Experimental

4.1. Pathogenicity test of *Glomerella cingulata* on different tea varieties

Varietal resistance test of tea against *Glomerella cingulata* was carried out with thirty two clonal varieties and five seed varieties released by Tocklai Experimental Station, Jorhat, Assam; Darjeeling Tea Research Centre, Kurseong, Darjeeling and UPASI Tea Research Centre, Valparai, Tamilnadu, 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 2-7, Figs. 1 & 2 and Plate VI (figs. A, B & C).

4.1.1. Detached leaf

Detached leaf inoculation of 13 Tocklai varieties, 15 Darjeeling varieties and 9 UPASI varieties was carried out. Disease assessment and symptom development were done after 48, 72 and 96h of inoculation on the basis of percent drop that resulted in lesion production. Fifty leaves of each variety were inoculated for each experiment. Results revealed that, in case of Tocklai varieties, TV-18 was most susceptible, followed by Teenali 17/1/54, TV-20, TV-25 and TV-23 while, TV-26 and TV-29 were found to be most resistant followed by TV-30, TV-9, TV-28, TS-449, TV-27 and TV-22 . After 96h of inoculation 67% lesion production was obtained in TV-18 while in TV-26 approximately 36% lesion production was observed (Table - 2, Fig.1). On the basis of significance tests (t-test), the varieties were grouped into highly susceptible (TV-18, Teenali 17/1/54 and TV-20), moderately susceptible (TV-25 and TV-23) and resistant (TV-26, TV-29, TV-30, TV-9, TV-28, TS-449, TV-27 and TV-22). Among the UPASI varieties, UPASI 9 and UPASI 3 were found to be most susceptible followed by UP-26, UP-8, BSSI, BSS 2 and BSS3 which were moderately susceptible, while UPASI 2 was resistant and UPASI-17 was moderately resistant (Table-3). Maximum lesion production among UPASI varieties was 93% in (UPASI-9) and minimum was 17% in UPASI-2 after 96h of inoculation. In case of Darjeeling varieties, B-157 and T-78 were found to be most susceptible, followed by TTV_1, KI/I, B777, BS/7A/76, HV-39 and B668 while BT-15, and CP-I were most resistant followed by P-312, T-135, AV-2, RR-17 and P-1258 (Table-4). In the most resistant vartiety BT-15, only 16% lesion production was obtained after 96h of inoculation, as compared to 90% in the most susceptible variety, B-157.

Among all the 37 varieties of tea tested, maximum and minimum susceptibilities were exhibited by UPASI-9 and BT-15 respectively.

Table 2 :Pathogenicity test of *G.cingulata* on Tocklai varieties of tea following detached leaf inoculation

Variety	Percentage lesion production[a] Hours after inoculation		
	48	72	96
TV-9	28.06 ± 1.2	34.77 ± 2.3	*41.20[c]±1.8
Teenali 17/1/54	34.66 ± 1.7	47.20 ± 3.5	57.66[b] ± 1.9
TV-18	53.33 ± 1.9	59.20 ± 3.9	66.45[b] ± 1.9
TV-20	45.00 ± 0.6	57.65 ± 2.1	57.30[b] ± 3.7
TV-22	35.90 ± 0.8	42.20 ± 3.2	47.31[c] ± 2.5
TV-23	30.55 ± 0.8	41.66 ± 1.7	50.90 ± 1.5
TV-25	45.45 ± 3.3	54.36 ± 0.9	51.60 ± 1.8
TV-26	26.08 ± 1.3	32.60 ± 3.3	36.20[c] ± 3.6
TV-27	31.25 ± 0.7	36.30 ± 0.8	45.05[c] ± 1.2
TV-28	29.30 ± 1.7	35.00 ± 2.1	41.42[c] ± 2.8
TV-29	26.85 ± 2.1	34.29 ± 2.6	40.00[c] ± 2.2
TV-30	26.99 ± 3.1	35.79 ± 1.8	40.60[c] ± 3.7
TS-449	25.90 ± 3.0	37.2 ± 2.0	42.33[c] ± 2.1

a Average of three separate trials;
50 leaves inoculated in each trial.
± Standard error.
*Difference in values with same superscript insignificant at P=0.01.

Table 3: Pathogenicity test of *G.cingulata* on UPASI varieties following detached leaf inoculation.

Variety	Percentage Lesion Production[a] Hours after inoculation		
	48	72	96
UP-2	0	5.55 ± 3.2	*16.66 ± 2.9
UP-3	75.00 ± 2.9	86.20 ± 2.5	88.60[b] ± 2.8
UP-8	36.84 ± 2.9	52.10 ± 3.1	57.36[c] ± 2.2
UP-9	85.71 ± 2.2	86.20 ± 2.7	92.85[b] ± 2.9
UP-26	50.00 ± 3.1	61.21 ± 2.1	69.14[c] ± 2.4
UP-17	14.39 ± 1.6	25.00 ±3.2	30.18 ± 2.1
BSS-1	49.12 ± 2.9	53.66 ± 2.7	57.22[c] ± 2.8
BSS-2	42.79 ± 3.2	46.77 ± 2.1	50.34[c] ± 2.3
BSS-3	35.19 ± 2.4	40.80 ± 2.9	42.93[c] ±3.1

a Average of three separate trials;
50 leaves inoculated in each trial.
± Standard error.
* Difference in values showing similar superscript in significant at p=0.01.

Table 4 : Pathogenicity test of *G. cingulata* on Darjeeling varieties of Tea following detached leaf inoculation.

Variety	Percentage lesion Production[a]		
	Hours after inoculation		
	48	72	96
B-668	33.33 ± 2.8	45.83 ± 3.2	*58.33 ± 2.9
B-777	41.37 ± 3.1	48.27 ±2.7	65.51 ± 3.1
P-312	7.40 ± 2.5	11.11 ± 2.7	18.51[c] ± 2.9
T-78	48.88 ± 3.2	62.22 ± 2.1	82.22[b] ± 2.8
P-1258	35.71 ± 2.1	42.85 ± 3.3	50.00 ± 2.7
B-157	77.27 ± 2.9	79.54 ± 2.2	90.90[b] ± 2.9
TTV[1]	55.00 ± 2.5	60.00 ± 2.4	75.00 ± 2.7
BT-15	0.00	5.28 ± 1.0	16.00[c] ± 1.0
AV-2	17.24 ± 2.6	20.68 ± 2.3	24.13[c] ± 3.1
BS/7A/76	34.48 ± 2.8	55.17 ± 2.7	62.06 ± 3.2
RR-17	9.67 ± 2.3	19.35 ± 3.1	32.25 ±2.4
HV-39	35.48 ± 2.7	51.61 ± 2.3	61.29 ± 2.9
K1/1	65.21 ± 3.2	69.56 ± 2.7	73.19 ± 3.1
T-135	13.79 ± 3.1	17.24 ± 2.8	20.68[c] ± 2.4
CP-1	6.18 ± 2.9	10.89 ± 1.4	17.34[c] ± 2.9

a Average of three separate trails
50 leaves inoculated in each trial.
± Standard error.
* Difference in values with same superscript insignificant at P = 0.01.

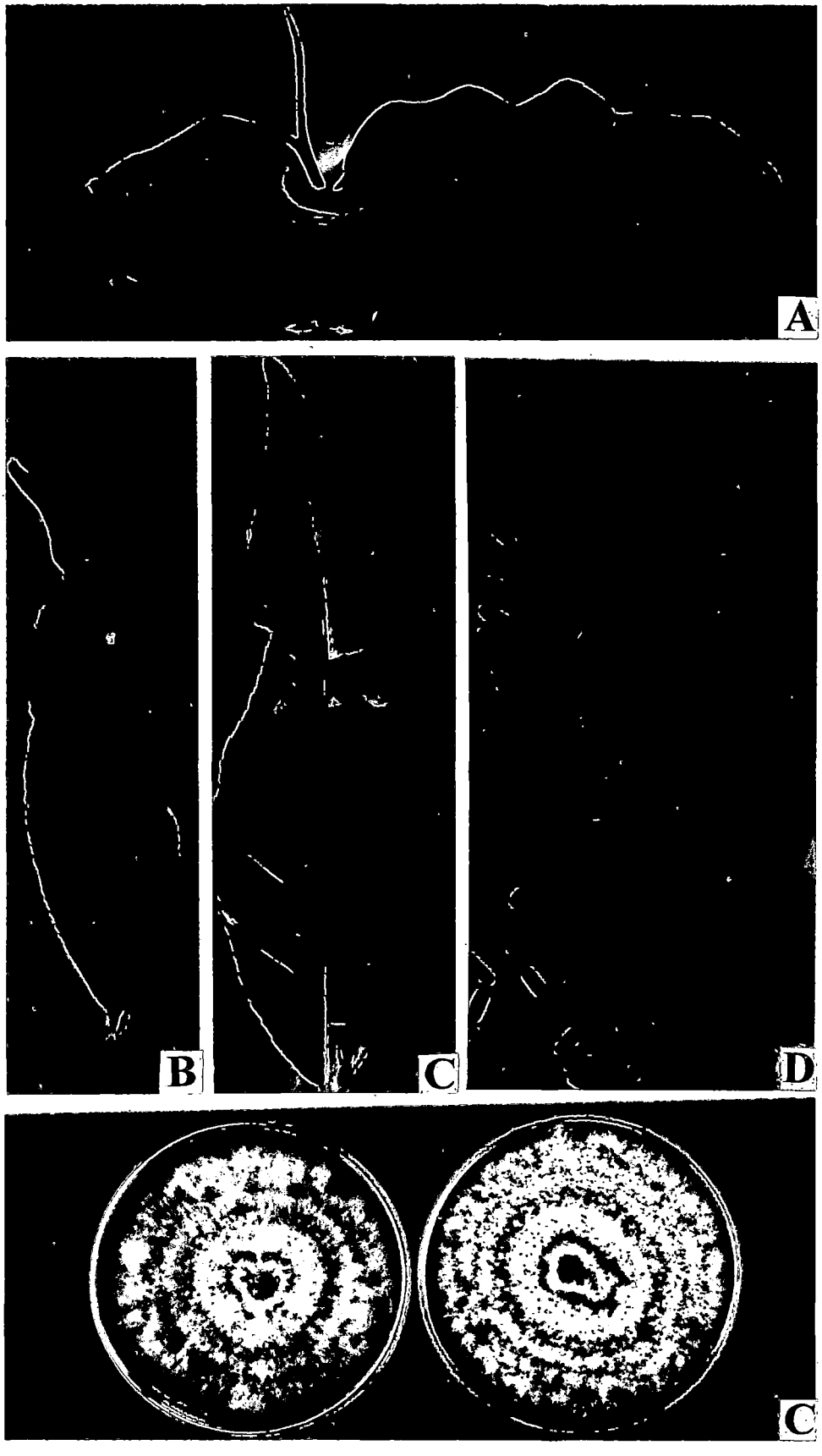


PLATE VI (figs.A-E). **A**- Twig of UPASI 9 artificially inoculated with *G. cingulata* ; **B & C**- detached leaves after artificial inoculation ; **D** -germinated conidia *G .cingulata* with appressoria ;**E**-*G.cingulata* grown on RM showing orange acervuli

Pathogenicity test of *Glomerella cingulata* on different varieties of tea (Detached leaf inoculation)

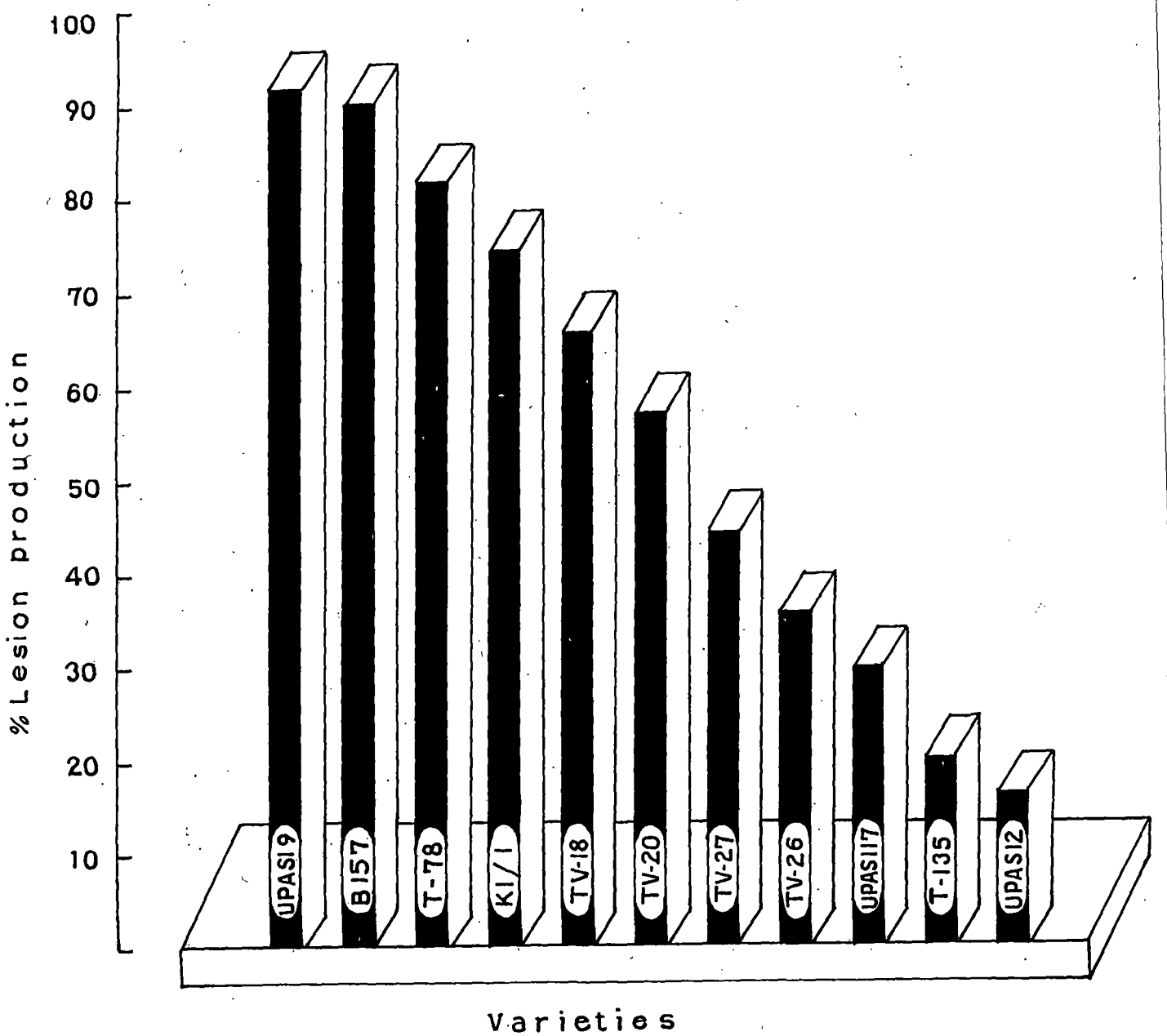


Fig. 1

4.1.2. Cut Shoot

Pathogenicity of *G cingulata* was also tested on 37 varieties of tea by cut shoot inoculation as described in Materials and Methods. Disease was assessed on the basis of mean number of lesion per shoot from which mean disease index per shoot was calculated. Disease symptoms appeared as early as 24h after inoculation. Results (Tables 5-7) confirmed the trend shown in detached leaf inoculation technique. Among the Tocklai varieties tested, highest disease index was evident in TV-18 and lowest in TV-26 (Table-5). In case of UPASI varities, UPASI-2 exhibited lowest disease index value, as revealed in Table-6 (5.90 and 1.00) respectively. A maximum disease index value of 6.6 was exhibited by B-157 and minimum of 0.75 by BT-15, among the different Darjeeling varieties tested (Table-7).

Among all the 37 varieties tested, UPASI 9 wasthe most susceptible, followed by B157 and TV-18 while BT-15 was the most resistant followed by CP-1, UPASI 2, and TV-26 on the basis of cut shoot inoculation (Fig. 2). This was in confirmity with result of detached leaf inoculation.

Table 5 : Pathogenicity test of *G.cingulata* on UPASI varieties of tea following cut shoot inoculation.

Variety	Hours after inoculation							
	24		48		72		96	
	Mean no. of lesion/ shoot	Mean Disease index/ shoot	Mean no. of lesion/ shoot	Mean Disease index/ shoot	Mean no. of lesion/ shoot	Mean Disease index/ shoot	Mean no. of lesion/ shoot	Mean Disease index/ shoot
UPASI 2	0	0	1.0	0.4	1.3	0.6	1.9	1.0
UPASI 3	5.0	3.0	6.0	3.9	6.5	4.5	7.0	5.2
UPASI 8	3.0	1.9	3.9	2.8	4.4	3.4	5.0	3.8
UPASI 9	6.6	4.0	7.0	4.2	7.5	4.8	8.0	5.9
UPASI 17	3.5	2.2	4.5	3.1	5.2	3.6	5.5	3.8
UPASI 26	4.0	2.0	5.0	3.0	5.6	3.8	6.0	4.0
BSS 1	3.7	2.6	4.8	3.3	5.6	3.9	5.9	4.0
BSS2	3.3	2.1	4.4	3.0	4.9	3.3	5.0	3.1
BSS3	3.1	2.0	4.3	2.9	4.8	3.2	4.9	3.0

* 50 shoots per treatment

Table 6 :Pathogenicity test of G. cingulata on Tocklai varieties of tea following cut shoot inoculation

Tea varieties	Hours after inoculation							
	24		48		72		96	
	Mean No. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean No. of lesion/ shoot	Mean dis-ease index/ shoot	Mean No. of lesion/ shoot	Mean dis-ease index/ shoot	Mean No of lesion/ shoot	Mean dis-ease index/ shoot
Teenali 7/1/54	6.0	3.0	7.4	3.6	8.0	4.2	9.2	5.2
TV-18	8.0	4.0	8.8	5.0	10.0	5.4	12.5	5.5
TV-20	5.0	2.7	6.5	3.2	7.0	3.8	8.2	4.5
TV-22	4.5	2.00	6.0	3.0	6.6	3.5	6.9	4.2
TV-23	6.2	3.5	8.0	4.3	8.3	5.0	7.9	6.3
TV-25	5.8	2.9	6.4	3.3	7.8	4.1	9.0	5.0
TV-26	2.1	0.5	2.5	0.8	4.0	1.0	5.3	1.2
TV-27	4.2	1.9	6.0	2.8	7.0	3.2	7.5	4.3
TV-28	3.5	1.2	4.1	1.7	5.7	2.0	6.0	2.6
TV-29	2.9	0.9	3.2	1.0	5.0	1.2	5.8	1.4
TV-30	3.0	1.0	3.5	1.2	5.2	1.5	6.0	2.0
TV-9	2.9	0.8	3.3	1.1	5.3	1.9	6.2	2.5
TS-449	3.1	1.2	3.3	1.5	4.9	2.1	5.2	2.5

* 50 shoots per treatment.

Table 7 : Pathogenicity test of *G.cingulata* on Darjeeling varieties of tea following cut shoot inoculation.

Variety	Hours after inoculation							
	24		48		72		96	
	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot	Mean no. of lesion/ shoot	Mean Dis-ease index/ shoot
B668	2.9	1.5	3.5	1.9	4.0	2.5	4.6	2.8
B777	4.2	2.2	5.0	2.8	5.6	3.0	6.0	3.3
P312	1.2	0.5	2.0	0.8	2.5	1.0	3.0	1.2
T-78	5.2	3.03	6.0	4.0	6.8	4.8	7.2	5.4
P1258	2.5	1.3	3.0	2.0	3.9	2.4	4.0	2.9
B157	6.0	3.9	7.5	4.5	8.2	5.4	9.0	5.6
TTV_1	5.0	3.0	5.5	3.8	6.0	4.3	6.3	5.0
BT-15	0.8	0.3	1.1	0.4	1.5	0.5	1.9	0.75
AV-2	1.9	0.9	2.2	1.4	3.0	2.0	3.2	2.6
BS/7A/76	3.7	2.0	4.0	3.0	4.6	3.5	5.0	3.8
RR17	2.0	1.1	2.8	1.9	3.0	2.3	3.4	2.9
HV-39	3.0	1.9	3.5	2.5	4.0	2.8	4.2	3.3
K1/1	4.6	2.6	5.0	3.0	5.6	3.6	6.0	4.0
T-135	1.6	0.7	2.0	0.9	2.6	1.2	3.0	1.5
CP-1	1.0	0.4	1.5	0.5	2.0	0.7	2.2	0.9

* Average of 50 shoots

Pathogenicity test of *Glomerella cingulata* on Darjeeling varieties of tea (Cut shoot inoculation)

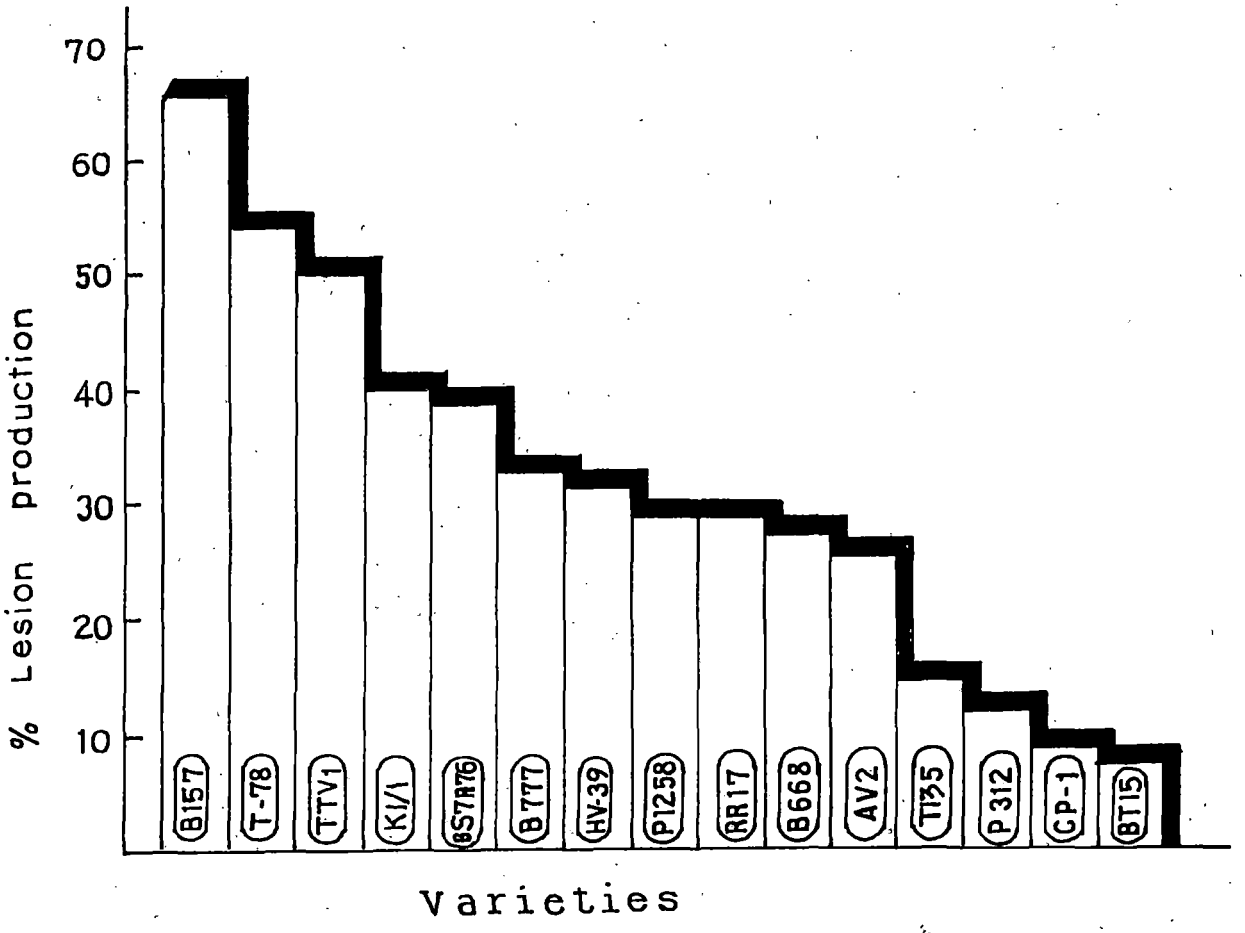


Fig. 2.

4.2. Cultural conditions affecting growth of *G.cingulata.*

Growth, sporulation and infectivity of any pathogen both **in vivo** and **in vitro** is dependent on several factors. *Glomerella cingulata* (isolate GC-1) usually had white mycelial growth which sometimes turned to brown or grey depending on the medium. Orange acervuli were characteristic of this fungus (Plate VI, Fig.E) but accrvuli production was dependent on light and temperature. Conidial morphology was studied under bright field and phase contrast of Leica Microscope (Plate VI, fig. D). Conidia appeared scantily on solitary phialides but normally in orange sporodochia; a basal stromatic cushion in covered with dense layer of cylindrical, slightly tapering phialides upto 20 μm long; sometimes these are interpersed with dark brown, tapering, blunt, septate setae. Conidia cylindrical with a rounded apex and slightly truncated base, hyaline, 12-18 μm x 3.5 μm; forming orange-red slimy masses, germinating by irregularly rounded brown appressoria.

4.2.1. Solid media

The fungus was grown in five different media, such as potato dextrose agar, Richard's agar, Flentze's soil extract agar, carrot juice agar and Czapekdox agar - medium and their growth and sporulation behaviour studied. Mycelial growth rate was recorded after 4,6,8 and 10 days of inoculation at 30$\pm$1°C. Maximum growth occurred in RMA (89.0 mm), followed by CJA (80.1 mm) as revealed in Table-8. Mycelial growth was fluffy and white in colour in RM, showing deep orange on the reverse side of the petridish. In PDA and CDA, grey white mycelia were observed on the upper side but black concentric rings with orange acervuli was prominent on reverse side of the petridish. Sporulation was very abundant in RMA and CJA, moderate in PDA and CDA while in FSEA sporulation was rare.

4.2.2. Liquid media

The growth of fungi both **in vitro** and in natural conditions is greatly influenced by different factors like temperature, pH of the surrounding medium and available nutrients. To determine the effect of such factors on mycelial growth of *G.cingulata* **in vitro**, the following experiments were undertaken. Effects of incubation period, pH of the medium, different carbon sources and nitrogen sources on the mycelial growth of *G.cingulata* studied.

Table 8: Effect of different media on mycelial growth and sporulation of *G.cingulata*

Medium	Diameter of mycelia (mm)[a] Incubation Period (days)				
	4	6	8	10	Sporulation[b]
Potato dextrose agar	30.1 ±1.25	42.6 ±1.22	62.3 ±1.69	88.9 ±1.23	+
Richard's medium	35.6 ±2.21	48.9 ±1.08	69.9 ±2.00	89.0 ±1.33	++
Czapekdox-agar	10.0 ±1.16	15.5 ±2.01	28.9 ±1.80	44.2 ±1.43	_
Flentze's soil extract-agar	22.5 ±2.5	35.9 ±1.23	49.6 ±2.8	58.6 ±2.6	_
Carrot juice agar	32.9 ±1.23	40.6 ±2.3	56.6 ±2.6	80.1 ±1.89	+

Temperature - 30 ± 1°C

a Average of three replicates

b Excellent (++) ; Good (+) ; No(-)

± Standard error.

