASI-9	+	±
UPASI-26	±	+
BSS-2	±	+
BSS-3	±	±
Tocklai varieties		
TV-9	+	+
TV-18	+	+
TV-20	±	+
TV-22	+	+
TV-23	+	+
TV-25	+	±
TV-26	±	+
TV-29	±	+
TeenAli-17/1/54	±	±
Pathogen		
C.theae isolate-Ct1	-	+
C.theae isolate-Ct2	-	±
Non -pathogen		
Fusarium oxysporum	-	-
Non-host		
Oryza sativa	-	-

\+ Common precipitin band present
± Weak precipitin band present
\- Common precipitin band absent

4.11.2. Direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA)

With the introduction of enzyme linked immunosorbent assay (ELISA) for assaying plant viruses, serological methods have been used in agricultural research and practice. ELISA in now routined for detection and diagnosis of plant pathogens (virus, bacteria and fungi). Application to the study of bacteria and fungi came much later, presumably because other methods of diagnosis were available for this microorganisms and they are generally much more complex in their make up. In DAC- ELISA antigens are linked to solid carrier, after which the antibody is allowed to bind to the antigen. To this antigen-antibody complex, the conjugate (an antibody conjugated to an enzyme) is added. Finally, a non coloured substrate is added which is converted to a coloured end product, which is generally detected by a reader. In the present study, DAC-ELISA formats has been used in all the experiments. Since ELISA depends on a number of factors and these varies from system to system, it was considered essential to optimize the conditions in this particular host (tea) and pathogen (*C.theae*) system.

4.11.2.1. Optimization of ELISA

Optimization of ELISA was done using IgG fraction of antisera raised against mycelial antigens of *C.theae.* Three variables such as enzyme dilution, dilutions of the antiserum and antigens were optimized. In all cases, ELISA reactions were carried out using PAb raised against *C.theae* (isolate Ct-1) and homologous antigens.

4.11.2.1.1. Enzyme dilution

In this study, antigen concentration (10 μg/ml) and antiserum dilution (1:125) were kept constant, and different dilutions of enzyme

(alkaline phosphatase) were used ranging from 1:10,000 to 1:40,000. On the basis of results, 1:10,000 of alkaline phosphatase was selected for further experiments.

4. 11.2.1.2. Antiserum dilution

PAb raised against *C.theae* (isolate Ct-1) were pooled in two batches. First bleeding and second bleeding were separately purified for IgG. These two batches of IgG were diluted ranging from 1:125 to 1:16,000 and then tested against homologous antigen (mycelial antigen of Ct-1 isolate) at a concentration of 10 µg/ml. Results are given in Table 21. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:16,000 prepared for IgG of first and second bleeding (Fig 10). Highest absorbance value (2.14) was obtained with second bleeding whereas 1.78 was obtained with first bleeding at 1:125 dilution. In all serological assays IgG prepared from second bleeding was considered.

Table 21 : ELISA reaction with various dilution of anti-*C.theae* antiserum and homologous antigen

Antiserum dilution	Absorbance at 405 nm	
	First bleeding	Second bleeding
1:125	1.782 ± 0.091	2.143 ±0.023
1:250	1.647 ± 0.032	1.950 ±0.034
1:500	1.572±0.055	1.812 ±0.065
1:1000	1.393±0.052	1.781 ±0.044
1:2000	1.165±0.044	1.563 ±0.058
1:4000	0.921 ±0.009	1.493 ±0.011
1:8000	0.869 ±0.006	1.036 ±0.003
1:16000	0.756 ±0.012	0.962 ±0.017

± Standard error

Effect of dilution of anti -*C.theae* on ELISA reaction with homologous mycelial antigen

