EXPERIMENTAL

surface (Plate 6 , figs B & C). They were ovoid to oblong, slightly dumbbell-shaped, 17μm in length and 2-5μm in width.

4.1. Brown blight – symptomatology and disease development

Causal organism of brown blight is *Colletotrichum gloeosporioides*, anamorph of *Glomeralla cingulata*. This kind of fungi are particularly hideous as these are difficult to detect and prediction of disease occurrence is quite impossible. In case of brown blight the fungus after settling on the tea leaf surface, remains as an asymptomatic pathogen. The disease expression is activated through tissue damage or senescence. The fungus affects nursery saplings under favourable environmental conditions (Plate 4 , figs. A-E). Natural brown blight infection in the field develops on the maintenance leaves of tea bush and is most revealing just after pruning (Plate 5, fig. C). It is usually restricted to the tea bushes pre-disposed to water stress. When the disease strikes, shoot initiation and productivity are much reduced, thus the yield is minimum. The usual symptoms of brown blight are the scorched leaves with minute fructifications in the form of black dots (Plate 5, figs. A & B). These are manifested in the form of necrotic lesions dark in colour in the middle portion of the leaf. Gradually, the lesions enlarge up to 10-15mm long and 5-12mm wide. They become tanned at the center with red, reddish-brown to yellow-orange borders, where the young spores develop (Plate 5, figs. D & E). Fruiting bodies, acervuli, can be observed as blackish spots in lesion area. The lesions lack concentric zonations and are randomly scattered. The lesions ultimately coalesce and the whole tea leaf is blighted.

Evaluation of the damage caused by the fungus in natural conditions on the leaf surface was performed by scanning electron microscopy (SEM) in comparison with healthy tea leaves. Events occurring on leaf surface during natural infection that leads to damage of tea leaves were studied in order to understand the damage-causing potentials of *G.cingulata*. Dorsal surface of healthy tea leaf shows a uniform layer of epidermal tissue with prominent resin ducts (Plate 6 , fig. A). The whole surface is rugate and these rugae exhibit a compact network. There are flakes of waxy deposition at some places. These wax plates are irregular and arranged in clusters. There was prominent absence of stomata or any appendages on adaxial surface. At the initial stage of infection, where acervuli were only beginning to form, spores were prominently visible on the dorsal surface (Plate 6 , figs B & C). They were ovoid to oblong, slightly dumbbell-shaped, 7-17μm in length and 2-5μm in width.

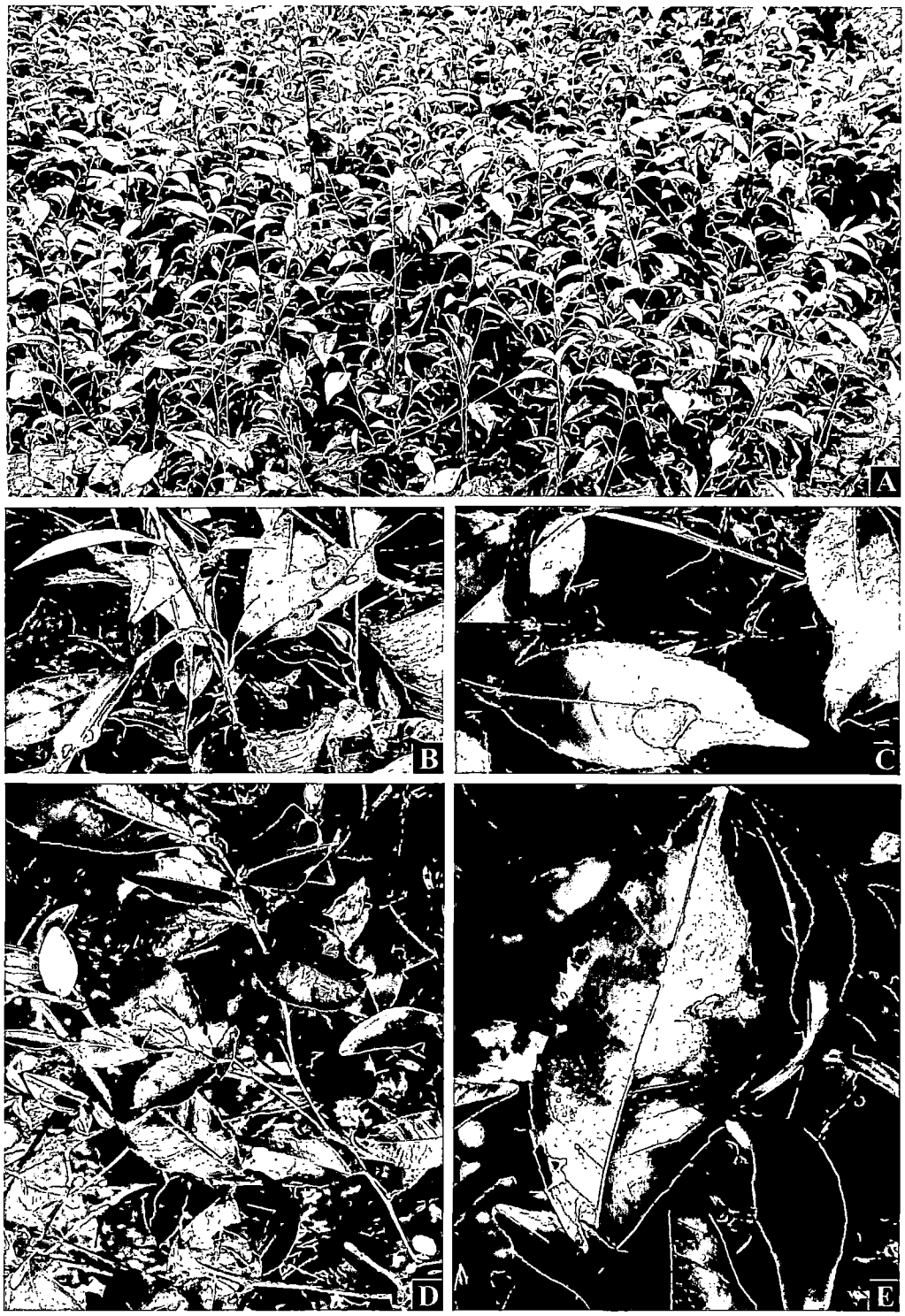


Plate 4 (A-E) : Tea saplings in the sleeves. (A) Healthy; (B-E) Natural infection (brown blight).

Plate 5 (A-E) : Symptoms of natural brown blight infection on tea leaves. (A) TV-22; (B) T-17/1/54; (C) UP-26; (D & E) CP-1/1.

In enlarged view (Plate 6, fig.D) distinct short erect conidiophores were also visible. In the leaf of final infection stage, where there was a distinct demarcation between healthy and infected portions, the magnified view shows the distinct junction formation (Plate 6, figs. E&F) However, no spores were visible. When the infected dry area was scanned, the black acervuli were visible as black patches and no further details were discernable (Plate 6, figs. G & H)

Ventral surface of healthy tea leaf just like the dorsal surface is rugate, with rugae exhibiting a compact network (Plate 7, figs. A & B). Wax deposits were scanty. In contrast to dorsal side, ventral side shows presence of numerous stomata and hairs (trichomes). Stomata are distinct paracytic (parallel-celled) type characterized by of two subsidiary cells lying parallel to the long axis of guard cells (Plate 7, fig.B). The hairs are simple unbranched and elongated with a broad base and narrow tip. The trichome joint (attachment to the leaf epidermis) is expanded.

The initial stage of the infected leaf tissue on the ventral side shows a distinct junction between healthy and infected portions, where the differences between the two regions were marked. At the later stages, subsidiary and epidermal cells in the infected portion were totally disorganized and no hairs were visible (Plate 7, figs. C & D). Numerous spores were formed in the region surrounding the stomata (Plate 7, fig. D).

It is thus clear from the above description that *G. cingulata* causes extensive damage to tea plants and is particularly difficult to control due to its complicated lifestyle.

4.2. Characterization of *G. cingulata* isolates

After thorough study of natural infection it was decided to characterize different isolates of *G. cingulata*. It is a well-known fact that variations at the morphological and molecular levels may exist within a particular species of fungi, especially so among the *Colletotrichum gloeosporioides* isolates. It was, therefore, considered worthwhile to compare the three selected sporulating isolates (GC-1, GC-2 and GC-3) for determining their cultural and molecular characters in order to note any variations existing within the species, since many strains are known to exist in nature for a single fungal species which may differ from each other.

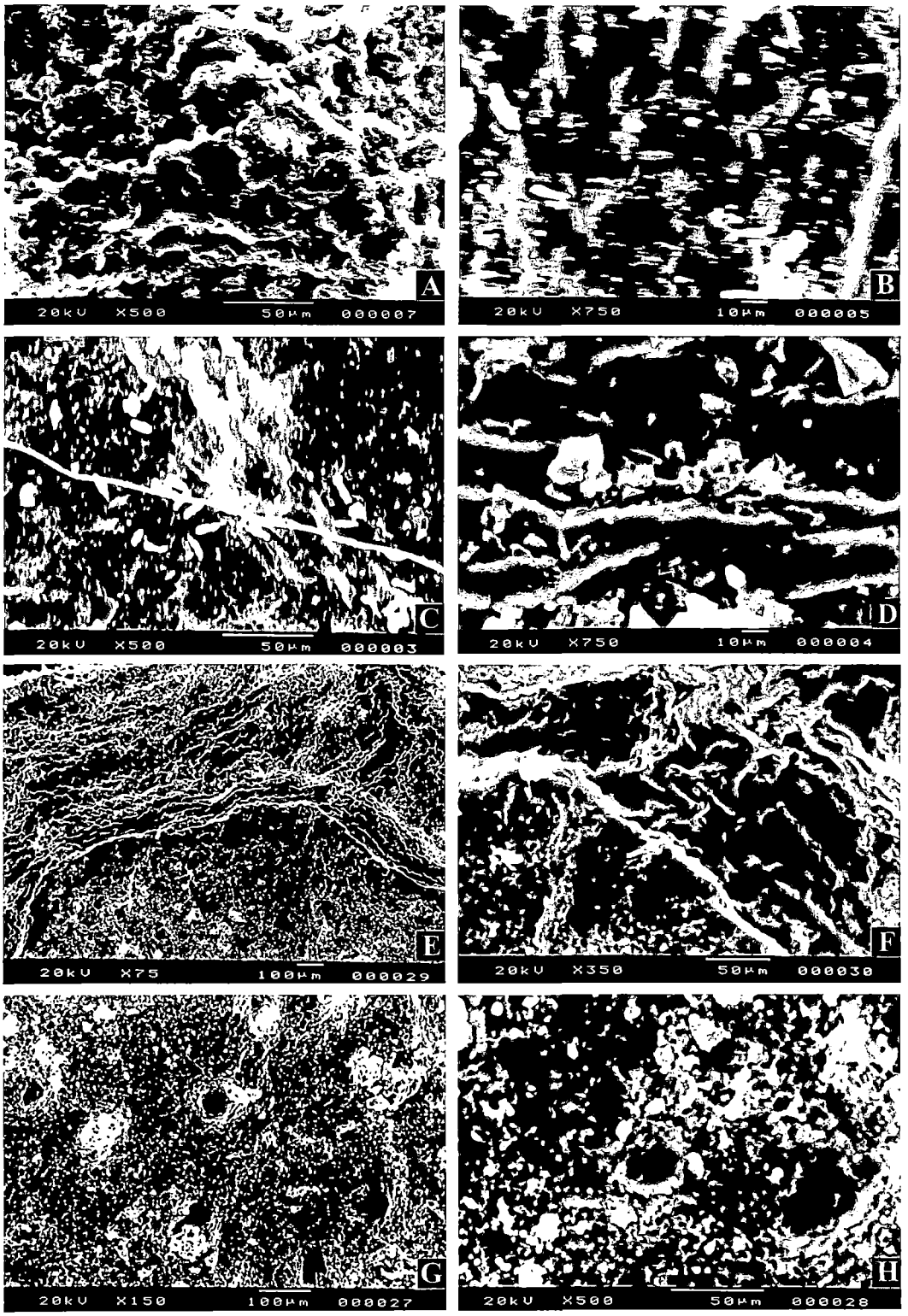


Plate 6 (A-H) : Scanning electron microscopy images of adaxial surface of tea leaf. (A) Healthy ; (B-H) Brown blight infected tea leaves.

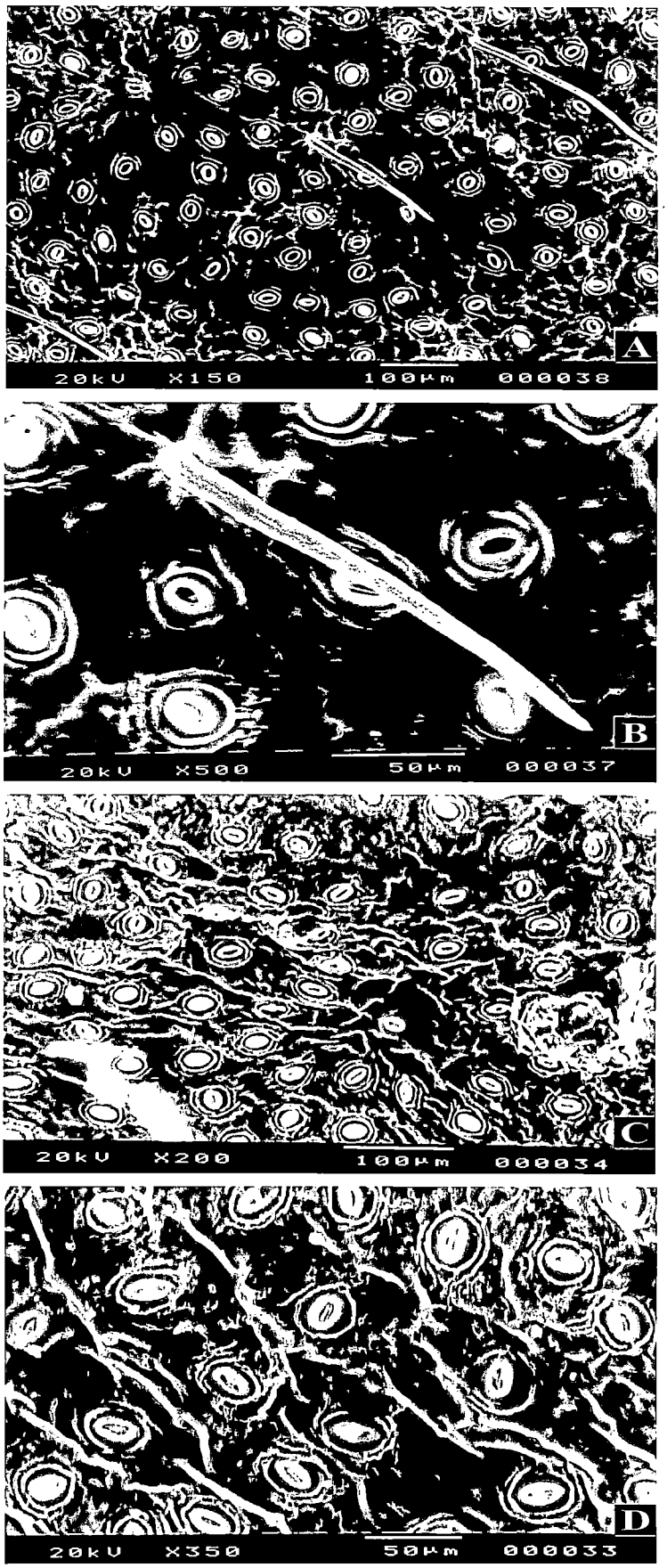


Plate 7 (A-D) : Scanning electron microscopy images of abaxial surface of tea leaf. Healthy (A & B) and brown blight infected (C & D).

The three isolates of *G. cingulata* were obtained from three different tea varieties of naturally brown blight infected tea bushes maintained in open field grown Tea Germplasm Bank. The isolate GC-1 was obtained from naturally infected TV-22, isolate GC-2 - from S-449 and isolate GC-3 from TV-26. These were all maintained on OMA slants. Koch's postulates were completed for confirmation of the disease causing capacity.

For assessment of growth rate and sporulation ability, different solid media (PDA, RMA and OMA) in Petridishes was inoculated with 4mm diameter plugs taken from the periphery of the young fungal culture of the specified medium, incubated at 25°C and growth rate was measured by taking readings every 24 h. Growth pattern was evaluated in 10 day-old cultures. While mycelial dry weight was determined for all three isolates grown in liquid Richard's Medium.

Spores of the isolates grown on PDA medium after14 days of incubation were measured with the help of ocular micrometer. The spores of each isolate were germinated separately on glass surface at +25°C and relative humidity at around 90% and their appressorial dimensions were noted after 24h. Conidiomata, sporulation patterns and the various perennating structures in solid media were studied under compound microscope.

Molecular characterization was also attempted for these isolates. Total soluble protein was extracted from the mycelial mats grown in RM and estimated . Protein profile was analysed by SDS-PAGE and compared for cultures of different ages. Following this, polyclonal antibodies (PAbs) were raised against the 8 day-old mycelial antigen of the selected isolate (GC-1) in white male rabbits. The titre of antibody was confirmed by immunodiffusion test. Besides, PTA-ELISA and dot blot were standardized using the three isolates for precise detection of *G. cingulata* in tea leaf tissues. For serological comparison of the isolates, western blot analysis was also performed. The single protein band which gave the strongest recognition signal, was identified.

4.2.1. Growth

Growth rates were measured in the three strains of *G. cingulata* in three different solid media – RMA, OMA and PDA. as described earlier. The results are presented in Table 2. It is evident that the isolates did not differ significantly with respect to their growth rates in solid media. However, there were observable differences

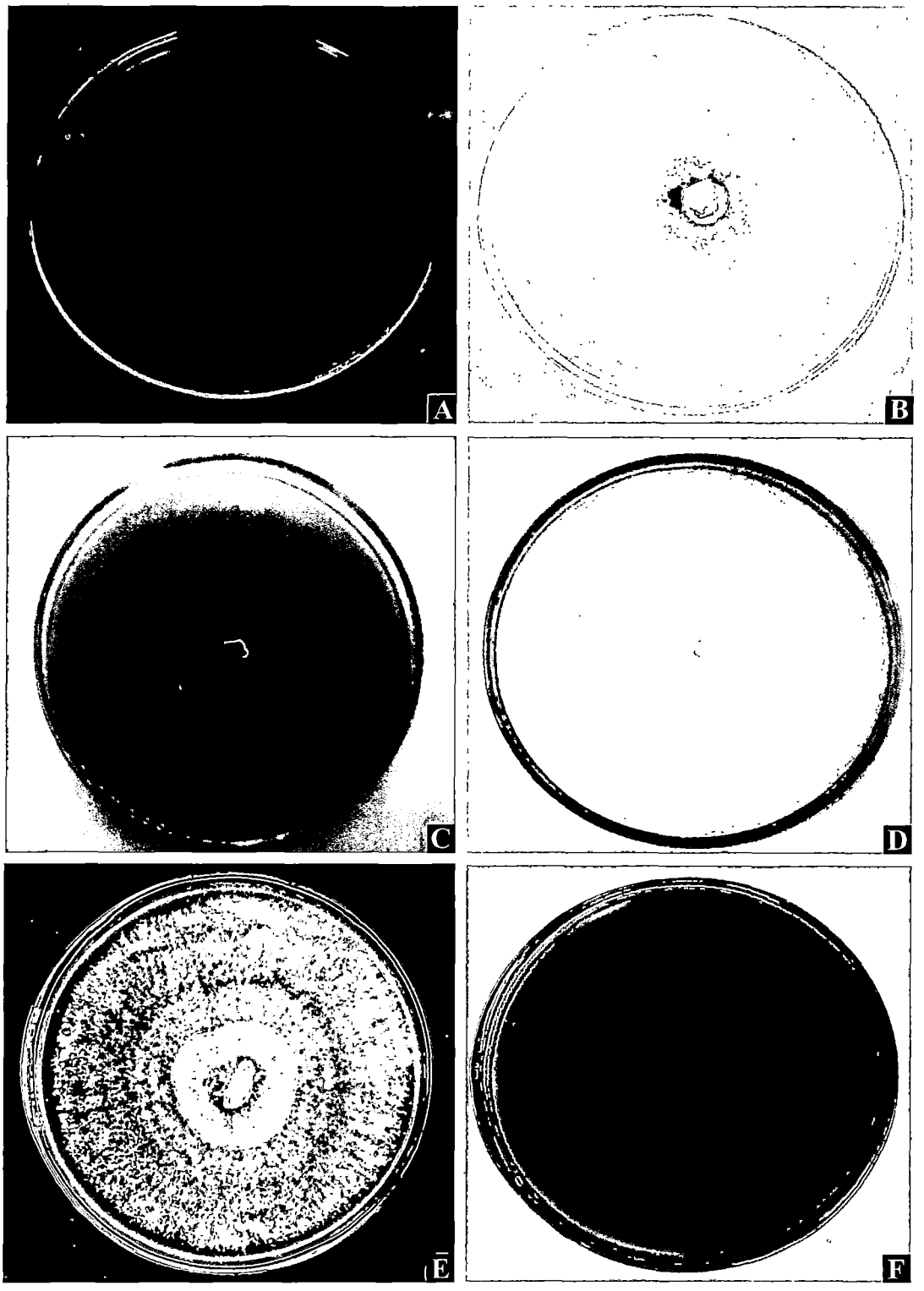


Plate 8 (A-F) : Mycelial growth of *G. cingulata* isolates. GC-1 (A, C & E); GC-2 (B & D) and GC-3 (F) on Richard's Medium Agar (RMA), Potato Dextrose Agar (C & D) and Oat Meal Agar (E & F).

with respect to the type of solid medium used. For all three isolates growth rate was highest in PDA, followed by RMA and least growth rate was observed in OMA. The growth rates ranged from 8.13 mm day $^{-1}$ (GC-2 in OMA) to 11.4 mm day $^{-1}$(GC-1 in PDA).

Table 2: Growth rates of *G. cingulata* isolates in different media.

Isolates	Growth rate (mm day^{-1})		
	Medium [a]		
	PDA	RMA	OMA
GC-1	11.4 ± 0.10	9.5 ± 0.13	8.24 ± 0.23
GC-2	11.0 ± 0.15	9.4 ± 0.32	8.13 ± 0.25
GC-3	11.0 ± 0.48	9.3 ± 0.54	8.17 ± 0.37

Means ±SE, n=3

[a]Incubation temperature 25°C

Analysis of variance

Source	D.F	S.S	M.S.	F	C.D (5%)
Medium	2	13.22	6.61	661	0.23
Isolates	2	0.09	0.045	4.5	
Error	4	0.04	0.01		
Total	8	13.35			

The patterns of growth also differed between the isolates and also depending on the medium used. In PDA mycelial colour was white to greyish-white, where growth pattern was raised and felty, excepting in GC-2, where it was raised and wooly (Plate 8, fig. D). In RMA, mycelial colour varied from white (GC-1) to greyish-white (GC-3) to black (GC-2) on the upper surface. Acervuli formation was observed on RMA (Plate 10, figs A & B). In OMA the mycelia were always digging and very sparse (Plate 8, figs. E & F). Instead, bright orange acervuli formation was evident on the upper surface forming concentric zonations.

Growth in liquid synthetic medium (RM) was measured for the three isolates of *G. cingulata* separately by taking dry weight. The resulting data is presented in Fig. 1(A). The dry weight accumulated was highest 16 days after inoculation in *G. cingulata*, irrespective of the isolate. However, the dry weight values at that point differed

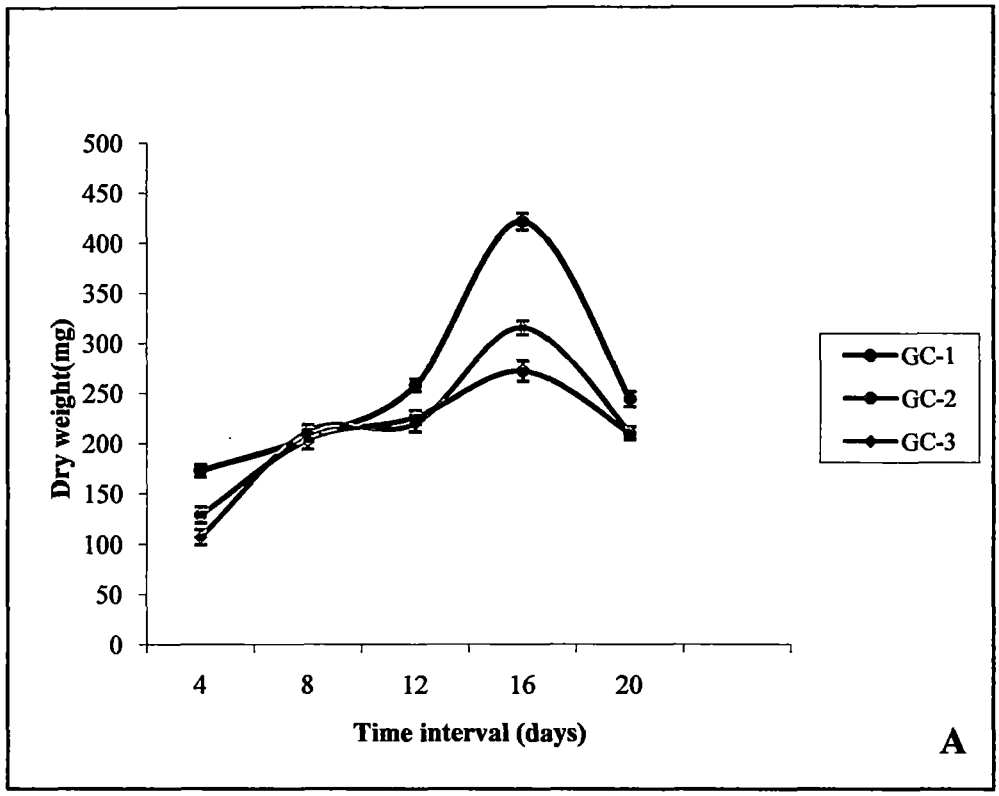
significantly among these isolates GC-1 had maximum weight (421mg), while GC-2-minimum (272mg).

4.2.2. Sporulation

Ability to form spores (conidiogenesis) in artificial medium is an important character for a fungal species. Spores so formed will be used for various experiments in the present investigation. Hence, sporulation was assessed in *G. cingulata* isolates in the different media (PDA, RMA and OMA) as described earier. The results are presented in Fig. 1(B). Lowest amount of sporulation was in PDA, which was similar in all the isolates tested. OMA was the best medium for sporulation. Next best medium was RMA. However, GC-1 exhibited highest sporulation among the three isolates in all the culture media tested.Conidia appeared either on solitary phialides but normally in light brown sporodochia in 14 day-old cultures. A basal stromatic cushion is covered with a dense layer of cylindrical, slightly tapering phialides 10 to 20μm long. Sometimes these are interspersed with dark brown, tapering septate setae (Plate 9, figs. G & H). Among the isolates studied, GC-1 and GC-3 showed distinct formation of sporodochia (Plate 9, figs A & B). The conidia were produced either on the tips of the hyphae in bunches (Plate 9,fig. D) or from the thickened fertile hyphae by thallic mode of conidiogenesis (Plate 9, fig E) so that they formed chains. In 20 day-old cultures, the mycelia formed perennating structures like chlamydospores in chains (Plate 10, fig. B) and also sclerotia (Plate 10, figs. C & D). Chlamydospores in chains were observed in cultures of GC-2 only. Sclerotia were found in GC-1 and GC-2. Conidia also formed microconidia (brown in colour) by transverse septa in these cultures (Plate 9, fig.A). Mycelia of GC-1 and GC-3 became greatly thickened and brown in colour forming appressoria (Plate 9, figs. C & F). In 14 day-old cultures of all the three isolates, these mycelia aggregated into acervuli with setae (Plate 9, figs. G & H). GC-1 formed pycnidia-like ovoid fruit bodies with an opening (Plate 10, figs.E & F), revealing presence of spores when squashed.

4.2.3. Spore morphology

Conidia were hyaline aseptate cylindrical with a rounded apex and slightly truncated base (Plate 9, fig I). Truncate base is especially evident in Scanning Electron Microscopy images (Plate 10, figs. J & K). Spore dimensions were always measured from spores grown in PDA. There was a great range in length of the conidia even within each isolate, as observed from standard deviation values in Table 3. Between the isolates,



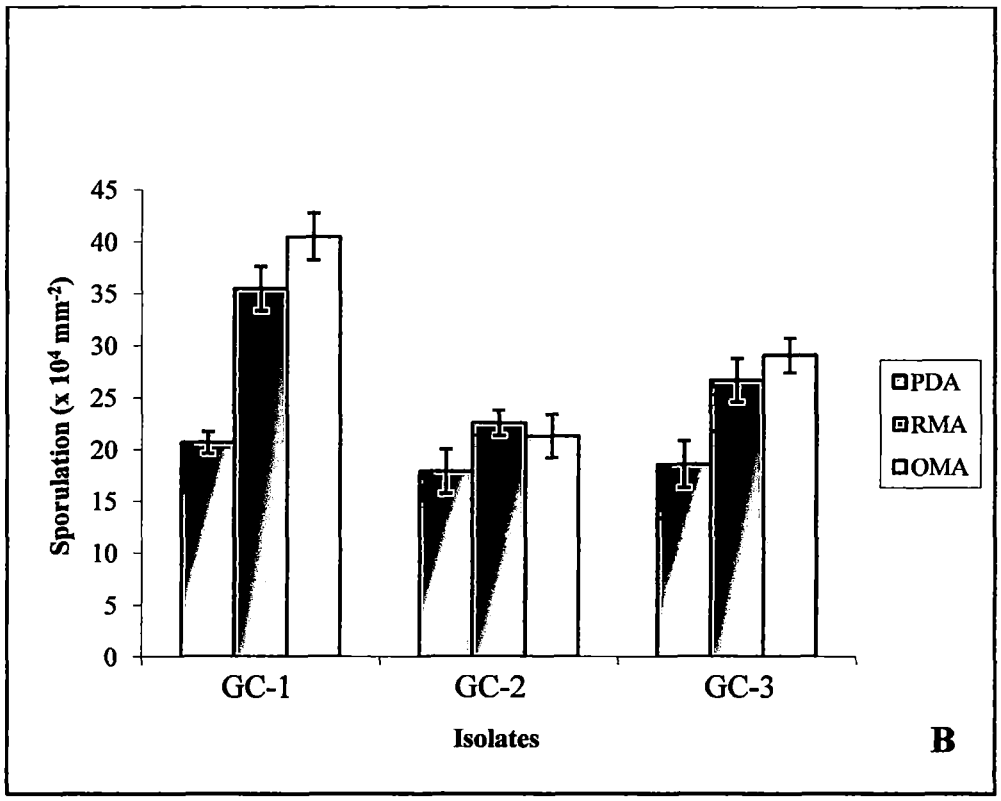


Fig.1 (A & B): Mycelial growth (A) and sporulation (B) of *G. cingulata* isolates

mean conidial length was shortest in GC-3 (11.93 μm) and longest in GC-1 (14.12 μm). The mean width of conidia did not vary much among the isolates, the widest being in GC-2 (5.22 μm) and the narrowest in GC-1 (4.91 μm).

Table 3: Spore size and dimensions of appressoria of *G. cingulata* isolates grown in PDA.

Isolate	Spore size[a,d](μm)		Appressorium size[b,c](μm)	
	Length	Width	Length	Width
GC-1	14.12 ± 1.1	4.91 ± 0.0	12.54 ± 0.2	7.56 ± 0.1
GC-2	13.60 ± 0.9	5.22 ± 0.1	10.40 ± 0.4	5.90 ± 0.1
GC-3	11.93 ± 1.2	5.00 ± 0.1	10.47 ± 0.3	5.95 ± 0.1

Means ± SD; Average of 300 spores per isolate in three independent experiments

[a]Spores used were the ungerminated conidia of *G. cingulata* from a 14 day-old culture grown on PDA [a]Incubation temperature 25°C

4.2.4. Appresssoria

Appressorial morphology and size serves as an important taxonomic character. Therefore, mean dimensions of these structures were measured. Besides, these are important for pathogenicity of the fungus. The appressoria were studied from glass surface germinated conidia after 24 h of incubation (Plate 10, figs G-I). These were all irregularly rounded and brown in colour. Mean dimensions of appressoria varied from 10.4 x 5.9 μm in GC-2 to 12.54 x 7.56 μm in GC-1 as presented in Table 3. Thus, it is evident that GC-1 exhibited largest appressoria.

4.2.5. Protein content

Quantitative protein analysis of the mycelial antigen of *G. cingulata* isolates prepared as described in Materials and Methods, was done at 4 day intervals, revealed that 12 day- old culture of GC-1 had the highest protein content of 25.3mg g^{-1} fresh tissue. The results are presented in Table 4. It is clear that GC-1 exhibited high total protein content from 4^{th} to 12^{th} day of incubation. On the other hand, for GC-2 and GC-3 total protein accumulation up to 12^{th} day of incubation was less than in GC-1.

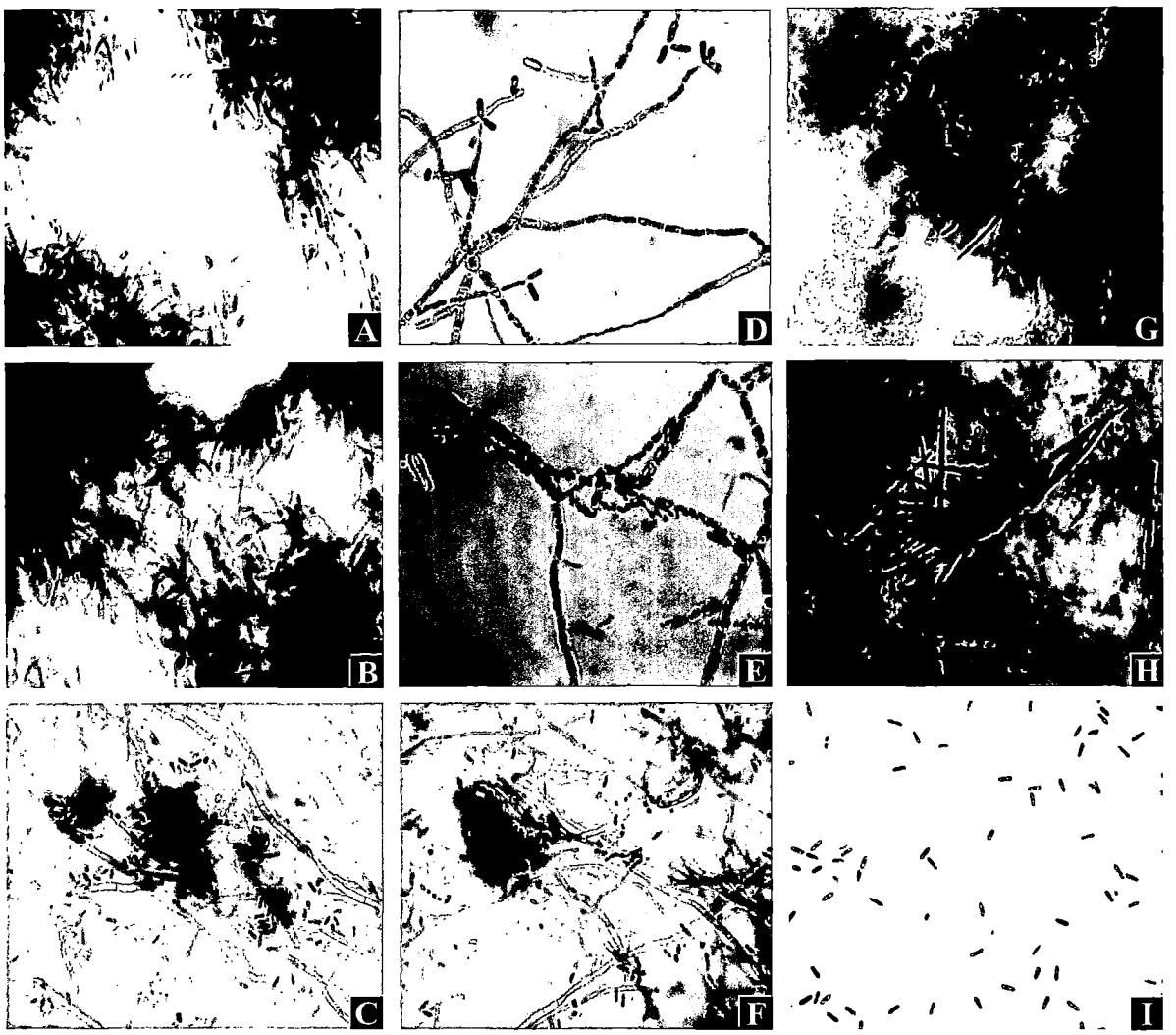


Plate 9 (A-I): Sporulation of *G. cingulata* isolates. (A & B) Formation of sporodochia (C & F) appressoriate mycelia; (D & E) conidia formation; (G & H) setose acervuli; (I) conidia . GC-1 (A, C, D, G, & J); GC-2 (H); GC-3 (B, E, F & H).

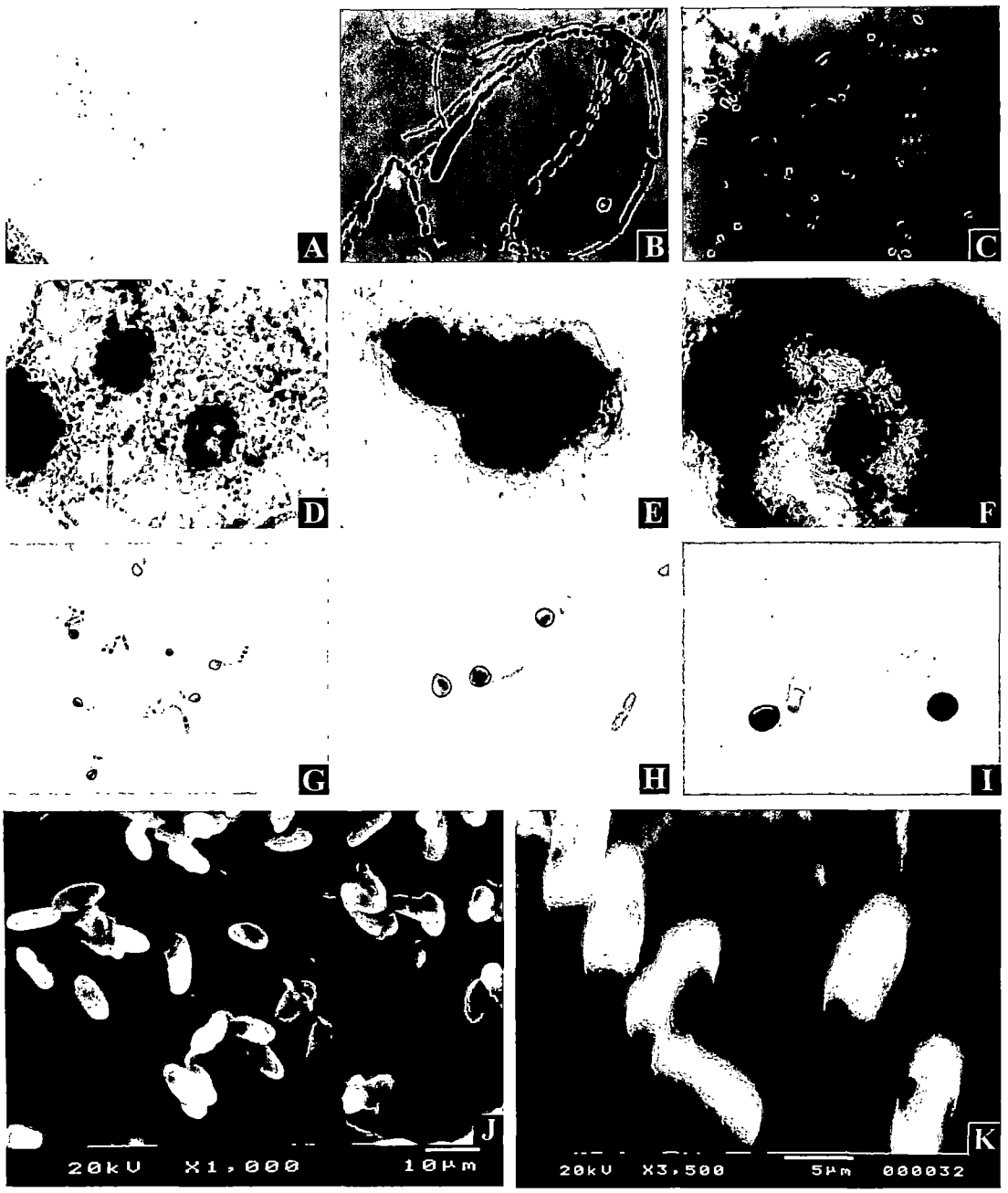


Plate 10 (A-K): Reproductive structures of *G. cingulata* isolates (A) microconidia; (B) chlamydospores; (C & D) sclerotia ; (E & F) pycnidia-like fruit bodies; (G-I) germinated conidia with appressoria under light microscope; (J-K) conidia under Scanning Electron Microscope ; GC-1 (D, E, G, J & K); GC-2 (A, B, C & H); GC-3 (F & I).

Table 4: Protein content of *G. cingulata* isolates at different time intervals.

Isolates	Protein content (mg protein g^{-1} fresh weight)				
	Incubation period (days)[a]				
	4	8	12	16	20
GC-1	12.0 ± 1.5	22.5 ± 1.3	25.3 ± 2.1	14.0 ± 1.7	12.6 ± 2.2
GC-2	09.5 ± 2.3	15.6 ± 1.6	20.3 ± 2.2	12.3 ± 1.3	10.6 ± 2.5
GC-3	07.8 ± 2.2	12.2 ± 2.5	20.1 ± 1.3	15.6 ± 2.3	11.2 ± 1.1

Means ±SE, n=3
[a]Incubation temperature 25°C

Analysis of variance

Source	D.F	S.S	M.S.	F	C.D (5%)
Incubation period	4	274.24	68.56	13.18	4.29
Isolates	2	47.32	23.66	04.55	
Error	8	41.61	05.20		
Total	14	363.17			

4.2.6. SDS-PAGE.

Protein patterns on SDS-PAGE were studied for the three strains at 4-day intervals. Qualitative analysis by SDS-PAGE showed that there are 10 soluble proteins in 4-days old culture of GC-1 (Plate 11, fig.A, Table 5). In GC-2 there were only eight bands and GC-3 only five. Thirteen more protein bands appeared in 8 day-old culture of GC-1 making a total of 23 bands. GC-3 exhibited a new band of ca 83.4 kDa, while bands of ca 116.0 and 97.4kDa were lacking (Plate 11, figs A-C). On the other hand, eight day-old culture of GC-2 revealed only 15 bands. In 12 day-old cultures, however, there was a loss of one protein band of 27.2kDa in GC-1 and GC-3. Isolate GC-2, on the other hand, showed recovery of many proteins at this stage and there were 21 bands observed (Plate 11, fig.B). Only 97.4 kDa band was absent in this case. Proteins extracted from 16 and 20 days old cultures indicated further deterioration of protein quality (Table 5). Identical protein profiles were exhibited by GC-1 and GC-2 isolates, while in GC-3 two low molecular weight proteins (14 and 21 kDa) were lacking. It is quite clear that GC-1 showed maximum number of protein bands in 8 day-old cultures.