4.2.2.1 Incubation time

G.cingulata was grown in Richard's medium (RM) for a period of 30 days. Mycelial growth was recorded after 5,10,15,20,25 and 30 days of growth. Maximum growth (591 mg) of *G.cingulata* was recorded after 10 days of incubation (Table-9). There was a steady decline in growth after 10 days. After 5 days of incubation there was a growth of only 61 mg. Therefore, the mycelial growth of 10 days was about 10 times that of 5 days. sporulation was abundant at 10 days' growth. Spores were also observed at 5 days' growth.

4.2.2.2 pH

It is well known that the pH of the medium usually plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH. In the present investigation buffer solutions with pH values ranging from 4.0 to 8.0 (4.0, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. The medium and the buffer was sterilized separately by autoclaving for 15 min. at 15 lb. P.S.I. pressure. Equal parts of the buffer solution and the medium (RM) were mixed

Table 9 : Effect of incubation period on mycelial growth of *G.cingulata*

Incubation period(days)	Mean mycelial dry wt.[a] (mg)
5	61.0 ± 2.21
10	591.0 ± 3.24
15	395.2 ± 3.21
20	365.5 ± 4.21
25	285.2 ± 3.8
30	262.8 ± 4.22

a Average of five replicates
± Standard error.
Temperature 30 ± 1°C.

before use. Each flask containing 50 ml of the medium was then inoculated with mycelial block of *G.cingulata* and incubated for 10 days at 30 ± 1°C. Results (Table-10) revealed that *G.cingulata* grew to a lesser or greater extent in all the pH tested. Maximum growth was recorded at pH 6.5, while minimum growth occurred at 4.0. Sporulation was very less in high pH 8.0; pH 6.0 and 6.5 supported good sporulation.

Table 10 : Effect of pH on mycelial growth of *G.cingulata.*

pH of medium	Mean mycelial dry wt.[a] (mg)
4.0	301.0 ± 3.61
5.0	362.5 ± 4.20
5.5	381.6 ± 2.71
6.0	421 5 ± 2.7
6.5	453.0 ± 4.2
7.0	369.0 ± 3.5
8.0	340.0 ± 3.41.

a Average of three replicates
± Standard error
Temperature 30 ± 1°C.
Incubation period 15 days.

4.2.2.3 Carbon sources

The ability of fungi to grow in different media depends on their capacity to utilize the available nutrients, of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In this investigation, 9 different carbon sources (fructose, sorbose, glucose, galactose, mannose, mannitol, sucrose, starch and maltose) were tested for their effect on the growth of *G.cingulata.* These were added separately to the basal, medium (0.2% as paragine, 0.1% KH_2PO_4, 0.05% $MgSO_4$, $7H_2O$, 2 ppm Zn^{++}, Mn^{++} and Fe^{+++}). Data were recorded after 3,6 and 9 days of incubation. A control set without any carbohydrate was also set up. Both mycelial weight and sporulation were determined. Results given in Table-11 revealed maximum growth and sporulation of *G.cingulata* using maltose as the carbon source. Sucrose and glucose also supported comparatively good growth. Good sporulation was observed in case of sucrose, glucose and mannose. Among the tested carbohydrates least growth occurred in mannitol. There was little growth in absence of any carbohydrate.

4.2.2.4. Nitrogen Source

Nitrogen is undoubtedly the most important single nutrient necessory for the growth of any organism. The availability of nitrogen depends to a great degree on the form in which it is supplied. Hence the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effect of inorganic nitrogen sources (calcium nitrate, sodium nitrate, ammonium sulphate, ammonium nitrate and potassium nitrate) as wellas complex organic sources (peptone, urea, yeast extract and beef extract) on the mycelial growth of *G. cingulata* was tested. A basal medium without any nitrogen source was considered as control. Data, recorded after 3,6 and 9 days of growth showed potassium nitrate to be optimum for growth of *G. cingulata,* followed by sodium nitrate, among all sources, both inorganic and organic (Table-12). Among the organic sources beef extract supported maximum growth. Only insignificant growth was noted without nitrogen. Sporulation was also maximum in potassium nitrate followed by sodium nitrate, calcium nitrate and beef extract.

Table 11 : Effect of different carbon sources on mycelial growth of *G.cingulata*

Carbon Source wt.(mg)	Incubation period					
	3 days		6 days		9 days	
	Dry[a] wt.(mg.)	Sporulation[b]	Dry[a] wt.(mg)	Sporulation[b]	Dry[a] wt.(mg.)	Sporulation[b]
Fructose	35.1 ±2.9	-	157.6 ±4.1	-	315.5 ± 2.6	±
Sorbase	38.4 ±4.21	-	117.2 ±2.21	-	270.2 ± 2.8	-
Glucose	42.2 ±3.39	±	150.8 ± 3.29	++	382.1 ±3.7	+++
Gallactose	35.9 ±3.3	-	212.6 ±2.21	-	350.2 ± 1.9	-
Mannose	32.2 ±1.29	±	225.6 ±3.2	++	340.5 ±2.9	+++
Mannitol	29.6 ±2.65	-	95.6 ±2.25	-	145.2 ±2.9	-
Sucrose	35.6 ±2.61	±	272.6 ±3.26	++	385.9 ±3.6	+++
Starch	25.6 ±2.91	-	190.3 ±3.26	-	230.9 ±3.8	-
Maltose	40.6 ±2.33	++	300.0 ±3.2	+++	416.9 ±2.6	++++
Control (without carbon)	11.2 ±2.3	-	18.5 ±3.3	-	22.4 ±2.7	-

a Average of three replicates.

b - = Nil; ± = Poor; ++ = Fair; +++ = Good; ++++ = Excellent.

Temparature 30 ± 1°c

Table 12 :Effect of different nitrogen sources on mycelial growth of *G. cingulata.*

Nitrogen Source	Incubation period					
	3 days		6 days		9 days	
	Dry[a] wt.(mg.)	Sporulation[b]	Dry[a] wt.(mg.)	Sporuloation[b]	Dry[a] wt.(mg.)	Sporulation[b]
Inorganic						
Calcium nitrate	23.3 ±3.3	-	256.0 ±1.9	+	430.6 ±2.1	+
Sodium nitrate	21.0 ±2.1	-	305.6 ±2.6	+	430.6 ±2.9	++
Ammonium sulphate	17.5 ±3.1	-	189.2 ±3.9	-	237.8 ±2.8	-
Ammonium nitrate	20.1 ±2.1	-	285.6 ±4.2	-	335.6 ±3.9	-
Potassium nitrate	25.6 ±2.5	-	321.5 ±3.8	++	435.6 ±4.2	+++
Organic						
Peptone	13.6 ±2.9	-	195.6 ±3.1	-	269.6 ±3.1	-
Urea	9.0 ±4.1	-	160.7 ±2.9	-	213.5 ±3.2	-
Yeast extract	15.6 ±2.6	-	172.5 ±5.2	-	205.7 ±4.0	-
Beef extract	12.3 ±2.6	-	190.9 ±5.2	+	293.6 ±4.0	++
Control (without nitrogen)	7.7 ±2.1	-	9.7 ±1.9	-	11.2 ±1.7	-

a Average of three replicates Temperature 30±1°C.

b - = nil ; ± = poor ; ++ = fair ; +++ = good; ++++ = excellent

4.3. Detection of cross reactive antigen between *G. cingulata* and tea varieties

The presence of cross reactive antigens among hosts and pathogenic organisms is a well documented phenomenon. Existing studies on plant hosts and pathogens suggests that whenever an intimate and continuing association of cells of hosts and pathogens occurs, partners of this association have a unique serological resemblences to one another involving one or more antigenic determinants.

Various method have been generally used to detect the presence of cross reactive antigens between hosts and parasite. Earlier techniques like immunodiffusion and immuno-electrophoresis are being currently replaced by more advanced techniques like ELISA and immunofluorescence. In the present investigation cross reactive antigens between *G. cingulata* and tea varieties have been detected using immunodiffusion, immunoelectrophoresis and ELISA. Series of experiments performed and results obtained have been presented below.

4.3.1. Immunodiffusion tests

The effectiveness of antigen preparation from tea leaves (TV-18 and CP-1) and *G. cingulata* (isolate GC-1) in raising antibodies was checked by homologous cross reaction following agar gel double diffusion technique.Control sets involving normal sera andantigens of both the host and parasite were all negative. The results are presented in Tables 13 to 15 and Plate VII, figs. A-D. Strong precipitin reactions occured when antiserum raised against mycelia of *G. cingulata* (GCIA) was reacted against its own antigen and the antigens of other isolates (Table-13; Plate VII,fig-B). When anti - *G. cingulata* antiserum was crossreacted with leaf antigens prepared from 37 tea varieties including 13 Tocklai, 15 Darjeeling and 9 UPASI varieties, 16 varieties (TV-18,TV-20, TV-22, TV-23, TV-25, TV-27, Teenali-17/1/54, UPASI 3, UPASI 9, UPASI 26, T-78, B157, TTV_1, K1/1, B668, B777) exhibited precipitin band in immunodiffusion test (Table-13). However, weak precipitin reactions were observed with antigens of 13 other varieties (TV-9, TV-28, TV-29, TV-30, TS-449, UPASI 8, UPASI 17, BSS 1, BSS 2, BSS 3, P-1258, BS/7A/76 and HV-39). No such precipitin reactions were observed in case of antigens of 8 varieties tested (TV-26, UPASI 2, P312, BT-15, AV-2, RR-17, T-135 and CP-1) as well as antigen preparations from three non host species viz. *Glycine max* (cv. soymax), *Cicer arietinum* (cv. JG-62) and *Camellia japonicum* and one non pathogen (*Fusarium oxysporum*).

To confirm the presence of common antigens between isolates of *G.cingulata* and tea varieties reciprocal cross reaction with antiserum of TV-18 was carried out with leaf antigens of host and non host as well as mycelial antigens of pathogen and non pathogen. Results are presented in Table-14. Strong precipitin reactions were observed in most of the cases with the antigens of Tocklai varieties except- TV-26 and TS-449. Whereas, antiserum of TV-18 exhibited weak precipitin band when reacted with the leaf antigens prepared from UPASI

Table 13 : Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti *G.cingulata* antiserum.

Antigen of host and parasite	Antiserum of *G. cingulata.* Pricipitin band	Antigen of host and parasite	Antiserum of *G. cingulata* Pricipitin band
Tocklai varieties		**Darjeeling varieties**	
TV 9	±	B 668	+
TV18	+	B777	+
TV20	+	P312	-
TV 22	+	T-78	+
TV 23	+	P-1258	±
TV 25	+	B157	+
TV 26	-	TTVI	+
TV 27	+	Bt-15	-
TV 28	±	AV2	-
TV 29	±	BS/7A/76	±
TV 30	±	RR17	-
TS 449	±	Hv39	±
Teenali 17/1/54	+	K 1/1	+
UPASI varieties		T-135	
UPASI-2	-	Cp-1	-
UPASI-3	+		
UPASI-8	±	**Pathogen:**	
		G.cingulata Isolates:	
UPASI-9	+	GC-1	+
UPASI-17	±	GC-2	+
UPASI-26	+	GC-3	+
BSS-1	±	GC-4	+
BSS-2	±	GC-5	+
BSS-3	±	GC-6	+
Non host		GC-7	+
Glycine max(Cv.Soymax)	-	GC-8	+
Cicer arietinum(Cv.JG-62)	-	GC-9	+
Camellia japonicum		**Non pathogen**	
		F.oxysporum	-

+ Common precipitin band present;

(±) weak precipitin band; (-) Common precipitin band absent.

Table 14 : Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti TV-18 antiserum.

Antigen of host and parasite	Antiserum of TV-18 Precipitin band	Antigen of host and parasite	Antiserum of TV-18 Precipitin band
Tocklai varieties		**Darjeeling varieties**	
TV 9	+	B 668	±
TV 18	+	B 777	±
TV 20	+	P 312	-
TV 22	+	T 78	+
TV 23	+	P 1258	±
TV 25	+	B 157	+
TV 26	±	TTV_1	±
TV 27	+	BT -15	-
TV30	+	AV2	±
TS-449	±	BS/7A/76	±
Teenali 17/1/54	+	RR 17	±
		HV 39	+
		K1/1	±
		T-135	±
		CP-1	±
UPASI varieties		**Pathogen :**	
UPASI 2	-	*G.cingulata*	
UPASI 3	±	*Isolates*	
UPASI 8	±	GC-1	+
UPASI 9	+	GC-2	+
UPASI 17	±	GC-3	+
UPASI 26	±	GC4	±
BSS-1	±	GC-5	±
BSS-2	±	GC-6	±
BSS-3	±	GC-7	+
Non host		GC-8	±
G. max(Soymax)	-	GC-9	+
C. arietinum(JG-62)	-	**Non Pathogen**	
C. japonicum	-	*F. oxysporum*	-

(+) Common precipitin band present;

(±) Weak precipitin band; (-) Common precipitin band absent.

Table 15: Detection of cross reactive antigens among tea varieties and *G.cingulata* in agar gel double diffusion test using anti-CP-1 antiserum.

Antigen of host and parasite	Antiserum of Cp-1 Precipitin band	Antigen of host and parasite	Antiserum of CP-1 Precipitin band
Tocklai varieties		**Darjeeling varieties**	
TV-9	+	B 668	-
TV-18	+	B 777	±
TV-20	+	P 312	-
TV-22	+	T 78	±
TV-23	+	P 1258	±
TV-25	+	B 157	±
TV-26	+	TTV_1	±
TV-28	+	BT-15	+
TV-29	+	AV-2	±
TV-30	±	BS/7A/76	+
TS-449	-	RR17	±
Teenali 17/1/54	-	HV-39	+
		K1/1	±
		T135	±
UPASI varieties		CP-1	+
UPASI-2	-	**Pathogen:**	
UPASI-3	±	*G.cingulata* isolates	
UPASI 8	-	GC- 1	-
UPASI 9	±	GC-2	-
UPASI 17	±	GC-3	-
UPASI 26	-	GC-4	-
BSS1	±	GC-5	-
BSS2	±	GC-6	-
BSS3	±	GC-7	-
		GC-8	-
Non host		GC-9	-
G. max(Soymax)	-	**Non pathogen**	
C. arietinum (JG-162)	-	*F. oxysporum*	-
Camellia japonicum	-		

(+) Common precipitin band present, (±) Weak precipitin band;

(-) Common precipitin band absent.

and Darjeeling varieties. In repeated trials with anti- TV-18 antiserum no such precipitation reaction could be observed in immunodiffusion test with antigens prepared from UPASI-2, P312, BT-15 (Plate VII, fig. A) and also non host species. It is interesting to note that among 9 isolates of *G. cingulata* tested, 4 isolates (GC-1, GC-2, GC-3, and GC-7) exhibited common precipitin band, but rest of the 5 isolates gave weak precipitin reaction in agar gel double diffusion test. (Table-14 & Plate VII, fig. C).

Reciprocal cross reaction using antiserum of CP-1 and antigens prepared from leaves of 37 tea varieties, 3 non host species, 9 isolates of *G. cingulata* (pathogen) and *F. oxysporum* (non pathogen) were also carried out. Results (Table-15) revealed that none of the isolates of *G. cingulata* could develop any precipitin reaction with anti CP-1 antiserum (Plate VII, fig. D). Non host species and non pathogen also failed to develop any precipitin line.

4.3.2. **Immunoelectrophoresis**

Results of immunodiffusion tests revealed the presence or absence of cross reactive antigens between isolates of *G.cingulata* and tea varieties. Many of the cross reactions in the immunodiffusion tests gave diffused precipitin bands which could not be clearly distinguished. It was also not clear whether precipitin reactions in all cases were due to single or several antigenic substances. Therefore, further resolution was attempted by subjecting the antigens to electrophoresis before exposing them to antisera. Details of immunoelectrophoretic technique have been described under Materials and Methods.

In this experiment, antigenic comparison among 6 selected varieties of tea in which 3 susceptible varieties (TV-18, UPASI-9 and B-157) and 3 resistant varieties (TV-26, UPASI-2 and CP-1), 4 isolates of *G.cingulata* (GC-1, 2, 3 and 4), 3 non host species (*G. max, C. japonicum and C. arietinum*) and one non pathogen (*F.oxysporum*) using antisera of host (TV-18, CP-1) and parasite (*G. cingulata*) were done following conventional set up of immunoelectrophoresis. Results are shown in Table-16.

Effectiveness of each antigen extract of TV-18 and CP-1 and isolate of *G.cingulata* (GC-1) in raising antibodies was checked by homologous cross reactions. Six precipitin arcs were formed by TV-18, CP-1 and GC-1 in homologous reaction. When anti TV-18 antiserum was cross reacted with the leaf antigens of 5 other tea varieties, they were antigenically very close to each other. In this reaction antigens of UPASI 9 and B 157 gave rise to 4 precipitin arcs, while UPASI-2 and CP1 and showed 2 precipitin arcs but TV-26 exhibited 3 arcs. No such precipitation was observed when antigens of non pathogen and non-hosts were cross

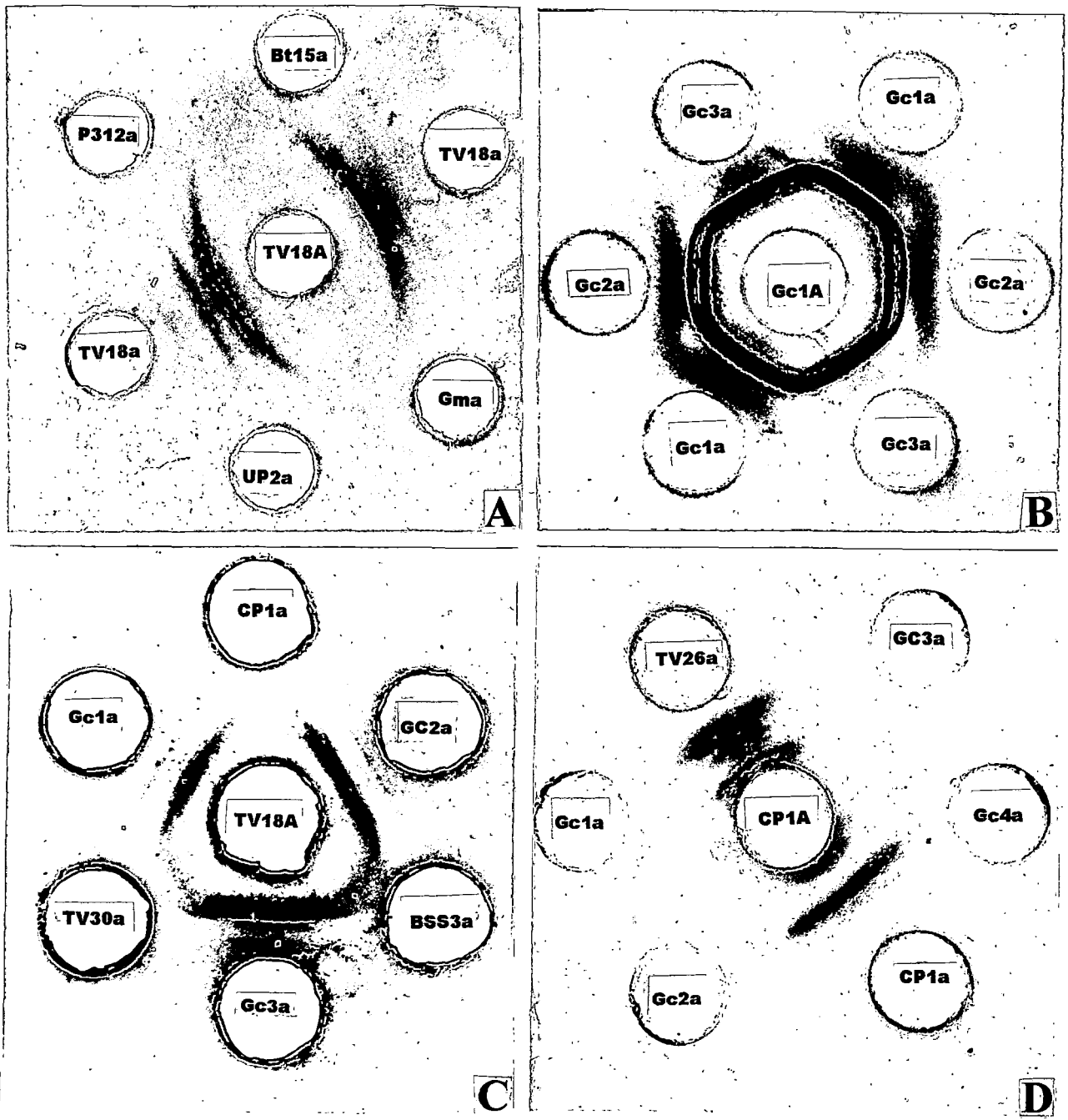


PLATE VII (figs.A-D). Agar gel double diffusion tests. Central wells contain antisera to *G.cingulata* **(B)**, TV-18 **(A&C)** and CP1 **(D)**. Peripheral wells contain antigens of *G.cingulata* (**B & C**), tea leaves **(A,C & D)** and non host (**A**)

reacted with antiserum of TV-18. It is interesting to note that all three isolates of *G.cingulata* (GC-1, 3 and 4) were antigenically more related to TV-18 by sharing 2 precipitin lines in common. Isolate GC-2 shared only 1 line in common.

Similarly, anti CP-1 antiserum was cross reacted with antigens of five different tea varieties. Antigens of UPASI 9, B 157 and UPASI 2 exhibited 3 precipitin arcs each, while 2 precipitin arcs were formed with antigens of TV-18 and TV-26. When antigens of non-pathogen (*F.oxysporum*) and non-host (*G.max, C.japonicum* and *C.arietinum*) were cross reacted with antiserum of CP-1, no precipitin arcs could be observed.

Reciprocal cross reactions with anti *G.cingulata* antiserum and antigens of all three susceptible varieties (TV-18, UPASI-9 and B 157) formed two precipitin lines but antigens of resistant varieties (TV-26, UPASI 2 and CP-1), non host and non pathogen failed to develop any precipitin lines.

4.3.3 Rocket immunoelectrophoresis

The results of some immunoelectrophoretic tests were verified by rocket immunoelectrophoresis. In this experiment antiserum of *G.cingulata* was cross reacted with its homologous antigens and leaf antigens of TV-18 and TV-26. Gel containing anti *G.cingulata* antiserum and homologous antigens resulted in 3 immunoprecipitate line while a single immunoprecipitin line was discerned for the susceptible variety (TV-18). No such line was observed in case of resistant variety (TV-26).

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

Enzyme linked immunosorbent assay in 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.

Table 16:Antigenic comparison of tea varieties, pathogens, non-pathogen and non hosts.

Antigen of host and parasite	Total no. of precipitin lines		
	Antisera of host and parasite		
	TV-18	**CP-1**	***G.cingulata***
Susceptible varieties			
TV-18	6	2	2
UPASI 9	4	3	2
B-157	4	3	2
Resistant varieties			
TV-26	3	2	0
UPASI-2	2	3	0
CP-1	2	6	0
Isolate of *G.cingulata*			
GC-1	2	0	6
GC-2	1	0	4
GC-3	2	0	5
GC-4	2	0	5
Non pathogen			
F. oxysporum	0	0	0
Non hosts			
G. max (Cv. Soymax)	0	0	0
C. japonicum	0	0	0
C. arietinum (Cv JG-62)	0	0	0

In the present investigation DAC ELISA has been used in most of the experiments. Since ELISA depends on a number of factors and this varies from system to system it was considered essential to optimize these conditions in the particular host pathogen system to be studied. Hence, initially a number of experiments were performed for optimization.

4.3.4.1. Optimization of ELISA

The optimum conditions for ELISA reaction were determined initially as a large number of experiments in this investigation have been carried out using ELISA. Optimization of ELISA was done using IgG fraction of antisera raised against antigen preparations from both mycelia and cell wall of *G. cingulàta*. In all cases experiments were repeated thrice.

4.3.4.1.1. Antiserum raised against antigen preparation from mycelia of *G.cingulata*

Optimization of ELISA was done by considering three variables, dilution of the enzyme, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity. In all cases, homologous ELISA reactions using antigen of *G.cingulata* was carried out as described under Materials and Methods.

4.3.4.1.1.1.Enzyme dilution

In this experiment, keeping the antigen and antiserum dilutions constant, different dilutions of alkaline phosphatase was used. Dilutions ranged from 1:10,000 to 1:40,000. Results (Table 17 . Fig.3) revealed that absorbance values in ELISA decreased with increasing dilution of the enzyme. Absorbance value at 1:10,000 dilution was approximately 3 times that at 1:40,000 dilution (1.9 and 0.65 respectively). 1:10,000 dilution was selected for further experiments.

Effect of enzyme dilution on ELISA reaction of anti - *G.cingulata* antiserum with homologous antigen

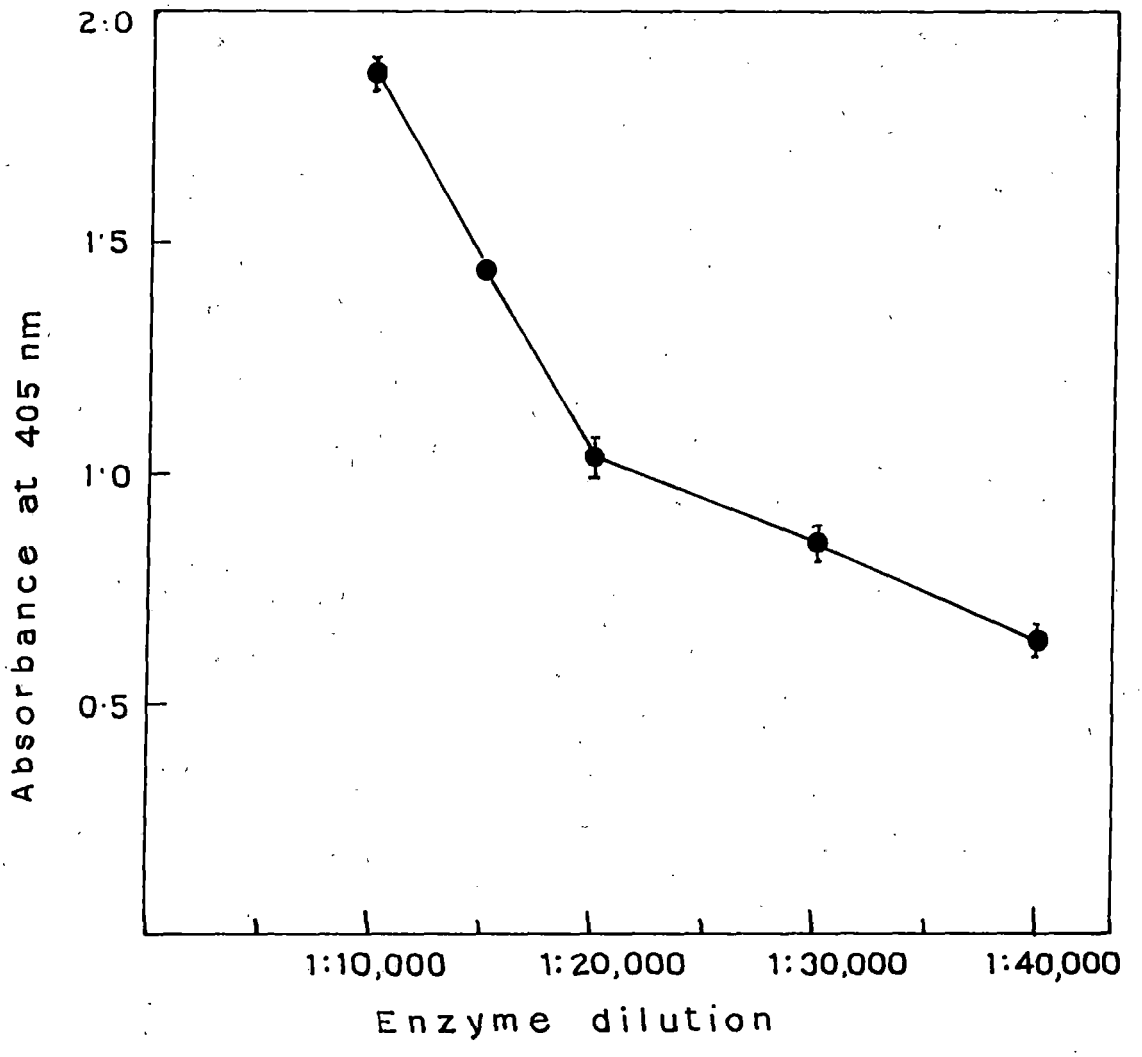


Fig. 3.