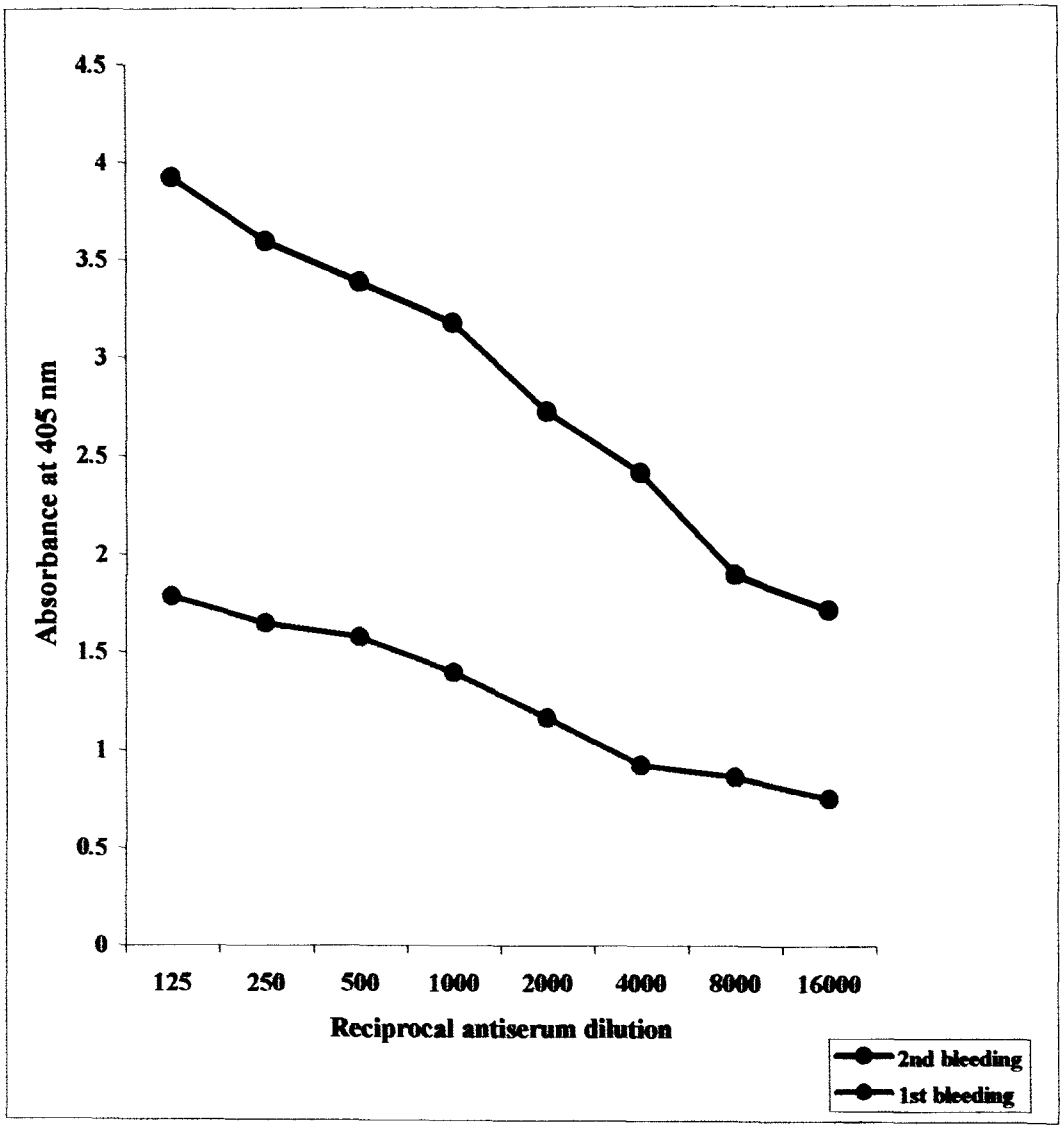


Fig.10

4.11.2.1.3. Antigen dilution

Antigen dilutions of *C.theae* ranging from 10,000 ng/ml to 78 ng/ml were tested against two antisera dilutions (1:125 and 1:250). ELISA values increased with the concomitant increase of antigen concentration (Table 22, Figure 11). Mycelial antigen concentrations as low as 78 ng/ml could also be easily detected by ELISA at both antisera dilutions.

Table 22 : ELISA reaction with various concentration of mycelial antigen of *C.theae* and homologous antiserum

Antigen dilution (ng/ml)	**Absorbance at 405 nm Antisera dilution (Second bleeding)**	
	1:125	**1:250**
10000	2.203± 0.081	1.986± 0.045
5000	1.961±0.074	1.755±0.023
2500	1.652±0.064	1.433±0.033
1250	1.230±0.066	1.026±0.009
625	0.945±0.007	0.783±0.014
312	0.892±0.005	0.622±0.009
156	0.714±0.006	0.535±0.012
78	0.634±0.017	0.507±0.007

± Standard error

Enzyme dilution = 1:10,000

Effect of dilution of mycelial antigen of *C.theae* on ELISA reaction with homologous antiserum