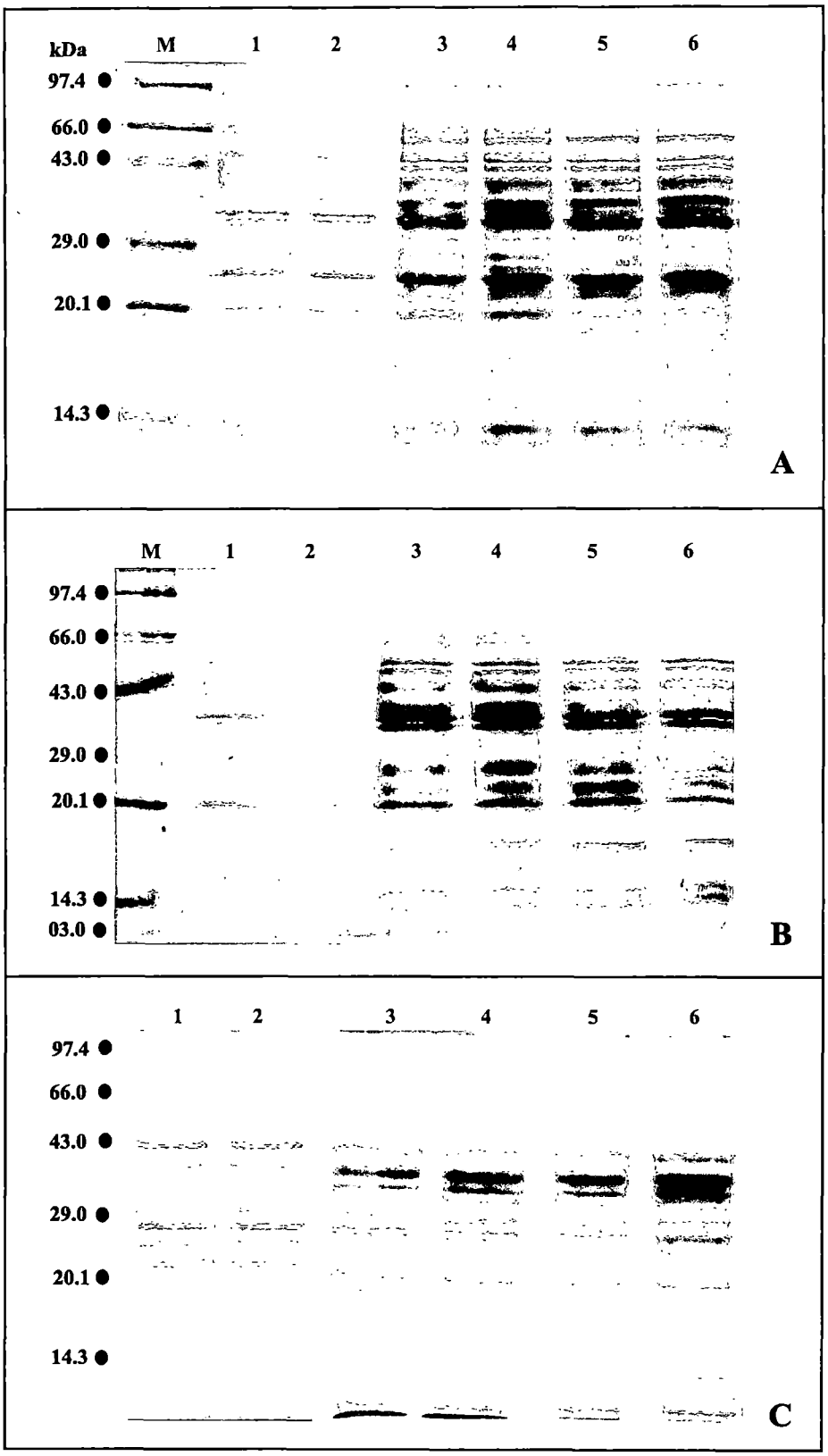


Plate 11 (A-C) : SDS-PAGE analysis of mycelial protein of *G. cingulata* isolates GC-1 (A), GC-2 (B) and GC-3 (C);Lanes 1 & 2 (4 day-old); Lanes 3 & 4 (8 day-old); Lanes 5 & 6 (12 day-old) M-Marker.

Table 5: Molecular weights of soluble proteins (kDa) of *G. cingulata* grown on RM as revealed by SDS-PAGE .

Isolate	Incubation period (days)	Molecular weight (kDa)
GC-1	4	68.4, 64.0, 60.3, 40.5, 39.0, 38.0, 26.0, 20.0, 17.3, 14.0 **(10)**
	8	116.0, 97.4, 68.4, 64.0 , 60.3, 52.6, 43.0, 40.0, 39.0, 38.5, 35.0, 32.5, 29.0, 27.2, 26.0, 24.0, 20.0,19.0, 17.3, 16.6, 15.9,14.0,6.5**(23)**
	12	116.0, 97.4, 68.4, 64.0, 60.3, 52.6, 43.0, 40.0, 39.0, 38.5, 35.0, 32.5, 29.0, 26.0, 23.3, 20.0, 19.0, 17.3, 16.6, 15.9, 14.0, 6.5**(22)**
	16	68.4, 60.3, 52.6, 39.0, 38.5, 20.0, 14.0 **(7)**
	20	60.3, 39.0, 20.0, 14.0 **(4)**
GC-2	4	64.0, 60.3, 40.5, 39.0, 38.5, 26.0, 20.0, 17.3 **(8)**
	8	52.6, 43.0, 40.0, 38.5, 35.0, 27.2, 26.0, 24.0, 20.0,19.0, 17.3,16.6, 15.9, 14.0, 6.5 **(15)**
	12	116.0, 68.4, 64.0, 60.3, 52.6, 43.0, 40.0, 39.0, 38.5, 35.0, 32.5, 29.0, 26.0, 24.0, 20.0, 19.0, 17.3, 16.6, 15.9, 14.0, 6.5**(21)**
	16	68.4, 60.3, 52.6, 39.0, 38.5, 20.0, 14.0 **(7)**
	20	60.3, 39.0, 20.0, 14.0 **(4)**
GC-3	4	60.3, 40.0, 39.0, 38.5, 20.0 **(5)**
	8	83.4, 64.0 , 60.3, 52.6, 43.0, 40.0, 38.5, 35.0, 27.2, 26.0, 24.0, 20.0,19.0, 17.3, 16.6, 15.9, 14.0, 6.5 **(18)**
	12	64.0, 60.3, 52.6, 43.0, 40.0, 39.0, 38.5, 35.0, 29.0, 26.0, 23.3, 21.0, 19.3, 17.3, 16.6, 15.9, 14.0, 6.5 **(17)**
	16	68.4, 60.3, 52.6, 39.0, 38.5 **(5)**
	20	60.3, 39.0 **(2)**

Values in parenthesis indicate the number of bands.

4.2.7. Serological relatedness

The antigen used for the preparation of polyclonal antibody was protein extracted from the 8 day-old mycelia of GC-1 , since it was found to be best both quantitatively and qualitatively. Besides, GC-1 was superior than the other two isolates regarding the characters like growth rate, sporulation potential and perithecia formation. PAbs were raised in white male rabbits as described earlier. IgG was purified and fractions collected. Protein concentration of each fraction was determined from the following formula:

Protein concentration (mg ml^{-1}) =1.55 x A 280 - 0.76 x A 260.

The fractions with protein concentration greater than 2 mg ml^{-1} were kept for further experiments, while others were discarded. Evaluation of IgG quality from different bleeds was done initially by immunodiffusion against GC-1, GC-2 and GC-3. Two tools for immunodetection - ELISA and dot blot were standardised for antigen and antibody concentration. Western blot of SDS-resolved antigen proteins was performed for the three strains.

4.2.7.1. Immunodiffusion

Polyclonal antibodies from 1^{st}, 2^{nd}, 3^{rd}, $4^{th}$and 5^{th} bleeds were evaluated. The PAbs from 3^{rd} and 4^{th} blood were found to be most effective. Therefore, IgGs obtained from 3^{rd} and 4^{th} bleeds were pooled and used for further immunological experiments. Comparison of immunodiffusion pattern of the three isolates (Plate 12, fig. A) indicates that GC-1 has maximum number of common antigenic determinants with this IgG, while GC-2 had the lowest.

4.2.7.2. Optimization of PTA-ELISA

Optimization of ELISA was done considering two variables – dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity.

4.2.7.2.1. Enzyme dilution

Antigen concentration (10μg/ml) and antiserum dilution (1:125) were kept constant, and different dilution of enzyme (alkaline phosphatase) were used ranging from 1:10,000 to 1:40,000. On the basis of results 1:10,000 of enzyme (alkaline phosphatase) dilution was selected for further experiments.

4.2.7.2.2. Antibody dilution

PTA-ELISA reactions with different dilutions of antibody ranging from 1 : 16,000 to 1 : 125 were determined at antigen concentration 60µg ml^{-1}. Absorbance values decreased with increasing dilution (Fig. 2 A) . Absorbance values in ELISA decreased from the dilution of 1:125 to 1: 16,000. Highest absorbance value (2.928) was obtained with 1 : 125 antiserum dilution. Thus, for optimum results, 1 : 125 dilution of antibody was used in all the immunological experiments .

4.2.7.2.3. Antigen dilution

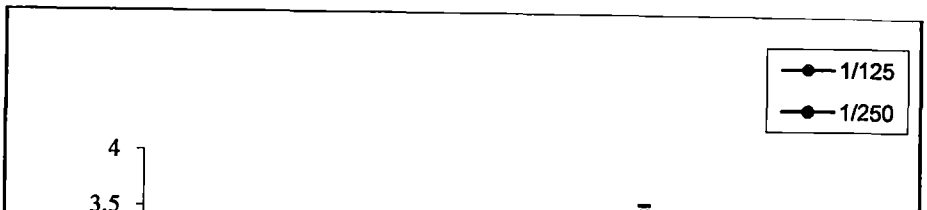
Doubling dilutions of *G. cingulata* mycelial antigen (extracted from 8 day-old GC-1 mycelia) ranging from 162.5 to 48,000ng/ml were tested against two antiserum dilutions (1:125 and 1: 250). The results are presented in Fig. 2B. ELISA values increased with increasing concentration of antigen. Absorbance value of 0.9 were obtained in case of 1 : 125 dilution for antigen concentration as low as 81.25 ng/ml. Higher values were obtained for 1 : 125 dilution than 1 : 250. The absorbance values greater than 3 were obtained when the saturation was reached. After that the accuracy reduced, as the absorbance decreased on increasing concentration of antigen. The standard error also increased from 60µg ml^{-1} onwards. Thus, this antigen concentration was taken to determine the optimum antibody dilution.

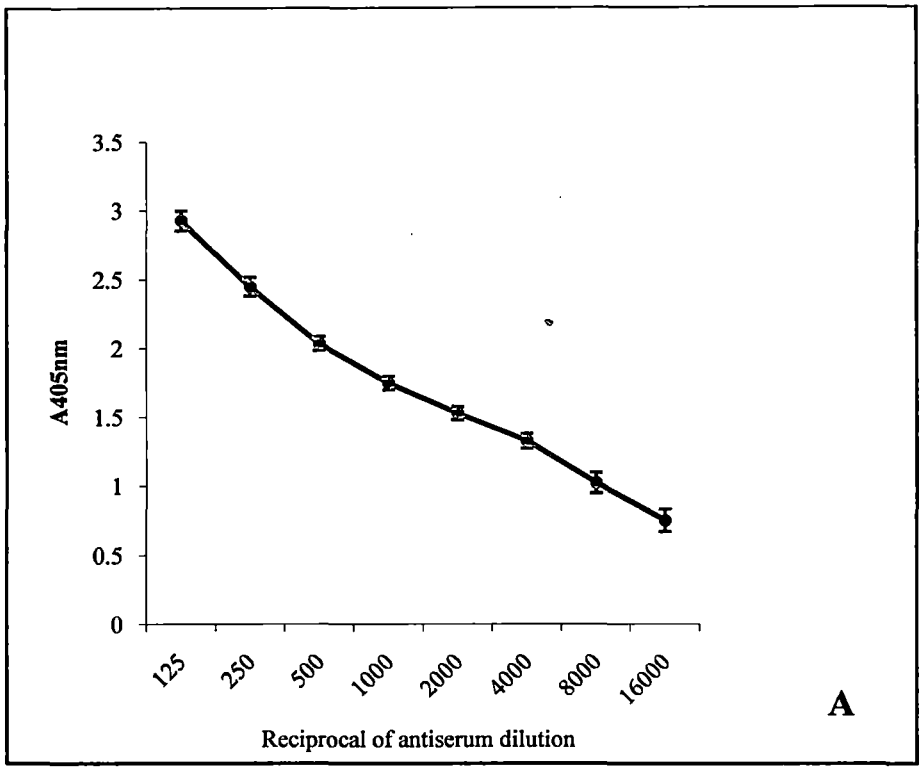
4.2.7.3. PTA-ELISA

After optimization of ELISA, PTA-ELISA was conducted against the three isolates separately . The results obtained (Table 6) show that the mean absorbance value was highest in case of GC-1.

4.2.7.4. Dot Blot

No differences in the dot intensity was revealed when *G. cingulata* isolates were probed separately with PAb of *G. cingulata* (Plate 12, fig.B). Therefore, it was concluded that PAb raised is sensitive enough to detect at least three starains of *G. cingulata*, in spite of their differences.





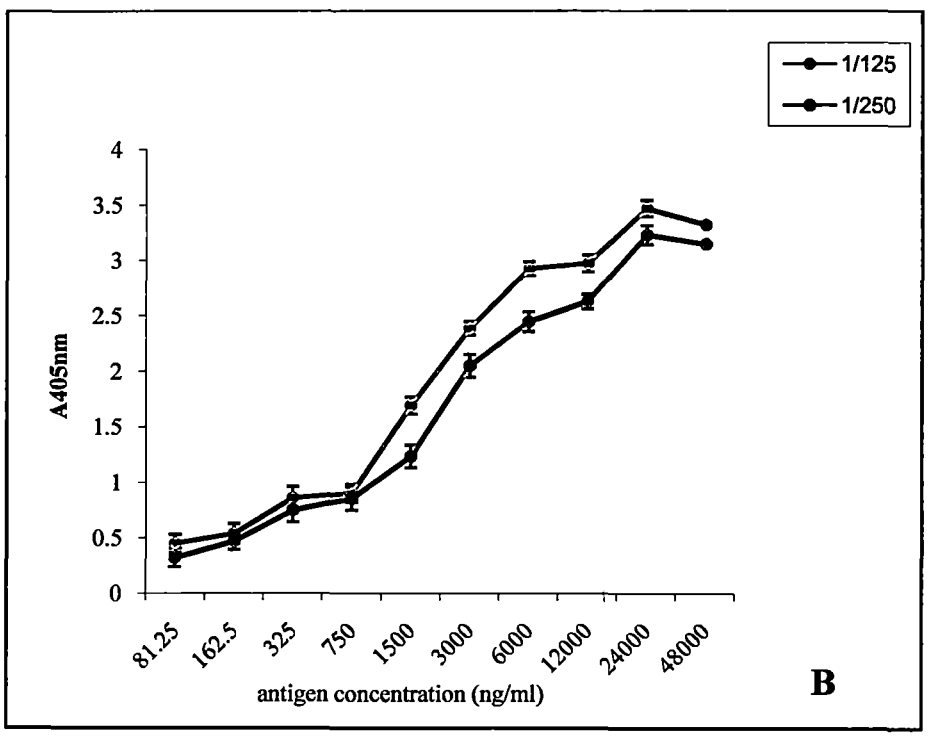


Fig. 2 (A & B): Optimization of PTA-ELISA for antiserum dilution (A) and antigen concentration (B).

Table 6: PTA-ELISA value and molecular weights of soluble proteins of *G. cingulata* isolates probed with PAb of GC-1.

Isolate*	PAb of GC-1	
	PTA-ELISA	Western Blot
	A_{405}	Molecular weight (kDa)
GC-1	2.84 ± 0.062	40.0, 38.5, 35.0, 32.0, 24.0, 20.0, 19.0 **(7)**
GC-2	1.68± 0.053	40.0, 38.5, 35.0, 24.0 **(4)**
GC-3	1.93 ± 0.067	40.0, 38.5, 35.0, 32.0, 24.0, 20.0, 19.0 **(7)**

* 8 day-old cultures grown on RM were used in each case ; Values in parenthesis indicate the number of bands

4.2.7.5. Western Blot

Antigens extracted from 8 day-old cultures of *G. cingulata* isolates revealed some differences on Western blots after probing with PAb of GC-1, which indicates the subtle differences in the antigenic properties of the isolates. The reference isolate (GC-1) exhibited maximum number of bands on the Western blot (Plate 12, fig.D). When compared with corresponding SDS-PAGE stained with coomassie blue (Plate 12, fig. C), the blotted bands were revealed as presented in Table 6. Around seven bands of ca 40.0, 38.5, 35.0, 32.0, 24.0, 20.0 and 19.0 kDa could be identified on the blot, but many of these were pale and diffuse.Two bands (ca 20 and 19 kDa) were totally lacking in GC-2, while very faint images of these were present in GC-3. Three bands (ca 24, 35.0 and 40 kDa) were prominent in all isolates. The 24 kDa protein was the most prominent, which gave the strongest recognition signal.

4.3. Screening of resistance of *Camellia sinensis* towards *G. cingulata*

It is well recognized that pathogenic variability poses difficulty in development and deployment of effective host resistance, which is a dependable and economic means of disease management. Besides, it is known to hinder the efforts to deploy host resistance as long-term plant disease control strategy. There should be efficient means of screening this resistance found naturally in the germplasm (pre-formed resistance).

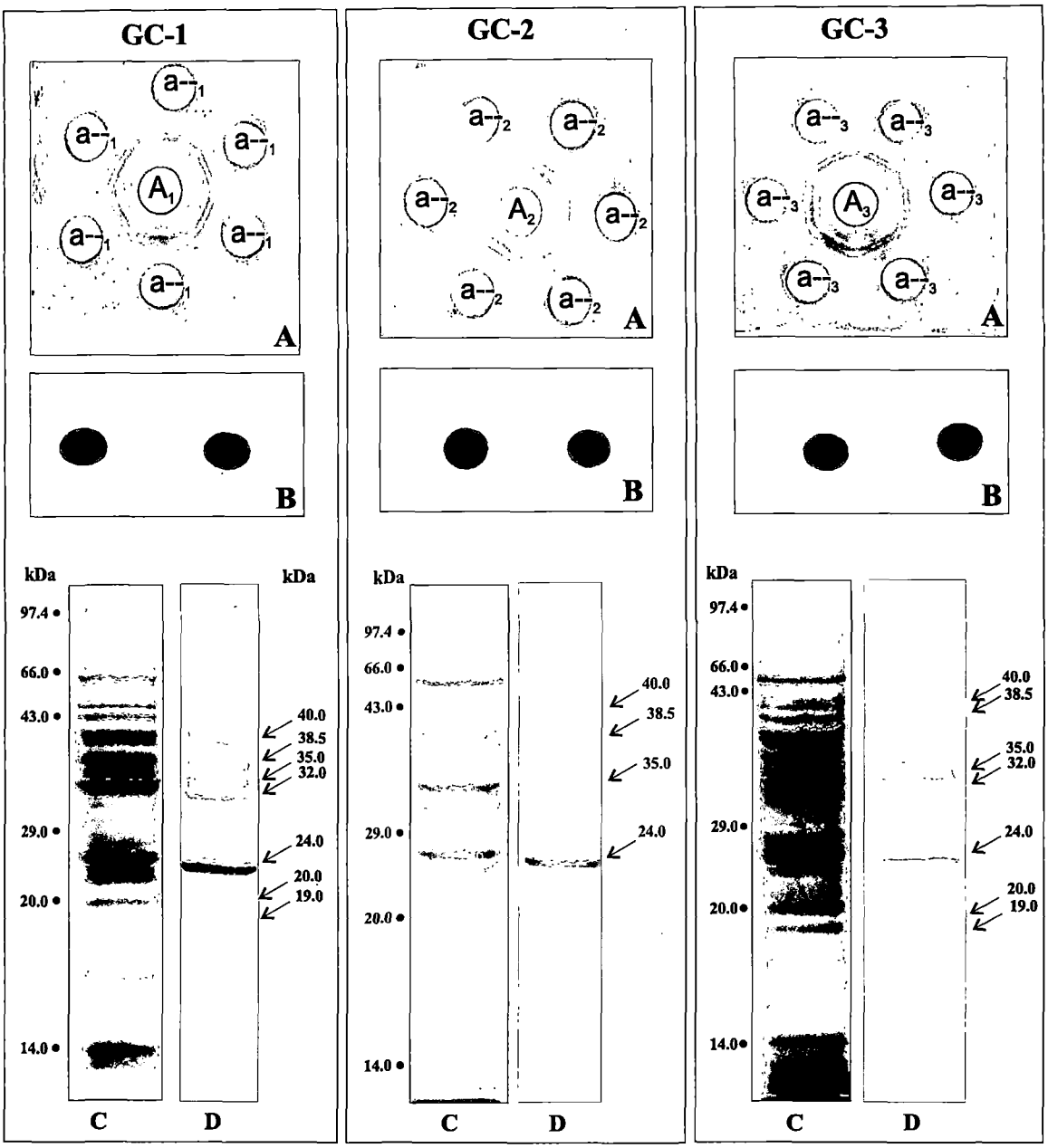


Plate 12 (A-C) : Serological comparison among isolates of *G. cingulata* using PAb of GC-1 and antigen of three isolates. (A) Immunodiffusion; (B) dot immunobinding assay; (C) SDS-PAGE profile of mycelial antigen; (D) Western Blot analysis [Central wells (A_1, A_2 & A_3) - PAb of GC-1; peripheral wells (a_1, a_2 & a_3)-mycelial antigen of isolates GC-1, GC-2 and GC-3 respectively].

Before going into molecular mechanism of plant-pathogen interaction, at the outset, eighteen tea varieties (TV-18, TV-22; TV-25, TV-26, TV-29, TV-30, T-17/1/54, CP-1/1, BS/7A/76, P-312, AV-2, TS-449, UP-2, UP-3, UP-9, UP- 26, BSS-2 and BSS-3) as mentioned in Materials and Methods, were used to identify the disease reaction towards the isolates of *G. cingulata*. The data was collected from 50 leaves of each variety after every interval separately for each isolate. Experiments were repeated thrice. For whole plant inoculation, disease incidence was scored by disease index. Twenty plants were used for screening in three independent experiments for each variety. Disease assessment was done after 25, 30 and 35 days of inoculation on the basis of appearance of symptoms as mentioned earlier. Screening was also done using immunoenzymatic formats – ELISA and Dot Immunobinding Assay (DIBA), which were less time consuming and more accurate. The results of pathogenicity tests and immunoenzymatic assays were compared and correlation was drawn on the basis of correlation studies (Karl-Person's Correlation coefficient).

4.3.1. Pathogenicity tests

4.3.1.1. Detached leaf

Artificial inoculation of detached leaves of eighteen tea varieties was carried out by spore suspension of *G. cingulata* (isolates GC-1, GC-2 and GC-3) separately as described presented in Plate 13, figs A-E . Assessment of inoculation infectivity and symptom development was done after 48, 72 and 96 hours after inoculation, on the basis of percent drops that resulted in disease lesion production. Results (Table 7, Fig. 3 A-C) revealed that T-17, TV-22, UP-26, BSS-3, AV-2 and CP-1/1 showed susceptibility on inoculation with all isolates. On the other hand, resistant reaction was shown by TV-30, TV-29, UP-2, BS/7A/76 and S-449. The remaining varieties showed moderately susceptible or resistant reactions. It is thus evident from Figure 3 (A-C) that TV-22 and T-17 were the most susceptible (exhibiting compatible reaction). TV-30, UP-2 and TS-449, on the other hand, were the most resistant (exhibiting incompatible reaction). There was a slight difference in the pathogenicity of different isolates. It is clear from the results that GC-1 is the most virulent, while GC-2 - the least virulent. Therefore, GC-1 was selected for further experiments.

4.3.1.1. Whole plant

Eighteen varieties of well established pot grown tea plants were inoculated with spore suspension of *G. cingulata* (GC-1). In TV-22, UP-26, BSS-3, AV-2 and CP-1/1 and T-17 disease intensity was high at each interval in comparison to the other varieties (Table 8 , Figure 4A). Restricted lesions, in contrast, were noticed in TV-30, UP-2 and BS/7A/76. The degree of susceptibility or resistance in the other varieties against *G. cingulata* was moderate. Besides, the Disease index (DI) of the 18 varieties was correlated with the percentage lesion formation in detached leaf method. The scatter diagram together with the regression line and regression equation is presented in Figure 4B. Linear relationship with positive slope is indicative of positive correlation between the values at the three intervals. Karl Pearson's correlation coefficient in this case was calculated as 0.719936, which was significant at P=0.05 level.

Results obtained from varietal resistance test performed on 18 tea varieties against *G.cingulata* (GC-1) following detached leaf and whole plant inoculation technique indicated that six varieties (TV-22, T-17/1/54, BSS-3, UP-26, CP-1/1 and AV-2) were highly susceptible, two varieties (TV-30 and BS/7A/76) were highly resistant, only one was moderately susceptible (UP-3), while the remaining nine varieties were moderately resistant.

4.3.2. Immunoenzymatic assays

G. cingulata is a pathogen with prolonged phase of quiescence or latency after ingress, which makes it difficult to detect. Whole plant inoculation screening requires around one month for symptoms to be significantly visible. The detection of fungal pathogen in tissues before the symptoms appear is possible by immunological methods like ELISA and Dot Immunobinding Assay.

4.3.2.1. PTA-ELISA

The antigen was extracted from healthy and *G. cingulata* inoculated tea leaf tissues of the 18 tea varieties as described in Materials and Methods. These were hybridized with the IgG raised against *G. cingulata* and screened by PTA-ELISA formats as described earlier. The antigen concentration was adjusted to 60μg/ml. The results presented in Table 9, were correlated with the pathogenicity test values in case infected

Table 7 : Pathogenicity test of *G.cingulata* isolates on detached tea leaves.

Variety	% Lesion formation									Disease Reaction
	Hours post inoculation									
	48			72			96			
	GC-1	GC-2	GC-3	GC-1	GC-2	GC-3	GC-1	GC-2	GC-3	
Tocklai										
TV-18	35.0 ±1.9	24.6 ±1.4	36.7 ±2.3	41.0 ±2.5	35.3 ±2.9	42.6 ±3.0	43.4 ±2.7	40.9 ±2.9	46.9 ±3.1	MS
TV-22	52.9 ±4.5	45.2 ±3.8	49.8 ±4.2	77.2 ±4.8	55.3 ±2.2	66.0 ±3.8	94.1 ±3.5	56.4 ±2.7	68.7 ±3.4	S
TV-25	26.7 ±1.8	05.0 ±1.8	11.6 ±2.1	32.0 ±2.6	12.9 ±2.7	15.8 ±3.5	32.6 ±4.0	22.4 ±2.5	27.8 ±3.3	MR
TV-26	19.0 ±2.7	10.9 ±3.5	15.2 ±3.1	39.0 ±2.6	17.5 ±2.0	24.3 ±1.8	40.2 ±3.3	24.1 ±2.2	35.9 ±3.8	MR
TV-29	15.9 ±2.3	09.8 ±3.5	10.3 ±3.2	25.5 ±2.1	11.6 ±3.2	13.6 ±2.7	36.4 ±4.2	12.1 ±3.5	15.9 ±3.4	MR
TV-30	06.3 ±0.5	01.3 ±3.8	06.3 ±3.5	20.4 ±3.7	13.5 ±2.2	15.9 ±3.5	23.4 ±2.9	13.6 ±3.4	22.5 ±3.0	R
T- 17	70.3 ±2.1	53.7 ±3.8	63.7 ±2.5	95.5 ±2.8	59.5 ±2.2	77.1 ±3.8	96.4 ±2.2	61.2 ±2.0	80.4 ±3.4	S
UPASI										
BSS-2	30.6 ±1.1	02.1 ±1.0	12.8 ±3.0	31.3 ±2.0	18.2 ±2.9	20.2 ±3.5	32.4 ±3.1	18.5 ±3.0	31.5 ±3.2	MR
BSS-3	52.7 ±4.3	40.9 ±2.8	42.3 ±4.2	64.4 ±3.9	48.5 ±2.2	49.1 ±3.8	67.2 ±2.5	50.3 ±4.1	60.7 ±3.0	S
UP-2	13.5 ±1.5	01.9 ±0.8	07.1 ±2.0	33.1 ±3.5	20.6 ±2.2	10.9 ±3.8	33.2 ±2.4	25.7 ±4.0	15.7 ±3.9	MR
UP-3	11.3 ±3.1	13.9 ±2.9	15.7 ±4.0	50.6 ±4.1	22.3 ±2.2	25.6 ±3.8	53.0 ±2.4	31.7 ±2.7	33.5 ±2.4	MS
UP-26	24.0 ±2.2	15.6 ±1.8	17.6 ±4.0	75.6 ±3.8	39.8 ±2.2	40.3 ±3.8	78.9 ±3.5	42.6 ±2.7	49.3 ±4.4	S
UP-9	15.1 ±3.7	10.9 ±2.8	11.3 ±4.0	40.0 ±4.5	12.9 ±2.2	22.9 ±3.8	41.9 ±1.9	21.4 ±1.7	31.6 ±3.9	MR
Darjeeling										
BS/7A/76	05.0 ±1.7	01.2 ±0.7	05.0 ±4.0	20.4 ±2.4	02.5 ±2.0	20.4 ±3.8	22.5 ±2.6	08.9 ±2.3	22.5 ±3.7	R
TS -449	7.2 ±1.5	05.1 ±2.0	06.3 ±4.0	31.0 ±3.1	15.9 ±2.9	25.4 ±3.8	33.1 ±1.4	28.4 ±2.7	32.6 ±2.4	MR
CP-1/1	42.3 ±2.2	32.8 ±4.1	20.1 ±4.0	63.2 ±3.2	46.3 ±2.2	47.6 ±3.8	67.3 ±2.1	54.1 ±2.8	65.3 ±3.5	S
P- 312	22.3 ±1.6	20.6 ±2.8	21.9 ±4.0	35.3 ±1.7	24.3 ±2.2	27.6 ±3.2	36.3 ±3.7	26.1 ±2.5	30.1 ±3.6	MR
AV-2	28.0 ±2.3	21.9 ±3.7	24.9 ±4.0	45.0 ±3.5	32.6 ±2.25	35.2 ±3.1	65.2 ±3.4	55.4 ±2.9	67.8 ±3.4	S

*Data are the mean of 200 inoculum droplets made on 50 leaves of each variety; ±SE; R - Resistant (0 – 25%) ; MR – Moderately resistant (26% - 45%)
MS – Moderately susceptible (46% - 65%) ; S – Susceptible(>65%)

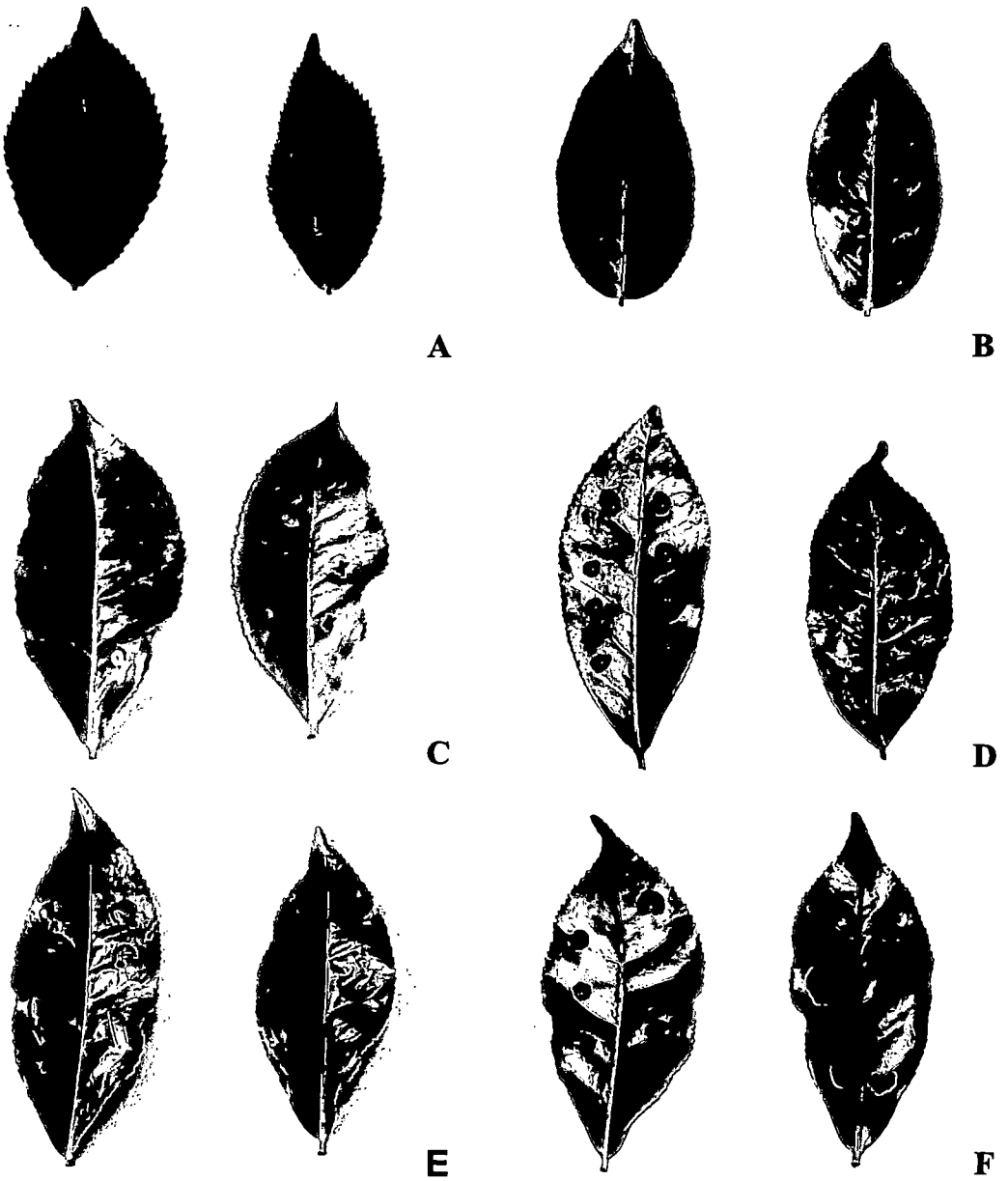


Plate 13 (A-F) : Lesion development on tea varieties P-312 (A), TV-29 (B), T-17/1/1 (C& F), TV-18 (D), and TV-22 (E) 48 hours post inoculation with *G. cingulata* isolates GC-1 (A & B), GC-2 (C & D) and GC-3 (E & F) ;[left - healthy; right inoculated with *G. Cingulata*].

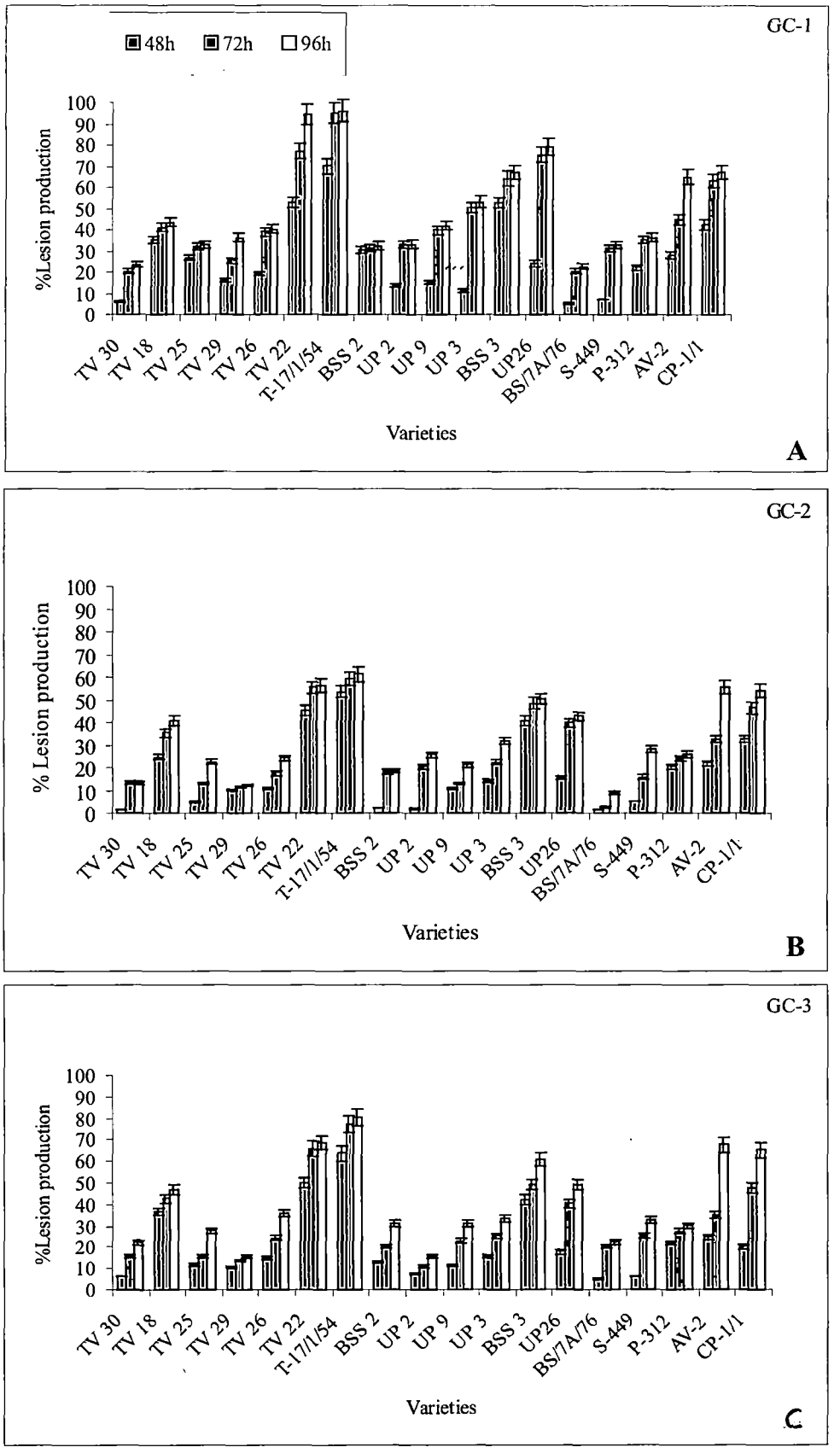


Fig. 3 (A-C): Screening of resistance of tea varieties against *G. cingulata* isolates.

leaves. There was a positive correlation coefficient (r= 0.986), which was significant at P=0.05 level. The scatter diagram and regression line are presented in Fig 4C. The varieties showing greater susceptibility had higher PTA-ELISA values, while those with greater resistance showed lower absorbance values.

Table 8: Screening of tea varieties against *G.cingulata* in potted plants.

Tea varieties	Mean disease index/plant		
	Days after inoculation		
	25	30	35
Tocklai			
TV-18	0.14 ± 0.04	0.84 ± 0.07	2.23 ± 0.07
TV-22	0.98 ± 0.17	4.48 ± 0.15	6.40 ± 0.06
TV-25	1.06 ± 0.09	3.67 ± 0.12	4.09 ± 0.13
TV-26	0.45 ± 0.14	2.49 ± 0.14	3.80 ± 0.16
TV-29	0.30 ± 0.05	1.48 ± 0.12	3.45 ± 0.16
TV-30	0.11 ± 0.07	0.38 ± 0.02	1.05 ± 0.17
T-17/1/54	1.35 ± 0.12	3.85 ± 0.16	5.64 ± 0.14
UPASI			
BSS-2	0.21 ± 0.04	0.75 ± 0.05	1.11 ± 0.11
BSS-3	1.26 ± 0.14	3.88 ± 0.16	5.58 ± 0.12
UP-2	1.18 ± 0.03	2.89 ± 0.04	3.96 ± 0.03
UP-3	1.11 ± 0.02	2.56 ± 0.03	4.89 ± 0.06
UP-9	1.32 ± 0.13	2.47 ± 0.11	3.48 ± 0.12
UP-26	1.21 ± 0.14	2.75 ± 0.05	6.10±0.11
Darjeeling			
BS/7A/76	0.16 ± 0.01	0.81 ± 0.04	0.98 ± 0.03
TS-449	1.34 ± 0.14	2.78 ± 0.16	3.47 ± 0.17
CP-1/1	1.45 ± 0.11	3.55 ± 0.17	4.96 ± 0.16
P-312	0.56 ± 0.21	1.86 ± 0.31	3.55 ± 0.22
AV-2	0.89 ± 0.10	2.87 ± 0.41	5.79 ± 0.13

± SE, n=3 ; ten plants were used per experiment

Analysis of variance

Source	D.F	S.S	M.S.	F	C.D (5%)
Varieties	17	60.13	3.54	06.32	1.24
Incubation period	2	85.80	42.90	76.61	
Error	34	19.10	0.56		
Total	53	165.03			

4.3.2.2. Dot Immmunobinding assay

For DIBA, total soluble protein extracts were prepared from healthy and artificially inoculated leaves of eighteen different tea varieties. Dot immunobinding assay was performed using these antigen preparation with IgG of *G. cingulata*. Antigens were carefully spotted on nitrocellulose paper and probed with this IgG. Results have been presented in Table 10. Clear and intense colour reactions were observed in case of homologous antigens of GC-1, GC-2 and GC-3, as noted in the prevoious chapter. In case of non-homologous reaction, there was wide variation in the intensity of the dots.

Greater colour intensity was noticed in TV-22 and T-17 varieties which showed susceptible reactions in varietal resistant test. TV-30 and BS/7A/76, on the other hand showed insignificant colour. These were incompatible with *G. cingulata.*

The 18 tea varieties showed differences in disease reaction against *G. cingulata* infection. The results obtained were similar whether assessed by the traditional methods or by immunological means, which conclusively proved that T-17 and TV-22 were highly susceptible while TV-30 and BS/7A/76 were highly resistant.