Effect of dilution of anti - *G.cingulata* antiserum on ELISA reaction with homologous mycelial antigen

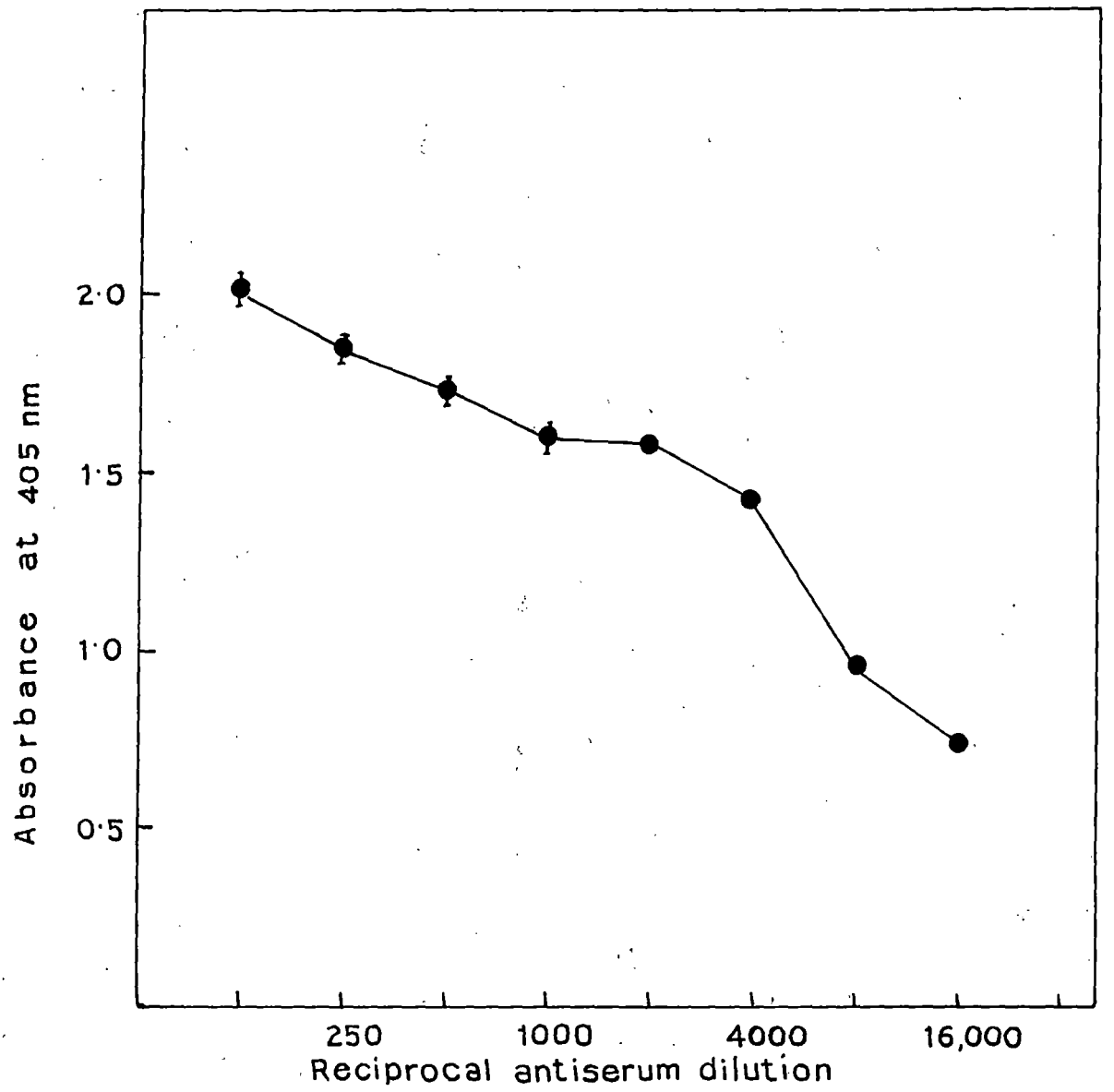


Fig. 4.

Table 17 : ELISA reaction of anti *G.cingulata* antiserum and homologous antigen with various dilution of alkaline phosphatase.

Enzyme[a] dilution	Absorbance of 405 nm			
	Anti *G.cingulata* antiserum (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
1:10000	1.890	1.900	1.892	1.894±.002
1:15,000	1.430	1.482	1.405	1.439±.022
1:20,000	1.002	1.098	1.011	1.037±.03
1:30,000	0.843	0.892	0.824	0.853±.022
1:40,000	0.623	0.679	0.645	0.649±.016

Mycelial antigen concentration - 5 μg/ml

a Alkaline phosphatase

± Standard error.

4.3.4.1.1.2 Antiserum dilution

Antiserum dilutions ranging from 1:125 to 1:16,000 were tested against homologous antigen at a concentration of 2μg/ml. Absorbance values in ELISA decreased with increasing dilution of the antiserum. At 1:125 dilution absorbance values were above 2 and it decreased to 0.743 in 1:16,000 dilution (Table 18, fig. 4). 1:250 dilution was selected for further experiments.

Table 18 :ELISA reaction with various dilution of anti *G. cingulata* antiserum(mycelia) and homologous antigen.

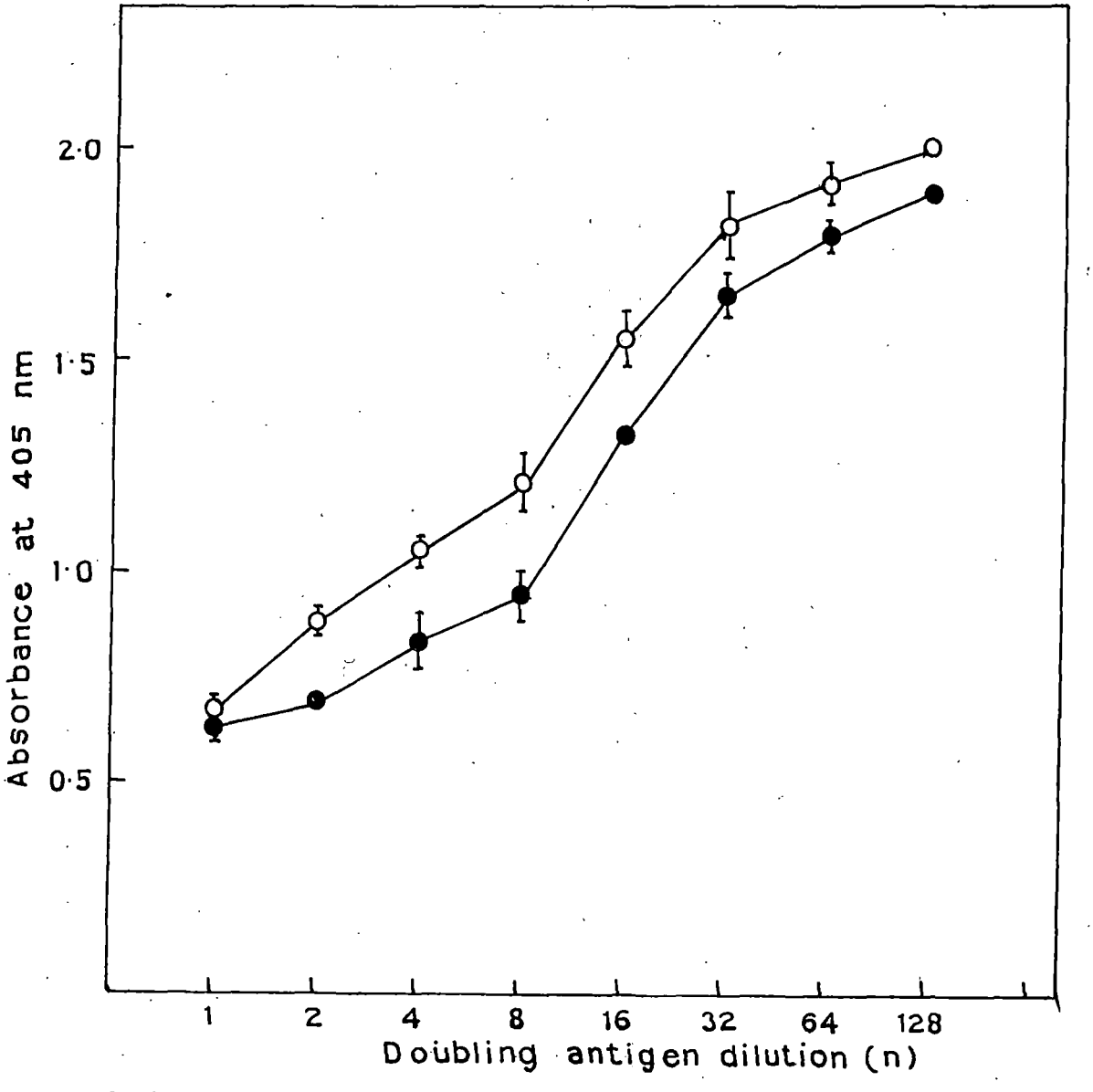
Antiserum dilution	Antigen concentration (2 μg/ml)			
	Expt.1	Expt.2	Expt.3	Mean
1:125	>2	>2	>2	>2
1:250	1.866	1.834	1.850	1.850 $\pm$ .009
1:500	1.730	1.779	1.750	1.750 $\pm$ .014
1:1000	1.624	1.607	1.599	1.610 $\pm$ .004
1:2000	1.616	1.601	1.604	1.607 $\pm$ .002
1:4000	1.410	1.419	1.413	1.414 $\pm$ .002
1:8000	0.975	0.926	0.949	0.950 $\pm$ .014
1:16000	0.722	0.764	0.743	0.743 $\pm$ .012

$\pm$ Standard error

4.3.4.1.1.3 Antigen dilution

To determine the effect of antigen dilution a doubling series of dilution of *G.cingulata* mycelial antigen from 3200 ng to 25 ng was made and tested against two antiserum dilutions (1:125 and 1:250). Results (Table 19, fig.5) revealed that ELISA values decreased with increasing dilution of antigen. However, concentration as low as 25 ng/ml could also be detected by ELISA. At a concentration of 3200 ng/ml and antiserum dilution of 1:125 absorbance values of above 2 was recorded.

Effect of dilution of mycelial antigen of *G.cingulata* on ELISA reaction with homologous antiserum



Antiserum o—o 1:125 , ●—● 1:250
n = from 25 ng/ml (1) to 3200 ng/ml (128)

Fig. 5.

Table 19 :ELISA reaction with various concentration of mycelial antigen of*G.cingulata* and homologous antiserum.

Antigen concentration (ng/ml)	Antiserum dilution	
	1:125	1:250
3200	>2	1.906 ± .001
1600	1.901 ± .034	1.804 ± .021
800	1.821 ± .046	1.663 ± .05
400	1.545 ± .035	1.349 ± .011
200	1.200 ± .032	0.949 ± .048
100	1.050 ± .021	0.838 ± .059
50	0.888 ±.03	0.682 ± .017
25	0.687 ± .029	0.646 ± .015

± Standard error.

4.3.4.1.2. Antiserum raised against antigen preparation from cell wall of *G. cingulata.*

Optimization of ELISA was also done using antiserum raised against cell wall preparations of *G. cingulata* and antigen prepared from cell wall. The effect of two variables, antiserum dilutuion and antigen dilution on ELISA reactivity, were determined.

4.3.4.1.2.1. Antigen dilution

Doubling dilution of *G. cingulata* cell wall antigen ranging from 3200 to 25 ng/ml were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with increasing concentration of antigen. Absorbance value of 0.9 was obtained at 25 ng/ml concentration and 1:125 antiserum dilution, and hence such low concentration was also detectable. At this antiserum dilution and antigen concentration of 3200 ng/ml absorbance value of >2 was obtai ned (Table 20 & Fig. 6).

Table 20 : ELISA reaction with various concentration of cell wall antigen of *G. cingulata* and homologous antiserum.

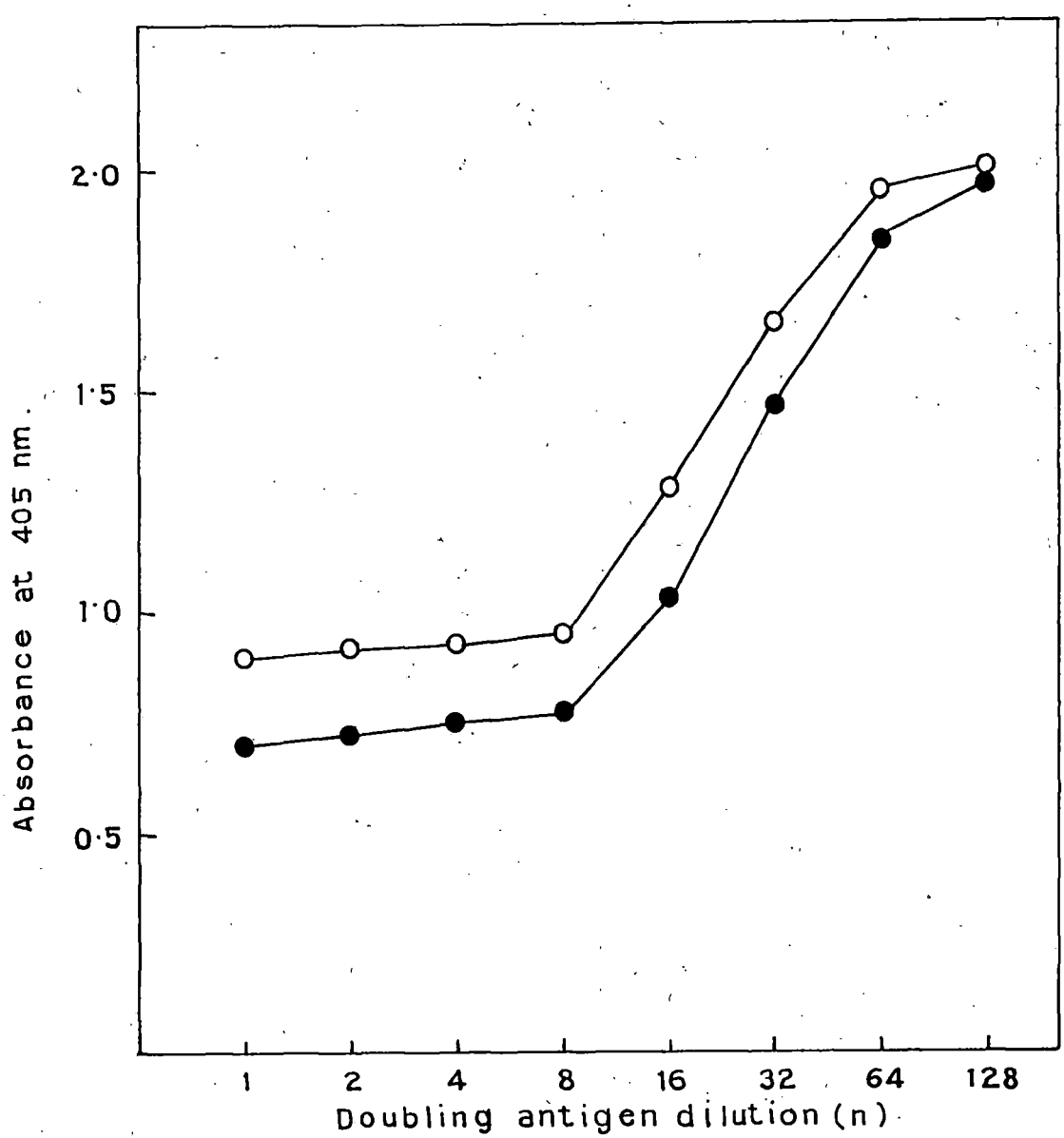
Antigen concentration	Absorbance at 405 nm[a] Antisera dilution	
(ng/ml)	1:125	1:250
3200	>2	1.984 ± .062
1600	1.952 ± .052	1.851 ± .018
800	1.662 ± .041	1.496 ± 029
400	1.292 ± .038	1.021 ± .072
200	0.952 ± .002	0.782 ± .063
100	0.932 ± .039	0.750 ± .039
50	0.917 ± .062	0.723 ± .018
25	0.906 ± .032	0.702 ±.042

a Average of three replicates
± Standard error.

4.3.4.1.2.2. Antiserum dilution

ELISA reaction with different dilution of antiserum ranging from 1:16,000 were determined at an antigen concentration 2 μg/ml. Absorbance values decreased with increasing dilution. At 1:125 dilution value of above 2 were obtained while at 1:250 dilution the value was 1.95. These value decreased to 0.88 at 1:16,000 dilution (Table-21, fig. 7). For further experiments 1:250 dilution was selected.

Effect of dilution of cell wall antigen of *G.cingulata* on ELISA reaction with homologous antiserum



Antiserum o—o 1:125 , ●—● 1:250
n = from 25 ng/ml (1) to 3200 ng/ml (128)

Fig. 6.

Table 21: ELISA reaction with various dilution of anti *G. cingulata* antiserum (cell wall) and homologous antigen.

Antiserum dilution (cell wall)	Absorbance at 405 nm			
	Antigen concentration (2μg/ml)			
	Expt. 1	Expt. 2	Expt. 3	Mean
1:125	>2	>2	>2	>2
1:250	1.952	1.912	1.975	1.946 ± 0.18
1:500	1.849	1.811	1.830	1.830 ± 0.01
1:1000	1.776	1.798	1.742	1.772 ± 0.016
1:2000	1.561	1.540	1.551	1.550 ± 0.006
1:4000	1.492	1.462	1.461	1.471 ± 0.010
1:8000	1.005	1.001	1.00	1.002 ± 0.001
1:16000	0.878	0.869	0.896	0.881 ± 0.038

± Standard error.

4.3.4.2. Comparison of ELISA reactivity among antigens of different tea varities against antiserum of *G. cingulata.*

Among 37 tea varieties tested for their resistance against *G. cingulata,* differential responses were obtained. Certain varieties exhibited high susceptibility, others high resistance while still others were either moderately susceptible or resistant. Conventional techniques for determination of host resistance or susceptibility are being replaced by more rapid and sensitive modern serological techniques. It was therefore considered worthwhile to determine the ELISA reactivity of different tea varieties against antiserum of the pathogen and compare the results obtained with those of the pathogenicity tests.

Effect of dilution of anti - *G.cingulata* antiserum on ELISA reaction with homologous cell wall antigen

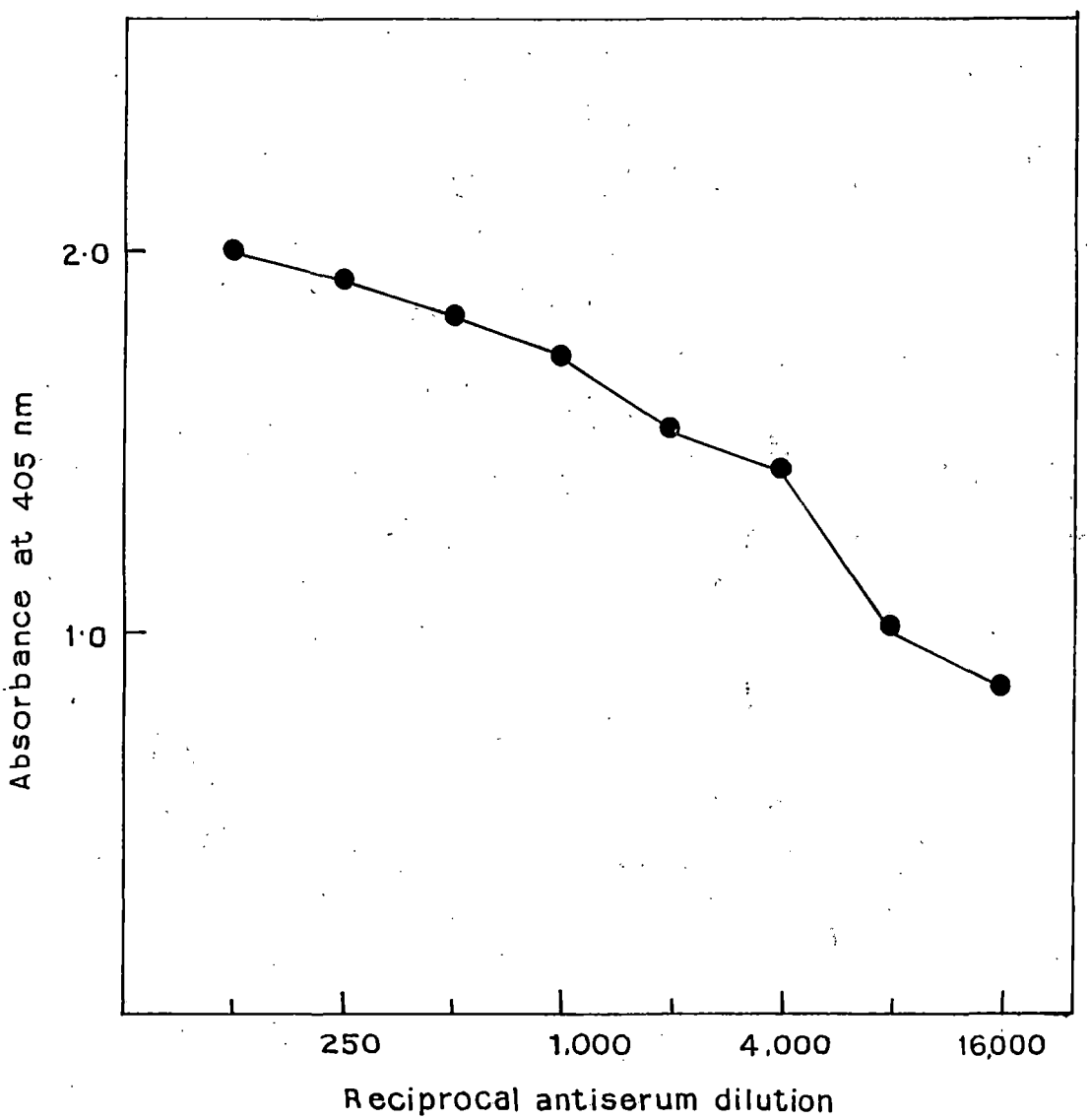


Fig. 7.

4.3.4.2.1. Mycelia

Antigens were prepared from tea leaves of all the 37 varieties as well as from 3 nonhost species (*G. max, C. arietinum, C. japonicum*), 9 isolates of the pathogen (*G. cingulata*) and 1 nonpathogen (*F. oxysporum*). Antigens at a concentration of 40 μg/ml were testedby DAC ELISA against purified mycelial antiserum of *G. cingulata* except incase of *G. cingulata* isolates, where a concentration of 5 μg/ml was used. In all cases experiments were repeated thrice under same condition. Results (Tables 22-24) revealed that absorbance value in ELISA varied with the different varieties. Among the Tocklai varieties tested TV-18 showed highest

Table 22: Indirect ELISA values (A_{405}) of tea leaf antigens (Darjeeling varieties) reacted with antiserum raised against mycelial antigen of *G.cingulata*

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
B 668	0.999	0.998	0.998	0.998±0.000
B 777	1.034	1.032	1.033	1.033±0.000
P 312	0.785	0.782	0.782	0.783±0.001
T-78	1.146	1.161	1.129	1.145±0.009
P-1258	0.967	0.921	0.944	0.944±0.013
B-157	1.350	1.481	1.408	1.413±0.037
TTV_1	1.117	1.190	1.123	1.143±0.023
BT-15	0.701	0.701	0.722	0.708±0.007
AV-2	0.833	0.827	0.833	0.831±0.002
BS/7A/76	0.757	1.012	1.034	1.023±0.088
RR 17	0.853	0.848	0.849	0.850±0.001
HV 39	1.034	1.003	1.017	1.018±0.008
KI/I	1.099	1.045	1.072	1.072±0.015
T-135	0.796	0.777	0.785	0.786±0.005
CP-1	0.738	0.804	0.801	0.781±0.021

± Standard error

A_{405} for normal serum 0.007

Table 23: Indirect ELISA values (A_{405}) of tea leaf antigens of UPASI varieties, non hosts, and mycelial antigens of *G.cingulata* isolates and *F.oxysporum* (non pathogen) reacted with anti-*G. cingulata* antiserum (mycelia).

Antigen concentration(40 μg/ml)	Antiserum of *G.cingulata* (GCIA) (1.250 dilution)			
	Expt.1	**Expt.2**	**Expt.3**	**Mean**
Tea varieties				
UPASI 2	0.729	0.768	0.747	0.748±0.011
UPASI 3	1.222	1.278	1.247	1.249±0.014
UPASI 8	0.945	1.008	0.975	0.976±0.025
UPASI 9	1.570	1.562	1.566	1.566±0.002
UPASI 17	0.864	0.824	0.844	0.844±0.011
UPASI 26	1.070	1.033	1.050	1.051±0.010
BSS1	0.947	1.004	0.974	0.975±0.016
BSS 2	0.899	0.958	0.954	0.937±0.019
BSS 3	0.860	0.905	0.881	0.882±0.013
Isolates of G.cingulata[a]				
GC-1	>2	>2	>2	>2
GC-2	1.885	1.927	1.825	1.879±0.040
GC-3	1.727	1.812	1.804	1.781±0.027
GC-4	1.779	1.751	1.736	1.755±0.0126
GC-5	1.892	1.801	1.877	1.856±0.028
GC-6	1.721	1.739	1.746	1.735±0.007
GC-7	1.776	1.712	1.801	1.763±0.026
GC-8	1.763	1.702	1.791	1.752±0.026
GC-9	1.733	1.756	1.800	1.763±0.0196
Non pathogen				
F. oxysporum	0.353	0.341	0.289	0.327 ± 0.019
Non host				
G.max	0.373	0.368	0.356	0.365 ± 0.005
C. arietinum	0.364	0.339	0.382	0.361 ± 0.010
C. japon icum	0.411	0.445	0.404	0.420 ± 0.012

A405 for normal sera = 0.005

± Standard error

a Concentration of antigen - 5μg/ml

absorbance followed by Teenali 17/1/54 & TV-20. Lowest absorbances were obtained in TV-26, TV-29 and TV-30 (Table-24). Similarly in the Darjeeling varieties highest and lowest absorbances were obtained in B157 and BT-15 respectively (Table-22). Of the 9 UPASI varities tested UPASI 9 exhibited highest reactivity while UPASI 2 exhibited lowest reactivity. Reactivity of the non host and non pathogen were low as evidenced by low absorbance values (Table-23). The different isolates of the pathogen tested also showed reactivity with the antiserum of one particular isolate (GC-1). Highest absorbance value(>2), however, was obtained in the homologus reaction. Absorbance values for normal serum controls were below the corresponding test values. Results of the ELISA tests revealed that the highest reactivitywas shown by UPASI 9 and the lowest by BT-15. These results were in confirmity with the results of the pathogenicity tests.