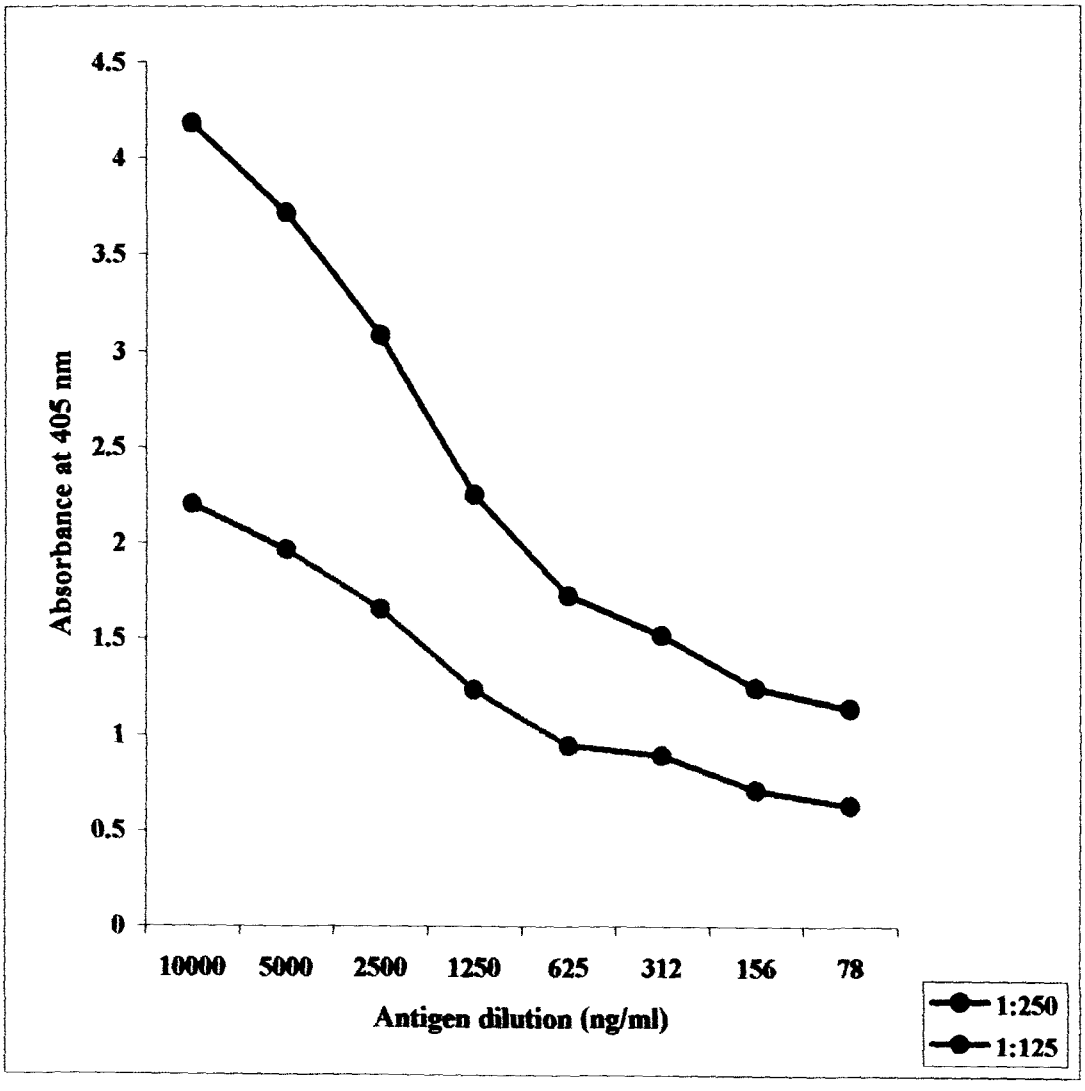


Fig.11

4.11.2.2. Comparison of ELISA reactivity among antigens of different tea varieties against antiserum of *C.theae*

Among 15 tea varieties tested for varietal resistance towards *C.theae*, diferental responses were obtained. Certain varieties were found to be highly susceptible, while others were moderately susceptible or moderately resistant. Disease development to some extent was visible in all the varieties tested. However, in biochemical analysis a definite conclusion has been drawn with reference to their antifungal properties, mainly antifungal phenolics for the development of resistant reaction toward *C.theae*. Conventional techniques for determination of host resistance or susceptibility are being replaced by more rapid, sensitive and reproducible modern serological techniques. Detection of pathogen in plant tissue in soil even before appearance of the disease symptom has become routine practice now a days in different agricultural station. It was therefore considered to find out serological cross reactivity of all the tea varieties against the pathogen (*C.theae*) using DAC-ELISA formats.

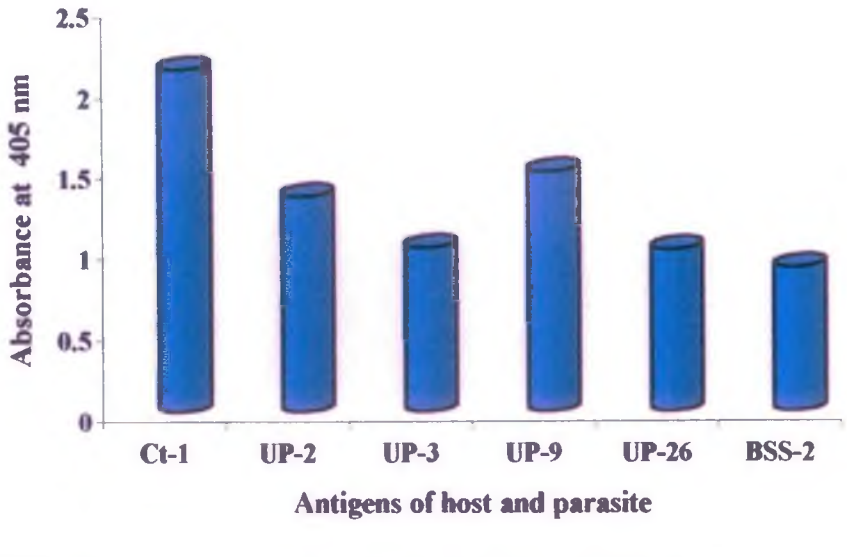
Tea leaf Antigens were prepared from 6 UPASI varieties (UPASI-2, UPASI-3, UPASI-9, UPASI-26, BSS-2, BSS-3) and 9 Tocklai varieties (TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26, TV-29 and TeenAli-17/1/54) , leaf antigens of non-host (*O.sativa*), mycelial and cell wall antigens two isolates of *C.theae* and non-pathogen (*F.oxysporum*). All of these antigens at a concentration of 40 μg/ml were tested against 1:250 dilution of PAb raised against *C.theae* (isolate Ct-1) by using DAC-ELISA formats. Experiments were repeated thrice keeping same concentrations of antigens and antisera under same incubation conditions. Results have been presented in (Table 23 and Fig. 12)

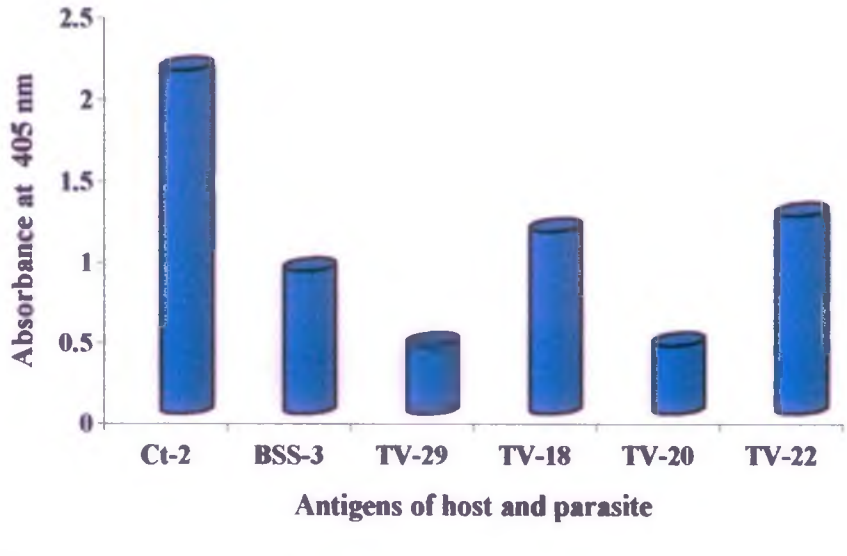
Reciprocal cross reaction involving antisera of TV-18 and TV-26 and antigen preparations from all fifteen tea varieties, two isolates of *C.theae* , non host and non pathogen were studied using DAC-ELISA formats. Results have been presented in Table 24 and Figures 13 and 14. Susceptible varieties showed positive reaction and highest absorbance values were detected in these cases. Whereas resistant varieties exhibited lower absorbance value than resistant varieties. Absorbance for the non host and non pathogen antigen prepartion with these antisera were always found to be low.