4.4. Disease reaction elicited by *G. cingulata* spore and cell wall and its characterization.

In the previous chapters the isolates were characterized, their virulence assessed and the single isolate (GC-1) was selected for further studies. It was now decided to study the disease reaction at light microscope level in susceptible and resistant (TV-30) leaf tissues. For the purpose, the tea leaves of TV-22 (susceptible variety) and TV-30 (resistant variety) were artificially inoculated separately on both-dorsal and ventral surfaces. The inoculated portions were cut out at 6,18,24 and 48hours after inoculation, cleared as described earlier and studied. Three samples were studied per treatment and the typical results presented. Percentage of germinated spores, appressoria formation and germ tube length were recorded in each case. A drop of same spore suspension was kept on glass surface and spore germination process recorded at same intervals. This served as surface control. Incubation temperature was maintained at

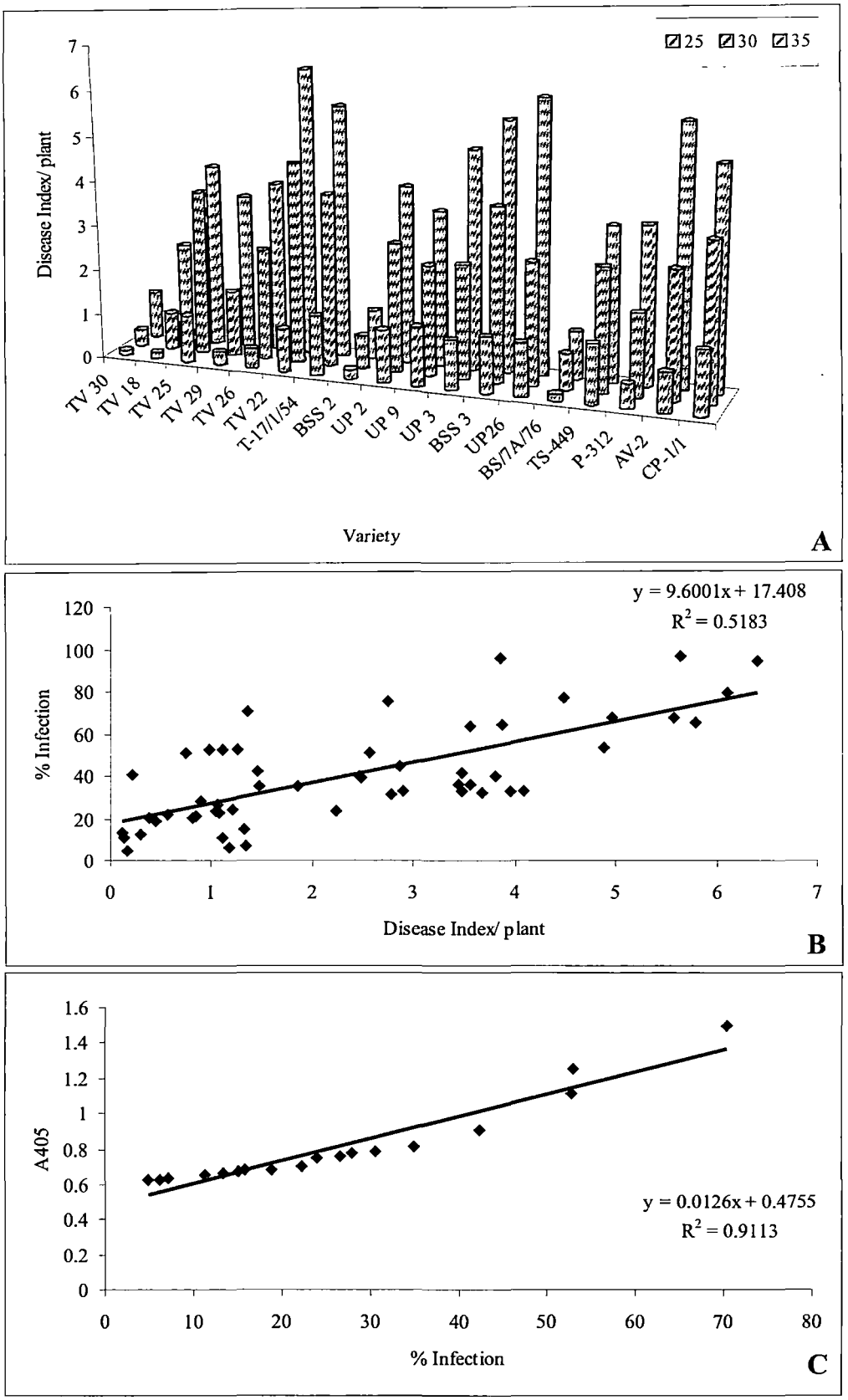


Fig. 4 (A-C): Screening of resistance of tea varieties against *G. Cingulata* (GC-1) (A) and regression analysis for the correlation between Disease index and percentage infection (B); PTA-ELISA and percentage infection.

+25°C and relative humidity at around 90% throughout the experiment to provide uniform and optimum germination conditions.

Table 9: PTA-ELISA reaction values of healthy and *G. cingulata* inoculated leaves of tea using PAb of *G. cingulata*

Antigen (50 μg ml^{-1})	PAb of *G. cingulata* A_{405}	
Leaf : Varieties	Healthy	Inoculated with *G. cingulata**
Tocklai		
TV-18	0.572 ± 0.048	0.996 ± 0.044
TV-22	0.699 ± 0.051	1.250 ± 0.051
TV-25	0.492 ± 0.055	0.955 ± 0.039
TV-26	0.459 ± 0.054	0.918 ± 0.058
TV-29	0.442 ± 0.047	0.927 ± 0.033
TV-30	0.356 ± 0.039	0.859 ± 0.035
T- 17/1/54	0.659 ± 0.031	1.506 ± 0.030
UPASI		
BSS-2	0.575 ± 0.066	1.023 ± 0.044
BSS-3	0.627 ± 0.063	1.280 ± 0.041
UP-2	0.414 ± 0.064	0.911 ± 0.035
UP-3	0.404 ± 0.061	0.902 ± 0.056
UP-9	0.436 ± 0.067	0.920 ± 0.047
UP-26	0.502 ± 0.055	0.954 ± 0.058
Darjeeling		
BS/7A/76	0.329 ± 0.062	0.828 ± 0.044
S -449	0.362 ± 0.063	0.867 ± 0.047
CP-1/1	0.579 ± 0.058	1.026 ± 0.036
P- 312	0.452 ± 0.049	0.920 ± 0.041
AV-2	0.562 ± 0.039	1.013 ± 0.038

*48 hours after inoculation with GC-1 isolate.

Note : Homologous reaction with antigen of *G. cingulata* (GC-1) and PAb of GC-1 gave ELISA value of 2.84 at A_{405}

Fungal cell wall is an important participant of plant-pathogen interactions, since it comes directly in contact with the plant tissues right after spore germination. Besides, cell wall fragments have been implicated as elicitors of antifungal compounds and recently, as inducers of resistance response. It was therefore, decided to examine whether any fungitoxic compounds were elicited by the cell wall in both- compatible and incompatible interactions. Besides, the kind of disease reaction, if any, elicited by the cell wall, was recorded. Lastly, the mycelial cell wall extract was analysed and characterized biochemically to confirm its nature.

Table 10: Dot immunobinding assay of healthy and *G. cingulata* inoculated leaves of tea using PAb of *G. cingulata*

Antigen (50 µg ml^{-1})	PAb of *G. cingulata* Colour intensity	
Leaf: Varieties	Healthy	Inoculated with *G. cingulata**
Tocklai		
TV-18	+	++
TV-22	++	++++
TV-25	±	+
TV-26	+	++
TV-29	+	++
TV-30	±	+
T- 17/1/54	++	++++
UPASI		
BSS-2	+	++
BSS-3	++	+++
UP-2	±	+
UP-3	+	++
UP-9	++	+++
UP26	++	++++
Darjeeling		
BS/7A/76	±	+
S -449	±	+
CP-1/1	+++	++++
P- 312	+	+++
AV-2	++	+++
Mycelia: *Glomerella cingulata*	++++	

Colour intensity of dots: ++ light violet; +++ violet; ++++ deep violet; ± insignificant ; – no colour reaction ; NBT/BCIP used as substrate ; PAb (1: 125)

*48 hours post inoculation with GC-1.

4.4.1. Spore of *G. cingulata*

The study of infection process was done *in vitro*. The mode of infection was studied in artificially inoculated detached leaves of two varieties – TV-22 (susceptible) and TV-30 (resistant) on dorsal and ventral surfaces as described earlier. The portions of inoculated tissues were decolorized after definite time periods, stained and observed under microscope. The data regarding the progress of spore germination is presented in Fig. 5(A-C).

The spores were found to have germinated producing distinct germ tubes 6 hours after inoculation (Plate 14, figs A & B) in case of glass slide germination as well as dorsal surfaces of tea leaves. Microconidiation was visible on the surface of resistant variety (Plate 14, fig. A). Germ tube length was also much smaller in this case. Germination was not initiated on the ventral surface of tea leaves in TV-30, while in TV-22 there was formation of germ tubes. Bipolar germination was observed on leaf surfaces. Many conidia became septate during germination. Numerous darkly melanized appressoria were produced 18 hours after inoculation (Plate 14, fig. D) on glass and TV-22 leaf surface. At 24 hours after inoculation the percentage of appressoria formation reached its peak. However, it was highest on ventral surface of TV-22 (74.2%) and lowest (15.4%) on the dorsal surface of TV-30. Surface of the susceptible variety was conductive for appressoria formation and germination started earlier (Plates 14 & 15). After the formation of appressoria, penetration hyphae were produced 48 hours after inoculation (Plate 14 & 15, figs G & H) and germ tubes were no longer distinct. Clear differences in the colonization pattern of ventral surface of tea leaves of resistant and susceptible varieties are exhibited in Plate 15, figs. A - H. Similar but not identical picture emerges on the dorsal surface of tea leaves. Events of secondary conidia formation sites of attempted penetration are visible on the surface of resistant variety (Plate 14 fig. G), but such structures were formed on the surface of the susceptible variety (Plate 14, fig. H). On the surface of TV-22, the susceptible variety, on the other hand, the penetration hyphae are produced and lead to necrosis of the tissues if the favourable conditions persist. This phase is exhibited by leaf tissues heavily colonized by the fungal mycelium at the site of formation of symptoms. Thus, it is clear from the present discourse that even though the spores of *G. cingulata* germinate on all the surfaces, the higher percentage of appressoria formation was associated with compatible

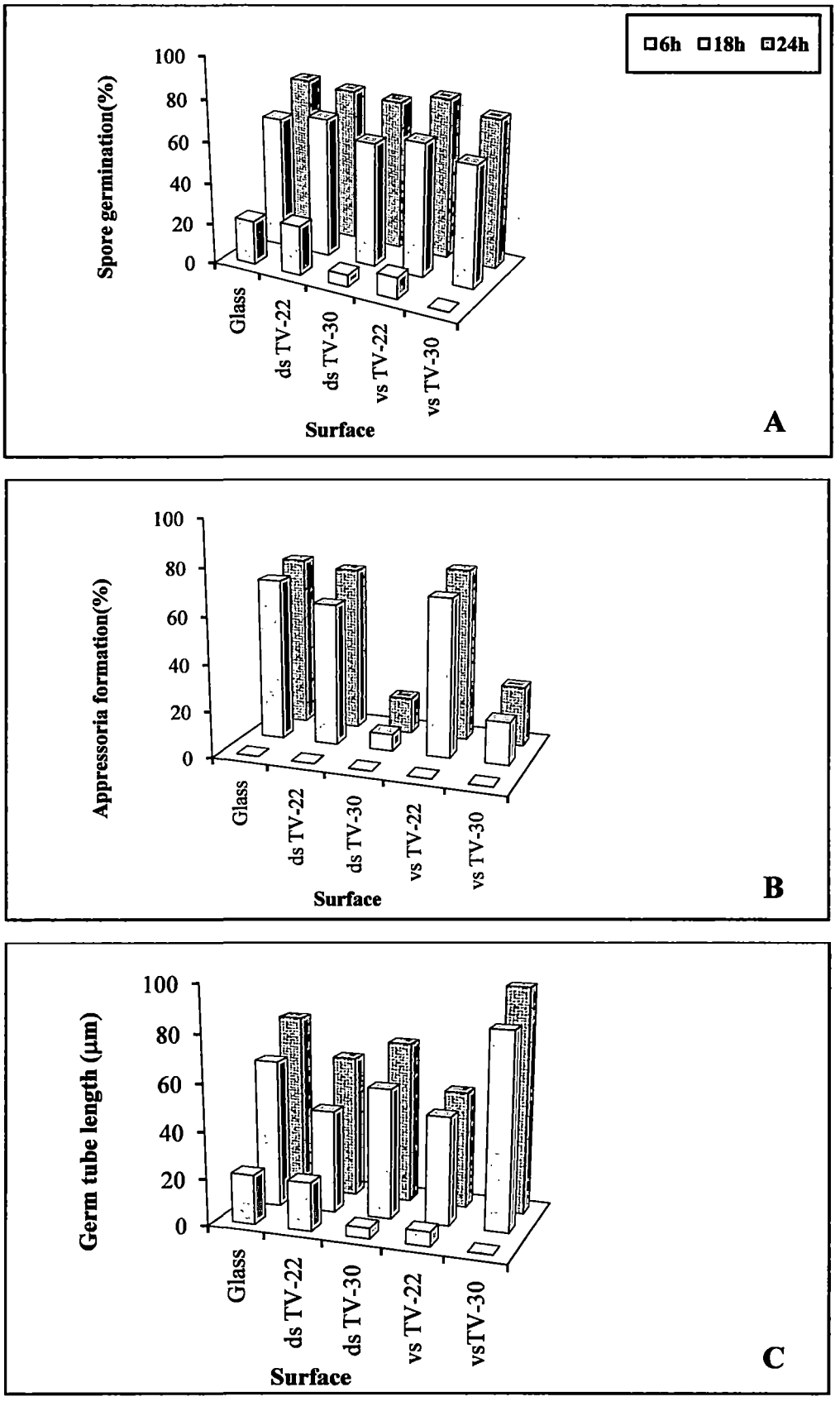


Fig.5 (A-C): Progress of *G. cingulata* spore germination (A), appressoria formation (B) and germ tube length (C) on dorsal surface and ventral surface (vs) of the varieties.

interaction. Besides, greater germ tube lengths and lower percentage of appressoria formation are characteristics of incompatibility under the present conditions.

4.4.2. Cell wall of *G. cingulata*

In order to determine the nature of disease reaction elicited by cell wall, initially, cell walls were isolated from *G. cingulata* cultures and the isolated cell walls were further extracted with NaOH as described earlier. Next, the detached leaves of one resistant (TV-30) and one susceptible (T-17/1/54) variety were slightly wounded and inoculated on adaxial surface separately with four different treatments such as – (a) spore suspension of *G. cingulata,* (b) mycelial wall extract preparation of *G. cingulata,* (c) spore suspension mixed with mycelial wall extract, (d) sterile distilled water.

Percentage lesion produced was calculated after 48, 72 and 96 hours after inoculation and the results (Table 11) revealed that the disease reaction observed with the spore suspension was the strongest, followed by spore suspension mixed with mycelial wall extract. Mycelial wall extract as such developed mild symptoms on tea leaves. Nevertheless, cell wall mimicked the symptoms elicited by the fungal spore suspension, developing higher percentage lesion formation in susceptible tea variety T-17/1/54 (20.1% at 96 hours after inoculation) and lesser percentage lesion formation in the resistant variety TV-30 (5.2% at 96 hours after inoculation) as evident from Table 11.

4.4.3. Bioassay of diffusible compounds elicited by cell wall

Since the mycelial wall extracts could elicit symptoms even in case of resistant variety, it was considered imperative to determine whether it elicited any antifungal compound. Bioassay of diffusible compounds collected after 48h from the adaxial surface of tea leaves of the two varieties (TV-30 and T-17/1/54) was performed as mentioned earlier following four treatments viz. distilled water, mycelial wall extract and distilled water, spore suspension and mycelial wall extract and spore suspension. Control sets of slide germinated spores were kept with mycelial wall extract and distilled water. Diffusible leaf compounds collected from leaf surface against all four treatments inhibited spore germination and appressoria formation markedly (Table 12). Besides, mycelial wall extract elicited diffusates collected from the surface of resistant variety (TV-30) were more fungitoxic than in case off susceptible variety (T-17/1/54). However, when the spores of *G. cingulata* were allowed to germinate on glass slides in presence of mycelial wall extract and distilled water separately, the germination percentage was

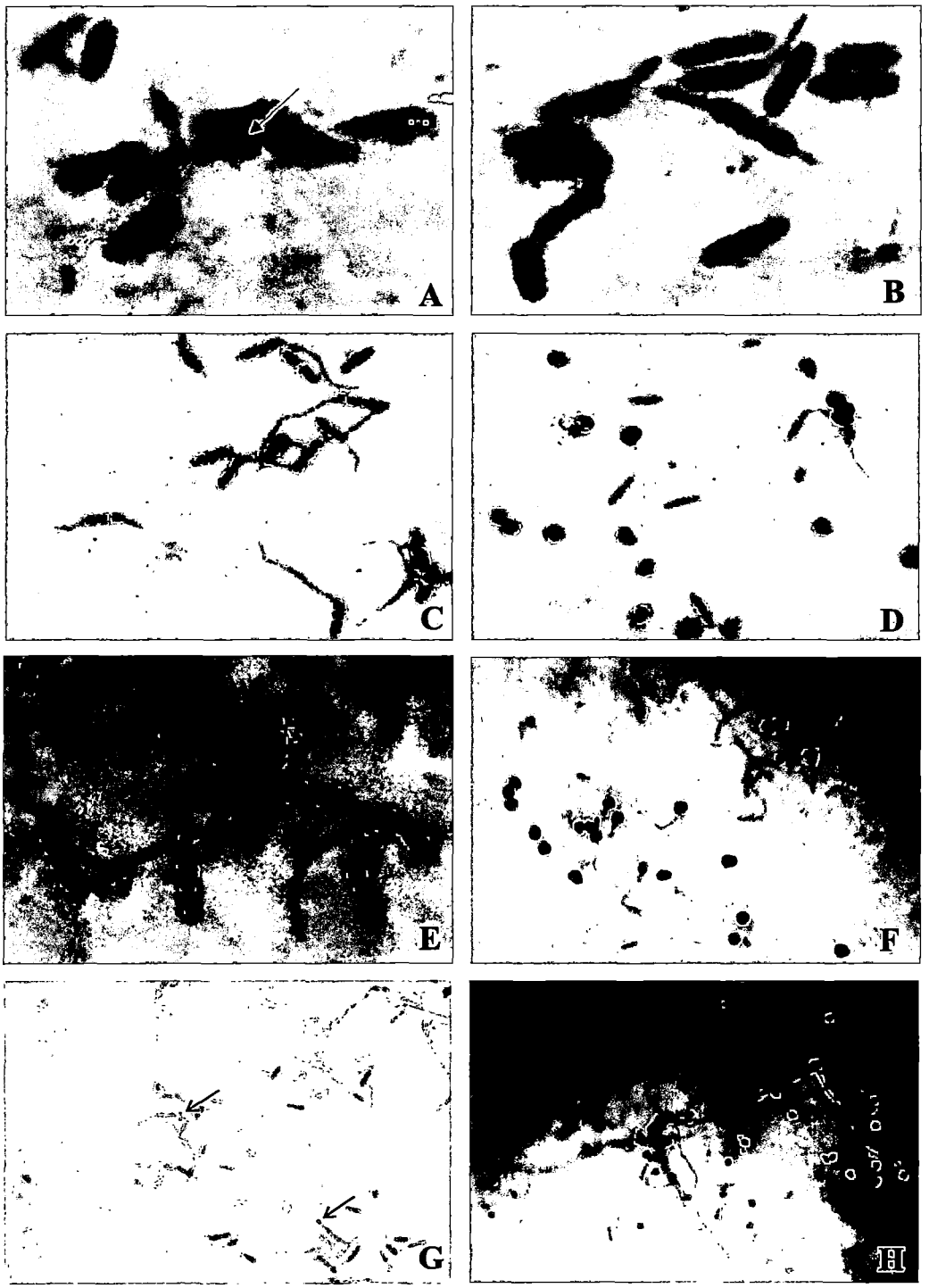


Plate 14 (A-H) : Progress of *G. cingulata* spore germination on the adaxial surface of tea varieties [TV-30 (A, C, E & G), TV-22 (B, D, F & H) at 6 h (A & B), 18 h (C & D), 24 h (E & F) and 48 h (G & H) post inoculation.

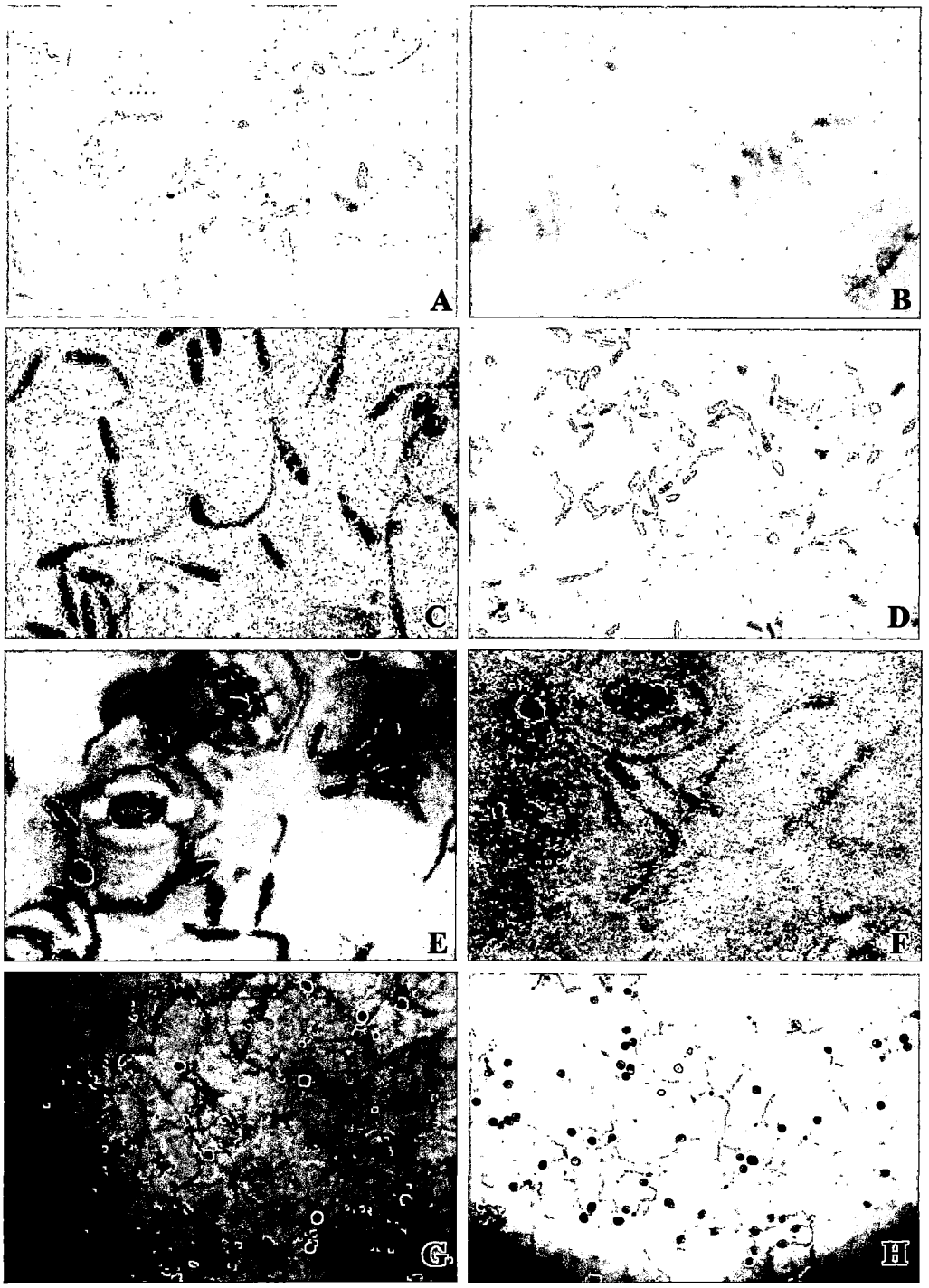


Plate 15 (A-H) : Progress of *G. cingulata* spore germination on the abaxial surface of tea varieties [TV-30 (A, C, E & G), TV-22 (B, D, F & H)] at 6 h (A & B), 18 h (C & D), 24 h (E & F) and 48 h (G & H) post inoculation].

similar in both the cases. Therefore, mycelial wall extract as such was not fungitoxic, but it elicited production of antifungal compounds on the host leaf surface.

4.4.4. Characterization of cell wall

4.4.4.1. Indirect Immunoflourescence

Indirect immunofluorescene of the isolated cell walls was conducted using PAb of *G. cingulata* as primary antibody and RITC-labeled anti-rabbit IgG conjugate as secondary antibody. Reddish fluorescence was observed on the cell walls (Plate 16, figs. B & C). This was compared with the fluorescence observed on mycelia labeled with FITC conjugates.

Table 11: Comparison of lesion production by mycelial wall extract and spore suspension of *G. cingulata* on detached tea leaves of resistant and susceptible varieties.

Variety	Treatment	Percentage lesion formation		
		Hours post inoculation		
		48	72	96
TV-30	Distilled water	0	0	0
	Mycelial wall extract	02.3 ± 1.45	03.8 ± 1.80	05.4 ± 1.74
	Spore suspension + Mycelial wall extract	10.1 ± 1.82	12.5 ± 2.44	14.6 ± 1.88
	Spore suspension	12.4 ± 2.13	19.3 ± 1.21	22.9 ± 0.94
T-17/1/54	Distilled water	0	0	0
	Mycelial wall extract	15.3 ± 3.01	18.5 ± 2.46	20.1 ± 2.77
	Spore suspension + Mycelial wall extract	52.8 ± 2.04	54.1 ± 2.11	55.8 ± 2.36
	Spore suspension	71.4 ± 3.25	94.3 ± 2.55	95.9 ± 1.47

Data are the mean of 200 inoculum droplets made on 50 leaves of each variety per experiment.
Values are means ± SE, n=3

Table 12: Spore germination bioassay of diffusible compounds elicited by mycelial wall extract of *G. cingulata*

Treatment	Variety	% Spore germination[a]	% Appressoria formation
Distilled water	TV-30	55.9 ± 2.08	52.1 ± 2.11
	T-17/1/54	62.4 ± 1.09	60.4 ± 1.55
Mycelial wall extract	TV-30	09.1 ± 1.29	0.7 ± 0.09
	T-17/1/54	44.5 ± 1.22	39.2 ± 1.43
Spore suspension	TV-30	13.9 ± 2.08	12.1 ± 2.11
	T-17/1/54	66.4 ± 1.13	65.1 ± 1.55
Mycelial wall extract + Spore suspension	TV-30	11.5 ± 1.28	10.2 ± 1.88
	T-17/1/54	52.7 ± 1.24	50.9 ± 1.79
Slide control: Distilled water		73.4 ± 1.64	70.3 ± 2.42
Mycelial wall extract		70.1 ± 3.06	68.6 ± 1.09

[a] Average of 500 spores per experiment
[b] Average of 60 germlings per experiment; Values are means ± SE , n=3

4.4.4.2. SDS-PAGE

Biochemical characterization of mycelial wall extract was done. Quantitative and qualitative estimation of total protein and carbohydrate content was performed in order to confirm its glycoprotein nature. The protein content in the isolated cell wall was estimated to be 10mg g^{-1} cell wall. Carbohydrate content was found to be 0.414 mg g^{-1} cell wall. This preparation was further analysed by SDS-PAGE. Mycelia, crude cell wall and cell wall extract were run on SDS-PAGE and stained for protein (Plate 16, fig. D) and carbohydrate (Plate 16, fig. E) . The results show that only a single high molecular weight protein band was visible in cell wall extract preparation as compared to the mycelial protein banding pattern. The same band was visible when stained for carbohydrate by PAS staining. The mycelial wall extract analysed before being

concentrated by PEG also shows presence of the same band, but it was very faint. No visible carbohydrate moiety was detected in mycelial preparation and crude cell wall. The molecular weight of the single glycoprotein band was found to be of ca 66.0 kDa.

4.5. Analysis of pathogen-induced proteins in tea varieties.

It is obvious from the results of the previous chapters that tea varieties showed differential responses towards infection by *G. cingulata*. Since proteins are the active components that participate in the various defence-related activities in the complex immune system of the plants, it was decided to characterise the total soluble proteins of the 18 tea varieties (TV-18, TV-22, TV-25, TV-26, TV-29, TV-30, T-17/1/54, CP- 1/1, BS/7A/76, P-312, AV-2, TS-449, UP-2, UP-3, UP-9, UP- 26, BSS-2 and BSS-3) biochemically and immunologically. At the onset, total soluble proteins were extracted from healthy and artificially inoculated tea leaves. Quantitative estimation of these was performed as described earlier in order to assess whether there are any changes in the total protein pool on infection and how these are related to resistance. These proteins extracted from tea leaves were next separated by SDS-PAGE. Protein profiles were analysed and the molecular weight of the bands revealed were determined. Following this, an attempt was made to find out if any of these proteins were of pathogen origin. For the purpose, the proteins from two susceptible (CP-1/1 and TV-22) and one resistant variety (TV-30) were transferred to nitrocellulose membrane, according to Western blot procedure described earlier, and probed with the PAb raised against *G. cingulata* mycelial antigen. The blot was compared with the corresponding SDS-PAGE and the molecular weights of the PIps -(Pathogen-Induced proteins) were determined in compatible and in the incompatible interactions.

4.5.1. Protein content of healthy and *G. cingulata* infected tea leaves

For analysis of total soluble proteins, the plant material was harvested at 48 hours after inoculation, when symptoms started appearing. Total soluble protein was extracted from healthy and artificially *G. cingulata* inoculated tea leaves of 18 varieties as described earlier. The results presented in Table 13 indicate that there was some variation in protein content of different varieties among themselves as well as due to inoculation. In the susceptible varieties (TV-22, T-17, UP-26, CP-1/1, AV-2) the protein content decreased significantly. On the other hand, in the resistant varieties (TV-18, TV-30,

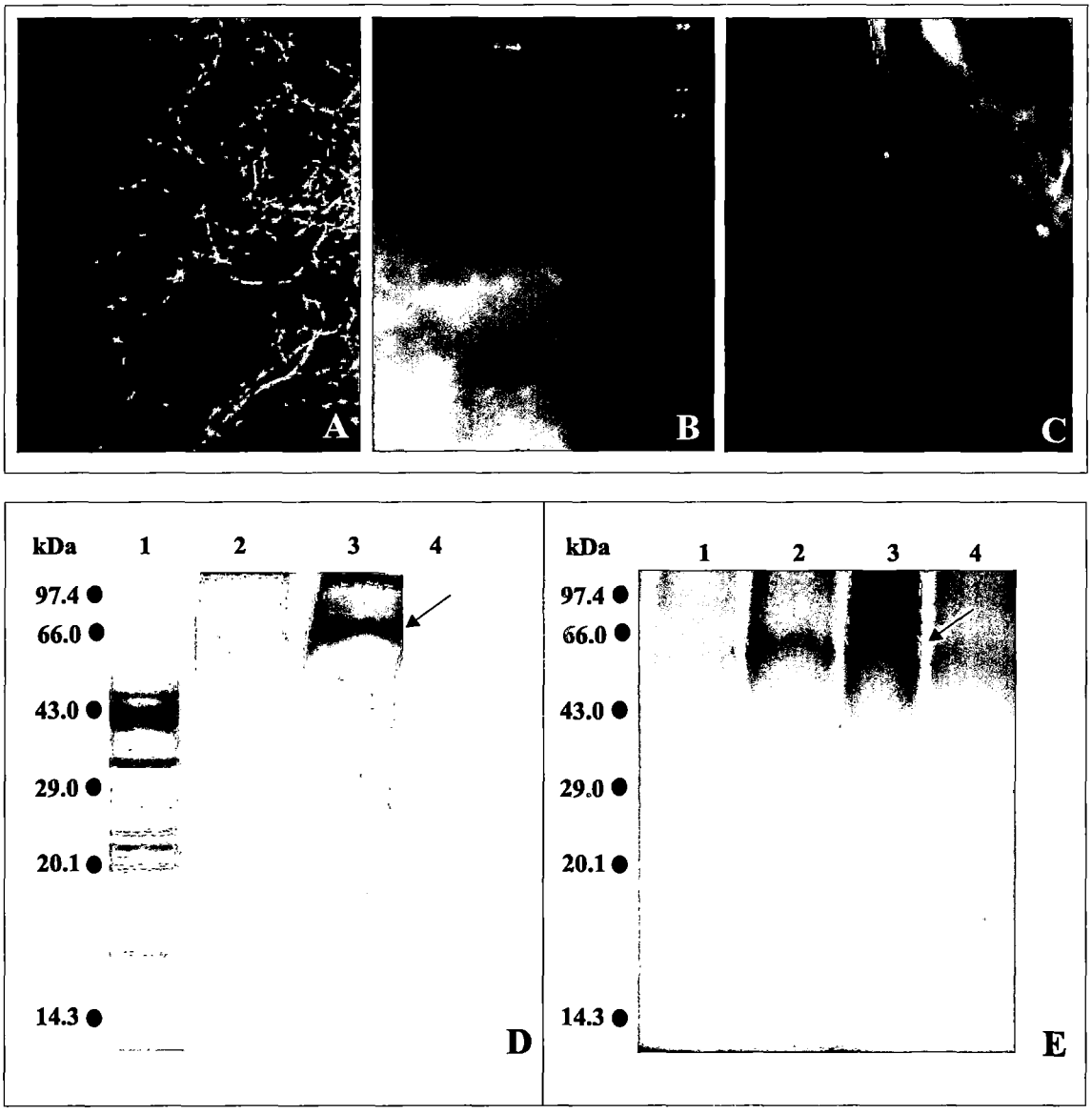


Plate 16 (A-D) : Indirect Immunofluorescence of hyphae (A) and cell wall (B & C) of *G. cingulata* treated with PAb of *G. cingulata* and labeled with FITC conjugates (A) and RITC conjugates (B & C) ; SDS-PAGE analysis of mycelial protein (Lane 1), undialized cell wall extract (Lane 2), cell wall extract after dialysis (Lane 3) and crude cell wall (Lane 4) after staining with coomassie blue (D) and periodic Schiff's reagent (E).

BSS-2, UP-9, BS/7A/76) there was a very insignificant increase in protein content on infection. However, no decrease in total protein was observed in such cases.

Table 13: Total soluble protein content of healthy and *G. cingulata* inoculated tea leaf tissues

Variety	Protein content (mg g tissue $^{-1}$)	
	Healthy	Inoculated**
Tocklai varieties		
TV-18	40.60 ± 1.50	43.10 ±1.80
TV-22	36.75 ± 1.23	*28.00 ± 1.15
TV-25	35.20 ± 1.17	40.00 ± 3.90
TV-26	43.50 ± 0.56	47.10 ±1.25
TV-29	45.72 ± 1.45	46.33 ± 2.96
TV-30	32.75 ± 1.24	37.00 ± 0.83
T- 17/1/54	39.25 ± 1.10	*23.21 ± 1.08
UPASI varieties		
BSS-2	18.00 ± 1.32	22.50 ± 2.25
BSS-3	22.80 ± 1.58	19.52 ± 2.47
UP-2	26.70 ± 0.80	27.32 ± 1.68
UP-3	32.81 ± 1.09	*23.60 ± 1.87
UP-9	22.00 ± 1.14	24.09 ± 2.92
UP-26	36.34 ± 1.07	*23.88 ± 0.56
Darjeeling varieties		
BS/7A/76	37.50 ± 1.27	42.70 ± 2.39
TS -449	26.81 ±1.02	27.23 ± 1.10
CP-1/1	32.12 ± 1.08	*25.64 ± 1.83
P- 312	30.51 ± 0.96	29.41 ± 0.75
AV-2	24.28 ± 0.86	20.30 ± 0.66

Means ± SE, n=3
* Difference between healthy and infected Significant at P=0.02 as tested by Student's t-test; ** 48 hours after inoculation with *G. cingulata* (GC-1).

4.5.2. SDS-PAGE of healthy and *G. cingulata* infected tea leaves

Proteins of the healthy and *G. cingulata* inoculated tea leaves of the 18 varieties as mentioned earlier were resolved on SDS-PAGE. Healthy and *G.cingulata* inoculated samples (48 hours after inoculation) were compared for any difference in the presence or absence of any particular protein bands. There were some differences in the banding patterns of different varieties (Tables 14-16, Plates 17 & 18). Also, pathogen infection

caused changes in the banding patterns. SDS- PAGE analysis of these extracted proteins reflected the protein content changes, with decreased content showing reduction in number of bands as well as general decreased intensity of protein bands (Plates 17 & 18). No reduction in intensity of band profiles was observed in resistant varieties on inoculation. In the most susceptible varieties (TV-22, T-17, UP-26) leaf proteins of ca. 97.4, 94.3 kDa were degraded by the fungus. New proteins were not induced. On the other hand, in case of the resistant varieties the same proteins were either newly induced (TV-25 and TV-26) or increased in intensity on inoculation with *G. cingulata* (TV-29). Thus, these seem to be associated with resistance. On the other hand, in some moderately resistant varieties (TV-29, BSS-2, BS/7A/76, AV-2) the banding pattern did not change at all. There were only slight changes in the intensity depending on the total protein content.

Table 14: Molecular weights of soluble proteins of healthy and *G. cingulata* inoculated tea leaf tissues in Darjeeling varieties.

Variety	Treatment	Molecular weight (kDa)
BS/7A/76	H	94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 29.0, 24.6, 20.1, 14.3, 3.0 **(11)**
	I	94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 29.0, 24.6, 20.1, 14.3, 3.0 **(11)**
S -449	H	94.3, 66.0, 56.8, 29.0, 24.6, 20.1, 14.3, 3.0 **(08)**
	I	94.3, 69.2, 66.0, 56.8, 47.6, 43.6, 29.0, 24.6, 20.1, 14.3, 3.0 **(11)**
CP-1/1	H	69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 20.1, 14.3, 6.0 **(11)**
	I	69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 20.1, 6.0 **(10)**
P- 312	H	69.2, 66.0, 56.8, 47.6, 40.0, 24.0, 20.1, 06.0, 3.0 **(9)**
	I	94.3, 69.2, 66.0, 56.8, 47.6, 40.0, 24.0, 20.1, 06.0, 3.0 **(10)**
AV-2	H	69.2, 66.0, 56.8, 47.6, 40.0, 24.0, 20.1, 06.0, 3.0 **(9)**
	I	69.2, 66.0, 56.8, 47.6, 40.0, 24.0, 20.1, 06.0, 3.0 **(9)**

Values in parenthesis indicate the number of bands; H – Healthy, I – Inoculated with *G. cingulata* (48 hours after inoculation).

4.5.3. Dot immunobinding assay.

The soluble proteins of the 18 tea varieties were initially characterized by dot immunobinding assay using PAb of *G. cingulata*. The results displayed in Plate 19, fig. B show that there was more intense reaction in case of the susceptible varieties (TV-22,

UP-26, CP-1/1, T-17/1/54) than in the resistant varieties (TV-30, BS/7A/76, BSS-2) in heathy as well as inoculated tea leaf tissues.

Table 15: Molecular weights of soluble proteins of healthy and *G. cingulata* inoculated tea leaf tissues in UPASI varieties.

Variety	Treatment	Molecular weight (kDa)
BSS-2	H	56.8, 40.0, 33.0, 24.6, 20.1, 14.3, 6.0, 3.0 **(8)**
	I	56.8, 40.0, 33.0, 24.6, 20.1, 14.3, 6.0, 3.0 **(8)**
BSS-3	H	97.4, 94.3, 66.0, 56.8, 20.1, 14.3, 6.0 **(7)**
	I	94.3, 66.0, 56.8, 20.1, 14.3, 6.0 **(6)**
UP-2	H	94.3, 66.0, 56.8, 43.0, 41.0, 40.0, 14.3, 11.5, 6.0 **(9)**
	I	94.3, 66.0, 56.8, 43.0, 41.0, 40.0, 15.2,14.3, 11.5, 6.0 **(10)**
UP-3	H	94.7, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0, 24.6, 14.3, 3.0 **(10)**
	I	47.6, 43.0, 40.0, 33.0, 29.0, 24.6, 3.0 **(07)**
UP-9	H	94.3, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0, 24.6, 14.3, 6.0, 3.0 **(11)**
	I	56.8, 41.0, 40.0, 33.0, 29.0, 24.6, 3.0 **(8)**
UP-26	H	97.4, 69.2, 66.0, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0, 20.1, 14.3, 6.0, 3.0 **(13)**
	I	66.0, 41.0, 40.0, 33.0, 29.0, 20.1, 14.3, 3.0 **(8)**

Values in parenthesis indicate the number of bands; H – Healthy, I – Inoculated with *G. cingulata* (48 hours after inoculation).

4.5.4. Western blot of healthy and *G. cingulata* infected tea leaves

The soluble proteins of three varieties were probed with antibody raised against *G. cingulata* by Western blot technique to reveal the presence of any common antigenic determinants between host and the pathogen. The most susceptible tea varieties – TV-22 and CP-1/1 indicated the presence of cross-reacting proteins with molecular weights ca. 66.0 and 56.8kDa even in the healthy leaf tissues (Plate 19, fig.C, Table 17). No such proteins were detected in the resistant variety TV-30. *G. cingulata* inoculated leaf tissues of CP-1/1, revealed two prominent bands of ca 41.0, and 43.0kDa, while TV-22 exhibited an additional band of ca 24.0 kDa. Thus, the differences in the proteins that could not be revealed by SDS-PAGE (Plate 19, fig.A), were obvious when probed with the PAbs. The different kinds of polyclonal antibodies are each raised against a specific epitope on the surface of the antigen. Thus, acting together, they may recognize protein(s) different from those against which the PAbs were raised, resulting in cross-reactivity. In the infected tissues this cross-reactivity may be due to the presence of the

fungal mycelia. The bands of ca 40.0 and 24.0 kDa revealed on Western Blot in leaf tissues may be identical to those of fungal protein. It may be recalled that the protein band with ca 24.0 kDa gave the strongest signal on probing with the same antibody.

Table 16: Molecular weights of soluble proteins of healthy and *G. cingulata* inoculated tea leaf tissues in Tocklai varieties.