Table 24: Indirect ELISA values (A_{450}) of tea leaf antigens of Tocklai varieties reacted with antiserum of *G. cingulata* (Mycelia)

Antigens of (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
TV-9	0.883	0.892	0.808	0.861 ± 0.026
TV-18	1.464	1.425	1.443	1.444 ± 0.011
TV-20	1.028	0.937	0.918	0.982 ± 0.026
TV-22	0.895	0.897	0.890	0.894 ± 0.001
TV-23	0.963	0.963	0.963	0.963 ± 0.000
TV-25	0.781	0.997	0.937	0.967 ± 0.061
TV-26	0.826	0.829	0.861	0.838 ± 0.011
TV-27	0.899	0.880	0.888	0.889 ± 0.005
TV-28	0.898	0.821	0.874	0.864 ± 0.020
TV-29	0.882	1.000	0.834	0.858 ± 0.040
TV-30	0.858	0.866	0.889	0.860 ± 0.009
Teenali 17/1/54	0.950	1.042	0.785	0.996 ± 0.070
TS-449	0.830	0.917	0.894	0.880 ± 0.026

± Standard error; A_{405} for normal sera = 0.007

4.3.4.2.2. Cell wall

Since cell walls of pathogen also play a major role in the recognition phenomenon antiserum raised against cell wall preparations of *G.cingulata* was also tested against leaf antigens of all the 37 varieties. As in the case with antiserum raised against mycelial antigen here also absorbance values in ELISA varied with the different varieties. Results have been presented in Tables 25-27. Among all the 37 varieties tested with the antiserum against cell wall preparation highest absorbance value was exhibited by UPASI-9 and lowest by BT-15. Low absorbance values were obtained with non hosts and non pathogen.

The overall trend in ELISA reactivity with the different tea varieties and antiserum of mycelia or cell wall preparations of *G.cingulata* were similar. However, absorbance values were higher in most cases with antiserum raised against cell wall preparations than those of antiserum raised against mycelial preparation of *G.cingulata* as evidenced in fig.8.

4.3.4.3. Comparison of ELISA reactivity among antigens prepared from tea leaves of various ages against antiserum of *G. cingulata.*

Since tea is a perennial plant being maintained year after year, individual tea bushes having a large number of leaves of various size and position created an interest to investigate whether serological resemblances among tea varieties and the isolates of *G. cingulata* as evidenced in earlier experiments also existed in case of the leaves of all ages. In order to perform this experiment, 11 Tocklai varieties (Teenali 17/1/54, TV-18, 20, 22, 23, 25, 26, 27, 28, 29 and 30) were selected. Leaf samples were collected from individual variety considering the position and size of the leaves and they were categorised into three groups (A, B and C). Uppermost young leaves (10-15 mm length x 6-10 mm breadth), mid-branches middle aged leaves (40-45 length x 25-30 mm breadth) and lower branches mature leaves (65-72 mm length x 35-40 mm breadth) were designated as A, B and C respectively. Antigens were prepared from the categories of leaves of each of 11tea varieties and using anti *G. cingulata* antisera raised against both mycelia and cell wall DAC - ELISA was performed.

4.3.4.3.1. Mycelia

Anti *G. cingulata* antiserum (1;250 dilution) raised against antigen preparations from mycelia were cross reacted with 40 μg/ml antigens prepared from various category of 11 varieties following DAC-ELISA. Homologous reaction involving mycelial antigen and antisera gave A_{405} value of >2, while highest absorbance values in heterologous reactions were in case of TV-18 and TV-23 followed by TV-29 and Teenali 17/1/54.However , TV-26, TV-25 and TV-20 showed absorbance values <1. It is interesting to note that leaf antigens prepared from all 3 categories gave positive response in ELISA reactions under identical condition (Table - 28)

Table 25: Indirect ELISA values (A_{405}) of tea leaf antigens of UPASI varieties,non host and mycelial antigen of *G. cingulata* and *F. oxysporum* reacted with cell wall antiserum of *G. cingulata*.

Antigen Concentration (40 μg/ml	Antiserum of *G. cingulata* (GCIA) (1:250 dilution)			
	Expt.1	Expt.2	Expt.3	Mean
Teavarieties				
UPASI 2	1.272	1.259	1.252	1.261 ± 0.005
UPASI 3	1.776	1.774	1.775	1.775 ± 0.005
UPASI 8	1.545	1.576	1.554	1.558 ± 0.009
UPASI 9	1.849	1.834	1.840	1.841 ± 0.004
UPASI 17	1.326	1.376	1.377	1.359 ± 0.016
UPASI 26	1.727	1.709	1.718	1.718 ± 0.005
BSS 1	1.574	1.577	1.643	1.568 ± 0.022
BSS 2	1.611	1.498	1.553	1.554 ± 0.032
BSS 3	1.483	1.569	1.526	1.526 ± 0.024
Isolates of *G. cingulata*[a]				
GC-1	>2	>2	>2	>2
GC-2	1.929	1.973	1.981	1.961 ± 0.021
GC-3	1.834	1.817	1.821	1.824 ± 0.042
GC-4	1.969	1.901	1.922	1.930 ± 0.020
GC-5	1.929	1.962	1.956	1.949 ± 0.010
GC-6	1.825	1.812	1.834	1.820 ± 0.006
GC-7	1.861	1.869	1.883	1.871 ± 0.006
GC-8	1.902	1.911	1.879	1.897 ± 0.009
GC-9	1.803	1.872	1.889	1.854 ± 0.026
Non pathogen				
F. oxysporum	0.378	0.329	0.386	0.364 ± 0.017
Non host				
G. max	0.399	0.378	0.351	0.376 ± 0.013
C. arietinum	0.311	0.363	0.351	0.376 ± 0.015
C. japonicum	0.412	0.426	0.404	0.414 ± 0.005

A_{405} for normal serum = 0.009.

± Standard error

a Antigen concentration 5 μg/ml

Table 26: Indirect ELISA values (A_{405}) of tea leaf antigens of Darjeeling varieties reacted with cell wall antiserum of *G. cingulata* .

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt. 1	Expt. 2	Expt. 3	Mean
B668	1.592	1.657	1.618	1.622 + 0.018
B777	1.635	1.692	1.653	1.660 ± 0.016
P312	1.343	1.222	1.281	1.282 ± 0.050
T-78	1.771	1.761	1.748	1.760 ± 0.006
P-1258	1.573	1.534	1.543	1.550 ± 0.011
B-157	1.803	1.849	1.826	1.826 ± 0.013
TTV_1	1.750	1.793	1.716	1.753 ± 0.022
BT-15	1.118	1.196	1.171	1.161 ± 0.023
AV-2	1.299	1.363	1.256	1.306 ± 0.031
BS/7A/76	1.668	1.645	1.686	1.666 ± 0.011
RR17	1.432	1.417	1.423	1.424 ± 0.004
HV-39	1.663	1.603	1.684	1.650 ± 0.024
K1/1	1.734	1.772	1.753	1.753 ± 0.010
T-135	1.363	1.269	1.247	1.293 ± 0.028
CP-1	1.365	1.205	1.285	1.285 ± 0.046

± Standard error.

A_{405} for normal sera = 0.007

Table 27: Indirect ALISA values (A_{405}) of tea leaf antigens of Tocklai varieties reacted with antiserum of *G. cingulata* (cell wall).

Antigens of host (40 μg/ml)	Antiserum of *G. cingulata* (1:250 dilution)			
	Expt. 1	Expt. 2	Expt. 3	Mean
TV-9	1.458	1.502	1.502	1.487 ± 0.014
TV-18	1.672	1.680	1.676	1.676 ± 0.002
TV-20	1.556	1.560	1.582	1.566 ± 0.008
TV-22	1.538	1.568	1.553	1.553 ± 0.008
TV-23	1.560	1.569	1.563	1.564 ± 0.002
TV-25	1.586	1.626	1.527	1.570 ± 0.015
TV-26	1.382	1.306	1.344	1.344 ± 0.021
TV-27	1.515	1.502	1.579	1.532 ± 0.023
TV-28	1.535	1.503	1.519	1.519 ± 0.009
TV-29	1.433	1.407	1.435	1.425 ± 0.009
TV-30	1.493	1.506	1.498	1.499 ± 0.003
Teenali 17/1/54	1.578	1.528	1.645	1.583 ± 0.017
TS-449	1.484	1.555	1.518	1.519 ± 0.019

± Standard error.

$A_{(405)}$ for normal serum = 0.005.

ELISA responses of leaf antigens of different tea varieties against cell wall and mycelial antisera of *G.cingulata*

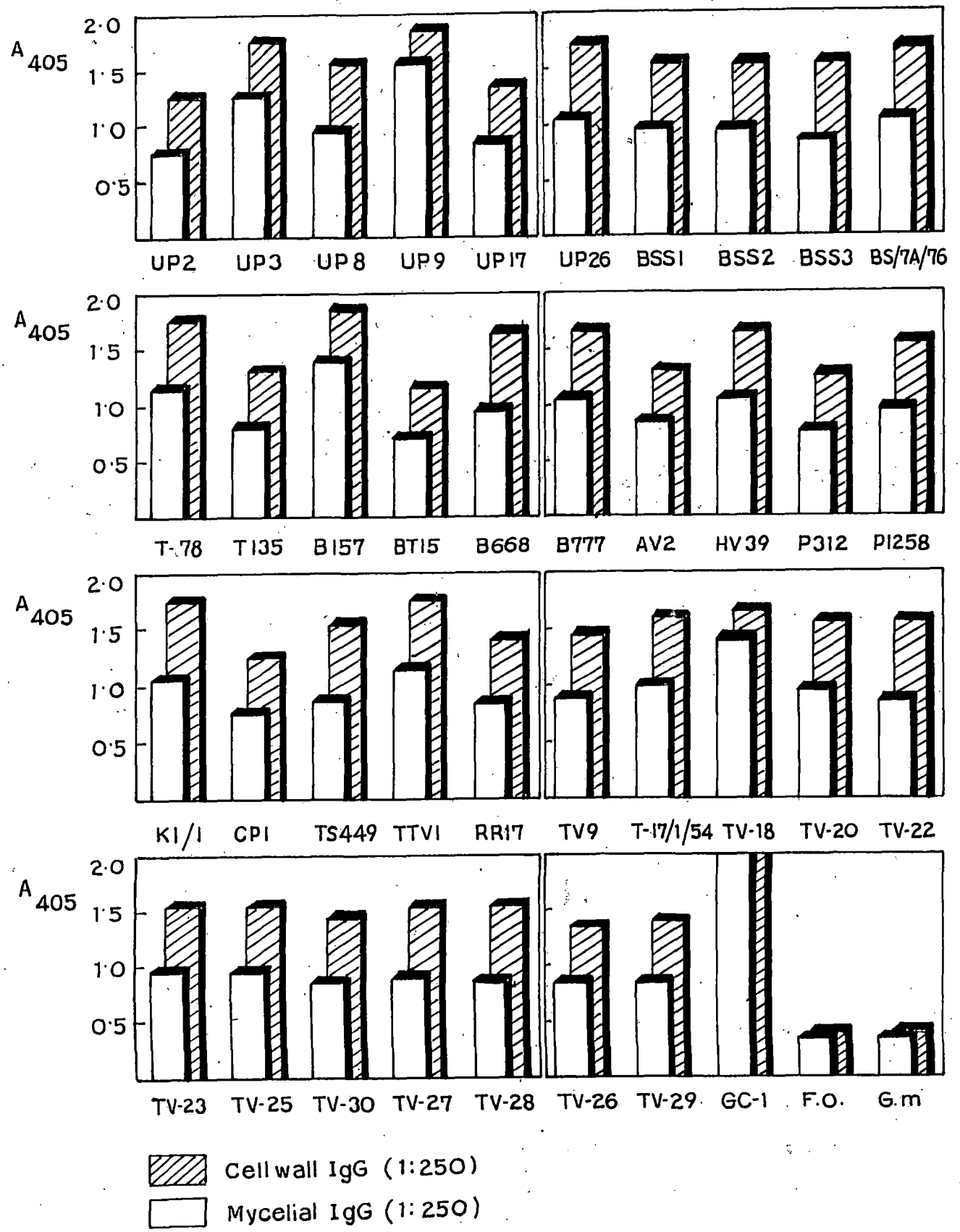


Fig. 8.

Table 28: Indirect ELISA values (A_{405}) of antigens prepared from tea leaves of various ages against antiserum of *G. cingulata* (Mycelia)

Antigen concentration 40 μg/ml	Mycelial antiserum of *G. cingulata* (1:250 dilution)		
	Position and size of tea leaves*		
	A	B	C
Teenali 17/1/54	1.151 ± 0.016	1.100 ± 0.013	1.126 ± 0.013
TV-18	1.225 ± 0.029	1.512 ± 0.027	1.565 ± 0.014
TV-20	0.996 ± 0.002	1.101 ± 0.025	1.210 ± 0.011
TV-22	1.015 ± 0.013	0.955 ± 0.018	1.109 ± 0.003
TV-23	1.214 ± 0.031	1.160 ± 0.047	1.109 ± 0.017
TV-25	0.996 ± 0.014	1.095 ± 0.015	1.078 ± 0.031
TV-26	0.726 ± 0.013	0.980 ± 0.012	1.065 ± 0.021
TV-27	1.063 ± 0.012	1.082 ± 0.006	1.145 ± 0.020
TV-28	1.096 ± 0.013	1.051 ± 0.021	1.100 ±0.012
TV-29	1.151 ± 0.024	1.075 ± 0.013	1.232 ± 0.017
TV-30	1.068 ± 0.008	1.113 ± 0.010	1.152 ± 0.016

Note: $A_{(405)}$ valueof homologous reaction >2; for normal serum = 0.006

*A = Uppermost young leaves;

B = Mid branches middle aged;

C = Mature old leaves; lower branches;

± Standard error.

4.3.4.3.2. Cell wall

When antiserum of *G. cingulata* (1:250 dilution) raised against cell wall antigens were cross reacted with three categories of leaf antigens (40 μg/ml) prepared from 11 Tocklai varieties, young leaves of TV-18 followed by TV-23, TV-20, TV-28, TV-22, showed greater absorbance values than Teenali 17/1/54, TV-25 and TV-27. Here also lowest A_{405} value was obtained in case of TV-26 (Table-29). Recognition of leaf antigens with cell wall antiserum of *G. cingulata* was always better than the antiserum of the pathogen raised against mycelial preparation. In this experiment, It was notised that there was no correlation among the antigen preparation either from young or middle or mature leaves with respect to the individual variety tested. However, irrespective of verieties or categories A_{405} values were always higher with cell wall antiserum of the pathogen than the anti *G. cingulata* antiserum raised against the mycelial antigen (Fig. 9).

4.3.4.4. Reciprocal cross reaction of antisera of tea varieties and non pathogen with leaf antigens (host and non host) and mycelial antigens (pathogen and non pathogen).

In previous experiments following DAC-ELISA serological relationship between tea leaves and *G.cingulata* have been detected using antiserum of the pathogen raised against mycelia and cell wall. 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 leaf antigens prepared from two varieties of tea TV-18 and CP-1 which were found to be most susceptible and resistant respectively in pathogenicity test, were cross reacted with leaf antigens prepared from 14 Tocklai varieties (CP-1, TS-449, Teenali 17/1/54, TV-18, 20, 22, 23, 25, 26, 27, 28, 29 and 30), 3 non-host species (viz. *G.max, C.arietinum* and *C. japonicum*) as well as mycelial antigen prepared from pathogen (*G.cingulata*, isolate GC-1) and non pathogen (*F.oxysporum*). Simultaneously, antiserum raised against *F.oxysporum* (non-pathogen) was also tested against the antigens of the above materials. Results have been presented in Table-30. In this experiment, A_{405} values in ELISA reaction involving antigens and antisera of CP-1, TV-18 and *F.oxysporum* were 1.642, 1.712 and 1.821 respectively. When anti CP-1 antiserum was cross reacted with various antigens of host, non host, pathogen and non pathogen, TV-9, TS-449. Teenali 17/1/54 and TV-23 gave higher absorbance than the other 10 varieties of tea tested. Anti TV-18 antiserum gave higher absorbance in TS-449, TV-23, TV-28 and Teenali 17/1/54 than the other varieties tested. It is interesting to note that in cross reaction with anti TV-18 antiserum and mycelial antigen of *G.cingulata* higher absorbance (1.132) was obtained. But anti CP-1 antiserum gave lower

Table 29: Indirect ELISA values (A_{405}) of antigen prepared from leaves of various ages against antiserum of *G. cingulata* (Cell wall)

Antigen concentration 40 μg/ml	Cell wall antiserum of *G. cingulata* (1:250 dilution)		
	Position and size of tea leaves*		
	A	B	C
Teenali 17/1/54	1.615 ± 0.004	1.765 ± 0.009	1.771 ±0.008
TV-18	1.750 ± 0.05	1.912 ± 0.070	1.862 ±0.029
TV-20	1.725 ± 0.005	1.692 ± 0.030	1.751 ± 0.015
TV-22	1.721 ± 0.004	1.728 ± 0.007	1.730 ± 0.004
TV-23	1.728 ± 0.015	1.750 ± 0.016	1.738 ± 0.012
TV-25	1.635 ± 0.005	1.729 ± 0.031	1.600 ± 0.009
Tv-26	1.002 ± 0.006	1.065 ± 0.070	1.231 ± 0.020
Tv-27	1.612 ± 0.006	1.803 ± 0.012	1.609 ± 0.014
Tv-28	1.725 ± 0.023	1.731 ± 0.043	1.800 ± 0.021
Tv-29	1.310 ± 0.025	1.415 ± 0.009	1.611 ± 0.017
Tv-30	1.211 ± 0.016	1.226 ± 0.017	1.512 ± 0.024

Note: A_{405} valueof homologous reaction >2; for normal serum = 0.006 ; ± Standard error.
*A = Uppermost young leaves; B = Mid branches middle aged; C = Mature old leaves;

absorbance value (0.673) when reacted with the mycelial antigen of the pathogen. Antiserum raised against non pathogen (*F.oxysporum*) did not react significantly with the tea leaf antigens, as lower absorbance values were detected in all cases. However, A_{405} value of 0.621 was obtained, when this antiserum was reacted with the antigen of *G.max* since, *F.oxysporum* is pathogenic to *G.max* (cv. Soymax). Non host and non pathogen antigens also did not react with anti TV-18 and anti CP-1 antiserum (Table-30).

ELISA reactivity among antigens prepared from tea leaves of various ages against cell wall and mycelial antisera of *G.cingulata*

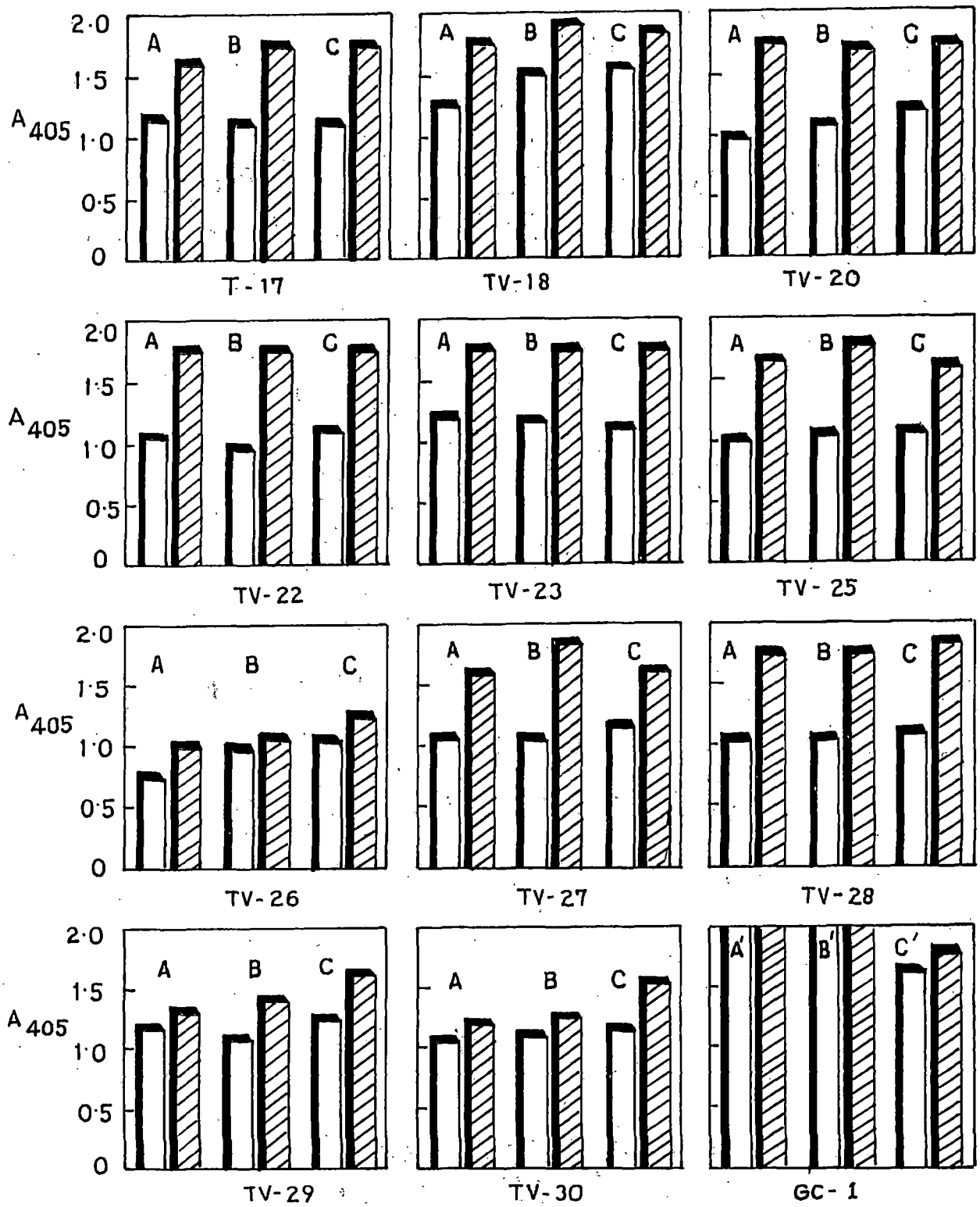


Fig.9

Table 30: Indirect ELISA values (A_{405}) of leaf antigens (host and non host) and mycelial antigens (pathogen and non pathogen) reacted with antisera of tea varieties, resistant (CP-1) and susceptible(TV-18) to *G.cingulata* and non-pathogen (*F.oxysporum)*

Antigens of host and parasite	Antisera dilution (1:250)		
	Tea varieties		Non-pathogen
	(CP1A)	(TI8A)	*F. oxysporum*
Tea varieties			
CP-1	1.642±0.001	0.895±0.001	0.301±0.001
S-449	1.055±0.016	1.122±0.016	0.312±0.016
TV-9	1.109±0.021	0.968±0.021	0.326±0.021
Teen Ali-17/1/54	1.015±0.008	1.060±0.020	0.346±0.009
TV-18	0.985±0.016	1.712±0.041	0.311±0.021
TV-20	0.848±0.008	0.935±0.032	0.319±0.036
TV-22	0.977±0.002	0.896±0.061	0.349±0.006
TV-23	1.069±0.019	1.102±0.047	0.361±0.029
TV-25	0.782±0.016	0.865±0.016	0.329±0.006
TV-26	0.993±0.021	0.981±0.023	0.261±0.011
TV-27	0.977±0.002	0.827±0.006	0.299±0.061
TV-28	0.812±0.061	1.004±0.019	0.301±0.070
TV-29	0.796±0.002	0.809±0.016	0.326±0.042
TV-30	0.818±0.018	0.874±0.009	0.316±0.039
Pathogen			
G.cingulata(GC-1)	0.673±0.006	1.132±0.019	0.381±0.006
Non pathogen			
F.oxysporum	0.198±0.031	0.140±0.011	1.821±0.042
Non host			
G.max	0.245±0.016	0.270±0.036	0.621±0.002
C.arietinum	0.267±0.021	0.259±0.029	0.269±0.019
C.japonicum	0.258±0.031	0.176±0.019	0.312±0.022

± Standard error

A_{405} for normal serum = 0.007

4.3.5. Double antibody sandwich (DAS) ELISA

Detection of cross reactive antigens shared between isolates of *G. cingulata* and tea varieties by indirect ELISA using the format of DAS ELISA was further confirmed by using another format of direct ELISA i.e., double antibody sandwich (DAS) ELISA. In this experiment, antigens were prepared from 5 selected varieties of tea - (viz., Teenali 17/1/54, TV -18, TV-23, CP-1 and TV-26), 3 non host species (*G. max, C. japonicum and C. arietinum*), an isolate of *G. cingulata* (GC-1) and *F. oxysporum* (non pathogen). Anti *G. cingulata* antiserum at 1:250 dilution was reacted with the antigen preparations (40 μg/ml) as mentioned

Table 31: Reaction of anti *G. cingulata* antiserum against tea leaf antigens, non hosts, mycelial antigen of *G. cingulata* (isolate GC-1) and *F. oxysporum* (non pathogen) using double antibody sandwich (DAS) ELISA.

Antigen concentration (40 μg/ml)	Anti-*G. cingulata* antiserum (at 1:250 dilution) Absorbance at 450 nm			
	Expt. 1	**Expt. 2**	**Expt. 3**	**Mean**
Pathogen				
G. cingulata	1.409	1.415	1.407	1.410 ± 0.002
Host				
Teenali-17/1/54	1.105	1.054	1.101	1.086 ± 0.016
TV-18	1.254	1.255	1.217	1.242 ± 0.012
TV-23	0.982	0.923	0.975	0.960 ± 0.018
CP-1	0.738	0.746	0.797	0.760 ± 0.018
TV-26	0.866	0.706	0.676	0.749 ± 0.050
Non hosts				
C. japonicum	0.391	0.372	0.392	0.385 ± 0.006
G. max	0.377	0.477	0.411	0.421 ± 0.029
C. arietinum	0.328	0.315	0.312	0.318 ± 0.004
Non pathogen				
F. oxysporum	0.396	0.426	0.361	0.394 ± 0.018

above following DAS ELISA. Details of the method have been described under Materials and Methods. Results have been presented in Table 31. Antigen and antisera of *G. cingulata* were highly reactive. Mean A_{405} values for TV-18 and Teenali 17/1/54 were 1.242 and 1.086, while, for TV-26 and CP-1 these were 0.749 and 0.760 respectively. Anti *G. cingulata* antiserum had a significantly higher absorbance mean reaction ($P < 0.01$) to susceptible varieties (TV-18 and Teenali 17/1/54) than to resistant varieties (TV 26 and CP-1). Antigen prepared from non host and non pathogen gave lower absorbance values.

4.4. Detection of *G.Cingulata* in infected leaf tissue by indirect ELISA

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 readings between infected and healthy antigens indicates 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 *G.cingulata.* In the next series of experiments attempts have been made to detect *G.cingulata* in infected leaf tissues of different varieties as well as after different hours of inoculation.

4.4.1 Artificially inoculated leaves

Leaves of all the tea varieties used in previous studies were inoculated with *G.cingulata* as described in Materials and Methods. Antigens were extracted after 72 h of inoculation as by this time symptoms were very well established on the leaves. Antigens prepared from inoculated leaves as well as from corresponding healthy leaves,at concentrations of 40μg/ml were reacted with anti *G.cingulata* antiserum at 1:250 dilution. Results (Tables 32-34, Figs. 10 and 11) revealed that absorbance values for inoculated leaf antigen preparation of all varieties were significantly higher than their respective healthy leaf extracts. However, healthy leaf extracts also showed quite high absorbance values due to the presence of cross reactive antigens.