Table 23 Indirect ELISA values (A 405 nm) of leaf antigens (host and non-host) and mycelial antigens (pathogen and non-pathogen) reacted with PAb raised against *C.theae* (isolate Ct-1)

Antigens of host and parasite (40 μ g/ml)	Polyclonal antibody of *C.theae* Dilutions	
	1: 125	1:250
Tea varieties		
UPASI – 2	1.432±	1.325±
UPASI-3	1.119±	1.004±
UPASI-9	1.653±	1.472±
UPASI-26	1.115 ±	0.989±
BSS-2	1.103±	0.875±
BSS-3	0.973±	0.885±
TV-9	0.566±	0.423±
TV-18	1.448±	1.128±
TV-20	0.492±	0.421±
TV-22	1.455±	1.223±
TV-23	1.723±	1.574±
TV-25	1.204±	1.041±
TV-26	0.998±	0.821±
TV-29	0.876±	0.711±
TeenAli-17/1/54	1.025±	0.951±
Pathogen		
C.theae (isolate Ct-1)	2.801±	2.114 ±
C.theae (isolate Ct-2)	2.032±	1.706 ±
Non-pathogen		
F.oxysporum	0.471±	0.267±
Non-host		
O.sativa	0.330±	0.205±

ELISA responses of leaf antigens of different tea varieties against antiserum of *C.theae*





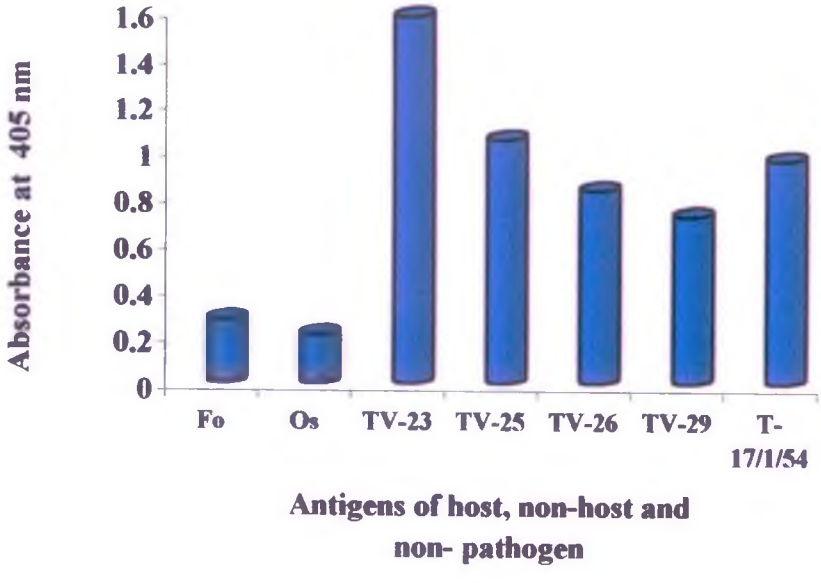


Fig.12

Table 24: Indirect ELISA values (A 405 nm) of leaf antigens (host and non-host) and mycelial antigens (pathogen and non-pathogen) reacted with PAb raised against tea leaf antigens (TV-26 and TV-18)

Antigens of host and parasite (40 μ g/ml)	Antisera of tea varieties (1:125 dilution)	
	TV-26	TV-18
Tea varieties		
UPASI – 2	1.132±	1.217±
UPASI-3	1.149±	1.044±
UPASI-9	1.614±	1.734±
UPASI-26	1.135 ±	1.239±
BSS-2	0.998±	1.384±
BSS-3	0.977±	0.926±
TV-9	0.566±	0.995±
TV-18	1.448±	1.868±
TV-20	0.892±	1.112±
TV-22	1.455±	0.992±
TV-23	1.723±	1.046±
TV-25	1.204±	0.897±
TV-26	1.982±	1.075±
TV-29	1.876±	1.028±
TeenAli-17/1/54	1.145±	0.912±
Pathogen		
C.theae (isolate Ct-1)	0.577±	0.924 ±
C.theae (isolate Ct-2)	0.482±	0.839 ±
Non-pathogen		
F.oxysporum	0.271±	0.265±
Non-host		
O.sativa	0.323±	0.216±

Reciprocal ELISA responses of leaf antigens of different tea varieties against antiserum of tea (TV-18)