Variety	Treatment	Molecular weight (kDa)
TV-18	H	69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0,20.1, 16.0, 14.3, 13.0, 11.2, 6.0, 3.0 **(16)**
	I	*94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0,20.1, 16.0, 14.3, 13.0, 11.2, 6.0, 3.0 **(17)**
TV-22	H	94.7,69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 20.1, 14.3, 6.0 **(12)**
	I	69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 14.3 **(09)**
TV-25	H	69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0,20.1, 14.3, 13.0, 11.2, 6.0, 3.0 **(15)**
	I	*97.4, *94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0,20.1, 14.3, 13.0, 11.2, 6.0, 3.0 **(17)**
TV-26	H	94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 24.6,29.0,20.1, 16.0,14.3, 13.0, 11.2, 6.0, 3.0 **(18)**
	I	*97.4, 94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 24.6,29.0,20.1, 16.0,14.3, 13.0, 11.2, 6.0, 3.0 **(19)**
TV-29	H	97.4, 94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 24.6,29.0,20.1, 16.0,14.3, 13.0, 11.2, 6.0, 3.0 **(19)**
	I	97.4, 94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 24.6,29.0,20.1, 16.0,14.3, 13.0, 11.2, 6.0, 3.0 **(19)**
TV-30	H	94.7,69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 20.1 **(10)**
	I	94.7,69.2, 66.0, 56.8, 41.0, 40.0, 29.0, 25.4, 24.0, 20.1, 14.3, 6.0 **(12)**
T- 17/1/54	H	94.3, 69.2, 66.0, 56.8, 47.6, 43.0, 41.0, 40.0, 33.0, 29.0,20.1, 16.0, 14.3, 13.0, 11.2, 6.0, 3.0 **(17)**
	I	66.0, 47.6, 40.0, 33.0, 29.0, 20.1, 3.0 **(7)**

Values in parenthesis indicate the number of bands; H – Healthy, I – Inoculated with *G. cingulata* (48 hours after inoculation).

The resistant variety TV-30 did not show any bands in healthy protein extract, while only 66.0 kDa protein was observed on inoculation with *G. cingulata*. This protein seems to be aiding in general pathogenesis, at the time of symptom appearance in both

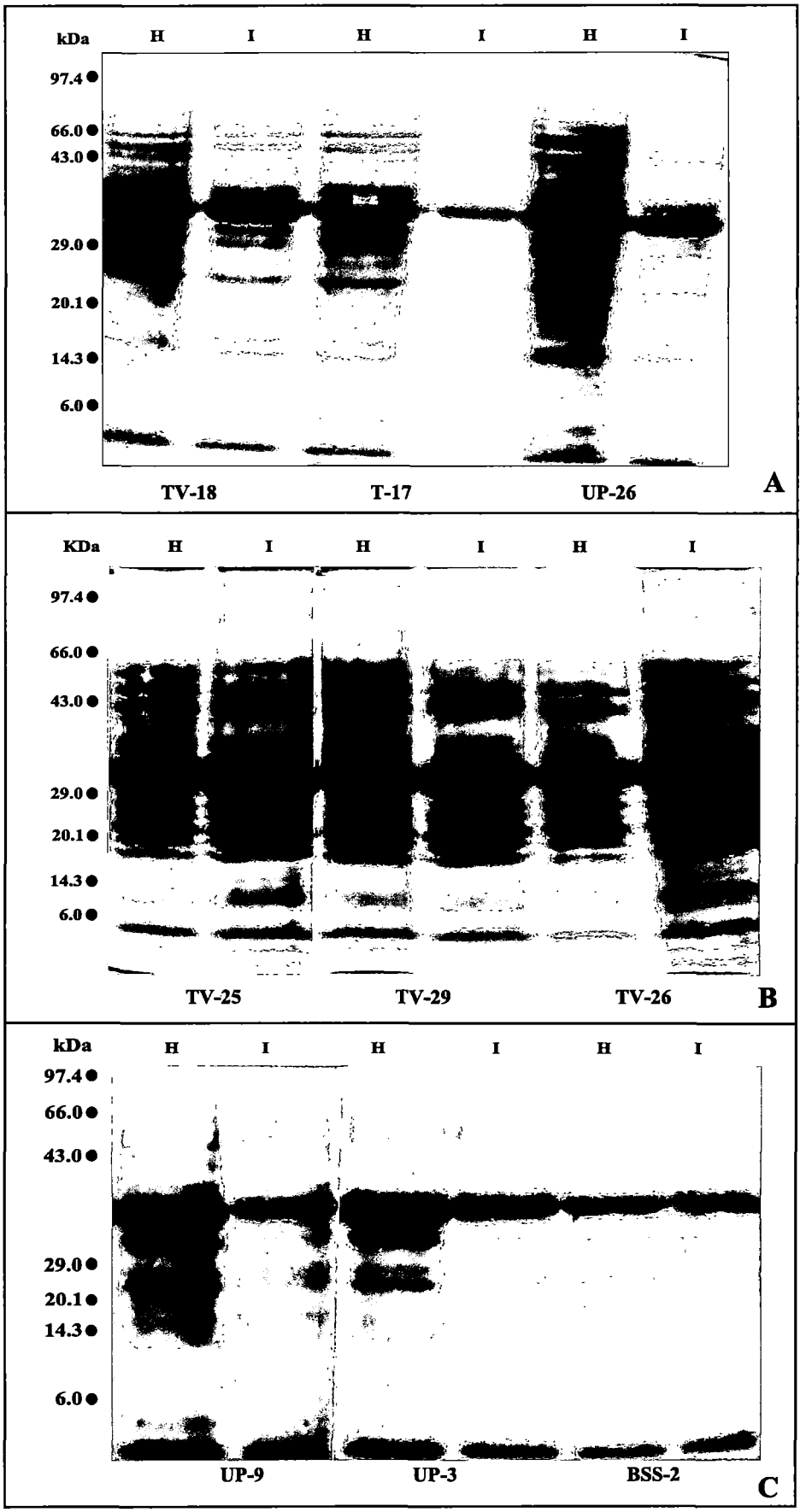


Plate 17 (A-C): SDS-PAGE analysis of soluble proteins from healthy (H) and *G. cingulata* inoculated (I) leaf tissues of tea varieties.

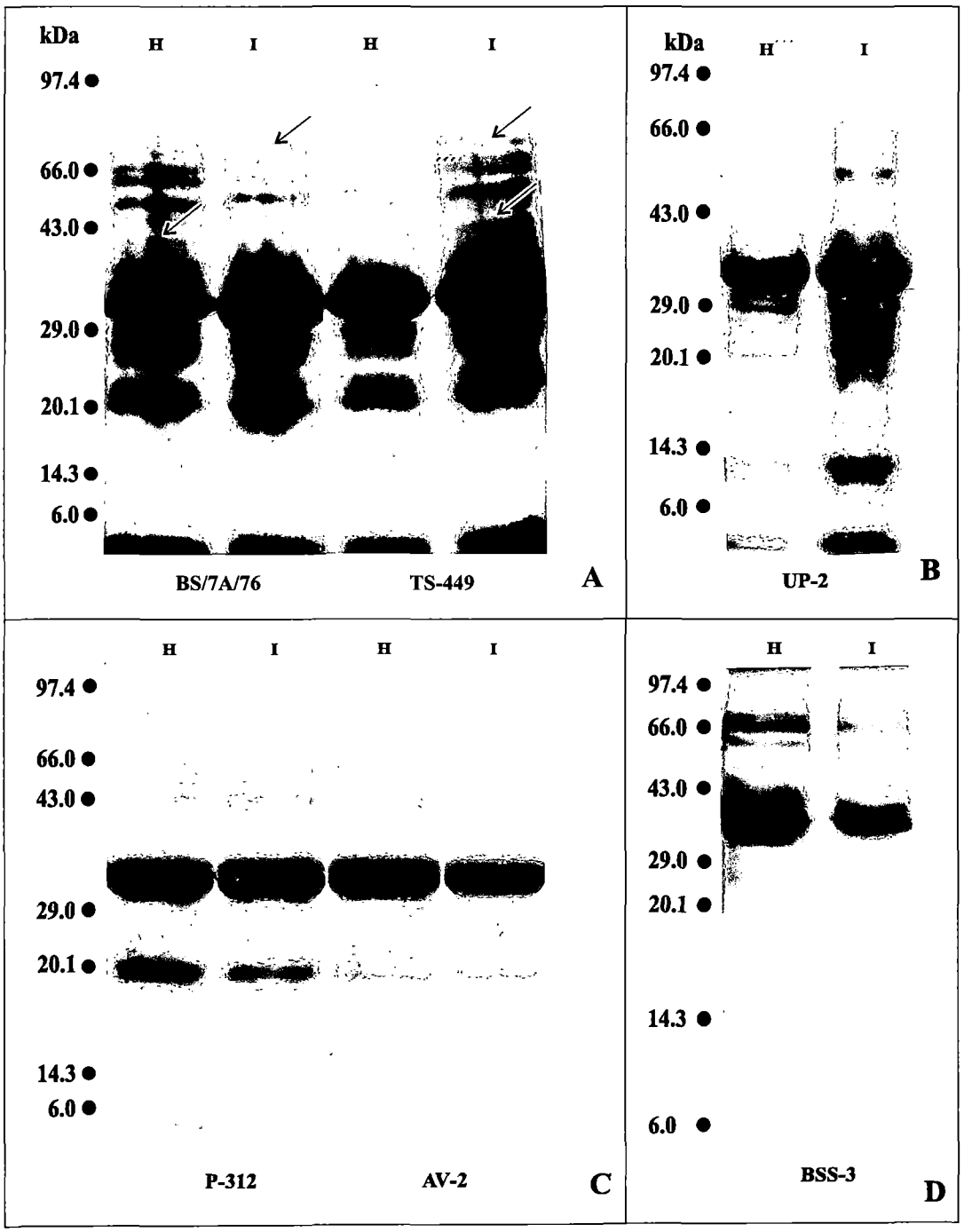


Plate 18 (A-D): SDS-PAGE analysis of soluble proteins from healthy (H) and *G. cingulata* inoculated (I) leaf tissues of tea varieties.

the susceptible and resistant interactions. Cell wall of *G. cingulata* was also found to be possessing the same molecular mass of 66.0 kDa.

Table 17: Western blot analysis of Pathogen-induced proteins in healthy and *G. cingulata* inoculated tea leaves

Variety	Treatment	Molecular weight (kDa)
CP-1/1	H	66.0, 56.8 (2)
	I	66.0, 56.8, 43.0, 40.0 (4)
TV-22	H	66.0, 56.8 (2)
	I	66.0,56.8, 43.0, 40.0, 24.0 (4)
TV-30	H	- (0)
	I	66.0 (1)

Values in parenthesis indicate the number of bands; H – Healthy, I – Inoculated with *G. cingulata.* (48 hours after inoculation).

4.6. Determination of activity of defense enzymes in tea varieties triggered by *G.cingulata*

Detailed analysis regarding the alteration of proteins has been carried out quantitatively as well as qualitatively in the previous section. Enzymes are the most important proteins that catalyse crucial biochemical reactions. Numerous enzymes have been identified to be involved in defense reactions of the plants. Four defense enzymes – peroxidase (POX), polyphenol oxidase (POX), phenyl alanine ammonia lyase(PAL) and tyrosine ammonia lyase (TAL) have been selected for the present study due to their involvement with phenolic biosynthesis (PAL and TAL) and phenolic oxidation (POX and PPO). Phenolics have always been in focus of studies on *Camellia sinensis*. POX is an important pathogenesis-related protein of PR-9 family. PPO catalyses the oxidation of polyphenols into simple phenolics that are important components of defence specially in tea plants. PAL and TAL catalyse the initial steps in phenyl propanoid pathway and their importance in defense mechanism is immense. Therefore, in the present investigation, eighteen tea varieties as mentioned earlier were inoculated by detached leaf inoculation method and time course accumulation of the above-mentioned four enzymes was conducted. Healthy control and *G. cingulata* inoculated tea leaf samples were harvested at 0, 24, 48 and 72 hours after inoculation, enzymes extracted and specific activity estimated.

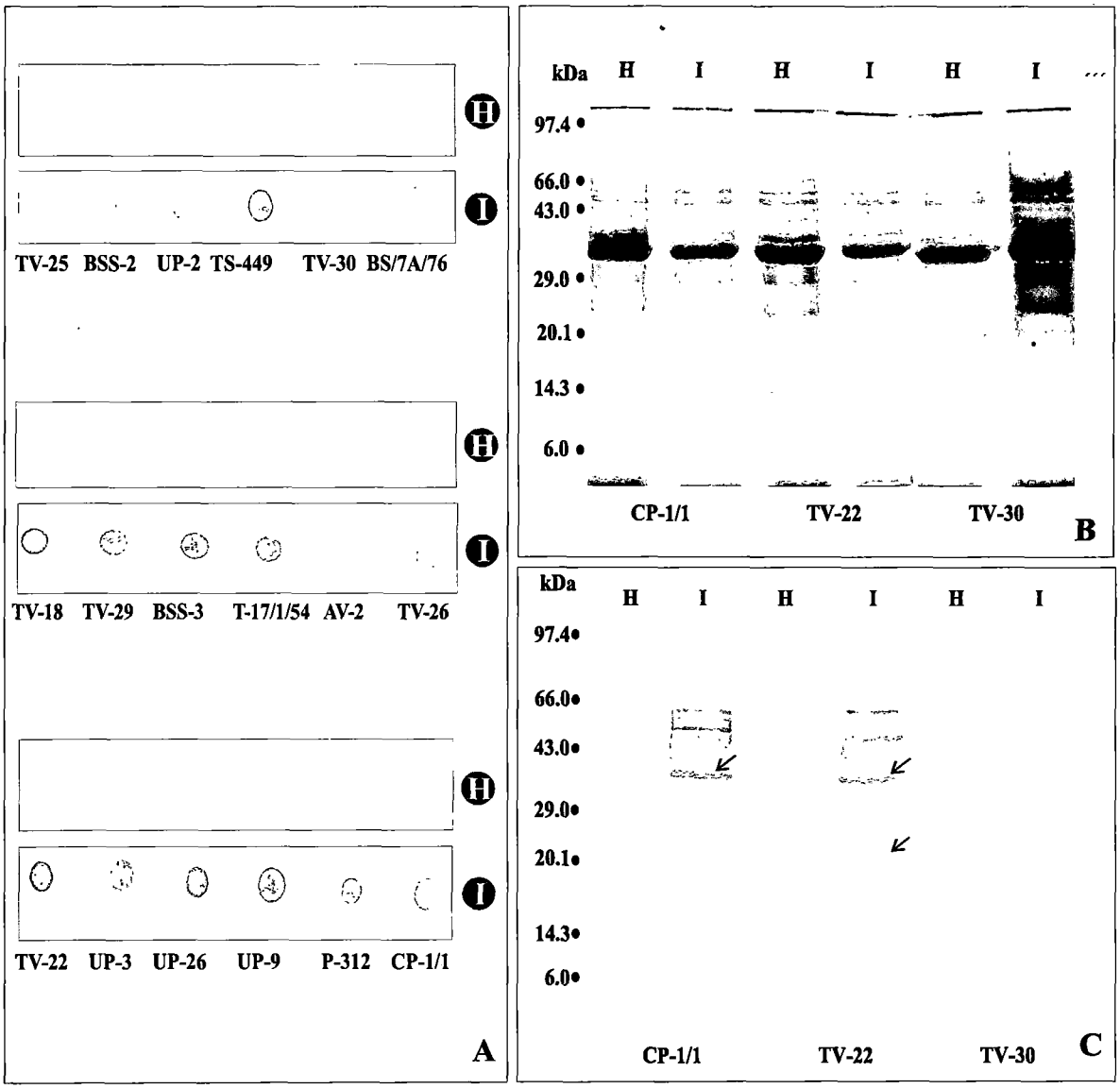


Plate 19 (A-C) : SDS-PAGE analysis (B) , Dot immunobinding assay (A) and Western Blot analysis (C) of antigens prepared from healthy (H) and *G. cingulata* inoculated (I) leaf tissues of tea varieties probed with PAb of *G. cingulata* (A & C).

spectrophotometrically as described earlier. Protein content of the enzyme extracts was measured as mentioned earlier and specific activity was calculated.

Multiple forms of enzymes (isozymes) are known to be present in case POX and PPO. These two enzymes have been reported to be associated with the potential quality of tea clones in relation to fermentation during tea processing. Anionic isoperoxidases and isopolyphenoloxidases - constitutive and induced on *G. cingulta* inoculation, were revealed by Native PAGE as described earlier.

The results of enzymes analysed were compared with disease reaction of the varieties as determined from the earlier experiments in order to relate the role of each enzyme in defense mechanism of *C. sinensis* - *G. cingulata* interaction.

4.6.1 Peroxidase

Peroxidases are members of a large group of heme-containing glycoproteins that catalyze oxidoreduction between hydrogen peroxide and various reductants. They have an absolute requirement of hydrogen peroxide as electron donor. Peroxidases are implicated to play multiple roles in plant-pathogen interactions. In case of peroxidase activity (quantitative analysis) o-dianisidine was used as substrate and its oxidation was monitored spectrophotometrically. For qualitative profiling of POX isozymes benzidine, another aromatic diamine, was used as a substrate. It gave rise to distinct blue coloured bands after separation of enzyme extract by Native PAGE and staining.

At the onset, peroxidase specific activity was assessed in healthy and *G. cingulata* inoculated tea leaf tissues at definite time intervals as mentioned above for the eighteen tea varieties. The results are presented in Table 18, from which it is clear that at the constitutive level there was no correlation between POX activity and resistance to *G. cingulata*.

The specific activity of peroxidase in the highly resistant varieties (TV-30 and BS/7A/76) as well as moderately resistant varieties (TV-18, TV-25, TV-26, TV-29, BSS-2, UP-2, UP-9, TS-449 and P-312) followed a pattern (Fig. 6A, C & E), in contrast to the highly susceptible varieties (TV-22, T-17/1/54, BSS-3, UP-26,CP-1/1 and AV-2) as illustrated in Fig. 6 (B, D & F). In resistant interactions, the activity slightly decreased 24 hours after inoculation in the inoculated samples relative to control, except in case of UP-2, where it slightly increased. However, there was recovery of the activity in such cases 48 hours after inoculation when it shot up to as much as 5.25 mg^{-1} protein min^{-1} in

BSS-2. Thus, significant differences between healthy and *G. cingulata* inoculated samples were observed only 48 hours after inoculation in the incompatible interactions (Table 18).

On the other hand, in compatible interactions the activity increased significantly with respect to control at 24 hours after inoculation in the inoculated samples. It shot up to as much as 6.05 unit mg^{-1} protein min^{-1} in TV-22, the highly susceptible variety. However, there was a dramatic drop in this activity at 48 hours after inoculation in these cases as observed in the Fig. 6 (B, D & F), except the moderately susceptible UP-3, in which there was only slight reduction in activity.

Thus, the activity of peroxidase reached its peak 24 hours after inoculation or 48 hours after inoculation in the inoculated samples, depending on the compatibility with the pathogen, and dropped thereafter i.e. at 72 hours after inoculation in case of all the 18 varieties studied. Peak activity was always higher in the inoculated samples than the healthy control. Thus, in the present findings, in all the interactions POX increased on infection. However, the timing of increase was not same. At 24 hours after inoculation POX activity is associated with susceptibility, while at 48 hours after inoculation high activity is associated with resistance. Karl-Pearson's Correlation coefficient between percentage infection, and POX activity was found to be -0.74178, which was highly significant at $P = 0.05$ level. The scatter diagram and the regression line are represented in Fig. 8A. Therefore, at the time of symptom appearance the POX activity was high in the incompatible interactions, where the percentage infection was low. Thus, at this time point POX contributes to resistance.

The highest activity in case of inoculated samples, 6.05 units mg^{-1} protein min^{-1}, was recorded in TV-25, the resistant variety. The lowest activity, 0.92 units mg^{-1} protein min^{-1}, was recorded in the inoculated samples of CP-1/1, the susceptible variety.

4.6.1.1. Peroxidase isozyme analysis

Isoperoxidase variations have been reported to be used as genetic markers in case of different levels (genus, species, varieties) within a taxon. At the onset, it was therefore, decided to study whether any isozyme variations exist at the constitutive level in *Camellia sinensis* (L.) O. Kountze varieties under the present investigation. In order to reveal changes in the isozyme patterns on infection, native polyacrylamide gelelectrophoresis was performed as described earlier in the 18 varieties as mentioned

Table 18: Peroxidase activities in different tea varieties at various time intervals after inoculation with *G.cingulata*

Variety	Treatment	Peroxidase activity (ΔA_{460nm} mg protein^{-1} min.$^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
TV-18	H*	3.28 ± 0.41	2.82 ± 0.52	2.71 ± 0.23	1.93 ± 0.42
	I	3.73 ± 0.44	3.45 ± 0.91	**4.66 ± 0.33	2.05 ± 0.72
TV-22	H	1.95 ± 0.63	1.83 ± 0.22	1.67 ± 0.54	0.97 ± 0.17
	I	2.12 ± 0.16	**6.05 ± 0.92	2.25 ± 0.35	**2.34 ± 0.36
TV-25	H	4.42 ± 0.27	3.73 ± 0.13	3.55 ± 0.26	2.54 ± 0.19
	I	4.55 ± 0.32	3.69 ± 0.63	**5.07 ± 0.34	2.58 ± 0.54
TV-26	H	4.04 ± 0.44	2.70 ± 0.34	2.56 ± 0.27	1.95 ± 0.46
	I	4.12 ± 0.71	**3.73 ± 0.39	**4.74 ± 0.28	2.26 ± 0.83
TV-29	H	2.05 ± 0.37	1.63 ± 0.26	1.17 ± 0.19	0.68 ± 0.28
	I	2.25 ± 0.29	1.93 ± 0.60	**3.95 ± 0.43	1.93 ± 0.85
TV-30	H	1.82 ± 0.20	1.52 ± 0.19	1.63 ± 0.35	1.58 ± 0.16
	I	2.30 ± 0.50	1.65 ± 0.43	**4.93 ± 0.58	4.30 ± 0.85
T-17	H	1.36 ± 0.23	1.18 ± 0.29	1.02 ± 0.30	0.75 ± 0.12
	I	1.55 ± 0.35	**5.58 ± 0.77	2.23 ± 0.56	1.05 ± 0.23
BSS-2	H	3.72 ± 0.43	3.03 ± 0.20	3.25 ± 0.35	2.57 ± 0.39
	I	3.76 ± 0.35	3.16 ± 0.37	**5.25 ± 0.70	3.52 ± 0.53
BSS-3	H	3.35 ± 0.23	3.02 ± 0.15	2.16 ± 0.35	1.83 ± 0.30
	I	3.68 ± 0.70	**5.02 ± 0.15	2.19 ± 0.74	1.92 ± 0.16
UP-2	H	2.26 ± 0.35	2.06 ± 0.34	1.45 ± 0.43	1.06 ± 0.22
	I	2.52 ± 0.54	2.54 ± 0.80	**4.34 ± 0.41	1.23 ± 0.17
UP-3	H	1.26 ± 0.40	1.31 ± 0.19	1.03 ± 0.23	0.91 ± 0.35
	I	1.52 ± 0.37	**3.84 ± 0.46	**2.61 ± 0.35	1.31 ± 0.71
UP-9	H	2.82 ± 0.33	2.55 ± 0.14	2.63 ± 0.37	1.55 ± 0.60
	I	3.76 ± 0.60	3.27 ± 0.51	**5.26 ± 0.29	**3.07 ± 0.81
UP-26	H	2.23 ± 0.52	1.72 ± 0.53	2.15 ± 0.23	1.84 ± 0.15
	I	2.56 ± 0.35	**4.59 ± 0.49	2.23 ± 0.91	1.86 ± 0.22
BS/ 7A/ 76	H	1.96 ± 0.33	1.65 ± 0.26	1.63 ± 0.27	1.55 ± 0.18
	I	2.22 ± 0.10	1.62 ± 0.33	**3.83 ± 0.69	1.89 ± 0.41
TS-449	H	1.12 ± 0.24	0.64 ± 0.34	1.05 ± 0.35	0.89 ± 0.16
	I	1.27 ± 0.34	1.02 ± 0.17	**3.90 ± 0.23	1.02 ± 0.61
CP-1/1	H	0.87 ± 0.36	0.65 ± 0.23	0.54 ± 0.19	0.50 ± 0.28
	I	1.01 ± 0.55	**4.19 ± 0.63	**1.46 ± 0.16	0.92 ± 0.42
P-312	H	2.37 ± 0.39	2.19 ± 0.18	1.95 ± 0.55	1.72 ± 0.44
	I	2.56 ± 0.26	2.29 ± 0.46	** 4.25 ± 0.71	**3.56 ± 0.19
AV-2	H	2.25 ± 0.45	1.75 ± 0.33	1.79 ± 0.24	1.72 ± 0.25
	I	2.35 ± 0.65	**2.92 ± 0.44	1.83 ± 0.29	1.84 ± 0.37

***H- healthy control; I- inoculated**

Means ± S.E, n=3 ; ** Difference between healthy and infected Significant at P=0.01 as tested by Student's t-test

earlier. Since the peak POX activity was observed 24 hours after inoculation and 48 hours after inoculation, these intervals were selected for the detailed analysis of isozyme profile in the inoculated samples. It is clear from the earlier discourse that the peroxidase specific activity did not increase in any of the healthy control samples in time course

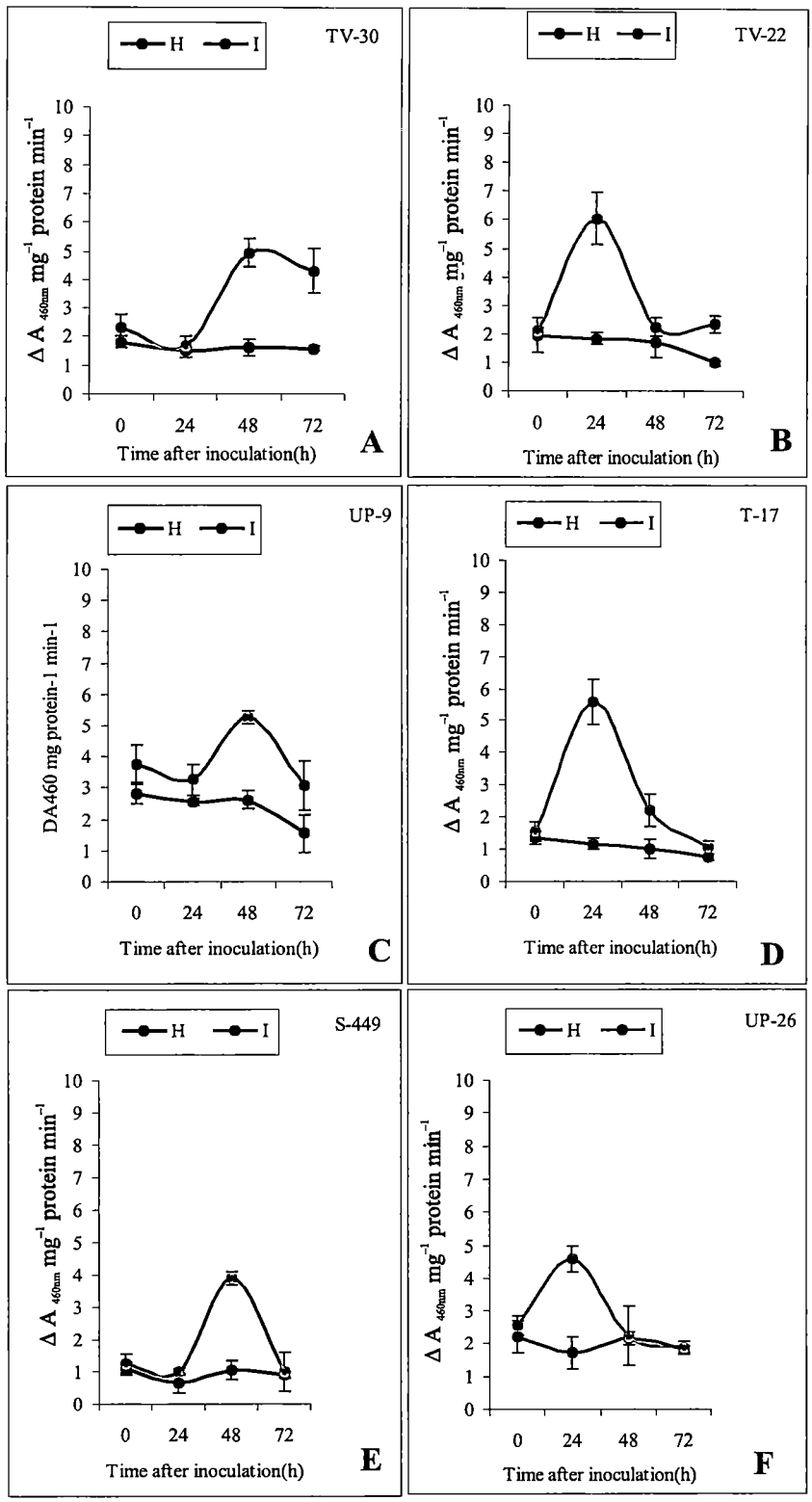


Fig. 6.(A-F): Peroxidase activity in healthy and *G. cingulata* inoculated tea varieties

experiments. Thus, only a single healthy control was run for different varieties separately and compared with the profiles of samples 24 hours after inoculation and 48 hours after inoculation. The results are presented in Tables 19-21 and Plate 20. Isozyme analysis recorded the presence of 8 anodic (acidic) isozymes (I_1 to I_8) ranging between R_m 0.11 and 0.83.

In case of constitutive expression (Table 19, Plate 20, fig. A) it easily observed that two isozymes of R_m=0.28 (I_3) and R_m=0.47 (I_5) are common to all varieties. It is, however, interesting to note that extra bands were present in some varieties (Table 19). The slow migrating isozyme of R_m=0.20 (I_2) was observed in BSS-2, UP-2 and TV-29, which were moderately resistant varieties. The isozyme with R_m=0.41 (I_4) was specific to BS/7A/76, a resistant variety. Among the susceptible varieties examined, UP-3 indicated presence of isoperoxidase with R_m=0.83(I_8). Further studies with inoculated samples were conducted to reveal changes in isozyme profile and detect whether any of the isoforms are associated with resistance or susceptibility and whether these isozymes can be used as markers of resistance.

In order to reveal changes in the isozyme patterns on infection, Native Polyacrylamide Gel Electrophoresis was performed in the 18 varieties and stained for POX isozyme analysis. None of the highly susceptible varieties like UP-26, T-17/1/54, TV-22, CP-1/1, AV-2 and BSS-3 showed any extra isoform accumulation on infection with *G. cingulata*. Involvement of isoperoxidases I_6 (R_m=0.60) and I_7 (R_m=0.72) in disease resistance was especially prominent, with I_6 being formed in all resistant interactions (TV-25, TV-26, TV-30, UP-2, BS/7A/76 and P-312) and only one moderately susceptible interaction in case of UP-3. The isozyme I_1 (R_m=0.11) was observed only in the highly resistant variety TV-30 on inoculation. Another isomorph with R_m=0.83 (I_8) was observed after *G. cingulata* inoculation of moderately resistant and susceptible varieties (TV-18, TV-26, BSS-2 and UP-3) but not in the highly resistant or susceptible ones. Therefore, it seems that each isoform has a definite function assigned to it with respect to resistance to infection. It is noteworthy that the isozyme profile of the highly susceptible varieties did not change on infection. Besides, they exhibited only two isozyme bands throughout the study. Peroxidase isozyme induction is important for the present system.

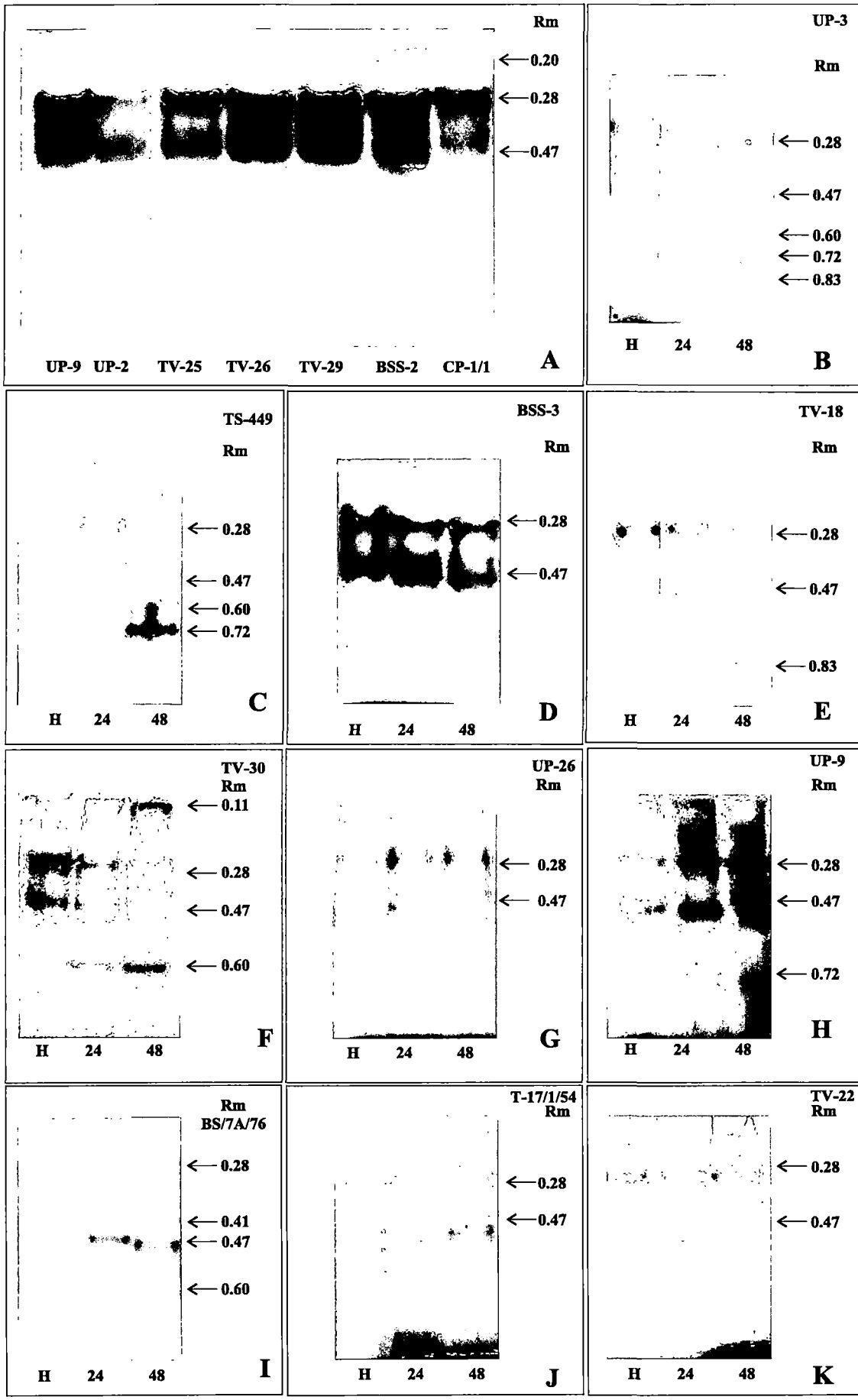


Plate 20 (A-K) : Isozymes of peroxidase expressed constitutively in leaf tissues of tea varieties (A); after 24 & 48 h of inoculation with *G. cingulata* (B-K).

Table19. Isozymes of peroxidase expressed constitutively in leaf tissues of tea varieties:

Variety	Number of isozymes	R_m values of isozymes
TV-18	2	0.28, 0.47
TV-22	3	0.28, 0.47
TV-25	2	0.28, 0.47
TV-26	2	0.28, 0.47
TV-29	3	0.20*, 0.28, 0.47
TV-30	2	0.28, 0.47
T-17/1/54	2	0.28, 0.47
BSS-2	3	0.20*, 0.28, 0.47
BSS-3	2	0.28, 0.47
UP-2	3	0.20*, 0.28, 0.47
UP-3	3	0.28, 0.47, 0.83*
UP-9	2	0.28, 0.47
UP-26	2	0.28, 0.47
BS/ 7A/ 76	3	0.28, 0.41*, 0.47
TS-449	2	0.28, 0.47
CP-1/1	2	0.28, 0.47
P-312	2	0.28, 0.47
AV-2	2	0.28, 0.47

* The extra isozymes bands that are present only in some varieties

Table 20: Isozymes of peroxidase expressed constitutively in leaf tissues of Tocklai tea varieties and after 24 and 48 hours of inoculation with *G.cingulata.*

Variety	Treatment	Number of isozymes	R_m values of isozymes	Disease reaction
TV-18	H	2	0.28, 0.47	MR
	24	2	0.28, 0.47	
	48	3	0.28, 0.47, 0.83*	
TV-22	H	2	0.28, 0.47	S
	24	2	0.28, 0.47	
	48	2	0.28, 0.47	
TV-25	H	2	0.28, 0.47	MR
	24	2	0.28, 0.47	
	48	2	0.28, 0.47, 0.60*	
TV-26	H	2	0.28, 0.47	MR
	24	3	0.28, 0.47, 0.60*	
	48	4	0.28, 0.47, 0.60*, 0.83*	
TV-29	H	3	0.20, 0.28, 0.47	MR
	24	3	0.20, 0.28, 0.47, 0.72*	
	48	3	0.20, 0.28, 0.47, 0.72*	
TV-30	H	2	0.28, 0.47	R
	24	3	0.28, 0.47, 0.60*	
	48	4	0.11*, 0.28, 0.47, 0.60*	
T-17/1/54	H	2	0.28, 0.47	S
	24	2	0.28, 0.47	
	48	2	0.28, 0.47	

*Isozymes induced on infection with respect to uninoculated control

4.6.2. Polyphenol oxidase

PPO catalyses the oxygen-dependent oxidation of phenols to quinones. They also possess various isoforms of differing relative mobility. In view of its importance in defense, it was analysed quantitatively and qualitatively and it role in brown blight infection ascertained.

Time course accumulation of PPO specific activity was estimated in healthy and *G. cingulata* inoculated tea leaf tissues of the 18 different tea varieties as mentioned

Table 21: Isozymes of peroxidase expressed constitutively and in *G.cingulata* inoculated leaves of Darjeeling tea varieties.

Variety	Treatment	Number of isozymes	R_m values of isozymes	Disease reaction
BS/ 7A/ 76	H	3	0.28, 0.41, 0.47	R
	24	4	0.28, 0.41, 0.47, 0.60*	
	48	4	0.28, 0.41, 0.47, 0.60*	
TS-449	H	2	0.28, 0.47	MR
	24	3	0.28, 0.47, 0.72*	
	48	4	0.28, 0.47, 0.60*, 0.72*	
CP-1/1	H	2	0.28, 0.47	S
	24	2	0.28, 0.47	
	48	2	0.28, 0.47	
P-312	H	2	0.28, 0.47	MR
	24	2	0.28, 0.47	
	48	4	0.28, 0.47, 0.60*, 0.72*	
AV-2	H	2	0.28, 0.47	S
	24	2	0.28, 0.47	
	48	2	0.28, 0.47	

*Isozymes induced on infection with respect to uninoculated control.

earlier. Results are presented in Table 22 and Fig. 7(A-F). In general, the activity profile of PPO was on the increasing trend in all the varieties. Significant differences between healthy and inoculated samples were found as tested by Student's t-test at 24 hours after inoculation in the resistant varieties (TV-29, TV-30, BSS-2, UP-2, UP-9, TS-449 and P-312) as evident from Table 22. On the other hand, at 48 hours after inoculation there was significant increase in all the varieties tested, irrespective of compatibility with pathogen. The high activity of PPO in *G. cingulata* inoculated samples persisted or even increased further in the resistant varieties 72 hours after inoculation (TV-18, TV-25, TV-26, TV-29, TV-30, BSS-2., UP-2, UP-9, BS/7A/76, TS-449, P-312) and the difference between activity in healthy and inoculated leaf samples was statistically significant Table 22, Fig. 7(A, C & E). High PPO activity was noticed in some susceptible varieties also (TV-22, T-17/1/54, UP-3 and CP-1/1) at this point, but these were not as high as in the

incompatible interactions. Karl-Pearson's Correlation coefficient between percentage infection at 72 hours after inoculation and PPO activity was found to be -0.64833, which was found to be highly significant at P = 0.05 level. The scatter diagram and the regression line for the same is presented in Fig. 8B.

In the remaining susceptible varieties there was a sharp fall in PPO activity. Thus, it is evident that this enzyme is of vital importance in defense mechanism. The differences in the activity profiles of PPO in compatible and in the incompatible interactions is illustrated in Fig. 7(A-F) indicating that the variation in PPO specific activity is most evident and the highest at 72 hours after inoculation, going up to 7.30 units mg^{-1} protein min^{-1} , in the resistant TV-30.

4.6.2.2. Polyphenol oxidase isozyme analysis

At the onset, it was decided to study whether any PPO isozyme variations exists at the constitutive level in *Camellia sinensis* (L.)O. Kountze varieties under the present investigation. In order to reveal changes in the isozyme patterns of peroxidase samples, Native polyacrylamide gel electrophoresis was performed as described earlier and stained for PPO isozyme analysis. There were some minor variations in the isozyme patterns of the 18 varieties (Plate 21, fig. A & Table 23), however, the patterns could not be associated with the reaction towards the pathogen. The two isozymes with R_m=0.12 and 0.25 were present in all the varieties, while the third one with R_m= 0.81 was absent in leaves of TV-18, TV-25, T-17/1/54, BSS-3, UP-26 and BS/7A/76.