Table 32: ELISA values showing reaction of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Tocklai varieties (absorbance at 405 nm)

Tea varieties	Antigen concentration (40 μg/ml)	
	Healthy	Inoculated[a]
TV-9	0.942±0.012	1.233±0.011
Teenali 17/1/54	0.999±0.011	1.342±0.023
TV-18	1.020±0.018	1.469±0.012
TV-20	0.899±0.029	1.062±0.011
TV-22	0.927±0.020	1.116±0.018
TV-23	0.989±0.021	1.217±0.039
TV-25	0.971±0.008	1.116±0.013
TV-26	0.729±0.017	0.929±0.017
TV-27	0.853±0.031	1.002±0.001
TV-28	0.796±0.003	0.989±0.035
TV-29	0.763±0.016	0.962±0.028
TV-30	0.792±0.-012	1.020±0.008
TS-449	0.783±0.007	1.006±0.027

Anti *G.cingulata* antiserum used at 1:250 dilution

[a]3d after inoculation with *G.cingulata*

Difference between healthy and inoculated significant at p = 0.01 in all varieties

± Standard error.

Table 33: ELISA values (A_{405}) of healthy and *G.cingulata* inoculated (72h) leaf antigens of different Darjeeling varieties reacted with pathogen antiserum.

Tea variety	Leaf antigens (40 μg/ml)	
	Healthy	Inoculated[a]
B 668	0.733±0.002	0.977±0.021
B 777	0.751±0.019	1,084±0.002
P 312	0.722±0.023	0.991±0.006
T-78	0.811±0.005	1.176±0.009
P-1258	0.784±0.014	1.018±0.016
B-157	0.897±0.021	1.215±0.039
TTV_1	0.801±0.001	0.916±0.005
BT-15	0.716±0.003	0.903±0.039
AV-2	0.719±0.016	0.967±0.024
BS/7A/76	0.804±0.005	1.010±0.002
RR17	0.733±0.007	1.012±0.062
HV-39	0.735±0.019	0.914±0.003
K1/1	0.753±0.006	1.113±0.040
CP-1	0.721±0.016	0.927±0.070
T-135	0.723± 0.009	1.002±0.039

Anti *G. cingulata* antiserum used at 1:250 dilution.

[a]3 d after inoculation with *G.cingulata*

Differences between healthy and inoculated significant at p=0.01 in all varieties.

± Standard error

Table 34: ELISA values showing reaction of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of UPASI varieties (absorbance at 405 nm)

Tea variety	Antigen concentration (40 μg/ml)	
	Healthy	Inoculated[a]
UPASI-2	0.629 ± 0.002	0.759 ± 0.009
UPASI-3	0.687 ± 0.012	1.073 ± 0.019
UPASI-8	0.635 ± 0.006	0.869 ± 0.017
UPASI-9	0.739 ± 0.021	1.242 ± 0.016
UPASI-17	0.630 ± 0.022	0.763 ± 0.012
UPASI-26	0.670 ± 0.036	1.049 ± 0.020
BSS-1	0.668 ± 0.017	0.892 ± 0.010
BSS-2	0.679 ± 0.022	0.919 ± 0.021
BSS-3	0.652 ± 0.016	0.879 ± 0.006

Anti *G.cingulata* antiserum used at 1:250 dilution.

a - 3d after inoculation with *G.cingulata*.

Differences between healthy and inoculated significant at p=0.01

± Standard error.

ELISA responses of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Tocklai varieties

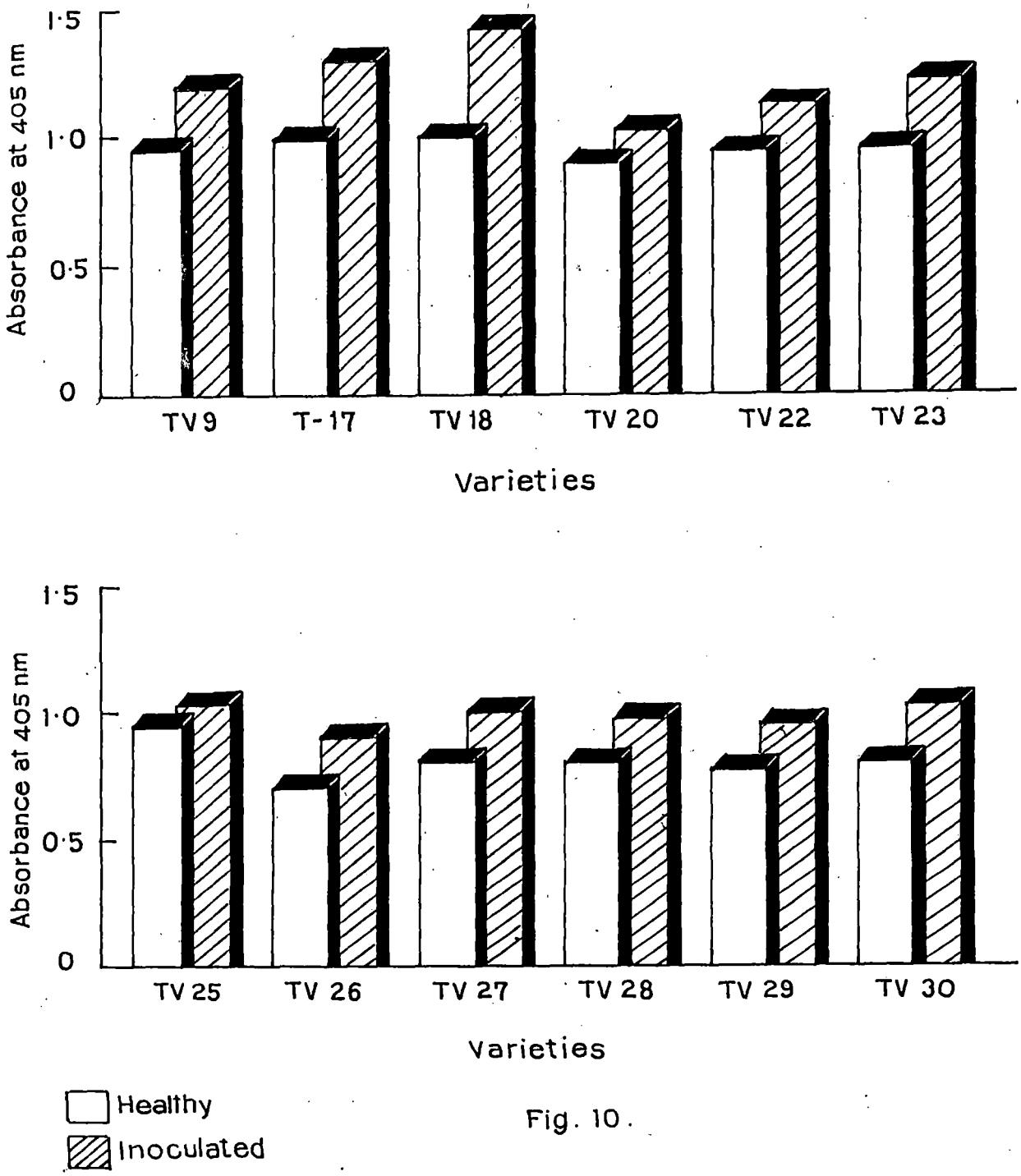


Fig. 10.

ELISA responses of antiserum of *G.cingulata* with antigens of healthy and inoculated tea leaves of Darjeeling varieties

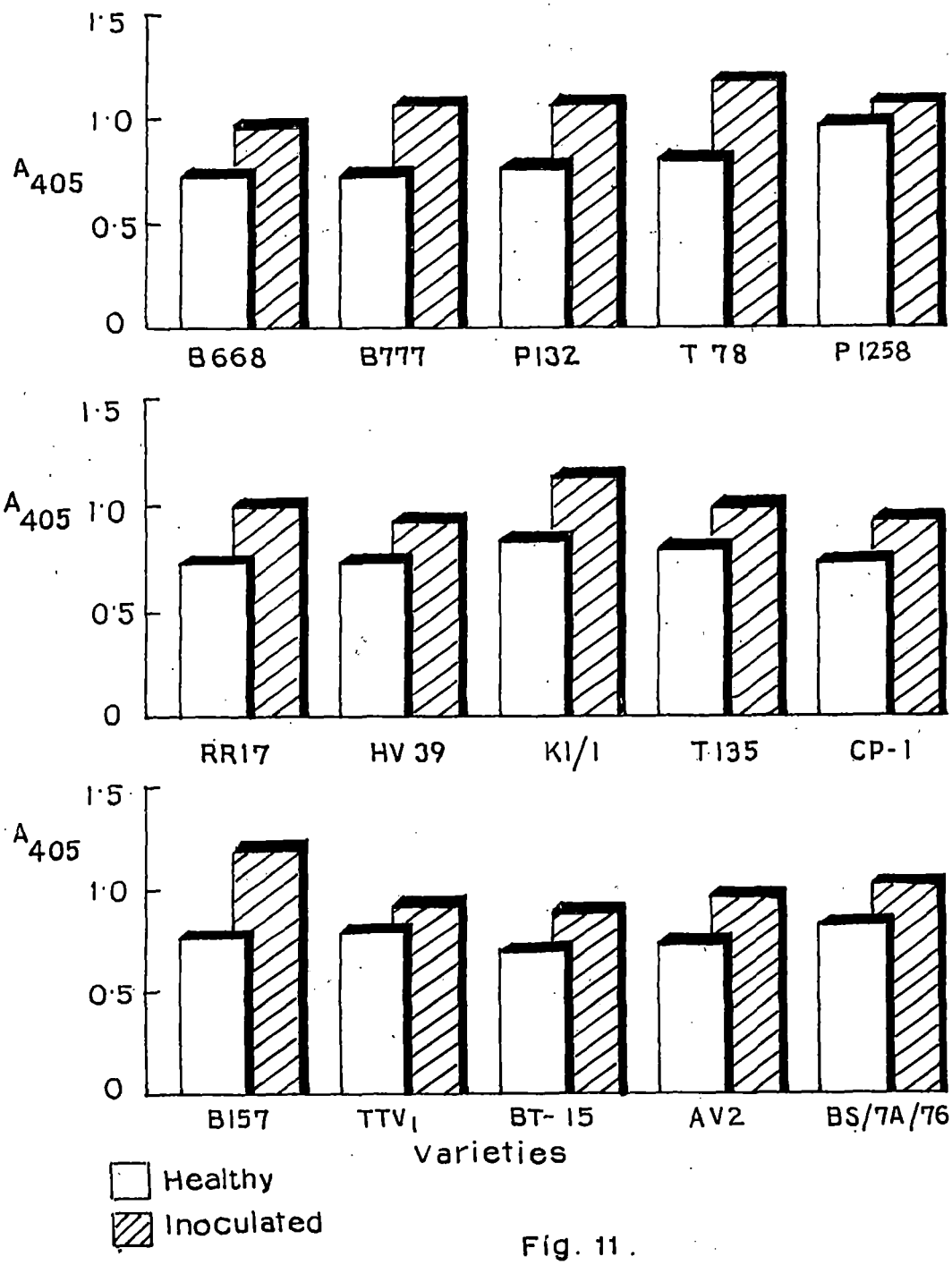


Fig. 11.

4.4.2 Naturally infected leaves

In the previous experiment anti *G.cingulata* antiserum was used to detect infection in artificially inoculated tea leaves. Subsequently in the next experiment it was decided to test whether natural brown blight infection could be detected with this antiserum. For this, tea leaves of different UPASI varieties showing brown blight infection in the field were collected and antigens were prepared from these leaves. Using the antigen extracts from healthy and naturally infected tea leaves ELISA was performed against anti *G.cingulata* antiserum. Results presented in Table-35 revealed that infected extracts of all varieties had significantly higher absorbance values in ELISA in comparison to the healthy extracts.

Table 35: ELISA values showing reaction of antiserum of *Glomerella cingulata* with antigens of healthy and naturally infected tea leaves of UPASI varieties.

Tea varieties	Antigen concentration (40 μg/ml)	
	Healthy	Infected[a]
UPASI clones		
UPASI-2	0.619 ± 0.017	0.713 ± 0.011
UPASI-3	0.690 ± 0.001	1.020 ± 0.015
UPASI-8	0.647 ± 0.012	0.964 ± 0.017
UPASI-9	0.795 ± 0.007	1.154 ± 0.029
UPASI-17	0.700 ± 0.019	1.092 ± 0.019
UPASI-26	0.675 ± 0.027	1.019 ± 0.022
Seed varieties		
BSS-1	0.592 ± 0.002	1.012 ± 0.017
BSS-2	0.612 ± 0.023	1.023 ± 0.022
BSS-3	0.724 ± 0.015	1.000 ± 0.037

Anti *G.cingulata* antiserum used at 1:125 dilution

a - Naturally infected tea leaves.

Difference in values between inoculation and healthy leaves significant at p=0.01.

± Standard error.

4.4.3 Different times after inoculation

G.cingulata was detected in infected tea leaves of different varieties using antiserum raised against *G.cingulata*. Since in these cases antigens were extracted after 72h of inoculation, symptoms were already well developed at the time of antigen preparation. As ELISA has been considered to be an important tool for early detection of pathogen in infected tissues even before appearance of symptoms it was decided to perform an experiment to determine the earliest hour after inoculation when *G.cingulata* could be detected in artificially inoculated leaf. For this experiment leaves of a susceptible and a resistant variety (B 157 and BT-15 respectively) of tea were inoculated with *G.cingulata* as described in Materials and Methods. Antigens were extracted at 6h intervals till the period of 96h. These antigens were then tested against both anti *G.cingulata* mycelial antiserum and anti *G.cingulata* cell wall antiserum.

4.4.3.1 Anti *G.cingulata* antiserum (Mycelia)

Antigens from healthy and infected leaves were tested against purified antiserum (IgG) raised against *G.cingulata* mycelial antigen. Experiments were repeated thrice in all cases. Results revealed that in ELISA, absorbance values were higher for infected extracts in comparison to healthy extracts. In the susceptible variety (B 157) differences between healthy and infected extracts were statistically significant from 6h onwards while in resistant variety (BT-15) differences were statistically significant only from 30h onwards. Hence, on the basis of significantly higher absorbance values of infected leaf extracts in comparison to healthy extracts the earliest period for detection of infection was 6h after inoculation in case of susceptible variety and 30h in case of resistant variety (Table-36). In both the varieties no lesion production was evident at the earliest time of pathogen detection.

Table 36: ELISA values obtained by reaction of anti *G.cingulata* antiserum (mycelial) and tea leaves after different hours of inoculation with *G.cingulata.*

Hours after inoculation	Anti *G.cingulata* antiserum (1:250 dilution)			
	B 157[a]		BT-15[a]	
	H	I	H	I
6	0.750±0.003	0.823±0.003	0.709*±0.030	0.795±0.022
12	0.752±0.001	0.901±0.016	0.711*±0.021	0.800±0.032
18	0.709±0.009	1.101±0.012	0.750*±0.207	0.801±0.027
24	0.765±0.003	1.150±0.017	0.761*±0.034	0.811±0.029
30	0.750±0.013	1.152±0.012	0.709± 0.009	0.820±0.017
36	0.732±0.012	1.155±0.019	0.702± 0.012	0.869±0.029
42	0.782±0.017	1.159±0.020	0.750± 0.031	1.050±0.031
48	0.785±0.016	1.195±0.009	0.761± 0.021	1.072±0.009
54	0.751±0.008	1.198±0.001	0.752± 0.051	1.082±0.021
60	0.800±0.006	1.198±0.009	0.732± 0.002	1.155±0.015
66	0.750±0.001	1.223±0.016	0.751± 0.019	1.200±0.002
72	0.762±0.002	1.229±0.012	0.795± 0.017	1.209±0.012
78	0.763±0.001	1.250±0.002	0.762± 0.024	1.216±0.032
84	0.750±0.020	1.270±0.009	0.713± 0.032	1.202±0.051
90	0.761±0.016	1.301±0.050	0.723± 0.039	1.153±0.002
96	0.732±0.012	1.253±0.034	0.750± 0.014	1.101±0.051

a Leaf antigen used at 40 μg/ml concentration.

* Difference in values between healthy and inoculated extracts not significant at p=0.01 ; all the rest significant.

± Standard error.

4.4.3.2 Anti *G.cingulata* antiserum (Cell wall)

Antiserum raised against cell wall preparations of *G.cingulata* were also used to determine the earliest period when infection could be detected in the leaf tissues. Healthy leaf extracts as well as antigen extracted at 6h intervals after inoculation with *G.cingulata* were used in ELISA. Absorbance values in infected extracts were higher than those of healthy

Table 37: ELISA values obtained by reaction of anti *G.cingulata* antiserum (cell wall) and tea leaves after different hours of inoculation with *G.cingulata.*

Hours after inoculation	Anti *G.cingulata* antiserum (1:250 dilution)			
	B 157[a]		BT-15[a]	
	H	I	H	I
6	0.672 ± 0.021	0.850 ± 0.009	0.750* ± 0.025	0.861 ± 0.016
12	0.663 ± 0.059	0.853 ± 0.022	0.756* ± 0.031	0.846 ± 0.029
18	0.701 ± 0.042	0.923 ± 0.034	0.711 ± 0.034	1.050 ± 0.017
24	0.785 ± 0.022	0.962 ± 0.032	0.752 ± 0.018	1.069 ± 0.009
30	0.750 ± 0.008	0.983 ± 0.052	0.711 ± 0.002	1.092 ± 0.060
36	0.732 ± 0.001	0.995 ± 0.051	0.709 ± 0.009	1.099 ± 0.029
42	0.783 ± 0.030	1.016 ± 0.039	0.803 ± 0.021	1.116 ± 0.032
48	0.772 ± 0.034	1.082 ± 0.034	0.761 ± 0.029	1.136 ± 0.026
54	0.762 ± 0.022	1.159 ± 0.008	0.742 ± 0.007	1.212 ± 0.021
60	0.801 ± 0.021	1.185 ± 0.016	0.750 ± 0.012	1.229 ± 0.006
66	0.750 ± 0.034	1.232 ± 0.012	0.750 ± 0.015	1.232 ± 0.014
72	0.795 ± 0.031	1.332 ± 0.019	0.762 ± 0.025	1.250 ± 0.016
78	0.782 ± 0.056	1.350 ±0.009	0.755 ± 0.032	1.261 ± 0.025
84	0.800 ± 0.021	1.369 ± 0.023	0.732 ± 0.039	1.251 ± 0.009
90	0.750 ± 0.021	1.400 ± 0.032	0.716 ± 0.024	1.226 ± 0.021
96	0.762 ± 0.003	1.342 ± 0.009	0.800 ± 0.026	1.200 ± 0.029

a Leaf antigen used at 40 μg/ml concentration.

* Difference in values between healthy and inoculated extracts not significant at p=0.01 ; all the rest significant.

± Standard error.

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens (B-157) at different periods

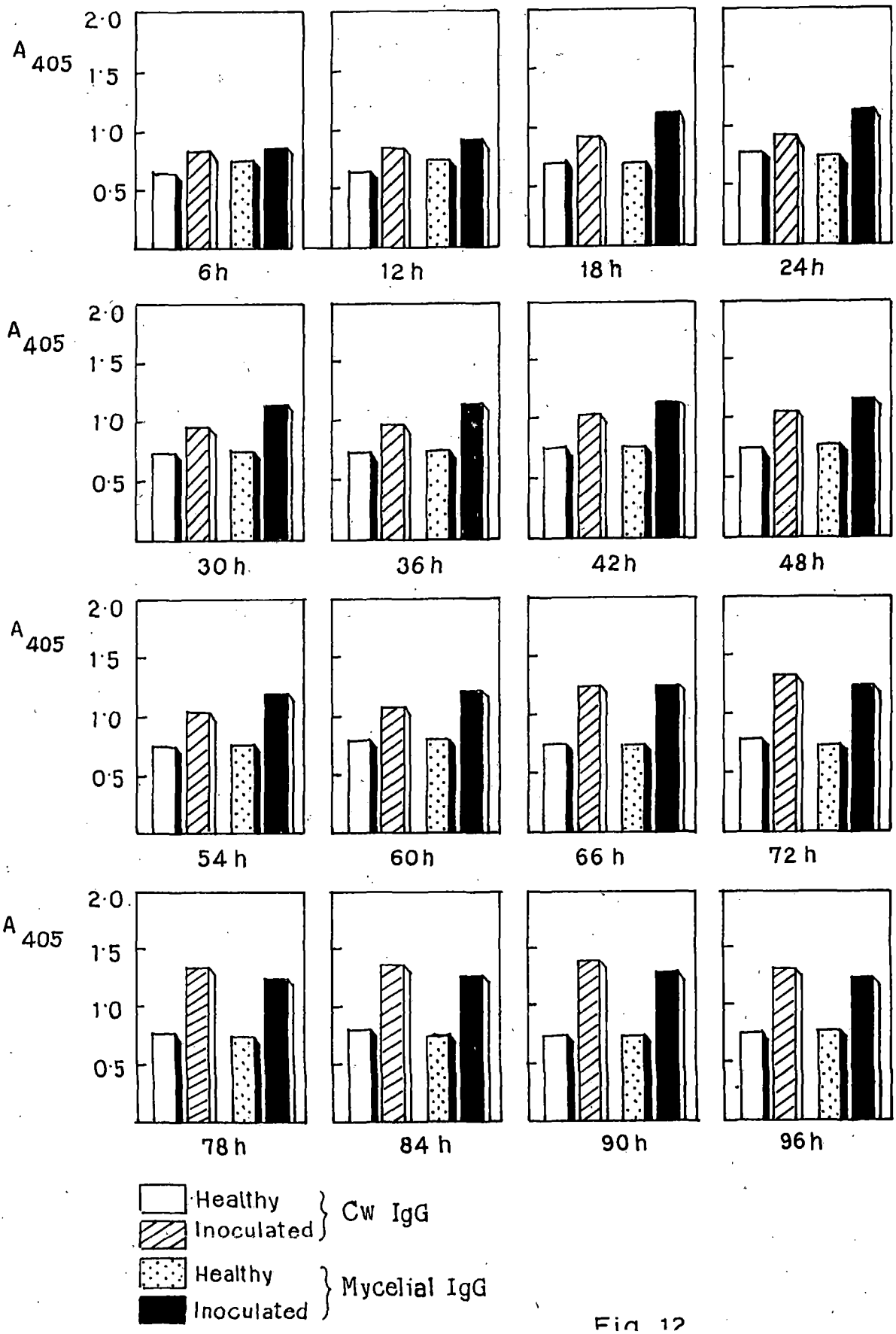


Fig. 12

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens (BT-15) at different periods

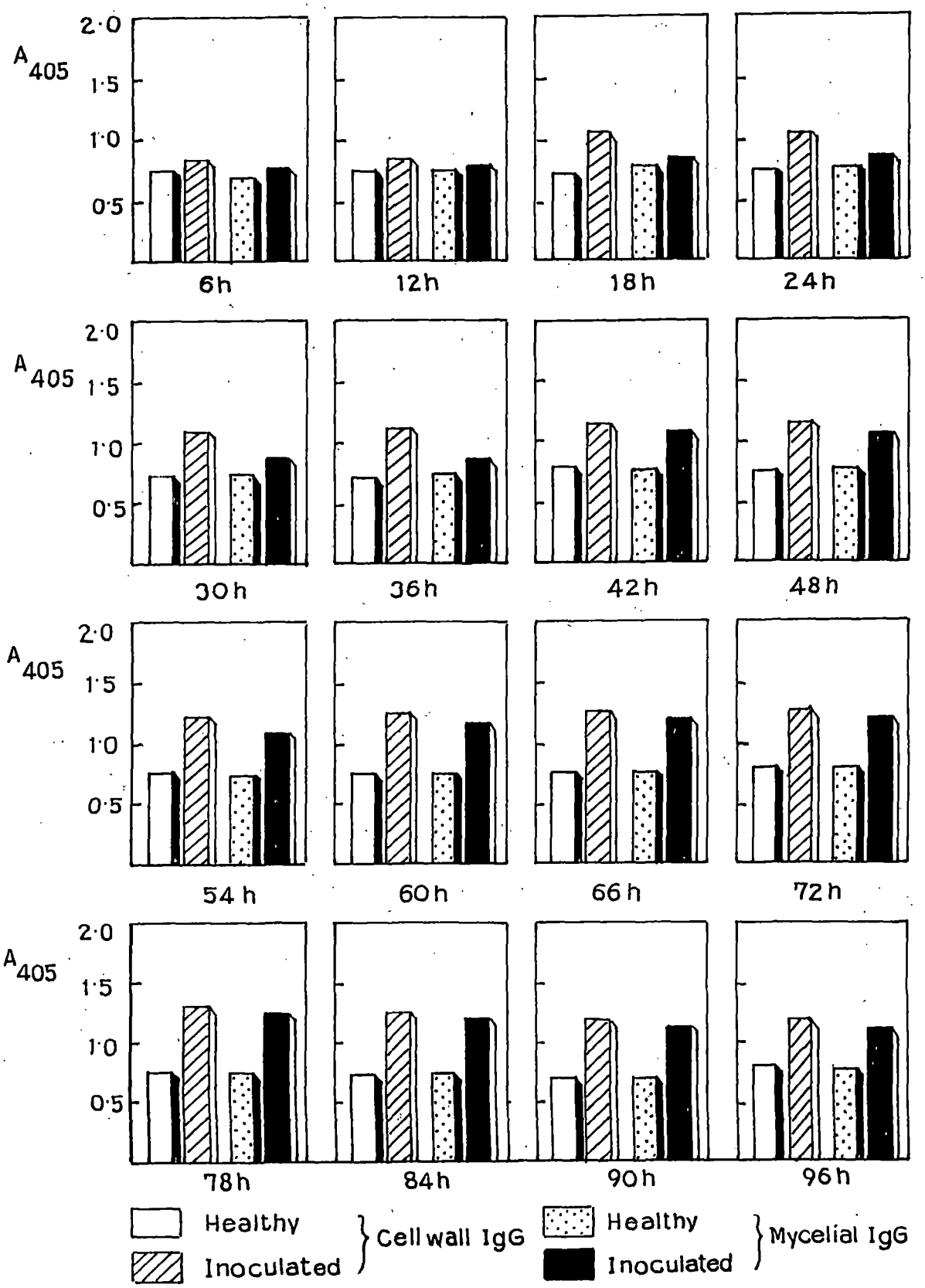


Fig. 13

extracts. In B157 the difference in values between infected and healthy extracts were significant at p=0.01 from 6h of inoculation onwards. In case of BT-15 such statistically significant difference was obtained from 18h onwards. (Table-37)

The above results indicate that *G.cingulata* can be detected in the susceptible variety as early as 6h of inoculation using antiserum raised against either mycelial or cell wall preparation. (Fig.12). In the resistant variety antiserum raised against cell wall preparation could detect *G.cingulata* from 18h onwards while antiserum raised against mycelial preparation could detect *G.cingulata* only after 30h of inoculation. (Fig. 13)

4.4.4. Different antigen concentration

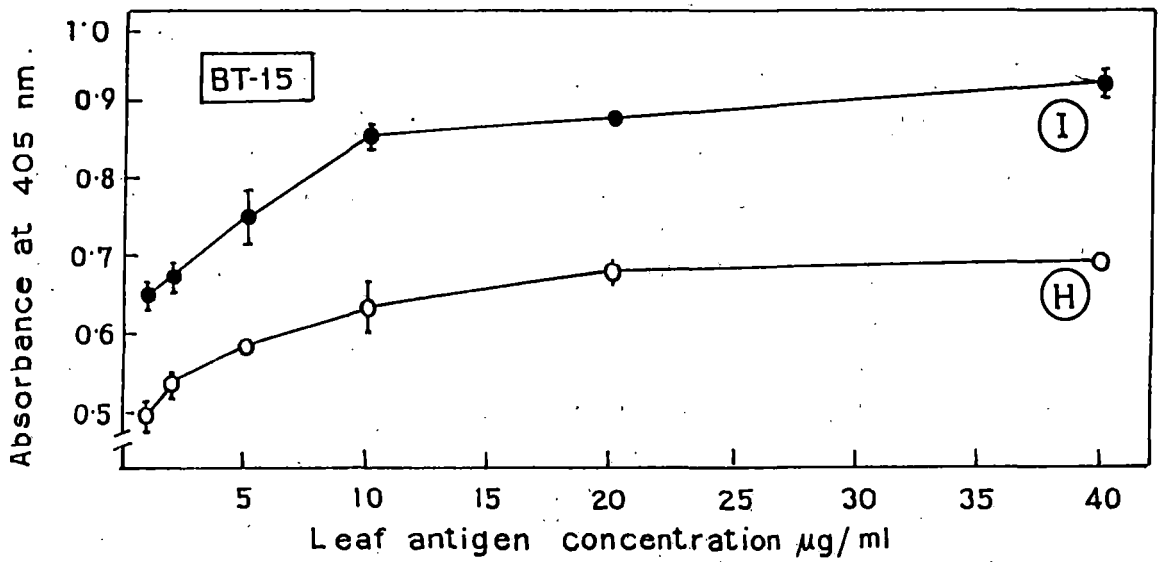
After determining the earliest period after inoculation when infection could be detected in leaf tissues it was decided to determine the lowest concentration of infected leaf extract in which infection could be detected using antiserum raised against mycelial antigen of *G.cingulata*. Infected leaf extract of two varieties (B 157 and BT-15) at dilution ranging from 40-1 μg/ml were tested in ELISA. Results (Table 38, Fig.14) revealed that differences in absorbance values between infected and healthy leaf extracts for both the varieties were significant at p=0.01 in all concentrations tested. Hence by ELISA *G.cingulata* could be detected in infected leaf extracts at a concentration as low as 1 μg/ml.