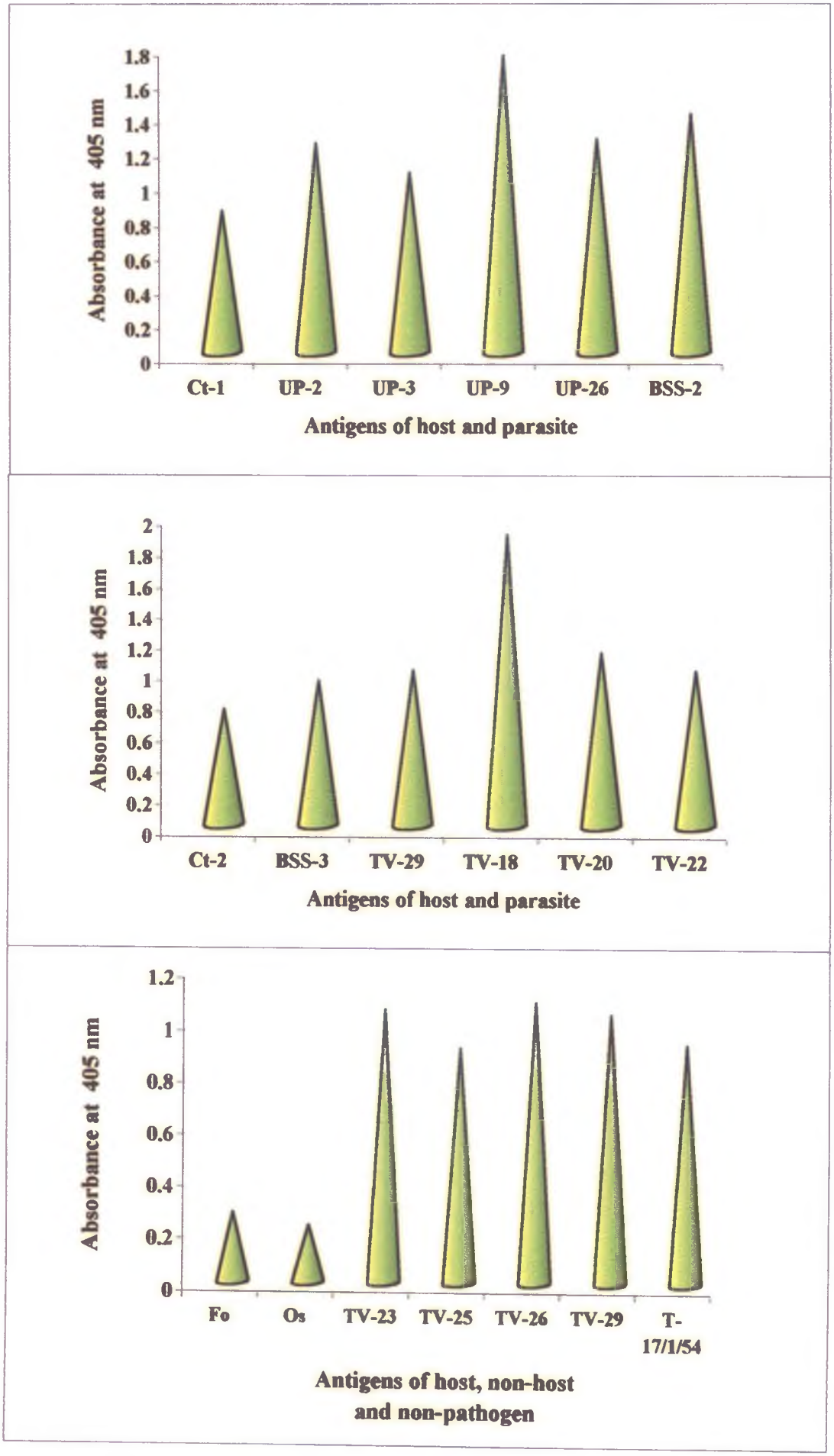
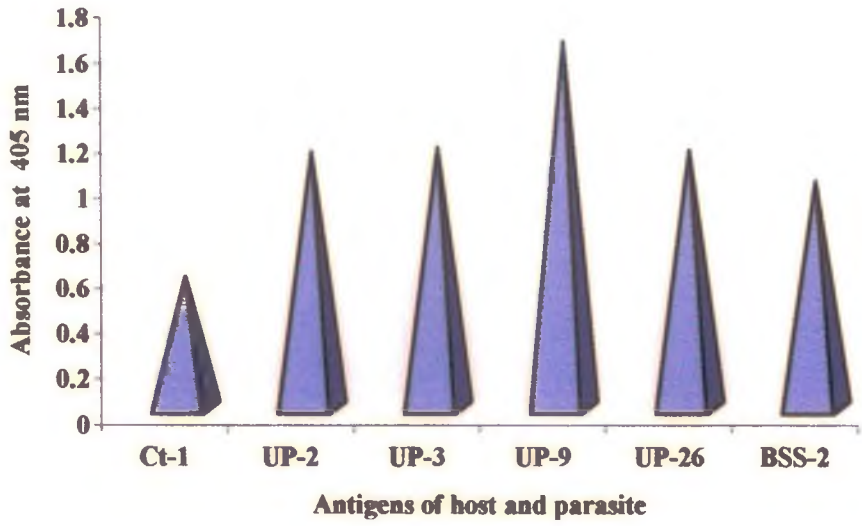
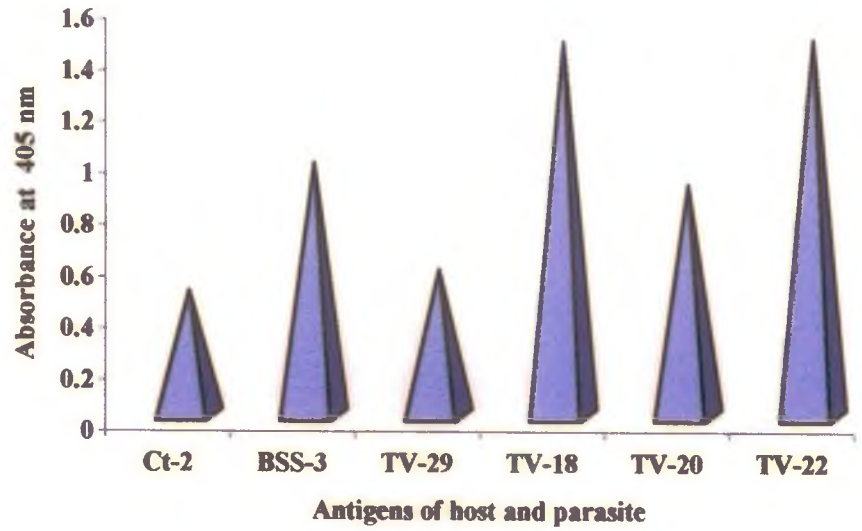


Fig.13

Reciprocal ELISA responses of leaf antigens of different tea varieties against antiserum of tea (TV-26)