Since PPO activity increased significantly and the differences were particularly noticeable 72 hours after inoculation, this point was selected for the detailed analysis of isozyme profile. PPO isozyme analysis was done using native PAGE as described earlier in the 18 tea varieties. Infection with *G. cingulata* caused changes in the isozyme patterns of PPO. There was loss of some isozymes especially in the compatible interactions (Table 22). This may be because of the destruction of leaf tissue. The susceptible varieties TV-22, UP-26, CP-1/1 and AV-2 each had lost one isozyme on inoculation with *G. cingulata*. Only one moderately resistant TV-25 behaved similarly. No changes were observed in isozyme profiles on inoculation in three susceptible (T-17/1/54, BSS-3 and UP-3) and four moderately resistant (TV-26, TV-29, UP-9, TS-449) varieties. However, all the moderately susceptible varieties in this case possessed three constitutive isozymes, all of which were present during the infection. The isozyme of

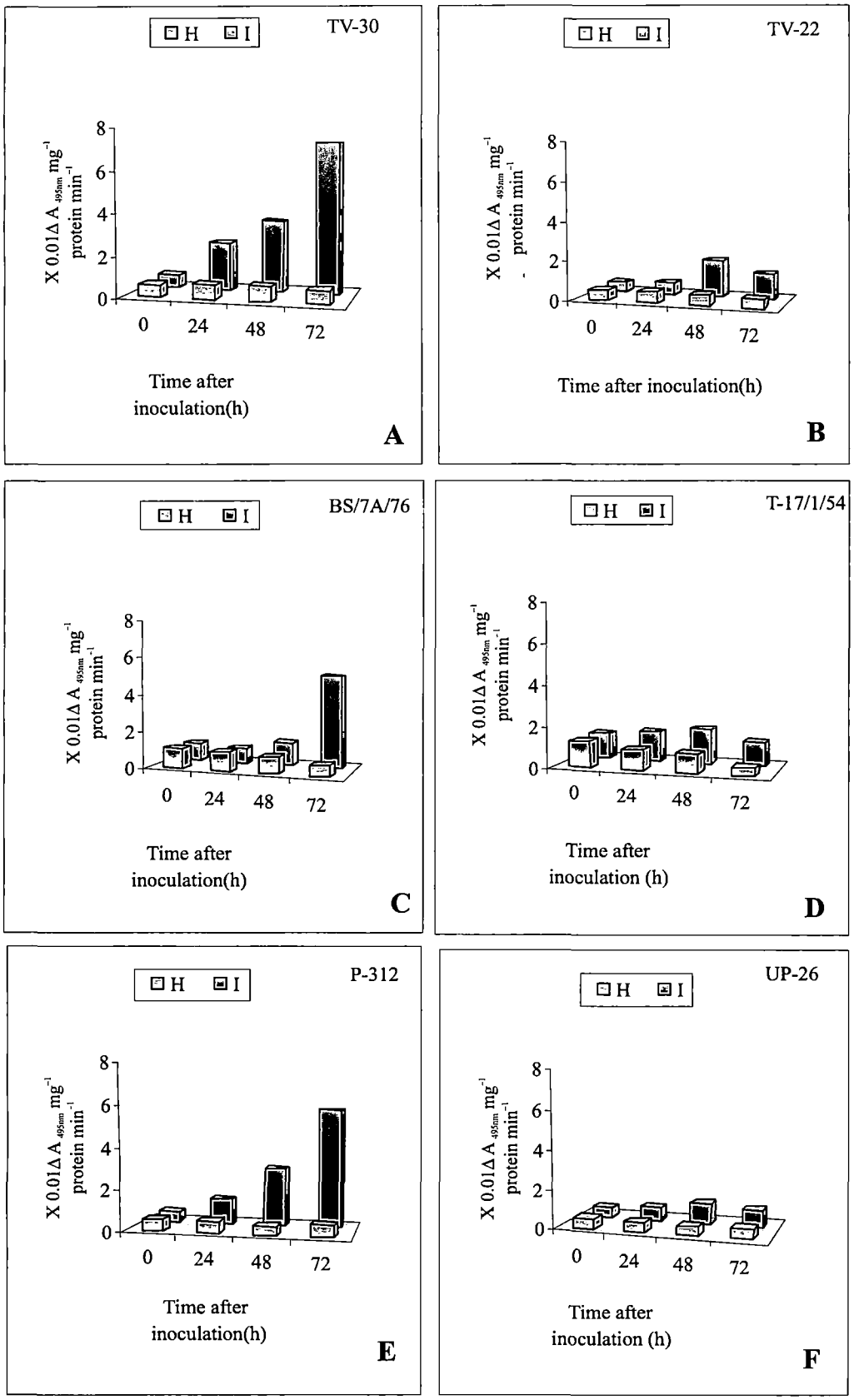


Fig. 7 (A-F): Polyphenoloxidase activity in healthy and *G. cingulata* inoculated tea varieties.

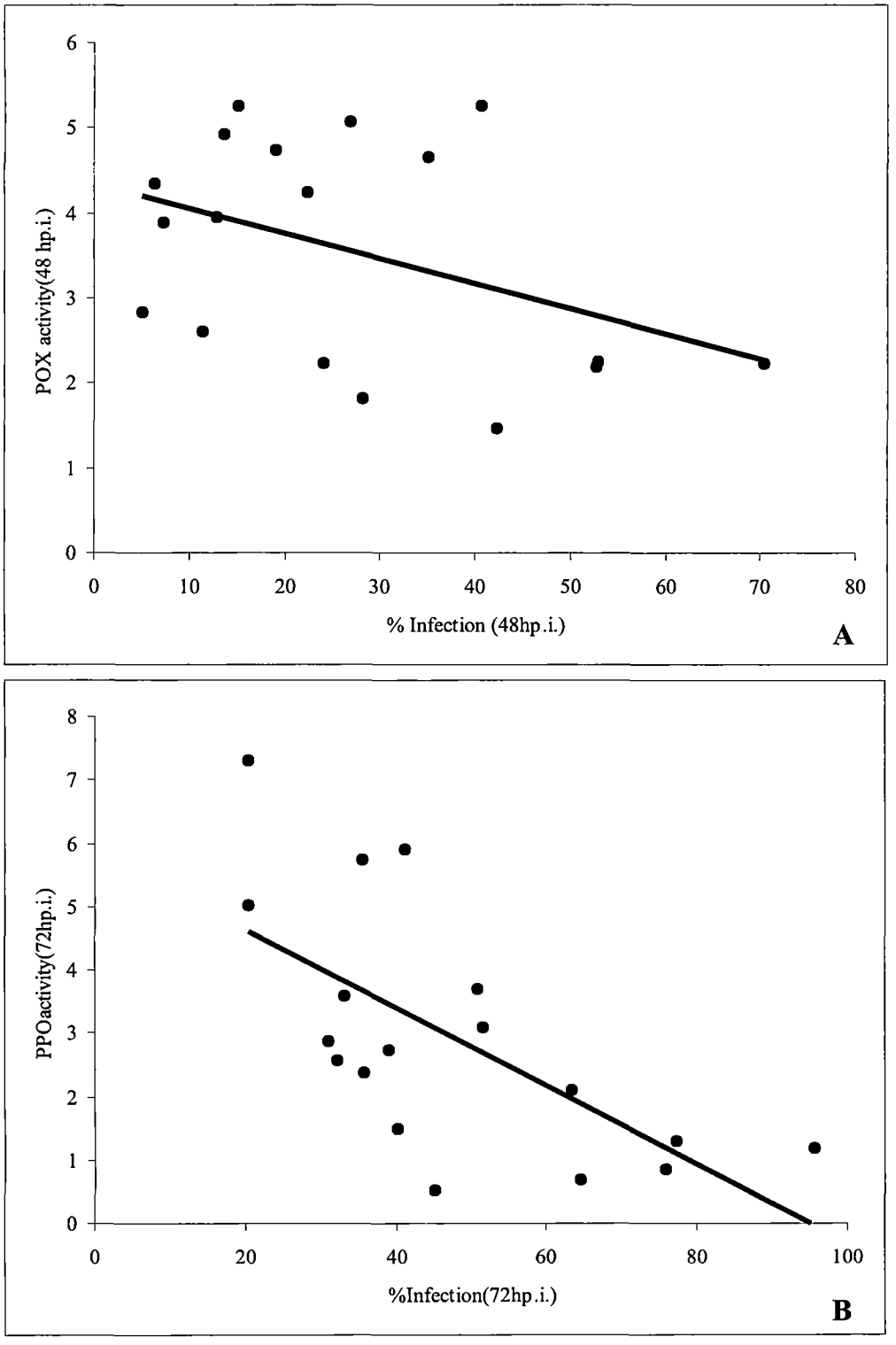


Fig. 8 (A&B): Scatter diagram and regression line for the correlation between the percentage infection and enzyme activity for peroxidase (A) and polyphenoloxidase (B).

R_m=0.55 was induced only in the resistant tea leaf tissues (TV-30, TV-18, TV-25, BSS-2, UP-2, BS/7A/76 and P-312) on infection. This particular isozyme, therefore, is associated with defense reaction in tea.

Table 22: PPO (Polyphenoloxidase) activities in tea leaf tissues of different varieties at various time intervals after inoculation with *G.cingulata*

Variety	Treatment	Polyphenoloxidase activity (x0.01 ΔA_{495nm} mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
TV-18	H	1.40 ± 0.02	1.65 ± 0.04	1.48 ± 0.04	1.20 ± 0.05
	I	1.30 ± 0.09	1.80 ± 0.04	**2.10 ± 0.02	**5.90 ± 0.07
TV-22	H	0.52 ± 0.09	0.56 ± 0.03	0.54 ± 0.06	0.47 ± 0.07
	I	0.51 ± 0.02	0.59 ± 0.08	**1.90 ± 0.03	**1.30 ± 0.06
TV-25	H	0.55 ± 0.10	0.42 ± 0.09	0.47 ± 0.03	0.38 ± 0.07
	I	0.58 ± 0.08	0.76 ± 0.10	0.83 ± 0.06	**2.56 ± 0.04
TV-26	H	0.63 ± 0.08	0.82 ± 0.05	1.00 ± 0.08	0.57 ± 0.06
	I	0.65 ± 0.07	0.83 ± 0.05	1.70 ± 0.03	**2.74 ± 0.08
TV-29	H	0.40 ± 0.01	0.36 ± 0.05	0.32 ± 0.07	0.30 ± 0.09
	I	0.41 ± 0.02	**1.23 ± 0.02	1.56 ± 0.02	**2.36 ± 0.05
TV-30	H	0.60 ± 0.07	0.69 ± 0.05	0.74 ± 0.03	0.65 ± 0.02
	I	0.65 ± 0.02	**2.36 ± 0.02	3.50 ± 0.01	**7.30 ± 0.07
T-17	H	1.30 ± 0.08	1.00 ± 0.05	0.90 ± 0.07	0.40 ± 0.04
	I	1.32 ± 0.09	1.50 ± 0.09	1.73 ± 0.06	**1.20 ± 0.03
BSS-2	H	0.64 ± 0.02	0.69 ± 0.03	0.36 ± 0.10	0.36 ± 0.01
	I	0.68 ± 0.09	**1.30 ± 0.02	1.50 ± 0.04	**3.10 ± 0.04
BSS-3	H	0.54 ± 0.03	0.58 ± 0.02	0.55 ± 0.07	0.49 ± 0.04
	I	0.56 ± 0.02	0.59 ± 0.01	1.00 ± 0.14	0.68 ± 0.07
UP-2	H	3.51 ± 0.03	2.32 ± 0.07	2.52 ± 0.10	2.40 ± 0.04
	I	3.57 ± 0.05	**4.05 ± 0.02	4.85 ± 0.12	**3.60 ± 0.07
UP-3	H	3.20 ± 0.07	3.50 ± 0.02	3.00 ± 0.11	2.45 ± 0.09
	I	3.00 ± 0.05	3.50 ± 0.03	3.90 ± 0.01	**3.70 ± 0.03
UP-9	H	0.58 ± 0.09	0.88 ± 0.07	0.40 ± 0.09	0.22 ± 0.01
	I	0.63 ± 0.07	**1.48 ± 0.09	3.16 ± 0.05	1.50 ± 0.03
UP-26	H	0.49 ± 0.02	0.47 ± 0.01	0.46 ± 0.03	0.51 ± 0.10
	I	0.52 ± 0.06	0.69 ± 0.05	**1.05 ± 0.04	0.85 ± 0.07
BS/ 7A/ 76	H	1.10 ± 0.07	1.02 ± 0.06	0.90 ± 0.02	0.60 ± 0.09
	I	1.00 ± 0.07	0.82 ± 0.09	**1.26 ± 0.05	** 5.02 ± 0.04
TS-449	H	0.63 ± 0.08	0.60 ± 0.03	0.50 ± 0.02	0.52 ± 0.04
	I	0.65 ± 0.10	1.00 ± 0.04	**1.54 ± 0.07	**2.87 ± 0.05
CP-1	H	2.30 ± 0.04	2.00 ± 0.07	1.90 ± 0.05	1.70 ± 0.10
	I	2.60 ± 0.05	2.05 ± 0.05	**2.67 ± 0.09	2.09 ± 0.09
P-312	H	0.56 ± 0.03	0.55 ± 0.05	0.42 ± 0.08	0.58 ± 0.08
	I	0.50 ± 0.05	**1.20 ± 0.06	**2.80 ± 0.04	**5.74 ± 0.03
AV-2	H	0.60 ± 0.08	0.58 ± 0.10	0.54 ± 0.06	0.49 ± 0.07
	I	0.61 ± 0.10	0.64 ± 0.03	0.98 ± 0.09	0.52 ± 0.08

H- healthy control; I- inoculated

Means ± S.E, n=3. ; ** Difference between healthy and infected Significant at P=0.01 as tested by Student's t-test.

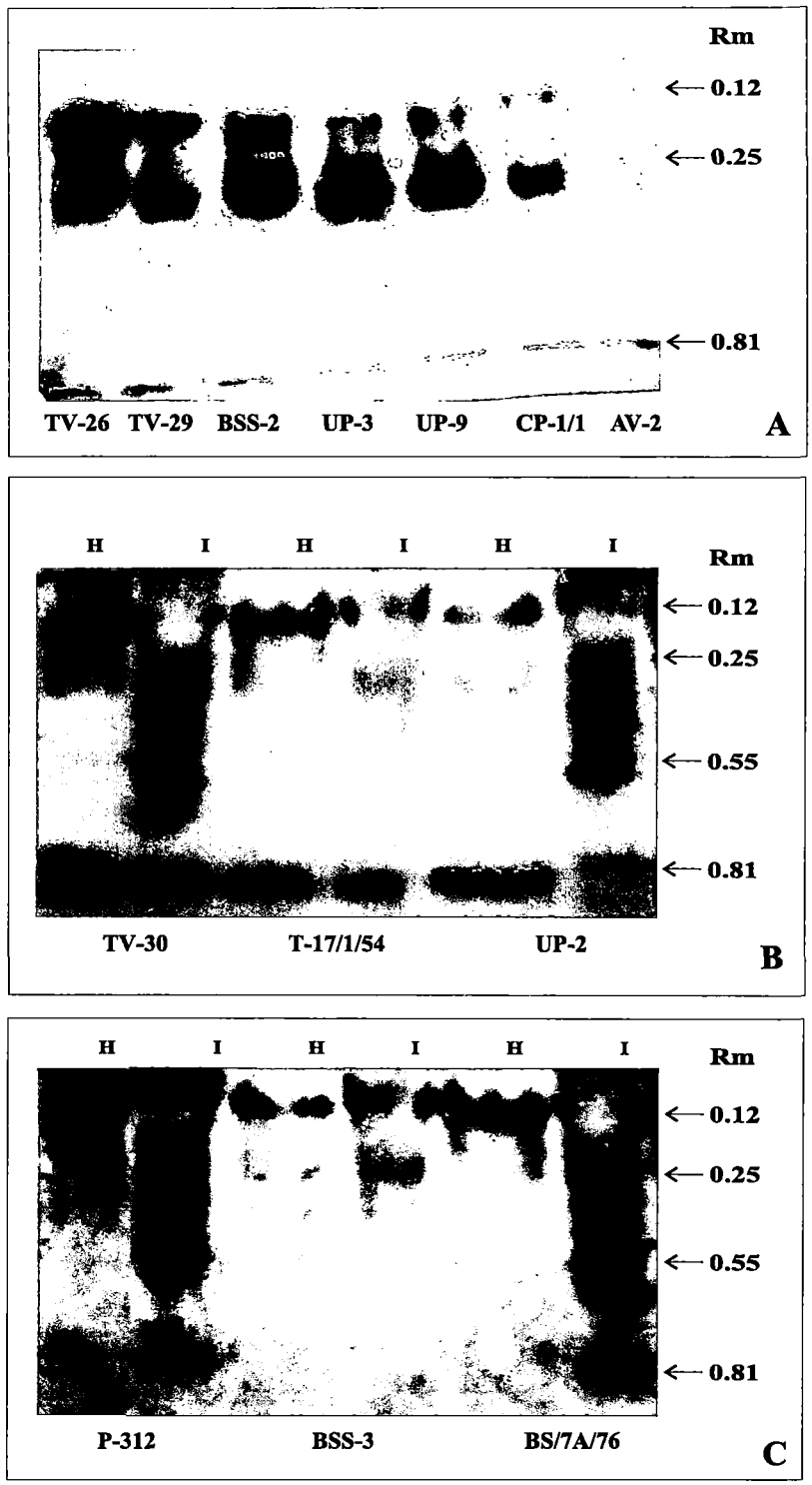


Plate 21 (A-C): Isozymes of polyphenoloxidase expressed constitutively in leaf tissues of tea varieties (A); after 48 h of inoculation with *G. cingulata* (B-K).

Table 23. Isozymes of polyphenoloxidase expressed constitutively and on *G.cingulata* inoculation of tea leaves of Tocklai varieties.

Variety	Treatment	Number of isozymes	R_m values of isozymes	Disease reaction
TV-18	H	2	0.12, 0.25	MR
	I	3	0.12, 0.25, 0.55	
TV-22	H	3	0.12, 0.25, 0.81	S
	I	2	0.12, 0.25	
TV-25	H	2	0.12, 0.25	MR
	I	3	0.12, 0.25, 0.55	
TV-26	H	3	0.12, 0.25, 0.81	MR
	I	3	0.12, 0.25, 0.81	
TV-29	H	3	0.12, 0.25, 0.81	MR
	I	3	0.12, 0.25, 0.81	
TV-30	H	3	0.12, 0.25, 0.81	R
	I	4	0.12, 0.25, 0.55, 0.81	
T-17/1/54	H	2	0.12, 0.25	S
	I	2	0.12, 0.25	
BSS-2	H	3	0.12, 0.25, 0.81	MR
	I	4	0.12, 0.25, 0.55, 0.81	
BSS-3	H	2	0.12, 0.25	S
	I	2	0.12, 0.25	
UP-2	H	3	0.12, 0.25, 0.81	MR
	I	4	0.12, 0.25, 0.55, 0.81	
UP-3	H	2	0.12, 0.25	MS
	I	2	0.12, 0.25	
UP-9	H	3	0.12, 0.25, 0.81	R
	I	3	0.12, 0.25, 0.81	
UP-26	H	2	0.12, 0.25	S
	I	1	0.25	
BS/ 7A/ 76	H	2	0.12, 0.25	R
	I	4	0.12, 0.25, 0.55, 0.81	
TS-449	H	3	0.12, 0.25, 0.81	MR
	I	3	0.12, 0.25, 0.81	
CP-1/1	H	3	0.12, 0.25, 0.81	S
	I	2	0.12, 0.25	
P-312	H	3	0.12, 0.25, 0.81	MR
	I	4	0.12, 0.25, 0.55, 0.81	
AV-2	H	3	0.12, 0.25, 0.81	S
	I	2	0.12, 0.25	

H- healthy control; I- inoculated

4.6.3. Phenylalanine ammonia lyase

Activity of phenylalanine ammonia lyase was measured at 0, 24, 48 and 72 hours after inoculation in healthy and *G. cingulata* infected samples. Results are presented in Table 24. Infected tissues exhibited an increase 24h after challenge in all the varieties. This was followed by a rapid decline. Highest activity was found in infected leaves of

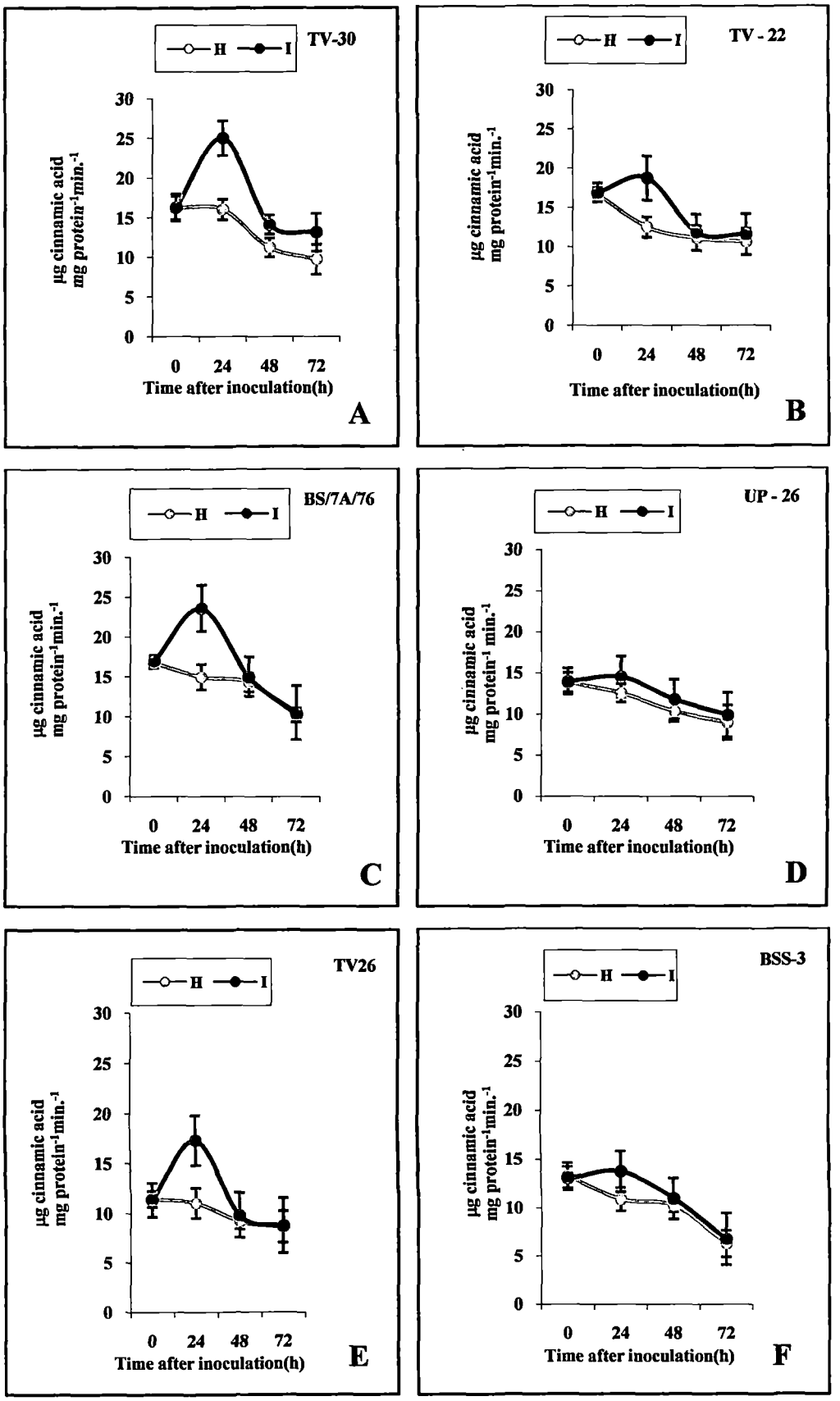


Fig.9 (A-F): Phenylalanine ammonia lyase activity in healthy and *G.cingulata* inoculated tea varieties.

Table 24: PAL (Phenyl alanine ammonia lyase) activities in different tea varieties at various time intervals after inoculation with *G. cingulata*

Variety	Treatment	Phenyl alanine ammonia lyase activity (µg cinnamic acid mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
TV-18	H	16.6 ± 1.5	12.0 ± 1.3	09.5 ± 1.2	04.8 ± 1.9
	I	16.7 ± 1.7	**18.0 ± 2.2	11.1 ± 1.2	06.3 ± 2.4
TV-22	H	16.7 ± 0.9	12.5 ± 1.3	11.1 ± 1.6	10.6 ± 1.7
	I	17.0 ± 1.2	**18.8 ± 2.8	11.8 ± 2.3	11.6 ± 2.6
TV-25	H	18.2 ± 1.1	18.6 ± 0.9	12.7 ± 1.3	12.1 ± 1.7
	I	18.5 ± 0.8	20.3 ± 2.1	**16.9 ± 2.6	15.8 ± 2.4
TV-26	H	11.4 ± 0.8	11.1 ± 1.5	09.3 ± 0.8	08.7 ± 1.6
	I	11.4 ± 1.7	**17.3 ± 2.5	09.9 ± 2.3	08.9 ± 2.8
TV-29	H	10.2 ± 1.1	10.1 ± 0.5	06.7 ± 0.7	06.3 ± 1.9
	I	10.5 ± 2.2	**19.4 ± 2.3	09.5 ± 1.2	09.2 ± 2.0
TV-30	H	16.3 ± 0.7	16.1 ± 0.5	11.3 ± 1.3	09.8 ± 1.2
	I	16.3 ± 1.2	**25.0 ± 2.5	14.2 ± 2.1	13.2 ± 1.7
T-17	H	16.6 ± 0.8	16.5 ± 1.5	12.0 ± 0.7	11.9 ± 1.4
	I	16.7 ± 0.9	19.1 ± 1.9	12.5 ± 1.6	12.1 ± 2.3
BSS-2	H	12.9 ± 1.2	13.2 ± 1.3	09.8 ± 2.1	07.6 ± 1.0
	I	13.1 ± 0.9	** 21.1 ± 1.2	10.6 ± 1.4	10.9 ± 2.4
BSS-3	H	13.3 ± 1.3	10.9 ± 1.2	10.3 ± 0.7	06.3 ± 1.4
	I	13.0 ± 1.2	13.8 ± 2.1	11.0 ± 2.1	06.8 ± 2.7
UP-2	H	16.6 ± 1.3	13.3 ± 0.7	12.9 ± 1.1	12.2 ± 1.4
	I	16.6 ± 0.5	**18.8 ± 1.2	14.4 ± 2.1	13.7 ± 1.7
UP-3	H	11.7 ± 0.7	12.0 ± 1.2	08.7 ± 1.1	08.2 ± 0.9
	I	11.9 ± 0.5	14.8 ± 1.3	08.8 ± 1.0	06.8 ± 2.3
UP-9	H	11.9 ± 0.9	11.5 ± 0.7	08.0 ± 1.9	07.2 ± 2.1
	I	12.2 ± 0.7	13.2 ± 1.9	10.7 ± 2.5	10.3 ± 2.3
UP-26	H	13.8 ± 1.2	12.8 ± 1.1	10.4 ± 1.3	09.0 ± 2.1
	I	14.0 ± 1.6	14.5 ± 2.5	11.8 ± 2.4	09.9 ± 2.7
BS/ 7A/ 76	H	16.8 ± 0.7	15.0 ± 1.6	14.3 ± 1.2	10.2 ± 0.9
	I	17.0 ± 0.7	**23.6 ± 2.9	15.0 ± 2.5	10.5 ± 3.4
S-449	H	17.1 ± 0.8	11.1 ± 1.3	10.1 ± 2.4	08.6 ± 2.4
	I	16.8 ± 1.1	**18.8 ± 2.4	11.0 ± 1.7	09.0 ± 2.5
CP-1	H	15.3 ± 0.4	15.2 ± 0.7	11.3 ± 1.5	08.8 ± 2.1
	I	15.5 ± 0.5	16.6 ± 1.5	13.7 ± 1.9	12.9 ± 2.7
P-312	H	13.5 ± 0.7	13.0 ± 0.5	12.3 ± 1.8	08.2 ± 2.8
	I	13.4 ± 0.5	16.0 ± 1.6	13.0 ± 2.4	10.1 ± 2.3
AV-2	H	06.9 ± 0.8	07.0 ± 1.1	04.0 ± 1.6	03.2 ± 2.7
	I	06.8 ± 0.9	**10.5 ± 1.3	06.0 ± 0.9	05.5 ± 0.8

Means ± S.E, n=3. ; ** Difference between healthy and infected Significant at P=0.01 as tested by Student's t-test

Table 25: TAL (Tyrosine ammonia lyase) activities in tea varieties at various time intervals after inoculation with *G. cingulata*

Variety	Treatment	Tyrosine ammonia lyase activity (μg coumaric acid mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
TV-18	H	72.0 ± 1.5	63.7 ± 2.3	62.1 ± 2.2	61.3 ± 1.9
	I	72.9 ± 1.7	64.2 ± 2.2	**90.3 ± 3.2	67.0 ± 2.4
TV-22	H	44.0 ± 2.9	41.3 ± 2.3	41.0 ± 1.6	40.8 ± 1.7
	I	44.7 ± 2.2	41.8 ± 1.8	42.1 ± 1.3	41.8 ± 2.6
TV-25	H	42.7 ± 2.1	41.6 ± 1.9	40.9 ± 2.3	40.2 ± 1.7
	I	43.0 ± 2.8	41.5 ± 1.1	59.5 ± 2.6	41.9 ± 2.4
TV-26	H	41.7 ± 2.8	41.4 ± 2.5	41.9 ± 1.8	41.0 ± 2.6
	I	42.1 ± 1.7	41.5 ± 2.5	49.9 ± 2.3	45.1 ± 2.8
TV-29	H	40.2 ± 2.1	40.1 ± 2.5	36.7 ± 2.7	36.3 ± 1.9
	I	40.5 ± 2.2	**49.4 ± 1.2	**59.5 ± 2.2	**45.5 ± 2.0
TV-30	H	40.1 ± 2.7	39.5 ± 2.5	38.4 ± 2.3	35.5 ± 2.2
	I	40.1 ± 1.2	41.8 ± 1.2	**82.0 ± 2.1	35.9 ± 2.7
T-17	H	35.9 ± 1.8	35.0 ± 2.5	30.8 ± 2.7	28.7 ± 1.4
	I	27.0 ± 1.9	35.8 ± 1.9	35.8 ± 2.6	30.8 ± 2.3
BSS-2	H	54.0 ± 2.2	50.9 ± 2.3	45.7 ± 1.1	45.1 ± 1.3
	I	54.5 ± 1.9	53.7 ± 1.2	**65.5 ± 1.4	46.3 ± 2.4
BSS-3	H	53.3 ± 2.3	52.9 ± 1.2	53.3 ± 2.7	53.3 ± 2.4
	I	53.9 ± 1.2	53.8 ± 2.1	54.0 ± 2.1	52.8 ± 2.7
UP-2	H	27.6 ± 2.3	25.6 ± 2.7	24.8 ± 2.1	23.6 ± 2.4
	I	27.7 ± 2.5	29.8 ± 1.2	**43.4 ± 2.1	23.8 ± 1.7
UP-3	H	21.7 ± 1.7	22.0 ± 2.2	18.7 ± 2.1	18.1 ± 2.9
	I	21.9 ± 1.5	24.8 ± 1.3	25.8 ± 1.0	19.8 ± 0.3
UP-9	H	35.7 ± 1.9	32.1 ± 1.7	27.9 ± 1.9	26.4 ± 2.1
	I	35.8 ± 1.7	35.5 ± 1.9	**37.0 ± 1.5	28.0 ± 2.3
UP-26	H	30.2 ± 2.2	26.0 ± 2.1	25.3 ± 2.3	23.5 ± 2.1
	I	30.6 ± 1.6	26.3 ± 1.5	** 46.9 ± 1.4	23.9 ± 1.7
BS/ 7A/ 76	H	43.6 ± 2.7	41.4 ± 1.6	41.0 ± 1.2	36.5 ± 1.9
	I	43.8 ± 1.7	41.7 ± 1.9	**59.5 ± 2.5	36.4 ± 2.4
S-449	H	37.1 ± 2.8	36.1 ± 2.3	35.9 ± 1.2	34.5 ± 1.4
	I	36.8 ± 1.1	38.8 ± 1.4	**51.0 ± 1.7	**39.0 ± 1.5
CP-1/1	H	35.3 ± 1.4	34.2 ± 1.7	34.3 ± 2.5	28.8 ± 1.1
	I	35.5 ± 1.5	35.7 ± 1.5	36.0 ± 3.9	32.9 ± 1.9
P-312	H	23.5 ± 1.3	23.0 ± 1.5	22.3 ± 2.8	18.6 ± 2.8
	I	23.4 ± 2.5	22.9 ± 1.6	26.1 ± 2.4	20.1 ± 2.3
AV-2	H	26.9 ± 3.8	26.0 ± 2.1	24.0 ± 2.6	23.2 ± 1.7
	I	26.8 ± 2.1	**20.5 ± 2.3	26.0 ± 2.9	24.5 ± 1.8

Means ± S.E, n=3. ; ** Difference between healthy and infected Significant at P=0.01 as tested by Student's t-test.

TV-30, a resistant line, 24 hours after inoculation (25.0 units mg^{-1} protein min^{-1}). The lowest activity was found in healthy leaves of AV-2 (3.2 units mg^{-1} protein min^{-1}). The susceptible varieties like UP-26 exhibited very low increase in activity on inoculation. Significantly (P=0.05) higher activities were observed in the inoculated leaf samples as

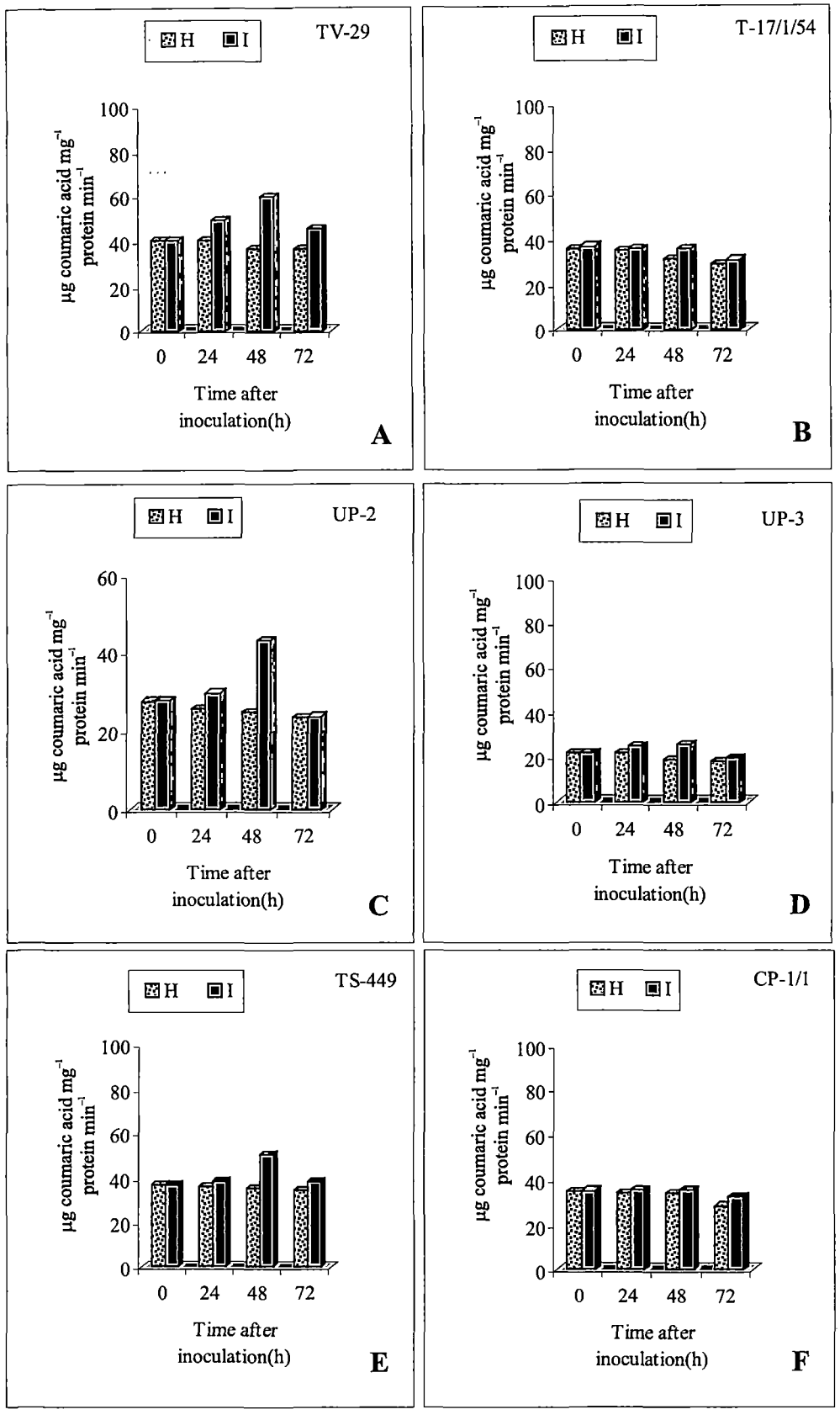


Fig. 10 (A-F): Tyrosine ammonia lyase activity in healthy and *G. cingulata* inoculated tea leaf tissues.

tested by Student's t-test in the resistant varieties (Table 23) such as TV-26, TV-29, TV-30, BSS-2, UP-2, BS/7A/76, TS-449 and CP-1/1.

PAL, therefore, was induced especially in the incompatible reactions before the onset of symptoms. The comparison in the activity profile of this enzyme in compatible and in incompatible reactions is illustrated in Fig. 9 (A-F).

4.6.4. Tyrosine ammonia lyase

Activity profile of tyrosine ammonia lyase was studied at 0, 24, 48 and 72 hours after inoculation in the 18 tea varieties in healthy and *G. cingulata* inoculated samples. The activity of TAL increased 48 hours after inoculation significantly as compared to uninoculated control tissues in the resistant varieties (TV-18, TV-30, BS/7A/76, TV-25, BSS-2, UP-2), sometimes becoming three-folds of the initial activity as in UP-2. The results are presented in Table 25 & Fig. 10 (A-F). In the susceptible ones there was a statistically very insignificant increase and the activity remained at the control level. Thus, it seems that TAL is induced only 48 hours after inoculation, in the tea leaf tissues and it has a definite role in resistance mechanism.

4.7. Analysis of diffusible compounds in tea varieties following infection with *G.cingulata*

Tea plants possess very high amounts of phenolics that have been implicated in the resistance of the tea bushes to the pathogens. Phenolics constitute the most abundant class of secondary metabolites and share a common origin in the phenylpropanoid biosynthetic pathway, in which the enzymes like PAL and TAL, are involved. Among their biological properties, phenolics are involved in stress responses by acting as chemical deterrents to pathogens and also potential scavengers of active oxygen species. Study involving tea plants would be incomplete without touching this major aspect. Besides, the study of biological activity of diffusible compounds that may interfere with spore germination on leaf surface, was also conducted.

4.7.1. Changes in levels of phenolics in healthy and *G. cingulata* inoculated tea leaves

Phenolics are known to accumulate in numerous plant species following infection with fungal pathogens and in many cases they are associated with resistance. Besides, level of phenolics has been reported to be quite high in tea plants particularly. In plants,

these are found to be both – constitutive as well as inducible (level increases following infection) in nature during defense reaction. These compounds are also known to act as active oxygen species scavengers during host-pathogen interactions. Therefore, extraction and analysis of both total and ortho-dihydroxy phenols were performed in 18 selected tea varieties in healthy and *G. cingulata* inoculated tea leaves 48 hours after inoculation. The results presented were average of three independent experiments.

4.7.1.1. Total phenol

Total phenols were extracted and estimated in tea leaves of healthy and *G. cingulata* inoculated tea leaves separately, 48 hours after inoculation. Results have been presented in Table 26. Total phenol content decreased or remained same following inoculation with *G. cingulata* in the susceptible varieties. However, there was an increase in the total phenol content of resistant varieties following inoculation with *G. cingulata.* Among the varieties tested, TV-30 and BS/7A/76 showed maximum and significant increase (tested by t-test) in total phenol following inoculation with *G. cingulata,* while T-17/1/54 and TV-22 showed maximum and significant decrease (tested by t-test) when inoculated with *G. cingulata.* Thus, it is quite clear that the resistant varieties accumulated high amounts of this antifungal substance, which was not the case in susceptible varieties.

4.7.1.2. Ortho-dihydroxy phenol

Orthodihydroxy phenols were also extracted and estimated in eighteen tea varieties. Leaves of healthy and *G. cingulata* inoculated tea varieties were harvested separately for estimation 48hp.i. Results presented in Table 26 revealed that orthodihydroxy phenol content decreased in the most susceptible varieties (TV-22 and T-17) and increased in resistant varieties (TV-30 and BS/7A/76) following inoculation with *G. cingulata.* Therefore, results were similar to total phenol variation on inoculation.

4.7.2. Studies on biological activities of leaf diffusates of tea

Phenolic compounds are present within the tea leaf tissues. These act after the pathogen has gained access into the host. The differential resistance of tea varieties in

Table 26: Phenolic content in healthy and *G. cingulata* infected tea leaves of different tea varieties.

Variety	Phenol content (mg caffeic acid g^{-1} leaf tissue)			
	Total		Ortho-dihydroxy	
	Healthy	Infected	Healthy	Infected
Tocklai				
TV-18	39.9 ± 1.3	*47.6 ± 1.2	16.8 ± 2.3	17.4 ± 1.1
TV-22	51.4 ± 1.3	46.5 ± 1.8	10.8 ± 0.7	*05.9 ± 1.0
TV-25	50.7 ± 0.8	48.9 ± 0.7	10.5 ± 0.7	11.2 ± 0.9
TV-26	43.8 ± 1.4	47.3 ± 1.1	10.1 ± 1.1	13.5 ± 1.3
TV-29	44.9 ± 0.7	46.8 ± 0.7	11.3 ± 1.2	13.7 ± 1.2
TV-30	40.0 ± 1.4	*46.4 ± 1.9	11.5 ± 0.9	14.6 ± 1.3
T-17/1/54	50.6 ± 0.8	*43.1 ± 2.1	12.3 ± 1.4	08.2 ± 1.2
UPASI				
BSS-2	35.7 ± 1.3	40.5 ± 0.6	09.9 ± 1.5	*15.9 ± 1.2
BSS-3	35.2 ± 1.1	36.5 ± 1.1	09.4 ± 1.1	08.4 ± 1.5
UP-2	38.3 ± 1.2	42.1 ± 1.2	11.1 ± 1.2	13.4 ± 1.5
UP-3	36.8 ± 1.4	37.2 ± 1.3	12.4 ± 1.4	11.5 ± 1.2
UP-9	41.6 ± 1.4	44.2 ± 1.1	10.2 ± 1.2	12.2 ± 1.6
UP-26	39.4 ± 1.3	37.4 ± 1.3	12.7 ± 1.6	*08.0 ± 1.3
Darjeeling				
BS/7A/76	39.7± 2.3	*46.4 ± 2.3	11.6 ± 0.6	*14.6 ± 1.0
S-449	43.5 ± 1.5	46.1 ± 1.2	15.4 ± 1.1	16.4 ± 1.1
CP-1/1	45.4 ± 1.4	*40.6 ± 1.5	14.7 ± 1.1	15.4 ± 1.3
P-312	33.6 ± 1.3	*38.4 ± 1.0	12.3 ± 1.1	11.6 ± 1.5
AV-2	30.1 ± 0.8	29.3 ± 0.8	06.9 ± 0.9	07.5 ± 1.0

± SE, n=3

* Difference between healthy and infected Significant at P=0.02 as tested by Student's t-test

response to infection with *G. cingulata* as evident by varietal resistance test and immunological techniques, may be attributed to differences in their ability to produce antifungal compounds on leaf surface after inoculation with pathogen. Therefore, leaf diffusates were collected following drop diffusate method as described under Materials and Methods, and their biological activities were tested. Leaf diffusates were collected separately from adaxial surface of the four tea varieties – two susceptible (T-17/1/54, TV-22) and two resistant (TV-30 and BS/7A/76) and their biological activities were evaluated on spore germination of *G. cingulata*. Percentage spores germinated and percentage appressoria formed were calculated. Distilled water was kept as control. The

results presented in Table 27 indicate that diffusates collected from TV-30 and BS/7A/76 were much more fungitoxic than those from T-17/1/54 and TV-22. Percentage inhibition in spore germination and appressoria formation with respect to distilled waster control was much higher in case of the resistant varieties. Thus, the resistant reaction in case of brown blight is also exhibited due to secretion of diffusible compounds directly on the leaf surface in presence of water. Susceptible plants also try to ward off the pathogen, but these diffusible compounds are not fungitixic enough to inhibit spore germination to a great extent.