Table 38: ELISA response obtained with different concentrations of artificially inoculated leaf extracts and anti - GC antiserum.

Antigen concentration (μg/ml)	Absorbance of 405 nm			
	BT-15		B157	
	Healthy	Inoculated	Healthy	Inoculated
40	0.699±.006	0.954±.021	0.864±..012	1.159±.002
20	0.694±.017	0.854±0.008	0.840±.009	1.093±.009
10	0.634±.042	0.851±0.016	0.740±.017	1.000±.016
5	0.586±.003	0.757±.0.042	0.667±.021	0.863±.022
2	0.565±.017	0.671±.0 021	0.653±.022	0.807±.026
1	0.500±.019	0.654±.0235	0.542±.022	0.719±.031

Anti *G.cingulata* antiserum used at a dilution of 1:250. ; ± Standard error.
Difference between healthy and infected leaf extracts significant at p=0.01 at all concentrations

Effect of dilution of healthy and inoculated tea leaf antigens on ELISA responses with anti - *G.cingulata* antiserum



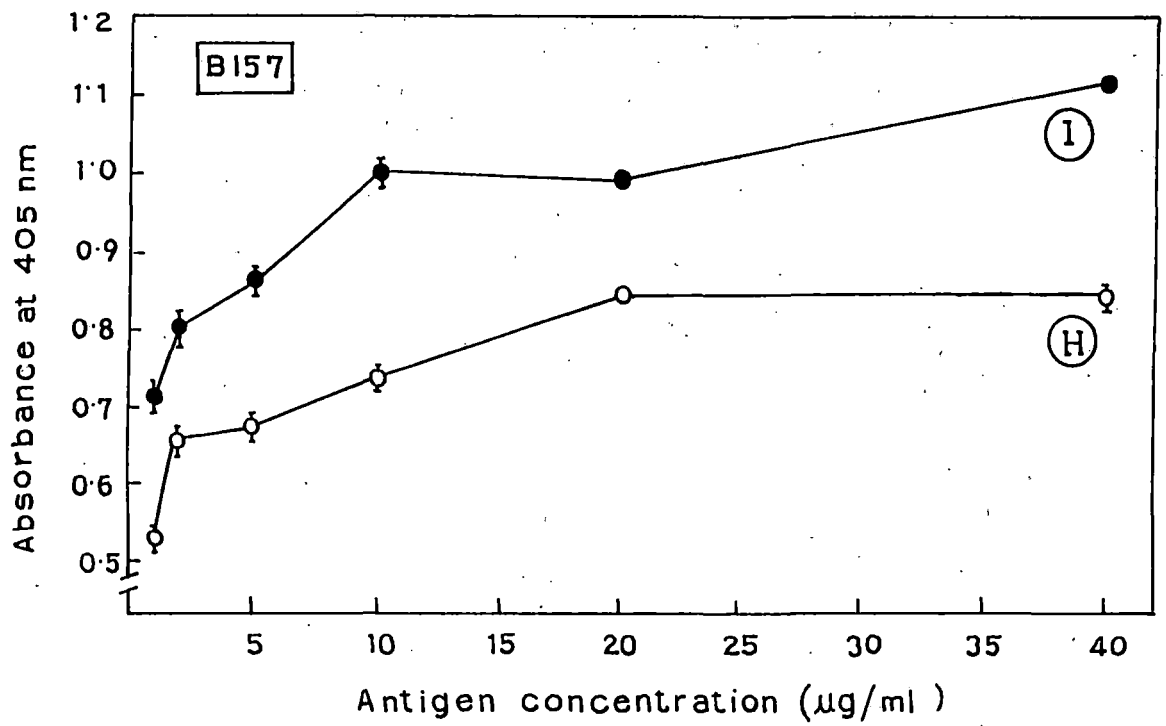


Fig. 14

4.5. Detection of infection with other pathogens using anti *G.cingulata* antiserum.

It has been observed that under field condition brown blight of tea caused by *G.cingulata* is sometimes associated with other diseases such grey blight caused by *Pestalotiopsis theae* or black rot caused by *Corticium invisum.* It was therefore, decided to test whether antiserum raised against *G.cingulata* could detect other infection. For this, two types of ELISA reactions were tested by DAC-ELISA and competition ELISA.

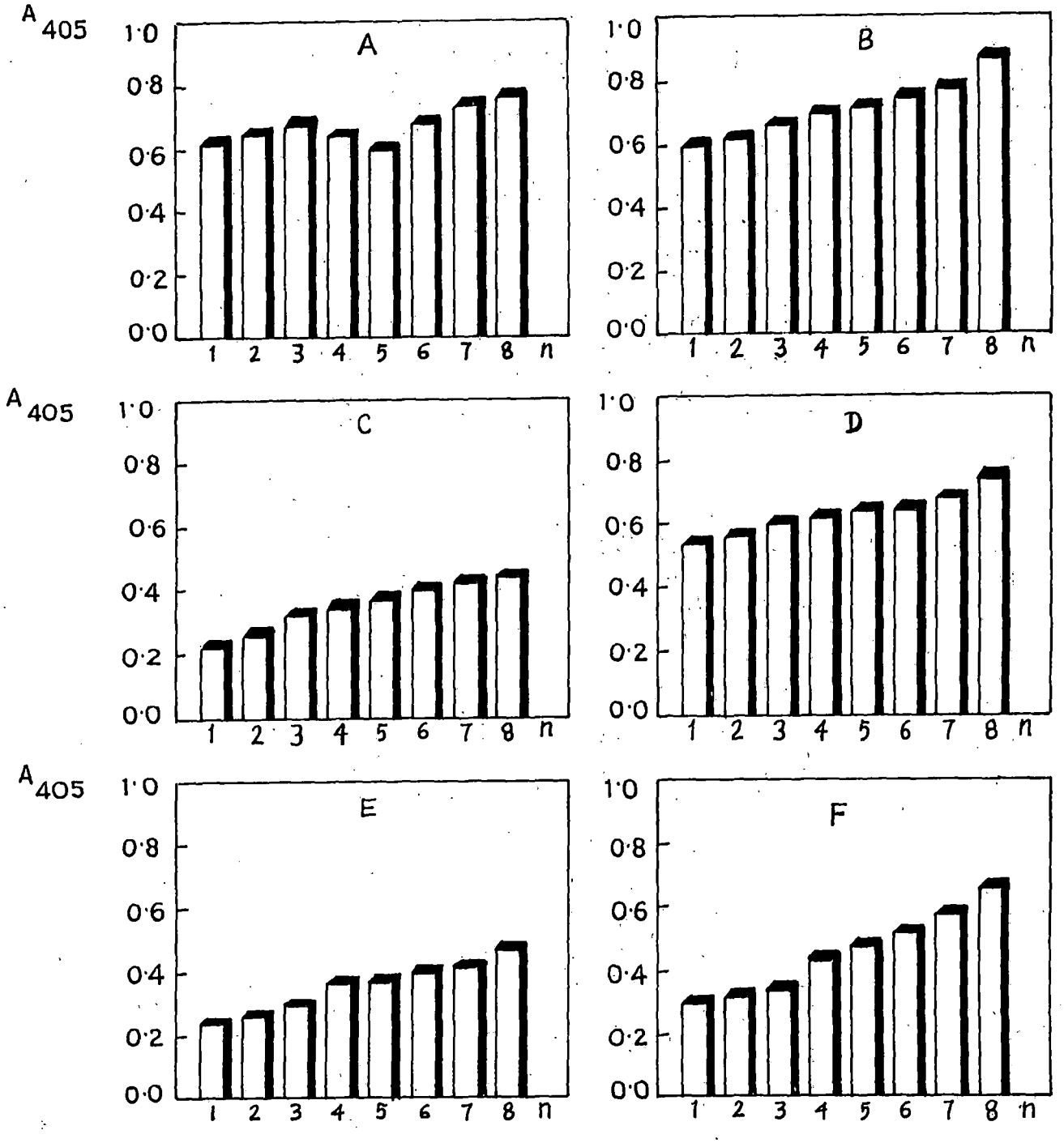
4.5.1 DAC - ELISA

Tea leaves of two varieties (AV-2 and HV-39) were inoculated with *G.cingulata, P.theae* and *C.invisum* separately and antigens were extracted after 72h of inoculation. These antigens along with corresponding healthy leaf antigens were tested against anti *G.cingulata* antiserum in DAC - ELISA. Two antigen concentrations (40 and 20μg/ml) were used and antiserum was used at a dilution of 1:250. Absorbance values in extracts of leaves inoculated with *G.cingulata* were higher than those with the other two pathogens. Significantly higher values for infected extracts in comparison to healthy extracts were only obtained when leaves were inoculated with *G.cingulata.* However, anti *G.cingulata* antigen did show a certain amount of reactivity with the extracts of leaves inoculated with other pathogens which was more or less similar to that obtained with healthy leaf extracts (Table-39).

4.5.2 Competition ELISA

One modification of the commonly used DAC-ELISA is the competition or inhibition ELISA which can detect particular antigens from mixed infections at very low concentrations. Since very often brown blight occurs as part of mixed infection specific detection of this pathogen using anti *G.cingulata* antiserum sometimes becomes difficult. In the previous experiment it was demonstrated that anti *G.cingulata* antiserum did not react significantly with antigen of leaves inoculated with other pathogens. However, such clear cut significant differences is not obtained in case of mixed natural infection. To detect the specific pathogen from mixed infection it was decided to use the competition ELISA format. Detailed procedure for competition ELISA has been outlined in Materials and Methods.

Absorbance values of doubling dilutions of healthy and infected tea leaf (UPASI-3) antigens using competition ELISA



n = antigen dilution from 1:100 to 1:12800
Left column - G. cingulata antiserum
Right column - P. theae antiserum

Fig·15

A – B = healthy leaf antigens
C – D = naturally infected leaf antigens
E – F = artificially infected leaf antigens

Table 39: Reaction of anti *G.cingulata* antiserum with antigens of tea leaves infected with other pathogens.

Tea varieties	Pathogen	Absorbance of 405 nm	
		40 μg/ml	20 μg/ml
AV-2	Uninoculated	0.660±0.031	0.418±0.012
	G.cingulata	0.960±0.031	0.718±0.029
	P.theae	0.643±0.022	0.389±0.009
	C.invisum	0.584±0.029	0.373±0.026
HV-39	Uninoculated	0.600±0.012	0.402±0.021
	G.cingulata	0.900±0.029	0.720±0.025
	P.theae	0.573±0.003	0.487±0.043
	C.invisum	0.543±0.016	0.459±0.061

Anti *G.cingulata* antiserum used at a dilution of 1:250.

± Standard error.

Results of competition ELISA are presented in (Table 40, Fig.15). Antigens of healthy tea leaves (UPASI-3), tea leaves artificially inoculated with *G.cingulata* and *P.theae* and naturally infected tea leaves were tested against anti *G.cingulata* and anti *P.theae* antisera. Test plates were initially bound with antigens of the two pathogens separately. Doubling dilution of leaf antigens ranging from 1:100 - 1:12,800 were used for binding with the two antisera. ELISA values decreased with increasing reactivity as this was a competition ELISA. Positive reactions were recorded when the absorbance value of 1:100 was less than approximately 50% of the value for the extract diluted 1:12,800. Positive reactions on the above basis was recorded in case of both naturally infected leaf extracts as well as leaves artificially inoculated with *G.cingulata* using anti *G.cingulata* antiserum. Positive responses were not recorded in healthy leaf extracts using either of the two antiserum or naturally infected extracts with anti *P.theae* antiserum. The presence of *G.cingulata* as the major pathogen in the natural infection was therefore confirmed by competition ELISA.

Table 40: Absorbance values of doubling dilutions of healthy and inoculated tea leaf antigens in competition ELISA.

Leaf antigen[a]	Antigen dilution	Antiserum (1:125 dilution)	
		G.cingulata	*P.theae*
Healthy	1:100	0.619	0.601
	1:200	0.669	0.618
	1:400	0.685	0.661
	1:800	0.647	0.688
	1:1600	0.602	0.717
	1:3200	0.672	0.747
	1:6400	0.738	0.782
	1:12800	0.770	0.867
Naturally infected			
	1:100	0.224	0.555
	1:200	0.274	0.579
	1:400	0.303	0.599
	1:800	0.342	0.622
	1:1600	0.366	0.635
	1:3200	0.397	0.633
	1:6400	0.418	0.696
	1:12800	0.433	0.756
Artificially inoculated[b]			
	1:100	0.238	0.309
	1:200	0.265	0.313
	1:400	0.298	0.353
	1:800	0.366	0.440
	1:1600	0.374	0.480
	1:3200	0.400	0.510
	1:6400	0.420	0.587
	1:12800	0.464	0.661

a Variety UPASI-3 ;

b Inoculated with *G.cingulata*

4.6. Determination of cross-reactivity of anti *G.cingulata* antiserum.

Cross reactivity of the antiserum raised against *G.cingulata* (isolate GC-1) was tested against 8 other isolates (isolates GC 2-9). Since *Glomerella cingulata* is a telomorph of *Colletotrichum gloeosporoides* it was decided to test the cross reactivity of the antiserum raised against *G.cingulata* with other species of *Colletotrichum* (2 strains of *C.gloeosporiodes,* 2 strains of *C.lindemuthianum* and one species of *C.papayae*). Cross reactivity was also tested against other foliar pathogens of tea i.e. *P.theae* and *C.invisum.*

For cross reactivity tests, antigens were prepared from the mycelia of all the above species and isolates and were tested against anti *G.cingulata* antiserum. Results revealed that among the different isolates of *G.cingulata* tested GC-5 showed 91% homology with GC-1. Of all the isolates minimum homology of 35% was exhibited by GC-8 (Table-41). Hence, all the isolates showed some homology with the isolate GC-1. In reactions involving other species of *Colletotrichum* homology ranged from 27-35% (Table-42). Hence, the different species did not cross react with the antiserum raised against *G.cingulata.* Antigens of *P.theae* and *C.invisum* exhibited homologies of 28 and 11% respectively.

Table 41: ELISA values showing reaction of antiserum of *Glomerella cingulata* (GC-1) at 1:250 dilution, with antigens of other isolates of *G.cingulata* (GC 1 to 9)

Pathogen	Isolate	Absorbance at 405 nm			
		Expt.1	Expt.2	Expt.	Mean
Glomerella cingulata	GC-1	1.894	1.899	1.920	1.904(100%)
	GC-2	0.703	0.729	0.729	0.720(37.9%)
	GC-3	1.038	1.084	1.076	1.066(55.0%)
	GC-4	1.245	1.209	1.243	1.232(64.7%)
	GC-5	1.744	1.715	1.740	1.733(91.0%)
	GC-6	0.817	0.854	0.866	0.845(44.3%)
	GC-7	1.220	1.231	1.263	1.238(65.0%)
	GC-8	0.681	0.640	0.694	0.671(35.24%)
	GC-9	0.959	0.955	0.959	0.954(50.10%)

Values in parenthesis represents homology as a percentage of that of *G.cingulata* (GC-1) which is designated as 100.

Anti *G.cingulata* antiserum used at a dilution of 1:250.

Table 42: ELISA reaction of different species of genus *Colletotrichum* against antisera of GC-1 (IgG 1:250).

Pathogens	Absorbance values at 405 nm			
	Expt.1	Expt.2	Expt.3	Mean
Colletotrichum gloeosporioides (1809)	0.505	0.502	0.511	0. 506(27.26%)
C.gloeosporioides(1726)	0.604	0.598	0.627	0.609(32.8%)
C.lindemuthianum(1119)	0.643	0.662	0.667	0.660(35.5%)
C.lindemuthianum(1764)	0.501	0.506	0.505	0.504(27.15%)
C.papayae (1269)	0.672	0.634	0.664	0.656(35.34%)
G.cingulata	1.916	1.829	1.823	1.856(100%)
P.theae	0.521	0.506	0.554	0.527(28.39%)
C.invisum	0.198	0.244	0.184	0.208(11.20%)

Values in parenthesis represents homology as a percentage of that of *G.cingulata* (GC-1) which is designated as 100.

Anti *G.cingulata* antiserum used at a dilution of 1:250.

4.7 Estimation of mycelia in infected tea leaf tissues

The measurement of fungal growth in tissues cannot be effectively done by direct methods like quantitative microscopy. A recent approach to the estimation of fungal biomas is the use of serological techniques such as ELISA. In the present study, initially a standard curve of known mycelial fresh weight of *G.cingulata* against absorbance value in ELISA was prepared. Mycelial growth in leaf tissues were estimated from the standard curve.

4.7.1 Different hours after inoculation

In order to estimate the growth of *G.cingulata* in tea leaves after different hours of inoculation, ELISA was carried out using healthy and inoculated tea levves of 2 varieties (B 157 and BT-15). In case of inoculated tea leaves antigens were prepared after every 6h of inoculation till 96h. The antigens were used at a concentration of 40 μg/ml against anti *G.cingulata* antiserum at 1:250 dilution. Results revealed that the absorbance values of infected leaf extracts were always higher than those of corresponding healthy extracts (Fig. 16). Difference in ELISA values between healthy and infected extracts were plotted on the previously prepared standard curve and the mycelial fresh weight was determined in each case (Fig. 17). Results revealed that in the susceptible variety (B 157) mycelial fresh wt. within the leaf tissue increased till 90h of inoculation. However, in the resistant variety (BT-15) mycelial fresh weight decreased after 78h (Table-43).

4.7.2. Different varieties

Fungal biomass was also determined in infected leaf extracts of different tea varieties from the standard curve as mentioned above. Results (Table 44) revealed that the susceptible varieties showing higher percentage of lesion production had maxinum fungal biomass within the tissue. Among the 15 varieties tested (UPASI-2, 3, 8, 9 and 26; TV-18, 23, 26, 29 and Teenali 17/1/54; B668, T-78, AV-2, CP-1, BS/7A/76), after 72 h of inoculation, UPASI-9 showed maximum lesion production of 67%. Maximum mycelial fresh weight as determined by ELISA was also in this variety (2.5 mg/g leaf tissue). Varieties showing low percentage of lesion production (5 %) also had lower mycelial fresh weight (0.85 mg/g leaf tissue).

Table 43: Estimation of biomas s of *G. cingulata* by ELISA within tea leaf tissues after different hours of inoculation.

Hours after inoculation	Mycelial fresh wt. (mg/g)	
	B 175	BT-15
6	0.006	0.004
12	0.186	0.118
18	0.483	0.514
24	0.651	0.669
30	1.760	2.287
36	1.767	2.732
42	2.349	2.943
48	2.609	3.193
54	2.796	3.565
60	3.385	3.664
66	4.166	3.742
72	4.259	3.918
78	4.823	4.550
84	6.088	4.283
90	6.621	4.147
96	6.503	3.961

Table 44: Percentage lesion production and growth of mycelia in different varieties of tea leaves inoculated with *G. cingulata.*

Tea varieties	72h after inoculation	
	Percent lesion production (%)	mycelial fresh weight mg/g leaf tissue[a]
Tocklai varieties		
Teenali 17/1/54	49.2	2.16
TV-18	60.0	2.02
TV-23	43.2	1.78
TV-26	29.9	0.09
TV-29	33.0	1.02
Darjeeling varieties		
B668	43.2	1.05
T-78	60.1	2.28
AV2	35.0	1.05
CP1	21.0	1.22
BS/7A/76	54.4	1.07
UPASI varieties		
UPASI-2	4.8	0.84
UPASI-3	62.4	1.94
UPASI-8	51.0	1.04
UPASI-9	66.7	2.05
UPASI-26	63.4	2.03

[a] Determined on the basis of ELISA response

ELISA reactions of anti-*G.cingulata* antisera with healthy and inoculated tea leaf antigens at different periods

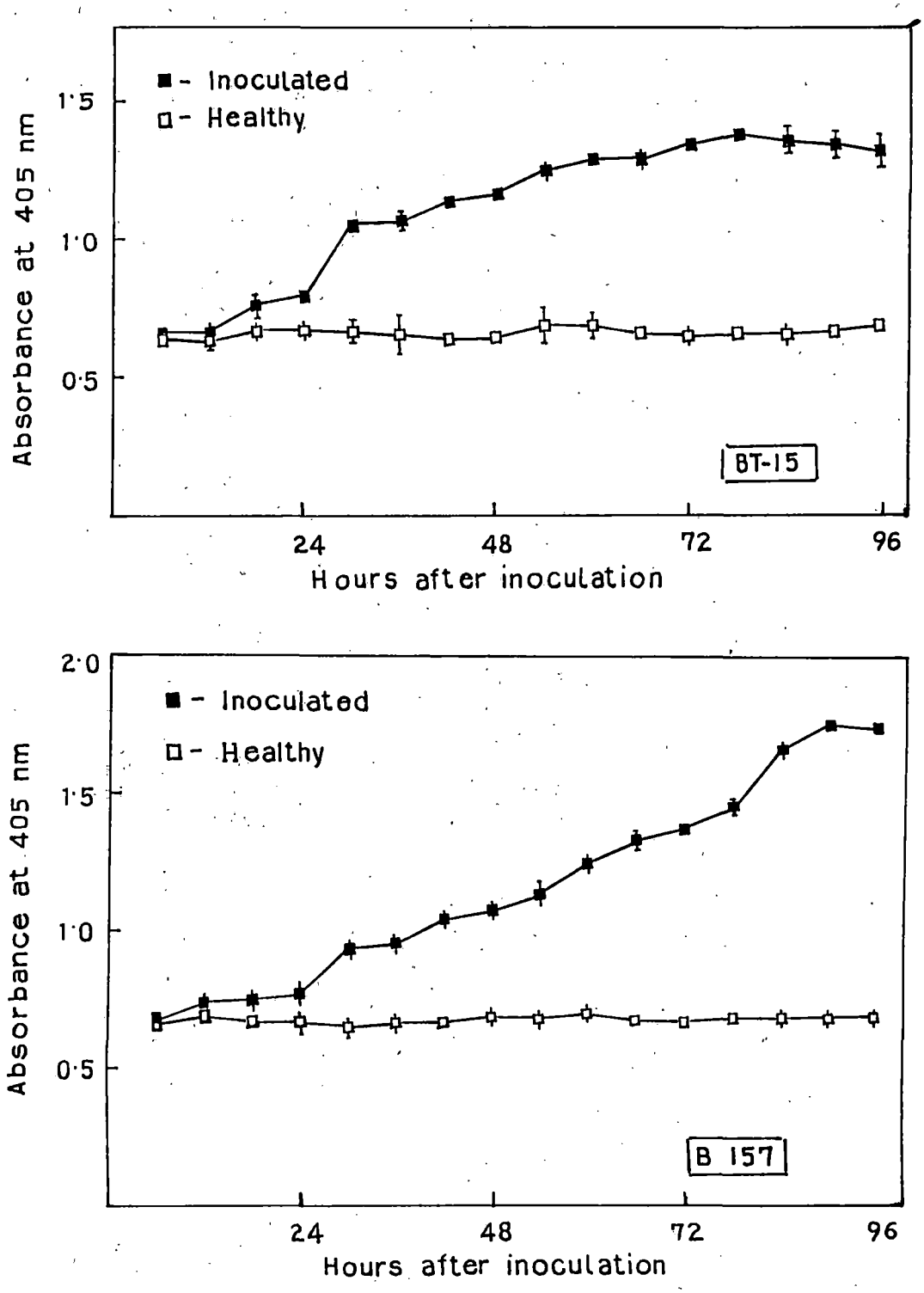


Fig. 16

Fungal biomass in tea leaves after different hours of inoculation with *G.cingulata*

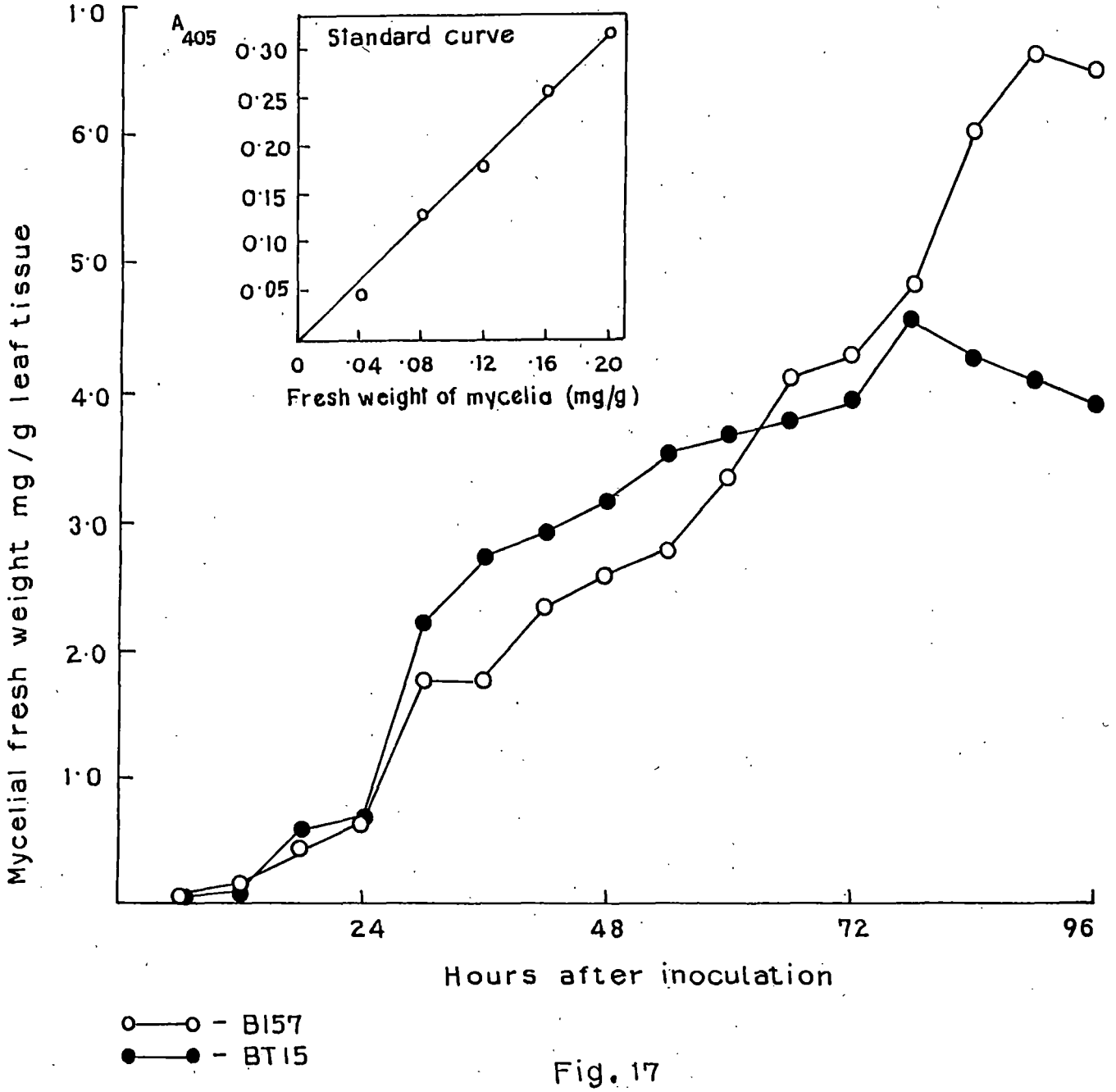


Fig. 17

4.8. Purification of cross reactive antigen (s) from mycelia of *G. cingulata.*

Having established that cross reactive antigen is present in *G. cingulata.* it was decided to purify the protein (s) from the crude extract which are actually responsible for the antibody production. A series of steps outlined in Materials and Methods were performed for this purification.