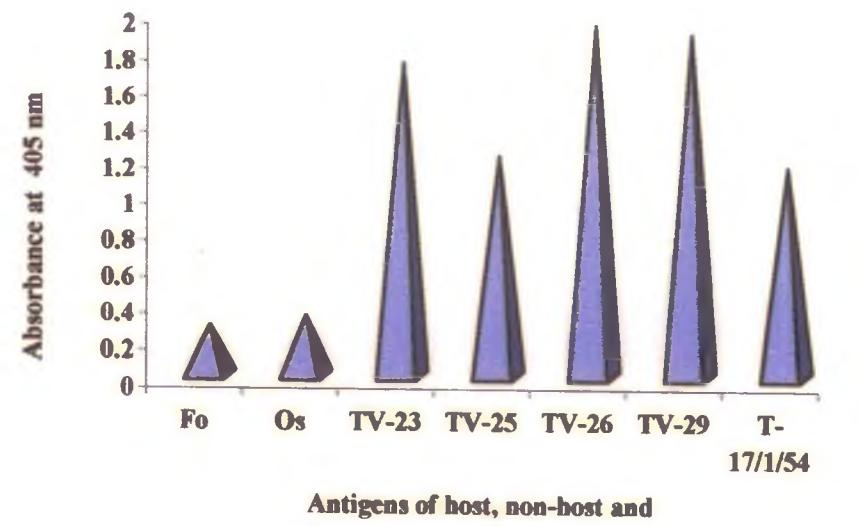


Fig.14

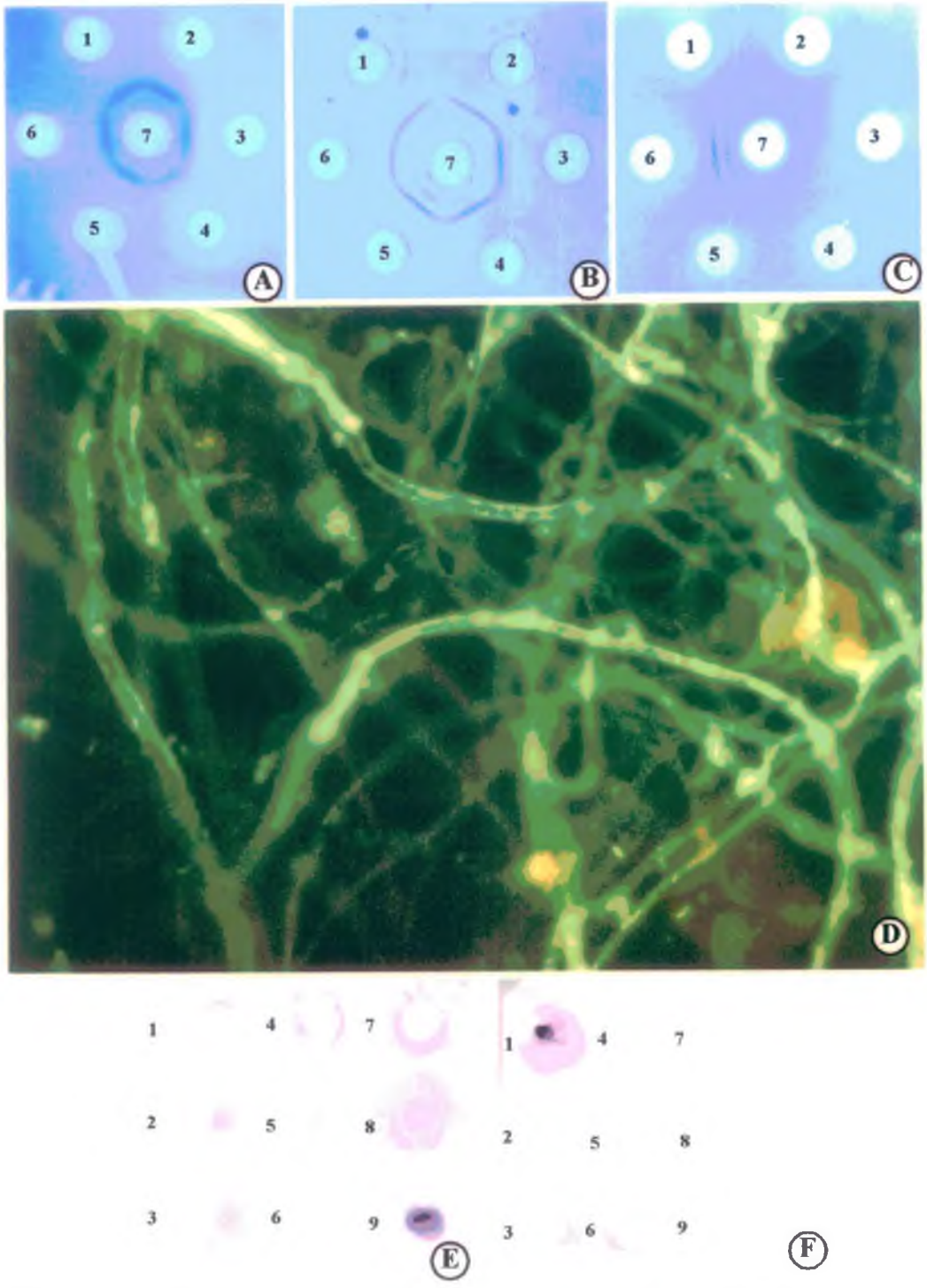


Plate 8(Figs. A-F) : Serological assays with antigens and antisera of host (tea leaf)and parasite (*C.theae*). (**A-C**) Immunodiffusion test with antisera of *C.theae*(A&C) and tea leaf (B) in the central well (7) and antigens in peripheral wells (1-6). A: mycelial antigens (1-6) ; B: leaf antigens of TV-18 (1-6), C: TV-18(6), TV-26 (5), TeenAli (4), non-host (3), and non-pathogen (1&2). (**D**) Immunofluorescence of *C.theae* mycelia treated with antisera of *C.theae* (isolate Ct-1) followed by FITC labelling. (**E&F**) Dot-blot assay with antigens of tea leaves and mycelial antigens of *C.theae* reacted with anti-*C.theae* antiserum. (**E**) Antigens of isolate Ct-1 (9) ; TV-18 (1), TV-23(2) , TV-22 (3), UPASI-2 (4) , TV-9(5), TV-20 (6) , BSS-2 (7), TV-29 (8) and (**F**) Antigens of Ct-2 (1), TV-18(2), UPASI-3 (3), TV-20 (5) BSS-3 (6), TeenAli-17 (7), TV-25 (8) and TV-9 (9).