Table 27: Effect of leaf diffusates of different tea varieties on spore germination of *G. cingulata*

Variety	%spore Germination[a]	%inhibition in spore germination[c]	%appressoria formation[b]	%inhibition in appressoria formation[c]
TV-30	31.6 ± 1.42	59.20	27.5 ± 2.18	63.40
BS/7A/76	33.3 ± 1.60	57.00	29.7 ± 2.54	60.45
T-17/1/54	63.2 ± 2.03	18.45	58.3 ± 2.15	22.40
TV-22	61.9 ± 1.86	20.12	56.6 ± 1.94	24.63
Distilled water (control)	77.5 ± 0.84	N.A.	75.1 ± 3.42	N.A.

Means ± SE , n=3; 300 conidia for each treatment per experiment

Diffusates collected 48 hours after inoculation
Incubation temperature ± 25°C. R.H. 90%

Time of incubation : 24h

[a] Average of 300 spores

[b] Average of 300 germinated spores

[c]Inhibition in relation to control

4.8. Induction of resistance in tea plants against brown blight disease and associated changes in defense enzymes

The mechanism of disease resistance in plants is a very versatile and complex phenomenon. Many-a-times the resistant varieties are not available for use or they may be low-yielding. Besides, tea plant is a perennial crop and once planted, a tea bush will be used for as many as fifty years or even more so in case of seed varieties. This feature makes the use of resistant cultivars all the more difficult. Replantations are costly and unnecessary. Besides, organic tea is in high demand now-a-days, while use of fungicides and insecticides is still high due to absence of any alternative. Presently, biodegradable compounds that offer wide range and long-lasting protection are in focus of the scientists. These are the inducers or elicitors of defense response. The principle behind the use of such compounds is the mechanism of Systemic Acquired Resistance (SAR), according to which even susceptible plants can be protected from diseases by enhancing their own defense mechanism. There is a signal originating at the point of elicitor application that moves throughout the plant and makes it resistant to subsequent attack by the pathogen. This phenomenon is related to accumulation of defense enzymes such as chitinase (CHT), glucanase (β-GLU) and peroxidase (POX). In this chapter an attempt has been made to induce resistance in the susceptible tea varieties and study the changes in the defense enzymes. Besides, changes in catalase (CAT) and ascorbate peroxidase (APX), the two important antioxidant enzymes, were also monitored.

For the purpose of inducing resistance three compounds have been chosen:
(i) signal compound hydrogen peroxide (H_2O_2) ; (ii) nitric oxide (NO) donor sodium nitroprusside and (iii) salicylic acid analogue benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH). The optimum doze for application was determined for each inducer by observing the changes in morphology of two year-old potted tea plants. Visible deviations from normal growth were noted.

Induction period (IP) is the optimal time required for building up of maximum resistance after treatment with inducer. Tea plants of three varieties – two susceptible (TV-22 and T-17/1/54) and one resistant (TV-30) were used in the present experiment. After spraying the tea plants with the optimum concentration of inducer, were next inoculated with spore suspension while maintaining a gap of 24h, 48h and 72h between treatment and inoculation. Disease intensity was assessed by disease index per plant 35

days after inoculation and percentage of protection calculated as compared to untreated control plants. The particular induction period for each inducer was determined and maintained throughout the study for each treatment.

Time course changes in specific activity of defense enzymes on treatment with the three inducers separately, while maintaining the optimum time gap between treatment and inoculation, were conducted after 2, 6, 12, 24 and 48 hours after treatment. The enzymes analysed were peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), chitinase (CHT) and glucanase (β-GLU). The level of these enzymes was also assessed 48 hours after inoculation with *G. cingulata*. In such cases untreated healthy (UH), untreated inoculated (UI), treated healthy (TH) and treated inoculated (TI) sets of each variety were compared.

4.8.1. Induction of resistance with hydrogen peroxide

Hydrogen peroxide is a candidate molecule for the function of second messenger for SAR activation. Therefore, direct foliar application of this signal molecule was considered. However, since hydrogen peroxide is light-sensitive, the plant was left in the dark for 8 hours after its application. Sixteen hour photoperiod was maintained throughout.

Potted and field-grown plants treated with different concentrations of H_2O_2 (3%, 0.3% and 0.15%) were observed for changes in vegetative growth. Definite wilting was observed in most of the potted plants as early as one day after application of 3% solution. On the other hand, 0.15% solution had enhanced the growth of shoots in 10 year-old tea bushes and even dormant buds gave rise to young shoots after 7 days of application of solution. (Plate 22, figs. G & H). Therefore, 0.15% concentration of hydrogen peroxide was used for further experiments.

4.8.1.1. Bioassay of hydrogen peroxide.

Hydrogen peroxide has been reported to possess antimicrobial properties. Therefore, it was decided to study the effect of different concentrations of this signal molecule on spore germination of *G. cingulata*. The bioassay was performed as described earlier. The results are presented in Table 28. Appressoria formation was totally stopped by 0.01% solution of hydrogen peroxide. Interestingly, percentage of spore germination and germ tube length were enhanced in this concentration. Further increase in concentration of hydrogen peroxide decreased spore germination to control

levels, but the germ tube length was still quite high (Plate 22, figs. C-F). In spite of further fall in germination percentage, germ tube length remained significantly higher than in the control set. Only at 1% hydrogen peroxide concentration the germ tube length did not differ significantly from control value. Besides, it is noteworthy that germination was totally inhibited by 3% solution.

Table 27: Effect of H_2O_2 on spore germination of *G.cingulata*

Concentration of H_2O_2 (%)	% spore germination	germ tube length(μm)
0.00	75.5 ± 5.0	52.1 ± 4.3
0.01	80.3 ± 7.5	105.3 ± 10.9
0.03	73.5 ± 6.1	71.5 ± 7.4
0.15	65.6 ± 3.6	73.4 ± 6.8
0.30	46.1 ± 3.0	75.1 ± 7.3
1.00	37.9 ± 2.3	52.8 ± 6.9

Means ± SE , n=3; 300 conidia for each treatment per experiment
Incubation temperature ± 25°C. R.H. 90%

Time of incubation: 24h

[a] Average of 300 spores

[b] Average of 300 germinated spores

Thus, it is obvious that H_2O_2 gave rise to unfavourable environment for spore germination at high concentration. Low concentration, however, did not inhibit spore germination. On the contrary, spore germination was enhanced at low concentration. Absence of appressoria formation in the H_2O_2 treated spores indicates that this signal molecule inhibits appressoria formation and hence the infectivity potential of the spore is decreased.

4.8.1.2. Assessment of disease intensity and determination of induction period in tea varieties following treatment with hydrogen peroxide and inoculated with *G. cingulata*

Induction period for 0.15% H_2O_2 was determined as mentioned above. The effect of hydrogen peroxide on disease development was ascertained by whole plant inoculation method as described earlier. Disease intensity was assessed by disease index per plant 35 days after inoculation. Percentage protection was calculated with respect to untreated inoculated control plants. Three time gaps between inducer treatment and challenge inoculation were tested – 24h, 48h and 72h. The results are presented in Table 29. In case of the resistant TV-30 percentage protection over control was very low. However, in the two susceptible varieties there were significant differences between the treated and untreated plants. It is clearly observed that the best protection was offered by H_2O_2 when it was sprayed 48h before inoculation. Even in TV-30 there was 18.1% protection over control when this interval was maintained. The treatment offered 45.6% protection in case of T-17/1/54 and 40.8% protection in TV-22 (Table 29). Hydrogen peroxide, therefore, increased resistance of tea plants against brown blight pathogen. Therefore, the time period of 48h was selected as the optimum time gap between inducer treatment and challenge inoculation. This gap (Induction Period) was maintained throughout the study of resistance induction with hydrogen peroxide.

4.8.1.3. Analysis of level of defense enzymes on treatment of tea plants with hydrogen peroxide and inoculation with *G. cingulata*

Hydrogen peroxide is has a double role of active oxygen radical as well as a signal molecule that activated defense responses. Therefore, time course accumulation studies of three antioxidant enzymes (peroxidase, catalase and ascorbate peroxidase) and two cell wall degrading enzymes (chitinase and β-1,3-glucanase) were conducted in untreated healthy and treated healthy tea plants. For the purpose, leaves were harvested at different intervals after treatment for the treated and control plants. These were processed as described earlier for extraction and assay of different enzymes.

Inoculation of induced and uninduced potted plants was performed 48 hours after treatment. Level of antioxidant enzymes and cell wall degrading enzymes was assessed similarly in the tea plants 48h after challenge inoculation.

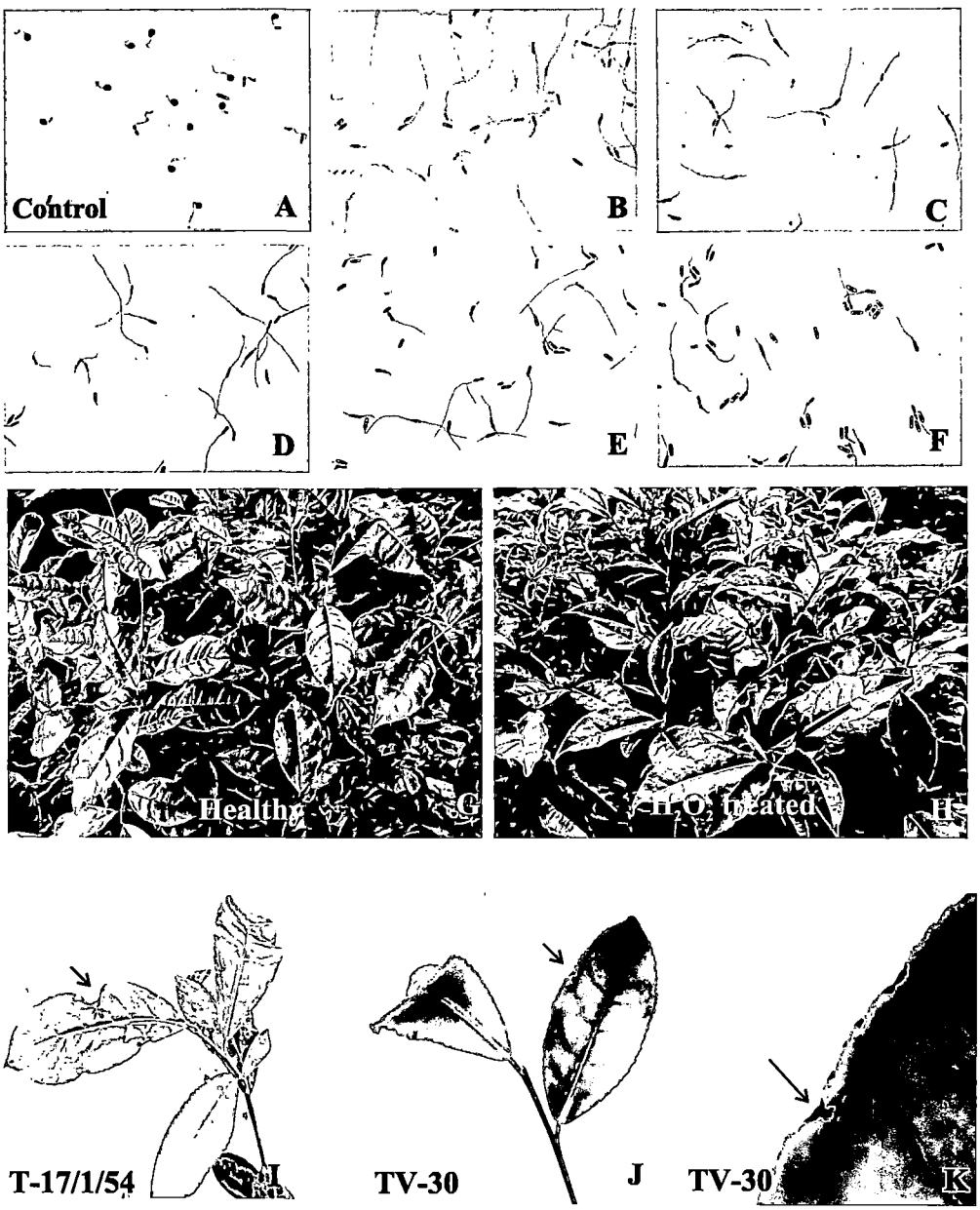


Plate 22 (A-K) : *In vitro* effect of hydrogen peroxide on conidial germination of *G. cingulata* (A-F) and effect of hydrogen peroxide (H) and SNP (sodium nitroprusside) (I, J & K) on tea plants.

Table 29: Effect of time gap between treatment with hydrogen peroxide and challenge inoculation on disease index per plant.

Variety	Time (h) after treatment with H_2O_2 (0.15%)	Disease index/plant*	% protection over control
T-17/1/54	24	4.33 ± 0.15	23.6
	48	3.07 ± 0.10	45.8
	72	4.13 ± 0.14	27.2
Untreated control	0	5.67 ± 0.12	-
TV-22	24	5.04 ± 0.12	21.6
	48	3.79 ± 0.15	41.1
	72	4.73 ± 0.10	26.4
Untreated control	0	6.43 ± 0.03	-
TV-30	24	1.00 ± 0.10	04.7
	48	0.86 ± 0.05	18.1
	72	1.03 ± 0.10	01.9
Untreated control	0	1.05 ± 0.10	-

± SE, n=3 ; 10 plants were used per experiment per treatment

* Evaluated 35 days after inoculation with *G. cingulata* (GC-1).

4.8.1.3.1. Peroxidase

Peroxidase specific activity was measured in control and hydrogen peroxide (0.15%) – treated tea plants between 2 and 48 hours post treatment. It is evident from Fig. 11 (A-C) that the tea varieties used responded to treatment with H_2O_2 in terms of peroxidase specific activity. Initially, there was no significant change in activity up to 12 hours after treatment. However, peroxidase activity increased significantly when compared to untreated control as tested by Student's t-test 24 hours after treatment in the tea plants. Further increase was evident 48 hours after treatment in case of T-17, TV-22 and TV-30. This increase was steeper in the two susceptible varieties (127% in T-17/1/54, 128% in TV-22) than in the resistant (83% in TV-30). There were no obvious

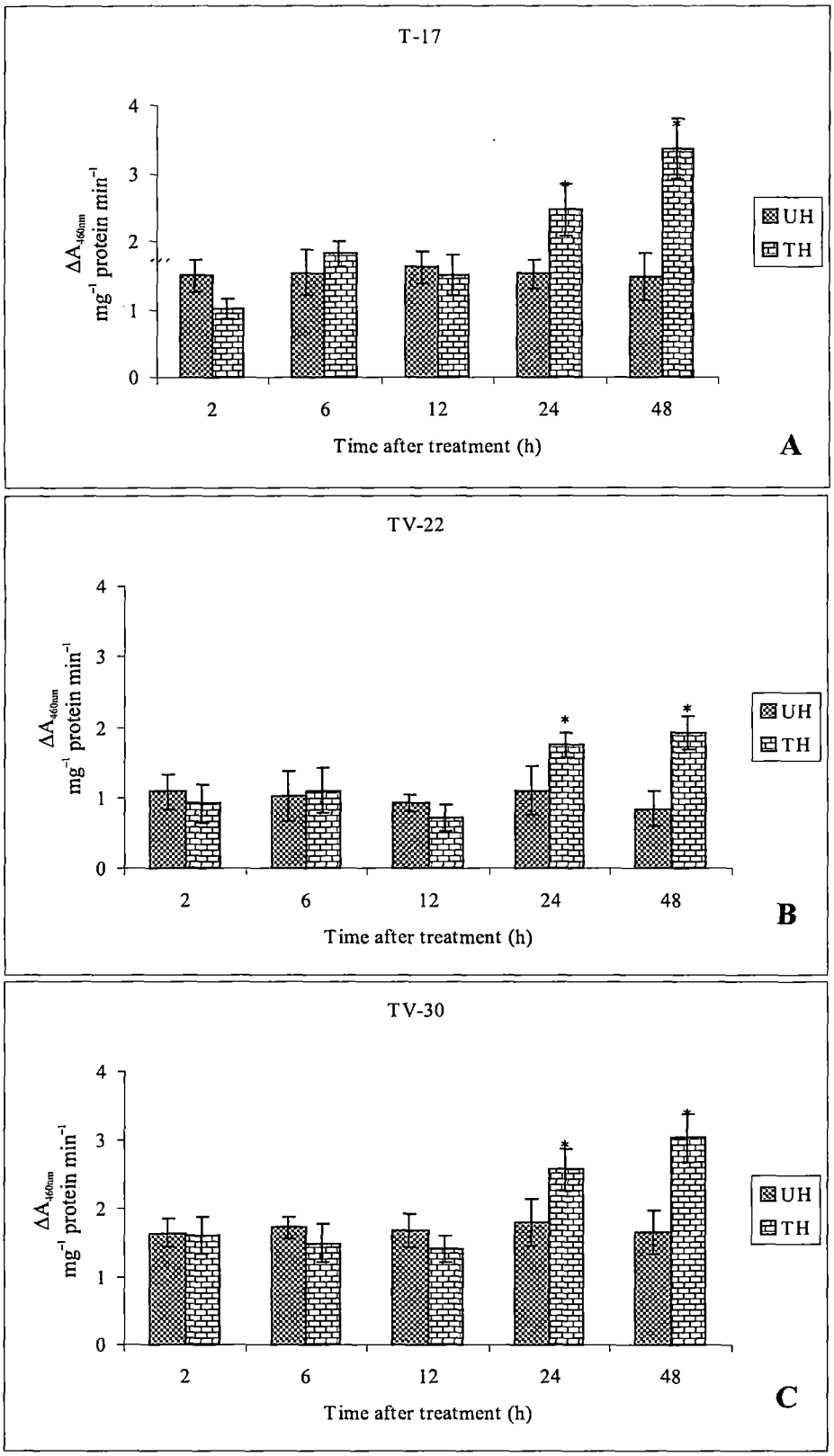


Fig. 11 (A-C): Effect of hydrogen peroxide treatment on peroxidase activity in tea varieties.

differences in response between resistant and susceptible interactions. At the time of peroxidase level assessment the activity was still high in the treated healthy plants, except in case of the susceptible TV-22, which registered an insignificant decline. Inoculation of the treated tea plants with *G. cingulata* further stimulated peroxidase activity (Table 31) when determined 48 hours after inoculation.

4.8.1.3.2. Catalase

Catalase is known to act as a major hydrogen peroxide scavenger. Therefore, it was of a major interest to know its effect after delivering excess of its substrate on to tea leaves. Catalase specific activity was measured in control and 0.15% hydrogen peroxide – treated tea leaves between 2 and 48 hours post treatment. The results of these studies are presented in Table 30.

Specific acivity of catalase was found to be was highest in T-17 (2.44 units mg^{-1} protein $min.^{-1}$) and lowest (1.25 units mg^{-1} protein $min.^{-1}$) in TV-30 at the constitutive level. Statistically significant differences were found 12 hours after treatment in TV-22 and TV-30 as well as 24 hours after treatment in TV-30. On the other hand, plants of T-17 did not show any significant variation in catalase activity throughout the study. Plants of TV-22, on the other hand, registered an increase (37.6%) with respect to control at 12 hours after treatment. TV-30 behaved in a totally different way. There was a sharp and significant increase (97.6%) in activity 12 hours after treatment, after which gradual decline followed.The level of the major hydrogen peroxide scavenging enzyme did not change much in the susceptible varieties on treatment. In contrast, in the resistant variety addition of the external H_2O_2 stimulated catalase to metabolise and scavenge the radical, which has the potential to damage the plant cell. However, after the extra amount has been scavenged, there was decrease. The constitutively high presence of catalase in the varieties examined may account for the tolerance of tea plants to such high dozes of hydrogen peroxide.

Inoculation of the untreated susceptible varieties (T-17/1/54 and TV-22) led to decreased CAT activity as revealed 48 hours after inoculation (Table 31). The incompatible interaction, however, showed a statistically significant increase in CAT activity. It appears that the high amounts of this enzyme are needed for resistance in order to combat the oxidative stress occurring during pathogen invasion. Level of CAT in case of treated healthy tissues was not statistically different from the untreated healthy

ones. Inoculation of the treated plants, however, triggered increase in CAT specific activity, which was significantly different from untreated healthy and treated healthy in TV-22 and TV-30.

Table 30: Time course accumulation of catalase in leaves of hydrogen peroxide-treated tea plants.

Variety	Treatment	. Catalase activity (ΔA_{240nm} mg^{-1} protein $min.^{-1}$) . Time after treatment (h)				
		2	6	12	24	48
T-17	UH	2.32 ± 0.01	2.44 ± 0.05	2.22 ± 0.04	2.28 ± 0.07	2.35 ± 0.04
	TH	2.26 ± 0.09	2.41 ± 0.03	2.63 ± 0.13	2.49 ± 0.17	2.57 ± 0.19
TV-22	UH	2.09 ± 0.06	2.16 ± 0.06	1.97 ± 0.06	2.07 ± 0.05	1.82 ± 0.04
	TH	2.11 ± 0.15	2.16 ± 0.13	*2.71 ± 0.19	2.23 ± 0.22	2.35 ± 0.08
TV-30	UH	1.26 ± 0.05	1.48 ± 0.06	1.26 ± 0.04	1.25 ± 0.06	1.27 ± 0.06
	TH	1.35 ± 0.12	1.55 ± 0.22	*2.49 ± 0.23	*2.31 ± 0.16	1.52 ± 0.09

Values are means ±S.E., n=3; UH-Untreated Healthy; TH – Treated Healthy; *Difference with untreated healthy (UH) significant at P=0.02 tested by Student's t-test

4.8.1.3.3. Ascorbate peroxidase

Ascorbate peroxidase, another AOS scavenger, was investigated in the present discourse. Specific activity of APX was measured in control and 0.3% hydrogen peroxide – treated tea leaves sampled as mentioned earlier. Pattern of specific activity of ascorbate peroxidase was found to be similar in all the three varieties tested in case of treatment. (Fig. 12 A - C). There is a gradual increase with respect to control up to 48 hours after treatment in the treated healthy plants.

Inoculation of the treated plants increased the activity to a great extent in TV-30. The other two varieties showed a very low increase. Treated healthy samples did not register activity different from the untreated tissues as detected 48 hours after inoculation. Besides, inoculated untreated plants, did not show any significant changes from the constitutive levels in the two susceptible varieties. In contrast, *G. cingulata* inoculated plants of TV-30, a resistant variety, indicated dramatic and significant

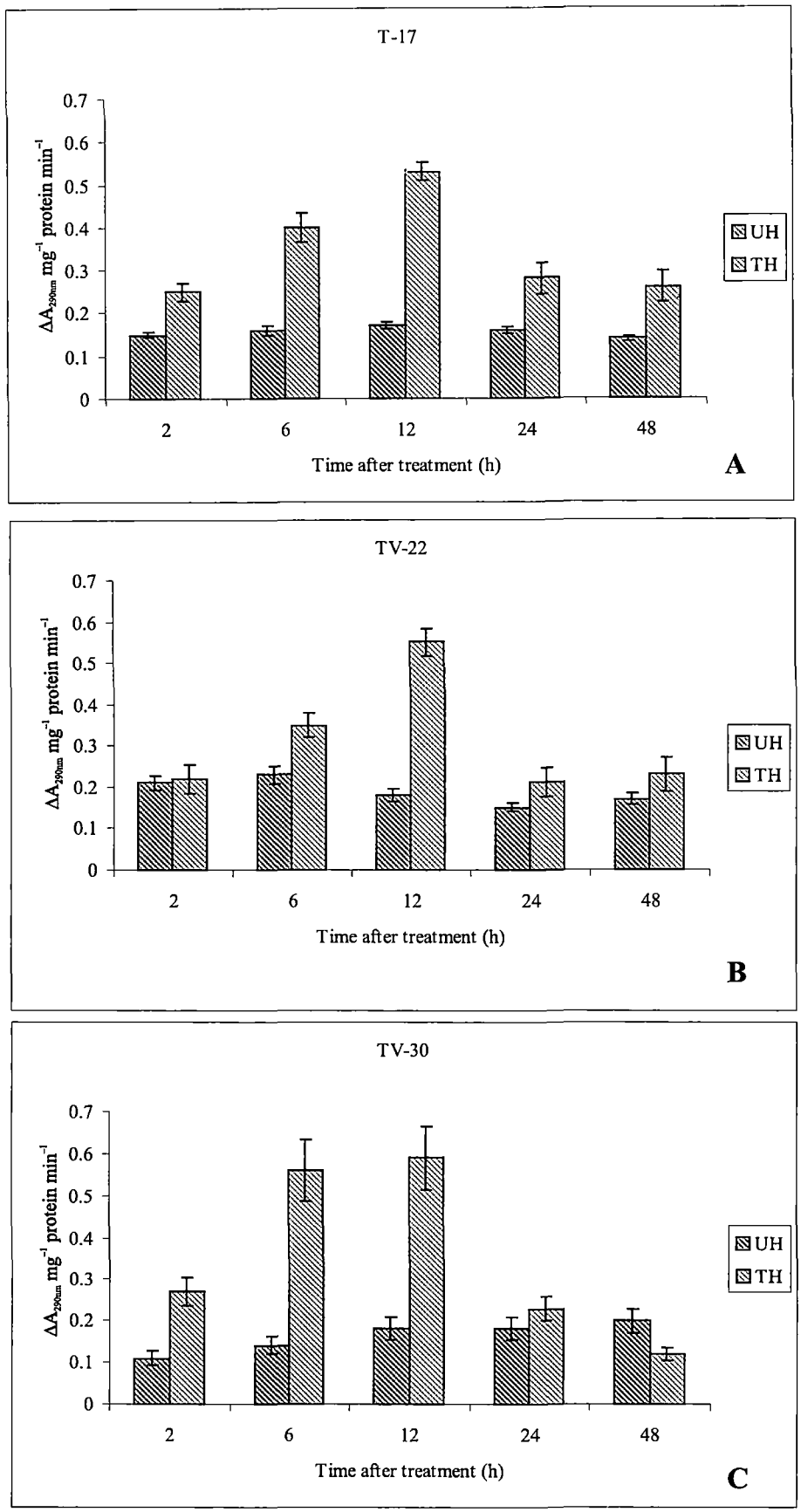


Fig. 12 (A - C): Effect of hydrogen peroxide treatment on ascorbate peroxidase activity in tea varieties.

increase. Thus, APX can also be considered as a defense enzyme, since its increase is related to defense process.

Table 31: Effect of hydrogen peroxide on activity of defense enzymes in healthy and *G.cingulata* – inoculated tea plants of different varieties.

Enzyme	Variety	Treatment			
		UH	UI	TH	TI
Peroxidase	T-17/1/54	1.45 ± 0.24	1.90 ± 0.19	3.01 ± 0.27	3.57 ± 0.39
(ΔA_{460nm} mg^{-1}	TV-22	1.13 ± 0.22	1.92 ± 0.29	1.81 ± 0.28	2.50 ± 0.37
protein $min.^{-1}$)	TV-30	1.66 ± 0.23	2.76 ± 0.26	3.04 ± 0.37	3.54 ± 0.34
Catalase	T-17/1/54	2.26 ± 0.02	1.43 ± 0.12	2.32 ± 0.10	3.48 ± 0.09
(ΔA_{240nm} mg^{-1}	TV-22	2.13 ± 0.02	1.77 ± 0.11	2.45 ± 0.08	2.97 ± 0.13
protein $min.^{-1}$)	TV-30	1.33 ± 0.03	2.77 ± 0.11	1.25 ± 0.09	3.08 ± 0.10
Ascorbate	T-17/1/54	0.17 ± 0.01	0.35 ± 0.03	0.37 ± 0.03	0.78 ± 0.04
peroxidase	TV-22	0.20 ± 0.02	0.41 ± 0.04	0.20 ± 0.05	1.11 ± 0.04
(ΔA_{290nm} mg^{-1}	TV-30	0.16 ± 0.02	1.12 ± 0.04	0.15 ± 0.04	1.32 ± 0.05
protein $min.^{-1}$)					
Chitinase	T-17/1/54	0.24 ± 0.03	0.25 ± 0.08	0.31 ± 0.09	0.32 ± 0.07
(mg GlcNAc	TV-22	0.26 ± 0.03	0.27 ± 0.07	0.31 ± 0.08	0.34 ± 0.05
g^{-1}tissue $h.^{-1}$)	TV-30	0.21 ± 0.03	0.35 ± 0.06	0.29 ± 0.06	0.36 ± 0.06
β-1,3-Glucanase	T-17/1/54	23.7 ± 1.3	25.7 ± 2.3	31.2 ± 2.4	36.4 ± 2.1
(mg glucose	TV-22	24.3 ± 1.5	26.3 ± 1.9	31.6 ± 2.6	37.1 ± 3.1
g^{-1}tissue $min.^{-1}$)	TV-30	34.1 ± 1.8	40.5 ± 1.5	34.4 ± 3.0	38.0 ± 2.4

Values are means ± SE, n=3 ; UH- Untreated Healthy; UI- Untreated Inoculated; TH-Treated Healthy; TI-Treated Inoculated

4.8.1.3.4. Chitinase

Chitinase is included in PR-3 group of pathogenesis-related proteins (PR-3) and these are elicited by the SAR inducers. In the present work, it was found out that application of hydrogen peroxide enhanced the basal level of chitinase significantly in tea plants only after a lag of 48h, when the activity increased as compared to the constitutive level in T-17, TV-22 and TV-30 (Table 32) separately.

Treated inoculated susceptible varieties indicated an increase in CHT activity, which was similar and statistically not different from the values obtained in case of untreated inoculated resistant variety (Table 31). Thus, CHT is associated with the induced resistance in tea plants.

Table 32: Time course accumulation of chitinase in leaves of hydrogen peroxide-treated and untreated tea plants.

Variety	Treatment	Chitinase activity (mg GlcNAc g^{-1} tissue $h.^{-1}$) Time after treatment (h)				
		2	6	12	24	48
T-17	UH	0.254 ± 0.05	0.212 ± 0.06	0.245 ± 0.02	0.230 ± 0.04	0.250 ± 0.06
	TH	0.253 ± 0.03	0.208 ± 0.05	0.164 ± 0.06	0.256 ± 0.06	*0.461 ± 0.05
TV-22	UH	0.276 ± 0.05	0.254 ± 0.04	0.279 ± 0.05	0.263 ± 0.03	0.281 ± 0.05
	TH	0.231 ± 0.04	0.257 ± 0.06	0.224 ± 0.04	*0.386 ± 0.03	*0.491 ± 0.08
TV-30	UH	0.216 ± 0.03	0.204 ± 0.05	0.222 ± 0.04	0.201 ± 0.04	0.218 ± 0.03
	TH	0.203 ± 0.01	0.164 ± 0.02	0.119 ± 0.04	*0.357 ± 0.07	*0.409 ± 0.05

Values are means ±S.E. , n=3; UH-Untreated Healthy; TH – Treated Healthy;
*Difference with untreated healthy (UH) significant at P=0.02 tested by Student's t-test

4.8.1.3.5. β-1,3-glucanase

β-1,3-glucanase is another PR-protein, included in PR-2 family. The time course accumulation on treatment is illustrated in Fig.13 (A – C). Significant differences from the untreated is noticed in case of T-17 and TV-22. However, no such differnces were observed in TV-30.

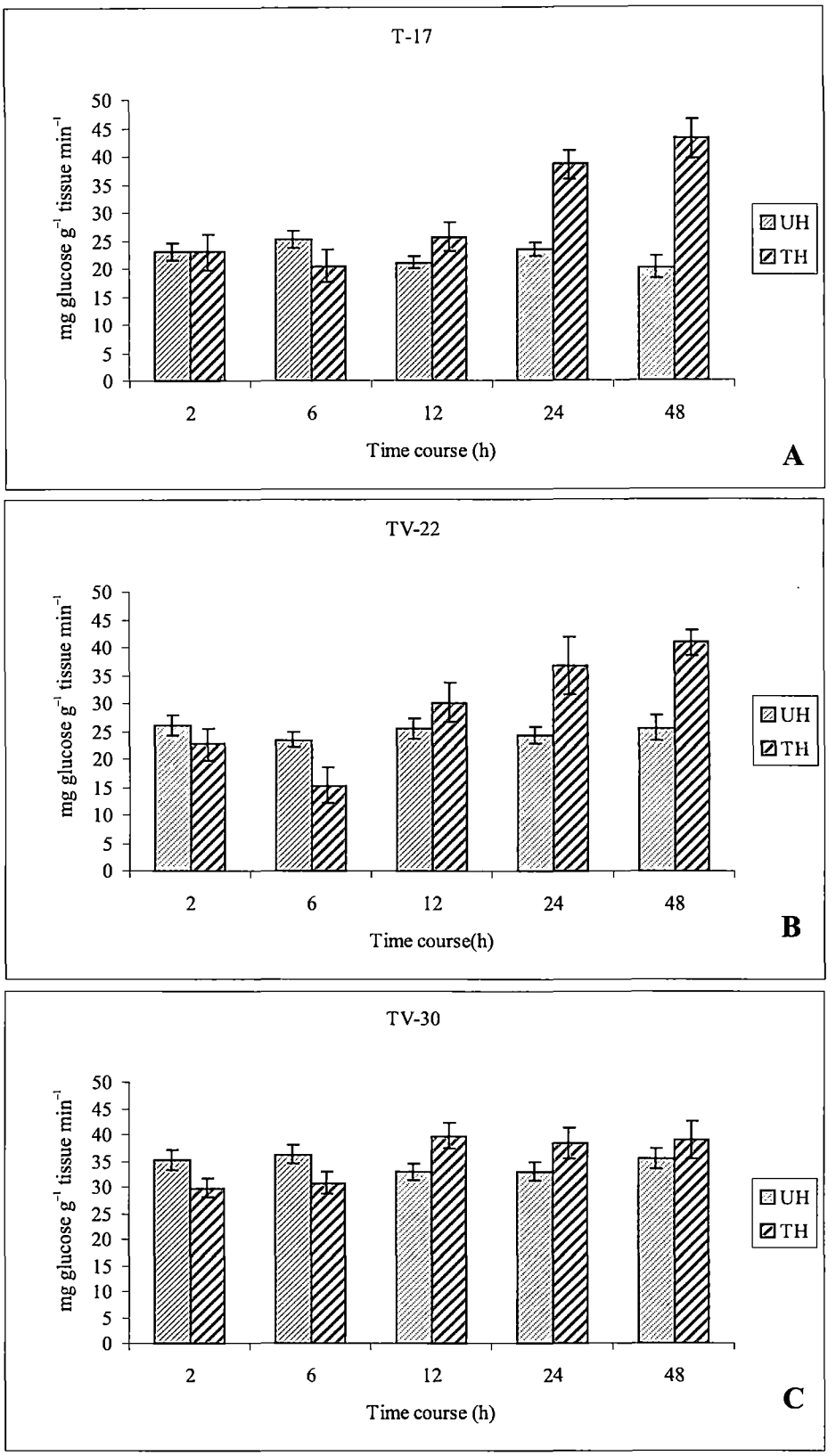


Fig 13 (A - C): Effect of hydrogen peroxide treatment on β-1,3 glucanase activity in tea varieties.

Inoculation with *G.cingulata* enhanced the level of this important enzyme in all the interactions, especially obvious in case of the treated susceptible varieties (Table 31), which was comparable to the healthy inoculated of resistant variety (TV-30).

It is quite clear from the results that hydrogen peroxide acts via POX and APX as ROS scavengers through increase in CHT and βGLU, which are important biochemical markers of induced resistance.

4.8.2. Induction of resistance with sodium nitroprusside

Sodium nitroprusside (SNP) or sodium pentacyanonitrosoferrate generates nitric oxide when it comes in contact with the vascular system in presence of light and a reducing agent. NO has been recently identified as a molecule that mediates hypersensitive cell death, but its exact role in the resistance mechanism is unclear. In the present experiment, an attempt has been made to induce resistance against *G. cingulata* in tea plants and to ascertain its effect on the various defense enzymes as mentioned earlier.

Initially, the optimum doze was determined by treating the potted plants with 1mM, 0.1mM and 0.01mM concentrations of SNP as described earlier. No adverse effects on the growth of the plants were observed for 0.1 and 0.01mM concentrations. However, some dark spots resembling HR were observed on the edges and also on the middle portions of leaf lamina 3 days after the spray with 1mM solution of sodium nitroprusside (Plate 22, figs. I-K). These spots reduced and completely vanished 10 days later. However, since the quality of the young tea leaves is industrially important, next higher concentration (0.1mM) was used for the present study.

4.8.2.1. Assessment of disease intensity and determination of induction period in tea varieties following treatment with sodium nitroprusside and inoculated with *G. cingulata*

The potted tea plants of TV-30, TV-22, T-17/1/54 varieties were sprayed with 0.1mM SNP solution and inoculated with *G. cingulata* spore suspension as described earlier while keeping a gap of 24h, 48h and 72h after treatment. The DI (Disease Index) was assessed 35 days after inoculation. The results presented in Table 33 indicate that the best protection was offered to the tea plants when the gap between treatment and inoculation was 24h. The efficacy of the treatment reduced with the increasing time

Table 33: Effect of time gap between treatment with sodium nitroprusside and challenge inoculation with *G. cingulata* on disease index per plant.

Variety	Time (h) after treatment with SNP (1mM)	Disease index/plant*	% protection over control
T-17/1/54	24	3.20 ± 0.16	45.0
	48	4.22 ± 0.12	25.2
	72	5.04 ± 0.09	11.6
Untreated control	0	5.64 ± 0.11	-
TV-22	24	3.59 ± 0.08	51.7
	48	4.11 ± 0.12	35.8
	72	5.76 ± 0.04	10.0
Untreated control	0	6.40 ± 0.12	-
TV-30	24	0.93 ± 0.30	09.7
	48	0.97 ± 0.25	05.8
	72	1.01 ± 0.13	01.9
Untreated control	0	1.03 ± 0.15	-

± SE, n=3 ; ten plants were used per experiment per treatment

* Evaluated 35 days after inoculation with *G. cingulata* (GC-1).

period. In the susceptible TV-22, DI reduced by 57.1%, while in T-17/1/54, it reduced by 45% with respect to the untreated control plants. In the resistant TV-30 maximum reduction in DI was only 9.7%. It is therefore, clear that the IP (Induction Period) is 24h for the present system. This time gap between treatment and inoculation was maintained throughout the study of SNP effect on tea plants.

Table 34: Effect of sodium nitroprusside on activity of defense enzymes in healthy and *G. cingulata* – inoculated tea plants of different varieties.

Enzyme	Variety	Treatment			
		UH	UI	TH	TI
Peroxidase	T-17/1/54	1.52 ± 0.14	1.90 ± 0.19	1.78 ± 0.16	3.18 ± 0.24
(ΔA_{460nm} mg^{-1}	TV-22	1.10 ± 0.26	1.92 ± 0.33	1.32 ± 0.17	2.52 ± 0.34
protein $min.^{-1}$)	TV-30	1.80 ± 0.34	2.70 ± 0.35	2.18 ± 0.40	3.26 ± 0.41
Catalase	T-17/1/54	2.28 ± 0.07	2.86 ± 0.09	2.58 ± 0.16	3.37 ± 0.10
(ΔA_{240nm} mg^{-1}	TV-22	2.07 ± 0.05	2.44 ± 0.07	2.34 ± 0.15	2.95 ± 0.05
protein $min.^{-1}$)	TV-30	1.25 ± 0.06	2.56 ± 0.11	1.45 ± 0.20	3.18 ± 0.10
Ascorbate	T-17/1/54	0.16 ± 0.01	0.37 ± 0.03	0.24 ± 0.03	1.26 ± 0.10
peroxidase	TV-22	0.15 ± 0.01	0.45 ± 0.04	0.20 ± 0.02	0.88 ± 0.06
(ΔA_{290nm} mg^{-1}	TV-30	0.18 ± 0.02	1.17 ± 0.04	0.23 ± 0.04	1.24 ± 0.06
protein $min.^{-1}$)					
Chitinase	T-17/1/54	0.23 ± 0.03	0.25 ± 0.05	0.75 ± 0.10	0.80 ± 0.05
(mg GlcNAc	TV-22	0.26 ± 0.04	0.29 ± 0.03	0.82 ± 0.09	0.85 ± 0.06
g^{-1}tissue $h.^{-1}$)	TV-30	0.20 ± 0.03	0.43 ± 0.04	0.45 ± 0.11	0.67 ± 0.03
β-1,3-Glucanase	T-17/1/54	23.31 ± 1.34	26.57 ± 2.31	39.52 ± 2.55	43.24 ± 2.71
(mg glucose	TV-22	24.34 ± 3.62	29.30 ± 1.45	30.31 ± 5.26	45.71 ± 3.14
g^{-1}tissue $min.^{-1}$)	TV-30	33.03 ± 3.72	37.85 ± 2.54	48.14 ± 1.90	50.80 ± 2.43

Values are means ± SE, n=3 ; UH- Untreated Healthy; UI- Untreated Inoculated; TH-Treated Healthy; TI-Treated Inoculated

4.8.2.2. Analysis of level of defense enzymes on treatment of tea plants with sodium nitroprusside and inoculation with *G. cingulata*

Sodium Nitroprusside acts by releasing NO (Nitric oxide) into the plant system. NO is an important gaseous ROS that regulates cellular processes in living organisms. It was, therefore, considered worthwhile to examine time course accumulation of the ROS scavenger enzymes – peroxidase, catalase and ascorbate peroxidase on treatment with SNP as well as on inoculation with *G. cingulata*. Besides, since some amount of protection was offered by the compound, the activities of two PR-proteins (Pathogenesis-related proteins) – PR-2 (β-1,3-glucanase) and PR-3 (chitinase) were also assessed in treated and untreated plants 48h post inoculation.

4.8.2.2.1. Peroxidase

Peroxidase specific activity was found to be decreasing initially right after treatment in the tea plants as shown in Fig 14 (A-C). However, significant decrease was found only in T-17/1/54 at 6 and 12 hours after treatment. The plants of this variety were more responsive even in hydrogen peroxide treatment as compared to TV-22. The specific activity increased significantly at P=0.01 as tested by Student's t-test 48 hours after treatment in all the three varieties. The increase was to the extent of 127%, 85.7% and 190.9% in T-17, TV-22 and TV-30 respectively, when compared to their corresponding controls (untreated healthy). Therefore, after a lag phase of around 12 h, POX activity increased on treatment with the NO releasing agent, SNP (sodium nitroprusside).

Further, activity of this enzyme in the inoculated untreated plants 48 hours after inoculation also increased when compared to the untreated healthy control (Table 34). Treated inoculated plants exhibited maximum activity. These plants were induced for resistance and hence the increase in this defense enzyme. Thus, in this interaction also POX defends the plants against the potential threat by increase in specific activity.

4.8.2.2.2. Catalase

The level of this important ROS scavenger shows an increasing accumulation in the two susceptible varieties T-17 and TV-22, maximum being at 6hpt, up to 27.8% and 24.5% respectively relative to the corresponding control (Table 35). However, for the rest of the time intervals there are no significant changes in any of the varieties.