4.8.1. Ammonium sulphate fractionation

Initially from the crude mycelial protein preparation of *G. cingulata* ammonium sulphate fractionation were carried out. Each ammonium sulphate fraction after centrifugation and dialysis was tested by agar gel double diffusion and indirect ELISA, against homologous (anti *G. cingulata* antiserum) and heterologous antisera. The heterologous antiserum was raised against a susceptible tea variety, TV-18.

4.8.1.1. Agar gel double diffusion

The different ammonium sulphate saturated (0-20%, 20-40%, 40-60%, 60-80% and 80-100%) fraction were tested for cross reactivity by agar gel double diffusion tests. Tests involving antisera of either *G. cingulata* or TV-18 showed common precipitin bands with the crude extract and 80-100% saturated fraction (Plate VIII, figs. A & C). No precipitin bands were detect with any of the other fractions.

4.8.1.2. ELISA

To further confirm the result of agar gel double diffusion tests ELISA were performed with the different fractions and the two antisera as mentioned above. Results revealed that in both homologous and heterologous combinations 80-100% fraction gave highest absorbance values which was comparable to the value obtained with the total protein extract (Table-45).

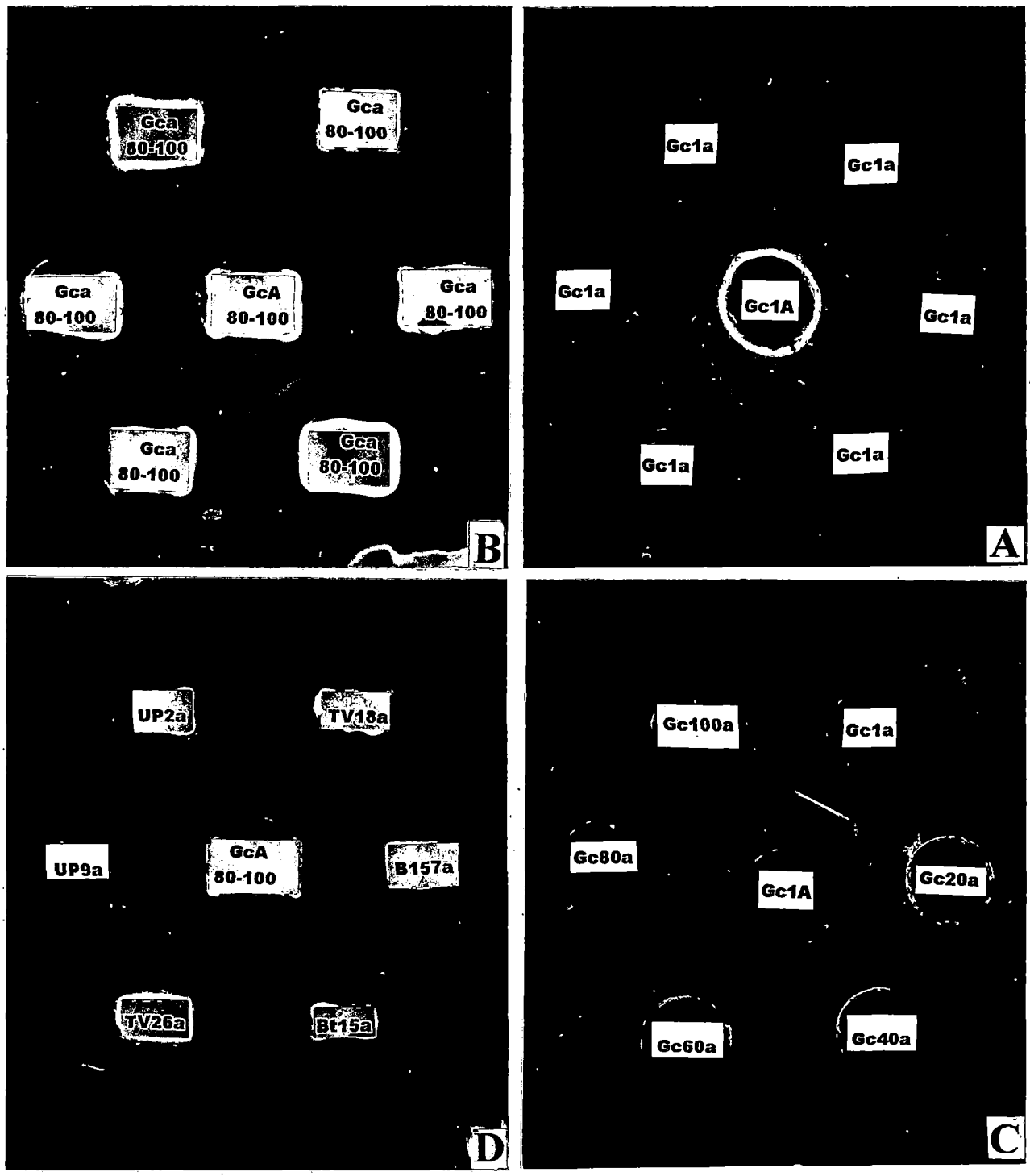


PLATE VIII (figs.A-D) . Agar gel double diffusion tests.Central wells contain antisera to crude **(A & C)** and partially purified **(B&D)** mycelial antigen (80-100% SAS) of *G.cingulata* . Peripheral wells contain *G.cingulata* crude antigen **(A)** , 80-100% SAS fraction **(B)** , ammonium sulphate fraction **(C)** and tea leaf antigens **(D)**

4.8.2. DEAE - Sephadex chromatography

Since both agar gel double diffusion and ELISA tests revealed that the major cross reactive antigens were present in 80-100% SAS of *G. cingulata* this fraction was further purified by DEAE - Sephadex chromotography. Details of the procedure used for the purification have been given in Materials nad Methods. Twenty fractions each were collected from the three buffers (0.05 MTris-HCl, 0.05 M NaCl in 0.05 M Tris-HCl and 0.05 M to 0.25 M NaCl in 0.05 M tris-HCl). Absorbance values of all the fractions were taken at 280nm and these were plotted on a graph paper. Analysis of the OD values revealed three distinct peaks corresponding to the three buffer systems (Fig. 18). In the third buffer the OD values were much lesser than the previous two.

4.8.2.1. DAC - ELISA

Indirect ELISA was performed with all the fractions obtained from DEAE Sephadex chromotography and antisera of *G. cingulata* and TV-18. When tested against antiserum of *G. cingulata* high absorbance were obtained in four fractions (5,6,7,8) of the second buffer system (0.05 M NaCl in 0.05 M Tris-HCl). Readings in all other fractions were insignificant (Table-46). Similar results (Table-47) were obtained against antiserum of TV-18 but in this case high absorbance values were recorded in two-5 and 6 (Table-47) fractions. These two purified fractions therefore contained the major cross reactive antigens.

4.8.2.2. SDS - PAGE

In order to determine the molecular weight of the purified antigen SDS-PAGE was performed as described previously. The antigenic fractions were pooled and run along with 80-100% SAS protein as well as crude protein extract and a set of molecular markers. Following electrophoresis, staining and destaining revealed a prominent band. This corresponded to a molecular weight of approximately 10 KDA (Fig. 19). Hence the antigenic protein seems to be a low molecular weight protein.

Elution pattern of 80-100% SAS fraction of *G.cingulata* antigen in different buffers by DEAE-Sephadex column chromatography

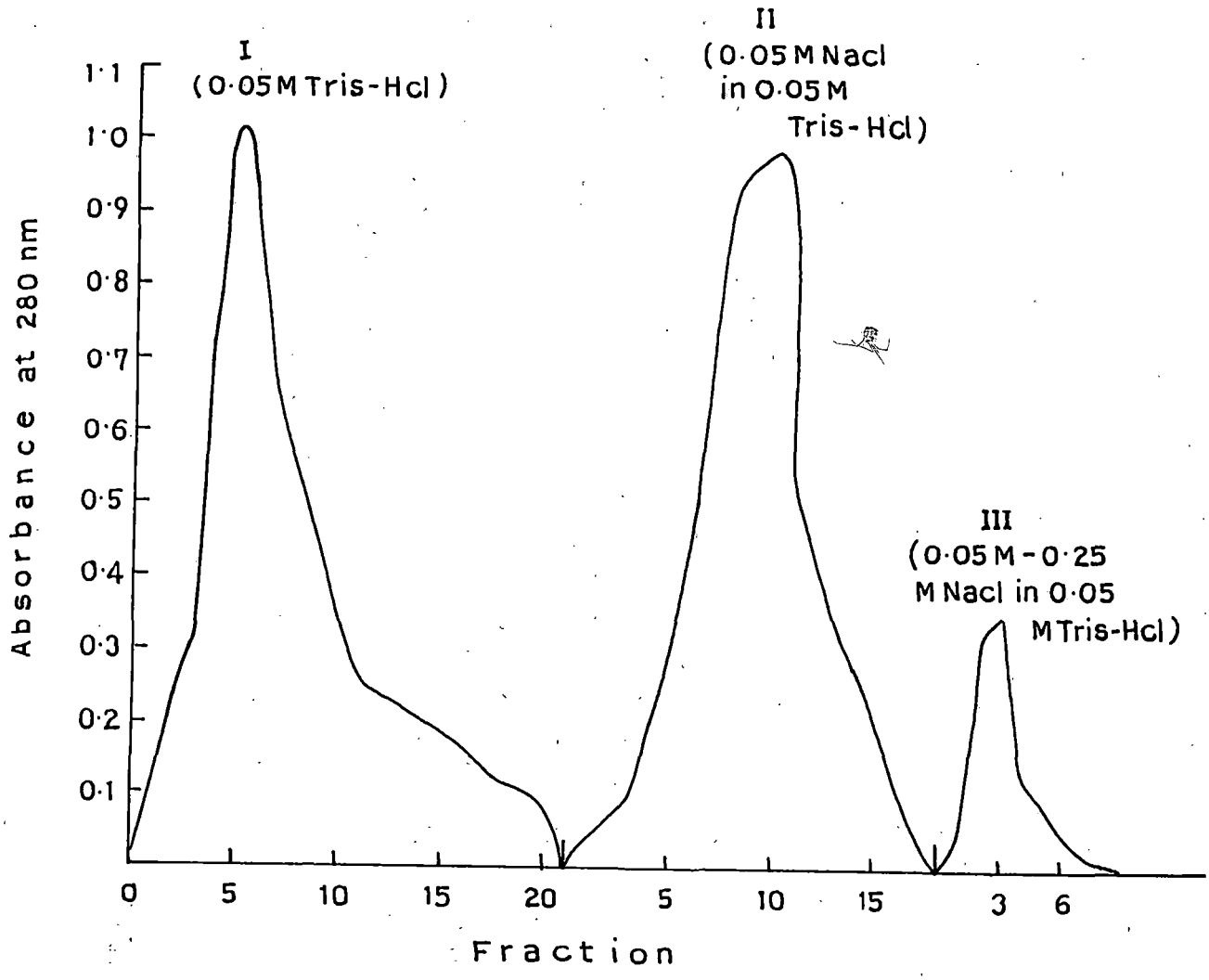


Fig. 18

Table 45: Absorbance values obtained in ELISA reactions of ammonium sulphate saturated fraction of *G.cingulata* antigen with anti *G.cingulata* and anti TV-18 antiserum.

Antigen	Absorbance at 405 nm[c]	
	Antisera (1:250 dilution)	
	G.cingulata	TV-18
G.cingulata[a] fractions:		
0-20% SAS	0.684±0.003	0.593±0.031
20-40%	0.644± 0.017	0.469±0.041
40-60%	0.607±0.003	0.547±0.003
60-80%	0.726±0.009	0.707±0.047
80-100%	1.860± 0.007	1.356±0.013
100%	1.889±0.037	1.427±0.006
TV-18[b]	ND	1.648±0.070

SAS - Saturated ammonium sulphate precipitation

a Antigen concentration - 5 μg/ml

b Antigen concentration - 40 μg/ml

c Average of three experiments.

± Standard error.

Table 46: ELISA responses obtained for reactions of different fractions from DEAE Sephadex chromatography of partially purified *G.cingulata* antigen against anti *G.cingulata* antiserum.

Antigen fractions	Anti *G.cingulata* antiserum (1:250 dilution)		
	Absorbance at 405 nm		
	Buffer 1[a]	Buffer 2[b]	Buffer 3[c]
1.	ND	ND	ND
2.	0.366	0.360	0.349
3.	0.379	0.351	0.386
4.	0.374	0.347	0.360
5.	0.369	0.918	0.371
6.	0.386	1.040	0.327
7.	0.355	1.060	0.339
8.	0.397	0.829	0.342
9.	0.360	0.785	0.361
10.	0.375	0.685	0.356
11.	0.395	0.356	ND
12.	0.366	0.304	ND
13.	0.365	0.305	ND
14.	0.376	0.367	ND
15.	0.382	0.371	ND
16.	0.393	0.363	ND
17.	0.377	0.373	ND
18.	0.368	0.323	ND
19.	0.357	0.318	ND
20.	0.325	ND	ND

a 0.05 M Tris - HCl

b 0.05 M NaCl in 0.05 M Tris HCL

c 0.05 - 0.025 M NaCl in 0.05M Tris HCl.

ND - Not detected.

Table 47: ELISA responses obtained for reactions of different fractions from DEAE Sephadex chromatography of partially purified *G.cingulata* antigen against anti TV-18 antiserum.

Antigen fractions	Antisera dilution 1:250 (TV-18 IgG)		
	Absorbance at 405 nm		
	[a] Buffer 1	[b]Buffer 2	[c] Buffer 3
1.	0.341	0.386	0.392
2.	0.348	0.378	0.327
3.	0.373	0.362	0.342
4.	0.400	0.381	0.336
5.	0.370	0.941	0.361
6.	0.362	0.952	0.351
7.	0.424	0.317	0.362
8.	0.368	0.315	0.361
9.	0.370	0.335	0.351
10.	0.329	0.372	0.341
11.	0.340	0.396	ND
12.	0.321	0.366	ND
13.	0.322	0.362	ND
14.	0.335	0.354	ND
15.	0.352	0.350	ND
16.	0.363	0.325	ND
17.	0.331	0.320	ND
18.	0.395	0.311	ND
19.	0.395	0.312	ND
20.	0.393	0.319	ND

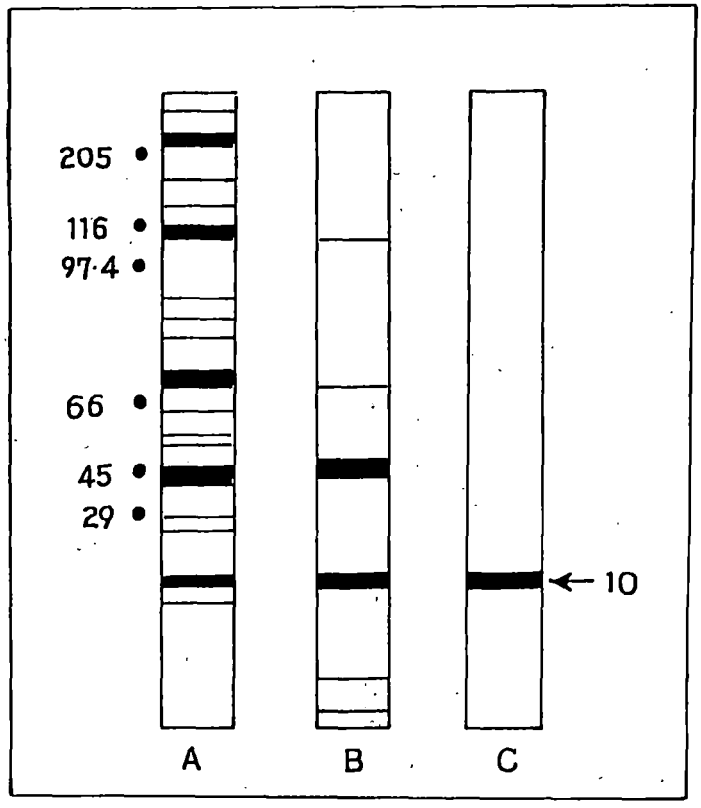
a 0.05 M Tris HCl

b 0.05 M NaCl in 0.05 M Tris-HCl

c 0.05-0.25M NaCl in 0.05 M Tris HCl.

ND Not detected

SDS-PAGE analysis of crude and purified mycelial antigen of *G.cingulata*



A = Crude mycelial antigen .

B = 80-100% SAS fraction .

C = Antigenic fraction from DEAE-Sephadex Column.

Fig. 19 .

4.9 Evaluation of antisera raised against purified mycelial antigens of *G.cingulata*

In the previous experiments antisera were raised against antigenic preparations from mycelia of *G.cingulata* and these were used for various tests. Subsequently from the crude mycelial preparations antigens were purified by ammonium sulphate fractionation and ion exchange chromatography. It was evident from immunodiffusion and ELISA tests that 80-100%. SAS fraction contained major cross reactive antigens. Hence, antiserum was raised against this fraction (80-100% SAS) following the protocol as described previously. This antiserum was further tested by immunodiffusion and ELISA against both homologous and heterologous combinations.

4.9.1. Immunodiffusion tests

In agar gel double diffusion tests the antiserum raised against 80-100% SAS was placed in the central well and in the peripheral well either homologous antigens (80-100% SAS precipitate) or heterologous antigens (TV-18, UPASI-9, B-157, UPASI-2, BT-15 and TV-26) were placed. Strong precipitation was observed in the homologous reactions (Plate VIII, fig.B). In reactions involving tea leaf antigens precipitation was evident against susceptible varieties (TV-18, UPASI-9 and B 157) while no such reactions occurred with the resistant varieties UPASI-2, BT-15 and TV-26 (Plate VIII, fig. D).

4.9.2. ELISA

The antiserum raised against 80-100% SAS was also tested by ELISA to determine whether it could detect CRA among *G.cingulata* and tea varieties and also whether infection could be detected by this antiserum. Both DAC and DAS ELISA was performed and results are given below.

4.9.2.1 Detection of CRA among selected tea varieties

Cross reactive antigens have been detected between tea varieties and *G.cingulata* by ELISA using purified antiserum (IgG) raised against whole mycelial antigen as described earlier. Since the antiserum raised against 80-100% SAS gave positive results in immunodiffusion tests they were further tested by ELISA. For ELISA the antisera were initially purified and the IgG was used.

4.9.2.1.1 DAC ELISA

Antigenic preparations from tea leaves (varieties Teenali 17/1/54, TV-18, 23, 26, CP-1), non hosts (*G.max, C.arietinum* and *C.japonicum*), non pathogen (*F.oxysporum*) as well as mycelia of *G.cingulata* (80-100%) were tested against the above antiserum by DAC ELISA. Experiments were repeated thrice and results were represented in Table-48. High absorbance values were noted in homologous reactions followed by reactions against susceptible varieties (TV-18, 23, Teenali 17/1/54). ELISA reactions with either non host or non pathogen were marked low. Thus, the antiserum raised against partially purified antigen could detect the presence of cross reactive antigens between host and pathogen.

4.9.2.1.2 DAS ELISA

Using similar antigenic preparations as in DAC ELISA, DAS ELISA was also performed to confirm the results obtained in DAC ELISA. Procedures for DAC ELISA have been outlined in Materials and Methods. In this case also absorbances were high in reactions with homologous antigens as well as with susceptible varieties (Table 49). Low absorbances were evident against non host and non pathogen.

4.9.2.2 Detection of *G.cingulata* in artificially inoculated tea leaves of selected varieties

The efficacy of the antiserum raised against 80-100% SAS fraction of *G.cingulata* mycelial antigen was further tested by its ability to detect the pathogen in infected tissues by ELISA. For this both DAC ELISA and DAS ELISA were performed and results are given below.

4.9.2.2.1 DAC ELISA

Antigenic preparations from healthy and inoculated tea leaves of 8 UPASI varieties (UPASI 1,2,3,8,9,26; BSS 1,2,3) were tested against the above antiserum. A_{405} values of inoculated extracts were significantly higher than the corresponding healthy extracts at p=0.01 (Table-50).

Table 48: Absorbance values in DAC ELISA for reactions of selected tea varieties with antiserum raised against 80-100% SAS fraction of *G.cingulata* mycelium.

Antigen	Absorbance at 405 nm[a]			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
Teenali 17/1/54	0.850	0.934	0.824	0.869±0.033
TV-18	1.030	1.129	1.018	1.059±0.035
TV-23	0.859	0.878	0.815	0.850±0.018
TV-26	0.613	0.624	0.598	0.611±0.007
CP-1	0.676	0.620	0.665	0.653±0.015
Pathogen				
G.cingulata	1.697	1.702	1.668	1.689±0.010
Non pathogen				
F.oxysporum	0.223	0.312	0.238	0.253±0.027
Non host				
C.japonicum	0.362	0.392	0.325	0.359±0.019
G.max.	0.334	0.337	0.286	0.319±0.016
C.arietinum	0.326	0.355	0.386	0.355±0.017

a Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

± Standard error

Table 49: Absorbance values in DAS ELISA for reactions of selected tea varieties with antiserum raised against 80-100% fraction of *G.cingulata* mycelium.

Antigen	Absorbance at 405 nm[a]			
	Expt.1	Expt.2	Expt.3	Mean
Tea varieties				
Teenali 17/1/54	1.139	1.128	1.099	1.112±0.011
TV-18	1.239	1.244	1.284	1.255±0.014
TV-23	0.940	1.032	1.005	0.992±0.027
TV-26	0.695	0.700	0.682	0.692±0.005
CP-1	0.710	0.721	0.749	0.726±0.011
Pathogen				
G.cingulata	1.408	1.457	1.437	1.434±0.014
Non pathogen				
F.oxysporum	0.305	0.210	0.287	0.267±0.029
Non host				
C.japonicum	0.389	0.382	0.384	0.385±0.002
G.max	0.368	0.407	0.324	0.366±0.024
C.arietinum	0.371	0.390	0.393	0.384±0.016

a Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

± Standard error

Table 50: Absorbance values in DAC ELiSA for reactions of antiserum raised against 80-100% SAS of *G.cingulata* mycelia with healthy and inoculated tea leaf antigens.

Antigen (40 μg/ml)	Absorbance at 405 nm[a,b,c]	
	Healthy	Inoculated*
UPASI-2	0.616±0.011	0.763±0.016
UPASI-3	0.685±0.009	1.090±0.015
UPASI-8	0.636±0.017	0.863±0.013
UPASI-9	0.738±0.012	1.142±0.029
UPASI-26	0.668±0.002	1.046±0.020
BSS 1	0.648±0.016	1.012±0.024
BSS 2	0.633±0.021	1.001±0.017
BSS 3	0.630±0.032	0.848±0.012

* 72h after inoculation

a Average of three experiments

b Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

c Difference between healthy and inoculated extracts significant at p=0.01 in all cases.

4.9.2.2.2 DAS ELISA

Healthy and infected tea leaf extracts were tested as in DAC ELISA mentioned above. Results of ELISA reactions revealed significantly higher absorbance values for infected extracts as compared to healthy extracts in all the varieties tested (Table - 51). Both immunodiffusion tests and ELISA revealed that antiserum raised against partially purified mycelial preparation (80-100% SAS) was as effective as the antiserum raised against whole mycelial preparation in detection of cross reactive antigens as well as infection.

Table 51: Absorbance values in DAS ELISA for reactions of antiserum raised against 80-100% SAS of *G.cingulata* mycelia with healthy and inoculated tea leaf antigen.

Antigen (40µg/ml)	Absorbance at 405 nm[a,b,c]	
	Healthy	Inoculated*
UPASI-2	0.526±0.003	0.653±0.012
UPASI-3	0.670±0.032	1.015±0.009
UPASI-8	0.569±0.015	0.826±0.021
UPASI-9	0.740±0.024	1.061±0.013
UPASI-26	0.612±0.031	0.823±0.017
BSS 1	0.606±0.017	0.816±0.016
BSS 2	0.601±0.021	0.802±0.022
BSS 3	0.593±0.032	0.693±0.035

* 72h after inoculation ; a Average of three experiments.

b Anti *G.cingulata* antiserum (raised against 80-100% SAS fraction) at 1:250 dilution.

c Difference between healthy and inoculated extracts significant at p=0.01 in all cases.

4.10. Characterization of mycelial and conidial wall of *G.cingulata*

Since, both cell walls of mycelia and conidia play important roles in host pathogen interaction phenomena and as earlier experiements also proved cell walls to be effective in raising antesera, it was decided to characterize the cell walls and to determine their chemical nature.

4.10.1. Mycelial wall

Cell walls were isolated from *G. cingulata* and the isolated cell walls were further extracted with NaOH as described earlier. Carbohydrate and protein content of cell wall preparations from *G.cingulata* were 11 mg/ml 9.25 mg/ml respectively. This preparation was further analysed by SDS-PAGE and confirmed by binding with fluorescein labelled concanavalin A.