4.11.2.3. Detection of *C.theae* in tea leaf tissue by DAC-ELISA

The efficacy of the antiserum raised against *C.theae* were tested for its ability to detect the pathogen in infected tissue by DAC-ELISA. For this experiment the leaves were artificially inoculated with *C.theae* and incubated for 48h. After that antigens were prepared from healthy as well as infected tea leaves of five varieties and tested by using DAC-ELISA formats. Results have been presented in Table 25. Higher absorbance values were recorded in all the tested varieties after 48 h of inoculation in comparison to healthy leaf antigens.

Table 25 : Absorbance values in DAC-ELISA for reactions of antiserum of *C.theae* with healthy and inoculated tea leaf antigen.

Antigens (40µg/ml)	Absorbance values at A_{405}	
	Healthy	**Inoculated**
TV-18	1.423±0.12	1.889±0.17
TV-22	1.434±0.22	1.763±0.07
UPASI-2	1.348±0.42	1.712±0.09
BSS-2	1.103±0.22	1.648±0.14
TV-23	1.723±0.52	1.973±0.12

4.11.2.4. Detection of *C.theae* by Dot blot

The antigen preparations were spotted on nitrocellulose paper carefully and tagged with antiserum of *C.theae*. Finally probed with the conjugates. Results have been presented in Plate 8 (figures E&F). Clear and intense colour reactions were observed in case of mycelial antigens preapred from both the isolates (Ct-1 and Ct-2) and alongwith that the leaf antigens preapred from susceptible varieties.

4.12. Cellular location of CRA using immunofluorescence

Fluorescent antibody labelling with 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 following immunodiffusion, DAC-ELISA as well as Dot-blot the presence of CRA shared by *Camellia sinensis* and *C.theae* has been detected. It was decided to determine the tissue and cellular location of CRA in tea leaf tissue as well as mycelia and sclerotia of *C.theae.*

4.12.1. Tea leaf tissue

Cross sections of tea leaves (TV-22 and TV-23) were treated separately with normal serum, homologous and pathogen antisera, then reacted with FITC.Leaf sections exhibited a natural autofluorescence under UV light on the cuticle (Plate 9, figure A). Same observation was noted when the leaf sections were treated with normal serum and FITC. Leaf sections treated with antiserum of *C.theae* and then reacted with FITC developed bright fluorescence which was distributed throughout the leaf tissue, mainly in the epidermal and mesophyll tissues (Plate 9, figures B&C).

4.12.2. Mycelia

Mycelia of both the isolates were not auto-fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of mycelia of *C.theae* with homolgous antiserum and FITC showed a general fluorescence that was more intense on young hyphal tips (Plate 8, figure D).

4.12.3. Sclerotia

Sclerotia of both the isolates were also not auto-fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of young germinated sclerotia of *C.theae* with homolgous antiserum and FITC showed a general fluorescence that was also more intense on young hyphal tips (Plate 10).