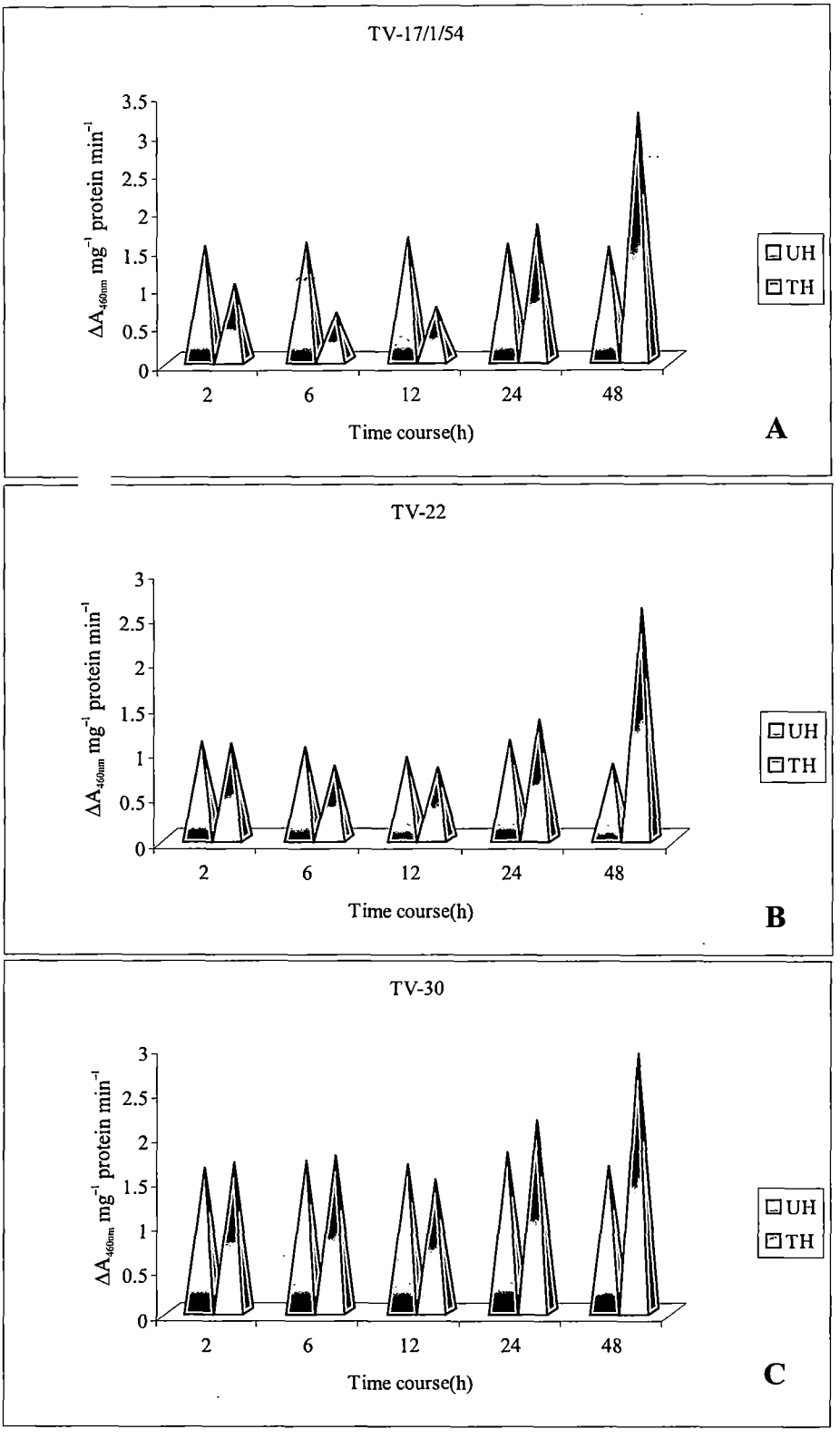


Fig. 14 (A - C): Effect of sodium nitroprusside treatment on peroxidase activity in tea varieties

Table 35: Time course accumulation of catalase in leaves of sodium nitroprusside-treated tea plants.

Variety	Treatment	. Catalase activity (ΔA_{240nm} mg^{-1} protein $min.^{-1}$) . Time after treatment (h)				
		2	6	12	24	48
T-17	UH	2.32 ± 0.03	2.44 ± 0.05	2.22 ± 0.04	2.28 ± 0.07	2.35 ± 0.04
	TH	3.06 ± 0.11	*3.12 ± 0.11	2.36 ± 0.14	*2.58 ± 0.15	2.16 ± 0.10
TV-22	UH	2.09 ± 0.06	2.16 ± 0.06	1.97 ± 0.06	2.07 ± 0.05	1.82 ± 0.04
	TH	2.54 ± 0.12	*2.69 ± 0.13	2.31 ± 0.19	*2.34 ± 0.15	2.05 ± 0.08
TV-30	UH	1.26 ± 0.05	1.48 ± 0.06	1.26 ± 0.04	1.25 ± 0.06	1.27 ± 0.06
	TH	1.58 ± 0.11	1.64 ± 0.18	1.51 ± 0.19	1.45 ± 0.20	1.44 ± 0.07

Values are means ± SE, n=3 ; UH- Untreated Healthy;TH-Treated Healthy; *Difference with untreated healthy (UH) significant at P=0.02 tested by Student's t-test

Thus, the effect of NO production via SNP application is indicated in CAT activity profile. Probably, NO produced acts by increasing level of hydrogen peroxide. In any case, the indication is that the extra hydrogen peroxide might have been produced initially, mainly within the first 6 hours after treatment.

Inoculation with *G. cingulata* increased the activity of CAT irrespective of the disease index of the varieties. Significant increase however, was noted in TV-30, the resistant variety (Table 34). Similar increase was found on treatment with SNP, which brought the level of CAT in the susceptible varieties to the level similar to that in the resistant variety.

4.8.2.2.3. Ascorbate peroxidase

It is clear from Fig. 15 (A – C) that APX activity is induced in all SNP-treated plants up to 12 hours after treatment. The greatest increase was 12 hours after treatment to as much as 211.8%, 100% and 61.1% in T-17, TV-22 and TV-30 respectively relative to their corresponding controls. This means that high amounts of endogenous H_2O_2 are actually produced by NO, which are required to be scavenged, and APX is the enzyme

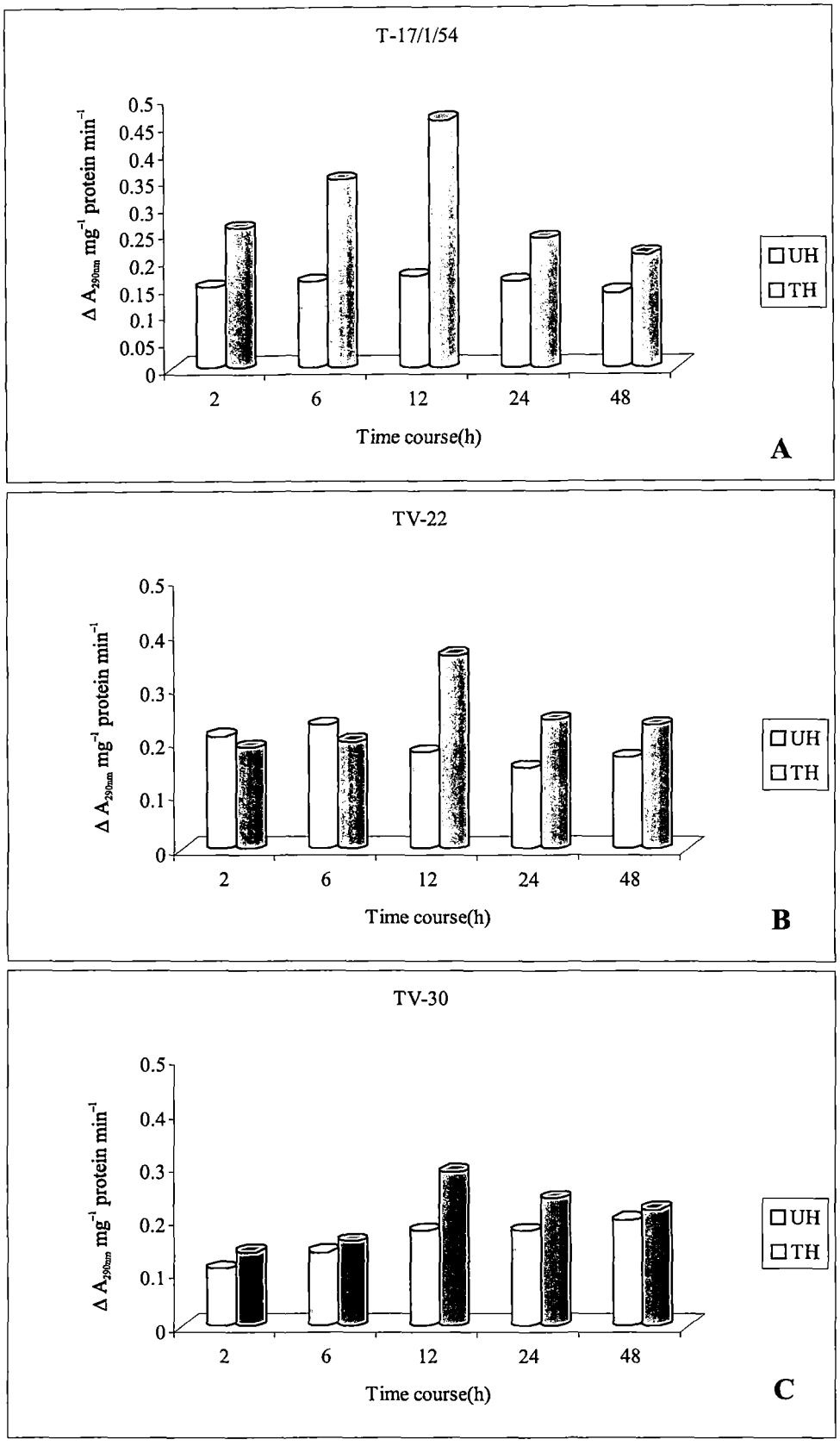


Fig. 15 (A - C): Effect of sodium nitroprusside treatment on ascorbate peroxidase activity in tea varieties.

undertaking this function in tea plants. Probably, after CAT stops acting APX takes off from that point to scavenge the radical.

Inoculation of the untreated tea plants did not change activity of APX significantly in the susceptible plants 48 hours after inoculation (Table 34). However, significant increase of this enzyme was noticed in the inoculated resistant varietiy TV-30 (irrespective of treatment) as well as in SNP-treated inoculated susceptible varieties. The high APX levels may be necessary for the resistance to develop.

4.8.2.2.4. Chitinase

Chitinase activity was measured in control (untreated healthy, UH) and 0.1mM SNP – treated (treated healthy, TH) (T-17, TV-22 and TV-30) are presented in Fig. 16 (A – C). It is quite clear that the CHT activity was at its highest in all the SNP-treated tea plants at 24 hours after treatment. There was a progressive increase 6 hours after treatment onwards up to 24 hours after treatment, after which there was a decline. This increase was significant with respect to the untreated control plants at 24 and 48 hours after treatment in the varieties examined.

Inoculation of tea plants with *G. cingulata* increased the level of this important PR protein drastically in case of the resistant variety (TV-30) with respect to the untreated healthy control (Table 34). In the susceptible untreated inoculated varieties no such significant increase was noted. However, treatment with SNP increased the level of this enzyme, which persisted even 48 hours after inoculation in the susceptible T-17/1/54 and TV-22. In the resistant TV-30, on the other hand, there was a further increase with respect to the healthy control in the treated inoculated samples. It is interesting to note that the level of this enzyme in the induced resistant plants was much higher than in the resistant TV-30.

4.8.2.2.5. β-1,3-glucanase

β-1,3-glucanase activity was measured in control (untreated healthy, UH) and 0.1mM SNP – treated (treated healthy, TH) tea leaves sampled between 2 and 48 hours post treatment with 0.1mM SNP solution. The results are presented in Table 36. Specific activity of this enzyme reached its peak at 48 hours after treatment in all the susceptible tea varieties examined (T-17/1/54 and TV-22). There was a progressive increase in such cases 6 hours after treatment onwards up to 48 hours after treatment. Statistically significant differences between untreated and treated tea plants was noted 12 hours after treatment in TV-22 and 24 hours after treatment in T-17/1/54. The

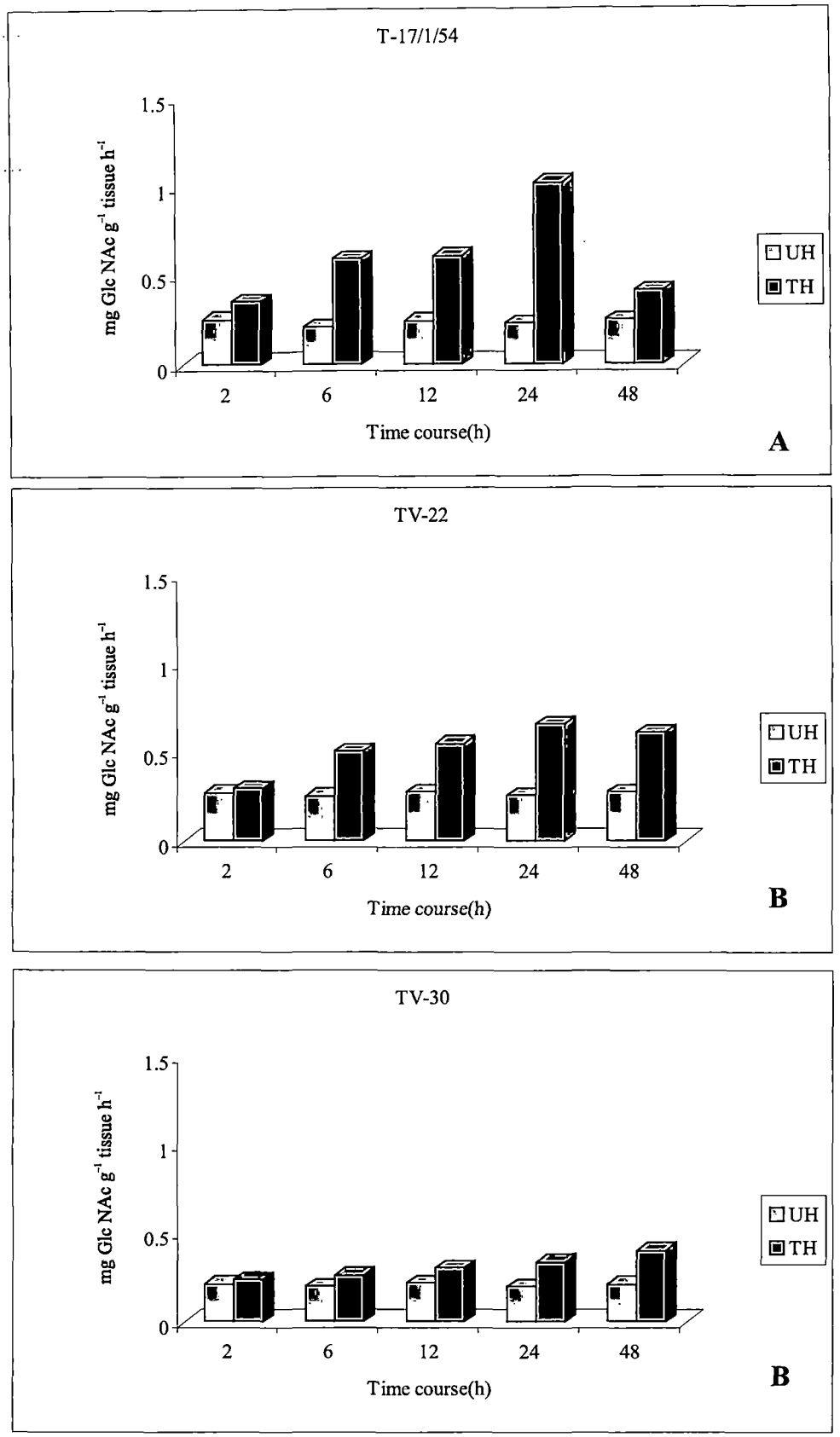


Fig. 16 (A - C): Effect of sodium nitroprusside treatment on chitinase activity in tea varieties.

Table 36: Time course accumulation of β-1,3-glucanase (mg glucose g^{-1} tissue $min.^{-1}$) in leaves of sodium nitroprusside-treated and untreated tea plants.

Variety	Treatment	Time after treatment (h)				
		2	6	12	24	48
T-17	UH	22.9 ± 4.7	25.1 ± 1.5	21.0 ± 1.1	23.3 ± 1.3	20.1 ± 4.9
	TH	19.8 ± 3.2	24.6 ± 1.0	23.8 ± 1.7	*29.5 ± 2.5	*35.3 ± 3.5
TV-22	UH	26.1 ± 3.8	23.4 ± 1.3	25.4 ± 2.8	24.3 ± 3.6	25.6 ± 5.4
	TH	22.4 ± 2.9	23.7 ± 3.3	*30.2 ± 3.4	*32.3 ± 5.2	*34.6 ± 2.2
TV-30	UH	35.1 ± 1.9	36.2 ± 2.8	32.8 ± 3.6	33.0 ± 3.7	35.4 ± 2.9
	TH	*28.3 ± 1.8	*30.9 ± 1.2	35.4 ± 1.4	*38.1 ± 1.9	38.5 ± 5.5

Values are means ± SE, n=3 ; UH- Untreated Healthy; TH-Treated Healthy;

*Difference with untreated healthy (UH) significant at P=0.05 tested by Student's t-test

resistant TV-30, however, behaved differently. Significant decrease in activity occured initially as early as 2 hours after treatment in this case. However, there was an abrupt recovery 12 hours after treatment and finally activity reached its peak 48 hours after treatment, which was much higher than in the SNP-treated susceptible varieties.

The untreated plants of the three varieties inoculated with *G. cingulata* spore suspension, exhibited a slight increase with respect to the healthy control plants. Treatment with NO-releasing molecule (sodium nitroprusside) enhanced the activity of βGLU notably (Table 34) in all the interactions.

It is, therefore, clear from these results that whatever little protection was offered to the susceptible tea varieties, was due to the induction of the two cell wall degrading enzymes – chitinase and β-1,3-glucanase. Besides, status of the antioxidant enzymes – catalase and ascorbate peroxidase was enhanced on inoculation especially in SNP-treated plants. The regulatory enzyme, peroxidase, however, increased in all the interactions. Its involvement needs more detailed investigation.

4.8.3. Induction of resistance with benzothiadiazole

SAR activator, salicylic acid analogue, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) was initially tested for its effect on tea plants at different concentrations viz. 0.01%, 0.1% and 1% diluted with distilled water and stirred on

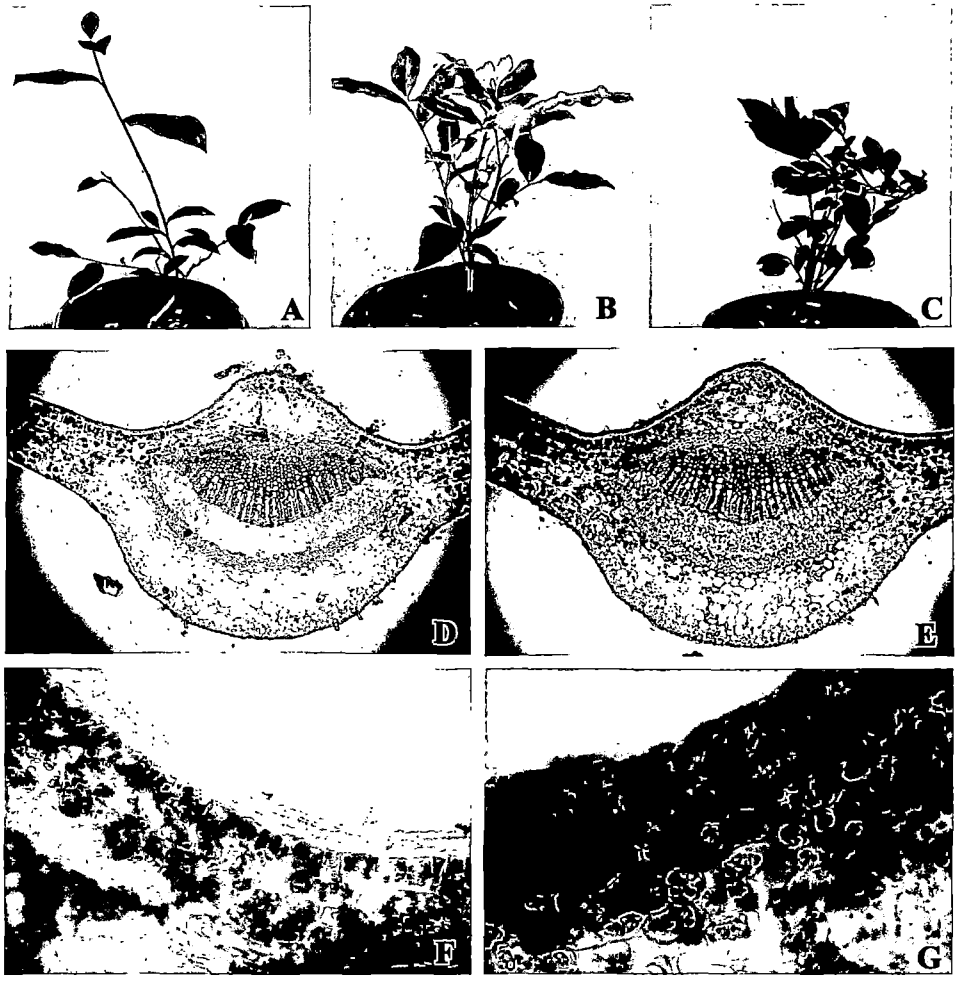


Plate 23 (A-G): Effect of BTH (benzothiadiazole) treatment. Untreated (A) and BTH-treated (B & C) tea plants (T-17/1/54) and cross sections of healthy (D & F) and 0.1% BTH-treated leaves (E&G).

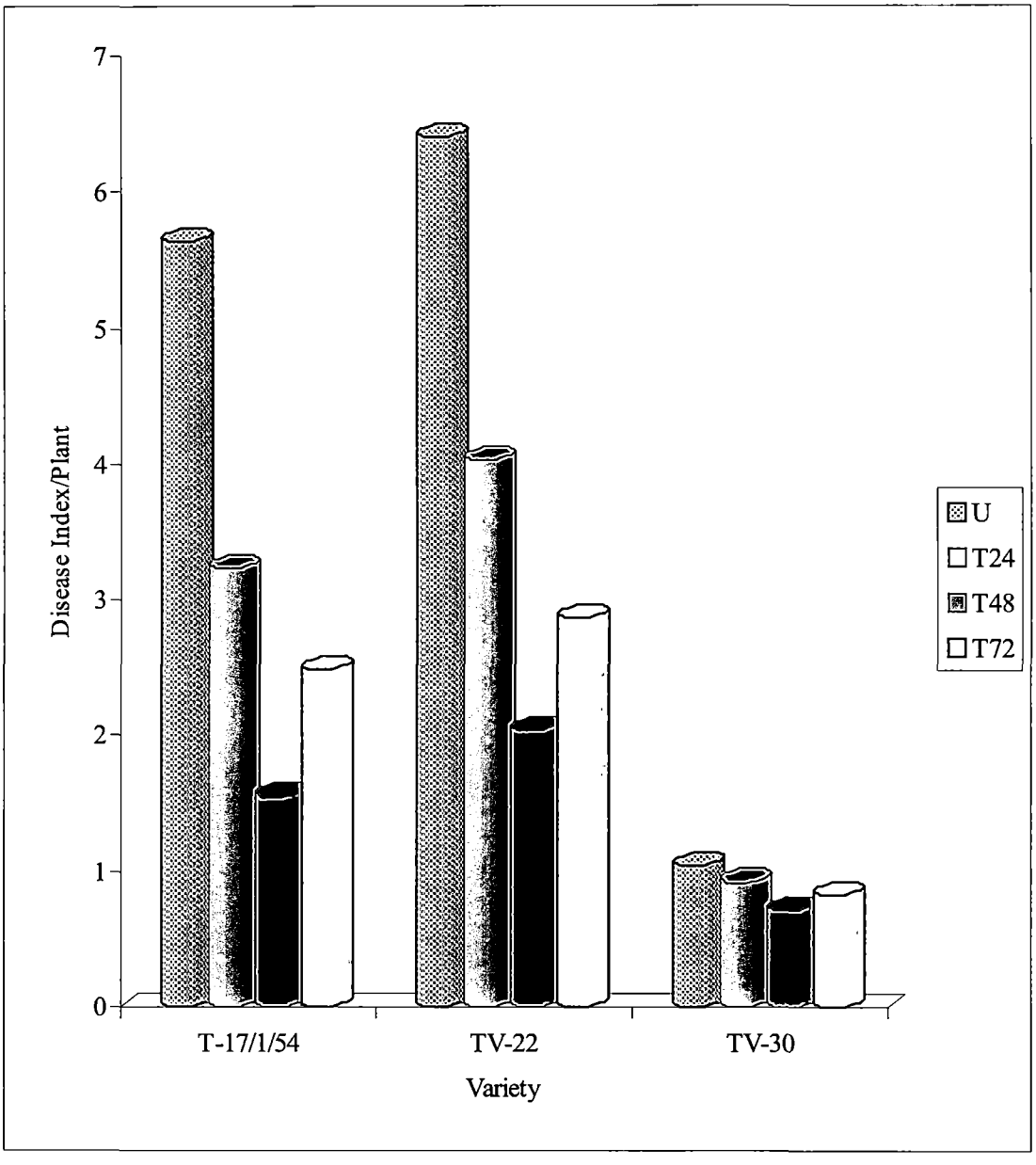


Fig. 17: Effect of time gap between treatment with benzothiadiazole and challenge inoculation with *G. cingulata* on Disease development.

magnetic stirrer until the solution was homogenous. It was noted that the plants sprayed with 1% solution were visibly stunted with respect to the untreated plants one month after the spray. Their buds did not exhibit the expected growth as compared to the plants treated with the two lower concentrations (Plate 23, figs. A - C). Therefore, the next highest concentration (0.1%) was used for induction of resistance in the present experiment.

4.8.3.1. Assessment of disease intensity and determination of induction period in tea varieties following treatment with benzothiadiazole and inoculated with *G. cingulata*

Initially, just as in the previous treatments, optimum time gap between treatment and inoculation with *G. cingulata*, was standardized. Disease intensity was assessed by whole plant inoculation technique. The results are presented in and Figure 17. It is evident from these findings that the time gap of 48 h between treatment and challenge inoculation controlled the disease to the best extent. Protection was as much as 72.52% in case of the susceptible variety T-17/1/54 and 68.13% in TV-22. In the resistant TV-30 it was 32.38%.. In view of the best protection offered by this compound out of the three tested, field trials were conducted with BTH. The treatment with the inducer visibly reduced the brown blight infection during its occurrence.

4.8.3.2. Anatomical changes

Cross-sections of the untreated and long-term BTH-treated (4 consecutive sprays) tea leaves were observed under microscope and visible changes in the anatomy. There was thickening in the mesophyll cells of the tea leaves (Plate 23, figs E & G) that was absent in the untreated leaves (Plate 23, figs D & F).

4.8.3.3. Analysis of level of defense enzymes on treatment of tea plants with benzothiadiazole and inoculation with *G. cingulata*

Studies on the levels of defense enzymes, POX, CAT, APX, CHT and GLU were conducted as described for the earlier two inducers. Initially, time course accumulation changes in the enzyme level were recorded and subsequently these were assessed after inoculation in BTH- pre-treated and untreated tea plants and compared to the respective controls.

4.8.3.3.1. Peroxidase

Time course accumulation of POX in the BTH-treated tea plants indicated a statistically significant increase in POX specific activity 48 hours after treatment in T-17,

TV-22 and TV-30 (Table 37). Thus there was a very gradual increase in activity on treatment in all the three tea varieties examined. No significant differences in POX activity pattern was observed between the different varieties.

Inoculation of the tea plants with *G. cingulata* did not change the level of specific activity of POX to any significant extent. However, there was a rise in enzyme content on treatment (Table 39). Treated inoculated plants did not show activity different from the treated healthy. Therefore, activity of POX did not reveal much changes in this case.

4.8.3.3.2. Catalase

Catalase specific activity reduced and differed significantly when tested by Student's t-test at 24 hours after treatment in the two susceptible varieties, T-17 and TV-22 (Table 38). It seems that BTH, similar to SA, inhibited CAT activity in tea.

Inoculation of untreated tea plants with *G. cingulata* caused a slight but insignificant increase in activity in case of the susceptible varieties T-17 and TV-22.

Table 37: Time course accumulation of peroxidase in leaves of benzothiadiazole treated tea plants.

Variety	Treatment	Peroxidase activity (ΔA_{460nm} mg^{-1} protein $min.^{-1}$) Time after treatment (h)				
		2	6	12	24	48
T-17	UH	1.54 ± 0.26	1.62 ± 0.24	1.52 ± 0.33	1.48 ± 0.24	1.50 ± 0.26
	TH	1.57 ± 0.14	1.59 ± 0.32	1.58 ± 0.24	1.94 ± 0.27	*2.46 ± 0.31
TV-22	UH	1.03 ± 0.22	1.12 ± 0.31	0.98 ± 0.25	1.11 ± 0.21	0.88 ± 0.16
	TH	0.98 ± 0.27	1.15 ± 0.24	1.18 ± 0.25	1.28 ± 0.28	*1.47 ± 0.26
TV-30	UH	1.64 ± 0.15	1.72 ± 0.19	1.68 ± 0.28	1.73 ± 0.24	1.69 ± 0.33
	TH	1.67 ± 0.24	1.70 ± 0.23	1.65 ± 0.17	*2.09 ± 0.22	*2.30 ± 0.27

Values are means ± SE, n=3 ; UH- Untreated Healthy; TH-Treated Healthy;
*Difference with untreated healthy (UH) significant at P=0.05 tested by Student's t-test

Table 38: Time course accumulation of catalase in leaves of benzothiadiazole-treated and untreated tea plants.

Variety	Treatment	Catalase activity (ΔA_{240nm} mg^{-1} protein $min.^{-1}$) Time after treatment (h)				
		2	6	12	24	48
T-17	UH	2.22 ± 0.02	2.21 ± 0.06	2.22 ± 0.05	2.28 ± 0.07	2.35 ± 0.07
	TH	2.26 ± 0.04	2.22 ± 0.06	2.05 ± 0.04	*1.93 ± 0.04	*1.46 ± 0.09
TV-22	UH	1.97 ± 0.04	2.06 ± 0.03	1.97 ± 0.08	2.07 ± 0.08	1.98 ± 0.10
	TH	2.05 ± 0.05	2.05 ± 0.07	1.77 ± 0.09	*1.66 ± 0.05	*1.22 ± 0.10
TV-30	UH	1.26 ± 0.03	1.24 ± 0.06	1.26 ± 0.06	1.25 ± 0.09	1.27 ± 0.10
	TH	1.20 ± 0.07	1.25 ± 0.08	1.26 ± 0.09	1.23 ± 0.11	1.02 ± 0.08

Values are means ± SE, n=3 ; UH- Untreated Healthy; TH-Treated Healthy;

*Difference with untreated healthy (UH) significant at P=0.05 tested by Student's t-test

On the contrary, inoculation of TV-30 under the same conditions caused a markedly significant increase in activity (Table 39). Inoculation of the induced plants with the pathogen caused a recovery of the lost catalytic activity. Therefore, the ultimate level of CAT remained same independent of treatment. It may be that after hydrogen peroxide is utilised for some sort of signal reactions, level of CAT returns to its basal level. Reduction in CAT may be needed for increasing hydrogen peroxide concentration in the tea tissues.

4.8.3.3.3. Ascorbate peroxidase

APX behaves in a manner similar to CAT. BTH treatment gradually reduced level of the enzyme in tea tissues, especially 24 and hours after treatment (Fig.18 A – C). APX specific activity was also measured in *G. cingulata* inoculated control (untreated inoculated, UI) and 0.1% BTH-treated (treated inoculated, TI) tea leaves 48 hours after inoculation. These were compared with the uninoculated controls as well. The results are presented in Table 39. It is evident that just as in case of CAT, there was a recovery of this antioxidant enzyme in the treated susceptible varieties on inoculation. There was only an insignificant increase on inoculation in the uninduced tea plants (T-17 and TV-22). When these plants were inoculated after pre-treatment with BTH, level of APX was significantly greater than in the untreated inoculated plants of the susceptible

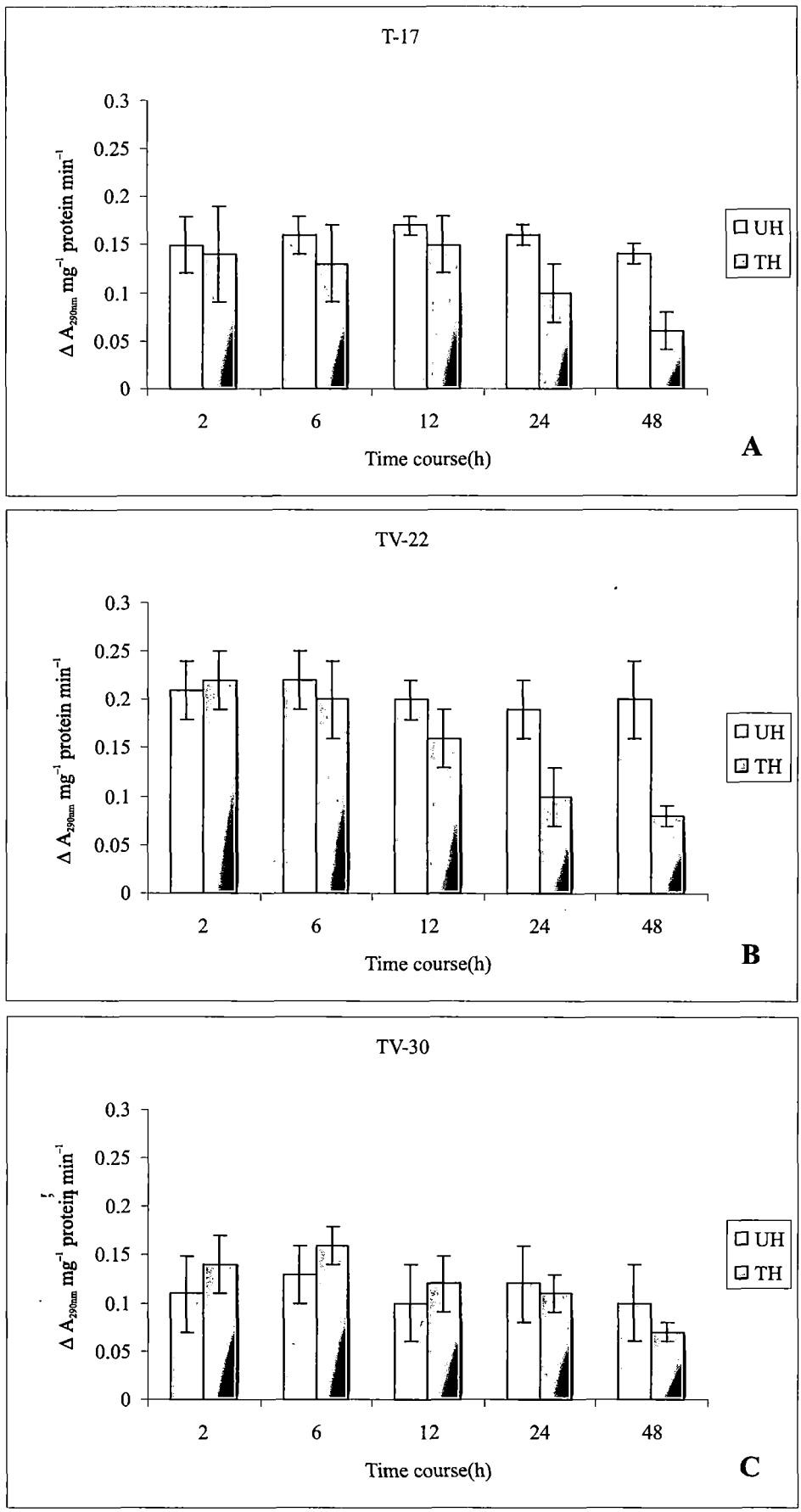


Fig. 18 (A -C): Effect of benzothiadiazole treatment on ascorbate peroxidase activity in tea varieties.

varieties (T-17 and TV-22). The resistant variety, however, did mot register any significant changes in the enzyme level with respect to untreated inoculated. In this resistant variety, APX level did not differ between UI and TI. Therefore, role of APX in resistant reaction is quite obvious, as treatment enhanced the level of APX in the susceptible varieties on inoculation.

4.8.3.3.4. Chitinase

Citinase, the enzyme considered to be an important marker if induced resistance, was measured in the leaf tissues of different varieties on treatment with BTH. Time course studies indicated significantly enhanced the level of this enzyme in the susceptible varieties only (T-17 and TV-22), which was found to be highest 48 hours after treatment (Fig. 19 A – C). No significant difference was found in the resistant TV-30 variety with respect to control throughout the study. CHT activity was also measured in control (untreated inoculated, UI) and 0.1% BTH-treated (treated inoculated, TI) tea leaves 48 hours after inoculation. These were compared with the uninoculated control plants as well. The results are presented in Table 39. It is evident that pre-treatment has enhanced CHT activity in the susceptible varieties (T-17 and TV-22) on inoculation significantly with respect to untreated plants. On the other hand, the untreated plants of TV-30 variety, registered a dramatic and significant increase on inoculation with *G. cingulata*. Therefore, CHT plays a definite and important role on resistance induced with BTH.

4.8.3.3.5. β-1,3-glucanase

β-1,3-glucanase activity was initially measured in control (untreated healthy, UH) and BTH – treated (treated healthy, TH) tea leaves sampled between 2 and 48 hours after treatment. The results are presented in Fig. 20 (A – C). Significant increase in activity of this enzyme was observed at 24 and 48 hours after treatmen in the susceptible varieties (T-17 and TV-22) with respect to the control. The resistant variety, TV-30, however, did not show any significant increase on treatment throughout this study. βGLU activity was measured in control (untreated inoculated, UI) and BTH-treated (treated inoculated, TI) tea leaves 48 hours after inoculation with *G.cingulata*. These were compared with the uninoculated controls. The results are shown in Table 39. The level of this hydrolysing enzyme indicated a trend similar to CHT. Pre-treatment with BTH enhanced βGLU activity in the susceptible varieties T-17 and TV-22 after inoculation.

The same pre-treatment also changed the level of βGLU to a great extent in the inoculated resistant variety (TV-30) with respect to control. On the contrary, inoculation of the untreated resistant variety (incompatible reaction) significantly increased the level of βGLU. In untreated susceptuble varieties (T-17 and TV-22) inoculation (compatible reactions) did not change the level of βGLU to any significant extent. Thus, only when the level of βGLU came to the level found in the inoculated resistant variety, there was induced resistance in tea plants.

Table 39: Effect of benzothiadiazole on activity of defense enzymes in healthy *G. cingulata* – inoculated tea varieties.

Enzyme	Variety	Treatment			
		UH	UI	TH	TI
Peroxidase	T-17/1/54	1.52 ± 0.14	1.79 ± 0.27	2.46 ± 0.33	2.43 ± 0.41
(ΔA_{460nm} mg^{-1}	TV-22	0.99 ± 0.13	1.16 ± 0.19	1.47 ± 0.24	1.40 ± 0.37
protein $min.^{-1}$)	TV-30	1.69 ± 0.04	1.88 ± 0.03	2.79 ± 0.04	2.82 ± 0.04
Catalase	T-17/1/54	2.35 ± 0.05	2.86 ± 0.09	1.46 ± 0.12	2.81 ± 0.11
(ΔA_{240nm} mg^{-1}	TV-22	1.98 ± 0.07	2.44 ± 0.07	1.22 ± 0.05	2.51 ± 0.12
protein $min.^{-1}$)	TV-30	1.27 ± 0.03	2.76 ± 0.10	1.02 ± 0.07	2.26 ± 0.08
Ascorbate	T-17/1/54	0.14 ± 0.02	0.33 ± 0.01	0.06 ± 0.01	1.08 ± 0.05
peroxidase	TV-22	0.20 ± 0.03	0.39 ± 0.04	0.08 ± 0.01	0.62 ± 0.03
(ΔA_{290nm} mg^{-1}	TV-30	0.10 ± 0.01	1.15 ± 0.03	0.07 ± 0.01	1.26 ± 0.02
protein $min.^{-1}$)					
Chitinase	T-17/1/54	0.25 ± 0.03	0.25 ± 0.05	1.21 ± 0.07	1.47 ± 0.05
(mg GlcNAc	TV-22	0.28 ± 0.03	0.29 ± 0.07	1.04 ± 0.05	1.26 ± 0.02
g^{-1}tissue $h.^{-1}$)	TV-30	0.22 ± 0.04	0.96 ± 0.04	0.94 ± 0.03	1.23 ± 0.09
β-1,3-Glucanase	T-17/1/54	22.60 ± 1.03	24.32 ± 2.35	56.83 ± 3.42	55.33 ± 3.13
(mg glucose	TV-22	26.50 ± 1.15	25.40 ± 1.81	37.92 ± 3.63	56.3 ± 2.77
g^{-1}tissue $min.^{-1}$)	TV-30	34.41 ± 1.80	57.3 ± 2.91	46.7 ± 3.71	55.4 ± 3.07

Values are means ± SE, n=3 ; UH- Untreated Healthy; UI- Untreated Inoculated; TH-Treated Healthy; TI-Treated Inoculated

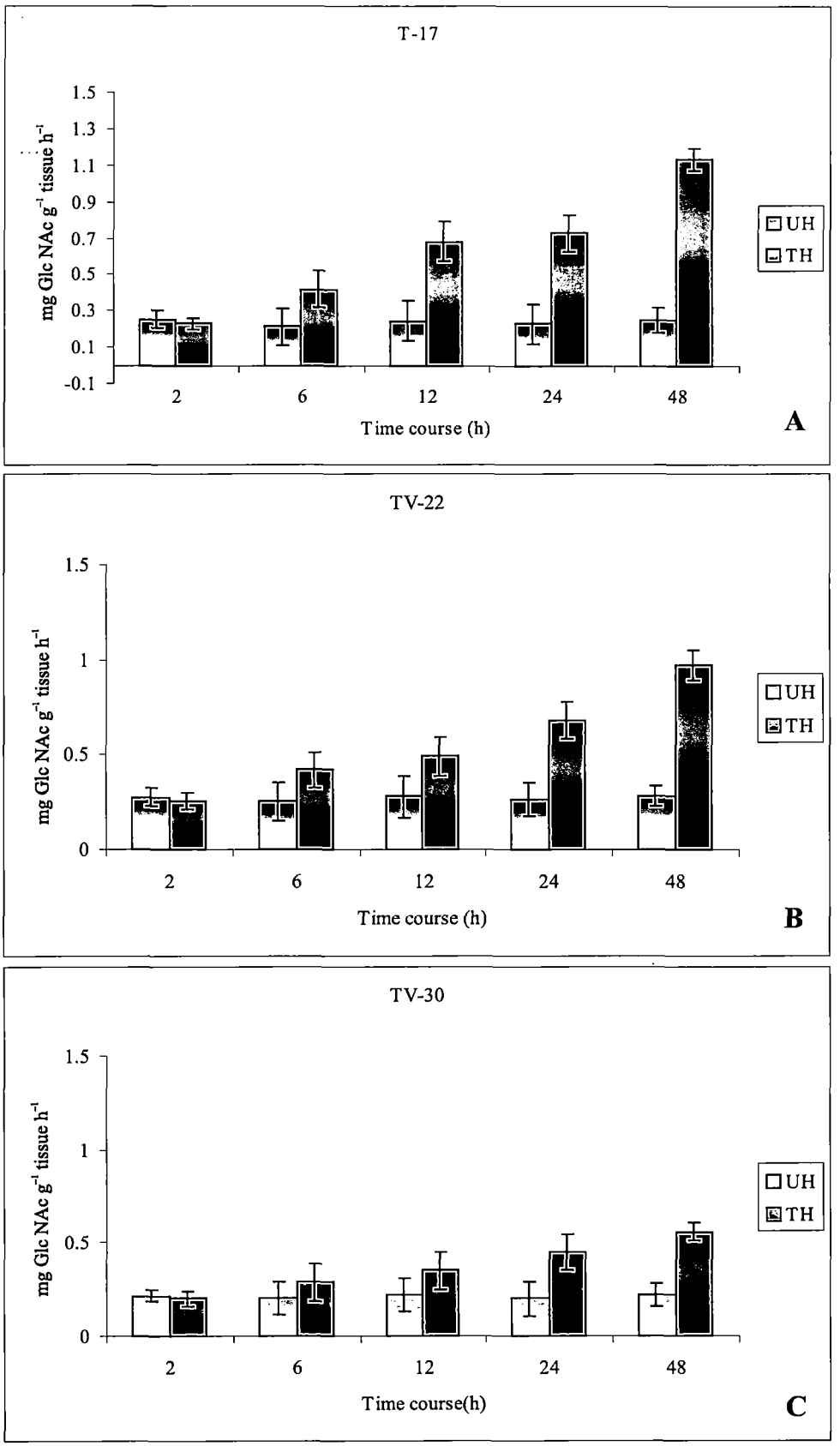


Fig. 19 (A -C): Effect of benzothiadiazole on chitinase activity in tea varieties.