4.10.1.1. ConA-FITC binding

In order to detect the nature of the cell walls, mycelia or isolated cell walls of *G.ingulata* were treated with FITC labelled conA and observed under the microscope as described under Materials and Methods. Strong fluorescence was observed under the microscipe in both mycelia and cell wall of *G.cingulata* (Plate IX, figs. A & B). The occurrence of conA binding substance in cell walls confirmed the glycoprotein nature of mycelial wall of *G.cingulata.*

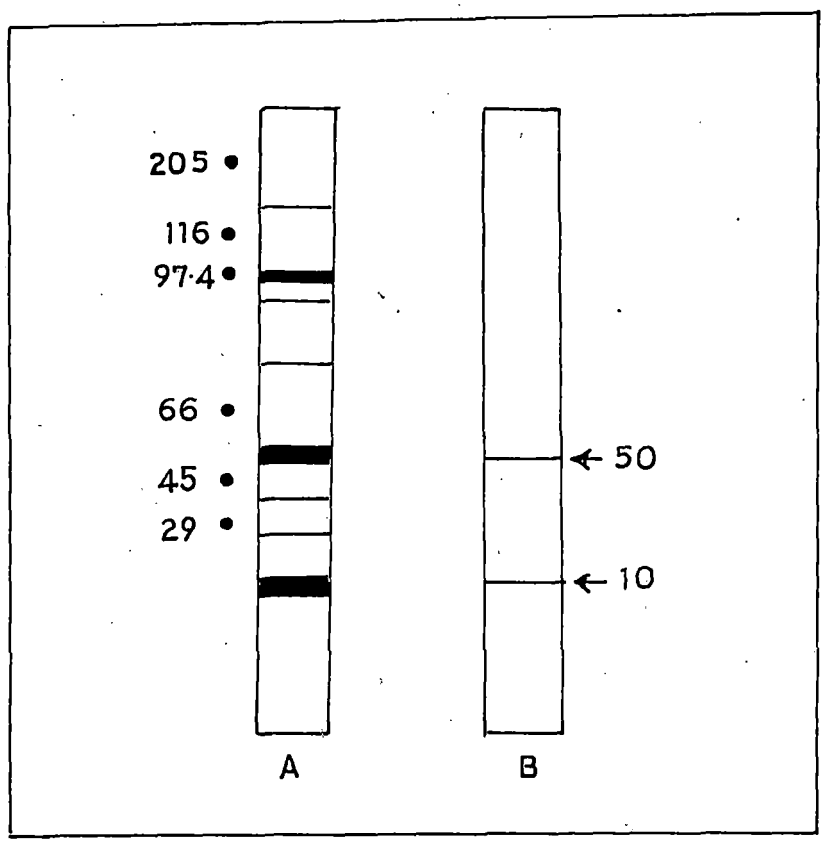
4.10.1.2. SDS-PAGE

The cell wall preparation of *G.cingulata* were further resolved in SDS-PAGE in order to confirm their glycoprotein nature. Gels were fixed in appropriate solution and stained either with coomassie blue R250 or with Periodic acid Schiff's reagent respectively for protein and carbohydrate detection. Staining of gels with coomassie blue exhibited eight protein bands. Carbohydrate staining on the other hand showed two bands at molecular weights 10 and 50 KD. These two bands coincided with two of the bands of proteins. The coincidence of coomassie blue and PAS staining indicated the presence of two glycoproteins of molocular weight of 10 and 50 KD in the mycelial wall extract of *G.cingulata* (Fig. 20).

4.10.2. Conidial wall

Since conidial wall plays an important role in fungal morphogenesis and development it may also be involved in recognition phenomena leading to host pathogen interaction. Lectiins are proteins of non immune origin which agglutinate cells and /or precipitate glycoconjugates by specifically inteacting with sugar moieties. Lectins have proved to be useful tools for identifying glycoconjugate components on cell surfaces. In the present investigation initially mycelial wall was characterized following which attempts were made to characterize then conidial wall by studying the agglutination effect of 7 lectins (Con A, HPA, UAE1, WGA) on conidia. Agglutination procedure has been

SDS-PAGE analysis of cell wall extract of *Glomerella cingulata*



A = Cell wall extract (protein)

B = Cell wall extract (carbohydrate)

Fig . 20 .

described in Materials and Methods. Agglutination were examineed and arbitrarily scaled from 0 to 4 (0 = no agglutination ; 1 = 1-25% ; 2 = 26-50% ; 3 = 51-75% ; 4 = 76-100% agglutination). Microscopical examination revealed that conidia were strongly agglutinated by conA and to slightly lesser degree by UAE 1. No agglutination was evident by HPA and WGA (Table 52, Plate X, figs. A-C). Con A interacts specifically with α-D mannosyl (and α-D glucosyl residues) while HPA interacts with N-acetyl α-D galactoseaminyl residues. Strong agglutination with conA suggests that the surface components are glycoconjugate containing α-D glucopyranoside and /or α-D mannopyranoside residues. Agglutination with UAE 1 lectins which has affinity for L-fucose indicated that residues of this sugar was also located on the surface of conidia.

Table 52 : Agglutination response of conidia of *G.cingulata* to different lectins

Name of lectins	**Agglutination extent of conidia of *G.cingulata* ***
Concanavalin A (conA)	4
Helix pomatia agglutinin (HPA)	0
Ulex europaeus agglutinin I (UEA 1)	3
Wheat-germ agglutinin (WGA)	0

* 0 - No agglutination
1 - 1-25%
2 - 26-50%
3 - 51-75%
4 - 76-100%.

PLATE IX (figs. A&B) . Fluorescence of hyphae (**A**) and isolated cell walls (**B**) of *G . cingulata* after staining with FITC - Con A

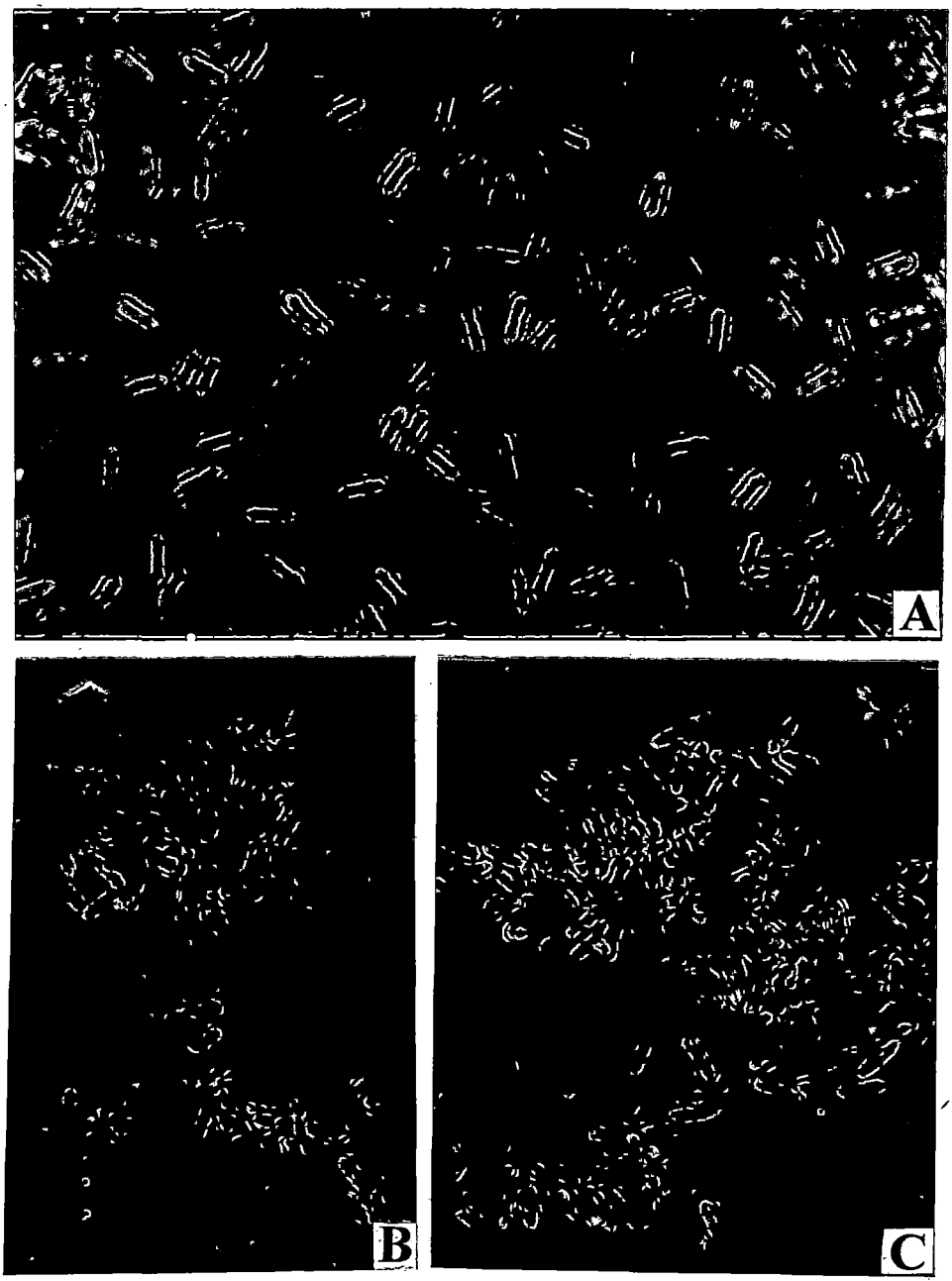


PLATE X (figs. A - C) . Agglutination of spores of *G.cingulata*;
A - control ; **B & C** - after treatment with Con A

4.11. Callus induction

Having established the presence of cross reactive antigens between tea leaves and *G.cingulata* it was decided further to detect the cellular location of the CRA by immunofluorescence test in leaf tissues, mycelia and conidia of *G.cingulata* as well as in loosened cells. For obtaining loosened cells, initially induction of callus from stem segments of susceptible variety (TV-18) was done. TV-18 was selected as it was one of the highly suceptible varieties and in field tests was the most suceptible one. It was observed that within 10 days of transferrring the stem segments to MS medium (as described in Materials and Methods) callus initiation started and well developed calli were, formed within one month (Plate XI, fig. A). Young calli thus formed were fragile and within a period of 7 days these were tranferred to liquid MS medium and agitated gently to obtained loosened cells.

4.12. Immunofluoresence

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 following immunodiffusion, immunoelectrophoresis, DAC-ELISA as well as DAS-ELISA the presence of CRA shared by *C. sinensis* and *G.cingulata* have been detected. It was decided to determine the tissue and cellular location of CRA in fragile callus and leaf tissues of tea varieties as well as mycelia and conidia of *G.cingulata* Detailed methods of antibody staining of leaf sections and fungal cells have already been discussed under Materials and Methods. Leaf sections and mycelial preparations were photographed under UV- fluoresence.

4.12.1. Loosened cells

Fragile callus or loosened cells (Plate XI fiig. B&C) were prepared from the stem segments of susceptible variety (TV-18). The loosened cells were treated separately with normal serum, antiserum raised against TV-18 and *G.cingulata* and finally reacted with FITC. These cells were not autofluorescent nor did they fluoresce when reacted with normal serum, but bright fluorescence of these cells under UV-light were noticed when reacted with antiserum of TV-18. It is interesting to note that when cells were treated with anti *G.cingulata* antiserum and then reacted with FITC, bright fluorescence was noticed (Plate XII, figs. A & B).

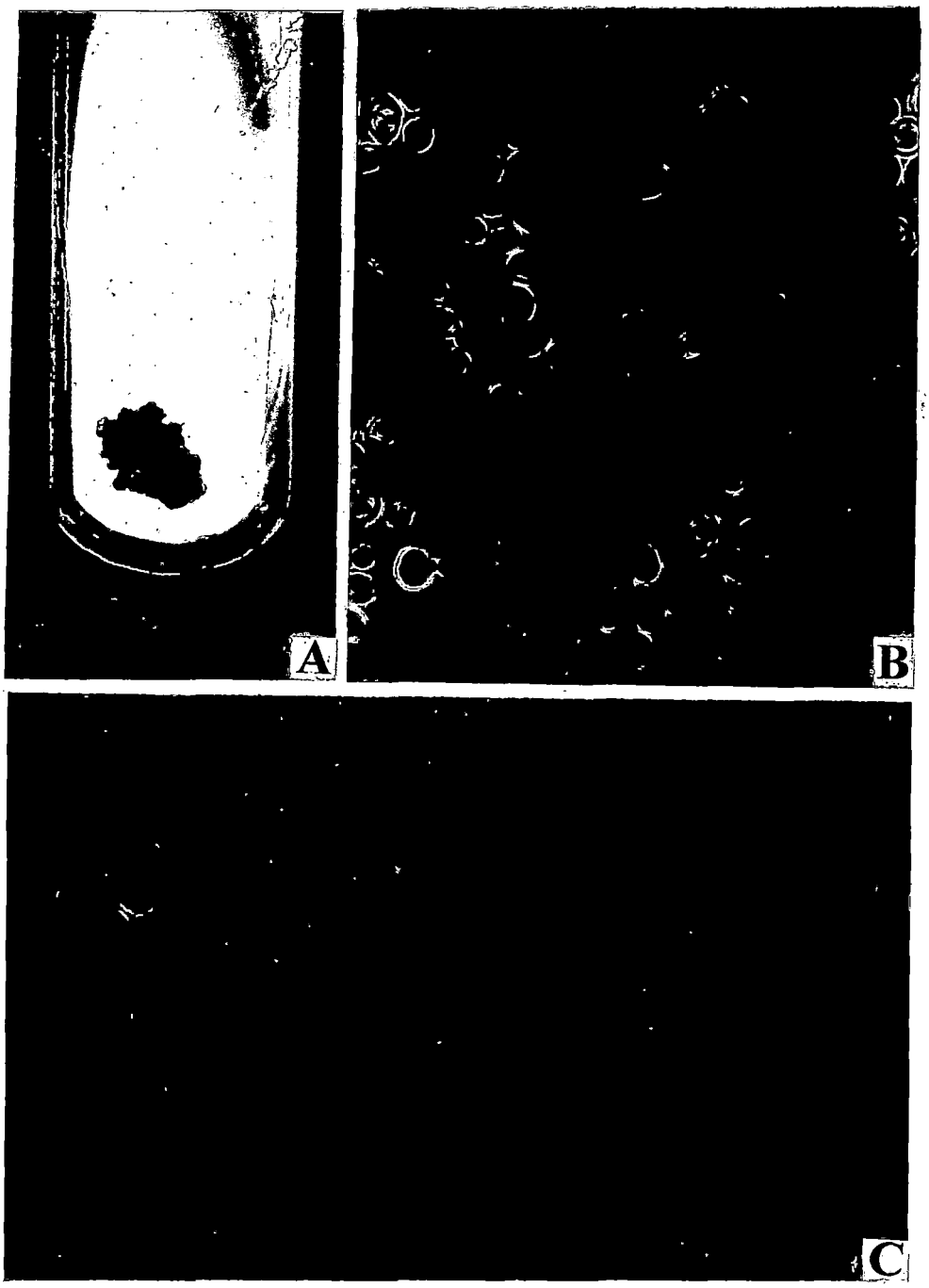


PLATE XI (figs. A-C) .Callus from stem segments of tea (TV -18); **B & C** - loosened cells from callus observed under microscope

4.12.2. Leaf tissues

Cross sections of tea leaves (Tv-18) 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 XIII, fig. A). Same observations were noted when the leaf sections were treated with the normal serum and FITC. Leaf sections treated with antiserum of TV-18 and then reacted with FITC, developed bright fluorescence which was distributed throughout the leaf tissue, mainly in the epidermal cells and mesophyll tissues. Of much significance was the strong reaction of anti *G.cingulata* antiserum with leaf tissues of TV-18. CRA was concentrated mainly around epidermal cells and mesophyll tissues (Plate XIII, fig.B).

4.12.3. Mycelia and conidia

Mycelia and conidia of *G. cingulata* were not auto fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of mycelia and conidia of *G. cingulata* with homologous antisera and FITC showed a general fluorescence that was more intense on young hyphae (Plate XIV, fig. A) and throughout the surface of the conidia (PlateXIV, figs. B & C). When fungal cells were reacted with antiserum to leaves of TV-18 and treated with FITC, bright fluorescence was apparent on young hyphae and throughout the surface of some conidia.

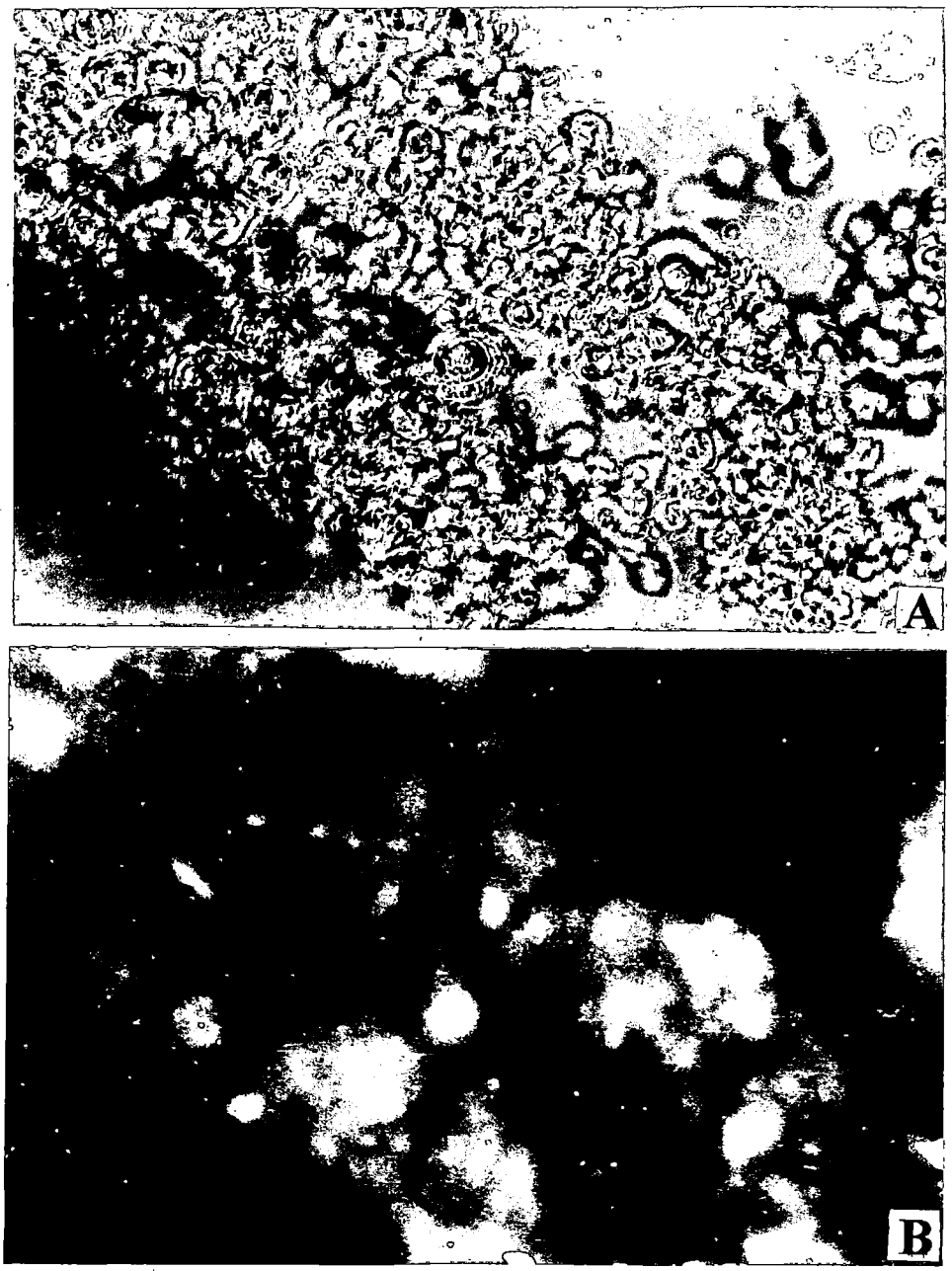


PLATE XII (figs. A & B) . Loosened cells from callus of tea (TV-18) . **A**- under bright field microscope ; **B**- after treatment with antiserum to TV-18 followed by FITC labelling

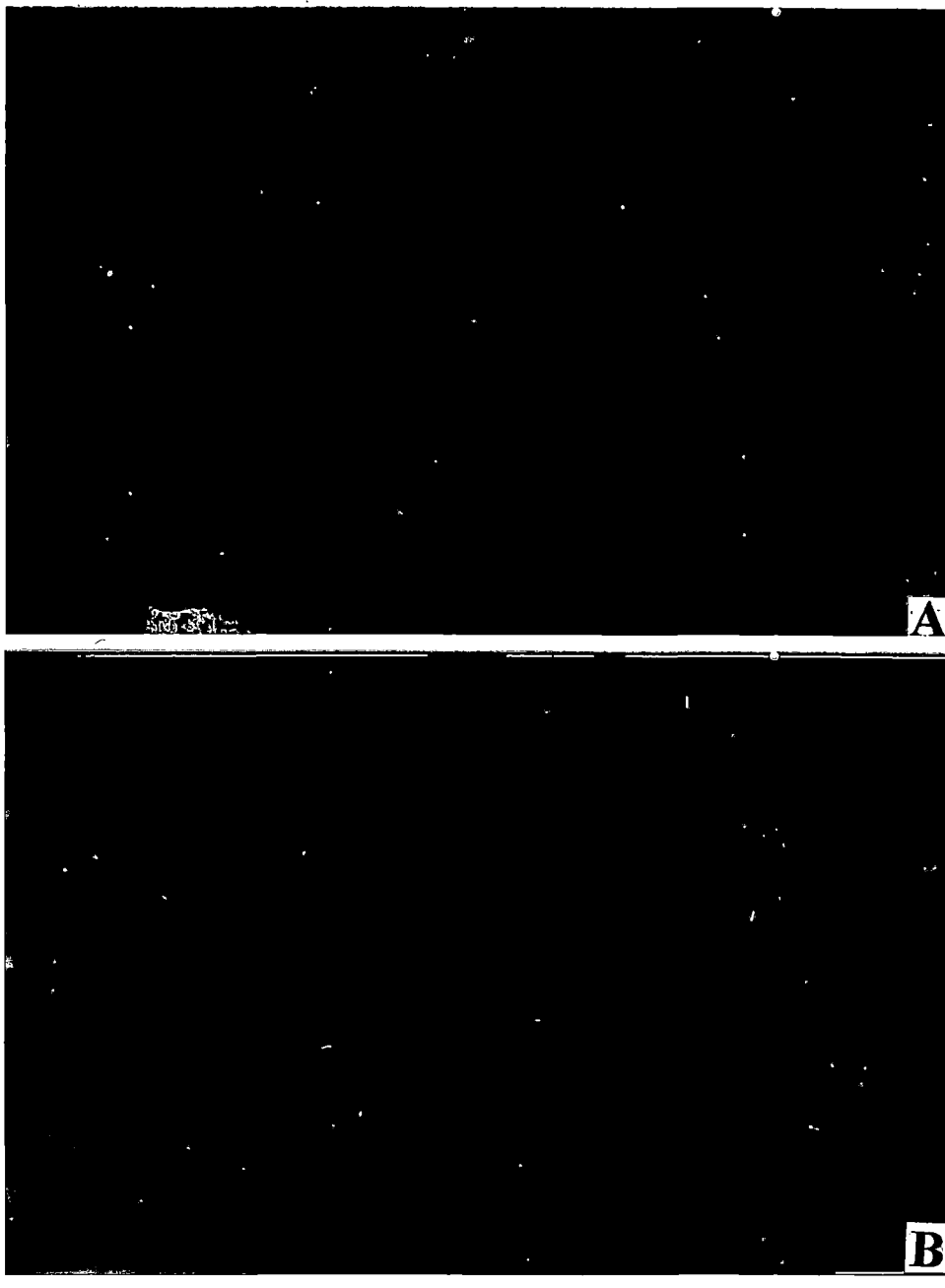


PLATE XIII (figs. A & B) . FITC antibody staining of tea leaf tissues (TV - 18) for cross reactive antigens shared with *G.cingulata* ; **A** - autofluorescence of unstained leaf section; **B** - leaf section treated with antiserum to *G.cingulata* and FITC antibodies of goat specific for rabbit globulin

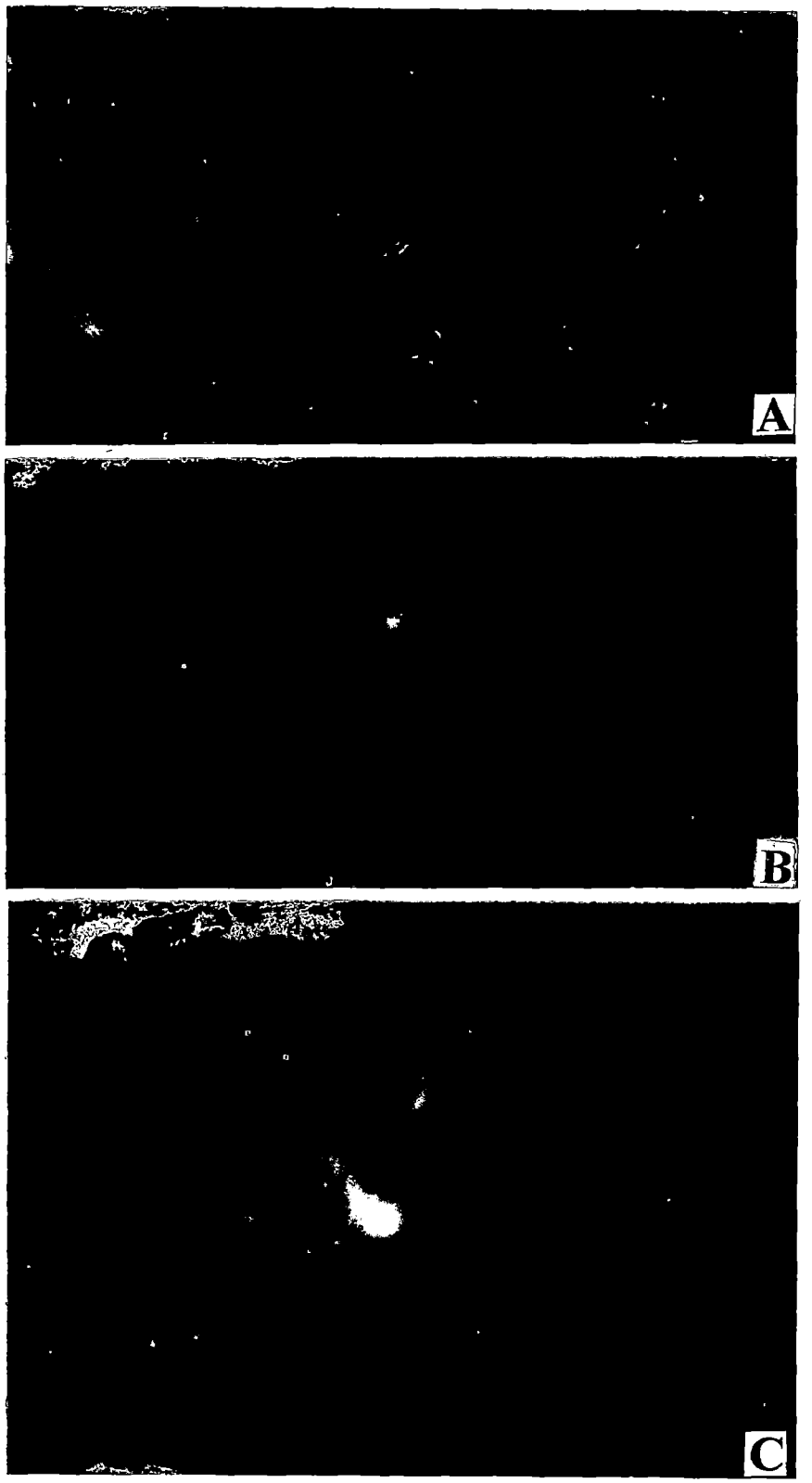


PLATE XIV (figs. A-C) . Hyphae **(A)** and conidia **(B&C)** of *G.cingulata* treated with antiserum to *G.cingulata* and FITC antibodies of goat specific for rabbit globulin