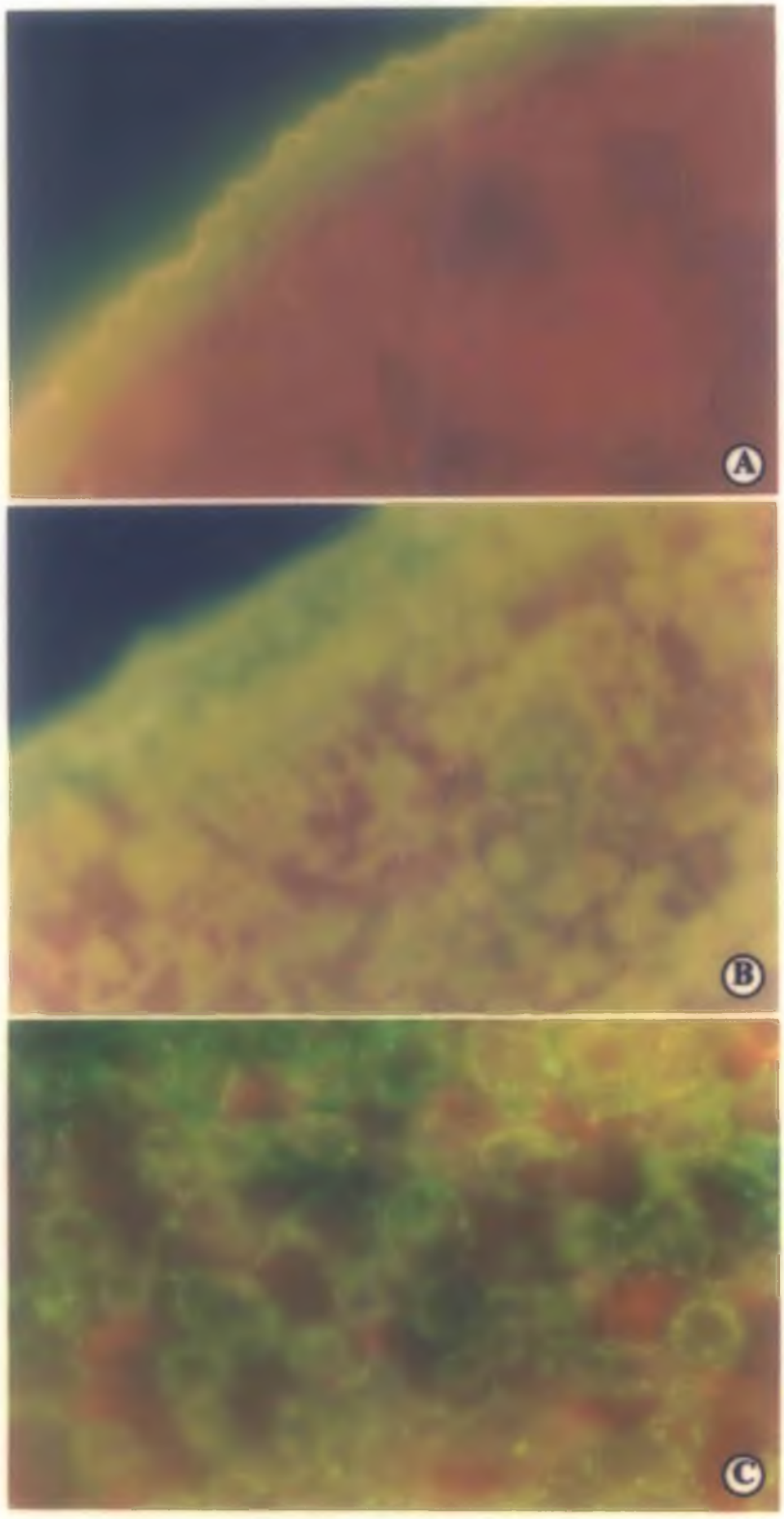


Plate 9 (Figs. A-C) :FITC antibody staining of tea leaf tissues (TV-23) for cross reactive antigens shared with *C.theae*. (A) Autofluorescence of unstained leaf section; (B&C) leaf section treated with antiserum of *C.theae* and FITC antibodies of goat specific for rabbit globulin.

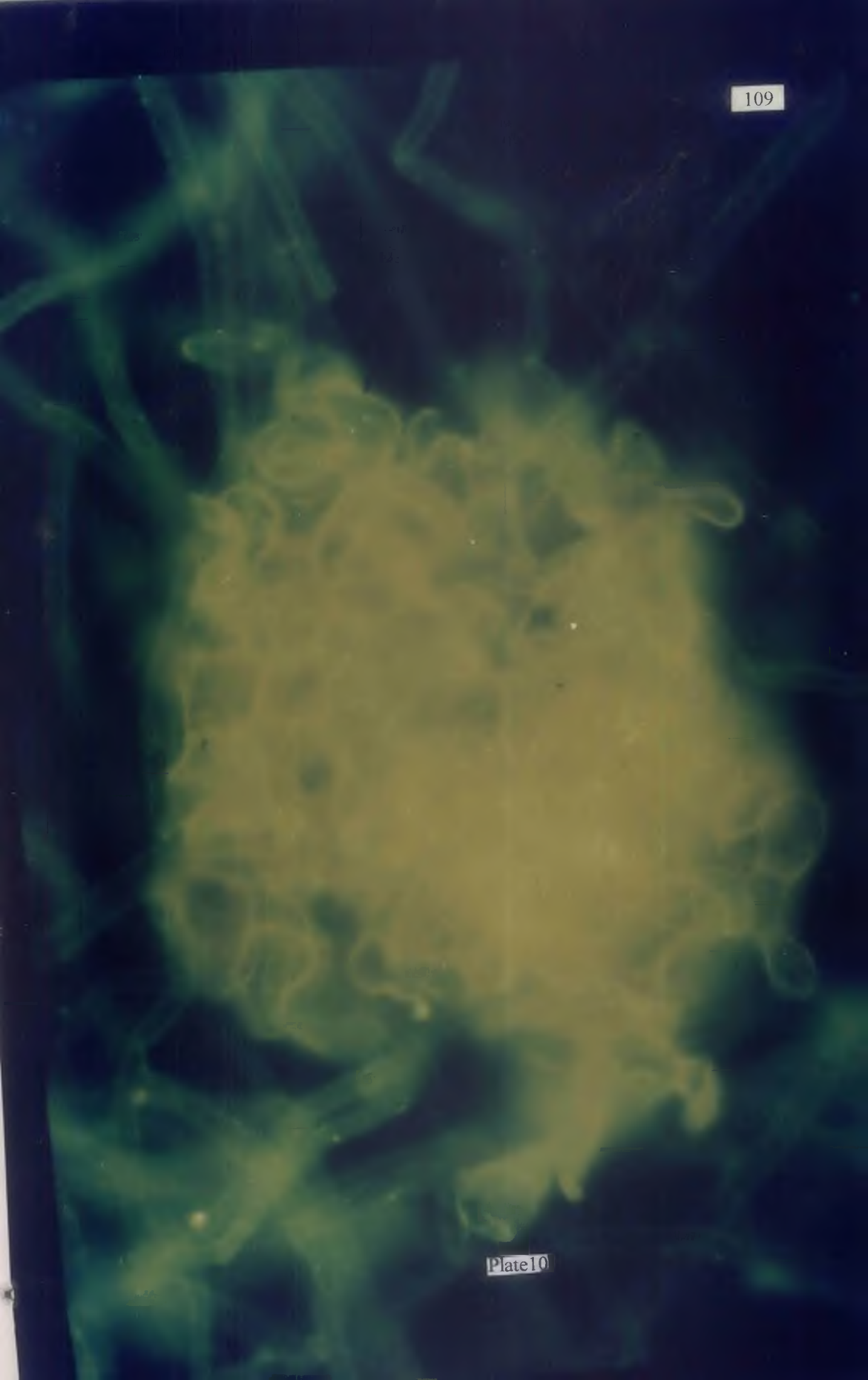


Plate10