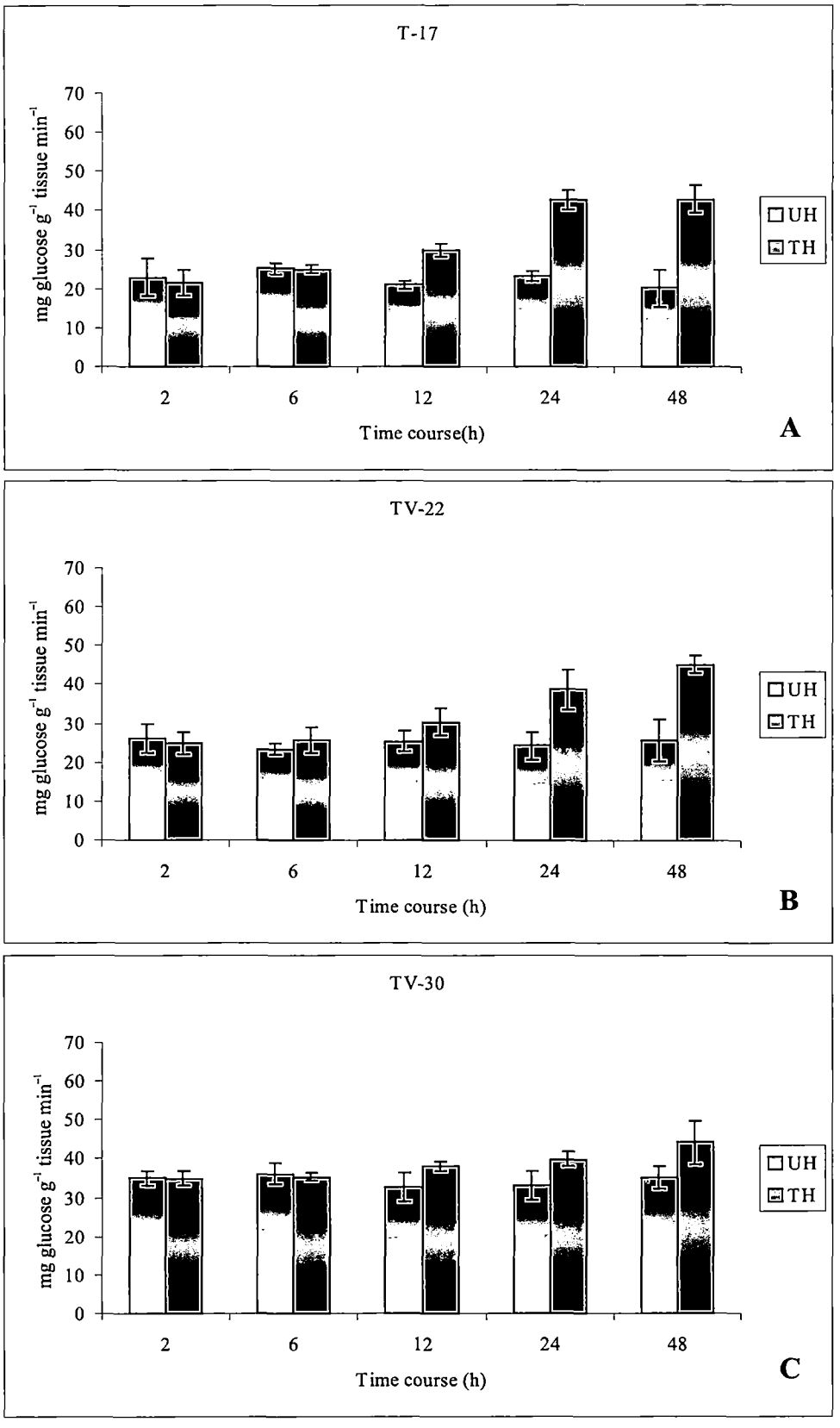


Fig. 20 (A -C): Effect of benzothiadiazole on β-1,3 glucanase activity in tea varieties.

4.9. Peroxidase isozyme analysis of tea plants following induction of resistance towards *G. cingulata*

Peroxidase is included in PR-9 family of pathogenesis-related proteins. It is an important regulator of various cellular processes and is very diverse regarding its substrate. Activity profile on induced resistance has already been investigated in the previous section. Besides, activity and isozyme analysis was conducted in healthy and *G. cingulata* inoculated leaf tissues of different varieties. Therefore, it was decided to conduct isoperoxidase profile analysis by Native PAGE on induced resistance. POX isozyme analysis was conducted 6, 12, 24 and 48 h post treatment with the inducing agent (hydrogen peroxide, sodium nitroprusside or benzothiadiazole). Besides, analysis was done for treated healthy (TH), treated inoculated (TI), untreated healthy (UH), and untreated inoculated (UI) 48 hours post inoculation with *G. cingulata*. Induction period was maintained as 48 h for H_2O_2 and BTH, while for SNP 24 h was maintained throughout the study as per earlier findings.

Table 40: Relative mobility (R_m) values of isoperoxidases in healthy control and hydrogen peroxide treated tea plants.

Variety	Hours post treatment	Number of isozymes	R_m values of isozymes
T-17/1/54	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	3	0.28, 0.47, 0.60
	48	4	0.28, 0.47, 0.60, 0.72
TV-22	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	3	0.28, 0.47, 0.60
	48	4	0.28, 0.47, 0.60, 0.72
TV-30	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	2	0.28, 0.47
	48	3	0.28, 0.47, 0.60

Table 41: Effect of treatment with hydrogen peroxide and inoculation with *G. cingulata* on peroxidase isozyme profile.

Variety	Treatment	Number of isozymes	R_m value of isozymes
T-17/1/54	UH	2	0.28, 0.47
	UI	3	0.28, 0.47, 0.92
	TH	3	0.28, 0.47, 0.85
	TI	6	0.20, 0.28, 0.47, 0.72, 0.85, 0.92
TV-22	UH	2	0.28, 0.47
	UI	3	0.28, 0.47, 0.92
	TH	3	0.28, 0.47, 0.85
	TI	6	0.20, 0.28, 0.47, 0.72, 0.85, 0.92
TV-30	UH	2	0.28, 0.47
	UI	4	0.11, 0.28, 0.47, 0.60
	TH	3	0.28, 0.47, 0.85
	TI	4	0.20, 0.28, 0.47, 0.60

4.9.1. Hydrogen peroxide

As evident from Plate 24 (fig. A), and Table 40, there was induction of two new isozymes with R_m=0.60 and 0.72. Presence of two constitutive isozymes in all the tea plants with R_m=0.28 and 0.47 at all the stages of experimentation is easily observed. The isozyme with R_m=0.60 was expressed as early as 24 hours after treatment in the two varieties susceptible to brown blight disease (T-17 and TV-22). In the resistant TV-30, on the other hand, the same isozyme was expressed only 48 hours after treatment.

Inoculation of tea leaves of the susceptible varieties with *G. cingulata* spore suspension induced expression of the peroxizyme with R_m=0.92. Resistant TV-30 registered appearance of isozyme with R_m=0.85 at 48 hours after inoculation (Plate 24, figs. B-D). Inoculation of the treated plants with *G. cingulata* was done 48 hours after treatment in this case. Thus, the isozyme patterns presented for treated healthy plants were 96 hours after treatment. There was appearance of an isozyme with R_m=0.85 at this point in all the three varieties examined. It is noteworthy that the same isomorph with R_m=0.60 was observed in the resistant plants on inoculation as well as in the susceptible plants on treatment with the inducer (Table 41). Thus, costitutively resistant and induced resistant plants showed the involvement of the same POX isozyme in resistance process. It may be recalled that the treatment with hydrogen peroxide offered 45.6% protection in case of T-17/1/54 and 40.8% protection in TV-22.

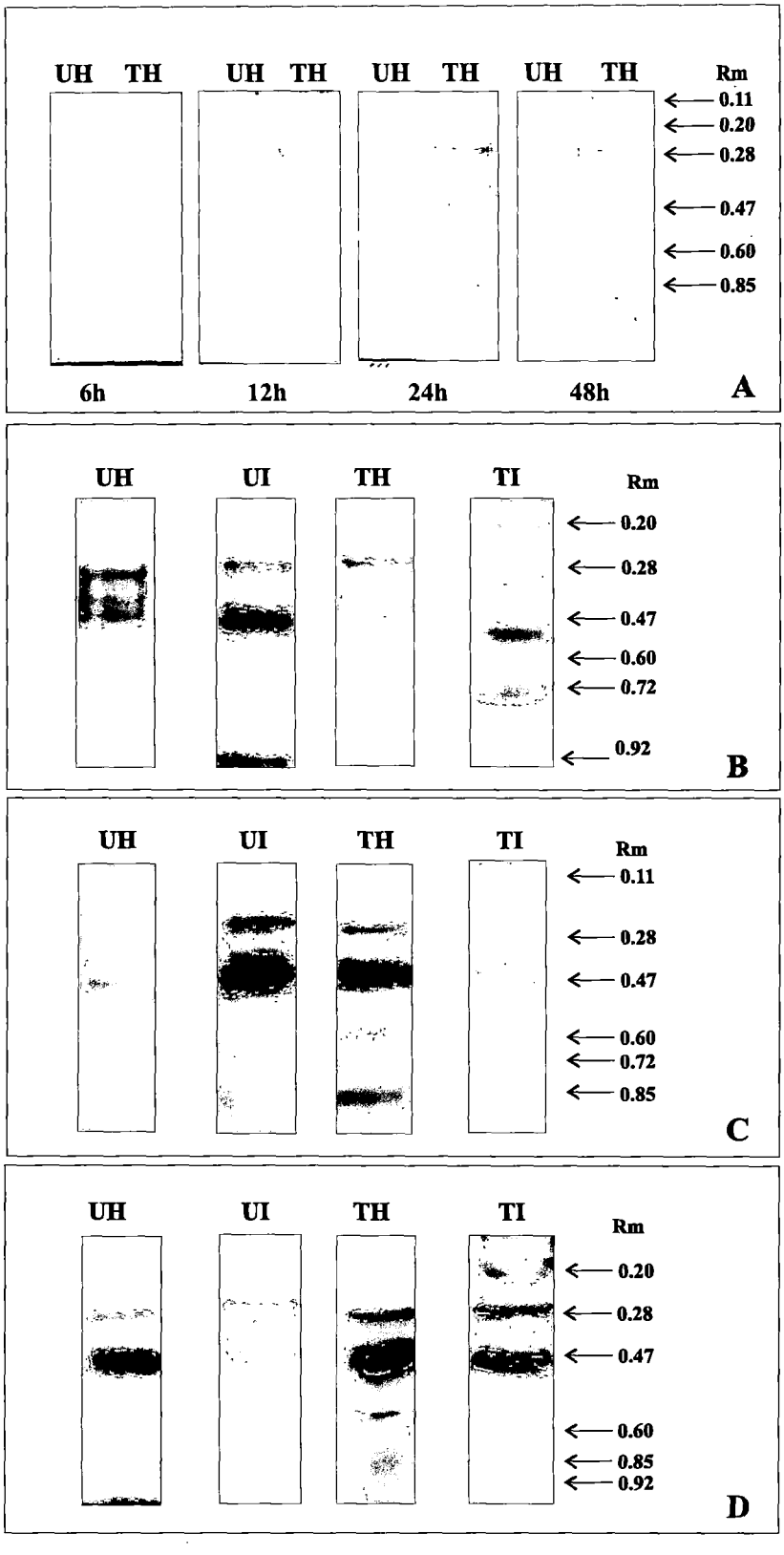


Plate 24 (A-D): Peroxidase isozyme analysis of leaf tissues of tea varieties [A & B T-17/1/54; C TV-22 ; D TV-30] following treatment with hydrogen peroxide (A) and after 48h of inoculation with *G. cingulata* (B-D); [UH: Untreated healthy; TH : treated healthy; TI : treated inoculated ; UI : untreated inoculated.]

Table 42: Relative mobility (R_m) values of isoperoxidases in healthy control and sodium nitroprusside treated tea plants.

Variety	Hours post treatment	Number of isozymes	R_m values of isozymes
T-17/1/54	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	4	0.28, 0.47, 0.60, 0.72
	48	5	0.11, 0.28, 0.47, 0.60, 0.85
TV-22	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	4	0.28, 0.47, 0.60, 0.72
	48	5	0.11, 0.28, 0.47, 0.60, 0.72
TV-30	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	3	0.20, 0.28, 0.47
	48	4	0.20, 0.28, 0.47, 0.60

Table 43: Effect of treatment with sodium nitroprusside and inoculation with *G. cingulata* on peroxidase isozyme profile.

Variety	Treatment	Number of isozymes	R_m values of isozymes
T-17/1/54	UH	2	0.28, 0.47
	UI	3	0.28, 0.47, 0.92
	TH	4	0.28, 0.47, 0.60, 0.72
	TI	5	0.20, 0.28, 0.47, 0.60, 0.72
TV-22	UH	2	0.28, 0.47
	UI	4	0.28, 0.47
	TH	3	0.28, 0.47, 0.85
	TI	5	0.11, 0.28, 0.47, 0.60, 0.72
TV-30	UH	2	0.28, 0.47
	UI	3	0.28, 0.47
	TH	5	0.20, 0.28, 0.47, 0.60, 0.92
	TI	5	0.20, 0.28, 0.47, 0.60, 0.72

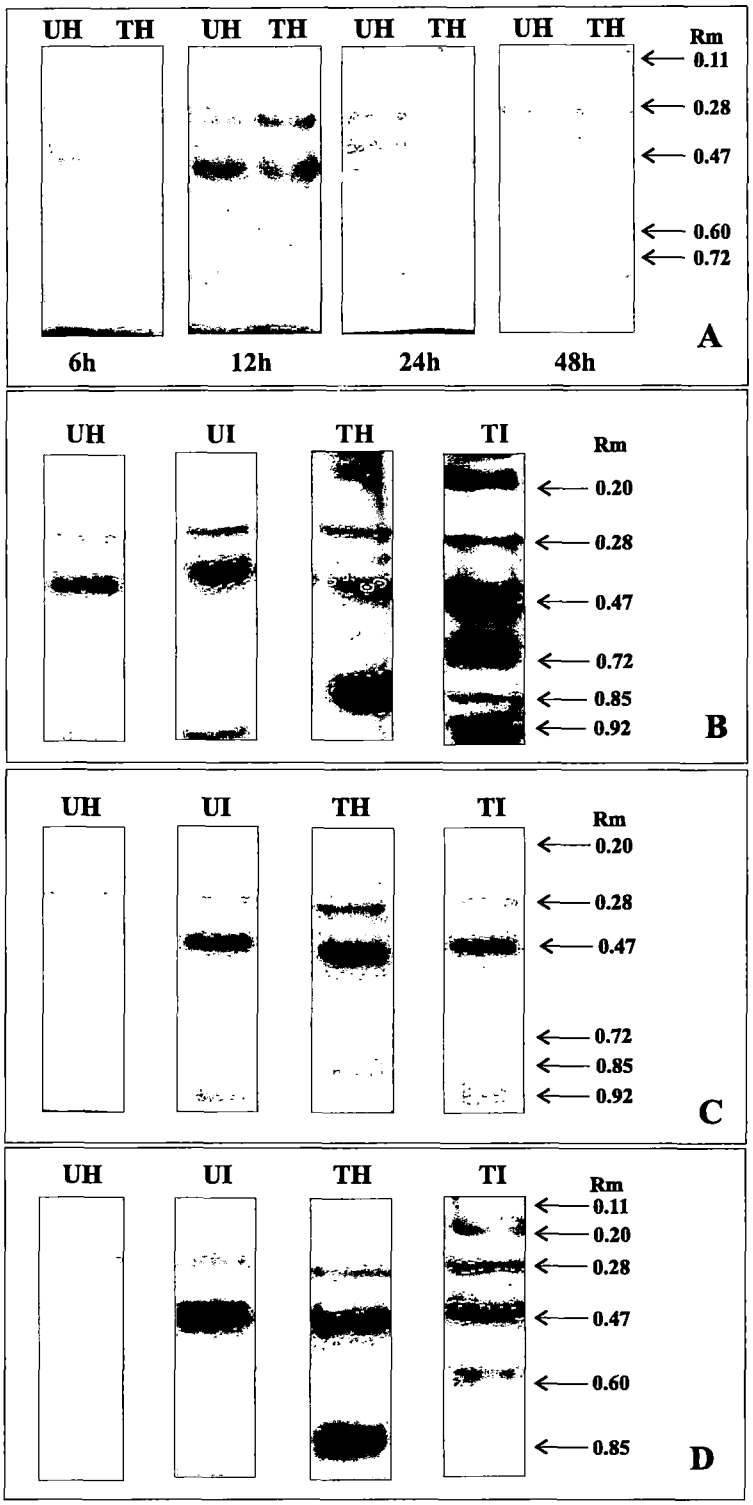


Plate 25 (A-D):Peroxidase isozyme analysis of leaf tissues of tea varieties [A & B T-17/1/54; C TV-22 ; DTV-30]following treatment with sodium nitroprusside (A) and after 48h of inoculation with *G. cingulata*(B-D);[UH: Untreated healthy; TH: treated healthy; TI: treated inoculated; UI : untreated inoculated.]

4.9.2. Sodium nitroprusside

Similar experimental pattern was followed in case of SNP used as inducer of resistance. It is evident from Table 42, that several bands appear in the SNP-treated tea plants. The band with R_m=0.11 was induced on treatment 48 hours after inoculation only in the susceptible varieties T-17 and TV-22. However, 72 h after treatment (TH plants) this isozyme disappears in both the varieties (Plate 25). It may be recalled that this particular isozyme was associated with resistance in the earlier experiments. There appearance of this isozyme in treated inoculated plants of TV-22. In TV-30 there isozyme with R_m=0.20 was prominently induced by SNP treatment. SNP had offered protection level similar to hydrogen peroxide. However, in this case no particular isozyme could be attributed to protective action.

4.9.3. Benzothiadiazole

Treatment of tea plants with salicylic acid analogue, benzothiadiazole offered best protection to tea plants among the three inducers tested. Presence of the protective isozyme with R_m=0.11, is prominent throughout the study. Besides, the same band was retained as late as 96 hours after treatment (Plate 26, fig. A, Table 45) i. e. 48 hours after inoculation in the treated healthy plants in case of the susceptible T-17 variety. However, TV-22 showed appearance of this band only 48 hours after treatment (Tables 44 & 45).

This isozyme with R_m=0.11 was also induced in the untreated inoculated plants of resistant variety (TV-30). Besides, the same band persisted in the treated inoculated plants of the same variety (Plate 26, figs. B-D ; Table 45). Therefore, in this case, different isozymes participate in the constitutive and induced resistance. It may be however recalled that the same isozyme (R_m=0.11) was expressed in the healthy tea leaves of three varieties that were resistant or moderately resistant to *G. cingulata*.

Table 44: Relative mobility (R_m) values of isoperoxidases in healthy control and BTH treated tea plants.

Variety	Hours post treatment	Number of isozymes	R_m values of isozymes
T-17/1/54	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	4	0.11, 0.28, 0.47, 0.60
	48	5	0.11, 0.28, 0.47, 0.60, 0.72
TV-22	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	3	0.28, 0.47, 0.72
	24	3	0.28, 0.47, 0.72
	48	4	0.11, 0.28, 0.47, 0.60, 0.72
TV-30	Control	2	0.28, 0.47
	6	2	0.28, 0.47
	12	2	0.28, 0.47
	24	2	0.28, 0.47
	48	3	0.28, 0.47, 0.60

Table 45 : Effect of treatment with benzothiazole and inoculation with *G. cingulata* on peroxidase isozyme profile.

Variety	Treatment	Number of isozymes	R_m values of isozymes
T-17/1/54	UH	2	0.28, 0.47
	UI	3	0.28, 0.47, 0.92
	TH	4	0.11, 0.28, 0.47, 0.60
	TI	6	0.11, 0.20, 0.28, 0.47, 0.60, 0.85
TV-22	UH	2	0.28, 0.47
	UI	3	0.28, 0.47, 0.92
	TH	4	0.28, 0.47, 0.60, 0.72
	TI	6	0.20, 0.28, 0.47, 0.60, 0.72, 0.85
TV-30	UH	2	0.28, 0.47
	UI	4	0.11, 0.28, 0.47, 0.60
	TH	2	0.28, 0.47
	TI	4	0.11, 0.28, 0.47, 0.85

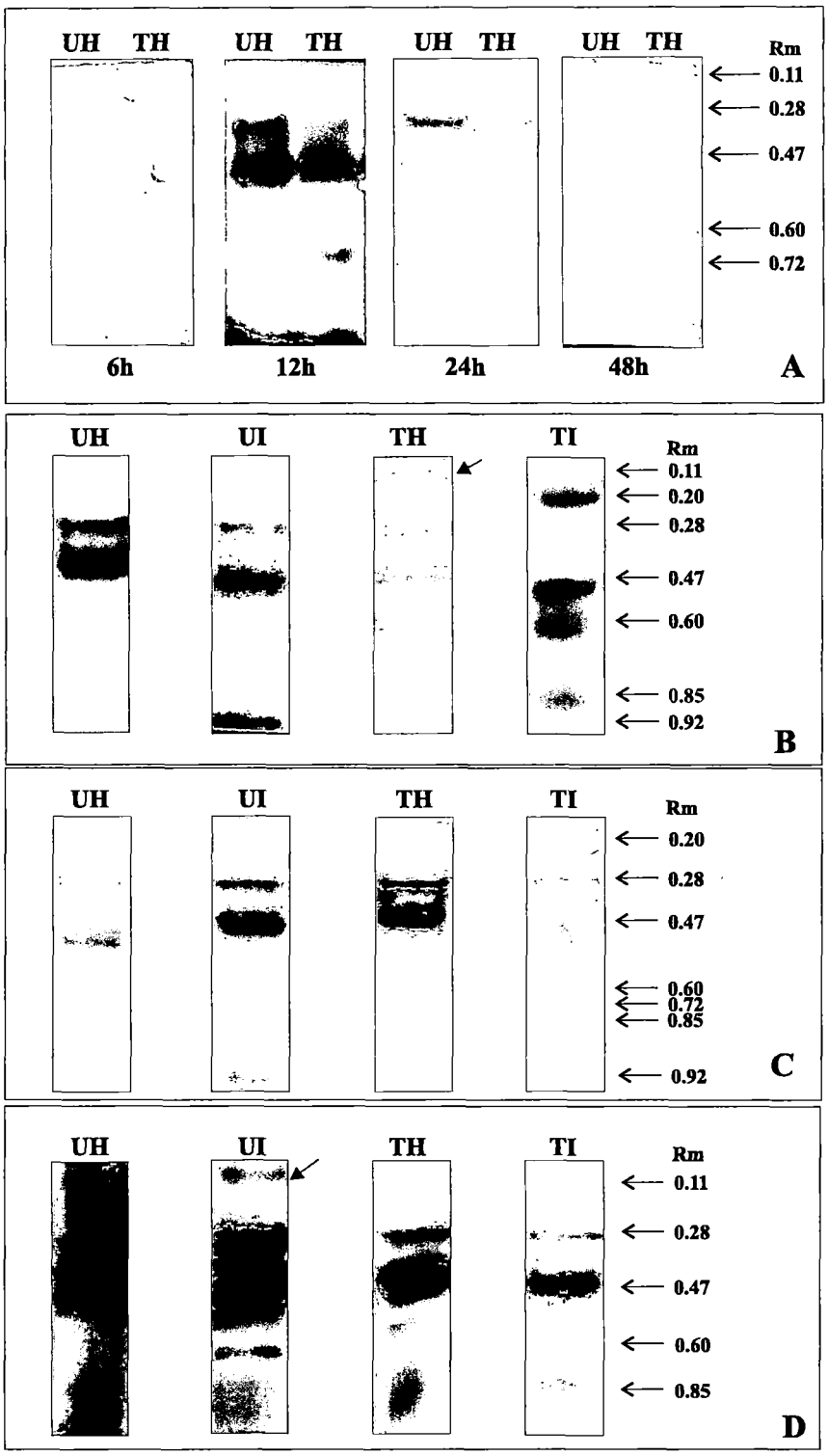


Plate 26 (A-D) : Peroxidase isozyme analysis of leaf tissues of tea varieties [A & B T-17/1/54; C TV-22 ; D TV-30] following treatment with benzothiadiazole (A) and after 48h of inoculation with *G. cingulata* (B-D); [UH: Untreated healthy; TH : treated healthy; TI : treated inoculated ; UI : untreated inoculated.

4.10. Immunolocalization of defense enzymes in tea leaf tissues

Induced resistance acts via definite mechanisms and can be manifested in different ways. Alterations in spore germination process of *G. cingulata* were observed *in vitro* on the surface of induced tea leaves. Besides, immunolocalization of defense enzymes – chitinase and β-1,3-glucanase in tea leaf tissues (*in vivo*) of susceptible (TV-22) and induced resistant (BTH-treated) was conducted using PAb of chitinase and β-1,3-glucanase obtained from the stock of Immuno-Phytopathology Laboratory; FITC and RITC-labeled secondary antibodies were used in this investigation. Besides, Western blot analysis for chitinase was also done using PAb of chitinase.

4.10.1. Spore germination on leaf surface

After the successful induction of susceptible tea plants with the SAR inducer BTH, the infection process occurring on the tea leaf surface was followed in order to find out the mechanism of resistance at the microscopic level, if any. The process of infection and the structures associated with the constitutive resistance, has already been elucidated in detail earlier. Presently, leaves obtained from susceptible tea plants (TV-22) as well as BTH-treated were inoculated with *G. cingulata* and percentage spore germination and appressoria formation calculated from the cleared leaf tissues. The results have been presented in Table 46 and Plate 27. It is clear from these results that on induced resistant tea leaves of TV-22, there was suppression of spore germination percentage, appressoria formation and germ tube length. Besides, there was formation of microconidia (Plate 27, fig.E) and cell necrosis at the site of penetration (Plate 27, fig. H). No such structures were visible on the surface (Plate 27, figs. A-D) of the uninduced TV-22 tea leaves. 48 hours after inoculation there was heavy colonization of tea leaf tissues of untreated TV-22 (Plate 27, fig. D). BTH-treated TV-33 plants showed formation of microconidia (Plate27, fig. E), sites of HR-like response(Plate27, fig. F) and sites of attempted penetration (Plate27, fig. H).

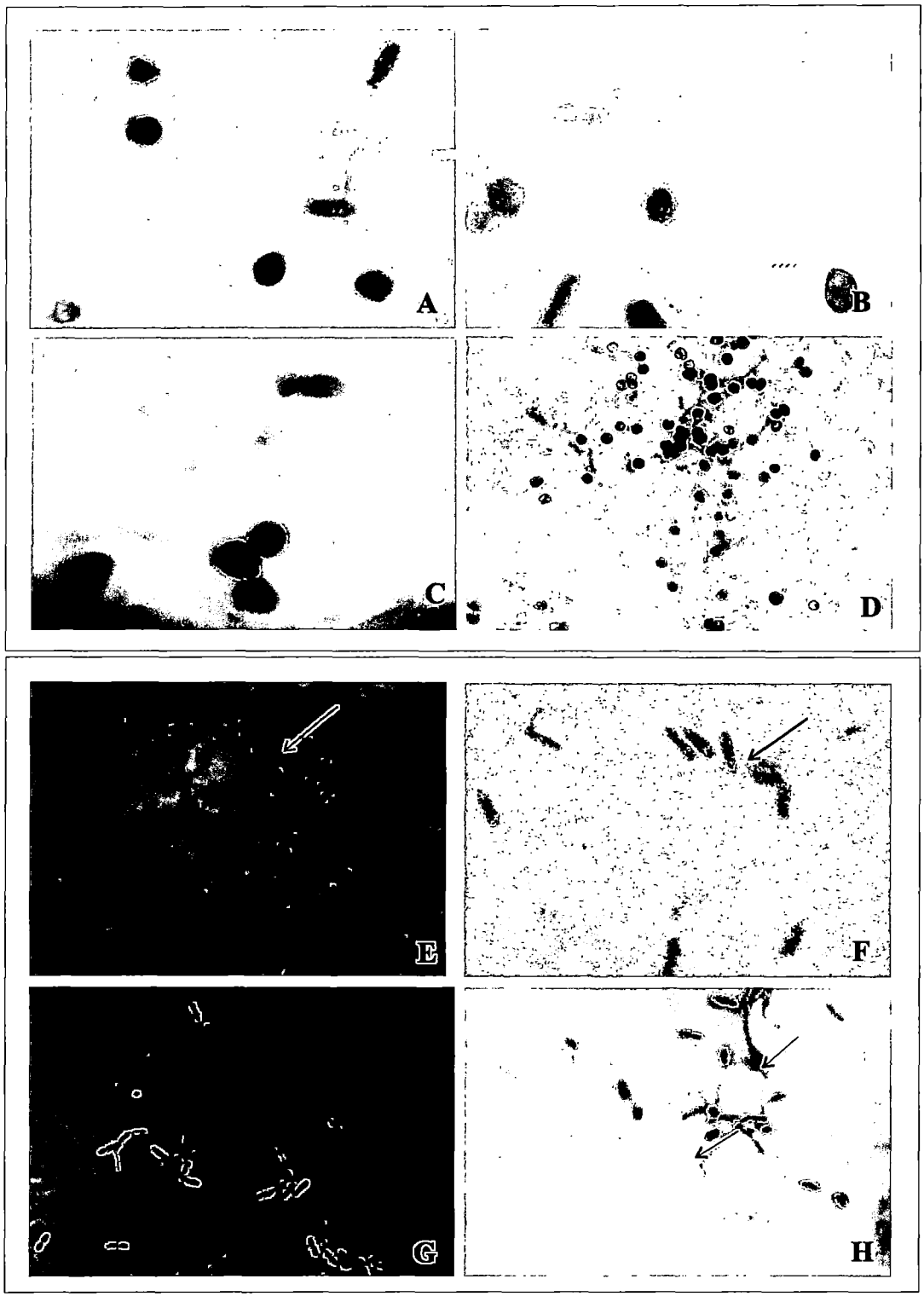


Plate 27 (A-H) : Progress of spore germination of *G. cingulata* on(A - D) untreated and BTH (benzothiadiazole) treated (E - H) leaf surface of tea (TV-22) at 6 (A & E), 18 h (B & F), 24 h (C & G) and 48h (D & H) post inoculation.

Table 46: Effect of induced resistance on spore germination, percentage appresoria formation and germ tube length of *G. cingulata* on tea leaf surface .

	% spore germination[a]	% appressoria formation [a]	Germ tube length(μm)[b]
Susceptibe (Untreated TV-22)	73.1 ± 3.2	66.8 ± 2.2	61.2 ± 2.8
Resistant (TV-30)	65.3 ± 1.9	25.4 ± 2.0	64.3 ± 2.3
Induced resistant (BTH-treated TV-22)	57.4 ± 2.7	22.7 ± 5.2	44.6 ± 3.3

Means ± SE, n=3

Incubation temperature ± 25°C. R.H. 90%

[a] Average of 300 spores per experiment

[b]Average of 60 germlings per experiment

4.10.2. Western Blot analysis

The untreated and treated tea plants were inoculated with the spore suspension of *G. cingulata* and total protein homogenates were extracted from the tea leaves and run on SDS-PAGE (Plate 29, fig. A). The single low molecular weight protein with ca 12 kDa was found to be present in the leaf homogenates of all the treated plants that was absent in the untreated healthy and inoculated plants.

The separated proteins were next blotted on to Nitrocellulose membrane and the proteins were probed with PAb of chitinase It is clear that the treated plants had increased levels of chitinase as assayed earlier, that was visible as a distinct band of molecular weight ca 29 kDa, especially in the treated inoculated plants (Plate 29, fig. C). Thus chitinase showed greater involvement in defense reaction of tea plants associated with BTH-induced resistance than β-1,3-glucanase.

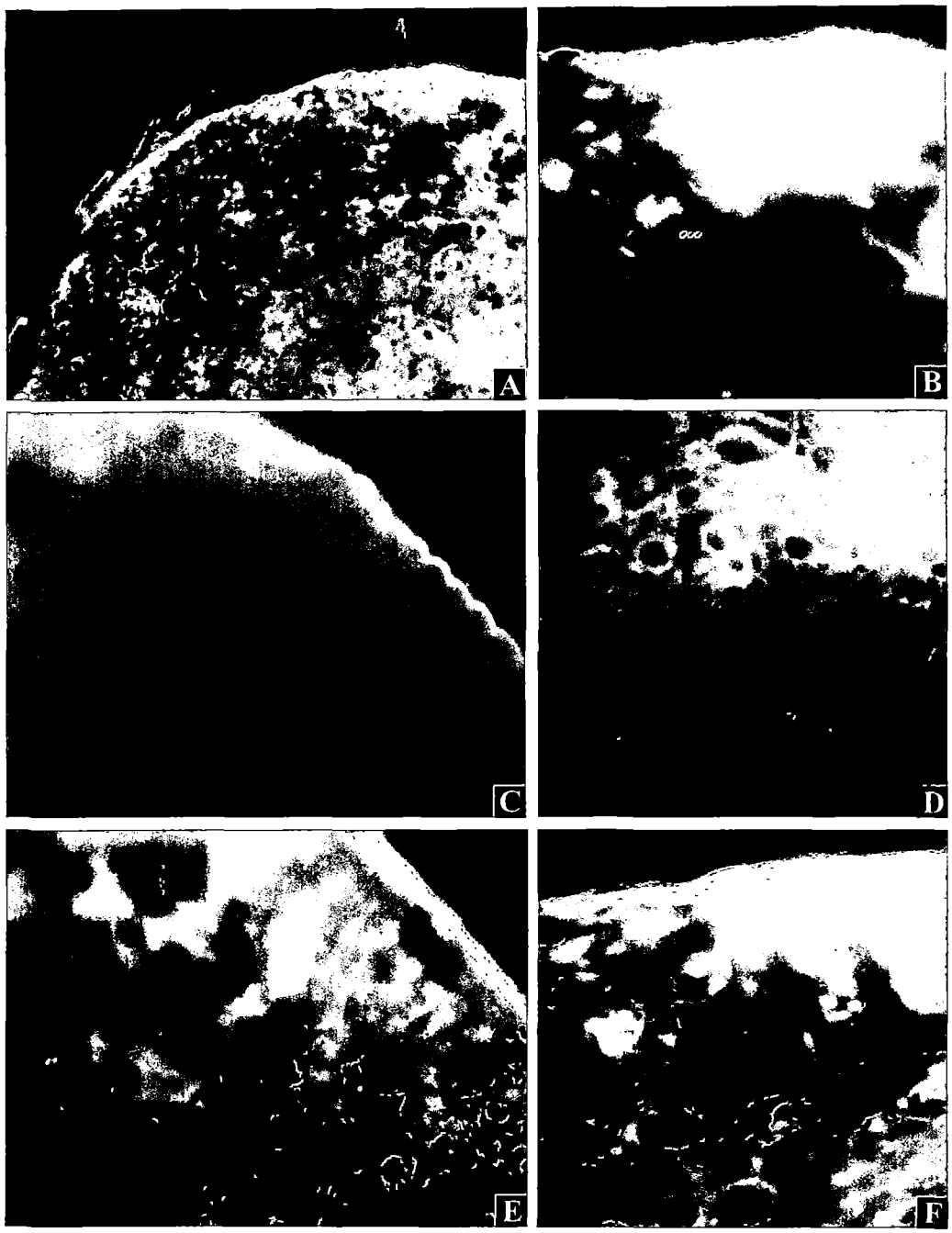


Plate 28 (A-F) : Cross-sections of BTH (benzothiadiazole)-treated tea leaf tissue (TV-22) reacted with PAb of β-1,3-glucanase and treated with FITC-labeled conjugate (B, D-F); untreated (A & C).

4.10.3. Indirect Immunofluorescence

Immunolocalization of β-1,3-glucanase was conducted in untreated and BTH-treated tea leaf tissues of TV-22 (susceptible variety). The activity of β-1,3-glucanase that was measured earlier, was reflected in the indirect immunofluorescence studies. There was a faint fluorescence in the untreated samples (Plate 28, figs. A & C). However, in the BTH-treated samples excellent apple-green fluorescence was observed when treated with FITC-labeled secondary antibody, especially so in the mesophyll tissues (Plate 28, figs. D-F).

Immunolocalization of chitinase in untreated healthy and untreated *G. cingulata*-inoculated tea leaf tissues (Plate 29, figs. B & F) indicated very low amount of enzyme, the fluorescence observed was mainly from the autofluorescent phenolics. Treatment with BTH enhanced the level of apple-green fluorescene (Plate 29, fig. D) that was confirmed by using RITC-labeled secondary antibody (Plate 29, figs. G & H). Challenge inoculation of induced tea plants with *G. cingulata* further increased the level of fluorescence (Plate 29, fig. E).

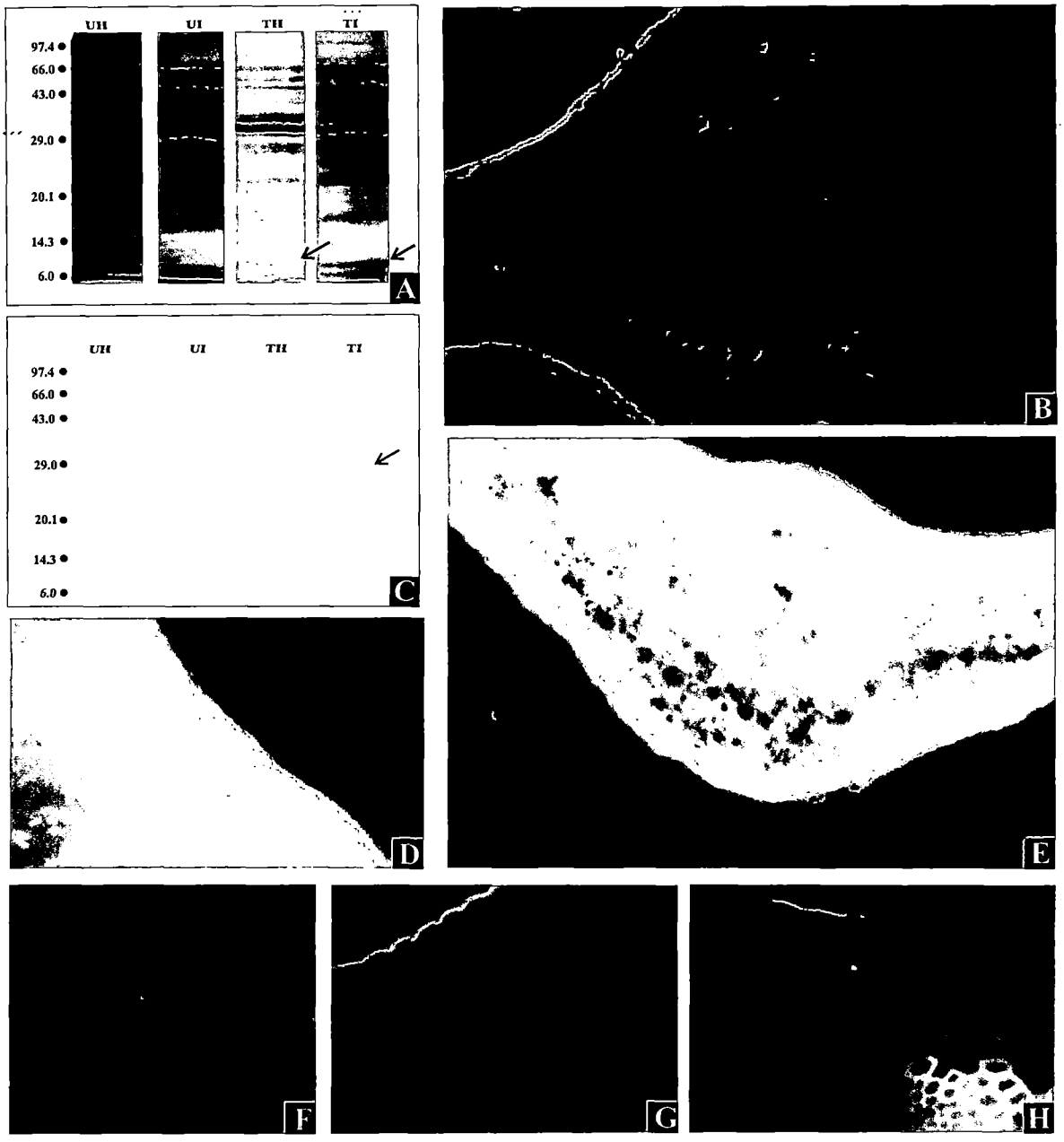


Plate 29 (A-H) :SDS-PAGE (A) Western Blot analysis (C) and immunolocalization (B, D-H) of defense enzyme using PAb of chitinase and reacted with FITC (B, D & E) and RITC (F-H) conjugate of untreated and BTH-treated tea (TV-22)leaf tissue.